The impact of angelicae sinensis radix and its herb-pairs in embryonic development

Ting Ting Xiao

Hong Kong Baptist University

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The Impact of Angelicae Sinensis Radix and Its Herb-pairs in Embryonic Development

XIAO Ting Ting

A thesis submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Principal Supervisor: Dr. XU Min
Hong Kong Baptist University
September 2015
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature:

Date: September 2015
ABSTRACT

Background and purpose:

Angelicae Sinensis R unplcd (Chinese Angelica, *Dang Gui*, DG), the dry root of *Angelica sinensis* (Oliv.) Diels, is one of the most popular herbs used around the world. It has been named as the “female ginseng” and served as an indispensable herb to treat many obstetrical and gynecological diseases. Traditionally, DG was recommended to pregnant women to ease delivery and to eliminate complications. It is believed that the body of DG (*Dang Gui Shen*, DGS) is superior in nourishing blood, while the tail of DG (*Dang Gui Wei*, DGW) is commonly used to remove blood stagnation. Clinically, DG is commonly combined with *Paeoniae Radix Alba* (White Peony Root, *Bai Shao*, BS) and *Rehmanniae Radix* (Unprocessed Rehmannia Root, *Sheng Di Huang*, SDH) to treat disorders during pregnancy as it may not only strengthen therapeutic effects but also eliminate adverse effects caused by each single herb. However, it is contradictory that DG may increase the risk of miscarriages reported by previous studies: the use of DGS among pregnant women, while avoiding using DGW has always been recommended since ancient times to avoid miscarriage. To date, there is no clear evidence to identify the safety of DG in pregnant women and to support the theory that different pharmaceutical effects are attributed to chemical difference between DGS and DGW. Furthermore, little is known regarding the specific effects of DG on fetal bone while limited research has been done to explore herb-herb interactions between DG and other herbs. The aims of this project are (1) to identify the safety of DG in maternal and fetal health; (2) to compare the chemical composition of DGS and DGW and their cytotoxicity; (3) to analyze the integrated role of herb-pair (DG plus BS or SDH); (4) to investigate the mechanism of specific impact of herb-herb interaction emphasis on embryonic development. Based on the theory of traditional Chinese medicine, our project is believed to provide experimental evidence to rationalize clinical use of DG in pregnant women.
Method:

(1) For the herbal quality control, aqueous extracts of DG, DGS, DGW, BS and SDH were prepared respectively, and their reference marker compounds were quantitatively authenticated by HPLC. In addition, pesticide residues and heavy metals in DG extract were examined by GC-MS and ICP-MS. Moreover, comparison of composition of DGS and DGW extract in terms of main constituents was performed by GC-MS and LC-MS analysis.

(2) In-vivo mouse study (Segment II study), pregnant mice were randomly assigned into different dosage groups: oral administration of either distilled water as negative control, or DG extract of 2, 8, 16, 32 g/kg/day, or BS extract of 2, 16, 32 g/kg/day, or SDH extract of 2, 16, 32 g/kg/day, or DG (32 g/kg/day) plus BS (32 g/kg/day), or DG (32 g/kg/day) plus SDH (32 g/kg/day), respectively from the gestation day (GD) 6 to 15; another group mice were treated with vitamin A (200,000 IU) on the GD7, 9 and 11 as positive control. The mice were sacrificed for assessing parameters on GD18.

(3) In-vitro assay using embryonic stem cell (ESC) and fibroblast 3T3 cell was conducted to investigate the cytotoxicity of DG, Z-LIG, FA, DGS, DGW, BS and SDH by MTT test, according to European Centre for the Validation of Alternative Methods.

(4) For mechanistic study of DG impacts and herb-herb interactions, the expression of a characteristic set of bone formation/resorption markers, and some site-specific bone regulatory factors in fetal tissues and amniotic fluids on the GD15 were measured by ELISA.

Result:

(1) In the study to evaluate the safety of DG extract, maternal body weight (BW), gravid uterine weight, corrected BW change, live fetus/litter, mean fetal body weight in the group of DG (32 g/kg/day) were significantly lower than those
of the negative control (p < 0.05); while resorption site/litter, post-implantation loss (PIL)/litter, percentage of abnormal skeleton were significantly higher than those of the negative control (p < 0.05). Although there was no statistical difference between IC₅₀ values of ESCs (IC₅₀ ESC) and 3T3 cells (IC₅₀ 3T3) after treatment with DG, Z-LIG and FA samples respectively, the IC₅₀ Z-LIG was significantly less than IC₅₀ FA in both ESCs and 3T3 cells (p < 0.05). It was indicated that DG extract (32 g/kg/day) might result in adverse impacts to maternal function and fetal development in mice. Z-LIG in DG extracts might be less safe compared to FA in in-vitro cultured cells and its potential impacts should be further examined its potential impacts in in-vivo studies.

(2) In the study to compare the composition of main constituents from DGS and DGW water extract, HPLC quantitative analysis indicated that the ratio of FA and Z-LIG between extract from DGS and DGW is 1:1.83 and 1:1.35, respectively. Sathulenol (1), 3-butylphthalide (2), Z-butylidenephthalide (3), benzeneacetic acid (4), Z-LIG (5) and E-LIG (6) were identified by GC-MS analysis. The peak area of compound 5 in DGW extract was close to 5 times of that in DGS extract. The amounts of compound 2 and 3 in DGW extract were respectively over 20 times and 2 times higher than that in DGS extract, respectively. Except for compound 3, 5, 6, additional three compounds: coniferyl ferulate (7), FA (8), senkyunolide A (9) were identified by LC-MS analysis. The amount of compound 3, 5, 6, 7, 8, and 9 in DGW extract was higher than that in DGS extract. The peak area of compound 3 and 5 in DGW extract was over 2 times of that in the DGS extract. In MTT assay, the effect of DGS and DGW water extract on inhibition of cell viability of cultured ESCs and 3T3 cells was in a dose-dependent manner, respectively. The difference between IC₅₀ ESC and IC₅₀ 3T3 after DGS extract treatment was statistically significance (p < 0.05), however no statistical significance was identified in DGW (p > 0.05). Both IC₅₀ ESC and IC₅₀ 3T3 values of DGW were much lower than those of DGS (p < 0.05).
(3) In the study to evaluate the role of DG plus BS or SDH, expectedly DG extract (32 g/kg/day) resulted in significant abnormalities in maternal and fetal parameters when compared with the negative control. Whereas BS or SDH extracts at a dosage of 2, 16, or 32 g/kg/day did not result in any adverse effect for both maternal health and embryonic development. There was no statistically significant difference between the IC$_{50}$ ESC and IC$_{50}$ 3T3 value in the cytotoxicity assays of BS or SDH extracts ($p > 0.05$). It was indicated that the use of BS or SDH extract should be safer than DG extract in pregnant mice. More importantly, the treatment with DG plus BS or DG plus SDH extract could significantly correct abnormalities caused by DG extract alone as seen in the corrected BW change, mean fetal body weight, live fetus/litter (%), resorption site/litter (%), PIL/litter (%), skeletal variation (%), etc. ($p < 0.05$) in pregnant mice.

(4) In the study to analyze the mechanism of herb-herb interactions, the mean values of PICP, ALP-Bone, osteocalcin, BMPs and GDF-5 in fetal tissues were significantly lower in mice treated with DG extract (32g/kg/day) alone when compared with the negative control ($p < 0.05$); while there was no significant difference among the mice treated respectively with BS, SDH, DG plus BS and DG plus SDH extracts with the same dosage. The outcome was similar to those of the negative control ($p > 0.05$). In addition, there were no significant differences in the mean value of ICTP in both fetal tissues and amniotic fluids among all mice groups ($p > 0.05$).

**Conclusion:**

(1) High dosage and long-term use of DG water extract may result in adverse effects on embryonic development including fetal bone malformations, hence its use is considered as not safe in pregnant women. As DG extract in this study was not contaminated by pesticide residues and heavy metals, the embryonic toxicity of DG extract can be considered as due to the intrinsic constituents of the herb.
(2) As seen in cytotoxicity assay, that water extract of DGW had the lower IC₅₀ value, hence it is believed that the higher phthalides level (3-butylphthalide, Z-butylideneephthalide, senkyunolide A Z-LIG and E-LIG) contributes to a more toxicity on both ESC and 3T3 cells.

(3) Herb-pair extract of DG plus BS or SDH could significantly correct abnormalities caused by DG extract alone in pregnant mice. Therefore, herb BS or SDH not only has beneficial effects when used for treating pregnant disorders safety, but also has attenuated effects for DG when used together as herb-pair extract.

(4) At the molecular biomarker level, DG extract might significantly affect bone formation rather than bone resorption. However, it could be ameliorated when applied combination with either BS or SDH.

These results should be valuable for further analysis on the integrated effects of herb-herb interactions and complex mechanism of formula therapies in Chinese herbal medicine.
ACKNOWLEDGEMENTS

Completion of this doctoral dissertation was impossible without the guidance of my advisors, help from friends, and support from my family. I owe my sincere gratitude to all those people who have made the impossible possible, making the past four years cherishable. This study was supported by the scholarship of the University Grants Committee of Hong Kong, the project grants of Hong Kong Baptist University.

I would like to express my deepest gratitude to my principal supervisor, Dr. Xu Min, School of Chinese Medicine, Hong Kong Baptist University. Dr. Xu is one of the best mentors I have had in my life. He sets high standards for his students and encourages and guides them to meet those standards. He introduced me to traditional Chinese medicine and his talk always inspired me. I am deeply grateful for the long discussions which guide me and helped me refine the technical details of my experiments and problems during the research. His support was crucial for me to overcome many critical situations and assisted the completion of my doctoral dissertation.

I would also like to express my heartfelt gratitude to my co-supervisor, Prof. Zhao Zhong Zhen and Prof. David Fong Wang-fun, School of Chinese Medicine, Hong Kong Baptist University, for their valuable advices, inspiration and encouragement in all aspects of my PhD study.

I would give my special thanks to Dr. Tian Xiao Ying, who gave me valuable advices, continual encouragement and support in those hard times. I was very much privileged to learn from Dr. Shen Xiang Chun, Research Division of Pharmacology, Guiyang Medical University, who consistently extended his research expertise throughout my study and I owe a lot to him for this achievement.
Also, some faculty members of the University and other institutes have been kindly assisted in different aspects of this research, whenever I approached them, and I hereby acknowledge all of them. I would like to thank Dr. Kelvin Sze-Yin Leung, Department of Chemistry, Hong Kong Baptist University, for his professional guidance in the respect of chemical analysis. Special thanks go to Ms. Yang Xiao Hong, Guangzhou Institute of Traumatic Surgery, and Dr. Li Zhi, Department of Pathology, Sun Yat-Sen University, for morphological slide preparation. I am also very grateful to Ms. Irene Koo Ching, Mr. Alan Ho Hing Man, Dr. Zhou Li Min, and other staffs in School of Chinese Medicine, for their technical supports throughout the period of my PhD study.

I am also thankful to Dr. Chen Lei, Dr. Cao Hui Hui, and Mr. Chow Yeuk Lung, who as good friends, always gave me support and encouragement. It would have been a lonely lab without them. Many thanks to Dr. Yu Hua, Dr. Xiao Hai Tao, Dr. Zhang Jing Lu, Dr. Wang Yuan Yuan and Mr. Deng Bin, they were always willing to help and give their best suggestions.

I would like to express my thanks to Mr. Lo Kam Fai, Graduate School of Hong Kong Baptist University, who bothered to solve a lot of practical problems for me in emergency situations during the last year.

Finally, I owe a lot to my parents and my loved one, who encouraged and helped me at every stage of my personal and academic life, and longed to see this achievement come true. They were always there cheering me up and stood by me through the good and bad times.
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>As</td>
<td>Arsenic</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BMPs</td>
<td>bone morphogenetic proteins</td>
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<tr>
<td>BS</td>
<td>Bai Shao</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>Cd</td>
<td>Cadmium</td>
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<tr>
<td>CHM</td>
<td>Chinese herbal medicine</td>
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<tr>
<td>CMM</td>
<td>Chinese material medica</td>
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<tr>
<td>CS</td>
<td>Chi Shao</td>
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<tr>
<td>CX</td>
<td>Chuan Xiong (Ligusticum chuanxiong)</td>
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<tr>
<td>DAD</td>
<td>diode-array detector</td>
</tr>
<tr>
<td>DG</td>
<td>Dang Gui</td>
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<td>Dang Gui Wei</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<td>EB</td>
<td>embryo bodies</td>
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<tr>
<td>ECD</td>
<td>electron capture detector</td>
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<tr>
<td>ECVAM</td>
<td>European Centre for the Validation of Alternative Methods</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>EMF</td>
<td>external malformed fetus</td>
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<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ERF</td>
<td>early resorbed fetus</td>
</tr>
<tr>
<td>ERS</td>
<td>early resorption site</td>
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<tr>
<td>ESCs</td>
<td>embryonic stem cell</td>
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<tr>
<td>EST</td>
<td>embryonic stem cell test</td>
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<tr>
<td>EU</td>
<td>European Union</td>
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<tr>
<td>FA</td>
<td>ferulic acid</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GC</td>
<td>gas chromatography-mass spectrometry</td>
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<tr>
<td>GD</td>
<td>gestation day(s)</td>
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<tr>
<td>HE</td>
<td>haematoxylin-eosin</td>
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<td>Hg</td>
<td>mercury</td>
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<td>HKCMMS</td>
<td>Hong Kong Chinese Materia Medica Standard</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>ICH</td>
<td>International Conference on Harmonization</td>
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<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
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<tr>
<td>ICR</td>
<td>Institute for Cancer Research</td>
</tr>
<tr>
<td>ICTP</td>
<td>crosslinked carboxyl terminal telopeptide of type I collagen</td>
</tr>
<tr>
<td>Ihh</td>
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</tr>
<tr>
<td>IL-6</td>
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<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
</tr>
<tr>
<td>LD</td>
<td>lethal dose</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LIG</td>
<td>ligustilide</td>
</tr>
<tr>
<td>LOAEL</td>
<td>lowest observed adverse effect level</td>
</tr>
<tr>
<td>LRF</td>
<td>late resorbed fetus</td>
</tr>
<tr>
<td>LRS</td>
<td>late resorption site</td>
</tr>
</tbody>
</table>
LSD  least significant differences
MBW  mean fetal body weight
MDA  methane dicarboxylic aldehyde
mLIF mouse leukemia inhibiting factor
MM   limb bud micromass
MS   mass spectrometry
MTD  maximum tolerated dose
MTT  3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NEAA non-essential amino acid
NMDAR N-methy-D-aspartate receptor
OECD the Organization for Economic Cooperation and Development
Pb   lead
PBS  phosphate buffered solution
PICP carboxyl terminal propeptide of type I procollagen
PIL  post-implantation loss
PIL  post-implantation loss
PTHrP parathyroid hormone-related peptide
RF   resorbed fetus
SDH  *Sheng Di Huang*
SOD  super oxygen dehydrogenises
TCM  traditional Chinese medicine
TGF-β transforming growth factor -β
TNF-α tumor necrosis factor-alpha
TXA2 thromboxane A2
UPLC ultra-performance liquid chromatography
Vit A vitamin A
WEC  
rodent whole embryo culture

WHO  
World Health Organization
CHAPTER ONE

Literature Review
1.1 Developmental toxicity

It was believed that congenital malformation was caused by genetic factor rather than by tragedy in the late 1950s and early 1960s. During this period, up to 12,000 babies with severe defects were born following thalidomide injection by pregnant women [1]. It was one of the first evidence to indicate that not only genetic factor contribute to congenital malformation but the xenobiotics stimulation during pregnancy can cause undesirable birth outcome. The thalidomide catastrophe had a major impact in the field of developmental toxicology. Since then, evaluating the safety of substances during pregnancy was required before license can be granted for each pharmaceutical product.

Developmental toxicity is defined as the "adverse effects induced during pregnancy, or as a result of parental exposure" [2]. Undesired human pregnancy outcome occurs at a very high frequency, including post-implantation loss (31%), major (6-7%) and minor (14%) birth defects, low birth weight (7%), neurologic dysfunction (16-17%), and death under age of 1-year-old (1.4%). By considering the above data, more than half of all pregnancy results in an abnormal birth. To date, various human developmental toxicants were recorded and banned for use during pregnancy. Among these, ethanol, tobacco smoke, cocaine, retinoids, angiotensin converting enzyme inhibitors and angiotensin receptor antagonists exposure represent a significant risk to the pregnant women and infant evident in previous human experience and animal experiments [3].

1.1.1 Embryotoxicity

Embryotoxicity can be defined as the adverse effects on the embryonal development, growth, and/or viability or as presence of abnormal structure. This terminology was given by National Library of Medicine as consequences of substances that enter the maternal system and cross the placental barrier or of radiation during the first period of pregnancy between conception and the fetal stage,
causing embryo death, malformations and/or variations, dysfunctions, altered growth, and functional defects [4].

Before birth, there are three critical periods, pre-implantation, implantation to time of organogenesis, and fetal to neonatal stage. After fertilization, the embryonal development and implantation into the wall of the uterus begins, gastrulation then occurs to form the ectoderm, mesoderm, and endoderm. The formation of the neural plate represents the beginning, while the closure of the secondary palate means the end of organogenesis [3].

1.1.2 Study guideline

1.1.2.1 In-vivo segment II study

A series of international accepted guidelines has been established for detecting developmental toxicity after the thalidomide disaster. The general goal of these guidelines is to identify the no-observed-adverse-effect level (NOAEL) of the test agents, which means the maximum dose level that does not result in significantly adverse effects on the offspring [3].

Testing protocols for developmental toxicity had been established by organizations such as U.S. Food and Drug Administration (FDA) [4], the Organization for Economic Cooperation and Development (OECD) [5], and International Conference on Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [6]. All the guidelines mentioned above rely on the investigators to meet the primary requirements and perform general reproductive and developmental toxicity study, which could be adjusted in different Countries and institutes. Currently, majority of scientists conduct and publish the test results on the basis of segment I, II, III study introduced by FDA.

Since 1988, FDA established three basic types of studies, namely, segment I, II, and III, which are based on exposure during sequential phases of the reproductive
cycle. This guideline has been updated every few years with increasing knowledge about the metabolism and pharmacokinetics of chemicals, and the latest version was updated in 2000. Segment I study focuses on reproductive and fertility study. Male rodents are treated for 70 days before mating, while females are dosed for 14 days before mating. The treatment lasts for the rest of gestation until the beginning of organogenesis. In segment II study, treatment of pregnant females covers through major organogenesis. Segment III study concerns peri-natal and post-natal study, pregnant animals are exposed to test agent from late pregnancy to weaning. Each segment study should be done as a stand-alone study. If needed, these studies can be combined accordingly, to become part of a multi-generation reproduction study. Complete multi-generation studies may be required for anticipating low-level chronic exposure to populations like environmental pollutants and food supplements.

Segment II study attentively evaluates structural growth and in-utero development of conceptus, including death, malformations, functional deficits, and growth retardation [4]. Key elements of representative guidelines for evaluation of developmental toxicity are provided in Table 1.1 [3].

In segment II study, the test substance is administered to pregnant animals at least from the day of implantation to the end of the closure of the secondary palate, and also the time of embryo developing into fetus. All animals should be sexually mature, primiparous female with comparative age and size. At least 16 pregnant animals should be used to ensure that enough litters are produced to evaluate the toxic potential of the test agent. The exposure of chemical covers the major organ development (GD6 - GD15 for rodent). At least three doses and a concurrent control should be used. The dose levels should be spaced to produce a gradation of toxic effects. Unless limited by the physical/chemical nature or biological properties of the test agent, the highest dose should be chosen to induce some developmental and/or maternal toxicity, but not death. And one intermediate dose should produce
minimal detectable toxic effects. The lowest dose level should not produce maternal or developmental toxicity [7].

During gestation, an assessment can be made in view of whether the observed maternal side effects are transient or permanent. Test animals should be sacrificed one day prior to the expected day of delivery. Fetal evaluations generally take place at term. For many test chemicals, the manifestation of the altered development is more obvious with the increasing dose (i.e., from no effect to lethality). Relevant historical control data (i.e., source, strain, identical species, age, vehicle, route and duration of administration, operating personnel) may be used to avoid genetic drift in the experimental animal population and increase the understanding of the study results [8].

**Table 1.1 Guidelines of developmental toxicity.**

<table>
<thead>
<tr>
<th>Study</th>
<th>Exposure</th>
<th>Endpoints</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA Segment II</td>
<td>Implantation (or mating) through end of organogenesis (or term)</td>
<td>Viability, weight, and morphology (external, visceral, and skeletal) of conceptuses just prior to birth</td>
<td>Shorter exposure to prevent maternal metabolic adaption and to provide high exposure to the embryo during gastrulation and organogenesis. Earlier dosing option for bioaccumulative agents or those impacting maternal nutrition. Latering dosing option covers male reproductive tract development and fetal growth and maturation</td>
</tr>
<tr>
<td>ICH 4.4.3: effects on embryo/fetal development</td>
<td>Implantation till end of organogenesis</td>
<td>Viability and morphology (external, visceral, and skeletal) of fetuses just prior to birth</td>
<td>Similar to Segment II study</td>
</tr>
<tr>
<td>OECD 414: prenatal developmental</td>
<td>Implantation (or mating) thorough day prior to cesarean section</td>
<td>Viability and morphology (external, visceral, and skeletal) of fetuses just prior to birth</td>
<td>Similar to Segment II study</td>
</tr>
</tbody>
</table>
Recently, a positive control or a comparative control is recommended to be employed during segment II study by guidelines [4,5]. To date, various human development toxicants such as ethanol, cocaine, retinoids, angiotensin converting enzyme inhibitors were identified [3]. As we know, vitamin A plays an essential role in the various aspects of reproductive and developmental physiology. However, clinical studies reported that when pregnant women take vitamin A as retinol/retinyl esters above 25,000 IU/day during pregnancy, it could induce birth defects. It demonstrated that high dose level of vitamin A is teratogenic to a number of species [9], and the pattern of birth defects caused by vitamin A is called retinoic acid syndrome which represents malformation in central nervous, craniofacial site, cardiovascular system, and thymus [10]. In this study, vitamin A was used as a positive control in the animal experiments.

1.1.2.2 In-vitro embryonic stem cell test

In addition to segment II guidelines for developmental toxicity test in animals, alternative test system has been developed and proposed to reduce the use of animal and to obtain more rapid information concerning the suspicious developmental toxicants. To date, in-vitro embryonic stem cell test (EST) is the most recommended alternative studies for developmental toxicology by European Centre for the Validation of Alternative Methods (ECVAM). Two permanent cell lines (embryonic stem cell and fibroblast cell) are used to mimic the embryonic and adult tissues, respectively. Through EST, the test agents could be classified into three classes of embryotoxicity (non-embryotoxicity, weak and strong embryotoxicity) [11]. Owing to the high-throughout screen and time-effective features, EST has good pore-prospect in pharmaceutical industry.

1.1.3 Fetal bone impairment

Maternal toxicants intake during pregnancy might result in impairments not
only in general growth but also in fetal skeletal development, both of which is well accepted as developmental toxicity endpoints. Development of the fetal skeleton begins with the condensation of mesenchymal cells in the embryo, which subsequently differentiate into chondrocyte, osteoblast and osteoclast, eventually forming cartilage and bone [12]. During the whole process of bone development, consecutive phases such as proliferation, extracellular matrix maturation, and mineralization are represented by a characteristic set of genes expression. Besides, several bone growth regulatory factors do individual or collaborative works in this process, regulating precisely skeletal development. Hence, any disruption of factors’ expression caused by toxicants will affect bone structure, and the skeletal sites vary in their sensitivity to toxic effects.

1.1.4 Maternal toxicity

Developmental toxicity not only results from direct impacts on embryo/fetus, but also may be caused by indirect impacts through toxicity of the agent to the mother [13]. Developmental toxicity in the absence of maternal toxicity is commonly regarded as most serious because the incidence is thought to be the result of greater sensitivity of the developing organism [4]. Since materials exchange between mother and its conceptus, not only limited to nutrients but toxic substances as well, maternal toxicity should not be ignored. Maternal toxicity in developmental study usually represents a decrease in food and water consumption, a decrease in body weight gain, and clinical signs such as vaginal bleeding, diarrhea, piloerection, organ toxicity, and mortality. When maternal toxicity is observed, the developmental toxicity might be indirect because maternal factors such as diseases, malnutrition and stress, for the most part, will affect embryo/fetus development.

1.2 Chinese herbal medicine

Traditional Chinese Medicine (TCM) is a unique system originated in China
and has developed for thousands of years. TCM shows particular concerns with the body’s function rather than the anatomical structures. TCM is a holistic approach to health that strives for harmony in body, mind and spirit. It is believed that the functions of the body are based on *Yin* (陰, means negative, dark, and feminine) and *Yang* (陽, means positive, bright, and masculine), and also the “five-element theory” consisting of wood, fire, earth, metal, and water.

Chinese material medica (CMM) accounts for the majority of treatments of TCM. CMM consists of drugs deriving from herbs, minerals and animals. Chinese herbal medicine (CHM) is an indispensable part of CMM. *Divine Husbandman’s Classic of the Materia Medica* (*Shen Nong Ben Cao Jing*, 《神农本草经》) is considered as the oldest CHM text. It classified 365 herbs into 3 therapeutic categories based on properties and relative toxicities. Later, 1,892 drugs are recorded in *Compendium of Materia Medica* (*Ben Cao Gang Mu*, 《本草綱目》) compiled by Li Shizhen in the Ming Dynasty. Treatises of CHM provide traditional information of usages, toxicities, cautions, precautions and contraindications.

### 1.2.1 Herbal therapy for women health

CHM are widely used for women health. They can be used to treat common diseases like irregular menstruation, infertility, menopause, hypermenorrhea, amenorrhea, etc. Besides, they can be used to treat the illnesses during pregnancy such as threatened miscarriage, vaginal bleeding, gestational hypertension, etc. The ultimate treatment goal of TCM in obstetrics is to maintain the health of both mother and the fetus. Through the knowledge of the anatomy, physiology, pathology and diagnosis of the female reproductive system, a specific theoretic system about gynaecology and obstetrics was developed in TCM. The system mainly involved heavenly tenth (*Tian Kui*, 天癸), blood vigour (*Qi Xue* 氣血), thoroughfare vessel (*Chong Mai*, 衝脈), conception vessel (*Ren Mai*, 任脈), governor vessel (*Du Mai*, 督脈), belt vessel (*Dai Mai*, 帶脈) and uterus (*Bao Gong*, 胞宮). Meanwhile, it is
of vital importance to pay much attention to the function of kidney, liver and spleen. Moreover, harmonising Qi and Xue is the priority in treatment [14].

CHM has been used in gynaecology and obstetrics to treat various diseases for thousands of years. Some formulae have been commonly used over years, such as Si Wu Decoction, Tai Yuan Decoction, Shou Tai Pill, Shao Fu Zhu Yu Decoction, etc.

1.2.2 Herbal therapy for miscarriage

In TCM, miscarriage is a common obstetrical disease, including threatened miscarriage (Tai Lou and Tai Dong Bu An), namely, actual miscarriage and habitual miscarriage in western medicine. In Chinese medicine, miscarriage also takes different names as follows according to the time it occurs during pregnancy [15], including vaginal bleeding (胎漏), restless fetus (胎動不安), hidden labour (暗產), falling fetus (墮胎), small labour (小產) and habitual miscarriage (滑胎).

For the treatment of threatened miscarriage, identifying the cause and the mechanism of the disease is the first criterion. From the perspectives of TCM, the commonest reason is Yin and/or Xue deficiency, and consequently causing overabundance of Yang. Secondly, spleen deficiency causes inadequate engendering transformation, in which may lead to malnutrition to fetus. Meanwhile, kidney deficiency causes insufficiency of kidney essence, in which triggering insecurity of fetus. Lastly, stagnation of Qi may result in water retention. Therefore, to prevent miscarriage, the principal is to calm the fetus by tonifying the kidney and spleen, meanwhile regulating Qi and nourishing blood as well as cooling blood. The most common traditional medicines used during pregnancy have the properties to improve these functions and to meet the requirements [16].

According to our previous researches, the frequent use of CMM which kept the fetus safe and healthy was systematically counted. Table 1.2 [16] showed the top twenty most frequently used CMM during pregnancy.
Table 1.2 Top 20 TCM used in threatened miscarriage.

<table>
<thead>
<tr>
<th>No.</th>
<th>Chinese Name</th>
<th>Pharmaceutical Name</th>
<th>Frequency (n)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>菟絲子</td>
<td>Cuscutae Semen</td>
<td>507</td>
<td>72.0</td>
</tr>
<tr>
<td>2</td>
<td>續斷</td>
<td>Dipasaci Radix</td>
<td>506</td>
<td>71.9</td>
</tr>
<tr>
<td>3</td>
<td>白朮</td>
<td>Atractylodis Macrocephalae Rhizoma</td>
<td>493</td>
<td>70.0</td>
</tr>
<tr>
<td>4</td>
<td>桑寄生</td>
<td>Herba Taxilli</td>
<td>464</td>
<td>66.0</td>
</tr>
<tr>
<td>5</td>
<td>阿膠</td>
<td>Asini Corii Colla</td>
<td>387</td>
<td>55.0</td>
</tr>
<tr>
<td>6</td>
<td>白芍</td>
<td>Paeoniae Radix Alba</td>
<td>366</td>
<td>52.0</td>
</tr>
<tr>
<td>7</td>
<td>黨參</td>
<td>Codonopsis Radix</td>
<td>362</td>
<td>51.4</td>
</tr>
<tr>
<td>8</td>
<td>杜仲</td>
<td>Eucommiae Cortex</td>
<td>351</td>
<td>49.0</td>
</tr>
<tr>
<td>9</td>
<td>甘草</td>
<td>Glycyrrhiza Radix et Rhizoma</td>
<td>338</td>
<td>48.0</td>
</tr>
<tr>
<td>10</td>
<td>地黃</td>
<td>Rehammiae Radix</td>
<td>297</td>
<td>42.2</td>
</tr>
<tr>
<td>11</td>
<td>黃芪</td>
<td>Astragali Radix</td>
<td>282</td>
<td>40.1</td>
</tr>
<tr>
<td>12</td>
<td>黃芩</td>
<td>Scutellariae Radix</td>
<td>275</td>
<td>39.1</td>
</tr>
<tr>
<td>13</td>
<td>當歸</td>
<td>Angelicae Sinensis Radix</td>
<td>246</td>
<td>34.9</td>
</tr>
<tr>
<td>14</td>
<td>山藥</td>
<td>Dioscoreae Rhizoma</td>
<td>190</td>
<td>27.0</td>
</tr>
<tr>
<td>15</td>
<td>砂仁</td>
<td>Amomi Fructus</td>
<td>176</td>
<td>25.0</td>
</tr>
<tr>
<td>16</td>
<td>素麻根</td>
<td>Boehmeriae Rhizoma et Radix</td>
<td>106</td>
<td>15.1</td>
</tr>
<tr>
<td>17</td>
<td>艾葉炭</td>
<td>Artimisiae Argyi Folium</td>
<td>98</td>
<td>13.9</td>
</tr>
<tr>
<td>18</td>
<td>川芎</td>
<td>Chuanxiong Rhizoma</td>
<td>96</td>
<td>13.6</td>
</tr>
<tr>
<td>19</td>
<td>枸杞子</td>
<td>Lycii Fructus</td>
<td>95</td>
<td>13.5</td>
</tr>
<tr>
<td>20</td>
<td>茯苓</td>
<td>Poria</td>
<td>89</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Note: * Percentage (%) = Frequency (n)/704 × 100%.

1.2.3 Herbal safety in pregnant female

CHM is experienced to treat pregnancy for thousands of years. According to documentations of CHM, the medicines carrying embryotoxicity mainly result in Tai Dong Bu An, Hua Tai and also Duo Tai, etc. These herbs were listed into the category of forbidden herbs to pregnancy. In Divine Husbandman’s Classic of the Materia Medica, it mentioned some herbs causing abortion, e.g. Niu Xi (Achyranthis Bidentatae Radix). Compendium of Effective Prescriptions for Women
(Fu Ren Da Quan Liang Fang, 《婦人大全良方》) recorded that the medicine used under great caution and the forbidden herbs to pregnancy in a systemic way. Also, other treatises also have the record of contraindications during pregnancy, such as Synopsis of Prescriptions of the Golden Chamber (Jing Gui Yao Lue, 《金匱要略》), Essential Prescriptions Worth a Thousand Gold for Emergencies (Bei Ji Qian Jin Yao Fang, 《備急千金要方》), etc. In Compendium of Materia Medica, there are 84 kinds of herbs that are banned during pregnancy. In Chinese pharmacopoeia (2010), there are 69 types of herbs which are forbidden or should be used with great caution during pregnancy. For instance, Yuan Hua (Genkwa Flos), Jiang Da Ji (Euphorbiae Pekinensis Radix), Cao Wu (Aconiti Kusnezoffii Radix), Chan Su (BufonisVenenum), Da Huang (Rhei Radix et Rhizoma), Bing Pian (Borneolum Syntheticum), Bai Ji Li (Tribuli Fructus), etc. are the forbidden absolutely, while Di Long (Pheretima), Gan Jiang (Zingiberis Rhizoma), Da Fu Pi (Arecae Pericarpium), Bai Guo (Ginkgo Semen), Bu Gu Zi (Psiraloeae Fructus), Chang Er Zi (Xanthii Fructus), Dang Zhu Ye (Lophatheri Herba), etc. should be used with great caution, as they are believed to cause vaginal bleeding during pregnancy.

