INTRODUCTION

During S-nitrosylation, covalent addition of nitric oxide group (-NO) to the thiol side chain of cysteine in protein forms S-nitroso thiol (SNO) (Hess and Stamler, 2012). Controlled S-nitrosylation regulates activities and functions of several proteins (Jaffrey et al., 2001), while dysregulated S-nitrosylation is involved in pathophysiology, including cellular senescence and cell death (Nakamura and Lipton, 2011; Iyer et al., 2014). Steady-state concentrations of protein SNO (PSNO) depend on the balance of S-nitrosylation and denitrosylation. Nitric oxide synthase (NOS) catalyzes the production of NO, and nitrosylases directly transfer NO group to protein (Seth et al., 2018), while denitrosylases induce the S-denitrosylation through the enzyme S-nitrosoglutathione reductase (GSNOR).

Previous studies have reported that NO-induced protein S-nitrosylation triggers mitochondrial fragmentation and dysfunction, accumulation of damaged mitochondria, endoplasmic reticulum stress, and protein misfolding, causing bioenergetic compromise (Hess and Stamler, 2012; Raju et al., 2015). GSNOR decreases excessive protein S-nitrosylation in primary cells undergoing senescence, as well as in mice and human cells during their life span (Rizza et al., 2018). GSNOR deficiency promotes mitochondrial nitrosative stress, leading to excessive S-nitro-
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sulation of dynamin-related protein 1 (Drp1) and Parkin, the two components involved in mitochondrial fission and clearance, thereby impairing mitochondrial quality control system.

After maturation, the oocytes are arrested in metaphase II (MII) till fertilization in the oviduct or in culture medium. Unless fertilized within the optimal time, MII oocytes undergo degradative process called post-ovulatory aging (Lord and Aitken, 2013). This is not the same as the oocyte aging through the aging of the mother. During the production of transgenic animals and assisted reproduction of humans, the in vitro maturation time of oocytes is often prolonged due to unavoidable reasons. As a result, oocytes undergo aging process. Post-ovulatory aging adversely affects various cellular structures, including cortical granules (Ducibella et al., 1990; Xu et al., 1997), zona pellicuda (Dodson et al., 1989; Xu et al., 1997), maturation-promoting factor (MPF) and MAPK levels (Kikuchi et al., 2002), cytoskeleton, and chromosomes (Wakayama et al., 2004).

A previous study reported that nitric oxide (NO) signals mediate the aging of oocytes (Premkumar and Chaube, 2015). However, very limited research has focused on the effect of NO-related redox signaling on oocyte quality during oocyte aging. Another report shows that NO-related redox signaling is involved in S-nitrosylation of proteins, affecting morphology and function of mitochondria, releasing calcium stored in the endoplasmic reticulum, regulating cellular autophagy and apoptosis (Sun et al., 2007), and is closely related to ROS-related redox signaling. Therefore, exploring the changes and mechanisms of NO-related redox signaling during oocyte aging is important for delaying, if not prevention of oocyte aging.

**MATERIALS AND METHODS**

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Co., Inc. (St. Louis, MO, USA) and all experimental manipulations were performed on a heated stage, maintained at 38.5°C.

**Collection and in vitro maturation of cattle oocytes**

All experimental protocols were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Chungbuk National University Laboratory Animal Center, Cheongju, South Korea.

Ovaries from Hanwoo cattle were collected from a local slaughterhouse, and transported in saline at 38.5°C to the laboratory. In vitro oocyte maturation was performed as previously described (Hwang et al., 2016; Choudhury et al., 2017; Hassan et al., 2017; Choi et al., 2019; Jeon et al., 2019). Cumulus oocyte complexes were aspirated from the follicles using a syringe, washed three times in Tyrode’s lactate-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and cultured in tissue culture medium 199 (TCM 199) supplemented with 10% fetal calf serum (10082-147; Gibco, USA), 1 μg/mL β-estradiol (E2758; Sigma, USA), 10 μg/mL follicle-stimulating hormone (F2293; Sigma), 10 ng/mL epidermal growth factor (E4127; Sigma), 100 IU/mL penicillin/streptomycin, 22 μg/mL sodium pyruvate (P5280; Sigma), and 95 μg/mL L-cysteine (C7352; Sigma) at 38.5°C in a humidified atmosphere with 5% CO₂ and 95% air.

