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Correlations between beclin-1 and transforming growth factor-β1 in letrozole induced polycystic ovary syndrome

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In the pathogenesis of polycystic ovary syndrome (PCOS), despite the importance of autophagy and transforming growth factor-β1 (TGF-β1), there is scarce information about their inter-relationship. Therefore, here we assessed the correlations between beclin-1, a cornerstone in autophagy, and TGF-β1 in a letrozole-induced PCOS rat model. Accordingly, a total of 45 female adult albino Wistar rats were randomly assigned into control, vehicle (carboxymethyl cellulose), and PCO groups. To establish the PCOS model, letrozole (1.0 mg/kg body wt., p.o.) was given once daily for three successive weeks. Circulating levels of luteinizing hormone, follicle-stimulating hormone, testosterone, estradiol, and progesterone were assayed along with ovarian total antioxidant capacity (TAC), protein carbonyl content (PCC), beclin-1 level, and TGF-β1 level. Ovarian morphology and ultrastructure were examined by hematoxylin and eosin staining and electron microscopy, respectively. Compared to control groups, hormonal levels and ovarian morphology in the letrozole-exposed animals indicated the successful construction of the PCOS model. Further, the PCO group exhibited an oxidative stress status reflected by a significant decrease in ovarian TAC and a significant elevation in the PCC. Moreover, ovarian beclin-1 and TGF-β1 levels were significantly increased with an enhancement of autophagy as revealed by electron microscopy. In multiple linear regression models, only TGF-β1 was observed in the final model where it explained the 62.3% variability of Beclin-1. In conclusion, the ovarian level of TGF-β1 might be a determinant factor of beclin-1 level in PCOS which may provide new insight into the pathophysiology and therapy of the disease.

Keywords: Autophagy, Estradiol, Follicle-stimulating hormone, Luteinizing hormone, Oxidative stress, Progesterone, Protein carbonyl content, Reactive oxygen species (ROS), Testosterone, Total antioxidant capacity

Polycystic ovary syndrome (PCOS) is a prevalent condition affecting up to 20% of reproductive-aged women. It is characterized mainly by chronic anovulation, polycystic ovaries, and hyperandrogenism. With multiple faceted endocrinal, reproductive, metabolic, and psychological features, PCOS puts a significant strain on healthcare resources which might be attributed to unsatisfactory management[1].

PCOS has been strongly related to oxidative stress (OS) with subsequent deleterious alterations in the cell signaling process, excessive apoptosis of granulosa cells, and infertility[2]. Increased total oxidant status and oxidative stress index alongside decreased total antioxidant status have been reported previously in PCOS patients compared to controls suggesting the crucial role of OS in PCOS pathogenesis[3].

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Reactive oxygen species (ROS), the direct triggers of OS, were shown to be the main inducers of autophagy *via* mediating various signaling pathways in the process of autophagosome formation[4]. Autophagy is involved in the physiological and pathological processes of reproduction[5]. In PCOS, Li *et al*[6] demonstrated that autophagy was activated in the ovarian tissues of diseased humans and rats compared to the corresponding controls. Further, it was reported previously that the accumulation of autophagosomes could induce apoptosis of granulosa cells that may, in turn, account for anovulation and steroidogenesis observed in PCOS[7].

Beclin-1, encoded by the *BECN1* gene, is one of a limited number of AuTophaGy-related (ATG) genes that highly regulate autophagy[8]. Beclin-1 is the mammalian ortholog of the Atg6 protein in yeast required to initiate autophagy because of its importance for the localization of autophagic proteins to a pre-autophagosomal structure[9].

Transforming growth factor-beta1 (TGF-β1) is a multifunctional cytokine engaged in the molecular regulation of several reproductive processes spanning oocyte development and ovulation, early embryogenesis, embryo implantation, and placental morphogenesis[10]. Further, the role of TGF-β1 in the pathogenesis of PCOS and reproductive dysfunction has been demonstrated in knockout mice[11].

The potential cross-talk between autophagy and TGF-β has received a great deal of attention in different diseases[12-14], but this is not the case with PCOS. Thus, in the current study, we investigated the relationship between TGF-β1 and beclin-1, as a cellular autophagy marker, in a letrozole-induced PCOS model to provide an impetus for identifying therapeutic strategies in the management of the disease.

Material and Methods

Chemicals

Carboxymethyl cellulose (CMC) was purchased from Sigma-Aldrich Co (MO, USA), and Letrozole was purchased as Femara® tablets manufactured by Novartis Pharma (Stein, Switzerland). All other chemicals and solvents used were of the highest purity grade available.

Animals

Forty-five female adult albino Wistar rats aged 6 weeks and with average weights of 180 g were obtained from the Laboratory Animal Research House, Medical Research Center, Faculty of Medicine, Ain Shams University, Cairo, Egypt. Animals were housed in a well-ventilated room with a 12/12 h light/dark cycle in polypropylene cages at 25±2°C where they were kept on a standard diet and provided with food and water *ad libitum*. Animals were adapted to the laboratory conditions for one week before experimentation.

Experimental design

Animals were randomly divided into three groups, each of fifteen animals, as follows: Group I served as a normal control group, Group II (vehicle control group) received 1% of CMC aqueous solution (2.0 mL/kg body wt.) once daily per oral route (p.o.) for three successive weeks, and Group III (PCO group) administered letrozole dissolved in 1% CMC at a dose of 1.0 mg/kg body wt. once daily p.o. for three successive weeks[15].

