The Importance of Cited-1 and HIF-1α Immune Activity of Granulosa Cells in IVF Treatment

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Abstract

Aim: The aim of this study was to investigate the Cited-1 and HIF-1α immune activity in granulosa cells in follicular development in patients who underwent IVF for infertility.

Methods: This study was conducted on 40 patients who were admitted to the assisted reproductive program with the complaint of infertility at the Gazi Yaşargil Training and Research Hospital Obstetrics and Gynecology Clinic IVF center between January 2022 and November 2022 and had primary or secondary infertility while starting the Ovum-Pick-Up (OPU) procedure. The fluid containing the granulosa cells was centrifuged at 3000 rpm for 10 min. The samples were fixed and processed for routine paraffine wax tissue embedding protocol. Sections were taken from paraffin blocks and immune stained with Cited-1 and HIF-1α. The preparations were examined under the microscope.

Results: HIF-1α expression was positive in membrane of granulosa cells. The nuclei were apoptotic and pyknotic. Cited1 expression was positive in membrane of granulosa cells. the cells were pyknotic

Conclusions: The high level of HIF-1α immunopositivity and negative Cited1 immunoreactivity in the immunohistochemical staining after the granulosa cells around the oocytes collected from female patients admitted to the IVF clinic and diagnosed with infertility showed that granulosa cell viability may be important on oocyte quality.

Keywords: *Granulosa cells, Oocyte, Cited-1, HIF-1α, Infertility*

1. Introduction

In vitro fertilization (IVF) is a complex series of procedures that is used in fertility. Simply, it is joining of sperm and egg in the laboratory dish. IVF is used to achieve fertility and genetic problem in people who cannot get pregnancy in normal ways. A population of somatic cells are granulosa cells that produced progesterone. During IVF procedure, granulosa cells were picked up from the patients. These cells were further analyzed for specific genes.[1] During IVF protocol, psychological and chemical stress occur and result in oxidative stress. This stress on granulosa cells also affect the oocyte and its quality. External and internal stimuli elevated oxidative stress rate, inducing apoptosis in granulosa cells.[2]

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Granulosa cells are follicular cells around the ovarian follicles. These cells close to the oocyte and their interaction support growth and maturation of oocytes. Granulosa cells surrounds the ovarian follicles and provide maturation of follicles and reacts to stimuli from adjacent cells. They secrete hormones including sex hormones estrogen and progesterone.[3] During maturation of follicles, granulosa cells also proliferated rapidly and maintains its support to developing follicles.[4] Through follicle growth, mechanism of granulosa cells proliferation rapidly is still not clear. The embryological origin of granulosa cells is still unknown.[5] Fan et al studied apoptosis rate of granulosa cells in IVF patients. They found that higher apoptosis rate in granulosa cells was a result of low ovarian reserve, with low egg and embryo numbers in IVF patients. They also stated early apoptosis can affect clinical complication in pregnancy.[6] McKenzie et al investigated a biochemical marker during embryo development to increase success rate in IVF patients. Their results showed that some genes can give clues about morphological and physiological characteristics of embryos. These can help to predict the follicular and embryonic health.[7]

HIF-1α is an important transcriptional factor that regulates cell survival in mammals when hypoxia conditions occur.[8] When HIF-1α is activated, cell metabolism is reprogrammed by the downstream expression of a number of genes. Moreover, HIF-1α can also modulate the induction of autophagy to ensure metabolic balance.[9] Little is known about the role of HIF-1α in granulosa cells, and these cells play a very important role in healthy oocyte excretion in infertility. The CITED family are transactivators with a glutamic acid/aspartic acid-rich C-terminal domain. CITED1 plays an important role in the embryonic development process, especially in the development of the ureter, placenta and brain.[10] Abnormal embryonic development occurs in the absence of CITED1.

In this study, we investigated the Cited-1 and Hif-1α immune activity in granulosa cells in ovary during follicular development in women who applied for IVF treatment.

