Rosiglitazone affects the progression of surgically-induced endometriosis in a rat model

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Abstract. Endometriosis is closely associated with inflammatory reactions and angiogenesis. Whether PPARγ is a target for the treatment of endometriosis remains unknown. The present study was designed to investigate the impact of a PPARγ agonist (rosiglitazone, RSG) on endometriosis in a rat model and to identify the underlying mechanism. The endometriosis model was established in rats. The pathological state of the endometrium was examined using hematoxylin-eosin staining. The microstructures of interest were visualized using electron microscopy. Western blot analysis and reverse transcription-quantitative polymerase chain reaction were used to detect PPARγ and MAT2A expression. VEGF and caspase-3 expression were investigated using immunohistochemistry. Pathological analysis revealed transparent and red nodules in the model group, and that vasoganglions were present all over the nodules. Endometrial epithelial hyperplasia was observed in the model group, and the shape was columnar. Increased interstitial cell numbers, with compact structure and abundant blood supply, were detected in the model group. Compared with the model group, incomplete epithelial structures with sparse interstitial cells and loose structure were observed in the pathological images from RSG treatment groups. Numerous inflammatory cells and poor blood supply were observed in the endometrial tissues, and the gland was filled mostly with vacuolar cells. Electron microscopy revealed that the tissue structure was integrated. Many vacuoles were formed within the endometrial tissue and the classical morphological changes of apoptotic cells were observed in RSG-treated groups. Caspase-3 and PPARγ expression increased and expression of VEGF and MAT2A decreased in RSG-treated groups. Taken together, these results revealed that RSG impacts the development and progression of endometriosis likely by inhibiting angiogenesis and inducing apoptosis.

Introduction

Endometriosis is a progressive disease that critically impacts the physical and psychological health of females. Endometriosis may also lead to, or be a cause of, infertility. The symptoms of endometriosis can only be temporarily improved, and endometriosis has a high reoccurrence rate (1). Therefore, it is of great clinical significance and social value to develop drugs with novel mechanisms and improved efficacy for endometriosis treatment. Immunological tolerance is involved in the paroxysm of endometriosis, and would be induced because the ectopic endometrium is not eliminated by the immune system (2). Enhanced macrophage activity and attenuated cellular immune function are commonly observed in endometriosis, particularly including the dysfunction of the cytotoxic reaction mediated by T cells and natural killer cells. The factors released by macrophages, including prostaglandins, cytokines and growth factors, may induce ectopic endometrium growth. These results indicated that ectopic endometrial growth may be inhibited by decreasing macrophage activity (3).

PPARγ is involved in multiple physiological and pathological processes, including lipid metabolism, glucose metabolism, cell proliferation and differentiation, tumorigenesis, inflammation, and immune responses. Recently, the effects of PPARγ on gynecological disease have gained increasing amounts of attention. PPARγ is expressed in the endometrium (4), and activating PPARγ may lead to inflammatory inhibition and anti-angiogenesis effects (5). As endometriosis is associated with inflammation and angiogenesis, PPARγ may become a novel target for endometriosis treatment (6). Liu et al (7) reported that macrophage activity and proliferation was inhibited by PPARγ endogenous ligands in a dose-dependent manner (7). Additionally, the inhibitory effect of PPARγ activation on ectopic endometrial growth is associated with the inhibition of the activity and proliferation of macrophages within the endometriosis lesion (4). Rosiglitazone (RSG), a PPARγ agonist, not only inhibits the progress of endometriosis (8), but also reduces the symptoms (9).
The present study examine the effects of RSG on the development and progression of endometriosis in a rat model. The results presented here will allow for deeper understanding of the mechanism of endometriosis, its treatment, and the identification of improved clinical diagnostic markers.

Materials and methods

Ethic statements. All animal experiments were authorized by the Ethical Committee of The First Affiliated Hospital of Nanchang University and performed following the guidelines for the care and use of laboratory animals and the principles of laboratory animal care and protection.

Preparation of an endometriosis model in rats. Female rats (n=30; weight ~220 g; 3 months) were purchased from Hunan SJA Laboratory Animal Co., Ltd and housed in a specific pathogen-free environment that was automatically maintained at a temperature of 23±2°C, a relative humidity of 45-65%, and with a controlled 12 h light/dark cycle. The animals had free access to food and water. The endometriosis model was prepared as previously described (10). The estrous cycles of rats were confirmed by vaginal smears. The rats in estrus were anesthetized by isoflurane (5%) and fixed on the operating table.