There are “Six Prohibitions” concluded according to the documentations of TCM and clinical experiences, namely, active blood, break Qi, purgative, great heat, great cold and poison. Besides, several herbal categories should be prohibited during pregnancy. They are “harsh expellants”, “aromatic substances that open the orifices”, “substances that extinguish wind and stop tremors”, “herbs that warm the interior and expel the cold” and “herbs that invigorate the blood”, etc.

During the past decades, increasing studies were conducted to identify the safety of CMM to pregnancy, but most of them mainly focused on the impacts of miscarriage. In the recent twenty years, the study extended to embryotoxicity and genotoxicity. Bing Lang (Arecae Semen) may decrease the sperm activity in the male mice and cause abnormality in the mice fetus [17]. It was demonstrated that Zhu Sha (Cinnabar) causes genotoxicity, using micronucleus assay and comet
assay [18], as well as compromising sperm quality of rats [19]. The oil extracts of Jing Jie (Schizonepetae Herba), Xi Xin (asari Herba), and Hua Jiao (Zanthoxyli Pericarpium) contain safrrole, which is genotoxic to the animals [20]. Furthermore, Gou Gu Ye (Ilicis Cornutae Folium) [21] and Hong Hua (Carthami Flos) [22] decoctions may cause, to some degree, embryo-fetus developmental toxicity. Ban Xia (Pinelliae Rhizoma) decoction may induce maternal and embryo toxicity [23]. E Zhu (Curcumae Rhizoma) decoction may produce developmental neurotoxicity in rats [24], and serum containing Tu Si Zi (Cuscutae Semen) has impact on embryonic limb buds cartilage development of rats [25].

1.2.4 Herb-herb interaction

1.2.4.1 Herbal formula

In order to treat complicated conditions, or to gain the most efficacy of each herb, herbalists use several kinds of herbs to treat individual patients. Nowadays, the application of herbal formulae was much more common than using single herb. The basic structure of a formula is sovereign (Jun, 君), minister (Chen, 臣), assistant (Zuo, 佐) and courier (Shi, 使) medicines [26,27].

<table>
<thead>
<tr>
<th>Different role</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sovereign</td>
<td>medicine provides the principal curative action on the main pattern or primary symptom</td>
</tr>
<tr>
<td>Minister</td>
<td>medicine strengthens the principal curative action</td>
</tr>
<tr>
<td>Assistant</td>
<td>medicine treats the combined pattern/syndrome, and relieves secondary symptoms or tempers the action of the sovereign medicine when the latter is too potent</td>
</tr>
<tr>
<td>Courier</td>
<td>medicine directs action to the affected meridian/channel</td>
</tr>
</tbody>
</table>

There are several popular formulae used in clinic during pregnancy for a long time in China. Amongst all the formulae studied for miscarriage, Dang Gui, Bai Shao, and Di Huang frequently occur in various formulae (Table 1.3).
### Table 1.3 Chinese herbal formulae used in pregnancy.

<table>
<thead>
<tr>
<th>Formula name</th>
<th>Source</th>
<th>Herbal ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si Wu Decoction</td>
<td>《和剂局方》</td>
<td>當歸、白芍、熟地黃、川芎</td>
</tr>
<tr>
<td>Ba Zhen Decoction</td>
<td>《正體類要》</td>
<td>當歸、白芍、熟地黃、川芎、黨參、白朮、茯苓、甘草</td>
</tr>
<tr>
<td>Bao Chan Wu You Decoction</td>
<td>《傅青主女科》</td>
<td>當歸、白芍、川芎、荊芥穗、炙黃芪、艾葉、厚樸、枳殼、菟絲子、川貝母、羌活、甘草、生薑</td>
</tr>
<tr>
<td>Jiao Ai Decoction</td>
<td>《金匱要略》</td>
<td>當歸、生地黃、白芍、川芎、艾葉、阿膠、甘草</td>
</tr>
<tr>
<td>Sheng Yu Decoction</td>
<td>《蘭室秘藏》</td>
<td>當歸、生地黃、熟地黃、川芎、人參、黃芪</td>
</tr>
<tr>
<td>Tai Shan Pan Shi San</td>
<td>《景嶽全書》</td>
<td>當歸、白芍、熟地黃、川芎、人參、黃芪、續斷、白朮、黃芩、砂仁、炙甘草</td>
</tr>
<tr>
<td>Tai Yuan Yin</td>
<td>《景嶽全書》</td>
<td>當歸、白芍、熟地黃、人參、杜仲、白朮、陳皮、炙甘草</td>
</tr>
</tbody>
</table>

### 1.2.4.2 Herb-pair

Herb-pairs are commonly used in majority of formulae. TCM practitioners usually consider adding paired-herb to perfect the original formulae rather than adding a single herb. For example, adding Tao Ren-Hong Hua into Si Wu Decoction to form Tao Hong Si Wu Decoction, adding Ren Shen-Huang Qi to form Sheng Yu Decoction. That’s why herb-herb interactions are regarded as being more and more important, not only in reducing side effects, but also increasing pharmacological efficiency.

The first treatise of herb-pair was Lei Gong herb-pairs (Lei Gong Yao Dui, 《雷公藥對》), which was composed over a thousand years ago. Herb-pair is the smallest usage of combination of herbs. Although usually two herbal medicines were selected, it can still achieve the goal of effective pharmaceutical uses. Herb-pair involves the fusion of “seven-emotion” [27].
<table>
<thead>
<tr>
<th>七情</th>
<th>Seven-emotion</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>單行</td>
<td>Single use</td>
<td>using a single medicine to achieve the desired therapeutic effect</td>
</tr>
<tr>
<td>相須</td>
<td>Mutual</td>
<td>two medicines with similar properties used in combination reinforce each other’s functions</td>
</tr>
<tr>
<td>相使</td>
<td>Mutual</td>
<td>two medicines with certain similarities in property and function used in combination to increase therapeutic effect: one acts as the sovereign, the other as an assistant</td>
</tr>
<tr>
<td>相畏</td>
<td>Mutual</td>
<td>two medicines used in combination: toxic reactions or side effects from one can be neutralized by the other</td>
</tr>
<tr>
<td>相殺</td>
<td>Mutual</td>
<td>two medicines used in combination: one can weaken the toxicity or side effects of the other</td>
</tr>
<tr>
<td>相惡</td>
<td>Mutual</td>
<td>two medicines used in combination: one reduces the therapeutic functions of the other</td>
</tr>
<tr>
<td>相反</td>
<td>Mutual</td>
<td>two medicines used in combination may cause toxic reactions or side effects</td>
</tr>
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There are lots of popular herb-pairs frequently used in formula treatment for gynecological diseases. *Synopsis of Golden Chamber* suggested using *E Jiao* (Asini corii Colla) and *Ai Ye* (Artemisiae Argyi Folium) to treat vaginal bleeding during threatened miscarriage. *Dang Gui-Bai Shao* and *Dang Gui-Sheng Di Huang* are famous herb-pairs are commonly presented in a plenty of formulae used for preventing miscarriage.

1.3 Angelicae Sinensis Radix

1.3.1 General information

The genus Angelica (Umbelliferae family) is perennial apiaceous plant. About 90 species have been found around the world and mainly distributed in Asia (China, Japan, South and North Korea) and New Zealand. Up to 45 species are planted in
China, therein, 32 species and 2 varieties are quite peculiar to China [28]. Medicinal herbs belonging to Angelica genus include *Angelica sinensis* (Oliv.) Diels, *Angelica pubescens* Maxim. F. *biserrata* Shan et yuan, *Angelica dahurica* (Fisch. ex hoffm.) Benth, et Hook. f., etc., which have long been used in traditional medicine in the Far East. Angelicae Sinensis Radix is the dried root of *Angelica sinensis* (Oliv.) Diels. Its use was first recorded in the ‘Divine Husbandman’s Classic of the Materia Medica’. *Dang Gui* (DG) is the Chinese name, literally means “state of return,” which refers to its ability to regulate “Qi” and “Xue” to maintain a normal state of well-being, which has been used for more than 2000 years in China. DG is a popular traditional herbal medicine mainly produced in Gansu, Sichuan, Yunnan, Shaanxi, and Hubei province [29]. Nowadays, in addition to the common usage in Far Eastern countries, DG is also being used as a functional food in Germany, UK and the U.S.A., etc.

CMM processing is a pharmaceutical technique to fulfill the different requirements of therapy, which is well-known to have its unique function, e.g., to reduce toxicity, to enhance efficacy, and to stabilize bioactive ingredients. Different processing methods of TCM have been developed for thousand years, which could be classified into the following categories: stir-frying without additional adjuvants, stir-frying with solid adjuvants, such as fine powder of terra, rice, sand, etc., stir-frying with liquid adjuvants, such as yellow rice wine, vinegar, ginger, etc., steaming, calcining, boiling, etc. [30]. The Chinese pharmacopeia records three methods applied for processing DG, including dry-fried with yellow rice wine or with earth, and calcining. DG under different processing methods will be used for producing different pharmacological effects. For instance, dry-frying DG increases its warmth, which makes it an ideal agent to tonify the blood without diarrhea. DG stir-fried with wine could activate blood circulation and stimulate menstrual discharge; DG stir-fried with earth is regarded to enrich blood and tonify spleen; DG dry-fried with grain-based liquor more strongly moves the blood and is
commonly used to treat menstrual pain, traumatic bruises; Charred DG is used for stopping bleeding and metrorrhagia.

DG has been chemically extensively analyzed, and over 70 compounds have been isolated and identified from DG [31]. The major constituents of DG include volatile oil and water-soluble components. Although the former accounts for only 0.62% of the whole chemicals, it is responsible for far-ranging pharmacological effects. There are three different properties oil in DG, which are phenolic, neutral, and acid oil, respectively. Ligustilide (LIG), one of the neutral volatile oil, is the major ingredient accounts for over 60% of all the volatile oil. The water-soluble constituents consist of various types of coumarins, organic acids, amino acids, polysaccharides, and flavonoid derivates. Furthermore, ferulic acid (FA) is regarded as the representative ingredient in DG selected for quality control of DG until now. However, given that the complex chemical constituents of DG, it is currently recommended to assess the quality of DG through FA and LIG together. With the development of analytical technology, quality evaluation from analysis of a few components to fingerprinting of DG will become reality.

1.3.2 Pharmacological study

Increasing evidences indicate that volatile oil, organic acids and their esters, polysaccharides from DG are main bioactive components related its pharmacological properties including promoting the production of blood and improve angiogenesis [32,33], dual-direction regulatory effect on uterus [34], and protecting against nervous system injury of fetus from hypoxia [35], etc.

1.3.2.1 Effects on the hematological and hematopoietic systems

DG has been regarded as an effective herbal medicine for enriching the blood. Modern pharmacological studies indicate that DG promote hematopoietic function of marrow and spleen cell, significantly increasing hemoglobin and erythrocyte and
activating the hemopoietic stem cell in the periphery region. Evidences suggested that polysaccharides, the major constituents from DG, play a role in hematopoiesis [33]. Animal experiment-based study indicated that subcutaneous administration of polysaccharides could significantly enhance the proliferation and differentiation of pluripotent hemopoietic stem cells and hematopoietic progenitors to granulocytes and monocytes, thus promoting the synthesis of hemoglobin and the formation of erythrocyte. Besides, polysaccharides stimulate macrophages in a direct or indirect way and promote the synthesis and secretion of hematopoietic modulating factors by handling gene and protein. Besides, iron, vitamin B₁₂, nicotinic acid, folic acid, folinic acid, and biotin, from DG could contribute to hematosis to some extent [32]. Based on its significant anti-platelet aggregation and anti-thrombotic effects, DG is well-known for invigorating the blood circulation. Studies indicate that DG could improve rheological behavior of blood, and correct the irregular process so as to inhibit thrombus formation. Moreover, FA has anti-platelet aggregative effect, as well as inhibiting thromboxane A₂ activity and synthesis, therefore increases prostaglandin I₂ bioactivity. Investigation on mice and rabbits treated with polysaccharides and sulfuric acid ester shows blood coagulation time, thrombin formation time and partial thromboplastin activation time were significantly prolonged, while bleeding time was decreased, and prothrombin forming time was not interrupted [36].

1.3.2.2 Effects on uterus

Several researches indicated that DG demonstrates double effects, “contraction” and “relaxation”, on the smooth muscle of the uterus, the aqueous and alcohol extracts tend to stimulate the uterus, while the volatile oil inhibits it [34]. Furthermore, one of the in-vitro studies observed that different concentrations of volatile oil perform the opposite impact on normal uterus. Lower dose of volatile oil (≤ 20 mg/L) showing excitation to normal uterus of rat ex-vivo even those effect
is not significant, while higher dose (≥ 160 mg/L) suppressing the amplitude and intension of contraction remarkably. Moreover, the volatile oil of DG could be divided into three parts including acidic (A₁), phenolic (A₂), and neutral parts (A₃). It demonstrated that A₁ (≤ 160 mg/L), A₂ (≤ 10 mg/L) stimulated the uterus, while A₁ (≥ 320 mg/mL), A₂ (≥ 20 mg/L) suppressed the uterus. On the other hand, the A₃ part (range from 10-160 mg/L) showed extraordinary uterine suppression with a potency that is 3.7 times greater than that of total DG volatile oil.

Furthermore, the dual action of DG on uterus is also associated with the status of uterine function. When the uterus is in a state of relaxation, DG could induce contraction. Conversely, if the uterus is in the contracted state, DG might promote relaxation [37].

1.3.2.3 Effects on fetal development

Intrauterine hypoxia causes mental retardation, and the expression of nestin in decidual cells has been found to be related to hypoxia. It was found hypoxia could cause an increase in nestin expression. Pregnant rats given saline (8 mL/kg) by intravenous injection through tail vein could result in intrauterine hypoxia. However, the expression of nestin decreased significantly (p < 0.01) if pregnant rats were pre-given 250 g/L DG injection (8 mL/kg) intravenously [38].

N-methy-D-aspartate receptor (NMDAR) expression in 0-day old rats’ brain tissue was analyzed after pregnant rats suffered from intrauterine hypoxia and in the comparable group where 250 g/L DG injection (8 mL/kg) was given simultaneously via intravenous injection between gestation day 14 to 20. The results showed that expression of NMDA1 mRNA of hippocampal CA3 in-situ hybridization in DG group (33.05 ± 4.32) was significantly decreased (p < 0.05) when compared with pregnant rats without DG administration (56.07 ± 6.85). Another experiment analyzed expression of GFAP and VEGP mRNA in hippocampal CA3 and observed the level of GFAP in dentate gyrus of rats. It
demonstrated DG injection could protect nervous system injury of fetal rats from intrauterine hypoxia [35].

1.3.2.4 Protection of cardiac system

Experimental evidences showed that organic acid from DG could increase coronary arterial blood flow, decrease myocardial oxygen consumption, and relieve the injury caused by cardiac ischemia. Sodium ferulate might stabilize the cardiac myocyte membrane of mice in the atmosphere of hypoxia, and the mechanism of protective effect is related to integrity of mitochondria and lysosome, and stronger ability of anti-hypoxia [39]. In-vivo and in-vitro studies demonstrated that DG elicits a typical cardioprotective effect on doxorubicin-induced oxidative stress [40].

1.3.2.5 Protection of liver

It is said that DG could protect hepatocytes, reduce transaminases and stabilize ATPases activity in liver failure model. Patients administrated with DG showed lower levels and activities of lipid peroxide and glutamic-oxalacetic transaminase compared with pre-administration. Besides, DG with antioxidant property could protect against liver fibrosis induced by various stimuli (e.g., carbon tetrachloride and bovine serum albumin) via increasing the content of SOD and decrease the level of MDA [41].

1.3.2.6 Protection of kidney

The renal function of rats with nephritis administered with sodium ferulate was improved as evident by less urinary protein loss. DG exhibited protective effect on renal pure ischemia-reperfusion injury of rabbit, and the possible mechanism involved regulation of cytokines such as tumor necrosis TNF-α, IL-6, and basic fibroblast growth factor (bFGF) [42], etc.
1.3.2.7 Effects on immune system

It is well known that B-lymphocytes mediate humoral immune and T-lymphocytes mediate cell immunity. Increasing evidence demonstrates that polysaccharides extracted from DG have immunomodulatory activity by regulating expression of Type I T helper lymphocytes (Th1) and Th2 related cytokines. DG polysaccharides significantly increased the production of Th1 related cytokines interleukin (IL)-2 and interferon (IFN)-γ, while decreased the production of Th2 related cytokine IL-4 [43]. The polysaccharides could increase phagocytic activity of macrophage in mice, promoting the proliferation of macrophage and increasing the production of cytokines such as NO, tumor necrosis factor (TNF)-α. Moreover, four water soluble fractions, not only polysscharides, but also oligosaccharides (Angelica oligosaccharide and Angelica sucrose) and Angelica total amino acid served as immunomodulator by interacting with peritoneal macrophages in mice [44].

1.3.3 Clinical application

The Chinese pharmacopeia records the pharmacological effects of DG including hemopoiesis and promotion of blood circulation, regulating menses, releasing pain, and relaxing bowel, etc. DG has been used alone, or in combination with other herbal medicines (e.g., Ren Seng, Shu Di Huang, Bai Zhu, Bai Shao, etc.) to treat diseases, ranging from gynecological disorders and cardiovascular diseases to neurodegenerative diseases and traumatic injuries in clinical practice. For instance, DG has not only been intensively and extensively used to treat gynaecological illness (such as premenstrual syndrome, menopausal symptoms, and menstrual cramps, etc.), but also used for obstetrical diseases. It is believed that DG could tonify uterus and release pain during delivery, recovery from childbirth, among others. There are approximately 80 formulae used for gynecological diseases recorded in ancient medical book Fu Qingzhu's Obstetrics and
DG is most commonly used in formula for liver Qi stasis and spleen deficiency causing various diseases. It is believed to work best in patients with Yin profile, owing to its mildly warm property. DG is thought to return the body to proper condition by harmonizing vital energy and tonifying the blood [45]. In addition to gynaecological and obstetric diseases, patients with other common diseases, such as coronary artery disease, arhythmia, acute ischemic stroke, neurodynia, psoriasis, constipation, etc. also answer to DG treatment to some extent. For instance, Xue Fu Zhu Yu Decoction was applied for coronary heart disease, prosopalgia, and neurosis induced by stagnation of Qi and Xue; Bu Yang Huan Wu Decoction was applied to treat for cerebrovascular diseases such as cerebral infarction, thrombosis, and arteriosclerosis, etc. Besides, it also can be taken by patients with hemiplegia, chronic nephritis, and vasculitis caused by Qi and Xue stagnation in clinic. The popular formula Dang Gui Bu Xue Decoction composed of DG and Huang Qi is recommended for fatigue, blood deficiency, and constipation after delivery; Dang Gui Long Hui Decoction is used as the laxative for chronic constipation of the aged and debilitated-induced liver and gall bladder damp-heat; Dang Gui Si Ni Decoction is in treatment with psoriasis.
In addition to aforementioned prescribed formulae, DG alone also has been used for treatment of various diseases. A case report demonstrated that a patient suffered from persistent atypical polypoid adenomyoma and infertility had a successful live birth after DG and low-dose aspirin intervention [46]. A clinical study of DG injection in the treatment of acute cerebral infarction showed that the total effective rate was 78.7% after two weeks of treatment, which was significantly higher than those in the other control groups (salvia, 63.6%; low molecular dextran injection, 59.3%, p < 0.05) [47]. The anti-platelet effect of DG injection was observed in patients with ulcerative colitis. After 3 weeks treatment, data indicated that the platelet activation was inhibited, and the vascular endothelial cell injury was ameliorated and microcirculation was improved [48].

1.3.4 Safety assessment

1.3.4.1 Adverse reaction and contraindication

Most of the cases about the side effect of TCM caused by the bias of medicines (such as nature and flavor, ascending and floating, and settlement) should attract our attention. As we know, using drugs without symptom differentiation, consideration of contraindication, or with long-term or excessive consumption could lead to adverse reaction, and the symptom and incidence vary with individuals. Although DG is relatively safe to be used for many diseases, adverse effects still occur including bloating, loss of appetite, chronic diarrhea, rash, photosensitivity, and gynecomastia [49]. More serious adverse effects such as anaphylactic shock had been reported previously. Due to its anti-coagulation and estrogen-like effect, DG should not be taken during gestation and lactation period or used in conjunction with anticoagulants such as warfarin; patients with hormone-sensitive conditions such as breast cancer, uterine cancer, ovarian cancer, endometriosis, or uterine fibrosis might deteriorate with exposure to DG; Patients with atopic diseases such as asthma should pay much attention to supplementary
food containing DG since recent study found that DG could release a higher amount of histamine from basophils in the patient than in health control [50]. In addition, from the perspectives of TCM, patients with abdominal distention, spleen deficiency, or patients with loose stools or diarrhea should use DG with caution. Overall, it is not recommended to use DG in excess, or for patients with Yin-deficient heat sign [51].

1.3.4.2 Toxicological study

LIG contributes largely to the side effects produced by DG. It is possible that lipid soluble LIG could cross the blood brain barrier and affect the central nervous system. The central motor nerve system, respiratory system and reproductive system could also be affected by LIG. The LD<sub>50</sub> of the DG in mice ranges from 80 g/kg to 100.6 g/kg; sodium ferulate 1.71 g/kg; DG volatile oil 1 mL/kg by intravenous administration induced intracranial pressure decrease and respiratory depression. DG ether extract 0.06 mL/kg, and 0.02 mL/kg by intravenous injection could be fatal for dogs and cats, respectively. After mice had been administered with 108 g/kg DG ethanol extract, pulmonary and renal haemorrhage was observed. When rats were exposed to LIG 50 μL/kg for 30 days, the index of uterus decreased significantly, and mice administered with LIG 375 μg/kg 15 days, both index of uterus and ovary decreased extraordinarily.

1.3.5 Comparison of different part

According to a famous ancient herbal book ‘Zhen Zhu Nang，《珍珠囊》’, it describes that different part of DG possesses different bio-actions with respect to Xue, the root stocks (Dang Gui Tou, DGT) was superior in stopping bleeding, the branching roots (Dang Gui Wei, DGW) in promoting blood, and the main roots (Dang Gui Shen, DGS) in harmonizing blood.

According to Chinese pharmacopeia (2010), the DGT 1.5-4 cm in diameter,
appears to be annulated, apex obtuse and rounded or with several obvious protrudent rhizome scars, showing purple or yellowish-green remains of stems and leaf sheaths; the DGS lumpy on the surface; while the DGW 0.3-1 cm in diameter, the upper portion thick and the lower portion thin, mostly twisted and exhibiting a few rootlet scars. The modern pharmacological studies demonstrated that relative percentage content of components (FA, essential oil, metallic elements, tannin, amino acids, etc.) in different parts of DG had significant difference, which accounts for the extracts from different parts of DG possessing different and specific pharmacological and toxicological actions. For example, the contents of calcium, copper, zinc in DGT were much higher than those in DGS and DGW, and the contents of potassium and iron were markedly higher than those in DGT and DGS [52]. It is well accepted that calcium promotes blood clotting and hemoglobin solidification. In addition, study showed that the content of tannin in DGT was higher than that in the other part of DG [53]. The bioactive tannin is regarded as a stringent extracted from herbal medicine. Hence, the higher calcium and tannin levels in DGT might do better than the other parts of DG in controlling haemorrhage, consistent with the theory proposed thousands of years ago. Sodium ferulate is one of the anti-platelet ingredients, and the content of FA in DGW is higher than that in DGS and DGT [54]. The blood-activating effect of DGT decoction was stronger than those found in other parts, which supports its application in promoting as found in ancient literatures.

### 1.3.6 Commonly used herb-pair

In clinical application, DG is always prescribed with other herb such as *Bai Shao*, *Di Huang*, etc., as the optimal herb-pair for special purpose based on syndrome differentiation.

BS is cool in terms of thermal property, which can nourish the blood and preserve *Yin*, and is the best used for blood deficiency with heat. DG and BS are
always used as herb-pair in traditional Chinese medicine formulae. This herb-pair is mainly used to treat obstetric syndrome. Some cases which include the usage of this herb-pair are shown in Table 1.3.

SDH can enrich Yin, remove pathogenic heat from blood and promote the production of body fluids [55], benefit the kidney. DG can tonify and harmonize the blood, emolliate the liver and regulate the menstruation. They are usually used together as herb-pair in order to harmonize the nutrient and blood.

A simple formula called Er Yi Wan, Dang Gui Di Huang Gao or Da Bu Xue Wan in different classics, using equal portions of DG and SDH for tonifying the kidney and replenishing Yin, treating deficiency of Yin and blood. Other formulae also consist of both DG and SDH, such as Si Wu Decoction, Yi Guan Jian, Bo Zi Ren San from Peaceful Holy Benevolent Prescriptions (Tai Ping Sheng Hui Fang, 《太平聖惠方》) [56,57].

<table>
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<th>Table 1.4 General information of BS and SDH.</th>
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<td><strong>Aspects</strong></td>
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<td>Herb Name in Chinese</td>
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<tr>
<td>Family</td>
</tr>
<tr>
<td>Botanical Latin name</td>
</tr>
<tr>
<td>Pharmaceutical name</td>
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<tr>
<td>English name</td>
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<tr>
<td>Properties</td>
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<td>Meridians</td>
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1.3.6.1 Paeoniae Radix Alba

The genus *Paeonia lactiflora* Pall. (Ranunculaceae family) widely distributed in Asia, North America and Europe, was divided into three sections, including sect. *Paeonia*, sect. *Moutan* and sect. *Onaepia*. In China, 15 species and 4 varieties from *Paeonia* L., were identified. More specifically, the sect. *Paeonia* consists of 9
species and 2 varieties [58]. Paeoniae Radix Alba (*Bai Shao*, BS) is the steamed and dried root of cultivated *Paeonia lactiflora* Pall., which is superior in nourishing the blood.

BS is effective to tonify blood and preserve *Yin*, and best for nourishing the liver to calm liver *Yang* and release pain. Hence, BS is usually combined with *Di Huang*, DG, *E Jiao*, *He Shou Wu* to treat hepatic blood deficiency or anemia. In addition, BS nourishes blood, softens the liver and is an important herb to regulate menstruation and alleviate pain. BS combined with different herbs targeted different symptoms, for example, combined with *Wu Ling Zhi* (*Excremenum Trogopteri seu Pteromi*), *Pu Huang* (*Typhase Pollen*), *Gan Cao* (*Glycyrrhizae Radix*), etc. for menstrual disorders with blood stagnation, with *Huang Qin* (*Scutellariae Radix*), *Huang Lian* (*Coptidis Rhizoma*), and *Mu Dan Pi* (*Moutan Cortex*) for menstrual disorders with heat, with *Du Zhong* (*Eucommiae Cortex*), *Xu Duan* (*Dipsaci Radix*), *Sang Ji Sheng* (*Taxilli Herba*), and *Tu Si Zhi* (*Cuscutae Semen*) for lower back pain or restless fetus due to kidney deficiency, with *E Jiao*, *Mo Han Lian* (*Ecliptae Herba*), *Xian He Cao* (*Agrimoniae Herba*), and *Hai Piao Xiao* (*Endoconcha Sepiae*) to stop abnormal uterine bleeding. Combined BS with DG, *Chai Hu* (*Bupleuri Radix*), *Bai Zhu* (*Atractylodis Macrocephalae Rhizoma*) and *Gan Cao* (*Glycyrrhizae Radix et Rhizoma*) for pre-menstrual syndrome relieves liver *Qi* stagnation, and nourishes the blood and stops pain.

From TCM theory, BS is contraindicated in case of *Yang* deficiency in middle *Jiao* with cold; in postpartum patients with blood stagnation, or for those who are still bleeding; in patients with eczema or rashes from exterior wind attack with incomplete expression [59]. In clinical practice, BS is incompatible with *Li Lu* (*Veratrum nigrum L.*).

Acute toxicological study showed that the LD$_{50}$ of the BS in mice is 81.1 g (crude drug)/kg given by gavage, and the LD$_{50}$ of total glycosides of BS is 159 mg/kg by intravenous administration, and 230 mg/kg by intraperitoneal
administration. The LD$_{50}$ of peoniflorin in mice is 3.53 g/kg via intravenous injection and 9.53 g/kg via intraperitoneal injection [51].

1.3.6.2 Rehmanniae Radix

There are 6 kinds of plants in the genus *Rehmannia* Libosch. ex Fisch. et Mey (Scrophulariaceae family), which have very high medicinal value in China. Rehmanniae Radix, the fresh or dried root tuber of *Rehmannia glutinosa* (Gaert.) Libosch. ex Fisch. Et Mey, the former is known as *Xian Di Huang* (Fresh Rehmannia Root), and the latter known as *Sheng Di Huang* (SDH, Unprocessed Rehmanniae Root). It is recorded in ‘*Shen Nong Ben Cao Jin*’ as “Gan Di Huang”.

According to the Chinese Pharmacopoeia (2010), *Rehmannia glutinosa* (Gaert.) Libosch. ex Fisch. et Mey is the only statutory source of *Di Huang*. It is usually classified as heat-clearing and *Yin*-nourishing herb.

SDH is the most significant medicinal herb to clear heat, cool the blood, nourish *Yin* and engender body fluids. It is commonly used to treat complications of excess heat in which heat is drying body fluids. This herb is especially effective in treating *Xiao Ke* syndrome (similar to diabetes) with *Huang Qi*, *Ge Gen*, and *Tian Hua Fen*. SDH is an excellent herb for treatment of chronic disorders with deficiency heat symptoms such as tidal fever, flushed cheeks, poor appetite, when usually combined with *Zhi Mu*, *Qing Hao* and *Bie Jia*. SDH also had good performance in clinical studies, such as treatment of arthritis, urticaria, etc., up to 90 g of SDH per day given as herbal decoction to patients.

According to the TCM theory, because of sweet and stagnation in nature, SDH is contraindicated in patients with spleen and stomach *Qi* or *Yang* deficiencies or those with abdominal fullness or diarrhea.

Aqueous or methanol extract of SDH 60 g/kg intraperitoneal administration 3 days did not induce any mice death. 18 g/kg aqueous or alcohol extract of SDH administration by gavage for 15 days did not cause fatalities in rats, and no
abnormality was found in internal organs. Oligosaccharide of SDH preparation administration by oral (49.6 g/kg) once or ultrasonic atomization inhalation (9.6 g/kg) for 3 times in 1 day did not produce any toxic effects in mice. When administered by tail intravenous injection for once, LD$_{50}$ is 7.88 g/kg [60].

1.4 Hypothesis and study objectives

Rationale for selection of the tested herbs and herb-herb interaction

DG is one of the world famous herbs frequently used not only as a medicine but also as a food supplement for various purposes. DG has been called the “female ginseng” and served as a health food supplement for women health during pregnancy. Besides, based on the systematic review of clinical data on Chinese medicine for preventing abortion, the frequency usage of DG was ranked as 13th among more than 200 herbs used during pregnancy [61]. Thus, the selection of DG for the study of safety of developmental toxicity is significant. The information on safety of dose-ranging is important for both clinic practitioners and self-therapy consumers.

DG belongs to blood-tonifying herbs with property of warm, and enters the heart, liver and spleen meridian, which could invigorate the blood, nourish the heart and liver, and moisten the intestine. Even so, the different parts of DG should be used for different medicinal purposes according to TCM theory. The head of DG is recommended for the purpose of stopping bleeding, the bottom for promoting blood, and the body for harmonizing blood. It is suggested that different parts of DG possess specific medicinal properties and pharmacological activities, even toxicities. In addition, based upon the theory of herb-pair, which indicates that the two types of combinations [62] are two herbs complement to each other (i.e., herbs with similar function, herb with the same meridian, etc.) and two herbs opposite to each other (i.e., cold or cool herb with hot or warm herb, strong herb with calm herb, mobilizing herb with setting herb, etc.). Therefore, the rationale for selection of
herbs for exploring the herb-herb interaction is crucial. The herb should be better to (1) have opposite property (cold or cool) to balance either deficiency or excessiveness of the body; (2) have similar function (tonifying) in consideration of application during pregnancy; and (3) have the same meridian (heart, liver and spleen). BS and SDH are cool in property. BS tonifies the blood and pacifies the liver, harmonizes internal (organs) and enters liver and spleen meridian. SDH could nourish the Yin of the blood and enter heart and liver meridian, when it is used together with DG, it reinforces tonifying the blood. In addition, BS and SDH are also commonly used for treatment of miscarriage (BS: 6th; SDH: 10th) (Table 1.2), while DG-BS and DG-SDH are herb-pair models recorded in plenty of formulae used during pregnancy (Table 1.3). Therefore, DG-BS and DG-SDH could be suitable and typical herb-pair to reflect the theoretical essence of TCM in herbal feature and functions, and also could be the most representative practice in traditional experience since ancient times.

Hypothesis

Research of the xenobiotics on developmental toxicity in rodents is a well acceptable method. Based on the literature review in chapter one, we set the scientific hypothesis as follows:

1) Herbs may have different effects on maternal healthy and embryo-fetal development;
2) Herb (DG) may have dose-dependent impact on embryo-fetal development;
3) The different part of DG (body and tail) may have different impact on embryo-fetal development, which might be attributed to different composition of chemical components from the different part.
4) Herb-herb interaction with an opposite trend may neutralize their actions on embryo-fetal development;
5) The mechanism of developmental toxicity induced by single herb may be
attenuated while two herbs used together.

Objectives of the study

Even though Chinese herbal medicine has been used for thousands of years, the safety of herbal medicine had been scientifically tested for decades. However, researches about safety assessment of herbs on developmental toxicity were not enough. Based upon the above hypothesis, the objectives of present study are expected to be achieved as follows:

1) To evaluate the safety of DG on maternal health and embryonic development in mice

As described in chapter 1.1.3, there is a high degree of correlation between the status of embryo/fetus and maternal condition, which is marked obvious at a certain toxic level. Take maternal toxicity into account while assessment of embryo/fetus developmental toxicity is necessary. DG as “female ginseng” is a world-wide food supplement during pregnancy. Although clinical adverse effects of DG are rarely reported, the evaluation of DG safety during pregnancy remains crucial and closely relevant to the women health and their next generation. Assessment of the safety of DG used during the gestation in mice would provide important toxicological information for safe medication practice.

