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NITRIC OXIDE EXTENDS THE OOCYTE TEMPORAL WINDOW FOR OPTIMAL FERTILIZATION

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Abstract

Deteriorating oocyte quality is a critical hurdle in the management of infertility, especially one associated with advancing age. In this study, we explore the role of nitric oxide (NO) on the sustenance of oocyte quality post-ovulation. Sibling oocytes from superovulated mice were subjected to intracytoplasmic sperm injection (ICSI) with cauda-epididymal spermatozoa following exposure to either the NO donor, *S*-nitroso *N*-acetyl penicillamine (SNAP, 0.23 μ M/min); an NO synthase (NOS) inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME, 1 mM), or an inhibitor of soluble guanylyl cyclase (*sGC*), 1H-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one (ODQ, 100 μ M) and their sibling oocytes were subjected to ICSI either before (young) or after culture for the corresponding period of time (old). Outcomes of normal fertilization, cleavage and development to the morula and blastocyst stages were compared. Embryos from each subgroup were also subjected to TUNEL assay for apoptosis. A significant deterioration in the ability of the oocytes to undergo normal fertilization and development to morula and blastocyst stages occurred among oocytes aged in culture medium compared to their sibling cohorts subjected to ICSI immediately after ovulation ($P < 0.05$). This deterioration was prevented in oocytes exposed to SNAP. In contrast, exposure to L-NAME or ODQ resulted in a significant compromise in fertilization and development to the morula and blastocyst stages ($P < 0.05$). Finally, apoptosis was noted in embryos derived from aged oocytes and those exposed to L-NAME or ODQ, but not in embryos derived from young oocytes or oocytes exposed to SNAP. Thus, NO is essential for sustenance of oocyte quality post-ovulation.

Keywords

Apoptosis; fertilization; intracytoplasmic sperm injection (ICSI); nitric oxide; oocyte postovulatory aging; oocyte quality; oocyte temporal window

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INTRODUCTION

Nitric oxide (NO) is a ubiquitous free radical that plays a significant role in various physiological systems [1]. Its role in reproduction is particularly noteworthy and multifaceted since NO has been implicated in a wide array of processes including, but not limited to, spermatogenesis, penile erection, sexual behavior, folliculogenesis, ovulation and embryo development, as well as pregnancy, placental function and labor [2]. Particularly, NO is reported to play a role in early development such as oocyte activation, oocyte maturation, embryonic development and implantation [3–7]. The role of NO has also been implicated in fertilization, and suggested to be the sperm factor that activates oocytes at fertilization [4]. Nevertheless, the role of NO as a sperm factor in oocytes from other species remains controversial [8].

NO is generated by NO synthases (NOSs) which typically utilize molecular oxygen (O_2), NADPH, and tetrahydrobiopterin (H_4B) to convert L-arginine (L-Arg) to NO and citrulline. The potential role of NO during early embryogenesis was evidenced by higher consumption of arginine in mouse and human preimplantation embryos [9]. Similarly, the role of NO in early development was elucidated using N^G-nitro-L-arginine methyl ester (L-NAME), NOS inhibitor, during oocyte and embryo culture, which inhibited both mouse oocyte maturation and blastocyst development in a concentration dependent manner [5,7,10–12]. A similar dose dependent effect of L-NAME was reported in rat implantation as well [13]. Thus, oocytes and embryos need to be NO-sufficient to ensure development.

We have recently identified a novel and unique role of NO in maintaining the quality and integrity of recently ovulated oocytes [14]. In this study, depriving the oocytes of NO lead to deterioration in oocyte quality, while supplementing the oocytes with NO delayed this process [15–18,19]. In contrast, reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hypochlorous acid enhance oocyte aging [20].

We, therefore, hypothesized not only that NO insufficiency is a mechanism leading to compromised oocyte quality [16]. Thus, supplementing oocytes with NO *in vivo* or *in vitro* or activating the downstream cGMP pathway could be a possible strategy to rescue oocytes from deteriorating in quality. In the current study, we compare oocyte fertilizability and developmental potential in sibling oocytes allowed to age in culture, either with or without supplementation with an NO-donor, a NOS inhibitor, or a sGC inhibitor.

MATERIALS AND METHODS

Materials

All the chemicals and reagents used were of the highest purity grades available and obtained from either Sigma or Aldrich (St. Louis, MO).

Study Design—The study was approved by the Animal Investigation Committee of Wayne State University. The study design comprised of exposure of sibling M II oocytes obtained from superovulated mice to either an NO-donor (SNAP versus controls, experiment, groups A, B and C, set 1), or a NOS inhibitor (L-NAME versus controls, experiment, groups D and E, set 2), or an sGC inhibitor (ODQ versus controls, groups F and G, experiment set 3) versus culture medium (M-16, Sigma) under controlled conditions (37°C, 5% CO_2 in air, 2–3 h). All oocytes were then subjected to ICSI with mouse cauda epididymal spermatozoa, and allowed to develop to the blastocyst stage. Outcomes were compared for rate of oocyte survival, normal fertilization (2 pronuclei, 2 polar bodies), cleavage divisions, and development to morula and blastocysts. Finally, some embryos at their respective final stages of development (arrested cleavage stage or blastocysts) were

also subjected to the TUNEL assay for apoptosis (experiment set 4). Outcomes were compared using statistical methods.

