Nitric Oxide Delays Oocyte Aging†

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Received April 18, 2005; Revised Manuscript Received June 29, 2005

ABSTRACT: Nitric oxide (NO) is a ubiquitous signaling molecule that plays a crucial role in oocyte maturation and embryo development. However, its role in oocyte aging is unclear. To examine how NO affects oocyte aging, we retrieved young and relatively old mouse oocytes and exposed them to increasing concentrations of NO donor *S*-nitroso acetyl penicillamine (SNAP). Aging related phenomena of ooplasmic microtubule dynamics (OMD), cortical granule (CG) exocytosis, zona pellucida (ZP) hardening, and spindle/chromatin integrity were studied at each SNAP concentration using fluorescence immunocytochemistry and confocal microscopy and compared with respective unexposed controls. Exposure of both young and old oocytes to NO resulted in a significant diminution in OMD and ZP dissolution time, whereas spontaneous CG loss decreased in old NO exposed oocytes compared to controls ($P < 0.001$ for all). Furthermore, NO exposure decreased the rate of spindle abnormalities in oocytes compared to unexposed controls. Interestingly, in old oocytes, the positive influence of NO was attenuated beyond 0.23 μM/min and disappeared at 0.46 μM/min NO. Overall, a significant dose−response relationship was noted between NO exposure and markers of aging with between 50 and 100 μM SNAP (0.11−0.23 μM/min NO, $P < 0.0001$). Collectively, our results demonstrate for the first time that exposure to NO delays oocyte aging and improves the integrity of the microtubular spindle apparatus in young and old oocytes.

Mammalian oocytes have a limited fertilizable lifespan typically called the "temporal window for optimal fertilization" (*1*). Oocytes that are not fertilized within this "temporal window" undergo characteristic changes akin to postfertilization events, which may lead to parthenogenetic activation and eventual fragmentation (*2*−*4*). Collectively, these changes are referred to as "oocyte post-ovulatory or postmaturation aging". Aged oocytes have a significantly increased likelihood of failed or abnormal fertilization and/or cleavage divisions, as well as chromosomal abnormalities in the embryo (*2*, *3*).

The changes of postovulatory aging may be particularly enhanced in oocytes that have undergone preovulatory aging, as in individuals with advanced chronological age. Preovulatory oocyte aging thus affects the fertilizable lifespan of an oocyte (*5*, *6*). Therefore, oocytes remaining unfertilized beyond this "temporal window for optimal fertilization" are doomed to failure, causing abnormal fertilization and development, eventually resulting in atresia and fragmentation (*2*−*4*). Aging of gametes may also contribute to several cellular/molecular pathways that affect development. Furthermore, aging of gametes is also postulated to have remote effects on the conceptus, in the form of diseases during later life (*7*).

Control of the processes of oocyte maturation and aging is particularly critical in modern techniques of assisted reproduction (ART).[1] In vivo or in vitro matured oocytes, for instance, are typically utilized for in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and nuclear transfer. Postmaturation aging significantly affects the results of these procedures (*2*−*4*, *7*). Therefore, controlling oocyte aging could have many rewards in ART.

Some of the pathophysiological mechanisms involved in oocyte aging are well-defined. Accordingly, deterioration in oocyte quality following maturation/ovulation is most likely due to derangement of intracellular Ca^{2+} homeostasis, with secondary activation of the cell cycle machinery (*8*−*10*). This process results in a drop in the activity of the cell cycle factors, namely, M phase promoting factor (MPF) and mitogen activated protein kinase (MAPK) (*9*, *10*). The latter two are pivotal for completion of meiosis and initiation of normal embryo development (*11*). Thus, drops in MPF and MAPK result in premature advancement of the cell cycle prior to fertilization. This is also accompanied by other

† This work was supported by National Institutes of Health Grant RO1 HL066367 to H.M.A.-S. and by the Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI.

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[1] Abbreviations: NO, nitric oxide (nitrogen monoxide); SNAP, *S*-nitroso acetyl penicillamine; OMD, ooplasmic microtubule dynamics; CG, cortical granule; ZP, zona pellucida; MPF, M phase promoting factor; ART, assisted reproductive technology; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; MAPK, mitogen activated protein kinase; NOS, nitric oxide synthase; H_4B, tetrahydrobiopterin; L-NAME, N^{ω}-nitro-L-arginine methyl ester; 8-Br-cGMP, 8-bromoguanosine 3′:5′-cyclic monophosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate.

10.1021/bi050711f CCC: $30.25
Published on Web 08/05/2005

phenomena such as cortical granule (CG) loss, zona pellucida (ZP) hardening, and changes in spindle and ooplasmic microtubule dynamics (*10*, *12*−*14*), as well as a tendency to parthenogenetic activation (*15*). In an M II stage oocyte the microtubules are in a stage of dynamic instability. Paclitaxel exposure decreases the critical concentration of tubulin polymerization, contributing to a net increase in ooplasmic microtubule dynamics. Paclitaxel stimulated ooplasmic microtubules are readily visible in aged oocytes in comparison to young oocytes (*14*), and this is used as a marker to identify aged oocytes in our current study.