2) To evaluate the dose-response of DG on embryonic development

Based on the dose level chosen, at least three dose levels were used. The highest dose level should produce some developmental or maternal toxicity, and the lowest dose level should not result in any sign of toxicity. Natural herbs may be toxic at certain level, which is similar to poison, being harmful at different aspects while harmless at low level. Thus, the range between being toxic and non-toxic is challenging, which determines the safe spectrum of its medical application. In
Chinese Pharmacopoeia (2010), it mentions that the daily usage of DG by oral route is 9-12 g for adult per day. Actually, during TCM experience, the usage of DG is variable based on the syndrome differentiation. Moreover, DG, a food supplement and over-the-counter medicine, could be taken by people without specialist recommendation. Therefore, DG over-dose can potentially occur with adverse effects, especially during sensitive period such as pregnancy. Research on toxicity impact of DG on embryonic development in mice is vital in analyzing and revealing dose-response relationship.

3) **To compare the DG body and tail in the aspect of chemical composition, and to evaluate their respective cytotoxicity on embryonic stem cell**

The theory about different part of DG possesses different medicinal properties was first proposed as early as hundred years ago. Comparative composition analysis of DG body and tail has demonstrated they have different medicinal functions or pharmacological activities because of various compositions of chemical ingredients. Therefore, it is possible that different part of DG shows different impact on the embryo-fetal development. Research on safety of different part of DG on embryonic stem cell and comparison of constituent composition from each part would give a valuable clue to identify the toxic-related chemicals.

4) **To examine herb-herb interaction on embryonic development**

Identification of DG effect in mice embryonic development would be critical for description during pregnancy. However, clinical use of herbal medicine usually consist of paired or multiple herbs rather than as single herb. Single herb application aims to exert its function, and herb-pair application aims to complement to each other and decrease the adverse effects of each other as well. Thus, it is necessary to explore whether or not developmental toxicity induced by single herb could be attenuated or reduced by combination with another herb.
5) To investigate the impaired bone development mechanism emphysis on DG-induced developmental toxicity

It is well accepted that intrauterine growth retardation and skeletal variation are developmental toxicity endpoints. Embryonic toxicity can be represented by abnormal bone development. Investigating bone markers’ change would provide valuable information for assessment of DG safety on embryonic development in mice.
CHAPTER TWO
Research Design and Methodology
2.1 Material

(1) Materials for preparation of herb decoction


(2) Materials for high-proformance liquid chromatography (HPLC) analysis

Instruments: Agilent 1100 series, Alltima C\textsubscript{18} column (250 mm × 4.6 mm, 5 μm), DAD Detector, 0.45 μm microporous filters, volumetric flasks.

Chemicals and reagents: reference standards (ferulic acid (FA), Z-ligustilide (Z-LIG), paeonflorin, catalpol) were purchased from the National Institute for the Control of Pharmaceutical and Biological products (NICPBP) (Beijing, China). Methanol, acetonitrile, phosphate acid, acetic acid (HPLC grade), deionized water were obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA).

(3) Materials for heavy metal and pesticide residues analysis

Instruments: Shimadzu GCMS-QP 2010 equipped with a DB-17MS (30 m × 0.25 mm) capillary column. Gel permeation chromatography (Waters 515 HPLC pump equipped with a 2487 Dual λ Absorbance Detector) performed by a Water Envirogel\textsuperscript{TM} GPC Cleanup column (Waters. USA). Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) (Perkin-Elmer Elan DRC-II, USA).

Chemicals and reagents: 20 pesticide standards (α-hexachlorobenzene, heptachlor, lindane, pentachloroaniline, quintozene, β-hexachlorocyclohexane, δ-hexachlorocyclohexane, γ-hexachlorocyclohexane, aldrin, oxychlordane, methyl pentachlorophenyl sulphide, heptachlor epoxide, cis-chlordane, trans-chlordane, p,p’-DDE, endrin, dieldrin, p,p’-TDE, p,p’-DDT, o,p’-DDT) were obtained from Chem Service (PA, USA). 2,4,5,6-tetrachloro-m-xylene (internal
standard) was purchased from Aldrich (Shanghai, China). Pesticide grade acetone, dichloromethane, ethyl acetate, diethyl ether, iso-octane and n-hexane (VWR, BDH Prolabo, UK) were used in the experiment. Four internal standards (cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As)) were purchased from GBW. Analytical grade nitric acid and deionized water were obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA).

(4) Materials for gas chromatography–mass spectrometry (GC-MS) analysis

Instruments: Shimadzu QP2010 GCMS (Shimadzu, Japan) equipped with an AOC-20i auto sampler. A DB-5 ms capillary column (0.25 μm × 30.0 m × 0.25 μm), pasteur pipettes, volumetric flasks, micro syringes, 0.02 μm microporous filters, 2.0 mL glass vials with autosampler vital glass insert.

Chemicals and reagents: 95% n-Hexane (RCI Lab-scan, Bangkok, Thailand).

(5) Materials for liquid chromatography–mass spectrometry (LC-MS) analysis

Instruments: Waters Acquity™ ultra-performance liquid chromatography (UPLC) system (Waters Corp., Milford, USA) consisting of a binary pump, autosampler, thermostated column compartment and diode array detector (DAD). A Bruker MicrOTOFQ system by an electrospray ionization (ESI) interface (Bruker Daltonics, Bremen, Germany). A Waters BEH C₁₈ column (1.7 μm, 2.1 mm × 100 mm, Waters Corp.) with a VanGuard™ pre-column (BEH, C18, 1.7 μm, 2.1 mm × 5 mm).

Chemicals and reagents: Chromatographic grade formic acid (Fluka, Buchs, Switzerland), chromatographic grade acetonitrile (RCI Lab-scan, Bangkok, Thailand), deionized water obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA).
(6) Materials for animal experiments

Lab wares: electronic scale, stereomicroscope, magnifier, ruler, scalpel, dissecting scissors, haemostatic forceps, tissue forceps, tray, beakers, cylinders, syringe (1 mL, 5 mL), test tube (15 mL, 50 mL), feeding needles (No.12).

Chemicals and reagents: 100% ethanol, saline, formaldehyde, 4% paraformaldehyde, potassium hydroxide, picric acid, glycerol, glacial acetic acid, Alizarin red powder, vitamin A pills (25,000 IU/pill, Guangzhou Chem Reagents Ltd., Guangzhou, China), xylene, hematoxylin, eosin (Sigma, USA), deionized water obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA).

(7) Materials for in-vitro study

Instruments: incubator (37 ± 1°C, humidified, 5 ± 1% CO₂ in air), laminar flow cabinet, monochromatric microtiter plate reader, water bath (37 ± 1°C), benchtop centrifuge, refrigerator (4°C, -20°C) and freezer (-80°C), liquid nitrogen storage tank, hemocytometer, electronic scale, disposable plastic pipettes (2, 5 and 10 mL), pasteur pipettes, T-flask, tissue culture dishes, 24-well plate and 96-well plate.

Chemicals and reagents: embryonic stem (ES) cell: E14.1 (Cyagen Biosciences Inc. China), Embrynic Fibroblasts (3T3 cells) (ATCC). Dulbecco’s Modified Eagle’s Medium (DMEM) powder (sigma, USA), Sodium Hydrogencarbonate (NaHCO₃) (Tianjin Fuchen Chemical Reagents Factory, China), L-Glutamine (Beijing Dingguo Biotech Co. Ltd), penicillin-streptomycin antibiotic mixture (GIBCO), β-Mercaptoethanol (Beijing Dingguo Biotech Co. Ltd, China), non-essential amino acid (NEAA) (GIBCO), fetal calf serum (FCS) (Hangzhou Sigijing biological Engineering Materials Co. Ltd, China), mouse leukemia inhibiting factor (mLIF) (ESGRO, Millipore), 0.25% trypsin/EDTA solution (GIBCO), phosphate buffered solution (PBS) (GIBCO), 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (sigma, USA), dimethyl
sulphoxide (DMSO) (sigma, USA), deionized water obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA).

(8) Materials for protein expression detection by enzyme-linked immunosorbent assay (ELISA)

Instruments and labware: monochromatric microtiter plate reader, water bath (37 ± 1°C), benchtop centrifuge, refrigerator (4°C, -20°C) and freezer (-80°C), disposable plastic pipettes (2, 5 and 10 mL), Pasteur pipettes, 96-well plate, dissecting scissors, haemostatic forceps, tissue forceps, tray, beakers, cylinders, syringe (1 mL), test tube (15 and 50 mL), feeding needles (No.12), microcentrifuge tube (0.2 and 1.5 mL).

Chemicals and reagents: Milli-Q water, physiological saline solution. Mouse carboxy-terminal telopeptide type I collagen (ICTP) ELISA Kit (Shanghai Lichen Co. Ltd, China); Mouse carboxy-terminal propeptide of type I collagen (PICP) ELISA Kit (Shanghai Lichen Co. Ltd, China); Mouse bone morphogenetic proteins (BMPs) ELISA kit (Shanghai Lichen Co. Ltd, China); Mouse bone specific alkaline phosphatase B (ALP-B) ELISA kit (Shanghai Lichen Co. Ltd, China); Mouse growth differentiation factor 5 (GDF-5) ELISA kit (Shanghai Lichen Co. Ltd, China); Mouse osteocalcin ELISA kit (Shanghai Lichen Co. Ltd, China). Mouse bone morphogenetic protein 8 (BMP-8) ELISA kit (Shanghai Lichen Co. Ltd, China); Mouse bone morphogenetic protein 11 (BMP-11) ELISA kit (Shanghai Lichen Co. Ltd, China); Mouse bone morphogenetic protein 6 (BMP-6) ELISA kit (Shanghai Lichen Co. Ltd, China). Pierce BCA protein assay kit (Thermo scientific, USA).

2.2 Herbal quality control

The herbs of Angelicae Sinensis Radix (Chinese Angelica, Dang Gui, DG) (Batch number: 201112-01/201304-01), tail of DG (Dang Gui Wei, DGW) (Batch
number: 201304-02), Paeoniae Radix Alba (White Peony Root, *Bai Shao*, BS) (Batch number: 201112-02), Rehmanniae Radix (Unprocessed Rehmannia Root, *Sheng Di Huang*, SDH) (Batch number: 201112-03) were purchased from Kang Mei Pharmaceutical Co., LTD in Guangzhou, China. Essential process was conducted according to the clinical application to make them identical to human usage.

(1) **Pharmacognostical analysis**

All the herbs were authenticated by experts of pharmacognosy in School of Chinese Medicine, Hong Kong Baptist University, where voucher specimens were deposited. The pharmacognostical analysis of all the herbs was performed according to Chinese Materia Media (1999) and/or ‘*Xin Bian Zhong Yao Zhi*, 《新编中药志》’ [63].

(2) **Phytochemical analysis**

In phytochemical analysis, HPLC was applied to quantitatively authenticate the main bioactive ingredients in aqueous extracts. The procedure was followed according to Chinese Pharmacopoeia (2010) for BS and SDH, and by Hong Kong Chinese Materia Medica Standard (HKCMMS) [64] for DG and DGW.

(3) **Heavy metal and pesticide residue analysis**

Determination of pesticides and heavy metals was performed according to the HKCMMS guideline [64] for maximum levels of pesticides and heavy metals in medicinal plants. These assays were done only for DG.
2.3 *In-vivo* study

2.3.1 Preparation

(1) Herbal decoction

The water extract was prepared by boiling 2800 g herb (DG, BS, SDH) with distilled water enough for covering the herb in an electronic pot three times each (1 h each time). The total water extract was filtered through gauze (4 layers), concentrated in vacuum below 70°C in a rotary evaporator to prepare 2 g/mL herb water extract (herbal weight)/mL. All of the concentrated extract was transferred to 2 L reagent bottles, sealed the extracts bottle with paraffin, and stored at 4°C for 24 h, -20°C for 24 h, and moved them to -80°C.

(2) Positive control agent (vitamin A emulsion)

Vitamin A oil was mixed with Tween-80 to increase the solubility in Milli-Q water. The mixture was then diluted by Milli-Q water to make up concentration of 200,000 IU/mL vitamin A.

2.3.2 Animal

(1) Selection of animals

It is recommended that test should be performed in the most relevant species. Mice are small, easy to care for, and most importantly, have historically provided consistent results that can be extrapolated to hypothesize human effects. Besides, mice have high fecundity and are highly sensitive to teratogens and embryotoxicity [65]. During experiment, mice are resistant to infection and have relatively longer existing period of vaginal plug compared with rats. Outbred/Swiss ICR mice have relatively consistent genes but with less mutations. Therefore, ICR mice purchased from the Experimental Animal Centre in Chinese University of Hong Kong were selected in this study.
(2) Housing and feeding conditions

In the experimental animal room, temperature was maintained at 22 ± 3°C for rodents. The relative humidity was kept at 50-60%, with 30% and 70% as the lower and upper limits. Provision of artificial light was required, with a control of 12 h dark and light alternatively. Conventional laboratory diets were fed and drinking water was unlimitedly provided.

(3) Mating and grouping

The female mice were allowed to mate with the male mice in a ratio of 2:1 or 3:1 in each cage. Mating was allowed to begin from 4:30 p.m. to 8:30 a.m. in the next day, total of 17 h. Confirmation of pregnancy was carried out at 8:30 a.m. by identification of the presence of vaginal plug which is a white waxy mass from mixture of secretions from coagulation glands, seminal vesicle and vaginal mucus. Once vaginal plug was found at the orificium vaginae, the mice were recognized as pregnant and timed as day 0 of gestation (GD0). It was then separated and put into a new cage in which only pregnant mice were placed. The weight of the pregnant mice was recorded daily. Marking was drawn on the surface of the fur to provide identification of the mice. For the remaining non-pregnant mice, they were separated from the male mice at that time and were allowed to have another mating at 4:30 p.m. on that day by random matching. The process was repeated until the target number of pregnant mice was reached. The total duration of this was about 4 weeks.

The pregnant mice have been randomly assigned into several groups: negative control (distilled water), low-dose group, medium-dose group, high-dose group, even very high-dose group, and positive control (vitamin A). A positive control group was used for the testing method validation.
2.3.3 Treatment

Treatment has been administered by gavage with 1 mL disposable syringe and No. 12 feeding needle.

Table 2.1 Treatment period of Segment II study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Gestation day (GD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control group</td>
<td>Fed with distilled water</td>
<td>From GD6 to 15</td>
</tr>
<tr>
<td>Herb treatment groups</td>
<td>Fed with herb water extract</td>
<td>From GD6 to 15</td>
</tr>
<tr>
<td>Positive control group</td>
<td>Fed with vitamin A (200,000 IU).</td>
<td>GD7, 9, 11</td>
</tr>
<tr>
<td></td>
<td>Fed with distilled water</td>
<td>GD6, 8, 10 and 12-15</td>
</tr>
</tbody>
</table>

2.3.4 Endpoint

Endpoints of maternal toxicity

- Clinical signs and mortality daily
- Body weight twice weekly
- Organ weights & histopathology
- Food and water consumption

Endpoints of developmental toxicity

- Count of resorption, live and dead implantations
- Fetal body weight
- Gross necropsy and histopathology
- External, visceral, and skeletal examination of fetuses

Formulae of terms used as endpoints

- BW change = maternal BW on GD18 - maternal BW on GD0
- Corrected body weight change = maternal BW on GD18 - maternal BW on GD0 - gravid uterine weight
- Organ weight (%) = absolute organ weight/(maternal BW on GD18 - gravid uterine weight) × 100
• Live (dead) fetus/litter (%) = live (dead) fetus per litter/implantation site per litter × 100
• RF/litter (%) = RF (resorbed fetus) per litter/implantation site per litter × 100
• ERF (early resorbed fetus)/litter (%) = ERF per litter/implantation site per litter × 100
• LRF (late resorbed fetus)/litter (%) = LRF per litter/implantation site per litter × 100
• PIL (post-implantation loss)/litter (%) = PIL per litter/implantation site per litter × 100
• EMF (external malformed fetus)/litter (%) = external malformed fetus per litter/total live fetus per litter × 100
• MBW (mean fetal body weight)/litter = total live fetal body weight per litter/live fetus number per litter
• Skeletal variation (%) = skeletal variation (malformation) fetus/total fetus per group × 100
(1) **Typical abnormalities in the segment II study**

The representative pictures of abortion, resorbed and dead fetus, external and skeletal variations in segment II study were shown in Fig.2.1 - Fig.2.8. The external malformations only happened in positive control group.

**Fig.2.1** Typical vaginal bleeding and abortion. (A, B and C, the premature expulsion of the products of conception from the uterus). Normal (D).

**Fig.2.2** Resorptions and death in the segment II study. Early resorption (A, evidence of implantation without recognizable embryo/fetus), late resorption (B, dead embryo or fetus with external degenerative change), dead fetus (C).
Fig.2.3 Typical external malformations in the positive control group. Normal (A), exencephaly (B), short tail (C).

Fig.2.4 Lumbar vertebrae and rib of fetuses of GD18. Control (A), rudimentary rib (B, C) as arrows indicated.
**Fig.2.5** Hindlimb and forelimb skeletons of fetuses of GD18. Normal (A, B). Observe the relative hypoplasia and absence of metacarpals and phalanges of fetuses in the DG treatment group and positive control group (C, D).

**Fig.2.6** Forelimb and hindlimb skeletons of fetuses of GD18. Normal (A, C), arrows indicate the miss of ulna (B) and fibula (D).
Fig. 2.7 Ventral chest wall of fetuses of GD18. Representative six sternebrae forming the sternum in the negative control group (A), observe the scrambling (B), absence of the sternebra (C, D).

Fig. 2.8 Skull of fetuses of GD18. Normal (A, C). Observe the parietal, interparietal and occipital bone defect in positive control group (B, D).
The criteria of fetal skeletal variations identification in mice

According to the previous studies, inappropriate recording of unossified sternebrae or phalanges may increase the control incidence to a very high proportion of fetuses affected, which would then reduce the sensitivity for detecting treatment effects. It is believed that recording high incidences of incomplete ossification at some sites are not worth the effort involved. As we know, the ossification at various sites is highly correlated, therefore reduced to reasonable levels (1 - 6% of control fetuses) and the criteria should be made more objective [66] and the terms must be carefully and clearly defined by each study report. In our case, the criteria were made for recording skeletal alterations with respect to different sites including sternebrae, rib, phalanges and occipital bone as indicated by following figures:

- Ribs

Note: The mice with 12-14 pairs of ribs are regarded as normal development. However, once the rib with irregular development such as asymmetric, particularly in the last pair of rib should be classified as abnormal.
● Sternebrae

Examples should be classified into normal category:

[Images showing normal and incomplete fifth sternebra ossification]

Examples should be classified into abnormal category:

[Images showing fifth sternebra missing, scrambling, and split]

Note: Over 3 sternebra defects (including delayed ossification, split, confusion, and missing) was considered to be abnormality in sternum.
Occipital bone

Note: The completely occipital bone ossification (A) and slightly delayed occipital bone ossification (B) (commonly at the upper site) is classified into the normal development. External malformations such as encephalocele or nosencephalia are commonly accompanied by missing cranial bones.

Phalanges

Note: Abnormal phalangeal development is only considered if delayed ossification was observed in all the phalanges. Thus, figures A, B, C, D, E, F should be classified into normal phalanges development.

Stability of baseline in negative controls

It is well accepted that a concurrent negative control should be included in toxicological studies for detecting non-specific changes and provid a baseline for the assay endpoints, is indispensable. Data changes over time, this may be due to changes in operating personnel in collecting data, changes in environmental
conditions, and genetic alterations in the strain of the species used. Therefore, appropriate cumulative historical negative control data are very useful during the interpretation of endpoints. In our test system, comparison of data from concurrent negative controls provided comparison with cumulative historical data. To establish this data-pool, the maternal and fetal parameters from concurrent negative control were added to previous data-pool. Total 54 pregnant mice were sacrificed as negative controls at the end of this study, and the data were presented in the Table 2.2 and 2.3. Data presented in negative control statistically used for each herb or herb-pairs animal study consisted of the randomly chosen data from cumulative historical negative control and the data from concurrent negative control.

### Table 2.2 Maternal parameters of negative control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant female</td>
<td>54</td>
</tr>
<tr>
<td><strong>Body weight (BW, g)</strong></td>
<td></td>
</tr>
<tr>
<td>BW on GD0</td>
<td>28.59 ± 2.01</td>
</tr>
<tr>
<td>BW on GD18</td>
<td>55.60 ± 4.46</td>
</tr>
<tr>
<td>Gravid uterine weight</td>
<td>19.98 ± 2.63</td>
</tr>
<tr>
<td><strong>BW change</strong></td>
<td></td>
</tr>
<tr>
<td>BW change</td>
<td>27.02 ± 3.48</td>
</tr>
<tr>
<td>Corrected BW change</td>
<td>7.04 ± 1.94</td>
</tr>
<tr>
<td><strong>Organ weight (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7.31 ± 0.59</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.19 ± 0.09</td>
</tr>
<tr>
<td>Heart</td>
<td>0.40 ± 0.03</td>
</tr>
</tbody>
</table>

**Note:** ▲Gravid uterine weight on GD18; BW change = maternal BW on GD18 - maternal BW on GD0; Corrected body weight change = maternal BW on GD18 - maternal BW on GD0 - gravid uterine weight; Organ weight (%) = absolute organ weight/(maternal BW on GD18 - gravid uterine weight) × 100.

### Fetal skeleton examination

Alizarin Red S is an anthraquinone derivative used to identify calcium in tissue and cell. Calcium forms an Alizarin Red S-calcium complex in a chelation process, and shows the end product with orange-red color.
(1) Preparation

Solution of Alizarin red: Glacial acetic acid (2.5 mL), glycerol (5 mL) and 1% chlорal hydrate (30 mL) were mixed together and added alizarin red powder until saturation, stored in brown bottle. Before use, the solution was diluted 1000 fold with 1% potassium hydroxide solution (KOH) to prepare Alizarin Red S solution.

Solution A: Mixture of 1.25% KOH and 100% glycerol (in 4:1 v/v).

Solution B: Mixture of 100% glycerol and distilled water (in 1:1 v/v).

Solution of Bouins: Saturated picric acid (750 mL), 40% formaldehyde (200 mL), and glacial acetic acid (50 mL) were mixed together.

Table 2.3 Fetal parameters of negative control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fetus/group</td>
<td>733</td>
</tr>
<tr>
<td>Implantation site/dam</td>
<td>14.30 ± 1.71</td>
</tr>
<tr>
<td>Total fetus/litter</td>
<td>13.57 ± 1.80</td>
</tr>
<tr>
<td>Live fetus/litter</td>
<td>13.52 ± 1.78</td>
</tr>
<tr>
<td>Live fetus/litter (%)▲</td>
<td>94.71 ± 7.24</td>
</tr>
<tr>
<td>Dead fetus/litter</td>
<td>0.06 ± 0.23</td>
</tr>
<tr>
<td>Dead fetus/litter (%)▲</td>
<td>0.38 ± 1.59</td>
</tr>
<tr>
<td>RF/litter</td>
<td>0.72 ± 1.06</td>
</tr>
<tr>
<td>RF/litter (%)▲</td>
<td>4.92 ± 7.33</td>
</tr>
<tr>
<td>ERF/litter</td>
<td>0.69 ± 1.07</td>
</tr>
<tr>
<td>ERF/litter (%)▲</td>
<td>4.65 ± 7.38</td>
</tr>
<tr>
<td>LRF/litter</td>
<td>0.04 ± 0.19</td>
</tr>
<tr>
<td>LRF/litter (%)▲</td>
<td>0.27 ± 1.42</td>
</tr>
<tr>
<td>PIL/litter</td>
<td>0.78 ± 1.04</td>
</tr>
<tr>
<td>PIL/litter (%)▲</td>
<td>5.29 ± 7.24</td>
</tr>
<tr>
<td>EMF/litter</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>EMF/litter (%)▲</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>MBW/litter (g)▲</td>
<td>1.27 ± 0.10</td>
</tr>
</tbody>
</table>

Note: ▲Live (dead) fetus/litter (%) = live (dead) fetus per litter/implantation site per litter ×100; RF/litter (%) = RF (resorbed fetus) per litter/implantation site per litter × 100; ERF (early resorbed fetus)/litter (%) = ERF per litter/implantation site per litter × 100; LRF (late resorbed fetus)/litter (%) = LRF per litter/implantation site per litter × 100; PIL (post-implantation loss)/litter (%) = PIL per litter/implantation site per litter × 100; EMF (external malformed fetus)/litter (%) = external malformed fetus per litter/total live fetus per litter × 100; MBW (mean fetal body weight)/litter = total live fetal body weight per litter/live fetus number per litter.
(2) Procedure

Live fetuses were stored in 95% ethanol for at least 2 weeks. The fetuses were then being washed with tap water and put in 1-2% aqueous potassium hydroxide for 48 h until the flesh was sufficiently clear for making skeleton visualized. The specimens were immersed in alizarin red-S solution for at least 48 h to allow adequate staining, and the solution was shaken gently a few times a day. The specimen was then immersed in solution A for several days to remove excessive staining. After that, the specimen was then placed in solution B until the soft tissue become transparent. Stereomicroscope was used for the observation of fetus skeleton. All skeletal malformations were recorded and analyzed.

Fetal visceral examination

Method one: The fetuses on GD18 were fixed in Bouin’s solution of saturated solution of picric acid, 37% formaldehyde solution and glacial acetic acid (15:5:1 v/v/v). The fetuses were immersed for 14 days and then washed twice in tap water and stored in 70% ethanol until examination. According to Wilson, the initial horizontal section is made through the naris, the remaining part of the head is dissected by a number of frontal cuts but the body is dissected by a series of transverse cuts as shown below:
Fig.2.9 Wilson’s soft tissue sectioning. (A-I indicated several sectioning).

Method two: The fetuses on GD18 were fixed in 4% paraformaldehyde for 24 h, followed by two tap water washes. The fetuses were immersed in 20% solution of glycerol/egg white (1:1 v/v) for 3 days. Before frozen section, the fetuses were washed by ddH$_2$O. Then, the fetuses were dissected into section using Wilson’s method. The next step was as follows:

**Fetal frozen sections preparation**

Sectioning: Piece of fetus was embedded with O.C.T, frozen sectioning by cryostat, and fixed in slides for staining.

H/E Staining: Slides were immerged in hematoxylin 6 min, subsequently in ddH$_2$O 1 min (observation under microscope), 70% ethanol (1% HCL) 1 min, running water 5 min, eosin 5 s, ddH$_2$O 30 s.

Rehydration: Slides were immerged in 75% ethanol 1 min, subsequently in 95% ethanol 1min, 100% ethanol 1min twice, xylene 2 min twice.

Mounting: Slides were mounted with Canada Balsam and a cover glass.

**Maternal organ (liver, heart and kidney) examination**

**Specimen preparation**

Dehydration process: Samples were immerged in 75% ethanol for 12 h,
subsequently 80% ethanol for 30 min, 96% ethanol for 30 min, 100% ethanol for 30 min, 100% ethanol for 1 h, xylene for 15 min, xylene for 30 min, xylene & paraffin for 1 h (in oven), paraffin for 1 h (in oven), and were embedded in paraffin as blocks.

Sectioning: Samples (4 μm) were sectioned by a rotary microtome. The section was washed by 30% ethanol and placed in 40°C water bath to let them lie flat. Then slides were fixed for staining.

Dewax: Slides were immersed in xylene for 10 min, subsequently in xylene for 5 min, 100% ethanol for 5 min twice, 95% ethanol for 5 min twice, 85% ethanol for 3 min, 75% ethanol for 2 min, ddH₂O for 1 min.

H/E Staining: Slides were immersed in hematoxylin for 6 min, subsequently in ddH₂O for 1 min, 70% ethanol (1% HCL) for 1 min, tap water for 3 min, eosin for 5 s, ddH₂O for 30 s.

Rehydration: Slides were immersed in 75% ethanol for 1 min, subsequently in 95% ethanol for 1 min, 100% ethanol for 1 min twice, xylene for 2 min twice.

Mounting: Slides were mounted with Canada Balsam and a cover glass.

(2) ELISA
1) Embryonic and amniotic fluid specimen preparation

All pregnant mice on GD15 were sacrificed 60 min post final exposure. Amniotic fluid samples were collected from each individual fetal sac using 1mL syringe [67]. During suction process, one hand with hook bending forceps gently squeezed the embryo to one side of the amniotic cavity, and the other hand with syringe made the needle inserted into the middle of the lacuna filled with amniotic fluid. The amniotic fluid was transferred to a microcentrifuge tube for storage at -80°C. The amniotic fluid contaminated with blood was excluded for further use. Each fetus with connected placenta was removed rapidly, and immediately rinsed with ice-cold water to wash off excess blood and to cool the tissue. Then, it was transferred into a 1.5 mL tube individually and quickly frozen in liquid nitrogen.
prior to storage at -80°C [68]. Embryos and the relevant amniotic fluid of each dam were collected via caesarian section in order of proximity to the birth canal alternating between right and left uterine horns [69]. Before usage, embryos were thawed and then homogenized in physiological saline solution (1 g : 1 mL) with cut inside type homogenizer on ice (20 s/ time, 30 s interval, 3 - 5 times), followed by centrifuged at 12,000 g for 10 min, and the supernatant was collected for further use. Each thawed amniotic fluid was centrifuged at 12,000 g for 20 min at 4°C and the supernatant was collected for further use.

2) **Protein quantification**

Total protein content in each homogenate sample was determined using dye reagent (BCA) according to the manufacturer’s protocol (Pierce BCA Protein Assay Kit, Thermo Scientific). Before quantification, the fetus homogenate was diluted 20 times by physiological saline solution (100 μL:1900 μL). Then, the diluted homogenate was further used for ELISA.

Microplate procedure: pipette 25 μL of each standard or unknown sample replicate into a microplate well. Add 200 μL of the working reagent to each well and mix plate thoroughly on a plate shaker for 30 s. Cover plate and incubate at 37°C for 30 min. Cool plate at room temperature. The absorbance was measured at 562 nm on a plate reader.

3) **Preparation of a set of standard**

PICP Kit: Standard (800 ng/mL) with standard diluent concentration was followed by: 5, 25, 50, 100, 200, 300, 400 ng/mL; ICTP Kit: Standard (800 pg/mL) with standard diluent concentration was followed by: 5, 25, 50, 100, 200, 300, 400 pg/mL

BMPs Kit: Standard (48 ng/mL) with standard diluent concentration was followed by: 0.1, 0.4, 1, 4, 8, 16 ng/mL. GDF-5 Kit: Standard (1600 pg/mL) with standard diluent concentration was followed by: 50, 80, 100, 160, 200, 400 pg/mL.
ALP-B ELISA Kit: Standard (16 ng/mL) with standard diluent concentration was followed by: 0.5, 0.8, 1, 1.6, 2, 3.2 ng/mL. Osteocalcin Kit: Standard (80 ng/mL) with standard diluent concentration was followed by: 2.5, 5, 8, 10, 16, 20 ng/mL. BMP-8 Kit: Standard (12 ng/mL) with standard diluent concentration was followed by: 0.5, 0.8, 1, 1.6, 2, 3.2 ng/mL. BMP-11 Kit: Standard (320 pg/mL) with standard diluent concentration was followed by: 10, 20, 32, 40, 80, 160 pg/mL. BMP-6 Kit: Standard (960 pg/mL) with standard diluent concentration was followed by: 30, 60, 96, 120, 240, 480 pg/mL. The standard curve for each marker was produced as below.
Fig. 2.10 Standard curve of bone biomarkers.
4) **Procedure**

Add 50 μL different concentrations of standard solution to each well while nothing was added to Blank wells. Add 10 μL testing sample (amniotic fluid or fetal tissue homogenate) to each well, then add 40 μL sample diluent to each testing sample well. Add 100 μL HRP-conjugate reagent to standard wells and sample wells, cover with an adhesive strip and incubate for 60 min at 37°C. Aspirate each well and wash, repeating the process 4 times for a total of five washes. Wash by filling each well with 400 mL wash solution by multi-channel pipette. Complete removal of liquid at each step was ensured. After the last wash, remove any remaining wash solution by aspirating or decanting. Add 50 μL chromogen solution A and 50 mL chromogen solution B to each well. Gently mix and incubate for 15 min at 37°C. Protecting them from light, and add 50 μL stop solution to each well. The absorbance is measured at 450 nm on a plate reader within 15 min.

5) **Quality assurance and control**

In order to express the precision, or repeatability, of ELISA results, measurement of the intra-assay coefficient of variability (CV) was conducted. The intra-assay CV is an average value calculated from the individual CV for all the duplicates. Four individual samples of fetal homogenates (0.5 mL) from the negative controls were mixed into 2 mL as control to evaluate the intra-assay CV%. The plate means for control from 3 results were calculated and then used to calculate the overall mean, SD, and CV%. Overall CV% = SD of plate means / mean of plate means × 100. The average of the control CV% is reported as the intra-assay CV. In the present study, different plates were randomly selected for intra-assay. The coefficients of variance evaluated by assaying 3 replicates of each sample in a single assay, ranged from 2.13% - 5.11%.
2.4 In-vitro study

2.4.1 Preparation

(1) Cell culture medium for ESC maintenance

A bag of DMEM powder with 3.7 g NaHCO₃ was dissolved in 1000 mL ultrapure water. DMEM with L-glutamin (200 mM), all cell culture media were freshly supplemented with glutamine. Penicillin/streptomycin solution (10,000 U/mL penicillin and 10,000 μg/mL streptomycin). Mercaptoethanol (10 mM): to prepare 10ml of mercaptoethanol, add 7 μL of it to a final volume of 10 mL PBS. All cell culture media should be freshly supplemented with mercaptoethanol. Murine leukemia inhibitory factor (mLIF, 10⁶ U/mL), LIF was provided as a solution added directly to the culture plates/T-flasks during maintenance of mESCs. Heat-inactivated FCS: firstly, thaw bottle of serum overnight at 4°C and then warm the bottle in a water bath to 56°C. Allowing the serum to heat inactivate for 30 min at 56°C (Mixing the serum approximately every 10 min to avoid gelling of serum proteins. When the 30 min heat inactivation period was completed, serum was removed from the water bath and cooled rapidly by placing it in an ice water bath) [70].

