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Expression of genes involved in the inflammatory response in human granulosa cells in short-term in vitro culture

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Abstract

The essential function of granulosa cells is to maintain the proper course of oogenesis and folliculogenesis. The immune system is an additional local regulator of ovarian function, with cytokines necessary for the proper function of the ovaries, including the secretion of steroid hormones

This study aimed to analyze the expression of genes in human GCs in short-term primary culture and define the difference in the expression of *IL1β*, *IL6,* and *TNFα* genes at 48h and 72h of culture compared to the 24h control. Total RNA was isolated using the Chomczyński and Sacchi protocol. RNA samples were treated with DNase I and reverse transcribed (RT) into cDNA. The determination of transcript levels of the mentioned genes was performed using the Light Cycler® 96 Real-Time PCR kit, Roche Diagnostics GmbH (Mannheim, Germany).

The present study proved that granulosa cells in a short-term primary *in vitro* culture express *IL-1β, IL-6*, and *TNFα*. The tested genes show a decrease in expression at 24h of culture and a subsequent slight increase at 72h, not exceeding the initial levels. The expression changes the most for IL1β and the least for TNFα.

The fluctuations in the amount of transcript may be influenced by factors stored in granulosa cells before the IVM procedure, the procedure of *in vitro* fertilization, as well as factors related to the process of primary culture. More research is needed to understand the details of these occurrences.

Running title: The inflammatory response in human granulosa cells

Keywords: granulosa cells (GCs), IL1β, IL6, i TNFα

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Introduction

There are several complex processes occurring in ovaries, the female gonads, including oogenesis - the formation and maturation of eggs, and folliculogenesis - the formation of ovarian follicles. The ovarian follicle consists of oocytes and granulosa cells (GCs) [1,2]. The basic function of the granulosa is to maintain the proper course of oogenesis and folliculogenesis [3], while more specifically, their functions include: communication with the oocyte, production of hormones (conversion of androgens to estrogens), and production of progesterone by co-formation of the corpus luteum [1]. There are several types of follicular GCs in the ovarian follicle: mural GCs that line the inner part of the ovarian follicle, adjacent to the basal lamina; followed by the layer forming the cumulus oophorus, the cells immediately surrounding the oocyte [4].

Studies carried out over many years provide information that the immune system is a local regulator of ovarian function, due to mutual relationship between polypeptides of immune origin, cytokines and the reproductive system [5]. At the time of innate activation or immune response, cells begin to produce cytokines, chemokines and growth factors [6]. In 1999, the research of Richards [7] showed that cytokines are necessary for the proper function of the ovaries, influencing the secretion of steroid hormones [7]. Moreover, they affect the development and regression of the functions of the corpus luteum in humans and during embryonic development and implantation, they help in vascularization, cell differentiation and the penetration of trophoblast into the endometrium [8]. During ovulation in the ovary, an inflammation-like reaction is observed, with the follicular fluid containing interleukins, e.g. IL1 [5].

Many researchers have analyzed the presence of cytokines in the follicular fluid [6], correlation them to viral and bacterial infections in the context of ovarian or uterine diseases [9]. Molecular factors related to human granulosa cells are still of interest, but this is a very broad range that requires continuous improvement and expansion of research.

It has also been proven that the level of cytokines in the ovaries is influenced by various factors, e.g. in bovine granulosa cell, liposaccharides (LPS) from gram-negative bacteria bind to the toll-like receptor 4 (TLR4), causing the translocation of the nuclear factor-kB (NF-κB) from the cytoplasm to the nucleus, which increases the level of pro-inflammatory cytokines (IL1β, IL6, IL8 and TNFα) [10]. Research by Lei et al. also showed that LPS increased the levels pro-inflammatory cytokines (IL1β, IL6, IL8 and TNFα) in murine granulosa cells [11]. On the other hand, the analysis of gene expression in buffalo granulosa cells showed that IGF-1 significantly decreased LPS-induced expression of IL1β, TNF-α and IL6 [10].