Experimental groups. Following the induction of endometriosis, the rats were allowed to recover for 4 weeks, during which time they were not administered with any medication. Subsequently, 30 rats were divided into the following five experimental groups: Model, saline (equal volume of normal saline for each rat); 5 µM RSG; 10 µM RSG; and 20 µM RSG. The abdomen of each rat was cut open to locate the uterus. Two sides of the uterus were ligated, along with the vessels, by twine and ~1 cm was left in the middle. This 1-cm section of tissue was cut using scissors and placed in a sterile culture dish containing normal saline. The endometrium was removed using sterile microscopic tweezers and cut into fragments measuring 5x5 mm. These fragments were attached to the abdominal wall. The incision was stitched and smeared with iodophor. Rats were placed onto heating pads to regain consciousness quickly.

Immunochemistry. The endometrium was separated and fixed in 4% paraformaldehyde for ~1 week at 4°C. The tissues were placed into a plate filled with pre-cooled normal saline. The tissue was embedded in paraffin, sectioned into 20 µm. The sections were cut into ultrathin sections (100 nm) and the slides were stained with lead citrate and uranyl acetate at room temperature for 5 min. Ultrastructural endometriosis changes were observed and images were captured using transmission electron microscopy (magnification: x1,000; JEM-1230; JEOL, Ltd.).

Transmission electron microscopy. The endometrium was collected after the animals were executed and was fixed with 2.5% glutaraldehyde for over 2 h at room temperature and washed with 0.1 M phosphoric acid solution three times. Next, the tissues were fixed with 1% osmic acid for 2-3 h at room temperature and washed with 0.1 M phosphoric acid three times. The tissues were then washed with 50% ethyl alcohol, 70% ethyl alcohol, 90% ethyl alcohol, 90% ethyl alcohol and 90% acetone (v:v:1:1), and 90% acetone successively for 15-20 min. Next, the tissues were incubated with 100% acetone at room temperature three times for 15-20 min each. Acetone (100%) and the embedding solution were incubated with the tissues for 3-4 h at room temperature. Finally, the tissues were successively embedded in 0.01% epoxy resin at 37°C overnight, 45°C for 12 h, and 60°C for 48 h. The solid tissue was cut into ultrathin sections (100 nm) and the slides were stained with lead citrate and uranyl acetate at room temperature for 5 min. Ultrastructural endometriosis changes were observed and images were captured using transmission electron microscopy (magnification: x1,000; JEM-1230; JEOL, Ltd.).

Hematoxylin & eosin (H&E) staining. Endometrium was collected from each animal and fixed in 4% paraformaldehyde for ~1 week at 4°C. The endometrium was dehydrated using 70, 80 and 90% ethanol solutions successively and mixed with equal quantities of ethanol and xylene. After 15 min incubation, the tissue was mixed with an equal quantity of xylene for 15 min. These steps were repeated until the tissue appeared transparent. The tissue was then embedded in paraffin at room temperature for 5 min and sectioned at a thickness of 10 µm, and stained with H&E for 3 min at room temperature. Images were acquired using an inverted microscope (magnification, x200; Olympus Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from the collected endometrium cells was obtained using an RNA Extraction kit (Takara Biotechnology Co., Ltd.) and according to the manufacturer's protocols. Extracted RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies). cDNA was synthesized at 30°C for 10 min according to the instructions of the reverse transcriptase kit (CoWin Biosciences). SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) and an Applied Bio-Rad CFX96 Sequence Detection system (Applied Biosystems) was used for real-time PCR. The cycling protocol was as follows: Pre-denaturation at 95°C for 10 min, 40 cycles of 95°C for 12 sec, 58°C for 30 sec and 72°C for 30 sec. PPARγ and MAT2A expression levels were determined by the threshold cycle (Ct), and relative expression levels were calculated using the 2^-ΔΔCT method after normalization with reference to the
Table I. Primer sequences.

| Genes   | Sequences (5'-3')                  | Length of the primer (bp) | Length of the product (bp) | Annealing temperature (°C) |
|---------|-----------------------------------|---------------------------|---------------------------|---------------------------|
| PPARγ F | CCCAGGTTTGGCGAATGTG               | 18                        | 197                       | 57.8                      |
| PPARγ R | TGTCTGTCTCCGTCTCTCTTGAT           | 20                        |                           |                           |
| MAT2A F | TTGGGCCTGGAAATACTT                | 19                        | 102                       | 57                        |
| MAT2A R | CCCCAACCGCCATAAGT                 | 17                        |                           |                           |
| GAPDH F | CAATGACCCCTTCATTTGACC             | 20                        | 106                       | 57.2                      |
| GAPDH R | GAGAAGCTTCCGTCTTCAG               | 20                        |                           |                           |

expression of U6 small nuclear RNA (11). GAPDH expression levels were used as negative controls. Primer information is listed in Table I.

