

IMPAIRED FUNCTIONS OF HUMAN GRANULOSA CELL LINE COV434 UNDER HYPOXIC AND INFLAMMATORY CONDITIONS

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Received on April 20, 2023

Presented by N. Atanassova, Corresponding Member of BAS, on June 27, 2023

Abstract

Premature ovarian insufficiency (POI) affects 1% of women under 40 years old and represents one of the main causes of infertility in females of childbearing age. POI is a primary ovarian defect resulting from follicular depletion and/or ovarian dysfunction caused by a compromised ovarian somatic cell compartment. Etiologically, ovarian dysfunction could be caused by certain diseases, including infections and autoimmune disorders. There could also be iatrogenic factors such as chemotherapy, radiation, or surgery. The related surgical risks are due to the possibility of disrupting the blood supply to the ovary and/or a subsequent inflammatory reaction. To date, it is unclear whether and how the impaired oxygen delivery and inflammatory environment may influence the basic functions of human ovarian somatic cells. In this study, we used an immortalized human granulosa cell line (COV434) as a model to investigate the effect of exposure to low oxygen levels (hypoxia) and inflammatory conditions on granulosa cell proliferation, apoptosis, and steroidogenesis. The results showed that both hypoxia (5% O₂) and proinflammatory cytokine treatment (a combination of TNF- α , IL-1 β , IFN- γ and IL-6) significantly increased apoptosis, suppressed proliferation, and affected estradiol secretion in COV434 cells. Our

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This research was funded by the Bulgarian National Science Fund, Ministry of Education and Science, Bulgaria, under grant No KP-06-H51/2.

DOI:10.7546/CRABS.2023.08.05

data suggest that granulosa cells could be particularly sensitive to changes in the local environment caused by oxygen deprivation and chronic inflammation.

Key words: granulosa cells, POI, COV434 cell line, hypoxia, proinflammatory cytokines, proliferation, apoptosis, steroidogenesis

Introduction. A deficiency in the functioning of the ovary as a reproductive and endocrine organ occurring before the age of 40 is referred to as premature ovarian insufficiency (POI) [1]. This condition affects approximately 1–2% of women under the age of 40 and 0.1% of women younger than 30. Although POI is a heterogeneous disorder with a wide range of causes and phenotypes, two key mechanisms eventually contribute to the insufficiency of ovarian function: follicle depletion or follicle dysfunction [2]. Follicle depletion may result from the inability to establish a sufficient initial pool of primordial follicles in gestation, accelerated follicle consumption, or autoimmune or toxic follicle destruction. Follicle dysfunction, in contrast, means that the follicles are present in the ovary but fail to undergo normal maturation, probably due to a compromised ovarian somatic cell compartment.

The ovarian follicle, which is the basic functional female reproductive unit in mammals, consists of an oocyte and two types of specialized somatic cells: granulosa cells (GCs) and theca cells [3]. The primary result of the highly organized, complex, and dynamic process of folliculogenesis is the production of a mature oocyte, whose quality and developmental competence are crucial for reproductive success and heavily rely on the bidirectional physical interaction and hormonal support provided by the surrounding GCs [4]. The granulosa cells are therefore seen as an essential functional component of the ovary that is vital to each stage of follicular growth and development, thereby ensuring the successful realization of its reproductive potential. In this context, it is evident that any disruption in the development and functions of GCs would have serious negative effects, such as aberrant hormonal secretion, disorders in the maturation of follicles or their growth arrest, and even follicular atresia.

Various diseases are associated with ovarian dysfunction, with infections and autoimmune disorders being among the main causes. Other factors that are linked to the condition include iatrogenic causes such as chemotherapy, radiotherapy, or surgical procedures [4]. Whatever their nature, many of these causes are related to the onset of a local inflammatory response or impaired blood flow, which, in turn, results in nutrient and oxygen deficiency in the ovaries. Recent findings indicate that ovarian reserve is negatively affected by chronic inflammatory disorders such as Crohn's disease, systemic lupus erythematosus and endometriosis.

