Effects of Bai zhu san (*Atractylodis macrocephalae*) decoction on cellular immunity and Th1/Th2 cytokine ratio in a Mifepristone-induced murine abortion model

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ABSTRACT: Chinese herbal medicine has been used as an anti-abortive treatment in farm animals for thousands of years. To investigate the anti-abortive functions and mechanisms of Bai zhu san (BZS) on mifepristone (RU-486)-induced abortion, pregnant mice were allocated to different groups as follows: control group receiving neither RU-486 nor BZS; mice receiving RU-486 only; mice treated with both RU-486 and BZS. The results showed that the concentrations of IL-10 and IL-4 in uterine lysates were significantly higher in mice of the BZS + RU-486 group compared with the mice of the RU-486 group. The IL-10 and IL-4 levels in serum were several folds higher than that in uterus lysates. The IFN-γ concentrations in uterine lysate decreased significantly in mice of the BZS + RU-486 group vs. the RU-486 group. There were no significant differences in IL-2 concentrations between the mice of BZS + RU-486 or RU-486 groups and controls in the uterus and serum. The numbers of $CD4^+$, $CD8^+$T lymphocytes or macrophages in the uterus decreased in the BZS + RU-486 group compared with the RU-486 group. These results indicate that the Chinese herbal formula Bai zhu san inhibits RU-486-induced abortion and modulates the Th1/Th2 cytokine balance at the maternal-foetal interface.

Keywords: Bai zhu san; mifepristone; cytokine; anti-abortive; $CD4^+$, $CD8^+$T cells

Successful embryo development has been immunologically attributed to a T-helper 2 phenomenon and studies have confirmed the existence of a T-helper 2-type pattern deficiency in missed abortions (Paradisi et al. 2003; Wang et al. 2010; Yang et al. 2011). Sporadic spontaneous abortion, recurrent spontaneous abortions, early pregnancy loss or implantation failures pose serious problems for mammalian reproduction. Many studies in the last decades (Zhao et al. 2011a; Zhao et al. 2011b) have attempted to address the mechanisms that allow for successful pregnancy, but many unanswered questions remain. Successful pregnancy outcome has been attributed to the immunotrophism theory of the foetal allografts (Joachim et al. 2003; Zenclussen et al. 2003; Chaouat et al. 2009). The self-nonself model of immune recognition during maternal pregnancy is now widely accepted. Maternal immune suppression has been suggested to explain a successful mammalian pregnancy in this model and the balance of Th1/Th2 cytokines during pregnancy has been intensively studied (Paradisi et al. 2003; Zenclussen et al. 2003; Quinn et al. 2004; Curry et al. 2008; Fukui et al. 2008). Thus, the balance of Th1 and Th2 cytokines appears to be very important for the acceptance of the foetus by the maternal immunity (Wegmann et al. 1993; Saito 2000).

Several studies have provided strong evidence that maternal T cells are aware of foetal alloantigens during pregnancy (Zhou and Mellor 1998). Other studies in humans have also investigated numbers of blood lymphocytes during pregnancy (Watanabe et al. 1997; Luppi 2003). Some data suggest that in rats, the innate immune system is activated during pregnancy, too (Faas et al. 2003). Some other reports revealed substantial changes in blood leukocytes during pregnancy. Taking into account what is thus far known, it appears certain that lympho-

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cyte counts in the uterus change from early to late pregnancy. The present report extends previous findings in rats by showing that the decline in lymphocytes is a combined effect resulting from a decrease in $CD4^+$ and $CD8^+$ T cells. Some reports suggest that peripheral $CD8^+$T lymphocytes have enhanced systemic cytotoxic capacity as a manifestation of maternal anti-foetal rejection (Jacques and Qureshi 1998). As for macrophages, a recent study has demonstrated that $F4/80^+$ macrophages are required for the differentiation of antigen-specific $CD8^+$ regulatory T cells (Lin et al. 2005).

