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Exposing Mouse Oocytes to Necrostatin 1 During In Vitro Maturation Improves Maturation, Survival After Vitrification, Mitochondrial Preservation, and Developmental Competence

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Abstract

Necrostatin 1 (Nec1) is widely used in disease models to examine the contribution of receptor-interacting protein kinase 1 in cell death. The biological actions of Nec1 are blocking necrotic cell death. The purpose of this study was to investigate whether adding Nec1 into in vitro maturation (IVM) media, followed by vitrification procedures, could enhance the survival and developmental competency of oocytes. Germinal vesicle oocytes were matured in IVM medium containing 2 different doses of Nec1 (0.5 and 1 μmol/L). After IVM, the oocytes were vitrified using a 2-step exposure to equilibrium and vitrification solutions. After warming, the rates of survival, fertilization, embryonic development up to blastocyst in vitro, morphology of spindle and chromosome, membrane integrity, mitochondria integrity, and several gene expressions were evaluated. The survival and developmental competency of oocytes were higher in the 1 μmol/L Nec1-treated group than control. The proportion with intact spindles/chromosomes and stable membranes was similar in all the groups. The mitochondrial integrity of all Nec1-treated groups showed a higher score with strong staining. The 1 μmol/L Nec1 showed significantly increased expressions of *Mad2*, *Gdf9*, and *Bcl2*. The *Cirp* level had a tendency to be downregulated in the 0.5 μmol/L Nec1 but upregulated in the 1 μmol/L Nec1, compared with the control. The *Mtgenome* expressions were significantly decreased in both Nec1 groups. The supplementation of 1 μmol/L Nec1 into the IVM medium could be beneficial for the survival and development of immature oocytes after vitrification.

Keywords

in vitro maturation, vitrification, necrostatin 1, oocyte, spindle, mitochondria

Introduction

Oocyte vitrification has been a significant strategy to preserve fertility for teenage girls and young women without male partners. A variety of improved cryoprotocols and tools have conspicuously enhanced oocytes survival and contributed to increased pregnancy and live birth rates.[1-7] There are a few effective clinical options for preserving female fertility. Traditionally, embryo cryopreservation was the common method, followed by in vitro fertilization (IVF). However, many young reproductive-age female patients with cancer do not benefit in terms of preserving their fertility using embryo cryopreservation. In addition, ovarian stimulation with gonadotropins in the IVF cycle is not suitable in some patients with cancer who are having breast cancer or who require immediate chemotherapy. In most fertility centers, retrieved immature oocytes are usually allowed to mature in vitro and are then vitrified at metaphase II (MII).[8,9] Although the survival rate of immature oocytes seems improved compared with MII oocytes, the poor maturation and fertilization of oocytes and the poor embryonic development up to the blastocyst stage are the major problems related to immature oocyte freezing.[10,11] In addition, according to Cao et al,

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oocytes vitrified at the germinal vesicle (GV) stage and at the MII stage exhibit similar survival rates, and oocytes vitrified at the GV stage showed substantially less maturation rates than oocytes vitrified at the MII stage.[12] Because of these problems, vitrification at the MII stage seems to be a more proper method according to previous researches.

In cell culture systems, safe and essential factors such as consistent temperature, growth factors, and nutrients are important to success culture. Normally, most researchers have tried to find the best culture system, which is similar in conditions compared to in vivo for their experiments. However, most culture systems have been always exposed from harmful circumstances because it could not be the same as in vivo. Hence, more effective culture methods need to be developed for avoiding harmful effect such as apoptosis or necrosis on in vitro cultured cell.

Necrostatin 1 (Nec1) is widely used in disease models to examine the contribution of receptor-interacting protein kinase 1 (RIP1) in cell death and inflammation. There are 2 principal types of cell death according to morphological appearance, apoptosis and necrosis.[13] "Necroptosis" is a recently coined term that refers to 1 specific form of programmed necrosis that is induced by stimulating death receptors with agonists such as tumor necrosis factor α (TNF-α), Fas ligand, and TNF-related apoptosis-inducing ligand. Necroptosis has its own unique signaling pathway, which requires the involvement of RIP1 and RIP3. Receptor-interacting protein kinase 1, best known for its function in nuclear factor κB activation,[14,15] also plays a critical role in necroptosis. Receptor-interacting protein kinase 3, a RIP1 family member, has recently been implicated in necroptosis.[16-18] According to 1 recent research, Nec1 suppressed the apoptosis and cleaved caspase 3 protein levels in a mouse intracerebral hemorrhage model. Also, Nec1 treatment improved the expression of Bcl2 protein in mouse model.[19] Although the function of Nec1 is not fully understood, Nec1 might regulate the apoptosis-related gene and protein expression in mouse cells. In addition, it is known to be a potent inhibitor of RIP1 kinase activity and is able to inhibit the interaction between RIP1 and RIP3 upon the activation of necroptosis.

In this study, we added Nec1 to in vitro maturation (IVM) medium used for the maturation of immature mouse oocytes for the first time. Our aim was to determine whether Nec1 supplementation during IVM improves the rates of survival, fertilization, and developmental competence up to blastocyst formation of oocytes matured in vitro and embryo production after cryopreservation. To evaluate the effect of Nec1, we used Nec1-treated oocytes and nontreated control oocytes that survived after vitrification for examining spindle morphology, testing membrane integrity, staining for mitochondrial membrane potential, and testing for the expression of genes related to oocyte development and spindle formation.

Methods

Animals

B6D2F1 female mice (5-6-week-old) were used in all experiments. The Institutional Animal Care and Use Committee (IACUC) of Seoul National University Bundang Hospital approved this study. Animals were cared for according to the guidelines of IACUC.

Retrieval of Immature Oocytes

We injected mice intraperitoneally with 7.5 IU pregnant mare's serum gonadotropins (Sigma-Aldrich, St Louis). After 48 hours, the stimulated mice were killed by cervical dislocation, and ovaries were dissected and placed in 1 mL of washing (collection) medium. The washing medium used was mouse tubal fluid (MTF)[20] supplemented with additional components (modified MTF [mMTF]). The mMTF contained MTF plus 0.4% (w/v) bovine serum albumin (BSA; Sigma), 1% (v/v) nonessential amino acids (11140-050; Gibco, Grand Island), 1% (v/v) essential amino acid (R7131; Sigma), 0.5% (v/v) L-glutamine (25030-081; Gibco), and 0.25% (v/v) multivitamins (11120-052; Gibco). By puncturing the antral follicles, we isolated intact cumulus–oocyte complexes (COCs).