Twenty-four hours after receiving the last dose, rats were anesthetized with urethane after an overnight fasting period. Blood was collected by the retro-orbital puncture into a vacutainer tube with clot activator, left to clot for 10 min, and centrifuged at 3000 rpm for 10 min to separate serum which was subsequently divided into several aliquots and stored at −70°C for further use. After the completion of blood sampling, animals were sacrificed by cervical dislocation and laparotomy was performed to expose the abdominal viscera. The gut was displaced to reveal the ovaries that were excised, immediately cleaned of fat, washed with saline, dried with filter papers, and weighed. Ovaries were homogenized in ice-cold phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffered with 0.15 M sodium chloride, pH 7.4) to form 10% w/v homogenate for biochemical assays. A small part of the ovaries was fixed in aqueous Bouin's fluid for histopathological examination, whereas a third part was cut into ~1 mm sections and fixed in 4% buffered glutaraldehyde solution for electron microscopy.

Hormonal analyses

Serum levels of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were quantitatively determined using commercial competitive enzyme-linked immunosorbent assay (ELISA) kits (Cat# CSB-E06869r, and Cat# CSB-E12654r, respectively) according to manufacturer's instructions (Cusabio, TX, USA). The lower limit of detection (LLD) of the assays was <0.07 mIU/mL for FSH and <0.15 mIU/mL for LH. The intra-assay and inter-assay coefficients of variation (CV) of the assays were <15%. The test was performed on Stat Fax® 303 plus microstrip reader (Awareness Technology Inc., FL, USA).

Serum levels of gonadal hormones including total testosterone, estradiol (E2), and progesterone were quantitatively determined by the VIDAS® immunoassay kits based on enzyme-linked fluorescent assay (ELFA) (BioMérieux Diagnostics, Marcy-l'Étoile, France) using VIDAS® Testosterone II kit, VIDAS® Estradiol II kit, and VIDAS® Progesterone kit, respectively. The lower limit of quantitation (LLoQ) of the testosterone assay was 0.05 ng/mL while the functional detection limit of the E2 assay was 25 pg/mL and the detection limit of the progesterone assay was 0.25 ng/mL. The intra-assay and inter-assay CV of the assays were <8% and <10%, respectively. The test was performed on VIDAS® 30 immunoassay system (BioMérieux Diagnostics, NC, USA).

Assessment of ovarian oxidative stress

Ovarian homogenates were used to estimate oxidative stress parameters. Total antioxidant capacity (TAC) and the protein carbonyl (PC) content, the most commonly used marker of protein oxidation caused by ROS, were determined in rat ovarian tissues using colorimetric assay kits (Cat# ab65329 and Cat#ab126287, respectively) provided by Abcam. Protein concentration was determined in rat ovarian tissues using a colorimetric assay kit (Cat# ab102535) provided by Abcam (MA, USA) based on the method of Bradford.

Determination of beclin-1 and TGF-β1 levels

Ovarian beclin-1 and TGF-β1 levels were determined using sandwich ELISA kits (Cat# CSB-EL002658RA and Cat# CSB-E04727r, respectively) according to the manufacturer's instructions (Cusabio, TX, USA). The test was performed on Stat Fax® 303 plus microstrip reader (Awareness Technology Inc., FL, USA).

Protein-protein interaction network analysis

To construct the protein-protein interaction (PPI) network, beclin-1, TGF-β1, and their connected proteins in autophagy and TGF-β1 signaling pathway were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) under the entry ID of map04140 and hsa04350, respectively (Supplementary figure 1). The protein set was then imported into the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string.embl.de/) to build up and visualize the interaction network using a minimum required interaction score of medium confidence ≥0.4.

Histopathology

Immediately after dissection, autopsy samples were taken from ovarian tissues, cut into small pieces, and fixed in freshly prepared aqueous Bouin's fluid for 24 h. Samples were then dehydrated in graded concentrations of alcohol, cleared in terpineol, and embedded in parablast. Blocks were cut at 5 μm thicknesses on a rotary microtome, mounted on slides, and stained with hematoxylin and eosin. Slides were evaluated for histological changes under light microscopy.

Electron microscopy

Small pieces of the rat ovary were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. The tissues were then washed in 0.1 M phosphate buffer containing 5% sucrose and post-fixed in Osmium tetraoxide (OsO_4) for 2 h at 4°C. After that, the samples were dehydrated in an ascending ethanol series, treated with propylene oxide, infiltrated, and embedded in epoxy resin at 60°C. Semi-thin sections (1 μm) were cut with the RMC-MT7 ultra-microtome (Boeckeler Instruments Inc., AZ, USA), stained with toluidine blue, examined, and photographed. Ultra-thin sections were cut, stained with uranyl acetate and lead citrate, and then examined under the JEOL 1200EX II transmission electron microscope (JEOL Ltd., Tokyo, Japan) at the Electron Microscope Unit, The Central Laboratory, Faculty of Science, Ain Shams University.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM Corp, NY, USA). All data were expressed as mean±SEM. Statistical significance of differences among the different studied groups was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Pearson's correlation analysis was used to assess the relationship between two variables. After a significant crude correlation was found, multiple linear regression models with a subsequent backward stepwise procedure were used to assess the association of beclin-1 with different parameters. All stepwise selection models used a P <0.05 level for entry and a P <0.10 for removal; the variables to be included in these models were based on previous simple regression analyses. All P values were two-sided and a P value <0.05 was taken as a criterion for a statistically significant difference.

Results

Hormonal levels

Among the hormonal analytes, the serum levels of LH and testosterone turned out to be significantly elevated in letrozole-induced group compared to normal control (by 1.34 fold and 9.2 fold, respectively; P <0.001) as well as to CMC vehicle group (by 1.65 fold and 5.37 fold, respectively; P <0.001).

