

Altered expression of Cx26/Cx32 of cumulus cells by β -adenoceptor drugs in women with polycystic ovary syndrome and poor response

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Altered expression of Cx26/Cx32 of cumulus cells by β -adenoceptor drugs in women with polycystic ovary syndrome and poor response

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Short Title:Cx26/Cx32, PCOS/POR

Abstract

Background

This experimental study was conducted to evaluate and compare the pharmacological effects of beta-adrenergic drugs on the Cx26 and Cx32 gene expression and protein levels of cultured cumulus cell junctions in women with PCOS and POR undergoing IVF. Noradrenaline (NA) plays a fundamental role in the processes of folliculogenesis and steroidogenesis. Continuous beta-adrenergic stimulation by NA leads to receptor desensitization and down-regulation, resulting in impaired downstream signaling and reduced connexin expression.

Methods

This experimental study included 45 women in three groups: control (oocyte donors) and two infertile women (PCOS and POR) groups, undergoing in vitro fertilization. Following controlled ovarian stimulation and oocyte retrieval (via ovarian puncture), cumulus cells (CCs) were isolated and cultured. After three passages (1, 3 and 5 days), the cells were treated with the β -adrenergic agonist (isoproterenol) and its antagonist (propranolol) at a dose of 100 nM. Total RNA was extracted, and complementary DNA (cDNA) was synthesized. The expression levels of Cx26 and Cx32 were quantified using real-time polymerase chain reaction (real-time PCR), while protein expression was evaluated by Western blot analysis. Apoptosis was assessed by flowcytometry.

Results

Baseline expression levels of Cx26 and Cx32 were significantly lower in the study groups than control ($p < 0.001$). Treatment with isoproterenol significantly reduced both gene expression levels in all groups ($P < 0.001$), whereas propranolol treatment significantly increased it ($P < 0.001$).

Western blot findings were consistent with the gene expression results. Furthermore, isoproterenol increased apoptosis, while propranolol reduced it.

Conclusion

Inhibition of beta-adrenergic receptors confirmed the existence of a potential response for up-regulating Cx26 and Cx32 gene expression in cumulus cells. This approach represents a novel therapeutic strategy for improving ovarian function and oocyte competence in women with PCOS and POR, as well as an adjunct therapy in assisted reproductive technologies (ART).

Keywords: Connexin 26 and 32, cumulus cells, infertility, β -adrenoceptor, polycystic ovary syndrome, poor response

1. Introduction

Background

Cellular connections & follicle growth

In oocytes, connexin gene expression exhibits a unique pattern that regulates metabolic, hormonal, and developmental processes. Connexins (Cxs) and pannexins (Pxs) are transmembrane proteins that form large-pore channels involved in intercellular communication. Gap junction channels, which are primarily composed of connexins, establish direct communication pathways between neighboring cells, and play essential roles in cellular homeostasis, growth, differentiation, and migration [1, 2]. In the ovary, gap junction-mediated communication is critical for folliculogenesis, oocyte growth, oocyte maturation, and granulosa cell proliferation. Consequently, disruption of gap junction function can impair follicular development and contribute to female infertility [1]. Alterations or mutations in

several connexin isoforms, including Cx26, Cx32, Cx37, Cx40, and Cx43, have been associated with reproductive dysfunction and impaired fertility [3]. Therefore, a better understanding of connexin-mediated signaling pathways may provide valuable insights into the mechanisms underlying female reproductive disorders.

Since the identification of connexin family members by Beyer and colleagues in 1987, at least 21 connexin isoforms have been characterized based on their molecular weights and amino acid sequences. Structural diversity among connexins is largely determined by variations in the cytoplasmic loop and C-terminal domains, which influence channel properties and regulatory functions [4]. Connexins exhibit rapid turnover compared with many other membrane proteins, making them attractive therapeutic targets. Their short half-life enables cells to rapidly modify gap junction communication in response to changing physiological and environmental conditions [4-7]. Connexin proteins are synthesized in the endoplasmic reticulum and oligomerize into hexameric structures known as connexons or hemichannels. These hemichannels are subsequently transported to the plasma membrane, where they dock with connexons from adjacent cells to form complete gap junction channels. The resulting channels permit passive diffusion of molecules with molecular weights generally below 1 kDa, thereby facilitating direct intercellular communication [6]. The rapid assembly, turnover, and degradation of connexin channels enable dynamic regulation of gap junction intercellular communication (GJIC), allowing tissues to respond efficiently to physiological demands and environmental changes. Connexin turnover is tightly regulated through multiple cellular mechanisms controlling channel assembly, internalization, recycling, and degradation, thereby ensuring appropriate levels of intercellular communication under both physiological and pathological conditions (Fig1) [7].

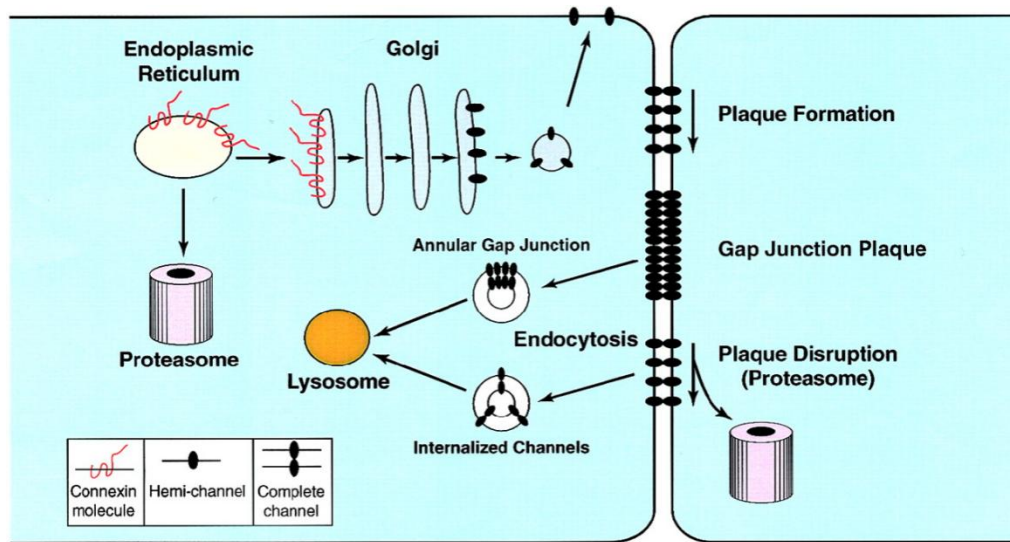


Fig1. Model of intracellular pathways involved in connexin trafficking and degradation [7].

Second messengers, including cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), Ca^{2+} , adenosine, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and various metabolites, are critical intracellular signaling molecules that transmit signals from membrane receptors to downstream effector pathways, thereby regulating cellular responses. These molecules amplify intracellular signaling and coordinate diverse physiological processes [8].

In addition to their localization at the plasma membrane, connexins have been identified in non-canonical cellular compartments, including mitochondria and the nucleus, where they contribute to the regulation of cell growth, differentiation, and survival [9]. Connexin hemichannels (connexons) exhibit rapid turnover and dynamic regulation, allowing tissues to adapt quickly to changing physiological conditions [10]. For example, Cx43 hemichannels display a very low opening probability under physiological conditions, thereby maintaining controlled inter cellular communication [11].

Alterations in connexin expression have been associated with chronic inflammation and tissue injury. Several studies have demonstrated that inflammatory conditions can modify the expression and

activity of Cx26, Cx32, and Cx43, resulting in altered gap junction communication and increased hemichannel activity [12]. Mugisho et al. demonstrated that Cx43-mediated ATP release contributes to the initiation and amplification of inflammatory responses through an autocrine feedback mechanism. Consequently, connexin hemichannels have been implicated in the pathogenesis of several inflammatory and ischemic disorders [13]. Within the ovary, connexins are essential for maintaining bidirectional communication between the oocyte and surrounding somatic cells, thereby supporting follicular growth and oocyte maturation. Dysregulation of these communications have also been associated with numerous pathological conditions, including liver diseases [14], retinal injury [15], cancer [16], and disorders of the reproductive system [17]. Although Cx37 and Cx43 have been extensively studied in the cumulus–oocyte complex (COC), increasing evidence indicates that Cx26 and Cx32 are also expressed in ovarian tissues and may participate in follicular development and cellular signaling during oogenesis [18, 19]. The distribution of connexins varies throughout follicular development. Cx26, Cx37, and Cx43 are expressed in primordial, primary, and secondary follicles, whereas Cx32 expression is predominantly detected in antral follicles. In addition, Cx26 and Cx37 are present in ovarian blood vessels, while Cx26 is also expressed within stromal tissues [18]. These localization patterns suggest distinct but potentially complementary roles for individual connexins during folliculogenesis and ovarian function. The distribution of Cx26, Cx32, Cx37, and Cx43 in various regions of the ovary is summarized in Table 1, and illustrated in Figure 2.

