Effects of a Kidney-Tonifying Herbal Formula on Type I Osteoporosis

LIONG, Ching

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List of thesis committee:
Professor LIN Zhixiu (Chairman)
Professor CHE Chun-tao (Supervisor)
Professor LIU Wing-keung Ken (Committee member)
Professor WONG Man-sau (External examiner)

Abstract

Osteoporosis is a skeletal disorder which leads to an increased risk of bone fracture, disability or even death. It has become a major public health threat and the worldwide incidence of osteoporotic fracture is projected to increase two fold within the next 50 years. Postmenopausal women, being affected by a lack of estrogen, face a much higher risk of the disease. This study would therefore focus on type I osteoporosis (i.e. postmenopausal osteoporosis). Although current medications can slow down the bone deterioration process, their side effects and high cost had impaired patients' compliance with long term treatment.

In search for safe, effective and low-priced medicine, the public have turned their attention to Traditional Chinese Medicine (TCM). Extensive experience has been accumulated in TCM regarding the diagnosis and treatment of osteoporosis, which often involves the prescription of kidney-tonifying herbs (補腎中藥). Therefore, the aim of the study, firstly, was to explore the association of the incidence of postmenopausal osteoporosis and Kidney-Vacuity Syndromes (腎虛證候) in TCM, so as to formulate a rational kidney-tonifying herbal formula for osteoporosis research (OPR). Secondly, the effect of the

formula was evaluated by *in-vitro* and *in-vivo* studies. Thirdly, the Osteoporosis-Targeted Quality of Life Questionnaire was linguistically validated from English to Chinese, which was expected to be one of the outcome measurement tools in future clinical trials. Lastly, a pilot clinical study was performed, which revealed some potential hazards of the formula on human beings which have not been shown in previous works.

Association of the incidence of postmenopausal osteoporosis and Kidney-Vacuity Syndromes in TCM was investigated with the aid of a Kidney-Vacuity Syndromes scoring questionnaire. In the study, postmenopausal women, who suffered from deficiency of kidney "qi" (腎氣虛證) and kidney "essence"(腎精不足證), had a significantly higher incidence of osteoporosis. These findings strongly supported that replenishing kidney qi and kidney essence was a logical therapeutic principle in the formulation of OPR.

The effect of OPR for the treatment of postmenopausal osteoporosis was then evaluated by *in-vitro* and *in-vivo* studies. In the *in-vivo* study, an osteoporosis model was established by performing ovariectomy on the four-week-old C57BL/6 mice. A high bone turnover rate was induced and OPR successfully slowed down the high turnover rate of bones by decreasing bone formation and resorption process without increasing the uterine linings. However, its beneficial effect on bones could not be detected on bone mineral density measurement.

The potential mechanism of action of OPR on bones was explored by *in-vitro* study. OPR was shown to induce cell proliferation and differentiation of osteoblast-like UMR 106 cells. Furthermore, the estrogenic activity of OPR was detected by MCF-7 cell line, which has been stably transfected with estrogen responsive elements (ERE). OPR was shown to possess an estrogenic activity in a dose dependent manner and was comparable to the positive

control at a concentration of 200 and 1000 μg/ml. The induced estrogenic activity by OPR may be associated with the presence of phytoestrogen within the herbal formula. These findings suggested that the beneficial effect of OPR on bones might relate to its direct positive effect on osteoblast and its estrogenic-like activity.

After the *in-vivo* and *in-vitro* studies, a double-blinded, randomized, placebo-controlled clinical trial (RCT) was planned. Due to a lack of a Chinese version of instrument to measure osteoporosis-specific quality of life, an English version of the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) was translated into Chinese and linguistically validated according to the standard guideline. The newly formed Chinese OPTQoL can be used to assess the impact of new interventions on quality of life among Chinese osteoporosis patients.

A pilot clinical trial was conducted after the *in-vivo* and *in-vitro* studies. Eight subjects fulfilled the inclusion criteria were recruited. However, the liver function tests of three subjects out of eight were found to be abnormal with elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level, which was not reported in previous toxicity test. The trial was suspended immediately and a follow-up test showed that the elevated AST and ALT level had reverted back to normal within one month after termination of OPR intake. Although we could not accomplish a RCT, the pilot study revealed potential hepatotoxicity of OPR on human beings and it would raise the safety awareness of investigators on the use of herbal remedies in future clinical studies.

In conclusion, this study investigated the use of TCM on the treatment of postmenopausal osteoporosis in a systematic manner. It started from herbal formulation, basic science studies to clinical trial. It revealed beneficial effects of OPR on bones through *in-vivo* and *in-vitro*

studies and demonstrated certain possible mechanism behind. On the other hand, the hepatotoxicity of OPR on human beings was also exposed and had not been reported in previous toxicity tests. The study provided valuable clinical data for other investigators on the potential hazards of herbal remedies although they had been validated as safe and effective in pre-clinical stage.

摘要

骨質疏鬆症是一種全身性骨骼疾病，它使患者骨折風險增加，嚴重限制患者的活動，甚至縮短壽命。骨質疏鬆症已經成為全球關注的健康問題，有數據顯示骨質疏鬆症所引致的骨折在下半個世紀將大幅增加兩倍。絶經後的婦女由於卵巢合成的激素降低，比男性更易於患上骨質疏鬆症。因此，研究重點將放於絶經後骨質疏鬆症上。雖然現今已有藥物可有效減慢骨量的流失，但其副作用、潛在風險及高昂的醫療費用往往使患者卻步。為找尋安全、有效和價廉的替代療法，公眾已逐漸將焦點放於傳統中醫中藥上。源遠流長的中醫藥學一直有記載骨質疏鬆症相類似疾病的診斷及治療方法，當中尤其以補腎中藥最為常用。所以本研究目的將設定為：第一、探討中醫腎虛證與骨質疏鬆症發生的關聯性，這將有助擬定一條合理的補腎處方；第二、以體外實驗和動物實驗為補腎處方治療骨質疏鬆症提供科學佐證；第三、以標準的語文檢定方式將英文版本的《骨質疏鬆症的生活質量問卷》翻譯成市面缺乏的中文版本，並將其作為日後臨床實驗的其中一項檢定指標。最後進行一項臨床預實驗以揭示補腎處方對人體可能的潛在風險。

中醫腎虛證與骨質疏鬆症發生的關聯性可以透過中醫腎虛症狀資料收集量表的協助，客觀地量化患者腎虛證的程度。結果顯示具有較嚴重腎氣虛證及腎精不足證的絶經後婦女會有較高機會罹患骨質疏鬆症。由此可見補腎氣、填腎精是治療骨質疏鬆症的合理治法，補腎處方亦根據這一治法擬定。補腎處方的護骨作用在體外實驗中表現為對擁有成骨細胞特徵的 UMR106 細胞有加速其細胞分化及增值的作用，其雌激素樣作用亦已由帶有雌激素應答序列 (ERE) 的 MCF-7 細胞所肯定。在動物實驗中，4 周大的 C57BL/6 小鼠在去勢後作為絶經後骨質疏鬆症的動物模型。去勢後的小鼠表現為高轉換型骨代謝，其骨吸收（骨鈣素）及骨形成（I 型膠原羧基前肽）指標均升高，股骨上端的骨量由於雌激素缺乏而急速丟失，子宮亦隨之萎縮。經過補腎處方的六周治療，結果顯示補腎處方可有效減緩骨重建循環但不影響子宮內膜的增生。但其護骨作用並未能反映在骨

礦密度上。

由於坊間仍缺乏針對骨質疏鬆症的中文版本生活質量量表，英文版本的《骨質疏鬆症的生活質量問卷》以標準的語文檢定方式被翻譯成中文版本，本意是作為日後臨床實驗的其中一項檢定指標。雖然最後未有採用，但日後亦可提供予其他研究員用於估量各種骨質疏鬆症治療方法在華人社區中對生活質量的影響。

最後進行一項臨床預實驗揭示了補腎處方對人體可能存在的潛在風險。八位服用補腎處方的受試者中，有三位在服用一個月後的例行抽血檢測中出現不同程度的肝功能異常，表現為血清內的穀草轉胺酶(AST) 和穀丙轉胺酶(ALT)升高。雖然所有患者的肝功能在停藥後的一個月內都已回復正常水平，但這次的實驗數據已為日後的研究員提出警示，不可輕視中藥複方的潛在肝毒性。

總括而言，結果顯示具有較嚴重腎氣虛證及腎精不足證的絕經後婦女會有較高機會罹患骨質疏鬆症。因此補腎氣、填腎精是治療骨質疏鬆症的合理治法，並以此擬定補腎處方。補腎處方加速具有成骨細胞特徵的 UMR106 細胞分化及增值，其雌激素樣作用亦已由帶有雌激素應答序列 (ERE) 的 MCF-7 細胞所肯定。補腎處方可有效減緩去勢後小鼠的高轉換型骨代謝，但不影響子宮內膜的增生。英文版本的《骨質疏鬆症的生活質量問卷》以標準的語文檢定方式被翻譯成坊間缺乏的中文版本，可用於估量各種骨質疏鬆症治療方法在華人社區中對生活質量的影響。臨床預實驗揭示補腎處方對人體的潛在風險，可見不能忽視中藥複方的潛在肝毒性。

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List of Abbreviations

ALT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
AST	Aspartate Aminotransferase
ATCC	American Type Culture Collection
BMD	Bone Mineral Density
DAD	Diode Array Detector
DMEM	Dulbecco's Modified Eagle Medicum
DXA/DEXA	Dual-Energy X-Ray Absortiometry
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
GGT	Gamma-Glutamyl Transferase
HBSS	Hanks' Balanced Salt Solution
HPLC	High Performance Liquid Chromatography
ICC	Intra-Class Correlation Coefficient
IGF	Insulin-Like Growth Factor
IL	Interleukin
LD_{50}	Lethal Dose, 50%
KVS	Kidney-Vacuity Syndromes
luc	Luciferase Reporter Gene
M-CSF	Macrophage Colony-Stimulating Factor
MEM	Minimum Essential Medium
MS	Mass Spectrometer
MTD	Maximum Tolerable Dose
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
OBs	Osteoblasts
Ocs	Osteoclasts
OPG	Osteoprotegerin
OPR	Kidney-Tonifying Herbal Formula for Osteoporosis Research
OPTQoL	Osteoporosis-Targeted Quality of Life Questionnaire
OVX	Ovariectomy

P/S	Penicillin & Streptomycin
PBS	Phosphate Buffered Saline
pCm	Proprietary Chinese Medicines
PMS	Phenazine Methosulfate
pQCT	Peripheral Quantitative Computed Tomography
PRO	Patient-Reported Outcomes
PTH	Parathyroid Hormone
QCT	Quantitative Computed Tomography
QoL	Quality of Life
RANK	Receptor Activator of Nuclear Factor Kappa B
RANKL	Ligand of Receptor Activator of Nuclear Factor Kappa B
RCT	Randomized Controlled Trial
rOPG	Recombinant Osteoprotegerin
SD	Standard Deviation
SEM	Standard Error Mean
SERMs	Selective Estrogen Receptor Modulators
SFCA	Surfactant Free Cellulose Acetate
TGF	Transforming Growth Factor
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
Trypsin-EDTA	Trypsin-Ethylenediaminetetraacetic Acid
WHO	World Health Organization

1. General Introduction

Osteoporosis, literally means porous bone (**Figure 1-1**), is a disease characterized by low bone mass and structural deterioration of bone tissue. It leads to bone fragility and an increased susceptibility to fractures of the hip, spine, and wrist (International Osteoporosis Foundation, 2008). In 2000, National Institute of Health (NIH) Consensus Development Conference on "Osteoporosis Prevention, Diagnosis, and Therapy" stated that osteoporosis is "a skeletal disorder characterized by compromised bone strength that increases the risk of fracture" (NIH Consensus Statement, 2000). However, bone mineral density (BMD) assessed by dual-energy X-ray absortiometry (DXA) remains the golden standard for the diagnosis of osteoporosis (World Health Organization, 1994).

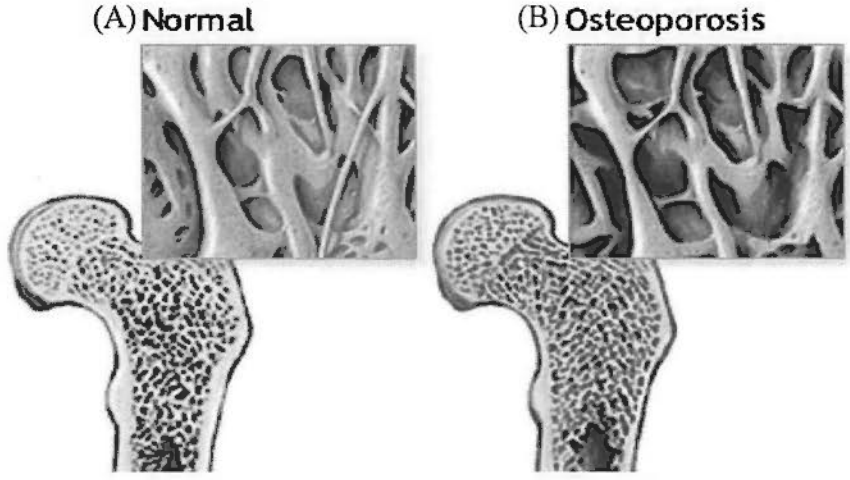


Figure 1-1 Comparison of a normal bone (A) with an osteoporotic bone (B).
(Adopted from National Library of Medicine under the National Institutes of Health, 2007)

1.1. Classification of osteoporosis

1.1.1. Primary and secondary osteoporosis

"Primary" osteoporosis is by tradition a skeletal disorder of postmenopausal women (postmenopausal osteoporosis) or of older men and women (senile osteoporosis). "Secondary" osteoporosis refers to bone loss resulting from specific, defined clinical disorders, e.g. glucocorticoid-induced osteoporosis. As primary osteoporosis occurs in both genders and is an ultimate consequence of aging, we would mainly focus on primary osteoporosis in later studies.

1.1.2. Type I and type II osteoporosis

Primary osteoporosis can be further divided into type I osteoporosis and type II osteoporosis. Type I osteoporosis signifies to a loss of trabecular bone after menopause, dominantly seen in menopausal women, whereas type II osteoporosis represents a loss of cortical and trabecular bone in both sexes as the result of age-related bone loss (Riggs et al., 1982). The characterization of type I and type II osteoporosis is shown in **Table 1-1**.

As postmenopausal women are affected by both factors of the lack of estrogen and later aging, they become the major sufferers in bone loss and result in much higher incidence of osteoporosis. Type I osteoporosis, or more commonly referred as postmenopausal osteoporosis, would become our main scope of study.

Table 1-1 Comparison of two main types of primary osteoporosis (Robert Marcus, 2001)

	Type I osteoporosis	Type II osteoporosis
Age (years)	51-75	>70
Sex ratio (Female:Male)	6:1	2:1
Type of bone loss	Mainly trabecular	Trabecular and cortical
Rate of bone loss	Accelerated	Not accelerated
Major fracture sites	Vertebrae (crush) and distal radius	Vertebrae (multiple wedge) and hip
Parathyroid function	Decreased	Increased
Estrogen effects	Mainly skeletal	Mainly extraskeletal
Main causes	Menopause plus individual predisposing	Factors related to aging including the effects of estrogen

1.2. Epidemiology of osteoporosis worldwide

Osteoporosis is a major public health threat worldwide. It affects 75 million people in Europe, USA and Japan (Lindsay et al., 1997). A life time risk of a white woman over age of 50 experiencing osteoporotic fractures is about 40% (Melton et al., 2005). It is estimated that the incidence of hip fracture and other osteoporotic fractures will increase fourfold worldwide during the next 50 years (Riggs and Melton, 1995). By 2050, the worldwide incidence of hip fracture is projected to increase by 240% in women.

Although low bone mineral density confers increased risk for fracture, most fractures occur in postmenopausal women (Pasco et al., 2006; Siris et al., 2004; Sornay-Rendu et al., 2005). In women over 45 years of age, osteoporosis accounts for more days spent in hospital than many other diseases, including diabetes, myocardial infarction and breast cancer (Kanis et al., 1997). However, the situation may be still underestimated. There is evidence to suggest that many women who sustain a fragility fracture are not appropriately diagnosed and treated for probable osteoporosis (Freedman et al., 2000; Siris et al., 2001). In every six Canadian women aged over 65 years after screened by bone mineral density testing, one previously undiagnosed case of osteoporosis was detected (Sawka et al., 2006). A survey conducted in eleven countries showed that many postmenopausal women is lack of dialogue about osteoporosis with their doctors, and with restricted access to diagnosis and treatment before the first fracture. It results in under-diagnosis and under-treatment of the disease (International Osteoporosis Foundation, 2000).

1.2.1. Osteoporosis in Asia

According to the Asian Osteoporosis Study, there is moderate variation in the incidence of hip fracture among Asian countries with the highest rate in urbanized areas. With rapid economic development in Asia, hip fracture will become a major public health challenge (Lau et al., 2000). It has been projected that more than 50% of osteoporotic hip fractures will occur in Asia by the year 2050 (Dennison et al., 2006; Gullberg et al., 1997).

1.2.2. Osteoporosis in Hong Kong

In the past three decades, the incidence of osteoporotic hip fracture increased in the Chinese population in Hong Kong by two-fold to reach an incidence of approximately 10 per 1000 in the age group of 70 or older (Lau et al., 1999). Taking one Hong Kong hospital as an example, 497 patients with osteoporotic vertebral collapse and aged 65 to 94 years old, were admitted between 1989 and 1994,. The mean hospital stay was five days, with an additional 6 to 23 days in a convalescent hospital. In 1996, the acute hospital care cost of hip fracture per year amounted to HK$17 million (Lau, 2001). Despite the general benign nature of the problem, the high morbidity and the long hospital stays undoubtedly are significant drains on health care resources (Lee and Yip, 1996).

1.3. Epidemiology of osteoporotic fractures

Osteoporosis, an indication of low bone mineral density, is positively co-related with a high fracture risk (**Figure 1-2**). The most common fractures associated with osteoporosis occur at hip, spine and wrist (**Figure 1-3**). Epidemiologic studies from North America

estimated the remaining lifetime risk for common fragility fractures to be 17.5% for hip fracture, 15.6% for clinically diagnosed vertebral fracture, and 16% for distal forearm fracture among white women aged 50 years (Dennison et al., 2006). Age-associated fracture are both found in men and women (Boonen et al., 1997; Hinton and Smith, 1993; Nevitt et al., 2005; Tanno et al., 2001).

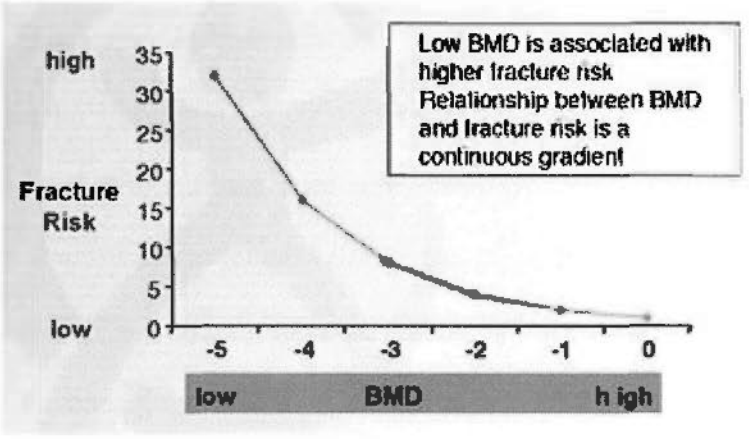


Figure 1-2 Relationship of bone mineral density and fracture risk

(Adopted from International Osteoporosis Foundation, 2008)

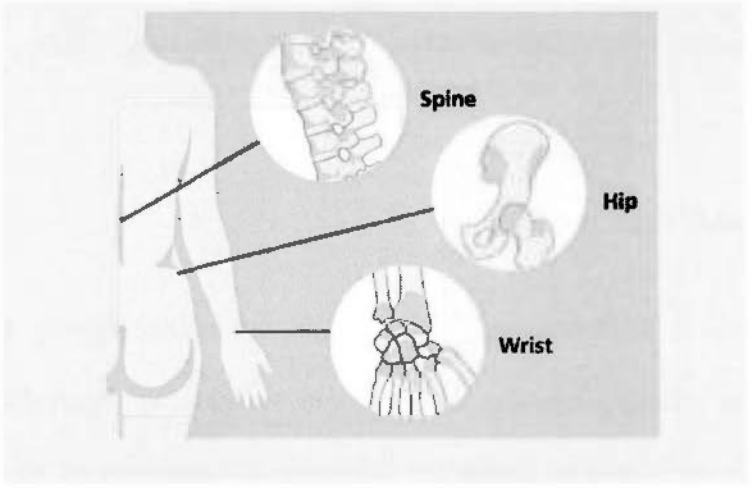


Figure 1-3 Common sites of fractures

(Adopted from International Osteoporosis Foundation, 2008)

1.4. Mortality, morbidity and economic cost of osteoporosis

Osteoporotic fractures exert a terrible toll on the population with respect to morbidity,

cost, and to a lesser extent mortality. Moreover, fractures rate will increase dramatically with the growing elderly population. All osteoporotic fractures are associated with significant morbidity, but hip and vertebral fractures also are associated with excess mortality (Dennison et al., 2006). Recent data suggest that the annual cost of fragility fractures in Europe is 13 billion Euros, mainly accounted for hospitalization after fracture (Dennison et al., 2005). In the US, an estimated 10% of patients are disabled by hip fracture, and 19% require institutionalization, accounting for almost 140,000 nursing home admissions annually in this country. Distal forearm and vertebral fractures less commonly result in nursing home placement, but about 10% of postmenopausal women have vertebral deformities that cause chronic pain, and a substantial minority has poor function after forearm fracture. These fractures interfere greatly with the activities of daily living, and all of them will have a substantial negative impact on the quality of life. Annual expenditures for osteoporotic fracture care in the United States (US$ 17.5 million in 2002) are dominated by hip fracture treatment, but vertebral fractures, distal forearm fractures, and importantly, the other fractures related to osteoporosis contribute one-third of the total (Melton, 2003).

1.5. Symptoms of osteoporosis

Osteoporosis is a silent disease. One might not realize having the disease until bone fracture one day. Back pain, decrease in height or dorsal kyphosis ("the dowager's hump") could be a sign of vertebral fracture. However, a bone mineral density test is the most reliable way to monitor bone health (The American Board of Orthopaedic Surgeons, 2008).

1.6. Diagnosis of osteoporosis

Bone mineral density (BMD), assessed by dual-energy X-ray absortiometry (DXA),

remains the golden standard for the diagnosis of osteoporosis (World Health Organization, 1994). Biochemical markers of bone turnover, measured in serum or urine, act as indicators of bone turnover rates (International Osteoporosis Foundation, 2008). By combining BMD with Biochemical markers, fracture prediction in osteoporosis patients can be improved (Akesson et al., 2005; Sarkar et al., 2004; Takahashi et al., 2003; Weisman and Matkovic, 2005).

1.6.1. Bone mineral density

Dual-Energy X-ray Absorptiometry (DXA or DEXA) or bone densitometry, is an enhanced form of x-ray technology that is used to measure bone mineral density (BMD). DEXA is today's established standard for measuring BMD. An x-ray (radiograph) is a painless medical test that helps physicians diagnose and treat medical conditions. Radiography involves exposing a part of the body to a small dose of ionizing radiation to produce pictures of the inside of the body. X-rays are the oldest and most frequently used form of medical imaging. DEXA is most often performed on the lower spine and hips. Portable DEXA devices, including some that use ultrasound waves rather than x-rays, measure the wrist, fingers or heel and are sometimes used for screening purposes (Radiological Society of North America, 2008).

BMD can be expressed as:

- T-score (the number of standard deviations, SD) above or below the mean BMD values for a young healthy adult)
- Z-score (the number of standard deviations above or below the mean BMD values for a population of the same age and gender)

Based on the 1994 WHO report (**Table 1-2**), osteoporosis in women is defined as a BMD value at least -2.5 SD below the mean value of a young healthy population (T-score$\leq$-2.5).

Table 1-2 WHO definition of osteoporosis based on bone density level

Definition	Bone density level
Normal	Bone density is within 1 SD (+1 or -1) of the young adult mean.
Low bone mass	Bone density is 1 to 2.5 SD below the young adult mean (-1 to -2.5 SD).
Osteoporosis	Bone density is 2.5 SD or more below the young adult mean (> -2.5 SD).
Severe osteoporosis	Bone density is more than 2.5 SD below the young adult mean and there have been one or more osteoporotic fractures.

1.6.2. Biochemical markers of bone turnover

Biochemical marker can act as an early estimation of treatment effect. Significant changes in biochemical markers can already be seen after anti-osteoporosis therapy for a few weeks; whereas individual monitoring with DXA usually requires 1-2 years to observe significant changes (Black et al., 2006; Kraenzlin et al., 1996; Papanastasiou et al., 2006; Reginster et al., 2008).

Biochemical markers are based upon the measurement of peptides, enzymes and other small molecules, synthesized by osteoclasts and/or osteoblasts, or of osteoclast-generated degradation products of bone matrix (International Osteoporosis Foundation, 2008).

Bone markers fall into two main categories, markers of bone resorption and bone formation, as listed in **Table 1-3** (Pearson and Miller, 2002; Reginster et al., 2008; Vasikaran, 2008).

Table 1-3 Biochemical markers of bone turnover

Formation markers usually only measured in serum	Resorption markers usually measured in urine, but some also measured in serum
Alkaline phosphatase (ALP): Total and bone specific ALP	Hydroxyproline (OHP)
Osteocalcin (OC)	Galactosyl hydroxylysine (GHyL)
Propeptides of type I collagen: N-terminal and C-terminal	Tartrate resistant acid phosphatase (TRACP)
	Collagen crosslinks includes actual crosslinks (Deoxypyridinoline and Pyridinoline) and Peptide bound crosslinks (N-terminal and C-terminal telopeptide)

1.7. Pathogenesis of osteoporosis

Bone is a living tissue which undergoes a continuous process of bone remodeling throughout lifetime. When bone resorption overtakes bone formation, it results in a loss of bone mass (osteoporosis) and disruption of architecture (fracture) (Seeman, 2003). There are multiple mechanisms underlying the regulation of bone remodeling, and these involve not only the osteoblastic (bone formation cells) and osteoclastic (bone resorption cells) cell lineages but also other marrow cells, in addition to the interaction of systemic hormones, local cytokines, growth factors, and transcription factors. Estrogen deficiency is known to play a critical role in the development of osteoporosis, while calcium and vitamin D deficiencies and secondary hyperparathyroidism also contribute. Fortunately, it is now possible to diagnose osteoporosis, assess fracture risk, and reduce that risk with anti-resorptive or other available therapies (Raisz, 2005).

1.7.1. Bone remodeling

1.7.1.1. Bone structure and composition

Bone is composed of type I collagen fibres, crystals of hydroxyapatite and ground substance. Based on structural differences, bone can be subdivided into cortical and trabecular compartments. Human skeleton consists of 80% cortical bone and 20% trabecular bone. Cortical bone (the outer layer) is composed of a thick and dense layer of calcified tissue, whereas trabecular bone (the central part) is composed of thin trabeculae forming a robust, though slightly flexible, framework, as shown in **Figure 1-4** (Norrdin et al., 1977).

Cortical bone predominates in the shafts of long bones while trabecular bone is more

dominate in the vertebrae, the epiphyses of long bones and the iliac crest. Trabecular bone, having a larger surface area, is metabolically more active and more affected by factors leading to bone loss (Allen et al., 2007). During bone scan, the proximal tibia metaphysis is a preferred site of region of interest as this site is rich of trabecular bone and reacts with the greatest magnitude of change to interventions such as ovariectomy and parathyroid hormone therapy (Gasser, 1995; Gasser, 1996; Helfrich and Ralston, 2003).

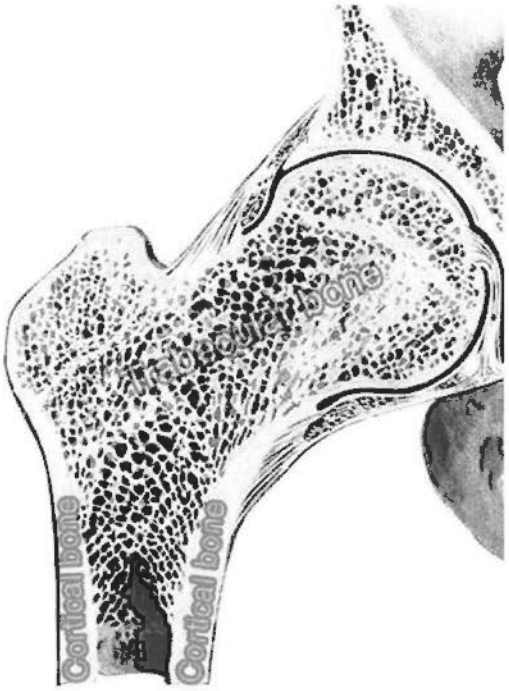


Figure 1-4 Cross-section of femur with both cortical and trabecular bone

(Adopted from website of The University of Aberdeen, Department of Orthopaedics)

1.7.1.2. The action of cells and cytokines

Bone remodeling consists of two distinct stages: bone resorption and bone formation. During bone resorption, oeteoclast precursors, recruited by cytokines and hormones, are activated by osteoblasts (OBs) to form mobile multinuclear osteoclasts (OCs) as described in section 1.7.1.3. OCs move along the bone surface, resorpting bone and releasing the cytokines (e.g. IGF, TGF-β), which are originally embedded in the bone matrix (Horowitz, 1998; Jilka, 1998; Li et al., 2006). During bone formation, the released cytokines from bone

resorption process will then recruit OBs, which lay down osteoid (an organic matrix of bone with collagen as the principal component) and embed cytokines back into the bone matrix to form new bone (Devernejoul and Marie, 1993; Horowitz, 1998; Lorenzo, 1991; Marie and Devernejoul, 1993; Riancho and Mundy, 1995; Robling et al., 2006; Steeve et al., 2004). The process is illustrated in **Figure 1-5**.

Usually, bone resorption and bone formation are linked so that they occur in close sequence and remain balanced. An imbalance in the bone remodeling may eventually lead to bone loss (osteoporosis). The loss during the menopause is due to the increase in OCs activity and affects mainly trabecular bone; the later loss in both sexes with increasing age is due to decreased OBs numbers and affects mainly cortical bone (Marcus et al., 2001).

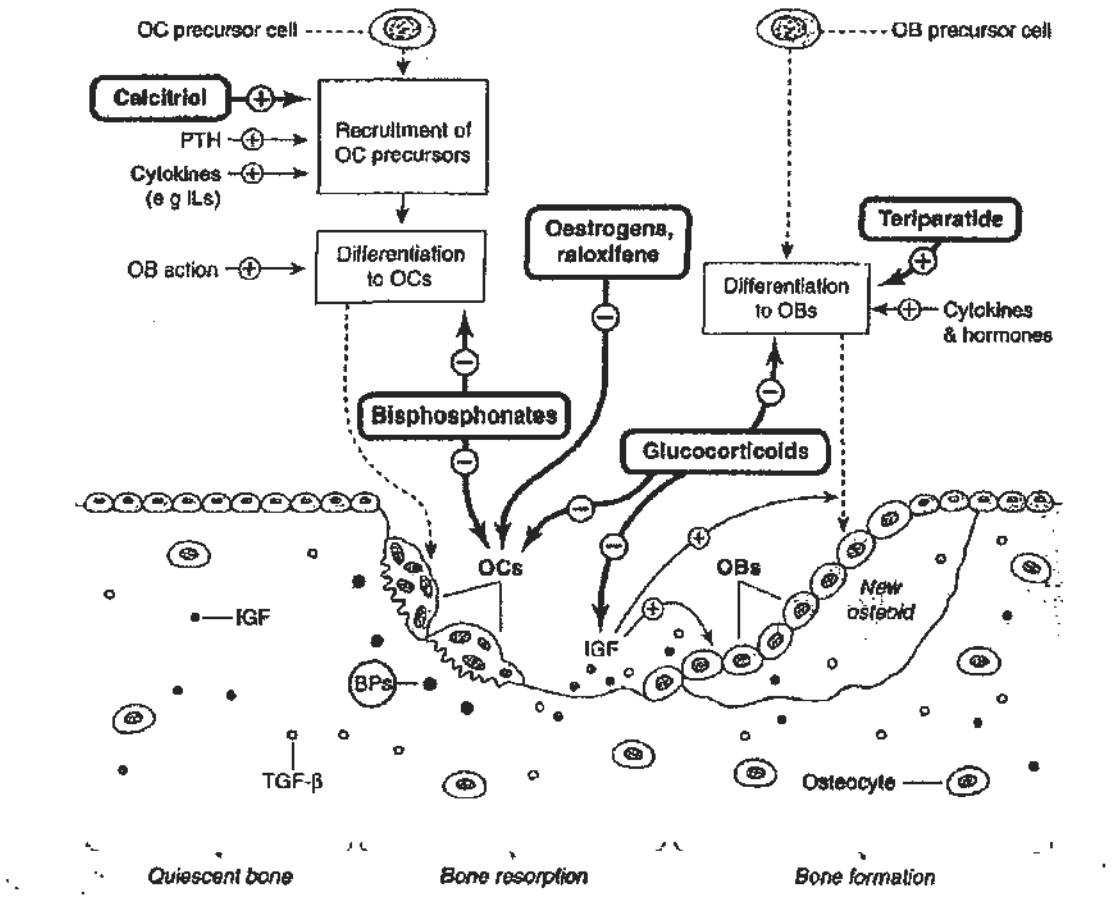


Figure 1-5 Bone-remodeling cycle and the action of hormones, cytokines and drugs.

Action of drugs in bone-remodeling process: Glucocorticoids. Inhibit osteoblast differentiation and activity, and stimulate osteoclast action - leading to osteoporosis; Estrogens, raloxifene inhibit the cytokines that recruit OCs, and oppose the bone resorpting & calcium mobilizing action of Parathyroid Hormone – protect bone from osteoporosis; Bisphosphonates (BPs): Pharmaceutical BPs promote OCs apoptosis, and interfere the attachment of OCs on bone. – protect bone from osteoporosis.

Abbreviations: IL, Interleukin; PTH, parathyroid hormone; OCs, osteoclasts, OBs, osteoblasts; IGF, Insulin-like growth factor; TGF-β, transforming growth factor; BPs, Bisphosphonates

(Adopted from Rang, 1999)

1.7.1.3. Osteoclastogenesis

Osteoclastogenesis is a differentiation and activation process of osteoclasts by osteoblasts and cytokines. When osteoblasts (OB) stimulated by calcitriol, parathyroid hormone (PTH) and interleukins (ILs), they will express a surface ligand called Ligand of Receptor Activator

of Nuclear Factor Kappa B (RANKL). RANKL expression is increased by various inerleukins, parathormone, tumor necrosis factor (TNF)-α, prostaglandin E_2 and glucocorticoids. RANKL interacts with a receptor on the osteoclast (OC) – an OC differentiation and activation receptor termed RANK. This, with macrophage colony-stimulating factor (M-CSF) released by the OB, causes differentiation and activation of the osteoclast progenitors to form mature osteoclasts. Fusion of OCs occurs to give giant multinucleated bone-resorpting cells and start bone resorption. On the other hand, the stromal cell and OB synthesize and release a molecule termed osteoprotegerin (OPG) and able to bind with RANKL. It inhibits RANK-RANKL binding and interferes the differentiation and activation of OC (Heinrich et al., 2005; Hofbauer, 2006; Li et al., 2000; Liu et al., 2006; Rios Moreno et al., 2007). The process is illustrated in **Figure 1-6**.

The ratio of RANKL to OPG is critical in the formation and activity of OCs and becomes markers of osteoclastogenesis. These markers are helpful in the evaluation of the mechanism of action and efficacy of novel drugs acting on osteoclast functions (Cao et al., 2002; Heinrich et al., 2005; Liu et al., 2006; Rucci et al., 2007).

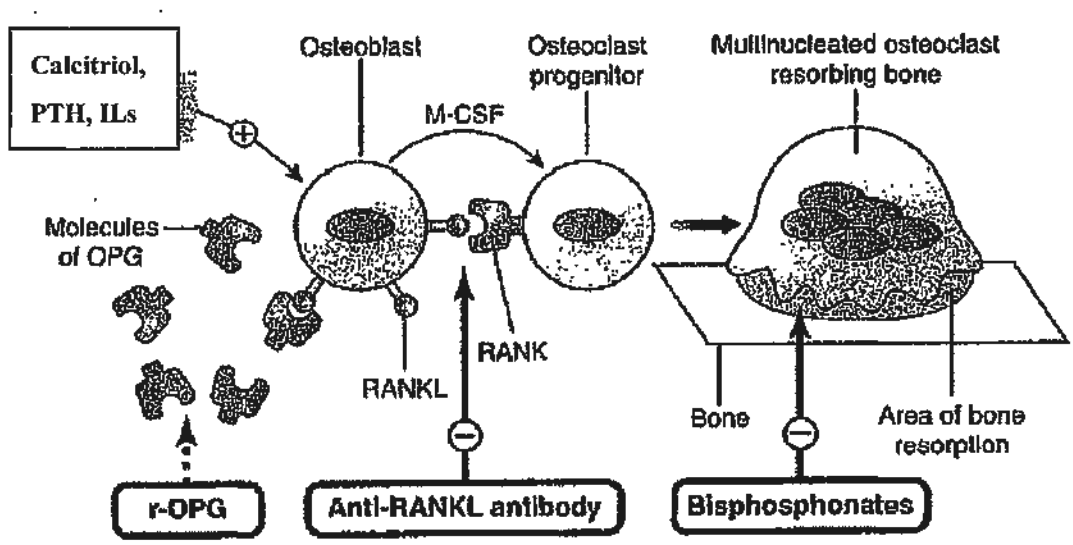


Figure 1-6 Osteoclastogenesis

Action of antiresorptive drugs: Bisphosphonates inhibit bone resorption by action on osteoclasts; . Anit-RANKL antibodies bind RANKL and prevent the RANK-RANKL interaction. The osteoblast also release 'decoy"molecules of osteoprotegerin (OPG), which can bind RANKL and prevent activation of the RANK receptor; Recombinant OPG (r-OPG) inhibits RANK-RANKL binding and interferes the differentiation and activation of OC

Abbreviations: PTH, parathyroid hormone; ILs, interleukins; RANKL, RANK ligand; RANK, receptor activator of nuclear factor Kappa B; M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin (Adopted from Rang, 1999).

1.7.2. Relationship of hormonal changes at menopause and bone loss

The mechanism(s) of the effects of estrogen on bone appears to be particularly complex as it involves the regulated production of cytokines from hematopoietic cells and bone cells and the responsiveness of stromal cells to these cytokines. In addition, the contribution of specific factors to postmenopausal bone loss appears to vary as the system adapts over time to the hormonal withdrawal.

However, some mechanisms of estrogen on bone remodeling have been revealed. As mentioned before, bone remodeling is coupled by hormones and cytokines. Cytokines are released during activation of the osteoblast and after bone resorption from the skeletal matrix.

In premenopausal women, estrogen activates the estrogen receptor in the osteoblast and then suppresses the release of cytokine-activating factors (**Table 1-4**). Conversely, withdrawal of estrogen triggers a response element in the cytokine receptor, which stimulates cytokine synthesis and release. The increased cytokine production stimulates osteoclastogenesis, which mediate osteoclast activation and result in bone loss during early postmenopausal period. However, some evidences revealed that the increase of some cytokine level is time-dependent. It suggests that accelerated bone turnover after estrogen deprivation is self-limited, and that bone loss later in life (beyond age 65 years) is a complex result of dietary intake (e.g. Calcium and vitamin D) and hormonal factors (e.g. parathyroid hormone) with postmenopausal osteoporosis (Rosen and Kessenich, 1997).

Table 1-4 Effects of cytokines on osteoclasts and its response to estrogen

Cytokine	Modulated by Estrogen	Effect on osteoclastogenesis	Effect on osteoclast activation
IL-1	++	+	++
IL-6	++	++	±
IL-11	++	++	±
CSF	++	++	-
TNF	++	+	++
TGF-beta	+	↓	↓
IGF-I, -II	+	+	-

Abbreviations: "+" = modest effect; "++ "= marked effect; "↓ " = suppression; "±" = not clear; "- "= slight effect; IL, interleukin; CSF, colony-stimulating factors; TNF, tumor necrosis factor; TGF, transforming growth factor; IGF, insulin like growth factor. (Rosen and Kessenich, 1997)

1.8. Current approach for osteoporosis

1.8.1. Current medication for osteoporosis

Currently, bisphosphonates (alendronate and risedronate), calcitonin, estrogens, parathyroid hormone and raloxifene are approved by the US Food and Drug Administration (FDA) for the prevention and/or treatment of osteoporosis. Bisphosphonates (alendronate and risedronate), calcitonin, estrogens and raloxifene affect the bone remodeling cycle and are classified as anti-resorptive medications. Anti-resorptive medications slow down or stop the bone-resorbing portion of the bone-remodeling cycle but they do not slow the bone-forming portion of the cycle. As a result, new formation continues at a greater rate than bone resorption, and bone density may increase over time. Teriparatide, a form of parathyroid hormone, is a newly approved osteoporosis medication. It is the first osteoporosis medication to increase the rate of bone formation in the bone remodeling cycle. It stimulates new bone formation and significantly increases bone mineral density (Information from website of the National Institute of Arthritis and Musculoskeletal and Skin Diseases).

1.8.2. Side effects of current medication

Estrogen replacement therapy is often unsuitable for many postmenopausal women, especially for those with chronic steroid administration. Significant evidences show that long-term estrogen replacement therapy increases the risk of breast cancer, endometrial cancer, endometriosis and venous thrombo-embolism (Information from Medline Plus under NIH).

Selective estrogen receptor modulators (SERMs), as an alternative to estrogen replacement therapy (Gennari et al., 2007; Haynes and Dowsett, 1999; Mitlak and Cohen,

1999; Thiebaud and Secrest, 2001), also have a concern over breast cancer.

Ralosifene, a newer SERMs, eliminates the concerns over endometrial stimulation that was not addressed by first generation SERMs (Mitlak and Cohen, 1999). However, women receiving ralosifene had increased risk of venous thromboembolus against placebo (Ettinger, 1999).

The main side effect of calcitriol (used in osteoporosis to suppress PTH secretion and reduce bone turnover) is hypercalcemia (Sambrook et al., 1993). In a study on the effect of PTH(1-34) on BMD and fractures, there were a greater number of withdrawals because of adverse events in the PTH(1-34) group compared to placebo (Neer et al., 2001). Nausea and headache were the commonest reported adverse events. Osteosarcoma has been found in rats given lifetime daily injections of PTH(1-34) and an increased cortical porosity has been reported.

In Hong Kong, prescription of bisphosphonate and SERMs are common for most postmenopausal osteoporosis women. However, around 60% of the cohort studies expressed their concern regarding the high cost that may impair patient's compliance for long term treatment. The annual cost amounted to US$460 and US$650 for bisphosphonate and SERMs therapy, respectively (Ip et al., 2004). This poses a very heavy burden on most retired elderly.

1.9. Traditional Chinese Medicine and osteoporosis

In search for safe and effective medicine, the public has increasingly turned their attention to complementary and alternative medicines (CAM). According to The National

Center for Complementary and Alternative Medicine NIH, 62% of adults in the United States were using some form of CAM, not most often (~11%) used to treat back pain or problems associated. Most elderly encounter not only osteoporosis, but also several health problems (e.g. hypertension, cardiovascular disease, and diabetes mellitus). They urge for an alternative treatment that can tonify their body condition to a harmonized stage. Approximately 1% of adult CAM users also utilize CAM to treat cholesterol, hypertension, and/or menopause (Information from the website of The National Center for Complementary and Alternative Medicine, NIH). In this respect, extensive experience has been accumulated in Traditional Chinese Medicine (TCM) regarding diagnosis and treatment of osteoporosis. TCM is considered as a potential candidate in treating osteoporosis (Chen et al., 1999; Ping, 2001; Wang et al., 2005a; Zhang et al., 1994).

1.9.1. TCM diagnosis in osteoporosis

"Osteoporosis" is a diagnostic term coined by modern medicine, it does not appear in the original texts of traditional Chinese Medicine (TCM). However, the clinical symptoms place osteoporosis in the categories of bone wilting (骨痿), joints pain and deformity (痹證), vacuity taxation (虛勞) or lumbar pain (腰痛) (Chen et al., 1999; Ping, 2001). According to TCM theory, the kidney system governs the bone, stores the qi (氣) and engenders bone and marrow. Vacuity in kidney system is highly correlated with the incidence of osteoporosis (Chen et al., 1999; Wang et al., 2005a). Consumption of kidney tonifying herbs or formulae can readjust the balance of bone remodeling process and hence reduce bone loss as described in section 1.9.2. Thus, a close relationship exists between the kidney system and bone as perceived by TCM theories.

In Traditional Chinese Medicine theory, diagnosis is given based on differential diagnosis

(辨證論治). One of the differential diagnosis methods is by visceral organs system analysis (臟腑辨證). It is believed that five visceral organ systems hold the key of the important functions of life, they are namely, Heart System, Liver System, Spleen System, Lung System and kidney system (Kong, 2005). *The Inner Canon* states "the kidney governs the bones and engenders marrow". The kidney system is therefore essential in the development of firm, strong bones in the youth whereas degeneration is reflected in the increasing bone brittleness in age (Wiseman and Ye, 1998). Some studies have shown that deficiency in kidney system results in a higher incidence of osteoporosis and/or a decreased bone mineral density (Chen et al., 1999; Wang et al., 2005a; Xu et al., 2005). However, limited information is available for quantitative determination of severity of deficiency in kidney system (Langevin et al., 2004; Lee et al., 2007), and further investigation of the correlation with the incidences of osteoporosis is warranted.

1.9.2. TCM treatment in osteoporosis

In Chinese community, kidney tonifying herbs are commonly used to treat bone fracture and prevent osteoporosis. Some scientific studies have been done to support the anti-osteoporotic effect of these herbs. Three commonly reported kidney-tonifying herbs are Fructus Psoraleae (補骨脂), Herba Epimedii (淫羊藿) and Fructus Ligustri Lucidi (女貞子). Fructus Psoraleae extract decreases urinary calcium excretion and serum osteocalcin in ovariectomized rats, resulting in positive effects on bone mineral density as well as bone formation (Tsai et al., 2007). Its extracts exhibit osteoblastic proliferation stimulating activity in osteoblastic-like cells cultured in vitro (Wang et al., 2001) Aqueous extract of Herba Epimedii increases serum alkaline phosphatase activity and prevents the increase in trabecular separation in ovariectomy-induced osteoporosis rats (Gau et al., 1999; Nian et al., 2006b). Its flavonoids extract could stimulate the proliferation and enhance the osteogenic

differentiation of marrow stromal cells (Lai et al., 2007). Fructus Ligustri Lucidi prevents OVX-induced high bone turnover rate by suppression of both serum osteocalcin and urinary deoxypyridinoline levels. In addition, its extract can prevent OVX-induced loss of calcium in rats by increasing the intestinal calcium absorption rate, suppressing urinary calcium excretion as well as increasing bone calcium content (Zhang et al., 2006b; Zhang et al., 2007a). It also improves bone properties of cortical and trabecular bone in aged rats possibly via its direct action on osteoblastic cells (Zhang et al., 2008). Other herbs like *Radix Rehmanniae* (熟地黃), it replenishes yin and benefit blood (滋陰補血) as stated in Pharmacopoeia of the People's Republic of China, 2000. Its extracts also exhibit antiosteoporotic effect by stimulating the proliferation and activities of osteoblasts, while inhibiting the generation and resorptive activities of osteoclasts (Oh et al., 2003). Although a few well-controlled scientific studies have confirmed the efficacy of some of the herbal therapies and some basic studies have been performed on various herbal components (active ingredients), less studies have assessed the composite effects of many herbal remedies. The assessment of herbal formulation is important as it is the most common and traditional form of intake by general TCM consumers. Moreover, most antiosteoporotic TCM formulas are investigated in case-study mode (Ding et al., 1995; Li et al., 2001; Liang et al., 1994; Mingyue et al., 2005; Shi et al., 1997; Xie et al., 1997; Zhang et al., 2004; Zhang et al., 2005; Zhao et al., 2003), a well designed research evaluating the clinical efficacy and mechanism of action of various TCM interventions for osteoporosis is still in great need. Therefore, a double blind, randomized, placebo-control clinical trial to evaluate the antiosteoporotic effect of TCM is expected to be a hot topic for researchers to work on.

1.10. Aim and scope of the Study

The overall objective of this study was to develop a kidney-tonifying herbal formula for

osteoporosis research (OPR), which is safe, effective and compatible to Hong Kong postmenopausal osteoporosis patients. The safety, efficacy and mechanism through *in vivo* and *in vitro* studies and pilot clinical studies are investigated. In order to achieve the goals, the research plan includes the following:

(1) Study on the association between postmenopausal osteoporosis and TCM Kidney-Vacuity Syndrome in order to formulate a kidney-tonifying herbal prescription for clinical evaluation.

