RESEARCH ARTICLE

Apoptotic markers and antioxidant enzymes have altered expression in cumulus and granulosa cells of young women with poor response to ovarian stimulation

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Background: The objective of this study was to evaluate apoptosis and oxidative stress markers in Granulose (GC) and Cumulus Cells (CC) of women with low response to Controlled Ovarian Stimulation (COS) and controls which underwent an IVF treatment.

Methods: mRNA concentrations of BCL-2, BAX, caspase-3, caspase-9, Superoxide Dismutase (SOD) and catalase were evaluated in patients (n=30) and donors (n=45) using qRT-PCR analysis on CG and CC.

Results: The concentrations of mRNA coding for BCL-2, BAX, caspase-3, and caspase-9 were significantly higher in GC from women with low response compared to women with normal response ($p<0.001$).

In contrast, concentrations of catalase mRNA were significantly increased in normoresponders women ($p< 0.05$). The levels of catalase mRNA were also significantly higher in CCs from normoresponders women compared to patients with low response ($p<0.003$), and the levels of BAX mRNA in CC were significantly increased in patients ($p<008$).

Conclusions: In addition, we found a correlation between the expression of genes regulating OS and apoptosis and response to ovarian stimulation, which could mean that an increase in ovarian oxidative stress can reduce the number of oocytes obtained after ovarian stimulation.

Key Words: *Low ovarian reserve; apoptosis; oxidative stress; cumulus cells; granulosa cells*

The goal of Controlled Ovarian Hyper-stimulation (COH) is the recruitment of multiple follicles that may yield good-quality fertilizable oocytes, allowing an optimal early embryo development and a successful embryo transfer.

Ovarian response to COH varies widely amongst individuals, extending from a poor to a strong response to gonadotropins. A poor response to ovarian stimulation typically results in fewer retrieved oocytes with a lower rate of pregnancy.

Recently, a change of the definition for Poor Ovarian Response (POR) has been proposed. Previously it was founded on a combination of diverse norms, which has now been transformed into a concept of low prediction [1]. In any case, poor ovarian response in young women remains a therapeutic challenge without a clear explanation for human reproduction specialists.

The association between low ovarian response and antioxidant enzyme function continues to be a promising area. We have showed lately that the levels of Reactive Oxygen Species (ROS) scavenging effectiveness in the follicular fluid undergo a meaningful reduction in young low responding patients compared to normoresponders women, as a consequence of variations in some antioxidant enzymatic actions. Changes in the follicular microenvironment of patients with low ovarian reserve might alter the metabolism of cumulus and granulose cells [2].

On the other hand, it has been shown that ovarian oxidative stress (OE) can increase the apoptosis of the Cumulus (CC) and Granulose Cells (GC) [3-5].

Apoptosis is a genetically controlled event that can be initiated by two major pathways, which include the death receptor, and the mitochondrial pathways. Members of the BCL2 (B-cell Leukemia/Lymphoma-2) protein family regulate apoptosis through the mitochondrial pathway and acting at the effector stage [6].

Apoptosis of cells surrounding developing oocytes can notionally be used as a marker of oocyte competence, since we now know that GC apoptosis is an important event in follicular atresia [7]. Increased GC apoptosis has been related to reduced pregnancy outcome when using IVF [8,9]. Moreover, increased CCs apoptosis has been related to impaired oocyte maturation and fertilization as well as reduced pregnancy outcome [10]. In this respect, the most significant characteristic involves the increase of oxidative stress damage, which has been related with a progressively lower microcirculation nearby the principal follicle [11].

However, the clinical usefulness of CC apoptosis as a marker for oocyte competence remains controversial and our understanding of the mechanisms that regulate GC apoptosis in follicular atresia is limited.

CC and GC transcriptome has been studied to identify several genes that could be differentially expressed in CC and GC surrounding a good-versus a poor quality oocyte. Several microarray studies have also been performed in order to identify potential biomarkers that could be associated with oocyte quality [12].

However, to date there is no consensus as to which genes are the most useful biomarkers of oocyte competence. On the other hand, there is scarce information about the relationship between oxidative stress and apoptosis in the ovary with the low response to ovarian stimulation.

The objective of this study was to analyze the possible relationship between the levels of oxidative stress markers and apoptosis in Granulose (GC) and Cumulus Cells (CC) and the low response to ovarian stimulation in young women. Consequently, we compared the mRNA expression coding for antioxidant enzymes and apoptotic markers in women with low response undergoing *In vitro* Fertilization (IVF) and in young normoresponders women with proven fertility.

SUBJECT AND METHODS

Study participants

This was a prospective study conducted at Clinica Tambre at Clinica Tambre from June 2016 to February 2017, which was approved by the Ethical Review Board of the Hospital de la Princesa (Madrid, Spain) (reference PI-648). The study included 45 normoresponders women (fertile oocyte donors), and 30 patients with low response to ovarian stimulation (<5 oocytes recovered) in at least one previous ovarian stimulation cycle. There was included only the first stimulation cycle from each patient.

