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Original Article

Effects of jnk inhibitor on inflammation and fibrosis in the ovary tissue of a rat model of polycystic ovary syndrome

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Abstract: Objective: In our study, we aimed to investigate the effects of Jun N-terminal kinase inhibitor (SP600125) on fibrosis and inflammation in rats with polycystic ovary syndrome (PCOS). Method: 50 Wistar-albino rats were divided into five groups (n=10 each): control group, sham group, PCOS group, SP600125+ PCOS group and SP600125 group. In the estradiol valerate (EV)-treated group in which PCOS was injected with a single 4 mg/kg i.p. of EV in 0.2 ml sesame oil and the rats were sacrificed on day 60. The estradiol valerate (EV)-treated + SP600125-treated group was injected with a single 4 mg/kg i.p. of EV in 0.2 ml sesame oil. As of day 60, the treatment group was additionally given 15 mg/kg i.p. of SP600125 once daily for 4 consecutive days and the rats were sacrificed on day 65. Histopathological findings (ovarian morphology, edema, inflammatory cell infiltration, vascular congestion and hyperemia) and collagen type IV immunoexpression were assessed. Results: The SP600125+ PCOS group showed a significant level of improvement in ovarian follicle morphology, edema, inflammatory infiltrate, vascular congestion and hyperemia as compared with the PCOS group. Furthermore, collagen type IV immunoexpression showed a significant reduction in staining intensity on the theca cell layer and ovary stroma as compared to the PCOS group. Conclusion: This study demonstrates the therapeutic effect of SP600125 in the prevention of PCOS in an experimental model.

Keywords: Fibrosis, inflammation, Jun N-terminal kinase inhibitor, polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) affects approximately 6% of women at childbearing age [1]. PCOS is a common metabolic and endocrine disorder, of which the pathogenesis is extremely complicated. The primary chronic complications that cause abnormal ovulation are interstitial fibrosis and thickening of the capsule. PCOS is characterized by excessive ovarian androgen production and polycystic ovaries with a thickened, fibrotic tunica albuginea and a subcortical band composed of many cystic follicles at various stages of growth and atresia [2]. Despite a wide-range of research being conducted, the mechanisms for the pathological changes that are underlying anovulation in PCOS remain unclear. Recently, many aspects of PCOS have been studied, including the Hypothalamic pituitary-ovarian and adrenal axis dysfunction, obesity, insulin resistance and genetics. However, the definitive cause is not completely understood. Dumesic et al. showed that the local ovarian bioactive factors play an important role in polycystic ovary formation [3].

One of the main characteristics of PCOS is hyperandrogenism, which is 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 a more highly collagenized cortex, hyperplasia of the theca interna and a thicker theca cell layer in comparison with the control group [4].

Hyperinsulinemia, the potential factor driving increased androgen production, is widespread in PCOS [5, 6]. Treatment of ovarian organoids with insulin and insulin-like growth factor-1 results in hyperplasia of the ovarian surface epithelium and alterations in the organization of collagen [7]. Fibrotic states in various organs have several etiologies; however, fibrosis typically originates from chronic persistent inflammation. Currently, low-grade chronic inflammation has been demonstrated to be involved in the pathophysiological mechanisms of PCOS [8-10]. Hu et al. demonstrated that PCOS patients have a higher level of serum-transforming growth factor-beta 1 (TGF-β1) [11], which appears to be the key factor in fibrosis [12, 13]. All of these pieces of evidence support the view that hyperfibrosis may develop in patients with PCOS.

Collagen fibers in the ovarian follicles are altered during ovulation as a consequence of the pre-ovulatory increase of collagenolytic activities. The collagenous components of the ovarian stroma develop in interstitial collagen type I, III and IV in tunica albuginea as well as the extracellular matrix (ECM) of the stroma [14]. They provide the structural strength for ovaries and support the matrix in which follicular maturation, ovulation and formation of corpus luteum take place [14, 15].

Because of evidence based on both DHEA-induced rat models of PCOS and human clinical investigation of PCOS, it is likely that fibrosis might involve polycystic ovaries or other relevant organs. Fibrosis is consistently associated with the accumulation of ECM and a remarkable number of molecular factors are required for ECM formation, such as connective tissue growth factor (CTGF) [16] and epidermal growth factor [17].

Chronic low-grade inflammation has appeared to be a key contributor to the pathogenesis of PCOS. It is now clear that PCOS is a pro-inflammatory state, and emerging data suggest that chronic low-grade inflammation contribute to ovarian dysfunction. Most importantly, a strong correlation exists between hyperandrogenism and inflammation in PCOS. Recent data show that diet-induced inflammation may directly invoke hyperandrogenism in PCOS [18].

The chronic low-grade inflammation observed in PCOS forms the genetic basis of the disease. Various pro-inflammatory genotypes, including those that encode TNF-α and type 2 TNF receptor, as well as interleukin-6 (IL-6) and its signal transducer, are associated with PCOS [19-21].

