Autophagic activation in vitrified–warmed mouse oocytes

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Abstract

Vitrification involves the use of cryoprotectants (CPAs) and liquid nitrogen (LN₂), which may cause osmotic damage and cryoinjury to oocytes. Autophagy is widely recognized as a survival or response mechanism elicited by various environmental and cellular stressors. However, the induction of autophagy in vitrified–warmed oocytes has not been examined. In this work, we investigated whether the vitrification–warming process induces autophagy in mouse oocytes. Metaphase II (MII) oocytes that were vitrified and stored in LN₂ for at least 2 weeks were used in the study. In RT-PCR analyses, we observed that several *Atg* genes such as *Atg5*, *Atg7*, *Atg12*, *LC3a* (*Map1lc3a*), *LC3b* (*Map1lc3b*), and *Beclin1* were expressed in MII mouse oocytes. Slight reduction in mRNA levels of *Atg7* and *Atg12* in vitrified–warmed oocytes was noted, and expression of these genes was not significantly influenced. Confocal live imaging analysis using oocytes from GFP-LC3 transgenic mice revealed that vitrified–warmed oocytes had a significantly higher number of GFP-LC3 puncta in comparison to fresh oocytes. The expression of BECLIN1 protein was also increased in vitrified–warmed oocytes. Treatment with 3-methyladenine, an inhibitor of autophagy, did not significantly affect the rates of oocyte survival, IVF, and embryonic development after warming and IVF. The results suggest that the observed autophagic activation in vitrified–warmed oocytes is a natural adaptive response to cold stress. Collectively, we show for the first time that vitrified–warmed mouse oocytes exhibit autophagic activation during warming and that this response is not induced by CPA-containing solutions. The induction of autophagy by cold temperature is first reported herein.

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Introduction

Autophagy is an important intracellular lysosomal degradation system that can degrade and recycle macromolecules and organelles (Klionsky & Emr 2000). Autophagy, along with apoptosis and necrosis, is known as a cell death mechanism, but it is now widely accepted as an adaptive response used for cell survival under conditions of stress (Mizushima 2007). Various macromolecules and subcellular organelles can be engulfed by double-membrane vesicles called autophagosomes. Fusion of these vesicles with lysosomes results in the formation of the autophagolysosome, where internalized materials are subsequently degraded (Klionsky *et al.* 2007). Extracellular environmental (nutrient starvation, hypoxia, high temperature) and intracellular (accumulation of damaged cytoplasmic components) stressors are well-known inducers of autophagy (Levine & Klionsky 2004).

Basal autophagy plays a critical role in preserving cellular homeostasis (Mizushima *et al.* 2008). Autophagy also serves multiple functions in disease, aging, antigen presentation, and bacterial invasion (Klionsky 2010).

Evidence regarding the role of autophagy in embryonic development and survival is mounting (Mizushima & Levine 2010). Tsukamoto *et al.* (2008) showed that the basal level of autophagy is upregulated in early mouse embryos after fertilization. During this period, autophagy degrades old maternal proteins and the macromolecular building blocks are recycled to produce new zygotically expressed proteins (Tsukamoto *et al.* 2008). Previously, we have shown that autophagic activation increases the viability of dormant blastocysts in a delayed implantation mouse model and inhibition of autophagy compromises survival of dormant blastocysts (Lee *et al.* 2011).

Oocyte cryopreservation is an efficient technique that can preserve the reproductive potential of female organisms. There has been a sustained effort to improve protocols since the first successful human birth derived from frozen oocytes (Chen 1986). The American Society for Reproductive Medicine asserted that fertilization and pregnancy rate are similar to IVF/ICSI with fresh oocytes when vitrified–warmed oocytes are used as part of IVF/ICSI for young women (2013). Many IVF centers store

