

## Corticosterone induced apoptosis of mouse oviduct epithelial cells independent of the TNF- $\alpha$ system

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**Abstract.** It has been reported in recent studies that restraint stress on pregnant mice during the preimplantation stage elevated corticotrophin-releasing hormone (CRH) and glucocorticoid levels in the serum and oviducts; furthermore, CRH and corticosterone (CORT) impacted preimplantation embryos indirectly by triggering the apoptosis of oviductal epithelial cells (OECs) through activation of the Fas system. However, it remains unclear whether TNF- $\alpha$  signaling is involved in CRH- and/or glucocorticoid-induced apoptosis of OECs. In the present study, it was shown that culture with either CRH or CORT induced significant apoptosis of OECs. The culture of OECs with CRH augmented both FasL expression and TNF- $\alpha$  expression. However, culture with CORT increased FasL, but decreased TNF- $\alpha$ , expression significantly. Although knocking down/knocking out FasL expression in OECs significantly ameliorated the proapoptotic effects of both CRH and CORT, knocking down/knocking out *TNF- $\alpha$*  expression relieved only the proapoptotic effect of CRH but not that of CORT. Taken together, our results demonstrated that CRH-induced OEC apoptosis involved both Fas signaling and TNF- $\alpha$  signaling. Conversely, CORT-induced OEC apoptosis involved only the Fas, but not the TNF- $\alpha$ , signaling pathway. The data obtained are crucial for our understanding of the mechanisms by which various categories of stress imposed on pregnant females impair embryo development, as well as for the development of measures to protect the embryo from the adverse effects of stress.

**Key words:** Apoptosis, Corticotrophin-releasing hormone (CRH), Glucocorticoids, Oviductal epithelial cells, TNF- $\alpha$  signaling (J. Reprod. Dev. 67: 43–51, 2021)

**A**lthough it is established that various kinds of stress can impair pregnancy in both humans [1, 2] and animals [3, 4], the mechanisms by which such stress, acting on pregnant females, impairs embryo development are largely unknown. While prior research has revealed that the initial stages of pregnancy are more vulnerable to stress than the later stages [5, 6], and that the preimplantation and peri-implantation stages are among the most sensitive periods [7, 8], detailed studies on the effects of maternal stress during the preimplantation stage on embryo development are limited [9, 10].

Zheng and colleagues [11] observed that restraint stress on pregnant mice during the preimplantation stage resulted in a rise in the serum and oviduct levels of corticotrophin-releasing hormone (CRH) and glucocorticoids. However, it was unknown whether the stress-associated elevation of glucocorticoids and CRH would act on embryos directly or indirectly through oviductal epithelial cells (OECs). It is known that the oviduct and uterine epithelia are the pivotal tissues instrumental in the establishment of a healthy pregnancy

[12]. For example, the oviduct and uterus secrete growth factors that stimulate the development of preimplantation embryos [13]. Furthermore, TNF mRNA has been detected in mouse OECs [14], and Fas protein was expressed by both ciliated cells and secretory cells in the bovine oviduct [15].

However, while Tan and colleagues [16] showed that CRH and corticosterone (CORT) affected preimplantation embryos by triggering apoptosis of OECs via activation of the Fas system, Drew and Chavis [17] demonstrated that cortisol inhibited the release of TNF- $\alpha$  from microglial cells that were treated with lipopolysaccharide to induce apoptosis. Thus, whether CRH and/or glucocorticoids induce apoptosis of OECs by activating TNF- $\alpha$  signaling remains to be explored.

The objective of the present study was to investigate whether CRH and/or glucocorticoids induce the apoptosis of OECs by activating TNF- $\alpha$  signaling. To this end, mouse OECs were first cultured in the presence of CRH or CORT before evaluating the rate of apoptosis as well as FasL and TNF- $\alpha$  expression. The *FasL* and *TNF- $\alpha$*  genes in OECs were then knocked down by RNA interference before culture with CRH or CORT and examined for apoptosis. Finally, OECs obtained from generalized lymphoproliferative disorder (*gld*) mice harboring a *FasL* mutation and from *TNF- $\alpha$*  knockout mice were cultured in the presence of CRH or CORT before examination for apoptosis. Our results demonstrated that while CRH triggered the apoptosis of OECs by activating both the FasL system and the TNF- $\alpha$  system, glucocorticoids did so by activating Fas but not the TNF- $\alpha$  system.

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## Materials and Methods

Animal care and handling were conducted according to the guidelines approved by the Shandong Agricultural University Animal Care and Use Committee, P. R. China (Permit number: SDAUA-2019-004). Unless otherwise mentioned, all the chemicals and reagents employed in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Experimental animals

Mice of the Kunming strain were employed in most experiments conducted for the present study; they were originally derived from ICR (CD-1) mice. The Kunming mice were bred in our laboratory (Shandong Agricultural University). Generalized lymphoproliferative disorder (gld) mice with a germline mutation F273L in *FasL* in a C57BL/6J genomic background were obtained from the Key Laboratory of Stem Cell Biology, Shanghai Institute of Biological Sciences, China.  $\text{TNF-}\alpha^{-/-}$  mice with a C57BL/6J genomic background were purchased from the Model Animal Research Center of Nanjing University, Nanjing, China. Wild-type C57BL/6J mice were purchased from Shandong University Center for Laboratory Animals, Jinan, China. All the mice were kept in rooms under a photoperiod of 14-h light/ 10 h darkness, with lights off at 2000 h.