In Vitro Maturation

Collected good-quality COCs were incubated in IVM medium for 18 hours at 37°C in a humidified chamber with 5% CO_2. Basal medium was commercial TCM-199 (Invitrogen, Carlsbad), which we supplemented with 20% fetal bovin serum (FBS; Invitrogen), recombinant follicle-stimulating hormone and human chorionic gonadotropins (75 mIU/mL and 0.5 IU/mL; Serono, Geneva, Switzerland), and recombinant epidermal growth factor (10 ng/mL; Sigma). For experimental groups, 0.5 or 1 μmol/L of Nec1 (N9037; Sigma) was added to the IVM medium. We used a group culture system. Ten oocytes were cultured in a drop under mineral oil. After the maturation period, we denuded all COCs by applying 85 IU/mL hyaluronidase to confirm maturation, which was evaluated based on the extrusion of the first polar body. This observation was performed under an inverted microscope (200× magnification). The nonvitrified control group oocytes were matured, fertilized, and developed up to blastocyst stage.

Vitrification and Warming of Oocyte

We performed oocyte vitrification in equilibrium solution (ES) and vitrification solution (VS). Base solution (BS) consisted of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered TCM-199 medium supplemented with 20% (v/v) FBS (Invitrogen). Equilibrium solution contained 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) 1,2–propanediol (PROH) in BS solution. Vitrification solution contained 15% (v/v) EG, 15% (v/v) PROH, and 0.5 mol/L sucrose in BS solution. The oocytes were submerged in ES for 5 minutes and then transferred to VS for 45 to 60 seconds at room temperature (RT). Approximately 5 to 6 oocytes were placed on a CryoTop (Kitazato, Shizuoka, Japan) and then plunged immediately into liquid nitrogen for storage.

For warming oocytes, we prepared a warming solution (WS), which was BS containing sucrose at 1.0 mol/L (WS1), 0.5 mol/L (WS2), and 0.25 mol/L (WS3). The CryoTops containing the oocytes were immersed directly in WS1 at 37°C for 1 minute. The processed oocytes were transferred to WS2 and WS3 for 3 minutes each and then washed twice with BS (washing solution). Warmed oocytes were transferred to fertilization medium at 37°C and maintained with 5% CO_2 in humidified air. To assess the survival rate of oocytes, they were cultured for 1 hour more. We counted the oocytes that survived, which were identified morphologically based on the appearance of membrane integrity and discoloration of the ooplasm. The surviving oocytes were used for subsequent experiments.

In Vitro Fertilization

The oocytes that survived after warming were fertilized using epididymal sperm. We used epididymal spermatozoa obtained from the cauda epididymis of 8- to 10-week-old male B6D2F1 mice. For capacitation, the collected spermatozoa were preincubated for 1.5 hours in fertilization medium (mMTF supplemented with 0.8% BSA). Sperm at a final dilution of 2×10^6/mL were used to inseminate oocytes, which were incubated at 37°C in humidified air with 5% CO_2. After 6 hours, inseminated oocytes were washed twice by moderate pipetting and then placed in embryo maintenance medium (Global medium supplemented with 10% human serum albumin; Life Global, North Carolina). To assess fertilization, development of the 2-cell stage was measured 1 day after insemination. The cleaved embryos were transferred to new embryo maintenance medium, and their development up to blastocyst formation was evaluated on day 5 after insemination.

Spindle and Chromosome Staining

We incubated the oocytes that survived after vitrification for 1 hour in humidified air with 5% CO_2 for spindle reassembly and chromosomal distribution after warming. The cultured oocytes were fixed with 4% paraformaldehyde for 10 minutes and were then washed in PBS; the washed oocytes were transferred to 0.25% Triton X-100 in PBS for 10 minutes at RT and then washed twice before blocking. For blocking, the oocytes were submerged in 2% BSA in PBS for 1 hour at RT. After rinsing in PBS, the oocytes were incubated overnight at 4°C with β-tubulin polyclonal antibody (1:100 dilution; Cell Signaling, United Kingdom). The oocytes were then washed twice in PBS and stained in the dark (1 hour, 37°C) with fluorescein isothiocynate (FITC)-conjugated antirabbit immunoglobulin G (Abcam, United Kingdom). The stained oocytes were rinsed twice in PBS and then mounted on glass slides with coverslips; we used Vectorshield mounting medium (Vector Laboratories, Burlingame, California) containing 0.5 μg of 4′,6-diamidino-2-phenylindole (DAPI). To assess spindle and chromosomal localization, we used a fluorescence microscope (Leica DMIL, Germany) equipped with a Hamamatsu digital camera imaging system and observed the samples under 400× magnification. The spindle and chromosomes were considered to be intact when oocytes displayed barrel-shaped microtubules and centrally aligned chromosomes between the 2 poles. Damaged or missing meiotic spindles and chromosome misalignments were considered abnormal (Figure 1).

Membrane Integrity Evaluation

Warmed oocytes that survived were incubated in PBS for membrane staining. To these oocytes, we added 5 mg/mL BSA, 1 mg/mL fluorescein diacetate (FDA; Sigma), and 50 mg/mL propidium iodide (PI; Sigma) in PBS for 10 minutes.[21] After being exposed to FDA, most live cells that are intact and normal accumulate fluorescein intracellularly. Thus, the stained oocytes appeared green under fluorescence microscopy when they were intact. Propidium iodide has 2 characteristics, the dye potently stains nuclei and PI can be used to detect entire nonintact cells or parts of them. We regarded oocytes stained by both FDA and PI to be alive but to have damaged parts.[22]

Oocytes Mitochondrial Staining by JC-1

All oocytes in controls and Nec1 groups were stained by the ΔΨm-specific probe JC-1 (Invitrogen). JC-1 was diluted to final concentration of 5 μg/mL in mMTF. All oocytes were stained in a humidified incubator containing 5% CO_2 at 37°C for 30 minutes. The JC-1 reaction was conducted in darkness. The stained oocytes were examined by epifluorescence microscopy in the FITC and rhodamine-B isothiocyanate (RITC) channels using narrow band path filter sets anddivided into 3 grades which consisted of strong, moderate, and weak staining groups, respectively. Because there were no standard references, we divided the grades into 3 criteria based on our own discretion. In a recent study, we divided 3 evaluation standards according to the characteristics of JC-1 (strong, moderate, and weak staining). In the strong red per green staining, the red fluorescence located around the periphery of oocytes represents a higher mitochondrial membrane potential, whereas the green fluorescence dispersed within the oocytes represents a lower mitochondrial membrane potential (Figure 2). The moderate red per green staining is similar to strong grade, but the red fluorescence is weaker than in the strong staining. The weak red per green staining has far weaker red fluorescence than moderate group. In weak grade, the red fluorescence was almost not detected in the oocyte periphery site or cytoplasm. These staining grades are presented in Figure 2. We counted each of oocyte of red per green staining according to 3 grades. The determination method using JC-1 on oocyte staining has not yet been standardized. So, we had to make our score criterion. To add accuracy, 5 researchers evaluated each oocyte and calculated average scores.

