Dehydroepiandrosterone induces ovarian and uterine hyperfibrosis in female rats

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STUDY QUESTION: Do dehydroepiandrosterone (DHEA)-treated rats with polycystic ovary syndrome (PCOS) demonstrate a high level of fibrosis in ovarian and uterine tissues?

SUMMARY ANSWER: DHEA induces ovarian and uterine hyperfibrosis in rats, probably involving a transforming growth factor-β (TGF-β)-dependent mechanism.

WHAT IS KNOWN ALREADY: Chronic inflammation is the typical cause of fibrosis and is involved in the pathophysiological process of PCOS. Patients with PCOS are reported to have a higher serum level of TGF-β, a well-characterized key pro-fibrotic factor. Fibrillin-3, a protein capable of interacting with TGF-β, has been reported to be partially responsible for the fetal origin of PCOS.

STUDY DESIGN, SIZE, DURATION: Female Sprague–Dawley rats were treated with a vehicle control or DHEA for 35 days, with subsequent analyses of changes in morphology and gene expression in ovarian and uterine tissues. Rescue groups treated with metformin or simvastatin and their corresponding controls were also analyzed. A total of 80 rats were included.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The PCOS model was induced by daily administration of DHEA s.c. to 3-week-old female rats, and the rescue groups were injected daily with either metformin or simvastatin in addition to DHEA. Serum steroid hormone levels were measured by enzyme-linked immunosorbent assay. Samples were stained with hematoxylin and eosin for histological morphology, and Sirius Red and immunohistochemistry for revealing collagens. The expression of fibrosis-related genes was analyzed both at mRNA (real-time RT–PCR) and protein (western blot) levels.

MAIN RESULTS AND THE ROLE OF CHANCE: DHEA-induced rats with PCOS exhibited significantly higher levels of fibrosis (collagen IV) in both ovarian and uterine tissues. In ovarian tissue, the expression of connective tissue growth factor (CTGF) increased following DHEA treatment at both mRNA and protein levels (P < 0.05, P < 0.001 versus controls, respectively). Similar results versus controls were obtained at a protein level for TGF-β (P < 0.01) and mRNA level for fibronectin (P < 0.05) and angiotensin-II (P < 0.05). Likewise, in uterine tissue, the protein levels of both CTGF and TGF-β were higher than controls following DHEA treatment (P < 0.05). Treatment with either metformin or simvastatin attenuated the fibrosis progression induced by DHEA exposure, as evidenced by a reduction of TGF-β, plus CTGF or not, in both ovarian and uterine tissues.

LIMITATIONS, REASONS FOR CAUTION: The particular mechanism involved in the DHEA-induced fibrosis was not fully revealed.

WIDER IMPLICATIONS OF THE FINDINGS: Ovarian and uterine hyperfibrosis may occur in patients with PCOS and result in anovulation or other PCOS-related phenotypes. Anti-fibrotic therapy, for example metformin treatment, may be beneficial for patients with PCOS.

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Key words: dehydroepiandrosterone / polycystic ovary syndrome / fibrosis / metformin / simvastatin

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**Introduction**

Polycystic ovary syndrome (PCOS) is one of the most common reproductive and endocrinial diseases, with a prevalence of 7.4% in women of child-bearing age in China (Qiao, 2008). Early in 1982, Hughesdon found that the tunica increased by 50%, which was accompanied by a sharp increase in cortical stromal thickness and subcortical stroma in Stein-Leventhal ovaries, or polycystic ovaries as they were later called. One of the key characteristics of PCOS is hyperandrogenism, which has been associated with ovarian fibrosis. The ovarian morphology of long-term androgen-treated female-to-male transsexuals meets the criteria for the diagnosis of polycystic ovaries, and is associated with theca interna hyperplasia and a thicker, more collagenized cortex when compared with the controls (Pache et al., 1991). Hyperinsulinemia, the potent factor driving increased androgen production, is common in PCOS (Diament-Kandarakis et al., 1995; Silen et al., 2003). Treatment of ovarian organoids with insulin and insulin-like growth factor-1 results in ovarian surface epithelium hyperplasia and altered organization of collagen (King et al., 2013). Fibrotic conditions in different organs have different etiologies but fibrosis typically results from chronic persistent inflammation. Recently, chronic inflammation has been demonstrated to participate in the pathophysiological process of PCOS (Kelly et al., 2001; Boullan et al., 2004; Xiong et al., 2011). One study showed that PCOS patients have a higher level of serum transforming growth factor-beta 1 (TGF-β1) (Hu et al., 2004), which appears to be the key factor in the pathogenesis of fibrosis (Schiller et al., 2004; Schaafsma et al., 2011). All these pieces of evidence suggest that hyperfibrosis may occur in patients with PCOS.

