

*Full Length Research Paper*

# Anti-abortion effect of quercetin and bornyl acetate on macrophages and IL-10 in uterus of mice

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The objective of this work was to investigate the significance of macrophages and IL-10 in uterus in early embryo loss (or resorption), and to elucidate the anti-abortion effect and the immunological modulation of maternal-fetal interface with quercetin and bornyl Acetate. Lipopolysaccharide (LPS) (0.10 µg/mouse) was injected via the tail vein in order to induce abortion in 7-day-gestation mice which received quercetin and bornyl acetate at days 4 to 7 of gestation. Levels of IL-10 in uterus supernatant were measured using enzyme-linked immuno-absorbent assay (ELISA), and uterine macrophages of each group (n=10) were detected by immunohistochemistry. The levels of IL-10 declined significantly in uterus with LPS treatment. The amount of macrophages in the uterus of LPS-induced abortion mice was much higher than that of the control mice. When quercetin and bornyl acetate were used to prevent LPS-induced abortion, the effect of quercetin combined with bornyl acetate on anti-LPS-induced abortion was more significant, and the IL-10 content was close to normal and the amount of macrophages was decreased to  $16.199 \pm 0.802$ , which was significantly different from that of LPS-induced abortion group. The decrease of IL-10 and the increase of macrophage number in the LPS-treated mice uterus were associated with the embryo loss, and quercetin and bornyl acetate has the anti-abortion effect through modulation of maternal-fetal interface immunity balance.

**Key words:** Embryo resorption, lipopolysaccharide, macrophages, IL-10, mice.

## INTRODUCTION

Abortion is one of the most common complications in mammals, especially in early pregnancy, involving a complicated immunomodulating mechanism. Successful gestation depends on an immune balance between the mother and the embryo. In recent studies, localized immunity *in utero* relevant to embryo implantation and growth has become a focus in reproductive immunology, and it is a main concern for maintaining maternal immunological tolerance to the fetus.

Macrophages are one of the main immunocytes in the uterus, which engulf pathogenic microorganisms and apoptose trophoblastic cells and other abnormal cells. Macrophages can protect the embryo from infection by secreting manifold cytokines [tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1] and some biotic factors

(nitrogen monoxidum, rennin and prostaglandin) (Abrahams et al., 2004). However, if overactivated, macrophages will produce nitric oxide (NO) and TNF- $\alpha$ , which can induce embryo resorption or abortion (Clark et al., 1998). The qualitative characteristics of immune responses are regulated by T cell subsets through their production of distinctive cytokines. Two well-characterized T cell subsets are Th1 cells which, via production of IFN- $\gamma$ , promote cell-mediated responses against bacteria, and Th2 cells which, by producing IL-4, IL-5 and IL-13, induce the anti-parasite mast cell and eosinophil responses (Abbas et al., 1996). IL-10 is secreted by the Th2 cells and is a potent immune-regulating cytokine and inhibitor of inflammatory cytokine synthesis. IL-10 is a central regulator of the inflammatory response, acting to limit inflammation-induced tissue pathology by terminating monocyte and macrophage synthesis of TNF- $\alpha$  and an array of other proinflammatory cytokines and chemokines (Moore et al., 2001). Experiments in rodent models and humans implicate IL-10 in controlling inflammatory processes in pregnancy. IL-10 is

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**Table 1.** Gestation results of different treatments (n = 10).

Group	Drug or PBS i.g. (ml) (day 4 to 7 of gestation)	LPS or PBS i.v. (ml) (day 7 of gestation)	Abortion rate (%)	Rate of resorption (%)
A	PBS 0.4	PBS 0.2	20.0 (2/10) <sup>Bb</sup>	24.4 <sup>Bb</sup>
B	PBS 0.4	LPS 0.2	100.0 (10/10) <sup>Aa</sup>	100.0 <sup>Aa</sup>
C	Que 0.4	LPS 0.2	50.0 (5/10) <sup>ABb</sup>	53.2 <sup>Bb</sup>
D	BA 0.4	LPS 0.2	60.0 (6/10) <sup>ABb</sup>	60.4 <sup>ABb</sup>
E	Que + BA 0.4	LPS 0.2	30.0 (3/10) <sup>Bb</sup>	28.6 <sup>Bb</sup>

Values followed by different capital letters are significantly different ( $p < 0.01$ ); values followed by different small letters are different ( $P < 0.05$ ); values followed by small letters are not different ( $P > 0.05$ ).

expressed abundantly in the decidual and placental tissues in mice (Lin et al., 1993; Chaouat et al., 1999).