Some Chinese herbal formulas that have been used clinically for centuries in Asian countries, especially in China, have been reported to be effective in the preventing certain types of abortion in animals (Ma et al. 2012). BZS is a typical example and is documented in the 2010 Edition of China's Veterinary Pharmacopeia. In this formulation the important herbs are Bai zhu (White atractylodes) and Huang qin (Scutellaria) and the two herbs together are required for the anti-abortive activities (Zhong et al. 2002). The anti-abortion mechanisms of BZS are still unclear although it has been used successfully in clinical practice for thousands of years in China. It is well known that some herbal medicines can regulate the immune system *in vivo* and affect the function of cytokines (Chiu et al. 2008; Li et al. 2009; Chang et al. 2011; Chatterjee et al. 2011; Yeap et al. 2012). Whether the anti-abortive mechanisms of BZS are related to changes in uterus immunity or the balance of Th1/Th2 cytokines was thus far unknown as there has been a lack of data on the effects of BZS on immune cells in the uterus during successful or failed pregnancies.

The present study was undertaken to examine the anti-abortive effects of BZS on mifepristone-induced abortion and the production of inflammatory cytokines, up-regulation of Th2-cytokines and immune cell counts in the murine uterus during pregnancy. These results constitute a reference point for the clinical use of herbal medicines in the prevention of animal embryo loss especially in bovines.

MATERIAL AND METHODS

Preparation of BZS. The herbs used in this study were provided by Tong Ren Tang Herbal Shop (Beijing, China). Our formula contained the following herbs: Atractylodes 30 g, Chinese Angelica 20 g, Ligusticum 15 g, Codonopsis 30 g, Licorice root 15, Amomum 20 g, Prepared Rehmannia 30 g, Citrus 25 g, Perilla 25 g, Scutellaria 25 g, Peony 20 g, and Asinum 30 g. The total weight of the raw materials was 290 g and they were used as described by Xie and Preast (2010).

The herbs were soaked in 2030 ml of distilled water (seven times in a volume corresponding to the total weight) for one hour and the mixture was then boiled for two hours. The herbal liquid was separated from the herbal residues before the soup volume was reconstituted to 145 ml with distilled water. Additional distilled water (870 ml, three times in a volume corresponding to the total weight) was added to the herbal residue, and then the 2 h boiling process was repeated. The collected soup volume was reconstituted to 145 ml, and combined with the first decoction to achieve a final volume of 290 ml. The final decoction of the BZS (final concentration of 1 g/ml) was stored at 4 °C until used.

Preparation of mifepristone. Mifepristone (RU-486) was purchased from Sigma Chemical Co. (USA) and was dissolved in propylene glycol at a final concentration of 0.25 μg/ml and stored at 4 °C.

Animals and treatments. Male and female BALB/c mice (8–10 weeks) were purchased from the Chinese Experimental Animal Centre, Chinese Academy of Sciences (Beijing, China) and subsequently maintained in a Laboratory Animal Facility with a 12-h light : 12-h dark cycle. The housing and handling of the experimental animals were in accordance with the guidelines of the Chinese Council for Animal Care. After overnight cohabitation with males, the females were determined by vaginal plug, as previously described (Zhong et al. 2002; Zhong et al. 2008). The mice with vaginal plugs were separated from the males and the day of vaginal plug detection was considered Day 0 of gestation.

All pregnant females were then randomly divided into different experimental groups. The control group received PBS via oral administration (0–7 days of gestation; n = 10). Mice in the RU-486 group were given mifepristone via subcutaneous injection at Day 7 of gestation (0.1ml or 0.025 μg/mouse; n = 10). The BZS + RU-486 group was given mifepristone via subcutaneous injection at Day 7 of gestation (0.025 μg/mouse) after receiving an oral gavage of BZS extract (0.5 ml or 0.5 g/mouse) from Day 1 to Day 7 of gestation (n = 10) (Zhong et al. 2002).

Abortion rate and the rate of embryo resorption. The abortion rate (AR) of mice was calculated using the formula:

AR (%) = A/S × 100%

where:

A = represents the number of mice which had aborted,

S = represents the total number of mice (Baines et al.1996).

