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Presence of encircling granulosa cells protects against oxidative stress-induced apoptosis in rat eggs cultured in vitro

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Abstract Increased oxidative stress (OS) due to in vitro culture conditions can affect the quality of denuded eggs during various assisted reproductive technologies (ARTs). Presence of intact granulosa cells may protect eggs from OS damage under in vitro culture conditions. The present study was aimed to investigate whether encircling granulosa cells could protect against hydrogen peroxide (H_2O_2)-induced egg apoptosis in ovulated cumulus oocyte complexes (COCs) cultured in vitro. The OS was induced by exposing COCs as well as denuded eggs with various concentrations of H_2O_2 for 3 h in vitro. The morphological changes, total reactive oxygen species (ROS) as well as catalase expression, Bax/Bcl-2, cytochrome c levels and DNA fragmentation were analysed in COCs as well as denuded eggs. Our results suggest that H_2O_2 treatment induced morphological apoptotic features in a concentration-dependent manner in denuded eggs cultured in vitro. The 20 μM of H_2O_2 treatment induced OS by elevating total ROS level, reduced catalase and Bcl-2 expression levels with overexpression of Bax and cytochrome c and induced DNA fragmentation in denuded eggs cultured in vitro. The presence of encircling granulosa cells protected H_2O_2-induced morphological apoptotic features by preventing the increase of Bax, cytochrome c expression levels and DNA fragmentation in associated egg. However, 20 μM of H_2O_2 was sufficient to induce peripheral granulosa cell apoptosis in COCs and degeneration in few denuded eggs cultured in vitro. Taken together our data suggest that the presence of encircling granulosa cells could be beneficial to protect ovulated eggs from OS damage under in vitro culture conditions during various ART programs.

Keywords Rat egg · Granulosa cells · Hydrogen peroxide · Bax/Bcl-2 · DNA fragmentation · Apoptosis

Introduction

Mammalian ovary generates meiotically competent oocytes required for successful fertilization and early embryonic development [1, 2]. Follicular oocytes are arrested at diplotene stage for long time from birth to puberty. Meiotic competency in these oocytes starts with resumption of meiosis from diplotene arrest and ends with achievement of metaphase-II (M-II) stage. During this period, oocytes are encircled by several layers of granulosa cells that provide nutrients and growth factors required during the achievement of developmental competency inside the follicular microenvironment [2]. These encircling granulosa cells generate several signal molecules, survival factors and a cross talk between encircling granulosa cells and oocytes are essential for survival of both the cell types under in vivo and in vitro culture conditions [3–5].

Generation of reactive oxygen species (ROS) has been reported in cumulus oocyte complexes (COCs) cultured in vitro [4, 5]. The removal of granulosa cells from diplotene-arrested oocytes triggers susceptibility of immature oocytes towards apoptosis in vitro [6, 7]. Our recent studies suggest that granulosa cell apoptosis leads to egg apoptosis in rat [8]. Generation of ROS and/or depletion of

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antioxidant system could result oxidative stress (OS) and thereby egg apoptosis in rat [9–12]. Based on these studies, we propose that encircling granulosa cells could protect against ROS-induced egg apoptosis cultured in vitro. ROS act as signal molecules in wide variety of cell types including mammalian germ cells [13, 14]. A moderate increase of ROS is beneficial for meiotic cell cycle progression [13–16], while their sustained high levels induce cell cycle arrest and apoptosis [6, 17–19]. In addition, exogenous supplementation of H_2O_2 in culture medium induces apoptosis in oocytes and zygotes cultured in vitro [6, 20, 21]. Recent studies from our laboratory suggest that high intracellular level of ROS trigger egg apoptosis [22, 23]. Under in vitro culture conditions, several factors can stimulate egg apoptosis. A minor change in culture conditions or culture medium may cause the generation of ROS in cultured cells [4, 24], a possibility exist that the handling of denuded eggs under in vitro culture conditions during various assisted reproductive technologies (ARTs) can generate ROS [25–27], and deteriorate egg quality by inducing apoptosis. Based on these studies, we propose that the generation of ROS particularly hydrogen peroxide (H_2O_2) could be one of the factors affecting egg quality as well as in vitro fertilization rate. Further, presence of encircling granulosa cells could protect egg from OS-induced apoptosis under in vitro culture conditions. The 20 µM of H_2O_2 treatment for 3 h in vitro has been reported to induce OS-mediated apoptosis in rat eggs cultured in vitro [6, 9]. Hence, in the present study, we have used this concentration and exposure time to induce OS in COCs as well as denuded eggs under in vitro culture conditions and protective effects of encircling granulosa cells were analysed. The morphological changes, total ROS, catalase, Bax/Bcl-2, cytochrome c levels as well as DNA fragmentation were analysed in COCs as well as denuded eggs cultured for 3 h in vitro.

Materials and methods

Chemicals and reagents

Chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Culture medium-199 (HiMedia Laboratories, Mumbai, India) was prepared as per company manual protocol. The culture medium was supplemented with sodium bicarbonate (0.035% w/v) and then pH was adjusted to 7.2 ± 0.1 and osmolarity was found to be 290 ± 5 mOsmol. The culture medium was supplemented with L-glutamine, penicillin and streptomycin (GPS; 1 µl/ml: HiMedia Laboratories, Mumbai, India) antibiotics before use. The final concentrations of H_2O_2 (0, 2.5, 5, 10 and 20 µM) were prepared by diluting in medium-199. Addition of H_2O_2 at final concentrations did not alter the osmolarity and pH of culture medium used in the present study.

