

**EFFECTS OF VITRIFICATION TEMPERATURES AND
CONCENTRATION OF CRYOPROTECTIVE AGENTS ON
THE CYTOSKELETON IN BOVINE IMMATURE OOCYTES**

**Shiyu Zhao, Jiaqi Liu, Xiaojing Wang, Yiheng Liu,
Xiaoxia Li, Zhiqian Xu, Yinghua Li, Xueli Yu[#]**

Received on December 13, 2022

Presented by H. Najdenski, Corresponding Member of BAS, on February 28, 2023

Abstract

The objective of the research was to evaluate the effect of vitrification temperatures (VTs) and concentration of cryoprotective agents (CPAs) on the cytoskeleton of bovine immature oocytes (BIOs). Bovine cumulus oocyte complexes (COCs) were collected and divided randomly into five groups: (a) fresh control group, (b) oocytes vitrified in liquid helium (LHe; -269°C with 5.6 M CPAs, (c) oocytes vitrified in LHe (-269°C with 6.6 M CPAs, (d) oocytes vitrified in liquid nitrogen (LN; -196°C with 5.6 M CPAs, (e) oocytes vitrified in LN (-196°C with 6.6 M CPAs. The results showed that the developmental level of BIOs in the LHe 5.6 M group was the highest among the four vitrification groups, and the maturation rate and cleavage rate were significantly higher than the other three vitrification groups ($P < 0.05$). With further experiments, the normal rate of microfilament and spindle in the LHe 5.6 M group was the highest compared with the other three vitrification groups. It was concluded that lower VT (-269°C and lower CPAs (5.6 M) could significantly reduce damage to the cytoskeleton, which had a positive effect on the development of oocytes after vitrification.

Key words: oocyte, cytoskeleton, vitrification temperatures, concentration of cryoprotective agents

[#]Corresponding author.

This project was supported by the National Natural Science Foundation of China (Grant No 31872354).

DOI:10.7546/CRABS.2023.04.18

Introduction. For mammals, the available oocytes especially large ruminants are very limited. Cryopreservation showed great improvement in the utilization of oocyte resources. Vitrification of oocytes is a powerful method to protect the genetic diversity of endangered animal species and farm animals by establishing oocyte banks [1]. Compared with slow freezing, vitrification damages cells less and does not require expensive equipment [2]. Therefore, vitrification technology has been one of the best choices for the cryopreservation of oocytes in recent years. However, compared to sperms [3] and embryos [4,5], oocytes were a challenging sample because of their huge size and the complicated internal structure. Liquid nitrogen (LN) was used for the vitrification in most studies, in which working temperature was -196°C . YU et al. [6] used liquid helium (LHe) for vitrification of oocytes for the first time, and the working temperature of LHe was -269°C . Cryoprotective agents (CPAs) are a compound solution that protects cells during vitrification. To achieve vitrification, oocytes were exposed to a high concentration of cryoprotective agents and frozen at a very high cooling rate, resulting in an ice-free, amorphous vitrification state. In our team's previous research, different concentrations of cryoprotective agents (5.6 M and 6.6 M) could affect the expression of some mRNA transcriptome on the cytoskeleton in bovine immature oocytes (BIOs) after vitrification. The cytoskeleton was distributed in the cytoplasm of the oocyte. The developmental competence of oocytes was an extremely complex dynamic process, in which organelles need to migrate and relocate. Microfilaments and microtubules played an extremely important role in these life activities. The subsequent developmental competence of oocytes after vitrification and warming was still far less compared to that of fresh oocytes, showing a lower maturation rate, a lower fertilization rate, and even abnormal embryonic development in the later stage. This was closely associated with the physical and chemical properties and structural changes of oocytes, and the influence of vitrification on the cytoskeleton was one of the important factors that cannot be ignored. Therefore, the objective of this research was to evaluate the effect of two kinds of VTs (-269°C and -196°C and two kinds of CPAs (6.6 M and 5.6 M) on the cytoskeleton of BIOs.

Materials and methods. All chemicals were obtained from Sigma Chemical Company (St.). Open Pulled Straw (OPS) were prepared in our laboratory by using 0.25 mL French straws (IMV, L'Aigle, France).

Oocyte collection. For COCs collection, ovarian follicles between 2–8 mm in diameter were punctured using a 12-gauge needle set to a 10 ml syringe. Only COCs with three or more layers of cumulus cells and which displayed homogeneous cytoplasm were selected for this experiment's further processing. The COCs were selected and randomly assigned to the following experimental groups: (a) fresh control group. (b) LHe5.6M group, (c) LHe6.6M group, (d) LN5.6M group, (e) LN6.6M group.

