Free Radical Research, March 2008; 42(3): 212–220

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healthcare

Calcium ionophore-induced egg activation and apoptosis are associated with the generation of intracellular hydrogen peroxide

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Accepted by Dr J. Keller

(Received 8 August 2007; revised 5 December 2007)

Abstract
The present study was designed to investigate whether calcium ionophore-induced activation and apoptosis are associated with the generation of hydrogen peroxide (H_2O_2) in rat eggs cultured *in vitro.* Culture of metaphase-II (M-II) arrested eggs in Ca^{2+}/Mg^{2+}-deficient medium did not induce egg activation, while a second polar body was observed in 20% of eggs when cultured in Ca^{2+}/Mg^{2+}-supplemented medium. In Ca^{2+}/Mg^{2+}-deficient medium, lower concentrations of calcium ionophore (0.2,0.4 and 0.8 μM) not only induced egg activation in a dose-dependent manner but also generation of intracellular H_2O_2 (84.40 ± 0.50 ng/egg) when compared to control eggs (80.46 ± 1.34 ng/egg). The higher concentration of calcium ionophore (1.6 μM) induced apoptosis and pronounced generation of intracellular H_2O_2 (92.43 ± 0.93 ng/egg) in treated eggs. Conversely, cell-permeant antioxidant such as 2(3)-tert-butyl-4-hydroxyanisole (BHA) reduced intracellular H_2O_2 level (81.20 ± 1.42 ng/egg) and protected against calcium ionophore-induced morphological changes characteristics of egg activation and apoptosis. These results clearly suggest that calcium ionophore-induced activation and apoptosis are associated with the generation of intracellular H_2O_2 in rat eggs.

Keywords: *Calcium ionophore, egg activation, intracellular H_2O_2 level, apoptosis*

Introduction

The role of calcium in the physiology of oocyte from oogenesis to maturation and fertilization has been well documented [1–4]. The transition from one meiotic phase to the following is regulated by cell cycle control checkpoints, which are in turn modulated by a transient increase of intracellular calcium ion $[Ca^{2+}]i$ [4,5]. Egg activation that comprises several events, including cortical granule exocytosis, pronuclei formation, exit from M-II arrest and extrusion of second polar body, is driven by increase of $[Ca^{2+}]i$ initiated by fertilizing sperm [4,6–8]. If the fertilization does not occur, aged unfertilized eggs undergo spontaneous egg activation in mouse [9], rat [10,11], rabbit [12], pig [13], porcine [13–17], bovine [18] and human [19].

The intracellular calcium homeostasis is very important in maintaining the normal function of a cell. Changes in $[Ca^{2+}]i$ level modulate various aspects of cellular function such as meiotic cell cycle, apoptosis and/or cell death [2,4,20]. A transient increase of $[Ca^{2+}]i$ signals egg activation, while high sustained level induces egg apoptosis [20–22]. Conversely, an abnormally high $[Ca^{2+}]i$ level results into cell death [23]. Under *in vitro* culture conditions, calcium ionophore A23187 has been used to induce egg activation in mouse [24], rat [25,26], pig [13,27],

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760701868352

porcine [13,27,28–30], human [31] and apoptosis in pig oocytes and bovine embryos [18,32,33].

The possible mechanism(s) by which calcium ionophore A23187 (hereafter calcium ionophore) induces egg activation or apoptosis remains obscure. This ionophore has been reported to increase $[Ca^{2+}]i$, possibly by depleting essentially all internal calcium pools from endoplasmic reticulum and mitochondria leading to egg activation or apoptosis [4,32,33]. However, it is not yet known whether calcium ionophore A23187-induced these two events are associated with the generation of intracellular hydrogen peroxide (H_2O_2) in eggs cultured *in vitro*. If yes, whether cell-permeant antioxidant such as 2(3)-tert-butyl-4-hydroxyanisole (BHA) could reduce generation of intracellular H_2O_2 and protect against calcium ionophore-induced morphological changes characteristic of egg activation or apoptosis. Using rat liver mitochondria, it has been reported that the addition of low concentration of free Ca^{2+} (0.2 μM) resulted in stimulation of H_2O_2 generation and induced cell death through apoptosis. The production of H_2O_2 was more pronounced when a high concentration of free Ca^{2+} (20 μM) was added and resulted in cell death through necrosis [34]. Similarly, it is possible that the generation of a distinct level of intracellular H_2O_2 might be associated with calcium ionophore-induced morphological changes characteristic of activation and apoptosis in rat eggs cultured *in vitro*.