(2) Development of a kidney-tonifying herbal formula for osteoporosis research (OPR).

(3) Evaluation of the safety of OPR by carrying out acute and 90 days oral toxicity studies on rats.

(4) Evaluation of the efficacy of OPR in osteoporosis animal model using C57BL/6 inbred mice. Bone mineral density and Biochemical markers of bone turnover would be employed as outcome parameters.

(5) Detection of extrogenous estrogen-like activities of OPR by measuring the MCF-7 estrogen response element-luciferase reporter.

(6) Study of the mechanism of OPR on osteoporosis by employment of osteoblast-like UMR-106 cells.

(7) Validation of the first Chinese osteoporosis-specific Quality of life (QoL) questionnaire, named "The Chinese Osteoporosis-Targeted Quality of Life Questionnaire" (The Chinese-OPTQoL).

(8) Analysis of the adverse events appeared in the pilot clinical study and investigation of similar adverse events involved in the use of TCM.

2. Association of the Incidence of Postmenopausal Osteoporosis and Kidney-Vacuity Syndromes

2.1. Introduction

In Traditional Chinese Medicine theory, kidney system is a key of healthy bone development as stated in *Inner Canon of the Yellow Emperor* written in the first century, AD. Many scientific studies have proven this relationship by showing a positive linkage between the deficiency in kidney system and higher incidence of low bone mass or even osteoporosis (Chen et al., 1999; Wang et al., 2005a; Xu et al., 2005). However, the TCM diagnosis is usually retained in a subjective manner.

In previous studies, TCM diagnoses are not standardized and tend to depend on individual TCM practitioner experiences, based on the "four diagnostic methods" (inspection, listening & smelling, inquiring and palpation), clinical judgment and intuition (Kaptchuk, 2000). Studies in which several practitioners examined the same subjects often showed considerable variability in diagnosis (Hogeboom et al., 2001; Zell et al., 2000). Therefore, TCM diagnoses have always been criticized for lacking objectivity and reliability.

In 2002, The Ministry of Health, People's Republic of China, released a document known as "Chinese Medicine New Drug Clinical Research Guidelines". Although the guideline has listed the diagnostic criteria of Kidney-Vacuity Syndromes, the diagnosis still depend on the experience of TCM practitioners and it will vary from one practitioner to the other. Besides, without systemic scoring, the degree of deficiency of kidney system cannot be revealed. Few published studies have tried to develop scoring diagnostic questionnaires to generate a TCM

diagnosis objectively (Langevin et al., 2004; Lee et al., 2007). Yet none of them were specially designed for evaluating Kidney-Vacuity Syndromes.

With the support of The National Natural Science Foundation of China, The DME (design, measurement and evaluation) Center of Guangzhou University of TCM has developed a series of syndrome diagnosis questionnaires, one of which is focused on kidney system (Lai et al., 2005). By adopting the kidney system questionnaire on osteoporosis patients, we may be able to quantify the degree of severity of Kidney-Vacuity Syndromes and correlate with the incidences of postmenopausal osteoporosis.

The aim of this study is to investigate the association between the incidence of postmenopausal osteoporosis and Kidney-Vacuity Syndromes (KVS), with the aid of a KVS scoring questionnaire.

2.2. Materials

The TCM KVS scoring questionnaire is designed to quantify the degree of severity of KVS of patients according to TCM theory. The questionnaire was kindly contributed by Professor S.L. Lai and his team at The DME Center of Guangzhou University of TCM. A sample of the questionnaire is shown in **Appendix I**.

2.3. Methods

With collaboration of the Kwong Wah Hospital, Tung Wah Group of Hospitals, and under the approval of the Clinical Research Ethics Committee of the Kowloon West Cluster,

seventy-three postmenopausal women were admitted into the study. They were aged 55 or above in age and were postmenopausal for at least two years. Secondary osteoporosis patients were excluded.

When patients were admitted in the study, TCM practitioner would help them to fill in the KVS scoring questionnaire. The questionnaire is interviewer-administered and consists of 98 questions. One question represents one kidney system related symptom (e.g. tinnitus 耳鳴, nocturia 夜尿). These symptoms are closely related to six KVS, they are "Deficiency of kidney qi (腎氣虛證)", "Deficiency of kidney yin (腎陰虛證)", "Deficiency of kidney yang (腎陽虛證)", "Deficiency of kidney yin and yang (腎陰陽兩虛證)", "Deficiency of kidney essence (腎精不足證)" and "Kidney failing to absorb qi (腎不納氣證)". We assigned codes to these syndromes (KVS 1 – 6) as shown in **Table 2-1** for easier referral in later paragraphs. During interview, TCM practitioner would ask the patient if they had these 98 symptoms or not. Practitioner recorded the symptoms of each patient and later used for the calculation of their KVS scores.

Table 2-1 Codes of 6 Kidney-Vacuity Syndromes

Code	6 Kidney-Vacuity Syndromes	
KVS1	Deficiency of kidney qi	腎氣虛證
KVS2	Deficiency of kidney yin	腎陰虛證
KVS3	Deficiency of kidney yang	腎陽虛證
KVS4	Deficiency of kidney yin and yang	腎陰陽兩虛證
KVS5	Deficiency of kidney essence	腎精不足證
KVS6	Kidney failing to absorb qi	腎不納氣證

Each symptom carries weighted indexes which contribute a mark to six KVS. The marks with decimals indicate the weighed indexes of a symptom contribute to six KVS.

These weighed indexes were determined by TCM experts from different professional areas during the formulation of the questionnaire (Lai et al., 2005). Take one of the 98 symptoms, tinnitus (耳鳴), as an example, it contributes marks of 9.06, 7.3, 9.01 and 5.19 to KVS1, KVS2, KVS4 and KVS5 respectively as shown in **Table 2-2.** A higher mark represents a higher possibility of having that particular KVS. Take nocturia (夜尿) as another example, it contributes marks of 8.28 and 5.85 to KVS3 and KVS4 respectively. If a patient had both tinnitus and nocturia, she would have six KVS scores by simple summation: KVS1 (9.06), KVS2 (7.3), KVS3 (8.28), KVS4 (14.86), KVS5 (5.19) and KVS6 (0). Same summations were carried out on 98 symptoms listed in the questionnaire. By simple addition, 6 KVS scores of individual patient can be derived. A higher score indicates a higher degree of vacuity in a particular category of KVS. By using this system, the degree of severity of six KVS of patients can be quantified with less bias.

Table 2-2 Calculation of six Kidney-Vacuity Syndromes scores

Symptoms		Six Kidney-Vacuity Syndromes (KVS) scores						
		KVS 1	KVS 2	KVS 3	KVS 4	KVS 5	KVS 6	
Tinnitus		9.06	7.3		9.01	5.19		} +
Nocturia				8.28	5.85			}
	=	9.06	7.3	8.28	14.86	5.19	0	

Bone mineral density (BMD) measurement was carried out by medical specialists using Dual X-ray Bone Densitometer (DEXA). Diagnosis of osteoporosis was confirmed by a physician when the patients' BMD values were at least -2.5 SD below the mean value of a young healthy population (i.e. T-score≤-2.5) according to World Health Organization guideline. The documentation of questionnaire was performed by a TCM practitioner blinded from BMD and osteoporosis results.

2.4. Statistical analysis

Statistical analyses were performed using the SPSS 10.0 statistics package (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SEM (Standard Error of Mean) unless otherwise noted. All variables were analyzed for difference between osteoporosis and non-osteoporosis subjects. All comparisons were made two-tailed, and P-values < 0.05 were considered to be statistically significant.

Independent samples t-test, logistic regression modeling and Fisher's exact test were used. Independent samples t-test was performed to compare the six KVS mean scores between the osteoporosis and non-osteoporosis groups. Logistic regression is a model used for prediction of the probability of occurrence of an event by fitting data to a logistic curve. It makes use of several predictor variables that may be either numerical or categorical. Logistic regression was used to investigate how the six KVS (predictor variables) determine the probability of the incidence of osteoporosis. Fisher's exact test is a statistical significance test used in the analysis of categorical data where sample sizes are small. It is used to examine the significance of the association between two variables in a 2 x 2 contingency table. The Fisher's exact test was applied to investigate the association between 98 kidney system symptoms and the incidence of osteoporosis.

2.5. Results

2.5.1. Demographic data

The demographic data of osteoporosis and non-osteoporosis subjects are summarized in

Table 2-3. Seventy-three menopausal women aged between 58 and 87 (mean: 70 ± 6.19) were enrolled. Forty-one women were diagnosed as osteoporosis (with BMD 2.5 standard deviations below the healthy youths of same sex, according to WHO guideline). Both the mean age of non-osteoporosis and osteoporosis subjects were 70 years old. Non-osteoporosis subjects were significantly heavier (P=0.002) and slightly taller (P=0.058) than the osteoporosis subjects. More than a half of the subjects in both groups delivered three babies or more and both groups had health problems such as hypertension, diabetes mellitus and cardiovascular disease, with similar rate of incidence.

Table 2-3 Demographic data

	Non-osteoporosis	Osteoporosis	P-value^
Sample size	32	41	
Age (year)	70.03±1.049	70.59±1.007	0.707
Body weight (kg)	61.99±1.870	54.57±1.370	0.002*
Body height (cm)	152.19±1.228	149.56±0.730	0.058
With hypertension	15	21	0.815
With diabetic mellitus	4	7	0.746
With cardiovascular disease	1	5	0.221
Number of child(ren) delivered ≥3	19	27	0.63

Total n=73, *P<0.05, ^ by t-test

Bone mineral density (BMD) of the subjects in the osteoporosis and non-osteoporosis groups were expressed in T-score and shown in **Table 2-4** and **Figure 2-1**. BMD of the osteoporosis group was much lower than that of non-osteoporosis group as expected. Both lumbar and femur mean BMD values of the non-osteoporosis group were less than the golden standard of osteoporosis (-2.5 SD from normal youth). For the osteoporosis group, the lumbar mean BMD fulfilled osteoporosis criteria but not at the proximal femur.

Table 2-4 Comparison of bone mineral density expressed in T-score

	Non-osteoporosis	Osteoporosis
Sample size	32	41
Lumbar bone mineral density	-0.73±0.17	-3.03±0.15
Proximal femur bone mineral density	-0.61±0.15	-1.79±0.13

Total n=73

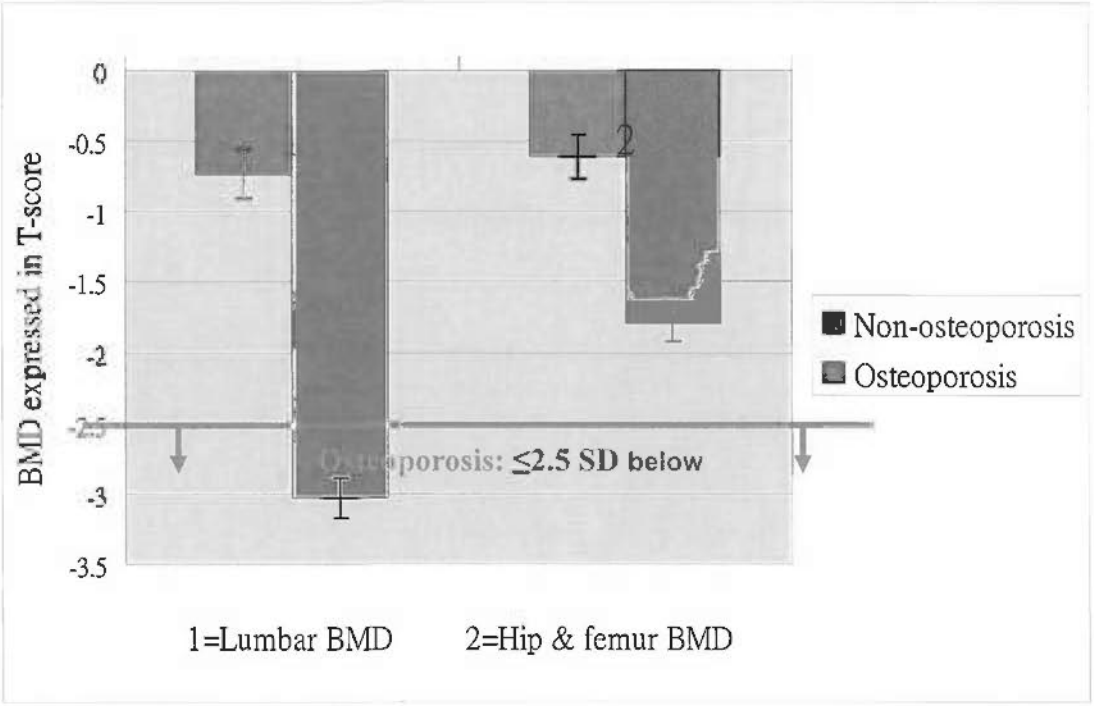


Figure 2-1 Bone mineral density expressed in T-score

2.5.2. Association of six Kidney-Vacuity Syndromes with osteoporosis

Independent samples t-test was performed to compare the six KVS mean scores between the osteoporosis and non-osteoporosis groups. Data are summarized in **Table 2-5** and **Figure**

2-2. Mean KVS scores of the osteoporosis subjects were generally higher than those of the non-osteoporosis subjects in all six Kidney-Vacuity Syndrome. It indicated that osteoporosis group had a higher deficiency in kidney system when compared with the non-osteoporosis group. Score difference between osteoporosis and non-osteoporosis group was largest in the syndrome "Kidney failing to absorb qi", and then followed by "Deficiency of Kidney qi", "Deficiency of Kidney essence", "Deficiency of Kidney yin & yan", "Deficiency of Kidney yin" and the smallest difference was observed in the syndrome "Deficiency of Kidney yin". Osteoporosis group had significantly higher scores in three syndromes namely, "Deficiency of kidney qi (腎氣虛證)" (P= 0.017), "Deficiency of kidney essence (腎精不足證)" (P= 0.012) and "Kidney failing to absorb qi (腎不納氣證)" (P= 0.02) when compared with the non-osteoporosis group. It indicated that subjects with higher deficiency in these three syndromes would have a higher probability of osteoporosis.

Table 2-5 Association of six Kidney-Vacuity Syndromes with osteoporosis

Code	6 Kidney-Vacuity Syndromes		Non-osteoporosis (n=32)	Osteoporosis (n-=41)	Mean Score difference	P-value^	P-value#
KVS1	Deficiency of kidney qi	腎氣虛証	28.77±2.34	37.88±2.9	9.11	0.017*	0.892
KVS2	Deficiency of kidney yin	腎陰虛証	18.35±2.39	24.45±2.13	6.1	0.06	0.913
KVS3	Deficiency of kidney yang	腎陽虛証	18.19±2.22	23.50±2.32	5.31	0.10	0.943
KVS4	Deficiency of kidney yin and yang	腎陰陽兩虛証	22.27±2.35	28.56±2.3	6.29	0.06	0.851
KVS5	Deficiency of kidney essence	腎精不足証	28.00±1.99	34.91±1.79	6.91	0.012*	0.194
KVS6	Kidney failing to absorb qi	腎不納氣証	12.83±2.09	22.81±3.63	9.98	0.02*	0.319

Total n=73, *P<0.05. ^ by independent-sample t-test, # by logistic regression

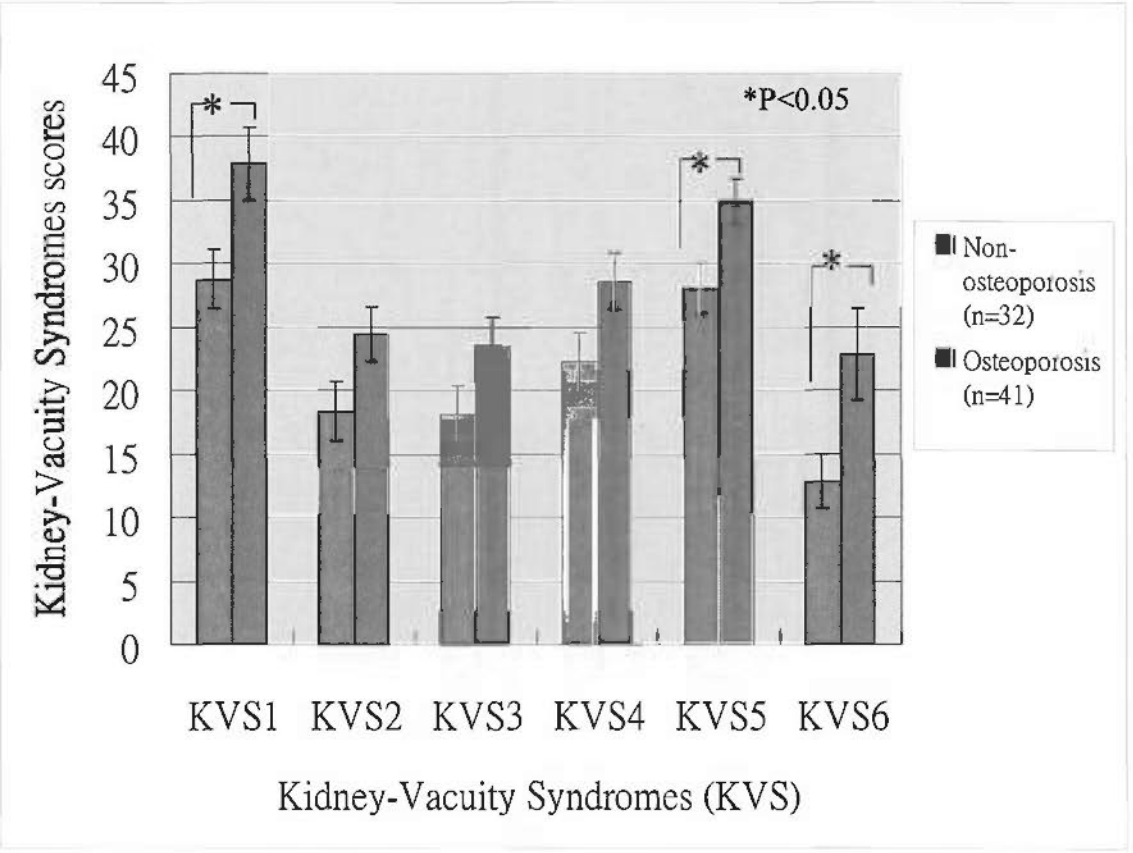


Figure 2-2 Association of six Kidney-Vacuity Syndromes with osteoporosis

Abbreviation: KV1= Deficiency of kidney qi; KV2= Deficiency of kidney yin; KV3= Deficiency of kidney yang; KV4= Deficiency of kidney yin and yang; KV5= Deficiency of kidney essence; KV6= Kidney failing to absorb qi.

After performing t-test, we further performed another statistic analysis, logistic regression, on six KVS scores with the incidence of osteoporosis. Logistic regression is a model used for prediction of the probability of occurrence of an event by fitting data to a logistic curve. It makes use of several predictor variables and is able to show the inter-relationship of multi-variables on the occurrence of an event. We performed logistic regression model to investigate how the six KVS (predictor variables) determined the probability of the incidence of osteoporosis (occurrence of an event)

Logistic regression model was performed to investigate how the six KVS predicted the probability of osteoporosis and result is shown in **Table 2-5** (P-value marked with #). Analysis showed that none of the six KVS is a determining factor to predict the probability of osteoporosis.

2.5.3. Association of ninety-eight kidney system symptoms with osteoporosis

We performed Fisher's exact test to investigate the association between 98 kidney system symptoms and the incidence of osteoporosis. Among the 98 kidney system symptoms, nine symptoms showed significant association with the incidence of osteoporosis. Results are shown in **Table 2-6**.

Table 2-6 Association of ninety-eight kidney system symptoms with osteoporosis

Symptoms	Non-osteoporosis (n=32)	Osteoporosis (n=41)	P-value^
起病緩慢	7	24	0.002*
喘促氣不接續	0	7	0.016*
視物黑花飛舞	10	25	0.018*
發病年齡中老年人	9	23	0.020*
平素體質偏虛	4	15	0.030*
面部烘熱	0	6	0.032*
舌苔乾	10	24	0.033*
短氣	1	9	0.036*
胸悶	3	12	0.045*
耳鳴	4	14	0.054
智能減退	9	21	0.058
體形偏廋	9	21	0.058

Total n=73, *P<0.05

^ by Fisher's exact test

2.6. Discussion and conclusions

Kidney system governs the bone health as stated in *Inner Canon*. In this study, significant association was observed between TCM KVS and a common bone disease - postmenopausal osteoporosis. Postmenopausal women, who were insufficient in kidney qi and essence, had a higher probability of osteoporosis. It was very important as TCM KVS might serve as diagnostic indications for osteoporosis.

In this study, we utilized the TCM KVS scoring questionnaire to quantify the degree of severity of six KVS. Demographic data showed that osteoporosis and non-osteoporosis subjects were at similar age and health status except bone aspect. This can minimize the effects of other health problems to interfere the incidence of osteoporosis. Non-osteoporosis subjects were heavier than osteoporosis subjects as they are determining factors in occurrence of the disease (Mascarenhas et al., 2003; Newberry et al., 1990).

Six KVS scores were not predicting factors in occurrence of osteoporosis in multivariate analysis may due to two reasons. Ninety-eight TCM kidney system symptoms were shared by six KVS in the questionnaire. Composition of individual KVS had more or less parallel symptoms with another (i.e. one symptom might both appear in two or more KVS. Overlapping symptoms diminished the distinctive value of each KVS in determining the occurrence of osteoporosis. Moreover, some KVS were similar to the other (e.g. "Deficiency of kidney qi 腎氣虛證" and "Kidney failing to absorb qi 腎不納氣證"), it further blurred the boundaries among six KVS.

To bypass the two reasons mentioned above, we turned round to investigate the 98 kidney system symptoms, which served as raw data in the calculation of KVS scores. Nine

symptoms significantly appeared more frequently in osteoporosis patients, or in other word, they were less frequently seen in non-osteoporosis patients. The nine symptoms that showed significant association with osteoporosis may serve as useful markers in osteoporosis screening. Among these nine symptoms, some were very distinctive, like hot flashes (面部烘熱). It only appeared in KVS2 "Deficiency of kidney yin (腎陰虛證)" but not shared by other KVS. However, KVS2 could not stand out in performing t-test and was not significantly associated with the occurrence of osteoporosis. It further verified our assumption that marks contributed by symptoms to KVS scores cannot reflect its weight properly.

Some of these TCM symptoms were not generally included in western medicine history taking (e.g.喘促氣不接續), but they had diagnostic values for osteoporosis. Some TCM kidney symptoms were similar to musculoskeletal (e.g. 腰痛、腰痠、骨骼痿軟), cognitive (e.g. 智能減退、健忘) and general weakness (e.g. 短氣、喘促氣不接續) symptoms in western medicine, which when occur, might be indicative of a need to screen for osteoporosis. Integrative TCM and Western medicine diagnostic approach opens a new horizon in the holistic understanding of osteoporosis.

In conclusion, normalizing and evaluating the kidney qi and kidney essence state might be useful in treating and monitoring the progress of osteoporosis. Replenishing kidney qi and kidney essence is a logical therapeutic principle in the treatment of osteoporosis with TCM.

3. A Kidney-Tonifying Herbal Formula for Osteoporosis Research

3.1. Rationale

A kidney-tonifying herbal formula for osteoporosis research (OPR) was formulated according to information from three aspects: Firstly, extensive literature reviews suggested that Fructus Psoraleae, Herba Epimedii and Fructus Ligustri Lucidi are three kidney-tonifying herbs that show antiosteoporotic effect (Lai et al., 2007; Nian et al., 2006b; Tsai et al., 2007; Zhang et al., 2006b; Zhang et al., 2007a; Zhang et al., 2008) as mentioned in Section **1.9.2**. Therefore, these three herbs became the major components of OPR. Secondly, according to the study of the association of postmenopausal osteoporosis and KVS in TCM as described in previous Chapter, postmenopausal women who were insufficient in kidney qi (氣) and essence (精) would have a higher probability of osteoporosis. Herbs which can replenish kidney qi and essence, were chosen to be part of the OPR components. Details of the herbs' nature and function used in OPR are listed in **Appendix II**. Finally, OPR is an innovative formula developed by the collaboration of TCM experts and clinical physician in School of Chinese Medicine, The Chinese University of Hong Kong and the Kwong Wah Hospital. The interpretation of ten herbs used in OPR is stated in **Appendix III** according to TCM theory. Based on the information from literature reviews and experts' opinions, and taking into consideration results of the linkage between kidney system and osteoporosis, OPR was formulated with ten component herbs as shown in **Table 3-1**.

Table 3-1 Composition of OPR

Pharmaceutical name	Chinese Name	Quantity (g)
Fructus Psoraleae	補骨脂	15
Herba Epimedii	淫羊藿	15
Radix Rehmanniae Preparata	熟地黃	25
Fructus Corni	山茱萸	12
Rhizoma Dioscoreae	山藥	15
Carapax Et Plastrum Testudinis	龜甲	30
Fructus Ligustri Lucidi	女貞子	15
Rhizoma Polygonati	黃精	15
Rhizoma Atractylodis Macrocephalae	白术	12
Cortex Moutan	牡丹皮	12

3.2. Safety information

Safety information and Lethal Dose, 50% (LD_{50}), of ten herbs in OPR from previous published studies (Chen et al., 2001; Huang, 1994; Li, 2004; Yao et al., 1997a; Yao et al., 1997b; Zhang, 2003; Zhang, 2004; Zhang and Ping, 2002) are shown in **Table 3-2**. Dosages of ten herbs used in OPR are far smaller than the LD_{50} values.

Table 3-2 Comparison of dosage used in OPR with LD_{50}

Pharmaceutical name	Chinese name	Quantity of OPR		LD_{50} in animal	Dose/LD_{50}
		Human dose (g/70kg/d)	Animal dose		
Fructus Psoraleae	補骨脂	15	0.0375g/20g/d (mice)	0.754 g/20g/d (mice)	1/20
Herba Epimedii	淫羊藿	15	0.0375g/20g/d (mice)	0.72 g/20g/d (ip)	1/20
Radix Rehmanniae Preparata	熟地黃	25	0.0625g/20g/d (mice)	1.2 g/20g/d (mice)	1/20
Fructus Corni	山茱萸	12	0.03g/20g/d (mice)	2 g/20g/d (mice)	1/66
Rhizoma Dioscoreae	山藥	15	0.0375g/20g/d (mice)	-	-
Carapax Et Plastrum Testudinis	龜甲	30	1.125g/20g/d (mice)	-	-
Fructus Ligustri Lucidi	女貞子	15	1.05g/1.5kg/d (rabbits)	75 g/1.5kg/d (rabbits)	1/70
Rhizoma Polygonati	黃精	15	0.0375g/20g/d (mice)	9 g/20g/d (mice)	1/240
Rhizoma Atractylodis Macrocephalae	白术	12	0.03g/20g/d (mice)	0.266 g/20g/d (mice)	1/9
Cortex Moutan	牡丹皮	12	0.03g/20g/d (mice)	-	-

Reference: 《常用中藥成分與藥理手冊》，中國醫藥科技出版社 (1994)

3.3. Preparation of OPR

The Eu Yan Sang (Hong Kong) Ltd. was responsible for the preparation of OPR granules and quality assurances and safety tests on both the raw materials and granules. Chemical analysis of OPR decoction was performed at the School of Chinese Medicine, The Chinese University of Hong Kong.

3.3.1. Raw materials of OPR

Raw materials were authenticated by Eu Yan Sang (Hong Kong) Ltd. according to the methods described in the Pharmacopoeia of PRC (The State Pharmacopoeia Commission of the P.R.China, 2000). Fructus Psoraleae (補骨脂), Fructus Ligustri Lucidi (女貞子) and Cortex Moutan (牡丹皮) were further confirmed by Thin Layer Chromatography (TLC) identification of marker compounds. Marker compounds, i.e. psoralen and isopsoralen in Fructus Psoraleae were identified by TLC method as showed in **Figure 3-1**. Oleanolic acid in Fructus Ligustri Lucidi and paeonol in Cortex Moutan were identified and the TLC patterns are shown in **Figure 3-2** and **Figure 3-3**, respectively. TLC results showed that three herbs fulfilled authentication requirements of the Pharmacopoeia of PRC.

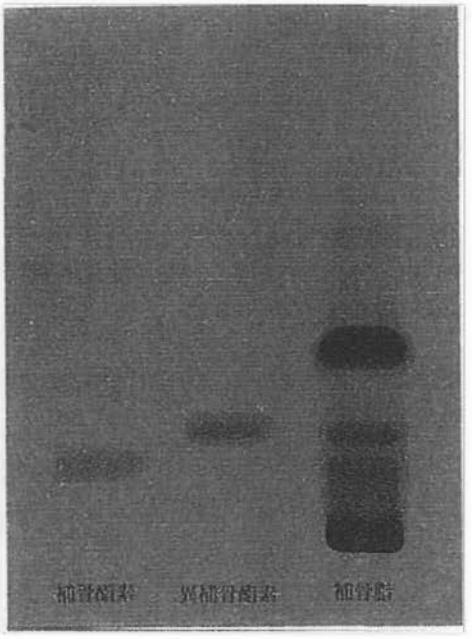


Figure 3-1 Thin layer chromatography identification of Fructus Psoraleae.

From left to right: Standard marker psoralen, Standard marker isopsoralen and sample of Fructus Psoraleae

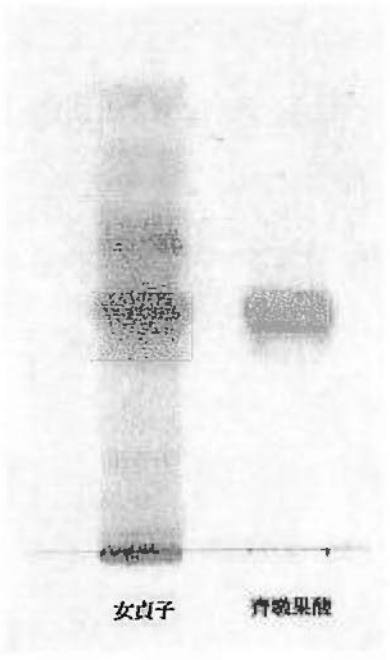


Figure 3-2 Thin layer chromatography identification of Fructus Ligustri Lucidi.

From left to right: Sample of Fructus Ligustri Lucidi, Standard marker Oleanolic acid.

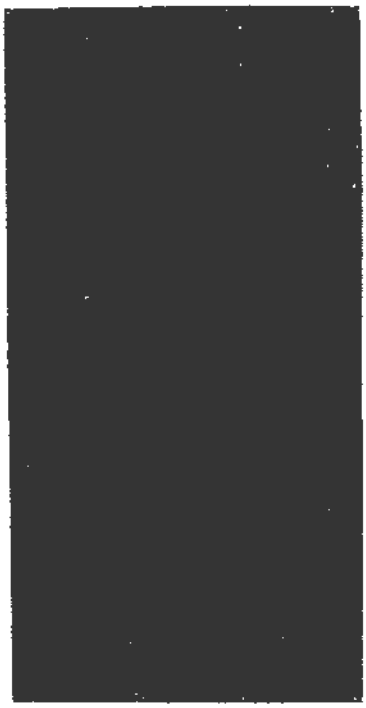


Figure 3-3 Thin layer chromatography identification of Cortex Moutan.

From left to right: Sample of Cortex Moutan, Standard marker paeonol.

3.3.2. Decoction of OPR

Samples of crude drugs were obtained from Eu Yan Sang (Hong Kong) Ltd. Chemical analysis of OPR decoction was completed at the School of Chinese Medicine, The Chinese University, using High Performance Liquid Chromatographic system (HPLC) - Mass Spectrometer (MS).

3.3.2.1. Sampling of Crude Drugs

3.3.2.1.1. Methods

Before sampling, labeling details and package outlook were recorded. Details of

labeling include: Chinese and Pharmaceutical name (品名), Source (產地), Net weight (淨重), Quality level (等級), Form of package (包件式樣，如紙盒、塑膠袋、麻包袋); Details of package include: Intactness (完整性), Cleanliness (清潔程度), Contamination of moisture or moulds (有無水跡、霉變), Contamination of foreign matters (有無其他物質污染). During sampling, one hundred to three hundred grams of samples were collected from the same lot of herbs as shown in **Table 3-3.**

Table 3-3 Quantity of samples collected for each herb

Pharmaceutical name	Chinese Name	Quantity (g)
Fructus Psoraleae	補骨脂	100
Herba Epimedii	淫羊藿	100
Radix Rehmanniae Preparata	熟地黃	300
Fructus Corni	山茱萸	100
Rhizoma Dioscoreae	山藥	100
Carapax Et Plastrum Testudinis	龜甲	300
Fructus Ligustri Lucidi	女貞子	100
Rhizoma Polygonati	黃精	100
Rhizoma Atractylodis Macrocephalae	白术	100
Cortex Moutan	牡丹皮	100

3.3.2.1.2. Results

Herbs were imported from different provinces of mainland China with a shelf life of two years. They were packed in white nylon bags as outer and transparent plastic bags as inner

package. Packages were intact, clean and without contamination. Herbs were stored in dry cool area, however, humidity and temperature were not recorded. To note that some herbs had been redistributed into smaller package and the original labeling were missing, they are marked as "-" in the **Table 3-4**.

Table 3-4 Summary of crude drug sampling

Pharmaceutical name	Chinese name	Source	Net weight (kg/pack)	Manufacture date	Lot number	Self-life (years)
Fructus Psoraleae	補骨脂	-	-	-	-	-
Herba Epimedii	淫羊藿	-	-	-	-	-
Radix Rehmanniae Preparata	熟地黃	河南	10	5-Aug-02	2.01E+08	2
Fructus Corni	山茱萸	浙江	10	5-Jul-13	2.01E+08	2
Rhizoma Dioscoreae	山藥	-	-	-	-	-
Carapax Et Plastrum Testudinis	龜甲	-	-	-	-	-
Fructus Ligustri Lucidi	女貞子	浙江	10	5-Aug-03	2.01E+08	2
Rhizoma Polygonati	黃精	-	-	-	-	-
Rhizoma Atractylodis Macrocephalae	白术	浙江	10	5-Aug-06	2.01E+08	2
Cortex Moutan	牡丹皮	安徽	9.6	5-Aug-04	2.01E+08	2

3.3.2.2. Establishment of OPR fingerprint as an index for quality control

3.3.2.2.1. Chemicals and reagents

Ethanol (AR grade) and acetonitrile (HPLC grade) were purchased from BDH Laboratory Supplies (Spoole, England). Reference compounds of psoralen, isopsoralen, Icarrin, paeoniflorin, ursolic acid and paeonol were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

3.3.2.2.2. Preparation of herbal decoction

Herbal samples were supplied by Eu Yan Sang (Hong Kong) Ltd, powdered, and mixed in ratios as shown in **Table 3-1.** One-hundred grams of herbal mixture were boiled in 600 ml of water for two hours. The extract was filtered and the herbal residue was extracted again with 300 ml of water for one hour. The extracts were combined and concentrated to 200 ml. Three-hundred ml of ethanol was added into the concentrated extract and allowed to stand for 24 hours. The extract was filtered and analysed by HPLC and MS.

3.3.2.2.3. Instrumentation

3.3.2.2.3.1. High Performance Liquid Chromatographic system

An Agilent 1100 series chromatographic system consisted of a vacuum degaser, a binary pump, an auto-sampler, a column oven and a Diode Array Detector (DAD). Chromatographic separation was achieved on an Alltech Previa C18 column with an inner diameter of 4.6 mm, 250 mm in length, and 5 micron particle size, at 25°C. The mobile phase consisted of water (A) and acetontrile (B) using a gradient elution of 0%-80% (B) in 0-60 min at 0.7 ml/min. Samples were injected by an auto-sampler. The injection volume of samples was 10 µl. HPLC chromatograms were monitored with DAD at 210 nm, 230 nm, 254 nm and 270 nm.

3.3.2.2.3.2. Mass Spectrometer

The HPLC system was coupled to Agilent MSD/Trap SL through a Brucker APCI

interface. Temperatures of dry gas and vaporizer were set at 300°C and 400°C, respectively. The nebulizer gas pressure was set at 70 psi and the dry gas flow rate set at 6 L / min. To increase sensitivity of detector, the HPLC system flow was directly connected to the MS without stream splitting. APCI-MS spectra were collected over a scan range of *m/z* 50-800 in positive mode.

3.3.2.2.4. Results

There were 31 peaks found in the chromatograms of OPR decoction as detected at 210 nm, 230 nm, 254 nm and 270nm (**Figure 3-4**). The peaks were identified by comparison of the UV and MS spectra with literature data. Components found in the decoction and the contribution herbs are summarized in **Table 3-5**. Peaks 1 › 20 › 24 › 28 › 29 and 30 were identified to be ursolic acid, paeoniflorin, icariin, psoralen, isopsoralen and paeonol.

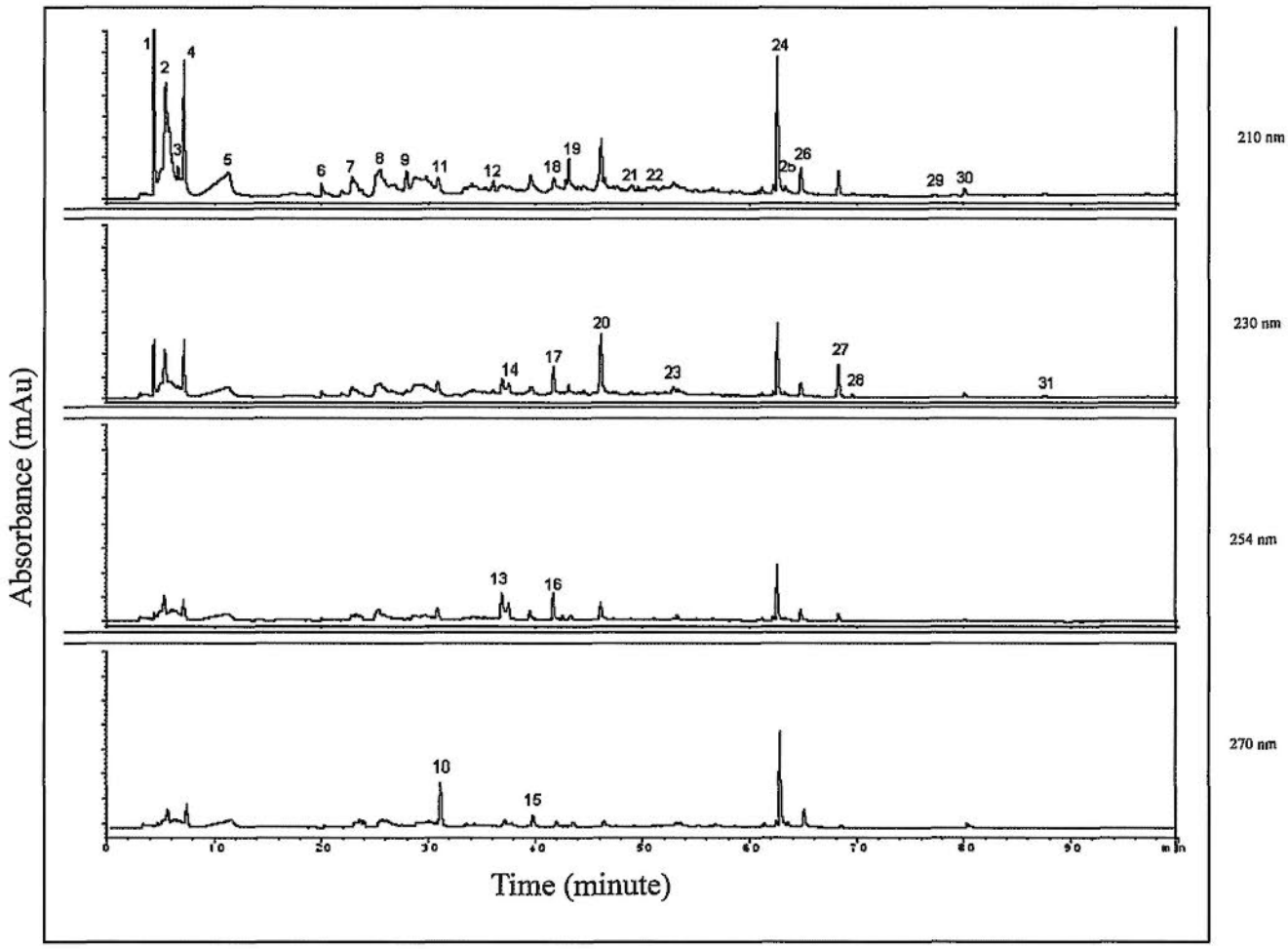


Figure 3-4 High performance liquid chromatography chromatograms of OPR decoction

Table 3-5 Components in decoction and their contributing herbs

Pharmaceutical name	Chinese Name	Peak number
Fructus Psoraleae	補骨脂	27，28，29
Herba Epimedii	淫羊藿	6，16，17， 24，25，26
Radix Rehmanniae Preparata	熟地黃	7，9，11
Fructus Corni	山茱萸	1，5，12，19，20
Rhizoma Dioscoreae	山藥	8
Carapax Et Plastrum Testudinis	龜甲	7，8
Fructus Ligustri Lucidi	女貞子	21，22，23
Rhizoma Polygonati	黃精	3，4，10
Rhizoma Atractylodis Macrocephalae	白术	2，15，31
Cortex Moutan	牡丹皮	13，14，18，20，30

3.3.3. OPR granules

3.3.3.1. Quality control

The extraction rate and yield of granule were 22.41% and 90% respectively. The granule product was sealed in aluminum bag and the packing size was 28 g ± 5%. To ensure the quality, the physical characteristics, moisture content and identification of marker compounds in the granules were carried out by Eu Yan Sang Research and Analysis Laboratory. Results showed that OPR granules passed in all tests. The results are shown in **Table 3-6**.

Table 3-6 Quality test results of OPR granules

Test items	Results
Characteristics	Brown small granules
Moisture content	1.99%
Identification of Oleanolic acid	Present
Identification of Paeonol	Present

3.3.3.2. Safety tests

To ensure safety, a series of tests for heavy metals, microbial counts, organochlorine pesticides residues and acute toxicity were carried out by the CMA testing and Certification Laboratories. Results showed that the OPR granule passed the permitted level of all tests. The product therefore complied with the Technical Guidelines for Tests on the Safety of Proprietary Chinese Medicines (pCm), sanctioned by the Chinese Medicine Council of Hong Kong. Therefore, OPR granules were safe for oral intake under TCM practitioners' prescription. The report from CMA testing and Certification Laboratories is shown in **Appendix IV**.

3.3.3.3. Acute toxicity test

The acute toxicity test was carried out by CMA testing and Certification Laboratories. Results showed that the maximum tolerable dose (MTD) of OPR granules on mice was 120g/kg, which was equivalent to 120 times of clinical dose. Details of acute toxicity test is shown in **Appendix V**.

3.3.4. Conclusions

OPR was established according to a logical therapeutic principle as revealed in Chapter 2, and its formulation was also supported by extensive literature reviews and experts' opinions. Quality control was executed throughout the manufacturing process from raw materials, decoction to granules production of OPR. For the raw materials, crude drugs were authenticated according to the methods described in the Pharmacopoeia of PRC. Three out of tem herbs in OPR formula: Fructus Psoraleae (補骨脂), Fructus Ligustri Lucidi (女貞子) and Cortex Moutan (牡丹皮) were further confirmed by Thin Layer Chromatography (TLC) identification of marker compounds. For the OPR decoction, an OPR fingerprint was established using High Performance Liquid Chromatographic system (HPLC) - Mass Spectrometer (MS), which served as an index in quality control during production process. To ensure safety of the OPR granules, a series of tests for heavy metals, microbial counts, organochlorine pesticides residues and acute toxicity were carried out. Results showed that the OPR granules passed the permitted level of all tests. Therefore, OPR granules were safe for oral intake and were used in later *in-vitro*, *in-vivo* and clinical studies.

4. Effects of a Kidney-Tonifing Herbal Formula on Osteoblastic Cell Line, UMR 106

4.1. Introduction

Osteoblast proliferation and differentiation are crucial in bone remodeling cycle as they dominate in bone formation process and indirectly alter bone resorption process. UMR 106 cell line is a clonal derivative of a transplantable rat osteosarcoma, which expresses many phenotypic characteristics of normal osteoblast and becomes a surrogate of it.

UMR 106 cells have been widely used to screen for the anti-osteoporotic action of various interventions. Fructus Ligustri Lucidi, one of the components of OPR, has been shown to increase osteoblastic differentiation in rat osteoblast-like UMR-106 cells by enhancing the mineralization process of the cells (Zhang et al., 2008). UMR 106 cells were also used to determine the potential mechanism of the anti-osteoporotic action of various herbs. Herbal Ramulus Sambuci Williamsii (接骨木) inhibits osteoclastogenesis by modulating the expression of osteoprotegrin (OPG) and receptor activator of NF-kappaB ligand (RANKL) mRNA in osteoblastic UMR 106 cells. Therefore, its protection effect on bone was mediated by the decrease in osteoclastogenesis (Xie et al., 2005b). Another medication amine-carboxyboranes have been shown to prevent osteoporosis and loss of bone mass in rodents. Its derivatives significantly reduce the loss of intracellular calcium from UMR 106 cells. In another word, calcium incorporation into these cells was accelerated with the presence of amine carboxyboranes (Rajendran et al., 1995). From these studies, osteoblastic-like UMR 106 cells will prove to be a mature in vitro model to screen for anti-osteoporotic action of various interventions and it was also used to investigate part of the

action mechanism.

In the present study, the effects of a kidney tonifing herbal formula on osteoblast-like cells were studied for cell proliferation (Cell Proliferation Assay) and differentiation (Alkaline Phosphatase Activity). Protocol of culturing and treatment of UMR 106 cells is shown in **Table 4-1.**

4.2. Materials

4.2.1. Cell line

UMR 106 cell line from American Type Culture Collection (ATCC) was kindly provided by the Institute of Chinese Medicine, The Chinese University of Hong Kong.

4.2.2. Culture medium

Powder of Dulbecco's Modified Eagle Medium (DMEM) with high glucose was obtained from Invitrogen (California, USA). The powder (13.4 g) was reconstituted in 1 L distilled water and buffered with 3.7 g/L sodium bicarbonate (Sigma, Missouri, USA). The pH value was adjusted to 7.2 by 1M NaOH or 1M HCl with the aid of pH meter (SCHOTT Instruments, UK). Medium was sterilized by filtration through a 0.22 μm vacuum driven bottle top filter (Corning, NY, USA) under aseptic condition. The pH value will rise 0.1-0.3 after filtration and become the final desired working pH of 7.4. DMEM was supplemented with 1% of Penicillin (100 U/ml) and Streptomycin (100 mg/ml) obtained from Invitrogen and was kept at 4°C for future use. Five percent of fetal bovine serum (Invitrogen) was added in DMEM just before use.

Table 4-1 Protocol of culturing and treatment of UMR 106 cell line

Culturing medium	1)Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin 100 U/ml and streptomycin 100 mg/ml (1%P/S) 2)Phenol red free DMEM supplemented with 2% Charcoal/Dextran Treated Fetal Bovine Serum (DCC-FBS) and 1%P/S	
Sub-culture	Subcultivation ratio of 1:4 to 1:8 Typsinization: Rinse with Hanks' Balanced Salts Solution (HBSS) twice and add in 1 ml trypsin and incubate at 37℃ for 3 mins	
Assay kits	CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) Alkaline Phosphatase LiquiColor® (Stanbio) Protein assay kit (Bio-Rad Laboratories)	
Day 1	Seed cells	Seed cells on microtiter plate for 24hr:
Day 2	Switch medium	Switch culture medium to non-phenol red DMEM with 2% Charcoal/Dextran Treated FBS for another 24 h.
Day 3	Treatment	Treat with OPR solution at 1, 10, 100, 1000 μg/ ml for 24 h.
Day 4	Collect cells	Perform different assay protocols

Another culture medium used was DMEM with high glucose but without phenol red (Invitrogen). It is a ready-to-use medium without the process of dissolution and sterilization as mentioned above. It cultures cells directly with a supplement of 1% Penicillin and Streptomycin and 2% Charcoal/Dextran Treated fetal bovine serum purchased from HyClone (Logan, USA.).

4.2.3. Wash buffer

Hanks' Balanced Salt Solution (HBSS) powder was purchased from Sigma (Missouri, USA). The powder (9.50 g) was reconstituted in 1 L distilled water and buffered with 0.35 g/L sodium bicarbonate (Sigma) with a pH value adjusted to 7.4. The pH value adjustment and filtration steps are mentioned before in medium preparation. HBSS wash buffer was kept at 4°C for future use.

4.2.4. Assay kits

CellTiter 96® AQueous One Solution Cell Proliferation Assay is a commercial kit obtained from Promega (Madison, USA). It determines the number of viable cells in proliferation by colorimetric method. The kit reagent is composed of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt] and PMS (phenazine methosulfate). MTS is bioreduced by the cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan product at 490 nm can be measured directly. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product is quantified by measuring the absorbance at

490 nm, which is proportional to the number of living cells in culture. . The cell proliferation assay is also known as MTS assay.