Lipopolysaccharide (LPS) is one main component of cell envelope of Gram-negative bacillus. Previous studies have shown that LPS given intravenous (i.v.) injection could induce embryo resorption in pregnant animals (Zhong et al., 2008). Endotoxin is the name given to a group of heat-stable LPS molecules present in the cell envelopes of gram-negative bacteria that possess toxic effects (Burell, 1994). Lipopolysaccharide (0.10 µg/mouse) was injected via the tail vein in order to induce an abortion model in 7-day-gestation mice. Quercetin is an herbal flavonoid derived from various foods and plants. In Chinese herbal medicine, quercetin is a component of many herbs such as, *Semen cuscutae*, *Herba Taxilli* and *Cortex Eucommiae*. Bornyl acetate is an abundant component in the oil of *Fructus Amomi*. The Chinese Veterinary Pharmacopoeia (2005 Edition) records that *Cuscuta chinensis* Lain, *Herba Taxilli*, *Eucommia ulmoides* Oliver and *F. Amomi* all play an anti-abortion role. Therefore, it is worth studying, the anti-abortion effect of quercetin and bornyl acetate, and the immunological regulation at the maternal-fetal interface. In this study, we used LPS to induce embryo resorption and measured the amount of uterine macrophages and IL-10 contents with the aim of elucidating the mechanism of LPS-induced abortion and the anti-abortion effects of quercetin and bornyl acetate.

## MATERIALS AND METHODS

LPS (lipopolysaccharide, Sigma Chemicals) from *Escherichia coli* was dissolved in sterile phosphate-buffered saline (PBS) (0.01 M, pH 7.4) to a final concentration of 0.5 µg/mL. Quercetin (Que, Sigma products, purity = 98%) alone or bornyl acetate (BA, Fluka products, purity ≥ 99%) alone or the two combined was first dissolved in small amount of dehydrated alcohol, then diluted in PBS. The final concentration of Que was 2.5 mg/ml, and final concentration of BA was 2.5 µl/ml.

### Treatment of animals and groups

BALB/c mice aged 10 weeks were purchased from the Laboratory Animal Center, Hebei Medical University, China. The mice were

given free access to mouse chow and water, with a 12 h light cycle from 7:00 to 19:00. One virgin female mouse was housed with one male mouse, was checked every day in the early morning, and the pregnancy was confirmed by the presence of a vaginal plug. The day of appearance of the vaginal plug was marked as day 0 of pregnancy (Zhong et al., 2008; Zhong et al., 2002). The animals were treated humanely following our provincial government guidelines for using experimental animals.

Then, the pregnant mice were divided randomly into five groups. Group A was the control group, group B was the LPS model group, group C was the quercetin (Que) group, group D was the bornyl acetate (BA) group and group E was the Que plus BA group. Briefly, mice in groups B, C, D and E were given an intravenous injection (i.v.) of LPS via the lateral tail vein at a dose of 0.2 ml (0.1 µg) on day 7 and received a gavage of PBS, Que, BA and Que+BA, respectively at a dose of 0.4 ml at days 4 to 7. Animals in group A received an oral gavage of PBS on the same days of gestation as in group C, and were administered with PBS at 0.4 ml at day 7 of gestation (Table 1). The gravid females were sacrificed by cervical dislocation at the day 9 of gestation. The uterine samples were collected for further assays.