Although the cellular changes of oocyte aging and related mechanisms are more or less understood, the exact mechanisms that initiate these changes are enigmatic. Involvement of secondary messengers and reactive oxygen species has been hypothesized, but are as yet unproven. Nitric oxide (NO) is a ubiquitous molecule and forms a vital component of the oocyte microenvironment from folliculogenesis through early embryo development (*16*−*23*). At physiological concentrations, NO is essential for meiotic maturation, and may be involved in processes that determine oocyte quality and embryonic developmental potential (*18*, *19*). Furthermore, NO has been proposed to prevent atresia and apoptosis in developing follicles (*20*). NO is generated enzymatically by three distinct isoforms of NO synthase (NOS): neuronal (nNOS, or NOS-1), inducible (iNOS, or NOS-2), and endothelial (eNOS, or NOS-3) (*24*). All three isoforms typically require molecular oxygen, β-nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (H_4B), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and calmodulin to convert L-arginine to NO and citrulline (*24*, *25*). NO activates soluble guanylate cyclase by coordination to a heme associated with the enzyme, and resultant increase in cGMP induces smooth muscle relaxation affecting blood flow through vessels (*26*, *27*).

Although mounting evidence implicates NO at every step of oocyte release, maturation, and fertilization, the role of NO in oocyte aging is still unclear. Characteristic events of aging such as Ca^{2+} dysregulation, Ca^{2+}-calmodulin kinase activation, and secondary phenomena suggest that NO may be playing a role in oocyte aging. In this report, we evaluate the potential physiological role of NO in attenuating oocyte aging. Our results demonstrate for the first time that exposing oocytes to NO results in a significant delay in the process of postovulatory aging in mouse oocytes.

MATERIALS AND METHODS

Materials. N^{ω}-Nitro-L-arginine methyl ester (L-NAME), 8-bromoguanosine 3′:5′-cyclic monophosphate (cGMP), and β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma (St. Louis, MO). Other chemicals and reagents were of the highest purity grades available and obtained from either Sigma or Aldrich.

Study Design. This study was approved by Wayne State University's Animal Investigation Committee. Experiments were performed in two sets. In both sets, normal appearing M II stage oocytes obtained from superovulated B6D2F1 mice at 13.5 and 17.5 h after hCG (groups A and B, respectively) were exposed to different concentrations (5−300 μM) of an NO donor *S*-nitroso acetyl penicillamine (SNAP) for 3 h. Sibling control oocytes were allowed to age in a medium without SNAP. In set 1, aging related phenomena of ooplasmic microtubule dynamics (OMD) and cortical granule loss were determined in exposed and control oocytes; while in set 2, zona pellucida (ZP) dissolution time and spindle and chromatin status were determined in another set of test and control oocytes. Oocytes exposed to each concentration of SNAP were statistically compared to respective controls.

Superovulation and Oocyte Retrieval. Four- to six-week-old B6D2F1 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were adjusted to the 14 h light−10 h dark cycle for at least one week prior to superovulation with 7.5 IU each of pregnant mare's serum gonadotropin (PMSG) and hCG (Sigma, Saint Louis, MO), administered ip 48−52 h apart. Mice were sacrificed at 13.5 (group A, young) and 17.5 h (group B, old) after hCG injection, and oocytes were retrieved from oviductal ampullae. The cumuli were treated with 0.1% hyaluronidase (w/v) in M2 medium (Sigma) for 2−3 min at 37 °C to release oocytes, which were subsequently denuded to remove all cumulus-corona cells with a narrow bore pulled glass Pasteur pipet. Oocytes were thoroughly rinsed in M2 medium, inspected to rule out abnormal morphology, and were kept ready in M16 medium (Sigma) preequilibrated with 5% CO_2 in air at 37 °C in a common pool before random assignment into test (treatment with the NO donor SNAP) and control groups.

Exposure to SNAP. The young and the old oocytes were exposed to 5, 10, 50, 100, 200, and 300 μM of the NO donor, SNAP, that generates 0.015, 0.031, 0.11, 0.23, 0.46, and 0.7 μM/min NO, respectively, as determined by the NO capturing method oxyhemoglobin assay (*28*). SNAP was prepared as stocks of 100 mM and 1 mM in DMSO, and desired concentrations were freshly prepared from stocks in M2 medium. Oxyhemoglobin assay was performed on conditioned medium (exposed to oocyte and cumulus cells). The media used in this study are the standard preparations formulated to mimic in vivo conditions that enable the culture of an oocyte to fertilization and the blastocyst stage. Control sibling oocytes were allowed to age in culture in M16 without SNAP. The test and control were incubated for 3 h, rinsed, and subjected to acid Tyrode treatment for ZP removal and fixation in 4% paraformaldehyde (37 °C) following paclitaxel (Sigma) treatment in experiment set 1, and without paclitaxel in experiment set 2. Following the identical procedures described above, young and old oocytes were exposed to 5, 10, 50, 100, 200, and 300 μM of penicillamine, SNAP byproduct. These results indicated that exposure to penicillamine has no significant effect on oocyte aging.

Paclitaxel Treatment and Tubulin Fluorescence Immunocytochemistry. Paclitaxel 1 mM stock solution was prepared in DMSO and stored at −20 °C. Just prior to experiments, it was diluted with M2 medium containing 10% fetal bovine serum (FBS, Life Technologies) to a working concentration of 5 μM. Paclitaxel treatment and tubulin staining were performed by a technique previously used by Goud et al. (*11*).

