







INVESTIGATION OF ENDOPLASMIC RETICULUM SIGNAL PROTEINS IN CUMULUS CELLS WITH NORMAL, HYPERRESPONSIVE, HYPORESPONSIVE AND POLYCYSTIC OVARY SYNDROMES

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Abstract: *The study aimed to investigate Endoplasmic reticulum dependent Unfolded Protein Response signal molecules in cumulus cells of the patients presenting normal, hyperresponsive, hyporesponsive, and polycystic ovary syndrome. Cumulus cells were provided during oocyte retrieval. Of the patients applying for in vitro fertilization treatment, three subtypes of patient groups were distinguished according to their response to follicle stimulations. The first group composed of normoresponsive (n=8) was considered as retrieval of 10-20 oocytes. The second group composed of hyporesponsive (n=8) as retrieval of 6 or less oocytes. Retrieval of 30 or more oocytes as a response to the same stimulus was considered hyperresponsive (n=8), which included polycystic ovary syndromes. (n=8). We analyzed the protein expressions of glucose-regulated protein 78 and mRNAs levels of the X-box binding protein-1 and splicing X-box binding protein-1 by Western blot and Reverse Transcriptase PCR, respectively, in the cumulus cells from different patient groups. All data were loaded to the software Sigma Stat 3. Differences between groups were evaluated with one-way ANOVA post hoc TURKEY test. We found that the protein expression of glucose-regulated protein 78 was two-fold higher in cumulus cells from the hyporesponsive group than the other groups. Reverse Transcriptase PCR results showed us, the mRNA expression of splicing X-box / X-box binding protein-1 level was 1,5 fold higher in the cumulus cells from the hyporesponsive group compared to others. Furthermore, we have shown that when the cumulus cells were exposed to signal pathway molecules that related to endoplasmic reticulum stress; a decrease in cell proliferation and/or increase in apoptosis can occur. We have shown that an increased endoplasmic reticulum stress in the cumulus cells of the ovarian follicle from the patients forming the hyporesponsive group. We can conclude that increased endoplasmic reticulum stress or impaired protein folding mechanism in cumulus cells may affect oocyte maturation, therefore, the agents may be used to decrease pathological endoplasmic reticulum stress in the hyporesponsive patients.*

Keywords: *Endoplasmic Reticulum Stress, Unfolded Protein Response, Cumulus Cells*

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1. Introduction

Endoplasmic reticulum (ER) is an essential organelle plays important roles in cells vital functions. ER regulates intracellular calcium concentration, protein synthesis, and homeostasis and it also enables lipid biosynthesis [1,2]. Several factors may affect the aggregation of unfolding proteins in the ER

lumen. The accumulation of unfolding or misfolding proteins in the ER lumen induces an ER-dependent cellular reaction cascade which is called Unfolding Protein Response (UPR) [3,4].

There are three ER stress transducers, inositol requiring enzyme (IRE) 1, protein kinase RNA-like ER kinase (PERK), and activating transcription factor (ATF) 6 that involve in UPR signaling pathway. These three ER stress sensors sense the levels of unfolded proteins in the ER lumen [5,6]. GRP78 is also called binding protein (BIP) is a major regulatory protein of UPR. Under normal conditions, BIP binds to the luminal domains of IRE1, PERK, and ATF6 receptors and keeps them in the inactive state. In response to the ER stress induced by accumulation of unfolded proteins in the ER lumen, BIP released from the luminal domains of PERK, IRE1, and ATF6 receptors to activate them [7,8].

PERK is an ER-related transmembrane protein kinase. Accumulation of unfolded proteins in the ER lumen, PERK dimerization and trans-autophosphorylation lead to activation of its eIF2 α kinase activation [9]. PERK also induces transcription of about 1/3 of the UPR-dependent genes [10].

