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TASK-2 Expression Levels are Increased in Mouse Cryopreserved Ovaries

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ABSTRACT

Cryopreservation affects osmotic tolerance and intracellular ion concentration through changes in expression levels of water and ion channels. Control of these changes is important for cell survival after cryopreservation. Relatively little is known about changes in K^+ channel expression compared to water channel expression. This study was performed to investigate changes in TASK-2 channel (KCNK5: potassium channel, subfamily K, member 5), a member of two-pore domain K^+ channel family, in cryopreserved mouse ovaries. Cryopreservation increased TASK-2 mRNA expression in mouse ovaries. In addition, TASK-2 protein expression was upregulated in vitrified and slowly frozen ovaries. TASK-2 protein was expressed in all area of granulosa cells that surround the oocyte within the follicle, except nucleus. Viability of cells overexpressed with TASK-2 was higher than that of vector-transfected cells. Our results found that TASK-2 expression was increased by cryopreservation and overexpression of TASK-2 decreased cryopreservation-induced cell death. These results suggest that TASK-2 upregulation might reduce cryodamage.

(Key words : cryopreservation, ovary, TASK-2 channel)

INTRODUCTION

Cryopreservation has been used as an effective method for preservation of mammalian cells and tissues (Wallace *et al.*, 2015; Shikanov *et al.*, 2011). A number of live young offsprings have been produced from the transfer of cryopreserved embryos or ovarian tissue. Cryopreservation is appearing as a strong approach to preserve fertility for human and animals that are losing ovarian function (Smith *et al.*, 2014). However, many challenges remain to produce successful cryopreservation in order to increase viability, number of offsprings, and ovarian function. In particular, there are many issues to be resolved in successful ovarian tissue cryopreservation.

Slow-freezing and vitrification are representative methods for the cryopreservation of embryos and ovarian tissues (Hasler, 2010). Intracellular ice formation, which is induced by water in the cells, is one of the lethal factors that reduce cell viability during cryopreservation (Rubinsky *et al.*, 1980). Permeability of the plasma membrane to water and cryoprotectants is crucial for cell survival during cryopreservation (Tan *et al.*, 2015). Cryopreservation affects osmotic balance and intracellular ion concentration (Blässe *et al.*, 2012). Water movement may change ion concentration in the presence of cryoprotectants, and control of water and ions transported across a cell membrane is an important concern for cell viability of cryopreserved cells. Water movement may be accomplished by K^+ channel as well as aquaporin channels when cells are exposed to cryoprotectant solution (CS).

Among K^+ channels, TASK-2 (KCNK5: potassium channel, subfamily K, member 5), a member of the two-pore domain K^+ channel (K_{2P}) family, is sensitive to changes in the osmotic potential of the extracellular solution and is involved in cell volume regulation (Niemeyer *et al.*, 2001: Barriere *et al.*, 2003). TASK-2 currents are increased by hypotonic cell swelling and decreased by cell shrinkage (Niemeyer *et al.*, 2001). Intracellular K^+ concentration is increased in cryopreserved bovine

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sperm, which expresses TASK-2 channel (Blässe *et al.*, 2012).

This study was performed to identify the role of TASK-2 channel and the changes in TASK-2 expression in cryopreserved mouse ovaries. Our results showed that cryopreservation increased TASK-2 expression in mouse ovaries, and that overexpression of Chinese hamster ovary (CHO) cells with TASK-2 increased cell viability.

MATERIALS AND METHODS

1. Chemicals

All components of the medium and other chemicals were tissue culture grade and were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise specified.

2. Animals and Ovary Isolation

Female mice (ICR strain, age 6 weeks) were purchased from Koatech Co. (Animal Breeding Center, Korea). Animal experiments were performed in accordance with the guidelines of the Gyeongsang National University Animal Care and Use Committee (GLA-090520-M0061). The animals were housed under a 12-h light/dark cycle in a pathogen-free area, with food and water freely available. To collect the ovaries, mice were sacrificed by cervical dislocation. Isolated ovaries were randomly assigned to three experimental groups: fresh control, slow-freezing, and vitrification.