This process was followed by the staining for cortical granules with a technique described earlier using rhodamine conjugated lens culinaris agglutinin (LCA, Vector Laboratories; *29*). The oocytes were thoroughly rinsed once again with the PBS TX 0.3% BSA solution prior to mounting in Vectashield with DAPI (Vector Laboratories), which con-

tained 4′,6-′diamidino-2-phenylindole (DAPI), a fluorescent chromatin stain. The oocytes were stored in the mounting medium in glass chambers at 4 °C until processing with confocal microscopy, image processing, and 3-D reconstructions (LSM 310; Carl Zeiss Inc., Thornwood, NY).

Confocal Microscopy, Assessment of Microtubules, and Cortical Granules. The cortical granules were stained fluorescent red, which was distinct from the fluorescent green staining of the microtubules (MT) and fluorescent blue staining of chromosomes. Individual oocytes and control oocytes were closely examined for spindle/ooplasmic microtubules and cortical granule status. In experiment set 1, ooplasmic MT dynamics in response to paclitaxel was evaluated and graded into the following three categories of microtubule dynamics, namely, minimal or negligible, moderate, and increased. The first category, minimal/negligible, included oocytes with MT restricted to the spindle and occasional MT organizing center (MTOC), no free MT in the cortex; the second category, moderate, included oocytes displaying some free microtubules in the cortex in addition to the MTOC, without formation of dense MT network. The third category of (markedly) increased MT included oocytes with extensive dense cortical microtubules extending into the rest of the ooplasm.

Similarly, cortical granule status in each oocyte was categorized as intact CG, minor CG loss, and major CG loss. Those with intact CG had a rim of CG visible all along the oolemma without aggregation of exocytosis in all optical sections examined. An exception was the CG free domain in the vicinity of the spindle apparatus. Those categorized into the minimal loss category had <10% loss in CG in one or more optical sections. Oocytes with >10% CG loss were categorized into the major CG loss group. The categorization of oocytes based on MT and CG status was also confirmed by an independent observer blinded to treatment group assignment, who used comprehensive evaluation of the individual optical sections and the 3-D reconstructed images.

Experiment Set 2. The experiment set 2 involved the same design of oocyte retrieval, preparation, and exposure to SNAP. However, oocytes were not treated with paclitaxel to evaluate specifically the spindles and chromosome metaphase plates. Furthermore, time required for dissolution of the ZP (seconds) in acid Tyrode's solution (Sigma) was determined in 5 μL droplets under oil at 37 °C in 2−3 oocytes at a time using timer clocks. Oocytes were then thoroughly rinsed and subjected to the above-mentioned fixation as well as α-tubulin and DAPI staining protocol to evaluate spindles and chromosome metaphase plates using confocal microscopy. Specific consideration was given to spindle morphology and orientation, condensation status of chromatin, and location of chromosomes in relation to the metaphase plate. As in experiment set 1, an observer blinded to treatment groups conducted scoring of ZP dissolution, as well as spindle and chromosome metaphase plates.

Statistical Tests. Statistical analysis was performed using SPSS version 11.0 (SPSS Inc., Chicago, IL). The frequency data in each test and control subgroup were analyzed using Chi Square tests. Frequencies of microtubule dynamics, CG status, and spindles in individual subgroups with SNAP exposure within groups A and B were compared to their respective sibling control oocyte subgroups using the Fisher's exact test. The data on zona pellucida dissolution timings were compared between test and control subgroups using the Student's unpaired *t* test. Differences in ZP dissolution timings among subgroups exposed to individual concentrations were analyzed using one-way ANOVA and the Student−Newman−Keuls post hoc test. Where appropriate, raw data underwent log transformation prior to statistical analysis. Data were represented as mean ± SD. Significance was defined as $P < 0.05$.

Table 1: Oocyte Numbers, SNAP Concentrations, and the Rate of Nitric Oxide Released by SNAP, as Determined by the Capturing Method Oxyhemoglobin Assay (*28*), in Individual Groups and Controls within Each Experiment Set

SNAP concn (μM)	rate of NO released by SNAP (μM/min)	expt set 1		expt set 2	
		group A	group B	group A	group B
0[a]	0	198	188	183	204
5	0.015	36	40	30	32
10	0.031	32	38	29	38
50	0.11	32	29	30	36
100	0.23	40	43	31	32
200	0.46	34	34	35	40
300	0.70	28	40	32	40

[a] Total of control oocytes in each group unexposed to nitric oxide donor.

RESULTS

In total, 812 and 792 oocytes were used in 12 different experiments in sets 1 and 2, respectively. In experiment set 1, the ooplasmic microtubule dynamics in response to paclitaxel was tested in young (group A, $n = 400$) and old (group B, $n = 412$) oocytes exposed to different concentrations (5−300 μM) of SNAP. Overall, 202 oocytes in group A and 224 oocytes in group B were exposed to 5−300 μM SNAP, while 198 and 188 oocytes from groups A and B were used as controls, respectively, that underwent identical treatment without SNAP exposure. The oocytes exposed to each SNAP concentration are presented in Table 1. In experiment set 2, ZP dissolution time and spindle/chromatin morphology were studied in NO exposed and control young (group A, oocytes retrieved at 13.5 h following hCG, NO exposed, $n = 185$, and controls, $n = 185$) and old oocytes (group B, retrieved at 17.5 h after hCG, NO exposed, $n = 218$, and controls, $n = 204$).