Jun N-terminal kinase (JNK) is a stress-activated protein kinase. It can be induced by inflammatory cytokines, bacterial endotoxin, osmotic shock, UV radiation, and hypoxia. JNK is a serine/threonine protein kinase that phosphorylates c-Jun [22, 23], a component of the transcription factor activator protein-1 (AP-1). AP-1 regulates the transcription of numerous genes, including cytokines [e.g., IFN-gamma, IL-2 and tumor necrosis factor (TNF)-α], growth factors [e.g., vascular endothelial growth factor (VEGF)], immunoglobulins (e.g., kappa light chain), inflammatory enzymes (e.g., COX-2), and matrix metalloproteinase (e.g., MMP-13). JNK is a member of the mitogen-activated protein kinase (MAPK) family, which includes the extracellular signal-regulated kinases (ERKs) and p38 kinases. It regulates multiple activities in cell development and function. JNK-1 and JNK-2 are present in numerous tissues, whereas JNK-3 is restricted to neuronal tissues, heart, pancreatic islets and testis. Current studies have shown that JNK plays a central role in the modulation of insulin action and is a critical component of the pathogenesis of obesity, fatty liver disease, and type 2 diabetes [24]. Moreover, JNK controls major inflammatory response pathways, and it is activated by a variety of stress signals [25].

Unlike apoptosis and inflammation, data available for JNK in tissue fibrosis are limited. In vitro studies show a role for JNK signaling in TGF-β1-induced fibronectin production in fibroblasts, and in TGF-β1-induced connective tissue growth factor (CTGF) production [26, 27]. Transforming growth factor (TGF-β1) is one of the factors that have been found to demonstrate more extensive biological activity in comparison with their originally named predecessor. It has been proven that TGF-β1 plays a very important role in the organs and tissues of fibrotic disease. Therefore, it is a key cytokine that is recognized by most scholars as a starting hub for fibrosis formation and development [28].

CTGF is a downstream factor of TGF-β1, which may be induced by TGF-β1 and mediate some of the pro-fibrotic actions of TGF-β1, such as

increasing the synthesis and accumulation of the extracellular matrix [29].

Plantevin Krenitsky et al. showed that potent and selective aminopurine-based Jun N-terminal kinase (JNK) inhibitors demonstrated pharmacological efficacy in animal models of acute inflammation and tissue damage [30]. In addition, SP600125, an early JNK inhibitor with modest selectivity for JNK over p38 and ERK MAP kinases, showed efficacy in several animal models of fibrosis [31].

JNK inhibitor not only inhibits the JNK pathway, but it also reduces superoxide production. It has a cytotoxic effect in T-cell proliferation. Experimental studies reported that it blocked cell proliferation in CD4+ cells. JNK inhibitor (SP600125) shows a potent effect on DNA. JNK plays an important role in the activation of transcription factors, mRNA stabilization and apoptotic processes, as well as cell proliferation. JNK inhibition has an important potential in acute cell disorders due to cell death and inflammatory diseases [32-34].

In the present study, we aimed to investigate if the development of inflammation and fibrosis in the ovarian tissues of PCOS-induced female rats and the ovaries of rats with PCOS could be prevented. PCOS could be prevented. Our study is the first to investigate the histopathological effects of a JNK inhibitor on rats with PCOS.

Materials and methods

Study design

The study was conducted at the Animal Center of Yüzüncü Yıl University. The study was approved by the Yüzüncü Yıl University Experimental Medical Research Center's Experimental Animals Ethics Committee, Van, Turkey. All of the rats were housed in the translational medical Centre of Yüzüncü Yıl 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 Wistar Albino rats were randomly assigned into 5 groups: sham animals (group 2) were injected with only 0.2 ml sesame oil while control animals (group 1) received no injections. In the estradiol valerate (EV)-treated group (group 3), PCOS was injected with a single 4 mg/kg i.p. of EV (Progynon Depot, Schering, Buenos Aires, Argentina) in 0.2 ml sesame oil, and the rats were sacrificed on day 60 (n=10). The estradiol valerate-treated + SP600125-treated group (group 4) was injected with a single 4 mg/kg i.p. dose of EV (Progynon Depot, Schering, Buenos Aires, Argentina) in 0.2 ml sesame oil. As of day 60, the treatment group was additionally given 15 mg/kg i.p. of SP600125 (Tocris Bioscience, Philadelphia, USA) once daily for 4 consecutive days, and the rats were sacrificed on day 65 (n=10). The SP600125 treated group (group 5) was injected with 15 mg/kg i.p. of SP600125 once daily for 4 consecutive days and the rats were sacrificed on day 5 (n=10).

During the experiment stage, one experimental animal in the control group, two animals from the sham group, two animals from the PCOS group and one animal from the PCOS+ SP600125 group died.