The aim of this study was to analyze gene expression in human GCs, obtained from patients undergoing *in vitro* fertilization (IVF) procedures, during short-term primary culture. Moreover, we have aimed to define the difference in the expression of IL1β, IL6 and TNFα genes, responsible for the induction of the inflammatory process, at 48 h and 72 h of culture compared to the 24h control. The obtained results may broaden the knowledge of the molecular processes occurring in human granulosa.

Materials and methods

Patients and collection of granulosa cells

The experimental group included follicular fluid collected from 2 patients, aged 18-40, were undergoing *in vitro* fertilization (IVF) at the Department of Infertility and Reproductive Endocrinology, Poznań University of Medical Sciences. The collection procedure was based on a controlled ovarian hyperstimulation protocol that was adapted to the patient's initial infertility and ovarian response testing. Cetrorelix acetate (Cetrotide, Merck Serono) was injected at the appropriate dose to suppress the pituitary gland. Subcutaneous injection of 6500 U hCG (Ovitrelle, Merck-Serono) triggered ovulation. Alveolar fluid containing GC was obtained during transvaginal collection of oocytes under ultrasound guidance, 36 h after administration of human chorionic gonadotrophin. From follicles larger than 16 mm in diameter, the sampled contents were immediately forwarded to an embryologist who isolated the oocyte and collected the GC-containing fluids from each ovary. Fresh vesicle fluid was centrifuged at 200 g for 10 minutes to separate and collect GC. Patients with polycystic ovary syndrome (PCOS), endometriosis and decreased ovarian reserve (serum anti-mullerian hormone levels below 0.7 ng / ml and / or serum FSH levels above 15 mU / ml on day 2-3 and / or antral follicle count less than 9) excluded from the study.

Primary cell culture

Harvested cells were purified twice by centrifugation at 200 xg for 10 min at room temperature in culture medium. The medium consisted of Dulbecco's Modified Eagle's Medium (DMEM / F12, Sigma-Aldrich, St. Louis, MO, USA), 2% FBS Fetal Bovine Serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA), 200 mM L-glutamine (Invitrogen, Carlsbad, California, USA), 10 mg / ml gentamicin (Invitrogen, USA), 10,000 units / ml penicillin, and 10,000 μg / ml streptomycin (Invitrogen, USA). Cells were grown at 37 ° C under aerobic conditions (5% CO_2). At appropriate time intervals, the cells were detached with 0.05% trypsin-EDTA (Invitrogen, USA) for 1-2 minutes and counted in a "Nebauer Improved" counting chamber (ISO LAB Laborgerate GmbH, Wertheim, Germany, DIN EN ISO 9001 CERTIFICATE). The GCs

were grown for 72h days. Finally, total RNA was isolated from the GC after 24 hours, 48 hours and 72 hours of culture.

Total RNA isolation Reverse Transcription

Total RNA was isolated at three time periods, after 24 h, 48 h and 72 h of culture. For the isolation of total RNA, we have used the improved Chomczyński-Sacchi method [15]. First, GCs were suspended in 1 ml of a mixture of guanidine thiocyanate and phenol in a monophasic solution (TRI Reagent®, Sigma-Aldrich, St. Luis, MO, USA). In the next step, chloroform was added and centrifuged to separate the mixture into three phases. In the upper aqueous phase contained RNA with little or no contaminating DNA and protein. The last step was stripping the RNA with 2-propanol (Sigma-Aldrich, St. Luis, MO, catalog number I9516) and washing with 75% ethanol. RNA samples were treated with DNase I and reverse transcribed (RT) into cDNA.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

RT-qPCR was performed on a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using Eva Green as detection dye, and the relative quantification method. The study focused on 3 genes: IL1β, IL6, TNFα, as pro-inflammatory factors. Two biological samples of each group were used for the analysis, and each biological test was performed in two technical replications. The following were used as reference genes: β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GADPH) and hypoxanthine phosphoribosyltransferase 1 (HRPT1). The q-PCR primers were designed using the Primer3Plus software (http:// primer3plu s.com/cgi-bin/dev/prime r3plus .cgi). In order to avoid possible amplification of genomic DNA fragments, the exon-exon design method was used. The Esembl database was also used to design primers using the sequence of several variants of transcripts of selected genes. For amplification of 1 µl of cDNA solution, 9 µl of the prepared mix of Eva Green detection dye and primers shown in **table 1** were added.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. The present study has been approved by Poznan University of Medical Sciences Bioethical Committee with resolution 558/17.