So far, the precise pathophysiological mechanisms underlying hypoxia- or inflammation-induced follicle dysfunction remain largely unknown, and, in particular, whether and how the inflammatory environment and disrupted oxygen delivery may affect the basic functions of human ovarian somatic cells. In the

present study, we assessed the effects of exposure to low oxygen levels (hypoxia) and inflammatory conditions (a mixture of proinflammatory cytokines TNF- α , IL-1 β , IFN- γ and IL-6) on granulosa cell proliferation, apoptosis, and steroidogenesis. As a model system, we used human granulosa cell line COV434, which possesses a number of properties considered essential for natural human granulosa cells, such as 17-estradiol production and expression of FSH receptors [5].

Materials and methods. Cell culture. The immortalized granulosa cell line COV434 originally established from a solid primary human granulosa cell tumour was used as a model of GCs and was purchased from the European Collection of Authenticated Cell Cultures (ECACC; Sigma-Aldrich). COV434 cells were cultured and maintained in low-glucose (1.0 g/L) Dulbecco's modified Eagle's medium (LG-DMEM; Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% antibiotic/antimycotic solution (Sigma-Aldrich), and were incubated under standard culture conditions (humidified atmosphere, 37°C, 5% CO₂), with the cell culture media being changed every 3 days. On reaching confluence, cells were harvested and expanded in 75 cm² flasks or used for further experiments.

For hypoxia exposure, COV434 cells were cultured in a gas mixture consisting of 5% O₂, 5% CO₂, and nitrogen, at 37°C within a CO₂-O₂-N₂-regulated incubator. Control cells were maintained under a normal culture atmosphere (21% O₂ and 5% CO₂ at 37°C).

To evaluate the effects of the inflammatory microenvironment, COV434 granulosa cells were cultured in growth medium (LG-DMEM with 10% FBS and antibiotics/antimycotics), supplemented or not (control cultures) with a mixture of human recombinant proinflammatory cytokines tumour necrosis factor alpha (TNF- α ; 50 ng/ml), interferon gamma (IFN- γ), interleukin-1 beta (IL-1 β ; 10 ng/ml) and interleukin-6 (IL-6; 10 ng/ml).

Cell proliferation and population doubling time (PDT). COV434 cells were seeded in quintuplicates on 96-well plates at a density of 1.0×10^4 cells/well and cultured for 14 days in a reduced oxygen atmosphere or in the presence of proinflammatory cytokines, as previously described. AlamarBlue (AB) assay was performed to assess the cellular growth following exposure to hypoxic or inflammatory conditions. Cell proliferation was evaluated every 48 h by measuring the reduction of alamarBlue fluorometric growth indicator according to the manufacturer's instructions (Bio-Rad Laboratories). The population doubling time (PDT) was calculated using the formula:

$$\text{PDT} = \frac{(t - t_0) \times \log 2}{\log C - \log C_0},$$

where $t - t_0$ is the culture time (h), C is the final cell concentration, and C_0 is the initial cell concentration.

Apoptosis assay. COV434 cells were plated at a density of 1.0×10^6 cells per well into 6-well plates in triplicates and exposed to inflammatory cytokines or hypoxia. Control cultures were maintained in standard growth medium (LG-DMEM with 10% FBS and antibiotics/antimycotics) and normal culture conditions (21% O₂; 5% CO₂; 37 °C), respectively. At several intervals (48, 96, and 144 h) during the culture period, cells were harvested and subjected to a flow cytometric apoptosis assay using Annexin V-fluorescein isothiocyanate (FITC) staining (FITC Annexin V Apoptosis Detection Kit I; BD Biosciences) according to the manufacturer's instructions. Data were collected on a FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro software (BD Biosciences) was used to determine the percentage of apoptotic cells. The mean apoptosis rates were calculated on the basis of three independent experiments at each interval.

Steroid hormone estimation. To evaluate the estradiol production, COV434 cells were seeded on 24-well plates at a density of 1.0×10^6 cells/well and androstenedione (10 µM; Sigma-Aldrich) was added to the culture media as a substrate for aromatase activity. After 48, 96, and 144 h of exposure to hypoxia or proinflammatory cytokines, cell culture media were collected, centrifuged, and stored at -80 °C. The levels of secreted 17β-estradiol in the conditioned media were measured by an electrochemiluminescence immunoassay (ECLIA) on the Roche Elecsys 2010 Immunoassay Analyzer (Roche Diagnostics). All analyses were carried out in triplicate, and mean values were presented.