Experiment 1. Oocytes were processed for LHe vitrification and LN vitri-

fication cooling according to CHEN et al. [7]. The morphology, maturation rate, and cleavage rate of frozen-thawed oocytes were examined and the developmental capacity of oocytes after in vitro maturation (IVM) and in vitro fertilization (IVF) were evaluated, respectively.

In vitro maturation (IVM) of COCs. An appropriate amount of mature medium (TCM-199 supplemented with 10% [v:v] FBS and 3% [v:v] follicular fluid) was taken and 5 microdroplets (50 μ L) were made in a sterile Petri dish, and an appropriate amount of mineral oil was injected to completely cover the microdroplets. Each group of COCs was carefully transferred into the droplets after washing, and 20 ~ 25 COCs were put in each droplet. Subsequently, they were transferred to a CO₂ incubator (conditions set at 38.5 °C, 100% humidity, and 5% CO₂ concentration) for 22–24 h. Cumulus-free oocytes with visible first polar body (PB) were defined as MII oocytes.

In vitro fertilization (IVF). Oocytes were washed three times with IVF medium (consisting of 6 mg/mL bovine serum albumin, 10 IU/mL heparin, and 5 mM caffeine), transferred to a sperm suspension (50 μ L) under mineral oil for fertilization (approximately 20 oocytes/drop), and then incubated at 38.5 °C with 5% CO₂ in air at maximum humidity for 20 h. The concentration of sperm (Nanyang yellow cattle) used for fertilization was 1×10^6 sperm/mL.

Experiment 2. All microfilament distribution, microtubule distribution, and chromosome arrangement were evaluated through immunofluorescent staining after in vitro maturation. There were five replicates performed for each treatment (see below for details). After IVM, spindle morphology, chromosome morphology, and microfilament morphology were classified according to the criteria described by ROJAS et al. [8].

Staining of microtubule (assemble spindle). Each group of oocytes was fixed in 4% paraformaldehyde for 1 h at room temperature, followed by penetration with 1% Triton X-100 for 1 h. Then oocytes were blocked with 2% BSA at 4 °C overnight. Then the oocytes were incubated with an anti- α -tubulin antibody (Santa Cruz, sc-23948) diluted in PBS at a concentration of 1:100 at 4 °C for 10 h. Subsequently, the oocytes were incubated with fluorescein-conjugated Goat Anti-Mouse IgG (Wuhan Sanying, China) diluted in PBS at a concentration of 1:80 at room temperature for 2 h in dark conditions. The chromosomes were stained with Hoechst 33342 diluted in PBS at a concentration of 10 μ g/mL at room temperature for 10 min.

Staining of microfilament. Each group of oocytes was fixed in 4% paraformaldehyde for 1 h at room temperature, followed by penetration with 1% Triton X-100 for 1 h. Then oocytes were blocked with 2% BSA at 4 °C overnight. Then the oocytes were incubated with FITC-phalloidin (Shanghai Yisheng, China) diluted in PBS at a concentration of 1 μ g/mL at room temperature for 1 h in dark conditions. The chromosomes were stained with Hoechst 33342 diluted in PBS at a concentration of 10 μ g/mL at room temperature for 10 min.

T a b l e 1

Effects of VTs and CPAs on the developmental ability of BIOs ($n = 5$, mean $\pm$ SEM)

Group	No of oocytes	N/(%) Morphologically normal oocytes	N/(%) Maturation	N/(%) Cleavage
Fresh control	270	264/(97.8 $\pm$ 1.8) ^a	203/(75.2 $\pm$ 3.5) ^a	148/(54.8 $\pm$ 3.6) ^a
LHe5.6M	260	229/(87.9 $\pm$ 2.3) ^b	160/(61.4 $\pm$ 3.0) ^b	89/(34.5 $\pm$ 2.4) ^b
LHe6.6M	264	224/(84.8 $\pm$ 2.2) ^b	133/(50.4 $\pm$ 2.5) ^c	67/(25.4 $\pm$ 2.7) ^c
LN5.6M	268	195/(72.7 $\pm$ 2.1) ^d	76/(28.3 $\pm$ 3.4) ^e	23/(8.6 $\pm$ 1.9) ^e
LN6.6M	258	205/(79.5 $\pm$ 2.2) ^c	100/(38.8 $\pm$ 3.0) ^d	46/(17.8 $\pm$ 3.5) ^d