Microtubule Dynamics in Oocytes Exposed to Nitric Oxide. In experiment set 1, enhancement of spindle microtubules was evident in all oocytes irrespective of SNAP treatment, or postovulatory age. This was related to paclitaxel treatment, which can be used to differentiate old from young oocytes (*23*). Significantly higher numbers of NO-exposed oocytes exhibited minimal or no ooplasmic microtubules in groups A (5−300 μM SNAP) and B (5−200 μM SNAP), as compared to their respective control oocytes ($P < 0.001$, Figures 1A and 2). Similarly, significantly lower numbers of NO-exposed oocytes exhibited moderate or markedly increased microtubules (group A, 5−300 μM and group B, 5−200 μM SNAP, respectively; Figure 2). On the other hand, oocytes in group B that were exposed to 300 μM SNAP exhibited no difference in ooplasm microtubule dynamics compared to controls (Figure 2).

Therefore, exposure of oocytes to NO-donor SNAP prevented postovulatory age related increase in OMD in both young and old oocytes, and in the older oocytes, this

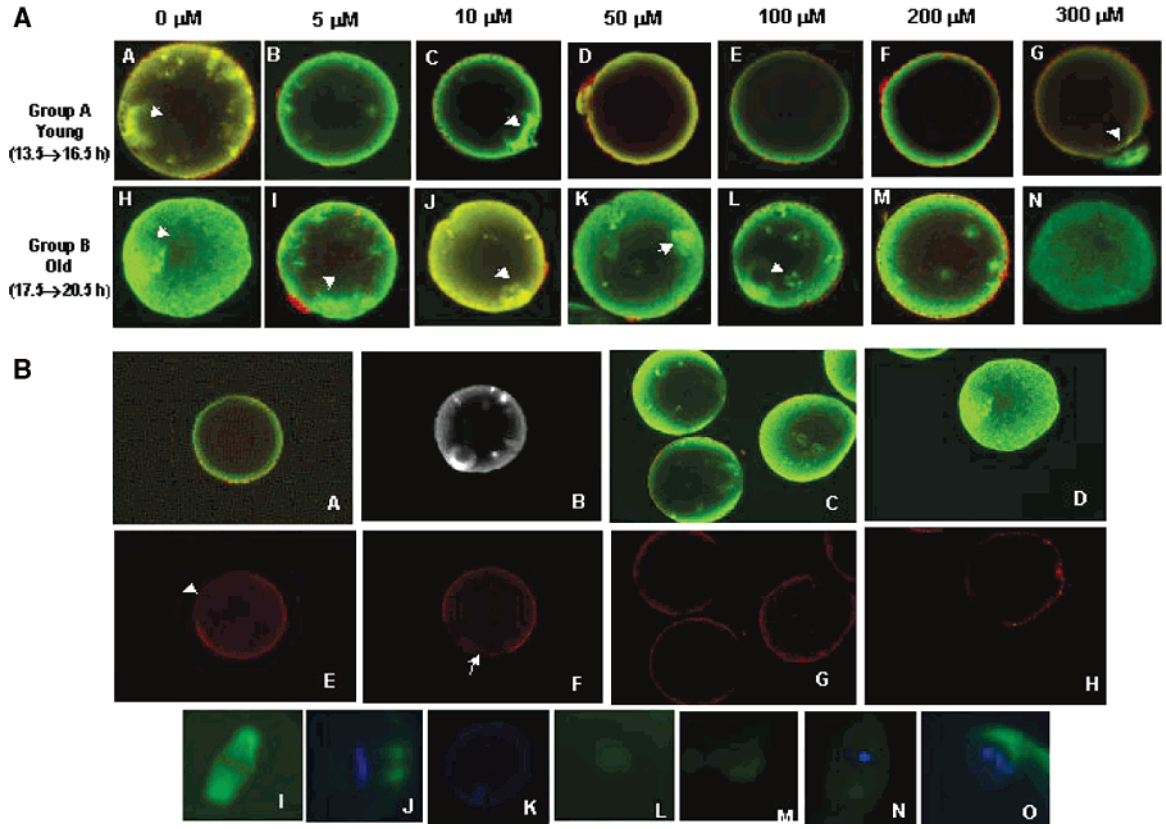

FIGURE 1: (A) Composite of photomicrographs that depicts confocal microscope generated optical sections of oocytes exposed to increasing concentrations of SNAP and unexposed controls. Microtubules are visible in the spindle in each oocyte, in MTOC in certain oocytes, and in ooplasm in certain others. Photomicrographs in A through G and those from H through N belong to group A and B, respectively. White arrowheads point to microtubular spindles that have undergone paclitaxel induced broadening. Also seen are MTOC in some oocytes in the form of bright specks in the ooplasm (A, B, I−M). Oocytes in A, I, L, and M represent moderate MT; those in B−G, J, and K display minimal/negligible MT, and oocytes in H and N have markedly increased MT. (B) Composite of photomicrographs reveals optical sections from oocytes that were either young or relatively old and either were untreated controls or were exposed to different concentrations of NO donor, SNAP. The oocytes were stained for α-tubulin with FITC conjugated antibody, for cortical granules with a fluorescent lectin, and for chromatin using DAPI (refer to text for technical details). The green (FITC), red (rhodamine), and blue (DAPI) colors, therefore, depict the microtubules, cortical granules, and chromosomes, respectively. Oocytes in A, B, C, and D are presented again using a different filter in E, F, G, and H, respectively, to highlight tubulin and CG staining in the same respective optical sections. Oocytes depicted in A and B (and E and F) were young oocytes exposed to 0−300 μM SNAP and reveal minimal/negligible OMD (A and B) and intact cortical granules (E and F). Oocytes in C and G were untreated control old oocytes displaying moderate OMD (B) and minimal to moderate CG loss (F). The oocyte depicted in D and H was a control old oocyte aged for a further 3 h without NO. Increased OMD (D) and major CG loss (H) are evident. Arrows in E and F indicate CG free domains that naturally lack CGs. Initial magnification = 400−600×; scale bars represent 50 μm. Fluorescent photomicrographs in I through O are spindles and/or chromosome metaphase plates of old oocytes treated with NO (I−K) and untreated controls (L−O). Normal spindle and chromosome metaphase morphology is evident (I−K), whereas abnormalities of spindle and chromosome metaphases can be seen in L−O. Original magnification 400−600×; scale bars represent 10 μm.

