Glucocorticoids impair oocyte competence and trigger apoptosis of ovarian cells via activating the TNF- α system

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Abstract

Mechanisms by which female stress and particularly glucocorticoids impair oocyte competence are largely unclear. Although one study demonstrated that glucocorticoids triggered apoptosis in ovarian cells and oocytes by activating the FasL/Fas system, other studies suggested that they might induce apoptosis through activating other signaling pathways as well. In this study, both *in vivo* and *in vitro* experiments were conducted to test the hypothesis that glucocorticoids might trigger apoptosis in oocytes and ovarian cells through activating the TNF- α system. The results showed that cortisol injection of female mice (1.) impaired oocyte developmental potential and mitochondrial membrane potential with increased oxidative stress; (2.) induced apoptosis in mural granulosa cells (MGCs) with increased oxidative stress in the ovary; and (3.) activated the TNF- α system in both ovaries and oocytes. Culture with corticosterone induced apoptosis and activated the TNF- α system in MGCs. Knockdown or knockout of TNF- α significantly ameliorated the pro-apoptotic effects of glucocorticoids on oocytes and MGCs. However, culture with corticosterone downregulated TNF- α expression significantly in oviductal epithelial cells. Together, the results demonstrated that glucocorticoids impaired oocyte competence and triggered apoptosis in ovarian cells through activating the TNF- α system and that the effect of glucocorticoids on TNF- α expression might vary between cell types.

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Introduction

Stresses of various types can affect female reproduction in both humans and animals. For example, thin women with a poor psychological profile are at increased risk of giving birth to preterm infants when depressed during pregnancy (Neggers *et al.* 2006), and the state anxiety of women had a significant influence on their outcome of IVF treatment (Csemiczky *et al.* 2000, Kee *et al.* 2000, Klonoff-Cohen *et al.* 2001, Schröder *et al.* 2004). In dairy cows, the heat stress-induced alterations in small antral follicles can lead to compromised maturation and developmental capacity of ovulating oocytes (Roth 2017). Furthermore, exposure of pregnant mice and rats to restraint stress, an effective procedure to mimic the human psychological stress (Paré & Glavin 1986, Glavin *et al.* 1994), impaired function of corpora lutea and decreased pregnancy rates and litter size (Wiebold *et al.* 1986, Sugino *et al.* 1994). However, evidences on the direct effect of stress on the oocyte are limited. Although recent studies indicated that heat stress of dairy cows (Roth 2017) and restraint stress of female mice (Zhang *et al.* 2011) significantly impaired oocyte

developmental potential, the underlying mechanisms are largely unknown.

It is known that stresses activate the hypothalamus-pituitary-adrenal (HPA) axis and increases secretion of glucocorticoids from the adrenal cortex. For example, heat stress in workers can lead to an increase in blood levels of stress hormones including cortisol with cognitive performance impairment (Mazlomi *et al.* 2017). Heat stress in dairy cows significantly increased blood cortisol and reduced milk yield irrespective of the genetic makeup (Ihsanullah *et al.* 2017). Furthermore, mice and rats exposed to various stresses showed significant elevation in serum corticosterone (MacNiven *et al.* 1992, Kim *et al.* 2008, Gong *et al.* 2015) and cortisol (Yin *et al.* 2007, Zhang *et al.* 2011, Gong *et al.* 2015). However, although the previously mentioned data suggested that stressors impaired oocyte competence through activating the HPA axis and increasing secretion of glucocorticoids, treatment of mouse oocytes directly with physiological or stress-induced concentrations of cortisol (Andersen 2003, Zhang *et al.* 2011) or corticosterone (González *et al.* 2010) during *in vitro* maturation did not affect nuclear maturation and embryo development. Thus,

the mechanisms by which glucocorticoids damage the oocyte are worth exploring.

Glucocorticoids triggered Leydig cell death with activation of the Fas system and increased ROS generation (Gao *et al.* 2003). Treatment of osteoblasts with dexamethasone and tumor necrosis factor (TNF)- α promoted their apoptosis (Almeida *et al.* 2011). Furthermore, our previous studies demonstrated that the restraint-induced CRH elevation impaired developmental potential of mouse oocytes by triggering apoptosis of ovarian cells and oocytes within the ovary through activating the Fas system (Liang *et al.* 2013, Li *et al.* 2018). We thus hypothesized that glucocorticoids might also impair oocyte competence indirectly by inducing apoptosis of ovarian cells and oocytes during their development within the ovary. We tested this hypothesis by injecting female mice with cortisol, and the results confirmed that glucocorticoids impaired oocyte competence by triggering apoptosis in ovarian cells including the oocyte through activating the FasL/Fas system (Yuan *et al.* 2016). However, recent studies have shown that restraint stress caused apoptosis in oviductal (Zheng *et al.* 2016) and spermatogenic cells (Xiao *et al.* 2019) as well in the gld mice with a FasL mutation, although to a less severe degree compared with that in the WT mice. This suggests that glucocorticoids may induce apoptosis in ovarian cells and oocytes by activating other signaling pathways in addition to the Fas system.

Both the TNF receptor (TNFR) and the Fas receptor are members of the TNFR superfamily, which can induce cell apoptosis through binding their respective ligands (Kavurma *et al.* 2008). Previous studies have shown that the TNF- α system is active in mammalian ovaries. For instance, human oocytes and cumulus cells express TNF- α and its receptor TNFR2 both at the mRNA and protein levels (Naz *et al.* 1997). Rat oocytes, granulosa cells, and interstitial cells express TNFR and rat oocytes can produce TNF- α (Marcinkiewicz *et al.* 2002). Expression of TNF- α system members was observed in bovine ovarian follicles (Silva *et al.* 2017). *In vitro* treatment with TNF- α impaired maturation of bovine oocytes leading to compromised development of embryos (Soto *et al.* 2003). Mouse oocytes express both TNFR1 and TNFR2 and they are therefore sensitive to TNF-induced cell death (Greenfield *et al.* 2007). Furthermore, treatment of bovine oocytes with 9-cis retinoic acid improved developmental potential with downregulated TNF- α expression (Deb *et al.* 2011).