Quantitative Real-Time Polymerase Chain Reaction Analysis

We used the Dynabeads messenger RNA (mRNA) DIRECT micro kit (Dynal Asa, Oslo, Norway) to extract mRNA from vitrified-warmed oocytes from experimental and control

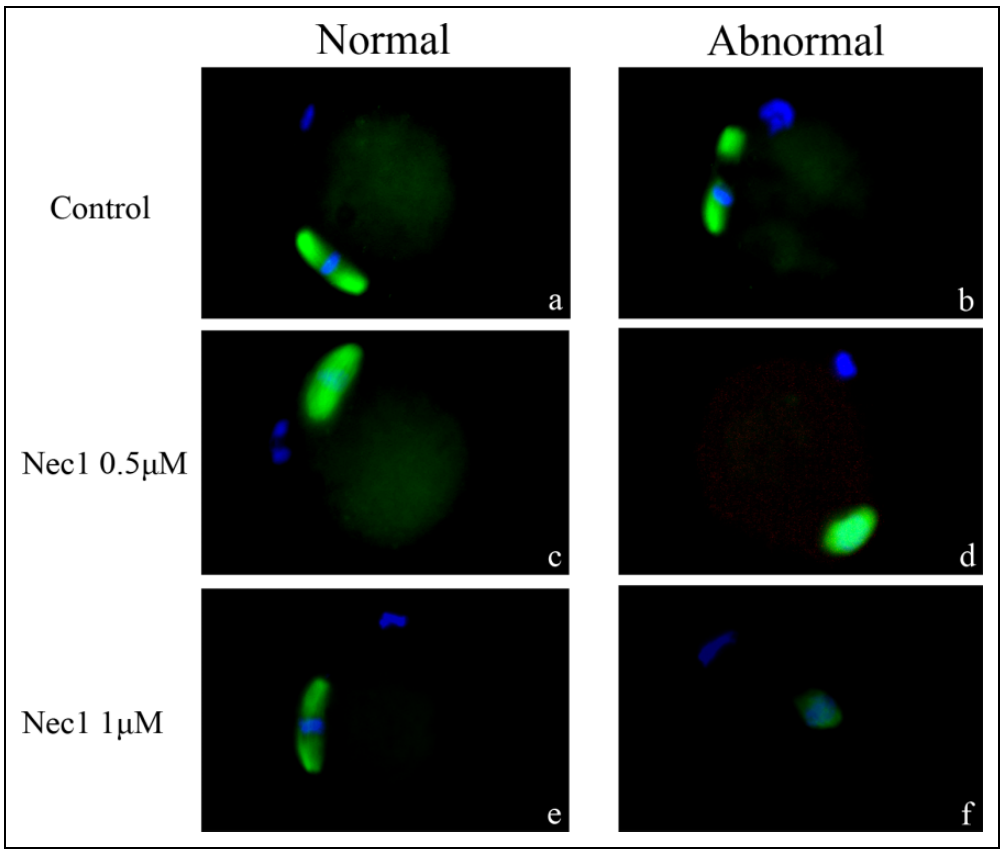

Figure 1. Representative microphotographs showing morphology of meiotic spindle organization and chromosome alignment in vitrified-warmed oocytes (×400). Nec1 indicates necrostatin 1.

groups. Each of mRNA sample used 5 oocytes. All extraction steps were completed according to the manufacturer's instructions. After extracting and purifying RNA, we synthesized complementary DNA (cDNA) from the mRNA using 50 ng/mL random hexamer primers designed following the SuperScript protocol. When calculating the amount of mRNA in groups, we used iCycler (Bio-Rad, Hercules, California) to assess our quantitative real-time polymerase chain reaction (PCR) results. We used the iCycler iQ real-time detection system software to evaluate the results. The reaction mixture contained 20 pmol of primers (reverse and forward), cDNA, and SYBR Green Supermix2 (50 U/mL iTaq DNA polymerase, 6 mmol/L $MgCl_2$, 100 mmol/L KCl, SYBR Green I, 40 mmol/L Tris-HCl, pH 8.4, 20 nmol/L fluorescein, 0.4 mmol/L of each deoxynucleotide triphosphate, and stabilizers). Templates were amplified for 45 cycles as follows: denaturation at 95°C for 10 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 20 seconds. Until the PCR amplifications were completed, we continuously monitored the fluorescence while slowly heating the samples from 55°C to 95°C at 0.5°C intervals. To identify any nonspecific amplification products, we used the melting curves obtained. We performed quantitation of gene amplification to determine the cycle threshold (C_T) that was based on fluorescence detected within the geometric region of the semilog amplification plot. All mRNA expressions were normalized to that of mouse glyceraldehyde 3-phosphate dehydrogenase mRNA. We used the comparative C_T method to evaluate relative quantitation of target gene expression. The experiments were repeated at least 6 times using different sets of oocytes. All sequence information in these experiments are presented in Table 1.

Differential Staining of Blastocysts

Blastocysts were fixed in 4% paraformaldehyde (Sigma) for 20 minutes at RT and stored in PBS containing 0.5% BSA (Sigma) at 4°C until the staining was performed. The blastocysts were incubated overnight in 0.5% Triton X-100 (Sigma) in PBS at 4°C. Subsequently, the blastocysts were washed 3 times for 2 minutes in PBS–BSA. On the second day, blastocysts were transferred to the blocking solution. Blocking occurred overnight in 10% goat serum (Invitrogen) in PBS at 4°C. The test blastocysts were washed and incubated in the ready-to-use primary OCT3/4 antibody (Invitrogen) for 1 day at 4°C. After another wash step (2 times 15 minutes), blastocysts were labeled with secondary Alexa 594-conjugated antibody diluted 1:100 (Invitrogen) for 1 day at 4°C. After washing in PBS, the samples were mounted onto a slide under a coverslip in Vectorshield mounting medium (Vector Laboratories) containing 0.5 μg/mL of DAPI.

