Efficacy and Safety of Chinese Medicines for Pregnancy

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Publications

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Scholarships/Academic Awards

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Table of contents

List of figures

List of tables

Table Titles

Thesis Abstract

For more than 3,000 years of history, Traditional Chinese Medicine has been used in pregnant women. Nowadays, it is principally applied in Mainland China but has become more and more widely used worldwide to promote both mothers' and fetuses' health and treat common pregnancy disorders.

To date, no data are available to provide an overview of Chinese medicines in pregnancy. In the first part of this study, the clinical applications, and therapeutic effects and safety of Chinese medicines for pregnancy were reviewed and studied. In the second part of this study, the safety of most commonly used Chinese medicine during pregnancy was studied in pregnancy animal models, in vivo and in vitro.

Over 3,000 publications were identified and selected to assess Chinese medicines for pregnancy, including their indications, contraindications, formulae, individual medicines, regimes, effectiveness, efficacy, safety, adverse effects and toxicity. Amongst all clinical applications, threatened miscarriage was the most common clinical indication. Shou Tai Pill, containing 4 major herbal medicines *Chinese Dodder Seed, Chinese Taxillus Twig, Donkey-hide Glue,* and *Himalayan Teasel Root*, was the most commonly used formula in preventing miscarriage and promoting pregnancy. The top 10 most frequently prescribed single herbal medicines in wide variety of formulas were identified. The average clinical dose for each medicine ranged from 6g to 28g daily, however the dosage of Chinese medicines was not significantly correlated with its overall efficacy. Randomized controlled trials evaluating the effectiveness of the medicines were selected for meta-analysis. The results showed Chinese medicines in combination with other pharmaceuticals were more effective to improve the clinical outcomes of threatened miscarriages than other pharmaceuticals. No specific safety problem was reported, but potential adverse and toxic effects on reproductive system by certain medicines were identified from other publications.

A safety study was then carried out in vivo in normal pregnant mice. The most commonly used single herb, *Largehead Atractylodes Rhizome,* at relevant clinical doses was administered orally to the pregnant animals at different or throughout gestational periods, beneficial and adverse effects on both mothers and offspring were studied. At higher clinical dosage, significant decreased maternal weight, fetal/neonatal growth and weight; and significant increased incidences of fetal resorption, congenital caudal regression and hip dysplasia were recorded. Bone CT examination and skeleton staining confirmed congenital skeleton abnormality, including shoulder joint dislocation, congenital absence of ulna and distal digits, oligodactyly, long bone shortening, congenital hip dislocation and caudal regression In vitro whole embryo culture confirmed that Largehead Atractylodes Rhizome induced abnormal limb development during early development. Molecular study suggested the effects of *Largehead Atractylodes Rhizome* on *Tbx* suppression for early limb development.

摘要

傳統中醫運用中草藥治療妊娠婦人已經有 3000 多年。如今，中草藥在促進母嬰健康和治療常見妊娠疾病上，主要應用於中國內地，同時也在全球被越來越廣泛地使用。

到目前為止，還沒有科學數據可以提供一個中草藥在妊娠時期應用的全面概述。所以這項研究將首先對中草藥在妊娠期的臨床應用，療效以及安全性進行綜述和研究。同時開展在體動物實驗和體外全胚胎培養實驗來檢驗妊娠期間最常用中草藥的安全性。

我們從 3,000 多篇中草藥在妊娠期應用的文獻，從適應症，禁忌症，方劑，單藥，複方，實效性，有效性，安全性，不良反應和毒性等方面進行探討。在所有的臨床應用中，先兆流產是最常見的臨床指徵。壽胎丸是在預防流產和促進懷孕上最常用的方劑，其基本方包含 4 味單藥：菟絲子，桑寄生，阿膠與續斷。我們還明確了在眾多方劑中使用最多的前十位單藥。各種單藥的平均臨床劑量為每日 6 克至 28 克，但其用量與總體療效沒有顯著相關性。研究中藥療效的隨機對照試驗被選作 Meta-analysis，結果表明：相比其他藥品，中草藥能更有效得改善先兆流產的臨床症狀。文獻中沒有關於安全問題的具體報告，但有提及個別中草藥的潛在的對生殖系統的毒性影響。

我們將最常用的單味中藥白朮的提取物，就正常妊娠老鼠進行了在體安全性研究。通過在整個妊娠期或者妊娠的不同階段，給妊娠動物模型灌予相關臨床劑量白朮提取物，對母體和後代的益處和害處進行研究。在較高的臨床用量組中發現，顯著降低孕鼠體重和胎兒或新生兒體重及明顯增加吸收胎率，胎兒骨骼畸形，先天性髖關節發育不良，尾骨退化等。骨骼 CT 和染色檢查證實了手骨先天性發育異常，比如指骨缺失和尺骨缺失，長骨短縮，髖關節錯位和尾骨退化。全胚胎體外培養也證實了白朮粗提物可以引起早期的肢體發育異常。基因表達研究表明白朮對 Tbx 基因在肢體發育上有抑制影響。

Chapter I

Chinese Medicine

1.1 Definition of Medicine

The word *medicine* is derived from the Latin "*ars medicina*", meaning *the art of healing* (Tribhuwan RD, 2009; Etym Online). It encompasses a range of health care practices evolved to maintain and restore health by the prevention and treatment of illness. In the old times, healing art was practiced in accordance with alchemical treatments and ritual practices that developed out of religious and cultural traditions (Healthcare Products Focused Sites).

Nowadays, "medicine" means more the science of healing. Particularly in western countries, scientific medicine applies health science, biomedical research, and medical technology to diagnose and treat injury and disease, typically through medication, surgery, or some other forms of therapy. The term "Western Medicine" stands for scientific and science-based practices to distinguish it from "Eastern Medicine", which typically refers to traditional or anecdotal practices in eastern countries (Loudon I, 1997).

1.2 Introduction of Chinese Medicine

Early records on medicine have been discovered from ancient Egyptian Medicine, Babylonian Medicine, Ayurvedic Medicine (in the Indian subcontinent), Chinese Medicine (predecessor to the modern Traditional Chinese Medicine), Greek Medicine and Roman Medicine. China has a history of 5,000 years, and Chinese Medicine has been widely used to promote health and treat illnesses since then (Loudon I, 1997).

1.3 Development of Chinese Medicine

1.3.1 Ancient Chinese Medicine

The first record of Chinese Medicine can be dated back to over 3,000 years ago, but it is believed that the origin of Chinese Medicine was more than 5,000 years, as long as the recorded history of China (Li et al., 2005). According to legend, the origin of Chinese Medicine started from three legendary emperors/mythical rulers: Fu Xi, Shen Nong, and Huang Di (Yellow Emperor) (Roberts J, 2009). Historians believe that Fu Xi and Shen Nong were early tribal leaders (Schinz A, 1996). Chinese Medicine has a longer history than any other nation. It can be summarized by a list of important practitioners/doctors and books below (Li et al., 2005), some of which are also well known in western countries.

- As the first record of Chinese Medicine, the textbook Huangdi Neijing (黃帝內經), or called Yellow Emperor's Inner Canon, written by Yellow Emperor (2697 – 2597 BC), was regarded as the fundamental and most representative medical context in which the most basic knowledge and questions related to Chinese Medicine were systematically recorded, standardized, and developed. It's a basis of the unique theoretical system of Chinese Medicine.
- Warring States Period (5th century BC – 221 BC): Silk manuscripts recorded channels and collaterals, Moxibustion Classic of the Eleven Channels of Legs and Arms (足臂十一脈灸經), and Moxibustion Classic on the Eleven Yin and Yang Channels (陰陽十一脈灸經). The latter was part of a cache of texts found in Mawangdui in the 1970s, as the earliest record of Acupuncture.
- Han Dynasty (206 BC – 220 AD) to Three Kingdoms Period (220 – 280 AD):
    - Classic of Moxibustion and Acupuncture Preserved in a Pillow (鍼灸枕中經) by Huà Tuó (華佗), a prominent eastern physician who was famous for his contributions on the development of surgery in China.
    - Treatise on Cold Pathogenic and Miscellaneous Diseases (傷寒雜病論) by Zhāng Zhòngjǐng (張仲景), which contained the earliest known reference to Neijing Suwen. It included two contexts: Treatise on Cold Damage (傷寒論, focusing on febrile conditions attributed to "Cold"),

and Essentials of the Golden Cabinet (金匱要略, focusing on "miscellaneous illnesses").

- Jin Dynasty (265 – 420 AD): Systematic Classic of Acupuncture and Moxibustion (鍼灸甲乙經), the first book in acupuncture, by Huángfǔ Mì (皇甫謐), as practitioner and advocate of acupuncture and moxibustion.

- Tang Dynasty (618 – 907 AD), notable advancement in Chinese Medicine during the Middle Ages.
    - Emergency Formulas Worth a Thousand in Gold (備急千金要方) and Supplement to the Formulas Worth a Thousand in Gold (千金翼方) by Sūn Sīmiǎo (孫思邈), which provided great amount of formulae for clinical medicine.
    - Arcane Essentials from the Imperial Library (外臺秘要) by Wang Tao (王燾), which had important reference value basis of large-scale medical literatures.
    - Emperor Gaozong (649 – 683 AD) of the Tang Dynasty commissioned the scholarly compilation of a *Materia Medica* (本草) in 657 that documented 833 medicinal substances taken from stones, minerals, metals, plants, herbs, animals, vegetables, fruits, and cereal crops (Unschuld et al., 1985).

- Song Dynasty (960 – 1279 AD):
    - Illustrated Manual of the Practice of Acupuncture and Moxibustion at the Transmission (銅人腧穴鍼灸圖經), by Wáng Wéiyī (王惟一), was a famous guidance book for application of acupuncture, which vividly and exactly marked all the acu-points on a Bronze Figure.

- Yuan Dynasty (1271 – 1368 AD): The book Exposition of the Fourteen Channels (十四經發揮), written by Huá Shòu (滑壽), based on the flow injection order of the twelve meridians and the Renduermo (十二经脉和任督

二脉) to describe the corresponding organs function, acupoints and main diseases.

- Ming Dynasty (1368 – 1644 AD): golden age of acupuncture and moxibustion.
  - Compendium of Acupuncture and Moxibustion (鍼灸大成) by Yáng Jìzhōu (楊繼洲), completed in 1601, which is recommended as the most important reference book by many practitioners.
  - Compendium of Materia Medica (本草綱目) by Lǐ Shízhēn (李時珍), the most complete and comprehensive pre-modern book (completed in 1578 AD), and also be considered as an index book for Chinese herbs or herbal medicines.

- Qing Dynasty (1644 – 1912 AD):
  - Golden Mirror of the Medical Tradition (醫宗金鑒) was compiled by Wu Qian (吳謙) under imperial commission. The essence of Chinese Medicine, from the Warring States Period down to the Ming and Qing Dynasties was collected in this medical textbook.
  - The Source of Acupuncture and Moxibustion (鍼灸逢源) by Li Xuechuan (李學川), which collected all abstracts of ancient acupuncture literatures.
  - Systematized Identification of Warm-factor Disorders (溫病條辨) compiled by Wu Jutong (吳鞠通) in 1798. This book was a master work on summary of clinic work and provided good references for warm-factor disorders.

1.3.2 Classical and Modern Chinese Medicine

The term "Classical Chinese Medicine" often refers to medical practices that rely on theories and methods before the fall of the Qing Dynasty (1911 AD) (Huyssteen WV, 2003). It is usually considered as transition from ancient Chinese Medicine to modern

Chinese Medicine during the period which has been less influenced by Western Medicine and political agendas.

Modern Chinese Medicine now refers to Traditional Chinese Medicine (TCM), and the modern practices have been systematized in the 1950s, as the government has supported the medical reform to develop both Western Medicine and Chinese Medicine by sending some doctors who graduated with clinical experience of Western Medicine to learn Chinese Medicine theory and therapy, and participant in Chinese Medicine practices, after the establishment of People's Republic of China (Taylor et al., 2005).

1.4 Theories of Chinese Medicine

Although Chinese Medicine has such wide applications as an important part in daily medical care throughout Asia, it is mostly accepted as an alternative medical method in many nations of the western world.

Chinese Medicine has a unique system to make diagnosis and cure illness. The clinical diagnosis and treatment in Chinese Medicine are mainly based on Yin-Yang, five Phases, the human body Meridian/Channel system, Zang Fu organ theory, six Confirmations, four Layers, etc. Chinese Medicine claims to be rooted in meticulous observation of "Cosmos", "Nature", and "Body" (Schipperges H, 1997; Lu GZ, 2009). "Cosmos", Chinese Medicine considers the world is a big universe while human is a small universe. "Nature" refers to the environment on which human life relies, and with which huamn activities interact. "Body" is a unique model of the human body, notably concerned with the meridian system. Unlike the western anatomical model which divides the physical body into parts, the Chinese model is more concerned with function. For instance, the "Spleen" is not a specific piece of flesh, but an aspect of function related to transformation and transportation of essences within the body, and also an aspect of the mental functions of thinking and learning.

During development of Chinese Medicine, the theories on basic knowledge, principles and applications were established in several schools of thought. There are significant regional and philosophical differences between practitioners and schools which in turn can lead to differences in clinical theory and practice, which also has hindered its modern acceptance in the West.

1.4.1 Yin-Yang

The meanings of Yin and Yang in Chinese are dark and bright sides of an object (Hutchison JA, 1975; Kuang ZR, 1992) (Figure 1.1). It includes four basic principles (Li et al., 2005):

Figure 1.1 Tai Ji – sign for Yin and Yang

1, Opposition, which represents a wider range of opposite properties for every thing in the universe; i.e. heaven and earth, stillness and movement, cold and hot, lower and upper, etc.

2, Interdependence, which means that the nature of Yin and Yang is relative, but they can not be able to exist in isolation. If you mention the Yin side of an object, there must be a Yang side stands for different nature, effect or function.

3, Growth and Decline. The opposition between Yin and Yang is not static, but always

in a process of growth and decline, which achieves a dynamic balance.

For example, daytime is referred to as Yang while nighttime is represented by Yin (Li et al., 2005). Human beings work and play during the daytime and rest and sleep during the night time. If there is no sleep at nights, the balance of Yin and Yang is broken, signs and symptoms related to excess of Yang or deficiency of Yin develop.

4, Conversion. Under certain conditions, the two sides could transform into each other. For example, cold in limbs could weaken the pulse suddenly when the patient is suffering from a sustained high fever, this is transformed from Yang to Yin.

The theory of Yin and Yang has been extended to include body structures, physiological functions, and pathological changes for guidance of diagnoses and treatments.

1.4.2 Five Elements

The relationship within the human body and between the human body and the external environment (like the relationship between nature, climate and diet), is described as the Five Elements, i.e. Wood, Fire, Earth, Metal and Water (Li et al., 2005). They constitute the basic material elements of the universe. All substances can be classified as one of these Five Elements, such as seasons, organs, emotions, etc (Moss, 2010; Kuang ZR, 1992) (Table 1.1).

Each Element has its own characters and interactions, and each can not exist without the others. One can promote or encourage another while one can restrict or inhibit another (Figure 1.2). There are also balances among these elements, and if there is failure to keep the balance, health problems will arise.

1.4.3 Zang-Fu

Zang-Fu refers to Five Zang (五臟), Six Fu (六腑), and Six Extraordinary Organs (奇恆之腑) (Li et al., 2005). Five Zang includes "Heart", "Liver", "Spleen", "Lung", "Kidney", storing different and necessary substances for human life (Li et al., 2005). Six Fu means "Gallbladder", "Stomach", "Large Intestine", "Small Intestine", "Bladder" and "Triple Energizer" (三焦, which is closely associated with the "Spleen" functions of transformation and transportation, particularly the metabolism of incoming food), controlling the absorption of food and excretion of waste (Li et al., 2005). Extraordinary Organs, includes Brain, Marrow, Bone, Veins, Gallbladder, Uterus (also called Womb), which are relatively close organs that store the Qi (Chi, 氣) (Li et al., 2005).

Table 1.1 Associations of five elements with different aspects of nature and human

	Wood	**Fire**	**Earth**	**Metal**	**Water**
Orientation	East	South	Middle	West	North
Season	Spring	Summer	Late Summer	Autumn	Winter
Climate	Wind	Summer Heat	Dampness	Dryness	Cold
Cultivation	Germinate	Grow	Transform	Reap	Store
Yin Organ	Liver	Heart	Spleen	Lung	Kidney
Yang Organ	Gall Bladder	Small Intestine	Stomach	Large Intestine	Bladder
Orifice	Eye	Tongue	Mouth	Nose	Ear
Tissues	Tendons	Vessels	Muscles	Skin & Hair	Bones
Emotions	Anger	Joy	Pensiveness	Grief	Fear
Colour	Blue/ Green	Red	Yellow	White	Black
Taste	Sour	Bitter	Sweet	Pungent	Salty
Voice	Shout	Laugh	Sing	Cry	Groan

Modified from: http://www.txtchineseclinic.co.uk/chinese_medication_theory.php

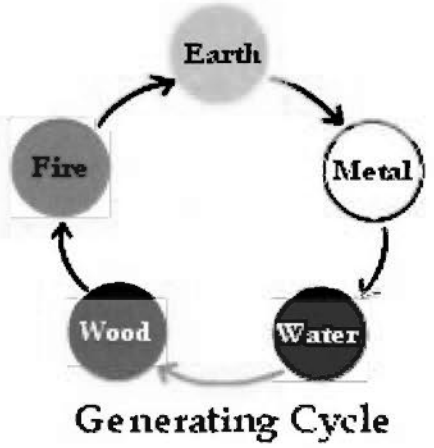


Figure 1.2 Relationship among the five elements

Formation of Zang-Fu theory is based on three parts (Xia T, 2001; Ross, 1985): the ancient anatomical knowledge, long-term observation on human physiological and pathological phenomena, and summary of long history of medical practice. Therefore, although some terminology is similar to the anatomic organs used in Western Medicine, Zang Fu organs include different meanings on physiological and pathological functions. For example, the Heart, besides its physiology function of regulating the blood in the body which is the same as in Western Medicine, is also in charge of the spirit and motion. The clinical manifestations of insomnia, dreaminess, amnesia, delirium, and even coma are firstly considered as the dysfunction of Heart in Chinese Medicine.

Like the organs in Western Medicine, all the Zang and Fu have very close relationships to each other, interdependent and constrained, and work together coordinately and harmoniously as a whole within the human body. Generally speaking, the physiological function of a Zang or Fu in Chinese Medicine may refer to the physiological functions of 2-3 organs in modern anatomy. Take Liver as an example, in Chinese Medicine the function of Liver is clearing and smoothing the Qi,

improving the other organs with digestion activity, adjusting the mood and emotion, and regulating the reproductive function. The function of an organ in modern anatomy may be involved in several Zang and Fu as well, like the mental activities are controlled by both Brain and Heart in Chinese Medicine.

1.5 Causes of Disease in Chinese Medicine

All diseases have their causes in Chinese Medicine, but the classification is different from Western Medicine. Chinese Medicine mainly uses "Evils" and "Emotions", instead.

Six Evils (六邪) means Wind, Cold, Heat, Wet, Dry and Fire (風，寒，暑，濕，燥，火) (Li et al., 2005; Maciocia, 1997). In daily life, there are only 6 different climates in nature, called "Six Gas" (六氣), which not only have no harms to human, but also are the most essential conditions for the development of all living things in universe. Sudden change of the climates or environment, if mankind cannot adapt or are too weak to the changes, these "Six Gas" would become the causes of diseases, and are considered as "Six Evils".

Seven Emotions (七情) refer to Happiness, Anger, Worry, Thinking, Sadness, Fear and Surprise（喜，怒，憂，思，悲，恐，驚） (Li et al., 2005; Maciocia, 1997), which also are the normal feelings of human beings. But if one of the emotions becomes stronger than the others, or persists for a longer time by certain stimulation, and exceeds the tolerence level, humans will suffer from illness.

Other causes include food and diet, activity and rest, and injuries (trauma, burn, frost, injury or attacks, etc) (Li et al., 2005; Maciocia, 1997). What's more, the pathological products or wastes from other systems, such as sputum and gallstone, could also result in problems in health (Li et al., 2005; Maciocia, 1997).

1.6 Clinical Diagnosis in Chinese Medicine

There are four steps of diagnosis in Chinese Medicine: Observe (望), Hear and Smell (聞), Ask (問) and Touch (切) (Li et al., 2005; Teng et al., 1999).

Observe: A doctor will carefully look at the whole and part of a patient, including mental status, color of tongue, face, skin, appearance and activities to have an impression of the general health condition.

Hear and Smell: Chinese Medicine believes that the individual voice and odor change during the physiological and pathological activities of Zang and Fu (Teng et al., 1999). So doctors will have an idea on the conditions within human body by listening to the voice, words, breath, cough, hiccough and vomit, and by smelling the breath, sweat, and wastes (such as sputum, urine, stool, etc.)

Ask: History taking is almost similar as in Western Medicine. Physicians will not only ask the chief complaints and the symptoms, but also ask other information like history, the previous treatments and medicines, the efficacy and side effects, etc.

Touch: It includes two methods: Pulsation and Deep Palpation (Teng et al., 1999). Pulsation is a unique diagnosis method in Chinese Medicine. A doctor will first touch the lateral part of the wrist on the radial side and feel the pulse to study the cause, lesion and severity of the illness from the body. Deep Palpation is the same as the physical examination in Western Medicine, and doctor will touch and press certain parts of the body to get better understanding of the medical problem.

1.7 Therapies in Chinese Medicine

Therapies in Chinese Medicine include several different treatments, which are applied quite differently, but they are all based on the same understandings of assumptions

and insights in the nature of the human body. Main therapeutic approaches are listed as follows (Lu HC, 1994; Wang HH, 2003; Li et al., 2005).

1, General Acupuncture (針灸). It actually refers to 2 types of treatments, Acupuncture (針療) and Moxibustion (灸療) (Li et al., 2005). These two treatments are always used together and applied by stimulating certain areas of the external body or internal site, called Acupuncture Point (acu-point, 穴位). The former is usually performed by inserting fine needles into the special acu-points, while the latter by burning a dried herb, Chinese Mugwort (艾草, Artemisia Vulgaris) (Li et al., 2005), on the surface of certain acu-points with or without acupuncture needles. The intended effect is to regulate or improve the circulation and balance of the Qi within the body.

2, Chinese medicines (中藥). Most Chinese medicines are derived from nature, including plants, animals and minerals. Herbal medicines from plants are much more commonly applied than the others. In China, it is not only considered as a primary therapy for treatment, but also as a supplementary therapy to promote health in general.

3, Food therapy (食療). Dietary recommendations are provided and certain foods and herbs are prescribed to make the balances of the "Five Flavors" in the body (see below).

4, Qi Gong (氣功). It is related to special breathing and meditation exercise. Qi Gong tries to restore the orderly flow inside the network through the regulation of Qi in the body. Continually doing this exercise could enhance the physical condition, so it is considered as one important means of producing health and preventing disease in Chinese Medicine.

5, Tai Chi Exercise. It not only requires the movements of muscles and the activities of related joints, but also specifically requests participants try to be calm and with high concentration, which could benefit the central nervous system and functions of

other systems and organs. There are different types of Tai Chi exercises. Tai Chi Chuan (太極拳) is the most famous one, and another well known exercise is Tai Chi Sword (太極劍).

6, Tui Na (推拿). It is a form of massage on the surface of the body which evolved from Shiatsu (指壓按摩療法). This massage could clear the meridians and improve the blood flow to maintain the body's Yin and Yang balance, so people can feel the relaxation of muscle, better flexibility of joint, and elimination of fatigue. It helps to maintain good health.

7, Cupping (拔罐). It is a special type of Chinese massage. It produces thermal, mechanical, and relatively mild stimulation to the body, which can regulate Yin and Yang, dredge channels, and relieve blood stasis and swelling pain.

8, Die Da (跌打). It is very commonly applied in Hong Kong and is usually practiced by professional orthopedic doctors to treat injures in limbs. Its main priniciple is clearing the meridians, regulating Qi, activating Blood, dispelling cold, and relieving pain. It improves the blood running by means of direct stimulation on the body surface while speeding up the flow of Qi and Blood with the generated heat effect by hands.

9, Gua Sha (刮痧). It is a folk therapy for certain disorders, scaling the skin to stimulate specific acu-points until mild to moderate subcutaneous hemorrhage, and to excrete the Evils from the body.

Most of the above therapeutic techniques in Chinese Medicine have spread aboard since the sixth century BC (Wang SM, 2007), and in most western countries, acupuncture and herbal medicines are the two most popular therapies of Chinese Medicine nowadays.

1.8 Acupuncture

Acupuncture, with the advantages of easy operation, fast and significant effectiveness, has been widely used since it has been recorded in literature, and spread quickly overseas afterwards. More than three hundred acu-points are identified as special parts for Qi of organs and meridians importing from the body surface, most of which exist in the places with dense nerve endings or where the thicker nerve fibers passing by (Yin et al., 2010). Each acu-point has its own therapeutic action. For example, the point Ren Zhong (人中穴), located on the midline between the root of the nose and the margin of the upper lip (Shao XN, 2000), is mainly used for emergencies, such as epilepsy, stroke, shock, poisoning, coma, etc. And the most common case is to awake a patient who suffered from heat stroke.

The operation of acupuncture is not complex, but one should be well trained on accurate use of his hand, finger and strength before applying the needles to patients. The principle of operation is the same; however, treatment plans, frequency and duration depend on the cause, presentation and diagnosis of the disease. A common course of treatment usually includes 10-15 operations and 3-5 days rest before beginning the next course (Yin et al., 2010).

The effectiveness of an acupuncture treatment has become a hot discussion since it spread to western countries. One explanation for its effectiveness is that, inserting needles into the acu-points stimulates the nerves inside the body, then the brain will release different neurotransmitters which relieves the corresponding symptoms and cure the diseases (Shi XM, 2004). So the needling skills and techniques of the practitioners will also have great effects on the outcome. However, as acupuncture is a minimal invasive operation and its principles for treatments are still controversial, it is not widely accepted by the public. Most patients often consider it as the last option for their chronic problems (especially if the patients believe in Western Medicine), and that also influences its effectiveness and efficacy.

It is worth mentioning that most developed countries have paid more and more attention to acupuncture, and have established lots of collaborations to further study

the theories and applications of acupuncture. France is the first western country to do research on acupuncture (Li et al., 2005), and with the "Acupuncture Rush" around 1970s, America, Canada, Germany, Japan, Korea and Singapore set up different associations to study its effectiveness and safety, and allow the private clinicians to undertake acupuncture practices (Li et al., 2005).

1.9 Chinese Medicines

1.9.1 Literatures

In primitive society, people inevitably eat some harmful "substances" during their food hunting, which results in vomiting, diarrhea and other reactions and even poisoning. Occasionally, all or some of the symptoms are relieved or disappear when taking other "substances". Through experience, people gradually realized the nutritious value and toxicity of the "substances" from nature, and had the first idea about "drug". Most knowledge of the "drug" passed from generation to generation verbally, then in written forms. In Chinese history, the first record of "drugs" was in a book called Shuo Wen Jie Zi (說文解字) (Xu S, 1970), in which "drug" was used to treat diseases and most of them are plants. Over 100 plants, animals, and minerals were recorded to treat around 10 diseases in the later famous book "Chinese Bestiary" (山海經) (Guo P, 1939).

The more acceptable word "Ben Cao" came from the completion of "Shen Nong's Herbal Classic" (神農本草經) (Wu et al., 1995), which is considered the oldest book on oriental medicines, which roughly classifies 365 species of roots, grass and woods from plants, furs, animals and stones into three categories (finest grade, moderate grade and lowest grade) by toxicity, effectiveness and functions on treatment. "Ben Cao" has been developed widely and quickly in China, and has been comprehensively studied for about 2,000 years. Another world famous book is "Compendium of Materia Medica" (本草綱目), which is also called "the Encyclopedia of China" (Li et

al., 1994), which was considered the most important and comprehensive review to study the herbs by classification, names, property, identification, function, application, formula and so on. It opened the door to the world for the herbal medicines in Chinese Medicine since it was translated into different languages in the 17th century (Li et al., 1994).

In late 19th century, with the influence of foreign scientific and technical influences, Western Medicine spread to China, and it has been in coexistence with Chinese Medicine since then (Wang SM, 2007). Correspondingly, the community and medical societies defined a new concept of "Chinese herbal medicine" from "Ben Cao", and "Chinese medicines" as different from "Western medicines" (Zhang JR, 1997) to identify the corresponding medicines. After the establishment of People's Republic China, there comes the faster development of Chinese herbal medicines. "Chinese Pharmacopoeia" (中國藥典) and "Chinese Herbal Medicine" (中華本草), were published and considered as two most useful and important reference books as bibles of Chinese Medicine. In "Chinese Pharmacopoeia" over 3,700 Chinese medicines were listed while in "Chinese Herbal Medicine" over 8,900 different medicinal substances were recorded. Approximately 600 Chinese herbs are widely used while about 250 or so are commonly used in clinical practice (Chan et al., 2010). These two bibles give all the details and information on individual herbs, animals and mineral products, including formal names, different names, common names, species, source, original plant, cultivation (aquaculture) point, harvest processing, medicine and marketing, medicine identification, chemical composition, pharmacology, processing, properties, effects, application indications, compatibility, usage, dosage, precautious, preparation, clinical research, medicine theory, annals, notes and references. They also conclude on special topics about history, resources, storage, chemistry and pharmacology in Chinese medicines.

1.9.2 Properties of Chinese medicines

There are some basic properties of Chinese medicines:

Four Gas (四氣), namely hot (熱), warm (溫), cold (寒), and cool (涼), refers to four temperature characteristics of the herbs (Li et al., 2005; Wang DR, 1996). There is another character called neutral (平), which means the existence of both hot and cold (Li et al., 2005).

Five Flavors (五味), namely sour (酸), bitter 苦), sweet (甘), spicy (辛), and salty (咸), refers to five taste properties of the herbs (Li et al., 2005).

Lifting-Dropping and Floating-Sinking (升降浮沉) applies the elevation-elimination and outward-inward tendencies of the herbs in body. For example, good controlling of Qi and Blood is the basic way in stroke treatment in Chinese Medicine, then a doctor may give Chinese Thorowax Root（柴胡）and Immature Bitter Orange（枳實）as basic formula (Zhang JR, 1997; Yan ZH, 2006; Li et al., 2005). With the lifting function of Thorowax Root, it can clear up the gas accumulated in liver. Depends on the dropping effect of Bitter Orange, it can sort out the gas in the intestine, then make the gas flow smoothly within the body.

1.9.3 Characterises

Chinese medicines are widely accepted and applied worldwide. As in acupuncture, many factors will affect the outcomes:

1, Place of origin. China has very diverse natural and geographical conditions. Water, soil, climate, sunshine, and ecological environment are not identical, or even have large variation in different regions. So it is important to collect the medicines from the places where they could be harvested with highest qualities. For example, Ji Lin, is the best place for the very common used medicine, Ginseng (人參) ("Chinese Herbal Medicine").

2, Collection. It means to collect the right part of a medicine for medical use. In plants, therapeutic usages of flower, leaf, branch, stem, bark, root, bud and fruit are quite different. Take Lotus as an example, its flower is used to prevent heat stroke, its nut is applied to treat hypertension, its root can improve gastrointestinal function, and its stem has anti-miscarriage effects (Wang TQ, 2008).

3, Processing. To reduce side effects and assure safe usage, to enhance the function and improve the clinical application, to adjust the property or effectiveness to meet the needs and conditions, to change the characteristics for better and easier storage, and to remove the non-medicinal parts for higher purity or accurate amount, the methods and skills of medicinal processing are very important to prepare Chinese herbal medicines.

4, Preparation. There are different methods to prepare Chinese medicines, as with Western medicines. Chinese medicines are usually processed into different types for easier storage, such as gas, solid, semisolid, liquid for better and quicker efficacy; and are administrated in different routes, such as tablet, powder, and granules by gastrointestinal delivery or inhalation and aerosol by respiratory delivery (Zhang JR, 1997).

5, Compatibility. Chinese Medicine commonly uses formula, compatibility selects two or more Chinese medicines as the key therapeutic medicines as the main and basis for the composition of prescriptions for the patients.

1.9.4 Application

As in Western Medicine, medicines are often applied singly or combined as cocktail that with the same or complementary function. It is important to understand that each

medicine has its own effect and function, but in most cases, instead of being prescribed individually, formulae are commonly used by Chinese Medicine practitioners as therapy to different kinds of health related problems. And herbs within a formula always work as a combination of properties and temperatures and may reach one main therapeutic effect to work together in human body. A formula usually contains no less than four herbs as basic prescriptions and up to 25 herbs for complementing and subtracting (加减方) (Li J, 2006).

In practice of Chinese Medicine, the very first formula is based on identified health problems according to the basic diagnosis theories, that is, the Chinese medical practitioners will choose one basic formula (consisting of 4 to 20 herbs) from all traditional formulae related to this disease. Then the doctors will add some other herbal medicines into or subtract other herbal medicines from the basic formula, mostly according to their own experiences. For example, if a patient is suffering from headache, the Chinese Medicine practitioner will prescribe a formula called "Yin Qiao San (銀翹散)" as basic formula (Li J, 2006) for the patient who is catching a cold (eg. 外感风热). The practitioner will further identify if the patient needs some more medicines, such as if the patient has a heavy headache, Mulberry Leaf (桑葉, Folium Mori) will be added into the basic formula (Li J, 2006). Sometimes, the practitioners will also make changes to the dosages of some herbal medicines or the whole formula to meet the specific needs of individual patients. For example, if the patient is a child, the doctor may consider reducing the total dosage and duration of the formula.

Chapter II

Chinese Medicine and Pregnancy

2.1 Obstetrics & Gynaecology in Chinese Medicine

2.1.1 Chinese Medicine for Women

In Chinese Medicine, the harmonium of human health is based on the organs, meridians, Qi and Blood, the activities of which are the similar in both men and women. But women have a special organ called "Uterus (胞宮)", with special physiological features like menstruation, pregnancy, parturition, breast, feeding and so on. These differences constitute the particular physical characteristics of women (Ma et al., 2006).

2.1.2 Physiology Basis

The performance of "Uterus" is under the biological functions of organs, meridians, Qi and Blood. According to theories of Chinese Medicine, Uterus not just refers to the organ as Western Medicine, but also stands for the whole female reproductive system (Ma et al., 2006). Qi and Blood are the foundations for normal female physiology, organs functioned as the sources of Qi and Blood, while meridians provides the pathways for interactions among the organs and the running of the Blood in the body.

2.1.2.1 Four pulses and uterus

The physiology of Uterus is closely related to four pulses, Chong Pulse (衝脈), Ren Pulse (任脈), Du Pulse (督脈), and Dai Pulse (帶脈). Chong Pulse is the fortress of the Blood in the circulation, also called "Sea of Blood" (血海) (Ma et al., 2006). Only if there is enough energy and blood in Chong Pulse, can the Uterus maintain the normal female physiological functions for menstruation and pregnancy. Ren Pulse is in charge of Yin in the body, which provides the basis for pregnancy and essence to the fetus. While Du Pulse represents the Yang side and mainly effects on the Uterus, keeping balance of Yin Yang in the body and providing the best environment for pregnancy. The function of Dai Pulse is to properly restrict the effects of 3 other pulses and to maintain the normal physical activities of Uterus.

2.1.2.2 Zang, fu and uterus

All the important factors for human health, ie. Qi and Blood, nurtrition and defense, fluid and spirit, are generated by the Zang and Fu, and the functions of Zang and Fu are the essences of life. The physiological features of a woman are achieved by the nourishment of these organs.

Kidney is a fundamental organ for human growth, development and in charge of reproduction. As all the features of Uterus are also related to reproductive activity, Kidney and Uterus show the same functions. Therefore, Kidney and Uterus have the closest relationships due to their meridian contacts and functional consistency. Liver, with the functions of storing Blood and adjusting the blood volume, has important regulation effects on the Uterus. The main function of Spleen is for haemopoiesis, as well as regulating the Blood, so it is considered as the basis for menstruation and pregnancy. Stomach is also essence for normal function of Uterus, as it is in charge of conversion of Qi and Blood. Heart controls the mental activity and all Blood channels, and maintains the daily physiology activity of Uterus. Lung dominates the Qi in the body, and all the necessary materials for Uterus transfer and adjustment.

2.1.2.3 Qi and blood

Qi and Blood are the basic elements for all living activities. They generate from the organs, transferred by the four Pulses and arrival at Uterus, to provide the important materials for accomplishing the particular physiological activities for reproduction.

2.1.2.4 Heavenly tenth (天癸, Tian Gui)

Tian Gui is a special term related to both male and female reproduction. As in women, it forms the physiological basis of menstruation, originated from the Kidney (Ma et al., 2006). From the growth of a child to teenage, Tian Gui matures and functions in the reproductive activities directly, to develop reproductive organs and to maintain reproductive function. Tian Gui is the essential materials for menstruation and

pregnancy in women, and we can also consider it as a kind of sex-stimulating element as in Western Medicine.

2.1.3 Physiology

2.1.3.1 Moon water

Periodic bleeding of Uterus in every month is known as menstruation (Ma et al., 2006). In Chinese Medicine, because this regular feature is similar with the different phases of the moon and the fluctuations of the sea tide, it is called "Moon Water" (月水). The mechanism of menstruation could be concluded as "Kidney - Tian Gui – Chong and Ren Pulses - Uterus" principle. As benefited from the Qi in Kidney, Tian Gui becomes mature and promote the concentration of Blood from other organs to Uterus. Under the regulations of Chong and Ren Pulses and the restrictions of Du Pulse, the Sea of Blood is fulfilled and the Moon Water overflows in each month, then menstruation occurs.

2.1.3.2 Dai xia

The definition of Dai Xia (帶下) is a kind of sticky liquid in the vagina, which is the same of leucorrhea in Western Medicine. However, Chinese Medicine states that Dai Xia is controlled by Ren Pulse, which commands all the liquid in the body. The mechanism of physiological leucorrhea is basis on the Qi in Kidney too. When the Qi of Kidney is abundant, it converts into Tian Gui and promotes the concentration of all the water substance from the body firstly to uterus then to vagina, and the leucorrhea occurs. The process is also regulated by Du and Dai Pulses.

2.2 Pregnancy in Chinese Medicine

The clinical book Zheng Zhi Zhun Shen (證治準繩) by Yuan Liaofan, first recorded that there must be a best timing for being conceived in every month, called "Di Hou (的候)" (Wang KT, 1998), which is the same period as ovulation in Western Medicine.

As to the clinical features of pregnancy, it is described as the same as Western Medicine, such as the breast swelling and vomiting at early pregnancy, fetal movement from the 4th month of pregnancy, and lower limb edema during late pregnancy, and so on.

The fetal development process for each gestational stage is similar to Western Medicine too. Embryogenesis in the 1st month of pregnancy, embryo development in the 2nd month, development of spine in the 3rd month, forming of basic body in the 4th month, fetal movement in the 5th month, growth of bone in the 6th month, occurrence of body hair in the 7th month, formation of organs in the 8th month, and Qi filling into organs in the 9th month, which is very important as Qi is considered as basic material for organs and human body. The basic structures of the body are ready and fetus could easily survive in the 10^{th} month.

Another feature of pregnancy in Chinese Medicine is the Pregnancy Pulse. The "Slippery Pulse (滑脈)" is always felt by pulsation around the 2^{nd} and 3^{rd} month of pregnancy. Western Medicine also believes that after 11 weeks of pregnancy cardiac output increases, which is consistent with the occurrence of Slippery Pulse.

2.2.1 Parturition

The signs of birth, the signs of labor and the clinical courses and treatments of labor are same as Western Medicine. Most of the mothers start with irregular uterine contractions, relief of upper abdomen, and appearance of shows before delivery. Important signs for labor include regular and gradual uterine contraction for at least 30 seconds with 5 to 6 minutes interval, accompanied by progressive flattening and expansion of cervical canal and cervical dilation and the decend of presenting part. The features of three stages and the durations of labor are similar with Western Medicine, but it is emphasized to control the environment, including room temperature and quietness, in Chinese Medicine.

2.2.2 Postpartum and Nursing

Because of heavy sweating and blood loss during delivery, there are losses of Yin Liquid, ie. Blood. The whole physiological characteristic is of "Blood Deficiency due to sudden decrease of Yin and floating of Yang (陰血驟虛，陽氣易浮)". Most women will suffer from a mild fever for 1 or 2 days after childbirth, accompanied with some other symptoms, such as spontaneous perspiration, which are of no risk, and will disappear generally within a short time.

During the postpartum period, the Qi and Blood stored in the Spleen and Stomach partly supply the nutrition for maternal needs, as well as the milk for infant. Therefore, in the lactation period, most Blood is used for the milk production, and usually there won't be any menstruation, also difficult to conceive.

2.3 Diseases in pregnancy

2.3.1 Causes

All Six Evils could cause gynecological and obstetrical diseases, women are much easier to be affected by Cold, Heat and Wet, which interact more with Blood (Ma et al., 2006; Liu MR, 2001). Cold, stands for Yin Evil, is divided into external, internal, excess and deficiency in Cold (外寒，內寒，實寒，虛寒). The related diseases include dysmenorrhea, gynecological disease, abdominal pain, infertility, etc. Heat, represents for Yang Evil, can also be divided into external, internal, asthenic and sthenic heat (外熱,內熱,實熱,虛熱), which could result in early menstruation, vaginal bleeding, threatened miscarriage, fetal irritability, postpartum fever, etc. There are only external and internal Wet (外濕，內濕) according to the body parts attacked by the Wet Evil. The related gynaecological diseases include leucorrhea problem, pruritus vulvae, and infertility. In pregnancy, vomiting and edema may occur.

If pregnant women are over-stimulated, the emotional changes will affect the balance of Yin and Yang in Qi and Blood, and will cause organs dysfunctions and illnesses. Internal injuries among the Anger, Thinking and Fear of Seven Emotions always have more significant impacts to the health of women. Excessive Anger mainly affects the Kidney, and could cause illnesses such as menstruation, dysmenorrhea, amenorrhea, vomiting, etc. Too much Thinking may harm the functions of Spleen and Stomach, and induce amenorrhea and irregular menstruation. Fears, like the long term influence of war, could lead to injury in the Kidney, and menorrhagia, bleeding, fetal irritation, abortion, and preterm labors were recorded (Ma et al., 2006; Liu MR, 2001).

Environmental factor (Ma et al., 2006; Liu MR, 2001), to a certain extent, is an important reason for physical impact and also affects the physical conditions. It includes frequent sexual activity, multiparity, malnutrition, restlessness, injuries, and drug abuse, and so on. The antenatal health conditions and nutrition status of each pregnant woman are different, so body resistance to disease may vary in individuals too. This is another important cause for diseases during pregnancy (Ma et al., 2006).

2.3.2 Mechanism

Physiologically, Uterus is linked to the meridians through the Chong and Ren Pulses, the pathological dysfunction of Organs only occurs when Qi and Blood dysfunctions influrence the functions of Chong and Ren Pulses, then lead to various diseases.

There are three key mechanisms to describe the pathology changes during pregnancy. First, dysfunctions of Organs cause the imbalance of Qi and Blood, subsequently affect the functions of Ren Pulse, Du Pulse and the Uterus, therefore induce diseases. The main involved Organs are Kidney, Liver, Spleen, Heart, and Lung, and the major responding illnesses are infertility, menstrual disorder, abortion, amenorrhea and renal diseases The second mechanism is Qi disorders casued by Emotion changes and Blood disorders caused by Evils, as mentioned in former sections. The third

mechanism is the direct injuries to Uterus, including falling, operations, and sex during menstrual period, etc. These three kinds of pathological mechanisms are not isolated but interrelated, interacted and interdependent. Dysfunction of organs can lead to Qi and Blood disorders while imbalance of Qi and Blood could also cause harms to organs. Similarly, direct damages to Uterus, may result in loss of physiological functions of the others and cause problems in reproduction.

2.4 Chinese Medicines for Pregnancy

2.4.1 Development in China

Chinese medicines have now become very popular and are widely applied to different kinds of medical conditions (Li J, 2006). It promotes both mothers' and fetuses' health, relieves and cures common disorders in women (Li J, 2006). It has been used as a main medicine in China with a longer history than Western medicines.

The first record of Chinese medicines treatment related to reproductive medicine was firstly in A Chinese Bestiary (山海經) 3,000 years ago during Xia, Shang and Zhou era (Li et al., 2005; Guo P, 1939). Since then, Gu Rong (菁蓉) was commonly used for contraception (Li et al., 2005; Guo P, 1939). In the following centuries, lots of milestones have been developed in Obstetrics and Gynaecology.

During the Warring States Period, Chinese Medicine theories in pregnancy are mainly on eugenics and embryology. The concept of consanguinity leading to abnormal offspring was introduced, and the stages and characteristics of embryo development were roughly described. Inner Canon of Yellow Emperor (黄帝内经) firstly described basic knowledge in female anatomy, menstrual physiology, and pregnancy (Ma et al., 2006).

In Qin and Han Dynasty, "woman's doctor" (女医) as obstetrics and gynecology specialist firstly appeared as a professional Chinese Medicine practitioner. There was the first record of application of Chinese Medicine for therapeutic abortion, conjoined fetus, and stillbirth. A prominent Eastern Han physician, Hua Tuo (140–208 AD), anesthetized patients with a formula of wine and powdered Marijuana (大麻), and successfully carried out hysterectomy and removed a dead fetus with maternal survival (Ma et al., 2006).

In Wei and Sui Dynasty, the Chinese Medicine practitioners advocated late marriage and birth control. They also described in detail the characteristics of embryos at different developmental stages and recommended maternal support in each month of a pregnancy, which was the basic concept for early Perinatology. The first induction of labor by needles was also recorded at that time (Ma et al., 2006).

Department of Imperial Doctor (禦醫院) was the highest educational and medical institution, which trained medical personnels in Tang Dynasty. One important feature of this era is that lots of medical text books were specialized for pregnancy, and Jing Xiao Chan Bao (经效产宝) was an existing obstetrical monograph with complete theoretical knowledge (Zan Y, 1995).

In Song Dynasty, Chinese Medicine in Obstetrics had developed into an independent specialist department, and the development of Chinese medicines was notablly faster than Western medicines in foreign countries. One famous and useful text book at this stage was Fu Ren Da Quan Liang Fang (妇人大全良方) by Chen Ziming (Chen ZM, 1999). He summarized the knowledge from over 30 textbooks and the experience from different Chinese Medicine practitioners, and systematically discussed the common diseases during pregnancy and parturition.

During Jin and Yuan Dynasty, various medical schools started to rise, Liu, Zhang, Li, and Zhu (Ma et al., 2006) were the four most famous schools and contributed significantly in Obstetrics and Gynecology. Zhang Zihe ardvocated taking food to

preserve health and having medicines to treat illnesses in the book Ru Men Shi Qin (儒門事親) (Zhang CZ, 1999; Xiao GG, 1998). The book also recorded a case that successfully used a hook to deliver stillbirths, which set up the precedent of Chinese midwife, considered as the embryonic form of scalp traction.

Considerable progress was achieved both in clinical theory and practices during Ming Dynasty. Some famous Chinese medicine formulae were created at this period, some of which became popular later and widely applied nowadays, such as Liu Wei Di Huang Wan (六味地黃丸) stores the "Water" and Ba Wei Di Huang Wan (八味地黃丸) increases the "Fire" in kidney to prevent miscarriage (Zhou QY, 2000).

Due to historical factors of the late Qing Dynasty, and the influence of Western Medicine under the Renaissance, development of Chinese Medicine was less prominent (Ma et al., 2006). However, some textbooks were completed and considered as references for future studies and researches in Chinese medicines for pregnancy. One of them was Fu Qing Zhu Nv Ke (傅青主女科) (Fu S, 1997), which serve a good guidance for modern Chinese Medicine in Obstetrics & Gynaecology.

After the establishment of People's Republic of China, with the great efforts of lots of Chinese Medicine practitioners and researchers, 6th edition of the textbook "Traditional Chinese Medicine in Obstetrics and Gynecology" (中医妇科学) (Ma et al., 2006) and lots of reference books and monographs have been published and used for daily teaching, training and self-learning. Apart from medical educations in Chinese Medicine, researches in collaborations with Chinese medicines and Western medicines have been raised to a new level and lots of meaningful conclusions have been drawn. For example, it was reported that combined Chinese medicines and Western medicines for ectopic pregnancy was more effective than conventional treatment (Jiang YZ, 2009; Li CZ, 2009), and the method of combined medicines has been well studied and applied widely since then.

2.2.2 Development in Foreign Countries

Chinese Medicine in China has a long history, but its development for pregnant women in other countries is just within recent centuries. In foreign nations, the most common treatments are Acupuncture and Chinese herbal medicines. Other therapies of Chinese Medicine, which could be used during pregnancy, began to spread to the world in very late 20th century, such as Tui Na Massage and Die Da (Ma et al., 2006).

Acupuncture first spread to nearby Asian countries, such as Korea and Japan in the 6th century (Wang et al., 2007). Around 14th century, acupuncture was recorded in Marco Polo's Biography, and had been known by Europe since then (Wang et al., 2007). However, until 17th century, European practitioners began to learn and apply acupuncture. It was firstly used in the European royal families only (Wang et al., 2007), until 1950 with the famous application of acupuncture as an anesthesia technique (Wang et al., 2007), it became popular and quickly spread worldwide. A well known case for its application as a narcotic was reported during the visit of President Nixon to China in 1972 (Reston J, 1971; Prensky WL, 1995) by journalist James Reston, who received acupuncture during an emergency appendectomy. So as with Cesarean section, the main clinical application of acupuncture is to relieve the pain.

Chinese herbal medicines spread to the world earlier than acupuncture but only widely applied lately, due to the early advancement and modernization of Western medicines in foreign countries (Wang et al., 2007). For instance, the "European Pharmacopoeia" had been locally well-developed, and Chinese herbs as medicines were not attractive to the practitioners and patients. With the advantages of Chinese medicines, including less side effects and greater effectiveness in some chronic diseases (such as infertility and irregular menstruation) than Western medicines, it was gradually accepted by foreigners and now has been spread to over 160 countries (Li et al., 2005). More and more foreign researchers and clinical doctors seriously have interests in it and come to China for further study.

2.5 Application and Efficacy of Chinese Medicines for Pregnancy

With a long history of application of Chinese medicines to treat pregnant disorders, and large amounts of case reports and clinical trials have been reported (Duke, 2000; Cochrane Review, 2010). However, until now, no data are available to overview Chinese medicines for pregnancy, including applications, formulae, dosage, therapeutic efficacy, and so on.

Chinese medicines are prescribed in formulae, and the Chinese medicine practitioners decide the formula according to the clinical presentation. Based on medical knowledge and personal experience, some use original or traditional formula, the others have individual prescription as personalized medicine. The prescribed formulae vary a lot, some formulae even lack unified theory and scientific evidence.

Therefore, it is meaningful and worthy to carry out systemic reviews to study the claimed efficacy of Chinese herbal medicines for pregnancy. The results will not only provide useful background information about the concerns of application of Chinese herbal medicines for pregnant women in the public health and health service systems, but also provide scientific supports and useful references for its clinical applications for pregnancy.

2.6 Safety of Chinese Medicines forPregnancy

Safety is always the biggest issue in daily medical practice, and the issue is also a major concern to pregnant women. Chinese herbal medicines have been used to treat diseases and complications during pregnancy, and it is apparently well accepted as with fewer side effects.

There are 31 Chinese herbal medicines that were classified as toxic and contraindicated during pregnancy, which have been listed in many textbooks and the website of Chinese Medicine Council of Hong Kong (CMCHK, 中醫藥條例). Further studies of these Chinese herbal medicines have been carried out in the last 20 years, and have demonstarted their adverse effects on both/either mothers and/or newborns. For example, Kansui Root (Radix Kansui, 甘遂) is prohibited in pregnancy because it can poison the fetus and stimulate uterine contraction (Wu et al., 1990).

On the other hand, numbers of clinical trials have also been carried out to assess the safety of some Chinese herbal medicines in pregnancy and associated conditions, or to compare the adverse effects of Chinese herbal medicines with other medicines. Amongst the commonly used Chinese medicines, there are not too many studies of their potential harmful effects however. No systematic data is available to record the potential adverse effects and safety issues, which are very important to patients and clinical practitioners.

Chapter III

Aims & Objectives

With a long history and as a culture, Chinese Medicine has formed its unique theoretical system to establish its way on healing diseases and maintaining health. Amongst all therapeutic applications, Chinese medicine is the most commonly and widely accepted method. Chinese medicine for pregnancy is well accepted as conventional treatment in China and as alternative treatment in foreign countries, and such use appears to be effective and to have better tolerated and less side effects. However, its efficacy and safety claims still has no scientific proof.

To understand Chinese medicines for pregnancy, we carried out systematic reviews on the use of Chinese medicines during pregnancy and also screened the safety of Chinese medicines in pregnant animals. The aim of the study was to provide general information about the clinical applications of Chinese medicines and scientific evidences on their efficacy and safety for pregnancy.

The objectives of the study included:
Firstly, we reviewed all the available literature on the clinical applications of Chinese medicines for pregnancy in Chapter 4 to study its usage, prevalence, clinical application, dose, dosing and commonly used formulae and individual herbal medicines.

Secondly, we selected the most common application of Chinese medicines for pregnancy, and carried out meta-analysis in Chapter 5 to review the therapeutic effects of Chinese medicines for pregnancy.

Thirdly, we also conducted systematic review on the safety data of most common application of Chinese medicines for pregnancy in Chapter 6 to study their potential adverse effects to mothers, fetuses and newborns.

Fourthly, we applied pregnancy models in animals in vivo and in vitro in Chapter 7 to determine the safety of the most commonly used Chinese medicine during pregnancy.

Lastly, we further studied the congenital malformation induced by the studied Chinese medicine in Chapter 8 to understand its underlying molecular effects on the developmental defects in mice.

Chapter IV

Chinese Medicines for Pregnancy: Systematic Reviews

4.1 Introduction

4.1.1 History of Chinese Medicines for Pregnancy

Since the first records, Gu Rong (莪蓉) has been used for contraceptionin for 3,000 years (Li et al., 2005; Guo P, 1939). Chinese medicine has been widely studied and applied in obstetrics. Thereafter the application of Marijuana (大麻) on anesthetized patients for cesarean section by Hua Tuo 2,000 years ago (Ma et al., 2006), Chinese medicine has been developed for obstetrical surgery. As the mainstream of medicine and therapy in China, large amount of clinical trials have been carried out during the past hundreds of years. Chinese medicines have been applied to treat all kinds of diseases and complications during pregnancy, after labor and the following development of the baby. For example, Liu Wei Di Huang Wan (六味地黄丸) and Ba Wei Di Huang Wan (八味地黄丸) can be used to improve the health conditions of mothers to prevent miscarriage without any adverse effect on fetus (Zhou QY, 2000).

4.1.2 Modern Chinese Medicines for Pregnancy

Nowadays, Chinese medicines originating from China are well-accepted as the mainstream of medical care throughout East Asia, and are considered as complementary and alternative medicine in Western world (Colmant et al., 2004). Another hot trend is applying to improve the physical condition and cure common medical problems in pregnancy, such as cold or cough. In South Mainland China and Hong Kong, many families are used to having soups in their meals and some Chinese medicines are always added into the soup. For example, Largehead Atractylodes Rhizome (白术） is usually added into chicken or duck soup for pregnant women to nourish energy and improve blood circulation (Gao CX, 2009). As well as the soups, another common way is to takie Chinese medicines as beverages. For example, Hemp Fruit (火麻仁) is popular to be served as drink for constipation and dry stool during pregnancy and after delivery (Chan LN, 2005).

4.1.3 Popularity of Chinese Medicines for Pregnancy

Chinese medicines and other herbal medicines or botanicals are widely used to promote maternal and fetal health and to relieve medical disorders during pregnancy. As originated in China, Chinese medicines have been accepted and favored by both the doctors and patients as medical treatments, and also have been frequently applied in daily activities.

Over 9% of pregnant women had consumed Chinese medicines or associated products. The prevalence varied in different countries and was higher in Asia Pacific countries. As medical therapy, surveys reported that in Shanghai, over 46% pregnant patients received Chinese medicines for different kinds of disorders and complications during their gestational periods (Wang et al., 1995). The prevalence is about 51-61% in Canada (Hollyer et al., 2002), 58% in UK (Holst et al., 2009), 56% in Hong Kong (Ong et al., 2005), 50.9% in Japan (Mantani et al., 2003), 46% in China (Wang et al., 1995), 7-45% in United States (Hepner et al., 2002 & Low et al., 2009), 36% in Norway (Nordeng et al., 2004) and Australia (Della et al., 2006), 33% in Taiwan (Chuang et al., 2009), 14% in Finland (Hemminki et al., 1991), 12% in Nigeria (Gharoro et al., 2000), 9.1% in Rhode Island (Gibson et al., 2001), and 1% in Sweden (Holst et al. 2009). As food therapy, Ginseng has been used in 10% of Asian women during their pregnancies (Chan et al., 2003). In western countries, 9.1-15% of the population have attempted Chinese medicines during their pregnancy (Chan et al., 2003). It is regarded by the public and some health care providers as gentle and safe (Marcus et al., 2005). A survey of our local population indicated that the main reasons for the use of Chinese medicines in pregnancies included "good for pregnancy and fetus", "good for general health", and common cold.

Although both the safety and efficacy claims have no scientific basis, still there are

vast numbers of individuals who use Chinese medicines in an effort to maintain good health and reduce the need for medical intervention (Westfall et al., 2001). In recent years, a number of clinical trials have been carried on to assess the value of Chinese medicines in pregnancy. However, no data is available to overview the clinical applications of Chinese medicines for pregnancy.

4.1.4 Systematic Reviews

A systematic review is a literature review focused on a subtantive question, several primary studies and substantial uncertainty, and aims to provide an exhaustive summary of research evidence – literature – relevant to that research question, and transparent approach for research synthesis to minimize bias. It dose not limit to medicine, but is also quite common in other science areas, such as psychology, nursing, physical therapy, educational research, sociology and business management. An understanding of systematic reviews and how to implement them in practice is becoming mandatory for all professionals involved in the delivery of health care. An overview of systematic review process includes definition of a proper healthcare question, literature search, study assessment, result combination, and discussion and appropriate placing findings in context, and usually a meta-analysis is also involved (More details could be read in Chapter V),

4.2 Aims and Objectives

The aim of this chapter was to access and review the available literature on the applications of Chinese medicine for pregnancy, and to provide valuable references to clinical workers and researchers for practices and studies.

The specific objectives were:

1, To locate the clinical studies of Chinese medicines as treatment for disorders during

pregnancy from different databases.

2, To identify the most common clinical application of Chinese medicines for pregnancy.

3, To evaluate the most commonly used formulae and individual Chinese medicines and its clinical dose and dosing for pregnancy.

4.3 Methods

4.3.1 Search Method

We searched the titles of all published literatures on Chinese medicines for pregnancy. The following databases were searched: EMBASE (1980 to Dec. 2010); Cumulative Index to Nursing and Allied Health Literature (CINAHL) (1982 to Dec. 2010); Chinese Biomedical Database (CBM) (1978 to Dec. 2010); Medline (and PreMedline) (1950 to Dec. 2010); China Journal Net (CJN) (1915 to Dec. 2010); China National Knowledge Infrastructure (CNKI) (1915 to Dec. 2010); Wiley Inter Science (1966 to Dec. 2010) and Wan Fang Database (Chinese Ministry of Science & Technology) (1980 to Dec. 2010).

We also explored the searches in the reference parts which were listed in these clinical trials and reports identified. If there was any literature related to the clinical use and/or studies on effects and functions of Chinese medicines for miscarriages, but not repeated with our first screening, we also included these papers. We also screened bibliographies of local articles, and searched by hand for any internet inaccessible articles.

4.3.2 Search Strategies

Search strategy for EMBASE

1. exp PREGNANCY/

2. exp CHINESE HERB/

3. (chin* adj6 herb*).af

4. ((china OR chinese) AND (tradition* adj4 medicine*)).af

5. 2 OR 3 OR 4

6. 1 AND 5

Search strategy for CINAHL

1. exp PREGNANCY/

2. (chin* adj6 herb*).af

3. ((china OR chinese) AND (tradition* adj4 medicine*)).af

4. DRUGS, CHINESE HERBAL/

5. 2 OR 3 OR 4

6. 1 AND 5

Search strategy for MEDLINE (PreMedline)

1. exp Pregnancy/

2. exp Drugs, Chinese Herbal/

3. (chin$ adj 6 herb$).mp.

4. 2 OR 3

5. 1 AND 4

Search Strategies for other databases

We used the similar search strategy for CBM, CJN, CNKI, WILEY and WanFang Database, searching by subject heading/keyword/abstract with the following:

- Traditional Chinese Medicine / Chinese Medicine

Or could be included or replaced by similar words:

- herbal medicines

4.3.3 Inclusion Criteria

1. Type of studies: All clinical studies reporting the applications of Chinese medicines to treat illnesses and complications during pregnancy were included. All the titles and abstracts were further reviewed. Case reports, commentary studies and review articles were excluded.

2. Participants: All women regardless of the age, gestational age, parity, nationality of the participants, receiving Chinese medicines during their pregnancy.

3. Interventions: Chinese medicines were administered as interventions in the clinical trials.

4. Outcome measures: in this chapter, we aimed to identify the clinical applications, formulae and individual Chinese medicines for pregnancy, no outcome of the clinical studies was studied.

5. Publication: No restriction on the languages was applied. Publications without full text available but with abstracts only were also included

4.3.4 Exclusion Criteria

1. Other participants: To focus on the clinical application for disorders related to pregnancy, for non-pregnancy related and other gynaecology illness and complications were excluded.

2. Other Chinese therapies: To focus on the implication of Chinese medicines, acupuncture and massage were excluded. If the intervention combined Chinese medicines with other therapies, the clinical trial were included.

3. Other type of studies: To focus on the clinical studies of Chinese medicines, animal

and molecular and chemical studies were excluded.

4.3.5 Data Extraction and Analysis

We designed extraction forms to extract data quantitatively. The numbers of the publication in different databases within each decade were counted. The total numbers of excluded papers and the exclusion criteria were presented in flow charts. The total numbers of included studies were summarized. To identify the common clinical applications of Chinese medicines during pregnancy, the clinical indication of each clinical study was recorded and compared. To identify the most commonly used Chinese medicine formulae and individual Chinese medicines, the frequency of each formula or individual medicine used in the clinical studies was calculated. The clinical daily dose and dosing, the formula and individual Chinese medicines and their effective rate were recorded.

4.4 Results

4.4.1 Study Identification

Up to 31st Dec. 2010, 301,547 literatures reported studies of Traditional Chinese Medicine for all applications were identified (Figure 4.1). Most of the literatures were mainly found in CNKI and CJN Full-Text Database (49.8%) and WanFang Database (36.7%), but much less in PubMed (5.7%), Cochrane Library (3.1%), EMBASE (2.4%), MEDLINE (1.6%) and WILEY Inter Science (0.7%) (Table 4.1 and Figure 4.2) Most of the literatures (78.6%) were published in Chinese whilst few of the literatures (21.4%) were published in English and other language. All of the literature provided the abstracts either in Chinese (64.9%) or English (35.1%), full texts were

available in 56% (168,866) literatures.

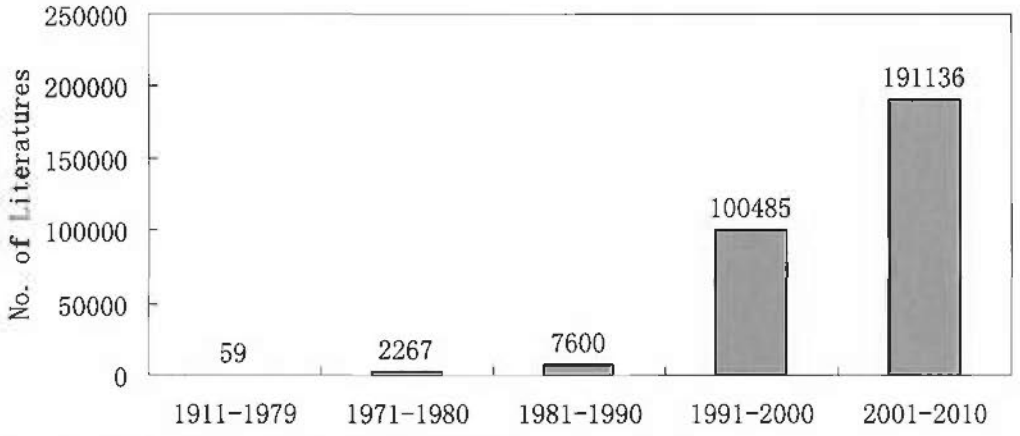


Figure 4.1 Chinese Medicine literatures published in decades

Numbers on the top: total numbers of literature in each decade.

Table 4.1 Chinese Medicine literatures in different databases

Database	Literatures (%)
CNKI and CJN Full-Text Database	150170 (49.8)
WanFang Database	110667 (36.7)
PubMed	17188 (5.7)
Cochrane Library	9348 (3.1)
EMBASE	7237 (2.4)
MEDLINE	4824 (1.6)
WILEY Inter Science	2110 (0.7)
Total	**301547 (100)**

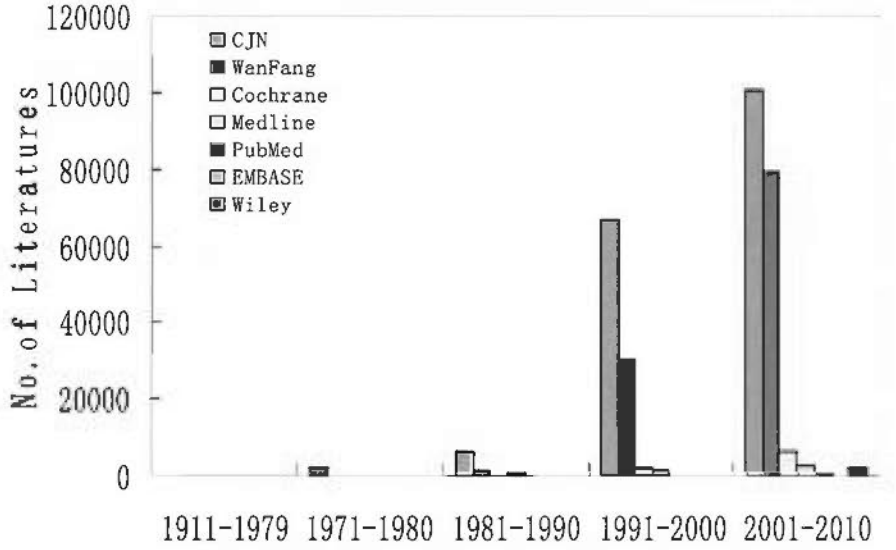


Figure 4.2 Databases published Chinese Medicine in pregnancy

Amongst all the studies, 11,158 (3.7%) literature studied Traditional Chinese Medicine for pregnancy or pregnancy related applications were included (Figure 4.3). Other clinical applications (92.2%) of non pregnancy applications were excluded.

Chinese medicines and Acupuncture were the two most commonly used therapeutic approaches of Traditional Chinese Medicine for pregnancy. 40.7% acupuncture was excluded. 48.4% of literature studied Chinese medicines on animal, chemical and basic research were excluded. 4 (0.1%) and 11 (0.3%) studies which respectively employed other medicines not included by the Chinese Pharmacopeia and included the pharmanutrients from various herbal biological agents and products were further excluded.

2,751 (84.8%) studies used herbal medicines for intervention were included. Other studies used medicines originated from animals 317 (9.8%) and minerals 160 (4.9%) were also included. In total, 3,228 literatures were included (Figure 4.3).