We therefore proposed that glucocorticoids might impair oocyte competence by triggering apoptosis of oocytes and ovarian cells through activating the TNF- α system during oocyte growth and maturation within the ovary. Both *in vivo* and *in vitro* experiments were conducted to test this hypothesis. In the *in vivo* experiments, female mice were injected with cortisol

before examination for oocyte developmental potential and the redox state, apoptosis, and activation of the TNF- α system in oocytes and ovarian cells. In the *in vitro* experiments, mural granulosa cells (MGCs) were cultured with corticosterone before examination for apoptosis and activation of the TNF- α system. Finally, TNF- α was knocked down in MGCs *in vitro* by RNAi and knocked out *in vivo* by using the TNF- α $-/-$ mice to confirm the role of the TNF- α system in mediating the pro-apoptotic effects of glucocorticoids on oocytes and MGCs.

Materials and methods

Procedures for animal care and handling were carried out exactly in accordance with the guidelines approved by the Shandong Agriculture University Animal Care and Use Committee, P. R. China (Permit number: SDAUA-2019-004). If not mentioned otherwise, all the chemicals and reagents used in this study were purchased from Sigma Chemical Co.

Animals and treatment

Most of the experiments in this study used the Kunming mice, which were bred in this laboratory. The TNF- α ^{-/-} mice with a C57BL/6J genomic background were obtained from Model Animal Research Center of Nanjing University, Nanjing, China, and the WT C57BL/6J mice were purchased from Shandong University Center for Laboratory Animals. The mice were raised in rooms under a 14 h light:10 h darkness photoperiod, with lights turned off at 2000 h. At age of 8–12 weeks after birth, female mice were injected intra-peritoneally with 10 IU of eCG, and at 24 h after eCG injection, while cortisol-injected mice were injected with 50 mg/kg cortisol, the ethanol-injected control mice were injected with 50% ethanol vehicle. This dose of cortisol injection was chosen based on our previous study, which tested 0, 10, and 50 mg/kg and found that cortisol levels in both serum and ovaries were similar between mice injected with 50 mg/kg cortisol and the restraint stressed mice but were significantly higher than that in mice injected with 10 mg/kg bodyweight (Yuan *et al.* 2016). Cortisol was dissolved in 50% alcohol in saline and was administrated intra-peritoneally. Procedures for preparation of cortisol stock solution and injection were conducted exactly as we reported previously (Yuan *et al.* 2016).

Collection of ovaries, oocytes, and mural granulosa cells (MGCs)

At 24 h after cortisol injection or 48 h following eCG injection, mice were killed to recover ovaries. The large follicles on the ovary were ruptured in M2 medium to release oocytes. Only oocytes with more than three layers of unexpanded cumulus cells, containing oocytes larger than 70 μ m in diameter, and with a homogenous cytoplasm were used for experiments. The MGCs sheets released into M2 medium at puncture of follicles were collected for further use.

In vitro maturation of oocytes

Oocytes recovered were washed three times in M2 medium and once in the maturation medium. Then, 20–30 oocytes were cultured in a 100 μ L drop of maturation medium at 37.5°C in a humidified atmosphere of 5% CO₂ in air. The maturation medium used for routine *in vitro* maturation was TCM-199 (Gibco) containing 10% (v/v) fetal calf serum (Gibco), 1 μ g/mL of 17 β -estradiol, 24.2 mg/L of sodium pyruvate, 0.05 IU/mL of FSH, 0.05 IU/mL of LH, and 10 ng/mL of EGF. However, in the maturation medium used to prepare conditioned medium for TNF- α assay, serum, growth factor, and hormone (SGH) were omitted and 24.2 mg/L of sodium pyruvate and 0.3 mg/mL of polyvinyl alcohol were supplemented, because our previous studies had shown that the adverse effect of stress or glucocorticoids on oocytes could be observed immediately after *in vitro* maturation only when oocytes were matured in a poor medium without SGH (Liang *et al.* 2013, Yuan *et al.* 2016).

In vitro fertilization

Male mice were killed by cervical dislocation at 10–12 weeks after birth and cauda epididymidis and vas deferens were removed and placed in 35-mm sterile plastic dishes containing simple PBS. Caudae epididymides and vasa deferentia were cut several times with the edge of an injection needle and squeezed to release sperm masses. Then, the sperm masses were transferred to a drop of 1 mL T6 medium with 10 mg/mL BSA and incubated at 37°C in a CO₂ incubator to allow spermatozoa to swim out. After the sperm concentration of the sperm suspension was adjusted with the same medium to 2–4 $\times 10^7$ sperm/mL, the spermatozoa were incubated for 2 h for capacitation. After being washed in the fertilization medium (T6 with 20 mg/mL BSA), oocytes recovered at 14 h of maturation culture were placed in the fertilization drops (about 20 oocytes/40 μ L drop). Capacitated spermatozoa were added to the fertilization drops to give a final sperm concentration of about 1 $\times 10^6$ mL. After 6 h of incubation, zygotes with two pronuclei and the second polar body were selected for embryo culture.

Oocyte activation

After maturation culture for 24 h, oocytes were freed of cumulus cells by repeatedly pipetting in M2 containing 0.1% hyaluronidase. After being washed twice in M2 and once in the activating medium, the oocytes were incubated in the activating medium for 6 h at 37.5°C in a humidified atmosphere with 5% CO₂ in air. The activating medium was Ca²⁺-free CZB medium supplemented with 10 mM SrCl₂ and 5 μ g/mL cytochalasin B. At the end of the activation treatment, oocytes were examined for activation under a microscope. Oocytes were considered activated when each contained one or two well-developed pronuclei.