### Culture of oviduct epithelium cells (OECs)

Female mice (8–12 weeks old) were injected with eCG (10 IU/mouse); at 48 h after eCG injection, the mice were sacrificed to recover oviducts. The oviducts recovered from three mice were cut into segments, and each segment was squeezed using forceps to release the oviduct epithelium tissue. The tissue blocks obtained were digested with 0.25% trypsin at 37°C for 30 min and washed twice by centrifugation (200 g, 5 min). The pellets were resuspended in 2 ml of Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; Gibco, Beijing, China) supplemented with 10% fetal calf serum (Gibco) and 0.5% penicillin–streptomycin solution (Gibco). The final suspension was added to two wells (1 ml per well) of a 12-well culture plate and cultured at 37.5°C under a humidified atmosphere of 5% CO<sub>2</sub> in air. When they grew to about 80–90% of confluence at 18–24 h of culture, the cells in each well were digested with trypsin, resuspended in 2 ml DMEM/F12, and cultured in two wells (1 ml per well) of a 12-well culture plate. When the subcultures reached approximately 50% confluence at 24–36 h of culture, the OECs were cultured for 48 h in the presence of CRH or CORT before further analysis. The concentrations of CRH and CORT used were  $2 \times 10^{-6}$  M and  $1 \times 10^{-5}$  M, respectively, which can induce apoptosis of OECs both efficiently and reversibly [16]. To prepare stock solutions, CRH (10 mM) was dissolved in water, and CORT (10 mM) was dissolved in ethanol. The stock solutions were stored at –20 °C until use.

### Apoptosis assessment of OECs by Hoechst 33342 staining

Cultured OECs were stained *in situ* in wells of a 12-well plate using 0.01 mg/ml of Hoechst 33342 for 5 min in the dark. The stained cells were examined under a Leica DMLB fluorescence microscope at a magnification of 400 $\times$ . Six fields were randomly observed in each well. The heterochromatin was heavily stained and was identified by its characteristic bright fluorescence. While

the apoptotic cells showed pyknotic nuclei full of heterochromatin, healthy cells showed normal nuclei with sparse heterochromatin spots. To reduce subjectivity, percentages of the apoptotic cells were always calculated in a double-blind approach by two investigators.

### Apoptosis assessment of OECs by flow cytometry

Annexin/PI staining was performed using a BD Pharmingen FITC Annexin V Apoptosis Detection Kit (BD Biosciences, 0027279). Briefly, the spent medium was collected, to the maximum possible extent, from wells with cultured OECs and preserved for future use. After the OECs were washed twice with cooled PBS, 200  $\mu$ l PBS containing 0.25% trypsin was added to each well. Digestion was carried out at 37.5°C for 3 to 4 min, and terminated by adding the spent medium collected previously. The cells were then dispersed by repeatedly pipetting in PBS, and the resultant suspension was centrifuged at 200  $\times$  g for 5 min. After the supernatant was removed, the cells were resuspended in cooled PBS and washed twice by centrifugation at 200 g for 5 min. Thereafter, cells were resuspended in 100  $\mu$ l of 1  $\times$  staining buffer, 5  $\mu$ l Annexin V-FITC, and 5  $\mu$ l PI staining solution, and stained for 15 min at 37°C in the dark. At the end of staining, 400  $\mu$ l of 1  $\times$  staining buffer was added and the mixture was cooled on ice. Flow cytometry was performed on the stained cells using BD LSR Fortessa™ within 1 h after staining to assess apoptosis. We analyzed the data using FlowJo software (FlowJo 10 LLC; Treestar, Ashland, 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. The lower left quadrant contains the healthy (double-negative) population, the lower right quadrant contains the early apoptotic (annexin V-positive/PI-negative) population, the upper left quadrant contains the damaged (annexin V-negative/PI-positive) cells, and the upper right quadrant contains the late apoptotic and necrotic (double-positive) cells.

### Real-time PCR

To isolate RNA, the spent medium was removed to the maximum possible extent from wells with cultured OECs, and 1 ml Trizol reagent was added to each well. The cells were pipetted for 5 min to facilitate lysis. Thereafter, RNA was resuspended in DEPC-dH<sub>2</sub>O before digestion with RNase-free DNase 1 (Takara Biotechniques, Dalian, China). The RNA obtained was spectroscopically quantified at 260 nm. The ratio of A260:A280 (1.8–2.0) was determined and electrophoresis in 1% agarose was carried out to assess RNA purity and integrity.

Reverse transcription was conducted using a total volume of 20  $\mu$ l and Superscript III Reverse Transcriptase (Invitrogen Australia Pty., Mulgrave, Australia). First, 2  $\mu$ l of RNA sample, 1  $\mu$ l Oligo dT18 (Takara), and 10  $\mu$ l DEPC-dH<sub>2</sub>O were mixed in a reaction tube, and the mixture was incubated at 65°C for 10 min in a PCR instrument (Thermo Scientific, Hudson, NH, USA). Second, the reaction tube was cooled for 2 min on ice before a brief centrifugation (200  $\times$  g, 4°C) step. Third, 5 $\times$  reverse transcription buffer (4  $\mu$ l), RNase inhibitor (0.5  $\mu$ l), dNTP (2  $\mu$ l), and Superscript III Reverse Transcriptase (0.5  $\mu$ l) were added to the reaction tube. The mixture was incubated at 42°C for 1 h, at 70°C for 15 min, and stored at –80°C until use.