oocytes using this technique and have reported good clinical outcomes (Porcu *et al.* 2000, Borini *et al.* 2004). Two approaches that differ in their cooling speed are available. Slow freezing is the more traditional approach, and vitrification, the rapid cooling method, is more recently employed method (Edgar & Gook 2012). Some studies suggest that the vitrification method of oocyte cryopreservation is more effective than slow freezing (Kuleshova & Lopata 2002), leading to better survival and pregnancy rates in humans (Katayama *et al.* 2003, Yoon *et al.* 2003, Lucena *et al.* 2006, Smith *et al.* 2010, Cobo & Diaz 2011). In human oocytes, vitrified-warmed oocytes tend to recover cellular volume and spindle structure faster than slow-frozen oocytes (Martinez-Burgos *et al.* 2011). In addition, the ultrastructural features and the intracellular calcium response are better maintained in vitrified-warmed oocytes than in slow frozen oocytes (Gualtieri *et al.* 2011).

Vitrification protocols employ combinations of membrane-impermeable (e.g., sucrose, trehalose) and permeable cryoprotectants (CPAs) (e.g., ethylene glycol (EG), DMSO, propanediol (PROH)). A mixture of two permeable CPAs (DMSO and EG) is generally used (Katayama *et al.* 2003, Kyono *et al.* 2005, Lucena *et al.* 2006, Selman *et al.* 2006, Antinori *et al.* 2007). EG has emerged as a popular CPA, as it has a low molecular weight and low toxicity (Sommerfeld & Niemann 1999, Emiliani *et al.* 2000). CPA-treated oocytes are placed in cryo-containers and are exposed to liquid nitrogen (LN₂) (Gosden 2011, Saragusty & Arav 2011). Several indicators have been used to assess the quality of vitrified-warmed oocytes, including the embryonic developmental rate after IVF, the state of the meiotic spindle, DNA damage, the production of reactive oxygen species, and ultrastructural changes (Bromfield *et al.* 2009, Gualtieri *et al.* 2011, Martinez-Burgos *et al.* 2011, Tatone *et al.* 2011). However, the status of autophagy in vitrified-warmed oocytes has not been studied. Because these oocytes are exposed to LN₂ and CPA, the process of vitrification causes certain degrees of osmotic damage and cryoinjury to oocytes. Thus, we hypothesized that autophagy might play a role in the adaptive response to the potential cellular damages incurred during vitrification and warming. Using multiple approaches, we show, for the first time, that warming after vitrification induces autophagy in vitrified-warmed metaphase II (MII) mouse oocytes.

Materials and methods

Mice

Four-week-old virgin female mice and 7-week-old male ICR mice were purchased from Orient-Bio (Gyunggido, Korea). GFP-LC3 transgenic mice (GFP-LC3 tg) (Mizushima *et al.* 2004) were obtained from the RIKEN BioResource Center (Ibaraki, Japan). GFP-LC3^{tg/+} mice were obtained by breeding LC3^{tg/+}

with WT mice. All mice were used in accordance with the policies of the Konkuk University Institutional Animal Care and Use Committee (IACUC). The study was approved by the Konkuk University IACUC (approval number KU12081). To genotype the transgenic mice, the following primers were used: GFP-LC3 tg (5'-TTC TGC TGG AGT TCG TGA CCG-3' and 5'-TTG CGA ATT CTC AGC CGT CTT CAT CTC TCT CGC-3'); and LC3 internal control (5'-TGA GCG AGC TCA TCA AGA TAA TCA GGT-3' and 5'-GTT AGC ATT GAG CTG CAA GCG CCG TCT-3').

Collection of in vivo matured oocytes

Four- to six-week-old female mice were superovulated using i.p. injection of 5 IU PMSG (Sigma-Aldrich), followed by injection with 5 IU hCG (Sigma-Aldrich) 48 h later to induce superovulation. At 13–14 h post-hCG injection, cumulus-oocyte complexes (COCs) were retrieved from oviducts. MII stage oocytes were collected in Quinn's Advantage Medium with HEPES (Sage, *In Vitro* Fertilization; Trumbull, CT, USA) containing 20% fetal bovine serum (FBS, Gibco). The cumulus cells were removed enzymatically using 300 µg/ml hyaluronidase (Sigma-Aldrich).

