APOPTOSIS OF GRANULOSA CELLS IN WOMEN WITH IMPAIRED REPRODUCTIVE FUNCTION AND EXTRAGENITAL PATHOLOGY

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Granulosa cells feed the oocyte during its maturation and protect it. Aberrant apoptosis in these cells is known to ultimately impair oogenesis. The current knowledge of how extragenital inflammation affects apoptosis in granulosa cells is incomprehensive, which is the root of an urgent problem connected to the spread of inflammatory diseases and the growing level of female infertility. This study aimed to assess the intensity of granulosa cell apoptosis in women with impaired reproductive function that suffer from chronic respiratory and/or digestive system diseases of inflammatory origin, and to identify the link, if any, between the studied factor and dysfunction of the reproductive system in the test group. The group included 60 women with a history of respiratory and/or digestive system inflammatory pathology that underwent IVF in 2021–2022. The women were donors of the granulosa cells from the follicular fluid collected through transvaginal puncture of preovulatory follicles. We studied the apoptosis process with the help of flow cytometry. For statistical analysis, we used the Fisher's F-test and the Kruskal–Wallis test. Twenty participants without extragenital pathology in their medical histories, the first subgroup, had the level of apoptosis in granulosa cells at 0.0088 ± 0.0062%, which is significantly lower than in twenty donors with a history of chronic inflammatory digestive system diseases, the second subgroup (granulosa cell apoptosis at 0.0140 ± 0.0099%, $p = 0.015$), and the subgroup of women suffering from inflammatory diseases of the respiratory system (granulosa cell apoptosis at 0.0650 ± 0.0391%, $p = 0.033$); the efficacy of IVF was higher in the first subgroup.

Keywords: apoptosis, granulosa cells, infertility, flow cytometry

Author contribution: LN Rogova — study planning, data analysis and interpretation; DS Lipov — manuscript authoring, analysis of the study data; VN Perfilova, MV Kustova — determination of the level of apoptosis in granulosa cells by flow cytometry; AV Mukhina — collection of the granulosa cell samples from patients; DA Churzin — analysis of the published papers, statistical processing of the obtained data.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Volgograd State Medical University (Minutes № 2021/053 of May 27, 2021) and conducted in compliance with the ethics principles of the WMA Declaration of Helsinki (2000). All donors have voluntarily signed the participant consent forms.

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УРОВЕНЬ АПОПТОЗА ГРАНУЛЕЗНЫХ КЛЕТОК У ЖЕНЩИН С НАРУШЕНИЕМ РЕПРОДУКТИВНОЙ ФУНКЦИИ И ЭКСТРАГЕНИТАЛЬНОЙ ПАТОЛОГИЕЙ

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Гранулезные клетки питают и защищают ооцит во время его созревания. Известно, что абберантный апоптоз в этих клетках может привести к нарушению оогенеза. На современном уровне знаний нет исчерпывающей информации о влиянии экстрагенитального воспаления на апоптоз в гранулезных клетках, что становится актуальной проблемой из-за распространения воспалительных заболеваний и роста бесплодия у женщин. Цель исследования — оценить уровень апоптоза гранулезных клеток у женщин с нарушением репродуктивной функции, имеющих в анамнезе хронические заболевания дыхательной и/или пищеварительной систем воспалительного генеза, а также определить наличие взаимосвязи между изучаемым параметром и репродуктивной дисфункцией в исследуемой группе. Исследовали образцы гранулезных клеток 60 женщин, имеющих патологию воспалительного генеза дыхательной и/или пищеварительной систем в анамнезе, проходивших лечение бесплодия методами ЭКО с 2021 по 2022 г. Образцы клеток были собраны из фолликулярной жидкости, полученной во время трансвагинальной пункции преовуляторных фолликулов. Оценку апоптоза проводили методом проточной цитометрии. Для статистического анализа использовали F-критерий Фишера и критерий Краскела–Уоллиса. Установлено, что у женщин без экстрагенитальной патологии в анамнезе ($n = 20$) уровень апоптоза гранулезных клеток составил 0,0088 ± 0,0062%, что достоверно ниже, чем у женщин группы с воспалительными заболеваниями пищеварительной системы в анамнезе ($n = 20$) — 0,0140 ± 0,0099% ($p = 0,015$) и группы женщин с воспалительными заболеваниями дыхательной системы в анамнезе — 0,0650 ± 0,0391% ($p = 0,033$), а результативность ЭКО была выше у представительниц первой группы.

