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RESEARCH ARTICLE

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Influence of *Radix scutellariae* on Th1/Th2 cytokine balance in RU486-induced abortion in mice

Abstract The aim of this study is to investigate the significance of Th1/Th2 cytokine balance in the uterus in the early embryo loss (or resorption), and to elucidate immunological modulation at the maternal-fetal interface with Chinese herbal medicine *Radix scutellariae* (*Huang Qin*) and its constituents (Baicalin and Baicalein). Mifepristone (RU486) was given via subcutaneous injection in the scapular area to induce abortion in mice at day 7 of gestation. The levels of uterine Th1 cytokines (IFN-γ, IL-2) and Th2 cytokines (IL-4, IL-10) were analyzed by enzyme-linked immunosorbent assay (ELISA), respectively. The mean values of Th1 cytokines in the uterus of RU486-treated abortion mice were significantly higher ($P < 0.05$) than that of the control mice, but no significant difference was observed regarding to the contents of Th2 cytokines of different groups ($P > 0.05$). However, when the *Radix scutellariae* and its constituents were used to prevent RU486-induced abortion, the levels of IFN-γ and IL-2 decreased while that of IL-4 and IL-10 increased. The embryo loss induced by RU486 was closely related to the Th1/Th2 immune balance at the maternal–fetal interface. *Radix scutellariae* and its constituents have an anti-abortive effect through restoring the Th1/Th2 balance at the maternal-fetal interface.

Keywords *Radix scutellariae*, Mifepristone, abortion, Th1/Th2, Baicalin, Baicalein

1 Introduction

Early embryo loss, including failure of embryo implantation and embryo resorption soon after implantation, has been paid much attention to in animal reproduction. Studies have shown that the embryo resorption rate in mammals is high especially in ruminants, i.e., the resorption rate of sheep, cattle, and swine is up to 25%, which is much higher in horse (Zhang, 1997). Furthermore, in embryo transplantation, the successful rate of fecundation of in vitro fertilization is only 30% to 35%. Early embryo loss plays a bad role in animal reproduction and breeding, causing economical losses in the animal farming industry. Therefore, it is important to study the immune relation between the mother and the fetus at the maternal-fetal interface and to find out the preventive measures of early embryo loss.

Pregnancy is usually referred to as an "immunological paradox" because tissue allografts are generally rejected by an immunocompetent host, while the conceptus is not rejected by the maternal immune system. The survival of the fetus in the face of potentially hostile maternal immune system has been postulated to be due to immunomodulation at the maternal–fetal interface (Chaouat et al., 1997), and a consequent lack of strong maternal cell-mediated anti-fetal reactivity of the Th1 type (Raghupathy, 1997). Th1 and Th2 cells are the major functional subsets of T helper cells (Mosmann and Coffman, 1989; Mosmann and Sad, 1996). Th1 cells secrete interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which are detrimental to pregnancy (Haddad et al., 1995; Raghupathy, 1997). Th2 cells are associated with help for B-cell antibody production and secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, which are assumed to be a pregnancy-preferred Th2 bias (Wegmann et al., 1993). Studies have shown that significantly increased production of both IL-2 and IFN-γ and reduced production of IL-10 characterized pathologic pregnancies and distinguished them from normal pregnancies, suggesting that a type 2 cytokine profile may be associated with normal human pregnancy, whereas the lack of a dominant type 2 cytokine profile may be indicative of a pathologic pregnancy (Marzi et al., 1996).

The Chinese herbal medicine *Huang Qin* (*Radix scutellariae*) has been used as a sedative to a restless fetus for thousands of years. The famous physician Danxi Zhu in the Chinese Jin Dynasty (A.D.1115–1234) pointed out that *Huang Qin* and *Bai Zhu* are the best herbs for fetal restlessness. A

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great deal of animal experimentation and modern pharmacological research show that Chinese herbal medicine has a positive influence on the cytokine balance in pregnancy, suggesting that it may be feasible to investigate the anti-abortive effects of *Huang Qin* and it's constituents on Th1/Th2 immune balance.

The study was designed to elucidate the anti-abortive effects of *Huang Qin* and its constituents Baicalin and Baicalein on RU486-treated mice at the maternal–fetal interface by measuring uterine IFN-γ, IL-2, IL-4, and IL-10 levels.

2 Materials and methods

2.1 Animals

BALB/c mice aged ten weeks were purchased from the Animal Laboratory of Hebei Medical University, China. The animals were given free access to mouse chow and water, with a 12 h light cycle from 7:00 to 19:00. Pregnancies were obtained by housing one virgin female with one male, and the females were examined each day in the early morning for the presence of a vaginal plug. The day when the vaginal plug was detected was designated as day 0 of pregnancy.