Vitrification procedure and experimental groups

The vitrification procedure was based on the method described by Cha *et al.* (2011). A combination of EG (Sigma-Aldrich) and DMSO (Sigma-Aldrich) was used as the CPA in the vitrification solution. PBS was used as the base medium in all of the vitrification and warming solutions. Oocytes were pre-equilibrated with 1.3 M EG, 1.1 M DMSO, and 20% FBS for 2.5 min, and then equilibrated with 2.7 M EG, 2.1 M DMSO, 20% FBS, and 0.5 M sucrose (Fisher Scientific; Fair Lawn, NJ, USA) for 20 s. Equilibrated oocytes were loaded onto an electron microscope (EM) copper grid (Ted Pella, Inc., Redding, CA, USA) and submerged into LN₂. Vitrified oocytes were stored in LN₂ for at least 2 weeks. For warming, the grids loaded with vitrified oocytes were transferred to 0.5, 0.25, 0.125, and 0 M sucrose at 2.5 min intervals. The vitrified-warmed oocytes were washed and cultured in HEPES with 20% FBS in an incubator at 37 °C in 5% CO₂ for 1–2 h (recovery time). The oocytes were divided into three groups (Fig. 1). Group 1: fresh MII oocytes; Group 2: fresh MII oocytes equilibrated with CPAs and warmed, but not vitrified (solution-treated oocytes); and Group 3: vitrified-warmed oocytes that had been equilibrated with CPAs, loaded onto an EM copper grid, and stored in LN₂.

RNA isolation and RT-PCR

Total RNA was extracted from MII oocytes (50 oocytes/sample) or tissues using TRIzol Reagent (Life Technologies, Invitrogen) according to the manufacturer's protocol. Rabbit α -globin RNA (10 pmol/sample, Sigma) was used as an external control for the RNA isolation (Kang *et al.* 2011). To isolate the minute amounts of RNA in oocytes, 3 M sodium acetate (pH 5.2) and glycogen were used in a modified protocol. Resuspended RNA was treated with RNase-free DNase I (Roche) for 20 min at room temperature to remove any genomic DNA. The RNA was

Figure 1 A schematic diagram of the procedure and experimental groups. MII oocytes from several ICR mice were pooled and divided into three groups. Group 1: fresh oocytes without exposure to any solution; Group 2: fresh oocytes treated with vitrification and warming solutions; and Group 3: vitrified-warmed oocytes.

reverse transcribed to cDNA using MMLV reverse transcriptase (Beamsbio, Seoul, Korea) and random hexamer primers (Roche). Equal amounts of cDNA from the three groups were used as the template in our PCR analysis. The genes and primer sets are listed in Table 1. Ribosomal protein L7 (*Rpl7*) was used as an internal control.

Live imaging and quantitative analysis of GFP-LC3 puncta in mouse oocytes

To observe the status of autophagy in oocytes by live imaging, MII oocytes from GFP-LC3^{tg/+} mice were imaged live. Oocytes were stained with LysoTracker Red to stain acidified endosomes (Lee *et al.* 2011). Three groups of oocytes (fresh, solution-treated, and vitrified-warmed oocytes, see Fig. 1) were cultured in 100 nM LysoTracker Red (Molecular Probes, Invitrogen) in HEPES with 20% FBS for 30 min. Live images of LysoTracker Red and GFP-LC3 were observed using the Olympus FV1000

spectral confocal microscope (Olympus, Tokyo, Japan). In the first set of experiments, oocytes were imaged after warming and 1 h of recovery time. In the second set of experiments, oocytes of each group were imaged after the warming process, without recovery time. Live images were captured at 20 min intervals for a 1 h period. For each oocyte, the number of GFP-LC3 puncta was quantified (Fig. 3C).