Dehydroepiandrosterone (DHEA), a metabolic intermediate in the biosynthesis of androgen, is one of the many drugs, including testosterone and letrozole, utilized to build animal models of PCOS by prenatal or prepubertal exposure (Luchetti et al., 2004; Manneras et al., 2007). The disease pathogenesis of PCOS has been largely investigated in rodent models. Miao et al. (2008) have revealed that TGF-β1 is highly expressed in the theca cells of antral follicles and stromal cells in DHEA-induced PCOS rats. No results on fibrosis have been reported in rodent models of PCOS induced by drugs other than DHEA.

Accumulating evidence suggests that PCOS may have fetal origins. Genetic studies have found strong linkage and association of D19S884, a microsatellite in the fibrillin-3 gene, with PCOS (Stewart et al., 2008). Fibrillin-3 is an extracellular matrix (ECM) glycoprotein and has been reported to be highly expressed at a critical early stage in the developing fetal ovaries, suggesting an important role for this molecule in the pathogenesis of PCOS (Hatzirokos et al., 2011). Given that fibrillin can regulate TGF-β activity through interaction with latent TGF-β-binding proteins and TGF-β can modulate fibrillin expression (Kenney et al., 2003; Massam-Wu et al., 2010), a linkage between TGF-β and PCOS may possibly be established.

In view of such evidence based on both human clinical investigation of PCOS and DHEA-induced rat models of PCOS, it is likely that fibrosis might have taken place in polycystic ovaries or other relevant organs. Fibrosis is always characterized by the accumulation of ECM and quite a number of molecular factors are involved in ECM formation, such as TGF-β (Roberts et al., 1986; Risser et al., 1998; Schiller et al., 2004), connective tissue growth factor (CTGF) (McLennan et al., 2004) and epidermal growth factor (Lembach, 1976). Among these, the pro-fibrotic proteins TGF-β and CTGF are considered master switches for induction of the fibrotic program (Verrecchia and Mauviel, 2007).

Metformin is an oral insulin sensitizer widely used for type 2 diabetes, and has been recommended for the treatment of anovular PCOS, mainly by reducing serum insulin levels and insulin resistance and improving ovulatory function (Palomba et al., 2005; Legro et al., 2007; Palomba et al., 2009). Given the fact that metformin has great potential in attenuating the progress of fibrosis in various organs (Qiang et al., 2010; Xiao et al., 2010; Burla et al., 2013), we hypothesized that DHEA-induced fibrosis could be decreased by metformin treatment, which may account for its clinical efficacy for PCOS patients. Meanwhile, the use of statins recently has emerged as a novel therapeutic approach for PCOS. Many clinical trials have shown that simvastatin exerts positive and protective effects in patients with PCOS, as it improves the endocrine/clinical aspects of PCOS and contributes to improved lipid profiles (Duleba et al., 2006; Banaszewska et al., 2007, 2009; Kazerouni et al., 2010). Interestingly, simvastatin also had an anti-fibrosis effect (Rodrigues Diez et al., 2010; Abe et al., 2012), which may also be a mechanism for its effectiveness in the clinical trials.

In summary, we hypothesized that the level of fibrosis was significantly higher in ovarian and uterine tissues in DHEA-induced PCOS rats, and could be attenuated by treatment with metformin and/or simvastatin.