protective effect was lost at 300 μM of SNAP. Interestingly, the percentages of oocytes with minimal and increased OMD in control oocytes from group A (postovulatory age 13.5 + 3 = 16.5 h) were comparable to those from group B (postovulatory age 17.5 + 3 = 20.5 h) exposed to 5−200 μM SNAP.

Cortical Granule Status in Oocytes Treated with SNAP. In group A, the numbers of oocytes displaying intact cortical granules were similar between the SNAP treated and control oocytes at all SNAP concentrations (5−300 μM). Similarly, there was no difference between oocyte numbers displaying major or minor CG loss at all SNAP concentrations (Figures 2 and 3). However, in the older oocytes (group B), there was an over 2-fold significant increase in numbers of oocytes displaying intact CG compared to controls, and a significant decrease in oocytes displaying major CG loss after exposure to 5−200 μM SNAP (Figure 2). In oocytes from group B exposed to 300 μM SNAP, the CG status was similar to that of their respective control oocytes.

Zona Pellucida Dissolution Time. A significant difference in ZP dissolution timings was noted between NO exposed and respective controls at all concentrations of SNAP studied in group A, and at 5−200 μM SNAP in oocytes from group B. Significant dose−response relationships between NO exposure and ZP dissolution timings were noted in groups A and B (Figure 4). Nonetheless, optimal response for group A was noted at 100 μM SNAP and that for group B was observed at 10−100 μM SNAP concentrations (Figure 4). The ZP dissolution timings at these concentrations significantly differed compared to other concentrations ($P = 0.002$, Figure 4). The differences between NO exposed and control oocytes in group B disappeared at 300 μM SNAP.

Spindle and Chromosome Metaphase Plates. The number of oocytes within each subgroup of NO exposure in group A with spindle/metaphase integrity was not significantly different than their respective controls. However, the oocytes with intact or abnormal spindle/metaphase morphology were significantly different in group A compared to group B in

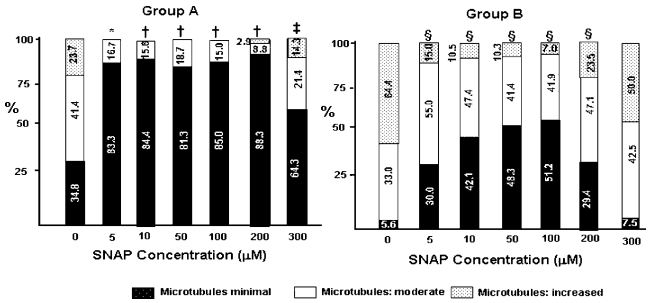

FIGURE 2: Bar charts show oocyte percentages with minimal, moderate, and increased ooplasmic microtubule dynamics. Individual SNAP concentrations are indicated at the bottom of each bar chart. Overall, a significant increase in oocytes with minimal MT is seen with serially increasing NO exposure in groups A (5−200 μM SNAP, corresponding to 0.015−0.46 μM/min NO) and B (5−100 μM SNAP, corresponding to 0.015−0.23 μM/min NO). A relative drop in the percentage of minimal microtubules noted with increase in SNAP concentration from 5 to 100 μM is seen in groups A and B, but was significant only in group B. Numbers of oocytes with minimal and increased microtubules are significantly different from respective controls ($^{*}P = 0.003$; $^{\dagger,\S}P < 0.0001$; $^{\ddagger}P = 0.045$). The oocyte numbers are presented in Table 1.