Immunofluorescence staining and confocal microscopy

Immunofluorescence staining of the MII oocytes was carried out using a drop culture system as described previously (Shin *et al.* 2013). The oocytes were fixed in 4% paraformaldehyde and 0.1% Triton X-100 in PBS for 20 min. Then, the oocytes were washed in 0.1% Triton X-100 in PBS and blocked in 2% BSA in PBS for 60 min. The oocytes were incubated with the primary antibody in 2% BSA/PBS at 4 °C overnight. The oocytes were next washed in 2% BSA/PBS and incubated with secondary antibody in 2% BSA/PBS for 40 min. After washing, the oocytes were counterstained with TO-PRO-3-iodide (1:250, Life Technologies) to label DNA. Finally, the oocytes were washed in 2% BSA/PBS and directly placed onto a slide and covered with a glass coverslip. Rabbit IgG was used as a mock control. The oocytes were visualized using the Olympus FV1000 spectral confocal microscope (Olympus). Images were obtained and analyzed using Fluoview software (version 1.5), a platform associated with the confocal microscope. The primary antibodies used were rabbit polyclonal anti-LC3b (4 µg/ml; Abcam, Cambridge, UK) and rabbit polyclonal anti-Beclin1 (1:200; Novus Biologicals, Littleton, CO, USA). The secondary antibody used was Alexa Fluor 488 chicken anti-rabbit IgG (1:250, Molecular Probes).

3-Methyladenine treatment of vitrified oocytes

3-Methyladenine (3-MA) (Sigma-Aldrich) was solubilized in distilled water (DW) by gentle heating. Vitrified oocytes were warmed in a four-step series of warming solution containing 10 mM 3-MA, washed, and cultured in HEPES with 20% FBS and 10 mM 3-MA. Vitrified oocytes in the control group were

Table 1 Primers used for RT-PCR.

Gene name	Sequence (5'–3')	Product size (bp)	GenBank accession no.
<i>Atg5</i>	F: GAC AAA GAT GTG GCT TCG AGA TGT G R: GTA GCT CAG ATG CTC GCT CAG	772	NM_053069.5
<i>Atg7</i>	F: ATG CCA GGA CAC CCT GTG AAC TTC R: ACA TCA TTG CAG AAG TAG CAG CCA	350	NM_028835.4 NM_001253718.1 NM_001253717.1
<i>Atg12</i>	F: TTC GGT TGC AGT TTC GCC R: CCA TGC CTG TGA TTT GCA GTA	310	NM_026217.3
<i>Beclin1 (Atg6)</i>	F: CTG AAA CTG GAC ACG AGC TTC AAG R: CCA GAA CAG TAT AAC GGC AAC TCC	989	NM_019584.3
<i>Map1lc3a (Atg8)</i>	F: AGC TTC GCC GAC CGC CGT AAG R: CTT CTC CTG TTC ATA GAT GTC AGC	276	NM_025735.3
<i>Map1lc3b (Atg8)</i>	F: CGG AGC TTT GAA CAA AGA GTG R: TCT CTC ACT CTC GTA CAC TTC	279	NM_026160.4
<i>Rpl7</i>	F: TCA ATG GAG TAA GCC CAA AG R: CAA GAG ACC GAG CAA TCA AG	246	NM_011291.5
Rabbit α -globin	F: GCA GCC ACG GTG GCG AGT AT R: GTG GGA CAG GAG CTT GAA AT	257	NM_001082389.2

F, forward; R, reverse.

warmed and cultured in warming solution and HEPES with 20% FBS containing the same amount of DW.