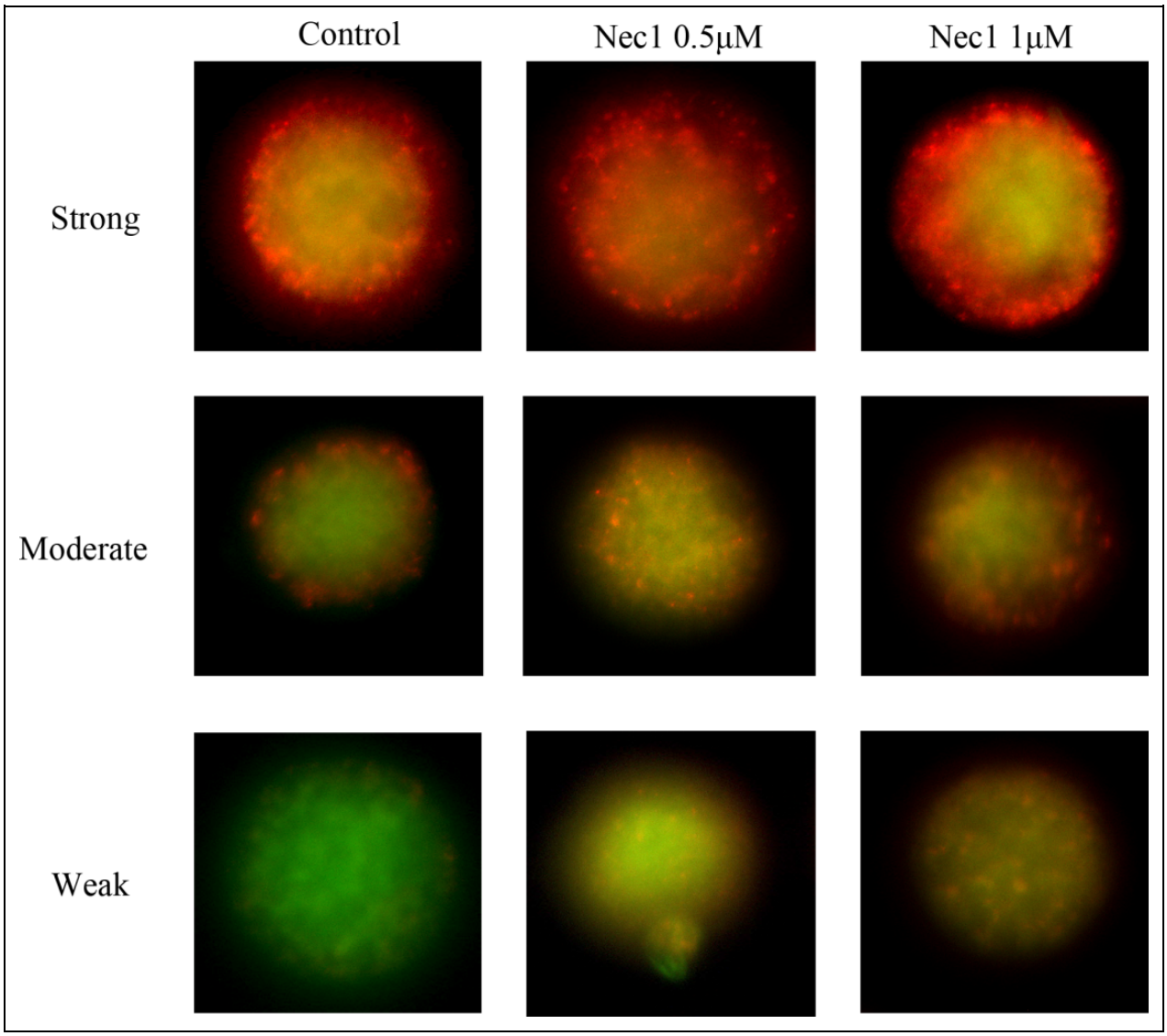

Figure 2. Microphotographs showing fluorescent JC-1 staining of oocytes derived from vitrified-warmed mouse oocytes. Upper panel, strong grade. Medium panel, moderate grade. Under panel, weak grade.

Caspase Staining of Blastocysts

We stained the apoptotic parts of blastocysts using the CaspaTag Pan Caspase in situ assay kit according to the manufacturer's instructions (Millipore, New Bedford). To set up positive controls, blastocysts were immersed in 0.1% (v/v) H_2O_2 in PBS for 1 minute. Negative controls were not exposed to the dye used for staining; instead, the blastocysts were incubated in PBS only. Stained blastocysts were mounted on slides and examined using fluorescence microscopy. Caspase-positive cells were identified by their green fluorescence, and nuclei were identified by DAPI staining. Caspase positivity was measured as the ratio of caspase-positive blastomeres to the total number of nuclei (Figure 3).

Statistical Analyses

These experiments of developmental competence and spindle staining were repeated at least 4 times with different pools of samples. The membrane integrity and mitochondrial membrane potential test were repeated at least 3 times. Means and standard deviations were compared using the Student *t*-test. The proportions were compared using the chi-square tests. Relative mRNA abundance differences in control group and Nec1-treated groups were analyzed using 1-way analysis of variance (ANOVA) test. Significant results in the ANOVA were further analyzed by the Tukey test. Differences were considered statistically significant when $P < .05$. All data were analyzed using the SPSS for Windows (Version 18.0; SPSS, Inc, Chicago, Illinois).

Table 1. Primer Sequences and Their Condition for Real-Time PCR Analysis.

Gene	Primer Forward (5′-3′) Primer Reverse (5′-3′)	Accession No.	Annealing Temp, °C	Cycles
Cirp	cca agt atg ggc aga tct ccg a ctg ccg ccc gtc cac aga ct	NM_007705	55	45
Rbm3	agc ttt ggg cct atc tct gag g ccc atc cag gga ctc tcc at	NM_016809	55	45
Bax	tgc aga gga tga ttg ctg ac gat cag ctc ggg cac ttt ag	NM_007527	55	45
Bcl2	ttc ggg atg gag taa act gg tgg atc caa ggc tct agg tc	X83574	55	45
Mad2	aag tcc cag aaa gcc ata cag tca gca gat caa agg aac aag a	U83902	55	45
Mtgenome	ggg cat gag gag gac tta ac tag agt gag gga tgg gtt gta	V00711	55	45
MtDNA9	act atc ccc ttc ccc att tg tgt tgg tca tgg gct gat ta	U47506	55	45
Gdf9	tcc caa acc cag cag aag tc gga gga gga aga ggc aga gtt g	NM_008110	55	45
GAPDH	gtg gag tca tac tgg aac atg tag aat ggt gaa ggt cgg tgt g	NM_008084	55	45

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction.