Under non-stress conditions, Ire1 protein kinase is maintained in an inactive state interaction with BIP. Upon aggregation of unfolded/misfolded proteins in the ER lumen, IRE1 is released from BiP and undergoes homodimerization and trans-autophosphorylation to activate its RNase activity [11] XBP1 mRNA is a substrate for the endoribonuclease activity of IRE1 [12]. Upon activation of the UPR, the IRE1 RNase activity initiates removal of a 26 nucleotide intron from XBP1 mRNA. Splicing X-box binding protein-1 (sXBP-1) is a transcriptional activator and activates transcription of the genes that involve in UPR. sXBP-1 increases protein folding capacity in the ER lumen [13,14].

Under unstressed conditions, ATF6 is localized at the ER membrane and bound to BIP. In response to ER stress, BIP dissociation permits ATF6 to translocate to the Golgi where ATF6 is cleaved by two proteases the serine protease site-1 protease (S1P) and S2P. S1P cleaves ATF6 in the luminal domain and N terminal portion is cleaved by the S2P [15]. Two ATF6-like molecules that are cleaved by S1P and S2P to activate UPR transcription [16].

Tunicamycin (TM) is highly toxic for mammals [17]. TM induces ER stress in plants and eukaryotic cells. Tauroursodeoxycholic acid (TUDCA), is a hydrophilic bile acid found in small amounts in humans. TUDCA has been found to reduce the apoptotic effect [18,19].

The study aimed to investigate ER-dependent UPR signal molecules in the cumulus cells (CCs) of the patients presenting normal, hyperresponsive, hyporesponsive and polycystic ovary syndrome (PCOS).

2. Materials and Methods

2.1. Tissue Samples

CCs were provided during oocyte retrieval from patients under 35 years old without endometriosis. Informed consent was obtained from all individual participants included in the study. 32 patients were distinguished according to their response to follicle stimulations. Follicle stimulation was performed according to the procedure described previously [20]. Normoresponsive (n=8) and hyporesponsive (n=8) groups were considered as retrieval of 10-20 and 6 or fewer oocytes, respectively. Retrieval of 30 or more oocytes responded to the same stimulus was considered as hyperresponsive (n=8), which included PCOS (n=8). Human experimental procedures were carried out under protocols approved by Gazi University Medical Faculty Institutional Review Board Ankara, TURKEY.

2.2. Cumulus Cell Culture

5ml of DMEM: F12 medium was added on the CCs and distributed evenly in petri dishes. The cell medium was changed every two days. The CCs were checked until they reached a density of 60% -

70% saturation. CCs were washed with PBS and 5 ml trypsin was added into the CCs dishes and incubated at 37°C for 5 minutes to separate the cells from the adherent surface. After checking the cells under phase-contrast microscopy, 5 ml of DMEM: F12 medium was added to eliminate trypsin activity and transferred to the falcon tube. Then it was centrifuged at 1800 rpm for 10 minutes. After centrifugation, the supernatant was removed and 5 ml of DMEM: F12 medium was added to the remaining cell pellet, and homogenized by pipette, and then distributed in equal amounts to the cell culture dishes. The cell medium was changed every two days and trypsinized when the cells reached a density of 80%-90%. To eliminate trypsin activity, the medium was added again and transferred to the falcon tube. Then it was centrifuged for 10 minutes at 1800 rpm. After the supernatant was removed and homogenized by adding 5 ml of medium to the pellet, the cells were transferred to 96-well culture dishes. Serum-free medium was applied to the cells for 24 hours. At the end of 24 hours, proliferation and apoptosis indices were determined by treatment with different UPR inhibitors and ER stress stimulating substances.

2.3. MTT Proliferation Technique

Cell proliferation was performed by using Cell Titer 96 Aqueous non-radioactive cell proliferation kits (Promega, Madison, WI, USA). 500,000 cells / 100 µl of cultured CCs were transferred into each well of the 96-culture plate. CCs were divided into four groups. Control: CCs were treated with serum-free medium; TUDCA: CCs were treated with 0.001 µl / ml TUDCA ; TM: CCs were treated with 0.5 µg/ ml TM (Sigma Aldrich St. Louis MO, USA) and TM+TUDCA: CCs were treated with both 0.5 µg/ ml TM and 0.001 µl/ ml TUDCA for 48 h [18,21]. The cells were incubated with 100 µl media contained 10 µl MTS solution for 4 h at 37°C. The plates were read at 490 nm wavelength and the mean of their optic density was calculated.

