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Intracellular levels of hydrogen peroxide and nitric oxide in oocytes at various stages of meiotic cell cycle and apoptosis

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Abstract

The objective was to find out the functional roles of hydrogen peroxide (H_2O_2) and nitric oxide (NO) during various stages of meiotic cell cycle and apoptosis in rat oocytes. For this purpose, 30 oocytes from each stage such as diplotene, metaphase-I (M-I), metaphase-II (M-II) and apoptosis were collected and intracellular H_2O_2, total nitrite level and inducible nitric oxide synthase (iNOS) expression were analysed. This study demonstrated that generation of a tonic level of H_2O_2 induces meiotic resumption in diplotene-arrested oocytes and further increase may lead to apoptosis. Conversely, reduction in iNOS expression and total nitrite level are associated with meiotic resumption in diplotene-arrested oocytes, but induce apoptosis in aged oocytes. These results suggest that generation of a tonic level of H_2O_2, reduced iNOS expression and total nitrite level are associated with meiotic resumption, while more generation of H_2O_2 and sustained reduced total nitrite level are linked with oocyte apoptosis in rat.

Keywords: *Hydrogen peroxide, total nitrite level, oocyte, meiotic cell cycle, apoptosis, rat*

Introduction

The reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) such as nitric oxide (NO) are produced as byproducts of normal cellular metabolism. They serve as key signal molecules in the physiological processes such as meiotic resumption, but also have a role in pathological processes such as cell death [1,2]. These free radicals have a biphasic role since a tonic level may serve as a key signal molecule in various physiological processes from oocyte maturation to fertilization, while a higher level may lead to negative consequences [1–8].

The role of free radicals during meiotic resumption comes from the observations that free radical scavengers inhibit resumption of meiosis in immature oocytes cultured *in vitro* [9–11]. Generation of a tonic level of ROS is beneficial to increase developmental potential of oocytes during maturation [5,6,12]. This is further supported by our observations that exogenous exposure of low concentration of H_2O_2 induces meiotic resumption in immature oocytes cultured *in vitro* [7]. On the other hand, higher concentrations of H_2O_2 induce cell cycle arrest and apoptosis in rat oocytes and mouse embryos [7,13–15]. However, it remains unclear whether generation of H_2O_2 is associated with meiotic cell cycle progression/arrest and apoptosis in rat oocytes.

Nitric oxide (NO) is an inorganic free radical gas that freely diffuses through biological membranes. The NO is a ubiquitous signalling molecule synthesized by several ovarian cells and regulates various ovarian physiology including oocyte maturation and ovulation in mammals [16]. The NO is produced by

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three different Nitric Oxide Synthase (NOS) isofoms, namely neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The eNOS and iNOS but not nNOS expressions have been reported in rat ovarian follicles [17]. The iNOS has been detected in rat follicles [17,18] and mice oocytes [19]. Further, hCG reduces iNOS expression and production of NO metabolites in gonadotropin-primed immature rats [18], suggesting the involvement of iNOS-NO mediated pathway during gonadotropin-induced meiotic maturation and ovulation [18,20].

The NO plays an important role in the regulation of meiotic maturation in rat oocytes. The higher level of NO inhibits oocyte meiotic resumption [18], maintains oocyte quality [21] and prevents post-ovulatory ageing [22]. On the other hand, lower level induces resumption of meiosis at diplotene stage [20]. Depriving the oocytes of NO can cause deterioration in the oocyte quality post-ovulation [21]. In contrast, generation of a tonic level of ROS such as H_2O_2 induces resumption of meiosis [23] and enhances oocyte ageing [24]. The possibility exists that the higher level of NO maintains meiotic arrest at diplotene stage for a long time in follicular oocytes. During superovulation induction in rat, hCG reduces iNOS expression thereby production of NO metabolites and induces meiotic maturation and ovulation. Another possibility exists that sustained lower level of NO in aged oocytes after ovulation may reduce quality by inducing oocyte apoptosis in rat.

Gametes are very sensitive to damage by free radicals and must be protected against to improve the quantity and quality of oocytes. Hence, it is important to delineate the biphasic actions of free radicals by analysing intracellular levels of H_2O_2 and total nitrite in oocytes. Therefore, our objective was to find out the intracellular levels of H_2O_2 and total nitrite at various stages such as diplotene, metaphase-I (M-I), Metaphase-II (M-II) of meiotic cell cycle and apoptosis to understand their functional roles in meiotic cell cycle progression and apoptosis in rat oocytes.

