Effect of Shoutai Pills on Th1/Th2 Cytokines in Serum and Endometrium of Rats with Stimulated Ovulation*

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Summary: In our previous study, we found that Shoutai pills could improve the embryo implantation rate as well as the levels of estrogen, progesterone and estrogen receptor in rats with stimulated ovulation. However, the mechanism is not clear. This study was designed to investigate the effect of Shoutai pills on the levels of Th1 and Th2 cytokines in rats with stimulated ovulation and the mechanism. The rat model of stimulated ovulation was established by combined injection of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG). Then the rats were randomly divided into model group (M), Shoutai pills group (S), progesterone group (P) and normal group (N). All the pregnant rats were treated from the first day. The S and P groups were administrated with gavage of Shoutai pills and injection of progesterone respectively, and N and M groups were given the same volume of normal saline and distilled water respectively. After treatment for 7 days, the animals were executed for serum and uterine tissues. The ELISA method was adopted to detect the contents of Th1 cytokines [interferon-γ (INF-γ), interleukin-2 (IL-2)] and Th2 cytokines (IL-4, IL-6, IL-10). The expression of leukemia inhibitory factor (LIF) and leukemia inhibitory factor receptor (LIFR) was detected by Western blotting and real-time PCR. As compared with N group, the expression levels of IFN-γ and IL-2 in M group were significantly increased, and those of IL-4, IL-6, IL-10, LIF and LIFR were significantly decreased (P<0.05). As compared with M group, the levels of IL-4, IL-6, IL-10, LIF and LIFR in S group were significantly increased (P<0.05), and those of IFN-γ and IL-2 were significantly decreased (P<0.05). It was suggested that Shoutai pills can increase the levels of IL-4, IL-6, IL-10, LIF and LIFR as well as reduce the levels of INF-γ and IL-2 in rats with stimulated ovulation. The Shoutai pills may improve endometrial receptivity and promote embryo implantation by maintaining the balance of Th1/Th2 cytokines.
Key words: Shoutai pills; Th1/Th2; cytokines; rats

Many families have benefited from the rapid development of assisted reproductive technology (ART) in recent years. Kupka *et al* showed that the number of reported ART cycles has been increased by 2.4% since 2009, reaching a total of 550 296, based on the 14th European IVF-monitoring report in Europe during 2010[1]. Yang *et al* reported that the proportion of births from ART in mainland China was about 1.013% in 2011[2]. However, the pregnancy rate of ART is still 30% to 40%[3].

A successful implantation depends on a coordinated series of events that would allow establishment of a timely dialogue between a receptive endometrium and an intrusive blastocyst. The members of the molecular repertoire that make endometrium receptive to implantation are gradually being recognized. Among these are cytokines, which may be an appropriate signal for the closure of the "implantation window"[4]. Several studies indicated that in normal pregnancy, there is a shift in the cytokine pattern from Th1 towards Th2, suggesting that successful pregnancy is a Th2 phenomenon. Th1 cytokine family members, such as interleukin (IL)-2, interferon (INF)-γ and tumor necrosis factor (TNF)-α, etc., which participate in cell-mediated cytotoxic immune responses, play a main role in immune killing. On the other hand, Th2 cytokine family members such as IL-4, IL-5, IL-6 and IL-10, etc. take effect on immune protection and

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nutrition during pregnancy[5, 6]. Leukemia inhibitory factor (LIF) is a IL-6 type cytokine family, and binds to the same receptor[7]. Recent studies have shown that LIF plays an important role in embryo implantation and the process of adhesion, and put forward determination of the relative endometrial CLDN4/LIF expression in a spontaneous menstrual cycle preceding an IVF treatment can be used as a biomarker of endometrial receptivity[8].

Shoutai pills, which came from Zhang Xichun's Records of Chinese Medicine with Reference to Western Medicine in Qing dynasty, consist of dodder, mistletoe, teasel and gelatin, being the primary formula for miscarriage. It is widely used in the treatment of miscarriage in clinic. Huang and Li *et al* reported that Shoutai pills can improve the pregnancy rate of ART, but the mechanism is still unknown[9, 10]. In our previous study, we have investigated the effects of different dosages of Shoutai pills on the levels of estrogen and progesterone, as well as embryo implantation in rats with stimulated ovulation. The results showed that Shoutai pills could improve the levels of estrogen and progesterone, estrogen receptor, progesterone receptor, together with the number of blastocyst implantation points[11].