A certain threshold level of reactive oxygen species (ROS) has been reported as a potential marker for predicting success rate of *in vitro* fertilization [35,36]. The generation of ROS is beneficial to increase developmental potential of bovine oocytes during *in vitro* maturation [37]. A previous study has indicated that lower concentrations (2.5 and 5 μM) of H_2O_2 induce meiotic cell cycle progression whereas higher concentrations (10 and 20 μM) induce apoptosis in rat oocytes cultured *in vitro* [38]. On the other hand, cell-permeant antioxidant 2(3)-ter-butyl-4-hydroxyanisole BHA inhibits resumption of meiosis in rat oocytes cultured *in vitro* [39]. Hence, we hypothesize that calcium ionophore-induced morphological changes characteristic of egg activation might be associated with increased intracellular H_2O_2 level under physiological range, while more pronounced generation could promote egg apoptosis *in vitro*. Further, BHA could reduce intracellular H_2O_2 level and protect against calcium ionophore-induced spontaneous egg activation (SEA) and egg apoptosis *in vitro*. Therefore, objectives of our study were to find out effects of calcium ionophore with or without pre-incubation of BHA on morphological features characteristic of egg activation and apoptosis. Further, intracellular H_2O_2 levels were quantified to correlate with calcium ionophore-induced egg activation or apoptosis.

Materials and methods

Reagents

All reagents unless otherwise noted were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of calcium ionophore working solutions

The calcium ionophore was initially dissolved in 100 μl of dimethyl sulphoxide (DMSO) and then in distilled water to get a final concentration of 1 mg/ml (stock solution). The stock solution was aliquoted and kept at $-20^{\circ}C$ until use. The stock solution of calcium ionophore (1 mg/ml) was further diluted in serum-free and Ca^{2+}/Mg^{2+}-deficient medium-199 (HiMedia Laboratories, Mumbai, India) to get final concentrations of calcium ionophore (0.2, 0.4, 0.8, 1.6 and 3.2 μM). The freshly prepared working solutions of calcium ionophore were kept at $37^{\circ}C$ for 5 min before use. Addition of calcium ionophore, at final concentration, did not alter the osmolarity (290 ± 5 m Osmol) and pH (7.2 ± 0.2) of culture medium used in the present study. Since DMSO was used as a solvent in the calcium ionophore stock solution, an equivalent dilution of the highest concentration (0.1% DMSO) was used in the control group.

Animals

Holtzman-derived rats were housed in air-conditioned, light controlled rooms, with food and water available *ad libitum*. Twenty-three–25-day-old female rats were primed with single subcutaneous injection of 10 IU pregnant mare's serum gonadotropin (PMSG) for 48 h followed by 10 IU human chorionic gonadotropin (hCG) for 14 h. All procedures confirmed to the stipulations of the Institutional Animal Ethical Committee of National Institute of Health and Family Welfare (New Delhi, India).

Collection and culture of eggs

Ovulated cumulus-enclosed eggs were isolated in serum-free and Ca^{2+}/Mg^{2+}-deficient medium-199 under a dissecting microscope (Carl Zeiss, Oberkochen, Germany) by puncturing oviduct using 26-gauge needle attached to a 1 ml syringe. The serum-free and Ca^{2+}/Mg^{2+}-deficient medium-199 was used in the present study unless stated otherwise. All ovulated cumulus-enclosed eggs were picked up using microtubing (inner diameter 2 mm) attached with glass micropipette (Clay Adams; B&D and Co., NJ) and transferred to culture medium containing 0.01% hyaluronidase at $37^{\circ}C$. After 3 min of treatment, denuded eggs were removed and washed three times with culture medium. The average time for isolation and preparation for culture of denuded eggs were 4 ± 2 min and 5 ± 2 min, respectively. For entire

in vitro experiments, 15–20 denuded eggs were maintained in each petri dish and cultured in a CO_2 incubator (Thermo Pharma Company, Ohio) at 37°C in 100% humidity with 5% CO_2.

Effect of calcium ionophore on morphological changes in eggs

A group of 15–20 eggs was cultured either in Ca^{2+}/Mg^{2+}-supplemented medium (HiMedia Laboratories, Mumbai, India) or Ca^{2+}/Mg^{2+}-deficient medium with or without various concentrations of calcium ionophore (0.2, 0.4, 0.8, 1.6 and 3.2 μM) for 3 h. To induce egg activation, 50 μM calcium ionophore was used for 5 min in a Ca^{2+}/Mg^{2+}-supplemented fertilization medium [33]. In the present study, we reduced the concentration (≤ 3.2 μM) and increased treatment time for calcium ionophore in order to achieve a shift from egg activation to apoptotic cell death so that a correlation could be made between these morphological changes with intracellular H_2O_2 level. At the end of the incubation period, eggs were removed, washed three times with culture medium and transferred on to a grooved slide with 100 μl of culture medium and then examined for morphological changes using a phase-contrast microscope (Nikon, Eclipse; E600, Tokyo, Japan) at 400 × magnification.