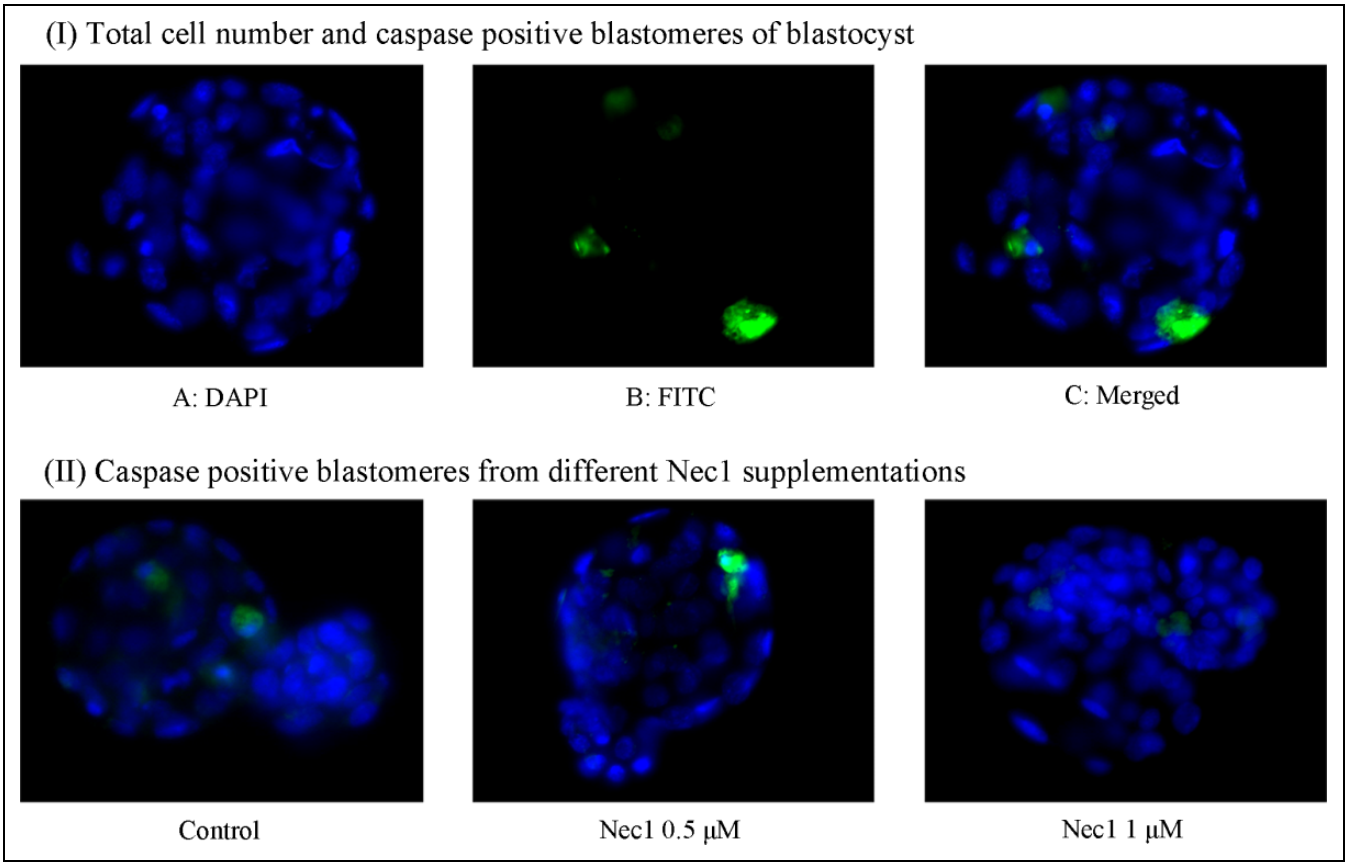

Figure 3. Microphotographs showing fluorescent caspase staining of blastocysts derived from vitrified-warmed mouse oocytes. (I) Apoptotic blastomeres appear green; (II) caspase-positive blastomeres from different Nec1 supplementations and control group (×400). (I, A) 4′,6-diamidino-2-phenylindole (DAPI), (B) fluorescein isothiocynate (FITC), (C) merged; (II) control (without Nec1), Nec1 0.5 μmol/L, and Nec1 1 μmol/L. Nec1 indicates necrostatin 1.

Table 2. Developmental Competency of Vitrified-Warmed Mouse Oocyte.[a]

	Control (Nonvitrified)	Control (Without Nec1)	Nec1 0.5 μmol/L	Nec1 1 μmol/L
Initiated GV oocyte	214	181	245	256
Matured	185 (86.4%)	154 (85.1%)	225 (91.8%)[b]	236 (92.2%)[b]
Survived	–	138 (89.6%)	213 (94.7%)	226 (95.8%)[b]
Cleaved (per survived)	168 (90.8%)	92 (66.7%)	164 (77.0%)[b]	170 (75.2%)
Blastocyst (per cleaved)	131 (77.9%)	21 (22.8%)[c]	55 (33.5%)[c]	56 (32.9%)[c]
Blastocyst (per survived)	–	21 (11.6%)	55 (22.5%)[b]	56 (21.9%)[c]
Blastomere count per blastocyst	73.3 ± 12.7[d]	62.5 ± 11.0[e]	66.3 ± 11.4[f]	65.3 ± 9.1[g]
No. of caspase positive blastomeres	91/1539 (5.9%)	51/687 (7.4%)	130/2123 (6.1%)	125/2284 (5.5%)
No. of trophectoderms in blastocyst	68.1 ± 9.7[h]	47.9 ± 7.2[i]	51.3 ± 7.0[j]	56.2 ± 7.0[k]
No. of inner cell masses in blastocyst	11.5 ± 2.5[h]	11.9 ± 1.7[i]	12.5 ± 1.8[j]	11.7 ± 1.3[k]

Abbreviations: GV, germinal vesicle; Nec1, necrostatin 1; SD, standard deviation.
[a]Each group comprises 4 replicates. Mean ± SD. Number of blastocyst tested: [d] = 21 [e] = 11, [f] = 32, [g] = 35, [h] = 19, [i] = 10, [j] = 23, and [k] = 21.
[b]$P < .05$ versus control (without Nec1).
[c]$P < .05$ versus control (nonvitrified).