3. Slow-Freezing

Ovaries were slowly frozen using a programmable freezer (Model CL 863, Biogenics Co., Napa, CA, USA). The CS was composed of 10% fetal bovine serum (FBS), 1.8 mol/l ethylene glycol, 5% (w/v) polyvinylpyrrolidone (PVP, MW 40,000), and 0.05 mol/l galactose prepared in Dulbecco's phosphate buffered saline (D-PBS). Ovaries were equilibrated in the CS for 30 min at 4℃ with slow shaking, and the ovaries were transferred into a 1.8 ml cryotube containing 1 ml CS. The tubes were cooled in a programmable freezer as following: cooled from 4℃ at −2℃/min to −7℃; seeded manually; hold for 10 min at −7℃; cooled at −0.3℃/min to −35℃ and −1℃/min to −85℃; immersed into liquid nitrogen (LN_2) and stored for future use.

4. Vitrification

D-PBS supplemented with 10% FBS, 40% (v/v) ethylene glycol, 18% (w/v) ficoll (MW 70,000), and 0.3 mol/l sucrose was used as CS for vitrification. Ovaries were first exposed to CS for 1 min at room temperature (20～24℃), then transferred into a 1.8 ml cryotube containing 1 ml CS. The cryotubes were then placed 1 to 2 cm above the surface of the LN_2 for 10 sec, plunged into LN_2 tank, and stored for future use.

5. Thawing and Dilution

After storage for 1～10 days, thawing was done by agitating the cryotube in 37℃ water after exposure for 10 second in air. The cryotube was dried with a paper towel, and the CS was removed from ovaries in one-step. The contents were expelled into a 4-well dishes containing 1 ml of 0.5 M saccharide (galactose or sucrose) in D-PBS supplement with 20% FBS, and kept for 4 min, transferred to 1 ml D-PBS for additional 5 min to rehydrate in D-PBS. Finally, the ovaries were incubated in M16 medium containing 10% FBS at 37℃ in a humidified atmosphere containing 5% CO_2 for future use. Processes of dilution were conducted at room temperature.

6. Immunostaining

The ovaries were washed in 0.1 M PBS, fixed with 4% (w/v) paraformaldehyde in 0.1 M PBS, and processed into paraffin sections cut 4 μm thick. In order to examine the expression and localization of TASK-2, the sections were processed for immunostaining. Sections were air-dried on gelatin-coated slides, deparaffinized, washed with PBS three times, permeabilized with 0.2% Triton X-100 for 20 min at room temperature, and incubated with an anti-TASK-2 antibody (1:200 dilution, Alomone Lab, Jerusalem, Israel) at 4℃ overnight. After three washes in PBS, the sections were incubated in the dark for 1 h with FITC-conjugated anti-rabbit IgG fluorescent secondary antibody diluted 1:500 in PBS, or incubated with streptavidin peroxidase for 25 min and submerged with diaminobenzidine (DAB, Vectastain ABC kit, Vector Laboratories, CA, USA) solution for 5 min. Finally, the sections were washed and stained with propidium iodide (PI) for nuclei staining. The stained sections were wet-mounted and observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan). The negative controls (NCs) were analyzed by omitting the primary antibody.

7. Annexin-V-FLUOS Staining

To identify cell membrane alterations, ovary sections were

stained with Annexin-V-FLUOS and PI solutions, and incubated 15 min at 25℃. The stained sections were wet-mounted and observed using a confocal laser scanning microscope (Olympus).

8. Transfection

Chinese hamster ovary (CHO) cells were seeded at a density of 2×10^5 cells per 35 mm dish 24 h prior to transfection in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. CHO cells were co-transfected with DNA fragments encoding mouse TASK-2 (AF319542.1) and green fluorescent protein (GFP) in pcDNA3.1 using LipofectAMINE2000 and OPTI-MEM I Reduced Serum Medium (Life technologies, Grand Island, NY, USA). Cells transfected with only GFP were used as a positive control for transfection. Cells were cryopreserved 2 days after transfection.

9. Cell Viability Assay

Cell viability was determined colorimetrically using 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Duchefa, Haarlem, Netherlands). The MTT assay procedures were performed as described previously (Park *et al.*, 2012). Briefly, CHO cells vitrified were seeded (4×10^5 cells/ml) in a 24-well plate. After 24 h, 20 μl of 5 mg/ml MTT solution was added to each well (0.1 mg/ml) and incubated for 4 h. The supernatants were then aspirated, the formazan crystals in each well were dissolved in 200 μl of dimethyl sulfoxide (DMSO) for 30 min at 37℃, and the 24-well plates were read at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Data are expressed as an optical density (OD).

10. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First-strand cDNAs were synthesized from total RNA isolated from ovaries using oligo dT (SuperScript™ First-Strand Synthesis System; Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was used as a template for PCR amplification. Specific primers for mouse aquaporin-3, TASK-2, and GAPDH were used with Taq polymerase (G-Taq, Cosmo Genetech, Seoul, Korea). Details of primer sequences are shown in Table 1. The PCR conditions for RT-PCR were as follows: initial denaturation at 94℃ for 5 min; 30 cycles at 94℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 30 sec; and a final extension step at 72℃ for 10 min. The products were electrophoresed on 1.5% (w/v) agarose gels to verify the product size. The DNA fragments were directly sequenced with the ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, CA, USA).

11. Statistical Analysis

The differences among the groups were analyzed using the one-way ANOVA test and post hoc comparisons using Tukey's test (SPSS18 software; SPSS, Inc., Chicago, IL, USA). The data are represented as mean±S.D. A $p<0.05$ was considered to be significant.

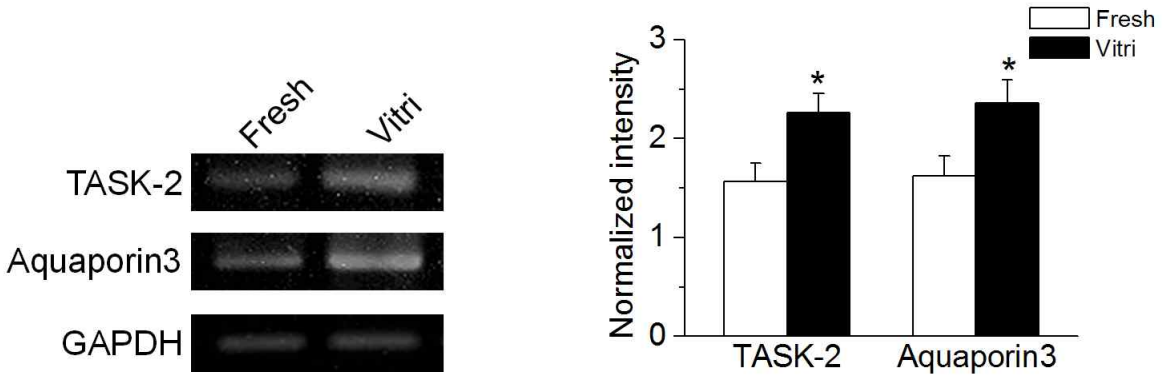
RESULTS

1. Upregulation of TASK-2 Expression in Cryopreserved Mouse Ovaries

RT-PCR data showed that vitrification increased TASK-2 mRNA expression (Fig. 1A). In agreement with earlier studies, aquaporin-3 mRNA was upregulated in vitrified ovaries. TASK-2 mRNA was increased by 44% in vitrified ovaries, similar to that with aquaporin-3 (Fig. 1B). The increase in TASK-2 mRNA expression was validated at the protein levels. Immunohistochemistry showed that the TASK-2 protein level was increased in cryopreserved ovaries (Fig. 2A). In slowly frozen ovaries, TASK-2 was predominantly expressed in oocytes. As shown in Fig. 2B, the TASK-2 protein expression level was increased

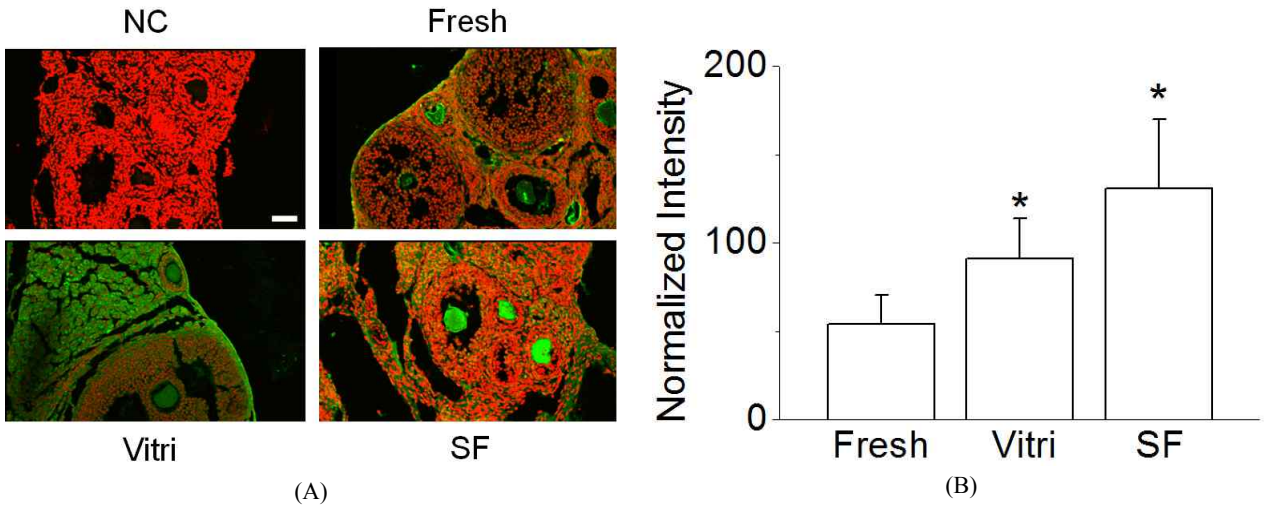
Table 1. Primer sequences used for RT-PCR