Histopathological analysis

One ovary per rat was removed, cleansed of adherent connective fat tissue, fixed in 10% formaldehyde buffer and embedded into paraffin blocks. With the aid of a microtome, 4-micron sections were cut out from the blocks. As per standard protocols, all of the tissues were stained with hematoxylin and eosin. The slides were evaluated using an Olympus (Tokyo, Japan) BX51 microscope.

Histopathological examination

All of the ovarian tissues were evaluated for ovarian morphology, edema formation, inflammatory cell infiltration, and vascular congestion.

In cases in which ovum degeneration or at least one pyknotic granulosa cell was observed, the follicle populations were classified as atretic; otherwise, they were classified as healthy. Morphological characteristics of follicular atresia included inter alia, scattered pyknotic nuclei in the granulosa cell layer, detachment of the granulosa cell layer from the basement membrane, fragmentation of the basal lamina, and the presence of cell debris in the antrum of the follicle.

Scoring

To evaluate inflammation, edema and vascular congestion-hyperemia under light microscopy, a semiquantitative grading was designed

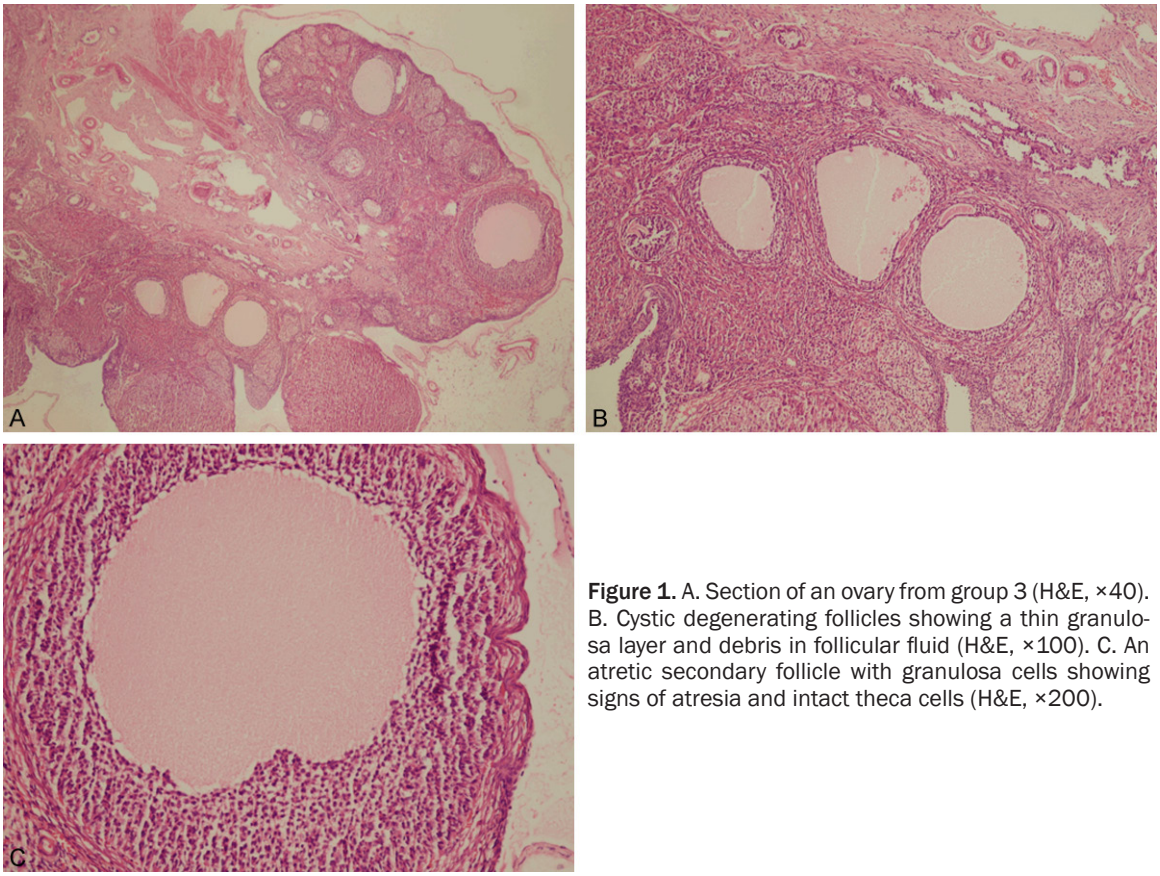

Figure 1. A. Section of an ovary from group 3 (H&E, ×40). B. Cystic degenerating follicles showing a thin granulosa layer and debris in follicular fluid (H&E, ×100). C. An atretic secondary follicle with granulosa cells showing signs of atresia and intact theca cells (H&E, ×200).

according to the degree of inflammation, edema or vascular congestion: grade 0 (none), grade 1 (mild), grade 2 (moderate) or grade 3 (severe). To qualitatively evaluate the severity of inflammation, infiltration of polymorphonuclear leucocytes (PMNL) and lymphocytes was taken into consideration. They were graded as 0 (none; infiltration of 1-20 lymphocytes, no PMNL), 1 (mild; infiltration of 21-50 lymphocytes, 1-2 PMNLs), 2 (moderate; 51-80 lymphocytes, 3-10 PMNLs) and 3 (severe; infiltration of 81-120 lymphocytes, >10 PMNLs).

