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Original Article

# Assessment of Gene Expression on the Gap Junction Connexin of Cumulus Cells on Infertile Women With Polycystic Ovary Syndrome and Poor Ovarian Response: The Novel Role of Propranolol

**Farideh Zafari Zangeneh; Pharm-D, Ph.D.[1], Mohammad Mehdi Naghizadeh; Ph.D.[2], Mina Jafarabadi; M.D.[1], Masoumeh Dehghan; Ph.D.[1], Maryam Bagheri; Ph.D.[1]**

1 Vali-E-Asr Reproductive Health Research Center, Family Health Research Institute, Tehran University of Medical Sciences, Tehran, Iran
2 Non-communicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran

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## Abstract

**Objective:** Building upon prior research, our investigation focused on examining changes in gene expression of Connexins 37 and 43 (Cx) influenced by β2-adrenergic agents in cumulus cell cultures from women with polycystic ovarian syndrome (PCOS) and poor ovarian response (POR), all of whom were candidates for in vitro fertilization (IVF).

**Materials and methods:** This experimental study was conducted between April 2021 and November 2023, involving three groups: a control group (donated eggs) and two study groups (POR and PCO). All three groups received ovulation stimulation drugs. Following oocyte puncture, cumulus cells (CCs) were isolated and placed in a culture medium. After three passages, CCs were exposed to the ADR-β2 agonist isoproterenol and its antagonist propranolol (100nM for both drugs). RNA extraction was performed, and cDNA was synthesized. Real-time PCR was used to determine gene expression, and protein levels were measured through the Western blotting method.

**Results:** The gene expression of Cx 37/43 was significantly reduced in all three groups (P<0.001). For women with PCO and POR, Isop notably decreased expressions (P<0.001), while Prop increased them (P<0.001). Western Blot results confirmed these findings.

**Conclusion:** The findings of this in-vitro study suggest that the beta-2 adrenergic antagonist propranolol could upregulate gene expression of Cx37/43 in the cellular connections of CCs among infertile women. Consequently, propranolol may enhance communication between CCs and oocytes, facilitating the transfer of signalling messengers and other essential agents required for oocyte development. This novel discovery could have significant implications for oocyte growth and maturation, offering valuable perspectives on drug treatment and assisted reproductive technology. This novel discovery could have significant implications for oocyte growth and maturation, offering valuable perspectives on drug treatment and assisted reproductive technology.

**Keywords:** Cumulus Cells; Gap Junction Cx37/43 Expressions; Polycystic Ovarian Syndrome; Poor Ovarian Response; Beta 2 Adrenergic Receptors; Propranolol

## Introduction

**Connexin/Pannexin channels & cell to cell communication signaling**

Cell-to-cell and cell-to-matrix communication are crucial in oocytes. As intercellular membrane channels, gap junctions provide a direct route for the passage of signaling molecules and essential agents

**Correspondence:**
*Dr. Farideh Zafari Zangeneh*
*Email: zangeneh14@gmail.com*

between cells. These channels, formed by Cx or Px, link the intra- and extracellular compartments and permit the release of paracrine signals, such as ATP, playing a significant role in regulating and coordinating physiological mechanisms (1) (Figure 1).

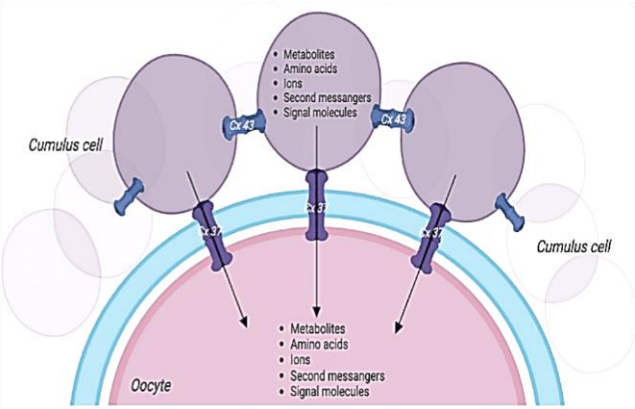


**Figure 1:** Bidirectional cumulus–oocyte complex (COC) connection. This communication through gap junctions facilitates the crosstalk between oocyte and surrounding somatic cells for passage of low-molecular-weight molecules. Created with BioRender.com (accessed on 25 January 2024) (1)