### Calculation of embryo loss rate and abortion rate

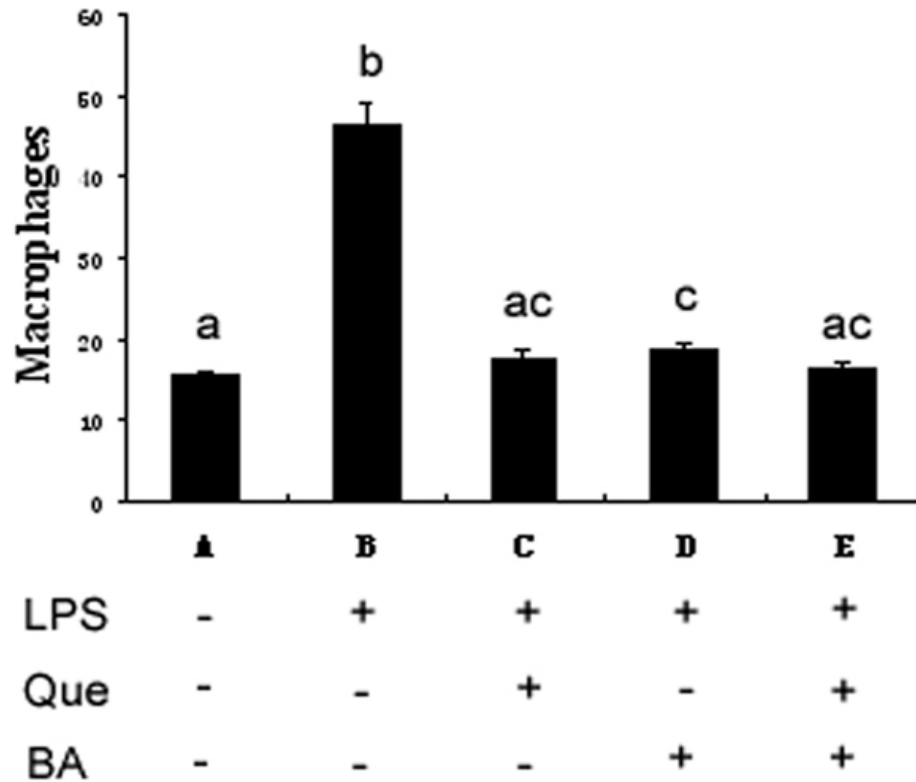
All the female mice were sacrificed by cervical dislocation at day 9 of gestation and the contents of uterus were examined for viable and resorbing embryos. The viable embryos (V) were well-oxygenated (pink) and showed a well-defined embryonic capsule and placenta. The resorbing embryos (R) were much smaller, showed signs of ischemia, haemorrhage and were often macerated and black without identifiable embryo or placenta. The embryo loss was presented as a percentage of the contents of the uterus [ $100 \cdot R / (V+R)$ ]. The incidence of abortion was calculated as a percentage of the contents of the miscarriage ( $100 \cdot \text{abortive mice} / \text{total mice}$ ).

### ELISA assay for IL-10

The left parts of uterine horns were carefully cleansed of fat, and the embryos were removed. Uterine lysates were prepared in PBS (pH 7.4) containing PMSF (0.75 µg/mL) on ice, centrifuged for 15 min at 12000 r/min at 4°C, and the supernatants were collected. IL-10 levels in the supernatants were measured by IL-10 ELISA kit detection (Biosource, USA) according to the manufacturers' instructions.

### Immunohistochemistry

The right uterine horn were fixed in Bouin's fluid, dehydrated in



**Figure 1.** Change of macrophages in uterus of mice in different groups. Results are mean  $\pm$  SD (n = 10). Significant difference ( $p < 0.05$ ) is denoted by different letters.

graded ethanol, and embedded in paraplast. Then serial sections of 6  $\mu\text{m}$  thick were prepared. After properly deparaphinization and hydration, the uterine sections were stained using streptavidin immunoperoxidase technique. Briefly, the tissue sections were overlaid with macrophage antibody (rat anti-mouse F4/80 macrophage monoclonal antibody, eBioscience, USA) after citrate pretreatment, and incubated overnight in a humid chamber at 4°C. Then the sections were allowed to react with a biotinylated rabbit anti-rat IgG (H+L) antibody (Vector, U.K.) in 10% normal calf serum, followed by incubation with horseradish peroxidase conjugated streptavidin (Vector, U.K.). The tissue sections were washed thoroughly with PBS before each procedure. Finally, the sections were incubated with 0.03% DAB containing 0.05% hydrogen peroxide and mounted.

Macrophages were observed under a light microscope (magnification 40 $\times$  for the objective lens). All positive cells in campus visualis were counted. The mean value was pooled from 20 high power fields in each sample, and averaged from ten mice. Differences in macrophages and IL-10 contents were assessed by one-way analysis of variance. Statistical analysis of abortion rate and embryo resorption rate was conducted using  $\chi^2$ -test ( $P < 0.05$  was taken as significant).