Gene-specific primers used are as follows: For *Bcl2*, forward 5'- TTC GGG ATG GAG TAA ACT GG -3', reverse 5'- TGG ATC

CAA GGC TCT AGG TG-3'; for *Bax*, forward 5'- TGC AGA GGA TGA TTG CTG AC-3', reverse 5'- TGG ATC CAA GGC TCT AGG TG -3'; and for *Gapdh*, forward 5'- AAA CCR GCC AAG TAT GAT GA-3', reverse 5'- GTG GTC CAG GGT TTC TTA CT-3'. A Mx3005P Real-Time PCR System (Stratagene, Valencia, CA, USA) was used to conduct mRNA quantification in a 10- $\mu$ l reaction volume including 1  $\mu$ l of cDNA, 5  $\mu$ l of  $2 \times$  SYBR Green Master Mix (Stratagene), 0.15  $\mu$ l of  $500 \times$  diluted reference dye, 3.25  $\mu$ l of RNase-free water, and 0.3  $\mu$ l each of forward and reverse gene-specific primers (10  $\mu$ M). Cycle amplification conditions included denaturation at 95°C for 10 min, 40 cycles at 95°C for 5 sec, and 58°C for 20 sec. Expression of all genes was first normalized to *Gapdh* (internal control), following which all values were expressed relative to the calibrator samples using the  $2^{-\Delta\Delta CT}$  method.

#### Enzyme-linked immunosorbent assay (ELISA)

The ELISA for FasL and TNF- $\alpha$  in OECs was carried out using Mouse FasL Elisa kit (BG191223MUC) and Mouse TNF- $\alpha$  Elisa kit (BG190628MUC), respectively. The FasL and TNF- $\alpha$  ELISA kits were purchased from BlueGene, Shanghai, China. The OECs were digested with 0.25% trypsin and dispersed by repeatedly pipetting in PBS, as described above. Assay procedures were performed according to the manufacturer's instructions.

#### Transfection of OECs with siRNAs

The targeting siRNAs and negative control siRNAs were designed and synthesized by RiboBio (Shanghai, China). The sense strands of targeting siRNAs for FasL and TNF- $\alpha$  included FasL siRNA-1 (5'-GCA GTA TTC AAT CTT ACCA-3'), FasL siRNA-2 (5'-GGC CTT GTG ATC AAC GAAA-3'), FasL siRNA-3 (5'-CCG TGA GTT CAC CAA CCAA-3'), TNF- $\alpha$  siRNA-1 (5'-GAC AAC CAA CTA GTG GTGC-3'), TNF- $\alpha$  siRNA-2 (5'-CCA ACG GCA TGG ATC TCAA-3'), TNF- $\alpha$  siRNA-3 (5'-CGT CGT AGC AAA CCA CCAA-3'), and siR-Ribo<sup>TM</sup> for negative control. Transfection with 100 nM siRNAs was carried out using Lipofectamine RNAiMAX reagent (Invitrogen/Life Technologies). When the OEC subcultures attained 60–70% of confluence, the spent medium in the wells was replaced with 900  $\mu$ l of fresh DMEM/F12, and the cells were transfected by the forward transfection method. To prepare the transfection complex, 5  $\mu$ l of a 20  $\mu$ M solution of each siRNA was diluted in 45  $\mu$ l of Opti-MEM medium (Invitrogen) and mixed with 3  $\mu$ l of lipofectamine RNAiMAX reagent (Invitrogen) diluted in 47  $\mu$ l of Opti-MEM medium. Following a 5-min incubation at room temperature, the transfection complex was added to the wells and incubated for 48 h at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> in air.

#### Data analysis

Each treatment was repeated at least three times. The percentage data were arcsine transformed before analysis. Data were analyzed using one-way ANOVA. A Duncan multiple comparison test was performed to determine differences during ANOVA. The software used was the Statistics Package for Social Science (SPSS 20, SPSS). Data are expressed as mean  $\pm$  SEM, and the difference was considered significant when the P value was less than 0.05.

## Results

#### Effects of *in vitro* exposure to CRH or CORT on apoptosis of OECs

When mouse OECs harvested 48 h post eCG injection attained 70–90% of confluence (Fig. 1A), they were sub-cultured. When the subcultures reached approximately 50% confluence (Fig. 1B), the OECs were cultured for 48 h in the presence of  $2 \times 10^{-6}$  M CRH or  $1 \times 10^{-5}$  M CORT before the assessment of apoptosis. Control OECs were cultured for 48 h without CRH or CORT. Hoechst staining showed that the percentage of apoptotic cells was significantly higher in OECs cultured with CRH/CORT than in control OECs (Figs. 1C, D, E, and F). Our flow cytometry results showed that while the percentage of cells undergoing early or late apoptosis/necrosis was significantly higher (Figs. 2A to D), the ratio of *Bcl2/Bax* mRNAs was significantly lower (Fig. 2E) in OECs cultured with than without CRH or CORT. Consequently, it was inferred that the culture of OECs with either CRH or CORT induced severe apoptosis.

