Effect of Endocrine Disruptors on the Synthesis of Estrogen and Corticotrophin-Releasing Hormone *In Vitro* and *In Vivo*

HUANG, Hui

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Thesis committee

Professor Franky L. Chan (Committee Chairman)

Professor L.K. Leung (Thesis supervisor)

Professor Ronald C.C. Wang (Committee member)

Professor Jennifer M.F. Wan (External examiner)

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ABSTRACT

Estrogens and corticotrophin-releasing hormone (CRH) produced by the placenta are important in the maintenance of pregnancy and control of parturition in human. Human placenta is the primary source of estrogen after the ninth week of gestation. Aromatase or CYP19 is the enzyme which is primarily responsible for catalyzing the conversion of estrogen from its precursor in the placental cells. The overall objective of this study was to investigate the effect of bisphenol-A and genistein on the expression of genes responsible for the synthesis of estrogen and corticotrophin-releasing hormone.

Bisphenol-A is an industrial contaminant whose estrogenic property has been reported in many studies. Because of its ubiquitous existence in our environment, the safety of bisphenol-A has been drawn much attention. By employing real-time PCR, bisphenol-A was shown to down-regulate aromatase and up-regulate CRH transcription in JEG-3 cells. The promoter activities of the two genes were evaluated by employing electromobility shift assays and gene reporter assays. The results were consistent with the mRNA expression. Furthermore, we demonstrated that bisphenol-A could affect the estrogen metabolism and CRH expression in mice. These data are indeed useful for illustrating bisphenol-A's toxicological properties.

Genistein is a phytoestrogen isolated from soyabeans. Given the differential interactions with different estrogen receptor isoforms, genistein is generally considered to be a selective estrogen receptor modulator (SERM). While Genistein is commercially available as nutraceutical for women; however, the potential safety issues have not been fully addressed. In the trophoblast cells SGHPL, exon II (90%) was the overwhelming promoter used with trace levels of usage from promoter I.3, I.4 and I.1. Real-time PCR indicated that genistein down-regulated aromatase through promoter II. CRE-driven *CRH* transactivity appeared to be compromised upon genistein treatment. Western blot analysis

also signified MAPK activation in these placental cells. An *in vivo* study also demonstrated that genistein could affect normal parturition in LPS-sensitized mice. This information may provide some scientific basis for establishing the safety level of genistein intake during pregnancy.

摘要

由胎盤產生的雌激素和促腎上腺皮質激素釋放激素（CRH）對維持人體妊娠和控制分娩起到很重要的作用。在妊娠後第 9 週， 胎盤成為雌激素的主要來源。芳香酶（CYP19）的主要功能是促進人體胎盤細胞中的雌激素前體轉換成雌激素。本研究的總體目標是探討雙酚 A 和染料木素對負責雌激素和促腎上腺皮質激素釋放激素合成的基因表達的影響。

雙酚 A 是一種工業污染物，而它的雌激素的特性已經在許多研究中被廣泛報導。由於雙酚 A 廣泛存在我們周圍的環境，它的安全問題已引起普遍關注。使用實時 PCR 技術發現，在人的胎盤癌細胞 JEG-3 中，雙酚 A 能夠抑制芳香酶，同時增加 CRH 的轉錄。電泳移動偏向分析法和基因報告系統用來檢測這兩個基因的啟動子活性。結果與信使 RNA 表達的結果一致。我們進一步證明，雙酚 A 能會影響小鼠雌激素代謝和 CRH 表達。這些實驗結果對闡述雙酚 A 毒理學性質有重要作用。

染料木素是一種從大豆中分離的植物雌激素。由於它能與各種雌激素受體亞型有不同的相互作用，它被認為是一種選擇性雌激素受體調節劑（SERM）。在商業上，染料木素可作為婦女保健品，但它潛在的安全問題沒有得到完全解決。在滋養層細胞 SGHPL 細胞中，啟動子 PII 的使用率（90％）是最高的，其次是啟動子 PI.3，PI.4 和 PI.1。實時 PCR 結果顯示，染料木素能夠通過調控啟動子 PII 降低芳香酶的合成。而由 CRE 調控的 CRH 轉錄活性也受到染料木素的影響。蛋白质印迹分析實驗進一步證明了在這些胎盤細胞中，染料木素對絲裂原活化蛋白激酶活性的影響。動物實驗還表明，染料木素可能會影響 LPS 致敏小鼠的正常分娩。這些信息可對確立在懷孕期間攝入的染料木素的安全水平提供一定的科學依據。

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CHAPTER 1
GENERAL INTRODUCTION

Pregnancy induces many physiological changes in a woman's body, and hormones produced in the placenta are the major contributors to most of these changes. Steroid hormones, like estrogens, progesterone, cortisol, adrenocorticotropin hormone (ACTH) and CRH affect various pregnancy events, such as implantation, fetus development, maternal adaptation and birth initiation (Shmagel & Chereshnev, 2004). Estrogen concentration is significantly increased during the course of pregnancy and is primarily produced by the placenta. Progesterone and adrenal hormones, such as cortisol and aldosterone, are also raised during this period (Marzi *et al.*, 1996). Estrogen and progesterone regulate placental growth and differentiation (Hoffmann & Schuler, 2002). Maternal plasma level of CRH increases progressively during pregnancy. CRH receptor has been located in most female reproductive tissues, including the ovary, uterus, and placenta (Kalantaridou *et al.*, 2004).

1.1 Pregnancy and Hormone Metabolism

1.1.1 Timeline of Pregnancy

Pregnancy is typically divided into three periods or trimesters in describing the changes that take place over time. A typical time-span of human pregnancy is around 40 weeks. The first trimester is 6–12 weeks after conception, the second trimester is

13–28 weeks of gestation and the third trimester is 29–40 weeks of gestation.

Parturition occurring prior to 37 weeks of gestation is considered as preterm birth. Preterm birth is the major cause of perinatal mortality and morbidity. Preterm birth is responsible for more than 80% of neonatal deaths and more than 50% of long term morbidity in the surviving infants (Rush *et al.*, 1976; Goldenberg *et al.*, 2008).The incidence of preterm birth ranges from 6-8% in Europe, Australia and Canada to 9-12% in Asia, Africa and the United States (Yuan *et al.*, 2010). The biochemical mechanism of preterm delivery is still unknown (Lopez Bernal, 2003).

In the first trimester of pregnancy, the corpus luteum is responsible for the secretion of progesterone, estradiol, which is subsequently taken over by the placenta (Schindler, 2004). In a primary cell study, exogenous corticotropin-releasing hormone (CRH) can stimulated first-trimester trophoblast proliferation and may have an important role in early placental development and successful pregnancy (Choy *et al.*, 2004).

1.1.2 Pregnancy and Estrogen Metabolism

Estrogen is important in the physiological function of many tissues, including skeleton, central nervous and reproductive systems. It has anti-inflammatory and vascular protective effects particularly for those postmenopausal women (Xing et al., 2009). In fetal development, estrogen triggers the maturation of lungs, kidneys, liver, adrenal glands and other organs (Albrecht & Pepe, 1999). It also maintains pregnancy by stimulating and regulating the production of progesterone over the full term (Yallampalli et al., 1994). Since progesterone is important to the well-being of the fetus, keeping proper estrogen supply is important. Estrogen treatment before delivery can

stimulate uterine contraction effectively for preventing postpartum hemorrhage and shortening the birth process in women undergoing induced abortion (Huddleston et al., 2003; Zhou et al., 2007). Reduced estrogen levels during pregnancy often correlates with miscarriage (Tuckerman et al., 2004). The increase of androgens and/or the lack of estrogens in maternal could change the fetal programming of insulin sensitivity (Guercio et al., 2009). Progesterone was proved to promote uterine quiescence and estrogen may promote myometrial activation (Kilarski *et al.*, 1993).

Two isoforms of estrogen receptors (ERs): ER-alpha (ERα) and ER-beta (ERβ) have been identified. They are DNA-binding transcription factors that are potentially contributing to overall estrogen action (Levin, 2005). A study indicates that ERα is the predominant estrogen receptor in primary placental cells and placental tissue, while ERβ is detectable at very low levels. In primary placental cells, E2 inhibits CRH synthesis through ERα-mediated mechanisms (Ni *et al.*, 2002). In normal placenta, the increased estrogen level during pregnancy may stimulate cytotrophoblast cells differentiation via the ERα.

1.2 Estrogen Metabolism and Aromatase

In premenopausal women, the ovaries are the principle source of estrogen (Simpson, 2003) while in postmenopausal women, estrogen is produced by the peripheral tissues such as breast, bone, vasculature, and brain (Simpson & Davis, 2001).

Cytochrome p450 I family enzymes are responsible for the hydroxylation of estrogen (Tsuchiya *et al.*, 2005). The conversion of cholesterol to the estrogens, estrone (E1) and estradiol (E2) is shown in **Fig.1.1**. Androstenedione, testosterone, and estrone sulfate are the major precursors for estrogen synthesis (Bell *et al.*, 2008).

CYP11A1 catalyses the first reaction in the pathway, by which the cholesterol side chain is cleaved and pregnenoione is formed (Kristensen & Borresen-Dale, 2000). *CYP17A1* mediates the 17a-hydroxylation of pregnenoione (Voutilainen & Miller, 1986; Picado-Leonard & Miller, 1987), and 17b-Hydroxysteroid dehydrogenates (HSD17B) converts estrone to estradiol (Kristensen & Borresen-Dale, 2000). Aromatase is the rate-limiting enzyme for estrogen biosynthesis, it catalyses the conversion of testosterone to estradiol, androstenedione to estrone, and 16a-hydroxylated dehydroepiandrosterone to estriol (Meinhardt & Mullis, 2002). *CYP1A1* is a highly inducible member of cytochrome P450 family. It catalyses the C-2 hydroxylation of estradiol (Nedelcheva & Gut, 1994). *CYP1B1* catalyses the C4-hydroxylation of estradiol to the corresponding catechol estrogen, a documented carcinogenic agent in animal models (Hayes *et al.*, 1996). Catechol-O-methyltransferase (*COMT*) inactivates 2-hydroxy-estradiol and 4-hydroxy-estradiol by methylation (Hamajima *et al.*, 2001).

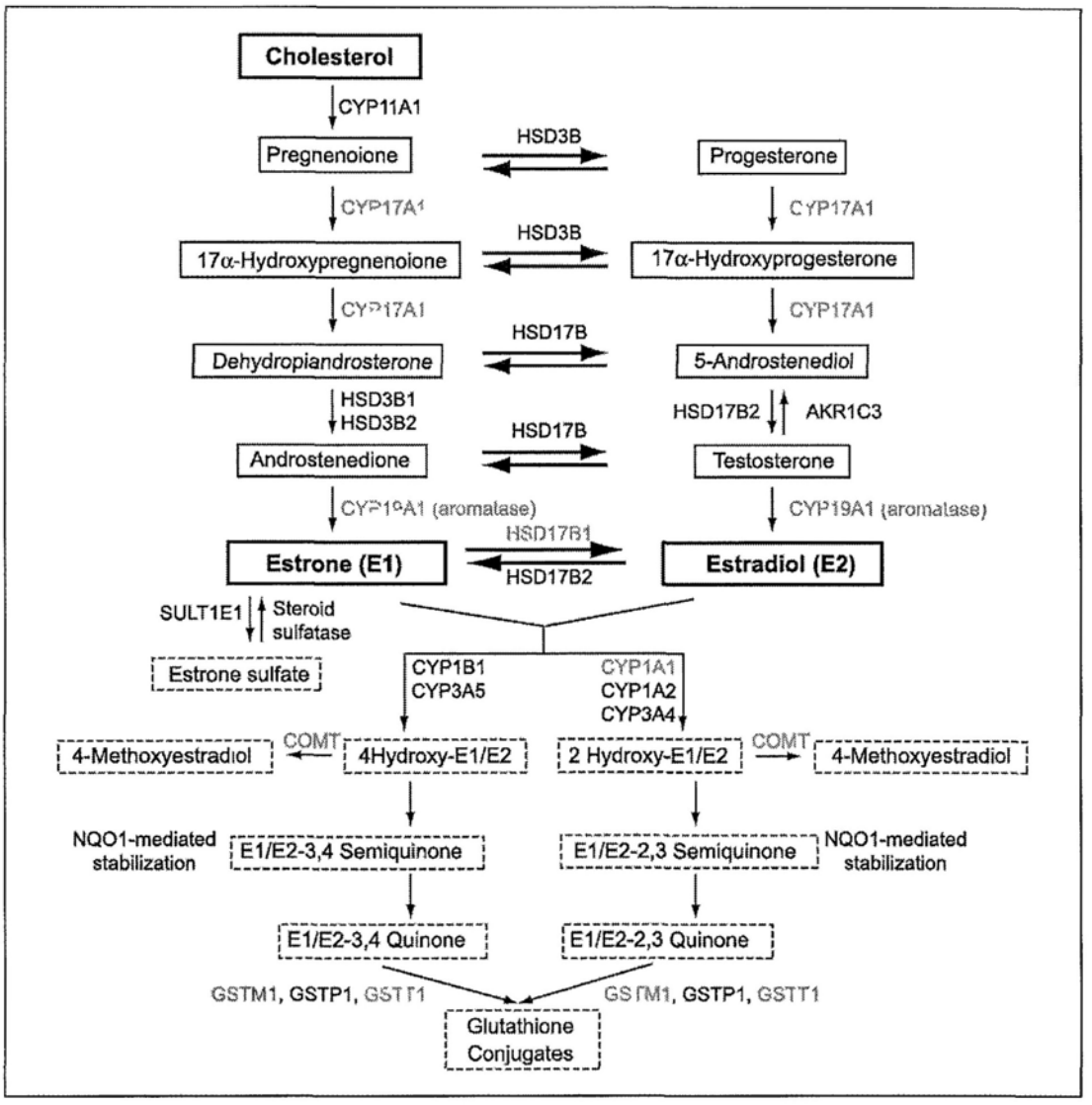


Fig.1.1 Estrogen biosynthesis and metabolism. (This figure is adopted from (Bell *et al.*, 2008).

1.3 Tissue Specific Promoter for Aromatase Expression

1.3.1 Promoter of Aromatase

Aromatase belongs to the P450 superfamily and is encoded by the *CYP19* gene localized at chromosome 15q21.2 (Simpson *et al.*, 1997). The encoding gene is

approximately 130 kb (Kamat *et al.*, 1998). The coding region comprises ten translated exons, and a number of untranslated first exons lie upstream of the coding region (Kamat *et al.*, 2002). These first exons are spliced onto a common acceptor site in exon II. Each first exon is associated with a unique promoter region (Ellem *et al.*, 2004) and aromatase synthesis is controlled by tissue-specific promoters and alternative splicing mechanisms (Mahendroo *et al.*, 1993; Simpson *et al.*, 1994; Harada *et al.*, 2003).

Aromatase is broadly expressed in different tissues, including gonads, brain, adipose tissue, skin and placenta syncytiotrophoblast, ovarian granulosa cells, and adipose stromal cells (Kamat *et al.*, 1998; Nelson & Bulun, 2001). Up to now, ten distinct tissue-specific promoters have been indentified on the coding region and are designated as PI.1 (placenta, major), PI.2 (placenta, minor), PI.3 (adipose/breast cancer), PI.4 (skin/adipose), PI.5 (fetal tissue), PI.6 (bone), PI.7 (endothelial), PI.f (brain), PII (ovary/breast cancer/endometriosis), P2a (placenta, minor) (Shozu & Simpson, 1998; Sebastian & Bulun, 2001; Sebastian *et al.*, 2002) as shown in **Fig.1.2**. In placenta and corpus luteum, PI.1 is overwhelmingly responsible for *CYP19* gene expression (Sun *et al.*, 1998). In ovary and granulosa cells, the aromatase expression is predominantly regulated by PII (Michael *et al.*, 1995; Simpson *et al.*, 2000; Sharma *et al.*, 2009).

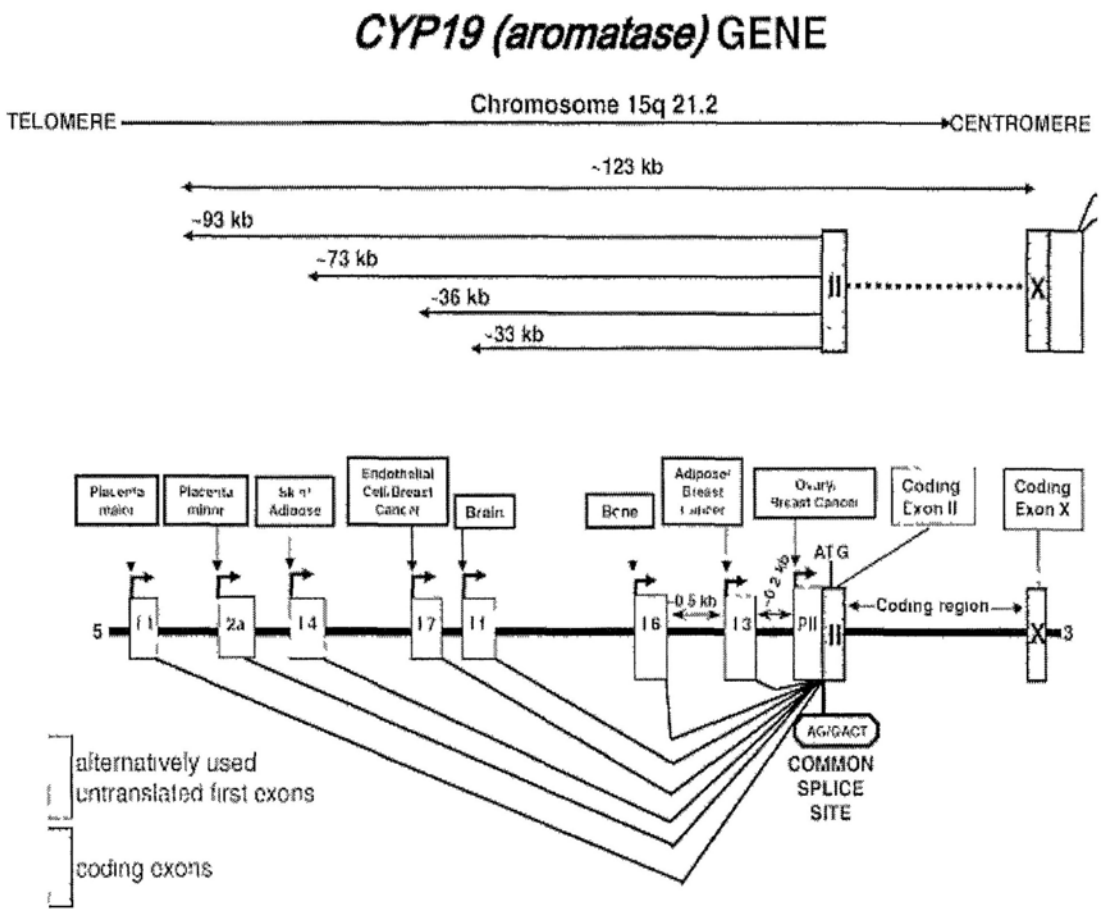


Fig.1.2 Aromatase (*CYP19*) gene structure. (This figure is adopted from (Chen *et al.*, 2009).

1.3.2 Abnormal Regulation of Aromatase Expression

A distinct set of transcription factors regulates each promoter of aromatase in a tissue-specific manner (Bulun *et al.*, 2005). The risk of three common female cancers: breast, endometrium and ovarian cancers could be raised by abnormally high estrogen. In these cancers, aromatase expression is regulated by transcription factors, by which the activity of promoter could be altered (Mah *et al.*, 2007).

In normal adipose tissue, aromatase expression is driven only by promoter I.4

(Zhao *et al.*, 1995a; Zhao *et al.*, 1995b; Zhao *et al.*, 1996). The presence of a carcinoma in breast causes a switch of promoter use from I.4 to II/I.3 in the adipose tissue (Nelson & Bulun, 2001). Additionally, the endothelial-type promoter I.7 is also up-regulated in breast tumor tissue (Sebastian *et al.*, 2002). Aromatase is active in prostate epithelial tumor cells while showing no activity in benign epithelial cells. Change from PII to promoter I.4 in promoter usage occurs in prostate malignancy (Ellem *et al.*, 2004).

Malignant epithelial cell-conditioned medium induces C/EBPβ binding to the C/EBP site in promoter I.3/II region and increases its transactivation in adipose fibroblasts. In contrast, benign epithelial cell conditioned medium increases C/EBPα and δ interacting with the same site and suppress the transactivation (Zhou *et al.*, 2001). Similarly, C/EBPα and CREB binding to the CRE could activate the transcriptional activity of promoter II of aromatase in endometriotic cells, whereas C/EBPβ and CREB binding to the same site in promoter II will function as a co-inhibitor with HDAC (**Fig. 1.3**) (Yang *et al.*, 2002). In breast cancer, endometrial cancer and uterine fibroids, transcription factors prefer binding to co-activator rather than co-repressor and initiate transcription of aromatase (Bulun *et al.*, 2005).

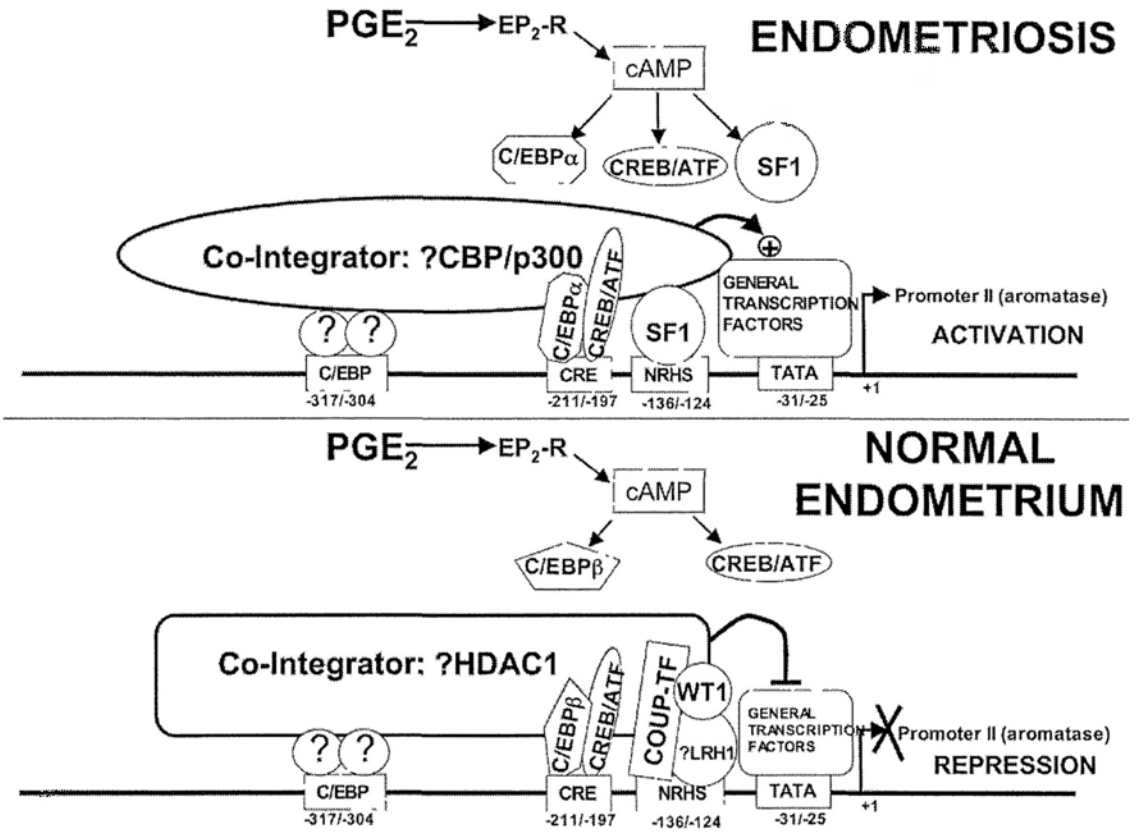


Fig.1.3 Differential binding of transcription factors to aromatase promoter II in endometriotic versus normal endometrial stromal cells. (This figure is adopted from (Bulun *et al.*, 2005).

1.4 Corticotrophin-releasing Hormone and Estrogen

1.4.1 Introduction

Corticotrophin releasing hormone (CRH) is a 41-amino acid peptide hormone which is first identified in the hypothalamus as the principal mediator of the hypothalamic-pituitary-adrenal axis (HPA) response to stress (Chrousos & Gold, 1992). In human and rodent choriocarcinoma cell lines, the placental CRH expression

is species-specific (Scatena & Adler, 1996). CRH is also highly produced in the placenta during pregnancy and correlates to the length of pregnancy (Shibasaki *et al.*, 1982). The CRH hormone have two receptors, CRH receptor type 1 (CRH-R1) and CRH receptor type 2 (CRH-R2) and a binding protein named CRH binding protein (CRH-BP). CRH-BP could neutralize the activity of human CRH both in placenta and blood (Behan *et al.*, 1995; Petraglia *et al.*, 1996). The CRH/CRH-BP interaction may play a role in the initiation of human parturition (Reis *et al.*, 1999).

1.4.2 CRH and Estrogen Metabolism

Estrogens and CRH produced by the placenta play a part in the control of parturition in human. There is a strong correlation between maternal CRH and estrogen concentrations throughout gestation.

Some studies have shown that estrogen have an inhibitory effect on placental CRH expression through an ERα-mediated mechanism (Ni *et al.*, 2002). Estrogen and progesterone treatment could significantly reduce CRH mRNA and protein in the paraventricular nucleus (PVN) of ovariectomized monkeys (Bethea & Centeno, 2008). On the contrary, estradiol is shown to induce CRH expression through ERα and/or ERβ recruitment to act on the CRH promoter (Chen *et al.*, 2008; Lalmansingh & Uht, 2008).

CRH could also mediate the metabolism of estrogens, adrenal steroids, and prostaglandins, builds a positive-feedback loops in the control of parturition and fetal maturation in humans (McLean & Smith, 2001). In the placenta, CRH is able to activate the placental production of estrogens and cortisol (Power & Schulkin, 2006; You *et al.*, 2006). CRH stimulates estrogen biosynthesis by increasing the mRNA levels of CYP19A1 and HSD17B1 (You *et al.*, 2006).

1.5 Corticotrophin-releasing Hormone and Parturition

1.5.1 CRH and Parturition

Placenta is a critical source of hormones, cytokines and growth factors. Placental CRH acts as a biological clock that activates the onset of parturition (Karteris *et al.*, 1998). Fetal maturation may also be influenced by the hormone in early stage of gestation (Wadhwa *et al.*, 2004). Exogenous CRH can strongly stimulated human trophoblast proliferation in first trimester. CRH-deficient offspring cannot survive at birth with dysplastic lungs and this could be prevented by prenatal maternal glucocorticoid treatment (Venihaki *et al.*, 2000). Abnormally high placental expression of CRH will associate with preterm labor, intrauterine growth restriction (IUGR) and preeclampsia (Power & Schulkin, 2006).

1.5.2 CRH and Preterm Delivery Diagnosis

The probability of preterm delivery (PTD) is around 6% of all pregnancies and still the major cause of perinatal mortality and morbidity (McCormick, 1985).

There are four primary pathogenic mechanisms of preterm delivery: activation of the maternal or fetal hypothalamic-pituitary-adrenal (HPA), systemic inflammation, decidual hemorrhage and pathologic distention of the myometrium. Each of them has a distinct clinical profile and unique biophysical pathways in initiating parturition (Lockwood & Kuczynski, 1999). Recent approaches of predicting PTD were designed to detect various biomarkers, like human chorionic gonadotropin (hCG), cytokines, fetal fibronectin (Yuan *et al.*), matrix metalloproteinases (MMPs), estrogens, CRH, chorion, amnion and uterine decidua. These are more sensitive biomarkers than

traditional methods (Lockwood & Kuczynski, 1999; Moore, 1999; Meczekalski, 2006).

CRH secreted by the placenta is a well studied biomarker to predict preterm labor. Many researchers found that elevated CRH level is associated with preterm delivery (McLean *et al.*, 1995; Korebrits *et al.*, 1998; Wadhwa *et al.*, 1998), and CRH-BP and CRH-BP/CRH dimer-complex index are decreased throughout all three trimesters (Hobel *et al.*, 1999b). Women delivering pre-term infants have increased maternal CRH level earlier than those deliver at term. In contrast, those who deliver post-term would have a delayed surge of CRH level (Pearson *et al.*, 2001). Glucocorticoids may participate in the processes of birth at term and preterm by altering synthesis of CRH (Coleman *et al.*, 2000).

In early 1996, Kaga (Kaga *et al.*, 1996) developed a LPS-induced PTD mice model for the assessment of drugs against PTD. However, the pathogenesis of PTD in this model is not clear, although inflammation could be the causative factor. Intrauterine or intraperitoneal administration of LPS in normal mice at mid gestation induces PTD dependent on the expression of inflammatory cytokines (Bizargity *et al.*, 2009). In contrast, interleukin-1b (IL-1b), IL-6 and tumor necrosis factor-α (TNF-α) are elevated in the amniotic fluid in PTD patients (Mitsuhashi *et al.*, 2000). The addition of LPS in trophoblast explants increases CRH and CRH-R1 mRNA expression (Torricelli *et al.*, 2010). CRH concentrations were higher in PTD compared with controls. LPS significantly increased CRH and CRHR1 expression in *ex vivo* tissues from African Americans (Menon *et al.*, 2008). The toxicological effect of morphine (Javadi-Paydar *et al.*, 2009) and delta9-tetrahydrocannabinol (Asghari-Roodsari *et al.*, 2010) were evaluated in this LPS-induced PTD mouse model in recent publication.

1.5.3 The Usage of CRH Antagonists

The potential application of CRH antagonists have been used experimentally to clarify its role in anxiety and depression, addictive behavior, inflammatory disorders and preterm labor (Zoumakis *et al.*, 2006). Antalarmin, a CRH antagonist, delays spontaneous delivery by 7 days in sheep (Chan *et al.*, 1998) . In rats, the administration of antalarmin could protect the fetus against maternal stress and/or premature labor and delivery (Makrigiannakis *et al.*, 2001). Clinically, maternal plasma CRH concentrations may be useful in identifying women at high risk of preterm delivery and administration of CRH antagonist may be useful in preventing preterm labor (Smith *et al.*, 2009).

1.6 Signaling Pathways

Mitogen-activated protein kinase (MAP kinase) is a key family of signal transduction proteins which transmit signals involved in both cell proliferation and apoptosis (Santen *et al.*, 2002). MAP kinase family is a group of well-conserved serine/threonine kinases that control a wide variety of physiological functions in organisms ranging from yeast to mammals (Pearson *et al.*, 2001; Kuida & Boucher, 2004). The ultimate signaling cascade would converge to transcriptional factor activation. Activated transcriptional factors with binding sites located in the promoter region, could up-regulate or down-regulate the target gene expression (Pawson, 1993). The kinase sequence cascade is usually regulated by three analogous proteins, i.e. MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAPKK) and MAP kinase

(MAPK) (Santen *et al.*, 2002). There are three major MAP kinase cascades in mammalian (**Fig.1.4**). Raf-1 is the initial kinase in one of the cascade sequences, and MEK1/2 and ERK1/2 are the downstream kinases. Another cascade is initiated by the activation of c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and p38 MAP kinase activates the third cascade pathway (Pearson *et al.*, 2001). The MAP kinases are negatively regulated by mitogen activated protein kinase phosphatases (MKPs) (Haagenson & Wu, 2010).

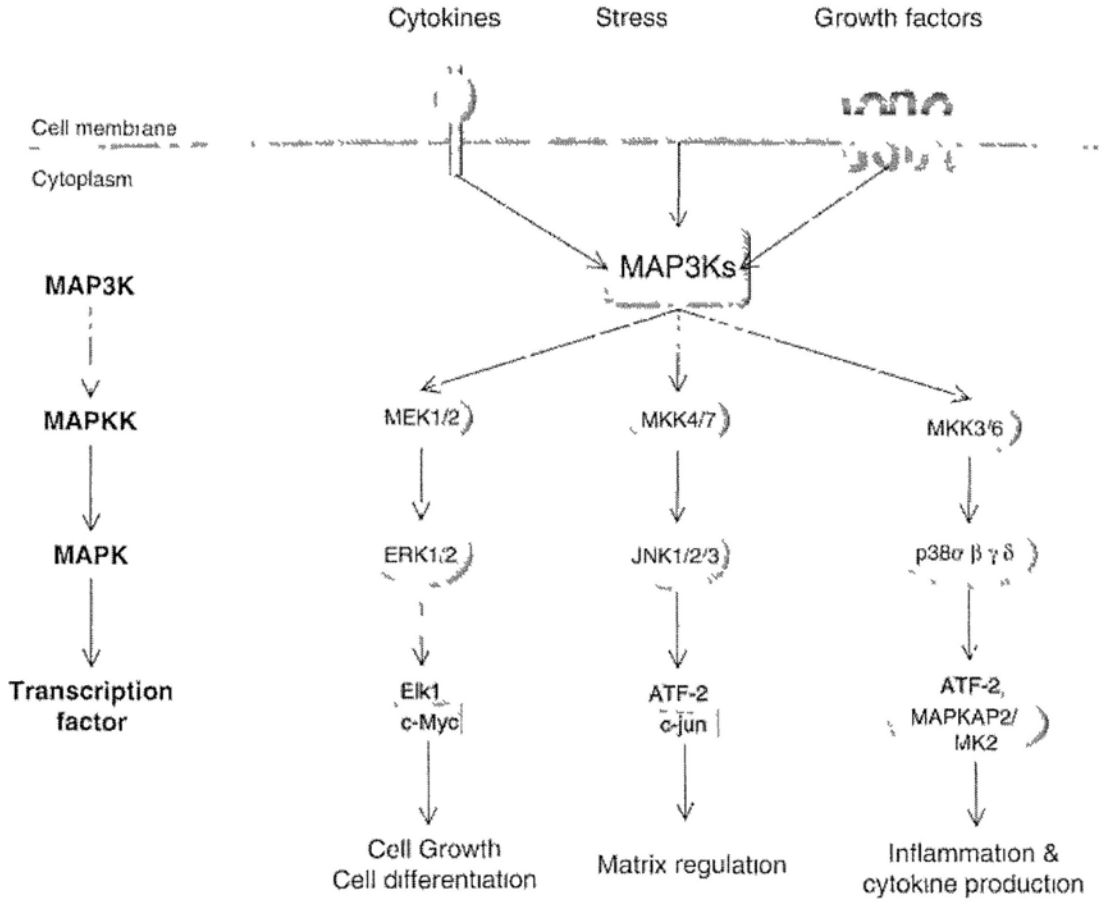


Fig. 1.4 The mitogen-activated protein kinase (MAPK) signalling cascade. (This figure is adopted from (Waldburger & Firestein, 2009).

1.6.1 Estrogen-related Pathways

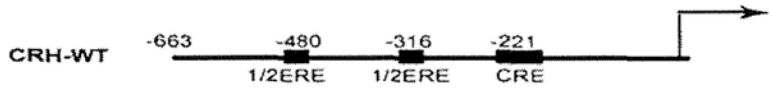
In the signaling cascade, estrogen crosses the cell membrane and binds to the estrogen receptor in the cytoplasm. The ligand-bound ER re-localizes to the nucleus, and is recruited to regulate the associated genes (Osborne *et al.*, 2001). During trophoblast differentiation, estrogen/ERα forms a positive feedback system and induces human *CYP19* gene transcription by promoting histone modifications (Kumar *et al.*, 2009). GPR30/SF-1 pathway merged with ER signaling increases local estrogen concentration, and mediates the proliferative effects of synthetic estrogens, such as tamoxifen, in promoting endometriosis and endometrial cancers (Lin *et al.*, 2009). Estrogen activates ERK1/2 in midbrain astrocytes but not neurons (Ivanova *et al.*, 2001). ERK signaling is necessary for estradiol to induce axon growth and this activation is mediated by a membrane bound estrogen receptor (Carrer *et al.*, 2005). Insulin-like growth factor (IGF)-I enhances cancer progression through ERα activation via the Raf/Ras/mitogen-activated protein kinase cascade (Thordarson *et al.*, 2004). Ras-MAPK cascade of the growth factor signaling pathways can phosphorylate ER and increase its transcriptional efficiency (Kato *et al.*, 1995). TGF-beta1 also affects human placental functions and pregnancy by mediating the transcription of aromatse (Zhou *et al.*, 2009). Aromatase can be induced by prostaglandin E2 via a cAMP-PKA-dependent pathway (Mah *et al.*, 2007).

1.6.2 CRH-related Pathways

Several endogenous factors are responsible for regulating placental *CRH* gene expression. Promoter analysis indicates that the *CRH* gene contains several putative transcription regulatory elements, including two TATA boxes, CAAT boxes, a cAMP response element (CRE) (Stamatelou *et al.*), two half-estrogen responsive elements (ERE) (Vamvakopoulos & Chrousos, 1994; Yao & Denver, 2007) and a caudal-type homeobox response element (CDXRE) (Nicholson *et al.*, 2004). The CRH promoter has been highly conserved in different species (Chen *et al.*, 2008). Locations and sequences of the major transcription factor binding sites are shown **in Fig.1.5A** and **Fig.1.5B**, respectively.

