Lysyl oxidase blockade ameliorates anovulation in polycystic ovary syndrome

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STUDY QUESTION: Is overexpression of lysyl oxidase (LOX), an enzyme responsible for the cross-linking of collagens, a cause of anovulation in polycystic ovary syndrome (PCOS)?

SUMMARY ANSWER: LOX overexpression was present in PCOS ovaries, due at least in part to interleukin-1β (IL-1β), and inhibition of LOX activity with β-aminopropionitrile (BAPN) ameliorated polycystic ovary morphology and anovulation.

WHAT IS KNOWN ALREADY: Aberrant ovarian extracellular matrix (ECM) remodeling and inflammation may contribute to the development of PCOS. It remains unknown whether proinflammatory IL-1β is a contributing factor for LOX overexpression in PCOS ovaries and whether inhibition of LOX can improve PCOS conditions.

STUDY DESIGN, SIZE, DURATION: LOX and IL-1β abundance in the granulosa cells and follicular fluid was compared between non-PCOS (n = 30) and PCOS (n = 39) patients. The effect and mechanism of IL-1β on LOX expression was examined in cultured primary human granulosa cells. The improvements in PCOS conditions by LOX inhibition with BAPN was investigated in a dehydroepiandrosterone (DHEA)-induced PCOS rat model.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The abundance of LOX and IL-1β was measured with quantitative real-time polymerase chain reaction (qRT-PCR), LOX activity assays and enzyme-linked immunosorbent assays (ELISA), respectively. The effect of IL-1β on LOX expression was examined in the presence or absence of inhibitors for signaling molecules and small interfering RNA-mediated knockdown of the putative transcription factor. Chromatin immunoprecipitation assays were conducted to further identify the responsible transcription factor. The role of LOX in ovulation was investigated in a DHEA-induced PCOS rat model with administration of the LOX inhibitor BAPN. The numbers of retrieved total oocytes and MII oocytes were recorded upon ovarian stimulation.

MAIN RESULTS AND THE ROLE OF CHANCE: Increased abundance of LOX (P < 0.05) and IL-1β (P < 0.05) was observed in the granulosa cells and follicular fluid in PCOS patients. IL-1β increased LOX expression via activation of ERK1/2 and JNK and subsequent activation of the transcription factor c-Jun. Inhibition of LOX with BAPN ameliorated irregular estrous cyclicity (P < 0.05), polycystic ovary morphology and anovulation (P < 0.05) in PCOS rats, but appeared to be ineffective in the improvement of oocyte quality.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Ovarian tissue-directed specific inhibition of LOX in combination with oocyte quality-improving drugs may be more effective in the treatment of PCOS.

WIDER IMPLICATIONS OF THE FINDINGS: Inflammation of the ovary is a contributing factor for the aberrant expression of LOX in the PCOS ovary, and inhibition of LOX together with anti-inflammatory therapy may improve the core features of PCOS.
Excessive LOX impairs ovulation in PCOS

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine diseases affecting reproductive-age women. Depending on the criteria used for definition and ethnicity of the disease, the prevalence of PCOS ranges from 9% to 18% (Azziz et al., 2004; March et al., 2010). The core features of PCOS include hyperandrogenism, oligomenorrhea/chronic anovulation and polycystic ovarian morphology. Among these, aberrant follicular development with accumulation of small antral follicles, failure in dominant follicle selection and consequent anovulation are central to the endocrine disorders and infertility associated with PCOS (Franks et al., 2000). Despite intensive research, the mechanisms underlying arrested follicular development and anovulation in PCOS remain mostly obscure.

In addition to the intimate interaction between the follicular cells (granulosa and theca cells) and the oocyte, successful development of ovarian follicles from primordial to early antral stages and beyond also requires a tightly coordinated interaction between the follicle and extracellular matrix (ECM). The ECM not only influences the structure and endocrine functions of follicular cells but also affects the process of ovulation (Richards et al., 1998; Woodruff and Shea, 2007; Young and McNeilly, 2010). Collagenous components of the ECM, such as collagen types I and IV, are crucial infrastructure of the ovarian stroma including the follicular basement membrane and theca luteal stroma (Auersperg et al., 1994; Huet et al., 1997; Rodgers et al., 2000; Lind et al., 2006). Collagen I is widely distributed in the ovary with high concentrations seen at the ovarian epithelial surface and in the follicular compartments (Berkholtz et al., 2006), while collagen IV is more concentrated in the follicular basement membrane, which undergoes dramatic remodeling during follicular development and in the process of ovulation, thus allowing adaptation of the ECM to the growing follicle and rupture of the follicle during ovulation (Harlow et al., 2003; Lind et al., 2006).

