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GAMETE BIOLOGY

An insufficient increase of cytosolic free calcium level results postovulatory aging-induced abortive spontaneous egg activation in rat

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Abstract
Purpose The present study was aimed to find out whether postovulatory aging-induced abortive spontaneous egg activation (SEA) is due to insufficient increase of cytosolic free Ca^{2+} level.
Methods Immature female rats (22–24 days old) were subjected to superovulation induction protocol. Eggs were collected 14, 17 and 19 h post-hCG surge to induce in vivo egg aging. The eggs were collected 14 h post-hCG surge and cultured in vitro for 3, 5 and 7 h to induce in vitro egg aging. The morphological changes, rate of abortive SEA, chromosomal status and cytosolic free Ca^{2+} levels were analyzed.
Results Postovulatory aging induced morphological features characteristics of abortive SEA in a time-dependent manner in vivo as well as in vitro. The extracellular Ca^{2+} increased rate of abortive SEA during initial period of culture, while co-addition of a nifedipine (L-type Ca^{2+} channel blocker) protected against postovulatory aging-induced abortive SEA. However, CI induced morphological features characteristics of egg activation (EA) in a dose-dependent manner. As compare to control, an increase of cytosolic free Ca^{2+} level (1.42 times) induced abortive SEA, while further increase of cytosolic free Ca^{2+} level (2.55 times) induced EA.
Conclusion Our results show that an insufficient cytosolic free Ca^{2+} level is associated with postovulatory aging - induced abortive SEA, while furthermore increase is required to induce EA in rat.

Capsule Postovulatory aging induces insufficient increase of cytosolic free Ca^{2+} level leading to abortive spontaneous egg activation, while further increase of Ca^{2+} level is required to induce egg activation.

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Keywords Cytosolic free calcium · Abortive spontaneous egg activation · Postovulatory aging · Rat eggs

Introduction

In most of the mammalian species, ovulated eggs are arrested at metaphase-II (M-II) stage of meiotic cell cycle until fertilization. The fertilizing spermatozoa increases cytosolic free calcium (Ca^{2+}) level and induces egg activation (EA), which is morphologically characterized by an exit from M-II arrest, extrusion of second polar body (PB-II), cortical granules exocytosis and pronuclei formation [1, 2]. The inositol 1,4,5-triphosphate-sensitive stores are the primary source for an increase of cytosolic free Ca^{2+} level during EA [3–5]. Calcium ionophore A23187 (CI) increases cytosolic free Ca^{2+} level and induces EA in vitro [6–9].

In the absence of fertilization, postovulatory aging results in abortive spontaneous egg activation (SEA) in several mammalian species including human [2, 10–16]. However, the possible factor(s) that drive postovulatory aging-induced abortive SEA is not yet known. An increase of cytosolic free Ca^{2+} level is one of the important factors that triggers EA under in vivo as well as in vitro conditions, a possibility exists that, similar to EA, an increase of cytosolic free Ca^{2+} level may also trigger postovulatory aging-induced abortive SEA. However, there is no evidence to support this possibility. Postovulatory aging-induced abortive SEA deteriorates the egg quality and limits assisted reproductive technologies (ART) such as in vitro fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT) during animal cloning [14, 17–19]. Several drugs such as proteasome inhibitor, MG-135 [20, 21], melatonin [22], verapamil [16] and pyruvate [23] have been used to prevent postovulatory aging-induced abortive SEA in vitro [16, 24].

Rat is an interesting animal model for the study of SEA [14, 16, 25–28], since this process is rapid [29] and abortive [30]. After extrusions of PB-II, rat egg neither form pronuclei and nor proceed to interphase, instead gets arrested at metaphase-III (M-III) like stage [14, 24, 27, 28]. Postovulatory aging is one of the important factors that induce abortive SEA in rat. For example, freshly ovulated eggs recovered after 14 h post human chorionic gonadotropin (hCG) surge are less likely to undergo abortive SEA, while postovulatory aging induces abortive SEA [16, 31–33]. However, factor(s) that drives postovulatory aging-induced abortive SEA are not fully understood. We hypothesize that an insufficient increase of cytosolic free Ca^{2+} level could be one of the factors associated with postovulatory aging-induced abortive SEA. Hence, present study was designed to investigate the involvement of cytosolic free Ca^{2+} level during postovulatory aging-induced abortive SEA in rat. For this purpose, post-ovulatory egg aging was induced in vivo as well as in vitro and morphological changes such as extrusion of PB-II, meiotic status and cytosolic free Ca^{2+} level were analyzed.

Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Preparation of CI and Fluo-3 AM working solutions

CI was initially dissolved in 100 μL of dimethyl sulfoxide (DMSO) and then in distilled water to get a final concentration of 1 mg/ml (stock solution). The stock solution was further diluted in Ca^{2+}-free medium (Medium-199, AL043A, HiMedia Laboratories, Mumbai, India) to get working concentrations of CI (0.2, 0.3, 0.4 and 0.5 μM). The freshly prepared working concentrations were pre-warmed at 37 °C for 5 min before use. Addition of CI did not alter the osmolarity (290±5 m Osmol) and pH (7.2±0.2) of culture medium. Fluo-3 AM was initially dissolved in 100 μL of DMSO and then in media to get a final concentration of 1 mg/ml (stock solution). The stock solution was further diluted using Ca^{2+} -free medium to get working concentration (50 μM) of Fluo-3 AM. Since DMSO was used as a solvent for the preparation of CI and Fluo-3 AM stock solutions, an equivalent volume of the highest concentration (0.1 % DMSO) was used in the control group.

Experimental animals

Immature female rats (22–24 days old) of Charles-Faster strain were housed in light-controlled room, with food and water available ad libitum. In order to collect maximum number of ovulated eggs, rats were subjected to superovulation protocol by priming with subcutaneous injection of 15 IU pregnant mare's serum gonadotropin (PMSG) for 48 h followed by 15 IU hCG for various times depending on the experiments (i.e. 14 h for in vitro studies and 14, 17, and 19 h for in vivo studies). All procedures confirmed to the stipulations of the Institutional Animal Ethical Committee of Banaras Hindu University, Varanasi, UP, India.

Collection and culture of eggs

Ovulated cumulus-enclosed eggs were collected from oviduct using a 26-gauge needle and transferred in pre-warmed Ca^{2+}-free medium. All ovulated cumulus-enclosed eggs were picked up using micropipette (Clay Adams; B&D and Co., NJ) and transferred to fresh culture medium containing 0.01 % hyaluronidase at 37 °C for 3 min for the removal of cumulus cells. The denuded eggs were washed three times with fresh culture medium and then 15–20 denuded eggs were transferred 200 μl of culture medium for in vitro studies. The eggs used for all in vitro experiments were arrested at the M-II stage showing first polar body (PB-I).

Effect of postovulatory aging on morphological changes in egg in vivo

Ovulation occurs in immature rats (22–24 days old) subjected to superovulation induction protocol after 14 h post-hCG surge. In order to induce postovulatory aging, eggs were collected 14, 17 and 19 h post-hCG surge. These eggs were quickly denuded as described above and then observed for their morphological changes using phase-contrast microscope (Nikon, Eclipse; E200, Japan) at 400x magnification.

Effect of extracellular Ca^{2+} on morphological changes in eggs in vitro

A group of 15–20 eggs (collected 14 h post-hCG surge) was cultured either in Ca^{2+} -free medium-199 or Ca^{2+} (1.80 mM) supplemented medium (Medium-199, Cat. no. AL014A; HiMedia Laboratories, Mumbai, India) containing with or without 200 μM nifedipine (a known L-type Ca^{2+} channel blocker). This concentration of nifedipine has been reported to inhibit SEA in rat eggs cultured in vitro [29]. All cultures were maintained at 37 °C for 3, 5 and 7 h in humidified chamber. At the end of the incubation period, eggs were examined for abortive SEA using a phase-contrast microscope at 400x magnification.