2.2 Reagents

RU486 (Sigma Chemicals), dissolved in ethanol at a concentration of 0.01 mol, and then distilled water was added up to the concentration of 150 μg/mL.

Baicalin (Aldrich products) was dissolved to a concentration of 2.5 mg/mL with distilled water.

Huang Qin decoction: *Radix scutellariae* (*Huang Qin*) purchased from a herbal shop in Baoding City and authenticated by the Hebei Provincial Bureau of Herbal Medicine was soaked in distilled water for one hour and then boiled for an hour. After removing the supernatant, the herb was boiled again for another hour in distilled water. Then the two parts of the supernatant were mixed and simmered to the desired volume, i.e., 0.5 g/mL.

2.3 Animal treatments in different groups

Ten-week-old BALB/c mice were divided into five groups randomly: "A" as control group, "B" as RU486 model group, "C" as Baicalin group, "D" as Baicalein group, and "E" as *Huang Qin* group. Mice in groups B, C, D, and E were given a subcutaneous injection of RU486 in the scapular area at a dose of 0.4 mL (60 μg) at Day 7 of pregnancy. Mice in groups C, D, and E received an oral gavage of Baicalin (1 mg), and herbal decoction (0.2 g) respectively at a dose of 0.4 mL at days 4–7. Animals in Group A received a distilled water gavage on the same days of gestation as in Group C and were administrated with distilled water at 0.4 mL at Day 7 of gestation. The gravid females were killed by cervical dislocation at Day 9 of gestation.

2.4 Preparation of uterine lysate and ELISA for cytokines

All the pregnant mice were sacrificed by cervical dislocation at Day 9 of gestation. The uterus was carefully cleaned of fat, and the fetus was removed. Uterine lysates were prepared in PBS (pH 7.4) containing phenylmethanesulfanyl fluoride (PMSF, 0.75 μg/mL), centrifuged for 15 min at 12 000 r/min at 4°C and the supernatants were collected for ELISA. Using commercial kits IFN-γ, IL-2, IL-4, and IL-10 (Endogen Company, USA), ELISA was performed according to the manufacturer's instructions, respectively.

2.5 Calculation of embryo loss rate and abortion rate

All the gravid females were killed by cervical dislocation at Day 9 of gestation and the levels of uterus were examined for viable and resorbing embryos. The viable embryos (V) were well-oxygenated (pink) and showed a well-defined embryonic capsule and placenta. The resorbing embryos (R) were usually smaller, showed signs of ischemia, haemorrhage, and often were macerated and black in color without identifiable embryo or placenta. The incidence of embryo loss was presented as a percentage of the levels of the uterus ($100R/(V+R)$). The incidence of abortion was calculated as a percentage of the levels of the miscarriage (100·abortive mice/total mice).

2.6 Statistical analysis

Statistical analysis of data was conducted using Excel 2000 and SPSS 11.0, analyzed with One-way Analysis of Variance and χ^2-test ($P < 0.05$ was taken as significant).

3 Results

3.1 Anti-abortive effects of *Radix scutellariae* and its constituents Baicalin and Baicalein

Group A, pretreated with distilled water as a control, showed a natural abortion rate of 33.3%. Mice in Group B were sacrificed at Day 9 of gestation in which the abortion rate was 80.0%, the embryo resorption rate was 85.7%. The resorbed conceptus was severely macerated and black in color. Mice in Group C, pretreated with Baicalin, showed a resorption of 29.4% and abortion 30.0%. The number of mice in Group D was eight and three were aborted and the resorption rate was 33.3%. Mice in Group E pretreated with herbal soup and showed a 31.6% resorption and a 40.0% abortion (Table 1).

3.2 Uterine Th1/Th2 cytokines in different groups

The ratio of IL-4/IFN-γ in Groups A to E was 14.019 ± 4.185; 7.582 ± 1.472; 13.192 ± 5.204; 13.839 ± 4.637; and 14.499 ± 7.853, respectively. The statistically significant difference

Table 1 Anti-Abortive effects of *Radix scutellariae* and its constituents Baicalin and Baicalein on RU486-treated mice

Group	Medicine i.g. (G 4–7)	Medicine s.c. (G 7)	Abortion rate/%	Fetal resorption/%
A	Distilled water	Distilled water	33.3 (3/10)^{B}	7.1 (2/28)^{B}
B	—	RU486	80.0 (8/10)^{A}	85.7 (30/35)^{A}
C	Baicalin	RU486	33.3 (3/10)^{B}	29.4 (5/17)^{B}
D	Baicalein	RU486	37.5 (3/8)^{B}	33.3 (7/21)^{B}
E	Herbal decoction	RU486	40.0 (4/10)^{B}	31.6 (6/19)^{B}

Notes: Data with different superscripts have significant differences at $P<0.01$ (capital letters) or at $P<0.05$ (small letters); G4-7 means Days 4–7 of gestation; G7 means Day 7 of gestation.

