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Protective effect of anti-abortive herbal medicine on embryo implantation and the changes of serum progesterone, IFN-γ and IL-4 in cows after artificial insemination

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Early embryo death is a serious problem in most mammals including cows. To investigate the protective effect of Chinese herbal medicine against embryo loss during implantation, 100 healthy multiparous cows were divided randomly into five groups: Control (CO) group, *Taishan Panshi* (TP) powder group, *Baotai Wuyou* (BW) powder group, *Huangqin* (HQ) group and baicalin (BC) group. The cows in the control group were fed routinely, each cow in the other four groups was given TP, BW, HQ or BC powder respectively into their ration daily from day 7 to day 14 after artificial insemination (AI), then blood serum was collected on days 7, 14, 24 and 54 after AI. The contents of progesterone and Th2/Th1 cytokines, interleukin-4 (IL-4) and interferon-γ (IFN-γ) were detected with commercial kits. Results showed that with increasing days after AI, the levels of progesterone and IL-4 increased steadily, while the contents of IFN-γ increased first but decreased afterwards in all the five groups. Among the five groups, treatment with TP and BW powder significantly increased the number of pregnant cows and elevated the levels of progesterone and IL-4 increased steadily, while the contents of IFN-γ increased first but decreased afterwards in all the five groups. The results indicate that TP and BW powder, which represent two formulas, would exert their effect at the maternal-fetal interface in pregnant cows by elevating the levels of progesterone and regulating the secretion balance of Th2/Th1 cytokines, these effects, in other words, would be beneficial to a successful embryo implantation and survival.

Key words: Herbal medicine, progesterone interferon-γ (IFN-γ), interleukin-4 (IL-4), cow, implantation.

INTRODUCTION

The Chinese herbal medicine has been used for the treatment of restless fetus for hundreds of years in China. It had been ascertained that the anti-abortive herbal medicine could be beneficial for the maternal health and fetal development especially in the condition of threatened abortion. The knowledge of the anti-abortive herbal medicine was collected from both human and animal. In animal husbandry, in order to raise the mammalian performance and increase the economic benefit of breeding, herbs with the action of nourishing and invigorating the Qi, blood and preventing miscarriage were often prescribed during pregnancy, such as cows, pigs, sheeps, etc.

In Chinese Veterinary Pharmacopoeia (2005), *Taishan Panshi* (TP) powder and *Baotai Wuyou* (BW) powder are recorded for treating disorders such as abnormal oestrous cycle, embryo death in trimester, threatened abortion, physically weak of offspring in cows, pigs, sheeps, etc. The ingredients of TP and BW powder are all mainly consists of the herbs with the action of replenishing both Qi and blood with warming effect and the herbs with the action of preventing miscarriage.

Abbreviations: CO, Control; TP, *Taishan Panshi* powder; BW, *Baotai Wuyou* powder; HQ, *Huangqin*; BC, baicalin.

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According to the theory of traditional Chinese medicine, the fetus will be safe and sound in the abdomen of its mother with sufficient supply of both Qi and blood and consolidation of the kidney Qi.

The Chinese herbal medicine Radix Scutellariae (Huangqin, HQ) is a well-known anti-abortive herb and baicalin is one of the major components in HQ. In traditional Chinese medicine, herbal drugs often exert their action in a way that put several drugs together, thus ensuring a full play of their advantages and inhibiting the disadvantages. It is remain to be confirmed that whether or not a single drug or a component in a drug would produce ideal result in the preventing or treatment of a disease. In present study, Taishan Panshi (TP) powder, Baotai Wuyou (BW) powder, Huangqin (HQ) and baicalin (BC), which represent two compound prescriptions, a single drug and a component in HQ were applied respectively to the cows after artificial insemination to ascertain their protective effects on embryo implantation.

Early embryo death in pregnancy is a serious problem in most mammals. Many studies revealed that most pregnancy loss occurred during the early gestation period within day 42 post-insemination (Prvanovic et al., 2009). It is known that both the production of cytokines and the population of immune cells within the uterus change during early pregnancy. Evidence obtained mainly from mice indicates that these changes are important for implantation and in preventing a very strong maternal immune response to the conceptus (Leung et al., 2000).