On Day 9 of gestation, the mice were sacrificed, the uteri were removed and the resorption sites were identified by their small size accompanied by their necrotic hemorrhagic appearance compared with normal embryos. The percentage of resorption was calculated using the formula:

ER (%) = R/(R + N) × 100%

where:

ER (Rate of Embryos Resorption) = the percentage of resorptions relative to the total number of effective implantation sites,

R = the number of resorbed embryos,

N = the number of normal embryos (Baines et al. 1996).

Cytokine assay. The concentrations of interferon-γ (IFN-γ), interleukin (IL)-2, IL-4 and IL-10 in uterine lysates and serum were measured using cytokine assay kits (purchased from Sigma Chemical Co., USA), following the manufacturer's instructions. The results of (IFN-γ + IL-2)/(IL-4 + IL-10) ratio was calculated to simulate the balance of Th1/Th2 cytokines in the uterus and serum of pregnant mice.

CD4$^+$, CD8$^+$ T cell immunohistochemistry. All mice were sacrificed and 1/4 of the uteri were fixed in Bouin's fluid for 2 h and embedded in paraffin. Serial 5 μm sections were cut and mounted on glass slides. All sections were washed in PBS, followed by blocking of the intrinsic peroxidase activity with 3% H_2O_2 at room temperature for 8 min. After blocking for 30 min in 10% goat serum and incubation with rat monoclonal antibody against the CD4$^+$ or CD8$^+$ antigen (1 : 50; eBioscience) overnight at 4 °C, the slides were further incubated with biotin-labelled rabbit anti-rat IgG antibody (1 : 50; Zhongshan Golden Bridge Biotech Company Ltd, Beijing, China) at 37 °C for 30 min. After washing with 0.01M PBS 3 times, the sections were incubated with horseradish peroxidase-labelled streptavidin (HRP, 1 : 50; Zhongshan Golden Bridge Biotech Company Ltd, Beijing, China) at 37 °C for 30 min. The HRP-binding sites were detected in 0.005% 3,3-diamiobenzidine 4 HCl and 0.001% H_2O_2 in 0.1M Tris-HCl buffer.

Macrophage assay. Serial 5 μm sections were prepared as described above. All sections were incubated with a monoclonal antibody against F4/80$^+$ antigen (1 : 50; eBioscience) overnight at 4 °C. The remaining steps were the same as in the CD8$^+$ T cell assay described above.

Statistical analysis. All data were analysed using SPSS 16.0 software. A *P*-value of less than 0.05 was considered significant, and a *P*-value of less than 0.01 was considered highly significant.

RESULTS

Abortion rate and the rate of embryo resorption of the mice

The abortion rate after administration of Mifepristone in the RU-486 group was 100% (Figure 1A).

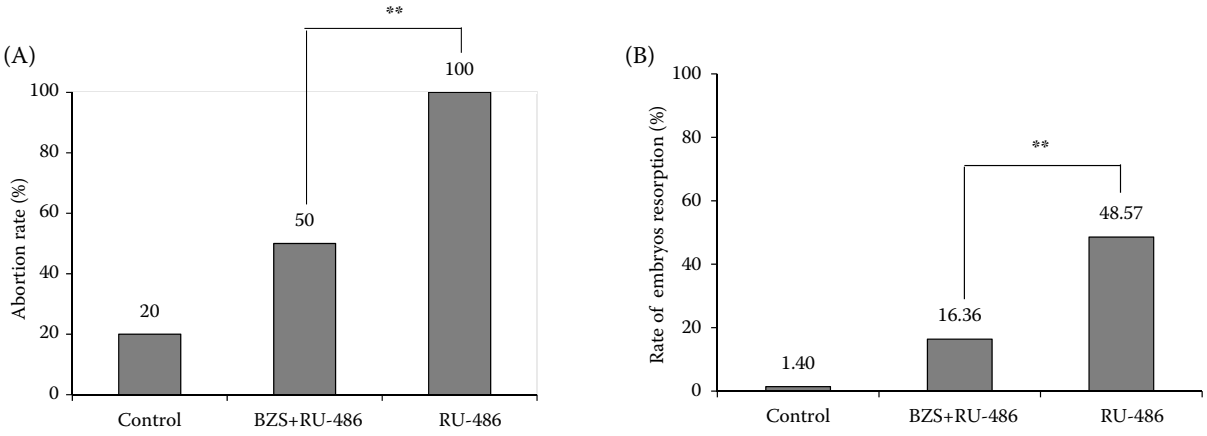

Figure 1. Abortion rate and the rate of embryo resorption of the mice. (**A**) The abortion rate of the mice. (**B**) The rate of the embryo resorption of the mice. The *P*-values for the comparison of abortion rate and the rate of embryo resorption of the mice in the BZS + RU-486 and RU-486 groups are also shown (n = 10 in each group)