Px proteins, a group of ATP-release channels, play an essential role in oocyte growth, providing the high energy sources needed. Cx proteins modulate gene expression, growth, and cell migration. Further research on the critical function of Cx in all reproductive organs in human females could be significant for maintaining women's reproductive health. For example, Cx channels play an essential role in regulating uterine blood flow and exhibit an adaptive response to pregnancy. Mutated Cx channels, such as Cx26, Cx32, Cx37, Cx40, and Cx43, may lead to possible female reproductive problems (2). Cx protein channels (gap junctions) also play significant roles in cell proliferation and electrical communication patterns in excitable tissues, such as the heart muscle (3). The conductance and permeability of Cx channels for each isotype of Cx differ depending on the charge and size of the perfused molecule (4). Recent suggestions propose that inflammation is more likely mediated through the opening of hemichannels of Cx43 (5). Therefore, Cx channels are essential for establishing a correct pattern of intercellular passage for signaling pathways for oocyte secreted factors (OSFs) which are the main means of intercellular communication in the COC (6, 7). Oocytes can release OSFs by microvilli, and they can shorten the time required for diffusion in CCs. Thereby control the time and frequency of release. This is a necessary process for the oocyte to achieve its central domination in folliculogenesis (8). Many studies confirm that oocytes by secreting OSFs prevent CC luteinization to control steroid and inhibin synthesis and suppress LHR expression (9, 10) (Figure 2).

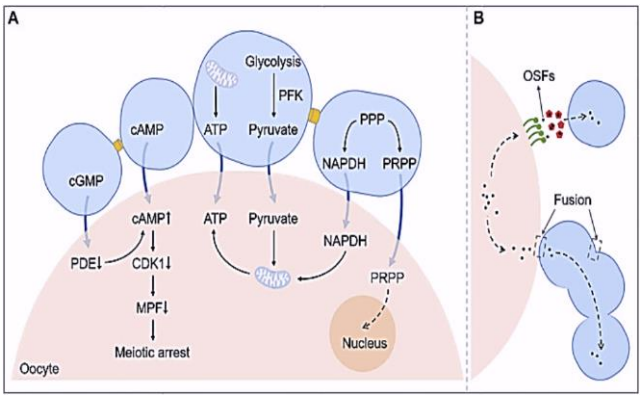


**Figure 2:** A schematic diagram of intercellular communication in COC (10)
A: Demonstrations connected pathways of metabolism and meiosis in COC, B: shows role of OSFs which can be transported by EVs or directly get into cumulus cells through the membrane fusion
PPP: Pentose phosphate pathway; NADPH: Nicotinamide adenine dinucleotide phosphate; cAMP: Cyclic adenosine monophosphate; cGMP: Cyclic guanosine monophosphate; CDK1: Cyclin-dependent kinase 1; OSFs: Oocyte secreted factors; PRPP: phosphoribosyl pyrophosphate; PDE: phosphodiesterase; MPF: maturation promoting factor; ATP: Adenosine triphosphate

## Connexin channels as a responsible target in therapeutic

Under physiological conditions, Cx43 channels open only at very low speeds (11). While some studies indicate that injuries, such as those causing chronic inflammation, may alter Cx expression in tissues, particularly Cx26, Cx32, and Cx43, most reports suggest that hemichannel numbers increase in response to injury or inflammation. In cases of chronic inflammation, studies have shown that the expression of Cx26, Cx32, and Cx43 is altered, leading to an increase in the number of hemichannels (12). In vitro studies by Mugisho et al. in 2018 demonstrated that Cx43 plays a significant role in the early events of the inflammation process. Continued hemichannel opening of Cx43 facilitates ATP release and inflammatory cytokines, providing an available mechanism for amplifying and

sustaining the inflammasome response in cell culture (13). Gap junction disconnection, similar to hemichannel activation, results in cumulative oxidative stress, which has been implicated in age-related macular degeneration (14). Disruptions to these channels can occur in pathological conditions and may become the main target of treatment, especially for chronic diseases.

**Design of the therapeutic approaches Cx hemichannels in the target tissue**

Therapeutic agents can act at different levels: 1) Px channels, 2) Cx channels/gap junctions, 3) hemichannels of Cx in the plasma membrane, and 4) hemichannels in mitochondria. Tenofovir, an antiviral drug, down-regulates adenosine levels in the liver and skin of patients with chronic hepatitis B, acting as a Panx1-mediated ATP release inhibitor. Drugs can also affect Cx channels by acting on the hemichannels or modulating the opening status of gap junctions for therapeutic purposes (15). Morphological studies using histofluorescence methods have shown that ovarian sympathetic nerve fibers are mostly noradrenergic (15). Lara et al. demonstrated that a single dose of estradiol valerate can alter ovarian catecholamine homeostasis, leading to down-regulation of β-2 adrenal receptors and an increase in ovarian nerve growth factor (NGF) (16, 17). Ovarian NA content and NA release from ovarian nerve terminals play roles in down-regulating beta-adrenoceptors in granulosa and theca-interstitial cells, and beta-2 adrenergic signaling can modulate various pathways for oocyte growth and survival (18, 19). Chronic cold stress in rats has been shown to increase TRH mRNA expression in the magnocellular neurons of the PVN, correlating with biochemical indices of sympathetic activity in the ovary (20, 21). Our 2011 findings revealed that 3 weeks of chronic cold stress in rat models before estradiol injection inhibited PCO induction in rats. We suggested that this inhibitory effect of PCO induction by cold stress was due to a reduction in ovarian NA content, which could be explained by up-regulation of β2-adrenoceptors (22). Changes in ovarian catecholamine homeostasis can impact ADRB-2-mediated actions in follicle growth. The desensitization of receptor responses involves three separate steps: 1) receptor phosphorylation, 2) interaction with arrestins (scaffolding proteins), and 3) internalization. The phosphorylation pattern depends on numerous elements, including cell type, receptor expression, and receptor uptake (20-22).

