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Full Length Research Paper

Influence of quercetin on transforming growth factor β1 in the uterus following Lps-induced abortion in pregnant mice

Qian Yang, Wan-yu Shi and Xiu-hui Zhong*

College of Animal Science and Technology, Agricultural University of Hebei, Baoding, Hebei 071001, China.

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To investigate the significance of transforming growth factor β1(TGF-β1) in the uterus in early embryo loss (or resorption) and to elucidate the anti-abortive effect and the immunological modulation at the maternal-fetal interaction of quercetin (Que). Lipopolysaccharide (LPS) (0.10 μg/mouse) was injected via the tail vein in order to induce abortion in 7-day-gestation mice which also received quercetin at days 1 to 7 of gestation. Uterine TGF-β1 of each group (n=10) was detected by *in situ* hybridization. The amount of TGF-β1 in the uterus of LPS-induced abortion mice was much lower (P<0.01) than that of the control mice. When quercetin was used to prevent LPS-induced abortion, more TGF-β1mRNA was counted. The effect of high concentration quercetin on LPS-induced abortion was more significant, and the rate of TGF-β1mRNA was increased to 13.52±0.58, significantly higher than that of LPS-abortion group (P<0.01). The change of TGF-β1mRNA in the mouse' uterus could be associated with the embryo loss, and quercetin exerts an anti-abortive effect through the modulation of the immune balance at the maternal-fetal interface.

Key words: Embryo resorption, transforming growth factor-β1, lipopolysaccharide, quercetin, *in situ* hybridization.

INTRODUCTION

Pregnancy is a complex physiological process. In terms of reproductive immunology, pregnancy is somewhat like organ transplantation. The embryo carrying the paternal antigen is a semi-allograft to the mother. The maternal immune system should identify the allograft, and reject it. However, in terms of the outcome, pregnancy progress differs from that in organ transplantation. The maternal can recognize the implanting fetus, and produce protective immune responses or immunological tolerance. Therefore, the balance between immunological rejection and immunological tolerance at the maternal-fetal interface plays an essential role in the maintenance of normal pregnancy. The maternal can endure semi-allogeneic fetus with the paternal and maternal genes during pregnancy. It is an extremely complex and subtle regulation of the immunological phenomenon. Transforming Growth Factor (TGF-β1) is one member of the TGF-β superfamily network system ligand-receptor (Adra et al., 2010). It is formed by a group of similar structure and function associated multifunctional polypeptides and plays the vital role in embryo development, cell proliferation and differentiation and immunoregulation (Cheng and Cao, 2005). Recent studies suggested that TGF-β1 was involved in female reproductive processes, and participated in the regulation of embryo implantation and embryo development. It has been reported that during the implantation process, the rodent endometrium undergoes a complex process of cell decidualization that results in expressing a variety of cytokines. Together with the progesterone and estrogen, these cytokines regulate proliferation and differentiation of uterus cells, and of them, TGF-β1 is one important cytokine (Pollar, 1990). Research has shown that at the site of implantation, TGF-β1 produced by decidual cells inhibits the proliferation of trophoblasts which change into no-infiltration of syncytiotrophoblasts. In addition, TGF-β1 also decreases the activity of matrix metalloproteinases and tissue plasminogen protease to prevent the

*Corresponding author. E-mail: zxh8078@163.com

excessive infiltration of the trophoblasts (Sharkey et al., 1995) and then keep the immunological balance at the maternal-fetal interface. Quercetin is found to be the most active of the flavonoids in studies with high medicinal value. It is the main substance of dodder and Chinese taxillus herb.

The Chinese Veterinary Pharmacopoeia, 2005 edition, records that dodder and Chinese taxillus herb have the effects of miscarriage prevention. Research has elucidated that quercetin and bornyl acetate has anti-abortive function in pregnant mice induced by lipopolysaccharide (Wang et al., 2008; 2011). However, there have been few reports on how quercetin plays its effect during implantation. So, this investigation was designed to test the TGF-β1 mRNA following the administration of quercetin through *in situ* hybridization, and to describe the mechanism from the molecular level.

MATERIALS AND METHODS

Chemicals and reagents

Lipopolysacchride (LPS) purchased from Sigma Co. (USA) was diluted with 0.01 mol/l, pH 7.4 phosphate-buffered saline (PBS, filtered and sterilized by bacterial filter) to 0.5 mg/l. The purity of the quercetin (Que) obtained from Sigma Co.(USA) was 98%. The agent was diluted with PBS at the concentrations of 0.5 mg/ml (low dose), 1 mg/ml (medium dose), 1.5mg/ml (high dose), respectively. TGF-β1 *in situ* hybridization kit was obtained from Wuhan Boster Biotech Wuhan, China).

