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ORIGINAL ARTICLE

Role of Klotho and N-acetylcysteine in Oxidative Stress Associated with the Vitrification of Ovarian Tissue Cytoprotective Function of Klotho in Cryopreservation

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Abstract

BACKGROUND: Cryopreservation can cause mechanical and chemical stress, ultimately leading to the formation of reactive oxygen species (ROS) and oxidative stress. ROS inhibits the expression of antioxidant enzymes in cells, resulting in increased DNA fragmentation and apoptosis. In this paper, we used a vitrification method that has the advantage of producing less ice crystal formation, cost-effectiveness, and time efficiency during cryopreservation. The objective of this paper is to evaluate the degree of protection of ovarian tissue against oxidative stress when N-acetylcysteine (NAC) and Klotho proteins are treated in the vitrification process of ovarian tissue.

METHODS: The control group and the cryopreservation groups were randomly assigned, and treated NAC, Klotho, or the combination (NAC + Klotho). The cell morphological change, DNA damage, senescence, and apoptosis of each group after the freeze–thaw process were compared using transmission electron microscopy, immunohistochemistry, and western blot analysis.

RESULTS: Both NAC and Klotho were found to be more effective at protecting against DNA damage than the control; however, DNA damage was greater in the NAC + Klotho group than in the group treated with NAC and Klotho, respectively. DNA damage and cellular senescence were also reduced during the vitrification process when cells were treated with NAC, Klotho, or the combination (NAC + Klotho). NAC increased apoptosis during cryopreservation, whereas Klotho inhibited apoptosis and NAC-induced apoptosis.

CONCLUSION: This study highlights Klotho's benefits in inhibiting DNA damage, cell senescence, and apoptosis, including NAC-induced apoptosis, despite its unclear role in vitrification.

Keywords Klotho · N-acetylcysteine · Vitrification · Oxidative stress · Ovarian tissue

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1 Introduction

In the United States, 89,000 women between the ages 15 and 39 are diagnosed with cancer each year, accounting for 5% of the total cancer prevalence. This number cannot be ignored. Further, cancer treatment should be performed with sufficient consultation and discussion regarding the preservation of fertility [1]. According to the World Health Organization (WHO), 48 million couples and 186 million individuals are diagnosed with infertility worldwide, an important problem that must be overcome in modern society with low fertility rates [2]. Owing to this social trend, the need and importance of cryopreservation of ovarian tissue are emerging.

Ovarian tissue cryopreservation can be performed using slow-freezing or vitrification. Slow freezing involves freezing ovarian tissue by adjusting the cooling rate using a programmed freezer; however, this method is time-consuming and expensive. In contrast, vitrification involves rapid freezing of the ovarian tissue using a supercooling liquid. Compared to the slow freezing method, vitrification is more important cryopreservation method due to its reduced cell damage by preventing ice crystal formation in the cell. In addition, vitrification offers cost-effectiveness and time efficiency compared to slow freezing [3]. These benefits have contributed to the growing trend of utilizing vitrification in cryopreservation application. In this study, considering the aforementioned benefits of vitrification, we employed this method for cryopreservation purposes. Cryopreservation itself can cause mechanical and chemical stress, resulting in damage to the phospholipid membrane of the ovarian cell, morphological changes, and mitochondrial damage, ultimately leading to the formation of reactive oxygen species (ROS) and oxidative stress. ROS inhibits the expression of antioxidant enzymes produced in cells, resulting in increased DNA fragmentation and apoptosis [4]. Identifying substances that can reduce oxidative stress-induced damage to the ovarian tissue has garnered remarkable interest in recent years. N-acetylcysteine (NAC) is the precursor of L-cysteine. When NAC is combined with glycine, glutathione, which functions as an antioxidant that balances cell redox processes, immune responses, and inflammatory reactions within cells, is produced. NAC is well known as an antioxidant that not only functions as a free radical scavenger but also as a barrier against free oxygen stress by maintaining an appropriate concentration of glutathione, a metabolite [5]. Quadrilatero and Hoffman-Goetz injected NAC into the muscle cells of exhausted mice and confirmed that glutathione concentrations were maintained for a longer period, and mitochondrial destruction and apoptosis were suppressed. Such a finding suggests that NAC may have anti-apoptosis functions and antioxidant functions [5]. Klotho is a type of protein that plays a crucial role in various physiological processes within the human body. Klotho protein exists in two main forms: membrane-bound form and secreted form. Membrane-bound Klotho serves as a co-receptor for FGF23, regulating phosphate and vitamin D metabolism, which is vital for maintaining proper mineral balance. Secreted Klotho is a circulating hormone with pleiotropic effects on various organs, associated with anti-aging, antioxidative, anti-inflammatory, and anti-fibrotic properties [6]. A comparison of the life of Klotho-deficient mice and Klotho-overexpressing mice by Hiroshi Kurosu et al. revealed that the life of Klotho-overexpressing mice increased and aging rapidly progressed in Klotho-deficient mice. Since then, the Klotho protein is known as an anti-aging protein. Klotho exists in three forms and has different functions. Membrane Klotho interacts with FGF-23 to regulate the progression of aging and chronic disease, secreted Klotho controls oxidative stress and growth factor signaling, and intracellular Klotho protects organs and inhibits cell aging-related inflammatory reactions [7].