Superovulation and Oocyte Retrieval

Four to six week-old B6D2F1 mice were obtained from Jackson Laboratories (Bar Harbor, ME), and were adjusted to the 12 h light-12 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, St. Louis, MO), administered IP 48–52 h apart. Mice were sacrificed at 13.5 h (young) 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 pipette. Oocytes were thoroughly rinsed in M2 medium, inspected to rule out abnormal morphology, and were kept ready in M16 medium (Sigma) pre-equilibrated with 5% CO₂ in air at 37 °C in a common pool before randomly assigning them into test and control groups.

Exposure to S-Nitroso Acetyl Penicillamine (SNAP, Group C)

The young and the old oocytes were exposed to 100 µM of the NO donor, SNAP, that generated 0.23 µM/min NO, as determined by the NO capturing method oxyhemoglobin assay [21,22]. Freshly SNAP was prepared as stocks of 100 mM and 1 mM in DMSO and desired concentrations were freshly prepared in M2 medium. Oxyhemoglobin assay was performed on conditioned medium [14] to determine the exact amount of NO that the oocytes were exposed during the experiment. 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 intracytoplasmic sperm injection (ICSI) with cauda epididymal spermatozoa (group C, n=26) [23]. Control sibling oocytes were either subjected to ICSI immediately (group A, n=24) or were allowed to age in culture in M16 without supplementation for 3 h and then subjected to ICSI (group B, n=28). End product of SNAP, penicillamine, has no significant effect in comparison to culture medium alone [14].

Exposure to NOS Inhibitor, N^ω-Nitro-L-Arginine Methyl ester (L-NAME, Group E)

L-NAME was prepared as stocks of 1 mM dissolved in distilled water and stored at –20 °C. Oocytes retrieved at 13.5 h after hCG were exposed to L-NAME in M16 medium (10 µM, 3 h, 37 °C, 5% CO₂, group E, n=22), rinsed in M2 medium, while their sibling oocytes were cultured in M-16 without L-NAME, but otherwise, identical conditions as above for 3 h (group D, n=23). Finally, oocytes from both groups were subjected to ICSI with epididymal spermatozoa, under identical conditions described below.

Exposure to 1H-[1,2,4] Oxadiazolo [4,3-a] Quinoxalin-1-One (ODQ, Group G)

In experiment set 3, the NO-sensitive soluble guanylyl cyclase (sGC) inhibitor, ODQ (prepared as a 10 mM stock solution in DMSO). Sibling M II oocytes (retrieved 13.5 h post-hCG) were then exposed either to ODQ (100 µM, and controls, at 37 °C, and 5% CO₂, for 3 h, group G, n=28) or culture medium (controls, n=25). The ODQ desired concentration was selected on the basis of previous related studies [14,15]. Subsequently, test and control oocytes were rinsed and subjected to ICSI with cauda epididymal spermatozoa as below.

Preparation of Spermatozoa

Spermatozoa were prepared by the method of Kimura and Yanagimachi [23], with some modification. Briefly, 8–12 weeks old B6D2F1 male mice were used to obtain the spermatozoa. The cauda epididymis was located and cut out from the testis and placed in HEPES-BWW medium prepared in-house [24]. It was cut in several places to allow the

sperm to disperse in the medium, a drop on this suspension was transferred in the medium and incubated for 5–15 min at 37 °C to allow the spermatozoa to disperse evenly in the medium. The sperm were then transferred in the central drop containing 7% PVP solution and kept on the microscope stage, which was pre-cooled at 17–18 °C for at least one h prior to beginning the ICSI procedure. The percentage of motile spermatozoa dropped to 30–40% after incubation with cooled PVP solution.

Injection of the Mouse Oocytes

Before the injection procedure, a group of 12 to 15 sibling oocytes belonging to treatment and control groups in each experiment set were transferred in the adjoining drops of M2 medium laid out around the central sperm drop at 17 °C for 15 min pre-incubation [22,23]. The injection procedure was done at the same temperature. The injection needle had inside and outside diameters of 6 and 8 µm, respectively (Cook Medical). The holding pipette had an inner and an outer diameter of 5 and 90 µm, respectively, the small opening of the holding pipette was important for survival of the mouse oocytes during the injection procedure. The single spermatozoon was immobilized by touching the tail with the injection needle, and the sperm was withdrawn in the pipette tail first, and then the hooked head was manipulated in the needle. The injection of the oocytes was performed with the polar body at 6 o'clock position with the smallest amount of injection medium as possible. The medium in the vicinity of the injected sperm was retrieved to extent possible. The needle was then withdrawn gently.

Culture of the Injected Mouse Oocytes

After injection, the mouse oocytes were kept for 10 min at 17 °C, followed by 10 min at room temperature, after which they were transferred to M16 at 37 °C. The oocytes were cultured at 37 °C and 5% CO₂. The oocytes microinjected with spermatozoa were closely examined for membrane damage or lysis under Nomarsky contrast at 400–600x magnification, and intact oocytes were cultured in M-16 medium for 5 days. Fertilization check was performed at 16–18 h, and subsequent embryo development was monitored daily to the blastocyst stage. The rate of normal fertilization as seen by two pronuclei, and two polar bodies, was noted during each experiment. Similarly, rates of cleavage divisions to the 2–4 cell stage, and subsequent development to morula and blastocyst stages, were documented for later comparisons between treatment (SNAP, ODQ or L-NAME) versus control groups.