$^{**}P < 0.01$

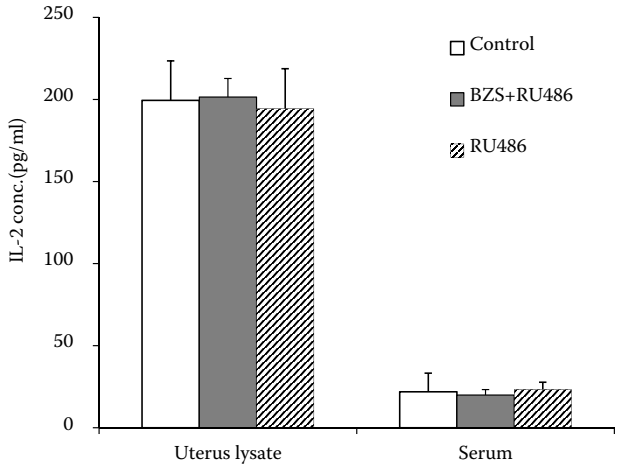

Figure 2. Comparison of the concentrations of IL-2 in the uterine lysate and serum

More embryos showed a necrotic haemorrhagic appearance compared with normal embryos in the control group. The rate of embryo resorption in the RU-486 group was 48.57% (Figure 1B). On the other hand, in the BZS + RU-486 group, the abortion rate and the rate of embryo resorption was dramatically lower, 50% and 16.36%, respectively. These differences were highly significant (Figures 1A and 1B).

The concentrations of cytokines in uterine lysate and serum

IL-2 concentrations in uterine lysates and serum in RU-486 and BZS + RU-486 groups showed no significant differences compared with the control group. IL-2 concentrations in uterine lysates were about 6- to 10-fold higher than in serum (Figure 2).

The IFN-γ concentrations in uterus lysates were significantly increased in the mifepristone group (Figure 3A). This increase in IFN-γ concentrations in uterine lysate was blocked when the mice received an oral gavage extract of BZS (Figure 3A). Similar changes occurred in the serum (Figure 3B). There was a very marked divergence in IFN-γ concentrations between the uterine lysates and serum. The serum IFN-γ concentrations were about 13- to 22-fold higher than in uterine lysates.

The IL-4 concentration in the uterine lysates of the control group was 1.26 ± 0.20 pg/ml. This value was significantly lower in the RU-486 group. In contrast, the IL-4 concentrations in the uteri of the BZS + RU-486 group showed a dramatic elevation and were significantly compared to the RU-486 group (Figure 4). The difference in IL-4 concentrations in uterine lysate between the BZS + RU-486 group and the control was also significant (Figure 4). There was also a significant elevation in the serum IL-4 concentrations in the BZS + RU-486 group compared to the RU-486 group, but the difference between the BZS + RU-486 group and the control was not significant (Figure 4). There were also divergences in IL-4 concentrations between uterine lysates and serum, with the values being about 2-fold higher in uterine lysates.

The uterine lysate IL-10 concentration in the control group was 35.56 ± 4.75pg/ml, while the value was 39.64 ± 2.02 pg/ml in serum. There was no significant change in the IL-10 concentrations after RU-486 administration (Figure 5). However, the uterine lysate IL-10 concentration increased significantly in the BZS + RU-486 group compared with the control, reaching 42.25 ± 3.98 pg/ml. The serum IL-10 concentration was 34.00 ± 6.95 pg/ml