NAC is well known as a strong antioxidant and Klotho is an anti-aging protein. Previously, our team conducted a study evaluating the efficacy of NAC and Klotho proteins in ovarian tissue cryopreservation using the slow freezing method, and their results demonstrated that Klotho proteins are effective antioxidants. However, the efficacy of NAC and Klotho proteins in vitrification is still unclear. Hence, we evaluated the degree of protection of the ovarian tissue against oxidative stress by cryopreservation with vitrification following treatment with NAC and Klotho.

2 Results

2.1 Effect of the antioxidants on morphology after vitrification

After four weeks of cryopreservation, mouse ovarian tissue was thawed according to the specified protocol. Hematoxylin and eosin (H&E) staining confirmed the status of the frozen and thawed mouse ovarian tissue (Fig. 1, top). Transmission electron microscopy (TEM) was performed to confirm the primordial follicle morphology (Fig. 1, bottom). TEM revealed that the basal layer structure and mitochondria of primordial follicles were mainly destroyed upon vitrification (Fig. 1, bottom). Vitrification can effectively preserve isolated cells as the cryoprotectant formulation is very viscous, enabling relatively easy injection and removal of the cryoprotectant. However, for tissues, rapid absorption and removal of the highly viscous cryoprotectant are difficult to achieve. Thus, tissue vitrification results in a volume change associated with tissue

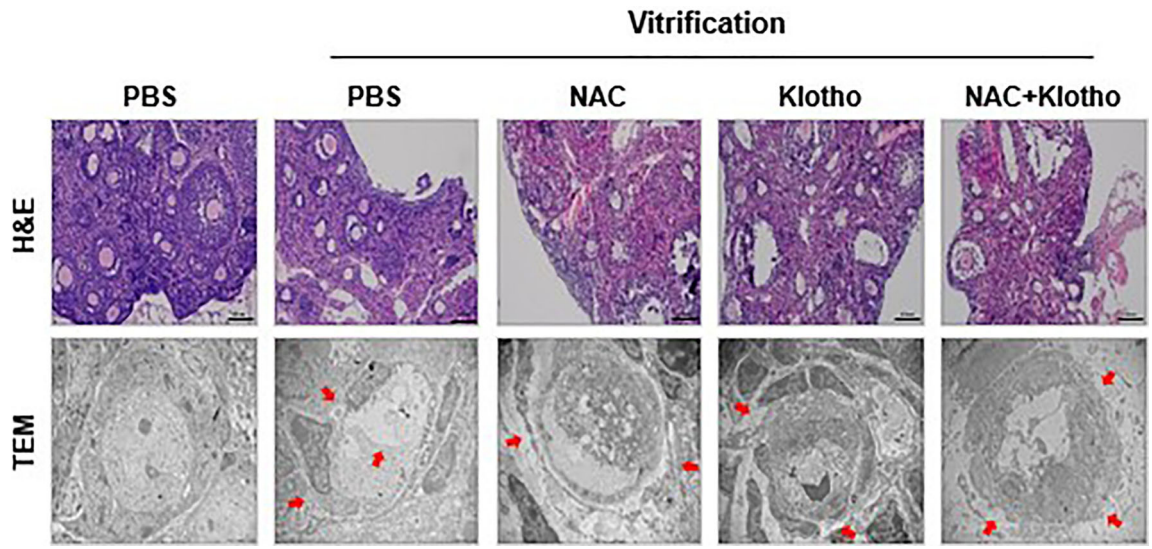


Fig. 1 Effect of Klotho and NAC on mouse primordial follicle morphology after vitrification (top) H&E staining was performed to determine the morphological changes in mouse ovarian tissues after vitrification $n = 10$ per group (bottom). Electron micrograph showing the mitochondria in mouse primordial follicles (5000 × magnification). $n = 2$ per group. The damaged part of the TEM is marked with a red arrow

expansion or contraction during cooling. As the cooling rate increases, the potential risk of tissue cracking and structural damage increases [9]. Such findings suggest that Klotho and NAC did not have a positive effect on the extrinsic damage caused by vitrification.