2. Materials and methods

This study was conducted on 40 patients who were admitted to the assisted reproductive program with the complaint of infertility at the Diyarbakır Gazi Yaşargil Training and Research Hospital Obstetrics and Gynecology Clinic IVF center between January 2022 and November 2022 and had primary or secondary infertility while starting the Ovum-Pick-Up (OPU) procedure. The patient with male factor was excluded. The fluid containing the granulosa cells was centrifuged at 3000 rpm for 10 min. Half formaldehyde and alcohol were added to the samples. The samples were centrifuged at 3000 rpm for 5 minutes and kept at +4°C overnight. The next morning, the excess liquid in the samples were poured and 1-2 drops of plasma liquid was added to the samples. The samples were placed on filter paper and 1-2 drops of eosin were dropped on it. Afterwards, the samples were taken into routine histology follow-up. Blocked tissue samples cut 3-5 microns thick with a microtome, placed on slides, and put in preparation boxes. Routine histochemical staining and immune-histochemical staining were performed on tissue samples taken from each subject and cut at 5-micron thickness. Biochemically, Prolactin, Follicle stimulating hormone (FSH), estradiol (E2), luteinizing hormone (LH), anti-mullerian hormone (AMH), thyroid stimulating hormone (TSH) were detected in blood samples.

2.1. Immunohistochemical Analysis

After the granulosa cells were fixed in 10% formalin for histopathological analysis, histological follow-up procedures were performed and paraffin blocks were prepared. For immunohistochemical staining, 4-5 micron thick sections were taken from paraffin blocks. The sections taken from the slide were removed from paraffin and alcohol. Antigen retrieval was performed in a 700 W microwave for 15 minutes. After the sections were left to cool, they were washed with PBS and endogenous peroxidase blockade was performed with 3% hydrogen peroxide. It was then incubated with Ultra V blocking (catalog no. TA-015UB, ThermoFischer, USA). Primary antibodies were incubated with Cited-1 and HIF-1α (AFG Bioscientific, USA, 1/100) overnight at +4°C. Secondary antibody (TP-015-BN, ThermoFischer, USA) was then applied for 20 minutes. It was exposed to streptavidin-peroxidase (TS-015-HR, ThermoFischer, USA) for 20 minutes.

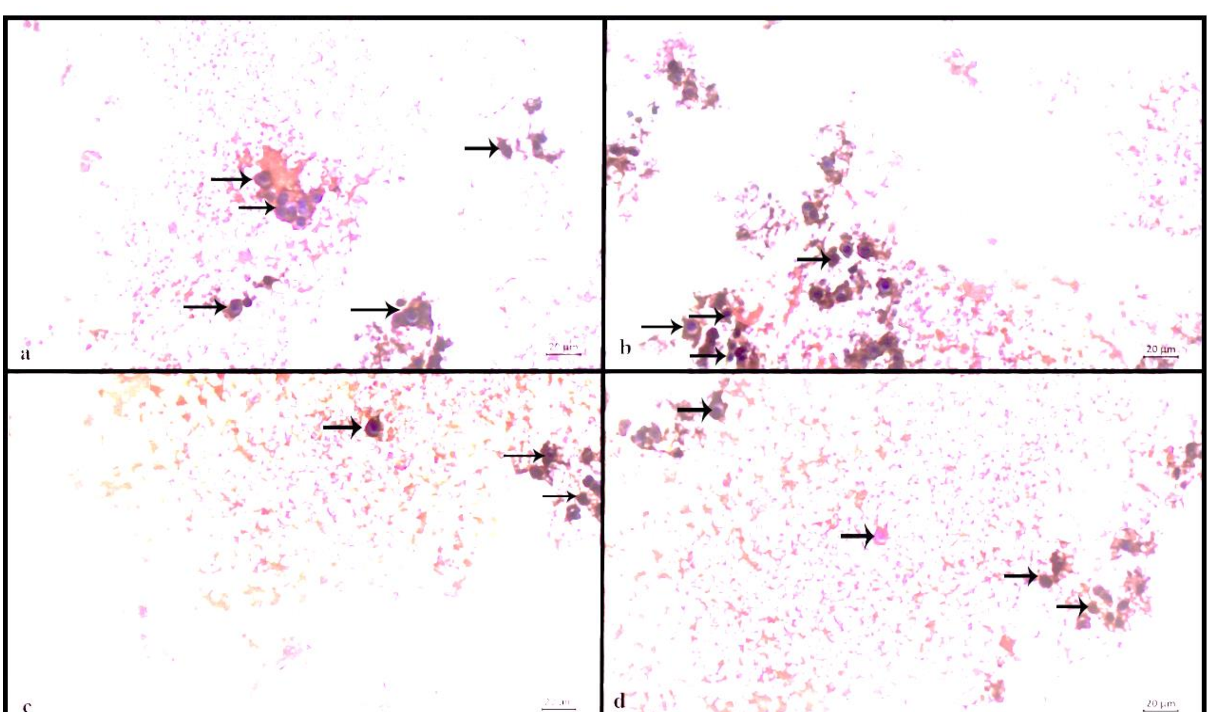
Finally, it was reacted with DAB (TA-001-HCX, ThermoFischer, US) chromogen. Hematoxylin was used for background staining11. All stained tissue samples were evaluated under Zeiss AXIO Scope 1 brand research microscope and photographed with a digital camera (Axio Cam ICc 3).