MAPK signal transduction pathway plays a pivotal role in the regulation of *CRH* gene expression in human placenta (Cheng & Handwerger, 2005b). CRH induces vascular endothelial growth factor release from human mast cells via the cAMP/PKA/p38 pathway (Cao *et al.*, 2006). The species specific expression of placental CRH could be mediated by the differential transcriptional response to CRE which is conserved in mouse, rat, and human (Scatena & Adler, 1996; Cheng & Handwerger, 2005b). The PKC and PKA pathways are possible stimuli on CRE transactivations in placental cells (Yue *et al.*, 2008). The proximal promoter element, cAMP analogues, or forskolin, could stimulate the PKA-dependent pathway to elevate the endogenous hypothalamic and placental CRH (Dorin *et al.*, 1993). Phorbol ester, a PKC activator, also stimulates *CRH* gene transcriptional activity through activates the CRE regulatory element in placental cells (Yue *et al.*, 2008). Inducible cAMP early repressor (ICER) causes a rapid inhibition of CRH transcription in a CRE-dependent manner in the hypothalamic cell line 4B (Liu *et al.*, 2006).

A



B

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          -490
human     CACCTGGTCAGGGAGGTTAGGAGAAGGGGCATCCAGGTCCACCCCCTCCA
mouse     CAACCTGACAGAAGAGTTAGGTGGGGGTGC-TGAGGCACCAGGAAATGTC
  rat     CAACATGACAGAAAAGTTAGGTGGGAGTGG-TGAGACACCAGGAAATGTC
            (-480 ERE half site)
human     ACTGGCTGCTGCTTTCCTGGCAGGGCTGCACTGGGACAC-CTCACTTCCT
mouse     CAGATCCACCCCTTTAATGGTAACTACCTTCTTGGAAGCTCTGCACTCCC
  rat     CAAATCCACCCCCTTAATGGTGGCTACCTTCTTGGCAGCTCTGCACTCCC

human     TCCCACTTCCCCTTCCTCCTCCCATTCGCTGTCTCTTTGCACACCCCTAA
mouse     CACCTCTTCTTCTTTCCCCTCCCACTCTCTGTCTCTTTTCTGGTCCGTA-
  rat     CACCTCTTTCTCT-----CTCCCACT--CTGCCTCTTTTCTGGTCTGTA-

human     TATGGCCTTTCATAGTAAGAGGTCAATATGTTTTC-ACACTTGGGAAATC
mouse     TCTGGCCTATCATAGTAAGAGGTCAGTCTGTTTTCCACACTTGGATAGTC
  rat     TCTGGCCTATCATAGTAAGAGGTCAGTATGTTTTCCACACTTGGATAATC
                          (-316 ERE half site)
human     TCATTCAAGAATTTTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTT
mouse     TCATTCAAAAATTTTTGTCAATGGACAAGTCATAAGAAACCCTTCCATTT
  rat     TCATTCAAGAATTTTTGTCAATGGACAAGTCATAAGAAGCCCTTCCATTT

human     TAGGGCTCGTTGACGTCACCAAG
mouse     TCGGGCTCGTTGACGTCACCAAG
  rat     TAGGGCTCGTTGACGTCACCAAG
                    (-221 CRE)  -216
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Fig.1.5 Schematic diagrams of wild-type *CRH* promoters (**Fig.1.5A**). Comparison of the DNA sequences of the promoter regions of *CRH* genes of human, mouse and rat. Transcription binding sites of *CRH* promoter and the major regulatory elements are shown in (**Fig.1.5B**). The figure is adopted from (Chen *et al.*, 2008).

1.7 Endocrine Disrupting Agents

Endocrine disruptors are exogenous hormone-like substances interfering with the maintenance of homeostasis, reproduction, development, and behavior. They are widely spread in the environment and some display estrogenic, anti-estrogenic or anti-androgenic activity.

1.7.1 Xenoestrogens

Xenoestrogens are synthetic substances with estrogenic property. They represent a diverse group of chemicals including natural compounds, pharmaceuticals, industrial chemicals and environmental pollutants (Stefanidou *et al.*, 2009), such as bisphenol-A(BPA), benzo[a]pyrene (B[a]P) and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). Their presence in the environment correlates to the increased incidence of endocrine and reproductive problems (Sharpe & Skakkebaek, 1993; Sumpter, 1995; Eertmans *et al.*, 2003). Identifying the properties of various xenoestrogens remains a major area in toxicological research.

BPA has drawn much attention for the safety concern and its ubiquitous existence in our environment. BPA has estrogenic properties which might cause diseases relating reproductive dysfunction (Hunt *et al.*, 2003; Darmani & Al-Hiyasat, 2004). In 2002, the European Food Safety Authority re-evaluated the safety of BPA with the focus on reproduction and the endocrine system, and settled the Tolerable Daily Intake (TDI) from 5 to 50 micrograms/kg body weight/day.

BPA alters morphogenesis of the mammary glands and disrupts estrous cyclicity in exposed animals (Markey *et al.*, 2005). BPA also impairs sexual differentiation of

exploratory behavior and increases depression-like behavior in rats with prenatal exposure (Fujimoto *et al.*, 2006). Perinatal BPA exposure can induces mammary intraductal hyperplasia and alter the morphology in adult CD-1 mice (Vandenberg *et al.*, 2008). Early oogenesis could be disrupted by the exposure to BPA during fetal development (Susiarjo *et al.*). Daily intake of 68.84 mg BPA during days 1-4 of gestation could terminate all pregnancies (Berger *et al.*, 2007).

1.7.2 Phytoestrogen

Phytoestrogens are plant chemicals with estrogenic activity, and many of them exhibit inhibitory effect on aromatase. Resveratrol is found in the skin of red grapes and red wine, it is a potential inhibitor in the hormone synthesis and interfered with the normal functioning of placental cells (Wang & Leung, 2007). The isoflavones biochanin A, genistein which can be isolated from soybean and red clover are another class of phytoestrogen. Biochanin A can be rapidly converted into the demethylated metabolite genistein in vitro and in vivo (Moon *et al.*, 2006). Genistein is involved in variant cellular events, including proliferation, differentiation and apoptosis (Chan, 2009). Genistein could raise estrogen level and promote endometriosis and endometrial cancers (Lin *et al.*, 2009). Both genistein and biochanin A can work as a inhibitor of ovarian aromatase activity (Pelissero et al., 1996) while genistein can increase aromatase activity in human endometrial stromal cells (Edmunds *et al.*, 2005). It has been reported that genistein may alter ovarian function, causing abnormal estrous cycles, early reproductive senescence and infertility in mice (Jefferson et al., 2005). Neonatal treatment with genistein induces prolonged estrous cyclicity and adverse consequences on ovarian development and reproductive function (Jefferson *et*

al., 2006).

1.8 Aim of Study and Hypothesis

Endocrine disrupting agents can be synthetic and natural substances. Many of them possess estrogenic activity. Unlike the hormone estrogen, these substances are not steroidal compounds. Isoflavones have shown anti-proliferative properties, like delaying tumor progression and anti-inflammatory and previous research has substantiated that they are safe to consume as food supplements (Reiter *et al.*, 2009). In fact, these isoflavones are the major ingredients in an over-the-counter supplement gearing for treating perimenopausal symptoms.

Xenoestrogens may potentially affect parturition because of their estrogenic property (Hoppe & Carey, 2007; Reiter *et al.*, 2009). They may damage the system and mimic the biological activities of the female hormone estrogen (Sonnenschein & Soto, 1998). Exposure to these endocrine disruptors could induce morphological abnormality associated with reproduction functions in female mice (Toda *et al.*, 2002). Exposure to estrogenic agents during the postnatal period may lead to an increased incidence of neoplastic transformation in the prostate gland of the aging male (Prins *et al.*, 2007).

In this project, we attempted to determine the effect of endocrine disruptors including genistein, BPA, TCDD and B[a]P on estrogen and CRH metabolism during pregnancy. The underlying molecular mechanisms of these agents on CYP19 and CRH transcriptional regulation were also addressed. The background and hypothesis indicate that certain endocrine disruptors may affect pregnancy via their actions on

estrogen and CRH metabolism. The aim of the study was therefore to investigate how the estrogen and CRH metabolism would be affected using cell lines, pregnancy animal model as well as human placenta tissue.

CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals and Materials

2.1.1 Chemicals

Bishphenol A was a gift from Dr. KM. Chan (Department of Biochemistry, the Chinese University of Hong Kong, HKSAR, China) and it was dissolved in dimethylsulphoxide (DMSO) at a concentration of 100 mM as stock. Genistein and lipopolysaccharide (Escherichia coli LPS, serotype 055: B5) were obtained from Sigma Chemicals, St Louis, MO, USA. The mitogen-activated protein (MAP) kinase inhibitor U0126, the p38 MAP kinase inhibitor SB203580, the protein kinase A (PKA) inhibitor myristoylated 14-22 amide and the protein kinase C (PKC) inhibitor bisindolylmaleimide I were obtained from Calbiochem (San Diego, CA). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). All primers were synthesized by Invitrogen (Carlsbad, California, USA). ICI 182 780 was a gift from Dr. Y. Huang (School of Biomedical sciences, the Chinese University of Hong Kong, HKSAR, China). All other chemicals, if not stated, were acquired from Sigma Chemical.

2.1.2 Plasmids

Mammalian expression vector pcDNA3.1+ was a gift from Dr. Y.Y. Ho (Department of Biochemistry, the Chinese University of Hong Kong, HKSAR, China). Firefly luciferase reporter plasmid pGL3 and renilla luciferase control plasmid pRL

were purchased from Promega Crop. (Madison, WI, USA).

2.2 Cell Culture

Human placental choriocarcinoma cell line JEG-3 was generously provided by Prof. Stephen Shiu (Department of Physiology, the University of Hong Kong, Pokfulam, Hong Kong). The first trimester trophoblast cell lines SGHPL and TEV, and third trimester trophoblast cell line PL30 were generously provided by Dr. Ronald C.C. Wang and Dr. M.Y.Choy (Department of Obstetrics & Gynaecology, the Chinese University of Hong Kong, Shatin, N.T., Hong Kong). The cells were maintained in PRMI-1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine plasma (Invitrogen Life Technology, Rockville, MD), and incubated at 37 °C with 5 % carbon dioxide. Cells were routinely subcultured when reaching 80% of confluency. Chemicals were administered in the solvent vehicle dimethyl sulphoxide (DMSO), and the concentration was limited to 0.1% v/v. Three days before experiment, the cells were seeded in phenol-red free RPMI 1640 supplemented with 2 mol/L L-glutamine, 1% Penicillin-Streptomycin (P/S) and 5% charcoal/dextran treated fetal bovine plasma (FBS) (Hyclone).

The choriocarcinoma cell lines JEG-3 and BeWo were derived from the first-trimester trophoblast and have been used as models to study calreticulin (Wainwright *et al.*, 1998) and hyperglycemia (Weiss *et al.*, 2001). In early 1998, the human choriocarcinoma cell line JEG-3 was already used to test the effects of some environmental contaminants on aromatase activity (Drenth *et al.*, 1998). SGHPL cell line was also widely used to investigate signal transduction (Sonderegger, et al. 2010). A brominated derivative of cAMP, was found to inhibit the aromatase activity and further decreased the production of estradiol in SGHPL cells (Rodway, et al. 1990).

2.3 Aromatase Activity Assay

2.3.1 'In-cell' Aromatase Assays

Cellular aromatase assays were performed as previously described (Grube *et al.*, 2001). In brief, JEG-3 cells were seeded and allowed 1 day for attachment. Assays were started by replacing the culture medium with serum-free medium containing [1β-^{3}H(N)]-androst-4-ene-3,17-dione (NET-926, PerkinElmer Life and Analytical Sciences, Boston MA, USA) and testing compound. The final concentration of androstenedione was controlled at 25nM, and the reaction was incubated at 37°C for 1 hr. An aliquot of the medium was then mixed with equal volume of chloroform, followed by a 10,000 × g centrifugation at 4°C for 10 min. The aqueous phase was removed into a new tube containing 500μl of 5% activated charcoal suspension. After 30 min incubation, an aliquot of the supernatant fraction was taken out for scintillation counting. The protein content of the cells, on the other hand, was determined by using a BCA kit (Sigma Chemicals) after dissolving the cells in 0.5 mol/l NaOH.

2.3.2 Aromatase Assay on Recombinant Supersomes

In addition to the 'in-cell' assay, inhibition assay was also performed on recombinant aromatase expressed in insect microsomes (human CYP19 Supersomes®, Gentest Corp, Woburn, MA, USA). Similar experimental procedures were applied except that cells were replaced by the recombinant protein (Ciolino *et al.*, 2000). The assay performed in a total volume of 250 μl with the following additions: 25 nM [1β-^{3}H(N)]-androst-4-ene-3, 17-dione, 2 pmol Supersomes®, 3.3 mM $MgCl_2$, 100 mM

KH_2PO_4 (pH 7.4), 2.5 μl testing compounds and 1.3 mM NADPH. The reaction was initiated by the addition of NADPH and incubated at 37 °C for 15 min. After the incubation, the reaction was stopped by adding 1.4 ml chloroform and 0.35 ml 0.9% NaCl. After vortexing and centrifugation at 10,000 × g for 10 min, 0.4 ml of the aqueous phase was removed and mixed with 0.4 ml 5% activated charcoal in a new eppendorf tube. The charcoal-contacting aqueous extract was then vortexed and incubated for 30 min at room temperature and subjected to a centrifugation at 15,000 × g, 4 °C for 15 min. An aliquot was removed and used for scintillation counting in 4 ml scintillation cocktail (Perkin Elmer).

2.4 Measurement of Cell Proliferation

Cell number was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) staining as described by (Mosmann, 1983). Briefly, JEG-3 cells were seeded in 96-well plates at 10^4 cells per well and maintained in phenol red free PRMI-1640 supplemented with 10% charcoal dextran-treated serum (Hyclone, Logan, UT, USA). The cultures were allowed 24 h for attachment. Afterwards, the cells were treated with various concentrations of phytochemicals. At the end of the treatment, 50 μl of 1 mg/ml MTT was added to the cells and incubated at 37 °C for 4 h. Absorbance at 544 nm was the index of cell viability.

2.5 Real Time PCR

2.5.1 RNA Isolation and cDNA Synthesis

After cells were treated with different concentrations of compounds for 24 h, total RNA was isolated from the cells using TRIzol reagent (Invitrogen) according to the manufacture's protocol. The concentration and purity of RNA were determined by spectrophotometry at 260/280 nm. The integrity of RNA was assessed by agarose gel electrophoresis.

Total RNA (3μg) was denatured at 70 °C for 5 min in the presence of 0.025 μg/μl oligo-dT (Invitrogen). The samples were cooled on ice. Complementary DNA (cDNA) was synthesized using 100 units M-MLV Reverse Transcriptase (USB Corporation) in a total volume of 20 μl, according to the manufacturer's instructions. Briefly, the reactions were conducted in the presence of 1×M-MLV reaction buffer and 0.5 mM dNTP mixture (Invitrogen) at 37 °C for 60 min and consequently inactivated at 70 °C for 10 min. The cDNA generated was used as a template in real-time PCR reactions.

2.5.2 Quantitative Real Time PCR Assay

Target mRNA levels were determined by real-time PCR using the OpticonTM 2 System (MJ research, Waltham, MA, USA). Real-time PCR Master Mix Reagent kit was obtained from Applied Biosystems and PCR reactions were set up as described in the manual. A typical reaction contained 200 nmol/l of forward and reverse primer, 2 μl cDNA and the final reaction volume was 20μl. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 45 amplification cycles were then carried out with 15 s denaturation at 95°C and 1 min annealing and extension at 58°C. Target fragments were quantified by real-time

PCR, and a DNA Engine Opticon II (MJ Research, Inc., Waltham, MA) was employed for this assay.

The primers and FAM-labeled probes of *CYP19* and five *CYP19* exons were all purchased from Applied Biosystems (**Table 2.1**). Taqman/VIC minor groove binder probes and primers for human and mouse genes (Assay on-Demand™) (**Table 2.2**) and real-time PCR Taqman Universal PCR Master Mix were all obtained from Applied Biosystems. PCR reactions were set up as described in the protocol, which was validated by the manufacturer. Signals obtained for GAPDH were used as a reference housekeeping gene to normalize the amount of total RNA amplified in each reaction.

The gene expression of samples relative to control is determined by $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The mean fold change in expression of target gene compared with control was calculated with the equation: Relative target gene expression = $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$= (CTtarget – CT house-keeping) sample - (CTtarget – CT house-keeping) control.

Table 2.1 The forward primer, reverse primer and reporter sequences of *CYP19* Taqman probes (Applied Biosystems).

CYP19	Assay No	CYP19-F165
	Forward primer	GGAGAATTCATGCGAGTCTGGAT
	Reverse primer	GGAACATACTTGAGGACTTGCTGAT
	Reporter	TCTGGAGAGGAAACACTC
Exon I.1	Assay No	EXONIA.1-JUN
	Forward primer	CTGTGCTCGGGATCTTCCA
	Reverse primer	CATCTTGTGTTCCTTGACCTCAGA
	Reporter	ACGTCGCGACTCTAAAT
Exon I.3	Assay No	EXONI3-J68
	Forward primer	AAATTAGTCTTGCCTAAATGTCTGATCACA
	Reverse primer	CCAAAACCATCTTGTGTTCCTTGAC
	Reporter	TTATAAAACAGACTCTAAATTGCC
Exon I.4	Assay No	EXONI4-J60
	Forward primer	GTCCCTGGCACTGGTCAG
	Reverse primer	CATCTTGTGTTCCTTGACCTCAGA
	Reporter	CCCATCAAACCAGGACTC
Exon II	Assay No	EXONII-J79
	Forward primer	GCAACAGGAGCTATAGATGAACCTT
	Reverse primer	CATCTTGTGTTCCTTGACCTCAGA
	Reporter	CCACAGGACTCTAAATTG
Exon I.2	Catalog No	4331348

Table 2.2 The Taqman probes (Applied Biosystems) of human and mouse genes.

Human Gene	Cat. No.	Mouse Gene	Cat. No.
GAPDH	Hs99999905_m1	*Gapdh*	Mm99999915_g1
CYP19	Hs00240671_m1	*Cyp19*	Mm00484049_m1
ERα	Hs00174860_m1	*Erα*	Mm00433149_m1
ERb	Hs01100353_m1	*Erb*	Mm00599821_m1
CRH	Hs00174941_m1	*Crh*	Mm01283830_m1
CRHBP	Hs01075815_m1	*Crhbp*	Mm01283830_m1
CRHR1	Hs01062289_m1	*Crhr1*	Mm00432671_m1
CRHR2	Hs01120860_m1	*Crhr2*	Mm01326460_m1
CREB1	Hs00231713_m1	*Pgr*	Mm00435628_m1
CEBPA	Hs00269972_s1		
AP-1	Hs00277190_s1		
Sp1	Hs00412720_m1		

2.6 Measurement of Promoter Activity

2.6.1 Construction of Reporter Plasmids for CRH Promoter and Its Truncated Constructs

The pGL-3 basic reporter vector was purchased from Promega. *CRH* promoter plasmid was constructed by subcloning of *CRH* promoter into the mammalian expression vector pGL-3 (Invitrogen Life Technology). The construct was confirmed using restriction analysis and DNA sequencing. Primers for constructing *CRH* promoter reporter plasmid are listed as follows:

The forward primer of *CRH* containing a KpnI site:

5' CGCGGTACCGAGAGACGTCTCCGGGGGC 3'

Reverse primer *CRH* containing a Xho1 site:

5' GCGCTCGAGGGCTCATAACTCCTTTATGTGCTTGC 3'

Several fragments localized in the 5'flanking region of *CRH* was investigated. These regions contain two ERE half sites and one CRE (Chen *et al.*, 2008). GenBank accession number of human CRH gene promoter sequence is AF48855, The amplified genomic fragments (-663/+124bp, -442/+124bp, -315/+124bp, -188/+124bp) were inserted into the pGL-3 basic reporter vector. All constructs were confirmed using restriction analysis and DNA sequencing. Primers for constructing truncation CRH promoter plasmids are listed in **Table 2.3**. Underlined bases are the restriction site.

Table.2.3 Primer sequences for constructing CRH truncation plasmids

Oligonucleotide	Sequences
-663 forward	CGCGGTACC GGCTCATAACTCCTTTATGTGCTTGC
-442 forward	CGCGGTACC CCAACTGGCTGCTGCTTTCCT
-315 forward	CGCGGTACC ATATGTTTTCACACTTGGGAAATC
-188 forward	CGCGGTACCTGTGAGATTCAGTGTTGAGATAGC
+124 reverse	GCGCTCGAG GAGAGACGTCTCCGGGGGC

2.6.2 Transient Transfection and Dual-Luciferase Assay

1×10^5 per well JEG-3 cells were seeded in 24-well plates for 24 h. Then transient transfection was performed using 1 μl Lipofectamine and 2 μl Plus reagent (Invitrogen) per well. 0.25 μg reporter plasmid (containing DNA fragments derived from aromatase promoter region) with or without 0.1 μg expression vector

(pcDNA3.1-CRH or pcDNA3.1) were transfected into cells in serum-free medium. The renilla luciferase vector pRL-CMV (Promega) was cotransfected as an internal correction for transfection efficiency. After 5 h incubation, cells were cultured in phenol red free PRMI-1640 supplemented with 5% charcoal-dextran treated FBS. One day after transfection, various concentrations of testing compounds were added and incubated at 37°C for 24 h. The cells were lysed in lysis buffer (Promega) and stored at -80°C until assay.

Cell lysate was scraped and transferred to 1.5 ml eppendorf tube, and was centrifuged at 4°C at full speed for 5 min. 20μl of the supernatant was assayed in a dual luciferase reporter assay system (Promega). The luciferase activity was read by a FLUOstar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany), and expressed as relative light units of firefly/renilla.

2.7 Western Blot

JEG-3 or SGHPL cells were seeded in 6-well plate at a density of 5 x 10^5 cells per well. Before the day of testing compounds treatment, the cells were replaced with fresh phenol red-free RPMI 1640 medium supplemented with 5% charcoal-dextran treated FBS. The cells were treated with testing compounds for 24 hrs and then harvested for protein detection. During harvesting, cells were washed twice with PBS and lysed by 0.1 ml RIPA lysis buffer (25mM Tris-HCl pH 8.8, 50mM NaCl, 0.5% NP40, 0.5% Deoxycholate, 0.1% SDS). After 10-minute incubation on ice, cells were scraped and cell lysate was collected in a 1.5-ml microfuge tube. The lysate was then subjected to sonication on ice for 30 seconds with cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA). Clear supernatant was collected by centrifugation at 13,000 rpm for 5 minutes and was transferred to a new 1.5-ml microfuge tube.

Proteins were then used immediately or stored at -80˚C for long-term storage.

Protein concentration was determined by Bicinchoninic Acid (BCA) Assay (Pierce Biotechnology, Inc., Rockford, IL, USA.) with bovine serum albumin (BSA) as standards. 50 μg proteins were separated by sodium dodecylsulpahte-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% resolving gel and were transblotted to Immobilon-P polyvinylidene diflupride (PVDF) membrane (Millipore Corp., Bedford, MA, USA) at 15 V for 90 minutes. The membranes were probed with primary antibodies at 4˚C overnight. Anti-phospho-ERK (Cell Signaling Technology, Inc., Boston MA, U.S.A.), anti-CYP19 (Abcam plc, Cambridge, U.K.), anti-p-MEK (Cell Signaling), anti-p-ERK, anti-p-PKC, anti-p-PKA, anti-p-P38, anti-p-JNK, anti-CREB-1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-actin primary (Sigma Chemicals) and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz) were used for protein detection. The membranes were briefly washed and were then probed with appropriate secondary antibodies conjugated with horseradish peroxidase (Zymed Laboratories, South San Francisco, CA, USA) at room temperature for two hours. After probing the membranes were washed thoroughly. The antigen-antibody complexes were detected using Enhanced Chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ, USA) and were visualized by autoradiography. β-Actin was used for protein normalization. The membrane was reprobed with anti-actin primary antibody at 1:5000 dilutions.

2.8 Electrophoretic Mobility Shift Assay (EMSA)

2.8.1 Nuclear Protein Extraction

JEG-3 or SGHPL cells were seeded in 75 cm^2 flask and were replaced with phenol red-free RPMI 1640 medium supplemented with 5% charcoal-dextran treated FBS the day of experiment. Cells were treated with testing compound for 24 hours and nuclear protein was extracted using Novagen's NucBuster™ Protein Extraction Kit (EMD Biosciences, Inc., San Diego, CA, USA). Cells were washed with PBS, trypsinzed and pelleted by centrifugation at 1,000 rpm for 5 minutes. The cell pellet was resuspended in 150 µl NucBuster Reagent 1 with vigorous vortexing for 15 seconds. The homogenous suspension was then chilled on ice for 5 minutes, vortexed at high speed for another 15 seconds and chilled on ice again. The cell suspension was centrifuged at 13,000 rpm for 5 minutes at 4˚C. Supernatant was discarded and the pellet was washed with 500 µl cold PBS. The pellet was then resuspended by vortexing for 15 seconds in 75 µl NucBuster Reagent 2 supplemented with 1 µl 100X protease inhibitor and 1 µl 100 mM DTT. The suspension was then chilled on ice for 5 minutes, vortexed for another 15 seconds and chilled on ice again. Nuclear protein was collected in the supernatant by centrifugation at 13,000 rpm at 4˚C for 5 minutes and was kept at -80˚C for future experiments.

2.8.2 Electrophorectic Mobility Shift Assay

Complementary strands of oligonucleotides with CRE binding site on promoter II regulatory region (-211 to -197) of *CYP19* and with CRE binding site (-243 to -205) on *CRH* promoter regulatory region were commercially synthesized. The sequence of

CRE for *CYP19* is: 5- GAATGCACGTCACTCTACCCACT-3. The sequence of CRE for *CRH* is: 5-TTTAGGGCTCGTTGACGTCACCAAGAGGCGATAAATAT-3.

The oligonucleotides were annealed and labeled at 3'end with digoxigenin (DIG) using DIG Gel Shift Kit (Roche Applied Science, Indianapolis, IL, USA). Labeled probes were diluted to 1.55 fmol/µl for binding reactions. 10 µg nuclear extract was mixed with 1 µl DIG-labeled probe and the binding reaction was carried out at 25°C for 30 minutes according to the following composition:

5X binding buffer	4 µl
poly dI-dC (1µg/µl)	1 µl
poly L-lysine (0.1µg/µl)	1 µl
DIG-labeled probe (1.55 fmol/µl)	2 µl
10 µg nuclear protein	X µl
sterile ddH_2O	12-X µl
Total	20 µl

For supershift experiment, CRE-binding protein 1/activating transcription factor (CREB-1) and C/EBP antibodies from Santa Cruz Biotechnology were used. The CREB-1 antibody is reactive against CREB-1, CREB, CREM-1 and ATF-1 proteins, while the C/EBP β antibody recognizes C/EBP β, C/EBP α, C/EBPδ and C/EBP ε proteins. The antibodies were added and incubated with the reaction mixture for 45 minutes at room temperature. After incubation, the reaction mixture was resolved by 5% non-denaturing polyacrylamide gel electrophoresis at 8 V/cm. The gel was then transblotted to a positively charged nylon membrane at 15 V for 60 minutes and UV cross-linked 3 times at 120 mJ in an UV crosslinker (UV stratalinker 1800, Stratagene). The membrane was soaked in Blocking Buffer at room temperature for 1 hour; the membrane was probed with anti-DIG antibody for 30 minutes at room

temperature, followed by washing in Washing Buffer 3 times. The membrane was then equilibrated in Detection Buffer for 10 minutes at room temperature and the DNA-protein complexes were detected by incubating at 37°C for 10 minutes in the CSPD® solution. The chemluminescence emitted was visualized by autoradiography.

2.9 Animal Experiment Design

The oral effect of the various endocrine disruptors on pregnancy outcome was investigated by using pregnancy mice model. The experiments designed to test the hypothesis that endocrine disruptors treated animals had affect on CRH expression and estrogen metabolism.

2.9.1 Animal Model for BPA Study

Female ICR mice (Laboratory Animal Service Centre, Hong Kong Chinese University) were mated at 6–8 weeks of age (vaginal plug = Embryo day 0.5). The mice were acclimated to the laboratory environment for 3 days before the experiment. The pregnant mice were randomly assigned to two treatment groups: control and BPA treatment group. The BPA group was sub-divided into three BPA dosage groups. BPA was freshly prepared by dissolving in ethanol in (10 mg/ml BPA), and subsequently diluted to the final concentrations with corn oil. BPA was administered per orally from Embryo day 13(E13) to E16 at three dosages, 2, 20, and 200 mg/kg body weight respectively. Control group received corn oil only. The mice were sacrificed at E17 between 9:00 to 11:00 a.m. Serum was collected by cardiac puncture and placental tissue samples were excised and stored at -80°C. The protocol adopted in the present study had been approved by Animal Experimentation Ethics Committee of Chinese University of Hong Kong.

2.9.2 Animal Model for Genistein Study

Female ICR mice (Laboratory Animal Service Centre, Hong Kong Chinese University) were mated at 6–8 weeks of age (vaginal plug = day E0.5). The mice were acclimated to the laboratory environment for 3 days before the experiment and had free access to a phytoestrogen-free diet (**Table 2.3**). Normal lab chow might contain soy products. The pregnant mice were randomly assigned to five treatment groups. Genistein was freshly prepared by dissolving in distilled water and was administered per orally from E13 to E16 at three dosages, 40, 200, and 400 mg/kg body weight respectively. Control group received distilled water only. An intraperitoneal injection of 6.6 mg/kg of LPS dissolved in phosphate-buffered saline (PBS) was administered at E16.5. The mice were sacrificed 8 h after LPS injection, i.e. at E17 between 9:00 to 11:00 a.m. Serum was collected by cardiac puncture and placental tissue samples were excised and stored at -80^{o}C. The protocol adopted in the present study had been approved by Animal Experimentation Ethics Committee of Chinese University of Hong Kong.

Table 2.3 Phytoestrogen-free diet composition

Phytoestrogen-free diet	
Corn oil	54g
Casein	276g
Sucrose	100g
Mineral mix	35g
Vitamin mix	10g
Choline	4g
DL-methionine	3g
Corn starch	518g
Total	1kg

2.9.3 Quantitative Real Time RT-PCR Assay

Frozen placental tissues were pulverized in a Dounce homogenizer. Liquid nitrogen was constantly added in keeping the tissue in the frozen state throughout the homogenization. The pulverized samples were stored in −80 °C until assayed. Total RNA was extracted from the sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined by absorbance at 260/280 nm. First DNA strands were synthesized from 3 μg total RNA using oligo-dT primers and moloney murine leukemia virus reverse transcriptase (USB Corporation, Cleveland, OH, USA). Target fragments were quantified by real-time PCR, and a DNA Engine Opticon II (MJ Research, Inc., Waltham, MA) was employed for this assay. Taqman/VIC minor groove binder probes and primers for mouse *Crh, Crhbp,*

Crhr1, Crhr2 and *Gapdh* (Assay-on-Demand™) and real-time PCR Taqman Universal PCR Master Mix were all obtained from Applied Biosystems (Foster City, CA, USA). PCR reactions were set up as described in the protocol, which was validated by the manufacturer. Signals obtained for actin were used as a reference housekeeping gene to normalize the amount of total RNA amplified in each reaction. Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.9.4 Plasma CRH Determination

Blood samples were obtained from pregnant mice from heart on E17.5. All blood samples were taken in 1 hour to minimize the difference. Blood samples were collected into chilled test tubes containing heparin and aprotinin (0.6 TIU per ml blood; Phoenix Pharmaceuticals, California, USA). Maternal plasma was isolated from whole blood by centrifugation at 5000×g for 10 min and stored at 80°C until analysis. Plasma CRH concentrations were measured using ELISA kit from Phoenix Pharmaceuticals (Belmont, CA). Plasma samples (50 µl), primary antiserum and biotinylated peptide were added into a 96-well plate pre-coated with secondary antibody. After 2-hour incubation at room temperature, the plate was washed and incubated 1-hour with streptavidin-horseradish peroxidase (SA-HRP) which interacts with biotinylated peptide. The plate was washed and developed in the dark for 60 minutes with the substrate solution 3,3,5,5-Tetramethylbenzidine (TMB). Finally the plate was read at 450 nm using a plate reader (BMG Labtechnologies GmBH, Offenburg, Germany). The amount of CRH could be read against a standard curve constructed with the hormone provided in the kits.

2.9.5 Plasma Estradiol Determination

Blood samples were collected into chilled test tubes containing heparin and aprotinin (0.6 TIU per ml blood; Phoenix Pharmaceuticals, California, USA). Maternal plasma was isolated from whole blood by centrifugation at 5000×g for 10 min and stored at 80°C until analysis. The plasma estradiol was measured using Estradiol ELISA kit according to the company's protocol (Cayman Chemical Company, Ann Arbor, Michigan USA). Plasma samples (50 µl) were added into a 96-well plate coated with antibody raised against estradiol. After 1-hour incubation at room temperature, the plate was washed and then developed in the dark for 60-90 minutes. Finally the plate was read at 410 nm using a plate reader (BMG Labtechnologies GmBH, Offenburg, Germany). The amount of estradiol in plasma was quantitated by competing with estradiol tracer (estradiol linked to an acetylcholinesterase) for binding to the antibody. The absorbance representing the amount of plasma estradiol could be read against a standard curve constructed with the estradiol provided in the kit.

2.9.6 Plasma Progesterone Determination

Blood samples were collected into chilled test tubes containing heparin and aprotinin (0.6 TIU per ml blood; Phoenix Pharmaceuticals, California, USA). Maternal plasma was isolated from whole blood by centrifugation at 5000×g for 10 min and stored at 80°C until analysis. The plasma progesterone was measured using Progesterone ELISA kit according to the company's protocol (Cayman Chemical Company, Ann Arbor, Michigan USA). Plasma samples (50 µl) were added into a 96-well plate coated with antibody raised against progesterone. After 1-hour

incubation at room temperature, the plate was washed and then developed in the dark for 60-90 minutes. Finally the plate was read at 410 nm using a plate reader (BMG Labtechnologies GmBH, Offenburg, Germany). The amount of progesterone in plasma was quantitated by competing with progesterone tracer (progesterone linked to an acetylcholinesterase) for binding to the antibody. The absorbance representing the amount of plasma progesterone could be read against a standard curve constructed with the progesterone provided in the kit.

2.9.7 Plasma Testosterone Determination

Blood samples were collected into chilled test tubes containing heparin and aprotinin (0.6 TIU per ml blood; Phoenix Pharmaceuticals, California, USA). Maternal plasma was isolated from whole blood by centrifugation at 5000×g for 10 min and stored at 80°C until analysis. The plasma testosterone was measured using Testosterone ELISA kit according to the company's protocol (Cayman Chemical Company, Ann Arbor, Michigan USA). Plasma samples (50 µl) were added into a 96-well plate coated with antibody raised against testosterone. After 2-hour incubation at room temperature, the plate was washed and then developed in the dark for 60-90 minutes. Finally the plate was read at 410 nm using a plate reader (BMG Labtechnologies GmBH, Offenburg, Germany). The amount of testosterone in plasma was quantitated by competing with testosterone tracer (testosterone linked to an acetylcholinesterase) for binding to the antibody. The absorbance representing the amount of plasma testosterone could be read against a standard curve constructed with the testosterone provided in the kit.

2.10 Statistical Analysis

A Prism® 5.0 (GraphPad Software, Inc., CA, U.S.A.) software package was utilized for statistical analysis. For multiple group analysis, results were analyzed by *One-way ANOVA* followed by Bonferroni's Multiple Comparison Test if significant differences ($P<0.05$) were observed. The association of early delivery and treatment in **Fig.6.5** was determined by Chi-Square (χ^2) test for trend in the animal study of genistein. Significance was also set at $p<0.05$.

CHAPTER 3
BISPHENOL-A DOWNREGULATES CYP19 TRANSCRIPTION IN JEG-3 CELLS

3.1 INTRODUCTION

Bisphenol-A is a chemical used in the synthesis of polycarbonate plastic, which is the material used in manufacturing baby bottles, reusable water bottles and food containers, plastic food wrappers, and the inner coating of food cans. Concerns have been raised because the chemical leaches from polycarbonate plastic food containers. As reviewed in an expert panel report (Chapin *et al.*, 2008), the compound is weakly estrogenic and binds to the estrogen receptor (ER) with an affinity of EC_{50} at the sub-micromolar range. It may also trigger some physiological changes and introduce adverse effects on reproductive and developmental health. This ubiquitous endocrine disruptor can be found in the serum of maternal and fetal plasma (Ikezuki *et al.*, 2002; Schonfelder *et al.*, 2002a) as well as in placental tissue and breast milk (Sun *et al.*, 2004). Studies on mouse have shown that prenatal exposure to BPA could change the gross ovarian anatomy with a reduced number of corpora lutea and an increase in unilateral or bilateral blood-filled ovarian bursae (Markey *et al.*, 2003). Others have reported that BPA exposure increases aneuploidy in oocytes or meiotic disturbances in mice (Hunt *et al.*, 2003). Aneuploidy of the ovum has been linked to miscarriages, and a similar correlation has also been reported in humans (Sugiura-Ogasawara *et al.*, 2005).