TUNEL Assay

In experiment set 4, some randomly selected embryos, including arrested cleavage stage embryos and blastocysts that were generated after subjecting oocytes to ICSI from either groups, were subjected to the TUNEL assay. The embryos that arrested during development as, well as those that developed to the blastocyst stage, were then processed for apoptosis assessment (blastocysts, n=27; arrested embryos, n=24). Out of them, 21 blastocysts belonged to groups A and C, and the other 6 to groups B, D and F. Apoptosis was assessed using the TUNEL assay (*In Situ* Cell Death Detection Kit, Fluorescein; Roche) as follows: Briefly, blastocysts and arrested embryos were fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4), followed by permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate (w/v), freshly prepared, on ice (2–8 °C) for 2 min. The embryos are then subjected to the TUNEL reaction, by adding the enzyme terminal deoxynucleotidyl transferase (TdT) and the fluorescein labeled nucleotides, suitable negative and positive controls incorporation were included. The specimens were, subsequently, mounted in Vectashield® (Vector) and processed for confocal microscopy as above by an independent examiner.

Statistical Analysis

Statistical analysis was performed using SPSS® version 14.0 (SPSS Inc., Chicago IL). The frequency data in each test and control subgroup were analyzed using Chi Square tests. Frequencies of oocyte lysis after ICSI, normal fertilization (2 pronuclei and 2 polar bodies), development to 2–4 cells, morulae and blastocysts in individual subgroups of SNAP and ODQ exposure were compared to their respective sibling control oocyte subgroups using the Fisher's exact test. Significance was defined as $P < 0.05$.

RESULTS

Oocyte Survival and Fertilization Following ICSI

Overall, 176 oocytes were subjected to the experimental conditions consisting of SNAP, L-NAME or ODQ, while the respective sibling oocyte groups were subjected to media without any of these agents. Of these, 78, 53 and 55 oocytes were subjected to ICSI in the first, second, and third experiment sets, respectively. The overall survival of oocytes among oocytes combined from all groups was 70.5%. Moreover, there were no differences among the rates of oocyte survival following ICSI when all the subgroups were compared within experiment sets and also when compared to the oocytes pooled from all the subgroups. The ICSI procedure is depicted in Figs. 1A and 1B. In experiment set 1, a significant decline was nonetheless observed in the rate of normal fertilization (2 pronuclei and 2 polar bodies, Figs. 1C and 1D among the control oocytes that were subjected to ICSI after allowing to age in culture for 3 h, compared to the oocytes that were subjected to ICSI soon after ovulation (young oocytes, 13.5 h post-hCG, $P < 0.05$).

Interestingly, the time dependent decline in the rate of normal fertilization was prevented among the oocytes exposed to SNAP despite the post ovulatory age as the fertilization rate was similar in this subgroup compared to postovulatory young oocytes (Fig. 2). On the other hand, a significant decline in the rate of normal fertilization occurred among oocytes subjected to L-NAME compared to their sibling control oocytes ($P < 0.05$). Similarly, oocytes pretreated with ODQ had a significant diminution in the rate of normal fertilization compared to their sibling control oocytes.

Development of Fertilized Oocytes Following ICSI

Among the oocytes that underwent normal fertilization, further culture resulted in the first cleavage division in almost all the zygotes, barring one oocyte each in group D and G that underwent a developmental block and subsequent fragmentation. Thus, there were no significant differences in this regard.

Nonetheless, further culture revealed diminished developmental potential in several embryos that underwent developmental blocks at various stages at and beyond the 2–4 cell stage. Eventually, a significant decline in the rate of development to the morula stage was observed among the control aged oocytes (group B) compared to groups A and C. Similarly, a significant decline in development beyond 2–4 cells occurred among embryos from oocytes that were exposed to L-NAME (group E) or ODQ (group G) compared to their respective sibling control oocytes unexposed to ODQ or L-NAME (groups D and F, respectively, $P < 0.05$ for both). Finally, there was a further decline to blastocyst stage development among embryos in groups B, E and G compared to their respective control groups ($P < 0.05$). On the other hand, exposure to SNAP resulted in superior rates of fertilization and development despite allowing to age *in vitro* (Figs. 1 and 2).

In summary, deterioration in the rate and quality of fertilization and development were noted among post-ovulatory aged oocytes, and the deterioration worsened among oocytes exposed

to ODQ or L-NAME with virtually a total lack of development to the blastocyst stage. Conversely, supplementation of the oocyte medium with an NO donor significantly sustained the oocyte ability to result in normal fertilization and further development to the blastocyst stage.

Apoptosis in Embryos

Evidence of apoptosis was noted among embryos that underwent arrest in the 2–16 cell stage in all those that were exposed to L-NAME (group E) or ODQ (group G), but in only 4 out of 12 embryos among their sibling controls (100.0 and 33.3% in groups E+G and D+F, respectively). Similarly, apoptosis was noted among the embryos produced from the control old oocytes (group B, 55.5%). However, there was no evidence of apoptosis among all embryos from group A irrespective of developmental stage or arrest. Similarly, no apoptosis could be detected among all embryos tested from group C irrespective of the stage of development (Fig. 3, group C).