The purpose of this study was to explore the relationship between Shoutai pills and the levels of Th1/Th2 cytokines, and discuss its possible mechanism.

1 METERIALS AND METHODS

1.1 Experimental Animals

Experiments were performed on virgin female Wistar rats aged 10 weeks and weighing 200 to 300 g, and the male rats weighing 250 to 300 g with SPF grade. The animals were provided by the Center for Disease Control (CDC) of Hubei Province, China (the animal certificate SCXK No. 2008-0005) and fed in the barrier system according to the institutional guidelines established by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. They were housed in polypropylene cages and maintained under standard laboratory conditions with a 12-h light-dark cycle and free access to standard rat pellet diet and drinking water. This study was approved by the Animal Ethical Committee of the Central Theater General Hospital of Chinese People's Liberation Army (Approval ID: 2014021).

1.2 Chemicals and Reagents

Pregnant mare serum gonadotropin (PMSG) was purchased from Hangzhou Animal Medicine Factory, China (No. 110204564). Human chorionic gonadotropin (HCG) was purchased from Livzon Pharmaceutical Group, China (No. H440206673). Progesterone injection was purchased from Xianju Pharma, China (No. H33020828). Trizol was purchased from Invitrogen, USA. Rat IL-2, IL-4, IL-6, IL-10 and IFN-γ ELISA kits were purchased from Neobioscience, China. Shoutai pills consisting of dodder, mistletoe, teasel and gelatin with a proportion of 2:1:1:1 were purchased from Hubei Provincial Hospital of Traditional Chinese Medicine, China. Traditional decoction method was used to concentrate the crude drug content to l.2 g/mL. The decoction was stored at 4°C.

1.3 Treatment and Grouping

All the female rats were acclimatized to laboratory conditions for 7 days before starting the experiment. PMSG was combined with HCG to establish the stimulated ovulation model during the late estrus: 25 U PMSG was injected into the female rats' abdominal cavity, and 48 h later, 200 U HCG was injected at the same way, then these female rats were mated with male rats at 6:00 pm with the scale of 2:1[14]. The females were examined at 8:00 am next day for the presence of sperm via vaginal smear. The detection day of the sperm or pessary was designated as day 1 of pregnancy. Then pregnant rats were randomly divided into model group (M) (n=6), Shoutai pills group (S) (n=6) and progesterone group (P) (n=6). The rats in normal group (N) (n=6) had natural pregnancy given no interference. All the pregnant rats were treated at the first day. The S group was given intragastric Shoutai pills (1 mL/100 g), the P group was injected with progesterone (4 mg/kg), and the N and M groups were given the same volume of normal saline and distilled water respectively. All the groups were treated for 7 days. At the 8th day, the animals were executed for serum and uterine tissues. Blastocyst implantation in endometrium was added to Trizol solution, then specimens were preserved in a –80°C refrigerator.

1.4 ELISA Quantification

The serum levels of IL-2, IL-4, IL-6, IL-10 and IFN-γ in rats were determined by ELISA using quantikine ELISA kits according to the manufacturer's instructions.

1.5 Real-time PCR for LIF and LIFR mRNA Expression

The total RNA of blastocyst implantation endometrial tissues was obtained by Trizol, and subjected to reverse transcription process according to the reverse transcription kit manual instructions. The cycling parameters were 95°C for 10 s, annealing at 60°C for 20 s, and at 72°C for 20 s for a total of 40 cycles. The primers for LIF amplification were 5′-AGCCTCCCTGACCAACATCACCT-3′ (forward) and 5′-GCAGCCCAACTTCTTCCTTT-3′ (reverse). The primers for LIFR amplification were 5′-CCGCC-CTCTTATCCATCTTTATG-3′ (forward) and 5′-CCC-ACCAGTC CCGTTATCCTTCC-3′ (reverse). And the primers for β-actin amplification were 5′-CGTTGA-CATCCGTAAAGACCTC-3′ (forward) and 5′-TAGG-

AGCCAGGGCAG TAATCT-3′ (reverse). Relative quantitative calculation was done by the $2^{-\Delta\Delta CT}$ method.