In addition to the interaction with estrogen receptor, BPA also displays binding activity to the thyroid hormone receptor in rat liver extracts and to the human thyroid

hormone receptor expressed in human embryonic kidney 293 cells (Moriyama *et al.*, 2002). In a rat model, BPA increases serum thyroxine and upregulates RC3/neurogranin in the brain of postnatal pups (Zoeller *et al.*, 2005). Iwamuro et al. (Iwamuro *et al.*, 2006) also have demonstrated that this compound may block the upregulation of thyroid hormone receptor expressions induced by triiodo-thyronine in cultured Xenopus tail. These studies demonstrate that BPA could be a pleiotropic endocrine disruptor.

Aromatase or CYP19 is a cytochrome P450 enzyme responsible for the synthesis of estradiol, estriol, and estrone from androgenic steroidal compounds. It is encoded by a single gene mapping to 15q21.2 (Nelson *et al.*, 1996). Human *CYP19* gene encompasses ten exons, i.e. exon I to X. The coding sequence is distributed over nine exons, i.e. from exon II to X with a length of around 123 kb. Regulation of this gene is complex and is dictated by alternate splicing over a span of 90-kb immediately upstream of the coding sequence, i.e. exon I and part of exon II (Simpson *et al.*, 1993; Sebastian & Bulun, 2001; Simpson & Davis, 2001). Ten alternate spliced sequences are identified within this region, and each one is associated with a distinctive promoter giving rise to the unique tissue-specific regulation. After translation and glycosylation, CYP19 protein is localized at the endoplasmic reticulum.

Flanked by its own unique promoter region, exon I is alternate spliced onto a common splice junction immediately upstream of the start of translation. Hence, the open reading frame of each transcript (exon II to X) and the translated protein are identical, irrespective of the promoter used and site of expression. Some distinct promoters proximal to sequences in exon I are identified and used in different tissues; for instance, the promoters proximal to exons I.1 (PI.1), I.3 (PI.3), I.5 (PI.5), and II (P.II) control CYP19 expression in placenta, adipose, fetal tissue, and ovary,

respectively (Shozu *et al.*, 1998; Sebastian & Bulun, 2001; Bulun *et al.*, 2004). Different promoters may be under the control of different transcription factors, for example CREB and C/EBP are involved in the PI.3 and PII (Ye *et al.*, 2008; Ye, 2009), whereas a NF-IL6 binding site located at -2141 to -2115 in PI.1 (Toda & Shizuta, 1994).

During the course of human pregnancy, the placenta produces estrogen and is the primary source starting from the ninth week of gestation. Only humans express and maintain a high level of aromatase expression in placenta, and recruitment of the distal promoter of exon I may have an evolutionary implication (Sebastian & Bulun, 2001). The significance of aromatase in the maintenance of pregnancy is not clear; however, the administration of fadrozole, an aromatase inhibitor, to late pregnant rats can cause fetal injury. The injury may arise from the disruption of uterine expression of matrix metalloproteinase-1 and lysyl oxidase, which, in turn, affects the cross-linking of collagen (Tamada *et al.*, 2004). After replenishing the rats with exogenous estrogen, restoration of the uterine condition and no fetal injury have been observed. In another study, fadrozole administration delayed the initiation of implantation by one or two days in female rats but the viability of embryo was not affected (Tamada *et al.*, 2003). These studies have illustrated the role of aromatase in reproduction.

BPA suppresses rm-cyp19a mRNA expression in the hermaphroditic fish Rivulus marmoratus (Lee *et al.*, 2006). It has been shown to inhibit aromatase enzyme activity in JEG-3 and embryonic 293 cells (Nativelle-Serpentini *et al.*, 2003), but no transcriptional changes have been reported. In the present study, we hypothesized that BPA could downregulate aromatase transcriptional activity in human placental cells.

In placenta, transcriptional control has been established to be mainly dictated by the region proximal to exon I.1 (Yamada *et al.*, 1995), and the promoter is designated to be PI.1. Being under the same promoter control for aromatase expression, JEG-3 cells were the model of choice in the current study.

3.2 Results

3.2.1 Enzyme Inhibition Assay Performed on Recombinant Human CYP19 Protein

To verify the aromatase enzyme inhibition as reported in previous investigation, we examined BPA in a purified system. **Fig.3.1** indicated that BPA could inhibit recombinant aromatase activity. A significant inhibition was observed at 25 μM and the IC_{50} value was around 50 μM.

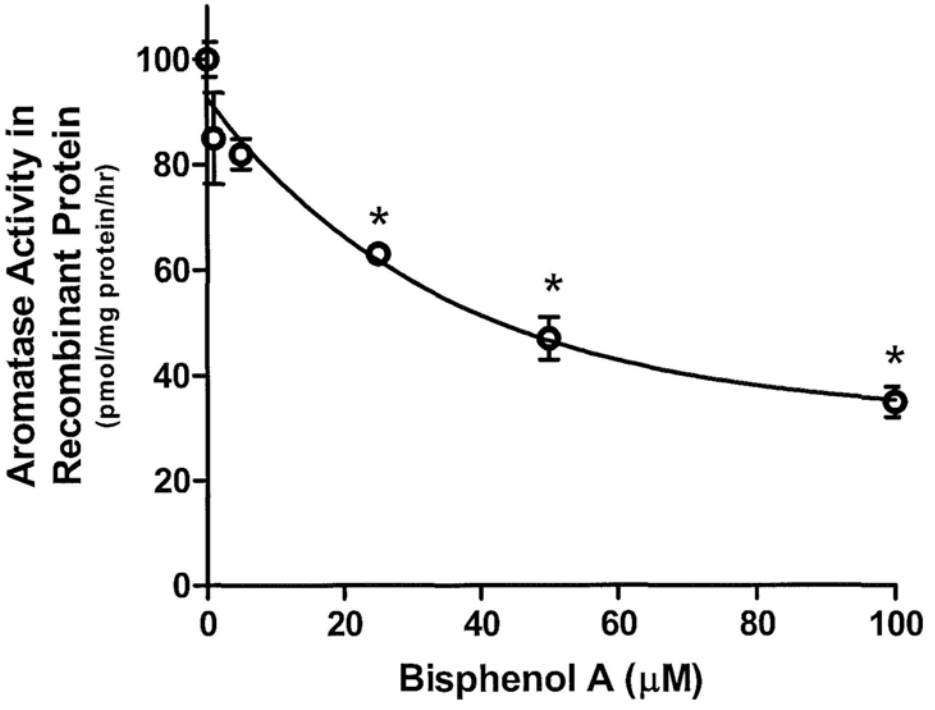


Fig.3.1 Recombinant CYP19 was inhibited by BPA. Recombinant CYP19, tritiated androstenedione and BPA were mixed and incubated for 1 hr. The IC_{50} value of BPA was determined to be ~50 μM. Values are means ± SEM, n=3. (*) Means were significantly ($p<0.05$) different from that of the control.

3.2.2 BPA Reduced Aomatase mRNA Expression in JEG-3 and PL30 Cells

Since aromatase is regulated differently in a cell-specific manner, BPA's effect on its expression in JEG-3 and PL30 cells was investigated. Quantitative RT-PCR indicated that the mRNA expression of aromatase was dose-dependently reduced by BPA. Cultures treated with 25 μM BPA revealed a 25% drop in aromatase expression, and 50 μM of the compound could decrease the expression by 70% (**Fig. 3.2A**). A comparable result was observed in PL30 cells (**Fig.3.2B**), and these results indicated that BPA could downregulate CYP19 expression in placental cells. JEG-3 cells were used in further investigations because PL30 cells had a limitation in transfecting reporter plasmids. Because the number of cells might be altered by the treatment of BPA, an MTT assay was carried out. The result illustrated that the treatment did not significantly affect the cell survival (data not shown).

3.2.3 Aromatase Activity and Protein Expression in JEG-3 Cells Treated with BPA

Since the mRNA abundance in JEG-3 placental cells, the aromatase activity was also evaluated and found to be significantly reduced by 5, 25, 50 and 100 μM BPA (**Fig.3.3A**). The decrease was consistent with the reduced amount of CYP19 protein expression (**Fig. 3.3B**).

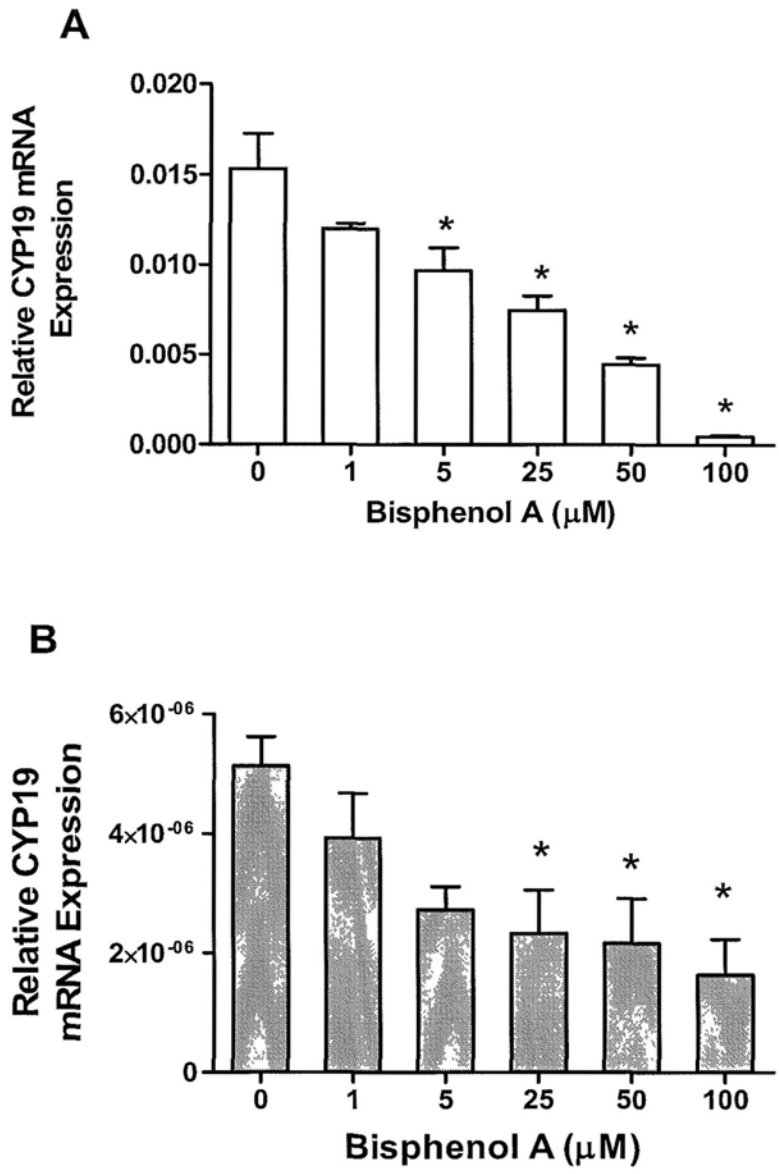


Fig.3.2 Message RNA expressions of aromatase in JEG-3 and PL30 cells treated with BPA. Cells were seeded in 6-well plates and maintained in phenol red-free RPMI medium supplemented with 10% charcoal dextran-treated serum. BPA was administered to the cultures for 24 hr. The effect of BPA on the *CYP19* expression was determined by real-time RT-PCR in JEG-3 (**Fig.3.2A**) and PL30 (**Fig.3.2B**) cells. Values are means ±SEM, n=3. (*) Means values were significantly ($p<0.05$) different from that of the control cultures with no BPA treatment.

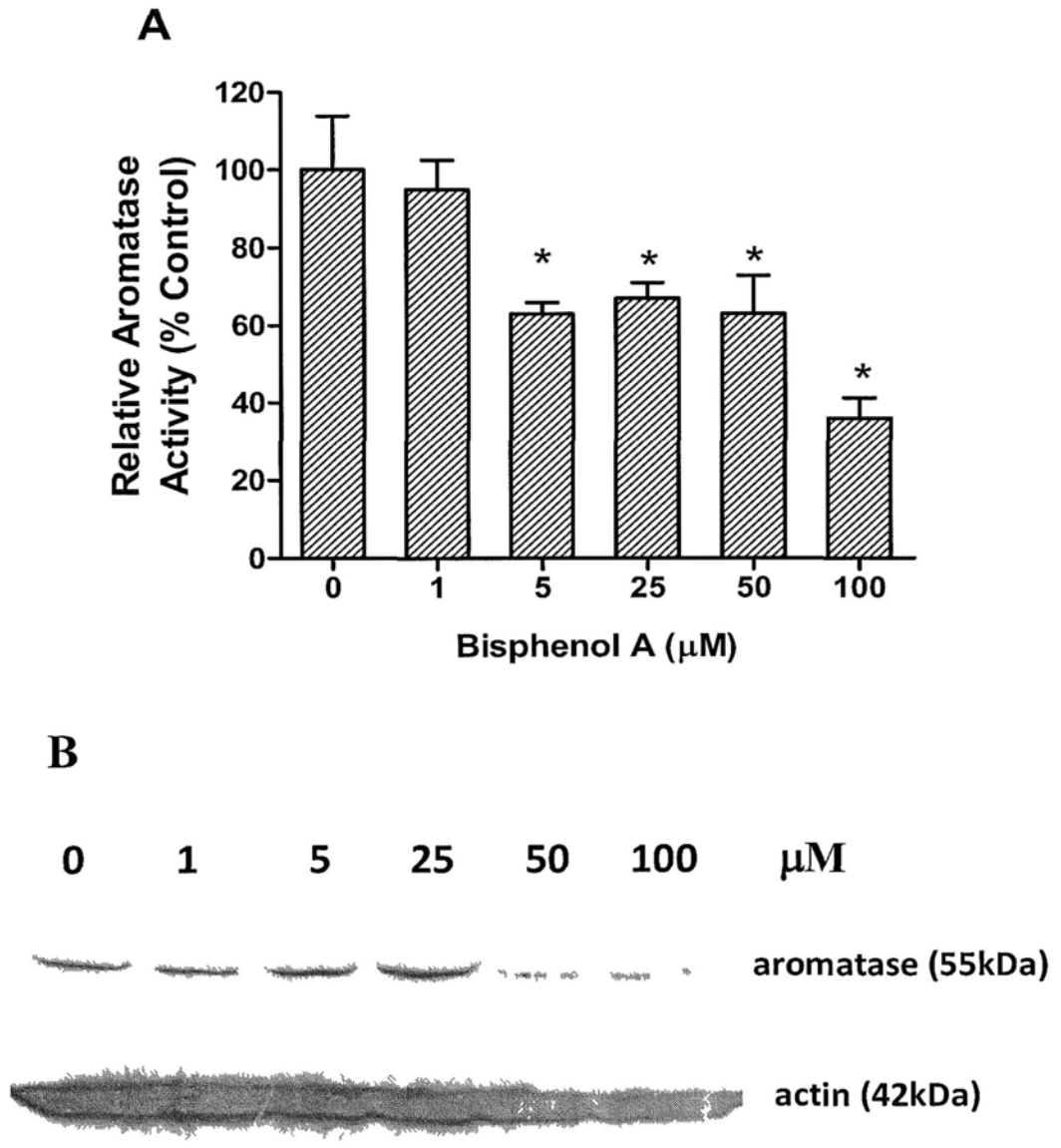


Fig.3.3 Effect of BPA on CYP19 enzyme activity and protein expression in JEG-3 cells. JEG-3 cells were maintained in phenol red-free RPMI medium and treated with BPA. CYP19 enzyme activity (**Fig.3.3A**) and immunoblotting (**Fig.3,3B**) were performed on the treated cultures. In **Fig.3.3A**, values are means ± SEM, n=3. (*) Means were significantly ($p<0.05$) different from that of the control. In **Fig.3.3B**, lanes 1-5 were samples treated with 0, 5, 25, 50, and 100 μM of BPA, respectively.

3.2.4 Transactivity of Promoter I.1 in JEG-3 cells

Since differential mRNA expression was observed between control and BPA-treated cultures, the transcriptional activities of *CYP19* were investigated. As mentioned above, the promoter usage of *CYP19* is proximal to exon I.1. This assay was performed to verify the suppression of the active promoter in JEG-3 cells. The luciferase reporter gene activity driven by promoter I.1 was significantly reduced by the administration of BPA at or above 25 μM (**Fig.3.4**). This illustrated that BPA downregulated CYP19 at the transcriptional level in JEG-3 cells.

3.2.5 Effect of BPA on Exon I.1-spliced mRNA Species of *CYP19* in JEG-3 Cells

Total CYP19 mRNA included all alternate-spliced transcripts, but those with exon I.1-spliced are dominating the CYP19 mRNA pool in placental cells. After confirming the suppression on the active promoter in JEG-3 cells, we investigated the abundance of the alternate spliced RNA species of exon I.1. Real-time PCR confirmed that the amount of exon I.1-spliced mRNA species was significantly reduced by BPA ranging from 5 to 25 μM (**Fig.3.5**). Since the corresponding promoter has been documented to be the primary control for *CYP19* regulation in placental cells, this and the result presented in **Fig.3.5** reinforced that BPA could downregulate the enzyme's transcriptional activity.

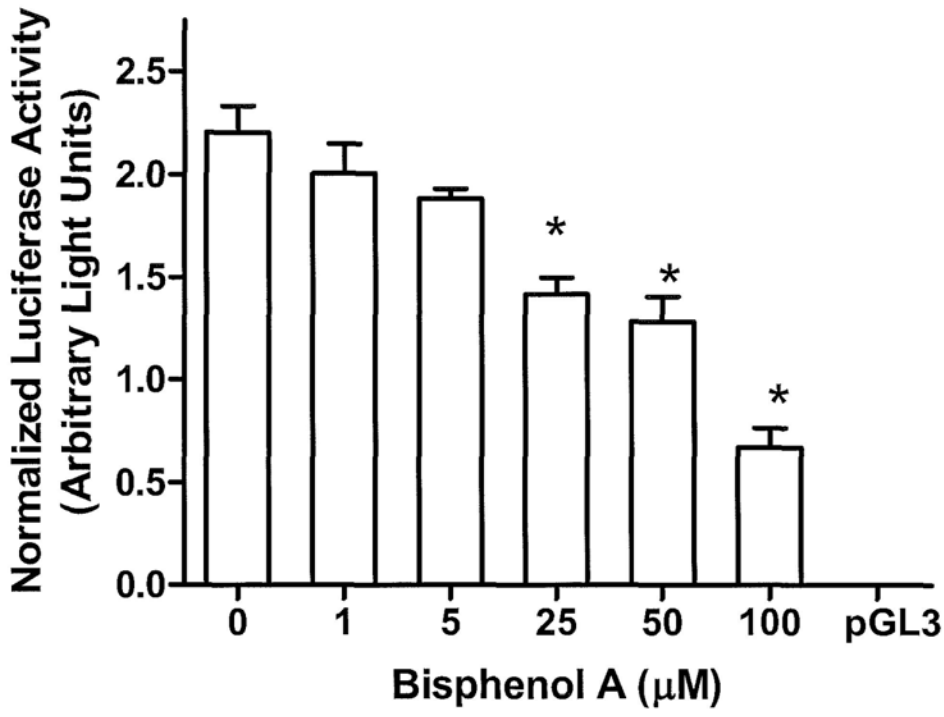


Fig.3.4 BPA suppressed *CYP19* promoter driven luciferase activity in JEG-3 cells. JEG-3 cells were seeded in 24-well plates and maintained in RPMI medium and switched to serum-free medium for the transfection of Promoter I.1-specific reporter plasmid. JEG-3 cells were transiently transfected with *CYP19* reporter plasmid and treated with BPA. The activities of the luciferases were determined in the cell lysate. Values are means ± SEM, n=3. (*) Means were significantly ($p<0.05$) different from that of the control (0 μM). pGL3basic is the negative control without Promoter I.1 insert.

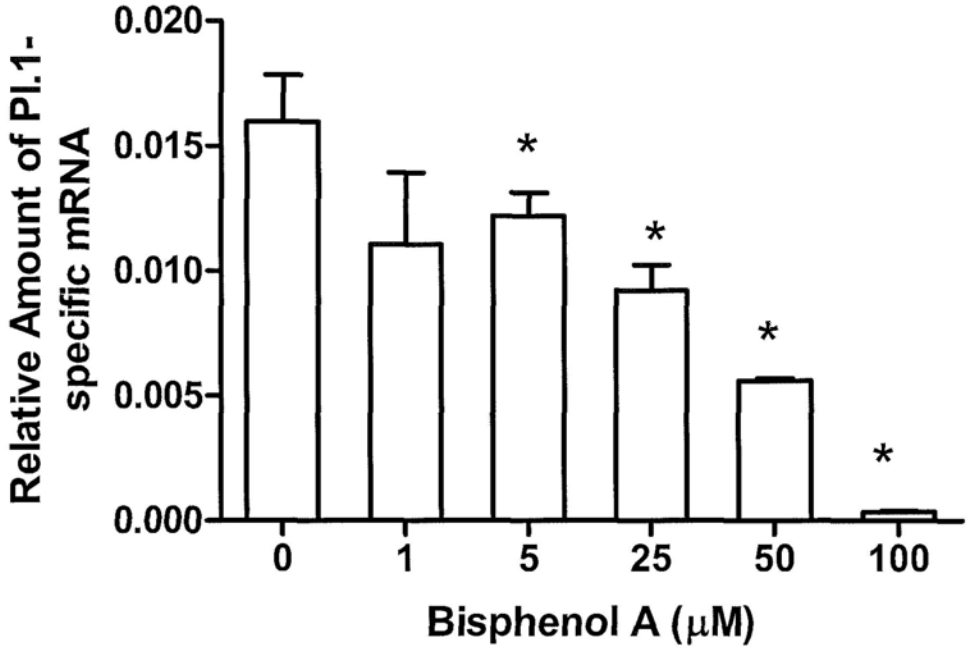


Fig.3.5 Exon I.1-spliced mRNA species in JEG-3 cells treated with BPA. JEG-3 cells were seeded in 6-well plates and maintained in RPMI medium supplemented with 10% charcoal dextran-treated serum. BPA was administered to the cultures for 24 hr. The effect of BPA on the abundance of Exon I.1-spliced CYP19 mRNA was determined by real-time RT-PCR. Values are means ±SEM, n=3. (*) Means values were significantly ($p<0.05$) different from that of the control cultures (0 μM).

3.2.6 Aromatase Activity Cells Treated with endocrine disruptors

In the present study, we also examined the ability of the toxicant in suppressing the transcription of CYP19. We measured the aromatase activity in JEG-3 cells treated with different agents, biochanin A and BP can slightly inhibited aromatase activity. Genistein and TCDD seems has no effect on aromatase activity (**Fig3.6**).

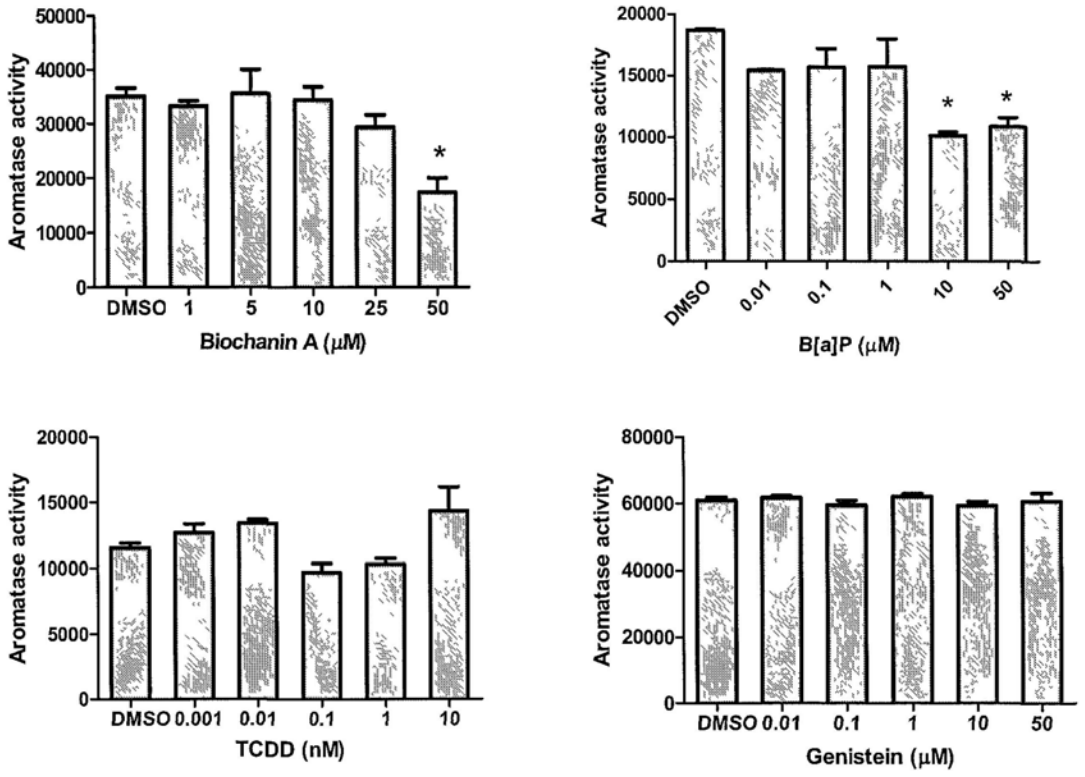


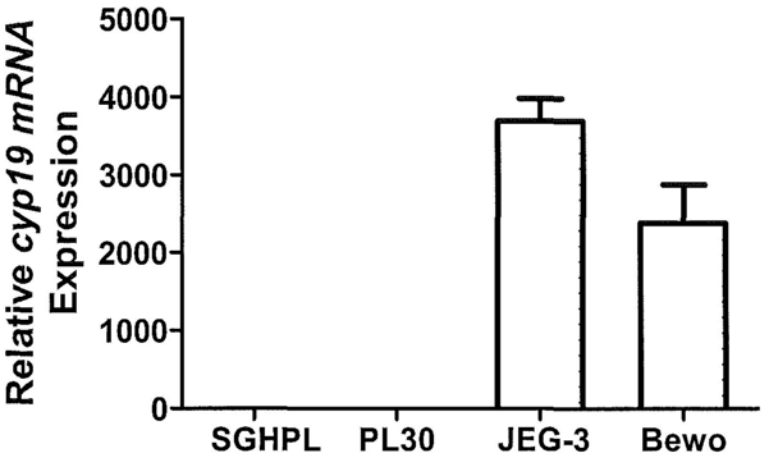
Fig.3.6. Inhibitory effect of endocrine-disrupting agents on CYP19 in JEG-3 cells. JEG-3 cells were seeded in 24-well plates and maintained in RPMI medium and switched to serum-free medium and treated with different Endocrine-disrupting agents. . CYP19 enzyme activity was performed on the treated cultures. Values are means ± SEM, n=3. (*) Means were significantly ($p<0.05$) different from that of the control.

3.2.7 CYP19, Promter I.1, Promter II, CRH and ERs expression in four trophoblast cell lines

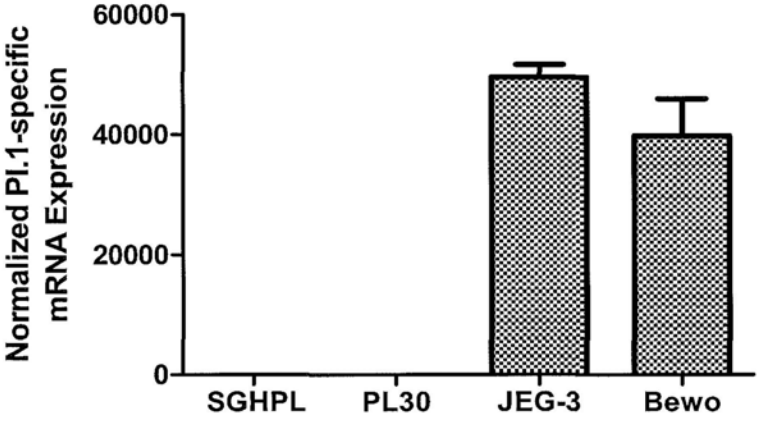
Four tropholblast cell lines were cultured in phenol red free medium for 24 h. Quantitative real time PCR was performed by using TaqMan probe. Quantitative RT-PCR indicated that the mRNA abundance of *CYP19* was almost 3000 fold higher in JEG-3 and Bewo cells than that in SGHPL and PL30 cells (**Fig.3.7A**). The alternate spliced mRNA species of exons I.1 was 40000 fold higher in JEG-3 and Bewo cells than that in SGHPL and PL30 cells (**Fig.3.7B**), whereas exon II-specific mRNA was only 5 to 10 fold higher in JEG-3 and Bewo cells that in SGHPL and PL30 cells (**Fig.3.7C**). This result suggested that the promoter usage of trophoblast cells SGHPL and PL30 was different from that of choriocacinoma cells JEG-3 and Bewo.

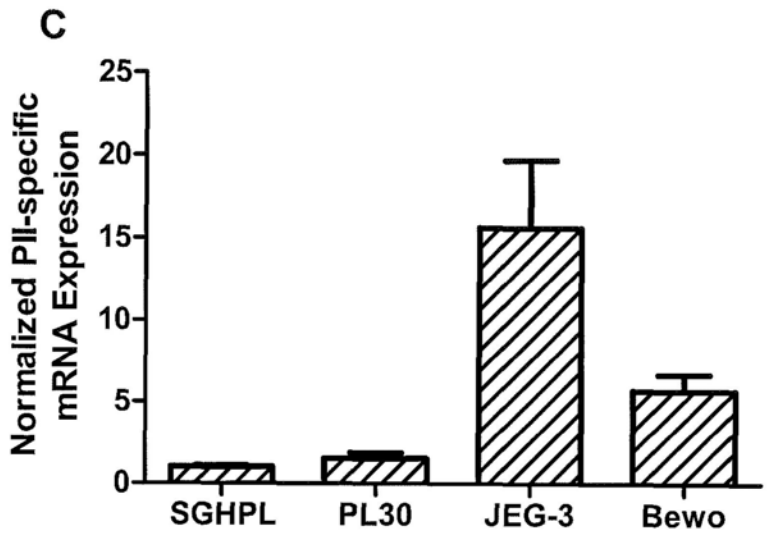
CRH expression was higher in SGHPL than the other three, while Bewo exhibited the lowest CRH expression level in four cell lines (**Fig.3.7D**). ER isoforms are detectable in four cell lines, and JEG-3 and BeWo cells have lower expressions compared with SGHPL and PL30 cells (**Fig.3.7E**). The first trimester and term trophoblast cells appear to respond differently to some stimulation that may reflect differential cellular function as gestation progresses when compared with placental choriocarcinoma cell lines (Lash *et al.*, 2003).

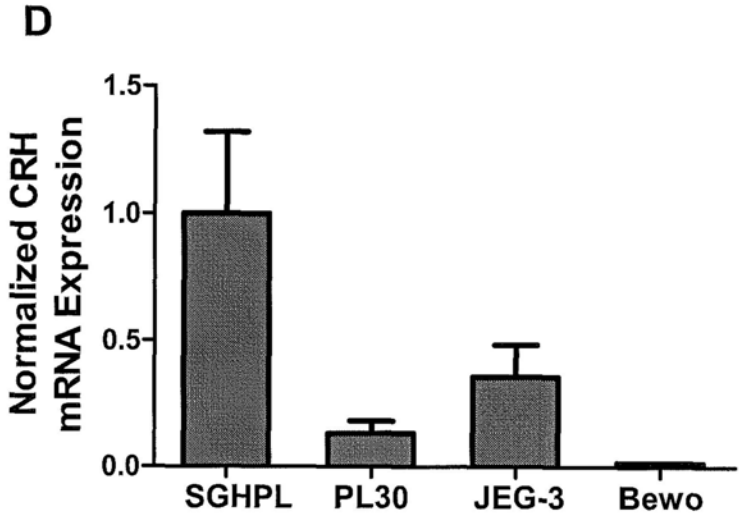
A


Relative cyp19 mRNA Expression
5000
4000
3000
2000
1000
0
SGHPL
PL30
JEG-3
Bewo

B


Normalized PI.1-specific mRNA Expression
60000
40000
20000
0
SGHPL
PL30
JEG-3
Bewo


C
Normalized PII-specific mRNA Expression
25
20
15
10
5
0
SGHPL
PL30
JEG-3
Bewo


D
Normalized CRH mRNA Expression
1.5
1.0
0.5
0.0
SGHPL
PL30
JEG-3
Bewo

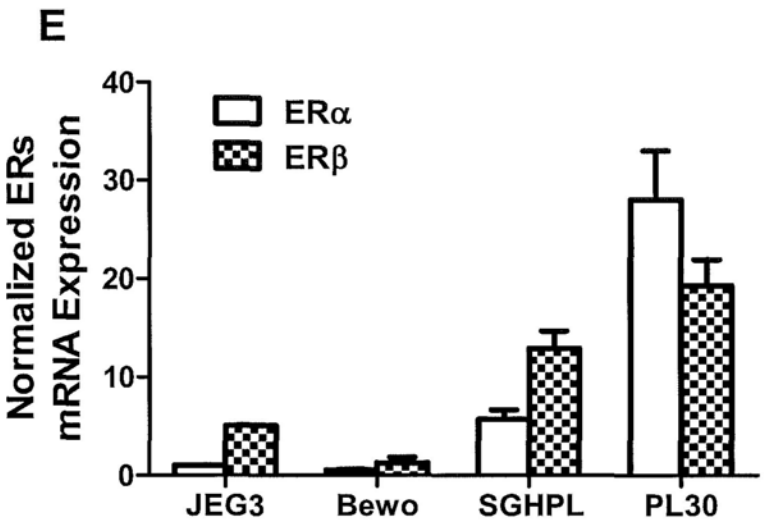


Fig.3.7 Relative *CYP19*, promoter PI.1, PII, CRH and ERs expression in four cell lines. Placental cells were seeded in 6-well culture plates and treated with genistein. Messenger RNA was extracted and quantitated by real-time PCR. **Fig.3.7A**, *CYP19* expression in four cell lines (*) represents a significant ($p<0.05$) increase in expression of cancer placenta cell lines when compared with the normal trophoblast cell lines. The expression differences were also observed in PI.1 (**Fig. 3.7B**), PII (**Fig. 3.7C**), CRH (**Fig. 3.7D**) and ERs (**Fig. 3.7E**). Values are means ±SE, n=3.

3.3 Discussion

In this study, we demonstrated that BPA suppressed CYP19 mRNA expression in the placental JEG-3 cells, and that the promoter I.1-driven transactivity was decreased by the chemical. In contrast to a previous report on CYP19 enzyme inhibition only (Nativelle-Serpentini *et al.*, 2003), BPA was actually acting at two different levels - enzyme and gene expression - on the aromatase in these cells. The enzyme inhibition can be a rapid and short-lived outcome, whereas suppression of gene expression may contribute to the delayed or relatively long-lasting effect.

Regulation of the transcription of aromatase is complicated. Many factors have been documented to induce the transcriptional activity, such as cyclic AMP, phorbol esters, dexamethasone, prostaglandin (PG) E2, transforming growth factor- , and -interferon (Simpson *et al.*, 1997), whereas cyclooxygenase inhibitors suppress CYP19 expression and inhibit the enzyme activity (Diaz-Cruz *et al.*, 2005). In JEG-3 cells, Yamada et al. (Yamada *et al.*, 1995) have shown that the first 301 bp of promoter I.1 introduces the placenta-specific expression of this *CYP19* gene. The present study found that BPA might interrupt the promoter I.1 activity. However, the transcriptional factor which was responsible for the downregulation was not identified. Further research on the mechanism of transcriptional regulation is justified.

Previous in vitro studies have demonstrated the inhibitory effect of BPA on aromatase. 25 μM of BPA reduces aromatase activities in JEG and embryonic 293 cells by 34%, and 15%, respectively (Nativelle-Serpentini *et al.*, 2003). However, the same study did not detect any changes in mRNA expression of CYP19 by non-quantitative RT-PCR. Another study also has shown the inhibitory effect of BPA

on aromatase activity in placental microsomes and 293 embryonic cells with other xenobiotic or by itself (Benachour *et al.*, 2007). However, the groups failed to identify the downregulation of aromatase transcription. In the present study, we first showed that BPA could suppress the transactivity of promoter I.1 and reduce the abundance of CYP19 transcripts in placental cells.

Other adverse effects have been documented for the action of BPA on pregnancy. In a mouse model, BPA exposure during gestation and lactation periods suppresses the activities of macrophages isolated from the mothers and their infants (Pyo *et al.*, 2007). The compound also reduces the number of embryos, uterine weight, and the intervillous spaces of the placenta in pregnant mice. Ultimately, the survival rate of the mouse neonates is decreased (Tachibana *et al.*, 2007). An influence on embryonic development has also been documented (Takai *et al.*, 2000). In pregnant rats, messenger RNA expression of placental prolactin-growth hormone and their stimulating genes Pit-1a/b are repressed upon BPA exposure (Lee *et al.*, 2005).