DISCUSSION

In clinical assisted reproduction treatment (ART), oocytes need to be fertilized within a narrow “temporal window” for optimal success. Delay in inseminating these oocytes beyond the optimal temporal window could result in deterioration in fertilization and development, with consequent abnormalities. Similarly, in the *in vivo* situation, oocytes await penetration by spermatozoa in the oviducts for a variable, but finite time, and a delay in this process could result either in failure or abnormality in fertilization and development, resulting in pre- or post-implantation embryo loss, miscarriage or even remote effects on the embryo or fetus [25,26]. Recently, this temporal window was studied among couples trying to conceive, and those with shorter ones took significantly longer to conceive [27]. The temporal window may be narrowed also in conditions such as uncontrolled diabetes and advanced age, as evidenced by accelerated aging of the oocytes pre- and post-ovulation [15,28,29]. Similar enhancement of preovulatory aging has also been noted after exposures to certain environmental toxins [30]. On the other hand, improved folliculogenesis and sustenance of oocyte quality post-ovulation will result in an extended temporal window for optimal fertilization. This strategy could help prevent reproductive loss and infertility related to oocyte aging. Evidence regarding improvement in oocyte quality and extension of the oocyte temporal window has been indirect based on certain markers in the oocyte.

In the current study, we investigate the impact of NO on the oocyte directly by studying its fertilizability and developmental potential under three distinct conditions, namely, with or without supplementation with an NO-donor, NOS inhibitor, or GC inhibitor. Our results clearly indicate that the NO supplementation not only resulted in sustained ability of oocytes to undergo normal fertilization and development to the blastocyst stage, but also prevented the onset of apoptosis in the embryos that occurred with fertilization of aged oocytes. Inhibition of NO synthesis or downstream mediators significantly worsened fertilization and development to the blastocyst stage and enhanced arrest and apoptosis in the embryos. Collectively, our results provide the first direct evidence regarding the role of NO in sustenance of oocyte quality post-ovulation by preventing the deterioration in oocyte fertilizability and developmental potential.

NO plays a critical role during folliculogenesis, oocyte maturation, and fallopian tubal transport [7,31–33]. It also enhances the processes of embryo development and implantation [5,7,34]. Nonetheless, the exact role of NO during fertilization has been debated recently. Kuo et al. discovered that in sea urchins, fertilizing spermatozoa deliver NOS to the oocyte. resulting. This process possibly involves activation of cGMP and stimulation of the ryanodine receptors with intracellular Ca^{2+} release and oocyte activation [4]. These findings

are somewhat contrary to subsequent studies by Hyslop et al. in ascidian oocytes [8], where simultaneous measurements of intracellular Ca^{2+} and NO failed to detect a notable rise in NO within the oocyte at fertilization. Subsequent studies by Leckie et al. revealed that a rise in NO follows the release of Ca^{2+} within the oocyte and may, in fact, determine the duration of the Ca^{2+} transients during fertilization [35]. This finding highlights the Ca^{2+} regulatory role of NO since altered spatiotemporal characteristics of Ca^{2+} release at fertilization is a hallmark of aged oocytes [36–38]. Irrespective of the debate, NO is essential for the sustenance of oocyte quality, no matter whether NO is provided by the sperm or the oocyte or a combination of both [6,7,14,15,20].

A model describing the role of NO in the regulation of oocyte quality and optimal fertilization is shown in Fig. 3. This model consists of two cycles: a productive cycle where regulation of Ca^{2+} release ensures optimal oocyte quality; and a nonproductive cycle where disruption in Ca^{2+} release leads to apoptosis. In the productive cycle, NO binds and activates GC-Fe(II) which, subsequently, enables the enzyme to catalyze the conversion of GTP to cGMP . Enhancement of the cGMP level activates the cGMP -dependent protein kinase, which in turn, catalyzes the phosphorylation of the InsP_3 or ryanodine receptor leading to regulation of Ca^{2+} release Fig. 3. Regulation of the spatiotemporal pattern of Ca^{2+} release prevents Ca^{2+} dysregulation associated with oocyte aging. In the nonproductive cycle, inactivation GC-Fe(II) through NO deficiency, exposure oocytes to the NOS inhibitor, L-NAME, or cGC inhibitor, ODQ, accelerated oocyte aging and significantly compromised fertilization and development. Supplementation with the SNAP delayed oocyte aging and improved fertilization and development to blastocyst stage (Fig. 3).

Different NOS isoforms play different roles in the various reproductive events. The participation of NO in mediating sGC signaling pathway in oocyte [14,15] suggests the involvement of eNOS and nNOS during normal fertilization and development. However, higher NO levels generated by iNOS associated with higher $\text{O}_2^{\bullet-}$ production [20,39], a characteristic feature of aging, may promote oocyte fragmentation and apoptosis. Consistent with this hypothesis, eNOS-deficient mice have shown prolonged estrous cycle and reduction ovulation [6,40], while iNOS-deficient mice display an inhibitory role in fertilization [41].

The occurrence of oocyte aging has been related to a time dependent partial exit from M II arrest, possibly secondary to a decline in the activity of cell cycle regulators, namely, M-phase promoting factor (MPF) and mitogen activated protein kinase (MAPK). A significant decline in these factors, in parallel with deterioration in oocyte quality, has been reported to occur as early as at 16 h following the hCG trigger [42,43]. One possible mechanism could be declining bioavailability of NO secondary to either underproduction or consumption or both. Consistent with this hypothesis, NO mediated delay in oocyte aging was reconfirmed as the age dependent decline in oocyte fertilizability and developmental potential were prevented in oocytes pretreated with NO. Production of NO in the oocyte microenvironment is known to undergo a dynamic alteration in expression in the follicles, as well as oviducts in relation to the timing of ovulation and key events of oocyte maturation regulated by the cell cycle factors [31,44–46]. NO may, thus, act as a regulator of MPF and MAPK activity, optimizing oocyte maturation and preventing aging.

