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Effects o follicular fluid oxidative status on human mural granulosa cells, oocyte competency and ICSI parameters

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ABSTRACT

Purpose: The aim of the present study was to understand the molecular and genetic alterations involved in follicular fluid oxidative process by investigating human mural granulosa cells and to find possible biomarkers for oocyte competency and ICSI outcome measures.
Methods: A total of 166 patients were included in the study. Total antioxidant and oxidant levels of follicular fluids were measured on the day of oocyte pick-up and oxidative status were calculated. Expression profiles of three potential target proteins in cases of oxidative stress (Hsp70, Tgf-β, Notch1), DNA status and chromatin integrity of mural granulosa cells were analyzed.
Results: TAS levels were positively correlated with the Hsp70 and Tgf-β expression patterns of mural granulosa cells. Mature oocyte rate and fertilization rates were affected negatively by the presence of oxidative stress and a significant positive correlation was found with the oxidative status and the fertilization rate, whereas no correlation with the remaining ICSI parameters in the overall group. Conclusions: Oxidative stress detected in follicular fluid adversely affects fertilization rates post-ICSI however no effect on the remaining parameters including embryo quality, pregnancy, and implantation rates. DNA damage, chromatin integrity were increased, whereas Hsp70 and Tgf-ß were decreased in mural granulosa cells in cases of oxidative stress which may indirectly reflect the oocyte competency and may be used as biomarkers for ICSI outcome measures.

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Introduction

Infertility burden millions of people all around the world, yet current treatment strategies are capable of remedying only 40 % of them [1,2]. Intracytoplasmic sperm injection (ICSI) is one of the most commonly used assisted reproduction techniques for the treatment of infertility in couples with conception failure.

Oxidative stress (OS) which is defined as a consequence of an imbalance between the amount of reactive oxygen species (ROS) produced, and the capability of antioxidant mechanisms to eliminate these oxidizing species, has been suggested as one of the causative factors of female infertility [3–6] according to the recent data. Important physiological roles of oxidative status have been demonstrated to affect a wide variety of female reproductive functions including oocyte maturation and quality, formation of corpus luteum, ovarian steroidogenesis, fertilization process, embryo development, and pregnancy [3–5]. ROS are known to be side-products of normal metabolic activity and are proved to have various beneficial effects on the organism at physiological concentrations [7]. However, the imbalance between ROS production and its elimination is reported to cause damage to the components of cellular structures such as lipids, nucleic acids, carbohydrates, and proteins [8,9].

Studies regarding the impact of oxidative stress on the female reproductive capacity mostly focus on the microenvironment of the developing oocyte, ovarian follicles and in particular, levels of ROS and antioxidants within the follicular fluid with conflicting results [3,10–16]. Although there is also some evidence for the role of reactive oxygen species (ROS) in the pathophysiology of infertility and assisted fertility [17,18] with special role on oocyte quality and impaired oocyte fertilization [12,18–20] the existing data are conflicting and the effect of oxidative stress on the outcome of IVF is not clear [15,21]

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Ovarian follicles are sites of developing oocytes in which complicated network of systemic and local signaling pathways crosstalks with each other. Within these follicles, the oocyte becomes mature which is essential for its normal fertilization process and the competence of the embryo [22]. Follicular fluid is a mixture that contains proteins, metabolites, polysaccharides, steroids, reactive oxygen species, and antioxidant enzymes [23,24] which act as signaling molecules and contribute to proper oocyte maturation. Molecular interactions that regulate the follicle and oocyte development until ovulation, require a suitable environment provided by the local and systemic interactions between follicular cells and fluid. Mural granulosa cells are important components of ovarian follicles that surround developing oocytes and which are the sources of cumulus oophorous and corona radiata cells. These cells act as a provider of different molecules such as sugars, amino acids, signaling molecules and nucleotides for the oocyte [25]. Furthermore, these cells supply glutathione which is an important antioxidant for the oocyte [26,27], cysteine for the synthesis of glutathione [28] hence defend the oocyte against oxidative stress [29,30] and regulate maturation of oocytes by producing regulatory signals, induce meiosis for progression and alter the cytoskeleton of the oocyte as well [31].

As the oocytes appears as the most common factor (70–80 %) that is responsible for implantation failure [32] and quality of the oocyte reflects the intrinsic developmental potential of an oocyte, it is of great importance to understand the nature of the molecular and cellular processes that may affect the quality and competence of the oocyte. Assessment of oocyte quality depends only on morphologic examination and not on the molecular competency during an assisted reproductive technique (ART) program. Several indirect methods that evaluate the competency of oocytes have been the subject of several studies mostly focusing on cumulus cells as the reflectors of the oocytes [33,34]. In the light of the data about the potential role of granulosa cells as regulators of oocyte competence and as biomarkers for oocyte/embryo quality or pregnancy outcome [35–41], alterations in the metabolism and protein composition of follicular cells is suggested to cause problems in oocyte maturation and competence [42].

Folliculogenesis is a complex event which include the crosstalk of many cells in the follicular environment and activation of many pathways in those cells in a strictly coordinated manner. This event control the oocyte and thus developing embryos' fate which is expected to change under different follicular circumstances. Among those proteins, three of which are important regulators of cells fate (Hsp70, Tgf-β, Notch1) and possibly target of oxidative damage were analyzed in the recent study.

Heat shock proteins (Hsps) are a protein family that may be potential candidate to affect oocyte competence functioning as chaperones in the cell [43]. Their expression increase as a result of various stress inducers including oxidative stress [44]. Hsp70 is the most abundant, highly conserved, and sensitive protein of the superfamily of Hsps [45,46] which interacts with many intracellular pathways that control cell proliferation, differentiation, and cellular death [47]. Hsps were shown to be expressed in follicular cells [45,48–55], and shown to have a role in ovarian physiology and follicular development [56,57].

Transforming growth factor beta (Tgf-β) is a multifunctional cytokine belonging to the transforming growth factor superfamily that function as a growth factor in follicular development [58]. Tgf-β was identified at mRNA and protein level in preantral follicles of various species including human, rodents, cattle, and sheep [59–63] and local Tgf-β members were shown to regulate preantral follicle growth positively [64].