Meanwhile, serum levels of FSH, progesterone, and estradiol were significantly decreased in PCOS animals compared to both the control group (by 33.85%, p=0.031, 41.96%, p=0.006, and 46.44%, p=0.010; respectively) and the CMC vehicle group (by 30.98%, p=0.047, 39.43%, p=0.008, and 40.59%, p=0.033; respectively) (Table 1).

Table 1 — Hormonal and biochemical characteristics of the studied groups

	Control group	Vehicle (CMC) group	PCO (Letrozole) group
FSH (mIU/mL)	1.92±0.17	1.84±0.21	1.27±0.11[a,b]
LH (mIU/mL)	0.92±0.11	0.8±0.1	2.12±0.19[a,b]
Testosterone (ng/mL)	0.05±0.00	0.05±0.00	0.51±0.09[a,b]
Estradiol (pg/mL)	30.17±5.52	27.20±1.76	16.16±2.00[a,b]
Progesterone (ng/mL)	6.22±0.81	5.96±0.49	3.61±0.36[a,b]
Ovarian PCC (nmol/mg protein)	17.61±0.68	18.07±1.40	23.33±0.95[a,b]
Ovarian TAC (µmol Trolox/mg protein)	4.509±0.416	4.343±0.340	3.307±0.169[a,b]
Ovarian Beclin-1 (ng/mg tissue)	2.00±0.11	2.00±0.08	2.45±0.07[a,b]
Ovarian TGF-β1 (Pg/mg tissue)	90.42±4.86	86.74±4.62	159.00±5.93[a,b]

[Data are expressed as mean±SEM. n=15 for each experimental group. CMC: Carboxymethyl cellulose, PCO: Polycystic ovary, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, PCC: Protein carbonyl content, TAC: Total antioxidant capacity, TGF-β1: Transforming growth factor-beta1. In multiple comparison, [a]P <0.05 *vs.* normal control group and [b]P <0.05 *vs.* vehicle group]

Oxidative stress parameters

Although the ovarian PC content was significantly increased in PCO group in comparison with normal and CMC vehicle groups (23.33±0.95 *vs.* 17.61±0.68 and 18.07±1.40 nmol/mg protein, respectively; p=0.003), letrozole produced only a slightly significant decrease in TAC in the ovarian tissue of the induced group by 26.61% (p=0.027) and 23.73% (p=0.044) compared to normal and CMC vehicle groups, respectively (Table 1).

Ovarian levels of beclin-1 and TGF-β1

Table 1 shows that the ovarian level of beclin-1 in the PCO group was significantly increased by 22.50% compared to control and CMC vehicle groups (2.45± 0.07 ng/mg tissue *vs* 2.00±0.11 and 2.00±0.08 ng/mg tissue, respectively; P <0.001). In the same context, a significant increase in the ovarian TGF-β1 level by 75.85% was observed in the PCO group compared to the control group (159.00±5.93 pg/mg tissue *vs.* 90.42±4.86 pg/mg tissue, P <0.001) and 83.31% (159.00±5.93 pg/mg tissue *vs* 86.74±4.62 pg/mg tissue, P <0.001) compared to the CMC vehicle group.

Correlation between beclin-1 and various factors

The increased beclin-1 level in ovarian tissue of PCOS animals correlated positively and significantly with TGF-β1 whereas, it showed a negative significant correlation with TAC (Table 2). When a significant crude correlation was found, multiple linear regression models with a subsequent backward stepwise procedure were used to assess the association between beclin-1 and different parameters. Table 3 shows the initial model, an intermediate model, and the final model of the multiple linear regression models. The final model revealed just one relationship; it was a positive relationship between beclin-1 and TGF-β1 that explained the 62.3% variance in beclin-1.

Table 2 — Correlation analysis of Beclin-1 levels with biochemical parameters in the studied groups

Variable	Control		Vehicle (CMC)		PCO (Letrozole)	
	Ovarian Beclin-1 (ng/mg tissue)					
	r	p	r	p	r	p
Serum FSH (mIU/mL)	0.307	0.359	0.417	0.138	0.172	0.540
Serum LH (mIU/mlL)	0.267	0.428	0.359	0.207	0.059	0.835
Serum testosterone (ng/mL)	–0.055	0.872	0.424	0.141	–0.146	0.560
Serum progesterone (ng/mL)	0.046	0.894	0.198	0.497	0.116	0.681
Serum estradiol (pg/mL)	–0.102	0.765	0.554	0.040	0.062	0.825
Ovarian PCC (nmol/mg protein)	0.767	0.006	0.452	0.105	0.251	0.367
Ovarian TAC (µmol Trolox/mg protein)	–0.318	0.341	–0.172	0.557	–0.703	0.003
Ovarian TGF-β1 (pg/mg tissue)	0.892	<0.001	0.554	0.040	0.781	0.001

[CMC: Carboxymethyl cellulose, PCO: Polycystic ovary, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, PCC: Protein carbonyl content, TAC: Total antioxidant capacity, TGF-β1: Transforming growth factor-beta1]

Table 3 — Backward stepwise multiple regression models with Beclin-1 as a dependent variable

Variable	Initial model (All variables) R^2 = 0.651		Intermediate model R^2 = 0.648		Final model R^2 = 0.623	
	β	p	β	p	β	p
Ovarian PCC (nmol/mg protein)	0.029	0.851				
Ovarian TAC (µmol Trolox/mg protein)	–0.052	0.684				
Serum LH (mIU/mL)	–0.153	0.337	–0.157	0.313		
Serum testosterone (ng/mL)	–0.148	0.261	–0.155	0.225		
Ovarian TGF-β1 (pg/mg tissue)	0.974	<0.001	1.006	<0.001	0.790	<0.001

[PCC: Protein carbonyl content, TAC: Total antioxidant capacity, LH: Luteinizing hormone, TGF-β1: Transforming growth factor-beta1. Beclin-1 and TGF-β1 were independently and positively associated. TGF-β1 explains the 62.3% variability of Beclin-1. In the backward stepwise procedure, the variables were sequentially removed as follows: PCC, TAC, LH, and testosterone. Only one of the intermediate models (three variables) obtained through this procedure was reported]

Moreover, the integrated protein set obtained from KEGG was mapped into the PPI network complex using the STRING online database (Fig. 1). The results confirmed that the TGF-β1 signaling pathway interacts with autophagy *via* beclin-1 as a connection point (PPI enrichment *P* value <1.0e-16).