Informed consent statement

Informed consent has been obtained from all individuals included in this study.

Results

In the present study, the transcripts of IL1β, IL6 and TNFα were analyzed during short-term *in vitro* culture of human granulosa cells. RT-qPCR showed that in the studied time intervals of the cell culture granulosa cells expressed the mentioned genes involved in the pro-inflammatory response. The obtained results are presented below (**Fig. 1**).

It was observed that the tested genes (IL1β, IL6 and TNFα) show a similar pattern of expression, exhibiting the highest levels at 24h of culture, with a following decrease at 48h. The biggest downregulation was observed for IL1β and the smallest for TNFα. At 72h of cultivation, the expression increases slightly in relation to 48h, however, it does not exceed the level of the 24h reference.

TABLE 1 Oligonucleotide sequences of primers used for RT-qPCR analysis

GENE	PRIMER SEQUENCE (5'–3')	PRODUCT SIZE (BP)
IL1β	**F** GGGCCTCAAGGAAAAGAATC **R** TTCTGCTTGAGAGGTGCTGA	205
IL6	**F** ACAACCTGAACCTTCCAAAGA **R** ACAACCTGAACCTTCCAAAGA	179
TNFα	**F** AGCCCATGTTGTAGCAAACC **R** AGCCCATGTTGTAGCAAACC	157
ACTB	**F** AAAGACCTGTACGCCAACAC **R** CTCAGGAGGAGCAATGATCTTG	132
GAPDH	**F** TCAGCCGCATCTTCTTTTGC **R** ACGACCAAATCCGTTGACTC	90
HPRT	**F** TGGCGTCGTGATTAGTGATG **R** ACATCTCGAGCAAGACGTTC	141