Table 3. Meiotic Spindle Organization and Chromosome Alignment of Vitrified-Warmed Mouse Oocyte Supplemented With Necrostatin 1.[a]

	Control (Nonvitrified)	Control (Without Nec1)	Nec1 0.5 μmol/L	Nec1 1 μmol/L
No. of oocytes examined	84	78	74	83
Normal	81 (96.4%)	56 (78.8%)[c]	59 (79.7%)[c]	58 (69.9%)[c]
Abnormal	3 (3.6%)	22 (21.2%)	15 (20.3%)	25 (30.1%)

Abbreviation: Nec1, necrostatin 1.
[a]Each group comprises 4 replicates.
[b]$P < .05$ versus control (without Nec1).
[c]$P < .05$ versus control (nonvitrified).

Results

Effect of Nec1 on the Developmental Competence of Oocytes

Oocyte maturation rates were significantly higher in the Nec1-treated groups (91.8%, 0.5 μmol/L Nec1; 92.2%, 1 μmol/L Nec1) than in the untreated group (85.1%) and nonvitrified control group. These results suggest that supplementing the IVM medium with Nec1 alleviates the detrimental effects of environmental factors during the IVM process. This was especially true for Nec1 used at 1 μmol/L. After IVM, successfully matured oocytes were vitrified and warmed. In this study, we determined that oocyte survival rate in the group treated with 1 μmol/L Nec1 was significantly higher than that in the untreated group (95.8% vs 89.6%). After warming, the oocytes that survived and nonvitrified control group were fertilized with motile sperm in IVF medium for 6 hours. After washing off the sperm, the oocytes were transferred to IVF medium and incubated. After incubation for 1 day, the cleavage rates were determined to be significantly higher in the group treated with 0.5 μmol/L Nec1 than in the control (without Nec1) group (77.0% vs 66.7%). Treatment with 1 μmol/L Nec1 resulted in a higher cleavage rate than control (without Nec1), but it was not significantly different from the 0.5 μmol/L Nec1 group (75.2% vs 77.0%; 1 μmol/L Nec1 vs 0.5 μmol/L Nec1). Cleaved embryos were transferred to cleavage media and maintained for 4 days until they reached the blastocyst stage. Blastocyst formation rates (blastocyst per surviving oocyte) were significantly higher in the 0.5 and 1 μmol/L Nec1-treated groups than in the control (without Nec1) group (22.5% and 21.9% vs 11.6%). Total blastomere and trophectoderm and inner cell mass cell counts were similar across the control groups and Nec1-treated groups. Caspase positivity was slightly decreased in the Nec1-treated groups (Figure 3), but this was not statistically different (Table 2).

Effect of Nec1 on the Meiotic Spindle and Chromosome Morphology of Vitrified-Warmed Oocytes

We observed similar proportions of oocytes with normal spindle and chromosome morphologies in the control (without Nec1) and Nec1-treated groups (78.8% vs 79.7% vs 69.9%; control vs 0.5 μmol/L Nec1 vs 1 μmol/L Nec1). However, experimental groups (Nec1-treated groups) did not reach the results of nonvitrified control group (normal spindle: 96.4%). These results reflected that the cleavage rates after fertilization were not corroborated by adding Nec1 during IVM. Hence, our results suggest that adding Nec1 during IVM does not enhance spindle protection and chromosome conservation after vitrification (Table 3).

Effect of Nec1 on Membrane Integrity

We treated oocytes with FDA and PI to determine the "live and intact" status and "live with some of damage" status of

Table 4. Membrane Integrity of Vitrified-warmed Mouse Oocyte Supplemented with Necrostatin1.[a]

	Control (Nonvitrified)	Control (Without Nec1)	Nec1 0.5 μmol/L	Nec1 1 μmol/L
No. of oocytes examined	91	72	102	70
Live (intact), %	89 (97.8%)[c]	62 (86.1%)[c]	88 (86.3%)[c]	60 (85.7%)[c]
Live (damaged), %	2 (2.2%)	10 (13.9%)	14 (13.7%)	10 (14.3%)

Abbreviation: Nec1, necrostatin 1.
[a]Each group comprises 3 replicates.
[b]$P < .05$ versus control (without Nec1).
[c]$P < .05$ versus control (nonvitrified).

Table 5. Mitochondrial Integrity of Vitrified-Warmed Mouse Oocyte Supplemented With Necrostatin 1.[a]

	Control (Nonvitrified)	Control (Without Nec1)	Nec1 0.5 μmol/L	Nec1 1 μmol/L
Strong	41 (78.8%)	27 (39.1%)[b]	44 (59.5%)[b,c]	41 (58.6%)[b,c]
Moderate	8 (15.4%)	24 (34.8%)[b]	24 (32.4%)[b]	19 (27.1%)
Weak	3 (5.8%)	18 (26.1%)[b]	6 (8.1%)[c]	10 (14.3%)
Total	52	69	74	70

Abbreviation: Nec1, necrostatin 1.
[a]Each group comprises 3 replicates.
[b]$P < .05$ versus control (nonvitrified).
[c]$P < .05$ versus control (without Nec1).

oocytes. Live and intact oocytes exhibited only green fluorescence, whereas oocytes that were alive but had damaged parts presented both green and red fluorescence.[22] The results of membrane integrity tests showed that the percentages of live (intact) oocytes in the control (without Nec1) and experimental groups were similar (86.1%, 86.3%, and 85.7%). However, experimental groups (Nec1-treated groups) did not reach the results of nonvitrified control group (intact live oocytes: 97.8%; Table 4).

Effect of Nec1 on Mitochondrial Membrane Potential

We evaluated the mitochondrial membrane potential ($\Delta\Psi m$) in mouse oocytes using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide); for these experiments, we used warmed oocytes that had survived. Previously, $\Delta\Psi m$ was reported to be a sensitive index to measure oocyte damage, which is because $\Delta\Psi m$ is easily affected by the environmental conditions.[23] The specific characteristics of JC-1 are the following. When the mitochondrial membrane potential is a low, JC-1 exists as a monomer, and green fluorescence can be detected in the FITC channel. By contrast, when the potential increases, JC-1 monomers assemble into arrays termed J-aggregates, which exhibit red fluorescence; thus, at high a mitochondrial membrane potential ($\Delta\Psi m$), red fluorescence is detected in the RITC channel.[24] JC-1 is an especially suitable probe for measuring $\Delta\Psi m$ because the red fluorescence and green fluorescence of JC-1 rely only on $\Delta\Psi m$ and are unaffected by other factors such as the size of oocytes and the number mitochondria. In the test for mitochondrial integrity, significantly higher proportions of oocytes with strong red fluorescence were observed in the 0.5 and 1 μmol/L Nec1 groups than in the control group (without Nec1). In the 0.5 μmol/L Nec1-treated group, the weak red fluorescence signals were significantly lower than the control (without Nec1) and 1 μmol/L Nec1-treated groups. However, experimental groups (Nec1-treated groups) did not reach the results of nonvitrified control group in strong signal proportion (Table 5).