The tensile strength of collagen fibers depends not only on their abundance but also on their cross-linking which is catalyzed by lysyl oxidase (LOX), a copper-dependent amine oxidase (Erl er et al., 2009). Cross-linking not only strengthens collagen fibers but also enables them to be more resistant to protease degradation (Vater et al., 1979). Downregulation of LOX expression appears to be a prerequisite for the normal development of the follicle and ovulation (Harlow et al., 2003), whereas increased LOX expression and overcollagenized follicle cysts are often found in PCOS (Fienberg, 1963; Henmi et al., 2001; Papachroni et al., 2010). However, it remains unknown whether blockade of LOX can improve ovulation in PCOS and what causes the aberrant expression of LOX in the ovary in PCOS.

Accumulating evidence indicates that chronic low-grade inflammation is present in the ovary in PCOS, which is believed to be a contributing factor of ovarian dysfunction (Gonzalez et al., 2006; Gonzalez, 2012). Several proinflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), have been demonstrated to be increased in the follicular fluid in PCOS (Pellicer et al., 1999; Gonzalez, 2012). Of note, IL-1β has been shown to inhibit rather than stimulate LOX expression in human amnion and synovia fibroblasts and aortic smooth muscle cells (Aoki et al., 2009; Zhang et al., 2017a, 2017b). These observations are contradictory to what would be expected since enhanced LOX expression is seen in the ovary in PCOS. By contrast, there are also reports that IL-1β stimulates LOX expression in ligament fibroblast cells (Xie et al., 2013). Therefore, it is necessary to clarify the effects of IL-1β on LOX expression in ovarian cells in order to better understand whether chronic inflammation is a contributing factor of LOX overexpression and excessive collagen deposition in PCOS. We anticipate that cell-specific upregulation of LOX expression by IL-1β in ovarian granulosa cells may contribute to excessive ovarian collagen deposition in PCOS, and inhibition of LOX may ameliorate anovulation in PCOS. Here, we examined this hypothesis in samples from patients with PCOS and in a rat model of PCOS.

Materials and Methods

Collection of follicular fluid and granulosa cells from human subjects

Ovarian granulosa cells and follicular fluid were collected at oocyte retrieval from PCOS and non-PCOS patients who underwent IVF or ICSI at the Center for Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai jiao Tong University. The samples were collected under a protocol approved by the Ethics Committee of Ren Ji Hospital, with informed consent from the patients. In order to minimize the heterogeneity of PCOS patients, patients meeting all the three criteria (oligo- and/or anovulation, hyperandrogenism and polycystic ovaries) according to the revised Rotterdam consensus (Rotterdam, 2004) were recruited into the PCOS group. Women with tubal factor and/or male factor-related infertility were enrolled as the non-PCOS group. The basal serum levels of hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), estradiol (E2), prolactin (PRL) and anti-Mullerian hormone (AMH) were determined using chemiluminescence assay kits (Beckman Access Health Co.) and a standard glucose oxidase method (Beckman Access Health Co.), respectively. The homeostasis model of assessment for insulin resistance (HOMA-IR) (fasting serum insulin [μIU/ml] × fasting serum glucose [mmol/L] /22.5) was calculated.

Ovarian stimulation and oocyte retrieval were performed under the same GnRH antagonist protocol for both non-PCOS and PCOS groups. After adequate follicle development, ovulation was triggered with hCG (Livzon Pharmaceutical Group Co., Guangdong, China). Upon oocyte retrieval, the follicular fluid was collected from the size-matched dominant follicle (18–20 mm) and the granulosa cells in the follicular fluid were pelleted followed by purification with Ficoll-Paque (GE Healthcare

**Key words:** polycystic ovary syndrome / lysyl oxidase / extracellular matrix / anovulation / interleukin-1β / granulosa cells

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Bio-Sciences, NJ, USA) density centrifugation. After digestion with hyaluronidase (Sigma, Chemical Co., St Louis, MO, USA), cells from a single subject were used for RNA extraction without culture and cells from three or four normal subjects were pooled for culture in Dulbecco’s modified Eagle medium/Ham’s F12 (DMEM/F12) containing charcoal-stripped 10% fetal bovine serum (FBS) (Gibco) and antibiotics (Invitrogen, CA, USA). The identity of granulosa cells was verified with immunofluorescence staining for the FSH receptor (Fig. S1). The follicular fluid without cells was stored at −80°C for later determination of LOX activity and IL-1β levels.