Treatment of animals and medication

Ten-week-old naive female and male BALB/c mice were purchased from the Experimental Animal Center of Hebei Medical University. The average weight was 24.32±0.63 g. Three or four female mice were kept in one cage, and 1 male in one cage. The mice were fed with standard rat chow and tap water *ad libitum*. The animals were housed under controlled lighting (12 h light and dark) and temperature (21 to 22°C) conditions. Studies started after one week adaptation. Pregnancies were obtained by housing one estrous female with one male overnight, and the females were examined each day in the early morning for the presence of a vaginal plug. The day of detection of the vaginal plug was designated as day 0 of pregnancy.

The pregnant mice were randomly divided into 5 groups (10 mice/group); the control (A), LPS (B), low-dose Que (C), medium-dose Que (D) and the high-dose Que (E). The mice in Group A were treated with 0.2 ml PBS by tail intravenous injection (Gendron et al., 1990) at day 7 of pregnancy. Mice in groups B, C, D and E were treated with 0.2ml (0.10μg) LPS by tail intravenous injection at day 7 of pregnancy, respectively.

Every mouse in Groups A and B was orally given 0.4 ml PBS at days 1 to 7 of gestation. Mice in Groups C, D and E were treated with Que by gavage at 0.5 mg/l (low dose), 1 mg/l (medium dose) and 1.5 (high dose) mg/l at day 7 of pregnancy in 0.4 ml, respectively. On day 9 of gestation, the mice were sacrificed by cervical dislocation, and the rates of embryo absorption observed. The uterus was weighed with the embryos removed. The left horn of the uterus was quick-frozen in liquid nitrogen and stored at -80°C. The right horn was fixed in 4% paraform phosphate buffer (0.1 M, pH 7.4) prepared with diethypyrocarbonate (DEPC), subjected to *in situ* hybridization.

Calculation of abortion rate and embryo resorption rate

The absorbed embryos were smaller, showed signs of ischemia, haemorrhage, and often macerated and black in color without identifiable embryo or placenta. In contrast, the normal embryos were big and well-oxygenated (pink) observed under anatomical microscope, and showed a well-defined placenta without hemorrhage or congestion. The abortion rate and embryo resorption rate of each group were calculated as follows:

Abortion rate = The number of abortion/The total number×100%.

Embryo resorption rate = The amount of absorbed embryos/ (absorbed embryos+ the normal)×100%.

In situ hybridization

In situ hybridization was carried out on cross-sections of uterus to detect TGF-β1 mRNA as described in the kit instruction. The fixed samples of uterus were embedded in paraffin and serial sections of 5 μm were prepared. The sections were deparaphinized and hydrated and then washed with distilled water 3 times after the endogenous peroxidase activity was neutralized by 3% H_2O_2 for 15 min at room temperature. Then the tissue was digested with fresh pepsin diluted with 3% citric acid at 37°C for 20 min. The slides were washed with *in situ* hybridization liquid 3 times, and then with fresh water 1 time. Then prehybridization buffer (20 μl) was added to each section, and the sections put into a wet chamber at 38 to 42°C for 3 h. Excess buffer was gotten by wiping around the sections with a cloth or tissue. Each section was overlaid with 20 μl hybridization buffer, and covered with parafilm at 38°C over night. The parafilm was removed from tissue sections and rinsed three times with 37°C 2 × citric acid buffer and 0.5 × citric acid buffer. Excess buffer was gotten off the tissue section after incubation with the quenching solution at 37°C for 30 min without wash. Adequate biotinylated mouse anti-digoxin solution was added to each section at 37°C for 60 min and then the tissue was rinsed. Tissue sections were overlaid with streptavidin-biotin (SABC) complex and kept at 37°C for 20 min and then the tissue was rinsed. Then the sections were incubated with biotinylated peroxidase at 37°C for 20min. After the sections were rinsed by PBS, the reactions were made visible with metal-enhanced diaminobenzidine (DAB).

Statistical analysis

The positive granules counting were observed under the 40× objective lens, and 10 visual fields of every section randomly selected to calculate the number of positive granules. Significant differences were compared among groups by one-way analysis of variance (ANOVA). Using X^2 test, the differences of abortion rate and the embryo resorption rate were compared among the 5 groups.