2.2 Effects of antioxidants on DNA damage inhibition

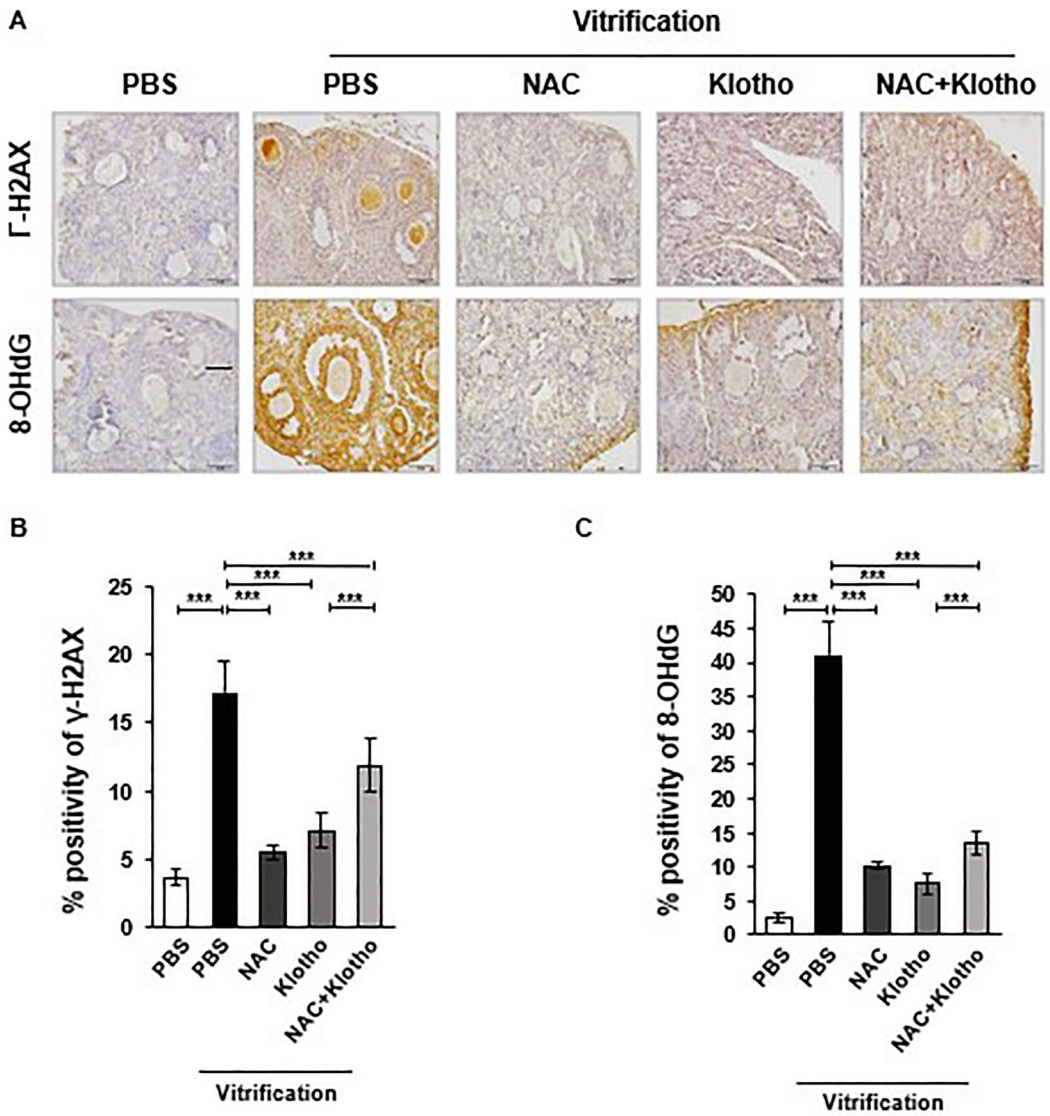
To determine whether vitrification-induced DNA damage is mediated by oxidative stress in ovarian tissue, γ-H2AX, a DNA double-strand break (DSB) marker, and 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker for oxidative stress, were analyzed using immunohistochemistry (IHC). Vitrified mouse ovarian tissue had significantly increased levels of γ-H2AX and 8-OHdG compared to the control ovarian tissue. In contrast, the groups treated with Klotho, NAC, or the combination (Klotho + NAC) showed significantly decreased vitrification-induced γ-H2AX and 8-OHdG (Fig. 2A-C). Vitrification-induced γ-H2AX was significantly decreased by 67, 58, and 31% in the NAC-, Klotho-, and combination-treated groups, respectively (Fig. 2A top, B). Furthermore, the increased 8-OHdG in vitrified mouse tissues was inhibited by 75, 81, and 67% owing to treatment with NAC, Klotho, and the combination, respectively (Fig. 2A bottom, C). Accordingly, the remarkable increase in DNA damage and oxidative stress during ovarian tissue vitrification can be regulated by treatment with Klotho and NAC.

2.3 Effects of Klotho on aging induced by cryopreservation

To determine the effects of Klotho and NAC on aging induced by cryopreservation, p16INK4a, a biological senescence marker, was examined using IHC (Fig. 3). p16INK4a levels were the highest in vitrified control tissues and were significantly reduced in groups treated with Klotho, NAC, or the combination (Fig. 3A, B). Compared with p16INKa in tissues vitrified without antioxidants, a significant reduction of 52, 78, and 59% was observed in the NAC-, Klotho-, and combination-treated groups, respectively (Fig. 3B). Unlike the slow freezing group, the NAC-treated group displayed suppressed aging during vitrification. Comprehensive data also revealed that Klotho could prevent cryopreservation-induced senescence in ovarian tissues.

