ROS

RESEARCH ARTICLES

Inability to Maintain Metaphase-II Arrest due to Increase of Reactive Oxygen Species in Rat Eggs

Shail K. Chaube, Karuppanan V. Premkumar, Shilpa Prasad, Meenakshi Tiwari, Anumegha Gupta, Alka Sharma, Kankshi Sahu, and Pramod K. Yadav

Cell Physiology Laboratory, Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi-221005, U.P., India

Correspondence: shailchaube@bhu.ac.in (S.K.C.)

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ABSTRACT | Maintenance of metaphase-II (M-II) arrest until fertilization is required to maintain the haploidy and presence of first polar body (PB-I) is a morphological indicator of egg quality. The environmental changes, stress and other factors could induce spontaneous exit from metaphase-II arrest (EM-II), a first sign of abortive spontaneous egg activation (SEA) that deteriorates egg quality in few mammalian species including rat. The reactive oxygen species (ROS) and calcium (Ca^{2+}) play a major role in oocyte meiosis but their role in abortive SEA remains ill understood. We examined ROS, Ca^{2+}, and maturation promoting factor (MPF) levels. We observed that postovulatory aging induced EM-II in vivo as evident by initiation of extrusion of second polar body (PB-II) but never completed. The ROS and cytosolic free Ca^{2+} levels were increased that resulted in MPF destabilization and thereby EM-II. In summary, our study suggests that inability of rat eggs to maintain M-II arrest may be due to premature onset of Ca^{2+}- and/or ROS-mediated MPF destabilization that deteriorates egg quality. The poor-quality egg directly impacts reproductive outcome in several mammalian species.

KEYWORDS | Calcium; Exit from metaphase-II arrest; Maturation promoting factor; Rat eggs; Reactive oxygen species

ABBREVIATIONS | Cdk1, cyclin-dependent kinase 1; COCs, cumulus oocyte complexes; CTCF, corrected total cell fluorescence; EM-II, exit from metaphase-II arrest; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; M-II, metaphase-II; MPF, maturation promoting factor; PB-II, second polar body; ROS, reactive oxygen species; SEA, abortive spontaneous egg activation

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1. INTRODUCTION

In mammals, ovulated eggs are generally arrested at metaphase-II (M-II) stage and possess first polar body (PB-I) [1-3]. These eggs are the right choice for in vitro fertilization (IVF) [2-4]. Normally, fertilizing spermatozoa triggers meiotic exit from M-II arrest and thereby egg activation process in mammals [5]. Surprisingly, ovulated eggs in several mammalian species do not wait for fertilization and quickly undergo spontaneous exit from metaphase-II arrest (EM-II) by initiating the extrusion of second polar body (PB-II), a first morphological feature of spontaneous egg activation (SEA) under in vitro culture conditions [3, 5-8]. Although extrusion of PB-II starts quickly in these eggs, it never gets completed and pronucleus is also not formed [6, 7]. The chromosomes are dispersed in the egg cytoplasm leading to metaphase-III (M-III)-like arrest, the so called abortive SEA [3, 5]. The abortive SEA was reported for the first time by Keefer and Schuetz in 1982 in rats [9] and thereafter in mice [10], rats [1, 11], hamsters [12], pigs [13], bovines [14], camels [15], and humans [16]. Abortive SEA has made somatic cell nuclear transfer (SCNT) and IVF programs challenging, and limits assisted reproductive technology (ART) outcome in mammals [3, 5, 17, 18].