Data analysis and statistics. The Student's *t*-test or non-parametric Mann-Whitney U rank sum test was applied to evaluate the statistical significance of the difference between mean values. The statistical analyses were performed using SigmaPlot software (v12.5, Systat Software, Inc.), and a threshold of *p*-value ≤ 0.05 was considered statistically significant. The results are reported as mean values ± standard deviation (SD). Regression analyses were carried out using CurveExpert Basic (version 1.4, Hyams Development) statistical software.

Results. COV434 cell proliferation dynamics under hypoxic and inflammatory conditions. Using an AB assay, the proliferative capacity of COV434 cells was evaluated during the course of a 14-day culture period. As demonstrated by the growth curves (Fig. 1A, B), both reduced oxygen levels and treatment with proinflammatory cytokines resulted in a significantly slower proliferation pattern of COV434 granulosa cells throughout the whole observation period, in comparison with that of the control cultures. The average population doubling time (PDT) of COV434 cells cultured in the presence of proinflammatory cytokines was calculated to be 177.2 h (Fig. 1D), which is 2.2-fold higher than that of the controls (82.1 h, *p* < 0.01; Fig. 1D). Cell cultures grown under hypoxic conditions had an average PDT of 89.9 h, compared to 78.7 h (*p* < 0.01) for the control cells (Fig. 1C).

Apoptotic changes in hypoxic and inflammatory culture environment. The percentage of apoptotic COV434 cells (FITC-Annexin V positive)

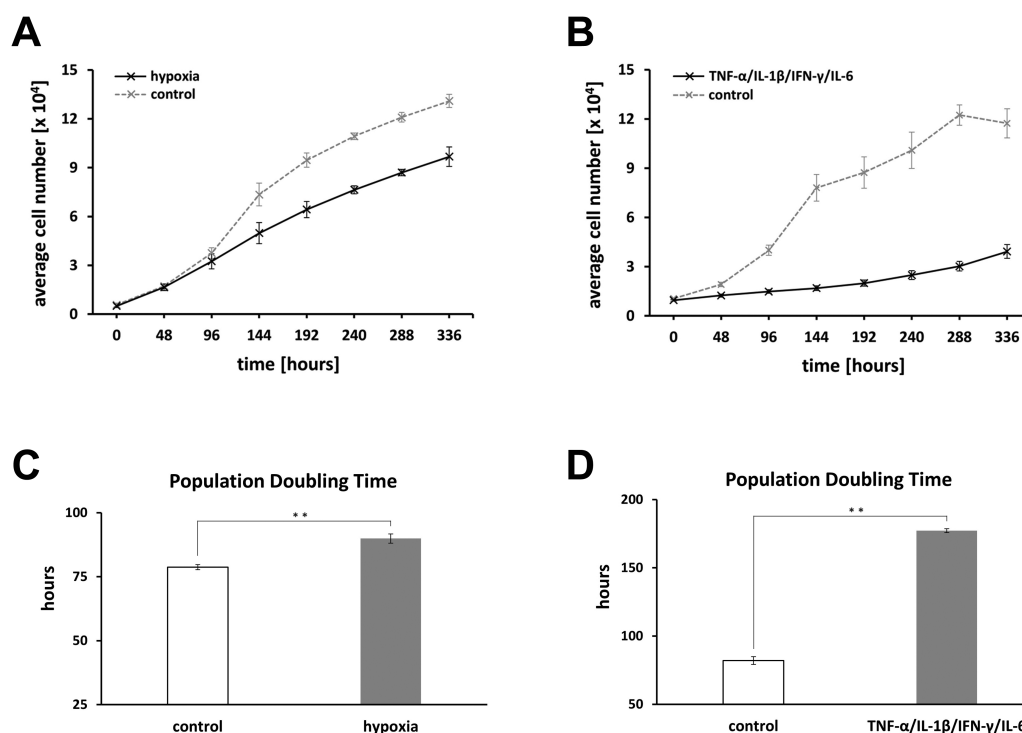


Fig. 1. Effects of hypoxic (A and C) and inflammatory (B and D) conditions on the proliferation profiles (upper panels) and average population doubling times (lower panels) of COV434 cells. The data are presented as mean $\pm$ SD from five individual experiments; ** $p < 0.01$