**Materials and Methods**

**Animal maintenance and treatment**

Sprague-Dawley rats (n = 80, 3 weeks old) were obtained from Qinglongshan, Inc., Nanjing, China. The experiments were approved by the ethical review committee of Nanjing University. All the rats were housed in the translational medical centre of Nanjing University at 21 °C with a 12 h light/12 h dark cycle. Free access to a standard rat diet and water was provided. The rats were randomly assigned into 11 groups: the blank group, i.e. no treatment (n = 6); the vehicle (0.2 ml sesame oil) group (n = 10); the DHEA group in which PCOS was induced by injection of DHEA (n = 8); two groups in which vehicle-treated rats were treated with saline (n = 6) or metformin dissolved in saline (n = 9); two groups in which DHEA-treated rats were given saline (n = 5) or metformin dissolved in saline (n = 8); two groups in which vehicle-treated rats received phosphate-buffered saline (PBS) (n = 6) or simvastatin dissolved in PBS (n = 9); and two groups in which DHEA-treated rats were treated with PBS (n = 5) or simvastatin dissolved in PBS (n = 8).

PCOS was induced by administration of 6 mg/100 g body weight trans-Dehydroandrosterone s.c. (DHEA, Sigma-Aldrich (Shanghai) Trading Co., Ltd) dissolved in 0.2 ml sesame oil (Zhu et al., 2010). 1.1-Dimethylbiguanide hydrochloride (metformin; Sigma-Aldrich) was diluted in 1.0 ml saline and given at 30 mg/100 g body weight by gavage. Simvastatin (Admas beta, Admas Reagent Co., Ltd) was diluted in 0.2 ml PBS (Gibco, invitrogen) and 1 mg/100 g body weight was injected i.p. Saline (1.0 ml) and PBS (0.2 ml) were administered by gavage and by i.p. injection, respectively. The administration of metformin and simvastatin was carried out immediately after the injection of DHEA. The daily treatments lasted for up to 33 days consecutively. Thereafter, estrous cycles of all rats were determined by analyzing the cell types in vaginal smears (Marcondes et al., 2002) for 1 week. All rats were killed by the injection of chloral hydrate and ovaries and uteri were harvested for the following studies.
Serum hormone assays and estrous cycle determination

After treatment for 35 days, all the rats were anesthetized and blood samples were obtained from the inferior vena cava. Sera were immediately separated and kept at −20 °C for subsequent hormone determination by enzyme-linked immunosorbent assay (ELISA) (testosterone, estradiol, FSH and LH) (rat testosterone/estradiol/FSH/LH ELISA Kit; Yunhan Biotechnology Co., Ltd, Shanghai). Serum fasting insulin levels, fasting blood glucose, homeostasis model assessment of insulin resistance (HOMA-IR, an index commonly used to quantify insulin resistance and β-cell function) and lipid profiles [total glycerin, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein and lipoprotein-a (LP-a)] were also measured.

Tissue sample collection and histopathology

Immediately following the collection of blood samples, the uterus and both ovaries were quickly removed and cleaned. Thereafter, one ovary was fixed in 10% formalin solution and stored at 4 °C for staining. After fixation in 10% formalin for ~24 h, the ovarian and uterine tissues were dehydrated with ethanol and xylene, embedded in paraﬃn and sliced into 5-μm sections, stained with hematoxylin and eosin (H&E) or Sirius Red and analyzed with an optical microscope (Olympus Bx50).