Alkaline Phosphatase LiquiColor® was obtained from Stanbio (Boerne, USA). It is designed for the kinetic quantitative determination of Alkaline phosphatase. Alkaline phosphatase in cell lysate hydrolyzes 4-nitrophenyl phosphate (in working reagent) to form nitrophenol and phosphates. 4-nitrophenol is yellow in color, at pH 10.4 with an absorbance peak at 405 nm. The formation rate of 4-nitrophenol is directly proportional to alkaline phosphatase activity.

Bio-Rad Protein assay kit was obtained from Bio-Rad Laboratories (California, USA). It determines the concentration of solubilized protein based on the method of Bradford (www.bio-rad.com). It involves the addition of an acidic dye to protein solution, and subsequent measurement of absorbance at a wavelength of 595 nm. Comparison to a standard curve provides a relative measurement of protein concentration.

Other materials include Trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) and Trypan blue solution. They were obtained from Invitrogen.

4.3. Methods

4.3.1. Cell culture

Propagation: UMR 106 cells were cultured in a culture flask with Dulbecco's Modified Eagle Medium (with phenol red), which was supplemented with 5% Fetal Bovine Serum and

1% Penicillin and Streptomycin. The cells were kept in a humidified 5% CO_2 atmosphere with a temperature at 37°C. The medium was renewed 2-3 times per week and sub-cultured up to 80-90% confluence.

Sub-culturing: Medium was removed and rinsed twice with HBSS. Afterwards, 1-2 ml of Trypsin-EDTA solution was added and allowed the flask to sit at 37°C until the cells detach (~3 min). Culture medium was added and the cell suspension was centrifuged (Hermle Z383) at 1500 x g for 3 min. Supernatant was removed and cells were re-suspended in fresh culture medium. Cells were sub-cultivated in a ratio of 1:4 to 1:8 according to the need.

Seeding Cells: Cells were seeded on a 96-well microtiter plate (Corning) and 24-well plate at a density of 5 x 10^3 and 2.5 x 10^5 cells per well respectively as shown in **Table 4-2.** Cells were seeded at a known cell numbers by using hemocytometer. Trypan blue stain was used to recognize viable cells in a population.

Table 4-2 Culture condition of UMR 106-cells for different assay protocols

	Cell Proliferation Assay	Alkaline Phosphatase Activity Assay
Type of multiwell plate	96-well	24-well
Working volume/well (ml)	0.1	0.5
Cells number (cells/well)	5 x 10^3	2.5 x 10^5

Cells were allowed to equilibrate in DMEM (with phenol red) for 24 hours, followed by phenol red free DMEM. Phenol red free DMEM was supplemented with 1% Penicillin & Streptomycin (P/S) and 2% Charcoal/Dextran Treated Fetal Bovine Serum. Then, cells were treated with OPR solution with a series of concentrations.

4.3.2. Preparation of OPR

4.3.2.1. Stock solution

Fifty milligram of OPR granule, which was manufactured by Eu Yan San Ltd. described in Chapter 4, was dissolved in 1ml of HBSS (i.e. 50 mg/ml). The suspension was spilled down and supernatant was filtered with a 0.22 μm syringe filter (Corning) with a membrane material of Surfactant Free Cellulose Acetate (SFCA), which is compatible to aqueous solvent. This stock solution was prepared just before use.

4.3.2.2. Series dilution

Filtered stock solution, with a concentration of 50 mg/ml, was serially diluted to 1000, 100, 10, 1 μg/ml by DMEM (without phenol red). Serial dilution steps are shown in **Table 4-3**.

4.3.3. Treatment with OPR solution

After switching medium to non-phenol red DMEM for 24 hours, cells were treated with OPR solution at 1000, 100, 10, 1 μg/ml for another 24 hours. The negative control group was treated with medium only. Cells were then collected to perform different assay protocols.

Table 4-3 Serial dilution of OPR stock solution

	Concentration	Volume extracted from previous solution (μL)		Volume of non-phenol red DMEM added (μL)	Dilution ratio
	50 mg/ml ↓ 10 mg/ml	100	+	400	1 to 5
	↓ 1000 μg/ml	400	+	3600	1 to 10
Working Solution	↓ 100 μg/ml	400	+	3600	1 to 10
	↓ 10 μg/ml	400	+	3600	1 to 10
	↓ 1 μg/ml	400	+	3600	1 to 10

4.3.4. Assays

4.3.4.1. Cell proliferation assay

Cells were seeded in 96-well plate at a density of 5 x 10^3 cells/well in order to perform cell proliferation assay. After treatment for 24 hours, 20 µl of CellTiter 96® AQueous One Solution reagent (Promega) was added directly in each culture well, cells were allowed to incubate at 37°C for 1~2 hour(s). The absorbance at a wavelength of 492 nm was recorded twice by a microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) at the end of 1st and 2nd hour of incubation. The culture plate was shaken gently for a while before measurement to avoid un-uniform color change within the well.

4.3.4.2. Cellular alkaline phosphatase activity

Cells were seeded in 24-well plate at a density of 2.5 x 10^5 cells/well. Measurement of Alkaline phosphatase (ALP) activity includes 3 steps: 1) Collection of cell lysate; 2) ALP activity assay and 3) Protein assay.

Collection of cell lysate: Protocol for collection of cell lysate is shown in **Figure 4-1**. Cells were rinsed twice with ice-cold HBSS and 0.3 ml of ice-cold lysis buffer was added in each well. The lysis buffer is a 10 mM of Tris-HCl (pH 7.2) solution supplemented with 2 mM $MgCl_2$ and 0.05% of Triton X-100. Samples were scraped from the bottoms of the wells by rubber policeman (Corning) and were placed in microcentrifuge tubes (Molecular Bio Products, San Diego, CA). Cell suspensions were sonicated on ice by ultrasonic processor (SONICS, Newtown, USA) and then centrifuged (Eppendorf Centrifuge 5415R) at 16 110 x g for 10 min at 4°C. Supernatants were collected and aliquoted to 2 parts for measurement of ALP activity and protein content.

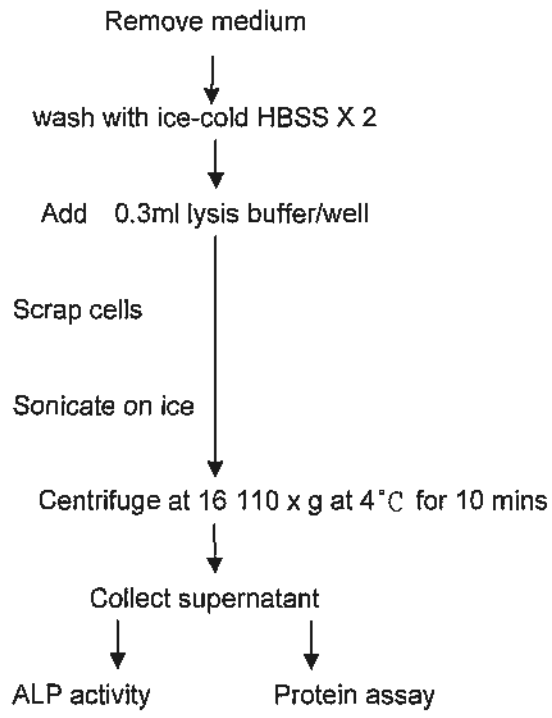


Figure 4-1 Protocol for collection of cell lysate.

Measurement of alkaline phosphatase activity: Alkaline Phosphatase LiquiColor® (Stanbio) was used to measure cellular ALP activity. Aliquote of cell lysate was diluted 10-fold by distilled water. Working reagent from the assay kit was warmed to 37°C for 3 min. One hundred microlitre of working reagent from the assay kit and 10 µl of diluted samples were added in 96-well plate and mixed gently. Absorbance at a wavelength of 405 nm was recorded at 1, 2 and 3 min after reagent and samples were mixed. They were incubated continually at 37°C during the record of absorbance.

4.3.4.3. Protein assay

Bio-Rad Protein assay (Bio-Rad Laboratories) can determine concentration of solubilized protein by comparison to a standard curve based on the method of Bradford. Aliquote of cell lysate prepared was diluted 2-fold by distilled water. Bovine serum albumin was diluted with

distilled water to serve 6 dilutions of protein standard. The standard concentration ranged from 0.05 mg/ml to 0.5 mg/ml as shown in **Table 4-4** and **Figure 4-2**. Dye reagent was diluted 5-fold with distilled water before use. Ten-microlitre of diluted samples and standards were mixed with 200 µl diluted dye in 96-well plate. The mixture was allowed to incubate at room temperature for at least 5 min but not more than 1 hour. Absorbance was recorded at a wavelength of 595 nm after incubation.

Table 4-4 Dilution of bovine serum albumin in protein assay

Bovine Serum Albumin of 1mg/ml (µl)	H_2O (µl)	Protein Standard Concentration (mg/ml)
5	95	0.05
10	90	0.1
20	80	0.2
30	70	0.3
40	60	0.4
50	50	0.5

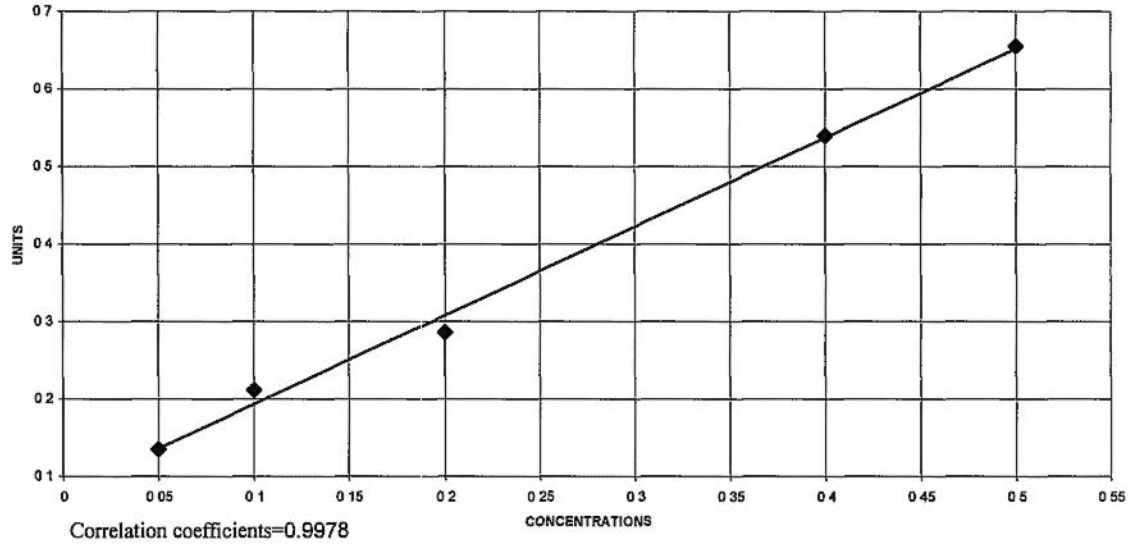


Figure 4-2 Protein standard curve

4.3.5. Statistical analysis

Data were presented as fold of control ± S.E.M unless otherwise noted. Data were analyzed with GraphPad Prism 4. Results were evaluated by one-way analysis of variance (ANOVA) to .determine differences of various OPR dosages with negative control group. Dunnett's Multiple Comparison Test was used whenever necessary as the post-hoc analysis. A value of $P<0.05$ was considered as statistically significant.

4.4. Results

4.4.1. Cell proliferation assay

In cell proliferation assay, absorbance is directly proportional to the number of living cells in culture. Absorbance was measured twice after incubation for the 1st and 2nd hour and the absorbance was expressed in fold of control ± S.E.M in compare to the group treated with medium only (negative control group). Data are summarized in **Table 4-5** and expressed in graph format in **Figure 4-3**.

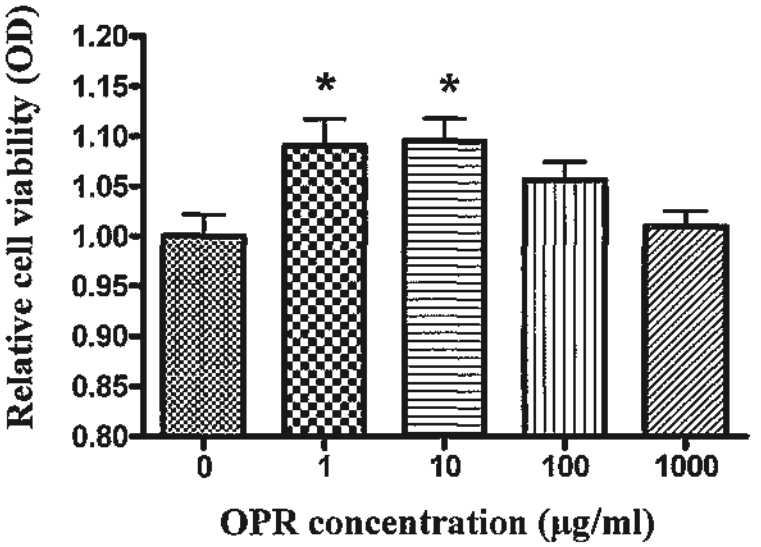
One or two hours of incubation period showed similar result. OPR did not show cytotoxic effect on UMR 106 cells in four concentrations tested. It increased cell proliferation by almost 10 % at 1 and 10 μg/ml concentrations, which was significantly higher than the negative control group ($P<0.05$). The rate of proliferation reached the peak at a concentration of 10 μg/ml and the rate dropped afterwards as concentration increased. At a concentration of 1000 μg/ml, number of living cells was almost the same as that in the negative control group.

Table 4-5 Effects of OPR on cell proliferation of UMR 106 cells

Concentration of OPR (μg/ml)	Cell viability relative to control	
	1-hour incubation	2-hour incubation
	Fold of control ± S.E.M	
0 (Negative control)	1 ± 0.0213	1 ± 0.0198
1	1.090 ± 0.0266 *	1.079 ± 0.0177 *
10	1.095 ± 0.0225 *	1.080 ± 0.0149 *
100	1.056 ± 0.0180	1.062 ± 0.0112
1000	1.009 ± 0.0152	0.959 ± 0.0248

*P<0.05 based on one-way ANOVA, Dunnett's Multiple Comparison Test by comparison with control. Total n= 5 ~ 6

(A)



(B)

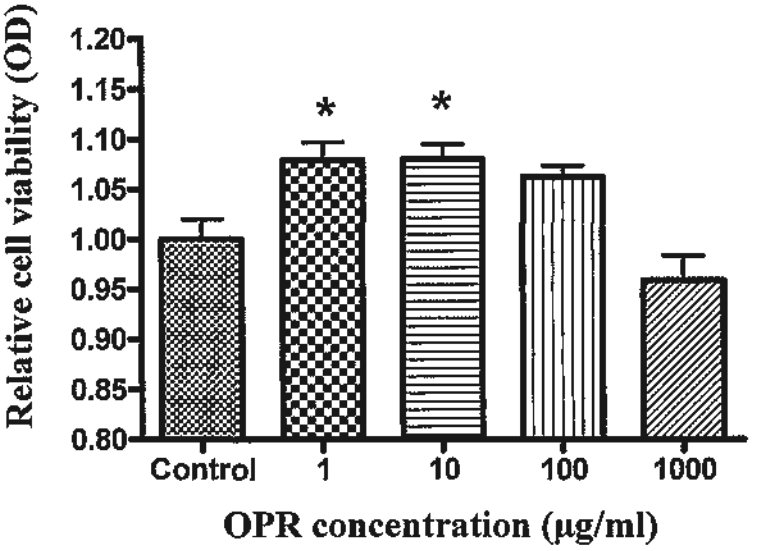


Figure 4-3 Cell proliferation of UMR 106 cells induced by OPR with an incubation period of (A) 1 hour and (B) 2 hour

*P<0.05 versus control group

4.4.2. Cellular alkaline phosphatase activity

Alkaline phosphatase (ALP) activity is expressed in Units per liter (U/L), which is a measurement of the amount of ALPase which can produce one mM/L of 4-nitrophenol from 4-nitrophenyl phosphate per minute. ALP activity (U/L) is calculated by the following formula:

U/L = (rate of absorbance change/absorptivity of 4-nitrophenol at 405 nm) x (Total volume/Sample volume).

ALP activity was normalized with the cell number by measuring protein content. Concentration of solubilized protein (g/L) was obtained directly by comparison to a standard curve. ALP activity was expressed in U/g after normalization. Data are summarized in **Table 4-6** and expressed in graph format in **Figure 4-4**.

OPR increased ALP activity of UMR 106 cells by more than 20 % at a concentration of 10 μg/ml, which was significantly higher than the negative control group ($P<0.05$). However, ALP activity reached the peak at 10 μg/ml and the rate dropped afterwards as concentration increased.

Table 4-6 Effects of OPR on alkaline phosphatase activity of UMR 106 cells

Concentration of OPR (μg/ml)	Alkaline phosphatase activity relative to control
	Fold of control ± S.E.M
0 (Negative control)	1 ± 0.0549
1	1.087 ± 0.0525
10	1.216 ± 0.0545*
100	1.164 ± 0.0370
1000	0.957 ± 0.0340

*P<0.05 based on one-way ANOVA, Dunnett's Multiple Comparison Test by comparison with control. Total n= 3 ~ 4

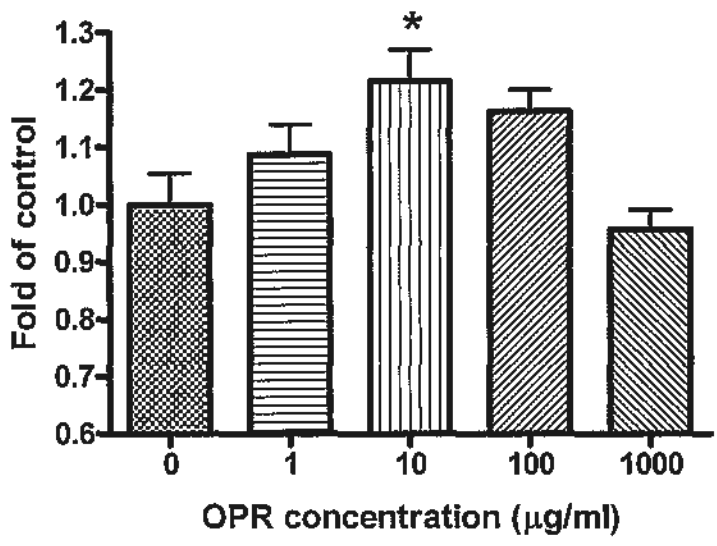


Figure 4-4 Effects of OPR on alkaline phosphatase activity of UMR 106 cells

n=3~4, *P<0.05 versus control group.

4.5. Discussion and conclusions

Cell proliferation and differentiation of UMR 106 cell line were measured by cell proliferation assay (MTS assay) and alkaline phosphatase activity respectively. Alkaline phosphatase is a membrane bound enzyme that is used as a marker for osteoblast

differentiation as its activity is detected at an early stage of osteoblast differentiation and continues to increase during osteoblast maturation(Fragale et al., 1999). In this study, UMR 106 cells responded to OPR in a biphasic manner and reached the peak at a concentration of 10 μg/ml.

UMR 106 cells are osteoblastic-like cells which were reported to have estrogen-regulated biologic responses and proven to contain estrogen receptor (ER) (Davis et al., 1994). The biphasic response of the cells to OPR may be due to an excessive and prolonged stimulation of ER at concentrations higher than 10 μg/ml, which in turn resulted in a down regulation of the receptor (Miller-Martini et al., 2001). Another reason may be due to an inconsistent estrogen responsiveness of UMR 106 (Davis et al., 1994; Gray et al., 1987). Multiple factors, such as serum, estradiol, and cell density, influence the ER levels in the cells and probably cause fluctuations in the abundance of receptors available and result in a biphasic response to OPR. In conclusion, the direct beneficial effects of OPR on bone cells were demonstrated at concentrations at 10 μg/ml.

In the study, OPR enhanced cell proliferation and differentiation of UMR 106 cells. However, the mechanism remains unknown. Report on Fructus Ligustri Lucidi, which is one of the major components of OPR, showed that its extract could accelerate the formation of calcified matrix and increase extracellular calcium and phosphate depositions in time (3-6 days) and dose (1–100mg/ml) dependent manner. Its enhancement of mineralization process on osteoblastic cells might be one of the reasons of its beneficial effect on bone (Zhang et al., 2008). Also, UMR 106 cells possess the expression of both osteoprotegrin (OPG) and receptor activator of NF-kappa B ligand (RANKL), it therefore serves as an easy tool to monitor the rate of osteoclastogenesis. Another major component of OPR is *Herba epimedii*. Its extract modulated osteoclastogenesis by increasing osteoprotegrin (OPG)

mRNA and decreasing receptor activator of NF-kappa B ligand (RANKL) mRNA expression in UMR 106 cells, resulting in a dose-dependent increase in OPG/RANKL mRNA ratio. Therefore, the beneficial effect of Herba epimedii on bone mass might due to its increase in osteoblastic activities and decrease in osteoclastogenesis (Xie et al., 2005a).

As estrogen related studies were performed, several experimental conditions needed to be adjusted. Phenol red free culture medium was used as phenol red mimics the action of estrogen (Wesierska-Gadek et al., 2007). Charcoal/dextran-treated FBS was used because a steroid hormone, which is present in FBS, was removed (Tsang et al., 2001). Therefore, cells were incubated in phenol red free DMEM supplemented with charcoal/dextran treated FBS for 1 day prior to treatment.

In conclusion, OPR induced differentiation and proliferation of UMR 106 cells in a biphasic manner and reached the peak at a concentration of 10 μg/ml. Although other related studies have suggested that induction of mineralization process and reduction of osteoclastogenesis might be the potential mechanism of achieving a positive effect on osteoblast-like cells, more studies need to be done to confirm the statement. Presence of estrogen receptor (ER) on UMR 106 cells initiates us to detect the phytoestrogen effect, if any, present in OPR and the study is described in chapter 5.

5. Detection of Estrogenic-Like Activities of a Kidney-Tonifying Herbal Formula by MCF-7 Reporter Gene Assay

5.1. Introduction

Estrogen regulates skeletal homeostasis and plays an important role in bone remodelling as described in Section **1.7.2**. Deficiency of estrogen in postmenopausal women leads to a higher risk of osteoporosis. Raloxifene, a marketed medication for postmenopausal osteoporosis patients, is a selective estrogen receptor modulator (SERM). It mimics estrogen in bone tissue and is indicated for the prevention and treatment of postmenopausal osteoporosis. Phytoestrogens, which are steroid-like plant compounds, mimic or act as precursors to sex hormones. They may be a potential candidate of treating postmenopausal osteoporosis.

Prevention and treatment effects of phytoestrogen on osteoporosis have been verified in many studies. Aqueous extract of black tea (*Carmellia sinensis*) increases the serum estradiol level of ovariectomized rats and significantly diminishes ovariectomy-induced decaying changes in bone (Das et al., 2005). Another study shows that premenopausal women with a dietary intake which is rich of phytoestrogens would have a higher bone mineral density (total and trabecular) and bone strength strain index, compared with those practicing dietary style with less phytoestrogens (Di et al., 2000). A phytoestrogen-rich herbal formula. Xianlinggubao (仙靈骨葆), containing genistein 510 μg/g and daidzein 250 μg/g, was able to prevent ovariectomy-induced deterioration of musculoskeletal tissues at the hip without causing uterine stimulation (Qin et al., 2005). From these studies, the positive

effect of phytoestrogens on bones has been established.

Detection of phytoestrogen in TCM is not a new topic. Phytoestrogens were found in some common TCM such as *Angelica sinensis* 當歸 (31 mg diadzein/kg) and *Spatholobus suberectus* 雞血藤 (31 mg daidzein/kg) by using HPLC (Li et al., 2004). In Herba Epimedii, one of the components of OPR, its derived phytoestrogen has shown to have a preventive effect on osteoporosis induced by ovariectomy in rats with stimulation of bone formation as well as inhibition of bone resorption (Peng et al., 2008). Phytoestrogens in complex herbal formulas can also be detected by transfected cell line. Utilizing an oestrogen-sensitive chimeric receptor/reporter gene element which has been stably transfected into HeLa cells, the presence of phytoestrogen in Bupleurum & Peony Formula (加味逍遙散) was revealed by inducing an expression of the reporter gene (Miller-Martini et al., 2001).

In earlier study, OPR was shown to induce a cell differentiation and proliferation of osteoblastic-like UMR 106 cells. Presence of estrogen receptor (ER) on UMR 106 cells initiates us to detect phytoestrogen, if any, present in OPR. In the present study, a human mammary epithelial carcinoma cell line MCF-7, which has been stably transfected with estrogen responsive elements (ERE) and luciferase reporter gene (luc), is employed to detect estrogenic-like activities of OPR. Protocols of culturing and treatment of MCF-7 cells are shown in **Table 5-1**.

Table 5-1 Protocol of culturing and treatment of MCF-7 cell line

Culturing medium	1)Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin 100 U/ml and streptomycin 100 mg/ml (1%P/S) 2)Minimum Essential Medium Alpha (MEMα) supplemented with 2% Charcoal/Dextran Treated Fetal Bovine Serum (DCC-FBS), penicillin 100 U/ml and streptomycin 100 mg/ml (1%P/S)
Sub-culture	Subcultivation ratio of 1:3 to 1:6 Typsinization: Rinse with Phosphate buffered saline (PBS) twice and add in 1 ml trypsin and incubate at 37℃ for 1 min
Assay kits	CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) Luciferase Assay kit (Applied Biosystems) Protein assay kit (Bio-Rad Laboratories)

Day 1 & 2	Switch medium	Switch culture medium to non-phenol red DMEM with 2% Charcoal/Dextran Treated FBS for 48 h in culture flask.
Day 3	Seed cells	Seed cells on microtiter plate for 24hr:
Day 4	Treatment	Ttreat with MEM alpha (negative control), OPR solutions and 17β-Estradiol for 24 hours.
Day 5	Collect cells	Perform different assay protocols

5.2. Materials

5.2.1. Cell line

A human mammary epithelial carcinoma cell line MCF-7 which has been stably transfected with estrogen responsive elements (ERE) and luciferase reporter gene (luc) was used. The transfected ERE-luc MCF-7 reporter gene assay was kindly provided by Professor Karl W. K. Tsim (Department of Biology, The Hong Kong University of Science and Technology).

5.2.2. Culture medium

Minimum Essential Medium (MEM) powder was obtained from Invitrogen (California, USA). The powder was dissolved and filtered by the same procedure as described in Section **4.2.2** except it was buffered with 2.2 g/L sodium bicarbonate (Sigma). The medium was supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin (equivalent to 1% P/S) and 10% of FBS. The culture reagents were purchased from Invitrogen. Antibiotic G418 (Sigma) at a concentration of 300 μg/ml was added in the medium before cell culturing.

Another medium used was MEM alpha medium (Invitrogen), which has no phenol red and can be used directly for cell culture with the supplement of 1% P/S (Invitrogen) and 2% charcoal/dextran treated fetal bovine serum (Hyclone) only.

5.2.3. Wash buffer and chemicals

Phosphate buffered saline (PBS) at a pH of 7.4 was used as wash buffer, which was purchased from Sigma. 17β-Estradiol was purchased from Sigma. Its chemical structure is shown in **Figure 5-1**. Its molecular weight is 272.4. It is sensitive to air and needs to be prepared freshly just before use.

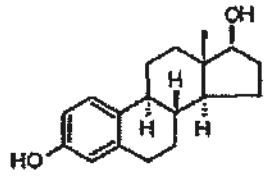


Figure 5-1 Chemical structure of 17β-Estradiol

5.2.4. Assay kits

CellTiter 96® AQueous One Solution cell proliferation assay (also known as MTS assay) was obtained from Promega (Madison, USA). MTS assay is used to detect the cytotoxic effect of OPR on transfected MCF-7.

Luciferase Assay kit was purchased from Applied Biosystems (Massachusetts, USA), which is a bioluminescent reporter gene assay system for the detection of luciferase presence in reporter gene. It composes of lysis solution and two working substrates, which are essential in the collection of cell extracts and bioluminescent detection.

Protein assay kit was purchased from Bio-Rad Laboratories (California, USA). Luciferase activity was normalized with the cell number by measuring protein content.

Other materials include Trypsin- ethylenediaminetetraacetic acid (Trypsin-EDTA) and Trypan blue solution. They were obtained from Invitrogen. Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific, UK. DL-Dithiothreitol (DTT) was obtained from Promega.

5.3. Methods

5.3.1. Cell culture

Propagation: Stably transfected MCF-7 cell line was cultured in MEM (with phenol red) supplemented with 10% FBS and 300μg/ml of antibiotic G 418. The cells were kept in a humidified 5% CO_2 atmosphere and a temperature at 37°C. The medium was renewed 2-3 times per week and sub-cultured up to 70% confluence.

Sub-culturing: Medium was removed and rinsed twice with PBS. Sub-culturing process was the same as described in Section **4.3.1** except the trysination time is 1 min for MCF-7. Cells were sub-cultured in a ratio of 1:3 to 1:6.

Seeding Cells: Two days before cell seeding, the medium was switched to MEM alpha (without phenol red), which was supplemented with 2% charcoal/dextran treated FBS. Afterwards, cells were seeded on a 96-well and 24-well microtiter plates (Corning) at a density of 5000 and 5 x 10^4 cells per well respectively as shown in **Table 5-2.** Cells were allowed to equilibrate for 24 hours before treatment. They were then treated with 17β-Estradiol or OPR solution with a series of concentrations.

Table 5-2 Cell culture of MCF-7 cells for different assay protocols

	MTS Assay	Luciferase Assay/Protein assay
Type of multiwell plate	96-well	24-well
Working volume/well (ml)	0.1	0.5
Cells number (cells/well)	5000	5×10^4

5.3.2. Preparation of 17β-Estradiol

17β-Estradiol (Sigma) is in a form of white powder, which is sensitive to air, light and insoluble in water. It was freshly prepared just before use and its stock solution was stored in brown microcentrifuge tube (Jencons, UK). The stock solution was prepared by dissolving 272 mg of 17β-Estradiol in 1 ml of DMSO, which is equivalent to a molarity of 10 mM. Stock solution was freshly prepared just before use and it was serially diluted to prepare a range of concentrations from 10^{-4} to 10^{-11} M (10-fold dilution).

5.3.3. Preparation of OPR

Preparation of OPR stock solution has been described in Section **4.3.2.1.** Stock solution of OPR at a concentration of 50 mg/ml was serially diluted by MEM alpha (without phenol red) to prepare concentrations of 1, 2 and 4 mg/ml (2-fold dilution) for MTS assay and concentrations of 1.6, 8, 50, 200, 1000 μg/ml (5-fold dilution) for Luciferase Assay/Protein assay.

5.3.4. Treatment

Transfected MCF-7 cells were treated with MEM alpha (negative control), OPR solutions and 17β-Estradiol for 24 hours. Cells were then collected to perform different assays.

5.3.5. Optimized concentrations of 17β-Estradiol and OPR

Optimized concentrations (with no cytotoxic effect) of 17β-Estradiol and OPR on transfected MCF-7 cell line could be determined by MTS assay. Transfected MCF-7 cells were seeded on two 96-well plates at a density of 5000 cells per well. Cells on the first plate were used to determine the optimized concentration of 17β-Estradiol and therefore treated with 17β-Estradiol at 4 concentrations of 1, 10, 100, 1000 μM (10-fold dilution). Cells on the second plate were used to determine the optimized concentration of OPR and therefore treated with OPR solution at 3 concentrations of 1, 2, 4 mg/ml (2-fold dilution). MTS assay was carried out as described in Section **4.3.4.1**.

5.3.6. Responsiveness of transfected MCF-7 cells to 17β-Estradiol

The sensitivity of transfected MCF-7 assay system to 17β-Estradiol was determined by luciferase assay kit. Cells were treated with a series of concentrations of 17β-Estradiol. Transfected MCF-7 cells were seeded in 24-well plates at a density of 5×10^4 cells per well for 24 hours. Afterwards, cells were treated with 17β-Estradiol at 6 concentrations from 1 to 10^6 pM (10-fold dilution) for another 24 hours. Then, medium was removed and rinsed with PBS once. One hundred microlitre of lysis buffer (100 mM potassium phosphate at pH 7.8 complementary with Triton X-100, 1 mM dithiothreitol) was added to each well and the plate was shaken for 20 min at 4°C. Cells extract were transferred to microcentrifuge tubes

(Molecular Bio Products) and vortexed for 5 min at 4°C. They were then centrifuged at 16 110 x g for 5 min at 4°C. Supernatant (cell lysate) was collected to perform luciferase assay and protein assay. For Luciferase Assay, cell lysate was transferred to an opaque assay plate (Falcon, Becton Dickinson Labware, Franklin Lakes, USA). After adding working substrates, a light signal was emitted and it was measured by a plate reader ((FLUOstar OPTIMA). Luciferase activity was proportional to the light intensity and need to be normalized by the protein content Protein assay was carried out as described in Section **4.3.4.3**.

5.3.7. Detection of luciferase activity of OPR

Detection of luciferase activity of transfected MCF-7 induced by OPR was measured by luciferase assay kit. MEM alpha and 17β-Estradiol were used as negative and positive controls respectively. Transfected MCF-7 cells were seeded in two 24-well plates at a density of 5 x 10^4 cells per well for 24 hours. Afterwards, transfected MCF-7 cells were treated with MEM alpha (negative control), 17β-Estradiol (positive control) and 5 concentrations of OPR solutions (1.6, 8, 40, 200, 1000 μg/ml) for another 24 hours. Then, luciferase activity was measured as described in section **5.3.6.**

5.3.8. Statistical analysis

Data were presented as fold of control ± S.E.M unless otherwise noted. They were analyzed with GraphPad Prism 4. Results were evaluated by one-way analysis of variance (ANOVA) to determine differences of various OPR dosages with negative and positive control groups. Dunnett's Multiple Comparison Test was used whenever necessary as the post-hoc analysis. A value of $P<0.05$ was considered statistically significant.

5.4. Results

5.4.1. Optimized concentrations of 17β-Estradiol and OPR

To select an optimized concentration of OPR and 17β-Estradiol for later studies, MTS assays were performed to determine a maximum concentration that had no cytotoxic effect on transfected MCF-7 cells. Number of viable cells was comparable to those in negative control group at concentrations of 1 mg/ml and 1 μM for OPR and 17β-Estradiol respectively. Therefore, we take these concentrations as the highest tolerable concentration for the detection of luciferase activity in subsequent experiments. MTS assay results are shown in **Table 5-3** and **Table 5-4** and expressed in graph in **Figure 5-2.**

Table 5-3 Cytotoxic effects of OPR on transfected MCF-7 by MTS assay

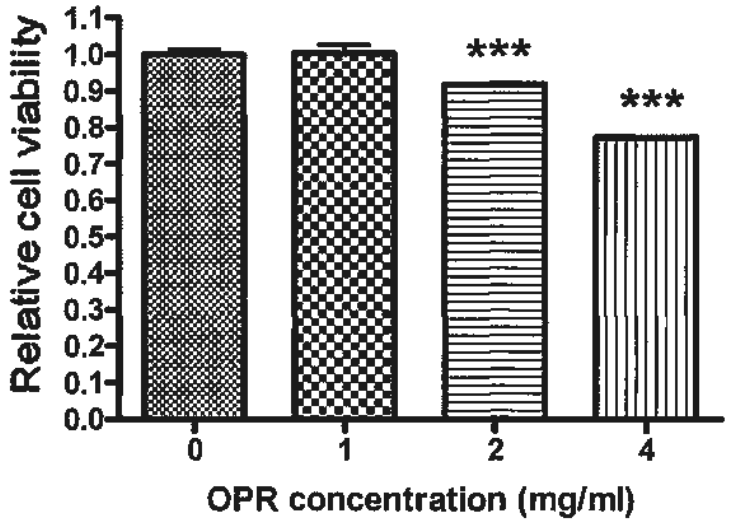
Concentration of OPR (mg/ml)	Cells viability relative to control
	Fold of control ± S.E.M
0 (Negative control)	1 ± 0.0136
1	1.004 ± 0.0230
2	0.918 ± 0.0024***
4	0.7734 ± 0.0017***

Table 5-4 Cytotoxic effects of 17β-Estradiol on transfected MCF-7 by MTS assay

Concentration of 17β-Estradiol (μM)	Cells viability relative to control
	Fold of control ± S.E.M
0 (Negative control)	1 ± 0.0136
1	0.9541 ± 0.0131
10	0.851 ± 0.0071***
100	0.635 ± 0.0103***
1000	0.4061 ± 0.0156***

***P<0.01 based on one-way ANOVA, Dunnett's Multiple Comparison Test by comparison with control. n= 3

(A)



(B)

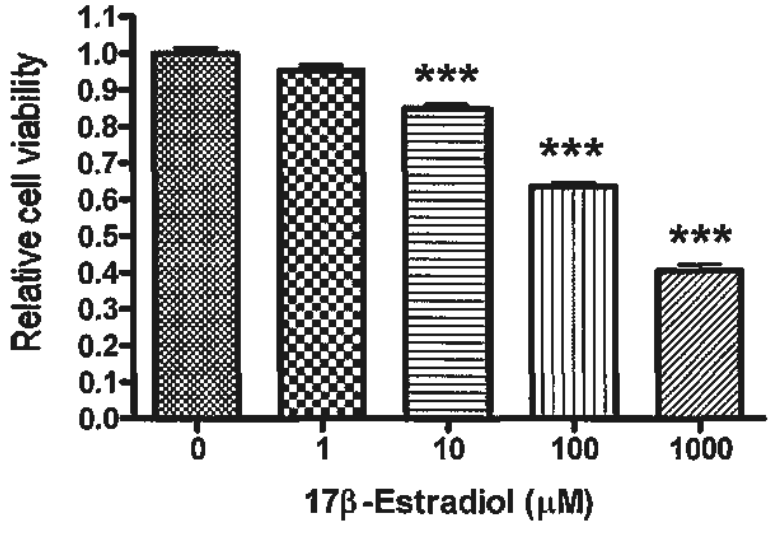


Figure 5-2 Cytotoxic effects of **(A)** OPR and **(B)** 17β-Estradiol MCF-7 cells

***P<0.01 based on one-way ANOVA, Dunnett's Multiple Comparison Test by comparison with control. n= 3

5.4.2. Responsiveness of transfected MCF-7 cells to 17β-Estradiol

In order to determine the sensitivity of the assay system to oestrogenic compounds, transfected MCF-7 cells were treated with various concentrations of 17β-Estradiol. From the result of MTS assay, 1 μM (i.e. 10^6 pM) was the highest tolerable concentration for MCF-7 cells. Therefore, cells were treated with 7 concentrations from 0 to 10^6 pM (10-fold dilution), results and shown in **Table 5-5** and expressed in graph in **Figure 5-3**. Maximal induction of luciferase activity was achieved at concentrations of 10^3, 10^5, 10^6 pM, which induced an increase of luciferase activity for more than 50% compared with the negative control group ($P < 0.01$). As there is no statistical difference of the luciferase activity induced by these 3 concentrations, we chose the lowest but effective concentration (i.e. 10^3 pM = 1 nM) as the positive control in subsequent experiments. Using the lowest concentration can minimize the content of DMSO added to the treating cells. Significant induction of luciferase activity could be observed at a concentration as low as 10^2 pM ($P < 0.05$).

Table 5-5 Luciferase activity of transfected MCF-7 cells induced by 17β-Estradiol

Concentration of 17β-Estradiol (pM)	Luciferase activity relative to control
	Fold of control ± S.E.M
0	1 ± 0.0873
10	1.279 ± 0.1467
10^2	1.491 ± 0.0491 *
10^3	1.534 ± 0.0978 ***
10^4	1.495 ± 0.1515 *
10^5	1.601 ± 0.0688 ***
10^6	1.63 ± 0.0035 ***

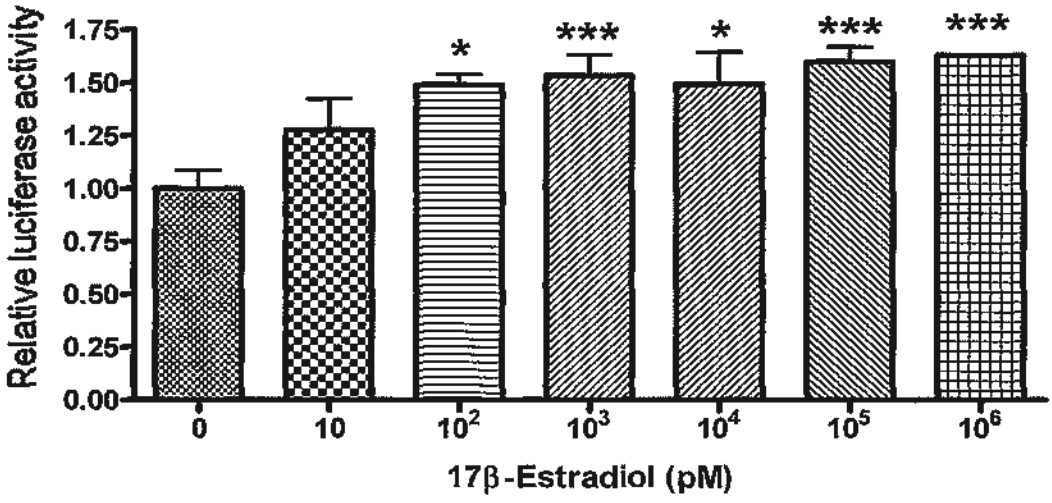


Figure 5-3 Luciferase activity of transfected MCF-7 cells induced by 17β-Estradiol *P < 0.05, ***P<0.01 based on one-way ANOVA, Dunnett's Multiple Comparison Test by comparison with control. n= 3

5.4.3. Detection of luciferase activity of OPR

From the result of previous study, the highest tolerable concentration of OPR on transfected MCF-7 cells was 1000 μg/ml, and for the positive control (17β-Estradiol) was 1 nM. Concentrations of OPR, ranging from 1.6, 8, 40, 200 to 1000μg/ml, were assayed for their ability to induce the estrogen responsive elements (ERE) and luciferase reporter gene (luc) in the MCF-7 cells line. As shown in **Table 5-6** and **Figure 5-4**, OPR at 200 and 1000 μg/ml induced an increase of luciferase activity by approximately 80% and 130% respectively compared with the negative control group ($P < 0.01$). The induction of luminescence by OPR at 1000 μg/ml was 50% more than that of the positive control.

Table 5-6 Luciferase activity of transfected MCF-7 cells induced by OPR

Concentration of OPR (μg/ml)	Luciferase activity relative to control	
	Fold of control ± S.E.M	
0	1 ± 0.0803	
1.6	1.114 ± 0.0555	
8	1.355 ± 0.0615	
40	1.427 ± 0.1200	
200	1.78 ± 0.1587	***
1000	2.363 ± 0.2418	***
17β-Estradiol	1.865 ± 0.1665	***

***P<0.01 based on one-way ANOVA, Dunnett's Multiple Comparison Test by comparison with control. n= 3

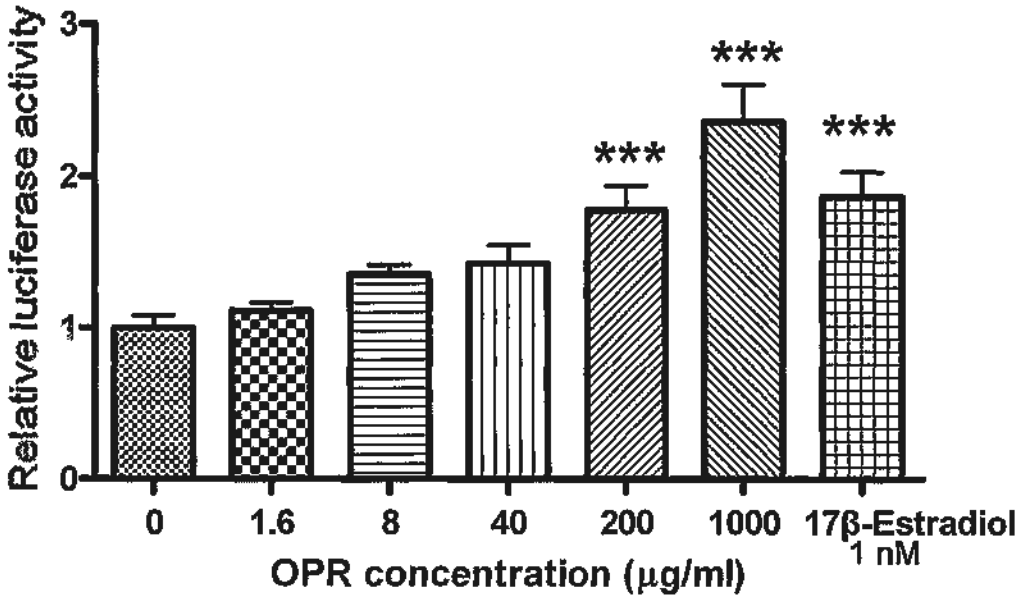


Figure 5-4 Luciferase activity of transfected MCF-7 cells induced by OPR

***P<0.01 based on one-way ANOVA, Dunnett's Multiple Comparison Test by comparison with control. n= 3

5.5. Discussion and conclusions

Estrogen inhibits bone turnover by reducing osteoblast-mediated bone resorption and enhancing osteoblast-mediated bone formation. Action of estrogen is triggered by binding to estrogen receptors (ERs) located in the nucleus. This nuclear estrogen-ER complex binds to estrogen response element (ERE) in the target gene and sequences directly or indirectly to start a series of physiological responses (Deroo and Korach, 2006). The estrogen receptor mediates bone protective effects of estrogen and raloxifene is a typical example to exert its protective effect on bone by binding to estrogen receptors (Silverman et al., 2007).

In Traditional Chinese Medicine, many herbs were reported to have estrogenic-like properties. In this study, a MCF-7 cells line, which was stably transfected with estrogen responsive elements (ERE) and luciferase reporter gene (luc), was employed to detect estrogenic-like activities of OPR. This bioassay utilizes two different components for the detection of cells' responses to estrogenic substances. The first component is a ligand binding domain of the estrogen receptor (ER) linked to a transcription factor; the second component is a luciferase reporter gene. Expression of the luciferase reporter gene is dependent upon the ligand-dependent activation of the estrogen receptor, so that transfected MCF-7 cell line is a reliable receptor/reporter gene assay as it is based on the measurement of a primary transcriptional response which reflects ligand-dependent activation of the estrogen receptor (Gao et al., 2007). Other traditional assays for screening estrogenic compounds, which are mostly based on uterotrophic activity in vivo or mitogenic activity towards breast tumor cell line in vitro, are not reliable because the mechanisms for these biological responses are complex and poorly understood. Those assays require long period of hormone treatment and their results may be interfered by metabolism or bioconversion of the estrogenic compounds being tested (Miksicek, 1993). Therefore, we employed transfected MCF-7 cell

line in identifying receptor-specific ligands with potential estrogenic activities.

Estrogen, which are used in hormone replacement therapy (HRT) on treating postmenopausal syndromes, have been criticized for its increased risk of breast cancer and coronary artery disease shown in subject-based studies (Coombes et al., 2005; Rippy and Marsden, 2006; [Anon], 2006; [Anon], 2007). Although phytoestrogen also process estrogenic-like activities, consumption of phytoestrogns was widely accepted to decrease risk of breast and prostate cancer and cardiovascular diseases. Although some studies have shown that phytoestrogens may produce mild estrogen-like effects on vaginal cytology (Baird et al., 1995), other studies reveal that weak estrogens found in plant can function paradoxically by protecting estrogen receptors from activation and therefore reduce breast cancer risk (Wattenberg, 1983). Therefore, low concentration of phytoestrogen is still considered as a safe supplement for menopausal women.

In this study, OPR was proven to possess estrogenic activity in a dose dependent manner. OPR at concentrations of 200 and 1000 μg/ml induced a significant increase of luciferase activity by approximately 80% and 130% respectively compared with negative control group. The induced estrogenic activity by OPR may be due to the presence of phytoestrogens within the herbal formula. Phytoestrogens are plant compounds with estrogen-like biological activity. Many botanicals contain phytoestrogens that are capable of eliciting agonist or antagonist responses possibly via a mechanism of action comparable to that of estrogen. Thus, phytoestrogens are defined functionally and include known compounds that belong to the structural groups of isoflavones, flavonoids, lignans, phytosterols and coumestans. They were identified by their ability to bind to the estrogen receptor and to induce series of oestrogenic responses (Murkies et al., 1998).

Among the ten herbs in OPR, Herba Epimedii, Fructus Psoraleae were reported to possess estrogenic-like activities (Zhao et al., 2007a). For Herba Epimedii, many studies have investigated the different classes of flavonoids within this herb (Chen et al., 2008b; Chen et al., 2008a; Huang et al., 2007). Study shows that water extract of Herba Epimedii significantly increased the serum estrogen level of postmenopausal women after a six-month treatment. (Yan et al., 2008). For Fructus Psoraleae, isoflavonoid compounds were found which include daidzein (Hsu et al., 2001). Daidzein belongs to the isoflavone class of flavonoids and classified as a phytoestrogen since it is a plant-derived nonsteroidal compound that possesses estrogen-like biological activity. Daidzein stimulate the expression of the ERE-dependent reporter in a MCF-7-derived cell line, MVLN cells (Zierau et al., 2006). Daidzein has been found to have both weak estrogenic and weak anti-estrogenic effects. (National Cancer Institute, NIH). It is therefore not surprise that Herba Epimedii and Fructus Psoraleae process both estrogenic effects (when administered indepently) and antiestrogenic effects (when administered together with diethylstilbestrol) and such bidirectional effects depends on the internal estrogen level (Zhao et al., 2007a). Further study is needed to investigate the source of estrogenic activities in OPR.

