

Activation of endoplasmic reticulum stress mediates oxidative stress–induced apoptosis of granulosa cells in ovaries affected by endometrioma

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Running title: Endoplasmic reticulum stress in ovary with endometrioma

Abstract

Endometriosis exerts detrimental effects on ovarian physiology and compromises follicular health. Granulosa cells from patients with endometriosis are characterized by increased apoptosis, as well as high oxidative stress. Endoplasmic reticulum (ER) stress, a local factor closely associated with oxidative stress, has emerged as a critical regulator of ovarian function. We hypothesized that ER stress is activated by high oxidative stress in granulosa cells in ovaries with endometrioma and that this mediates oxidative stress-induced apoptosis. Human granulosa-lutein cells (GLCs) from patients with endometrioma expressed high levels of mRNAs associated with the unfolded protein response (UPR). In addition, the levels of phosphorylated ER stress sensor proteins, inositol-requiring enzyme 1 (IRE1) and double-stranded RNA-activated protein kinase-like ER kinase (PERK), were elevated in granulosa cells from patients with endometrioma. Given that ER stress results in phosphorylation of ER stress sensor proteins and induces UPR factors, these findings indicate that these cells were under ER stress. H₂O₂, an inducer of oxidative stress, increased expression of UPR-associated mRNAs in cultured human GLCs, and this effect was abrogated by pre-treatment with tauroursodeoxycholic acid (TUDCA), an ER stress inhibitor in clinical use. Treatment with H₂O₂ increased apoptosis and the activity of the pro-apoptotic factors caspase-8 and caspase-3, both of which were attenuated by TUDCA. Our findings suggest that activated ER stress induced by high oxidative stress in granulosa cells in ovaries with endometrioma mediates apoptosis of these cells, leading to ovarian dysfunction in patients with endometriosis.

Key words: apoptosis, endometrioma, endometriosis, endoplasmic reticulum stress, follicular microenvironment, granulosa cell, ovary, oxidative stress, unfolded protein response

Introduction

Endometriosis affects 6–10% of women of reproductive age and is closely related to infertility (Giudice *et al.*, 2010): from 25% to 50% of infertile women have endometriosis, and 30–50% of women with endometriosis are infertile (Missmer *et al.*, 2004). The biological mechanisms that may link endometriosis and infertility remain unclear. However, endometrioma, a primary manifestation of the disease, as well as endometriosis at sites other than the ovary, exert detrimental effects on ovarian physiology and compromises follicular health (de Ziegler *et al.*, 2010). The proportion of atretic follicles in ovarian cortex is elevated in ovaries with endometrioma (Kitajima *et al.*, 2014). Atresia of follicles beyond the late preantral stage initially involves apoptosis of granulosa cells (Morita and Tilly, 1999; Sanchez *et al.*, 2014a); on the other hand, granulosa cells from patients with endometriosis exhibit high levels of apoptosis (Goud *et al.*, 2014; Jana *et al.*, 2010; Nakahara *et al.*, 1997; Sanchez *et al.*, 2014a; Sanchez *et al.*, 2016; Toya *et al.*, 2000).

Among several pathophysiologic factors associated with endometriosis, it is expected that oxidative stress contributes to the induction of apoptosis in granulosa cells, although the underlying mechanism remains unclear (Sanchez *et al.*, 2016). Levels of oxidative stress are elevated in the ovarian cortex surrounding endometrioma (Di Emidio *et al.*, 2014; Matsuzaki and Schubert, 2010). Higher levels of oxidative stress are also observed in the follicular fluid of follicles adjacent to endometrioma, as well as in follicles of endometriosis patients, regardless of the presence or absence of endometrioma (Da Broi *et al.*, 2016a; Regiani *et al.*, 2015). Furthermore, granulosa-lutein cells (GLCs) harvested at IVF from endometriosis patients exhibit higher levels of oxidative stress markers (Seino *et al.*, 2002). In this context, we hypothesized that higher levels of oxidative stress in granulosa cells in ovaries with endometrioma will activate endoplasmic reticulum (ER) stress, leading to apoptosis of these cells.

ER stress is a local factor that is closely related to oxidative stress (Cao and Kaufman, 2014; Hasnain *et al.*, 2016). It arises due to the accumulation of unfolded or misfolded proteins in the ER, which can be caused by a variety of physiological and pathological conditions, including oxidative stress and inflammation, which increase the demand for protein folding or attenuate the protein folding capacity of the ER. ER stress results in activation of several signal transduction cascades, collectively termed the unfolded protein response (UPR), which play a

primary role in stress adaptation and affect a wide range of cellular functions, ultimately inducing apoptosis in the affected cells when the stress cannot be resolved (Ozcan and Tabas, 2012; Walter and Ron, 2011). We previously demonstrated that ER stress is activated in granulosa cells of growing follicles, and that activated ER stress in granulosa cells regulates cellular functions and thus contributes to the various pathologies, including ovarian hyperstimulation syndrome, obesity-related progesterone deficiency, polycystic ovary syndrome (PCOS), and advanced glycation end products (AGEs)-related poor oocyte developmental competence (Harada *et al.*, 2015; Takahashi *et al.*, 2016; Takahashi *et al.*, 2017a; Takahashi *et al.*, 2017b; Azhary *et al.*, 2019; Takahashi *et al.*, 2019).

To test our hypothesis, we first examined apoptosis and oxidative stress status in granulosa cells in ovaries affected by endometrioma. We then examined activation of ER stress using GLCs from patients with endometrioma and their affected ovaries. In *in vitro* experiments utilizing human GLCs, we examined the effects of oxidative stress on activation of ER stress and its intermediary role in oxidative stress-induced apoptosis. We also tested the anti-apoptotic effect of an ER stress inhibitor, tauroursodeoxycholic acid (TUDCA). We selected TUDCA as the ER stress inhibitor for this present study because its safety in humans has already been demonstrated (Hetz *et al.*, 2013).

Materials and Methods

Ethical approval

All experimental procedures were approved by the relevant institutional review boards. All patients provided signed informed consent (authorization reference number: 3594-(6)).

Human specimens

GLCs and follicular fluid were aspirated from patients undergoing oocyte retrieval for IVF at the University of Tokyo Hospital, Matsumoto Ladies Clinic, Hamada Hospital, and Akihabara ART Clinic. Isolated GLCs from this group of patients were used for *in vitro* experiments with hydrogen peroxide (H₂O₂) and TUDCA treatment. For comparisons of mRNA levels of UPR factors in GLCs, 16 control patients and seven patients with endometrioma confirmed by ultrasonography were examined. For terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and immunohistochemical analysis, ovaries from seven patients with endometrioma and seven control patients were collected. Ovaries with endometrioma were obtained from patients with or without infertility during laparoscopic or abdominal salpingo-oophorectomy. For control samples, normal ovaries were obtained from women with regular menstrual cycles without hormonal treatment who underwent radical or extended hysterectomy for carcinoma of the uterine cervix or endometrium. It was confirmed during surgery that these control patients had no endometriosis lesion. The ovaries were stored in 10% formalin neutral buffer solution and sectioned at 5 μm before being subjected to staining. At least six randomly-selected sections from each sample were analyzed.