Experimental animal

The immature female rats (22–24 days old) of Charles Foster strain were subjected to superovulation induction protocol (20 IU pregnant mare's serum gonadotropin for 48 h followed by 20 IU human chorionic gonadotropin for 14 h, intramuscular injection) to collect COCs and ovulated eggs. This study was approved by Institutional Animal Ethical Committee of the University (vide letter No. F.Sc/IAEC/2014-15/0248 dated 27/08/2014).

Collection of COCs and denuded eggs

Ovary along with oviduct was collected from experimental animals subjected to superovulation induction protocol. The ampulla was punctured using 26 gauge needle attached to 1 ml tuberculin syringe and ovulated COCs were isolated in medium-199 under a dissecting microscope (Nikon dissecting microscope, model C-DS; Tokyo, Japan). For collection of eggs, half number of ovulated COCs were picked up using microtubing (inner diameter 2 mm) attached with disposable glass micropipette (inner diameter, 100 µm for denuded eggs; 300 µm for COCs; Clay Adams, NJ) and transferred to culture medium containing 0.01% hyaluronidase at 37 °C and manually pipetted to denude the eggs quickly. The denuded eggs were removed and washed three times with culture medium.

In vitro effects of H_2O_2 on morphological changes

A group of 12–14 COCs and denuded eggs were transferred separately in a culture medium with various concentrations (0, 2.5, 5, 10 and 20 µM) of H_2O_2 in CO_2 incubator (Galaxy 170R New Brunswick, Eppendorf AG, Hamburg, Germany, UK) for 3 h in vitro. At the end of incubation period, COCs and denuded eggs were removed, washed three times with culture medium and transferred on to a grooved slide with 100 µl of culture medium and then analysed for morphological changes using a phase-contrast microscope (Nikon, Eclipse; E600, Tokyo, Japan) at ×400 magnification. The 20 µM of H_2O_2 induced apoptosis in majority of eggs cultured in vitro in the present study as well as previous studies [6, 9]. Hence, we selected 20 µM of H_2O_2 treatment for 3 h to analyse the protective effects of granulosa cells on egg apoptosis in present study.

Analysis of total ROS level in COCs as well as denuded eggs

Total ROS can be measured using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) dye, which is commonly used as cell permeable fluorescence-based probe that can be detected as green fluorescence in a treated cell. The total ROS level was detected using H_2DCFDA following previous published protocol [16] with minor modifications. In brief, control and 20 µM H_2O_2-treated COCs as well as denuded eggs (12–14) were exposed to H_2DCFDA (10 µM) for 15 min at 37 °C in CO_2 incubator. At the end of incubation period, COCs and denuded eggs were washed five times with pre-warmed PBS and then DCF fluorescence was measured at 485 nm excitation/520 nm emissions using fluorescence microscope (Model, Ni-U, Nikon Eclipse, Tokyo, Japan).

Detection of catalase, Bax, Bcl-2 and cytochrome c expressions

Immunofluorescence for catalase, Bax, Bcl-2 and cytochrome c expressions were analysed in COCs and denuded eggs using their highly specific antibodies purchased from Santa Cruz Biotechnology, CA, USA as per our published protocol with some modifications [18]. COCs as well as denuded eggs (12–14 in each group) were fixed with 4% buffered formaldehyde for 10 min at room temperature. Slides were washed 3 times with pre-warmed PBS and exposed to triton X-100 (0.01% in PBS) for 10 min at 37 °C for permeabilization. Slides were washed three times with pre-warmed PBS and then treated with sodium citrate solution (0.01 M) at 37 °C for 10 min for better antigen retrieval. Slides were again washed 3 times with pre-warmed PBS and then incubated with blocking buffer (2.5% PBS-BSA solution) at 37 °C for 30 min. Thereafter, slides were exposed to 100 µl of their respective primary antibodies [Catalase (H-9), mouse monoclonal antibody (sc-271803) specific for an epitope mapping between amino acids 471–503 near the C-terminous of catalase of mouse origin; Bax (B-9), mouse monoclonal antibody (sc-7480) raised against amino acids 1–171 of Bax of mouse origin; Bcl-2 (C-9), mouse monoclonal antibody (sc-7382) raised against amino acids 1-205 of Bcl-2 of human origin; Cytochrome c (A-8), mouse monoclonal antibody (sc-13156) raised against amino acids 1–104 of cytochrome c of horse origin; Actin (C-2) mouse monoclonal antibody (sc-8432) specific for an epitope mapping between amino acids 350–375 at the C-terminous of actin of human origin] (1:500 dilutions in blocking buffer) at 37 °C for 1 h. After 4–5 washes with PBS, slides were exposed to 100 µl of fluorescein isothiocyanate (FITC, sc-2010) or tetra methyl rhodamine isothiocyanate (TRITC, sc-3796) labeled secondary antibody (1:1000 dilution in blocking buffer) for 1 h at 37 °C in humidified chamber. After 1 h of incubation, slides were washed five times with pre-warmed PBS, mounted with fluorescence mounting medium and then observed under fluorescence microscope 465 nm (FITC) and 540 (TRITC) at ×400 magnification. FI of β-actin was analyzed in parallel as a control. The experiment was repeated three times to confirm the results.

The corrected total cell fluorescence (CTCF) of egg was carried out by taking the fluorescence intensity (FI) of egg cytoplasm for analysis. All parameters were kept constant for each egg and FI was analyzed using ImageJ software (version 1.44 from National Institute of Health, Bethesda, USA). For this purpose, minimum three different areas of each egg cytoplasm as well as its corresponding background were selected. Total fluorescence per egg was calculated on an Excel sheet by applying the measurements obtained from the analyzed cell using formula i.e. CTCF = Integrated density − (area of selected cell × mean fluorescence of background readings).