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In our study, the low responder patients were categorized, according to Poseidon, as Group 1: patients younger than 35 years with good ovarian pre-stimulation reserve (AFC R5, AMH ≥ 1.2 ng/mL) and with an unexpected low ovarian response [1].

Patients were also enrolled according to the following criteria: (i) normal reproductive system with normal basal hormonal levels; (ii) normal endocrine system absence of hyper pro-lactinaemia, thyroid dysfunction or polycystic ovary syndrome as defined by the Rotterdam criteria [13]; (iii) age of women between 28 and 35 years old; (vi) Body Mass Index (BMI) between 19 and 26 kg/m^2; and (vii) absence of any smoking history in women. All the patients signed the corresponding informed consent and did not receive any financial compensation for the study.

Women included as oocyte donors were informed of the donation process and fulfilled all requirements as donors. In brief, oocyte donors were 18–35 years old, with a complete medical history, which included the absence of current or past exposure to radiation or hazardous chemical substances, drug abuse and past reproductive history. All had a normal physical and gynecological examination, BMI between 19 and 26 kg/m^2 and no family history of hereditary or chromosomal diseases, normal karyotype and negative screening for Sexually Transmitted Diseases (STD). Neither the patients nor the donors took vitamins, antioxidants or any substance that could interfere in the study

Ovarian stimulation protocols

Both groups (patients and donors) received the same stimulation protocol: short protocol with GnRH antagonists and recombinant gonadotropins.

Ovarian stimulation was initiated with 225–300 UI/day rec-FSH (Gonal-F®; Merck, Madrid, Spain) from day 2 of the menstrual cycle, and the GnRH antagonist (Cetrotide; Merck, Madrid, Spain) was introduced according to a multiple-dose protocol (0.25 mg/day) when a leading follicle of 14 mm and/or estradiol concentrations of 400 pg/mL were reached. Recombinant human chorionic gonadotropin (HCG) (In patients, Ovitrelle®, Merck, Madrid,Spain) was applied when ≥ 2 follicles reached ≥ 17 mm and oocyte retrieval was performed under sedation at the 36th hour following HCG. In donors, triggering was performed with 0.2 mg of triptorelin (SC Decapeptyl, Ipsen Pharma, Barcelona, Spain) and oocyte retrieval was achieved under sedation at the 36th hour following GnRHa. In all groups, the first control (ultrasonography and serum estradiol) was carried out after 5 days of stimulation, and the daily dose of FSH was adjusted individually according to the ovarian response.

Cumulus and granulose cells isolation and processing

We equated the expression of mRNAs coding for antioxidant enzymes: Catalase (CAT) and Superoxide Dismutase (SOD) and apoptotic markers (Caspase 3, Caspase 9, BCL2-associated X protein (BAX) and B-cell Leukemia/Lymphoma gene-2 (BCL2) in 385 oocyte-cumulus and 314 oocyte-granulose recovered from 45 young healthy fertile oocyte donors, and 110 oocyte-cumulus and 98 oocyte-granulose complexes retrieved from 30 low responders patients after *In-vitro* Fertilization (IVF).

Oocytes were separated and placed into culture media, whereas follicular fluid was collected in flasks. Considerable care was taken to pool cells uncontaminated with flush medium or blood. Samples with blood contamination were rejected.

Granulose cells were processed according to the Ferrero et al. method [14]. Briefly, follicular fluids were filtered through a 40 µm cell strainer (Fisherbrand, Fisher Scientific, Madrid, Spain) and the clusters of GC were retained. The filter was washed with 12 mL G-MOPS (Vitrolife, Sweden), to eliminate the last vestiges of blood contaminants and the filter was then washed back with G-MOPS (Vitrolife, Sweden) to collect the GC. Subsequently, the suspension was incubated for ten minutes and aspirated through Pasteur pipettes to break the aggregates mechanically. The suspension was then filtered through a 70 mm cell filter (Fisherbrand, Fisher Scientific, Madrid, Spain) to remove unwanted material, which was retained in the filter. The cell suspension, collected after filtering the sample, was subsequently washed for 5 minutes at 600 g, after which the supernatant was removed and the pellet was suspended in 1 mL of G-MOPS (Vitrolife, Sweden).

Retrieved cumulus-oocyte complexes were placed in culture medium (G-MOPS, VitroLife, Sweden), and cumulus cells were dissected from the oocyte mechanically in the absence of hyaluronidase. Cumulus cells from each patient were pooled in a single Eppendorf tube (Denville Scientific Inc Holliston, MA., USA.). Samples were washed twice with 0.5 mL of 1X Phosphate-buffered Saline (PBS) and centrifuged at 1,000 g for 1 minute, with the supernatant removed after each wash. After the final wash, the cell pellet was suspended in 50 mL of SideStep Lysis and Stabilization Buffer (Stratagene Products Division, La Jolla, CA USA).