1.6 LIF and LIFR Protein Detected by Western Blotting

Blastocyst implantation endometrial tissues (50 mg) were cut into pieces, put in the homogenate machine, added with the extracted total protein, then homogenated for 5–20 min, subsequently run in 12% SDS-PAGE and transferred into nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat and incubated with LIF/LIFR rabbit anti-human (1:300). The relative intensities were quantified by Quantity One 4.62 vision image Analysis Software.

1.7 Statistical Analysis

Statistical analysis was performed by using Statistic Package for Social Science version17.0 software. Data were presented as median and range or mean standard deviation (SD). Difference of measurement data was compared by single factor analysis of variance with the Least significant-difference test. A probability of less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 Serum Levels of IFN-γ, IL-2, IL-4, IL-6 and IL-10 Detected by ELISA

As compared with N group, the expression of IFN-γ and IL-2 in M group was increased significantly, and that of IL-4, IL-6 and IL-10 decreased significantly. As compared with M group, the levels of IFN-γ and IL-2 in S group and P group decreased significantly, and those of IL-2, IL-4, IL-6 and IL-10 increased significantly. However, there was no significant difference between S group and P group (fig. 1).

2.2 Expression of LIF and LIFR mRNA in Endometrium of Pregnant Rats

The expression of LIF and LIFR mRNA in M group was significantly decreased as compared with that in N group. As compared with M group, the expression of LIF and LIFR mRNA in S group and P group was increased significantly. However, there were no statistically significant differences in the expression levels of LIF and LIFR mRNA between S group and P group (fig. 2).

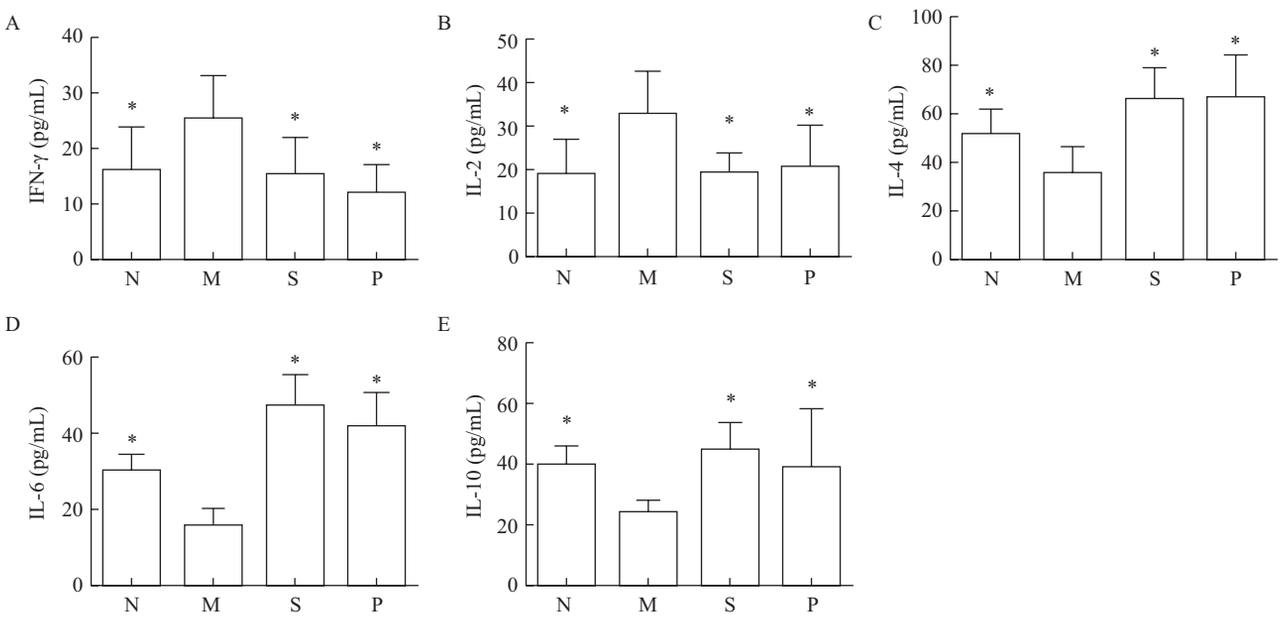

Fig. 1 The serum levels of IFN-γ (A), IL-2 (B), IL-4 (C), IL-6 (D) and IL-10 (E) in each group
Values are expressed as mean±SD. *P<0.05 *vs.* M group