RNA isolation, RT–PCR and real-time RT–PCR

Total RNA was extracted from ovarian and uterine tissues using the RNAPure kit (BioTeke, BioTeke Corporation) according to the manufacturer’s instruction. RNA concentration and purity were measured with a NanoDrop 2000 spectrophotometer (Thermo scientiﬁc). Five hundred nanograms of total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa) in a Takara PCR Thermal Cycler Dice (TP600; Takara Bio, Inc., Japan). Reverse transcription was carried out as follows: 37 °C for 15 min and 85 °C for 5 s. Real-time PCR was performed in a total volume of 20 μl with 10 μl of 2× SYBR Premix Ex Taq (TaKaRa), 0.4 μl of 50× Rox dye (TaKaRa), 0.5 μl of each primer (3 pmol each), 2.0 μl of cDNA and 6.6 μl ddH2O and was conducted in triplicate for subsequent hormone determination by enzyme-linked immunosorbent assay (ELISA) (testosterone, estradiol, FSH and LH) (rat testosterone/estradiol/FSH/LH ELISA Kit; Yunhan Biotechnology Co., Ltd, Shanghai). Serum fasting insulin levels, fasting blood glucose, homeostasis model assessment of insulin resistance (HOMA-IR, an index commonly used to quantify insulin resistance and β-cell function) and lipid profiles [total glycerin, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein and lipoprotein-a (LP-a)] were also measured.

Immunohistochemistry

Formalin-ﬁxed, paraﬃn-embedded sections (4 μm thick) were analyzed by immunohistochemistry using a standard protocol. Antigen retrieval was performed in 1 mmol/l EDTA (pH 8.0) in a microwave oven for 15 min.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Forward primer sequence (5′→3′)</th>
<th>Reverse primer sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin-II</td>
<td>CACCCAGAAAAAGGATGAAAGA</td>
<td>AGACTCAGCACAAACACAAACA</td>
</tr>
<tr>
<td>CTGF</td>
<td>CATTAAAGAGGCGAAAGGACTGC</td>
<td>CAACCCCAAGGAATTTAGCC</td>
</tr>
<tr>
<td>TGF-β</td>
<td>TACGGTCTAGCTCCACAGAGA</td>
<td>CAGAGCAAGTGGCATGGAGT</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>TGACAATCGGCCGACCTGGG</td>
<td>TACTGGTTGAGGTGTTGCGCG</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>TTGGTGTGGTTTCGGAAACTGA</td>
<td>CTGTATGGCTCGAATCTAGA</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAGGCCAACCGTGGAAAGAT</td>
<td>ACCAGGGCATACGGGACA</td>
</tr>
</tbody>
</table>

CTGF, connective tissue growth factor; TGF-β, transforming growth factor-β.

Table II The serum hormone levels in control and dehydroepiandrosterone (DHEA)-treated rats with PCOS.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Vehicle</th>
<th>DHEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (ng/ml)</td>
<td>9.13 ± 0.36</td>
<td>9.39 ± 1.16</td>
<td>12.90 ± 1.05*</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>41.34 ± 2.70</td>
<td>36.49 ± 1.23**</td>
<td>30.90 ± 1.29*</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>13.60 ± 1.25</td>
<td>14.33 ± 1.13</td>
<td>22.17 ± 1.23*</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>3.83 ± 0.23</td>
<td>4.51 ± 0.20**</td>
<td>5.79 ± 0.34*</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>3.56 ± 0.48</td>
<td>3.18 ± 0.25</td>
<td>3.84 ± 0.26*</td>
</tr>
</tbody>
</table>

*Signiﬁcantly different from blank and vehicle (P < 0.05).
**Signiﬁcantly different from blank (P < 0.05). P < 0.05 was considered signiﬁcantly different. Data are mean ± SD.
Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 10 min. After incubation in blocking buffer (goat serum) for 30 min, the sections were exposed to primary antibody (collagen IV, Abcam, rabbit anti-rat, 1:2000) overnight at 4°C. The slides were then washed in PBS for 5 min × 3 and incubated with secondary antibody (ZSGB-Bo, goat anti-rabbit) for 20 min at 37°C. Incubation with the chromogen (3,3′-diaminobenzidine) was carried out for 1 min. Images were captured with an optical microscope (Olympus Bx50).

Statistical analysis
Data were presented as either mean ± SD or mean ± SE. One-way analysis of variance was used for data analysis within different groups using the Origin 8.5 software. A P < 0.05 was considered to be statistically significant.