The physiological dosage equivalent used in the present study is not known. However, our result could provide additional information when the safety limit for the exposure of BPA is reviewed. Domoradzki et al. (Domoradzki *et al.*, 2003) have shown that gavaging at 10 mg /kg body weight would induce a maximum plasma concentration of 1 mg/L (around 5 μM) in rats at 17-21 gestation days. The range is within the concentration that would alter CYP19 expression based on the results of the present study.

In summary, we are first to report that BPA downregulates the mRNA expression of CYP19 in placental cells. Further study on the transcription factor(s) involved is warrant. Findings of the present study enrich the scientific database of BPA, which may facilitate a better estimation for its toxicity in the future. In the

present study, we examined the ability of the endocrine disruptor biochanin A, B[a]P, Genistein and TCDD in regulating the transcription activity of CYP19. The CYP19, Promter I.1, Promter II, CRH and ERs expression in four trophoblast cell lines were also evaluated by RT-PCR.

CHAPTER 4
EFFECT OF BISPHENOL-A ON PLACENTAL CORTICOTROPIN-RELEASING HORMONE EXPRESSION AND ESTROGEN METABOLISM IN VITRO AND IN VIVO

4.1 INTRODUCTION

Bisphenol-A (BPA) is an endocrine-disrupting contaminant, which is used for the manufacture of consumer products, like food container and dental sealants (Calafat *et al.*, 2005). BPA exposure has been correlated to the increased incidence of endocrine and reproductive problems (Eertmans *et al.*, 2003). This has raised safety concern for its ubiquitous existent in our environment. The Tolerable Daily Intake (TDI) is determined to be ranged from 5 to 50 micrograms/kg body weight/day by the European Food Safety Authority. The maximum non-toxic dose of BPA administered to rodent is around 200 mg/kg/day (Takahashi & Oishi, 2003). BPA at doses comparable to human exposure can induce malformation of the reproductive organ in developing rodents (Schonfelder *et al.*, 2002b).

Prenatal BPA exposure leads to long-term adverse reproductive and carcinogenic effects, such as atypical hyperplasia and stromal polyps of the uterus, sarcoma of the uterine cervix and mammary adenocarcinoma, in murine female at environmentally relevant doses (Savabieasfahani *et al.*, 2006; Newbold *et al.*, 2009). Prenatal exposure to BPA also elicits an endometriosis-like phenotype in female mice offspring (Signorile *et al.*, 2010), and alters the development and tissue organization

of the mouse mammary gland (Markey *et al.*, 2001). Perinatal and postnatal exposure to BPA increases murine adipose tissue mass and serum cholesterol level which facilitates the development of obesity and hyperlipidemia later in life (Miyawaki *et al.*, 2007).

Embryonic and fetal exposure to BPA causes underdevelopment of the brain, kidney and testis in the newborn (Kabuto *et al.*, 2004) and disruption of placental functions and the neonates' survival in mice (Tachibana *et al.*, 2007). BPA exposure also reduces sperm counts in mice (Al-Hiyasat *et al.*, 2002). On the other hand, low concentration BPA has been reported to have some beneficial effect on health. BPA may prevent ovarian degeneration and bone loss in aromatase gene knockout mice (Toda *et al.*, 2002).

CRH is expressed in placenta and the amount increases progressively toward the end of human pregnancy (Usui *et al.*, 1988). Placental CRH determines the length of gestation and acts as a trigger for parturition in humans (McLean *et al.*, 1995). The expression of CRH is controlled by several endogenous factors (Yue *et al.*, 2008). Glucocorticoids inhibit *CRH* gene expression in the hypothalamic paraventricular nucleus (PVN) while stimulate the expression in the placenta (King & Nicholson, 2007). Several neurotransmitters and peptides, like norepinephrine, acetylcholine, angiotensin II, interleukin-1 and oxytocin, stimulate the release of immunoreactive CRH from placental cells (Petraglia *et al.*, 1989).

Steroid hormones are able to regulate *CRH* gene expression through activating different signaling pathways and their downstream response elements (Ni & Nicholson, 2006). In the placenta cells, cAMP induces CRH expression via phosphorylating PKA and transcription factor AP-1 (Uh *et al.*, 2009). In cultured human placental trophoblasts, CRH inhibits progesterone production in a

PKC-dependent pathway (Yang *et al.*, 2006); in contrast, CRH induces a key proinflammatory mediator VEGF release via the cAMP/PKA/p38 MAPK signaling pathway in mast cells (Cao *et al.*, 2006).

There is a strong correlation between CRH and estrogens secretions during pregnancy. CRH can stimulate estrogen biosynthesis and estrogen activates other hormone secretion in the placenta (You *et al.*, 2006; Klimkova *et al.*, 2010). Reversely, estrogen can also regulate CRH expression through an estrogen-receptor dependent pathway. Ultimately, the downstream CRE binding site on CRH promoter region is affected (Chen *et al.*, 2008; Lalmansingh & Uht, 2008; Stamatelou *et al.*).

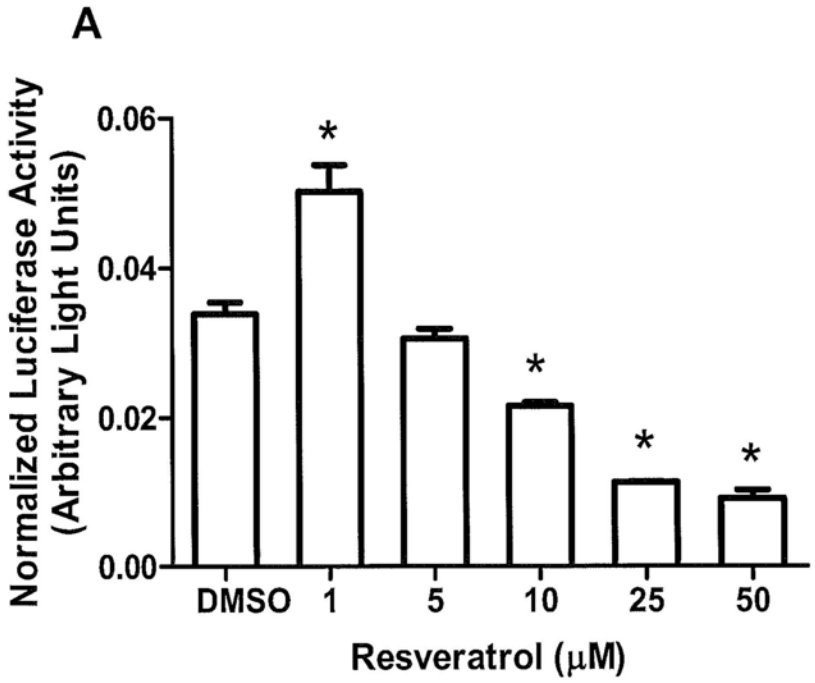
Progesterone is another important steroid hormone in human pregnancy. In early pregnancy, corpus luteum is the major source of progesterone. After 6–8 weeks of pregnancy, the placental trophoblasts take over (Tuckey, 2005). Placental progesterone continues to increase up to term until the placenta is lost following parturition (Mesiano, 2001). The circulating concentrations of progesterone in maternal plasma will return to normal after parturition (Csapo, 1956).

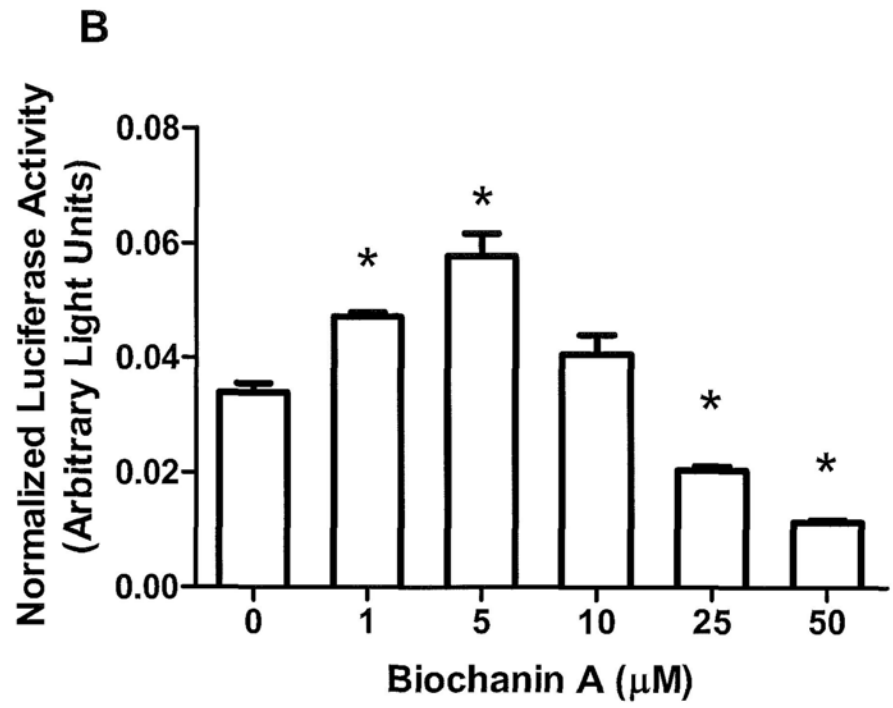
In the current study, the effect of BPA on placental CRH metabolism was investigated in the choriocarcinoma cell line JEG-3. We further studied the effect on steroid hormone expression, secretion and metabolism in pregnant.

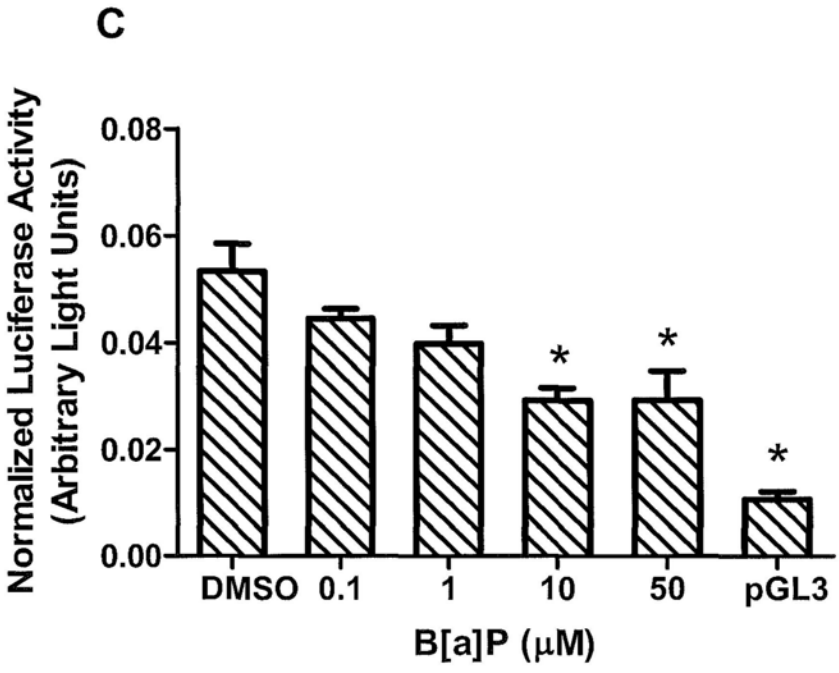
4.2 Results of Cell Culture Experiments

4.2.1 BPA Induced CRH Transactivity in JEG-3 Cells

The transcriptional activities of CRH treated with endocrine-disrupting agents were investigated. Both resveratrol and biochanin A have biphasic response on promoter activity, low concentration show elevated luciferase activity, while high concentration decreased promoter activity (**Fig.4.1.A,B**). B[a]P exerted moderate inhibition of CRH promoter activity (**Fig.4.1.C**). BPA could dose-dependently increase the luciferase light units as CRH transcriptional activity in JEG-3 cells (**Fig.4.1D**).




B
Normalized Luciferase Activity
(Arbitrary Light Units)
0.08
0.06
0.04
0.02
0.00
*
*
*
*
0
1
5
10
25
50
Biochanin A (μM)


C
Normalized Luciferase Activity
(Arbitrary Light Units)
0.08
0.06
0.04
0.02
0.00
*
*
*
DMSO
0.1
1
10
50
pGL3
B[a]P (μM)

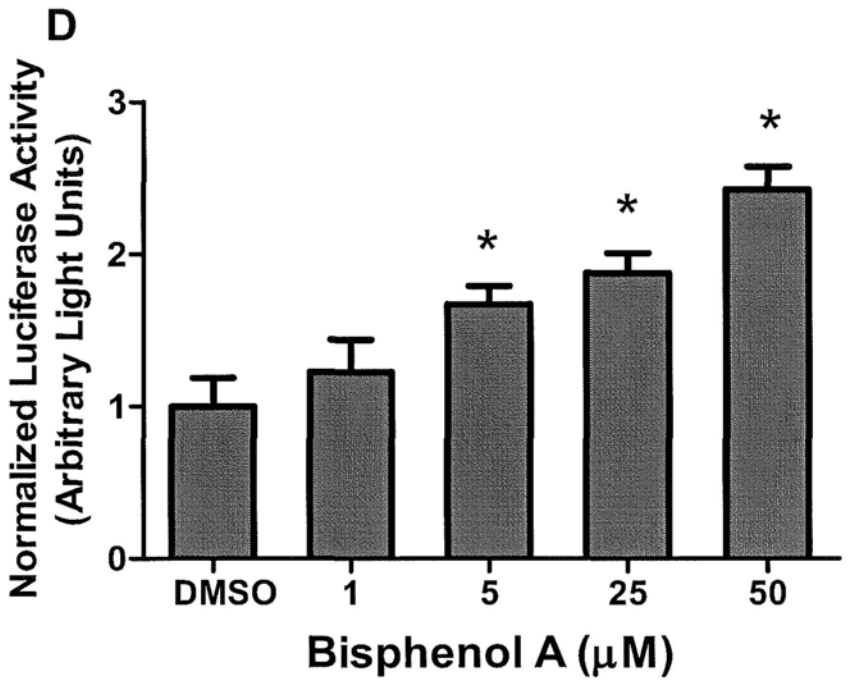


Fig.4.1.Endocrine-disrupting agents suppressed CRH promoter driven luciferase activity in JEG-3 cells. JEG-3 cells were seeded in 24-well plates and maintained in RPMI 1640 medium and switched to serum-free medium. JEG-3 cells were transiently transfected with CRH reporter plasmid and treated with different agents. The activities of the luciferases were determined in the cell lysate. Values are means ± SEM, n=3. (*) Means were significantly ($p<0.05$) different from that of the control (0 μM). pGL3 basic is the negative control without promoter CRH insert.

4.2.2 BPA Induced CRH mRNA Expression in JEG-3 Cells

As the CRH transactivity was induced by BPA, we subsequently determined the effect on CRH mRNA expression. 25 μM BPA or above increased CRH expression significantly.

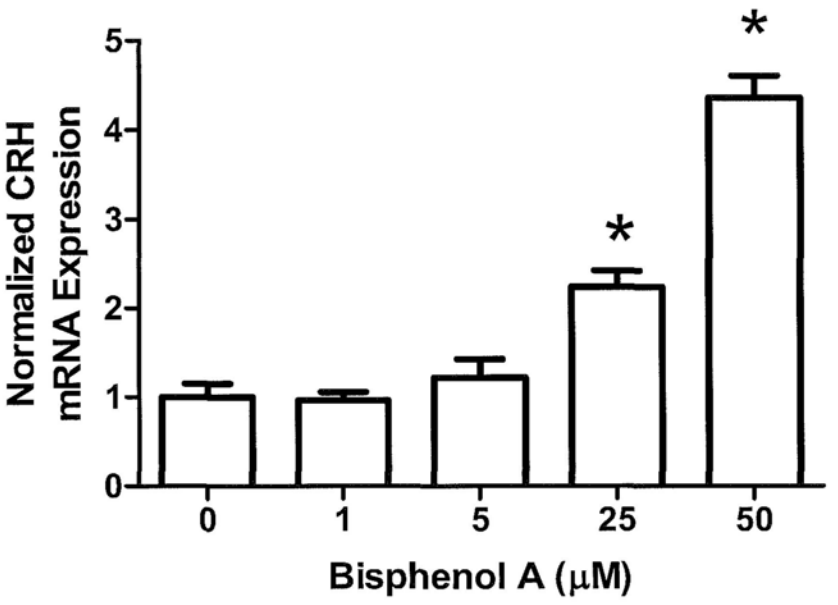
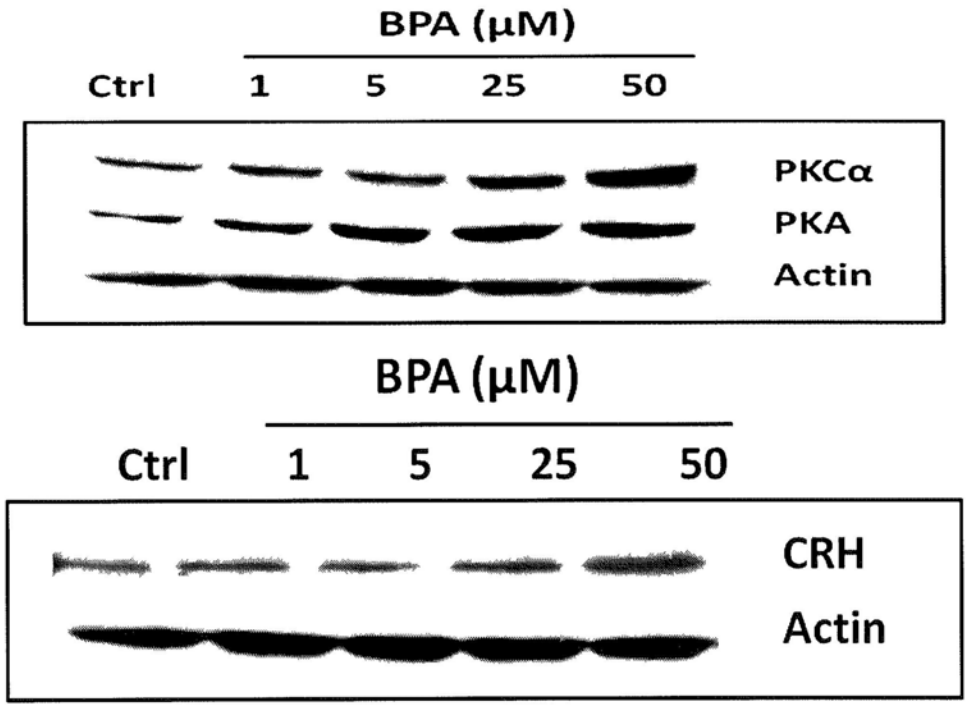


Fig.4.2 Effect of BPA on CRH mRNA expression in JEG-3 cells. JEG-3 cells were seeded in 6-well plates and maintained in phenol-red free RPMI 1640 medium supplemented with 10% charcoal dextran-treated FBS. Cells were treated with BPA for 24 h. The amount of CRH mRNA was determined by real time PCR and was normalized by GAPDH mRNA. Values are means ±SEM, n=3. (*) Means values were significantly ($p<0.05$) different from that of the control cultures.

4.2.3 Activation of Protein Kinases in JEG-3 Cells Treated with BPA

As various experiments demonstrated that CRH was upregulated by BPA, we further looked into the possible regulatory mechanisms. Many MAPKs were previously implicated in the regulation of CRH in placental cells. Proteins of CRH, p-PKCα, p-ERK-1/2, p-P38, p-MEK and p-JNK in cell lysates were determined by western blot analysis. Phosphorylated forms of PKCα and PKA were found increased as shown in **Fig.4.3A**, while others have no change. Optical density of the images illustrated that the amount of these activated kinases were significantly increased (**Fig.4.3B**). BPA activated protein kinases concurrently with the induction of the CRH protein (**Fig.4.3A**).

A



B

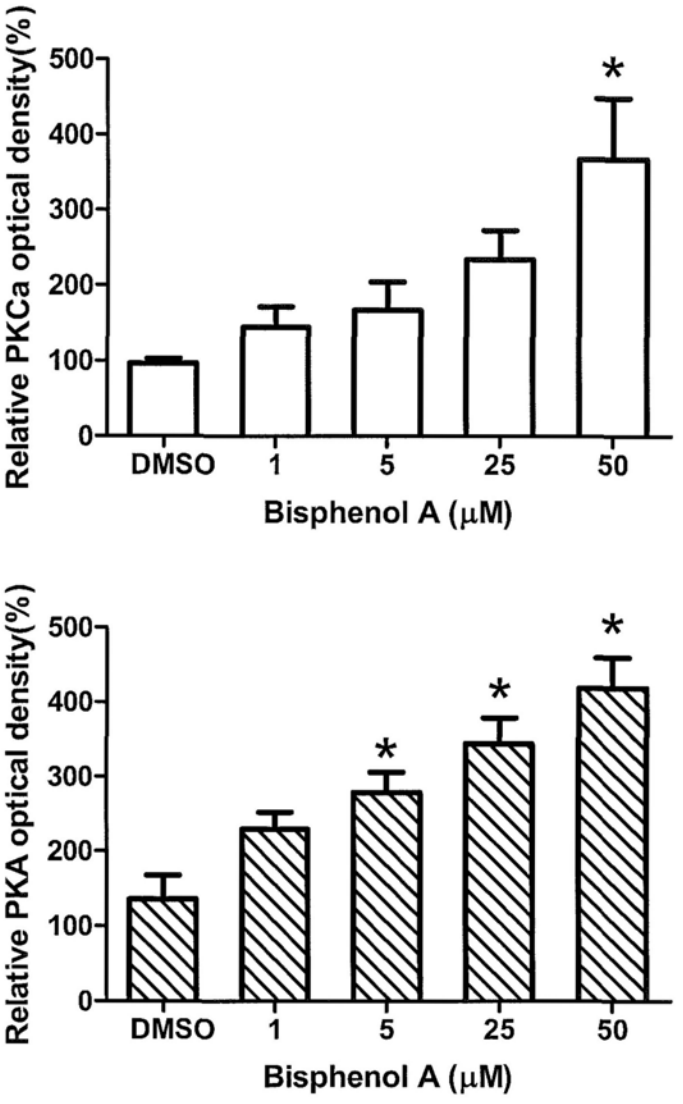


Fig.4.3 Immunoblot of signaling proteins in JEG-3 cells treated with BPA. JEG-3 cells were seeded in 6-well culture dishes and treated with BPA for 24 h. Protein expression of CRH, p-PKCα and p-JNK in cell lysates was determined by western blot analysis. The images in **Fig.4.3A** are representations of three independent experiments, and **Fig.4.3B** displays the optical density readings of the proteins. Values are means ±SE, n=3. Mean with (*) is significantly ($p<0.05$) different from control.

4.2.4 Reporter Gene Assay on CRH Promoter in JEG-3 Cells

BPA could increase CRH promoter activity in JEG-3 cells. To locate the sequence responsible for the enhancement, a series of truncate promoters were prepared of CRH promoter and were inserted into the reporter plasmid. Cells transfected with (-663/+124), (-442/+124) and (-315/+124) driven reporter plasmids displayed increased luciferase activity by 25 μM BPA treatment (**Fig.4.4**). Since the three sequences all contain CRE, the increased transactivity might reveal that CRE is necessary for the CRH promoter response to BPA treatment.

4.2.5 BPA Increased CRE Binding in CRH Promoter Fragment

Subsequently, EMSA assay also verified that the binding of CRE was increased by BPA dose-dependently (**Fig.4.5**). The specificity of the band was demonstrated by the competition for binding upon incubating with the antibody of CREB.

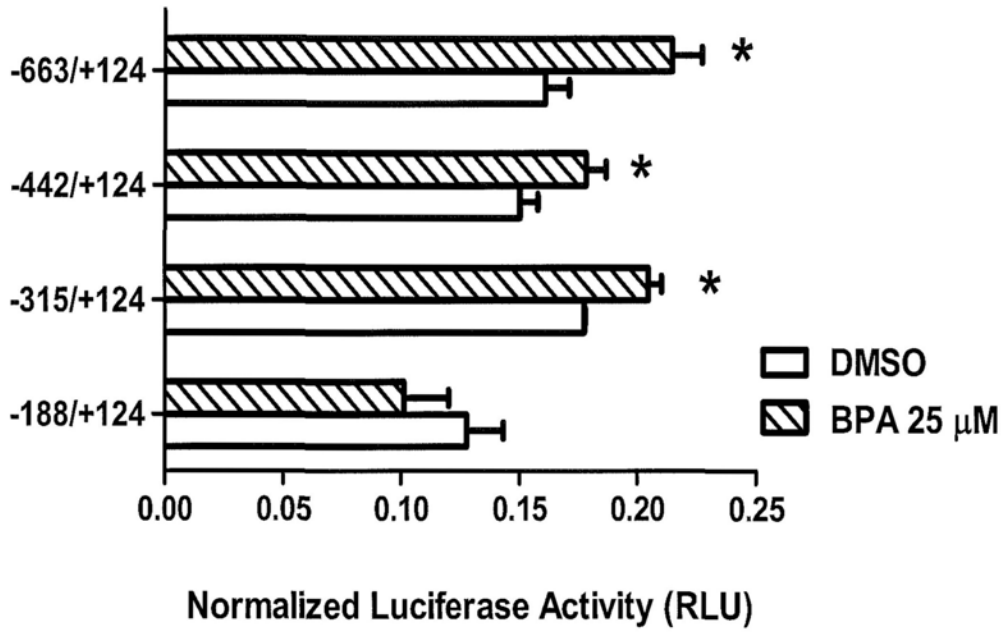


Fig.4.4 Reporter gene assay of CRH promoter in JEG-3 cells. JEG-3 cells were seeded in 48-well plates and maintained in RPMI 1640 medium. The cells were switched to serum-free medium and a series of truncation reporter plasmids of CRH promoter were constructed and transfected into JEG-3 cells. The cells were then treated with 25μM BPA and assayed for luciferase activities. Values are means ± SEM, n=4. (*) Means were significantly ($p<0.05$) different from that of the control (0 μM).

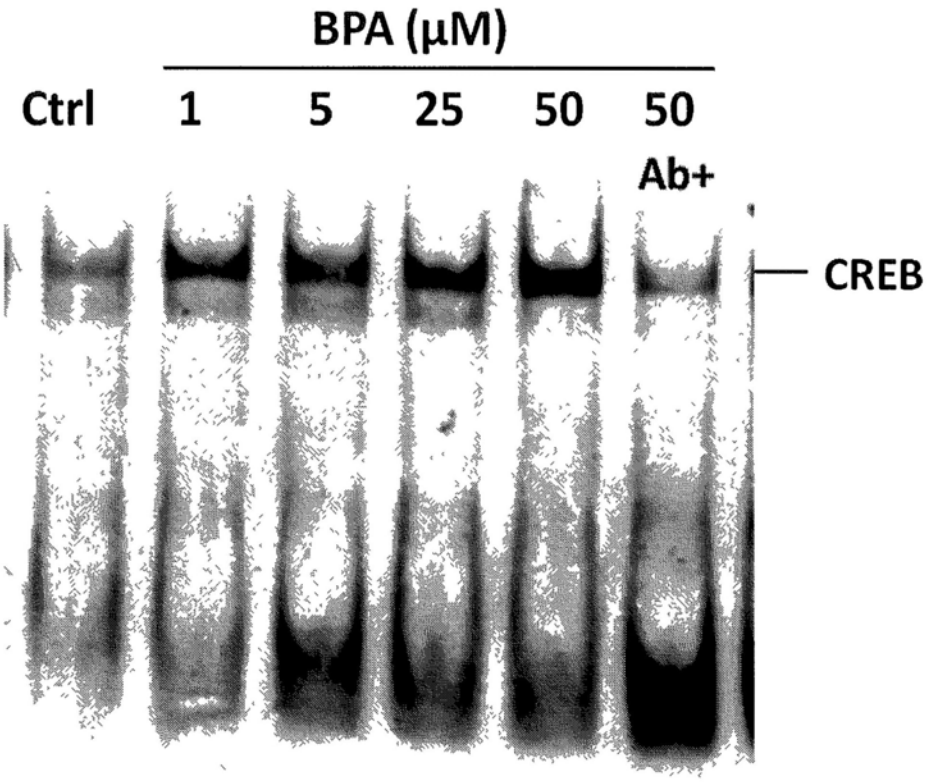


Fig.4.5. BPA treatment increased the electromobility shift of CRE. Nuclear extracts were prepared from JEG-3 cells treated with BPA. Samples were treated with BPA at 0, 1, 5, 25 and 50 μM; lane 6 is a sample treated with 50 μM BPA incubating with CREB antibody. Data represents one of two independent experiments with comparable results.

4.2.6 Western Blot on Nuclear CREB in BPA Treated Cells

The kinetics of CREB phosphorylation in response to various activators such as kainite, carbachol and basic fibroblast growth factor (bFGF), corresponds to that of MAPK activation (Pende *et al.*, 1997). Western analysis indicated that increased CREB protein was phosphorylated in nuclear extracts isolated from BPA treated cells in a dose dependent manner (**Fig.4.6**).

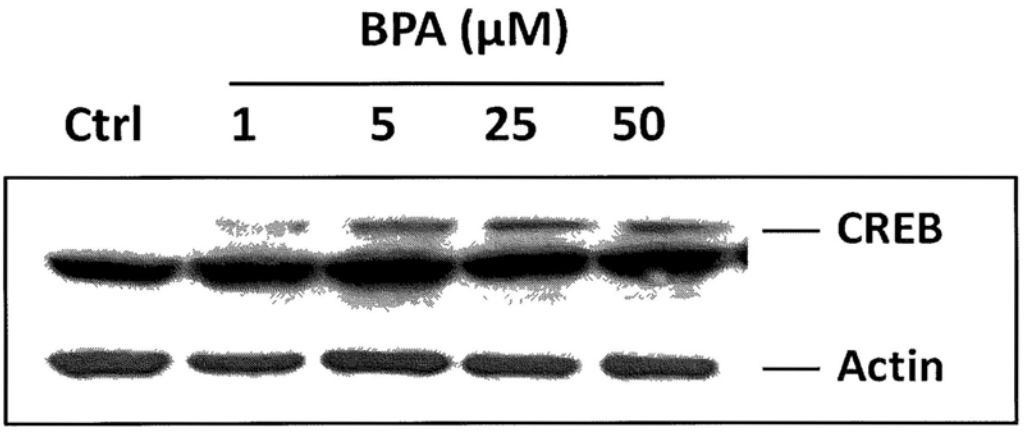


Fig.4.6. Western analysis on nuclear CREB under BPA treatment. JEG-3 cells were treated with BPA for 24 h. Nuclear extracts were isolated and immunoblotted for CREB was performed. BPA was administered at 0, 1, 5, 25 and 50 μM.

4.3 Result of Animal Experiment

4.3.1 Effect of BPA on Placental CRH mRNA Expression in Mice

As BPA could induce CRH expression in placenta cells, the same was examined in a mice model. Messenger RNA expression of *Crh* in the placental tissue of mice treated with BPA at 200 mg/kg was elevated (**Fig.4.7**).

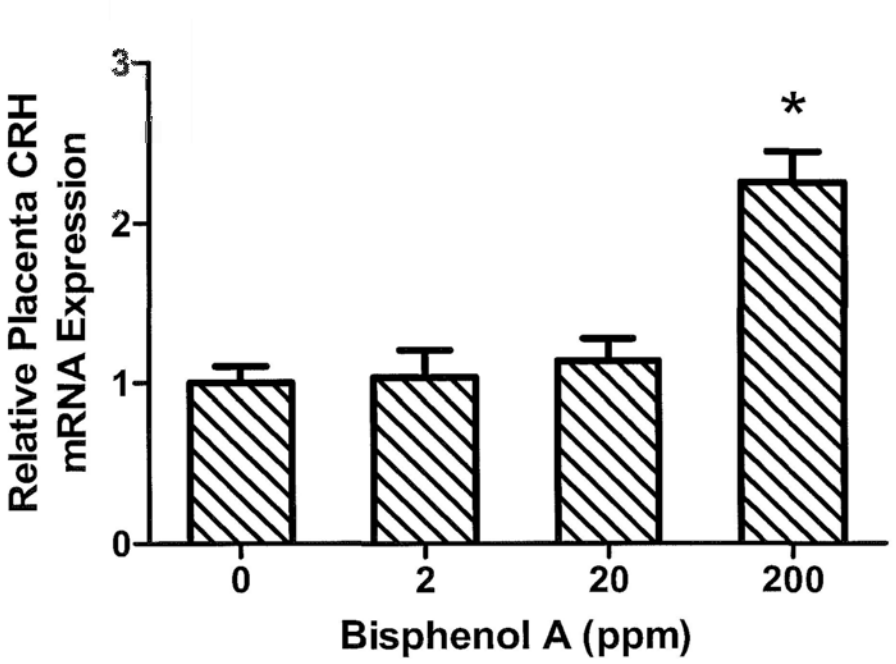


Fig.4.7 Effect of BPA on placental Crh mRNA expression in mice. Pregnant ICR mice were gavaged with BPA. Total RNA was extracted from the placental tissue and CRH expression was quantified by real-time RT-PCR. Values are means ±SE, n=5. Mean with (*) is significantly ($p<0.05$) different from control.

4.3.2 Plasma Crh in Maternal Circulation

Since mRNA of placental Crh increased, the hormone in maternal circulation was also determined. Plasma Crh in the 200 mg/kg BPA treatment group was significantly greater than that of the control (**Fig.4.8**). This pattern was highly consistent with the mRNA expression of placental Crh.

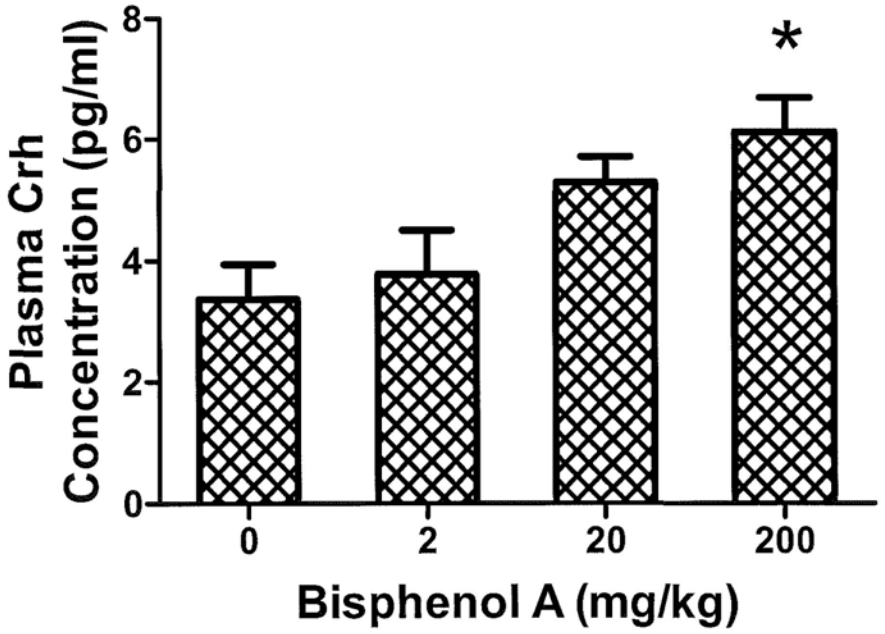
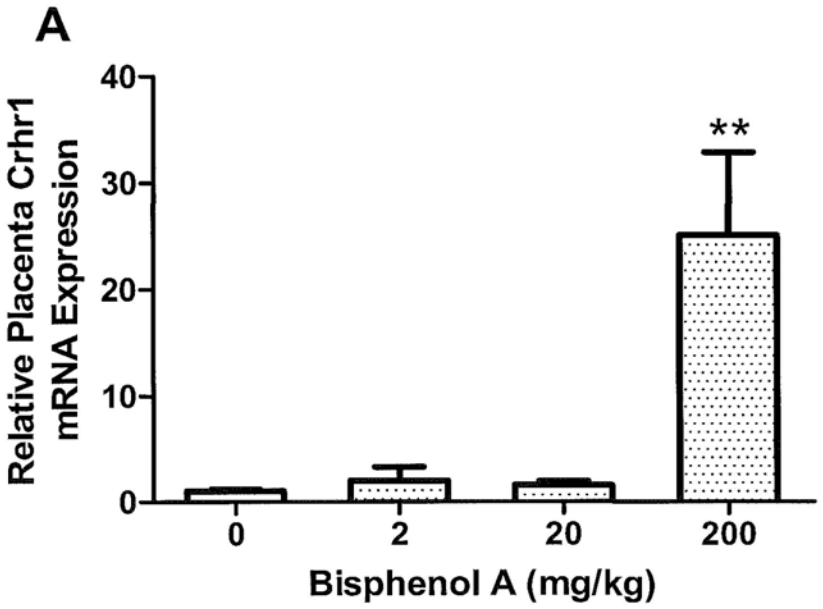
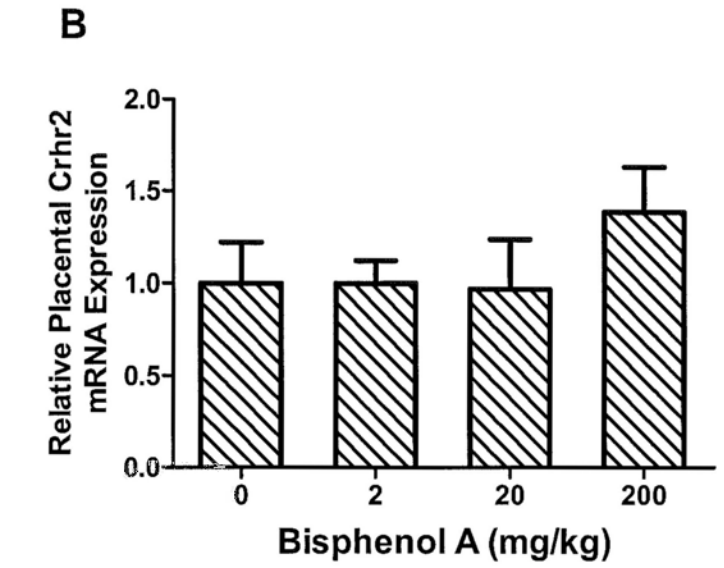


Fig.4.8 BPA induced plasma Crh in maternal circulation. Pregnant ICR mice were gavaged with BPA. Blood was drawn and assayed for Crh by ELISA. Values are means ±SE, n=5. Mean with (*) is significantly ($p<0.05$) different from Control.