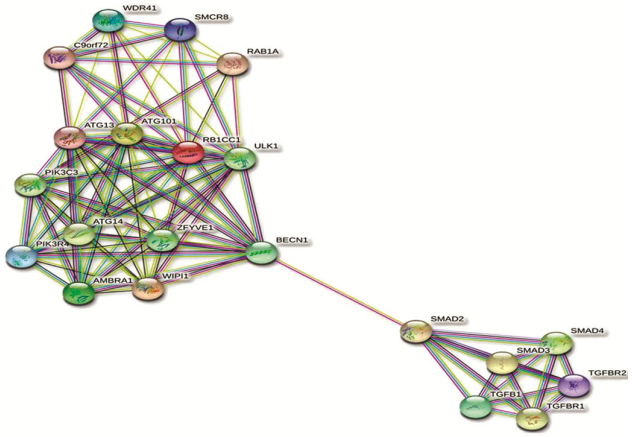

Fig. 1 — Protein-protein interaction (PPI) network between autophagy and TGF-β1 signaling pathway using STRING tool.

Histopathological examination

In the control and vehicle groups, ovaries exhibited a typically normal appearance with follicles and corpora lutea in different stages of development and regression (Fig. 2A). The ovaries of such groups had mostly primary, secondary, and tertiary follicles. The oocyte was surrounded by a single layer of cuboidal granulosa cells in the primary follicle; whereas, it was surrounded by a few layers of granulosa cells with no visible antral space in the secondary follicle. Moreover, the tertiary follicles were characterized by numerous layers of granulosa cells with small antral space (Fig. 2B).

On the other hand, follicular cysts in the ovarian surface were evident macroscopically in the letrozole-induced group. Further, the ovaries displayed typical PCO-like changes that are characterized by the presence of numerous atretic follicles (cysts) which were seen as fluid-filled sacs under the ovarian surface (Fig. 2C). In addition, the primary, secondary, and Graafian follicles, as well as corpora lutea, were significantly lower than those of the control and vehicle groups and had severe histopathological

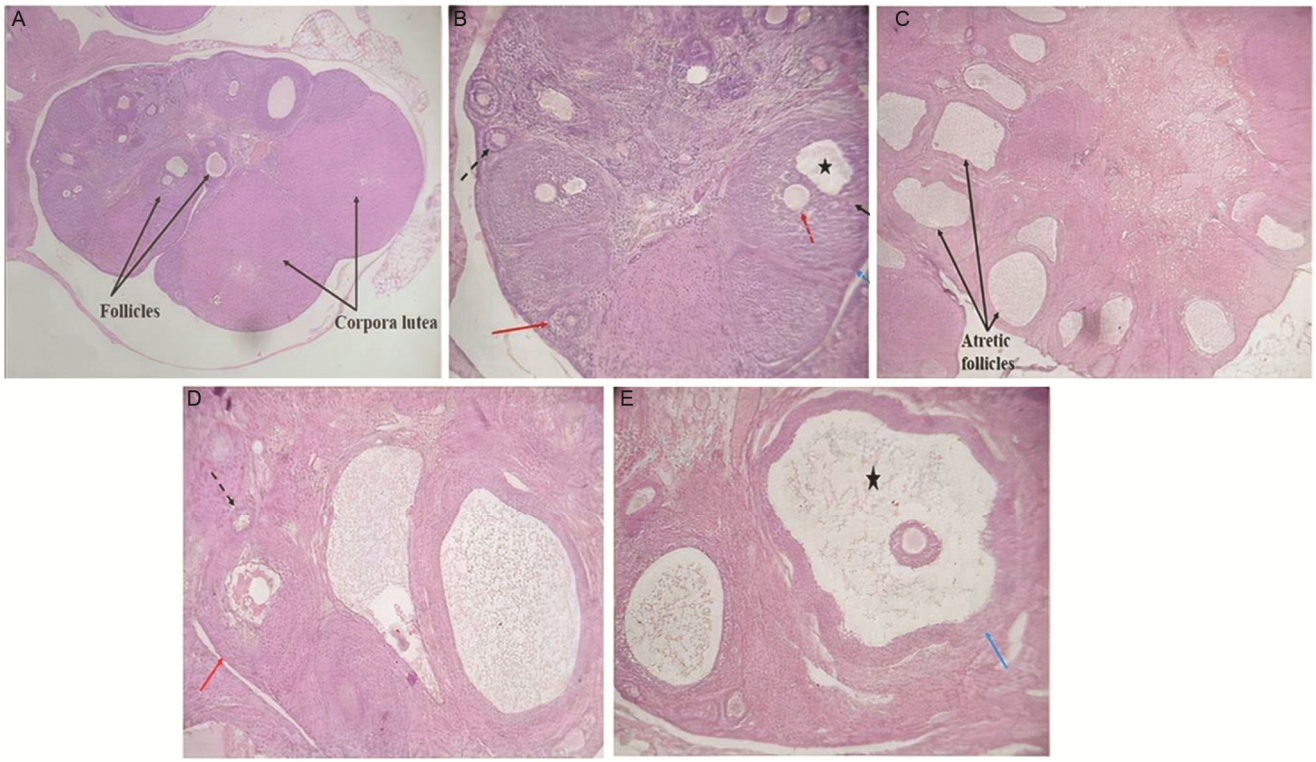

Fig. 2 — Photomicrographs of hematoxylin and eosin stained sections of ovarian tissues. [Ovaries of control groups exhibited a typically (A) normal appearance of follicles and corpora lutea (4X) ; (B) primary (➸), secondary (➞), and tertiary (⇢) follicles, in addition to granulosa cells (➞), antral space (★), and oocyte (⇢) (10X); and (C) Ovarian tissue in polycystic ovary (PCO) group showed a typical PCO with numerous atretic follicles (4X), altered primary (➸) and secondary (➞) follicles (D; 10X), and altered Grafiaan (➞) follicles (E; 10X)]