Embryo culture

Zygotes obtained from *in vitro* fertilization or activated oocytes generated by Sr²⁺ activation were cultured in regular CZB

medium (about 30 zygotes/oocytes per 100 μ L drop) at 37.5°C in humidified atmosphere containing 5% CO₂ in air. Glucose (5.5 mM) was added to CZB when embryos developed beyond 3- or 4-cell stages. At the end of the embryo culture, embryo development was examined and some of the blastocysts were stained with Hoechst 33342 for cell counting.

Assays for intra-oocyte reactive oxygen species (ROS)

Intra-oocyte ROS was measured by detecting H₂O₂ concentrations using 2',7'-dichlorodihydro-fluorescein diacetate (DCHFDA). To prepare a stock solution (1 mM), DCHFDA was dissolved in DMSO and the stock solution was stored in the dark at –20°C. Immediately before use, the stock solution was diluted to 10 μ M in M2 medium and oocytes that had been freed of cumulus cells were stained in the resultant solution at 37°C for 10 min. Following staining, the oocytes were washed several times in M2, placed on a slide, and examined under a confocal microscope (Leica, TCS SP2) with fluorescence obtained by excitation at 488 nm. All the photographs were taken using the same microscopic parameters. A Leica software was used to analyze the fluorescence intensity of each oocyte.

Measurement of oocyte mitochondrial membrane potential (MMP)

Oocyte MMP was measured using a MMP detection kit (Beyotime Biotechnology Research Institute, China). Cumulus-free oocytes were washed three times in M2, placed in a drop containing 1 mL M2 and 1 mL JC-1 dye working solution, and incubated at 37°C for 25 min. After being washed three times with a JC-1 staining buffer, the oocytes were observed under a Leica confocal microscope. The same oocytes were observed through TRITC channel (red fluorescence) and FITC channel (green fluorescence). The aggregate JC-1 (red fluorescence) was detected at an emission wave length of 590 nm, while the monomeric JC-1 (green fluorescence) was monitored at 529 nm. The ratio of aggregate/monomeric JC-1 was calculated to quantify changes in MMP, and a decreased red/green JC-1 ratio represented depolarization of the mitochondria.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

We dispersed MGCs by pipetting in M2 medium. We concentrated the dispersed MGCs by centrifugation at 200 *g* for 5 min after washing them three times in M2. We then resuspended the pellets with 20 μ L M2 and smeared 5 μ L of the suspension on a slide. After the slides were dried thoroughly in air, we stained them using an *in situ* cell death detection kit (KGA 703, KeyGEN) as follows: (1.) fixation for 5 min in 4% paraformaldehyde at room temperature; (2.) treatment for 5 min with 1% cold Triton X-100; (3.) incubation for 5 min in 50 μ L terminal deoxynucleotidyl transferase (TdT) reaction solution (45 μ L equilibration buffer, 1 μ L biotin-11-dUTP, and 4 μ L TdT enzyme) at 37°C in darkness; (4.) incubation for 30 min in 50 μ L streptavidin-fluorescein labeling solution (45 μ L labeling buffer and 5 μ L streptavidin-fluorescein) at

37°C in darkness; and (5.) incubation for 10 min in 10 µg/mL Hoechst 33342 to stain the nucleus. We then covered the stained smears with coverslips and observed them under a Leica fluorescence microscope. In each smear, we observed three fields to calculate percentages of TUNEL-positive cells from the number of Hoechst-stained nuclei. We determined numbers of the TUNEL-positive cells by using the Image J software.

Preparation for blood serum and ovarian homogenates

Trunk blood was collected from mice, put into ice-cooled centrifugal tubes, and centrifuged (1700g, 10 min, 4°C) to separate serum. The serum obtained was stored at -80°C until use. For homogenization, ovaries were snap-frozen in liquid nitrogen immediately after removal from the mice. The frozen ovaries were weighed and transferred to an electrical homogenizer (ULTRA TURRAX IKA T18 basic) with proper amount of homogenization solution. Homogenization was performed while cooling on ice. The homogenates obtained were centrifuged (15,000g, 10 min, 4°C), and the supernatant was collected for immediate use or stored at -80°C until use.

Culture and treatment of MGCs and oviductal epithelial cells (OECs)

Sheets of MGC released into M2 medium when follicles were punctured were recovered. Oviducts recovered at the same time as ovaries were collected, cut into small pieces, and squeezed to extrude the mucosal tissue. The cell pellets obtained were centrifuged at 200 g for 5 min. After centrifugation, the pellets were digested for 10 min with 0.25% trypsin at 37.5°C and were washed twice by centrifugation (200 g for 5 min). The pellets were then resuspended in DMEM/F12 (Gibco) supplemented with 10% (v/v) fetal calf serum (Gibco) and 0.5% (v/v) penicillin-streptomycin solution (Gibco). After the cell concentration was adjusted to 1×10^5 cells/mL, 2 mL of the final suspension was added to each well of a 6-well culture plate and cultured at 37.5°C in a humidified atmosphere of 5% CO₂ in air. To study the effects of corticosterone supplementation on cultured MGCs, when cells grew to 70–80% of confluence, the medium was renewed and cells were cultured for 24 h in serum- and antibiotics-free DMEM/F12 medium with or without 10^{-5} M (2 µL) of corticosterone and/or of RU486. To prepare stock solutions, corticosterone (10^{-2} M) was dissolved in dimethyl sulfoxide (DMSO) and RU486 (10^{-2} M) was dissolved in absolute alcohol. The stock solutions were stored at -20°C until use. Control cells were cultured in serum- and antibiotics-free DMEM/F12 medium containing 2 µL of DMSO and/or ethanol.