## RESULTS

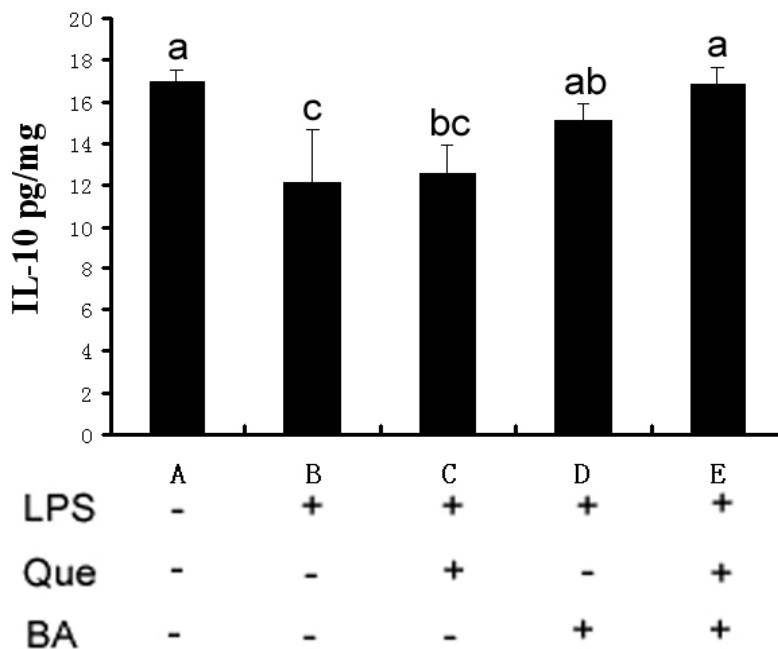
### The anti-abortion effects of quercetin and bornyl acetate

Group A, pretreated with PBS as a control, showed a

natural abortion rate of 20.0%. Mice in group B were sacrificed at day 9 of gestation. Both the abortion and the embryo resorption rates were 100.0%. The resorbed conceptus was severely macerated and black in color. Mice in group C, pretreated with quercetin, showed an abortion rate of 50.0% and a resorption rate of 53.2%. Six out of 10 mice in group D were aborted and the resorption rate was 60.4%. Mice in group E pretreated with quercetin and bornyl acetate showed a 28.6% resorption and a 30.0% abortion (Table 1).

### Influence of quercetin and bornyl acetate on uterine macrophages

Macrophages were distributed in the endometrium of the uterus. The cell number in the control group (group A) was  $15.531 \pm 0.493$ . Mice treated with LPS in group B, had a much larger uterine macrophage quantity and the difference was found statistically significant ( $p < 0.01$ ) between groups A and B. When quercetin and bornyl acetate were used to prevent LPS-induced abortion, less macrophages were counted. The effect of quercetin combined with bornyl acetate on LPS-induced abortion was significant, and the amount of macrophages decreased to  $16.199 \pm 0.802$ , lower than that of LPS-abortion group ( $p < 0.01$ ) (Figures 1 and 3 to 7).



**Figure 2.** IL-10 contents of different constituents of uterus lysate in different groups. Results are mean  $\pm$  SD (n = 10). Significant difference ( $p < 0.05$ ) is denoted by the different letters.