Different letters in the same column indicate significant differences ($P < 0.05$)

Abbreviations: LHe – liquid helium; LN – liquid nitrogen; SEM – standard error of the mean

Statistical analysis. Data were analyzed with SPSS 20.0 and evaluated using a one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results. Experiment 1. The morphological normal rate, maturation rate, and cleavage rate for the five groups were summarized in Table 1. The morphological normal rate, maturation rate, and cleavage rate in the LHe5.6M group were significantly higher compared to those in the LN5.6M group ($P < 0.05$). The morphological normal rate, maturation rate, and cleavage rate of the LHe6.6M group were significantly higher than those of the LN6.6M group ($P < 0.05$). Furthermore, the developmental level of BIOs in the LHe5.6M group was the highest among the four vitrification groups.

Experiment 2. The effects of VTs and CPAs on the microfilaments were shown in Table 2. The abnormal rate and deletion rate of microfilaments in the LHe5.6M group were significantly lower than those in the LN5.6M group ($P < 0.05$). In addition, the normal rate of microfilaments in the LHe5.6M group was the highest among the four vitrification groups. The details of microfilament distribution were shown in Fig. 1 (A1–A4). At the MII stage, microfilaments are mainly enriched around the first polar body (Fig. 1 A1).

T a b l e 2

Effect of VTs and CPAs on microfilaments of BIOs ($n = 5$)

Group	No of oocytes	Normal (%)	Abnormal (%)	None (%)
Fresh control	145	124(85.5) ^a	19(13.1) ^a	2(1.4) ^a
LHe5.6M	141	82(58.2) ^b	44(31.2) ^c	15(10.6) ^d
LHe6.6M	149	62(41.6) ^c	64(43.0) ^b	23(15.4) ^c
LN5.6M	150	39(26.0) ^c	78(52.0) ^b	33(22.0) ^b
LN6.6M	145	47(32.4) ^c	72(49.7) ^b	26(17.9) ^c

Different letters in the same column indicate significant differences ($P < 0.05$)

Abbreviations: LHe, liquid helium; LN, liquid nitrogen

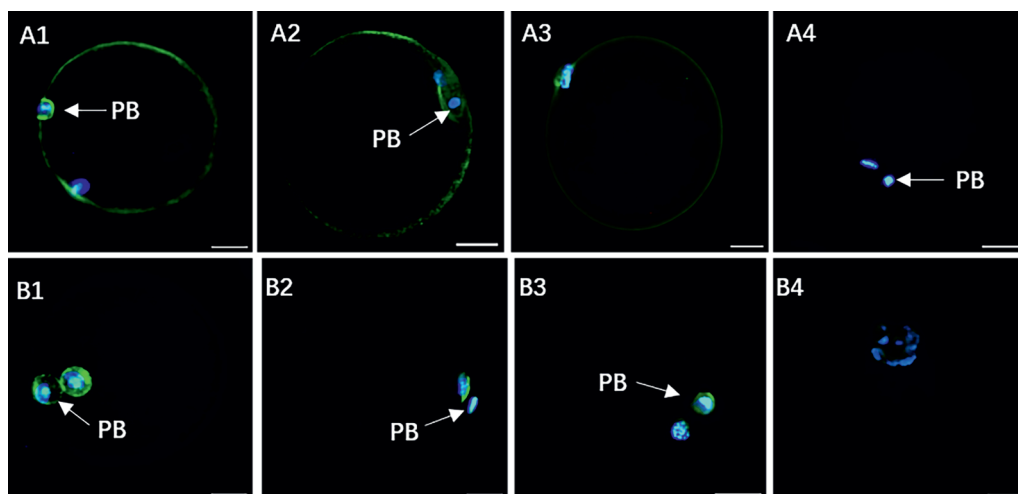


Fig. 1. Immunocytochemistry of microfilaments and spindle in bovine oocytes. Microfilaments and spindle were stained green, and chromosomes were stained blue. The arrows point to the polar bodies. Scale bar represents 20 μm . PB – polar body (A1) Normal microfilament. (A2) Dispersed microfilaments. (A3) Diffused microfilaments. (A4) Microfilaments are absent. (B1) Normal spindle and chromosomes. (B2) Shriveled spindle and disorganized chromosomes. (B3) Chromosomes aligned normally, but spindle microtubules almost disappeared. (B4) Dispersed chromosomes in the absence of spindles