The environmental changes, loss of natural habitats, and limited resources cause stress in several mammalian species including human [19]. Stress generates reactive oxygen species (ROS) in the ovary, induces anovulation [20], and limits ART outcome [21]. However, under experimental conditions, collection of cumulus oocyte complexes (COCs), removal of cumulus cells (shear force), and other physical factors may accelerate meiotic resumption from M-II arrest and thereby SEA [22-24]. Although a moderate increase of ROS does not cause oxidative stress, it is good enough to modulate meiosis in rat eggs cultured in vitro [2, 3, 25, 26]. ROS induce release of Ca^{2+} from the endoplasmic reticulum [27, 28]. The elevated levels of ROS as well as Ca^{++} trigger maturation promoting factor (MPF) destabilization and thereby exit from diplotene as well as M-II arrest in vitro [2, 3, 27, 28]. Similar to in vitro studies, we postulated that a moderate increase in ROS as well as Ca^{++} levels may drive EM-II and thereby abortive SEA in vivo too [6-8]. To test this hypothesis, the present study was designed to investigate whether increases in ROS as well as Ca^{++} levels could induce MPF destabilization and thereby EM-II under in vivo conditions using rats as an experimental animal model.

2. MATERIALS AND METHODS

Chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. The culture medium (M-199) was purchased from HiMedia Laboratories (Mumbai, India) and prepared as per the company manual protocol. The freshly prepared culture medium M-199 having a pH of 7.2 ± 0.2 and an osmolarity of 290 ± 10 mOsmol was used in the present study. All procedures were approved by the Institutional Animal Ethical Committee (wide letter no. F.Sc./IAEC/2014-15/0248) and the experimental protocols were approved by the Institutional Animal Ethical Committee, Institute of Science, Banaras Hindu University and performed in accordance with the Institutional guidelines.

2.1. Experimental Animal and Stimulation of Ovary

Sexually immature female rats (*Rattus norvegicus*) of Charles-Foster strain (22–24 days old; 45 ± 5 g body weight) were separated from existing colony and kept in light-controlled room with food and water ad libitum. Experimental animals were maintained in 12 h light/12 h dark conditions and divided into two groups. The first group was given a single injection of 20 IU pregnant mare serum gonadotropin

(PMSG) for 48 h followed by a single injection of 20 IU human chorionic gonadotropin (hCG) for 14 h, and the second group received a single injection of 20 IU PMSG for 48 h followed by a single injection of 20 IU hCG for 17 h. After that, the rats were euthanized and the ovary along with oviduct was collected in a 35-mm Petri dish having 2 ml of pre-warmed medium.

2.2. Collection of Eggs

Eggs were collected by puncturing the ampulla using 26-gauge insulin syringe in a pre-warmed M-199 medium. The freshly ovulated COCs were collected after 14 h post-hCG surge from the first group of experimental animals. To induce abortive SEA, COCs were collected from the second group of experimental animals after 17 h post hCG surge. These COCs were exposed to 0.01% hyaluronidase for 3 min at 37ºC and then denuded by repeated manual pipetting. Eggs were analyzed for their morphological changes under 400× magnification with a light microscope (Nikon Eclipse E200, Tokyo, Japan). For each experiment, five rats were subjected to a superovulation induction protocol and approximately 180–200 eggs were collected for each experiment. The experiment was repeated three times to confirm the results.

2.3. Analysis of Meiotic Status of Eggs

The meiotic status of oocytes was confirmed by using propidium iodide (PI) staining. A group of 12–14 eggs were collected and denuded by repeated manual pipetting. The eggs were washed 3 times with phosphate-buffered saline (PBS) and then exposed to PBS containing PI (1 mg/ml) for 10 min at room temperature. The eggs were washed 10 times with PBS and then analyzed for their meiotic status using a fluorescence microscope (Model, Ni-U, Nikon Eclipse, Tokyo, Japan) at 540 nm. Three independent experiments using 36–48 eggs were conducted to confirm the meiotic stage.