Effect of pre-incubation of BHA on calcium ionophore-induced morphological changes in eggs

Eggs were first pre-incubated in Ca^{2+}/Mg^{2+}-deficient medium containing 100 μM of BHA for 5 min. After 5 min of pre-incubation, eggs were washed with Ca^{2+}/Mg^{2+}-deficient medium and then a group of 15–20 eggs were incubated in Ca^{2+}/Mg^{2+}-deficient medium containing various concentrations of calcium ionophore (0.2, 0.4, 0.8, 1.6 and 3.2 μM) for 3 h. Untreated eggs and eggs pre-incubated with 100 μM of BHA alone for 5 min were also cultured for 3 h simultaneously as controls. This concentration of BHA has been reported to inhibit resumption of meiosis in rat oocytes cultured *in vitro* [39]. At the end of the incubation period, eggs were removed, washed three times with Ca^{2+}/Mg^{2+}-deficient medium and transferred on to a grooved slide with 100 μl of culture medium and then examined for morphological changes characteristic of egg activation and apoptosis using a phase-contrast microscope (Nikon, Eclipse; E600, Tokyo, Japan) at 400 × magnification.

DNA fragmentation analysis by TUNEL assay

To confirm calcium ionophore-induced egg apoptosis, control eggs that had normal morphology and 1.6 μM calcium ionophore-treated eggs that had morphological apoptotic features were used for DNA fragmentation analysis using terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) kit purchased from R&D Systems Inc. (MN). To prevent the loss of eggs, poly L-lysine-coated glass slides were used for the analysis of DNA fragmentation using TUNEL assay. Control and calcium ionophore-treated eggs (10–15 eggs from each group) for 3 h were washed three times with phosphate buffer saline (PBS), transferred onto slide and then fixed in buffered formaldehyde (3.7%) for 15 min at 18–20°C. Slides were washed three times with PBS and then air dried at room temperature (18–20°C). All procedures were carried out at 18–20°C unless stated otherwise. The TUNEL assay was performed as per company manual protocol as described briefly elsewhere [38].

Quantitative estimation of intracellular H_2O_2 levels in eggs

To find out the association between calcium ionophore-induced egg activation and apoptosis with the generation of intracellular H_2O_2 levels, eggs from various groups were used for the analysis of intracellular H_2O_2 level using H_2O_2 assay kit purchased from R&D Systems Inc. (MN). In brief, 25 eggs from various groups, i.e. control eggs (untreated eggs showing first polar body with normal morphology), activated eggs (0.8 μM calcium ionophore-treated eggs showing second polar body and pronuclei), apoptotic eggs (1.6 μM calcium ionophore-treated eggs showing morphological apoptotic features) and eggs pre-incubated with BHA (100 μM) for 5 min and then incubated with 1.6 μM of calcium ionophore for 3 h were washed three times with PBS and transferred to a microcentrifuge tube containing 100 μl hypotonic lysis buffer (5 mm Tris, 20 mm EDTA, 0.5% Triton X-100, pH 6.0). After 1 h of incubation on ice, lysates were centrifuged at 10 000 rpm at 4°C for 15 min and a clear supernatant was immediately stored at −30°C until use. Samples were diluted by 5-fold with sample diluent (provided with the kit) and then processed for the quantitative estimation of H_2O_2 by calorimetric assay as per company manual protocol. In brief, all reagents, working standards and samples were brought to room temperature before use. The 50 μl of sample diluent was added to the blank wells and 50 μl of H_2O_2 standards or samples to remaining wells in duplicate. Thereafter, 100 μl H_2O_2 colour reagent was added to each well and the plate was incubated for 30 min at room temperature after tapping the side of the plate gently for 10 s. At the end of the incubation period, optical density (OD) was determined using a microplate reader set to 550 nm. All samples were run in one assay to avoid inter-assay variation and intra-assay variation was 3%.

Statistical analysis

Data are expressed as mean ± SE of mean (SEM) of triplicate samples. All percentage data were subjected to arcsine square-root transformation before statistical analysis. Data were analysed by either Student's *t*-test or one-way analysis of variance (ANOVA) using SPSS software, version 11.5 (SPSS, Inc. Chicago, IL). A probability of $p < 0.05$ was considered to be statistically significant.