Samples of each patient and donor were stored at −80°C for a maximum of 2 weeks, until assessed for Superoxide Dismutase (SOD), Catalase, Caspase 3, Caspase 9, BAX and BCL2.

RNA isolation and reverse transcription

To assess mRNA concentrations of SOD, Catalase, Caspase 3, Caspase 9, BAX and BCL2, we performed qRT–PCR (Real Time-Polymerase Chain Reaction) analysis on GCs and CCs from pooled follicular aspirates of each patient.

The RNA was obtained according to the method described by Voge [15]. Briefly, after adding 500 µL of Trizol, each sample was incubated for 5 minutes at 25°C. Then, 100 µL of chloroform were added and samples were incubated for 3 minutes at 25°C. After centrifugation (3500 g for 30 minutes at 4°C) the aqueous phase was collected in another tube and 250 µL of 100% Isopropyl alcohol were added. Samples were then incubated for 10 minutes at room temperature, the supernatant was discarded and 500 µL of 75% Ethanol was added. Samples were centrifuged at 3500 g for 5 minutes at 4°C, the resulting supernatant was discarded and 10 µL of Molecular Biology grade water were added to each sample. RNA purity and concentration were evaluated by spectrophotometry using µL Biodrop (Isogen Life Science, De Meern, Netherlands) at 260/280 nm (ratio > 2.0). The reverse transcription of RNA was performed using the RETROscript® Reverse Transcription Kit (Ambion, Life Technologies, Texas, USA) following the manufacturer's instructions. The preamplification of each sample was performed mixing 250 ng of cDNA with the TaqMan® PreAmp Master Mix (Applied Biosystems, Warrington, UK). 10 preamplification cycles were performed in an Applied Biosystems 7500 Fast apparatus following the manufacturer's instructions. qRT-PCR was performed using the TaqMan® Universal Master Mix II (Applied Biosystems, Warrington, UK) and 1 µL of the corresponding TaqMan® Assay. The housekeeping gene 18s was used as an internal control. Relative changes in gene expression were calculated using the 2-ΔΔCT method [16].

Statistical analysis

The patients' characteristics were compared using the Student's t test and the Chi-Square test as appropriate. The relative expressions of mRNAs in cumulus and granulose cells were compared across poor and normal responders using the Student's t-test. p-values of <0.05 were considered statistically significant. The SPSS version 22.0 statistical package was used in this study.

RESULTS

The clinical characteristics of the study population are showed in Table 1. No statistical difference was found in the amount of total gonadotropins used for ovarian stimulation for poor responders, as compared to the normal responders group. Serum estradiol concentrations on the day of HCG injection were lower in poor responders (p=0.01) (Table 1). As the response levels were defined according to oocyte numbers, the number of retrieved oocytes was significantly different between poor and normal responders (p=0.001).

Table 1

Patients characteristics in the low responders (<5 oocytes)(n=30), and control groups)(n=45).

Variable	Low responder patients	Control (donors)	p-value
Age (years)	31.4 ± 2.1	29.6 ± 3.1	NS
BMI (kg/m^2)	23.5 ± 2.2	22.4 ± 1.8	NS
AMH (ng/mL)	3.76 ± 1.2	5.74 ± 1.3	NS
Basal FSH (IU/l)	6.2 ± 1.2	5.9 ± 3.2	NS
Total FSH administered (IU)	1977 ± 156	1862 ± 246	NS
E2 value of hCG/GnRH day (pg/mL)	1447 ± 199a	2573 ± 321	0.01
Total oocytes retrieved	3.4 ± 1.3b	12.5 ± 3.8	0.001

Values are mean ± SD; BMI: Body Mass Index; AMH: anti-Mullerian hormone; The value of Estradiol and number of retrieved oocytes are significantly different among the poor and non-poor responder patients.

Antioxidant enzymes

Using qRT-PCR analysis it was obtained that mRNA expression for CAT was significantly higher in GC from oocyte donors (1.47 ± 0.11) compared to low responder patients (0.87 ± 0.06) ($p<0.05$) (Figure 1A). The expression of CAT mRNA in CC was also significantly higher in donors (1.66 ± 0.14) compared to young patients with low response (0.99 ± 0.06) ($p=0.0037$) (Figure 1B).

No significant differences were found between groups in the mRNA expression of SOD in GC (Figure 2A), neither in CC (Figure 2B).

Apoptotic markers

Using qRT-PCR analysis it was obtained that mRNA expression for BAX was significantly increased in GC from young women with low response (2.11 ± 0.2) when compared with oocyte donors (1.19 ± 0.02) ($p=0.018$) (Figure 3A). The mean expression of BAX mRNA in CC was also significantly increased in patients (2.95 ± 0.19) when compared to oocyte donors (1.26 ± 0.06) ($p=0.08$) (Figure 3B).