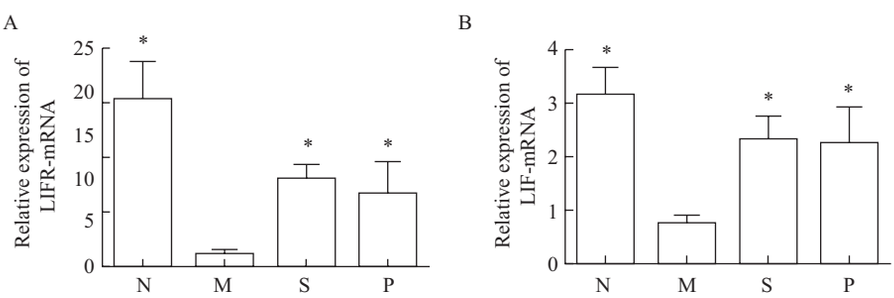

Fig. 2 The expression of LIFR (A) and LIF (B) mRNA in endometrium of pregnant rats
*P<0.05 *vs.* M group

2.3 Expression of LIF and LIFR Proteins in Endometrium of Pregnant Rats

The expression of LIF and LIFR proteins in M group was decreased significantly as compared with that in N group. The expression of LIF and LIFR proteins in S group and P group was increased significantly as compared with M group. Interestingly, LIFR protein levels were significantly higher in S group than in P group (P<0.05), but no significant difference was found in LIF protein expression between S group and P group (fig. 3).

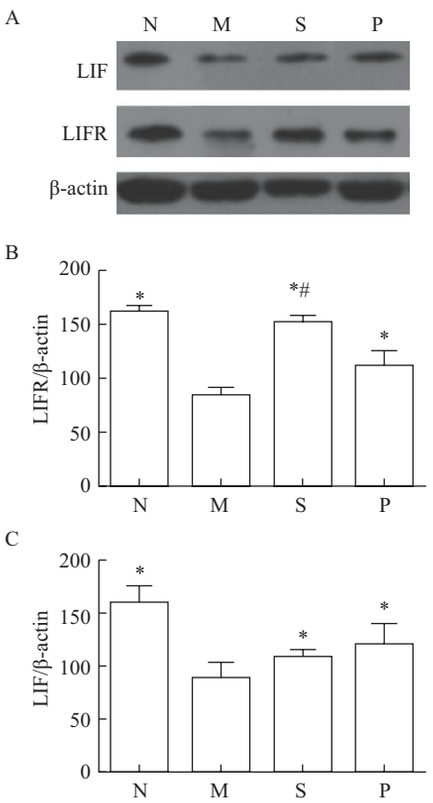

Fig. 3 The expression of LIF and LIFR proteins in endometrium of pregnant rats
A: the expression of LIF and LIFR in all groups; B and C: Gray-scale value of straps visually revealed the expression of LIFR (B) and LIF (C) in endometrium.
$^{*}P$<0.05 *vs.* M group, $^{\#}P$<0.05 *vs.* P group

3 DISCUSSION

In mammalian reproduction, the embryo and placenta are all recognized as non-self by the maternal immune system, and are vulnerable to immunological attack. An active system to prevent rejection must exist when conceptus and maternal tissues first come into contact at implantation. These factors include: (1) appropriate cytokine balance; (2) correct phenotype of endometrial leukocytes to enable Treg cell activation; (3) sufficient estrogen and progesterone to stabilize and strengthen Treg cell phenotype, and (4) appropriate priming of Treg cell populations by male partner seminal fluid. Among them, the paramount factor in determining the nature and quality of immune balance is the cytokines environment[12].