**Sympathetic innervation role in the ovarian aging**

Ovarian reserve, a significant parameter of female fertility, permits assessing the number of functional oocytes and is crucial in pregnancy planning and assisted reproductive technology (ART) applications. The accurate causes of pathological changes leading to a decline in ovarian reserve and its volume are unclear. As women age, their ovarian reserve tends to diminish. Many factors determine ovarian reserve, including physiological, hormonal, iatrogenic, genetic, and other factors. Genetic profiles, such as adrenoceptors (ADR-α1, 2, ADR-β2), are crucial in determining ovarian reserve (23). The Noradrenergic (NA) system dominates the ovary (14). In the thecal zone, nerve fibers accompany vasculature and ovarian follicles (24). In the ovaries, approximately 90% of noradrenaline (NA) is derived from the sympathetic nervous system (SNS). NA acts on β2-adrenoceptor present in ovarian theca and granulosa cells, stimulating androgen production, increasing follicle recruitment, and thereby growing the probability of cystic follicle formation, which can cause PCOS (25, 26). The celiac ganglion of the sympathetic nervous system (SNS) projects through two diverse routes to the rat ovarian blood vessels and to the superior ovarian nerve (SON) into the follicles (27, 28). Unmyelinated C fibers associate with blood vessels to regulate ovarian blood flow, which is necessary for regulating steroidogenesis and follicular development. As the negative feedback between the ovary and the hypothalamus-pituitary unit (HPU) declines with age, the primary effects of aging can present with a progressive increase in FSH levels, followed by noticeable cycle irregularities. Declining levels of Anti-Mullerian hormone (AMH) are significantly linked to a slow decline in the size of antral follicles (29).

**Management of ovarian response to gonadotropins**

The aim of clinical management has been essentially focused on maximizing oocyte produce as to increase the possibility of yield at least one euploid embryo for transfer. Indices such as FORT (follicle output rate) and FOI (follicle-to-oocyte index) may be used to determine if the ovarian reserve was properly explored during a previous ovarian stimulation (27). In this regard, the response to gonadotropins must be accompanied by fully developed follicles to induce the resumption of meiosis and ovulation in the body and hence knowledge about ovarian targets appears desirable.

The our previous studies showed that ovarian target for NA may enhance communication between CCs and oocytes, facilitating more signalling messengers and other essential agents for oocyte development. This novel discovery could have significant implications for the growth and maturation of oocytes and may offer a valuable perspective on drug treatment and assisted reproductive technology.

## Materials and methods

***Participants:*** In this exprimental study, 30 women were divided into three groups: the control group (egg donor women), the women with POR (Poor Ovarian Response), and the women with PCO (Polycystic Ovary). The inclusion criteria were as follows: age between 20 and 40 years old and a body mass index (BMI) of less than 28 kg/m. These women were recruited from the Vali-e-Asr Clinic of Tehran University of Medical Sciences between April 2021 and November 2023. The diagnosis of POR was based on the joint criteria of the European Society of Human Reproduction and Embryology (ESHRE) Bologna and PCOS was diagnosed according to the Rotterdam ESHRE/American Society of Reproductive Medicine (ASRM) 2004 criteria (28). The study was approved by the ethical committee under the code IR.TUMS.VCR.REC.2016.1329. Consent was obtained from all participants for this study.

***Collection and culture of cumulus cells:*** After the diagnosis, all groups including POR, PCOS, and control 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 folitopin 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.

***Cumulus survival and growth culture:*** The CC samples were washed several times with PBS (Phosphate Buffer Saline) buffer containing antibiotics. Subsequently, the CCs were incubated in DMEM (Dulbecco's Modified Eagle Medium) medium with 10% FBS (Fetal Bovine Serum) in a 3 cm plate coated with 0.2-2% gelatin solution (Sigma Gelatin Solution, Cat. No. G1393) at a density of 0.1 mg/cm2. The cultures were maintained at 37 °C and 5% CO2. After 48 and 72 hours, the CCs' growth was monitored, and the culture medium was changed. When approximately 80% of the flask surface was covered by CCs, the cells were passaged at a ratio of 1:3 using trypsin 0.2% and EDTA. At the end of the culture period, all surviving CCs were collected and frozen until the determination of DNA concentration using fluorescence oligonucleotide indices (Figure 3).

Then CCs were divided into nine groups each group of 5 cells for treatment of isoproterenol (β2-agonist), propranolol (β2-antagonist) and control groups.