Table1. Localization of Cx26, Cx32, Cx37, and Cx43 in ovarian compartments [18].

Ovarian compartment	Protein
Primordial follicles	Cx26, Cx37 and Cx43

Primary follicles	Cx26, Cx37 and Cx43
Secondary follicles	Cx26, Cx37 and Cx43
Antral follicles	Cx26, Cx32, Cx37 and Cx43
Blood vessels	Cx26 and Cx37
Stromal tissues	Cx26

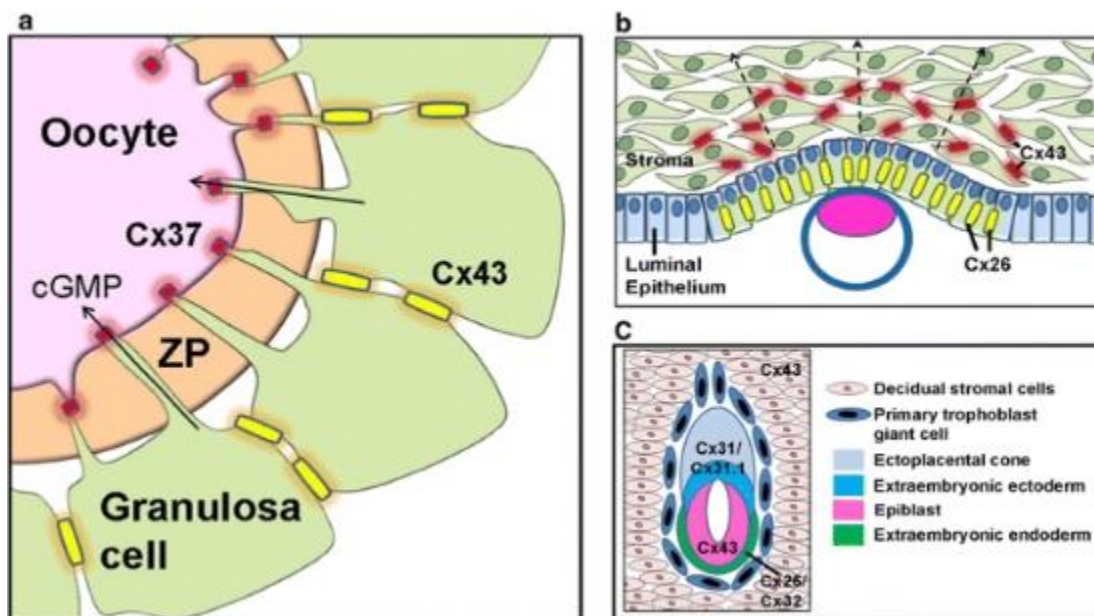


Fig2. Localization and physiological roles of connexins in female reproduction. (a) Schematic representation of gap junction communication within the ovarian follicle, illustrating Cx43-mediated coupling between cumulus cells (CCs) and Cx37-mediated communication between granulosa cells (GCs) and the oocyte. (b) Proposed role of connexins during embryo implantation. Cx26 expression is induced in the luminal epithelium, whereas Cx43 expression is upregulated in stromal cells undergoing decidualization. (c) Differential expression of

connexins during early embryonic development, showing Cx43 localization in the epiblast and Cx26/Cx32 expression in the extraembryonic endoderm [19].

The ovary receives extensive autonomic innervation, including sympathetic nerve fibers that regulate follicular growth, steroid hormone production, ovulation, and ovarian blood flow [20, 21, 22, 23, 24]. Increasing evidence suggests that dysregulation of sympathetic nervous system activity contributes to several reproductive disorders, particularly polycystic ovary syndrome (PCOS). Increased sympathetic tone and increased ovarian noradrenaline (NA) concentrations have been reported in women with PCOS and in laboratory animal models, suggesting a role for adrenergic signaling in the pathophysiology of this disease. Increased sympathetic tone and elevated ovarian noradrenaline (NA) concentrations have been widely reported in women with PCOS and in laboratory animal models, indicating the role of adrenergic signaling in the pathophysiology of this disease [20, 21].

Adrenergic signaling is mediated through α - and β -adrenergic receptors, which belong to the G protein-coupled receptor (GPCR) superfamily. Among these receptors, the β -adrenergic receptor (β -AR) has attracted considerable attention because of its involvement in follicular development, steroidogenesis, and ovarian function [24, 25]. Activation of β -AR stimulates adenylate cyclase activity, leading to increased intracellular cAMP production and activation of signaling pathways that can regulate gene expression, cellular metabolism, and intercellular communication [8, 21]. Recent studies have demonstrated the presence of β -AR in human and non-human primate ovarian follicles, particularly in granulosa and CCs surrounding the developing oocyte [25-27]. These findings suggest that β -AR-mediated signaling may contribute to the regulation of follicular maturation and oocyte competence. Over stimulation of the beta receptor can cause receptor

desensitization and its down-regulation, which leads to impaired cellular responsiveness and altered ovarian function [28-30]. Such mechanisms may be particularly relevant in reproductive disorders characterized by chronic sympathetic over activity. Given the critical role of β -adrenergic signaling in ovarian function and the importance of connexin-mediated communication in oocyte development, and investigation of the relationship between β -AR activity and connexin expression may provide novel insights into the molecular mechanisms underlying infertility associated with PCOS and poor ovarian response (POR). Our study was designed to evaluate the effects of β -adrenergic receptor modulation on the expression of Cx26 and Cx32 in cultured CCs obtained from infertile women with PCOS and POR undergoing assisted reproductive treatment. Increased ovarian NA content and enhanced neurotransmitter release from ovarian sympathetic nerve terminals have been associated with the down-regulation of β -adrenergic receptors in granulosa cells and theca-interstitial cells. Beta-adrenergic receptor blockade increased expanding the subfertility window and can promote successful fertility planning in aging women. In morphological analysis, they reported an increase in the number of healthy antral follicles, no change in atretic antral follicles, and a decrease in the size and number of cystic follicles. Serum androstenedione levels were increased with propranolol [28]. Alterations in adrenergic receptor signaling may contribute to impaired follicular development and ovarian dysfunction. Down-regulation of α 2A- and α 2B-adrenergic receptor subtypes involves an increased rate of receptor degradation accompanied by a reduction in receptor synthesis [29]. Similarly, persistent stimulation of β -adrenergic receptors induces receptor desensitization and down-regulation, resulting in impaired G protein coupling, receptor internalization, and subsequent recycling or degradation. These processes ultimately reduce receptor mRNA and protein expression levels [30]. The process of beta receptor phosphorylation typically results in two safety outcomes for the cell: 1) desensitization for termination of signaling to prevent over stimulation of the cell,

and 2) internalization for entry of the receptor into the cell membrane (down-regulation). Desensitization of β -adrenergic receptor (β -AR) signaling is a tightly regulated process that prevents excessive receptor stimulation and maintains cellular homeostasis. The classical desensitization pathway consists of three sequential steps: (1) receptor phosphorylation, (2) interaction with arrestin proteins, and (3) receptor internalization. The phosphorylation pattern of the receptor is influenced by several factors, including cell type, receptor expression level, and the nature and duration of agonist exposure.

Duration in the exposure agonist includes short- and long-term stress-induced adaptations in norepinephrine (NE) containing cell bodies may contribute to these conditions. The short-term response is immediate and primary action of CRF signaling during stressors exposure via electrophysiological properties on the membrane depolarization but Long-term response is the intracellular signaling cascades that promote noradrenergic signaling in the brain stem nucleus locus coeruleus (LC as a smaller noradrenergic nuclei) in CNS [31].

Our study on the process of inducing polycystic ovaries in rats demonstrated that chronic cold stress, applied for three weeks prior to estradiol administration, was able to prevent the development of poly cystic ovaries. Based on the results of the study, we suggested that this lack of modeling may be due to reduced expression of beta-adrenergic receptors in the rat ovary [32, 33]. More recently study on the chronic stress adversely affects female reproductive function by reducing the proportion of mature oocytes and impairing oocyte developmental competence [34]. Gein's et al. demonstrated the role of beta-adrenergic receptors, as 10-min cold stress increased stimulated ROS production, while 60-min cold stress increased it. The β -receptor blocker was able to equalize the effect of 10-min stress in stimulated cultures and the effect of 60-min stress in spontaneous cultures. They

suggested that β -adrenergic receptors are directly involved in the regulation of cytokine production [35, 36], and microbicidal potential of macrophages in acute cold stress [35]. Subsequent studies have further elucidated the role of β -arrestins in receptor signaling. β -Arrestins are multifunctional intracellular scaffolding proteins involved in numerous physiological processes, including cell proliferation, differentiation, migration, and apoptosis. β -arrestins regulate receptor trafficking, signal attenuation, receptor turnover, and downstream signaling cascades. Furthermore, β -arrestins play important roles in immune regulation and inflammatory signaling pathways. Yang et al. reported that β -arrestin-1 interacts with distinct phosphopeptide patterns that mimic phosphorylation sites within the C-terminal region of β -ARs targeted by GRK2, GRK6, or protein kinase A (PKA). These interactions generate specific phospho-interaction profiles that are associated with different functional outcomes. This observation led to the development of the “barcode hypothesis,” which proposes that GPCRs can be phosphorylated at multiple sites in a context-dependent manner, producing unique phosphorylation signatures that determine the nature of β -arrestin recruitment and downstream signaling responses. GPCR kinases (GRKs) and arrestins interact with agonist-bound GPCRs for promoting receptor desensitization and downregulation. The trigger signaling cascades by arrestin distinct from heterotrimeric G proteins. Biased agonists for GPCRs that favor either heterotrimeric G protein or GRK/arrestin signaling are of profound pharmacological interest because they could usher in a new generation of drugs with greatly reduced side effects [37].

