**Original Article**

# The Intervention Effect of Rosiglitozone in Ovarian Fibrosis of PCOS Rats*

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**Abstract**

**Objective** To explore the Intervention effect of Rosiglitozone in ovarian fibrosis of PCOS rats.

**Methods** 60 female SD rats were randomly divided into 3 groups: control group, model group and treatment group. The model and treatment groups were established by subcutaneous injection of DHEA, while the treatment group was given RGZ. The serum hormone values, pathohistology of ovarian structure of rats, ovarian ultrastructure and the expressions of TGF-$\beta_1$ and CTGF were detected.

**Results** The PCOS model was established successfully. The expression intensity of TGF-$\beta_1$ and CTGF in Oocytes of the PCOS groups was 9.545±2.954 and 9.665±2.400, respectively and was significantly higher than that of the control group 6.636±2.264 and 7.036±2.133; after treatment with rosiglitazone, the expression was significantly decreased 6.980±2.421 and 6.642±2.721 as compared with that of the model group ($P$<0.05, $P$<0.001). The values in serum of the PCOS groups were 3.749±2.054 and 0.265±0.129, and 1.914±1.801 and 0.096±0.088 in the control group which had statistically significant difference ($P$<0.05, $P$<0.001). After treatment with rosiglitazone, the values were 2.3100±1.825 and 0.112±0.187 and were significantly different with those of the model group ($P$<0.05, $P$<0.001).

**Conclusion** TGF-$\beta_1$ and CTGF play an important role in the development of ovary fibrosis in PCOS. However, RGZ may postpone the development of fibrosis by decreasing the levels of TGF-$\beta_1$ and CTGF.

**Key words:** Polycystic ovary syndrome; Transforming growth factor-$\beta_1$; Connective tissue growth factor; Rosiglitozone

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## INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a common metabolic and endocrine disorder that affects women of childbearing age and its pathogenesis is extremely complicated. Interstitial fibrosis and thickening capsule are the main chronic complication causing abnormal ovulation. The mechanism for the pathological change is unclear. Over the past years it has been studied in many aspects including the hypothalamic-pituitary-ovarian and adrenal axis dysfunction, insulin resistance, obesity and genetics. The exact cause has not yet fully understood. The recent research shows that the local ovarian bioactive factors play an important role in polycystic ovary formation[1].

Transforming growth factor (TGF-$\beta_1$) is one of the factors which are found to have more extensive

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biological activity than their originally named one. It has been proved that TGF-$\beta_1$ plays a very important role in the organs and tissues of fibrosis diseases; it is such a key cytokine that is recognized by most scholars as a start-hub for fibrosis formation and development[2].

Connective tissue growth factor (CTGF) is a downstream factor of TGF-$\beta_1$, which may be induced by TGF-$\beta_1$ and mediate some of the pro-fibrotic actions of TGF-$\beta_1$, such as increasing the synthesis and accumulation of extracellular matrix[3].

However, it has not been reported whether the pathological change of ovarian capsule and interstitial fibrosis in PCOS are related with the abnormal expression of TGF-$\beta_1$ and CTGF.

Rosiglitazone (RGZ) has an action of controlling blood glucose and regulating Immune function, and exerts anti-inflammatory and anti-fibrosis effects[4]. In order to clarify the mechanism of ovarian fibrosis and hardening capsule in PCOS, and explore the function of RGZ, the expressions of TGF-$\beta_1$/CTGF, and the intervention effect of RGZ on PCOS rats deserve an intensive study.

## MATERIALS AND METHODS

### *Materials*

Spragne-Dauley (SD) rats were clean, 22 days of age, weight 40-50 g, purchased from Guangdong Laboratory Animal Research Center, (Certification NO. 0026414). Rosiglitazone maleate Tablets (Avandia) were provided by GlaxoSmithKline Pharmaceutical Co. Ltd.

### *Animal Model*

The rats were housed in a light (12 h on/12 h off) and temperature- controlled environment, with food and water accessible; 21-day-old weaned, female rats were identified and fed in separated cages. Sixty female SD rats were randomly divided into three groups, i.e. control, model and treatment groups. The model and treatment groups were injected hypodermically dehydroepiandrosterone (DHEA) 6 mg/100(g·d) for 20 days as a model of PCOS, the control group were subcutaneous injected equivalent solvent, and the treatment group was additionally given RGZ 3 mg/ (kg·d). Both the model and control groups were orally given distilled water at the same time.