2.4. TUNEL

Cultured CCs were transferred into 4-well culture plates. CCs were divided into two groups. Control: CCs were treated with 1 ml serum-free media for 48 h and TM: CCs were treated with 0.5 µg/ ml TM for 48 h. Apoptosis index was determined by using TUNEL kit (Roche, In Situ Cell Death Detection Kit, Mannheim, Germany). CCs were washed with PBS and incubated with permeabilization solution (0.1% sodium citrate solution containing 0.1% triton X-100) for 5 minutes at +4°C. The slides were incubated with the TUNEL solution for 1h at 37°C. Cells were incubated with streptavidin-alkaline phosphatase enzyme for 30 minutes. The cells were incubated with Fast-Red substrate for 10 minutes to visualize the TUNEL positive cells. The discrimination and count of positive and negative cells were performed, and the apoptosis rates of cells after inhibition of the UPR mediated signal pathway estimated.

2.5. Western Blot

Total protein from CCs was extracted by using lysis buffer and protease inhibitors. 20 µg of protein was loaded into each hole. Samples were separated on 10% polyacrylamide gel at 80V in 1X running buffer for 1h. Samples were transferred to the nitrocellulose membrane inside the transfer buffer for one night at 32 mA +4°C. The membrane was incubated in TBS-T (0.05% Tween- in TBS) prepared with 5% oil-free dry milk for 1 h at room temperature. GRP78 (Cell Signaling Technology, Beverly, MA, USA) primer antibody was incubated for overnight at +4 °C. Secondary antibody (Peroxidase Labeled Anti-Rabbit, Vector Labs, Burlingame, CA, USA) stained with peroxidase was incubated. After being immunofixation with ECL for 1 minute, it was exposed to Bio Max film. The results were normalized by taking the ratio of GRP78 to β-actin expression levels.

2.6. Reverse Transcriptase PCR (RT-PCR)

CCs from each group put in the 100 μ l lysis buffer of RNAqueous Micro kit (Ambion, Austin, TX, USA). We obtained total RNA from CCs according to the RNAqueous Micro kit protocol. The genomic DNA is removed by using DNase1 (Ambion, Austin, TX, USA). The RNA quality and concentration were determined by measuring absorbance at 260 and 280 nm. Amplifications first applied after denaturation at 94 $^{\circ}$ C, 20 μ l of 1X PCR buffer, each 0.125 mM dNTP, 0.5 μ M each primer and 2 units of Super Taq polymerase. Semi-quantitative RT-PCR was performed using specific XBP1 and sXBP1.

The primers are human XBP1: sense, 5'-ACACGCTTGGGAATGGACAC-'3 antisense5'-CCATGGGAAGATGTTCTGGG-'3 and human β -actin: sense, 5'-CGGATGTCAACGTCACACTT -'3, antisense, 5' - TGGGTGACATCAAAGAGAAG-'3. PCR products and molecular weight markers were carried out, 1.5 % for the β -actin and 3.5% for XBP-1 in agarose gel that contains 10 mg ml⁻¹ ethidium bromide and visualized under ultraviolet light. The intensity of each band was normalized with β -actin band of each sample and compared in the semi-quantitative analysis between samples.