2. Materials and Method

2.1. Participants

This experimental study included 45 women who were divided into three groups: a control group (oocyte donors), a poor ovarian response (POR) group, and a polycystic ovary syndrome (PCOS)

group. The inclusion criteria were age between 20 and 40 years and a body mass index (BMI) <28 kg/m². Women with systemic diseases or a history of medication use affecting ovarian function were excluded from the study.

Participants were recruited from Vali-e-Asr Infertility Clinic, Tehran University of Medical Sciences, between November 2023 and December 2024. The diagnosis of POR was established according to the European Society of Human Reproduction and Embryology (ESHRE) Bologna criteria, whereas PCOS was diagnosed based on the joint recommendations of the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine (Rotterdam ESHRE/ASRM 2003) [38, 39].

The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences (Approval No. IR.TUMS.VCR.REC. 2022/6/15), and written informed consent was obtained from all participants prior to enrollment.

2.2. Collection and culture of cumulus cells

After the diagnosis, all groups (control, PCOS and POR) were prescribed ovulation stimulant drugs from the second or third day of the menstrual cycle, along with gonadotropins and gonadotropin-releasing hormone (GnRH) analogs (antagonists) to prevent premature luteinizing hormone (LH) surge during ovarian stimulation. Ovarian desensitization was confirmed when estradiol levels were less than 0.05 nmol/L and follicles were less than 5 mm in diameter. Subsequently, they were treated with 150-300 IU of gonadotropin, specifically follitropin α (Gonal F; Merck Serono S.p.A., an affiliate of Merck KGaA, Darmstadt, Germany). Once a dominant follicle measuring ≥ 12 -14 mm in diameter was observed via sonographic monitoring, a GnRH antagonist, cetrorelix acetate (Cetrotide; Asta Medica AG, Frankfurt, Germany), at a dose of 0.25 mg subcutaneously was administered. HCG (human chorionic gonadotropin) was prescribed when two follicles reached a size of 16-18 mm,

after which a follicle puncture was performed. Cumulus cells (CCs) were isolated and counted with a Neobar lamella, and then added to the culture medium.

Following oocyte retrieval, cumulus–oocyte complexes (COCs) were collected, and cumulus cells (CCs) were enzymatically isolated. The isolated cells were counted using a Neubauer hemocytometer and transferred to culture medium for further experiments.

2.3. Cumulus survival and growth culture

Isolated CCs were washed several times with phosphate-buffered saline (PBS) containing antibiotics. The cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 3-cm culture dishes coated with gelatin solution (0.1 mg/cm²; Sigma-Aldrich, Cat. No. G1393).

Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cell growth and morphology were monitored after 48 and 72 h of incubation. When cell confluence reached approximately 80%, the cells were passaged at a ratio of 1:3 using 0.2% trypsin–EDTA solution.

At the end of the culture period, viable cumulus cells were harvested and stored at –80°C until further molecular analyses.

2.4. Study groups

Cultured cumulus cells obtained from the control, POR, and PCOS groups were randomly allocated to three experimental subgroups:

1. **Untreated group** (culture medium only);
2. **Isoproterenol-treated group** (β -adrenergic receptor agonist);
3. **Propranolol-treated group** (β -adrenergic receptor antagonist).

Isoproterenol (Monico, Italy) and propranolol (Toliddaru, Iran) were added to the culture medium at a final concentration of **100 nM** and incubated for **72 h**. These concentrations were selected based on previous studies demonstrating effective modulation of β -adrenergic receptor activity under in vitro conditions. The experimental design and treatment conditions are summarized in Table 2.

Table 2-1. Arrangement of study groups

Group symbole	Extracted source	Treatment
N	Normal Ovary	Without treatment
N-IPR	Normal Ovary	Isoproterenol β - agonist
N-Pro	Normal Ovary	Propranolol β - antagonist
POR	Poor response ovary	Without treatment
POR-IPR	Poor response ovary	Isoproterenol β - agonist
POR-Pro	Poor response ovary	Propranolol β - antagonist
PCOS	Typical PCOS	Without treatment
PSOC-IPR	Typical PCOS	Isoproterenol β - agonist
PCOS-Pro	Typical PCOS	Propranolol β - antagonist

2.5. RNA Extraction and cDNA Synthesis

Total RNA was extracted from cultured cumulus cells using standard RNA isolation procedures according to the manufacturer's instructions. The concentration and purity of the extracted RNA were determined using a UV spectrophotometer by measuring absorbance at 260 and 280 nm. Only RNA samples with acceptable purity were used for subsequent analyses.

Complementary DNA (cDNA) was synthesized from the extracted RNA using a reverse transcription kit according to the manufacturer's protocol. The resulting cDNA samples were stored at -20°C until further analysis.

2.6. Real-time polymerase chain reaction (real-time PCR, or qPCR Quantitative)

The mRNA expression levels of Cx26 and Cx32 were quantified by real-time polymerase chain reaction. PCR amplification was performed using 35 cycles consisting of denaturation at 94°C for 60 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. The primer sequences used for amplification are presented in Table2.

Table2. Primer sequences used for real-time PCR analysis.

Type of connexin mRNA	Primer probes	Nucleotide sequences
Cx 26	Sense	5' CCGAAGTTCATGAAGGGAGAGAT
	Antisense	5' GGTCTTTTGGACTTCCCTGAGCA
Cx 32	Sense	5' CTGCTCTACCCGGGCTATGC
	Antisense	5' CAGGCTGAGCATCGGTCGCTCTT

Relative gene expression levels were calculated after normalization to the housekeeping gene and expressed as fold changes relative to the control group.

2.7. Protein Expression Analysis by Western Blotting

Protein expression levels of Cx26 and Cx32 were evaluated by Western blot analysis. The primary antibodies used were anti-Cx26 (GTX54817), anti-Cx32 (SC-59948), and anti-GAPDH (GTX100118). Secondary antibodies included rabbit secondary antibody (BA1054-2) and mouse secondary antibody (SC-516102).

Western blotting was performed according to the Laemmli and Towbin methods with minor modifications to improve the detection of low-abundance proteins. Protein bands were visualized and quantified using densitometric analysis, and expression levels were normalized to GAPDH.

2.8. Apoptosis Assay

Apoptosis of cultured cumulus cells was evaluated using flow cytometry according to the manufacturer's protocol. The percentage of apoptotic cells was determined and compared among the experimental groups.

3. Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD). Differences among the experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). A P-value < 0.05 was considered statistically significant.

4. Results:

2.1. Analysis of Cumulus Cell Growth

Phase-contrast microscopy observations (Figures 3 - 1 to 3 - 4) revealed that treatment with isoproterenol, a β -adrenergic receptor agonist, reduced the growth and proliferation of cumulus cells (CCs) in all study groups. This inhibitory effect was most pronounced in the POR group. In contrast, treatment with propranolol, a β -adrenergic receptor antagonist, enhanced CC growth compared with untreated cells, suggesting a beneficial effect of β -adrenergic receptor blockade on cumulus cell survival and proliferation.