alterations (Fig. 2 D & E). Graafian follicles generally possessed a single large antral space of follicular fluid.

Ovarian ultrastructure

Results obtained from the electron microscopy showed that oocytes in the control and CMC vehicle groups conserved their junctions and maintained a close relationship with the granulosa cells (Fig. 3A). The oocytes' cytoplasm was homogeneous and contained mitochondria, endoplasmic reticulum, microtubules, and a nucleus with prominent nucleolus (Fig. 3 B & C).

In the PCO group, the oocytes in the cell death process had altered junctions with granulosa cells leading to the detachment of the latter from the wall of the former (Fig. 3D). Moreover, the chromatin in the nucleus began to condense (Fig. 3E). An interesting ultrastructural characteristic in the altered oocytes was the presence of numerous dispersed autophagic vesicles in the cytoplasm, which contain cytoplasmic structures in different phases of degradation (Fig. 3 F & G).

Discussion

Being the most widespread metabolic and endocrine disorder, PCOS has become immensely prevalent among women of fertile age[16]. Kumariya *et al*.[17] demonstrated that autophagy plays a significant role in the pathogenesis of PCOS. Moreover, the TGF-β1 signaling pathway has been associated with reproductive dysfunction[10]. Although autophagy can be induced by a variety of stimuli under different conditions, the relationship between TGF-β signaling and autophagy has been poorly understood, therefore, the current study aimed to explore the putative link between the cellular autophagy marker beclin-1 and TGF-β1 in a letrozole-induced PCOS model.

In the current study, the administration of letrozole caused a significant elevation in serum testosterone level accompanied by a significant decrease in serum estradiol level. This can be attributed to the blocking effect of letrozole, as a non-steroidal aromatase inhibitor, on the conversion of testosterone into estrogens[18]. Hyperandrogenism is the main characteristic of PCOS; it plays a pivotal role in the