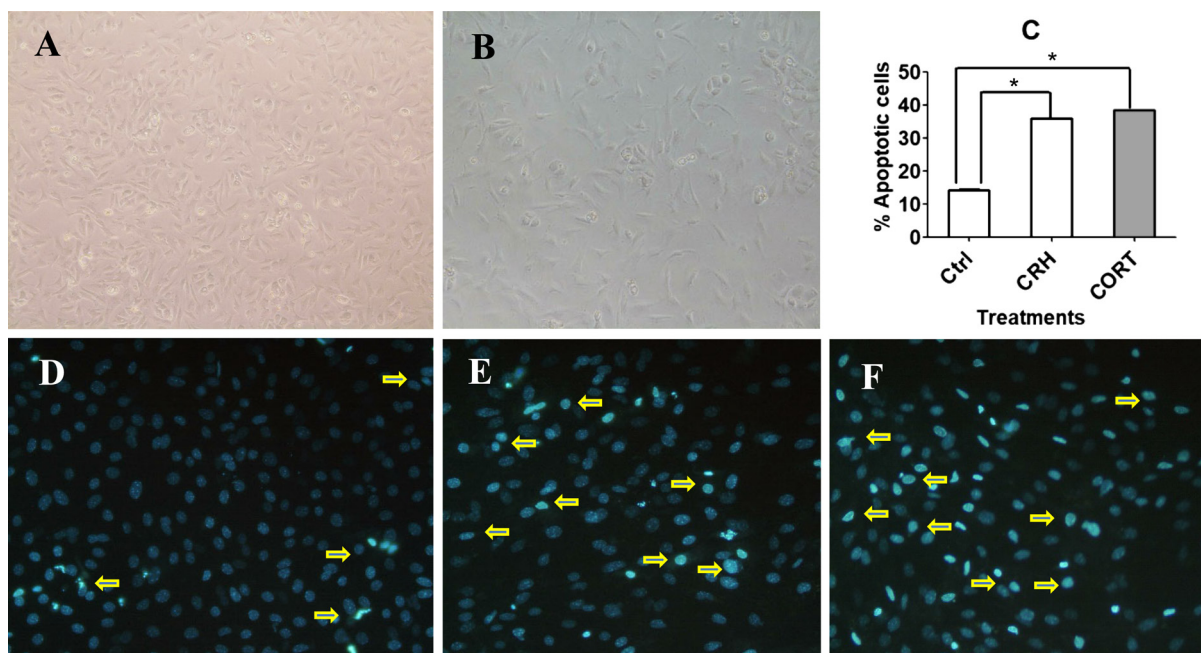
#### Effects of *in vitro* exposure of OECs to CRH or CORT on the FasL and TNF- $\alpha$ expression

The OECs were cultured for 48 h in the presence of CRH or CORT before ELISA-based measurement of FasL and TNF- $\alpha$  levels. Culture with either CRH or CORT significantly enhanced FasL expression (Fig. 3A). However, while culture with CRH augmented TNF- $\alpha$  expression, culture with CORT significantly diminished TNF- $\alpha$  expression (Fig. 3B). Thus, while CRH triggered OEC apoptosis through both the Fas system and TNF- $\alpha$  signaling, CORT did so through Fas signaling but not through TNF- $\alpha$  signaling.

#### Effects of knocking down FasL or TNF- $\alpha$ on CRH- or CORT-induced apoptosis of OECs

To evaluate the regulatory role of FasL and TNF- $\alpha$  in CRH- or CORT-induced apoptosis of OECs, *FasL* and *TNF- $\alpha$*  were knocked down by RNA interference. To assess the efficiency of different siRNA sequences, FasL and TNF- $\alpha$  levels were measured by ELISA in cells transfected with *FasL* siRNA and *TNF- $\alpha$*  siRNA, respectively. Levels of FasL and TNF- $\alpha$  declined significantly following transfection with *FasL* siRNA-2 and *TNF- $\alpha$*  siRNA-2 and -3 compared to the corresponding levels following transfection with negative control siRNA (Figs. 4A and B). Thus, *FasL* siRNA-2 and *TNF- $\alpha$*  siRNA-2 were used in the subsequent experiments.

When cultured in the presence of CRH, the percentage of cells undergoing early or late apoptosis/necrosis was significantly lower (Figs. 4C to F) and the ratio of *Bcl2/Bax* mRNAs was significantly higher (Fig. 4G) in OECs transfected with either *FasL* siRNA-2 or *TNF- $\alpha$*  siRNA-2 than in those transfected with negative control siRNA. The results indicated that knocking down either FasL or TNF- $\alpha$  significantly ameliorated the proapoptotic effects of CRH on OECs. When cultured in the presence of CORT, however, only transfection with *FasL* siRNA-2 decreased the percentage of cells undergoing early and late apoptosis/necrosis (E + L/N) significantly compared to that following transfection with negative control siRNA. However, transfection with *TNF- $\alpha$*  siRNA-2 did not impact the percentages of E + L/N cells (Figs. 4H, J, K, and L). While transfection with *FasL* siRNA-2 increased the ratio of *Bcl2/Bax* mRNAs,



**Fig. 1.** Culture of oviductal epithelial cells (OECs) and Hoechst staining for apoptosis assessment. Micrographs A and B show primary culture of OECs with 70–90% of confluence and subculture of OECs with approximately 50% of confluence, respectively. Micrographs D, E, and F show OECs following Hoechst staining after culture without (Ctrl) or with corticotrophin-releasing hormone (CRH) or corticosterone (CORT), respectively. Arrows indicate apoptotic cells with pyknotic nuclei heavily stained by Hoechst 33342. The micrographs were taken at an original magnification of  $\times 400$ . Graph C shows percentages of apoptotic cells as observed by Hoechst staining after OECs were cultured without (Ctrl) or with CRH or CORT. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice. \* indicates significant differences ( $P < 0.05$ ) between two groups.

transfection with *TNF- $\alpha$*  siRNA-2 decreased the ratio significantly (Fig. 4I). Taken together, the results indicated that CRH-induced OEC apoptosis involved both Fas signaling and *TNF- $\alpha$*  signaling; conversely, CORT-induced OEC apoptosis involved only Fas signaling but not *TNF- $\alpha$*  signaling.