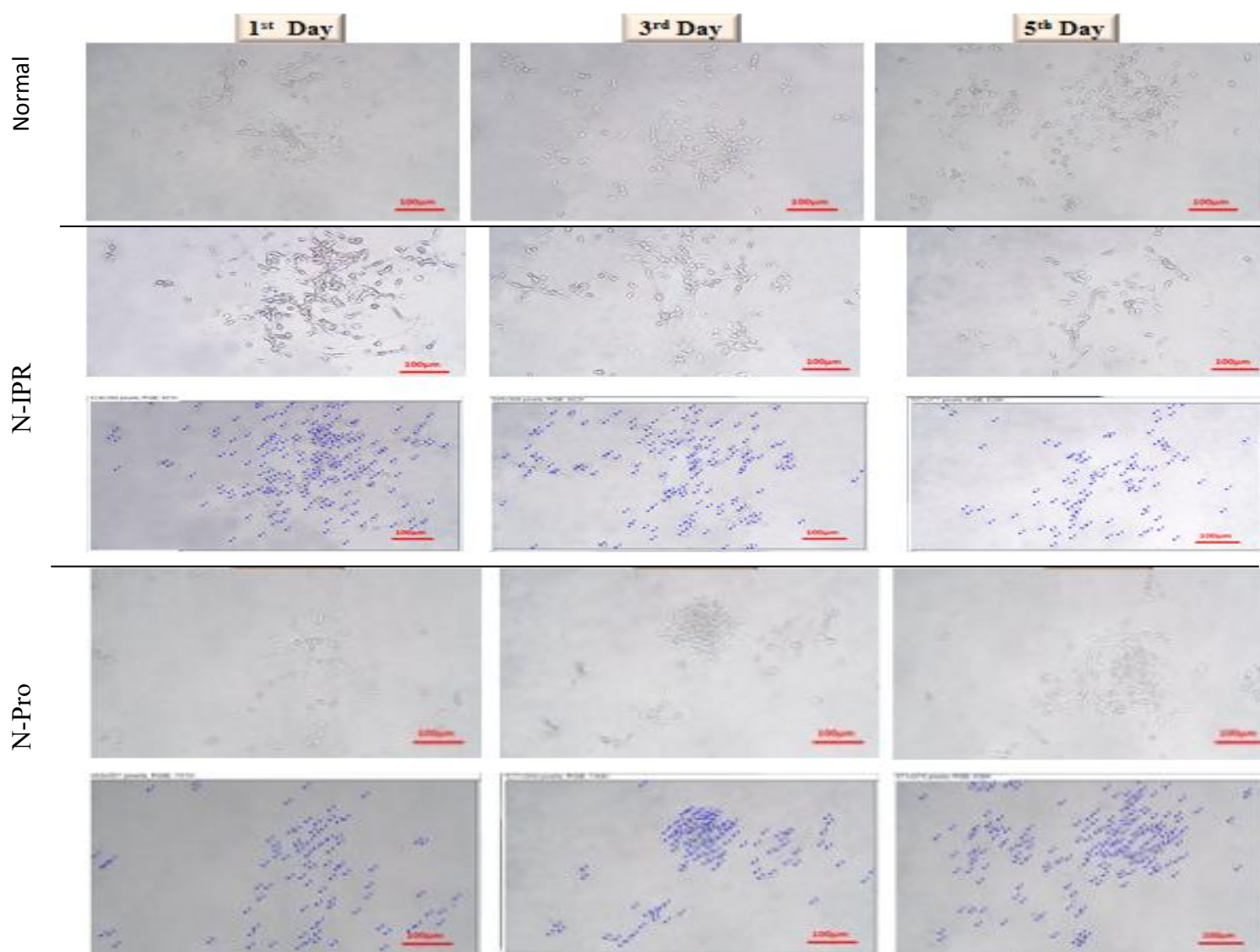


Fig3-1. CCs growth in control group. Treatment with IPR decreased cell growth while treatment with Pro increased it.

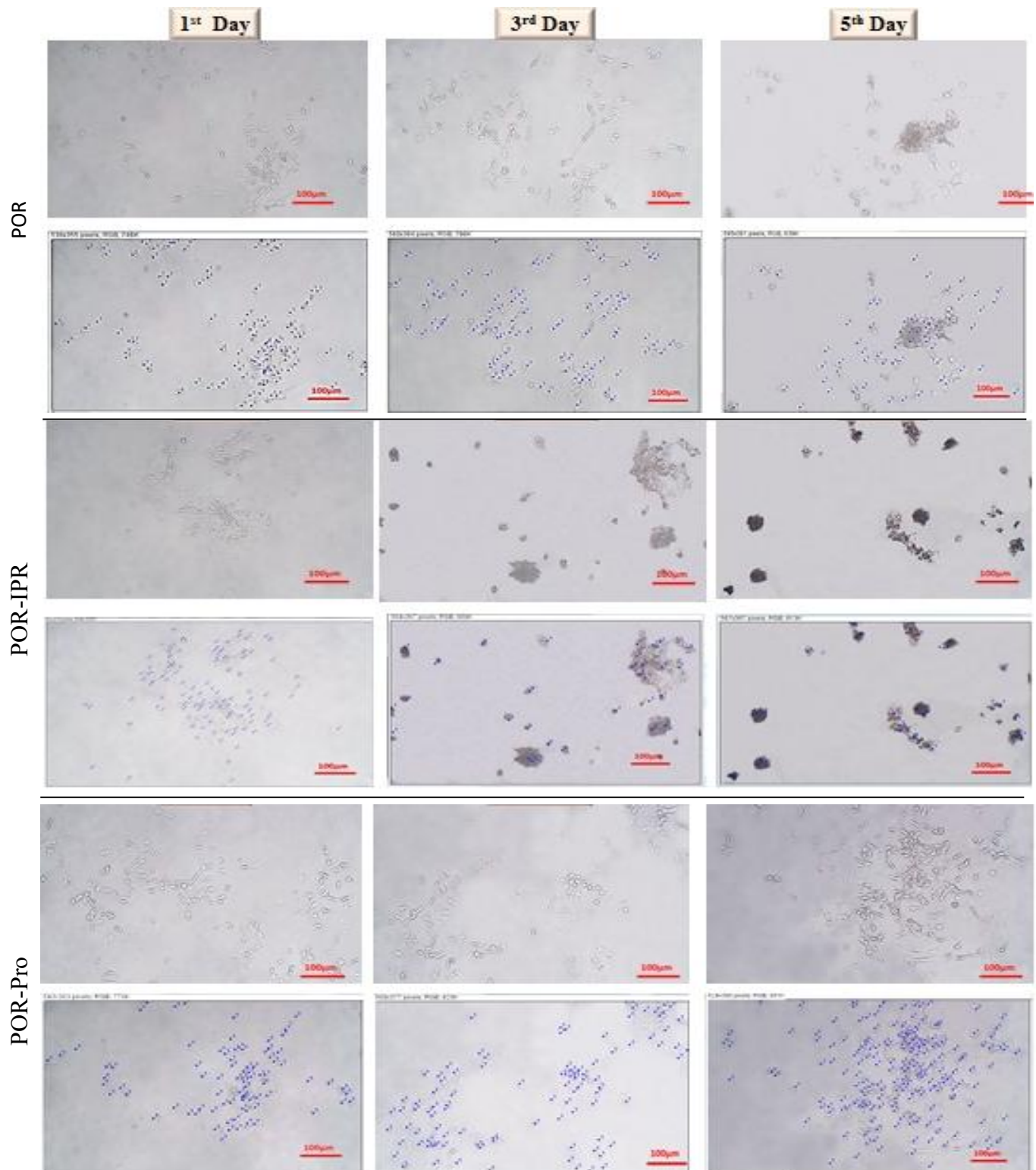


Fig3-2. CCs growth in POR group. Treatment with IPR decreased cell growth while treatment with Pro increased it.