Ключевые слова: апоптоз, гранулезные клетки, бесплодие, проточная цитометрия

Вклад авторов: Л. Н. Рогова — планирование исследования, анализ и интерпретация данных; Д. С. Липов — подготовка рукописи, анализ полученных данных; В. Н. Перфилова, М. В. Кустова — определение уровня апоптоза гранулезных клеток методом проточной цитометрии; А. В. Мухина — сбор образцов гранулезных клеток у пациенток; Д. А. Чурзин — анализ литературы, статистическая обработка полученных данных.

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГБОУ ВО ВолгГМУ Минздрава России (протокол № 2021/053 от 27 мая 2021 г.), проведено с соблюдением этических принципов Хельсинской декларации Всемирной медицинской ассоциации (2000). Все участники исследования подписали добровольное информированное согласие на участие в исследовании.

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Today, infertility is an urgent and not fully resolved problem affecting both men and women. According to the World Health Organization, there are 50 to 80 million couples in the world suffering from dysfunction of the reproductive system. In our country, the researchers estimate that up to 15% of couples have problems with fertility.

Female infertility is a matter that researchers pay significant attention to because of the structural and physiological complexity of the female reproductive system and the crucial role it plays in human procreation [1]. There are several factors that are typically mentioned as impairing female fertility: age, chronic diseases, lifestyle, environmental toxins, genetic characteristics etc [2]. Lately, scientists have been actively investigating the relationship between chronic inflammatory extragenital pathology and development of infertility. Extragenital pathology is known to significantly affect women's reproductive system and, consequently, lead to fertility disorders [2, 3]. There is a number of diseases, including diabetes mellitus, autoimmune disorders, thyroid diseases, dysfunction of the immune and hypothalamic-pituitary-ovarian axis, that can have a negative effect on the uterus, ovaries and the process of maturation of oocytes [2, 3]. Most researchers believe that violation of the said process is one of the main causes of female infertility [3, 4].

Oogenesis is a complex and multistage process guided by the interactions of various types of cells, hormones, growth factors, and signaling molecules. The quality of the oocyte is of paramount importance for successful conception and pregnancy, therefore, any disruption in this process can lead to a host of problems with fertility [4].

Oocyte maturation largely depends on the interaction of somatic cells surrounding it, including granulosa and cumulus cells [5]. Although histologically similar from the origin viewpoint, these cells have different functions. Granulosa cells, in particular, drive production of estrogen and participate in regulation of the follicle stimulating hormone that is crucial for development of the follicle [6, 7]. Recent studies have shown that granulosa cells directly affect quality of the oocytes, since they produce growth factors and other signaling molecules that shape oocyte maturation [8].

Cumulus cells are specialized cells found next to the maturing oocyte. They support the developing egg physically and biochemically, generate a number of growth factors and other signaling molecules (hyaluronic acid etc) [9, 10].

Some researchers have provided quantitative and qualitative assessments of apoptosis in granulosa and cumulus cells, and evaluated its effect on the processes of oocyte maturation. It was noted that inhibition of apoptosis in granulosa cells promotes growth of follicles and improves quality of the oocytes [8]. Other authors have shown that selective apoptosis of granulosa cells during oocyte maturation is a necessary prerequisite of successful ovulation [11]. The regulation of apoptosis was found to be influenced by the complex interaction of signaling pathways, including the Fas/FasL system and the Bcl-2 protein family [12]. Many experts agree that investigation of the mechanisms behind apoptosis in granulosa and cumulus cells is necessary for the development of targeted treatment regimens for infertility and other reproductive disorders [12, 13].

However, it should be noted that most studies used cells taken from animals (mice, rats, pigs), and human samples were collected only in isolated cases. With this in mind, seeking to analyze the process of oogenesis and its role in female fertility in greater detail, we used granulosa cells donated by the patients whose infertility was treated with the help of assisted reproduction technologies (ART).

This purpose of this study was to assess the intensity of granulosa cell apoptosis in women with impaired reproductive function that suffer from chronic respiratory and/or digestive system diseases of inflammatory origin, and to identify the link, if any, between the studied factor and the dysfunction of the reproductive system in the test group.

The results of the study can give insight into the mechanisms underlying pathogenesis of infertility, which can help develop new treatments and targeted therapies aimed at improvement of the reproductive outcomes.

METHODS

This work is a multidirectional cohort study; the Figure shows its design. We analyzed samples of granulosa cells collected from 60 patients who underwent ART infertility treatment in Clinic № 1 of the VolgSMU in 2021–2022. The study involved women of reproductive age with a history of inflammatory extragenital pathology of digestive or respiratory system. These systems and this sort of pathology were chosen because of their high prevalence in the population and the results of the earlier studies that point to the negative effect such combinations have on the outcomes of ART-enabled treatments [14].