Results
DHEA induces PCOS in rats
Serum hormone levels and estrous cycle alteration: as shown in Table II, the serum testosterone and LH levels and the LH/FSH ratio were significantly higher in DHEA-exposed rats than in controls, while the estradiol level was lower (all comparisons P < 0.05). Compared with controls, the DHEA-induced PCOS rats had higher levels of fasting insulin (17.36 ± 1.49 versus 13.00 ± 1.77 mU/l, P < 0.05) and HOMA-IR (4.09 ± 0.62 versus 2.73 ± 0.51, P < 0.05). For the lipid profile changes, LDL (0.63 ± 0.24 versus 0.19 ± 0.07 mmol/l) and LP-a (72.62 ± 3.44 versus 62.76 ± 8.46 mmol/l) were significantly higher in DHEA-treated rats than controls (all comparisons P < 0.05). The control rats had a normal estrous cycle of 4–6 days, while the DHEA-exposed rats were completely acyclic or had prolonged estrous cycles. The prolonged estrous cycle was not precisely determined in DHEA-exposed rats because of the limited duration of observation for 7 days. DHEA-induced rats had significantly reduced fecundity when compared with the controls (personal communication).

The PCO morphology in DHEA-exposed rats: no cystic follicle or other structural abnormalities were detected in the ovaries of control rats. Follicles at different stages and corpora lutea were observed in these control rats (Fig. 1A, D; B, E). While in DHEA-exposed rats, an increasing number of fluid-filled cystic follicles was recorded (Fig. 1C and F).

Figure 1  Histological sections of representative ovaries from dehydroepiandrosterone (DHEA)-exposed rats and controls following hematoxylin and eosin (H&E) and Sirius Red staining. (A–C) Representative sections from ovaries of a blank control rat (A), a vehicle-treated rat (B) and a DHEA-treated rat (C). Ovaries from control rats (A and B) had corpora lutea and follicles of different stages, but without cystic follicles. Ovaries from the DHEA-exposed rat (C) showed an increasing number of cystic follicles. H&E staining × 40. (D–F) Higher magnification views corresponding to A–C. Cystic follicles from a DHEA-exposed rat showed a thicker theca cell layer (arrows). H&E staining × 100. (G–I) Representative sections from ovaries of a blank control rat (G), a vehicle-treated rat (H) and a DHEA-treated rat (I). Ovaries from the DHEA-exposed rat showed a markedly higher level of collagen, especially in regions around the follicles (shown in red). Sirius Red staining × 200. The insets in G–I were higher magnification.
DHEA induces ovarian and uterine fibrosis

H&E staining of ovarian tissues from DHEA-exposed rats and controls showed that ovaries from rats with PCOS possessed a relatively enhanced proportion of stromal tissue, and a thickened theca cell layer (Fig. 1A–F). Sirius Red staining revealed a markedly higher level of collagen in DHEA-exposed ovaries, especially at the region around the follicles (Fig. 1G–I). In uterine tissues, the effect of DHEA was also obvious: uteri of the DHEA-exposed rats exhibited a thinner layer of endometrium, a thicker layer of myometrium (Fig. 2A–F) and a higher level of collagen fibrosis (Fig. 2G–I).

Immunohistochemical analysis confirmed the results revealed by Sirius Red staining. Collagen IV was mainly expressed in the theca cell layer. Accompanying with the thickened theca cell layer was an increased accumulation of collagen IV in the ovaries of the DHEA-exposed rats compared with control rats (Fig. 3A–F). In uterine tissues, collagen IV was mainly present in the myometrium layer and vessels in endometrium. The levels of collagen IV were substantially higher in DHEA-exposed rats than the control rats (Fig. 3G–L).

The content of CTGF in ovarian tissue significantly increased in the DHEA group at both mRNA and protein levels compared with control (Fig. 4A, E and F). Similar results were obtained at a protein level for TGF-β (Fig. 4G and H) and mRNA levels for fibronectin and angiotensin-II (Fig. 4B and C), another two potential pro-fibrotic factors. No significant difference was detected in the TGF-β mRNA level between the control and DHEA groups (Fig. 4D). Similarly, protein levels of both CTGF and TGF-β were significantly higher in the uterine tissue following DHEA treatment compared with controls (Fig. 4I–L).