6. Therapeutic Effect of a Kidney-Tonifying Herbal Formula on Osteoporosis Mice Developed by Ovariectomy

6.1. Introduction

The Food and Drug Administration (FDA) has established "Guidelines for preclinical and clinical evaluation of agents used in the prevention or treatment of postmenopausal osteoporosis" in April, 1994 (FDA, 1994). It stated that "in addition to the toxicity studies required for all new drugs, preclinical studies of bone quality should be performed for drugs to be used in the prevention and intervention of osteoporosis." The World Health Organization (WHO) also acknowledged the value of these animal models as being highly predictive for drug action in human postmenopausal osteoporosis with regard to bone mass, remodeling, bone architecture, and strength (Bonjour et al., 1999). Animal studies provide an opportunity to directly examine bone mass, architecture and strength, and demonstrate potential deleterious effects on bone quality after long term treatment, both of which cannot be easily validated clinically. Therefore, we perform a preclinical animal study as a preparation for future clinical trial.

A wide range of animals can serve as potential candidates of osteoporosis model. Animal models for osteoporosis can be classified as either modeling (mice, rats) or remodeling (examples include dogs, ewes, and primates). Modeling of bone is the method by which bone grows and is shaped. In remodeling species, including adult humans, bone undergoes a continuous coordinated process of bone resorption, followed by formation of new bone. At the present time, an experimental model that precisely mimics the pathophysiology of

postmenopausal osteoporosis is unavailable. Although several risk factors for osteoporosis have been identified, there is a predominant association with estrogen-deficiency. Hence, ovariectomized animals are the preferred animal models to provide insight into the clinical outcome of anti-osteoporotic agents (Kimmel, 2001).

Mice are now widely used in biomedical research because of their ease of breeding, reproductive capacity and relatively low cost. In osteoporosis study, mice possess a more important characteristic of short life span (Rosen et al., 2001). For estrogen-depleted women, the phase of accelerated estrogen-depletion bone loss lasts 5-8 years. The time from attainment of peak bone mass until the development of fragility fractures is 30 years or longer (Kimmel, 2001). A short lifetime span allows mice to be an effective animal model as it significantly compresses the time frame from experiencing peak bone mass to postovariectomy bone loss. In recent years, increasing numbers of osteoporosis model have successfully induced in young ovariectomized mice with an age ranging from 4-12 weeks (Chiba et al., 2003; Fujioka et al., 2004; Jochems et al., 2005; Wang et al., 2003) compared with 6-12 months old rat model used in former studies (Kim et al., 2008; Srivastava et al., 2008; Uyar et al., 2008; Zhu et al., 2008). Moreover, the efficacy of the tested agents can be shown within a treatment period of 4-12weeks (Wang et al., 2005b). A study even developed an osteoporosis mice model in a short period of 2 weeks after ovariectomy and demonstrated the action of antiosteoporotic agents with a treatment period of 2 weeks only (Xiang et al., 2006). Therefore, mice were employed as the animal model in our osteoporosis *in-vivo* study.

Traditional Chinese Medicine has been used to treat bone fracture and prevent osteoporosis for several centuries as described in session **1.9**. Some scientific studies have confirmed the antiosteoporotic action of individual herb extract by evaluating the efficacy on

ovariectomized rodent model (Jiang et al., 2002; Kim et al., 2008; Nian et al., 2006b; Tsai et al., 2007; Wang et al., 2003) and some basic studies have been performed to assess the underlying mechanism (Zhang et al., 2006a; Zhang et al., 2006b). However, fewer studies (Nian et al., 2006a; Zhao et al., 2007b) had assessed the composite effects of herbal remedies. The assessment of herbal formulation is thus important as it is the most common and traditional form of therapy by general TCM consumers.

In the study, we had two aims: Firstly, we could develop osteoporosis model by performing ovariectomy on four-week-old C57BL/6 mice which should exhibit skeletal responses similar to those in post-menopausal women. These responses include high bone turnover and subsequent bone loss similar to the human post-menopausal condition, and can be prevented by estrogen replacement. Secondly, we try to investigate the antiosteoporotic effect of a kidney-tonifying herbal formula, OPR, on the osteoporosis mice model and evaluate the efficacy by bone mineral density and biochemical markers of bone turnover measurements.

6.2. Materials

6.2.1. Animals

Thirty virgin female C57BL/6 mice of 4 weeks old were obtained from and housed in the Laboratory Animal Services Centre of The Chinese University of Hong Kong. Mice were housed in allergen-free condition with 12 hours light dark cycle at a temperature around 22°C. They received water ad libitum and control diet (0.6% Calcium, 0.65% Phosphorus) obtained from Harlan Teklad, Wisconsin, U.S.A. License to Conduct Experiments had been obtained from the Department of Health, HKSAR under the Animals (Control of Experiments) Regulations.

6.2.2. Preparation of OPR granules and vehicle

Granules of OPR formula were produced by Eu Yan Sang Ltd as described in Chapter 3. OPR granules were prepared at a dosage of 7 g/kg/daily for mice and were dissolved in warm distilled water at a concentration of 1.16 g/ml, which was equivalent to five times of normal clinical dose. Distilled water was used as vehicle.

6.2.3. Chemicals

Isoflurane (ATTANE, Minrad Inc., NY) is an inhalation anesthetic agent used before cardiac puncture. Ketamine and xylazine were obtained from Alfasan International, Woerden, Holland, which were used as surgical anesthesia before ovariectomy. Antibiotics used after surgery was mainly composed of trimethoprin and sulfadoxine, which was obtained from Dopharma Holding (Raamsdonksveer, the Netherlands).

6.2.4. Assay kits

Both Rat-MID Osteocalcin Enzyme linked Immunosorbent Assay (ELISA) and RatLaps ELISA were ordered from Nordic Bioscience (Herlev, Denmark).

6.3. Methods

6.3.1. Protocol

Thirty C57BL/6 mice of 4 weeks old were sham operated or ovariectomized (OVX). Ovarictomy procedures are described in Section **6.3.2.** Operated mice were then randomly

assigned to one of the three treatment groups. The groups were: 1) sham-operated control group treated with vehicle (i.e. distilled water); 2) OVX group treated with vehicle (negative control) and; 3) OVX group treated with OPR at a dosage of 7 g/kg/day, which is equivalent to 5 times of the clinical dose. After a recovery period of two weeks from operation, a six weeks treatment started. Body weight and diet consumption were recorded every week throughout the study period. Mice were fed by forced feeding with orogastric gavage at a volume of 0.3 ml/10g. Three groups were treated with same volume of vehicle or OPR solution once a day and 7 times a week for six weeks. When treatment ended, mice were fasted 24 hours before scarified. Fasting blood samples (~300 μl) were collected through cardiac puncture after deep inhalation anesthesia, which were used in bone markers measurement. Left femur was collected for bone mineral density measurement and uteri wet weight was recorded. The study protocol is shown in **Figure 6-1**.

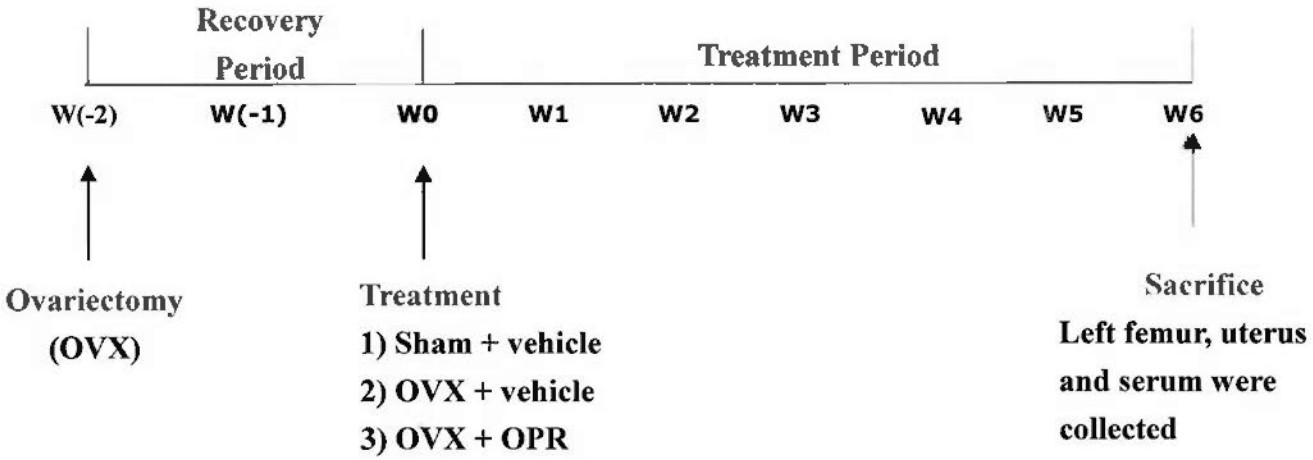


Figure 6-1 Protocol of *in-vivo* study

6.3.2. Ovariectomy

Anesthesia was induced by intraperitoneal injection of 100 mg/kg ketamine (Alfasan) and 10 mg/kg xylazine (Alfasan). After the onset of anesthesia, the lumbar dorsum was shaved bilaterally to prepare for exposed skin for aseptic surgery. For each ovary a 3/4 cm dorsal flank incision penetrating the abdominal cavity was made. The parovarian fatty tissue was identified and retracted. The exposed ovary and associated oviduct (**Figure 6-2**) were severed and removed. A 5-0 absorbable suture (ETHICON Inc., New Jersey) around the severed ovarian vasculature was required to maintain haemostasis. The incision was closed with 5-0 non-absorbable suture (ETHICON Inc., New Jersey) in an interrupted pattern.

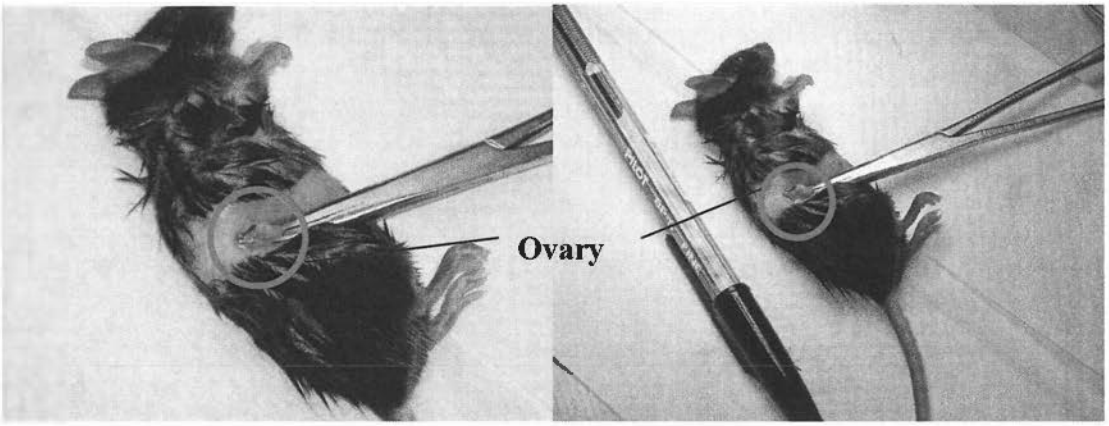


Figure 6-2 Ovariectomy

6.3.3. Samples handling

Blood samples were allowed to clot at room temperature and serums were separated from whole blood by centrifuging (Eppendorf Centrifuge 5415R, Eppendorf, Hamburg Germany) at 16 110 x g for 10 minutes at 4°C. Serum samples were stored at -80°C until analysis. Left femurs were wrapped in wet gauze soaked with saline and stored at -20°C before bone mineral density measurement. Wet uteri weights were measured at once after dissection.

6.3.4. Bone turnover markers measurement

Serum osteocalcin (bone formation marker) and serum C-terminal telopeptides of type I collagen (bone resorption marker), in short form CTx, were measured by Rat-MID Osteocalcin ELISA and RatLaps ELISA respectively. Both kits were purchased from Nordic Bioscience, Denmark. Experiments were done by following instruction in the user's guides and standard ELISA kits procedures.

6.3.5. Bone mineral density measurement

Bone mineral densities (BMD) at the head and distal of femur were measured ex vivo by Peripheral Quantitative Computed Tomography (pQCT) with XCT 2000 (Norland, Germany).

6.4. Statistical analysis

Data were presented as mean ± S.E.M unless otherwise noted. Data were analyzed with GraphPad Prism 4. Results were evaluated by one-way analysis of variance (ANOVA) to determine differences of OPR group with control groups. Bonferroni's Multiple Comparison Test was used whenever necessary as the post-hoc analysis. All comparisons were made two-tailed, and P-values < 0.05 were considered to be statistically significant.

6.5. Results

Twenty-three out of 30 mice completed the experiment. Six mice die during or after (within 48 hours) the operation due to anesthetic overdose or internal bleeding. Twenty-four mice were enrolled in the treatment period and one mouse died during tube-feeding at the first

week.

6.5.1. Body weight and diet consumption

Body weight and diet consumption were measured weekly after treatment began. Changes of body weight during the 6 weeks treatment period are shown in **Table 6-1** and **Figure 6-3**.

Table 6-1 Changes in body weight

Week	Body weight (g)			
	Shamvehicle (n=6)	OVX+vehicle (n=9)	OVX+OPR (n=8)	P-value^
W0	16.33 ± 1.116	17.11 ± 1.16	18.25 ± 0.366	0.4118
W1	17.33 ± 1.116	18.11 ± 1.160	19.25 ± 0.366	0.4118
W2	16.83 ± 0.872[a]	18.89 ± 0.889[ab]	20.00 ± 0[b]	0.0269 *
W3	18.50 ± 0.806	18.67 ± 0.667	19.25 ± 0.366	0.6817
W4	18.50 ± 0.342	19.67 ± 0.167	18.25 ± 0.675	0.0672
W5	18.67 ± 0.333	19.33 ± 0.527	18.50 ± 0.627	0.4978
W6 (fasting)	17.83 ± 0.4014	19.00 ± 0.6236	18.00 ± 0.5	0.2786

^Based on means among three groups using one way ANOVA, for *P<0.05, means in a row without a common letter are statistically differ.

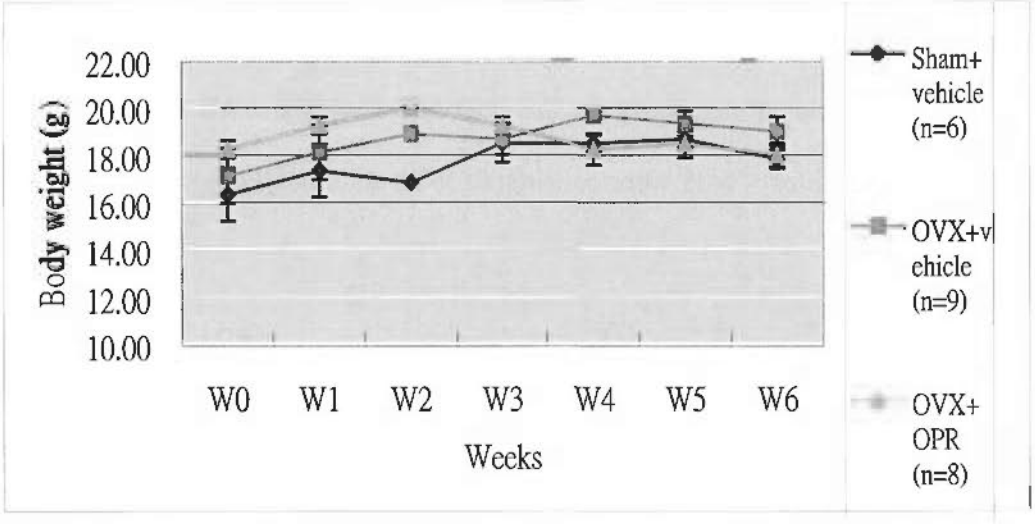


Figure 6-3 Changes in body weight during a study period of six weeks

Three groups of mice shared similar body weight when treatment started. The body weight was not significantly different in all groups except at week 2. At week 2, mice of OVX group treated with OPR were heavier (P=0.0269) compare with those in the sham group. The discrepancy soon disappeared and no significant difference was recorded till the end of treatment. Sham and OVX mice treated with vehicle remained to grow steadily throughout the treatment period while body weight of mice treated with OPR started to drop by 2-5% from week 2 and generally become constant afterwards.

The diet consumption of both sham and OVX mice treated with vehicle shared similar trend and remained quite steadily throughout the treatment period. While diet consumption of OPR treated group dropped almost 10% at week 3 and their food intake started to increase till week 5. Mice of the same group were housed in 1 cage only, so no mean of standard error (SEM) can be obtained. Data are shown in **Table 6-2** and **Figure 6-4.**

Table 6-2 Diet consumption

	Diet consumption (g)		
Week	Sham+vehicle (n=6)	OVX+vehicle (n=9)	OVX+OPR (n=8)
W1	12.33	14.00	14.50
W2	13.33	12.89	12.13
W3	15.00	13.42	14.44
W4	15.00	14.67	10.69
W5	13.00	13.22	10.38
W6	15.50	14.78	12.88

Total n=23

Mice of same group were housed in the same cage, no SEM can be obtained.

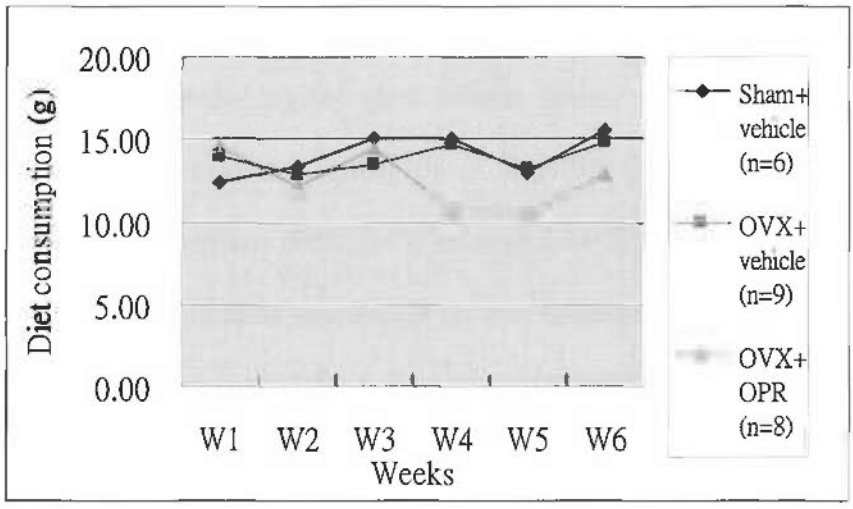


Figure 6-4 Diet consumption

6.5.2. Biochemical markers of bone turnover

Ovariectomy raised the serum osteocalcin level to nearly double ($P<0.01$) in comparison with the sham group, and the level reduced by 30% after 6 weeks treatment of OPR. OPR treatment lowered the osteocalcin level in the OVX group comparative to that of the sham group. In serum C-terminal telopeptides (CTx) measurement, ovariectomy also raised the CTx level by 60% but the increase was not statistically significant ($P=0.142$). Following the same trend as in osteocalcin level, treatment of OPR lowered the CTx level by one-third to a range very near to the sham-operated group. Data are shown in **Table 6-3**, **Figure 6-5** and **Figure 6-6**.

Table 6-3 Level of serum osteocalcin and C-terminal telopeptides

Bone markers	Sham+vehicle (n=6)	OVX+vehicle (n=9)	OVX+OPR (n=8)	P-value^
Osteocalcin (ng/ml)	31.83 ± 4.38^a	62.15 ± 6.20^b	43.95 ± 6.20^a	0.004***
C-terminal telopeptides (ng/ml)	9.15 ± 0.62	15.68 ± 3.20	11.68 ± 1.27	0.142

Based on means among three groups using one way ANOVA, for ***P<0.01, means in a row without a common letter are statistically differ. Total n=23

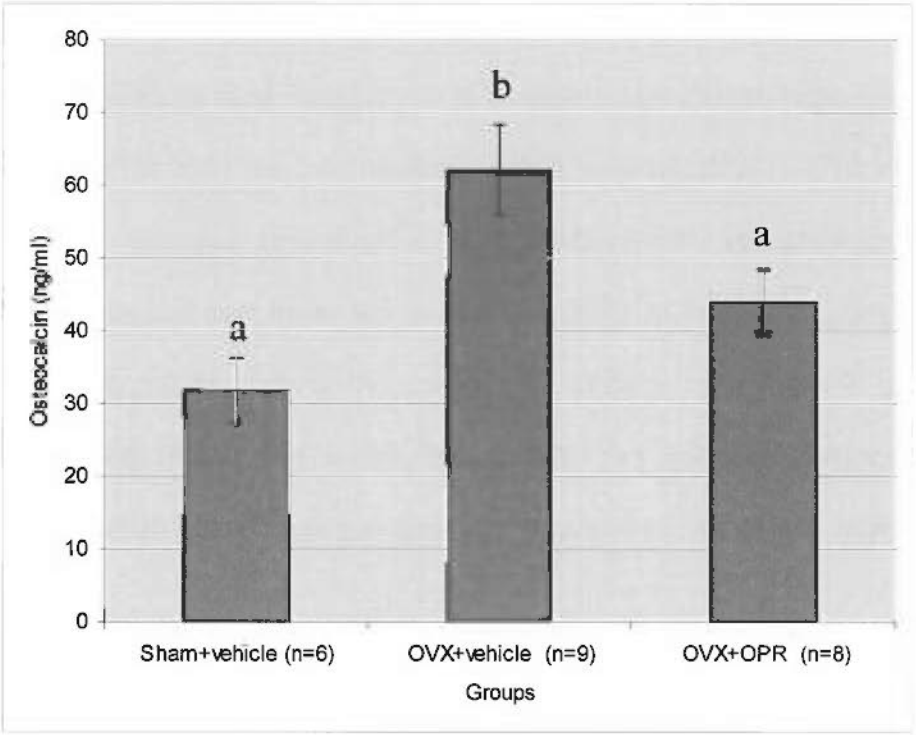


Figure 6-5 Serum osteocalcin level

Means value not sharing a common letter were significantly different (P<0.01).

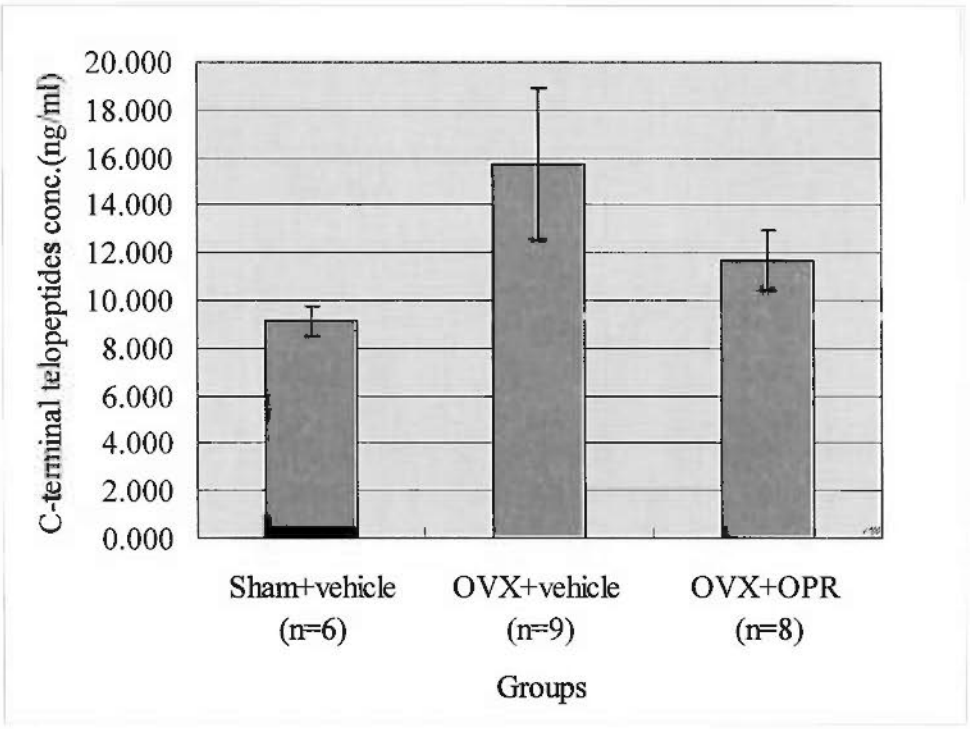


Figure 6-6 Serum C-terminal telopeptides level

6.5.3. Bone mineral density

Bone mineral density was measured at two regions: head and distal of femur (**Table 6-4** and **Figure 6-7**). At the head of femur, marked bone loss ($P<0.05$ by t-test) occurred after ovariectomy, compared with the sham group. The density drop was reduced from 8% to 2% after six weeks treatment of OPR. However, the result was insignificant ($P=0.092$) when performing a more strict analysis of one-way ANOVA. At the distal femur, individual difference within the group was larger in comparison with the measurement at the proximal region. Bone density level of three groups were not significantly different ($P=0.185$).

Table 6-4 Bone mineral density measurement

Bone mineral density (mg/ccm)				
Position	Sham+vehicle (n=6)	OVX+vehicle (n=9)	OVX+OPR (n=8)	P-value^
Head of femur	483.63 ± 12.52	444.84 ± 9.39*	472.83 ± 14.71	0.092
Distal femur	429.04 ± 17.75	420.70 ± 14.46	378.16 ± 25.59	0.185

^ Based on one-way ANOVA, P-value is >0.05; *Based on t-test, P<0.05 compare with sham group.
Total n=23.

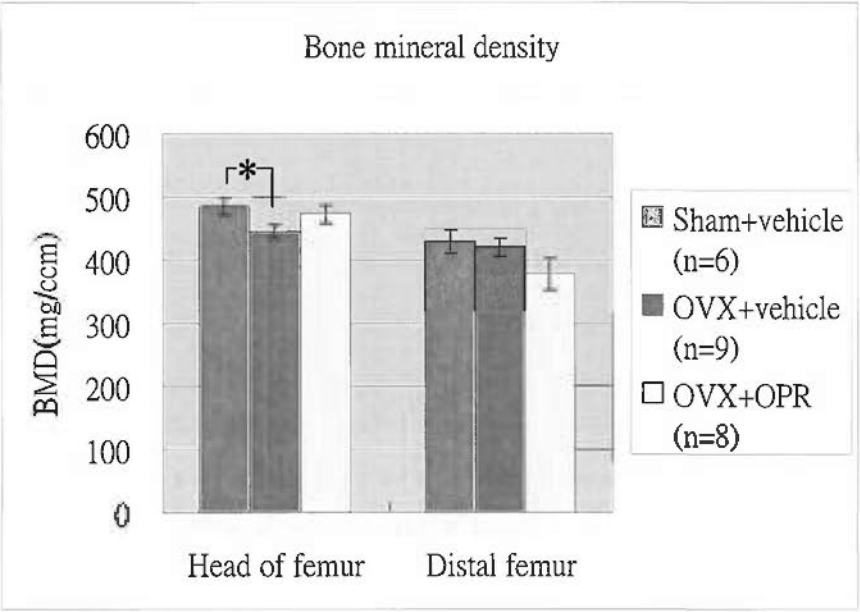


Figure 6-7 Bone mineral density measurement

*P<0.05 compare with sham group by t-test

6.5.4. Uterus weight

Wet uteri weights were expressed in a ratio over body weight. Removal of ovaries reduces the uterine weight drastically (P<0.0001) and treatment of OPR did not affect the uterine weight (**Table 6-5** and **Figure 6-8**).

Table 6-5 Uterus weight per body weight

Uterus weight/body weight ratio (g/g)			
Sham+vehicle (n=6)	OVX+vehicle (n=9)	OVX+OPR (n=8)	
0.117 ± 0.00715^{a}	0.073 ± 0.00408^{b}	0.059 ± 0.00611^{b}	###

Total n=23

^ Based on one-way ANOVA, ###P<0.0001.

For ###P<0.0001, means in a row without a common letter are statistically differ.

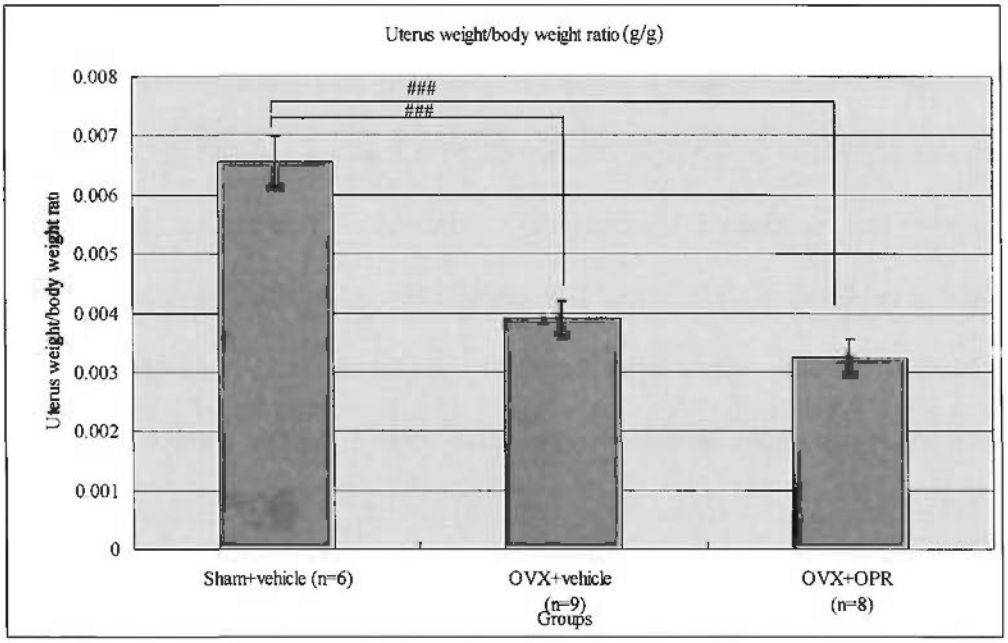


Figure 6-8 Uterus weight

P<0.0001 versus control group

6.6. Discussion and conclusions

In this study, an osteoporosis model was established on 4 weeks-old C57BL/6 mice by performing ovariectomy. A high bone turnover rate was induced on mice, which was indicated by an increase in bone formation marker (osteocalcin) and bone resorption marker level (C-terminal telopeptides of type I collagen). Subsequent bone loss at the head of femur and recession of uterus due to lack of estrogen were also developed. OPR successfully slowed down the high turnover rate of bone by decreasing bone formation and resorption process without increasing the uterine linings. However, its beneficial effect on bone could not be detected on bone mineral density measurement.

In the present study, we have demonstrated the beneficial effect of OPR on bone in ovariectomized mouse model. Study should be performed on other animal species in the future. Although mice exhibit many clinical symptoms and outcomes similar to humans, which include cancellous bone loss after ovariectomy and suspension of bone loss by estrogen replacement, they also possess dissimilarities with humans. Mice are modeling species, their bone grows and is shaped continuously. Human is remodeling species and their bone undergoes a continuous coordinated process of bone resorption, followed by the formation of new bone. Women experience regular menstruations mediated by hypothalamic -pituitary-regulated cycle, which is absence in mice. At the present time, an experimental model that precisely mimics the pathophysiology of postmenopausal osteoporosis is unavailable. Study should be conducted first in ovariectomized rodent model and second in a non-rodent model. Examination of bone quality in two species is necessary to adequately investigate the effectiveness and safety of drugs for osteoporosis (FDA, 1994).

As the study was aimed at the intervention for the treatment of osteoporosis instead of

prevention, the treatment was started two weeks after ovariectomy. A recovery period of two weeks not only provides a healing time, but also allows significant cancellous bone loss following ovariectomy (FDA, 1994). Therefore, a longer recovery period is recommended in later animal study in order to demonstrate a greater decline on bone density prior treatment.

Our findings indicated that diet consumption and body weights of the control and treatment groups were not consistent. The reason was due to a lack of pair feeding. When pair-fed, the daily consumption of diet would be steadily controlled among groups, so as the calcium intake (Furuhata et al., 2002). For diet consumption, data shown in **Table 6-2** might not reflect the true food intake of individual mouse in the group. This is because 3-4 mice were housed in one cage and a large variation of food intake might exist within the group. However, these data could still reveal a correlation between diet consumption and change of body weight. The greatest drop of 5% of body weight at week 3 might be explained by a drop of 10% diet intake in the ovariectomized mice treated with OPR during the same period of time. OPR suspension, which was five times of clinical dose, was turbid and thick in texture. Mice were fed by forced feeding with orogastric gavage at a volume of 0.3 ml/10g which was not far away from the maximum tolerable dose of 0.4 ml/10g (Shi, 1980). Therefore, the texture and dosage of OPR might affect the appetite of the treated mice and it resulted in a drop in diet consumption and body weight.

End point parameters used in this study include measurement of bone mineral density and biochemical markers of bone resorption and formation. General details of these two parameters have been described in Sections **1.6.1** and **1.6.2**. Measurement of bone mineral density by quantitative computed tomography (QCT) is one of the techniques suggested by FDA. QCT is unique amongst methods of bone mineral measurement as it provides separate estimates of trabecular and cortical bone mineral density as a true volumetric mineral density

value. In addition, QCT can measure geometric properties of cortical bone with great accuracy and predict some mechanical properties with remarkable precision. In the study, peripheral quantitative computed tomography (pQCT) was employed to detect bone density level. pQCT is a special type of computed tomography and is initially used to assess bone density at peripheral sites (e.g. wrist) in human. Now XCT 2000, as one of the commercial brand of pQCT, is adapted for use in small rodents (Helfrich and Ralston, 2003).

Tests for bone markers detect products of bone resorption and formation. They are signs of the bone turnover process and provide information of skeletal level. In this study, serum osteocalcin and degradation products from C-terminal telopeptides of type I collagen (CTx) were measured. Osteocalcin is a major non-collagenous protein of bone matrix and it is a specific product synthesized by osteoblasts. After production, it is partly incorporated into the bone matrix and partly delivered to the circulatory system. Circulating osteocalcin is associated with changes in bone turnover and regarded as a specific marker for bone formation (Joffe et al., 1994; Schaller et al., 2004). Serum osteocalcin level increased by 2-fold after ovariectomy and this estrogen-deficiency-induced state was shown to be prevented by treatment of OPR. Another common marker is peptide fragment deriving from collagen type I (CTx) and it is a resorption marker. CTx is an 8-amino acid fragment from the C-telopeptide of type I collagen. Its rate of release from bone is a useful index of the resorbing activity of osteoclasts (Bone, 1993). In the present study, ovariectomy raised the CTx level by 60% whereas OPR lowered the CTx level by one-third, to a level comparable to the sham-operated group. A large individual difference between mice within the same group results in a large standard error. The data were therefore insignificant and similar result was also appeared in other study (Jochems et al., 2005). Choosing osteocalcin and CTx as bone markers is because both of them are bone specific and sensitive markers and can be measured from serum sample.

In this study, only a slight increase in level of C-terminal telopeptides of type I collagen (CTx) is expected as the rapid phase of bone loss that follows OVX will slow down in established menopausal model. No significant difference in uterine weight between OVX vehicle and OVX treatment group indicates that OPR exerts beneficial effects on bone without inducing potentially harmful proliferation in reproductive tissues. A longer recovery period and treatment period may be needed to amplify the BMD difference between sham and OVX groups.

Traditional Chinese Medicine has been used to treat and prevent osteoporosis and osteoporotic fracture for centuries. Many herbs are potential candidates in treating osteoporosis (Kim et al., 2008; Leung et al., 2001; Wang et al., 2008; Wang et al., 2003b; Xie et al., 2005b; Yang et al., 2007; Zhang et al., 2007b). Fructus Ligustri Lucidi, Herba Epimedii and Fructus Psoraleae are three frequently reported herbs with beneficial effects on bones. They are therefore selected as the major components of OPR. Although the beneficial effect of OPR on bone had been demonstrated on ovariectomized mice, the mechanism behind was still unknown. Other published studies on these 3 herbs might provide a clue for the answer. Zhang and his colleagues have done extensive studies on Fructus Ligustri Lucidi (FLL). They reported that FLL extract could prevent OVX-induced high bone turnover rate. FLL increases the intestinal calcium absorption rate and can prevent OVX-induced loss of calcium in rats as well as increasing bone calcium content (Zhang et al., 2006b). Fructus Ligustri Lucidi significantly reduced fecal calcium excretion and induced apparent calcium absorption rate in OVX rats fed with medium and high calcium diet. They showed that the improvement in calcium balance is by increasing serum 1,25 (OH)(2)D(3) level and inducing expression of vitamin D-dependent calcium binding proteins (Zhang et al., 2007a).

Aqueous extract of Epimedii has a definite antiosteoporotic effect and has been assessed

in different aspects which include biochemical test, bone mineral density and histomorphometric parameters in many OVX-induced osteoporosis rats models (Nian et al., 2006b). It suppresses the bone loss possibly by increasing osteoblastic activities and decreasing osteoclastogenesis (Xie et al., 2005a). Researchers believe that the flavonoids contained in Epimedii are the effective component for this activity. Many basic studies, therefore, were performed on flavonoids extract. Flavonoids of Epimedii have a beneficial effect on trabecular bone and the effect is possibly associated with stimulation of bone formation as well as inhibition of bone resorption (Peng et al., 2008). Study shows that total flavonoids of Epimedii improve the bone density, enhance estrogen level and decrease the Interleukin-6 (IL-6) concentration in serum (Jiang et al., 2002). Unlike Fructus Ligustri Lucidi, its antiosteoporotic effect was independent of the enhancement in intestinal calcium absorption (Zhang et al., 2006a). Some flavonoid extracts induce the production of active metabolites in rats, the rat serum would therefore enhance the development of osteoblast-like cells (Chen et al., 2004). Some flavonoids have been identified as the active component responsible for anti-osteoporotic effect. Icariin, one of the major flavonoids of *Epimedium* stimulates the proliferation and enhances the osteogenic differentiation of rat bone marrow stromal cells and is regarded as an effective component for bone-strengthening activity (Chen et al., 2005).

Compared to the two herbs described previously, reports on Psoraleae were relatively fewer. However, its beneficial effect on bone has been suggested. Extract of Psoraleae preserves bone mineral density by increasing bone formation (Tsai et al., 2007). Crude fractions of an acetone extract of Psoraleae showed a significant elevation of the serum inorganic phosphorus bone calcification in non-operated rats (Miura et al., 1996). The anti-osteoporotic action of different Psoraleae fractions has been compared and result showed that no matter the crude ethanol extract, ethyl acetate fraction, or flavonoid fraction would all

stimulate bone formation to a similar extent (Xiong et al., 2003). Similar to Fructus Ligustri Lucidi and Herba Epimedii, some flavonoids of Psoraleae are identified as the active components for stimulating bone formation. However, more studies are needed to confirm such findings (Wang et al., 2001). The combined action of these three herbs have also been studied and they were shown to preserve bone mineral density at the femur neck in a dose-dependent manner on ovariectomy- and calcium deficiency-induced osteoporotic rats (Sun et al., 2008).

In conclusion, OPR was able to slow down the rate of bone turnover induced by ovariectomy in an C57BL/6 mouse model. Results of the study provided scientific evidence of Chinese medicine on the treatment of osteoporosis disease and it may facilitate the use of Traditional Chinese Medicine clinically in treating osteoporosis patients.

7. Linguistic Validation of the Osteoporosis-Targeted Quality of Life Questionnaire

7.1. Introduction

Osteoporosis has long been considered a major public health problem with its sequence of fracture. There has been increasing recognition that osteoporosis represents an important burden as a common cause of morbidity, mortality, and health-care expenditure, especially in postmenopausal women (Dennison et al., 2006). Although the long-term outcomes of osteoporosis such as fracture, kyphosis, and pain are well known, the physical, psychological, and social consequences, beyond fracture and pain, have not been fully evaluated (Martin et al., 2002). These are aspects of quality of life (QoL). Health-related quality of life covers physical, mental, and social well-being. Quality of life may be measured for evaluation of treatment effects in clinical trials, for the assessment of the burden of the disease of osteoporosis, and for estimates of the cost-effectiveness of different treatment scenarios in health care policy (Lips and van Schoor, 2005). According to the Guidelines of Osteoporosis Trials released in 1996 and endorsed by the US National Osteoporosis Foundation (Sambrook et al., 1997), quality of life is considered to be one of the outcome measures for osteoporosis trial. It recommended the QoL instruments should encompass not only for general disease (e.g. SF-36) but also osteoporosis-specific. Six available osteoporotic-specific questionnaires of different focus are available (Tosteson and Hammond, 2002), but none of them consist of a Chinese version.

According to the projection of WHO, the annual number of hip fractures is expected to total 6.3 million by 2050, with 51% of the fractures occurring in Asia alone (Cooper et al.,

1992b). The prevalence of vertebral fracture in elderly women was found to reach 30% in Hong Kong. Using similar protocols for radiography and fracture definition, the prevalence of vertebral fracture in Hong Kong Chinese was found to be similar to American Caucasians (Kung, 2004; Lau, 1997). As more osteoporotic fractures are expected to happen in Asia due to urbanization and aging, evaluation of the impact of osteoporosis on quality of life among Asian is in need. Chinese, being one of the most popular languages in Asia, is chosen to be translated first. A Chinese osteoporosis-specific QoL instrument, therefore, is in a great need to deal with the remarkable potential osteoporosis patients in Chinese population.

The Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) aimed at assessing the physical difficulty, fears, and adaptations to one's daily life. The OPTQoL was developed by E. Lydick and her colleague in Merck Research Laboratories, U.S.A. It contains 22 scored items in three domains – 7 questions in physical function, 9 in adaptations, and 6 in fears – and 3 non-scored questions relating to osteoporotic changes and 7 on health and demographics. OPTQoL has passed the validity and reliability tests and is able to act as a cross-sectional survey tool for assessing the impact of osteoporosis on quality of life in women living in the community. The questionnaire has been translated and culturally adapted into seven languages (Canadian-French, Dutch, French, German, Italian, Spanish and UK English) to allow cross-cultural studies of the community impact of osteoporosis (Chandler et al., 1998). This instrument is unique among osteoporosis-targeted questionnaires because it attempts to measure the total impact of the disease on quality of life within a population at a single point in time (Lydick et al., 1997). Besides, it includes "fear" as one of the domain whereas osteoporosis-related fears have been proven to exert significant percentage of the variation in quality of life for women in midlife (Lydick et al., 1996). The reasonable length (32 questions) and simple layout (3 domains) are favorable to the elderly. OPTQoL has been used in the OFELY study in France to assess the impact of osteoporosis and related factors on

women (Martin et al., 2002). Due to the above reasons, English OPTQoL was chosen to be converted into a Chinese version.

The conversion process is known as linguistic validation. Linguistic validation is a process to produce a target language version that is conceptually equivalent to the source Patient-Reported Outcomes (PRO) instruments and allows data pooling and/or comparison across countries (European Research Group on Health Outcomes, 1996). It is different from translation because it is not simply converting instruments from one language to one or several others which is highly subjective (Chassany et al., 2002; Maria et al., 2005; Ring et al., 2005; Szende et al., 2005). Linguistic validation addresses the same concepts of PRO instruments in all languages in order to make it possible to pool data and compare results across countries in international studies.

This study was a collaboration project between School of Chinese Medicine, The Chinese University of Hong Kong and Department of Occupational Therapy, Tai Po Hospital. The aim of the study is to linguistically validate an English Osteoporosis-Targeted Quality of Life Questionnaire into Chinese OPTQoL and employ it to assess the treatment effects of OPR in later clinical study.

7.2. Methods

According to standard guideline (Acquadro et al., 2004; Chassany et al., 2002), linguistic validation process comprises forward translation, backward translation and cognitive debriefing (**Figure 7-1**). An approval from authors of OPTQoL was obtained. English version of the OPTQoL is shown in **Appendix VI**.

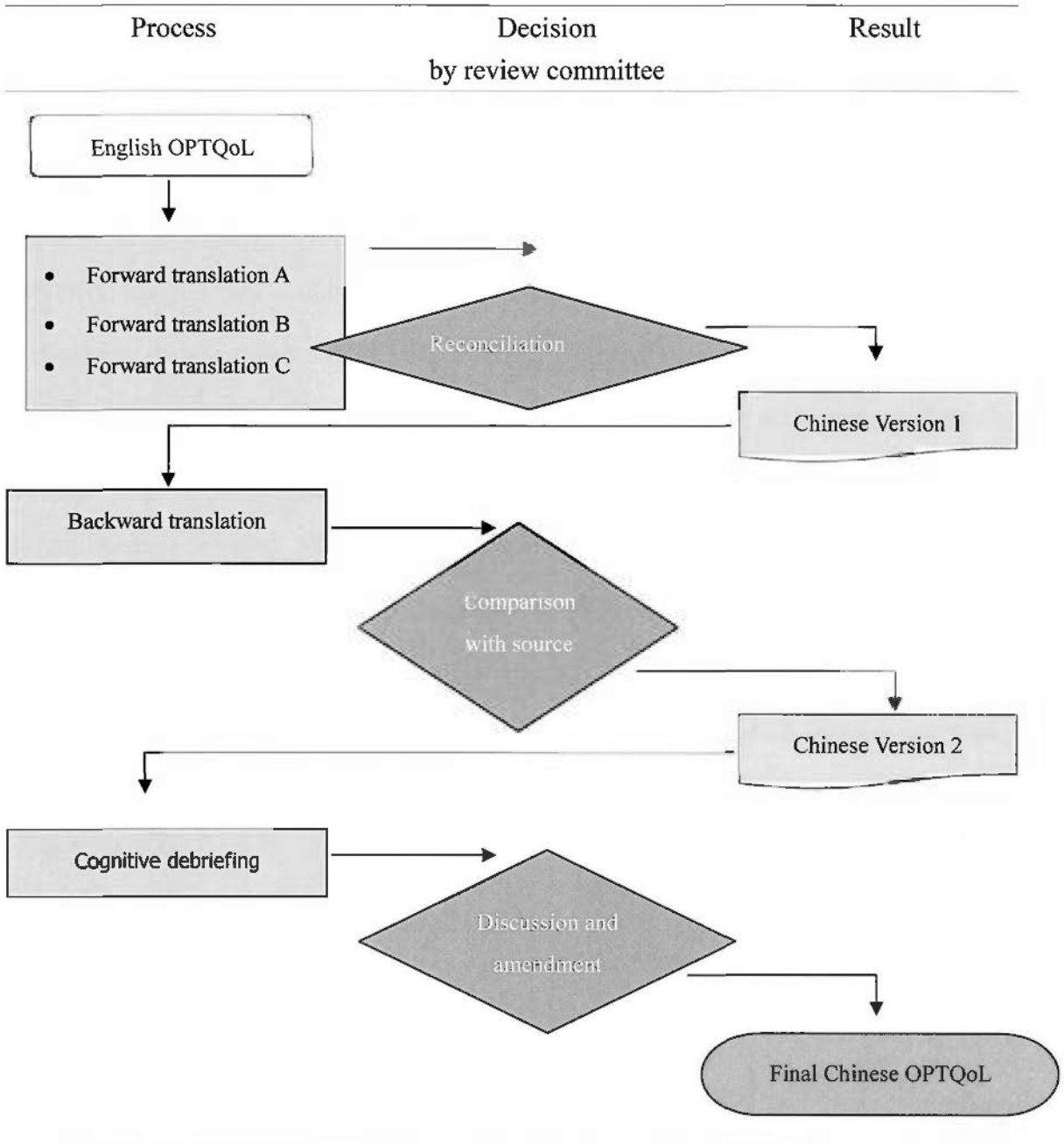


Figure 7-1 Processes of linguistic validation

7.2.1. Forward and backward translation

The Chinese version of OPTQOL was developed through forward-backward translation techniques and committee review. The original English version of OPTQoL was translated into Chinese, and then backward translated into English. The original OPTQOL was translated independently into Chinese by three native Chinese speakers with good command of English and medical background. One of the three translators and one external reviewer, who is a Chemist, formed a review committee to evaluate the quality of three different versions of the translation. This common version of the forward translation was then backward translated into English by a bilingual speaker in Chinese and English. The backward translation was reviewed again by the review committee for conceptual equivalence with the original form. A final Chinese version of the OPTQOL was determined through discussion and comparisons among the original, the forward, and the backward translations by the review committee.

7.2.2. Cognitive debriefing

Cognitive debriefing is a process to collect opinion from a sample of respondents and the Clinician's review. It gets input from people representing future users of the questionnaire, i.e., experts in the therapeutic areas in question, as well as the eventual respondents/patients. There are two procedures occurring in parallel. The aim of the process is to assess the clarity, intelligibility, appropriateness, and cultural relevance of the targeted instrument. The Chinese-OPTQoL was assessed by a group of postmenopausal women (targeted population) and clinicians from orthopaedic and rehabilitation disciplines.