TUNEL staining

Apoptotic cells in human ovaries were detected by TUNEL staining using the In Situ Cell Death Detection Kit, POD (Roche, Mannheim, Germany). Briefly, sections were deparaffinized in xylene and rehydrated in alcohol. Then sections were incubated with proteinase K (Wako, Osaka, Japan) for 8 minutes at room temperature; for the positive control, an additional 10-minute incubation was performed with deoxyribonuclease 1 (Thermo Fisher Scientific, Waltham, MA, USA). After washing with PBS, the sections were incubated at room temperature

for 1 hour with TUNEL reaction solution containing label solution and enzyme solution. For the negative control, only label solution was used. Subsequently, the tissues were incubated with converter-POD (horse-radish peroxidase: HRP) solution for 30 minutes at room temperature. After PBS washing, TUNEL-positive cells were stained with 3,3'-diaminobenzidine (DAB) (Dako, Tokyo, Japan). TUNEL staining was performed three times independently using different sections from the same patients.

Immunohistochemistry

Human ovarian sections were immunostained with anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody (1:20, N45.1, JaICA, Shizuoka, Japan), anti-phospho-inositol-requiring enzyme 1 (IRE1) (1:500, ab48187, Abcam, Cambridge, UK), and anti-phospho-double-stranded RNA-activated protein kinase-like ER kinase (PERK) antibody (1:100, ab192591, Abcam), using the EnVision + Dual Link System/HRP (DAB) kit (Dako). Isotype-specific IgG served as the negative control. Antigen retrieval was performed using target retrieval solution (Dako). Immunohistochemistry was performed three times independently using different sections from same patients.

Quantitative analysis of immunohistochemistry and TUNEL staining

The ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for quantitative analysis (Schneider *et al.*, 2012). Positively stained granulosa cells were counted in a cross section from each follicle of five randomly selected follicles per women, and from seven individual controls and patients. We examined the staining of granulosa cells from growing follicles in the present study. The growing follicles were distinguished from atretic follicles by the morphology. Atretic follicles were determined by the following criteria; oocyte degeneration, irregular shapes of follicles or oocytes, and nuclear pyknosis in granulosa layers.

Isolation and culture of human GLCs

Isolation of GLCs was performed as reported previously (Takahashi *et al.*, 2017b; Azhary *et al.*,

2019; Takahashi *et al.*, 2019). Follicular fluids were centrifuged at 430 g, 20°C for 10 minutes, resuspended in 0.2 w/v% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS, and incubated at 37°C for 30 minutes. The suspension was layered over Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) and centrifuged at 700 g, 20°C for 30 minutes. GLCs were collected from the middle layer and washed with PBS. GLCs were cultured with Dulbecco's Modified Eagle Medium F-12 (DMEM/F12) (Thermo Fisher Scientific) containing 10 % fetal bovine serum (Sigma-Aldrich) and antibiotics (100U/mL penicillin, 0.1 mg/mL streptomycin, and 250 ng/mL amphotericin B; Sigma-Aldrich). GLCs were adjusted to a density of 2×10^5 cells/mL and cultured in 6-well or 48-well plates. All GLCs were precultured for 3–5 days prior to treatment at 37°C in a humidified atmosphere containing 5% CO₂.

Treatment of human GLCs

To evaluate the effect of oxidative stress on activation of ER stress, human GLCs from patients who were not suffering from endometriosis were pre-incubated with a reactive oxygen species (ROS), namely H₂O₂ (Wako), at 200, 400 or 600 μM for 12, 24, or 48 hours; these conditions were chosen on the basis of previous studies using human granulosa cells (Akino *et al.*, 2018, Bar-Joseph H *et al.*, 2014; Fu *et al.*, 2014, Nakahara *et al.*, 2012). Based on the results shown below, H₂O₂ exerted the maximum effect at a concentration of 400 or 600 μM over 24 hours. Hence, 24-hour stimulation with 400 μM H₂O₂ was used for the following experiments. To evaluate the effect of an ER stress inhibitor on ROS-induced apoptosis and expression of UPR factors, GLCs were pre-incubated with TUDCA (Tokyo Chemical Industry, Tokyo, Japan) at 1 mg/mL for 24 hours, followed by treatment with H₂O₂. Pre-treatment with TUDCA was performed as described previously (Takahashi *et al.*, 2016; Takahashi *et al.*, 2017b; Azhary JMK *et al.*, 2019).

RNA extraction, reverse transcription, and real-time quantitative PCR

Synthesis of cDNA templates from human GLCs were performed using the SuperPrep Cell Lysis & RT kit for qPCR (TOYOBO, Osaka, Japan). To quantitate mRNA levels of UPR genes, real-time quantitative PCR (qPCR) was performed on a Light Cycler system (Roche

Diagnosics GmbH, Mannheim, Germany). The UPR genes activating transcription factor 4 (*ATF4*), *ATF6*, spliced X-box-binding protein 1 (*sXBP1*), heat shock 70kDa protein 5 (*HSPA5*), and C/EBP homologous protein (*CHOP*) were examined as markers of ER stress activation. Human *GAPDH* mRNA was used as an internal standard for RNA loading. Primer sequences were as follows: human *ATF4* (sense, 5'-GGCTGGCTGTGGATGGGTTG-3'; antisense, 5'-CTCCTGGACTAGGGGGGCAA-3'), *ATF6* (sense, 5'-TCAGACAGTACCAACGCTTATGC-3'; antisense, 5'-GTTGTACCACAGTAGGCTGAGA-3'), *sXBP1* (sense, 5'-TCCGCAGCACTCAGACTAC-3'; antisense, 5'-TCCAAGTTGTCCAGAATGCC-3'), *HSPA5* (sense, 5'-GACATTTGCCCCAGAAGAAA-3'; antisense, 5'-CTCATGACATTCAGTCCAGCA-3') and *CHOP* (sense, 5'-GGAGAACCAGGAAACGGAAAC-3'; antisense, 5'-TCTCCTTCATGCGCTGCTTT-3'). PCR conditions were as follows: for *GAPDH*, *ATF4*, *ATF6*, *HSPA5*, and *CHOP*, 40 cycles of 98°C for 10 seconds, 60°C for 10 seconds, and 68°C for 30 seconds; for *sXBP1*, 40 cycles of 98°C for 10 seconds, 62°C for 10 seconds, and 68°C for 30 seconds. Calculation and quantification of PCR products was conducted by using the (2-delta, delta C_T) method. These products were confirmed by electrophoresis as well. All samples from GLCs were analyzed in triplicate or quadruplicate.