To prepare 50 mL of ESC maintenance medium, following solutions were mixed: 38.25 mL of DMEM, 10 mL of heat-inactivated FCS, 0.5 mL of L-glutamine, 0.5 mL of NEAA, 0.5 mL of 10 mM mercaptoethanol and 0.25 mL of penicillin/streptomycin. mLIF is added directly to the plate (4 μL mLIF in 4 mL of medium).

(2) Cell culture medium for 3T3 maintenance

One bag of DMEM powder with 3.7 g NaHCO₃ was dissolved in 1000 mL ultrapure water. DMEM with L-glutamin (200 mM), all cell culture media should be freshly supplemented with glutamine. Penicillin/streptomycin solution (10,000 U/mL penicillin and 10,000 μg/mL streptomycin). Heat-inactivated FCS (same
preparation as mentioned previously).

To prepare 50 mL of 3T3 maintenance medium, following solutions were mixed: 43.75 mL of DMEM, 5 mL of heat-inactivated FCS, 1 mL of L-glutamine, and 0.25 mL of penicillin/streptomycin.

(3) Herb aqueous extract and standards for cytotoxicity

The stock of DG aqueous extract (2 g/mL) was diluted by maintenance medium to obtain final concentrations of 500, 250, 125, 50, 25, 6.25, 2.5, 1.25, 0.1 and 0.05 mg/mL. 200 mg FA was dissolved in 1 mL DMSO and stored in -20 °C. The stock of FA (200 mg/mL) was diluted with maintenance medium to obtain final concentrations of 0.1, 0.2, 0.24, 0.28, 0.3, 0.32, 0.34, 0.36, 0.4, 0.6 mg/mL. 100 mg Z-LIG was dissolved in 1 mL DMSO and stored in -20°C. The stock of Z-LIG (100 mg/mL) was diluted with maintenance medium to obtain final concentrations of 0.002, 0.004, 0.005, 0.006, 0.008, 0.01, 0.014, 0.016, 0.02 mg/mL. The stock of aqueous extract from body of DG (Dang Gui Shen, DGS) and DGW (1 g/mL) was diluted with maintenance medium to obtain final concentrations of 50, 25, 12.5, 6.25, 5, 3.125, 2.5, 1.25 and 0.625 mg/mL. The stock of BS aqueous extract (2 g/mL) was diluted with maintenance medium to obtain final concentrations of 250, 25, 10, 7.5, 6.75, 6.25, 5, 2.5 and 0.5 mg/mL. The stock of SDH aqueous extract (2 g/mL) was diluted with maintenance medium to obtain final concentrations of 20, 10, 4, 2, 1, 0.5, 0.4, 0.2 and 0.1 mg/mL.

(4) Working agent for MTT assay

MTT solution was prepared by dissolving MTT powder into PBS at the concentration of 5 mg/mL and then filtered with 0.2 μm cellulose filter.

2.4.2 Cell culture

Instead of classic embryotoxicity testing performing in-vivo using pregnant
females or cultured embryos or embryonic tissues from pregnant females *ex-vivo*, the permanent ESC and fibroblast 3T3 cell are employed to detect the potential developmental toxic effect of chemicals, which represent embryonic tissue and adult tissue, respectively. Three endpoints were included: the inhibition of ESC differentiation, cytotoxicity of ESC and 3T3 cell. The ESC stays pluripotent in the presence of the LIF. In the absence of the LIF, the ESC will differentiate into various cell types under appropriate conditions. In the present study, ESC line (E14.1) and embryonic fibroblast 3T3 cell line were used.

(1) **Thawing**

Cells taken from liquid nitrogen were thawed in 37°C water bath until all frozen material was melt. Suspended the cells in 4 mL DMEM and centrifuged at 1,000 rpm, 4°C for 5 min to remove DMSO. Discarded the supernatant and suspended the cell pellet in 2 mL cell culture maintenance medium. Added the cells suspension to a 25 cm² tissue culture flask (T-flask) and incubated the cells at 37°C in a humidified 5% CO₂ atmosphere for 3 days.

(2) **Routine culture**

After 3 days of incubation, the cells should approach 80% confluence in the T-flask and were removed by trypsin. The medium in the T-flask was decanted and the cultures were washed twice with 2 mL of PBS. 1 mL of trypsin/EDTA solution was added to the monolayer for 1 min (ESC) or 3 min (3T3 cells). Cell culture maintenance medium were added to stop trypsinization. The cell suspension was transferred to a centrifuge tube and centrifuged in room temperature at 1,000 rpm, room temperature for 4 min. The supernatant was then discarded and the cell pellet was dispersed in 2 mL cell culture maintenance medium by gentle trituration to obtain give single cell suspension for cell counting. After cell number was determined, the cells were subcultured or used in an assay. ESC and 3T3 cells
were routinely passaged at cell density of $5 \times 10^4$ cells/mL every 3 days in 25 cm$^2$ T-flasks.

![Fig.2.11 Cell morphology under light microscope, undifferentiated ESC (left, × 40), fibroblast 3T3 cell (right, × 40).](image)

### 2.4.3 Treatment

For cytotoxicity test, ESC and 3T3 cells growing exponentially were seeded on 96-well plate in the absence of LIF, and cells are treated with several concentrations of test agent 2 h later. After 7 days of incubation with test agent, the MTT assay is performed. The absorbance was interpreted using 570 nm microplate reader [70].

**Day 0**

Add 200 μL PBS into the peripheral wells of a 96-wells plate to create a moist environment for culture. Cell suspension of 500 cells/100 μL was prepared in maintenance medium (for ESC without mLIF). 100 μL cell suspension was dispensed into the remaining wells and incubated for 2 h in a humidified 5% CO$_2$ atmosphere at 37°C. 100 μL test solution was added into each appropriate well and further incubated for 3 days.

**Day 3 & Day 5**

The medium (containing the test compound in an appropriate concentration)
was replaced on day 3 and day 5, respectively.

Day 7

Add 20 μL MTT solution to each well of plate (except peripheral wells). Incubate at the same atmosphere for 4 h. The solutions were decanted in each well carefully. 100 μL DMSO was added to each well. The 96-wells plate was shaked thoroughly for 10 min until the solution is clear and no more clumps were visible. The absorption of each well was measure using a 570 nm microplate reader.

2.4.4 Endpoint

The percentage viability of the treated cells was calculated from the percentage MTT conversion in the test solution treated cells relative to the vehicle control (100% viability). The following equation was used: percentage viability = (individual OD solution treated cells/ mean OD solvent control) × 100% [72].

2.5 Statistical analysis

Data analysis was done using SPSS software (version 16.0). Data presented as mean ± SD among the negative control, herbal decoction treatment groups were compared by one-way ANOVA, and the least significant difference (LSD) test using Post hoc method was applied to analyze the difference between every two groups. Pearson’s χ²-test was used for analyzing category data such as skeletal alterations among the negative control, herbal decoction treatment groups, and bonferroni correction was applied to compare every two groups. The analysis of cytotoxicity assay was conducted to obtain R² values and IC₅₀ values. A student t-test was also used for a comparison between two-group data such as the positive control group versus the negative control group, IC₅₀ for ESC value versus 3T3 cell value, and DG treatment group versus the negative control groups. A p value < 0.05 was considered as statistically significant difference.
CHAPTER THREE

The Study of Angelicae Sinensis Radix
3.1 Introduction

Angelicae Sinensis Radix (Chinese Angelica, *Dang Gui*, DG), the dried root of *Angelica sinensis* (Oliv.) Diels (Fig. 3.1), is ranked as a medium grade drug in *Divine Husbandman's Classic of the Materia Medica*. The nature of DG is sweet, acrid, bitter and warm, and it influences meridians of heart, liver and spleen. DG has been served as herbal medicine for thousands of years. According to ancient herbal books and literatures, DG has been used to tonify the blood and regulate the menses, also for treatment of blood-deficient pattern with symptoms such as a pale ashen face, tinnitus, blurred vision, palpitations and constipation, etc. Besides, DG could invigorate and harmonize the blood, help stop pain from congealed blood, hence commonly used as treatment for abdominal pain, traumatic injuries, and carbuncles, etc. [73].

Traditional Chinese medicine (TCM) believes that DG can treat all blood related disorders. It is said that women’s health is often dependent on the status of *Xue* since ancient times, so DG gained the reputation as the “female ginseng” due to its features in *Xue* tonification and promotion of *Xue* production. DG is one of the most popular herbal medicines recommended by practitioners to treat infertility, easing delivery, reducing pain, recovery from childbirth, etc. among countries of the Far East.

*Fig. 3.1* Medicinal herb, DG.
A recent clinical systematic review about TCM treating threatened or recurrent miscarriage found that the frequency of DG usage was ranked 13th amongst more than 200 traditional medicines. However, the single use of DG was not recommended during pregnancy. So far the precise mechanism is unclear.

Due to the lack of evidence regarding the safety of DG use during pregnancy, this study aimed to investigate the impact of DG aqueous extract on embryonic development in outbred/ICR mice using FDA segment II study. In addition to in-vivo study, in-vitro embryonic stem cell test using embryonic stem cells (ESCs) and the reference 3T3 fibroblast cells was performed to investigate the cytotoxicity of DG water extract and its representative compounds (ferulic acid (FA) and Z-liguistilide (Z-LIG)) based on the European Centre for the Validation of Alternative Methods (ECVAM) guidelines.

3.2 Experiment

3.2.1 Dosage design

According to the Chinese Pharmacopoeia (2010), the maximum daily dosage for a 60 kg human is 12 g.

Human Dosage per day: 12/60 = 0.2 g/kg.

The following formula of dosage conversion was used [74]:

\[ D_2 = D_1 \times \frac{R_2}{R_1} \times \left(\frac{W_1}{W_2}\right)^{1/3}, \]

where

- \( D_1 \) = Human dosage (g/kg)
- \( D_2 \) = Mice dosage (g/kg)
- \( R_1 \) = Relative shape coefficient for human (\( R_1 = 0.1057 \))
- \( R_2 \) = Relative shape coefficient for mice (\( R_2 = 0.0898 \))
- \( W_1 \) = Human body weight (kg) (\( W_1 = 60 \))
- \( W_2 \) = Mice average body weight (kg) (\( W_2 = 0.03 \))

Mouse dosage (\( D_2 \)) = \( 0.2 \times 0.0898/0.1057 \times (60/0.03)^{1/3} \)

= 2.141 g/kg (equal to human daily dosage)
(1) *In-vivo study*

<table>
<thead>
<tr>
<th>Group</th>
<th>Comparison with human daily dosage (times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-dose (2 g/kg) (n=18)</td>
<td>2/2.141 = 0.93</td>
</tr>
<tr>
<td>Mid-dose (8 g/kg) (n=18)</td>
<td>8/2.141 = 3.74</td>
</tr>
<tr>
<td>High-dose (16 g/kg) (n=18)</td>
<td>16/2.141 = 7.47</td>
</tr>
<tr>
<td>Very High-dose (32 g/kg) (n=18)</td>
<td>32/2.141 = 14.94</td>
</tr>
</tbody>
</table>

As the accurate concentration of aqueous exact is 2 g/mL, dilution is required for the low, medium, high, and very high-dose groups. The feeding volume is 0.5 mL per feed per day.

(2) *In-vitro study*

The stock of DG aqueous extract (2 g/mL) was diluted using maintenance medium to obtain final concentrations of 500, 250, 125, 50, 25, 6.25, 2.5, 1.25, 0.1 and 0.05 mg/mL. 200 mg FA was dissolved in 1 mL DMSO and stored in -20°C. The stock of FA (200 mg/mL) was diluted with maintenance medium to obtain final concentrations of 0.1, 0.2, 0.24, 0.3, 0.32, 0.34, 0.36, 0.4, 0.6 mg/mL. 100 mg Z-LIG was dissolved in 1 mL DMSO and stored in -20°C. The stock of Z-LIG (100 mg/mL) was diluted with maintenance medium to obtain final concentrations of 0.002, 0.004, 0.005, 0.006, 0.008, 0.01, 0.014, 0.016, 0.02 mg/mL.

3.2.2 Quality control

3.2.2.1 Herbal authentication

The purchased DG was planted in Minxian County, Gansu Province in China. Methodology of herbal authentication was complied with description in chapter 2.2.

3.2.2.2 Aqueous extract for chemical analysis

(1) High-proformance liquid chromatography (HPLC) analysis

The HPLC - diode detector (DAD) analytical technique was used to determine
the content of FA and Z-LIG in DG in order to control the quality of DG.

Condition: according to Hong Kong Chinese Materia Medica Standard (HKCMMS) [64], setting as: 0.1% acetic acid/ACN = 81/19, wavelength: 280 nm, flow rate: 1.0 mL/min, temperature: room temperature, injection Volume: 20 µL.

Test solution preparation: 2.8 mg FA was weighed and added into a 10 mL volumetric flask. Methanol was then added up to 10 mL and mixed thoroughly. 2.1 mg/L, 10.5 mg/L, 105 mg/L, 210 mg/L, 420 mg/L FA standard were prepared by methanol for use. Z-LIG: 10 mg Z-LIG was added into a 10 mL volumetric flask. Methanol was then added up to 10 mL and mixed thoroughly. 0.5 mg/L, 25 mg/L, 50 mg/L, 100 mg/L Z-LIG standard were prepared by methanol for use. 2 g/mL DG water extract was diluted into 200 mg/mL and 400 mg/mL respectively, and then filtered through 0.45 µm syringe filters for injection.

Procedure: 20 µL of each standard solution and sample solution were injected respectively into the column. Data were collected for calculation.

Method validation: No validation was practiced by following HKCMMS.

(2) Heavy metal analysis

Heavy metals including their respective compounds may be present as a result of contamination. They are often absorbed and accumulated in medicinal herbs. Arsenic, cadmium, lead and mercury are the common ones with relatively high toxicity to human beings.

The quantity of heavy metal can be verified by using ICP-MS with indium as an internal standard.

The criteria of the ICP-MS system are consistent with those listed by HKCMMS, namely, resolution equal or more than 0.7 amu at 10% peak height; mass ranging from 6 to 240 amu and a mass accuracy of ± 0.05 amu. Four standard solutions were prepared in dilute nitric acid (3%, v/v), containing all the targeted
heavy metals at concentrations suitable for plotting calibration curves. The concentration of the internal standard in the test solution was the same as in the standard solutions.

The ICP-MS was optimized according to the manufacturer’s recommended procedures. The isotopes recommended for monitoring the heavy metals are listed as below. The amount of heavy metals in herbs should not exceed the limits according to HKCMMS [64], i.e. Arsenic, cadmium, lead and mercury need to be below 2.0, 1.0, 5.0, and 0.2 mg/kg, respectively.

(3) Pesticide residue analysis

There are different types of pesticide, a synthetic chemical or a biological substance, or a mix.

The quantity and quality of pesticide can be verified by using GC-MS with 2,4, 5,6-tetrachloro-m-xylene as an internal standard.

The criteria of GC-MS system recommended by HKCMMS are: R value of any analyte peak with the adjacent peak: > 1.5; n value: ≥ 105 for the peak of α-BHC; RSD of the peak area: ≤ 55. A capillary column: 0.25 mm × 30 m. Carrier gas: helium. The relative retention time (RTT) and monitoring ions of the targeted pesticides were obtained by GC-MS. The column was maintained at 100°C for 2 min, then raise to 160°C at a rate of 15°C/min, afterwards to 270°C at a rate of 5°C/min and maintain for 10 min. It maintained the temperature of the injector port at 250°C and the temperature of the ion source at 230°C.

3.2.3 In-vivo study

As described in chapter 2.3, the pregnant mice were administered with dose-graded DG water extract. These pregnant mice were observed daily for clinical toxicity signs. The maternal body weight (BW) was recorded on GD0, 6, 12 and 18. The maternal uterus with contents and organs (liver, heart and kidney) were
weighed on GD18 after being sacrificed. Specimen such as liver, kidney and heart of pregnant mice were fixed with 4% paraformaldehyde and sections were stained with haematoxylin-eosin (HE) later for morphological and pathological examination under light microscope or dissecting microscope.

After removal of uterus, the early and late resorptions, dead and live fetuses, implantations were identified and counted. Fetal BW was weighed. All live fetuses were examined for external malformations immediately. Then most of live fetuses were preserved in 95% ethanol for two weeks, eviscerated with 1% KOH for 48 h, then placed in solution of alizarin red-S for further 48 h, and washed with increased concentrations of glycerol for detection of skeletal malformations. The rest of fetuses were fixed in Bouin's solution for visceral assessment.

3.2.4 In-vitro study

As described in chapter 2.4, for cytotoxicity test, ESC and 3T3 cells were treated with several concentrations of test agent. On day 7, MTT assay was performed to test the viability of the treated cells, which were calculated from the percentage MTT conversion in the test solution treated cells relative to the vehicle control (100% viability).

3.3 Result

3.3.1 Herbal evaluation

3.3.1.1 Pharmacognostical analysis

Pharmacognostical analysis was demonstrated that DG reached the required quality standard. Heavy metal and pesticide residues analysis showed that DG aqueous extract met the requirement of quality standard.

The detected contents of DG aqueous extract are well below the regulatory limits of Chinese Pharmacopoeia and HKCMMS, showing no heavy metals contamination, as shown in Table 3.1.
Table 3.1 Heavy metal detection of DG water extract.

<table>
<thead>
<tr>
<th>Heavy Metal</th>
<th>Lead (μg/g)</th>
<th>Cadmium (μg/g)</th>
<th>Mercury (μg/g)</th>
<th>Arsenic (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content</td>
<td>0.11</td>
<td>ND</td>
<td>ND</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Note: ND = Not detected or below limit of detection.

3.3.1.2 Phytochemical analysis

The HPLC chromatograms showed that FA and Z-LIG, two representative chemicals of DG, were presented at retention time around 10.456 and 43.848 min in the reference and 10.676 and 43.905 min in the aqueous extract samples (Fig.3.2).

Fig.3.2 HPLC chromatograms of FA and Z-LIG in the reference samples (upper) and water extract of DG (bottom).
Table 3.2 The concentration of FA in DG water extract.

<table>
<thead>
<tr>
<th>Concentration of DG aqueous extract (mg/mL)</th>
<th>Retention time (min)</th>
<th>Peak Area (mAU*s)</th>
<th>FA Conc. in DG sample (mg/mL)</th>
<th>(mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>9.996</td>
<td>5158.9</td>
<td>0.106884</td>
<td>0.53</td>
</tr>
<tr>
<td>400</td>
<td>9.97</td>
<td>10044.4</td>
<td>0.209713</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Average content of FA in DG sample 0.53

Table 3.3 The concentration of Z-LIG in DG water extract.

<table>
<thead>
<tr>
<th>Concentration of DG aqueous extract (mg/mL)</th>
<th>Retention time (min)</th>
<th>Peak Area (mAU*s)</th>
<th>Z-LIG Conc. in DG sample (mg/mL)</th>
<th>(mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>43.256</td>
<td>132.9</td>
<td>0.0008813</td>
<td>0.00441</td>
</tr>
<tr>
<td>400</td>
<td>43.354</td>
<td>182.1</td>
<td>0.0015269</td>
<td>0.00382</td>
</tr>
</tbody>
</table>

Average content of LIG in DG sample 0.00411

According to the standard curve of FA and Z-LIG, the linear regression equations were $y = 47.511x + 80.711$ ($R^2 = 0.9997$) and $y = 76.209x + 65.737$ ($R^2 = 0.9997$), respectively. The FA and Z-LIG accounted for 0.529 mg/g and 0.411 μg/g of the aqueous extract sample, respectively (Table 3.2 and 3.3).

3.3.2 Maternal evaluation

Treatment with DG water extract did not interrupt the progress of gestation nor induced any clinical toxicity on the pregnant mice. The food consumption decreased slightly during treatment period compared with pre-treatment period, but it steady increased during post-treatment period in G1 - G6 groups, and there was no statistical difference among G1 - G6 groups. The steady water intake occurred in all groups without difference between the groups during gestation. No mortality and morbidity were observed throughout this study. There was no abnormal observation at autopsy.

In Table 3.4, the gravid uterine weight of group G2 - G6 was significantly lower than that in the G1 group ($p < 0.05$). The BW change in each DG treatment...
group was significantly lower than that in the G1 group. Besides, the corrected BW change in the G5 group was significantly lower than that in the G1, G2, and G3 group (p < 0.05). In particular, there was significant difference of corrected BW change between DG extract treatment groups and G1 indicating a negative correlation suggesting that dose-dependent inhibitory effect of DG extract on pregnant mice. Besides, the corrected BW change in G6 group was significantly lower than that in the G1 group (p < 0.05).

The difference of organ weight (%) between the G1 group and DG treatment groups was found to be unremarkable. The histopathological examination of organ sample (heart, liver and kidney) from different groups was also conducted to be sure of no organ toxicity. Noticeably, despite significantly higher kidney- and heart-weight (%) was recorded in G6 group when compared with G1 group (p < 0.05), there was no microscopic difference observed.

3.3.3 Fetal evaluation

As shown in Table 3.5, the live fetus/litter (%), mean fetal BW/litter were markedly reduced in the G5 group when compared with the G1 group (p < 0.05). Meanwhile, the resorbed fetus (RF)/litter (%), early resorbed fetus (ERF)/litter (%), post-implantation loss (PIL)/litter (%) were increased in the G3 - G5 groups compared with the G1 and G2 groups, especially significant higher in the G5 group (p < 0.05). Although no typical external malformation was observed in the G1 and all the DG treatment groups, the incidence of external malformation was higher in the G6 group with typical external malformation features including exencephaly and short tail, etc.

As seen in Table 3.6, the skeletal malformation was not observed in any of the groups. However, skeletal variation was observed in all the groups including the G1 group (4.88%), and G6 group (47.31%) with more obvious features. Some fetuses had more than one type of variation. The increased incidence of skeletal variation
correlated with the increase in dosage of DG treatment, ranging from 5.71% - 19.39%. The incidence of skeletal variation was higher in the G4 and G5 group (p < 0.05) when compared with the G1 group. Abnormal ribs, hypoplasia of sternebrae, scrambing sternebrae, hypoplasia and absence of phalanges were found in G1 - G6 groups while occurrence of sternebrae hypoplasia and split, phalanges hypoplasia and absence were dramatically higher in the G5 group (p < 0.05 compared with the G1 group).

Alteration to soft tissue was not observed in the G1 - G6 groups by macroscopic as well as by microscopic observation (Fig.3.3).
Table 3.4 Maternal parameters of DG water extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 (G2)</td>
<td>8 (G3)</td>
<td>16 (G4)</td>
</tr>
<tr>
<td>Pregnant female</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td><strong>Body weight (BW, g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW on GD0</td>
<td>29.87 ± 2.27</td>
<td>30.54 ± 3.05</td>
<td>30.58 ± 3.55</td>
</tr>
<tr>
<td>BW on GD18</td>
<td>57.41 ± 4.11</td>
<td>53.51 ± 4.53</td>
<td>52.39 ± 4.62</td>
</tr>
<tr>
<td>Gravid uterine weight**</td>
<td>20.41 ± 2.18</td>
<td>17.97 ± 4.10</td>
<td>17.04 ± 3.09</td>
</tr>
<tr>
<td>BW change**</td>
<td>27.54 ± 3.01</td>
<td>22.97 ± 5.14</td>
<td>21.81 ± 3.64</td>
</tr>
<tr>
<td>Corrected BW change**</td>
<td>7.13 ± 1.97</td>
<td>4.99 ± 1.72</td>
<td>4.77 ± 1.61</td>
</tr>
<tr>
<td><strong>Organ weight (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7.46 ± 0.68</td>
<td>7.30 ± 0.20</td>
<td>7.42 ± 0.31</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.24 ± 0.11</td>
<td>1.28 ± 0.06</td>
<td>1.28 ± 0.14</td>
</tr>
<tr>
<td>Heart</td>
<td>0.40 ± 0.03</td>
<td>0.39 ± 0.03</td>
<td>0.39 ± 0.03</td>
</tr>
</tbody>
</table>

**Note:** Data were presented as M ± SD, and data from the G1 to G5 group were analyzed by one-way ANOVA.

* p < 0.05 when compared with the G1 by LSD post-hoc test.

# p < 0.05 compared with the G1 and G2 group by LSD post-hoc test.

* p < 0.05 - 0.001 compared with the G1 by student t-test.

**Gravid uterine weight on GD18; BW change = maternal BW on GD18 - maternal BW on GD0; Corrected body weight change = maternal BW on GD18 - maternal BW on GD0 - gravid uterine weight; Organ weight (%) = absolute organ weight/(maternal BW on GD18 - gravid uterine weight) × 100.
Table 3.5 Fetal parameters of DG water extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 (G2)</td>
<td>8 (G3)</td>
</tr>
<tr>
<td>Total fetus/group</td>
<td>246</td>
<td>210</td>
<td>208</td>
</tr>
<tr>
<td>Implantation site/dam</td>
<td>14.44 ± 1.34</td>
<td>12.39 ± 2.78 a</td>
<td>12.61 ± 2.30 a</td>
</tr>
<tr>
<td>Total fetus/litter</td>
<td>13.72 ± 1.84</td>
<td>11.94 ± 2.97 a</td>
<td>11.56 ± 2.41 a</td>
</tr>
<tr>
<td>Live fetus/litter</td>
<td>13.67 ± 1.81</td>
<td>11.67 ± 3.00</td>
<td>11.56 ± 2.41</td>
</tr>
<tr>
<td>Live fetus/litter (%)</td>
<td>94.94 ± 7.72</td>
<td>93.54 ± 8.03</td>
<td>91.41 ± 6.65</td>
</tr>
<tr>
<td>Dead fetus/litter</td>
<td>0.06 ± 0.24</td>
<td>0.28 ± 0.47</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Dead fetus/litter (%)</td>
<td>0.37 ± 1.57</td>
<td>2.33 ± 4.12</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>RF/litter</td>
<td>0.72 ± 1.07</td>
<td>0.44 ± 0.62</td>
<td>1.06 ± 0.80 b</td>
</tr>
<tr>
<td>RF/litter (%)</td>
<td>5.14 ± 7.82</td>
<td>4.13 ± 5.90</td>
<td>8.59 ± 6.65</td>
</tr>
<tr>
<td>ERF/litter (%)</td>
<td>0.72 ± 1.07</td>
<td>0.33 ± 0.59</td>
<td>0.50 ± 0.77 b</td>
</tr>
<tr>
<td>ERF/litter (%)</td>
<td>5.78 ± 7.82</td>
<td>3.24 ± 5.80</td>
<td>8.19 ± 6.95</td>
</tr>
<tr>
<td>LRF/litter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.11 ± 0.33</td>
<td>0.06 ± 0.24</td>
</tr>
<tr>
<td>LRF/litter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.89 ± 2.66</td>
<td>0.40 ± 1.68</td>
</tr>
<tr>
<td>PIL/litter (%)</td>
<td>0.78 ± 1.06</td>
<td>0.72 ± 0.85</td>
<td>1.06 ± 0.08</td>
</tr>
<tr>
<td>PIL/litter (%)</td>
<td>5.51 ± 7.72</td>
<td>6.46 ± 8.03</td>
<td>8.59 ± 6.65</td>
</tr>
<tr>
<td>EMF/litter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>EMF/litter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>MBW/litter (g) (%)</td>
<td>1.26 ± 0.09</td>
<td>1.23 ± 0.08</td>
<td>1.21 ± 0.09</td>
</tr>
</tbody>
</table>

**Note:** Data were presented as M ± SD, and data from the G1-G5 groups were analyzed by one-way ANOVA.

* *p < 0.05 compared with the G1 group by LSD post-hoc test.
* 1p < 0.05 compared with the G1 and G2 groups by LSD post-hoc test.
* 2p < 0.05 - 0.001 compared with the G1 by student t-test.

▲ Live (dead) fetus/litter (%) = live (dead) fetus per litter/implantation site per litter ×100; RF/litter (%) = RF (resorbed fetus) per litter/implantation site per litter ×100; ERF (early resorbed fetus/litter (%)) = ERF per litter/implantation site per litter ×100; LRF (late resorbed fetus/litter (%)) = LRF per litter/implantation site per litter ×100; PIL (post-implantation loss/litter (%)) = PIL per litter / implantation site per litter ×100; EMF (external malformed fetus/litter (%)) = external malformed fetus per litter/total live fetus per litter ×100; MBW (mean fetal body weight)/litter = total live fetal body weight per litter/live fetus number per litter.
Table 3.6 Skeletal variations of DG water extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 (G2) 8 (G3) 16 (G4) 32 (G5)</td>
<td></td>
</tr>
<tr>
<td>Fetuses (litters)</td>
<td>246 (18)</td>
<td>210 (18) 208 (18) 205 (18) 196 (18)</td>
<td>93 (17)</td>
</tr>
<tr>
<td>Skeletal variation</td>
<td>12</td>
<td>12 17 22 38</td>
<td>44</td>
</tr>
<tr>
<td>Skeletal variation (%)▲</td>
<td>4.88</td>
<td>5.71 8.17 10.73 a 19.39 aa</td>
<td>47.31*</td>
</tr>
</tbody>
</table>

**Variation in▲**

<table>
<thead>
<tr>
<th></th>
<th>Occipital</th>
<th>Rib</th>
<th>Sternebrae</th>
<th>Limb</th>
<th>Coccyx</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>G2</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>G3</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>G4</td>
<td>0</td>
<td>0</td>
<td>17 a</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>G5</td>
<td>0</td>
<td>0</td>
<td>32 a</td>
<td>20 b</td>
<td>0</td>
</tr>
<tr>
<td>G6</td>
<td>0</td>
<td>0</td>
<td>36 *</td>
<td>10 *</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Data were analyzed by χ²-test.

▲ Skeletal variation (%) = skeletal variation (malformation) fetus/total fetus per group × 100; Fetus might have more than one type of skeletal variation. Limb variation consists of ulna, metacarpal and phalangeal variation.
Fig.3.3 Fetal visceral examination (J). According to Wilson’s soft tissue sectioning technique, (A) a slice at the half length of nares, posterior view; (B) a slice at the a point of the eye bulges, posterior view; (C) a slice at the half of the brain, posterior view; (D) a slice at the beginning of the nasal passage just behind the nares, inferior view; (E) a slice at the neck, superior view; (F) a slice at the xiphoid, inferior view; (G) a slice at the middle of xiphoid and umbilicus, superior view; (H) a slice at the umbilicus, superior view; (I) a slice at the anterior superior spine, superior view.

3.3.4 Cytotoxicity evaluation

Figs.3.4 - 3.5 showed the result of cytotoxicity assays by MTT method. The effect of DG water extract, bioactive components including FA and Z-LIG on inhibition of cell growth (cell viability) of cultured ESCs and 3T3 cells was in a dose-dependent manner, and the IC$_{50_{ESC}}$ was $6.10 \pm 0.23$ mg/mL ($n = 6$, $R^2 = 0.9852 - 0.993$) and IC$_{50_{3T3}}$ DG was $5.43 \pm 0.33$ mg/mL ($n = 6$, $R^2 = 0.9745 - 0.9945$) respectively, when treatment with DG water extract. The differences between IC$_{50}$
ESC and IC₅₀ 3T₃ were, however, statistically insignificant (p > 0.05). Likewise, cultured ESCs and 3T3 cells were treated with Z-LIG and FA individually. The IC₅₀ FA and IC₅₀ Z-LIG were 0.31 ± 0.04 mg/mL (n = 6, R² = 0.9387 - 0.9621) and 2.46 ± 0.56 μg/mL (n = 6, R² = 0.9475 - 0.9871) respectively on ESCs, and 0.26 ± 0.05 mg/mL (n = 6, R² = 0.9508 - 0.9857) and 4.89 ± 0.36 μg/mL (n = 6, R² = 0.9696 - 0.9945) in 3T3 cell culture. There was no significant difference between IC₅₀ ESC and IC₅₀ 3T₃ of FA, as well as those of Z-LIG. However, as shown in Fig.3.5, both IC₅₀ ESC and IC₅₀ 3T₃ of Z-LIG were much lower than those of FA, which indicated that Z-LIG was a potentially toxic agent in DG.

![Graphs](image)

**Fig.3.4** The relative viability of ESC and 3T3 cell exposed to DG water extract (left), FA (middle), and Z-LIG (right). ESCs and 3T3 cells exposed to DG water extract (0.05 - 500 mg/mL), FA (0.1 - 0.6 mg/mL) and Z-LIG (0.002 – 0.02 mg/mL) individually. The IC₅₀ DG, IC₅₀ FA and IC₅₀ Z-LIG were 6.10 ± 0.23 mg/mL (n = 6, R² = 0.9852 - 0.993), 0.31 ± 0.04 mg/mL (n = 6, R² = 0.9387 - 0.9621) and 2.46 ± 0.56 μg/mL (n = 6, R² = 0.9475 - 0.9871) respectively on ESCs, which were 5.43 ± 0.33 mg/mL (n = 6, R² = 0.9745 - 0.9945), 0.26 ± 0.05 mg/mL (n = 6, R² = 0.9508 - 0.9857) and 4.89 ± 0.36 μg/mL (n = 6, R² = 0.9696 - 0.9945) in 3T3 cell culture. There is no significant difference between IC₅₀ ESC and IC₅₀ 3T₃ of DG water extract, as well as those of FA and Z-LIG.
3.4 Discussion

The Chinese herbal medicine (CHM) is often considered to be safe as it was taken naturally from the plant earth. However, the natural products do not mean safe. Although the common usage of herbs in TCM is in the form of herbal formula, we should not ignore the potential toxicity of any single herb, especially the frequently-used ones. The study of CHM in developmental toxicity would be an important criterion to assess the safety of individual herbs, which could provide toxicological information for the usage of each herb and also as the guidance for the description dosing.