7.2.2.1. Review by target population

The targeted population was Chinese-speaking postmenopausal women who lived in Hong Kong for more than 20 years and were not able to read and speak English. They were asked if they could understand the questions in OPTQoL and rephrase the questions in their own word. Comments from respondents were critically reviewed and questions were modified in syntactical or structural aspects.

7.2.2.2. Review by clinician

A group of bilingual expert panels from Tai Po Hospital who should have at least ten years of working experience in orthopedic and rehabilitation were invited to complete a questionnaire, which aimed at evaluating both face and content validity of the OPTQoL. The questionnaire was designed to study the adequacy, accuracy and ease of understanding of translation, face and content validity of the Chinese-OPTQoL. This questionnaire was sent together with a covering letter and both OPTQoL and Chinese-OPTQoL for reference. A sample of the expert review form is shown in **Appendix VII**. Panel members were invited to judge whether they agreed the translation of each question was accurate or not, by choosing "Agree" or "Disagree". Qualitative comments for modification of translation were also requested if "Disagree" was rated for that item. The percentage of agreement in each question was calculated, and 85 % agreement was selected as the cut-off score for accurate translation. Comments from all panels were critically reviewed and descriptive statistics such as percentage of experts' agreement on the accuracy of translation was also analyzed. Basing on the evaluation by the expert panels, professional translators then further modified and finalized the Chinese-OPTQoL.

7.3. Results

The copyright of OPTQoL belongs to Merck Research Laboratories, Pennsylvania, US and approval had been gained from Dr Barbara P. Yawn. The final version of the Chinese OPTQoL is shown in **Appendix VIII**.

7.3.1. Forward and backward translation

Terminology was the most debatable part during forward and backward translation. Problems encountered when several Chinese terms, which share similar meaning but different wording (志工 *vs.* 義工；擔心 *vs.* 憂慮), were available for forward translation. These wording originated from different regions (Taiwan or Mainland China) and some are commonly used in Hong Kong. Advices from professional translators and linguistic scholars were critical in solving these problems.

7.3.2. Review by target population

Twenty postmenopausal women aged from 58 to 72 (Mean: 70±6.10) were recruited in the pilot test. Among the 20 participants, 15 are osteoporosis, four are osteopenia and one has a normal bone mineral density. Demographic data of the 20 participants are shown in **Table 7-1**. Each question in the questionnaire was cognitive debriefed by 5 subjects, with a total of 20 subjects to review the questionnaire. Subjects agreed that the questionnaire was clear, relevant, and appropriate to the condition. Minor modifications were done by the review committee after the debriefing.

7.3.2.1. Review by clinician

7.3.2.1.1. Linguistic validity

Linguistic validity results are summarized in **Table 7-2**. Three questions in the Chinese OPTQoL (A4, B6 and B9) showed mean scores for clear presentation and understandability below the cut-off score, which required refinement to enhance the questions' clarity and understandability. Another briefing meeting then was held, and the expert panel agreed all subsequent modifications would increase the degree of clarity, comprehensiveness, and comprehensibility of the Chinese-OPTQoL.

7.3.2.1.2. Face and Content Validity

Level of agreement of the panel members on the translation of items of the Chinese-OPTQoL was showed in **Table 7-3**, ranging from 71.4 % to 100%. As 85 % agreement was selected as the cut-off score for accurate translation, five questions (question A7, B2, B5, B9 and C1) with only 71.4 % agreement in the Chinese-OPTQoL were considered not accurate enough, and needed to be further modified.

For content relevancy, the Chinese-OPTQoL was evaluated to see whether the questions were relevant to the quality of life for patients with osteoporosis. As summarized in **Table 7-4**, results showed that panel members agreed that all items were relevant to the content area. The percentage of agreement ranged from 85.7% to 100%, and the median scores were ranged from 4 to 5 in each item. The twenty-two items in the Chinese OPTQoL can be regarded as good items. The results are summarized in **Table 7-5**. The percentage of agreement was 100% and the median score was 4, which indicated all panel members agreed that these twenty items have covered the major constructs and could represent a person's perceived quality of life.

Table 7-1 Demographic data of 20 participants in pilot test

Subject No.	Age	Spine BMD (L1-L4 T-Score)	Hip BMD (Total T-Score)	Diagnosis
1	62	-1.61	-1.94	Osteoporosis
2	77	-2.97	-1.09	Osteoporosis
3	63	-2.69	-2.07	Osteoporosis
4	72	-3.50	-1.28	Osteoporosis
5	72	-3.47	-2.73	Osteoporosis
6	76	-4.31	-1.30	Osteoporosis
7	72	-2.79	-2.16	Osteoporosis
8	77	-2.97	-2.33	Osteoporosis
9	77	-3.51	-2.16	Osteoporosis
11	71	-3.60	-1.80	Osteoporosis
14	73	-5.60	-3.40	Osteoporosis
15	77	-3.00	-2.00	Osteoporosis
16	67	-3.00	-2.20	Osteoporosis
19	58	-2.50	-1.50	Osteoporosis
20	73	-4.50	-3.00	Osteoporosis
10	67	-2.20	-1.80	Osteopenia
13	66	-1.40	-1.10	Osteopenia
17	73	-1.50	-1.00	Osteopenia
18	58	-1.50	-1.50	Osteopenia
12	69	-0.90	-0.30	Normal

Table 7-2 Mean scores of linguistic validity of 22 items of the Chinese OPTQoL

Item No.	Clear Presentation	Understandability	Relevancy to Concept
A1	8.47	8.53	8.67
A2	8.47	8.53	8.13
A3	8.47	8.53	8.13
A4	7.58*	7.07*	8.90
A5	8.47	8.53	8.13
A6	8.02	8.35	7.83
A7	8.02	8.35	7.83
B1	8.73	8.07	8.93
B2	8.73	8.07	8.93
B3	8.42	8.88	8.70
B4	8.97	8.77	8.42
B5	8.97	8.77	8.42
B6	7.30*	7.32*	8.17
B7	9.00	8.85	8.92
B8	8.75	8.22	8.43
B9	7.68*	7.68*	8.45
C1	8.32	8.13	8.07
C2	8.92	8.77	7.10
C3	8.02	8.35	7.83
C4	8.73	8.07	8.93
C5	8.73	8.07	8.93
C6	8.42	8.88	8.70

*Items below the cut-off score.

Table 7-3 Percentage of agreement of 22 items of the Chinese OPTQoL

Item No.	Agree	Disagree	% of Agreement
A1	7	0	100
A2	6	1	85.7
A3	6	1	85.7
A4	7	0	100
A5	6	1	85.7
A6	6	1	85.7
A7	5	2	71.4*
B1	6	1	85.7
B2	5	2	71.4*
B3	6	1	85.7
B4	7	0	100
B5	5	2	71.4*
B6	6	1	85.7
B7	7	0	100
B8	7	0	100
B9	5	2	71.4*
C1	5	2	71.4*
C2	7	0	100
C3	6	1	85.7
C4	6	1	85.7
C5	6	1	85.7
C6	6	1	85.7

*Items below the cut-off score.

Table 7-4 Results of content relevancy of the 22 items of the Chinese OPTQoL

	% of Agreement			
Item	Agree	Neutral	Disagree	Median score*
A1	100	0	0	4.5
A2	100	0	0	4.5
A3	100	0	0	4.5
A4	100	0	0	4
A5	85.7	14.3	0	4
A6	85.7	14.3	0	4.5
A7	85.7	14.3	0	4.5
B1	85.7	14.3	0	4
B2	100	0	0	4.5
B3	85.7	14.3	0	4.5
B4	85.7	14.3	0	4
B5	100	0	0	4.5
B6	100	0	0	4.5
B7	100	0	0	5
B8	100	0	0	5
B9	100	0	0	5
C1	100	0	0	4
C2	85.7	0	14.3	4
C3	100	0	0	4
C4	100	0	0	4
C5	100	0	0	4
C6	100	0	0	4

*Median score represented the degree of clear presentation and content understandability of the Chinese OPTQoL.

Table 7-5 Results of content representativeness of 22 items of the Chinese OPTQoL

	% of Agreement			
	Agree	Neutral	Disagree	Median score
Items 1-20	100	0	0	4

7.4. Discussion and conclusions

In the study, the English version of Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) was successfully translated into Chinese and linguistic validated according to the standard guideline (Acquadro et al., 2004). The English OPTQoL was forward, backward and forward translated to Chinese OPTQoL and cognitively debriefed by 20 postmenopausal women and clinicians in the orthopaedic and rehabilitation disciplines. Final version was obtained after moderate adjustment from the review panel. Abstract of the study had been presented in Conference on Quality of Life Research in Asia, 2006 (**Figure 7-2**). The Chinese OPTQoL could be used in future osteoporosis clinical trial to assess the benefits of other interventions on quality of life among Chinese population.

Due to a lack of validated Chinese language osteoporosis-specific questionnaire, the burden of the disease on Chinese population has been under-studied in health services stream (Ip et al., 2004). Recognition of the impact of osteoporosis-related factors on quality of life (QoL) can help health-care providers more fully appreciate the importance of prevention and treatment. Furthermore, a linguistically validated Chinese OPTQoL allows Chinese speaking regions to be included in international multi-centre studies as the QoL data obtained can now be pooled together. Linguistic validation enables the language version obtained to be conceptually equivalent to the original instrument and to one another; they are culturally relevant and acceptable to the target population within each target country, and they are

psychometrically comparable (Mapi Research Institute, 2007).

During forward and backward translation, it is recognized that some common phrases used in everyday life can be expressed in different wordings. Although Chinese characters are uniform, different spoken language and social settings exist in mainland China, Hong Kong, Taiwan and overseas Chinese communities. Adjustments on the Chinese OPTQoL must be done before using the questionnaire in different countries.

In order to evaluate how well a questionnaire is culturally adapted to the target language, a second phase called psychometric validation can be carried out. It is a statistical evaluation of the properties of the target language versions and can be complemented to the linguistic validation. However, it was not included in the present study as it requires a large sample size to complete. In order to carry out psychometric validation, a group of osteoporosis patients should be recruited. They will be aged from 65 to 85, with no previous major trauma history and mental function deficiency. They will be asked to complete the Chinese OPTQoL on the day after admission to the clinic and again two days after their admission. Such a short interval can ensure there is no significant difference in their perceived quality of life. The reliability of the Chinese-OPTQoL is determined by intra-class correlation coefficient (ICC) and analyzed by the model of one way random. Through power analysis and effect size estimation, one hundred subjects are required. And for further factor structure estimation, sample size of hundred was marginally satisfactory in factor analysis by principal component analysis for construct validation. When the Linguistic Validation process is complemented to a psychometric validation, the questionnaire is said to be cultural adapted to the target language version and the whole process will be completed

In conclusion, the study had linguistically validated an English Osteoporosis-Targeted Quality of Life Questionnaire into Chinese and it is believed to be the only Chinese osteoporosis specific QoL questionnaire available at this time. The Chinese OPTQoL can help other investigators to study the quality of life among Chinese osteoporosis patients and quantify the efficacy of a new interventions on quality of life. Psychometric validation of the Chinese OPTQoL will be carried out later on to refine the validity of the questionnaire.

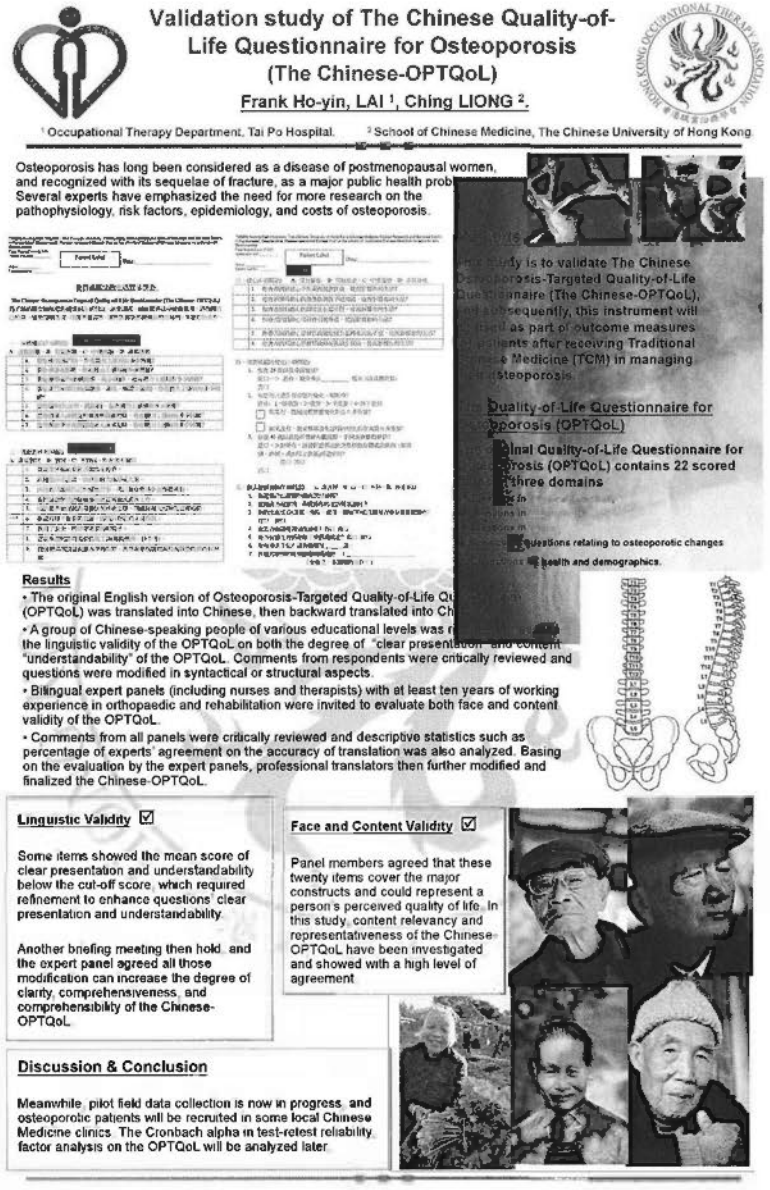


Figure 7-2 Poster presentation of the study on the Chinese OPTQoL

8. A Pilot Clinical Study of a Kidney-Tonifying Herbal Formula on Postmenopausal Women with Osteoporosis

8.1. Introduction

Osteoporosis is a global public health problem. Worldwide, the lifetime risk for a woman to have an osteoporotic fracture is 30-40%. In Asia, hip fracture is expected to be a major public health challenge in this century (Lau et al., 2001). In Hong Kong, the incidence rate of osteoporotic hip fracture had been doubled in the past 3 decades to a ratio of 10 per 1000 in 70 year-old women or older. It is expected that half of the hip fractures in the world will occur in Asia in a very near future (Lau and Cooper, 1996).

With rapid ageing of the Asian population, osteoporosis has become one of the most prevalent and costly health problems. About 20% of patients sustaining an osteoporotic hip fracture will die within a year and almost two-thirds remain disabled (Cummings et al., 1985). As a result, over 1% of the total hospital budget in Hong Kong has been used on osteoporotic fractures and it was projected that this cost will rise exponentially as the population ages (Lau, 2002). Although many medications are approved to treat osteoporosis, side effects of long term treatment shown by many studies are always a concern for most Asian women (Section **1.8.2**). Moreover, drugs for treating osteoporosis are expensive, it is doubtful if many countries in Asia can sustain the high costs for ageing populations. In Hong Kong, bone scan (diagnosis) and anti-osteoporosis medications (treatment) are self-financed. The high cost discourages elderly from enjoying proper medical care on their bone health.

Traditional Chinese Medicine (TCM) has been widely accepted to treat and prevent osteoporosis and related fracture for centuries among the Chinese population in many Asian countries. Clinical effects of TCM herbal formula on osteoporosis has been widely reported (Huang and Chen, 1996; Ma et al., 2005; Putnam et al., 2007; Qiu et al., 2004; Shen et al., 1994; Shen and Shi, 2005; Sun and Shen, 2004; Wang et al., 2007a; Wang et al., 2006; Wang et al., 2007b; Xu and Lawson, 2004a; Xu and Lawson, 2004b; Yao and Jiang, 2003; Zhu et al., 1999). Wang and his colleagues had done a comprehensive study on a TCM formula, Gushukang *in-vitro*, *in-vivo*, and clinically (Li et al., 2001; Wang et al., 2007b). However, the clinical study did not follow proper trial design and lack of a control group for comparison. Moreover, the herbal formula was not a fixed prescription which is not favorable for a scientific study (Xu and Lawson, 2004a).

A more scientific study was an eight month-paired study, which was carried out in Australia to study the effect of a herbal formula, Shu Di Shan Zha. It was a cross over study and the menopausal women participated would switch from treatment group to control group, or *vice versa*, after a period of treatment. As osteoporosis is a slow bone deteriorating process, the prolonged effect of herbal medicine, which was believed to be the privilege of TCM, could not be shown in a cross over study (Xu and Lawson, 2004a).

Traditional Chinese Medicine, regarded as part of the Complementary and Alternative Medicine (CAM), has been criticized of its placebo-related effects. CAM therapy is often regarded as "not tested in large, randomized, placebo-controlled, double-blind clinical trials published in high-quality peer-reviewed medical journals" (http://www. newsweek.com). Dr R. Barker Bausell, former director of research at the University of Maryland's Center of Complementary and Alternative Medicine also had made a strong statement that CAM therapies have an impact on health ONLY by placebo-related effects (Bausell, 2007).

Therefore, a well-planned double-blinded, randomized, placebo-controlled clinical trial (RCT) is desirable in order to provide evidence for the efficacy of TCM on postmenopausal osteoporosis.

In previous *in-vivo* and *in-vitro* study, OPR was shown to be effective in treating postmenopausal osteoporosis. Water extract of OPR increased the rate of cell differentiation and proliferation of osteoblastic cells, UMR 106, in comparison with the negative control group. Although the mechanism of OPR on osteoblast is still unknown, the presence of estrogen receptors on UMR 106 cell line (Davis et al., 1994) suggested the possibility of the presence of estrogenic activity of OPR. To further our study (Chapter 5), we employed a MCF-7 reporter gene assay, which had been transfected with an estrogen responsive element (ERE), as a screening tool to verify the presence of estrogenic-like activity of OPR. OPR was shown to trigger the luciferase activity of the reporter gene in a dose dependent manner. For *in-vivo* study, high bone turnover rate was induced by ovariectomy in young C57BL/6 mice which mimic to a state of post menopause. OPR was shown to slow down the estrogen-depleted condition by decreasing the serum osteocalcin and serum C-terminal telopeptides of type I collagen without altering the growth of uterine lining. Although animal studies on bone might demonstrate certain extend of efficacy, clinical trial must be performed. When natural products or agents already approved for human use, a human trial are likely to be the next logical step.

The study aimed to: (1) record the potential adverse effects, if any, of OPR on the general health of postmenopausal women, (2) document abnormal effects of OPR on human hematologic, hepatic and renal functions, and (3) disclose potential problems encountered when performing clinical trial on Traditional Chinese Medicine.

8.2. Ethics approval

The study captioned "A randomized double blinded, placebo-controlled clinical trial in the effects of traditional Chinese medicine for patients with osteoporosis" was approved by the Clinical Research Ethics Committee of the Kowloon West Cluster (KWC-CREC) on 3 March 2005 with a CREC reference of KW/FR/04-028 (**Figure 8-1**).

8.3. Protocol

The protocol was developed by the collaboration between the clinical investigators and colleagues in the School of Chinese Medicine, The Chinese University of Hong Kong and Kwong Wah Hospital.

8.3.1. Study designs

The study follows a double-blind, placebo-controlled, randomized clinical trial design. Patients fulfilling the inclusion criteria will be randomized into the treatment group and control group. Subjects in two groups receive study treatment or control treatment. Patients in the two arms will be assessed per month throughout the whole trial to compare the outcome. The clinical trial is designed to evaluate the efficacy of the herbal formula, OPR on postmenopausal osteoporosis women.

醫院管理局
HOSPITAL AUTHORITY

群策群力精神人・優質醫護合林
Quality Patient-Centred Care Through Teamwork

Our Ref : ( ) in KWC/GR/REC/FR/04-028

14 March 2005

Dr CHAN Ming-hoang
Consultant
Department of Medicine & Geriatrics
Kwong Wah Hospital

Dear Dr CHAN,

CREC Reference : KW/FR/04-028
A Randomized, Double-blind, Placebo-controlled Clinical Trial in the Effects of Traditional Chinese Medicine for Patients with Osteoporosis

I am pleased to inform you that the above-mentioned research application and the following documents have been reviewed and approved by the Clinical Research Ethics Committee of the Kowloon West Cluster (KWC-CREC) on 3 March 2005 through a full review process.

You are required to adhere to the following conditions:

1. Do not deviate from or make changes to the research protocol without prior written approval of the KWC-CREC except when it is necessary to eliminate immediate hazards to the research subjects or when the changes involve only logistical or administrative aspects of the research.
2. Report to the KWC-CREC in the event of the following:
   a) change in research protocol or consent documents;
   b) unanticipated problems or serious adverse events; and
   c) new information that may adversely influence the risk / benefit ratio or affect a subject's willingness to continue participation in the research.
3. Report research progress to the KWC-CREC at 12-monthly intervals or upon research closure if the research duration is less than 12 months.
4. Send a copy of your final report to the KWC-CREC for record upon research completion.

Please quote the CREC Reference *(KW/FR/04-028)* in all your future correspondences with the KWC-CREC, including submission of progress reports and requesting for amendments to the research protocol.

If you have any enquiry, please feel free to contact Miss Dawn LEUNG on 2990 1039. Thank you for your attention.

Yours sincerely,

(Dr TSAO Yen-chow)
Chairperson
Clinical Research Ethics Committee
Kowloon West Cluster

c.c. COS(M & O), KWH

Secretariat of Clinical Research Ethics Committee, Kowloon West Cluster
Room 133, Block J, Princess Margaret Hospital, Lai Chi Kok, Kowloon, Hong Kong Tel (852) 2990 1039 Fax (852) 2990 1059

Figure 8-1 Approval letter of the clinical trial

8.3.2. Subjects' recruitment

Postmenopausal women were recruited from two sources: Referral from out-patient clinic of Kwong Wah Hospital, Tung Wah Groups of Hospitals (T.W.G.Hs.) and recruitment activities in T.W.G.Hs. Wong Cho Tong District Elderly Community Centre. Referral form and poster of recruitment are shown in **Table 8-1** and **Figure 8-2**. Subjects' recruitment flowchart is shown in **Figure 8-3**.

Figure 8-2 Poster for subjects' recruitment

Table 8-1 Referral form for subjects' recruitment

Referral of patients to KWH CRSC TCM research: "A Randomized, Double-blind, Placebo-controlled Clinical Trial on the effects of Traditional Chinese Medicine for patients with Osteoporosis"

Subject's details

Patient label

Referral

The above patient is now referred to a clinical research held by KWH CRSC named:
"A Randomized, Double-blind, Placebo-controlled Clinical Trial on the effects of Traditional Chinese Medicine for patients with Osteoporosis"

Assessments/ tests have been done:

Proposed assessments need to be done

Assessment	
Vital Signs, Height Measurement & Incidence of fracture	( )
SF-36 Health Status Questionnaire	( )
TCM data collection	( )
Lab test	
BMD measurement	( )
Bone marker test (serum)/ml	( )
Complete blood test/ml	( )
Liver and renal function test/ml	( )
Serum calcium/ml	( )

This form is completed by: _____ Signature: _____

8.3.2.1. Inclusion criteria

1. Women aged 55 or above;
2. Women who was postmenopausal for at least two years;
3. Women who was osteoporotic at lumbar spine or total hip using WHO criteria;
4. A written informed consent was signed before initiation of trial;
5. Women who fulfill the diagnostic criteria of Kidney-Vacuity Syndromes listed in the "Chinese Medicine New Drug Clinical Research Guidelines" published by The Ministry of Health, People's Republic of China, 2002.
   中醫肝腎不足證證候診斷標準 -
   主症：腰脊疼痛，酸軟少力
   次症：不能持重，目眩，舌質或偏紅或淡

8.3.2.2. Exclusion criteria

1. Major medical problems that would preclude participating in a trial lasting for two years;
2. Severe malabsorption syndrome;
3. Prior treatment on bone pathology within one month;
4. Severe psychiatric or addictive disorders;
5. Poor compliance to the study protocol.

8.3.3. Randomization method

Subjects are randomized using the following method:

1. Use the PROC PLAN program in the SAS computer software, according to ratio of 1:1 in number of subjects between the two groups, and then divided subjects into two groups.
2. Prepare randomization cards (including a sequence number, randomization number, group, treatment method), according to the result
3. Prepare opaque envelops with the same sequence numbers as the randomization cards, put the randomization cards into the envelops and keep the envelops according to the sequence number

4. All envelops are managed by one person who does not involve the assessment of subject. When a subject is recruited into the study, according to the sequence number, take out one envelope, assign patient into one group and give treatment as prescribed by that randomization card.

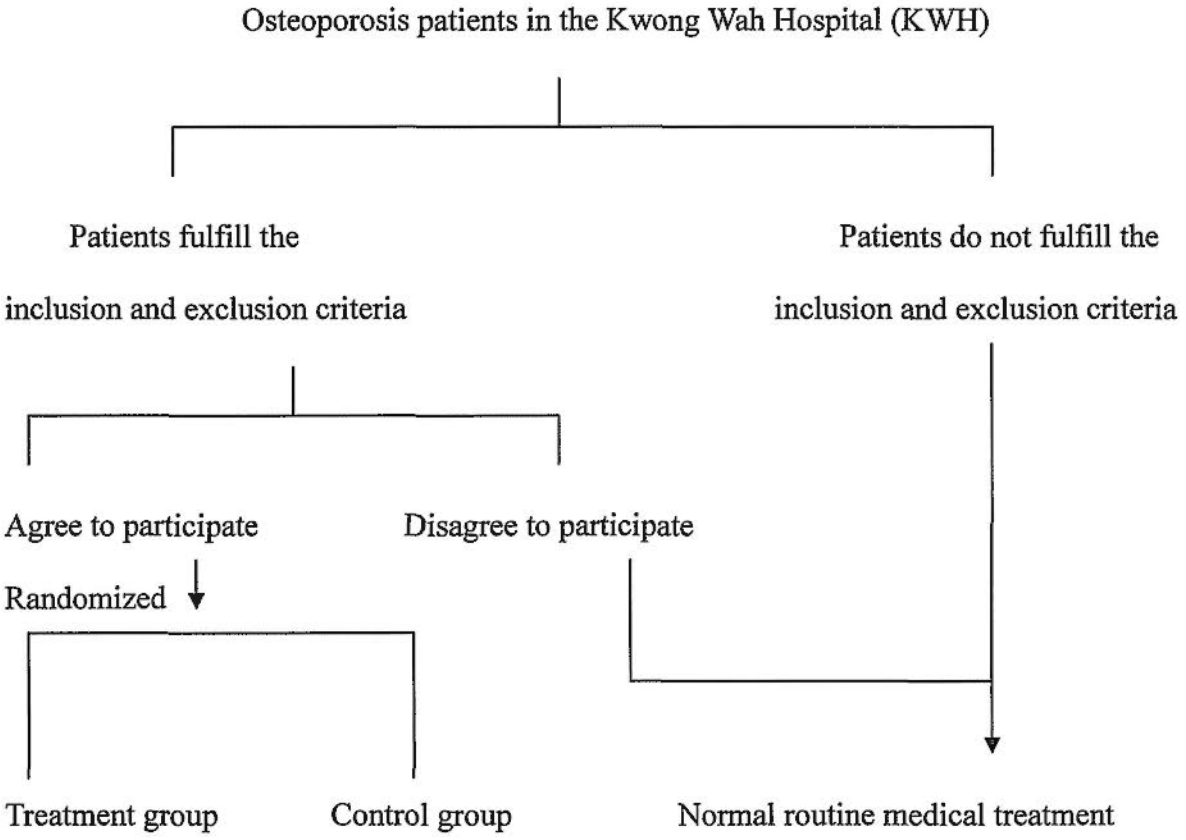


Figure 8-3 Subjects' recruitment flowchart

8.3.4. Sample size

By summarizing statistical data in published studies related to the use of TCM herbal formula on osteoporosis patients (Section **1.9.2**), a sample size of 40 subjects in each group was calculated and it would provide a power of 80% and a 5% significance level to detect a 20% difference in bone mineral density between treatment and control groups.

As a lost rate of 10% was predicted, the sample size was estimated as 45 in each group with a total of 90. These 90 subjects will be recruited and randomized into the treatment and control groups in a ratio of 1:1.

8.3.5. Treatment

For the TCM treatment group, Chinese herbal powder will be given. For the control group, placebo in a powder form with an appearance and the dosage comparable to the Chinese herbal powder will be given to the patients. Patients in both groups need to take in one pack twice a day. All participants will be evaluated at scheduled time slots every 2 weeks. Patients recruited in the research will need to finish a treatment period of 6 months. The research will last for 2 years.

8.3.6. Problem of drug interaction

As the recruited subjects are patients of age 55 or above, they may suffer chronic disease like cardiovascular disease, diabetic mellitus etc. They may need to take related medication. These drugs may or may not interact with the Chinese herbal preparation. Instruction needed to be given to the patients for proper intake of Western and Chinese medicine. Patients are required to take Chinese medicine at least 2 hours apart from western medicine. Moreover, all the dosage and kinds of drugs patients taken during the study period will be recorded, so as to evaluate the treatment outcome and the possible adverse reaction.

8.3.7. Outcome measurements

1. Bone mineral density (BMD)

BMD changes at lumbar and hip were measured by Hologic QDR-4500SL Dual X-ray Bone Densitometer (HOLOGIC, Massachusetts, US). BMD measurements need to be done before and after the treatment (i.e. baseline and six months after the treatment). Also, two more bone scan will be done after the treatment stop for 1 and 2 years, which is to detect any prolonged effect of the herbal powder.

2. Biochemical markers of bone turnover

Two bone resorption markers (type I collagen cross-linked: N-telopeptide & C-telopeptide) and two bone formation markers (osteocalcin and bone-specific alkaline phosphatase) both in serum were measured at the Department of Pathology, Kwong Wah Hospital by using commercial kits before and after the treatment. Schedule of BMD and bone markers measurement is shown in **Table 8-2**.

Table 8-2 Schedule of measurement of bone density and bone markers

	Baseline (T0)	1st month (T1)	3rd month (T3)	6th month (T6)	12th month (T12)	24th month (T24)
	Treatment				No treatment	
BMD Measurement	✓	✗	✗	✓	✓	✓
Bone marker test (Serum)	✓	✗	✗	✓	✗	✗
Bone marker test (serum)	✓	✗	✗	✓	✗	✗

✓ represents the test will be carried out; ✗ represents the test will not be carried out.

3. Incidence of new fractures: Detection of new fractures by the use of X-ray.
4. Health outcome questionnaires: SF-36 Health Status Questionnaire, Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL)
5. Height and stature: Height can be used as a surrogate for vertebral fracture as loss of height is a feature in vertebral osteoporosis.
6. TCM Kidney-Vacuity Syndromes scoring questionnaires will be used to assess the disease progress
7. Other laboratory tests: Serum calcium and hematologic, hepatic and renal functions were carried out regularly.

8.3.8. Assessment methods

Subjects' recruitment and assessment are carried out by doctors in the KWH. Although placebo control is available in this study, in order to ensure no bias in the study, all the assessments of outcome measurements will be done by individual blinded assessors. The specific diagnoses and symptoms in traditional Chinese medicine will be assessed and recorded by the traditional Chinese medicine practitioner in the TWGHs Kwong Wah Hospital – The Chinese University of Hong Kong, Chinese Medicine Clinical Research and Services Centre (CRSC).

8.3.9. Adverse events

Any adverse effects during study period will be recorded and treatment will be given when necessary. Subjects with abnormal laboratory blood results will be followed up until blood result become normal. All adverse effects will be assessed to see if they are related to the treatment in the study.

8.3.10. Treatment compliance

Patients will receive guidance about drug administration method. The dosage and frequency of drug taken will be recorded. Missing dose of TCM herbal formula, if any, will also be recorded.

8.3.11. Ethical consideration

In this study, subjects receive adequate information: include the benefit and adverse effect they may have after they take the herbal formula (**Appendix IX**). They also understand that they will not deliver herbal powder if they are allocated to the placebo group. The information provided enable subjects to make a decision.

As investigators, we totally respect the decisions of the individual and try our best to protect them from harm and secure their well-being by following strictly to the study protocol. Adverse event, if any, will be handled immediately and our experienced medical staffs will provide professional guidance or treatment if necessary.

The selection of trial subjects is completely fair and able to represent of the population that is likely to benefit from the research. No patient will be recruited if he/she has participated in another clinical trial. All subjects must sign the informed consent (**Appendix X**) before being recruited into the study, if subject are illiterate they must agree all the detail about the study, before they sign the consents by their guardian. This study will seek approval from the ethical committee before commencement.

8.3.12. Data record and analysis

The study will have a uniform case report forms (**Appendix XI**) for data collection. All data will be analyzed by SPSS program. Data analysis details and methods:

1. Baseline variables comparison and analysis
2. Intra-group pre and post treatment variables comparison and analysis
3. Inter-groups treatment outcome data comparison and analysis
4. Combined comparison and analysis in all inter-groups data during different assessments periods
5. Intention-to-treat analysis
6. Comparison between treatment outcome and TCM diagnoses, signs and symptoms

The statistical methods used in this study will be Wilconxon, Wilconxon ran-sum test, Chi-square test, student's t test, repeated measure analysis, etc.

8.4. Study progress

Due to a series of adverse events occurred, the clinical trial was suspended after recruiting eight subjects for two months. Although the trial could not be accomplished, several preparation and follow up have been done and they were described as follow.

8.4.1. Preparation of OPR for clinical trial

Eu Yan Sang (Hong Kong) Ltd. was responsible for the preparation of OPR granules as described in Chapter 3. There were several adjustments on the granules before clinical use.

8.4.1.1. Determination of clinical dosage of OPR granules

With an extraction rate of 22.41% (Section **3.3.1**) from raw materials to granules, a daily granule uptake for each subject should be 56 g. However, Eu Yan Sang suggested to reduce the daily dosage by half as they believed that modern extraction method could extract more useful substances from the crude herbs compare with traditional decocting method. To verify

their suggestion, we had performed High Performance Liquid Chromatographic system (HPLC) profiles on both the decoction and the granules to compare the content of 2 major markers: isopsoralen(異補骨脂素) and icariin (淫羊藿苷) from Fructus Psoraleae and Herba Epimedii respectively. Fructus Psoraleae and Herba Epimedii are two major components in OPR formula.

The objective of the study is to determine a clinical dose for OPR granules by comparing the markers content in decoction and granules by using HPLC profiles. HPLC profiles of the OPR decoction from crude herbs and OPR granules produced by Eu Yan Sang are shown in **Figure 8-3** and **Figure 8-4.** Two markers: isopsoralen and icariin are marked at the corresponding peaks.

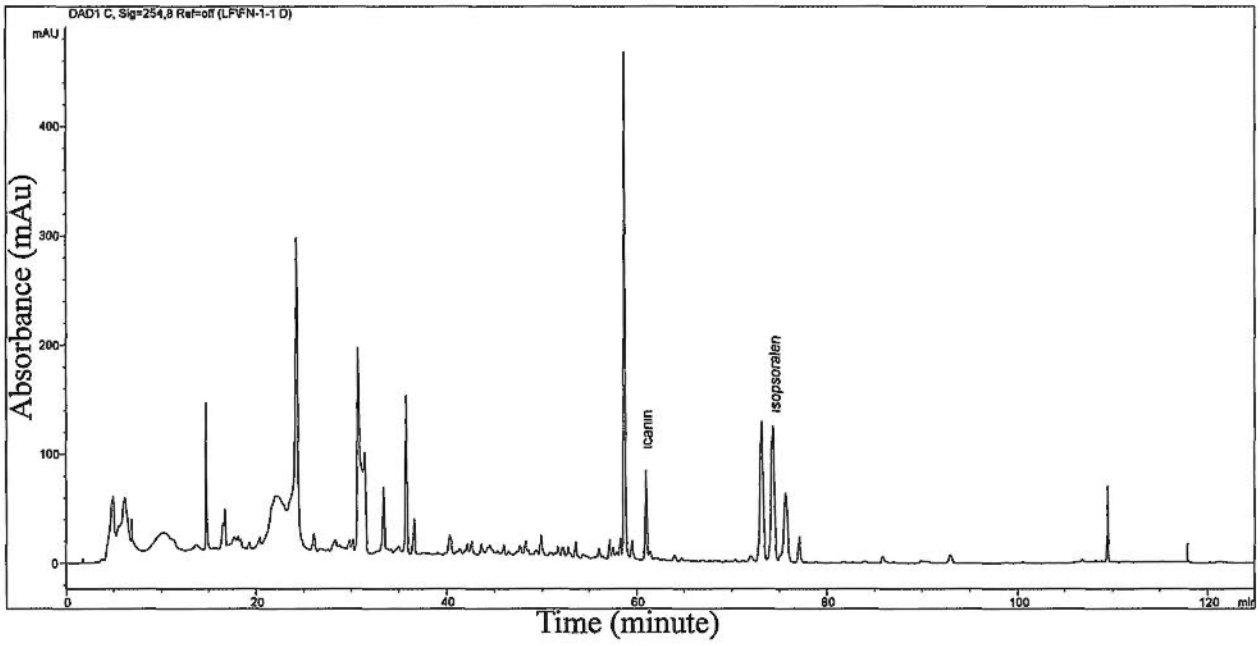


Figure 8-4 HPLC profiles of OPR decoction

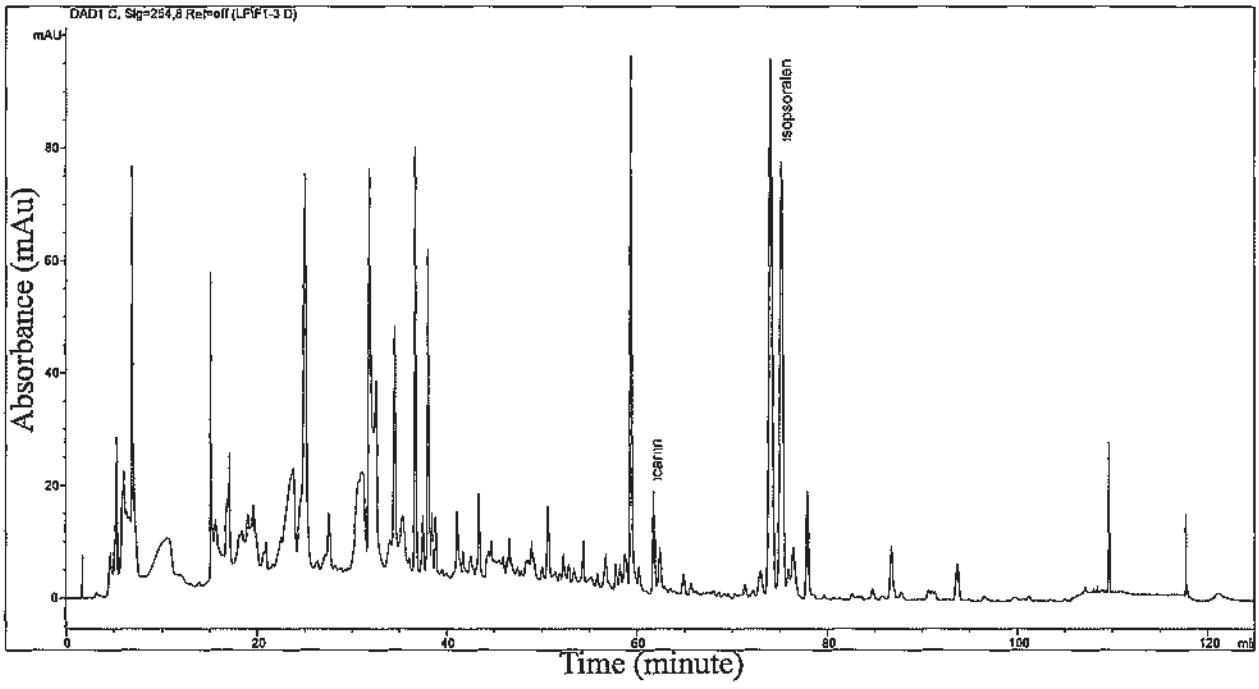


Figure 8-5 HPLC profiles of OPR granules

Table 8-3 Comparison of the content of two markers in the decoction and the granules

	Decoction/day	Granules/g	Granules/day
Isopsoralen (units)	550400	18470	29.80
Icariin (units)	198400	2290	86.64

Table 8-3 shows that when OPR formula was prepared in traditional decocting method, the content of isopsoralen taken by a subject per day was 550400 units. As the content of isopsoralen in 1 g of granule was 18470 units, a dosage of (550400 units/18470 units) 29.80 g/day of OPR granules would already achieve the same marker content as OPR decoction, which was nearly a half of the original proposed dose of 56 g. However, the result of another marker icariin was opposite. A dose of 86.64 g/day of OPR granules was needed in order to achieve the same icariin content as the decoction. As the results were not consistent, a clinical dose for OPR granules was maintained as 56 g/day after intensive discussion.

8.4.1.2. Rejection of lactose as pharmaceutical additive

Lactose was used as a stabilizing additive of OPR granules at the initial phase of the trial. Symptoms appeared on 7 out of 8 patients between 30 minutes to 2 hours after taking the granules. Symptoms included abdominal distention and diarrhea. Since the drug is lactose-based, and around 15-20% Chinese population has lactose intolerance (Sung, 1972), it was suspected that the side effect was caused by lactose. To exclude the possibility that the side effect was caused by the OPR formula itself, OPR decoctions were distributed to the 8 subjects involved in last trial phase. The OPR decoctions were prepared by the electronic decoction system at The Chinese Medicinal Clinical Research and Services Centre at Kwong Wah Hospital, which mimic the traditional decocting method. New consent form was signed and the subjects were under intensive monitoring. Trial would be suspended immediately if diarrhea or other adverse events occurred. No adverse event was observed on the subjects after taking the decoction for five days. Eu Yan Sang was liaised and dextrin was used as the stabilizing additive instead of lactose for the second lot granule production.

8.4.1.3. Package of OPR

Granules were fully sealed in aluminum packages with a net weight of 28 g per pack. Project code, expiry date and instruction for intake were clearly printed on package labels as shown in **Table 8-4**.

Table 8-4 Label of OPR granules

項目代號 Project Code: OPR
過期日 Expiry Date: 02.2008
用量：每天兩次，每次壹包，或按醫師指示服用。
用法：撕開小包，將顆粒放入容器中，加熱水攪拌至溶解後，即可服用。
儲存：置於陰涼乾燥處
注意：避免兒童服用。
Dosage: Take one sachet twice a day, or as directed by practitioner.
Direction: Empty the contents of one sachet into a cup. Stir granules in hot water until dissolve.
Storage: Store in a cool and dry place.
Caution: Keep medicine out of reach of children.

8.4.2. Preparation of placebo for clinical trial

To prepare for a double-blinded, randomized, placebo-controlled clinical trial (RCT), a placebo which can "blind" the subjects successfully is a key to success. The quality of placebo in an RCT can affect not only patients' physical and psychological response to a particular intervention, but also the trial setting, the success of patient blinding to the intervention, and therefore the outcome of the study and the efficacy of treatment in general (Brinkhaus et al., 2008).

The placebo used in a trial with herb as an intervention should be able to mimic the unique appearance, smell and taste of herb but without herbal component. Amount of artificial additive should be minimized for the benefit of subjects' health. Several pharmaceutical companies had provided placebo samples for consideration. Comparison had been done and report was as follow.

8.4.2.1. Rejection of decaffeinated coffee powder as placebo

PuraPharm, a Chinese Medicine production company, provided decaffeinated coffee powder as a placebo for the clinical trial. As decaffeinated coffee is not caffeine-free (McCusker et al., 2006), presence of caffeine in placebo was confirmed by Gas chromatography-mass spectrometry (GC/MS) and the content of caffeine was determined by capillary gas chromatography equipped with an FID detector by colleagues at School of Chinese Medicine, The Chinese University of Hong Kong. The content of Lipton yellow label tea bag was also determined for parallel comparison. Result showed that caffeine in a daily intake of 56 g placebo (daily dosage of OPR granules) is equal to that in 17.8 grams of Lipton Yellow Label tea bag. Details of the experiment are shown in **Appendix XII**.

Role of caffeine plays in osteoporosis is controversial (Hallstrom et al., 2006; Lloyd et al., 1997). It is thought that by increasing the urinary excretion of calcium that the consumption of caffeine may reduce bone mineral density and subsequently increase the risk of fracture (Barrettconnor et al., 1994), whereas some studies show that caffeine intake is not a significant risk factor for osteoporosis, particularly in women who consume adequate calcium (Conlisk and Galuska, 2000; Cooper et al., 1992a). As the role of caffeine on osteoporosis is still unknown, the use of decaffeinated coffee powder as the placebo was rejected.

8.4.2.2. Evaluation of placebos

Two placebos were provided by Eu Yan Sang (Hong Kong) Ltd. and comparison with OPR granules was done. Twelve voluntary participants were invited in the comparison test. Placebo 1, placebo 2 and OPR granules were placed on 3 identical dishes. Participants were asked to compare one of the placebos with the OPR granules but they were blinded from the

samples' identity. Participants were requested to fill in a questionnaire as shown in **Table 8-5**. Granules' smell, color, size, texture and taste were evaluated before and after dissolution, and a mark was given instantly. The mark ranged from 1 to 5 and "5" represented the most dissimilar and a mark of 4 or above was set as a cutoff point, which represented a fail in the item listed in the questionnaire. All granules were dissolved in warm water at a concentration of 100 mg/ml. At the end of the test, participants would make a guess of the "true" herbal granules on the two samples tested.

Results (**Table 8-6**) shown that placebo 1 failed in 6 items, it was differ from the OPR granules in the aspects of smell, color and taste no matter before or after the dissolution. Placebo 2 had a better performance but still failed in the color and taste aspects. About one-third of the participants believed placebo 2 as herbal granules whereas only 12.5% of participants had the same thought on placebo 1.

The test indicated that placebo 1 was very different from OPR granules and could not convince people to consider itself as a herbal product. For placebo 2, it could not mimic the unique taste of herbs but it might have a higher chance to persuade others as a "true" medicine when standing alone. In conclusion, modification was needed on taste, color and smell aspects to increase the similarity of placebo with OPR granules. Progress of placebo preparation stopped at the present stage as trial was terminated due to adverse events occurred.

Table 8-5 Evaluation of the similarity between placebo and herbal granules

(安慰劑與中藥顆粒的相似度測試)

Please compare the 2 specimens given and circle the most appropriate answer, "1" represents the most similar, "5" represents the most different.

請比較兩個樣本的異同後於各項目中圈出最恰當的答案，"1"代表完全相同，"5"代表完全不同．

	Same 相同				Different 不相同	Not sure 不確定
After opening 拆開後						
Granule smell 顆粒的氣味	1	2	3	4	5	N
Granule color 顆粒的色澤	1	2	3	4	5	N
Granule size 顆粒的大小	1	2	3	4	5	N
Granule texture 顆粒的質感	1	2	3	4	5	N
Granule taste 顆粒的味道	1	2	3	4	5	N
After dissolution 溶解後 (80°C, 100 ml)						
Smell 氣味	1	2	3	4	5	N
Colour 色澤	1	2	3	4	5	N
Concentration 濃稠度	1	2	3	4	5	N
Taste 味道	1	2	3	4	5	N
Others (Please specify) 其他，請說明						

Table 8-6 Comparison test of placebo 1, placebo 2 and OPR granules

Before dissolution 溶解前	Placebo 1	Placebo 2
Granule smell 顆粒的氣味	4.4	3
Granule color 顆粒的色澤	4.6	4.3
Granule size 顆粒的大小	2.1	1.9
Granule texture 顆粒的質感	2.5	3.2
Granule taste 顆粒的味道	4.3	4.4
After dissolution 溶解後		
Smell 氣味	4.8	3.8
Colour 色澤	4	3.8
Concentration 濃稠度	2	2.3
Taste 味道	4.6	4.3
Believe it as herbal granules	12.5%	33.3%

n=12, "1"represents the most similar, "5"represents the most different.
Items with mark ≥ 4 are defined as fail in the similarity test.