**In vitro aging and SNP treatment**

After removing cumulus cells by repeated pipetting in 1 mg/mL hyaluronidase, only the oocytes with first polar bodies were used for the present studies. For post-ovulatory oocyte aging, the collected oocytes at metaphase II (MII) were cultured in fresh in vitro maturation medium (IVM) covered with mineral oil, for 24 or 48 h at 38.5°C in humidified atmosphere (5% CO₂). SNP was dissolved in DMSO to prepare 200 mM stock solutions. To determine whether NO-induced protein S-nitrosylation was harmful during oocyte aging process, MII oocytes were cultured in fresh IVM with or without 160 μM SNP.

**Biotin–switchd assay for detection of S–nitrosylated proteins**

The biotin-switch method for detecting S–nitrosylated proteins was used as described previously (Haun et al., 2013; Lee et al., 2013). In brief, oocytes from the different experimental groups were fixed in 3.7% paraformaldehyde at room temperature overnight, washed three times with HEN (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) containing 0.1% Triton X-100 for 5 min. Thiol groups were then blocked with 20 mM methyl methane thiosulphonate (MMTS), a thiol-reactive agent in the same buffer at 4°C for 30 min. The oocytes were then washed three times with HEN, and incubated with 1 mM ascorbate to reduce the S-nitrosothiols and with 0.4 mM MTSEA-Texas Red, a fluorescent derivative of MTSEA in HEN at room temperature for 1 h. Excess dye was removed.
by repeated washing of the oocytes with HEN containing 0.1% Triton X-100. Stained oocytes were then mounted on glass slides in prolonged antifade mounting medium.

ROS measurements

Total ROS levels in oocytes were determined using 2′, 7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA, Cat # D399, Molecular Probes, USA) as previously described (Nasr-Esfahani et al., 1990; Park et al., 2017; Fang et al., 2018; Jeon et al., 2019). Briefly, oocytes were incubated for 15 min in PBS/PVA containing 10 μM H$_2$DCF-DA at 38.5°C, and washed three times with PBS/PVA. Fluorescence signals were captured using a digital camera (DP72; Olympus, Tokyo, Japan) connected to a fluorescence microscope (IX70, Olympus). Total ROS levels were quantified by analyzing the fluorescence intensity of the oocytes using Image J version 1.44g software (National Institutes of Health, Bethesda, MD, USA).

Total and active mitochondrial staining

Oocytes were incubated with 500 nM MitoTracker Red CMXRos (Cat # M7512, Invitrogen) at 38.5°C for 30 min. After three washes with PZM-5 medium, TOM20 staining was carried out as described in the Immunofluorescence and confocal microscopy subsection. After this, the oocytes were stained with 10 μg/mL Hoechst 33342 for 10 min, washed three times with PBS/PVA, mounted on slides, and examined under a confocal microscope (Zeiss LSM 710 META). Images were processed using Zen software (version 8.0, Zeiss).

Immunofluorescence and confocal microscopy

Immunostaining was performed as previously reported (Kim et al., 2019). After washing three times with PBS/PVA, oocytes were fixed in 3.7% paraformaldehyde at room temperature for 30 min, permeabilized with 0.5% Triton X-100 at room temperature for 30 min and incubated in 1.0% BSA at room temperature for 1 h. These oocytes were then incubated overnight at 4°C with either anti-Caspase 3, anti-GSNOR or anti-TOM20 antibodies, diluted in blocking solution. After washing three times with PBS/PVA, the oocytes were incubated at room temperature for 1 h with Alexa Fluor 488™ Donkey anti-Mouse IgG (H + L) (1:200; Cat # A21202, Invitrogen), or Alexa Fluor 546™ Donkey anti-Rabbit IgG (H + L) (1:200; Cat # A10040, Invitrogen). After this, the oocytes were stained with 10 μg/mL Hoechst 33342 for 10 min, washed three times with PBS/PVA, mounted on slides, and examined under a confocal microscope (Zeiss LSM 710 META). Images were processed using Zen software (version 8.0, Zeiss).