Group 1: Control (N without treatment)
Group 2: Control (N-Isop)
Group 3: Control (N- Prop)
Group 4: PCOS without treatment
Group 5: PCOS-Isop
Group 6: PCOS-Prop
Group 7: Poor without treatment
Group 8: Poor-Isop
Group 9: Poor-Prop

Isoproterenol (Monico, Italy) and propranolol (Toliddaru, Iran) as potent beta-adrenoceptor agonists and antagonists respectively by the concentration of 100 nM in 72 hours as selective drugs were added to the culture medium (Johnsson and Regardh, 1976; Colangelo et al., 1992).

**RNA extraction & cDNA production**
**Quantitate real-time PCR (RT-qPCR)**
**Quantification with UV spectrophotometer**

The RNA assessment by the reaction quantitative real-time PCR (RT-qPCR): PCR steps were complicated 35 cycles of denaturing (at 94 °C for 60 sec), annealing (at 60 °C for 30 sec), and extension (at 72 °C for 60 sec) (Table 1).

Western Blotting (The protein immunoblot) was used for assessment of proteins (Table 2), and (Figure 4). The Western blotting method employed in this study is based on the Laemmli and Towbin technique, with certain modifications applied to detect low protein doses effectively.

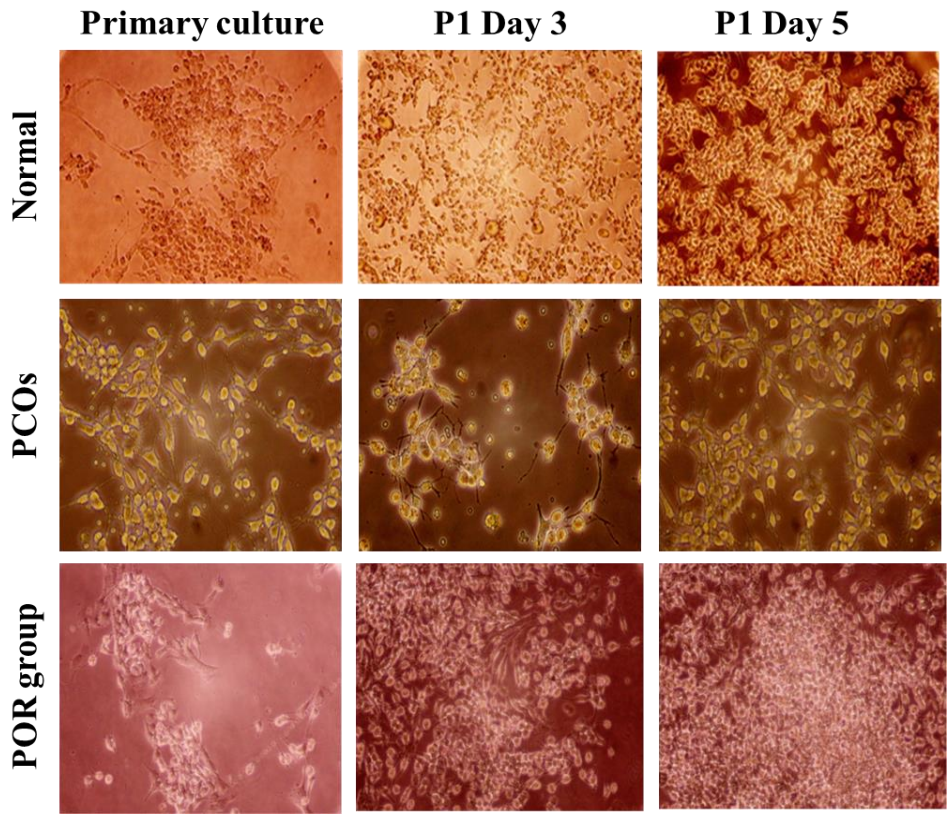


**Figure 3:** Morphological images of three groups of cumulus cells during three passages in the culture medium. Upper row control for group, middle for PCOs group, lower for Poor-responder PCOs group

***Statistical analysis:*** After performing the necessary tests, the gene expression was analyzed using Kruskal-Wallis nonparametric test for overall comparison and Mann-Whitney test for multiple comparison. mRNA expression levels and mean, median and standard deviation were presented. Statistical analysis was performed using IBM SPSS version 26(IBM SPSS Co., Armonk, NY). A significance level of P < 0.05 was considered statistically significant.

## Results

### Analysis of gene expression at the mRNA level

***The results of Cx37 gene expression:*** The expression of Cx37 in overall comparison with Kruskal-Wallis nonparametric test had significance difference between study groups (P = 0.001). The multiple comparison showed that the expression of Cx37 in the control group was significantly decreased by the treatment of isoproterenol (P = 0.037) and propranolol (P =0.037). In PCO women, Cx37 expression was significantly reduced in all three groups: without treatment (P =0.037), isoproterenol treatment (P = 0.049), and propranolol treatment (P = 0.049) compared to the control group. However, in the PCO group with treatments, Cx37 expression decreased with isoproterenol treatment (P = 0.049), while propranolol increased it (P = 0.049) compared to the group without treatment (Table 3). Immunocytochemical results confirmed these findings (Figure 4).