Notch signaling is another pathway regulating proliferation, differentiation and apoptosis, and controlling cell-fate decisions between adjacent cells [57,65]. It has been reported that the members of Notch signaling are expressed in mammalian ovaries, but exact functions of this pathway in follicular development are not clearly identified. One of the suggested functions is the regulation of granulosa cell proliferation during follicular development. Notch genes are reported to be actively expressed by cumulus cells during folliculogenesis [66].

All three proteins analyzed in the recent study determine the faith of the cell by activating signaling pathways for survival or apoptosis. Because it is reported that oocyte maturation, fertilization, quality of the resultant embryos, number of oocytes obtained, fertilization rate, and pregnancy outcomes following in vitro fertilization are associated with the apoptosis of cumulus cells [67–69] understanding the behavior of these proteins in different circumstances will provide clues for understanding the molecular basis of oxidative stress in follicular fluid and for predicting oocyte and embryonic competence.

The aim of the present study was to understand the molecular and genetic mechanisms involved in follicular fluid oxidative process by investigating human mural granulosa cells and to find possible biomarkers of oocyte competency and ICSI outcome measures. Data obtained, may provide us clues about the effects of oxidative status on oocyte and embryo development causing a successful pregnancy.

Materials and methods

Study participants and study design

This prospective clinical study was conducted at Medicana Çamlıca Hospital, IVF center between January 2017 to June 2018 on 166 cycles that were undertaken ICSI treatment because of unexplained infertility. Patient exclusion criteria were as follows: 1) For both gender: having any anatomic and physiologic abnormality of the reproductive system as revealed by their physical and clinical examinations, having chromosomal abnormality and a smoking history. 2) For women: poor responders according to ESHRE consensus, absence of polycystic ovary syndrome as defined by the Rotterdam criteria [[70]] and endometriosis, age <25 and >37 years, body mass index (BMI) <19 and >26 kg/m^2. 3) For men: any sperm abnormality as defined by the WHO 2010 criteria [71], varicocele history. ICSI cycles with preimplantation genetic diagnosis, assisted hatching, total fertilization failure, no embryo transfer regardless of the reason, and coasting were also excluded from the study. Patients were informed about the study and written informed consent form were taken from the couples. The study was approved by the Ethical Review Board of the Istanbul Medipol University ethics committee on 27 September 2017.

Follicular fluids of 166 patients were collected and total oxidant and total antioxidant levels were measured on the day of oocyte pick-up (OPU). To analyze the effects of oxidative stress on mural granulosa cells, three potential target proteins' (Hsp70, Tgf-β, Notch1) expression levels, their intracellular localization, DNA fragmentation levels, and chromatin integrities were determined. Oocyte quality, number of oocytes collected, normal fertilization (2 P N) rate, embryo development rate, embryo qualities, pregnancy rate, implantation rate, clinical pregnancy, and spontaneous miscarriage rates were assessed for each patient in order to define the effects of oxidative stress on ICSI outcome measures.

Granulosa cells collection

Follicular aspirates of patients were collected separately from each follicle by a single lumen follicle puncture needle (Swemed, Sweden) on the day of oocyte pick-up without performing flushing. Follicular samples with blood contamination were discarded.

Cumulus-oocyte complexes (COC) within the aspirates were collected under stereo-microscope and placed into a previously equilibrated culture dish. All of the follicular fluids were collected individually, numbered and the fluids of mature oocytes were pooled in a separate sterile container after oocyte denudation because only the mature oocytes which are checked for the presence of 1 st polar body (metaphase II), were used for microinjection. Pooled aspirates were centrifuged at 600*g* for 10 min and supernatants and sedimented pellets were seperated. Aspirates were used for oxidative status analysis and the sedimented pellet were used as the source of granulosa cells. Granulosa cells were divided into two portions, first portion for preparing the smears for immunocytochemistry, immunofluorescence, toluidine blue, and TUNEL analysis, and the remaining portion was stored at −20 °C for protein extraction.

Ovarian stimulation and ICSI procedures

All women were undertaken ovulation induction using a short or long gonadotrophin-releasing hormone (GnRH) analog suppression protocol or a GnRH antagonist protocol and human menopausal gonadotrophins or recombinant follicle-stimulating hormone (FSH). Oocyte-cumulus complexes (OCC) were recovered 36 h after the administration of 5000 IU of human chorionic gonadotrophin (Ovitrelle, Merck Serono, Italy).

Cumulus and corona cells surrounding the oocytes were removed enzymatically (Hyase-10X^{TM}, Vitrolife, Sweden) by using denudation pipettes. Nuclear maturation of the oocytes was assessed under inverted microscope with X40 magnification. Oocytes with a polar body (PB) in their first microscopic examination after denudation and oocytes that extrude their polar body within 4 h after denudation were defined as a mature oocyte in Metaphase II stage. The oocytes were classified into two categories as good and poor morphology according to their cytoplasmic status, PB morphology, zona morphology, shape, and size. Oocytes with a diameter of 120−130 μm, an intact shape, a homogeneous cytoplasm, no vacuolization, granulation and refractile body formation, non-fragmented polar body, and a zona pellucida below 10 μm were classified as 'good morphology', while the remaining were classified as 'poor-morphology' (Fig. 1). Oocyte quality was denoted as the percentage (%) of good morphology oocytes per total number of mature oocytes.

Mature oocytes were microinjected with a motile sperm as described previously by Van Steirteghem et al., 1993 [72]. Further culture of injected oocytes was performed in 25 μL microdrops of culture medium (IVF, Vitrolife, Sweden) under light paraffin oil (OVOIL, Vitrolife, Sweden). Fertilization was confirmed after 16–18 h by the observation of two distinct pronuclei (2 P N) and two polar bodies. Fertilization rate was calculated as the number of fertilized oocyte divided by the total number of mature oocytes for each couple. Oocytes with 2 P N were observed for embryonic development on day 2 and 3 following microinjection. Embryo development rates were calculated as the number of embryos developed divided by the total number of mature oocytes, for each couple. Quality of the embryos at cleavage stage was assessed under an inverted microscope with X40 magnification according to the criteria of Staessen et al., 1992 [73]. Grade A and B embryos were grouped as good quality embryos and grade C and D embryos were grouped as poor quality embryos. All ICSI procedures including microinjection, embryo, and oocyte grading was performed by the same embryologist. Good quality embryo rate was calculated as the number of good quality embryos divided by the total number of embryos, for each couple. Embryos with 4-cell in day 2 were grouped as normally cleaved embryos and embryos with less or more than 4-cell in day 2 were grouped as abnormally cleaved embryos. Normally/abnormally cleaved embryo rates were calculated as the number of normally/abnormally cleaved embryos developed divided by the total number of embryos, for each couple.

