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The effect of leflunomide on the transplanted endometriosis lesions in SD rats

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Summary

Objective: To investigate the effects of the leflunomide (LEF) on the size of the transplanted endometriosis (EMS) lesions and transforming growth factor (TGF) -β1gray level in SD rats. *Materials and Methods:* EMS was surgically induced in rats by autologous transplantation and the focal volume was also measured. The rats were divided into three groups: group A: normal SD rats, group B: rats irrigated by one ml·kg^{-1}·d^{-1} saline for three weeks, and group C: rats irrigated by 35 mg·kg^{-1}·d^{-1} LEF for three weeks. The rats were then sacrificed and measured their focal volume and TGF-β1 gray value with immunohistochemical method. *Results:* The sizes of the focal volume in group C were significantly reduced compared to the rats before feeding, and the volume in group C was smaller than group B after feeding and so was the TGF-β1. *Conclusion:* LEF could be a new therapeutic drug for EMS.

Key words: Ectopic endometriosis model in rats; Endometriosis; Leflunomide; TGF-β1.

Introduction

Endometriosis (EMS) is a common and frequently occurring disease in Gynecology. The main methods to cure EMS are medicine and surgery [1]. Medicine mainly includes sex hormone suppression therapy [2], and these drugs can inhibit the EMS lesion's growth by reducing the estrogen level of patients. Although it achieves certain effects, these drugs cannot cure EMS and the drugs' side effects limit its long-term use. Therefore searching for new non-hormonal drugs becomes the hottest research [3]. EMS is a kind of immune disease [4]. Some researchers reported they had achieved obvious results which the immune preparation used in SD rats EMS model, such as interferon, etanercept levamisole [5-7]. In this study, the authors established the SD rats EMS model, observed the impact of the immune modulators of LEF on lesion morphology, the TGF-β1 of rats' EMS, discussed LEF inhibition mechanism of growth of uterine endometriosis lesions, and provided new ideas for clinical treatment of EMS.

Materials and Methods

Establish the rats' model of EMS

Test animal: The experiment included 60 female, mature, healthy non-pregnant Sprague-Dawley (SD) rats of nine weeks, weighing (200 ± 20 grams). The rats were fed for a week before the experiment with the feeding conditions: 18°C to 25°C, humidity 55% ~ 65%, illumination 14 hours, and darkness alternating ten hours, free feeding, drinking water, while maintaining the pad dry. Forty-five SD rats were selected to establish animal model by autotransplantation.

Test procedure: The authors injected benzoate (200 g.kg^{-1},im) into rats 24 hours before the operation in order to induce the rats' estrus state that can improve the success rate of established model. Then we disinfected skin, fixed the rats on operation bedplate, anesthetized intraperitoneal with 10% chloral hydrate (0.03ml. kg^{-1}), cut length 2~3 cm longitudinal incision on urethral mouth about one cm into the abdominal, exposed the Y type of uterus, ligated a period about one cm right uterine horn from the 0.5cm distal to the proximal direction, placed it into physiological saline, incited uterine cavity longitudinally and rapidly, cut five-mm long uterine fragment, and sewed it onto the uterine lateral abdominal wall with a 4-0 absorbable seam, attempting to make its intimal surface face the abdominal wall and the major abdominal vascular to pass through the uterine mucosa underneath. The remained tissue was used for histological pathology to confirm graft for uterine tissue, and the TGF-β1 gray value of transplanted tissue was tested with immunohistochemical method. Intraperitonea was checked to ensure that there was no obvious bleeding, indwelling penicillin sodium solution was in abdominal cavity, and abdominal was incited with layered suture with no. 0 thread. Animal was delivered in separate cage for natural recovery. General breeding was conducted. To prevent infection the authors injected penicillin needle (80,000 U/d, im, qd × three days). In order to promote intimal growth and to increase the successful rate of model establishment, the authors injected benzoate (50 g.kg^{-1}.d^{-1}, im, qd × five days) from the fifth day after the operation of estradiol.

Identification of animal models

The authors continued with the second operation at the third week after the transplant operation; graft lesions were growing in the rat lateral pelvic wall, and their transplant focal volume was also increasing. Most of the surface of the transparent lesions was light or red saccate, and some of them was with small nodules.

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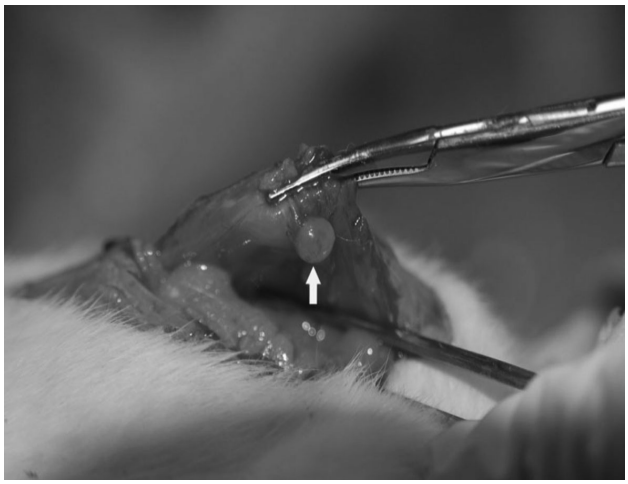

Figure 1. — Ectopic endometrium in Group C after modeling.