The mean expression of Caspase 3 mRNA in GC was significantly higher in patients (0.71 ± 0.049) as compared to oocyte donors (0.34 ± 0.01) ($p=0.0006$) (Figure 4A), whereas no significant differences between groups were found in mRNA expression coding for Caspase 3 in CC (Figure 4B).

The mean expression of Caspase 9 mRNA in GC was significantly increased in patients (1.37 ± 0.1) as compared to oocyte donors (0.81 ± 0.06) ($p=0.009$) (Figure 5A), whereas no significant differences between groups were found in mRNA expression coding for Caspase 9 in CC (Figure 5B).

The mean expressions of mRNA coding for BCL2 was significantly higher in GC from patients (1.46 ± 0.09) when compared to oocyte donors (1.02 ± 0.02) $p=0.006$) (Figure 6A). However, no significant differences between patients and donors were found in mRNA expression coding for BCL2 in CC (Figure 6B).

DISCUSSION

Our results show that there is a lower antioxidant capacity and an increase of apoptosis in the cumulus and granulose cells of women with a low response to ovarian stimulation compared to women with normal response. It is not clear whether poor oocyte quality induces apoptosis in CC and GC or whether poor oocyte quality is induced by CC with high levels of apoptosis [12]. However, numerous studies that demonstrate the dependence between the oocyte and the cells that surround it, suggest that appropriate functioning of these cells is essential for oocyte maturation [17,18].

mRNA expression profiles in GC are associated with the maturity of adjacent oocytes, and the modulation of these profiles may regulate oocyte maturation [19]. Gasca et al. [20,21], indicated that the medium in which the oocyte is found inside the follicular fluid can influence the gene expression of the cumulus cells. Moreno et al. [22] and Diez-Fraile et al. [23] have described

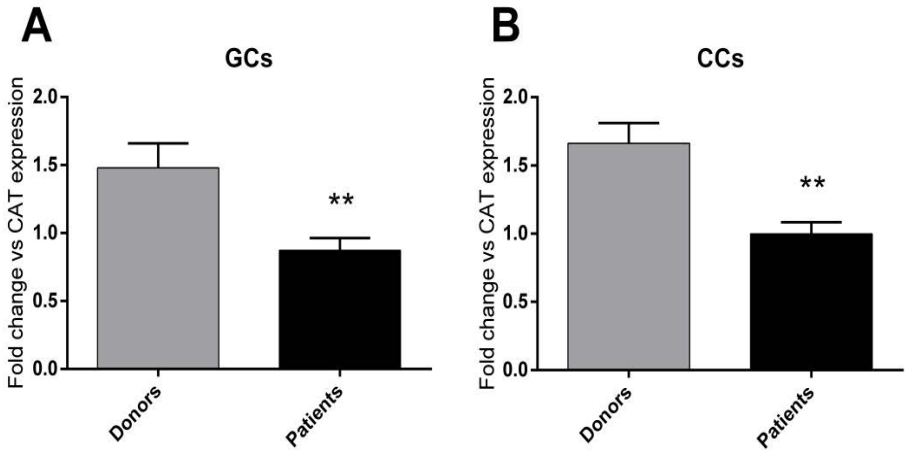

Figure 1) Bar graphs represent catalase mRNA expression levels in granulose cells (1A) and cumulus cells (1B) from control and patients.
Data are expressed as mean ± SEM.
Mean relative expressions of mRNAs coding for CAT were significantly higher in GCs (1.47 ± 0.11) and in CCs (1.66 ± 0.14) from oocyte donors when compared to young women with low response (0.87 ± 0.06, $p<0.05$ and 0.99 ± 0.06, $p=0.0037$ respectively).

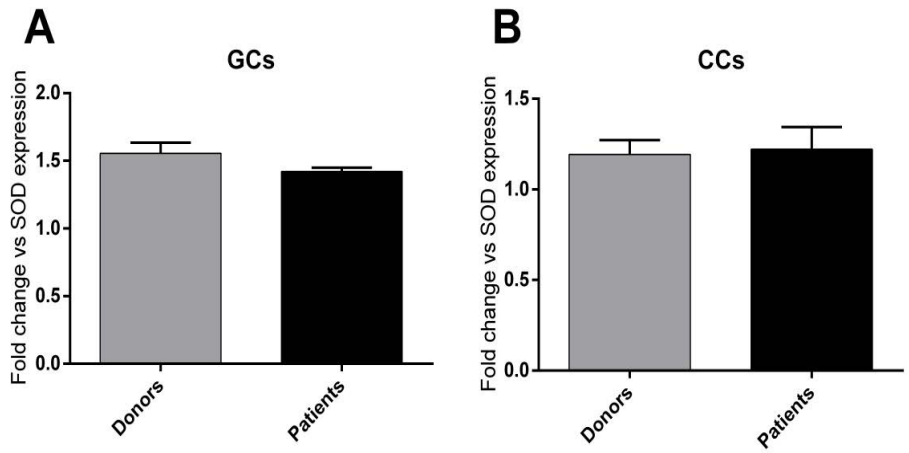

Figure 2) Bar graphs represent Superoxide Dismutase (SOD) mRNA expression levels in granulose cells (2A) and cumulus cells (2B) from control and patients.
Data are expressed as mean ± SEM. No significant differences were found between groups in the mRNA expression of SOD in GCs and CCs.