Enzyme-linked immunosorbent assay (ELISA)

Total antioxidant status (TAS) and total oxidant status (TOS) in serum and ovarian homogenates were measured using Mouse TAS ELISA Kit (SU-B20662) and Mouse TOS ELISA Kit (SU-B20663) purchased from Quanzhou Kenuodi Biological Technology Co., Ltd. Levels of TNF-α in serum, ovarian homogenates, conditioned medium, MGCs, and OECs were

measured by ELISA using a mouse tumor necrosis factor α (TNFα) Elisa kit (BLUE GENE, Shanghai, China). The intra-assay CV(%) and inter-assay CV(%) for all the kits were less than 10%. The detection limit for TOS/TAS assays was 0.1 U/mL and that for TNF-α assay was 1 pg/mL. Cultured MGCs were dispersed by repeatedly pipetting in PBS containing 0.25% trypsin and the cells were then lysed by the freeze-thaw method to release proteins. To conduct the ELISA assay, (1.) 100 µL of standards or samples were added in duplicate to wells of a micro-titer plate that had been coated with mouse monoclonal antibodies; (2.) 50 µL of conjugate was added to each well and incubated for 60 min at 37°C; (3.) the micro-titer plate was washed using the wash solution and dried using paper towels; and (4.) 50 µL of substrate A and 50 µL of substrate B were added to each well and incubated for 15 min at 25°C. The optical density was measured at 450 nm using a plate reader (BioTek-ELx808, BioTek Instruments, Inc.) within 15 min after the reaction was terminated by 50 µL of stop solution. The concentrations of TAS, TOS, and TNF-α were calculated against their respective standard curves.

Western blot

The MGCs were placed in a microfuge tube with 50 µL RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethyl sulfonyl fluoride, and 50 mM Tris, pH 8) and were lysed for 30 min. After a 5-min centrifugation at 300g, supernatant was recovered and frozen-stored at -80°C until use. To extract protein, 20 µL of the supernatant and 6.67 µL 4× SDS-PAGE loading buffer were put in each microfuge tube and the tubes were heated to 100°C for 5 min. SDS-PAGE was run on a 12% polyacrylamide gel to separate total proteins, and the proteins obtained were transferred electrophoretically onto polyvinylidene fluoride membranes. The membranes were then (1.) washed in TBST (150 mM NaCl, 2 mM KCl, 25 mM Tris, and 0.05% Tween 20; pH 7.4); (2.) blocked with TBST containing 3% BSA at 37°C for 2 h; (3.) incubated at 4°C overnight with primary antibodies; (4.) washed in TBST; and (5.) incubated for 1.5 h at 37°C with secondary antibodies. The primary antibodies used included rabbit anti TNFR polyclonal antibodies (1:1000, ab19139, Abcam Co., Ltd) and mouse anti-GAPDH antibodies (1:1000, CW0100M, CWBio Co., Ltd), and the secondary antibodies included goat anti-rabbit IgG (1:2000, CW0111, CWBio Co., Ltd, Beijing, China) and goat anti-mouse IgG (1:2000, CW0110, CWBio Co., Ltd). Signals were detected using a 5-bromo-4-chloro-3-indolyl phosphate/tetranitroblue tetrazolium chloride alkaline phosphatase color development kit (Beyotime Institute of Biotechnology). The sum density of each protein band image was analyzed using an Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD, USA). The density value of each sample was normalized to that of internal control, GAPDH, to get its relative quantity value.

Quantification of TNFR1 in oocytes by immunofluorescence microscopy

Cumulus-free oocytes were (1.) fixed for 30 min with 3.7% paraformaldehyde contained in PBS; (2.) treated with 0.5%

protease for 10 s to remove zona pellucida; (3.) permeabilized for 10 min with 0.1% Triton X-100 in PBS at 37.5°C; and (4.) blocked for 30 min with 3% BSA in PBS at 37.5°C; (5.) incubated at 4°C overnight with rabbit polyclonal anti-TNF-R1 (IgG, 1:100, Abcam) in 3% BSA in M2 medium; (6.) incubated for 1 h with Cy3-conjugated goat-anti-rabbit IgG (1:800, Jackson ImmunoResearch) in 3% BSA in M2; and (7.) incubated for 10 min with 10 μ g/mL Hoechst 33342 in M2 to stain chromatin. As negative controls, samples with the primary antibody omitted were also processed. The stained oocytes were mounted on glass slides and observed under a Leica laser scanning confocal microscope (TCS SP2). Blue diode (405 nm) and helium/neon (543 nm) lasers were used to excite Hoechst and Cy3, respectively. Fluorescence was detected with bandpass emission filters: 420–480 nm for Hoechst and 560–605 nm for Cy3. The captured signals were recorded as blue and red, respectively.

Flow cytometry

Annexin/PI staining was performed using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, 556547). Briefly, spent medium was collected as much as possible from each well with cultured MGCs in a 6-well plate and was kept for future use. After being washed twice with cooled PBS, 200 μ L of PBS containing 0.25% trypsin were added to each well. Digestion was carried out at 37°C for 3 to 4 min before termination by adding the spent medium collected previously. The MGCs were then dispersed by repeatedly pipetting in PBS, the resultant suspension was collected into a centrifuge tube and centrifuged at 200 g for 5 min. After supernatant was removed, the cells were resuspended with cooled PBS and washed twice by centrifugation (200 g for 5 min). Then, the cells were resuspended with 100 μ L 1 $\times$ staining buffer, 5 μ L Annexin V-FITC, and 5 μ L PI staining solution and stained for 15 min at 37°C in darkness. At the end of staining, 400 μ L 1 $\times$ staining buffer were added and the mixture was cooled on ice. The stained cells were subjected to flow cytometry (BD LSR Fortessa™) within 1 h after staining to assess apoptosis. Data obtained were analyzed using FlowJo software (FlowJo 7.6 LLC, OR, USA). The cell populations were gated according to the gating information of non-dyed control cells and Annexin V- or PI-dyed control cells.