Metformin attenuates DHEA-induced rat ovarian and uterine fibrosis

Metformin treatment reversed the PCOS pathology observed following DHEA administration, such as acyclicity, prolonged estrous cycles and fibrosis. A significant reduction of CTGF and TGF-β protein in both ovarian and uterine tissues was noted after metformin treatment.
following DHEA exposure (Fig. 5), while no change was observed after metformin treatment in the vehicle-treated rats.

**Simvastatin decreases DHEA-induced rat ovarian and uterine fibrosis**

Simvastatin treatment following DHEA exposure significantly reduced the level of the pro-fibrotic protein TGF-β, and showed a tendency to down-regulate CTGF, although this did not reach statistical significance (Fig. 6). No change was observed after simvastatin treatment in the vehicle-treated rats. Ayclicity and prolonged estrous cycles induced by DHEA were not reversed by simvastatin treatment.

**Discussion**

Our study demonstrated for the first time that DHEA can augment the fibrosis process on ovarian and uterine tissues in female rats, possibly in a TGF-β-dependent manner. Dramatic changes in the morphology of ovaries and uteri in DHEA-induced PCOS rats were observed. The ovaries demonstrated a thickened layer of theca cells and a higher level of collagen accumulation. The uteri showed a thinner layer of endometrium, a thicker layer of myometrium and a higher level of collagen accumulation, all of which suggested a higher level of fibrosis in rats with PCOS. Consistently, two key pro-fibrotic factors, TGF-β and CTGF, showed up-regulated expression in both ovaries and uteri of the rat...
Figure 4  Rats with DHEA-induced PCOS show an up-regulated level of fibrotic factors in both ovarian and uterine tissues. A dose of 6 mg/100 g body weight DHEA dissolved in sesame oil was daily administered s.c. for up to 35 consecutive days and the control group received nothing or an equal volume of vehicle. Ovaries and uteri were harvested for the following quantitative PCR (A–D) and western blotting (E–L) assay. (A–D) mRNA expression levels of various targets as indicated in rat ovarian tissues. mRNA levels of connective tissue growth factor (CTGF) (A), fibronectin (B) and angiotensin (Ang)-II (C) were significantly higher in DHEA-induced PCOS rats than controls; no difference in the transforming growth factor-β (TGF-β) was detected (D). (E–H) Detection of CTGF (E and F) and TGF-β (G and H) in ovarian tissues by western blot. Pooled quantification data (E and G) and original representative blots (F and H) demonstrated significant up-regulation of fibrosis in ovarian tissues of DHEA-exposed rats. (I–L) Detection of CTGF (I and J) and TGF-β (K and L) in uterine tissues by western blot. Pooled quantification data (I and K) and original representative blots (J and L) demonstrated remarkable up-regulation of fibrosis in uterine tissues of DHEA-exposed rats. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle-treated rats; #P < 0.05, ##P < 0.01 versus rats in the blank group.
model of PCOS. Similarly, mRNA levels of fibronectin and angiotensin-II were also increased.

In the last few decades, extensive studies have been carried out on the role of many cytokines and growth factors that can contribute to fibrosis. Among them, TGF-β and CTGF are considered the key mediators of fibrosis. Many studies based on animal models support an important role of TGF-β in the induction of fibrosis. TGF-β over-expression in various tissues results in overwhelming fibrosis. Transient over-expression of porcine TGF-β1 in rat lungs using the adenovirus transfection system induced prolonged lung fibrosis (Sime et al., 1997). Expressing elevated levels of active TGF-β1 in the heart by genetic modification results in atrial fibrosis (Nakajima et al., 2000; Verheule et al., 2004). Consistently, TGF-β inhibition attenuates fibrosis progression of various organs and tissues, for example the heart (Kuwahara et al., 2002). The most widely recognized pathway involved in the pathogenesis of fibrosis in various tissues was the canonical TGF-β/Smad3 pathway (Zhao et al., 2002). Much attention has recently been focused on CTGF which was discovered a decade ago and considered as a downstream effector of TGF-β. TGF-β exerts its effect, at least in part, through the induction of its downstream mediator CTGF (Duncan et al., 1999), and CTGF