**Table 1:** Sequence of specific primers for Real Time PCR

| Gene Name | Primer Sequence (Forward, FW; Reverse, RV) | Product Length (bp) | NCBI Accession Number |
|---|---|---|---|
| Connexin37 (Cx37) Gap Junction Protein Alpha 4 (GJA4) | FW: GTCACTCCGGCCATCGT<br>RV: AGCAACTTCTCCAGGAAGCC | 101 | NM_002060.3 |
| Connexin34 (Cx34) Gap Junction Protein Alpha 1 (GJA1) | FW: TGAGCAGTCTGCCTTTCGTT<br>RV: CCAGAAGCGCACATGAGAGA | 94 | NM_000165.5 |

**Table 2:** Primary antibodies used for Western blotting and tissue-cell staining

| Gene Name | Antibody | Description | Molecular weight (KDa) |
|---|---|---|---|
| Connexin37 (Cx37) | Antibody (42-4400) | Anti-alpha 2C Adrenergic Receptor antibody - C-terminal (ab151618) | 37 |
| Connexin43 (Cx43) | Antibody (13-8300) | Connexin43 Antibody Thermofisher | 50 |

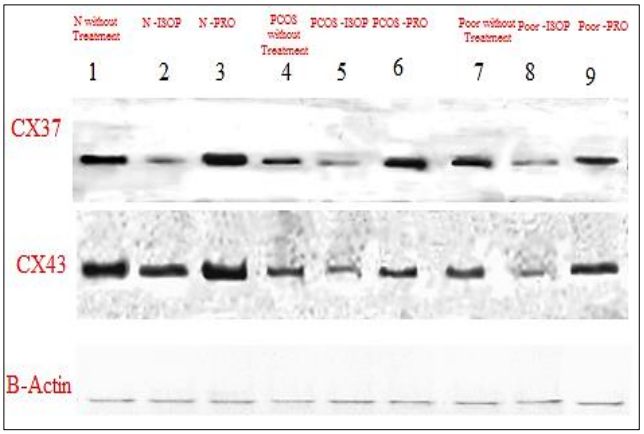


**Figure 4:** Western blot images of proteins of two Cx37 and 43 with Beta actin: Beta-actin has been considered as the home protein of the foot and control of the experiment

In patients with POR, untreated individuals (P =0.037) and those receiving propranolol treatment (P =0.049) or isoproterenol treatment (P =0.049) showed significant reductions compared to the control group. However, within this POR group, the propranolol treatment increased Cx37 gene expression (P =0.049), while the isoproterenol treatment decreased it compared to the non-treatment group (P =0.049). These results indicate that the decrease in Cx37 expression in the POR group was more significant than in the PCO group.

***The results of Cx43 gene expression:*** The expression of Cx34 in overall comparison with Kruskal-Wallis nonparametric test had significance difference between study groups (P = 0.001). The multiple comparison showed that in the control group isoproterenol treatment had no significant effect on Cx43 expression (P = 0.487) in CCs culture, while propranolol decreased it significantly (P = 0.037).

In PCO women, Cx43 expression was significantly reduced in all three groups: without treatment (P = 0.037), isoproterenol treatment (P = 0.049), and propranolol treatment (P = 0.049), compared to the control group. However, within this group, isoproterenol was found to significantly decrease Cx43 expression (P = 0.049), while propranolol increased it (P = 0.049).

For women with POR, the results in all three groups were as follows: no treatment (P =0.037), treatment with isoproterenol, and treatment with propranolol (P =0.049), all of which significantly reduced Cx43 expression compared to the control group. In these women, treatment with isoproterenol decreased Cx43 expression compared to the untreated group (P =0.049), while treatment with propranolol significantly increased it (P =0.049) (Table 4). Western Blot results confirmed these findings (Figure 4).

**Table 3:** Changes of the Cx37 gene expressions in the control, PCOS, and POR groups

| Groups | CX37 $2^{-\Delta\Delta C_T}$ | | | | P-value[1] | P-value[2] | P-value[3] |
|---|---|---|---|---|---|---|---|
| | N | Mean | SD | Median | | | |
| Control (N without treatment) | 3 | 1.000 | 0.000 | 1.000 | | | |
| Control (N- ISOP) | 3 | 0.641 | 0.058 | 0.653 | 0.037[a] | | |
| Control (N- Prop) | 3 | 0.901 | 0.035 | 0.905 | 0.037[a] | | |
| PCOS without treatment | 3 | 0.366 | 0.016 | 0.370 | 0.037[a] | | |
| PCOS-ISOP | 3 | 0.232 | 0.005 | 0.232 | 0.049[b] | 0.049 | |
| PCOS-Prop | 3 | 0.482 | 0.021 | 0.482 | 0.049[c] | 0.049 | |
| POR without treatment | 3 | 0.308 | 0.024 | 0.307 | 0.037[a] | | |
| POR-ISOP | 3 | 0.003 | 0.003 | 0.002 | 0.049[b] | | 0.049 |
| POR-Prop | 3 | 0.570 | 0.021 | 0.581 | 0.049[c] | | 0.049 |

P-value[1]-a: In comparison with Non-PCOS without treatment group. b: In comparison with Control Isoproterenol group. c: In comparison with Non-PCOS Propranolol group.
P-value[2]: In comparison with PCOs without treatment
P-value[3]: In comparison with poor responder without treatment group
All comparison was done with Mann-Whitney test.