4.3.3 BPA Induced Placental Crhr1 Expression

Since both Crh mRNA expression and secretion in serum was found to be elevated by BPA treatment, the expressions of Crhr1/Crhr2 and binding protein of Crh (Crhbp) were also determined. The amount of Crhr1 mRNA was significantly increased in the mice treated with 200 mg BPA /kg (**Fig.4.9A**). However, this induction was not seen in Crhr2 (**Fig.4.9B**) and Crhbp (**Fig.4.9C**).





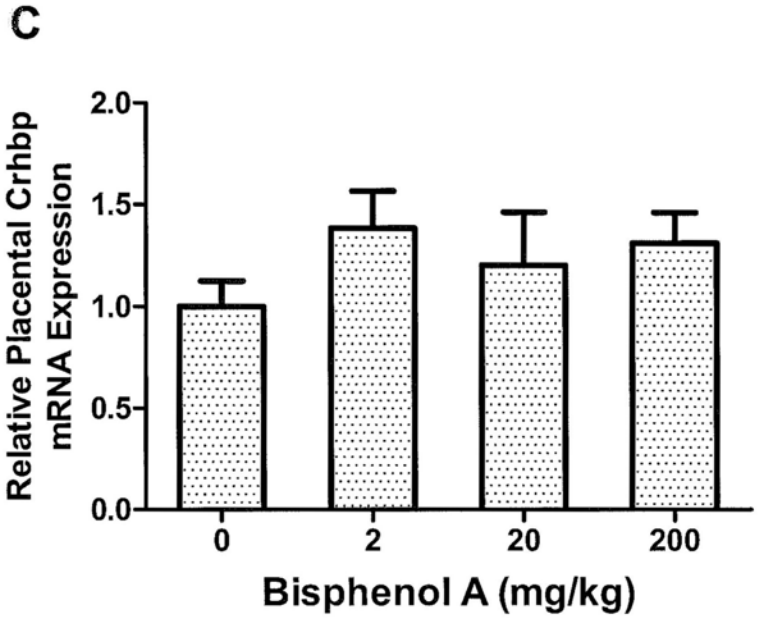


Fig.4.9 Placental Crhr1 expression was induced by BPA. ICR mice were gavaged with BPA. Total RNA was extracted from the placental tissue and Crhr1, Crhr2 and Crhbp expressions were quantified by real-time RT-PCR. Values are means ±SE, n=5. Mean with (*) is significantly ($p<0.05$) different from control.

4.3.4 Plasma Estrogen Concentration in Mice Treated with BPA

Previous study has shown that estrogen could regulate CRH expression (Stamatelou *et al.*, 2009), and endogenously produced CRH stimulates estrogen biosynthesis in human (Klimkova *et al.*, 2010). In addition, BPA could disrupt normal cell functioning by acting as an estrogen receptor agonist (Prasanth *et al.*, 2010). Estradiol is the most active estrogen. The plasma estradiol was significantly increased in the 20 mg/kg and 200 mg/kg BPA treatment group compared with the control group (**Fig.4.10**).

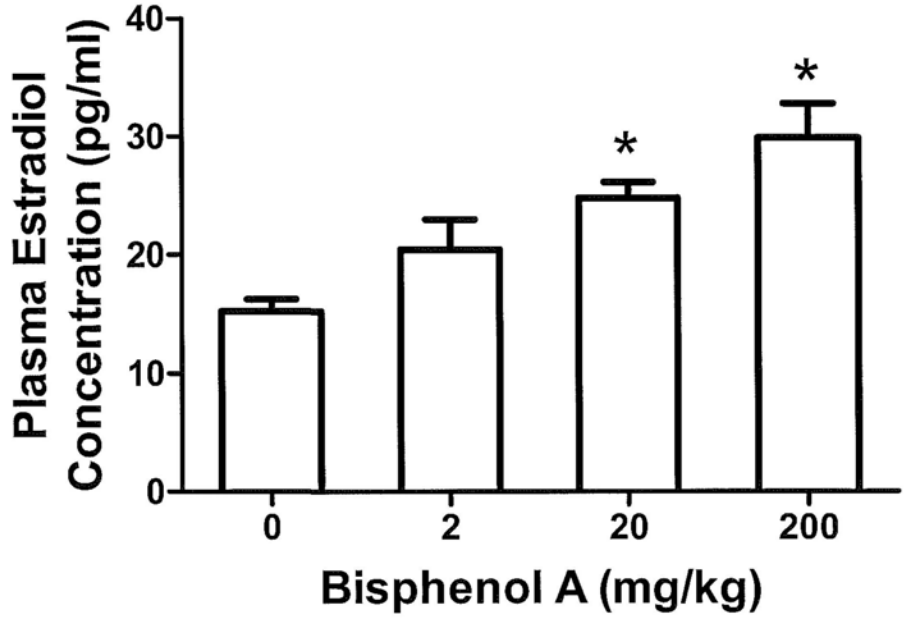


Fig.4.10 Plasma estrogen concentration in pregnant mice treated with BPA. Pregnant ICR mice were gavaged with BPA and sacrificed at E17. Blood was drawn and assayed for estradiol by ELISA. Values are means ±SE, n=5. Mean with (*) is significantly ($p<0.05$) different from Control.

4.3.5 Plasma Testosterone and Progesterone Concentrations in Mice Treated with BPA

Estradiol is converted from testosterone by aromatase. Previous study has demonstrated that serum testosterone level was escalated by BPA exposure in mice (Kawai *et al.*, 2007). In the present study, plasma testosterone concentration was increased in the group treated with 20 mg/kg and 200 mg/kg BPA (**Fig.4.11A**). It appeared that the increased estradiol seen in **Fig.4.10** was result of the increasing of testosterone.

20mg/kg BPA group displayed the highest mean value of progesterone. However, differences among the four treatment groups did not reach statistical significance (**Fig. 4.11B**).

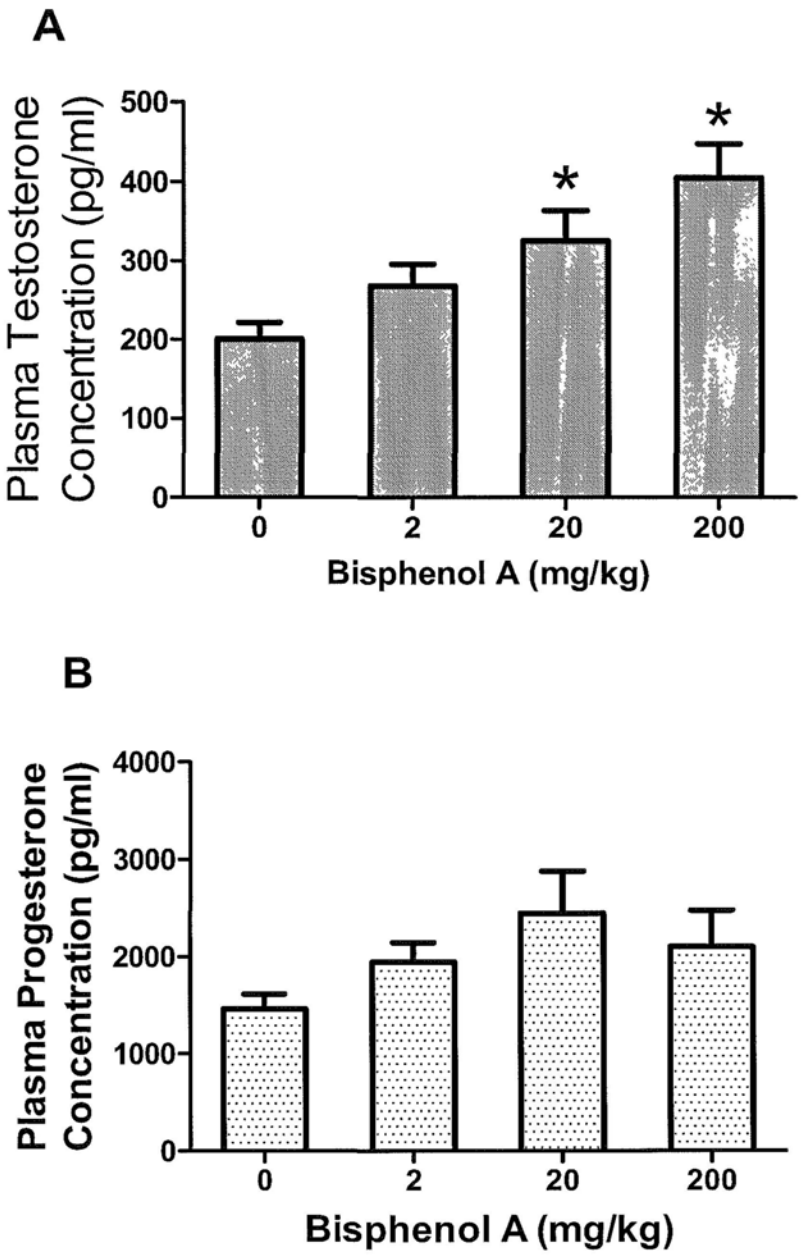
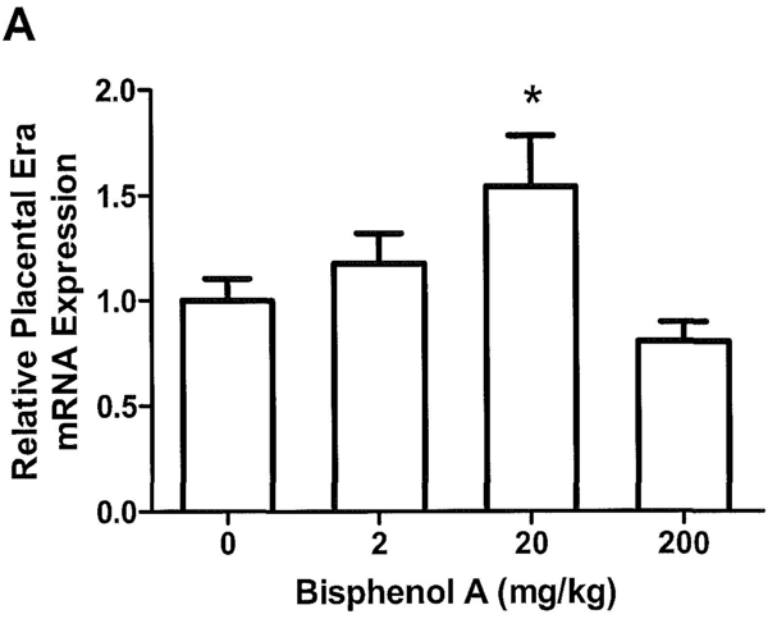


Fig.4.11 Plasma testosterone and progesterone concentrations in pregnant mice treated with BPA. Pregnant ICR mice were gavaged with BPA. Blood was drawn and assayed for testosterone (**Fig.4.11A**) and progesterone (**Fig.4.11B**) by ELISA. Values are means ±SE, n=5. Mean with (*) is significantly ($p<0.05$) different from Control.

4.3.6 Erα and Progesterone Receptor (Pgr) mRNA Expression in Mice Treated with BPA

Previous study has show that BPA could bind to the estrogen receptors and regulate the expression of estrogen responsive genes in placenta (Imanishi *et al.*, 2003). The Erα, Erβ and Pgr expression in mouse placenta, and the Erα mRNA level was the highest in 20 mg/kg treatment group and was significant increased than the control (**Fig.4.12A**), while the trend of Pgr mRNA expression was similar to that of Erα (**Fig.4.12B**). No change was observed in Erβ expression (Data not shown).



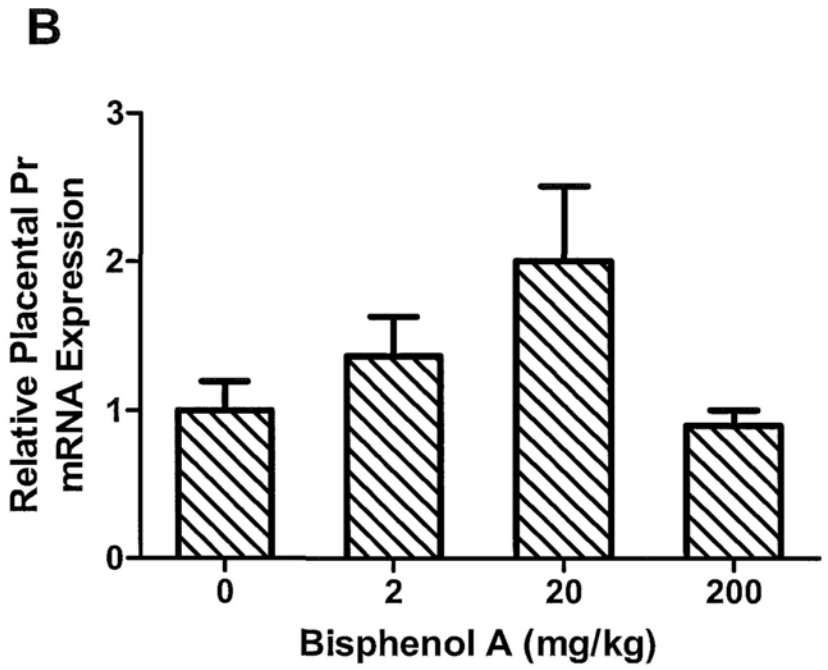


Fig.4.12. Placental Erα and Pgr expression by BPA treatment. ICR mice were gavaged with BPA. Total RNA was extracted from frozen placental tissue and Crhr1 expression was quantified by real-time RT-PCR. Values are means ±SE, n=5. Mean with (*) is significantly ($p<0.05$) different from Control.

4.4 Discussion

In cell culture study, we demonstrated that the activation of MAPK is important in *CRH* gene expression in human placental cells. We further demonstrated that oral administration of BPA increased the production of estrogen and Crh in maternal plasma of pregnant ICR mice. Induction of placental Crh expression and concurrent upregulation of Crhr1 expression were responsible for the increased of plasma Crh. Increased testosterone in circulation might be the cause of estrogen elevation. BPA appeared to increase the substrate of estrogen biosynthesis without affecting the enzyme aromatase.

Many signaling transduction pathways have been described in stimulating CRH expression. Activation of MAPK (Cheng & Handwerger, 2005b), PKA (Uh *et al.*, 2009), PKC (Yue *et al.*, 2008) can upregulate CRH expression through CRE binding. Various factors like PMA (Yue *et al.*, 2008), Ultraviolet B (UVB) (Zbytek *et al.*, 2006), cAMP (Uh *et al.*, 2009), etc. may stimulate CRH transactivity via these signal transduction pathways. The present study demonstrated that BPA induced CRH expression by activating PKA and PKC signaling pathways.

Placental CRH may participate in the maintenance of pregnancy and determining the length of human gestation (Torricelli *et al.*, 2006). Two distinct CRH receptors CRH-R1 and -R2, and one CRH binding protein called CRHBP have been identified in human placenta (Florio *et al.*, 2000). Plasma CRH-BP inactivates CRH to prevent its peripheral action in human (Hobel *et al.*, 1999b). Preterm birth patients have elevated CRH, lower CRH-BP, and a reduced CRH-BP/CRH dimmer complex index throughout the gestation period (Hobel *et al.*, 1999b). At the onset of labor, CRH-R1 mRNA is increased significantly by activated cAMP signaling cascade in myometrial tissue (Markovic *et al.*, 2007). CRH can inhibit spontaneous

contractions through CRH-R1 in women not undergoing labor (Zhang *et al.*, 2008). Besides, CRH-R1 antagonist drugs can relieve depression and anxiety symptoms (Holsboer, 2001). Our results indicated that BPA treatment caused increases of Crh and Crhr1 mRNA expressions in the placenta. These might contribute to the increased amount of Crh hormones in plasma.

Crh increases serum estradiol and corticosterone levels in hypophysectomized rats (Murase *et al.*, 2002). There is also a strong correlation between maternal CRH and estrogen concentrations throughout gestation. In the placenta, CRH stimulate estrogen production by increasing mRNA levels of CYP19A1 and HSD17B1, the key enzymes for estrogen synthesis (You *et al.*, 2006). BPA disrupts normal cell functioning by acting as an estrogen agonist and an androgen antagonist (Prasanth *et al.*, 2010). The present study showed that plasma estradiol was elevated dose-dependently by BPA treatment.

Testosterone can be converted to estradiol by aromatase. In pregnant ICR mice, the testosterone level in serum was elevated under BPA exposure, and the expression of Erα and β are increased at 5 and 13 weeks (Kawai *et al.*, 2007). In male mice, oral administration of BPA decreases the testosterone level dramatically (Takao *et al.*, 1999). Our result indicated that BPA increased plasma testosterone level at dosages 20 mg/kg and 200 mg/kg. The increased amount of testosterone may contribute to the increase of estrogen in plasma by providing ample amount of substrate for the synthetic reaction.

Progesterone is an important steroid hormone in maintaining human pregnancy (Hill *et al.*, 2010). Placental CRH can inhibit progesterone production (Yang *et al.*, 2006), which could trigger the initiation of labor in human term trophoblasts (Jeschke *et al.*, 2005). Progesterone synthesis could be stimulated by the

addition of estradiol in the human placenta minces (Shanker & Rao, 1997). Acute exposure of BPA could significantly decrease urinary progesterone excretion, and has no effect on estradiol levels (Berger *et al.*, 2008). Our result showed that the progesterone concentration was not altered upon BPA treatment.

BPA is able to change the mRNA level of nuclear receptor and disrupts the functions of placenta (Imanishi *et al.*, 2003). BPA exposure increases estrogen receptor expression in the brain at 5 and 13 weeks of the new born mice (Kawai *et al.*). Prolonged exposure of BPA could induce ERα and progesterone receptor expression in the fetal luminal epithelium of the endometrium and subepithelial stroma (Markey *et al.*, 2005)., In contrast, BPA could decrease progesterone receptor expression during early gestation (Berger *et al.*, 2010). In our study, the Ers and Prg expressions did not vary significantly. Both the Era and Prg mRNA reached the highest levels at 20 mg BPA/kg and then fall down at higher dosage, and the trend of Prg mRNA expression was similar to that of the progesterone level in maternal plasma.

In the present cell culture study, we demonstrated that BPA increased CRH transactivity and expression, and the regulatory element CRE in the *CRH* gene promoter was responsible for CRH activation through PKC/PKA signaling pathways. In the animal study, we demonstrated that BPA could increase plasma estrogen concentration and the expression of placental CRH.

CHAPTER 5
EXPRESSION OF CYP19 MRNA IS REDUCED BY THE SOY ISOFLAVONE GENISTEIN IN HUMAN PLACENTAL CELLS

5.1 Introduction

Genistein may alter the metabolism of estrogen *in vitro* and *in vivo*. Our lab previous has found that genistein inhibits cytochrome P450 CYP1A1 and CYP1B1 (Leung *et al.*, 2005). Genistein induces cell proliferation at an EC_{50} value of 32nM or inhibit aromatase at an IC_{50} value of 3.6 μM in human primary mammary fibroblasts and MCF-7 cells (van Meeuwen *et al.*, 2007). Biochanin A, the metabolic precursor of genistein, is more effective than genistein inhibiting aromatase activity and expression (Wang *et al.*, 2008b).

5.2 Result

5.2.1 CYP19 Promoter Utilization in First Trimester Cells

We employed real-time PCR to measure the alternate spliced *CYP19* mRNA species in placental cells. Differences in utilization of I.1-, II-, I.2-, I.3-, I.4-, and 2a-specific exons were quantified. PI.1 spliced mRNA was the dominating species, followed by PII as shown (**Fig.5.1**). This indicated that Exons PI.1 and II were controlling the transcriptional activity of *CYP19* in this cell type.

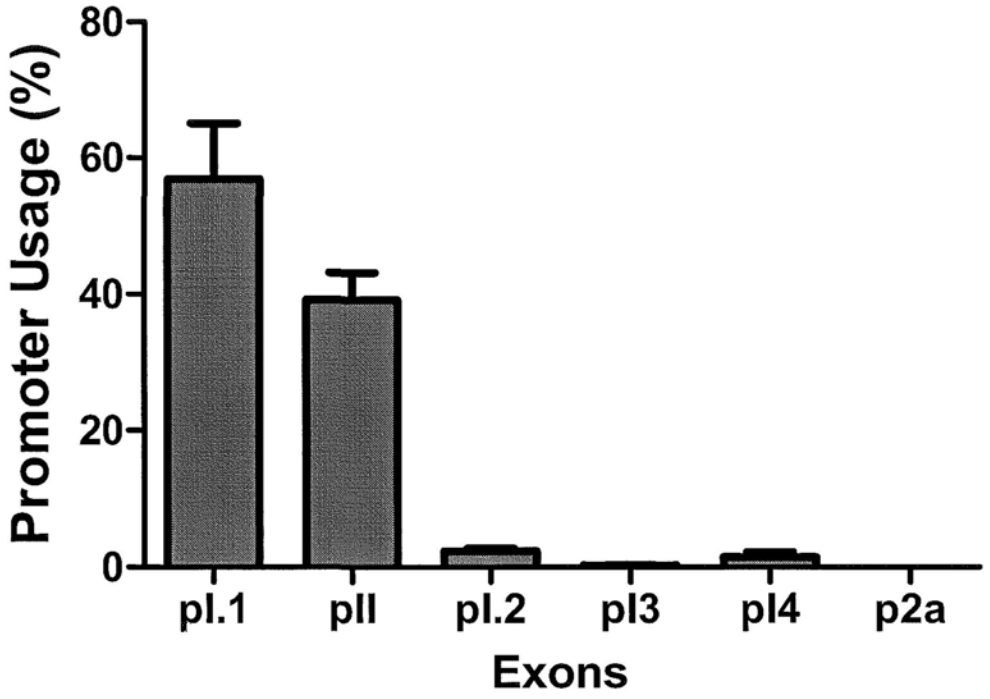
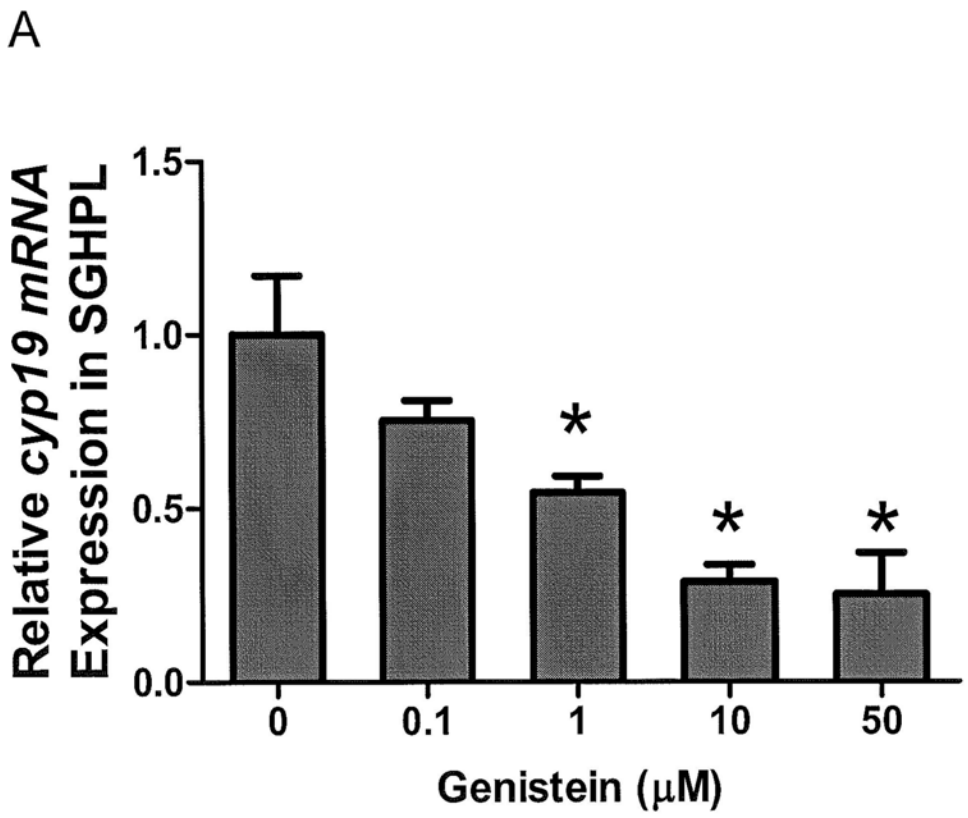


Fig.5.1. Exon I-specific promoter usage in SGHPL cells. Total RNA was extracted and reverse transcribed. Relative quantitative real time PCR using exon I-specific primers were performed. GAPDH was amplified as a house keeping gene. Promoter usage levels were calculated by using $2^{-\Delta\Delta}$CT method and expressed as a percentage of total CYP 19 expression. Values are means ± SE, n = 3.

5.2.2 Effect of Genistein on Aromatase and Promoter I.1 and II mRNA Expression in SGHPL Cells

Quantitative RT-PCR indicated that the mRNA abundance of aromatase coding region was dose-dependently suppressed by genistein (**Fig.5.2A**). Subsequent measurement of alternate spliced mRNA species showed significant decrease in exons II-specific mRNA (**Fig.5.2B**) where as exon I.1 specific mRNA was not down-regulated (**Fig.5.2C**). This indicated that the suppressive effect of genistein on CYP19 mRNA might act through the control of promoter II transcription.



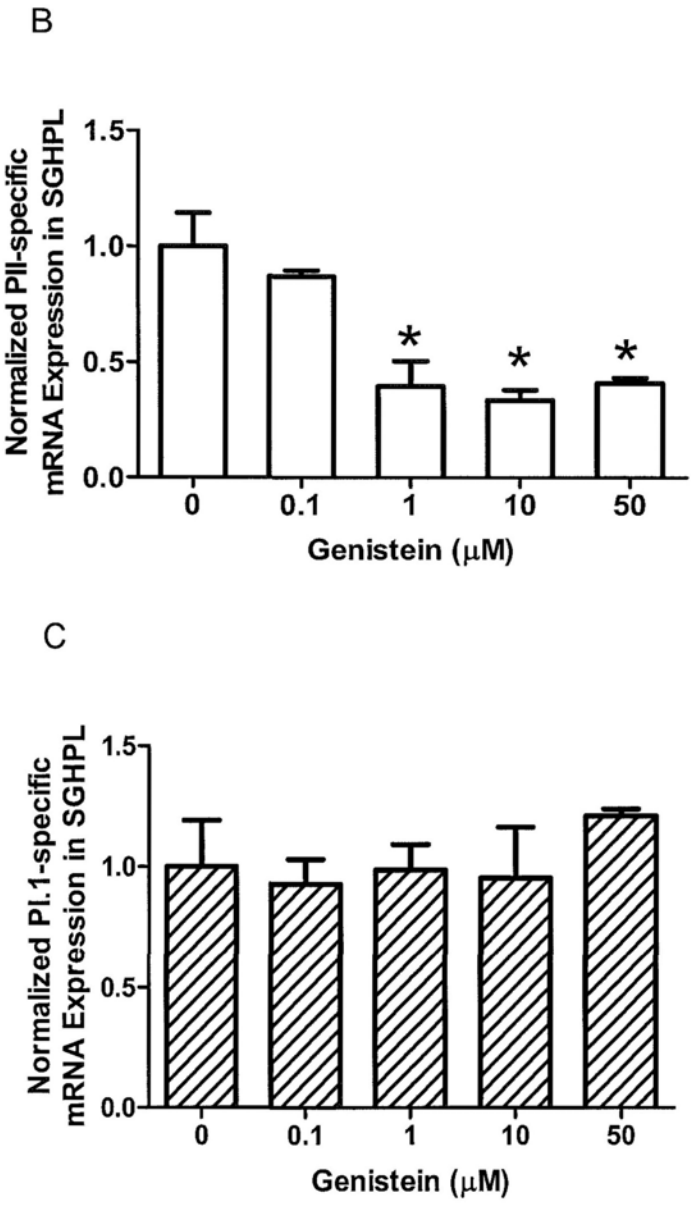
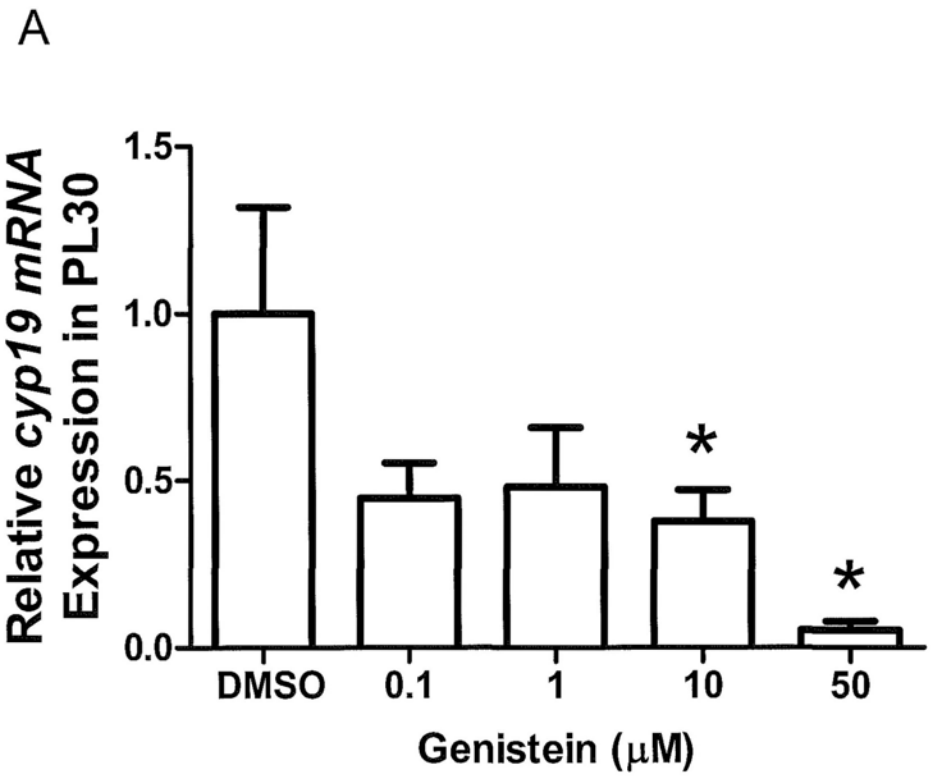


Fig5.2. Genistein inhibited *CYP19* gene expression and aromatase exon II mRNA expression in trophoblast SGHPL cells. SGHPL cells were seeded in 6-well culture plates and were treated with genistein. Messenger RNA was extracted and quantitated by real-time PCR. **Fig.5.2A**, genistein decreased *CYP19* expression. (*) represents a significant ($p<0.05$) increase in expression when compared with the control cultures. Genistein downregulated the alternate spliced mRNA driven by PII (**Fig.5.2B**) but not PI.1 (**Fig.5.2C**). Values are means ±SE, n=3.

5.2.3 Effect of Genistein on Aromatase and Promoter I.1 and II mRNA Expression in PL30

Similar results were observed in PL30 cells. Total *CYP19* mRNA expression was suppressed by genistein (**Fig.5.3A**). Exon II-specific mRNA was decreased (**Fig.5.3B**), but not exon I.1 specific mRNA (**Fig.5.3C**). This result was consistent with that observed in SGHPL cells.



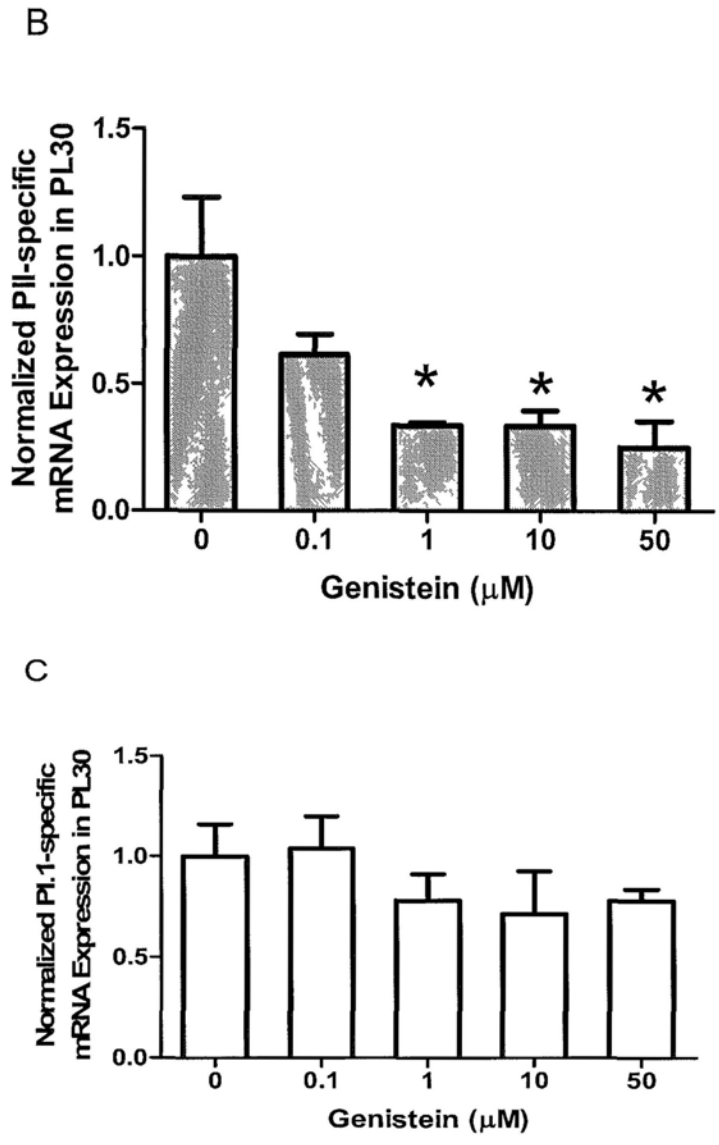
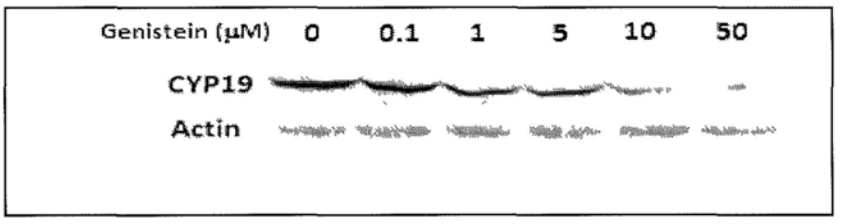


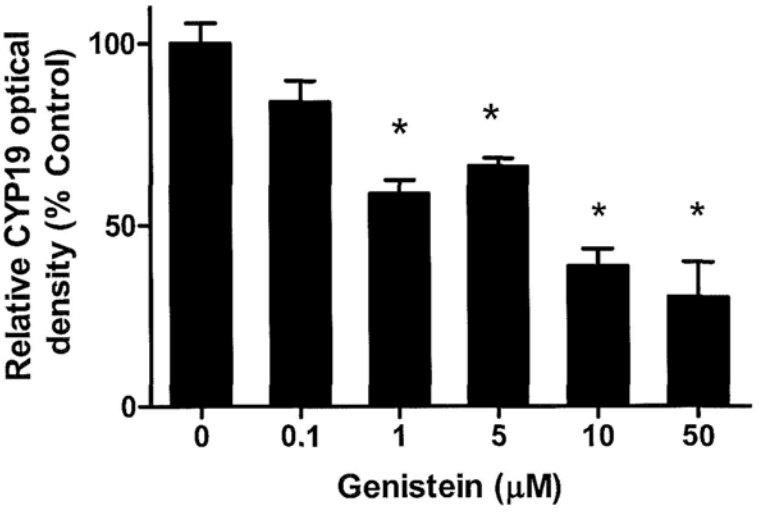
Fig.5.3. Genistein suppressed total *CYP19* mRNA expression, exon I.3- and II-specific mRNA expressions in trophoblast PL30 cells. PL30 cells were seeded in 6-well culture plates and were treated with genistein. Messenger RNA was extracted and quantitated by real-time PCR. **Fig.5.3A**, genistein decreased CYP19 expression. (*) represents a significant ($p<0.05$) increase in expression when compared with the control cultures. Genistein downregulated the alternate spliced mRNA driven by PII (**Fig.5.3B**) but not PI.1 (**Fig.5.3C**). Values are means ±SE, n=3.