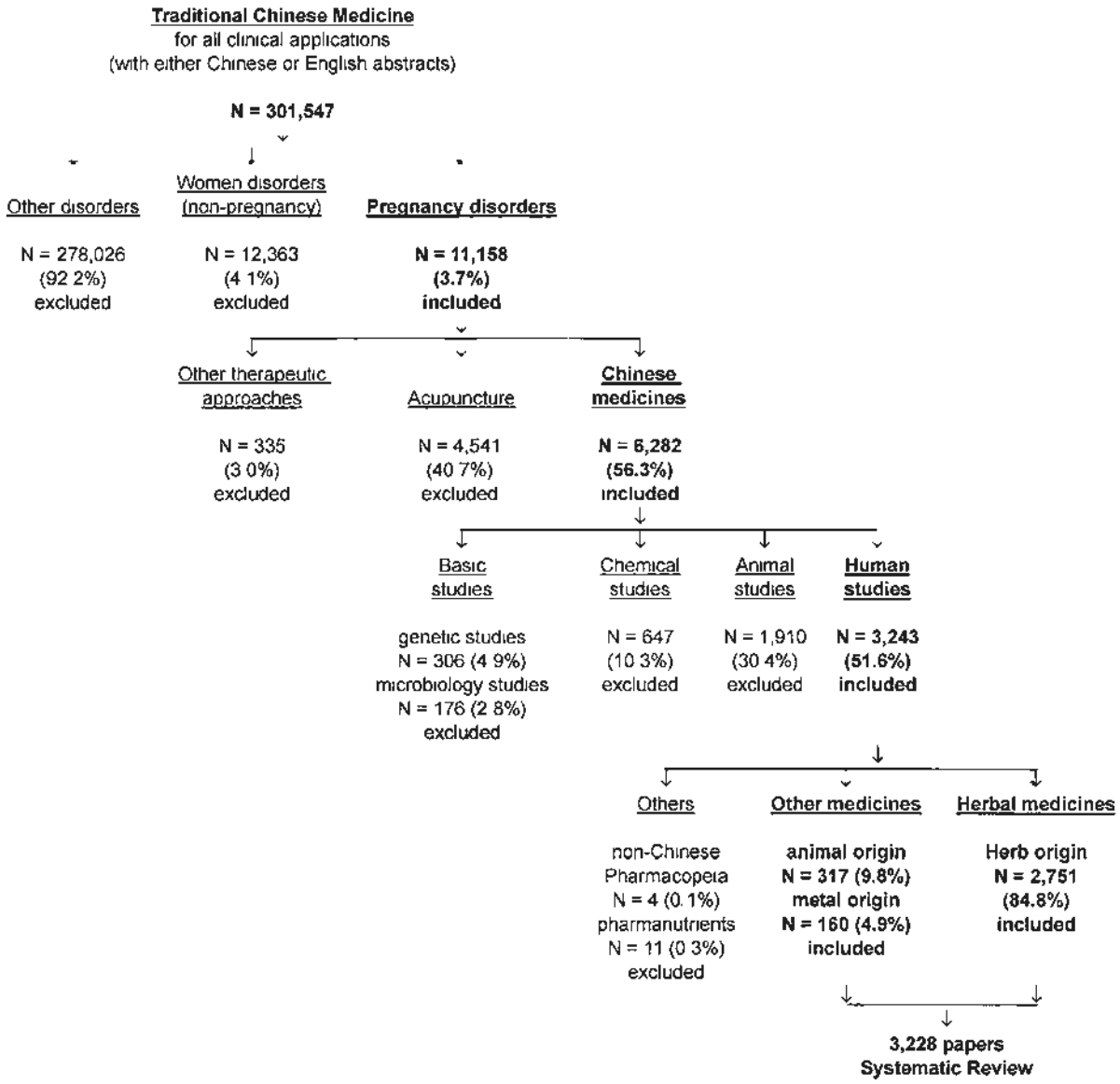


Figure 4.3 Study identification

4.4.2 Study Eligibility

Chinese medicines have been widely used for pregnant women complicated with common colds, low back pain, miscarriage, preterm labour, low fetal weight, growth restriction, placenta previa, preeclampsia, malpresentation and other obstetrics problems. Amongst all included literatures, miscarriage (43.8%) was the most common clinical application of the Chinese medicines for pregnancy (Figure 4.4, Table 4.2). Less common clinical applications included infertility, therapeutic abortion,

maternal medical problems, and other obstetric complications (Figure 4.4, Table 4.2).

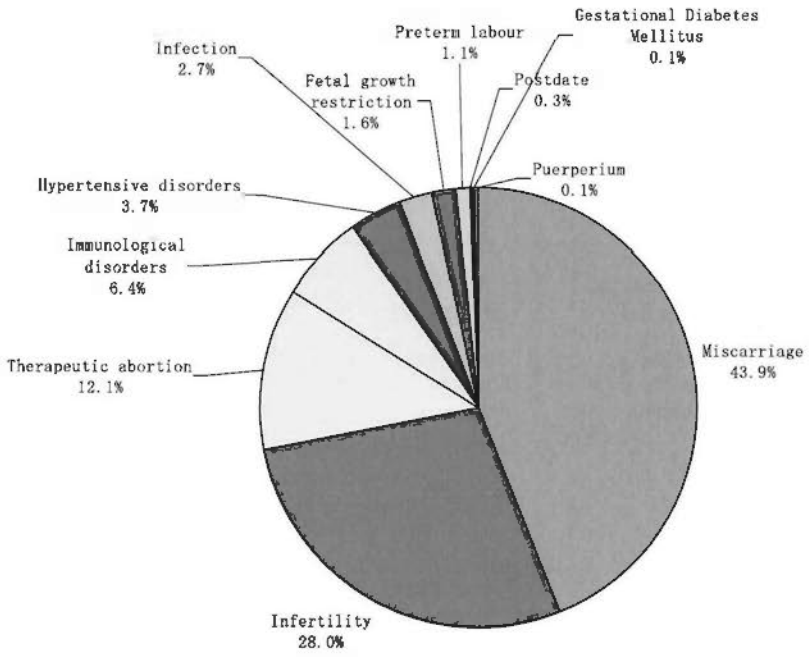


Figure 4.4 Chinese herbal medicines in pregnancy related disorders

Amongst 1,414 records of Chinese medicines for miscarriages, more studies (50.5%) employed Chinese medicines to prevent inevitable miscarriage from threatened miscarriage (28.4%) and recurrent miscarriage (22.3%), while fewer studies (49.5%) employed the Chinese medicines to enhance uterine contractions and expel products of conceptus for complete (31.9%), incomplete (12.7%), inevitable (3.0%), and missed miscarriage (1.6%) (Figure 4.5 and Table 4.3).

Whereas only the therapeutic management of threatened and recurrent miscarriage were expectant and involved promotion of maternal and fetal health and maintenance of pregnancy, but recurrent miscarriage involved a very different disease etiology and mechanism. To further study the Chinese medicines for pregnancy, in particular of its effectiveness and safety in early pregnancy, we decided to include clinical studies of Chinese medicines for threatened miscarriage as the indexed clinical application of

Chinese medicines.

Table 4.2 Applications of Chinese medicines for pregnancy

Clinical applications	Frequency (%)	Therapeutic Applications
Miscarriage	1,414 (43.8)	Prevent abortion, relieve clinical signs
		improve women's health
Infertility	899 (27.9)	elevate the female fertility
		diminish lethal effect on embryos
		decrease incomplete abortion rate
Therapeutic abortion	392 (12.1)	improve vaginal irregular bleeding
Immunological disorders	206 (6.4)	inhibit the release of inflammatory molecules
		promote vasodilatation
		increase blood flow
Hypertensive disorders	126 (3.7)	decrease platelet aggregation
Infection	87 (2.7)	decrease intrauterine transmission
		improve uteroplacental circulation
Fetal growth restriction	53 (1.6)	promote fetal growth
Preterm labour	36 (1.1)	inhibit uterine contractility
Postdate	9 (0.3)	accelerate labor process
		improve insulin levels
Gestational Diabetes Mellitus	4 (0.1)	enhance glucose metabolism
		improve hormone levels
		promote lactation and uterine contraction
Puerperium	2 (0.1)	heal perineum injuries
Total	3,228 (100)	

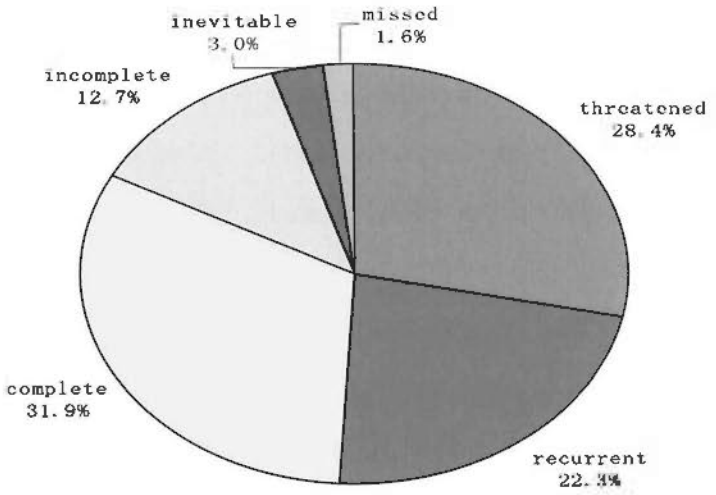


Figure 4.5 Clinical applications of Chinese medicines for miscarriages

Table 4.3 Application of Chinese medicines for miscarriages

Clinical applications	Frequency (%)	Therapeutic Applications
Miscarriage	**1,414 (100)**	
Expectant management	718 (50.5)	improve maternal health promote embryo-fetal development
threatened	402 (28.4)	
recurrent	316 (22.3)	
Active management	696 (49.5)	promote uterine contraction reduce irregular vaginal bleeding
complete	451 (31.9)	
incomplete	179 (12.7)	
inevitable	43 (3.0)	
missed	23 (1.6)	

4.4.3 Common Applications of Chinese Medicines for Pregnancy

There were 402 clinical studies of Chinese medicines for threatened miscarriages. In the 402 trials, 97 (24.1%) were case reports, 65 (16.2%) were commentary articles, and 41 (10.2%) were review articles other than systematic reviews. Since case reports only involved with very small number of participants, mostly with only one case and all less than 5 individuals, which could hardly represent the general application of Chinese medicines. Commentary articles focused on the theory and hypothesis without details and data for further study. Other review articles contributed to summary and conclusion on clinical topics, other than systematically review the clinical trials. We further excluded the case reports, commentary articles and the review articles. Another 2 papers were duplicated. Therefore, 197 clinical trials (An LY, 2000; Ban YH, 2003; Cai XF, 2005; Chen JF, 1987; Chen YF, 1999; Chen CQ, 1997; Chou HG, 2002; Cui SH, 1998; Ding QY, 1999; Fan LL, 1995; Fan SX, 2003; Gu YZ, 2002; Gu YZ, 2001; Han H, 1997; He YP, 1997; Hou RX, 1996; Huang HQ, 2001; Huang L, 2000; Huang XJ, 2003; Huang XL, 2007; Jiang CX, 1997; Jiang JN, 1997; Jiang JN, 2002; Jiang JN, 1997; Jiang DS, 1995; Jiang XY, 1991; Kang YW, 2003; Leng YH, 1991; Li J, 2002; Li GY, 1990; Li MY, 1989; Li MD, 1997; Li QW, 2001; Li XH, 1995; Liang J, 1996; Lin AM, 2003; Liu MX, 2002; Liu SX, 1989; Liu

XR, 1998; Liu XY, 1997; Long T, 1994; Ma BZ, 1998; Mao MR, 1982; Peng GY, 2007; Qi GC, 1997; Sha YQ, 1997; Sheng H, 1998; Shi RH, 1983; Song YL, 2006; Wang YP, 1992; Wang F, 2000; Wang FS, 1987; Wang HD, 2002; Wang HR, 2007; Wang RH, 1998; Wang XH, 1987; Wang Y, 1990; Wang YB, 2007; Wang MZ, 2000; Wu G, 1994; Wu ZG, 1987; Xie N, 1998; Xu L, 2002; Xu R, 2000; Xu HM, 2007; Xu RS, 1990; Xu XM, 1996; Xue YY, 2001; Yang CX, 1997; Yang JP, 1996; Ying Y, 1994; Yu BG, 1999; Yu BX, 1997; Zeng JE, 2001; Zhan HF, 1996; Zhang AX, 1998; Zhang DH, 1998; Zhang LH, 1999; Zhang LX, 1998; Zhang XS, 1999; Yu ZR, 1996; Zhang FC, 1999; Zou XD, 2005; Chen LX, 2002; Li XH, 1995; Wang FS, 1987; Yao JS, 2005; An LY, 2000; Chen JF, 1987; Chen YF, 1999; Chen CQ, 1997; Chou HG, 2002; Cui SH, 1998; Ding QY, 1999; Fan LL, 1995; Fan SX, 2003; Gu YZ, 2002; Gu YZ, 2001; Han H, 1997; He YP, 1997; Hou RX, 1996; Huang HQ, 2001; Huang L, 2000; Huang XJ, 2003; Huang XL, 2007; Jiang CX, 1997; Jiang JN, 1997; Jiang JN, 2002; Jiang JN, 1997; Jiang DS, 1995; Jiang XY, 1991; Kang YW, 2003; Leng YH, 1991; Li J, 2002; Li GY, 1990; Li MY, 1989; Li MD, 1997; Li QW, 2001; Li XH, 1995; Liang J, 1996; Lin AM, 2003; Liu MX, 2002; Liu SX, 1989; Liu XR, 1998; Liu XY, 1997; Long T, 1994; Ma BZ, 1998; Mao MR, 1982; Peng GY, 2007; Qi GC, 1997; Sha YQ, 1997; Sheng H, 1998; Shi RH, 1983; Song YL, 2006; Wang YP, 1992; Wang F, 2000; Wang FS, 1987; Wang HD, 2002; Wang HR, 2007; Wang RH, 1998) were selected for quantitative analysis in this chapter (Figure 4.6)

4.4.4 Common Formulae for Threatened Miscarriage

Traditional Chinese Medicine in clinical practice, it is very common to combine different Chinese medicines as standard formulae for treatment. Amongst all the formulae reported in the 197 literatures for threatened miscarriage, "Shou Tai Pill" (壽胎丸) was the most frequently used formula (80.2%) to prevent inevitable miscarriage and promote continuation of pregnancy (Ding et al., 1997) (Figure 4.7).

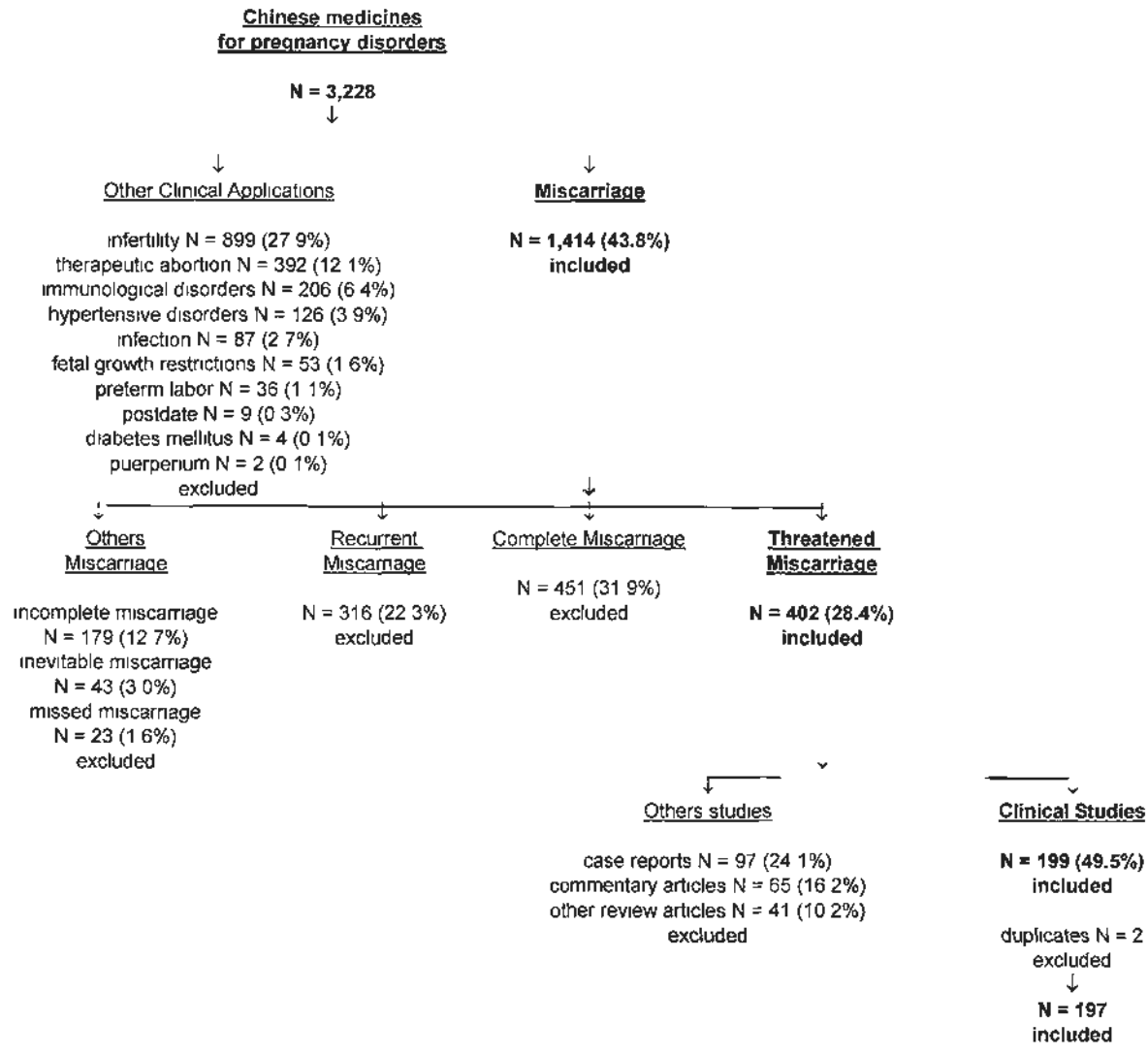


Figure 4.6 Study eligibility of Chinese medicines for threatened miscarriage

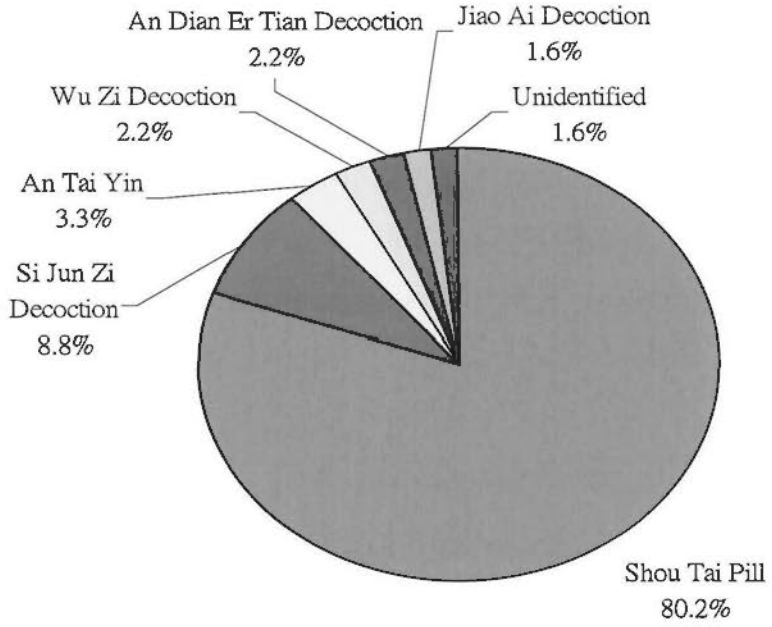


Figure 4.7 Chinese medicinal formulae prescribed in threatened miscarriage

The formula Shou Tai pill consisted of Chinese Dodder Seed (菟絲子, *Cuscuta Chinensis*), Chinese Taxillus Twig (桑寄生, *Taxillus chinensis*), Donkey-hide Glue (阿膠, *Colla corii asini*), and Himalayan Teasel Root (續斷, *Radix Dipsaci*) as four basic herbal medicines in the regime (Table 4.4). Largehead Atractylodes Rhizome (白朮, *Rhizoma Atractylodis Macrocephalae*) and Pilose Asiabell Root (党參, *Radix Codonopsis*) are commonly included into the formula Shou Tai pill to enhance the therapeutic effects by improving the "Qi" of pregnant women (Ding et al., 1997). The other medicines might be included according to the clinical presentation of patients. If the patient was diagnosed with "Kidney Yang Deficiency", Eucommia Bark (杜仲, *Euonymus Ulmoides*), Sharpleaf Galangal Fruit (益智仁, *Zingiber Nigrum*), Argy Wormwood Leaf (艾葉, *Artemisia Vulgaris*), Malaytea Scurfpea Fruit (補骨脂, *Psorales corylifolia*), and Rhizome of Scythian Lamb (狗脊, *Cibotium barometz*) were included. Shou Tai pill has also been applied to recurrent miscarriage, malpresentation, growth restraction, irregular menstruation, and dysmenorrhea.

Less frequently used formula included "Si Jun Zi Decoction" (四君子湯) (Zhang et al., 2000), "An Tai Yin" (安胎飲) (Yao GM, 2003), "Wu Zi Decoction" (五子湯) (Liu ZW, 1999), "An Dian Er Tian Decoction" (安奠二天湯) (Zhou SP, 2008), and "Jiao Ai Decoction" (膠艾湯) (Liu HX, 1998), in which the regime commonly contains Largehead Atractylodes Rhizome (白朮, *Rhizoma Atractylodis Macrocephalae*), Ginseng (人參, *Panax Giaseng*), and Baical Skullcap Root (黃芩, *Scutellaria. baicalensis*).

4.5.5 Individual Chinese Medicines for Pregnancy

There were 134 kinds of individual Chinese medicines recorded in the 197 selected clinical trials for threatened miscarriage. To identify the importance of individual Chinese medicine as the key medicine for expectant supportive treatment of threatened miscarriage, the frequency of use of individual Chinese medicine in all the regimes were analysed (Table 4.5).

Table 4.4 Standard Chinese medicinal formulae for threatened miscarriage

Name	Frequency (%) *	Main composition	Therapeutic dose & dosing[a]	Other applications[b]
Shou Tai Pill (壽胎丸)	158 (80.2%)	*Colla Corii Asini* *Herba Taxilli* *Radix Dipsaci* *Semen Cuscutae*	9g~60g 1:1:1:2 by weight QD or BID	Abdomen distension, Lower abdomen pain, Dizziness, Frequent urination, Urinary incontinence, Tinnitus, Lower-limb weakness
Si Junzi Decoction (四君子湯)	17 (8.8%)	*Giseng, Poria* *Radix Glycyrrhizae* *Rhizoma Atractylodis Macrocephalae*	3g~30g 2:2:1:2 by weight QD or BID	Chronic Gastritis, Gastric ulcer, Duodenal ulcer, Anti-tumor
An Tai Decoction (安胎飲)	6 (3.3%)	*Cortex Eucommiae* *Folium Artemisiae Argyi* *Giseng, Radix Dipsaci* *Poriacocos(schw)Wolf* *Radix Angelicae Sinensis* *Radix Astragali* *Radix Paeoniae Alba* *Radix Rehmanniae* *Praeparata, Cyperi* *Radix Scutellariae* *Rhizoma Atractylodis Macrocephalae Rhizoma*	3g~100g QD or BID	Vitiligo
Wu Zi Decoction (五子湯)	4 (2.2%)	*Caulis Perillae* *Colla Corii Asini* *Cortex Eucommiae* *Fructus Lycii, Fructus Rubi* *Fructus Schisandrae Chinensis, Herba Taxilli* *Radix Dipsaci* *Radix Scutellariae* *Semen Cuscutae*	9g~120g QD or BID	Uterine hypoplasia, Male infertility
An dian Er tian Decoction (安奠二天湯)	4 (2.2%)	*Eucommia ulmoides Oliver* *Fructus Corni, Praeparata* *Fructus Lycii, Giseng* *Radix Glycyrrhizae* *Radix Rehmanniae* *Rhizoma Atractylodis Macrocephalae* *Rhizoma Dioscoreae*	5g~150g QD or BID	Postmenopausal bleeding
Jiao Ai Decoction (膠艾湯)	3 (1.6%)	*Colla Corii Asini* *Folium Artemisiae Argyi* *P. Lactiflora Pall* *Radix Angelicae Sinensis* *Radix Glycyrrhizae* *Radix Rehmanniae Praeparata*	3g~150g QD or BID	Thrombocy to penic Purpura, Abdominal pain
Others	3 (1.6%)	*Non standard Formulae*		

* Frequency calculation = number of literatue of each formula/total amount of literatures * 100

[a] Therapeutic dose & dosing refers to the dose and dosing of the formulae per regime for threatened miscarriage

[b] Other applications refers to the applications of the formulae for other disorders during pregnancy, except threatened miscarriage

Table 4.5 134 Chinese medicines for threatened miscarriage

No.	Chinese names	English names	Biological names	Frequency (%)*	Remmended Dose# min-max (g)	Daily Mean Dose*± SD (g)
1	白术	Largehead Atractylodes Rhizome	*Rhizoma Atractylodis Macrocephalae*	59 (41%)	6-12	12.7 ± 4.40
2	菟丝子	Chinese Dodder Seed	*Semen Cuscutae*	55 (38%)	6-12	21.8 ± 8.83
3	续断 (川断)	Himalayan Teasel Root	*Radix Dipsaci*	55 (38%)	9-15	15.3 ± 5.63
4	阿胶	Donkey-hide Glue	*Colla Corii Asini*	49 (35%)	5-10	6.3 ± 2.02
5	桑寄生	Chinese Taxillus Twig	*Herba Taxilli*	48 (34%)	9-15	17.9 ± 7.81
6	甘草	Liquorice Root	*Glycyrrhizae,Radix Et Rhizoma Glycyrrhizae Praeparata Cum Melle*	48 (34%)	1.5-9	6.2 ± 12.07
7	黄芪	Mongolian Milkcetch Root	*Radix Astragali*	44 (31%)	9-30	22.9 ± 5.50
8	白芍	White Paeony Root	*Radix Paeoniae Alba*	42 (29%)	6-15	15.5 ± 2.58
9	当归	Chinese Angelica	*Radix Angelicae Sinensis Radix Et Rhizoma*	40 (28%)	6-12	10.1 ± 2.67
10	黄芩	Baical Skullcap Root	*Radix Scutellariae*	35 (24%)	9-30	10.1 ± 2.36
11	杜仲(杜仲炭,炒杜仲)	Eucommia Bark	*Cortex Eucommiae*	35 (24%)	6-9	14.7 ± 4.47
12	熟地（熟地黄）	Steamed Rehmannia Root	*Radix Rehmanniae Praeparata*	28 (20%)	9-15	21.6 ± 2.65
13	党参	Pilose Asiabell Root/ Szechwon Tangshen Root	*Radix Codonopsis*	27 (19%)	9-30	18.8 ± 3.89
14	山药（怀山药）	Common Yam Rhizome/ Wingde Yan Rhizome	*Rhizoma Diosscoreae*	23 (16%)	15-30	20.8 ± 2.67
15	砂仁	Villous Amomrum Fruit	*Fructus Amomi*	23 (16%)	3-6	6.5 ± 2.13
16	地黄（生地黄，生地)	Rehmannia Root	*Radix Rehmanniae*	22 (15%)	9-15	19.1 ± 2.54
17	川穹	Szechuan Lovage Rhizome	*Rhizoma Chuanxiong*	17 (12%)	3-9	7.4 ± 2.26

18	艾叶（艾叶炭）	Chinese Mugwort Leaf	*Folium Artemisiae Argyi*	17 (12%)	3-9	8.7 ± 2.44
19	益母草	Motherwort Herb	*Herba Leonuri*	15 (10%)	10-15	14.5 ± 1.05
20	陈皮	Tangerine Peel	*Pericarpium Citri Reticulatae*	14 (10%)	3-9	8.5 ± 2.93
21	丹参	Danshen Root	*Miltiorrhizae*	14 (10%)	9-15	11.1 ± 7.05
22	太子参	Heterophylly Falsestarwort Root	*Radix Pseudostellariae*	14 (10%)	9-30	17.6 ± 2.77
23	旱莲草	Giant St.John's Wort Herb	*Herba Ecliptae Eclipta prostrala L*	14 (10%)	15-30	16.8 ± 5.10
24	紫苏梗 (苏梗,紫苏)	Perilla Stem	*Caulis Perillae*	14 (10%)	5-9	9.9 ± 3.40
25	苎麻根	Ramie Root	*Radix Boehmeriae*	14 (10%)	5-30	18.8 ± 5.65
26	人参	Gin Seng	*Radix Ginseng*	11 (8%)	3-10 or 10-30	20 ± 10.41
27	茯苓	Indian Buead	*Poria*	10 (7%)	9-15	13.6 ± 5.84
28	升麻（炙升麻）	Largetrifoliolious Bugbane Rhizome	*Rhizoma Cimicifugae*	10 (7%)	3-9	7.1 ± 1.52
29	柴胡	Chinese Thorowax Root	*Radix Bupleuri*	10 (7%)	3-9	7 ± 1.14
30	赤芍	Red Paeony Root	*Radix Paeoniae Rubra*	9 (6%)	6-12	11 ± 2.43
31	女贞子	Glossy Privet Fruit	*Fructus Ligustri Lucidi*	8 (6%)	6-12	15.8 ± 8.78
32	仙鹤草	Hairyvein Agrimonia Herb and Bud	*Herba Agrimoniae*	8 (6%)	6-12	22.8 ± 10.24
33	竹茹（姜竹茹）	Bamboo Shavings	*Caulis Bambusae in Taenia*	8 (6%)	4.5-9	10.7 ± 4.33
34	山萸肉，山茱萸	Common Macrocarpium Fruit	*Fructus Corni*	7 (5%)	6-12	13.9 ± 2.43
35	蒲黄（生蒲黄，炒蒲黄）	Cattail Pollen	*Pollen Typhae*	7 (5%)	5-9	10 ± 3.46
36	糯米	Polished Glutinous Rice	*Semen Oryzae Glutinosae*	7 (5%)	30-60	30 ± 0.00
37	郁金	Turmeric Root Tuber	*Radix Curcumae*	7 (5%)	3-9	10 ± 3.03
38	补骨脂	Malaytea Scurfpea Fruit	*Fructus Psoraleae*	6 (4%)	6-9	14.4 ± 3.45
39	泽兰	Hiraute Shiny Bugleweed Herb	*Herba Lycopi*	6 (4%)	6-12	10 ± 4.64
40	枸杞 （杞果）	Barbary	*Fructus Lycii*	6 (4%)	6-12	15.1 ± 8.01

		Wolfberry Fruit				
41	香附	Nutgrass Galingale Rhizome	*Rhizoma Cyperi*	6 (4%)	6-9	11.2 ± 7.54
42	鸡血藤	Suberect Spatholobus **Stem**	*Caulis Spatholobi*	5 (3%)	9-15	18.3 ± 7.09
43	茜草根	India Madder Root	*Radix Et Rhizoma Rubiae*	5 (3%	6-9	13.2 ± 7.66
44	桂枝	Cassia Twig	*Ramulus Cinnamomi*	5 (3%)	3-9	6 ± 0.96
45	**鹿角（鹿角胶霜）**	Deerhorn	*Colla Cornu Cervi ,Cornu Cervi Degelatinatum*	5 (3%)	5-10	10 ± 2.89
46	酸枣仁	Spina Date Seed	*Semen Ziziphi Spinosae*	5 (3%)	9-15	13.7 ± 8.22
47	**延胡索(延胡，元胡)**	Yan Hu Suo	*Rhizoma Corydalis*	5 (3%)	3-9	11 ± 1.89
48	小茴香	Fennel Fruit	*Fructus Foeniculi*	4 (3%)	3-6	3 ± 0.88
49	巴戟（巴戟天）	Medicinal Indianmulberry Root	*Radix Morindae Officinalis*	4 (3%)	3-9	12.4 ± 2.50
50	地榆(地榆炭,炒地榆）	Garden Burnet Root	*Radix Sanguisorbae*	4 (3%)	9-15	18.5 ± 3.54
51	麦冬	Dwarf Lilyturf **Tuber**	*Radix Ophiopogonis*	4 (3%)	6-12	13 ± 1.41
52	荆芥（荆芥炭）	Fineleaf Schizonepeta Herb	*Herba Schizonepetae (Carbonisatum)*	4 (3%)	5-10	10.7 ± 3.08
53	栀子	Cape Jasmine **Fruit**	*Fructus Gardeniae*	4 (3%)	6-9	8.2 ± 2.03
54	香橼	Citron Fruit	*Fructus Citri*	4 (3%)	3-6	5 ± 0.35
55	莲房炭	Lotus Seed Pot	*Receptaculum Nelumbinis*	4 (3%)	5-10	5 ± 0.0
56	益智仁	Sharpleaf Galangal Fruit	*Fructus Alpiniae Oxyphyllae*	4 (3%)	3-9	17.4 ± 7.64
57	五灵脂	Trogopterus **Dung**	*Faeces Trogopterori*	4 (3%)	5-10	8 ± 1.15
58	川谏（木）子	Szechwan Chinaberry Fruit	*Fructus Toosendan*	3 (2%)	4.5-9	9.25 ± 2.58
59	五味子	Chinese Magnoliavine **Fruit**	*Fructus Schisandrae Chinensis*	3 (2%)	1.5-6	10 ± 1.24
60	半夏 (法半夏)	Pinellia Tuber	*Rhizoma Pinelliae, Rhizoma Pinelliae praeparatum*	3 (2%)	3-9	9.77 ± 2.19

61	白豆蔻	Round Cardamom Fruit/ Java Amomum Fruit	*Fructus Amomi Rotundus*	3 (2%)	3-6	12 ± 6.83
62	生牡蛎	Oyster Shell	*Concha Ostreae*	3 (2%)	9-30	27 ± 7.58
63	何首乌	Tuber Fleeceflower Root	*Radix Polygoni Multiflori,Radix Polygoni Multiflori Praeparata Cum Succo Glycines Sotae*	3 (2%)	6-12	16.1 ± 5.77
64	桃仁	Peach Seed	*Semen Persicae*	3 (2%)	4.5-9	6 ± 0.00
65	乌贼骨	Cuttlefish Bone	*Endoconcha Sepiae*	3 (2%)	5-9	16.7 ± 5.26
66	黄柏	Amur Corktree Bark	*Cortex Phellodendri Chinensis*	3 (2%)	3-12	11.3 ± 2.05
67	淫羊藿	Epimedium Herb	*Herba Epimedii*	3 (2%)	3-9	15.3 ± 3.43
68	藕节（炭）	Lotus Rhizome Node	*Nodus Nelumbinis Rhizomatis*	3 (2%)	9-15	11.3 ± 2.58
69	丹皮	Tree Peony Bark	*(牡丹皮) Tree Peony Bark*	3 (2%)	6-9	6.7 ± 1.43
70	三七（粉）	San Chi	*Radix Et Rhizoma Notoginseng*	3 (2%)	1-3	3.67 ± 1.77
71	肉苁蓉（淡大芸）	Desertliving Cistanche	*Herba Cistanches*	3 (2%)	6-9	12 ± 0.00
72	棕榈炭（棕榈）	Fortune Windmillpalm Petiole	*Petiolus Trachycarpi*	2 (1%)	3-9	14.8 ± 6.53
73	木香	Costustoot	*Radix Aucklandiae*	2 (1%)	1.5-6	6.86 ± 2.65
74	乌梅（炭）	Dark Plum Fruit	*Fructus Mume*	2 (1%)	6-12	10 ± 0.00
75	藿香	Wrinkled Gianthyssop Herb	*Herha Agastaches.*	2 (1%)	6-10	12.5 ± 3.44
76	红花	Safflower	*Flos Carthami*	2 (1%)	3-9	3 ± 0.00
77	牡丹	Tree Peony	*Cortex Moutan*	2 (1%)	6-12	-
78	羌活	Incised Notopterygium Rhizome/ Forbes Notopterygium Rhizome	*Rhizoma Et Radix Notopterygii*	2 (1%)	3-9	6 ± 0.00
79	知母	Common Anemarrhena Rhizome	*Rhizoma Anemarrhenae*	2 (1%)	6-12	13.3 ± 3.33
80	狗脊	East Asian Tree Fern Rhizome	*Rhizoma Cibotii*	2 (1%)	6-12	15.7 ± 1.89
81	莲子	Lotus Seed	*Semen Nelumbinis*	2 (1%)	6-15	11.3 ± 2.03

82	墨旱莲	Yerbadetajo Herb	*Herba Ecliptae*	2 (1%)	6-12	15 ± 0.00
83	生龙骨	Drgon's Bones	*Os Draconis*	2 (1%)	11-18	30 ± 0.00
84	茯神	Indian Bread with Pine/ Tuckahoe with pine	*Poriacocos(schw.)Wolf*	2 (1%)	9-15	11.7 ± 2.27
85	茵陈	Capillary Wormwood Herb	*Herba Artemisiae Scopariae*	2 (1%)	6-15	22.5 ± 6.25
86	干姜（炭）	Dried Ginger	*Rhizoma Zingiberis*	2 (1%)	3-9	3 ± 0.00
87	生山楂	Chinese Hawthorn Fruit	*Fructus Crataegi*	2 (1%)	9-12	25 ± 0.00
88	大青叶	Indigowoad Leaf	*Folium Isatidis*	1 (1%)	9-15	-
89	大枣	Chinese Date	*Fructus Jujubae*	1 (1%)	6-15	10
90	石菖蒲	Grassleaf Sweelflag Rhizome	*Rhizoma Acori Tatarinowii*	1 (1%)	3-9	9
91	龙眼肉	Dried Longan Prip	*Arillus Longan*	1 (1%)	9-15	15
92	玄参	Figwort Root	*Radix Scrophulariae*	1 (1%)	9-15	-
93	地骨皮	Chinese Wolfberry Root Bark	*Cortex Lycii*	1 (1%)	9-15	-
94	合欢皮	Silktree Albizzia Bark	*Cortex Albiziae*	1 (1%)	6-12	15
95	炒麦芽	Malt	*Fructus Gordei Germinatus*	1 (1%)	9-15	13
96	远志	Thinleaf Milkwort Root-bark	*Radix Polygalae*	1 (1%)	3-9	10
97	苍术	Swordlike Atractylodes Rhizome / Chinese Atractylodes Rhizome	*Rhizoma Atractylodis*	1 (1%)	3-9	-
98	芡实	Gordon Enryale Seed	*Semen Euryales*	1 (1%)	9-15	9
99	芦根	Reed Rhizome	*Rhizoma Phragmitis*	1 (1%)	15-30	-
100	忍冬藤	Japanese Honeysuckle Stem	*Caulis Lonicerae Japonicae*	1 (1%)	9-30	-
101	枇杷叶	Loquat Leaf	*Folium Eriobotryae*	1 (1%)	6-9	10
102	侧柏（炭）	Chinese Arborvitae Twig	*Cacumen Platycladi*	1 (1%)	6-12	15

103	金银花	Honeysuckle Flower	*Flos Lonicerae Japonicae*	2 (1%)	6-15	10
104	鱼腥草	Heartleaf Houttuynia Herb	*Herba Houttuyniae*	1 (1%)	15-25	-
105	**泽泻**	Oriental Waterplantain Rhizome	*Rhizoma Alismatis*	1 (1%)	6-9	10
106	珍珠母	Nacre	*Concha Margaritifera*	1 (1%)	10-25	30
107	**枳壳**	Bittet Orange	*Fructus Aurantii*	1 (1%)	3-9	9
108	**厚朴**	Officinal Magnolia Bark	*Cortex Magnoliae Officinalis*	1 (1%)	3-9	-
109	**钩藤**	Gambir Plant	*Ramulus Uncariae Cum Uncis*	1 (1%)	3-12	15
110	桑叶	Mulberry Leaf	*Folium Mori*	1 (1%)	5-9	18
111	桑螵蛸	Mantis Egg-case	*Ootheca Mantidis*	1 (1%)	5-9	-
112	**黄精**	Manyflower Solomonseal Rhizome / Siberian Solomonseal Rhizome / King Solomonseal Rhizome	*Rhizoma Polygonati*	1 (1%)	9-15	11
113	**贯众**	Cyrtomium Rhizome	*Rhizoma Cyrtomii*	1 (1%)	5-10	20
114	苏叶（紫苏槿叶）	Perilla Leaf	*Folium Perillae*	1 (1%)	5-9	7.5
115	**紫草**	Redroot Gromwell Root	*Radix Arnebiae*	1 (1%)	5-9	15
116	蒲公英	Mongolian Dandelion Herb	*Herba Taraxaci*	1 (1%)	9-15	-
117	椿根皮	Tree-of-heaven Ailanthus Bark	*Cortex Ailanthi*	1 (1%)	6-9	10
118	覆盆子	Palmleaf Raspberry Fruit	*Fructus Rubi*	1 (1%)	6-12	16
119	沙参（北沙参）（南沙参）	Coastal Glehnia Root (North) Ladybell Root (South)	*Radix Glehniae* *Radix Adenophorae*	1 (1%)	10-15	15
120	败酱草	Dahurian Patrinia Herb / Whiteflower Patrinia Herb	*Herba Patriniae*	1 (1%)	3-9	-
121	**茜草**	India Madder Root	*Radix Rubiac Cordifoliae*	1 (1%)	10-15	-
122	胡麻仁(火麻仁)	Hemp Fruit	*Fructus Cannabis*	1 (1%)	9-15	12

123	石莲子	Seed of Whiteflower Cacalia	*Semen Caesalpiniae Minacis*	1 (1%)	30-50	10
124	佛手	Finger Citron	*Fructus Citri Sarcodactylis*	1 (1%)	3-9	10
125	石斛	Dendrobium	*Herba Dendrobii Nobilis*	1 (1%)	6-12	10
126	紫河车	Human Placenta	*Placenta Hominis*	1 (1%)	2-3	3
127	金樱子	Cherokee Rose Fruit	*Fructus Rosae Laevigatae*	1 (1%)	6-12	15
128	柏子仁	Platycladi Seed	*Semen Platycladi*	1 (1%)	3-9	15
129	白及(白芨)	Tuber of Hyacinth Bletilla	*Rhizoma Platantherae Chloranthae*	1 (1%)	6-15	20
130	桑椹	Mulberry Fruit	*Fructus Mori*	1 (1%)	9-15	15
131	花麦肾	花麦肾	-	1 (1%)	-	15
132	薏仁	薏仁	-	1 (1%)	-	6
133	蜜丸	蜜丸	-	1 (1%)	-	-
134	生晒参	生晒参	-	1 (1%)	-	-

* Frequency time is referred to number of literatue of each formula/total amount of literatures * 100

Recommended dose is referred to the recorded dose of each Chinese medicine in "Chinese Pharmacopiea"

*Daily mean dose is referred to the dose of each Chinese medicine in all published literatures.

4.4.6 Common Individual Chinese Medicines for Threatened Miscarriage

The top 10 most commonly used single Chinese medicines included Largehead Atractylodes Rhizome, Chinese Dodder Seed, Himalayan Teasel Root, Donkey-hide Glue, Chinese Taxillus Twig, Mongolian Milkcetch Root, White Paeony Root, Chinese Angelica, Liquoric Root and Baical Skullcap Root in descending order (Figure 4.8 and Table 4.6). Largehead Atractylodes Rhizome was the most commonly prescribed single Chinese medicine for threatened miscarriage. The frequency of its clinical usage was as high as 42.4% amongst all Chinese medicines. The clinical applications and therapuetic actions of the top 10 herbs are listed as in Table 4.6.

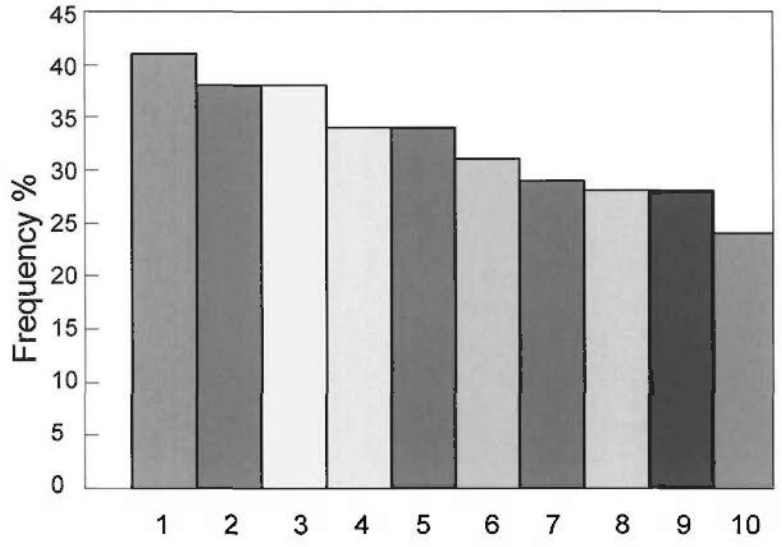


Figure 4.8 Top 10 most commonly used single Chinese medicines

1: Largehead Atractylodes Rhizome; 2: Chinese Dodder Seed; 3: Himalayan Teasel Root; 4: Donkey-hide Glue; 5: Chinese Taxillus Twig; 6: Mongolian Milkcetch Root; 7: White Paeony Root; 8: Chinese Angelica; 9: Liquoric Root: 10: Baical Skullcap Root.

4.4.7 Clinical Dose and Dosing

A large range of clinical dosage of the Chinese medicines was recorded in the reported clinical trials. The mean and median daily dose for each medicine ranged from 6 g/day to 23 g/day. In about 90.9% of Chinese medicines, 10 to 20 g/day was recommended while in 9.1% less than 5 g/day or more than 25 g/day was recommended. For example, Fructus Amomi has been prescribed as small as 2 g/day only while Rehmannia Root has been used more than 150 g/day. In 95% of selected clinical trials, the Chinese formulae were taken once a day, while 4% twice a day and 1% three times a day.

Table 4.6 Ten most commonly used single Chinese medicines for threatened miscarriage

No.	Chinese Name	English Name	Biological Name	Frequency (%)*	Mean daily dose[a]	Therapeutic actions[b]	Other Applications[c]
1	白朮	Largehead Atractylodes Rhizome	*Rhizoma Atractylodis Macrocephalae*	59 (42.4%)	12.7 g	Prevent miscarriage	—
2	菟絲子	Chinese Dodder Seed	*Semen Cuscutae*	55 (39.6%)	21.8 g	Prevent miscarriage and pre-labor	Cataract, Diarrhea, Sperm abnormality, Chronic Prostatitis.
3	續斷	Himalayan Teasel Root	*Radix Dipsaci*	55 (39.6%)	15.3 g	Stop vaginal bleeding Prevent miscarriage	Fractures and injuries, Lower back pain.
4	阿膠	Donkey-hide Glue	*Colla Corii Asini*	49 (35.3%)	6.3 g	Increase platelet count, Stop vaginal spotting	Chronic bleeding, Anemia, Tuberculosis, Uterine fibroids, Endometriosis.
5	桑寄生	Chinese Taxillus Twig	*Herba Taxilli*	48 (34.5%)	17.9 g	Prevent miscarriage Lower high blood pressure	Lower back pain, Tendons atrophy.
6	黃芪	Milkvetch Root	*Radix Astragali*	44 (31.7%)	22.9 g	—	chronic nephritis, diabetes mellitus, Diuresis.
7	白芍	White Peony Root	*Radix Paeoniae Alba*	42 (30.2%)	15.5 g	Regulate menstruation	Abdomen and limb pain, Check sweating.
8	當歸	Chinese Angelica	*Radix Angelicae Sinensis*	40 (28.2%)	10.1 g	Improve blood circulation Regulate menstruation	General pain, Bowels overactivity.
9	甘草	Liquorice Root	*Radix Et Rhizoma Glycyrrhizae*	40 (28.2%)	6.2 g	—	Detoxification, Dispel phlegm, Coughing, Spasmodic pain.
10	黃芩	Baical Skullcap Root	*Radix Scutellariae*	37 (26.6%)	10.1 g	Stop vaginal bleeding Prevent miscarriage	Detoxification.

* Frequency calculation = number of literatue of each formula/total amount of literatures * 100

[a] We calculated the mean of the reported daily dose in all included clinical trials.

[b] The reported function of Chinese herbal medicines as treatment to threatened miscarriages.

[c] Other reported functions of Chinese herbal medicines as treatment to other disorders except threatened miscarriages.

4.5 Discussion

4.5.1 Literature Search

4.5.1.1 Database

In the early years of Chinese Medicine studies, the literatures could be only obtained from Chinese databases. With the development of Chinese Medicine and its spread to foreign countries, more and more western scientists and clinical workers have interests in Chinese Medicine, various studies have been carried out and could be identified in English databases since late 70s. An increasing trend that Chinese Medicine was studied by foreign researchers in the following decades. From 2000 onwards, more English database recorded Chinese Medicine on different area and topics, covering clinical trials for various applications, animal studies for toxicity tests, laboratory research on chemical components, and commentary articles for theories of Chinese Medicine. Due to the differences in language and theory, most literatures of Chinese Medicine studies are still identified in Chinese database, however. There are some major limitations in identifying the publications from various databases. As there are some discrepancies in the translation of Chinese Medicine from Chinese to English, and lots of medical terms of Chinese Medicine are difficult to interpret, searches are always inefficient when searched by an English subject headings and keywords in Chinese database. On the other hand, 80% of the literatures were overlapped in two famous Chinese database, CJN and WanFang, and it is time consuming to double check because of limited resources and tools. One advantage in Chinese database is that over 80% of the texts can be accessed, which provides lots of convenience for detailed literature studies.

4.5.1.2 Number of publication

Large numbers of literatures have been identified during the study search. Chinese Medicine has been extensively studied. Before 1970, there were only 59 Chinese Medicine studies published in CJN database. From 1970 to 1980, more studies were carried out and published in different databases, but still mainly in Chinese databases. In 90s, Chinese Medicine studies increased 3 folds. From 1990 to 2000, with the hot topic on integrative medicines study, the publications were 12 times more than the former decade, and then doubled in this decade. The study on Chinese Medicine is blooming in these years. However, as the mainstream medicine in China, over 86% of the publications were found in Chinese Databases only. This largely limits the researchers and scientists in western countries to obtain the information and knowledge. Although some of the publications were with English abstracts but in most cases English full texts are not available. It is very difficult for foreigners to understand the Chinese Medicine.

4.5.1.3 Chinese Medicine studies

Various records of Chinese Medicine applications for different medical disorders were found, and only 3.7% were related to pregnancy. Acupuncture and Chinese medicines were two major choices of therapies in Chinese Medicine, and Chinese medicines have a higher record of publications than acupuncture, which indicated that Chinese medicines are more widely applied and accepted.

Different kinds of studies were found. It included human, animal, chemical, cellular and molecular studies. Human studies were very common, people always have high caution about pregnant women and the efficacy and safety of Chinese medicines as a treatment during pregnancy. Large numbers of animal studies were also carried out, as human studies on pregnancy are difficult and animal models are considered as a choice of pregnancy study.

Nearly 85% of the reported Chinese medicines are of herbal origins, while less than 10% and 5% are originated from animals and minerals. Over 99% of the reported Chinese medicines could be checked in "Chinese Pharmacopiea", represented Chinese Medicine researchers complied the basis theories and application of Chinese Medicine accordingly, though their formulae vary a lot from each other or even within a same treatment course.

4.5.1.4 Clinical applications for pregnancy

To pregnant women, Chinese medicines can be used as therapy for miscarriage, infertility, therapeutic abortion, immunological disorders, and other pregnancy complications were recorded, amongst which miscarriage was the most common applicaition of Chinese medicines during pregnancy. Similar number of studies on either expectant managements for threatened miscarriage or active managements for complete miscarriage were recorded for its application for miscarriage. The Chinese medicines can not be used for the other miscarriage, otherwise it would result in opposite outcomes.

4.5.2 Miscarriage

In our initial systematic review, miscarriage is found as the most commonly studied clinical application of Chinese medicines for pregnancy.

In Western medicine, miscarriage is defined as spontaneous abortion without medical or mechanical means to terminate a pregnancy before the fetus is sufficiently developed to survive (Cunningham et al., 2005). It denotes termination of pregnancy prior to completion of the 20th gestational week, or 139 days, counting from the first day of the last normal menses (DeCherney et al., 2007). The incidence of miscarriage

is commonly stated as 10%-15% of all pregnancies, and it is the most common complication during pregnancy (Petrozza et al., 2006). However, the incidence is difficult to determine precisely, since as many as 30% may go unrecognised, and these can occur very early during a pregnancy.

Miscarriage can be classified as threatened, inevitable, incomplete, missed or recurrent. Recurrent miscarriage is generally defined as spontaneous abortions repeated consecutively over three or more times (Cunningham et al., 2005). Recurrent and threatened miscarriages will become inevitable when gross rupture of fetal membranes occur along with severe vaginal bleeding and cervical dilatation; imminent fetal loss is almost certain in these cases (Cunningham et al., 2005). Incomplete miscarriage refers to the internal cervical os remaining open and allows passage of blood, and the products of conception could remain entirely in utero or partially extrude. (Cunningham et al., 2005). Missed miscarriage is used to describe dead fetus and placenta that remained for days or weeks in the uterus with a closed cervical os, and/or without any symptoms of abortion (Schorge et al., 2008).

Threatened miscarriage, the most common presentation of miscarriage, presents with vaginal bleeding or any bloody vaginal discharge during early pregnancy, whereas the bleeding is frequently slight, but it may persist for days or weeks without cervical dilatation and fetal loss (Cunningham et al., 2005). Vaginal bleeding during early gestation occurs in 20-25% pregnancies and may last for days or weeks, and nearly half of these pregnancies will result in abortion (Cunningham et al., 2005). If abortion is avoided, the mothers are likely to suffer another miscarriage (Bhattacharya et al., 2008), while the fetuses are still at high risk of preterm labor (Batzofin et al., 1984), low birthweight (Funderburk et al., 1980), and perinatal death (Weiss et al., 2004).

More than 80% of miscarriages occur in the first 12 weeks of pregnancy (Loue et al., 2004). There are many factors to make a pregnancy at a high risk of miscarriage, including genetic defects, such as chromosomal anomalies which contribute to at least half of miscarriage at early stage of pregnancies, and it could depend on paternal,

maternal and fetal factor; immunological dysfunction, such as maternal fetal incompatibility happens because of ABO and Rh blood group antigen (Bandyopadhyay et al., 2010); other maternal factors, such as parity, maternal and paternal age (Gracia CR, 2005), chronic infection, anatomic defects, endocrine deficiencies, toxin exposure, immunologic disorders, and physical or emotional trauma (DeCherney et al., 2007), laparotomy, chronic debilitating disease, nutrition, high fever, tobacco drug abuse, and alcoholism (Lyttleton et al., 2004); and environmental factors.

In Chinese Medicine, miscarriage is defined as "fetal irritability" or "fetal restlessness" (胎動不安), while recurrent miscarriage is called "stirring fetus" (滑胎). Miscarriage shares the same clinical signs and symptoms as in Western Medicine, nowadays the same laboratory examinations are used for diagnosis of miscarriage. The presentations of miscarriage are similar, mainly with abdominal pains and vaginal bleeding. But unlike mainstream Western Medicine, Chinese Medicine has an unique medical theory to understand miscarriage. To make the diagnosis and guide the treatment, "Qi" and "Blood" are the two basic elements involved. The major causes of threatened miscarriage include "Kidney Deficiency" (腎虛), "Qi Deficiency" (氣虛), "Blood Deficiency" (血虛), "Blood Heat" (血熱), "External Injury" (外傷), and "Wei Jia" (癥瘕傷胎, refers to ectopic pregnancy, which is considered as a cause of threatened miscarriage in Chinese Medicine). The diagnosis and treatment are based on different causes and varied a lot in different patients.

4.5.3 Chinese Medicines for Miscarriage

In Western Medicine, treatments for miscarriage are rather empirical. Bed rest does not alter the course and progress of miscarriage significantly (Aleman et al., 2005). Acetaminophen-based analgesia may have some effects on relieving the pains (Lede et al., 2005). Western medicines were commonly used, such as Human chorionic

gonadotropin (HCG) which maintains the luteotrophic effects after Luteinizing hormone (LH) secretion decreases, to support continued secretion of estrogen and progesterone and preventing menstruation (Devaseelan et al., 2010; Wahabi et al., 2011). Repeating the evaluation is necessary to guide subsequent management.

In Chinese Medicine, Chinese medicines are prescribed according to the "Jun, Chen, Zuo, Shi" principle (which stands for the characters as Monarch, Minister, Assistant and Guide) (Li et al., 2005). As each of the herbal medicines has their own properties and potential interactivity, the application of this principle will decrease or avoid the side effects of the other herbs, enhance the therapeutic actions of some herbs and collaborate all the herbs to create a more harmonious effect on the human body and more enhanced and direct impact on treatments.

Among all the systems, deficiency in the "Kidney" and "Liver" functions are particularly important in the pathology and mechanism of miscarriage. "Kidney" stores the essential "Qi" that warms up and activates all the other systems in the body. It is responsible for growth, development, and reproduction (Li et al., 2005). "Liver" stores the "Blood" that regulates the flow of "Qi" and maintains reproductivity (Li et al., 2005). As mentioned, the main causes of miscarriage include "Qi" deficiency, "Blood" heat, "Blood" deficiency and "Kidney" deficiency (Ma et al., 2006). Amongst all of these, "Kidney Deficiency" is the most frequent clinical type in miscarriage (Lyttleton et al., 2004); women with "Kidney Deficiency" tend to miscarry earlier in the pregnancy (Lyttleton et al., 2004). The principles of Chinese medicines treatments are to supplement the lacking element and regulate the balance of total "Qi", "Blood" and the function of each organ on mothers, and enhance the survivals of fetuses, so as to relieve clinical signs, promote pregnancy and prevent miscarriage. Besides, its application as expectant management for threatened and recurrent miscarriages, Chinese medicines are also used for missed, incompleted and complete miscarriages as active managements, such as Kansui Root (甘遂) and its formula Kansui powder (Wu et al., 1990), mainly stimulate uterine contractions and empty the uterus, then

result in loss of the fetus. From our literature study, the applications of Chinese herbal medicines as expectant and active management are nearly the same, 50.5% to prevent inevitable miscarriage, and 49.5% to induce abortion.

4.5.4 Common Formulae for Threatened Miscarriage

Shou Tai pill was the most common formula in our literature study. The main function of Shou Tai pill is to enhance the function of "Kidney" and regulate the "Qi" in the human body, then to improve the health condition of mothers and benefit the fetus. It's basic formula includes four individual Chinese medicines, Chinese Dodder Seed (菟絲子, *Cuscuta Chinensis)*, Chinese Taxillus Twig (桑寄生, *Taxillus chinensis*), Himalayan Teasel Root (續斷, *Radix Dipsaci*), and Donkey-hide Glue (阿膠, *Colla corii asini*). The former three medicines mainly improve the "Qi" in maternal body while Donkey-hide Glue mainly regulates the blood circulation of mother. Therefore, the therapeutic effects of Shou Tai pill are mainly for the pregnant women to improve body condition of mothers and the fetuses. Addition and subtraction of Largehead Atractylodes Rhizome (白朮, *Rhizoma Atractylodis Macrocephalae*) and Pilose Asiabell Root (党參, *Radix Codonopsis*) can enhance the therapeutic effects by improving the "Qi" of pregnant women (Bai MX, 2008). Since most of the individual Chinese medicines in Shou Tai pill are gentle and moderate in properties and slight sweet taste. It is now prepared in pills for convenience, Shou Tai pill was become very popular.

Other popular formulae, including "Si Jun Zi Decoction" (四君子湯), "An Tai Yin" (安胎飲), "Wu Zi Decoction" (五子湯), "An Dian Er Tian Decoction" (安奠二天湯), and "Jiao Ai Decoction" (膠艾湯), were also recorded. The therapeutic effects of Si Jun Zi Decoction in the treatment of miscarriage are to improve the functions of "Spleen" and "Stomach" and regulate "Qi" (Zhang et al., 2000). An Tai Yin improves the function of "Kidney", then regulates "Qi" and "Blood" (Yao GM, 2003). An Dian

Er Tian Decoction relieves the clinical signs such as vaginal bleeding, and is mainly applied for recurrent miscarriage (Zhou SP, 2008). Jiao Ai Decoction enhances "Kidney" and regulates "Blood" to improve the health condition of mothers and also to relieve vaginal bleeding (Liu HX, 1998). These regimes all contains Largehead Atractylodes Rhizome (白朮, *Rhizoma Atractylodis Macrocephalae*), Ginseng (人參, *Panax Giaseng*), and Baical Skullcap Root (黃芩, *Scutellaria. Baicalensis*), which are mostly used to supplement and regulate the "Qi". The combination of Largehead Atractylodes Rhizome and Baical Skullcap Root are highly recommended for their effects to benefit and survive the fetus (Ma et al., 2005).

In our literature searches, we still found that there were lots of other formulae, which were not recorded in either "Chinese Pharmacopeia" or the Chinese Medicine textbooks. They were prescribed according to the experience of physicians and clinical presentations of the patients. For example, *Radix rehmanniae* (Rehmania root), *Fructus lycii* (Lycium fruit) and *Semen cuscutae* (Cuscuta fruit) are added into the basic formula of Shou Tai pill and prescribed as a new formula, which was used to correct "Kidney Deficiency" in treatment of miscarriage (Liu MX, 2002).

4.5.5 Common Individual Chinese Medicines for Threatened Miscarriage

Chinese medicines are usually given in formulae. Characteristics of each individual herb have been recorded in "Chinese Pharmacopiea". It includes all Chinese medicines, including plants, animals, and mineral, which have pharmaceutical activities. The collection is still pending, because new Chinese medicines will be included. "Chinese Pharmacopeia", acknowledged by World Health Organization (WHO) as the official pharmacopeia for Chinese medicines, records 1,146 different Chinese medicines. It provides information on the herbs with their characteristics, identity, impurity, contents, extractum, analysis, property and channel (性味與歸經), therapeutic action, pharmacological data, dose and dosing, precautious and storage.

And in our literature search, 134 individual Chinese medicines have been applied for threatened miscarriages.

Largehead Atractylodes Rhizome is the most commonly used Chinese medicine in the treatments of threatened miscarriage. It is also considered and accepted as a moderate herbal medicine to evaluate the therapeutic effects of formulae for various pregnancy conditions. With little sweet taste, its function is mainly to improve the Spleen and Kidney, which are key organs related to most pregnancy disorders; to improve the blood circulation, which could relieve the gestational edema and efficient for the threatened miscarriage caused by Blood and Kidney Deficiency. Its moderate warm herbal nature has diuretic effect to eliminate dampness and sweating and to enhance the synergetic therapeutic effects of other herbal medicines. Its main effect for pregnant women is to improve the functions of "Spleen", "Kidney" and "Qi" to prevent inevitable miscarriage.

Besides Largehead Atractylodes Rhizome, Chinese Dodder Seed, Himalayan Teasel Root, Chinese Taxillus Twig, and Baical Skullcap Root, the less commonly used Chinese medicines for threatened miscarriage are able to prevent miscarriage and/or relief the clinical signs, while White Paeony Root and Chinese Angelica can regulate menstruation.

4.6 Summary

In this chapter, we identified over 300,000 literatures studying Chinese Medicine in large range of clinical applications. Only a small number of literatures studied Chinese medicines for pregnancy, most of which are Chinese publications. The most common clinical application of Chinese medicines for pregnancy is threatened miscarriage. The most commonly used formula is Shou Tai pill while the most frequently used individual Chinese medicines is Largehead Atractylodes Rhizome.

The average clinical dose range for individual Chinese medicines is 6g - 20g, which are usually taken once a day.

Chapter V

Efficacy of Chinese Medicines for Pregnancy: Systematic Reviews & Meta-Analysis

5.1 Introduction

In the last chapter, we overviewed the available literatures related to clinical applications of Chinese medicines for pregnancy. The most common application of Chinese medicines for pregnancy was threatened miscarriage. In current clinical practice, surgical and non-surgical interventions are used in the management of miscarriage. However, in threatened miscarriage, non-surgical interventions, which prevent inevitable consequence and pregnancy loss, are rather empirical (Tien et al., 2007). Bed rest and avoidance of sexual intercourse, though commonly advised, are without sufficient proven benefit (Aleman et al., 2005). Supportive care may reduce inevitable miscarriage (Tien et al., 2007). Dydrogesterone support was helpful to reduce the incidence of miscarriage (El-Zibdeh et al., 2009). So far, the therapeutic effects of Chinese medicines in early pregnancy are still largely unknown. In this chapter, in order to study the efficacy of Chinese medicines for pregnancy, we systematically evaluated the clinical trials of Chinese medicines for threatened miscarriage.

A systematic review was carried out in this Chapter, and the rationale (Mulrow CD, 1994) for the review included (1) large amount of information was reduced into palatable pieces for digestion, and only most important and relevant information on the effectiveness, meaningfulness, feasibilities and appropriateness of the healthcare intervention were included for further study; (2) critical exploration, evaluation, and synthesis methods were applied to separate the insignificant and included the studies worthy of reflection; and (3) meta-narrative approaches were used to overcome the problems of methodological and epistemological heterogeneity in the diverse literatures exsiting on certain subjects. Forth, integration of the critical pieces of available biomedical information is needed to meet various decision makers.

5.2 Aims and Objectives

This chapter was aimed to provide more scientific evidences of the therapeutic effects of Chinese medicines in the treatment of threatened miscarriage, in order to provide general information on the effectiveness of Chinese medicines to both Western and Chinese practitioners.

The specific objectives were:

1, To select and evaluate the clinical trials of Chinese medicines as treatment for threatened miscarriages.

2, To determine the effectiveness of Chinese medicines as treatment for threatened miscarriage.

5.3 Methods

5.3.1 Study Inclusion

5.3.1.1 Types of studies

All randomized controlled clinical trials were included in this study, regardless of publication status with or without full text, evaluating the effectiveness of Chinese medicines for the treatment of threatened miscarriage. Quasi-randomized controlled clinical trials (quasi-random method of allocating participants were used, such as by date of birth, medical record number, etc.), as well as cluster-randomized trials (participants are recruited in randomized groups, i.e. the group is randomized, not the individuals) were also studied. We identified the studies which compared Chinese medicines with other pharmaceuticals. We had not applied any language restrictions.

5.3.1.2 Types of participants

All women in the clinical trials had a viable pregnancy complicated with threatened miscarriage, regardless of its underlying causes. No treatment was given before interventions. Fetal viability was assessed by ultrasound to ensure exclusion from this study of women with inevitable, incomplete, or missed miscarriage. Vaginal bleeding after the 20th week of pregnancy was excluded. We included women regardless of whether the pregnancy was singleton or multiple, and irrespective of the maternal age and parity.

5.3.1.3 Types of interventions

All types of Chinese medicines in either standard or new formulae for the treatment of threatened miscarriage regardless of the dose or duration of administration were compared with other pharmaceuticals. The comparisons were made as follows:

- Chinese medicines versus no treatment (including bed rest).
- Chinese medicines versus placebo.
- Chinese medicines alone versus other pharmaceuticals.
- Combined Chinese medicines and other pharmaceuticals versus other pharmaceuticals.

5.3.1.4 Types of outcome measures

Primary outcomes

(1) Effectiveness of intervention: continuation of pregnancy after 28 weeks of gestation.
(2) Effectiveness of intervention: continuation of pregnancy immediate after treatment .

Secondary outcomes

Mother

During treatment

(2) No relief of clinical signs (vaginal bleeding and abdominal pain).

(3) No improvement in laboratory investigations (urinary and serum ß-HCG titer).

After treatment

(4) Repeated threatened miscarriage before 28th week of the same pregnancy (current miscarriage signs and symptoms remitted after intervention but relapsed in the same pregnancy).

(5) Preterm labour.

(6) Any other adverse pregnancy outcomes reported, including side effects, toxicity, etc

Fetus

(7) Preterm birth.

(8) Stillbirth.

(9) Neonatal death.

(10) Fetal structural malformations.

(11) Any other adverse perinatal outcomes reported, including side effects, toxicity, etc.

5.3.2 Study Exclusion

5.3.2.1 Types of studies

Clinical trials without randomization were excluded from this review, but if the randomization method was not clearly stated or was doubted, we contacted the author for confirmation.

5.3.2.2 Types of participants

In Chinese Medicine, threatened miscarriage is closely related to recurrent miscarriage, and these two complications during pregnancy are sometimes studied together. If the participants were diagnosed with both threatened miscarriage and recurrent miscarriage, the studies were excluded.