Western blot analysis. The Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology) was used to isolate proteins from the tissues. After determining the concentration using the BCA method, approximately 35 µg protein per lane was separated on 12% SDS-polyacrylamide gels. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (EMD Millipore). The membrane was blocked with 5% skimmed dry milk in TBST (Tris buffered saline/0.1% Tween-20, pH 7.4) for 1 h at room temperature and incubated overnight with primary rabbit anti-human antibodies against PPARγ (cat. no. AP6284; Afinity Biosciences; dilution 1:1,500) and MAT2A (cat. no. ab189208, Abcam; dilution 1:1,000). A horseradish peroxidase-conjugated antibody against rabbit IgG (cat. no. ZB-2305, OriGene Technologies, Inc.; dilution 1:5,000) was used as a secondary antibody. Blots were incubated with ECL reagents (Beyotime Institute of Biotechnology) and exposed using a Tanon 5200-multi to detect protein expression.

Statistical analysis. The data are expressed as the mean and standard deviation with six repeats. Statistically significant differences for continuous variables were determined using a one-way analysis of variance with the least significant difference test for normally distributed data. All testing was performed using GraphPad Prism 5 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of RSG on the pathological states of the endometrium. The endometriosis rat model in each group is shown in Fig. 1. Compared with the model group, the tumor weight in the RGS group was significantly decreased (vs. model; P<0.05). This decrease was more significant at 10 and 20 µM doses of RSG.

To investigate the impact of RSG on the pathological changes of endometria extracted from experimental rats, H&E staining was performed. Three RSG dosages were used: 5, 10 and 20 µM. Transparent and red nodules were observed in the model group, and vasoganglione were present all over the nodules (Fig. 2). Columnar shaped endometrial epithelial hyperplasia was observed in the model and saline groups. Increased numbers of interstitial cells, with compact structure and abundant blood supply, were detected in model and NS groups. Compared with the model group, incomplete epithelial structure with sparse interstitial cells and loose structure was observed in the pathological images from RSG-treated groups. Inflammatory cells were involved in the tissues, and mainly included neutrophil granulocytes. The cells were filled with edema, with poor blood supply. The gland was filled with more vacuolar cells. These pathological results indicated that the endometrium was notably damaged by different RSG dosages.

Effects of RSG on the physiological structure of the endometrium. To investigate the effects of RSG on the growth of the endometrium, the subcellular structure in the tissues was observed using transmission electron microscopy. The results demonstrated that the tissue structure was integrated in the model and saline-treated groups (Fig. 3). Numerous vacuoles were formed within the endometrial tissue and classical morphological changes in apoptotic cells were observed in the RSG-treated groups.

Effects of RSG on VEGF and caspase-3 expression levels in the endometrium. To further investigate the potential mechanism of RSG on endometriosis, VEGF and cleaved caspase-3 expression levels were assessed by immunohistochemistry. As shown in Fig. 4, compared with the model group, expression of VEGF in endometria from rats treated with different RSG dosages decreased significantly (vs. model; P<0.05) and caspase-3 expression increased (vs. model; P<0.05; Fig. 4).

Effects of RSG on PPARγ and MAT2A expression levels in the endometrium. To investigate the effects of RSG on the endometriosis at the molecular level, the expression of MAT2A, a major clinical biomarker for endometriosis, was assessed using RT-qPCR and western blotting. Compared with the model group, the PPARγ expression in the endometria of rats treated with RSG increased significantly (vs. model, P<0.05; Fig. 5). These results indicated that RSG, which is a PPARγ agonist, may upregulate PPARγ expression in this tissue. By contrast, MAT2A expression was decreased in the endometrium of rats treated with RSG, compared with that observed in the model group (P<0.05).

Discussion

Numerous factors, including immunoreactions, inflammation and angiogenesis, contribute toward the development and
Figure 1. Endometriosis rat models in each treatment group. (A) Endometriosis-related morphological changes. (B) Representative images of tumors; scale bar, 8 mm. (C) Quantitative data of the tumor weight. *P<0.05, vs. model group.