Effect of CI on morphological changes in eggs in vitro

We used CI as a positive control in the present study to compare morphological changes characteristics of EA with postovulatory aging-induced abortive SEA. For this purpose, 15–20 eggs collected after 14 h post-hCG surge were cultured in Ca^{2+}-free medium with or without various concentrations of CI (0.2, 0.3, 0.4 and 0.5 μM) for 3 h in humidified chamber at 37 °C. At the

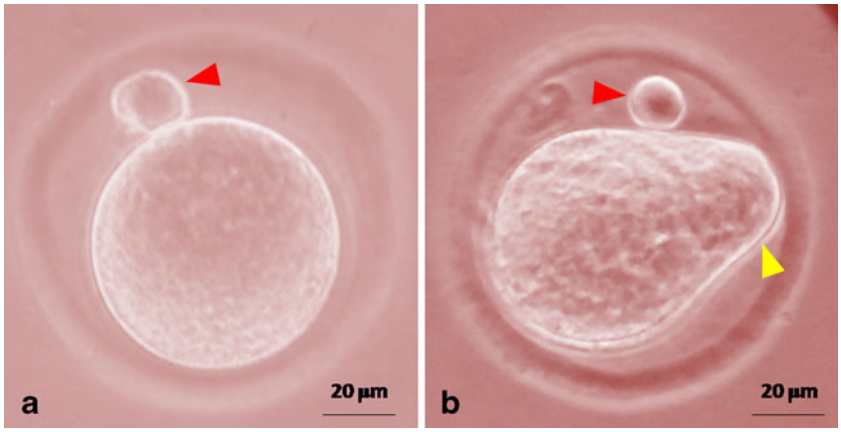

Fig. 1 Representative photographs showing postovulatory aging-induced abortive SEA in vivo. **a** Egg collected 14 h post-hCG surge showing extruded PB-I. **b** Eggs collected 19 h post-hCG surge showing PB-I (*red arrow head*) and incomplete extrusion of PB-II (*yellow arrow head*). Bar =20 µm

end of the incubation period, eggs were removed, washed with fresh medium and then examined for morphological changes using phase-contrast microscope at 400x magnification.

Analysis of meiotic status of eggs

To analyze the meiotic status, 14–15 eggs (eggs at various stages of meiotic cell cycle) were incubated in medium containing Hoechst stain (0.5 µg/ml of distilled water) for 10 min at 37 °C. At the end of the incubation period, eggs were removed and washed three times with phosphate buffer saline and then examined for chromosomal status under Epi-fluroscence Microscope (Nikon, Eclipse; E-80i, Japan) using $_{Ex}$350 nm and $_{Em}$460 nm at 400x magnifications.

Analysis of cytosolic free Ca^{2+} level using Fluo-3

The cytosolic free Ca^{2+} level was analyzed in eggs at various stages of meiotic cell cycle following published protocol with some modifications [3, 34, 35]. In brief, eggs (14–15 eggs in each group) were cultured in Ca^{2+}-free medium with or without CI (0.5 µM) and 50 µM Fluo-3 AM for 1 h at 37 °C in humidified chamber. At the end of the incubation period, eggs were removed and washed three times with Ca^{2+}-free medium and observed under Epi-fluroscence Microscope (Nikon, Eclipse; E-80i, Japan) using $_{Ex}$488 nm and $_{Em}$525 nm. The corrected total cell fluorescence (CTCF) was calculated following the method published earlier [34] using Image J Software (version 1.44 from National Institute of Health, USA).

Statistical analysis

Data are expressed as mean±standard error of mean (SEM) of triplicate samples. All percentage data were subjected to arc-sine square-root transformation before statistical analysis. Data are analyzed either by Student's *t*-test or by one-way ANOVA followed by post hoc (Bonferroni) analysis using SPSS software, Version 13 (SPSS, Inc. Chicago, IL). A probability of $P<0.05$ was considered as statistically significant.

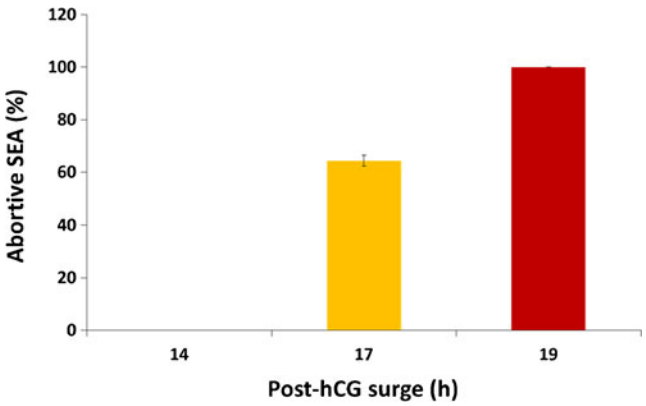

Fig. 2 Postovulatory aging-induced abortive SEA in vivo. Eggs were collected 17 and 19 h post-hCG surge and morphological features of abortive SEA were analyzed. Data are mean (%)±SEM of three replicates and data were analyzed by one-way ANOVA