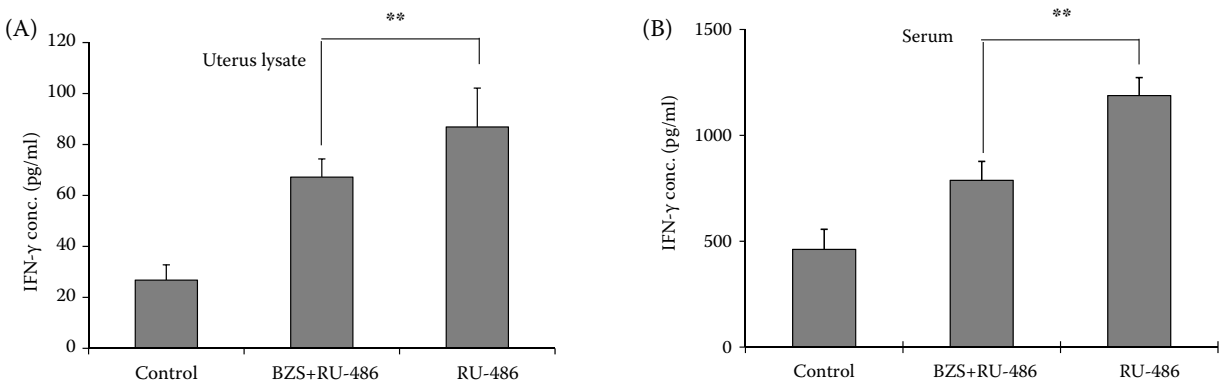

Figure 3. Comparison of the concentrations of IFN-γ in the uterine lysates and serum. (**A**) The IFN-γ concentrations in uterine lysate. (**B**) The IFN-γ concentrations in serum (n =10/group; pg/ml ± S.E.M.)

**$P < 0.01$

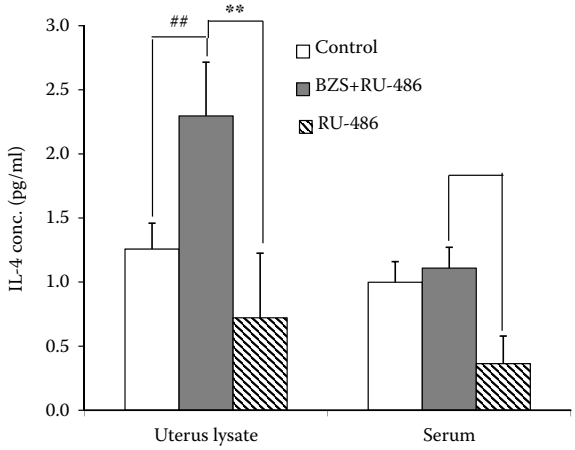

Figure 4. Comparison of the concentrations of IL-4 in the uterine lysates and serum (n = 10/group; pg/ml ± S.E.M.)

$^{**}P < 0.01$ vs. RU-486; $^{\#\#}P < 0.01$ vs. control

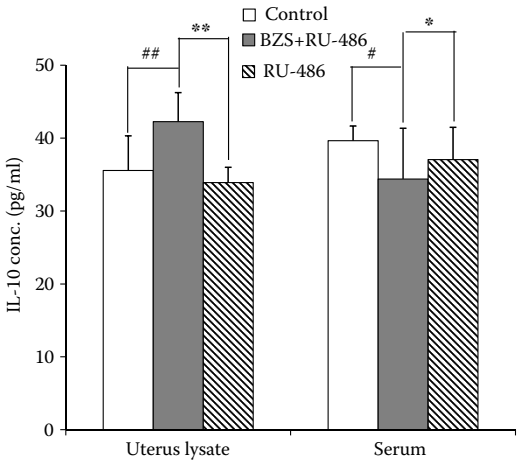

Figure 5. Comparison of the concentrations of IL-10 in the uterine lysates and serum (n = 10/group; pg/ml ± S.E.M.)

$^{*}P < 0.05$ vs. RU-486; $^{**}P < 0.01$ vs. RU-486; $^{\#}P < 0.05$ vs. control; $^{\#\#}P < 0.01$ vs. control

in the same group, significant lower vs. the control (Figure 5).