Immunohistochemical procedure

Immunohistochemical examination was performed on a Leica Bond-Max automated IHC/ISH platform (Model: Bond-Max, Serial No: M211-708 Made in: Melbourne, Australia). Four-micrometer paraffin sections were dewaxed in a Bond Dewax solution, rehydrated in alcohol and Bond Wash solution (Leica Microsystems). Antigen retrieval was performed using a high-pH (ER2) retrieval solution for 15 minutes followed by endogenous peroxidase blocking for 5 minutes on the machine. Anti-Collagen IV antibody (ab6586, Abcam, in dilution 100 µg at 1 mg/ml) was applied at a 1:200 dilution for 60 minutes at room temperature. Detection was performed using a Bond Polymer Refine Red Detection system (Leica Microsystems) with a 15-minute post-primary step followed by 25 minutes incubation with alkaline phosphatase-linked polymers. The sections were then counterstained with hematoxylin on the machine, dehydrated in alcohol, and mounted with mounting medium (Bio Mount HM, 20134, Milan, Italy). The tissues were observed by histopathologists who were blinded to the experimental study groups. The percentage of positive cells was estimated using light microscopy. The staining intensity was also assessed semi-qualitatively as follows: +, weak; ++, moderate; +++, strong.

Statistical analysis

The descriptive statistics for the emphasized characteristics are expressed in numbers and percentages. Chi-squared test was used to determine the association among categorical

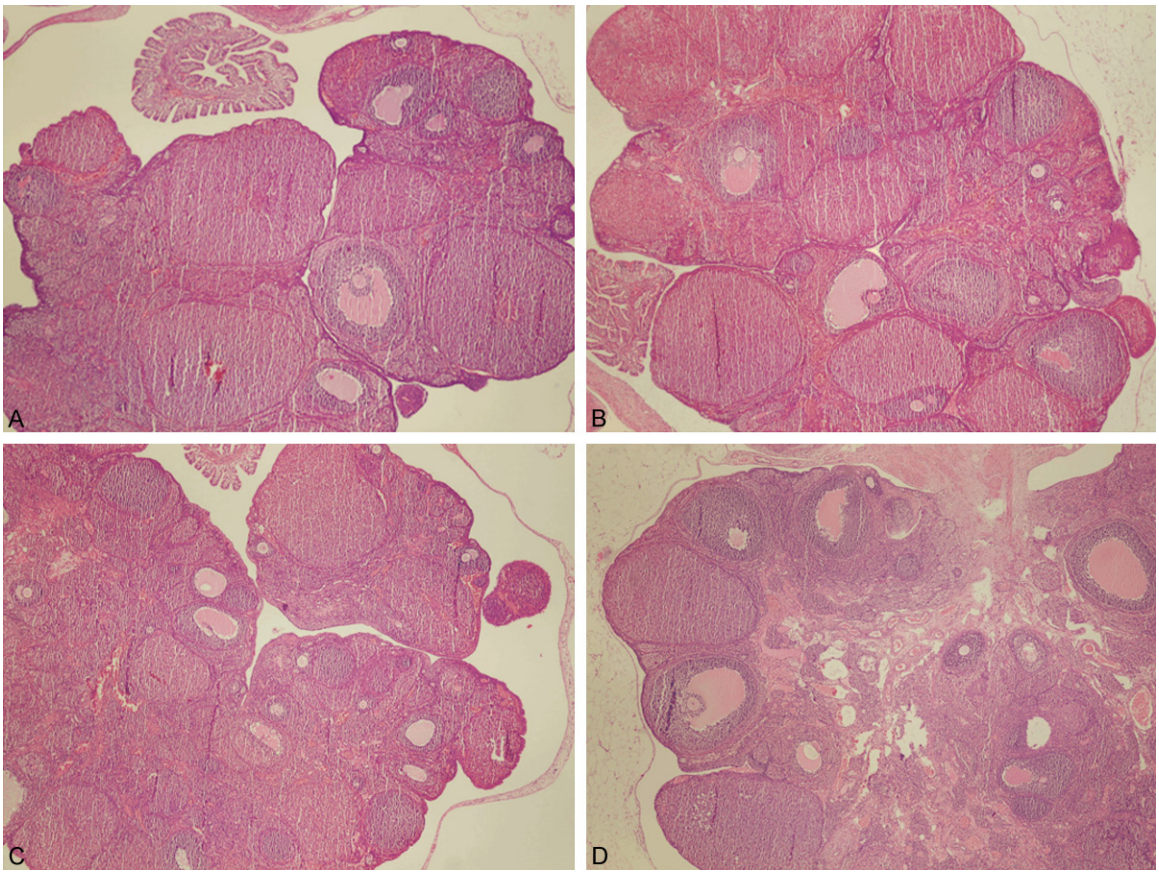

Figure 2. Histological examination of normal ovaries. Different developmental stages of follicles were detected in group 1 (A) (H&E, ×40), group 2 (B) (H&E, ×40), group 4 (C) (H&E, ×40), group 5 (D) (H&E, ×40).

variables, and a Z test and Fisher's exact test were used to compare the ratios. The statistical significance level was accepted as 5% for calculations. The statistical package software MINITAB was used for calculations.