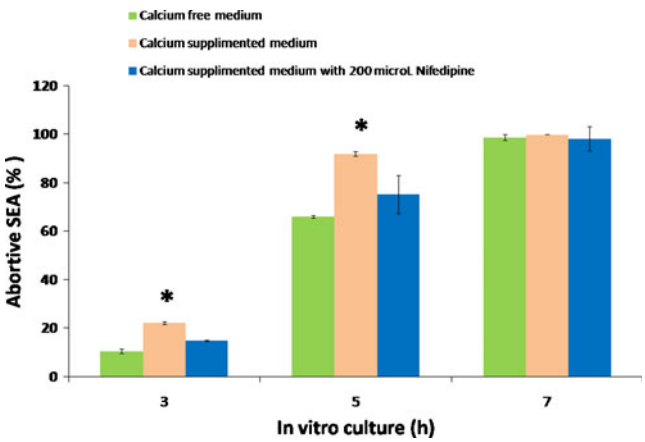

Fig. 3 Postovulatory aging-induced abortive SEA in vitro. Eggs collected 14 h post-hCG surge were cultured in Ca^{2+}-free medium or Ca^{2+}-supplemented medium with or without nifedipine (200 µM) for various times (3, 5 and 7 h). Data are mean (%)±SEM of three replicates. Data were analyzed by one-way ANOVA followed by post hoc test (Bonferroni). "*" Denotes significant ($P<0.05$) increase as compare to Ca^{2+}-free medium

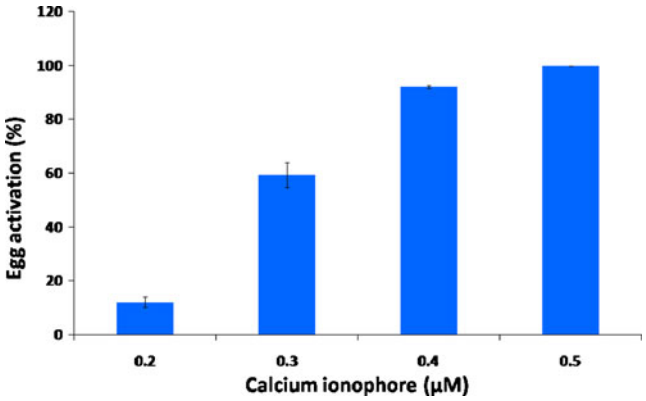

Fig. 4 CI-induced morphological changes characteristics of EA in vitro. Eggs collected 14 h post-hCG surge were cultured in Ca^{2+} -free medium containing various concentrations of CI for 3 h in vitro. Data are mean (%)±SEM of three replicates and analyzed by one-way ANOVA

Results

Postovulatory aging induces abortive SEA in vivo

As shown in Fig. 1, newly ovulated eggs collected after 14 h post-hCG surge were arrested at M-II stage of meiotic cell cycle and extruded PB-I (Fig. 1a). Postovulatory aging induced exit from M-II arrest and incomplete extrusion of PB-II, a morphological feature of abortive SEA (Fig. 1b). The rate of abortive SEA was increased in a time-dependent manner if the egg were collected after 17 and 19 h post-hCG surge (One-way ANOVA; F=1863.4, P<0.001; Fig. 2). All eggs underwent abortive SEA if collected after 19 h of post-hCG surge.

Extracellular Ca^{2+} has a role during abortive SEA in vitro

As shown in Fig. 3, postovulatory aging in vitro induced abortive SEA a time-dependent manner in eggs cultured either in Ca^{2+}-deficient medium (one-way ANOVA; F=4578.5, P< 0.001) or Ca^{2+} supplemented medium (one-way ANOVA; F= 3154, P<0.001). Presence of extracellular Ca^{2+} induced significantly higher rate of abortive SEA during 3 and 5 h of in vitro culture. Almost all eggs underwent abortive SEA after 7 h of in vitro culture in either media suggesting the role of extracellular Ca^{2+} during postovulatory aging-induced abortive SEA in vitro. However, nifedipine (200 μM) protected extracellular Ca^{2+} -induced abortive SEA in eggs cultured in Ca^{2+} supplemented medium (Fig. 3).