Materials and methods

Chemicals and preparation of culture medium

All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. The culture medium (M-199) was prepared as per company manual protocol. The pH of culture medium was adjusted to 7.2 and osmolarity was found to be 290 ± 5 m osmol. The culture medium was supplemented with sodium bicarbonate (0.035% w/v), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

Animals

Holtzman-derived rats were housed in air-conditioned, light controlled rooms, with food and water available *ad libitum*. Twenty-three-to-25-day-old female rats were separated out and subjected to various experimental protocols to collect oocytes (30 oocytes in each group) at different stages of meiotic cell cycle and apoptosis. The collected oocytes were photographed using phase-contrast microscope (Nikon, Eclipse; E600, Tokyo, Japan) at 400 × magnification. All procedures were in conformation to the stipulations of the University Animal Ethical Committee of Banaras Hindu University, Varanasi and in keeping with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication).

Collection of diplotene stage oocytes

Immature female rats were separated from our existing colony and maintained in normal husbandry conditions with food and water *ad libitum*. To obtain diplotene stage oocytes that had germinal vesicle (GV) and nucleolus, rats (24–25 days old; 50 ± 5 gm body weight) were given a single subcutaneous injection of 20 IU pregnant mare's serum gonadotropin (PMSG) in 100 μl of sterile normal saline to promote growth of a cohort of healthy antral follicles. After 48 h of PMSG injection, rats were killed by euthanasia and ovaries were removed and transferred to a 35 mm petridish containing 2 ml of sterile culture medium. Ovarian follicles (≥ 0.8 mm diameter) were punctured with a sterile 26-gauge needle attached to a 1 ml syringe. Cumulus oocyte complexes (COCs) were collected, washed and maintained at 37°C in culture medium. The immature COCs were treated with 0.1% hyaluronidase in culture medium to strip their cumulus cells by repeated pipetting through a narrow-bore pipette in culture medium. A group of 30 immature denuded diplotene stage oocytes were transferred to a microcentrifuge tube containing 100 μl of hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, 0.5%TritonX-100, pH8.0) and lysate was immediately frozen at −30°C for the analysis of intracellular free radicals such as H_2O_2 and total nitrite levels.

Collection of M-I arrested mature oocytes

To obtain mature oocytes that were arrested at M-I stage (germinal vesicle break down; GVBD stage) of meiotic cell cycle, rats (24–25 days old; 50 ± 5 gm body weight) were given a single subcutaneous injection of 20IU PMSG in 100 μl of sterile normal saline for 48 h followed by another injection of 20IU hCG for 10 h. Thereafter, rats were killed by euthanasia and ovaries were removed and transferred to a 35 mm petridish containing 2 ml of sterile washing medium. The denuded oocytes were

collected using a similar method described for the collection of diplotene stage oocytes. A group of 30 M-I stage denuded oocytes were transferred to a microcentrifuge tube containing 100 µl of hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, 0.5%TritonX-100, pH 8.0) and lysate was immediately frozen at −30°C for the analysis of intracellular free radicals such as H_2O_2 and total nitrite levels.

Collection of M-II arrested mature oocytes

To obtain oocytes that were arrested at M-II stage of meiotic cell cycle, rats (24–25 days old; 50 ± 5 gm body weight) were subjected to superovulation induction protocol (single injections of 20IU PMSG for 48 h followed by 20 IU hCG for 14 h). Thereafter, rats were killed by euthanasia and oviducts were removed and transferred to a 35 mm petridish containing 2 ml of sterile washing medium. The oviduct was punctured with a sterile 26-gauge needle attached to a 1 ml syringe and ovulated COCs were collected, washed and maintained at 37°C in culture medium. The denuded oocytes were collected using a similar method described for the collection of diplotene stage oocytes. Denuded oocytes (30 oocytes) that had first polar body (considered to be at M-II stage of meiotic cell cycle) were transferred to a microcentrifuge tube containing 100 µl of hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, 0.5%TritonX-100, pH8.0) and lystae was immediately frozen at −30°C for the analysis of intracellular free radicals such as H_2O_2 and total nitrite levels.