Detection of apoptosis in human GLCs in vitro using the annexin V-FITC/propidium iodide (PI) double-staining assay

Oxidative stress-induced apoptosis was detected by flow cytometry using the annexin V-FITC apoptosis detection kit 1 (BD Biosciences, San Jose, CA, USA). Cultured human GLCs were treated with H₂O₂ and TUDCA, trypsinized, and collected by centrifugation at 430g, 4°C for 4 minutes. After resuspension in annexin V-FITC binding buffer, annexin V and PI were added (5 µL per sample) and incubated for 15 minutes in the dark at room temperature. The samples were analyzed using BD FACSCalibur (BD Biosciences) within 60 minutes of double staining.

Caspase-8 activity assay

Caspase-8 activity was measured using the Caspase-8 Assay Kit, Colorimetric (K113-25, Bio Vision, Milpitas, CA, USA). Briefly, following treatment, the GLCs were washed with PBS and resuspended in chilled Cell Lysis Buffer provided in the kit. After incubation on ice for 10 minutes, the samples were centrifuged at 4°C for 1 min at 10,000 g, and the supernatant was collected. Reaction buffer containing dithiothreitol (DTT) and IETD-p-nitroanilide (IETD-pNA) substrate was added, and the samples were incubated for 90 minutes at 37°C in the dark. Activity was determined at 405 nm using a microplate spectrophotometer (Epoch, Bio Tek Instruments, Winooski, VT, USA).

Western blot analysis

GLCs were lysed in lysis buffer (Merck, Darmstadt, Germany) and centrifuged at 19,300 g for 10 minutes at 4°C to remove insoluble material. The supernatants were recovered and the protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of lysate protein (20 µg) were loaded onto Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) and electrophoretically transferred onto Trans-Blot Turbo Transfer Packs (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). After blocking with 5% skim milk for 1 hour at room temperature, the membranes were probed overnight at 4°C with anti-cleaved caspase-3 antibody (1:1000; #9664, Cell Signaling Technology, Danvers, MA, USA). Then, the blots were incubated with the secondary antibody (1:2000, anti-rabbit IgG, #7074S, Cell Signaling Technology) for 1 hour at room temperature, and visualized using the ECL Plus Western blotting detection reagents (GE Healthcare). The images were acquired on an Image Quant LAS 4000 mini luminescent image analyzer (GE Healthcare). β-actin (1:3000; A2228, Sigma-Aldrich) was used as a loading control. All samples were analyzed in triplicate.

Statistical analysis

All statistical analyses were performed using the JMP pro 14 software (SAS Institute Inc., Cary, NC, USA). All data are shown as mean ± SEM. For qPCR and the caspase-8 activity assay,

values are shown as relative to the control; the average value of each group was divided by that of control group. The Student's t-test was used for comparisons between pairs of samples, and the Tukey–Kramer honest significant difference test for multiple comparisons. A p-value < 0.05 was considered statistically significant. All experiments were repeated at least three times independently.

Results

No difference in patient characteristics (age, type of controlled ovarian stimulation, basal FSH concentrations, anti-Müllerian hormone concentrations, and number of oocytes retrieved) was observed between the groups (Table I).

Apoptosis is elevated and oxidative stress is activated in granulosa cells from ovaries with endometrioma

To confirm the increase in apoptosis in granulosa cells of ovaries affected by endometrioma, we performed TUNEL staining. As shown in Fig. 1, the ratio of TUNEL-positive apoptotic cells was upregulated in granulosa cells of antral follicles in ovaries with endometrioma compared to those without endometrioma (37.2 ± 1.68 % versus 22.9 ± 3.80 %, $p=0.0011$). Next, we examined immunostaining of 8-OHdG, an oxidized nucleoside of DNA that serves as an oxidative stress marker. 8-OHdG expression was elevated in granulosa cells of antral follicles in the ovaries with endometrioma relative to those without endometrioma (52.0 ± 1.80 % versus 20.4 ± 1.71 %, $p<0.0001$, Fig. 2).

ER stress is activated in granulosa cells of ovaries affected by endometrioma

To examine activation of ER stress, we compared mRNA expression levels of UPR genes in granulosa cells of patients with endometrioma with those in patients without endometrioma. *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP* were used as ER stress markers: all of these markers were upregulated in GLCs harvested from patients with endometrioma ($p=0.0303$, $p=0.0449$, $p=0.0234$, $p=0.0361$, $p=0.0439$, respectively. Fig. 3). To confirm the activation of ER stress in granulosa cells of ovaries affected by endometrioma, we performed immunohistochemical analysis of the activated ER stress sensor proteins phospho-IRE1 and phospho-PERK. As shown in Fig. 4, the immunoreactivity of phospho-IRE1 and phospho-PERK was elevated in granulosa cells of antral follicles from patients with endometrioma (Fig. 4f, 33.1 ± 1.48 % versus 21.9 ± 3.65 %, $p=0.0088$; Fig. 4k, 42.2 ± 0.88 % versus 23.8 ± 3.76 %, $p=0.0002$).

Oxidative stress induces ER stress in cultured human GLCs

To determine whether oxidative stress induces ER stress in cultured human GLCs, we measured mRNA expression levels of the same UPR factors mentioned above: *ATF4*, *ATF6*, *sXBPI*, *HSPA5*, and *CHOP*. First, we treated cells with H₂O₂, an oxidative stress inducer, at various times (12, 24, 48 h) and concentrations (200, 400, and 600 μM). As shown in Fig. 5, oxidative stress induced mRNA expression of the UPR factors, with a maximum effect at 24 hours with 400 or 600 μM. Then, we treated GLCs with 400 μM of H₂O₂ for 24 hours to induce oxidative stress. Pre-treatment with the ER stress inhibitor TUDCA abrogated upregulation of UPR gene expression upon H₂O₂ treatment. As shown in Fig. 6, upregulation of *ATF4* mRNA level decreased by 50% in the TUDCA plus H₂O₂ group compared to the H₂O₂ group (2.40 versus 1.10, $p < 0.0001$), for *CHOP* there was a 40% decrease (2.01 versus 1.23, $p = 0.0235$), and for *ATF6*, *sXBPI* and *HSPA5* a 30% decrease (2.09 versus 1.54, $p = 0.003$; 1.86 versus 1.25, $p = 0.0197$; 4.58 versus 3.06, $p = 0.0225$, respectively), confirming that oxidative stress induced UPR factors via activation of ER stress. Collectively, these findings demonstrate that oxidative stress induces ER stress in cultured human GLCs.