5.3.2.3 Types of interventions

As our review focused on Chinese medicines, any other therapies of Chinese Medicine would be excluded, such as acupuncture, massage, and so on. If the intervention combined Chinese medicines and other therapies, the clinical trials were excluded from our study.

5.3.2.4 Types of outcome measures

If the trials concluded that Chinese medicines were effective but no data was shown, we also excluded the studies or contacted the author for details if the quality of the study method was good.

5.3.3 Search Methods

Search methods included electronic searches in different databases through internet, handsearches, and expended searches in the reference from published studies, unpublished articles, and communications with the authors if necessary. Electronic searches followed the guidelines of Cochrane Database of Systematic Reviews for reporting electronic database search strategies and search strategies from the Cochrane Handbook 2009.

5.3.3.1 Electronic searches

We contacted the Trials Search Co-ordinator to search the Cochrane Pregnancy and Childbirth Group's Trials Register (first search in May 2010, and an update search in Dec 2010).

1. quarterly searches of the Cochrane Central Register of Controlled Trials (CENTRAL);

2. weekly searches of MEDLINE;

3. handsearches of journals and the proceedings of major conferences;

4. weekly current awareness alerts for further journals plus monthly BioMed Central email alerts.

The search strategies for CENTRAL and MEDLINE, hand-searched journals and conference proceedings, followed the 'Specialized Register' section from the Cochrane Pregnancy and Childbirth Group.

In addition, we searched the following databases: Chinese Biomedical Database (CBM) (1978 to Dec. 2010); Cumulative Index to Nursing and Allied Health Literature (CINAHL) (1982 to Dec. 2010); EMBASE (1980 to Dec. 2010); International Clinical Trials Registry Platform (2007 to Dec. 2010); Pubmed (1980 to Dec. 2010); Wiley Inter Science (1966 to Dec. 2010); Chinese Clinical Trial Registry (2007 to Dec. 2010); China Journal Net (CJN) (1915 to Dec. 2010); China National Knowledge Infrastructure (CNKI) (1915 to Dec. 2010); and Wan Fang Database (Chinese Ministry of Science & Technology) (1980 to Dec. 2010).

5.3.3.2 Searching other resources

(1) References from published studies

We searched the reference lists of relevant trials and reviews identified, and also screened bibliographies of all located articles for some unidentified articles.

(2) Unpublished literature

As some of the trials indicated in the publications that there would be on-going studies, we tried to contact the authors for more details if the studies were completed and results became available. We contacted the pharmaceutical companies for more information of the relevant medicines/products.

(3) Personal communications

If the information of individual medicines in formulae were unclear in the clinical trials, we contacted organisations, individual experts working in the field, and medicinal herbal manufacturers in order to obtain additional references.

5.3.3.3 Search strategies

Search strategy for EMBASE

1. exp PREGNANCY/
2. (spontaneous adj2 abortion*).af
3. (threat* adj3 (pregnancy ADJ loss)).af
4. (abortion* adj3 threat*).af
5. (spontaneous adj3 (pregnancy ADJ loss)).af
6. miscarriage*.af
7. exp CHINESE HERB/
8. (chin* adj6 herb*).af
9. ((china OR chinese) AND (tradition* adj4 medicine*)).af
10. 2 OR 3 OR 4 OR 5 OR 6
11. 7 OR 8 OR 9
12. 1 AND 10 AND 11
13. effect* OR efficacy
14. 12 AND 13

Search strategy for CINAHL

1. exp PREGNANCY/
2. (spontaneous adj2 abortion*).af
3. (threat* adj3 (pregnancy ADJ loss)).af
4. (abortion* adj3 threat*).af
5. (spontaneous adj3 (pregnancy ADJ loss)).af
6. miscarriage*.af
7. (chin* adj6 herb*).af
8. ((china OR chinese) AND (tradition* adj4 medicine*)).af
9. DRUGS, CHINESE HERBAL/
10. 2 OR 3 OR 4 OR 5 OR 6
11. 7 OR 8 OR 9
12. 1 AND 10 AND 11
13. effect* OR efficacy
14. 12 AND 13

Search strategy for PUBMED

1. (("Chin Med"(Journal) OR ("chinese"(All Fields) AND "medicine"(All Fields)) OR "chinese medicine"(All Fields)) OR ("medicine, traditional"(MeSH Terms) OR ("medicine"(All Fields) AND "traditional"(All Fields)) OR "traditional medicine"(All Fields) OR ("traditional"(All Fields) AND "medicine"(All Fields)))) OR ("Trends Cardiovasc Med"(Journal) OR "Case Manager"(Journal) OR "tcm"(All Fields))
2. ((("therapy"(Subheading) OR "therapy"(All Fields) OR "treatment"(All Fields) OR "therapeutics"(MeSH Terms) OR "therapeutics"(All Fields)) OR ("therapy"(Subheading) OR "therapy"(All Fields) OR "therapeutics"(MeSH Terms) OR "therapeutics"(All Fields))) OR clinical(All Fields)) OR application(All Fields)

3. 1 AND 2
4. ("abortion, spontaneous"(MeSH Terms) OR ("abortion"(All Fields) AND "spontaneous"(All Fields)) OR "spontaneous abortion"(All Fields) OR "miscarriage"(All Fields)) OR ("abortion, induced"(MeSH Terms) OR ("abortion"(All Fields) AND "induced"(All Fields)) OR "induced abortion"(All Fields) OR "abortion"(All Fields))
5. threatened/abrupt(All Fields) OR threatened/actual(All Fields) OR threatened/actually(All Fields) OR threatened/endangered(All Fields) OR threatened/exposed(All Fields) OR threatened/forced(All Fields) OR threatened/injured(All Fields) OR threatened/involved(All Fields) OR threatened'(All Fields)
6. ("therapeutics"(MeSH Terms) OR "therapeutics"(All Fields) OR "therapeutic"(All Fields)) OR ("recurrence"(MeSH Terms) OR "recurrence"(All Fields) OR "recurrent"(All Fields)) OR complete(All Fields) OR incomplete(All Fields) OR missed(All Fields) OR inevitable(All Fields)
7. 4 AND 5 BUT 6
8. 3 AND 7
9. effect(All Fields) OR efficacy(All Fields)
10. 8 AND 19

Search strategy for CNKI and CJN (Chinese)

1. (subject =miscarriage) OR (subject =abortion)
2. (subject= threatened)
3. (subject =therapeutic) OR (subject = recurrent) OR (subject = recurrent) OR (subject = complete) OR (subject= incomplete) OR (subject= inevitable) OR (subject = missed)
4. 1 AND 2 BUT 3

5. (subject= Chinese medicine*(therapy application+ clinical use)) OR (subject= traditional medicine) OR (subject=TCM)
6. 4 AND 5
7. (subject =effect) OR (subject = efficacy) OR (subject = effectiveness)
8. 6 AND 7

Search strategy for WanFang Database (Chinese)

1. TCM OR (traditional medicine) OR (Chinese medicine)
2. application OR (clinical use) OR therapy
3. 1 AND 2
4. miscarriage OR abortion
5. (threatened abortion) OR (threatened miscarriage)
6. 4 AND 5
7. 3 AND 6
8. (effect) OR (efficecy) OR (effectiveness)
9. 7 AND 8

Search Strategies for More Clinical Trials from CBM and Wiley Inter Science

Search by subject heading/keyword/abstract with:

- Traditional Chinese Medicines
- threatened miscarriage treatment
- western medicines
- comparisons studies
- randomized controlled trials
- meta-analysis
- effect
- effectiveness

Or could be included or replaced by similar words:

- herbal medicines
- pharmaceuticals
- miscarriage
- spontaneous abortion
- therapy

Medical Subject Headings (MeSH)

Miscarriage, Abortion, Threatened (*drug therapy); Traditional Chinese Medicines, Herbal medicines, (*therapeutic use); Randomized Controlled Trials (*methods/topic); Meta analysis (*method/topic)

Search Strategy for International & Chinese Clinical Trials Regsitry

Keywords of "Threatened", "Threatened miscarriage", "Threatened abortion", "abortion", "Chinese medicine", "herbal medicine" were searched in the title list of registered clinical trials in the databases.

Search Strategy for Hand-search Journals

Acta Chinese Medicine And Pharmacology (1980 to Dec 2010)

Beijing Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Central Plains Medical Jouranl (1980 to Dec 2010)

China Medical Herald (1980 to Dec 2010)

China's Naturopathy (1980 to Dec 2010)

Chinese Archives Of Traditional Chinese Medicine (1980 to Dec 2010)

Chinese Journal Of Information On Tcm (1980 to Dec 2010)

Chinese Journal Of Medicine (1980 to Dec 2010)

Chinese Journal of Obstetrics and Gynecology (1953 to Dec 2010)

Chinese Journal of Perinatal Medicine (1999 to Dec 2010)

Chinese Journal of Practical Gynecology and Obstetrics (1986 to Dec 2010)

Chinese Medicine And Materia Medica (1980 to Dec 2010)

Clinical Journal Of Anhui Traditional Chinese Medicne (1980 to Dec 2010)

Clinical Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Forum On Traditional Chinese Medicine (1980 to Dec 2010)

Gansu Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Guizhou Medical Journal (1980 to Dec 2010)

Hebei Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Heilongjiang Medicine And Pharmacy (1980 to Dec 2010)

Henan Medical Information (1980 to Dec 2010)

Henan Traditional Chinese Medicine (1980 to Dec 2010)

Hubei Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Hunan Guiding Journal Of Traditional Chinese Medicine And Pharmacology (1980 to Dec 2010)

Hunan Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Jiangsu Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Jiangxi Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Jounal Of Hubei College Of Trditional Chinese Medicine (1980 to Dec 2010)

Jouranl Of Guangzhou Uiversity Of Traditional Chinese Medicine (1980 to Dec 2010)

Journal Of Anhui Traditional Chinese Medical College (1980 to Dec 2010)

Journal Of Changzhi Medical College (1980 to Dec 2010)

Journal Of Chinese Medicinal Materials (1980 to Dec 2010)

Journal Of Chinese Rural Physician (1980 to Dec 2010)

Journal Of Guangzhou University Of Traditional Chinese Medicine (1980 to Dec 2010)

Journal Of Guiyang College Of Traditional Chinese Medicine (1980 to Dec 2010)

Journal Of Handan Medical College (1980 to Dec 2010)

Journal Of Jinzhou Medical Coilege (1980 to Dec 2010)

Journal Of Nanjing University Of Traditional Chinese Medicine (1980 to Dec 2010)

Journal Of New Chinese Medicine (1980 to Dec 2010)

Journals of Practical Obstetrics and Gynecology (1986 to Dec 2010)

Journal Of Practical Traditional Chinese Medicine (1980 to Dec 2010)

Journal Of Tianjin College Of Traditional Chinese Medicine (1980 to Dec 2010)

Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Journal Of Traditional Chinese Medicine And Chinese Materia Medica Of Jilin (1980 to Dec 2010)

Journal Of Youjiang Medical College For Nationalities (1980 to Dec 2010)

Liaoning Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Maternal And Child Health Care Of China (1980 to Dec 2010)

Modern Journal Of Integrated Traditional Chinese And Western medicine (1980 to Dec 2010)

Modern Traditional Chinese Medicine (1980 to Dec 2010)

Nei Mongol Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

New Journal of Traditional Chinese medicine (1971 to Dec 2010)

Ningxia Medical Journal (1980 to Dec 2010)

Practical Clinical Medicine (1980 to Dec 2010)

Primary Journal Of Chinese Materia Medica (1980 to Dec 2010)

Progress in Obstetrics and Gynecology (1994 to Dec 2010)

Qinghai Medical Journal (1980 to Dec 2010)

Shaanxi Journal Of Traditional Chinese Mediticne (1980 to Dec 2010)

Shanghai Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Shanxi Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Sichuan Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Sponsored By Gubei College Of Traditional Chinese Medicine (1980 to Dec 2010)

The Practical Journal Of Integrating Chinese With Modern Medicine (1980 to Dec 2010)

Tianjin Journal Of Traditional Chinese Medicine (1980 to Dec 2010)

Traditional Chinese Medicinal Research (1980 to Dec 2010)

Zhejiang Journal of Traditional Chinese medicine (1964 to Dec 2010)

5.3.4 Meta-Analysis

5.3.4.1 Study selection

To determine the clinical trials to be involved for meta-analysis, we screened the titles, abstracts, and keywords of the included trials. Two reviewers (Li Lu and one from Cochrane PCG group) assessed each trial for inclusion independently, any disagreements were discussed. If the disagreements could not be resolved, we contacted the trial authors for clarification. We did not blind the review authors to the journal of origin or institution.

5.3.4.2 Data extraction and management

We designed a form to extract data, and two reviewers (Li Lu and one from Cochrane PCG group) extracted the data using the agreed form for study eligiblity. We resolved discrepancies through discussion or consulted the third review author (supervisor). We entered data into Review Manager software (RevMan 2010), and checked for accuracy, and assessed the abstracts in the same way as full papers, then included them in the analyses. For some trials that there were doubts about the eligibility of the study, we excluded them and remarked the characters of excluded studies (for those studies or the author's replies not met our designed criteria) and awaited studies (for those studies that the authors not replied until the publication of this review). However, we attempted to contact authors of the original reports and reconsidered for inclusion if the authors provided more information or once the full publication become available to confirm our queries.

5.3.4.3 Assessment of risk of bias in included studies

Two review authors (Dou and Li) independently assessed the risk of bias for each study using the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2009). It included seven parts as follows. Any disagreements were resolved by discussion or by the third assessor.

5.3.4.3.1 Sequence generation (checking for possible selection bias)

We described for each included study the method used to generate the allocation sequence in sufficient detail to allow an assessment of whether it produced comparable groups.

We assessed the method as:

- adequate (any truly random process e.g. random number table; computer random number generator);
- inadequate (any non random process e.g. odd or even date of birth; hospital or clinic record number); or
- unclear.

5.3.4.3.2 Allocation concealment (checking for possible selection bias)

We described for each included study the method used to conceal the allocation sequence in sufficient detail and determine whether intervention allocation were foreseen in advance of, or during recruitment, or changed after assignment.

We assessed the methods as:

- adequate (e.g. telephone or central randomisation; consecutively numbered sealed opaque envelopes);
- inadequate (open random allocation; unsealed or non-opaque envelopes; alternation; date of birth);

- unclear.

5.3.4.3.3 Blinding (checking for possible performance bias)

We described for each included study the methods used to blind study participants and personnel from knowledge of which intervention a participant received. We judged studies at low risk of bias if they were blinded, or if we judged that the lack of blinding not affected the results. We assessed blinding separately for different outcomes or classes of outcomes.

We assessed the methods as:

- adequate, inadequate or unclear for participants;
- adequate, inadequate or unclear for personnel;
- adequate, inadequate or unclear for outcome assessors.

5.3.4.3.4 Incomplete outcome data (checking for possible attrition bias through withdrawals, dropouts, protocol deviations)

We described for each included study, and for each outcome or class of outcomes, the completeness of data including attrition and exclusions from the analysis. We stated whether attrition and exclusions were reported, the numbers included in the analysis at each stage (compared with the total randomised participants), reasons for attrition or exclusion where reported, and whether missing data were balanced across groups or were related to outcomes. Where sufficient information was reported, or can be supplied by the trial authors, we re-included missing data in the analyses which we undertook.

We assessed methods as:

- adequate;
- inadequate;
- unclear.

If data for more than 20% of participants were missing, we excluded the outcome or study from the analysis.

5.3.4.3.5 Selective reporting bias

We described for each included study how we investigated the possibility of selective outcome reporting bias and what we found.
We assessed the methods as:

- adequate (where it was clear that all of the study's pre-specified outcomes and all expected outcomes of interest to the review had been reported);
- inadequate (where not all the study's pre-specified outcomes had been reported; one or more reported primary outcomes were not pre-specified; outcomes of interest were reported incompletely and so cannot be used; study failed to include results of a key outcome that would have been expected to have been reported);
- unclear.

5.3.4.3.6 Other sources of bias (compliance and baseline similarity)

We described for each included study any important concerns we had about other possible sources of bias. For example, if the trial stopped early, or if there was a baseline imbalance (e.g. severe blood loss before intervention) or differential diagnosis (e.g. pattern/syndrome differentiation for individualised treatment) between the comparing groups.

We assessed whether each study was free of other problems that could put it at risk of bias:

Compliance

- good: >95% participants received the treatment exactly following the physicians' instructions;
- fair: 95-90%;
- poor: 90-80%;
- failed: <80%.

Baseline Similarity

- yes: All participants were inpatients or outpatients, with the symptoms, signs, examinations or diagnosis related to threatened miscarriage and suitable for each study, then be randomly selected for different study groups;
- no;
- unclear.

5.3.4.3.7 Overall risk of bias

We made explicit judgements about whether studies were at high risk of bias, according to the criteria given in the Handbook (Higgins et al., 2009). With reference the above, we assessed the likely magnitude and direction of the bias and whether we considered it is likely to impact on the findings. We explored the impact of the level of bias through undertaking sensitivity analyses (see below).

5.3.4.4 Measures of treatment effect

Statistical analysis was performed by using RevMan 5. We presented results as summary risk ratio with 95% confidence intervals for dichotomous data.

5.3.4.5 Unit of analysis issues

Trials with up to three arms (Chinese medicines alone, Western medicines alone, combined Chinese and Western medicines) were analysed. We input the data separately for meta-analysis by RevMan5. Review Manager (RevMan) is a software set up by Cochrane Collaboration to prepare and maintain protocol and full reviews, including text, characteristics of studies, comparison tables, and study data. It can perform meta - analysis and graphically present the results after data input. Version 5.0 (RevMan 5) is the latest version provided by Cochrane Collabration.

5.3.4.6 Dealing with missing data

For included studies, we noted levels of attrition, and explored the impact of included studies with high levels of missing data for the overall assessment of treatment effect by using sensitivity analysis. For all outcomes we carried out analyses, on an intention-to-treat basis; we attempted to include all participants randomized to each group in the analyses. The denominator for each outcome in each trial was the total number of participants randomized minus any participants whose outcomes were known to be missing.

5.3.4.7 Assessment of heterogeneity

We assessed statistical heterogeneity in each meta-analysis using the T^2, I^2 and χ^2 statistics. We regarded heterogeneity as substantial if T^2 was greater than zero and

either I^2 was greater than 30% or there was a low P-value (< 0.01) in the χ^2 test for heterogeneity.

5.3.4.8 Data synthesis

We carried out statistical analysis using RevMan 5, and used fixed-effect inverse variance meta-analysis for combining data when the studies were estimating the same underlying treatment effect and the populations and methods of the trials were judged sufficiently similar. For clinical heterogeneity sufficient to expect that the underlying treatment effects differ between trials, or if substantial statistical heterogeneity was detected, we used random-effects meta-analysis to produce an overall summary if an average treatment effect across trials was considered clinically meaningful. We treated the random-effects summary as the average range of possible treatment effects and we discussed the clinical implications of treatment effects differing between trials. For the average treatment effect not clinically meaningful, we did not combine trials. For random-effects analyses, we presented the results as the average treatment effect with its 95% confidence interval, and the estimates of T^2 and I^2.

5.3.4.9 Subgroup analysis and investigation of heterogeneity

We carried out the following subgroup analyses:

1. maternal age below 35 versus 35 and above;
2. primipara versus multipara;
3. threatened miscarriage in first trimester versus second trimester;
4. referred herbal medicines versus non-referred herbal medicines, according to the formulary stated in Pharmacopeia;
5. short-term treatment (one course only) versus long-term treatment (more than one course);

6. quasi-randomised clinical trials versus randomised clinical trials.

We used the following outcome in subgroup analysis:

- continuation of pregnancy after 28 weeks of gestation.

For fixed-effect meta-analyses we conducted planned subgroup analyses classifying whole trials by interaction tests as described (Deeks et al., 2001). For random-effects meta-analyses we assessed differences between subgroups by inspection of the subgroups' confidence intervals; non-overlapping confidence intervals indicated a statistically significant difference in treatment effect between the subgroups.

5.3.4.10 Sensitivity analysis

We carried out sensitivity analysis to explore the effect of trial quality for important outcomes in the review. Sensitivity analyses on results were performed on look at the possible contribution of:

(1) high risk of bias in the allocation of participants to groups associated with a particular study (Schulz et al., 1995); or

(2) high levels of missing data (Higgins et al., 2009).

However, no trials had any high risk of bias in the allocation of participants to groups or significanty of missing data were identified, so the sensitivity analysis was not carried out. If a sufficient number of trials are found in the future update, we will further specify sensitivity analyses.

5.4 Results

5.4.1 Efficacy in General

Before randomized controlled trials for meta-analysis, all clinical trials were studied to evaluate the efficacy in general. From the 197 clinical studies of Chinese medicines for threatened miscarriages, the efficacy rate of interventions from 131 records ranged from 75% to 100%. Among all the records, over 97.7% of the studies exceeded the 80% effectiveness rate, 84.6% exceeded 90%, while 24.3% exceeded 95%, and another 2 studies reported 100% effectiveness. Two herbal medicines, Bark of Chinese Corktree (2.16% frequency and 11.3 g/day mean daily dose) (Long T, 1994) and Trogopterus Dung (2.88% and 8 g/day) (Zhou J, 1993), were reported with 100% efficacy. For example, in Song's study (Song YL, 2006), "Shou Tai Pill" was applied and the efficacy rate was 93.3%.

5.4.2 Formula and Individual Chinese Medicines

In the 131 records, we found that Shou Tai Pill was the most commonly used formula for threatened miscarriage, with an average effiacy rate of 91.3% (Figure 5.1). The other top 10 Chinese medicines, Chinese Dodder Seed, Himalayan Teasel Root, Donkey-hide Glue, Chinese Taxillus Twig, Mongolian Milkcetch Root, White Paeony Root, Chinese Angelica, Liquoric Root and Baical Skullcap Root, were also with satisfied efficacy rates in the treatment to threatened miscarriage, 92.8%, 92.1%, 92.1%, 92.2%, 92.4%, 93.2%, 92.7%, 92.0%, 92.6%, respectively. However, no significant correlation was found between usage frequency and efficacy ($r = 0.1086$, $p = 0.335$).

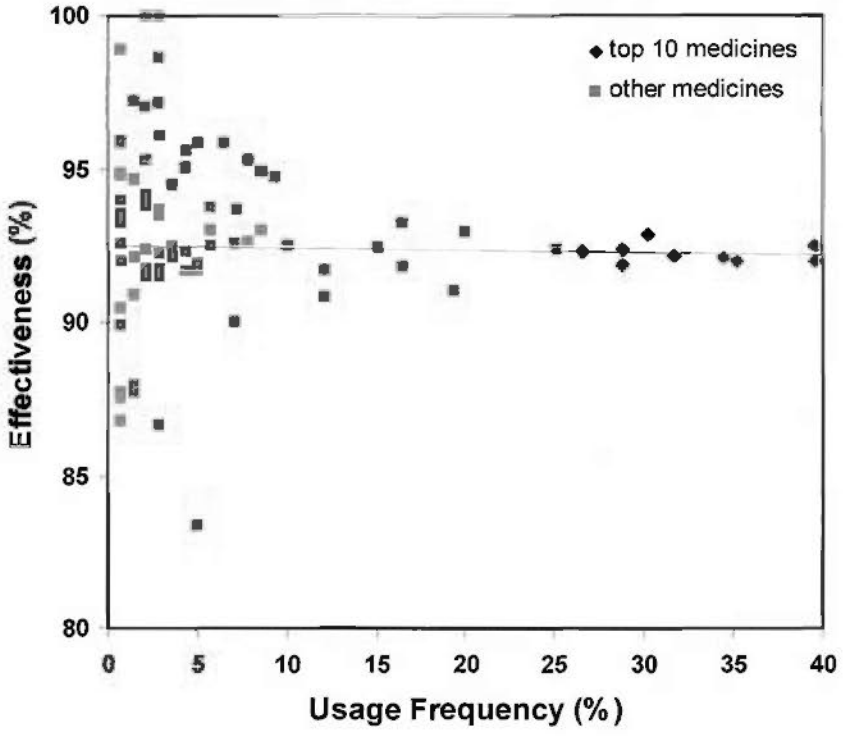


Figure 5.1 Efficacy of individual Chinese medicines

5.4.3 Dosage and Efficacy

A large range of clinical doses for Chinese medicines for threatened miscarriage was recorded in the clinical studies. To evaluate the effectiveness of Chinese medicines in the treatment of threatened miscarriage on its dosage, we recorded the daily dose of each individual herb and correlated with the effective rate of the intervention. Distribution of the dose was normally distributed (Figure 5.2). About 90.9% of Chinese medicines the studied dose was between 10 to 20 g/day while 9.1% less than 5 g/day or more than 25 g/day was recorded in the studies. For example, Fruit of Villous Amomum was prescribed as small as 2 g/day (Xu L, 2002), while Prepared Rhizome of Adhesive Rehmannia was used more than 150 g/day (Wang Y, 1990).

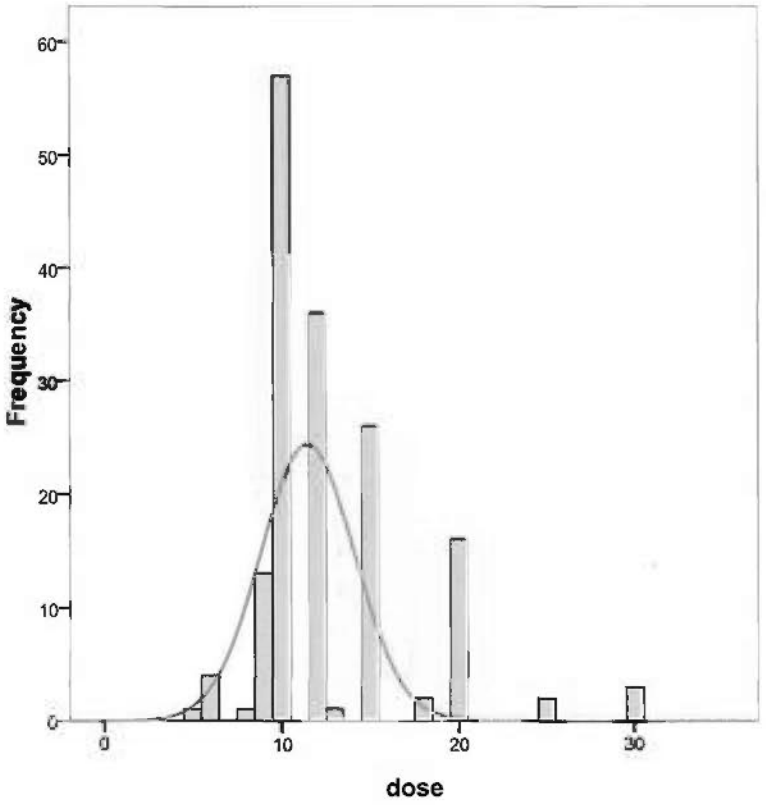


Figure 5.2 Distrbution of Largehead Atractylodes Rhizome dose

Number of Records: totally 197 clinical studies are involved, some of which has no daily dose records of Largehead Atractylodes Rhizome, some of which had two or three records, as the authors listed the changes of formulae and the clinical doses.

Chinese medicines with 95% or higher efficacy rate were less frequently used, 0.72% - 7.91%, but Chinese medicines with 90% or lower effective rate were not less frequently used. The herbs with 20 g or higher mean daily dose, the efficacy ranged from 83.33% – 95.83%. However, there was no significant correlation between mean daily dose and efficacy ($r = 0.2324$, $p = 0.513$) (Figure 5.3).

5.4.4 Dosing and Efficacy

There were also different dosing records in different clinical studies when Chinese medicines were applied by different authors. There was no significant difference

between effective rate and the daily dosing times (Figure 5.4; ANOVE, $p>0.05$). In 95% of selected clinical trials, the soups were taken once a day, while in 4% twice a day and in 1% three times a day.

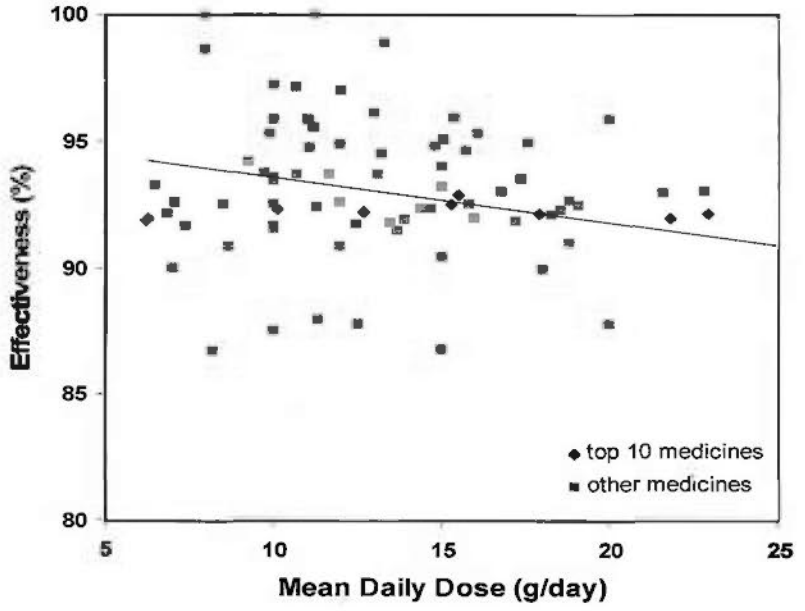


Figure 5.3 Dosage and efficacy

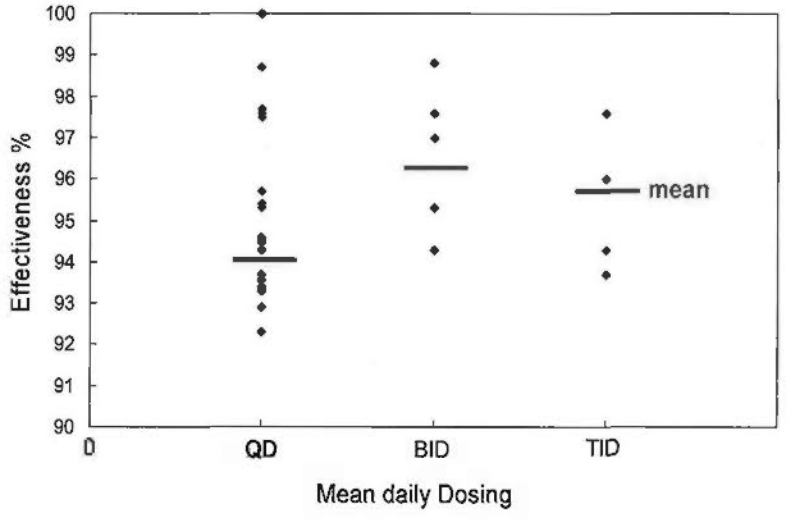


Figure 5.4 Dosing and efficacy

QD: once per day; BID: twice per day; TID: three times per day.

5.4.4 Meta-Analysis

5.4.4.1 Selection of trials

Amongst 197 selected clinical studies of Chinese herbal medicines for threatened miscarriage, we excluded 88 studies that only studied Chinese herbal medicines without comparing any intervention; 64 studies in shich the participants in the control group had normal pregnanices; 2 studies that included habitual abortion (recurrent miscarriage), 1 trial that did not report clinical outcomes of the intervention, and 1 trial that included participants at very late gestation (28-37 weeks). Therefore, 35 suitable randomized clinical studies were included for meta-analysis. (Figure 5.5)

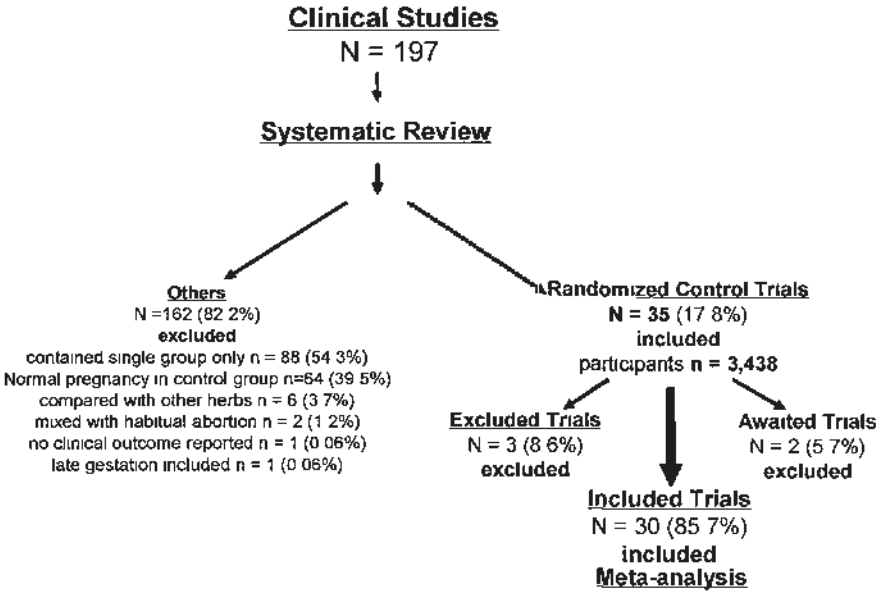


Figure 5.5 Selection of randomized clinical studies

5.4.4.2.1 Interventions

Comparisons with placebo and no treatment were not found in the 35 selected trials, but only with other interventions. All studies compared Chinese medicines with pharmaceuticals, mostly Western medicines. So three comparisons were included:

- Chinese medicines alone versus Western medicines alone,
- Chinese medicines alone versus combined Chinese and Western medicines,
- combined Chinese and Western medicines versus Western medicines alone.

Western medicines included tocolytic drugs (e.g. salbutamol and magnesium sulfate), hormonal supplementations (e.g human chorionic gonadotrophin and progesterone), immunotherapy (e.g IgG immunization and anti-phospholipid antibodies) and supportive supplements (e.g. vitamin E and folic acid).

5.4.4.2.2 Characteristics of studies

35 randomized clinical trials were included for this review, but only 30 of these trials were included for meta-analysis (Figure 5.5). The quality assessment and characteristics of each included study was summarized in Table 5.1 and Table 5.2, respectively. 3 trials studied different Western medicines in combined medicines group and Western medicines alone group or combined Kampoo medicines with Western medicines (Table 5.3); another 2 did not confirmed by the author were further excluded (Table 5.4).

Table 5.1 Quality assessments of included studies

No	Studies	Randomization[a]	Blinding[b]	Follow-up[c]	Follow-up duration	Compliance[d]	Baseline Similarity[e]	Overall quality[f]
1	Chen 2002	Unclear	Open	Adequate	Good	Adequate	Yes	Good
2	Chen 2003	Adequate	Open	Adequate	Good	Adequate	Unclear	Good
3	Cai 2002	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
4	Deng 2009	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
5	Feng 1997	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
6	Fu 2006	Unclear	Open	Adequate	Good	Adequate	Yes	Good
7	Kuang 2007	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
8	L 2004	Adequate	Open	Adequate	Good	Adequate	Unclear	Good
9	Li 2005	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
10	Li 2006	Adequate	Open	Adequate	Good	Adequate	Unclear	Good
11	Li 2009a	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
12	Li 2009b	Unclear	Open	Adequate	Good	Adequate	Yes	Good
13	Liu 2008	Adequate	Open	Adequate	Good	Adequate	Yes	Good
14	Liu 2009	Unclear	Adequate	Adequate	Good	Adequate	Unclear	Good
15	Lv 2007	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
16	She 2008	Unclear	Open	Adequate	Good	Adequate	Yes	Good
17	Song 2005	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
18	Song 2007	Adequate	Open	Adequate	Good	Adequate	Yes	Good
19	Sun 2003	Unclear	Open	Adequate	Good	Adequate	Yes	Good
20	Wang 2005	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
21	Wang 2007	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
22	Xiao 2008	Adequate	Open	Adequate	Good	Adequate	Unclear	Good
23	Xu 2005	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
24	Xun 2008	Unclear	Open	Adequate	Good	Adequate	Yes	Good
25	Yang 2001	Unclear	Open	Adequate	Good	Adequate	Yes	Good
26	Yang 2006	Adequate	Open	Adequate	Good	Adequate	Unclear	Good
27	Zhang 2007	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
28	Zhang 2008a	Adequate	Open	Adequate	Good	Adequate	Unclear	Good
29	Zhang 2008b	Unclear	Open	Adequate	Good	Adequate	Unclear	Good
30	Zhong 2002	Unclear	Open	Adequate	Good	Adequate	Unclear	Good

[a]adequate clearly by computer, envelope or telephone, uncertain reported randomization but without any approach and methods inadequate no randomization

[b]double-blinded single blinded open or unclear with blinding of participants, caregivers and administrating treatment and outcome assessors

[c]adequate <5% loss fair 5-10% loss, poor 10-20% loss and excluded from this review, inadequate >20% loss and exclude from this review, unclear not reported

[d]good >95% participants received the treatment exactly following the physicians instructions, fair 95-90%, poor 90-80% failed <80%

[e]good no significant difference (p>0.05) between the participants in intervention groups and control group, unclear not reported

f Newcastle Ottowa Scale

NA not applicable

Table 5.2 Characteristics of included studies

Chen et al., 2002	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone
Participants	84 inpatients or outpatients from People's Hospital of Ning Xia were recruited. Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines 1) The Chinese medicine formula was Shou Tai Pill, including Chinese Dodder Seed 30g, Himalayan Teasel Root, Chinese Taxillus Twig 20g, and Donkey-hide Glue 15g 2) Formula changes Qi deficiency: Pilose Asiabell Root, Mongolian Milkcetch Root 15g each, and Liquorice Root 6g were added Blood Deficiency: Chinese Angelica, and White Paeony Root 10g were added Blood Heat: Baical Skullcap Root 10g was added Yin Deficiency: Rehmannia Root 15g, Glossy privet fruit 10g, and Yerbadetajo Herb 10 were added Severe Vomiting: Tangerine Peel 10g, Villous Amomrum Fruit 10g, and Perilla Stem 10g were added Spleen Deficiency: Largehead Atractylodes Rhizome 10g was added Severe Bleeding: Hairyvein Agrimonia 15g, Chinese Arborvitae Twig 12g, Dragon Bone 30g, and Fortune Windmillpalm 10g were added 3) Decoction: po. BID till 12 weeks or 5 months of pregnancy 4) Western medicines were received at the same time, including HCG 2000U, im, qd, Progesterone 20~40mg, im. bid, Vitamin E 100mg, po, qd, Folic Acid 5mg, po, tid, Salbutamol Sulfate 2 pills, po, tid, 25 % Magnesium Sulfate 60ml ivgtt Control group was treated with Western medicines alone
Outcomes	Symptoms subsided and pregnancy maintained till delivery were considered as effective. The effectiveness rate of combined medicines group was 96.07%, and Western medicines group was 69.69% ($p<0.05$)
Notes	2 arms RCT
Allocation concealment	Unclear: no information was available from the author

Chen et al., 2003	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone
Participants	83 inpatients from Affiliated Hospital of Heng Dong Nurse School were recruited

	(1999-2002). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Tai Shan Pan Shi Yin, including Pilose Asiabell Root 12g, Mongolian Milkcetch Root 10g, Largehead Atractylodes Rhizome 10g, Liquorice Root 5g, Chinese Angelica 12g, Szechuan Lovage Rhizome 3g, White Paeony Root 9g, Steamed Rehmannia Root 12g, Himalayan Teasel Root 10g, Baical Skullcap Root 8g, and Villous Amomrum Fruit 3g. 2) Formula changes Heat excess: Villous Amomrum Fruit was removed and Baical Skullcap Root was increased to 16g. 3) Decoction: po, BID. 4) Western medicines were received at the same time, including HCG 1000U, im, qd till bleeding stopped; Vitamin E 20mg, po, tid. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 93.33%, and Western medicines group was 76.32% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, randomized into two groups by visiting sequence.

Cui et al., 2002	
Methods	Randomized controlled trial of Chinese herbal medicines compared with Western medicines.
Participants	70 inpatients or outpatients from Affiliated Hospital of Shan Xi Chinese Medicine College were recruited (1995 Mar-2000 Dec). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Chinese herbal medicine group. 1) Tai Er An decoction: mainly used Chinese Taxillus Twig, Chinese Dodder Seed, Himalayan Teasel Root, 15g each; Cattail Pollen, Trogopterus Dung 9g each; Danshen Root, Villous Amomrum Fruit, Perilla Stem, Donkey-hide Glue, White Paeony Root 10g each; and Liquorice Root 6g. 2) Decoction: po, TID. The Western medicines group used progesterone 20mg, im, qd; 3 days as a course, usually 5 courses.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of Chinese medicines group was 98%, and Western medicines group was 65% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Deng et al., 2009	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	200 participants at the Department of Obstetrcis & Gynaecology, Chinese Medicine Hospital of Foshan (Guangzhou, China) were recruited. Participants were all diagnosed as threatened miscarriage by HCG and ultrasound examination, and suffered with vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula: included Donkey-hide Glue, Pilose Asiabell Root, White Paeony Root, Himalayan Teasel Root 15g each; Prepared Rhizome of Adhesive Rehmannia, Chinese Taxillus Twig, 20g each; Chinese Dodder Seed 30g; and Chinese Mugwort leaf and Liquorice Root 10g each. 2) Formula changes Significant low back pain: Eucommia Bark 15g was added, Chinese Taxillus Twig was increased to 30g. Hard Stool: Desertliving Cistanche 10g was added. Combined with habitual abortion: Lotus Seed, and Ramie Root 10g were added. 3) Decoction: po, QD 4) Western medicines were received at the same time, including HCG 1000U, im, qd for 10 days then decline the dosage; Vitamin E 100mg, po, tid; Folic Acid 0.4mg, QD. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided and pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 95% while Western medicines group was 72% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was provided, can only quoted "the patients were randomized divided into 2 groups"

Feng et al., 1997	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	108 outpatients from Second Affiliated Hospital of Hunan University of Chinese Medicine were recruited (1995 Jan- 1996 Nov). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Shou Tai Pill, included Chinese Dodder Seed 30g, Himalayan Teasel Root 20g, Chinese Taxillus Twig 20g, Donkey-hide Glue 10g; Pilose Asiabell Root, White Paeony Root, Himalayan Teasel Root 15g each; Prepared Rhizome of Adhesive Rehmannia, Chinese Taxillus Twig 20g each; Chinese Dodder Seed 30g; and

	Chinese mugwort leaf and Liquorice Root 10g each. 2) Formula changes Qi Deficiency: Pilose Asiabell Root 15g, Mongolian Milkcetch Root 15g, and Liquorice Root 6g were added. Blood Deficiency: Chinese Angelica 10g and White Paeony Root 10g were added. Blood Heat: Baical Skullcap Root 10g was added. Yin Deficiency: Rehmannia Root 15g, Glossy Privet Fruit 10g, and Yerbadetajo Herb 10g were added. Increased Vomiting: Tangerine Peel 10g, Villous Amomrum Fruit 10g, and Perilla Stem 10g were added. Spleen Deficiency: Largehead Atractylodes Rhizome 10g was added. Increased Bleeding: Herb of Hairyvein Agrimonia 15g, Chinese Arborvitae Twig 10g, Dragon Bone 30g were added. 3) Decoction: po, QOD 4) Western medicines were received at the same time, including Vitamin E, Folic Acid, Progesterone (regular dosage but not listed in the paper). Control group was treated with Western medicines alone.
Outcomes	Symptoms subsided and pregnancy maintained till delivery were considered as effective. The effectiveness rate of combined medicines group was 95.08%, and Western medicines group was 70.21% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Fu et al., 2006	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	87 inpatients from First Affiliated Hospital of Zhong Shan University were recruited (2004 May-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Si Wu soup, included Steamed Rehmannia Root 12g, Chinese Angelica 9g, White Paeony Root 9g, Szechuan Lovage Rhizome 6g, Donkey-hide Glue, Pilose Asiabell Root, White Paeony Root, Himalayan Teasel Root 15g each, Prepared Rhizome of Adhesive Rehmannia, Chinese Taxillus Twig 20g each, Chinese Dodder Seed 30g, and Chinese Mugwort Leaf and Liquorice Root 10g each. 2) Formula changes Kidney Deficiency: Chinese Dodder Seed 15g, Chinese Taxillus Twig 15g, Himalayan

	Teasel Root 15g, and Donkey-hide Glue 10g were added, Szechuan Lovage Rhizome was decreased (no information on the dosage)
	Qi Deficiency Pilose Asiabell Root 30g, Mongolian Milkcetch Root 15g, Donkey-hide Glue 10g, and Villous Amomrum Fruit 6g were added
	Blood Deficiency: Chinese Angelica was increased to 18g, Szechuan Lovage Rhizome was removed, Donkey-hide Glue 15g, Himalayan Teasel Root 15g, and Chinese Taxillus Twig 15g were added.
	Blood Heat: Szechuan Lovage Rhizome removed, Rehmannia Root 15g replaced Steamed Rehmannia Root, Dwarf Lilyturf Tuber, and Cochinchinese Asparagus Root 15g, Donkey-hide Glue 10g, and Ramie Root 20g were added
	3) Decoction. po, QD
	4) Western medicines were received at the same time, including Progesterone 10mg, im, qd, HCG 2000U, im, qd, Allylestrenol 5mg, Ritodrine 10mg, po, tid, Vitamin C 100mg, po, qd, Folic Acid 0.4mg, tid.
	Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 97.87%, and Western medicines group was 87.50% (p<0 05)
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Kuang et al , 2007

Methods	Randomized controlled trial of comparisons among combined medicines, Chinese herbal medicines alone, and Western medicines alone
Participants	180 inpatients or outpatients from Second Affiliated Hospital of Hunan University of Chinese Medicine were recruited (2004 May-2006 May) Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains
Interventions	Chinese herbal medicine group
	1) mainly used Pilose Asiabell Root 10g, Mongolian Milkcetch Root, White Paeony Root, Indian Buead and Tuber Fleeceflower Root 10g each, Prepared rhizome of Adhesive Rehmannia, Chinese Dodder Seed, Himalayan Teasel Root and Chinese Taxillus Twig 15g each, and Liquorice Root 5g.
	2) Formula changes:
	Vaginal bleeding. Cuttlefish bone 30g, Dark Plum Fruit 15g and Tuber of Hyacinth Bletilla 10g were added, , and prepared Himalayan Teasel Root before add into the formula
	Dry mouth and Hard Stool White Paeony Root was increased to 30g, Liquorice Root was increased to 10g, and Chinese Thorowax Root, Baical Skullcap Root, Dwarf Lilyturf Tuber and Root of Lobed Kudzuvine 10g each were added
	3) 7-day as a course, and usually took 1-3 courses (Details of administration were not

	available) The Western medicines group used Vitamin E capsules 0.1g, po, BID, HCG 1000U, im, QD, then alternative injection after vaginal bleeding stopped. All 3 groups had standard care for pregnancy (prohibit sexual activity, psychotherapy, and regulate ultrasound tests).
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of Chinese medicines group was 68.3%, Western medicines group was 63.3%, and combined medicine group was 91.7%. Significant difference was found between combined medicine and Chinese medicines or Western medicines alone ($p<0.05$). No statistic difference was found between Chinese herbal medicines and Western medicines groups ($p>0.05$).
Notes	3 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Li et al., 2004	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	50 inpatients or outpatients from Affiliated Hospital of An Hui University of Chinese Medicine were recruited (1999 Jun-2003 Dec). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Bu Shen An Tai Yin, included Chinese Dodder Seed, Eucommia Bark, Chinese Taxillus Twig, Himalayan Teasel Root, Heterophylly Falsestarwort Root, Mongolian Milkcetch Root, Largehead Atractylodes Rhizome, Baical Skullcap Root, White Paeony Root, Steamed Rehmannia Root and Ramie Root, each 10g. 2) Formula changes (no information on the dosage) Blood Heat: Baical Skullcap Root was increased. Kidney Deficiency: Chinese Dodder Seed was increased. 3) Decoction: po, bid, 10 days as a course. 4) Western medicines were received at the same time, including Progesterone 10mg, im, qd, Vitamin E 100mg, po, qd, Folic Acid 5mg, tid. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 96%, and Western medicines group was 80% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, "Layered method", but no further information was available.

Li et al., 2005	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone
Participants	90 inpatients from First Affiliated Hospital of Guang Zhou University of Chinese Medicine were recruited (2003 Apr-2004 Jun). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Shou Tai Pill, included Chinese Dodder Seed 15g, Chinese Taxillus Twig 15g, Himalayan Teasel Root 15g, Donkey-hide Glue 10g, Pilose Asiabell Root 15g, Mongolian Milkcetch Root 15g, Largehead Atractylodes Rhizome 10g, White Paeony Root 15g, and Liquorice Root 5g. 2) Formula changes Increased Bleeding: Baical Skullcap Root, Lotus Rhizome Node and Garden Burnet Root, each 10g were added. Low Back Pain: Eucommia Bark and Palmleaf Raspberry Fruit, each 10g were added. Stool dehydration: Desertliving Cistanche and Mulberry Fruit, each 10g were added. 3) Decoction: po, BID 4) Western medicines were received at the same time, including Vitamin E 50 mg, po, bid, Folic Acid 0.4 mg, po, qd, HCG 2000 U, im, qd, Allylestrenol, 5mg, po, tid, if with a history of over twice habitual abortion. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 95%, and Western medicines group was 76.67% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Li et al., 2006	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	89 inpatients or outpatients from Affiliated Hospital of Guang Xi College of Chinese Medicine were recruited (2003 Jan-2005 Sep). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Bu Shen Gu Tai soup, included Chinese Dodder Seed 30g, Barbary Wolfberry Fruit 30g, Chinese Taxillus Twig 20g, Himalayan Teasel Root 20g, Pilose Asiabell Root 30g, Common Yam Rhizome 15g, Eucommia Bark 20g, White Paeony Root 20g, and Liquorice Root 5g.

	2) Formula changes Dry Mouth: Glossy privet fruit 15g and Yerbadetajo Herb 20g were added. 3) Decoction: po, BID, for 10 days. 4) Western medicines were received at the same time, including HCG 2000U and Progesterone 20mg, im, qod for 10 days. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 77.78%, and Western medicines group was 70.50% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, Randomized Number Table

Li et al., 2009 a	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	138 inpatients or outpatients from Zhao Qing People's Hospital were recruited (2004 May-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Shou Tai Pill, included Chinese Dodder Seed 20g, Himalayan Teasel Root 15g, Chinese Taxillus Twig 15g, Pilose Asiabell Root 15g, Donkey-hide Glue 10g, Largehead Atractylodes Rhizome 12g, Steamed Rehmannia Root 12g, Common Macrocarpium Fruit 10g, White Paeony Root 15 g, Eucommia Bark 15g, and Liquorice Root 6g. 2) Formula changes Bleeding: Fineleaf Schizonepeta Herb 10g,Garden Burnet Root 10g were added. Yin Deficiency: Pilose Asiabell Root replaced by Heterophylly Falsestarwort Root, Baical Skullcap Root were added. Qi Deficiency: Pilose Asiabell Root 30g was added. Vomiting: Villous Amomrum Fruit, Pinellia Tuber and Bamboo Shavings were added. Stool dehydration: Desertliving Cistanche and Hemp Fruit were added. 3) Decoction: po, BID, 7 days as a course. 4) Western medicines were received at the same time, including HCG 2000U, im, qod, Progesterone 20mg, im, qd. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical

	examinations shows pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 93.1%, and Western medicines group was 80.3% (p<0.05).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Li et al., 2009 b	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	189 inpatients from Wu Yi Chinese Medicine Hospital were recruited (2005 Sep-2007 Sep). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Shou Tai Pill, included Chinese Dodder Seed 15g, Himalayan Teasel Root 10g, Chinese Taxillus Twig 15g, Donkey-hide Glue10g, Baical Skullcap Root 6g, Pilose Asiabell Root 15g, Largehead Atractylodes Rhizome 12g, White Paeony Root 15g, and Liquorice Root 6g. 2) Formula changes Vomiting: Bamboo Shavings 6g, Villous Amomrum Fruit 6g and Perilla Stem 6g were added. Bleeding: Ramie Root 15g and Hairyvein Agrimonia 15g were added. Low Back Pain: Eucommia Bark 10g and Barbary Wolfberry Fruit 15g were added. Stool dehydration: Hemp Fruit 15g and Desertliving Cistanche 10g were added. 3) Decoction: details not provided. 4) Western medicines were received at the same time, including Vitamin E 50mg, po, bid; Folic Acid, 0.4mg, po, qd; HCG 2000 IU,im, qd. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 91.75%, and Western medicines group was 75% (p<0.05).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Liu et al., 2008	
Methods	Randomized controlled trial of Chinese herbal medicines compared with Western medicines.
Participants	90 outpatients from Shan Xi Service Centre for Pregnancy were recruited (2006 Jun-2007 Oct). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.

Interventions	Treatment group received Chinese herbal medicines. 1) The Chinese medicine formula was given according to different subtype of diagnosis of threatened miscarriage. i. Kidney Deficiency Subtype: used Shou Tai Pill, included Chinese Dodder Seed 12g, Chinese Taxillus Twig 12g, Himalayan Teasel Root 12g, Donkey-hide Glue 9g, Pilose Asiabell Root 9g, and Mongolian Milkcetch Root 9g. ii. Qi Deficiency subtype: used Tai Yuan Yin, included Pilose Asiabell Root 9g, Mongolian Milkcetch Root 9g, Largehead Atractylodes Rhizome 9g, Chinese Angelica 6g, White Paeony Root 12g, Chinese Taxillus Twig 12g, and Villous Amomrum Fruit 6g. iii. Trauma subtype: used Sheng Yu Soup, included Chinese Angelica 6g, Szechuan Lovage Rhizome 3g, White Paeony Root 12g, Rehmannia Root 9g, Pilose Asiabell Root 9g, and Mongolian Milkcetch Root 9g. iv. Blood Heat: used Bao Yin Jian, included Rehmannia Root 9g, Mongolian Milkcetch Root 12g, Bark of Chinese Corktree 9g, and White Paeony Root 9g. 2) Decoction: po, QD 3) Western medicines were received at the same time, including Progeserone 20mg, im, and Vitamin E 100mg, po, tid. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained at 12 weeks were considered as effective. The effectiveness rate of combined medicines group was 91.4%, and Western medicines group was 75.5% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, "Randomization according to visiting date"

Liu et al., 2009	
Methods	Randomized controlled trial of Chinese herbal medicines compared with Western medicines.
Participants	45 outpatients from Shen Zhen Women??s Hospital were recruited (2006 Aug-2007 Sep). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Chinese herbal medicine group: 1) mainly used Chinese Dodder Seed, Chinese Taxillus Twig, Pilose Asiabell Root each 20g; Himalayan Teasel Root, Donkey-hide Glue, Largehead Atractylodes Rhizome, Herb of Hairyvein Agrimonia each 15g; Villous Amomrum Fruit, Rhizome of East Asian Tree Fern each 10g, and Liquorice Root 5g. 2) Decoction: po, BID for 2 weeks. The Western medicines group used Progesterone 20mg, im, qd; HCG 2000U, im, qod for 2 weeks.

	Both 2 groups had standard care for pregnancy (prohibit sexual activity, psychotherapy, and regulate ultrasound tests).
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations shows pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 90%, and Western medicines group was 66.7% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Lv et al., 2007	
Methods	Randomized controlled trials of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	98 inpatients from Tian Jin Chinese Medicine University were recruited (2004 May-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula: included Chinese Dodder Seed 20g, Himalayan Teasel Root, Chinese Taxillus Twig, Pilose Asiabell Root, Common Yam Rhizome each 15g, Largehead Atractylodes Rhizome 12g, Common Macrocarpium Fruit 10g, Steamed Rehmannia Root 10g, and Liquorice Root 6g. 2) Formula changes Bleeding: Donkey-hide Glue, Baical Skullcap Root and Ramie Root were added. Abdomen Pain: White Paeony Root was added. Blood Heat: Baical Skullcap Root was added. Insomnia: Spina Date Seed was added. Low Back Pain: Eucommia Bark was added. Vomiting: Villous Amomrum Fruit was added. 3) Decoction: po, QD 4) Western medicines were received at the same time, including Progesterone 20mg, im, qd, Vitamin E 100mg, po, tid. Control group was treated with Western medicines alone.
Outcomes	Symptoms subsided and pregnancy maintained till delivery were considered as effective. The effectiveness rate of combined medicines group was 91.38%, and Western medicines group was 70% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

She et al., 2008	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	100 inpatients or outpatients from Chinese Medicine Hospital of Gui Lin were recruited. Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Shou Tai Pill, included Chinese Dodder Seed 15g, Chinese Taxillus Twig 10g, Himalayan Teasel Root 10g, Donkey-hide Glue 10g, Pilose Asiabell Root 20g, and Largehead Atractylodes Rhizome 15g. 2) Formula changes Qi and Blood Deficiency: Mongolian Milkcetch Root 15g, Steamed Rehmannia Root 10g and White Paeony Root 10g were added. Blood Heat: Rehmannia Root 15g, Baical Skullcap Root 12g and Bark of Chinese Corktree 6g were added. 3) Decoction: po, BID, 10 days as a course. 4) Western medicines were received at the same time, including Progesterone 20mg, im, qd; HCG 2000U, im, qod; Vitamin E 100mg, po, qd; Folic Acid 0.4mg, qd. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 93.3%, and Western medicines group was 70% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Song et al., 2005	
Methods	Randomized controlled trial of Chinese herbal medicines compared with Western medicines.
Participants	243 outpatients from Ning Xia Women's Hospital were recruited (2001 May-2003 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Chinese herbal medicine group. 1) Zhi Xue An Tai Yin: mainly used Himalayan Teasel Root, Chinese Dodder Seed, Chinese Taxillus Twig, Perilla Stem, Villous Amomrum Fruit, White Paeony Root, Largehead Atractylodes Rhizome, Common Macrocarpium Fruit, Sharpleaf Galangal Fruit, Mongolian Milkcetch Root, Heterophylly Falsestarwort Root, Chinese Arborvitae Twig, Garden Burnet Root, India Madder Root, and Donkey-hide Glue 10g each. 2) Formula changes:

	Heat Sign: Baical Skullcap Root was added. Cold Sign: Chinese Mugwort Leaf and Ginger each 6g were added. Vomiting: Pinellia Tuber, Clove, Persimmon Calyx and Receptacle each 10g, and Ginger 6g were added. 3) po, TID, for 7 days. The Western medicines group used Progesterone 20mg, im, qd; Vitamin E 100mg, po, tid; Vitamin K 8mg, tid; An Luo Xue, 5mg, po, tid.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained (HCG normal and LH> 200mIU/ ml) were considered as effective. The effectiveness rate of combined medicines group was 76.98%, and Western medicines group was 43.59% (p<0.05).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Song et al., 2007	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	105 outpatients from Ning Xia Women's Hospital were recruited (2005 Oct-2006 Aug). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Zhi Xue Bao Tai Yin, included Himalayan Teasel Root, Chinese Dodder Seed, Chinese Taxillus Twig, Perilla Stem, Villous Amomrum Fruit, White Paeony Root, Largehead Atractylodes Rhizome, Common Macrocarpium Fruit, Sharpleaf Galangal Fruit, Mongolian Milkcetch Root, Heterophylly Falsestarwort Root, Chinese Arborvitae Twig, Garden Burnet Root, India Madder Root, and Donkey-hide Glue, 10g each. 2) Formula changes: Heat Sign: Baical Skullcap Root was added. Cold Sign: Chinese Mugwort Leaf and Ginger each 6g were added. Vomiting: Pinellia Tuber, Clove, Persimmon Calyx and Receptacle 10g each, and Ginger 6g were added. 3) Decoction: po, TID, for 7 days. 4) Western medicines were received at the same time, including Progesterone 20mg, im, qd; Vitamin E 100mg, po, tid; Vitamin K 8mg, tid; An Luo Xue, 5mg, po, tid. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained (HCG normal and LH increased) were considered as effective. The effectiveness rate of combined medicines group was 81.5%,

	and Western medicines group was 43.1% (p<0.05).
Notes	2 arms RCT
Allocation concealment	Unclear, "Randomization according to first visit date"

Sun et al., 2003	
Methods	Randomized controlled trial of comparisons among combined medicines, Chinese herbal medicines alone, and Western medicines alone.
Participants	105 inpatients or outpatients from Xiang Tan Chinese Medicine Hospital were recruited (2004 May-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Chinese herbal medicine group: 1) mainly used Heterophylly Falsestarwort Root 15g, Mongolian Milkcetch Root 12g, Largehead Atractylodes Rhizome 10g, White Paeony Root 15g, Barbary Wolfberry Fruit 15g, Chinese Taxillus Twig 15g, Himalayan Teasel Root 15g, Chinese Dodder Seed 15g, Common Yam Rhizome 15g, and Liquorice Root 6g. 2) Formula changes: Severe Bleeding: Garden Burnet Root 10g and Yerbadetajo Herb 10g were added. Blood Heat: Mongolian Milkcetch Root removed, Baical Skullcap Root 6g were added. Abdomen Pain: White Paeony Root 20-30g and Largetrifoliolious Bugbane Rhizmome 10g were added. Vomiting: Perilla Stem 10g, Bamboo Shavings 10g and Tangerine Pee 16g were added. 3) po, BID, 5-day as a course, and usually 1-3 courses. The Western medicines group used Vitamin E 100mg, po, tid; Folic Acid 0.4mg, po, qd; HCG 1000U, im, qd.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of Chinese medicines group was 94%, Western medicines group was 71%, and combined medicine group was 98%. Significant difference was found between combined medicine or Chinese medicines and Western medicines alone (p<0.05). No statistic difference was found between Chinese herbal medicines and combined medicines groups (p>0.05).
Notes	3 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Wang et al., 2005	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	140 outpatients from Hui An Chinese Medicine Hospital were recruited (2002 Oct-2004 Sep). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.

Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Zhu Ma An Tai soup, included Ramie Root 30g ,Chinese Taxillus Twig 20g, Himalayan Teasel Root 20g,Chinese Dodder Seed 20g, Largehead Atractylodes Rhizome 10g, Baical Skullcap Root 10g, White Paeony Root 12g, Rehmannia Root 15g, Yerbadetajo Herb 30g, and Liquorice Root 3g. 2) Formula changes Severe Bleeding: Chinese Arborvitae Twig and root of Common Euscaphis were added. Severe Vomiting: Bamboo Shavings and Villous Amomrum Fruit were added. Stool dehydration: Desertliving Cistanche and Platycladi Seed were added. 3) Decoction: po, QD 4) Western medicines were received at the same time, including Progesterone 20mg, im, qd; HCG 2000U, im, qod; Vitamin E 100mg, po, qd; Folic Acid 0.4mg, qd, (add Zhi Xue Min if bleeding hardly). Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 95%, and Western medicines group was 50% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Wang et al., 2007	
Methods	Randomized controlled trial of Chinese herbal medicines compared with Western medicines.
Participants	96 inpatients or outpatients from Nan Yang Chinese Medicine College were recruited (2003 Mar-2006 Mar). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Chinese herbal medicine group: 1) Bu Shen Gu Chong soup: mainly used Chinese Dodder Seed 30g, Himalayan Teasel Root 15g, Chinese Taxillus Twig 30g, Largehead Atractylodes Rhizome 18g, Donkey-hide Glue 10g, Eucommia Bark 15g, White Paeony Root 15g, Pilose Asiabell Root 30g, Fineleaf Schizonepeta Herb 9g, and Liquorice Root 6g. 2) Formula changes: QI Deficiency: Pilose Asiabell Root removed, Heterophylly Falsestarwort Root and Mongolian Milkcetch Root, each 30g were added. Severe Bleeding: Herb of Hairyvein Agrimonia 30g, Male Fern Rhizome 15g and Garden Burnet Root 30g were added.

	Vomiting: Villous Amomrum Fruit 8g and Perilla Stem 9g were added. Heat Sign: Baical Skullcap Root 9g and Ramie Root 30g were added. Abdomen Pain: White Paeony Root 30g was added. 3) po, BID, 7-day as a course. The Western medicines group used HCG 1000U, im, qd, 7-day as a course.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 95.8%, and Western medicines group was 89.5% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Xiao et al., 2008	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	60 inpatients from Xin Shao People's Hospital were recruited (2005 Oct-2007 Oct). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Yan Xue Yi Shen soup, included Chinese Dodder Seed 10g, Chinese Taxillus Twig 15g, Himalayan Teasel Root 10g, Eucommia Bark 15g, Mongolian Milkcetch Root 20g, Ginseng 10g, Common Yam Rhizome 15g, White Paeony Root 15g, Donkey-hide Glue 15g, Steamed Rehmannia Root 10g and Tangerine Pee l5g. 2) Decoction: po, BID 3) Western medicines were received at the same time, includingProgesterone 20mg, im, qd; HCG 2000U, im, qd for 10 days then decline the dosage; Vitamin E 50mg, Ritodrine 10mg, po, tid; Folic Acid 0.4mg, QD, An Luo Xue, Vitamin K, Vitamin C if bleeding hardly (no detailed administration). Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 90%, and Western medicines group was 66.7% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, "Randomized Number Method"

Xu et al., 2005	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	62 inpatients or outpatients from Affiliated Hospital of Zhe Jiang University were recruited.

	Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Yun Kang decoction (Further information of herbs and dosages were not available.) 2) Decoction: 20ml, po, BID, for 14 days. 3) Western medicines were received at the same time, including Progesterone 40mg, im, qd; Vitamin E 1 pill, po, qd. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 90.6%, and Western medicines group was 86.6% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Xun et al., 2008	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	75 inpatients or outpatients from Jiang Su East West Medicine Hospital were recruited (2004 May-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula, included Pilose Asiabell Root 10g, Mongolian Milkcetch Root 10g, Largehead Atractylodes Rhizome 10g, White Paeony Root 10g, Steamed Rehmannia Root 10g, Chinese Taxillus Twig 12g, Donkey-hide Glue 10g, and Tangerine Peel 10g. 2) Decoction: po, BID 3) Western medicines were received at the same time, including Progesterone 20mg, im, qd; HCG 2000U, im, qd, qod if vaginal bleeding stopped. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 93.3%, and Western medicines group was 80% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Yang et al., 2001	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western

	medicines) compared with Western medicines alone.
Participants	55 inpatients or outpatients from Jiang Xi Ning Dou Chinese Medicine Hospital were recruited. Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Shou Tai Pill, included Chinese Dodder Seed 30g, Chinese Taxillus Twig 12g, Himalayan Teasel Root 12g, Donkey-hide Glue 15g, Pilose Asiabell Root 30g, *Largehead Atractylodes Rhizome 10g, Common Yam* Rhizome 15g, and Liquorice Root 6g. 2) Decoction: po, QD 3) Western medicines were received at the same time, including Progesterone 20mg, im, qd; Vitamin K_3 4mg, po, bid. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 90.5%, and Western medicines group was 61.5% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Yang et al., 2006	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	150 inpatients or outpatients from Guang Zhou Qin Zhou Chinese Medicine Hospital were recruited (2004 Jan-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Jiao Ai soup, included Donkey-hide Glue 15g, Chinese Mugwort Leaf 12g, Steamed Rehmannia Root 20g, Szechuan Lovage Rhizome 5g, White Paeony Root 10g, Chinese Angelica 10g, and Liquorice Root 6g. 2) Formula changes Qi and Blood Deficiency: Mongolian Milkcetch Root 30g and Pilose Asiabell Root 20g were added. Kidney Deficiency: Chinese Taxillus Twig 15g, Eucommia Bark 15g, Himalayan Teasel Root 15g and Chinese Dodder Seed 15g were added. Blood Heat: Baical Skullcap Root 10g was added. Adominal Dstension: Villous Amomrum Fruit 3g and Tangerine Peel 6g were added. 3) Decoction: po, QD 4) Western medicines were received at the same time, including HCG 1000U, im, qd for 10

	days then decline the dosage, Vitamin E 100mg, po, tid, Folic Acid 0.4mg, qd. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 95%, and Western medicines group was 72% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, "2:1 ratio randomization"

Zhang et al., 2007	
Methods	Randomized controlled trial of Chinese herbal medicines compared with Western medicines.
Participants	102 inpatients from Shan Dong Ji Nan Hospital were recruited (2004 May-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Chinese herbal medicine group: 1) Shou Tai Pill: mainly used Chinese Dodder Seed 10g, Himalayan Teasel Root 15g, Chinese Taxillus Twig 15g, Donkey-hide Glue 10g, Deer Horn 10g, and Morinda Root 10g. 2) Formula changes: Low Back Pain: Eucommia Bark was added. Abdominal Distension: Mongolian Milkcetch Root and Largetrifoliolious Bugbane Rhizmome were added. Bleeding: Herb of Hairyvein Agrimonia, Garden Burnet Root and Lotus Seed Pot were added. 3) Decoction: po, qd. The Western medicines group used Vitamin E 0.1g, po, qd; Progesterone 20~40 mg, im, qd; Folic Acid 5mg, po, qd; HCG, 2000IU, im, qod.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 97.1%, and Western medicines group was 84.85% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Zhang et al., 2008 a	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	96 inpatients or outpatients from Liu Yang Central Hospital were recruited (2006 Oct-2008 Jan). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.

Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Shou Tai Pill, included Chinese Dodder Seed 20g, Chinese Taxillus Twig 25g, Donkey-hide Glue, Himalayan Teasel Root, Steamed Rehmannia Root, Pilose Asiabell Root and Largehead Atractylodes Rhizome, each 15g, and Liquorice Root 10g. 2) Formula changes Qi Deficiency: Mongolian Milkcetch Root 30g was added. Severe Bleeding: Herb of Hairyvein Agrimonia 30g, Male Fern Rhizome 15g, Garden Burnet Root 30g were added. Vomiting: Villous Amomrum Fruit 8g and Perilla Stem 9g were added. Heat Sign: Baical Skullcap Root 9g and Ramie Root 30g were added. Abdomen Pain: White Paeony Root 30g was added. Insomnia: Spina Date Seed 8g was added. 3) Decoction, po, BID, 7-day as a course. 4) Western medicines were received at the same time, including HCG 2000U, im, qod; Vitamin E 100mg, po, qd; Folic Acid 2.5mg, po, qd. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 90.0%, and Western medicines group was 87.1% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, "Randomized Number Table"

Zhang et al., 2008 b	
Methods	Randomized controlled trial of combined medicines (Chinese herbal medicines + Western medicines) compared with Western medicines alone.
Participants	100 inpatients or outpatients from Second Affiliated Hospital of Hunan University of Chinese Medicine were recruited (2004 Jan-2006 Dec). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Treatment group received Chinese herbal medicines combined with Western medicines. 1) The Chinese medicine formula was Bao Tai Yin, included Steamed Rehmannia Root 20g, Common Macrocarpium Fruit 15g, Donkey-hide Glue 15g, Himalayan Teasel Root 15g, White Paeony Root 10g, Chinese Dodder Seed 15g, Chinese Taxillus Twig 15g, Eucommia Bark15g, Largehead Atractylodes Rhizome 15g, Mongolian Milkcetch Root 20g, Pilose Asiabell Root 10g, and Liquorice Root 6g. 2) Formula changes

	Blood Heat: Bamboo Shavings, Mulberry Leaf and Towel Gourd Vegetable Sponge each 10g were added. Abdominal Distension: Villous Amomrum Fruit 3g and Tangerine Pee 16g were added. 3) Decoction: po, tid, 1 or 2 weeks. 4) Western medicines were received at the same time, including Progesterone, 20mg, qd; Vitamin E 100mg, po, tid. Control group was treated with Western medicines alone.
Outcomes	Symptoms such as vaginal bleeding and abdominal pains subsided, and clinical examinations showed pregnancy maintained were considered as effective. The effectiveness rate of combined medicines group was 92%, and Western medicines group was 78% ($p<0.05$).
Notes	2 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Zhong et al., 2002	
Methods	Randomized controlled trial of comparisons among combined medicines, Chinese herbal medicines alone, and Western medicines alone.
Participants	90 inpatients or outpatients from Guang Zhou Second People's Hospital were recruited (2004 May-2006 May). Participants were all diagnosed as threatened miscarriage due to vaginal bleeding and abdominal pains.
Interventions	Chinese herbal medicine group: 1) mainly used Pilose Asiabell Root, Largehead Atractylodes Rhizome, Common Yam Rhizome, Chinese Taxillus Twig, Chinese Dodder Seed, Himalayan Teasel Root and Baical Skullcap Root (no information on the dosage). 2) Formula changes: (no information on the dosgae) Blood Deficiency: Donkey-hide Glue was added. Cold Sign: Chinese Mugwort Leaf was added. Blood Heat: Garden Burnet Root was added. Abdomen Pain: White Paeony Root, Nutgrass Galingale Rhizome and Perilla Stem were added. Vomiting: Bamboo Shavings, Villous Amomrum Fruit and Tangerine Peel were added. Dry Mouth: Rehmannia Root, Glossy Privet Fruit and Yerbadetajo Herb were added. 3) Decoction: po, QD. The Western medicines group used HCG 2000U, im, qod, then decline the dosage after 12 weeks of pregnancy.
Outcomes	Symptoms subsided and pregnancy maintained till delivery were considered as effective. The effectiveness rate of Chinese medicines group was 90%, Western medicines group was

	73.3%, and combined medicine group was 93.3%. Significant difference was found between Chinese herbal medicines and Western medicines alone ($p<0.05$). No statistic difference was found between Chinese herbal medicines and combined medicines ($p>0.05$).
Notes	3 arms RCT
Allocation concealment	Unclear, no information was available from the author.

Table 5.3 Characteristics of excluded studies

Lu et al., 2007 a	
Reason for exclusion	Comparison was made between combined medicine and Western medicines. However, the women in two groups received different Western medicines. So the review authors considered that these two groups were not comparable and doubt if the trial author could get his conclusion that combined medicine was better than Western medicines. Decision was made to exclude this paper.

Ushiroyama et al., 2006	
Reason for exclusion	Comparisons were made between a Kampo medicine and Western medicines. The Kampo medicines is Japanese traditional medicine, which may originate from China. To avoid the confusion of readers on the topic of our review, which is about Chinese herbal medicines, we decided to exclude this trial from our further analysis.

Zhang et al., 2006	
Reason for exclusion	Data were collected from 1995 to 2005, using 10 years. So the review authors doubt if this is a real RCT. What's more, the amount of participants in each group differed. There were 140 participants in combined medicine group, but 128 participants in Western medicines group, and the author did not give further information on the randomization method. Thus, decision was made to exclude this paper.

Table 5.4 Characteristics of studies awaiting classification

Chen et al., 1999	
Methods	Randomized controlled trial of Chinese herbal medicines combined with Western medicines compared with Western medicines alone.
Participants	720 participants diagnosed with threatened miscarriage were 2:1 divided into 2 groups. (480 for combined medicine group, and 240 for Western medicines group)
Interventions	Comparison between combined medicines and Western medicines was made.
Outcomes	The effectiveness of combined medicine is higher than Western medicines. Chinese herbal medicine could help and improve the treatment of Western medicines alone.
Notes	Dates of data collection started from 1991, and the result was published in 1999, without mentioning the duration that the 720 patients were involved in the treatments. So the review authors doubt if this was a real RCT, and have been awaiting response from authors for further information.

Guan et al., 2008	
Methods	Randomized controlled trial of Chinese herbal medicines combined with Western medicines compared with Western medicines alone.
Participants	100 participants diagnosed with threatened miscarriage were divided into two groups.
Interventions	Comparison between combined medicines and Western medicines was made.
Outcomes	The effectiveness of combined medicines is higher than Western medicines. Chinese herbal medicines could help and improve the treatment of Western medicines alone.
Notes	Dates of data collection was from March 1999 to March 2006, using 7 years. So the review authors doubt if this was a real RCT, and have been awaiting response from authors for further information.

5.4.4.3 Outcomes

5.4.3.3.1 Primary outcomes (continuation of pregnancy after 28 weeks of gestation)

1) Effectiveness of Chinese medicines alone versus Western medicines alone

Only one trial (Zhong et al., 2002) was selected, with 60 participants, half of which were randomized to receive Chinese medicines while the other half received Western medicines. Though the effectiveness rate of Chinese medicine was higher than Western medicine as treatment for threatened miscarriage, 90.0% versus 73.3%, respectively, there was no statistically significant difference (RR = 1.23; 95% CI 0.96 to 1.57) (Figure 5.6).

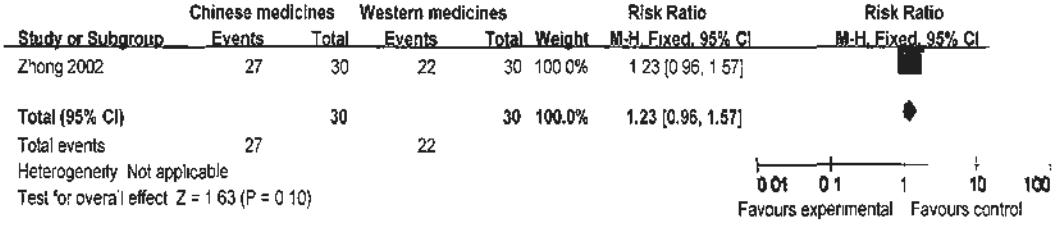


Figure 5.6 Forest plot of comparison: Chinese medicines versus Western medicines

Outcome: Effectiveness of intervention (continuation of pregnancy after 28 weeks of gestation).

2) Effectiveness of Chinese medicines alone versus combined Chinese and Western medicines

Only one trial (Zhong et al., 2002) was selected, with 60 participants, 30 of which were randomized to receive Chinese medicines alone while the others received combined Chinese and Western medicines. Although the effectiveness rate of combined medicines was higher than Chinese medicines alone, 93.3% versus 90.0%,

respectively, no statistically significant difference was found (RR = 0.96; 95% CI 0.83 to 1.12) (Figure 5.7).

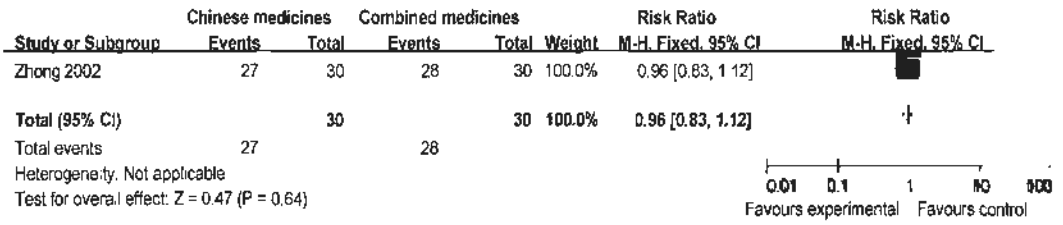


Figure 5.7 Forest plot of comparison: Chinese medicines versus combined medicines

Outcome: Effectiveness of intervention (continuation of pregnancy after 28 weeks of gestation).

3) Effectiveness of combined Chinese and Western medicines versus Western medicines alone

4 clinical trials with 350 participants were included (Chen et al., 2002, Feng ZR, 1997, Lv et al., 2007, Zhong et al., 2002). There was no siginificant heterogeneity ($\chi^2 = 0.29$, df = 3, $I^2 = 0\%$), while test for overall effect was significant ($p < 0.00001$). The result showed that combined Chinese herbal and Western medicines were more effective than Western medicines alone as treatment for threatened miscarriages, 94.0% versus 70.7%, respectively (RR = 1.33; 95% CI 1.19 to 1.48) (Figure 5.8).

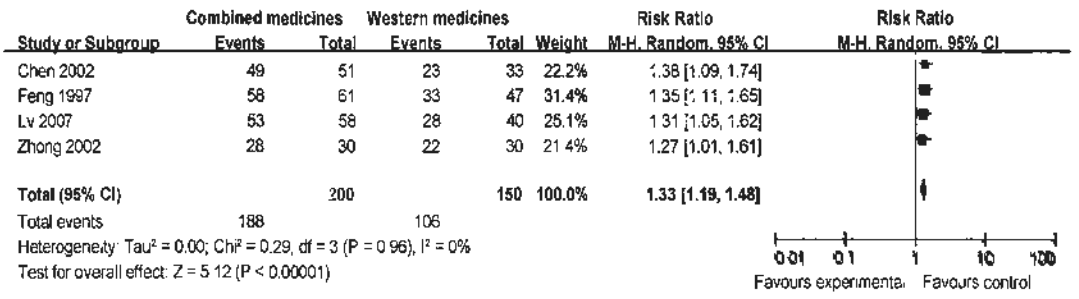


Figure 5.8 Forest plot of comparison: Combined medicines versus Western medicines

Outcome: Effectiveness of intervention (continuation of pregnancy after 28 weeks of gestation).

5.4.4.3.2 Secondary outcomes

1) No relief of clinical signs (vaginal bleeding and abdominal pain)

One trial (Zhong et al., 2002) compared Chinese medicines alone with Western medicines alone, the incidences of no relief of clinical signs were 10% and 26.7%, respectively (RR = 0.38; 95% CI 0.11 to 1.28) (Figure 5.9).

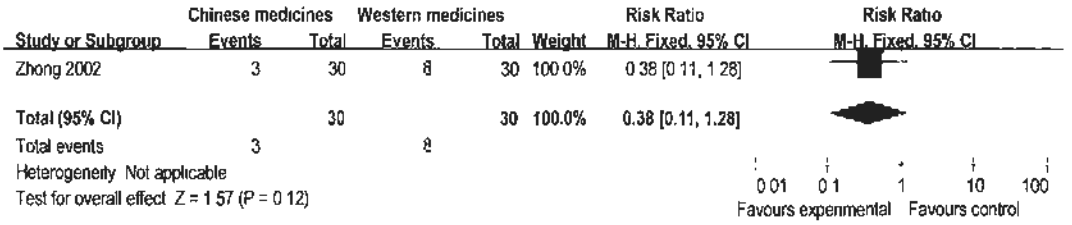


Figure 5.9 Forest plot of comparison: Chinese medicines alone versus Western medicines alone

Outcome: No relief of clinical signs.

This trial also compared Chinese medicines alone with combined medicines, the incidences of no relief of clinical signs were 10% and 6.7%, respectively (RR = 1.50; 95% CI 0.27 to 8.34) (Figure 5.10).

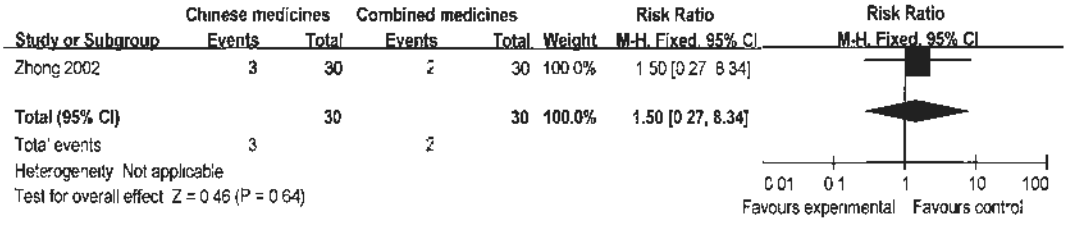


Figure 5.10 Forest plot of comparison: Chinese medicines alone versus combined medicines

Outcome: No relief of clinical signs.

Four trials (Chen et al., 2002, Feng ZR, 1997, Lv et al., 2007, Zhong et al., 2002) compared combined medicines with Western medicines alone. 200 participants were randomized into combined Chinese herbal medicines group while 150 were in Western medicines group. There was no siginificant heterogeneity ($\chi^2 = 1.06$, df = 3, $I^2 = 0\%$), while test for overall effect was significant ($p < 0.00001$). The result showed that combined medicines treatment was more effective in the relief of clinical signs than Western medicines treatment, the incidences were 6% and 29.3%, respectively (RR = 0.21; 95% CI 0.12 to 0.39) (Figure 5.11).

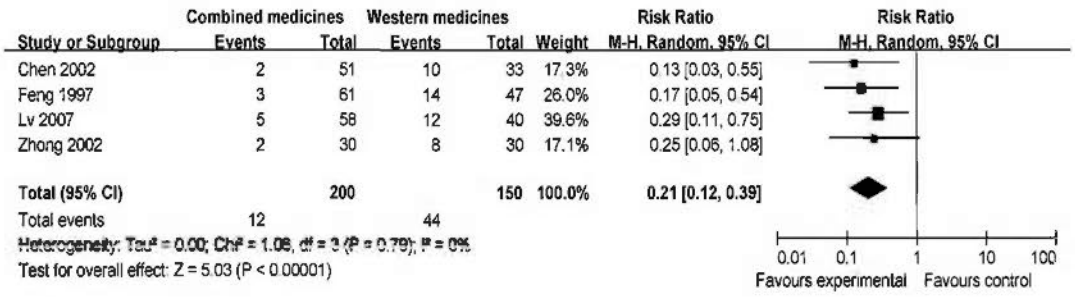


Figure 5.11 Forest plot of comparison: Combined medicines versus Western medicines alone

Outcome: No relief of clinical signs.

2) No improvement in laboratory investigations (urinary and serum ß-HCG titer).

Only one trial (Lv et al., 2007) mentioned serum ß-HCG and progestrone levels during the treatments. However, the author did not report the data, so we could not include the study for further analysis.

3) No other secondary outcomes (repeated threatened miscarriage, preterm labour, adverse pregnancy outcomes, preterm birth, stillbirth, neonatal death, fetal structural malformations, and other adverse perinatal outcomes) could be analysed due to a lack of data from the included studies.

5.4.4.3.3 Other outcomes

There were 26 randomized controlled trials concluded the outcomes right after the courses of treatment, without follow up until 28 weeks of gestation. Therefore, as a supplement to the primary and secondary outcomes, we also included these clinical trials and analysed as non-prespecified outcomes, i.e., continuation of pregnancy after treatment.

1) Effectiveness of Chinese medicines alone versus Western medicines alone

In total, 9 clinical trials with 960 participants were included. Test for heterogeneity and test for overall effect were both significant (χ^2 = 33.94, df = 8, I^2 = 76%, p < 0.0001; p = 0.0002). The result showed significant effectiveness with the use of Chinese medicines alone to prevent inevitable miscarriage and continue the pregannncy when compared to that of Western medicines, 86.5% verse 62.7%, respectively (RR = 1.33; 95% CI 1.14 to 1.54) (Figure 5.12).

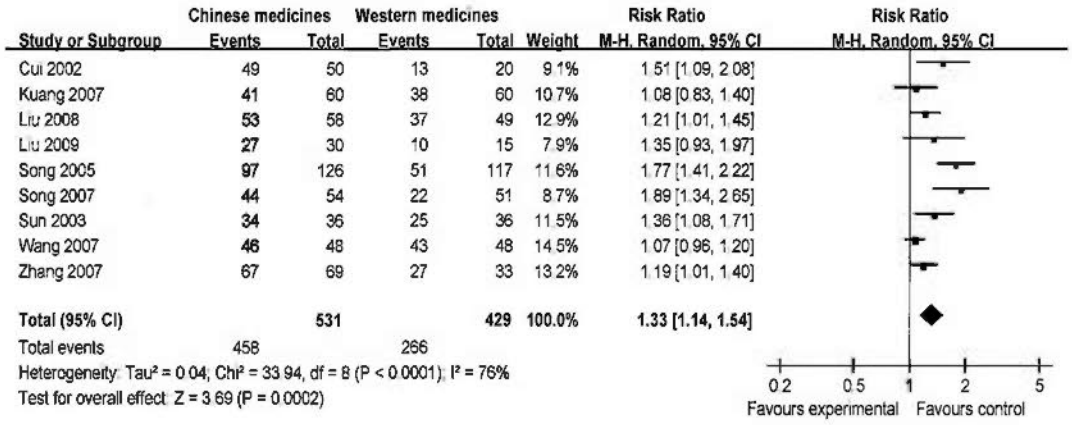


Figure 5.12 Forest plot of comparison: Chinese medicines alone versus Western medicines

Outcome: Effectiveness of intervention (continuation of pregnancy after treatment).

2) Effectiveness of Chinese medicines alone versus combined Chinese and Western medicines

In total, 2 clinical trials with 172 participants were included. Test for heterogeneity was significant (χ^2 = 9.46, df = 1, I^2 = 89%, p =0.002) while there was no significant overall effect. The result indicated that combined Chinese and Western medicines was not significant effective to prevent inevitable miscarriage and continue the preganncy than Chinese medicines alone as treatment for threatened miscarriage, 92.7% verse 78.1%, respectively (RR = 0.87; 95% CI 0.62 to 1.20) (Figure 5.13).

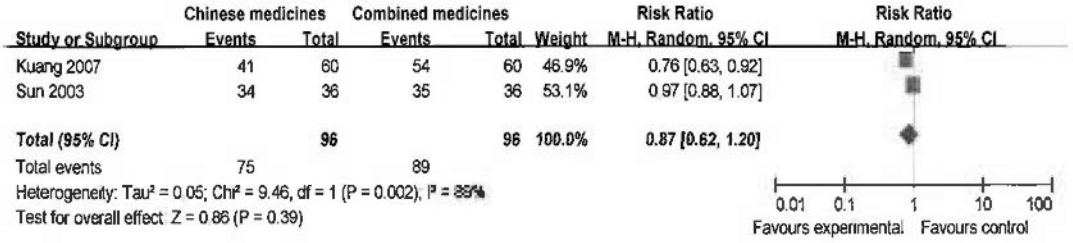


Figure 5.13 Forest plot of comparison: Chinese medicines versus combined medicines

Outcome: Effectiveness of intervention (continuation of pregnancy after treatment).

3) Effectiveness in combined Chinese and Western medicines versus Western medicines alone

In total, 19 clinical trials with 1,956 participants were included. Test for heterogeneity and test for overall effect were both significant (χ^2 = 34.41, df = 18, I^2 = 48%, p = 0.01; p < 0.00001). The result showed that combined Chinese and Western medicines was significant effective to prevent inevitable miscarriage and continue the preganncy than Western medicines alone as treatment for threatened miscarriages, 92.8% verse 73.0%, respectively (RR = 1.24; 95% CI 1.17 to 1.32) (Figure 5.14).

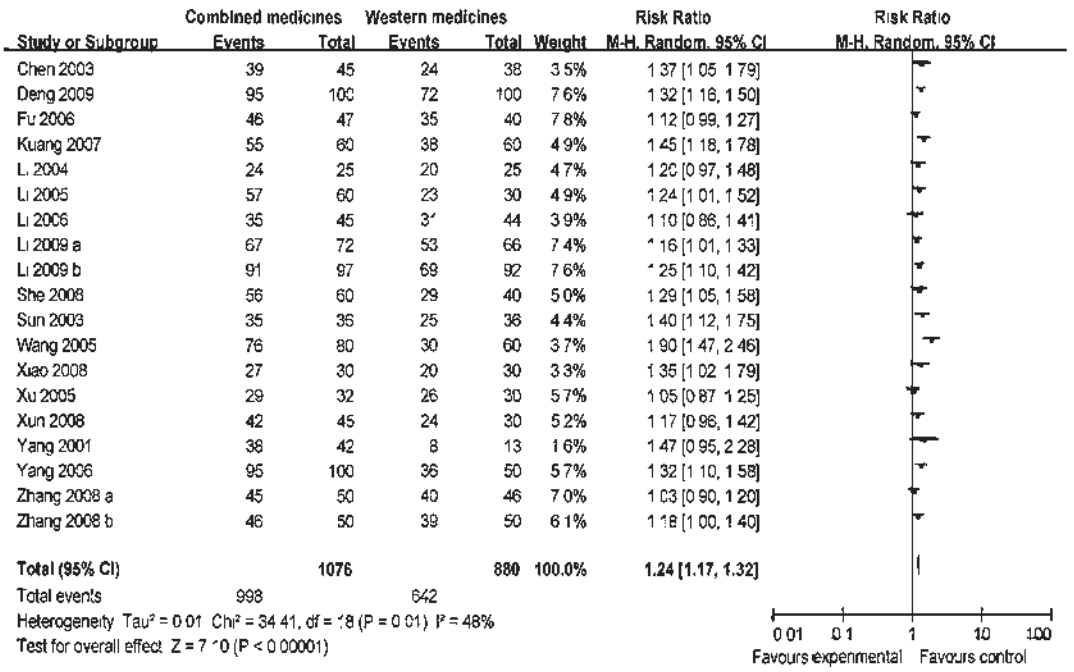


Figure 5.14 Forest plot of comparison: Combined medicines versus Western medicines alone

Outcome: Effectiveness of intervention (continuation of pregnancy after treatment).

5.4.4.3.4 Subgroup analysis

Due to insufficiency of data, analysis for all designed subgroups was not employed. Mean maternal age and/or range in each group were reported in all the trials, however, comparison between below 35 year-old and above 35 year-old was not possible. All the clinical trials reported the overall parity of all participants but did not provide details about the parity in each groups, so further comparisons were not possible. Gestational age at threatened miscarriage was not avaliable, so comparison between first trimester and second trimester was not possible. All of the Chinese medicines and the supplements were standard formulae as stated in the "Chinese Phamacopeia", so no subgroup analysis was carried out. As to the treatment course, Chen's study (Chen et al., 2002) stopped the treatments around 12 or 20 weeks of gestation, Feng's (Feng ZR, 1997) study did not report the termination of treatment but evaluated the

effectiveness after the relief of clinical signs, Lv's study (Lv et al., 2007) reported one week as one course but did not give details on the total amount of the courses for the treatment, Zhong's study (Zhong et al., 2002) reported that the treatment was stopped at 2 weeks after clinical sign relieved. Therefore, it's difficult to extract the data and carry out analysis on the duration of intervention for the effectiveness. There was no quasi-randomised clinical trial in the included studies.

5.5 Discussion

5.5.1 Efficacy in General

Chinese medicines have been claimed to be effective and are well accepted as an alternative treatment for pregnancy. Amongst the 133 selected clinical trials of Chinese medicines for threatened miscarriages, the overall average efficacy rate is over 90%. Chinese medicines apparently can relieve the clinical signs, improve the laboratory outcomes, promote pregnancy and increase the survival of fetuses. As Chinese medicines are prescribed in formulae, its efficacy may only represent the effect of a formula as a whole, or may be due to the effect of individual medicine in the formula. In further analysis, there is no correlation between efficacy rates and dose and dosing of individual Chinese medicines. Thus, the most commonly used Chinese medicine formulae may not necessarily be associated with high efficacy, while frequent administration may not necessarily produce better efficacy.

5.5.2 Effectiveness

This chapter aimed to evaluate the therapeutic effects of Chinese medicines for threatened miscarriage. However, no placebo controlled trials could be reached, such

as bed rest, therefore, we could only partially studied on the effectiveness of Chinese medicine treatments, through the comparions amongst Chinese medicines, Western medicines, and combined medicines.

Finally only 4 randomized clinical trials were included in the meta-analysis. There were insufficient data to support the hypothesisthat Chinese medicines alone were more effective than Western medicines, and indicated that combined Chinese and Western medicines were more effective than other pharmaceuticals (Chinese medicines alone or Western medicines alone) in the treatment of threatened miscarriage to prevent inevitable miscarriage and continue pregnancy after 28 weeks of gestation. Another 26 trials, which did not follow up women till 28 weeks of pregnancy, indicated that Chinese medicines alone were more effective than Western medicines alone to treat threatened miscarriage. Chinese medicines alone or Chinese medicines combined with Western medicines were more effective than Western medicines alone in relieving the clinical signs of threatened miscarriage, including vaginal bleeding, low back pain and abdomen pains. The result confirmed the therapeutic effects of Chinese medicines alone and combined with other pharmaceuticals for threatened miscarriage.

Though the review favored Chinese medicines for threatened miscarriage, it must be remembered that different studies used different Chinese medicines. Most Chinese Medicine practitioners slightly modify the standard prescriptions depending on the presentations of individual patients. In the 30 selected clinical trials, 23 trials of which used a common prescription of Shou Tai Pill as basic formula, while the other 7 trials used different prescriptions. Some Chinese medicines have been added into or removed from the standard formula during the treatment. Therefore, it further suggested that the effectiveness of Chinese medicines for threatened miscarriage could only represent for the general effects of Chinese medicines, but not the effects of one Chinese formula or individual Chinese medicines.

One thing needs to be emphesised is that Western medicines, such as Human chorionic gonadotropin (HCG) which maintains the luteotrophic effects after Luteinizing hormone (LH) secretion decreases, to support continued secretion of estrogen and progesterone and preventing menstruation, were not considered as claasical therapies for threatened miscarriage, (Devaseelan et al., 2010; Wahabi et al., 2011). So the conclusion of the effectiveness of Chinese medicines is limited. As in most cases, doctors would suggest the patients to have bed rest first, although it also has no significant effects in altering the course and progress of miscarriage (Aleman et al., 2005). Therefore, more placebo controlled trials are necessary to give overall view on the effectiveness of Chinese medicines.

5.5.3 Adverse Effects

Chinese medicines have been commonly applied to treat threatened miscarriage in attempts to promote maternal health and embryo-fetal development. Most of the included clinical trials did not report whether there were side effects of Chinese medicines during or after the treatments. Very few clinical trials followed-up the pregnancy after birth. So no conclusion on the safety of Chinese medicines for mothers and fetuses could be achieved in this review. Xiao's study (Xiao ZX, 2008) mentioned that all the participants were followed up after 1 month of delivery, but did not provided further information in the result. Zhang's study (Zhang H, 2008b) reported that no complications occured during the deliveries and all the infants developed well both mentally and physically in the half year following-up. Song's study (Song et al., 2007) reported that there were no congenital malformations of the newborns and no adverse effects occured during the treatments, but did not provided the detailed data. Li's study (Li et al., 2006) reported detailed information and data on the percentage of follow-ups, term delivery and preterm delivery, Apgar scores of newborns and average newborn weight. Thus, only 1 in 4 trials was available but no further analysis could be carried out. Therefore, the confirmation of its safety claims

is still pending. It is also necessary to study the potential adverse effects on mothers and fetuses of the Chinese medicines.

5.5.4 Study Limitation

Regarding the design of the included clinical studies in Chinese medicines for threatened miscarriage, there are still some limitations. Firstly, well-conducted randomized controlled trials are important for meta-analysis. All the selected trials in this review have inadequate methodology quality. What's more, the quality of each clinical trial was obviously not at the same level. As to the 4 included trials for the primary outcome analysis, the authors all mentioned that the participants were randomized into either the control groups or intervention groups, but none of them reported the detailed methods for randomisation. As to the other non-prespecified 26 trials, only 8 trials reported with adequate sequence generation, "visiting sequence" (Chen LZ, 2003), "layered method" (Li et al., 2004), "randomized number table" (Li et al., 2006, Xiao ZXet al., 2008, Zhang XM, 2008a), and "visiting date" (Liu SM, 2008, Song et al., 2007), "2:1 ratio randomization" (Yang MQ, 2006). None of the 30 trials reported the blinding method, but from the descriptions of methods reported in each trial we believed that all studies are open to both the researchers and the patients. Therefore, it would be greatly helpful to improve the quality of analysis if the authors were adequately trained to carry out and report such clinical trials according to the international standard, including sufficient randomization method and adequate allocation concealment, double-blinded participants and researchers or outcome assessors, participants' classifications, and effects assessments. Secondly, a better clinical trial should also provide some essential information, such as the average days or weeks of the treatments, the changes on the medicines dosage and compositions, the amount of participants with a successful pregnancy till 28 weeks or afterwards, and the mortality and follow-ups of newborns, which would be helpful to examine the effects of Chinese herbal medicines in the treatment. Last but not least, small numbers

of qualified clinical trials and insufficient information in this review did limit us to conduct further sub-group analysis, more details and information of the clinical trials are essential to further understand the effects of Chinese medicines in the analysis.

5.6 Summary

No placebo controlled trials could be identified. No conclusion can be made to assess the effectiveness of Chinese Medicines for threatened miscarriage. The potential harm and long-term effects to the mother or child, or both, with the use of Chinese herbal medicines in the treatment of threatened miscarriage was lacking.

Chapter VI

Safety of Chinese Medicines for Pregnancy: Systematic Review & Meta-analysis

6.1 Introduction

Chinese medicines have been applied in China as one of the main medical therapies in various clinical areas for a long time. However, it is mostly considered as an alternative method of treatment in other countries where Western medicines domainate. One important factor, to limit its wide application as a formal medical method, is that most Western Medicine practitioners have little knowledge, in particular on its maternal and fetal toxicity.

6.1.1 Toxicity of Chinese Medicines

Toxicity is the degree to which a substance can harm humans or animals (Osweiler GD, 1996; Hodgson E, 2010). Toxicity can refer to the effect on a whole organism, such as bacterium, animal, or plant; as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ (organotoxicity), for example, the liver (hepatotoxicity). A central concept of toxicology is that specific toxic effects are dose-dependent (ECETOC, 2004); even water can lead to water intoxication when taken in large enough doses. In Traditional Chinese Medicine, toxicity has the same meaning as in Western Medicine, referring to the degree that a substance can harm human and/or animal.

Before the western Han Dynasty, regardless of "Drug" (藥) and "Poison" (毒), all medicines will be referred to as "Toxicant" (毒藥) (Yan ZH, 2006). In eastern Han Dynasty, all medicines were divided into "Toxic" (有毒) and "Non-toxic" (無毒) according to the strength of their properties recorded in "Herbal Classic" (本經) (Yan ZH, 2006). With the progress and development of medical practice, people gradually found that some medicines could cure illness but at the same time may harm the body. Therefore, after the eastern Han Dynasty, all the medicines cause harm were labeled with "Toxic", and documented the performances of its toxicity in ancient herbal medicine books (Li et al. 2005).

6.1.2 Causes of Chinese Medicine Toxicity

6.1.2.1 Over dose

If the Chinese medicines are taken in a higher than conventional dosage or a very large dose in a single administration, toxicity effects occur very quickly. For example, it was reported that diarrhea occurred when overdosing Barbary Wolfberry Fruit (枸杞子, Fructus Lycii) (Li et al., 2005).

6.1.2.2 Prolonged administration

Accumulated toxicity could happen, if the duration of Chinese medicine treatment is too long. For example, long term use of Threewingnut Root (雷公藤, Radix et Rhizoma Tripterygii) can do harm to reproductive glands, and lead to amenorrhea in women and impotence in men (Zhang L, Song WX, 2010).

6.1.2.3 Improper processing

Most toxic Chinese medicines are still in use to treat certain illnesses, because their toxic properties can be removed or reduced after processing. With a wrong processing method, its toxicity will occur. For example, Monkshood Mother Root (川乌, Radix Aconiti) is a toxic Chinese medicine and must be cooked for long time before use to degrade the toxic substance inside the root (Jiang ZB, 2007).

6.1.2.4 Incorrect compatibility

Chinese Medicine practitioners use "Inquiry, Inspection, Hearing, Palpation" to make diagnoses and prescribe Chinese medicines in formulae according to the "Jun, Chen, Zuo, Shi" Principle (which stands for the character of each involved single medicine as Monarch, Minister, Assistant and Guide) (Li J, 2006). As each of the Chinese medicine has its own property and potential interactivity with other medicines, the application of this principle will decrease or avoid the side effects of some herbs, enhance the therapeutic functions of some herbs and make all the herbs collaborate to create a more harmonious therapeutic effect on the human body and more direct impact for the treatments. Incompatibility could occur when the combination of certain herbs produce side effects or become poisonous, which may lead to reduction of the efficacy, or even

produce adverse reactions.

The general rules for incompatibility are Shi Ba Fan (十八反) and Shi Jiu Wei (十九畏), according to the property of each Chinese medicine involved (Li et al; 2005).

In Shi Ba Fan, the following Chinese medicines should be contradicated to apply in one formula, as the property of one medicine would cause opposite function to another medicine, and severe toxicity has been reported.

(1) Liquorice Root (甘草, Radix Glycyrrhizae) should not be used together with Gansui Root (甘遂), Knoxia Root (大戟), Seaweed (海藻), Lilac Daphne Flower Bud (芫花). For example, when Liquorice Root was used together with Gansui Root, different degree of heart damages would be caused according to the ratios of each medicine (Zhang et al., 2007).

(2) Monkshood (烏頭) should not be used together with Bulb of Thunberg Fritillary (貝母), Snakegourd Fruit (瓜蔞), Pinellia Tuber (半夏), Japanese Ampelopsis (白蘞), Common Bletilla Pseudobulb (白及). The enzyme activity CYP1A2 and CYP2E1 decreased under intervention of combined Monkshood and Bletilla Pseudobulb, which would further induce the dysfunction of digestion in rats (Jin et al., 2007).

(3) Falsehellebore Root and Rhizome (藜蘆) should not be used together with Gin Seng (人參), Lightyellow Sophora Root (苦參), Coastal Glehnia Root (沙參), Dan Shen Root (丹參), Figwort Root (玄參), Manchurian Wildginger (細辛), Paeony Root (芍藥). For example, animal death was reported when Manchurian Wildginger and Falsehellebore Root were prepared together (Zhou ZX, 2008).

In Shi Jiu Wei, 19 herbal medicines are not recommended to be used together with other medicines, as the property of one medicine inhibit the function of another medicine, and side effects were recorded.

(1) Sulphur (硫黃) and Po Qiao (朴硝, Mirabilite), otherwise severe abdominal pain could be caused, as both have the function as cathartic drugs (Chang et al., 1985).

2) Mercury (水銀) and Arsenic (砒霜) could cause severe chemical reaction if used together (Li et al., 1998).

(3) Chinese Stellera Root (狼毒) and Lithargite (密陀僧), dysphoria were recorded in mice studies (Chang et al., 1985).

(4) Croton Seed (巴豆) and Pharbitis Seed (牽牛). When these two medicines were applied together, toxicity of each medicine was increased, and over 90% mortality was recorded in mice studies (Chang et al., 1985).

(5) Clove (丁香) and Turmeric Root-tuber (鬱金). Diarrhea was observed in mice experiments (Chang et al., 2005).

(6) Snakegourd Fruit (川烏), Kusnezoff Monkshood Root (草烏) and Black Rhinoceros (犀角). When the latter two medicines were used together, tachypnea and dysphoria were recorded in mice experiments and high mortality was observed (Chang et al., 2005) and dysfunction of immune system was also recorded (Mao et al., 1997).

(7) Ya Qiao (牙硝, Mirabilite) and Burreed Rhizome (三棱). All study mice died in 12 hours of intraperitoneal injection with this combination (Chang et al., 2005).

(8) Chinese cassia (官桂) and Red Halloysite (赤石脂). Loss of appetite and quiescence were observed after combination and administration to mice (Chang et al., 2005).

(9) Gin Seng(人參) and Trogopterus Dung (五靈脂). Each of them has the therapeutic function on anti tiredness; however, the overall effect of the combination was reported worse than singly used (Guo et al., 1994).

6.1.2.5 Preparation error

Chinese medicines usually are prepared as soup, pill, tablet, granule, wine, cream, needle and syrup, but actually some Chinese medicines can present different medical or toxic effects if prepared

improperly. For example, the toxicity of Monkshood Daughter Root (附子, Radix Aconiti Laterlis) will significantly increase if prepared as wine (Wu XL, 2010).

6.1.2.6 External usage

Chinese medicines can be absorbed through skin and mucous membranes resulting in toxicity, if used externally for long term or big area. For example, purging Croton Fruit (巴豆, Fructus Crotonis), usually externally used to relieve pimples, but long term usage could cause skin hurt.(Li et al. 2005).

6.1.2.7 Mistaken or misused

Some Chinese medicines look similar. For example, Gin Seng is a very common Chinese medicine also in western nations, and is famous for its various benefits to human body and health. But it looks similar to Pokeberry Root (商陆, Radix Phytolaccae), which was reported to cause thrombocytopenia with frequent gum bleeding and epistaxis, then unconsciousness and polyuria in rabbits (Li et al., 2005), and even death in cats (Li et al., 2005). If mistaken, it might result in severe adverse outcomes.

6.1.3 Classification of Toxicity in Chinese Medicines

Most Chinese Medicine practitioners, and also Western Medicine practitioners, agreed with "Chinese Pharmacopoeia" on the classification of toxicity for herbal medicines. "Chinese Pharmacopoeia" divides all Chinese medicines into three toxicity levels (Chan et al., 2010).

(1) "Severe Toxicity" -- the toxic symptoms are very severe, result in damage to vital organs and death, such as Snakegourd Root (天花粉, Radix Trichosanthis Kirilowii);
(2) "Mild Toxicity" -- the toxic symptoms happen due to long term usage or abuse of drugs, result in injuries to important organs, even lead to deaths, such as Realgar (雄黄, Realgar);
(3) "Little Toxicity" -- the toxic symptoms are slight, not leading to injuries to organs, such as Bitter Apricot Seed (苦杏仁, Semen Armeniacae Amarum).

The rest of medicines were considered as "Non-Toxic", such as Baical Skullcap Root (黄芩, Radix Scutellariae).

Despite the classification mentioned above, there was no standard approach to identify the toxicity of Chinese herbal medicines. Fortunately, by personal experience, Chinese practitioners summarized some Chinese medicines that should be forbidden or cautiously used during pregnancy, which have been recorded in "Chinese Pharmacopoeia", which have been now widely accepted and took as references by most clinical workers in their daily work.

6.1.4 Reproductive Toxicity and Embryotoxicity of Chinese Medicine

6.1.5.1 Reproductive toxicity

According to United Nations Economic Commission for Europe (UNECE), reproductive toxicity has been defined as adverse effects of chemicals that would interfere with on the male and/or female reproductive ability or capacity," including effects on lactation. FDA apprises the potential hazards to a fetus associated with taking the category D (positive evidence of human fetal risks based on adverse reaction data from investigations or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks) and category X (demonstrated fetal abnormalities in animals or humans and/or there is positive evidence of human fetal risk based on adverse reaction data from investigations or marketing experience, and the risks involved in use of the drug in pregnant women clearly outweigh potential benefits). The European Medicines Agency (EMEA) restricts the use of drugs which are known to be teratogenic during pregnancy and prevention of pregnancy may be the only option. The British National Formulary (BNF) recommends all drugs should be prescribed in pregnancy only if the expected benefit to the mother is thought to be greater than the risk to the fetus. Otherwise it should be avoided if possible during the first trimester. No related reproductive toxicy of Chinese medicine is available unfortunately.

6.1.5.2 Embryotoxicity

Organisms during early development undergo rapid and complex changes within a relatively short period of time. Therefore, in mammals any agent administered during pregnancy to the mother may interfere with embryonic development and induce embryo lethality, growth retardation or teratogenic effects, with structural and functional abnormalities in the offspring. Such pathological outcomes persist and are defined as embryotoxic effects (Cunningham et al., 2005).

In Western Medicine, embryotoxicity of pharmaceuticals can be classified into "Strongly Embryotoxic", "Weakly Embryotoxic", and "Non-Embryotoxic". However, there is no definite criterion for classification of embryotoxicity in Chinese Medicine. It is also considered that the Chinese medicines with either severe or moderate toxicity on embryos should be prohibited during pregnancy.

6.2 Aims and Objectives

In this chapter, we aimed to review the available data of the Chinese medicines for threatened miscarriage and its potential adverse events, including side effects and toxicity to mothers and fetuses in order to to give overall information about the safety of Chinese herbal medicines and no regulation is established to monitor and control its clinical application, particularly during pregnancy.

The specific objectives were

1, To select and evaluate the clinical studies of Chinese medicines for threatened miscarriage with reported side effects and toxicity.

2, To determine the adverse effects and toxicity of Chinese medicines for threatened miscarriage.

6.3 Methods

6.3.1 Search Strategies

Since threatened miscarriage was the most common application of Chinese medicines during

pregnancy (Chapter V), we focused on the adverse outcomes with Chinese medicine treatments for threatened miscarriage in this chapter. Our search methods and strategies were same as previous chapter, adverse effects of the clinical trials were further evaluated and selected for analysis.

Search strategy for Cochrane

1. (Chinese medicine) or (traditional medicine) or (TCM)
2. (application) or (clinical use) or (therapy) or (treatment)
3. (disorder) or (illness) or (complication) or (disease)
4. 1 and 2 and 3
5. women or woman or female
6. pregnancy or obstetric or gestation
7. 5 and 6
8. 4 and 7
9. herb or herbal medicine or drug or Chinese drug
10. acupuncture or massage
11. 8 and 9 but 10
12. (human study) not (animal study) not (chemical study) not (gene study) not (microbiology study)
13. 11 and 12
14. miscarriage or abortion
15. (threatened abortion) or (threatened miscarriage)
16. therapeutic or recurrent or complete or incomplete or inevitable or missed
17. 14 and 15 but 16
18. (toxic) or (safety) or (adverse effect) or (side effect)
19. 17 AND 18

Search strategy for PREMEDLINE and MEDLINE

1. exp Medicine/ or exp Drugs, Chinese Herbal/ or exp Medicine, Chinese Traditional/ or exp Plants, Medicinal/ or Chinese medicine.mp. or exp Medicine, East Asian Traditional/
2. application.mp. or exp Medical Informatics Applications/ or exp Emergency Treatment/
3. 1 and 2

4. exp Pregnancy/
5. exp obstetric/
6. exp gestation/
7. 4 or 5 or 6
8. 3 and 7
9. exp Drugs, Chinese Herbal/
10. (chin$ adj 6 herb$).mp.
11. herb.mp. or exp Plants, Medicinal/ or exp Herb-Drug Interactions/ or exp Plant Extracts/ or exp Drugs, Chinese Herbal/
12. 9 or 10 or 11
13. 12 and (Humans/)
14. abortion, threatened.mp.
15. exp Abortion, Spontaneous/
16. miscarriage.mp.
17. spontaneous near abortion.mp.
18. spontaneous near pregnancy loss
19. threatened near pregnancy loss
20. abortion near threatened
21. 14 or 15 or 16 or 17 or 18 or 19 or 20
22. 13 and 21
23. (toxicity.mp.) or (safety.mp.) or (adverse.mp.) or (side.mp.)
24. 22 AND 23

Search strategy for PUBMED

1. (("Chin Med"(Journal) OR ("chinese"(All Fields) AND "medicine"(All Fields)) OR "chinese medicine"(All Fields)) OR ("medicine, traditional"(MeSH Terms) OR ("medicine"(All Fields) AND "traditional"(All Fields)) OR "traditional medicine"(All Fields) OR ("traditional"(All Fields) AND "medicine"(All Fields))))
2. ((("therapy"(Subheading) OR "therapy"(All Fields) OR "treatment"(All Fields) OR "therapeutics"(MeSH Terms) OR "therapeutics"(All Fields)) OR ("therapy"(Subheading) OR

"therapy"(All Fields) OR "therapeutics"(MeSH Terms) OR "therapeutics"(All Fields))) OR clinical(All Fields)) OR application(All Fields)

3. ((("disease"(MeSH Terms) OR "disease"(All Fields) OR "disorder"(All Fields)) OR illness(All Fields)) OR ("disease"(MeSH Terms) OR "disease"(All Fields))) OR complication(All Fields)
4. 1 AND 2 AND 3
5. ((("pregnancy"(MeSH Terms) OR "pregnancy"(All Fields)) OR ("pregnancy"(MeSH Terms) OR "pregnancy"(All Fields) OR "gestation"(All Fields))) OR obstetric(All Fields)) NOT ("gynaecology"(All Fields) OR "gynecology"(MeSH Terms) OR "gynecology"(All Fields))
6. (((((herb(All Fields) OR ("herbal medicine"(MeSH Terms) OR ("herbal"(All Fields) AND "medicine"(All Fields)) OR "herbal medicine"(All Fields))) OR (herbal(All Fields) AND drug(All Fields))) OR "chinese"(All Fields)) AND herb(All Fields))) NOT ("massage"(MeSH Terms) OR "massage"(All Fields))) NOT ("acupuncture"(MeSH Terms) OR "acupuncture"(All Fields) OR "acupuncture therapy"(MeSH Terms) OR ("acupuncture"(All Fields) AND "therapy"(All Fields)) OR "acupuncture therapy"(All Fields))
7. ("humans"(MeSH Terms) OR "humans"(All Fields) OR "human"(All Fields)) OR (("humans"(MeSH Terms) OR "humans"(All Fields) OR "human"(All Fields)) AND ("clinical trials as topic"(MeSH Terms) OR ("clinical"(All Fields) AND "trials"(All Fields) AND "topic"(All Fields)) OR "clinical trials as topic"(All Fields) OR "study"(All Fields)))
8. ("animals"(MeSH Terms:noexp) OR animal(All Fields)) OR ("J Mol Catal A Chem"(Journal) OR "chemical"(All Fields)) OR ("genes"(MeSH Terms) OR "genes"(All Fields) OR "gene"(All Fields)) OR ("microbiology"(Subheading) OR "microbiology"(All Fields) OR "microbiology"(MeSH Terms))
9. 6 AND 7 NOT 8
10. ("abortion, spontaneous"(MeSH Terms) OR ("abortion"(All Fields) AND "spontaneous"(All Fields)) OR "spontaneous abortion"(All Fields) OR "miscarriage"(All Fields)) OR ("abortion, induced"(MeSH Terms) OR ("abortion"(All Fields) AND "induced"(All Fields)) OR "induced abortion"(All Fields) OR "abortion"(All Fields))
11. threatened/abrupt(All Fields) OR threatened/actual(All Fields) OR threatened/actually(All Fields) OR threatened/endangered(All Fields) OR threatened/exposed(All Fields) OR

threatened/forced(All Fields) OR threatened/injured(All Fields) OR threatened/involved(All Fields) OR threatened'(All Fields)

12. ("therapeutics"(MeSH Terms) OR "therapeutics"(All Fields) OR "therapeutic"(All Fields)) OR ("recurrence"(MeSH Terms) OR "recurrence"(All Fields) OR "recurrent"(All Fields)) OR complete(All Fields) OR incomplete(All Fields) OR missed(All Fields) OR inevitable(All Fields)
13. 1 AND 2 BUT 3
14. ("toxicity"(MeSH Terms) OR ("safety"(MeSH Terms) OR "adverse effect"(All Fields) OR "side effect"(All Fields)
15. 13 AND 14

Search strategy for EMBASE

1. TCM.mp.
2. exp traditional medicine/
3. exp Chinese medicine/
4. 1 or 2 or 3
5. application.mp.
6. clinical.mp.
7. therapy.mp. or THERAPY/
8. treatment.mp
9. 5 or 6 or 7 or 8
10. disorder.mp.
11. illness.mp. or exp "general aspects of disease"/
12. disease.mp. or exp "general aspects of disease"/
13. exp COMPLICATION/
14. 10 or 11 or 12 or 13
15. 4 and 9 and 14
16. (women or woman or female).mp. (mp = title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer)
17. exp PREGNANCY/
18. exp OBSTETRICS/th (Therapy)

19. exp gestation.mp. /ae, co, dt (Adverse Drug Reaction, Complication, Drug Therapy) /
20. 15 and (16 or 17 or 18 or 19)
21. herb or herbal or Chinese herb*).mp. (mp = title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer)
22. (drug or medicine).mp. (mp = title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer)
23. 20 and (21 and 22)
24. exp spontaneous abortion/
25. exp abortion/
26. 1 OR 2
27. (threatened or threatened msicarriage).mp. (mp = title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer)
28. 26 and 27
29. (toxicity).mp. (mp = title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer)
30. (safety).mp. (mp = title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer)
31. 28 AND 29 AND 30

Search strategy for CJN (Chinese)

1. (subject = Chinese medicine*(therapy application+ clinical use)) OR (subject = traditional medicine) OR (subject = TCM)
2. (subject = pregnancy) OR (subject = female) OR (subject = gestation) OR (subject = obstetric*(complication))
3. 1 AND 2
4. (subject = miscarriage) OR (subject = abortion)
5. (subject = threatened)
6. (subject = therapeutic) OR (subject = recurrent) OR (subject = recurrent) OR (subject = complete) OR (subject = incomplete) OR (subject = inevitable) OR (subject = missed)
7. 1 AND 2 BUT 3

8. (subject = toxicity) OR (subject = safety) OR (subject = adverse effect) OR (subject = side effect)
9. 7 AND 8

Search strategy for WanFang Database (Chinese)

1. TCM OR (traditional medicine) OR (Chinese medicine)
2. application OR (clinical use) OR therapy
3. 1 AND 2
4. women OR woman OR female
5. pregnancy OR obstetric OR gestation
6. 4 AND 5
7. 3 AND 6
8. miscarriage OR abortion
9. (threatened abortion) OR (threatened miscarriage)
10. 8 AND 9
11. toxicity OR safety OR (adverse effect) OR (side effect)

To further characterise the pharmacological and toxicology data of the Chinese medicines, several online national and public resources on World Wide Web were also referred. It included Center for Food Safety and Applied Nutrition (CFSAN) from US Food and Drug Administration (FDA, (http://vm.cfsan.fda.gov/~dms/ supplmnt.html), National Center for Complementary and Alternative Medicine (NCCAM) from US National Institute of Health (NIH, http://nccam.nih.gov), Agricultural Research Service (ARS) from US Department of Agriculture (USDA, http://www.ars-grin.gov/duke), Medical Dictionary for Regulatory Activities (MedDRA) from International Federation of Pharmaceutical Manufacturers and Associations (IFPMA, http://www.meddramsso.com), National Council Against Health Fraud (NCAHF) from a private health agency (http://www.ncahf.org), Quackwatch from an American non-profit organisation (http://www.quackwatch.com), HerbMed from Alternative Medicine Foundation (http://www.herbmed.org) and ConsumerLab from an independent laboratory (http://www.consumerlab.com), accessibility verified until 31st Dec. 2010 (Michael et al., 2001).

Search Strategies for Online Databases

Search by subject heading/keyword/abstract/full text with:

1. Traditional Chinese Medicines
2. threatened miscarriage treatment
3. western medicines
4. comparisons studies
5. safety
6. toxicity

Or could be included or replaced by similar words:

1. herbal medicines
2. pharmaceuticals
3. miscarriage
4. spontaneous abortion
5. therapy

Only clinical trials which assessed the adverse pregnant outcomes of the Chinese medicines were further selected for meta-analysis.

6.3.2 Study Criteria

6.3.2.1 Types of studies

All published clinical studies that evaluated the safety of Chinese medicines for threatened miscarriage were considered. Studies of Chinese medicines for other clinical applications and in animal, chemical and basic research were excluded. The therapeutic practices of Traditional Chinese Medicine other than Chinese medicines were also excluded. Case reports, commentary articles and non-systematic reviews were then excluded. Clinical studies with no evaluation or incomplete records of adverse pregnancy outcome were further excluded. Only case controlled studies with or without randomisation were included for meta-analysis. For randomized studies, blinded randomized, quasi-randomised and cluster-randomised, were included. Clinical studies without case controlled, including observational and prospective cohorts, were also included for

pooled analysis if there would have been an invalid meta-analysis due to no or too few case controlled studies.

Language of the publications is was restricted to English. Literature with either Chinese or English abstract should be available for initial search. No translation was required for Chinese articles as all review assessors can read Chinese and understand Traditional Chinese Medicine and Chinese medicines thoroughly. Translations were only sought from the language facilities of the university for articles written in English and Chinese.

6.3.2.2 Types of participants

All pregnant women with a viable pregnancy diagnosed with threatened miscarriage and no treatment before interventions were studied. Fetal viability was assessed by ultrasound to ensure exclusion from this review of studies which included women with inevitable, incomplete, missed or recurrent miscarriage and vaginal bleeding before the 20^{th} week of pregnancy. Women regardless of whether the pregnancy was singleton, twin or multiple, and irrespective of the maternal age and parity were included. All the pregnancies followed up immediately after interventions or until delivery were recorded.

6.3.2.3 Types of interventions

Only Chinese medicines recorded by the Chinese Pharmacopeia with well characterised principles of pharmacological and medicinal applications were included. Other pharmanutrients from various herbal agents and products, e.g. green tea and ginger, widely used as daily pharmanutrients for general health were excluded. Since Chinese medicines are crude drugs of plant, animal and mineral origins, not only those Chinese medicines originated from plants or herbs but also those from animals and minerals were included. All types of Chinese medicine in either standard or combined formulas used in the treatment of threatened miscarriage regardless of the dose or duration of administration. Selected clinical studies which compared Chinese medicines with placebo, no treatment, or other pharmaceuticals were identified.

6.3.2.4 Types of outcome measures

Adverse pregnancy outcomes in both mothers and fetuses/infants will be recorded. Maternal outcomes included (1) toxicity (e.g. renal failure, liver failure, neurological impairment and death); (2) side-effects (e.g. anaphylaxis, gastrointestinal disturbance, hypertension/hypotension, cardiac arrhythmia, gestational diabetes, etc); (3) pregnancy loss (e.g. late abortion, intrauterine death and still birth); and (4) pregnancy complications (e.g. preterm/postdate labour, placenta previa, placenta abruptio, etc). Fetal outcomes included (5) perinatal mortality (including prenatal and postnatal death); (6) toxicity (e.g. fetal compromise, neurological consequences, hydrops fetalis, etc); (7) congenital malformations; and (8) neonatal complications (e.g. jaundice, infection, hypoglycaemia, etc).

6.3.3 Data Collection and Analysis

To determine the clinical studies to be involved in this review, all review assessors firstly screened the titles, abstract sections, and keywords of every record to exclude the duplicates and obvious false positive. Secondly, full text of potentially eligible studies was assessed for inclusion or exclusion. If there was sufficient information and it met the inclusion criteria, the study was included in the analyses. Two review assessors assessed the studies for inclusion independently; any disagreement was resolved by discussion among all the review authors. The study authors were contacted for clarification if there were doubts about the eligibility of the study and the disagreement could not be resolved. The review authors were not blinded to the journal of origin or institution.

6.3.4 Data Extraction and Management

Extraction form was designed and used to extract data. For eligible studies, two review authors extracted the data, any discrepancy was resolved through discussion or the third person was consulted. For each selected literature, publication year, study population, participant numbers, maternal age, gestation age, symptoms and signs, clinical diagnosis, examination and laboratory results, disease course, study intervention, standard or modified Chinese medicine formulas,

individual medicine, immediate and follow-up outcomes were recorded. All the extracted data were entered into Review Manager software (RevMan5 2010) to ensure the accuracy, comprehensiveness and consistency for analysis.

6.3.5 Quality Controls and Risk of Bias in Included Studies

Two review authors (Li Lu and one from Cochrane PCG group) independently assessed the quality and the risk of bias for each selected study according to the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2009). Quality control of the randomized controlled studies were assessed using a 11-item checklist modified from the revised Consolidated Standards of Reporting Trials (CONSORT) statement (Moher et al., 1998), with 2 items specific requirement for Chinese Medicine studies including herb preparation form and quality control of herbs (Bian et al., 2006). Quality control of the non-randomized controlled studies, the risk of bias in sequence generation, allocation concealment, blinding, incomplete outcome data and selective reporting bias were assessed. Any disagreement was also resolved by discussion or by involving the third assessor. Only the controlled clinical studies with at least "good" overall quality and non-controlled clinical studies with at least "fair" overall quality were included for data analysis.

6.3.6 Data Analysis

For dichotomous outcomes, we counted the number of adverse events and the involved participants in each study. For continuous outcomes, we calculated the mean and standard deviation of the measures if appropriate. Dichotomous data were expressed as relative ratio (RR) with 95% confidence intervals (CI) while continuous data were expressed as weighted mean differences (WMD) by the meta-analysis in RevMan5. We assessed statistical heterogeneity using the T^2, I^2 and λ^2 statistics. We regarded heterogeneity as substantial if T^2 is greater than zero and either I^2 is greater than 30% or there was a low ($p<0.10$) in the λ^2 test for heterogeneity. Potential bias was tested using the funnel plot or other corrective analytical methods depend on the number of clinical studies included in the systematic review (Egger et al., 1997).

For potential sub-group analysis, classifying whole studies by interaction tests were conducted for fixed-effect meta-analyses. Differences between subgroups by inspection of the subgroups' confidence intervals; non-overlapping confidence intervals indicate a statistically significant difference in treatment effect between the subgroups were assessed of random-effects meta-analyses. To explore the effect of trial quality for important outcomes in the review, sensitivity analysis was conducted as well when sufficient clinical studies were available.

6.4 Results

6.4.1 Search Results and Study Inclusion

Since the most common applications of Chinese medicines for pregnancy was threatened miscarriage, we focused on the safety of Chinese medicines as treatment to threatened miscarriage. As from previous chapter, 197 (49.0%) clinical trials were included for further study inclusion and exclusion (Figure 6.1). Most clinical trials (68.5%) were not controlled in that no other intervention group were included for comparison. There were 62 (31.5%) controlled trials whilst 44 (71%) were randomized controlled and 18 (29%) were case controlled. Unfortunately, most of the clinical trials did not record any adverse pregnancy outcome, 88.9-95.5% for controlled trials and 92.6% for non-controlled trials. Finally, only 1 randomised controlled study and 1 case-controlled study with complete records in each intervention group were selected for meta-analysis; and 10 cohort studies were selected for quantitative analysis.

For the selected clinical trials of Chinese medicines for threatened miscarriage, fetal viability were assessed by ultrasound to ensure exclusion from this review of studies which includes women with inevitable, incomplete, missed or recurrent miscarriage and vaginal bleeding before the 20^{th} week of pregnancy. All the pregnancies were followed up after interventions and the adverse pregnancy outcomes until delivery were recorded. Both the quality of the controlled trials and the non-controlled trials were good (Table 6.1).

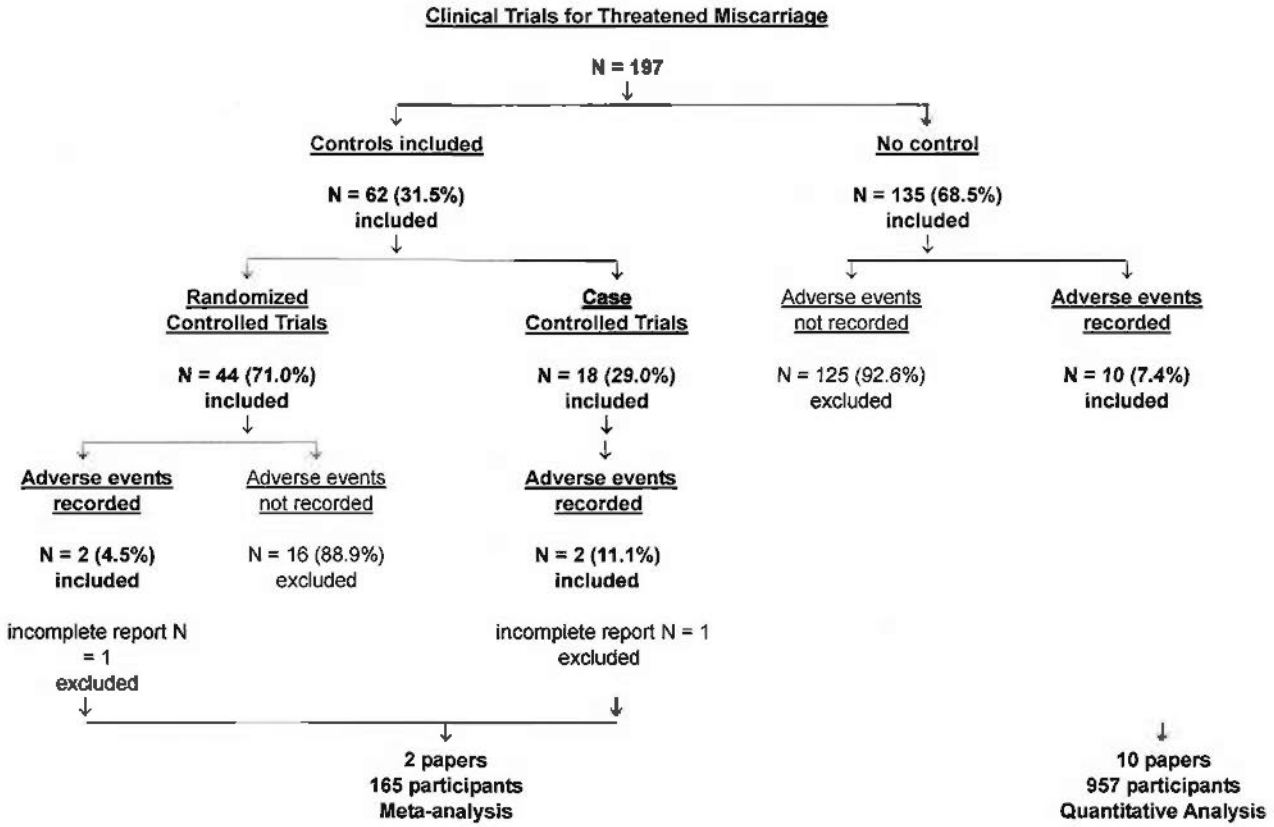


Figure 6.1 Study selection and inclusion

Table 6.1 Quality assessment of clinical trials

Studies	Randomization[a]	Blinding[b]	Follow-up[c]	Follow-up duration	Compliance[d]	Baseline Similarity[e]	Overall quality[f]
Controlled Clinical Trials							
Zhou Y 2006	Inadequate	Open	Good	delivery	Good	Good	Good
Song & Zhu 2007	Adequate	Open	Good	delivery	Good	Good	Good
Non-controlled Clinical Trials							
Wu ZG 1987	Inadequate	Open	Good	delivery	Good	NA	Good
Leng YH 1991	Inadequate	Open	Good	delivery	Good	NA	Good
Tian & Li 1991	Inadequate	Open	Good	delivery	Good	NA	Good
Wu & Ji 1994	Inadequate	Open	Good	delivery	Good	NA	Good
Zhou Y 1997	Inadequate	Open	Good	delivery	Good	NA	Good
Cui SH 1998	Inadequate	Open	Good	delivery	Good	NA	Good
Chen et al 2001	Inadequate	Open	Good	before delivery*	Good	NA	Good
Chou HG 2002	Inadequate	Open	Good	after delivery#	Good	NA	Good
Luo et al 2007	Inadequate	Open	Good	delivery	Good	NA	Good
Gu et al 2008	Inadequate	Open	Good	3 courses	Good	NA	Good

[a]adequate: clearly by computer, envelope or telephone; uncertain: reported randomization but without any approach and methods; inadequate: no randomization

[b]double-blinded, single-blinded, open or unclear with blinding of participants, caregivers and administrating treatment and outcome assessors.

[c]good: <5% loss; fair: 5-10% loss; poor: 10-20% loss and excluded from this review; failed: >20% loss and exclude from this review.

[d]good: >95% participants received the treatment exactly following the physicians' instructions; fair: 95-90%; poor: 90-80%; failed, <80%.

[e]good: no significant difference (p>0.05) between the participants in intervention groups and control group; unclear: not reported.

[f] Newcastle Ottowa Scale.

NA: not applicable.

* 15-18 weeks of treatment

Mostly after delivery, one case was followed up for 20 years

6.4.2 Adverse Pregnancy Outcomes

In 2 selected controlled studies, there were in total 165 participants compared Chinese herbal medicines with other intervention, mostly pharmaceuticals (Table 6.2). No placebo or without interventions was included for comparison. Comparison between Chinese medicines and pharmaceuticals (mainly Western medicines) were identified in the included controlled studies, however no adverse pregnancy outcome occurred in either group (Figure 6.2).

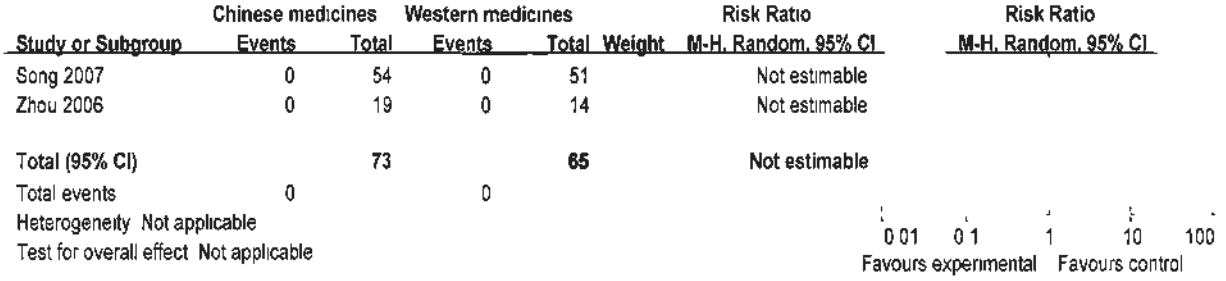


Figure 6.2 Forest plot of comparison: Chinese medicines alone versus Western medicines alone
Outcome reported adverse effects

Comparison between combined Chinese and Western medicines and Western medicines alone was identified in 1 case-controlled study. There was only one case reported of neonatal death due to preterm labor in combined intervention group (RR 2.14, 95% CI 0.09 – 49.08) (Figure 6.3). Heterogeneity analysis was not possible, hence meta-analysis and sub-group analysis could not conclude the safety of Chinese medicines for threatened miscarriage.