2.4. Analysis of ROS Level Using H_2DCFDA

The ROS level was analyzed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; a stable dye that is converted to H_2DCF by intracellular esterases) following our previously published protocol [28]. In brief, approximately, 12–14 eggs were used in each group and three independent experiments were conducted using 36–42 eggs to confirm the observations. For corrected total cell fluorescence (CTCF) analysis, at least 12–14 eggs/group were analyzed following our previously published protocol [27] using ImageJ software (version 1.44 from the National Institutes of Health, Bethesda, MD, USA). For this purpose, a minimum of three different areas of each egg cytoplasm as well as its corresponding background were selected. The total fluorescence per egg was calculated using the following formula: CTCF = (integrated density) – (area of selected cell × mean fluorescence of background readings). All parameters were kept constant. For each egg, the whole cytoplasmic area was selected, and the fluorescence intensity was analyzed using the aforementioned ImageJ software.

2.5. Analysis of Cytosolic Free Ca^{2+} Level Using Fluo-3

The cytosolic free Ca^{2+} level was analyzed following a previously published protocol [5]. In brief, 12–14 eggs from each group were exposed to Fluo-3 AM (50 µM) for 30 min at 37ºC in a CO_2 incubator. We conducted three independent experiments to confirm our observations using 36–48 eggs and representative photographs were shown in the Results section. A group of 12–14 eggs were used for CTCF analysis from each experiment following the published protocol [5, 27].

2.6. Analyses of Total and Specific Phosphorylated Cdk1 as well as Cyclin B1 Levels

The specific as well as total phosphorylation status of Cdk1 and cyclin B1 levels were analyzed using their specific antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunofluorescence analysis was carried out following our previous published protocol [27] under a fluorescence microscope (Model, Ni-U, Nikon Eclipse) at 465 nm for FITC and 540 nm for TRITC at 400× magnification. In brief, 12–14 eggs from each group were exposed to their specific antibodies i.e., anti-pThr-161(sc-12341), anti-pThr-14/Tyr-15 (sc-12340) and cdc2 p34 PSTAIRE antibody (sc-53), and anti-cyclin B1 antibody (sc-752). We conducted three independent experiments to confirm our observations

and approximately 36–42 eggs were used for CTCF analysis. The CTCF of the egg was carried out by taking the immunofluorescence intensity of the egg cytoplasm for analysis. All parameters were kept constant for each egg and immunofluorescence intensity was analyzed using ImageJ software (version 1.44). For this purpose, a minimum of three different areas of each egg cytoplasm as well as its corresponding background were selected.

2.7. Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM) of three independent experiments. Data obtained using CTCF were analyzed by Student's t-test using the SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

In mammals, freshly ovulated eggs are arrested at M-II stage and possess PB-I [29, 30]. However, in several mammalian species ovulated eggs under in vitro culture conditions are unable to maintain M-II arrest until fertilization and undergo EM-II [28, 29]. The inability to maintain meiotic arrest results in EM-II thereby abortive SEA that deteriorates egg quality and limits reproductive outcome in several mammalian species [29]. This is a typical situation in mammals, and factors that drive EM-II thereby abortive SEA need to be addressed at greater detail in order to protect the precious mammalian species that are either threatened or at the verge of extinction. Our results revealed that the freshly ovulated rat eggs were arrested at M-II stage and exhibited PB-I (**Figure 1A**). The meiotic stages such as M-II arrest (**Figure 1C**) and EM-II (**Figure 1D**) were confirmed using PI staining in the present study.

In rats, the superovulation induction protocol results in ovulation after 14 h post hCG surge [2, 3]. In the present study, ovulated eggs were collected after 14 h post hCG surge (freshly ovulated) or after 17 h post hCG surge (aged in vivo) from the ampulla of fallopian tube. We observed that postovulatory aging induced EM-II and thereby abortive SEA in the majority of ovulated eggs. The initiation of PB-II extrusion in the presence of PB-I (**Figure 1B**) indicated abortive SEA as compared to control eggs that showed PB-I only (**Figure 1A**). Although extrusion of PB-II was quickly initiated but never got completely extruded and pronucleus was not seen in any egg that underwent abortive SEA. These data further support previous studies that the postovulatory aging leads to EM-II and abortive SEA in rat eggs cultured in vitro [1-3, 5-7, 11]. The inability of eggs to extrude PB-II completely results in M-III-like arrest without forming pronuclei in rat eggs in vitro [1-3, 27, 28].