Developmental block at the 2-cell stage has been well studied in the mouse model. One important contributor to this could be the failure to activate the embryonic genome [47,48]. Similarly, developmental block among the embryos that developed from oocytes subjected to the pretreatment with ODQ or L-NAME may have been secondary to failure of embryonic genome activation, either secondary to Ca^{2+} dysregulation, or deficiency of ooplasmic factors critically required for genome activation or both. This possibility further

underscores a novel and critical role played by NO improving and sustaining the oocyte quality. NO may also have prevented temporal decline in oocyte factors involved in optimal sperm chromatin decondensation [49], which is critical for gene expression during development [50–55].

In vitro aging of the oocytes as well as exposure of the oocytes to the sGC inhibitor, ODQ resulted in significantly poorer rates of fertilization and development (Fig. 2). These findings are indicative of a significant decline in the oocyte quality or ability to undergo normal fertilization and development after competitive inhibition of NO action. ODQ is a potent and selective competitive inhibitor of sGC and induces a rightward shift of the concentration-response curves with NO donors, thus, competitively blocks the NO-sensitive sGC at concentrations that significantly augment the occurrence of aging phenomena of zona pellucida (ZP) hardening, ooplasm microtubule dynamics (OMD) as well as cortical granule (CG) loss [15,56,57]. Furthermore, the NO-competitive inhibition of sGC by ODQ results in an apparently irreversible oxidation of the prosthetic heme group [57].

Treatment of oocytes with NO also prevented the onset of apoptosis in the embryos, which occurred exclusively in embryos derived from postovulatory old and L-NAME or ODQ treated oocytes. Apoptosis in the preimplantation embryo may occur due to chromosomal and nuclear abnormalities [58,59], imbalance of growth and survival factors, or due to the exposure to damaging factors such as the reactive species acting directly or via their influence of the developmental potential of the oocyte [20,60–62]. Similarly, dysregulation of intracellular Ca^{2+} and depletion of endoplasmic reticulum stores associated with oocyte aging may be involved in triggering apoptosis [38,63,64]. NO may have, thus, prevented apoptosis in the embryos by regulating the Ca^{2+} release and maintaining the ER Ca^{2+} stores and preventing the activation of the upstream mediators of apoptosis (Figs. 1 and 3).

In conclusion, NO extends the 'oocyte temporal window for optimal fertilization and development'. These findings not only indicate the physiological role of NO in the oocyte and its microenvironment, but also suggest a mechanistic role of NO insufficiency to explain deterioration of oocyte quality in conditions, such as in aging and diabetes mellitus [15].

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ABBREVIATIONS

CG	cortical granules
H₄B	tetrahydrobiopterin
ICSI	intracytoplasmic sperm injection
L-NAME	N -nitro-L-arginine methyl ester
MAPK	mitogen activated protein kinase
MPF	M phase promoting factor
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide

ODQ	1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one
OMD	ooplasmic microtubule dynamics
sGC	soluble guanylyl cyclase
SNAP	S-nitroso acetyl penicillamine
ZP	zona pellucida

REFERENCES

- [1]. Ignarro LJ. Nitric oxide: a unique endogenous signaling molecule in vascular biology. *Biosci. Rep.* 1999; 19:51–71. [PubMed: 10888468]
- [2]. Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Hum. Reprod. Update.* 1998; 4:3–24. [PubMed: 9622410]
- [3]. Petr J, Rajmon R, Rozinek J, Sedmíkova M, Jeseta M, Chmelíkova E, Svestkova D, Jílek F. Activation of pig oocytes using nitric oxide donors. *Mol. Reprod. Dev.* 2005; 71:115–122. [PubMed: 15736132]
- [4]. Kuo RC, Baxter GT, Thompson SH, Stricker SA, Patton C, Bonaventura J, Epel D. NO is necessary and sufficient for egg activation at fertilization. *Nature.* 2000; 406:633–636. [PubMed: 10949304]
- [5]. Gouge RC, Marshburn P, Gordon BE, Nunley W, Huet-Hudson YM. Nitric oxide as a regulator of embryonic development. *Biol. Reprod.* 58:875–879. [PubMed: 9546715]
- [6]. Jablonka-Shariff A, Olson LM. The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock-out mouse oocytes. *Endocrinology.* 1998; 139:2944–2954. [PubMed: 9607805]
- [7]. Sengoku K, Takuma N, Horikawa M, Tsuchiya K, Komoro H, Sharifa D, Tamate K, Ishikawa M. Requirement of nitric oxide for murine oocyte maturation, embryo development, and trophoblast outgrowth in vitro. *Mol. Reprod. Dev.* 2001; 58:262–268. [PubMed: 11170266]
- [8]. Hyslop LA, Carroll M, Nixon VL, McDougall A, Jones KT. Simultaneous measurement of intracellular nitric oxide and free calcium levels in chordate eggs demonstrates that nitric oxide has no role at fertilization. *Dev. Biol.* 2001; 234:216–230. [PubMed: 11356031]
- [9]. Lamb VK, Leese HJ. Uptake of a mixture of amino acids by mouse blastocysts. *J. Reprod. Fertil.* 1994; 102:169–175. [PubMed: 7799310]
- [10]. Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.* 101:746–752. [PubMed: 1706208]
- [11]. Chen HW, Jiang WS, Tzeng CR. Nitric oxide as a regulator in preimplantation embryo development and apoptosis. *Fertil. Steril.* 2001; 75:1163–1171. [PubMed: 11384644]
- [12]. Nishikimi A, Matsukawa T, Hoshino K, Ikeda S, Kira Y, Sato EF, Inoue M, Yamada M. Localization of nitric oxide synthase activity in unfertilized oocytes and fertilized embryos during preimplantation development in mice. *Reproduction.* 2001; 122:957–963. [PubMed: 11732991]
- [13]. Biswas S, Kabir SN, Pal AK. The role of nitric oxide in the process of implantation in rats. *J. Reprod. Fertil.* 1998; 114:157–161. [PubMed: 9875168]
- [14]. Goud AP, Goud PT, Diamond MP, Abu-Soud HM. Nitric oxide delays oocyte aging. *Biochemistry.* 2005; 44:11361–1138. [PubMed: 16114873]
- [15]. Goud AP, Goud PT, Diamond MP, Gonik B, Abu-Soud HM. Oocyte aging in diabetes mellitus: role of NO-mediated cGMP signaling pathway. *Biochemistry.* 2006; 45:11366–11378. [PubMed: 16981697]
- [16]. Eichenlaub-Ritter U, Boll I. Nocodazole sensitivity, age-related aneuploidy, and alterations in the cell cycle during maturation of mouse oocytes. *Cytogenet. Cell Genet.* 1989; 52:170–176. [PubMed: 2535312]

