DOCTORAL THESIS

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

Xiao, Tingting

Date of Award:
2015

ABSTRACT

Background and purpose:

Angelicae Sinensis Raidx (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 the 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 IC_{50} values of ESCs ($IC_{50\ ESC}$) and 3T3 cells ($IC_{50\ 3T3}$) after treatment with DG, *Z*-LIG and FA samples respectively, the $IC_{50\ Z\text{-}LIG}$ was significantly less than $IC_{50\ FA}$ 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 $IC_{50\ ESC}$ and $IC_{50\ 3T3}$ after DGS extract treatment was statistically significance ($p < 0.05$), however no statistical significance was identified in DGW ($p > 0.05$). Both $IC_{50\ ESC}$ and $IC_{50\ 3T3}$ 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 IC_{50} value, hence it is believed that the higher phthalides level (3-butylphthalide, Z-butylidenephthalide, 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.

TABLE OF CONTENTS

CHAPTER THREE

CHAPTER FOUR

CHAPTER SIX

CHAPTER SEVEN