### Influence of quercetin and bornyl acetate on uterine IL-10 contents

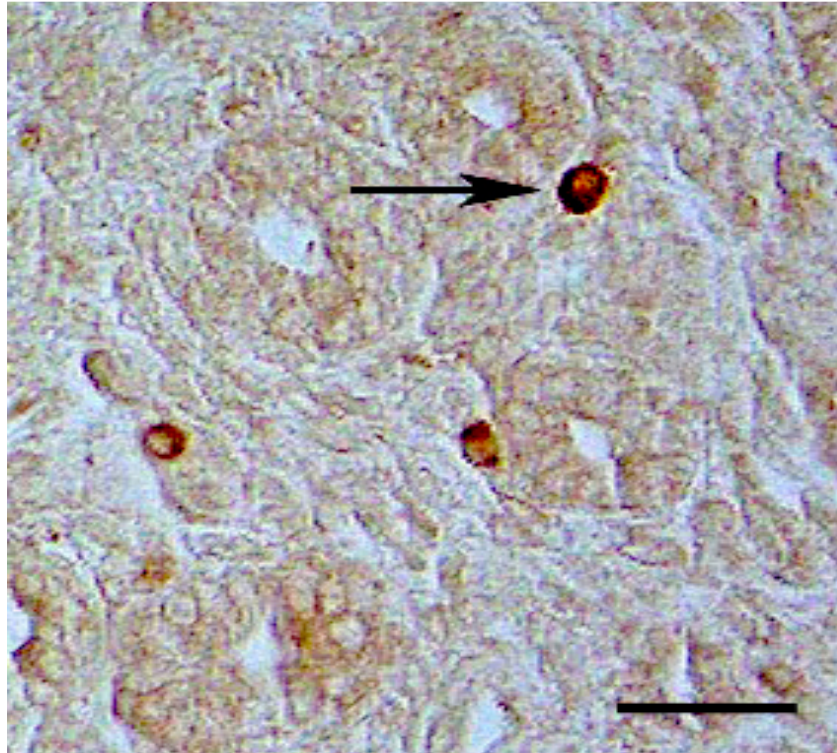
The IL-10 contents in all the control mice treated with sterile PBS (group A) were relatively stable and there was no significant variance between individuals. The mean value was  $17.004 \pm 4.013$  pg/mg protein. When the mice were given an injection of LPS, the IL-10 values were down-regulated greatly. The mean value was  $12.087 \pm 2.296$  pg/mg protein. However, pretreatment with quercetin and bornyl acetate prevented the IL-10 contents from decreasing; suggesting that quercetin and bornyl acetate had the effect of anti-LPS induced abortion (group E). When pretreated with quercetin (group C), the level of IL-10 was as low as that of LPS treated group, indicating that the anti-abortion effect of quercetin was weak. The mice of group D was pretreated with bornyl acetate, and the IL-10 content was  $15.118 \pm 3.119$  which was similar to group E (Figure 2).

### DISCUSSION

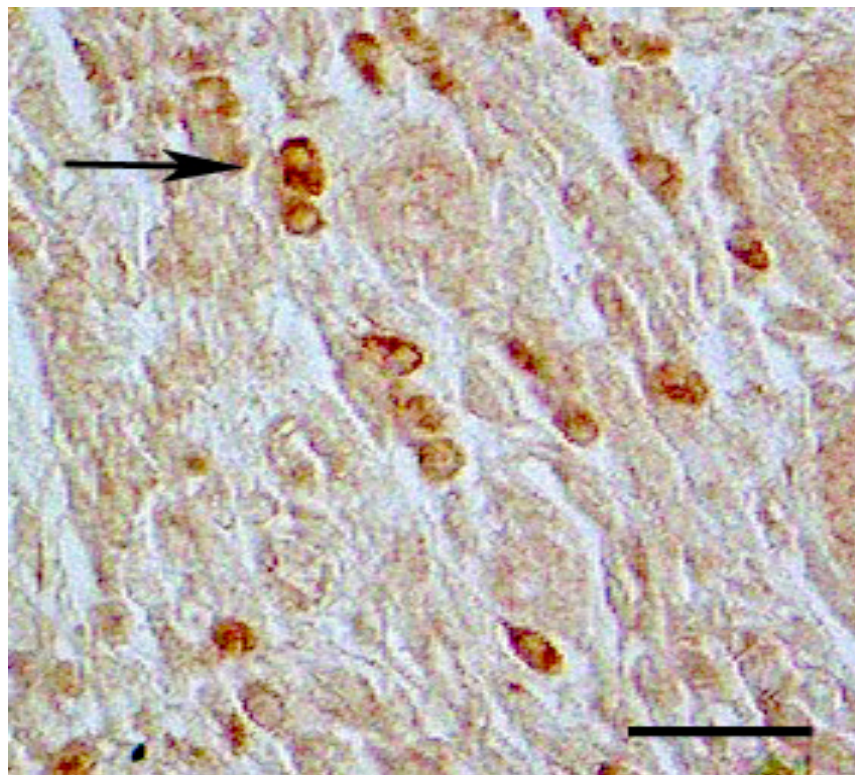
In this study, both abortion rate and embryo resorption rate were 100% in gestation mice injected  $0.10 \mu\text{g}$  LPS via the tail vein indicating that LPS was suitable for abortion model in studying the anti-abortion effects. Quercetin is the constituent of *S. cuscutae*, *H. Taxilli*, *C. Eucommiae* and etc., and bornyl acetate is the constituent of *F. Amomi*. Modern pharmacological