Gene name	GenBank Acc. No.	Primer sequences (5′–3′)	Expected size (bp)
Aquaporin-3	BC027400	Sense: GAACCTCCATGGGCTTCAAT Antisense: GGTGGGAACCTAATGCCCTA	419
KCNK5 (TASK-2)	AF319542	Sense: CCTCATCAAACAGATTGGGAAGAAGG Antisense: TCAGGTTGTCCTCCACAGAAGACTTAG	428
GAPDH	GU214026	Sense : CTA AAG GGC ATC CTG GGC Antisense: TTA CTC CTT GGA GGC CAT	201



(A) (B)

Fig. 1. Increase in TASK-2 mRNA expression in vitrified mouse ovaries (A) RT-PCR analysis. RT-PCR products for TASK-2 and aquaporin-3 derived from mouse ovaries. GAPDH was used as a loading control. (B) Normalized mRNA levels of TASK-2 and aquaporin-3. The mRNA expression of TASK-2 and aquaporin-3 was normalized to that of GAPDH. Each bar represents mean±S.D. of five experiments. * $p<0.05$ compared to the corresponding value obtained from fresh ovaries. Vitri, vitrification.



(A) (B)

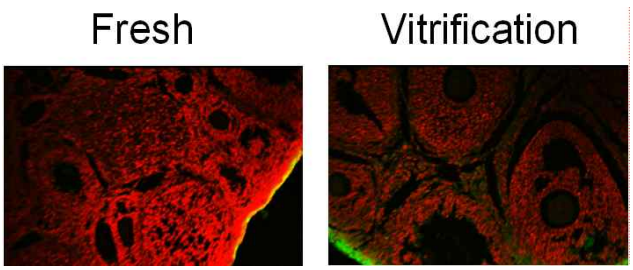
(C) (D)

Fig. 2. Microphotographs of sections of cryopreserved mouse ovaries. (A) TASK-2 immunostaining of fresh and cryopreserved ovary section. Fresh, vitrified, and slowly frozen ovary sections were immunostained with anti-TASK-2 antibody. Scale bar, 50 μm. (B) Normalized intensity of TASK-2 protein expression in fresh and cryopreserved ovaries. The fluorescence intensity of ovaries was normalized to that of negative control, which was produced by omission of anti-TASK-2 antibody. Each bar represents mean±S.D. of five experiments. * $p<0.05$ compared to the fresh ovaries. (C) Localization of TASK-2 in vitrified ovary section. Expanded scale of vitrified ovaries in (A). Scale bar, 50 μm. (D) Ovary sections stained with DAB. The images were captured from DAB stained glass slides with 20X objective lens. NC, negative control; Vitri, vitrification; SF, slow-freezing.