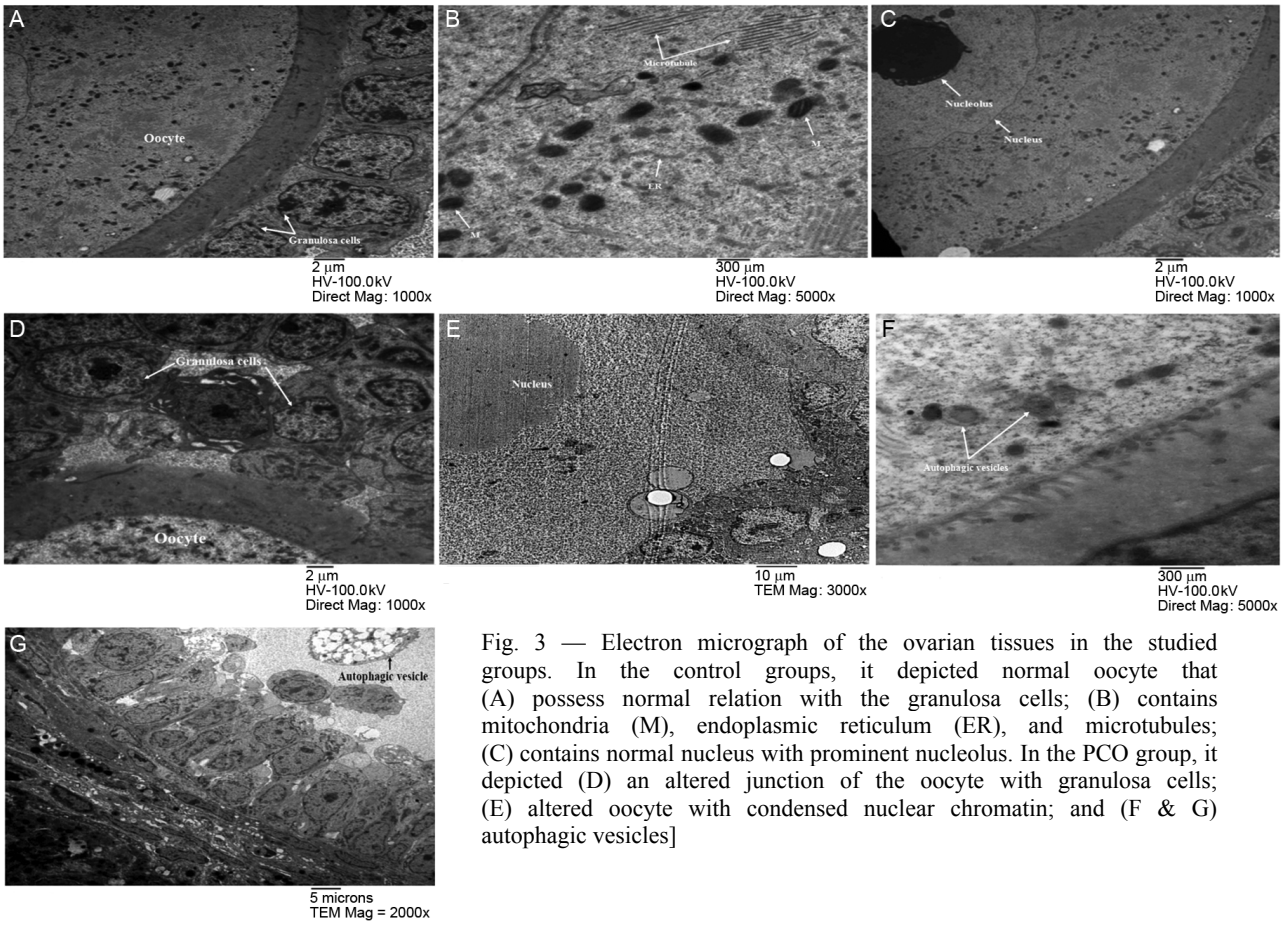

Fig. 3 — Electron micrograph of the ovarian tissues in the studied groups. In the control groups, it depicted normal oocyte that (A) possess normal relation with the granulosa cells; (B) contains mitochondria (M), endoplasmic reticulum (ER), and microtubules; (C) contains normal nucleus with prominent nucleolus. In the PCO group, it depicted (D) an altered junction of the oocyte with granulosa cells; (E) altered oocyte with condensed nuclear chromatin; and (F & G) autophagic vesicles]

pathogenesis of the disease and may contribute to reproductive and metabolic dysfunctions[19]. Moreover, hyperandrogenism impairs the feedback signaling from ovarian sex hormones to the hypothalamus-pituitary axis affecting, in turn, the normal release of gonadotropin-releasing hormone, LH, and FSH[20]. This is supported by the results of the present work that showed a significant increase in serum LH level in the letrozole-induced group compared to control groups. This elevation in the LH level might prevent follicular maturation and lead to ovulation failure[21].

In the same context, the significant reduction of serum progesterone level observed in letrozole-exposed animals indicates anovulation and echoes the results reported by Begum *et al*.[22]. Further, the numerous atretic follicles found in the histo-pathological examination could be a result of disturbed folliculogenesis and oogenesis[23].

Furthermore, it was reported that hyperandrogenism induces OS status in the ovary[24]. The elevated OS interferes with many pathological conditions of reproduction such as infertility, abortion, preeclampsia, endometriosis, and PCOS[25]. Herein, a disrupted equilibrium was observed in the letrozole-induced PCO group between endogenous antioxidant capacity, as pointed from the diminished ovarian TAC, and toxic oxygen-derived products that are insufficiently detoxified leading inevitably to oxidative burden and harmful consequences targeting cellular biomolecules, as evident by the elevated ovarian PC content. This is in agreement with the results of previous studies conducted on both humans and animals[26,27].

The imbalanced redox potential has been suggested to enhance OS, this effect might be associated with increased autophagy. Among all autophagy-associated proteins, beclin-1 appears to be a key regulator[28]. Studies on the beclin-1 levels in PCOS are relatively sparse and offer contradictory findings. Li *et al*.[29] observed an increase in the *BECN1* mRNA abundance in ovarian granulosa cells from PCOS patients compared to non-PCOS patients. Similarly, Yi *et al*.[30] found that the increased beclin-1 protein level was linked to PCOS pathogenesis. This is in accord with the results of the present study that showed a significant increase in ovarian beclin-1 level in the letrozole-exposed animals compared to controls. Moreover, electron microscopy revealed an increased autophagosomes number in the PCO group. However, Li *et al*.[6] demonstrated a slightly, but not significant, increasing trend in the expression of beclin-1 protein in human PCOS ovarian tissues in comparison with normal ovaries, although the authors reported that autophagy was activated in the ovarian tissue of PCOS patients and dehydroepiandrosterone (DHEA)-induced PCOS rats. On the other hand, Sumarac *et al*.[31] reported that the endometrial gene expression level of *BECN1* was significantly reduced in anovulatory PCOS patients compared to the control group.

In the same direction, the increased production of both ROS and OS was connected to TGF-β1 activation and production, indicating their crucial role in the fibrotic process[32]. Consequently, a relationship between the TGF-β1 level and the pathogenic events in PCOS was suggested[33]. TGF-β1 was proven to increase the production of the proinflammatory cytokines and profibrogenic molecules responsible for the marked inflammatory responses and fibrosis in PCOS[34].

In the current work, letrozole induced a significant increase in the ovarian TGF-β1 level compared to control groups. This concurs with the results of Raja-Khan *et al*.[35], who reported a high level of TGF-β1 in PCOS patients. Further, Kabel *et al*.[36] reported that ovarian TGF-β1 level was significantly elevated in letrozole-induced PCOS animals.

Notably, the increased ovarian beclin-1 level in the present study showed only a positive relationship with ovarian TGF-β1 level in the final regression analysis model, which seems consistent with the proautophagy effects of TGF-β1 demonstrated previously[37,38].

TGF-β binds to type I and II receptors and transduces signals through receptor-regulated Smad2/3 and common-partner Smad4, and it was reported previously that TGF-β1/Smad3 pathway induces beclin-1[39]. Further, TGF-β activated autophagy by inducing the accumulation of autophagosomes and increasing the mRNA expression levels of several autophagy-related genes including *BECN1* in human hepatocellular carcinoma cell lines. The knockdown of Smad2/3 or Smad4 attenuated the TGF-β-induced autophagy[14]. Moreover, Li *et al*[12] found that fucoidan, a long chain sulfated polysaccharide, inhibits TGF-β1 that, in turn, reduces the phosphorylation of Smad2/3 and impedes the transfer of the latter to the nucleus to combine with specific DNA sequences of *BECN1* gene, therefore, Beclin-1 could not promote the autophagosome

biogenesis and autophagy was eventually hindered. This concurs with the results of the current work's STRING analysis that confirmed the interaction of TGF-β1 signaling with autophagy *via* Beclin-1.