ER stress mediates oxidative stress-induced apoptosis in cultured human GLCs

Next, to determine whether ER stress mediates oxidative stress-induced apoptosis, we monitored apoptotic cell death by annexin V-FITC/PI double-staining assay. As shown in Fig. 7a, early and late apoptotic cells were determined as annexin V-positive /PI-negative (lower right panel in each group) and annexin V-positive/PI-positive (upper right panel), respectively. Fig. 7b shows the total amounts of early and late apoptotic cells. Relative to the controls, the proportion of apoptotic cells was two-fold higher in the H₂O₂ group (14.7 ± 2.11 % versus 7.37 ± 0.99 %, $p = 0.0123$). Pre-treatment with TUDCA suppressed the oxidative stress-induced apoptosis (14.7 ± 2.11 % versus 7.79 ± 0.59 %, $p = 0.0179$). These results suggest that ER stress mediates oxidative stress-induced apoptosis in cultured human GLCs.

Activation of caspase-8 and caspase-3 is involved in ER stress-mediated apoptosis induced by oxidative stress

To elucidate the underlying mechanism by which H₂O₂-activated ER stress mediates apoptosis in human granulosa cells, we evaluated the expression and activation of two pro-apoptotic factors, caspase-8 and caspase-3. First, we measured the mRNA levels of both caspases. As shown in Fig. 8a and b, expression levels of caspase-8 and caspase-3 mRNA were increased by treatment with H₂O₂ (1.00 ± 0.056 versus 5.76 ± 0.189 ; 1.00 ± 0.173 versus 6.00 ± 0.462 , respectively $P < 0.0001$) and attenuated by pre-treatment with TUDCA (5.76 ± 0.189 versus 3.01 ± 0.198 , $p = 0.0002$; 6.00 ± 0.462 versus 1.59 ± 0.228 , respectively $P < 0.0001$). Next, we examined the activities of caspase-8 and caspase-3, respectively, by caspase-8 activity assay (Fig. 8c) and western blotting for cleaved caspase-3, an active form of caspase-3 (Fig 8d and Supplementary Fig. S1 for full blot). As with the mRNA levels, the activity of caspase-8 was upregulated by treatment with H₂O₂ (1.00 versus 1.31 ± 0.040 , $p = 0.0007$) and attenuated by pre-treatment with TUDCA (1.31 ± 0.040 versus 1.12 ± 0.057 , $p = 0.0195$). Western blotting for cleaved caspase-3 showed the same trend.

Discussion

Our results show that ER stress is activated in granulosa cells in ovaries affected by endometrioma, with concomitant activation of oxidative stress and elevated levels of apoptosis. In cultured human GLCs, oxidative stress activates ER stress, which mediates oxidative stress-induced apoptosis by activating caspase-8 and caspase-3.

In GLCs of patients with endometrioma, the expression of the UPR genes *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP* was upregulated. The UPR is a group of signal transduction cascades that are activated by ER stress. Elevated expression of UPR-associated genes is indicative of ER stress in granulosa cells of patients with endometrioma. Activation of ER stress is sensed by ER stress sensor proteins, such as IRE1 and PERK, which are phosphorylated upon activation, leading to induction of the UPR. The histological findings presented here in human ovaries, which exhibited an increase in immunoreactivity for phospho-IRE1 and phospho-PERK in granulosa cells of antral follicles in ovaries affected by endometrioma, confirm the activation of ER stress in these cells. We previously showed that under physiological conditions, ER stress is activated in granulosa cells of follicles after the preantral stage (Harada *et al.*, 2015), and its

activation is more prominent in various pathologies, including obesity and PCOS (Takahashi *et al.*, 2017a; Takahashi *et al.*, 2017b). Moreover, other works showed that locally accumulating lipids in obese patients and hyperandrogenism in PCOS patients contribute to the activation of ER stress in granulosa cells (Yang *et al.*, 2012; Azhary *et al.*, 2019). Oxidative stress and inflammation, which are closely related to ER stress and commonly observed in the follicular microenvironment of obese women and patients with PCOS, are also speculated to activate ER stress in these cells. On the other hand, elevated levels of oxidative stress were observed in the follicular fluid of follicles adjacent to endometrioma, as well as in the follicular fluid and GLCs of patients with endometriosis (Regiani *et al.*, 2015; Da Broi *et al.*, 2016a; Seino *et al.*, 2002). In this study, the findings that immunoreactivity of an oxidative stress marker 8-OHdG is upregulated in granulosa cells in the ovaries with endometrioma relative to without endometrioma, further confirm the increase in oxidative stress in the follicular microenvironment of patients with endometrioma. Endometrioma and endometriosis may induce oxidative stress in granulosa cells by any of several mechanisms. First, ROS in endometrioma permeate the surrounding tissue because of their highly diffusible character. The absence of a real anatomic capsule that can serve as a physical barrier further promotes this permeation, as endometriomas are surrounded by a thin wall composed of ovarian cortex or fibrotic tissue (Giacomini *et al.*, 2017; Sanchez *et al.*, 2014b). Alternatively, pelvic endometriotic lesions produce higher levels of pro-oxidant agents, which induce disease progression and alteration of the pelvic environment (Ngô *et al.*, 2009; Ruder *et al.*, 2008). Furthermore, oxidative stress activated by endometriotic lesions reaches the systemic circulation and affects the follicular microenvironment (Da Broi *et al.*, 2016a; Da Broi and Navarro, 2016b). In *in vitro* studies, we showed that stimulation of human GLCs with the oxidative stress inducer H₂O₂ increased mRNA expression levels of the UPR genes *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP*, whereas pre-treatment with the ER stress inhibitor TUDCA abrogated this effect. Collectively, the findings of this study indicate that ER stress is activated in granulosa cells in ovaries affected by endometrioma, and that this can be attributed to the high levels of oxidative stress in these cells.

Treatment with the oxidative stress agent H₂O₂ induced apoptosis of human cultured GLCs, and this effect was attenuated by pre-treatment with the ER stress inhibitor TUDCA; together, these observations indicate that ER stress activated by oxidative stress mediates

apoptosis in these cells. In addition, we showed that the expression and activity of caspase-8 and caspase-3 are upregulated by treatment with H₂O₂ and that again this effect is attenuated by pre-treatment with TUDCA; thus, the ER stress activated by oxidative stress utilizes the extrinsic pathway of apoptosis. Very few studies to date have examined the underlying mechanisms by which oxidative stress induces apoptosis in human granulosa cells. Yang demonstrated that oxidative stress activates c-Jun N-terminal kinase (JNK) and p53, thereby inducing activation of an intrinsic pathway of apoptosis (Yang *et al.*, 2017). Oxidative stress also promotes acetylation of Forkhead box O1 (FoxO1), which drives expression of pro-apoptotic factors, such as Fas ligand (FasL) and Bim, which are involved in the extrinsic and intrinsic pathway, respectively (Zhang *et al.* 2017). In this study, we showed that oxidative stress activates ER stress, which induces activation of the extrinsic pathway of apoptosis. The apoptotic programs induced by ER stress are highly complex and may depend on the nature of the stimulus, as well as the cell types affected (Urrea *et al.*, 2013). We previously demonstrated that testosterone activates ER stress in human GLCs, and that this ER stress induces apoptosis via the extrinsic pathway (Azhary *et al.*, 2019). In conjunction with the findings of this study, these observations suggest that activation of the extrinsic pathway represents a common mechanism underlying ER stress-induced apoptosis in human GLCs, independent of the stimulus that activates the stress, although it remains to be determined whether the intrinsic pathway is also involved.