Expression of Candidate Target Genes in Mouse Oocytes After Vitrification

Expression levels of *Mad2*, *Gdf9*, and *Bcl2* were significantly higher in the 1 μmol/L Nec1-treated group than in the control (without Nec1) or 0.5 μmol/L Nec1-treated groups. By contrast, the expression of the *Mtgenome* was significantly lower in both Nec1 groups. All candidate target genes were analyzed using real-time PCR (Figure 4).

Discussion

When IVF centers retrieve immature oocytes, they usually prefer to perform the IVM first and then the vitrification at MII.[8,9] Because most researchers think that GV-stage oocyte vitrification cause a loss of maturation capability, in the present study, we showed that pretreatment with Nec1 during IVM might be beneficial to improve the developmental competence of oocytes. Because of Nec1 treatments, our oocyte vitrification protocol could be more effective in vitrified-warm oocyte survival rates. For deciding the concentration of Nec1 to oocyte, we divided 4 groups (0.1, 0.5, 1, and 5 μmol/L, respectively). In our results, 100 nmol/L could not improve the maturation and survival rates compared to 0.5 and 1 μmol/L. In addition, 5 μmol/L group showed the lower survival rate compared to 1 μmol/L groups. According to these results, we performed the

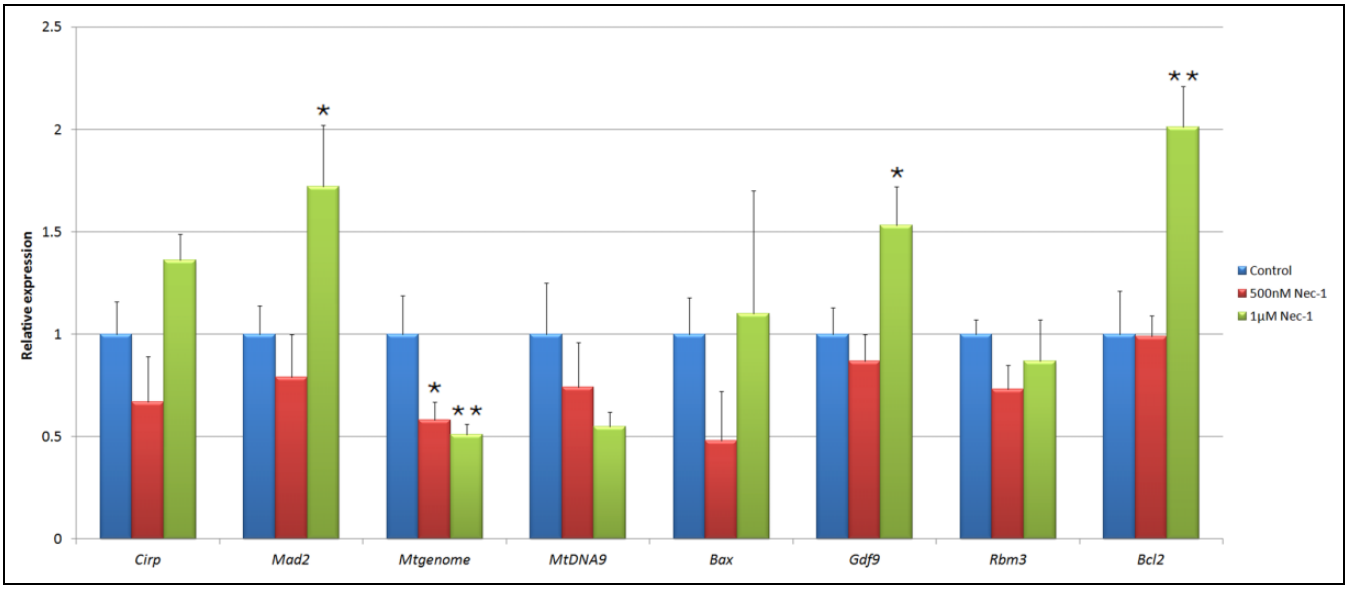

Figure 4. Quantitative real-time polymerase chain reaction (PCR) analysis of candidate genes from vitrified-warmed mouse metaphase II (MII) oocytes.

further study by nontreated control, 0.5 μmol/L Nec1, and 1 μmol/L groups, respectively.

Once we determined the concentration ranges of Nec1, we needed to test to decide the effective periods when we exposed the oocytes to Nec1. First of all, we added Nec1 to vitrification medium only following IVM. In this experiment, we did not obtain the positive effects on oocyte survival rates after warming (87.3%, 0.5 μmol/L Nec1; 86.8%, 1 μmol/L Nec1). Second, we supplemented Nec1 to IVM and vitrification medium. At this time, enhanced maturation (90.7%, 0.5 μmol/L Nec1; 93.3%, 1 μmol/L Nec1) and survival rates (90.8%, 0.5 μmol/L Nec1; 91.3%, 1 μmol/L Nec1) were observed compared to nontreated control group. Finally, we added Nec1 to IVM medium only. The last experiment denoted that exposing Nec1 to oocytes during IVM presented the highest survival rate (94.7%, 0.5 μmol/L Nec1; 95.8%, 1 μmol/L Nec1) and developmental competence. Interestingly, the first experiment could not show enhanced survival and fertilization rates. It might be explained that enough exposing time of Nec1 to oocyte was essential to improve the results. In addition, second test showed elevated survival and fertilization rates compared with the first test. However, it did not reach the results of survival and developmental competence of the third test. It could be suggested that optimal exposure of oocyte to Nec1 might be important. Hence, we adapted Nec1 to IVM medium only for further studies.

In the present study, significantly higher proportions of oocytes with maturation and survival rates were observed in the Nec1-treated group. This result suggests that Nec1 supplementation could help to preserve the oocyte from a variety of harsh conditions during the oocyte maturation periods and subsequent vitrification. This is the first study on the effect of Nec1 pretreatment on oocyte vitrification outcomes, and improved oocytes survival and fertilization and subsequent embryonic development were observed in the Nec1-treated groups.