CI induces morphological features characteristics of EA in vitro

To confirm the role of Ca^{2+} in inducing morphological features characteristics of EA, we used CI, a known drug that releases Ca^{2+} from internal stores. As shown in Fig. 4, CI induced morphological features characteristics of EA in a dose-dependent manner (one-way ANOVA; F=247.23, P< 0.001). Egg collected 14 h post-hCG surge were arrested at M-II stage exhibiting PB-I (Fig. 5a). However, an exit from M-II arrest, complete extrusion of PB-II and pronuclei formation were the morphological features associated with CI-induced EA (Fig. 5b).

Postovulatory aging induces M-III like arrest

The meiotic status was confirmed during abortive SEA and CI-induced EA using Hoechst stain. As shown in Fig. 6a, control eggs were arrested at M-II stage as evidenced by condensed haploid set of chromosomes in egg cytoplasm as well as in PB-I. Eggs that underwent postovulatory aging–induced abortive SEA showed scattered chromosome in the egg cytoplasm suggesting M-III like arrest and condensed haploid set of chromosomes in PB-II (Fig. 6b). However, eggs underwent CI-induced EA showed pronuclei formation, condensed chromosomes in PB-I and PB-II suggesting the completion of meiosis (Fig. 6c).

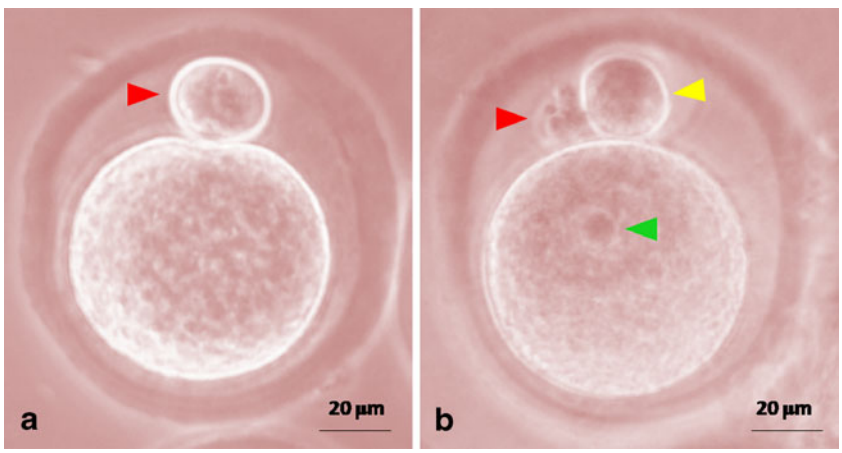

Fig. 5 Representative photographs showing CI-induced morphological changes characteristics of EA in vitro. **a** Control egg showing PB-I. **b** CI-induced EA as evidenced by degenerating PB-I (*red arrow head*), complete extrusion of PB-II (*yellow arrow head*) and formation of pronuclei (*green arrow head*). Bar =20 μm