Treatment of human granulosa cells

Three days after plating, the cells were treated with IL-1β (Sigma) to study the effects of IL-1β on LOX abundance and phosphorylation of mitogen-activated protein kinases (MAPKs) and c-Jun. The concentrations of IL-1β and incubation time for each study are given in the corresponding figures.

The involvement of MAPKs in the regulation of c-Jun phosphorylation and LOX expression by IL-1β (0.1 ng/ml) was examined in the presence or absence of SB203580 (5 μM, a p38 inhibitor), PD98059 (2 μM, an Erk1/2 inhibitor) and SP600125 (5 μM, a JNK inhibitor) (all from Selleckchem, Houston, TX, USA).

To study the role of c-Jun, small interfering RNA (siRNA) (GenePharma Co., Shanghai, China) against c-Jun (5′-GAAAGUCAUGAACCACGUUTT-3′) was transfected into granulosa cells with electroporation. Granulosa cells were mixed with 50 nM siRNA in Opti-MEM (Life Technologies Inc., Grand Island, NY, USA).

Table I Demographic features of recruited subjects with and without PCOS.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Non-PCOS</th>
<th>PCOS</th>
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<tr>
<td>n</td>
<td>30</td>
<td>39</td>
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<tr>
<td>Age (y)</td>
<td>28.43 ± 2.50</td>
<td>27.82 ± 2.88</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>21.66 ± 2.69</td>
<td>23.44 ± 2.91*</td>
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<tr>
<td>Cycle length (days)</td>
<td>28.67 ± 1.52</td>
<td>56.72 ± 22.29*</td>
</tr>
<tr>
<td>Basal antral follicle count</td>
<td>14.23 ± 4.07</td>
<td>27.67 ± 6.33*</td>
</tr>
<tr>
<td>Basal FSH (mIU/ml)</td>
<td>6.50 ± 1.12</td>
<td>6.28 ± 1.15</td>
</tr>
<tr>
<td>Basal LH (mIU/ml)</td>
<td>5.23 ± 1.33</td>
<td>8.56 ± 3.11*</td>
</tr>
<tr>
<td>Basal E₂ (pg/ml)</td>
<td>33.01 ± 22.88</td>
<td>34.79 ± 13.66</td>
</tr>
<tr>
<td>Basal T (nmol/l)</td>
<td>0.93 ± 0.49</td>
<td>1.70 ± 0.53*</td>
</tr>
<tr>
<td>Basal PRL (μg/l)</td>
<td>17.50 ± 5.51</td>
<td>18.17 ± 9.23</td>
</tr>
<tr>
<td>AMH (ng/ml)</td>
<td>5.47 ± 1.92</td>
<td>11.92 ± 5.19*</td>
</tr>
<tr>
<td>Fasting glucose, mmol/ml</td>
<td>4.76 ± 0.76</td>
<td>5.11 ± 0.55</td>
</tr>
<tr>
<td>Fasting insulin, IU/ml</td>
<td>6.07 ± 0.71</td>
<td>12.06 ± 6.19*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.29 ± 0.28</td>
<td>2.78 ± 1.52*</td>
</tr>
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Data are mean ± SD values.

*P < 0.05 vs. non-PCOS.

Figure 1 Changes of LOX abundance in the granulosa cells and follicular fluid from PCOS patients and in the ovaries of DHEA-induced PCOS rats. (A and B) LOX mRNA in ovarian granulosa cells and LOX activity in the follicular fluid from non-PCOS (n = 30) and PCOS (n = 39) patients. (C) Immunohistochemical staining of LOX in the ovaries from control and DHEA groups. The bar graph on the right is the semi-quantitative data of the immunohistochemical staining. (D) Western blotting analysis of LOX protein abundance in the ovaries from control (n = 9) and DHEA (n = 10) groups. Blots on the left are representative and the bar graph on the right is the average data. *P < 0.05, **P < 0.01 vs. non-PCOS patients (data are means ± SD) or control group (data are means ± SEM).
Island, NY) and the cells were electroporated at 175 V for 5 ms using the NEPA21 electroporator (Nepa Gene Co., Ltd., Chiba, Japan). Randomly scrambled siRNA served as the negative control. After dilution with DMEM/F12 containing charcoal-stripped 10% FBS, the cells were transferred to the culture plate to incubate for 72 h before IL-1β treatment.