IVF and embryo culture

IVF was carried out following the procedure of Cha *et al.* (2011). The epididymal sperm suspension was obtained from 8- to 12-week-old male mice and incubated for 90 min in Quinn's Advantage Fertilization Medium (human tubal fluid, HTF, Sage) containing 10% substitute protein serum (SPS, Sage) at 37 °C in 5% CO₂ to ensure sperm capacitation. For IVF, capacitated sperms were added to oocytes in HTF containing 10% SPS and incubated for 6 h. The oocytes were washed three times in HTF containing 10% SPS and washed in KSOM-AA medium (Millipore, Danvers, MA, USA) lastly. The oocytes were cultured in KSOM-AA at 37 °C in 5% CO₂ overnight. The following morning, oocyte fertilization was confirmed by the presence of two blastomeres, and fertilized embryos were cultured for 5 days to analyze embryonic development.

Statistical analysis

All analyses and graphing were carried out by using GraphPad Prism (version 5) software (La Jolla, CA, USA). Statistical significance was assessed by a Student's *t*-test (one-tailed), and *P* values <0.05 were considered significant.

Results

Expression of autophagy genes in vitrified–warmed mouse oocytes

We hypothesized that osmotic damage and cryoinjury incurred during the vitrification and warming procedures would induce autophagy in MII mouse oocytes. Using RT-PCR, we first examined whether the expression of autophagy (*Atg*) genes is affected during vitrification and warming, focusing on genes encoding the key proteins that mediate autophagosome biogenesis (*Atg5*, *Atg7*, *Atg12*, *Map1lc3a* (*LC3a*), *Map1lc3b* (*LC3b*), and *Beclin1*) (Boya *et al.* 2013). As shown in Fig. 2, all of the selected *Atg* genes are detected in mouse MII oocytes. Expression of *Atg5*, *Map1lc3a*, *Map1lc3b*, and *Beclin1* do not show significant difference among the three groups (fresh, solution-treated, and vitrified–warmed oocytes). The expression of *Atg7* and *Atg12* was slightly reduced in Group 3 (vitrified–warmed oocytes) in comparison to Group 1 (fresh oocytes). Thus, several *Atg* genes are expressed in MII mouse oocytes, but the expression of most of these genes is not strongly affected by the vitrification and warming procedure.

Autophagic activation in vitrified–warmed mouse oocytes

GFP-LC3 transgenic mice ubiquitously expressing a GFP-LC3 fusion protein are a useful tool to monitor

Figure 2 mRNA expression of *Atg* genes in MII mouse oocytes. (A) The mRNA expression of *Atg* genes, *Atg5*, *Atg7*, *Atg12*, *Map1lc3a*, *Map1lc3b*, and *Beclin1*, was examined by RT-PCR. For each group, 50 oocytes were pooled for the preparation of RNA. RNA from mouse brain tissue was used as a positive control. Brain RNA without RT was used as a negative control. Ribosomal protein L7 (*Rpl7*) and α -globin were used as internal and external loading controls respectively. (B) The expression of *Atg* genes was normalized to the level of *Rpl7* in each reaction. The expression of *Atg7* and *Atg12* was slightly reduced in vitrified–warmed oocytes. The values represent the mean $\pm$ s.d. ($n=4$). Statistical significance was assessed by a Student's *t*-test (one-tailed). * $P<0.05$, ** $P<0.01$.

autophagy *in vivo* (Mizushima *et al.* 2004). We previously used these mice to observe autophagy in mouse blastocysts (Lee *et al.* 2011). In this study, we used oocytes from GFP-LC3^{tg/+} mice to monitor autophagic activity in vitrified–warmed oocytes. GFP-LC3^{tg/+} MII oocytes of the three experimental groups (as described in Fig. 1) were placed in the media for 1 h at 37 °C and were subjected to live confocal imaging. Oocytes were counter-stained with LysoTracker Red, which stains acidified endosomes (Lee *et al.* 2011). GFP-LC3 puncta that colocalized with LysoTracker Red staining (yellow)