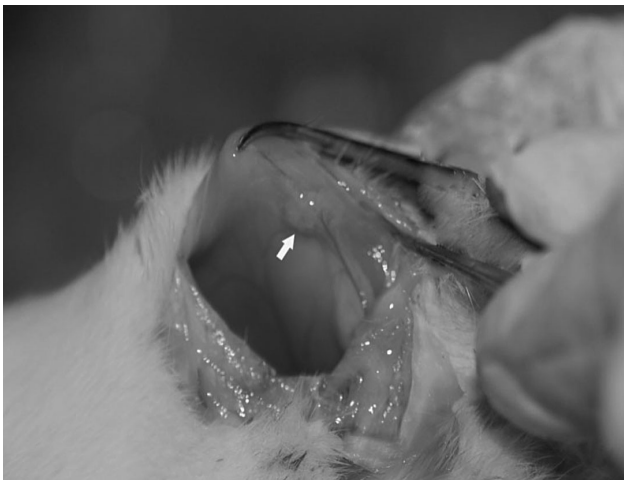

Figure 2. — Ectopic endometrium in Group C after feeding.

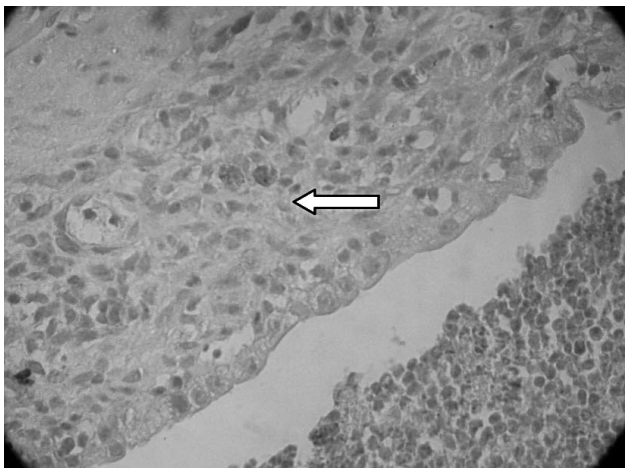

Figure 3. — Expression of TGF-β1 in ectopic in Group C after feeding (SP×400).

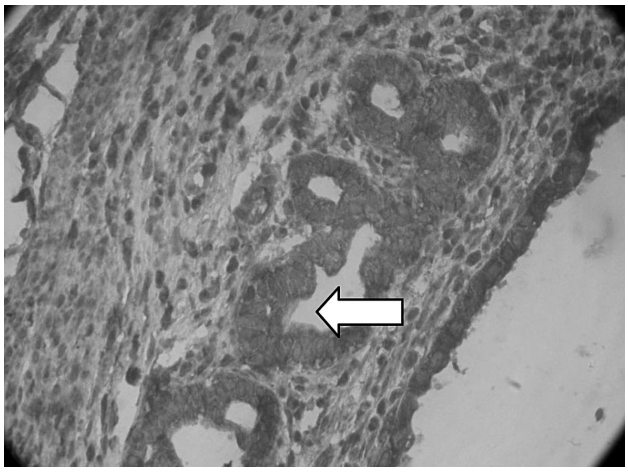

Figure 4. — Expression of TGF-β1 in ectopic in Group B after feeding (SP×400).

They were covered by connective tissue, and there was blood vessels formation. The authors measured lesion's length, width, and height. Some tissue was used to histological pathology to confirm graft for uterine tissue and they measured the TGF-β1 gray value with immunohistochemical method.

The rats were divided into three groups randomly. A) The normal control group (n=15): normally reared under the same conditions of female SD rats without model; B) The model control group (n=16): modeled rats which received gavage with saline, one ml.kg^{-1}.d, continuous medication for three weeks; and C) LEF treatment group (n=16): modeled rats which treated with LEF and 35 mg. kg^{-1}.d^{-1}, continuous medication for three weeks. During the treatment, the authors closely observed their activity, hair color, daily food intake, stool, weight, and the side effect of LEF was evaluated. After three weeks, the rats were sacrificed and the lesion volume and the TGF-β1 gray value wee both measured.

Detection of experimental indexes

1) The lesion's volume was measured according to the formula to calculate the volume: V=0.52× length × width ×height mm^3, calculating the lesions' volume of group B and C before and after treatment with drugs; 2) the TGF-β1 gray value of EMS lesion of group A, B and C was measured with immunohistochemical method before and after modeling and after treatment with drugs; 3) statistical parameters were analyzed by SPSS16.0. Comparison between two groups was done via t-test, and comparison of gray value among multiple groups was done via single factor variance analysis.