Collection of apoptotic oocytes

Calcium ionophore A23187 (1.6 µM) was used to induce oocyte apoptosis by following our published protocol [23]. In brief, M-II-arrested oocytes exhibiting first polar body with normal morphology (collected after superovulation induction protocol as described above) were treated with calcium ionophore A23187 (1.6 µM) for 3 h *in vitro*. A group of 30 oocytes that exhibited morphological apoptotic features were washed three times with PBS and then transferred to a microcentrifuge tube containing 100 µl of hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, 0.5%TritonX-100, pH 8.0) and lysate was immediately frozen at −30°C for the analysis of intracellular free radicals such as H_2O_2 and total nitrite levels.

Quantitative estimation of intracellular H_2O_2 level

The intracellular H_2O_2 level in oocytes was analysed using H_2O_2 assay kit purchased from R&D Systems (MN) as per company manual protocol. The 30 oocytes from each group such as diplotene-arrested (GV stage), M-I arrested (GVBD stage), M-II arrested (with first polar body) and oocytes showing morphological apoptotic features were transferred to a microcentrifuge tube containing 100 µl of hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, 0.5%TritonX-100, pH 8.0) for 1 h on ice for lysis. The lysates were centrifuged at 10000 rpm at 4°C for 15 min. A clear supernatant was removed from each group and stored at −30°C until use. Frozen samples were thawed immediately and diluted by 5-fold with sample diluent and then processed for the quantitative estimation of H_2O_2 by calorimetric assay following our published protocol [23]. 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, the optical density (OD) was determined using a microplate reader set at 550 nm. The samples were run in triplicate and all samples were run in one assay to avoid inter-assay variation and intra-assay variation was 3%.

Quantitative estimation of intracellular total nitrite level

The total intracellular NO was measured indirectly by analysing more stable NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-). Nitrite is a water-soluble metabolite of NO. Therefore, nitrate is converted into nitrite using nitrate reductase and total nitrite is measured as a function of total intracellular NO level using Griess method. The total nitrite level in oocytes was analysed using NO assay kit purchased from R&D Systems (MN) as per company manual protocol. For total nitrite assay, samples (30 oocytes in each group) were prepared as described above for H_2O_2 assay. Frozen samples were thawed immediately and then processed for the quantitative estimation of nitrite level in oocytes by calorimetric assay following company manual protocol. The lysates were diluted with reaction diluent at least 5-fold (50 µl sample + 200 µl diluent buffer) prior to use as per company manual protocol. In brief, all reagents, working standards and samples were brought to room temperature before use. The 50 µl of reaction diluent was added to the blank well and 50 µl of nitrate standards or samples to remaining wells. The 25 µl of NADH and then 25 µl of diluted nitrate reductase were added to all wells. The plate was incubated at 37°C for 30 min. At the end of incubation, 50 µl of Griess reagent I and then 50 µl of Griess reagent II were added. The content was mixed well by tapping the side of the plate and then incubated for 10 min at room temperature. At the end of the incubation period, the optical density (OD) was determined using a microplate reader set at 540 nm with wavelength correction at 690 nm. The samples were

run in triplicate and all samples were run in one assay to avoid inter-assay variation and intra-assay variation was 2.5%.

In situ *detection of iNOS*

The immunostaining for iNOS was carried out by following a previous published protocol [17] with some modifications. In brief, 10 oocytes from each group such as diplotene, M-I, M-II and apoptosis were fixed in 3.7% formaldehyde solution in PBS (0.01 M phosphate, 0.14 M NaCl, pH 7.4). After washing twice with distilled water for 5 min each time, slides were treated with 0.3% hydrogen peroxide in absolute methanol for 15 min to quench endogenous peroxidase activity. Thereafter, slides were washed twice with distilled water and twice with PBS. All subsequent incubations with immunochemicals were performed in a humidified chamber.