Results

Ovarian morphology

A significant decrease was observed in the number of primary follicles, antral follicles, corpora lutea, primordial follicles and preantral follicles in group 3 (**Figure 1A**). In addition, some large cystic follicles with a thin granulosa of 2-3 cell layers were observed (**Figure 1B**, **1C**). In these groups, no corpus luteum, which is a sign of ovulation, was detected. In groups 1, 2, 4 and 5, there were no ovarian cysts; however, several follicles at different stages of development, as well as corpora lutea, were distinguishable (**Figure 2A-D**). These results were indicative of a complete induction of the PCOS phenotype. In group 4, the number of primordial and preantral follicles and corpora lutea in the ovaries increased. Additionally, the number of cysts was significantly lower than that of group 3.

In regard to the assessment of edema of ovarian stroma among groups 1, 2, 4, 5 and 3, there were significant differences ($P < 0.05$) (**Figure 3**).

The assessment of vascular congestion and hyperemia among the groups revealed significant differences ($P < 0.05$) (**Figure 4**).

When we examined the specimens under a light microscope, a comparative observation of groups 1, 2, 4, 5 and 3, significant differences in inflammation were observed ($P < 0.05$) (**Figure 5**).

Immunohistochemical findings

In our samples, collagen type IV was detected primarily in the stroma of group 3, where the

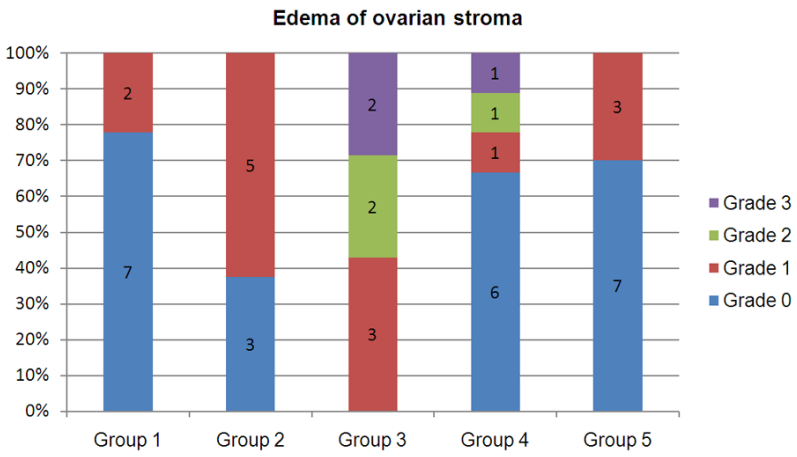

Figure 3. Distribution of degree of edema of ovarian stroma.

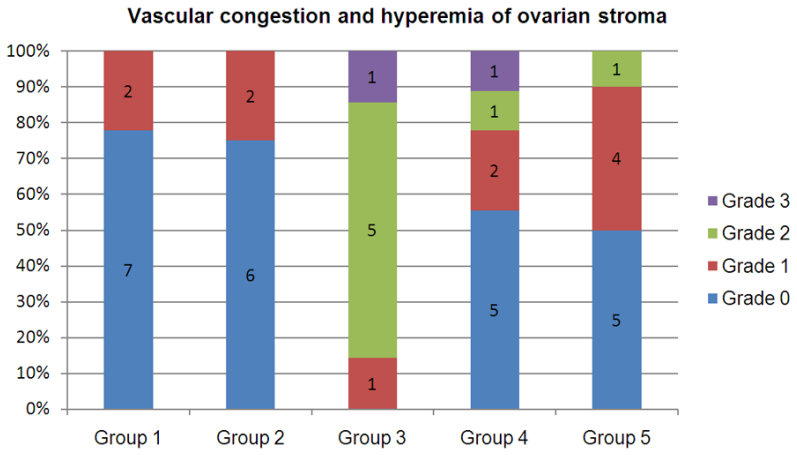

Figure 4. Distribution of degree of vascular congestion and hyperemia.