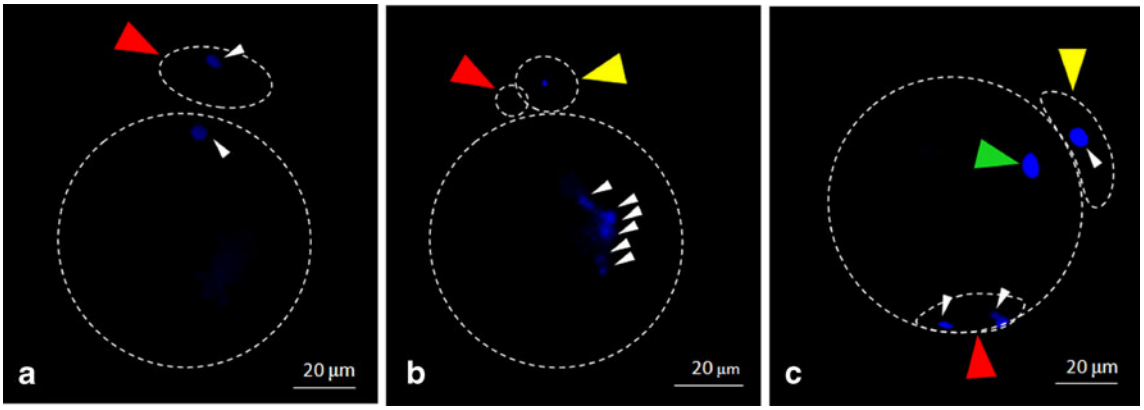

Fig. 6 Representative photographs showing meiotic status of eggs. **a** Control egg showing condensed chromosome (*white arrow heads*) in egg cytoplasm as well as in PB-I (*red arrow head*) characteristics of M-II arrest. **b** Egg showing scattered chromosomes (*white arrow heads*) in the cytoplasm suggesting M-III like arrest, extrusion of PB-II (*yellow arrow head*) and degenerating PB-I (*red arrow head*). **c** Egg showing pronuclei formation (*green arrow head*), complete extrusion of PB-II (*yellow arrow head*), condensed chromosome (*white arrow heads*) in PB-I (*red arrow head*) and PB-II suggesting the completion of meiosis. Bar =20 µm

Postovulatory aging increases cytosolic free Ca^{2+} level

To confirm the role of cytosolic free Ca^{2+} during postovulatory aging-induced abortive SEA and CI-induced EA, fluorescence intensity of Fluo-3 was analyzed. As shown in Fig. 7, a rise in fluorescence intensity of Fluo-3 was observed in eggs underwent postovulatory aging-induced abortive SEA (Fig. 7b) as compare to control eggs (Fig. 7a). However, further increase of fluorescence intensity was noticed in eggs that underwent CI-induced EA (Fig. 7c). The CTCF analysis using Image J software (Version 1.3) of three independent experiments further confirm that the postovulatory aging significantly ($P<0.001$) increased cytosolic free Ca^{2+} level (1.42 times) and induced abortive SEA, while further increase (2.55 times) was associated with CI-induced EA (Fig. 8).

Discussion

Postovulatory aging -induced SEA has been reported in several mammalian species [14, 16, 25, 27, 30]. However, rat is an interesting model because the process of SEA is abortive [30] and aged eggs are arrested at M-III like stage [24]. The freshly ovulated eggs (14 h post-hCG surge) are less likely to undergo abortive SEA as compare to aged eggs in vivo [28, 31, 33]. In the present study, freshly ovulated eggs (collected 14 h post-hCG surge) were arrested at M-II stage and showed extrusion of PB-I. However, postovulatory aging in vivo (egg collected after 17 and 19 h post-hCG surge) as well as in vitro (freshly ovulated eggs collected after 14 h post-hCG surge and their culture under in vitro conditions for 5 and 7 h) induced abortive SEA in a time-dependent manner. These data further support previous

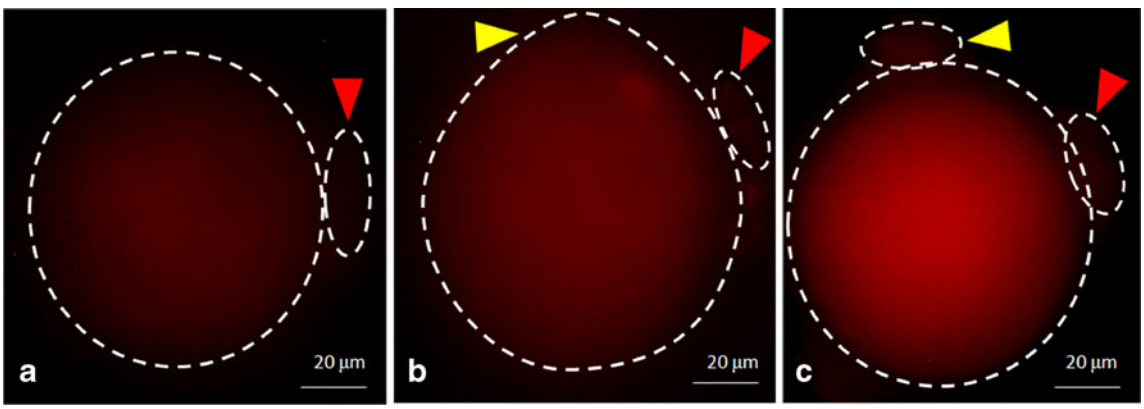

Fig. 7 Representative photographs showing fluorescence intensity of Fluo-3 in eggs. **a** Control egg arrested at M-II stage. **b** An increase of fluorescence intensity in postovulatory aging-induced abortive SEA. **c** The fluorescence intensity was further increased in eggs that underwent CI-induced EA. PB-I (*red arrow head*), PB-II (*yellow arrow head*). Bar =20 µm