CD4⁺, CD8⁺ T cell and macrophage localisation in mice uteri

The number of CD4⁺ T cells in mice uteri increased vs. control after administration of RU-486, and the majority of these cells were distributed over the endometrium (Figure 6A; CD4⁺/RU-486, Figure 6B). In contrast, there were no significant increases in the number of CD4⁺ T cells in the BZS + RU-486 group (Figure 6A; CD4⁺/BZS, Figure 6B). A similar trend could be observed for CD8⁺ cells after administration of RU-486, with more cells distributed over the endometrium and myometrium (Figure 6; CD8⁺/RU-486, Figure 6B). On the other hand, the number of CD8⁺ T cells in the BZS + RU-486 group was increased vs. the control (Figure 6; CD8⁺/BZS, Figure 6B), but was significantly less than the number of CD8⁺ T cells in the RU-486 group. As to the macrophages, there were no significant differences in the number of cells between the BZS + RU-486 group and the control. However, macrophage numbers were higher in the RU-486 group vs. the control. Similar changes, as in the RU-486 group, could be observed for CD4⁺ T cells (Figure 6; F4/80⁺, Figure 6B).

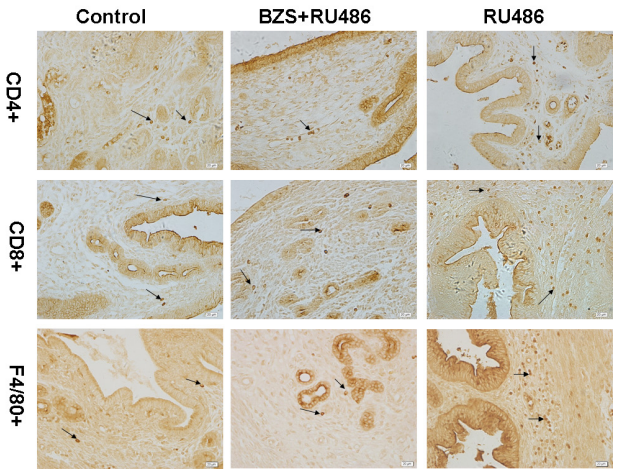

Figure 6A. CD4⁺, CD8⁺ T cells and F4/80⁺ macrophage localisation in mice uteri. All images were obtained at × 40 with an Olympus B × 51 micro telescope. Ten fields from sections were randomly chosen in order to locate and count the number of cells. The arrows show the CD4⁺, CD8⁺ T cells and macrophages, respectively. The arrows denote the positive cells. The BarStaff gauge is at 20 μm

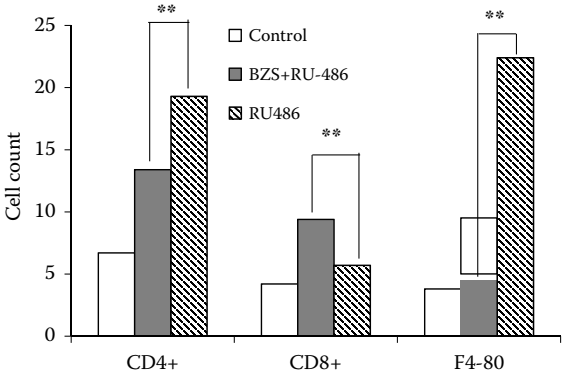

Figure 6B. Cell counts of CD4⁺,CD8⁺ T lymphocytes and F4/80⁺ macrophages in the uterine tissue of mice

$^{**}P < 0.01$

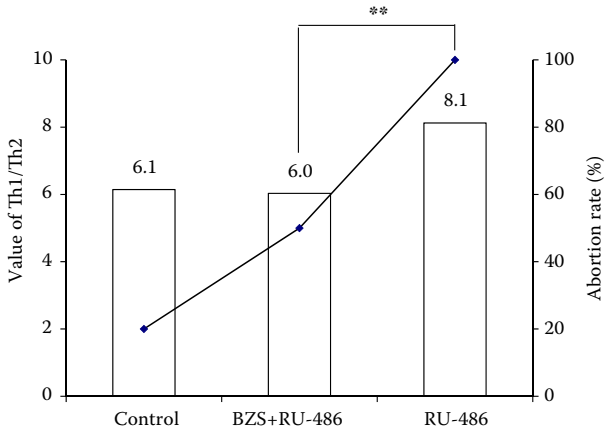

Figure 7. The relationship between the (IFN-γ + IL-2)/(IL-4 + IL-10) ratio and the abortion rate. The (IFN-γ + IL-2)/(IL-4 + IL-10) ratio was analysed to simulate the value of Th1/Th2 cytokines in the uterus of pregnant mice. The cytokine values are denoted by the numbers above each bar