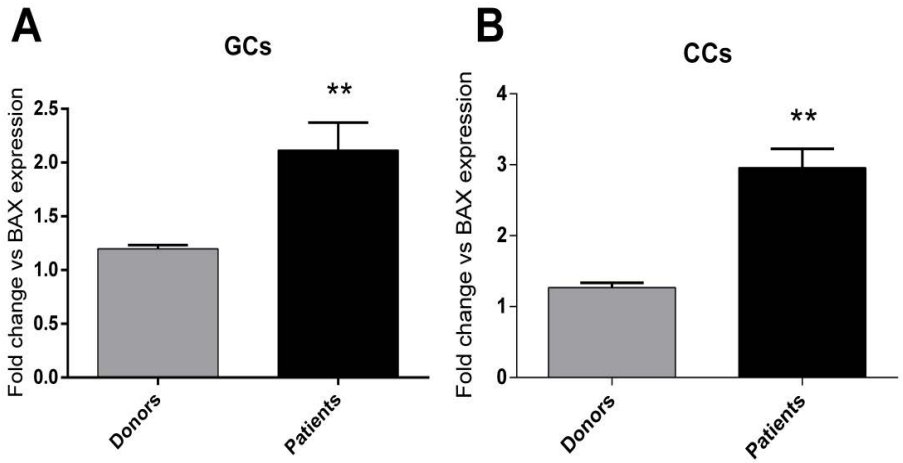

Figure 3) Bar graphs represent BAX mRNA expression levels in granulose cells (3A) and cumulus cells (3B) from control and patients.
Data are expressed as mean ± SEM.
Mean relative expressions of mRNAs coding for BAX were significantly higher in GCs (2.11 ± 0.2) and in CCs (2.95 ± 0.19) retrieved from young women with low response when compared to oocyte donors (1.19 ± 0.02, p=0.018 and 1.26 ± 0.06, p=0.08 respectively).

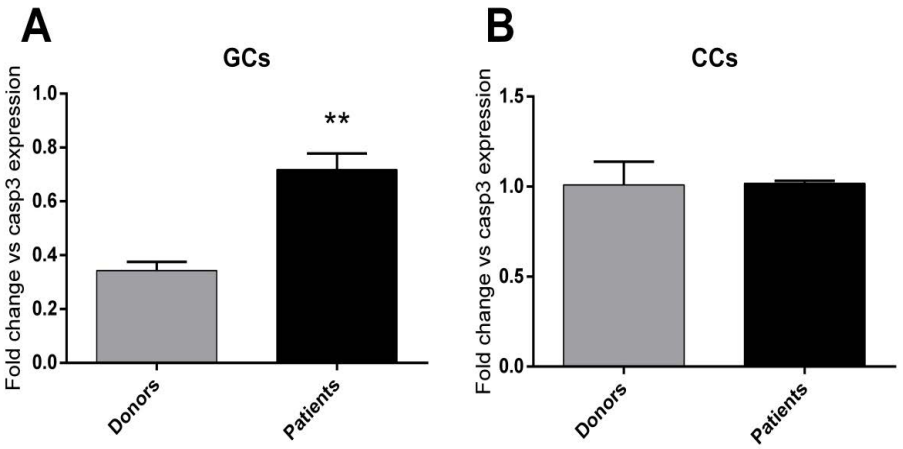

Figure 4) Bar graphs represent caspase 3 mRNA expression levels in granulose cells (4A) and cumulus cells (4B) from control and patients.
Data are expressed as mean ± SEM. Mean relative expressions of mRNAs coding for caspase 3 were significantly higher in GCs retrieved from young women with low response (0.71 ± 0.049) as compared to oocyte donors (0.34 ± 0.01, p=0.0006). No significant differences between groups were found in CCs.