The environmental changes and habitat loss may induce stress-mediated generation of ROS and abortive SEA in rats [19]. To confirm this possibility, we analyzed ROS level using an ROS-specific fluorescence-based cell permeable probe, i.e., H_2DCFDA in the present study. Our data suggested that the ROS level was increased in eggs that underwent EM-II in vivo (**Figure 1E2**) as compared to M-II-arrested control eggs (**Figure 1E1**). The CTCF analysis further strengthened the above findings (**Figure 1E3**; $p < 0.001$). These results confirm previous findings that a moderate increase of ROS induces abortive SEA in rat eggs cultured in vitro [28] and may reduce female fertility in humans [21, 31].

The increased ROS level could modulate membrane potential and induce Ca^{2+} release from internal stores to the cytosol [32]. This possibility is further supported by our observations that the elevated ROS level resulted in the increase of cytosolic free Ca^{2+} level in eggs that underwent EM-II in vivo (**Figure 1F2**) as compared to M-II-arrested control eggs (**Figure 1F1**). The CTCF analysis of fluorescence intensity of the respective egg further confirms above finding (**Figure 1F3**; $p < 0.001$). These results suggest that increased level of ROS could modulate membrane potential of internal stores resulting in the increase of cytosolic free Ca^{2+} in rat eggs in vitro [2, 3, 5, 25, 26] and EM-II in rat eggs in vitro [27].

Ca^{2+} is one of the major signal molecules that modulate cell physiology by operating downstream signaling pathways in eggs [2, 3, 5, 27]. MPF has a catalytic subunit. i.e., cyclin-dependent kinase 1 (Cdk1) and a regulatory subunit, i.e., cyclin B1 [6, 7, 33, 34]. However, changes in Cdk1 activity as well as MPF destabilization may also occur due to increase of mitogen-activated protein kinase (MAPK) [35]. This statement is supported by the observations that MAPK modulates Cdk1 activity and destabilizes MPF during spontaneous exit from M-II arrest [22, 23]. The increased cytosolic free Ca^{2+} level could modulate specific phosphorylation of Cdk1 and cy-