Transfection of MGCs with siRNAs

The siRNAs targeting mRNAs and the negative control siRNA were designed and synthesized by RiboBio (Guangzhou, China). The sense strands of targeting siRNAs for the TNF- α gene included siRNA-1 (5'-GACAACCACTA GTG GTGC-3'), siRNA-2 (5'-CCA ACG GCA TGG ATC TCAA-3'), and siR-Ribo™ for negative control. Transfection with 100 nM siRNAs was performed using lipofectamine RNAiMAX reagent (Invitrogen/Life Technologies). Briefly, when cells grew to 50–60% of confluence, the spent medium in the wells was replaced by 90 μ L of fresh TCM-199 medium and the cells were transfected by the forward transfection method. Approximately

0.5 μ L of a 20 μ M solution of each siRNA were diluted in 4.5 μ L of Opti-MEM medium (Invitrogen) and mixed with 0.3 μ L of Lipofectamine RNAiMAX reagent (Invitrogen) diluted in 4.7 μ L of Opti-MEM medium. After incubation for 5 min at room temperature, the transfection complex was added to the wells and incubated for 48 h at 37°C in a humidified 5% CO₂ atmosphere.

Real-time PCR

The MGCs recovered from two TNF-/- or C57 mice were treated to extract RNA using the PicoPure RNA Isolation Kit (Applied Biosystems) with on-column DNase treatment (Qiagen). RT was performed in a total volume of 20 μ L using Transcriptor Reverse Transcriptase (Roche). Briefly, 2 μ L of each RNA sample was mixed in a 0.2 mL reaction tube with 1 μ L Oligo dT18 (Fermentas) and 10 μ L DEPC-dH₂O and the mixture was incubated in a PCR instrument at 65°C for 10 min. As soon as the incubation ended, the reaction tube was cooled on ice for 2 min and then centrifuged (200 g for 10 s at 4°C) for a few seconds. Then, 4 μ L of 5 $\times$ RT buffer, 0.5 μ L RNase inhibitor (Roche), 2 μ L dNTP (Fermentas), and 0.5 μ L Transcriptor Reverse Transcriptase were added to the reaction tube. The mixture was then incubated at 55°C for 30 min, followed by incubation at 85°C for 5 min before stored at –20°C until use.

Quantification of mRNA was conducted using the Mx3005P real-time PCR instrument (Stratagene). Amplification reactions were performed in a 10- μ L reaction volume containing 1 μ L of cDNA, 5 μ L of 2 $\times$ SYBR Green Master Mix (Agilent), 0.15 μ L of ROX (reference dye), 3.25 μ L RNase-free water, and 0.3 μ L each of forward and reverse gene-specific primers (10 μ M). Cycle amplification conditions comprised an initial denaturation step at 95°C for 3 min followed by 40 cycles at 95°C for 20 s and 60°C for 20 s. Gene-specific primers used are as follows. For *Gapdh*, forward 5-AAG GTG GTG AAG CAG GCAT-3, reverse 5-GGT CCA GGG TTT CTT ACT CCT-3; for *Bcl-2*, forward 5-TTC GGG ATG GAG TAA ACTGG-3, reverse 5-TGG ATC CAA GGC TCT AGGTG-3; and for *Bax*, forward 5-TGC AGA GGA TGA TTG CTGAC-3, reverse 5-GAT CAG CTC GGG CAC TTTAG-3. Gene expression was normalized to the *Gapdh* internal control. All values were then expressed relative to calibrator samples using the $2^{-(\Delta\Delta CT)}$ method.

Statistical analysis

We repeated each treatment at least three times. Percentage data were arc sine transformed before being analyzed using one-way ANOVA when each measure contained more than two groups or using independent sample *t*-test when each measure had only two groups. We used Duncan multiple comparison test to locate differences during the ANOVA. We used Statistics Package for Social Science (SPSS 20, SPSS, Inc.) to conduct the analysis. We express data as means $\pm$ S.E.M. and considered data significant when the *P* value was less than 0.05.

Results

Cortisol injection of female mice impaired oocyte developmental potential and mitochondrial membrane potential (MMP) with increased oxidative stress

This experiment was conducted to examine the effects of cortisol injection of female mice on oocyte developmental potential and oxidative stress. At 24 h following cortisol injection, both cortisol- and ethanol-injected mice were killed to recover oocytes and the oocytes collected were either inseminated *in vitro* or used for assessment of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). While percentages of fertilized oocytes and 4-cell embryos did not differ, rates of blastocysts and cell number per blastocyst were significantly lower in oocytes recovered from cortisol-injected mice than from control mice injected with ethanol (Fig. 1A). While intra-oocyte ROS increased (Fig. 1B and D), MMP of oocytes decreased significantly (Fig. 1C and E) in cortisol-injected mice compared to that in the ethanol-injected mice. The results confirmed that cortisol injection of female mice significantly impaired oocyte developmental potential with increased oxidative stress.

Cortisol injection of female mice induced apoptosis in MGCs with increased oxidative stress in the ovary

To test whether cortisol injection increases oxidative stress and triggers apoptosis of ovarian cells, both cortisol- and ethanol-injected mice were killed to collect ovaries for assessment of apoptosis in MGCs and oxidative stress index (OSI) in the ovary. Our TUNEL analysis indicated that percentages of apoptotic MGCs were significantly higher in mice injected with cortisol than in control mice injected with ethanol (Fig. 2A and B). Total oxidative status (TOS) and OSI in both serum and ovarian homogenates were significantly higher in cortisol-injected mice than in control mice injected with alcohol (Fig. 2C and D). The results suggested that an elevation in cortisol triggered apoptosis of ovarian cells with increased oxidative stress.