Embryo transfers were performed by the same clinician on day 3 or 5 according to the number and quality of developing embryos. Wallace catheter (1816 N, Smiths Medical, USA) were used for embryo transfer. β-hCG level in the blood was measured twelve days after embryo transfer and a ≥50 mIU/mL ß-hCG value on day 12, indicated a positive pregnancy. Biochemical pregnancy (positive hCG without a gestational sac), implantation rate (number of gestational sacs/number of embryos transferred), clinical pregnancy rate (intrauterine pregnancy with fetal heart activity), and first-trimester spontaneous miscarriage (pregnancy failure after visualization of intrauterine gestation sac), were also assessed.

Follicular fluid oxidative status determination

Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) levels in follicular fluids were determined by using Rel assay kits (Rel Assay Diagnostics, Turkey). For TAS levels, Total Antioxidant Status Assay Kit was used and the absorbances were measured using a spectrophotometer (Molecular Devices SpectraMax i3 Multi- Mode Microplate reader).

TOS levels were measured by Total Oxidant Status Assay Kit, whose method is as follows: oxidants present in the sample oxidize ferrous-o-dianisidine complex into the ferric ions [74]. The absorbances were measured at 530 nm. The results are expressed in micromoles hydrogen peroxide equivalent per liter (H2O2eq μmol/μL).

TAS levels were measured by Total Antioxidant Status Assay Kit whose method is as follows: the hydroxyl radical as the product of Fenton reaction reacts with colorless o-dianisidine to form a

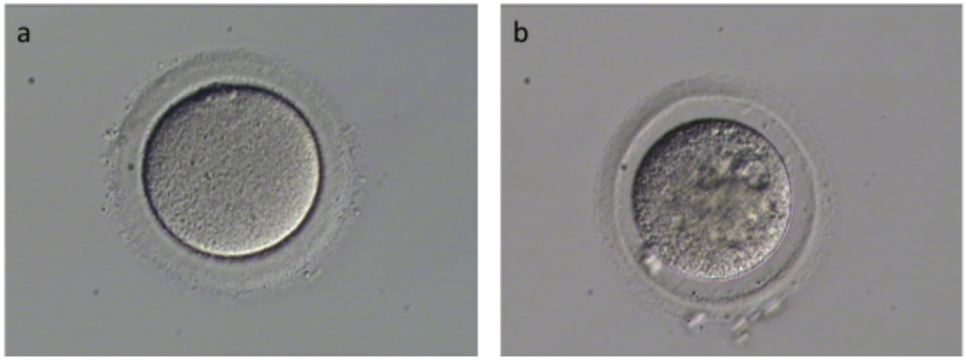

Fig. 1. **(a)** A good quality oocyte with a homogeneous, bright ooplasm and an intact polar body **(b)** A poor quality oocyte with many vacuoles, granulation and a large perivitelline space (X40).

radical bright yellowish brown dianisyl substrate. The absorbances were measured at 660 nm. The measurement results were expressed in Trolox equivalent of millimoles per liter (TroloxEq μmol/μL) [75].

The intraassay %CV values for the TAS measurement were 2.36 % for the 0.50 (0.35–0.65) mmol Trolox equiv / L and 2.24 % for the 2.0 (1.7–2.3) mmol Trolox equiv / L. The intraassay CV% values for TOS measurements were 3.57 % for 5.5 (3.0–8.0) μmol/L and 5.17 % for 19.5 (16–23) μmol/L. Oxidative Stress Index (OSI) was calculated with the formula: Total Oxidant Status (TOS) / Total Antioxidant Level (TAS).

Protein analysis by Immunocytochemistry and immunofluorescence

Immunocytochemistry analysis was performed to determine the expression and immunolocalization of Hsp70, Tgf-β and Notch1 proteins (Fig. 2). Immunofluorescence analysis was performed to determine the expression levels by measuring relative staining intensity, and western blot was performed in order to confirm the results (Fig. 3).

For immunocytochemistry, granulosa cells were fixed with freshly prepared 4% paraformaldehyde (PFA) at room temperature for 20 min. Cells were washed with phosphate buffered saline (PBS) during the whole procedure. To inhibit endogenous hydrogen peroxidase activity, slides were incubated with 3% hydrogen peroxide solution for 20 min. Cells were incubated with boiling citrate buffer for 5 min for antigen retrieval. Protein blocking solution was applied for 10 min at room temperature in a humidified chamber. The cells were then incubated with antibodies (anti-Hsp70 antibody (1:200, sc-24, Santa Cruz, CA, USA), anti-Tgf-β antibody (1:200, sc-146, Santa Cruz), anti-Notch1 antibody (1:200, sc-6014-R, Santa Cruz)), overnight at +4 °C. After washing with PBS, biotinylated goat anti-polyvalent solution was performed for 10 min at room temperature, and the samples were incubated with streptavidin-peroxidase for another 10 min. DAB (3, 3-diaminobenzidine) was applied for counterstaining for 10 min, and hematoxylin was applied for 5 min. Slides were then rinsed with tap water and dehydrated by using series of alcohol with increasing concentration and then mounted. Images were obtained using Nikon Eclipse Ni (Nikon, Japan) microscope with 100X oil-immersion objective.