research has shown that the quercetin, a major active component of *S. cuscutae*, has comprehensive biological actions. Lin et al. (2004) found out that quercetin could produce a protective action on TNF- $\alpha$ -induced cultured vascular endothelial cell (VEC)-304 and its mechanism of action may be related to the decrease of NO; antioxidant effect of lipids. However, antioxidant effect might be exerted through nuclear factor-kappa B (NF-kappa B) activation pathway. Research showed that the over-rodution of TNF- $\alpha$  and NO by LPS stimulated macrophages was markedly inhibited by quercetin (Manjeet and Ghosh, 1999). Bornyl acetate has the action of anti-diarrhea, analgesia, dephlogistication, depressing spasm, etc. By far, it has not been reported whether quercetin and bornyl acetate have the effect of immunoregulation on pregnant mice. In this study, LPS induced embryo resorption in mice, while pretreated with quercetin and bornyl acetate, the abortion rate was decreased markedly. The effect of quercetin combined with bornyl acetate on LPS-induced abortion was more significant, because the abortion rate was decreased to 30%; lower than that of LPS-abortion group ( $p < 0.01$ ). These results indicated that quercetin and bornyl acetate have the effect of anti-abortion in mice.

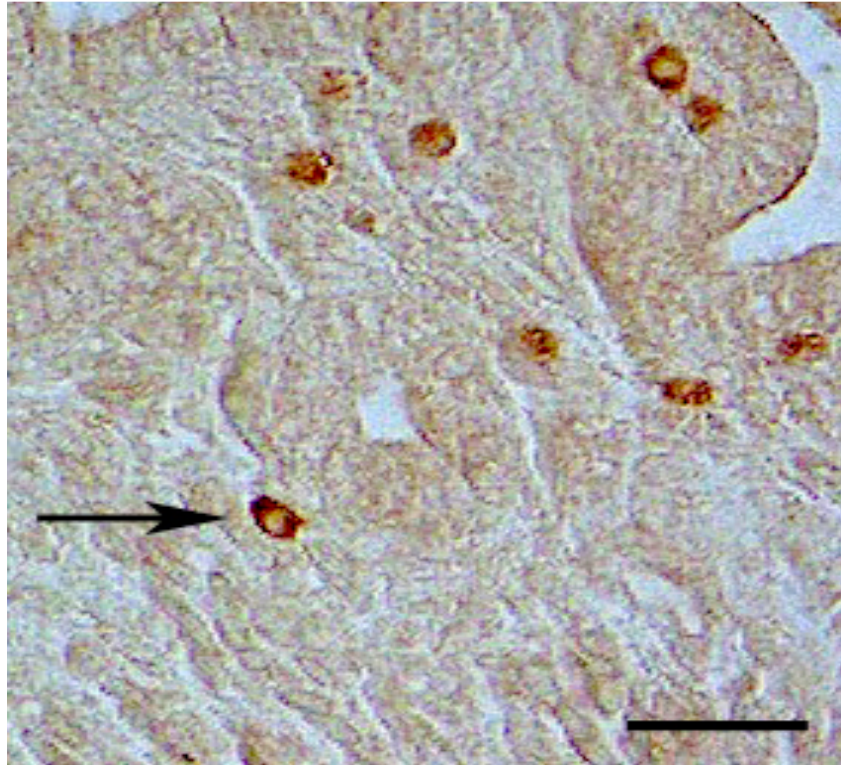
In the gestation period, some immunocytes migrated to the deciduas; they mainly include NK cells, macrophages and T cells, and 20 to 30% of them were macrophages (Piccinni, 2003), which are chiefly recruited from peripheral blood mononuclear cells. Macrophages could clear up effectively pathogenic microorganisms and