### Measurement of LOX activity and IL-1β

LOX activity and IL-1β in the follicular fluid and LOX activity in the culture medium from granulosa cells were measured with an Amplite™ Fluorimetric Lysyl Oxidase Assay Kit (AAT Bioquest Inc., CA, USA) and an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, MN, USA), respectively, following the protocols provided by the manufacturers as described previously (Liu et al., 2016; Zhang et al., 2017a, 2017b).

### Extraction of RNA for quantitative RT-PCR

Total RNA was extracted from cells using a total RNA extraction kit (Foregene Co., Ltd., Sichuan, China) and mRNA was reverse-transcribed to cDNA using a PrimeScript RT Master Mix Perfect Real Time kit (TaKaRa, Dalian, China). The target mRNA was determined with quantitative real-time polymerase chain reaction (qRT-PCR) and β-actin was amplified in parallel for internal loading control. The absolute mRNA in each sample was calculated according to a standard curve set up using serial dilutions of known amounts of PCR products against corresponding cycle threshold (Ct) values. The primer sequences used for PCR are listed in Supplementary Table SI.

### Extraction of protein and analysis with western blotting

The cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Active Motif, Carlsbad, CA) containing a protease inhibitor cocktail (Sigma) and a phosphatase inhibitor (Active Motif). Protein (50 μg) from each

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**Table II** Correlation between LOX activity and PCOS characteristics.

<table>
<thead>
<tr>
<th>PCOS Characteristics</th>
<th>P</th>
<th>R²</th>
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<tbody>
<tr>
<td>Basal antral follicle count</td>
<td>&lt;0.001</td>
<td>0.2981</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>&lt;0.001</td>
<td>0.3152</td>
</tr>
<tr>
<td>Basal serum T</td>
<td>0.0610</td>
<td>0.0514</td>
</tr>
<tr>
<td>BMI</td>
<td>0.3064</td>
<td>0.0156</td>
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**Figure 2** Inhibition of LOX with BAPN improves irregular estrous cyclicity, LH/FSH ratio and polycystic ovary morphology in DHEA-induced PCOS rats. (A–D) Effects of DHEA and DHEA+BAPN on estrus cycles, the number of cycles completed in 10 days, plasma LH levels and the LH/FSH ratio (n = 10 for each group). (E) Ovarian morphology showing multiple cystic follicles (arrow) in the DHEA group but few in the DHEA+BAPN group. In addition, a few corpus lutea (CL, asterisk) were observed in the DHEA+BAPN group but not in the DHEA group. Images are representative of 10 animals for each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group. #P < 0.05, ##P < 0.01 vs. DHEA group and the data are means ± SEM. D, di-estrus; P, pro-estrus; E, estrus; M, met-estrus.
sample was electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose blot. After blocking, the blot was incubated with antibodies against LOX (Abcam), IL-1β (Proteintech, Wuhan, China), collagen IV A1 (Novus), collagen IV A5 (Proteintech), total p38, phosphorlylated p38 at Thr180/Tyr182, total Erk1/2, phosphorlyated Erk1/2 at Thr202/Tyr204, total JNK, phosphorlyated JNK at Thr183/Tyr185, total c-Jun and phosphorlyated c-Jun at Ser73 (all from Cell Signaling, MA, USA) at 1:1000 dilutions and were then followed by incubations with secondary antibodies. An enhanced chemiluminescent detection system (Millipore, Billerica, MA) was used to detect the bands. The same blot was probed for β-actin (1:1000, Proteintech) for internal loading control. The bands were visualized using a G-Box Chemi Chemiluminescence Image Capture System (Syngene, Frederick, MD, USA).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChiP) was performed to examine the binding of c-Jun to the LOX promoter after treatment with IL-1β (0.1 ng/ml, 12 h). The granulosa cells were fixed with 1% formaldehyde. After termination with glycine, the cells were lysed with 1% SDS supplemented with a protease inhibitor cocktail. The lysed cells were sonicated to shear the chromatin DNA. After preclangering, sheared DNA was immunoprecipitated with the antibody against c-Jun. Equal amounts of preimmune IgG served as the negative control. The immunoprecipitate was then incubated with Magna ChiP Protein A Agrose Magnetic Beads (Millipore) and pulled down using a magnetic stand. After reverse cross-linking and digestion with ribonuclease A/proteinase K, the sheared DNA was extracted and analyzed with qRT-PCR with the following primers amplifying the putative AP-1 binding site at the LOX promoter: 5′-CTTCCGATAAAGCGAGA-3′ (forward) and 5′-GCCAAACATGTGACGAGA-3′ (reverse). The same amount of sheared DNA without antibody precipitation served as the input control. The ratio of DNA precipitated by c-Jun antibody over input control was obtained to indicate the amounts of transcription factors bound to the LOX promoter.