DNA fragmentation analysis

The DNA fragmentation was detected as our previous published protocol [8] using acridine orange/ethidium bromide (AO/EtBr) staining. In brief, COCs as well as denuded eggs (12–14 in each group) were fixed in 4% buffered formaldehyde for 15 min and then air dried. After washing, COCs as well as denuded eggs were labeled using the nucleic acid binding dye mix, 100 µl of 1:1 mixture of AO/EtBr solutions (4 µg/ml) for 1 min. Slides were then washed with PBS and photographs were taken using fluorescence microscope. The viable cells and apoptotic cells were identified depending on their color due to binding of EtBr to the fragmented DNA. Normal cells with intact DNA had green fluorescence of AO. The EtBr binding to fragmented DNA changed the color from green to yellow.

H_2O_2-induced DNA fragmentation and its protection by encircling granulosa cells were further confirmed using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit purchased from Trevigen Inc. MD. A group of COCs as well denuded eggs (12–14 eggs/group) was transferred and fixed in 4% buffered formaldehyde for 15 min. These slides were subjected to TUNEL assay for DNA fragmentation as per company manual protocol. The experiment was repeated three times to confirm the results.

Statistical analysis

Data are expressed as mean ± standard error of mean (S.E.M) of three independent experiments. All percentage data were subjected to arcsine square-root transformation before statistical analysis. Data were analyzed by One-way

analysis of variance (ANOVA) using SPSS software, version 17.0 (SPSS, Inc. Chicago, IL, USA) followed by Bonferroni post hoc analysis. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Encircling granulosa cells protect against H_2O_2-induced morphological changes

Figure 1 shows effects of H_2O_2 on morphological apoptotic features in denuded eggs cultured for 3 h in vitro. Freshly ovulated M-II arrested eggs showed first polar body (PB-I) with normal morphology (A1). Culture of these eggs for 3 h in vitro induced initiation of extrusion of second polar body (PB-II), which is a morphological feature characteristic of spontaneous egg activation (SEA) (data not shown). However, various concentrations of H_2O_2 inhibited SEA and induced morphological apoptotic features particularly shrinkage and cytoplasmic granulation (A2) followed by degeneration (A3). The H_2O_2 induced morphological apoptotic features in a concentration-dependent manner (A4: One-way ANOVA followed by Bonferroni post hoc analysis; $F = 353.765$, $p < 0.001$). The 20 µM of H_2O_2 induced morphological apoptotic features in majority of eggs ($92.33 \pm 1.45\%$), while few eggs underwent degeneration ($7.66 \pm 1.45\%$) after 3 h of in vitro culture. On the other hand, presence of encircling granulosa cells protected against H_2O_2-induced morphological apoptotic changes in denuded eggs collected from COCs exposed to 20 µM of H_2O_2 cultured for 3 h in vitro. These denuded eggs (B3) were similar to control denuded eggs (A1) showing normal morphology with PB-I. However, 20 µM of H_2O_2 altered encircling granulosa cells morphology (B2) as compare to encircling granulosa cells of control COCs (B1). Presence of encircling granulosa cells prevented H_2O_2-induced morphological apoptotic changes in majority of eggs collected from treated COCs, while granulosa cells could not prevent apoptosis in few eggs ($6.67 \pm 0.88\%$) if exposed to higher concentrations of H_2O_2 for 3 h in vitro (B4: One-way ANOVA followed by Bonferroni post hoc analysis; $F = 39.100$, $p < 0.001$). Three independent experiments using 36–42 eggs/COCs were conducted to confirm the observations.