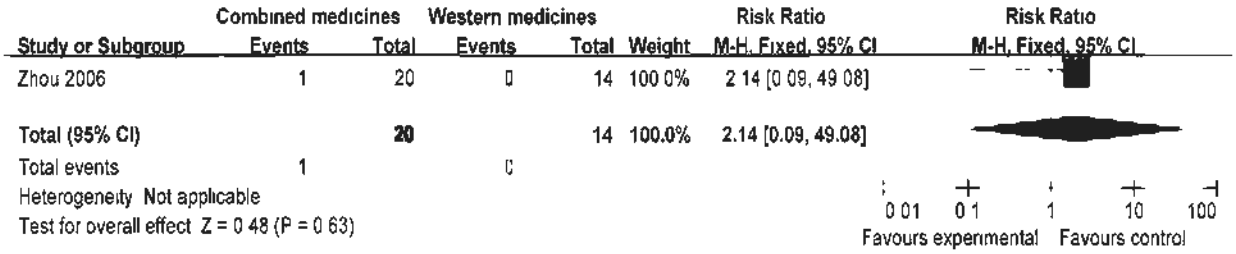


Figure 6.3 Forest plot of comparison: combined Chinese and Western medicines versus Western medicines alone
Outcome: reported adverse effects

Table 6.2 Adverse pregnancy outcomes of Chinese medicines in controlled clinical trials for threatened miscarriage*

Study	Control method (comparison#)	Gestational age mean±SD or range	Maternal age mean±SD or range	Chinese medicines dose range & dosing /day (participants)	Pharmaceuticals dosage & dosing /day (participants)	Adverse Outcomes incidence n/N (%)
Zhou et al., 2006	cohort (combined)	53±2 days	29±5 yr	Shou Tai Pill & Shi Xiao Pill 6-15g, QD, po (TCM: N = 19, combined: N = 20)	HCG 2000U, QD, im Progesterone 20mg, QD, im (N = 14)	preterm labor and death in combined group 1/20 (5)
Song et al 2007	randomized controlled (TCM vs WM)	5-8 weeks	20-38 yr	Zhi Xue Bao Tai Yin 2-10g, TID, po (N = 54)	Progesterone 20mg, QD, im Vit E 100mg, TID, po (N = 51)	no side effects 0/54 (0)

* 2 selected controlled clinical trials of Chinese medicines for threatened miscarriage with adverse outcomes recorded are included.

TCM: Chinese medicines group; WM: pharmaceutical group; combined: combined Chinese medicines and pharmaceutical group.

Table 6.3 Adverse pregnancy outcomes of Chinese medicines in non-controlled clinical trials for threatened miscarriage*

Studies	Gestational age mean±SD or range	Maternal age mean±SD or range	Chinese medicines Dose range & Dosing /day (participants)	Adverse Outcomes incidence n/N (%)	
				Maternal	Perinatal
Wu et al., 1987	NA	23-36 yr	New formula 10-20g, QD, po (N = 40)	NA	aspiration pneumonia & death, 1/40 (2.5)
Leng et al., 1991	NA	23-35 yr	Shou Tai Pill 15-20g, BID, po (N = 44)	NA	intra-uterine death, 3/44 (6.8)
Tian et al., 1991	1-4 months	24-41 yr	An Tai Yin 10-30g, QD, po (N = 34)	NA	oligohydramnios & stillbirth, 1/34 (2.9)
Wu et al., 1994	6-12 weeks	23-35 yr	Shou Tai Pill 6-30g, BID, po (N = 45)	no side effects 0/45 (0)	NA
Zhou et al., 1997	< 90 days	25-35 yr	An Tai Yin 6-12g, BID/TID, po (N = 305)	NA	preterm labor & death, 2/305 (0.7) epilepsy, 1/305 (0.3) mental retardation, 1/305 (0.3)
Cui et al., 1998	NA	24-32 yr	Shou Tai Pill 10-15g, QD, po (N = 47)	NA	preterm labor & death, 3/47 (6.4) premature rupture of membranes, asphyxia & death, 1/47 (2.1) incompatible blood group, jaundice & death, 1/47 (2.1)
Chen et al., 2001	6-8 weeks	NA	Zi Shen Yu Tai Pill 5g, TID, po (N = 231)	nausea, 9/231 (3.9) dry mouth, 2/231 (0.9) anorexia, 2/231 (0.9) constipation, 2/231 (0.9)	NA
Chou et al., 2002	NA	22-34 yr	New formula 10-30g, TID, po (N = 118)	NA	malformation (no details reported), 1/118 (0.9)
Luo et al., 2007	6-20 weeks	20-35 yr	Tai Er An Pill 10g/pack, TID, po (N = 58)	NA	hypertension & preterm labor, 1/58 (1.7)
Gu et al., 2008	8-16 weeks	22-33 yr	Le Yun Ning Decoction 10ml, TID, po (N = 35)	no side effects 0/35 (0)	NA

*10 selected non-controlled clinical trials of Chinese medicines for threatened miscarriage with adverse outcomes recorded are included.

Table S1 for detailed study quality assessments.

NA: not available.

In 10 selected non-controlled studies, there were in total 957 participants (Table 6.3). Quantitative analysis revealed that perinatal adverse outcomes were less frequent but more severe than maternal adverse outcomes. The incidence of every adverse event in pregnancies and newborns was calculated. The overall perinatal mortality and prematurity occurred in 8 (0.84%) and 7 (0.73%) of live births, respectively. The overall incidences of congenital malformation (non-specific in the article) and complicated neurological consequences (mental retardation and epilepsy) were just 1 (0.1%). Maternal nausea occurred in 0.9% of pregnant women and dry mouth, anorexia, constipation developed in 0.2% and hypertension was detected in 0.1% of pregnant women under the Chinese herbal medicines treatment. (Figure 6.4)

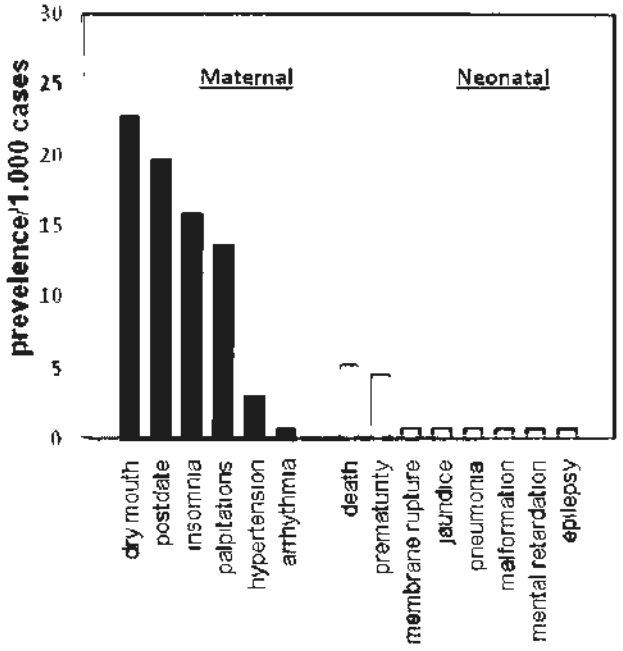


Figure 6.4 Adverse outcomes with use of Chinese herbal medicines for threatened miscarriages

Adverse pregnancy outcomes for other pregnancy disorders, we have also reviewed the other 1,012 clinical trials that used Chinese herbal medicines alone, and/or combined with Western medicines to treat other pregnancy disorders except threatened miscarriages. 21.9% of the studies did not report the adverse effects, while 77.3% reported no adverse effects (Figure 6.5). Only 8 clinical trials (0.8%) have assessed the safety of Chinese medicines for pregnancy, reported different side effects after the treatments, and followed up the pregnancy until birth. (Table 6.4)

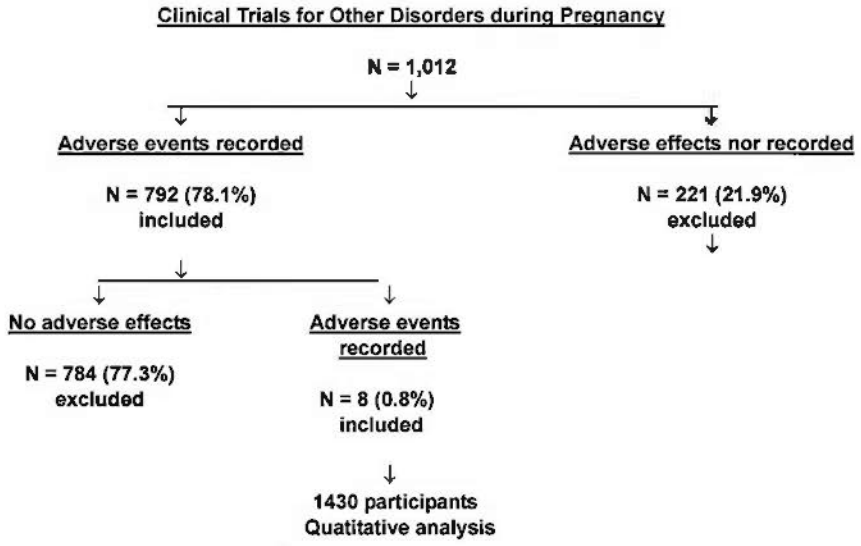


Figure 6.5 Study selection and inclusion

Quantitative analysis revealed that maternal adverse effects were with higher incidence than fetal adverse effects. The incidence of every adverse event in pregnancies and newborns were calculated. The most common maternal side effects on mothers during pregnancy were gastrointestinal reaction and nausea/vomiting, with the incidence of 1.4% and 1.2%, respectively. Allergic reaction occurred in 0.7% of pregnant women. The incidence of postpartum fever was 0.3%. Liver dysfunction and preterm labor were reported in 0.2% patients while oral ulcer, spontaneous abortion, and postpartum hemorrhage were detected in 0.1% patients under the Chinese medicines treatment. Fetal side effects on fetuses were reported with 0.9% fetus distress and 0.2% premature rupture of membranes. (Figure 6.6)

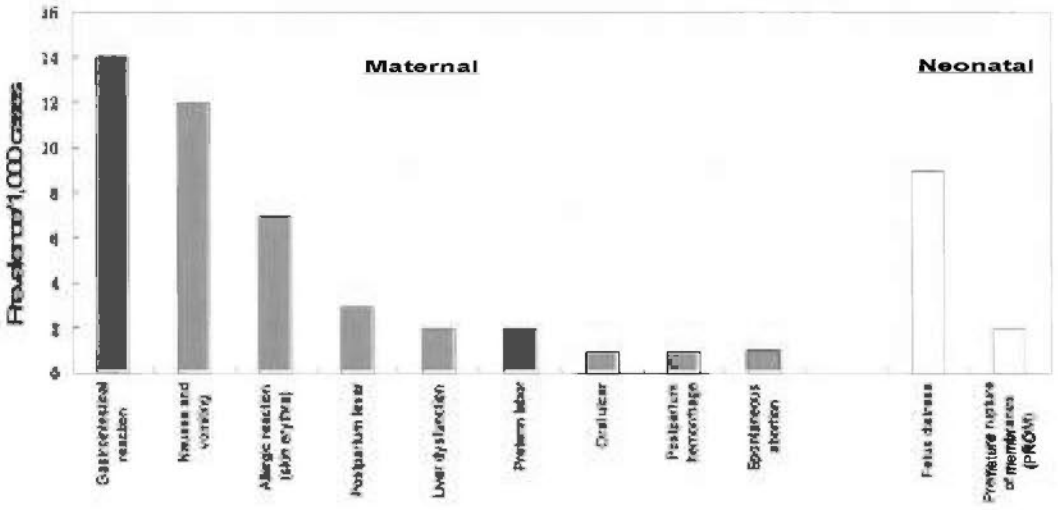


Figure 6.6 Adverse outcomes with use of Chinese medicines for other pregnancy disorders

Table 6.4 Adverse outcomes with use of Chinese medicines for other pregnancy disorders *

Studies	Disorders	Gestational age (mean±SD or range)	Maternal age (range)	Pharmaceuticals Formulae or Medicines	Dosage/day	Dosing/day	Incidence % (n/N)	Adverse Outcomes
Bai et al., 2010	infertility	32-57 days	18-43 yr	Western medicines: (MTX, Mifepristone)	50 mg/m^2, 50 mg	QD, im., BID, po.	15.6 (5/32)	Gastrointestinal reaction
							6.3 (2/32)	Liver dysfunction
				New formula:	9~15g	1 pack, QD, po.	3.1 (1/32)	Oral ulcer
Lin et al., 2010	infertility	36-68 days	18-40 yr	Gong Wai Yun Formula II	10~15g	1 pack, QD, po.	20 (1/5)	Allergic reaction (skin erythra)
Mao et al., 2007	infertility	ND	21-38 yr	New Formula I	6~15g	1 pack, TID, po.	3.7 (2/54)	Gastroenteric discomfort but complete the treatment course
				New Formula II	6~20g	1 pack, QD, external use		
Hou et al., 2006	Ectopic pregnancy	33-60 days	23-40 yr	Gong Wai Yun Formula	ND	1 pack, QD, po.	16.7 (4/24)	Nausea
				Western medicines (MTX+NS)	100mg+20ml	QD, iv		
Su et al., 2006	Ectopic pregnancy	>6 weeks	20-31 yr	New formula	10~30g	1 pack, BID, po.	6 (6/100)	Gastrointestinal reaction and/or Allergic reaction (skin erythra)
Zhang et al., 2006	Ectopic pregnancy	24-36 weeks	21-30 yr	New formula	15~20g	300ml, BID, po	11.1 (5/45)	Nausea and vomiting
Zhang et al., 2005	Ectopic pregnancy	< 6months	20-35 yr	Qing Zhi Soup	6~15g	1 pack, BID, po.	2.2 (2/90)	Nausea and vomiting
							1.1 (1/90)	Spontaneous abortion
							2.2 (2/90)	Premature rupture of membranes (PROM)
							2.2 (2/90)	Preterm labor
							8.89 (8/90)	Fetus distress
							1.1 (1/90)	Postpartum hemorrhage
Meng et al., 1988	Ectopic pregnancy	ND	ND	Pai Mo Decoction	6~10g	1 pack, BID, po.	4.2 (3/71)	Postpartum fever

Another 8 case controlled trials of Chinese medicines for other pregnancy disorders followed-up the outcomes are included

ND, not determined

6.4.3 Side Effect and Toxicity

6.4.3.1 Chinese medicines for pregnancy

There were 134 (11.5% of total records in Chinese Pharmacopeia) Chinese medicines have been used in the 197 clinical trials related to threatened miscarriages. Among all the recorded Chinese medicines, 8 were originated from animals, which were considered with toxicity potentials; 4 were not recommended for use and 1 should be avoided for pregnant women were also recorded (Table 6.5).

Amongst the top 10 most commonly used Chinese herbal medicines, only some minor side-effects have been reported (Table 6.6). It included constipation, allergic reactions, oedema, muscle pain and numbness.

Table 6.5 Precautious of recorded Chinese medicines for pregnancy

No.	Drugs	English names	Biological names	Precaution/Not racommended
1	益母草	Motherwort Herb	*Herba Leonuri*	induce miscarriage; forbidden for pregnancy
2	桃仁	Peach Seed	*Semen Persicae*	induce or increase vaginal bleeding; not recommonded for pregnancy
3	三七（粉）	San Chi	*Radix Et Rhizoma Notoginseng*	induce miscarriage or increase vaginal bleeding; not recommonded for pregnancy
4	红花	Safflower	*Flos Carthami*	induce or increase vaginal bleeding; not recommonded for pregnancy
5	枳壳	Bittet Orange	*Fructus Aurantii*	induce or increase vaginal bleeding; not recommonded for pregnancy
6	阿胶	Donkey-hide Glue	*Colla Corii Asini*	Animal origin from donkeys
7	五灵脂	Trogopterus Dung	*Faeces Trogopterori*	Animal origin from flying squirrels
8	生牡蛎	Oyster Shell	*Concha Ostreae*	Animal origin from oysters
9	乌贼骨	Cuttlefish Bone	*Endoconcha Sepiae*	Animal origin from squid
10	珍珠母	Nacre	*Concha Margaritifera*	Animal origin from mussels
11	桑螵蛸	Mantis Egg-case	*Ootheca Mantidis*	Animal origin from mantis
12	紫河车	Human Placenta	*Placenta Hominis*	Placenta from human
13	生龙骨	Drgon's Bones	*Os Draconis*	heavy metal in nature from fossils

Table 6.6 Adverse effects of 10 most commonly used single Chinese medicines for threatened miscarriage

No.	English Name	Latin Name	Chinese Name	Frequency (%)	Mean daily dose	Therapeutic actions	Other Applications	Side effects
1	Largehead Atractylodes Rhizome	*Rhizoma Atractylodis Macrocephalae*	Bai Zhu	59 (42.4%)	12.7 g	Prevent miscarriage	—	—
2	Chinese Dodder Seed	*Semen Cuscutae*	Tu Si Zi	55 (39.6%)	21.8 g	Prevent miscarriage and pre-labor	Cataract, Diarrhea, Sperm abnormality, Chronic Prostatitis	constipation
3	Himalayan Teasel Root	*Radix Dipsaci*	Xu Duan	55 (39.6%)	15.3 g	Stop vaginal bleeding Prevent miscarriage	Fractures and injuries, Lower back pain	—
4	Donkey-hide Glue	*Colla Corii Asini*	E Jiao	49 (35.3%)	6.3 g	Increase platelet count, Stop vaginal spotting	Chronic bleeding, Anemia, Tuberculosis, Uterine fibroids, Endometriosis	—
5	Chinese Taxillus Twig	*Herba Taxilli*	Sang Ji Sheng	48 (34.5%)	17.9 g	Prevent miscarriage Lower high blood pressure	Lower back pain, Tendons atrophy	—
6	Milkvetch Root	*Radix Astragali*	Huang Qi	44 (31.7%)	22.9 g	—	chronic nephritis, diabetes mellitus, Diuresis	—
7	White Peony Root	*Radix Paeoniae Alba*	Bai Shao	42 (30.2%)	15.5 g	Regulate menstruation	Abdomen and limb pain, Check sweating	incompatible *with* ***Rhizoma et Radix Veratri***
8	Chinese Angelica	*Radix Angelicae Sinensis*	Dang Gui	40 (28.2%)	10.1 g	Improve blood circulation Regulate menstruation	General pain, Bowels overactivity	—
9	Liquorice Root	*Radix Et Rhizoma Glycyrrhizae*	Gan Cao	40 (28.2%)	6.2 g	—	Detoxification, Dispel phlegm, Coughing, Spasmodic pain	Edema, Incompatible with *Radix Euphorbiae Pekinensis, Flos Genkwa* and *Radix Kansui*
10	Baical Skullcap Root	*Radix Scutellariae*	Huang Qin	37 (26.6%)	10.1 g	Stop vaginal bleeding Prevent miscarriage	Detoxification	—

6.4.3.2 Other Chinese medicines

Other Chinese medicines, which were not commonly used for pregnancy, were also surveyed and further characterized from other literatures and resources. As to pregnant women, due to physiological characteristics and changes, all drugs must be used with high attention, especially the ones reported that could lead to abortion or other negative effects on pregnancy health and fetal development.

The herbs with strong toxicity to human or potential effects in resulting abortions or severe malformations will be considered as known reproductive toxicity and are contraindicated for pregnant women (Zheng XY, 2005; Friedman JM, 2000). According to "Chinese Pharmacopoeia", the recorded 28 Chinese medicines which should be forbidden during pregnancy include Croton Seed (巴豆), Lilac Daphne Flower Bud (芫花), Gansui Root (甘遂), Knoxia Root (大戟), Pokeberry Root (商陸), Pharbitis Seed (牽牛子), Snakegourd Fruit (瓜蒂), Falsehellebore Root and Rhizome (藜蘆), Dried Lacquer (乾漆), Common Burreed Rhizome (三棱), E Zhu (莪朮), Leech (水蛭), Gadfly (虻蟲), Musk (麝香), (穿山甲), (皂莢), Mercury (水銀), Arsenic (砒霜), Cochinchina Momordica Seed (木鱉子), Large Blister Beetle (斑蝥), Snakegourd Fruit (川烏), Kusnezoff Monkshood Root (草烏), Common Monkshood Daughter Root (生附子), Mercurous Chloride (輕粉), Realgar (雄黃), Strychnos Seed (馬錢子), Dried Venom of Toads (蟾酥), Blue Vitriol (膽礬).

All these herbs have been proven reproductive and embryo-fetus toxic to both mother and fetus by animal studies or clinical reports. Examples were given in Table 6.7. Leech and Scolopendra, from animal origins, was highly embryotoxic, and induced severe congenital malformations in multiple organs (Zheng XY, 2005; Friedman JM, 2000). Semen Strychni and Pollen Typhae were associated with intrauterine death and mortality (Zhou AX, 1998). Coptidis Rhizoma and Aconiti Kusnezoffii led to maternal weight loss, fetal growth restriction and skeleton malformation (Chuang CH,

2006).

Other four Chinese medicines, remarked in "Chinese Pharmacopoeia" with "not recommended" and one as "contraindicated" for pregnancy, were recorded in the selected studies: Peach Seed, Pseudoginseng Root, Salfflower, Bitter Orange and Motherwort Herb (Table 6.8). Gin Seng Root should be avoided during pregnancy and lactation because of its documented hormonal activity (Newall et al., 1996) and Bitter Orange induced hypoglycemia and hypersensitivity (Wang JM, 1955; Fiebach et al., 2007), while Peach Seed (Zhang ZP, 1982) and Salfflower (Lin et al., 1998) increased bleeding tendencies and even were lethal. Motherwort Herb was well tolerated in animals but was reported causing deaths in human (Jia XS, 1989). These side effects were often associated with overdoses, precautions should be made for its use.

There were 6 Chinese medicines with serious reported maternal and embryo-fetus toxicity (Table 6.7). Hirudo and Scolopendra are highly embryotoxic, which induced severe multiple congenital malformations (Yu GM, 1994). Semen strychni and Pollen typhae were associated with intrauterine death (Li et al., 2005). Coptidis rhizoma and Aconiti kusnezoffii led to maternal weight loss, fetal growth restriction and skeleton malformation (Chuang CH, 2006). The toxicity doses of these Chinese medicines were very close to the maximum clinical doses used for other clinical applications.

What's more, in 1999, Department of Health (The Government of the Hong Kong Special Administrative Region) had revoked the license of 5 Chinese herbal medicines which contained the aristolochic acid that could exerted its carcinogenicity and mutagenicity and caused aristolochic acid nephropathy (AAN), and banned the productions of related drugs (Concumer Councsl, 2004). They were Dutchmanspipe Fruit (馬兜鈴, *Fructus Aristolochiae*)、Dutchmanspipe Vine (天仙藤, *Caulis Aristolochiae*)、Slender Dutchmanspipe Root (青木香, *Radix Aristolochiae*)、Fang Chi

Root (廣防己, *Radix Aristolochiae Fangchi*)、Manshurian Dutchmanspipe Stem (關木通, *Caulis Akebiae*).

Table 6.7 Reproductive toxicity of other Chinese medicines

	Toxicity	Pharmaceutical Name	English Name	Chinese Name	Clinical Applications	Clinical Dose	Developmental toxicity	Species	Exposure Range	LD50 (mice)
1	Strongly	*Realgar*	Realgar	雄黄	tinea, scabies, phlegm, tumors, leukemia	0.15-0.6g (2.5-10mg/kg)	anencephalus, cardiac eversion, skeleton defect, mortalityviscera bareness	human	5mg/kg until 28 wk p.o.	3.2g/kg
2		*Hirudo Seu Whitmania*	Leech	水蛭	thrombosis, salvage compromised microvascular free-tissue transfers, replanted digits, ears, lips and nasal tips due to venous congestion	1.5～3g (25-50mg/kg)	fetal resorption, growth retardation, still birth, tongue, palate, limb defect	mice	500-1000mg/kg at E7-E11 p.o.	15.28g/kg
3		*Radix Aconitikusnezoffi*	Kusnezoff Monkshood Root	草乌	chronic muscle or joint pain and tightness, chest and abdominal pain, lower limb edema and coldness	1.5-12g (25-200mg/kg)	IUGR liver, kidney, heart and gonad histological changes	rat mice	8.3g/kg at E7-16 p.o. 200-400mg/kg i.p for 4 days	0.4-5.7g/kg
4	Weakly	*Pollen Typhae*	Cattail Pollen	蒲黄	angina, high blood cholesterol, cerebralvascular accident Hemorrhoids, neonatal rash	3-10g (50-150mg/kg)	resorption, still birth, miscarriage, decreased fetal weight	mice	5-20g/kg p.o.	35.57g/kg
5		*Flos Carthami Tinctorii*	Safflower	红花	coronary diseases, menstruation disorders, thromboangitis obliterans, joint dislocation	0.5-2g (8-30mg/kg)	no malformation	rat	4g/kg at E8-10, p.o.	20.7g/kg
6		*Rhizoma Coptidis*	Figwort Flower	黄连	diabetes, inflammation of intestine, diarrhea caused by bacterial infection, high fever, restlessness and insomnia, spitting blood, nose bleeding , red eyes and excess stomach acid, tooth ache, boils	1.5-20g (20-300mg/kg)	low birth weight, SGA	human	10-20g/kg, p.o.	24.3 mg/kg

Table 6.8 Other Chinese medicines with precautious – not recommended and contraindicated

Not recommended:				
Peach Seed (桃仁)	Amenorrhea	bleeding tendency (rabbits) death (human)	1g/ml, po, QD for 7-8 days (rabbits) 20g, po (human)	LD50 = 5g/kg, po (mice)
Pseudoginseng Root (三七)	Bleeding, Miscarriage, Pain, Amenorrhoea, Dysmenorrhoea, Angina pectoris, Hyperlipidemia; Chronic infectious hepatitis	hypoglycaemia (rabbits)	1g/kg, po, QD for 1 month (rabbits)	LD50 = 0.45g/kg, po (mice) LD50 = 0.8g/kg, iv (mice)
Safflower (红花)	Irregular menstrual, Amenorrhoea, Trauma	bleeding tendency (mice) weight lose (mice), death (mice)	200mg/kg, po, QD for a week (mice) 8~10% powder, po (mice)	LD 50 = 2.4g/kg, ip (mice) LD50 = 20.7g/kg, po (mice)
Bitter Orange (枳殼)	Food stagnation and indigestion, Pelvic prolapse	hypersensitivity (rabbits) uterine hyperstimulation (rabbits)	skin contact >10g/kg (rabbits) 0.1ml, 83 times to non-pregnant uterus in vitro (rabbits)	LD50 = 0.2g/kg, po (mice) LD50 > 5g/kg, po (rats) LD50 > 10g/kg, po (rabbits)
Contraindicated:				
Motherwort Herb (益母草)	Nephropathy, Hematuria, Irregular menstruation, Eczema	prevent implantation (mice) uterine hyperstimulation (rabbits) death (human)	200-250mg, po, QD (mice) 0.2~2.0 μg/mL to uterus in vitro (rabbits) 400g, po, BID (human)	LD50 = 31.9g/kg, po (mice)

* 108 clinical trials for threatened miscarriages are included

6.4.3.3 Precautious

The herbs, which are with potential toxicity to pregnancy but the adverse effects could be avoided if properly prepared, or matched correctly in a formulae, or administrated with suitable dosage and duration to control and reduce the side effects (Li J, 2006), including Immature Bitter Orange (枳實), Betel Nut (檳榔), Peach Seed (桃仁), Safflower (紅花), Tree Peony Bark (丹皮), Cowherb Seed (王不留行), (乳香), Myrrh (沒藥), Cattail Pollen (蒲黃), Twotooth Achyranthes Root (牛膝), Trogopterus Dung (五靈脂), Sappan Wood (蘇木), Lilac Pink Herb (瞿麥), Jackinthepulpit Tuber (天南星), Monkshood Daughter Root (附子), Cassia Bark (肉桂), Antifeverile Dichroa Root (常山), Turmeric (薑黃), Chinese Rhubarb (大黃), Aloes (蘆薈), Mirabilite (芒硝). Take Monkshood Daughter Root as example, besides the proper preparation, another way to reduce its toxicity is to match it with Dried Ginger (干薑, Rhizoma Zingiberis) in a formulae (Li J, 2006). Because the macromolecular compounds in Dried Ginger, such as chloroform and petroleum ether extracts, formed a colloid solution, which could obviously reduce the hydrolysis of aconitine (the main toxic compound of Monkshood Daughter Root) (Li J, 2006).

Chinese medicines of animal origins were often associated with allergic reactions. Eight herbal medicines were recorded from the literatures: Donkey-hide Glue, Deglatined Deer Horn, Trogopterus Dung, Os Sepiae, Oyster Shell, Drgon's Bones, Nacre, and Mantis Egg-case (Table 6.9). Deglatined deerhorn induced fibrous peritonitis (Deerhorn, 2011), Oyster shells led to mucosal irritation, silicosis and even cardiopulmonary disturbance (Manjeshwar SB, 2004). Donkey glue, one of the top 10 commonly used Chinese medicines, caused allergic reactions (Wang CR, 2001). Dung and Nacre were associated with organ deformities (WMB 2010) and anaemia and hyperuricemia, respectively (WMB 2010).

Table 6.9 Other Chinese medicines with precautious – animal origins

English Name (Chinese Name)	Other clinical applications	Toxicity (species)	Nature of exposure (species)	LD50 (species)
Animal origins:				
Donkey-hide Glue (阿膠)	Thromobocytosis, vaginal spotting; Chronic bleeding; Anemia; Tuberculosis; Uterine fibroids; Endometriosis	allergic reaction (human)	10ml, po (human)	—
Deglatined Deerhorn (鹿角)	Irregular menstruation; Arthritis; Breast cancer; Mammary gland hyperplasia	fibrous peritonitis (rats)	9.27mg/kg, ip for 2 weeks (rats)	LD50>20g/kg, po (mice)
Trogopterus Dung (五靈脂)	Pain; Bleeding; Angina pectoris; Dysmenorrhea; Menorrhagia; Metrorrhagia; Peptic ulcer; Snake, Scorpion, and Centipede bites; Trauma	organ deformities (mice)	240mg, ip (mice)	LD50 >10g/kg, po (mice)
Os Sepiae (烏賊骨)	Hemostasis; Stomachache	—	—	LD50 = 0.2g/kg, po(rats)
Oyster Shell (牡蠣)	Pain; Palpitations; Insomnia; Anxiety	irritations and mucus damage (human) silicosis (human) cardiopulmonary impairment (human)	eye contact (human) inhalation (human) long-term exposure (human)	LD50 = 6.5g/kg, po (rats)
Drgon's Bones (龍骨)	Bleeding; Insomnia; Anxiety; Emotional stress	—	—	LD50 = 9.9g/kg,po (mice) LD50 = 21.5g/kg, iv (mice)
Nacre (珍珠母)	Night blindness; Emotional stress	anaemia (rats) hyperuricemia (rats)	4.3mg, po, QD for 2 months (rats)	LD50 > 21.5g/kg, po (rats)
Mantis Egg-case (桑螵蛸)	Diabetes; Prostate disorders; Hyper-parathyroidism; Primary aldosteronism; Urinary incontinence; Mental functions; Emotional distress	—	—	LD50 > 320g/kg, po (mice)

108 clinical trials for threatened miscarriages are included

6.5 Discussion

The current review analyzed the adverse events of Chinese medicines used during pregnancy, particularly in threatened miscarriage since that is it's most common clinical application. As in other clinical pharmaceuticals in Western Medicine, Chinese medicines in Traditional Chinese Medicine not only can result in maternal manifestations then disturb fetal health indirectly, but also can render harm to fetus directly. Threatened miscarriage is a major pregnancy complication at early gestation (Curnningham et al., 2005). Chinese medicines are commonly applied to prevent inevitable miscarriage, through attempts to promote maternal health and embryo-fetal development. There is urgent need to study the safety issue of the Chinese medicines. In this chapter the clinical trials of Chinese medicines for threatened miscarriages were systematically reviewed and its potential adverse effects on mothers and fetuses were studied.

Unfortunately very limited data is available for analysis. Very few clinical trials followed-up the pregnancy until birth and primarily assessed the adverse effects of Chinese medicines in the treatments for threatened miscarriage. Most available clinical trials were not controlled. Though 2 controlled clinical trials, only 1 was properly randomised, are available for meta-analysis and sub-group analysis, the small sample size and the negative adverse outcome failed to conclude the safety of Chinese medicines during pregnancy. From the quantitative analysis of 10 non-controlled clinical trials, adverse pregnancy outcomes apparently were more common in mothers than in infants, however the recorded perinatal consequences were more severe. Mortality and congenital malformation were reported but the incidence rates were very rare and well below the rates in general population in China (Wang YP, 2010) and in western countries (Deaton A, 2007). The causes of the reported perinatal deaths included prematurity, oligohydramnios, and aspiration pneumonia. Stillbirth and intrauterine death were also identified, but early pregnancy

loss in failure of the Chinese medicines treatment could underestimate the adverse pregnancy outcomes. Nausea, dry mouth, anorexia and constipation in mothers under Chinese medicines intervention were recorded in a study but they were unlikely to be pathological. Since the sample size was too small, the control group was not included for comparison and same outcome measures were not recorded in every study, the low risk of neonatal complications cannot be concluded by any ascertainment bias of the individual studies. As to the application of Chinese medicines as treatments to other pregnancy disorders, mostly infertility, the adverse effects were more common in mothers than in infants, but more mild effects than severe toxicities were recorded. Maternal gastrointestinal reaction and nausea and vomiting were most common, with a higher incidence over 1%, but still lower than the clinical side effects caused by Western medicines. The incidence rates of allergic reaction, liver dysfunction, oral ulcer, postpartum fever and postpartum hemorrhage occurred on mothers were all below the rates in general population in China (Wang YP, 2010) and in western countries (Deaton A, 2007). No stillbirth or intrauterine death was identified, but early pregnancy loss and preterm labor, fetus distress and premature rupture of membranes were recorded. However, due to insufficient information in these studies, further subgroup analysis could not be conducted.

The active ingredients of the Chinese medicines are chemicals that are similar to prescription drugs. Chinese medicines are not free of risk and they have the same potential to cause adverse effects. Extensive literature searches on frequently used Chinese medicine for pregnancy is required to show the side-effects do occur and reveal some reproductive effects which may affect maternal and fetal health. Other Chinese medicines for pregnancy with well-characterised reproductive toxicity cannot be overlooked. Though these Chinese medicines are not commonly used in clinical practice, some of them could result in severe consequences when given in over dosages or are misused. In the communities which use Chinese medicines, special attention should be paid and precautions should be taken to prevent mistaken overdoses of the Chinese medicines. It should be acknowledged that some of the

studies from animals may not be comparable to human responses. Despite variations in clinical practice and therapeutic prescription, Chinese medication in Traditional Chinese Medicine should comply with modern pharmacological principles as in Western Medicine. Chinese medicines may be beneficial, but may also adversely affect both mothers and fetuses *in utero*. International regulations have not been designed or specified to categorise the Chinese medicines for use in pregnancy. Until now, no detailed reproductive toxicity and pharmacotoxicity studies are available to assess the potential risk of Chinese medicines during pregnancy, as much as true that conventional medications are not well tested in pregnancy too. Before the detailed studies become available, here we take the initiative in gathering information about the adverse effects and potential toxicity of the Chinese medicines from the literatures.

In this chapter, we have also identified some potential reproductive and embryotoxic effects of the Chinese medicines. Most of the top 10 Chinese medicines have no major adverse effects, except preterm labor when Chinese Angelica was over-dosed (Zhang et al., 1995). However, some other reports have addressed pathological and pharmacological data, amongst these 10 herbal medicines, that may affect the mother and fetus during pregnancy.

Largehead Atractylodes Rhizome

Although Largehead Atractylodes Rhizome reduced serum lipid peroxidation to prevent dysfunction of the immune system in mothers and infants, it inhibited Natural Killer cells and decreased interleukin 2, and activated T-helper 2 cytokine interleukin 10 in inhibition of maternal-fetal interface immunity (Qiu et al., 1996; Zhong XH, 2008). It also inhibited lymphocytes significantly (бухова Фармакол и Токсикол, 1961). Lymphocytes play an important role for embryo implantation and placental development, in early pregnancy (Tian XZ, Zhang LZ, 1998). Imbalanced placental humoral T-helper 1 and cellular T-helper 2 immune regulation are closely associated with unexplained recurrent spontaneous abortion, intrauterine growth retardation,

pregnancy induced hypertension, and premature rupture of the fetal membranes (Zhang J, Zhang WZ, 2005).

Chinese Dodder Seed

Dodder Seed improved hypothalamus-pituitary-ovary axis and ovarian functions, to relieve menopausal symptoms (Chan et al., 2006; Wang et al., 2002). It also activated platelet formation and raised platelet counts, to prevent severe bleeding in pregnancy, with idiopathic thrombocytopenic purpura complications (Shi YM, Wu QZ, 1991). However, platelet aggregation disorders can happen, in excessive high platelet counts and may result in postpartum haemorrhages (Rahman et al., 2008).

Himalayan Teasel Root

Teasel Root increased bone density and improved bone trabeculae to prevent maternal osteoporosis owing to intensive placenta and fetal development during pregnancy (Wong et al., 2007). It also improved insulin resistance and alleviated diabetes status by decreasing serum fasting insulin, C-reactive protein and increasing insulin sensitivity (Fan et al., 2006).

Donkey-hide Glue

Donkey-hide Glue significantly increased serum granulocyte macrophage colony-stimulating factors, and erythropoietin levels, and reduced serum transforming growth factor beta level, which prevented anemia and promotes hematopoiesis, by activating immature granulocyte and erythroid cells (Zhang Y, Zhang XY, 2005). However, increased red blood cells and hemoglobin can result in hemoconcentration and is associated with low birth weight and premature delivery (Zhou J, Li DJ, 2006). Though it improves ovulation failure, and reduces luteal phase defect, it may on the other hand lead to habitual abortion and female infertility (Casanueva et al., 2006).

Chinese Taxillus Twig

Taxillus Twig significantly reduced hemolysis and lowered perinatal mortality not

only for ABO-type but also for Rh-type maternal-fetal blood groups incompatibility (Bian XM, 1998). It enhanced adiponectin and insulin pathways and secretion, by improving insulin resistance and lowering glucose levels, which potentially develop, as a substitution therapy for insulin resistance conditions, such as gestational diabetes mellitus (Huang HP, 1965).

Mongolian Milkcetch Root

Milkcetch Root increased the bone calcium content and decreased urine deoxypyridinoline to improve the bone calcium metabolism particularly in late gestation (Zhang et al., 2005). It prevented insufficient placental blood supply, such as pregnancy-induced hypertension syndrome and intrauterine growth retardation by releasing nitric oxide into circulation, which accelerated the formation of placental vessels and kept placental circulation in low resistance (Li RM, 2006). However, adverse effects were reported, such as weight loss, dose-related incidence of loose stools, which were considered as dose-limiting gastrointestinal toxicity, and abscesses and ulcerative skin disease complications (Greenway, 2006).

White Paeony Root

White Paeony Root regulated menstruation for dysmenorrhea and irregular menses (Bensky et al., 1993). It also enhanced hippocampus recovery from stress, by decreasing taurine contents (Zhang Z, 2005). In subacute toxicity tests, increased proteinuria was recorded under low dosage, but decrease of body weight, erythrocyte, hemoglobin, and hematocrit (considered as macrocytic anemia) were reported under high doses. In rats, it should be noted that it induced maternal weight loss, small placentae and fetal growth retardation (Li J, 1991).

Chinese Angelica

Angelica enhanced nitric oxide synthase activity, and increased nitric oxide metabolic products in arterial walls against myocardial ischemia, in order to relieve coronary heart disease (Yin et al., 2005). Decreased nitric oxide is considered as part of

pathogenesis in pregnancy-induced hypertension (Granger et al., 2001). This medicine should be used with extreme caution when it has been used together with Donkey-hide Glue.

Liquorice Root

Liquorice Root inhibited interferon gamma and tumor necrosis factor alpha mediated cytotoxicity in thyroid cells (Shon YH, 2004). Fluctuations in body weight, dysfunction of the adrenal gland, hypernatremia and systemic contact-type hypersensitivity which is involved in producing the exfoliative dermatitis lesion was reported (Watanabe et al, 1977).

Baical Skullcap Root

In rats, Baical Skullcap Root markedly reduced embryonic resorption, fetal body weight, ossification and skeletal variation or anomalies induced by valproic acid (Minematsu S, 1990). In rabbits it resulted in sedation, coma and even death (Tang RY, 1958). In dogs it caused vomiting and diarrhea, neither teratogenic nor any other adverse effects were noticed (Lin JQ, 1958).

However, these classifications are still lack of experimental data, and actually the difference among the types of toxicity was not very obvious sometimes while "Non-Toxicity" did not refer to absolute non toxic in Chinese Medicine. All drugs generally have two kinds of properties, "Medical" (藥性) and "Toxic" (毒性) (Li J, 2006). Therefore, people always try to improve efficacy by reducing or avoiding toxic effects. Toxic effects of a drug often closely depend on dosage and duration. Overdose, or prolonged treatment, even if the "Non-toxicity" drugs will lead to toxic. For example, Gin Seng Poisoning Syndrome presents as hypertension, nosal bleeding, irritability, insomnia, nervousness, dizziness, headache, rash, seizures and convulsions (Park ID et al., 2008). Conversely, with strict controls of appropriate dose and administration duration, a toxic reaction may not occur. For example, Villous

Amomrum Fruit (砂仁, *Fructus Amomi*) is a weakly toxic herbal medicine but is applied in formulae to prevent miscarriage without any adverse effect (Li J, 2006). Thus, in Chinese Medicine, the types of toxicity are relative, and drugs are generally with potential toxicities, which is also the same principle as in Western medicine.

We hope more comprehensive and systematic experiments will be carried out. Until more reliable and scientific research data become available, clinicians should appraise both the risk and benefit before recommendations to women. Both Chinese and Western physicians should explicitly elicit and document the history of the use of any Chinese medications. This is to prevent and recognise potential serious problems associated with their use and should encourage their discontinuation. More studies and clinical trials in humans with a larger sample size are obviously mandatory. We do recommend more systematic basic investigation of the safety use of Chinese medicines.

6.6 Summary

Adverse effects of Chinese medicines for threatened miscarriage during early pregnancy cannot be concluded due to limited studies available. Some Chinese medicines have been reported harm to mothers and fetuses. Rigorous and scientific clinical studies are necessary to confirm the risk of Chinese medicines to mothers and fetuses.

Chapter VII

Safety Studies of Commonly used Chinese Medicine in Pregnant Animals

7.1 Introduction

Because of the claimed safety and efficacy of Chinese medicines, various formulae are recommended for use during pregnancy, such as Shou Tai Pill (寿胎丸) for prevention of miscarriage and promotion of pregnancy (Ding et al., 1997). However, as described in previous chapter, some of Chinese medicines had reported unexpected adverse effects to pregnant women and newborns. To extend our interests and further researches on the safety of Chinese medicines during pregnancy, we established research collaborations with the Institute of Chinese Medicine (ICM) in the Chinese University of Hong Kong (CUHK) to screen the toxicity of most commonly used Chinese medicines in pregnant animals.

7.1.1 Clinical Studies

Chinese medicines have benn utilized for a long time, unlike those pharmaceutical drugs not recommend for use during pregnancy because of known or suspected adverse or teratogenic effects made evident by clinical trials, few clinical trials of high quality could be identified from our systematic reviews (Chapter VI). There was not sufficient record or statistics regarding the adverse effects of the use of Chinese medicines on embryo-fetal development and prenatal and postnatal growth. The incidence of congenital malformation after taking Chinese medicines is not uncommon, the causative Chinese medicines and its chemical composition for the consequence is difficult to be identified, however (Cunningham et al., 2005; Awang DV, 1991; Holst et al., 2008). On the other hand, our local government authorities are not only calling for proof of Good Manufacturing Practice (GMP) of Chinese medicinal compounds; they also ask for more solid evidence of the claims, in particular safety. Trials for clinical treatment using Chinese medicine continue, but there is still insufficient scientific evidence to assess the adverse effects of Chinese medicines on mothers and their offsprings.

7.1.2 Animal Studies

Claiming safety for a certain herb is already unacceptable without evidence from proper clinical trials or scientific tests. However, the strategy for testing Chinese medicines in humans has not yet been established and regulated. Besides clinical trials, animal testing is the other alternative for toxicity tests (OECD 414), which is the use of non-human animals in experiments, such as zebrafish, frogs, mice, rats, birds, cats, dogs, etc. Toxicology testing for a drug or medicine is very important nowadays. The Congress of the United States passed the laws that required safety testing of drugs/substances on animals before they could be marketed in response to the Elixir Sulfanilamide disaster in 1937 in which the eponymous drug killed more than 100 users without animal tests (Times 1937). Other countries enacted similar legislation since then (FDA, 1981). Due to the concerns in the communities caused by Thalidomide in mid 20th century, further laws were passed requiring safety testing on animals before a drug can be sold (Burkholz, 1997). Therefore, similar to western medicines, before clinical trials in human subjects, the very first step in developing the guidelines on the assessment of quality and safety of Chinese medicines is to test in animals. It includes the scientific tests of maternal toxicity and embryotoxicity in pregnant animals.

7.1.3 In Vitro Methods

The principal objective of embryotoxicity testing is the identification of chemicals which place the fetus at special risk and which might not therefore be detected in other general toxicity testing schemes (Smith et al., 1983; European Commission 2009). In vivo tests are costly and time consuming, and the study medicines firstly affect the mothers, then affected the fetuses, so the exact effects of study medicines on the fetus after metabolism in the maternal body cannot be estimated. Therefore, in vitro tests, which could directly apply study medicines on the embryos or fetuses, have been established for embryotoxicity studies.

Primary cultures or established cell lines are commonly used to analyse the mutagenic, embryotoxic or teratogenic potential of environmental factors, drugs and xenobiotics in vitro. However, these cellular systems do not include developmental processes from early embryonic stages up to terminally differentiated cell types or individual organisms. In vitro embryotoxicity test methods have been developed and have proven their significance in studies of mechanisms of embryonic development and developmental toxicity. They have also been extensively validated and now widely accepted as routine embryotoxicity screening by international authorities for safety assessment of chemicals and drugs, including EU European Centre for the Validation of Alternative Methods (ECVAM) in Europe (Balls et al., 1990), US National Institute of Environmental Health Sciences (NIEHS) (NIEHS, 1999) and Organisation for Economic Co-operation and Development (OECD) in USA since 1997 (OECD, 1996).

Three methods for in vitro screening have been highly recommended by ECVAM as preferred alternative screening bioassay test for embryotoxicity study. They include (1) embryonic stem cell test (EST) using commercial available mouse embryonic cell lines; (2) embryonic cell micromass culture (MM) test employing primary cultures of dissociated limb bud cells of rat/mouse embryos; and (3) whole embryo culture (WEC) test using culture of whole rat/mouse embryos.

Further comparisons have been made amongst these three methods. WEC, EST, and MM tests were 80%, 78% and 71% consistent with the overall prediction between in vivo and in vitro results. As to medicines with strong embryotoxicity, all these methods have shown 100% consistent rate of predicted results (Genschow et al., 2002). Further studies have indicated that EST is a cell-based assay, and has the advantage if a permanent cell line is used. For embryo-based assays, only the MM test and WEC test are suitable. The MM test focuses on the differentiation from limb bud cells into cartilage-producing chondrocytes, which limits its application only for the medicines that specifically interfere with specific step chondrocyte differentiation

process (Wang et al., 2010). Compared with MM test, WEC test could overview all the possible mechanisms of malformations on the embryos as a whole.

7.1.4 Whole Embryo Culture (WEC) Test

WEC was first raised in the 1930s, by Nicholas and Rudnick, then improved and better developed by New NT since 1970s. WEC test, as a well established method to incubate mammalian embryos from the fertilized egg to the end of organ formation process, is of advantage on the following reasons. Firstly, the models for WEC test are the embryos at a period of extremely sensitive developmental window to exogenous substances and medicines, in which could accurately control the exposure dose and time of test substance, to clarify the dose effect and time effect. Secondly, WEC test solves the problem of non-viewable embryo development in the uterus and observes irreversible teratogenic damage. Thirdly, the WEC test excludes the side effects from the health condition of mothers and maternal metabolism of test substances, and indicate direct effects on embryos. The WEC test method has been widely used for toxicology, pharmacology, and physiology research to observe the growth and development of embryos dynamically and to screen for any teratogenicity, mutagenicity, and embryotoxicity. However, there is still limitation in this method. Because the duration of embryos in vitro culture is very short, around 24 hr to 72 hr, in which most of the organ development has not been completed, it is difficult to study the teratogenic mechanisms of some organs caused by certain medicines. The WEC test are mostly carried out on mice, rats and rabbits, the former two animal models are more common. However, as rat WEC assay has been proven with poorer predictive values with its in vivo tests (Kiyohito N, 1995), WEC test in mice is preferable.

7.2 Objectives

In this chapter, both in vivo and in vitro tests in animals were screened for the maternal and embryo/fetal toxicity of Chinese medicines. We aimed to screen and determine the safety profile of the Chinese medicines on the dams and offsprings during and after pregnancy. Since Largehead Atractylodes Rhizome was the most commonly used Chinese medicine during pregnancy (Chapter IV), the specific objective was to identify the potential developmental teratogenicity and special toxicokinetic data of Largehead Atractylodes Rhizome using pregnant models in vivo and WEC model in vitro.

7.3 Methods

7.3.1. Chinese Medicine

7.3.1.1 Largehead Atractylodes Rhizome

Anticipated the test Chinese medicines selected for toxicity screening should be especially in relation to the medicines used in pregnancy with clinical relevance. According to our earlier systematic reviews and meta-analysis of clinical data from literatures and reports,

Largehead Atractylodes Rhizome (白术, Rhizoma Atractylodis Macrocephalae) was the most frequently used individual Chinese medicine during pregnancy to promote pregnancy and fetal growth (Chapter IV). It also had been demonstrated that Largehead Atractylodes Rhizome can inhibit uterine contraction to prevent miscarriage and premature delivery (Ma et al., 2006). Therefore, it selected for the safety study in this chapter.

7.3.1.2 Sources

Concentrated Chinese medicines as powders were from two sources. One was provided by a local pharmacy company PuraPharm Nongs. Nongs prepared the medicine from standardized extraction methods using state-of-art concentration technologies in GMP standard. Their concentrated Chinese medicine are prepared in granules and sold in most local hospitals and clinics established by Hospital Authority (HA), Tertiary institutions in university, non-governmental organizations and drugstores in Hong Kong. The other source was from Institute of Chinese Medicine (ICM), The Chinese University of Hong Kong. The raw herbs were purchased from renowned herbal supplier in Hong Kong (Largehead Atractylodes Rhizome origins from Zhejiang, China) were used. We extracted and prepared the crude medicine into powders by standardized extraction method protocols, including soaking, refluxing, repeating the extraction, combination of extracts, concentration, lyophilization, weighing and estimation methods.

7.3.1.3 Quality Controls

To ensure the quality, safety and efficacy of Chinese medicines, quality control of Chinese medicinal product is essential. Quality of the raw Chinese medicines was controlled by recognized organoleptic authentications and the medicines have been clinically approved to have same degree of curative efficacy with natural taste and flavor. Chemical marker of Chinese medicine was employed for authentication and differentiation of species, stability assessment, diagnosis of intoxication and discovery of bioactive compounds (Li et al, 2008). Supercritical fluid extraction (SFE) was an improved method for approaching higher quality and purity of extracts of Chinese medicines (Mosihuzzaman et al., 2008). Microbiological test was used as a means to assess safety (Mosihuzzaman et al., 2008).

7.3.1.4 Extraction

500 g dried roots of Largehead Atractylodes Rhizome were cut into small pieces, powdered and extracted by electromantle (Barnstead Electrothermal, EM5000/C, UK) with about 10 times volume of distilled water twice, boiled for 1 hour for the first decoction, poured out the upper water extract and kept it in a flask, then was added another 10 times volume of distilled water and 45 minutes boiling for the second decoction, and we pooled the two water extracts together. At the first round of decoction, it started with room temperature, and the residue adjusted the extract to a higher temperature for the second decoction, so the decoction duration was a bit longer for the first round. The temperature of both decoction rounds was not too high, as it could destroy its volatile oil component. The hot water extract was then filtered by a multi-layers cloth which worked as filter paper and with better function to filter the sediment, and centrifuged at 2000 r.p.m for 10 min. The supernatant was then concentrated in vacuum (Buchi, Rotavapor R-220, Switzerland) and freeze-dried into crude extract (123 g) for the tests.

Unlike the pharmaceutical arena that contains pure chemicals only, Chinese medicines are mostly from plants or animal products and their constituents work together synergistically for health promotion and medical treatment. To avoid missing any components of the Chinese medicines with potentially an effect on pregnancy, whole crude extract of Largehead Atractylodes Rhizome were used, while purified medicinal extracts (both hydrophilic and lipophilic portions) and composing compounds were not used for screening in present study. If there was any positive findings during the screening, we would carry out further biological studies on the fractions of the tested Chinese medicine.

The dried crude extract (123 g) was firstly dissolved by heating at 60-70℃ in 800 ml distilled water. The solution was then partitioned with n-Butanol (800ml each time for two times) to give two fractions. For the lipophilic fraction, n-butanol layer was evaporated under reduced pressure to give a dark gummy residue (4.435 g). The hydrophilic fraction was concentrated in vacuum and freeze-dried to give a powder

(107.91g).

7.3.1.5 Preparation

Each concentrated medicine of Largehead Atractylodes Rhizome as crude extract was sealed and stored as powder for long term usage and was prepared fresh prior to tests. For in vivo test, the powders were weighed and dissolved in Milli Q water solution (5ml 5% PBS in 45ml Milli Q water) to the in vivo test dosages and shaking by roller (RM-500, roller tubes mixer, MRC Ltd., Sokolov, ISRAEL) at room temperature for 30 min or longer until fully dissolved. The extraction was stored at 4℃, and warmed up at 37℃ water bath for 30 min before administration. To avoid variation and ensure the quality in each preparation, one extraction was usually used for a whole gestational period (around 20 days) to be tested, and was discarded if stored longer than one month.

For in vitro test, the crude extract powder was weighed and dissolved in rat serum to the in vitro test dosages, which was used as medium for WEC. Because of the bacteria-free requirement for in vitro studies, the extraction was only prepared fresh inside the culture hood every time before use.

7.3.1.6 TLC analysis

TLC was performed on Silica gel 60 F254-precoated plates (Merck), Solvent 7: 3 of n-Hexane and Ethyl acetate as solvent, and four methods were applied for observation, including UV-254 nm, UV-365 nm, heat-treated plate (2% vanillin-sulphuric acid reagent (20% sulphuric acid in 50% ethanol) after heating at 100-110℃ for 2-5min) under visible light and heat-treated plate under UV-365 nm. Atractylenolide I and Atractylenolide III are two main bioactive components of Largehead Atractylodes Rhizome. So we used them as chemical markers for the detection and observation.

7.3.2 Animals

7.3.2.1 Mice

Pregnant Institute of Cancer Research (ICR) mice were used and obtained from the Laboratory Animal Services Centre in The Chinese University of Hong Kong. This mammalian species and strain are well defined with respect to their health, fertility, fecundity, genetic background and the consistency displayed from study to study. These mice also have advantages of fast metabolic rate, large colonies, short fetal period, low prevalence of abnormalities and embryo-fetal deaths.

Guidelines were followed for the use and care of all laboratory animals, as set in the university. Animal Ethics (06/073/MS, 09/073/MS, 09/015/GRF) were obtained for the animal studies. Mice were kept on a 12:12 hr light-dark cycle with the dark period from 11:00 pm to 11:00 am at 25℃, with standard water and rodent chow *ad libitum* in the university animal facility. Female mice were paired with male mice for 2 hr time mating schedule just before the commencement of the light cycle. At the end of 2 hr, female mice were checked for a copulation plug in vagina and mating was confirmed by vaginal smear examination. Fertilization was assumed to occur at 10:00 am, which was regarded as gestation day E0. To avoid variations from batch to batch, the age, weight and parity of the animals were controlled, only young (at 7-8 weeks old), healthy and mature adult virgin females at the time of mating were studied. Mice at defined gestational days were used for both in vivo and in vitro tests.

7.3.2.2 Rats and rabbits

As adverse outcomes may vary amongst species because of the different sensitivity in animals to tested Chinese medicine, rats and rabbits were also studied.

Pregnant Sprague-Dawley (SD) rats were used and obtained from the Laboratory

Animal Services Centre in The Chinese University of Hong Kong. The advantages of this species are large colonies, rapid growth and development, strong resistance to disease, particularly respiratory disease. Besides, lower incidence of spontaneous tumors makes the chance of gene mutation smaller, and high sensitivity to hormone can be used to study changes of hormones during pregnancy. SD rats are suitable for pharmacology and teratology research, especially perinatal toxicity study. As with mouse studies, only young, healthy and mature adult virgin females at 2.5 months at the time of mating were studied. Rats at specific gestational stage were used for in vivo test only.

The experiments on pregnant New Zealand White rabbits (NZW rabbits) were carried out in the Laboratory Animal Services Centre in The Chinese University of Hong Kong, as this species requires special feeding and breeding. NZW rabbits have the advantages of rapid growth and high fecundity. Besides, ovulation happens only after mating, therefore one can accurately calculate the ovulation time, suitable for reproductive physiology, embryology and contraceptive drug screening studies. Pregnant rabbits at specific gestational stage were used for in vivo test only. Since virgin pregnancy are usually associated with smaller litter size and increased prenatal mortality, multigravid animals were used only.

7.3.3 In Vivo Tests in Pregnant Mice

7.3.3.1 Experimental designs

Maternal and fetal or neonatal adverse effects of Largehead Atractylodes Rhizome were firstly screened by in vivo test in pregnant mice. The in vivo study was designed basis on the guideline of FDA for toxicity study on medicines or medical products (Detection of Toxicity to Reproduction for Medicinal Products, 2006-ICH-S5A), in order to determine the potential reproductive effects of Largehead Atractylodes Rhizome both on mothers and their offsprings.

The experimental design was shown in Table 7.1. We firstly selected the most commonly used individual Chinese medicine, Largehead Atractylodes Rhizome, as it was with highest frequency of application for pregnancy, especially for threatened miscarriage. Then we set up different doses groups and intervened with Largehead Atractylodes Rhizome extracts for different developing stages of gestation, to overview the potential effects of Largehead Atractylodes Rhizome. At the end of intervention, the response of mothers, and development of embryos, fetuses and newborns were evaluated.

7.3.3.2 Dosage

For in vivo tests, dosages and administration of Largehead Atractylodes Rhizome to animals were in the form of the same medication intended for human as applied in clinical practices. Reference clinical dosage for the test Chinese medicine was calculated from the literatures with references to recommended dosage from "Chinese Pharmacopeia". The mean relevant clinical dosage for animals was determined. The mean clinical dosage of Largehead Atractylodes Rhizome (12 g/person/day) from the previous quantitative analysis (Chapter IV) was regarded as 1x clinical dosage as calculated. Most of the medium dosages fell within the dose ranges, thus it was set as 1x clinical dose for in vivo tests.

To screen for the safety of the test medicine in pregnant animals, 1x, 2x, 3x clinical dose (or higher doses in an ascending sequence) were tested. Further lower dosages in a descending sequence were included if an effect of studied medicine to be detected at the lowest clinical dose. Whilst in this situation it was desirable to be able to determine a "no observed effect level (NOEL)" close enough to reveal any dosage-related trends that could be presented. The desirable dose administered to the animals was calculated from the reference clinical dosages of the actual body weight in animals in ratio to the doses of body weight in human. The recommended formula

(You et al., 2007) for the calculation was:

$$d_B=d_A\times(R_B/R_A)\times(W_A/W_B)^{1/3}$$

d_A stands for the known drug dosage per weight (mg/kg) while d_B stands for the unknown one. R_A and R_B are the body size factors, 59 for mouse, 90 for rat, 99 for guinea pig, 93 for rabbit, 82 for cat, 104 for dog, 111 for monkey and 100 for human, while W_A and W_B are the actual weights of animal A and B. The advantage of this formula is that it can be used for different species of animals, especially good for animals with different drug sensitivities. It also takes the actual weight of the study animals into consideration and could be used for overweight or extremely low body weight.

The medium clinical dose of the raw herb of Largehead Atractylodes Rhizome was 12g/day as 1x clinical dose. Based on previous experience of Institute of Chinese Medicine (ICM) in extraction, the average yield was 24%w/w. After extraction, a pregnant woman (assume 60kg at early gestation) should administer 4.8g extract powder daily. Following the body size factor, the human equivalent 1x daily clinical dose of the crude extract is 1.18g/kg for mice, 0.84g/kg for rats, and 0.64g/kg for rabbits.