Intriguingly, we also found that the ER stress inhibitor TUDCA, which is safe for humans, effectively ameliorates the apoptosis of GLCs induced by oxidative stress. TUDCA, marketed as Tauro in Italy and TauroLite in China, has been used to treat liver diseases and dissolve gallstones, and recent studies have shown that the molecule functions as a chemical chaperone to attenuate protein misfolding and decrease ER stress (Boatright *et al.*, 2009; Hetz *et al.*, 2013). Endometriosis exerts detrimental effects on ovarian physiology and compromises follicular health, thereby contributing to subfertility in patients with endometriosis (de Ziegler *et al.*, 2010). Granulosa cells from endometriosis patients exhibit elevated levels of apoptosis (Goud *et al.*, 2014; Jana *et al.*, 2010; Nakahara *et al.*, 1997; Sanchez *et al.*, 2014a; Sanchez *et al.*, 2016; Toya *et al.*, 2000), which we also confirmed in this study: the proportion of TUNEL-positive apoptotic cells was elevated in granulosa cells from antral follicles in ovaries with endometrioma relative to those without endometrioma. Atresia of follicles after the late

preantral stage initially involves apoptosis of granulosa cells (Morita and Tilly, 1999; Sanchez *et al.*, 2014a), and several lines of evidence show that an optimal follicular microenvironment consisting of healthy granulosa cells is associated with oocyte health (Da Broi *et al.*, 2018; Dumesic *et al.*, 2015). Accordingly, it is plausible that inhibition of apoptosis of granulosa cells may serve to improve ovarian physiology. In addition to endometriosis, recent studies demonstrated the association between oxidative stress and ovarian dysfunction, and the resultant decrease in fecundity associated with several pathologies, including PCOS and aging (Agarwal *et al.*, 2012). Several antioxidants, including vitamin C, E, melatonin, and myo-inositol, were tested to determine whether they can rescue ovaries from oxidative stress-related insults. However, no single antioxidant has demonstrated sufficient effectiveness to merit clinical application (Meldrum *et al.*, 2016; Showell *et al.*, 2018). In this study, we demonstrated that ER stress is activated by oxidative stress and mediates its effects in human GLCs. However, the relationship between ER stress and oxidative stress is bidirectional: in general, ER stress can contribute to activate oxidative stress (Cao and Kaufman, 2014). TUDCA and 4-phenylbutyric acid (PBA), another ER stress inhibitor in clinical use for primary biliary cirrhosis and urea-cycle disorders, demonstrated therapeutic potential in preclinical/clinical studies for several diseases associated with ER stress including insulin resistance and Alzheimer's disease (Almanza *et al.*, 2019; Cao and Kaufman, 2014). Given that ER stress and oxidative stress form vicious cycles and co-operatively play causal roles in various disease, a combination of clinically available ER stress inhibitors and antioxidants could exert therapeutic effects against oxidative stress-related ovarian dysfunction induced by endometriosis.

This study has some limitations. For the GLCs samples, we divided groups by the existence of endometrioma confirmed by ultrasonography, since the videolaparoscopy is not required for IVF patients. Accordingly, we were not able to exclude the possibility of having minimal endometriosis lesions in control patients. Second, we focused on the granulosa cells of follicles in their later stages, as the only human primary granulosa cells available for *in vitro* experiments are GLCs, which are a model of granulosa cells in later-stage follicles. Future studies utilizing rodent cells could clarify the roles of ER stress and oxidative stress in granulosa cells of follicles in earlier stages. Third, we demonstrated that activated ER stress mediates oxidative stress-induced apoptosis. However, the UPR, which is activated directly by ER stress, modulates various cellular functions, including cytokine production and steroidogenesis, as we

previously demonstrated (Takahashi *et al.*, 2016; Takahashi *et al.*, 2017a; Takahashi *et al.*, 2017b; Takahashi *et al.*, 2019). It remains to be elucidated whether ER stress activated by oxidative stress is involved in alteration of cellular functions other than apoptosis provoked by oxidative stress and associated with ovarian dysfunction in endometriosis. Another limitation is that the *in vivo* effects of an ER stress inhibitor were not examined. Future clinical studies exploring the effect of ER stress inhibitors, alone or in combination with antioxidants, on ovarian function and fecundity in patients with endometriosis will clarify the potential for clinical applications.

In summary, we demonstrated that ER stress is activated in granulosa cells in ovaries that are affected by endometrioma. Higher levels of oxidative stress in these cells contribute to the activation of ER stress, and activated ER stress mediates oxidative stress-induced apoptosis via the extrinsic pathway. Targeting ER stress with current clinically available ER stress inhibitors, or with these agents in combination with antioxidants, may serve as a novel strategy for rescuing endometriosis-associated ovarian dysfunction.

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Authors' roles

C.K. designed and performed experiments, analyzed and interpreted data, and wrote the article. M.H. contributed to conception of the work, analyzed and interpreted data, and wrote the article. N.T. contributed to conception of the work and performed experiments. J.M.K.A., A.K., E.N., N.O., and A.T. performed experiments. O.W.-H., T.H., Y.H., K.K., T.F., and Y.O. contributed to study design, data interpretation, and article revision.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1 Apoptosis is elevated in granulosa cells of ovaries affected by endometrioma.

TUNEL staining (brown), counterstained with hematoxylin (blue), was performed on the cross-sections of whole ovaries from seven control patients and seven patients with endometrioma. Representative sections are shown. Lower panels (d,e) show highly magnified views corresponding to (a,b). (c) shows the corresponding negative control, for which only label solution was used. (f) shows quantitative analysis of TUNEL staining. The ImageJ software was used for quantitative analysis. Positively stained granulosa cells were counted in a cross section from each follicle of five randomly selected follicles per women, from seven individual controls and patients. Values are shown as mean \pm SEM. The Student's t-test was used for statistical analysis. * $p < 0.05$. Scale bars: (a,b), 100 μm ; (c,d,e), 50 μm . C, control; E, ovaries affected by endometrioma; GC, granulosa cell layer; NC, negative control.