Having analyzed the medical documentation, we applied the following criteria to select participants of the study: age 20 through 45; confirmed history of a chronic inflammatory disease of the digestive system (gastritis, duodenitis, peptic ulcer of the stomach and (or) duodenum, pancreatitis) or the respiratory system (chronic pathology, like chronic bronchitis, or frequently recurring acute pathology (more than 4 times a year), like ARVI, influenza, bronchitis, laryngitis, tracheitis, pneumonia), with control group including women suffering no such condition; infertile for at least a year; signature on the informed voluntary participant consent form.

The exclusion criteria were as follows: a combined pathology of the respiratory and digestive systems; oncological diseases; patient's refusal to participate and allow processing of her personal data; patient's belonging to a vulnerable social group.

Overall, we selected 60 for the study and divided them into three groups: group 1 (n = 20) — women with no history of extragenital pathology; group 2 (n = 20) — women with a history of respiratory system inflammatory diseases (chronic pathology, like chronic bronchitis, or frequently recurring acute pathology (over 4 times a year) — ARVI, influenza, bronchitis, laryngitis, tracheitis, pneumonia); group 3 (n = 20) — women with a history of chronic digestive system inflammatory diseases (gastritis, duodenitis, peptic ulcer of the stomach and (or) duodenum, pancreatitis). Technical capability to analyze apoptosis in the granulosa cells was the factor limiting the number of participants.

The age of the women ranged from 21 to 43 years, with the mean age at 33.5 ± 4.7 years. The duration of infertility ranged from 4 to 16 years, with the average period being 7.4 ± 1.5 years. The causes of infertility were established with standard clinical and laboratory tests; the features of the extragenital pathology were described in the collected medical history. Stimulation of ovulation in all treatment cycles and all subsequent procedures were performed in strict accordance with the generally accepted clinical guidelines and protocols [15].

Granulosa cell samples were taken from follicular fluid collected via a transvaginal puncture of the preovulatory follicles. The cells were emerged in the buffer solution (heparin 10 IU/ml, 1% human albumin solution, 0.01% recombinant human insulin, gentamicin sulfate 10 μg/ml) and transported to the laboratory where apoptosis and the level thereof were investigated. Bringing the samples to the laboratory did not take more than 3 hours.