Table 7.1 Experimental protocol for in vivo screening

Drug	Study groups: Largehead Atractylodes Rhizome extracts Sham control group: 1x PBS in MilliQ water				
Dosage	P.O., QD Sham control group: 0x clinical dose Study group 1: 1x clinical dose Study group 2: 2 x clinical dose Study group 3: 3x clinical dose				
Animals	*Pregnant ICR mice*				
	Screening 1: defined gestational stages and developmental windows (Stage A – Stage D) Screening 2: throughout the whole gestational and postnatal periods (Stage E: E0- P28)				
Gestational days	Stage A E3-E6	Stage B E6-E8	Stage C E8-E15	Stage D E15-P0	Stage E E0-P28
Sample size	5	5	5	5	5
Single repeated dosing	From implantation through early gastrulation	From early through late gastrulation	From early through late organogenesis	From late organogenesis through the end of pregnancy	From birth to the end of lactation
Aim	Evaluate blastocyst formation, implantation and post-implantation embryos	Evaluate early decidualization, placentation, and embryonic development	Evaluate late decidualization, placentation, and embryo-fetal development	Evaluate development of conceptus	Evaluate development of offspring following maternal exposure
Assessments	Implantation Blastocyst Pre-gastrulated embryos	Embryofetal growth and development	Embryofetal growth and development	Prenatal viability Altered growth Structural changes	Postnatal survival Altered growth and development Functional deficits
Terminal Sacrifice	Around E6.5 of early gastrulation	Around E8.5 of late gastrulation	Around E15.5 of late organogenesis	Last administration before delivery Allow delivery and rearing to weaning (P21)	Allow delivery and rearing to weaning (P21)
Outcome measures during study	Once daily: Signs and mortality Body weight Food intake Early miscarriage Other observation as in other toxicity studies	As Stage A Late miscarriage	As Stage A Late miscarriage	As Stage A Premature delivery Prenatal mortality Still birth	As Stage A Parturition Duration of pregnancy Lactation Birth weight Postnatal mortality Physical development Sensory function Neurodevelopment and behavior
Outcome measures at terminal	Maternal blood collection Necrospy (macroscopic) Histological examination Morula staging Implantation rate and site	As Stage A Litter size Resorption rate Morphological staging Embryo abnormalities Placental pathology	As Stage B	As Stage B Fetal growth Fetal visceral alternation Fetal soft tissue and skeleton changes	As Stage B Visceral alternation Soft tissue and skeleton changes

Table 7.2 Dosage records of common Chinese medicines

Frequency Sequence[a]	Chinese Name	Latin Name	Pharmacopeia Dose g/person/day			Literature Dose g/person/day		Research Dose[f] g/person/day			Animal Dose[g] mg/kg/day		
			lowest[b]	highest[c]	mean[d]	mean	medium[e]	×1	×2	×3	×1	×2	×3
1	白术	Rhizoma Atractylodis Macrocephalae	6	12	9	13	12	12	24	36	17	34	51
2	菟丝子	Semen Cuscutae	6	12	9	22	20	20	40	60	29	57	86
3	续断	Radix Dipsaci	9	15	12	15	15	15	30	45	21	43	64
4	桑寄生	Herba Taxilli	9	15	12	18	15	15	30	45	21	43	64
5	黄芪	Radix Astragali,炙黄芪 Radix Astragali Praeparata Cum Melle	9	30	19.5	23	20	20	40	60	29	57	86
6	白芍	Radix Paeoniae Alba	6	15	10.5	16	15	15	30	45	21	43	64
7	当归	Radix Angelicae Sinensis	6	12	9	10	10	10	20	30	14	29	43
8	甘草	Radix Glycyrrhizae	1.5	9	5.25	6.2	6	6	12	18	9	17	26
9	黄芩	Radix Scutellariae	9	30	19.5	10	10	10	20	30	14	29	43
10	杜仲	Cortex Eucommiae	6	9	7.5	15	15	15	30	45	21	43	64
11	杜仲叶		10	15	12.5								
12	熟地（黄）	Radix Rehmanniae Glutinosae Conquitae	9	15	12	22	20	20	40	60	29	57	86
13	党参	Radix Codonopsis	9	30	19.5	19	15	15	30	45	21	43	64
14	砂仁	Fructus Amomi	3	6	4.5	6.5	6	6	12	18	9	17	26
15	地黄,生地(黄)	Radix Rehmanniae Glutinosae	9	15	12	19	17.5	18	35	53	25	50	75
16	鲜连翘		12	30	21	—	—	—	—	0	—	—	—
17	川芎	Rhizoma Chuanxiong	3	9	6	7.4	6	6	12	18	9	17	26
18	艾叶,艾叶炭	Folium Artemisiae Argyi	3	9	6	8.7	9	9	18	27	13	26	38
19	陈皮	Pericarpium Citri Reticulatae	3	9	6	8.5	9	9	18	27	13	26	38

20	丹参	Radix Salviae Miltiorrhizae	9	15	12	11	12	12	24	36	17	34	51
21	太子参	Radix Pseudostellariae	9	30	19.5	18	15	15	30	45	21	43	64
22	旱莲草	Herba Ecliptae Eclipta prostrata L	—	—	—	17	15	15	30	45	21	43	64
23	苏梗,紫苏梗,紫苏	紫苏梗 Caulis Perillae	5	9	7	9.9	10	10	20	30	14	29	43
24	兰麻根	Radix Boehmeriae	—	—	—	19	15	15	30	45	21	43	64
25	升麻,炙升麻	Rhizoma Cimicifugae	3	9	6	7.1	6	6	12	18	9	17	26
26	柴胡	Radix Bupleuri	3	9	6	7	6	6	12	18	9	17	26
27	赤芍	Radix Paeoniae Rubra	6	12	9	11	10	10	20	30	14	29	43
28	女贞子	Fructus Ligustri Lucidi	6	12	9	16	15	15	30	45	21	43	64
29	仙鹤草	Herba Agrimoniae	6	12	9	23	20	20	40	60	29	57	86
30	竹茹,姜竹茹	Caulis Bambusae in Taenia	4.5	9	6.75	11	10	10	20	30	14	29	43
31	山萸肉,山茱萸	Fructus Corni	6	12	9	14	13.5	14	27	41	19	38	58
32	(生)蒲黄,炒蒲黄	Pollen Typhae	5	9	7	10	10	10	20	30	14	29	43
33	糯米	糯米条 Abelia Chinensis R.Br.	—	—	—	30	30	30	60	90	43	86	128
34	补骨脂	Fructus Psoraleae	6	9	7.5	14	15	15	30	45	21	43	64
35	郁金	Radix Curcumae	3	9	6	10	10	10	20	30	14	29	43
36	泽兰	Herba Lycopi	6	12	9	10	10	10	20	30	14	29	43
37	茯苓	Poria	9	15	12	14	13.5	14	27	41	19	38	58
38	枸杞，杞果	Fructus Lycii	6	12	9	15	15	15	30	45	21	43	64
39	香附	Rhizoma Cyperi	6	9	7.5	11	10	10	20	30	14	29	43
40	鸡血藤	Caulis Spatholobi	9	15	12	18	15	15	30	45	21	43	64
41	茜草根，炭	Radix Et Rhizoma Rubiae	6	9	7.5	13	15	15	30	45	21	43	64
42	桂枝	Ramulus Cinnamomi	3	9	6	—	—	—	—	0	—	—	—

43	小茴香	Fructus Foeniculi	3	6	4.5	3	3	3	6	9	4	9	13
44	地榆	Radix Sanguisorbae	9	15	12	19	15	15	30	45	21	43	64
45	半夏，法，姜半夏	Rhizoma Pinelliae, 法 Rhizoma Pinelliae praeparatum	3	9	6	9.8	10	10	20	30	14	29	43
46	肉苁蓉	Herba Cistanches	6	9	7.5	12	10	10	20	30	14	29	43
47	巴戟天，巴戟	Radix Morindae Officinalis	3	9	6	12	10	10	20	30	14	29	43
48	何首乌,(制)首乌	Radix Polygoni Multiflori	6	12	9	16	15	15	30	45	21	43	64
49	莲子	Semen Nelumbinis	6	15	10.5	11	10	10	20	30	14	29	43
50	麦冬	Radix Ophiopogonis	6	12	9	13	13.5	14	27	41	19	38	58
51	狗脊	Rhizoma Cibotii	6	12	9	16	15	15	30	45	21	43	64
52	木香	Radix Aucklandiae	1.5	6	3.75	6.9	6	6	12	18	9	17	26
53	藕节（炭）	Nodus Nelumbinis Rhizomatis	9	15	12	11	10	10	20	30	14	29	43
54	墨旱莲(旱莲草)	Herba Ecliptae	6	12	9	15	15	15	30	45	21	43	64
55	淫羊藿	Herba Epimedii	3	9	6	15	13.5	14	27	41	19	38	58
56	荆芥炭,（荆芥炭）	Herba Schizonepetae (Carbonisatum)	5	10	7.5	11	11	11	22	33	16	31	47
57	乌贼骨(海螵蛸)	Endoconcha Sepiae	5	9	7	17	15	15	30	45	21	43	64
58	五灵脂	Faeces Trogopterori	—	—	—	8	8	8	16	24	11	23	34
59	侧柏炭	Fructus Rubi	6	12	9	15	12	12	24	36	17	34	51
60	覆盆子	Fructus Rubi	6	12	9	16	15	15	30	45	21	43	64
61	桑叶,DOUBT	Folium Mori	5	9	7	18	15	15	30	45	21	43	64
62	五味子	Fructus Schisandrae Chinensis	1.5	6	3.75	10	10	10	20	30	14	29	43
63	益智仁,DOUBT	Fructus Alpiniae Oxyphyllae	3	9	6	17	15	15	30	45	21	43	64
64	棕榈	棕榈 Petiolus Trachycarpi	3	9	6	15	15	15	30	45	21	43	64

	炭,DOUBT												
65	栀子	Fructus Gardeniae	6	9	7.5	8.2	9	9	18	27	13	26	38
66	贯众	Rhizoma Dryopteridis Crassirhizomatis 棉马贯众	5	10	7.5	20	15	15	30	45	21	43	64
67	生牡蛎	Concha Ostreae	9	30	19.5	27	30	30	60	90	43	86	128
68	*白豆蔻,DOUBT*	Fructus Amomi Rotundus	3	6	4.5	12	12	12	24	36	17	34	51
69	川楝(木)子	Fructus Toosendan	4.5	9	6.75	9.3	9.5	10	19	29	14	27	41
70	黄柏	Cortex Phellodendri Chinensis	3	12	7.5	11	10	10	20	30	14	29	43
71	乌梅炭	Fructus Mume	6	12	9	10	10	10	20	30	14	29	43
72	炒麦芽	Fructus Gordei Germinatus	9	15	12	13	15	15	30	45	21	43	64
73	炒枣仁	Semen Ziziphi Spinosae	9	15	12	14	15	15	30	45	21	43	64
74	远志	Radix Polygalae	3	9	6	11	10	10	20	30	14	29	43
75	知母	Rhizoma Anemarrhenae	6	12	9	13	10	10	20	30	14	29	43
76	茯神	Poriacocos(schw.)Wolf	—	—	—	12	10	10	20	30	14	29	43
77	三七粉	Radix Et Rhizoma Notoginseng	1	3	2	3.7	3	3	6	9	4	9	13
78	生龙骨	Os Draconis	—	—	—	30	30	30	60	90	43	86	128
79	黄精	Rhizoma Polygonati	9	15	12	11	11	11	22	33	16	31	47
80	鲨香		—	—	—	13	12.5	13	25	38	18	36	53
81	金樱子	Fructus Rosae Laevigatae	6	12	9	15	15	15	30	45	21	43	64
82	芡实	Semen Euryales	9	15	12	9	9	9	18	27	13	26	38
83	*生山楂,DOUBT*	Fructus Crataegi	9	12	10.5	25	25	25	50	75	36	71	107
84	延胡		—	—	—	10	10	10	20	30	14	29	43
85	延胡索	Rhizoma Corydalis	3	9	6	12	12	12	24	36	17	34	51
86	茵陈	Herba Artemisiae Scopariae	6	15	10.5	23	22.5	23	45	68	32	64	96

87	柏子仁	Semen Platycladi	3	9	6	15	15	15	30	45	21	43	64
88	石莲肉	Sinocrassula Indica (Decne.)	—	—	—	10	10	10	20	30	14	29	43
89	苏叶	紫苏叶 Folium Perillae	5	9	7	7.5	7.5	8	15	23	11	21	32
90	白及	Rhizoma Bletillae	6	15	10.5	20	20	20	40	60	29	57	86
91	陈棕炭	棕榈 Petiolus Trachycarpi	3	9	6	12	12	12	24	36	17	34	51
92	椿根皮	Cortex Ailanthi	6	9	7.5	10	10	10	20	30	14	29	43
93	干姜炭	Rhizoma Zingiberis	3	9	6	3	3	3	6	9	4	9	13
94	钩藤	Ramulus Uncariae Cum Uncis	3	12	7.5	15	15	15	30	45	21	43	64
95	合欢皮	Cortex Albiziae	6	12	9	15	15	15	30	45	21	43	64
96	鸡冠花	Flos Celosiae Cristatae	6	12	9	10	10	10	20	30	14	29	43
97	龙眼肉	Arillus Longan	9	15	12	15	15	15	30	45	21	43	64
98	枇杷叶	Folium Eriobotryae	6	9	7.5	10	10	10	20	30	14	29	43
99	桑椹	Fructus Mori	9	15	12	15	15	15	30	45	21	43	64
100	石菖蒲	Rhizoma Acori Tatarinowii	3	9	6	9	9	9	18	27	13	26	38
101	泽兰	Herba Iycopi	6	12	9	10	10	10	20	30	14	29	43
102	泽泻	Rhizoma Alismatis	6	9	7.5	10	10	10	20	30	14	29	43
103	蒺草	Radix Arnebiae	5	9	7	15	15	15	30	45	21	43	64
104	紫河车	Placenta Hominis	2	3	2.5	3	3	3	6	9	4	9	13
105	胡麻仁	Fructus Cannabis	9	15	12	12	12	12	24	36	17	34	51
106	花麦冬		—	—	—	15	15	15	30	45	21	43	64
107	佛手	Fructus Citri Sarcodactylis	3	9	6	10	10	10	20	30	14	29	43
108	蔻仁	SemenAlpiniae	—	—	—	6	6	6	12	18	9	17	26
109	石斛	Caulis Dandrobil	6	12	9	10	10	10	20	30	14	29	43
110	桃仁	Semen Persicae	4.5	9	6.75	6	6	6	12	18	9	17	26
111	红花	Flos Carthami	3	9	6	3	3	3	6	9	4	9	13

112	珍珠母	Concha Margaritifera	10	25	17.5	30	30	30	60	90	43	86	128
113	枳壳	Fructus Aurantii	3	9	6	9	9	9	18	27	13	26	38
114	苍术	Rhizoma Atractylodis	3	9	6	—	—	—	—	—	—	—	—
115	大青叶	Folium Isatidis	9	15	12	—	—	—	—	—	—	—	—
116	大枣	Fructus Jujubae	6	15	10.5	—	—	—	—	—	—	—	—
117	地骨皮	Cortex Lycii	9	15	12	—	—	—	—	—	—	—	—
118	厚朴	Cortex Magnoliae Officinalis	3	9	6	—	—	—	—	—	—	—	—
119	金银花	Flos Lonicerae Japonicae	6	15	10.5	—	—	—	—	—	—	—	—
120	芦根	Rhizoma Phragmitis	15	30	22.5	—	—	—	—	—	—	—	—
121	牡丹(皮)	Cortex Moutan	6	12	9	—	—	—	—	—	—	—	—
122	炮姜,(干姜)	Rhizoma Zingiberis Praeparatum	3	9	6	—	—	—	—	—	—	—	—
123	蒲公英	Herba Taraxaci	9	15	12	—	—	—	—	—	—	—	—
124	羌活	Rhizoma Et Radix Notopterygii	3	9	6	—	—	—	—	—	—	—	—
125	忍冬藤	Caulis Lonicerae Japonicae	9	30	19.5	—	—	—	—	—	—	—	—
126	鱼腥草	Herba Houttuyniae	15	25	20	—	—	—	—	—	—	—	—
127	败酱草	Herba Patriniae，'Whiteflower Patrinia Herb'	—	—	—	—	—	—	—	—	—	—	—
128	茜草	Radix Rubiae	—	—	—	—	—	—	—	—	—	—	—
129	桑螵蛸	Ootheca Mantidis	5	9	7	—	—	—	—	—	—	—	—

a. Frequency Sequence: descend sequence by the frequency of the individual Chinese medicines in 197 literatures with dosage record (Chapter IV). Totally 134 individual Chinese medicines were recorded, however, 5 of them were not recorded in "Chinese Pharmacopeia", so we only listed the other 129 Chinese medicines in this table.

b. Lowest: lowest dose for clinical application, recommended in "Chinese Pharmcopiea".

c. Highest: highest dose for clinical application, recommended in "Chinese Pharmcopiea".

d. Mean: for Pharmcopiea dose, mean refers to the calculated mean by (lowest +highest)/2, for literature dose, mean refers to the calculated mean by (sum of every reported dose in 131 literatures)/131.

e. Medium: for literature dose, medium refers to the medium dose of every reported dose in 131 literatures, calculated by excel formula.

f. Research dose: In most Chinese medicines, the medium was better involved in the recommended range of Chinese medicine in Chinese Pharmacopeia then mean, so we set up medium as 1 time clinical dose as reference to our animal study.

g. Animal dose: calculated by the standard formula for animal – human dose calculation, using research dose as human dose in the formula.

7.3.3.3 Administration

To avoid overload and regurgitation of the administrated crude extract, the volume of the extraction was adjusted to less than 0.5 ml in total. For in vivo test, the extraction was administered orally to the mice via a sterile gastric tubule (24 gauge/30mm long, Fine Science Tools, Foster City, California, USA). For rats, we used sterile feeding needles (20 gauge/75mm long, Braintree Scientific, Inc., Boston, USA). For rabbits, disposable pediatric feeding tube (Size 8, 40cm Length, Pacific Hospital Supply Co., Ltd., Taiwan) was used (Figure 7.1).

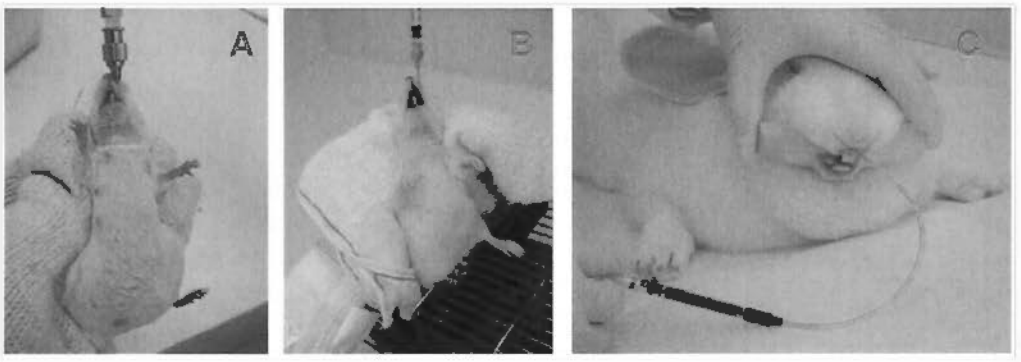


Figure 7.1 Orally administration of Largehead Atractylodes Rhizome to animals
A: mouse; B: rat; C: rabbit.

7.3.3.4 Study intervention (mice)

7.3.3.4.1 Groups

In mice, pregnant animals at various gestational stages, developmental stages, postpartum and postnatal period were randomized and grouped to receive different dosages (Figure 7.4), including Stage A (E3-E6, pregnant mice were fed from 3rd to 6th day of pregnancy), Stage B (E6-E8, pregnant mice were fed from 6th to 8th day of pregnancy), Stage C (E8-E15, pregnant mice were fed from 8th to 15th day of pregnancy), Stage D (E15-P28, pregnant mice were fed from 15th of pregnancy till

delivery while observations were continued till 28th day after delivery) and Stage E (E0-P28, pregnant mice were fed throughout the whole gestation while observations were continued till 28th day after delivery). Gestational milestones for each stage represented implantation, gastrulation, organogenesis, maturation and whole pregnant period, respectively.

As our focus was to identify the potential toxicity of Largehead Atractylodes Rhizome extracts during pregnancy, and to determine the specific gestational period and specific structure or organ affected under the treatment. We intervented with different pregnancy periods for in vivo tests as screening test for the safety of Largehead Atractylodes Rhizome extracts, including early pregnancy (E3-E6 study period), embryo fetal development (E6-E8 for gasturation period and E8-E15 for organogenesis period), prenatal and postnatal growth (E15-P28), and also whole gestational stage (E0-E18).

For each stage, the mice were administered with increasing clinical dosages (1x, 2x, 3x dose) as study groups, and vehicle control with normal drinking water (0x dose) was included as negative control group for comparison. Before administration, each animal was weighted and the calculated volume of extracted solution was administered.

7.3.3.4.2 Terminations

For Stages A-C, the pregnant mice were sacrificed at the end of each study period, i.e E6, E8 and E15, respectively. For Stages D and E, the pregnant mice were allowed to spontaneous deliver around E18 or E19. After birth, dams and pups were continually observed during the whole lactating period and scarified at the 28th day of postpartum and postnatal period.

7.3.3.4.3 Outcome measures

7.3.3.4.3.1 Maternal outcomes

Adverse pregnancy outcomes in the dams were recorded, including daily activities, food and water intake, body weight, miscarriage, preterm or postdate delivery and maternal mortality. Autopsy was performed if maternal death occurred. At the end of experiments, maternal plasma was collected for further pharmacological studies.

7.3.3.4.3.2 Fetal/neonatal outcomes

Specific fetal/neonatal outcomes for appropriate developmental stages were recorded (Table 7.1).

At E6 from Stage A group, litter size, resorption, fetal distribution, fetal size and developmental staging were recorded.

At E8 from Stage B group, litter size, resorption, fetal distribution, fetal size, crown-rump length (CRL), head length (HL), number of somite, growth restriction (GR), developmental staging and malformation were recorded. GR was defined as one of the body size parameters (CRL, HL, and somite number) was lower than mean – SD.

At E15 from Stage C group, litter size, resorption, fetal distribution, fetal size, crown-rump length (CRL), head length (HL), placenta diameters, GR, developmental staging and malformation were recorded. GR was defined as one of the body size parameters (CRL, HL, and placenta diameter) was lower than mean – SD.

At P0 from Stage D and E groups, litter size, birth weight, still birth, congenital malformation and developmental staging were recorded. Growth restriction was

defined as growth parameter (birth weight) was lower than mean – SD.

From P0 to P28 from Stage D and E groups, body weight, growth, activity, congenital anomalies and postnatal death were recorded. Growth restriction was defined as growth parameter (body wight) was lower than mean – SD.

7.3.3.5 Study intervention (rats and rabbits)

7.3.3.5.1 Groups

In rats, pregnant animals at gestational stage with sensitive toxicity period from E6 to E21 (from 6th day to 21st day of the pregnancy, suggested by Kuffman et al, 1998, as shown in Table 7.3), were randomly allocated and grouped to receive Largehead Atractylodes Rhizome extracts in different dosages. 3x and 6x daily clinical dosages were assigned as study groups, while feeding normal drinking water (0x dose) was included for drug effect controls as negative control group. Before administration, each animal was weighted and the calculated volume of extracted solution was administered.

Table 7.3 Comparative gestational milestones and developmental toxicity schedules for study animals

	Gestational Milestones[a]			Developmental toxicity testing schedule[a]	
Species	Differentiation	Organogenesis	Parturition	Exposure Period	Cesarean Section
Mouse	7-9	10-15	19-20	6-15	18
Rat	6-10	11-15	21-22	6-15	21
Rabbit	7.5-9	10-18	31-33	7-19	29
Human	7-21	21-56	266	NA[b]	NA

[a] In gestational days: day of confirmed mating = gestational day 0. [b] NA, not applicable.

Modified from Kaufman M. 1993

In rabbits, pregnant animals at gestational stage with sensitive toxicity period from E7 to E29 (from 7th day to 29th day of the pregnancy, suggested by Kaufman M, 1993, as shown in Table 7.3), were randomly allocated. However, due to limited number of matured does, we tested 3x clinical dose in 3 animals as study group, and sham control group without feeding intervention in 1 animal only. Each animal was weighted weekly to adjust the suitable volume of extracted solution for feeding, because the weight changes of does were not very significant daily, the slight changes of the solution were not necessary.

7.3.3.5.2 Terminations

In rats, the dams were fed daily and sacrificed at E21 by Caesarean section.
In rabbits, the does were fed daily and sacrificed at E29 by Caesarean section.

7.3.3.5.3 Outcome measures

7.3.3.5.3.1 Maternal outcomes

Adverse pregnancy outcomes in the dams/does were recorded, including daily activities, food and water intake, body weight, miscarriage, preterm or postdate delivery and maternal mortality. Autopsy was performed for maternal death. At the end of experiments, maternal plasma was collected for future pharmacological analysis.

7.3.3.5.3.2 Fetal outcomes

For fetuses of rats at E21 from all groups, litter size, resorption, fetal distribution, fetal size, crown-rump length (CRL), head length (HL), placenta weight, developmental staging and malformation were recorded.

For fetuses of rabbits at E29 from both sham control and 3x clinical dose groups, litter size, resorption, fetal distribution, fetal size, crown-rump length (CRL), head length (HL), ear length and width, indexes of limb (upper arm length, fore arm length, hand length and breadth, five fingers lengths, thigh length, leg length, knee hight, foot length and breadth, five toes lengths), placenta weight, developmental staging and malformation were recorded.

7.3.4 In Vitro Tests in Whole Mouse Embryos

7.3.4.1 Experimental designs

Potential embryotoxicty on embryo-fetal development, from early gastrulation to early organogenesis stages, were screened by the in vitro whole embryo culture system in mice, and the experimental design was shown in Figure 7.5.

ICR mouse embryos on the 7^{th} gestational day (E7) at the 1-5 somite stage were prepared. Interventions during this period may lead to general retardation of growth and development and/or to specific malformations in one and/or several organ systems, so as to have an overall understanding on the potential toxicity of the test medicine. Besides, whole embryo culture (WEC) is a good choice to have direct studies on the developing embryos or fetuses.

7.3.4.2 Dosage

We conducted an initial dose response experiment starting with at maximum of 0.1× in vivo animal dose with serial diluted lower concentrations covering the reference clinical dose in replicate *in vitro* until the critical concentrations (IC*vitro*) with embryotoxic effects to be identified (Figure 7.5).

Within the initial IC*vitro* range, we repeated the experiment in replicates with 5 test dosages per concentration in a maximum dose difference of a factor of 2 (Figure 7.5). We dissolved the extracts powder into 10 ml rat serum to prepare the desired solution, then serially diluted the dosage.

7.3.4.3 Administration

This dissolved Largehead Atractylodes Rhizome extracts was added by sterilized pipette into the heat treated rat serum with equilibrium, and kept in rolling rotor bottles at 37℃ for at least an hour.

7.3.4.4 Embryo dissection

Mouse embryos at E7.5 were removed from the uterus by caesarean section. Under a surgical microscope at low magnification, the uterus was separated and opened by sterilized scissors, decidua and Reichert's membrane were removed separately by sterilized forceps. The developmental stages of the embryos were evaluated and confirmed. Only embryos at primitive streak stage (E7.5-E7.75) during early gastrulation stage were selected for the tests. The damaged embryos and embryos at inappropriate developmental stage were discarded.

7.3.4.5 Groups

All embryos were randomized to study groups for different test doses, and 4 embryos in each group were pooled and cultured in a single rotor bottle for comparison. Embryos vehicle control supplemented with PBS supplement was included as negative control.

Figure 7.2 Experimental Protocol for in vitro screening

Drug	Study groups: Largehead Atractylodes Rhizome extracts Sham control group: 1x PBS in MilliQ water were added into the culture rat serum.
	↓
Dosage	Sham control group: 0x in vivo dose Study groups: 0.1x, 0.05x, 0.01x, 0.005x, 0.0025x, 0.0005 x in vivo dose
	↓
	IC vitro dose
	↓
	2x, 1x, 0.5x, 0.15x, 0.125x, 0.0625x in vitro dose
	↓
Animals	Pregnant ICR mice embryos
Gestational days	E7.5 - E9.5
Sample size	5
Supplementation	At E7.5 in culture media
	↓
Aim	Evaluate late embryonic development
	↓
Assessments	Late embryo growth and early system development
Outcome measures	Yolk sac size, Yolk sac circulation
	Crown rump length, Head length
	Morphological scoring (Maele-Fabry etal., 1990)
	Abnormalities or Malformations (Mal)
	Mal_{50}, $1/Mal_{50}$, $IC50_{mal}$, IC_{NOEC} (Wang et al., 2007)

7.3.4.6 In vitro culture

The culture serum together with the embryos were equilibrated with a gas mixture consisting of 5% O_2, 5% CO_2 and 90% N_2 for the first 24 hr, then with 20% O_2, 5% CO_2 and 75% N_2 for another 8 hr, and finally with a mixture consisting of 40% O_2, 5% CO_2 and 55% N_2 for further 16 hours or longer (Precision Incubator 317, B.T.C. Engineering, Milton, Cambridge, UK). The cultured embryos were harvested at E9.5.

7.3.4.7 Developmental assessments

During 48 hrs of culture, major steps of early organogenesis occur, including heart, neural tube, otic and optic, branchial bars, limbs and tails. Interference during this period may lead to development delay and/or specific congenital malformations in the organ systems. At the end of the embryo culture, growth, developmental and

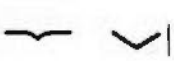
morphological parameters of the embryos were carefully recorded. The incidence of malformations (Mal) was recorded. Standard scoring system on each developmental hallmark was used to score each developing embryo (Table 7.4). Total morphological score (TMS) for each embryo at the test concentration was calculated and averaged, which could not only be applied to evaluate morphology of the whole developing embryo by the sum of the scores, but also helpful to determine the specific manifestation of the main developing organs (Xu et al., 2010). Embryotoxicity dose was expressed as the concentration of the supplemented extract induced malformation and decreased TMS in percentage in comparison to the mean TMS of sham controls.

7.3.4.8 Pharmacotoxicity measures

To determine the embryotoxic potentials of Largehead Atractylodes Rhizome extract by in vitro tests, a dose response curve was plotted by TMS percentage changes in the y-axis against logarithmic concentrations of Largehead Atractylodes Rhizome extract in the x-axis. All the embryotoxicity effects were compared with sham control, which was set as 100%. The potential embryotoxicity was determined by comparing the following pharmacotoxicity measures obtained from the dose response curve.

WEC IC_{NOEC} – the highest concentration that had no effect on the malformation rate in developing embryos after 48 hrs of treatment;
WEC IC_{50} – the concentration at which 50% of the developing embryos were malformed after 48 hrs of treatment;
WEC $IC_{NOECTMS}$ – the highest concentration that had no effect on mean TMS in developing embryos after 48 hrs of treatment;
WEC IC_{50}TMS – the concentration at which the mean TMS was reduced to 50% of developing embryos after 48 hrs of treatment.

Table 7.4 Morphological features of mice embryos for in vitro tests (scoring system)

Feature	0	1	2	3	4	5	6	Score*
Yolk Sac Circulatory System								4-5
Allantois								3
Flexion								5
Heart								4
Caudal neural tube								4
Forebrain								5
Midbrain								4
Hindbrain								5
Otic								4
Optic								4
Olfactory								2
Branchial bars								2
Maxillary process								2
Mandibular process								2
Forelimb								2
Hindlimb								1
Number of somites	0-5	5-10	11-15	16-20	21-25	26-30		21-25

* Score: The reference score for E9.5 embryo.

Furthermore, to evaluate the embryo toxicity of Largehead Atractylodes Rhizome, the recommended protocol from the European Center for the Validation of Alternative Methods (ECVAM) was applied by using the mean $IC50_{Mal}$ and $IC_{NOEC\ TMS}$ values as follows.

Function I (FN I) = $18.08 \times \log(IC50_{Mal}) - 11.56 \times \log(IC_{NOEC\ TMS}) - 10.19$,

Function II (FN II)=$21.55 \times \log(IC50_{Mal}) - 15.31 \times \log(IC_{NOEC\ TMS}) - 10.65$,

Function III (FN III)=$8.70 \times \log(IC50_{Mal}) - 8.53 \times \log(IC_{NOEC\ TMS}) - 2.53$.

If the result of FN I is higher than the results of both FN II and FN III, the test substance or medicine is classified as non-embryotoxic; if the result of FN II is higher than the ones of FN I and FN III, it is classified as weakly embryotoxic; if the result of FN III is higher than that of FN I and FN II, it is classified as strongly embryotoxic.

7.4 Statistical Analysis

T-test and Homogeneity of variance was tested by Bartlett's examination. Homogenous data were analyzed using the T-test, while non-homogenous data were analyzed using non-parameteric tests such as Mann-Whitney U test. The number of fetal resorptions, retailed fetuses and malformed fetuses were presented as in litter-based percentages and were analyzed by non-parametric methods. The incidence of the adverse effects was analyzed using a χ^2 test for all groups followed by Fisher's two-tailed test with Bonferroni correction for each treatment group versus the negative control. Significance was defined as $p<0.05$.

7.5 Pathological Examination

The study specimens, such as maternal organs and tissues, and embryos and fetuses with adverse effects were prepared for pathological examination.

7.5.1 Fixation

The specimens were preserved in 4% para-formaldehyde in PBS overnight at 4℃. After washed with DEPC-PBS for 3 times, the fixed tissues were dehydrated with 25%, 50%, 75% and 100% ethanol, kept in 70% ethanol, and finally embedded in paraffin wax. The paraffin blocks were then sectioned by microtome. The tissue sections were mounted on glass slides, then dry in open air or in oven.

7.5.2 H&E Staining

Hematoxylin and Eosin staining was performed in glass sections. The staining method was as described previously (Inouye, 1976; Kimmel et al., 1993). The dewaxing process rack with tissue mounted slides were immersed in 100% Xylene for 3 minutes, and repeated 3 times, then in 50:50 Xylene/100% ethanol for 3 minutes. Rehydration was performed by immersed the rack in 100% ethanol, 100% ethanol, 95% ethanol, 95% ethanol each for 3 minutes, then rinsed with distilled water for 2 or 3 times. The slides were put in Hematoxylin (Gill's 1X) for 5 minutes, followed by rinsing under running tap water in a staining box until the water is no longer colored. Then the slides were dig into Acid Alcohol Dunk (1% HCl in 70% ethanol) 2-3 times until the sections turn pink. After rinsing with tap water for 3-5 minutes and 5 or 6 slow dunks in Ammonia Water (1mL NH_4OH in 1L H_2O), the sections were darkened noticeably. Rinsing with tap water for another 3-5 minutes, the slides were immersed in Eosin Y for 1 minute, followed by rinsing under running tap water 3-4 times. The slides were dehydrated in 95% ethanol and 100% ethanol for 2 minutes, respectively, and repeated 3 times, then in 50:50 Xylene/100% ethanol for 2 minutes, and finally in 100% Xylene 3 times for 2 minutes.

7.6 Skeleton Staining

Alcian blue and Alizarin Red Staining method was used for specific staining on

skeleton and cartilage tissues in fetuses and neonates. The protocol was followed according to Kessel's method (Kessel et al., 1995). The fetuses and neonates showing skeleton malformations were scarified, eviscerated and fixed in 100% ethanol for a week, then dehydrated in acetone for 3 days. After rinsing with tap water for several times, the animals were immersed in the staining solution, which consisted of 0.3% alcian blue 8GX in 70% ethanol, 0.1% alizarin red S in 95% ethanol, 100% acenic acid, and 100% ethanol (1:1:1:17) for 10 days. The staining duration could be extended depend on the size of specimens and staining intensity. Rinse the animals with tap water again, then kept them in 20% glycerol/1% KOH in water at 37℃ for 16hr or 1-2 days for skin and muscle digestion until clear observation of the bone and no remains of the muscle. For larger animals, longer staining and digestion duration were needed. For long term storage, the stained fetuses and/or neonates were dehydrated with 50% and 80% glycerol respectively for 1-2 days, then kept in 100% glycerol.

7.7 Thin Layer Chromatography (TLC) Analysis

TLC is a chromatography technique to separate mixtures, as different analytes ascend with different rate on the TLC plate. The main constitutions of the crude extracts of the test Chinese medicine were primarily determined and quantified in this way.

After extraction of raw herbs, the dried crude extracts were fractionated into two parts: hydrophilic and lipophilic fractions. The dried extract was dissolved by heating at 60-70℃ in 800 ml distilled water. The solution was then partitioned with n-Butanol (800ml each time twice) to divide into two portions. For the lipophilic portion, n-butanol layer was evaporated under reduced pressure to give a dark gummy residue. The hydrophilic portion was concentrated in vacuum and freeze-dried as powder. Residue of the lipophilic fraction was dissolved in methanol. Residue of hydrophilic fraction was dissolved in methanol to give a methanolic solution and a precipitate.

A small spot of the solution containing the sample was applied to the silica plate, 1.5 cm away from the bottom edge, then the solvent was allowed to completely evaporate off. A small amount of an appropriate solvent was poured in to a glass beaker (separation chamber with filter paper inside) to a depth of less than 1 cm. The beaker was then closed with a cover glass and was left for a few minutes to allow the solvent vapors ascend the filter paper and saturate the air in the chamber. The solvent moved up the plate by capillary action, met the sample mixture and carried it up the plate. When the solvent front reached no higher than the top of the filter paper, the plate should be removed, to avoid misleading result due to continuation of the elution, then dried.

According to the different travelling rate of each chemical or compound, separation was achieved. In case of the chemicals or compounds being colorless, four common methods, including UV-254 nm, UV-365 nm, visible light after heating process and UV-365 nm after heating process, were applied to visualize the spots and further analysis. Chemical markers were obtained from an authorized pharmaceutical company (Shang Hai Tauto Biotech Co. Ltd China). Atractylenolide I (白朮內酯 I) and Atractylenolide III (白朮內酯 III) were included as reference for TLC analysis.

7.8 Results

7.8.1 Quality Control

7.8.1.1 Extraction yields

123g of crude extracts were obtained from 500 g of Largehead Atractylodes Rhizome dried root, the extraction yield was 24.6 %w/w, which is good and consistent with the common yield of the raw herbs of Largehead Atractylodes Rhizome in the ICM

ladoratory. The crude water extract was then further extracted and fractionated into two parts: hydrophilic and lipophilic portions. Finally 107.91g of hydrophilic fraction and 4.435g of lipophilic fraction were yielded, 87.7%w/w and 3.6%w/w, respectively. Though very limit lipophilic fraction was obtained, as the main components of Largehead Atractylodes Rhizome were hydrophilic, and the result was consistent with the Largehead Atractylodes Rhizome extraction in other laboratories.

7.8.1.2 TLC analysis

To confirm the constituents of Largehead Atractylodes Rhizome within the extracts, the crude extracts were further prepared for TLC analysis. The results showed the separation of the main constitutions of Largehead Atractylodes Rhizome retained in the extracts (Figure 7.3). Atractylenolide I and III in the lipophilic fraction were detected in UV-365 nm on the plate sprayed with 2% vanillin-sulphuric acid reagent after heat treating process (Figure 7.3D).

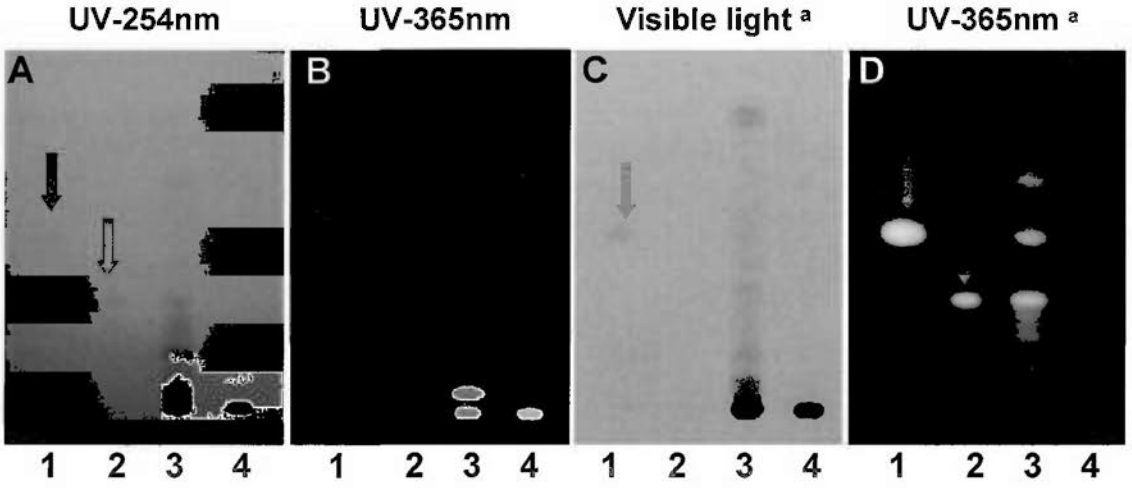


Figure 7.3 Constituents in Largehead Atractylodes Rhizome detected by TLC

a: the plate was heated at 100-110℃ for 2-5min before observation. Lane 1: standard Atractylenolide I (Rf 0.51); Lane 2: standard Atractylenolide III (Rf 0.32); Lane 3: hydrophilic portion of Largehead Atractylodes Rhizome extracts; Lane 4: lipophilic portion of Largehead Atractylodes Rhizome extracts. Arrows: signal from the standards.

7.8.2 In Vivo Safety Study in Mice

7.8.2.1 Effects on maternal weight

7.8.2.1.1 Gestational changes

During the implantation period, there was no significant difference in maternal weight gain or loss between treatment groups and control group (Figure 7.4; T-test, $p>0.05$; ANOVA test, $p>0.05$).

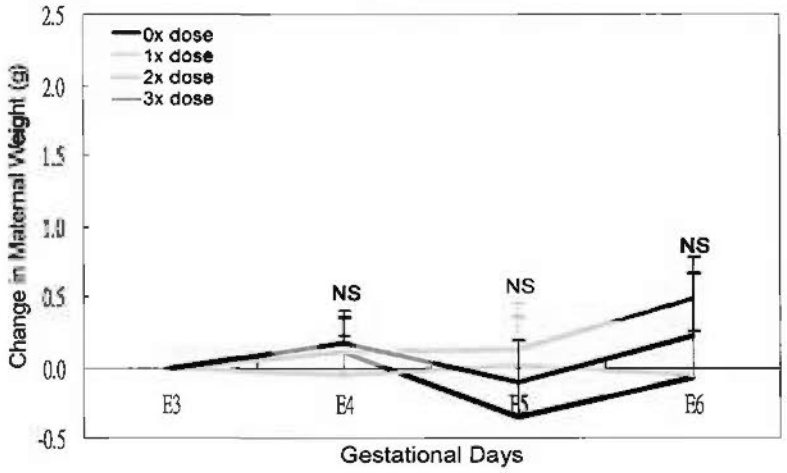


Figure 7.4 Maternal weight changes in implantation period

NS= not significant

During the gastrulation period, no significant difference was found between treatment groups and control group (Figure7.5; T-test, $p>0.05$; ANOVA test, $p>0.05$).

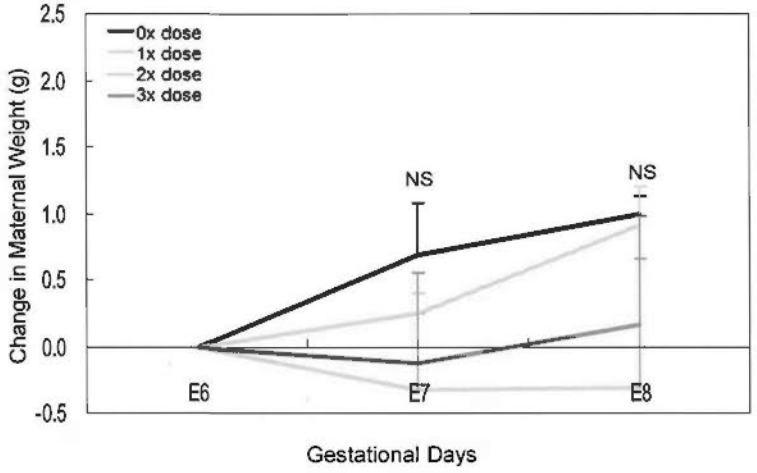


Figure 7.5 Maternal weight changes in gastrulation period

NS= not significant

During the organogenesis period, no significant difference was found between treatment groups and control group (Figure7.6; T-test, $p>0.05$; ANOVA test, $p>0.05$).

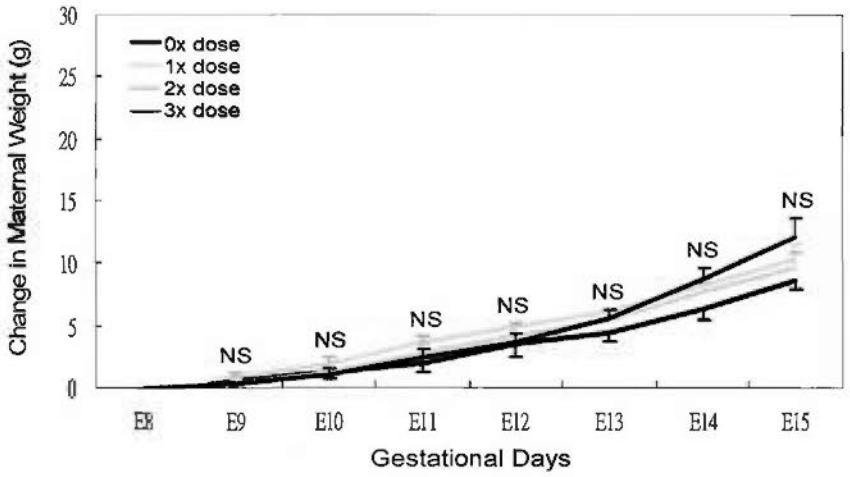


Figure 7.6 Maternal weight changes in organogenesis period

NS= not significant

During the maturation period, before the 17th day of gestation, no significant difference was found between treatment groups and control group (Figure7.7; T-test, $p>0.05$; ANOVA test, $p>0.05$). Prior to delivery, only maternal weights in 1x clinical dose group were significant lower than that in control group (Figure7.7; T-test, $p<0.05$; ANOVA test, $p>0.05$). However, no significant effects on maternal weight gain was found in higher clinical dose groups (T-test, $p>0.05$; ANOVA test, $p>0.05$).

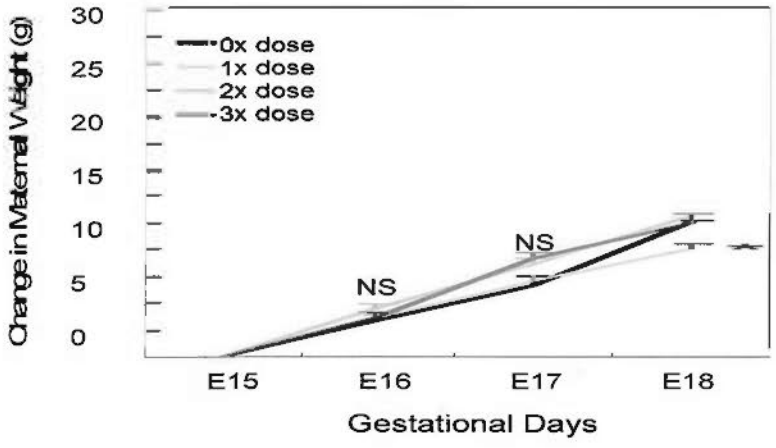


Figure 7.7 Maternal weight changes in maturation period

NS= not significant; * $p<0.05$.

During the whole gestational period, significant maternal weight loss was found in earlier pregnant stages on E1, E2, E3, and E5 (Figure7.8; T-test, $p<0.05$; ANOVA test, $p>0.05$). After 9th day of gestation, no significant difference was found between the study groups and control group (Figure7.8; T-test, $p>0.05$; ANOVA test, $p>0.05$), and no dose dependent effect was found for maternal weight change.

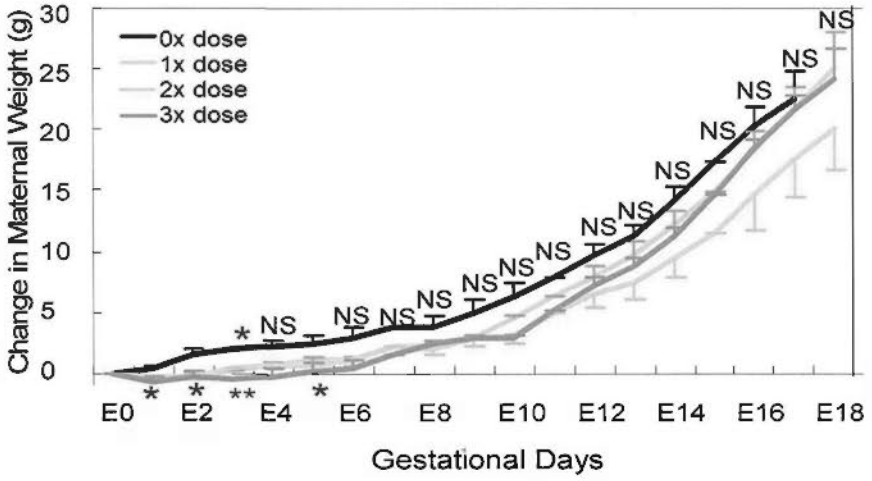


Figure 7.8 Maternal weight changes in whole gestational period

NS= not significant; * $p<0.05$

7.8.2.1.2 Postpartum changes

After administration during the maturation period, significant maternal weight loss in 2x and 3x clinical dose groups was found on 2nd and 3rd day after delivery (Figure7.9; T-test, $p<0.05$). In the 2nd week after delivery, maternal weight loss in all study groups were significant (T-test, $p<0.05$; ANOVA test, $p>0.05$). At the end of the 3rd week after delivery and lactation, no significant difference was found between study groups and control group (T-test, $p>0.05$; ANOVA test, $p>0.05$).

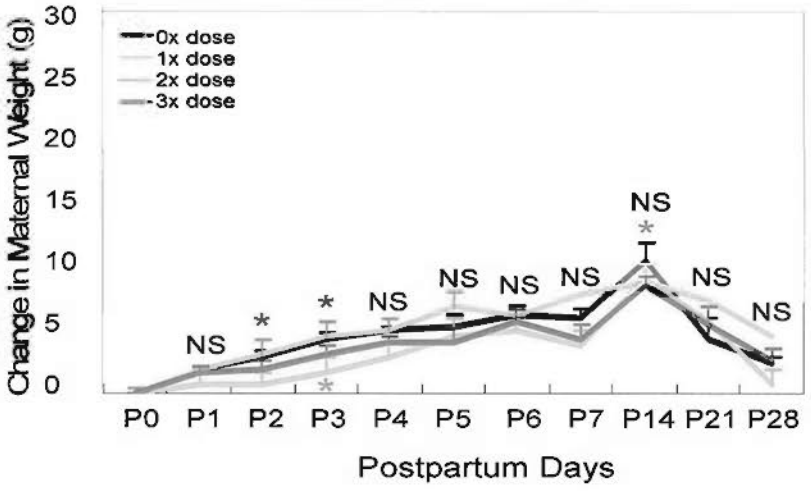


Figure 7.9 Postpartum maternal weight changes with intervention during maturation period

NS= not significant; * p<0.05 (study group versus control group).

After administration during the whole gestational period, significant difference was only found at the 1st and 2nd day after delivery (Figure7.10; T-test, p<0.05; ANOVA test, p>0.05). There was no significant difference amongst the study groups after different dose interventions in the first week and then after delivery (T-test, p>0.05; ANOVA test, p>0.05).

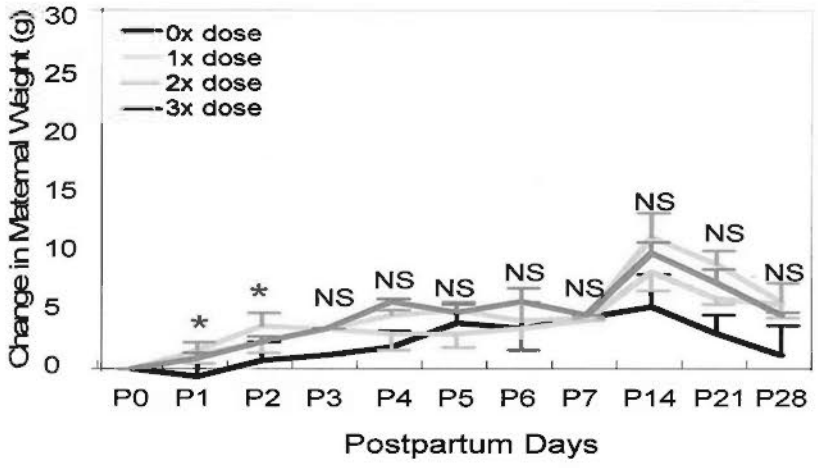


Figure 7.10 Postpartum maternal weight changes with intervention during whole gestational period

NS= not significant; * p<0.05.

7.8.2.2 Adverse outcomes on mothers

One pregnant mouse with intervention in whole gestational period (E0-P0) from control group suffered from diarrhea on E10 but recovered on E14 spontaneously; no maternal death was observed before delivery; 4 animals died at postpartum day P6, P6, P13, and P13, respectively, without abnormal finding at autopsy (Table 7.5). No significant difference was found between study groups and control group (Table 7.6; χ^2-test, $p>0.05$; ANOVA test, $p>0.05$).

7.8.2.3 Fetal resorption

Fetal resorption was significantly higher in 2x and 3x clinical dose groups (Figure 7.11; χ^2-test, $p<0.05$).

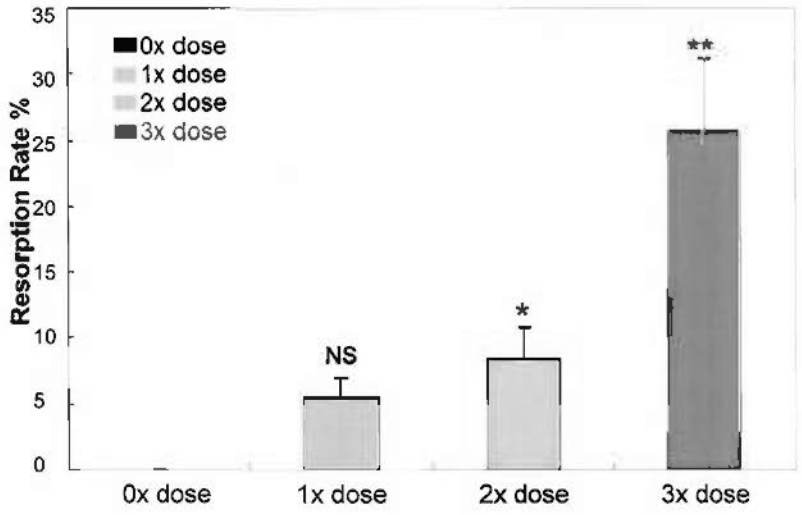


Figure 7.11 Resorption rate during implantation period

NS= not significant; * p<0.05; **p<0.01

Table 7.5 Records of maternal adverse outcomes

Intervention	Amount	Adverse Outcomes & Reason
Control	26	1 died at P6 without obvious cause (E15-P0) in maturation period
		1 died at P13 without obvious cause (E0-P0) in whole gestational period
1x dose	25	1 died at P6 without obvious cause (E15-P0) in maturation period
		1 died on P8 without obvious cause (E15-P0) in maturation period
2x dose	22	No adverse outcome
3x dose	26	1 suffered from diarrhea from E10 to E14 in whole gestational period, then stopped

Table 7.6 Postpartum mortality

Intervention	E3-E6	E6-E8	E8-E15	E15-P0	E0-P0
Period	Implantation	Gastrulation	Organogenesis	Maturation	Whole Gestation
control	Dam=8	Dam=8	Dam=8	Dam=5	Dam=4
	n=0, 0%	n=0, 0%	n=0, 0%	n=1, 3.8%#	n=1, 3.8%#
1x dose	Dam=8	Dam=8	Dam=8	Dam=5	Dam=5
	n=0, 0%	n=0, 0%	n=0, 0%	n=2, 8%#	n=0, 0%
2x dose	Dam=8	Dam=8	Dam=8	Dam=5	Dam=5
	n=0, 0%	n=0, 0%	n=0, 0%	n=0, 0%	n=0, 0%
3x dose	Dam=8	Dam=8	Dam=8	Dam=5	Dam=4
	n=0, 0%	n=0, 0%	n=0, 0%	n=0, 0%	n=0, 0%

Postpartum mortality = (number of dead maternal mice) / (number of maternal mice in the group) * 100

* statistics, $p<0.05$.

Abnormal deciduas and embryos observed in intervention during implantation period showed resorbed deciduas from study groups smaller and congested (Figure 7.12A). Figure 7.9B showed the embryos inside the deciduas. The embryos in 1x clinical dose group developed well as control group with normal egg cylinder structures, while embryos in 2x clinical dose group were less developed and abnormal growth of the deciduas vascular, and abnormal growth of deciduas vascularity in 3x clinical dose group were identified.

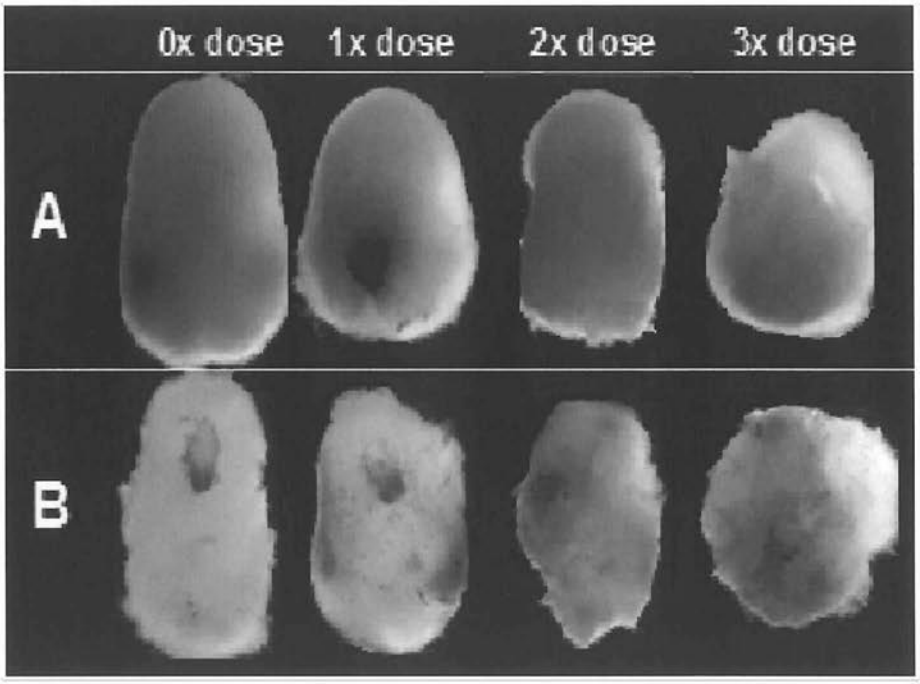


Figure 7.12 Deciduas and embryos with intervention during implantation period

The incidence of this resorption was not significantly difference between the study groups and the control group (Figure 7.13; χ^2-test, $p>0.05$). The resorptions resulted from intervention during gastrulation period showed the abnormal deciduas and embryos on both sides of uterus from 3x clinical dose group were smaller than control group at E8.5 gestation (Figure 7.14).

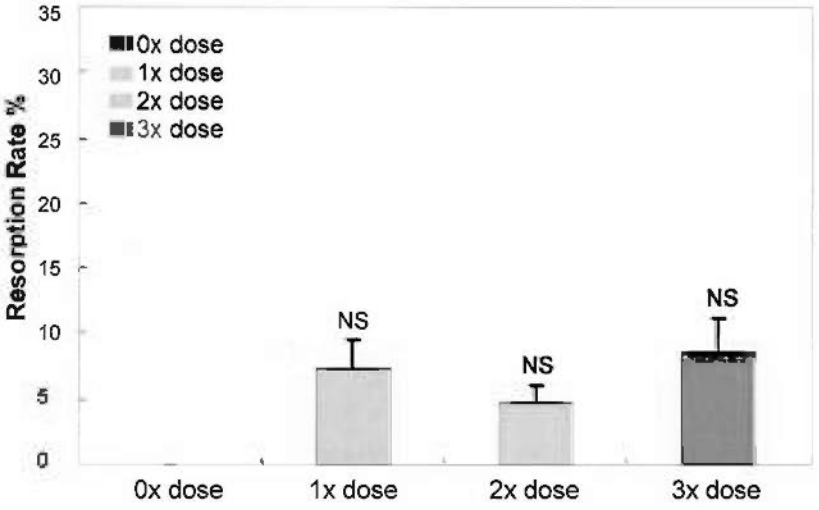


Figure 7.13 Resorption rate during gasturation period

NS= not significant; * p<0.05; **p<0.01

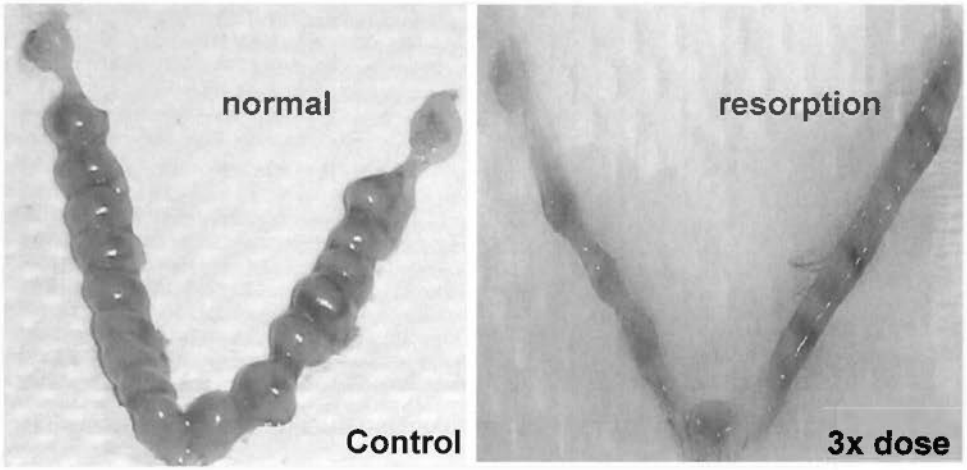


Figure 7.14 Resorption with intervention during gastrulation period

The incidence of this resorption was at the same level amongst different dose interventions, and no significant difference was found between the study groups and control group (Figure 7.15; χ^2-test, $p>0.05$). The resorpted fetuses with placentas whose mothers had intervention during organogenesis period showed small and underdeveloped fetuses and placentas. The body structures of resorpted embryos were difficult to recognize, and the placenta were pale and with less obvious vessel structures in higher clinical dose groups (Figure 7.16).

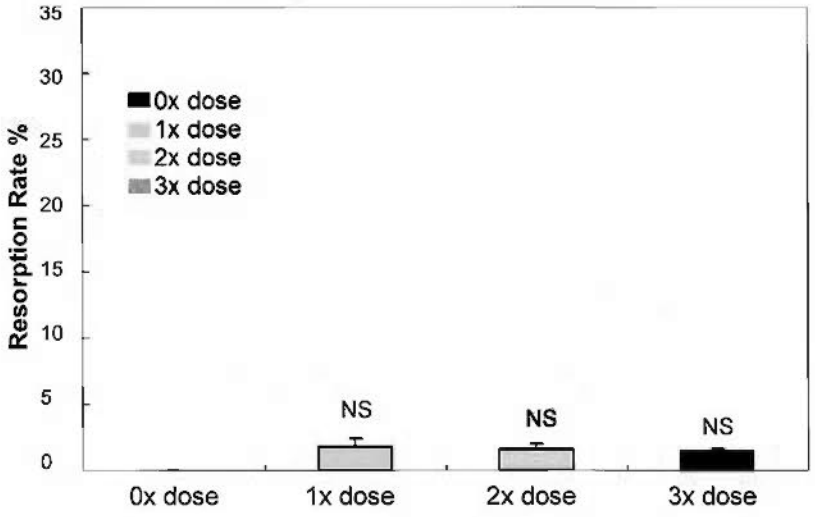


Figure 7.15 Resorption rate during organogenesis period

NS= not significant; * p<0.05; **p<0.01

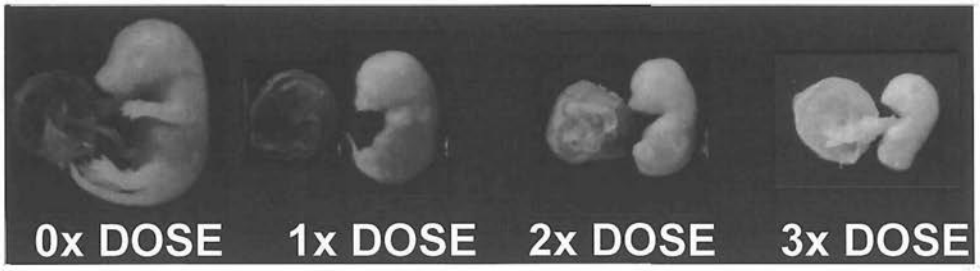


Figure 7.16 Fetuses with intervention during organogenesis period

Higher dose groups had significant higher resorption rates when interventions were applied in implantation period (Figure 7.11; χ^2-tests, $p<0.05$), while there was no significant difference between study groups and control group when interventions were applied in gastrulation period (Figure 7.13; χ^2-tests $p>0.05$) and organogenesis period (Figure 7.15; χ^2-tests, $p>0.05$).

7.8.2.4 Embryonic/fetal size

Embryonic size changes were observed in the embryos whose mothers were treated

with Largehead Atractylodes Rhizome extracts during gastrulation period. The key parameters, crown rump length (A), head length (B) and number of somite (C), were identified as shown in Figure 7.17. Means of CRL and HL were 1.7±0.23 and 0.6± 0.16 in control group. CRL below 1.47 mm and HL below 0.44 mm were defined as growth restricted embryos.

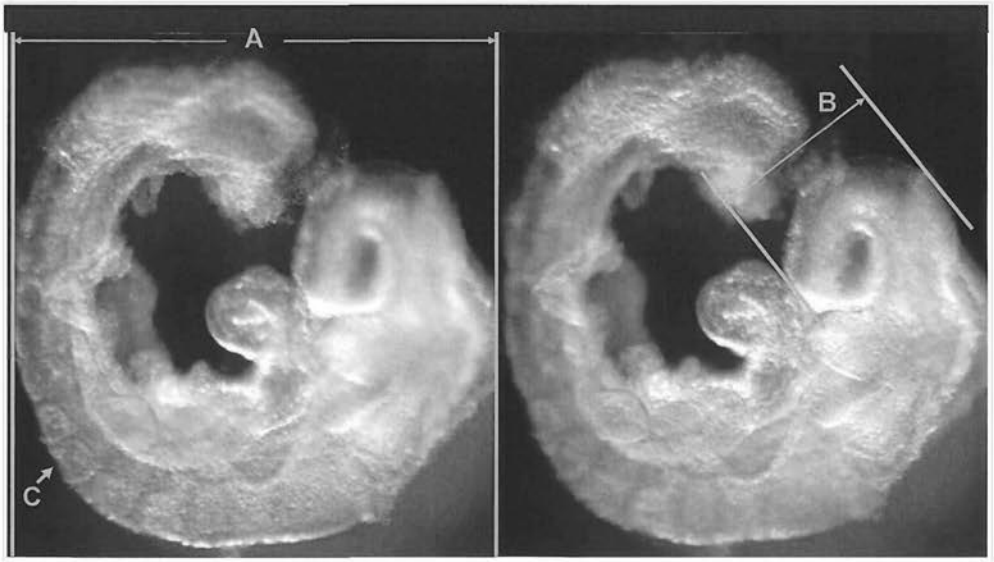


Figure 7.17 Evaluations on embryo size

A: crown rump length; B: head length; C: number of somite.

The crown rump lengths in 1x and 3x clinical dose groups were significantly shorter than control group (Figure 7.18; T-test, $p<0.05$; ANOVA test, $p>0.05$), while no significant difference was found in the embryos from 2x clinical dose group (T test, $p>0.05$; ANOVA test, $p>0.05$). No significant difference in the head lengths and numbers of somites was found between study groups and control group (T test, $p>0.05$; ANOVA test, $p>0.05$).

In the intervention during gastrulation period, the results of comparison on the incidence of growth restriction (GR) between study groups and control group were shown in Figure 7.19. All study groups were recorded with smaller embryos, and higher incidence of GR was found with the increase doses, however, no significant

smaller embryos were found between study groups and control group (χ^2-test, $p>0.05$).

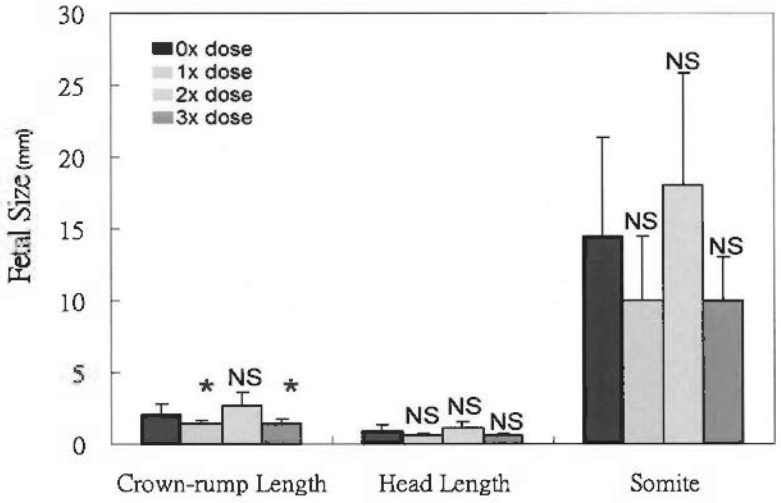


Figure 7.18 Changes of embryonic size with intervention in gastrulation period

NS= not significant; * $p<0.05$.

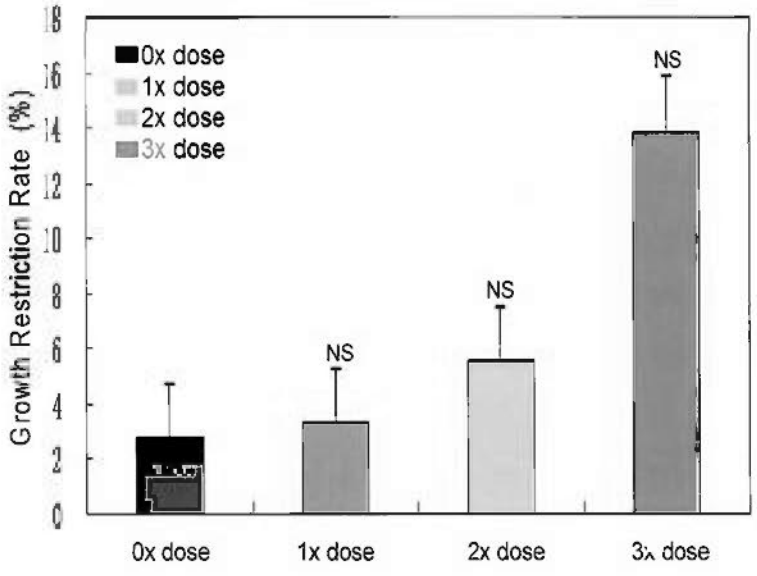


Figure 7.19 Incidence of embryo growth restriction with intervention in gastrulation period

NS= not significant.

Embryonic size changes were observed in the embryos whose mothers were treated with Largehead Atractylodes Rhizome extracts during organogenesis period. The key parameters, crown rump length (A), head length (B) and diameters of placenta (C), were identified as shown in Figure 7.20.

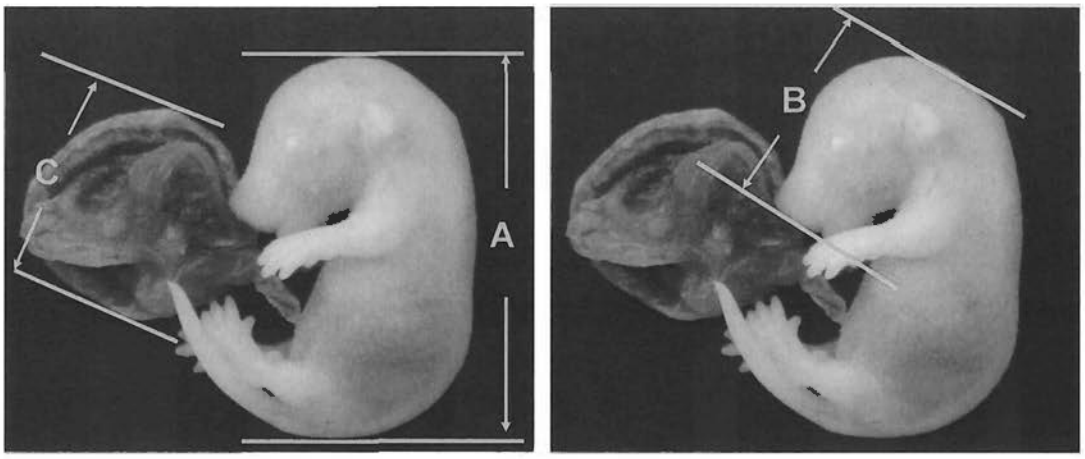


Figure 7.20 Evaluations on fetal size

A: crown rump length; B: head length; C: diameters of placenta.

Means of CRL, HL and placenta diameter were 16±1.36, 8.5±0.59 and 6.55±0.57 in control group. CRL below 14.7 mm, HL below 7.9 mm and placenta diameter below 6 mm were defined as smaller embryos. No significant difference in the crown rump length, head length and diameter of placenta was found between all study groups and control group (Figure 7.21; T test, $p>0.05$; ANOVA test, $p>0.05$).

In the intervention during organogenesis period, the results of comparison on the incidence of growth restriction (GR) between study groups and control group were shown in Figure 7.22. Obvious smaller fetuses were only observed in 1x clinical dose group, but no significant was found compared with control group (χ^2-test, $p>0.05$; ANOVA test, $p>0.05$).