Cytokines are believed to be important in maintaining pregnancy in humans and mice (Lim et al., 2000; White et al., 2004). The acceptance of the fetal allograft by pregnant mother seems to be associated with a shift from a Th1 dominated to a Th2 dominated immune response (Miyaura and Iwata, 2002). Many studies revealed that excessive expression of Th1 cytokines [interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), etc] would lead to pregnant failure (Marzi et al., 1996; Jenkins et al., 2000; Lin et al., 2000), and an adequate bias towards Th2 (IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13) would profit pregnancy (Hashih et al., 1998; Piccinni et al., 1998; Lin et al., 2003).

Moreover, progesterone plays an important role in the maintenance of pregnancy; the enrichment of progesterone in pregnancy favored the development of type 2 cytokines (Piccinni et al., 1995). But Arndt and his colleagues (2009) demonstrated that progesterone supplementation does not increase circulating levels of progesterone in the early stage of pregnant cows. Alternative methods to influence progesterone concentrations and/or early embryonic loss need to be investigated.

In our previous study, a remarkable deviation towards Th1 cytokine response was observed in abortion model mice (Zhong et al., 2007, 2008; Ma et al., 2009), while some Chinese herbal medicines could exert a positive influence on restoring the cytokine balance at maternal-fetal interface in pregnant mice (Zhong et al., 2008).

This study was designed to further investigate the protective effect of Chinese herbal medicine, in the way of compound prescription, single herb or an component, if any, which could affect the outcome of embryo implantation and the contents of serum progesterone and Th2/Th1 cytokines on the cows at day 7, 14, 24, and 54 after artificial insemination (AI).

MATERIALS AND METHODS

Reagents

Taishan Panshi (TP) powder and Baotai Wuyou (BW) powder are all documented in the Chinese Veterinary Pharmacopoeia. In both formulas, there are the same 9 herbal medicines, they are: Radix Codonopsis Pilosulae (Dangshen), Radix Astragali seu Hedysari (Huangqi), Radix Angelicae Sinensis (Dangguai), Radix Scutellariae (Huangqin), Rhizoma Liguistici Chuanxiong (Chuanxiong), Radix Paeoniae Alba (Baishaoh), Radix Rehmanniae Praeparata (Shu di), Rhizoma Aractylidios Macrocephalae (Baizhu) and Radix Glycyrrhizeae (Gancao). Apart from these drugs, the TP powder also consists of Radix Dipsaci (Xuduan) and Fructus Amomi (Shuren). The BW powder includes Fructus Aurantii (Zhiquiao), Pericarpium Citri Reticulatae (Chenpi), Caulis Perillae (Sugeng) and Folicum Artemisiae (Aiye). All these herbal medicines were purchased from Chinese Drug Market (Anguo, China) and identified by professional staff. Baicalin (BC) was produced from Slida Technology Co., LTD. (Tianjin, China) and its purity was 98%. These herbal medicines were grounded into fine powder and mixed thoroughly with feed stuffs when used. Bovine progesterone, IFN-γ and IL-4 enzyme-linked immuno-sorbent assay (ELISA) kits were all from RB company (USA). All the other chemicals used in the present study were of analytical grade.

Animals and treatments

The present study was performed in the Haocheng Breeding Co., LTD. (Dingzhou, China), one hundred healthy China holstein cows were collected for the experiment. All the cows have already calved; they were 3 to 7 years old and kept in the one stable in cattle farm. The cows were given AI at estrum (all the bull semen came from the same bull, whose number is 11101682 in Beijing Dairy Cow Center). Seven days after AI, the 100 cows were divided randomly into 5 groups: control (CO) group, TP group, BW group, Huangqin (HQ) group and baicalin (BC) group, each group containing 20 holstein cows.

The cows in the control group were fed routinely: each cow in TP group was given 250 g of Taishan Panshi powder, while each cow in BW group was supplied with 250 g of Baotai Wuyou powder into their ration. Each cow in Huangqin (HQ) group was feed with 50 g Huangqin powder and in the BC group were feed with 10 g baicalin (the dosages of TP, BW and HQ used in this study were determined according to the recommendation in Chinese Veterinary Pharmacopoeia, the dosage of BC was considered with the content of baicalin in crude huangqin and the data of our previous study). These treatments were carried out once a day from day 7 to day 14.