- [17]. Eichenlaub-Ritter U. Genetics of oocyte ageing. *Maturitas*. 1998; 30:143–169. [PubMed: 9871909]
- [18]. Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. Spindles, mitochondria and redox potential in ageing oocytes. *Reprod. Biomed. Online*. 2004; 8:45–58. [PubMed: 14759287]
- [19]. Eichenlaub-Ritter U. Genetics of oocyte ageing. *Maturitas*. 1998; 30:143–169. [PubMed: 9871909]
- [20]. Goud AP, Goud PT, Diamond MP, Gonik B, Abu-Soud HM. Reactive oxygen species and oocyte aging: Role of superoxide, hydrogen peroxide and hypochlorous acid. *Free Radic. Biol. Med.* 2008; 44:1295–1304. [PubMed: 18177745]
- [21]. Kelm M, Feelisch M, Spahr R, Piper HM, Noack E, Schrader J. Quantitative and kinetic characterization of nitric oxide and EDRF released from cultured endothelial cells. *Biochem. Biophys. Res. Commun.* 1988; 154:236–244. [PubMed: 3260776]
- [22]. Stone JR, Marletta MA. Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry*. 1996; 35:1093–1099. [PubMed: 8573563]
- [23]. Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. *Biol Reprod*. 1995; 52:709–720. [PubMed: 7779992]
- [24]. Whittingham DG. Culture of mouse ova. *J. Reprod. Fertil.* 1971; 14(Suppl.):7–21.
- [25]. Wilcox AJ, Weinberg CR, Baird DD. Post-ovulatory ageing of the human oocyte and embryo failure. *Hum. Reprod*. 1998; 13:394–397. [PubMed: 9557845]
- [26]. Tarín JJ, Perez-Albala S, Cano A. Consequences on offspring of abnormal function in ageing gametes. *Hum. Reprod. Update*. 2000; 6:532–549. [PubMed: 11129687]
- [27]. Keulers MJ, Hamilton CJCM, Franx A, Evers JLH, Bots RSGM. The length of the fertile window is associated with the chance of spontaneously conceiving an ongoing pregnancy in subfertile couples. *Hum. Reprod*. 2007; 22:1652–1656. [PubMed: 17449509]
- [28]. Santoro N, Isaac B, Neal-Perry G, Adel T, Weingart L, Nussbaum A, Thakur S, Jinnai H, Khosla N, Barad D. Impaired folliculogenesis and ovulation in older reproductive aged women. *J. Clinical. Endo. Metab.* 2003; 88:5502–5509.
- [29]. Eichenlaub-Ritter U, Boll I. Nocodazole sensitivity, age related aneuploidy and alterations in the cell cycle during maturation of mouse oocytes. *Cytogenet. Cell Genet.* 1989; 52:170–176. [PubMed: 2535312]
- [30]. Stoker TE, Jeffay SC, Zucker RM, Cooper RL, Perrault SD. Abnormal fertilization is responsible for reduced fecundity following thiram-induced ovulatory delay in the rat. *Biol. Reprod*. 2003; 68:2142–2149. [PubMed: 12606370]
- [31]. Tao Y, Fu Z, Zhang M, Xia G, Yang J, Xie H. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. *Mol. Cell Endocrinol.* 2004; 222:93–103. [PubMed: 15249129]
- [32]. Chun SY, Eisenhauer KM, Minami S, Billig H, Perlas E, Hsueh AJ. Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. *Endocrinology*. 1996; 137:1447–1456. [PubMed: 8625923]
- [33]. Tranguch S, Stuerwald N, Huet-Hudson YA. Nitric oxide synthase production and nitric oxide regulation of preimplantation development. *Biol. Reprod*. 2003; 68:1538–1544. [PubMed: 12606428]
- [34]. Khorram O. Nitric oxide and its role in blastocyst implantation. *Rev. Endocr. Metab. Disord.* 2002; 3:145–149. [PubMed: 12007291]
- [35]. Leckie C, Empsun R, Becchetti A, Thomas J, Galione A, Whitaker M. The NO pathway acts late during the fertilization response in sea urchin eggs. *J. Biol. Chem.* 2003; 278:12247–12254. [PubMed: 12540836]
- [36]. Igarashi H, Takahashi E, Hiroi M, Doi K. Aging-related changes in calcium oscillations in fertilized mouse oocytes. *Mol. Reprod. Dev.* 1997; 48:383–390. [PubMed: 9322251]
- [37]. Takahashi T, Saito H, Hiroi M, Doi K, Takahashi E. Effects of aging on inositol 1,4,5-trisphosphate-induced Ca^{2+} release in unfertilized mouse oocytes. *Mol. Reprod. Dev.* 2005; 55:299–306. [PubMed: 10657049]