3. Results

Figure 1 shows the HIF-1α and Cited1 immune staining of granulosa cells. HIF-1α expression is expressed at the membrane level. The nuclei of the granulosa cells are peripheral, and the apoptotic process is induced. The nucleus is pyknotic. Expression was generally considered positive for HIF-1α in the membrane (Figure 1a).

Figure 1

Immune staining of granulosa cells (arrows: positive cells).



With degenerative changes, the nuclei have taken on a pyknotic appearance. This shows that hypoxia induces the apoptotic process. The hypoxia event was first seen in the membrane and then in the nucleus (Figure 1b). In particular, expression of granulosa cells was Cited-1 negative, but Cited-1 expression was positive at the membrane level. In general terms, we can say that granulosa cells are affected especially at the membrane level (Figure 1c). In the study of an aggregated population, granulosa cells were found in the periphery and the Cited-1 expression was positive. Granulosa cells were observed in the pyknotic state (Figure 1d). The average age of the patients was determined as 30.37±4.44. The average body mass index is 22.64±4.16. Additionally, when the causes of infertility are examined, it has been seen those patients with PCOS, low ovarian reserve, endometriosis and unexplained infertility apply to the clinic. Among these, unexplained infertility was the leading cause with an average of 18 patients. The demographics of the patients are shown in Table 1. Age, cause of infertility, body mass index (BMI), serum FSH, prolactin, E2, AMH levels are given.

Table 1

Demographic characteristics of the harvests included in the study.

	Overall
Number of patients, n (%)	40
Age, mean (SD)	30,37 (4,44)
BMI (kg/m2), mean (SD)	22,64 (4,16)
Infertility cause, n (%)	
• Ovulatory	12 (22.89)
• Endometriosis	6 (4.57)
• PCOS	4 (17.25)
• Unexplained	18 (51.77)
AMH (ng/ml), mean (SD)	2,84 (1.24)
TSH, (mIU / L), mean (SD)	1,82 (1,1)
E2, (Pg/mL), mean (SD)	40,9 (12.8)
FSH, (mlU/ml), mean (SD)	5,67 (3,40)
LH, (IU/L), mean (SD)	6,84 (3,75)
Prolactin, (µg/L), mean (SD)	14,6 (9,35)

4. Discussion

Oocyte and granulosa cells have direct interaction between the each other. One study showed that there are gap junctions with macula adherens between granulosa cells and oocytes by freeze-fracture electron microscopy technique.[12] Granulosa cells are associated with oocytes and always interact with each other. Through these cellular lateral junctions, granulosa cells direct the maturation and growth of follicles and oocyte inside the follicles. Metabolic precursors and nutrients are also supplied by granulosa cells to oocyte. During the maturation of oocyte, granulosa cells also secrete molecules that regulate the oocyte growth.[13] Earlier findings was considered there were a oneway communication between the oocytes and granulosa cells. However, today we know that it is bidirectional. Some factors are secreted from oocyte and these directly regulate the maturation and proliferation of granulosa cells. these factors are as oocyte-derived growth factors which are growth differentiation factor (GDF-9) and bone morphogenetic protein 15 (BMP-15)[14].