5.2.4 Activation of Protein Kinases in SGHPL Cells by Treated with Genistein

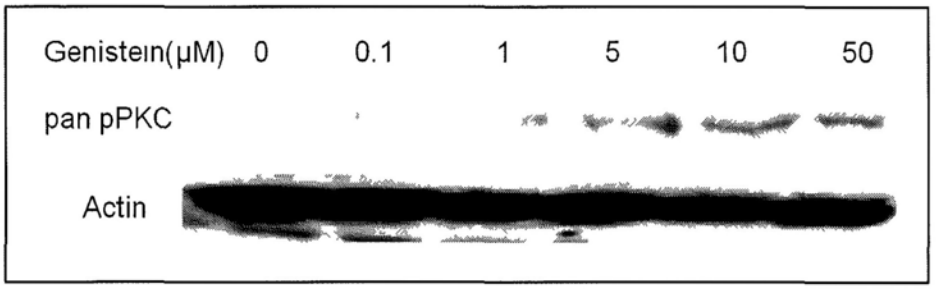
CYP19 mRNA expression was repressed by genistein, we also looked into the protein level of CYP19 and the possible regulatory mechanism involved. Because MAPKs were previously implicated in the regulation of CYP19 in placental cells, several signaling kinases were tested by western blot. Genistein activated some protein kinases concurrently with the reduction of the CYP19 protein (**Fig.5.4A**). Phosphorylated forms of pan PKC, P38 and ERK-1/2 were all induced as shown in **Fig.5.4B**. Optical density of the images illustrated that the amount of these activated kinases were significantly increased.

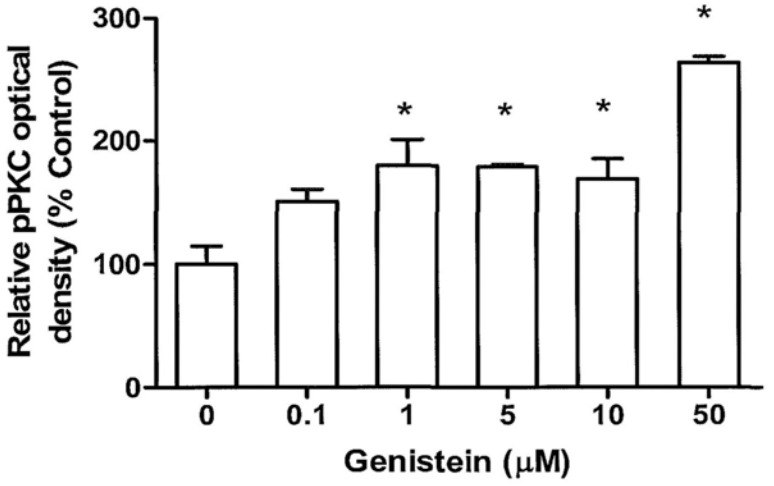
a



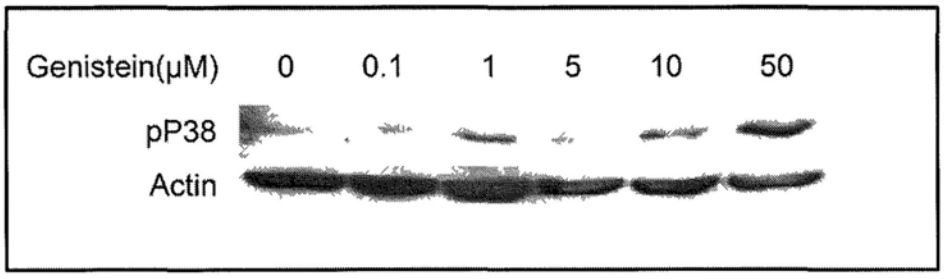


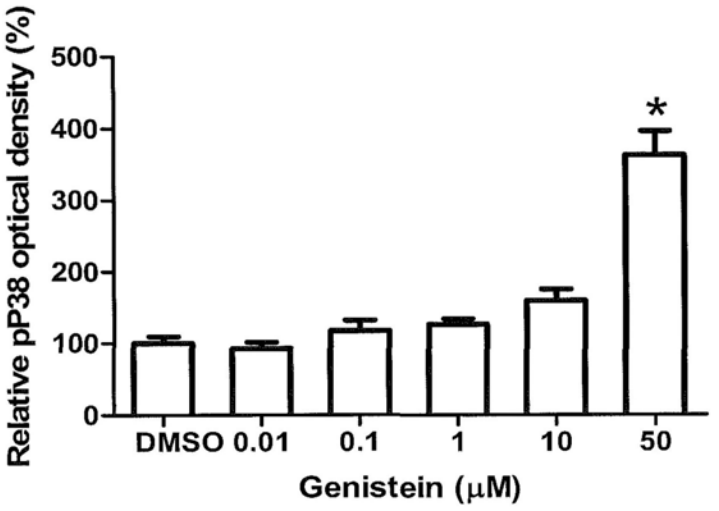
b


Genistein(μM) 0 0.1 1 5 10 50
pan pPKC
Actin

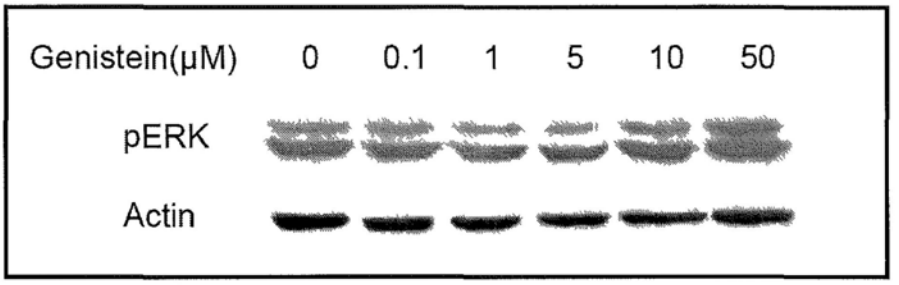

Relative pPKC optical density (% Control)
300
200
100
0
*
*
*
*
0 0.1 1 5 10 50
Genistein (μM)

c


Genistein(μM) 0 0.1 1 5 10 50
pP38
Actin


Relative pP38 optical density (%)
500
400
300
200
100
0
*
DMSO 0.01 0.1 1 10 50
Genistein (μM)

d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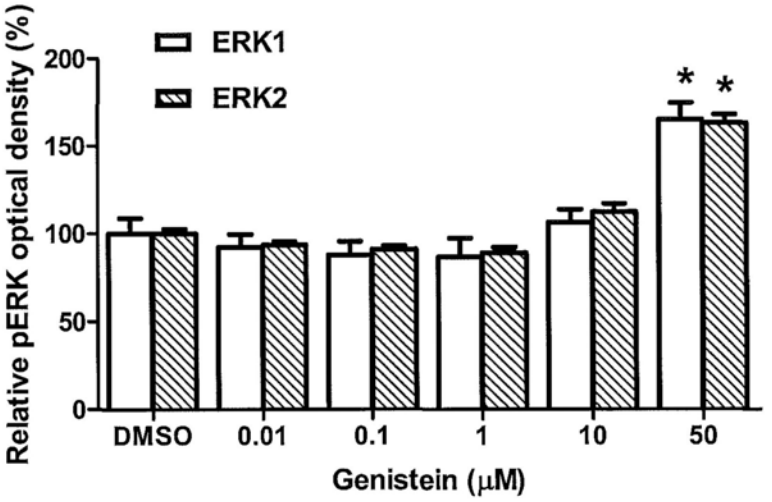
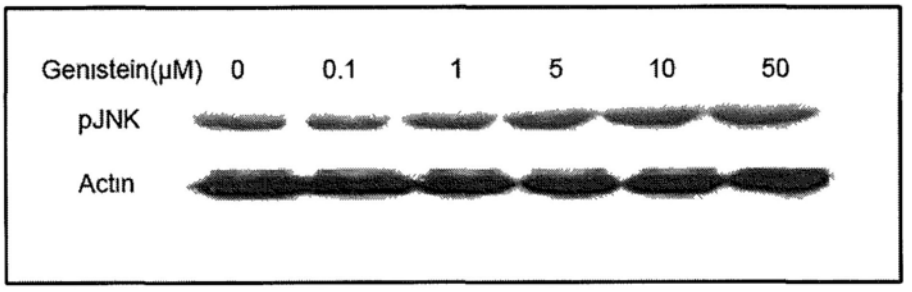
pERK
Actin


Relative pERK optical density (%)
200
150
100
50
0
ERK1
ERK2
* *
DMSO 0.01 0.1 1 10 50
Genistein (μM)

e


Genistein(μM) 0 0.1 1 5 10 50
pJNK
Actin

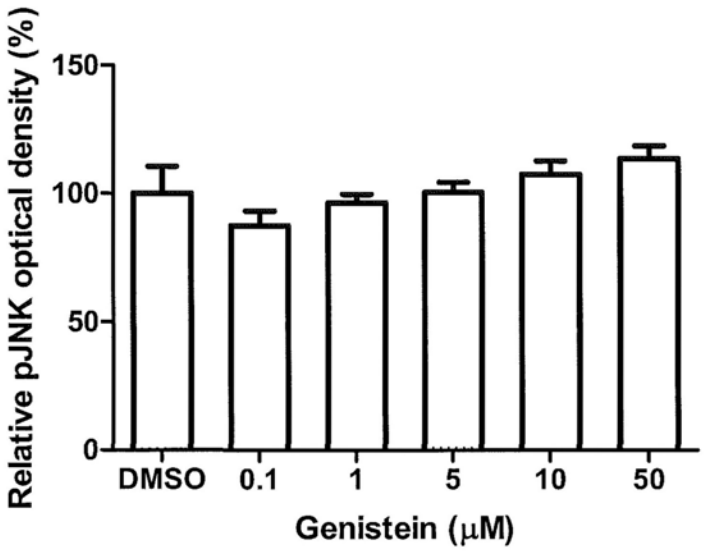


Fig.5.4. Immunoblot of signaling proteins in placental cells treated with genistein. Placental cells SGHPL were seeded in 6-well culture dishes and treated with genistein for 24 h. Protein expression of CYP19 (a), p-pan PKC (b), p-P38 (c), p-ERK-1/2 (d) and p-JNK (e), in cell lysates were determined by western analysis. The images in upper panels are representations of three independent experiments, and figures in lower panels are the optical density readings of the proteins. Values are means ±SE, n=3. Means with (*) is significantly ($p<0.05$) different from control.

5.2.5 Inhibiting PKC Reverts *CYP19* Expression Suppressed by Genistein in SGHPL Cells

Since genistein dose-dependently increased pPKC, p38 and pERK, we further verified these interactions by inhibiting the kinases. In cultures treated with 10 μM genistein and pre-treated with the inhibitor of PKC, the *CYP19* expression was downregulated (**Fig.5.5**). In contrast, the inhibitors of ERK and p38 could only slightly increase CYP19. These results complemented the data presented in **Fig.5.4**, and verified that genistein might suppress aromatase expression through activation of PKC.

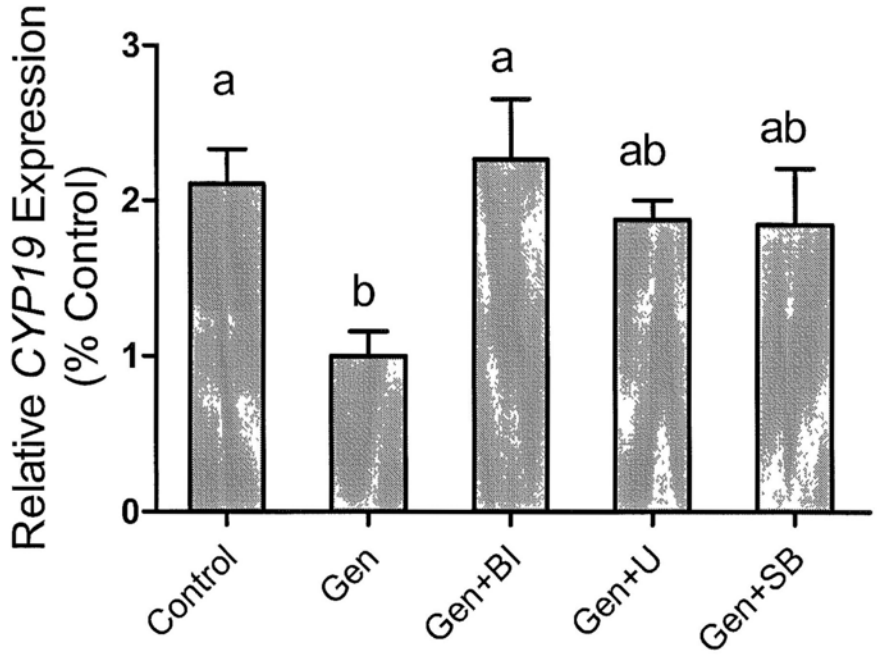


Fig.5.5. Verification of PKC, ERK and p38 involvement in genistein-regulated *CYP19* expression. SGHPL cells were seeded in six-well culture dishes and pre-treated with the PKC inhibitor bisindolyl-maleimide I (BI), the ERK inhibitor U0126 (U), and the P38 inhibitor SB203580 (SB), followed by 10μM genistein treatment. Messenger RNA was isolated and quantified for CYP19 expression after 24 h of incubation. Means labeled (*) represents a significant ($p < 0.05$) increase in expression or activity when compared with the control. Values are mean±S.E., n = 3. Data represents one of two independent experiments with comparable results.

5.2.6 Genistein Increased CREB Binding In Promoter II DNA Fragment

Nuclear extracts isolated from SGHPL cells showed a distinct band of DNA-protein complex. These complexes diminished using antibodies against CREB-1 or C/EBPβ. The intensity of the bands in samples treated with 1 and 10 μM genistein was increased compared with the control. On the other hand, the intensity of the major bands diminished when co-treated with the HDAC inhibitor Trichostatin A (TSA).

This experiment demonstrated that genistein stimulated the binding of transcription factor CREB (43kDa) and C/EBPβ (45kDa) to CRE with HDAC, to form a repressor complex in the promoter II of CYP19.

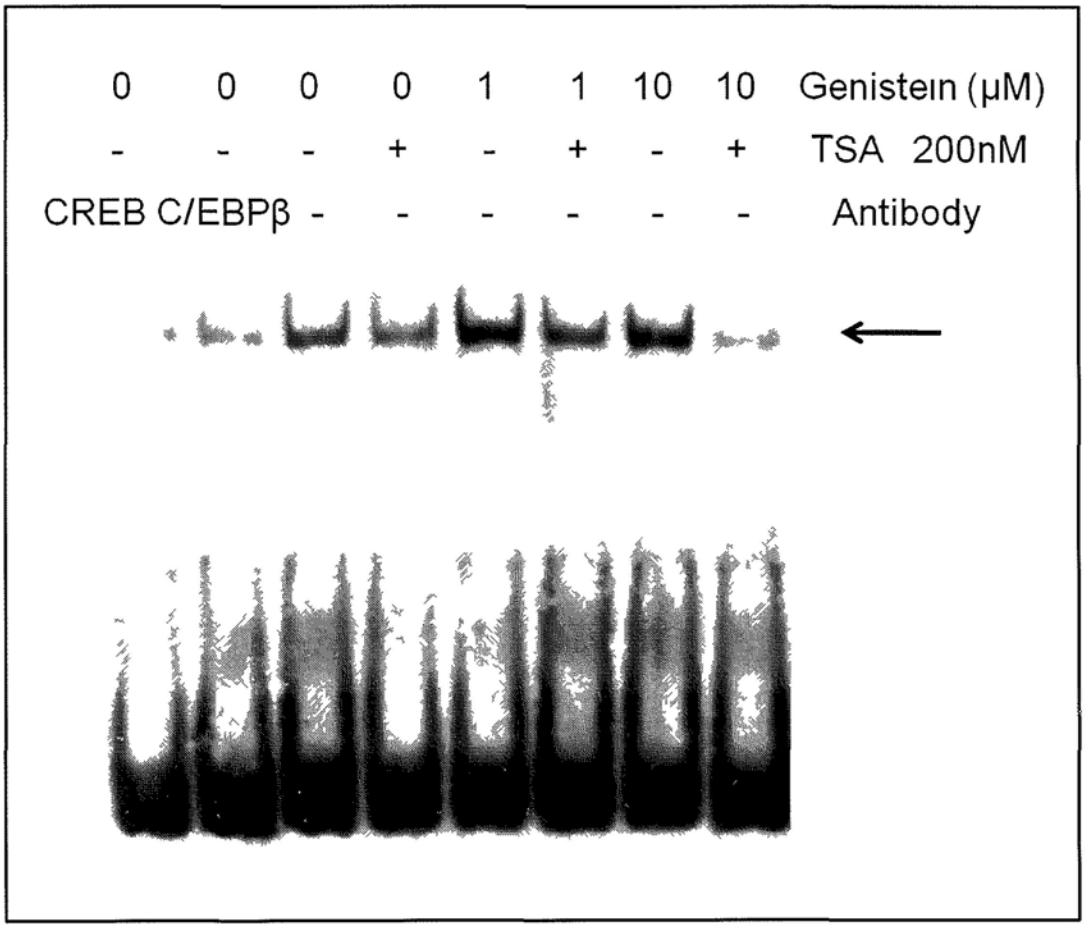


Fig.5.6 CREB and C/EBPβ were involved in genistein-induced CYP19 transcription. Nuclear extracts were prepared for EMSA from SGHPL cells treated with genistein. Samples were treated with genistein at 0, 1, 10 μM; with (+) or without (-) 200nM Trichostatin A (TSA) The autoradiograph is a representation of two independent experiments.

5.3 DISCUSSION

Genistein is a phytoestrogen isolated from soybeans, which are staple food to Asian population; however, the potential safety issues in reproduction have not been fully resolved. In the present study, effect of genistein on placental CYP19 expression was investigated in human trophoblast cell lines SGHPL and PL30. These cells were transformed from normal aborted placenta. The phytoestrogen blocked aromatase expression in these cells in low concentrations. In order to delineate the mechanisms involved, EMSA and Western blotting were performed. Results indicated that the suppression of CYP19 transactivation occurred in the promoter II region, and it could be related to increase in CRE binding. By tracing the signaling pathway, it appeared that the protein kinase PKC was activated. This illustrated that genistein might suppress CYP19 expression in normal placental cells through PKC. Previous study has shown that promoter I.1 is the major regulator of *CYP19* expression in human placental tissues; nevertheless, promoter II contributes a minor but significant part of the regulation (Ghai *et al.*, 2010). Since the estimated IC_{50} value for PII-specific expression is within physiologically achievable concentration in the current study, these findings might raise some concern in soy consumption during pregnancy.

The majority of aromatase transcripts are encoded by exon I.1 in placental trophoblast (Kamat *et al.*, 1998). Among all the promoters, Exon II is tightly related with the aromatase expression in gestation (Means *et al.*, 1991). Promoter II is the major promoter for expressing aromatase both in ovary and corpus luteum (Means *et al.*, 1991). In our previous study, we have verified the active promoter PI.1 and PII of CYP19 in JEG-3 cells and proven that the utilization of promoter I.1 was ten times

higher than promoter II (Wang & Leung, 2007). Partial shift in the promoter usage was observed in different stage of gestation in buffalo. Most transcripts driven by promoter I.1 in early stages, but the placenta promoter II was predominant in the mid-gestation period (Ghai *et al.*, 2010). In our experiment, real-time PCR assay result showed that PI.1 possessed 56.8% of promoter usage, followed by PII of 39% usage. This indicated that both PI.1 and II were controlling the transcriptional activity of CYP19 in the first trimester trophoblast cell line.

In aromatase excess syndrome patients, aromatase could be overexpressed in many tissues (Shozu *et al.*, 2003). As increased endometrial cancer risk has been linked to over-expression of aromatase, aromatase inhibitors become potential therapeutic agents (Bulun *et al.*, 2005). Aromatase mRNA has been markedly raised by increased promoter activity in breast cancer tissue compared with the normal breast tissue (Utsumi *et al.*, 1996). Our study indicated that the alternate spliced mRNA species of PI.1 were nearly 3000 to 4000 fold higher in JEG-3 and Bewo cells than those in SGHPL and PL30 cells, but only 5 to 10 fold differences in exon II-specific mRNA.

In various stages of placental development, exogenous stimulation may activate disparate apoptotic pathways (Crocker *et al.*, 2001). *CYP19* expression can also be regulated by differential methylation of promoters, and PII is hypomethylated both in gestation and term placenta (Ghai *et al.*, 2010). Interestingly, our results indicated that the alternate spliced mRNA of promoter II, but not promoter I.1, was suppressed by genistein dose-dependently in SGHPL cells. Similar result was also observed in a third trimester trophoblast cells PL30.

Contradicting results have been reported for genistein's action on aromatase. The amount of estrone (E1) is significantly reduced by genistein in MCF-7 cells

(Brooks & Thompson, 2005). Reduced aromatase activity has also been demonstrated in the ovarian and uterine tissues of genistein-treated rats (Iasinskaia & Rozanov, 2001). On the contrary, enhancing effect of genistein on aromatase expression has been reported (Lyou *et al.*, 2008). Similar potentiating effects have been observed in human endometrial stromal cells (Edmunds *et al.*, 2005), H295R human adrenocortical carcinoma cells (Sanderson *et al.*, 2004) and in human colon cancer epithelial cells (Fiorelli *et al.*, 1999). Our results illustrated that genistein was able to suppress both aromatase mRNA and the specific mRNA of promoter I.3/II in the first and trimester trophoblast cell lines while no effect was seen on JEG-3 and Bewo cells (data not shown).

Our laboratory has shown that genistein can activate several protein kinases, including PKC, P38, ERK-1/2 in HepG2 cells (Ye *et al.*, 2009). Current results also demonstrated that several protein kinases might be involved in genistein's action. pan-PKC was most likely pathway mediating the suppression.

The present study illustrated a suppression pathway of genistein on estrogen synthesis, contrasting to up-regulating the expression of CYP19 in HepG2 cells (Ye *et al.*). Previous study has uncovered that binding of C/EBP or CREB to the element at -211/-197 bp in aromatase promoter II are responsible for aberrant aromatase expression. C/EBP isoforms differentially display a stimulatory or inhibitory effect on the aromatase expression in endometriosis or normal endometrium (Yang *et al.*, 2002).

Related studies have shown that increased C/EBPα and CREB binding to the 211/197-bp CRE could form a co-integrator and activate the transcriptional activity of promoter II of aromatase in endometriotic cells, whereas increased C/EBPβ and CREB binding to the same site in promoter II will function as a co-inhibitor with

HDAC in endometrial cells (Yang *et al.*, 2002). The present study indicated that genistein could stimulate CREB and C/EBPβ binding to form a co-repressor.

In this study, we demonstrated that genistein suppressed CYP19 mRNA and protein expression in the first trimester cell SGHPL. This suppression was attributed to the reduced promoter II-driven transactivation.

CHAPTER 6
EFFECT OF GENISTEIN ON PLACENTAL CORTICOTROPIN-RELEASING HORMONE AND ESTROGEN METABLISM IN VITRO AND IN VIVO

6.1 INTRODUCTION

Corticotropin-releasing hormone (CRH) is a peptide hormone that expresses in central nervous system, adrenal medulla, sympathetic ganglia, etc. Humans share the same amino acid sequence homology of CRH with rodents (Majzoub, 2006). Its interactive receptors are G-protein-coupling CRH Receptor 1 and -Receptor 2. CRH acts on the hypothalamic-pituitary-adrenal (HPA) axis, and several physiological functions are regulated by this hormone, such as fetal organ development, glucose metabolism, immune response, etc (Majzoub, 2006).

Placenta also has the capacity of producing CRH. It is secreted at late gestational period, and its function is not completely known. Studies have shown that it may be involved in the initiation of parturition and fetal development (Korebrits *et al.*, 1998; McLean & Smith, 1999; Wadhwa *et al.*, 2004). The regulation of placental CRH secretion is also different from that of CRH in brain. Cortisol inhibits hypothalamus CRH secretion, whereas placental CRH appears to be stimulated by the same hormone (Robinson *et al.*, 1988).

Some diseases are linked to abnormal CRH production. Steroid hormones can control CRH production at the transcription level (Ni & Nicholson). For example,

prolactin potentiates forskolin-stimulated CRH transcription through ERK/MAPK pathway in hypothalamic cell cultures (Blume *et al.*, 2009). Other study has shown that estrogen is also a modulator for placental CRH expression (Ni *et al.*, 2002). Estrogen represses placental *CRH* gene expression through an ER alpha-mediated mechanism in JEG-3 cells (Ni *et al.*, 2002). CREB and ERK 1/2 phosphorylation can be controlled through activated CRH receptors (Kageyama *et al.*, 2007).

CRH in circulation is able to activate different signaling pathways in tissues. In pituitary corticotrope cells, CRH activates ERK1/ERK2 pathway to induce ACTH synthesis (Tsukamoto *et al.*, 2010). In rat microglia, CRH induces rapid phosphorylation of ERK and p38 kinases (Wang *et al.*, 2003). The MEK inhibitor, PD098059, could dose-dependently inhibit placental *CRH* gene expression (Cheng & Handwerger).

Genistein shares some common structure with the hormone estrogen. Despite the similarity, the relative binding affinity of genistein to estrogen receptor (ER)-α is only 0.05-1% of the binding affinity of 17β-estradiol (Shutt & Cox, 1972). In contrast, its binding affinity to ER-β is greater than that of estrogen. It is suggested that genistein may act as a selective estrogen receptor modulator (SERM) (Kuiper *et al.*, 1998). Epidemiologic studies have associated the consumption of isoflavonoids with a lower incidence of Cardiovascular disease (Adlercreutz *et al.*, 1993). In normal postmenopausal women, consuming whole soy foods with 60 mg isoflavones per day may help alleviating several key clinical risk factors for CVD (Scheiber *et al.*, 2001).

However, consumption of high dosages of genistein may pose some potential health threats. Previous studies have shown that genistein treatment could increase MCF-7 xenograft transplant in ovariectomized athymic mice (Hsieh *et al.*, 1998), and reduces the chemotherapeutic potential of the ER antagonist tamoxifen and the

aromatase inhibitor letrozole (Ju *et al.*, 2002; Ju *et al.*, 2008). Genistein may also induce abnormal estrous cycles, early reproductive deterioration in mice (Jefferson *et al.*, 2007). Placenta is a potential target organ for genistein's action during gestation in rodents (Soucy *et al.*, 2006).

The dietary relevance of the genistein dosages in human subjects has been reasonably established over many years of research. The major metabolite in women is in the glucuronide form, and the aglycone genistein only constitutes about 25% of total genistein present in plasma (Zhang *et al.*, 2003). However, previous study has illustrated that genistein at a concentration of 5000 ppm administered in mice would give rise to plasma concentration comparable to normal consumption in human (Hsieh *et al.*, 1998).

Lipopolysaccharide (LPS) is an endotoxin found in the cell membrane of gram-negative bacteria. These microbes are mostly human pathogens and causing inflammation upon infection. Previous study has shown that LPS may induce premature birth in rodents. In the present study, effect of genistein on LPS-induced preterm birth was investigated.

Given the endocrine disruptive potential of genistein, the current study was designed to explore the toxicological effect of this soy isoflavone in gestation and the dosage was chosen within the range of 5000 ppm.

6.2 Results of In Vitro Study

6.2.1 Transactivity of CRH Promoter in JEG-3 Cells

In order to investigate the effect of genistein on *CRH* transcriptional activity, reporter gene assay was carried out. A biphasic effect of genistein was observed on the *CRH* promoter transactivity in JEG-3 cells. Genistein induced the transactivity of CRH at low dosage while suppressed the transactivity at higher dosage (**Fig.6.1**).

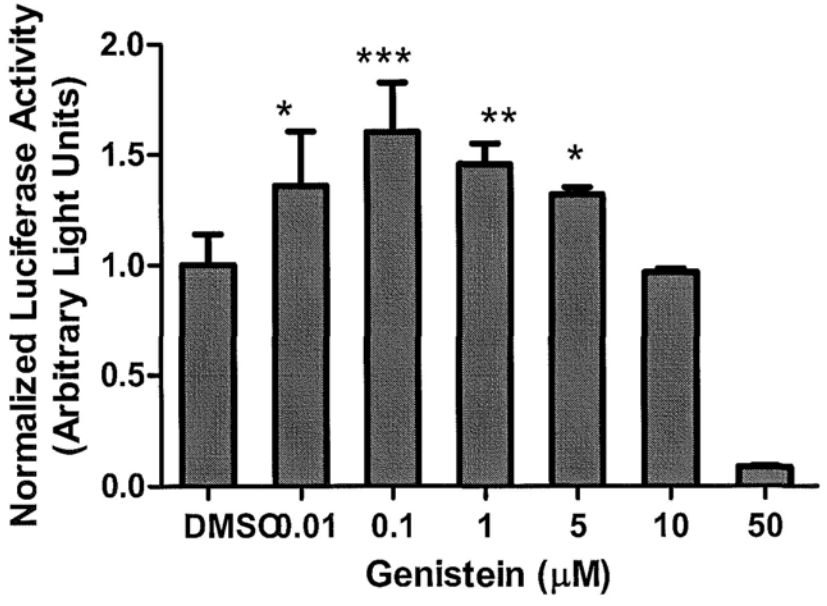


Fig.6.1 Genistein increase *CRH* promoter driven luciferase activity in JEG-3 cells. JEG-3 cells were seeded in 24-well plates and maintained in RPMI 1640 medium. The cultures were switched to serum-free medium and transfected with *CRH*-promoter driven reporter plasmid. The cells were then treated with genistein. Activities of luciferases were determined in the cell lysate. Values are means ± SEM, n=3. (*) Means were significantly ($p<0.05$) different from that of the control (0 μM). pGL3 basic was the negative control without CRH promoter insert.

6.2.2 Genistein Increased CRH mRNA Expression in JEG-3 Cells

JEG-3 cells were treated with genistein for 24 h. As shown in **Fig.6.2**, CRH mRNA expression was dose dependently increased by genistein while the expression was suppressed by 50 μM genistein in JEG-3 cells. The result was consistent with the dual luciferease assay, suggesting that genistein enhanced CRH expression in low dosages and suppressed CRH at higher dosages.

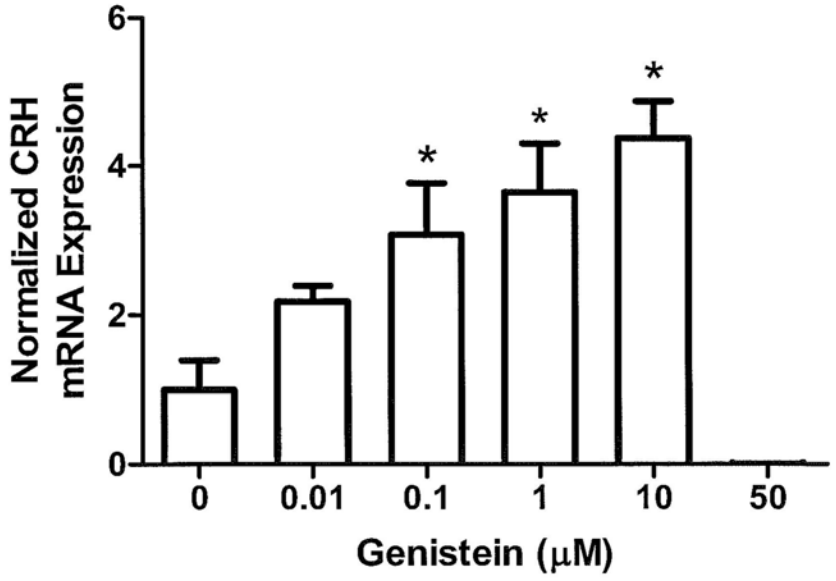


Fig.6.2 The effect of genistein on CRH mRNA expression in JEG-3 cells. JEG-3 cells were seeded in 6-well plates and maintained in phenol red free RPMI 1640 medium supplemented with 10% charcoal dextran-treated serum. Cells were treated with genistein for 24 h. The amount of CRH mRNA was determined by relative quantitative real time PCR and was normalized by GAPDH mRNA. Values are means ±SEM, n=3. (*) Means values were significantly ($p<0.05$) different from that of the control cultures.

6.2.3 Genistein Increased ERK1/2 Activation

As the mRNA experiments demonstrated that CRH was up-regulated by genistein, Subsequent experiment was designed to verify the signaling pathway. Protein expressions of CRH, p-PKC, p-ERK-1/2, p-P38, p-MEK and p-JNK were determined by western blot analysis. The CRH protein level in placenta cells was slightly increased (**Fig.6.3A**). Optical density of the images illustrated that the amount of the phosphorylation forms of ERK1/2 kinases were significantly increased (**Fig.6. 3B**).

6.2.4 Genistein Increased CREB Binding in Promoter Fragment of CRH

The CRE binding site (-432/-440) was identified by TFSEARCH, a software available on the web (http://www.cbrc.jp/research/db/TFSEARCH.html). A 38bp probe containing the CRE site was synthesized and labeled with DIG. EMSA was performed using nuclear extracts isolated from JEG-3 cells. A major complex was seen when the nuclear extracts incubated with the DIG-labeled CRE oligonucleotide. Genistein treatment could increase the intensity of the band (**Fig.6.4**). The specificity of the band was competed off upon incubating with the antibody of CREB.

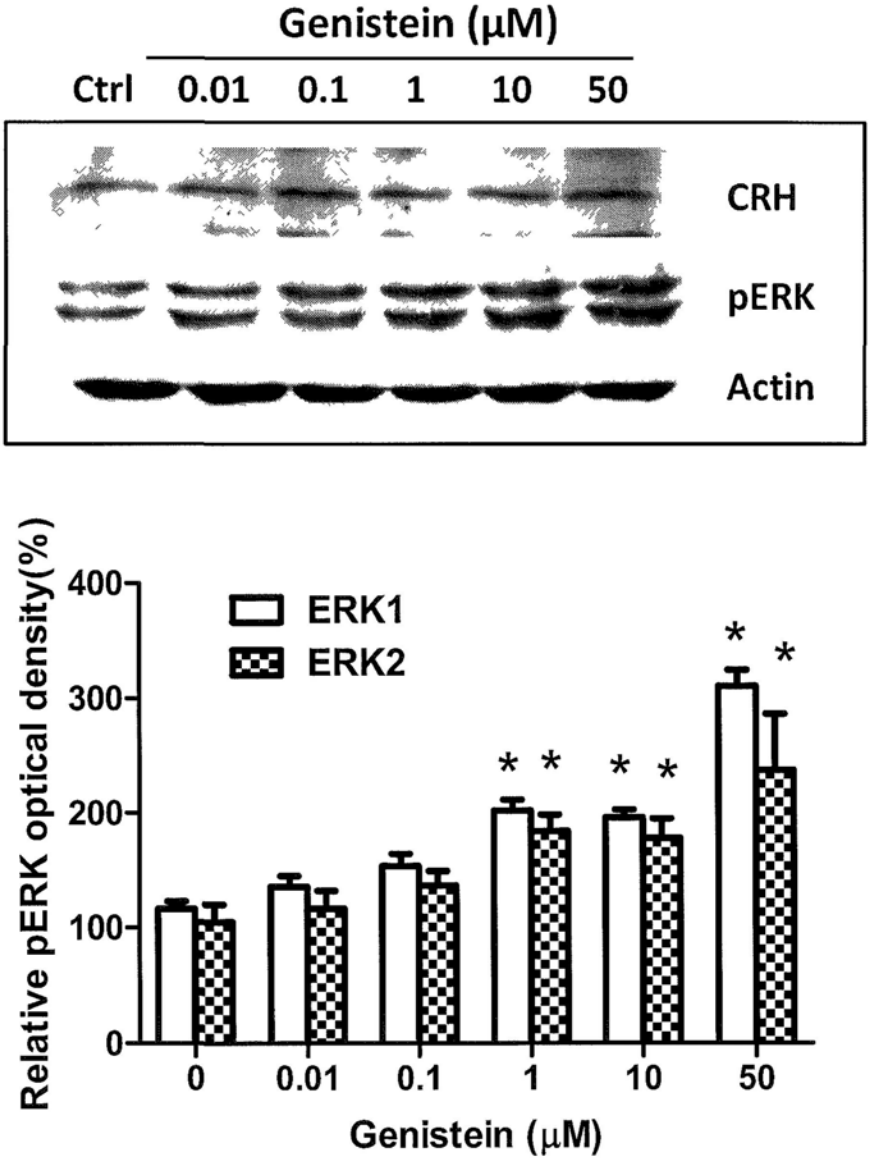


Fig.6.3 Immunoblot of p-ERK1/2 in placental cells treated with genistein. JEG-3 cells were seeded in 6-well culture dishes and treated with genistein for 24 h. Protein expression of CRH, pERK in cell lysates was determined by western blot analysis. The images in **Fig.6.3A** are representations of three independent experiments, and figures in **Fig.6.3B** are the optical density readings of the proteins. Values are means ±SE, n=3. Means labeled with (*) is significantly ($p<0.05$) different from control.