#### *Effects of knocking out FasL or TNF- $\alpha$ on CRH- or CORT-induced apoptosis of OECs*

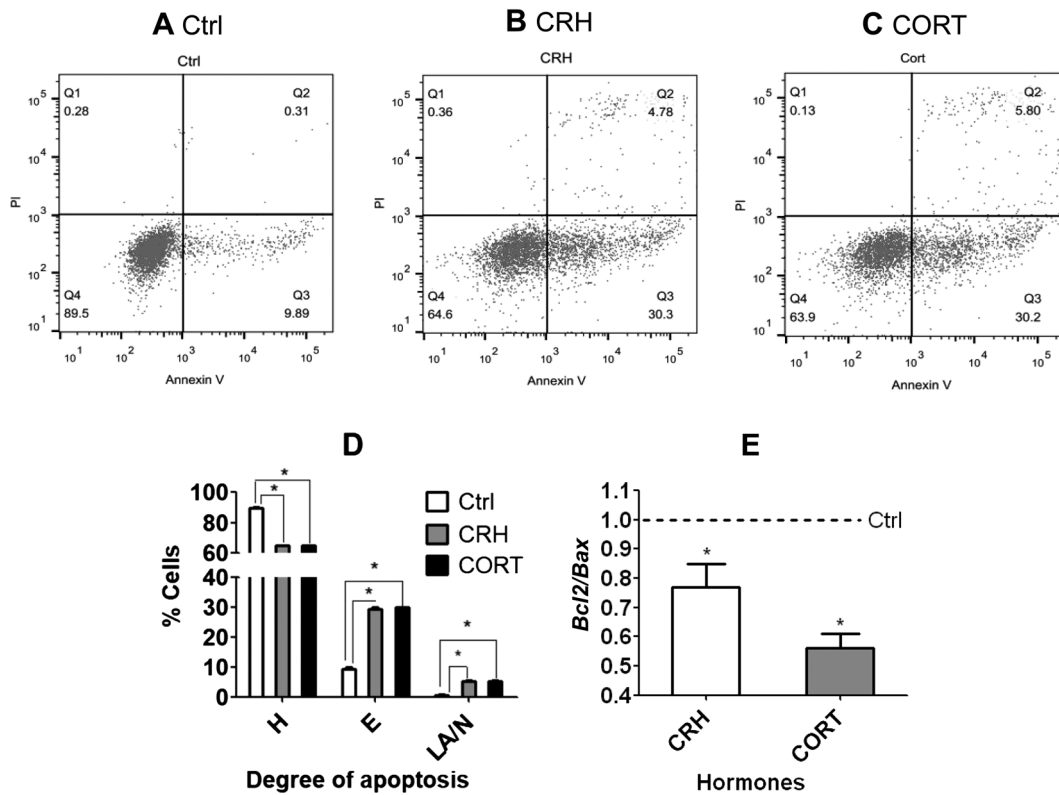
To further verify the role of FasL and *TNF- $\alpha$*  in mediating the CRH- or CORT-induced apoptosis of OECs, OECs recovered from the *gld* mice harboring a *FasL* mutation and those from the *TNF- $\alpha$*  knockout (*TNF- $\alpha$ <sup>-/-</sup>*) mice were cultured for 48 h in the presence of CRH or CORT before assessment for apoptosis by flow cytometry. OECs recovered from C57BL/6J mice were also cultured with CRH or CORT for the same period to serve as controls. When cultured with CRH, while the percentages of healthy cells were higher, those of early and late apoptotic/necrotic cells were significantly lower in both *gld* and *TNF- $\alpha$ <sup>-/-</sup>* OECs than in C57 OECs (Figs. 5A, C, D, and E). However, when cultured with CORT, while the percentages of early and late apoptotic/necrotic cells were lower in *gld* OECs than in the control C57 OECs, these percentages did not differ between the *TNF- $\alpha$ <sup>-/-</sup>* OECs and the control C57 OECs (Figs. 5B, F, G, and H). Taken together, the results further confirmed that while CRH induced apoptosis in OECs by activating both Fas signaling and *TNF- $\alpha$*  signaling, CORT triggered OEC apoptosis by activating the Fas system but not involving *TNF- $\alpha$*  signaling.

## Discussion

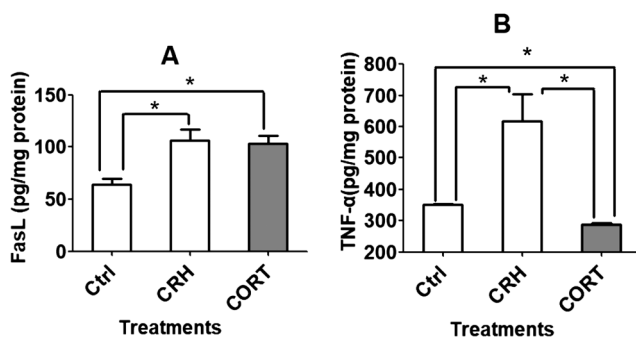
The findings of the present study demonstrated that culture with either CRH or CORT induced significant apoptosis of OECs. While treatment of OECs with CRH augmented both FasL expression and *TNF- $\alpha$*  expression, treatment with CORT increased FasL expression while concomitantly diminishing *TNF- $\alpha$*  expression. Although knocking down or knocking out *FasL* expression in OECs significantly ameliorated the proapoptotic effects of both CRH and CORT, knocking down or knocking out *TNF- $\alpha$*  expression relieved only the proapoptotic effect of CRH but not that of CORT. In summary, the results indicated that while CRH induced OEC apoptosis through both Fas signaling and *TNF- $\alpha$*  signaling, CORT induced OEC apoptosis only via Fas, without the involvement of *TNF- $\alpha$*  signaling.