Results

Effect of calcium ionophore on egg activation

Eggs collected from oviduct of immature female rat after 14 h post-hCG injection were at M-II stage and exhibited first polar body with normal morphology. Culture of these eggs in Ca^{2+}/Mg^{2+}-deficient medium did not induce any morphological change after 3 h (Figure 1A), while extrusion of second polar body but not pronuclei formation (Figure 1B) was observed in 20% eggs cultured in Ca^{2+}/Mg^{2+}-supplemented medium (Table I). On the other hand, supplementation of lower concentrations of calcium ionophore (0.2, 0.4, 0.8 μM) in Ca^{2+}/Mg^{2+}-deficient medium induced extrusion of second polar body (one-way ANOVA, $F = 288.99$, $p < 0.001$) and pronuclei formation (one-way ANOVA, $F = 53.12$, $p < 0.001$) in a dose-dependent manner (Figure 1C). The lowest concentration (0.2 μM) of calcium ionophore induced pronuclei formation in eggs that were arrested at M-II stage (Figure 2B). Further, 0.2 μM of calcium ionophore also induced initiation of cortical granules in few treated eggs near the peripheral area of egg cytoplasm after 1 h (Figure 2C) that was further increased after 3 h of treatment (Figure 2D), while control eggs had normal morphology with degenerating first polar body (Figure 2A). Conversely, pre-treatment of eggs with BHA (100 μM) for 5 min significantly inhibited calcium ionophore-induced egg activation and only 17% of eggs showed pronuclei formation and extrusion of second polar body (Table II).

Effect of calcium ionophore on morphological apoptotic changes in eggs

A shift from egg activation to apoptosis was observed if the eggs were exposed to higher concentrations of calcium ionophore (0.8 and 1.6 μM) for 3 h. The initiation of apoptotic features was observed at 0.8 μM of calcium ionophore and reached a maximum at 1.6 μM of calcium ionophore (42.85 ± 4.12%), while few eggs underwent degeneration (7.78 ± 0.56%; Table I). Further, a higher concentration of calcium ionophore (3.2 μM) induced degeneration in most of the treated eggs (90.67 ± 3.34%) after 3 h of culture. The calcium ionophore-induced first morphological apoptotic feature was shrinkage (Figure 3B) and membrane blebbing (Figure 3C) was observed as a second morphological apoptotic feature. The cytoplasmic granulation (Figure 3D) was the last morphological apoptotic feature observed just prior to degeneration (Figure 3E). The control eggs exhibited first polar body with normal morphology (Figure 3A). Conversely, pre-incubation of eggs with BHA (100 μM) for 5 min significantly inhibited 1.6 μM Calcium ionophore-induced morphological changes characteristic of apoptosis (20.67 ± 1.76%) and most of the eggs were morphologically similar to control eggs that had degenerating first polar body without pronuclei formation (Figure 3F). However, BHA could not protect higher concentration (3.2 μM) of calcium ionophore-induced egg degeneration.

TUNEL analysis for DNA fragmentation

In the present study, although membrane blebbing and cytoplasmic granulation were observed in few calcium ionophore-treated eggs, most of the treated eggs underwent either shrinkage or cytoplasmic granulation. Therefore, the TUNEL analysis was carried out using control eggs that had first polar body with normal morphology and 1.6 μM calcium ionophore-treated eggs that underwent either shrinkage or cytoplasmic granulation. The TUNEL analysis further confirmed our results that 1.6 μM calcium

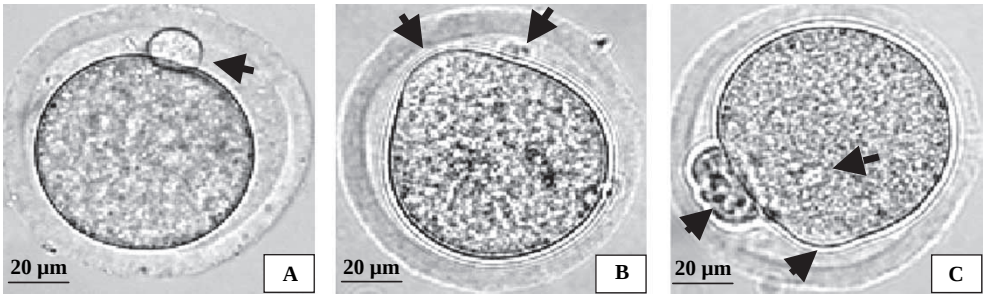

Figure 1. Representative photograph showing effect of calcium ionophore on morphological features characteristics of spontaneous egg activation. (A) Control egg showing first polar body (►) with normal morphology in Ca^{2+}/Mg^{2+}-deficient medium. (B) Activated egg showing degeneration of first polar body and extrusion of second polar body in Ca^{2+}/Mg^{2+}-supplemented medium (►). (C) Calcium ionophore (0.4 μM) treated eggs showing degeneration of first polar body, pronuclei formation and extrusion of second polar body (►). Bar = 20 μM.

Table I. Effect of various doses of calcium ionophore on morphological changes characteristics of activation and apoptosis in eggs cultured in Ca^{2+}/Mg^{2+}-deficient (−) or Ca^{2+}/Mg^{2+}-supplemented medium (+) *in vitro* for 3 h.