DG, as an indispensable ingredient in various formulae, has been commonly used in pregnant women for the past thousands of years. DG is used to treat gestation or postpartum disorders like abdominal pain, diarrhea during pregnancy, and postpartum bleeding in TCM. DG is believed to be of very low toxicity. The LD$_{50}$ of DG in mice is 100 g/kg by the injection route. Overdose can be suspected when patients develop symptoms such as fatigue, drowsiness, skin itching, stomach discomfort and abdominal pain [75]. The estrogen-like property of DG [76] attracted increasing attentions for its consumption in the different conditions such as peri- or post-menopausal syndrome, breast cancer, as well as other hormone-related diseases. Besides, the essential oil and water soluble components extracted from DG are found to have both uterine relaxing and stimulating effects. The
different dosage of oil components separated from DG has opposite action on uterus in mice. In addition, lines of studies demonstrated that Angelica polysaccharide suppresses the type-2 T helper (Th2) cytokines profile. As we know, the Th2 cytokines have a dominate advantage during a normal pregnancy. The increase of type-1 T helper (Th1) cytokines or the bias of Th1/Th2 cytokines ratio at fetal-maternal interface leads to pregnancy failure due to spontaneous mal-adaptation of immune system. From above mentioned pharmacological and toxicological activities observed in animal studies, the prescription of DG should be cautious in clinic. To date, there is no evidence-based safe dosage for pregnant women and their babies. In the present study, the segment II study was performed to evaluate the safety of DG on embryonic development of mice. Oral administration of DG water extract at the low dosage (2 g/kg/day, equal to human daily dosage) in pregnant mice did not cause any significant embryotoxic and teratogenic effects but did provide benefits such as increasing the fetal weight and decreasing the number of ERF. Thus, low dose of DG water extract should be safe to use in pregnant women. Very high dosage (32 g/kg/day) of DG (14.94 times to the daily usage of human) may have embryotoxic and teratogenic effects on the fetus when used during pregnancy, this is suspected even at the dosage of 16 g/kg/day. It may also result in maternal toxicity with decrease of BW. The most significantly toxic effects induced by high dose of DG would be suppression of fetal skeletal development. It's worth noting that herb quality analysis excluded the possibility of heavy metal and pesticide residue induced toxicity. This segment II study of DG provided valuable evidence in evaluating the embryotoxicity and teratogenic effects of DG, providing reference for future studies using non-rodent subjects, even human beings. Further chemical analysis is required to identify the toxic chemical components from DG.

In EST, two main active ingredients of DG, FA or Z-LIG were co-incubated with ESC and 3T3 cell to assess cytotoxicity. Due to higher sensitivity to Z-LIG than FA in both ESC and 3T3 cells, Z-LIG could be potentially more toxic to both
the embryonal and maternal health despite the lack of difference observed between IC$_{50}$ ESC and IC$_{50}$ 3T3 cell. As we know, the volatile oil from DG consists of LIG, butylideneephthalide, butylphthalide and senkyunoide-(A, I, F, H), etc. LIG, being one of phthalide components, accounts for up to 60% of the volatile oil. It is possible that the lipid soluble features LIG allowed passage across the blood brain barrier and impact upon the central nervous system. Toxicological studies indicated that rats with LIG 50 μL/kg administrated for 30 days was found to correlate with significant decrease in the index of uterus. On the other hand, mice administered with ligustilide 375 μg/kg for 15 days demonstrated reduction in index of both uterus and ovary. Besides, previous evidence indicated that LIG is phytotoxic to the monocots and therefore possess a weak antifungal ability [77]. LIG, Z-butylideneephthalide and butylphthalide have adulticidal features and the toxicity of LIG and Z-butylideneephthalide was higher than those found in commercial insecticides (thiamethoxam, imidacloprid and cypermethrin) [78]. In addition, butylideneephthalide had been demonstrated to possess acaricida activity [79]. Hence, it was reasoned that Z-butylideneephthalide and senkyunoide could also contribute to embryonic toxicity. Furthermore, α-pinene and β-ocimene can be extracted from DG, and both demonstrates insecticidal and repellent activities [80]. Despite the content of α-pinene and β-ocimene being much less than the amount of phthalide in DG, further studies to evaluate their role in DG-induced embryo-fetal development is still required.

Currently, we cannot identify the amount of toxic compounds being absorbed into maternal system or crossed the placenta barrier. Further studies are required to analyse the developmental toxicity using in-vivo and in-vitro models. 

Apart from the hypothesis that embryonic toxicity, are induced by presence of toxic substances, other causes relating to herbal features should also be taken into account. Based on the Chinese medical theories of physiology during pregnancy, “clearing during pregnancy and tonifying after birth” is one of the main principles
in obstetrics [81]. This principle suggests that pregnant female should avoid diets and herbs with hot and pungent features. However, this principle changes after childbirth. DG is a pungent and warm in property, and it is excellent as a food supplement for blood deficiency to treat diseases during pregnancy. However, it was commonly used in combination with other herbs with different features in traditional practice.

3.5 Summary

This study aimed to assess the safety of DG in embryonic development. DG is listed as one of the common CHM used for blood-tonification. However, its toxicological profile remains very limited. The quality of DG used in our study is premise. The animal experiment was performed to explore its development toxicity and the alternative in-vitro study was employed to quickly screen the contributing toxic agent.

Aqueous extract of DG was prepared for this study to mimic its clinical application. HPLC analysis of DG aqueous extract aimed to quality control the DG used in the present study, and two main chemical constituents were employed for quantitation as well. In addition, corresponding analysis was conducted according to HKCMMS to exclude potential bias from toxicity caused by contamination such as heavy metal and pesticide residue.

Previous animal experiments confirmed that intake of vitamin A 200,000 IU on GD 7, 9 and 11 resulted in adverse effects on pregnant mice and their offspring. This segment II study demonstrated adverse effects on pregnant mice and fetuses could be observed with oral administration of DG aqueous extract dose higher than 16 g/kg/day (equal to 7.47 times of human daily). However, segment I and III study are required to systematically explore the embryotoxicity of DG during other periods such as pre-mating, cohabitation and mating, early pregnancy or pre-natal period.
The toxicity of DG aqueous extract and the representative components were tested on ESC and fibroblast 3T3 cell in *in-vitro* study. There was no significant difference in IC$_{50}$ between the two cell lines for each test agent. However, data showed that the Z-LIG was more toxic to both two type cell lines than FA, which indicated that Z-LIG could be responsible for DG’s toxicity.
CHAPTER FOUR

The Comparison of Body and Tail of Angelicae Sinensis Radix
4.1 Introduction

The whole Angelicae Sinensis Radix (Dang Gui, DG) would be divided into three parts for different usage recorded in a famous ancient herbal book ‘Zhen Zhu Nang, 《珍珠囊》’ (Fig.4.1), which describes that the root stocks (Dang Gui Tou, DGT) is responsible for stopping bleeding, the branching roots (Dang Gui Wei, DGW) for promoting blood, and the main roots (Dang Gui Shen, DGS) for harmonizing blood. In current traditional medicine practice, DGS is used as an indispensable ingredient in various formulae for tonifying blood and regulating Qi, to treat diseases such as recurrent abortion, infertility, etc., has been reported in several case reports [82,83]. However, the DGW is not recommended to be used during pregnancy. More general option is selected for describing a formula containing DGW for promoting blood, to treat diseases such as cerebral infarction, hyperplasia, acne, etc., as reported in various clinical documentations [84,85,86]. However, it is still chosen to remove stagnant blood in womb after giving birth. Few researches have been done to investigate the potential mechanism emphasis on toxicology related to embryonic development so far.

Fig.4.1 The ancient herbal book ‘Zhen Zhu Nang’.
In TCM, each medicinal herb is complex in terms of its different chemical compositions which allow different clinical application of different parts. Even different ratio of the same chemical composition might contribute to different pharmacological activity, therapeutic actions and therefore, clinical indications. The content of phthalides, organic acids, polysaccharides, amino acids, metallic elements, etc. from the different part of DG was quite different. A study investigated the similarity between the DGT, DGS, DGW by fingerprint, which showed that the DGW is 0.987, and the DGT is 0.972, by using the composition of DGS as the reference 1.000 [87]. Studies on chemical analysis showed that the content of essential oil from different parts was also different. Furthermore, the majority of studies indicated that the contents of essential oil and ferulic acid (FA) were the highest in DGW, and the lowest in DGT [88]. Data showed that not only the ratio of each ingredient to total essential oil, but also the composition of chemicals varied between the different extraction methods used across different laboratories. As we know, the essential oil from DG including Z-ligustilide (Z-LIG), Z-butylidenephthalide, butylphthalide, senkyunoide-(A, I, F, H), etc. Each of them showed various and specific biological activities. It demonstrated that LIG, one of
the representative compounds in DG, exhibits a plenty of pharmacological effects (e.g., neuron protection, antioxidant effects, anti-inflammatory effects, etc.). Especially, LIG, with its strong cytotoxicity, earned a reputation for its anti-tumor property. It has been demonstrated that LIG inhibited the proliferation of several cancer cell lines including cervical cancer cell line (Hela cells), human glioblastoma cell line (T98G) [89], murine prostate cancer cell line (TRAMP C1) [90] and human hepatoma (HepG2-C8 cells) [91]. In addition, Z-butylidenephthalide showed *in-vitro* and *in-vivo* biological inhibitory effects on proliferation of human gliomas [92], HepG2-C8 cells [91] and angiogenesis [93]. Another essential oil fractions from DG such as 17-dien-12,14-diyn-1-yl-acetate, safrole-2’,3’-oxide, neodiligustilide, 11,16-dihydroxy-octadeca-9Z,3,8-falcarindiol, etc. also have demonstrated cytotoxic activities in previous studies [20,94].

In addition to essential oil, metallic elements analysis indicated that the content of calcium, copper and zinc was much higher in DGT than in DGS and DGW. Furthermore, the content of potassium and iron was markedly higher than in DGT and DGS [52]. In addition, one previous study evaluated the content of tannin in different parts of DG which showed the composition was highest in DGT than remaining parts of DG [53].

In chapter 3, the impact of DG on embryonic development was investigated. The result suggested that DG might not be safe to use in pregnant mice models and their offsprings. In addition, the cytotoxic effect of Z-LIG was higher than those in FA on both embryonic stem cell (ESC) and embryonic fibroblast 3T3 cell models. After reviewing available literatures, the aim of this study is to validate the hypothesis that the chemical composition of DGS is different from that DGW, which possibly contributes to their different applications in traditional Chinese medicine. This may also explain the different impacts of DGS and DGW on embryonic development. The comparison of main components of water extract from DGW and DGS will be conducted by chemical analysis using HPLC, GC-MS.
and LC-MS. Furthermore, cytotoxicity of water extract from DGS and DGW will be evaluated using two relative cell lines following the ECVAM guidelines.

4.2 Experiment

4.2.1 Herbal authentication

Methodology of herbal authentication was complied with description in chapter 2.2.

4.2.2 Herbal preparation

4.2.2.1 Separation of DGS

The DG samples were divided into three parts, namely DGT, DGS and DGW according to the appearance and texture as described in Chinese Pharmacopoeia (2010). In order to keep the exact part of DGS, the DGT and DGW, only a little part of DGS was removed by cutting. Qualitative and quantitative analysis of DGS and DGW parts were performed in this study.

4.2.2.2 Steam distillation

Steam distillation was performed to yield both aromatic water and water extract of DGS and DGW, respectively. The water extract was prepared by soaked in 1,000 mL of Milili-Q water for 24 h under room temperature and boiled in 1,000 mL of distille water 3 times (1 h each time). The total water extract was filtered through 4 layers of gauze and concentrated in vacuum below 70°C in a rotary evaporator to obtain 1 g/mL herb water extract.

4.2.3 Chemical analysis

4.2.3.1 High-performance liquid chromatography (HPLC) analysis

The HPLC - diode detector (DAD) analytical technique was used to determine the content of FA and Z-LIG in DGS and DGW water extract quality control.
Condition: described in chapter 3.2.2.2

Solution preparation: 5 mg FA was mixed with acetonitrile, and 6.25 mg/L, 12.5 mg/L, 25 mg/L, 50 mg/L, 100 mg/L FA standard were prepared for use. 10 mg Z-LIG was mixed with acetonitrile, and 6.25 mg/L, 12.5 mg/L, 25 mg/L, 50 mg/L, 100 mg/L Z-LIG standard were prepared for use. 1 g/mL DGS and 1 g/mL DGW water extract were diluted respectively into 200 mg/mL and filtered through 0.45 μm syringe filters for injection.

Procedure: 20 μL of each standard solution and sample solution were injected respectively into the column and data was collected for calculation.

Method validation: as the method followed the guideline set by HKCMMS, no validation was practiced.

4.2.3.2 Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS system is used to analyze the chemical profile of water extract from DGS and DGW.

Sample preparation: 200 mg/mL DGS (DGW) water extract was extracted by n-hexane for three times, total solutions were collected and centrifuged at 4,000 rpm for 4 min. The supernatant was filtered through 0.02 μm microporous filters for GC-MS analysis.

Condition: helium was the carrier gas with pressure 57.4 kPa. Total flow and column flow were 14.0 mL/min and 1.0 mL/min respectively. Linear velocity was 36.5 cm/s and purge flow was 3.0 mL/min. Total program time was 46 min in duration. Injection volume was 1 μL. Injector, interface and ion-source were kept at 250°C, 270°C and 200°C, respectively. Solvent cut time was 5.5 min. 909 scan speed was applied from 5.6 min to 46 min and the scan range was 40 - 450 amu. Through comparing the total ion chromatogram (TIC) with NIST mass spectral database, probable compound for each peak was identified. Only the compounds with compatibility greater than 90% were recorded.
Procedure: 0.5 μL of each sample was injected respectively into the column and data were collected for calculation.

4.2.3.3 Liquid chromatography-mass spectrometry (LC-MS) analysis

The LC-MS method was developed to analyze the chemical composition of water extract of different parts from DGS and DGW.

Solution preparation: 5 mg FA was mixed with methanol to obtain 25 mg/L FA standard. 10 mg Z-LIG was mixed with methanol to obtain 25 mg/L Z-LIG standard. 2 mL of 1 g/mL DGS (DGW) water extract was diluted into 200 mg/mL. The solution was centrifuged at 4,000 rpm for 4 min. The supernatant was then filtered through 0.02 μm microporous filters for LC-MS analysis.

Condition: the injection volume of samples and standards were 3 μL and detection was performed at 254, 210, 365 nm, respectively. An electrospray ionization (ESI) source was used for the conditions of MS analysis in the positive and negative ion mode as follows: drying gas (nitrogen), flow rate, 8.0 mL/min; gas temperature, 180°C; scan range, 100 - 1700 m/z; end plate offset voltage: -500V; capillary voltage, 4000 V; nebulizer press, 2.0 Bar.

4.2.4 In-vitro study

As described in chapter 2.4, for MTT test, ESC and 3T3 cells were treated with several concentrations of test agent. At day 7, the viability of the treated cells was calculated from the percentage of MTT conversion in the test solution treated cells, in relation to the vehicle control (100% viability).

4.3 Result

4.3.1 Pharmacognostical analysis

Pharmacognostical analysis was demonstrated that the required quality
standard for DGW was reached.

4.3.2 HPLC analysis

The HPLC chromatograms showed that FA and Z-LIG, two representative compounds of DG, were presented at retention time around 15.29 min and 46.50 min in the reference, 15.272 min and 46.499 min in the water extract from DGS, and 15.309 min and 46.502 min in the water extract from DGW (Fig. 4.3).

According to standard curve of FA and Z-LIG, the linear regression equations were $y = 39.18 \times (R^2 = 0.999)$ and $y = 22.20 \times (R^2 = 0.998)$, respectively. The content of FA and Z-LIG in DG water extract is calculated. FA accounted for 1.52 mg/g and 2.33 mg/g of the water extract from DGS and DGW, respectively (Table 4.1). And Z-LIG accounted for 0.99 mg/g and 1.71 mg/g of the water extract from DGS and DGW, respectively (Table 4.2). The content of FA and Z-LIG in DGW was higher than that in DGS water extract.
Fig. 4.3 HPLC chromatograms of FA (A) and Z-LIG (B) in the reference samples and water extract from DGS (C) and DGW (D).
| Table 4.1 HPLC result of FA concentration in DGS and DGW water extract. |
|------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Sample                | Concentration (mg/mL) | Retention time (min) | Peak Area (mAU*s) | FA conc. in sample (mg/mL) | Content Ratio |
| DGS                   | 200              | 15.272           | 2477.7           | 304.35          | 1.52            | 1 : 1.83       |
| DGW                   | 200              | 15.309           | 5125             | 445.61          | 2.23            |               |

| Table 4.2 HPLC result of Z-LIG concentration in DGS and DGW water extract. |
|------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Sample                | Concentration (mg/mL) | Retention time (min) | Peak Area (mAU*s) | Z-LIG conc. in sample (mg/mL) | Content Ratio |
| DGS                   | 200              | 46.499           | 827.1            | 198.89          | 0.99            | 1: 1.35        |
| DGW                   | 200              | 46.502           | 2065.3           | 341.46          | 1.71            |               |

4.3.3 GC-MS analysis

4.3.3.1 Characterization of the main volatile components in water extract from DGS and DGW

The components in water extract of DGS and DGW were separated by GC-MS. Some components are commonly found in both extract. Six compounds, mainly, sathulenol (1), 3-butylphthalide (2), Z-butylidenephthalide (3), benzeneacetic acid (4), Z-LIG (5) and E-LIG (6) were identified by comparing the TIC of DGS and DGW with NIST mass spectral database as well as spectral data and retention indices from previous literatures [95,96] (Fig.4.4). The compound 1 was found only in DGW water extract but not found in DGS water extract. The mass spectra of these compounds were shown in Fig.4.5.
4.3.3.2 Analytical comparison of the main components in water extract from DGS and DGW

In Table 4.3, the difference in ratio of the six identified compounds in DGS and DGW water extract was calculated by comparing the corresponding peak area. Higher amount of sathulenol, 3-butylphthalide, Z-butylideneephthalide, benzeneacetic acid, Z-LIG and E-LIG was detected in DGW water extract when compared with DGS. Z-LIG was the highest peak in the chromatogram and the main ingredient of the DG volatile oil, the peak area of Z-LIG in DGW extract was close to 5 times of those identified in DGS extract. The amount of Z-butylideneephthalide and 3-butylphthalide, also one of phthalides, was over 20 times and 2 times higher respectively in DGW than in DGS extract.
Fig. 4.5 Mass spectra of six identified compounds. Sathulenol (1), 3-butylphthalide (2), Z-butylidene phthalide (3), benzeneacetic acid (4), Z-LIG (5) and E-LIG (6).
Table 4.3 Six identified compounds in DGS and DGW water extract.

<table>
<thead>
<tr>
<th>Peak NO.</th>
<th>Chemical name</th>
<th>m/z</th>
<th>Formula</th>
<th>Retention time (min)</th>
<th>DGS Peak area</th>
<th>DGW Peak area</th>
<th>DGS/DGW Peak area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sathulenol</td>
<td>220</td>
<td>C_{15}H_{20}O</td>
<td>25.1</td>
<td>ND</td>
<td>4,474,551</td>
<td>/</td>
</tr>
<tr>
<td>2</td>
<td>3-butylphthalide</td>
<td>162</td>
<td>C_{11}H_{14}O</td>
<td>27.1</td>
<td>881,590</td>
<td>3,095,298</td>
<td>1 : 3.51</td>
</tr>
<tr>
<td>3</td>
<td>Z-butyldienephthalide</td>
<td>188</td>
<td>C_{12}H_{12}O_{2}</td>
<td>27.6</td>
<td>519,013</td>
<td>11,042,597</td>
<td>1 : 21.28</td>
</tr>
<tr>
<td>4</td>
<td>Benzeneacetic acid</td>
<td>208</td>
<td>C_{12}H_{16}O_{3}</td>
<td>28.7</td>
<td>2,781,442</td>
<td>10,896,406</td>
<td>1 : 3.92</td>
</tr>
<tr>
<td>5</td>
<td>Z-LIG</td>
<td>190</td>
<td>C_{12}H_{14}O_{2}</td>
<td>29.0</td>
<td>30,668,575</td>
<td>152,606,485</td>
<td>1 : 4.98</td>
</tr>
<tr>
<td>6</td>
<td>E-LIG</td>
<td>190</td>
<td>C_{12}H_{14}O_{2}</td>
<td>30.6</td>
<td>816,428</td>
<td>2,807,268</td>
<td>1 : 3.44</td>
</tr>
</tbody>
</table>

4.3.4 LC-MS analysis

4.3.4.1 Characterization of the main constituents in water extract from DGS and DGW

The constituents in water extract were separated by UPLC-DAD-QTOF-MS (Fig.4.6). The identified compounds and their corresponding data of (±) ESI-MS spectra were shown in Table 4.4. [M+H]^+, [M-H]^+ were observed to identify their quasi-molecular ion. By comparing the molecular weight and retention time with those of standard compounds, FA and Z-LIG attributed to peak 2 and 6, in the spectrum, respectively. In ESI-MS spectra, strong deprotonated molecular ion [M-H]^− peak at 193 (m/z) was found, and the characteristic fragments corresponding to [M+H-H_{2}O]^+ and [M+H-H_{2}O-CO]^+ at 173 and 145 (m/z) were also noted. Owing to the unavailability of authentic compounds, other peaks (1, 3, 4, 5) could only be tentatively assigned as coniferyl ferulate, Z-butyldienephthalide, senkyunolide A and E-LIG by comparing their molecular weight and characteristic mass fragments with previous literatures [97,98,99].
Fig.4.6 Chromatograms of water extract of DGW (yellow or purple) and DGS (blue or green). Base peak chromatogram (BPC) in negative ion mode (A) and BPC in positive ion mode (B). Chemical structure of coniferyl ferulate (1), FA (2), Z-butylideneephthalide (3), senkyunolide A (4), E-LIG (5) and Z-LIG (6).

Table 4.4 The on-line detected chromatographic and spectrometric data of six identified compounds.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Identified compounds</th>
<th>Retention Time (Min)</th>
<th>Negative (m/z)</th>
<th>Positive (m/z)</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coniferyl ferulate</td>
<td>3.4</td>
<td>355, 193</td>
<td></td>
<td>C_{20}H_{20}O_{6}</td>
</tr>
<tr>
<td>2</td>
<td>FA</td>
<td>4.7</td>
<td>193, 134</td>
<td></td>
<td>C_{10}H_{10}O_{4}</td>
</tr>
<tr>
<td>3</td>
<td>Z-butylideneephthalide</td>
<td>9.1</td>
<td></td>
<td>189, 171, 153</td>
<td>C_{12}H_{12}O_{2}</td>
</tr>
<tr>
<td>4</td>
<td>Senkyunolide A</td>
<td>11.1</td>
<td></td>
<td>193, 175, 147, 137</td>
<td>C_{12}H_{16}O_{2}</td>
</tr>
<tr>
<td>5</td>
<td>E-LIG</td>
<td>12.4</td>
<td></td>
<td>191, 173, 145</td>
<td>C_{12}H_{16}O_{2}</td>
</tr>
<tr>
<td>6</td>
<td>Z-LIG</td>
<td>12.7</td>
<td></td>
<td>191, 173, 145</td>
<td>C_{12}H_{16}O_{2}</td>
</tr>
</tbody>
</table>
4.3.4.2 Analytical comparison of the main constituents in water extract from DGS and DGW

The established LC-MS assay method was used to calculate the content of these present components in water extract from both DGS and DGW. The quantitative analytical results are summarized in Table 4.5. Generally, comparing the chemical components in DGW and DGS chromatograms, the amount of coniferyl ferulate, FA, Z-butylideneepthalide, senkyunolide A, E-LIG and Z-LIG in DGW was higher than in DGS water extract. The peak area of Z-butylideneepthalide and Z-LIG in DGW water extract was over 2 times more than in the DGS water extract.

Table 4.5 Comparative analysis on peak area of characteristic peaks in DGS and DGW water extract.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Chemical name</th>
<th>Area (DGW)</th>
<th>Area (DGS)</th>
<th>DGW : DGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coniferyl ferulate</td>
<td>544,073</td>
<td>279,049</td>
<td>1.95 : 1</td>
</tr>
<tr>
<td>2</td>
<td>FA</td>
<td>1,293,525</td>
<td>705,878</td>
<td>1.83 : 1</td>
</tr>
<tr>
<td>3</td>
<td>Z-butylideneepthalide</td>
<td>1,478,277</td>
<td>683,598</td>
<td>2.16 : 1</td>
</tr>
<tr>
<td>4</td>
<td>Senkyunolide A</td>
<td>266,789</td>
<td>179,874</td>
<td>1.48 : 1</td>
</tr>
<tr>
<td>5</td>
<td>E-LIG</td>
<td>989,685</td>
<td>735,396</td>
<td>1.35 : 1</td>
</tr>
<tr>
<td>6</td>
<td>Z-LIG</td>
<td>2,445,995</td>
<td>949,539</td>
<td>2.58 : 1</td>
</tr>
</tbody>
</table>

4.3.5 Cytotoxicity analysis

Fig. 4.7 showed the result of cytotoxicity assays by MTT method. The effect of DGS and DGW water extract on cell growth (cell viability) inhibition appears to follow dose-dependent manner using ESCs and 3T3 cells models. The IC$_{50}$ ESC was $2.772 \pm 0.440$ mg/mL ($n = 3$, $R^2 = 0.9865 - 0.9881$) and IC$_{50}$ 3T3 was $5.961 \pm 0.228$ mg/mL ($n = 3$, $R^2 = 0.9858 - 0.9882$), when treatment with DGS water extract. The IC$_{50}$ ESC was $1.949 \pm 0.190$ mg/mL ($n = 3$, $R^2 = 0.9856 - 0.9972$) and IC$_{50}$ 3T3 was
2.808 ± 0.577 mg/mL (n = 3, $R^2 = 0.9729 - 0.9888$) when treatment with DGW water extract. The differences between IC$_{50\ ESC}$ and IC$_{50\ 3T3}$ after DGS extract treatment were statistically significant ($p < 0.05$). However, no statistical significance between IC$_{50\ ESC}$ and IC$_{50\ 3T3}$ was found in DGW ($p > 0.05$) (Fig. 7 (upper)).

Both IC$_{50\ ESC}$ and IC$_{50\ 3T3}$ of DGW extract were much lower than those of DGS extract ($p < 0.05$), which indicated that DGW is a potential toxic part in DG (Fig. 7 (bottom)).
Fig. 4.7 The relative viability of ESC and 3T3 cell exposed to DGS and DGW water extract. ESCs and 3T3 cells exposed to DGS water extract (0.625 - 50 mg/mL) and DGW water extract (0.625 - 50 mg/mL) individually. The IC$_{50}$ ESC was 2.772 ± 0.440 mg/mL (n = 3, $R^2$ = 0.9865 - 0.9881) and IC$_{50}$ 3T3 was 5.961 ± 0.228 mg/mL (n = 3, $R^2$ = 0.9858 - 0.9882), when treatment with DGS water extract. The IC$_{50}$ ESC was 1.949 ± 0.190 mg/mL (n = 3, $R^2$ = 0.9856 - 0.9972) and IC$_{50}$ 3T3 was 2.808 ± 0.577 mg/mL (n = 3, $R^2$ = 0.9729 - 0.9888) when treatment with DGW water extract. The differences between IC$_{50}$ ESC and IC$_{50}$ 3T3 after DGS extract treatment were statistically significant (p < 0.05). However, no statistical significance between IC$_{50}$ ESC and IC$_{50}$ 3T3 was found in DGW (p > 0.05).

4.4 Discussion

In this study, the comparison of FA (Z-LIG) content in water extract from DGS and DGW showed quite similar results by integrating of three different chemical analytic methods. The content of FA (Z-LIG) in DGW water extract was higher than
DGS water extract. Different main components were identified despite which analytic method was applied. Six compounds, mainly sathulenol, 3-butylphthalide, Z-butylidenephthalide, benzeneacetic acid, Z-LIG and E-LIG were identified by GC-MS method. Except for Z-butylidenephthalide, Z-LIG and E-LIG, other three compounds including coniferyl ferulate, FA, senkyunolide A were characterized only by using the LC-MS method. The following compounds have been previously identified: coniferyl ferulate, FA, Z-butylidenephthalide, senkyunolide A, sathulenol, 3-butylphthalide, benzeneacetic acid, Z-LIG and E-LIG. By comparing the peak area between DGW and DGS water extracts, data showed higher ratio of the above compounds in DGW extract.

Three compounds, Z-butylidenephthalide, Z-LIG and E-LIG were identified by GC-MS, as well as by LC-MS. However, the ratio of each compound between DGS and DGW extracts was different recorded when using two different analytic methods. GC-MS results showed that the amount of Z-LIG in DGW was almost 5 times higher than DGS while the amount of Z-LIG was 2.5 times than DGS by LC-MS analysis. Moreover, the difference in ratio of Z-butylidenephthalide content between data from the two chemical analysis methods was statistically significant. As we know, LIG and some other phthalides are thermally labile and may be easily isomerized in high temperature. Moreover, senkyunolide I, H, angelicide, levistolide A are products from chemical changes of Z-LIG. Assessment of LIG using the GC-MS analysis can be inaccurate due to these potential chemical changes that occurred during this high temperature process. In addition, the ratio of FA between extract from DGS and DGW was 1:1.83 via HPLC-DAD analysis, consistent with the result from UPLC-QTOF-MS analysis. Based on previous literatures review, concentration of FA and Z-LIG in DG varies within the range of 0.211-1.75 mg/g and 1.26-37.7 mg/g, respectively, consistent with data produced by a variety of different extraction solvents and detection methods [100]. Although the steam distillation used for this studies underwent slight revision compared to
the Chinese Pharmacopoeia (2010), we ensured that the DG water extract remains the mainstream of administration, rather than aromatic water. This is consistent with majority of medicinal herbs administrations in current practice. The aims of the studies were not limited to chemical components analysis of DGS and DGW, but also focused on evaluating the difference in the toxic effects resulted from the different constituent composition between DGS and DGW.

Phthalides including Z-butylideneephthalide, 3-butylphthalide, senkyunolide A, Z-LIG and E-LIG, etc. are determined to be good systematic markers or chemical fingerprints for DG. Phthalides are also one of the most bioactive ingredients in DG, present in DG essential oil and used as an indicator of DG quality assessment and grading of plant material. Its strong aromatic odor is related to the presence of Z-LIG. Phthalide derivatives may have potential as a new natural pesticide as recent research indicates that a large number of phthalides have insecticidal, antifungal, phytotoxic, nematicidal, antimicrobial and acaricidal activities [101].

LIG greatly contributes large part to the side effects induced by DG. It is possible that lipid soluble LIG could cross the blood brain barrier and influence the central nervous system. The central motor nerve system, respiratory system and reproductive system would be affected by LIG. Toxicological studies indicated that rats exposed to LIG 50 μL/kg 30 days had significant reduction in uterine index, while mice administered with LIG 375 μg/kg 15 days had marked decrease in both uterine and ovarian indicies.

Besides, evidence indicates that LIG is phytotoxic to the monocots with weak in antifungal ability [77]. Phthalides including LIG, Z-butylideneephthalide and butylphthalide are identified with adulticidal activity, and the toxicity of LIG and Z-butylideneephthalide were higher than other commercial insectides (thiamethoxam, cypermethrin and imidacloprid) [78]. In addition, butylideneephthalide had been demonstrated to possess acaricidal activity [79].

The cytotoxicity evaluation showed that both ESC and 3T3 cells were more
vulnerable to DGW extract than to DGS extract. Moreover, chemical analysis showed that the relative amount of Z-butylideneptalide, butylphthalide, senkyunolide A and LIG in DGW water extract was higher than DGS. This finding indicated that DGW toxicity might be due to the higher level of those compounds by comparing the relative amount of the components and the toxic effects with DGS. Other studies on distinguishing DGW from DGS by chemical analysis indicated other than LIG, the content of Z-butylideneptalide, senkyunoide-(I, F, H), 6,7-epoxyligustilide extracted from DGW was 2 times of those measured in DGS. Therefore, other phthalides such as senkyunoide-(I, F, H), 6,7-epoxyligustilide as potential toxin should not be excluded. Furthermore, α-pinene and β-ocimene extracted from DG, also are identified in other herbs as the main constituents for their insecticidal and repellent activities [80,102]. Despite the level of α-pinene and β-ocimene were much less than phthalide in DG, their role in DG-induced toxicity on embryo-fetal development remain to be explored.

4.5 Summary

Study in this chapter focused on comparing the main constituents in water extract between DGS and DGW via quantitative analysis. By different chemical analytical methods, a total of nine compounds including sathulenol, 3-butylphthalide, Z-butylideneptalide, benzeneacetic acid, coniferyl ferulate, FA, senkyunolide A Z-LIG and E-LIG were identified and the relative amount of each compound was compared between DGS and DGW water extracts. Combining the result of cytotoxicity assay, water extract of DGW demonstrated lower IC_{50} value. It is believed that the higher phthalides level (3-butylphthalide, Z-butylideneptalide, senkyunolide A Z-LIG and E-LIG) contributes to toxicity on both ESC and 3T3 cells.