Analysis of MtDNA copy number

Each pool of 10 oocytes was transferred to a 0.2 mL tube containing 8 μL lysis buffer (20 mM TrisCl, 0.4 mg/mL proteinase K, 0.9% NP-40, and 0.9% Tween 20) and heated for 30 min at 65°C. Samples were diluted 1:25 in sterile ddH$_2$O before analysis. Subsequently, real-time quantitative PCR was performed by using WizPure™ qPCR Master (Super Green) mix (Cat # W1731-8, Wizbiosolution, Seongnam, South Korea) (Jeon et al., 2019; Lee et al., 2019a; Lee et al., 2019b). Amplification was conducted as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 25 s, and 72°C for 10 s, with a final extension at 72°C for 5 min. The target genes were ND1 (Forward primer: GAACCACTACGACCCGTAC, Reverse primer: ACGGCTAGGCTTGATATGGC). Relative quantification method was used for calculation of mtDNA copy number.

ATP measurements

ATP content was measured using luciferin–luciferase ATP assay in luminometer (CentroPRO LB 962; Berthold, ND, USA) according to the instructions of the manufacturer of the ATP determination kit (A22066, Molecular Probes). Briefly, 10 oocytes were collected in a 0.2 mL centrifuge tube containing 20 μL of lysis buffer (20 mM TrisCl, 0.9% Nonidet-40, and 0.9% Tween 20), and were homogenized by vortexing until they were completely lysed. Standard reaction solution was prepared according to the manufacturer’s instructions and was placed on ice in the dark before use. Before measurement, samples (5 μL) were added in 96-well plates and equilibrated for 10 s. Subsequently, 200 μL standard reaction solution was added into each well and the light signal was integrated for 10 s after a delay of 2 s. The light intensity in the control group was arbitrarily set as 1, and that in the treatment group was measured and expressed as relative values with respect to the control group.
Statistical analysis

Each experiment was repeated at least three times, and representative images are shown in the figures. The change of protein NO level, GSNOR expression, active mitochondria, and APT level during oocyte aging at 0, 24, and 48 h, as well as caspase 3 expression and ROS production in Fresh, Aging 24 h, and Aging 24 h + SNP were subjected to the multivariate analysis of variance (ANOVA), and differences among treatments were examined using the Duncan multiple range test. Other data were subjected to the Student’s t-test. All percentage data were subjected to arcsine transformation prior to statistical analysis and then presented as mean ± SEM. Significance was set at p < 0.05. All calculations were performed using SPSS software v.19 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Protein SNO level and GSNOR expression increases during oocyte aging

Status of the protein SNO was determined using the Biotin-switch assay. As shown in Fig. 1, the protein SNO level had significantly increased during 24 and 48 h aging of oocytes as compared to fresh oocytes, while the expression of GSNOR, the main denitrosylation enzyme was not changed after 24 h of aging. However, the GSNOR expression level increased significantly after 48 h of aging (Fig. 1B). Thus, markers of both nitrosylation as well as denitrosylation were upregulated in 48 h of oocyte aging.
Mitochondrial activity and function decrease during oocyte aging

To understand the relationship of mitochondrial function with the protein SNO level, active mitochondria were stained with the MitoTracker Red CMXRos at 0, 24, and 48 h of aging. The results showed that the fluorescence intensity of MitoTracker Red CMXRos decreased after 24 and 48 h of aging (Fig. 2A and B). Mitochondrial function was measured in terms of amount of ATP with luciferin-luciferase ATP assay. As shown in Fig. 2C, ATP level decreased after 24 and 48 h of aging, suggesting that the mitochondrial function was impaired with oocyte aging.