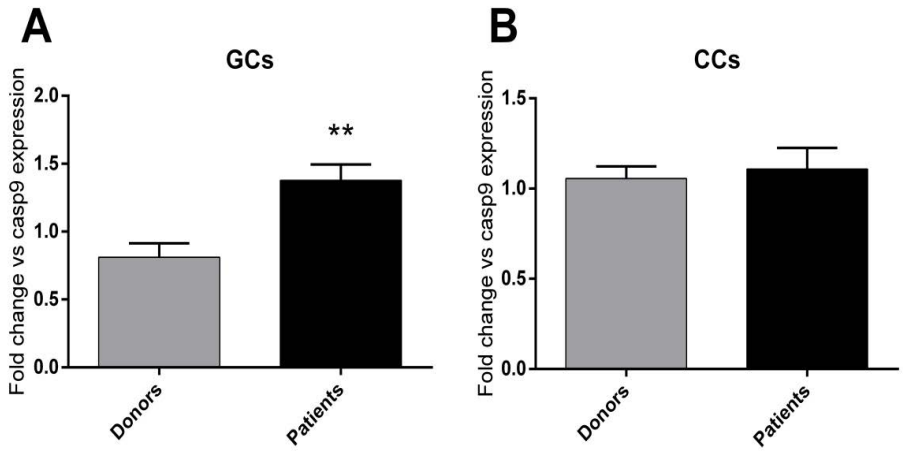

Figure 5) Bar graphs represent caspase 9 mRNA expression levels in granulose cells (5A) and cumulus cells (5B) from control and patients.
Data are expressed as mean ± SEM. Mean relative expressions of mRNAs coding for caspase 9 were significantly higher in GCs retrieved from young women with low response (1.37 ± 0.1) as compared to oocyte donors (0.81 ± 0.06, p=0.009). No significant differences between groups were found in CCs.

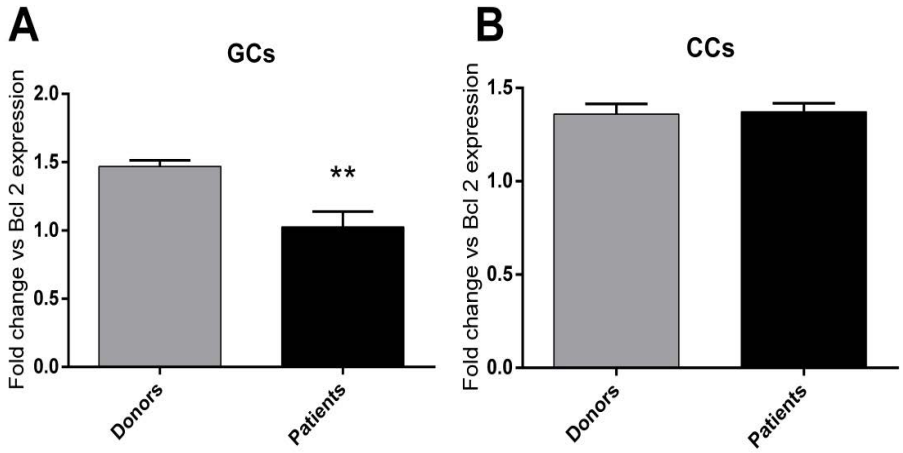

Figure 6) Bar graphs represent Bcl2 mRNA expression levels in granulose cells (6A) and cumulus cells (6B) from control and patients. Data are expressed as mean ± SEM. Mean relative expressions of mRNAs coding for Bcl2 were significantly higher in GCs retrieved from young women with low response (1.46 ± 0.09) when compared to oocyte donors (1.02 ± 0.02, p=0.006). No significant differences between patients and donors were found in CCs.

follicular fluid miRNA profiles depending on the age of the patient and the state of maturation of the oocyte. Low ovarian response of women undergoing IVF it has also been associated with altered microRNA profile expression in cumulus cells [24].

The intimate contact between the oocyte and the nearby GC is both scientifically [25] and clinically [26] significant. Our results suggest that GC from low responder patients suffer higher oxidative stress damage and apoptosis, and are taken as indication of lower defense against Reactive Oxygen Species (ROS).

The interaction between the cumulus and granulose cells and the oocyte is performed in both directions, and is essential for the regulation of oogenesis, ovulation and fertilization [27-29].

Although the importance of oxidative stress for oocyte quality is well known, the relationship between low ovarian response and oxidative stress is still a matter of debate. A study focusing on 8-hydroxy-2′-deoxyguanine, one of the major products of DNA oxidation observed that increased levels of this molecule in CC and GC were related with poorer oocyte fertilization rates and worse embryo quality [30]. As we have remarked previously, our group has demonstrated the relationship between low response to ovarian stimulation and oxidative stress [2]. This fact could be due to alterations in the metabolism in CC or GC that might be caused by changes in the ovarian environment of patients with low ovarian reserve.

The cells surrounding the oocyte produce antioxidant substances that protect it from oxidative stress. One of these substances, present in CC is Superoxide Dismutase (SOD). Superoxide Dismutase (SOD) is an enzymatic antioxidant that converts the pro-oxidant superoxide into hydrogen peroxide. The removal of hydrogen peroxide is catalyzed by catalase that reduces hydrogen peroxide to water. Therefore, the SOD and Catalase serve, together, as an important antioxidant protection. SOD levels in CC decrease in aging women while a higher SOD activity was associated with an increase in pregnancy rates in assisted reproduction techniques [31]. This suggests that oxidative stress is highly influencing oocyte viability highlighting SOD as a potential biomarker.