2.7. Statistical Analysis

All data were loaded to the software Sigma Stat 3. Data were shown as mean \pm SD. Difference between groups was evaluated with one-way ANOVA post hoc TUKEY test. P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. MTT assay results

As a result of the MTT analysis, the proliferation index of the cumulus cells of the control group was (0.142 \pm 0.01). In the TM-treated group, the proliferation index of CCs was significantly decreased (P < 0.05) compared to the control group (Figure 1). No significant difference was observed between the proliferation index of CCs-treated with TUDCA and of the cells from the control group (p > 0.05) (Figure 1). The proliferation index was (0.04 \pm 0.002) reduced in the cumulus cells induced by TUDCA and TM, compared to the control group. On the other hand, the proliferation index of the CCs was treated with both TUDCA and TM was two-fold higher than the CCs were treated with only TM (p > 0.001) (Figure 1).

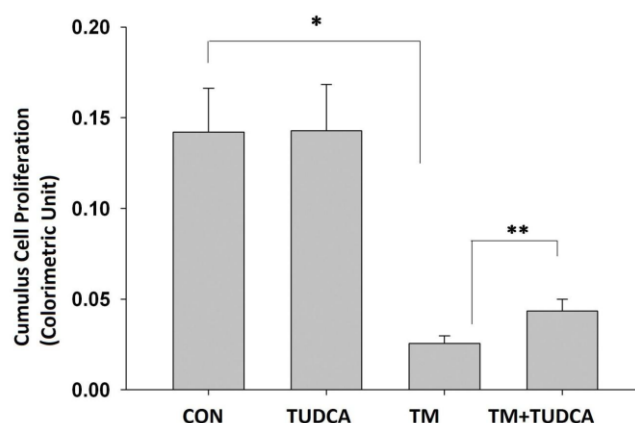


Figure 1. MTT cell proliferation in CCs culture. Control (CON): CCs were treated with serum-free media; TUDCA: CCs were treated with TUDCA; TM: CCs treated with TM; TM + TUDCA: CCs treated with both TM and TUDCA. *P < 0.05 and **P > 0.001. Bars indicate mean \pm standard error.

3.2. TUNEL results

According to the TUNEL results, we found that the apoptosis index of CCs from the control group was (0.108 ± 0.017) . On the other hand, when CCs were stimulated with ER stress inducer TM, there was a significant increase of apoptosis index of the CCs were treated with TM (0.334 ± 0.027) , compared to those of the control group ($P < 0.001$) (Figure 2a - c).