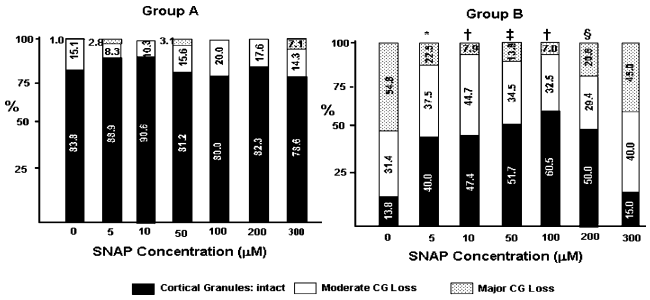

FIGURE 3: Bar charts represent the percentage of oocytes with minimal, moderate, and major CG loss in each subgroup of young and relatively aged oocytes exposed to NO, as well as the unexposed controls. Exposure to NO did not have significant effect on the number of oocytes with intact CG. However, a significant increase in the numbers of oocytes with intact CG is seen in group B among oocytes exposed to NO, compared to controls. Also seen is a concentration dependent increase in oocytes with intact CG in group B. Numbers of oocytes with intact CG are significantly different compared to respective controls: $^{*}P = 0.004$; $^{\dagger}P < 0.0001$; $^{\ddagger}P = 0.001$; $^{\S}P = 0.002$. The oocyte numbers are presented in Table 1.

control as well as NO exposed subgroups (Figure 5). Moreover, in group B, the number of oocytes with intact spindles in individual subgroups showed a trend to increase relative to their respective control subgroups ($P = 0.052$ and 0.06 at 50 and 100 μM SNAP concentrations, respectively). Nonetheless, combining data at all concentrations of SNAP, a significant improvement in spindle/metaphase integrity was noted after NO exposure in group B compared to controls ($P = 0.002$), while in group A, there was a trend toward improved spindle/metaphase integrity ($P = 0.057$).

Effect of L*-NAME (NOS Inhibitor), cGMP, and NADPH on Oocyte Aging*. To define the effect of NO and its mechanism of action, young and old oocytes were exposed to 1 mM L-NAME (NOS inhibitor) (Figure 6A), 20 mM 8-bromo guanosine *c*GMP (3 h) (Figure 6B), and microinjected with 2−4 pL of 1 μM NADPH (data not shown), with an estimated rise in the intraoocyte concentration of 5−20 nM. Again, aging related phenomena of OMD, CG exocytosis, and ZP hardening were studied for each group using fluorescence immunocytochemistry and confocal microscopy, and compared with respective unexposed controls.

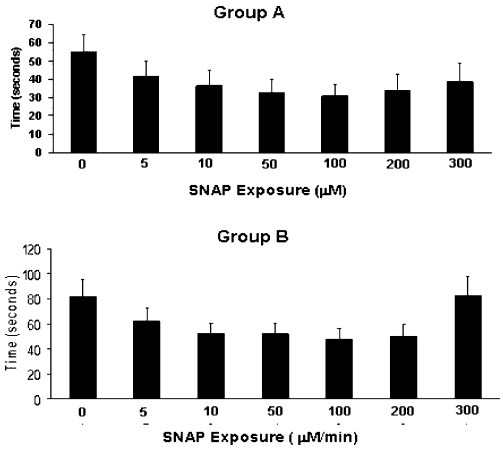

FIGURE 4: Bar charts depict the ZP dissolution timings in groups A and B, respectively. A significant decrease in the ZP dissolution time was noted after NO exposure in group A as well as B. Furthermore, a significant dose−response relationship was evident in both groups A and B, with optimal effect at 10−200 μM ($P = 0.002$). The oocyte numbers are presented in Table 1.

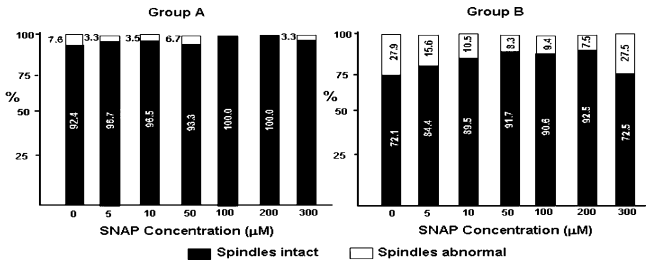

FIGURE 5: Bar charts represent the percentage of oocytes with intact spindles and abnormal spindles in each subgroup of young and relatively aged exposed to NO, as well as the unexposed controls. The oocyte numbers are presented in Table 1.

Collectively, a significant diminution in aging phenomena was noted after exposure to cGMP and NADPH compared to controls. Similarly, enhancement in aging phenomena was noted in young and old oocytes treated with L-NAME.

DISCUSSION

Oocyte postovulatory aging significantly contributes to abnormalities of fertilization, cleavage divisions, and chromosomal segregation, thereby leading to reproductive failure (*1*, *3*). However, very little is understood about the causative mechanisms involved in oocyte aging. Nitric oxide forms a vital component of the oocyte microenvironment during folliculogenesis, ovulation, and oviductal journey, and plays a positive role during oocyte maturation, fertilization, and beginning of embryo development (*17*−*23*). It is, thus, surmised that NO might also modulate oocyte aging. Information on this role of NO, however, is surprisingly unclear. In this report, we have demonstrated that exposing oocytes to increasing concentrations of NO led to a diminution of OMD, CG loss, ZP dissolution timing, and improved spindle as well as chromosomal integrity. We have employed amperometric integrated NO-selective microelectrodes (World Precision Instruments, Inc) to directly measure intraoocyte NO concentration in vivo. Our preliminary measurements indicate that the levels of NO during these in vitro exposures mimic or exceed those in vivo. Together, these studies support the notion that NO levels we used in the current studies are both physiologically and pharmacologically