Conclusion

This work highlights the relationship between TGF-β1 and beclin-1 ovarian levels in a letrozole-induced PCOS model indicating a significant increase in the ovarian beclin-1 and TGF-β1 levels with an enhancement of autophagy as revealed by electron microscopy. In multiple linear regression models, TGF-β1 appeared to be a determinant factor of beclin-1 and the increased level of the latter might be associated with the high level of the former. Thus, there is a need for additional studies to explore therapeutic strategies targeting TGF-β1signaling that may regulate autophagy and subsequently present promising remedies in the treatment of PCOS.

Declarations

Compliance with ethical standards

The investigation complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH) and the study protocol was approved by the local Ethics Committee of Faculty of Medicine, Ain Shams University, Egypt.

Conflict of interest

Authors declare no competing interests.

References

1 Louwers YV & Laven JSE, Characteristics of polycystic ovary syndrome throughout life. *Ther Adv Reprod Health*, 14 (2020) 2633494120911038.

2 Li Z, Peng A, Feng Y, Zhang X, Liu F, Chen C, Ye X, Qu J, Jin C, Wang M, Qiu H, Qi Y, Huang J & Yang Q, Detection of T lymphocyte subsets and related functional molecules in follicular fluid of patients with polycystic ovary syndrome. *Sci Rep*, 9 (2019) 6040.

3 Zhang R, Liu H, Bai H, Zhang Y, Liu Q, Guan L & Fan P, Oxidative stress status in Chinese women with different clinical phenotypes of polycystic ovary syndrome. *Clin Endocrinol* (Oxf), 86 (2017) 88.

4 Gao Q, Oxidative Stress and Autophagy. In: *Autophagy: Biology and diseases-Basic science* (Eds. Qin ZH; Science Press and Springer Nature, Singapore), 2019, 179.

5 Kanninen TT, de Andrade Ramos BR & Witkin SS, The role of autophagy in reproduction from gametogenesis to parturition. *Eur J Obstet Gynecol Reprod Biol*, 171 (2013) 3.

6 Li D, You Y, Bi FF, Zhang TN, Jiao J, Wang TR, Zhou YM, Shen ZQ, Wang XX & Yang Q, Autophagy is activated in the ovarian tissue of polycystic ovary syndrome. *Reproduction*, 155 (2018) 85.

7 Choi J, Jo M, Lee E & Choi D, Induction of apoptotic cell death *via* accumulation of autophagosomes in rat granulosa cells. *Fertil Steril*, 95 (2011) 1482.

8 Wang SY, Yu QJ, Zhang RD & Liu B, Core signaling pathways of survival/death in autophagy-related cancer networks. *Int J Biochem Cell Biol*, 43 (2011) 1263.

9 Tran S, Fairlie WD & Lee EF, BECLIN1: Protein structure, function and regulation. *Cells*, 10 (2021) 1522.

10 Ingman WV & Robertson SA, Defining the actions of transforming growth factor in reproduction. *BioEssays*, 24 (2002) 904.

11 Ingman WV, Robker RL, Woittiez K & Robertson SA, Null mutation in transforming growth factor beta1 disrupts ovarian function and causes oocyte incompetence and early embryo arrest. *Endocrinology*, 147 (2006) 835.

12 Li J, Chen K, Li S, Feng J, Liu T, Wang F, Zhang R, Xu S, Zhou Y, Zhou S, Xia Y, Lu J, Zhou Y & Guo C, Protective effect of fucoidan from Fucus vesiculosus on liver fibrosis via the TGF-β1/Smad pathway-mediated inhibition of extracellular matrix and autophagy. *Drug Des Devel Ther*, 10 (2016) 619.

13 Tian X, Zhang J, Tan TK, Lyons JG, Zhao H, Niu B, Lee SR, Tsatralis T, Zhao Y, Wang Y, Cao Q, Wang C, Wang Y, Lee VW, Kahn M, Zheng G & Harris DC, Association of β-catenin with P-Smad3 but not LEF-1 dissociates *in vitro* profibrotic from anti-inflammatory effects of TGF-β1. *J Cell Sci*, 126 (2013) 67.

14 Kiyono K, Suzuki HI, Matsuyama H, Morishita Y, Komuro A, Kano MR, Sugimoto K & Miyazono K, Autophagy is activated by TGF-beta and potentiates TGF-beta-mediated growth inhibition in human hepatocellular carcinoma cells. *Cancer Res*, 69 (2009) 8844.

15 Li C, Chen L, Zhao Y, Chen S, Fu L, Jiang Y, Gao S, Liu Z, Wang F, Zhu X, Rao J, Zhang J & Zhou X, Altered expression of miRNAs in the uterus from a letrozole-induced rat PCOS model. *Gene*, 598 (2017) 20.

16 Lemaitre M, Christin-Maitre S & Kerlan V, Polycystic ovary syndrome and adipose tissue. *Ann Endocrinol* (Paris), 84 (2023) 308.

17 Kumariya S, Ubba V, Jha RK & Gayen JR, Autophagy in ovary and polycystic ovary syndrome: role, dispute and future perspective. *Autophagy*, 17 (2021) 2706.

18 Xu J, Dun J, Yang J, Zhang J, Lin Q, Huang M, Ji F, Huang L, You X & Lin Y, Letrozole rat model mimics human polycystic ovarian syndrome and changes in insulin signal pathways. *Med Sci Monit*, 26 (2020) e923073.