Figure 3 Confocal live imaging and quantitative analysis of GFP-LC3^{tg/+} mice. (A and B) Confocal live imaging analysis of the oocytes collected from GFP-LC3^{tg/+} mice revealed that some oocytes in Group 2 (solution-treated) and Group 3 (vitrified-warmed) oocytes showed multiple green puncta, indicating autophagic activation. The majority of oocytes in Group 1 (fresh) did not have puncta. After warming, oocytes in (A) were given a recovery time of 1 h in 37 °C and were imaged at the end of culture. Oocytes were stained with 100 nM LysoTracker Red. Oocytes in (B) were imaged live during the process of warming at 20 min intervals for 1 h. Green, GFP-LC3; Red, LysoTracker Red. Scale bar, 20 μm. (C) Quantification of the number of GFP-LC3 puncta in the oocytes of each group. The bar graphs and table show the number and percentage of oocytes with or without GFP-LC3 puncta. The percentage of vitrified-warmed oocytes with puncta is significantly higher in Group 3 (vitrified-warmed) oocytes than any other group. Statistical significance was examined by a Student's *t*-test (one-tailed). **P*=0.0340 <0.05.

are generally considered as autolysosomes generated by the fusion of autophagosomes and lysosomes (Tsukamoto *et al.* 2008). As shown in Fig. 3A, several oocytes of Group 2 (solution-treated) and Group 3 (vitrified-warmed) showed GFP-LC3 puncta (green) that represent autophagic activation. Most of GFP-LC3 dots are negative for LysoTracker Red staining, suggesting these autophagosomes had not merged with lysosomes. The majority of Group 1 (fresh) oocytes did not possess GFP-LC3 puncta.

Next, to examine whether autophagic activation occurs during the warming process, we observed the

formation of GFP-LC3 puncta during the recovery time, 1 h after oocytes were placed in the warming solution. Live images of each oocyte were captured at 20 min intervals for 1 h. As shown in Fig. 3B, some oocytes of Group 2 (solution-treated) and Group 3 (vitrified-warmed) show heightened number of GFP-LC3 puncta, whereas most Group 1 (fresh) oocytes do not form puncta. We quantified the number of puncta in the oocytes of each group. Group 3 (vitrified-warmed) oocytes have a significantly higher number of GFP-LC3 dots than that of other groups (Fig. 3C). While 25.5% of Group 1 (fresh) oocytes showed GFP-LC3 puncta, 53.5%

of Group 3 (vitrified–warmed) oocytes had GFP-LC3 puncta. There was no significant difference between Group 1 (fresh) and Group 2 (solution-treated) oocytes. Autophagy induction in vitrified–warmed oocytes is apparently due to low-temperature cooling and warming processes itself, but is not associated with the solutions used.

BECLIN1 is the mammalian homolog of yeast Atg6. BECLIN1 interacts and forms a complex with class III phosphatidylinositol 3-kinase (PI3K). This complex is required to recruit the Atg12–Atg5 conjugate to the preautophagosome (Liang *et al.* 1999, Petiot *et al.* 2000, Kihara *et al.* 2001, Codogno & Meijer 2005). In Fig. 4, BECLIN1 is evenly distributed in the ooplasm of Group 1 (fresh) oocytes. Notably, in Group 2 (solution-treated) oocytes, osmosis-mediated shrinkage of the ooplasm occurred, causing the BECLIN1 signal to concentrate in the center of the ooplasm. The expression level of BECLIN1 protein in Group 3 (vitrified–warmed) oocytes is significantly increased compared with Group 1 (fresh) and Group 2 (solution-treated) oocytes. Similar to Group 2, the BECLIN1 signal is slightly concentrated in the center of the ooplasm. Upregulation of BECLIN1 in Group 3 (vitrified–warmed) oocytes, along with the observation of increased GFP-LC3 puncta in the same group (Fig. 3), indicates that there is an increased autophagic activation in oocytes during to the vitrification–warming process.