Treatment Calcium ionophore	Culture medium	Second polar body extrusion (%)	Morphological features (in %)		
			Pronuclei formation (%)	Apoptotic features (%)	Degeneration (%)
Control	(+)	20.0 ± 5.0	Nil	Nil	Nil
Control	(−)	Nil	Nil	Nil	Nil
0.2 μM	(−)	33.37 ± 1.41	36.45 ± 2.02	Nil	Nil
0.4 μM	(−)	53.33 ± 3.33	52.05 ± 3.57	Nil	Nil
0.8 μM	(−)	87.77 ± 1.92	78.39 ± 3.86	10.36 ± 1.50	Nil
1.6 μM	(−)	47.20 ± 1.21	43.37 ± 3.43	42.85 ± 4.12	7.78 ± 0.56
3.2 μM	(−)	Nil	Nil	7.93 ± 0.39	90.67 ± 3.34

Values are expressed as mean (%) ± SEM of three replicates. Data are analysed by one-way ANOVA.

ionophore-induced egg apoptosis. As shown in Figure 4, calcium ionophore induced DNA fragmentation in treated eggs that underwent morphological apoptotic changes such as shrinkage (Figure 4B) and cytoplasmic granulation (Figure 4C) as evidenced by TUNEL positive staining whereas control eggs that had normal morphology showed TUNEL negative staining (Figure 4A).

Intracellular H_2O_2 levels in activated and apoptotic eggs

As shown in Figure 5, calcium ionophore significantly ($p < 0.05$) increased intracellular level of H_2O_2 (84.40 ± 0.50 ng/egg) in activated eggs that exhibited second polar body with pronuclei as compared to control egg (80.46 ± 1.34 ng/egg) that had first polar body with normal morphology. The intracellular level of H_2O_2 was further increased ($p < 0.01$) in eggs (92.43 ± 0.93) that had morphological apoptotic features. Pre-incubation of eggs with 100 μM of BHA for 5 min significantly (< 0.01) reduced intracellular H_2O_2 level (81.20 ± 1.42 ng/egg) and it was comparable to control eggs.

Discussion

The involvement of calcium during meiotic cell cycle progression and apoptosis has been well documented [2–4,18,24,33,38,40]. Calcium ionophore elevates $[Ca^{2+}]i$ by depleting essentially all calcium pools and induces egg activation or apoptosis [4,7,18,32]. To induce egg activation, eggs are generally treated with calcium ionophore (5–50 μM) for a few minutes (5–15 min) under *in vitro* culture conditions [12,13,19,27,30,32]. In the present study, we reduced

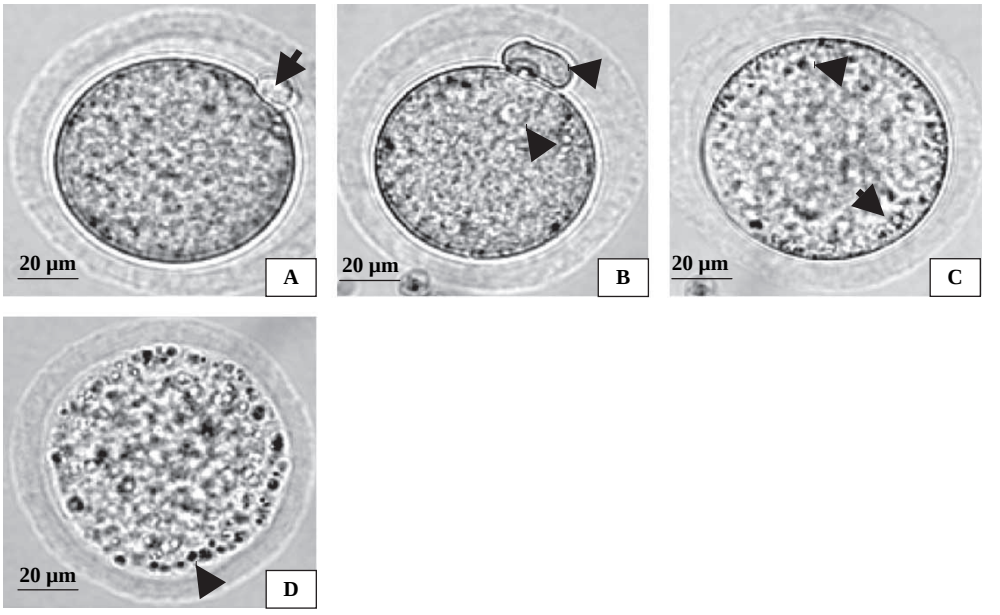

Figure 2. Representative photograph showing calcium ionophore-induced pronuclei formation and appearance of cortical granule in eggs. (A) Control egg showing degenerating first polar body with no sign of spontaneous activation in Ca^{2+}/Mg^{2+}-deficient medium (►). (B) Calcium ionophore (0.2 μM) induced pronuclei formation in M-II arrested egg (►). (C) Calcium ionophore (0.2 μM) induced appearance of cortical granules in M-II arrested egg cytoplasm after 1 h of culture (►). (D) Alignment of cortical granule structure in the peripheral area of treated egg after 3 h of culture (►). Bar = 20 μM.