The anti-abortion functions of BZS and the Th2 bias of the Th1/Th2 cytokine balance

To simulate the Th1/Th2 cytokine ratio in the uteri of pregnant mice, the formula (IFN-γ + IL-2)/(IL-4 + IL-10) was used to detect the relationship between the abortion rate of mice and the Th1/Th2 cytokine ratio after treatment with RU-486 or BZS + RU-486. Using this formula, the Th1/Th2 ratio was 8.1 in the RU-486 group (Figure 7). All mice were aborted in this group. The value was significantly lower (6.0) after treatment with BZS and RU-486. Likewise, the abortion rate of mice was only 50% in this group. There were significant differences in the Th1/Th2 ratio and abortion rate of mice between the BZS + RU-486 group and the RU-486 group. No significant differences were noted in the ratio of Th1/Th2 between the control and the BZS + RU-486 group (Figures 1A and 7).

DISCUSSION

Bai zhu san (BZS) is a traditional Chinese herbal formula used clinically for centuries in Asian countries, especially in China, for the prevention of certain types of abortion in domestic animals. Scutellariae and its three major components (baicalin, baicalein and wogonin) have shown anti-inflammatory effects (Lin and Shieh 1996), while baicalin and baicalein exhibit anti-tumour activity (Ikemoto et al. 2000; Kim et al. 2009). As a component of White atractylodes, atractylenolide I can antagonise Toll-like receptor 4 (TLR_4) and inhibit inflammatory reactions (Li and He 2006). However, how BZS exerts its anti-abortive function is as yet unknown. Many studies have demonstrated that cytokines play an important role during pregnancy, by creating microenvironments that modulate maternal immune responses (Jacques and Qureshi 1998; Jiang and Vacchio 1998; Paradisi et al. 2003; Chaouat et al. 2009; Dourado et al. 2010). Therefore, the interrelation between BZS and changes in the maternal immune response may be significant for the mechanism of action of BZS in preventing abortion.

Mifepristone is a potent antagonist of progesterone and glucocorticoid receptors, and is usually used for the termination of pregnancies of up to 49 days gestation in humans (Hapangama et al. 2001; Davey 2006). The medical termination of pregnancy with mifepristone has been used in clinical practice for 20 years (Davey 2006). To date, mifepristone has been approved in several countries for use in four indications: early termination of pregnancy (TOP), cervical dilatation prior to surgical TOP, preparation for prostaglandin-induced TOP during the second trimester, and expulsion of a dead foetus during the third trimester (Sitruk-Ware and Spitz 2003). In this study we used mifepristone to induce abortion in mice in order to study the possible anti-abortive mechanism of BZS. Indeed, our data showed that 100% mice aborted with 48.57% embryo resorption after mifepristone treatment (Figures 1A and 1B).

Our aim in these experiments was to investigate the effects of BZS on mifepristone (RU-486)-induced abortion, production of inflammatory cytokines, upregulation of Th2-cytokine levels and immune cells in pregnant mice. The results indicate that BZS inhibits Mifepristone-induced abortion in mice. To the best of our knowledge, this is the first report demonstrating the anti-abortive mechanisms of BZS in veterinary medicine.

IL-10 and IL-4 concentrations in uterine lysates were elevated after BZS administration (Figures 4 and 5). Although IL-4 concentrations were about 2-fold higher in uterine lysates compared to serum, there was no significant difference between the BZS group and the control group. The changes in IL-10 concentrations in uterine lysates after administration of BZS showed a similar tendency. As is well known, T-helper 2 cells primarily secrete IL-10, IL-4, but also IL-5, IL-6, IL-9 and IL-13 (Hill et al. 1995). Therefore, we evaluated the role of IL-10 and IL-4, the most representative cytokines of

the T-helper 2-type cytokine network (Dealtry et al. 2000) during abortion with the aim of evaluating the anti-abortive mechanism of BZS. Our data show that one of the major roles of BZS as an anti-abortive is to enhance Th2-cytokine production.