Safety issue of DGW should be taken into consideration seriously. Since DG
has been widely used to treat gynecological and obstetrical diseases, it is necessary to establish safety measurement regarding consumption of different parts of DG during pregnancy. Further animal studies are required to investigate the impact of DGW and/or DGS consumption in pregnant female and its offsprings. More specifically, looking at its impact on pre- and post-implantation loss, premature birth, low birth body weight, fetal developmental defects, etc. Safety dosage range of DGS and DGW used during pregnancy should also be determined.
CHAPTER FIVE

The Study of Paeoniae Radix Alba
5.1 Introduction

Paeoniae Radix Alba (White Peony Root, Bai Shao, BS) was not clearly distinguished from Paeoniae Radix Rubra (Red Peony Root, Chi Shao, CS) before the Song Dynasty. Together, these two herbs were categorized in medicinal herb Shao Yao, which is ranked as medium grade drug in Divine Husbandman’s Classic of the Materia Medica. According to Chinese Pharmacopoeia (2010), BS is the steamed and dried root of cultivated Paeonia lactiflora Pall. (Fig. 5.1), and CS is the dried root of wild Paeonia lactiflora Pall. or Paeonia veitchii Lynch. Because of their similarity in original plants and characteristic chemical compositions, their bioactivities and efficacies are similar as well. They both play a key role in treating blood diseases. However, they have different clinical applications in practice. BS is more superior in nourishing the blood while CS is superior in invigorating the blood circulation.

For thousands of years, BS has been long recognized as a valuable herbal medicine in the treatment of hepatic diseases and gynecopathies such as anemia, leukorrhea, and menstrual disorders [103]. Furthermore, BS, as an indispensable component in traditional Chinese medicine (TCM) prescriptions, has been used for miscarriage prevention given its anti-spasmodic and analgesic properties. BS is a bitter and sour medicinal herb and considered as slightly cold in its property, which was usually suggested for blood tonification as well as fetal calming effect. BS-containing descriptions such as Si Wu Decoction, Tai Shan Pan Shi San, Bao Chan Wu You Decoction, etc. have been previously tested and known for their good reputation as antiabortifacient. BS was ranked as the 6th herb for prevention and treatment for miscarriages amongst Chinese women as reported in previous systematic studies [16].
Recent pharmacological studies demonstrated that mice uterine contraction induced by oxytoxin could be significantly inhibited by BS aqueous extract [104]. It suggested that BS has phytoestrogenic effect and could increase the uterine-weight and the ratio of uterus to body weight, and stimulate the proliferation of human breast cancer MCF-7 cells [105]. However, toxicological studies on development suggested that total glucosides from BS might have side effects on pregnant rats, giving rise to the lower weight of placenta and fetal BW [106]. These observations made it difficult to conclude the impacts of BS on the maternal function and the embryo-fetal development.

Owing to the shortage of critical study on the safety assessment of BS, this study aimed to evaluate the impact of BS aqueous extract on embryo-fetal development in mice, and viability of embryonic stem cells (ESCs) and 3T3 fibroblast cells.

5.2 Experiment

5.2.1 Dosage design

According to the Chinese Pharmacopoeia (2010), the maximum daily dosage
for a 60 kg human is 15 g.

Human Dosage per day: 15/60 = 0.25 g/kg.

The formula of dosage conversion was the same as aforementioned [74]:

$$D_2 = D_1 \times \frac{R_2}{R_1} \times \left(\frac{W_1}{W_2}\right)^{1/3},$$

Mouse dosage ($D_2$) = $0.25 \times \frac{0.0898}{0.1057} \times \left(\frac{60}{0.03}\right)^{1/3}$

$$= 2.676 \text{ g/kg (equal to human daily dosage)}$$

(1) *In-vivo study*

<table>
<thead>
<tr>
<th>Group</th>
<th>Comparison with human daily dosage (times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-dose (2 g/kg)</td>
<td>2/2.676 = 0.747</td>
</tr>
<tr>
<td>Mid-dose (8 g/kg)</td>
<td>8/2.676 = 2.990</td>
</tr>
<tr>
<td>High-dose (32 g/kg)</td>
<td>32/2.676 = 11.958</td>
</tr>
</tbody>
</table>

As the accurate concentration of aqueous extract is 2 g/mL, dilution is required for the low, medium, and high-dose groups. The feeding volume is 0.5 mL per feed per day.

(2) *In-vitro study*

The stock of BS aqueous extract (2 g/mL) was diluted with maintenance medium into final concentrations of 250, 25, 10, 7.5, 6.75, 625, 5, 2.5 and 0.5 mg/mL, respectively.

5.2.2 Quality control

5.2.2.1 Herbal authentication

Methodology of herbal authentication was complied with description in chapter 2.2.
5.2.2.2 Aqueous extract for high-performance liquid chromatography (HPLC) analysis

The HPLC - diode detector (DAD) analytical technique was used to determine the content of paeoniflorin in BS in order to control the quality of BS.

Condition: according to Hong Kong Chinese Materia Medica Standard (HKCMMS), setting as: 0.1% phosphate acid/ACN = 86/14, wavelength: 230 nm, flow rate: 1.0 mL/min, temperature: room temperature, injection Volume: 10 µL.

Solution preparation: Paeoniflorin was mixed with methanol to obtain 0.303 mg/mL paeoniflorin standard. 2 g/mL BS water extract was diluted into 200 mg/mL and 50 mg/mL respectively and filtered through 0.45 µm syringe filters for injection.

Procedure: 10 µL of graded concentrations of standard solution and sample solution were injected respectively into the column and data were collected for calculation.

Method validation: as the method followed the Chinese Pharmacopoeia (2010), no validation was practiced.

5.2.3 In-vivo study

As described in chapter 2.4, dose-graded BS water extract was given to pregnant mice from GD6 to GD15. Clinical toxicity signs were observed daily throughout the whole gestation. The maternal body weight was recorded every three days. The maternal uterus with contents, liver, heart and kidney were weighed on GD18 after being sacrificed. Liver, kidney and heart of pregnant mice was then examined for morphological and pathological features.

The early and late resorptions, dead and live fetuses, and implantations were identified and counted. Fetal body weight was weighed on GD18. All live fetuses were examined for external malformations immediately. Then most of live fetuses were selected randomly and prepared for detection of skeletal malformations. Others were fixed in Bouin's solution for visceral assessment.
5.2.4  *In-vitro* study

ESCs and 3T3 cells growing exponentially were seeded on 96-well plate in the absence of mLIF 2 h later. Cells were treated with several concentrations of BS water extract. Medium with test agent was changed on day 3 and day 5. The procedure was the same as described in chapter 2.4.

5.3 Result

5.3.1 Herbal evaluation

5.3.1.1 Pharmacognostical analysis

Pharmacognostical analysis demonstrated that BS reached the required quality standard.

5.3.1.2 Phytochemical analysis

The HPLC chromatograms showed that paeonflorin, a representative chemical of BS, was presented at retention time around 21.629 min in the reference and 21.847 min in the aqueous extract samples (Fig. 5.2).

According to the standard curve of paeonflorin, the linear regression equation was $y = 10.31 \times - 36.16$ ($R^2 = 0.99$). Paeonflorin accounted for 10.52 mg/g of the aqueous extract sample (Table 5.1).

5.3.2 Maternal evaluation

The water and food consumption were not disturbed by the BS or vitamin A administration. During gestation, no abnormal manifestations such as vaginal bleeding, piloerection, or preterm were observed.
Fig. 5.2 HPLC chromatograms of paeonflorin in the reference samples (upper) and water extract (bottom).

Table 5.1 HPLC result of paeonflorin concentration in BS water extract.

<table>
<thead>
<tr>
<th>Concentration of BS water extract (mg/mL)</th>
<th>Retention time (min)</th>
<th>Peak Area (mAU*s)</th>
<th>Paeonflorin conc. in BS sample (µg/mL)</th>
<th>(mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>21.847</td>
<td>5425.44238</td>
<td>529.4309</td>
<td>10.59</td>
</tr>
<tr>
<td>200</td>
<td>21.809</td>
<td>21526.5</td>
<td>2090.216</td>
<td>10.45</td>
</tr>
</tbody>
</table>

Average content of paeonflorin in BS sample 10.52

The number of total implantations in all groups was comparable. Compared with the G1 group, no significant difference in body-weight recorded amongst the BS treatment groups. However, the increase in BW gain amongst females in the G5 group was not as obvious as in G1 group. When compared to G1 group, G5 group demonstrated significantly lower BW on GD18 with markedly lower corrected BW change and uterine weight in this group. The relative organ weight was not influenced by BS administration, and no significant difference was found when
compared with G1 group. Contrarily, the relative liver-, kidney-, and heart-weight in G5 group were markedly higher than those in NC group (Table 5.2). However, there was no pathological change observed under microscopy.

5.3.3 Fetal evaluation

The rates of resorptions and post-implantation loss in G5 group were 51.29 and 51.29%, respectively, these were significantly higher than those observed in other groups (p < 0.001); however there was no statistical difference amongst G1 and G2 - G4 groups (2.94 - 4.16%, 2.94 - 4.55%, p > 0.05). The rates of external malformed fetuses and skeletal malformed fetuses in G5 group were 48.72 and 48.25%, and were significantly higher than those in G1 group (p < 0.001); whereas there was no external malformation was detected, and no statistical difference in skeletal malformation amongst G1 and G2 - G4 groups (4.59 - 5.91%, p > 0.05) (Tables 5.3 and 5.4). The external malformations in positive control mice were presented as short tail and exencephaly.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant female</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td><strong>Body weight (BW, g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW on GD0</td>
<td>27.43 ± 1.43</td>
<td>27.09 ± 1.27</td>
<td>26.68 ± 1.44</td>
</tr>
<tr>
<td>BW on GD18</td>
<td>53.08 ± 3.77</td>
<td>53.19 ± 4.33</td>
<td>52.91 ± 5.27</td>
</tr>
<tr>
<td>Gravid uterine weight ▲</td>
<td>19.07 ± 2.16</td>
<td>19.30 ± 2.78</td>
<td>19.57 ± 3.36</td>
</tr>
<tr>
<td>Corrected BW change ▲</td>
<td>6.58 ± 2.11</td>
<td>6.80 ± 1.18</td>
<td>6.66 ± 1.68</td>
</tr>
<tr>
<td><strong>Organ weight (%) ▲</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7.11 ± 0.66</td>
<td>7.12 ± 0.48</td>
<td>7.17 ± 0.41</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.19 ± 0.13</td>
<td>1.17 ± 0.12</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>Heart</td>
<td>0.40 ± 0.03</td>
<td>0.38 ± 0.03</td>
<td>0.39 ± 0.03</td>
</tr>
</tbody>
</table>

**Note:** Data were presented as M ± SD, and data from the G1 to G4 group were analyzed by one-way ANOVA.

*p < 0.05 - 0.001 compared with the G1 by student t-test.

▲ Ditto.
Table 5.3 Fetal parameters of BS water extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fetus/group</td>
<td>237</td>
<td>218</td>
<td>226</td>
</tr>
<tr>
<td>Implantation site/litter</td>
<td>13.78 ± 1.99</td>
<td>12.56 ± 1.50</td>
<td>12.94 ± 2.18</td>
</tr>
<tr>
<td>Total fetus/litter</td>
<td>13.17 ± 1.76</td>
<td>12.11 ± 1.94</td>
<td>12.56 ± 2.12</td>
</tr>
<tr>
<td>Live fetus/litter</td>
<td>13.11 ± 1.75</td>
<td>12.11 ± 1.94</td>
<td>12.56 ± 2.12</td>
</tr>
<tr>
<td>Live fetus/litter (%)</td>
<td>95.45 ± 5.66</td>
<td>96.06 ± 6.64</td>
<td>97.06 ± 3.87</td>
</tr>
<tr>
<td>Dead fetus/litter</td>
<td>0.06 ± 0.24</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Dead fetus/litter (%)</td>
<td>0.40 ± 1.68</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>RF/litter</td>
<td>0.61 ± 0.85</td>
<td>0.44 ± 0.70</td>
<td>0.39 ± 0.50</td>
</tr>
<tr>
<td>RF/litter (%)</td>
<td>4.16 ± 5.72</td>
<td>3.94 ± 6.64</td>
<td>2.94 ± 3.87</td>
</tr>
<tr>
<td>ERF/litter</td>
<td>0.50 ± 0.86</td>
<td>0.44 ± 0.70</td>
<td>0.33 ± 0.49</td>
</tr>
<tr>
<td>ERF/litter (%)</td>
<td>3.35 ± 5.72</td>
<td>3.94 ± 6.64</td>
<td>2.57 ± 3.81</td>
</tr>
<tr>
<td>LRF/litter</td>
<td>0.11 ± 0.32</td>
<td>0.00 ± 0.00</td>
<td>0.06 ± 0.24</td>
</tr>
<tr>
<td>LRF/litter (%)</td>
<td>0.81 ± 2.38</td>
<td>0.00 ± 0.00</td>
<td>0.37 ± 1.57</td>
</tr>
<tr>
<td>PIL/litter</td>
<td>0.67 ± 0.84</td>
<td>0.44 ± 0.70</td>
<td>0.39 ± 0.50</td>
</tr>
<tr>
<td>PIL/litter (%)</td>
<td>4.55 ± 5.66</td>
<td>3.94 ± 6.64</td>
<td>2.94 ± 3.87</td>
</tr>
<tr>
<td>EMF/litter</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>EMF/litter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>MBW/litter (g)</td>
<td>1.29 ± 0.11</td>
<td>1.28 ± 0.07</td>
<td>1.25 ± 0.06</td>
</tr>
</tbody>
</table>

Note: Data were presented as M ± SD, and data from the G1 to G4 group were analyzed by one-way ANOVA.

* p < 0.05 - 0.001 compared with the G1 by student t-test.

▲ Ditto.
### Table 5.4 Skeletal variations of BS water extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuses</td>
<td>321(23)</td>
<td>218(18)</td>
<td>226(18)</td>
</tr>
<tr>
<td>Skeletal variation</td>
<td>15</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Skeletal variation (%)</td>
<td>4.67</td>
<td>4.59</td>
<td>5.75</td>
</tr>
</tbody>
</table>

#### Variation in ▲
- Occipital: 0, 0, 0, 0, 33*  
- Rib: 0, 0, 0, 1, 5*  
- Sternebrae: 11, 6, 7, 7, 45*  
- Limb: 6, 5, 6, 8, 36*  
- Coccyx: 0, 0, 0, 0, 8*  

**Note:** Data were analyzed by $\chi^2$-test.

* $p < 0.05 - 0.001$ compared with the G1 by Fisher’s exact test.

▲Ditto.
5.3.4 Cytotoxicity evaluation

The MTT result showed that BS inhibited the growth of both ESC and 3T3 cells in a dose-response manner. The $IC_{50}^{ESCs}$ and $IC_{50}^{3T3}$ in BS extract were $5.44 \pm 0.08$ mg/mL ($n = 6$, $R^2 = 0.9416 - 0.9838$) and $4.98 \pm 0.24$ mg/mL ($n = 6$, $R^2 = 0.9286 - 0.9766$) respectively. There was no significant difference between these two $IC_{50}$ values ($p > 0.05$) (Fig.5.3).

![Graph showing cell viability against BS concentration](image)

Fig.5.3 The relative viability of ESC and 3T3 cell exposed to BS water extract (0.5 - 250 mg/mL). The $IC_{50}^{ESCs}$ and $IC_{50}^{3T3}$ in BS extract were $5.44 \pm 0.08$ mg/mL ($n = 6$, $R^2 = 0.9416 - 0.9838$) and $4.98 \pm 0.24$ mg/mL ($n = 6$, $R^2 = 0.9286 - 0.9766$) respectively. There was no significant difference between these two $IC_{50}$ values ($p > 0.05$).

5.4 Discussion

BS has been perceived by the general public as relatively safe, posing fewer risks to the human and has been commonly used for treatment of various illnesses, especially those occurring during pregnancy. Currently, there is a lack of scientific data on safety evaluation and conceptus in terms of their long-term application during gestation using pregnant female animal models to investigate its reproductive and developmental toxicity.

In the present study, no abnormal manifestation was observed during the whole
treatment period (10-day from GD6 to GD15) in pregnant females administrated with BS aqueous extract (2, 8, 32 g/kg/day). It recommended that adult daily dosage for BS was 15 g/60 kg, which equates to 2 g/kg/day used in mice. Parameters of maternal evaluation including body-weight, uterine-weight, and relative organ-weight did not appear to be influenced by oral BS water extract administration. Furthermore, no pathological changes to maternal organ (heart, liver and kidney) was found under microscopic observation. There was no significant difference in live fetus/litter(%), resorbed fetus/litter(%), post-implantation loss/litter(%), or skeletal variation(%) among the negative control and BS groups. The external malformation was not detected after caesarean section amongst the negative control group and BS groups. In-vitro study showed there was no significant difference in IC$_{50}$ value between ESCs and 3T3 cells after incubated with BS water extract.

Because of its properties, BS has been frequently-used as an anti-spasmodic, analgesic medicinal herb during pregnancy for abdominal pain; as a blood-tonifying component during pregnancy with blood-deficiency issues and as a mild blood-clearing ingredient in herbal prescriptions for pregnancy with excessive heat. BS aqueous extract could inhibit the mouse oxytocin and estrogen induced uterine muscle contraction during ex-vivo studies. The effect of the mechanism was related to the increase in NO level and decrease in [Ca$^{2+}$]$_i$ in uterine tissue [104]. The effect of blood tonification of BS had been evident in previous studies. For instance, BS extracts could increase the number of erythrocyte, leukocyte, hemoglobin, and the weight of spleen and thymus, compared with those intraperitoneal cyclophosphamide injections [107]. The blood replenishing effect of BS was also tested in the other blood-deficient models caused by bloodletting and diet restriction. The result was promising [108]. In addition to its excellent in blood-enrichment, BS and its bioactive constituents such as total glucosides, paeoniflorin, etc. had demonstrated immune-regulating capability. It has been well accepted that the maternal immune status at maternal-fetus interface played important roles during
pregnancy. The shift from type I T helper cell (Th1) to Th2 profile was one of the prerequisites for the healthy pregnancy. Evidences indicated that the total glucosides from BS could affect both cellular and humoral immunity. They had consistent curative effects on autoimmune diseases such as rheumatoid arthritis and chronic abacterial prostatitis, etc. In these cases, the total glucosides efficiently results in a decrease in ratio between Th1 and Th2 cytokines [109]. This could explain the therapeutical effect of BS on miscarriage prevention.

Traditionally, BS had been used as an anti-abortifacient, and plenty of traditional Chinese herbal formulae containing BS had shown to have hormonal effects. A traditional Japanese herbal medicine preparation containing BS had shown to have stimulatory effects on the ovulatory process and the hypothalamic pituitary axis in human granulose cells in-vitro. This formula could promote steroidogenesis, cytokine, 17β-estradiol, and progesterone secretion in highly luteinized granulose cells from in-vitro fertilization patients [110]. A series of trial involving 20 young women was conducted in Japan, the low plasma progesterone levels were increased when they took the herbal prescription containing BS [111]. Another clinical trial with 126 pregnant Chinese women taking a traditional Chinese herbal description containing BS 180 g for the prevention of Rh type or ABO type maternal-fetal blood group incompatibility [112], there was no adverse effects reported during this case. Another clinical trial with 20 female volunteers in Korea using 0.5% paeoniflorin formulation showed that a statistically significant reduction in facial wrinkles, and no side effect was reported at the end of the 8-week trial [113].

The Species Paeonia lactiflora Pall is special because of the two herbs with different medicinal value under this category. BS and CS have been categorized in Paeonia lactiflora Pall until the Northern and Southern Dynasties. Since then, the differentiation between BS and CS was based on the color of the flour and root, their habitat and the process methods. The distinction between BS and CS remain
dependent on their individual nature and each of their medicinal superiority is important in different aspects. BS is used preferentially as a blood tonic and *Yin*-nourishing herbal medicine, while CS as an emmenagogue superior in invigorating and cooling blood. From this perspective, BS is recommended and prescribed for the purpose of calming fetus instead of CS.

Many factors such as the living place/planting environment, the season of collection, the process methods, etc. can potentially affect the characteristics and classification of herbs. The confusion of differentiation in current herbal market and traditional clinical practice remains a barrier for using BS correctly. Misusage of CS in inappropriate condition can lead to side effects. Hence, authentication is prerequisite for the standardization of herbal medicines. In the present study, the processing of BS aqueous extract is critical following the clinical application. Moreover, as we know today, the main bioactive constituents from BS are water soluble, some of which being absorbed and/or metabolized to be the medicinal composition. The manifestation and pathophysiological condition present true pharmacological and toxicological actions after oral administration of BS aqueous extract.

5.5 Summary

BS has been used for the treatment of various health conditions in TCM for centuries. The recommended dose of BS is based on historical practices. Traditionally, BS has been used as an anti-abortifacient and prescribed in plenty herbal preparations which have demonstrated hormonal effects. Currently, there is insufficient information on the adverse effects of BS. In the present study, the authenticated BS was tested with pharmacognostical and phytochemical analysis. Its standardized aqueous extract was used to evaluate the toxicity on embryo-fetal developmental in mice and in embryonic stem cell.
Results from this *in-vivo* segment II study suggested that oral administration of BS aqueous extract at/or lower than 32 g/kg/day to ICR mice during organogenesis from GD6 to GD15 did not cause significant maternal and embryo-fetal toxicity. This indicated that BS aqueous extract consumption, even when used at a high dosage of which was 12 times of those used in human per day, would not cause any fetal developmental toxicity. *In-vitro* embryonic stem cell test showed that there was no difference in IC$_{50}$ between ESCs and 3T3 cells, this can be interpreted as no specific toxicity was resulted from BS use on ESCs when compared with the reference cell line. Though there is no embryo-fetal developmental toxicity in present study, further studies are still required to verify the reproductive toxicity and post-natal toxicity of BS to guide its usage during late pregnancy and maternal lactation.
CHAPTER SIX

The Study of Rehmanniae Radix
6.1 Introduction

Rehmanniae Radix is ranked as a top grade drug in *Divine Husbandman’s Classic of the Materia Medica*. Unprocessed Rehmannia Root (*Sheng Di Huang*, SDH) is the dried root tuber of *Rehmannia glutinosa* (Gaert.) Libosch. ex Fisch. et Mey (Fig.6.1). Rehmanniae Radix Praeparata (*Shu Di Huang*) is the form of SDH after processing. Evidences indicate that they possess different pharmacological activities, and the quality and concentration of its chemical compounds may vary with the process. SDH is sweet in taste, cold in property, and it enter the heart, liver and kidney meridians. SDH can remove latent heat from the blood, nourish Yin and promote the production of body fluid [55]. SDH has been used as an antipyretic and hemostatic element in the medicinal prescription for treating conditions such as skin rash, diabetes, low-grade fever, etc., while Rehmanniae Radix Praeparata with its blood-tonifying properties and beneficial effects on Yin, it has been traditionally used to treat anemia, weakness, tinnitus, amenorrhea and metrorrhagia, etc.

A systematic review on clinical trials of the treatment of abortion with traditional Chinese medicine (TCM) indicated that Rehmanniae Radix was ranked as the 10th commonly used herb for treatment and prevention of abortion [16]. Based on the concept of TCM, the causative factors of abortion include blood and Yin deficiency, excessive Yang and Xue heat, stagnation of Qi and blood stasis, etc. In the view of the treatment based on syndrome differentiation, plenty of famous descriptions containing Rehmanniae Radix such as *Si Wu Decoction, Jiao Ai Decoction, Sheng Yu Decoction*, etc. have been used for thousands of years in China. To date, there is currently insufficient evidence to suggest or evaluate the safety of SDH in clinical application. Nowadays, TCM practitioners are concerned about the safety of SDH use on maternal function and embryo-fetal health, especially long-term usage during organogenesis, the very sensitive period.
Therefore, it is urgent to establish a suitable strategy and practical approach to systematically evaluate maternal toxicity and embryotoxicity of SDH as it is commonly used clinically among pregnant women. For this purpose in mind, the segment II study was carried out to evaluate the potential embryotoxicity. Meanwhile, the embryonic stem cells (ESCs) and the 3T3 fibroblast cells were used for assessing the cytotoxicity of SDH aqueous extract [7].

6.2 Experiment

6.2.1 Dosage design

According to the Chinese Pharmacopoeia (2010), the maximum daily dosage for a 60 kg human is 15 g.

Human Dosage per day: 15/60 = 0.25 g/kg.

The formula of dosage conversion was the same as aforementioned [74]:

\[ D_2 = D_1 \times \frac{R_2}{R_1} \times \left( \frac{W_1}{W_2} \right)^{1/3}, \]

Mouse dosage (\(D_2\)) = \(0.25 \times 0.0898/0.1057 \times (60/0.03)^{1/3}\),

\[ = 2.676 \text{ g/kg} \text{ (equal to human daily dosage)} \]
(1) *In-vivo* study

<table>
<thead>
<tr>
<th>Group</th>
<th>Comparison with human daily dosage (times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-dose (2 g/kg) (n=18)</td>
<td>1 \times 2.676 = 0.747</td>
</tr>
<tr>
<td>Mid-dose (8 g/kg) (n=18)</td>
<td>8 \times 2.676 = 2.990</td>
</tr>
<tr>
<td>High-dose (32 g/kg) (n=18)</td>
<td>32 \times 2.676 = 11.958</td>
</tr>
</tbody>
</table>

As the accurate concentration of aqueous extract is 2 g/mL, dilution was required for the low, medium, and high-dose groups. The feeding volume is 0.5 mL per feed per day.

(2) *In-vitro* study

The stock of SDH aqueous extract (2 g/mL) was diluted with maintenance medium into final concentrations of 20, 10, 4, 2, 1, 0.5, 0.4, 0.2 and 0.1 mg/mL, respectively.

6.2.2 Quality control

6.2.2.1 Herbal authentication

Methodology of herbal authentication was complied with description in chapter 2.2.

6.2.2.2 Aqueous extracts for high-performance liquid chromatography (HPLC) analysis

The HPLC - diode detector (DAD) analytical technique was used to determine the content of catapol in SDH in order to control the quality of SDH.

Condition: according to Hong Kong Chinese Materia Medica Standard (HKCMMS), setting as: 0.1% phosphate acid/ACN = 99/1, wavelength: 210 nm, flow rate: 0.6 mL/min, temperature: 25°C, injection Volume: 10 µL.

Solution preparation: Catalpol was mixed with methanol for preparing 0.299 mg/mL catalpol standard. The standard solution was diluted with methanol to
prepare for calibration curve. 2 g/mL SDH extract was diluted with distilled water to obtain 200 mg/mL and 50 mg/mL respectively, both concentrations then filtered through 0.45 µm syringe filters for injection.

Procedure: 10 µL of graded concentrations standard solution and sample solution were injected respectively into the column and data were collected for calculation.

Method validation: as the method followed the Chinese Pharmacopoeia (2010), no validation was practiced.

6.2.3 *In-vivo* study

The procedure was the same as the method described in chapter 2.3.

6.2.4 *In-vitro* study

The procedure was the same as the method described in chapter 2.4.

6.3 Result

6.3.1 Herbal evaluation

6.3.1.1 Pharmacognostical analysis

Pharmacognostical analysis demonstrated that SDH reached the required quality standard.

6.3.1.2 Phytochemical analysis

HPLC analysis was carried out on the reference and aqueous extract specimen according to Chinese Pharmacopeia (2010). The HPLC chromatograms showed the presence of catapol in both standard solution and SDH extracts. A remarkable peak occurred at the retention time of 21.590 min representing catalpol. The peak at
Retention time of 21.663 min revealed the presence of catalpol by comparing with reference material (Fig. 6.2).

**Fig. 6.2** HPLC chromatograms of catalpol in the reference samples (upper) and SDH water extract (bottom).

<table>
<thead>
<tr>
<th>Concentration of SDH (mg/mL)</th>
<th>Retention time (min)</th>
<th>Peak Area (mAU*s)</th>
<th>Catalpol conc. in sample (µg/mL)</th>
<th>(mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>21.011</td>
<td>373.21725</td>
<td>198.3710</td>
<td>3.97</td>
</tr>
<tr>
<td>200</td>
<td>21.663</td>
<td>1522.89954</td>
<td>844.9137</td>
<td>4.22</td>
</tr>
</tbody>
</table>

Average content of catalpol in SDH sample 4.10

According to the standard curve of catalpol, the linear regression equation was

\[ y = 1.778 x + 20.47 \ (R^2 = 0.999) \],

and catalpal accounted for 4.10 mg/g of the aqueous extract sample (Table 6.1).
6.3.2 Maternal evaluation

The water and food consumption taken by pregnant mice were not disturbed by the SDH or vitamin A oral administration. During the whole gestation, no abnormal manifestations such as death, abortion, vaginal bleeding, or preterm were observed amongst all the treatment groups.

The number of total implantations in all groups was comparable. There was no significant difference of maternal BW on GD0, 6, 12 and 18 among the G1 - G4 groups when compared with the NC group. However, the increase of BW gain among females in the G5 group was lower than remaining other groups (p < 0.05). The BW, corrected BW change, and uterine weight was significantly lower in the G5 group on GD18 (p < 0.05) when compared with the G1 group. The relative organ weight was not influenced by SDH administration, and no significant difference was found when comparisons were made with the negative control (p > 0.05). Although the relative liver-, kidney-, and heart-weight were higher in the G5 group compared with G1 group (p < 0.05) (Table 6.2), no pathological feature was found during microscopic assessment.
Table 6.2 Maternal parameters of SDH water extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 (G2)</td>
<td>8 (G3)</td>
</tr>
<tr>
<td>Pregnant female</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td><strong>Body weight (BW, g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW on GD0</td>
<td>27.54 ± 1.45</td>
<td>28.72 ± 2.30</td>
<td>28.34 ± 1.75</td>
</tr>
<tr>
<td>BW on GD18</td>
<td>53.17 ± 4.01</td>
<td>58.61 ± 5.43</td>
<td>57.42 ± 2.66</td>
</tr>
<tr>
<td>Gravid uterine weight▲</td>
<td>19.57 ± 2.90</td>
<td>21.34 ± 2.42</td>
<td>21.52 ± 1.93</td>
</tr>
<tr>
<td>BW change▲</td>
<td>26.19 ± 3.49</td>
<td>29.89 ± 3.78</td>
<td>29.08 ± 2.58</td>
</tr>
<tr>
<td>Corrected BW change▲</td>
<td>6.62 ± 1.86</td>
<td>8.56 ± 2.67</td>
<td>7.56 ± 1.45</td>
</tr>
<tr>
<td><strong>Organ weight (%)▲</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7.31 ± 0.41</td>
<td>7.21 ± 0.37</td>
<td>7.21 ± 0.33</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.18 ± 0.06</td>
<td>1.15 ± 0.10</td>
<td>1.16 ± 0.07</td>
</tr>
<tr>
<td>Heart</td>
<td>0.40 ± 0.03</td>
<td>0.38 ± 0.03</td>
<td>0.38 ± 0.07</td>
</tr>
</tbody>
</table>

Note: Data were presented as M ± SD, and data from the G1 to G4 group were analyzed by one-way ANOVA.

*p < 0.05 - 0.001 compared with the G1 by student t-test.
▲Ditto.
6.3.3 Fetal evaluation

The total number of implantation in all groups was comparable. The incidence of resorption/litter and post-implantation loss/litter in vitamin A group were up to (41.42 ± 34.07)% and (41.42 ± 34.07)%, respectively, which were significantly higher than those in other groups (P < 0.001); whereas there was no statistical difference among G1 - G4 groups (4.46 - 5.82%, 4.46 - 6.59%, p > 0.05). The incidence of skeletal variation in the G5 group was 44.00%, and was significantly higher than the G1 group (p < 0.001), but there was no statistical difference in skeletal variations amongst G1 - G4 groups (4.00 - 6.36%, p > 0.05) (Tables 6.3 and 6.4). Despite the lack of external malformation detected in the G1 - G4 groups, the external malformations such as short tail and exencephaly were still observed in mice treated with vitamin A.