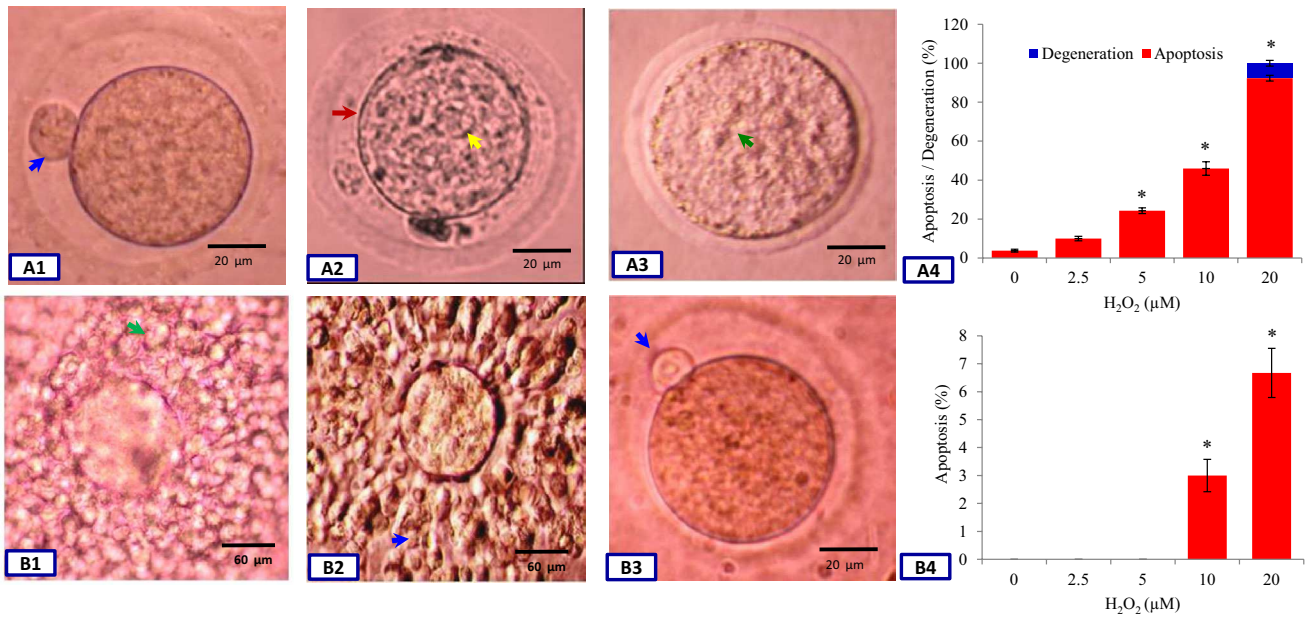

Fig. 1 Representative photograph showing the protective effects of encircling granulosa cells on H_2O_2-induced morphological apoptotic changes in eggs cultured for 3 h in vitro. H_2O_2-induced morphological apoptotic features (**A2**) such as shrinkage (*red arrow*) and cytoplasmic granulation (*yellow arrow*), followed by degeneration (**A3**, *green arrow*) as compare to control egg showing first polar body with normal morphology (**A1**, *blue arrow*). H_2O_2-induced morphological apoptotic features in a concentration-dependent manner (**A4**). Presence of encircling granulosa cells protected against H_2O_2-induced morphological apoptotic changes in denuded egg collected from treated COCs (**B3**) but the encircling granulosa cells showed deterioration in their morphology (**B2**, *blue arrow*) as compare to encircling granulosa cells of control COCs that showed normal morphology (**B1**, *green arrow*). Encircling granulosa cells prevented H_2O_2-induced morphological apoptotic features in treated eggs at lower concentrations, while higher concentrations induced apoptosis in few eggs cultured in vitro (**B4**). Data are mean ± SEM of three independent experiments using 36–42 eggs/COCs and analyzed by One-way ANOVA followed by Bonferroni post hoc analysis (*$p < 0.05$). Denuded egg, *Bar* 20 µm; COCs, *Bar* 60 µm. (Color figure online)

Granulosa cells protect against H_2O_2-mediated increase of total ROS and decrease of catalase expression in treated eggs

As shown in Fig. 2, H_2O_2 (20 µM) significantly increased total ROS level in encircling granulosa cells of treated COCs and denuded egg as evidenced by increased DCF FI (Fig. 2, A2) as compare to control COCs (Fig. 2, A1) and denuded egg (C, in box). Presence of encircling granulosa cells protected H_2O_2-mediated increase of total ROS level in denuded egg (H_2O_2 + GC, in box) of treated COCs (A2) and DCF FI was similar to denuded egg (C + GC, in box) collected from control COCs (A1). The total ROS level was comparatively high in denuded eggs (C and H_2O_2, in box) cultured in vitro as compare to denuded eggs (C + H_2O_2 and H_2O_2 + GC) collected from COCs of treated as well as control groups. On the other hand, catalase level was significantly decreased as evidenced by reduced catalase FI in denuded egg (H_2O_2, in box) as well as COCs cultured in vitro (B2) as compare to their respective denuded egg (C, in box) and COCs (B1) control. The catalase level was comparatively high in denuded eggs (C + GC and H_2O_2 + GC, in box) collected from COCs as compare to denuded eggs cultured in vitro (C and H_2O_2, in box).The CTCF analysis of 36–42 eggs/COCs from three independent experiments using One-way ANOVA followed by Bonferroni post hoc analysis further confirms the protective effects of granulosa cells on H_2O_2-mediated increase of total ROS level (A3) and decrease of catalase level (B3).

Granulosa cells protect against H_2O_2-mediated increase of Bax and cytochrome c level and decrease of Bcl-2 in treated eggs

As shown in Fig. 3, H_2O_2 (20 µM) increased FI of Bax (A2) as compare to control eggs (A1). Encircling granulosa cells protected H_2O_2-mediated increase of Bax expression in denuded eggs (A3) collected from treated COCs and FI was similar to control eggs (A1). On the other hand, H_2O_2 (20 µM) significantly reduced FI of Bcl-2 in treated eggs (B2) as compare to control eggs (B1). However, granulosa cells prevented the H_2O_2-mediated decrease of Bcl-2 expression in denuded eggs collected from treated COCs (B3). Further, cytochrome c expression was significantly