### *Collection and Preparation of Ovarian Tissue Specimens*

After 20 d, rats were aged 6-weeks. All the rats' ovarian tissues were detected in the morning fasting state respectively. The rats were anesthetized by an intraperitoneal injection of 0.35 mL/100 g, 10% cloralihydras; one side of fresh ovarian tissue was immediately fixed in 10% formalin, and then embedded in paraffin, sliced into 4 μm-thick sections, and mounted onto the poly-L-lysinecoated slide for immunohistochemical test. The other side of ovarian portions was fixed in 2.5% glutaraldehyde immediately, after putting in refrigerator at 4 °C for at least 2 h, the ovarian tissues were dissected about 1 mm × 1 mm × 2 mm at the wax plate filled with 2.5% of glutaraldehyde, and went on fixing in glutaraldehyde. Subsequently, the post-fixation was performed with osmium tetroxide, and then dehydrated, followed by penetration and embedding. When the tissue blocks were trim, semithin sections (1 μm) were made, then dyed with toluidine blue and observed under light microscopy to classify the follicles. The ultrathin sections (70 nm) were made contrasted with uranyl acetate and lead citrate to be observed with transmission electron microscopy (TEM) (Jeol 1011II/T, Japan).

### *Determination of Hormone Levels*

After the ovary was taken out, the chest was opened, about 4 mL blood was taken from the heart, and laid statically and put in 4 °C refrigerator overnight, and centrifuged at 2 318 ×g for 20 min; Serum hormone levels, including $E_2$, T, FSH, LH, LH/FSH, and FINS (fasting insulin,) were tested by microparticle enzyme immunoassay (AxSYM, USA).

### *Immunohistochemistry*

Streptavidin-biotin immunoperoxidase(S-P) method was used for immunochemical analysis. The test was performed according to standard procedure as SP immunohistochemical and 3', 3'-diaminobenzidine (DAB) colorimetrical reagent kit (Zhongshan Biotechnology Co. Ltd, Beijing). Briefly, the slides were deparaffinized in xylene and dehydrated through gradient ethanol. Endogenous peroxidase was blocked with 0.3% $H_2O_2$ in methanol for 10 min at room temperature and the slides were boiled in EDTA buffer in a microwave oven for 20 min for antigen retrieval, followed by incubating with normal goat serum for 10 min to reduce nonspecific binding. The primary rabbit anti-rat TGF-$\beta_1$ polyclonal antibody or rabbit anti-rat CTGF polyclonal antibody (BOSTER Biotechnology Co.Ltd, Wuhan) was applied to the slides at 4 °C overnight, and the slides were subsequently incubated with biotin-conjugated goat

against rabbit IgG at room temperature for 10 min, followed by incubation with streptavidin-horseradish peroxidase complex at room temperature for 10 min, staining with DAB for 2 min and counterstaining with Heamatoxylin (except the slides for image analysis). For negative control the primary antibody was replaced by PBS, the slides were dehydrated with gradient alcohol, made transparent with dimethylbenzene, covered with a glass coverslip and finally observed under light microscope.

### *Image Analysis*

After staining, all the slides were observed under microscopy (200×), and quantitatively evaluated by computer based image analysis system (Leica Qwin Germany). Rats' ovarian tissues per groups were observed, and 10 slides with equal interval were chosen randomly from every set of slides. Positive units (PU) were used to express positive response degrees and calculated by the formula: PU= | Gα-Gβ | /Gmax×100, where Gα and Gβmean gray level of the studying tissue structure and background respectively, Gmax is the maximum gray level of the instrument (256). The test of gray level should be performed in the same setting.

### *Detection of Serum TGF-$\beta_1$ and CTGF Levels*

Serum TGF-$\beta_1$ and CTGF ELISA Kit were purchased from USCN LIFE SCIENCE &TECHNOLOGY COMPANY, operations were carried out in strict accordance with specifications, quality control and calibration results were within the requirements. Assay procedure: all reagents were allowed to reach room temperature; Added 100 μL of standard, blank, or sample per well. Covered with the plate sealer. Incubated for 2 h at 37 °C. The liquid of each well was removed without washing. 100 μL of biotin-conjugated specific polyclonal TGF-$\beta_1$ or CTGF antibody working solution were added to each well. Covered again. Incubated for 1 h at 37 °C. Each well was aspirated and washed, the process being repeated three times for a total of three washes. Washed by filling each well with wash buffer (approximately 400 μL). Added 100 μL of Avidin conjugated to Horseradish Peroxidase (HRP) working solution to each well. Covered with a new plate sealer. Incubated for 1 h at 37 °C. Repeated the aspiration/wash as in step 4. Added 90 μL of substrate solution to each well. Covered with a new plate sealer. Incubated within 30 min at 37 °C. Protected from light. Added 50 μL of stop solution to each well. The optical density of each well was determined at once, using a microplate reader set to 450 nm.