RESULTS

Embryo resorption (loss) rate and abortion rate

The results indicated that quercetin had a reversal function on LPS induced abortion. The abortion rate decreased to 30 - 60% in quercetin treated groups. The embryo resorption and abortion rates of Group E were 30 and 29.1% and significantly different when compared with Group B ($p<0.01$). No significant difference was observed between Groups A and E (Table 1).

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

Group	Drug or PBS i.g. (ml) (Day 1 to 7 of gestation)	LPS or PBS i.v. (ml) (Day 7 of gestation)	Abortion rate (%)	Embryo resorption rate (%)
A	PBS 0.4	PBS 0.2	20.0 (2/10)^{Aa}	21.2^{Aa}
B	PBS 0.4	LPS 0.2	100.0 (10/10)^{Bb}	100^{Bb}
C	Que 0.4	LPS 0.2	60.0 (6/10)^{ABc}	66.2^{Bb}
D	Que 0.4	LPS 0.2	40.0 (4/10)^{Aa}	46.5^{Aa}
E	Que 0.4	LPS 0.2	30.0(3/10)^{Aa}	29.1^{Aa}

Values followed by different capital letters were significantly different (P<0.01); values followed by different small letters were different (P<0.05).

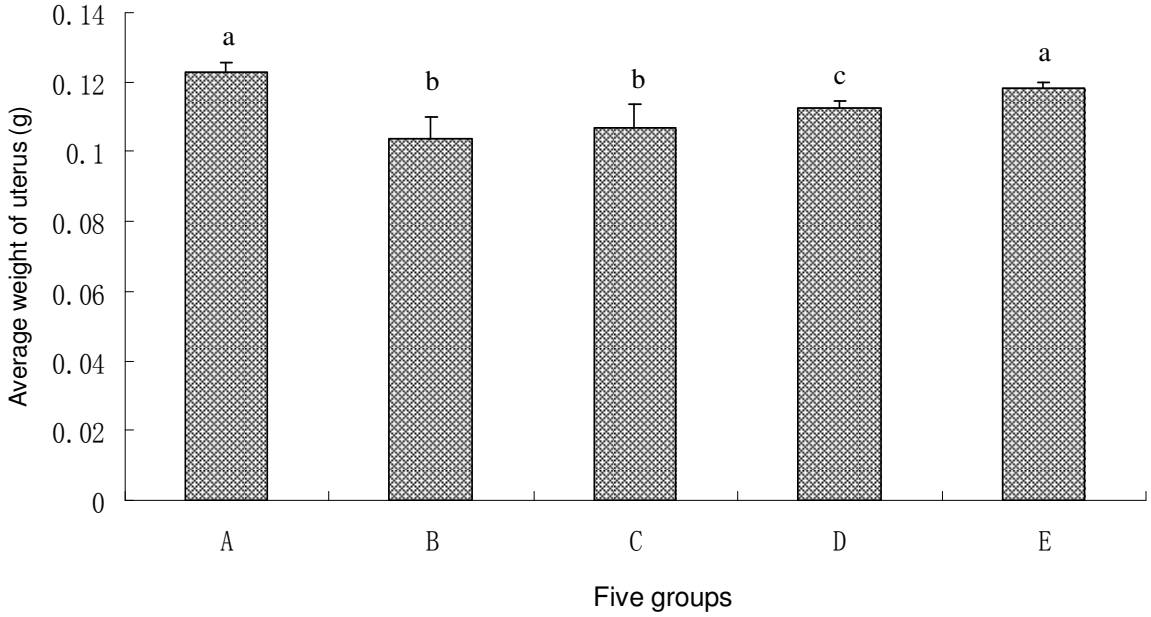

Figure 1. Average uterus weight in every group without embryos. Values followed by different capital letters were significantly different (P<0.01); values followed by different small letters were different (P<0.05).

Changes of uterus weight without embryos

In LPS Group, the average weight became light and degraded to 0.104 ± 0.020, significantly different when compared to Groups A, D and E (p<0.05). However, there was no difference between Groups B and C (p>0.05) (Figure 1).