Treatment with SNP increases protein SNO level and leads to accumulation of damaged mitochondria

As a NO donor, SNP increases the concentration of NO. Treatment with SNP for 24 h significantly promoted protein SNO production compared to control oocytes (Fig. 3A and B). This served as a model for oocytes with high NO. As reported previously, total and active mitochondria can be differentially labeled with TOM20 and MitoTracker Red CMXRos (Pendergrass et al., 2004). These labelling experiments showed that the TOM20 signal in SNP-treated oocytes was stronger than that in control, but the MitoTracker Red CMXRos signal in SNP-treated oocytes was weaker than that in controls (Fig. 3C). The fluorescence intensity of TOM20 significantly increased in treated oocytes compared to controls, suggesting the total number of mitochondria increased due to exposure to SNP (Fig. 3D). However, decreased intensity of fluorescence with MitoTracker Red CMXRos in treated oocytes (Fig. 3E), suggested that the number active mitochondria decreased due to SNP-treatment. In addition, the ratio between the fluorescence intensity of MitoTracker Red CMXRos and Hoechst/MitoTracker Red CMXRos

Fig. 2. Mitochondrial activity and function decrease during oocyte aging. Images (A) and fluorescence intensity of MitoTracker Red CMXRos (B) in fresh oocytes (n = 13), and those aged for 24 h (n = 13), and 48 h (n = 11). (C) ATP level in the fresh oocytes, and oocytes aged for 24 h and 48 h. Different letters indicate significant difference, p < 0.05.
Fig. 3. Treatment with SNP increases protein SNO level and causes accumulation of damaged mitochondria. Images (A) and protein SNO level (B) in the oocytes aged for 24 h (n = 14) and aged for 24 h+ treated with SNP (n = 11). (C) Colocalization of TOM20 and MitoTracker Red CMXRos in the oocytes aged for 24 h (n = 13) and aged 24 h+ treated with SNP (n = 20). Fluorescence intensities of TOM20 (D), MitoTracker Red CMXRos (E), and intensity ratio of MitoTracker Red CMXRos/TOM20 (F) in the oocytes aged for 24 h (n = 13) and aged for 24 h+ treated with SNP (n = 20) oocytes. (G) MtDNA copy number in oocytes aged for 24 h and aged for 24 h+ treated with SNP. (H) ATP levels in oocytes aged for 24 h and aged for 24 h+ treated with SNP. *p < 0.05, **p < 0.01, ***p < 0.001 versus Aging 24 h.
and TOM20 was significantly less after SNP-treatment (Fig. 3F). All of these results together suggested that even though the total mitochondrial contents increased due to exposure of oocytes to NO, the proportion of active mitochondria decreased, leading to an accumulation of damaged mitochondria. Mitochondrial DNA copy number as seen on real-time quantitative PCR also showed that SNP-treated oocytes contained more mitochondria after aging for 24 h in vitro (Fig. 3G). However, the ATP level, estimated using luciferin–luciferase ATP assay system, was reduced in SNP-treated oocytes, indicating a drop in the number of active mitochondria. Taken together, these results imply that increasing NO level caused accumulation of damaged and dysfunctional mitochondria, and reduction of active mitochondria.

Treatment with SNP increases ROS production and apoptosis level

To further investigate the effects of increase in NO level on oocyte aging, extent of ROS production and apoptosis were studied. ROS production was determined with H$_2$DCF-DA staining. As shown in Fig. 4A and B, ROS level increased drastically after 24 h of aging in vitro. Moreover, treatment with SNP further increased the ROS production. Apoptosis was estimated by staining with anti-Caspase-3 antibody. Although aging oocytes for 24 h alone did not change the level of apoptosis, treatment with SNP significantly increased it, compared to fresh and control oocytes. This suggested that aging for 24 h may not be enough to cause apoptosis, but further increase in NO level promoted apoptosis.