**Table 4:** Changes of the Cx43 gene expressions in the control, PCOS, and POR groups

| Groups | CX43 2^-ΔΔCT | | | | P-value[1] | P-value[2] | P-value[3] |
|---|---|---|---|---|---|---|---|
| | N | Mean | SD | Median | | | |
| Control (N without treatment) | 3 | 1.000 | 0.000 | 1.000 | | | |
| Control (N-Isop) | 3 | 0.638 | 0.024 | 0.649 | 0.037[a] | | |
| Control (N-Prop) | 3 | 1.013 | 0.023 | 1.012 | 0.487[a] | | |
| PCOS without treatment | 3 | 0.345 | 0.033 | 0.347 | 0.037[a] | | |
| PCOS-Isop | 3 | 0.211 | 0.032 | 0.207 | 0.049[b] | 0.049 | |
| PCOS-Prop | 3 | 0.489 | 0.015 | 0.496 | 0.049[c] | 0.049 | |
| POR without treatment | 3 | 0.313 | 0.017 | 0.309 | 0.037[a] | | |
| POR-Isop | 3 | 0.007 | 0.001 | 0.007 | 0.049[b] | 0.049 | |
| POR-Prop | 3 | 0.505 | 0.017 | 0.515 | 0.049[c] | 0.049 | |

P-value[1]-a: In comparison with Non-PCOs without treatment group. b: In comparison with Control Isoproterenol group. c: In comparison with Non-PCOs Propranolol group.
P-value[2]: In comparison with PCOs without treatment
P-value[3]: In comparison with POR without treatment group
All comparison were done with Mann-Whitney test

## Discussion

Successful ovulation requires the development of capable oocytes that can be released from the ovarian follicle at an appropriate time. The size of the primary follicle pool indicates fertility in women. High-quality mature oocytes are critical for supporting female fertility, achieved under normal conditions through fine-tuning oocyte meiotic arrest and resumption development.

In 1974, Bahr et al reported for the first time that autonomic innervation is the dominant nervous system in the ovarian organ (29). The association between the neuronal activity of intra-ovarian β-adrenoceptors and the stimulatory effect of noradrenaline (NA) on the secretion of steroids (30) supports the regulatory and complementary role of the sympathetic nervous system (SNS) in oogenesis and steroidogenesis. Beta-2 adrenergic signaling plays a modulatory role in various pathways for the growth and survival of oocytes (20). One of these pathways involves the activity of two-second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).

Numerous studies demonstrate the critical role of the sympathetic nervous system (SNS) and cGMP in maintaining oocyte meiotic arrest (31, 32). The meiotic arrest phase is regulated by high levels of cAMP in the oocyte (Figure 2) (10). Studies show that cAMP levels in oocytes decrease when they are isolated from the antral follicles, leading to an abrupt resumption of meiosis (33, 34).

Stimulation of beta-adrenergic receptors by catecholamine is recognized by the beta-adrenoceptor-adenylyl cyclase-protein kinaseA cascade, resulting in the accumulation of protein kinase A (PKA). In the adult mammalian ovary, the concentration of intra-oocyte cAMP plays an essential role in monitoring the arrest and resumption processes of the meiotic phase in oocytes (34). On the other hand, cGMP, synthesized by guanylyl cyclase in the CCs (cumulus cells) and mural granulosa cells, diffuses to the oocyte through gap junctions to maintain the oocyte meiotic arrest at the diplotene phase of prophase I in mice (34, 35). This process continues until luteinizing hormone (LH) is activated in the granulosa cells, as shown in Figure 5 (31).

In 2021 our previous study reported an increase in adrenergic gene expression (ADR-α1, 2, and β2) and protein levels induced by clonidine in the CC culture of women with PCO. This finding also supported the existence of ovarian SNS hyperactivity in these women. However, in women with POR, ADR-α1, 2 expressions decreased, which might contribute to ovarian aging in this population (36, 37).

Our current study demonstrates the effectiveness of using a beta-2 adrenergic receptor agonist/antagonist on the expression of Cx37 and Cx43 genes in cultured CCs of infertile women with PCO and POR. Consequently, the β2 adrenergic receptor emerges as a promising therapeutic target. We hypothesize that the down-regulation of beta2-adrenoceptor in women with PCO and POR may reduce oocyte growth and ovarian capacity (19, 21, 37, 38).