For immunofluorescence, granulosa cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Granulosa cells were washed with PBS during the whole procedure. Slides were permeabilized with 0.1 % Triton X-100 for 10 min at room temperature and were blocked with 10 % normal goat serum and

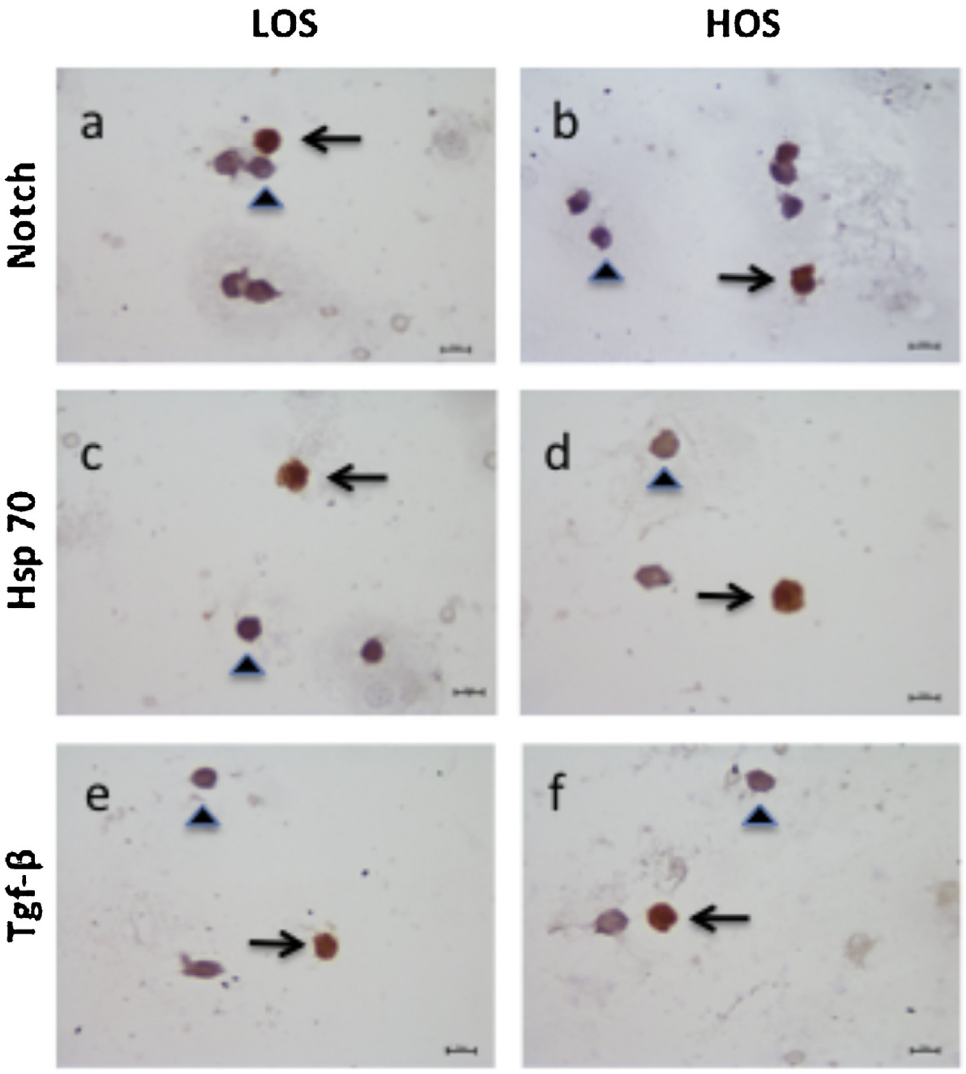

Fig. 2. Immunocytochemistry staining results for Notch1, Hsp70, and Tgf-β protein expressions in human mural granulosa cells of LOS and HOS groups (40X). Arrows indicate Notch1 (+) cells **(a)** in LOS group and **(b)** in HOS group. Arrows indicate Hsp70 (+) cells **(c)** in LOS group and **(d)** in HOS group. Arrows indicate Tgf-β (+) cells **(e)** in LOS group and **(f)** in HOS group. Arrowheads indicate (-) staining.

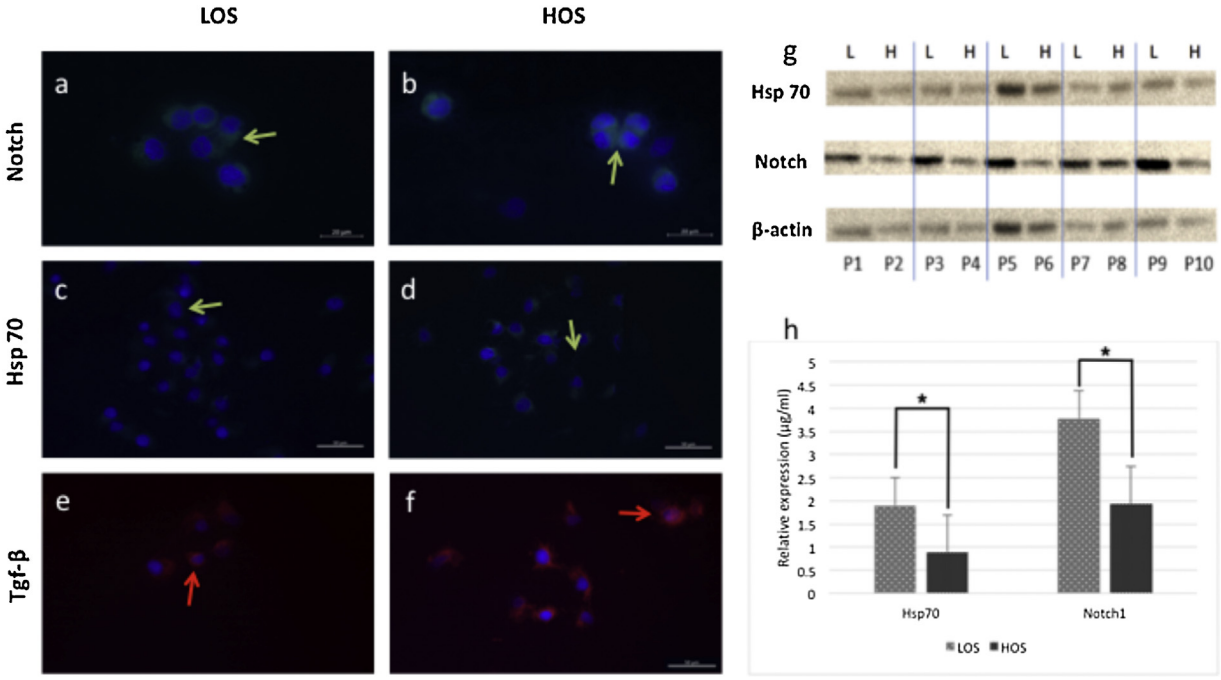

Fig. 3. Immunofluorescence staining results for Notch1, Hsp70, and Tgf-β protein expressions in human mural granulosa cells in LOS and HOS group (40X). Arrows indicate Notch1 (+) cells **(a)** in LOS group and, **(b)** in HOS group. Hsp70 (+) cells **(c)** in LOS group and **(d)** in HOS group. Tgf-β (+) cells **(e)** in LOS group and **(f)** in HOS group. **(g)** Representative protein bands for Hsp70, Notch1 and ß-actin (42 kDa) expression levels of 5 patients of LOS (L) and 5 patients of HOS (H). **(h)** Relative expression levels (mean densitometry) of Hsp70, and Notch1 protein obtained from western blotting analyses. Bars represent the means and error bars represent standard deviation of the quantified proteins. $^{*}p < 0.05$ (statistically significant).