Prior studies have reported that CRH triggers apoptosis in different cells *in vitro*. For instance, Zhang and colleagues [18] observed that CRH induced the apoptosis of hippocampal neurons *in vitro*, and its receptor (CRHR1) is involved in this process. Dermitzaki and colleagues [19] demonstrated that CRH triggered apoptosis in the PC12 rat pheochromocytoma cell line by activating p38 mitogen-activated protein kinase. Ock *et al.* [20] reported that CRH induced classical apoptosis in mouse microglia cultured *in vitro*. Jin *et al.* [21] showed that CRH promoted apoptosis in prostate cancer cells *in vitro*. Furthermore, treatment of cultured mouse mural granulosa cells (MGCs) with CRH induced significant apoptosis [22].

Glucocorticoids also trigger apoptosis in various cells *in vitro*. For



**Fig. 2.** Effects of *in vitro* exposure to corticotrophin-releasing hormone (CRH) or corticosterone (CORT) on the apoptosis of oviductal epithelial cells (OECs). A to C show flow cytometry graphs following annexin-V and PI staining of OECs cultured without (A), or with CRH (B), or CORT (C). Graph D shows percentages of healthy (H), early apoptotic (E), or late apoptotic and necrotic (LA/N) cells, as revealed by flow cytometry. \* indicates significant differences ( $P < 0.05$ ) between two groups. Graph E shows the ratio of *Bcl2/Bax* mRNAs as measured by RT-PCR in OECs in the Ctrl, CRH, and CORT groups. The ratio in the Ctrl group was set to 1 (dotted line), and the ratio in the treatment groups were expressed relative to it. \* indicates significant difference ( $P < 0.05$ ) from the control group. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice.



**Fig. 3.** Effects of *in vitro* exposure to corticotrophin-releasing hormone (CRH) or corticosterone (CORT) on FasL and TNF- $\alpha$  expression in oviductal epithelial cells (OECs). Graphs A and B show FasL expression and TNF- $\alpha$  expression (ELISA results), respectively, in OECs after culture with CRH or CORT for 48 h. Control (Ctrl) cells were cultured for 48 h with neither CRH nor CORT. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice. \* indicates significant differences ( $P < 0.05$ ) between two groups.

example, Schmidt and colleagues [23] found that *in vitro* treatment of human monocytes with glucocorticoids resulted in apoptosis in a time- and dose-dependent manner. Treatment with dexamethasone triggered apoptosis in MLO-Y4 osteocytes [24]. Culture of human fetal ovaries with dexamethasone significantly accelerated the apoptotic rates of germ cells [25]. Furthermore, culture of MGCs with corticosterone induced apoptosis [26].

Minas and colleagues [27] observed that CRH augmented FasL expression in ovarian cancer cells of OvCa3 and A2780 lines through CRHR1. Taliouri and colleagues [28] found that CRH could induce both transcription and translation of FasL in HeLa cells. Petsas and colleagues [29] reported that CRH induced FasL expression in human macrophages and enabled them to induce apoptosis of cocultured Fas-expressing cells. Li and colleagues [22] demonstrated that CRH significantly elevated the levels of FasL and Fas expression during culture of MGCs. Furthermore, Tan and colleagues [16] showed that CRH augmented the FasL and Fas expression in cultured OECs. However, there are relatively few studies reporting that CRH promotes TNF- $\alpha$  expression in different cells. Song and colleagues [30] reported that CRH facilitated TNF- $\alpha$  production in CD14+ cells and triggered apoptosis in endothelial cells. Zhao *et al.*