In mammals, accumulating evidence highlights the significant role of endogenous catecholamines in fundamental developmental processes, such as controlling cell proliferation, differentiation, migration, morphogenesis, hormonal responsiveness, embryogenesis, and preimplantation embryos (39).

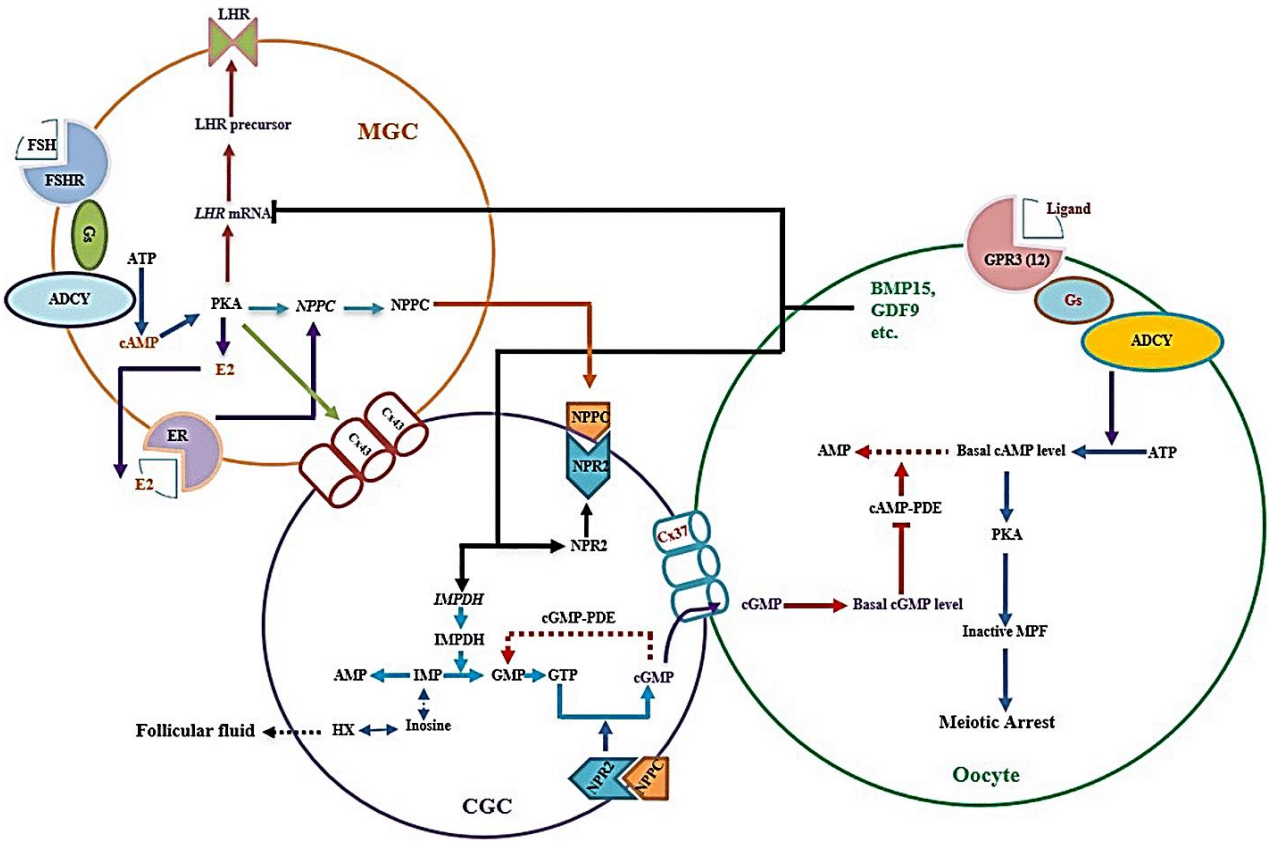


**Figure 5:** The Summarized Pathway of Oocyte-Derived Paracrine Factors Contributing to the Maintenance of Meiotic Arrest in Mammalian Oocytes

This figure presents the key pathways involving oocyte-derived paracrine factors, namely FSH/FHSR, Estrogen/ER, and NPPC/NPR2 that are essential for maintaining meiotic arrest in mammalian oocytes.

Orange Cycle: Within the mural granulosa cells (MGC), the binding of FSH to its receptor (FSHR) collaborates with Estrogen/ER, stimulating the production of NPPC. This, in turn, triggers the arrival of protein kinase A (mainly Cx43) in the cumulus granulosa cells (CGC), facilitating the cooperation between NPPC and NPR2 and activating the LH mRNA expression pathway in MGC.

Purple Cycle: In the cumulus granulosa cell (CGC), NPPC binds to its receptor NPR2, converting GTP into cGMP. Subsequently, cGMP diffuses into the oocyte through gap junctions (mainly Cx37), effectively inhibiting cAMP-PDE activity.