0.3 % Triton X-PBS for 30 min. The cells were then incubated with anti-Hsp70 antibody (1:200, sc-24, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Tgf-β antibody (1:200, sc-146, Santa Cruz), anti-Notch1 antibody (1:200, sc-6014-R, Santa Cruz). Bound antibodies were visualized with Alexa Fluor 568®-conjugated goat anti-mouse IgG (1:200, ab175473, Abcam, UK) for Hsp70, and Alexa Fluor 488®-conjugated goat anti-rabbit IgG (1:200, ab 150077, Abcam, UK) for anti-Notch1 and anti-Tgf-β. Fluorescence images were obtained with Zeiss Cell Observer SD Spinning Disk Time-Lapse Microscope (Carl Zeiss, Jena, Germany) with a magnification of ×400. In average, 100 cells were counted for each patient in every group. Negative controls were tested in each slide for quality control. Relative intensity was calculated as the ratio of intensity to area of each cell via tools that are provided by ZEN program (Carl Zeiss, Jena, Germany) in each slide.

Electrophoresis (SDS-PAGE) and Western blotting

Cell lysis procedure was performed subsequent to the thawing of the samples at room temperature. Samples were washed twice with cooled PBS by centrifuging at 2500 G for 10 min. A 400 µL lysis buffer solution (M-PER Mammalian Protein Extraction Reagent; Thermo Scientific, Wien, Austria) supplemented with 10 µL/mL protease-phosphatase inhibitor cocktail (Halt Protease Inhibitor Cocktail; Thermo Scientific) at 1:100 was added to the pellet on ice. The suspensions were mixed and left on ice for 20 min while shaking gently. Suspensions were then centrifuged for 15 min at 14,000 g, and the supernatant was transferred to a new tube. BCA assay (Pierce BCA Protein Assay Kit; Thermo Scientific) was used for protein assay according to the instruction manual. A mathematical curve was plotted by measuring serial BSA standards of pre-known dilutions. Protein concentrations of the unknown samples were calculated according to the standard curve obtained.

Extracted proteins (20 µg) were mixed with a 4X loading dye and run on a gel with a 4% concentration for 15 min at 90 V, and subsequently on a 10 % separating gel for 1.5 h at 100 V at room temperature. Marker (PageRulerTM Plus; Thermo Scientific) was loaded on each gel for molecular weight analysis. The gels were then transferred electrophoretically (100 V) on a PVDF membrane (Hybond-P; Amersham Biosciences, Buckinghamshire, UK) for 1 h. Membranes were blocked for 1 h in 5% (W/v) skim milk powder in Tris-buffered (20 mM) saline containing 1% Tween 20 (TBS-T) and were incubated overnight with primary antibodies: Hsp70/Hsc70 (sc24, Santa Cruz Biotechnologies, USA), Tgf-β1 (sc-146, Santa Cruz Biotechnologies, USA), Notch1(C-20)-R (sc-6014-R, Santa Cruz Biotechnologies, USA), ß-Actin (4967, Cell signaling, Netherlands). Membranes were washed three times and incubated for 1 h with the suitable secondary antibody (goat anti-mouse, ab175473, goat anti-rabbit, ab150077, Abcam, England), then exposed to HRP conjugated antibody (goat anti-mouse IgGHRP; Santa Cruz Biotechnologies, USA and anti-rabbit IgG HRP-linked antibody, Cell Signalling Technologies, Netherlands). After incubation, membranes were washed with TBS-T and the immunoreactive band intensities were analyzed with ChemiDoc Molecular Imager (Bio-Rad, Philadelphia, PA, USA). The integrated density values were calculated by comparing the signals of target proteins to that of the housekeeping Actin.

DNA fragmentation and chromatin integrity assessment

TUNEL test was used to evaluate DNA fragmentation levels and Toluidine blue (TB) staining was performed in order to determine the chromatin integrity of cumulus cells (Fig. 4). TB staining was evaluated under light microscope as positive (dark-stained) or negative (pale-stained) by comparing dye intake.

For TUNEL procedure, **s**lides were fixed in freshly prepared 4 % PFA at room temperature for 20 min. For permeabilization, 0.1 % Triton-X in PBS was used on ice for 2 min. DNA fragmentation was determined by TUNEL assay using a commercially available kit (In Situ Cell Death Detection Kit, fluorescein, Roche, Indianapolis, IN,