2.4 Klotho reduces the apoptosis induced by oxidative stress

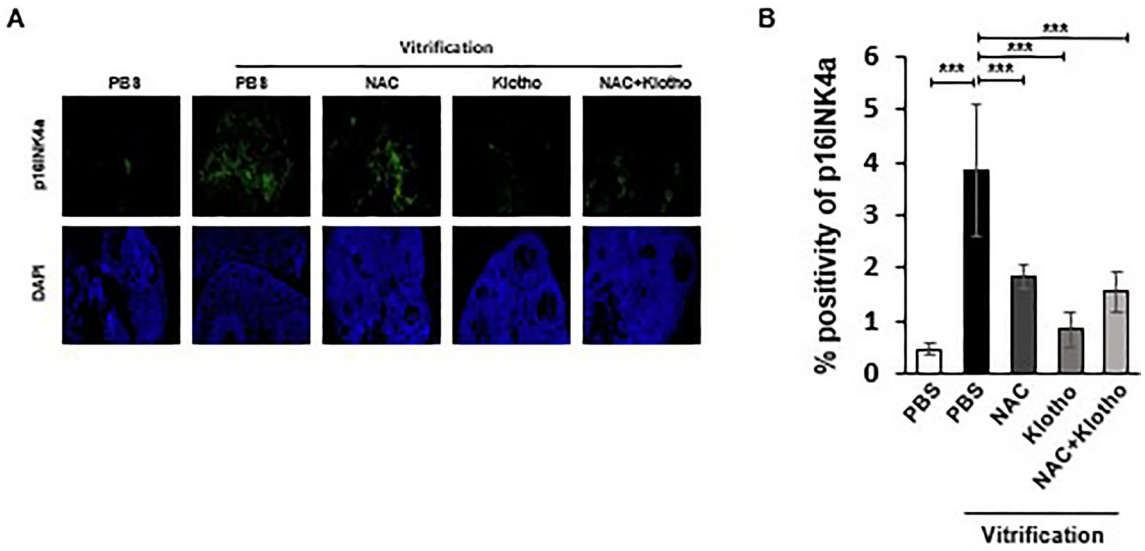
To determine the effects of Klotho and NAC on apoptosis during vitrification, immunoblotting analysis of apoptosis-associated proteins was performed. NAC was found to induce apoptosis of the ovarian tissue (Fig. 4A). The relative expression levels of apoptosis-mediated proteins, BAX and cytochrome C (Cyt C) in each group are presented in greater detail in Fig. 4B and 4C. As can be seen from the results in Figure 4B and 4C, treatment with NAC leads to an increase in the expression of apoptosis-mediated proteins BAX and Cyt C, suggesting the induction of



	Control-PBS	Vitrification-PBS	NAC	Klotho	NAC +Klotho
B. % positivity of γ-H2AX (%) M±SD	3.66±1.42	17.1±5.03	5.52±1.14	7.09±2.92	11.8±4.35

	Control-PBS	Vitrification-PBS	NAC	Klotho	NAC +Klotho
C. % positivity of 8-OHdG (%) M±SD	2.54±1.69	41.0±10.80	10.2±0.89	7.52±3.19	13.4±3.78

Fig. 2 Klotho and NAC reduce the increased DNA damage induced by vitrification. **A** Representative IHC images at 400 × magnification showing double-stranded breaks (DSBs) in the indicated conditions. Brown indicates DSBs-positive in the ovarian tissue (top). Representative IHC images at 400 × magnification showing 8-hydroxy-20-deoxyguanosine (8-OHdG) under the indicated conditions. Brown staining indicates 8-OHdG-positive regions in the ovarian tissue (bottom). **B** Graph of the average percentage of DSBs in the IHC images. **C** Graph of the average percentage of 8-OHdG in the IHC images. All data are presented as mean ± SD. The p values were determined by one-way analysis of variance (ANOVA). * $p < 0.05$; *** $p < 0.0001$. $n = 6$ per group. Each experiment was repeated three times



	Control-PBS	Vitrification-PBS	NAC	Klotho	NAC +Klotho
B. % positivity of P16INK4a (%) M±SD	0.46±0.25	3.84±2.83	1.82±0.51	0.85±0.74	1.57±0.84

Fig. 3 Klotho protects ovarian tissue from NAC-induced senescence. **A** Representative IF images of the ovarian tissues with p16INK4a at 400 × magnification. The positive area for these receptors is colored green. **B** Percentage of p16INK4a-positive cells. All data are presented as mean ± SD. The p values were determined using one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. $n = 6$ per group. Each experiment was repeated three times

apoptosis. In contrast, treatment with Klotho dose not show any significant changes in the expression of BAX and Cyt C. Interestingly, in the group treated with both NAC and Klotho, the expression of Cyt C, one of the apoptosis-mediated proteins, is reduced. This observation suggests that Klotho may exhibit anti-apoptosis agent functions (Fig. 4B, C). Furthermore, the expression of GADD45A, a growth arrest, and DNA damage-inducible protein, was significantly higher in NAC-treated ovarian tissues than in vitrified-frozen control tissues. Conversely, the group treated with Klotho showed significantly reduced NAC-induced GADD45A expression (Fig. 4D). These findings suggest that DNA damage and increased oxidative stress in ovarian tissues during vitrification could be regulated by treatment with Klotho.