($P<0.05$) was found in Groups C, D, and E in comparison to Group B (Table 2).

4 Discussion

One of the most intriguing immunological phenomena is for the semi-allogeneic embryo (or allogeneic embryo in surrogate mothers) to be exempt from immunological rejection. A dichotomous T-helper 1 (Th1) versus T-helper 2 (Th2) cytokine response to trophoblast has been proposed to mediate reproductive failure and success, respectively. Evidence in rodents suggests that successful pregnancy is associated with predominance of anti-inflammatory cytokines such as IL-4 and IL-10 in the uterine microenvironment while prevalence of Th1 cytokines such as IFN-γ and TNF-α may adversely affect pregnancy outcome (Wegmann et al., 1993; Chaouat et al., 1999). Studies on IVF (in vivo fertilized) patients showed that women with recurrent spontaneous abortion or implantation failure had significantly increased Th1 cytokine expression, i.e., low level of IL-10 and high level of TNF-α and IFN-γ (Gilman-Sachs, 2002). In the present study, we chose several major Th1 cytokines (IFN-γ, IL-2) and Th2 cytokines (IL-4, IL-10) to analyze their levels in the uterus and to relate the Th1/Th2 ratio with the pregnancy outcome. Our results are similar to Gilman-Sachs (2002).

Both NK cell and T cell (including CD4+Th1 and CD8+T) can produce IFN-γ, which can be a regulator to proliferation and differentiation of T cell and antigen presenting cell (APC) (Zhu et al., 1999; Wang and Zhu, 2002). IFN-γ primes and triggers macrophages, which secrete cytokines such as TNF-α and IL-2, the cytokines inducing infiltrating NK cells to become lymphokine-activated killer cells, which damage the placenta and fetus (Gifford and Lohmann-mathes, 1987). We found that the level of IFN-γ in Group B was significantly higher than in Group A ($P<0.05$), and the IFN-γ in Groups C, D, and E was significantly lower compared with Group B, respectively ($P<0.05$). There was no significant difference among Groups C, D and E ($P>0.05$). The results suggest that there is a close relationship between IFN-γ and early embryo loss.

IL-2 has been proved to be concerned with the rejection of fetal allografts. Studies showed that IL-2 played an important role in fetal resorption (Zhong et al., 2002). In humans, women in diminution of IL-2 production were prone to normal pregnancy (Taylor and Gercel-Taylor, 2004). Pregnant mice injected exogenously with IL-2 resulted in abortion (Shiraishi et al., 1996). In the present study, a significant difference of IL-2 expression in the uterus was observed between the RU486-induced abortion group and control group ($P<0.05$), and between the groups of *Huang Qin* or its constituents pretreated group and RU486-induced abortion group. The expression of IL-2 decreased in the herbal and its constituents pretreated group indicates that *Huang Qin*, Baicalin, and Baicalein reduce IL-2 levels in the uterus.

IL-4 is excreted by Th2 cells, which are the major modulating factor of humoral immunity. It can promote the proliferation of B cell to excrete special IgG and IgE, and can regulate the balance between Th1 cell and Th2 cell and so on (Klein et al., 2001). There was no significant difference in the level of IL-4 between Group B and Group A ($P>0.05$), while the IL-4 levels were increased sharply when pretreated with Baicalin, Baicalein or *Huang Qin* ($P<0.05$), especially in Group E which had higher level of IL-4 than the control group ($P<0.05$). So the RU486 induced pregnancy loss was not associated with IL-4, while the herb *Huang Qin* and its constituents have a positive influence on IL-4 secretion in the uterus.

IL-10 plays a complex role and is predominantly secreted by Th2 cells. It can stimulate effectively the differentiation and proliferation of B cell to express and produce IgM, IgG and IgA. On the other hand, it plays an important role in the balance of cytokine web, which can inhibit T cell and monocyte-macrophage to produce many cytokines including TNF-α, IL-1, IL-6, IL-12, and IFN-γ and so on, and reduce the ability of recognizing of CTL (Levings et al., 2001). In the present paper, a significant low level of IL-10 was observed