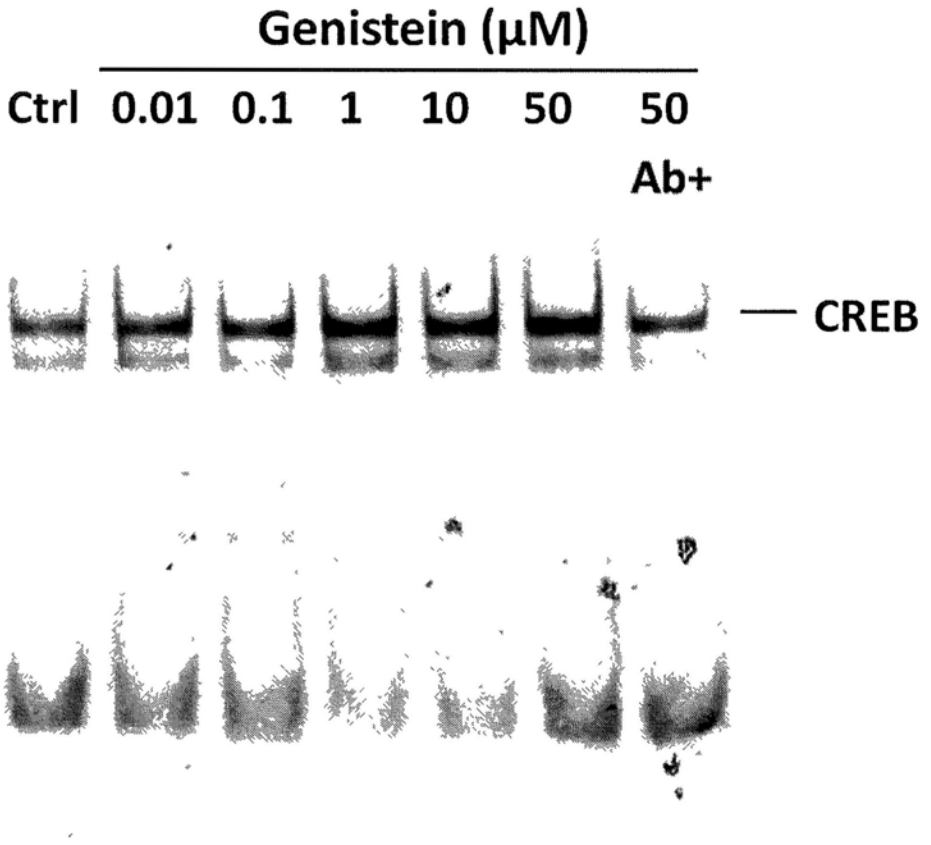


Fig.6.4 CREB was involved in genistein-induced CRH transcription. After identified the possible interacting segment of the CRH promoter, binding activity in this segment was investigated. Nuclear extracts were prepared from JEG-3 cells treated with genistein and EMSA was performed. Lanes 1–6 are samples treated with genistein at 0, 0.01, 0.1, 1, 10 and 50 μM; lane 6 is a sample treated with 10 μM genistein incubating with CREB antibody. Data represents one of two independent experiments with comparable results.

6.3 Result of Animal Study

6.3.1 Effect of Genistein and LPS on Early Delivery

Genistein could induce early delivery in mice sensitized with LPS. No animal gave birth early in the Control group. LPS alone and administered together with genistein significantly induced early delivery and an increasing trend was correlated with increased genistein dosages (**Fig.6.5**).

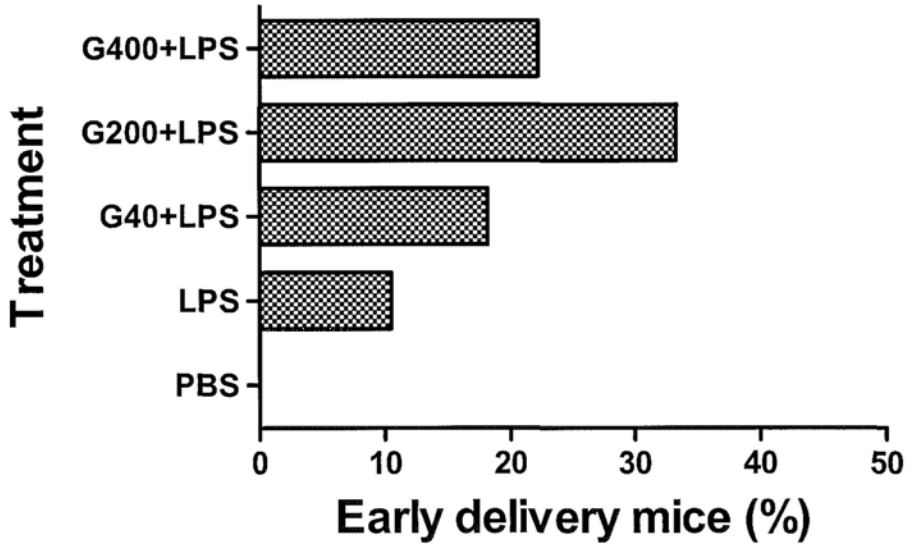
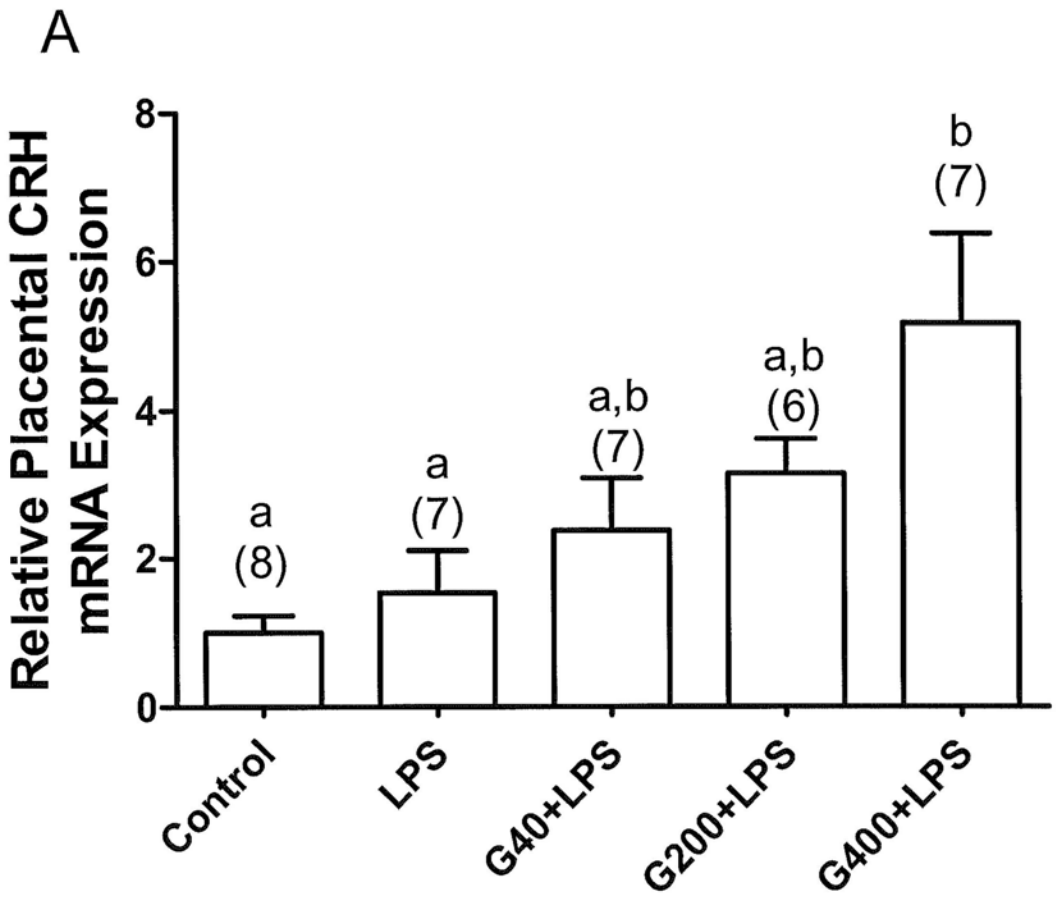


Fig.6.5 Contingency chart for early delivery data. Pregnant ICR mice were gavaged with genistein and injected with LPS. Early delivery rate in each group was recorded. Groups were labeled as: **Control –** mice received PBS; **LPS** – mice injected with lipopolysaccharide; **G40+LPS** – mice received genistein 40 mg/kg wt & LPS injection; **G200+LPS** – mice received genistein 200 mg/kg wt & LPS injection; **G400+LPS** – mice received genistein 400 mg/kg wt & LPS injection. Data was analyzed with Chi-Square test for trend.

6.3.2 Effect of Genistein on Placental CRH mRNA Expression in Mice

As genistein could introduce early delivery in LPS-treated mice, the hormone CRH which could be responsible for early delivery was examined. Messenger Expression of CRH in the placental tissue of mice co-treated with genistein at 400 mg/kg and LPS (G400+LPS) was elevated (**Fig.6.6A**). However, this induction was not seen in either LPS (**Fig.6.6A**) or genistein (**Fig.6.6B**) when administered alone.



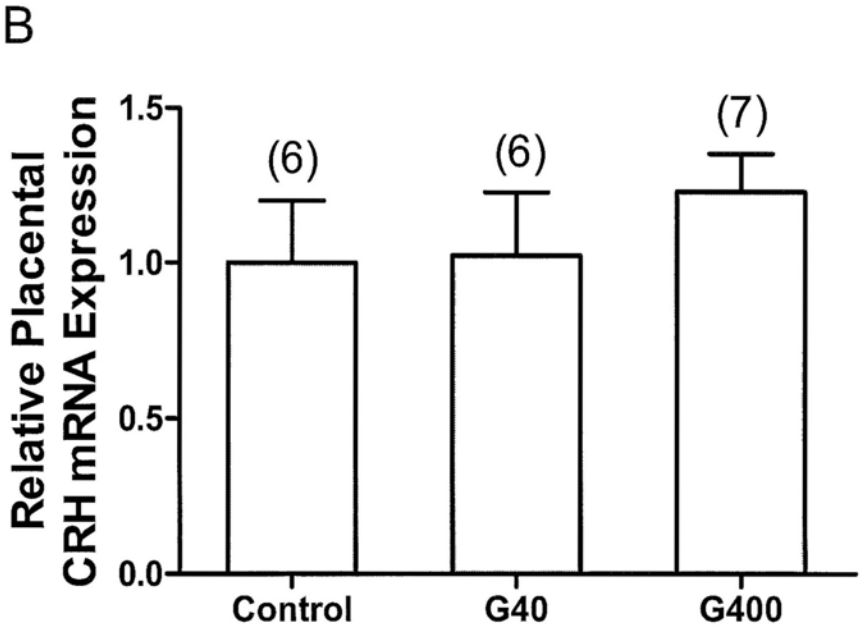


Fig.6.6 Effect of genistein on placental CRH mRNA expression in mice. Pregnant ICR mice were gavaged with genistein and injected with LPS (**Fig.6.6A**). Total RNA was extracted from frozen placental tissue and CRH expression was quantified by real-time RT-PCR. A separate experiment was performed on mice gavaged with genistein only (**Fig.6.6B**). Values are means ± SEM; the numbers of samples are given in the parentheses. Means labeled with various letters are significantly different.

6.3.3 Plasma CRH in Maternal Circulation

Since mRNA of placental CRH increased, the hormone amount in maternal circulation was also determined. Plasma CRH in the 400 mg genistein/kg co-treated group was significantly greater than that of the control (**Fig.6.7**). This pattern was highly consistent with the mRNA expression of placental CRH.

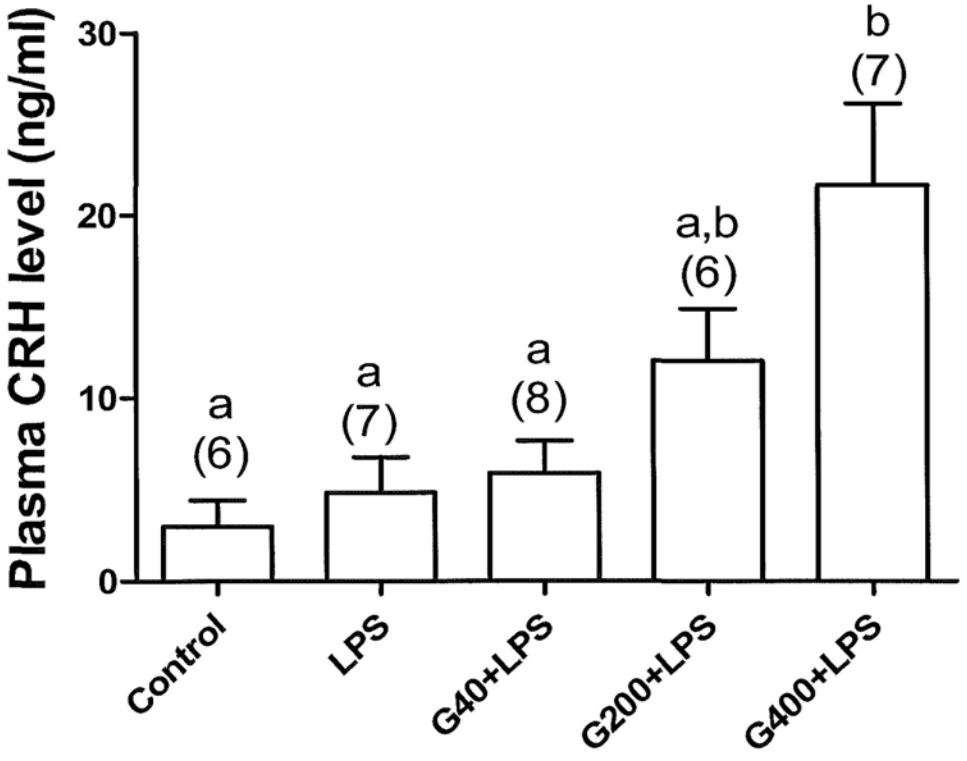


Fig.6.7 Genistein induced plasma CRH in maternal circulation. Pregnant ICR mice were gavaged with genistein and injected with LPS. Blood was drawn and assayed for CRH by ELISA. Values are means ± SEM; the numbers of samples are given in the parentheses. Means labeled with various letters are significantly different.

6.3.4 Genistein Reduced Placental CRH-BP Expression

CRH-BP could determine the activity of CRH. Since CRH expression was found to be elevated by genistein and LPS co-treatment, the expression of CRH-BP was also determined. The expression was suppressed in the group treated with G400+LPS (**Fig.6. 8**).

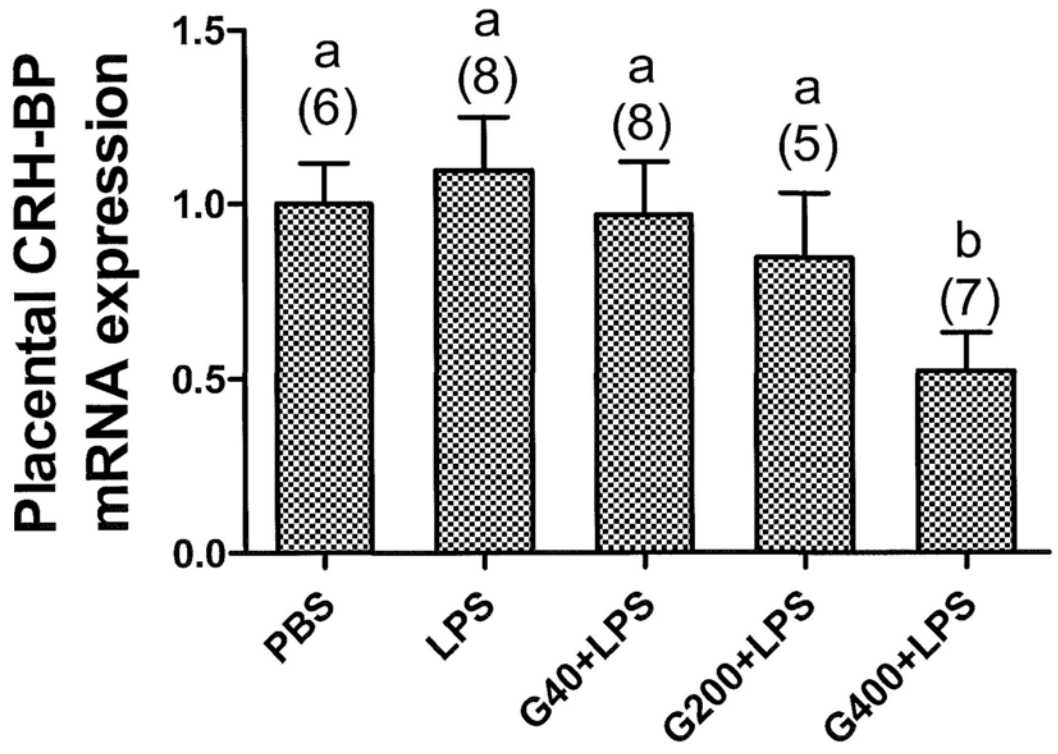


Fig.6.8 Placental CRH-BP expression was reduced by Genistein. Pregnant ICR mice were gavaged with genistein and injected with LPS. Total RNA was extracted from frozen placental tissue and CRH-BP expression was quantified by real-time RT-PCR. Values are means ± SEM; the numbers of samples are given in the parentheses. Means labeled with various letters are significantly different.

6.3.5 Plasma Estrogen and Progesterone Concentrations in Mice Treated with LPS and Genistein

Previous study has shown that estrogen could regulate the expression of CRH (Lalmansingh & Uht, 2008), and CRH may block progesterone release (Jeschke *et al.*, 2005). In addition, genistein may also alter estrogen and progesterone production in term tropholast cells (Richter *et al.*, 2009). The plasma concentrations of estradiol and progesterone were determined in these mice. No significant difference was observed in the plasma estradiol concentration (**Fig.6.9A**). Plasma progesterone concentration was decreased by LPS and was further lowered by genistein administration (**Fig.6.9B**). It appeared that the upregulation of CRH was independent of estrogen.

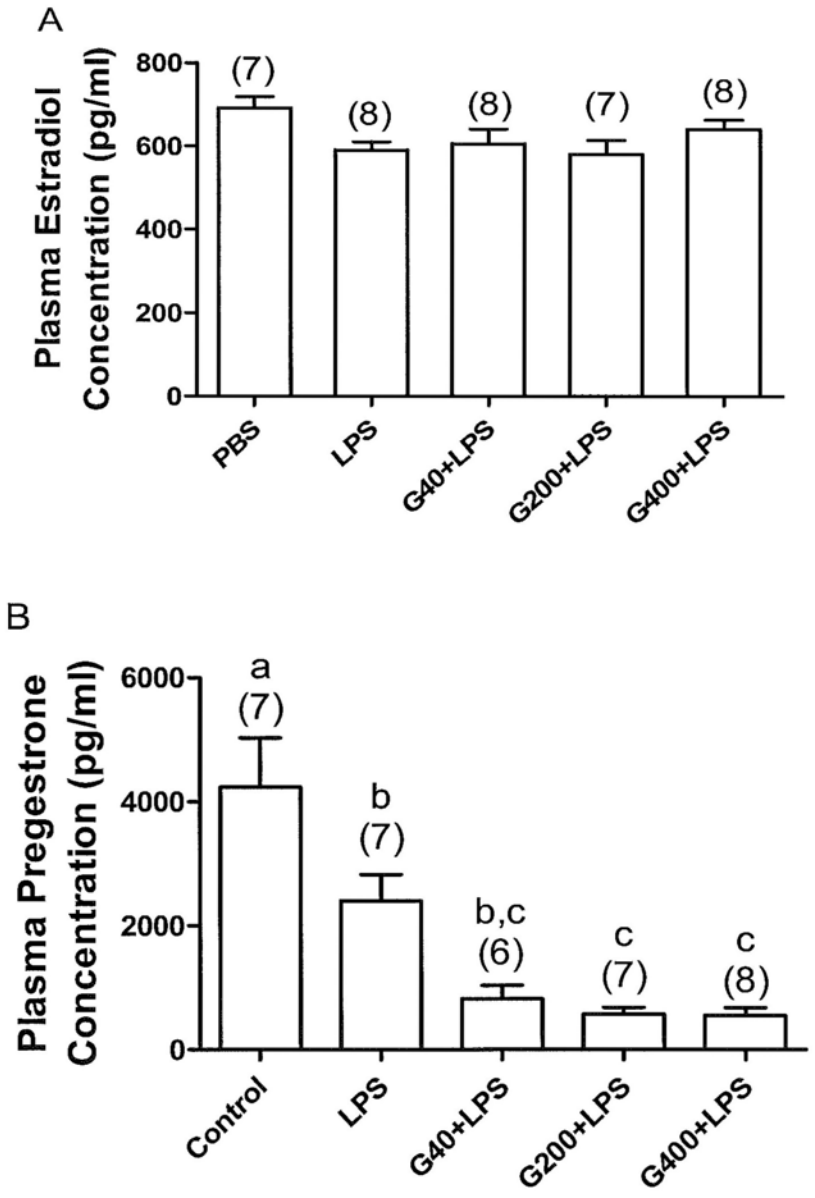


Fig.6.9 Plasma estrogen and progesterone concentrations in pregnant mice treated with genistein and LPS. Pregnant ICR mice were gavaged with genistein and injected with LPS. Blood was drawn and assayed for estradiol (**Fig.6.9A**) and progesterone (**Fig.6.9B**) by ELISA. Values are means ± SEM; the numbers of samples are given in the parentheses. Means labeled with various letters are significantly different.

6.4 Discussion

Genistein is a phytoestrogen isolated from soybeans, which are staple food to Asian population; however, the potential effect on reproduction has not been fully addressed. The animal study showed that genistein induced early delivery in pregnant mice sensitized with LPS. The present cell culture study demonstrated that genistein increased CRH transcriptional activity and expression through the activation of ERK. Induction of placental CRH expression and concurrent reduction of CRH-BP expression could be the underlying mechanisms. However, genistein administered alone could not induce CRH or early delivery (data not shown). Genistein and LPS appeared to exert a synergistic effect on inducing early delivery and placental CRH expression in gestation. Moreover, plasma progesterone concentration was reduced by the co-administration. Given the importance of progesterone in fetal maintenance, the decrease in progesterone by genistein might, at least, partially responsible for the increased preterm birth incidence observed.

CRH secreted by placental trophoblasts is responsible for the human parturition (McLean *et al.*, 1995). The ERK signal transduction pathway has been reported to be a regulatory factor of *CRH* gene expression in many tissues, but the relationship in placenta cells remains to be elucidated. CRH induces ERK activity in pituitary-derived cell line (Van Kolen *et al.*, 2010) and elevated levels of pERKs have been shown to be consistent with the increase of pCREB and CRH mRNA levels in neuroendocrine neurons (Cheng & Handwerger, 2005b). ERK1/2 activation may lead to the phosphorylation of the transcription factor CREB in vitro and in vivo (Xing *et al.*, 1998).

Steroid hormones could regulate *CRH* gene expression through binding to CRE (Ni & Nicholson). CRE-driven CRH promoter activity is stimulated by glucocorticoids and cAMP and inhibited by estrogen and estrogen receptor alpha in placental cells (King & Nicholson, 2007). Previous study has demonstrated that cAMP induces AP-1 and CRE promoter expression and activates CRH transcriptional activity in JEG3 cells (Uh *et al.*, 2009). The CRE in *CRH* gene can interact with transcriptional factors activated by multiple signal transduction pathways (Cheng & Handwerger, 2005b). In the present study, we demonstrated that genistein stimulated *CRH* promoter activity through ERK signaling pathway.

Increased placental CRH production has been associated with preterm delivery. As reviewed by Hobel at al. (Hobel *et al.*, 1999a), pre-mature birth is associated with elevated plasma CRH level, reduced CRH-binding protein (BP), and decreased CRH/CRH-BP interaction throughout the entire gestation period. CRH-BP is produced in the liver and placenta, and it may bind to the hormone and prevent it from interacting with CRH receptor. Reduced CRH-BP or CRH/CRH-BP complexes may suggest increased amount of free and active CRH in the blood. The reduced expression of CRH-BP observed in the present study might be responsible for some free CRH in blood.

Previous studies have shown that genistein alters hormone synthesis in reproductive tissues. Differential effect of genistein on estrogen synthesis has been reported in gonadal cells. In human endometrial stromal cells (Edmunds *et al.*, 2005) and immature rat ovarian follicles (Myllymaki *et al.*, 2005), genistein administration can increase the activity of estrogen synthesis. In contrast, Rice et al. (Rice *et al.*,

2006) have reported that the estrogen synthesis activity is reduced when human granulose-luteal cells are exposed to genistein.

The soya isoflavone also may have negative effect on pregnancy in a dose and stage dependent manner. Pregnant mice of 6 months of age delivered less live pups when treated with 0.5 to 5 mg/kg body wt genistein (Jefferson *et al.*, 2005). Reduced fertility was also seen in 2-month old mice which were given genistein 25 mg/kg or above starting from neonatal period. On the other hand, the successful rate of pregnancy with vaginal plug also reduced upon neonatal genistein treatment at 50 mg/kg in the same study. Moreover, the implantation sites in the genistein-treated pregnant mice were smaller and fewer in numbers than those observed in the control mice. No differences in serum hormones were evidenced among these treatments. The present study illustrated that genistein induced early parturition in mice exposed to LPS, possibly acting through CRH upregulation.

LPS is the endotoxin isolated from bacterial membrane and is mostly responsible for the pathophysiological effect of infection. Previous study has shown that LPS may induce preterm birth by increasing CRH in mice (Sarkar *et al.*, 2007; Wang *et al.*, 2008a). In the present study, LPS at the same dose did not increase CRH and had marginal effect on preterm birth. The strain difference might contribute to the variation in these results.

CRH receptor activity is another factor that may affect parturition (Cong *et al.*, 2009). The receptor is expressed in myometrium, and the amount appears to increase close to labouring period (Stevens *et al.*, 1998). However, no such increase was observed in the present study (data not shown) and the early delivery rate apparently was not related to the expression.

In summary, we verified that in placental cells, genistein stimulated CRH promoter activity by activating ERK and CRE binding. The animal study further demonstrated that co-administration of genistein and LPS induced the expression of placental CRH. We are the first to demonstrate that genistein could induce CRH in a synergistic manner. These results indicated that genistein might exacerbate the undesirable effect of LPS on pregnant mice by upregulating CRH expression. This elevation might affect normal parturition in mice.

CHAPTER 7
PROMOTER AND TRANSCRIPTIONLA FACTOR USAGE IN HUMAN PLACENTAL TISSUES

7.1 INTRODUCTION

The placenta is a structure that facilitates nutrients and waste exchange. The human placenta produces estrogens during pregnancy (Bonenfant *et al.*, 2000) which is important in the maintenance of pregnancy (Albrecht *et al.*, 2000). The biosynthesis of estrogen is catalyzed by aromatase which is highly expressed in placental cells (Mahendroo *et al.*, 1993). Various cytokines and lipids may regulate estrogen production in this organ (Harada *et al.*, 2003). During pregnancy, the aromatase expression increases (Kitawaki *et al.*, 1992). In placenta, the majority of CYP19 mRNA transcription is regulated by promoter I.1 (Mendelson & Kamat, 2007) with the minor usage promoter I.2 and 2a (Bulun *et al.*, 2003). The splicing is drastically different fro those of uterine leiomyomata and ovarian cells, which are regulated primarily by proximal promoter I.3/II (Imir *et al.*, 2007).

The transcriptional activity and usage of different promoter of aromatase is regulated in a tissue-specific and state-specific fashion. During the trophoblast differentiation, ERα recruits exon I.1 and induces *CYP19* gene (Kumar *et al.*, 2009). A recent study has demonstrated that the changes in buffalo *CYP19* gene expression during different stages of gestation might be due to partial shift in the promoter usage.

PI.1 only drives the *CYP19* gene expression in early stages of gestation while both the promoters I.1 and II are responsible for the expression of aromatase in post parturition of buffalo (Ghai *et al.*, 2010). In rabbit placenta, both PI.1 and PII are contribute to the aromatase transcription and the expression of PI.1 is much higher that PII (Bouraima *et al.*, 2001). The usage of aromatase promoters is altered from PI.4 in breast adipose tissues of cancer-free to PII and PI.3 in breast cancer patients, which is regulated via increased cAMP formation (Agarwal *et al.*, 1996).

Transcription factors (Schonfelder *et al.*) are proteins that bind to specific DNA sequences to regulate the transcription of the gene. The activation of these TFs can be stimuli- and /or tissure-specific (Latchman, 1997). TFs may bind to the DNA region by itself or with other proteins in a activator or repressor complex (Karin, 1990; Lee & Young, 2000). The TFs are important in signaling development and cell cycle, and mutations in these TFs are associated with many human diseases. Eukaryotic transcription factors can be classified into several families on the basis of their DNA-binding sequences (Karin, 1990). AP-1, C/EBP, CREB contains a highly conserved basic-leucine zipper (bZIP) domain at the C-terminus, and SP-1 contains a zinc finger protein motif (Stegmaier *et al.*, 2004) by which they are able to stabilize or block the binding of RNA polymerase to DNA (Gill, 2001).

Activator protein 1 (AP-1) transcription factors are dimers of JUN, FOS, MAF and activating transcription factor (ATF) family proteins (Schraml *et al.*, 2009). AP-1 mediates regulations of proliferation, differentiation, apoptosis and transformation (Hess *et al.*, 2004). The contribution of AP-1 to the cell fate critically depends on the composition of AP-1 dimers, the quality of stimulus, the cell type and the cellular environment (Hess *et al.*, 2004).

The cAMP responsive element binding protein (CREB) is a cellular transcription factor that modulates the transcription of genes with cAMP responsive elements(CRE) in their promoters (Silva *et al.*, 1998). CREB regulates a wide range of biological functions, ranging from spermatogenesis to circadian rhythms and memory (Silva *et al.*, 1998). CREB is important for the expression of many cAMP-responsive genes in different cell types in response to diverse signals (Mukherjee *et al.*, 1996). CREB is best known for its involvement in learning and memory. Manipulating CREB-regulated genes can be a potential treatment of neuropsychiatric conditions (Carlezon *et al.*, 2005).

CCAAT/enhancer-binding protein (C/EBP) family members are transcription factors involved in many physiological processes, such as cellular proliferation and differentiation, regulation of energy homeostasis, inflammation, and hematopoiesis (Kovacs *et al.*, 2003). The expression of the C/EBPs is regulated by a range of factors, including hormones, mitogens, cytokines, nutrients and certain toxins (Ramji & Foka, 2002). C/EBPs can recruit the coactivator CREB-binding protein and trigger its phosphorylation (Kovacs *et al.*, 2003).

Sp1 is a human transcription factor involved in gene expression. GC-boxes and related motifs are frequently found in many promoters and enhancers, the transcription factor Sp1 is the only factor acting through these motifs (Suske, 1999). Sp1 can interact with both aryl hydrocarbon receptor and/or the estrogen receptor (Wormke *et al.*, 2003). An architectural transcription factor HMGI-Y physically interacts with Sp1 and C/EBPβ and facilitates the binding of both factors to the insulin receptor promoter (Foti *et al.*, 2003).

The steroid hormone receptors constitute a large family of transcription factors, such as estrogen receptors, glucocorticoid receptor and progesterone receptor. The

binding of the hormonal ligands induces steroid receptors and leads to transcriptional activation (Xu *et al.*, 1999). Many other transcription factors also provide different functions. The Rel/NF-kappa B transcription factors may be a potential target for the treatment of chronic inflammatory diseases and certain malignancies (Grossmann *et al.*, 1999). The synthesis and releasing of proinflammatory factors are induced by oxidative stress through the p38 MAPK and nuclear factor-kappaB pathways and can be blocked by vitamins C and E in placental cells (Cindrova-Davies *et al.*, 2007).

The human placental samples were generously provided by Dr. Ronald C.C. Wang (Department of Obstetrics & Gynaecology, the Chinese University of Hong Kong, Shatin, N.T., Hong Kong) and stored in liquid nitrogen for further experiment. The 1st and 2nd trimester human placental tissues were collected from surgical termination of pregnancy and the 3rd trimester human placental tissues were collected from elective Caesarean section. We employed the human placental samples to investigate the aromatase expression and promoter usage with the development of gestation.

7.2 Result

7.2.1 Transcription Factor Analyze of Aromatase Promoters.

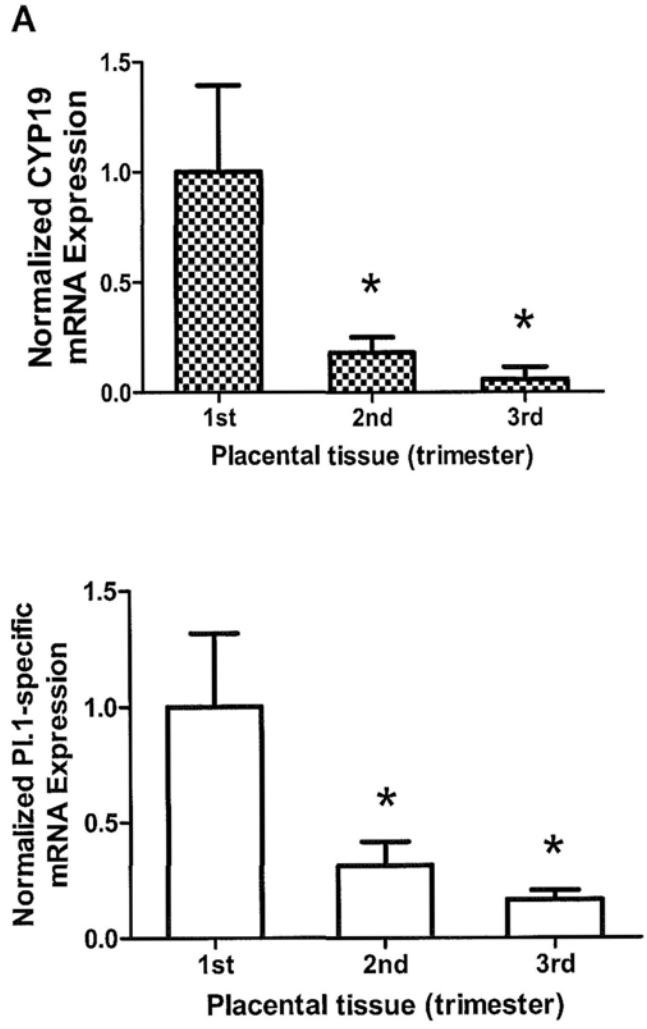
Each promoter of aromatase is regulated by a distinct set of regulatory sequences in the DNA. The putative transcription factors of aromatase promoters were identified from an online transcription factor database AliBaba2.1. The sequences were obtain from the GeneBank according to the accession numbers of the previously characterized promoters of the human *CYP19* gene: X55983 (placental-major, I.1), S52794 (ovary-specific promoter PII), S96437 (placenta-minor, I.2), D21241 (adipose/breast cancer, I.3), L21982 (skin and adipose, I.4) and D14473 (placenta-minor, 2a) (Sebastian & Bulun, 2001). The putative sites are summarized in **Table.7.1.** The distribution of Sp1 is extensive in PI.2 and PI.4. Binding sites for different members of C/EBP are found on the aromatase promoters.

Table 7.1. Number of seven major transcription factor binding elements on the promoters of aromatase. The sequences are listed in appendix I.

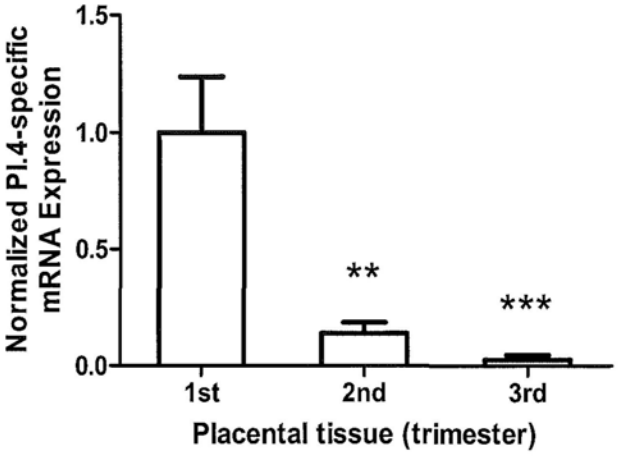
Promoter	*SP1*	*AP1*	*CREB-1*	*C/EBPa*	*C/EBPb*	*C/EBPg*	*C/EBP*
PI.1	**1**						
PI.2	**4**			**2**			
PI.3	**1**		**1**	**1**	**1**	**1**	
PI.4	**4**	**1**					
P2a						**1**	**1**
PII				**1**			

7.2.2 Aromatase Expression and Promoter Usage in Human Placental Tissues

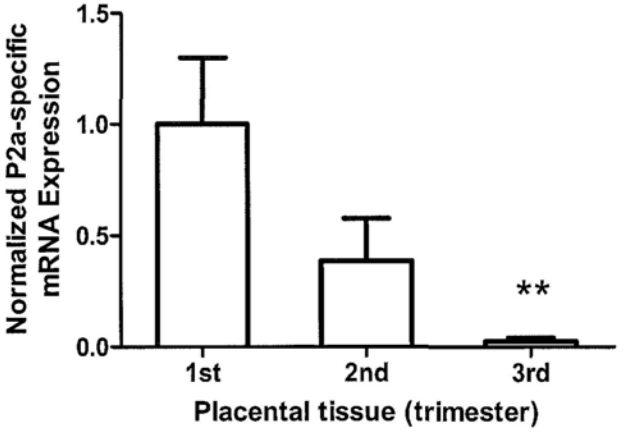
Real time PCR was performed to investigate the changes of aromatase expression and promoter usage of the 1st, 2nd and 3rd trimester human placental tissues. As indicated in **Fig.7.2A**, the CYP19 mRNA expression was gradually decreased with the progression of pregnancy. Six promoters were detected by real-time PCR. The promoter I.1, I.4 and 2a-specific mRNA species were decreased, while promoter I.2 expression increased a little in the 3rd trimester samples. The promoter II and I.3 expression remained at about the same level throughout the three trimesters.