3 Materials and methods

3.1 Animals and experimental design

All animal experiments were approved by the Korea University Institutional Animal Use Committee. The animals were housed in a pathogen-free state in a barrier facility (IACUC number KOREA2020-0136). All ovarian tissues were collected from 6-to 7-week-old BALB/c mice ($n = 30$) after sacrifice by carbon dioxide inhalation. Ovarian tissues were isolated from each group. Sixty ovarian tissue slices were randomly assigned to the control and cryopreservation groups and treated with PBS, NAC, Klotho, or the combination of NAC and Klotho. As previously mentioned, the term "PBS" denotes a group in which vitrification was conducted without any treatment involving NAC or Klotho. Ovarian tissue samples were cryopreserved and thawed in a cryopreservation solution containing the experimental reagent. The tissues were then evaluated for primordial follicle morphological changes, double-stranded DNA damage, senescence, and apoptosis after the freeze–thaw process.

3.2 Vitrification freeze–thaw protocol

Vitrification of mouse ovarian tissue was performed according to the vitrification freeze–thaw protocol used in previous studies [8]. Tissues from both ovaries of mice were frozen as whole ovaries, without fragmentation. Mouse ovarian tissue was incubated in an equilibrated solution (ES) comprising 65 mL HEPES (Gibco, Taiwan), 20 mL Human Serum Albumin (HSA), 7.5 mL ethylene glycol, and 7.5 mL Dimethyl sulfoxide (DMSO) for

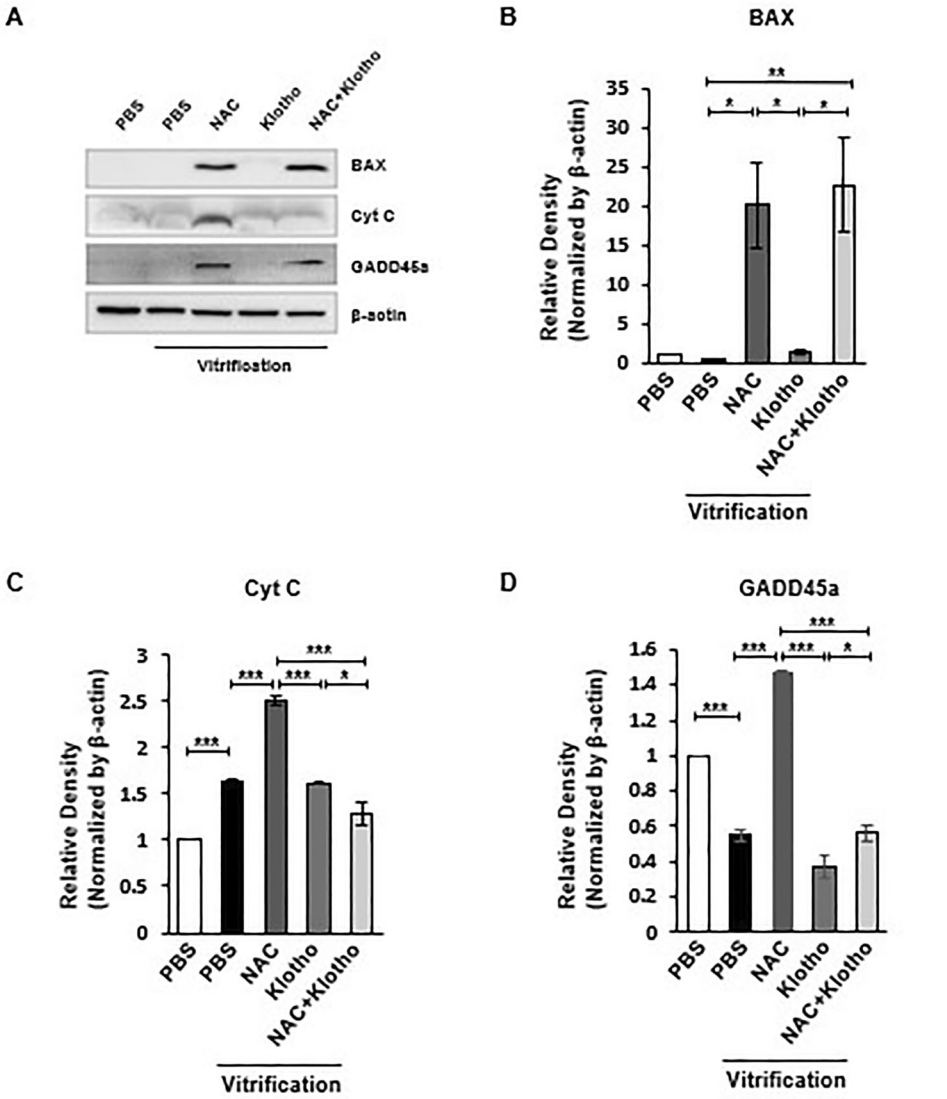


Fig. 4 Klotho reduces oxidative stress-mediated cell death caused by cryopreservation. **A** Expression of the apoptosis-related proteins, including the pro-apoptotic protein, Bax and Cyt C, was detected by immunoblotting. **B**-**D** Graphs of the fold-change value from the control after quantification based on the western blot analysis results. All data are presented as mean ± SD. The p values were determined using one-way ANOVA. ** $p < 0.01$; *** $p < 0.001$. $n = 6$ per group. Each experiment was repeated three times