cultured in a reduced oxygen atmosphere or in the presence of proinflammatory cytokines for different intervals (48, 96, and 144 h) was estimated by flow cytometry. Both hypoxic and inflammatory conditions were found to induce a substantial rise in the proportion of apoptotic cells (Fig. 2A, B). At each of the three intervals tested, hypoxia resulted in an average increase in the percentage of apoptotic cells that varied from 1.5 to 2.8 times that of the corresponding control (Fig. 2A). Regarding the effect of the inflammatory conditions, the observed significant average increase ($p < 0.01$; Fig. 2B) in the frequency of Annexin V⁺ apoptotic cells at all the analyzed intervals ranged from 2.3- to 4.7-fold compared to the respective controls (Fig. 2B).

Evaluation of the endocrine function of COV434 cells in hypoxic and inflammatory culturing conditions. For steroidogenesis analyses, COV434 cells were exposed to hypoxia or proinflammatory cytokines for 48, 96, and 144 h, and subsequently, the levels of secreted 17 β -estradiol in the conditioned media were quantitatively measured. Exposure of COV434 cells to inflammatory culture conditions caused a significant decrease ($p < 0.05$) in mean levels of secreted 17 β -estradiol compared to control cultures, regardless of treatment period duration

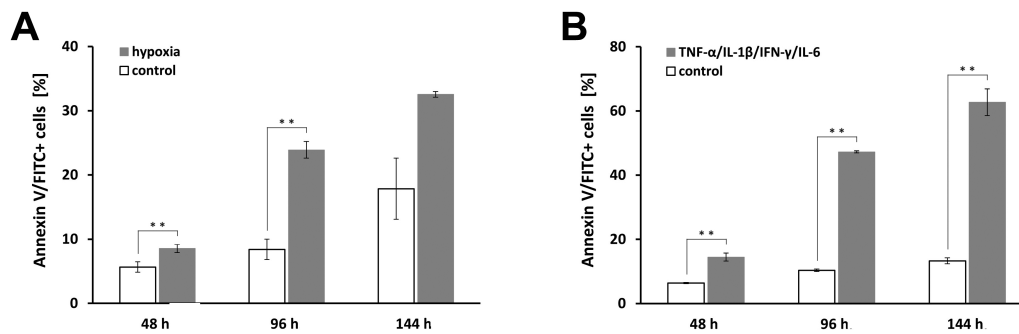


Fig. 2. Impact of hypoxia (A) and proinflammatory cytokines (B) on the percentage of COV434 cells undergoing apoptosis (Annexin V/FITC positive cells), estimated at three different intervals. Results are presented as mean $\pm$ SD of three separate experiments; ** $p < 0.01$

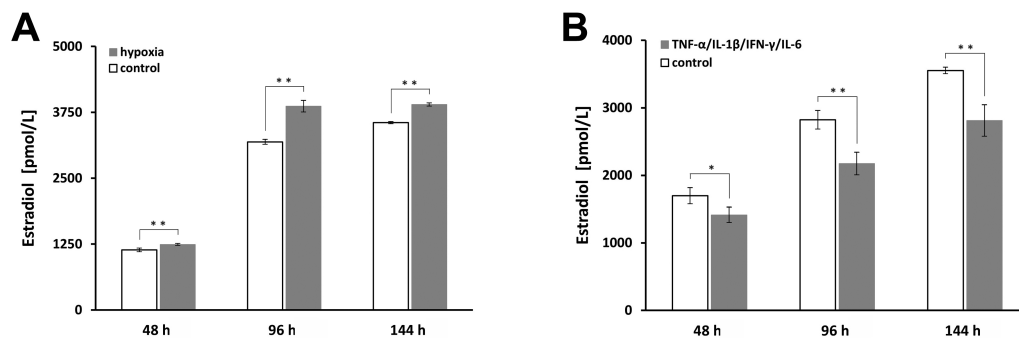


Fig. 3. Effects of hypoxia (A) and inflammatory conditions (B) on the estradiol production of COV434 cells measured by electrochemiluminescence at three different intervals. The bars represent mean $\pm$ SD of three independent experiments; * $p < 0.05$, ** $p < 0.01$

(Fig. 3B). In contrast, the effect of the reduced oxygen concentration on COV434 cell steroidogenesis was exactly the opposite. Under hypoxia, COV434 granulosa cells produced significantly higher mean 17β -estradiol levels compared to normoxic conditions ($p < 0.01$; Fig. 3A).