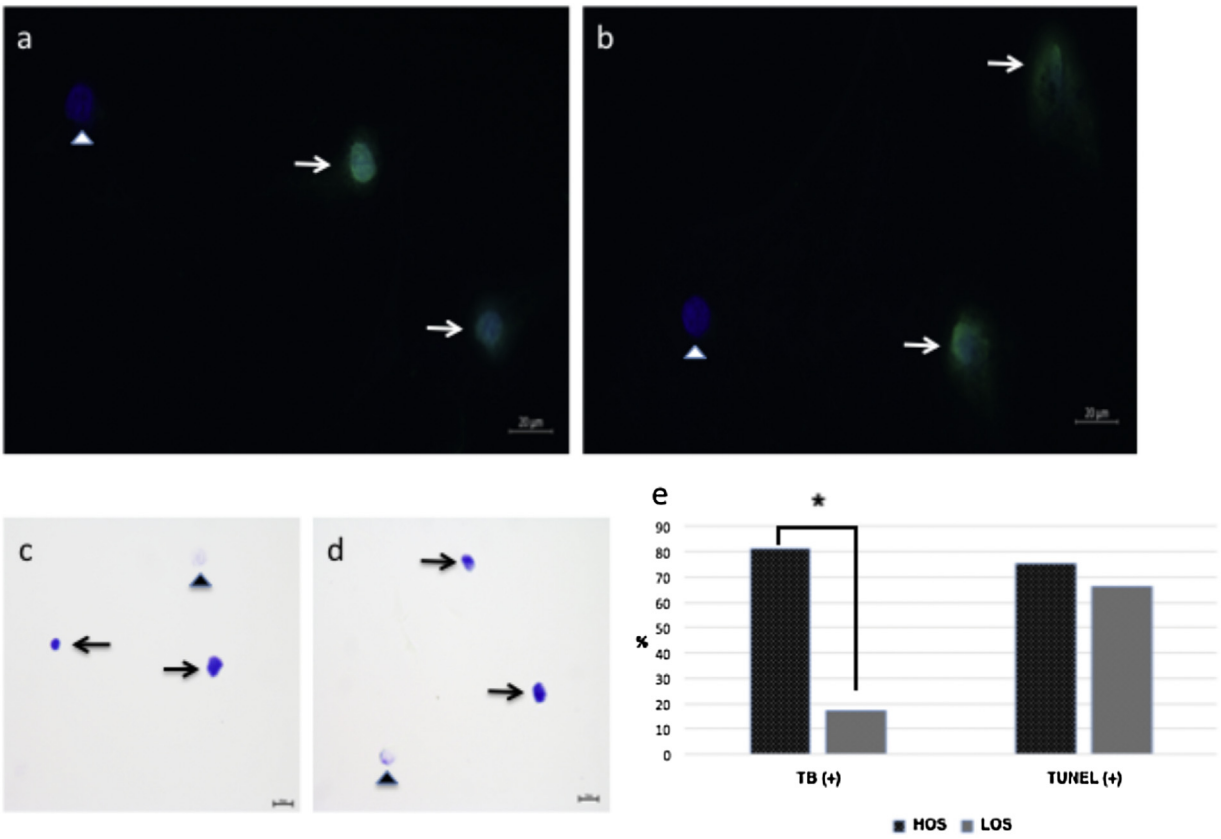

Fig. 4. Tunel (DNA fragmentation) and Toluidine Blue (chromatine integrity) staining results for Notch1, Hsp70, and Tgf-β protein expressions in human mural granulosa cells in LOS and HOS groups (40X). Tunel staining: Arrowhead represents Tunel (-) cells, arrows indicate Tunel (+) cells **(a)** in LOS and **(b)** in HOS group. Toluidine blue staining (40X). Arrows indicate Toluidine Blue (+) cells, arrowheads indicate Toluidine Blue (-) cells **(c)** in LOS and **(d)** in HOS group **e.** DNA fragmentation and chromatin integrity rates of LOS and HOS groups were shown in bars. Results were given as percentage (%). $^{*}p < 0.005$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

USA). Cells were incubated at 37 °C with TUNEL reaction solution (which is obtained from kit) for 1 h. Slides were rinsed three times with PBS. DAPI was used to label nuclei. Cells were examined under Zeiss Cell Observer SD Spinning Disk Time-Lapse Microscope (Carl Zeiss, Jena, Germany) with ×400 magnification. A minimum of 100 granulosa cells were chosen randomly in at least 10 microscopic fields with a magnification of 10X and the results were given as mean percentage (%). TUNEL-positive cells were counted to calculate the ratio of apoptotic cells in each group. Cells stained in green were those with fragmented DNA, and not-stained in green were without fragmented DNA.

For Toluidine blue staining, cells were fixed in freshly prepared 96 % ethanol-acetone (1:1) at 4 °C for 1 h and hydrolyzed in 0.1 N HCl at 4 °C for 5 min. Slides were rinsed three times in distilled water for 2 min and were stained with 0.05 % Toluidine blue for 5 min at room temperature. The slides were then rinsed thoroughly in distilled water before mounting. Images were obtained with Nikon Eclipse (Nikon, Japan) microscope by 100x oil-immersion objective.

Statistics

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS, Version 21 for Windows; SPSS, Inc., Chicago, IL, USA). Sample size was calculated for a significance level of 5% and a power higher than 80 %, in order to detect a difference between means higher than 15 %. StatMate for Windows (GraphPad Software, USA) program package was used for sample size calculations. Univariate (mean, standard deviation, and frequency) and bivariate (Student's t-test, Mann–Whitney-U and Wilcoxon tests) descriptive statistics were performed. Statistical comparisons for categorical variables were carried out using the chi-square test. The Kolmogorov–Smirnov test was used to assess compliance of the variables to normal distribution. Associations among quantitative variables were analyzed using the Pearson's correlation coefficient.

All tests were conducted using a p-value ≤ 0.05 defining statistical significance. The data were expressed as mean ± standard deviation for continuous variables and number of

Table 1
Oxidative parameters (TAC levels, TOC levels) according to LOS (<10 μmol/L) and HOS (>10 μmol/L) subgroups.

Oxidative parameters	LOS	HOS	*p* value
TAS level (mean O.D.)	309 ± 095	036 ± 026	0000 *
TOS level (mean O.D.)	1197 ± 373	1305 ± 531	054
Oxidative status (TOS /TAS ratio) (mean O.D.)	422 ± 197	6856 ± 77,	0000*

Results were given as mean O.D. ± SD.
Abbreviations: LOS, Low oxidative stress; HOS, High oxidative stress; TAS, Total Antioxidant Status; TOS, Total Oxidant Status ; O.D. Optical density. $^{*}p < 0.05$.

cases (n) and percentage of occurrence (%) for qualitative variables. Receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity values and area under curve.

Results

Mean antioxidant and oxidant levels within the follicular fluid in the overall group undergoing ICSI treatment were measured to be 1.64 and 12.32 OD (optical density) resp., and the mean level of oxidative status which indicates the balance between oxidants and antioxidants was calculated to be 41.49 OD. Patients were divided into two group according to their follicular fluid oxidative status as low oxidative stress (LOS, ≤10 OD) and high oxidative stress (HOS, >10 OD). Follicular fluid oxidative status within each group are presented in Table 1. Demographic data of the groups were similar between two groups in terms of maternal age, number of previous cycle, E2 level on the day of oocyte pick-up, controlled over stimulation period, number of oocytes collected, number of embryos transferred and the day of embryo transfer (Table 2, $p > 005$).