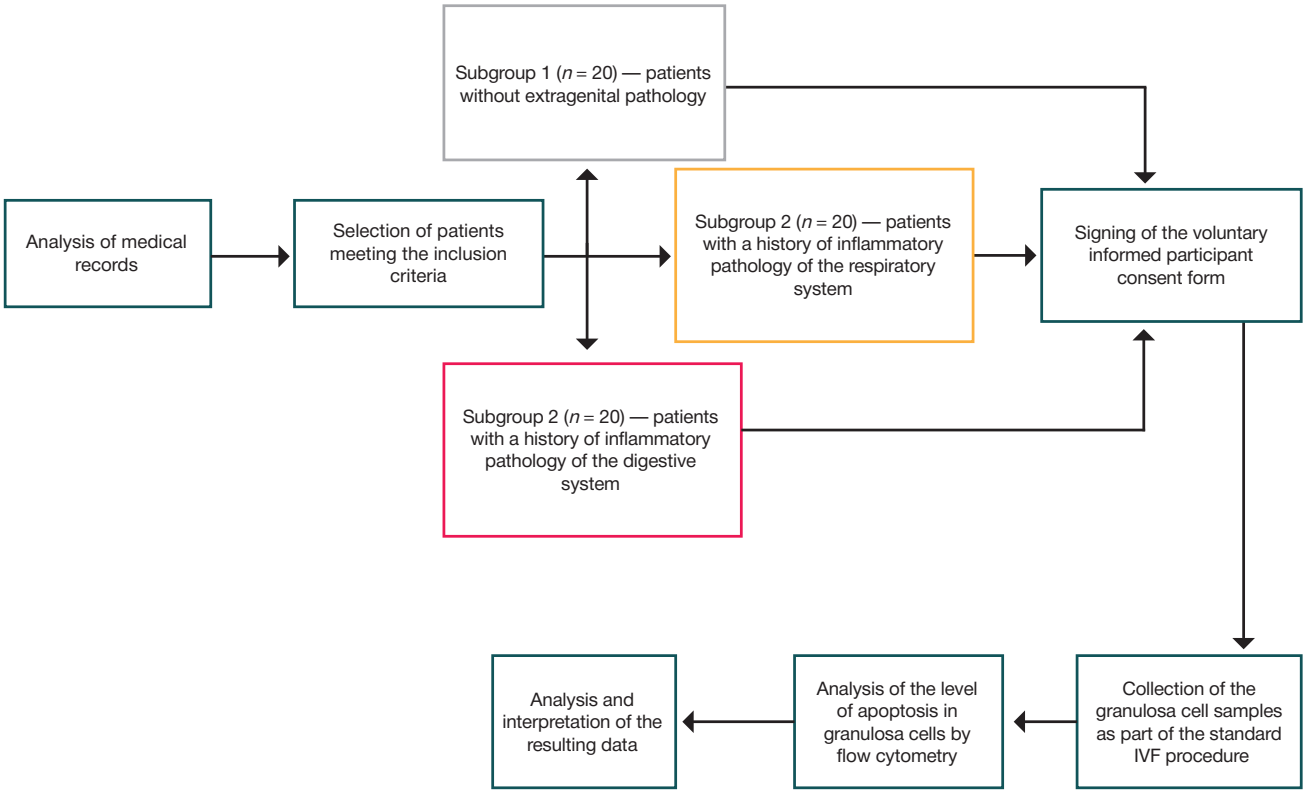

Fig. Research design

The amount of cells with signs of apoptosis was estimated with the help of the dead cell apoptosis kit with annexin V FITC and PI for flow cyrometry (Invitrogen, Thermo Fisher Scientific Inc.; USA). The cell suspension was washed with saline. The washed granulosa cells were counted, then resuspended in the annexin-binding buffer to the concentration of 1×10^6 cells per ml, incubated for 15 minutes at room temperature with annexin V FITC and propidium iodide (PI) as per the kit manufacturer's instructions. For the analysis, we used Attune® Acoustic Focusing Cytometer (Thermo Fisher Scientific Inc.; USA) (at least 10 thousand events). For interpretation of the results, we distinguished between living cells that did not fluoresce (Annexin V-FITC-/PI-), cells showing early signs of apoptosis (Annexin V-FITC+/PI-), and cells exhibiting signs of late apoptosis (Annexin V-FITC+/PI+).

Statistical processing of the results was done with StatTech v.2.8.8 software (Stattech, Russia). To assess conformity of the quantitative indicators to the normal distribution patterns we used the Kolmogorov-Smirnov test. The values that did follow the normal distribution patterns, they were described using arithmetic means (M) and standard deviations (SD) with CI at 95%. The values outside normal distribution were described with the help of the median (Me), lower and upper quartiles (Q_1–Q_3). To compare three or more groups by a quantitative indicator falling within normal distribution, we used one-way ANOVA; for post hoc comparisons, the tests of choice were the Fisher's test (with variances equal) and the Welch's test (with variances unequal). For comparison of three or more groups by the quantitative indicator with distribution outside of the norm we applied the Kruskal–Wallis test.

RESULTS

As shown in Table 1, the analysis of the level of apoptosis in granulosa cells has revealed the respective process to be most active in the women with a history of inflammatory pathology of the respiratory system, less active in women with inflammatory diseases of the digestive system and least active in the participants that had no pathology at all.

Seeking to understand the relationship between the degree of apoptosis in granulosa cells and oogenesis and fertilization, we counted the number of mature oocytes donated through transvaginal puncture of the preovulatory follicles and the number of eggs fertilized *in vitro*. It was established that no extragenital pathology in the medical history translates into best results of fertilization and the greatest number of mature oocytes, while the worst figures for these two indicators were registered in women that had inflammatory diseases of the respiratory system (Table 2).

DISCUSSION

This study allowed establishing that extragenital inflammatory pathology of the digestive system and the respiratory system affects oogenesis. This is confirmed by the greater number of oocytes in the follicle puncture samples collected from women that did not suffer from these diseases: they had 13.44 ± 2.60 eggs, while women with the considered inflammatory pathologies of respiratory and digestive systems had 4.47 ± 2.00 (p = 0.001) and 7.10 ± 1.85 (p = 0.001) oocytes, respectively, which is significantly lower. Inflammatory diseases of these systems are known to cause dynamic persistence of various inflammatory mediators in blood (interleukins, tumor necrosis factors etc) [16, 17]. There is published evidence showing that some cytokines, like Il6 and Il8, can negatively affect oogenesis: the higher their level in the blood, the less fertilizable eggs the woman has [18].