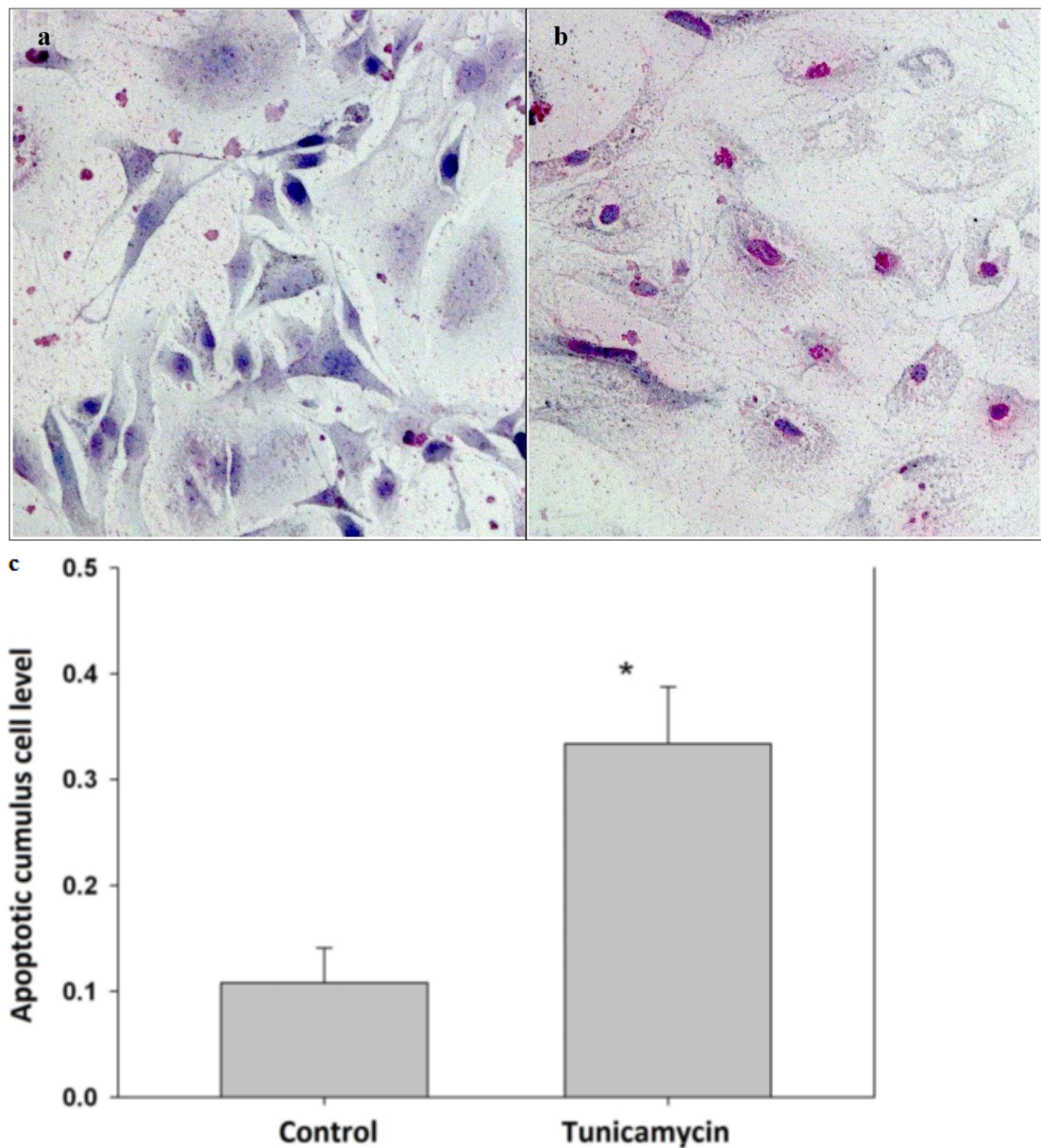


Figure 2a-c. The apoptotic effect of Tunicamycin in CCs culture. Control: CCs were treated with serum-free media (a). TM: CCs were treated with TM. Red color indicates TUNEL positive cells. Blue color indicates TUNEL negative cells (b). The quantitative analysis of CCs is observed. (c). * $P < 0.001$. Bars indicate mean \pm standard error. 3.3. Western Blot results

The protein expression of GRP78 was analyzed by Western blot method in the CCs of the normal, PCOS, hyporesponsive and hyperresponsive patients (Figure 3a). The results were normalized by taking

the ratio of GRP78 to β -actin expression levels. GRP78 expression levels, in CCs from normal, PCOS and hyperresponsive groups were 0.921 ± 0.048 , 0.835 ± 0.155 and 0.892 ± 0.118 respectively and no significant difference was observed between the groups (Figure 3b). On the other hand, the GRP78 expression level was two-fold higher (1.706 ± 0.288) in CCs from the hyporesponsive group than the other groups ($p < 0.05$).