The oxidative level was calculated as TOS/TAS ratio. TAS levels were significantly increased in HOS patients although TOS levels were similar (p = 0.000; p = 0.545 resp.). As a result, TAS levels were found to be the determinative parameter of oxidative stress level in both groups.

Expression profiles of three possible target proteins (Hsp70, Tgf-β, Notch1) of oxidative stress were analyzed in mural granulosa cells. According to the results of immunocytochemistry, Hsp70 and Notch1 were found to be expressed statistically lower in the HOS group whereas there was no difference in Tgf-β expression rates between the groups (Table 3, Fig. 2, $p = 0.0026$, $p = 0.0047$ resp.). The results obtained were further confirmed by immunofluorescence and western blot analysis (Fig. 3).

Chromatin integrity rates which is assessed by toluidine blue painting were significantly decreased in HOS group than LOS group (17.2 %; 81 % resp, $p = 0.0023$) although the difference in DNA fragmentation rates did not reach statistical significance level (75 %; 66.15 % resp. $p = 012$). (Fig. 4).

ICSI outcome parameters including oocyte quality, fertilization, embryo development, embryo quality, clinical pregnancy, implantation and miscarriage rates were also assessed to see the effect of oxidative status on ICSI outcome (Table 2). Mature oocyte rates and fertilization rates were the only two parameter that were affected negatively from the oxidative stress which were observed to be decreased significantly in HOS group ($p = 002$, $p = 0011$, resp.). Other ICSI outcome measures were not affected significantly by the presence of oxidative stress ($p > 005$, Table 2).

We then performed correlation analysis to analyze the correlation with the expression pattern of the three possible target proteins with TAS, TOS levels and ICSI outcome measures. We found a positive correlation between the TAS levels and the expressions of Hsp70 ($p = 0.001$, r = 0.488) although there were no correlation between TOS levels and expression profiles of investigated proteins ($p > 005$).

Among all ICSI parameters, fertilization rate is the only parameter that is found to be correlated with oxidative status of follicular fluid ($p = 0.001$, r = 0.518). There were no correlation with the remaining parameters including embryo development, embryo quality, clinical pregnancy, implantation and spontaneous miscarriage rates in the overall group ($p > 005$).

Discussion

Oxidative stress is known to play a major role in pathophysiology of infertility, yet the causes, effects, and the molecular mechanisms underlying this phenomenon is not well understood. There are several studies reporting the negative effects of follicular fluid oxidative stress on fertilization capacity [12,13,20,76] while the others had found no correlation [77]. Under physiological conditions the antioxidant defense mechanism of oocytes and ovaries, consisting of enzymatic antioxidants such as superoxide dismutase (SOD), and non-enzymatic antioxidants (GSH), detoxifies excess ROS maintaining the oxidant/antioxidant balance. However, increased levels of ROS beyond the physiological range may lead to OS and cause a wide range of molecular damages, including lipid peroxidation and protein and DNA damage resulting in deterioration of oocyte quality [78–81].

Granulosa cells are the common cell population that are exposed and thus are affected from the follicular microenvironment. Hence they act both as a bridge and a barrier between the oocyte and the follicular microenvironment. As their activity directly affects paracrine signaling with the oocytes they surround, understanding their behavior under different oxidative conditions may give valuable clues about the effect of oxidative status on oocyte's developmental competence and the success of ICSI cycle using these oocytes.

In this aspect, the expression patterns of three possible target proteins (Hsp70, Tgf-β, Notch1) of oxidative stress, which are involved in important regulatory signaling pathways in the cells were investigated. All three proteins were actively expressed in granulosa cells of patients but with different rates. Hsp70 and Notch1 were found to be expressed statistically lower in the HOS group whereas there was no difference in Tgf-β expression rates between the groups.

Table 2
Demographic data and ICSI outcome measures according to LOS (<10 μmol/L) and HOS (>10 μmol/L) subgroups.

ICSI outcome measures	LOS	HOS	*p* value
Maternal age	336 ± 615	344 ± 616	036
Number of previous IVF cycle	170 ± 098	176 ± 125	088
Estradiol Level	31,928 ± 19,038	51,678 ± 13,135	029
Controlled ovarian stimulation period (day)	652 ± 172	656 ± 131	038
Mean number of oocytes retrieved	753 ± 610	706 ± 508	062
Mean number of mature oocytes	576 ± 357	3,1 ± 2,5	002*
Normal fertilization rate (%) (# of 2 P N / # of mature oocytes)	7276 ± 2102	6254 ± 1416	0011*
Embryo development rate (%)(# of 6–8 cell on day 3/ # of 2 P N)	811 ± 112	731 ± 167	021
Top quality embryo rate (%)	451 ± 343	371 ± 446	025
Pregnancy rate (positive ß-hcG) (%)	502	396	030
Clinical pregnancy rate (%)	413	322	0,4
Implantation rate (%)	245	206	032
Spontaneous miscarriage rate (%)	532	912	018

Results were given as mean O.D. ± SD.
Abbreviations: LOS, Low oxidative stress; HOS, High oxidative stress; 2 P N, Two Pronucleus; MII, Metaphase II *$p < 0.05$.

Table 3
Expression rate and level of Hsp70, Tgf-ß, Notch1 according to according to LOS (<10 μmol/L) and HOS (>10 μmol/L) subgroups. Results were given as percentage (%) unless otherwise stated. Abbreviations: IF, Immunofluorescence $^{*}p < 0.05$.