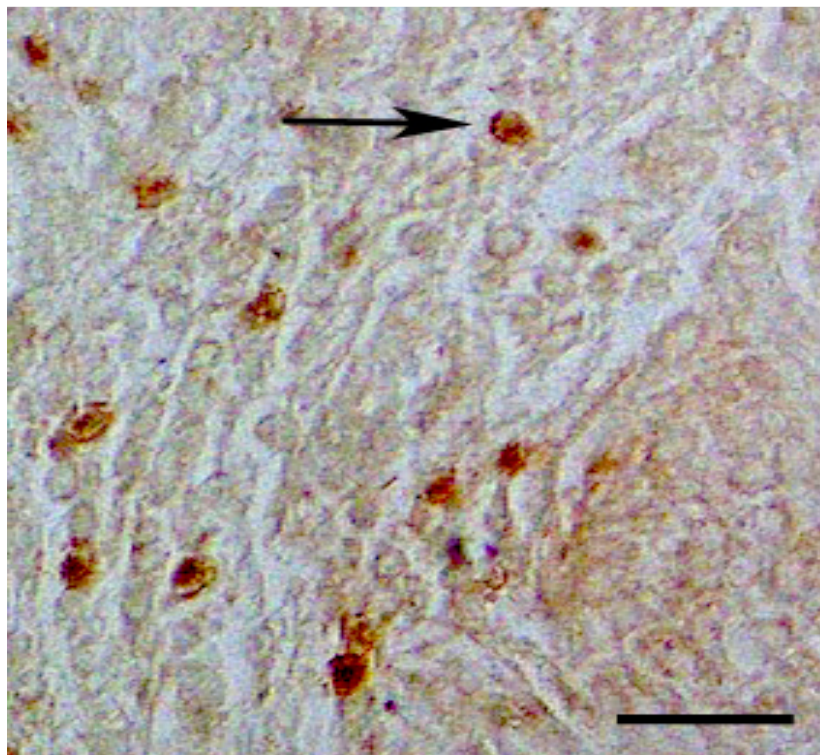
**Figure 3.** Few macrophages (→) found in the endometrium of normal pregnant mice. SP, DAB are stained. The bar is 25  $\mu$ m.



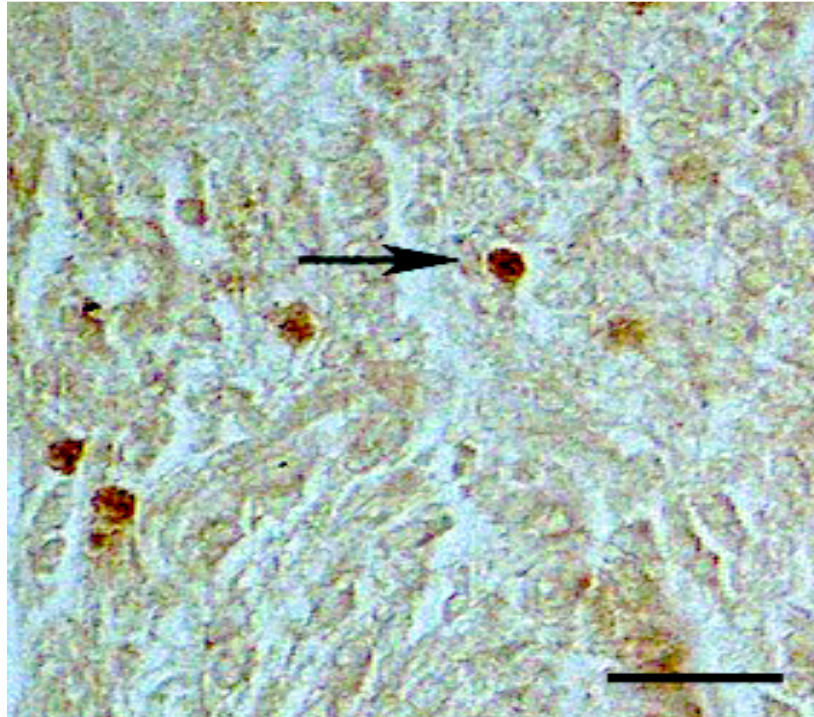
**Figure 4.** Many macrophages (→) found in the endometrium of LPS-induced abortion mice. SP, DAB are stained. The bar is 25  $\mu$ m.



**Figure 5.** Distribution of macrophages ( $\Rightarrow$ ) in mouse endometrium that received quercetin. SP, DAB are stained. The bar is 25  $\mu$ m.