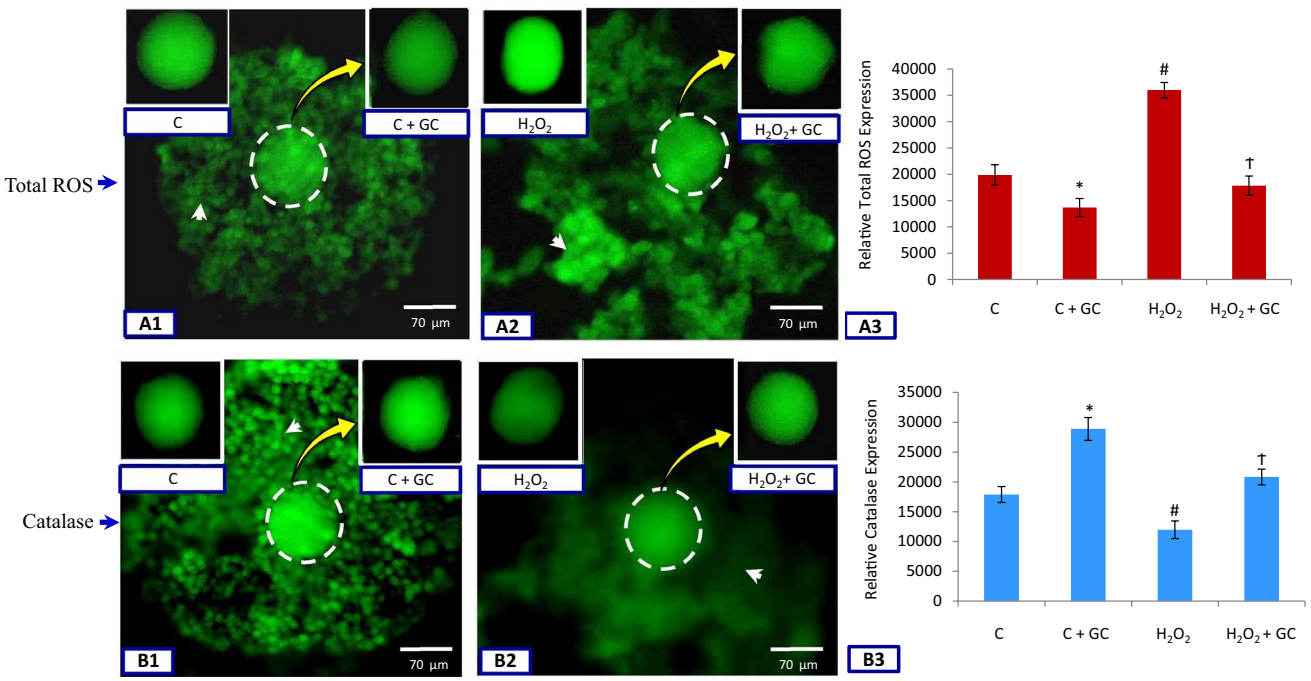

Fig. 2 Representative photograph showing total ROS and catalase expression levels in COCs as well as denuded egg cultured in vitro. H_2O_2 significantly increased total ROS level as evidenced by increased DCF FI in granulosa cells of treated COCs (**A2**, *white arrow*) as well as denuded egg (H_2O_2, in *box*) as compare to their respective control COCs (**A1**) and denuded egg (C, in *box*). Presence of encircling granulosa cells protected H_2O_2-mediated increase of total ROS level in denuded egg (H_2O_2 + GC, in *box*) of treated COCs and DCF FI was similar to denuded egg collected from control COCs (C + GC, in *box*). On the other hand, catalase level was significantly decreased as evidenced by reduced catalase FI in encircling granulosa cells of treated COCs (**B2**, *white arrow*) as well as denuded egg (H_2O_2, in *box*) as compare to their respective control COCs (**B1**) and denuded egg (C, in *box*). Presence of encircling granulosa cells protected ROS-mediated decrease of catalase level in denuded egg (H_2O_2 + GC, in *box*) of treated COCs. One-way ANOVA followed by Bonferroni post hoc analysis of CTCF data further confirms our results (**A3, B3**). Data are mean ± SEM of three independent experiments using 36–42 eggs/COCs. *$p < 0.05$ (C vs. C + GC), $^{\#}p < 0.05$ (C + GC vs. H_2O_2), $^{\dagger}p < 0.05$ (H_2O_2 vs. H_2O_2 + GC). *Bar* 70 µm