In humans, Th1 cells mainly synthesize IFN-γ, TNF-α and IL-2, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13[13]. In normal pregnancy, there is a shift in the cytokine pattern from Th1 towards Th2, called Th2 phenomenon, which is important for pregnancy. IFN-γ, which is derived from uterine NK cells, has been reported to involve in maternal arterial walls remodeling and inhibition of excessive trophoblast growth and invasion. Our research demonstrated the IFN-γ levels increased in M group, indicating the beneficial effect of IFN-γ in early stage of embryo hatching. Previous study had shown that IFN-γ at implantation sites was harmful to or incompatible with gestational success[14]. These findings support our hypothesis that the IFN-γ plays different roles in different stage of pregnancy. IL-2, an important immunoregulatory molecule, is the principal growth factor that enhances T lymphocyte proliferation after antigenic stimulation. IL-2 is known to have an beneficial antifertility effect, leading to pregnancy loss and higher expression in placenta[15]. Our findings that IL-2 levels increased in M group but decreased after progesterone stimulation also suggest IL-2 is beneficial to embryo implantation in early stage but harmful in late stage.

IL-4 plays a major role in embryo implantation. IL-4 and progesterone up-regulate LIF and IL-12[16]. Valeria has investigated that IL-6 is postulated as a regulatory factor as it promotes Th2 differentiation and inhibition of Th1 polarization[17]. IL-10 is known to selectively suppress Th1-mediated cellular immunity by inhibiting the production of inflammatory cytokines, such as IFN-γ, IL-1 and TNF-α[18]. And it is also linked with the regulation of dendritic cells and Treg cells to protect the embryo from immune rejection[19]. Our study showed the levels of Th2 cytokines were lower in M group but higher after progesterone treatment, indicating the Th2 cytokines act in the later stage of embryo implantation.

IL-6 can exert both pro-inflammatory and anti-inflammatory response though qp130. LIF is closely related with members of the gp130 signaling family of cytokines, and is a IL-6 type cytokine family[7]. This study showed that female mice lacking a functional LIF gene had problems with implantation even though they were fertile[20]. In clinical study, Serafini *et al* demonstrated that women with stronger LIF immunoreactivity during the window of implantation have greater probability of getting pregnant than those with weaker expression, thereby suggesting importance of LIF in IVF[8]. It's interesting that the LIF and LIFR expression was decreased in M group but increased

in S and P groups. Since the LIF and LIFR are the subfamily members of IL-6, we considered the LIF and LIFR play the similar role in Th2 cytokines. However, this hypothesis needs to be further addressed.

Shoutai, one of classical traditional Chinese medicine prescriptions, has a positive effects in tonifying kidney for preventing miscarriage. It has been widely used in the treatment of gynecological diseases, especially miscarriage. Research found that there were no obvious side effects in the aspects of acute and chronic toxicity, genetic and reproductive security[21–25]. Huang *et al* found that Shoutai pills combined with western medicine can more effectively maintain hormones levels during early pregnancy and promote the increases of hormones levels[26]. These findings combined with our results showing that the effect of Shoutai pills is similar to that of progesterone may support our assumption that the Shoutai pills can promote the pregnancy-related hormone secretion. Our assumption was also demonstrated by previous study that Shoutai pills could dose-dependently enhance the proliferative activity of trophoblastic cells, invasion and migration capacity, secretion of beta-HCG, and reduce the apoptosis of trophoblast cells, which might be one of mechanisms for Shoutai pills preventing and treating spontaneous abortion[10].

In this study, through the treatment of Shoutai pills, the levels of Th1 cytokines decreased significantly, and those of Th2 cytokines, LIF and LIFR increased. It is indicated that Shoutai pills may have the protective effect on embryo implantation by adjusting the balance of Th1/Th2. The balance of Th1/Th2 may play a different role at different stage. Shoutai pills may induce the embryo implantation and maintain the pregnancy by regulating the spatial and temporal expression of maternal-fetal interface Thl/Th2 type cytokines[27].

To sum up, Shoutai pills can balance the Th1 cytokines and Th2 cytokines, forming the Th2 immune bias, and improve the endometrial receptivity to a certain extent. The application of Shoutai pills provides a new method of improving the pregnancy rate in ART.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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