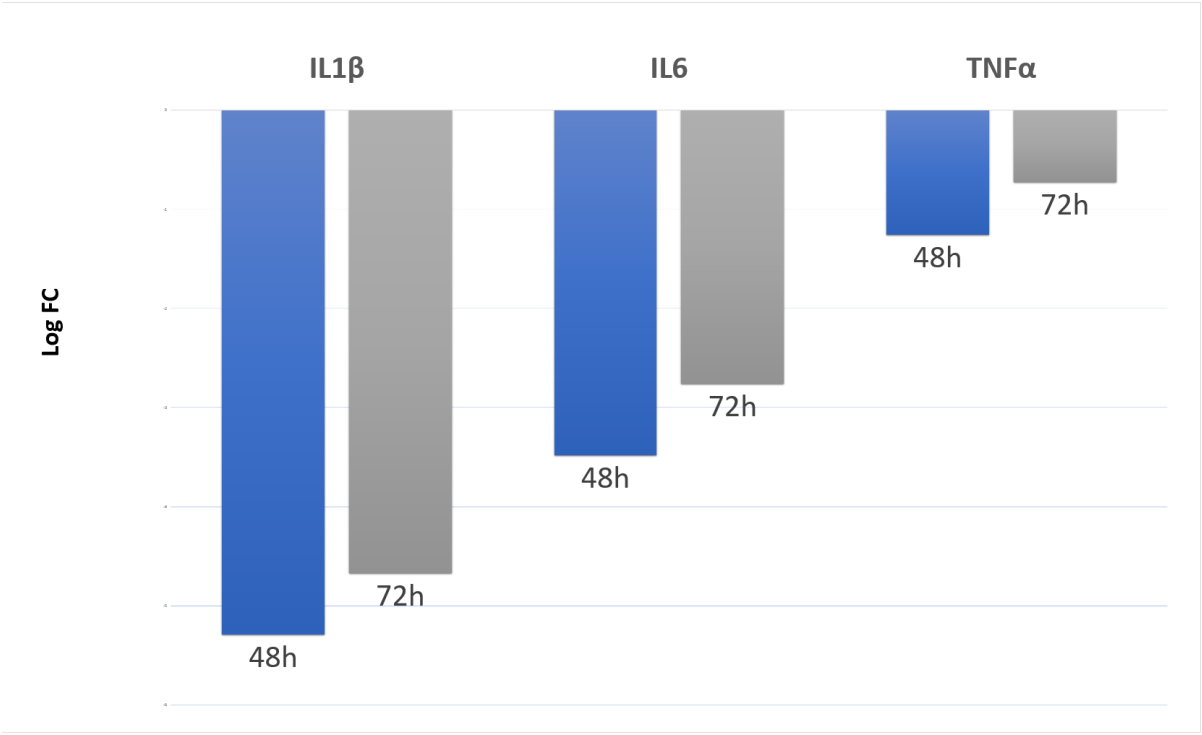


FIGURE 1 The change in the expression of IL1B, IL6 and TNFa genes in 48h and 72h in short-term *in vitro* culture of human ovarian granulosa cells, as compared to the expression in 24 hours of culture

Discussion

To better understand the molecular processes taking place in granulosa cells in primary *in vitro* culture, changes in transcript levels in human GCs from patients undergoing *in vitro* fertilization (IVF) were investigated. The main focus was on the expression of pro-inflammatory cytokines such as IL1β, IL6 and TNFα.

There have been many studies investigating the presence of these genes in the ovaries [12], follicular fluid [13] or granulosa cells [14], as well as experiments in this field conducted on cell cultures [15]. However, there are only few experiments explaining the molecular processes related to pro-inflammatory factors in for the *in vitro* primary culture of human granulosa cells. In this study, we confirmed the presence of the studied genes in the cells of interest during short-term primary culture.

Among the tested genes, IL1β showed the greatest decrease in expression in 48h and slight increase at 72h. However, it did not exceed the expression tested in 24h. Interleukin-1 beta (IL1β) belongs to the 11-member IL-1 family of cytokines [16]. The two main ligands of this family are IL-1α and IL-1β, which have 2 types of receptors: IL-1R1 and IL-1R2, and IL-1RA, which is a natural receptor antagonist [17]. IL1β is associated with chronic inflammatory diseases (CID) and is likely to be the major causative agent of CID. IL-1β is mainly synthesized by monocytes and macrophages, but may be produced by endothelial cells, fibroblasts, and epidermal cells in response to bacterial agents or innate factors. It is an interleukin produced as an inactive protein, with a mass of 31 kDa, transformed into its active form (17 kD) by caspase-1. In the case of the ovary, it is produced mainly by ovarian epithelial cells and stromal immune cells [16]. IL1-b produced in the ovary accumulates, inter alia, in the follicular fluid during ovulation, therefore it plays an important role in ovulation and steroidogenesis [18–20]. One of the functions of IL1b during ovulation is the production of progesterone in response to stimulation by luteinizing hormone [21–23]. According to Dang et al., IL-1b may increase the expression of steroidogenic acute regulatory protein (StAR) and stimulate progesterone biosynthesis by increasing CREB (cyclic adenosine monophosphate) phosphorylation, through activation of the ERK1 / 2 and p38 pathways in human granulosa cells [24]. The Martorati study shows that IL-1β is expressed in equine granulosa, with mRNA content fluctuating during final follicular maturation, and in the follicular fluid itself up to several hours after ovulation. The expression of this gene may be regulated by gonadotropins, and it may be involved in the ovulation process [17]. In our study, the expression in cell culture also changes dynamically, which may mean that in the cell culture conditions there are factors that affect the expression of this gene.