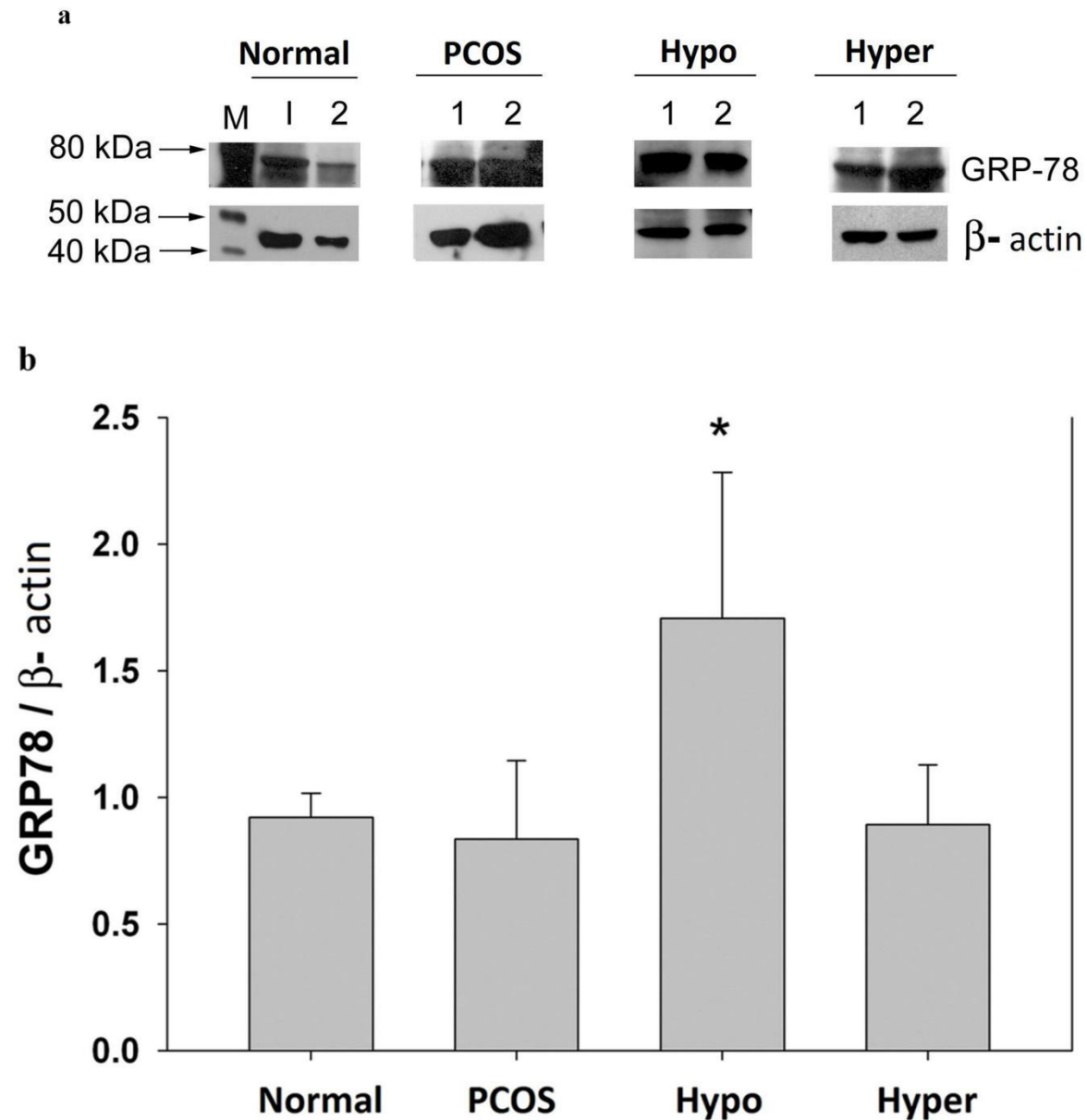


Figure 3a-b. GRP78 protein expression in CCs from normal, PCOS, hyporesponsive and hyperresponsive patients. Western blot analyzed of GRP78 and β - actin in CCs from different patient groups (a). Semi-quantitative analysis of GRP78 / β - actin levels of patient groups (b). PCOS, Hypo: hyporesponsive, Hyper: hyperresponsive. n = 8 patients per group. * P < 0.05. Bars indicate mean \pm standard error.

3.4. RT-PCR results

The mRNA levels of XBP-1 and sXBP-1 were analyzed by RT-PCR method in CCs of normal, polycystic ovary syndrome, hyperresponsive and hyporesponsive patients (Figure 4a).

sXBP-1/XBP-1 mRNA expression levels were 0.712 ± 0.256 , 0.622 ± 0.102 and 0.681 ± 0.291 in the CCs from normal, PCOS and hyperresponsive patients, respectively. No significant changes were observed in CCs between the groups (Figure 4b). On the other hand, sXBP-1/XBP-1 mRNA expression level was 1.5 fold higher (1.043 ± 0.165) in the CCs from the hyporesponsive group compared to others.