### *Statistical Analysis*

The data were expressed as mean±standard deviation (SD), difference were analyzed with SPSS10.0 software, single factor analysis of variance was used for comparison of the independent samples, and *q* test was used for the comparison of any two of the several samples. A difference with $P<0.05$ was considered significant.

## RESULTS

### *Pathological Observations*

The rat ovarian structure was in order. Follicles and corpus luteum were at different developmental stages, and a small amount of atretic follicles, instead of cystic follicles were seen in the control group. The ovarian structure of the rats in the PCOS model group was disordered, and there were large numbers of cystic dilatation and atresia follicle, among which some normal follicular developed, granular cell layers were reduced, theca cell layers were thicken and no luteal forming was observed in the majority of the ovarian structure. Since PCOS follicular collapsed easily, the figure offered following embedding and staining was different from each others.

In the treatment group, the ovarian structure was in order, and follicles and corpus luteum were at different developmental stages, a small amount of atretic follicles and cystic follicles were seen in the control group, and theca cell layers looked normal (Figure 1).

### *Serum Hormone Assays in the Three Groups*

Serum concentrations of T and FINS were significantly higher in the PCOS model group than those in the control group and the rosiglitazone treatment group ($P<0.01$, $P<0.05$), (Table 1).

### *The Cell Ultrastructure of the Three Groups*

Collagen fiber was increased in interstitial cells, and abundant of mitochondria, smooth endoplasmic reticulum (SER) and lipid droplet were seen in the theca-interstitial cells (TIC) in the model group. While collagen fiber was decreased in interstitial cell, and mitochondria, smooth endoplasmic reticulum (SER) and other organelles were normally distributed in TIC in the treatment group (Figure 2).

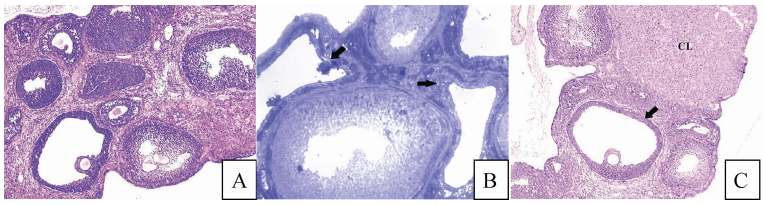


**Figure 1**. The pathological change in ovary of the three groups. A. Control group: Different developmental stages of follicles were detected in control group, HE ×50; B. Model group: The follicles were changed into cystic form ( ↑ ). Dyed with toluidine blue ×100; C. Treatment group: the ovarian structure was in order, and there were different developmental stages of follicles and corpus luteum(CL), a small amount of atretic follicles and cystic follicles in control group. HE ×100.

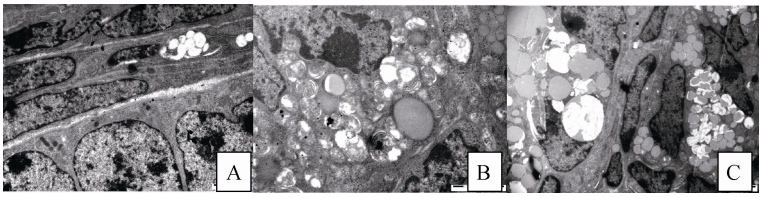


**Figure 2.** The ultra structure of the three groups. A. Control group: the theca-interstitial cells were in order; B. Model group: collagen fiber was increased in interstitial cells, and abundant of mitochondria, smooth endoplasmic reticulum (SER) and lipid droplet were seen in the theca-interstitial cells (TIC) of the model group; C. Treatment group: collagen fiber and the mitochondria, smooth endoplasmic reticulum (SER) and lipid droplet were decreased in the theca-interstitial cells (TIC).