For IL6, there was also a decrease in expression in 48h compared to the control 24h, and a slight increase in expression at 72h of culture. However,

the difference in transcript level was lower than for IL1b. Interleukin 6, as a cytokine, is mainly produced by monocytes, T lymphocytes, but also fibroblasts, keratinocytes and endothelial cells [25]. In the immune system, it is responsible for inflammation in response to an infection or injury. IL6 is a pleiotropic cytokine because, depending on its factors, it may have pro-inflammatory or anti-inflammatory properties [26]. However, it is mainly treated as an anti-inflammatory and immunosuppressive cytokine responsible for the inhibition of TNFa and IL1 [16]. In ovarian follicles, IL6 is produced by granulosa cells [27]. In pathological conditions, interleukin 6 may contribute to continuous inflammation that adversely affects the functioning of the ovaries. Kuang et al. in their study investigated the effect and mechanism of inflammatory cytokines in serum (including IL6) on PCOS sensitivity to insulin. Women with PCOS were affected by subclinical inflammation leading to insulin resistance contributing to abnormal ovulation and problems with fertilization [28]. Overexpression of this gene is also noticeable in malignant tumors of the ovaries, e.g. in cystic fluid [25].

The described experiment shows that TNFα was characterized by the smallest decreasing difference in gene expression. As in the case of the previous genes, a decrease in expression was observed in 48 hours, while in 72 hours of cultivation, the level of the transcript slightly increased, still not exceeding the control for 24 hours. TNFα, or tumor necrosis factor alpha, is a pro-inflammatory cytokine produced by macrophages and monocytes. It is produced during acute inflammation and in response to appropriate cellular signals that may lead to apoptosis or necrosis, and exerts its effect through binding to the TNF receptor (TNFR) 1 or 2 [29]. TNFα also plays an important role during the development of ovarian follicles in mammals, as well as during the processes taking place in them: ovulation, steroidogenesis, luteolin and atresia [30]. Moreover, it has been proven that it leads to apoptosis in the ovaries in many animal species, including humans. Many researchers have also tried to understand its exact function and its regulation in granulosa cells. Nakayama et al. showed intense TNFα and TRAF2 signals in healthy vesicles in the granulosa zone. They observed a significant number of proliferating cells and no apoptosis. However, in the case of the granulosa layer of arthritic vesicles, the situation was the opposite: a lot of apoptotic cells and decreased expression of TNFR2 were observed. This study showed that TNFα acts as a survival factor in granule cells during porcine follicle atresia [29]. In turn, Nakao et al., investigating intracellular signaling pathways in rat granular cells, demonstrated that TNFα-induced mRNA and LHR protein expression did not affect cAMP accumulation [31]. In turn, Yamada-Namoto et al. came to interesting conclusions that statin and insulin sensitizers can help in PCOS therapy by reducing the expression of Plasminogen activator inhibitor-1 (PAI-1) in GC and reducing the expression of TGF-β and TNF-α in peritoneal fluid mononuclear cells (PFMC) [32].

In the female reproductive system, viral or bacterial infections of the ovaries may occur. Hence, in human granulosa cells, an increased synthesis of pro-inflammatory cytokines can be observed due to immune response [9]. An example is the response of human ovarian granule cells to a synthetic viral double-stranded RNA analogue (polyinosinic acid-polycytidylic acid [poly (I: C)]). A study by Yan et al. showed that Poly (I: C) significantly increased the expression of pro-inflammatory cytokines, mainly TNF-a, IL6, IL-1b and interferon type I (IFN-a / b) [33]. Furthermore, infection with the mumps virus (MuV) can lead to inflammation of the ovaries and their dysfunction. Wang et al. conducted an experiment which showed that the Toll-like receptor 2 (TLR2) and retinoic acid induced gene I (RIG-I) initiate the innate immune response in mouse ovarian granulosa cells. Granulosa cells produced i.e. IL-1b, TNF-a, monocyte chemotactic protein 1 (MCP-1) and type 1 interferons (IFN-a and IFN-b) [34].

Conclusions

Summarizing, the present study proved that granular cells in short-term *in vitro* primary culture express genes encoding pro-inflammatory factors: IL-1β, IL-6, and TNF-α. The transcript level of these 3 genes was lower in 48h than in the 24h control, increasing slightly on the 3rd day of culture. Fluctuations in the amount of transcript may be influenced by factors stored in granulosa cells prior to extraction, activities related to the *in vitro* fertilization procedure and factors related to the process of primary culture. Hence, further studies are needed to understand these detailed mechanisms.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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