The process of maturation of female gametes is quite complex; it is controlled by a number of mechanisms and factors, including interaction of the oocyte with the somatic cells of its microenvironment. Since granulosa cells make the conditions optimal for oogenesis [6], excessive induction of apoptosis in them can increase the possibility of death of the

Table 1. Living granulosa cells and apoptosis in them as registered in the study subgroups. [1] — F, Fisher's test used for statistical analysis; [2] — Kruskal-Wallis test used for statistical analysis

Test group	Live granulosa cell index (%)[1]	Early granulosa cell apoptosis rate (%)[1]	Late granulosa cell apoptosis rate (%)[1]
Subgroup 1 (women with no history of extragenital pathology)	0.2673 ± 0.0151 p_1 (subgroup 1 — subgroup 2) = 0.001 p_2 (subgroup 1 — subgroup 3) = 0.001	0.0088 ± 0.0062 p_1 (subgroup 1 — subgroup 2) = 0.033 p_2 (subgroup 1 — subgroup 3) = 0.015	0.0028 [0.0012–0.0046] p_1 (subgroup 1 — subgroup 2) < 0.001 p_2 (subgroup 1 — subgroup 3) = 0.008
Subgroup 2 (women with a history of inflammatory diseases of the respiratory system)	0.1946 ± 0.0227 p (subgroup 2 — subgroup 3) = 0.008	0.0650 ± 0.0391 p (subgroup 2 — subgroup 3) = 0.026	0.0300 [0.0161–0.0393] p (subgroup 2 — subgroup 3) < 0.001
Subgroup 3 (women with a history of inflammatory diseases of the digestive system)	0.2195 ± 0.0154	0.0140 ± 0.0099	0.0132 [0.0102–0.0206]

Table 2. Results of ART treatment of the study participants. [1] — F, Fisher's test used for statistical analysis; [2] — Kruskal-Wallis test used for statistical analysis

Test group	Number of mature oocytes in the follicle puncture samples[1]	Number of fertilized eggs[2]
Subgroup 1 (women with no history of extragenital pathology)	13.44 ± 2.60 p_1 (subgroup 1 — subgroup 2) = 0.001 p_2 (subgroup 1 — subgroup 3) = 0.001	11.00 [9.00–12.00] p_1 (subgroup 1 — subgroup 2) < 0.001 p_2 (subgroup 1 — subgroup 3) = 0.020
Subgroup 2 (women with a history of inflammatory diseases of the respiratory system)	4.47 ± 2.00 p (subgroup 2 — subgroup 3) = 0.013	3.00 [2.50–3.00] p (subgroup 2 — subgroup 3) = 0.038
Subgroup 3 (women with a history of inflammatory diseases of the digestive system)	7.10 ± 1.85	5.50 [4.00–6.75]

egg or disruption of its normal maturation [19]. The growing blood levels of Il2, Il4, TNFα and other factors associated with inflammatory diseases of the respiratory and digestive systems can induce apoptosis by increasing the amount of reactive oxygen species and reducing the transmembrane mitochondrial potential, which can guide cells along the internal pathway of programmed death [20]. A possibly related fact: women without extragenital pathology have significantly more live granulosa cells (0.2673 ± 0.0151%) lower early and late apoptosis rate (0.0088 ± 0 .0062% and 0.0028% [0.0012–0.0046%]) than women with chronic inflammatory pathology of the digestive system (number of living cells — 0.2195 ± 0.0154%, early and late apoptosis rate — 0.0140 ± 0.0099% and 0.0132% [0.0102–0.0206%]) and chronic inflammatory pathology of the respiratory system (number of living cells — 0.1946 ± 0.0227%, early and late apoptosis rate — 0.0650 ± 0.0391% and 0.0300% [0.0161–0.0393%]).

It should also be noted that women with diseases of the respiratory system had the lowest number of living cells and the highest rate of early and late apoptosis in granulosa cells, and, accordingly, compared to the other two subgroups of participants, they had the worst results of oogenesis (lowest number of mature oocytes) and fertilization. This can probably be explained by the possible hypoxia developing against the background of the existing pathology, which can be an additional inducer of apoptosis and disruptor of oogenesis [21].

CONCLUSIONS

Extragenital inflammatory pathology of the respiratory and digestive systems drives up apoptosis in granulosa cells, which affects oogenesis and has an adverse effect on the women's fertility. 2) Extragenital inflammatory pathology of the respiratory system has a greater effect on the spread of apoptosis and viability of granulosa cells. This is why, compared to the control group, women in this subgroup have shown the poorest reaction to ART treatment. 3) The results of this study can be used in the development of new approaches aimed at optimization of preparation for *in vitro* fertilization of women with a history of chronic inflammatory diseases of the respiratory and digestive systems.

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