Table II. Effect of pre-incubation of BHA (100 μM) for 5 min on calcium ionophore-induced morphological changes characteristics of activation and apoptosis in eggs cultured in Ca^{2+}/Mg^{2+}-deficient medium *in vitro* for 3 h.

Treatment Calcium ionophore	Second polar body extrusion (%)	Morphological features (in %) Pronuclei (%)	Apoptotic features (%)	Degeneration (%)
Control	Nil	Nil	Nil	Nil
BHA (100 μM)	Nil	Nil	Nil	Nil
0.2 μM	Nil	Nil	Nil	Nil
0.4 μM	6.0±1.15	6.67±0.88	Nil	Nil
0.8 μM	17.67±1.45	17.67±0.88	Nil	Nil
1.6 μM	Nil	Nil	20.67±1.76	6.0±1.20
3.2 μM	Nil	Nil	8.0±1.15	92.0±1.15

Values are expressed as mean (%)±SEM of three replicates. Data are analysed by one-way ANOVA.

calcium ionophore concentrations (0.2–3.2 μM) and increased treatment time for 3 h in order to achieve a shift from egg activation to apoptotic cell death. Data of the present study revealed that eggs cultured in serum-free and Ca^{2+}/Mg^{2+}-deficient medium did not induce egg activation, while culture of eggs in Ca^{2+}/Mg^{2+}-supplemented medium induced extrusion of second polar body in 20% of eggs. A possibility exists that the presence of calcium in culture medium might have contributed at least in part towards the egg activation, particularly the extrusion of second polar body, but not other morphological features characteristic of egg activation such as pronuclei formation and cortical granule exocytosis. These results are in agreement with previous observations that the presence of calcium in culture medium induces extrusion of second polar body [11,24–30].

The role of exogenous calcium in inducing egg activation is further supported by data of the present study that the lower concentrations of calcium ionophore induced morphological features characteristic of egg activation such as extrusion of second polar body and pronuclei formation in a dose-dependent manner if cultured in Ca^{2+}/Mg^{2+}-deficient medium for 3 h. Theses results suggest that culture of eggs in medium containing lower concentrations of calcium ionophore might have increased $[Ca^{2+}]i$ level and thereby egg activation. Although we have not measured $[Ca^{2+}]i$ level, previous studies suggest that calcium ionophore increases intracellular