6.3.4 Cytotoxicity evaluation

Both ESCs and 3T3 cells were exposed to concentration gradients of SDH aqueous extract. The MTT result showed that SDH inhibited the growth of both cells in a dose-dependent manner. The IC_{50}^{ESC} and IC_{50}^{3T3} of SDH extract were 8.89 ± 0.56 mg/mL (n = 6, R^2 = 0.9219 - 0.9843) and 7.24 ± 0.37 mg/mL (n = 6, R^2 = 0.9327 - 0.9721) respectively. The IC_{50}^{ESC} and IC_{50}^{3T3} of SDH extract was then analyzed statistically and the result of χ^2-test showed p > 0.05, indicating little statistical significance (Fig.6.3).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>2 (G2)</th>
<th>8 (G3)</th>
<th>32 (G4)</th>
<th>Positive Control (G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fetus/group</td>
<td>238</td>
<td>250</td>
<td>244</td>
<td>220</td>
<td>125</td>
</tr>
<tr>
<td>Implantation site/litter</td>
<td>13.72 ± 1.13</td>
<td>14.67 ± 1.57</td>
<td>14.28 ± 1.23</td>
<td>12.83 ± 3.15</td>
<td>12.67 ± 3.29</td>
</tr>
<tr>
<td>Total fetus/litter</td>
<td>13.22 ± 1.35</td>
<td>13.89 ± 1.68</td>
<td>13.56 ± 1.46</td>
<td>12.22 ± 2.96</td>
<td>6.94 ± 4.77 *</td>
</tr>
<tr>
<td>Live fetus/litter</td>
<td>13.11 ± 1.28</td>
<td>13.89 ± 1.68</td>
<td>13.56 ± 1.46</td>
<td>12.17 ± 2.94</td>
<td>6.94 ± 4.77 *</td>
</tr>
<tr>
<td>Live fetus/litter (%)</td>
<td>95.57 ± 6.17</td>
<td>94.82 ± 6.94</td>
<td>94.91 ± 5.38</td>
<td>95.15 ± 6.55</td>
<td>55.80 ± 33.05 *</td>
</tr>
<tr>
<td>Dead fetus/litter</td>
<td>0.11 ± 0.32</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.06 ± 0.24</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Dead fetus/litter (%)</td>
<td>0.77 ± 2.23</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.40 ± 1.68</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>RF/litter</td>
<td>0.50 ± 0.79</td>
<td>0.78 ± 1.06</td>
<td>0.72 ± 0.75</td>
<td>0.61 ± 0.85</td>
<td>5.28 ± 4.34 *</td>
</tr>
<tr>
<td>RF/litter (%)</td>
<td>3.66 ± 6.25</td>
<td>5.18 ± 6.94</td>
<td>5.09 ± 5.38</td>
<td>4.46 ± 6.62</td>
<td>41.42 ± 34.07 *</td>
</tr>
<tr>
<td>ERF/litter</td>
<td>0.50 ± 0.79</td>
<td>0.78 ± 1.06</td>
<td>0.67 ± 0.77</td>
<td>0.44 ± 0.70</td>
<td>5.17 ± 4.34 *</td>
</tr>
<tr>
<td>ERF/litter (%)</td>
<td>3.66 ± 6.25</td>
<td>5.18 ± 6.94</td>
<td>4.70 ± 5.48</td>
<td>3.14 ± 5.43</td>
<td>40.62 ± 34.20 *</td>
</tr>
<tr>
<td>LRFLitter</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.06 ± 0.24</td>
<td>0.17 ± 0.51</td>
<td>0.11 ± 0.32</td>
</tr>
<tr>
<td>LRFLitter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.40 ± 1.68</td>
<td>1.32 ± 4.41</td>
<td>0.79 ± 2.31</td>
</tr>
<tr>
<td>PIL/litter</td>
<td>0.61 ± 0.78</td>
<td>0.78 ± 1.06</td>
<td>0.72 ± 0.75</td>
<td>0.67 ± 0.84</td>
<td>5.28 ± 4.34 *</td>
</tr>
<tr>
<td>PIL/litter (%)</td>
<td>4.43 ± 6.17</td>
<td>5.18 ± 6.94</td>
<td>5.09 ± 5.38</td>
<td>4.46 ± 6.62</td>
<td>41.42 ± 34.07 *</td>
</tr>
<tr>
<td>EMF/litter</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>2.61 ± 3.13*</td>
</tr>
<tr>
<td>EMF/litter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>48.72 ± 50.41 *</td>
</tr>
<tr>
<td>MBW/litter (g)</td>
<td>1.30 ± 0.13</td>
<td>1.24 ± 0.08</td>
<td>1.28 ± 0.08</td>
<td>1.26 ± 0.09</td>
<td>1.38 ± 0.20 *</td>
</tr>
</tbody>
</table>

**Note:** Data were presented as M ± SD, and data from the G1 to G4 group were analyzed by one-way ANOVA.

*p < 0.05 - 0.001 compared with the G1 by student t-test.

*Ditto.
Table 6.4 Skeletal variations of SDH water extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (G1)</th>
<th>Dose (g/kg/day)</th>
<th>Positive Control (G5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 (G2)</td>
<td>8 (G3)</td>
</tr>
<tr>
<td>Fetuses</td>
<td>321(23)</td>
<td>250(18)</td>
<td>244(18)</td>
</tr>
<tr>
<td>Skeletal variation</td>
<td>15</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Skeletal variation (%)^</td>
<td>4.67</td>
<td>4.00</td>
<td>4.92</td>
</tr>
</tbody>
</table>

**Variation in\^**

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>30 *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occipital</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rib</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Sternum</td>
<td>11</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>38 *</td>
</tr>
<tr>
<td>Limb</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>30 *</td>
</tr>
<tr>
<td>Coccyx</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

**Note:** Data were analyzed by $\chi^2$-test.

\*p < 0.05 - 0.001 compared with the G1 by Fisher’s exact test.

\^Ditto.
Fig.6.3 The relative viability of ESC and 3T3 cell exposed to SDH water extract (0.1 - 20 mg/mL). The IC\textsubscript{50} ESC and IC\textsubscript{50} 3T3 of SDH extract were 8.89 ± 0.56 mg/mL (n = 6, R\textsuperscript{2} = 0.9219 - 0.9843) and 7.24 ± 0.37 mg/mL (n = 6, R\textsuperscript{2} = 0.9327 - 0.9721) respectively. There was no significant difference between these two IC\textsubscript{50} values (p > 0.05).

6.4 Discussion

Rehmanniae Radix has been commonly used to treat threatened miscarriage since ancient times. Although previous research studies indicated that it has been well tolerated for long-term use, up to 1 year, there was no evidence-based review indicating its safety for pregnant females. Moreover, it was reported that oral administration with 0.05 - 2 mL Rehmania glutinosa Libusch twice a day could induce fertility toxicity in mice with decreased number of litters [114]. For fertility toxicity, one might refer to the FDA segment I study. Hence, research on the toxicity of fetal and post-natal development is urgent, for the guide of the clinical application. In this study, the systematical assessment for the safety of SDH on embryonic development was conducted following strict critical guidelines.

We examined the effects of SDH aqueous extract on embryo-fetal development using an in-vivo segment II study and in-vitro embryonic stem cell test (EST). The oral administration of SDH aqueous extract at or below 32 g/kg/day (equal to 11.96 times of human usage) did not cause maternal toxicity, without impact on food and water consumption, nor increase of BW, organ weight, etc. The
high dose (32 g/kg/day) treatment to pregnant mice would not cause the embryo-fetal developmental toxicity. ESCs and 3T3 cells had no significant difference in viability after SDH aqueous extract exposure, which indicated SDH had no specific toxic effect on ESCs.

SDH has been used as a mild immunosuppressive agent for rheumatoid arthritis, asthma, and urticaria, etc. for centuries [115]. As we know, immunosuppression is needed during pregnancy especially the early stage of implantation and growth of the embryo. Although most of herbal bioactivities changes after processing, it is believed that SDH could share some of pharmacological and toxicological activities presented by Rehmanniae Radix Praeparata. Lines of evidence indicated that Rehmanniae Radix Praeparata had phytoestrogen effect on ex-vivo mouse uterus and in-vitro MCF-7 cell line [116]. Certain level of estrogen plays an important role in regulating women reproductive system. Increased estrogen level not only helps to prepare the body for a possible pregnancy by changing the lining of the uterus, but also controls another important reproductive hormone during pregnancy, known as progesterone. Progesterone, commonly used as an anti-abortifacient, could inhibit smooth muscle contractions and decrease prostaglandin formation. Although Rehmanniae Radix Praeparata is superior in nourishing blood, and SDH is good at reducing heat in blood, actually hemotopoiesis of SDH should not be beyond our sight. Evidence indicated that SDH could stimulate blood cell production in mice mimicking hemorrhagic anemia, as well as promoting proliferation of bone marrow hematopoietic cells in-vitro [117].

In our study, teratogen vitamin A was used as a positive control. High level of vitamin A could produce birth defects such as central nervous, craniofacial, cardiovascular, and thymus malformations. Evidence indicated that vitamin A could cause mitochondrial swelling, release of lysosomal enzymes, and inhibiting DNA synthesis, the reason for its adverse effects. FDA recommended that the daily dose
of vitamin A should be lower than 8,000 IU, based on the evidences from lab animals and human experience. In our study, as a positive control to validate method, vitamin A produced typical embryotoxicity that was consistent with previous relevant reports from our and other laboratories.

6.5 Summary

The herbal medicinal prescriptions containing SDH were commonly used for the treatment of pregnancy with manifestations such as colporrhagia, abdominal pain, threatened abortion, etc. So far, there is a lack of sufficient data about the toxic effect of SDH on reproduction and development. Research on antifertility effects of natural product mentioned the possible effect of SDH. In the present study, the aqueous extract of SDH was prepared according to the preparation of herbs in clinic. The experiment of authenticated SDH was conducted to detect the maternal and embryo-fetal developmental toxicity in mice and its cytotoxicity on ESCs.

Previous studies reported that the Rehmania glutinosa Libosch could produce the anti-fertility effect in mice. From our study results, oral administration of SDH aqueous extract at a high dose of up to 32 g/kg/day did not result in toxicity on the pregnant mice and their conceptus. Further researches are required to verify this study, and to assess the safety of Rehmanniæ Radix Praeparata on embryonic development. Segment III studies to assess toxicity during post-natal period should also conducted to verify the safety of Rehmanniæ Radix.
CHAPTER SEVEN

The Study of Herb-pairs
7.1 Introduction

During thousand of years of traditional Chinese medicine (TCM) practice, herbs were used alone to treat diseases for certain conditions. This might increase the incidence of adverse effects. Then, one herb was combined with another as formula prescribed by TCM practitioners based on syndrome differentiation, as well as on principle of synergistical interaction and counteraction of potential side effects. There are approximately 365 herbs recorded in Divine Husbandman’s Classic of the Materia Medica, which established the basic theory of herbology, with more than 60% of them still in clinical use today. It defines the relationship between herbs in a formula. Individual herb in each formula served specific role such as sovereign, minister, assistant, or courier medicine. It also describes the interactions (e.g., mutual reinforcement, mutual assistance, etc.) between two herbs. It was demonstrated that, in previous chapter, when pregnant mice were treated with Angelica Sinensis Radix (Dang Gui, DG) aqueous extract over 16 g/kg/day during organogenesis, maternal and embryonic toxicity was observed. Based on the literature review regarding clinical application of DG for treatment of healthy problem during pregnancy, it was commonly used with other herb in pair, such as Paeoniae Radix Alba (Bai Shao, BS), Rehmanniae Radix (Di Huang, DH), Chuanxiong Rhizome (Chuan Xiong, CX), etc. Interestingly, these four herbs form a classical herbal formula, Si Wu Decoction, which is excellent in tonifying the blood. According to TCM theory, warm property of DG helps to neutralize the opposite nature of both BS and SDH. The side effect of one herb could be decreased by another herb with opposite property. As we know, one herb is complex owing to its chemical constituents, which could lead to not only pharmacological benefits but also side effects. After being decocted with one or more herbs, thousands of interactions might happen among these chemical compounds. One would influence others pharmacokinetically and/or pharmacodynamically inside the body. The metabolism would therefore be difficulties to predict. When the absorption,
distribution and metabolism of these constituents are altered, the manifestations of toxicity varies. This study aims to explore whether or not, maternal and embryonic toxicity induced by DG could be alleviated when it’s coupled with BS or SDH.

7.2 Experiment
7.2.1 Dosage design
(1) BS aqueous extract

According to the Chinese Pharmacopoeia (2010), the maximum daily dosage for a 60 kg human is 15 g.

Human Dosage per day: \(\frac{15}{60} = 0.25\) g/kg.

The formula of dosage conversion was the same as aforementioned [74]:

\[
D_2 = D_1 \times \frac{R_2}{R_1} \times \left(\frac{W_1}{W_2}\right)^{1/3},
\]

Mouse dosage \((D_2) = \frac{0.25}{0.0898/0.1057} \times \left(\frac{60}{0.03}\right)^{1/3} = 2.676\) g/kg (equal to human daily dosage)

(2) SDH aqueous extract

According to the Chinese Pharmacopoeia (2010), the maximum daily dosage for a 60 kg human is 15 g.

Human Dosage per day: \(\frac{15}{60} = 0.25\) g/kg.

The formula of dosage conversion was the same as aforementioned:

\[
D_2 = D_1 \times \frac{R_2}{R_1} \times \left(\frac{W_1}{W_2}\right)^{1/3},
\]

Mouse dosage \((D_2) = \frac{0.25}{0.0898/0.1057} \times \left(\frac{60}{0.03}\right)^{1/3} = 2.676\) g/kg (equal to human daily dosage)

<table>
<thead>
<tr>
<th>Group</th>
<th>Comparison with human daily dosage (times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS (32 g/kg)</td>
<td>32/2.676 = 11.96</td>
</tr>
<tr>
<td>SDH (32 g/kg)</td>
<td>32/2.676 = 11.96</td>
</tr>
</tbody>
</table>
7.2.2 In-vivo study

The pregnant mice were treated by oral administration with syringe and feeding needle. As the maximum feeding volume of mice was 1 mL, fasting was carried out at least 1.5 h before feeding. Feeding period was from GD6 to GD15.

1) Negative control group was treated with distilled water 1 mL once a day (n=18).
2) DG group was treated with 1 g/mL DG extract 1 mL once a day (n=18).
3) BS group was treated with 1 g/mL BS extract 1 mL once a day (n=18).
4) SD group was treated with 1 g/mL SDH extract 1 mL once a day (n=18).
5) DG-BS group was treated with 2 g/mL BS extract 0.5 mL and 1 g/mL DG extract 1mL 1.5 h later per day (n=18).
6) DG-SDH group was treated with 2 g/mL SDH extract 0.5 mL and 1 g/mL DG extract 1mL 1.5 h later per day (n=18).

The procedure was the same as the method described in chapter 2.3.

7.3 Result

7.3.1 Maternal evaluation

 Neither maternal death nor abortion was observed in any groups throughout the whole experimental period. There was no obvious signs of toxicity noted in any of the treatment groups. Water and food intake were the same among each group. The number of pregnant mice in all groups was comparable.

 As shown in Table 7.1, there was no significant difference amongst each treatment group of maternal BW at the beginning and the end of the experiment. Data showed similar gravid uterine weight between all the groups. It was worth noticing that the corrected BW change (maternal BW on GD18- maternal BW on GD0) in DG group was less than the negative control group (p<0.05). The corrected BW change was higher in the combination group with either BS or SDH, when compared with DG group (p<0.05).
The index of organs, including heart, liver and kidney, indicated no significant difference in all the herbal decoction treatment groups compared to the negative control group. The histopathological examination also did not show any abnormalities in liver, kidney and heart amongst negative control, DG, BS and SDH groups respectively (Fig. 7.1).
Table 7.1 Maternal parameters of herb-pair extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control</th>
<th>Dosage (32 g/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DG</td>
</tr>
<tr>
<td>Pregnant female</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Body weight (BW, g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW on GD0</td>
<td>27.69 ± 1.48</td>
<td>28.48 ± 2.88</td>
</tr>
<tr>
<td>BW on GD18</td>
<td>53.70 ± 4.11</td>
<td>51.00 ± 8.34</td>
</tr>
<tr>
<td>Gravid uterine weight▲</td>
<td>19.47 ± 2.22</td>
<td>18.19 ± 6.00</td>
</tr>
<tr>
<td>BW change▲</td>
<td>26.01 ± 3.69</td>
<td>22.63 ± 6.44</td>
</tr>
<tr>
<td>Corrected BW change▲</td>
<td>6.20 ± 1.80</td>
<td>4.42 ± 1.72</td>
</tr>
<tr>
<td>Organ weight (%)▲</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>7.18 ± 0.51</td>
<td>7.36 ± 0.59</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.17 ± 0.05</td>
<td>1.22 ± 0.07</td>
</tr>
<tr>
<td>Heart</td>
<td>0.41 ± 0.02</td>
<td>0.42 ± 0.04</td>
</tr>
</tbody>
</table>

Note: Data were presented as M ± SD, and analyzed by one-way ANOVA.

▲p < 0.01 compared with the negative control, BS, and SDH groups by LSD post-hoc test.

#p < 0.05 compared with the DG group by LSD post-hoc test.

▲Ditto.
Fig. 7.1 HE staining of maternal organs sections. (A) - (D) liver in the negative control, DG, BS, and SDH group; (E) - (F) kidney in the negative control, DG, BS and SDH group; (I) - (L) heart in the negative control, DG, BS and SDH group.

7.3.2 Fetal evaluation

The number of implantations per dam in all groups was comparable. It should be noticed that each parameter in BS and SDH group had no significant difference compared with the negative control group, which indicated that neither BS nor SDH impact the embryonic development (Table 7.2). The incidence (%) of live fetus/litter in DG group (85.33 ± 13.60) was much lower than the negative control group (95.61 ± 3.86, p < 0.01). The incidence (%) of live fetus/litter in DG-BS and DG-SDH groups was 94.17 ± 6.61 and 93.03 ± 8.86 respectively, the difference in both were statistically significant compared to the DG group (p < 0.05). Likewise, no obvious difference of resorption site/litter was found between every treatment group and the negative group, but significant difference of the incidence (%) of RF/litter, especially ERF/litter, was observed between DG and the negative group (p < 0.05). Combination of DG-BS or DG-SDH used could attenuate the toxicity caused by DG single use (p < 0.05). There was no significant difference in incidence of dead fetus/litter when compared between the groups. The PIL/litter (1.61 ± 1.29) and the incidence (14.67 ± 13.60)% were much higher in DG group than those in
the negative control group (0.67 ± 0.59, 4.39 ± 3.86, p < 0.05, p < 0.01). Herb-pair usage correlates with a decrease in the PIL/litter (%) compared with single DG administration (p < 0.05). Average fetal BW was lower in DG and DG-BS groups than the negative control group (p < 0.01, p < 0.05). The fetal BW was increased significantly in DG-SDH group when compared to the DG group. No external malformations or visceral abnormalities were observed in any of the six groups (Fig. 7.2). It was indicated that the embryotoxicity of DG could be reduced by combining BS and SDH.

As shown in Table 7.3, the fetal rates of skeletal variations in six groups ranged between 4.52% - 16.59%. There was no difference when compared with the negative control group, BS group and SDH group. The skeletal variations were more obvious in DG group reaching 16.59% when compared to the negative control group of 4.74% (p < 0.001). The variation induced by DG was alleviated by BS and SDH and the variation decreased to 9.61% in DG-BS and 9.48% in DG-SDH group, respectively. The major variations of skeleton included rudimentary rib, abnormal sternebrae structure, and absence or dysplasia of metacarpals and phalanges. The manifestations of abnormal sternebrae included extra, absence (dysplasia), split, scrambling, and fusion. The occurrence of abnormal sternebrae in DG group was much higher than the negative control group (p < 0.001), which could be lightened by the combined use of BS. Although the reverse effect of SDH was not significantly obvious when compared to BS, the occurrence rate remained lower than the DG group. The manifestation of dysplasia (absence) of limb (metacarpals and phalanges) was observed in all six groups, but the incidence rate was the highest in the DG group, and the lowest in the negative control group. Therefore, the combination of BS or SDH could decrease the rate (compared to negative control group p > 0.05 and p > 0.05) but the occurrence rate of rudimentary rib was small and no significant difference was found in all the groups.
Table 7.2 Fetal parameters of herb-pair extract treated groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control</th>
<th>Dosage (32 g/kg/day)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DG</td>
<td>BS</td>
<td>SDH</td>
<td>DG-BS</td>
<td>DG-SH</td>
</tr>
<tr>
<td>Total fetus/group</td>
<td>255</td>
<td>213</td>
<td>228</td>
<td>222</td>
<td>231</td>
<td>213</td>
</tr>
<tr>
<td>Implantation site/dam</td>
<td>14.72 ± 1.49</td>
<td>13.17 ± 3.82</td>
<td>13.11 ± 1.53</td>
<td>12.94 ± 3.19</td>
<td>13.61 ± 1.61</td>
<td>12.61 ± 2.35</td>
</tr>
<tr>
<td>Total fetus/litter</td>
<td>14.17 ± 1.34</td>
<td>11.83 ± 3.93</td>
<td>12.67 ± 1.50</td>
<td>12.33 ± 3.03</td>
<td>12.83 ± 1.92</td>
<td>11.83 ± 2.26</td>
</tr>
<tr>
<td>Live fetus/litter</td>
<td>14.06 ± 1.35</td>
<td>11.56 ± 3.93 c</td>
<td>12.61 ± 1.50</td>
<td>12.28 ± 3.01</td>
<td>12.83 ± 1.92</td>
<td>11.72 ± 2.30 c</td>
</tr>
<tr>
<td>Live fetus/litter (%)</td>
<td>95.61 ± 3.86</td>
<td>85.33 ± 13.60 a</td>
<td>96.34 ± 5.70</td>
<td>95.15 ± 6.55</td>
<td>94.17 ± 6.61 a</td>
<td>93.03 ± 8.86 a</td>
</tr>
<tr>
<td>Dead fetus/litter</td>
<td>0.11 ± 0.32</td>
<td>0.28 ± 0.96</td>
<td>0.06 ± 0.24</td>
<td>0.06 ± 0.24</td>
<td>0.11 ± 0.32</td>
<td>0.11 ± 0.32</td>
</tr>
<tr>
<td>Dead fetus/litter (%)</td>
<td>0.77 ± 2.32</td>
<td>2.06 ± 7.32</td>
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<td>0.40 ± 1.68</td>
<td>0.86 ± 2.51</td>
<td>0.95 ± 2.81</td>
</tr>
<tr>
<td>RF/litter</td>
<td>0.56 ± 0.62</td>
<td>1.33 ± 1.19</td>
<td>0.44 ± 0.78</td>
<td>0.61 ± 0.85</td>
<td>0.78 ± 0.88</td>
<td>0.78 ± 1.17</td>
</tr>
<tr>
<td>RF/litter (%)</td>
<td>3.62 ± 3.97</td>
<td>12.61 ± 13.48 a</td>
<td>3.23 ± 5.67</td>
<td>4.46 ± 6.62</td>
<td>5.83 ± 6.61 a</td>
<td>6.01 ± 8.76 a</td>
</tr>
<tr>
<td>ERF/litter</td>
<td>0.50 ± 0.62</td>
<td>1.22 ± 1.17 a</td>
<td>0.39 ± 0.78</td>
<td>0.44 ± 0.70</td>
<td>0.72 ± 0.83</td>
<td>0.78 ± 1.17</td>
</tr>
<tr>
<td>ERF/litter (%)</td>
<td>3.27 ± 4.00</td>
<td>11.82 ± 13.57 a</td>
<td>2.73 ± 5.52</td>
<td>3.14 ± 5.43</td>
<td>5.43 ± 6.28 a</td>
<td>6.01 ± 8.76 a</td>
</tr>
<tr>
<td>LRF/litter</td>
<td>0.06 ± 0.24</td>
<td>0.11 ± 0.32</td>
<td>0.06 ± 0.24</td>
<td>0.17 ± 0.51</td>
<td>0.06 ± 0.24</td>
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<tr>
<td>LRF/litter (%)</td>
<td>0.35 ± 1.47</td>
<td>0.79 ± 2.31</td>
<td>0.51 ± 2.14</td>
<td>1.32 ± 4.41</td>
<td>0.40 ± 1.68</td>
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</tr>
<tr>
<td>PIL/litter</td>
<td>0.67 ± 0.59</td>
<td>1.61 ± 1.29 a</td>
<td>0.50 ± 0.79</td>
<td>0.67 ± 0.84</td>
<td>0.89 ± 0.96 a</td>
<td>0.89 ± 1.18 a</td>
</tr>
<tr>
<td>PIL/litter (%)</td>
<td>4.39 ± 3.86</td>
<td>14.67 ± 13.60 a</td>
<td>3.66 ± 5.70</td>
<td>4.85 ± 6.55</td>
<td>7.08 ± 7.45 a</td>
<td>6.97 ± 8.86 a</td>
</tr>
<tr>
<td>EMF/litter (%)</td>
<td>0.00 ± 0.00</td>
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</tr>
<tr>
<td>EMF/litter (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>MBW/litter (g)</td>
<td>1.30 ± 0.13</td>
<td>1.18 ± 0.17 b</td>
<td>1.26 ± 0.11</td>
<td>1.25 ± 0.10</td>
<td>1.22 ± 0.09</td>
<td>1.27 ± 0.08 b</td>
</tr>
</tbody>
</table>

Note: Data were presented as M ± SD, and analyzed by one-way ANOVA.

* p < 0.01 compared with the negative control, BS, and SDH groups by LSD post-hoc test.

a p < 0.01 compared with the negative control and BS groups by LSD post-hoc test.

b c p < 0.01 compared with the negative control group by LSD post-hoc test.

*p < 0.05 compared with the DG group by LSD post-hoc test.

▲ Ditto.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control</th>
<th>Dosage (32 g/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DG</td>
</tr>
<tr>
<td>Fetus</td>
<td>548(42)</td>
<td>211(18)</td>
</tr>
<tr>
<td>Skeletal variation</td>
<td>2</td>
<td>35(^a)</td>
</tr>
<tr>
<td>Skeletal variation (%)(^▲)</td>
<td>4.74</td>
<td>16.59(^a)</td>
</tr>
</tbody>
</table>

**Variation in\(^▲\)**

<table>
<thead>
<tr>
<th></th>
<th>Rib</th>
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<th>0</th>
<th>1</th>
<th>0</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sternebrae</td>
<td>18</td>
<td>24(^a)</td>
<td>8</td>
<td>5</td>
<td>11(^*)</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Limb</td>
<td>10</td>
<td>17(^a)</td>
<td>3</td>
<td>7</td>
<td>12</td>
<td>6(^*)</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Data were analyzed by \(\chi^2\)-test.

\(^a\) \(p < 0.001\) compared with the negative control, BS, and SDH group by Fisher’s exact test.

\(^*\) \(p < 0.05\) compared with the DG group by Fisher’s exact test.

\(^▲\) Ditto.
Fig. 7.2 Gross fetal visceral examination and HE staining. (A, B) a slice at the beginning of the nasal passage just behind the nares; (C, D) a slice cross the neck; (E, F) a slice cross the xiphoid; (G, H) a slice cross the middle of xiphoid and umbilicus; (I, J) a slice cross the umbilicus; (K, L) a slice cross the anterior superior spine.

7.4 Discussion

This study demonstrated that DG aqueous extract (32 g/kg/day) could cause maternal and embryonic toxicity, which is evident in reduced maternal BW, lower fetal BW and the higher incidence of resorption/litter and post-implantation loss/litter, as well as skeletal variation. The toxicity relevant parameters in BS and SDH group did not show significant difference when compared with the negative control group, which indicated that these two herbal decoctions even at high dose level (32 g/kg/day) would not produce any side effect on embryonic development or result in maternal systematic toxicity. Meanwhile, EST indicated that ESC was not more sensitive to BS aqueous extract and SDH aqueous extract than fibroblast 3T3 cell. Interestingly, the maternal toxicity and embryotoxicity induced by DG could be alleviated by BS and SDH.

Herbal interactions can be synergetic and contradicting because of their different characteristics and function based on TCM theory. Chinese herbal medicine (CHM) has the basic features such as four Qi (cold, hot, warmth and coolness); five flavors (sourness, bitterness, sweetness, pungency and saltiness); four tendencies (upbearing, downbearing, floating and sinking); etc. [27]. All these features are difficult to be quantitatively assessed based on clinical experiments. With respect to the theory of TCM, briefly speaking, the side effects produced by
one herb will be possibly attenuated when two herbs with opposite properties are used in pair. Meanwhile, the evidence-based modern researches indicated that there is energy conversion such as electron transfer among herbs of hot, cold, warm or cool properly. For example, herbs with hot properties often have higenamine whose chemical structure is similar to catecholamine. These compounds are beta-receptor stimulants, which could increase heart rate, blood pressure, blood glucose level, and a general reaction of the sympathetic nervous system. Additionally, studies have previously demonstrated that some herbs with hot property could stimulate the system of hypothalamic-pituitary-gonadal axis while herb with cold property could inhibit the functions of adrenal cortex and gonads. In the present study, DG is warm and BS (SDH) is cool in nature. They might possess opposite regulatory effects on the endocrine systems. Thus, the combination of DG and BS (DG and SDH) makes them work coordinately and attenuate properly. This might contribute to the relevant mechanisms involved to decrease overall toxicity.

Current evidence supports chemical constituents in herbs attributing to the interactions between herbs. The mutual effect of which is similar to drug-drug or drug-herb interactions in traditional pharmaceuticals. The interaction may be of two general ways, namely, pharmacodynamic and pharmacokinetic pathways. With respect to pharmacodynamics, herbs used used as pairs may alter the pathways of affecting a target tissue, organ, system, receptor, etc. In terms of pharmacokinetics, herb-herb interactions occur due to altered absorption, metabolism, distribution and excretion of herbal ingredients. The underlying mechanisms on the change in concentration of bioactive constituents with concomitant ones are required to be determined. Usually, the induction or inhibition of hepatic and intestinal cytochrome P450s (CYPs) are thought to play a vital role. Studies suggested that a number of herbs or the chemical compounds from herbs may be substrates, inducers or inhibitors of intestinal and hepatic CYPs, leading to altered drug metabolism and clearance [118]. The CYPs commonly include CYP1A2, CYP2B6, CYP2C9,
CYP2C19, CYP2D6, CYP3A4, CYP2E1, etc. The expression of each CYP can be regulated by metabolic properties of drugs resulting in changes to the pharmacological effects of the drug itself or of other drugs when used in combination. Currently, 95% of drugs used in the market are metabolized by these subtypes of CYPs. In contrast, the expression of CYPs can be regulated by those herbs and chemical compounds. The regulation of CYPs may result in changes of pharmacokinetic parameters such as the AUC and maximum plasma concentration, etc., which is related to an underlying mechanism of the enhanced or reduced efficacy and/or toxicity of co-administered herbs. In the present study, the BS or SDH aqueous extract was orally taken after DG aqueous extract, the difference between the DG and herb-pair group should attribute to the direct influence of BS (SDH) on mice body responding to DG but not the change of chemical compounds produced by co-decocted. BS could increase the activity of P450, CYP3A, CYP2E1, and increase the mRNA expression of CYP2E1 [119]. SDH induces hepatic expressions of CYP3A, CYP2E1, CYP1A2 in rats [120]. As we know, the increased activities of CYPs can promote the metabolism of drugs, with the decrease of drugs plasma concentration.

In practice, oral administration is the main route for applying CHM, so herbal constituents are likely to be absorbed in the gastrointestinal tract to exhibit pharmacological effects. Therefore, the interactions of the compounds of herb during the intestinal absorption process are important. The over (under)-expression of P-glycoprotein (PgP), one of ATP-binding cassette transporters, is another main factor affecting oral bioavailability of compounds. The PgP is expressed in the several organs such as intestine, liver, kidney and blood-brain barrier, and it is thought that the increase in expression and activity of intestinal PgP can reduce the absorption of agents into the blood and increase their metabolism in gastrointestinal tract [121]. Evidence indicated that paeoniflorin induced high-expression of PgP in intestinal tract, especially in the ileum [122]. Besides, lots of active ingredients from
herbs are substracts of PgP, such as flavonoids, coumarins, alkaloids, etc. [123].

Research, focused on the combination of DG-\textit{Shao Yao San} constituent herbs, showed that combination of BS with DG-CX greatly decreased plasma level and bioavailability of ferulic acid (FA) and ligustilide (LIG) [124]. In the previous chapter, data suggested that Z-LIG was more toxic to both ESCs and 3T3 cell lines than FA, which demonstrated it might be the toxic ingredient of DG. Currently, we have no idea whether or not the absorption, distribution and metabolism of LIG have been changed by the combined usage of BS or SDH. This would be an interesting subject for future research.

7.5 Summary

After repeated assessment on the embryotoxicity of DG aqueous extract on pregnant mice, there is no doubt that DG aqueous extract higher than 16 g/kg/day given orally during pregnancy could produce side effects on embryonic development. However, administered with DG at the same dose level in combination with BS or SDH, both maternal and embryonic toxicity could be reduced. This data not only provided important toxicological information for clinical application of DG, but also indicated that BS or SDH aqueous extract could attenuate the toxicity induced by DG. It highlighted that herb-pair (DG-BS and DG-SDH) application could be safer than single DG application.
CHAPTER EIGHT

The Herbal Impact on Bone Development
8.1 Introduction

At the beginning of skeletogenesis, mesenchymal cells proliferate and migrate into the site. Condense and differentiation then occur to form three cell types, namely, chondrocyte, osteoblast and osteoclast, responsible for cartilage and bone formation. Mesenchymal cells condensation is an indispensable process in the skeletal development. Osteogenic and chondrogenic condensations are required for the formation of skeletal structure. Osteogenic condensation (intramembranous ossification) occurs during embryonic development of cranial bones and the clavicle by mesenchymal cell-derived osteoblasts. Chondrogenic condensation (endochondral ossification) involves the formation of intermediate cartilaginous anlagen, then ossification, eventually giving rise to the axial and limb skeletons [12]. Three dimensional patterning as a crucial event is simultaneous with mesenchymal condensations and osteoblast/chondrocytes differentiation for vertebrate skeleton development.

The developmental sequence of an osteoblast phenotype has another two consecutive phases after proliferation, including extracellular matrix maturation and mineralization. Each phase involves the expression of a characteristic set of genes and is necessary for the next [125]. The production of type I collagen (a major collagen constituent) takes place at an early stage of proliferation and differentiation of osteoblasts. The expression of alkaline phosphatase (ALP) characteristically starts immediately after the end of cell proliferation, and reaches a maximum before commencement of matrix mineralization [126]. Osteocalcin is produced by osteoblasts during the matrix mineralization phase, its expression is strongly controlled by 1,25-dihydroxy-vitamin D. Plasma osteocalcin is thought to be directly derived from osteoblasts. It has been estimated that up to 70% of the osteocalcin is secreted into the blood, and the rest is incorporated into the bone matrix [125]. Hence, osteocalcin is classified as a marker of osteoblast activity and a marker reflecting the metabolism of bone matrix. ALP and osteocalcin are two
non-collagenous indicators for osteoblast activity offering a complement to the procollagen propeptides. Owing to type I collagen, ALP and osteocalcin represent different developmental phases of the osteoblast. Measurement of these markers would be a valuable approach to investigate the status of the fetal skeleton development [125].