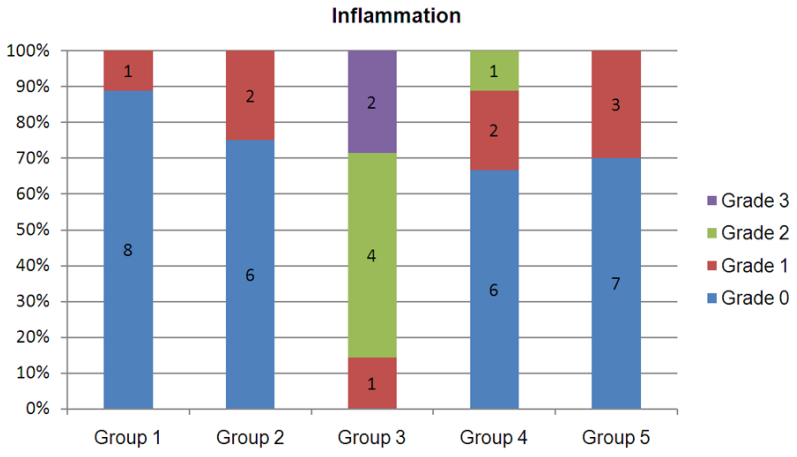

Figure 5. Distribution of degree of inflammation.

intensity ($P < 0.05$) of positive staining was higher in comparison with normal ovaries (groups 1-2-4-5) (**Figure 6**). Increased collagen type IV staining was also observed in the theca cell layer of group 3 in comparison with groups 1, 2, 4, and 5 ($P < 0.05$) (**Figures 7**, **8A-F**).

Discussion

PCOS is the most common reproductive and endocrinal disease. It also has hyperandrogenemia-related and reproductive impacts, and metabolic dysfunction is a common feature of PCOS. A high proportion of women with PCOS are marked by obesity and/or insulin resistance [35].

Several studies have demonstrated the development of fibrosis in rats with PCOS. The mechanisms that trigger fibrosis are currently under investigation. Fibrosis in the stroma and tunica albuginea of the ovary causes abnormal ovulation. Additionally, several studies have demonstrated the effects of low chronic inflammation rates on the development of PCOS.

The results of the present study indicate that the JNK signaling pathway is involved in ovarian interstitial inflammation and fibrosis processes. Pharmacological blockage of JNK activation strongly reduces fibrosis, edema, inflammation, vascular congestion and hyperemia in ovarian tissue. In ovaries with PCOS for which we provided SP600125 treatment, we observed thinning of the theca cell layer and decreased collagen accumulation in the stroma with collagen type 4 staining.

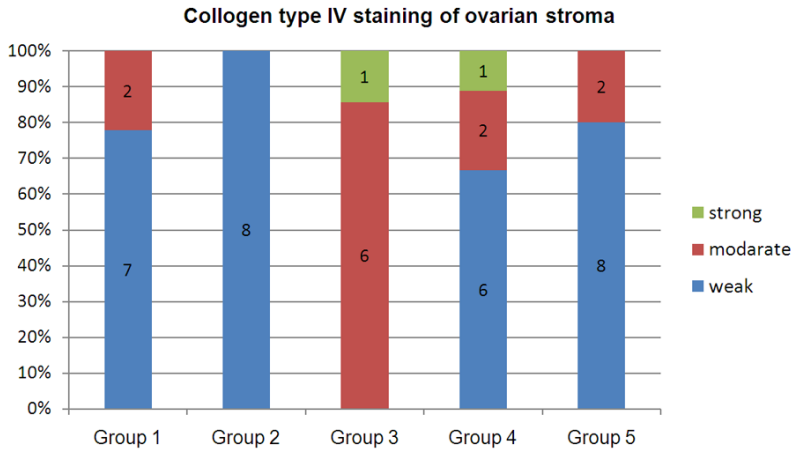

Figure 6. Distribution of staining intensities among groups with collagen type IV staining in the ovarian stroma.

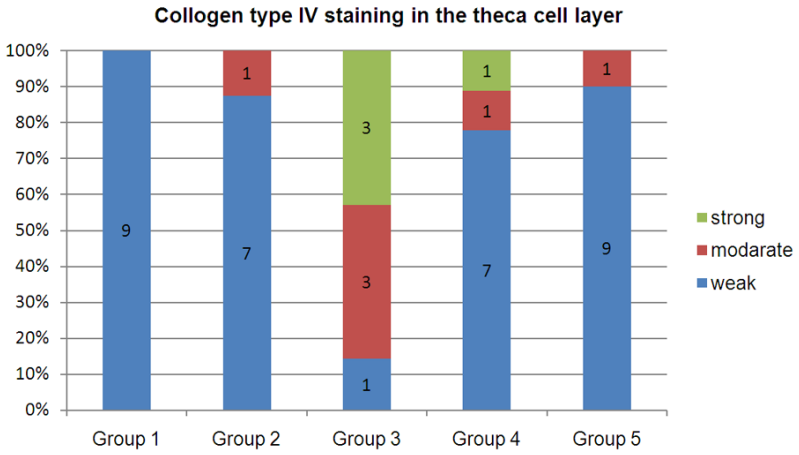

Figure 7. Distribution of staining intensities among groups with collagen type IV staining in the theca cell layer.