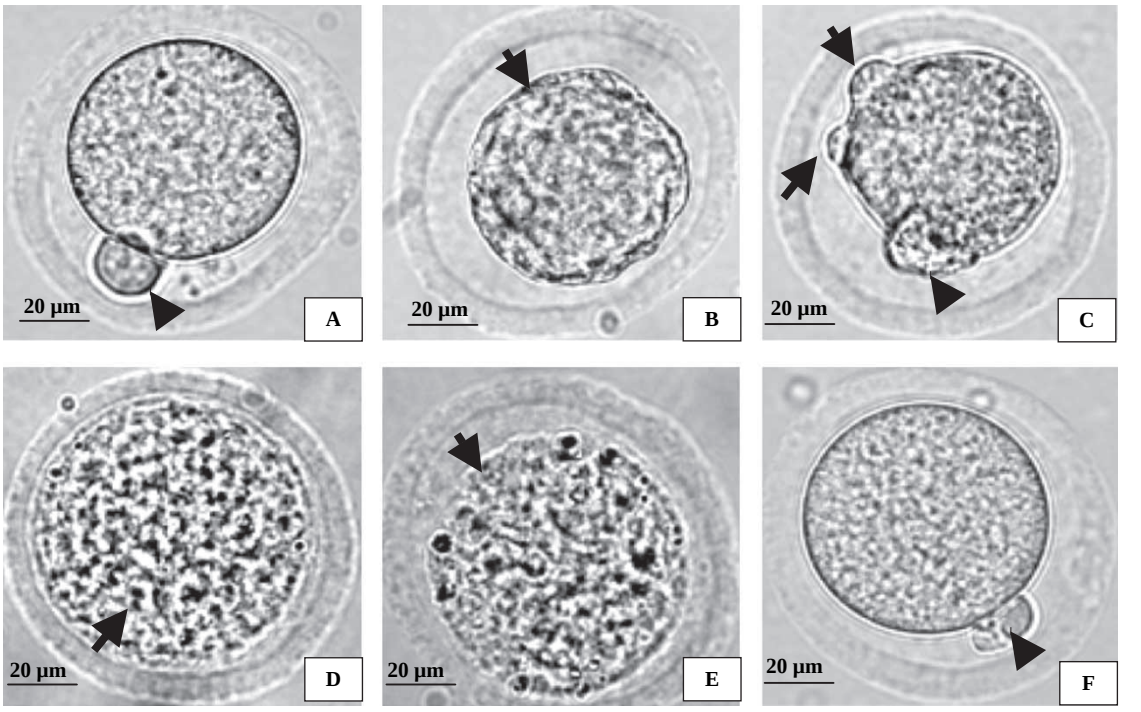

Figure 3. Representative photograph showing effect of pre-incubation of eggs with BHA (100 μM) on calcium ionophore-induced morphological features characteristic of egg apoptosis. (A) Control egg showing first polar body with no sign of spontaneous activation in Ca^{2+}/Mg^{2+}-deficient medium (►). (B) Calcium ionophore (1.6 μM) treated eggs showing shrinkage, (C) membrane blebbing, (D) cytoplasmic granulation prior to degeneration (E) (►). Egg pre-incubated with BHA (100 μM) for 5 min and incubated with calcium ionophore (1.6 μM) for 3 h showing degenerating first polar body with normal morphology. Bar = 20 μM.

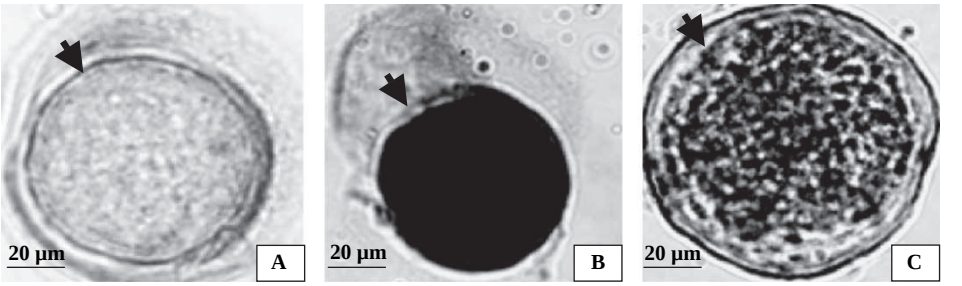

Figure 4. Representative photograph showing calcium ionophore-induced DNA fragmentation in eggs exhibiting morphological apoptotic features. (A) Control egg without any treatment showing TUNEL negative staining (►). (B) Calcium ionophore (1.6 µM) treated eggs exhibiting shrinkage and (C) cytoplasmic granulation showing TUNEL positive staining (►). Bar = 20 µM.

calcium level and induces egg activation in mouse and rat [24,25]. The lower concentration of calcium ionophore (0.2 µM) induced pronuclei formation and appearance of cortical granule even in M-II arrested eggs. The cortical granules were aligned in the peripheral area of the egg cytoplasm in the later stage of treatment. These findings are in agreement with previous observations that lower concentration of calcium ionophore increased $[Ca^{2+}]$i level and that was not adequate to induce meiotic resumption but good enough to induce appearance of cortical granules in treated eggs [7,24,41].

The $[Ca^{2+}]$i level seems to be crucial during egg activation or apoptosis. In most instances, a transient increase of $[Ca^{2+}]$i is required for a short period of time to induce egg activation [33,42]; prolonged high intracellular calcium levels lead to apoptosis [4,20–22]. In the present study, a shift from egg activation towards apoptosis was observed if the eggs were treated with higher concentrations of calcium ionophore (0.8 and 1.6 µM) for 3 h. The shrinkage was a first morphological apoptotic feature observed in most of the treated eggs. The membrane blebbing was a second morphological apoptotic feature observed in a few of the treated eggs during later stage of treatment, while cytoplasmic granulation was a third morphological apoptotic feature observed just prior to degeneration of egg. Similarly, previous studies suggest that calcium ionophore increased $[Ca^{2+}]$i level [20,21,43] and induced apoptosis in bovine oocytes and embryos [18,32]. In the present study, a higher concentration of calcium ionophore (3.2 µM) induced degeneration in more that 90% of eggs after 3 h of culture. The detrimental effects of calcium ionophore at its higher concentration (3.2 µM) might be due to complete endogenous burst of Ca^{2+} from internal source such as mitochondria as reported for rat somatic cell [23,34]. These results together with previous findings suggest that lower concentrations of calcium ionophore induce egg activation whereas higher concentrations induce morphological apoptotic features characteristics of apoptosis prior to degeneration.