Results

There was no significant statistical difference of endometriosis volume between groups B and C (Figure 1) before feeding drugs. After feeding LEF, the size of the focal volume in group C (Figure 2) was significantly reduced and

Table 1. — *The lesion volume' comparison before and after feeding in groups B and C.*

Group	Sample size (n)	Before feeding (m^3)	After feeding (m^3)
B	16	119.11+22.38	90.19+10.03
C	16	121.09+25.59	71.98+11.71
p		0.839	0.001

Table 2. — *TGF-β1 gray value in three groups.*

Group	Sample size (n)	Before modeling	After modeling	After feeding	*p*
A	15	96.45±7.26	95.73±5.81	96.02±3.28	>0.05
B	16	95.75±4.25	179.42±3.26	176.92±5.21	<0.05
C	16	96.02±2.21	181.63±4.90*	103.41±3.31	<0.05
p		>0.05	<0.05	<0.05	

*no significant difference between groups B and C.

also in group B ($p < 0.05$). The size of the focal volume in group C was less than group B after feeding drugs (Table 1).

There was no significant statistical difference of the TGF-β1 gray value among the three groups before feeding drugs. The gray value of TGF-β1 after modeling was higher than before modeling in groups B and C. After feeding LEF, the gray value of TGF-β1 in group C (Figure 3) was lower, and it was also lower than group B (Figure 4) after feeding. They had significant statistically differences ($p < 0.05$) (Table 2).

Discussion

The SD rats autotransplant model was established in 1984 by Jone [8]. This study established EMS rats model by autotransplantation, and the authors injected high doses of benzoate into rats 24 hours prior to the operation in order to induce the rats in estrus state. It also excluded the impact of estrus on operation, decreased the error, and improved the success rate of establish model [9, 10]. To benefit from the survival of transplantation, promote glandular growth of ectopic endometrium epithelial cell, and ensure a rich blood supply of endometrium, the authors injected low doses of benzoate five days after the operation. The success rate of experimental modeling was 80%. The method can establish an ideal animal model, while providing useful experimental basis for further study on EMS.

This study showed no significant statistical difference of endometriosis volume between LEF treatment group and model control group; the size of the focal volume was significantly reduced compared with that of the control group, before treatment and the model group. A report has shown that metabolites of LEF A771726 can block cell cycle progression [11], prevent the peripheral blood mononuclear cells from G1 phase of the cell cycle entering S phase, and cause lymphocyte dormancy in G1/S phase at the junction, and LEF A771726 significantly downregulating the TGF-β1 [12].

Meanwhile, LEF can interfere with the de novo pyrimidine biosynthesis pathway [13], inhibit the activity of protein tyrosine kinase, inhibit reversibly the proliferation of cells, especially of lymphocytes, and reduce the production of auto antibodies and cytokines [14, 15]. LEF also induces a dose- and time-dependent differentiation of these cells as characterized by growth inhibition, hemoglobin production, and erythroid membrane protein glycophorin A expression. This effect was dependent on depletion of the intracellular pyrimidine ribonucleotides (UTP and CTP), and preceded by a specific S-phase arrest of the cell cycle [16].

As EMS is an autoimmune disease, it suggests that LEF may inhibit the development of EMS through this way. Many experiments show that LEF can directly inhabit the produce of the antibody [13]. A771726 may exert its anti-inflammatory and immune effects by inhibiting the activity of macrophages in abdominal cavity. It can balance the mutual effect of the T lymphocyte and mononuclear phagocyte by changing the excessive secretion function of the macrophages [17]. For rheumatoid arthritis, LEF can obviously reduce TGF-β1 in macrophages, but the effect of LEF on TGF-β1 is opposite when it joined 17-betaestradiol [12]. It can be concluded that therapy effect of EMS with LEF inference may change at different menstrual period, but it has not been reported that the effects of different levels of estrogen may have a different therapeutic effect on LEF in EMS.

This study showed that there was no significant statistical difference of the TGF-β1 gray value among the three groups before feeding drugs. Studies have found that LEF may inhibit the growth of EMS and that it may protect kidney of rats with diabetes through the TGF-β1 [16]. The expression of TGF-β1 was reduced by treatment with LEF in diabetic rat kidney [18].

There are reports that showed that the main chemokine MCP-1 of mononuclear macrophages induced the monocytes/ macrophages infiltration, leading to the occurrence fibrosis of kidney by activating TGF- β1 of NF- K B [19]. It also found that the grave value of MCP-1, NF- Ka B, and TGF- β1 after LEF treatment were lower than before treatment, suggesting that LEF may also inhibit the development of EMS by the same way [20]; however its application in the field of EMS is still in the exploratory stage.

In summary, LEF plays a certain inhibitory effect on the growth of EMS rats, and inhibits the expression of its related cytokines of TGF-β1. It suggests that LEF may have a certain therapeutic effect in the treatment of EMS. It is expected to become a new way of treatment of EMS.

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