Cortisol injection of female mice activated the TNF- α system in ovaries and oocytes

The purpose of this experiment was to observe whether cortisol injection of female mice would activate the TNF- α system in ovaries. To this end, cortisol- and ethanol-injected mice were killed to collect blood and ovaries. First, TNF- α in serum and ovarian homogenates was measured by ELISA and TNFR1 in MGCs was assayed by Western blotting. The results showed that cortisol injection significantly increased expression of TNF- α in both serum and ovarian homogenates and expression of TNFR1 in MGCs compared to ethanol injection (Fig. 3A and B). The effects of cortisol injection on activation

Figure 1 Effects of cortisol injection of female mice on oocyte developmental potential, redox state, and mitochondrial membrane potential (MMP). Germinal vesicle (GV) stage oocytes were recovered from mice that had been injected with ethanol or cortisol and were used for *in vitro* maturation or for observation of redox state and MMP. (A) Percentages of fertilized oocytes, 4-cell embryos, blastocysts, and cell number per blastocyst following *in vitro* maturation and insemination of GV oocytes. Each treatment was repeated 5–6 times with each replicate containing 20–30 oocytes inseminated with spermatozoa from the same male mouse. Percentages of fertilized oocytes, 4-cell embryos, and blastocysts were calculated from oocytes inseminated, fertilized oocytes, and 4-cell embryos, respectively. (B) Levels (fluorescence intensity values, FIV) of reactive oxygen species (ROS) in oocytes freshly recovered from ethanol- or cortisol-injected mice. (C) MMP (red/green fluorescence intensity) as determined by staining with MMP-specific probe JC-1. In both graphs B and C, each treatment was repeated three times with each replicate containing 20 oocytes. * indicates significant difference ($P < 0.05$) from values of oocytes from ethanol-injected mice. (D) Confocal images showing the FIV of ROS in oocytes from ethanol- or cortisol-injected mice. Bar is 200 μ m. (E) Confocal images showing JC-1 staining intensity in oocytes from ethanol- or cortisol-injected mice. The same oocyte from ethanol- or cortisol-injected mice was observed either in TRITC channel (showing JC-1 aggregates of red fluorescence in the upper row) or in FITC channel (showing JC-1 monomers of green fluorescence in the lower row). Bar is 15 μ m.

of the TNF- α system in oocytes were then observed. To quantify oocyte expression of TNF- α and TNFR, TNF- α in medium conditioned by oocytes were measured by ELISA and TNFR1 in oocytes were quantified by immunofluorescence microscopy. Levels of both TNF- α (Fig. 3C) and TNFR1 (Fig. 3D, E, F and G) were significantly higher in oocytes from cortisol-injected mice than in oocytes from ethanol-injected mice. The results suggested that cortisol elevation significantly activated the TNF- α signaling in both ovaries and oocytes.

Figure 2 Apoptosis in mural granulosa cells (MGCs) and oxidative stress in the ovary after cortisol injection of female mice. Panel A shows images when smears of MGCs from ethanol- and cortisol-injected mice were observed under a fluorescence microscope following Hoechst and TUNEL staining. Original magnification: 400x. Graph B shows percentages of apoptotic cells revealed by TUNEL. Graphs C and D show levels of TAS, TOS, and oxidative index (OSI) in serum and ovarian homogenates, respectively, from mice injected with ethanol or cortisol. Each treatment was repeated nine times with each replicate including serum or ovarian homogenates from a single (the same) mouse. The OSI was calculated as follows: $OSI = TOS/TAS$. * indicates significant difference ($P < 0.05$) from the control (ethanol) values.

Culture of MGCs with corticosterone induced apoptosis and activated the TNF- α system

An *in vitro* experiment was conducted to further verify the effects of glucocorticoids on apoptosis and TNF- α activation of ovarian cells. At 48 h after eCG injection, mice were killed without cortisol or ethanol injection to collect ovaries. The MGCs recovered were cultured in DMEM/F12 medium containing corticosterone alone or with glucocorticoid receptor antagonist, RU486, before examination for apoptosis by flow cytometry, for TNF- α expression by ELISA, and for TNFR1 expression by Western blotting. The results showed that corticosterone significantly increased the percentage of apoptotic MGCs and expression of both TNF- α and TNFR1 and that RU486 completely reversed the pro-apoptotic effects of corticosterone to the level in control MGCs cultured in DMEM/F12 medium alone (Fig. 4). The results further confirmed that glucocorticoids triggered apoptosis and activated the TNF- α signaling in ovarian cells.

Knockdown or knockout of TNF- α significantly ameliorated the pro-apoptotic effects of glucocorticoids on oocytes and MGCs

Two experiments were conducted to verify that glucocorticoids induce apoptosis of oocytes and ovarian

Figure 3 Activation of the TNF- α system in ovaries and oocytes following cortisol injection of female mice. Female mice were injected with eCG and, 24 h later, the mice were injected with ethanol or cortisol. At 24 h after ethanol/cortisol injection, the mice were killed to collect serum and ovaries/MGCs/oocytes. Graph A shows ELISA measurement of TNF- α in serum and ovarian homogenates from ethanol- or cortisol-injected mice. Each treatment was repeated three times with each replicate including three mice. Graph B shows Western blot results of TNFR1 expression in MGCs from ethanol- and cortisol-injected mice. Each treatment was repeated three times with each replicate containing two mice. Graph C shows ELISA-measured TNF- α levels in medium conditioned for 14 h by GV oocytes from ethanol- and cortisol-injected mice in TCM-199 without SGH. Each treatment was repeated seven times with each replicate including 100 μ L medium conditioned by 30 oocytes. Graph D shows relative level of TNFR1/GAPDH (fluorescence intensity value) calculated from confocal microscopic images. * indicates significant difference ($P < 0.05$) from values of oocytes from ethanol-injected mice. Panels E, F, and G are merged laser confocal images showing negative control, ethanol-, and cortisol-treated oocytes, respectively. DNA and TNFR1 are colored blue and red, respectively. Bar is 20 μ m.