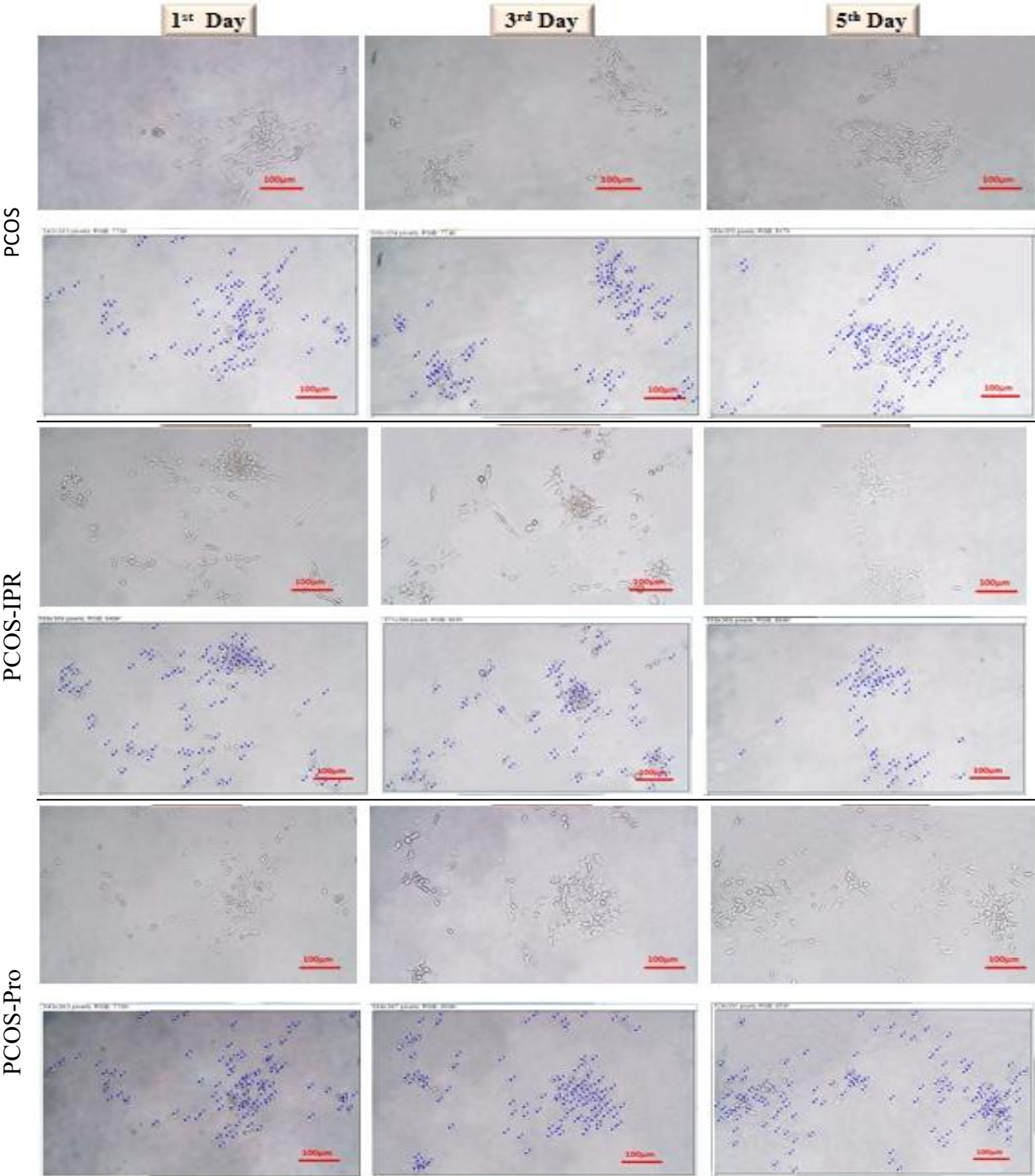


Fig3-3. CCs growth in the PCOS group. Treatment with IPR decreased cell growth while treatment with Pro increased it.

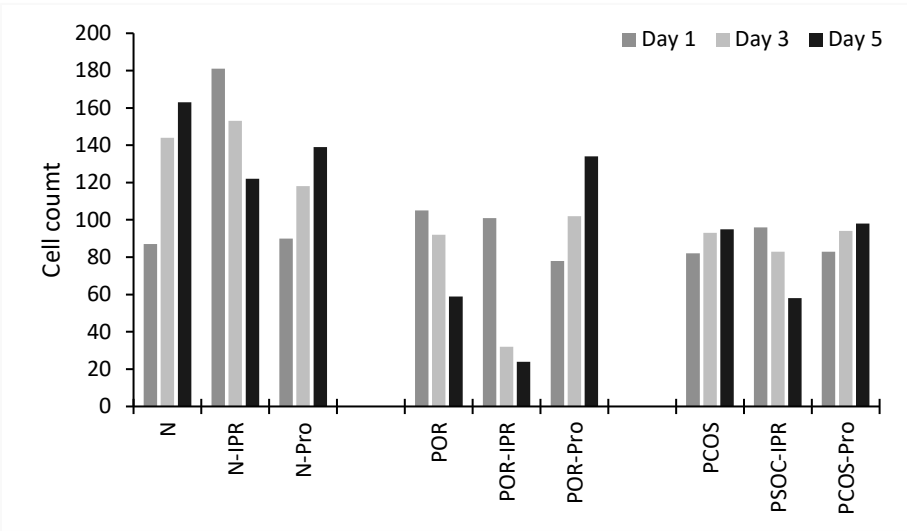


Fig3-4. Comparison of control (N), POR and PCOS. Treatment with IPR decreased cell growth specially in POR group. while treatment with Pro increased CCs growth.

2.2. Gene Expression Analysis

Analysis of gene expression revealed that the mRNA levels of both Cx26 and Cx32 in cumulus cells (CCs) obtained from the POR and PCOS groups were significantly lower than those observed in the control group. Treatment with isoproterenol (β -adrenergic receptor agonist) further reduced the expression of Cx26 and Cx32 mRNA in the control, POR, and PCOS groups. In contrast, treatment with propranolol (β_2 -adrenergic receptor antagonist) significantly increased the expression levels of both connexin genes in all study groups. Detailed gene expression data are presented in Table 3-1, and representative results are shown in Figure 3-5.

Table 3-1. Comparison of gene relative expression in the study groups

	Connexin 26			Connexin 32		
	Mean	Std. Deviation	Homogeneous subset	Mean	Std. Deviation	Homogeneous subset
N	1.00	0.05	a b	1.08	0.05	a b
N-IPR	0.75	0.06		0.74	0.04	
N-Pro	1.05	0.08	a	1.14	0.07	a
POR	0.16	0.07	d	0.24	0.07	c
POR-IPR	0.12	0.02	d	0.14	0.04	c
POR-Pro	0.44	0.04	d	0.52	0.05	b
PCOS	0.20	0.05	c	0.28	0.07	c
PSOC-IPR	0.14	0.02	d	0.17	0.04	c
PCOS-Pro	0.51	0.08	c	0.61	0.08	b

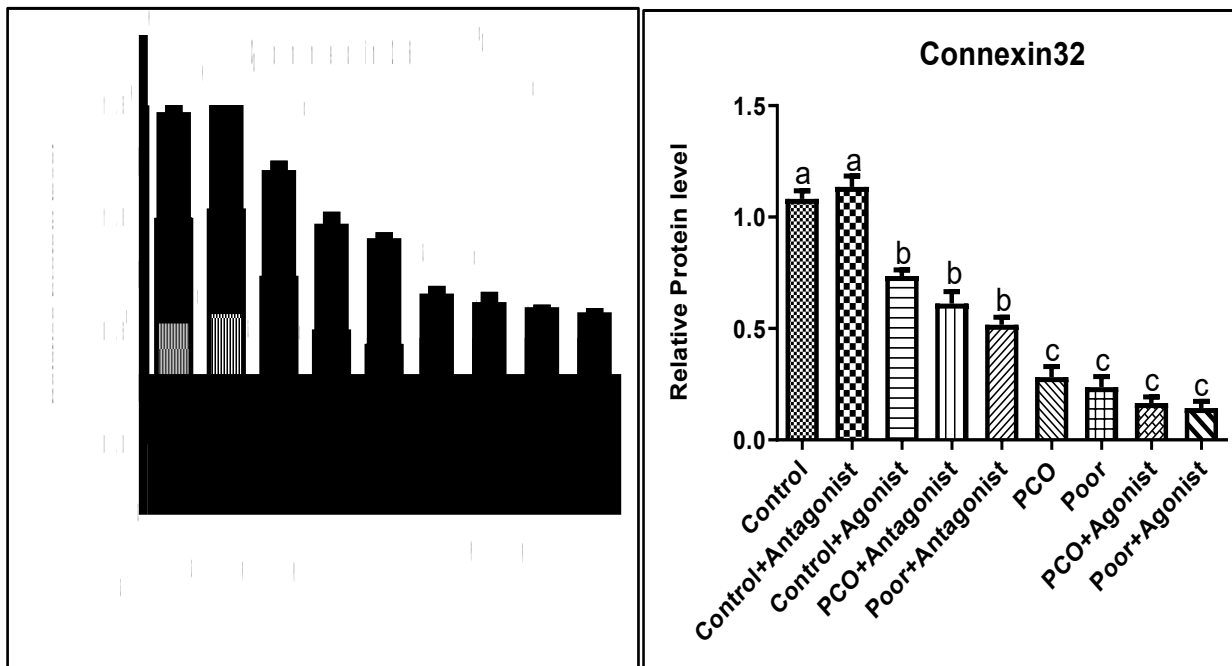


Figure 3-5. Relative expression levels of Cx26 and Cx32 mRNA in cumulus cells obtained from the control (N), POR, and PCOS groups following treatment with isoproterenol (IPR) or propranolol (Pro). Isoproterenol treatment decreased Cx26 and Cx32 gene expression in the POR and PCOS groups, whereas propranolol treatment increased their expression. Columns sharing the same lowercase letter are not significantly different, while columns with different letters indicate statistically significant differences ($P < 0.05$).

2.3. Protein Expression Analysis

Western blot analysis was performed to evaluate the protein expression levels of Cx26 and Cx32 in cultured cumulus cells. Representative immunoblot images are shown in Figure 3-6. The results demonstrated that the protein expression levels of both Cx26 and Cx32 in the POR and PCOS groups were significantly lower than those observed in the control group.

Treatment with isoproterenol (IPR; β_2 -adrenergic receptor agonist) further decreased the protein expression of Cx26 and Cx32 in the control, POR, and PCOS groups. In contrast, treatment with propranolol (Pro; β -adrenergic receptor antagonist) significantly increased the expression of both connexin proteins in all study groups. Representative Western blot images and quantitative densitometric analyses are presented in Figures 3-6 and 3-7 and Table 3-2.