Figure 1

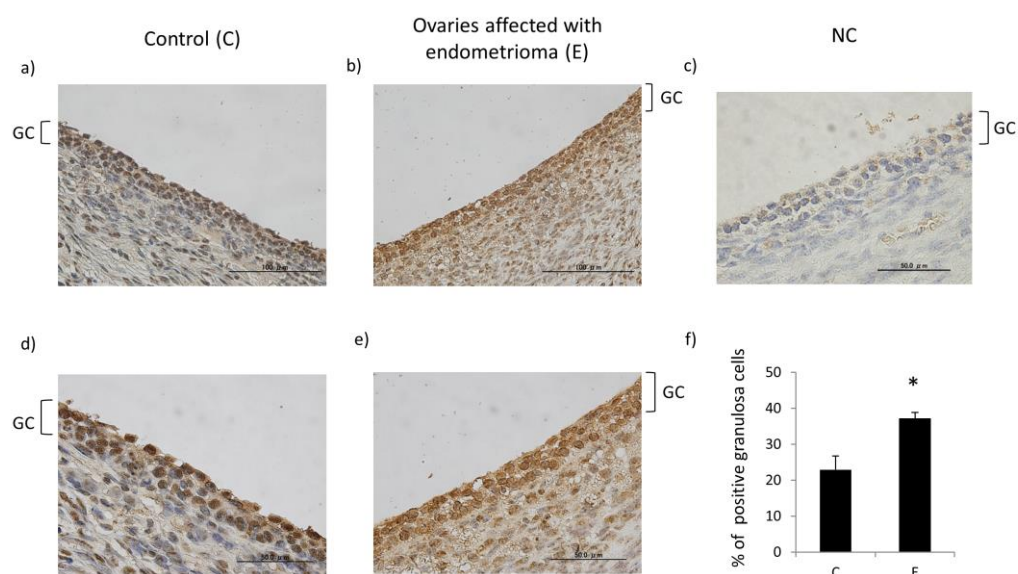


Figure 2 Oxidative stress is activated in granulosa cells of ovaries affected by endometrioma. Immunohistochemical detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was performed on ovaries from seven control patients and seven patients with endometrioma. Cross-sections of whole ovaries were stained with 8-OHdG antibody and counterstained with hematoxylin. Representative sections are shown. Lower panels (d,e) show highly magnified views corresponding to (a,b). (c) shows the corresponding negative control, for which only label solution was used. (f) shows quantitative analysis of immunohistochemical staining. The ImageJ software was used for quantitative analysis. Positively stained granulosa cells were counted in a cross section from each follicle of five randomly selected follicles per women, from seven individual controls and patients. Values are shown as means \pm SEM. The Student's t-test was used for statistical analysis. * $p < 0.05$. Scale bars: (a,b,c), 100 μm ; (d,e), 50 μm .

Figure 2

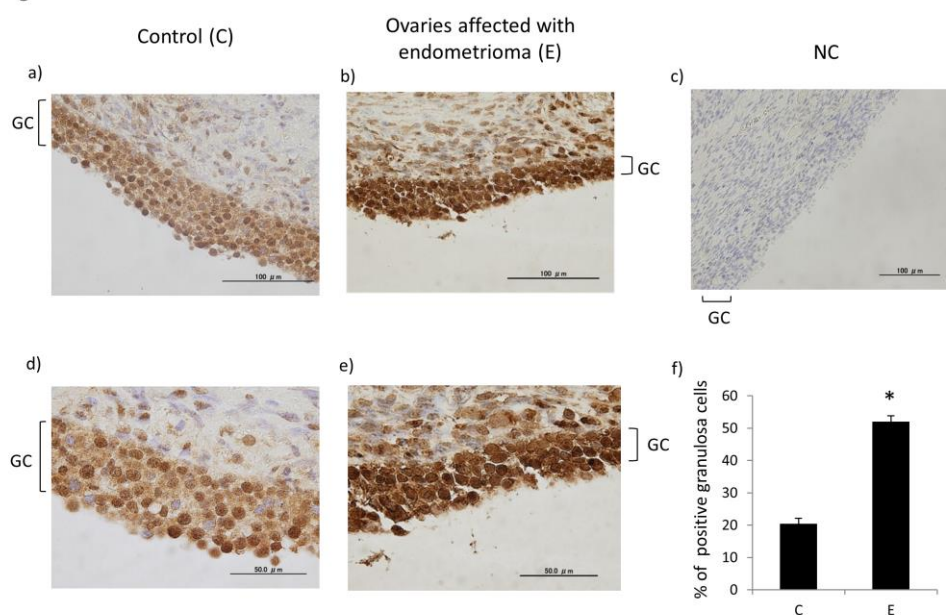


Figure 3 mRNA expression levels of *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP* in GLCs of control patients and patients with endometrioma.

mRNA levels of activating transcription factor 4 (*ATF4*), *ATF6*, spliced X-box-binding protein 1 (*sXBP1*), heat shock 70kDa protein 5 (*HSPA5*), and C/EBP homologous protein (*CHOP*) were examined by quantitative PCR and normalized against the corresponding levels of *GAPDH* mRNA (a–e). Values are shown as mean \pm SEM, relative to the control. The Student's t-test was used for statistical analysis. * $p < 0.05$. C, granulosa-lutein cells (GLCs) from control patients (n=16); E, GLCs from patients with endometrioma (n=7).

Figure 3

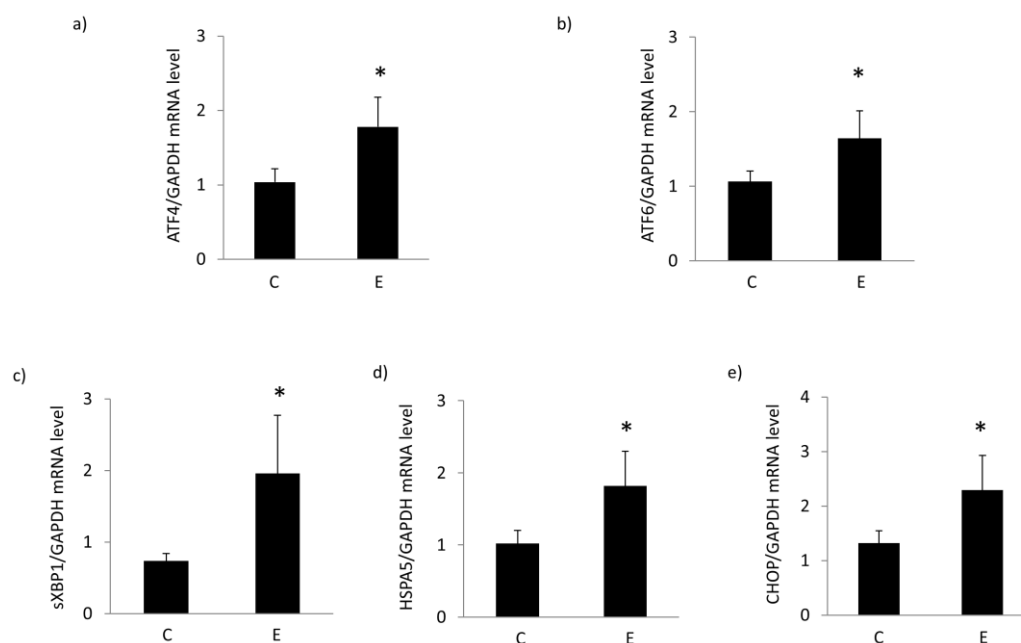


Figure 5 Effect of H₂O₂ on mRNA levels of *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP* in human GLCs.