Diversely, apoptosis is involved in regulation and selection of human ova from the primordial follicle pool. Apoptosis can be initiated in at least four different cell compartments in follicular development, including GC and CC. Apoptosis in GC and CC is associated with follicular atresia. Thus it has been suggested that the incidence of apoptosis in these cells may be useful to assess the fertility potential of oocytes recruited after controlled ovarian stimulation for *in vitro* fertilization [32, 33].

Apoptosis of GC is highly involved in the process of ovarian folliculogenesis [34] that is critical for normal ovarian physiology and plays a fundamental role in the cyclic function of the ovary. In ovarian follicles, CC are closely related to the oocyte, forming the cumulus–oocyte–complex (COC), and play a critical role in oocyte development and subsequent fecundation through bi-directional signals [26].

Moreover, analyzing mRNA and protein expressions in CC and GC may provide information of the oocyte microenvironment during the final stages of its maturation [12]. Assou et al. [35] associated some CC expression patterns with embryo quality and pregnancy outcome. These included the up- regulation of BCL2L, a pro-apoptotic molecule. Hence, they suggested that it could be used as biomarker to predict pregnancy [35]. The BCL-2 family proteins (BCL-2, BCL-XL BAD, and BAX) constitute critical components to regulate activation of intrinsic apoptotic pathways by governing mitochondrial membrane permeabilization and subsequent release of cytochrome. Caspase-3 is responsible for the activation of caspase-activated DNase (CADs) for DNA fragmentation and BAX is a pro-apoptotic gene member of BCL-2 family genes. Both of these genes are involved in early stages of apoptosis. Considering the latter, the measurement of BAX and BCL2 mRNA in ovarian cells would reveal changes in apoptosis [36].

CONCLUSIONS

In conclusion, our findings suggest a role for apoptosis and oxidative stress in human granulose cells and the pathogenesis of POR in women undergoing infertility treatment with COH-IVF. In this study we show a significant difference in mRNA expression of apoptotic and oxidative stress markers in cumulus and granulose cells in women with low response to ovarian stimulation compared to women with good response. More specifically, we found a correlation between the expression of genes regulating OS and apoptosis and response to ovarian stimulation, suggesting that oxidative stress could influence oocyte production. However, the relationship of some antioxidants and apoptotic markers in CC with the response to ovarian stimulation needs to be evidenced with studies on a larger scale. This knowledge could help us in the future development of treatments for these patients as, for example, specific antioxidant therapies.

AUTHORS' CONTRIBUTIONS

RNC and LR contributed to the conception and design of the study, data analysis, interpretation of the data and drafting of the manuscript. SC was responsible for the collection of follicular fluid samples and aided to draft the manuscript. CA helped to draft the manuscript and English revision. EV contributed to draft the article and revising it critically. PC was responsible for patient recruitment, and JFT was responsible for the final approval of the version to be published.