The blood was collected from jugular vein on day 7 (before herbal medication), 14 (0 day after herbal medication), 24 (10 days after herbal medication) and 54 (40 days after herbal medication) post-insemination both in the control and herbal treated groups. Blood serum was prepared for the detection of progesterone, IFN-γ and IL-4 following the kit instructions. All cows were treated humanely according to Chinese national guidelines. These tests
were made with two replications.

Statistics

Data were expressed as means ± SE. Differences between group treated with different medicines and control group were analyzed by two related samples of nonparametric test using the SPSS software package for Windows. In all tests, statistical significance was considered \( P<0.05 \) (*).

RESULTS

Pregnant outcome in control and herb medication groups

It had been ascertained through pregnancy diagnosis (by palpation per rectum), that 12 cows had conceived out of 20 in the control group. After supplement with TP or BW for 7 days, the number of pregnant cows increased to 16 and 15, respectively, which has significant difference compared with control. In the group treated with HQ or BC for 7 days, the number of pregnant cows was 13 and 12, respectively. After 275 to 290 days gestational period, all pregnant cows gave birth to healthy calves.

Changes of progesterone at different days

The progesterone concentrations increased significantly from day 7 to 54 post-insemination both in the control and herbal treated groups. In the control group, the contents of progesterone in serum were 20.66 nmol/L on day 7, while detected on day 14, 24 and 54 after AI, the concentrations increased to 29.95, 43.81 and 57.63 nmol/L, respectively. The hormonal fluctuation in other four groups was the same as the control group. As for the contents of progesterone in different groups in a definite day, results showed that in day 7, the serum progesterone levels was around 20 nmol/L in all the cows applied in this experiment. The significance between control and herbal groups appeared at day 14, 24 and 54 after AI. At day 14, the progesterone contents in the cows supplied with TP was 34.22 nmol/L, which had significance compared with control \( (P<0.05) \). At day 24 and 54, the hormonal concentrations remarkably augmented in both TP group \( (P<0.05) \) and BW group \( (P<0.05) \) compared with the control. While in HQ and BC groups, no remarkable variation existed (Figure 1).

Changes of IFN-γ at different days

From day 7 to day 24, IFN-γ secretion elevated steadily in the control group, yet the tendency ceased and turned to the negative direction from day 24 to 54, the more active secretion moment of IFN-γ was around day 24. The trend in other four groups was similar with the control group. As for the comparison among these five groups in certain time, Figure 2 showed that IFN-γ concentrations

![Figure 1. Progesterone contents in control and herbal groups at different days. Values are means ± SE (n = 20). \( *, \ P<0.05 \) as compared with control.](attachment:figure1.png)
in the TP group exceed slightly to control group at day 14, whereas at day 24 and 54, IFN-γ levels were slightly lower than control in the TP group. Nevertheless, no significant difference displayed between the control and TP powder groups, and neither between control and other three groups (Figure 2).

Changes of IL-4 at different days

The secretion pattern of IL-4 was similar with that of progesterone, as is shown in Figure 3. The concentrations of serum IL-4 upgraded from day 7 to 54 in all the five groups. As for the comparison between control and herbal groups, results showed that at day 14, 24 and 54, Serum IL-4 contents in TP and BW groups were all significantly elevated compared with the control group ($P<0.05$). Whereas, significant augmentation was never observed in HQ and BC groups on these days (Figure 3).

Changes of the ratio of IL-4/IFN-γ at different days

IL-4 and IFN-γ are important cytokines which represent the secretion mode of Th2 and Th1. Results showed that there were no significant difference in the ratio of IL-4/IFN-γ on day 7 and 14 in all the groups, but the ratio of IL-4/IFN-γ was significantly increased on day 24 and 54, especially in the cows treated with herbal prescriptions. After supplement with TP powder for 7 days, the ratio of IL-4/IFN-γ was remarkably elevated on day 24 and 54 compared with control group. Treatment with BW powder for 7 days, the ratio of IL-4/IFN-γ was augmented only on day 54. There was no significance in group treated with HQ or BC in this experiment (Figure 4).