Discussion. The present study was undertaken to investigate some of the key functional characteristics and the survival of granulosa cells in response to oxygen deficiency and in the presence of inflammatory mediators. Granulosa cells of the ovarian follicle are closely associated with the oocyte via gap junction connections, providing it with the essential signals and metabolites it needs for normal growth and development. Therefore, granulosa cell functional impairment due to any physiological or environmental factors could lead to follicle dysfunction and disrupted oocyte development. The ovaries are especially fragile and vulnerable to damage and any exposure to adverse environmental conditions and factors could be associated with disturbed folliculogenesis, chronic diseases, and reduced fertility [6]. Increasing evidence points to hypoxia and inflammation as two primary causes of compromised ovarian function. Previous animal model

studies have shown that hypoxia is an endocrine disruptor, leading to reproductive health issues, including impaired gamete quality and decreased fertilization success [7]. Similarly, proper folliculogenesis, oocyte maturation, and ovulation require a healthy inflammatory response, whereas, in pathological conditions, impaired inflammation may contribute to infertility. Accordingly, several studies have demonstrated the detrimental impact of chronic inflammation on follicular growth, ovarian reserve, and subsequent reproductive potential [8,9].

Currently, there is little information available about the functional activity and viability of human granulosa cells under hypoxic or inflammatory conditions and, given the pivotal role of these cells for proper ovarian function, any new data on the matter will improve our understanding of the precise pathophysiological mechanisms underlying ovarian follicular dysfunction.

For experimental studies, human granulosa cells are commonly collected from the preovulatory follicles of hormonally stimulated women during IVF procedures. Unfortunately, these terminally differentiated cells are few in number, proliferate very slowly, and have a limited life span, which significantly complicates their long-term culture in vitro and considerably limits their suitability as an in vitro research model system.

To overcome these challenges, in our study we used the immortalized granulosa cell line COV434 instead of primary cultures of freshly isolated human granulosa cells. Despite being isolated from a primary human granulosa cell tumour, these cells exhibit several characteristics that have established them as a useful in vitro model for human granulosa cell research. These include certain biochemical, morphological, and hormonal characteristics of normal granulosa cells, including the secretion of a number of biologically active substances [5].

Here we showed that hypoxic conditions and proinflammatory cytokine treatment induced a significant increase in cell apoptosis and suppressed proliferation of COV434 cells. Although these findings are consistent with previous reports suggesting the negative impact of hypoxia or inflammatory cytokines on the functionality and viability of various types of mammalian granulosa cells [10,11], to the best of our knowledge, there are almost no relevant reports to date on these issues regarding granulosa cells of human origin. However, JIAO et al. [12] have recently demonstrated that proinflammatory cytokines IFN- γ and TNF- α cooperatively promote apoptosis and inhibit the proliferation and steroidogenesis of human KGN cells in vitro. These data, while referring to a different human granulosa cell line, correspond to our findings, including those related to the suppressive effect of a combination of proinflammatory cytokines on estradiol secretion by human granulosa cells.

Regarding the effect of hypoxia, on the contrary, we found that, under our experimental conditions, a reduced oxygen atmosphere of up to 5% caused an increase in the estradiol secretion of the COV434 cell line. However, it is unclear whether this hypoxia-induced enhancement in estradiol production is a typical

characteristic of normal human granulosa cells or a unique feature of the COV434 immortalized granulosa cell line.

In summary, our results suggest that both hypoxia and proinflammatory cytokine treatment may be associated with the onset of granulosa cell dysfunction, manifested by increased apoptosis, suppressed proliferation, and impaired estradiol secretion. Revealing the vulnerability of these cells to changes in the local environment caused by oxygen deprivation and chronic inflammation could provide new insights into the pathophysiological mechanisms involved in follicle dysfunction and open the door to novel therapeutic approaches for patients suffering from related disorders, such as POI.

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