**Establishment of the rat PCOS model**

All rat experimentation were conducted according to the accepted standards of animal care, which was approved by the Institutional Review Board of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. Female Sprague–Dawley rats (Jiesijie Laboratory Animal, Shanghai, China) received daily subcutaneous injection of dehydroepiandrosterone (DHEA) (Yangzhou Pharmaceutical Co., Ltd., Zhejiang, China) 48 h later. Cumulus–oocyte complexes were collected from the oviducts at 16 h after hCG injection. Oocytes were freed from cumulus cells by digestion with 0.1% hyaluronidase (Vitrolife, Fröklunda, Sweden) for 5 min. Oocytes that reached MII were recorded and images were taken using a Zeiss microscope. The granulosa cells were isolated following a protocol as previously described (Dou et al., 2016). The granulosa cells from 5–6 rats were pooled for culture. Three days after plating, the cells were treated with IL-1β (0.1 ng/ml) in DMEM/ F12 containing 10% FBS for 24 h to investigate its effect on LOX expression.

**Determination of LOX and collagen abundance in the ovary of PCOS rats**

Immunohistochemical staining was performed to observe LOX distribution using an antibody against LOX (1:100, Abcam), which was quantified with the Image-pro plus 6.0 (Media Cybernetics, Rockville, MD, USA) by analyzing the average optical densities of the positive spots of 10 visual fields of each section at 200x magnification.

For histological evaluation of collagen abundance in the ovary, collagen volume fraction (CVF) was determined by the analysis of Masson trichrome stained ovarian sections (Lu et al., 2012). The section was scanned to format an image with the Case Viewer (3DHISTECH Ltd., Budapest, HUNGARY), and then the image was analyzed with the Image-pro plus 6.0 (Lu et al., 2012). The same intensity of blue-stained interstitial collagen fibers were highlighted giving a pixel count for the amount of collagen represented in each section. This was then expressed as a percentage of the overall number of pixels present in the picture giving a percentage area of interstitial collagen. Follicular cavity and vascular areas were excluded from the region of interest.

For western blotting analyses of LOX and collagen IV, the ovary tissue was snap-frozen in liquid nitrogen and then homogenized in the RIPA buffer with a Tissue Lyser (Bi Heng Biotechnology Inc., Shanghai, China). After centrifugation, the supernatants were used for Western blotting.

**Oocyte retrieval and culture of rat granulosa cells**

After 20 consecutive days of DHEA injections, superovulation were induced by intraperitoneal injection (i.p.) of pregnant mare serum gonadotropin (PMSG, 300 IU/kg) followed by hCG injection (300 IU/kg) (Ningbo Sansheng Pharmaceutical Co., Ltd., Zhejiang, China) 48 h later. Cumulus–oocyte complexes were collected from the oviducts at 16 h after hCG injection. Oocytes were freed from cumulus cells by digestion with 0.1% hyaluronidase (Vitrolife, Frölunda, Sweden) for 5 min. Oocytes that reached MII were recorded and images were taken using a Zeiss microscope. The granulosa cells were isolated following a protocol as previously described (Dou et al., 2016). The granulosa cells from 5–6 rats were pooled for culture. Three days after plating, the cells were treated with IL-1β (0.1 ng/ml) in DMEM/ F12 containing 10% FBS for 24 h to investigate its effect on LOX expression.

**Statistical analysis**

All data are reported as means ± SD or SEM. The Kolmogorov–Smirnov test was used to determine whether the continuous variables were of normal distribution. Paired or unpaired Student’s t-test or one-way analysis of variance (ANOVA) test followed by the Student–Newman–Keuls multiple comparison test was used where appropriate to assess significant differences. Correlation between the variables was performed using Pearson analysis. Significance was set at P < 0.05.