19 Guo Z, Jin F, Chen S, Hu P, Hao Y & Yu Q, Correlation between biochemical and clinical hyperandrogenism parameter in polycystic ovary syndrome in relation to age. *BMC Endocr Disord*, 23 (2023) 89.

20 Moore AM, Impaired steroid hormone feedback in polycystic ovary syndrome: Evidence from preclinical models for abnormalities within central circuits controlling fertility. *Clin Endocrinol* (Oxf), 97 (2022) 199.

21 Kanamarlapudi V, Gordon UD & López Bernal A, Luteinizing hormone/chorionic gonadotrophin receptor overexpressed in granulose cells from polycystic ovary syndrome ovaries is functionally active. *Reprod Biomed Online*, 32 (2016) 635.

22 Begum N, Manipriya K & Veeresh B, Role of high-fat diet on letrozole-induced polycystic ovarian syndrome in rats. *Eur J Pharmacol*, 917 (2022) 174746.

23 Rezvanfar MA, Rezvanfar MA, Ahmadi A, Shojaei-Saadi HA, Baeeri M & Abdollahi M, Molecular mechanisms of a novel selenium-based complementary medicine which confers protection against hyperandrogenism induced polycystic ovary. *Theriogenology*, 78 (2012) 620.

24 Wang D, Wang T, Wang R, Zhang X, Wang L, Xiang Z, Zhuang L, Shen S, Wang H, Gao Q & Wang Y, Suppression of p66Shc prevents hyperandrogenism-induced ovarian oxidative stress and fibrosis. *J Transl Med*, 18 (2020) 84.

25 Afshar Ebrahimi F, Foroozanfard F, Aghadavod E, Bahmani F & Asemi Z, The effects of magnesium and zinc co-supplementation on biomarkers of inflammation and oxidative stress, and gene expression related to inflammation in polycystic ovary syndrome: a randomized controlled clinical trial. *Biol Trace Elem Res*, 184 (2018) 300.

26 Darabi P, Khazali H & Mehrabani Natanzi M, Therapeutic potentials of the natural plant flavonoid apigenin in polycystic ovary syndrome in rat model: via modulation of pro-inflammatory cytokines and antioxidant activity. *Gynecol Endocrinol*, 36 (2020) 582.

27 Atef MM, Abd-Ellatif RN, Emam MN, Abo El Gheit RE, Amer AI & Hafez YM, Therapeutic potential of sodium selenite in letrozole induced polycystic ovary syndrome rat model: Targeting mitochondrial approach (selenium in PCOS). *Arch Biochem Biophys*, 671 (2019) 245.

28 Yang X, Yan X, Yang D, Zhou J, Song J & Yang D, Rapamycin attenuates mitochondrial injury and renal tubular cell apoptosis in experimental contrast-induced acute kidney injury in rats. *Biosci Rep*, 38 (2018) BSR20180876.

29 Li X, Qi J, Zhu Q, He Y, Wang Y, Lu Y, Wu H & Sun Y, The role of androgen in autophagy of granulosa cells from PCOS. *Gynecol Endocrinol*, 35 (2019) 669.

30 Yi S, Zheng B, Zhu Y, Cai Y, Sun H & Zhou J, Melatonin ameliorates excessive PINK1/Parkin-mediated mitophagy by enhancing SIRT1 expression in granulosa cells of PCOS. *Am J Physiol Endocrinol Metab*, 319 (2020) E91.

31 Sumarac-Dumanovic M, Apostolovic M, Janjetovic K, Jeremic D, Popadic D, Ljubic A, Micic J, Dukanac-Stamenkovic J, Tubic A, Stevanovic D, Micic D & Trajkovic V, Downregulation of autophagy gene expression in endometria from women with polycystic ovary syndrome. *Mol Cell Endocrinol*, 440 (2017) 116.

32 Radwan MI, Pasha HF, Mohamed RH, Hussien HI & El-Khshab MN, Influence of transforming growth factor-beta1 and tumor necrosis factor-alpha genes polymorphisms on the development of cirrhosis and hepatocellular carcinoma in chronic hepatitis C patients. *Cytokine*, 60 (2012) 271.

33 Xiong YL, Liang XY, Yang X, Li Y & Wei LN, Low-grade chronic inflammation in the peripheral blood and ovaries of women with polycystic ovarian syndrome. *Eur J Obstet Gynecol Reprod Biol*, 159 (2011) 148.

34 Raja-Khan N, Urbanek M, Rodgers RJ & Legro RS, The role of TGF-β in polycystic ovary syndrome. *Reprod Sci*, 21 (2014) 20.

35 Raja-Khan N, Kunselman AR, Demers LM, Ewens KG, Spielman RS & Legro RS, A variant in the fibrillin-3 gene is associated with TGF-β and inhibin B levels in women with polycystic ovary syndrome. *Fertil Steril*, 94 (2010) 2916.

36 Kabel AM, Al-Shehri AH, Al-Talhi RA & Abd Elmaaboud MA, The promising effect of linagliptin and/or indole-3-carbinol on experimentally-induced polycystic ovarian syndrome. *Chem Biol Interact*, 273 (2017) 190.

37 Zhang J, Jiang N, Ping J & Xu L, TGF-β1-induced autophagy activates hepatic stellate cells via the ERK and JNK signaling pathways. *Int J Mol Med*, 47 (2021) 256.

38 Wang Y, Lu H, Wang Z, Li Y & Chen X, TGF-β1 promotes autophagy and inhibits apoptosis in breast cancer by targeting TP63. *Front Oncol*, 12 (2022) 865067.

39 Hata A & Chen YG, TGF-β signaling from receptors to Smads. *Cold Spring Harb Perspect Biol*, 8 (2016) a022061.