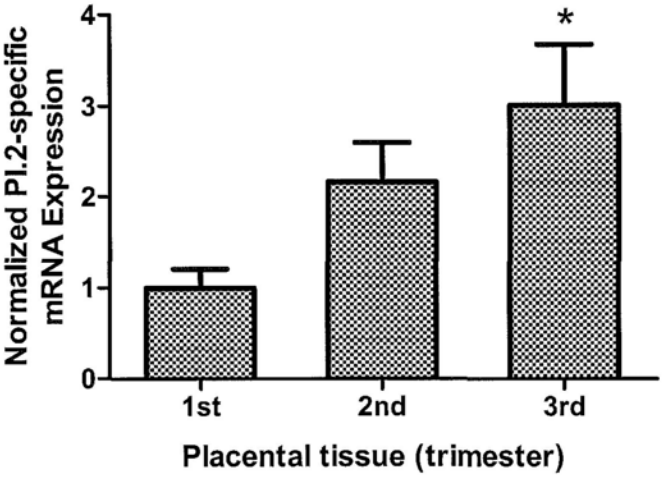
C


Normalized PI.4-specific mRNA Expression
1.5
1.0
0.5
0.0
**
***
1st
2nd
3rd
Placental tissue (trimester)

D


Normalized P2a-specific mRNA Expression
1.5
1.0
0.5
0.0
**
1st
2nd
3rd
Placental tissue (trimester)

E


Normalized PI.2-specific mRNA Expression
4
3
2
1
0
*
1st
2nd
3rd
Placental tissue (trimester)

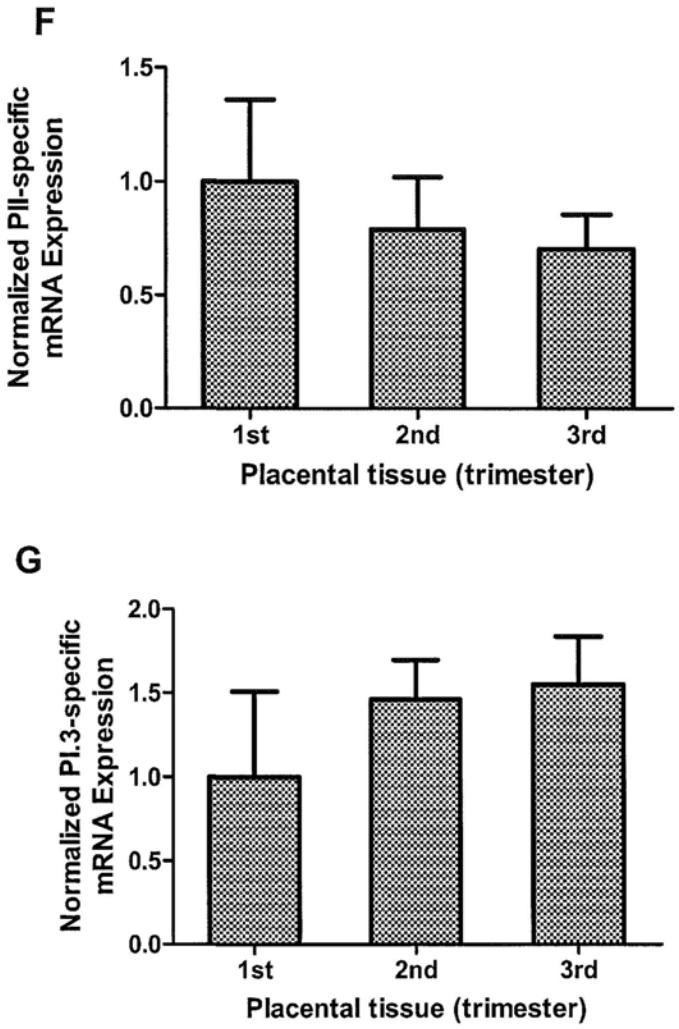
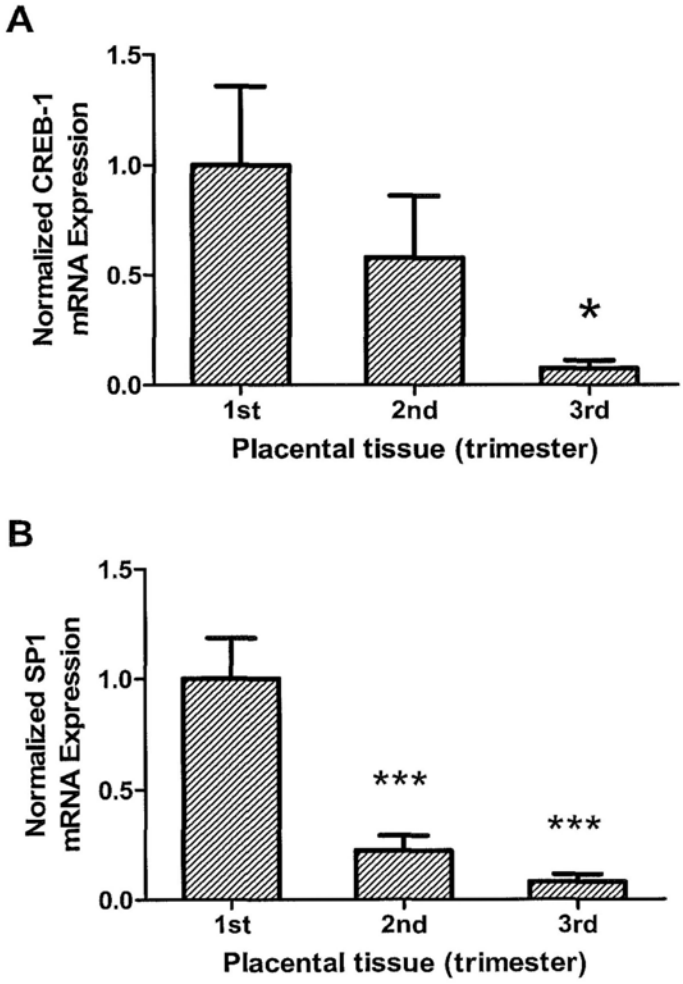


Fig.7.2 CYP19 mRNA and Exon I-specific promoter expression in human trimester tissues. Total RNA was extracted from human placental tissue and messenger RNA expression of genes was quantified by real-time PCR and normalized with GAPDH. These data was calculated by using $2^{-\Delta\Delta CT}$ method and analyzed by One-way ANOVA, (*) represents a significant ($p<0.05$), Values are means ± SE, n= 6.

7.2.3 Transcription Factor Expression in Human Trimester Tissues

Total mRNA was extracted from human placental samples and evaluated for the aromatase response transcription factors. Expression of four important transcription factors, CREB-1, Sp1, C/EBPα and JUN, were evaluated by using real-time PCR. As indicated in **Fig.7.3A** and **B**, gradually decrease in CREB and Sp1 mRNA level was observed with the maturation of fetal development. C/EBPa and JUN expressions not altered in these three phases of pregnancy.



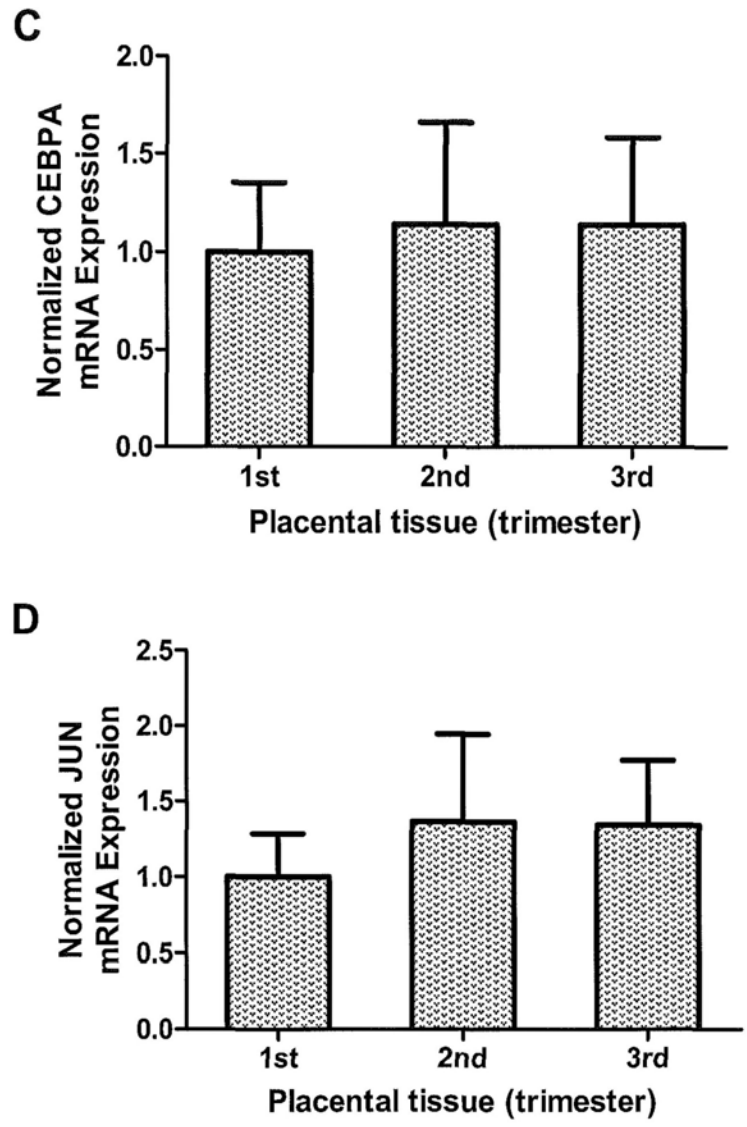


Fig.7.3 The transcription factor mRNA expression in human trimester tissues. Total RNA was extracted from human placental tissue and messenger RNA expression of these genes was quantified by real-time PCR. GAPDH was amplified for normalization. These data was calculated by using $2^{-\Delta\Delta CT}$ method and analyzed by One-way ANOVA, (*) represents a significant ($p<0.05$), Values are means ± SE, n= 6.

7.3 Summary

The biosynthesis of estrogen is catalyzed by aromatase (Adlercreutz *et al.*, 1993), and aromatase affects the placenta's function. Female pseudo-hermaphroditism has been found to be associated with placental aromatase deficiency (Shozu *et al.*, 1991). 17beta Estradiol, the major estrogen in human, stimulates actin protein expression (Sudha *et al.*, 1997) and regulates LDL-receptor levels in human placenta (Shanker *et al.*, 1998). It also suppresses the synthesis and secretion of human chorionic gonadotrophin(hCG) in first trimester human placenta (Sharma *et al.*, 1993). These studies have illustrated the physiological role of aromatase and estrogen in reproduction. Some transcription factors are responsible for the regulation of aromatase expression and the alteration of the activity of aromatase promoter in a signaling pathway- and tissue-specific manner (Bulun *et al.*, 2005; Mah *et al.*, 2007).

The transcription factor AP-1 responds simultaneously to a wide range of signal transduction pathways (Ameyar *et al.*, 2003). In placentas of women with preeclampsia, a Rac1-Jun aminoterminal kinase-c-Jun-dependent signal transduction pathway is downregulated (Hannke-Lohmann *et al.*, 2000). Jun proteins could bind to CRE in the PII promoter-proximal region and attenuate estrogen biosynthesis by directly downregulating transcription of the aromatase gene in ovarian granulosa cells (Ghosh *et al.*, 2005). It has also been reported that AP1 could regulate promoter II activity of the human *CYP19* gene in the ovary.

The inhibition of MAPK pathway partially prevents CREB phosphorylation and CREB can be phosphorylated by PKA in placental cells (Maymo *et al.*, 2010). Estradiol can activate a PKA signaling cascade that results in activation of the CREB

(Beyer & Karolczak, 2000). Prostaglandin E2 could enhance estrogen biosynthesis by stimulating aromatase mRNA (Yang *et al.*, 2002). The promoters I.3 and II are activated by prostaglandin E2 via a cAMP-PKA-dependent pathway (Mah *et al.*, 2007). Hormone-regulated expression of aromatase is mediated by the functional interactions, phosphorylation, and levels of CREB and SF1 mediate in gonadal cells (Fitzpatrick & Richards, 1994) (Carlone & Richards, 1997). The transcription factor Sp1 binds to the GC box in the 5'-flanking of aromatase exon I.1 (Kamat *et al.*, 1998). The GRE or Sp1 binding plays a role in the regulation of promoter 1.4-driven transcription in fetal hepatocytes (Zhao *et al.*, 1995a).

Several inhibitory transcription factors have also been described. The basic helix-loop-helix leucine zipper transcription factors upstream stimulatory factors 1 and 2 (USF1 and USF2) can bind to estradiol and estriol. USF1 could inhibit CYP19 expression and promoter activity in human trophoblast cells (Jiang & Mendelson, 2003). The lack of aromatase expression in eutopic endometrium is attributed to the binding of COUP-TF to the aromatase promoter (Zeitoun & Bulun, 1999).

In the present study, we analyzed six pregnancy-related promoters of aromatase, and many transcription factors have been identified. Four well-defined transcription factors were distributed over six promoters of aromatase. Our result showed that CYP19 mRNA expression was reduced with the progression of pregnancy in the human samples. Six promoter-specific species were detected and promoters I.1, I.4 and 2a driven mRNA expression were diminished progressively over the gestation period. The promoter II and I.3 expressions were maintained throughout the three trimesters. With the progression of pregnancy, the mRNA of CREB and SP1 was decreased gradually, whereas C/EBPα and JUN were not affected.

In summary, our result demonstrated that the CYP19 expression was reduced gradually in the human placental tissues accompanying the decrease of promoter I.1, I.4 and 2a expression in the progression of gestation. The transcription factors CREB-1 and Sp-1 may contribute to this decrease of aromatase expression.

CHAPTER 8
SUMMARY

Our results indicated that both genistein and bisphenol-A were able to affect the expression of aromatase and CRH, suggesting that these endocrine disruptors could change the estrogen metabolism and CRH expression, and potentially the outcome of pregnancy. The related MAPK signaling pathways were also investigated in the present study.

8.1 Molecular Regulation of Aromatase in Placental Cells by BPA and Genistein

JEG-3 cultures treated with bisphenol-A displayed a reduced aromatase activity. Real-time PCR analysis indicated that the compound significantly reduced the mRNA expression in these cells. As the transcriptional activity of CYP19 gene is controlled by the proximal promoter region of exon Ia in placental cells, the promoter activity of this gene fragment and exon-I.1-spliced mRNA abundance were also evaluated. Both assay results indicated that bisphenol-A repressed the transcriptional control of promoter Ia. The present study illustrated bisphenol-A potentially reduced estrogen synthesis by down-regulating CYP of placental cells.

We also evaluated the inhibitory effect of genistein on the aromatase expression and estrogen metabolism in the trophoblast cells. Our result indicated that the mRNA expression of aromatase was dose-dependently reduced by genistein in SGHPL cells

while it had no effect on aromatase expression in JEG-3 cells. **Fig.8.1** summarizes the cellular mechanism of genistein on CYP19 transcription in the SGHPL cell lines.

8.2 Molecular Regulation of CRH in Placental cells by BPA and Genistein

In the present study, we demonstrated that both BPA and genistein increased CRH transactivity and expression, and the regulatory element CRE in the promoter of *CRH* gene is responsible for the expression through PKC/PKA or ERK signaling pathways. **Fig.8.2** illustrates the two independent signaling pathways of BPA and genistein on controlling CRH transcription.

8.3 Determining the Physiological Role of Genistein and BPA Administrated in Mice

Many researchers have found that bisphenol-A and genistein might meddle pregnancy in rodents. The disturbance may be a collection of changes in estrogen and CRH production. The *in vivo* study demonstrated that the administration of BPA could change the estrogen metabolism and the expression of placental CRH, and that genistein increased placental CRH expression under LPS influence in mice. These findings imply a possible toxicity of genistein's toxicity on reproduction. In summary, this project showed that BPA and genistein could mediate estrogen and CRH biosynthesis in placental cell lines through MAPK signaling pathways.

In summary, this study described the investigation of the *in vitro* and *in vivo*

effects of bisphenol-A and genistein on the transcriptional regulation of two placental biomarkers, aromatase (CYP19) and CRH during pregnancy. Our results demonstrated that BPA and genistein could affect the estrogen biosynthesis via the transcriptional modulation on *CYP19 (cyp19)* and *CRH (crh)* in the mouse placenta and human placenta-derived cell lines. Their dietary consumption during pregnancy may affect the placenta function.

In the future, the promoter-regulating aromatase expression in the first and third trimester cell lines SGHPL and PL30 can be investigated. The differential regulation by various environmental agents may also be evaluated by using this established system.

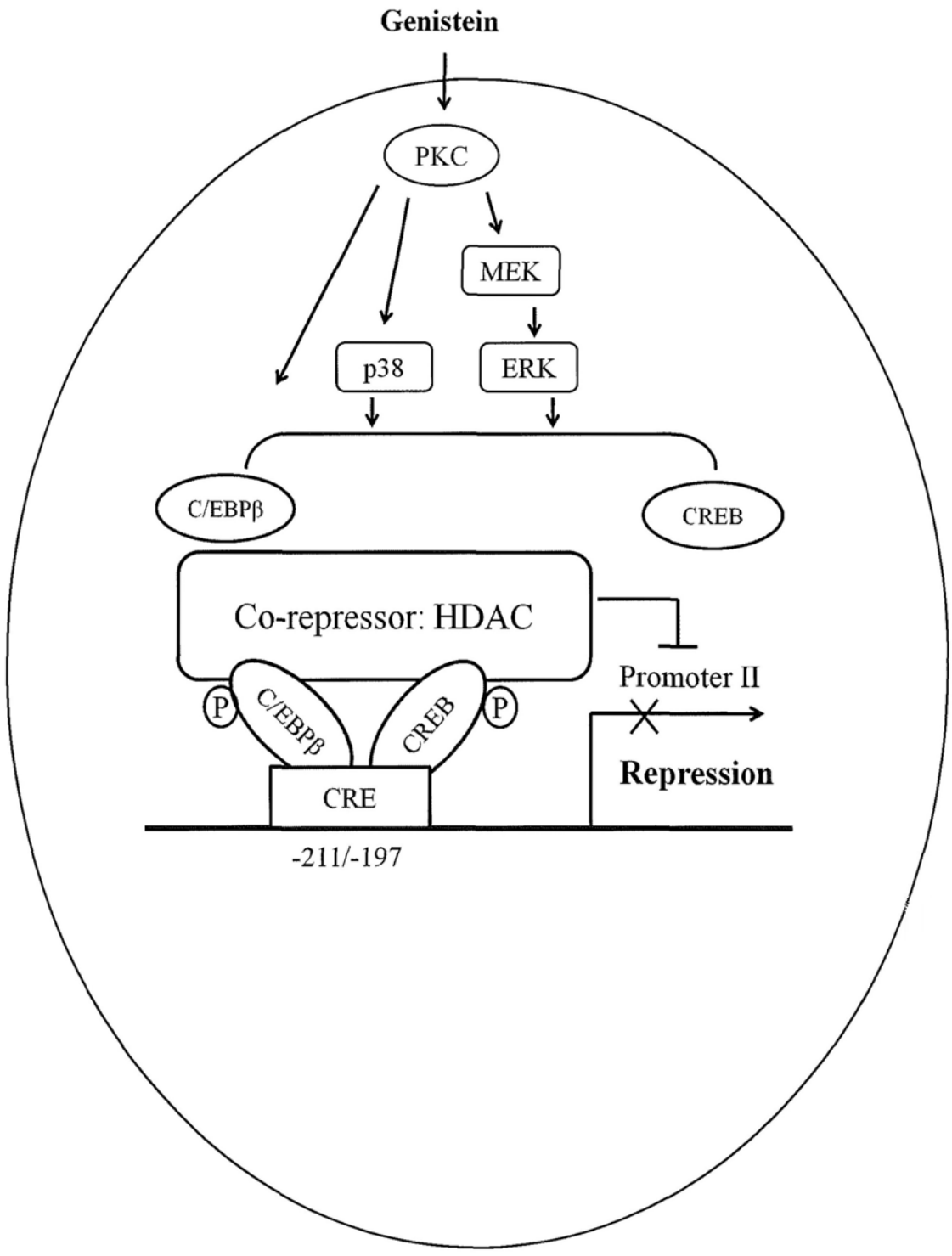


Fig.8.1 signaling pathways of genistein suppressing CYP19 expression in SGHPL cells.

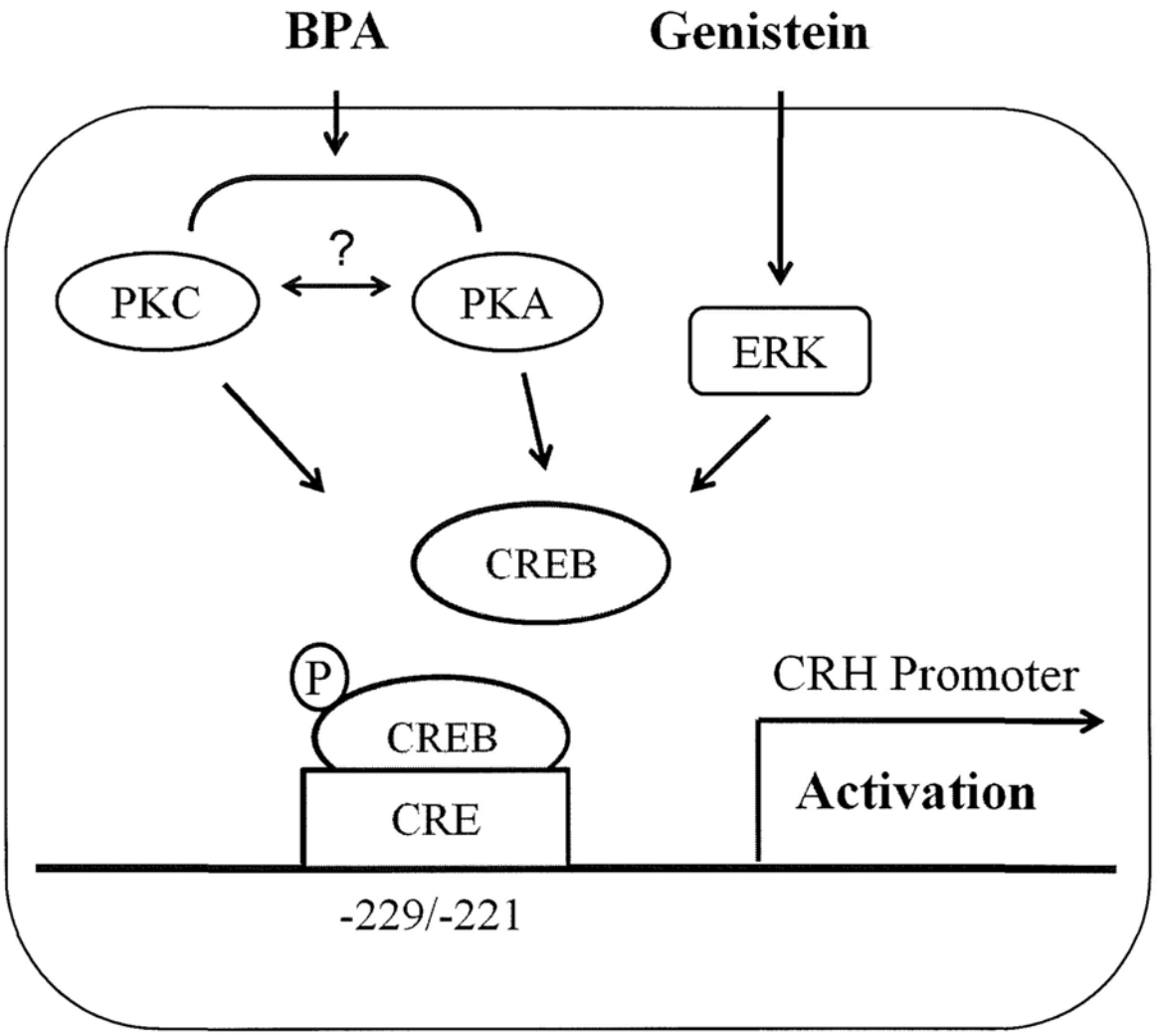


Fig.8.2 Signaling pathways of genistein and BPA activating CRH expression in JEG-3 cells.

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APPENDIX I.

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
AhR	Aryl Hydrocarbon Receptor
AP-1	Activator protein-1
Amp	Ampicilin
ATF	Activating transcription factor
B[a]P	Benzo[a]pyrene
Bcl-2	B-cell leukemia/lymphoma 2
BPA	Bisphenol-A
bFGF	Basic fibroblast growth factor
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic Acid
CDXRE	Caudal-type homeobox response element
C/EBP	CCAAT/enhancer binding protein
COMT	Catechol-O-methyltransferase
COX-2	Cyclooxygenase-2
COUP-TF	Chicken ovalbumin upstream transcription factor
CRE	cAMP response element
CREB	CRE binding protein
CRH-R	CRH receptor
CYP	Cytochrome P450
DAG	Diacylglycerol
dATP	Deoxyadenosyl triphosphate
dCTP	Deoxycytosinyl triphosphate
dGTP	Deoxyguanosinyl triphosphate
DMEM/F12	Dulbecco's Modification of Eagle's Medium/Ham's F12
DMSO	Dimethyl sulfoside
DNA	Deoxynucleic acid
dNTP	Deoxyribonucleotide Triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidunyl triphosphate
E1	Estrone
E2	17β-estradiol
E3	Estriol
E.coli	Escherichea coli
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal growth factor
EGFR	Epigermal growth factor receptor
ERE	Estrogen response element
ERK 1/2	Extracellular signal-regulated kinase 1/2
ER	Estrogen receptors
EtOH	Ethanol
FBS	Fetal bovine serum
fFN	Fetal fibronectin
FSH	Follicle stimulating hormone
FW	Formula weight
GR	Glucocorticoid response

HDAC	Histone deacetylase
HEPES	N-2-hydroxy-ethyl-piperazine-N'-2-Ethane-sulfonic acid
HPA	hypothalamus-pituitary-adrenal
ICER	Inducible cAMP early repressor
IGF	Insulin-like growth factor
IL	Interleukin
IP_3	Iinositol-1,4,5-triphosphate
IUGR	Intrauterine growth restriction
JNK	c-Jun N-terminal kinase
Jun	v-jun avian sarcoma virus 17 oncogene homolog
kb	Kilo-base
kDa	Kilo-dalton
LB	Luria-Broth Medium
LBD	Ligand binding domain
LPS	lipopolysaccharide
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MBD	Membrane binding domain
MEK	Mitogen-activated protein / Extracellular signal-regulated kinase kinase
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor kappa B
P450arom	Aromatase cytochrome P450
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP_2	Phosphoinositol-4,5-bisphoaphate
PI3 kinase	Phosphatidylinositol 3-kinase
POMC	Proopiomelanocortin
PKA	Protein kinase A
PKC	Protein kinase C
PTD	Preterm delivery
PVN	Hypothalamic paraventricular nucleus
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RPMI 1640 Medium	Rosewell Park Memorial Institute Tissue Culture Medium 1640
RT-PCR	Reverse transcription-polymerase chain reaction
SERM	Selective estrogen receptor modulator
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TCDD	2,3,7,8-tetrachlorodibenzo-para-dioxin
TDI	Tolerable daily intake
TGF	Transforming growth factor
Tris	Trizma base
TSA	Trichostatin A
UGT1A1	UDP-glucuronosyltransferase 1A1
XRE	Xenobiotic Response Element

APPENDIX II.

The major TF binding elements on the promoters of aromatase.

1. X55983 (placental- major, I.1), 103bp

```
seq(    0..   59)    ggagtttctggagggctgaacacgtggaggcaaacaggaaggtgaagaagaacttatcct
Segments:
1.3.1.2    17   26                    ====USF===
2.3.1.0    21   30                      ====Sp1===
3.5.2.0    31   40                            ===Elk-1==
2.2.1.1    57   66                                                    ===

seq(   60..  119)    atcaggacggaaggtcctgtgctcgggatcttccagacgtcgc
Segments:
2.2.1.1    57   66   GATA-1=
2.1.2.10   68   77         ====COUP==
9.9.590    84   93               =NF-kappaB
3.4.1.0    85   94               ====HSF===
3.5.1.2    97  106                        ===Adf-1==
```

2. S52794 (ovary-specific promoter PII), 125bp

```
seq(    0..   59)    gcaccctctgaagcaacaggagctatagatgaacctttttaggggattctgtaatttttct
Segments:
1.1.3.0    43   52                                                =C/EBPalp=

seq(   60..  119)    gtccctttgatttccacaggactctaaattgccccctctgaggtcaaggaacacaagatg
Segments:
9.9.853    96  105                                          =T3R-beta1
2.1.2.3    98  107                                           ====T3R===
2.1.2.2    99  108                                            ==RXR-beta

seq(  120..  179)    gtttt
```

3. S96437 (placenta-minor, I.2), 971bp

```
seq(    0..   59)    tggtctaagatctgatatgttccatagcctaagatgtacagctaagcctgctgaagaatt
Segments:
2.2.1.1    58   67                                                    ==

seq(   60..  119)    gtccttatttttccatttcagatattcccaaggttaggtagaggggtggcgttgaagag
Segments:
2.2.1.1    58   67   =GATA-1=
3.5.3.0    69   78         ===IRF-1==
9.9.590    79   88               =NF-kappaB
2.3.1.0   102  111                                    ====Sp1===

seq(  120..  179)    caggctctctagagccaatgctgtttattaataagctgtacagacacgcttcttccagga
Segments:
```

```
3.3.2.0      141   150                          ===SGF-1==
=============================================================================

seq(  180..  239)     atgtgcacttctgattctgctgtggtgataaagatactctgccttgcccattaagccatt
Segments:
=============================================================================

seq(  240..  299)     tgttggtggaaaacattacctttaaataaatgtctggttctatgtgagcaaagctagggc
Segments:
=============================================================================

seq(  300..  359)     tcccataatatgtgcattagatactctgaattcagctgaactgtttggcaactttgtcac
Segments:
     353   364
9.9.535      341   350                                              =====NF-1==
=============================================================================

seq(  360..  419)     tctggccatcatttcagatagttcccacagtgtgagaaatgacgcttcctatagtactgt
Segments:
=============================================================================

seq(  420..  479)     caagagtgtgaaaagagagctctttagcaaacacattatttatccatccttaaaaagctt
Segments:
1.1.3.0      443   452                          =C/EBPalp=
3.1.2.1      457   466                                 ===Pit-1a=
=============================================================================

seq(  480..  539)     gtcacctccagggtgcttgttcagaagtaatctcattacttaaatcatatcctagcatgt
Segments:
2.3.1.0      487   496          =====Sp1===
3.1.2.2      509   518                          ===Oct-1==
=============================================================================

seq(  540..  599)     ggaaaagctccctgagttccccgcctgccccacctgctgatttctcaggaccgctgataa
Segments:
2.3.1.0      558   570                    =======Sp1=====
2.3.1.0      565   574                       =====Sp1===
1.2.1.0      569   578                          ======E1===
1.2.2.0      569   578                          =====MyoD==
3.1.2.2      573   582                             ===Oct-1==
1.1.3.0      578   587                                =C/EBPalp=
=============================================================================

seq(  600..  659)     cagcttttcatgtccaacttgggattaatatcaagcaagccatggattttgtctccactg
Segments:
9.9.539      634   643                                      =====NF-1==
=============================================================================

seq(  660..  719)     aacttgggcatcatggacagtttccattccagcagttaagtcaagcaagccatggatttt
Segments:
9.9.539      704   713                                                 =====NF-1==
=============================================================================

seq(  720..  779)     gtctccactgaacttgggcatcatggacagtttccattccagcagttaagggcttcctga
Segments:
=============================================================================

seq(  780..  839)     ctttcaacagtggtgctgatcccagttctgaagagtggaacatcagagagcctcccctcc
Segments:
2.3.1.0      829   839                                                   ======Sp1===
=============================================================================

seq(  840..  899)     tcagccacttgtaagcataacaaactgaaaatgtaatctgctttcaaatattctgttgag
Segments:
=============================================================================

seq(  900..  959)     taaagattccttttaattactttaactgggtttgtagagacaagggaccgtctctggttg
Segments:
=============================================================================

seq(  960.. 1019)     gcaacttgtcc
```

4. D21241 (adipose/breast cancer, I.3), 204bp

```
seq(    0..   59)    gctttcaattgggaatgcacgtcactctacccactcaagggcaagatgataaggttctat
Segments:
1.1.1.6     18    27                    ==CRE-BP1=
9.9.51      18    27                    =====ATF====
2.3.1.0     22    31                      =====Sp1====
9.9.537     36    45                                 =====NF-1==
2.2.1.1     54    63                                              ===GAT

seq(   60..  119)    cagaccaagcgtctaaaggaacctgagactctaccaaggtcagaaatgctgcaattcaag
Segments:
2.2.1.1     54    63    A-1=
1.1.3.0     94   103                                 =C/EBPgam=
3.1.1.2    110   119                                           =====Ftz====
9.9.535    114   123                                              =====NF
9.9.539    114   126                                              ======N

seq(  120..  179)    ccaaaagatctttcttgggcttccttgttttgacttgtaaccataaattagtcttgccta
Segments:
9.9.535    114   123   -1===
9.9.539    114   126   F-1=====
1.1.3.0    169   178                                           =C/EBPalp=

seq(  180..  239)    aatgtctgatcacattataaaaca
Segments:
1.1.3.0    192   201          =C/EBPbeta
```

5. L21982 (skin and adipose, I.4), 323bp

```
seq(    0..   59)    aaccatgacagccacagtcaggacacaaaaaacaaagtgtccttgatcccaggaaacagc
Segments:

seq(   60..  119)    cctctggaatctgtgaaatctagaaacatagttgggaaaactctgacacccctgccccat
Segments:
4.1.1.0     91   100                          =NF-kappaB
2.3.1.0    105   117                                  ======Sp1=====
2.1.2.2    107   116                                   ==RXR-beta
1.3.1.2    114   123                                        =====US

seq(  120..  179)    gaccaaccaagactaagagtcccagaagattgaggtcacagaaggcagaggcctgccccc
Segments:
1.3.1.2    114   123   F====
2.1.1.4    150   159                          ======ER====
2.1.2.2    151   160                          =RXR-alpha
2.2.2.0    151   160                          =====Ttx====
2.1.2.10   152   161                            =====COUP==
2.3.2.1    170   179                                       ==Krox-20=
2.3.1.0    170   181                                       ======Sp1==

seq(  180..  239)    tctccaggagatccctgacccatgtggggtcatgggcggggcatgagtgatgtgatggga
Segments:
2.3.1.0    170   181   == AP-1 ==
1.3.1.2    206   215                       =====USF====
2.3.1.0    212   221                         =====Sp1====

seq(  240..  299)    aactggctcctggctccaagtagaacgtgaccaactggagcctgacaggaggtccctggc
Segments:
1.3.1.2    298   307                                                    ==

seq(  300..  359)    actggtcagcccatcaaaccagg
```

Segments:

1.3.1.2	298	307	==USF===
2.3.1.0	305	314	====Sp1===

6. D14473 (placenta-minor, 2a), 109bp

seq(0.. 59)	gcggtgtcagaaaccctgtggtgaaattcagcctgtggattccagaaatttggagtgttc		
Segments:			
1.1.3.0	17	26	=C/EBPgam=
4.1.1.0	19	28	=NF-kappa=
9.9.539	35	44	====NF-1==
3.4.1.0	40	49	=HSE-bind=
seq(60.. 119)	ttgggggggaaaaatccgcacacacaaagcaacatttggaaatccctgtg		
Segments:			
2.3.3.0	60	69	=CPE_bind=
3.1.2.2	89	101	======Oct-1===
1.1.3.0	92	101	===C/EBP==
4.3.2.0	92	101	====SRF===
9.9.213	95	104	===EBP-1==
9.9.590	96	105	=NF-kappaB
2.1.1.4	101	110	======ER===