Survival, fertilization, and development of oocytes treated with autophagy inhibitor

Induction of autophagy in vitrified–warmed oocytes could be a general stress response of oocytes to sudden changes in osmolality and temperature. Alternatively, autophagic activation could be required to actively reorganize subcellular structures and functions after cooling and warming. To address this issue, we monitored whether blocking autophagy during the vitrification–warming process adversely affects the developmental fate of vitrified–warmed oocytes. To inhibit autophagy during the warming process, we used 3-MA, a class III PI3K inhibitor that prevents

autophagosome formation (Kovacs *et al.* 1981, Seglen & Gordon 1982). 3-MA-treated vitrified–warmed oocytes were subjected to IVF. First, we determined the dose of 3-MA that effectively reduces GFP-LC3 puncta formation in oocytes. We confirmed that the treatment of 10 mM 3-MA during warming and subsequent incubation significantly reduces LC3 puncta formation (Fig. 5A). Next, we compared the rates of survival, fertilization, and development of the vitrified–warmed oocytes that were treated 3-MA with the control oocytes (Fig. 5B). After vitrification and warming, the survival rate of 3-MA-treated oocytes is not significantly different from that of control oocytes (3-MA-treated, 84.9%; control, 76.7%; $P=0.0990$). Thus, partial inhibition of autophagic activation using 3-MA in vitrified–warmed oocytes does not cause adverse effects on oocyte survival. There is no significant difference in the fertilization rate between 3-MA-treated vitrified–warmed oocytes and control vitrified–warmed oocytes (3-MA-treated, 56.7%; control, 61.8%; $P=0.2244$). The developmental rate in the 3-MA treated vitrified–warmed oocytes is similar to that of control vitrified–warmed oocytes (3-MA-treated, 35.5%; control, 49.4%; $P=0.1140$). The fertilization and developmental rates of the 3-MA-treated vitrified–warmed oocytes tend to be lower than those of control vitrified–warmed oocytes, but these differences have no statistical significance. Collectively, our results show that a partial blockage of autophagy by 3-MA does not cause detrimental effects on survival, fertilization, and development of vitrified–warmed oocytes.

Discussion

Cryopreservation of germ cells is a highly efficient and useful technique to preserve the reproductive potential of organisms during unfavorable circumstances. Efforts have continuously been made to improve and fine-tune this technique in many species including humans. It is evident that cells exposed to low temperature freezing would experience cellular damage caused by osmotic and low-temperature stresses. However, no information had been available as to whether cells cope with this stress with an autophagic

Figure 4 Immunofluorescence staining of BECLIN1 in vitrified–warmed oocytes. Oocytes were stained with rabbit polyclonal anti-Beclin1 antibodies (1:200) and probed with Alexa Fluor 488 chicken anti-rabbit IgG. DNA was stained with TO-PRO-3-iodide (1:250). Rabbit IgG was used at each staining as a mock control. The expression of Beclin1 is upregulated in Group 3 (vitrified–warmed) oocytes. Shrinkage of the ooplasm because of osmosis was observed in Group 2 (solution-treated) and Group 3 (vitrified–warmed) oocytes. Green, Beclin1; blue, DNA. Scale bar, 30 μ m.

Figure 5 Survival, fertilization and development of 3-MA-treated vitrified–warmed oocytes. (A) Immunofluorescence staining of LC3b in vitrified–warmed oocytes. Oocytes were stained with rabbit polyclonal anti-LC3b antibodies (4 µg/ml) and probed with Alexa Fluor 488 chicken anti-rabbit IgG. DNA was stained with TO-PRO-3-iodide (1:250). Rabbit IgG was used as a mock control. The number of LC3 puncta is reduced in oocytes treated 10 mM 3-MA during warming and following incubation. Green, LC3; blue, DNA. Scale bar, 20 µm. (B) The effect of 3-MA treatment during warming and incubation on the survival, fertilization and development of vitrified–warmed oocytes. Bar graphs show the percentage of 2-cell embryos and blastocysts after IVF. The table shows the total number and percentage of total oocytes that were used in IVF. There is no significant difference in the survival rate of the oocytes with or without 3-MA treatment ($P=0.0990 > 0.05$). The values represent the mean $\pm$ s.d. ($n=5$). Statistical significance was assessed by a Student's *t*-test (one-tailed).