In our previous reports, vitrification and warming procedures affected the spindle-chromosome integrity and membrane stability.[22,25] Especially, antifreeze protein supplementation of the vitrification medium brought protective effects on vitrified-warmed oocytes.[25] However, with regard to the results of spindle and membrane integrity in this report, Nec1 supplementation during IVM was not effective in protecting oocytes from the vitrification procedure. All groups presented similar results of normal or abnormal percentages of spindle-chromosome morphology. In addition, in the membrane integrity test, live but damaged oocytes were present in the same proportion among the groups. Hence, Nec1 supplementation might not protect the spindle and membrane in oocytes during vitrification.

Mitochondria play an important role in the metabolism of energy-containing compounds in the oocyte cytoplasm to provide adenosine triphosphate (ATP) for fertilization and preimplantation embryo development. A higher proportion of oocytes with strong periphery red fluorescence were observed in both Nec1-treated groups (Table 5). From these results, it can be inferred that Nec1 supplementation was beneficial in maintaining the mitochondrial membrane potential after warming.

The expression of various genes within the oocyte can be modulated by vitrification.[26] *Mad2* is a meiotic checkpoint protein that regulates anaphase onset and genome integrity in the oocyte.[27,28] In this study, we observed significantly higher levels of *Mad2* transcripts in oocytes treated with 1 μmol/L Nec1 than in untreated oocytes, suggesting that Nec1 elevates Mad2 mRNA expression. However, in the 0.5 μmol/L Nec1-treated group, the *Mad2* transcript level was slightly lower than that in the control (without Nec1). Considering our spindle and chromosomal results, Nec1 pretreatment during IVM might not be related to *Mad2* expressions and spindle conservation after vitrification.

Growth factor 9 (*Gdf9*) is a kind of growth factor made by ovarian somatic cells. It could affect oocyte for growth or function. During early folliculogenesis, *Gdf9* is considered as essential factor to complete ovarian growth.[29] In addition, *Gdf9* had various functions such as granulose cell, theca cell, and oocyte maturation.[30] In this study, we found that significantly higher expression levels of *Gdf9* in oocytes treated with 1 μmol/L Nec1 than in control (without Nec1) group. It suggested that potentially upregulated *Gdf9* at 1 μmol/L Nec1 group might help a more successful maturation and have better quality on immature oocyte.

Apoptosis-related genes such as *Bcl2* and *Bax* were examined in this study. *Bcl2* is an antiapoptotic gene that has been reported to promote cell survival by suppressing apoptosis.[31] Conversely, *Bax* is activated under proapoptotic conditions, and *Bax* function accelerates cell death through apoptosis.[32] In our study, *Bcl2* expression was significantly upregulated in the 1 μmol/L Nec1-treated group, but in the 0.5 μmol/L Nec1-treated group, it was similar to that in the control (without Nec1). *Bax* had a tendency to be downregulated in 0.5 μmol/L Nec1-treated groups and the 1 μmol/L Nec1-treated group showed similar expressions to that in the control (without Nec1) group (not significantly different). It could be assumed that 0.5 μmol/L was profitable considering normal theory. Although 1 μmol/L Nec1-treated group showed upregulated *Bax* expression compared with 0.5 μmol/L Nec1-treated group, developmental competency of 1 μmol/L Nec1 was better than 0.5 μmol/L Nec1. It might be presumed that more upregulated *Bcl2* expression offsets the upregulated *Bax*. Taken together, these results suggest that Nec1 may affect *Bcl2* expressions after vitrification and gives some protective effect to vitrified-warmed oocytes.

In this study, adding Nec1 to the medium did not affect the expression of *Cirp* and *Rbm3* in oocytes. This finding might be explained by the preservation of *Cirp* and *Rbm3* mRNA levels. *Cirp* and *Rbm3* are genes related to cold injury. *Cirp* has been recently introduced into mouse somatic cells under the cold condition of 32°C in vitro, and these 2 genes might have cryoprotective effects on cells.[33,34]

Thus, we examined whether Nec1 affects these genes and found that at concentrations of 0.5 and 1 mmol/L, Nec1 did not affect their expression relative to the controls. Interestingly, both *Cirp* and *Rbm3* expression levels were downregulated in 0.5 μmol/L Nec1-treated group compared with control (without Nec1) group and upregulated in 1 μmol/L Nec1-treated group. It might be assumed that optimal concentration of Nec1 had to be used for regulating gene expression. Further studies are needed to establish the gene expression patterns according to Nec1 concentrations.

The well-reserved mitochondria after cryopreservation is an important factor for further development.[35] In our present studies, mitochondria-related gene such as *Mtgenome* was investigated. The expression of *Mtgenome* was significantly decreased in both Nec1-treated groups. Surprisingly, the $\Delta\Psi$m staining results noted that both Nec1-treated groups had a relatively higher $\Delta\Psi$m value. It is possible to explain this, as a previous study by Steuerwald et al reported that the mitochondrial DNA copy number was increased in the oocytes of older women.[36] Such increase in mitochondrial biogenesis was attributed to a compensatory phenomenon to guarantee sufficient ATP production in the event of either an increased demand or a respiratory chain dysfunction. Considering these results, the decreased *Mtgenome* expression levels in both Nec1-treated groups, compared with the control (without Nec1) group, might be profitable consequences, although we did not perform the Western blotting tests. Because properly protected mitochondria in oocyte from vitrification and warming processes were already sufficient in the cytoplasm of Nec1-treated oocytes, it might be possible that gene such as *Mtgenome* might be potentially downregulated at gene levels.

In conclusion, we have demonstrated positive effects of Nec1 on the IVM and vitrified mouse oocytes for the first time. Supplementing IVM medium with Nec1 increased the maturation, survival, IVF, and embryonic developmental competence of immature oocytes. Thus, Nec1 might exhibit a cryoprotective effect under harsh in vitro conditions. The Nec1 was also observed to stabilize mitochondrial function in oocytes and affect the expression of various genes. Therefore, the positive effects of Nec1 on the survival and developmental competence of oocytes might be explained by enhanced mitochondrial integrity. Furthermore, Nec1-dependent modulation of the expression of *Mad2*, *Gdf9*, *Mtgenome*, and *Bcl2* may increase the fertilization and developmental competence of oocytes.

Authors' Note

JWJ contributed to conception and design, data acquisition, analysis, interpretation, drafting, and revision of the article. BCJ and JRL contributed to conception and design, data analysis, interpretation, and revision of the article. CSS contributed to conception and design, data analysis, interpretation, revision, and final approval of the article. SHK contributed to conception and design, data analysis, interpretation, and revision of the article. The submission of our manuscript has been approved by all of the authors. The authors have no actual or potential conflict of interest.

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