cells through activating the TNF- α system. In the first experiment, mice were killed at 48 h after eCG injection without cortisol or ethanol injection to collect ovaries. Expression of the TNF- α gene in MGCs was knocked down by transfection with TNF- α siRNAs. To evaluate the silencing efficiency of different siRNA sequences, TNF- α levels in the transfected cells were measured by ELISA. The ELISA results showed that the MGCs transfected with TNF- α siRNA-1 expressed significantly less TNF- α than MGCs transfected with negative control (NC) siRNA did (Fig. 5A). Then, the transfected MGCs were incubated

Figure 4 Levels of apoptosis, TNF- α , and TNFR1 following culture of MGCs with corticosterone. MGCs were recovered from unstressed mice and cultured in DMEM/F12 medium. When growing to 70–80% confluence, the cells were changed into DMEM/F12 alone (Ctrl), or with 10^{-5} M corticosterone (Cort) or Cort and 10^{-5} M RU486 (C+R), and cultured for 24 h before examination for apoptosis and expression of TNF- α and TNFR1. Panels A, B, and C show flow cytometry graphs following annexin-V and PI staining of MGCs in Ctrl, Cort, and C+R groups, respectively. While almost all of the cells are in the Q3 area in the Ctrl and C+R groups, quite a few cells are located in the Q4 and Q2 areas in the Cort-treated cells, indicating that, significantly, more cells underwent early or late apoptosis after Cort treatment and that RU486 treatment completely counteracted the pro-apoptotic effect by Cort. Graphs D and E show percentages of apoptotic cells revealed by flow cytometry and the TNF- α levels measured by ELISA, respectively, in MGCs in Ctrl, Cort, or C+R treatment groups. Each treatment was repeated three times with each replicate containing MGCs from one well of a 6-well culture plate. Graph F shows relative levels of TNFR1 expression detected by Western blotting in MGCs after different treatments. Each treatment was repeated three times with each replicate including MGCs from one well of a 12-well culture plate. a,b: Values with a different letter above bars differ significantly ($P < 0.05$).

with corticosterone before assessment of apoptosis by flow cytometry. Our flow cytometry indicated that transfection with TNF- α siRNA-1 significantly decreased the percentage of apoptotic MGCs compared to transfection with NC siRNA (Fig. 5B, C and D).

In the second experiment, TNF- α and WT mice with a C57BL/6J genetic background were injected with cortisol or ethanol before recovery of oocytes and MGCs for analysis of oocyte developmental potential and MGC apoptosis, respectively. Analysis of oocyte competence demonstrated that rates of oocyte maturation and activation did not differ among treatments. Percentages of 4-cell embryos/activated oocytes and blastocysts/4-cell embryos did not differ between WT and TNF- α mice following ethanol injection (Fig. 5E). Although cortisol injection significantly decreased the rates of blastocysts in both WT and TNF- α mice, blastocyst rates were significantly higher in TNF- α mice than in WT mice, suggesting that TNF- α knockout significantly relieved the adverse effects of cortisol injection on oocyte developmental potential. Similarly, our real-time PCR quantification showed that the level of Bcl2/Bax ratio in MGCs was significantly higher in TNF- α mice than in WT mice after injection with cortisol (Fig. 5F),

suggesting again that TNF- α knockout significantly relieved the pro-apoptotic effects of cortisol injection on cells. In summary, our TNF- α knockdown and knockout experiments further confirmed that glucocorticoids induced apoptosis of oocytes and ovarian cells through activating the TNF- α system.

Corticosterone treatment showed different effects on TNF- α expression between MGCs and oviductal epithelial cells

Because we found many papers reporting that glucocorticoids inhibit rather than increase TNF- α expression, which was in conflict with our results, we compared effects of corticosterone treatment on TNF- α expression between MGCs and oviductal cells. At 48 h after eCG injection, mice were killed without cortisol or ethanol injection to collect ovaries and oviducts. The monolayers of MGCs and oviductal epithelial cells were cultured for 24 h in DMEM/F12 medium with or without 10^{-5} M corticosterone before ELISA measurement for TNF- α concentrations. While the TNF- α level was significantly increased in MGCs, it was significantly decreased in oviductal epithelial cells following

Figure 5 Effects of knocking down or knocking out TNF- α on the pro-apoptotic effects of glucocorticoids on oocytes and MGCs. Graphs A and B show concentrations of TNF- α and percentages of apoptotic cells, respectively, after MGCs were transfected with negative control (NC), TNF- α siRNA-1 (TN1), or TNF- α siRNA-2 (TN2). TNF- α was measured by ELISA, and percentages of apoptotic cells (Q2 + Q4) were measured by flow cytometry, after the transfected MGCs were challenged with corticosterone. Each treatment was repeated three times with each replicate containing MGCs from one well of a 6-well culture plate. Panels C and D show flow cytometry graphs following annexin-V and PI staining of MGCs in NC and TN1 groups, respectively. Graph E shows percentages of activated oocytes (Act), 4-cell (4-C), and blastocyst (Blast) embryos after WT or TNF-/- mice were injected with ethanol (E) or cortisol (C). Each treatment was repeated four times with each replicate containing about 20 oocytes from two mice. Graph F shows relative level of Bcl2 and Bax and Bcl2/Bax (Bcl2/Bx) ratio in MGCs after WT or TNF-/- mice were injected with ethanol (E) or cortisol (C). Each treatment was repeated three times with each replicate containing MGCs from three mice. a–c: Values without a common letter above bars differ significantly ($P < 0.05$).

corticosterone treatment (Fig. 6). The results suggested that the effect of glucocorticoids on TNF- α expression might vary between cell types.