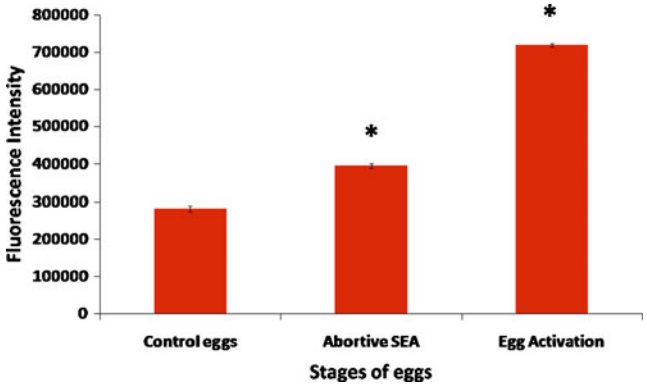

Fig. 8 The CTCF analysis of fluorescence intensity of Fluo-3 in eggs. Data are mean±SEM of fluorescence intensity using representative eggs of three different experiments. Data were analyzed by Students *t*-test, "*" Denotes significant ($P<0.001$) increase as compare to control eggs

observations that the residence of ovulated eggs in the oviduct or culture of freshly ovulated eggs under in vitro conditions induced morphological features associated with abortive SEA [4, 14, 25–27].

Ca^{2+} plays a major role in the modulation of egg physiology [36]. Data of the present study suggest that the extracellular Ca^{2+} increased rate of abortive SEA during initial period (3 and 5 h) of in vitro culture, while all eggs underwent abortive SEA if cultured for 7 h either in Ca^{2+}-deficient or Ca^{2+}-supplemented medium. Further, L-type Ca^{2+} channel blocker such as nifedipine (200 μM) protected against extracellular Ca^{2+}-induced abortive SEA. These data suggest that the L-type Ca^{2+} channels are still operated in ovulated eggs [37] and nifedipine blocks L-type Ca^{2+} channels and inhibits abortive SEA in rat eggs [29]. These data together with previous findings suggest that postovulatory aging induces abortive SEA probably by increasing cytosolic free Ca^{2+} level. The inhibitory effect of nifedipine suggests that it can be used to delay egg aging in vitro during various ART programs.

Intracellular Ca^{2+} homeostasis is very important in maintaining the physiology of an ovulated egg. An increase of cytosolic free Ca^{2+} (due to burst from internal stores) induces EA during fertilization [5, 38, 39]. Further, CI increases cytosolic free Ca^{2+} level [35] and induces EA in several mammalian species [11, 14, 16, 32, 40–42]. In the present study, we used CI as a positive control and data suggest that CI induced morphological features characteristics of EA in a dose-dependent manner. The complete extrusion of PB-II and formation of pronuclei were clearly observed in eggs that underwent CI-induced EA. However, postovulatory aging-induced abortive SEA showed incomplete extrusion of PB-II and did not show pronuclei formation. The chromosomes were scattered in the egg cytoplasm suggesting M-III like arrest in eggs that underwent postovulatory aging-induced abortive SEA. These results together with previous findings suggest that CI induced EA possibly by increasing cytosolic free Ca^{2+} level in rat egg cultured in vitro. A possibility exists that postovulatory aging-induced abortive SEA might be due to an increase of cytosolic free Ca^{2+} level. To test this possibility, we analyzed cytosolic free Ca^{2+} levels and results suggest that the postovulatory aging increased insufficient cytosolic free Ca^{2+} level (1.42 times as compared to control eggs arrested at M-II stage) and induced abortive SEA, while more increase of cytosolic free Ca^{2+} level (2.55 times) induced EA. Although the cytosolic free Ca^{2+} level during postovulatory aging-induced abortive SEA has not been reported in rat eggs so far, an increase of cytosolic free Ca^{2+} induces EA in mouse eggs [6].

Conclusions

Present study suggests that the postovulatory aging induces insufficient increase of cytosolic free Ca^{2+} (1.42 times) level leading to abortive SEA, while further increase in its level (2.55 times) induced EA. Further studies are requried to find out the cause(s) for postovulatory aging induced insufficient increase of cytosolic free Ca^{2+} level in order to protect abortive SEA and to improve ART outcome.

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