- [38]. Takahashi T, Takahashi E, Igarashi H, Tezuka N, Kurachi H. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Mol. Reprod. Dev.* 2003; 66:143–152. [PubMed: 12950101]
- [39]. Agarwal A, Allamaneni SS. Role of free radicals in female reproductive diseases and assisted reproduction. *Reprod. Biomed. Online.* 2004; 9:338–347. [PubMed: 15353087]
- [40]. Tempfer C, Unfried G, Zeillinger R, Hefler L, Nagele F, Huber JC. Endothelial nitric oxide synthase gene polymorphism in women with idiopathic recurrent miscarriage. *Hum. Reprod.* 2001; 16:1644–1647. [PubMed: 11473956]
- [41]. Yang JZ, Ajonuma LC, Rowlands DK, Tsang LL, Ho LS, Lam SY, Chen W. Y.; Zhou CX, Chung YW, Cho CY, Tse JY, James AE, Chan HC. The role of inducible nitric oxide synthase in gamete interaction and fertilization: a comparative study on knockout mice of three NOS isoforms. *Cell Biol. Int.* 2005; 29:785–791. [PubMed: 16087361]
- [42]. Kikuchi K, Izaike Y, Noguchi J, Furukawa T, Daen FP, Naito K, Toyoda Y. Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro. *J. Reprod. Fertil.* 1995; 105:325–330. [PubMed: 8568778]
- [43]. Xu Z, Abbot A, Kopf GS, Schultz RM, Ducibella T. Spontaneous activation of ovulated mouse eggs: time dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1, 4, 5- triphosphate sensitivity. *Biol. Reprod.* 1997; 57:743–750. [PubMed: 9314575]
- [44]. Takesue K, Tabata S, Sato F, Hattori MA. Expression of nitric oxide synthase-3 in porcine oocytes obtained at different follicular development. *J. Reprod. Dev.* 2003; 49:135–140. [PubMed: 14967938]
- [45]. Mitchell LM, Kennedy CR, Hartshorne GM. Expression of nitric oxide synthase and effect of substrate manipulation of the nitric oxide pathway in mouse ovarian follicles. *Hum. Reprod.* 2004; 19:30–40. [PubMed: 14688153]
- [46]. Lapointe J, Roy M, St-Pierre I, Kimmins S, Gauvreau D, MacLaren LA, Bilodeau JF. Hormonal and spatial regulation of nitric oxide synthases (NOS) (neuronal NOS, inducible NOS, and endothelial NOS) in the oviducts. *Endocrinol.* 2006; 147:5600–5610.
- [47]. Goddard MJ, Pratt HP. Control of events during early cleavage of the mouse embryo: an analysis of the '2-cell block'. *J. Embryol. Exp. Morphol.* 1983; 73:111–133. [PubMed: 6875454]
- [48]. Seshagiri PB, McKenzie DI, Bavister BD, Williamson JL, Aiken JM. Golden hamster embryonic genome activation occurs at the two-cell stage: correlation with major developmental changes. *Mol. Reprod. Dev.* 1992; 32:229–235. [PubMed: 1497872]
- [49]. Tamada H, Van Thuan N, Reed P, Nelson D, Katoku-Kikyo N, Wudel J, Wakayama T, Kikyo N. Chromatin decondensation and nuclear programming by nucleoplasmin. *Mol. Cell Biol.* 2006; 26:1259–1271. [PubMed: 16449640]
- [50]. Adenot PG, Szollosi MS, Chesne P, Chastant S, Renard JP. In vivo aging of oocytes influences the behavior of nuclei transferred to enucleated rabbit oocytes. *Mol. Reprod. Dev.* 1997; 46:325–336. [PubMed: 9041135]
- [51]. Aoki F, Worrad DM, Schultz RM. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev. Biol.* 1997; 181:296–307. [PubMed: 9013938]
- [52]. Adenot PG, Mercier Y, Renard JP, Thompson EM. Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. *Development.* 1997; 124:4615–4625.
- [53]. Goud PT, Goud AP, Rybouchkin AV, De Sutter P, Dhont M. Chromatin decondensation, pronucleus formation, metaphase entry and chromosome complements of human spermatozoa after intracytoplasmic sperm injection into hamster oocytes. *Hum. Reprod.* 1998; 13:1336–1345. [PubMed: 9647569]
- [54]. Goud PT, Goud AP, Laverge H, De Sutter P, Dhont M. Effect of post-ovulatory age and calcium in the injection medium on the male pronucleus formation and metaphase entry following injection of human spermatozoa into golden hamster oocytes. *Mol. Hum. Reprod.* 1999; 5:227–233. [PubMed: 10333356]