JNK protein is remarkable for its response to stress and it is therefore termed "Stress-Activated Protein Kinase". It is mostly associated with inflammation and apoptosis. The activation of inflammatory kinases, such as C-Jun N-terminal kinase (JNK), results in the impairment of insulin signal transmission. The insulin receptor is a member of the tyrosine kinases family. It is initially subjected to auto-phosphorylation with the binding of insulin; then, it phosphorylates the IRS-1 protein, which is mediated by tyrosines. It stimulates the IRS-1 insulin-specific signal pathways and enables the emergence of cellular responses. In human and animal models, it has been shown that there is a defect at this step of insulin resistance. JNK prevents the phosphorylation of tyrosine by serine phosphorylation of IRS-1, and JNK also suppresses signal transmission. It has been demonstrated that JNK-1 deficiency prevents serine phosphorylation of IRS-1 and development of insulin resistance induced by obesity in rats [8, 12, 36-38].

JNK is a member of the stress-activated family of MAP kinases, which is strongly activated by extracellular stimuli, including UV light, hypotonicity, and chemical toxins. In contrast, JNK is only moderately activated by growth factors. These kinases have a variety of functions within the cell, such as roles in growth, differentiation, survival and death. The best-characterized function of JNK is its role in furthering apoptosis. Persistent activation of JNK has been associated with apoptosis in diverse cell types, including HeLa cells [39], endothelial cells [40], rat mesangial cells [41], PC12 neuronal-like cells [42], and L929 fibrosarcoma cells [43]. Similarly, degenerating neurons from Alzheimer's disease patients have increased JNK activity [44]. However, in some cases, JNK activation has been associated with cell survival. For example, JNK activation has been demonstrated to protect myocytes from nitric-oxide-induced apoptosis, and to protect HeLa cells from apoptosis after photodynamic therapy [45]. Fundamentally, the effect of JNK activation on cell death and/or survival is probably sensitive to both the time course of activation and simultaneous molecular signaling events in the cell.

Turner et al. have shown that the 5-HT1A receptor can activate the c-Jun NH2-terminal kinase (JNK) in Chinese hamster ovary (CHO) fibroblasts. This activation was fast (within 10 min)