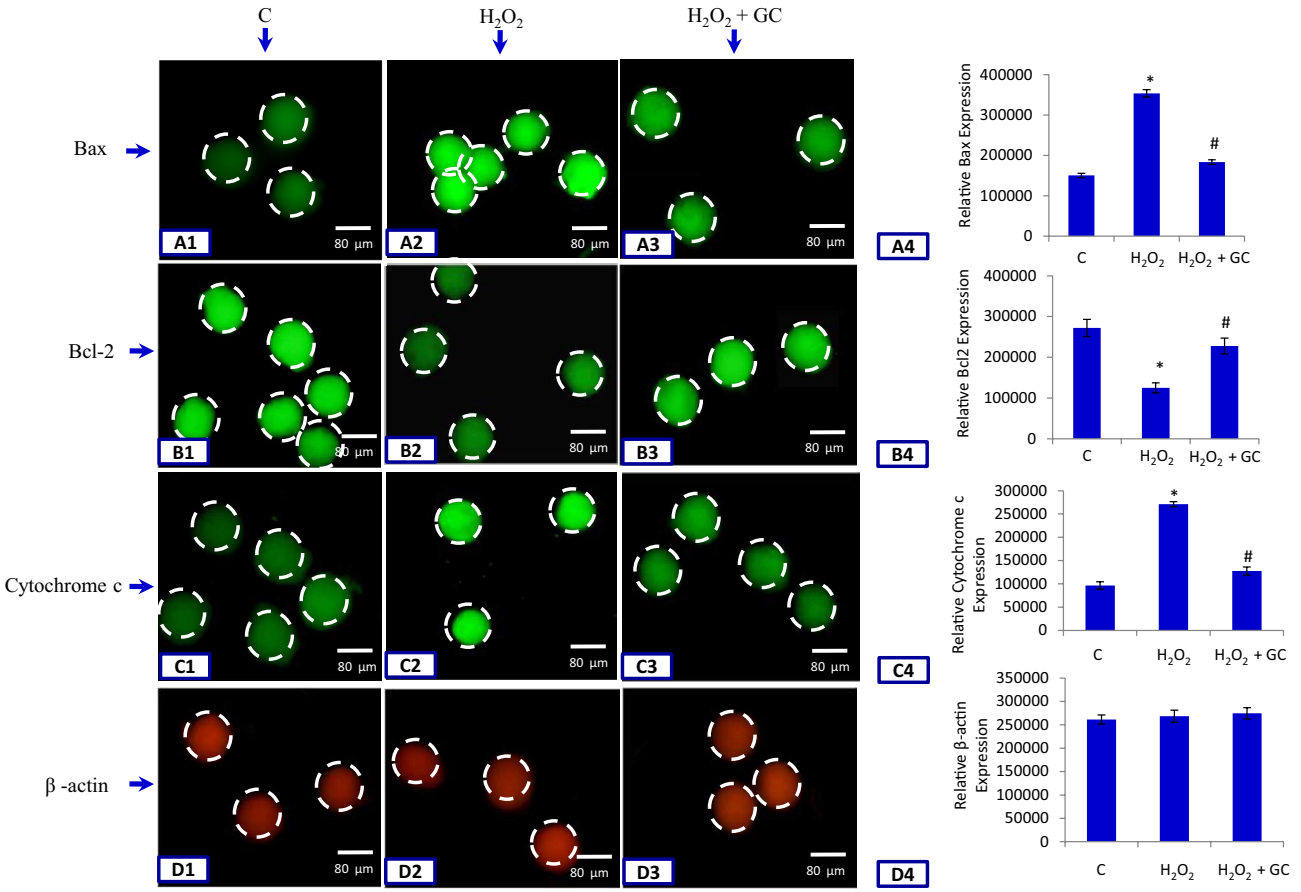

Fig. 3 Representative photograph showing the protective effects of encircling granulosa cells on Bax, Bcl-2 and cytochrome c expression levels in eggs cultured in vitro. H_2O_2 significantly increased FI of Bax in treated eggs (**A2**) as compare to control eggs (**A1**). Encircling granulosa cells protected H_2O_2-mediated increase of Bax expression in denuded eggs (**A3**) collected from treated COCs and FI was similar to control eggs (**A1**). On the other hand, H_2O_2 treatment significantly reduced FI of Bcl-2 in treated eggs (**B2**) as compare to control eggs (**B1**). However, granulosa cells prevented the decrease of Bcl-2 expression level in denuded eggs (**B3**) collected from treated COCs. The cytochrome c expression level was significantly increased in H_2O_2-treated eggs (**C2**) as compare to control eggs (**C1**). Encircling granulosa cells prevented H_2O_2-mediated increase of cytochrome c expression in denuded eggs (**C3**) collected from treated COCs and FI was similar to control eggs (**C1**). The lower panel shows β-actin FI as a control for upper panel photographs (**D1–3**). The CTCF analysis of FI using 36–42 eggs/COCs from three independent experiments further confirm above observations (**A4, B4, C4, D4**). Data are mean ± SEM of three independent experiments and subjected to One-way ANOVA followed by Bonferroni post hoc analysis. *$p < 0.001$ (C vs. H_2O_2), #$p < 0.001$ (H_2O_2 vs. H_2O_2 + GC). *Bar* 80 µm

increased in H_2O_2-treated eggs (C2) as compare to control eggs (C1) as evidenced by increased FI. Encircling granulosa cells prevented H_2O_2-mediated increase of cytochrome c expression in denuded eggs (C3) collected from treated COCs and FI was similar to control eggs (C1). However, the control protein β-actin expression level remains unchanged (D1–D3) suggesting that all parameters were kept constant during immunofluorescence studies. The CTCF analysis using 36–42 eggs/COCs from three independent experiments using One-way ANOVA followed by Bonferroni post hoc analysis further confirms the protective effects of granulosa cells on H_2O_2-mediated increase of Bax (A4), decrease of Bcl-2 (B4) and increase of cytochrome c levels (C4), while β-actin expression level did not change (D4).

Encircling granulosa cells protect against H_2O_2-induced DNA fragmentation in eggs

As shown in Fig. 4, H_2O_2 treatment induced DNA fragmentation as evidenced by yellow/orange color of EtBr (A2) and TUNEL fluor green fluorescence (B2) in treated eggs as compare to their respective controls (A1 and B1). On the other hand, encircling granulosa cells protected H_2O_2-mediated DNA fragmentation in eggs of treated COCs as evidenced by background green fluorescence of AO (A4) and TUNEL fluor negative staining (B4) in the cytoplasm of treated egg that showed FI similar to their respective controls (A3 and B3). Although encircling granulosa cells protected H_2O_2-induced DNA fragmentation in eggs but