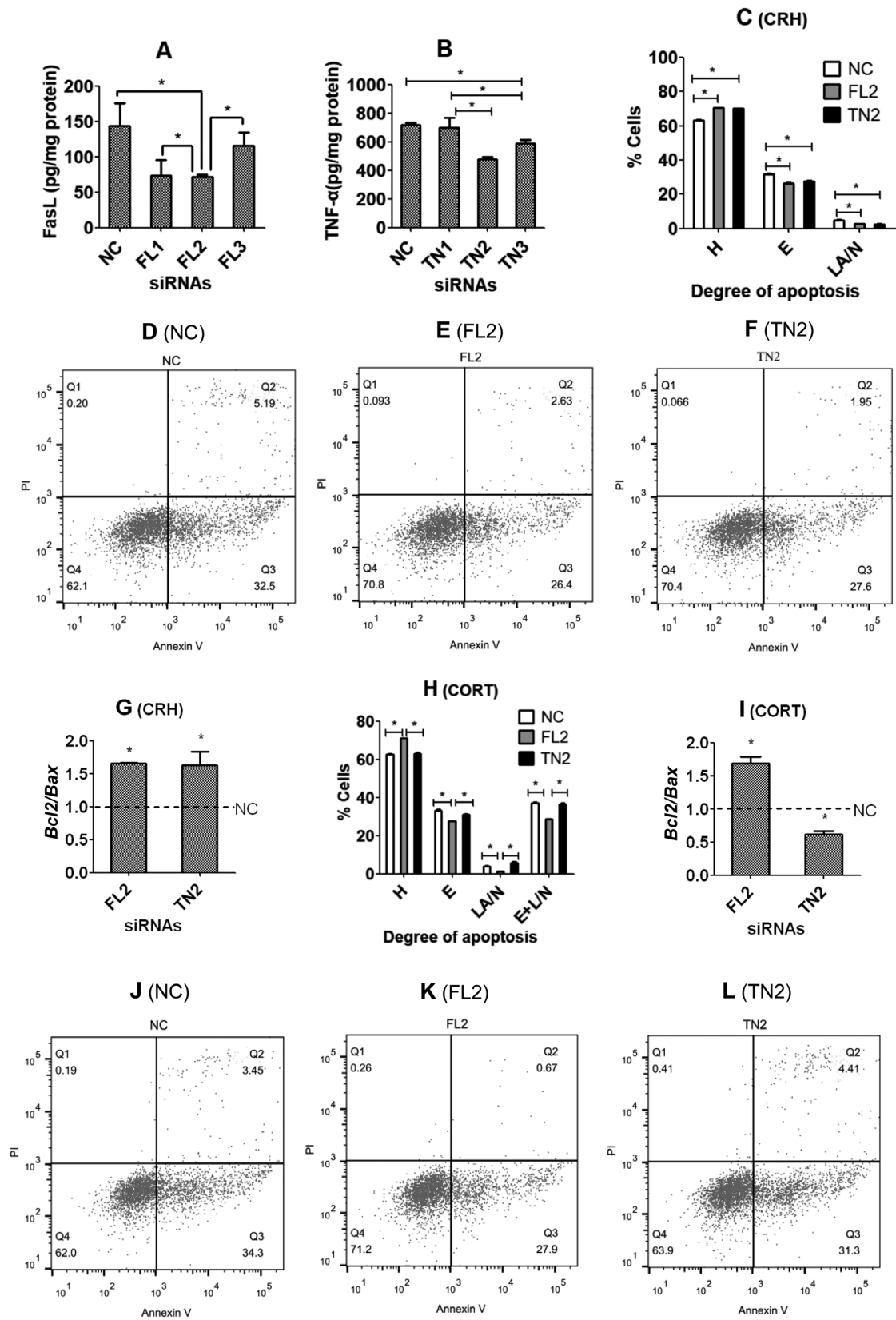
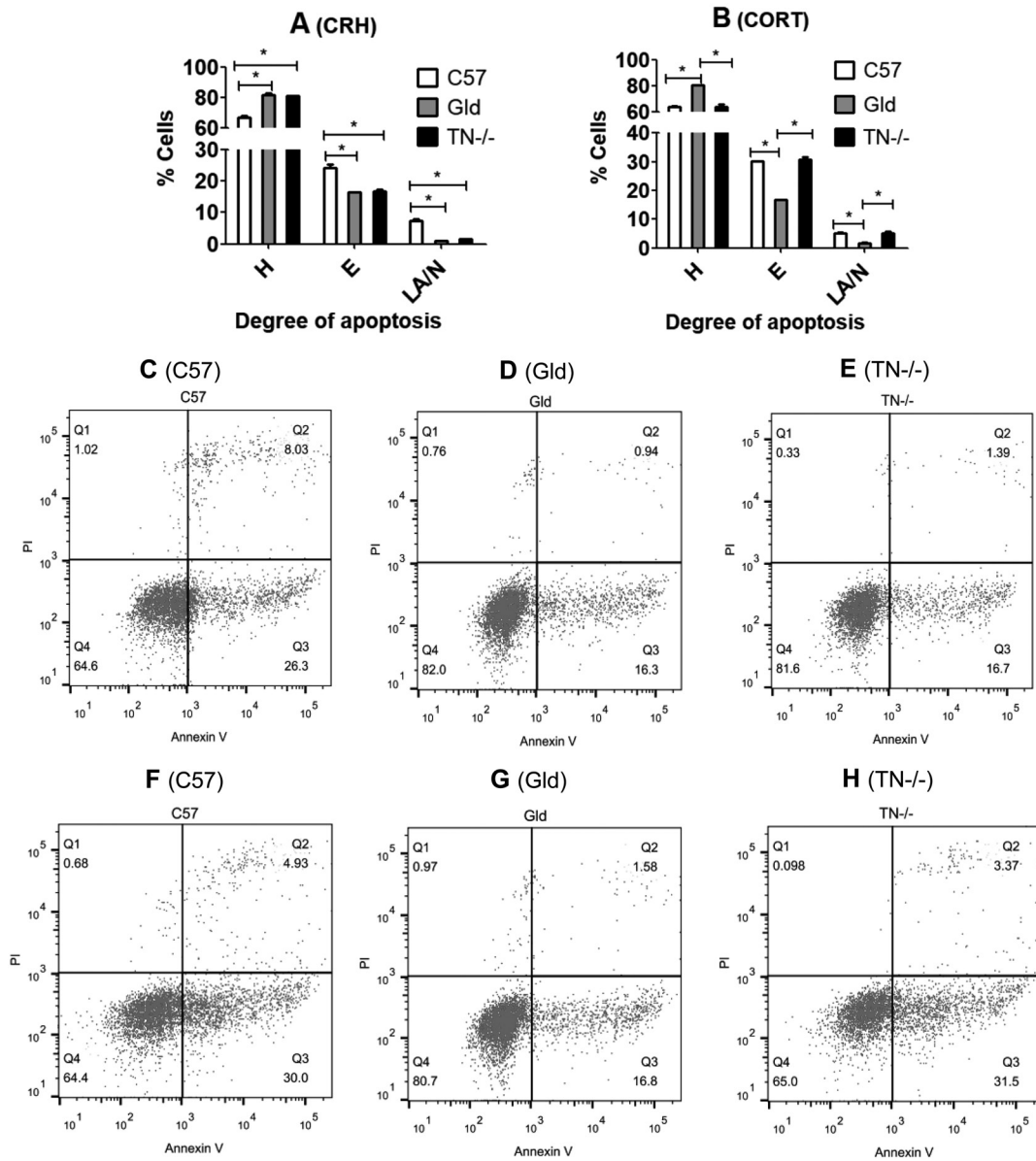