To reduce non-specific binding, slides were exposed to 100 µl of blocking buffer (PBS with 5% normal horse serum) at 25°C for 10 min and then incubated with 50 µl of diluted (1:100 dilution in blocking buffer) iNOS polyclonal rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 25°C for 1 h in humidified chamber. Thereafter, slides were washed three times with PBS and then incubated with diluted (1:200 dilution in blocking buffer) secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) tagged with horseradish peroxidase (HRP) at 25°C for 1 h. At the end of the incubation period, slides were washed three times with PBS and then exposed to 50 µl of freshly prepared diaminobenzidine (DAB) solution (1 µl of 30% H_2O_2 and 5 µl of DAB in 1 ml of PBS; R&D Systems Inc., MN) for 10 min. After that slides were washed four times in PBS and subsequently counterstained with 50 µl of 1% Methyl Green solution (R&D Systems Inc., MN) for 2 min. The slides were again washed three times with PBS and then mounted in distyrene plasticizer xylene (DPX). The mounted slides were analysed for DAB-positive staining under a phase-contrast microscope (Nikon, Eclipse; E600, Tokyo, Japan) at 400 × magnification. The experiment was repeated three times and a representative photograph is shown in the results section.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) of triplicate samples. Data are analysed by Student's *t*-test using SPSS software, version 11.5 (SPSS, Inc. Chicago, IL). A probability of $p < 0.05$ was considered to be statistically significant.

Results

Morphological assessment of meiotic cell cycle progression and apoptosis in oocytes

Oocytes collected from ovary after 48 h of PMSG (20 IU) injection were at diplotene stage of meiotic prophase-I and exhibited GV with nucleolus (Figure 1A). Collection of oocytes from ovaries of immature rats that were given single injections of 20 IU PMSG followed by 20 IU hCG for 10 h showed resumption of meiosis from diplotene-arrest which is morphologically characterized by GVBD (Figure 1B). If the treatment time for hCG was increased to 14 h, ovulation occurred and oocytes collected from the oviduct were at M-II stage with first polar body (Figure 1C). Treatment of these M-II arrested oocytes with calcium ionophore A23187 (1.6 µM) for 3 h induced morphological apoptotic features (Figure 1D).

Intracellular H_2O_2 level in oocytes at various stages of meiotic cell cycle and apoptosis

As shown in Figure 2, intracellular level of H_2O_2 in diplotene-arrested oocytes was 65.7 ± 1.68 ng/oocyte. Resumption of meiosis from diplotene to M-I stage (which is morphologically characterized by GVBD) was associated with a significant ($p < 0.05$) increase of intracellular H_2O_2 (76.7 ± 0.69 ng/oocyte) level. The intracellular H_2O_2 level did not significantly increase when the oocytes progressed from M-I to M-II stage (81.6 ± 1.10 ng/oocyte) exhibiting first polar body with normal morphology. However, further increase ($p < 0.05$) of intracellular H_2O_2 level (89.6 ± 1.47 ng/oocyte) induced appearance of morphological features characteristic of oocyte apoptosis as compared to M-II arrested oocytes (81.6 ± 1.10 ng/oocyte).

Intracellular nitrite level in oocytes at various stages of meiotic cell cycle and apoptosis

As shown in Figure 3, intracellular total nitrite level in diplotene stage oocytes was 1.33 ± 0.07 pmole/oocyte. Resumption of meiosis in diplotene-arrested oocytes was associated with a significant ($p < 0.05$) decrease of intracellular total nitrite level (0.73 ± 0.03 pmole/oocyte). The total nitrite level was found minimum in M-II arrested oocytes (0.40 ± 0.02 pmole/oocyte) that had first polar body with normal morphology. The intracellular total nitrite level in aged oocytes that underwent apoptosis (0.55 ± 0.11 pmole/oocyte) was comparable to M-II stage oocytes but it was significantly ($p < 0.05$) less than diplotene-arrested oocytes.

In situ *localization of iNOS in oocytes*

As shown in Figure 4, intracellular localization of iNOS was carried out in oocytes using polyclonal