Figure 2. Hematoxylin and eosin staining of ectopic endometrial tissue from rats in each group. Scale bar=100 μm; magnification, x200 (n=6).
Figure 3. Transmission electron microscopy images of ectopic endometrial tissue from rats in each group. Arrows indicate pathological changes.

Figure 4. Effects of RSG on VEGF and caspase-3 expression in the endometrium. (A) VEGF expression was detected by immunohistochemistry in ectopic endometrium tissue from rats in each group. (B) Caspase-3 expression was detected by immunohistochemistry in ectopic endometrial tissue from rats in each group. (C) Quantitative VEGF and caspase-3 expression. *P<0.05, vs. model group. RSG, rosiglitazone.
expression levels in ectopic model was successfully established in SD rats. The results of the present study indicated that the endometriosis variations in H&E staining results in the endometrium tissues, protuberances, were observed in the abdomen. Along with their abdominal wall. Clear endometriosis lesions, including was established in rats by transplanting the endometrium to cycle and high level of homology with humans. Endometriosis model. SD rats were selected because of their fast reproductive changes in the endometrium of the endometriosis animal model revealed that application of the RSG endometriosis model was successfully established in rats. The results indicated that the size of the endometrium was diminished as the RSG concentration increased. The observation of pathological states and sub-cellular structure revealed numerous vacuoles and the classic morphological changes of apoptotic cells in the endometrium following RSG treatment. The degeneration of endometrium may be induced by PPARγ activation (20). Nenicu et al reported that the therapeutic effects of Telmisartan on endometriosis in mice were associated with angiotensin II receptor inhibition and PPARγ signal pathway activation (19). In a mouse model of endometriosis, the volume of the endometriosis injury site in mice treated with RSG was much smaller than that in model mice (21). Lebovic et al reported that the growth of ectopic endometrial tissue was significantly inhibited in baboons by oral administration of RSG (22). These reports indicated that the growth of ectopic endometrium may be suppressed by PPARγ activation. These results demonstrated that the PPARγ signal pathway was activated by treatment with RSG, which inhibited the expression of MAT2A.

Immune cells, adhesion molecules, extracellular matrix metalloproteinase and pro-inflammatory cytokines activate/alter the peritoneal microenvironment, creating conditions for differentiation, adhesion, proliferation and survival of ectopic endometrial cells (23-25). Meanwhile, angiogenesis is an important factor that induces the paroxysm of endometriosis. The establishment and maintenance of the blood supply between ectopic endometrium and surrounding are a precondition for the establishment of endometriosis. The factors involved in angiogenesis within ectopic endometrium include epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), angiogenin-II and transformed growth factor β (TGFβ). Among these factors, VEGF is of great importance and may induce angiogenesis directly. VEGF is highly expressed in ectopic endometrial and mesenchymal cells (26) and VEGF expression in ectopic endometrial cells is significantly decreased by RSG treatment (27). RSG treatment also decreases the vessel density within the ectopic endometrium (21). In the present study, VEGF expression in ectopic endometrial tissues markedly declined following RSG treatment. Taken together, these results implied that the inhibition of ectopic endometrial growth by PPARγ activation may be associated with the decreased VEGF expression.

Caspase-3 is a key apoptotic protease in mammals, and serves a central role in the apoptotic cascade reaction pathway (28). Caspase-3 is activated when cells encounter apoptotic stimulants, which then activate other proteins in the apoptotic pathway to induce apoptosis (29). The therapeutic effects of cyclooxygenase-2 on endometriosis are associated with apoptosis mediated by caspase-3 activation (30). The ectopic endometrial cells refluxed to the pelvic cavity were still active because of the inhibition of ectopic endometrial cell apoptosis, which was beneficial to the plantation and growth.
of ectopic endometrium and induced endometriosis (31). In the present study, caspase-3 expression levels significantly increased following RSG treatment. These results indicated that the therapeutic effects of RSG on endometriosis may be associated with apoptosis in ectopic endometrial cells mediated by the activation of caspase-3. Nevertheless, the exact signaling pathways involved in RSG-induced apoptosis and more persuasive evidence to support the fact that angiogenesis should be tested in future.

In conclusion, RSG impacts the development and progression of endometriosis, likely by inhibiting angiogenesis and inducing apoptosis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
SZ, LZ, QL, XY, QM and MC performed the experiments and analyzed the data. SZ and QC designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments were authorized by the Ethical Committee of The First Affiliated Hospital of Nanchang University and performed following the guidelines for the care and use of laboratory animals and the principles of laboratory animal care and protection.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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