The effects of VTs and CPAs on the microtubules were shown in Table 3. The abnormal rate and deletion rate of the spindles in the LHe5.6M group were significantly lower than those in the LN5.6M group ($P < 0.05$). The abnormal rate of spindle in the LHe6.6M group was significantly lower compared to those in the LN6.6M group ($P < 0.05$). The abnormal rate and deletion rate of chromosomes in the LHe5.6M group were significantly lower compared to those in the LN5.6M group ($P < 0.05$). The abnormal rate of chromosomes in the LHe6.6M group was significantly lower than that in the LN6.6M group ($P < 0.05$). The normal rate of spindle and chromosomes in the LHe5.6M group was the highest among the four vitrification groups. The details of spindle and chromosome distribution patterns are provided in Fig. 1 (B1–B4). At the MII stage, microtubules formed meiotic spindles around the first PB, and normal chromosomes clustered forming a discoid shape covered by microtubules (Fig. 1 B1).

Discussion. In recent years, people have been committed to improving the cryopreservation of mammalian oocytes through vitrification technology. However, the freezing effect of cells will be affected by various factors: animal species [9], different stages of the oocytes [10], type and concentration of cryoprotectant [11], and vitrification temperature [6]. In fact, a sudden drop in temperature could disrupt temperature-sensitive cell structures and thus affect their growth and developmental functions after thawing [12]. A high concentration of cryoprotectant could help oocytes avoid the formation of harmful ice crystals during freez-

T a b l e 3
Effects of VTs and CPA on spindles and chromosomes of BIOs ($n = 5$)

Group	No of oocytes	Percentage of spindle configuration			Percentage of chromosome alignment		
		Normal (%)	Abnormal (%)	None (%)	Normal (%)	Abnormal (%)	None (%)
Fresh control	157	142(90.4) ^a	13(8.3) ^a	2(1.3) ^a	141(89.8) ^a	11(7.6) ^a	5(3.2) ^a
LHe5.6M	146	103(70.5) ^b	25(17.1) ^d	18(12.3) ^d	105(71.9) ^b	25(17.1) ^d	16(11.0) ^c
LHe6.6M	144	82(56.9) ^{bc}	31(21.5) ^c	31(21.5) ^c	85(59.0) ^b	32(22.2) ^c	27(18.8) ^c
LN5.6M	150	60(40.0) ^c	47(31.3) ^b	43(28.7) ^b	59(39.3) ^c	50(33.3) ^b	41(27.3) ^b
LN6.6M	153	75(49.0) ^c	43(28.1) ^b	35(22.9) ^c	76(49.7) ^c	48(31.4) ^b	29(19.0) ^{bc}

Different letters in the same column indicate significant differences ($P < 0.05$)

ing [13]. Theoretically, the higher the concentration of cryoprotectant, the greater the chance of inducing cell vitrification. However, the higher the concentration of cryoprotective agents, the higher the toxicity and osmotic pressure of cells will be. At the same time, the rapid cooling rate of vitrification technology shortens the time that cells are exposed to sensitive temperatures [14]. In the early stage, our research team found that LHe could be used for vitrifying oocytes, which could significantly improve the developmental ability of oocytes after vitrification [15]. Compared with LN, LHe could provide a lower vitrification temperature, which means a higher vitrification rate. Therefore, we hypothesized that the development of vitrification-thawed oocytes would be positively affected by lowering the vitrification temperature and appropriately reducing the concentration of cryoprotective agents.

The damage of cytoskeleton fibres (CSF) after vitrification was still the main cause of vitrification, the rapid change of osmotic pressure disrupted the equilibrium, resulting in damage to the cytoskeleton, and the cytoskeletons were also very sensitive to low temperatures. Microfilaments and microtubules were extremely sensitive to changes in external temperature, which were the main structural components of the cytoskeleton, and when they were in a low-temperature state, it would cause the depolymerization of microtubules and the disordered distribution of microfilaments. Lower freezing temperatures protected oocytes from reaching the temperature range of transition to vitrification at a very fast rate (around -120°C). In addition, the greater cryoprotectant concentrations disrupted cortical microfilaments and caused depolymerization and disorganization of spindle microtubules, leading to chromosomal scattering [16]. In a study of cryopreservation of rhesus monkey oocytes, it was found that the different concentrations of cryoprotective agents could cause different degrees of depolymerization of microfilaments and microtubules [17]. Excessively high cryoprotectant concentrations stimulated a transient increase in intracellular Ca^{2+} , which turns on blocking mechanisms in the plasma membrane or zona pellucida to impede fertilization. Vitrification and warming also resulted in immature oocytes showing 30–50% lysis within the cumulus cell, with necrosis of the nuclei, lysis of the cellular membrane, and swollen mitochondrial cristae and endoplasmic reticulum [18]. Some previous reports indicated that it might be that vitrification disrupted the cell membrane and increased the influx of Ca^{2+} [19]. The increased Ca^{2+} concentration induced a cascade reaction that activated protease C, which further caused changes of skeleton protein, affecting its reconstruction ability and further leading to the disruption or even loss of the skeletal fibre network [20].