In contrast, Th1-cytokine levels were down-regulated in the BZS group. Uterine lysate IFN-γ concentrations were significantly lower in the BZS group compared with the RU-486 group. A similar tendency was found in serum (Figure 3). The concentrations of IL-2 in the BZS group showed no significant differences compared with the control (Figure 2). We calculated the rate of (IFN-γ + IL-2)/(IL-4 + IL-10) to simulate the balance between Th1/Th2 cytokines in the uterus of pregnant mice. The (IFN-γ + IL-2)/(IL-4 + IL-10) ratio correlated closely with the abortion rate of the mice (Figure 7). This ratio was significantly lower after BZS treatment. At the same time, the abortion rate had reduced to 50% from 100% in the RU-486 group. Thus, one of the anti-abortion mechanisms of BZS may lie in its role in biasing the balance of Th1/Th2 towards Th2.

It is commonly thought that mammalian pregnancy is a state of immunological tolerance and immunological pregnancy complications might result from incomplete allo-tolerance. T regulatory cells (Tregs) were proposed to play an essential role in this process (Zenclussen 2005). It is reported that the granulocyte-macrophage colony-stimulating factor (GM-CSF) is one of an array of cytokines with pivotal roles in embryo implantation and subsequent development. The capacity of GM-CSF to abrogate foetal loss in the abortion-prone CBA/J × DBA/2J mating model is dependent on $CD8^+$ maternal T cells (Robertson 2007). Recently, a newly defined population of $CD4^+CD25^+$ regulatory T cells have been proven to play critical roles in both self-tolerance and allograft tolerance (Zhao et al. 2007). Therefore, we tested the effect of BZS on immune cells using immunohistochemistry. We found that the number of $CD4^+$ T cells in mice uteri exhibited no significant increase in the BZS group vs. the control (Figure 6). On the other hand, the number of $CD8^+$ T cells was elevated vs. the control in the BZS + RU-486 group (Figure 6, $CD8^+$/BZS + RU-486). As for macrophages, there was no significant difference in the number of cells between the BZS + RU-486 group and the control. However, the number of $CD4^+$, $CD8^+$ T cells and $F4/80^+$ macrophages were elevated when the mice underwent an abortion after RU-486 treatment.

Chinese herbal medicine generally involves combining two or more herbs into a formula that treats a specific disease. There are 12 herbs in the BZS formula in which Atractylodes (Bai zhu) and Scutellaria (Huang qin) play the main role in the anti-abortive action (Zhong et al. 2002). The three major components of Scutellaria, baicalin, baicalein and wogonin, all exhibited anti-abortive actions to various extents in our previous studies (Ma et al. 2009; Zhao et al. 2011a). Traditionally, it is believed that Atractylodes (Bai zhu) and Scutellaria (Huang qin) are the best herbs for foetal restlessness when used in combination. The ancient Chinese physician Danxi Zhu of the Jin dynasty (A.D. 1115–1234) identified Huang qin and Bai zhu as playing the main role in protecting against abortion. Most anti-abortive herbal formulas contain Huang qin, Bai zhu and other herbs. Other herbal constituents of BZS such as Codonopsis, Amomum, and Citrus contain quercetin, the anti-abortive actions of which have been demonstrated in our previous studies (Wang et al. 2010; Yang et al. 2011; Zhao et al. 2011b). Little is known regarding the roles of other herbs and whether BZS is the best of many anti-abortive herbal formulas. Further studies need to be carried to elucidate the pathway or target cells for these herbs in regulating immune balance.

Our present study demonstrates that BZS can lead to an up-regulation of Th2-cytokine levels, a down-regulation in the expression of Th1-cytokines, and steer the balance of Th1/Th2 towards a Th2-biased response. Our data also suggest that BZS also regulates immune cells to ensure a successful pregnancy. However, there may be other ways in which BZS exerts its anti-abortive functions and this requires further exploration in the future.

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