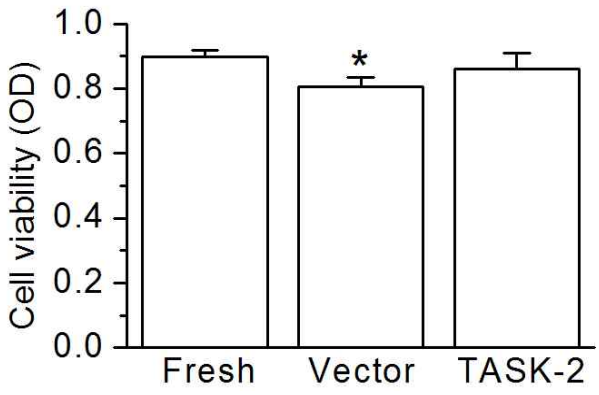
by 69% and 143% in vitrified and slowly frozen ovaries, respectively. Fig. 2C showed expanded scale of Fig. 2A vitrified ovaries. TASK-2 was localized at the perinucleus area and the plasma membrane of granulosa cells that surround the oocyte within the follicle. DAB staining also showed high TASK-2 expression in vitrified and slowly frozen ovaries (Fig. 2D).

2. Overexpression of TASK-2 Increases Cell Viability after Vitrification

To identify cryodamage at the plasma membrane, Annexin-V-FLUOS staining was performed to detect phosphatidylserine, which is translocated to outer layer from inner part of plasma membrane during early stage of apoptosis (Creutz, 1992). Annexin-V-FLUOS-positive cells are shown in the vitrified ovary section (Fig. 3A). However, the amount was not high. CHO cells transfected with mouse TASK-2 or vector pcDNA3.1 were vitrified, thawed, and cultured. The cultured cells were subjected to MTT assay. The cells overexpressed with TASK-2 showed higher cell viability than cells transfected with vector pcDNA3.1 (Fig. 3B).



(A)



(B)

Fig. 3. Increase in cell viability by TASK-2 overexpression. (A) Annexin-V-FLUOS staining of mouse ovary section. Scale bar, 100 μm. (B) MTT assay for CHO cells transfected with TASK-2. Each bar represents mean±S.D. of three experiments. * $p<0.05$ compared to the fresh ovaries.

DISCUSSION

To our knowledge, this is the first report to show that TASK-2 mRNA and protein expression levels are increased after cryopreservation in mouse ovaries. In addition, overexpression of TASK-2 increases cell viability after vitrification. We checked changes in aquaporin-3 mRNA expression as a positive control in cryopreserved mouse ovaries. The aquaporin-3 mRNA expression was increased in agreement with earlier studies. Aquaporin-3 is known as a water/solute channel that can transport water and glycerol. Movement of water and glycerol plays a key role in cell viability after cryopreservation. Overexpression of aquaporin-3 improves the survival of mouse oocyte after cryopreservation (Edashige *et al.*, 2003). The role of many members of aquaporin channels including aquaporin-3 has been intensively studied in cryopreserved cells and tissues (Sales *et al.*, 2013). However, the degree of cryodamage still remains to be resolved. In addition to aquaporin channels, other ion channels are involved in water movement, osmotic balance, and volume regulation. There is little report about changes in expression of ion channels in ovaries.

Here, we focused on TASK-2 channels, which are proposed to play an important role in volume regulation (Niemeyer *et al.*, 2001; Cid *et al.*, 2013). Cryopreserved bovine sperm expresses TASK-2 channel and shows increase in intracellular K^+ concentration. However, cryopreservation does not affect TASK-2 protein levels (Blässe *et al.*, 2012), indicating that other K^+ channels might be involved in increase in intracellular K^+ concentration. In this study, TASK-2 mRNA and protein expression levels were increased in mouse ovaries cryopreserved by vitrification and slow-freezing, suggesting that TASK-2 might have a role during the cryopreservation procedure. Cryoprotectants upregulate aquaporin-7 expression in mouse oocytes (Tan *et al.*, 2013), and DMSO in particular highly increased aquaporin-7 expression. We used ethylene glycol, PVP, galactose, ficoll, and sucrose as cryoprotectants, but not DMSO. The effect of each cryoprotectant was not investigated in this study. TASK-2 protein was increased in vitrified ovaries, which are cryopreserved using high concentration of ethylene glycol, ficoll, and sucrose. In addition, TASK-2 was also increased in CS containing ethylene glycol, PVP, and galactose for slow-freezing.

Upregulation of TASK-2 increased viability of vitrified CHO cell. The increase in TASK-2 expression level might protect cryodamage during vitrification. Higher expression of TASK-2 will facilitate water movement and osmotic balance with the CS during cryopreservation. Thus, overexpression of TASK-2 channel may improve the survival of cells. Elevation of TASK-2 may lead to successful cryopreservation of ovarian tissue, and could be effective for preserving fertility in animals and people who have concerns about fertility loss due to a variety of disease, accident, and extinction.

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