	Vitrification-PBS	NAC	Klotho	NAC +Klotho
B. relative Density of BAX (%) M±SD	0.46±0.04	20.0±5.29	1.36±0.35	22.6±6.07
	Vitrification-PBS	**NAC**	**Klotho**	**NAC +Klotho**
C. relative Density of Cyt C (%) M±SD	1.63±0.03	2.50±0.05	1.62±0.01	1.29±0.12
	Vitrification-PBS	**NAC**	**Klotho**	**NAC +Klotho**
D. relative Density of GADD45a (%) M±SD	0.54±0.03	1.47±0.01	0.36±0.06	0.56±0.04

25 min at room temperature (25 ± 2 ° C). After 25 min, the ES was removed using sterile gauze, and each sample was placed in HEPES buffer with 20% ethylene glycol, 20% DMSO, 20% HSA, and 0.5 M sucrose (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 15 min at room temperature. After incubation in vitrification solution (VS), the excess VS was removed by placing the tissue on sterile gauze. The tissues were individually placed in vials and rapidly plunged into fresh liquid nitrogen. Frozen tissues were stored in liquid nitrogen for four weeks. To thaw the tissues, the stored cryotube vials were transferred to an equal volume of wash solution containing 5% DMSO, 20% HSA in HEPES buffer. in a basic medium. The vials were incubated at room temperature for 10 min. After incubation, half of the supernatant was removed and replaced with an equal volume of washing solution, followed by incubation at room temperature for 5 min. NAC, Klotho, and the combination of NAC and Klotho were added to 1 ml media in the tubes at each vitrification step.

3.3 Transmission electron microscopy (TEM)

Mouse ovarian tissues were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4 °C overnight. The samples were then fixed with 1% osmium tetroxide, dehydrated, and embedded in an Epon mixture (Polybed 812 embedding kit/DMP-30, Polysciences, Inc., Warrington, FL, USA). Tissue Sects. (1 μm thick) were obtained using a Reichert-Jung Ultracut E ultramicrotome (Leica, Wetzlar, Germany) and stained with toluidine blue. Sections 60-nm thick were collected on copper grids and stained with uranyl acetate/lead citrate. The morphologies of the sections were then analyzed using a TEM H-7500 system (Hitachi, Tokyo, Japan) at 80 kV.

3.4 Immunohistochemistry (IHC)

Ovarian tissue samples from each group were fixed in 4% formaldehyde. The fixed samples were dehydrated in a step-by-step manner using ethanol (70–100%) and xylene. After paraffin embedding, sample slices (3 μm thick) were stained with H&E. Unstained and paraffin-embedded ovarian tissue slides were deparaffinized with xylene and rehydrated using graded ethanol. For antigen retrieval, slides were treated with Tris–EDTA (pH 9.0; Thermo Scientific) for 30 min at room temperature. The samples were blocked with a hydrogen peroxide solution (Cat# 88597, Sigma-Aldrich) for 10 min. Staining was performed using a Polink-2 Plus HRP Broad Kit (Cat#: D41-18, GBI Labs, Bothell, WA, USA) with DAB (3,3′-diaminobenzidine), according to the manufacturer's protocol. The slides were incubated overnight at 4 °C in a humid chamber with the following primary antibodies: γ-H2AX (1:500 dilution; Bethyl Laboratories, Montgomery, AL, USA), 8-OHdG (1:500, Cat# sc-66036, Santa Cruz, Dallas, TX, USA), and CDKN2A/p16INK4a (1:100, Cat# EPR20418, Abcam, Cambridge, MA, USA) in PBS containing 2% BSA. The slides were then incubated for 1 h with the following secondary antibodies: goat anti-rabbit IgG (H + L), Alexa Fluor 488 (1:1000, Invitrogen, Waltham, MA, USA), goat anti-rabbit IgG (H + L), and Alexa Fluor 594 conjugated (1:1000, Invitrogen). Counterstaining was performed using Mayer's hematoxylin (Cat# HMM125, Scytek, West Logan, WV, USA) and an antifade mounting medium containing DAPI (Cat# H-1200, Vector Laboratories, Burlingame, CA, USA). The stained samples were examined under a microscope (Olympus-BX53, Tokyo, Japan) at 400 × magnification.

3.5 Western blot analysis

Total protein (20 μg) was subjected to 8–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and membrane transfer. The membranes were incubated overnight at 4 °C with the following primary antibodies: β-actin (1:1000, Cat# 4970, Cell Signaling Technology), anti-cytochrome C (1:1000, Cat# AB13575, Abcam), anti-BAX (1:1000, Cat# ab32503, Abcam), and GADD45a (1:1000, Cat# 4632, Cell Signaling Technology, Danvers, MA, USA), followed by these secondary antibodies for 1 h: goat anti-rabbit (1:5000 dilution, Cat# ab6721, Abcam) and goat anti-mouse (1:5000, Cat# ab6789, Abcam). Protein expression was quantified using the BIO-RAD software. Western blotting was performed in triplicate.

3.6 Statistical analysis

The results from IHC and immunofluorescence (IF) staining were quantified using ImageJ software. Data are expressed as the mean of three independent experiments (± standard deviation) and are presented as tables and bars. Differences among groups were analyzed by one-way analysis of variance (ANOVA), and a p-value < 0.05 was considered statistically significant.