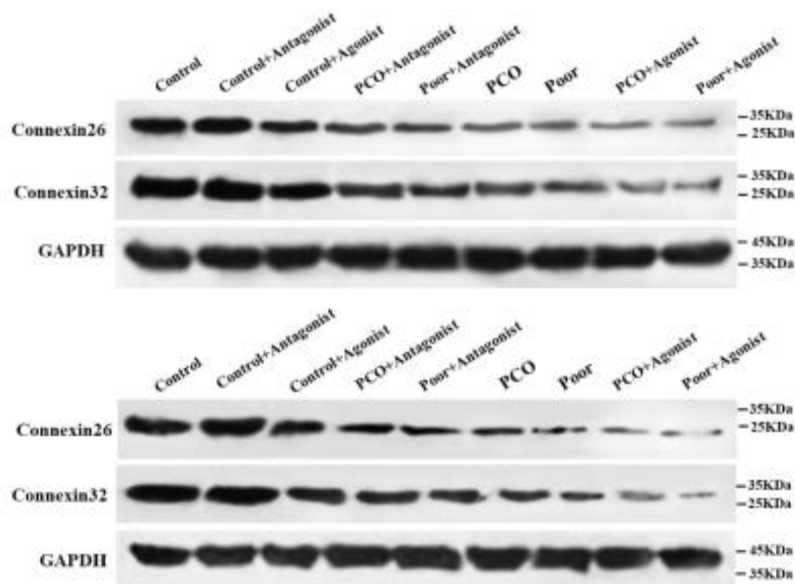


Fig3-6. Two experiment western blotting electrophoresis gel of protein expression for of connexin 26 and 32

Table 3-2. Comparison of protein relative expression in the study groups

	Connexin 26			Connexin 32		
	Mean	Std. Deviation	Homogeneous subset	Mean	Std. Deviation	Homogeneous subset
N	1.00	0.05	a	1.08	0.05	a
N-IPR	0.75	0.06	b	0.74	0.04	b
N-Pro	1.05	0.08	a	1.14	0.07	a
POR	0.16	0.07	d	0.24	0.07	c
POR-IPR	0.12	0.02	d	0.14	0.04	c
POR-Pro	0.44	0.04	d	0.52	0.05	b
PCOS	0.20	0.05	c	0.28	0.07	c
PSOC-IPR	0.14	0.02	d	0.17	0.04	c
PCOS-Pro	0.51	0.08	c	0.61	0.08	b

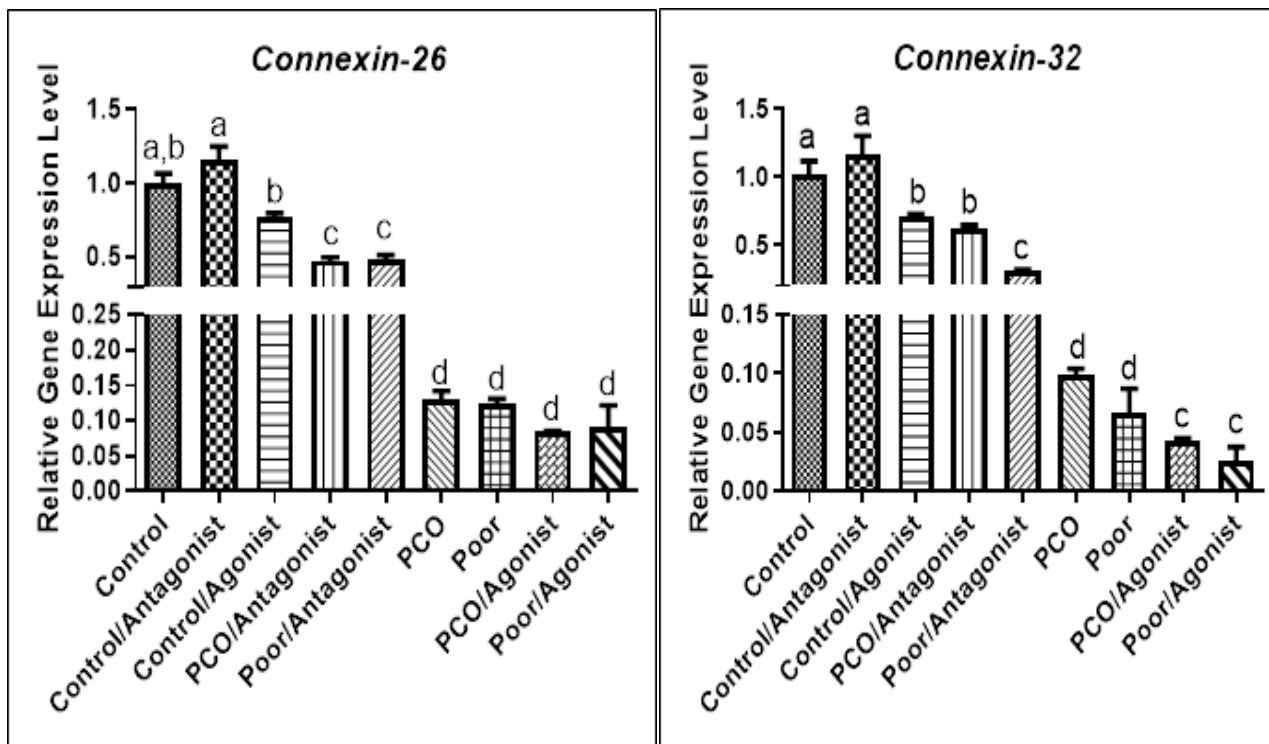
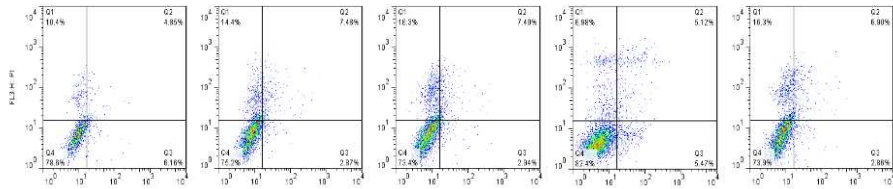


Fig3-7. Similar Latin letters do not show significant differences. Non-synonymous letters in each column show significant differences from the other column. Comparison of connexin 26 and connexin 32 protein expression in control (N), POR and PCOS. Treatment with IPR decreased protein expression while treatment with Pro increased it.

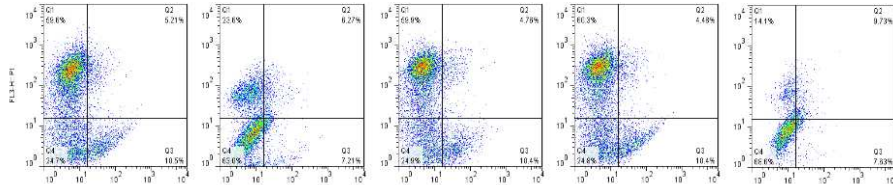
2.4. Apoptosis Assessment by Annexin V/PI Staining

Apoptosis was evaluated in cultured cumulus cells (CCs) using Annexin V/propidium iodide (PI) staining followed by flow cytometric analysis. The percentage of apoptotic cells was significantly higher in the POR and PCOS groups than in the control group. Treatment with isoproterenol (IPR; β_2 -adrenergic receptor agonist) further increased the proportion of apoptotic cells in the control, POR, and PCOS groups. In contrast, treatment with propranolol (Pro; β -adrenergic receptor antagonist) significantly reduced apoptosis in all study groups. Representative flow cytometry plots are shown in Figures 3-8a–c, whereas quantitative analysis of apoptotic cells is presented in Figure 3-9 and Table 3-3.

Normal



N-IPR (agonist)



N-Pro (antagonist)

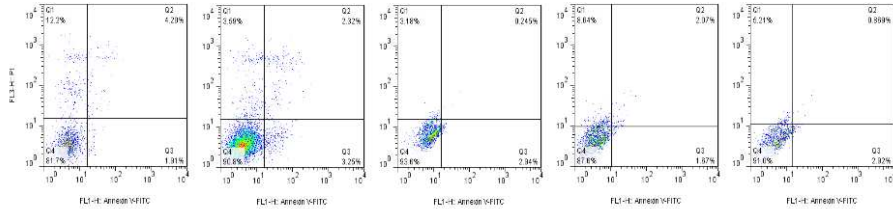
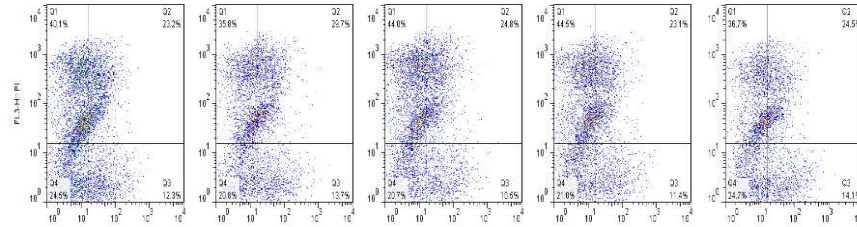


Figure 3-8a:

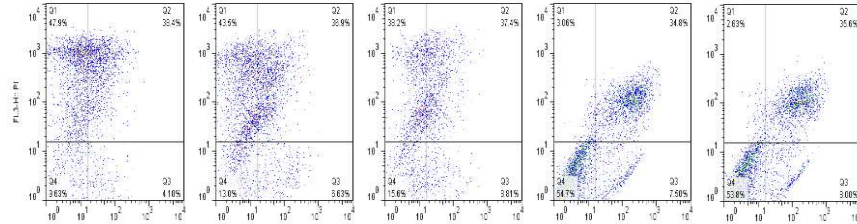
Flowcytometry result of apoptosis in cumulus cells in normal ovary (control) group.