**Results**

**Increased LOX abundance in PCOS**

The demographic features of recruited patients are given in Table I. Of the 69 recruited subjects, 30 were non-PCOS patients with tubal and/or male factor-related infertility, and 39 were PCOS patients. There were no significant differences between the two groups with regard to age and basal levels of FSH, E2 and PRL. Compared with non-PCOS patients, PCOS patients had significantly longer cycle lengths and
higher basal antral follicle counts, AMH, LH and T levels and body mass index (BMI). The fasting insulin levels and HOMA-IR were also significantly higher in PCOS patients than in non-PCOS patients.

LOX mRNA in ovarian granulosa cells (Fig. 1A) and LOX activity (Fig. 1B) in the follicular fluid were significantly higher in PCOS patients than in non-PCOS patients. Because the same ovarian stimulation protocol was used for both non-PCOS and PCOS groups, it is unlikely that the differences in LOX abundance are caused by the protocol. Correlation analysis revealed that LOX activity correlated positively with basal antral follicle count and HOMA-IR but not with basal serum T levels and BMI (Table II), suggesting that heterogeneity on these aspects may affect LOX abundance in PCOS.

Figure 3 Inhibition of LOX with BAPN improves anovulation in DHEA-induced PCOS rats. (A and B) Effect of DHEA and DHEA+BAPN on retrieved oocytes from ovarian stimulation. (A and C) Effect of DHEA and DHEA+BAPN on MII oocytes from ovarian stimulation. Black arrows indicate the MII oocytes and red arrows indicate the abnormal oocytes. n = 8–9 for each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group; #p < 0.05 vs. DHEA group and the data are means ± SEM.

Figure 4 Inhibition of LOX by BAPN reduces collagen deposition in the ovarian stroma in DHEA-induced PCOS rats. (A and B) Masson staining showed the distribution of blue-staining collagen in the ovaries from four groups of rats (n = 4 for each group). (C and D) Protein levels of collagen IV A1 and collagen IV A5 from ovaries of four groups of rats (n = 8–10 for each group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. DHEA group. Representative blots are shown and the data are means ± SEM.
To further investigate the role of LOX in PCOS, a DHEA-induced PCOS rat model was established, which was confirmed by irregular estrous cycles (Supplementary Fig. S2A), increased plasma LH levels and LH/FSH ratio (Supplementary Fig. S2B) as well as by ovarian morphological changes including multiple cystic follicles and few corpora lutea and mature follicles (Supplementary Fig. S2C). Immunohistochemical staining revealed intensive LOX staining in the granulosa cells and less staining in the theca cells and stroma of the ovary (Fig. 1C). Western blotting also showed that LOX protein abundance in the ovary was significantly increased in PCOS rats (Fig. 1D).

**Inhibition of LOX with BAPN ameliorates anovulation in the PCOS rat**

Treatment with BAPN improved the estrous cyclicity in 7 out of 10 PCOS rats (Fig. 2A and Supplementary Table SII). The number of estrous cycles completed in 10 days was increased by an average of 0.9 cycles by BAPN treatment when compared with the PCOS group (Fig. 2B). Moreover, BAPN also attenuated the increases in plasma LH levels and LH/FSH ratio (Fig. 2C and D) in PCOS rats. A few mature follicles and corpus lutea were observed in the ovary of BAPN-treated PCOS rats (Fig. 2E). The retrieved total and MII oocytes per rat upon...
ovarian stimulation were decreased, while abnormal oocytes were increased in PCOS rats (Fig. 3A–C, Supplementary Table SIII). BAPN ameliorated the decrease in retrieved total oocytes, but not the decrease in MII oocytes and the increase in abnormal oocytes (Fig. 3A–C, Supplementary Table SIII) in PCOS rats.

Masson staining revealed increased collagen abundance (blue) in the stroma surrounding the follicles and beneath the ovarian surface epithelium. The ovarian CVF was increased in PCOS rats, which was attenuated by BAPN (Fig. 4A and B). Western blotting revealed that the protein levels of collagen IV subunits (collagen IVA1 and collagen IVA5), the primary components of the follicular wall (Berkholtz et al., 2006), were significantly increased in the ovary of PCOS rats, and this was attenuated by BAPN as well (Fig. 4C and D).