Human GLCs from the control patients were incubated *in vitro* with 200 μ M H₂O₂ for 12–48 hours (a–e) and with 0–600 μ M H₂O₂ for 24 hours (f–j). Expression levels of *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP* mRNA were examined by quantitative PCR and normalized against the corresponding levels of *GAPDH* mRNA. Values are shown as mean \pm SEM of quadruplicates, relative to the vehicle control treated with PBS for 24 hours. Representative data are shown; at least three replicate experiments were performed on three different samples. For statistical analysis, the Student's t-test was used for comparisons between pairs of samples, and the Tukey–Kramer honest significant difference test for multiple comparisons. Different letters denote significant differences between groups. A p-value < 0.05 was considered statistically significant. *p<0.05.

Figure 5

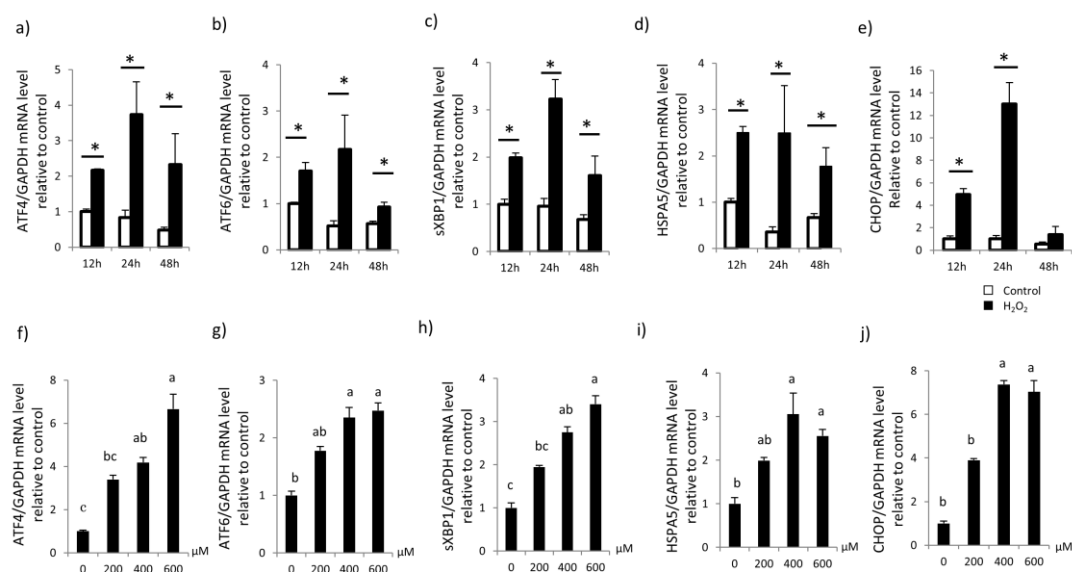


Figure 6 Effect of TUDCA on H₂O₂-induced mRNA expression of *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP* in human GLCs.

GLCs from the control patients were pre-incubated with tauroursodeoxycholic acid (TUDCA) 1 mg/mL for 24 hours, followed by incubation with H₂O₂ (400 μM) for 24 hours. In vehicle control, cells were treated with ethanol for 24 hours, followed by treatment with PBS for 24 hours. Expression levels of *ATF4*, *ATF6*, *sXBP1*, *HSPA5*, and *CHOP* mRNA were examined by quantitative PCR. Data were normalized against the corresponding levels of *GAPDH* mRNA. Values are shown as mean ± SEM of quadruplicates, relative to the control. Representative data are shown; at least three replicates were performed on three different samples. The Tukey–Kramer honest significant difference test was used for statistical analysis. Letters denote significant differences between groups. A p-value < 0.05 was considered statistically significant.

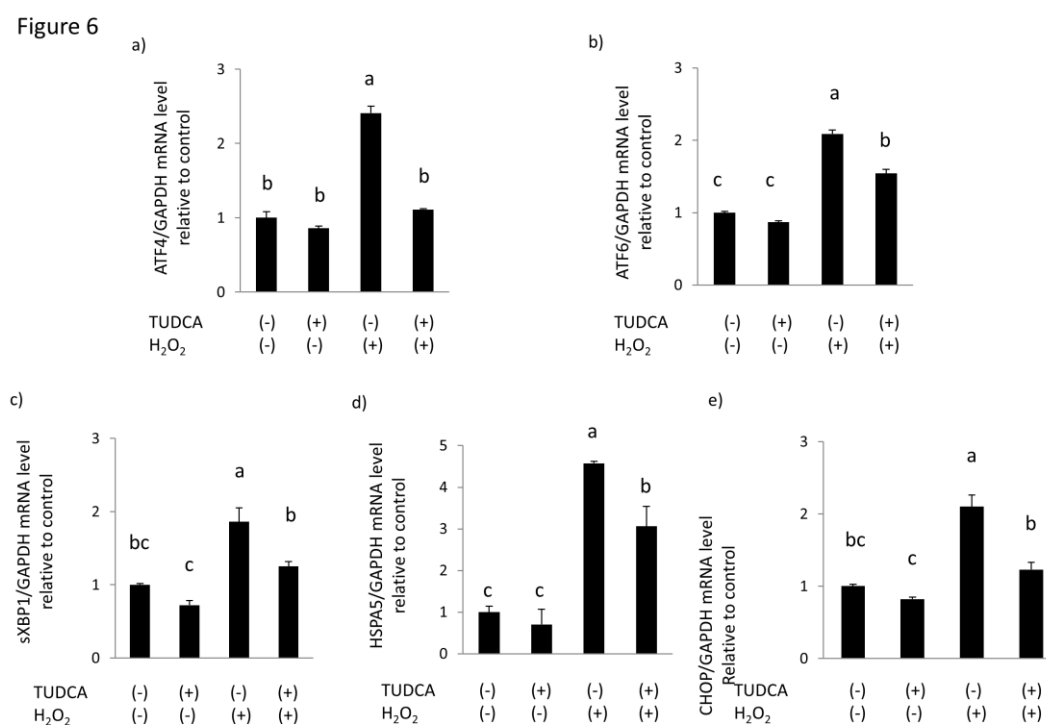


Figure 7 Effect of treatment with H_2O_2 and pre-treatment with TUDCA on induction of apoptosis in human GLCs.