**Figure 6.** Distribution of macrophages ( $\Rightarrow$ ) in mouse endometrium that received bornyl acetate. SP, DAB are stained. The bar is 25  $\mu$ m.



**Figure 7.** Macrophages ( $\rightarrow$ ) in mouse endometrium received quercetin and bornyl acetate. SP, DAB are stained. The bar is 25  $\mu$ m.

abnormal cells, protecting fetus from infection. Macrophages could produce collagenase, elastase, nitrogen monoxidum induced enzyme, etc., facilitating tissue restitution of fetus development and retaining uterus muscle relaxation. In addition, macrophages produce many kinds of cytokines (TNF- $\alpha$ , IL-1, etc.) and some biotic activators (nitrogen monoxidum, rennin and prostaglandin), protecting fetus from being infected. Macrophage infiltration and cellular activation are identified by increased expression of proto-oncogenes, and the production of cytotoxic macrophage products are closely linked to early embryo loss (Abrahams et al., 2004). These data add to the evidence that activated maternal macrophages may be directly responsible for spontaneous pregnancy failure (Duclos et al., 1996). Previous studies indicated that the increasing expression of TNF- $\alpha$  and NO due to macrophage overexpression, and cytokine-triggered thrombotic, inflammatory processes in maternal uteroplacental blood vessels caused abortion (Clark et al., 1998). In this study, the results of immunohistochemistry analysis showed that uterine macrophages increased in number in LPS-induced abortive mice than that of the normal pregnant mice. When quercetin was administrated with bornyl acetate to prevent LPS-induced abortion, less macrophages were counted. The effect of quercetin combined with bornyl acetate on LPS-induced abortion was more significant, and the amount of macrophages decreased to  $16.199 \pm 0.802$ , which was significantly lower than that of LPS-

abortion group ( $p < 0.01$ ).

Interleukin-10 is a potent immune-regulating cytokine for gestation and it is secreted by Th2 cells. IL-10 can stimulate B cells to proliferate and differentiate, producing IgM, IgG and IgA. Besides, IL-10 can stimulate T lymphocytes and mononuclear macrophages to down-regulate expression of pro-inflammatory cytokines TNF- $\alpha$ , IL-6, IL-1, IL-12 and IFN- $\gamma$ , protecting against inflammation-induced pathology in the implantation site. Therefore, IL-10 plays an accommodating role in the balance of maternal-fetal interface immunological network (Levings et al., 2001). IL-10 deficiency was accompanied by growth restriction in the remaining viable fetuses, with an approximately 10-fold reduction in the threshold dose for 100% abortion and recombinant, IL-10 rescued the increased susceptibility to LPS-induced fetal loss (Robertson et al., 2007). Previous study demonstrated that IL-10 could enhance the expression of HLA (human leucocyte antigen)-G mRNA and the secretion of human chorionic gonadotrophin (HCG) in trophoblasts, and played an important role in the formation of maternal-fetal immune tolerance (Sun and Wang, 2004). IL-10 relaxes decidual stromal cells by reducing the incorporation of  $\alpha$ -smooth muscle action into their stress fibers. This relaxing activity may be relevant to the maintenance of pregnancy (Kimatrai et al., 2005). In addition, IL-10 could inhibit CD4+ cell proliferation and the expression of mononuclear macrophage dependence antigen, and reduce the rejection of fetus (Howard and

O'Garra, 1992). IL-10 could restrain the composition and delivery of Th1 type cytokines, that is, IFN- $\gamma$ , TNF- $\alpha$ , IL-2, etc., and increase the expression of Th2 response at the maternal-fetal interface which is profitable for the establishment and maintenance of normal pregnancy (Dealtry et al., 2000). This results showed that the uterine IL-10 contents increased significantly, and almost achieved the normal level pretreated with quercetin and bornyl acetate, suggesting that IL-10 content in the uterus was related to maintenance of normal pregnancy.

## Conclusion

From the results, it can be concluded that the IL-10 content was decreased and the macrophages counting was elevated significantly in the LPS-induced abortion mice uterus, and quercetin and bornyl acetate had the anti-abortion effect through modulation of maternal-fetal interface immunity balance.

## ACKNOWLEDGEMENTS

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