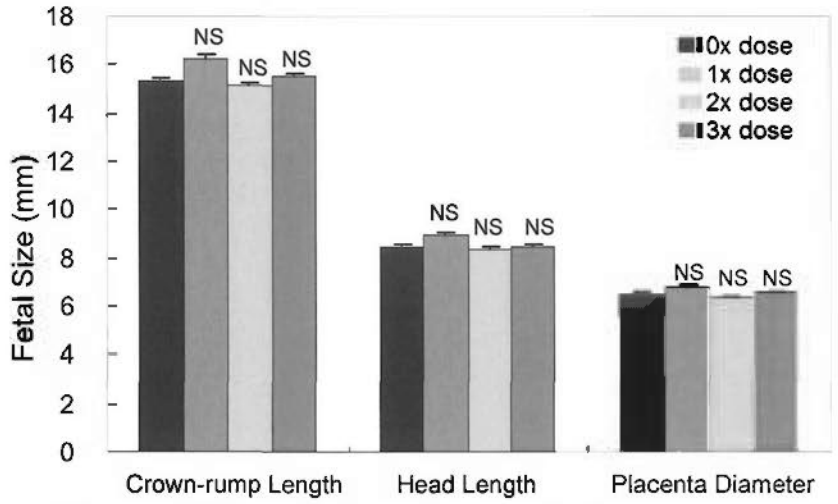


Figure 7.21 Changes of fetal and placental size with intervention in organogenesis period

NS= not significant

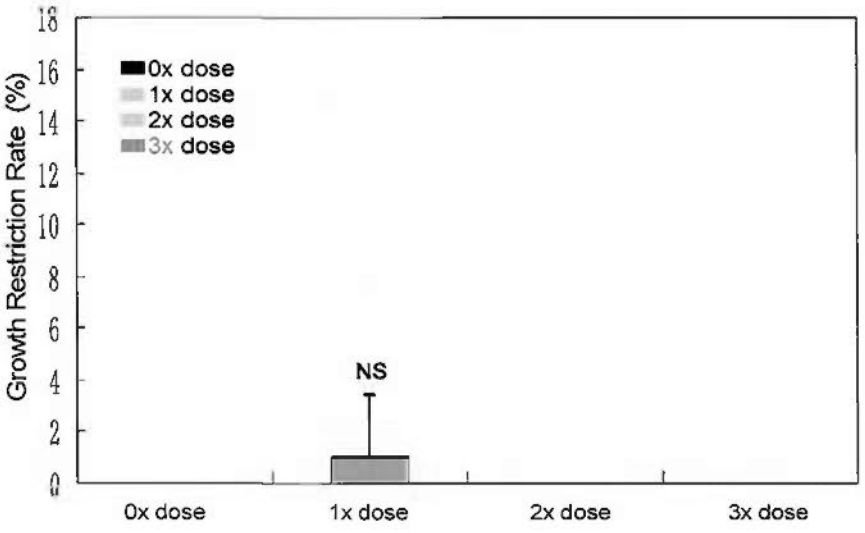


Figure 7.22 Incidence of fetal growth restriction with intervention in organogenesis period

NS= not significant.

7.8.2.5 Postnatal growth

With the intervention during maturation period, significant lower weight gain were

found in neonates of 2x clinical dose and 3x clinical dose groups from late 1st week to the end of 2nd week of postnatal development (T test, $p<0.05$ on P5, P6, P7 and P14; ANOVA test, $p>0.05$), but no significant difference was found between 1x clinical dose group and control group (Figure 7.23).

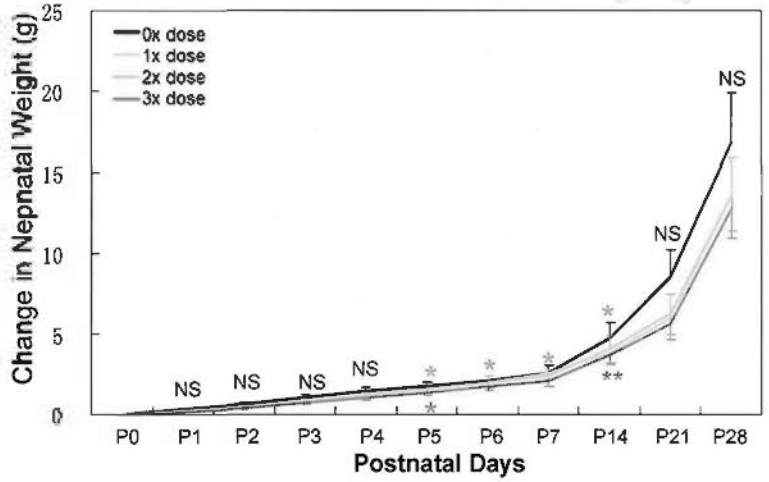


Figure 7.23 Fetal weight gain with intervention in maturation period

NS= not significant; * $p<0.05$; ** $p<0.01$

With the intervention during whole gestational period, in the first 5 days after birth, no significant difference between study groups and control group (Figure 7.24; T test, $p>0.05$; ANOVA test, $p>0.05$). In the following one week, no significant difference was found in all study groups (T test, $p>0.05$; ANOVA test, $p>0.05$).

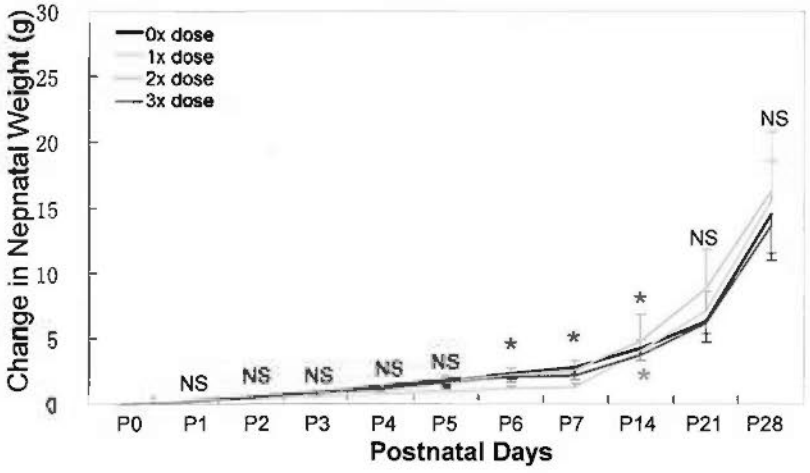


Figure 7.24 Fetal weight gain with intervention in whole gestational period

NS= not significant; * $p<0.05$.

7.8.2.6 Postnatal mortality

After intervention in maturation period (Figure 7.25A), no significant increase in postnatal mortality was found between study groups and control groups (χ^2-tests, $p>0.05$). After intervention during the whole gestational period (Figure 7.25B), significant increase in postnatal mortality was found between 2x clinical dose group and control groups (χ^2-tests, $p<0.05$) (Table 7.7).

Table 7.7 Postnatal mortality with intervention during maturation and whole gestational periods

Intervention Period	Dose	Amount of Neonates	Number of Deaths	Mortality (%)
Maturation Period	0x	120	13	10.8
	1x	95	12	12.6
	2x	71	20	28.2
	3x	90	22	24.4
Whole Gestational Period	0x	39	1	2.6
	1x	53	14	26.4
	2x	45	25	55.6
	3x	54	2	3.7

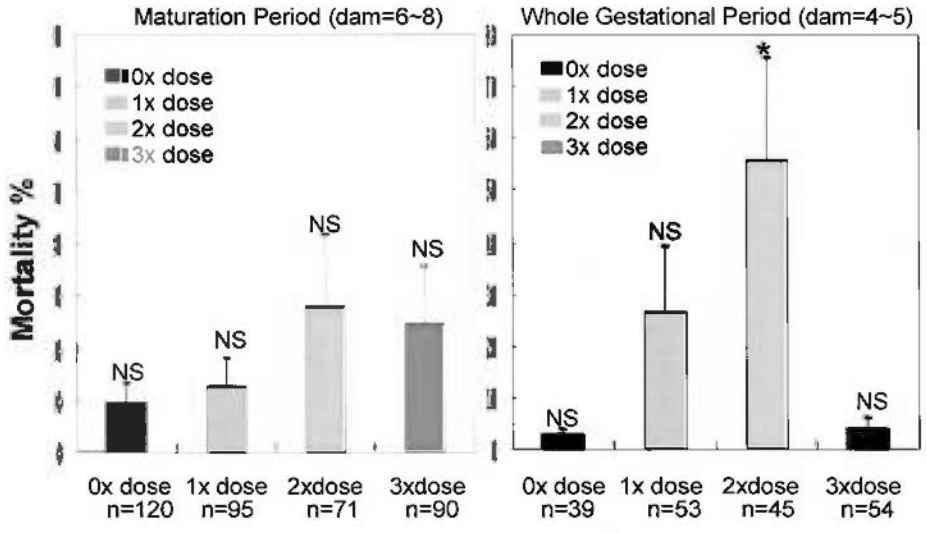


Figure 7.25 Postnatal mortality with different intervention periods on mothers

NS= not significant; * $p<0.05$.

7.8.2.7 Malformation

No malformations were found in control groups (Table 7.8). The incidence of malformation showed administration during maturation period (Figure 7.26A), significantly higher malformation rate in 1x and 2x clinical dose groups than control group (χ^2-tests, $p<0.05$), while no malformation was found in 3x clinical dose group. During the whole gestational period (Figure 7.26B), only 3x clinical dose group was recorded with malformation, and it was significantly different from control group (χ^2-tests, $p<0.05$).

Different types of exterior malformations on neonates were observed (Figure 7.27). Extended extension of upper and/or lower limbs was found in maturation period intervention groups, while extended adduction of upper limbs was found in whole gestational period intervention groups. No major birth defects in other organs was identified in autopsy.

Table 7.8 Neonatal malformation with intervention in maturation and whole gestational periods

Intervention Period	Dose	Amount of Neonates	Number of Malformation	Malformation (%)	Type of Malformation
Maturation Period	0x	120	0	0.0	
	1x	95	1	1.1	skeleton
	2x	71	2	2.8	skeleton
	3x	90	0	0.0	
Whole Gestational Period	0x	39	0	0.0	
	1x	53	0	0.0	
	2x	45	0	0.0	
	3x	54	2	3.7	skeleton

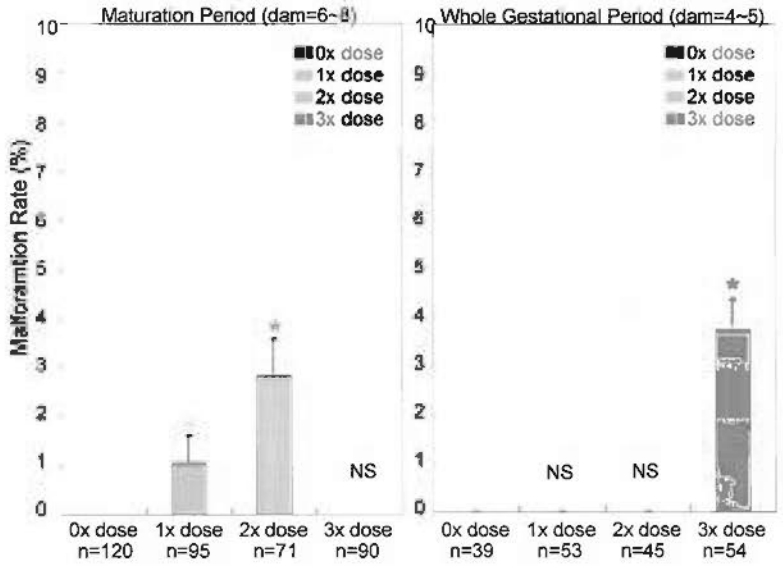


Figure 7.26 Neonatal malformation Rate with different intervention periods on mothers

NS= not significant; * $p<0.05$

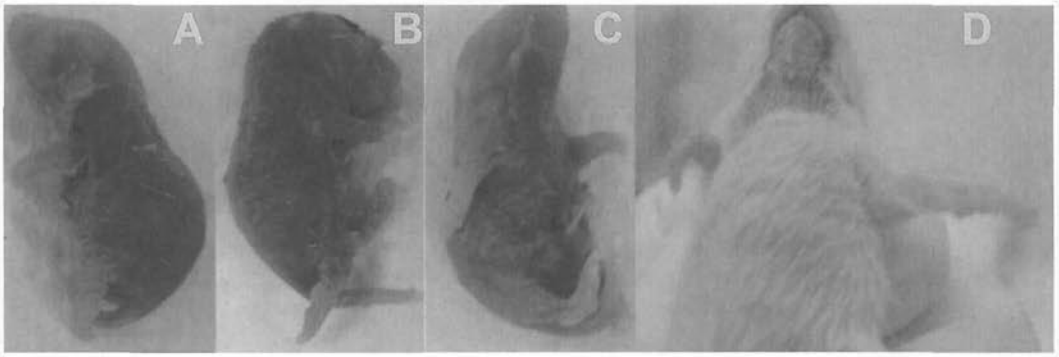


Figure 7.27 Skeleton malformation in Largehead Atractylodes Rhizome treated pups

A: Extended extension of left lower limb.

B: Extended extensions: right lower limb only, which was further confirmed with congenital hip dislocation and caudal regression by skeleton staining (Figure 7.30).

C: Extended extensions: right upper limb and left lower limb, which were further confirmed with shoulder joint dislocation, congenital hip dislocation and caudal regression by skeleton staining (Figure 7.30).

D: Extended adductions: right upper limb only, which was further confirmed with multiple malformation in limb development, such as long bone loss, limb shortening, and oligodactyly by CT scanning and skeleton staining (Figure 7.28, Figure 7.29).

CT scanning of the malformed neonates with extended adduction in right upper limb from 3x clinical dose group with Largehead Atractylodes Rhizome intervention on mothers throughout the whole gestational period (Figure 7.28), indicated congenital absence of ulna and distal digits, oligodactyly and long bone shortening were observed in the malformed limbs. 60% (3/5) of these malformed newborns died due to weaker adaptive ability, and only two survived and grew up. CT scanning and skeleton staining were further applied to determine the malformations.

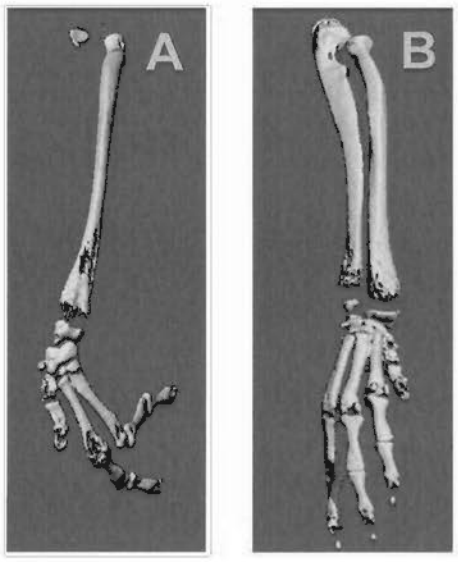


Figure 7.28 CT scanning for upper limbs

A: left upper limb from malformed mouse. B: right upper limb from normal mouse. Conparsion between malformed right upper limb and normal limb was shown in the figure. The CT scanning was carried out after sacrificed on P38.

Skeleton staining (Figure 7.29) further confirmed losses of ulna, finger and distal digits, shortening of humerus and radius in upper limb while shortening of fibula and tibia were well observed in lower limb.

Skeleton staining (Figure 7.30) confirmed posterior shoulder joint dislocation in the right upper limb of neonate from 2x clinical dose group with maturation period intervention, as the humerus was not in the right position and separated from the scapula at the glenohumeral joint, and the upper limb could only appeared backwards. Congenital hip dislocation and caudal regression were also observed. As the related

bones for the acetabulum, the pubic bone, the ischium and the hip bone, were not in the normal position, especially the pubic bone, and the lower limb appeared externally rotated, anterior hip dislocation was determined in this neonate. Fewer tail bones were observed in neonates from 2x clinical dose group, and the caudal vertebras in the tail were completely lost. Furthermore, the intervention also affected the formation and/or development of ankle, as the foot appeared backwards and could not move forwards.

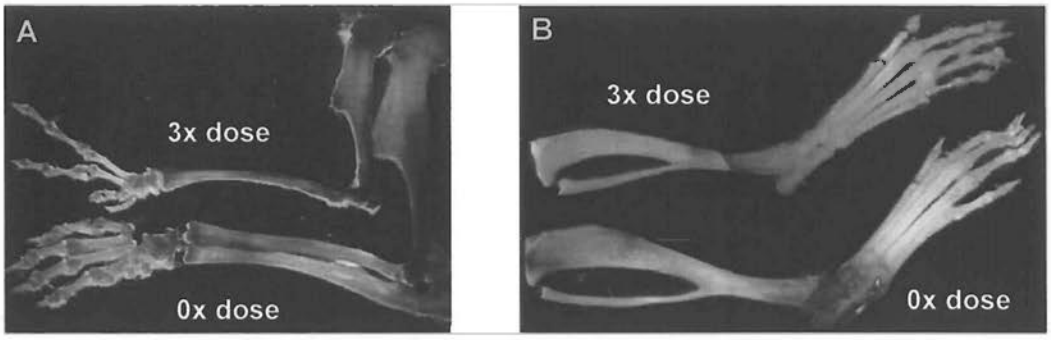


Figure 7.29 Skeleton staining of malformed mouse

A: Forelimbs from control group and the malformed mouse of 3x dose group; B: Hindlimbs from control group and the malformed mouse of 3x dose group. The pictures were taken after skeleton staining while the mouse was sacrificed on FP38.

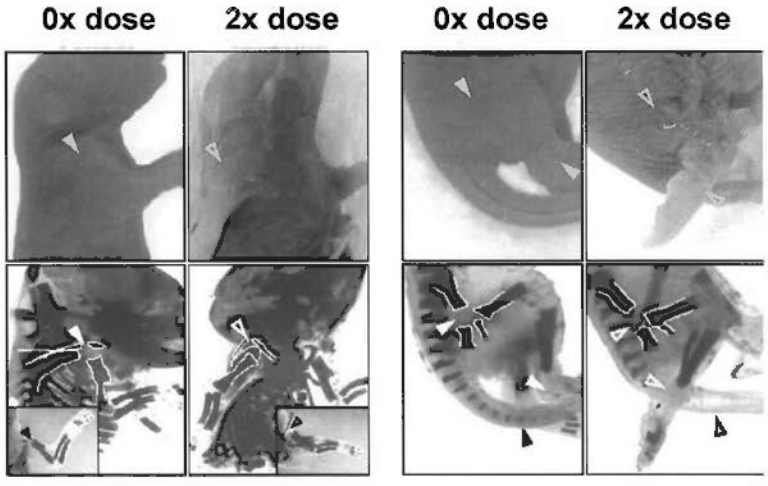


Figure 7.30 Skeleton staining for neonates from control group and Largehead Atractylodes Rhizome study group

These fetuses were collected from 0x and 2x clinical dose groups with oral administration on mothers from E15 till delivery (maturation period). The photos were taken on P5.

After sectioning and H&E staining of the elbows (Figure 7.31), a delay in endochondral ossification was found in 3x clinical dose group, including smaller bone size, less trabecular density and hypertrophic cell morphology. Flatten cells (or enlarged cartilage cells) arrest indicated they were underdeveloped and delayed in cartilage formation and development.

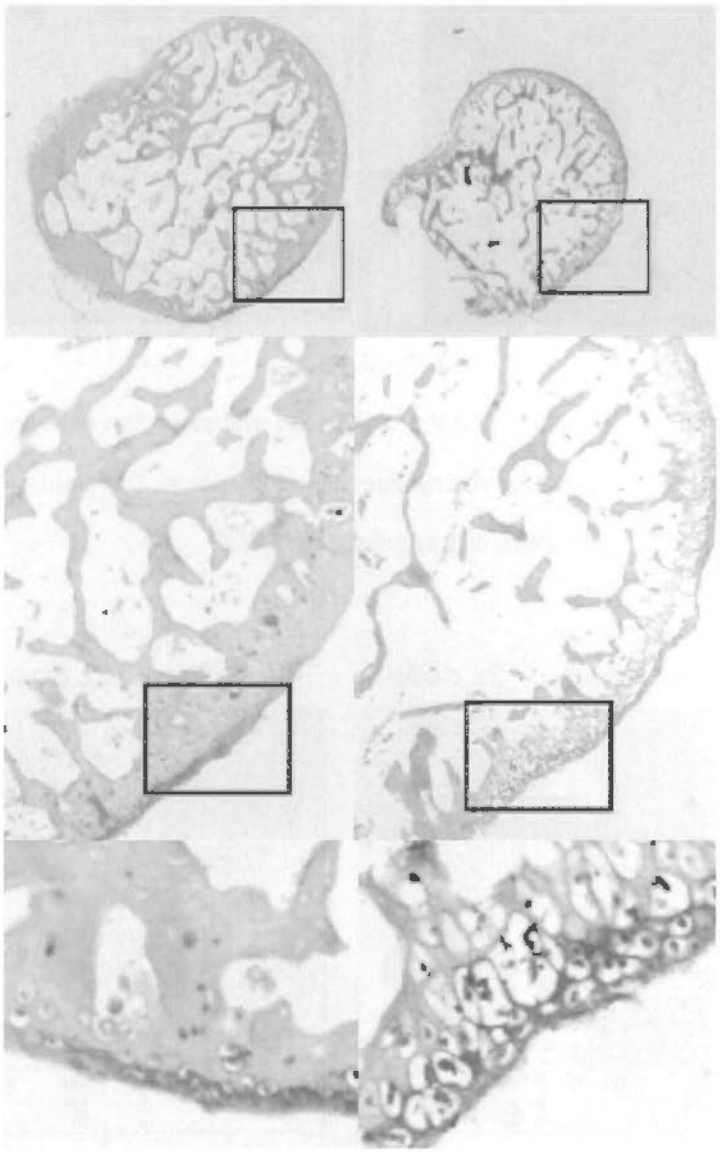


Figure 7.31 H&E staining for elbows from control group and Largehead Atractylodes Rhizome study group

7.8.3 In Vivo Safety Studies in Other Animals

7.8.3.1 Toxicity in SD rat

Comparisons were made on the E29 fetuses, whose mothers were orally administered with 3x and 6x clinical dose of Largehead Atractylodes Rhizome extracts during toxicity sensitive period. We compared body length, head length, body weight, placenta length and weight with controls (Table 7.9).

Table 7.9 Index for in vivo experiment on rats

	Body Length (cm)	Head Length (cm)	Body Weight (g)	Placenta Length (cm)	Placenta Weight (g)
Control	3.8 ± 0.15	1.5 ± 0.11	4.6 ± 0.43	1.3 ± 0.14	0.5 ± 0.06
3 x dose	3.9 ± 0.18	1.5 ± 0.12	4.9 ± 0.33	1.4 ± 0.14	0.6 ± 0.07
6 x dose	3.9 ± 0.28	1.5 ± 0.10	4.8 ± 0.66	1.4 ± 0.20	0.6 ± 0.06

No significant difference was found in fetal and placental size index between study groups and control group (Figure 7.32; T-test, $p>0.05$).

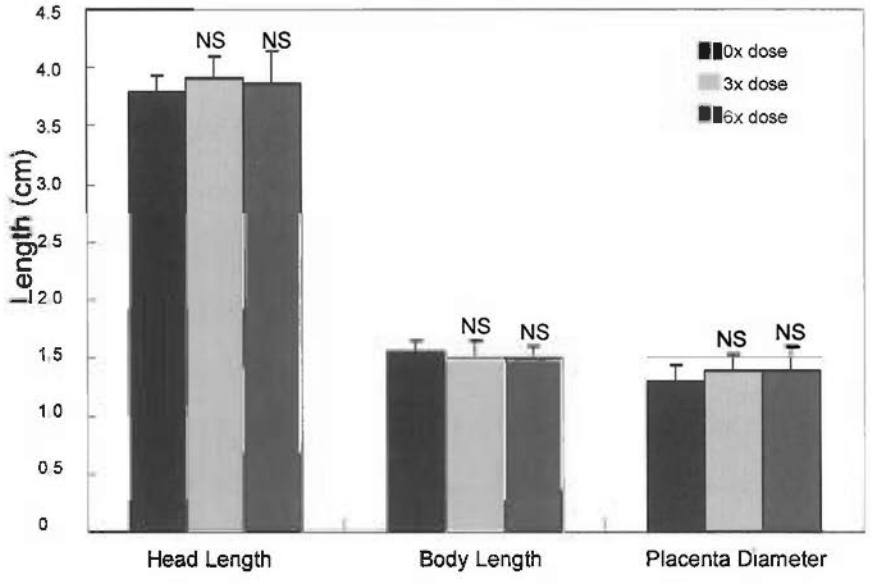


Figure 7.32 Changes of fetal and placental size with intervention in toxicity period

NS= not significant

No significant difference was found in body weight and placenta between study

groups and control group (Figure 7.33; T-test, p>0.05).

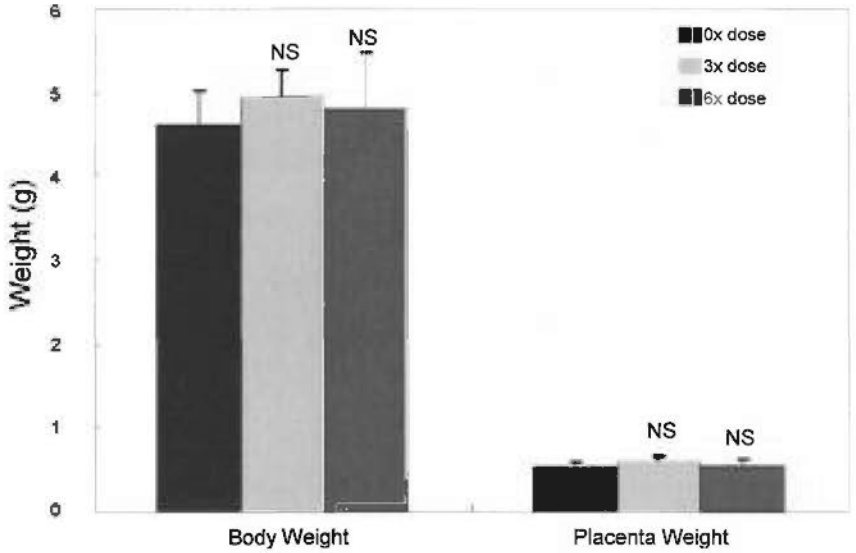


Figure 7.33 Changes of fetal and placental weight with intervention in toxicity period

NS= not significant.

7.8.3.2 Toxicity in NZW rabbit

Due to the limited number of does, three were treated with 3x clinical dose of Largehead Atractylodes Rhizome extract and were compared with two does without intervention during toxicity sensitive period. Adverse effects on fetal rabbits were observed (Table 7.10). Fetal resorptions (Figure 7.34A) and fetal hydrops (Figure 7.34B) were both recorded in the control group and 3x clinical dose group, while short ear (Figure 7.35) was only found in 3x clinical dose group. Resorbed fetuses were obviously underdeveloped compared with the fetus from control group, though the structure was still recognizable. Hydrops fetalis fetus was underdeveloped, with fluid accumulation in pleural and peritoneal. The placenta of hydrops fetalis was also smaller and calcified. Short ear was found on the left side of one fetus.

No significant difference in abnormal fetuses, resorption and hydrops was found between study group and control group (Figure 7.36; χ^2-tests, p>0.05).

Table 7.10 Adverse effects on fetal rabbits

Dose	Amount	Type	Number of Type	Mean Incidence (%)
		Normal	18	81.7
		Resorption	2	9.2
	Doe=2	Hydrops	2	9.2
sham	Neonates=22	Short Ear	0	0
		Normal	20	69.2
		Resorption	5	15.3
	Doe=3	Hydrops	4	12.8
3x dose	Neonates=30	Short Ear	1	2.8

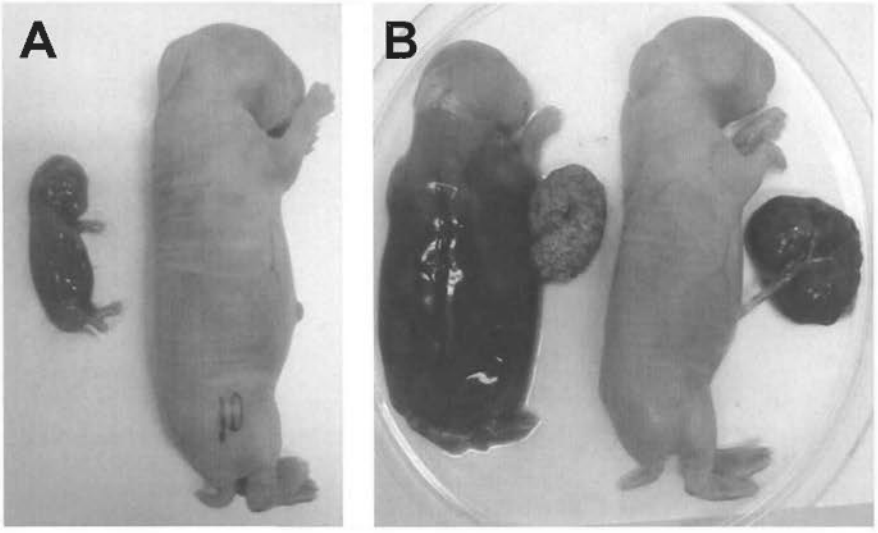


Figure 7.34 Resorpted fetal and hydrop fetalis (rabbit)

A: The left fetus from 3x dose group was considered as resorption, compared with the normal one on the right side from control group. Photos were taken on E29.

B: The left fetus from 3x dose group was hydrops fetalis, compared with the normal one on the right side from control group. Photos were taken on E29.

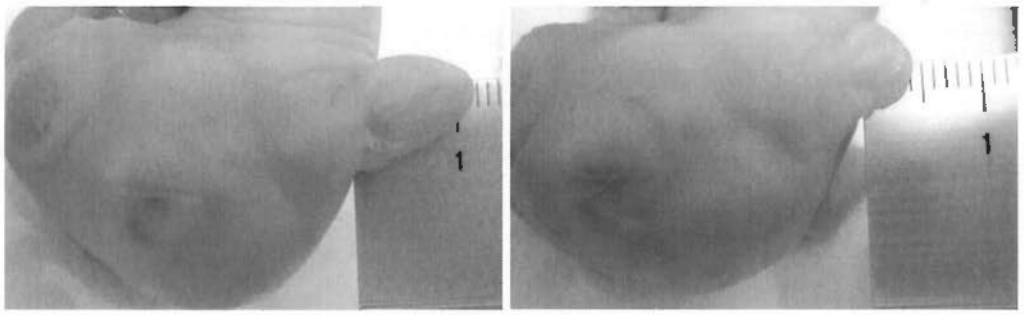


Figure 7.35 Short ear fetal rabbit

The right fetus from 3x dose group was with one side short ear, compared with the normal one on the left side from control group. Photos were taken on E29.

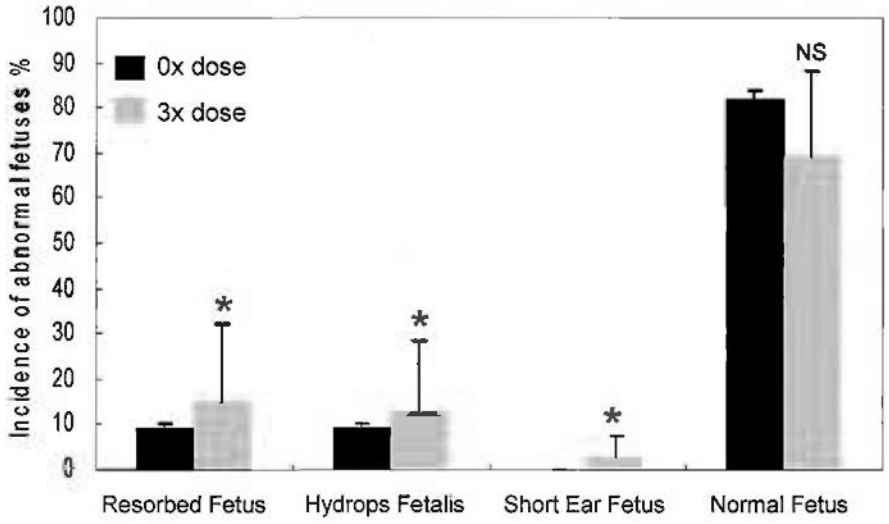


Figure 7.36 Incidence of abnormal fetuses

NS= not significant; * $p < 0.05$

X-ray examination showed no obvious difference in the skeleton structures between normal fetus and short ear fetus, but the hydrop fetalis seemed to have lower bone density, and the structure was more difficult to identify (Figure 7.37).

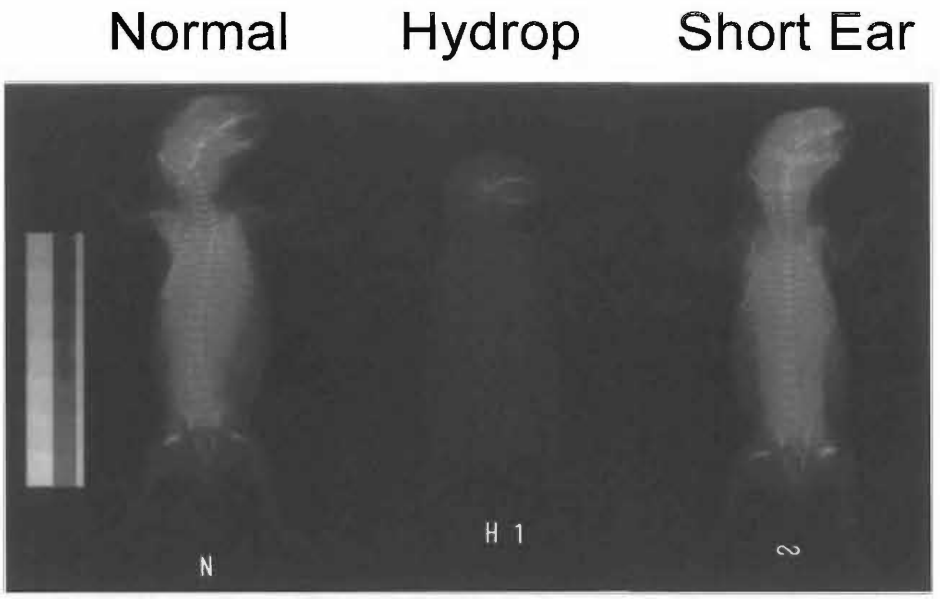


Figure 7.37 X-ray image of malformed fetal rabbits

Normal fetus was from control group, while hydrop and shot ear were from 3x clinical dose group. X-ray image was taken on E29.

CT images the hydrop fetalis was obviously underdeveloped, the bones was thinner, which is consistent with the finding in X-ray images (Figure 7.38). Other features included parietal bone dysostosis, occipital bone dysostosis, mandible dysostosis, as well as short limbs and lacking essential joint structures.

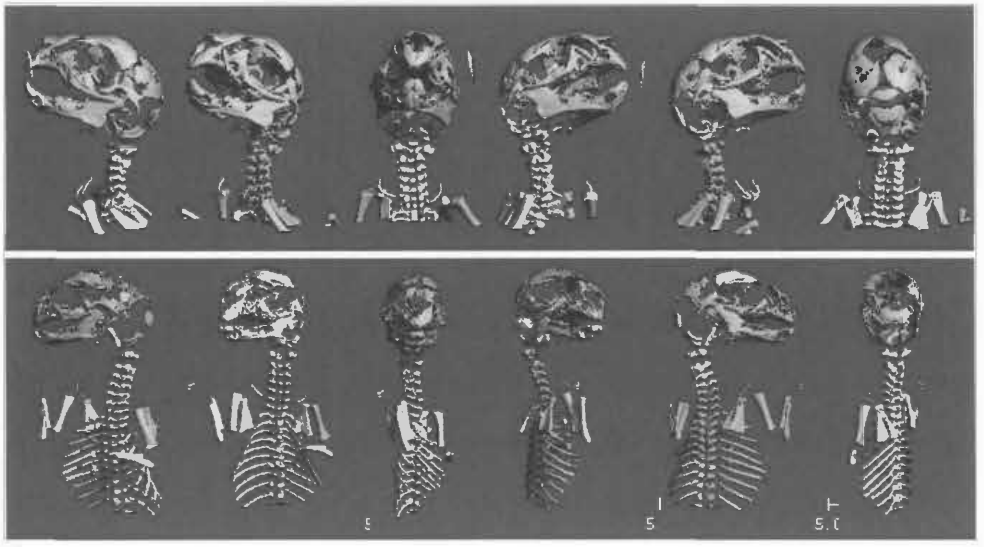


Figure 7.38 CT head and upper body image

Normal Fetus (upper) and Hydrops Fetalis (lower)

CT skull of 3x clinical dose intervention fetus with short ear was underdeveloped (Figure 7.39).

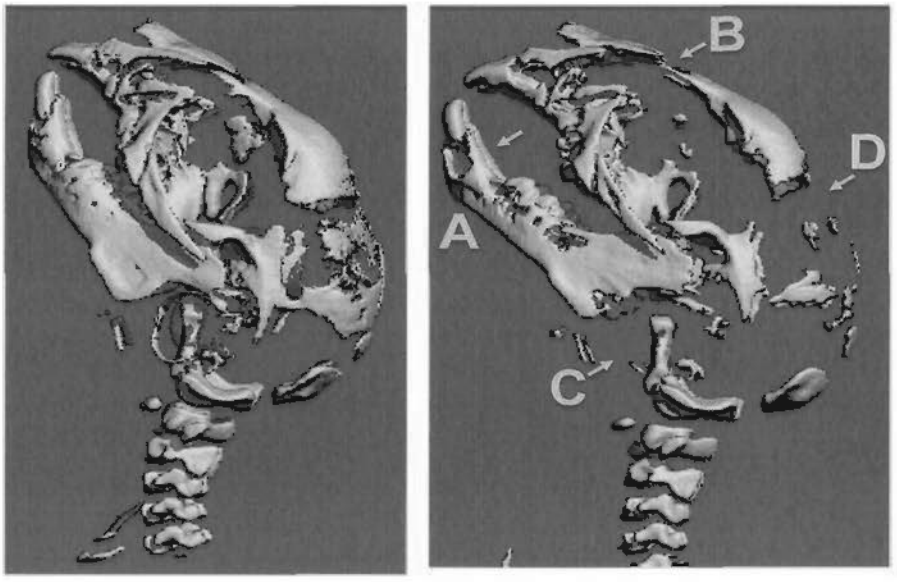


Figure 7.39 CT skull image

Normal fetus (left) and short ear fetus (right)

The short ear fetus was with uncompleted mandibular bone (A), missing bones in ethmoid (B), absence of Tympanic ring (C), and larger fontanel with less developed Parietal bone (D).

7.8.4 In Vitro Safety Study in ICR Mouse Embryos

7.8.4.1 Initial toxicity dose

We carried out test trials to identify the highest toxicity dose and dose range for in vitro experiments. In Figure 7.40, the embryos were collected from four independent runs, and the results showed that the developments and morphology of embryos were affected by Largehead Atractylodes Rhizome extracts at from 0.5mg/ml, and the main structures could hardly be recognized at 2mg/ml and 5mg/ml. Compared with the embryo from control group, the embryos in other dose groups were underdeveloped. The morphology features of the embryos from 2mg/ml group could still be evaluated by TMS, but hardly be identified from 5mg/ml group. Therefore, we set 2mg/ml as the highest test dose, and decided the initial toxicity dose with descending

concentration as 1mg/ml, 500ug/ml, 250ug/ml, 200ug/ml, 150ug/ml, 100ug/ml, 50ug/ml and 10ug/ml for in vitro test by WEC.

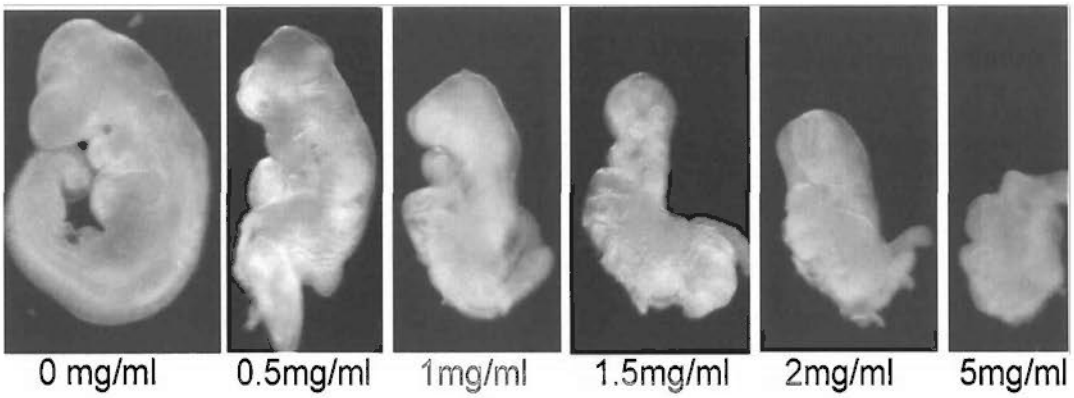


Figure 7.40 Dose range test

7.8.4.2 Malformations

The embryos from 1x PBS, 10ug/ml, 50ug/ml, 100ug/ml, 150ug/ml, 200ug/ml and 250ug/ml study groups were well developed, no significant difference in TMS was identified (Table 7.11; Figure 7.41). Underdeveloped embryos and malformed embryos were observed starting from 500ug/ml study group and higher doses. With 500ug/ml Largehead Atractylodes Rhizome extracts, anencephaly and underdeveloped forelimb and hindlimb buds were observed. As in 1mg/ml concentration, the embryos were obviously underdeveloped and multiple malformations were observed, including limb bud absence, viscera bareness, incomplete rotation, and hypoplasia of main external structures and internal organs. For 2mg/ml concentration, all the embryos were not developed.

The relative mean percentage differences in TMS were plotted against the individual test concentrations, and indicated that TMS was reduced by 50% (Mal_{50}) at the concentration of 2.31 mg/ml (Figure 7.42; regression equation: $y = -22.561x + 102.07$; correlation coefficient, $r = -0.9403$; $p < 0.001$).

Table 7.11 Records of morphological score (Mean±SD, n=3)

Parameters		Control	1x PBS	10 ug/ml	50 ug/ml	100 ug/ml	150 ug/ml	200 ug/ml	250 ug/ml	500 ug/ml	1 mg/ml	2 mg/ml
	yolk sac diameter A	6.2 ± 0.18	6.2 ± 0.25	6.1 ± 0.21	6.1 ± 0.26	6.2 ± 0.31	6.2 ± 0.40	6.2 ± 0.06	6.1 ± 0.19	5.8 ± 0.20	5.2 ± 0.30	3.4 ± 0.15
	yolk sac diameter B	5.6 ± 0.29	5.6 ± 0.24	5.5 ± 0.25	5.6 ± 0.20	5.5 ± 0.23	5.4 ± 0.17	5.5 ± 0.17	5.4 ± 0.25	5.2 ± 0.31	4.1 ± 0.22	3 ± 0.10
	CRL	5 ± 0.17	4.9 ± 0.29	5 ± 0.20	5 ± 0.24	5 ± 0.34	5 ± 0.30	5 ± 0.20	5 ± 0.21	4.2 ± 0.23	3.3 ± 0.20	2.1 ± 0.17
	HL	2 ± 0.08	2 ± 0.15	2 ± 0.18	2 ± 0.18	2 ± 0.12	2 ± 0.15	2 ± 0.07	2 ± 0.13	1.5 ± 0.17	1.1 ± 0.25	0.6 ± 0.26
A	yolk sac circulatory	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	4.5 ± 0.58	3 ± 0.00	2 ± 0.00
B	allantois	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	2 ± 0.00	1 ± 0.00
C	flexion	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	3 ± 0.00	2 ± 0.00
D	heart	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	3 ± 0.00	2 ± 0.00
E	caudal neural tube	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	3 ± 0.00	1 ± 0.00
F	hindbrain	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	4 ± 0.85	3 ± 0.00	2 ± 0.00
G	midbrain	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	3.5 ± 0.58	3 ± 0.00	2 ± 0.00
H	forebrain	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	3 ± 0.00	2 ± 0.00
J	otic	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	3.5 ± 0.58	3 ± 0.00	1 ± 0.00
K	optic	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	4 ± 0.00	3.5 ± 0.58	3 ± 0.00	1 ± 0.00
L	olfactory	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	1 ± 0.00	0 ± 0.00
M	branchial bars	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	3 ± 0.00	2.75 ± 0.50	1 ± 0.00	0 ± 0.00
N	maxillary process	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	1 ± 0.00	0 ± 0.00
O	mandibular process	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	1 ± 0.00	0 ± 0.00
P	forelimb	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00	1.5± 0.58	0 ± 0.00	0 ± 0.00
R	hindlimb	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	0 ± 0.00	0 ± 0.00
S	somites	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5 ± 0.00	5± 0.00	1 ± 0.00	0 ± 0.00
	no. somites	30.8 ± 0.96	31.5 ± 1.29	30.5 ± 1.00	30.5 ± 0.58	30.5 ± 1.00	30.5 ± 0.58	30.3 ± 0.50	30.3 ± 0.50	28.3 ± 1.35	9.3 ± 1.06	2.3 ± 0.05
	TMS	59 ± 0.00	59 ± 0.00	59 ± 0.00	59 ± 0.00	59 ± 0.00	59 ± 0.00	59 ± 0.00	59 ± 0.00	56.2 ± 3.49	34 ± 0.00	16 ± 0.00
	Relative Mean TMS %	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	93.6 ± 5.91	57.6 ± 0.00	27.1 ± 0.00

CRL: crown rump length; HL: head length; TMS: total morphology score (A-S score); units for yolk sac diameter, CRL, HL in mm, others in arbitrary unit.

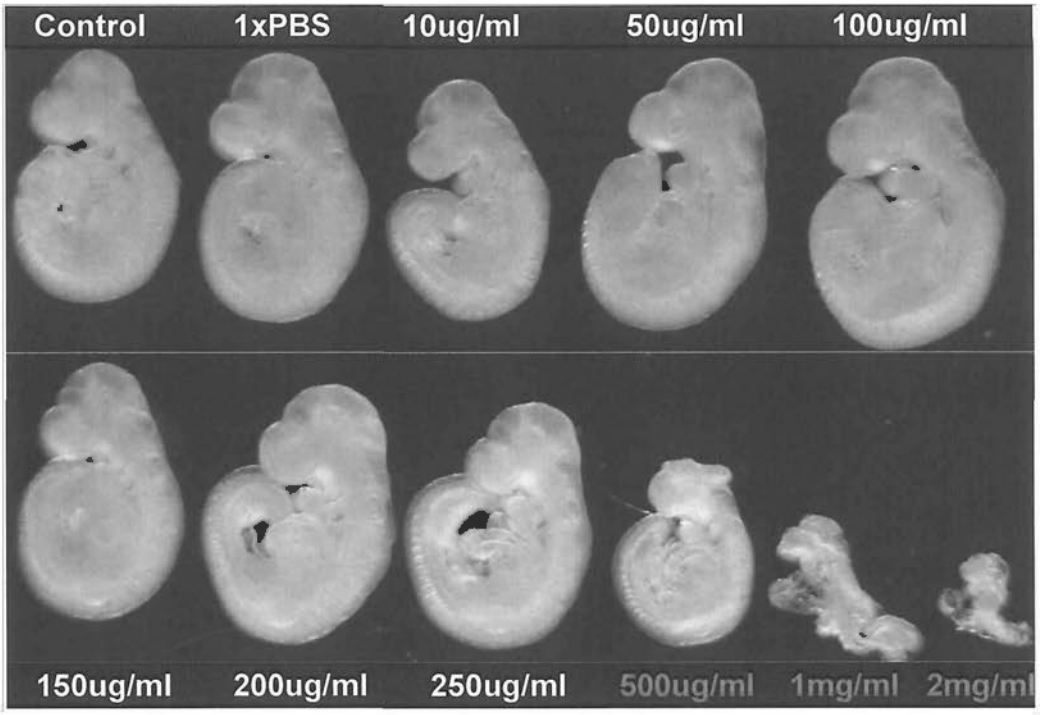


Figure 7.41 Malformations in embryo cultures with Largehead Atractylodes Rhizome intervention

The embryos were harvested from each intervention group as marked in the photo. Obvious multiple malformations could be observed from 500 ug/ml study group.

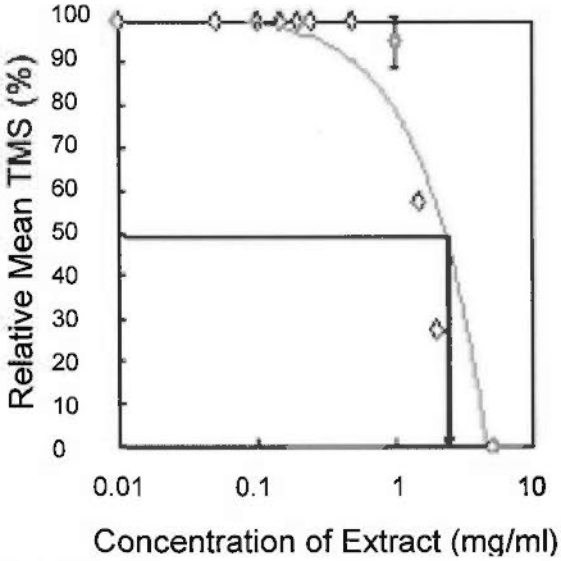


Figure 7.42 Dose response curve

Means ± SD of the TMS were presented and the minimum concentration which resulted in a 50% reduction in mean TMS (Mal50) was indicated.

$IC_{NOEC\ TMS}$ was also calculated from the formula, and it was 91.8 ug/ml. Furthermore,

according to the evaluation method of embryo toxicity classification, the results of calculation on function (FN) by mean $IC50_{Mal}$ and $IC_{NOEC\ TMS}$ values were listed as follows.

$IC50_{Mal}$= 2.31mg/ml = 2310ug/ml, log $IC50_{Mal}$= 3.36

$IC_{NOEC\ TMS}$=91.8 ug/ml, log $IC_{NOEC\ TMS}$=1.96

Function I (FN I) = $18.08 \times \log(IC50_{Mal}) - 11.56 \times \log(IC_{NOEC\ TMS}) - 10.19$

$= 60.75 - 22.66 - 10.19 = 27.9$,

Function II (FN II)=$21.55 \times \log(IC50_{Mal}) - 15.31 \times \log(IC_{NOEC\ TMS}) - 10.65$

$= 72.41 - 30.01 - 10.65 = 31.75$,

Function III (FN III)=$8.70 \times \log(IC50_{Mal}) - 8.53 \times \log(IC_{NOEC\ TMS}) - 2.53$

$= 29.23 - 16.72 - 2.53 = 9.98$.

The result indicated that Largehead Atractylodes Rhizome was classified as weakly-embryotoxic, because it had higher FN II results than both FN I and FN III.

7.9 Discussion

7.9.1 Largehead Atractylodes Rhizome

Largehead Atractylodes Rhizome (白术, Rhizoma Atractylodis Macrocephalae) was selected for the safety screening, instead of other individual herbs and common formulae. As we mentioned, from top 10 most commonly used individual Chinese medicines for threatened miscarriage, which was the most common application of Chinese medicines during pregnancy, Largehead Atractylodes Rhizome was the most frequently used individual Chinese medicine and was also one of the main individual medicines in the most commonly used formulae, Shou Tai Pill, for threatened miscarriage. In Chinese Medicine, it is also one of the most classical individual Chinese medicines for Qi Deficiency, which was considered as major cause of threatened miscarriage.

7.9.2 Decoction Method

Decoction is a common and classical way that Chinese medicines are prepared before administered. One package of the Chinese medicine formula is usually stewed with 500ml drinking water as daily use under simmered fire and the final soup is taken as medicine instead of the sediments of Chinese medicines. There are other methods of preparation and administration of Chinese medicines, such as to prepare the whole formula into powder then dissolved with boiled water, prepare into pills and taken with drinking water, etc. Amongst all the methods, decoction is traditional and considered as the best way to retain the property and biological activity of each individual Chinese medicine for the therapeutic effects. Largehead Atractylodes Rhizome was prepared by decoction as daily administration in clinical practices, and we applied this common method for animal studies. However, because of the decoctions are prepared by different Chinese Medicine practitioners, using different equipments, with different volumes of water, the decoction yield of formula differs and is difficult to calculate. In addition, there is no standard to determine and evaluate the concentration of the Chinese medicines in the final soups.

7.9.3 Quality Control of Largehead Atractylodes Rhizome

The crude extraction yield rate of Largehead Atractylodes Rhizome was 24.6%, which was consistent with the common extraction rate at the ICM laboratory. TLC analysis confirmed Largehead Atractylodes Rhizome extract contained the main bioactive constituents of Largehead Atractylodes Rhizome, Atractylenolide I and Atractylenolide III. It indicated the extraction quality during the processing and preparation. Atractylenolide I and Atractylenolide III were both exsited in lipophilic portions of Largehead Atractylodes Rhizome extracts. Atractylenolide I and Atractylenolide III regulate the gastrointestinal function, promote absorbing nutrients (Wang et al., 2010), and prevent inflammation and tumor (Cohen et al., 2002). Their effects on pregnancy and embryonic development were not studied. The adverse

pregnancy effects of Largehead Atractylodes Rhizome extracts were observed in our animal experiments, indicating that Largehead Atractylodes Rhizome extracts contain some unknown chemical component which induces the side effects. Further studies on the hydrophilic and lipophilic portions of Largehead Atractylodes Rhizome extracts are required to identify the active components for the outcomes.

7.9.4 In Vivo Animal Studies

7.9.4.1 Experimental designs

In our in vivo pregnant animal studies, intervention by oral feeding was employed. It shared the similar routine of administration as the daily clinical practice of Chinese medicines in patients. It should also cause less systematic side effects to mothers compared with injection (Karl et al., 2009).

Chinese medicines are mostly prescribed as in formula, and very few Chinese medicines are prescribed and applied individually, except Ginseng, which can be applied individually as a formula called Du Shen Soup (獨參湯) for cardiogenic shock (Wang Y, 1999) and postpartum hemorrhage (Zhang YM, 1998). But we used individual herb for the safety study. Our reasons are as follows.

Firstly, each Chinese medicine has its own property and therapeutic function, and the doctors will consider every character of each individual Chinese medicine then prescribe them together within one formula. The final effect of the formulae is a result of harmonies of each individual medicine, that is, not only the therapeutic function will be enhanced by the interactions of each other, but also the toxicity of each individual medicine will be decreased or the toxic substance inside a medicine has been degraded. Therefore, if formula was applied in our studies, we can only conclude the effects were mainly due to the formula. We still can not confirm which medicine actually caused the adverse effects. Secondly, one of the aims of toxicity studies is to

identify the chemical or substance responsible for the toxic effects. To determine the toxicity of Chinese medicine, study on the individual Chinese medicine can simplify the complexity of formula, but also can carry out the adverse studies on further Chinese medicine constituent and mechanism studies. Thirdly, Chinese Medicine practitioners usually prescribe the formulae based on the presentation of the patients. Individual herbs within the formula may be changed. In animal studies, the response of each animal after intervention is unknown. To avoid variation, individual Chinese medicine was the best choice for the first step of safety screening in animals.

As to toxicity study on animal models, researcheres usually screen with a large scale of different concentrations of western medicines (Lewis et al., 2007; Carmane at el., 2008), such as 1x, 10x, 100x, and 1000x. However, as to Chinese medicines, we set up 1x, 2x and 3x clinical dosages in our in vivo animal studies. Our reasons are as follows.

As we mentioned above, the most common method to prepare Chinese medicines formula is decoction, which could best retain the property and biological activity of each individual Chinese medicine for the therapeutic effects, but is difficult to calculate the decoction yield of a formula, and is hard to determine and evaluate the concentration of the whole formula as well as each individual Chinese medicine. We may further test the concentration in maternal blood, certain organs and/or placenta, however, the drug metabolism in individuals and different systems may vary a lot. Besides, the Chinese Medicine practitioners care the doses of individual Chinese medicines in a formula prescription before it was prepared, more than its concentration in the human body after metabolism. Therefore, clinical dosages are better than large scale of concentration for the studis that are applying raw herbs or Chinese medicine extractions.

As to an individual Chinese medicine, different from pure western drugs, it mostly contains a large number of chemical components, only a few of which are the real

active components that leads to the main therapeutic effects of this individual medicine. On further determination of those active components, we plan to set up the same scale of dosages for future animal studies.

7.9.4.2 Maternal adverse outcomes

Adverse outcomes were observed in maternal mice both during the pregnancy and lactation periods. Maternal weight gains in study groups were lower than that in control group during the whole gestational period, but significant differences were only found in early days (before E5). The decreased maternal weight gains in gestational periods resulted in the decreased fetal weight and/or placenta weight, but no dose dependent effect was observed. As to the other short intervention (the implantation period, the gastrulation period, the organogenesis period and the maturation period), no significant difference in maternal weight gain was observed between the study groups and control group. It's probably due to the rapid metabolism of the medicine, half life time of Largehead Atractylodes Rhizome is 15 hr ~ 302 hr (Zhu et al., 2010). Changes of maternal weight were more obvious in the groups where the oral administrations were given throughout the whole gestation period. For maternal weight change in postpartum period, if the mothers were administered within maturation period, significant decrease in weight gain were only recorded in the first 2 weeks after delivery, and higher doses resulted in lower maternal weight gain. In contrary, the mothers with oral administration throughout the whole gestation period, the mothers from study groups gained more weights than control group, but significant differences were only recorded on the first 2 days after labor. Therefore, the mothers may adapt to Largehead Atractylodes Rhizome after long term intervention, and there was no obvious effect on the maternal weight changes, but there was weight drops after short term treatment may be due to stress response and metabolism of the medicine.

Unexpected maternal deaths were only observed in control group and 1x clinical dose group. But there was no significant difference on maternal mortality between the study groups and control group, hence no dose dependent effect either. One of the mothers died on P13 from control group, who needed to raise 17 newborns, tiredness and exhaustion of energy may be one possible reason for the mortality. Because the 2nd week in lactating period is considered as a peek period for milk feeding, and the usual litter size in mice rarely exceed 12-15, with the increased demands of lactation as the fetuses are growing up, the maternal mouse may be exhausted to death. As to the other two mothers that died on P6 (from control and 1x clinical dose group) and one on P8 (from 1x clinical dose group), no obvious change on the appearances and activities of both the mothers and neonates was observed. No significant finding was observed in the stomach and intestine at autopsy, so we cannot reach a reasonable explanation for this maternal mortality.

7.9.4.3 Fetal and neonatal adverse outcomes

The adverse effects of Largehead Atractylodes Rhizome on embryos and fetuses were more severe, compared with their mothers. Embryonic resorptions were recorded in implantation period, both embryonic resorptions and growth restriction were found in gastrulation period, and fetal resorptions were recorded within organogenesis period. As to the resorptions, Largehead Atractylodes Rhizome could affect the embryos or fetuses in most developing stages, but significant dose-dependent effects were only found in implantation period with obvious dose-dependent effects. Largehead Atractylodes Rhizome may affect the implantation of the fertilized egg.

The CRL, HL, and number of somite were taken as parameters to evaluate embryo development in gastrulation period. Fetuses had shorter CRL and HL at 1x and 3x doses, but not in 2x dose group. But significant growth restriction was only found in CRL but not the other parameters. The incidence of growth restricted embryos was

obviously higher with the increased dose, but there was no significant difference compared with control.

The CRL, HL and placenta length were taken as parameters to evaluate embryo development in organogenesis period. Although Largehead Atractylodes Rhizome could lead to increase of the fetal size with 1x clinical dose, no significant difference was found. However, the incidence of growth restricted embryos was only found in 1x clinical dose group, and there was no significant difference compared with control group. No obvious difference could be obtained from the diameters of the placentas in all dose groups and the fetal size in higher dose groups. Significant growth restriction was only found in gasturation period, one possible reason is that the organogenesis period is more important for organ formation and development, while the gasturation period is more essential for the formation and differentiation of the main body structures in an embryo, which probably is more sensitive to Chinese medicine intervention and is more likely to affect the growth parameters.

Lower weight gains and higher postnatal mortality and malformation rate were the main findings on neonates of maternal mice administered with Largehead Atractylodes Rhizome during pregnancy. Reduced postnatal infant growth was observed in early and later postnatal development in newborns from both maturation period and whole gestational period treated groups. The fetuses in study groups were significantly lighter than control group in early postnatal development, mainly on P6, P7 and P14. Although no dose dependent relationship was found among three doses, high precautious should be exercised in the clinical application of Largehead Atractylodes Rhizome during pregnancy, for its potential long term adverse effects on neonatal growth and development.

In the postnatal development of groups with maturation period intervention, the mortality of neonates were identified. In the postnatal development of groups with whole gestational period intervention, the mortality of neonates was also recorded.

The mortality increased from 1x clinical dose, 2x clinical dose, but not in 3x clinical dose, and the changes were observed in both maturation period and whole gestational period intervention. The highest mortality was recorded at 2x clinical dose after both interventions, but significant difference was only recorded in the groups under whole gestational period. Hence, high dose of Largehead Atractylodes Rhizome could induce mortality of neonatal death.

During maturation period, the malformation rates increased with higher dose effects except 3x clinical dose groups. For whole gestational period interventions, malformation was only found in 3x clinical dose groups. It appeared that the effects were dose-dependent, that is the higher dose of Largehead Atractylodes Rhizome could significantly increase the incidence of neonatal malformation.

Exterior malformations in mice included elbow joint dislocation, congenital hip dislocation, caudal regression, limb shortening, absence of long bone and finger and distal digits loss. As to the skeleton defects caused by Largehead Atractylodes Rhizome, there must be some chemicals or constitutes within the extract, having potential toxicity on special skeleton formation and development, such as ossification process of bones, and/or inducing the mutation of skeleton specific genes.

To further determine if species sensitivity affects the above results and findings, we tested Largehead Atractylodes Rhizome on mice, rats and rabbits. There were different adverse outcomes in different animals. For SD rats, 3x and 6x clinical dose were applied as intervention to the mothers. No significant findings were observed in the fetuses, except slightly increase in fetal body length and diameters of placentas, fetal body weights and placenta weights and decrease head length, higher with Largehead Atractylodes Rhizome interventions. No resorption, growth restriction, stillbirth, malformed fetus and other adverse effect were found. So SD rats, as one of the most common animal species for toxicity studies, seem not sensitive to Largehead Atractylodes Rhizome.

For NZW rabbits, they were sensitive to Largehead Atractylodes Rhizome and severe adverse effects were observed on fetuses, including fetal resorption, short ear, and hydrops fetalis recorded in 3x clinical dose group, but resorption and hydrops were also observed in control group. To some degree, the adverse effects on rabbit fetuses were consistent with that on mice fetuses and neonates. The resorpted fetuses, which was also recorded as resorptions happened in gastrulation and organogenesis periods of mice, respresented fetal deaths at early pregnancy could be induced by Largehead Atractylodes Rhizome. The skeleton of the hydrop fetalis were thinner than control group. The skull of the fetus with short ear was underdeveloped. These confirmed the developmental skeleton toxicity of Largehead Atractylodes Rhizome. At least two species showed the specific adverse effects. It rised the concerns of safety use of Largehead Atractylodes Rhizome for pregnant women.

For the mouse, the main findings were involved with limb development, especially the bones. As to rabbit, high incidence of fetal death was recorded, including early fetal death such as resorption, and late fetal death such as hydrops fetalis. However, no obvious adverse effects were found in rats studies. Therefore, sensitivity of different species did affect the safety outcomes of Chinese medicine application, and should be taken into considerations when animal studies were designed and carried out, especially for toxicity screening.

7.9.5 In Vitro Animal Studies

WEC were studied to confirm the adverse effects of Largehead Atractylodes Rhizome on developing embryos. $IC_{NOEC\ TMS}$ was 91.8 ug/ml while IC_{50} was 2.31mg/ml according to the dose response curve. IC_{50} in vitro was equal to 0.1x of in vivo dose. Actually the adverse effects observed in in vivo and in vitro studies were different, and the calculated in vivo dose should not result in any adverse outcomes in vitro, higher dose were required to induce the outcome. So we conclude that there should be

some potential factors affect Largehead Atractylodes Rhizome extracts on the limb development in the mouse embryo. Because the Largehead Atractylodes Rhizome extracts were directly applied on the embryos or yolk sacs in vitro, there is drug metabolism mechanism of the mothers and the placenta barrier which could decrease the concentrations and activities of Chinese medicine extracts and its biological components leading to the side effects. Regarding to embryotoxicty, Largehead Atractylodes Rhizome was considered as weakly embryo toxic (lower $IC_{50\ Mal}$ and lower $IC_{NOEC\ TMS}$). These were important pharmatoxicity references for daily clinical applications of this Chinese medicine.

7.10 Summary

Largehead Atractylodes Rhizome, as the most commonly used Chinese medicine for threatened miscarriage treatment, various adverse effects were identified from in vivo animal studies. Significant decreased maternal weight, as well as deteriorated fetal and neonatal growth, reduced postnatal weight gain, increase fetal resorption rate, postnatal mortality and moderate to severe skeleton congenital malformations were observed. The embryotoxicty of Largehead Atractylodes Rhizome was further confirmed by in vitro test. Although Chinese medicines rarely be applied in single herbs, and it is claimed that Chinese medicine formulae can integrate the toxicity effects by the interactions of each involved individual Chinese medicine, we still suggest that Chinese Medicine practitioners should avoid to use this Chinese medicine, and pay high attention on its dose. We urge more relevant researches and studies to be carried out for detailed understanding on safety of Largehead Atractylodes Rhizome and other Chinese medicines.

Chapter VIII

Molecular Biology Studies

8.1 Introduction

8.1.1 Limb Development

8.1.1.1 Human development

In the human, limb formation occurs in the organogenesis period of development, usually at the 5th gestational week, begins in the limb field, as a limb "bud". Human upper limbs develop earlier than lower limbs. Upper limb buds appear approximately at the level of the 8th-12th somite. A distinct condensation of lower limb buds appears just at the end of the same stage, and become visible at the level of the 23rd-28th somite. Genesis of hands and feet take place in the 6th gestational week.

The upper limbs are divided into two regions, the proximal part consisting of the future limb-girdle and 'arm', and the more peripheral part forming into a circular or paddle-shaped 'hand plate'. It is considered as the earliest sign of finger formation that the hand plate is no longer circular but develops angles which correspond to the future digits at the 7th gestational week. Within the same period, the distal borders of the anterior and posterior footplates are indented and the digit widths and locations can be recognized. Some key joints, such as the 'elbow' and the 'wrist', generate and become identifiable at the same time. At the end of this stage, individual 'fingers' are visible and well developed distally, while deep indentations appear between the 'toes' which are not yet separated. In the 8th gestational week, the 'toes' separate, clearly divergent, then start to develop parallel. Nail primordia are visible, and long bones of the limbs are present and grow prominently later on. Pronation of hands and supination of feet make the limbs closer and can touch the other side, and spontaneous limb movements can be detected by ultrasound afterwards.

8.1.1.2 Mouse development

The key developmental characteristics in the development of mouse and human limbs were compared in Table 8.1. Appearance of upper limb buds occurs around E9 in mouse (Theiler Stage) and at gestational week 5 in human (Carnegie Stage). Distinct condensation of lower limb buds appears just at the end of E9 in mouse and later gestational week 5 in human. Genesis of hand plate and foot plate take place on E11 in mouse and within gestational week 6 in human. Earliest signs of fingers appear around E12 in mouse. The hand plate is no longer circular but with developing angles which is related to the future digits, while the foot plate is also distinguishable from the lower part of the leg. The same changes appear at gestational week 7 in human. Genesis of important joints of limbs, elbow, wrist, hip and knee, happens at E13 in mouse and within gestational week 7 in human. Well developed fingers are visible at E14 in mouse and by the end of gestational week 7 in human, while separated toes appear later of this stage. Arms, legs, hands and feet all lengthened after E15 in mouse and gestational week 8 in human. With the pronation of hands and supination of feet, the anterior and posterior terminals turn inward, come closer and touch the other side. When embryogenesis is finished, the precursors of all the major organs, the head, the body and the limbs have been created at the end of the 15th day and 10th week of gestational age in mouse and human, respectively.

The mouse limb structure is remarkably similar to the human limb (Javier et al., 2001). Since the similarity in mouse and human genomes molecular and development, mice are commonly used as an alternative model to study the limb development, and many developmental or invasive experiments can be done in mice but not in human. Transgenic techniques can study the gene functions during limb development (Zhu et al., 2010). It is easier, quicker and relatively cheaper to produce large quantities of mice for systematic studies. Thus, mouse models are likely to provide efficient accesses to perform experimental studies of limb development.