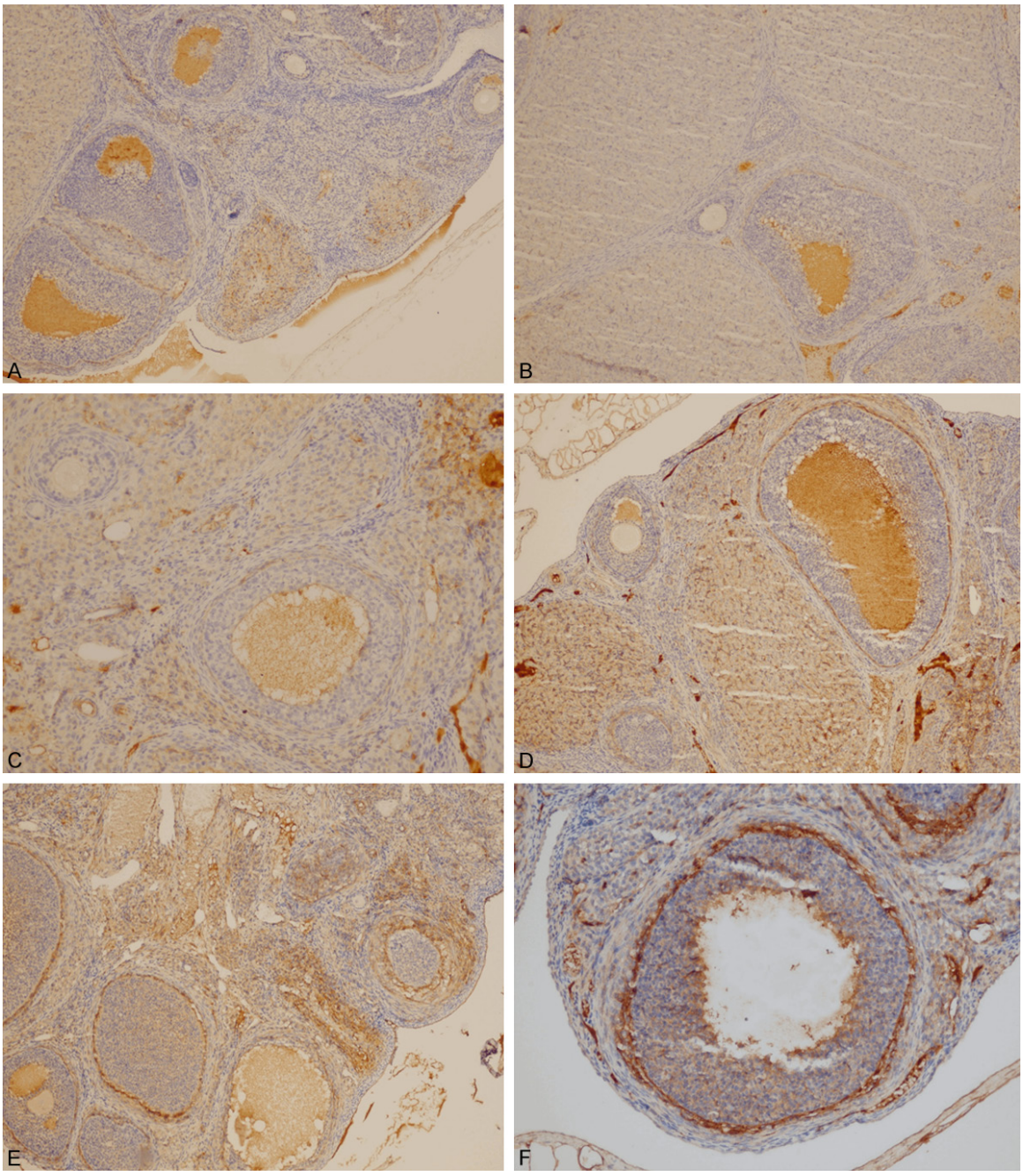

Figure 8. Expression of collagen type IV in rat ovaries. Group1 (A) (collagen type IV, ×100), group 2 (B) (collagen type IV, ×100) , group 5 (C) (collagen type IV, ×100), group 4 (D) (collagen type IV, ×100), group 3 (E, F) (collagen type IV, ×100, ×200).

and sustained, and resulted in a higher rate of programmed cell death, which was evident in the formation of apoptotic bodies and the observation of DNA fragmentation [46].

Recently, extensive studies have investigated the role of various cytokines and growth factors that can contribute to fibrosis. Among them, TGF-β and CTGF have been deemed to be the key mediators of fibrosis. Many studies based on animal models endorse the significant role of TGF-β in the induction of fibrosis. TGF-β over-expression in various tissues results in overwhelming fibrosis. Temporary over-expression of porcine TGF-β1 in rat lungs by means of the adenovirus transfection system induced pro-

longed lung fibrosis [47]. Expressing elevated levels of active TGF-β1 in the heart by genetic modification results in atrial fibrosis [48, 49]. Consistently, TGF-β inhibition extenuates fibrosis progression in a number of organs and tissues, e.g., the heart [50]. The most commonly acknowledged pathway involved in the pathogenesis of fibrosis in various tissues is the canonical TGF-β/Smad3 pathway [51]. Recently, growing attention has been focused on CTGF, which was discovered a decade ago and deemed to be a downstream effector of TGF-β. TGF-β exerts its effect, at least partially, via the induction of its downstream mediator CTGF [52], and CTGF could act as an enhancer of the TGF-β activity [53]. In our study, the significant decrease in the intensity of collagen type 4 staining in the ovary stroma and theca layer in the group treated with SP600125 shows that the JNK signaling pathway has a role in the fibrotic process of the ovary.

Inflammatory cytokines such as lymphokine, TNF-α and IL-6 are secreted by lymphocytes and macrophages. These cytokines consequently activate macrophages and lymphocytes to promote further cytokine secretion and thus participate in a vicious cycle. Because TNF-α and IL-6 could also induce insulin resistance, stimulate the production of androgen [54] and lead to hypothalamic-pituitary-ovarian axis secretion disorder [55]; the increased number of lymphocytes in the present PCOS group could be a triggering factor of chronic inflammation and disturbing the hormone spectrum.

Macrophages and lymphocytes are the primary white cells that are embedded in ovarian tissues. Their numbers are variable within the ovary and throughout the menstrual cycle. Developing follicles possess only a few of these cells, while are assembled in the progressive atretic follicles' granular and theca layers to the utmost extent [56]. Activated lymphocytes and macrophages secrete several cytokines that may induce apoptosis, e.g., IL-1, IL-6, IL-10, IL-12, TNF-α, GM-CSF and IGF-I [57]. Lymphocytes expedite cytotoxicity, mediate follicle cell apoptosis, and result in follicular atresia. Macrophages lead to phagocytosis of apoptotic follicular cells. Xiong et al. have shown that elevated macrophages and lymphocytes in the PCOS ovaries could induce cell apoptosis by various cytokines that act on granular and theca cells. As a result, dominant follicles cannot be generated [10]. We also observed a significant degree of improvement in the group treated with SP600125 in comparison with the PCOS group. It is known that inflammatory cytokines activate JNK, which is known as the "Stress Activated Protein Kinase". The fact that inflammation was reduced at a significant level in our study, thanks to the treatment with JNK inhibitor, supports the role of the JNK signaling pathway in the inflammatory process observed in PCOS.

The results of this study show that SP600125 treatment reduces estradiol valerate-induced alterations in the ovaries. This effect may be related to the limited inflammatory response and fibrotic process observed in the estradiol valerate+SP600125-treated rats. These data also suggest that inhibition of the JNK pathway may represent a novel therapeutic approach for preventing PCOS.

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Disclosure of conflict of interest

None.

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