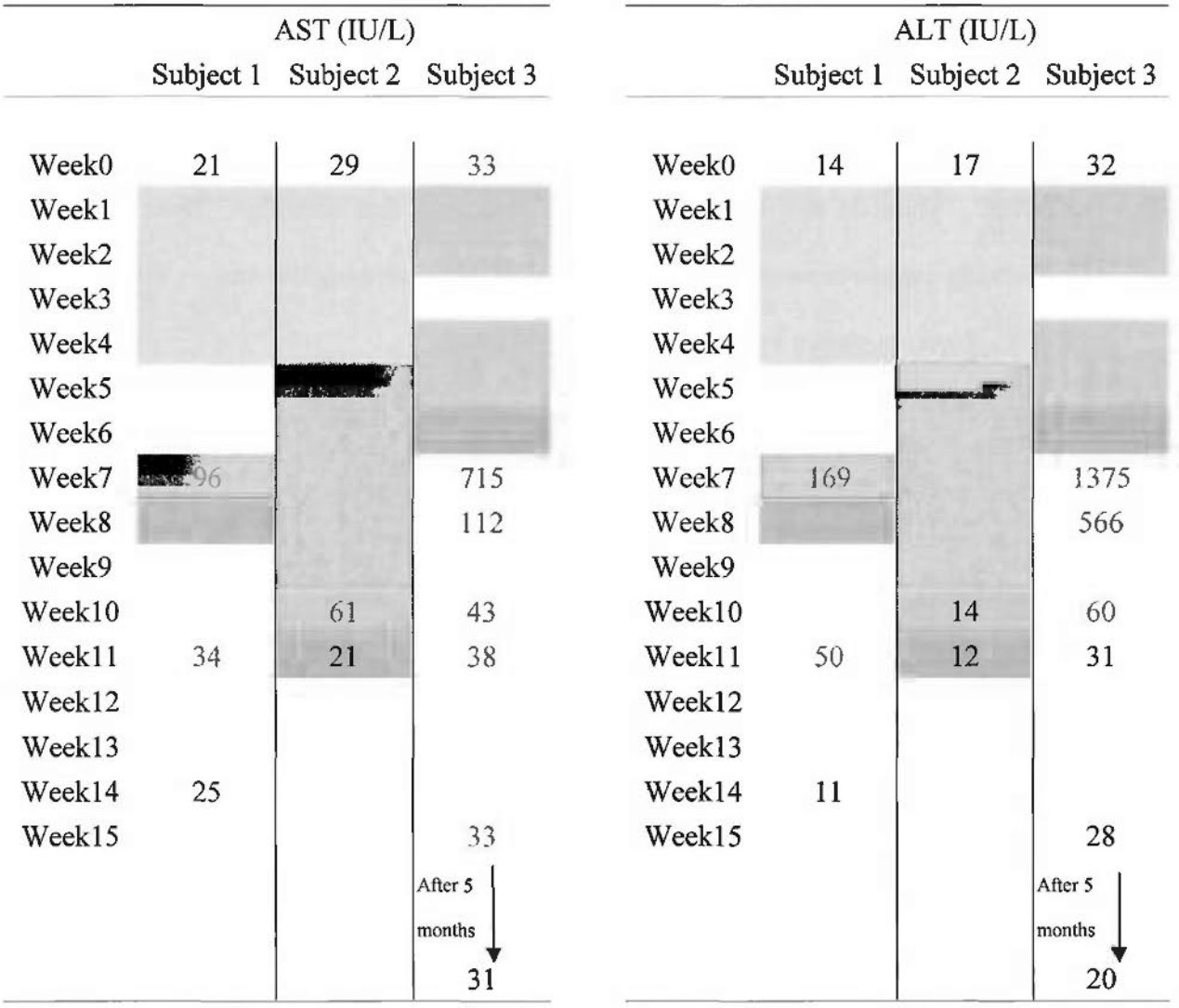
8.4.3. Pilot study

In view of poor tolerance of subjects to the granules previously, it was decided to start a pilot study in eight patients with half-dose of OPR granules. Besides those produced by Eu Yan Sang, granules produced by PuraPharm was used in another group (three patients) for comparison. PuraPharm supplies concentrated Chinese Medicine granules to mainstream hospitals and clinics in Hong Kong, which include clinics established by Hospital Authority and non-government organizations.

Eight subjects fulfilled the inclusion criteria were recruited, most patients developed stomach distention, constipation or insomnia after starting the treatment. Most of them withdraw from the study after 1-2 months due to the unpleasant response to OPR granules no matter manufactured by Eu Yan Sang or PuraPharm. Routine laboratory tests after 1 month treatment were performed, 3 out of 8 patients were found to be abnormal in liver function test

with elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level (two subjects from Eu Yan Sang group and one subject from PuraPharm group). Trial was suspended immediately on all patients and follow up monitoring was continued until the laboratory findings resumed to normal. All these three patients had normal liver function tests before the study. Hepatitis A and B status were negative. (Hepatitis C status was pending). They denied taking other neither herbal medicine nor recent change in western medicine. They do not have any sign of viral infection recently. Their liver function significantly improved or reverted back to normal after stopping the drug. Study progress of these 8 subjects is shown in **Table 8-7** and **Figure 8-6**.

Table 8-7 AST and ALT level of 3 subjects with abnormal liver function test



Key:

- Treatment of Eu Yan Sang OPR granules of half dose
- Treatment of PuraPharm OPR granules of full dose
- Treatment of PuraPharm OPR granules of half dose
- No treatment
- Withdrawal from study

Normal AST level: <31 IU/L; Normal ALT level: <34 IU/L

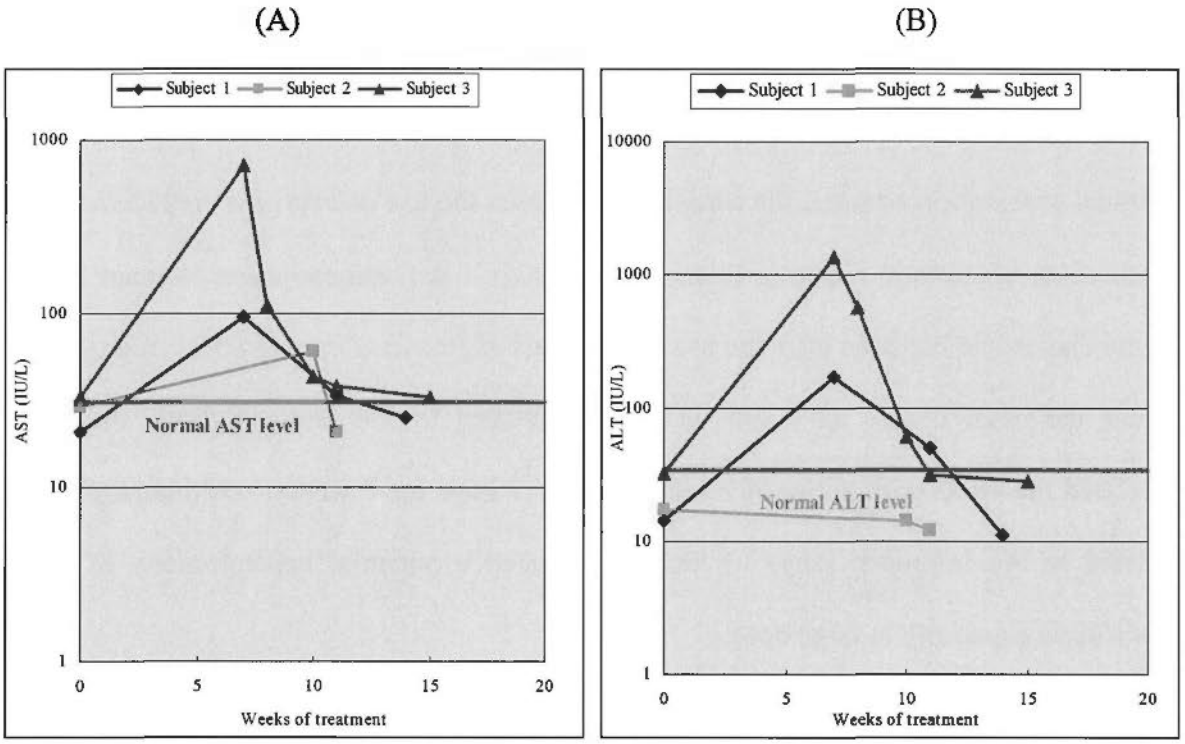


Figure 8-6 AST (A) and ALT (B) level of 3 subjects with abnormal liver function test

8.5. Discussion and conclusions

Abnormal liver function appears on both subjects taking OPR granules produced by Eu Yan Sang and PuraPharm. After reviewing the safety profile provided by these two companies, possibility of improper manufacturing process seems to be low. Quality control, safety test and acute toxicity report of OPR granules produced by Eu Yan Sang are listed in Section **3.3.3**. TCM products of PuraPharm meet the Good Manufacturing Practice (GMP) standards of China, Japan and Australia and pass the safety tests performed by independent agencies and have been widely used in TCM clinics in Hong Kong and China. Therefore, further investigation was focused on the toxicity from the herb itself or herbal remedies.

Safety information and Lethal Dose, 50% (LD_{50}) of ten herbs in OPR are described in Section **3.2**. Published studies of toxicity test on animals are summarized in **Table 8-8**. There was not much clinical report on the hepatotoxicity of individual herb within OPR. One clinical observation reported the association between the use of *Psoralea corylifolia* (補骨脂) with acute cholestatic hepatitis (Nam et al., 2005). A postmenopausal woman developed acute cholestatic hepatitis after the use of the seeds of *Psoralea corylifolia* in amounts over 10 times the usual dose. AST and ALT were elevated to 774 and 398 IU/L after she had ingested the seeds with a cup of black tea every 1 hour for 7 weeks. Although this case is related to the improper usage of herb, it revealed a potential hepatotoxicity of *Psoralea corylifolia* especially in large dose.

Table 8-8 Safety information of the component herbs in OPR

Pharmaceutical name	Chinese name	Safety information form published studies
Fructus Psoraleae	補骨脂	Study shows that kidney damage appeared only when mice are injected with 1mg Bakuchiol (補骨脂酚) extracted from 75 g of crude herbs for 7 days (Huang, 1994). The toxicity (LD50 and the injury on kidney) of the improved salt-baked product was lower than that of the salt-baked one, processing increases the efficacy and decreases the toxicity (Yao et al., 1997b).
Herba Epimedii	淫羊藿	Toxicity of methanol extract is very low, there is no adverse effect in mice (ig) up to 450 g/kg (Huang, 1994)
Radix Rehmanniae Preparata	熟地黃	No death or adverse effect reported on mice tested with water extract and methanol extract up to 60 g/kg/d for 3 d. No noticeable change in liver and kidney tissue was observed in rats (ig) at water and methanol extracts at 18 g/kg/d (Huang, 1994).
Fructus Corni	山茱萸	Study has been carried out to determine the embryo toxicity, acute toxicity, and accumulation toxicity of water extract . Result showed that the LD50 is larger than 10 g/kg which indicated that it has no acute toxicity, accumulation toxicity in mice, and no embryo toxicity in mice embryo (Zhang, 2004). The LD50 of pulp and acinus on mice are 53 g/kg and 90 g/kg. Both results suggested a low toxicity of *Fructus Corni*
Rhizoma Dioscoreae	山藥	The LD50 of one of the component of RD (淮山皂甙) in mice is 8715 mg/kg which indicates a low toxicity (Zhang, 2003).
Carapax Et Plastrum Testudinis	龜甲	Toxicity is so low that LD50 cannot be detected. The maximum tolerable dosage (MTD)on mice is 250 g/kg (Huang, 1994).
Fructus Ligustri Lucidi	女貞子	No toxic response in rabbit with a dosage of 75 g/1.5kg (ig) (Huang, 1994). No death report after injection of 5 mg extract in mice. Adverse response were found in clinical use which includes dizziness, mouth dryness, mild stomach pain and diarrhea and symptoms disappear after the termination of drug intake
Rhizoma Polygonati	黃精	No dealth was reported when 450 g/kg of water extract of baked *Rhizoma Polygonati* was used in mice (Huang, 1994). The aqueous extract of *Rhizoma Polygonati Odorati* does not cause seriously abnormal signs or death to animals in the acute toxicity test and in the 6-month chronic toxicity test. Neither was genetic toxicity found in the Ames test, the micronucleus test of bone marrow and the sperm malformation test in mice (Chen et al., 2001).

Table 8-8 (Continued)

Pharmaceutical name	Chinese name	Safety information form published studies
Rhizoma Atractylodis Macrocephalae	白术	A slight decrease in white blood cells (esp. lymphocytes) was found in rats after feeding 0.5 g/kg (ig) qd for 14 days. An increase of sensitivity to external environment stimulus was shown in animals after feeding the herb (Huang, 1994).
Cortex Moutan	牡丹皮	The LD50 of another component (牡丹酚磺鈉) in mice is 30 mg/kg (iv) which was equivalent to 15 times of a clinical dosage(Li, 2004a). The toxicity of *Cortex Moutan* and Paeonol are low. The LD50 of Paeonol in mice are 3430 (ig), 781(ip) and 196(iv) mg/kg. The LD50 of soluble Paeonol in 50% peanut oil extract in mice is 4.9 g/kg (ig). There was no noticeable change in liver and renal function of dogs with high blood pressure when treating them with Cortex Moutan (Huang, 1994).

More literature search was done on herbal remedies used to treat osteoporosis as they share similar component as OPR. One commercial available herbal capsule named Zhuang Gu Guan Jie Wan (壯骨關節丸) raised our attention as we found 13 reports (**Table 8-9**) and one systemic review (Cheng and Cai, 2000) on the hepatotoxicity of the capsule. It consists of 12 herbs while 3 herbs are the same as OPR, they are Fructus Psoraleae (補骨脂)、Herba Epimedii (淫羊藿)、Radix Rehmanniae Preparata (熟地黃). The systemic review showed that there were 156 cases of adverse reaction (ADR) induced by the capsule from 1992 to 1998. These cases were collected and reviewed by Beijing Centre for ADR Monitoring and Beijing Ditan Hospital. More than a half (64.7%) of the ADR was related to liver function impairment. Liver damages induced by the capsule were usually appeared on female elderly with an average age of 57±10.6. They were diagnosis as cholestatic hepatitis with ALP and GGT (Gamma-glutamyl transferase) elevated significantly. High fever and symptoms of gastrointestinal tract were not common. Latent period was in average 47 days and patients were hospitalized for 1-2 months. These clinical symptoms were alike with the subjects who had abnormal liver function in OPR trial except they did not show jaundice.

Another herbal remedies induced liver dysfunction is Xianlinggubao capsules (仙靈骨葆膠囊). It shared the same herb Herba Epimedii (淫羊藿) with OPR. Two patients developed liver dysfunction after treatment with Xianlinggubao capsules for osteoporosis (Yang and Zhou, 2007b). A 65-year old female consumed Xianlinggubao capsules of 1.5 g twice a day for six months and her ALT and AST levels elevated to 85 U/L and 93 U/L, respectively. Her ALT and AST levels decreased to 17 U/L and 28 U/L, respectively after cessation of the capsules for 20 months. Another 68-year old female patient also developed an increase of ALT and AST levels to 158 U/L and 123 U/L after two months treatment and the level dropped to 35 U/L (ALT) and 23 U/L (AST) after 6-month of time. Both remedies shared some common herbs

Table 8-9 Reports of adverse reactions caused by Zhuang Gu Guan Jie Wan

壯骨關節丸成份：狗脊、淫羊藿、獨活、骨碎補、續斷、補骨脂、桑寄生、雞血籐、熟地黃、木香、乳香、沒藥。

1. 陶勝來,王俊學. 壯骨關節丸引起肝臟損害一例報道. 肝臟 ,2003,(03)
2. 陳孝貞. 壯骨關節丸的不良反應. 福建醫藥雜志 ,1997,(01)
3. 郝玉娟,高玲麗. 壯骨關節丸致藥物性肝損傷1例. 護理研究 ,2003,(24)
4. 江曉靜，葛婭，靳桂敏. 壯骨關節丸致藥物性肝損害1例. 中國醫院藥學雜志 ,1994,(05)
5. 曹淑芬,陳一丸,程經華. 壯骨關節丸致肝損害15例. 藥物流行病學雜志 ,1995,(04)
6. 吳世強，孟令平，包從偉，鄭穎. 骨髓壯骨粉防治骨質疏松癥的臨床觀察. 中國骨質疏松雜志 ,1996,(02)
7. 吳卓智，潘薔. “壯骨關節丸”致急性肝損害4例報告[J]北京醫學 ,1994,(01) .
8. 孫功惠，尹宏怡. 壯骨關節丸致肝內膽汁瘀滯1例[J]安徽中醫學院學報 ,1995,(01) .
9. 齊荔紅,劉曄. 壯骨關節丸引起轉氨酶升高45例[J]海峽藥學 ,1995,(04) .
10. 齊荔紅,康魯平. 壯骨關節丸致高血壓患者血壓升高38例[J]海峽藥學 ,1996,(02) .
11. 劉曉彥，張栩，孫花芬，武菲菲. 壯骨關節丸致藥物性肝損害2例報告[J]寧夏醫學雜志 ,1995,(05) .
12. 柳奎榮. 壯骨關節丸引起藥疹1例[J]寧夏醫學雜志 ,1998,(02) .
13. 姜雨生. 壯骨關節丸引起藥疹1例[J]青島醫藥衛生 ,1995,(11) .

with OPR and induced an increase in ALP and AST level after 1-6 months of treatment. These laboratory findings dropped gradually to normal after cessation of treatment and the recovery period depended on the previous treatment time.

Due to the adverse events arose on humans after taking OPR granules for 1-2 months, a 90-day oral toxicity study (**Appendix XIII**) was therefore performed to determine if the same response would be induced on animal and investigate the reason behind. The aim of the study is to investigate potential health hazards likely to develop form repeated exposure of OPR granules on human over a 90-day period of time by using Sprague-Dawley rats as animal model. Thirty-seven female Sprague-Dawley rats (~200g) were randomly assigned to six groups: Control group (Vehicle), three OPR treated groups with a dose equivalent to one time, three-time and five-time of clinical dose (OPR-1X, 3X, 5X) and two major herbs, Fructus Psoraleae (補骨脂) and Herba Epimedii (淫羊藿) groups (FP-5X and HE-5X) equivalent to five time of clinical dose. Protocol of the 90-day oral toxicity study is show in **Figure 8-7**. Results showed that no obvious abnormal clinical signs were observed during the study. No statistical difference between control and treatment group on body weight and organ weight. No difference was found in liver and renal function tests between control and treatment group. Report of histopathology on organ tissues showed that no obvious difference was found between the tissues collected from control group and treatment group. These findings indicated that OPR did not induce any obvious toxic effect on SD rats' organs tissues.

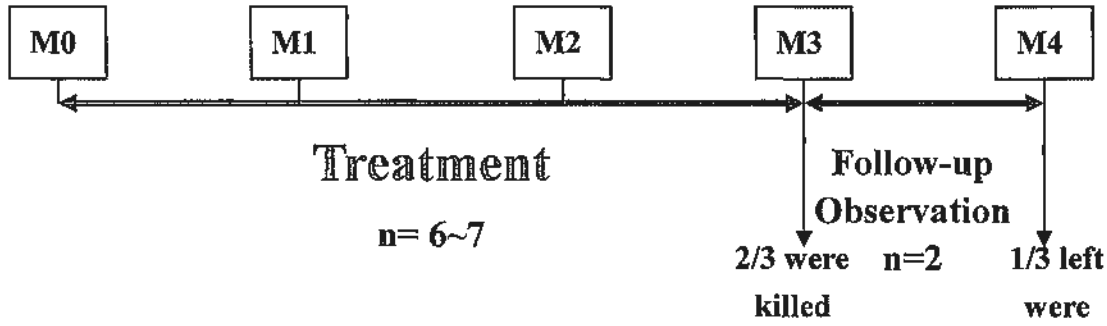


Figure 8-7 Ninety-day oral toxicity study protocol

Herbal drugs are widely used and recent reports have mentioned about hepatotoxicity of herbal remedies which ranges from mild liver enzyme alterations to chronic liver disease and liver failure (Kaplowitz, 1997; Peyrin-Biroulet et al., 2004; Stickel et al., 2001). Toxic effect of herbal remedies may be attributed to the chemical products of the plants associated with the system of cytochrome P450 enzyme (Shedlofsky et al., 1994), especially cyclical peptides and alkaloids as an offensive system to provide for preservation (Kaplowitz and Abboud, 2007). The liver, as an organ for metabolizing toxicity, may become exposed to these toxic invaders (Kaplowitz, 1997). Therefore, toxic hepatitis frequently occurs from taking different sorts of herbal remedies (**Table 8-10**) for a wide range of diseases or problems that are not easily resolved by modern medicines.

Table 8-10 Hepatotoxicity of natural substances

(Adopted from Kaplowitz, 1997)

Vitamins
Hypervitaminosis A
Niacin >3 g, slow-release form
Cocaine
Aspergillus (aflatoxins)
Mushroom (amatoxins)
Bacillus cereus (cereulide) rice syndrome
Cyanobacteria (microcystins)
Oriental hornet venom
Herbal remedies
Senecio, Crotalaria, *Symphytum*, comfrey, *Heliotropium* (pyrrolizidine alkaloids)
Pennyroyal oil (monoterpene)
Chaparral-Larrea, creosote bush (nordihydroguaiaretic acid)
Germander-teucrium (diterpenoids)
Senna (anthron)
Jin Bu Huan (levotetrahydropalmitine)
Atractylis gummifera (atractylate)
"Chinese herbals," complex mixtures of herbs
Probable: skullcap, valerian, mistletoe, sassafras

NOTE. Chemical responsible for toxicity is listed in parenthesis.

When conducting a TCM clinical trial, placebo production is a great challenge for most investigators. As randomized, placebo-controlled clinical trial favors the evaluation of an intervention that can be easily blinded and placebo-controlled, it is an excellent methodology

to evaluate the effect of one pill tested against one clinical endpoint. For example, it is easy to compare a new pill with an identical-looking placebo. But it is difficult to blind and develop appropriate placebos for herbal intervention trials as the unique appearance, taste and smell of herbs are very difficult to mimic. It would be more difficult if the medication used is in liquid or powder form. Wang and his colleagues (Wang, 2003a) shared their experience on placebo production of using food additives and natural agriculture products as raw materials. They employed balsam pear to mimic the bitter taste of herbal powder; Starch to increase the texture; Food additives to adjust the color of granules and dissolved solution. However, the taste is still a headache issue as herbal formula usually consists of 10 or more herbs which contribute a complicated and rich favor, which cannot be pretended by one single substance. Introduction of other herbs which do not impose therapeutic effect on the disease studied is a solution but this measure may be criticized by other investigators. A recent systemic review (Guan, 2006) on the use of placebo in TCM clinical trials showed that about half of the 77 TCM trials studied have mentioned the use of placebo. And less than 3% placebos had been validated properly on their competence on blinding. In our study, we have listed several tips in placebo and herbal granules production. A simple but useful checklist for evaluating a placebo was also developed. It serves as a simple tool for other investigator to evaluate their placebo.

Lactose had been used as a food additive in OPR granules production in the first stage and it caused stomach distention and diarrhea in 7 out of 8 patients tried it. It raised our awareness of lactose intolerance in Chinese population. Lactose intolerance is an inability to digest significant amounts of lactose. It is caused by a shortage of the enzyme lactase, which is produced by the cells that line the small intestine. Common symptoms, which range from mild to severe, include nausea, cramps, bloating, gas, and diarrhea. About 90 to 100 percent of Asian Americans are lactose intolerant (National Institute of Diabetes and Digestive and

Kidney Diseases, NIH). Some studies have found that people with lactose intolerance are at higher risk for osteoporosis, while others have not (Information from National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH). Therefore, lactose is not a feasible choice of additives in herbal granules production. Other stabilizing additives: dextrin, starch, cellulose, xylitol are good alternatives in granules production.

Although we could not accomplish a double-blinded, randomized, placebo-controlled clinical trial (RCT), the pilot study we carried out had revealed valuable clinical data and demonstrated several potential problems in TCM trial and provided feasible solutions for other investigators. Acute and chronic toxicity test on animals cannot guarantee a safety profile of the intervention tested. Unexpected adverse events would still be occurred on human during clinical trial. Abnormal liver function induced by OPR granules could raise the awareness of investigators on hepatotoxicity of herbal remedies. During the production of OPR granules, type of additive should be carefully selected. Consultation from experienced TCM products' manufacturers and clinical trial investigators is recommended. Quality of placebo is a key to success in a RCT because a "good" placebo could blind the patients successfully and raise the compliance to the intervention. Therefore, appearance, color, smell, taste and other sensory response of a placebo imposed on human should be systemic evaluated before applying in a RCT. Herbs without therapeutic effect on the disease studied may be a good substitute. In conclusion, the pilot clinical study on OPR had revealed the hepatotoxicity of a kidney-tonifying herbal formula and disclosed potential problems encountered when performing clinical trial on Traditional Chinese Medicine.

9. General Discussion and Conclusions

Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone tissue. It leads to bone fragility and an increased susceptibility to fractures of the hip, spine, and wrist (International Osteoporosis Foundation, 2008). As postmenopausal women are affected by both factors of the lack of estrogen and later aging, they become the major sufferers in bone loss and result in much higher incidence of osteoporosis. Therefore, postmenopausal osteoporosis would become our main scope of study. Osteoporosis is a major public health threat worldwide. It affects 75 million people in Europe, USA and Japan (Lindsay et al., 1997). It is estimated that by 2050, the worldwide incidence of hip fracture is projected to increase by 240% in women (Riggs and Melton, 1995). Osteoporotic fractures exert a terrible toll on the population with respect to morbidity, cost, and mortality. These fractures interfere greatly with the activities of daily living, and all of them will have a substantial negative impact on the quality of life. Although current medications can slow down the bone deteriorating process, their side effects (Gennari et al., 2007; Haynes and Dowsett, 1999; Mitlak and Cohen, 1999; Thiebaud and Secrest, 2001) and high cost (Ip et al., 2004) had hamper patient's compliance for long term treatment (Neer et al., 2001).

Public search for safe, effective and low-priced medicine and have turned their attention to Traditional Chinese Medicine (TCM). Extensive experience has been accumulated in TCM regarding the diagnosis and treatment of osteoporosis, which often involves the prescription of the kidney-tonifying herbs (補腎中藥). Therefore, the aim of the study, firstly, was to explore the association of the incidence of postmenopausal osteoporosis and Kidney-Vacuity Syndromes (腎虛証候) in TCM, so as to formulate a rational kidney-tonifying herbal formula for the study. Secondly, the effect of the formula on the

treatment of postmenopausal osteoporosis was evaluated by *in-vitro* and *in-vivo* studies. Thirdly, the Osteoporosis-Targeted Quality of Life Questionnaire was linguistically validated from English to Chinese, which was expected to be one of the outcome measurement tools in future clinical trials. Lastly, a pilot clinical study was performed, which revealed some potential hazards of the formula on human beings which have not been shown in previous toxicity tests.

In Traditional Chinese Medicine theory, kidney system is a key to healthy bone development. Many studies have provided scientific proof (Chen et al., 1999; Wang et al., 2005a; Xu et al., 2005) on this statement. However, the diagnosis on the "Deficiency in kidney system" was not standardized and the severity of the disease has not been properly quantified. Diagnosis in these studies are highly dependent on individual TCM practitioner's experiences. In the present study, the association between the incidence of postmenopausal osteoporosis and Kidney-Vacuity Syndromes (KVS) could be investigated in an objective manner with the aid of a "KVS scoring questionnaire". Seventy-three postmenopausal women were invited to fill in the questionnaire. They were classified into six KVS, which are "Deficiency of kidney qi (腎氣虛証)", "Deficiency of kidney yin (腎陰虛証)", "Deficiency of kidney yang (腎陽虛証)", "Deficiency of kidney yin and yang (腎陰陽兩虛証)", "Deficiency of kidney essence (腎精不足証)" and "Kidney failing to absorb qi (腎不納氣証)". Bone mineral densities of these subjects were measured. Results showed that osteoporosis group had generally a higher deficiency in kidney system when compared with the non-osteoporosis group. It further indicated that subjects with higher deficiency in kidney "qi "and kidney "essence" would have a higher probability of osteoporosis. These findings strongly supported that replenishing kidney "qi "and kidney "essence" was a logical therapeutic principle in the formulation of a herbal formula. It provided scientific support for the formulation of a kidney-tonifying herbal formula for osteoporosis research (OPR) and it

was not solely dependent on literature reviews and experts' opinions as in other researches.

The effect of OPR for the treatment of postmenopausal osteoporosis was then evaluated in the *in-vitro* and *in-vivo* studies. In the *in-vivo* study, an osteoporosis model was established by performing ovariectomy on four-weeks-old C57BL/6 mice. The model exhibited a high bone turnover rate and presented as an increase in bone formation marker (osteocalcin) and bone resorption marker level (C-terminal telopeptides of type I collagen). After a six weeks treatment of OPR, the high turnover rate of bones was slowed down by decreasing bone formation and resorption process without increasing the uterine linings. However, the change in bone mineral density measurement was not significant.

For the study design, there was room for improvement. A short lifetime span allows mice to be an effective animal model as it significantly compresses the time frame from experiencing peak bone mass to post-ovariectomy bone loss. However, they also possess dissimilarities with humans. Study should be conducted first in ovariectomized rodent model and second in a non-rodent model as stated by Food and Drug Administration, U.S.A. (FDA, 1994). Moreover, our findings indicated that diet consumption and body weights of the control and treatment groups were not consistent. Pair feeding should be introduced to ensure steadily calcium intake among groups (Furuhata et al., 2002). Furthermore, the difference in bone mineral densities between control and treatment group was not significant, a longer recovery period and treatment period might be needed.

Although the beneficial effect of OPR on bone had been demonstrated on ovariectomized mice, the mechanism behind was still unknown. The major components of OPR: Fructus Ligustri Lucidi, Herba Epimedii and Fructus Psoraleae are three frequently reported herbs with beneficial effects on bones. Some studies show that Fructus Ligustri Lucidi can

increase the intestinal calcium absorption rate and prevent OVX-induced loss of calcium in rats as well as increasing bone calcium content (Zhang et al., 2006b). Other publication suggested that Epimedii can increase osteoblastic activities and decrease osteoclastogenesis (Xie et al., 2005a), and Psoraleae can preserve bone mineral density by increasing bone formation (Tsai et al., 2007). Flavonoids of these three herbs are generally identified as the active components. This published information might provide a clue for the mechanism of action of OPR on bones.

The potential mechanism of action of OPR on bone was explored by *in-vitro* study. Osteoblast-like UMR 106 cells were used to determine the effect of OPR on bone formation cells. Water extract of OPR increased the rate of cell differentiation and proliferation of osteoblastic cells, UMR 106, in comparison with the negative control group. Although the mechanism of OPR on osteoblast is still unknown, the presence of estrogen receptors on UMR 106 cell line (Davis et al., 1994) suggested the possibility of the presence of estrogenic activity of OPR. In further study, a MCF-7 reporter gene assay transfected with an estrogen responsive element (ERE) was employed. It served as a screening tool to verify the presence of estrogenic-like activity of OPR. Results showed that OPR could trigger the luciferase activity of the reporter gene. Therefore, the estrogenic activity of OPR was proven and it might account for its protective effect on bones.

After the *in-vivo* and *in-vitro* studies, a clinical trial was planned to conduct. In the trial protocol, assessment on quality of life was intended to be one of the outcome measurements and was supplementary to the measurement of bone mineral density and biochemical markers of bone turnover. However, a Chinese version of instrument to measure osteoporosis-specific quality of life was unavailable. Due to a lack of validated Chinese language osteoporosis-specific questionnaire, the burden of the disease on Chinese population

has been under-studied in health services stream (Ip et al., 2004). Therefore, an English version of Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) was selected to be translated. The OPTQoL questionnaire aimed at assessing the physical difficulty, fears, and adaptations to one's daily life. It was developed in Merck Research Laboratories, U.S.A. and has been translated and culturally adapted into seven languages to allow cross-cultural studies of the community impact of osteoporosis (Chandler et al., 1998). In our study, the English OPTQoL was translated into Chinese and linguistically validated according to the standard guideline. It is believed to be the only Chinese osteoporosis specific Quality of Life questionnaire available at this time. Other investigators can assess the impact of new interventions on quality of life among Chinese osteoporosis patients with the newly formed Chinese OPTQoL.

In order to evaluate the effect of OPR clinically, a pilot clinical study was performed. Eight subjects fulfilled the inclusion criteria were recruited. However, the liver function tests of three subjects out of eight were found to be abnormal with elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level, which was not reported in previous toxicity test. The trial was suspended immediately and a follow-up test showed that the elevated AST and ALT level had reverted back to normal within one month after termination of OPR intake. Although we could not accomplish the trial, the potential hepatotoxicity of OPR on human beings was revealed. It raised the awareness of investigators on the use of herbal remedies in future clinical studies.

Some typical problems were encountered when conducting a clinical trial on Chinese Medicine. Placebo production is a great challenge for most investigators when herbal intervention is involved. It is difficult to blind and develop appropriate placebos for herbal intervention as the unique appearance, taste and smell of herbs are very difficult to mimic. It

would be more complicated if the medication used is in liquid or powder form. Feasible solutions include using food additives and natural agriculture products as raw materials for placebo production. Herbs without therapeutic effect on the disease studied may also be a good substitute. Validation of the placebo is another issue. Less than 3% placebos used in TCM clinical trials had been validated properly on their competence on blinding (Guan, 2006). Quality of a placebo is the key to success in a RCT because a "good" placebo could blind the patients successfully and raise the compliance to the intervention. Therefore, appearance, color, smell, taste and other sensation response of a placebo imposed on human should be systematically evaluated before applying in a trial. In our study, an extensive evaluation on placebos was done and a questionnaire was developed. It could serve as a simple tool for other investigator to evaluate their placebo used. Selection of food additive to be used in herbal granules production is also important. Improper food additive, e.g. lactose, might result in adverse events. Other stabilizing additives: dextrin, starch, cellulose, xylitol are good alternatives in granules production. Consultation from experienced TCM products' manufacturers and clinical trial investigators is recommended.

Although we could not accomplish a double-blinded, randomized, placebo-controlled clinical trial (RCT), the pilot clinical study had documented valuable clinical data of the use of a kidney-tonifying herbal formula and revealed its hepatotoxicity on human beings. It also presented several problems in conducting a TCM clinical trial, which include production and evaluation of placebo, and selection of suitable food additive for herbal granules production. Some feasible solutions were provided for other investigators.

In conclusion, the study demonstrated a positive co-relation of the incidence of postmenopausal osteoporosis and KVS. It provided strong evidence that replenishing kidney qi and kidney essence is a logical therapeutic principle in the formulation of an herbal formula.

The beneficial effect of OPR on bone might related to its increase on the rate of cell differentiation and proliferation of osteoblast-like UMR 106 cells and its estrogenic-like activity, which was detected by transfected MCF-7 reporter gene assay. For *in-vivo* study, OPR slowed down the estrogen-depleted condition induced by ovariectomy on young C57BL/6 mice, without altering the growth of uterine lining. English version of OPTQoL was linguistically validated into Chinese and it would be useful for other investigators to assess quality of life among Chinese osteoporosis patients. The pilot clinical study revealed the hepatotoxicity of OPR on human beings and it raised the safety awareness of investigators on the use of herbal remedies in future clinical studies.

Appendix I Kidney-Vacuity Syndromes scoring questionnaire

	是	否	指標名	註明
1			既往多病	
2			先天愚鈍	
3			先天稟賦不足	
4			發育遲緩	
5			平素體質偏虛	
6			發病年齡：中老年人	
7			起病緩慢	
8			病程較長	
9			他病遷延	
10			失治誤治	
11			形體偏瘦	
12			精神疲乏	尚可工作
13			精神萎靡	無法工作
14			倦怠乏力	
15			勞倦過度	
16			驚恐過度	
17			煩躁不安	
18			低熱	微熱:<38 度,或僅自覺發熱
19			潮熱	按時發熱,，或按時熱更顯
20			五心煩熱	心中煩熱伴兩手足心有發熱感
21			骨蒸發熱	其發熱自覺似從骨髓蒸發而出
22			面色潮紅	
23			面部烘熱	
24			盜汗	
25			自汗	
26			畏寒	
27			失眠	
28			夜寐多夢	
29			記憶力減退	
30			健忘	
31			智能減退	

	是	否	指標名	註明
32			皮膚乾枯不潤	
33			骨骼痿軟	
34			其他，請說明	
35			眩暈	
36			頭暈	
37			頭髮白	
38			毛髮不榮	
39			毛髮稀疏	
40			脫髮	
41			視物疲勞	
42			視物模糊	
43			視力減退	
44			視物黑花飛舞	
45			耳鳴	
46			聽力減退	
47			耳聾	
48			咽乾	
49			唇舌乾燥	
50			牙齒枯槁	
51			齒搖	
52			齒齦萎縮	
53			聲音低弱	
54			懶言	
55			少氣	呼吸微弱短促，言語無力
56			短氣	呼吸短促，如不能接續
57			喘促呼多吸少	喘促：呼吸時急促，氣逆不平
58			喘促動則尤甚	
59			喘促氣不接續	
60			喘促咳則遺溺	
61			咳嗽	
62			胸悶	
63			足跟痛	
64			肢冷	
65			水腫：全身各部位均可見，以腰下為甚，程度中重度，水腫發病特點起病緩慢	
66			水腫：程度輕度，水腫發病特點起病緩慢	

	是	否	指標名	註明
67			腰痛：性質隱隱作痛、綿綿不休，酸痛程度較輕，遇勞發作或加重，臥則減輕，喜按, 休息後緩解, 腰痛時間特點無明顯規律	
68			腰痛：遇寒發作或加重，得溫則減	
69			腰酸	
70			夜間尿多	
71			小便清長	
72			大便秘結	
73			便溏	
74			其他，請說明	
75			舌色紅	
76			舌色淡	
77			舌色淡紅	
78			舌苔少/無	
79			舌苔白	
80			舌苔剝	
81			舌苔乾	
82			舌苔薄	
83			舌體有裂紋	
84			舌體胖	
85			舌體瘦	
86			其他，請說明	
87			脈數	脈來急促，一息五～六至
88			脈遲	脈來緩慢，一息三～四至
89			脈弱	沉而細軟
90			脈沉	輕取不應，重按始得
91			脈沉弱	
92			脈沉細	
93			脈沉遲	
94			脈細	脈細如綫，應指明顯
95			脈細弱	
96			脈細數	
97			脈虛浮無根	舉之相對有餘，按之非常不足
98			其他，請說明	

Appendix II Monographs of ten herbs used in OPR (Chinese Pharmacopoeia 2000)

中華人民共和國藥典所列之藥性及用量

Fructus Psoraleae (補骨脂)

性味與歸經　：辛、苦温；歸脾、腎經．

功能與主治　：温腎助陽納氣止瀉．用於陽痿遺精遺尿尿頻腰膝冷痛腎虛作喘五更泄瀉．

用法與用量　：６－９ｇ

Herba Epimedii (淫羊藿)

性味與歸經　：辛、甘温；歸肝、腎經．

功能與主治　：補腎陽强筋骨祛風濕．用於陽痿遺精筋骨痿軟風濕痹痛麻木拘孿．

用法與用量　：３－９ｇ

Radix Rehmanniae Preparata (熟地黃)

性味與歸經　：甘微温；歸肝、腎經

功能與主治　：滋陰補血益精填髓．用干肝腎陰虛腰膝酸軟骨蒸潮熱盜汗遺精內熱消渴血虛萎黃心悸怔忡月經不調崩漏下血眩暈耳鳴鬚髮早白．

用法與用量　：９－１５ｇ

Fructus Corni (山茱萸)

性味與歸經　：酸、澀微温；歸肝、腎經．

功能與主治　：補益肝腎澀精固脫．用於目眩耳鳴腰膝遺精遺尿尿頻崩漏帶下大汗虛脫．內熱消渴．

用法與用量　：６－１２ｇ

Rhizoma Dioscoreae (山藥)

性味與歸經　：甘、平；歸肺、脾、腎經．

功能與主治　：補脾養胃生津益肺補腎澀精．用於脾虛食少久瀉不止肺虛喘咳腎虛遺精帶下尿頻虛熱消渴．

用法與用量　：１５－３０ｇ

Carapax Et Plastrum Testudinis (龜甲)

性味與歸經　：咸、甘微寒；歸心、肝、腎經．

功能與主治　：滋陰潛陽滋腎强骨養血補心．用於陰虛潮熱骨蒸盜汗頭暈目眩虛風內動筋骨痿軟心虛健忘．

用法與用量　：９－２４ｇ，先煎

Fructus Ligustri Lucidi (女貞子)
性味與歸經　：甘、苦涼；歸肝、腎經。
功能與主治　：滋補肝腎明目烏髮。用於眩暈耳鳴腰膝酸軟鬚髮早白目暗不明。
用法與用量　：６－１２ｇ

Rhizoma Polygonati (黃精)
性味與歸經　：甘、平；歸肺、脾、腎經。
功能與主治　：補氣養陰健脾潤肺益腎。用於脾胃虛弱體倦乏力口干食少肺虛燥咳精血不足內熱消渴。
用法與用量　：９－１５ｇ

Rhizoma Atractylodis Macrocephalae (白朮)
性味與歸經　：苦、甘、温；歸脾、胃經。
功能與主治　：健脾益氣燥濕利水止汗安胎。用於脾虛食少腹脹泄瀉痰飲眩悸水腫自汗胎動不安。
用法與用量　：６－１２ｇ

Cortex Moutan (牡丹皮)
性味與歸經　：苦、辛微寒。歸心、肝、腎經。
功能與主治　：清熱涼血活血化瘀。用於温毒發斑吐血衄血夜熱早涼無汗骨蒸經閉痛經痛腫瘡毒跌扑傷痛。
用法與用量　：６－１２ｇ

Appendix III Rationale of the formulation of OPR

方解

本方是以補骨脂、淫羊藿、熟地黃、山茱萸、山藥、龜甲、女貞子、黃精、白术、牡丹皮十味中藥組成，用於治療骨質疏鬆症屬於陰陽兩虛所致者．中醫學認為本病屬於虛勞范疇。主要病因是長期飲食不節，日久脾主運化功能下降，氣血生化之源不足，內不能濡養五臟六腑，外不能洒陳營衛經絡．加上該病患者大多年老體衰，肝腎虛弱，腎藏精主骨生髓的功能減退，故易發生骨質疏鬆症。《黃帝內經》云"大夫...七八肝氣衰，筋不能動，天癸竭，精少，腎臟衰，形體皆極."李東垣《脾胃論》說"大抵脾胃虛弱，陽氣不能生長,...則骨氣無力，是為骨萎,令人骨髓空虛，足不能履地."說明脾腎氣虛，精血虧損，骨失所養是老年人骨質疏鬆症的重要病因病機．據臨床觀察，婦女患有骨質疏鬆症者，每每見陰陽兩虛之象．此外，由於患者脾胃虛衰，加上平素缺乏鍛煉，肢體少動，使氣血凝滯，血行不暢，又可導致骨失所養，而成廢用性骨質疏鬆症．故本病往往伴隨氣滯血瘀的存在。因此骨質疏鬆症的特點是脾腎兩虛與血瘀同時存在。由此可見骨質疏鬆症的發生以"多虛多瘀"為其病機特點。治療應以 "補虛化瘀"為治則 除滋陰升陽、益氣健脾外，活血化瘀也是重要治法。

故方中重用補骨脂、淫羊藿補腎壯陽，強筋健骨為君藥；輔以熟地黃、山茱萸、山藥、女貞子、黃精補腎滋陰．除治陰虛之症外，並取《景岳全書．新方八陣》說"善補陽者，必於陰中求陽，則陽得陰助而生化無窮．"之意．其中山茱萸並能補益腎陽，為陰陽並補之品；山藥並能補脾氣、滋脾陰；黃精並能補血、益脾氣；龜板滋陰潛陽，補腎健骨，共為臣藥；白术益氣健脾；牡丹皮活血祛瘀，並退陰虛發熱，共為佐使藥．諸藥合用，陰陽並補，以補陽為主，兼以益氣健脾，活血化瘀，使腎陽旺，腎陰足，脾氣健，瘀血消，則骨質疏鬆症諸症自癒．

Appendix IV Safety tests report of OPR

Safety tests report of OPR 1 of 4

CMA Testing and Certification Laboratories
檢商會檢定中心

HOKLAS 004

TEST REPORT (測試報告)

Report No. 報告編號 : AF024192-001 Date : 2005 October 27 日期

Application No. 申請編號 : LF220378

Applicant 申請人 : Eu Yan Sang (Hong Kong) Ltd
Unit F, 2/F., Sunview Ind. Bldg.,
3 On Yip Street,
Chai Wan, Hong Kong.

Sample Description 樣品描述 : One (1) submitted sample stated to be TW-002
由申請人所提供的一個樣品是
Batch No. 批號 : TW-002
Expiry Date 有效日期 : 11-2007
Dose Form 劑型 : Granule without crude drug powder
Sample Status Upon Receipt 樣品收到時狀況 : Room Temperature 室溫

Date Received 收樣日期 : 2005 October 13.

Test Period 測試日期 : 2005 October 13 – 2005 October 17.

Test Requested 測試要求 :
1. Heavy metal or Toxic Elements test 重金屬及有毒元素測試
   (a) Lead Content 鉛含量
   (b) Cadmium Content 鎘含量
   (c) Arsenic Content 砷含量
   (d) Mercury Content 汞含量
2. Microbial Limit test 微生物限度測試
   (a) Aerobic Bacteria Count 總需菌數
   (b) Mold and Yeast Count 霉菌及酵母菌數
   (c) Escherichia coli 大腸桿菌
   (d) Salmonella species 沙門氏菌

For and on behalf of
CMA Industrial Development Foundation Limited

Authorized Signature 認可人簽名 :

May S.K. Ng
Deputy Manager
Chemical Division
吳婉嫦-化學部副經理

C.F. Wong
Specialist
Chemical Division
黃灼鳳-化學部化驗師

CMA Industrial Development Foundation Limited
Room 1302, Yan Hing Centre, 9-13 Wong Chuk Yeung St., Fo Tan, Shatin, N.T., Hong Kong.
Tel: (852) 2698 8198 Fax: (852) 2695 4177 E-mail: info@cmatel.com Web Site: http://www.cmatel.com

Safety tests report of OPR 2 of 4

CMA Testing and Certification Laboratories
廠商會檢定中心

HKLAS 004

TEST REPORT (測試報告)

Report No. 報告編號 : AF024192-001 Date : 2005 October 27 日期

Application No. 申請編號 : LF220378

Test Requested 測試要求 : 3.Organochlorine Pesticides Residue test 有機氯類殘留農藥測試

(a) Aldrin and Dieldrin 艾氏劑及狄氏劑
(b) Chlordane 氯丹
(c) DDT 滴滴涕
(d) Endrin 異狄氏劑
(e) Heptachlor 七氯
(f) Hexachlorobenzene 六氯苯
(g) Hexachlorocyclohexane 六六六
(h) Lindane 林丹
(i) Quintozene 五氯硝基苯

Test Method 測試方法 :
1. Inductively Couple Plasma Mass Spectrometry. 電感耦合等離子體－質譜法
2. Pharmacopoeia of the People's Republic of China 2000, Volume I, Appendix XIIIC (English Edition). 《中華人民共和國藥典》2000 年版一部附錄 XIIIC.
3. Gas Chromatography – ECD. 氣相色譜法及電子捕獲檢測器

Test Result 測試結果 : Refer to the results on page 3 to 4. 測試結果請參考第 3 頁到 4 頁

Conclusion 結論 : According to the said tests, the submitted sample was found to comply with heavy metal or toxic elements test; Microbial Limit test and Organochlorine Pesticide residue test as stipulated in the Technical guidelines for tests on the Safety of Proprietary Chinese Medicines (pCtn), sanctioned by the Chinese Medicine Council of Hong Kong.
按上述測試, 所提供的樣品符合香港中醫藥管理委員會所制定的中成藥註冊安全性測試技術指引中的有關重金屬及有毒元素測試, 微生物限度測試及有機氯類殘留農藥測試的限量標準.