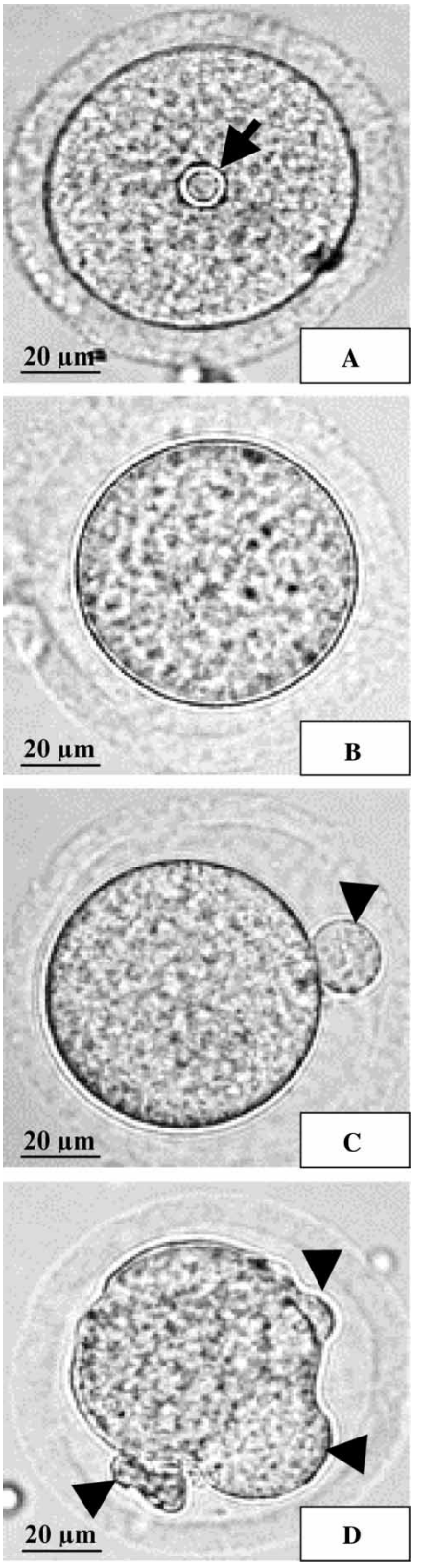

Figure 1. Representative photograph showing oocyte at various stages of meiotic cell cycle such as diplotene arrest (A) oocyte showing GV and nucleolus (►), M-I arrest (B) showing GVBD, M-II arrest (C) showing first polar body (►) and apoptosis (D) showing membrane blebbings (►). Bar = 20 µm.

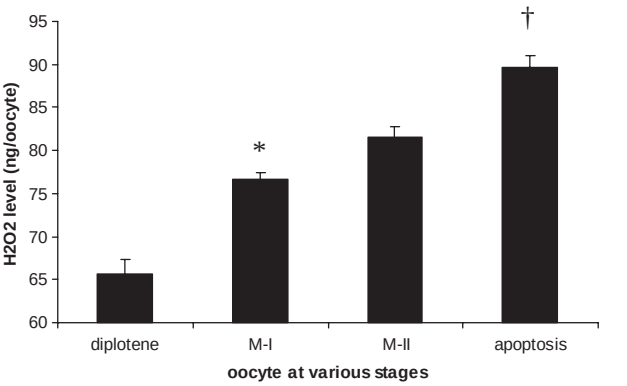

Figure 2. Intracellular level of H_2O_2 in oocytes at various stages of meiotic cell cycle and apoptosis. Data are mean ± SEM of three replicates. *Denotes significantly ($p<0.05$) higher as compared to diplotene stage oocytes. †Denotes significantly ($p<0.05$) higher as compared to M-II stage oocytes (Student's *t*-test).

rabbit antiserum and *in situ* detection technique. The diplotene arrested oocytes had maximum iNOS expression as evidenced by dark brown DAB positive staining in oocyte cytoplasm (Figure 4A). Resumption of meiosis in diplotene-arrested oocytes was associated with a reduced iNOS expression as evidenced by faint brown DAB positive staining in oocyte cytoplasm (Figure 4B and C). A mild but not significant increase of iNOS expression was observed in apoptotic oocytes as compare to M-II arrested oocytes (Figure 4D).