4 Discussion

In a four-week vitrification study, we assessed control, NAC, Klotho, and NAC + Klotho groups. The NAC and Klotho combination provided better protection against DNA damage and reduced cellular senescence more effectively than each treatment alone. Additionally, Klotho mitigated the apoptosis increase caused by NAC.

The cryopreservation methods are divided into slow freezing and vitrification. Slow freezing, which uses a freezer to control the freezing rate, is currently widely used as a freezing method for ovarian tissues. Several papers comparing the results of slow freezing have been published since the implementation of the two methods [3, 8]. Lee et al. performed xenotransplantation of frozen human ovarian tissue into immunodeficient mice using slow freezing and vitrification methods and compared the outcomes of oocytes. These researchers found more DNA damage and cellular apoptosis in vitrified ovarian tissue than in tissue subjected to slow-freezing [8]. However, when the freezing rate is slowed, ice crystals are formed in the space outside the cell, causing cell damage and creating an osmotic pressure difference between the intracellular and extracellular fluids, leading to cell dehydration and shrinkage. Thus, determining the optimal freezing rate is crucial. Vitrification involves freezing ovarian tissue at an ultrafast cooling rate, producing fewer ice crystals and less cell damage compared to slow freezing. Nevertheless, if the freezing rate isn't fast enough, ice crystal formation can still cause cell damage [3]. The absence of a standard vitrification protocol complicates consistent process control, and various environmental and laboratory factors may affect outcomes. Despite being less commercialized, vitrification's simplicity and cost-effectiveness enable widespread use, making it the chosen method for freezing ovarian tissue in this study.

As mentioned earlier, to address issues arising during the cryopreservation process, such as ice crystal formation, osmotic pressure differences, and ROS generation, CPAs, antioxidants, anti-apoptosis agents, and anti-senescence agents have gained attention, Their roles are briefly illustrated in Fig. 5. CPAs prevent issues like ice crystal formation and cell damage due to osmotic pressure differences. They are classified into permeable CPAs, such as DMSP and glycerol, which correct intracellular osmotic pressure [10, 11], and impermeable CPAs like antifreeze proteins that hinder ice crystal formation [12]. However, CPAs can be toxic at high concentrations, so appropriate amounts must be used when freezing ovarian tissue. Freezing causes oxidative stress, ROS that lead to cell damage, DNA damage, and dysfunction. Protecting frozen ovarian tissue from oxidative stress is crucial. Although CPAs can't protect against oxidative stress, antioxidants like glutathione, ascorbic acid, and salidroside can. Treating tissue with antioxidants before freezing reduces ROS, improves sperm motility, and increases pregnancy rate in frozen ovaries [13, 14]. Toker et al. used 1 mg of 0.67% thiabarbituric acid as antioxidants, which led to increased sperm motility as malondialdehyde (MDA) levels rose in sperm cells [13]. Ren et al. used 1.0mg/mL lycopene and 5μg/mL alpha-lipoic acid as antioxidants in goat sperm, resulting in improved motility and pregnancy rate for frozen ovaries. Lycopene is lipid-soluble, while alpha-lipoic acid is both water and lipid-soluble, addressing oxidative damage not covered by lycopene. However, alpha-lipoic acid's effectiveness varies across species, limiting its broader application despite its antioxidant benefits in goats [14]. In contrast, several studies have reported that the application of high dose of antioxidants can result in a decrease in sperm motility [15, 16]. Khor et al.'s study found that using 50mg/L or more of ascorbic acid during tissue freezing disrupted the ROS-antioxidant enzyme balance, inhibiting superoxide dismutase and catalase activity, and causing oxidative damage [15]. Banihani et al.

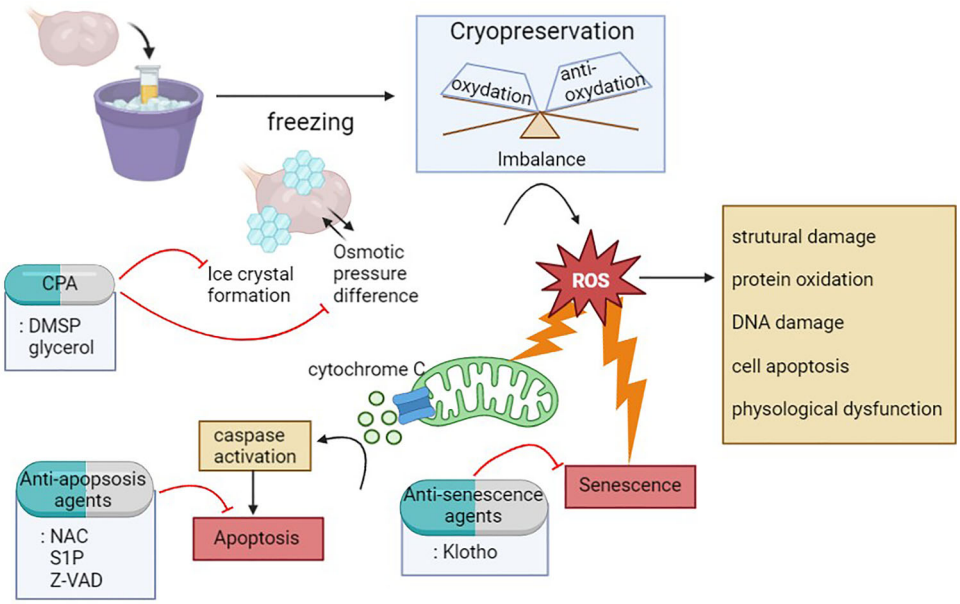


Fig. 5 The schematic representation of potential issues during cryopreservation and the helpful roles of CPAs, anti-apoptosis agents, and anti-senescence agents in there improvement