Treatment with IPR increased apoptosis while treatment with Pro decreased cumulus cell growth.

Poor



Poor-IPR
(agonist)



Poor-Pro
(antagonist)

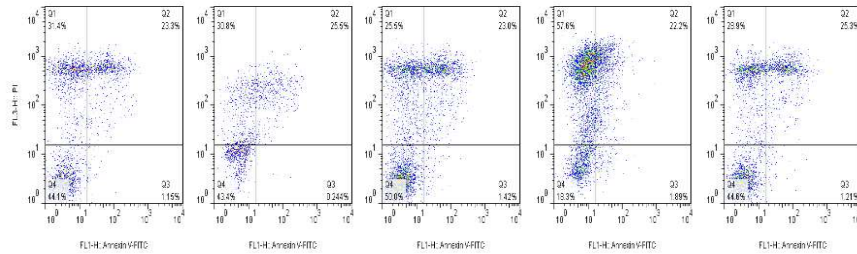


Figure 3-8b: Flowcytometry result of apoptosis in cumulus cells in poor group.

Treatment with IPR increased it while treatment with Pro decreased CCs growth

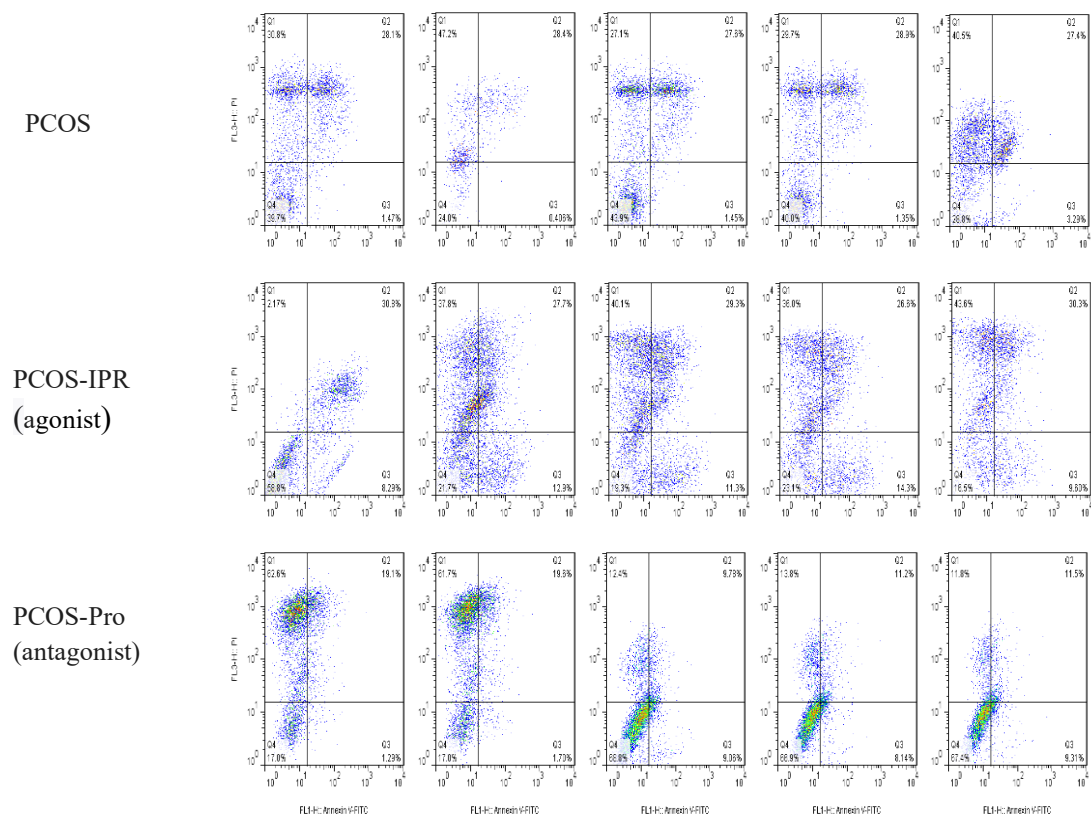


Figure 3-8c: Flowcytometry result of apoptosis in cumulus cells in PCOS group.

Treatment with IPR increased it while treatment with Pro decreased CCs growth.

Table 3-3. comparison of apoptotic cells (%) in the study groups

	Apoptotic cells (%)		
	Mean	Std. Deviation	Homogeneous subset
N	10.41	0.45	B
N-IPR	13.37	2.72	B
N-Pro	4.48	1.28	A
POR	37.46	3.67	F
POR-IPR	43.66	1.53	G
POR-Pro	25.04	1.04	D
PCOS	29.67	0.79	E
PSOC-IPR	40.22	0.73	Fg
PCOS-Pro	20.14	1.02	c

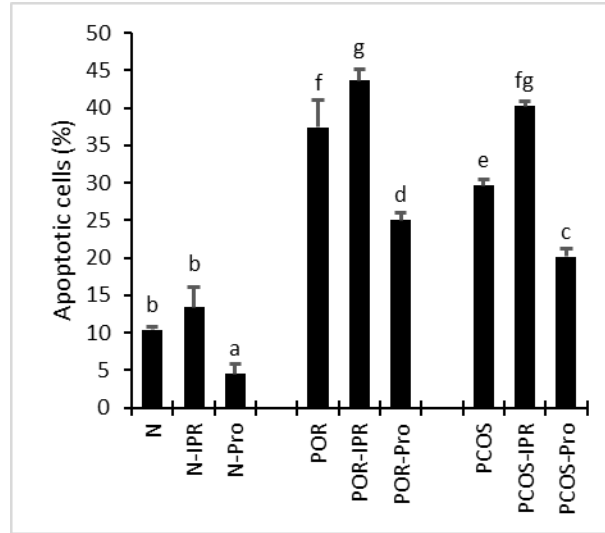


Fig3-9. Comparison of apoptotic CCS in control (N), POR and PCOS groups. Treatment with IPR increased apoptosis while treatment with Pro (propranolol β -antagonist) decreased it.

5. Discussion

Successful ovulation requires the development of competent oocytes that can be released from ovarian follicles at the appropriate stage of maturation. High-quality mature oocytes are essential for successful reproduction and are generated through the precise regulation of meiotic arrest, meiotic resumption, follicular development, and steroid hormone production. Previous studies have shown that noradrenaline (NA) as a neurohormone, plays a fundamental role in controlling follicular growth and ovarian steroid secretion. Garrido et al. demonstrated that ovarian NA content can recover following neonatal denervation in rats. However, despite restoration of ovarian NA levels, prolonged disruption of sympathetic innervation altered β -adrenergic receptor sensitivity and responsiveness, resulting in impaired follicular development and altered steroid hormone secretion. These changes ultimately affected both folliculogenesis and steroidogenesis, highlighting the importance of neural–ovarian communication in maintaining normal

reproductive function [40]. The regulatory mechanisms of receptor are essential for maintaining cellular homeostasis and preventing excessive receptor stimulation. This regulation occurs at β -adrenergic receptor (β -AR) through members of the G protein-coupled receptor (GPCR) super family in the cell membrane which is tightly regulated by feedback mechanisms—to control receptor responsiveness and signal transduction [41]. β -AR is one of the most extensively studied GPCRs and serves as a model for understanding receptor regulation. Upon agonist binding, β -AR undergoes conformational changes that promote coupling to heterotrimeric G proteins and activation of downstream signaling pathways, including the production of cyclic adenosine monophosphate (cAMP) as a second messenger [42]. Numerous studies have confirmed this role especially in polycystic ovary syndrome. In our previous in vivo and in vitro study 2022, we showed that clonidine, a selective α_2 -adrenergic receptor agonist, modulate sympathetic activity in women with PCOS. The intensity of clonidine effect showed a pattern for $ADR-\alpha_1 < ADR-\beta_2 < ADR-\alpha_2$. The main and central effect of clonidine is to reduce sympathetic nervous system (SNS) activity through stimulation of presynaptic α_2 -adrenergic autoreceptors, which consequently reduces the release of noradrenaline (NA) [43]. In addition to its effects on sympathetic tone, clonidine is now widely used in the management of conditions related to stress, anxiety, sleep disorders, and other neuropsychiatric disorders [44]. These findings further support the concept that modulation of adrenergic signaling pathways may influence ovarian function and reproductive outcomes in women with PCOS.