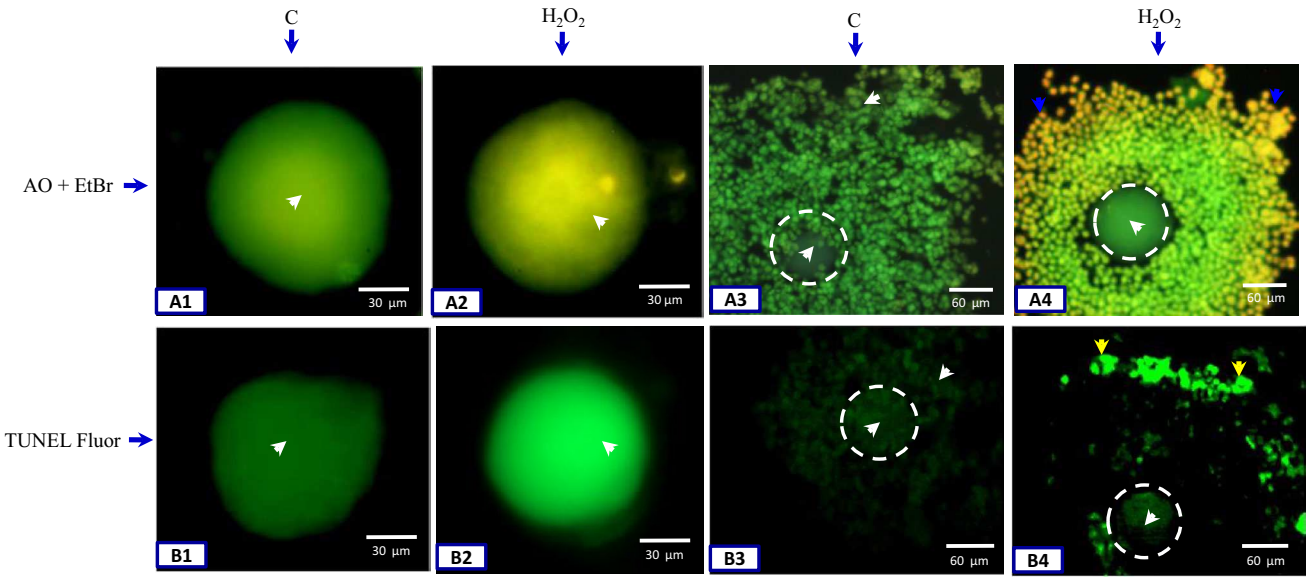

Fig. 4 Representative photograph showing H_2O_2-induced DNA fragmentation in eggs and its prevention by encircling granulosa cells cultured in vitro. H_2O_2 induced apoptosis as evidenced by yellow fluorescence of EtBr (**A2**, *white arrow*) and TUNEL fluor green fluorescence (**B2**, *white arrow*) in treated eggs as compare to their respective controls (**A1, B1**; *white arrow*). Presence of encircling granulosa cells protected H_2O_2-mediated DNA fragmentation in eggs of treated COCs as evidenced by green fluorescence of AO (**A4**, *white arrow*) and TUNEL fluor negative staining (**B4**, *white arrow*) that show FI similar to their respective controls (**A3, B3**; *white arrow*). However, peripheral encircling granulosa cells show DNA fragmentation as evidenced by *yellowish orange* color of EtBr (**A4**, *blue arrows*) as well as TUNEL fluor positive staining (**B4**, *yellow arrows*). Three independent experiments using 36–42 eggs/COCs were conducted to confirm the results. Denuded egg, *Bar* 30 µm; COCs, *Bar* 60 µm. (Color figure online)

peripheral granulosa cells themselves underwent apoptosis as evidenced by yellowish orange color of EtBr that indicates apoptosis (A4) and TUNEL fluor positive green staining (B4). Three independent experiments using 36–42 eggs/COCs were conducted to confirm the observations.

Discussion

Mammalian ovary is a dynamic organ that generates excess amount of ROS during final stages of folliculogenesis [13–17]. However, ovary has its own antioxidant system through which it maintains the redox status by scavenging free radicals generated during final stages of follicular development [28]. If the antioxidant system of the ovary fails to do so or increased level of ROS in the follicular fluid or under in vitro culture conditions may deteriorate egg quality by inducing apoptosis. This notion is supported by our previous study that H_2O_2 induces cell cycle arrest and apoptosis in rat immature oocytes cultured in vitro [6]. However, it remains unclear whether the increased level of ROS under in vitro culture conditions could affect egg quality by inducing apoptosis. If yes, whether encircling granulosa cells could protect H_2O_2-induced egg apoptosis? Data of the present study suggest that the majority of control eggs (55%) were arrested at M-II stage possessing PB-I, while remaining underwent spontaneous egg activation (45%) by extruding PB-II. The SEA in the present study could be due to generation of ROS under in vitro culture conditions [29]. Physical factors such as temperature, oxygen level and other in vitro culture conditions may also induce SEA [30, 31]. The removal of cumulus cells at the time of denudation (shear force) and handling of eggs under in vitro culture conditions may accelerate SEA in ovulated eggs [32–34].

Generation of ROS beyond physiological level under in vitro culture conditions or exogenous supplementation of H_2O_2 may cause OS, which could induce cell cycle arrest and apoptosis. This possibility was further strengthened by observations that exogenous H_2O_2 inhibited SEA and induce morphological apoptotic features in a concentration-dependent manner in denuded eggs, while few eggs underwent degeneration if exposed to 20 µM H_2O_2 for 3 h cultured in vitro. Presence of encircling granulosa cells protected H_2O_2-induced morphological apoptotic features in eggs of treated COCs. Only few eggs and encircling granulosa cells underwent apoptosis at higher concentrations of H_2O_2 (10 and 20 µM). These data suggest the protective effects of encircling granulosa cells on ROS-mediated apoptosis under in vitro culture conditions. Handling of cumulus enclosed oocytes instead of denuded oocytes could protect oocytes from OS damage due to in vitro culture conditions. Cumulus cells closely interact and provide

support to the maturing oocyte, shares the oocyte's microenvironment and minimize the damage by ROS [35]. Cumulus cells are able to produce antioxidants that protect oocyte from OS damage [36]. These observations corroborates previous findings that high level of H_2O_2 induces morphological apoptotic features in rat eggs cultured in vitro [9, 10, 18, 22] and presence of encircling granulosa cells prevent egg from ROS-mediated OS damage in mouse [37–40], rat [8, 41, 42], bovine [43, 44], porcine [4] and human [45] oocytes cultured in vitro.