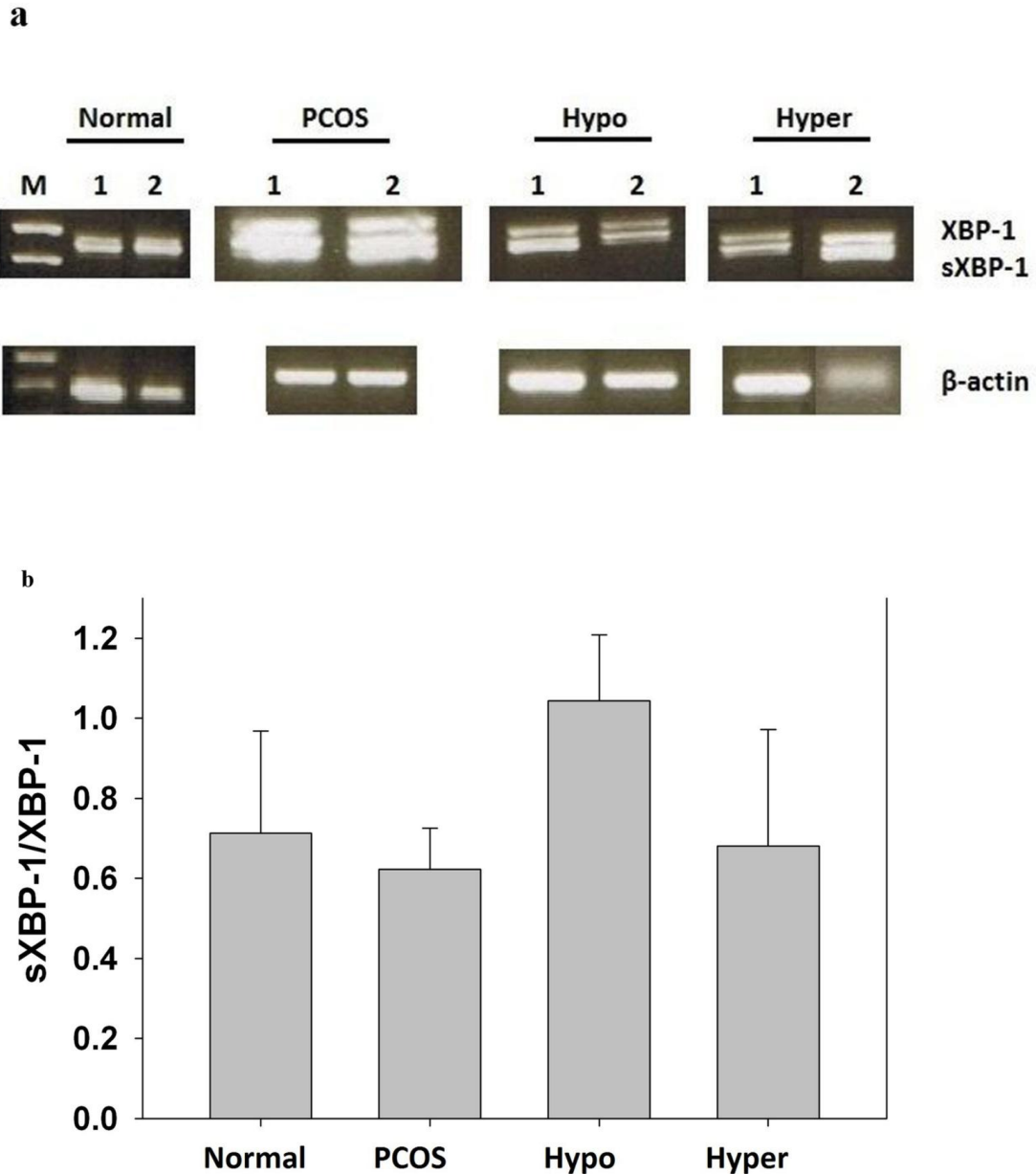


Figure 4a-b. sXBP-1/XBP-1 mRNA levels in CCs from normal, PCOS, hyporesponsive and hyperresponsive patients. RT - PCR samples of sXBP1, XBP1, and β -actin obtained from the groups (a). The semi-quantitative analysis of the ratio of sXBP1 / XBP1 to β - actin mRNA levels (b). PCOS, Hypo: hyporesponsive, Hyper: hyperresponsive. n = 8 patients per group. The bars indicate mean \pm standard error.

4. Discussion

Cell to cell communication between oocytes and CCs coordinates ovarian follicles development [10]. Modification, folding and transportation of newly synthesized secretion and transmembrane proteins take place in the ER lumen. Some conditions such as hypoxia, glucose deficiency, oxidative

stress, and viral infections, can cause the accumulation of unfolding proteins in the ER lumen. The accumulation of unfolded proteins in the ER lumen activates UPR [22].

It has been reported that excess production of vascular endothelial growth factor A (VEGFA) induces ovarian hyperstimulation syndrome (OHSS) and TUDCA administration prevented the OHSS development by reducing VEGFA production in the ovary of OHSS model rats. Additionally, TUDCA has been used in preimplantation embryos culture [23-25].

In our study, we investigated the effects of ER stress on CCs proliferation, we determined cell viability by MTT assay. Cultured CCs were treated with UPR inhibitor TUDCA and ER stress stimulator TM, for 48 h. We found that cell proliferation was decreased in CCs treated with TM compare to other groups.

When we suppressed ER stress by treating CCs with both TUDCA and TM, we showed a slight increase in cell proliferation compared to TM-treated group. These data indicate that the longer-term or high-dose use of the TUDCA may increase cell proliferation. We used the TUNEL assay, to understand the apoptotic effect of TM in CCs. We showed that apoptosis was significantly increased in CCs treated with TM compared to control.

It has been reported that fatty acid-induced ER stress disrupts protein secretion and mitochondrial membrane potential in mouse cumulus-oocyte complex [26]. It has been indicated that ER stress may be involved in OHSS development [27].