During proliferation and differentiation of osteoblasts, the cartilage template is being converted into bone. Precursor molecule type I collagen is synthesized in osteoblasts. Then activated by cleaving both termini, followed by carboxyl terminal pro-peptide of type I pro-collagen (PICP) splitting off from the precursor, in a 1:1 ratio [127]. The released PICP is incorporated into the extracellular fluid, becoming one of the markers of bone formation. Under normal conditions, bone formation and resorption are coupled processes [128]. The resorption of old bone brings about the cleavage of collagen by bone-specific proteases into smaller fragments, subsequently released into the extracellular fluid [129]. One of these fragments is crosslinked carboxyl terminal telopeptide of type I collagen (ICTP), mainly derived from bone, which make it a marker of bone resorption. Histomorphometry and calcium kinetic studies indicate that the specificity of levels of PICP and ICTP in circulation make them ideal markers in assessing bone change [130].

The entire of skeleton developmental process is complicated and is regulated by various growth factors, receptors and signaling molecules. For instance, embryonic bone development has been shown to be regulated by numbers of biological factors, including BMPs, fibroblast growth factors (FGFs), the Wnt family of secreted glycoproteins, and Indian hedgehog (Ihh)/parathyroid hormone-related peptide (PTHrP) signaling, etc. [131,132]. Therefore, bone morphogenetic proteins (BMPs) play dispensable roles in almost all aspects of bone generation.

BMPs, as growth factors, belong to the transforming growth factor (TGF)-β superfamily. BMPs are synthesized as inactive precursor in the plasma before being cleaved by special proteolytic enzymes. The bioactive dimeric BMPs are secreted
after dimerized with other BMPs. BMPs could bind to two major types of membrane-bound serine/threonine kinase receptors, type-I and type-II receptors. After binding with their receptor, the phosphorylated type-I receptors transduce the signals to downstream target proteins, such as Smad. Different Smad proteins could transduce signals of different members of TGF-β family. The BMPs-special Smad such as Smad1, 5, 8 transduce signals of BMPs. The phosphorylated Smad1, 5, 8 proteins oligomerize with Smad4 in the cytoplasm and then translocate into nucleus where they regulate transcription of various target genes related to osteoblast differentiation [12]. In addition to the well-characterized BMP-Smad signaling pathway, BMPs also activate the MAPK pathway, and the signaling molecules such as ERK1/2, p38 and stress-activated protein kinase/Jun N-terminal kinase, etc. are involved. These activated MAPK molecules have been demonstrated to be essential for osteoblast differentiation such as ALP activation and osteocalcin expressions [133]. Additionally, inhibitory Smad (Smad7, 8) with the ability of binding BMPR-I could compete with Smad1, 5, 8 for activation, resulting in inhibition of BMPs signals. There are also an array of extracellular antagonists including Glypican3, Noggin, Chordin, Cerberus, and Follistatin modulating the effects of BMPs by binding to BMPs and blocking their binding to receptors. Through those ways, the activation of BMPs signaling is well maintained, and the skeletal morphogenesis is regulated precisely. Since nearly every aspect of skeletogenesis is regulated by BMPs, including osteoblasts proliferation and differentiation, the synthesis of type I collagen is also influenced by BMPs signals. Runx2, one of the downstream effectors of BMPs, is an important Runt domain transcription factor that regulates osteoblast-specific gene expression [134]. It could induce expression of several bone matrix proteins including osteocalcin, type I collagen and ALP, etc., all of which serve as makers relevant to bone morphogenesis. In view of that, BMPs is crucial for the entire skeletal development process, and locates in upstream of signaling pathway. It is thought that abnormal expression of
BMPs family members might influence the downstream pathway and could be causative for the abnormal skeletogenesis.

The different BMPs family members are responsible for different part or stage during entire regulatory process. For example, the expression patterns of BMP-2, BMP-4, BMP-7 and BMP-9 are commonly referred to as the osteogenic BMPs, due to their potent bone-inducing activity [135,136]. Mice lacking in BMP-6 manifest as reduced long bones size and delayed sternal ossification [137,138]. BMP-11 exhibits key effects in skeletal patterning, as the normal anterior-posterior patterning of the axial skeleton is altered because of mutant of BMP-11 [139]. Mice knocking-out bmp-8 duplicated gene (bmp-8a and bmp-8b) showed no skeletal phenotype and defects in spermatogenesis or placental development [140]. These lines of evidence indicated that members of BMPs required for normal skeletal formation in the skull, axial skeleton and limbs.

Little is known, however, about the specific effects of Dang Gui (DG) on fetal bone. To date, only our previous study indicated that prenatal DG exposure impairs fetal bone formation, but the mechanism remains unclear. Therefore the typical phases and changes in key bone regulatory factors needs be investigated to explain the bone variations caused by DG and by its herb-pairs.

As we know, during in-utero development, the amniotic fluid is considered as an extension of fetal serum during the first half of gestation. Before epithelial keratinization in mid-gestation, water and substances may be exchanged across the immature skin surface directly. In the later half of gestation, most of the amniotic fluid is composed of fetal urine. Thus, there is a close relationship between embryo and amniotic fluid on the materials/body fluid exchange [141,142]. Enzyme-linked immunosorbent assay (ELISA) was employed to ensure accurate quantity of PICP, ICTP, osteocalcin, bone ALP, and BMP family members levels in amniotic fluid and fetus.
8.2 Experiment

The groups of pregnant mice were the same as mentioned in chapter 7. The embryo and amniotic fluid were collected on GD15, at the end of exposure day to test herbal decoction. After quantification of total protein in embryo homogenate and amniotic fluid, the samples were used to test the bone developmental and regulatory markers contents by ELSIA. All the procedures were performed in accordance with the manufacturing introductions. The methods were described in detail in chapter 2.3.

8.3 Result

The inter-assay coefficients of variance (CVs) were 5.27% - 5.49% as evaluated by assaying 4 replicates of each sample in a single assay.

The results of PICP, ICTP, ALP-Bone and osteocalcin level in both amniotic fluid and fetal tissue homogenate were shown in Table 8.1. Our data showed contents of bone formation marker PICP from the fetal tissue was lower in DG group than those in the negative control (p < 0.05), while they were increased in herbal pair groups (DG-BS and DG-SDH group). However, no significantly difference of PICP expression in amniotic fluid between DG group and the negative control. The bone degradation markers ICTP from amniotic fluid and fetus showed no difference among all the groups. The levels of ALP-Bone and osteocalcin from fetal tissue were significantly decreased when exposed to DG during organogenesis (p < 0.05 compared with the negative control), but the expressions were higher in herb-pair treated groups than DG treated group. There was no difference of ALP-Bone and osteocalcin expressions in amniotic fluid amongst BS, SDH, the herb-pair groups and the negative control group.

In both amniotic fluid and fetal tissue, concentrations of BMPs in DG group were decreased significantly compared the negative control (p < 0.05). No differences were spotted among BS, SDH, herb-pair groups and the negative control
in amniotic fluid or fetal tissue BMPs concentrations. The GDF-5 content from fetal tissue was significantly lower in the DG group than the negative control (p < 0.05). The expression of GDF-5 in herb-pair groups was higher than the DG group but no significant difference was observed in the corresponding amniotic fluids (Table 8.2).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative control (NC)</th>
<th>Dosage (32 g/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DG</td>
</tr>
<tr>
<td><strong>Fetal tissue (μg/g protein)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PICP</td>
<td>529.69 ± 39.03</td>
<td>439.84 ± 48.04\textsuperscript{a}</td>
</tr>
<tr>
<td>ICTP</td>
<td>0.46 ± 0.03</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>ALP-Bone</td>
<td>2.62 ± 0.09</td>
<td>2.35 ± 0.20\textsuperscript{a}</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>16.56 ± 0.47</td>
<td>13.42 ± 0.67\textsuperscript{a}</td>
</tr>
<tr>
<td><strong>Amniotic fluid (ng/mL)</strong></td>
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</tr>
<tr>
<td>PICP</td>
<td>587.31 ± 75.03</td>
<td>524.81 ± 88.93</td>
</tr>
<tr>
<td>ICTP</td>
<td>0.54 ± 0.09</td>
<td>0.54 ± 0.09</td>
</tr>
<tr>
<td>ALP-Bone</td>
<td>2.65 ± 0.35</td>
<td>2.40 ± 0.44</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>15.90 ± 2.20</td>
<td>15.22 ± 2.62</td>
</tr>
</tbody>
</table>

\textbf{Note:} Data were presented as M ± SD, and analyzed by one-way ANOVA.

\textsuperscript{a}p < 0.05 - 0.001 compared with the negative control by LSD post-hoc test.
Table 8.2 Bone regulatory markers in herbal water extract treated groups (M ± SD, n = 18).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative Control (NC)</th>
<th>Dose (32 g/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DG</td>
<td>BS</td>
</tr>
<tr>
<td><strong>Fetal tissue (μg/g protein)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMPs</td>
<td>25.04 ± 1.83</td>
<td>17.05 ± 3.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GDF-5</td>
<td>0.31 ± 0.01</td>
<td>0.27 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Amniotic fluid (ng/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMPs</td>
<td>32.18 ± 4.93</td>
<td>26.85 ± 6.73&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GDF-5</td>
<td>0.34 ± 0.04</td>
<td>0.33 ± 0.05</td>
</tr>
</tbody>
</table>

**Note:** Data were presented as M ± SD, and analyzed by one-way ANOVA.

<sup>a</sup>p < 0.05 - 0.001 compared with the negative control by LSD post-hoc test.
8.4 Discussion

In chapter 3, 4 and 7, the results showed that the DG aqueous extract at very high dose level (32 g/kg/day) could produce fetal development toxicity with significant skeletal variation. The obvious manifestation of skeletal structure abnormalities included sternebrae and digit formation defects, etc.; whereas the BS or SDH aqueous extract at high dose level (32 g/kg/day) did not cause developmental toxicity. Therefore, the combined use with BS or SDH decreased the toxicity induced by DG. Moreover, the incidence of fetal skeletal variation was also reduced. In this chapter, the level of bone formation marker, PICP, in both amniotic fluid and fetal tissue, was markedly lower in the DG 32 g/kg/day group when compared with the negative controls, but increased in the herb-pair groups. The data showed that the ICTP level in the amniotic fluid or fetal tissue was not affected significantly by herb treatment. PICP and ICTP represent by-product of type I collagen formation and degradation, respectively. It is well accepted that the extracellular matrix of bone is composed mainly of type I collagen. Thus, measurement of the rate of type I collagen may reflect the rates of bone metabolism formation and degradation. Although type I collagen are also present in other sites, including skin, connective tissue, and uterine tissue, bone histomorphometry studies have shown that type I collagen propeptides from most non-skeletal tissues contribute very little to the circulating propeptide pool [143,144,145]. Therefore, our data indicated that the DG administration influenced the fetal bone formation rather than resorption. Studies showed that during human gestation period, the markers of bone turnover in umbilical cord blood increases with gestational age. PICP decreases in early pregnancy, with a nadir at 12 weeks, followed by a steady rise throughout pregnancy, peaking at 38 weeks. ICTP collagen is maintained at a steady-state level until 14 weeks, after which it rises throughout pregnancy to peak
levels near term [146]. Some researches indicated that PICP and ICTP concentrations decreased in the amniotic fluid over gestation since the increasing ability of the maturing fetal kidney to retain the markers in blood or the decrease of permeability of maturing skin [147]. However, in the current study, because only one-time point collection of amniotic fluid and fetus, we have no idea in the changes of both bone markers during the entire gestation. However, our data showed that change of markers in the amniotic fluid was correlated with that in fetus because of herb exposure. A line of evidence suggested that a high turnover of bone matrix occurred during fetal life secondary to accelerated osteoclastic and osteoblastic activities. Any reduction in bone formation might compromise fetal growth, as measured by the bone marker PICP in the amniotic fluid, which was positively associated with fetal growth [147]. Our data showed that the fetal body weight was much lower in the DG treatment group than that in the negative control, and the fetal growth retardation was consistent with the abnormal bone formation.

ALP and osteocalcin produced by osteoblast can be used as indicators for different development phase, matrix maturation and mineralization respectively. At the same time, bone formation markers deserved equal attentions. In the present study, data showed that the contents of ALP and osteocalcin from fetal tissue and amniotic fluid were decreased by DG aqueous extract long-term exposure to pregnant females. It is suggested that oral administration of DG (32 g/kg/day) during organogenesis not only affected the proliferation of osteoblasts, but was also impacting on the extracellular matrix maturation and mineralization. The declined levels of PICP and osteocalcin suggested the decrease of both collagenous and non-collagenous constituents of the extracellular matrix of bone, which very likely resulted in abnormal skeletal development. Interestingly, the herb-pair showed different effects compared to treatment with DG alone. The level of ALP-Bone and
osteocalcin from the amniotic fluid in the DG-BS herb-pair approached to the level of that in the negative control, without significant difference. In the present study, the changes of bone marker were consistent with the observation.

The bone formation is under regulation by several growth factors, therein BMPs are one of the most important groups involved in nearly every physiological event. The abnormal expression or dysfunction might give rise to skeletal structure defect. In our study, the BMPs concentration from the amniotic fluid and fetal tissue was much lower in DG group. Compared to DG treatment group, the BMPs contents in both amniotic fluid and fetus increased when co-administered with BS, and there was no difference from those in the negative control. As mentioned previously, the lower expression of BMPs in DG group might induce undesired results, which was confirmed by the skeleton variants observed in DG aqueous extract group.

BMPs are critical determinants of the embryological development in mammal. During embryogenesis, BMPs regulate dorsal-ventral patterning, establishment of embryonic body plan, cell apoptosis, differentiation of neural cell, patterning of the limb bud and epithelial-mesenchymal interactions. In the present study, we pay more attentions to the toxic effects of DG on fetal skeletogenesis and its mechanism. The main and common skeleton variations produced by DG includes delayed ossification of sternum and phalanges, and loss of skeletal elements in the limbs. Therefore we focused on the expression of the specific BMP family members responsible for this abnormality. Lines of evidence demonstrated that mice lacking Gdf-5 showed severe reduction or loss of some skeletal elements in limbs, additional fusions between skeletal structures, scoliosis, and altered cartilage in the intervertebral joints of the spinal column [148]. Unfortunately, the experimental observation time ended before birth, so whether or not the impact on bone to be
normalized after birth is unknown. Moreover, the impact on function of reproductive system and nervous system remained to be explored in the future studies.

Moreover, our data showed that the levels of all these bone development markers in the amniotic fluid correlated with those from fetal tissue on DG15 in each group. Day 15 of the 18.5-day gestation, the keratinization was not yet completed, which meant solutes might be exchanged across the immature skin surface [147]. Thus, the change of solutes in the amniotic fluid also was reflected in fetal serum which is consistent with the present data.

8.5 Summary

Skeletal structure deficiency is one of the major manifestations of developmental toxicity. PICP and ICTP serving as markers of bone formation and degradation were employed to detect the status of fetal bone development in our study. Our data indicated the lower levels of PICP in the amniotic fluid and in fetus from DG group were consistent with the observation of the compromised bone formation in DG group. The decrease in ALP-Bone and osteocalcin content indicated interruption in bone matrix maturation and mineralization by DG. The co-administration of BS (SDH) might reduce the embryotoxicity on skeletal development induced by DG alone, as evidenced by the increased PICP, ALP-Bone and osteocalcin concentrations and fewer skeletal variations. There was no difference of ICTP concentrations among all the groups, which suggested that the bone degradation might not be influenced by all the herbs, even at very high dose level during organogenesis. As regulator of skeletal development, BMPs play dispensable roles in every aspect during developmental process. The low expression of BMPs in DG group resulted in decreased type I collagen synthesis, eventually
bone formation abnormalities. Fortunately, the expression of BMPs was upregulated by BS (SDH), and desired results, decrease of abnormal skeleton formations, were found during fetal skeleton observation. Individual BMP family members might have the specific expressed sites and work for different aspect of fetal skeleton development. Current skeleton screening further confirmed their functions. In addition, because of multi-roles of BMPs in embryogenesis, future studies are required to explore whether or not other systems are also involved.
CHAPTER NINE

General Discussion and Future Study
9.1 General discussion

9.1.1 Quality control of herbal preparation

Herbal medicines have been used worldwide for many centuries and are becoming increasingly popular in developed countries. The concerns regarding their safety and efficacy has grown as a result. However, before assessment of their safety and efficacy, it is thought that quality control is the prerequisites. As we know, the genera and species of medicinal herbs are variable, their chemical components are complicated, and compositions varied with the different processing and extraction methods. Besides, growing condition of the herbs such as climate, soil and harvest season, etc. is crucial. Their chemical constituents and amounts can be different. In the present study, the herbs were purchased from an authorized pharmaceutical company and authenticated by experts in traditional Chinese medicine from School of Chinese Medicine, Hong Kong Baptist University. The raw herbs were extracted with water over 3 times, strictly following the standardized preparation applied in clinic. The sign matters in each herb were qualified and quantified according to standard operation procedure using HPLC, such as ferulic acid (FA) and Z-ligustilide (Z-LIG) in Dang Gui (DG), paeoniflorin in Bai Shao (BS), catalpol in Sheng Di Huang (SDH), which was recommended by Chinese Pharmacopeia (2010) and The Hong Kong Chinese Materia medica Standards (HKCMMS). Only when the content of each reference material is measured up to the requirements can the herb be used in the following in-vivo and in-vitro studies. With respect to the complex factors such as agriculture, processing, etc., the external contamination with heavy metals and pesticide residues associating with herbal quality should be taken into account. In this regard, the herbal preparation was subjected to rigorous assessment of contaminants according
to HKCMMS once any slight toxic sign was observed after oral taken by experimental animals. In general, the DG decoction was found to be free from the common 20 target organochlorine pesticide residues and 4 heavy metals. Therefore, any observed toxicity in the studies could be resulted from the potential embryonic toxicity of the intrinsic constituents of DG.

9.1.2 Herbal impacts on maternal health

The developmental toxicity studies recommended by the guidelines of international agencies require the administration of high dose test agent that induce overt maternal toxicity, typically observed as significant reduction in food or water consumptions, a reduced maternal weight gain after subtraction of gravid uterus weight, clinical signs of adverse health effects, organ toxicity, and mortality. Actually, maternal toxicity is not always associated with abnormalities of the fetus, though maternal physiological function directly influences embryo/fetus development and the health in many cases. 22% failed to show any developmental toxicity in the presence of maternal toxicity among animal studies (rat, rabbit, and hamster) [149]. The general conclusion that developmental effects are secondary to maternal toxicity will decrease the significance of fetal toxicity [150]. The fetal endpoints obtained from the developmental study include mean body weight, resorption rate, number of anomalies, and mortality, etc. In the present study evaluating the toxicity of DG on embryonic development toxicity in mice, the developmental outcome such as a decrease of fetal mean weight, increase of post-implantation loss rate and birth defect, etc. occurred in the presence of significant reduction in maternal body weight gain as evident in the DG (≥ 16 g/kg/day) treatment group. It is hard to delineate clearly the role of maternal and direct
embryo/fetal toxicity on intrauterine development in our case.

9.1.3 Herbal impacts on fetal bone development

Among developmental outcomes (e.g., embryo death, morphological defects, malfunction, and growth retardation, etc.), skeletal alteration is one of the very important parameters for assessing embryotoxicity. As we know, during fetal intrauterine development, bone morphological changes are the most prominent. Once the embryo survived from the early environment factors’ challenge, the morphological defects and skeletal variations are the main manifestations. Commonly, malformation as permanent structural changes was considered to be harmful to survival, development, or function, while variation is a slight change different from the usual structural constitution, which may not adversely affect on survival or function and may occur relatively frequently in the control group [5]. There is no accurate classification to differentiate malformations and variations, to some degree, because there is a continuum of responses between normal and abnormal development [8]. In our previous work, the alterations could be mainly subdivided by distinguishing between alterations that results in developmental delays and structure changes. A delay in ossification is usually apparent and occurs frequently in regions such as fifth sternebra, phalanges, etc. These delays are commonly associated with decreases in fetal weight and maternal body weight gain because of high-dose test agent administration. Evidence from this study indicates that high-dose (32 g/kg/day) of DG water extract administration produced various skeletal variations including metacarpal missing, completely unossified phalangeal, and sternum with major anomalies which are the most prominent compared with the negative control.
The fetal skeletogenesis involves intramembranous and endochondral bone formation, in which intramembranous and endochondral ossification takes place. Intramembranous ossification occurs during embryonic development of the flat bones of the cranial vault and parts of the clavicle. Endochondral ossification is an indispensable process for axial and limb skeletons formation. Along with ossification, osteoblasts proliferate and differentiate, mature, and secrete collagen I and other bone-specific molecules, including alkaline phosphatase (ALP), osteocalcin indicating matrix maturation and mineralization (Fig. 9). In the present study, the level of PICP, one of the metabolites of collagen I, as well as ALP, osteocalcin were markedly lower in the DG 32 g/kg/day treatment group than the negative controls, which might indicated that the all three bone development phases: proliferation, matrix maturation and mineralization were influenced by DG administration during organogenesis. The change in bone markers coincided with the observation in skeleton detection.

Nearly every aspect of skeletogenesis is regulated by bone morphogenetic proteins (BMPs) signals. Each BMP family member has its special contribution or synergistic effects with another member during fetal bone development (Fig. 9).

Lines of evidence indicated that mice lacking Gdf-5 and Gdf-6 manifests as severe reduction of some skeletal elements in the limb. Furthermore, BMPs are also responsible for a series of physiological events during embryonic development. It has been demonstrated that BMP-8 knockout mice accompany with other altered biological consequence such as germ cell degeneration [151] and deficiency and infertility [152]. Except for controlling anterior-posterior patterning of the axial skeleton, BMP-11 also plays a key role in mesodermal and neuronal tissue pattering [153]. In the present study, the BMP-6 expression was significantly decreased by of DG extract treatment, which did not impact on the expression of BMP-8 and
BMP-11 in fetal tissue or amniotic fluid. Ossification delay is frequently seen in sternum and smaller long bone in DG treated group, but no abnormal structure of axial skeleton was observed, which could once again confirm the previous finding. Moreover, there was no pathological finding involving the reproductive system and nervous system of fetuses during visceral examination, which is in accordance with the negative results of BMP-8 and BMP-11 expression (as shown below).

<table>
<thead>
<tr>
<th>BMP family members</th>
<th>Negative control (NC)</th>
<th>DG 32g/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetal tissue (ng/g protein)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-11</td>
<td>59.72 ± 3.60</td>
<td>59.58 ± 4.80</td>
</tr>
<tr>
<td>BMP-6</td>
<td>143.95 ± 8.03</td>
<td>132.96 ± 12.20*</td>
</tr>
<tr>
<td>BMP-8</td>
<td>1229.33 ± 104.78</td>
<td>1220.32 ± 57.92</td>
</tr>
<tr>
<td><strong>Amniotic fluid (pg/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-11</td>
<td>63.04 ± 5.74</td>
<td>62.84 ± 4.75</td>
</tr>
<tr>
<td>BMP-6</td>
<td>151.91 ± 12.72</td>
<td>140.18 ± 11.65*</td>
</tr>
<tr>
<td>BMP-8</td>
<td>1300.06 ± 102.72</td>
<td>1410.11 ± 167.85</td>
</tr>
</tbody>
</table>

**Note:** Data were presented as M ± SD, n = 10.

*p < 0.05 - 0.001 compared with the negative control group by t-test.
Fig. 9.1 Bone markers during embryonic bone development. The developmental sequence of an osteoblast phenotype has three consecutive phases, including proliferation, extracellular matrix maturation and mineralization. Each phase involves the expression of a characteristic set of proteins.

9.1.4 Herbal impacts on EST

The *in-vivo* study may not suggest whether or not the embryo-fetal toxicity is secondary to the maternal toxicity. The recommended alternative *in-vitro* test using embryonic stem cell (ESC) and reference cell lines (embryonic fibroblast 3T3 cell) mimics the embryonic and adult tissues, this will provide opportunities to investigate the toxic effects of various test agents on embryo/fetus. Toxic effects can be due to either altered condition of pregnant female or directly on embryo/fetus. The inhibition of ESC differentiation (ID$_{50}$) and cytotoxicity of two cell lines (IC$_{50}$ ESC and IC$_{50}$ 3T3 cell) are served as the three experimental outcomes correlated significantly with embryotoxic potential. The test agents are tested for its non-embryotoxicity, or embryotoxicity. Although there was no significant difference in IC$_{50}$ of DG aqueous extract when compared between ESC and 3T3 cell, we may not rule out DG with specific toxicity on embryo *in-vitro* owing to lack of ID$_{50}$. The
difference between cytotoxicity of Z-LIG and FA on ESC and 3T3 cell indicates that Z-LIG was more toxic than FA to either ESC or 3T3 cell.

9.1.5 Herb-pair interaction

Herb-pair is the simplest form of formula representing the interaction and mutual influence of herbs. Six different compatibilities (mutual reinforcement, mutual assistance, mutual restraint, mutual detoxication, mutual inhibition, mutual antagonism) determine the way of combination of two herbs. In all, these combinations can be briefly classified into two categories based on purposes, one of which is for fewer side effects, the other one for better curative efficacies. This conception still affects the cognition of herb-herb interaction in modern Chinese medicine. Balance is one of the common criteria of herbs usage in pair, and several herbs are combined according to the properties of herb, e.g., warm paired with cool, ascending paired with descending, etc., so as to extend their abilities and counteract their toxicities or side effects. For example, DG and BS are both blood tonics. DG is warm and dispersing, and more suitable for blood-deficient patients with Yan-deficiency. On the contrary, BS is cool and stable, and is used for blood and Yin-deficient patients with heat [51]. A number of DG-containing herb-pair was used for treatment of various conditions during peri-natal period. Moreover, DG is one of the top 10 Chinese medicines frequently used for treatment of miscarriage. DG paired with Huang Qi is commonly used as a tonic for postpartum fatigue and weakness, and calming fetus, usually paired with Di Huang or Sang Ji Sheng for calming fetus, paired with Yi Mu Cao also for postpartum retention of lochia.

The development outcomes from our study such as a decrease of fetal body weight, an increase of post-implantation loss rate, and an increase of skeletal
variation occurred after DG water extract at or above 16 g/kg/day administration. In the present study, balance in nature of two paired herbs was the main criteria for selecting the paired herb for DG. According to traditional Chinese medical theory, DG is warm nature, whereas BS and SDH are opposite in nature. We investigated the difference in developmental outcomes between DG alone and DG-BS (SDH) treatment groups. The data suggested that DG paired with BS (SDH) could decrease the embryotoxic effects produced by DG used alone. In herb-pair groups, both maternal body weight gain and fetal body weight were increased, as well as the resorption rate. Besides, the skeletal variation rate was reduced when compared with that in the DG group.

In the tide of the modernization of Chinese medicine, the exact mechanism about synergism of herb pairs as the basic composition units of Chinese herbal formula attracts more attentions in the east and beyond. The pharmacodynamic and pharmacokinetic mechanisms are most relevant. The synergistic effects of herb pairs can be achieved by using a pair of herbs with one ingredient as an inducer or inhibitor of cytochrome P450s and other transporters such as P-glycoprotein, so as to enhance the therapeutic effects or reduce the toxic effects of another active ingredient by regulating the absorption, distribution, metabolism and excretion [154]. Besides, the one in paired-herb could alter the way the other herb affects a target tissue, organ, system, receptor, etc.

9.2 Future study

9.2.1 Study on chondrogenesis during fetal development

The vertebrate skeleton consists of bone and cartilage. Bone formation and chondrogenesis are initiated by mesenchymal cell proliferation and condensation.
The differentiated chondrocytes could convert into hypertrophic chondrocytes, which direct mineralization and vascular invasion. Concomitantly, the hypertrophic chondrocytes undergo programmed cell death and replaced by the bone matrix produced by osteoblasts that are transported by blood vessels during vascularization [132]. In this way, the hypertrophic chondrocytes could form either the articular cartilage or a template for long bone during endochondral ossification. Each stage of chondrocyte differentiation is characterized by amounts of extracellular matrix macromolecule production such as collagen and proteoglycans, etc. Collagens II, IX, and XI and aggrecan are maximally produced during resting, proliferation and hypertrophic maturation, whereas type X collagen is synthesized by chondrocytes after they have become hypertrophic and before mineralization of the extracellular matrix occurs [155].

Chondrogenesis is also dependent on several signals generated by cell-ECM and cell-cell adhesion interactions, which in turn are modified by the cell’s response to growth and differentiation factors [156]. Amounts of regulatory factors, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), insulin-like growth factor 1, Indian hedgehog, parathyroid hormone-related peptide, Runx, and Wnt, are in charge of discrete steps in chondrogenesis [132]. For example, BMP genes, Bmp-2, 4, 5, 6, and 7 transduced by heterodimers of type I receptors (Bmpr1a/b) with type II receptor (Bmpr2) is known to be indispensable for early events in chondrogenesis [157]. Bmpr1b-deficient mice exhibit phalangeal elements and appendicular joints defects [158]. Besides, Bmpr1a conditional knockout mice exhibit generalized chondrodysplasia with long bones shortened and delayed ossification. Importantly, Bmpr1/2 double mutants do not express Sox9, indicating that BMP signaling acts as an upstream activator of Sox9 expression [159]. Moreover, the expression of Bmp-8a was found in developing long bones
and the mouse achondroplasia locus, suggesting that it might be an important regulator of chondrogenesis [160]. In addition, Bmp-6 and Bmp-7 are also highly expressed during chondrogenesis [161], and Gdf-5 has been used to induce ectopic chondrogenesis *in-vivo* suggesting its role in chondrogenesis [162].

Up till now, we have focused most of our attention on the osteogenesis, which could be affected by DG during organogenesis though regulation of BMPs expression in fetus. This can also occur at the molecular level where collagen I metabolite (PICP), ALP, osteocalcin, etc. representing each stage of bone development can be affected. The delayed ossifications of sternum and phalanges were obvious in the DG treatment group. So far, plenty of published papers have provided evidence that chondrocyte hypertrophy and osteoblast differentiation are controlled by a variety of common signaling and factors, such as BMPs. In the further study, the chondrogenesis-toxic effect of DG could be investigated at different stage of chondrocyte differentiation, and the other regulatory factors such as Runx, Rox9, etc., need to be inspected during chondrogenesis.

9.2.2 Study on the immune-endocrine network during pregnancy

Apart from significant fetal bone formation inhibited by DG extract, the increased incidence of resorption was also obvious in the DG extract administration group. During recent decade research, evidence indicates that in addition to genetic abnormalities accounting for half of all miscarriages, the endocrine, immune infective thrombophilic conditions are other possible causes. The imbalance in type 1 T helper (Th1) cytokine /type 2 T helper (Th2) cytokine in feto-maternal interface is one of the causative factors for miscarriage. Th1 cytokines (e.g., interferon (IFN)-γ and tumor necrosis factor (TNF)-α) predominance leads to miscarriage, while Th2
cytokines (e.g., interleukin (IL)-4, IL-10, IL-13) are associated with pregnancy success [163,164]. DG is known as an immunoregulatory herb with interferon inducing activity, especially the polysaccharide isolated from herb [165]. Whether or not the immune condition of pregnant female was influenced by DG extract or DG herb-pair extracts or their active compounds remains unclear, further studies to clarify the mechanism are therefore required. Successful pregnancy not only results from precise immunoregulation between maternal body and fetus but also from the regulation of hormone secreted from endocrine system during pregnancy. The level of reproductive hormone is one of key regulators related to normal pregnancy. Increasing evidence indicated that DG might have estrogen-like effects in \textit{in-vivo} and \textit{in-vitro} [76,166]. A study using an \textit{in-vitro} competitive estrogen receptor (ER) binding assay illustrated that DG dose-dependently inhibited the binding of estradiol to ER. Thus, the authors hypothesized that it might compete with E\textsubscript{2} for ER binding sites, resulting in an inhibition of ER-mediated cellular event [167]. In contrast, \textit{in-vivo} study investigated the impact of DG aqueous extract on varian morphology, data of which showed that DG could promote folic maturation and ovulation by upregulating the expression of ER \beta in the ovarian granulose cell [168]. This discrepancy intrigues us to explore how and by which way DG might impact the endocrine system during this special period.

9.2.3 Embryonic stem cells differentiated osteoblasts

In order to prevent birth defects, toxicological studies have been designed to identify toxicities that may occur in human embryos. To reduce the spending of live animals, an alternative model, ESC, has been used for \textit{in-vitro} assay for testing embryotoxicity. This classic ESCs test assesses developmental toxicity of a given
test agent through counting of contracting cardiomyocytes agglomerates, which is one of the endpoints. The difference in sensitivity towards the cytotoxicity of agent between the adult and the ESC using MTT assay is the other two endpoints. ESCs are undifferentiated cells with the capability to regenerate into one or more committed cell lineages, such as cardiomyocyte, neurocyte, osteoblast, osteoclast, etc. The current evidence from in-vivo study indicated that over a certain dose of DG administration might be toxic to mice osteogenesis in utero. Hence, an experiment to differentiate ESC into osteoblasts is suitable for investigating bone toxicity in our further in-vitro cost-effective studies. ESC derived osteoblasts differentiation is presented along with two endpoints analysis including osteoblasts generation as well as their mineralization in culture.

9.2.4 Further testified in other species

More than one species (mostly rat and rabbit) used for reproductive toxicity testing is widely recommended as it provides better evaluations of its toxicology and side effects profile. Rat is one of the preferred species because toxicity data can be commonly obtained. Rabbits have different placenta and pregnancy physiology and is less similar to human physiology when compared with the rodents. However, historically, rabbits demonstrated limb-reduction and deformities when they received thalidomide and therefore can be considered as a potential species for further testing.
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PUBLICATIONS

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September 2015