Discussion

The ROS are a double-edged sword—they serve as key signal molecules in physiological processes such as meiotic cell cycle progression but also have a role in pathological processes such as apoptosis [1]. The dual role of H_2O_2 in oocyte physiology comes from the observations that exogenous supplementation of lower concentrations of H_2O_2 induce mei-

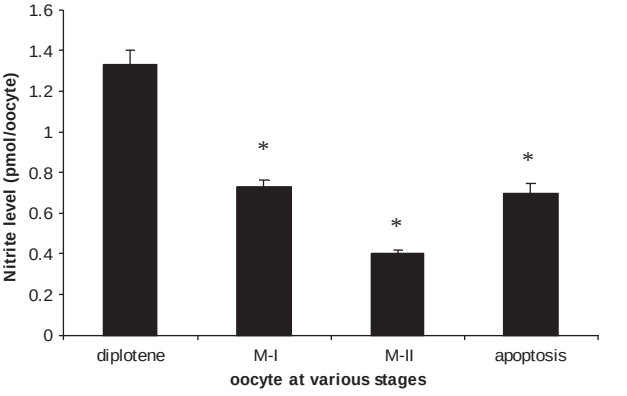

Figure 3. Intracellular level of total nitrite in oocytes at various stages of meiotic cell cycle and apoptosis. Data are mean ± SEM of three replicates. *Denotes significantly ($p<0.05$) lower as compared to diplotene stage oocytes (Student's *t*-test).

otic resumption, while higher concentrations induce apoptosis in rat and human oocytes cultured *in vitro* [7,15]. Based on these studies, we hypothesized that generation of a tonic level of H_2O_2 may be associated with resumption of meiosis in diplotene-arrested follicular oocytes, while high sustained level may induce apoptosis. This hypothesis is strengthened by data of the present study that a tonic generation of H_2O_2 is sufficient to induce meiotic resumption from diplotene-arrest to M-I and M-II stages, while further increases of H_2O_2 resulted in oocyte apoptosis. Taken together these results with previous findings suggest that generation of a certain level of H_2O_2 is required for meiotic cell cycle progression, while further more generation could induce apoptosis in rat oocytes. Indeed, intracellular H_2O_2 has a dual role—at lower concentration it serves as a key signal molecule in meiotic resumption but at higher concentration it induces oocyte apoptosis. Although we have not studied the source of H_2O_2 generation oocytes, previous findings suggest that H_2O_2 is produced via dismutation of superoxide by the enzyme superoxide dismutase [25]. The production of superoxide via mitochondrial, NADPH-oxidase and xanthine/xanthine oxidase systems during meiotic cell cycle progression and apoptosis have been reported in mouse embryo and human oocytes [25,26].

Nitric oxide has been identified as a major intracellular as well as intercellular signalling molecule involved in diverse physiological processes. The NO is generated from NO synthases (NOSs), but NO derived from iNOS seems to inhibit meiotic resumption in the rat. This hypothesis is supported by the observations that iNOS inhibitor induced meiotic resumption, while NO donor prevented this phenomenon [18,27,28]. Recently, it has been reported that the higher concentration of NO metabolite (nitrate/nitrite) is maintained in the ovary when the oocytes are arrested at diplotene stage of meiotic cell cycle. The LH-induced meiotic maturation and ovulation are associated with decreased concentrations of NO metabolite (nitrate/nitrite) in follicular fluid of rat ovary [20]. These studies in rats led us to hypothesize that maintenance of meiotic arrest in pre-ovulatory generation in oocytes is associated with higher intracellular total nitrite level. Reduction in total nitrite level might be associated with resumption of meiosis in diplotene-arrested rat oocytes. This hypothesis is