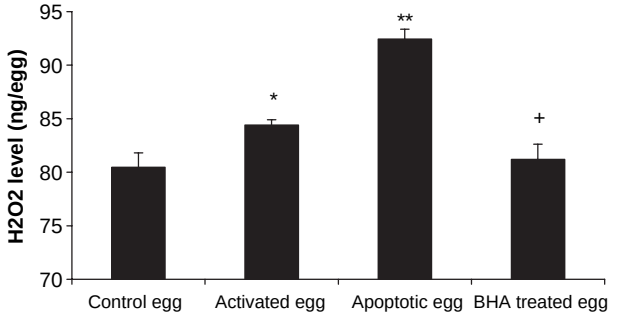

Figure 5. Effect of pre-incubation of BHA (100 µM) on calcium ionophore-induced generation of intracellular H_2O_2 level in eggs. Control egg, untreated egg with normal morphology; activated egg, calcium ionophore (0.4 µM) treated egg that had second polar body with pronuclei; apoptotic egg, calcium ionophore (1.6 µM) treated egg that had morphological apoptotic changes; BHA treated egg, egg was pre-incubated with 100 µM BHA for 5 min and then incubated with 1.6 µM of calcium ionophore for 3 h. Data are mean (%) ± SEM of three replicates. '*' and '**' denote significantly higher, compared to control, at $p < 0.05$ and $p < 0.01$ level, respectively. '+' denotes significantly lower, compare to apoptotic egg, at $p < 0.01$ level.

A unique biochemical event in apoptosis that precedes morphological changes is the activation of Ca^{2+}/Mg^{2+}-dependent endonuclease [44]. This enzyme cleaves genomic DNA at the enternucleosomal region resulting in 180–200 base-pair DNA oligonucleosomal fragments. These fragmented DNA can be detected either in a single cell using an *in situ* technique such as TUNEL assay or DNA gel-electrophoresis [35,45,46]. In the present study, TUNEL positive staining was observed in a higher concentration (1.6 µM) of calcium ionophore-treated eggs that had morphological apoptotic features such as shrinkage and cytoplasmic granulation. Similarly, the TUNEL positive staining has been reported in oocytes that had morphological apoptotic features [11,35,46,47].

A tonic generation of reactive oxygen species is beneficial for resumption of meiosis in bovine oocytes [37], while a cell-permeant antioxidant such as BHA inhibits resumption of meiosis in rat oocytes cultured *in vitro* [39]. The exogenous calcium supplementation induces generation of intracellular H_2O_2 and thereby apoptosis in rat somatic cell [34]. These observations suggest that calcium ionophore-induced egg activation or apoptosis may be associated with the

generation of a distinct level of intracellular H_2O_2 in treated eggs, while BHA could reduce intracellular H_2O_2 level and protect against calcium ionophore-induced egg activation and apoptosis. To test this possibility, we analysed intracellular H_2O_2 in eggs so that a correlation can be made between calcium ionophore-induced various morphological changes and intracellular H_2O_2 level in eggs. Data of the present study revealed that calcium ionophore induced generation of a tonic level of H_2O_2 in eggs that underwent egg activation. The pronounced increase of intracellular level of H_2O_2 was noticed in higher concentrations of calcium ionophore-treated eggs that exhibited morphological apoptotic features. Conversely, BHA not only reduced intracellular H_2O_2 level but also protected against calcium ionophore-induced egg activation and apoptosis. Taken together, these data suggest that lower concentrations of calcium ionophore induce generation of a tonic level (under physiological range) of intracellular H_2O_2 and thereby morphological features characteristic of egg activation, while higher concentrations of calcium ionophore induced generation of higher level of intracellular H_2O_2 and thereby morphological features characteristic of egg apoptosis. Conversely, BHA reduced intracellular H_2O_2 level and thereby calcium ionophore-induced egg activation and apoptosis in rat eggs cultured *in vitro*. Hence, generation of a distinct intracellular H_2O_2 levels may determine the fate of an egg undergoing activation or apoptosis.

In summary, data of the present study clearly suggest that the generation of distinct levels of H_2O_2 is associated with calcium ionophore-induced egg activation and apoptosis. The lower concentrations of calcium ionophore induce generation of a tonic level of H_2O_2 and thereby egg activation, while higher concentrations induce more pronounced generation of H_2O_2 and thereby egg apoptosis.

Acknowledgements

The authors are very much thankful to Mr Vinay K. Dubey and Mr Ravi S. Chaubey, Biotech India, Nandigram, Lanka, Varanasi, UP, India, for the generous gift of H_2O_2 kit. We are grateful to Professors Deoki Nandan (Director) and K. Kalaivani (HOD; RBM) from the National Institute of Health and Family Welfare, Baba Gang Nath Marg, Munirka, New Delhi-110067, India, for their keen interest and encouragement throughout the study.

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