![Fig. 4. Treatment with SNP increases ROS production and apoptosis level. Images (A) and ROS production (B) in the fresh oocytes (n = 10), and those aged for 24 h (n = 10), and aged for 24 h+ treated with SNP (n = 10). Images (C) and apoptosis level (D) in the fresh oocytes (n = 10), those aged for 24 h (n = 11), and aged for 24 h+ treated with SNP (n = 10). Different letters indicate significant difference, p < 0.05.](image-url)
DISCUSSION

Poor quality of oocytes, resulting from environmental pollution and advanced maternal age, has led to failure in conception, requiring assisted reproduction technology (ART) to help to conceive. Since 1978, many children have been born through ART. However, the efficiency of ART needs to be improved for its wider use. One of the main reasons for failure of ART is because of the extra time needed for the in vitro operation, which increases the time the mature oocytes are held in vitro, leading to oocyte aging. If the aging of the oocyte can be prevented, or at least, slowed down, fertilizable period of the oocyte can be extended. Previous studies have shown that the addition of antioxidants can reduce ROS levels, thereby delay the oocyte aging to some extent (Liu et al., 2012; Lord and Aitken, 2013; Li and Cui, 2016; Wang et al., 2017; Liang et al., 2018; Wang et al., 2019; Zhang et al., 2019), but this was not found to be sufficiently effective for clinical use. During the oocyte aging, the redox balance is disrupted, and the oxidative pressure triggers a series of events that ultimately reduce the quality of oocytes. It also induces mitochondrial dysfunction, leading to apoptosis (Tatone et al., 2011; Zhang et al., 2011; Lord et al., 2013), and oxidative damage to the cell (Guérin et al., 2001). Consequence of all these events are reflected in the lower rate of fertilization and poor quality of embryo, increasing the likelihood of abnormalities in the resultant offspring.

A previous study had reported that nitric oxide (NO) signals mediate the aging of oocytes (Premkumar and Chaube, 2015). However, very limited research has addressed the effects of NO-related redox signaling on oocyte quality during oocyte aging. To investigate the role of NO signals in oocyte aging, we studied several aspects of normal oocytes, including mitochondrial functions and contents, ROS production, and apoptosis. The results indicated a close correlation between NO signals and oocyte aging. Treatment with SNP significantly increased the protein SNO level, oxidative stress and mitochondrial dysfunction, accumulation of damaged mitochondria, and apoptosis.

NO-related redox signaling induces s-nitrosylation of proteins, affects morphology and function of mitochondria, increases the release of stored calcium from the endoplasmic reticulum, and affects autophagy and apoptosis (Sun et al., 2007). Mitochondria are essential for the normal development of oocytes, and mitochondrial quality control is an important protective step to ensure the steady state of mitochondria. Normally, damaged mitochondria are cleared by mitophagy, ensuring that only healthy mitochondria are present in the cells. In addition, mitochondrial biogenesis supports this process. However, when the quality control of mitochondria is weakened, damaged mitochondria start accumulating. In this study, after 24 h aging, SNP-treated oocytes contained more mitochondria, but the number of active mitochondria significantly reduced, indicating that the proportion of inactive mitochondria was increasing in these oocytes. In these oocytes, the damaged mitochondria were not cleared probably because their mitochondrial quality control system was faulty. The main reason for this effect may be due to S-nitrosylation of PINK1 and Parkin, components of the mitochondrial quality control system, which impairs their function and inhibits mitophagy. Due to the limited number of oocytes, it is difficult to determine the S-nitrosylation status of PINK1 and Parkin, but the increase in the number of damaged mitochondria is clearly seen.

The conclusion of this study is that during the oocyte aging process, the NO level increased, and it affected the quality control system of the mitochondria, particularly the mitophagy, resulting in the accumulation of damaged mitochondria, and production of ROS. Our results provide clues for new ways to delay oocyte aging. While preventing oxidative stress in oocytes, ensuring the steady state of NO signals will ultimately increase the efficiency of ART by delaying oocyte aging.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Xiang-Shun Cui, Ying-Jie Niu designed the experiment. Ying-Jie Niu conducted the experiments, analyzed the results, and wrote the article. Dongjie Zhou, Wenjun Zhou, Zheng-Wen Nie and Ju-Yeon Kim helped with the analyses of the results and figures. YoungJin Oh and So-Rim Lee conducted some of the experiments. Xiang-Shun Cui assisted in the analyses of the results and revised the manuscript.

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