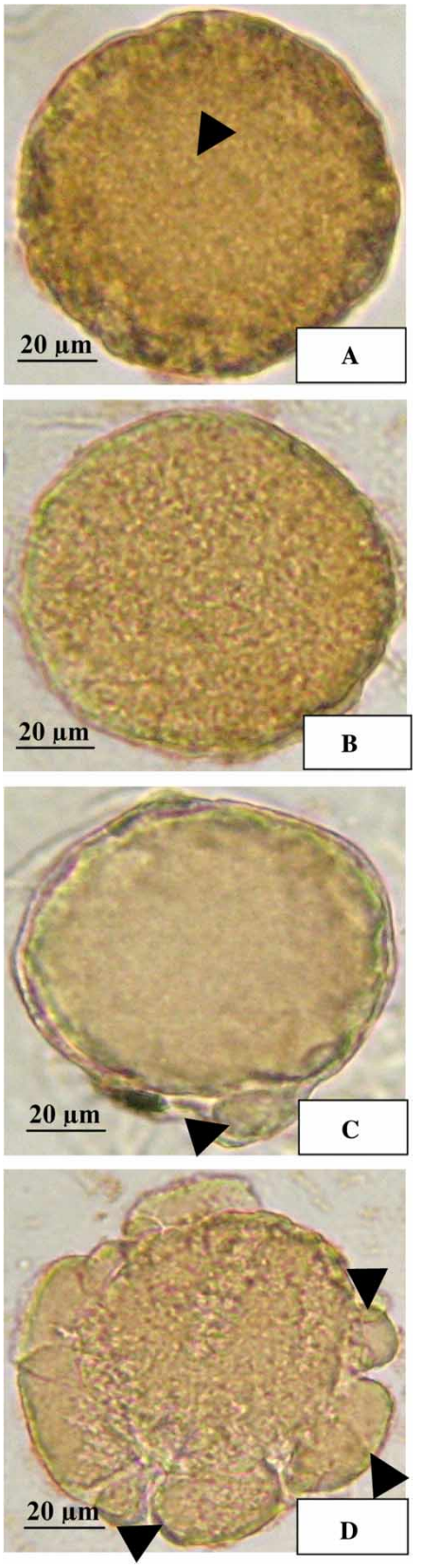

Figure 4

Figure 4. Representative photograph showing intracellular localization of iNOS in oocytes at various stages of meiotic cell cycle and apoptosis. The diplotene-arrested oocyte having nucleolus (►) showed maximum iNOS expression as evidenced by dark brown colour of DAB positive staining in oocyte cytoplasm (A). The brown colour intensity in oocyte cytoplasm reduced with the progression of meiotic cell cycle from diplotene to M-I (B) and M-II stage showing first polar body (C, ►). The mild increase in iNOS expression was seen in apoptotic oocyte that showed membrane blebbings (►) compared to M-II stage, as evidenced by increased brown colour of DAB positive staining in cytoplasm (D). Bar = 20 µm.

supported by our observations that diplotene-arrested oocytes showed maximum iNOS expression and had highest intracellular total nitrite level. Resumption of meiosis in diplotene-arrested oocytes (which is morphologically characterized by germinal vesicle breakdown and dissolution of nucleolus) was associated with significant decrease in iNOS expression and total nitrite level. The iNOS expression and total nitrite concentration were found to be minimum in M-II arrested ovulated oocyte that had first polar body with normal morphology. Taken together these data with previous observations suggest that a reduction in iNOS expression thereby total nitrite level is a prerequisite for resumption of meiosis in diplotene-arrested follicular oocytes in rats.

It has been recently identified that NO plays a novel and unique role in maintaining quality and integrity of newly ovulated oocytes [22]. Supplementing oocytes with NO inhibit meiotic resumption [18], essential for sustenance of oocyte quality post-ovulation [21] and delays deterioration of oocyte quality [29]. On the other hand, sustained reduced NO level reduces oocyte quality [22], possibly by inducing oocyte apoptosis. This possibility was further strengthened by data of the present study that a significantly reduced iNOS expression and total nitrite level as compared to diplotene-arrested oocytes induced morphological apoptotic features in aged M-II arrested oocytes. These data with previous findings suggest that an initial decrease of intracellular NO level may induce meiotic resumption in diplotene arrested oocytes, but depriving NO level in aged oocytes may induce apoptosis. However, the iNOS expression and total nitrite level in apoptotic oocytes was slightly higher as compared to M-II arrested oocytes in the present study. This mild increase of iNOS expression and total nitrite level in apoptosis oocytes might be due to nitrate/nitrite transport from outer fluid to oocytes *in vitro* for 3 h. Another possibility exists that calcium-sensitive anion channels and anion exchanger as well as aquaporins may facilitate nitrate/nitrite transport into oocytes during calcium ionophore A23187 induced apoptosis in rat oocytes cultured *in vitro*.

In conclusion, data of the present study clearly suggest that the generation of a tonic level of H_2O_2 is associated with meiotic resumption in diplotene-arrested oocytes, while further increase may induce apoptosis in M-II-arrested aged oocytes. In contrast, generation of NO through iNOS-mediated pathway is associated with the maintenance of meiotic arrest in diplotene-arrested oocytes. Reduction in iNOS expression and thereby total nitrite level is a prerequisite for meiotic resumption in diplotene arrested oocytes. Further, sustained reduced iNOS expression and intracellular NO level may induce apoptosis in aged M-II arrested rat oocytes cultured *in vitro*.

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