**DISCUSSION**

Early embryo loss in pregnancy is a serious problem in most mammals including cows. The economic loss resulting from each pregnancy failure was estimated at approximately $2,333, and this was largely due to an extended calving interval and increased culling (Lee and Kim, 2007). Many studies have revealed that the pregnancy loss occurred during early foetal period in which 80 to 90% foetal death occurred before day 42 post-insemination. Prvanović and his co-workers reported that 98% of the cows which had experienced embryonic
Figure 3. IL-4 Contents in control and herbal groups at different days. Values are means ± SE (n = 20). *, P < 0.05 as compared with control.

Figure 4. The ratio of IL-4 to IFN-γ at different days. Values are means ± SE (n = 20). *, P < 0.05 as compared with control.
mortality lost their embryos 17 to 24 days after Al (Prvanović et al., 2009). So, it is very important to take preventive measures against early embryo loss. Present study demonstrated that after artificial insemination, treatment with TP and BW powder would act on the maternal fetal interface, elevating the levels of progesterone and regulating the secretion balance of Th2/Th1 cytokines, these effects, in other words, would be beneficial to a successful embryo implantation and survival.

It is known that the presence of conceptus alloantigens necessitates changes in maternal immune function (Oliveira and Hansen, 2008), resulting in the production of cytokines, the distribution of immune cells and the endocrine system within the uterus diversifying during early pregnancy. Evidence obtained mainly from mice indicates that these changes are important for implantation and in preventing the maternal immune response to the conceptus (Leung et al., 2000).

With regard to fetal-placental hormones, progesterone seemed to exert an important immunosuppressive influence (Padua et al., 2005). In present study, the concentrations of serum progesterone elevated steadily with the increasing days in all the five groups with or without herbal medicine. The significant increases of serum progesterone appeared in the group supplied with TP or BW powder. The increase of progesterone secretion and the elevation of the pregnancy rate after TP and BW powder treatment suggest that the two formulas exert their positive influence through restoring the hormone at the maternal-fetal interface.

Studies demonstrated that the levels of local progesterone at human placenta directly affected T cell differentiation in the absence of other cell types and the development of Th1 cells was significantly suppressed by progesterone during pregnancy (Miyaura and Iwata, 2002). In mammals, the contents of progesterone were correlated with the secretion pattern of Th1 (IFN-γ, IL-2, TNF-α) and Th2 (IL-4, IL-5, IL-6, IL-10, etc.) cytokines and correlated with the success of pregnancy (Miyaura and Iwata, 2002). The acceptance of the fetal allograft by pregnant mother seems to be associated with a shift from a Th1 dominated to a Th2 dominated immune response (Marzi et al., 1996). This is to say, the enrichment of progesterone in pregnancy favored the development of Th2 cytokines. Among Th2/Th1 cytokines, IL-4 and IFN-γ exert quite important roles in regulating the expression of immune and non-immune cells during pregnancy (Farrar and Schreiber, 1993; Piccinni et al., 1995), so, IFN-γ and IL-4 was often chosen as the signature of Th1 and Th2 cytokines in the research of the mechanisms in pregnancy (Dixit et al., 2003). In present study, results showed that the secretion mode of IL-4 was similar with that of progesterone, with the contents elevated significantly in the groups supplied with TP and BW powder on day 14, 24 and 54. Although no significant difference appeared in IFN-γ levels between the control and the herbal groups, the ratio of IL-4/IFN-γ, which represents the secretion mode of Th2/Th1, significantly increased on day 24 and 54. This response seemed to be related with a shift from a Th1 dominated to a Th2 dominated immune response which has been confirmed by many reports (Marzi et al., 1996; Jenkins et al., 2000; Lim et al., 2000).

In our previous study, similar results were obtained through abortion model mice induced by RU486, lipopolysaccharide (LPS) or bromocriptine (Zhong et al., 2007, 2008; Ma et al., 2009) in which remarkable variation biased towards Th1 cytokine response in abortion mice, while many Chinese herbal medicines and some extracts could exert a positive influence on restoring the cytokine balance at maternal-fetal interface in pregnant mice (Zhong et al., 2008).