Table 8.1 Key morphological features of limb development in mouse and human

Development of key features	Development stage	
	Mouse	Human
Appearance of upper extremity buds	E9	30th gestational day
Appearance of lower extremity buds	E10	32th gestational day
Genesis of hand plate	E11	36th gestational day
Genesis of foot plate	E11	39th gestational day
Formation of the inter-digital zones	E12	41th gestational day
Visible finger radiations	E12	44th gestational day
Toe primordium		
Genesis of elbow and wrist joints	E13	46th gestational day
Genesis of hip and knee joints		
Arms in pronation	E14	49th gestational day
Separated fingers		
Lengthened hands and feet	E15	51-53th gestational day
Pronation of hands		
Supination of feet		
Well-developed toes		
Spontaneous limb movements be detected by ultrasound	—	63th gestational day

8.1.2 Cell Biology

Limb formation results from a series of epithelial-mesenchymal inductions between the limb bud mesenchymal cells and the overlying ectodermal cells (Scott et al., 2010). Cells from the lateral plate mesoderm and the myotome migrate to the limb field and proliferate to create the limb bud. The limb's cartilaginous and skeletal portions and structures depend on the lateral plate cells while the muscle comes from somitic myotome cells (Table 8.2).

8.1.2.1 Limb field

Limb fields are formed in somatic lateral plate mesoderm at specific sites along anterior/posterior (A-P) and dorsal/ventral (D-V) axes. It is a region specified by homeobox (Hox) gene expression. Limb field consists of limb morphogenetic field, of

which contains a population of cells committed to give rise to a particular organ when transplanted to a different part of the body. Cells of a morphogenetic field can regulate their fates to make up for missing cells in the limb field, and mark fate maps to identify the cells that participate in limb formation, eg. the ectodermal cells in apical ectodermal ridge (AER) (Lovejoy et al., 2003).

Table 8.2 Limb tissues originate from specific germ layer derivatives (Lauren et al., 1997)

Origins	Developments
Somatic lateral plate mesoderm	Connective tissues: cartilage, and later bone
Somites (myotome)	Skeletal muscle
Neural tube/Neural crest	Nerve (motor neuron innervate skeletal muscle)
Ectoderm	Skin (feathers, hairs, and nails)
More somatic mesoderm (dermatome)	Connective tissue: dermis

8.1.2.2 Limb bud

Early limb bud is composed of lateral plate mesoderm. Migratory cells invade the limb bud, including myoblasts from somites, pigment cells and schwann cells from the neural crest, while axons innervate the limb bud (Danton et al., 2010). The limb bud has a strict pattern and polarity. Development is organized in the A-P, D-V and proximal/distal (P-D) axes. The tissues of the limb bud will differentiate in a specific pattern that is defined in part by the existing embryonic regions: the apical ectodermal ridge (AER), the zone of polarizing activity (ZPA) and the progress zone (PZ) (Danton et al., 2010), as shown in Figure 8.1.

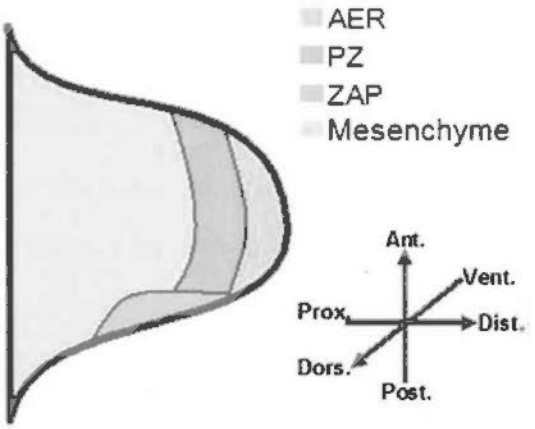


Figure 8.1 Embryonic regions of developing limb bud

modified from http://www.utm.utoronto.ca/~w3bio380/lecture19.htm

AER: apical ectodermal ridge; PZ: progress zone; ZPA: zone of polarizing activity; Ant: anterior; Post: posterior; Prox: proximal; Dist: distal; Dors: dorsal; Vent: ventral.

8.1.2.3 Apical ectodermal ridge (AER)

The lateral plate mesodermal cells secrete a fibroblast growth factor (FGF) to induce the overlying ectoderm to form a crucial organizing structure during limb development, called the apical ectodermal ridge (AER) (Danton et al., 2010; Yonei-Tamura et al., 1999), as marked with arrow and highlighted with pink color and shown in Figure 8.2. The AER creates a zone of cell proliferation and lays down the limb from proximal to distal. The time cells that leave the AER determines their positional value, proximal structures are formed earlier than distal structures.

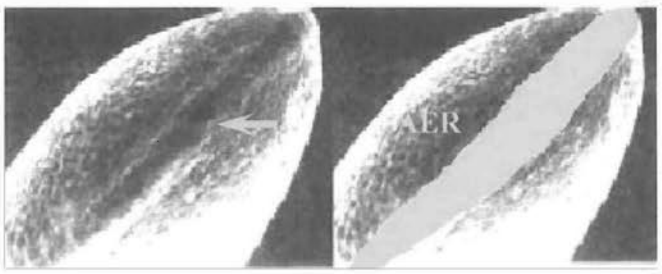


Figure 8.2 Scanning electron microscope picture of developing limb bud

AER: apical ectodermal ridge. The picture showed developing human limb buds at 1 month gestation, modified from http://www.utm.utoronto.ca/~w3bio380/lecture19.htm.

FGF is the developmental signal for AER, such as FGF2, FGF4, FGF8. AER controls limb development, and the problems with AER formation will result in different defects of limbs (Figure 8.3). Removal of AER leads to limb truncation (Yang YZ, 2009) or limbless mutant if the AER fails to form completely (Seghatoleslami et al., 2002). Splitting or addition of AER causes formation of a second limb (Richard et al., 1998).

8.1.2.4 Zone of polarizing activity (ZPA)

ZPA is an area origin from the mesenchyme that contains signals which organizes the developing limb bud to form along the A-P axis (Zeller et al., 2009). ZPA stimulate mesenchymal cell proliferation, controls its differentiation, and induce changes in the AER (Hinchiliffe et al., 1981). Grafted ZPA to the anterior part of limb bud results in supernumerary limbs (Imokawa et al., 1997). Limb bud development relies not only on ZPA, but also many different genes, signals mainly Sonic Hedgehog (Shh), and the AER, which are dependent on each other with ZPA (Kicheva et al., 2010).

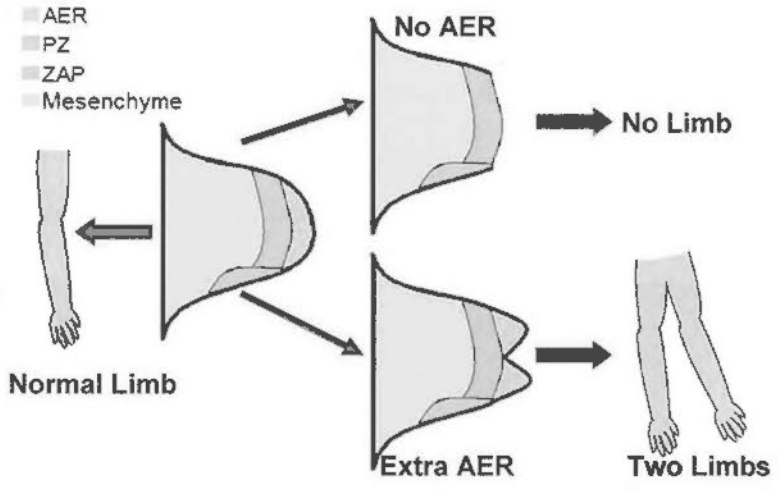


Figure 8.3 Limb defects due to changes of AER

modified from http://www.utm.utoronto.ca/~w3bio380/lecture19.htm

AER: apical ectodermal ridge; PZ: progress zone; ZPA: zone of polarizing activity.

8.1.2.5 Progress zone (PZ)

The progress zone is a layer for mesodermal cells immediately beneath the AER in limb bud development. The PD positional value of the mesodermal cells is thought to be dependent on the time it spends in the PZ during limb outgrowth (Galloway et al., 2009). Cells continually leave the progress zone, and if the duration of their stay in the zone was measured, it could specify their position along the axis (Weiss et al., 1998). Wingless-type MMTV integration site family (Wnt) give precise control of basic cellular processes such as cell proliferation and differentiation, and Wnt5a is the only Wnt gene expressed in PZ.

8.1.2.6 Cell death and digit formation

Programmed cell death, or apoptosis, is a normal developmental pathway to remove the "webbing" between the digits and joints. Bone Morphogenetic Proteins (BMP) signaling controls the interdigital cell death, which form paddle into individual digits, while homeobox proteins (Msx) and Retinoic acid receptors also play important roles in this process. Absence of cell death results in syndactyly (Figure 8.4).

8.1.2.7 Skeleton differentiation

Limb skeleton is derived from lateral plate mesoderm, and the differentiation is proximal to distal and posterior to anterior. The developing limb is organized into three regions: stylopod, zeugopod and autopod, which represent for humerus, radius and ulna, hand and foot in upper limbs, respectviely. The most important step is the formation of endochondral bone – chondrogenesis, and the key factors involved in this progress are Bone Morphogenetic Proteins (BMPs), Indian Hedgehog (IHH), and Growth differentiation factor-5 (Gdf-5) (Figure 8.5).

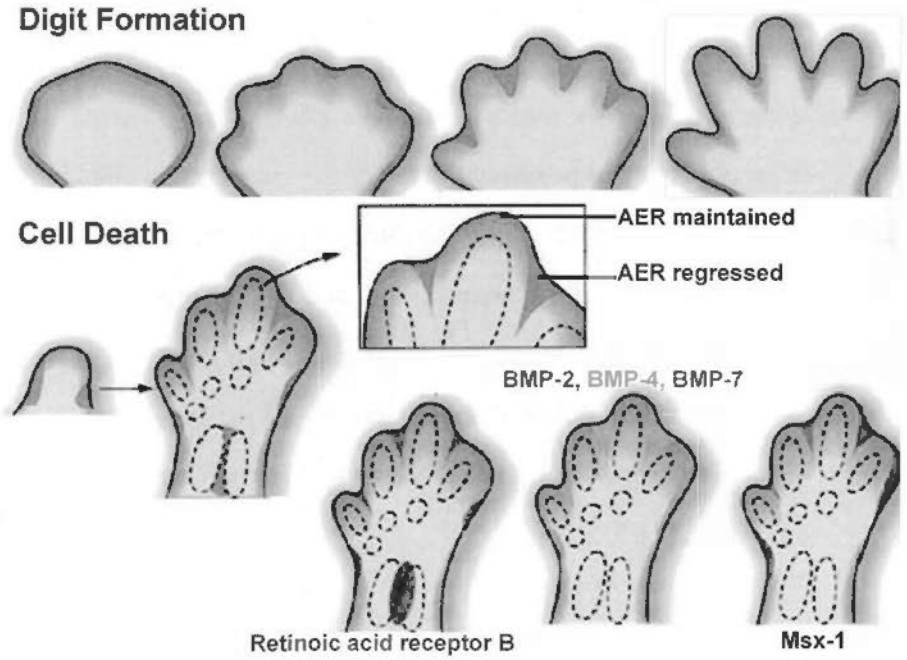


Figure 8.4 Genes regulate the cell death and digit formation

modified from http://www.utm.utoronto.ca/~w3bio380/lecture19.htm

AER: apical ectodermal ridge; BMP: bone morphogenetic Proteins; Msx: homeobox proteins.

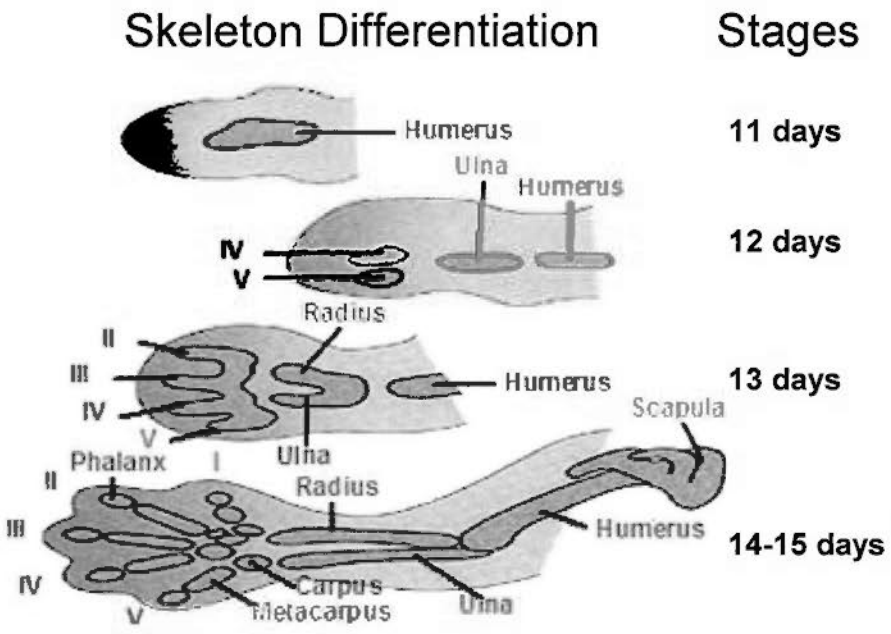


Figure 8.5 Skeleton differentiation in mouse

modified from http://www.utm.utoronto.ca/~w3bio380/lecture19.htm

I: thumb; II: index finger; III: middle finger; IV: ring finger; V: little finger.

8.1.2.8 Formation of cartilage and joint

Skeletal elements of limbs are prefigured by tight aggregates of mesenchymal cells called *precartilage condensations*, which are mediated by extracellular matrix and cell adhesion molecules (Goepfert et al., 2010). Chondrification, also known as chondrogenesis, is the process by which cartilage is formed. Cartilage is composed of specialized cells called chondrocytes, which are differentiated from mesenchymal stem cells (MSCs). In most tetrapod limb skeletons, the cartilage is replaced by bone in later developmental stage.

Transverse splitting of precartilage rod is a key step for joint formation (Gunin AG, 2010). Take the formation of synovial joint as an example, interzone mesenchyme differentiate into fibroblastic tissue. Fibroblasts differentiate into 3 layers and 2 cartilage layers with a dense connective tissue in between (Gunin AG, 2010). Central region forms menisci and ligament surrounded by the joint capsule. Vacuoles form and coalesce in the synovial cavity (Martinsen BJ, 2007).

8.1.3 Genes for Limb Development Defects

8.1.3.1 Fibroblast growth factors (FGFs)

FGFs, are a family of growth factors involved in embryonic development. FGFs play important roles in the processes of proliferation and differentiation of a wide variety of cells and tissues. The functions of FGFs in developmental processes include mesoderm induction, antero-posterior patterning (Koga et al., 1999), limb development, neural induction and neural development, (Bõttcher et al., 2005) and in mature tissues/systems angiogenesis, keratinocyte organization, and wound healing processes. 22 members of the FGF family have been identified in human.

In limb development, FGF2 supplied to the chick apical bud mesoderm after AER (which permits growth and elongation of amniote limb buds) removal will sustain

normal gene expression and cell viability, and allow relatively normal limb development. FGF4 and FGF8 regulate cell number in the nascent limb bud and are required for survival of cells located far from the AER to ensure the normal formation. Absence of both FGF4 and FGF8 activities lead to failure of limb development, both of which are essential to maintain the expression of Shh, as well as the FGF10 signaling (Norton et al., 2008).

Table 8.3 Genes for human limb development and the related diseases

Gene	Limb Expression	Associated Abnormalities in limb development
FGFs	AER: FGF 4&8	limbless
	Mesenchyme: FGF10	supernumerary limb
Hox	P/D mesoderm, A/P mesoderm	polysyndactyly
Shh	ZPA	absence of spinal column
		absence of ribs
		forelimb truncations
		hindlimb with single digit
Wnt	dorsal ectoderm	ventral sesamoid bone
BMPs	AER, ZPA, AM*	joint patterning defects
Noggin	condensing cartilage and immature chondrocytes	multiple joint fusion
Tbx	AER	human congenital malformation, eg. absence of ulna

* AM= anterior mesoderm; AER: apical ectodermal ridge; PZ: progress zone; ZPA: zone of polarizing activity; P/D: proximal/distal; A/P: anterior/posterior.

8.1.3.2 Homeobox genes (Hox)

Hox genes contribute to the specification of the stylopod, zeugopod and autopod. There are 39 Hox genes in the human Hox gene family, and the most specific genes for limb development are HoxD9, HoxD10, HoxD11, HoxD12 and HoxD13, which are mainly expressed in the bones of upper limb. Mutations in Hox genes lead to proximal/distal losses or abnormalities of limbs (Tschopp et al., 2010). HoxA11 or HoxD11 knockout mice were reported with absence of the radius and ulna in the forelimb. Mutation of Hox13 will lead to synpolydactyly (Taylor, 2000).

8.1.3.3 Sonic hedgehog genes (Shh)

ZPA in the limb bud has pattern-organizing activity by action of a morphogen gradient of Sonic hedgehog (Shh) (Tabata et al., 2004). Shh is both sufficient and necessary to maintain the AER, create the ZPA and specify the anterior/posterior pattern in the distal limb. Shh is turned on in the posterior through the early expression of Hox genes, and is maintained in the posterior though a feedback loop between the ZPA and the AER. The 3rd, 4th, and 5th digits are specified by a temporal gradient of Shh. The 2nd digit is specified by a long-range diffusible form of Shh while the 2st digit does not require Shh. Shh cleaves the Ci/Gli3 (GLI family zinc finger), loss of which leads to the formation of generic (unpatterned) digits in polydactyly (Chiang et al., 2001).

8.1.3.4 Wnt genes

19 members have been identified in human and mouse Wingless gene family, amongst which Wnt3, Wnt5a and Wnt7a are more relevant to limb development (Logan et al., 2004). Mutation of Wnt3 was recorded with loss of β-catenin, which could induce limb truncation defects, also with interference in formation and maintenance of AER (Niemann et al., 2004). Wnt5a was also reported with truncated limbs (Li et al. 2002). Wnt7a is important and essential for dorsalizing the limb (Akita et al., 1996). Wnt7a also influences the anterior/posterior axis, loss of which causes the dorsal side of limbs to become ventral sides and missing posterior digits. Replacing Wnt7a signals rescues this defect. Wnt7a is also required to maintain expression of Shh. The position of FGF10 expression is regulated by Wnt8c in the hindlimb and Wnt2b in the forelimb (Martin G, 2001).

8.1.3.5 Bone morphogenetic proteins (BMPs)

BMPs are a group of growth factors also known as cytokines, originally discovered by their ability to induce the formation of bone and cartilage, but are now considered to constitute a group of pivotal morphogenetic signals, orchestrating tissue architecture throughout the body (Denecke B, 2010). In regenerative medicine, BMPs are delivered to the site of the fracture, to allow bone formation (the stimuilus by BMPs must be localized and sustained for some weeks). Currently, two BMPs products have been approved by the Food and Drug Administration (FDA) for clinical applications in fractures of long bones and intervertebral disk regeneration (Mont A, 2004), though the implanted purified collagen matrix, i.e., BMP-2 (Medtronic) and OP-1 BMP-7 (Stryker Biotech) (Vukicevic et al., 2004). As to other known functions, BMP1, BMP5, BMP6 and BMP15 are related to cartilage or joint formation, BMP3, BMP4 and BMP7 are involved in bone growth while BMP2 and BMP8 are essential for both skeleton and cartilage developments (Nie X, 2006).

8.1.3.6 Noggin

Noggin, also known as NOG, plays a key role in neural induction by inhibiting BMP4. Mouse knockout experiments have demonstrated that NOG is involved in numerous developmental processes and plays a crucial role in bone development and joint formation, such as multiple joint fusion (Polymeropoulos et al., 1995).

8.1.3.7 T-box family

The T-box family of transcriptional factors is ancient and highly conserved among most species of animals; it provides instructions for making proteins called T-box proteins that play critical roles during embryonic development (Technau et al., 2001). These proteins are especially important for normal development of the arms, hands, and heart. Haploinsufficiency of multiple T-box proteins results in severe human

congenital malformation syndromes, involving craniofacial, cardiovascular, and skeletal defects (King M, 2006). These genes have major roles in embryogenesis, including the development of the limbs. Researchers have identified at least 17 genes in the T-box gene family, and recent studies have shown that Tbx1, Tbx2, Tbx3, Tbx4, Tbx5, Tbx15, and Tbx18 are all expressed in the limb buds (Table 8.4).

Table 8.4 Tbx genes for limb development and the related diseases

Gene	Limb Expression	Other Expression	Diseases
Tbx 1	Fore and hindlimbs	muscles and bones of the face and neck, large arteries, ear, and glands	DiGeorge syndrome
Tbx 2	Fore and hindlimbs	heart	cerebellar hypoplasia
Tbx 3	Fore and hindlimbs	apocrine gland, tooth, hair, and genital development	absence of ulna
Tbx 4	Hindlimb	trachea and esophagus	small patella syndrome
Tbx 5	Forelimb	development of the heart	Holt–Oram cardiac defect
Tbx 15	Fore and hindlimbs	heart, vertebral column and head	Cousin syndrome
Tbx 18	Fore and hindlimbs	formation of ureteral smooth muscle formation development of ear and heart	Branchio-Oto-Renal Syndrome

In particular to Tbx2, Tbx3, and Tbx5, the mutation of which are involved in absent ulna and oligodactylia (Todd et al 2003) in upper limbs as observed in our in vivo toxicity experiments. Since Tbx1 is more relevant to development of muscles and bones in the face and neck, and Tbx4 is mainly expressed in hindlimb and more relevant to the function and development of hindlimb (Hajduk et al., 2010), while Tbx15 (Singh et al., 2005) and Tbx18 (Trowe et al., 2008) are more related to the development of other structures. In this chapter, we focused on Tbx2, Tbx3 and Tbx5 genes in the toxicity of Largehead Atractylodes Rhizome in limb development.

8.2 Objectives

In our in vivo experiments, we observed limb malformations and other skeleton defects in higher dosage of the most commonly used Chinese herbal medicine

decoction (*Largehead Atractylodes Rhizome*).

In this chapter, we aimed to study the molecular mechanisms of the congenital malformation induced by *Largehead Atractylodes Rhizome*. Our objective was to test if Tbx gene expressions were dysregulated by *Largehead Atractylodes Rhizome* during early limb development.

8.3 Methodology

8.3.1 Mouse Embryos

Pregnant ICR mice were fed with 0x dose (PBS-Milli Q water solution) as control group and 3x clinical dose of *Largehead Atractylodes Rhizome* as study group from E7 to E10. Embryos were harvested on E10.5. The samples were further dissected in the DEPC treated ice-cold PBS. Limb buds (either left or right) or whole embryos were randomized collected and pooled from same litter for quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) and whole mount in-situ hybridization (WISH), respectively.

For qRT-PCR, the collected limb buds were immersed in 10 volumes of RNAlater RNA stabilizing Reagent (*Qiagen*) at 4℃ overnight to stabilize and protect cellular RNA, then were stored at -80℃ until use. For WISH, the collected whole embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4℃. The embryos were washed twice with PBT (1X PBS, 0.1% Tween-20). Dehydration was carried out by sequential immersion of the embryos in 25%, 50%, 75% and 100% methanol for 5 min each. The dehydrated embryos were then immersed in 100% methanol and stored at -20℃ before use.

8.3.2 RNA Extraction

Total RNA was prepared from frozen tissues using the RNeasy mini RNA kit (*Qiagen*). After discarding the RNAlater, tissues were disrupted and lysed in 600μl of buffer RLT with 1% beta-mercaptoethanol and homogenized by passing the lysate 10 times through a 19-gauge needle fitted to a RNase-free syringe, which was followed by 22-gauge and 26-gauge needles. The lysate was transferred directly onto a QIAshredder spin column placed in 2ml collection tube for further homogenization. The tissue lysate was mixed immediately with 600μl of 70% ethanol by pipetting to dissolve the nucleic acids. 700ul of Buffer RW1 was added to remove the residual ethanol, followed by washing twice with 500μl Buffer RPE for DNA and H_2O removal. Elution of total RNA was performed by adding 30μl of RNase-free H_2O. RNA purity and quality were checked by spectrophotometry. The extracted RNA was kept at -80℃ until use.

8.3.3 cDNA Synthesis

First-strand cDNA was synthesized using the SuperScript III Reverse Transcriptase (*Invitrogen*) according to the manufacturer's procedures. 10ng of total RNA was added to 1μl of 100mM oligo d(T)24, 1μl of 10mM dNTPs and sterile distilled water in a 13μl final volume. After 5 min at 65℃ for mixture and incubation on ice for 1 min to anneal the oligo-d(T) primer to polyA tail, the reaction was initiated with the addition of 1μl of 0.1M DTT, 1μl of RNaseOUT (40U/μl, *Invitrogen*), 4μl of 5X first strand buffer and 1μl of Superscript III Reverse transcriptase (200U/μl). After completely mixed by pipetting, reverse transcription (RT) was performed in a thermal cycler (ABI 9700, *Applied Biosystems*) at 50℃ for 60 min and inactivated by heating at 70℃ for 15 min. In total 20μl of cDNA solution was synthesized and kept at -20℃ for long term storage.

8.3.4 Tbx Cloning

8.3.4.1 PCR reaction

PCR was performed using the GeneAmp® PCR System 2700 (*Applied Biosystems*). Primers (*Applied Biosytems*) specific to Tbx2, Tbx3, and Tbx5 genes were used. PCR amplification was done in a 50µl final reaction volume, including 5µl 10x PCR buffer (200mM Tris-HCl at pH8.4 and 500Mm KCl), 1.5µl 50 mM $MgCl_2$, 1µl 10mM dNTP Mix, 1µl forward primer (10µM), 1µl reverse primer (10µM), 0.4µl Taq DNA polymerase (5U/µl), 2µl of cDNA as template, and 38.1µl autoclaved distilled water (ddH_2O). For all reactions, a master mix was prepared containing all ingredients to ensure homogeneity. Denaturation was done by heat reaction at 96℃ for 2 min, followed by 40 cycles included 10 sec at 96℃ and 30 sec at 55℃-60℃ for annealing, then 3 min at 72℃ for extension. PCR product was checked by agarose gel electrophoresis, and a single and discrete band could be observed.

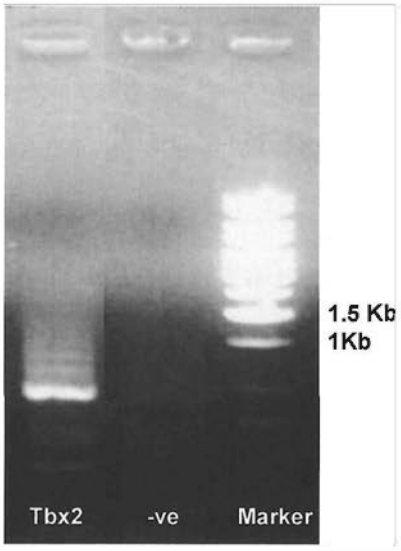


Figure 8.6 PCR amplification of Tbx2

-ve: negative; marker: 1Kb marker.

8.3.4.2 Transformation

TOPO® Cloning reaction for transformation into DH5α™-T1^R was performed with TOPO TA Cloning kit (Invitrogen). 6μl mixture of 4μl DNA, 1μl salt solution, 0.5μl TOPO® vector and 0.5μl water was incubated at room temperature for 5 min. 2μl of the mixture was added into a vial of DH5α™-T1^R, then was placed on ice. The cells were heat shocked at 42℃ for 30 sec, and immediately transferred to ice. After 250μl of super optimal culture (S.O.C.) medium was added, and the tube was shaked horizontally at 37℃ for 1 hour. 50μl of transformed cells was spread on a prewarmed agarose plate with antibiotics and incubated overnight at 37℃. 10 colonies were picked for mini preparations.

8.3.4.3 Mini-preparation of plasmid DNA

Transformed bacteria were grown in 10ml of LB broth (*Invitrogen*) with 80μg/ml ampicillin (*Roche*) at 37℃ overnight with agitation. cDNA plasmids were purified by using QIAprep® Miniprep Kit. The cells were harvested at 3000rpm for 5 min at 4℃. The cell pellet was resuspended in 250μl Buffer P1, to which 250μl Buffer P2 was added for lysing the cells and mixed thoroughly by inverting the tube 10 times. The mixture was neutralized by adding 350μl Buffer N3, mixed again immediately and thoroughly. The QIAprep spin column was washed by adding 0.5 ml Buffer PB and 0.75 ml Buffer PE, and centrifuged to remove trace nuclease activity. Then it was centrifuged for an additional 1 min to eliminate any chance of possible wash buffer carryover. The plasmid DNA was eluted with 50 μl sterile ddH_2O. The size and the quantity of plasmid DNA were checked by agarose gel electrophoresis and spectrophotometry, respectively. The prepared plasmid DNA was kept at -20℃.

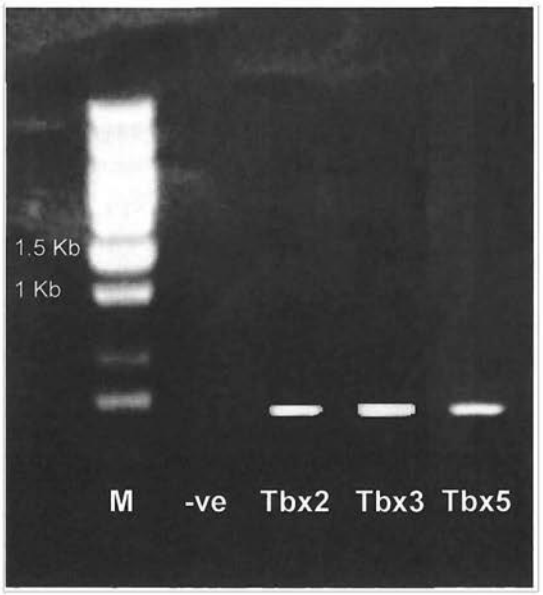


Figure 8.7 Plasmid DNA of Tbx genes

M: 1Kb marker; -ve: negative.

8.3.5 qRT-PCR

The qRT-PCR was performed using the ABI PRISM 7700 Sequence Detection System (*Applied Biosystems*). The pre-designed TaqMan® primers specific for Tbx 2, Tbx3 and Tbx5 genes were obtained from Applied Biosystems Gene expression assays (*Applied Biosystems*), the assay ID for Tbx 2, Tbx3, Tbx5 and *Gapdh* were Mm00436915_ml, Mm00809779_sl, Mm00803518_ml and Mm99999915_gl, respectively (Table 8.5). qRT-PCR amplification was done in a 25µl final volume, including 1µl of cDNA as template, 2.5µl of 10X PCR buffer, 3µl of 25mM magnesium chloride ($MgCl_2$), 0.5µl of dNTPs, 1µl of TaqMan probe and 0.1µl of AmpliTaq Gold ® DNA Polymerase (10U/µl; *Applied Biosytems*) and 16.9µl autoclaved water (ddH_2O). For all reactions, a master mix was prepared containing all ingredients to ensure homogeneity between wells. All reactions were carried out in triplicate for accurate quantification. For TaqMan® analysis the following two-step thermal profile was used: After 10 min of enzyme activation at 95℃, followed by 40 cycles included denaturation for 15 sec at 95℃, 1 min of extension at 60℃. The data

were analyzed quantitatively by ABI Prism®7000 SDS Software (*Applied Biosystems*). A negative control was run without cDNA template with every assay to assess the overall specificity.

Gapdh was used as the internal control for normalization. cDNA standards were co-amplified for the quantification of the amount of *Tbx* and *Gapdh* genes. The relative expression levels of *Tbx 2-5* in E10.5 limb bud were compared by one-way ANOVA analysis. All statistical analyses were carried out using SPSS software, with statistical significance level set at $p < 0.05$.

Table 8.5 Tbx primers for RT-PCR

Gene	Assay ID
Tbx2	Mm00436915_ml
Tbx3	Mm00809779_sl
Tbx5	Mm00803518_ml
Gapdh	Mm99999915_gl

8.3.6 In situ probe synthesis and precipitation

Antisense riboprobes were synthesized using MEGAscript® SP6 Kit (*Ambion*) according to the manufacturer's procedures. 10X Reaction buffer, 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP), Digoxigenin-11-dUTP (DIG-11-UTP), DTT, ddH_2O and SP6 polymerase were added to a total volume of 20μl. The mixture was incubated at 37℃ for 4 hours for in vitro transcription. The quality of DIG-labeled antisense riboprobes was checked by agarose gel electrophoresis and spectrophotometry. Then the probe was mixed with 2μl of 3M NaOAc (pH5.2) and 75μl of 100% ethanol, to precipitate the probes. The pellet was air-dried. Riboprobes were re-suspended with 100μl of DEPC H_2O. Probe concentration was determined by spectrophotometer.

Table 8.6 Pre-designed probes for WISH

Gene	Assay ID	Forward primer sequence	Reverse primer sequence	PCR Product Size
Tbx2	NM_009324.2	5'-GTTCCACCTCTCCCAGCATA-3'	5'-CGGACATGACTCCTCCTAGC-3'	948bp
Tbx3	NM_011535.2	5'-CTGCGTTACAGCCCCTATTC-3'	5'-CCAGCATCGGCTCTTAAAAC-3'	955bp
Tbx5	NM_011537.3	5'-CACGGAGCACCCCTATAAGA-3'	5'-CAACACTCAGCGAGGCAATA-3'	926bp

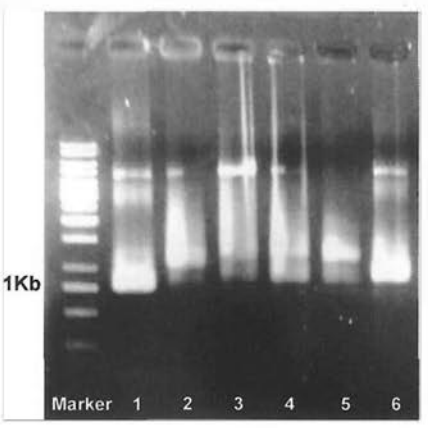


Figure 8.8 ISH probes for Tbx genes

1: Tbx2: sense; 2: Tbx2: antisense; 3: Tbx3: sense; 4: Tbx3: antisense; 5: Tbx5: sense; 6: Tbx5: antisense.

8.3.7 Dot blot analysis

Serial dilutions of the probe were prepared to select the best concentration of probe for hybridization by dot blot analysis. 1μl of each dilution was spotted onto a nylon membrane (Bio-Rad, Hong Kong), which was held between two filter papers and baked at 120℃ for 30 min. This membrane was then washed in MAB-T (0.1M maleic acid, 0.15M NaCl, 0.1% Tween-20 at pH 7.5) for 5 min. Blocking was carried out by immersed the membrane in 2% Boehringer blocking reagent (BBR; *Roche*) in 1X MAB-T for 30 min. The membrane then incubated in 2% BBR in MAB-T with anti-digoxigenin (Anti-DIG) conjugate antibody (1:5000; *Roche*) for another 30 min. After washing twice in MAB-T, purple color was developed with the addition of BM

Purple AP substrate (*Roche*) in darkness. Once sufficient color intensity was reached, membrane was washed in ddH2O and air-dried.

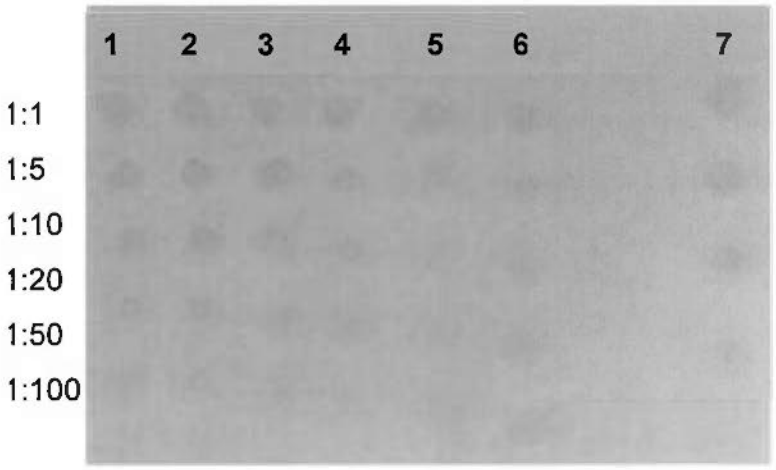


Figure 8.9 Serial dilutions of the probes

Dilutions: 1:1, 1:5, 1:10, 1:20, 1:50, 1:100.

1: Tbx2: sense; 2: Tbx2: antisense; 3: Tbx3: sense; 4: Tbx3: antisense; 5: Tbx5: sense; 6: Tbx5: antisense; 7: +ve.

8.3.8 Whole mount in-situ hybridization (WISH)

Embryos were rehydrated by reversing the dehydration process, followed by rinsing twice in PBT. 6% hydrogen peroxide (H_2O_2) in PBT was added for bleaching for 30 min, and rinsed twice with PBT. The bleached embryos were refixed in 0.2% gluteraldehyde in 4% PFA for 20 min. The embryos were washed twice with PBT before hybridized overnight at 70℃ with 1ng/ml DIG-labeled antisense riboprobes in hybridization buffer (50% formamide, 5X SSC at pH4.5, 1% SDS, heparin, and DEPC H2O). After hybridization, embryos were washed in 2X SSC with 1% SDS at 70℃ for 30 min, and followed by 0.2X SSC with 0.1% SDS at 70℃ for 30 min. The embryos were then washed twice in MAB-T and blocked for 1 hr at room temperature with 2% BBR (*Roche*) in MAB-T solution. Anti-digoxigenin-AP conjugate antibody (1:2000; *Roche*) in 2% BBR and 2% heat-inactivated sheep serum (*Jackson Immuno*

Research) was incubated overnight at 4℃. After washed twice with MAB-T for 1hr at room temperature, embryos were exposed to the BM Purple AP substrate (*Roche*) in darkness at 4℃ overnight. Reaction was stopped by washing with PBT for 1hr. The embryos were refixed at 4% PFA for 1hr, and then washed with PBT. Dehydration was performed with sequential soaking of embryos into 25%, 50%, 75%, 100% glycerol for imaging and long term storage.

8.4 Results

8.4.1 Tbx2

The relative quantifications of Tbx2 expression of E10.5 limb bud from 3 times clinical dose of *Largehead Atractylodes Rhizome* treated study group were compared with sham control group, as shown in Figure 8.10. Tbx2 were expressed in both forelimb and hindlimb. However, the expression level of Tbx2 in study group was significantly lower than control groups in both forelimb and hindlimb (one-way ANOVA, $p<0.05$).

At E10.5 of the control mouse embryos, *Tbx2* was expressed in the forebrain, midbrain, maxillary, first branchial arch, forelimb bud, hindlimb bud and also in tail, as shown in Figure 8.11. Tbx2 was highly expressed in both forelimb and hindlimb bud of E10.5 mouse embryo, which was consistent with the real time PCR results. However, the expression of Tbx2 was decreased in 3 time clinical dose study groups in both fore and hindlimb buds. In forelimb buds, the Tbx2 expression cannot be detected at proximal limb plate, and Tbx2 was less expressed in the peripheral limb plate (AER+PZ) (Figure 8.12). In hindlimb buds, the expression of Tbx2 could be hardly detected (Figure 8.13).

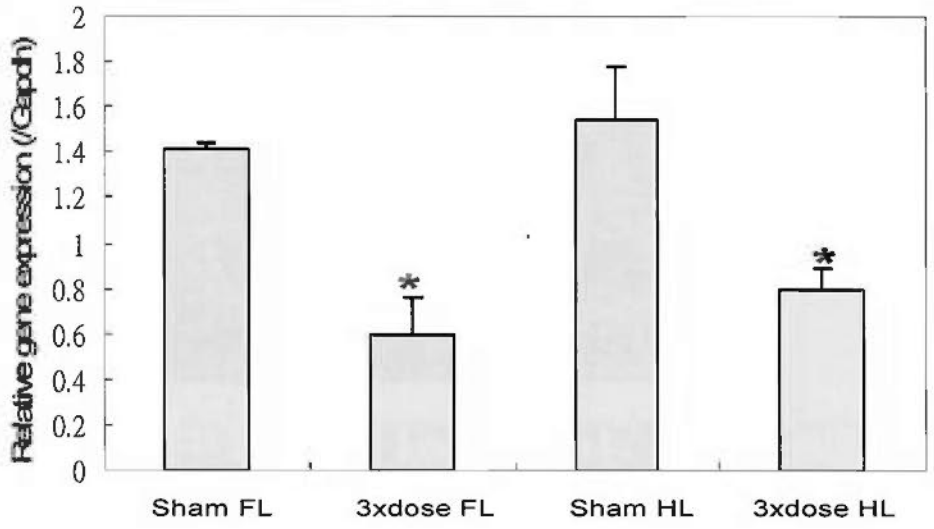


Figure 8.10 Expression level of Tbx2 in mouse limb bud after Largehead Atractylodes Rhizome treatment

FL= forelimb; HL= hindlimb; n=3; * $p<0.05$.

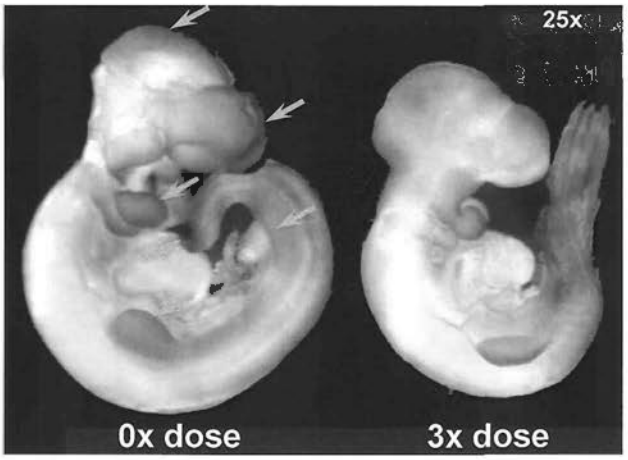


Figure 8.11 Tbx2 expression in mouse embryos at E10.5.

Lateral view of whole mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

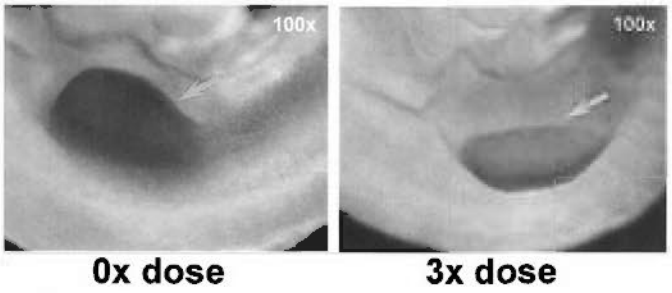


Figure 8.12 Tbx2 expression in forelimbs of mouse embryos at E10.5.

Forelimb buds of E10.5 mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

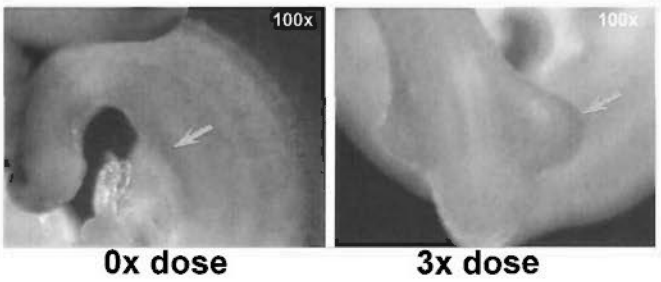


Figure 8.13 Tbx2 expression in hindlimbs of mouse embryos at E10.5.

Hindlimb buds of E10.5 mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

8.4.2 Tbx3

The comparison of the relative expression levels of Tbx3 in E10.5 limb bud between control group and 3x clinical dose of *Largehead Atractylodes Rhizome* treated study group were shown in Figure 8.14. Results indicated that Tbx3 were expressed in both forelimb and hindlimb. After 3x clinical dose of *Largehead Atractylodes Rhizome* interventions, the expression levels of Tbx3 significantly decreased in both forelimb and hindlimb (one-way ANOVA, $p<0.05$).

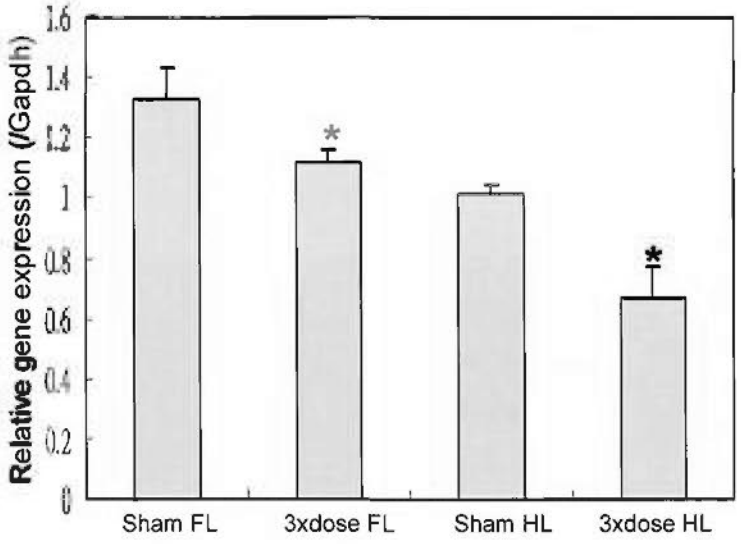


Figure 8.14 Expression level of Tbx3 in mouse limb bud

FL= forelimb; HL= hindlimb; n=3; * $p<0.05$.

At E10.5 of the mouse embryos, *Tbx3* was expressed in the brain (fore brain, middle brain and hind brain), otic system, first branchial arch, forelimb bud, hindlimb bud and tail, as shown in Figure 8.15. Tbx3 was highly expressed in both forelimb and hindlimb bud of E10.5 mouse embryos, which was consistent with the real time PCR results. However, the expression of Tbx3 could be hardly detected in 3x clinical dose intervention groups, either forelimb or hindlimb bud (Figure 8.16 & Figure 8.17).

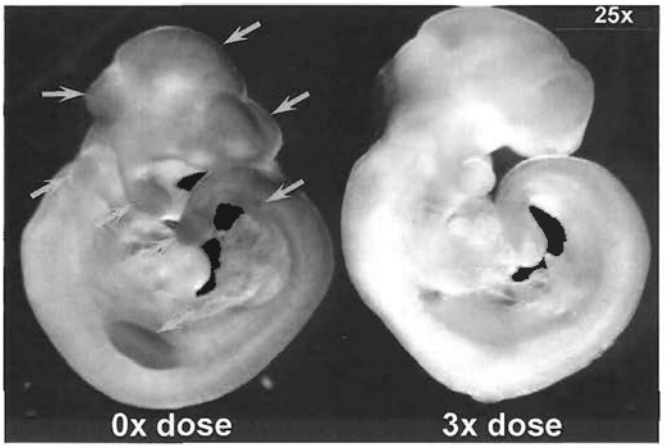


Figure 8.15 Tbx3 expression in mouse embryos at E10.5.

Lateral view of whole mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

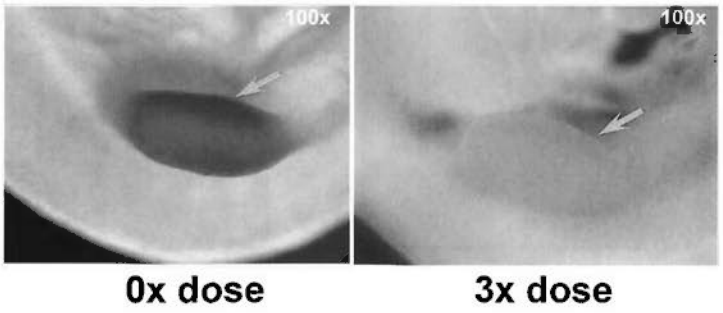


Figure 8.16 Tbx3 expression in mouse embryos at E10.5.

Forelimb buds of E10.5 mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

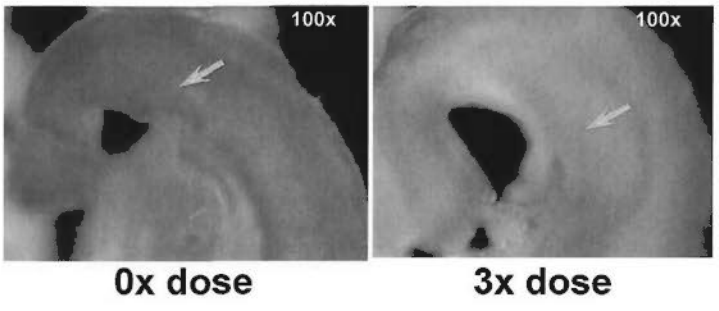


Figure 8.17 Tbx3 expression in mouse embryos at E10.5.

Hindlimb buds of E10.5 mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

8.4.3 Tbx5

The relative quantifications of Tbx5 expression of E10.5 limb buds from 3x clinical dose of *Largehead Atractylodes Rhizome* treated study group were compared with sham control group, as shown in Figure 8.18. Results indicated that Tbx5 was mainly expressed in forelimb but barely in hindlimb. After treatment with 3x clinical dose of *Largehead Atractylodes Rhizome*, the expression level of Tbx5 was significantly lower than control groups in forelimb (one-way ANOVA, $p<0.05$), while no expression was detected in hindlimb (one-way ANOVA, $p>0.05$).

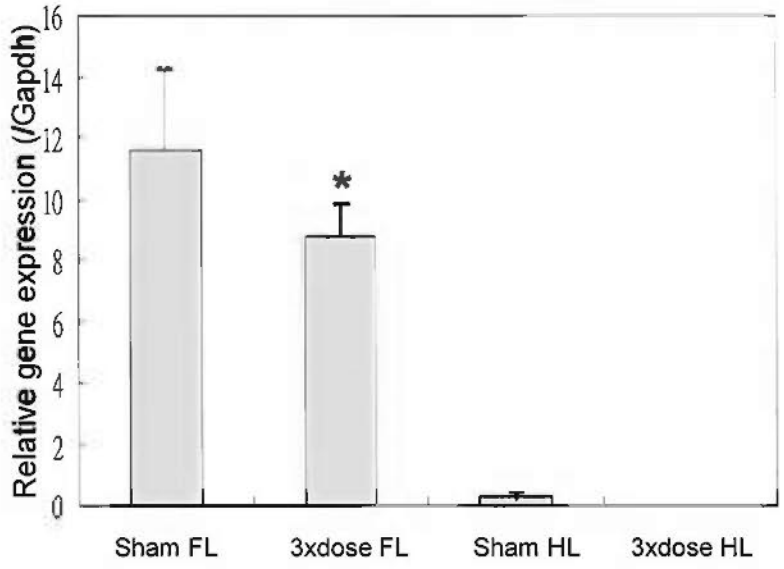


Figure 8.18 Expression level of Tbx5 in mouse limb bud

FL= forelimb; HL= hindlimb; n=3; * $p<0.05$.

At E10.5 of the mouse embryos, *Tbx5* was expressed in the brain (fore brain, middle brain and hind brain), otic system, maxillary, first branchial arch, forelimb bud, hindlimb bud and tail, as shown in Figure 8.19. Tbx5 was highly expressed in forelimb and barely expressed in hindlimb bud, which was consistent with the real time PCR results. However, the expression of Tbx5 declined in forelimb after giving 3x clinical dose intervention while there is no big difference of the expression in hindlimb (Figure 8.20 & Figure 8.21).

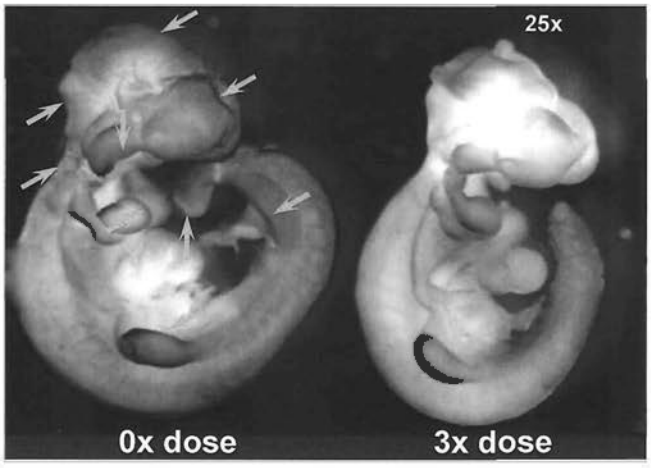


Figure 8.19 Tbx5 expression in mouse embryos at E10.5.

Lateral view of whole mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

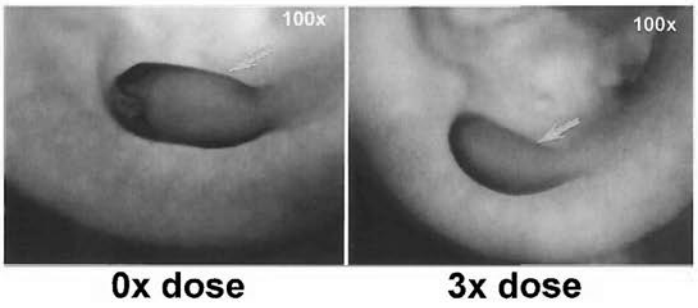


Fig. 8.20 Tbx5 expression in mouse embryos at E10.5.

Forelimb buds of E10.5 mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

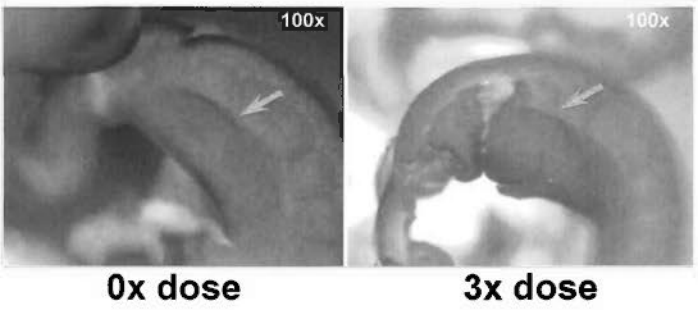


Fig. 8.21 Tbx5 expression in mouse embryos at E10.5.

Hindlimb buds of E10.5 mouse embryo from control group (left) and 3x clinical dose study group (right) are shown. Yellow arrows: positive hybridization signals.

8.5 Discussion

8.5.1 Experimental Design

As listed in Table 8.1, E9 - E15 is the specific stage for limb development in mouse, and the formation of limb buds usually start around E9. E9.5 or E10.5 is the best time to observe on both whole embryos, limb buds and other feature structures in early development. Therefore, we applied the intervention of *Largehead Atractylodes Rhizome* during E7-E10, to make sure the effects would occur at the beginning of limb bud formation, and to study the genes for molecular changes on limb development.

8.5.2 Tbx

Tbx2 could be detected from E8 embryo to newborns and adults (MGI database), expression studies indicate that Tbx2 may have a potential role in tumorigenesis as an immortalizing agent. During the formation and development of limb bud, Tbx2 is mainly expressed along the anterior and posterior edges of both forelimb and hindlimb buds from E9.5, and is essential for the digit development. Null mutation of Tbx2 led to deaths of mice due to cardiac malformations (Harrelson et al., 2004) and bilateral duplications of digit IV in the hindlimbs of the survivals (Suzuki et al., 2004). Tbx3 is expressed throughout the whole embryonic development and newborns and adults (MGI database). Embryonic lethal effects on the yolk sac resulted in Homozygous null mice death and limb dysmorphology (Davenport et al., 2003). Tbx3 also mainly expressed along the same sites in both forelimb and hindlimb buds as Tbx2, and plays an important role in the anterior/posterior axis of the tetrapod forelimb. Mutations in this gene cause ulna-mammary syndrome, affecting limb, apocrine gland, tooth, hair, and genital development, in which posterior forelimb deficiencies like absence of the ulna and fifth digit were involved (Davenport et al., 2003). The forelimb and hindlimb are specified by their position along the

anterior/posterior axis and possibly by Tbx5 and Tbx4, respectively (Ohuchi et al., 1998; Rodriguez et al., 1999). Tbx5, which is located on the long (q) arm of chromosome 12 at position 24.1, is essence for the formation of forelimb (Gilbert, 2000). During embryonic development, the Tbx5 protein turns on genes involved in the normal development of the arm and hand. The protein also activates genes that play an important role in the growth and development of the heart. This protein appears to be particularly important for the formation of the septum that separates the right and left sides of the heart. Mutation of Tbx5 results in abnormal development of the heart and upper limbs as in Holt-Oram syndrome (Basson et al. 1996; Li et al. 1996).

We compared the genes expressions of Tbx2, Tbx3, and Tbx5 in E10.5 mouse limb buds between 3x clinical dose of *Largehead Atractylodes Rhizome* treated study group and the sham control group. All these genes were under expressed in study groups, which indicate that *Largehead Atractylodes Rhizome* has adverse effects on the molecules important for mouse limb bud development, leading to the limb defects identified in our in vivo animal experiments. Tbx genes were essential for early limb bud formation and development and the depletion of these genes cause similar limb malformations as in our previous in vivo studies. Since various other genes were involved in limb development, further studies are needed to explain the other possible mechanism or pathways.

In controls, the expression of Tbx2 was observed in the whole limb bud area, including the AER, PZ, ZAP and the mesenchyme. But in study group, no expression was detected in ZAP and the mesenchyme of both forelimb bud and hindlimb bud. *Largehead Atractylodes Rhizome* intervention only resulted in lower expressions in forelimb and hindlimb buds for Tbx3 and mainly forelimb buds for Tbx5. As the ectoderm, including AER, PZ and ZAP, will develop into skeleton and muscles of the limb, the intervention of *Largehead Atractylodes Rhizome* dysregulate the expression of the specific genes and involved in specific defects in the limbs.

8.6 Summary

Tbx genes are essential to limb development. The decrease of Tbx genes expression was observed in developing limb bud after *Largehead Atractylodes Rhizome* intervention. It indicated that *Largehead Atractylodes Rhizome* could affect the expression of Tbx genes during limb development and may responsible for the various defects in limbs.

Chapter IX

Conclusions

Chinese medicines, as the most common therapy in Traditional Chinese Medicine, have become popular for therapeutic and complementary use in healing diseases and maintaining health, not only in China but also around the world. In this thesis, we focused on the application of Chinese medicines during pregnancy, and studies were covered from systematic reviews of clinical studies in human to animal experimentations in rodents, from effectiveness to toxicity, from physiology to developmental biology, in order to obtain background information for overall understanding on the applications of Chinese medicines, and provide scientific evidences on their efficacy and safety for pregnancy.

In Chapter IV, firstly we located all the relevant literatures on the clinical applications of Chinese medicines during pregnancy. The majority of publications were identified in Chinese databases, and only few clinical studies were selected for systematic reviews. The most common clinical application of Chinese medicines during pregnancy was as management of threatened miscarriage. The most commonly prescribed Chinese medicine formula to prevent miscarriage and promote the pregnancy was Shou Tai Pill, and the most frequently used individual Chinese medicine was Largehead Atractylodes Rhizome. The Chinese medicines for threatened miscarriage were mostly single dose per day, but the range of the dose was quite large.

In Chapter V, the overall effectiveness rate of Chinese medicines for threatened miscarriage in the selected clinical studies was over 90%, however, no direct correlation between efficacy and usage frequency, dose and dosing of the Chinese medicines. No placebo was identified to compare the effectiveness of Chinese medicines for threatened miscarriage. Basic on limited clinical trials, 4 qualified randomized clinical trials were included for meta-analysis to compare the effectiveness between Chinese medicines, Western medicines and combined medicines. It showed that combined Chinese and Western medicines were more effective than either Chinese medicines or Western medicines alone to prevent

inevitable miscarriage and continuous pregnancy until 28 weeks of gestation. Another 26 studies which did not follow-up till 28 weeks of pregnancy showed that Chinese medicines alone or combined medicines were more effective than Western medicines alone to prevent miscarriage and relieving the clinical signs. Since Western medicines were not used to treat threatened miscarriages, the result of systematic review had its limitations, and revealed a paucity of evidence on the effectiveness of Chinese medicines.

In Chapter VI, similar search strategies were applied on literature searches to identify the adverse events of Chinese medicines for threatened miscarriage. Unfortunately, only 1 randomized controlled clinical trial was available but no adverse pregnancy outcome was reported in either Chinese medicines or Western medicines treatment. Ten non-controlled clinical studies were identified, adverse pregnancy outcomes apparently were less common in infants but more severe than in mothers. Since the sample size was too small, control group was not included for comparison and same outcome measures were not recorded in every study, the low risk of neonatal complications cannot be concluded by any ascertainment bias of the individual studies.

Until now, no detailed reproductive toxicity and pharmacotoxicity studies are available to assess the potential toxicity of Chinese medicines during pregnancy except the already known toxic Chinese medicines, case reports and pharmacological researches on some formula and individual medicines. In Chapter VII, we used in vivo animal test to screen for the maternal and fetal toxicities of Largehead Atractylodes Rhizome, the most commonly used individual Chinese medicine for pregnancy. Significantly decreased maternal weight gain, increase fetal resorption rate in early gestation, increased incidence of growth retardation in mid gestation and increased incidence of congenital skeleton malformations in late gestation in mice were observed. The results were confirmed by embryotoxicity in vitro test. Although Chinese medicines rarely be applied in single herbs, and it is claimed that Chinese

medicine formulae can integrate the toxicity effects by the interactions of each involved individual Chinese medicine, the only conclusion we could made basic on recent studies and reported outcomes was that Largehead Atractylodes Rhizome be avoided to apply in pregnancy, and high attentions should be drawn on its clinical dose.

In Chapter VIII, we further studied an important gene family for limb development, Tbx2, Tbx3 and Tbx5 in related to the induced congenital malformation by Largehead Atractylodes Rhizome. Decrease in expression of all these genes were recorded after Largehead Atractylodes Rhizome intervention, which indicated that Largehead Atractylodes Rhizome could affect the expression of Tbx genes during limb development and may responsible for various defects in limbs.

As a conclusion, our systematic reviews suggested that Chinese medicines combined with Western medicines maybe effective to prevent miscarriage, while Chinese medicines alone may not be effective. However, large scales of randomized controlled trials and more scientific evidences, especially placebo controlled trials, are still necessary to confirm the effectiveness of Chinese medicines. Adverse effects of Chinese medicines during pregnancies are lacking. Our animal screening indicated one of the most commonly used Chinese medicines for pregnancy was potentially embryotoxic, further studies on common formulae may help to prove if the toxicity of this individual medicine could be relieved or abolished by the interactions within the formula.

These findings supplement background information about concerns over the use of Chinese medicines in pregnant women, provides useful information on the benefit and potential ham of the Chinese medicines to the mothers and offsprings, but also generates scientific support and useful references for further clinical studies and basic scientific research.

Chapter X

Future Studies

In my future studies, I will suggest four further studies.

Firstly, systematic review updates. Limited literatures were available for us to draw a conclusion on the efficacy and safety of Chinese medicines for pregnancy. Actually threatened miscarriage is not frequent, compared with other clinical areas in which the herbalists have more experience, such as back pain, nausea etc.

As to the efficacy and effectivness, over 100 experimental studies only few clinical tirals using Western medicines as comparisons were found, but no placebo and non-treatment controlled trials could be reached. This is insuffiencient to support the therapeutic effects of Chinese medicines and their advantages compared with Western medicines, as Western medicines are not the recommended treatment for threatened miscarriage, especially at its early stage.

The potential adverse effects identified probably are still unknown or unclear to majority of Chinese medicine healers. For example, as to threatened miscarriage, the Chinese Medicine practitioners might have used the herbs after the first trimester, which may not induce severe adverse effects. In addition, more Western practitioners have more concerns and interests in acupuncture, which is rather non-therapeutic approach to control adverse symptoms during pregnancy.

Systematic reviews not only provide the wide-ranging information to clinicians, nurses, therapists, healthcare managers, policy makers to well solve the conflicts with their busy clinical or professional workload, but also benefit for the consumers to specific the overwhelming amount of information, to avoid the false belief in unreliable information about efficacy and safety. Systematic reviews always provide the correct and trustful information if using an appropriate method. However, the figures and results require updating in a certain time, especially for some rapidly-changing fields of medicine (Shojania et al., 2007). A Cochrane study found that of 100 guidelines reviewed, 4% of systematic reviews required updating within a

year, and 11% after 2 years, and 7% needed updating at the time of publication (Shojania et al., 2007).

Therefore, systematic review is worthy to work on, and we will apply new literature search every year and keep updating the included clinical trials. If new randomized clinical trials with good quality were identified, we will update the results and conclusions about the clinical application of Chinese medicines, especially the efficacy and safety.

Secondly, clinical survey studies. Impressive advances of Chinese medicines are always claimed by the clinical workers, besides its lack of scientific evidence, current values of Chinese medicines could not really solve all problems. Although there are some well organised online clinical trial register systems become available in recent years, which are officially acknowledged by WHO, such as the former NHS Trusts Clinical Trials Register (2003) and Chinese Clinical Trial Registry (2005), to report the outcomes of different interventions in healthcare, it probably still need some time to improve and standardise the quality of clinical trials on Chinese Medicine. Therefore, a clinical survey may also be helpful for a general background of the application, efficacy and safety of Chinese Medicine therapies in clinical practices. This survey study should be first carried out in the countries or regions where both Chinese medicines and Western medicines are well developed and widely accepted by both patients and doctors. This survey will focus on certain disorders at early pregnancy, such as miscarriage prevention and infertility treatment.
Modern medicine has never stopped developing on this issue, and we also suggest to carry out animal studies to further confirm the efficacy and prove if Chinese medicines could offer effective means to prevent miscarriage.

Thirdly, further study of Largehead Atractylodes Rhizome (白术). Largehead Atractylodes Rhizome showed embryotoxic effects, and we suggest avoiding its usage during pregnancy according to the severe adverse outcomes observed in our animal

studies. However, further studies are still necessary to understand the pharmacokinetics and mechanism of the toxicity. Therefore, we will have two studies, including animal studies with formula as intervention for the former objective, and fractional bioassay to find out the active components that caused these side effects and the related Tbx gene molecular pathways.

For further study on Largehead Atractylodes Rhizome, we will first seperate the main chemical components and carry out the fractional assay. As we have proven that two main active components of Largehead Atractylodes Rhizome exsited in its lipophilic portion after further extraction, we will continue to isolate chemical components and other main active components by HPLC, then applied them to in vitro study to determine the ones that could lead to same adverse outcomes on whole embryos as caused by raw extraction. This study consists of 3 parts. In the first part, we will include the preparation of water crude extract of Rhizoma Atractylodis Macrocephalae, of which will be subjected to solvent partition (as primary phytochemistry separation) to fractionate into four fractions for the bioassay; *in vitro* micromass culture as primary screening bioassay test to identify the active extract in chondrocyte differentiation; and then whole embryo culture as secondary screening bioassay test to confirm the active extract in limb bud development. In the second prat, we will include chromatographic fractionation (as secondary phytochemistry separation) and to further isolate the pure sub-fraction/compound(s) of Rhizoma Atractylodis Macrocephalae by bioassay-guided fractionation; and again *in vitro* micromass and whole embryo culture as screening bioassay tests to identify and confirm the active sub-fraction/compound(s) in chondrocyte differentiation and limb development as above. In the third part, we will employ *in vivo* pregnant mouse model as validation test to confirm the embryotoxicity and to evaluate the pharmacological profiles of the the active sub-fraction/compound(s) in mother and fetuses.

Last but not the least, safety screening on other individual Chinese medicines.

Continual screening work on the safety of other most commonly used individual Chinese medicines should be carried out, as the interactions amongst these individual Chinese medicines within a formula are most difficult to explain. We will first screen the top 20 individual Chinese medicines, including Largehead Atractylodes Rhizome, Chinese Dodder Seed, Himalayan Teasel Root, Donkey-hide Glue, Chinese Taxillus Twig, Mongolian Milkcetch Root, White Paeony Root, Chinese Angelica, Liquoric Root, Baical Skullcap Root, Eucommia Bark, Steamed Rehmannia Root, Szechwon Tangshen Root, Common Yam Rhizome, Villous Amomrum Fruit, Rehmannia Root, Szechuan Lovage Rhizome, Chinese Mugwort Leaf, Motherwort Herb, Tangerine Peel. The methodology will be the same as our in vivo animal study. 1x, 2x and 3x clinical doses will be tested in mice first, and the administrations will be applied within different gestational stages and the whole gestational period, to identify and specify the potential adverse effects in both maternal and fetal health. If similar adverse outcomes were observed in different individual Chinese medicines, further studies on their common active components will be planned and designed.

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