**Upregulation of LOX by IL-1β in granulosa cells**

Western blotting showed that IL-1β protein abundance was significantly increased in the ovaries of PCOS rats (Fig. 5A). There was a positive correlation between IL-1β and LOX protein abundance in the rat ovaries (Fig. 5B). Consistently, PCOS patients had higher IL-1β levels in the follicular fluid (Fig. 5C) and IL-1β mRNA levels in the granulosa cells (Fig. 5D). The abundance of either LOX mRNA (Fig. 5E) in ovarian granulosa cells or LOX activity (Fig. 5F) in the follicular fluid was correlated positively with the abundance of IL-1β in the follicular fluid. In vitro studies showed that IL-1β increased LOX mRNA, protein and activity in a concentration-dependent manner in cultured human primary granulosa cells (Fig. 5G and H). Consistently, IL-1β also increased LOX protein abundance in the rat granulosa cells (Fig. 5I).

**Figure 6 Role of MAPK in the upregulation of LOX expression by IL-1β in human granulosa cells.** Time course effects of IL-1β (0.1 ng/ml) on the phosphorylation of (A) p38, (B) Erk1/2 and (C) JNK in human ovarian granulosa cells. (D–F) Effects of IL-1β (0.1 ng/ml, 24 h) on the protein abundance of LOX in the presence and absence of inhibitors for p38 (SB203580, 5 μM), Erk1/2 (PD98059, 2 μM) and JNK (SP600125, 5 μM). *P < 0.05, **P < 0.01 vs. controls; #P < 0.05 vs. IL-1β. Representative blots are shown and the data are means ± SEM from three or four experiments.

Role of MAPKs and c-Jun in the regulation of LOX by IL-1β in human granulosa cells

Time course studies showed that IL-1β (0.1 ng/ml) increased the phosphorylation of p38 (Fig. 6A), Erk1/2 (Fig. 6B), JNK (Fig. 6C) and c-Jun (Fig. 7A) in a time-dependent manner in human primary granulosa cells with the maximal phosphorylation observed at 15 min. The upregulation of LOX (Fig. 6D–F) and phosphorylation of c-Jun (Fig. 7B) by IL-1β (0.1 ng/ml, 24 h) were partially blocked by inhibitors for Erk1/2 (PD98059, 2 μM) and JNK (SP600125, 5 μM), but not by the inhibitor for p38 (SB203580, 5 μM). Moreover, small interfering RNA (siRNA)-mediated knockdown of c-Jun blocked the induction of LOX expression by IL-1β in granulosa cells (Fig. 7C). Bioinformatics analysis of LOX promoter revealed binding sites for AP-1 spanning the region between −708 and −696 bp (Fig. 7D). ChIP assays showed that IL-1β increased the enrichment of c-Jun at this region of the LOX promoter.
These data suggest that activation of Erk1/2 and JNK pathways is responsible for the phosphorylation of c-Jun and the subsequent induction of LOX transcription by IL-1β in granulosa cells.

**Discussion**

Although humans and rats belong to the monoovulatory and polyovulatory species, respectively, it appears that the ECM remodeling is crucial in the follicle development and ovulation in both humans (Yamada et al., 1999; Iwahashi et al., 2000; Papachroni et al., 2010; Heeren et al., 2015) and rats (Rajah and Sundaram, 1994; Frojdman et al., 1998; Slee et al., 2001). Hence, we believe that the PCOS rat model may recapitulate the human PCOS situations, including but not limited to ECM remodeling in the ovary. Indeed, the abundance of LOX was increased in the ovaries of both PCOS patients and rats in the present study.

In the current study, we revealed an important role of LOX, the enzyme which crosslinks collagens in PCOS etiology. We found that excessive LOX expression is present in the PCOS ovary, and inhibition of LOX activity with BAPN ameliorates irregular estrous cyclicity, polycystic ovary morphology and anovulation in PCOS. Since cross-linked collagens not only strengthen the mesenchymal tissue but are also resistant to the degradation by matrix metalloproteases (MMPs) (Casey and MacDonald, 1996), overexpression of LOX may thus lead to excessively tensile collagen deposition in the ovarian stroma, which may jeopardize the development of the dominant follicle and subsequent ovulation in PCOS. Of note, we found that inhibition of LOX with BAPN improved ovulation in PCOS, but not the quality of oocytes, suggesting that follicle development is a