**Table 1.** Comparison of Serum Hormone Value in Three Groups ($n$=20)

| Group | LH/FSH | T (ng/mL) | E2 (pg/mL) | FINS (μU/mL) |
|---|---|---|---|---|
| Control group | 1.036±0.460 | 0.780±0.440* | 28.956±11.133 | 13.636±6.264* |
| Model group | 1.115±0.654 | 1.215±0.702 | 37.105±16.400 | 18.945±7.950 |
| Treatment group | 1.081±0.561 | 0.836±0.498* | 36.200±12.721 | 14.480±5.421* |

***Note.*** * $P$<0.05 *vs* Model group.

### *Expression of TGF-$\beta_1$ and CTGF in the Ovarian Tissue of PCOS Rat*

As shown by the Immunohistochemical staining, TGF-$\beta_1$ protein was expressed strongly in oocytes cytoplasm of primordial and preantral follicles, while the expression was weaker in granulosa and theca-stromal cells. TGF-$\beta_1$ expression intensity was in parallel with follicle development, the expression intensity was gradually weakened, and it was significant ($P$<0.01, $P$<0.05) compared with antral follicles. There were no significant changes in granulosa or theca cells ($P$>0.05).

The expression intensity of TGF-$\beta_1$ and CTGF of oocytes in the PCOS model groups were 9.545±2.954 and 9.665±2.400 which were significantly higher than in the control group (6.636±2.264 and .036±2.133). After treatment with rosiglitazone, the expression was significantly decreased, and the value were 6.980±2.421 and 6.642±2.721, as compared with the model group, and the deference was statistically significant($P$<0.05, $P$<0.001) (Figure 3, Figure 4, and Figure 5).

### *Comparing the Expression of TGF-$\beta_1$ and CTGF in Sera of the Three Groups*

The expressions of TGF-$\beta_1$ and CTGF in serum of the model groups were 3.749±2.054 and 0.265±0.129, respectively, and those in the control group were 1.914±1.801 and 0.096±0.088, respectively, and the difference was statistically significant ($P$<0.05, $P$<0.001). The expressions were significantly decreased in the rosiglitazone treatment group, which were 2.3100±1.825 and 0.112±0.187, respectively and the difference was statistically significant as compared with the model group ($P$<0.05, $P$<0.001)(Figure 6 ).

## DISCUSSIONS

### *Mechanism of Ovarian Interstitial Fibrosis*

Chronic hepatitis virus infection can both result in hepatic fibrosis and cirrhosis. In recent years, with the increasing of patients suffering from this disease,

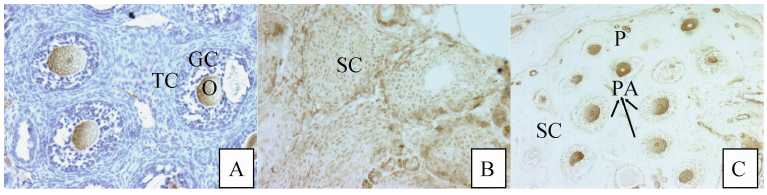


**Figure 3.** Expression of TGF-β1 in rat ovaries. immunohistochemical staining, SP method. A. Control group: Expression of TGF-β1 in the follicles of control groups, oocytes(O), granulosa cells(GC), theca cell(TC), Heamatoxylin staining. ×200; B. Model group: Expression of TGF-β1 in the interstitial of PCOS ovary. ×200.

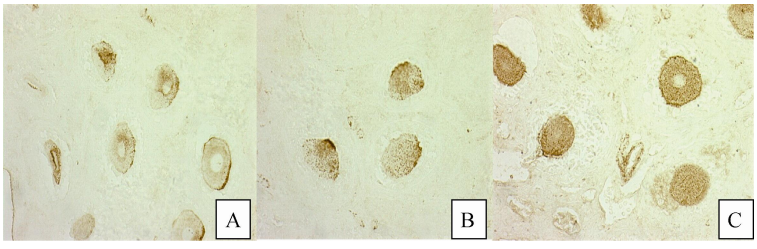


**Figure 4.** Expression of *CTGF* in rat ovaries. Immunohistochemical staining, SP method. A. Control group: *CTGF* protein was expressed in oocytes cytoplasm of all the follicles, ×200; B. Model group: Expression of *CTGF* in *PCOS* ovary, the normal oocytes decreased, ×200; C. Treatment group: *CTGF* was strongly expressed in the ovary of the treatment group, ×200.