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REFERENCES

1. Alviggi C, Andersen CY, Buehler K, et al. A new more detailed stratification of low responders to ovarian stimulation: From a poor ovarian response to a low prognosis concept. Fertil Steril. 2016;105(6):1452-3.
2. Nuñez-Calonge R, Cortés S, Gutierrez Gonzalez LM, et al. Oxidative stress in follicular fluid of young women with low response compared with fertile oocyte donors. Reprod Biomed Online. 2016;32(4):446-56.
3. Tajima K, Orisaka M, Hosokawa K, et al. Effects of Ovarian Theca Cells on Apoptosis and Proliferation of Granulosa Cells: Changes during Bovine Follicular Maturation. Biol Reprod. 2002;66(6):1635–39.
4. Suh CS, Jee BC, Choi YM, et al. Prognostic Implication of Apoptosis in Human Luteinized Granulose Cells During IVF–ET. J Assist Reprod Genet. 2002; 19(5):209-14
5. Acosta E, Peña O, Naftolin F, et al. Angiotensin II induces apoptosis in human mural granulosa-lutein cells, but not in cumulus cells. Fertil Steril. 2009; 91(5 Suppl):1984-89.
6. Reed JC. Double identity for proteins of the Bcl-2 family. Nature. 2009; 387:773–76.
7. Matsuda F, Inoue N, Manabe N, et al. Follicular growth and atresia in mammalian ovaries: Regulation by survival and death of granulosa cells. J Reprod Dev. 2012; 58(1):44-50.
8. Liu J, Li Y. Effect of oxidative stress and apoptosis in granulosa cells on the outcome of IVF-ET. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2010;35(9):990-4
9. Glamoclija V, Vilović K, Saraga-Babić M, et al. Apoptosis and active caspase-3 expression in human granulosa cells. Fertil Steril. 2005;83(2):426-31.
10. Jancar N, Kopitar AN, Ihan A, et al. Effect of apoptosis and reactive oxygen species production in human granulosa cells on oocyte fertilization and blastocyst development. J Assist Reprod Genet. 2007;24(2-3):91-7.
11. Dumesic DA, Meldrum DR, Katz-Jaffe MG, et al. Oocyte environment: Follicular fluid and cumulus cells are critical for oocyte health. Fertil Steril. 2015; 103(2):303-16.
12. Huang Z, Wells D. The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. Mol Hum Reprod. 2010;16(10):715-25.
13. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004. Revised 2003 consensus on diagnostic criteria and long term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004;19(1):41–47.
14. Ferrero H, Delgado-Rosas F, Garcia-Pascual CM, et al. Efficiency and purity provided by the existing methods for the isolation of luteinized granulosa cells: a comparative study. Hum Reprod. 2012; 27(6):1781-9.
15. Voge JL, Santiago CA, Aad PY, et al. Quantification of insulin-like growth factor binding protein mRNA using real-time PCR in bovine granulosa and theca cells: effect of estradiol, insulin, and gonadotropins. Domest Anim Endocrinol. 2004;26:241-58.
16. Livak KJ, Schmittgen TD, Kenneth J. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-408.
17. Tanghe S, Soom AV, Nauwynck H, et al. Functions of the cumulus oophorus during oocyte maturation, ovulation and fertilization. Mol Reprod Dev. 2002;61:414–424.
18. Wells D, Bermudez MG, Steuerwald N, et al. Expression of genes regulating chromosome segregation, the cell cycle and the apoptosis during human preimplantation development. Hum Reprod. 2005;20(5):1339–1348.
19. Cheng J, Huang J, Yuan S, et al. Circular RNA expression profiling of human granulosa cells during maternal aging reveals novel transcripts associated with assisted reproductive technology outcomes. PLoS One. 2017;12(6): e0177888.
20. Gasca S, Pellestor F, Assou S, et al. Identifying new human oocyte marker genes: a microarray approach. Reprod Biomed Online. 2007;14(2):175-83.
21. Gasca S, Reyftmann L, Pellestor F, et al. Total fertilization failure and molecular abnormalities in metaphase II oocytes. Reprod Biomed Online. 2008; 17(6):772–781.
22. Moreno JM, Núñez MJ, Quiñonero A, et al. Follicular fluid and mural granulosa cells microRNA profiles vary in *in vitro* fertilization patients depending on their age and oocyte maturation stage. Fertil Steril. 2015;104(4):1037-1046.
23. Diez-Fraile A, Lammens T, Tilleman K, et al. Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of *in vitro* fertilization. Hum Fertil (Camb). 2014;17(2):90-98.
24. Karakaya C, Guzeloglu-Kayisli O, Uyar A, et al. Poor ovarian response in women undergoing *in vitro* fertilization is associated with altered microRNA expression in cumulus cells. Fertil Steril. 2015;103(6):1469-76.
25. Kim YJ, Ku SY, Kim YY, et al. MicroRNAs transfected into granulosa cells may regulate oocyte meiotic competence during *in vitro* maturation of mouse follicles. Hum Reprod. 2013;28(11):3050–61.
26. Senbon S, Hirao Y, Miyano T. Interactions between the oocyte and surrounding somatic cells in follicular development: lessons from in-vitro culture. J Reprod Dev. 2003;49(4):259–269.
27. Eppig JJ, Down SM. Chemical signals that regulate mammalian oocyte maturation. Biol Reprod. 1984;30(1):1–11.
28. McNatty KP, Moore LG, Hudson NL, et al. The oocyte and its role in regulating ovulation rate: A new paradigm in reproductive biology. Reproduction. 2004;128(4):379–386.
29. Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and technologies to improve developmental potential *in vitro*. Theriogenology. 2007;67(1):6–15.
30. Seino T, Saito H, Kaneko T, et al. 8-hyroxy-2-deoxyguanosine in granulosa cells is correlated with quality of oocytes and embryos in an *in vitro* fertilization embryo transfer programme. Fertil Steril. 2002;77: 1184–1190.
31. Matos L, Stevenson D, Gomes F, et al. Superoxide Dismutase expression in human cumulus oophorus cells. Mol Hum Reprod. 2009;15(7):411–419.
32. Corn CM, Hauser-Kronberger C, Moser M, et al. Predictive value of cumulus cells apoptosis with regard to blastocyst development of corresponding gametes. Fertil Steril. 2005;84(3):627–633.
33. Host E, Gabrielsen A, Lindenberg S, et al. Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. Fertil Steril. 2002;77(3):511–515.
34. Jiang JY, Cheung CK, Wang Y, et al. Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. Front Biosci. 2003;8: d222–237.
35. Assou S, Haouzi D, Mahmoud K, et al. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. Mol Hum Reprod. 2008;14(12):711–719.
36. Antonsson B. Bax and other pro-apoptotic Bcl-2 family of 'killer proteins' and their victim, the mitochondrion. Cell Tissue Res. 2001;306(3): 347–361.