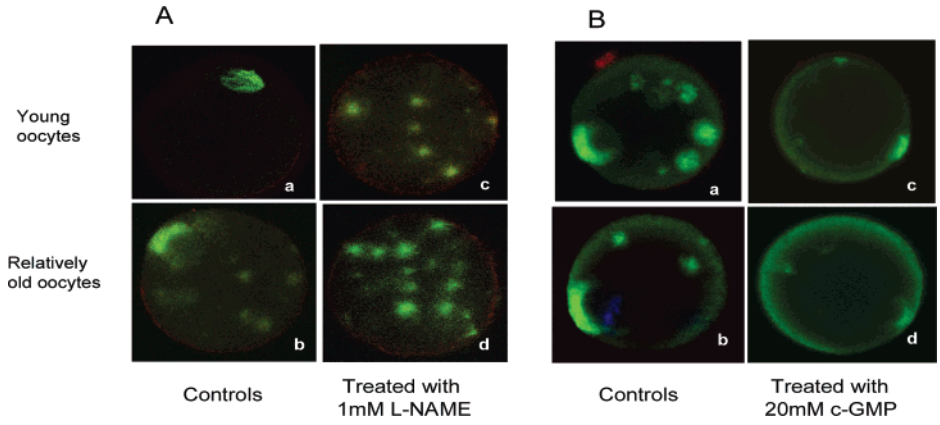

FIGURE 6: Young and relatively old control oocytes show mild, moderate microtubules, respectively. Microtubules are visible in the spindle in each oocyte, in MTOC in certain oocytes, and in ooplasm in certain others. The oocytes were stained for α-tubulin with FITC conjugated antibody, for cortical granules with a fluorescent lectin, and for chromatin using DAPI (refer to text for technical details). The green (FITC), red (rhodamine), and blue (DAPI) colors, therefore, depict the microtubules, cortical granules, and chromosomes, respectively. (A) Composite of photomicrographs that depicts confocal microscope generated optical sections of control young and old oocytes (a and b, respectively) and oocytes young and old exposed to 1 mM L-NAME (c and d, respectively). The oocytes in the L-NAME treated group have enhanced MTOC in the form of bright specks in the ooplasm. The relatively aged oocytes have markedly increased MT. (B) Composite of photomicrographs that depicts confocal microscope generated optical sections of control young and relatively old oocytes (a and b, respectively) and young and relatively old oocytes exposed to 20 mM cGMP (c and d, respectively). The young and relatively old oocytes treated with cGMP show decreased MTOC.

relevant.[2] These positive features clearly suggest that NO plays a crucial physiological role in attenuating oocyte aging, determining the oocyte quality and developmental potential, and preventing chromosome abnormalities as well as reproductive loss. On the basis of the known chemistry of NO, a multifunctional role of NO may account for its influence on oocyte aging.

Exposing young and relatively aged oocytes to NO resulted in a significant attenuation of OMD in both young and old oocytes and in a significant decrease in CG loss in old oocytes. Importantly, the OMD in NO exposed old oocytes (aged to 20.5 h after hCG) was comparable to a certain extent to that in unexposed young oocytes (aged to16.5 h after hCG), indicating that NO possibly delays the aging process. Ooplasmic microtubule dynamics are closely linked to the cell cycle stage and increase during interphase (*11*, *13*). During the M II arrest, despite paclitaxel stimulation, OMD are very minimal during the first few hours, but increase with advancing postmaturation age (*14*). Paclitaxel is a microtubule-stabilizing agent and lowers the critical concentration for tubulin polymerization (*30*), and it has previously been used to judge OMD in unfertilized oocytes (*14*). Accordingly, young M II stage oocytes do not display significant OMD despite paclitaxel stimulation. In contrast, older oocytes show increased OMD after paclitaxel exposure (*14*). Our data clearly demonstrates that the OMD were uniformly lowered after NO exposure as seen by an increase in the number of oocytes with minimal microtubules and a decrease in oocytes with increased microtubules compared to unexposed sibling control oocytes. These results indicate that NO diminishes the rate of tubulin polymerization in the ooplasm.

The ZP dissolution timings were significantly higher in older compared to the younger unexposed oocytes. Nevertheless, in both young and old oocytes, the ZP dissolution timings were significantly lower in oocytes exposed to NO compared to respective unexposed control oocytes. Hardening of ZP is known to be associated with oocyte aging and is associated with the cortical reaction (*1*, *12*). The ZP dissolution timings were more sensitive markers of aging compared to our technique of CG assessment in this regard.

Among the young and old oocytes which were exposed to the NOS inhibitor, L-NAME, a significant increase was noted in the OMD, CG exocytosis, and ZP dissolution time. These findings reveal that deprival of NO accelerated oocyte aging, further supporting the role of NO in preventing oocyte aging.