For and on behalf of
CMA Industrial Development Foundation Limited

Authorized Signature : 認可人簽名

May S.K. Ng
Deputy Manager
Chemical Division
吳婧錤 -化學部副經理

C.F. Wong
Specialist
Chemical Division
黃灼風 -化學部化驗師

CMA Industrial Development Foundation Limited
Room 1302, Yau Hing Centre, 9-13 Wong Chuk Yeung St., Fo Tan, Shatin, N.T., Hong Kong.
Tel: (852) 2698 8198 Fax: (852) 2695 4177 E-mail: info@cmatcl.com Web Site: http://www.cmatcl.com

Safety tests report of OPR 3 of 4

CMA Testing and Certification Laboratories
麻商會檢定中心

HKLAS 004

TEST REPORT (測試報告)

Report No. 報告編號 : AF024192-001

Date : 2005 October 27 日期

Application No. 申請編號 : LF220378

Test Result 測試結果 :

1. Heavy metal or Toxic Elements test 重金屬及有毒元素測試

Test Item 測試	Result 結果 (ppm)	Result 結果 (Daily Consumption* 每日服用量計算*)	Maximum permitted level 限值標準
a. Lead Content 鉛含量	0.38	23 (µg/day)	179 (µg/day)
b. Cadmium Content 鎘含量	<0.2	<12 (µg/dose)	3500 (µg/dose)
c. Arsenic Content 砷含量	0.68	41 (µg/day)	1500 (µg/day)
d. Mercury Content 汞含量	<0.2	<12 (µg/day)	36 (µg/day)

*Calculated as recommended: 60gram per dose, 1 dose per day, maximum daily intake 60 gram
*依產品建議服用量計算: 每劑 60 克, 每日 1 劑, 最高每日服用量 60 克

2. Microbial Limit test 微生物限度測試

Test Item 測試	Result 結果	Maximum permitted level 限量標準
a. Aerobic Bacteria Count 總細菌數 (CFU/g)	$<1.0 \times 10^1$	1.0×10^3
b. Mold and Yeast Count 霉菌及酵母菌數 (CFU/g)	$<1.0 \times 10^1$	1.0×10^2
c. Escherichia coli 大腸桿菌 (in 1 gram)	Not Detected 未驗出	Not Detected 不得檢出
d. Salmonella species 沙門氏菌 (in 1 gram)	Not Detected 未驗出	Not Detected 不得檢出

CMA Industrial Development Foundation Limited
Room 1302, Yau Hing Centre, 9-13 Wong Chuk Yeung St., Fo Tan, Shatin, N.T., Hong Kong.
Tel: (852) 2698 8198 Fax: (852) 2695 4177 E-mail: info@cmatel.com Web Site: http://www.cmatel.com

Safety tests report of OPR 4 of 4

CMA Testing and Certification Laboratories
廠商會檢定中心

HOKLAS 004

TEST REPORT (測試報告)

Report No. 報告編號 : AF024192-001

Date : 2005 October 27 日期

Application No. 申請編號 : LF220378

3. Organochlorine Pesticides Residue test 有機氯類殘留農藥測試

Test Item 測試	Result 結果 (mg/kg)	Maximum permitted level 限量標準(mg/kg)
a. Aldrin and Dieldrin 艾氏劑及狄氏劑	<0.02	0.05
b. Chlordane 氯丹 (cis-, trans-, oxychlordane)	<0.03	0.05
c. DDT 滴滴涕 (p,p-DDT, o, p-DDT, p,p-DDE and p,p' DDD)	<0.04	1.0
d. Endrin 異狄氏劑	<0.01	0.05
e. Heptachlor 七氯 (Heptachlor and Heptachlor epoxide)	<0.02	0.05
f. Hexachlorobenzene 六氯苯(HCB)	<0.01	0.1
g. Hexachlorocyclohexane 六六六(α-,β-,δ-BHC)	<0.03	0.3
h. Lindane 林丹(γ-BHC)	<0.01	0.6
i. Quintozene 五氯硝基苯 (pentachloronitrobenzene, Pentachloroaniline and Methyl pentachlorophenylsulphide)	<0.03	1.0

Note 註 :
1. ppm denotes part per million. ppm 即百萬份之一
2. µg/day denotes microgram per day. µg/day 即微克/每日
3. µg/dose denotes microgram per dose. µg/dose 即微克/每劑
4. < denotes less than. < 即少於
5. CFU/g denotes colony forming unit per gram. CFU/g 即個/克
6. in 1 gram 即每克
7. mg/kg denotes milligram per kilogram. mg/kg 即毫克/公斤
8. The Maximum permitted level of test items are based on Technical Guidelines for Tests on the Safety of Proprietary Chinese Medicines (pCm), sanctioned by the Chinese Medicine Council of Hong Kong.
限量標準參考香港中醫藥管理委員會所發出的中成藥註冊安全性測試技術指引

***** End of Report *****
***** 報告完結 *****

CMA Industrial Development Foundation Limited
Room 1302, Yan Hing Centre, 9-13 Wong Chuk Yeung St., Fo Tan, Shatin, N.T., Hong Kong.
Tel: (852) 2698 8198 Fax: (852) 2695 4177 E-mail: info@cmatel.com Web Site: http://www.cmatel.com

Appendix V Acute toxicity test report of OPR

Acute toxicity test report of OPR 1 of 2

CMA Testing and Certification Laboratories
廠商會檢定中心

TEST REPORT (測試報告)

Report No. 報告編號 : AF024192-002 Date : 2005 November 28 日期

Application No. 申請編號 : LF220378

Applicant 申請人 : Eu Yan Sang (Hong Kong) Ltd
Unit F, 2/F., Sunview Ind. Bldg.,
3 On Yip Street,
Chai Wan, Hong Kong.

Sample Description 樣品描述 : One (1) submitted sample stated to be TW-002.
由申請人所提供的一個樣品是
Batch No. 產品批號 : TW-002
Expiry Date 有效日期 : 11/2007
Sample Status Upon Receipt 樣品收到時狀況 : Room Temperature 室溫 ***

Date Received 收辦日期 : 2005 October 13.

Test Period 測試日期 : 2005 October 28 – 2005 November 19.

Test Requested 測試要求 : Acute toxicity test (急性毒性試驗)

Test Method 測試方法 : 依據衛生部<<中藥新藥研究指南>>.

Test Result 測試結果 : 本品以 KM 小鼠灌胃給藥, 最大耐受量爲 120 g/kg, 相當於臨床用藥量的 120 倍.

Conclusion 結論 : 本品按衛生部<<中藥新藥研究指南>>檢驗上述項目, 結果以 KM 小鼠灌胃給藥, 最大耐受量爲 120 g/kg, 相當於臨床用藥量的 120 倍.

***** End of Report *****

For and on behalf of
CMA Industrial Development Foundation Limited

認可人簽名 : _____ 總頁數 1 之 1
吳靖鐙
化學部副經理

CMA Industrial Development Foundation Limited
Room 1302, Yun Hing Centre, 9-13 Wong Chuk Yeung St., Fo Tan, Shatin, N.T., Hong Kong.
Tel: (852) 2698 8198 Fax: (852) 2695 4177 E-mail: info@cmatcl.com Web Site: http://www.cmatcl.com

Acute toxicity test report of OPR 2 of 2

CMA Testing and Certification Laboratories
廠商會檢定中心

Appendix 附件

TW-002 小鼠口服 最大耐受量(MTD)

Report No. 報告編號 : AF024192-002 Date : 2005 November 28 日期

Application No. 申請編號 : LF220378

1. 試驗目的
觀察受試物一日給於動物後所產生的急性毒性反應和死亡情況。

2. 試驗材料

2.1 受試物：TW-002, 數量：1.2 kg; 批號：TW-002; 由余仁生(香港)有限公司生產; 臨床用量：成人每日服 60 克。受試物用蒸餾水配制成高濃度的溶液作爲供試液(1.0 g/ml), 臨用前配制。

2.2 動物：SPF 級 KM 種小鼠, 體重 18-22g, 雌雄各半。由廣州中醫藥大學實驗動物中心提供, 實驗動物質量合格證明：粵監證字 2005A004。

2..3 實驗室條件：實驗室溫度爲 20-25°C, 相對濕度爲 40-70%。

3. 試驗方法與結果

3.1 實驗方法：取上述小鼠 20 只, 雌雄各半, 試驗前禁食 14 小時, 正常飲水, 按最大灌胃容量 40 ml/kg 的劑量給予供試液, 一日給藥 3 次, 每次間隔 4 小時, 給藥後連續觀察 7天, 記錄動物的反應、體重變化及死亡情況。

3.2 試驗結果：小鼠灌胃給予 TW-002 後無異常反應, 外觀、活動、飲食、糞便均正常, 體重增加。

4. 結論

KM 種小鼠灌胃給予 TW-002 的最大耐受量(MTD)爲 120 g/kg, 以成人體重爲 60 kg計算, 相當於成人臨床用藥量的 120 倍。

總頁數 1 之 1

CMA Industrial Development Foundation Limited
Room 1302, Yan Hing Centre, 9-13 Wong Chuk Yeung St., Fo Tan, Shatin, N.T., Hong Kong.
Tel: (852) 2698 8198 Fax: (852) 2695 4177 E-mail: info@cmatel.com Web Site: http://www.cmatel.com

Appendix VI English version of the Osteoporosis-Targeted Quality of Life Questionnaire

Section 1: Physical Function (7 items)

Responses: None; a little; a moderate amount; a lot

1. How much physical difficulty do you have pushing or pulling heavy doors?
2. How much physical difficulty do you have vacuuming?
3. How much physical difficulty do you have lifting something heavy, for example a shopping bag or small child?
4. How much difficulty do you have with leisure time activities, such as walking, swimming, bowling, golfing?
5. How much physical difficulty do you have shopping for clothes or presents?
6. How much physical difficulty do you have preparing meals for special gatherings of family or friends?
7. How much physical difficulty do you have visiting relatives or friends who do not live close by?

Section 2: Adaptations (9 Items)

Responses: Strongly agree; agree; disagree; strongly disagree

1. I need to carefully plan activities so I will not become exhausted.
2. I avoid travel because it's too much trouble or too tiring.
3. I have to avoid activities with a group because I can only do things at my own speed.
4. My physical health keeps me from doing community service or other volunteer work.
5. It is difficult to plan activities even a few days in advance because I never know what my physical health will be like.
6. I wear clothes I consider less attractive because that's all that fits.
7. I wear practical shoes for safety.
8. I use items, for example reachers and grabbers, to assist me when I reach for things.
9. It is difficult for me to reach up or down so I keep items more or less at eye level.

Section 3: Fears (6 Items)

Responses: No, not at all; A little; A moderate amount; A lot

1. Does the fear of falling unless holding on to something affect your life?
2. Does the fear of falling and not being able to get up affect your life?
3. Does the fear of falling and having a fracture (broken bone) affect your life?
4. Does the fear of pain from fractures affect your life?
5. Does the fear of future physical limitations due to osteoporosis affect your life?
6. Does the fear of osteoporosis because of lack of help affect your life?

Section 4: Osteoporotic Changes (3 Items)

1. Have you lost height since the age of 25? If yes, how many inches have you lost?
2. Have you noticed a change in your posture, such as a round back? If no, how important is it for you to avoid developing this change in your posture? If yes, how important is it for you to hide this change in your posture?
3. Have you had a hip, wrist, or spine fracture since age 45? If yes, were any of these fractures the result of a sudden move or a fall from a standing height or less (such as slipping, stumbling, or falling from bed)?

Section 5: Health and Demographics (7 Items)

1. How would you rate your overall health?
2. How would you rate your quality of life as regards your health?
3. Has anyone in your family (mother, sister, grandmother, aunt) or close friends/relatives had osteoporosis?
4. Have you ever had your bone density (a test for osteoporosis) measured?
5. Have you ever been told by a doctor that you had osteoporosis?
6. How old are you?
7. With which ethnic group do you most associate yourself?

Appendix VII Expert panel review form for the Osteoporosis-Targeted Quality of Life Questionnaire

Expert Panel Review on

Content Validation of The Chinese Osteoporosis-Targeted Quality of Life Questionnaire (The Chinese-OPTQoL)

Name of Reviewer : _____

Work Setting : _____

Year of Clinical Experience : _____

Date of Review : _____

INSTRUCTIONS TO REVIEWERS:

This document consists of two parts.

Part I :

1. Read the information sheet, which describes the purposes of this part of the study.
2. Give your consent for participating in the Expert Panel Review by signing the consent form in this section.

Part II :

1. Panel members are guided to fill in the questionnaire.
2. Final conclusion on the content relevance and representativeness will be drawn after discussion by experts.

Part I - Consent Form

Project Title: Development and Validation of a Discriminative Chinese Osteoporosis-Targeted Quality of-Life Questionnaire (The Chinese OPTQoL)

Principle Investigator: Mr Frank Ho-yin, LAI, Miss Ching, LIONG

The research project will validate The Chinese Osteoporosis-Targeted Quality of Life Questionnaire (The Chinese OPTQoL) in measuring the quality of life for women with osteoporosis. This expert panel review will help to evaluate the relevance and representativeness of The Chinese Osteoporosis-Targeted Quality of Life Questionnaire (The Chinese OPTQoL). The questionnaire contains 22 scored items in three domains – 7 questions in physical function, 9 questions in adaptations, and 6 questions in fears – and 3 non-scored questions relating to osteoporotic changes and 7 questions on health and demographics. This instrument is unique among osteoporosis-targeted questionnaires in that it attempts to measure the total impact of the disease on quality of life within a population at a single point in time.

I agree to attend the panel review and contribute my ideas in the content validation of The Chinese Osteoporosis-Targeted Quality of Life Questionnaire (The Chinese-OPTQoL)

This study carries no risk to me. There will be no direct benefits for me. My name will not be appeared in any documents or reports. All information collected in this study will be kept anonymous.

I am free to withdraw my consent and stop participating at any time. I have been given the chance to ask questions. I am satisfied that all my questions have been answered.

My signature means:

I have read this form,

I understand my involvement in this study; and

I voluntarily agree to participate.

I will be given a copy of this consent form. If I have any questions concerning this study, I can contact Mr Frank LAI at 2607-6505 or Miss LIONG Ching at 2603-7143.

Name of Participant : _____

Signature of Participant : _____ Date: _____

The Chinese Osteoporosis-Targeted Quality of Life Questionnaire

(The Chinese-OPTQoL)

Part II - An expert review on its semantic equivalence translation and content validity

Reference for Content Review

The original Osteoporosis-Targeted Quality of Life Questionnaire (The OPTQoL) contains 22 scored items in three domains – 7 questions in physical function, 9 questions in adaptations, and 6 questions in fears – and 3 non-scored questions relating to osteoporotic changes and 7 questions on health and demographics. This instrument is unique among osteoporosis-targeted questionnaires in that it attempts to measure the total impact of the disease on quality of life within a population at a single point in time. This study is to validate The Chinese Osteoporosis-Targeted Quality of Life Questionnaire.

I. Personal Information:

Name of Reviewer:_____
Occupation:_____
Work Setting:_____
Years of experience in rehabilitation:_____
Date of Review:_____

II. Study of Translation

Physical Function

Please indicate whether the Domain of **Physical Function** in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle):

Physical Function = 身體機能

Agree / Disagree, suggestions:_____

Please indicate whether the 7 items in the Domain of Physical Function in the Osteoporosis-Targeted Quality–of-Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle):

How much physical difficulty do you have pushing or pulling heavy doors?

當你推/拉重門時，你在體力上感到有多少困難?

Agree / Disagree, suggestions:_____

How much physical difficulty do you have vacuuming?

當你清潔家居時，你在體力上感到有多少困難?

Agree / Disagree, suggestions:_____

How much physical difficulty do you have lifting something heavy, for example a shopping bag or small child?

當你提舉重物(如購物袋、抱小孩)時，你在體力上感到有多少困難?

Agree / Disagree, suggestions:_____

How much physical difficulty do you have with leisure time activities, such as walking, swimming, bowling, golfing?

當你進行休閒活動(如散步、游泳、划船、打高爾夫球)時，你在體力上感到有多少困難?

Agree / Disagree, suggestions:_____

How much physical difficulty do you have shopping for clothes or presents?

當你購物(如衣物、禮品)時，你在體力上感到有多少困難?

Agree / Disagree, suggestions:_____

How much physical difficulty do you have preparing meals for special gatherings of family or friends?

當你為家人或朋友的聚會準備食物時，你在體力上感到有多少困難?

Agree / Disagree, suggestions:_____

How much physical difficulty do you have visiting relatives or friends who do not live close by?

當你探訪不住在附近的家人或朋友時，你在體力上感到有多少困難?

Agree / Disagree, suggestions:_____

Please also indicate whether the **"Response Scales"** in the Domain of **"Physical Function"** are adequately and accurately translated (Please circle) :

Agree / Disagree, suggestions:_____

None=沒有困難

Agree / Disagree, suggestions:_____

A Little=有點困難

Agree / Disagree, suggestions:_____

A Moderate Amount=中度困難

Agree / Disagree, suggestions:_____

A Lot=非常困難

Agree / Disagree, suggestions:_____

Study of Translation

Adaptations

Please indicate whether the Domain of **Adaptations** in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle) :

Adaptations = 適應性

Agree / Disagree, suggestions:_____

Please indicate whether the 9 items in the Domain of Adaptations in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle):

1. I need to carefully plan activities so I will not become exhausted.

為免過勞，我需要仔細地安排活動。

Agree / Disagree, suggestions:_____

2. I avoid travel because it's too much trouble or too tiring

由於怕會太麻煩或太累，我會避免外出旅遊。

Agree / Disagree, suggestions:_____

3. I have to avoid activities with a group because I can only do things at my own speed.

由於我只能按自己的速度行事，所以我會避免參加群體活動。

Agree / Disagree, suggestions:_____

4. My physical health keeps me from doing community service or other volunteer work.

我的健康狀況妨礙我參加社區服務或義務工作。

Agree / Disagree, suggestions:_____

5. It is difficult to plan activities even a few days in advance because I never know what my physical health will be like.

由於我不知道我的身體狀況將會怎樣，我很難幾天前預先安排活動。

Agree / Disagree, suggestions:_____

6. I wear clothes I consider less attractive because that's all that fits.

我選擇較不吸引的衣服，因為只有它們才適合我。

Agree / Disagree, suggestions:_____

7. I wear practical shoes for safety

我選穿實用的鞋子是為了安全。

Agree / Disagree, suggestions:_____

8. I use items, for example reachers or grabbers, to assist me when I reach for things

當我拿取物品時需要借助工具(如長柄夾、扶手等)。

Agree / Disagree, suggestions:_____

9. It is difficult for me to reach up or down so I keep items more or less at eye level.

我會把東西放在視線水平的位置是因為我拿取放在高位或低位的東西有困難。

Agree / Disagree, suggestions:_____

Please also indicate whether the **"Response Scales"** in the Domain of **"Adaptations"** are adequately and accurately translated (Please circle):

Response=評分

Agree / Disagree, suggestions:_____

Strongly Agree =非常贊同

Agree / Disagree, suggestions:_____

Agree=贊同

Agree / Disagree, suggestions:_____

Disagree=不贊同

Agree / Disagree, suggestions:_____

Strongly Disagree=非常不贊同

Agree / Disagree, suggestions:_____

Study of Translation

Fears

Please indicate whether the Domain of **Fears** in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle) :

Fears = 憂慮

Agree / Disagree, suggestions:_____

Please indicate whether the 6 items in the Domain of Fears in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle):

1. Does the fear of falling unless holding on to something affect your life?

你會否因為憂慮不扶東西就會跌倒，從而影響你的生活?

Agree / Disagree, suggestions:_____

2. Does the fear of falling and not being able to get up affect your life?

你會否因為憂慮跌倒及跌倒後不能爬起，從而影響你的生活?

Agree / Disagree, suggestions:_____

3. Does the fear of falling and having a fracture (broken bone) affect your life?

你會否因為憂慮跌倒並且引起骨折，從而影響你的生活?

Agree / Disagree, suggestions:_____

4. Does the fear of pain from fracture affect your life?

你會否因為憂慮骨折會引起疼痛，從而影響你的生活?

Agree / Disagree, suggestions:_____

5. Does the fear of future physical limitations due to osteoporosis affect your life?

你會否因為憂慮患骨質疏鬆症而引起將來活動不便，從而影響你的生活?

Agree / Disagree, suggestions:_____

6. Does the fear of osteoporosis because of lack of help affect your life?

你會否因為憂慮患骨質疏鬆症後缺少幫助，從而影響你的生活?

Agree / Disagree, suggestions:_____

Please also indicate whether the **"Response Scales"** in the Domain of **"Fears"** are adequately and accurately translated (Please circle) :

Response=評分

Agree / Disagree, suggestions:_____

None=沒有憂慮

Agree / Disagree, suggestions:_____

A Little=有點憂慮

Agree / Disagree, suggestions:_____

A Moderate Amount=中度憂慮
Agree / Disagree, suggestions:_____

A Lot=非常憂慮

Agree / Disagree, suggestions:_____

Osteoporotic Changes

Please indicate whether the Domain of **Osteoporotic Changes** in the Osteoporosis-Targeted Quality of Life Questionaire (OPTQoL) are adequately and accurately translated (Please circle):

Osteoporotic Change =骨質疏鬆的變化

Agree / Disagree, suggestions:_____

Please indicate whether the 3 items in the Domain of Osteoporotic Changes in the Osteoporosis-Targeted Quality–of-Life Questionaire (OPTQoL) are adequately and accurately translated (Please circle):

Have you lost height since the age of 25? If yes, how many inches have you lost?

你在 25 歲以後身高變矮? 若有，減少多少_____ 厘米

Agree / Disagree, suggestions:_____

Have you noticed a change in your posture, such as a round back? If no, how important is it for you to avoid developing this change in your posture? If yes, how important is it for you to hide this change in your posture?

你是否注意你有體態的變化，如駝背?
如果沒有，避免體態發生這樣的變化對你來說有多重要?
如果有，隱藏這種體態變化對你有多重要?

Agree / Disagree, suggestions:_____

Please also indicate whether the **"Response Scales"** in this Question in the Domain of **"Osteoporotic Change"** are adequately and accurately translated (Please circle) :

Agree / Disagree, suggestions:_____

None = 沒有多重要

Agree / Disagree, suggestions:_____

A Little = 有點重要

Agree / Disagree, suggestions:_____

A Moderate Amount = 中度重要

Agree / Disagree, suggestions:_____

A Lot = 非常重要

Agree / Disagree, suggestions:_____

Have you had a hip, wrist, or spine fracture since age 45? If yes, were any of these fractures the result of a sudden move or a fall from a standing height or less (such as slipping, stumbling, or falling from bed)?

你在 45 歲以後是否曾經有髖骨、手腕或脊椎的骨折? 如果有，該骨折是否由於突然移動身體或由跌倒 (如滑倒、絆倒、或由床上跌落)所造成的?

Agree / Disagree, suggestions:_____

Health and Demographics

Please indicate whether the Domain of **Health and Demographics** in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle):

Health and Demographics = 個人健康資料

Agree / Disagree, suggestions:_____

Please indicate whether the 7 items in the Domain of Health and Demographics in the Osteoporosis-Targeted Quality–of-Life Questionnaire (OPTQoL) are adequately and accurately translated (Please circle):

How would you rate your overall health?

你認為自己總的健康狀祝如何?

Agree / Disagree, suggestions:_____

How would you rate your quality of life as regards your health?

從健康方面來說，你認為你的生活質素如何？

Agree / Disagree, suggestions:_____

For Question 1 and Question 2, Please also indicate whether the **"Response Scales"** in this Question in the Domain of **"Health and Demographics"** are adequately and accurately translated (Please circle) :

Agree / Disagree, suggestions:_____

Very Well =非常好

Agree / Disagree, suggestions:_____

Well = 好

Agree / Disagree, suggestions:_____

Bad = 不好

Agree / Disagree, suggestions:_____

Very Bad =非常不好

Agree / Disagree, suggestions:_____

Has anyone in your family (mother, sister, grandmother, aunt) or close friends/relatives had osteoporosis?

你的家庭成員(母親、姐妹、祖母、姨嬸等)或近親有否患有骨質疏鬆症?

Agree / Disagree, suggestions:_____

Has you ever had your bone density (a test for osteoporosis) measured?

你是否做過骨質密度檢查?

Agree / Disagree, suggestions:_____

Have you ever been told by a doctor that you had osteoporosis?

是否有醫生告訴你患了骨質疏鬆症?

Agree / Disagree, suggestions:_____

How old are you ?

你有多大年紀?

Agree / Disagree, suggestions:_____

With which ethnic group do you most associated yourself?

你最常參與的社會團體或組織是：_____

Agree / Disagree, suggestions:_____

III. Study of Content Relevancy

Content Relevancy

Physical Function

Please indicate how far each of the following 7 items is relevant to the Domain of Physical Function in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL):

當你推/拉重門時，你在體力上感到有多少困難?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

當你清潔家居時，你在體力上感到有多少困難?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

當你提舉重物(如購物袋、抱小孩)時，你在體力上感到有多少困難?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

當你進行休閒活動(如散步、游泳、划船、打高爾夫球)時，你在體力上感到有多少困難?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

當你購物(如衣物、禮品)時，你在體力上感到有多少困難?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

當你為家人或朋友的聚會準備食物時，你在體力上感到有多少困難?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

當你探訪不住在附近的家人或朋友時，你在體力上感到有多少困難?

Totally Relevant 5 4 3 2 1 Totally Irrelevant
Comments:

Content Relevancy

Please indicate how far do these 7 items as a whole adequately relevant to the Domain of Physical Function in the Osteoporosis-Targeted Quality of Life Questionnaire:

Totally Relevant 5 4 3 2 1 Totally Not Relevant

Study of Content Relevancy

Content Relevancy

Adaptations

Please indicate how far each of the following 9 items is relevant to the Domain of Adaptations in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL):

1.	為免過勞，我需要仔細地安排活動。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

2.	由於怕會太麻煩或太累，我會避免外出旅遊。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

3.	由於我只能按自己的速度行事，所以我會避免參加群體活動。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

4.	我的健康狀況妨礙我參加社區服務或義務工作。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

5.	由於我不知道我的身體狀況將會怎樣，我很難幾天前預先安排活動。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

6.	我選擇較不吸引的衣服，因為只有它們才適合我。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

7.	我選穿實用的鞋子是為了安全。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

8.	當我拿取物品時需要借助工具(如長柄夾、扶手等)。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

9.	我會把東西放在視線水平的位置是因為我拿取放在高位或低位的東西有困難。

Totally Relevant 5 4 3 2 1 Totally Irrelevant

Comments:

_____

_____

_____

_____

Content Relevancy

Please indicate how far do these 7 items as a whole adequately relevant to the Domain of Adaptations in the Osteoporosis-Targeted Quality of Life Questionnaire :

Totally Relevant 5 4 3 2 1 Totally Not Relevant

Study of Content Relevancy

Content Relevancy

Fear

Please indicate how far each of the following 6 items is relevant to the Domain of Fears in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL):

1.	你會否因為憂慮不扶東西就會跌倒，從而影響你的生活?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

2.	你會否因為憂慮跌倒及跌倒後不能爬起，從而影響你的生活?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

3.	你會否因為憂慮跌倒並且引起骨折，從而影響你的生活?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

4.	你會否因為憂慮骨折會引起疼痛，從而影響你的生活?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

5.	你會否因為憂慮患骨質疏鬆症而引起將來活動不便，從而影響你的生活?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

6.	你會否因為憂慮患骨質疏鬆症後缺少幫助，從而影響你的生活?

Totally Relevant 5 4 3 2 1 Totally Irrelevant

Comments:

Content Relevancy

Please indicate how far do these 7 items as a whole adequately relevant to the Domain of Fears in the Osteoporosis-Targeted Quality of Life Questionnaire :

Totally Relevant 5 4 3 2 1 Totally Not Relevant

IV. Study of Content Representativeness

Content Representativeness

Physical Function

Please indicate how far each of the following 7 items can represent the Domain of Physical Function in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL):

當你推/拉重門時，你在體力上感到有多少困難?

Totally Represent 5 4 3 2 1 Totally Not Represent

當你清潔家居時，你在體力上感到有多少困難?

Totally Represent 5 4 3 2 1 Totally Not Represent

當你提舉重物(如購物袋、抱小孩)時，你在體力上感到有多少困難?

Totally Represent 5 4 3 2 1 Totally Not Represent

當你進行休閒活動(如散步、游泳、划船、打高爾夫球)時，你在體力上感到有多少困難?

Totally Represent 5 4 3 2 1 Totally Not Represent

當你購物(如衣物、禮品)時，你在體力上感到有多少困難?

Totally Represent 5 4 3 2 1 Totally Not Represent

當你為家人或朋友的聚會準備食物時，你在體力上感到有多少困難?

Totally Represent 5 4 3 2 1 Totally Not Represent

當你探訪不住在附近的家人或朋友時，你在體力上感到有多少困難?

Totally Represent 5 4 3 2 1 Totally Not Represent

Comments:

Content Representativeness

Please indicate how far do these 7 items as a whole adequately represent to the Domain of Physical Function in the Osteoporosis-Targeted Quality of Life Questionnaire:

Totally Represent 5 4 3 2 1 Totally Not Represent

Study of Content Representativeness

Content Representativeness

Adaptations

Please indicate how far each of the following 9 items can represent to the Domain of Adaptations in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL):

1. 為免過勞，我需要仔細地安排活動。

Totally Represent 5 4 3 2 1 Totally Not Represent

2. 由於怕會太麻煩或太累，我會避免外出旅遊。

Totally Represent 5 4 3 2 1 Totally Not Represent

3. 由於我只能按自己的速度行事，所以我會避免參加群體活動。

Totally Represent 5 4 3 2 1 Totally Not Represent

4. 我的健康狀況妨礙我參加社區服務或義務工作。

Totally Represent 5 4 3 2 1 Totally Not Represent

5. 由於我不知道我的身體狀況將會怎樣，我很難幾天前預先安排活動。

Totally Represent 5 4 3 2 1 Totally Not Represent

6.	我選擇較不吸引的衣服，因為只有它們才適合我。

Totally Represent 5 4 3 2 1 Totally Not Represent

7.	我選穿實用的鞋子是為了安全。

Totally Represent 5 4 3 2 1 Totally Not Represent

8.	當我拿取物品時需要借助工具(如長柄夾、扶手等)。

Totally Represent 5 4 3 2 1 Totally Not Represent

9.	我會把東西放在視線水平的位置是因為我拿取放在高位或低位的東西有困難。

Totally Represent 5 4 3 2 1 Totally Not Represent

Comments:

_____

_____

_____

_____

Content Representativeness

Please indicate how far do these 7 items as a whole adequately represent to the Domain of Adaptations in the Osteoporosis-Targeted Quality of Life Questionnaire:

Totally Represent 5 4 3 2 1 Totally Not Represent

Study of Content Representativeness

Content Representativeness

Fear

Please indicate how far each of the following 6 items can represent to the Domain of Fears in the Osteoporosis-Targeted Quality of Life Questionnaire (OPTQoL):

1. 你會否因為憂慮不扶東西就會跌倒，從而影響你的生活?

Totally Represent 5 4 3 2 1 Totally Not Represent

2. 你會否因為憂慮跌倒及跌倒後不能爬起，從而影響你的生活?

Totally Represent 5 4 3 2 1 Totally Not Represent

3. 你會否因為憂慮跌倒並且引起骨折，從而影響你的生活?

Totally Represent 5 4 3 2 1 Totally Not Represent

4. 你會否因為憂慮骨折會引起疼痛，從而影響你的生活?

Totally Represent 5 4 3 2 1 Totally Not Represent

5. 你會否因為憂慮患骨質疏鬆症而引起將來活動不便，從而影響你的生活?

Totally Represent 5 4 3 2 1 Totally Not Represent

6. 你會否因為憂慮患骨質疏鬆症後缺少幫助，從而影響你的生活?

Totally Represent 5 4 3 2 1 Totally Not Represent

Comments:

_____

_____

_____

_____

Content Representativeness

Please indicate how far do these 7 items as a whole adequately represent to the Domain of

Fears in the Osteoporosis-Targeted Quality of Life Questionnaire:

Totally Represent 5 4 3 2 1 Totally Not Represent

Others

Do you have any other comments on the Chinese Osteoporosis-Targeted Quality of Life Questionnaire (The Chinese-OPTQoL) ??

Thanks for your valuable effort and kind assistance!

End

Appendix VIII Final version of the Chinese Osteoporosis-Targeted Quality of Life Questionnaire

骨質疏鬆症的生活質量問卷

The Chinese Osteoporosis-Targeted Quality of Life Questionnaire (The Chinese OPTQoL)
為了幫助醫生瞭解您的健康和生活狀況，請您認真、如實填寫這份健康量表。請根據自己的第一感覺選填答案，并請不要漏答。請將答案填於題號前的空格內，多謝您的合作。

一、身體機能(7 項問題)

A: 沒有困難，B: 有點困難，C: 中度困難，D: 非常困難

	1. 當你推/拉重門時，你在體力上感到有多少困難?
	2. 當你清潔家居時，你在體力上感到有多少困難?
	3. 當你提舉重物(如購物袋、抱小孩)時，你在體力上感到有多少困難?
	4. 當你進行休閒活動(如散步、游泳、划船、打高爾夫球)時，你在體力上感到有多少困難?
	5. 當你購物(如衣物、禮品)時，你在體力上感到有多少困難?
	6. 當你為家人或朋友的聚會準備食物時，你在體力上感到有多少困難?
	7. 當你探訪不住在附近的家人或朋友時，你在體力上感到有多少困難?

二、適應性(9 項問題)

A: 非常贊同、B: 贊同、C: 不贊同、D:非常不贊同

	1. 我需要仔細地安排活動為免過勞。
	2. 我避免外出旅遊，因為怕會太麻煩或太累。
	3. 由於我只能按自己的速度行事，所以我會避免參加群體活動。
	4. 我的健康狀況妨礙我參加社區服務或義務工作。
	5. 由於我不知道我的身體狀況將會怎樣，我很難幾天前預先安排活動。
	6. 我選擇較不吸引的衣服，因為只有它們才適合我。
	7. 我選穿實用的鞋子是為了安全。
	8. 當我拿取物品時需要借助工具(如長柄夾、扶手等)。
	9. 我會把東西放在視線水平的位置是因為我拿取放在高位或低位的東西有困難。

三、憂慮(6 項問題)　A: 沒有憂慮.、B: 有點憂慮、C: 中度憂慮、D: 非常憂慮

	1. 你會否因為憂慮不扶東西就會跌倒，從而影響你的生活?
	2. 你會否因為憂慮跌倒及跌倒後不能爬起，從而影響你的生活?
	3. 你會否因為憂慮跌倒並且引起骨折，從而影響你的生活?
	4. 你會否因為憂慮骨折會引起疼痛，從而影響你的生活?
	5. 你會否因為憂慮患骨質疏鬆症而引起將來活動不便，從而影響你的生活?
	6. 你會否因為憂慮患骨質疏鬆症後缺少幫助，從而影響你的生活?

四、骨質疏鬆的變化(3 項問題)

1. 你在 25 歲以後身高變矮?
   是口－> 若有，減少多少_____ 厘米 (請具體填寫)
   否口
2. 你是否注意你有體態的變化，如駝背?
   評分: 1=很重要；2=重要；3=不重要；4=很不重要
   □ 如果有，隱藏這種體態變化對你有多重要?
   □ 如果沒有，避免體態發生這樣的變化對你來說有多重要?
3. 你在 45 歲以後是否曾經有髖骨、手腕或脊椎的骨折?
   是口－>如果有，該骨折是否由於突然移動身體或由跌倒 (如滑倒、絆倒、或由床上跌落)所造成的?
   是口 否口
   否口

五、個人健康資料(7 項問題)　A: 非常好、B: 好、C: 不好、D: 非常不好

1. 你認為自己總體的健康狀祝如何?
2. 從健康方面來說，你認為你的生活質素如何？
3. 你的家庭成員(母親、姐妹、祖母、姨嬸等)或近親有否患有骨質疏鬆症?
   有口　無口
4. 你是否做過骨質密度檢查? 有口 無口
5. 是否有醫生告訴你患了骨質疏鬆症? 有口 無口
6. 你有多大年紀? 請具體填寫_____歲。
7. 你最常參與的社會團體或組織是　：_____

《全卷完，多謝您的合作！》

Appendix IX Patient / Subject information sheet

TWGHs Kwong Wah Hospital – The Chinese University of Hong Kong
Chinese Medicine Clinical Research and Services Centre
Patient/Subject Information Sheet

A Case Study on the effects of Traditional Chinese Medicine for patients with Osteoporosis

You are invited to participate in a research*. Before you decide, it is important that you understand why the research is done and how you will be involved. Please read the information carefully and discuss it with friends, relatives and your family doctor if you wish. Ask if there is anything unclear or if you wish to obtain more information. Take time to decide whether you wish to participate in the research.
* The research has been reviewed by the Clinical Research Ethics Committee, Kowloon West Cluster.

Principal investigators of this research:
Dr. Chan Ming Houng - Consultant Physician, Medicine & Geriatrics, KWH

Background:
Osteoporosis (OP) is a disease characterized by low bone mass and structural deterioration of bone tissue, leading an increased chance to fractures. Osteoporotic fractures cause huge economic and social burden to patients and society. Conventional treatment in Osteoporosis includes medication, exercises and supplement intake. However, due to the adverse effect, limitation and high cost of the approved medication, there is an urgent need for alternative and safer therapeutic strategies. Traditional Chinese medicine (TCM) has been practicing in China for thousands of years. More and more researches show its positive effect on various diseases. It is hoped that osteoporosis patients can be benefited by the use of TCM.

Aim of research:
To study whether osteoporosis patients, receiving TCM Chinese herbal preparation, will have a significant decrease in Bone mineral density (BMD) drop in comparing with placebo group.

Criteria in choosing patients:
6. Women aged 55 or above
7. Postmenopausal for at least 2 years
8. Diagnosis as Osteoporosis according to the World Health Organization (WHO) definitions.
9. Fulfill the TCM sub-type requirement according to TCM specific diagnosis system.

Research method:

Participants will be randomized into two groups – treatment group and control group.
Treatment group: Chinese herbal medicine (orally in powder form) + Calcium & Vitamin D
Control group: Placebo herbal medicine (orally in powder form) + Calcium & Vitamin D
In this study, we plan a double-blind, placebo-controlled, randomized clinical trial.
Double-blind trial: In a double-blind trial, neither you nor the doctor knows which treatment group you are in.
Placebo: A placebo is a dummy treatment like pill which looks like the real thing but is not. It contains no active ingredient.
Randomized trial: Sometimes we do not know which treatment is the best. Therefore we need to make comparisons. Patients / Subjects will be selected by chance and divided into groups. Patients / Subjects in each group will have different treatments and the outcomes are then compared.

Research procedure:

All participants will be evaluated at scheduled time slots for every 2 weeks. Each evaluation will last for about 15-20 minute. Traveling expenses will not be available. About 148 patients will be involved in the research and they will need to take in the provided medication (or placebo) for 6 months. The research will last for 2 years. Conventional treatment will be provided after the research has ended.
The assessment includes the measurement of your lumber and hip BMD changes, recording your new fractures (if any), questionnaire survey, height measurement, blood and urine samples collection, physical examination and the TCM examination.
In the course of treatment, TWGHs Kwong Wah Hospital – The Chinese University of Hong Kong Chinese Medicine Clinical Research and Services Centre will provide Chinese herbal powder to you. You will be assessed at the centre mentioned above.

Current approach for Osteoporosis:

Currently, bisphosphonates (alendronate and risedronate), calcitonin, estrogens, parathyroid hormone and raloxifene are approved by the US Food and Drug Administration (FDA) for the prevention and/or treatment of osteoporosis. Exercises, Calcium and vitamin D treatment are also introduced.

Taking Traditional Chinese Medicine:

Please remember to take Chinese medicine powder as instructed, one pack each time, twice a day, 1 hour after meal and at least 4 hours apart. If you need to take western medicine due to other disease, please take Chinese medicine at least 2 hours apart from western medicine. You cannot take in any medication related to bone pathology once you participate in the research.

Chinese medicine my cause side effects such as gastrointestinal distress and allergic reactions. In case of discomfort, please call our centre (contact no: 3517-2634) at once or consult your family doctor. In case of emergency condition, please call 999 for immediate treatment. Drugs used in this research DO NOT contain any toxic herbs in the toxic and potent Chinese herbal medicines list.

Rights of participants:

It is up to you to decide whether to participate or not. If you decide to participate, you will keep this information sheet and sign a consent form. You will be free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

Your research doctor will inform you if new information about the treatment / drug under research is available. You may decide whether to continue to participate in the research or not. If you decide to continue, you will be asked to sign an updated consent form. If you choose to withdraw, your research doctor will arrange for your continued care. On the other hand, your research doctor may suspend your participation in the research should it be in your best interest. He / she will explain to you and arrange for your continued care.

Benefits of participation:

Osteoporosis patents will receive not only a conventional treatment, but also TCM herbal formula, which may decrease Bone mineral density (BMD) drop and new fracture risk.

Moreover, subjects can receive free bone scan, complete blood test, liver and renal function test, TCM consultation once they participate in the trial.

We hope the treatment may help you. However, it cannot be guaranteed. The information we get from this research may help us treat future patients with osteoporosis better.

Risks of participation:

Participation may affect your insurance status in future. Please check with the insurers in advance to ensure that your participation will not affect the medical insurance.

During assessment, we may uncover some conditions (e.g. high blood pressure, Diabetic Mellitus) which were unaware of previously, we will record your condition and provide treatment if necessary.

Staffs of the TWGs Kwong Wah Hospital – The Chinese University of Hong Kong, Chinese Medicine Clinical Research and Services Centre are under coverage of the Medical Institution Malpractice Indemnity Insurance and this insurance also covers claims arising out of clinician trials and clinical researches.

Confidential agreement:

All information about you collected during the research will be kept strictly confidential.

Should any information be sent outside the hospital, your name and address will be removed so that you cannot be identified.

Inquiry contact:
You can obtain further information about the research at TWGHs Kwong Wah Hospital – The Chinese University of Hong Kong Chinese Medicine Clinical Research and Services Centre.

Thank you for participation in the research. You will be given a copy of the information sheet and a signed copy of the informed consent form.

東華三院廣華醫院 – 香港中文大學
中醫藥臨床研究服務中心
中醫藥對改善骨質疏鬆症患者骨質密度及臨床症狀的研究

參加者須知

你已被邀請參與一項科研計劃，在你下決定之前，請細心閱讀以下內容，並了解本科研的目的及你需要作出的配合，你可以與親友及你的家庭醫生商議後才作出決定，如有不明白之處或需要更多資料，歡迎查詢，請閣下細心考慮。

主要研究人員：
陳銘洪醫生　－廣華醫院老人科顧問醫生

背景：
骨質疏鬆是以單位體積內骨量減少，骨的微結構退化，易於骨折為特徵的系統性骨骼疾病，骨質疏鬆引起的骨折已為病人以及社會帶來巨大的經濟、生理和心理負擔，過去十年，骨質疏鬆症的傳統治療：包括口服藥物、運動、補充劑等，由於具有多種局限性（藥費高昂、須符合適應症、副反應），未能廣泛應用於患者身上，患者都期盼有一種更安全、低侵擾性的另類療法可供選擇，中國醫學源遠流長，數千年來為人類的健康作出貢獻，近年來不斷有現代研究為中醫藥的療效提供科學憑證，我們期望骨質疏鬆症亦可在中醫藥的範疇內找到治療的新方向，

研究目的:
本研究探討中醫藥對骨質疏鬆症患者骨質密度及臨床症狀的改善是否優於鈣片及維生素丁內服組，

病人納入標準:
1) ５５歲或以上的女性
2) 停經兩年或以上
3) 符合世界衛生組織對骨質疏鬆症的診斷標準
4) 符合中醫証候診斷標準

研究方法:
閣下將被隨機分配入治療組或對照組：
治療組：口服中藥（粉劑）＋內服鈣片及維生素丁
對照組：口服安慰劑（粉劑）＋內服鈣片及維生素丁
此研究採用雙盲試驗、安慰劑對照、隨機方法進行，
雙盲試驗：在雙盲試驗中，無論醫生及病人都無權知悉受試對象被分配在治療組或對照組，

安慰劑：安慰劑是一種外觀與治療藥物近乎一樣的無藥效製劑，當中並無任何有效成份．
隨機：為找出那一種治療手段能帶給病人最大利益，我們需要進行分組比較．每位受試者將有等同機會被分配到不同的組別，並接受不同的治療手段以比較出療效的優劣．

研究程序:
閣下需要每兩個星期預約時間接受評估．每次評估大約需時１５至２０分鐘．來往評估地點的交通費須自行支付．參與此研究的病人預計有１４８名，每位病人需要連續服藥６個月．整個研究預計為兩年．你於研究結束後將繼續接受傳統療法治療．
定期評估的內容包括量度骨質密度、記錄服藥後（如有）的骨折次數、回答問卷、量度身高、收集血液及尿液樣本作測試、身體檢查和中醫四診資料收集等．
研究進行期間，東華三院廣華醫院 – 香港中文大學－中醫藥臨床研究服務中心會為閣下提供口服中藥（粉劑），你亦會於上述中心接受評估．

研究進展:
近年來，美國食物及藥物管理局逐步批准一些新藥作為預防及／或治療骨質疏鬆症的藥物．此外，運動、鈣片及維生素丁補充劑等亦於臨床配合使用．

服用中藥:
請謹記按時服藥，每天服藥兩次（最少相隔四小時），每次一包，飯後一小時溫服．如果需要同時服用西藥，請維持２小時的間隔時間．閣下在參與研究期間，將不能服用一切有關治療或預防骨骼疾病的藥物．另外，服用中藥有可能出現腸胃不適、過敏反應等副作用，如有任何不適，閣下可玫電本中心（電話：３５１７－２６３４）或聯絡閣下之家庭醫生．如發生任何突發事故，閣下可致電９９９要求緊急援助．本研究所採用之中藥均不屬於毒性／烈性中藥名單之內．

參與者之權利:
此項研究純屬自願參與，若閣下決定參與，請細閱此份參加者須知並簽署一份參加研究同意書．閣下可於任何時候無須任何原因退出研究，並且不會因此影響你得到醫療服務的機會及質素，亦不會由此負上任何法律責任．
如在研究期間有新開發的藥物或治療方法可供選擇，閣下之科研醫生會知會你，並由你自行決定是否繼續參與研究．若你決定繼續參與，你或者需要簽署一份新的同意書．若你選擇退出，科研醫生也會為你安排其他治療方法．但科研醫生也可能為著病人的最大利益而終止閣下參與此科研計劃，屆時他會向你解釋並為你安排其他治療．

參與者之得益:
閣下不但可接受骨質疏鬆症的傳統治療，還可以同時接受中醫藥治療，此治療有可能減慢患者骨礦密度的下降率及骨折的機會．患者一旦參與研究，將可免費接受本中心為受試者提供的骨質密度量度、血液樣本測試、肝腎功能檢查、身體檢查和註冊中醫醫療評

估，我們期望中醫藥治療可為閣下提供幫助，但本研究所提供之治療均無絕對療效保證，由本研究所收集的資料將能協助我們為日後的骨質疏鬆症患者提供更佳之治療方案。

參與者之風險:

參與此研究或會影響閣下之保險條款，請閣下務必知會你的保險公司有關參與本研究的決定，以確定受保範圍不受影響。

於評估期間，我們或會發現閣下一些未被知會的健康問題（如高血壓病、糖尿病等），這些健康問題將會被記錄並於有需要時給予相關治療。

東華三院廣華醫院 – 香港中文大學－中醫藥臨床研究服務中心已為屬下員工購買保證賠償保險，若果醫療失當事故不幸發生，病人便可就損失提出賠償要求。

保密協議:

所有個人資料將會絕對保密，可供識別你個人身份的資料（如姓名、地址）亦會在研究結束發放資料前被刪除。

查詢方法:

你可向東華三院廣華醫院 – 香港中文大學－中醫藥臨床研究服務中心索取更多有關本研究的資料。

感謝閣下參與這項研究，我們衷心感激。我們會把此參加者須知及閣下已簽署之參加研究同意書複印本給予閣下保存，以供查閱。

Appendix X Informed consent form

TWGHs Kwong Wah Hospital – The Chinese University of Hong Kong
Chinese Medicine Clinical Research and Services Centre
東華三院廣華醫院 – 香港中文大學　中醫藥臨床研究服務中心

Patient/Subject Informed Consent Form 參加研究同意書

A Case Study on the effects of Traditional Chinese Medicine for patients with Osteoporosis
中醫藥對骨質疏鬆症患者骨質密度及臨床症狀的研究

I, _____ have read and fully understood the Patient / subject Information Sheet and had the opportunity to ask questions. I understand that participation in the research is voluntary and I am free to withdraw at any time, without giving any reason and without any of the medical care or legal rights being affected. I understand that my medical notes may be read by other individuals concerned in the research.

I agree to participate in the research. For enquiries or complaints, I can contact TWGHs Kwong Wah Hospital – The Chinese University of Hong Kong Chinese Medicine Clinical Research and Services Centre (Contact number: 3517-2634).

本人 _____ 已細閱及清楚明白《參與者須知》的內容並已就相關問題提出質詢，本人明白此項研究純屬自願參與，可於任何時候無須任何原因退出研究，並且不會因此影響本人得到醫療服務的機會及質素，亦不會由此負上任何法律責任，本人之醫療記錄將會被用作研究用途，供研究人員翻閱。

本人同意參與此項研究，如有任何疑問或申訴，本人可致電（電話：３５１７－２６３４）與東華三院廣華醫院 – 香港中文大學　中醫藥臨床研究服務中心聯絡。

_____ _____

Participant Signature
參與者簽署

Participant Name
參與者姓名

_____ _____

Investigator Signature
研究者簽署

Investigator Name
研究者姓名

_____

Date　日期

Appendix XI Case report form

TWGHs Kwong Wah Hospital – The Chinese University of Hong Kong Chinese Medicine Clinical Research and Services Centre
A Randomized, Double-blind, Placebo-controlled Clinical Trial on the effects of Traditional Chinese Medicine for patients with Osteoporosis

Case Report Form (CRF)

Admission date:_____

Name:_____

Patient number: _____

Check List

Period	#T(-1)	T0	T1	T2	T3	T4	T5	T6	T12	T24
Date										
Administration										
Assessment Form	( )	( )								
Review of Incl/Excl. Criteria	( )	( )								
History	( )									
婦科病歷	( )									
Informed Consent		( )								
Randomization		( )								
Adverse events/reactions checklist (TCM-specific)										
Adverse events/ reactions report			( )	( )	( )	( )	( )	( )	( )	( )
Judgment criteria of ADR*										
Termination of trial*										
Assessment										
(1)BP(2)pulse(3)height measurement(4)Incidence of fracture		( )	( )	( )	( )	( )	( )	( )	( )	( )
Medication Record(HAPatient drug profiles+Private clinic record)		( )	( )	( )	( )	( )	( )	( )	( )	( )
每天服藥記錄表		( )	( )	( )	( )	( )	( )			
Drug Delivery (中西藥處方副本)		( )	( )	( )	( )	( )	( )			
SF-36 Health Status Questionnaire	( )		( )		( )			( )	( )	( )
TCM data collection	( )		( )		( )			( )	( )	( )
OP specific questionnaire	( )		( )		( )			( )	( )	( )
ITCM		( )	( )		( )			( )		
Lab test										
BMD measurement	( )							( )	( )	( )
Bone marker test (serum)/ml	( )							( )		
Complete blood test/ml	( )		( )		( )			( )		
Liver and renal function test/ml	( )		( )		( )			( )		
Serum calcium/ml	( )		( )		( )			( )		

*This form is optional; #T(-1) =screening period; T (0) =Baseline; T (X) =X month(s) after trial starts

Assessment Form

History:

Onset:_____

_____

_____

Past medical history:

_____

Please "✓" if yes and "✗" if no

☐ Renal stone history (excluded from trial if yes)

☐ High blood pressure disease
☐ Diabetic Mellitus
☐ Cardiovascular disease

Active medical problem:

_____

_____

Placement of follow up:

_____

_____

Medication:_____

_____

_____

Recent record of Hospital Admission: _____

Recent Episode of fall: _____

Social istory:_____

Subjective Examination

Main omplaint:_____

Objective Examination:

X-Ray eport:_____

Inclusion and Exclusion Criteria

Please "✓" in appropriate box.