- [55]. Goud P, Goud A, Van Oostveldt P, Van der Elst J, Dhont M. Fertilization abnormalities and pronucleus size asynchrony after intracytoplasmic sperm injection are related to oocyte postmaturity. *Fertil. Steril.* 1999; 72:245–252. [PubMed: 10438989]
- [56]. Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B. Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol.* 1995; 48:184–188. [PubMed: 7544433]
- [57]. Schrammel A, Behrends S, Schmidt K, Koesling D, Mayer B. Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol. Pharmacol.* 1996; 50:1–5. [PubMed: 8700100]
- [58]. Hardy K. Apoptosis in the human embryo. *Rev. Reprod.* 1999; 4:125–134. [PubMed: 10521149]
- [59]. Levy R. Genetic regulation of preimplantation embryo survival. *Int. Rev. Cytol.* 2001; 210:1–37. [PubMed: 11580204]
- [60]. Fabian D, Il'kova G, Rehak P, Czikkova S, Baran V, Koppel J. Inhibitory effect of IGF-I on induced apoptosis in mouse preimplantation embryos cultured *in vitro*. *Theriogenology.* 2004; 61:745–755. [PubMed: 14698063]
- [61]. Hardy K, Spanos G. Growth factor expression and function in the human and mouse preimplantation embryo. *J. Endocrinol.* 2002; 172:221–236. [PubMed: 11834440]
- [62]. Yang HW, Hwang KJ, Kwon HC, Kim HS, Oh KS. Detection of reactive oxygen species and apoptosis in human fragmented embryos. *Hum. Reprod.* 1998; 13:998–1002. [PubMed: 9619561]
- [63]. Kuang E, Wan O, Li X, Xu H, Liu O, Oi Y. ER Ca^{2+} depletion triggers apoptotic signals for endoplasmic reticulum (ER) overload response influenced by overexpressed reticulon 3 (RTN3/HAP). *J. Cell Physiol.* 2005; 204:549–559. [PubMed: 15799019]
- [64]. Gordo AC, Rodrigues P, Kurokawa M, Jellerette T, Exley GF, Warner C, Fissore R. Intracellular calcium oscillations signal apoptosis rather than activation in *in vitro* aged mouse eggs. *Biol. Reprod.* 2002; 66:1828–1837. [PubMed: 12021069]

Fig. 1.

Panels I and II, each depict a set of photomicrographs showing the process and sequel of intracytoplasmic sperm injection (ICSI) into mouse oocytes exposed to S-nitroso-acetyl penicillamine or allowed to age *in vitro* or exposed to the nitric oxide synthase (NOS) inhibitor (L-NAME) or the sGC inhibitor (ODQ). In panel I, photomicrographs A and B show the process of ICSI in progress. A sharp beveled glass micropipette containing culture medium and epididymal spermatozoon is being injected into an M II stage oocyte. Photomicrographs C and D depict fertilized zygotes obtained after ICSI. Arrowhead in D points to one of the two pronuclei and two arrowheads point at two polar bodies. First and subsequent embryonic cleavage divisions are depicted in E through H with no arrests at 1-cell and an occasional embryo arrest in 2-cell stage (arrowhead in G). Further development to blastocyst stage is depicted in I and J, while one hatched blastocyst is noted in K. In panel II, photomicrographs represent optical sections of embryos using both Nomarsky contrast, as well as fluorescence confocal microscopy. Embryos produced from postovulatory young (A, B) and relatively old oocytes (C, D) that had been treated with or without a NOS inhibitor (L-NAME, E, F); a sGC inhibitor (ODQ, G, H) and an NO donor (SNAP, I–L) were subjected to the TUNEL assay to study apoptosis. Apoptosis was prominently absent despite the stage of development in embryos produced from young or SNAP treated oocytes. On the other hand, embryos derived from relatively old oocytes and those derived from oocytes treated with L-NAME or ODQ prominently exhibited apoptosis (arrowheads in D–H).

Original magnification 200–600×; bar in H represents A, B, D and F and corresponds to 50 μm . Similarly, bars in C, E and G correspond to 100 μm .

Fig. 2.

A line diagram graphically represents fertilization and developmental outcomes among oocytes in different groups (A–G) subjected to ICSI at different postovulatory ages and after different treatments. A significant drop in fertilization and development was noted among oocytes that were either aged, or were exposed to the postovulatory age-related drop in the rates of fertilization and development was, nonetheless, prevented in oocytes exposed to (SNAP, group C).

Fig. 3.

A model to explain the action of NO in the oocytes is presented here. Accordingly, sustained production of NO within the oocyte sustains its integrity, and fertilization and developmental potential by regulating the intracellular release of Ca²⁺. NO is generated by the NOS, provided by the spermatozoa or the oocyte or a combination of the two and binds to GC-Fe(II) forming a six-coordinate ferrous-nitrosyl complex. This process causes the trans axial ligand to break, thus forming a five-coordinate GC-Fe(II)-NO complex which, subsequently, enables the enzyme to catalyze the conversion of GTP to cGMP. Enhancement of the cGMP level activates the cGMP-dependent protein kinase, which in turn, catalyzes the phosphorylation of the InsP₃ receptor leading to regulation of Ca²⁺ release. Regulation of the spatiotemporal pattern of Ca²⁺ release prevents Ca²⁺ dysregulation associated with oocyte aging. Exposing oocytes to the NOS inhibitor, L-NAME, or sGC inhibitor, ODQ, accelerated oocyte aging and significantly compromised fertilization and development. In parallel, supplementation with the NO-donor, SNAP delayed oocyte aging and improved fertilization and development to blastocyst stage.