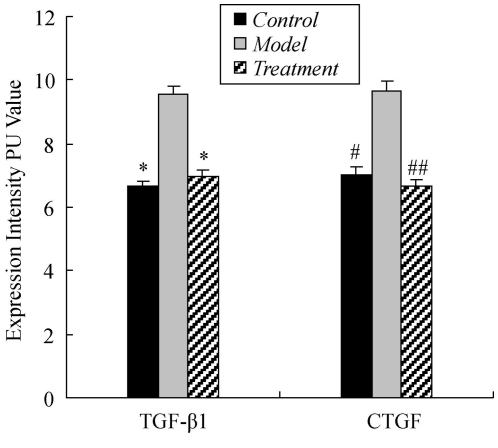


**Figure 5.** Expression intensity of TGF-$\beta_1$ and CTGF in Oocytes of three groups (PU). $^{*}P$<0.05, TGF-$\beta_1$ *vs* Model group; $^{\#}P$<0.05, CTGF *vs* Model group; $^{\#\#}P$<0.001, CTGF *vs* Model group.

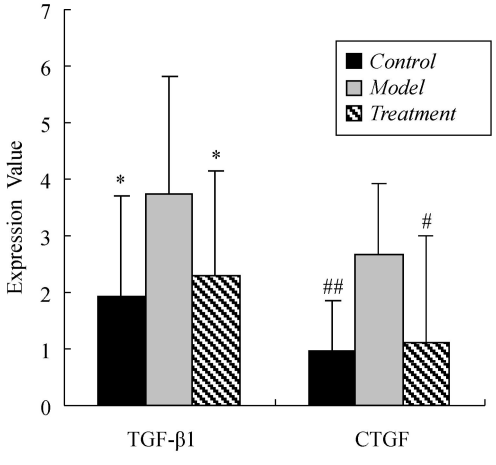


**Figure 6.** Comparison of TGF-$\beta_1$ and CTGF in serum of three groups (ng/mL). $^{*}P$<0.05, TGF-$\beta_1$ *vs* Model group; $^{\#}P$<0.05, CTGF *vs* Model group; $^{\#\#}P$<0.001, CTGF *vs* Model group.

"organ fibrosis" has been attracting attention of the biomedical investigators. Enormous researches have been undertaken in this field including liver fibrosis[5], pulmonary fibrosis[6], myocardial fibrosis[7], renal interstitial fibrosis[8], and pancreatic fibrosis[9]. With the development of modern medical technology, the study on organ fibrosis has made great progress. And the pathogenesis of fibrosis have been partially revealed and scholars have made an inference that all the tissues have the possibility of "fibrosis".

Although ovarian fibrosis has not been paid enough attention, fibrosis plays an important role in the physiological and pathological processes of ovarian. Ovarian fibrosis lesions have been commonly developed in women seriously affecting their reproductive health and quality of life. Ovarian fibrosis also decreases ovarian function and causes premature ovarian failure.

Ovaria experience growth, maturity and

degradation at the different stages of life, or in the process of reproductive cycle, which requires the constant remodel of extracellular matrix (ECM)[10]. The maintenance of ECM environment stability is largely dependent on the coordination of matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMPs); MMPs-TIMPs are the main proteinase system that is involved in ECM degradation and reconstruction[11]. In recent years, it has been increasingly reported that MMPs-TIMPs in ovaria play regulatory roles in follicle growth, atresia, ovulation, and corpus luteum formation or degeneration[12].

It has been proved that TGF-$\beta_1$ is closely related with *ECM* accumulation, and it is recognized as a key factor in the development of organ fibrosis. Studies show that TGF-$\beta_1$ can inhibit the expression of MMPs, and meanwhile can activate and up-regulate the expression of tissue inhibitors of MMP-TIMPs and other protease inhibitor, promote synthesis of ECM components such as I, III, IV collagen and fibronectin through autocrine and paracrine way, hindering *ECM* degradation[13].

As a downstream factor of TGF-$\beta_1$, CTGF has many similar biological functions with TGF-$\beta_1$. It can promote cell chemotaxis, adhesion and stimulate some cell proliferation, such as fibroblasts, epithelial cells, smooth muscle cells and endothelial cells. CTGF is also related to the synthesis and accumulation of ECM in scleroderma, atherosclerosis, systemic sclerosis and organ fibrosis formation to different degrees. It is closely related to disease occurrence and development[14].