The increased level of ROS causes OS damage hence, ovary protects oocytes from OS damage by several antioxidant enzymes [46, 47]. Encircling granulosa cells inside the follicular microenvironment protect oocytes from OS damage by activating its own enzymatic antioxidant system [4, 8, 24]. The total ROS can be measured using H_2DCFDA dye, which can be used as cell permeable fluorescence-based probe for ROS. H_2DCFDA is first deacetylated by endogenous esterases to dichlorofluorescein (DCFH), which can further react with several ROS to form the fluorophore DCF which can be measured fluorimetrically [48]. Studies suggest that there is a limit for the cumulus cells to protect against OS damage [49]. Hence, in the present study, we used 20 μM of H_2O_2 and protective effects of encircling granulosa cells were analysed under in vitro culture conditions. The DCF intensity in the present study suggest that 20 μM H_2O_2 increased total ROS level in the granulosa cells of treated COCs and denuded eggs cultured in vitro. However, total ROS level was significantly low in denuded eggs collected from control as well as treated COCs as compare to denuded eggs collected from both the groups after 3 h of in vitro culture. These data together with previous studies suggest that in vitro culture conditions generate ROS [24] and encircling granulosa cells prevent increase of ROS in associated egg [35, 36].

Catalase is one of the major enzymatic antioxidants that decompose H_2O_2 into non toxic molecular oxygen and water [50]. We propose that catalase could be one of the enzymes present in granulosa cells to scavenge H_2O_2 generated under in vitro culture conditions during various ART programs. Hence in the present study, we analysed catalase expression level in COCs as well as denuded eggs cultured in vitro. Data of the present study suggest that exogenous 20 μM H_2O_2 reduced catalase expression in encircling granulosa cells of COCs and denuded eggs cultured in vitro. A significant increase of catalase expression level in denuded eggs collected from treated COCs as compare to treated denuded eggs suggest the role of granulosa cell catalase in H_2O_2 scavenging process under in vitro culture conditions. Similarly, the protective effects of catalase from OS damage have been reported in maturing gilts and mouse oocytes cultured in vitro [8, 50]. On the other hand, the increased production of ROS has been associated with decreased viability of encircling cumulus cells and antioxidants level [40, 51].

The increased levels of ROS can modulate mitochondrial membrane potential and alter Bax/Bcl-2 ratio in the plasma membrane of treated eggs [6]. This notion is strengthened by our data that H_2O_2 treatment modulated Bax/Bcl-2 ratio by reducing expression of anti-apoptotic protein such as Bcl-2 and inducing the expression of proapoptotic protein such as Bax in treated eggs. However, presence of encircling granulosa cells prevented the decrease of Bcl-2 and increase of Bax protein expression in the present study suggesting that granulosa cells prevent ROS-mediated changes in Bax/Bcl-2 ratio, which is required for the initiation of apoptosis. The role of granulosa cells in the prevention of ROS-Bax/Bcl-2-mediated apoptosis has been reported in rat COCs cultured in vitro [9]. Change in Bax/Bcl-2 ratio within a cell disrupts mitochondria membrane potential and induces cytochrome c release, which initiate apoptosis in wide variety of cells [2, 11, 12, 52, 53]. Our data suggest that H_2O_2 treatment increased cytochrome c level in treated eggs. However, the presence of granulosa cells prevented increase of cytochrome c level in eggs collected from treated COCs suggesting the role of encircling granulosa cells in the prevention of ROS-induced Bax/Bcl-2-mediated apoptotic pathway in eggs cultured in vitro. Similarly, ROS-mediated increase of cytochrome c level has been reported to induce oocyte/egg apoptosis in rat [2, 5, 10]. In the present study, presence of encircling granulosa cells prevented H_2O_2-induced overexpression of Bax, underexpression of Bcl-2 and increased cytochrome c levels in eggs collected from treated COCs as compare to their respective controls. However, Bax and cytochrome c expression levels were still high and Bcl-2 expression level was still low as compare to their respective controls. A little high but insignificant increase of Bax and cytochrome c levels and decrease of Bcl-2 level suggest that the encircling granulosa cells can protect egg from OS damage upto certain limit [49]. Our data suggest that encircling granulosa cells are unable to protect egg if COCs are exposed to ≥20 μM H_2O_2 for 3 h under in vitro culture conditions.

The increased cytochrome c level in egg can induce apoptosis by activating upstream and downstream caspases [54]. Although we did not analyse caspases activity in the present study, our previous reports indicate that H_2O_2 supplementation induces caspase-9 as well as caspase-3 in rat oocytes cultured in vitro [6, 9, 18, 19]. The increased caspases activities may cleave key structural and regulatory proteins that result in DNA fragmentation in 180–200 base-pair, a hallmark feature of apoptosis [6, 8, 9]. Our results suggest that H_2O_2 induced DNA fragmentation as evidenced by AO/EtBr as well as TUNEL fluor positive staining in treated denuded eggs cultured in vitro. On the other hand, presence of encircling granulosa cells protected

egg apoptosis as evidenced by green background of AO and negative TUNEL fluor staining. However, 20 μM H_2O_2 was sufficient to induce peripheral granulosa cell apoptosis as evidenced by yellow/orange color of EtBr and TUNEL fluor positive staining in COCs cultured in vitro. These data support our observations that the encircling granulosa cells prevented OS-mediated egg apoptosis in vitro.

Conclusions

Our results suggest that the exogenous H_2O_2 induced ROS and thereby OS under in vitro culture conditions. The OS induced apoptosis through the activation of Bax/Bcl-2-cytochrome c-mediated apoptotic pathway in denuded eggs. However, presence of encircling granulosa cells prevented increase of ROS, Bax/Bcl-2 ratio, cytochrome c level and DNA fragmentation in eggs isolated from COCs cultured in vitro. Hence, we propose that presence of encircling granulosa cells could be beneficial to prevent ROS-mediated apoptosis and thereby deterioration of egg quality under in vitro culture conditions during various ART programs.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exist.

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