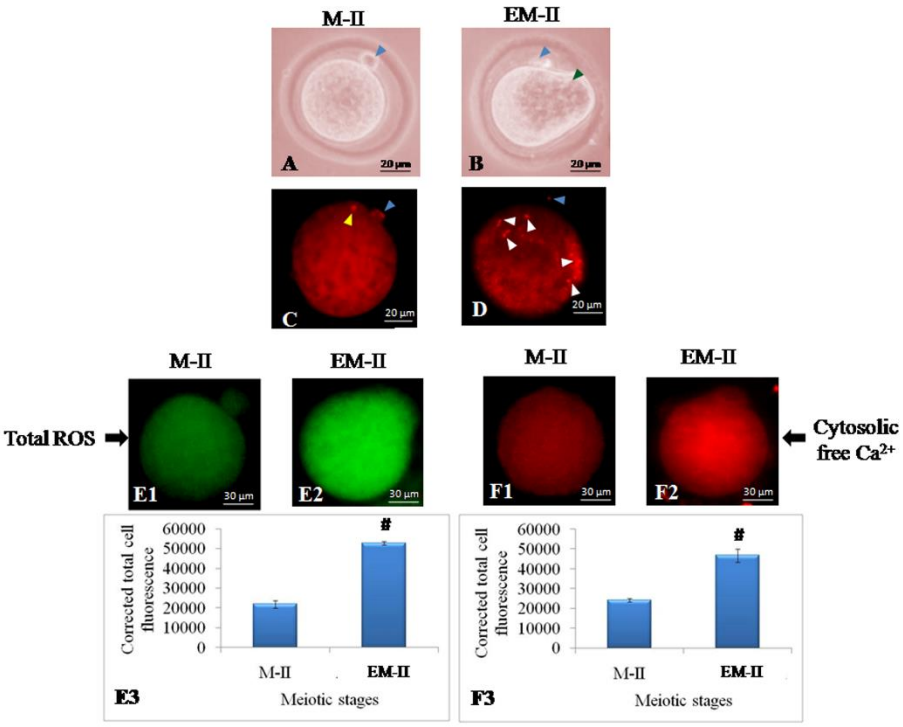

FIGURE 1. Morphological features of spontaneous exit from M-II arrest (EM-II), and intraoocyte levels of ROS and Ca^{2+} in eggs. (A, B) The EM-II is morphologically characterized by the initiation of extrusion of second polar body (PB-II) (green arrow head; 1B) as compared to a metaphase II (M-II)-arrested egg possessing PB-I (blue arrow head) (1A). (C) The M-II-arrested egg showing haploid genome in PB-I (blue arrow head) and in egg cytoplasm (yellow arrow head). (D) The EM-II showing haploid genome in PB-I (blue arrow head) and scattered haploid set of chromosomes throughout the egg cytoplasm (white arrow head). (E, F) Eggs showing increased level of total ROS (E2) and cytosolic free Ca^{2+} (F2) compared to their respective M-II-arrested controls (E1 and F1). The CTCF analysis of fluorescence intensity of respective eggs is shown in lower panel (E3 and F3). Data represent mean ± SEM of three independent experiments and analyzed by Student's t-test. #, $p < 0.001$. Microscopic pictures are representative of three independent experiments.

clin B1 degradation [6, 7, 36-38]. Our results suggest that high cytosolic Ca^{2+} increased Thr-14/Tyr-15 phosphorylated Cdk1 level in eggs that underwent EM-II in vivo (**Figure 2B2**) as compared to freshly ovulated M-II-arrested control egg (**Figure 2B1**). The CTCF analysis of its immunofluorescence intensity further strengthened our observations (**Figure 2B3**; $p < 0.001$). Furthermore, Thr-161 phosphorylated Cdk1 level was reduced (**Figure 2A1** and **2A2**), as evidenced by CTCF analysis of corresponding eggs (**Figure 2A3**; $p < 0.001$). However, total Cdk1 level remained unchanged (**Figure 2C1** and **2C2**) as evidenced by CTCF analysis (**Figure 2C3**; $p < 0.05$) in the present study. Similarly, increase of Thr-14/Tyr-15 and decrease of Thr-161 phosphorylated Cdk1 level resulted in EM-II and thereby abortive SEA in rat [5-7] and mouse eggs [37, 38] cultured in vitro. The modulation of Cdk1 specific phosphorylation status could induce cyclin B1 dissociation and degradation [33-38]. Our results suggested that cyclin B1 level decreased significantly during EM-II (**Figure 2D2**) as compared to control eggs (**Figure 2D1**) and further supported by CTCF analysis (**Figure 2D3**; $p < 0.001$).

A decrease of cyclin B1 level has been reported during EM-II and abortive SEA [5, 6, 27, 28, 33].