Table 2 Changes in Th1/Th2 cytokines in different groups

Group	IFN-γ	IL-2	IL-4	IL-10
A	19.409 ± 6.106^{bde}	288.453 ± 117.027^{b}	241.958 ± 55.666^{e}	9.154 ± 11.540^{e}
B	24.424 ± 5.920^{acde}	378.849 ± 92.509^{acde}	196.208 ± 46.421^{cde}	4.276 ± 5.073^{cdE}
C	20.212 ± 8.033^{b}	307.344 ± 28.282^{b}	298.986 ± 72.207^{b}	12.082 ± 4.337^{be}
D	22.109 ± 16.392^{ab}	320.748 ± 27.639^{b}	321.458 ± 58.408^{b}	12.864 ± 4.826^{be}
E	22.136 ± 16.392^{ab}	313.757 ± 44.939^{b}	365.236 ± 94.587^{ab}	22.377 ± 14.480^{aBcd}

Notes: Data with different superscripts have significant differences at $P<0.01$ (capital letters) or at $P<0.05$ (small letters).

in Group B compared to Group C ($P < 0.05$), Group D ($P < 0.05$) or Group E ($P < 0.01$). But there was no significant difference between Group B and the control group. In Groups C, D, and E, the IL-10 expression was predominant, suggesting that the high production of IL-10 was closely correlated with the protection of the fetus (Table 2).

It is now accepted that changes in the balance of Th1/Th2 type cytokines occur during pregnancy in the feto-placental unit. These changes contribute to the implantation of the embryo, development of the placenta and survival of the fetus to term (Dealtry et al., 2000). In the current study, the abortion rate was up to 80.0% and fetal resorption rate also increased sharply to 85.7%, when injected with RU486 on Day 7 of pregnancy. A high level of Th1 type cytokines (IFN-γ, IL-2) was found in RU486-induced abortion mice, while the Th2 type cytokine (IL-4, IL-10) levels were not influenced significantly. It is suggested that the mechanism of RU486-induced embryo loss might be through inducing a Th1 shift. Speculated in theory, the progesterone effects were blocked by RU486, in this condition, T cells polarized to Th1 type cells, resulting in the increase of Th1 cytokine secretion. On the one hand, the level of Th1 cytokines was enhanced, and on the other hand, Th2 cytokine expression was inhibited by Th1 shift. Thus, the Th1/Th2 immune balance was broken and prone to a Th1 bias, resulting in abortion occurrence.

The Chinese herbal medicine *Huang Qin* used for the restless fetus can be traced back to very early times in Chinese history. The famous physician in the Jin Dynasty (A.D.1115–1234), named Danxi Zhu, pointed out that *Huang Qin* and *Bai Zhu* were the best herbs for fetal restlessness. The available constituents of *Huang Qin* are flavone compounds involving Baicalin, Baicalein, Wogonin, wogoncside, skullcapflavone I and skullcapflavone II, etc. Modern pharmacological research has shown that the flavone of *Huang Qin* has comprehensive biological action, for example, many constituents of *Huang Qin* have antiradical and antioxidative actions (Gao et al., 1999; Gao et al., 1998; Chen et al., 2003), and that of Baicalein was the best (Ciesielska et al., 2002). Baicalin and Baicalein could inhibit IL-6 and IL-8 production as well as mRNA expression (Nakamura et al., 2003). However, it is seldom researched whether Baicalin and Baicalein influence the secretion of cytokine in pregnancy and modulate immune condition of the maternal–fetal interface.

In the recent study, it was found out that pretreatment with *Huang Qin* or its major components decreased sharply the embryo resorption rate ($P < 0.01$) and abortion rate ($P < 0.01$) compared to RU486-induced abortion in mice, suggesting that the herb and its components have a negative influence on RU486 distribution in the uterus. Here, we show that pretreatment with *Huang Qin*, and Baicalin, Baicalein decreased the level of Th1 cytokines (IFN-γ, IL-2) and increased the Th2 cytokine (IL-4, IL-10) levels, leading the Th1/Th2 balance to a shift towards Th2-type immunity in the uterus. Hence, the Chinese herbal medicine *Huang Qin* and its components (Baicalin, Baicalein) are shown to play an important role in anti-abortion, though the mechanism of action is not clear. One of the approaches might be the enhancement of prolactin receptor expression in the ovary and deciduas, which on one hand facilitates ovary development and progesterone secretion, and on the other hand, helps decidua formation and facilitates implantation, suggesting that anti-abortion effects of the herbs involve a complicated immune and endocrine network.

In conclusion, this study indicates that both IFN-γ and IL-2 participate in RU486-induced fetal resorption, and the Chinese herbal medicine *Huang Qin* and its constituents have an anti-abortive effect not only through inhibiting IFN-γ and IL-2 production, but also enhancing IL-4 and IL-10 expression in the uterus, therefore, restoring the Th2/Th1 cytokine balance. Further studies need to be carried out to make clear the mechanisms of herb and herbal ingredient effects since there is a complex immune regulation network during miscarriage especially recurrent spontaneous abortion.

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