Green Cycle: The oocyte responds to paracrine factors by increasing NPR2 expression in the cumulus cells, resulting in elevated cGMP levels both in CGC and the oocyte. Additionally, the second pathway involves inosine monophosphate dehydrogenase (IMPDH) in CGC, which boosts cGMP levels through inosine-5′-monophosphate (IMP). Notably, IMPDH also maintains a basal level of hypoxanthine (HX) in the follicular fluid, potentially serving as an oocyte phosphodiesterase inhibitor to enhance intracellular cAMP accumulation for the meiotic arrest process (31).

The adrenergic responsiveness of granulosa cells to gonadotropins, exemplified by isoproterenol (Iso) as a potent beta-adrenergic agonist, increases progesterone production in a time- and dose-dependent manner. Adrenergic agents enhance the effects of human chorionic gonadotropin (hCG) and prolactin (PRL) on progesterone production (40), but do not affect estrogen production (38).

Both animal and human studies demonstrate that an increase in ovarian NA levels due to higher NA release from the terminal of the ovarian nerve can lead to β-AR down-regulation in theca-interstitial cells (19) and granulosa cells (20). B-AR down-regulation reduces its expression, cellular interconnectivity, and synchronization during oocyte maturation and ovulation. Cellular interconnection depends on more complex communication among these cells, and that the oocyte itself orchestrates these conversation. Although ovarian development involves all three activities (paracrine, endocrine, and juxtacrine) between cells, only para- and juxtacrine signaling occurs in the oocyte-somatic cell complex. Juxtacrine communication between adjacent cells is mediated by membrane specializations called gap junctions, which allow cell-to-cell transmission of low molecular weight signaling or nutrient molecules (41).

The activity of gap junctions through which

cAMP enters the oocyte from the granulosa cells plays an important role in the regulation of meiosis. LH activates a signal transduction pathway leading to the resumption of oocyte meiotic maturation through a breakdown of communication between the oocyte and somatic cumulus and granulosa cells, ceasing the influx of cAMP molecules into the oocyte (42). LH surge-induced decrease in cGMP synthesis and/or increase in its hydrolysis further contribute to the lowering of cAMP levels in the oocytes through suppressing the activity of PDE3A, the enzyme responsible for cAMP degradation (16, 28).

Within the ovary, Cx channels in the CCs-oocyte complex play a crucial role in coordinating signaling pathways during oocyte maturation. This coordination relies on the autoregulation mechanism of Cx37 and Cx43 gap junctions through internally translated isoforms, which significantly reinforce ovarian meiotic maturation. Meiotic maturation is a dynamic and complex process essential for achieving the full capability of oocyte function and early embryonic development. B2-AR (beta2-adrenoceptor) plays a fundamental role in triggering these complex processes.

The findings of this study confirm several vital points:

The critical role of B2-AR in the brain-ovary axis communication (30, 40)

The significance of ovarian NA release (16)

The autoregulation mechanisms involving diffusion of cGMP into oocytes via Cx37 and Cx43 for oocyte meiotic resumption (19)

The critical roles of Cx37 and Cx43 in the folliculogenesis process (25, 41)

Treatment of Isop and Prop in CCs culture reveals that Cx37 and Cx43 hemichannels in the β2-AR response are essential factors for communication in CCs-oocyte complex cells. CCs are derived from mural granulosa cells (MGCs) and are closely related to the oocyte. The CCs-oocyte complex allows for the transfer of oocytes and CCs for gene expression and protein synthesis, facilitating oocyte maturation and leading to the differentiation and proliferation of CCs. CCs attach to the oocyte cytoplasm and enter the zona pellucida (ZP) through gap junctions. While CCs produce hyaluronic acid (HA) and become extensive, MGCs do not produce HA and therefore do not expand. These conditions create the cellular environment for the LH effect, ovulation, and subsequent fertilization in the oviduct's ampulla (42).

It should be noted that the physiological function of CCs and MGCs may vary at different follicle sizes and phases of the menstrual cycle. The decreasing permeability between CCs and oocytes through gap junctions induces meiosis resumption as well as cumulus extension, preparing the oocyte for ovulation and fertilization.

## Conclusion

Bidirectional cumulus-oocyte complex signaling is essential for establishing a dynamic intrafollicular microenvironment. This signaling system plays a crucial role in controlling the development of the primordial follicle among a group of growing follicles, from its initial selection to the eventual antral follicle chosen for ovulation in a healthy oocyte. The studies mentioned above have demonstrated that increased Cx expression of gap junctions in the CCs-oocyte complex is vital for meiosis resumption during oogenesis and folliculogenesis.

This study investigated alterations in the gene expression of connexins 37 and 43 hemichannels in the cumulus cells of women with PCOS and POR, utilizing beta-2 adrenergic agonists and antagonists. Our findings from the in-vitro study of propranolol treatment on CCs culture demonstrated that blocking beta2 adrenergic receptors could be a novel and effective strategy in the signaling pathway. However, gaining a deeper understanding of the signaling pathways during oocyte growth and maturation can significantly impact various drug treatment strategies, as well as improve the ART process.

## Conflict of Interests

Authors declare no conflict of interests.

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