Although NO overproduction is implicated in a range of diseases (*31*, *32*), our findings indicate that it protects against oocyte aging. Thus, in oocytes, NO may serve as an atypical antioxidant rather than a pro-oxidant. This could be attributed to its ability to scavenge cytotoxic reactive oxygen species and peroxyl lipid radicals. It is evident that a saturated amount of NO is required to retain the oocyte's capability of undergoing normal fertilization and development. Compromise in the production of NO may occur during oocyte aging. To test whether antioxdants play a potential role in delaying oocyte aging, we investigated the effect of NADPH in modulating the oxidative stress and aging related phenomena in both young and old oocytes. Oocytes microinjected with NADPH showed a significant decrease in OMD, indicating a significant potential modulatory/preventive role of NADPH in oocyte aging (P. Goud, unpublished results). The effect of NO supplementation on oocyte aging is somewhat similar in this regard. It is, thus, likely that supplementation with NO may have restored the balance between the bioavailability of NO and the overproduction of unwanted free radicals that contribute to the process of aging.

In related studies, Kikuchi et al. have previously shown that porcine aged oocytes have low MPF activity, which contributed at least in part to the aging phenomena (*9*). In these studies, the authors have demonstrated that treatment

[2] Anuradha P. Goud, Pravin T. Goud, Xueji Zhang, Semira Galijasevic, Michael P. Diamond, and Husam M. Abu-Soud, unpublished results.

of 72 h cultured aged oocytes with caffeine (last 10 h of culture) decreased the level of pre-MPF and elevated MPF activity (*9*). In comparison to untreated aged oocytes, caffeine treated oocytes displayed a significantly lower parthenogenetic activation rate and a lower percentage of fragmentation suggesting that caffeine remarkably delays the process of aging (*9*). It should be noted that there is increasing evidence that caffeine plays a contributory and even predominant role as an antioxidant molecule (*33*, *34*). Since NO shares phosphodiesterase inhibition and antioxidant properties of caffeine, it may have acted via a similar mechanism in delaying the aging phenomena.

Another mechanism by which NO could participate in delaying oocyte aging is through its activation of guanylate cyclase, which leads to an increased production of cGMP (*35*, *36*). Exposure of young and relatively old oocytes to 8-bromo cGMP significantly diminished ZP dissolution time and OMD. Furthermore, CG exocytosis was significantly reduced in oocytes exposed to 8-bromo cGMP. These findings implicate cGMP in inhibiting the aging phenomena, and suggest that activation of guanylate cyclase plays an essential role in preventing oocyte aging. Cyclic GMP by itself is known to stimulate resumption of meiosis in rat and hamster oocytes (*37*, *38*). Similarly, cGMP also activates specific phosphodiesterases, resulting in a decrease in cAMP (*39*), which in turn may also contribute to activation of MPF, which is a key regulator of M II phase arrest (*40*, *41*).

One of the major sources of oxidative stress is formation of superoxide radical ($O_2^{\bullet-}$) from oxygen by peroxidases. Under normal circumstances, $O_2^{\bullet-}$ is metabolized by superoxide dismutase and other mechanisms requiring NADPH as a cofactor. Metabolism of $O_2^{\bullet-}$ may be compromised in aging oocytes, e.g., secondary to NADPH depletion. Superoxide may, thus, accumulate in aging oocytes. Although NO is known to have some harmful effects as an oxidant, collectively, our current findings reveal a temporal sequence of NO participation in the chemical processes involved in aging, further suggesting that it may protect against oocyte aging, and also may paradoxically act as an antioxidant. Exposure to NO also protected the oocytes from spontaneous CG loss, which is a relatively late event in aging and is related to the onset of parthenogenetic activation (*42*). Loss of CG during aging could be related to elevating cytosolic Ca^{2+} and/or to other mechanisms such as activation of protein kinase C (*42*), both of which are normal phenomena during fertilization, but may occur in unfertilized oocytes due to aging (*43*). Nitric oxide may have prevented CG loss by rectifying the Ca^{2+} dysregulation via its "antioxidant" mechanism or by arresting the activation of protein kinase C.

There was a significant dose−response relationship among young and old oocytes exposed to NO. Both young and old oocytes showed optimal response to 0.11−0.23 μM/min of NO, and, in fact, in old oocytes, the beneficial influence of NO on oocytes disappeared at 0.70 μM/min. These findings were noted while studying all the aging phenomena in the current study. It is likely that the dose related improvement in response to NO in the first phase reached a peak, followed by a plateau, secondary to saturation. In older oocytes, increasing NO exposure may have contributed to an antagonistic effect. These observations are consistent with the biphasic effect of NO on oocyte meiotic maturation previously shown in cumulus enclosed and denuded mouse oocytes (*44*).

Supplementation with NO may have corrected its deficiency in aged oocytes. The fact that the positive effect of NO is plateaued beyond 0.46 μM/min of NO indicates that the oocytes were saturated beyond this level. The decrease in NO level combined with oocyte aging may be governed by a mechanism that involves one or more of the following: alteration in NOSs expressions, enzyme dysfunction, degradation in L-arginine and H_4B, or overproduction of NO scavenger. Thus, delivery of supplementary NO is unequivocally protective to the oocytes that have lost the protective effects of endogenous NO. Overall, NO supplementation could be an attractive therapeutic option to prevent oocyte aging, e.g., in ART and stem cell research to improve fertility and reproductive outcome in general.

ACKNOWLEDGMENT

The authors wish to thank Drs. Mohammed R. Haque and Kamiar Moin from the Confocal Microscopy Core, Department of Pharmacology; and Michael Kruger, Biostatistician, and Ramkumar Chidambaram, Department of Obstetrics and Gynecology, The C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, for their technical help.

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BI050711F