Fig. 4.



**Fig. 5.** Effects of knocking out FasL or TNF- $\alpha$  on corticotrophin-releasing hormone (CRH)- or corticosterone (CORT)-induced apoptosis of oviductal epithelial cells (OECs). OECs recovered from the generalized lymphoproliferative disorder (gld) mice with a FasL mutation, the TNF- $\alpha$  knockout (TN<sup>-/-</sup>) mice, and the control C57BL/6J (C57) mice were cultured for 48 h with CRH or CORT before apoptosis assessment by flow cytometry. Graphs A and B show percentages of healthy (H), early apoptotic (E), or late apoptotic/necrotic (LA/N) OECs following culture with CRH and CORT, respectively. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice. \* indicates significant differences ( $P < 0.05$ ) between two groups. Panels C to H show flow cytometry graphs following annexin-V and PI staining after OECs recovered from C57, gld, and TN<sup>-/-</sup> mice were cultured with CRH (panels C, D and E) or CORT (panels F, G and H).

**Fig. 4.** Effects of knocking down FasL or TNF- $\alpha$  on corticotrophin-releasing hormone (CRH)- or corticosterone (CORT)-induced apoptosis of oviductal epithelial cells (OECs). Graphs A and B show FasL concentration and TNF- $\alpha$  concentration, respectively, in OECs following transfection with negative control (NC), FasL siRNA-1 (FL1), -2 (FL2) or -3 (FL3), or with TNF- $\alpha$  siRNA-1 (TN1), -2 (TN2) or -3 (TN3). Graphs C and H show percentages of healthy (H), early apoptotic (E), or late apoptotic and necrotic (LA/N) cells as revealed by flow cytometry following culture of transfected cells in the presence of CRH and CORT, respectively. \* indicates significant differences ( $P < 0.05$ ) between two groups. In graph H, percentages of cells undergoing early apoptosis, late apoptosis and necrosis (E + LA/N) were calculated from E and LA/N cells. Graphs G and I show the ratio of Bcl2/Bax mRNAs as measured by RT-PCR after transfected OECs were cultured with CRH and CORT, respectively. The ratio in the NC group was set to 1 (dotted line), and those in the FL2 and TN2 groups were expressed relative to it. \* indicates significant difference ( $P < 0.05$ ) from the NC group. Each treatment was repeated thrice, with each replicate containing cells from one well of a 12-well plate from 3 mice. Panels D, E, F, J, K, and L show flow cytometry graphs following annexin-V and PI staining of transfected OECs after culture with CRH (D, E, and F), or CORT (J, K, and L).

[31] demonstrated that CRH enhanced TNF- $\alpha$  expression in ovarian cells. Thus, the present study provides evidence for the first time that CRH induces apoptosis of OECs by activating TNF- $\alpha$  signaling.

Treatment of mice with glucocorticoids significantly enhanced FasL expression in testicular germ cells [32], MGCs, and cumulus cells [33]. Culture with glucocorticoids activates the Fas/FasL system in osteocytes [24] and monocytes [34]. Furthermore, culture of mouse OECs with CORT significantly elevated Fas and FasL expression [16]. However, conflicting results have been reported on the effects of CORT treatment on TNF- $\alpha$  expression in different cells. For example, while Dinkel and colleagues [35] and Yuan and colleagues [26] observed the elevation of TNF- $\alpha$  expression in the rat central nervous system and mouse MGCs, respectively, following glucocorticoid treatment, Messmer *et al.* [36] and Zhang and colleagues [37] reported that glucocorticoids inhibited TNF- $\alpha$  expression in MCF-7 cells and in human subcutaneous adipocytes and preadipocytes, respectively. Thus, the present results demonstrated that glucocorticoids inhibit TNF- $\alpha$  expression in OECs and indicated that the effect of glucocorticoids on TNF- $\alpha$  expression may vary among cell types.

In summary, whether the effects of glucocorticoids on TNF- $\alpha$  expression vary among cell types is uncertain. Thus, we studied the roles of Fas and TNF- $\alpha$  systems in CORT- and CRH-induced apoptosis of mouse OECs by using an *in vitro* system involving gene knockdown and knockout techniques. The results showed that treatment with either CRH or CORT induced significant apoptosis of OECs. To elaborate, while CRH induced OEC apoptosis by activating both Fas signaling and TNF- $\alpha$  signaling, CORT induced OEC apoptosis by activating Fas signaling but not TNF- $\alpha$  signaling. The data obtained are crucial for our understanding of the mechanisms by which various categories of stress imposed on pregnant females impair embryo development, as well as for the development of measures to protect the embryo from the adverse effects of stress.

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