Changes of TGF-β1mRNA positive granules in each group

The TGF-β1mRNA positive granules of group A was 15.20 ± 0.58. Group B was exposed to LPS through the tail intravenously without quercetin pretreatment. Its positive granule number reduced to 3.30 ± 0.14, significantly different when compared to Group A (p<0.01). The positive granule number was increased in the testes of Groups D and E. There was no difference between Groups E and A (p>0.05) (Figure 2). The fetal development of Group A was fine with morphological integrity and there were considerable TGF-β1mRNA positive granules distributed uniformly in the decidua and endometrium (Figure 3A). The embryos of the LPS Group became blood lumps. Most fetal tissues were not discharged and the decidua structure was unclear. Only few positive granules were observed in the endometrium (Figure 3B). The low-dose quercetin treatment group had no much difference with the LPS group (Figure 3C). From Group D, it was concluded that the decidua structure was clear, and we could see some positive granules distributed in the decidua and endometrium (Figure 3D). We could observe a lot of TGF-β1mRNA positive granules from the decidua and endometrium of Group D

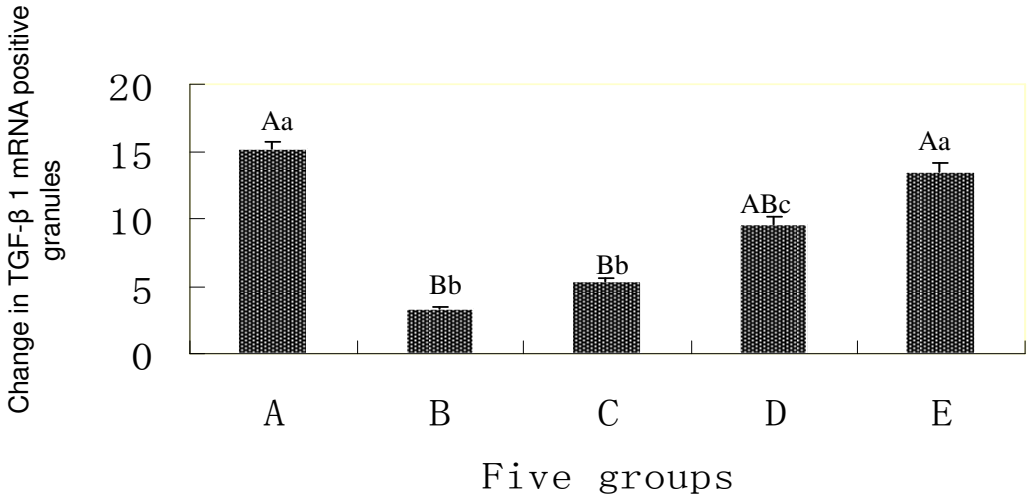

Figure 2. Change of TGF-β1 in uterus of mice in different groups (n=10).