In the present study, the concentrations of IFN-γ increased first but decreased afterwards, the more active stage of IFN-γ secretion was around day 24, and this time is just the key point the blastocyst touch on endometrium for implantation. It is well known that blastocyst implantation and successful establishment of pregnancy requires delicate interaction between the embryo and the maternal environment. Studies have shown that at the implantation site, edema and an increase in vascular permeability are signs of a local inflammatory reaction which prepares this part of the endometrium for implantation (Sammin et al., 2009).

It has been shown that a proper maternal immune response affects development of the fetal-placental unit by local production of cytokines, in which a shift from predominantly Th1 driven cell mediated immunity to predominantly Th2 driven humoral immunity, however it is not the case around implantation. Chaouat et al. (2003) proposed that Th2/Th1 concept is very efficient in the post implantation period; however, the phenomenon happening in the implantation period is different from the post implantation period. Indeed, IFN-γ and other Th1 or Th1 like cytokines have been shown to be critical for implantation by knock out technologies or by use of antibody neutralization (Robb et al., 1998; Stewart et al., 1992).

Taishan Panshi (TP) powder, a Chinese herbal formula, was from the book Jing Yue Quan Shu in the Ming dynasty (A.D. 1624), which mainly consists of herbs with the action of nourishing and invigorating the Qi, blood and preventing miscarriage during pregnancy. In the philosophy of Traditional Chinese Medicine, the drugs of Danshen, Baizhu, Huangqi and Gancao can be strengthening the spleen to invigorate Qi. Drugs such as Danggui, Shudi, Xuduan and Baishao are suitable for tonifying the liver and kidney, nourishing blood and regulating its flow. Huangqin can be clearing away heat toxin, working together with Baizhu to strengthen the spleen and remove pathogenic heat so as to prevent miscarriage. Sharen can be regulating Qi and the middle Jiao so as to ensure a successful gestation.
can be promoting the flow of Qi and coordinating blood. With all the drugs, TP powder exerts the effects of invigorating Qi, strengthening the spleen, nourishing blood and preventing abortion which will be suitable for the syndrome of miscarriage due to deficiency of both Qi and blood. In human beings, TP powder modified according to different symptoms and signs are clinically prescribed to treat disorders such as threatened abortion and repeated abortion.

The origination of Baotai Wuyou (BW) powder was from the book Da Sheng Pian, which was published in the early stage of the Qing dynasty (A.D. 1715) and gained a very high honor on the treatment of obstetric diseases. As for the ingredients of BW powder, except for the herbs prescribed in TP powder (except the herbs Xuduan and Sharren), which have the effects of invigorating Qi and blood, strengthening the spleen and preventing abortion, the herbs in BW powder also consist of Aiye, Zhiqiao, Chenpi and Sugeng. The actions and indications of Aiye are warm the uterus, arrest uterine bleeding due to deficiency-cold. Herbs of Zhiqiao, Chenpi and Sugeng are all the Qi regulating drugs, they can regulate the flow of Qi for treating stuffiness in the chest and distension of the abdomen, Sugeng can be prescribed for preventing abortion. In clinically, BW powder was often prescribed for the pregnant woman who suffering with threatened abortion or recurrent abortion.

In animal husbandry, TP and BW powder usually prescribed for treating mammal disorders such as abnormal oestrus cycle, embryo death in trimester, threatened abortion, physically weak of offspring in cows, pigs, sheeps, etc. Present study demonstrated that apart from the actions mentioned previously, TP and BW powder also exert their positive influence on embryo implantation. However, no difference was seen in the outcome of embryo implantation and the contents of serum progesterone and Th2/Th1 cytokines after treatment with HQ or BC, indicating that the single herb or an component are not the desirable selection for the regulation of embryo implantation in the process of early pregnancy.

Conclusion

In all, after supplement with TP and BW powder, the contents of progesterone were correlated with the secretion mode of Th2/Th1 cytokines, which showing a shift from Th1 dominated to Th2 dominated immune response in pregnant cows. This indicates that TP and BW powder would work on the maternal fetal interface in pregnant cows by elevating the levels of progesterone and shifting the secretion balance of Th2/Th1 to a Th2 mode, which would be beneficial to successful embryonic implantation and survival. Further studies are needed to explore more benefits of herbal medicine on the pregnant cows.

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