GRP78 play important roles in many cellular processes, including folding and combining of newly synthesized proteins, orienting misfolded proteins to ERAD, regulation of calcium homeostasis, and activation of ER stress sensors [10,19].

In our study, protein expression of GRP78 which is the major regulatory protein of UPR was analyzed by Western Blot. We showed that protein expression of GRP78 in CCs of the hyporesponsive group was two times higher than the other groups. According to these findings, we can suggest that ER stress may be significantly degraded in the protein folding mechanism in hyporesponsive patients.

XBP-1 is a transcriptional activator gene which prevents the accumulation of misfolded proteins, by activating transcription of the genes that involves in the UPR [28]. Recent studies investigated the role of UPR in granulosa cells (GC) and CCs. It has been shown that both sXBP1 and HSPA5 expressed in GC of the later stage than large secondary follicles. It was suggested that UPR was involved in the process of follicular growth and maturation [29].

To evaluate the ER stress at mRNA level; we analyzed XBP1 and sXBP1 mRNA level by the RT-PCR. In our study, when the quantity of sXBP1 mRNA in the whole form XBP1 mRNA compared to the other groups, it has only shown an increase in the CCs from hyporesponsive patients. We have demonstrated that an increased ER stress in the CCs of the ovarian follicle of the patients forming the hyporesponsive group and we have shown this stress by sXBP1 at the mRNA level.

To obtain healthy folliculogenesis and quality oocyte, we can suggest that the ER stress and UPR signaling pathway should be kept within physiological limits and a new treatment approach with the use of agents to reduce the level of pathological ER stress in hyporesponsive patients.

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Disclosure of interest

The authors report no conflict of interest.

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