**Figure 7** Role of c-Jun in the upregulation of LOX expression by IL-1β in human granulosa cells. (A) Time course effects of IL-1β (0.1 ng/ml) on the phosphorylation of c-Jun in human ovarian granulosa cells. (B) Effects of IL-1β (0.1 ng/ml, 15 min) on the phosphorylation of c-Jun in the presence and absence of inhibitors for p38 (SB203580, 5 μM), Erk1/2 (PD98059, 2 μM) and JNK (SP600125, 5 μM). (C) Effect of IL-1β (0.1 ng/ml, 24 h) on LOX protein levels in the presence and absence of siRNA-mediated knockdown of c-Jun in human granulosa cells. (D) The diagram shows the putative binding sites of AP-1 in the LOX promoter region. The arrows between P1 and P2 indicate the primer aligning positions in the ChIP assay used in this study. TSS, transcription start site. ChIP assays detected the enrichment of c-Jun at the AP-1 binding site of the LOX promoter in response to IL-1β (0.1 ng/ml, 12 h) treatment in human ovarian granulosa cells. IgG served as the negative control. *P < 0.05, **P < 0.01, ***P < 0.001 vs. controls; ##P < 0.01, ###P < 0.001 vs. IL-1β. Representative blots are shown and the data are means ± SEM from three or four experiments.
complex process under the fine control of multiple factors, and a single intervening approach may not be enough to correct any abnormalities.

Our results showed that proinflammatory cytokines, such as IL-1β, may contribute, at least in part, to the increased expression of LOX in PCOS ovaries. Accumulating evidence indicates that low-grade chronic inflammation is a contributing factor to the development of PCOS (Kelly et al., 2001). Proinflammatory cytokines such as IL-1β, TNF-α and IL-6 have been shown to increase in the follicular fluid of PCOS patients (Pellicer et al., 1999; Gonzalez, 2012). Findings in PCOS patients in this study are in line with such a role of IL-1β in the etiology of PCOS. Apart from the regulation by proinflammatory factors, LOX is also under the regulation of a number of endocrine factors in the ovary. FSH not only inhibits LOX activity by itself but also reverses the stimulating effects of TGF-β on LOX activity, and these effects are known to be associated with the normal development of follicles (Harlow et al., 2003). To this end, the upregulation of LOX expression in the ovary of PCOS patients may be a consequence of actions of multiple factors including proinflammatory cytokines and FSH. So, we speculate that impaired FSH functions, documented in granulosa cells (Oktay et al., 1997; Jonard and Dewailly, 2004; Pellatt et al., 2011), may contribute to the enhanced LOX expression at least in some of the PCOS patients despite the reports of normal or even enhanced FSH functions in some PCOS patients (Mason et al., 1994; Rosencrantz et al., 2010). These discrepancies in FSH functions may also contribute to the heterogeneity of LOX abundance in PCOS patients.

BAPN is a specific and irreversible inhibitor of LOX activity (Tang et al., 1983; Rodriguez et al., 2008). Because LOX knockout mice are lethal (Maki et al., 2002), currently researchers have to resort to this inhibitor to investigate the physiological and pathological functions of LOX (Levental et al., 2009; Koopathali et al., 2012; Nave et al., 2014). However, its wasting effects on muscle and bone over prolonged systemic usage (Ahsan et al., 1999; Turecek et al., 2008) have limited its therapeutic use. Hence, ovarian tissue-directed specific inhibition of LOX in combination with oocyte quality-improving drugs may become more fruitful approaches for treatment of PCOS in the future.

In conclusion, we have demonstrated in this study that overexpression of LOX is a contributing factor to acyclicity, polycystic ovary morphology and anovulation in PCOS patients, and low-grade chronic inflammation of the ovary accounts, at least in part, for the overexpression of LOX. Inhibition of LOX activity may provide a potential effective therapeutic approach to the treatment of PCOS.

**Supplementary data**

Supplementary data are available at Human Reproduction online.

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**Authors’ roles**

C.Y.Z. and K.S. designed the study. C.Y.Z., J.M. and W.S.W. produced the data. C.Y.Z. and K.S. analyzed the data. C.Y.Z. and Y.S. analyzed the clinical data. C.Y.Z. and K.S. contributed to the manuscript writing.

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**Conflict of interest**

All authors declare no conflict of interest in relation to this work.

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