The description of the studies conducted above and our findings suggest that impaired beta adrenergic signaling in women with POR, ,and PCOS may be associated with receptor adaptation desensitization, or down-regulation. Persistent receptor stimulation initiates a series of regulatory events, beginning with receptor phosphorylation, followed by β -arrestin recruitment, receptor

internalization, and ultimately receptor desensitization [45]. β -Arrestin binding sterically prevents further G-protein coupling, thereby attenuating receptor signaling and protecting cells from over stimulation[46].

Desensitization occurs through both short-term and long-term mechanisms. Short-term desensitization involves rapid receptor phosphorylation at the cell surface and can occur within minutes of receptor activation. In contrast, long-term desensitization develops over hours to days and is characterized by receptor internalization, degradation, and a reduction in cell-surface receptor expression [47]. These adaptive mechanisms are critical for preserving physiological homeostasis and may contribute to altered ovarian responsiveness under conditions of chronic sympathetic stimulation.

The pivotal role of cyclic adenosine monophosphate (cAMP) as an intracellular second messenger in regulating the arrest or resumption of meiosis during oocyte maturation is considered a key mechanism in the cellular communication within the oocyte-cumulus cell complex [48]. Previous studies have demonstrated that cAMP signaling can enhance gap junction communication between CCs and oocytes by modulating connexin expression and phosphorylation. Zhao et al. reported that cAMP promotes Cx43 phosphorylation and expression, thereby improving cumulus–oocyte gap junction function [49]. Although their study focused primarily on Cx43, these findings support the concept that adrenergic signaling pathways may regulate connexin-mediated intercellular communication through cAMP-dependent mechanisms. Consistent with this hypothesis, our results showed that activation of the beta-adrenergic receptor leads to a decrease in Cx26 and Cx32 expression, whereas blocking this receptor increases their expression. These observations suggest that β -adrenergic signaling may influence ovarian function, at least in part, through the regulation of gap junction proteins and intercellular communication within

the cumulus–oocyte complex. Dynamic membrane-surface activity during chronic exposure to noradrenaline reduces connexin stability a process mediated by beta-adrenergic receptors that shortens the half-life of Cx26/32 plaques in the cell membrane.

Based on these findings, we propose that persistent stimulation of β -adrenergic by its agonist (NA) may induce receptor desensitization and down-regulation, leading to impaired downstream signaling and reduced connexin expression [28, 30, 32, 34]. These observations support a potential role for β -adrenergic receptor in regulating ovarian responsiveness through modulation of connexin-mediated communication within the COC.

Maintaining cellular homeostasis in healthy cells involves finely tuned mechanisms that attenuate or neutralize G protein-coupled receptor (GPCR) signaling, thereby preventing excessive or prolonged receptor activation. These regulatory processes may occur rapidly and transiently or develop gradually during sustained receptor stimulation. Such mechanisms are essential for preserving normal cellular physiology and ensuring appropriate responsiveness to extracellular signals. Several molecular pathways contribute to GPCR desensitization and signal termination. One important mechanism involves receptor ubiquitination, which promotes the trafficking and degradation of agonist-activated receptors through lysosomal and proteasomal pathways. The ubiquitin–proteasome system (UPS), the primary pathway responsible for regulated intracellular protein degradation, plays a critical role in receptor turnover and cellular protein quality control. In addition to receptor ubiquitination, phosphodiesterases (PDEs), regulators of G-protein signaling (RGS) proteins, and A-kinase anchoring proteins (AKAPs) contribute to the modulation and termination of GPCR-mediated signaling pathways [50]. Together, these mechanisms regulate receptor sensitivity, prevent overstimulation, and maintain physiological homeostasis. Therefore, to establish this homeostasis, the short-term desensitization of β -AR receptors via

receptor phosphorylation causes the receptor to uncouple from downstream secondary messenger signaling pathways. In contrast, long-term desensitization involves receptor internalization and ubiquitination, ultimately leading to degradation of agonist-activated receptors and a reduction in receptor availability at the cell surface. Accumulating evidence indicates that endogenous catecholamine play important roles in mammalian reproductive physiology and developmental processes, including the regulation of cell proliferation, differentiation, migration, morphogenesis, hormonal responsiveness, embryogenesis, and preimplantation embryo development [51]. Granulosa and CCs are responsive to both gonadotropins and adrenergic stimuli. Previous studies have shown that isoproterenol (Iso), a potent β -adrenergic receptor agonist, stimulates progesterone production in a time- and dose-dependent manner. Furthermore, adrenergic agents enhance the effects of human chorionic gonadotropin (hCG) and prolactin (PRL) on progesterone secretion, while exerting little or no effect on estrogen production [52]. Adrenergic signaling plays an important role in ovarian physiology and steroidogenesis. Based on this evidence, we treated cultured CCs with isoproterenol (Iso) and propranolol (Pro) to examine the effects of β -adrenergic receptor activation and blockade on Cx26 and Cx32 expression.

Among the connexin family members, Cx43 is highly expressed in granulosa and CCs and contributes to follicular growth in response to follicle-stimulating hormone (FSH). In addition, Cx37 is essential for both nuclear and cytoplasmic maturation of oocytes and plays a critical role in maintaining communication between the oocyte and surrounding follicular cells. Zhao et al. provided evidence that cAMP directly regulates Cx37 gene expression and proposed a novel interaction between cAMP signaling and gap-junction communication during in vitro culture of ovine COC [53].

Numerous studies have shown that the influx and outflux of the second messengers cAMP/cGMP into oocyte via gap-junction channels are essential for normal follicular development and reproductive function. Previous studies have also demonstrated that cAMP participates in the transcriptional regulation of the β -AR gene through feedback mechanisms that modulate receptor expression and responsiveness [54]. Therefore, alterations in β -adrenergic homeostasis can be influenced on the ovarian function through multiple interconnected pathways, including regulation of intracellular signaling (cAMP/cGMP), gap-junction communication, and connexin expression. Consistent with this concept, our findings showed that β -adrenergic receptor activation reduced the expression of Cx26 and Cx32, whereas receptor blockade increased it in cultured CCs. These observations suggest that alterations in the sensitivity of beta-adrenergic receptors to NA—potentially linked to impaired connexin-mediated communication within the COC—may contribute to ovarian dysfunction in women with PCOS and POR.

Our findings show that in these women, Cx26/32 functions may be disrupted by two ways: reduction of the protein expression by impaired β -adrenergic homeostatic response to NA (desensitization receptor), and surly altered phosphorylation patterns. PKA (protein kinase A) activity associated with phosphorylation is reduced as a result of this down-regulation, because dynamic membrane-surface activity during chronic exposure to NA diminishes connexin stability—a process mediated by beta-adrenergic receptors—thereby shortening the half-life of Cx26/32 plaques in the cell membrane. Research evidence in this field and the analysis of this study's results indicate that in women with PCOS and POR, impaired gap junction communication—resulting from the disruption of the beta-adrenergic pathway—may be the primary factor underlying the arrest of follicular growth and oocyte immaturity. In the antral

follicle, this bidirectional communication between granulosa cells and COC established via gap junctions located in transzonal projections facilitates interdependent metabolic and signaling processes essential for follicular growth, fertilization, and subsequent embryonic development [54, 55].

6. Conclusion

Analysis of the study results indicates that in women with polycystic ovary syndrome and ovarian aging, impaired gap junction communication—resulting from a down-regulation of the beta-adrenergic receptors—is the primary cause of arrested follicular growth and oocyte maturation failure. This impairment disrupts the bidirectional communication between granulosa and cumulus cells, thereby blocking the transmission of cellular signals essential for continued oocyte development.

Overall, the present study provides new evidence that modulation of β -adrenergic signaling influences connexin-mediated inter cellular communication in CCs and may represent a promising avenue for future research aimed at improving ovarian responsiveness and reproductive outcomes. A better understanding of the signaling pathways regulating oocyte growth and maturation—specifically within the context of the proper interaction between cumulus cells and the oocyte—could pave the way for significant advancements in pharmacology and assisted reproductive technologies (ART). Further experimental and clinical studies are required to clarify the therapeutic potential of targeting β -adrenergic pathways in assisted reproductive technologies and the management of infertility associated with PCOS and POR.

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Conflict of Interest

The authors declare that they have no interest in competing.

Ethical statement

The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences (Approval No. IR.TUMS.VCR.REC. 2022/6/15), and written informed consent was obtained from all participants prior to enrollment.

Author Contributions

Farideh Zafari Zangeneh, Associate Professor, pharmacist and physiologist, Protocol/project development determining of the doses of Propranolol and Isoproterenol for treatment in animal and clinical trials, Data collection or management, writing – review. Mohammad Mehdi Naghizadeh, assistant Professor in bio statistics, and data analysis. Masoomah Dehghan Reproduction PhD. and Sara Zabihzadeh Biochemistry PhD. student performed cumulus cells isolation, and IVF services. Malihe Afiat Associate Professor , and infertile fellowship

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