response. One recent report on the preservation of stallion sperm have shown that autophagy is induced during refrigeration (Gallardo Bolanos *et al.* 2012). The present study shows for the first time that vitrified–warmed mouse oocytes exhibit autophagic activation during warming. Such a phenomenon was not observed in the group of oocytes treated with solutions only (Group 2), suggesting that the cooling process itself is the inducer of autophagy. CPAs used in the vitrification of oocytes or embryos play crucial roles in preventing cellular dehydration and ice crystal formation, but they are also responsible for causing osmotic injuries and toxicity (Huang *et al.* 2006). As our data show, the CPAs themselves do not induce autophagy (Fig. 3). Therefore, the cause of increased autophagy is the LN₂-mediated freezing process.

Previous work by Tsukamoto *et al.* (2008) showed that MII oocytes do not possess abundant GFP-LC3 puncta, but the number of puncta appears to increase in preimplantation embryos after fertilization. Thus, in oocytes not exposed to any stress, such as cooling, autophagic activation is rarely observed

(Fig. 3, Group 1). Consistent with this finding, our data showed that control MII oocytes showed no or a low number of puncta. A significantly increased number of GFP-LC3 puncta were present in vitrified–warmed oocytes, suggesting that the cooling–warming process is a previously unidentified inducer of autophagy. Our study also showed that the level of BECLIN1, an ATG protein essential for autophagy, is increased in vitrified–warmed oocytes (Fig. 4). BECLIN1 promotes autophagy when introduced into autophagy-deficient yeast and mammalian cells (Liang *et al.* 1999); however, this protein can also interact with the B-cell lymphoma 2 (BCL2) protein, an important regulator of apoptosis (Liang *et al.* 1998). Thus, BECLIN1 is considered to play roles spanning two important cellular processes, autophagy and apoptosis (Yue *et al.* 2003). Whereas our study showed autophagic activation in vitrified–warmed oocytes, induction of apoptosis during vitrification has also been reported in mouse MII oocytes (Rajaei *et al.* 2013). As autophagy and apoptosis, two cellular responses to stress, are often activated together (Xue *et al.* 1999, Ferri & Kroemer 2001), further analysis regarding the role of BECLIN1 in

the dual activation of these processes in oocyte vitrification warrant investigation.

We used 3-MA, an inhibitor of PI3K, to block autophagy during the vitrification and warming processes of oocytes. As shown in Fig. 5, 10 mM 3-MA provided a partial block of autophagic activation. Under these conditions, the rates of fertilization and development were slightly reduced, but not significantly. Therefore, it is plausible that the observed autophagic activation in vitrified-warmed oocytes is an adaptive response to cold stress and that it is not associated with vial developmental consequences in oocytes. Nonetheless, one cannot rule out the possibility that inhibition of autophagy by 3-MA was not complete enough to show the effects on fertilization and developmental rates. Alternatively, there could be unforeseen long-term developmental effects of inhibiting autophagy during warming of vitrified oocytes. All these possibilities require further investigation. In addition, the use of oocyte-specific knockout mice would generate concrete information as to whether the complete absence of a crucial autophagy gene affects survival, fertilization, and development of oocytes after the vitrification and warming processes.

Taken together, our study establishes, for the first time, that freezing induces autophagy in mouse oocytes. It is currently unclear whether autophagy is involved in regaining normal subcellular structures or to remove proteins that are damaged during freezing. Nonetheless, our study shows that autophagic activation seen in vitrified-warmed oocytes is a natural adaptive response induced during warming. Further investigation is warranted to examine whether this is a conserved response in oocytes and cells of other species and whether this increase in autophagic activation has any role in developmental potential of vitrified oocytes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H J Lim designed the experiments; S Bang and H Shin prepared samples and carried out the experiments; S Bang, H Shin, H Song, C S Suh, and H J Lim analyzed the data; S Bang and H J Lim wrote the paper.

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