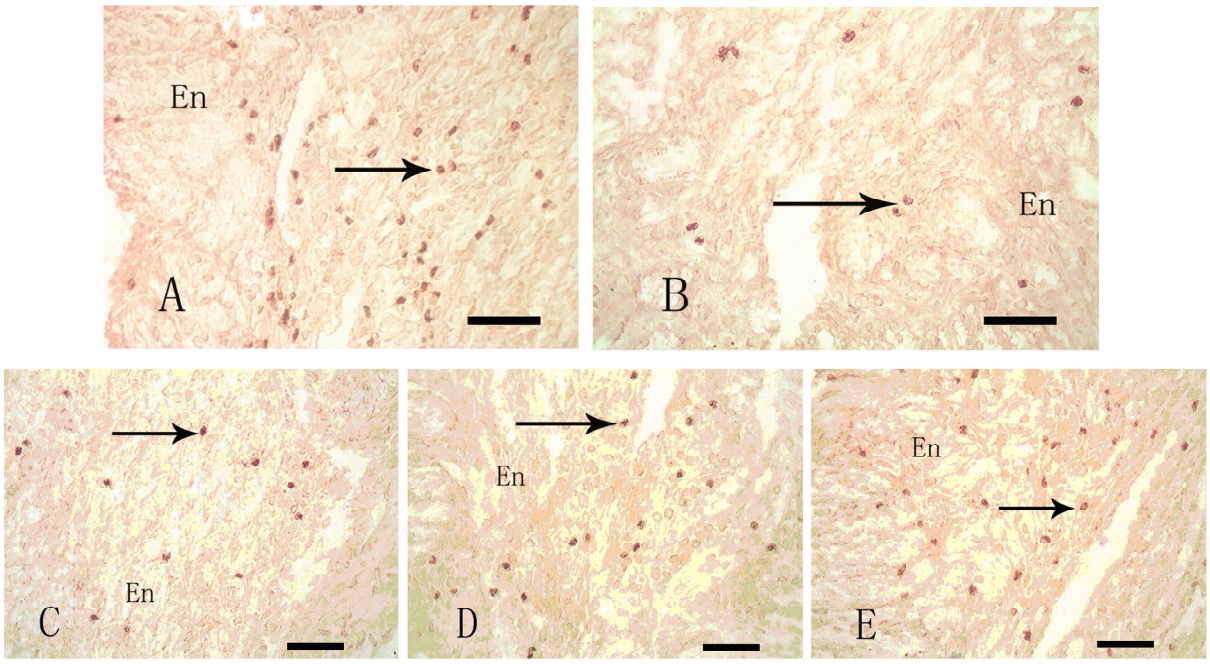

Figure 3. (A) A lot of TGF-β1 (↑) positive cells are found in the uterus of normal pregnant mice. *In situ* hybridization method. En: Endometrium. Bar = 30 μm. (B) A few TGF-β1 (↑) are found in the uterus of LPS-induced abortion mouse. (C) Distribution of TGF-β1 (↑) in mouse' uterus received low concentration [quercetin group]. (D) Distribution of TGF-β1 (↑) in mouse' uterus received medium dose quercetin. (E) TGF-β1 (↑) in mouse uterus received high dose quercetin.

(Figure 3E).

DISCUSSION

Bacterial endotoxin produced by gram negative bacteria is one of the stimulus. While LPS is the main component of endotoxin, and is also the cytotoxic factor, which induces inflammation by stimulating granulocytes, endometrial epithelial cells, interstitial cells and vascular endothelial cells secrete inflammatory cytokines (Iontcheva et al., 2004), and generates toxic and side-effects on organism' cells. Activation with LPS leads to significant increases in tumor necrosis factor-α (TNF-α), interleukin-1(IL-1), IL-6, IL-8 and interferon in mono-nuclear phagocytes and macrophages. These cytokines,

at an appropriate amount, activate the immune system and maybe through this pathway, LPS causes abortion (Guo et al., 2009).

Quercetin has many biological activities and can be associated with decreased risk of cardiovascular disease by stabilizing and protecting vascular endothelial cells against oxidative and proinflammatory insults (Reiterer et al., 2004). In our study, pregnant mice were treated with different concentrations of quercetin, then injected with LPS via the tail vein, and sacrificed at 9 days of pregnancy. The result revealed that after exposure to different concentrations of Quercetin, the abortion rate reduced to 60, 40 and 30%, and significant differences were observed in both Groups D and C when compared with LPS treated group ($p<0.05$). The expression of TGF-β1 mRNA in the mouse' uterus was investigated through *in situ* hybridization. The results demonstrated that the TGF-β1 mRNA expression level of three Que groups were higher than the LPS group. With the increase of the concentration of quercetin, the expression of TGF-β1 mRNA increased gradually. All the above results illustrates that quercetin has an anti-abortive effect which is dose-dependent, and consistent with previous studies (Wang et al., 2011).

TGF-β1 secreted by the decidual cells around the site of implantation inhibits the proliferation of trophoblast cells, and facilitates its transformation into non-invasive syncytiotrophoblasts. Moreover, TGF-β1 inhibits the activity of matrix metalloproteinase and organization of plasminogen to prevent excessive infiltration of the trophoblast and therefore provide a suitable environment for embryonic growth and development (Zhang et al., 2001). On the other hand, TGF-β1 has a strong immunosuppressive effect on the proliferation of T and B lymphocytes, and even inhibits the production of immunologic factors B lymphocytes so that the uterus is kept in a state of immune tolerance, and the embryos will escape from maternal immune rejection (Prud'homme and Piccirillo, 2000). In our studies, the TGF-β1 mRNA expression was greatly decreased in the LPS group. The reason may be that LPS induced quantitative IFN-γproduction was toxic to the embryos. The secretion of TGF-β1 by decidual cells was lowered accompanying with infiltrating of the trophoblasts, proliferating of the T and B lymphocytes. Therefore, Th1 cells increased and produced more Th1 cytokines, which damaged the embryo. However, oral administration of anti-abortive drugs in advance reduced the decidual injury caused by LPS at different levels and reversed the decrease of TGF-β1 mRNA expression in various degrees. TGF-β1 mRNA expression of the high-dose Que group increased and recovered to normal levels basically ($p>0.05$). Therefore, we can come to the conclusion that quercetin affects the local balance of the uterus immune network, improves the expression level of TGF-β1 mRNA in the uterus, inhibits the infiltration of syncytiotrophoblast cell, reduces the content of Th1 cytokine and plays a role in fetus protection.

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