Discussion

Both our *in vivo* and *in vitro* experiments showed that glucocorticoids induced apoptosis in MGCs and oocytes. Thus, injection of female mice with 50 mg/kg of cortisol, which increased serum cortisol to a restraint-induced level (1.4×10^{-7} M, Yuan *et al.* 2016), significantly impaired developmental potential of oocytes and induced apoptosis in MGCs while increasing oxidative stress. Culture with corticosterone (10^{-5} M) triggered apoptosis in MGCs. There are many reports that glucocorticoids can induce cell apoptosis (Tuckermann *et al.* 2005). For example, treatment of mice with 7 or 10 mg/kg of dexamethasone significantly increased apoptotic indexes in testicular germ cells (Khorsandi *et al.* 2008). Culture with dexamethasone (10^{-6} M) induced apoptosis with activation of the Fas/FasL system in monocytes (Schmidt *et al.* 2001) and osteocytes (Kogianni *et al.* 2004). Furthermore, it is well known that glucocorticoids can induce apoptosis in T lymphocytes (Herold *et al.* 2006).

The present results showed that glucocorticoids impaired oocyte competence and triggered apoptosis in MGCs with increased oxidative stress. Thus, cortisol injection of female mice impaired oocyte

Figure 6 Effects of corticosterone treatment on TNF- α expression in MGCs and oviductal epithelial cells (OECs). Monolayers of MGCs and oviductal epithelial cells were cultured for 24 h in DMEM/F12 medium with (Cort) or without (Ctrl) 10^{-5} M corticosterone before ELISA measurement for TNF- α concentrations. Each treatment was repeated three times with each replicate including OECs from one well of a 6-well plate. * indicates significant difference ($P < 0.05$) from values of control groups.

developmental potential and mitochondrial membrane potential and induced apoptosis in MGCs, both with increased oxidative stress. It has been reported that glucocorticoids can induce cell apoptosis by increasing oxidative stress. For example, glucocorticoids induced apoptosis of lymphocytes by reducing mitochondrial membrane potential and generating reactive oxygen species (ROS) (Zamzami *et al.* 1995). Treatment with dexamethasone and TNF- α promoted ROS production and apoptosis in osteoblasts (Almeida *et al.* 2011). Furthermore, treatment with betulinic acid or selenium decreased dexamethasone-induced apoptosis in mouse thymocytes (Yi *et al.* 2016) and in human osteoblast-like cell line (Yazici *et al.* 2018), respectively, by reducing oxidative stress.

However, because there are reports that agents that reduce the mitochondrial potential (e.g. uncouplers) may decrease ROS production as well (Toime & Brand 2010, Mailloux & Harper 2011), our results that cortisol injection reduced mitochondrial potential while increasing ROS require some explanations. There is evidence that the mitochondrial ROS is critical in initiating mitochondrial inner membrane permeabilization and mitochondrial swelling. For example, Peng and Jou (2004) observed that, in cells exposed to radiation, the intracellular ROS first increased, and then, swelling, which indicates the opening of the mitochondrial permeability transition pore, was observed in mitochondria that contained higher levels of ROS. When the mitochondrial ROS reached the highest level, a complete loss of mitochondrial membrane potential was observed. Furthermore, Lam *et al.* (2001) also reported that the formation of mitochondrial ROS was followed by inner membrane permeabilization, depolarization, and swelling of mitochondria.

This study demonstrated that glucocorticoids impaired oocyte competence and triggered apoptosis in ovarian cells through activating the TNF- α system. Thus, cortisol injection of female mice activated the TNF- α system in both ovaries and oocytes. Culture with corticosterone induced apoptosis and activated the TNF- α system in MGCs. Knockdown or knockout of TNF- α significantly ameliorated the pro-apoptotic effects of glucocorticoids on oocytes and MGCs. Despite of great efforts, however, we could retrieve fewer papers reporting that glucocorticoids upregulate the TNF- α signaling (Dinkel *et al.* 2003) than papers reporting that glucocorticoids inhibit TNF- α -induced apoptosis and TNF- α expression (Messmer *et al.* 2001, Zhang *et al.* 2001). We therefore conducted experiments to clarify this conflict. Monolayers of MGCs and oviductal epithelial cells were cultured for 24 h in DMEM/F12 medium with or without corticosterone before ELISA measurement for TNF- α concentrations. The results showed that, while TNF- α expression was increased in MGCs, it was decreased significantly in oviductal epithelial cells following corticosterone treatment,

suggesting that the effect of glucocorticoids on TNF- α expression might vary between cell types.

In summary, by both *in vivo* and *in vitro* experiments, we have studied the mechanisms by which glucocorticoids impair oocyte competence. The results demonstrated that *in vivo* and/or *in vitro* exposure to glucocorticoids significantly impaired oocyte competence and triggered apoptosis in MGCs with increased oxidative stress and enhanced TNF- α and TNFR expression. *In vitro* TNF- α knockdown by RNAi or *in vivo* knockout of TNF- α significantly relieved the adverse effects of glucocorticoids on oocytes and MGCs. However, *in vitro* exposure to glucocorticoids downregulated TNF- α expression significantly in oviductal epithelial cells. In conclusion, our results suggested that glucocorticoids impaired oocyte competence and triggered apoptosis in ovarian cells through activating the TNF- α system and that the effect of glucocorticoids on TNF- α expression might vary between cell types. The data are not only important for our understanding of the mechanisms by which stress impairs reproduction, but also relevant for our understanding of the role of glucocorticoids in the regulation of the inflammatory responses, as we have shown that the effect of glucocorticoids on TNF- α expression may vary between cell types.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H J Y, Z B L, X Y Z, G Y S, G L W, Y Q Z, and M Z conducted the experiments. H J Y, Z B L, and J H T analyzed the data. J H T designed the experiments and wrote the manuscript. All authors reviewed the manuscript.

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