Figure 5  Metformin inhibits the DHEA-induced hyper-fibrosis in rat ovarian and uterine tissues. A dose of 30 mg/100 g body weight metformin was given by gavage to rats immediately after administration of DHEA daily for up to 35 consecutive days. (A and B) Detection of CTGF (A) and TGF-β (B) in ovarian tissues by western blot. Pooled quantification data and original representative blots demonstrated significant down-regulation of fibrosis in ovarian tissues of metformin-treated PCOS rats. (C and D) Detection of CTGF (C) and TGF-β (D) in uterine tissues by western blot. Pooled quantification data and original representative blots demonstrated remarkable down-regulation of fibrosis in uterine tissues of metformin-treated PCOS rats. Data are mean ± SEM. *P < 0.05, **P < 0.01 versus saline-treated rats following DHEA exposure; ***P < 0.01 versus DHEA-exposed rats.
could act as an enhancer of the TGF-β action (Mon et al., 1999). Consistently, our data suggested a role for a TGF-β- and CTGF-dependent pathway in the process of DHEA-induced ovarian and uterine fibrosis in vivo.

Fibrillins are ECM glycoproteins present in all connective tissues (Sakai et al., 1986). Genetic studies have revealed a significant linkage between D19S884, a microsatellite marker in an intron of the fibrillin-3 gene, and susceptibility to PCOS (Stewart et al., 2006), indicating an important role of fibrillin-3 in PCOS etiology. In addition, a study on bovine and human fetal ovaries and human adult ovaries has revealed that fibrillin-3 is highly expressed at a critical stage early in the development of human and bovine fetal ovaries, and expression declines during gestation to very low levels (Hatzirodos et al., 2011). However, fibrillin-3 is localized only in the perifollicular stroma of primordial and primary follicles and follicles demonstrating morphological transition between them in adult ovaries, and ovaries of patients with PCOS have a significantly lower...

**Figure 6** Simvastatin inhibits the DHEA-induced hyper-fibrosis in ovarian and uterine tissues in rats. A dose of 1 mg/100 g body weight simvastatin was given i.p. immediately after administration of DHEA daily for up to 35 consecutive days. Thereafter, ovaries and uteri were harvested for western blotting assay. (A and B) Detection of CTGF (A) and TGF-β (B) in ovarian tissues by western blot. Pooled quantification data and original representative blots demonstrated down-regulation of fibrosis in ovarian tissues of simvastatin-treated PCOS rats, although reduction in CTGF protein did not reach a statistically significant level. (C and D) Detection of CTGF (C) and TGF-β (D) in uterine tissues by western blot. Pooled quantification data and original representative blots demonstrated down-regulation of fibrosis in uterine tissues of simvastatin-treated PCOS rats, although reduction in CTGF expression did not reach a statistically significant level. Data were presented as mean ± SEM. *P < 0.05 versus phosphate-buffered saline-treated rats following DHEA exposure; #P < 0.05 versus DHEA-exposed rats.
level of fibrillin-3-associated follicles (Jordan et al., 2010). Though not completely consistent, the two studies above suggest an important role of fibrillin-3 in follicle genesis and PCOS development. Given that fibrillins can regulate TGF-β activity through interaction with LTBP5s (Massam-Wu et al., 2010) and TGF-β can modulate fibrillin expression in vitro (Kenney et al., 2003), the existence of a strong association between TGF-β and PCOS as demonstrated in our present study is not a surprise. TGF-β and fibrillin-3 might interact to regulate stroma formation, thus facilitating the development of PCOS. Evidence from both hyperandrogenism and hyperinsulinaemia, the two most important factors in PCOS etiology, also strongly suggests ovarian fibrosis in PCOS (Pache et al., 1991; King et al., 2013). Significantly increased fibrosis levels in ovarian and uterine tissues of PCOS rats can possibly be compared with the numerous pathological changes in patients with PCOS. PCOS, which is always characterized by anovulation or oligo-ovulation, is a complex endocrine and gynecological disease with pathogenesis undetermined (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). In addition to the steroid endocrine abnormality that occurs in patients, here we propose that the histological changes of ovary might be partly responsible for the outcome of anovulation or oligo-ovulation. In our study in rats, the theca cell layer was thickened and an increased amount of collagen had accumulated around follicles, which might mechanically inhibit follicle rupture and ovulation. Patients with PCOS can have a higher rate of pregnancy loss (Khattab et al., 2006). Increased fibrosis in the uterus (and a thinner endometrium and a thicker myometrium) might partly affect the implantation process and thus lower the pregnancy rate.