showed that applying 150mM or more of ascorbic acid, 20mM or more of vitamin E, and 50mM or more of L-carnitine during cryopreservation resulted in a ROS-antioxidant imbalance, leading to cell damage and death from oxidative stress [16]. These findings demonstrate the need for further research not only to identify effective antioxidant agents during the freezing process but also to determine their optimal dosages. Numerous studies have demonstrated NAC's antioxidant efficacy against oxidative stress. Yang et al. reported NAC's effectiveness in reducing DNA damage in human lymphoid cells exposed to water-soluble cigarette smoke [17]. An in vitro study showed decreased lipid oxidation and toxicity in cancer cell lines treated with NAC [18]. Pollman et el. Found reduced vascular smoot muscle cell damage when treated with NAC after balloon angioplasty injury [19]. However, contrary to expectations, neither cell nor mitochondrial protection was observed in the Klotho-, NAC-, and combination-treated groups. To prevent cell damage, research has focused on controlling cooling rates during vitrification, with the most effective rate of 100 degrees per second, proposed by Luyet 80 years ago, to prevent ice crystal formation [20]. Apoptosis is divided into extrinsic and intrinsic (mitochondrial) pathways. The extrinsic pathway activates caspase 8 through Fas-associated death domain (FADD) binding upon Fas ligand binding to the cell surface apoptosis receptor. In the intrinsic pathway, stimuli like oxidative stress cause Bax protein relocation from the cytoplasm to the mitochondrial outer membrane, increasing permeability, releasing cytochrome C, and activating caspase 9. Activation of caspase 8 and 9 also triggers caspases 3, 6, and 7, leading to apoptosis [21]. To prevent apoptosis during cryopreservation, a study used anti-apoptotic agents like sphingosine-1-phophaste (S1P) and Z-VAD-FMK (Z-VAD) during ovarian tissue freezing and thawing. Apoptosis and DNA damage were significantly reduced [22]. NAC's effect on apoptosis has been widely studied [19, 23]. Pollman et al. reported inhibited apoptosis in a group with vascular balloon angioplasty injuries when NAC administered [19]. Erkkila et al.'s in vitro experiments showed inhibited apoptosis in human male germ cells treated with NAC [23]. However, in this study, apoptosis was increased by the vitrification of ovarian tissue treated with NAC. Another study indicated that NAC could induce apoptosis. Liu et al. found that apoptosis increased when NAC was administered and endoplasmic reticulum stress was applied to H9c2 cells [24]. The researchers used fluorescence assays with caspases to identify activated factors in the apoptosis cascade, confirming that caspase-8 in the extrinsic pathway was deactivated, while caspase-9 and caspase-3 were activated [24]. As the mechanisms of the apoptosis pathway due to oxidative changes remains unclear, further evaluations are necessary. During cryopreservation, cell senescence occurs. Kim et al. observed a significant reduction in telomere length and an increase in aging markers (p53, p16, p21) in ovarian tissue after slow freezing compared to before [25]. Due to increasing interest in Klotho and its role in cell senescence, research on Klotho protein function has been active. Xiao et al. examined the relationship between Klotho and human aging using serum. They found a significant decrease in Klotho with age, regardless of gender, and an association with human lifespan [26]. Additionally, studies have been published demonstrating Klotho's role in reducing the incidence of chronic diseases [27, 28]. In has been revealed that Klotho protein contributes to cell protection, cell aging prevention, and apoptosis prevention by inhibiting the release of inflammatory cytokines and suppressing ROS production [29–31]. Thus, we hypothesized that Klotho would contribute to achieving high-quality vitrification of ovarian tissues. In this study, despite cellular and mitochondrial damage observed in the Klotho-treated group, the protein exhibited inhibitory effects on cell senescence, apoptosis, and NAC-induced apoptosis. Further research is needed to understand the mechanism by which NAC causes apoptosis and how the interaction between NAC and Klotho results in cell damage.

Nevertheless, this study is significant as it is among the few to demonstrate that Klotho is an effective oxidative modulator in the cryopreservation of ovarian tissue through vitrification.

5 Conclusion

The groundbreaking functions of Klotho, including the inhibition of DNA damage, cell senescence, and apoptosis, as well as NAC-induced apoptosis, were confirmed in this study. By effectively utilizing and applying the advantages of Klotho and NAC to cryopreservation, it is possible to preserve fertility through improved ovary quality.

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Authors Contributions BK designed and performed the experiments. S-ML designed the study and analyzed data with analytic tools. S-JP designed and analyzed data. SL supervised the project, the main conceptual ideas and proof outline, and analyzed data. All authors wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical statement The study protocol was approved by the institutional review board of Korea University (Korea-2020-0136). Informed consent was confirmed by the IRB.

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