HIF-1α, a pleiotropic transcription factor, is important for the survival of mammalian cells. VEGF downstream influences the transcription of many factors such as glycolytic enzymes and glucose transporters. During the embryonic period, HIF-1α regulates vasculogenesis, tumor angiogenesis and ischemia[15]. Since cellular hypoxia develops during placenta formation, HIF-1α is activated, triggering the proliferation of trophoblasts and the formation of specific cell subtypes.[16] Tang et al. studied hypoxic cell culture model to mimic the rat follicular development model to show effect of HIF-1α. They found that hypoxia induces activation of HIF-1α and promoted rate of autophagy. By this, HIF-1α prevented the apoptosis in granulosa cells and support the follicular development.[17] Baddela et al. studied HIF-1α level in bovine granulosa cells. They stated that suppression of HIF-1α regulated the steroidogenesis. Additionally, HIF-1α affects transcription of many genes which play an important role in granulosa cells functionality.[18] In our study, granulosa cells were apoptotic and HIF-1α expression was positive. (Figure 1a-b)

Cited1, a transcriptional coactivator, likely regulates melanocyte pigmentation. It also initiates transcription, which estrogen regulates[19]. Cited1 consists of four nuclear proteins. Since there is no binding site in DNA, its role is mainly expressed as a transcriptional regulator.[20] Sriraman and his colleagues studied the progesterone receptor in granulosa cells in vitro and found that many genes that regulate the activation of the progesterone receptor in granulosa cells are controlled. It has been stated that one of these genes works depending on the progensterone receptor during ovulation and affects ovulation[21] When Hatzirodos et al. investigated the transcriptome profile of bovine granulosa cells, they found that the number of transcriptional regulators increased parallel to the growth of follicles. They found that one of the regulators was Cited1. In our study, it was observed that Cited1 expression decreased in granulosa cells with pyknotic nuclei (Figure 1 c-d).

HIF-1α is a key regulator of hypoxia-induced metabolism disruption. Many experimental studies have shown that HIF-1α ensures the survival of granulosa cells and the maintenance of follicular development in both mouse.[22] Kim et al. showed that inhibition of HIF-1α blocked hCG-dependent induced ovulation in a mouse model.[23] The findings obtained in this study also explain the cause of infertility. It showed that HIF-1a decreased in terms of immunoreactivity in granulosa cells collected from patients who came to the clinic with infertility problems. These findings showed that HIF-1α affected oocyte development as a result of hypoxia-induced cellular damage in granulosa cells and therefore played a protective role on the survival of granulosa cells.

In other experimental studies, it has been shown that FSH, another factor that facilitates the proliferation of granulosa cells and follicular development, plays a role as a critical regulator of HIF-1α activation and prevents the loss of mitochondrial balance through HIF-1α.[22,24] Another study found that oxidative stress increased after HIF-1α inhibition. They found that rat granulosa cells did not significantly increase their apoptosis when incubated under hypoxic conditions (3.0%), which are consistent with in vivo conditions. These findings indicate that granulosa cells may have a self-protective mechanism to protect themselves from hypoxia-induced apoptosis.[25] In our study results, the prominence of HIF-1a in terms of positivity suggests that CITED1 being positive may lead granulosa cells to apoptosis. Since there is no consistency in experimental studies on this subject, more comprehensive studies are needed.

5. Conclusion

As a result, it is important for individuals diagnosed with infertility to correctly diagnose the problems that prevent them from having children and to solve them with the right method. The aim of this study was to reveal one of the underlying causes of the problems encountered in the clinic and to define its relationship with infertility, if any. The findings indicate the duration of hypoxia to which granulosa cells are exposed. In this respect, the study can be made more comprehensive and the obstacle to an important problem can be removed with more clinical data. Considering that it may be an indicator of the effect of granulosa cells on the formation of quality eggs that can be fertilized, it is thought that HIF-1a and Cited-1 may be antibodies that may be among the important markers in egg development, affecting inflammation and cell apoptosis.

Conflict of interest statement

The authors declare that they have no financial conflict of interest with regard to the content of this report.

Statement of ethics

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved *Diyarbakır Gazi Yaşargil Training and Research Hospital Ethics Commitee for this study (07.05.2021, Decision No: 2021-760)*

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Author Contributions

Authors reviewed the results and approved the final version of the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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