GLCs from the control patients were pre-incubated with TUDCA 1 mg/mL for 24 hours, followed by incubation with H_2O_2 (400 μ M) for 24 hours. In vehicle control, cells were treated with ethanol for 24 hours, followed by treatment with PBS for 24 hours. Apoptosis of GLCs was examined by Annexin V–FITC/PI staining and flow cytometry. (a) A representative set of FACS figures is shown. Dots in the lower right panel in each group (annexin V–positive/PI–negative) show early apoptotic cells, and those in the upper right panel (annexin V–positive/PI–positive) show late apoptotic cells. Number (%) in each column represents the ratio of dots located in the panel relative to all dots in each group. (b) Total percentages of early and late apoptotic cells. Values are shown as mean \pm SEM relative to the control. Representative data are shown; six replicate experiments were performed in six different samples. The Tukey–Kramer honest significant difference test was used for statistical analysis. Different letters denote significant differences between groups. A p-value < 0.05 was considered statistically significant.

Figure 7

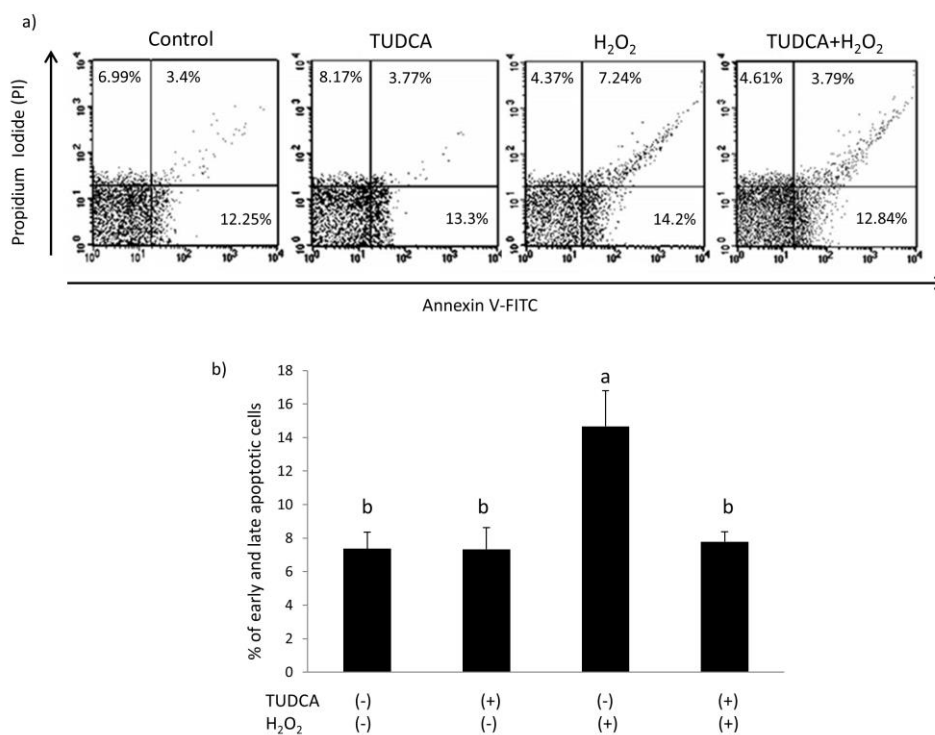


Figure 8 Effect of treatment with H₂O₂ and pre-treatment with TUDCA on mRNA expression and activity of caspase-8 and caspase-3 in human GLCs.

GLCs from the control patients were pre-incubated with TUDCA (1 mg/mL) for 24 hours, followed by incubation with H₂O₂ (400 μM) for 24 hours. Expression levels of caspase-8 and caspase-3 mRNA were examined by quantitative PCR and normalized against the corresponding levels of *GAPDH* mRNA (a,b). Caspase-8 activity assay (c) and western blot analysis with anti-cleaved caspase-3 antibody (d) were performed. Cleaved caspase-3 is an active form of caspase-3. β-Actin was used as a loading control. Values are shown as mean ± SEM of quadruplicates relative to the control. Representative data are shown; at least three replicate experiments were performed on three different samples. For statistical analysis, the Student's t-test was used for comparisons between pairs of samples, and the Tukey–Kramer honest significant difference test for multiple comparisons. Different letters denote significant differences between groups. A p-value < 0.05 was considered statistically significant. *p<0.05.

Figure 8

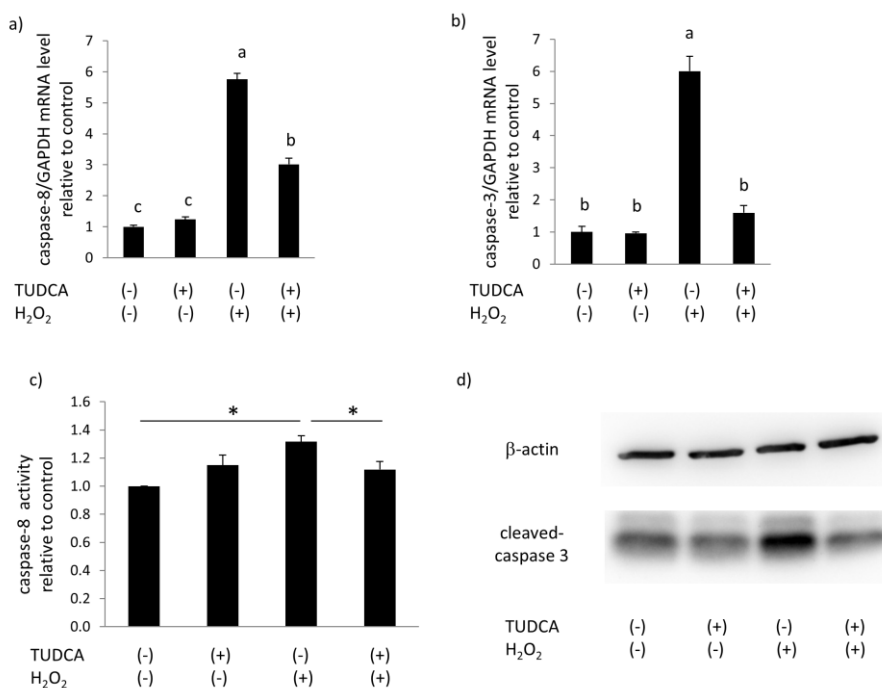


Table I Characteristics of the patients (controls and with endometriosis) and follicular fluid samples.

	Control	Follicle fluid from ovary with endometrioma	<i>p</i> -value
Number of patients	16	7	
Age (years), median (range)	36.5 (30–43)	37 (32–42)	0.425
COS protocols used	GnRH antagonist (10/16) Long (3/16) Short (2/16) FSH (1/16)	GnRH antagonist (6/7) Long (1/7)	0.865
Basal FSH concentration (mIU/mL), median (range)	8.2 (0.1–17.5)	11.4 (5.4–16.3)	0.120
AMH concentration (ng/mL), median (range)	2.745 (0.84–7.36)	1.19 (0.49–3.89)	0.893
Number of oocytes retrieved, median (range)	9 (0–21)	7 (4–9)	0.891

AMH: anti-Müllerian hormone, CC: clomiphene citrate, COS: controlled ovarian stimulation