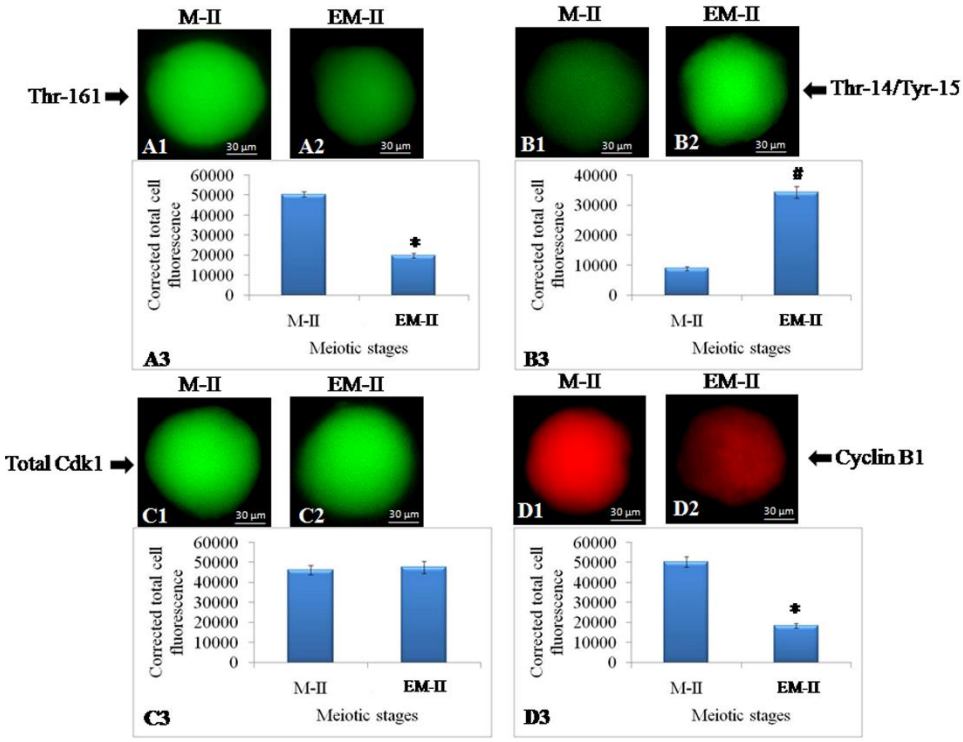

FIGURE 2. Specific as well as total phosphorylation status of Cdk1 and cyclin B1 in eggs. (A) Decrease of Thr-161 phosphorylated Cdk1 immunofluorescence intensity in an egg underwent EM-II (2A2) as compared to a freshly ovulated M-II-arrested control egg (2A1). (B) Increase of Thr-14/Tyr-15 phosphorylated Cdk1 immunofluorescence intensity in an egg underwent EM-II (B2) as compared to a freshly ovulated M-II arrested control egg (B1). (C) Total Cdk1 immunofluorescence intensity did not change (C1 and C2). (D) Decrease of cyclin B1 immunofluorescence intensity in an egg underwent EM-II (Figure D2) as compared to a freshly ovulated M-II-arrested control egg (D1). The CTCF analysis of fluorescence intensity of respective eggs is shown in lower panel (A3, B3, C3, and D3). Data represent mean ± SEM of three independent experiments and analyzed by Student's t-test. *, $p < 0.001$. Microscopic pictures are representative of three independent experiments.

Thus, data shown here together with previous findings suggest that moderate increase of ROS as well as Ca^{2+} may alter specific phosphorylation status of Cdk1, reduce cyclin B1 level, and induce MPF destabilization. The destabilized MPF drives EM-II that leads to abortive SEA in rat eggs in vivo. These eggs are of poor quality and unfit for fertilization due to scattered chromosomes in the egg cytoplasm and incomplete extrusion of PB-II [5, 27, 28]. Based on these results, we suggest that the incidence of abortive SEA in several mammalian species could be due to ROS/Ca^{2+}-mediated MPF destabilization in mammalian eggs.

4. CONCLUSIONS

In summary, an increase in ROS as well as cytosolic free Ca^{2+} levels may signal a downstream pathway to increase Thr-14/Tyr-15 phosphorylated Cdk1 level and induce cyclin B1 degradation to destabilize MPF. The MPF destabilization pushes ovulated eggs to quickly undergo abortive SEA, and chromosomes are scattered in the cytoplasm. This condition deteriorates egg quality and makes eggs unfit for fertilization. Based on our and previous studies carried out on other mammalian species, we propose that ROS/Ca^{2+}-mediated MPF destabilization and thereby

abortive SEA could be one of the driving forces for the gradual decline in reproductive outcome in several mammalian species.

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