	No	Yes
Inclusion criteria		
1.Women aged 55 or above		( )
2.Postmenopausal for at least 2 years		( )
3.Osteoporotic at lumbar spine or total hip using WHO criteria.		( )
4.Written informed consent		( )
Exclusion criteria		
1.Major medical problems that would preclude participating in a trial lasting 2 years	( )	
2.Severe malabsorption syndrome	( )	
3.Prior treatment on bone pathology within 1 month	( )	
4.Sever psychiatric or addictive disorders	( )	
5.May have poor compliance to the study protocol	( )	
6. Patients with history of renal stones.	( )	

Termination of trial*

Duration of drug taken: From: ___/___/___
To: ___/___/___

Q1. Premature termination of trial: ☐
No=0 (finished)
Yes=1(goes to Q2)

Q2. Reason for termination: ☐
Patient withdrawal=1
Suspected adverse events=2
(Specify: _____)
Other medical condition=3
(Specify: _____)
Others=4
(Specify: _____)

*This form is optional

Adverse events/ reactions Check List (TCM-specific)

Please "✓" in the appropriate box and fill in the details in "Adverse events/reactions report" if ADR appears

燥热 Hot Constitution			寒凉 Cold Constitution		
Yes	No		Yes	No	
		1. 皮膚乾燥 Dry/itchy skin			1. 臉色蒼白 Pale complexion
		2. 臉頰紅熱 Red & hot cheek			2. 浮腫 Oedema
		3 暗瘡 Acne			3. 口淡不渴 Low appetite but not thirsty
		4. 眼乾 Dry eye			4. 頭暈 Dizziness
		5. 口乾 Dry mouth			5. 頭重頭痛 Headache and heavy feeling
		6. 口苦 Bitter taste			6. 怕冷 Afraid of cold
		7. 口唇乾燥 / 乾裂 Dry /crack lips			7. 手腳冰冷 Cold limb
		8. 唇瘡 Cold sore			8. 小腹冷痛 Adbominal pain
		9. 喉嚨痛 Sore throat			9. 大便軟易瀉 Loose stool/diarrhea
		10. 牙齦腫痛 Sore gum			10. 白帶清稀 Dilute leukorrhea
		11. 流牙血 Gum bleeding			11. 疲倦 Tired
		12. 鼻乾鼻癢 Dry/itchy nose			
		13. 流鼻血 Episaxis			
		14. 頭痛 Headache	其他 Others		
		15. 怕熱 Afraid of hot			1. 嘔吐 Vomiting
		16. 心情煩躁 Emotional disturbance			2. 胃部不適 Stomach Discomfort
		17. 失眠 / 夜寐不佳 Insomina/poor sleeping			3. 病情加重 Symptomatic disease progression
		18. 便秘 Constipation			4 其他 others _____
		19. 痔瘡發作 Hemorrhoids	備註 Remarks		
		20. 尿短赤 Dark yellowish hypouresis			
		21. 尿痛 Dysuria			

Adverse events/ reactions report

1. Was there any adverse event(s)/ reaction(s) happened during the clinical trial?

No=0 (finished)

Yes=1(goes to Q2) ☐

2. Adverse event(s)/reaction(s)

項目[1]			
出現日期			
出現時間（用藥後多少小時或天後出現）			
持續時間（小時／天）			
除本科研用藥外，有否合併使用其他治療措施（請詳細列明)			
是否採取措施： 0＝未採取；1＝採取（請詳細列明)			
結果　0=消失;1=消失後見後遺症;　2=未消失			
停止日期			
嚴重程度[2]：1＝輕度；2＝中度3＝重度			
事後是否需要停藥[3]： 0＝不停藥;　3＝停鈣片及維生素丁; 1=暫時停藥;　4＝停止所有本研究藥物 2＝停中藥;			
備註			

[1]項目包括：腸胃不適、嘔吐、噁心、腹脹、便秘、腹瀉、皮疹、瘙癢、頭暈、頭痛或其他．

[2] 嚴重程度：

- 輕度－輕微副反應，症狀不明顯，一般不需治療
- 中度－中度副反應，症狀較明顯，經對症處理後，容易恢復
- 重度－重度副反應，包括：導致門診病人需入院治療，或延長住院病人的住院時間，或致殘、致畸、致癌，引起後遺症，甚者危及性命．

[3]事後是否需要停藥：0＝不停藥; 1=暫時停藥; 2＝停中藥; 3＝停鈣片及維生素丁; 4＝停止所有本研究藥物

Judgement Criteria of the relatedness of an ADR and the study

(不良事件／反應因果關係判斷標準)

被判斷的不良事件／反應：_____

1. 藥物/安慰劑

判斷指標	判斷結果*				
	肯定	很可能	可能	可疑	不可能
服藥時間與可疑 ADR 出現時間有否關聯	+	+	+	+	+
可疑的 ADR 是否所用藥物的已知 ADR	+	+	+	-	-
可疑的 ADR 能否以病者的病情、合併用藥的相互作用作解釋	-	-	+/-	+/-	+
停止服藥能否使可疑的 ADR 減輕或消失	+	+	+/-	+/-	-
恢復服藥後可疑的 ADR 有否再出現	+	?	?	?	-

2. 鈣片及維生素丁

判斷指標	判斷結果*				
	肯定	很可能	可能	可疑	不可能
服藥時間與可疑 ADR 出現時間有否關聯	+	+	+	+	+
可疑的 ADR 是否所用藥物的已知 ADR	+	+	+	-	-
可疑的 ADR 能否以病者的病情、合併用藥的相互作用作解釋	-	-	+/-	+/-	+
停止服藥能否使可疑的 ADR 減輕或消失	+	+	+/-	+/-	-
恢復服藥後可疑的 ADR 有否再出現	+	?	?	?	-

說明：+ 表示肯定 - 表示否定 +/-表示難以肯定或否定 ? 表示情況不明

*如判斷結果屬於"肯定"或"很可能"或"可能"，請填寫下表（可選擇多於一項）：

- ☐ ADR 可能由中藥引起
- ☐ ADR 可能由安慰劑引起
- ☐ ADR 可能由鈣片引起
- ☐ ADR 可能由維生素丁引起
- ☐ 無法區分 ADR 由何種施加項目(Intervention)引起

Appendix XII Determination of caffeine in placebo using capillary gas chromatography

1. Introduction

1.1. Presence of caffeine in placebo was conformed by GC/MS and the content of caffeine was determined by capillary gas chromatography equipped with an FID detector. The content of Lipton yellow label tea bag was also determined for parallel comparison.

2. Reagents

2.1. Chloroform, AR grade

2.2. Water: Milli-Q or equivale

2.3. Sodium Hydroxide (1%: dissolved 1.0g of sodium hydroxide in 100 ml water.

2.4. Caffeine reference standard (1.0 mg/ml: dissolved 10.0mg of caffeine in 10.0 ml of chloroform.)

3. Apparatus

3.1. Agilent 6890/5973N GC/MS system equipped with an FID detector.

3.2. HP-5ms capillary column (30m x 0.25mm x 0.25μm)

3.3. Pipette, 5.0 ml

3.4. Volumetric flask, 50 ml

3.5. RC syringe filter, 0.45μm

3.6. Syringe, 5 ml

3.7. Centrifugal tube, 50 ml

3.8. Balance, readable to 0.1 mg

3.9. Separating funnel, 50ml

4. Procedure

4.1. Extraction of ingredients

4.1.1. About 0.2 gram of sample was accurately weighed into a 50 ml centrifugal tube.

4.1.2. 5.0 ml hot water (85°C) was added into the flask and heated (85°C) for 20 minutes.

4.1.3. The mixture was filtered and allowed to cool down to room temperature.

4.1.4. The filtrate was alkalized by adding 1 ml 1% sodium hydroxide.

4.1.5. The mixture was then transfer into separation funnel and extracted with 5 ml of chloroform 3 times. The lower layer was collected and dried with anhydrate calcium chloride.

4.1.6. The fractions were combined, filtered, transferred to a 50 ml volumetric flask and made up to the mark.

4.1.7. Above steps were repeated once with another 0.2 gram of sample.

4.2. GC/MS Analysis

4.2.1. The chromatographic conditions were set according to the conditions listed in Appendix I.

.The standard solutions and samples were analyzed. Presence of caffeine was conformed by retention time and MS data.

4.3. GC/FID Analysis

4.3.1. The chromatographic conditions were set according to the conditions listed in Appendix I.

4.3.2. The standard solutions and samples solutions were analyzed. The retention time and peak area were measured.

5. Data and Calculations

Presence of caffeine was confirmed by GC/MS using library search. Chromatograms from GC/FID analysis were obtained and the results from GC/FID were summarized as follows:

Sample	Placebo		Tea bag	
Trial	1	2	1	2
Sample amount(g)	0.2054	0.2052	0.1962	0.2193
Peak area	6661446	6533868	21807465	21016169

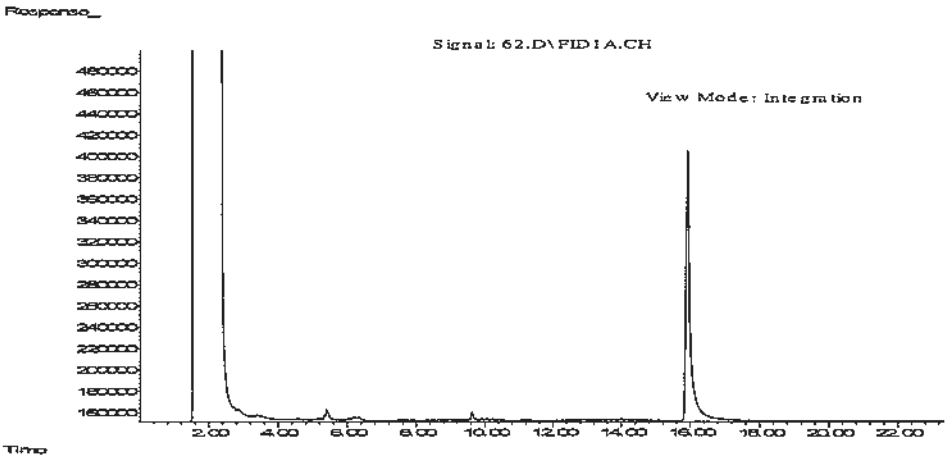


Fig. 1 GC/FID chromatogram of tea sample.

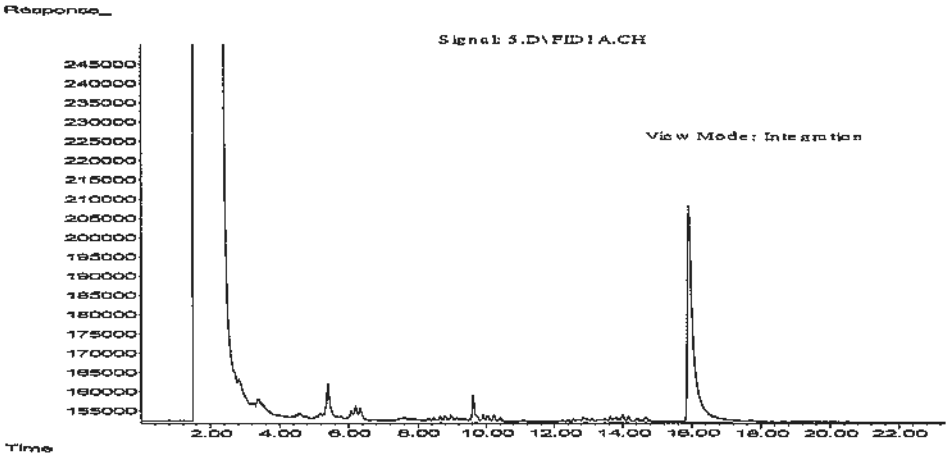
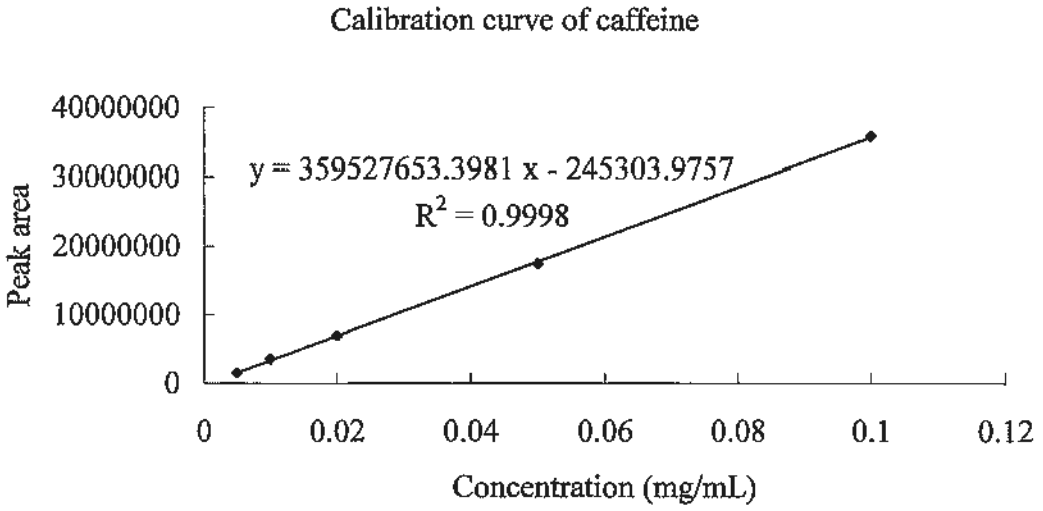


Fig. 2 GC/FID chromatogram of placebo sample.

A calibration curve of caffeine was established.



Concentration of caffeine, mg/g, in the testing sample was calculated according to the following equation:

$C = \frac{A \times V}{W}$, where

A = concentration of the caffeine in the sample solution, mg/ml

(determined from the calibration curve of the respective standard)

V = final volume of testing solution, i.e., 50 ml

W = sample weight, g

The value A was be given by:

A= (I-c) /m, where

I = peak area in the sample solution

c = y-intercept of the calibration curve

m = slope of the calibration curve.

6. Results

Concentrations of caffeine in testing samples were summarized as:

Sample	Placebo		Tea bag	
Trial	1	2	1	2
Concentration (mg/ml)	0.0192	0.0189	0.0613	0.0591
mg/g	4.68	4.59	15.63	13.48
mean(mg/g)	4.64		14.56	

From the summary, caffeine in a daily intake of 56 grams placebo is equal to that in 17.8 grams of Lipton Yellow Label tea bag.

Note

Typical GC/MS conditions

GC conditions

Column	:	HP-5ms capillary column (30mX0.25mmX0.25μm), Agilent
Carrier gas	:	Helium
Carrier gas flow	:	1.2 ml/min
Oven temp.	:	100℃→6℃/min→180℃ Post run: 280℃(10 min).
Injection volume	:	1μl
Injection mode	:	Splitless
Injection temp	:	210℃
Transfer line temp	:	280℃
Detector	:	MS

MS conditions

Solvent delay time	:	8 min
Ion source temp.	:	230℃
Quad. temp.	:	150℃
Mass scan range	:	*m/z* 50-550

Typical GC/FID conditions

Column	:	HP-5ms capillary column (30mX0.25mmX0.25μm), Agilent
Carrier gas	:	Nitrogen
Carrier gas flow	:	1.2 ml/min
Oven temp.	:	100℃→6℃/min→180℃ Post run: 280℃(10 min).
Injection volume	:	1μl
Injection mode	:	Splitless
Injection temp	:	210℃
Detector	:	FID
FID temp	:	230℃
Hydrogen flow	:	40 ml/min
Air flow	:	350 ml/min
Make up gas flow	:	35 ml/min

Appendix XIII Ninety-day oral toxicity study on OPR

Background

The study was started because of the adverse events arose on humans after taking OPR granules for 1-2 months. A ninety-day oral toxicity study was therefore performed to determine if the same response would be induced on animal and investigate the reason behind.

Objective

To investigate potential health hazards likely to arise form repeated exposure of OPR granules on human over a 90-day period of time by using Sprague-Dawley rats as animal model.

Materials and Methods

Animals

Thirty-seven female Sprague-Dawley rats (~200g) were obtained from and housed in Laboratory Animal Services Centre of The Chinese University of Hong Kong. Rats were housed in allergen-free conditions and received standard laboratory diet and water and libitum with 12 hours light: dark cycle. Ambient temperature was 22°C. License to Conduct Experiments had been obtained from Department of Health, HKSAR under the Animals (Control of Experiments).

Preparation of OPR granules and vehicle

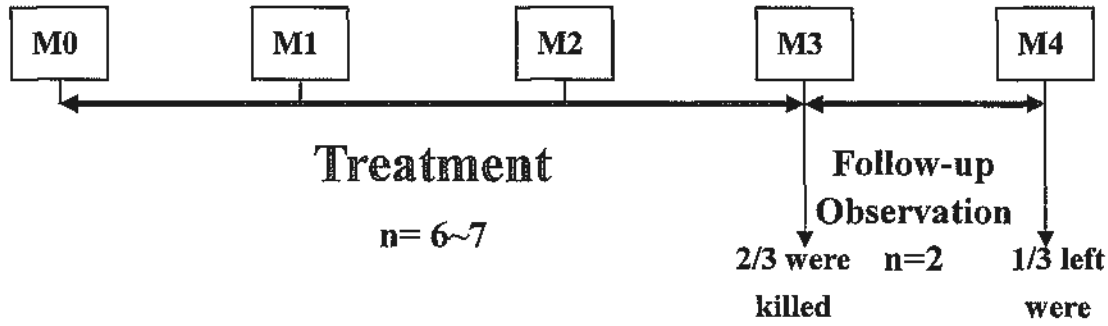
Granules of OPR formula, Fructus Psoraleae (補骨脂) and Herba Epimedii (淫羊藿) were obtained from PuraPharm. Granules of OPR were dissolved in distilled water at concentrations of 2.26g/kg, 6.768g/kg and 11.28 g/kg, which were equivalent to one time, three-time and five-time of clinical dose respectively (Shi, 1980). Granules of Fructus

Psoraleae and Herba Epimedii were dissolved in distilled water at a concentration of 0.929g/kg which were equivalent to five time of clinical dose. Distilled water was used as vehicle.

Protocol and administration of doses

Thirty-seven female Sprague-Dawley rats were randomly divided into six groups: Control group (Vehicle), three OPR treated groups (OPR-1X, 3X, 5X) and two major herb groups (FP-5X and HE-5X). Six to seven rats were distributed to each group and the dissolved solutions (or vehicle) were oral administrated to rats by forced feeding with orogastric gavage at a volume of 20ml/kg (i.e. 4ml/0.2g/day). Oral administration was completed once a day and 5 times a week for a period of 90 days. Administration of doses was completed after 90 days and two-third of rats in each group were scarified. The rest were killed after 4 weeks of follow-up observations without treatment. Protocol is shown in **Figure 1**.

Figure 1 Ninety-day Oral Toxicity Study protocol



Observation

General clinical observations were made once a day before administration of doses. Signs of morbidity and mortality were inspected, which include changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity.

Changes in movement, posture and response to handling were also recorded at baseline (M0), end of 1st (M1), 2nd (M2), 3rd (M3) and 4th (M4) month.

Body weight and organ weight

Rats were weighted once a week at about the same time before dosing. After killing, organs were dissected immediately from body and organs were washed in saline and sopped up the excess water by tissue paper before balance. Organ weight was expressed in organ/body weight ratios.

Liver and renal function tests

Blood samples were collected at baseline, 90 days (treatment completed) and 120 days (follow-up observation) after treatment started. Blood samples were allowed to clot at room temperature and serums were separated from whole blood by centrifuging (Eppendorf Centrifuge 5415R, Eppendorf, Hamburg Germany) at 16 110 x g for 10 minutes at 4°C. Serum samples were stored at -80°C until analysis. Liver and renal function tests were carried out at Department of Pathology, Kwong Wah Hospital by using SYNCHRON clinical system (Beckman Coulter Ireland Inc. 2003). Liver function tests include: Serum level of Total protein (TP), albumin (ALB), Total bilirubin (TBIL), Alkaline phosphatase (ALP), Aspartate transaminase (AST), Alanine transaminase (ALT). Renal function tests include: Serum level of electrolytes (Na, K, Cl), urea and creatinine.

Histopathology

Tissues were weighted and immediately preserved in 10% buffered neutral formalin for seven days. After formalin-fixation, tissue pieces were dehydrated and embedded in paraffin. Tissue sections of 5μm in thickness were stained by hematoxylin and eosin (HE staining) and observed under light microscope. Tissue sections prepared were delivered to Guangzhou University of Traditional Chinese Medicine for detailed histopathologic inspection. Heart, liver, spleen, lung, kidneys, uterus with oviducts, ovaries were collected. Extra tissues collected in control and high dose groups were: Brain, adrenal glands, pancreas, small

intestine (duodenum, jejunum, ileum) and colon.

Statistical Analysis

Data were presented as mean ± S.E.M unless otherwise noted. Data were analyzed with GraphPad Prism 4. Results were evaluated by one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered as statistically significant.

Results

In the study, 35 out of 37 rats completed the 90-day oral toxicity study, one rat from OPR- 5X group and one from control group die in week 2 and week 6 respectively during tube-feeding. The rat of OPR- 5X group was killed as the feeding tube had not been inserted deep enough to deliver thick OPR-5X suspension into its stomach. It resulted in a blockage at respiratory tract and finally led to death. The rat of control group was killed due to an improper feeding which led to lesion in esophagus. No abnormal sign was found on the death bodies after dissection.

No obvious abnormal clinical signs were observed during the study. No statistical difference between control and treatment group on body weight and organ weight (table 1 and table 2). No difference was found in liver and renal function tests between control and treatment group (Table 3). Report of histopathology (Attachment) on organ tissues showed that no obvious difference was found between the tissues (heart, liver, spleen, lung, kidneys, uterus, ovaries, brain, adrenal glands, pancreas, small intestine and colon) collected from control group and treatment group. These findings indicated that OPR did not induce any obvious toxic effect SD rats' organs tissues.

Table 1 Changes in body weight

	Changes in body weight (gram) Mean±SEM					
Month	Control	OPR(1X)	OPR(3X)	OPR(5X)	*Psoraleae*	*Epimedii*
M0	197.5±3.59	197.5±2.5	199.2±3.27	195±2.44	189.2±0.83	189.2±2.01
M1	220±2.58	210.8±5.23	210.8±2.01	208.3±3.80	205.8±2.01	214.2±2.39
M2	246±4.85	235.8±6.25	235.8±2.01	240±4.28	239.2±2.39	248.3±2.11
M3	238.3±4.41	216.3±5.54	236.3±5.54	227.5±5.20	241.3±3.15	251.3±1.25
M4#	255±10	262.5±12.5	245±5	267.5±2.5	262.5±12.5	265±5

#From M0 to M3, n=6-7. At M3, 2/3 rats were killed for analysis, therefore, n=2 at M4

Table 2 Organs weight / Body weight

Organs weight / Body weight Mean±SEM, n=6						
Organs	Control	OPR(1X)	OPR(3X)	OPR(5X)	*Psoraleae*	*Epimedii*
Brain	0.00753 ± 0.00011	-	-	0.00764 ± 0.00023	0.00758 ± 0.00005	0.00720 ± 0.00011
Heart	0.00309 ± 0.00012	0.00327 ± 0.00017	0.00316 ± 0.00022	0.00311 ± 0.00019	0.00314 ± 0.00007	0.00319 ± 0.00006
Liver	0.0269 ± 0.0010	0.0278 ± 0.0012	0.0268 ± 0.0014	0.0277 ± 0.0010	0.0281 ± 0.0013	0.0283 ± 0.0007
Spleen	0.00151 ± 0.00006	0.00160 ± 0.00006	0.00146 ± 0.00002	0.00167 ± 0.00005	0.00165 ± 0.00006	0.00182 ± 0.00006
Lung	0.00425 ± 0.00006	0.00440 ± 0.00016	0.00430 ± 0.00012	0.00467 ± 0.00019	0.00405 ± 0.00012	0.00409 ± 0.00028
Kidneys	0.00730 ± 0.00022	0.00792 ± 0.00022	0.00738 ± 0.00023	0.00737 ± 0.00029	0.00708 ± 0.00030	0.00780 ± 0.00017
Adrenal glands	0.000203 ± 0.000005	-	-	0.000226 ± 0.000033	0.000255 ± 0.000013	0.000327 ± 0.000017
Uterus with oviduc	0.00323 ± 0.00063	0.00263 ± 0.00056	0.00257 ± 0.00035	0.00263 ± 0.00019	0.00288 ± 0.00033	0.00317 ± 0.00018
Ovaries	0.00043 ± 4.79E-05	0.00038 ± 8.4E-05	0.00045 ± 3.2E-05	0.000461 ± 4.13E-05	0.000481 ± 2.83E-05	0.0004478 ± 2.664E-05

"-" indicates organs which were not collected unless in the high dose groups

Table 3 Liver and Renal Function Tests

		Liver and Renal Function Tests (gram) Mean±SEM, n=6					
	Parameters	Control	OPR(1X)	OPR(3X)	OPR(5X)	Psoraleae	Epimedii
W0	NA	137.50 ± 0.56	137.00 ± 0.68	137.67 ± 0.61	137.43 ± 0.20	138.33 ± 0.33	139.75 ± 0.48
	K	7.10 ± 0.18	7.10 ± 0.22	6.58 ± 0.10	6.91 ± 0.19	6.52 ± 0.18	6.18 ± 0.14
	CL	103.00 ± 0.77	102.83 ± 0.87	103.33 ± 0.33	103.43 ± 0.30	104.50 ± 0.56	104.50 ± 0.29
	UREAm	4.53 ± 0.30	4.87 ± 0.37	4.78 ± 0.25	5.00 ± 0.28	3.83 ± 0.22	4.00 ± 0.39
	CREm	39.67 ± 2.33	41.00 ± 1.21	39.33 ± 1.93	39.00 ± 1.96	32.17 ± 0.83	32.00 ± 0.71
	TPm	58.17 ± 1.08	56.67 ± 0.61	55.67 ± 1.15	56.29 ± 1.11	56.33 ± 0.71	55.17 ± 1.08
	ALBm	19.50 ± 0.34	19.00 ± 0.52	19.17 ± 0.48	19.14 ± 0.40	18.83 ± 0.54	18.83 ± 0.31
	TBIL	9.20 ± 0.84	11.23 ± 0.49	11.12 ± 0.86	10.09 ± 0.65	9.20 ± 0.50	9.52 ± 1.04
	ALP	171.67 ± 7.91	185.83 ± 11.97	171.67 ± 19.83	196.86 ± 14.28	323.00 ± 38.69	332.00 ± 37.91
	AST	114.00 ± 5.73	192.83 ± 49.83	186.33 ± 37.70	221.29 ± 75.09	115.00 ± 7.36	122.17 ± 7.61
	ALT	40.33 ± 1.41	46.83 ± 5.92	47.50 ± 5.21	53.14 ± 9.30	54.67 ± 1.91	54.50 ± 1.80
W4	NA	137.50 ± 0.43	137.00 ± 0.73	136.67 ± 0.42	136.50 ± 0.56	138.17 ± 0.31	137.67 ± 0.33
	K	6.88 ± 0.12	6.62 ± 0.14	6.25 ± 0.15	6.53 ± 0.32	5.90 ± 0.12	5.78 ± 0.07
	CL	102.67 ± 0.61	103.17 ± 0.48	102.33 ± 0.42	100.83 ± 0.31	103.17 ± 0.60	103.00 ± 0.37
	UREAm	5.07 ± 0.27	4.23 ± 0.17	5.72 ± 0.32	6.05 ± 0.27	4.97 ± 0.22	4.47 ± 0.12
	CREm	37.33 ± 1.45	39.00 ± 2.02	40.17 ± 2.39	44.50 ± 1.77	41.50 ± 0.50	38.17 ± 1.25
	TPm	60.83 ± 0.70	62.00 ± 0.97	61.50 ± 0.50	62.50 ± 1.69	59.17 ± 1.11	57.67 ± 0.61
	ALBm	21.50 ± 0.34	21.50 ± 0.56	22.33 ± 0.49	21.50 ± 1.09	20.50 ± 0.81	19.83 ± 0.40
	TBIL	8.83 ± 1.22	9.98 ± 0.70	9.68 ± 1.18	10.05 ± 0.97	10.08 ± 0.57	10.10 ± 0.62
	ALP	122.50 ± 18.01	143.83 ± 17.92	148.00 ± 29.36	143.17 ± 11.34	187.50 ± 14.15	166.83 ± 13.02
	AST	100.67 ± 7.83	96.33 ± 2.23	99.25 ± 8.42	94.67 ± 5.18	92.33 ± 5.42	105.33 ± 7.50
	ALT	45.50 ± 3.51	38.50 ± 1.80	41.33 ± 2.73	34.67 ± 2.01	34.67 ± 1.50	36.67 ± 1.41
W8	NA	135.60 ± 0.51	136.00 ± 0.37	133.67 ± 1.94	134.83 ± 0.40	137.00 ± 0.37	138.17 ± 0.31
	K	6.22 ± 0.27	6.20 ± 0.14	6.00 ± 0.22	5.93 ± 0.18	6.57 ± 0.24	6.07 ± 0.22
	CL	100.20 ± 0.66	100.83 ± 0.79	100.17 ± 1.35	99.67 ± 0.49	102.33 ± 0.56	104.17 ± 0.65
	UREAm	4.60 ± 0.20	4.27 ± 0.21	4.68 ± 0.28	4.85 ± 0.15	3.87 ± 0.32	4.63 ± 0.43
	CREm	39.80 ± 1.32	40.00 ± 1.00	40.67 ± 1.28	40.00 ± 1.32	37.17 ± 3.05	38.00 ± 3.21
	TPm	62.20 ± 1.69	63.00 ± 0.86	59.50 ± 1.61	65.50 ± 2.23	63.17 ± 1.33	61.83 ± 1.08
	ALBm	21.20 ± 0.86	21.33 ± 0.61	20.17 ± 0.75	21.83 ± 1.38	21.83 ± 0.79	21.67 ± 0.33
	TBIL	6.70 ± 0.71	7.23 ± 0.42	6.37 ± 0.88	6.45 ± 0.71	11.97 ± 0.53	10.18 ± 0.61
	ALP	129.80 ± 14.65	116.67 ± 11.64	122.17 ± 18.40	109.67 ± 9.03	118.67 ± 14.04	127.00 ± 11.01
	AST	102.60 ± 9.35	107.33 ± 8.50	114.83 ± 4.69	96.33 ± 3.26	107.67 ± 8.11	103.50 ± 5.98
	ALT	48.40 ± 2.01	48.17 ± 2.33	46.33 ± 1.96	42.67 ± 2.88	35.17 ± 1.49	30.83 ± 1.47

Table 3 (Continued)

	NA	137.20 ± 0.49	136.83 ± 0.31	136.67 ± 0.61	137.50 ± 0.22	135.67 ± 1.78	139.00 ± 0.63
	K	5.88 ± 0.10	6.00 ± 0.09	5.68 ± 0.32	5.73 ± 0.18	5.52 ± 0.41	5.07 ± 0.22
	CL	102.40 ± 0.40	102.17 ± 0.48	103.50 ± 0.56	103.17 ± 0.48	103.00 ± 1.18	104.67 ± 0.92
	UREAm	5.12 ± 0.27	5.28 ± 0.21	5.33 ± 0.35	5.13 ± 0.37	4.72 ± 0.36	4.78 ± 0.28
	CREm	44.20 ± 1.93	46.67 ± 2.63	43.17 ± 2.77	41.67 ± 1.94	38.33 ± 2.63	36.50 ± 2.26
W12	TPm	64.40 ± 1.29	63.67 ± 1.74	61.50 ± 1.57	66.50 ± 1.38	59.17 ± 2.60	59.67 ± 1.23
	ALBm	23.80 ± 0.58	22.17 ± 0.83	22.00 ± 1.13	22.67 ± 0.84	20.17 ± 1.01	20.50 ± 0.72
	TBIL	8.90 ± 0.91	9.43 ± 0.64	7.88 ± 0.48	9.67 ± 0.59	8.22 ± 1.07	10.63 ± 1.03
	ALP	74.60 ± 3.57	93.33 ± 7.02	105.00 ± 14.97	95.33 ± 6.87	120.67 ± 10.47	107.00 ± 6.16
	AST	101.80 ± 8.62	117.00 ± 14.23	114.17 ± 7.55	87.33 ± 3.08	125.50 ± 42.26	125.50 ± 12.64
	ALT	42.80 ± 8.83	32.33 ± 2.32	36.67 ± 2.46	26.83 ± 2.14	38.17 ± 3.51	43.33 ± 5.16
	NA	134.50 ± 1.50	134.00 ± 1.00	136.50 ± 0.50	136.50 ± 0.50	137.00 ± 1.00	136.00 ± 1.00
	K	5.95 ± 0.35	5.50 ± 0.10	4.80 ± 0.10	4.50 ± 0.30	4.65 ± 0.15	4.00 ± 0.10
	CL	103.50 ± 1.50	103.50 ± 0.50	103.00 ± 0.00	104.00 ± 0.00	105.00 ± 1.00	104.50 ± 1.50
	UREAm	4.85 ± 0.95	4.65 ± 0.05	5.05 ± 0.85	3.60 ± 0.00	3.55 ± 0.05	4.05 ± 0.15
	CREm	39.50 ± 3.50	34.00 ± 0.00	40.50 ± 9.50	37.00 ± 0.00	35.00 ± 1.00	38.00 ± 3.00
W16	TPm	64.50 ± 1.50	61.50 ± 2.50	60.00 ± 1.00	60.00 ± 1.00	56.00 ± 2.00	59.50 ± 1.50
	ALBm	24.00 ± 1.00	23.00 ± 0.00	21.00 ± 1.00	21.00 ± 1.00	18.50 ± 0.50	22.00 ± 1.00
	TBIL	9.15 ± 0.15	7.95 ± 0.95	8.35 ± 0.05	10.15 ± 0.25	8.05 ± 0.75	7.60 ± 1.20
	ALP	50.00 ± 3.00	58.50 ± 4.50	45.50 ± 1.50	46.00 ± 0.00	60.00 ± 2.00	71.50 ± 9.50
	AST	172.50 ± 72.50	128.00 ± 25.00	91.00 ± 12.00	107.00 ± 27.00	80.50 ± 11.50	82.00 ± 11.00
	ALT	101.50 ± 50.50	57.00 ± 1.00	50.50 ± 2.50	36.50 ± 1.50	40.00 ± 6.00	44.00 ± 8.00

W16 include last 2 rats only,which has stopped Tx for 4 weeks.

Attachment

OPR-PuraPharm 复方及单味中药在 SD 大鼠上的长期毒性试验病理组织检查报告

送检标本：对照组（A1-3）、高剂量组（Ｄ２－５）、单味药组-5 倍补骨脂（E1-4）、单味药组-5 倍淫羊藿(F1-4)，共 15 只 SD 大鼠。脑，心脏，肝脏，脾脏，肺脏，肾脏，肾上腺，胰脏，肠（十二指肠、回肠、结肠）子宫，卵巢　（共１１脏器）

大脑病理形态观察

正常对照组：3 例样本，脑膜未见充血，蛛网膜下腔未见性渗出物，脑组织内神经未见肿胀，胞浆未见空泡形成，未见核偏位；未见神经细胞坏死、神经卫星现象，未见软化灶形成；未见神经细胞及明显神经细胸凋亡；3 例样本均可见血管轻度淤血；未见炎细胞浸润及血管浸润套形成，未见间质水肿；未见胶质细胞增生及胶质小结形成；基质未见脱髓鞘，室管膜及脉络膜未见，未见脑室积水。

高剂量组：4 例样本除可见脑组织内血管轻度淤血外，脑膜及脑组织形态均未见明显异常。

补骨脂组：4 例样本除可见脑组织内血管轻度淤血外，脑膜及脑组织形态均未见明显异常。

淫羊藿组：4 例样本中除 3 例可见脑组织内血管轻度淤血外，脑膜及脑组织形态均未见明显异常。

结论：大脑组织病理检查，未显示药物对大鼠大脑组织明显的毒性作用。

小脑病理形态观察

正常对照组：3 例样本，脑膜未见充血，蛛网膜下腔未见性渗出物，脑组织内神经未见肿胀，胞浆未见空泡形成，未见核偏位；未见神经细胞坏死、神经卫星现象，未见软化灶形成；未见神经细胞及明显神经细胸凋亡；3 例样本中 2 例可见组织内血管轻度淤血；未见炎细胞浸润及血管浸润套形成，未见间质水肿；分子层、purkinie 神经细胞分布形态正常，白质未见脱髓鞘。

高剂量组：仅见 3 例样本，除其中 2 例可见组织内血管轻度淤血外，脑膜及小脑组织形态均未见明显异常。

补骨脂组：脑膜及小脑组织形态均未见明显异常。

淫羊藿组：4 例样本除均可见组织内血管轻度淤血外，脑膜及小脑组织形态均未见明显异常。

结论：小脑组织病理检查，未显示药物对大鼠小脑组织明显的毒性作用。

心脏病理形态观察

正常对照组：3 例样本心肌纤维束形态结构正常，心肌细胞胞浆红染，核居中，心肌横纹、纵纹清晰，心肌细胞未见细胞内水肿、脂肪变性；心肌细胞间闰盘隐约可见；2 例样本纤维组织增生（陈旧性小坏死灶）。心肌间质血管轻度扩张充血，未见炎细胞浸润，心肌间质未见脂肪浸润，未见纤维增生性改变。

高剂量组：4 例样本除心肌间质血管轻度扩张充血外，心肌组织形态均未见明显异常。
补骨脂组：4 例样本除心肌间质血管轻度扩张充血外，心肌组织形态均未见明显异常。
淫羊藿组：4 例样本除心肌间质血管轻度扩张充血外，心肌组织形态均未见明显异常。
结论：心脏组织病理检查，未显示药物对大鼠心肌组织明显的毒性作用。

肝脏病理形态观察

正常对照组：3 例样本均见肝细胞水肿，少量散在点状及小灶性坏死伴炎细胞浸润，1 例样本见汇管区少量炎细胞浸润。
高剂量组：2 例样本见少量散在点状及小灶性坏死伴炎症细胞浸润；1 例样本汇管区见少量炎症细胞浸润。1 例样本基本正常。
补骨脂组：2 例样本见少量散在点状及小灶性坏死伴炎症细胞浸润，伴汇管区见少量炎症细胞浸润；1 例样本见中央静脉及肝窦内淤血。
淫羊藿组：4 例样本均见少量散在点状及小灶性坏死伴炎症细胞浸润，伴汇管区见少量炎症细胞浸润；
结论：各给药组与正常对照组均能见肝细胞散在坏死灶及炎细胞浸润，给药组与正常对照组并无明显差异。肝组织病理检查，未显示药物对大鼠肝织明显的毒性作用。肝细胞的病理变化可能与感染或饲料等因素有关。

胰腺病理形态观察

正常对照组：2 例样本胰腺小叶结构清晰，未见上皮细胞萎缩、变性及溶解，胰岛大小形态正常，未见细胞萎缩、溶解，间质未见炎症细胞浸润，导管通畅未见结石及炎症。
高剂量组：4 例样本胰腺组织病理检查均未见明显异常。
补骨脂组：2 例样本胰腺组织内可见小灶性炎细胞浸润；2 例样本胰腺组织病理检查未见明显异常。
淫羊藿组：2 例样本胰腺组织内可见小灶性炎细胞浸润；2 例样本胰腺组织病理检查未见明显异常。
结论：补骨脂组和淫羊藿组 4 例样本中 2 例样本虽可见有小灶性炎细胞浸润，但胰腺外分泌腺及胰岛细胞形态正常。病理变化可能与感染相关的可能性更大。

十二指肠（空肠）病理形态观察

正常对照组：粘膜表面可见绒毛，被覆柱状上皮，内有吸收细胞和杯状细胞，吸收细胞表面可见纹状缘，未见上皮脱落；固有膜内可见许多肠腺，腺体形态正常，未见萎缩及增生，未见化生，间质内未见血管充血及炎细胞浸润；粘膜下层可见十二指肠腺，间质内未见血管充血及炎细胞浸润；肌层未见肥厚、萎缩或出血、坏死，未见充血、水肿及炎细胞浸润；浆膜未见充血、水肿及炎细胞渗出。
高剂量组：4 例样本十二指肠组织病理检查均未见明显异常。
补骨脂组：4 例样本十二指肠组织病理检查均未见明显异常。
淫羊藿组：4 例样本十二指肠组织病理检查均未见明显异常。
结论：十二指肠组织病理检查，未显示药物对大鼠十二指肠组织明显的毒性作用。

空肠病理形态观察

正常对照组：粘膜表面可见圆锥状绒毛，被覆柱状上皮，内有吸收细胞和杯状细胞，吸收细胞表面可见纹状缘，未见上皮脱落；固有膜内可见许多肠腺，腺体形态正常，未见萎缩及增生，未见化生，间质内未见血管充血及炎细胞浸润；粘膜下层未见血管充血及炎细胞浸润；肌层未见肥厚、萎缩或出血、坏死，未见充血、水肿及炎细胞浸润；浆膜未见充血、水肿及炎细胞渗出。

高剂量组：４例样本空肠组织病理检查均未见明显异常。

补骨脂组：４例样本空肠组织病理检查均未见明显异常。

淫羊藿组：４例样本空肠组织病理检查均未见明显异常。

结论：空肠组织病理检查，未显示药物对大鼠空肠组织明显的毒性作用。

回肠病理形态观察

正常对照组：粘膜表面可见指状绒毛，固有膜内可见肠腺，腺体形态正常，未见萎缩及增生，未见化生，间质内未见血管充血及炎细胞浸润，淋巴滤泡形态及分布正常；肌层未见肥厚、萎缩或出血、坏死，未见充血、水肿及炎细胞浸润；浆膜未见充血、水肿及炎细胞渗出。

高剂量组：４例样本回肠组织病理检查均未见明显异常。

补骨脂组：４例样本回肠组织病理检查均未见明显异常。

淫羊藿组：４例样本回肠组织病理检查均未见明显异常。

结论：回肠组织病理检查，未显示药物对大鼠回肠组织明显的毒性作用。

肾脏病理形态观察

正常对照组：３例样本肾皮、髓质结构均清晰。肾小球未见增生或萎缩、毛细血管未见坏死，管壁未见增厚。各级肾小管及集合管结构清晰，肾小管上皮细胞未见明显细胞水肿、玻璃样变性或坏死，肾小管腔内未见管型。均见肾间质内血管轻度充血，２例样本见少量炎细胞浸润。

高剂量组：４例样本均见肾间质内血管轻度充血，２例样本见少量炎细胞浸润。

补骨脂组：４例样本均见肾间质内血管轻度充血及少量炎细胞浸润。

淫羊藿组：４例样本均见肾间质内血管轻度充血。

结论：给药组及正常对照组均见肾间质内血管轻度充血及少量炎细胞浸润；间质内血管轻度充血与可能与动物处死时放血不尽有关，其间质性肾炎改变与上行性感染有关。未显示药物对大鼠肾组织明显的毒性作用。

肺组织病理形态观察

正常对照组：３例样本均能见肺泡壁毛细血管扩张、充血，肺泡壁轻度增厚，不同程度的炎细胞浸润，呈间质性肺炎的改变；未见间质及肺泡腔出血。

高剂量组：4 例样本均能见肺泡壁毛细血管扩张、充血，肺泡壁轻度增厚，及不同程度的炎细胞浸润，呈间质性肺炎的改变。

补骨脂组：4 例样本均能见肺泡壁毛细血管扩张、充血，肺泡壁轻度增厚，及不同程度的炎细胞浸润，呈间质性肺炎的改变。

淫羊藿组：4 例样本均能见肺泡壁毛细血管扩张、充血，肺泡壁轻度增厚，及不同程度的炎细胞浸润，呈间质性肺炎的改变。

结论：给药组及正常对照组均见肺泡壁毛细血管扩张、充血，水肿，肺泡壁轻度增厚，呈间质性肺炎等病理改变。但给药组与正常组之间并无明显差异。肺泡壁毛细血管扩张、充血、水肿及出血，肺胞壁增厚等改变可能与宰杀动物时对肺组织的损伤有关。其间质性肺炎改变可能与感染等因素有关。未显示该药对肺组织有毒性作用。间质性肺炎与感染有关。未显示药物对大鼠肺组织明显的毒性作用。

脾脏病理形态观察

正常对照组：3例样本脾脏红、白髓结构清晰。脾小梁未见增粗。动脉周围淋巴鞘清晰可见，细动脉未见病变；脾小体分布、形态正常，部分生发中心清晰，脾组织未见含铁血黄素沉积，未见含铁结节，为正常脾组织形态。

高剂量组：4例样本脾脏组织病理检查均未见明显异常。

补骨脂组：4例样本脾脏组织病理检查均未见明显异常。

淫羊藿组：4例样本脾脏组织病理检查均未见明显异常。

结论：脾脏组织病理检查，未显示药物对大鼠脾脏组织明显的毒性作用。

肾上腺病理形态观察

正常对照组：3例样本肾上腺皮、髓质结构清晰，未见出血。皮质各带未见增生、萎缩或坏死，皮质各带细胞形态及着色正常。髓质嗜铬细胞形态及分布正常，神经节细胞胞浆红染、核居中、散布其间。

高剂量组：4例样本肾上腺皮、髓质组织病理检查均未见明显异常。

补骨脂组：4例样本肾上腺皮、髓质组织病理检查均未见明显异常。

淫羊藿组：4例样本肾上腺皮、髓质组织病理检查均未见明显异常。

结论：肾上腺组织病理检查，未显示药物对大鼠肾上腺皮质和髓质明显的毒性作用。

子宫病理形态观察

正常对照组：3例样本子宫壁各层结构清晰可见。子宫内膜腺体呈增生期或分泌期改变，未见过度增生及异型增生，间质反应与腺体周期性改变同步，内膜未见变性或坏死，内膜可见轻度淤血及不同程度的炎细胞浸润；子宫肌层平滑肌细胞浆红染、核轮廓清晰，平滑肌细胞未见萎缩、细胞内水肿、脂肪变性或坏死，肌层血管壁未见增厚、管腔未见狭窄。

高剂量组：4例样本内膜均可见轻度淤血及不同程度的炎细胞浸润。

补骨脂组：4例样本内膜均可见轻度淤血及不同程度的炎细胞浸润。

淫羊藿组：4例样本内膜均可见轻度淤血及不同程度的炎细胞浸润。

结论：子宫病理检查，虽各组内膜均可见轻度淤血及不同程度的炎细胞浸润，但给药组与正常组之间并无明显差异。未显示药物对大鼠子宫壁各层组织明显的毒性作用。

卵巢病理形态观察

正常对照组：3例样本卵巢皮髓质结构清晰；可见原始卵泡、初级卵泡、次级卵泡，各期卵泡形态、数量正常，卵巢内见黄体结构。未见卵泡囊肿或黄体血肿。间质纤维未见增生。

高剂量组：4 例样本卵巢病理检查均未见明显异常。
补骨脂组：4 例样本卵巢病理检查均未见明显异常。
淫羊藿组：4 例样本卵巢病理检查均未见明显异常。

结论：卵巢病理检查，未显示药物对大鼠卵巢组织明显的毒性作用。

小结

各组动物大脑、小脑、心、肝、胰腺、十二指肠、空肠、回肠、肾、肺、脾、肾上腺、子宫、卵巢等脏器组织及细胞形态，在用药组与正常对照给之间比较无明显差异。上述病理形态观察结果未显示药物对 SD 大鼠大脑、小脑、心、肝、胰腺、十二指肠、空肠、回肠、肾、肺、脾、肾上腺、子宫、卵巢等脏器组织有明显的毒性作用。

签名：杜标炎 教授

（广州中医药大学）
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