Proteins of interest	LOS	HOS	*p* value
Hsp70	759 ± 146 1	5668 ± 2895 121 ± 0520.12 ± 013	0026 *
Immunostained cell (%)	18 ± 0.13		0.64
Expression level	0,2 ± 0,1		0.24
IF (Mean fluorescence intensity)			
Western blot (Mean densitometry)			
Tgf-β	8204 ± 1309185 ± 0830.24 ± 033	7319 ± 1838 205 ± 109 0.32 ± 0,3	0067 034 042
Immunostained cell (%)			
Expression level			
IF (Mean fluorescence intensity)			
Western blot (Mean densitometry)			
Notch1	78, 88 ± 1817 412 ± 1,10.4 ± 017	7217 ± 1459425 ± 1,7 0.26 ± 023	0047*
Immunostained cell (%)			0.43
Expression level			0.3
IF (Mean fluorescence intensity)			
Western blot (Mean densitometry)			

Hsp70 is one of the important members of Heat shock proteins (Hsp) that protect the cells against damages caused by stress conditions, and to mediate the transport of damaged proteins to target organelles for their repair or degradation. We found a lower Hsp70 expression rate in HOS group. It may be suggested that the oxidative follicular environment suppress Hsp70 expression which may, in turn, decrease the tolerance of cells to oxidative stress and cause them to become more vulnerable. There is no data in the literature analyzing the Hsp70 expression rate of granulosa cells under different oxidative stress conditions.

Tgf-β superfamily members have found to be selectively expressed by oocytes from primary follicles in rodents, and from primordial follicles in cows and sheep [59–63,78,79,82–84]. Various types were shown to be expressed by granulosa cells of the early follicle stages, making these cells potential targets for paracrine signaling and local Tgf-β from theca and granulosa cells implicated as positive regulators of preantral follicle growth [85]. Binelli and Murphy indicated an inhibitory effect of Tgf-β on primary follicle survival and/or progression to the late preantral/early antral stage while others indicated lack of any effects [86,87]. Tgf-β is reported to stimulate Follicle-stimulating hormone (FSH) receptor expression, amplify FSH-induced aromatase activity, inhibin production, progesterone production and luteinizing hormone (LH) receptor induction [69,88–91] and its expression were reported to change under different circumstances including female age [92]. We found no significant difference in Tgf-β expression rates between the groups which provides evidence that follicular fluid oxidative status do not have any effect on Tgf-β expressions in granulosa cells.

The other protein evaluated was Notch1, which is a transmembrane receptor and controls cell-fate decisions between tightly adjacent cells by modulating cell proliferation, differentiation, and survival [65]. Notch genes are shown to be actively expressed by cumulus cells during folliculogenesis [66]. Notch signaling is shown to be involved in ovarian follicle development by regulating granulosa cell proliferation [68]. We found a significant decrease in Notch1 expression rates in HOS patients which may influence the fate of granulosa cells and possibly the oocyte neighboring them. There is also no data about the effects of oxidative stress on granulosa Tgf-β expression rates.

ICSI outcome measures were analyzed to analyze the effects of oxidative status on ICSI outcome. Beyond all ICSI parameters including oocyte quality, embryo development, embryo quality, clinical pregnancy, implantation, and spontaneous miscarriage rates the two parameters including the oocyte maturation rate and the fertilization rate were negatively affected from the follicular fluid oxidative environment. Mature oocyte number were also found to be significantly lower in HOS group indicating the possible negative role of oxidative stress on oogenesis.

Hsp70 and Notch1 activity was significantly lower in granulosa cells of HOS patients according to our findings which may be correlated with the oocyte maturation. One of the possible suspected protein is Hsp70 which is shown to have role in regulating estradiol biosynthesis under stress conditions [93].

Results of this study provide evidence to explain the retarded oocyte maturation rate detected in HOS group of our study. Second suspected protein for retarded oocyte maturation is Notch1, which is known to regulate folliculogenesis thus oogenesis. As maturation and competency of an oocyte is closely related to the survival signals it receives, Notch1 may have effect on the developmental capacity of oocytes by paracrine signaling from granulosa cells.

This study shows a decrease in the activities of Hsp70 and Notch1 of granulosa cells with mature oocyte number under oxidative circumstance, thus providing indirect evidence for the role of these proteins in oocyte maturation. The other parameter that is influenced by oxidative stress was the fertilization rate. We observed a significantly decreased fertilization rate in HOS group indicating the possible role of oxidative status on pronucleus formation or gametes activation. One of the reasons may be the lower Hsp70 expression rates in HOS group which may not be enough to overwhelm the damage caused by oxidative stress. Increased oxidative attack and decreased cellular protection mechanisms may result in damaged or misfolded proteins which contribute the fertilization process. We found no significant difference for the other ICSI outcome measures analyzed including oocyte quality, embryo development, embryo quality, clinical pregnancy, implantation, and spontaneous miscarriage rates. The correlation analysis results also confirmed these findings by observing a significant positive correlation with the fertilization rate which, in turn, showed that higher Hsp70 and Notch1 expressions may be used as biomarkers of mature oocytes, leading to successful fertilization. Our results do not seem in accordance with several other studies which reported negative effects of oxidative status on embryo formation, quality, and pregnancy rate after ICSI [2,13,18]. The difference obtained may be because of differences in oxidative status determination methods and different patient population. Oxidative status of the follicular fluid is not found to be predictive for pregnancy assessment in an ICSI cycle in the recent study.

The genetic stability of these cells were also analyzed which is shown to affect oocyte progression [94–96]. We found an increased DNA fragmentation rates in HOS group although the difference did not reach statistical significance. But the chromatin integrity which is assessed by toluidine blue painting were significantly

decreased in HOS group probably addressing some problems on DNA repackaging. DNA methylation plays a central role in gene expression via epigenetic which modifies the genetic material and DNA packaging. The decreased Hsp70 may be one of the responsible proteins of DNA methylation which is reported to be negatively associated with the expression of Hsp70 under stress conditions in different tissues [97]. The decrease in fertilization rates in our study may be a result of this consequence which may affect fertilization process by altering chromatin rearrangement during pronucleus formation. However, this study was not designed to investigate the association of individual follicular fluid status with the quality of the corresponding oocyte and embryo. Further investigations are needed to evaluate individual follicles, other oxidative stress markers or the clinical characteristics of patients. Strategies aimed at reducing oxidative stress, including the addition of antioxidants in the oocyte incubation medium or embryo culture medium, may be tested to see whether these effects are disappeared or not. Further studies should focus on possible other pathways and crosstalks between them to understand the molecular mechanisms underlying the situation and the possible potential of Hsp70 and Notch1 to be used as biomarkers for ICSI outcome should be further investigated in the light of the data obtained in this study.

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Declaration of Comepting Interest

The authors declare no conflict of interest

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