Another important finding in our study was that both metformin and simvastatin, particularly the former, could attenuate DHEA-induced fibrosis. The protein levels of the two most important fibrotic factors, TGF-β and CTGF, were lowered by the treatment. Metformin, a potential drug for PCOS treatment, has recently been proposed to have an anti-fibrotic effect. In an experimental mouse model of left ventricular overload induced by transverse aortic constriction, metformin is shown to reduce the cardiac fibrosis area and inhibit pressure overload-induced TGF-β1 production in the mouse heart (Xiao et al., 2010). Meanwhile, metformin suppresses the phosphorylation of Smad3 in response to TGF-β1 in cultured cardiac fibroblasts (Xiao et al., 2010), suggesting that metformin exerts its anti-fibrotic effect via the TGF-β1/Smad3 signal pathway. Metformin is reported to improve the rate of ovulation, pregnancy and live births in patients with PCOS (Vandermolen et al., 2001; Morin-Papunen et al., 2012) but there is heated debate over whether it is time to recommend it for use in pregnancy. In addition to the beneficial effects on insulin resistance, hyperandrogenemia and obesity, does the anti-fibrotic effect of metformin contribute in part to the improvement of pregnancy in general? Of course to answer this question, rigorous experimental and clinical validation has to be carried out. Simvastatin, a member of the HMG-CoA reductase inhibitors, is demonstrated to inhibit the angiotensin-II-induced Smad activation in rat aorta via the intracellular signaling systems including ROCK and p38-MAPK (Ruperez et al., 2007; Rodrigues Diez et al., 2010). Coincidently, MAPKs are considered as one of the non-canonical signals involved in the fibrotic process and can be activated by TGF-β, suggesting a role of simvastatin in the process of fibrosis. In the study mentioned above (Rodrigues Diez et al., 2010), simvastatin exerts its beneficial effect in a TGF-β-independent manner; while pitavastatin, another kind of statin, is reported to decrease TGF-β production and Smad phosphorylation in the cardiac tissue, indicating a TGF-β-dependent mechanism (Yagi et al., 2008). Therefore, the underlying mechanisms by which statins affect fibrosis are still controversial. In the current study, we found that TGF-β, and CTGF to a lesser extent, were both down-regulated by simvastatin, suggesting that a TGF-β-dependent pathway was involved.

However, certain limitations may exist in our study. We did not determine the levels of Smad proteins and fibrosis-related molecules other than TGF-β and CTGF. Consequently, we could not determine whether the pathway involved in DHEA-induced fibrosis was Smad dependent or Smad independent. However, we confirmed that DHEA-induced PCOS rats developed higher levels of fibrosis in ovarian and uterine tissues in a TGF-β-dependent manner, and that both metformin and simvastatin could attenuate the pathological fibrotic process.

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Authors’ roles

X.Z. participated in the study design, method investigation, experiment performance and the preparation of the manuscript. C.Z. and S.S. performed the animal experiments, and C.Z. and Y.J.X. carried out the real-time RT-PCR analysis. X.Z. and C.Z. performed western blot analysis and did the statistical analysis together with L.Y. and Y.W. Q.G. and Y.W. were involved in the study design and the revision of the manuscript. All the authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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