PCOS is a common metabolic and endocrine disorder that affects women of childbearing age. Its pathogenesis is extremely complicated. Over the past years the PCOS pathogenesis has been studied in many aspects, including the hypothalamic-ituitary-ovarian and adrenal axis dysfunction, insulin resistance, obesity and genetic predisposition. Recent research shows that PCOS is a primary ovarian function disorder. The local ovarian bioactive factors play an important role in polycystic ovary formation. Before it was formally named, PCOS was intuitively described as a "hardening of cystic ovarian" showing that ovarian interstitial fibrosis, capsular thickening and hardening are the main pathological changes of PCOS.

Extensive literature demonstrates that PCOS also involves the damage of MMPs-TIMPs balance, and the ECM abnormal degradation. The abnormal expression of ovarian collagen in PCOS patients may be related to interstitial fibrosis and basement membrane thickening[15]. It suggests that increasing synthesis of extracellular matrix leads to interstitial fibrosis, capsular thickening and in the end causes ovulatory disorder.

The results of the present study have revealed that the expression of TGF-$\beta_1$ and CTGF in ovarian tissue and peripheral serum of the PCOS model group was significantly higher than that of the control group. It is also suggested that both of them may be related to the pathological changes of PCOS ovarian interstitial fibrosis. Based on the above results we can infer that TGF-$\beta_1$ and CTGF in PCOS are prompted by a variety of endogenous and exogenous factors, which affects MMPs-TIMPs balance subsequently and leads to an excessive accumulation of ECM in ovaries and causes ovarian interstitial fibrosis.

### *The Mechanisms of Rosiglitazone Inhibition of Rat Ovarian Interstitial Fibrosis*

Insulin sensitizers' rosiglitazone is a new synthetic anti-diabetic drugs which belongs to thiazolidinediones ketones (TZDs). It regulates the expression of lipid metabolism-related genes by activation of nuclear peroxisome proliferator- ctivator receptor gamma (PPARγ), and plays a key role in improving insulin sensitivity[16]. Some clinical data show that rosiglitazone can improve the reproductive and endocrine function of PCOS patients with insulin resistance by increasing insulin sensitivity, and also plays the roles of controlling blood glucose, immune regulation and anti-inflammation[17].

In recent years, it has been reported that some agonists of *PPARγ* can inhibit myofibroblast differentiation and collagen Ⅰ production induced by TGF-$\beta_1$ indicating that they might play an important role in organ fibrosis. Now, many animal and in vitro models of organ fibrosis show that TZDs have the effect of anti-organ hardening[18]. Rosiglitazone increases the expression of MMPI while inhibiting the expression of TIMP via the activation of PPARγ, thus preventing fatty liver fibrosis, and delaying the process of renal fibrosis and liver fibrosis, which indicates that rosiglitazone has an anti-fibrosis function[19].

This study shows that after treatment with rosiglitazone, the expression of TGF-$\beta_1$ and *CTGF* in rat ovarian tissue and serum were significantly decreased. Fibrosis is the interaction process of collagen fibers synthesis and degradation in case of collagen synthesis hyperthyroidism, the latter is inhibited, while there is fibrosis, and vice versa

fibrosis can be dissipated. Since there is fiber degradation in physiological processes in vivo, we believe that fibrosis is not irreversible, that has been confirmed by a large number of experimental and clinical studies. As it can be seen from the ultrastructure change of theca-interstitial cells, collagen fiber is decreased in interstitial cell after treatment with rosiglitazone. Moreover, mitochondria, smooth endoplasmic reticulum and other organelles are normally distributed in TIC indicating that their ability to synthesize testosterone is decreased. It demonstrates that rosiglitazone has the function of inhibiting ovarian interstitial fibrosis and capsule thickening, which may be carried out by the inhibition of TGF-$\beta_1$ and its downstream factor CTGF.

In recent years, rosiglitazone is widely used in treatment of diabetes and insulin resistance[20]. The results of this study suggest that rosiglitazone also has the function of reducing ovarian interstitial fibrosis, which will expand its application scope undoubtedly. Rosiglitazone as an old drug may have some development and research value for its new role.

However, further study is needed to explore how TGF-$\beta_1$/CTGF induces the formation of ovarian interstitial fibrosis.

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