In the present study, the results of immunofluorescence staining showed that the distribution of microfilaments and spindle bodies in vitrification-thawed oocytes was abnormal and even deleted. The damage to the cytoskeleton caused by low temperature and osmotic pressure will lead to incorrect migration of organelles, thus interfering with the normal self-repair and development capacity of oocytes.

This would affect the maturation of BIOs and sperm-egg binding, which could explain the reduction in the maturation and cleavage rates of mature in vitro BIOs after vitrification and thawing in Experiment 1.

Conclusion. In conclusion, VTs and CPAs had significant effects on the developmental capacity and cytoskeleton of vitrifying BIOs. When VTs were -269°C and CPAs were 5.6 M, the abnormal distribution of microfilaments and microtubules could be effectively reduced and the damage to the cytoskeleton was minimized, which might be partly responsible for the increased developmental capacity of vitrifying oocytes.

REFERENCES

- [1] SRIPUNYA N., T. SOMFAI, Y. INABA, T. NAGAI, K. IMAI et al. (2010) A comparison of cryotop and solid surface vitrification methods for the cryopreservation of in vitro matured bovine oocytes, *J. Reprod. Dev.*, **56**(1), 176–181.
- [2] MARTÍNEZ-BURGOS M., L. HERRERO, D. MEGÍAS, R. SALVANES, M. C. MONTÓYA et al. (2011) Vitrification versus slow freezing of oocytes: effects on morphologic appearance, meiotic spindle configuration, and DNA damage, *Fertil. Steril.*, **95**(1), 374–377.
- [3] MAZUR P., S. P. LEIBO, E. H. CHU (1972) A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells, *Exp. Cell Res.*, **71**(2), 345–355.
- [4] SMITH G. D., E. E. MOTTA, P. SERAFINI (2011) Theoretical and experimental basis of oocyte vitrification, *Reprod. Biomed. Online*, **23**(3), 298–306.
- [5] ZERON Y., M. TOMCZAK, J. CROWE, A. ARAV (2002) The effect of liposomes on thermotropic membrane phase transitions of bovine spermatozoa and oocytes: implications for reducing chilling sensitivity, *Cryobiol.*, **45**(2), 143–152.
- [6] YU X. L., Y. K. XU, H. WU, X. F. GUO, X. X. LI et al. (2016) Successful vitrification of bovine immature oocyte using liquid helium instead of liquid nitrogen as cryogenic liquid, *Theriogenology*, **85**(6), 1090–1096.
- [7] CHEN J. Y., X. X. LI, Y. K. XU, H. WU, J. J. ZHENG et al. (2014) Developmental competence and gene expression of immature oocytes following liquid helium vitrification in bovine, *Cryobiology*, **69**(3), 428–433.
- [8] ROJAS C., M. J. PALOMO, J. L. ALBARRACÍN, T. MOGAS (2004) Vitrification of immature and in vitro matured pig oocytes: study of distribution of chromosomes, microtubules, and actin microfilaments, *Cryobiology*, **49**(3), 211–220.
- [9] SUDIMAN J., A. LEE, K. L. ONG, W. Z. YUAN, S. JANSEN et al. (2019) Tolerance of lamb and mouse oocytes to cryoprotectants during vitrification, *Zygote* (Cambridge, England), **27**(1), 36–45.
- [10] WEI X., S. J. YE, W. B. ZENG, Q. XU, J. ZHENG et al. (2018) Cytoskeleton genes expression and survival rate comparison between immature and mature yak oocyte after OPS vitrification, *Anim. Biotechnol.*, **29**(4), 247–251.
- [11] SOMFAI T., Y. HIRAO (2021) Vitrification of immature bovine oocytes in protein-free media: The impact of the cryoprotectant treatment protocol, base medium, and ovary storage, *Theriogenology*, **172**, 47–54.

- [12] SMITH G. D., E. S. C. A. SILVA (2004) Developmental consequences of cryopreservation of mammalian oocytes and embryos, *Reprod. Biomed. Online*, **9**(2), 171–178.
- [13] FAHY G. M., D. R. MACFARLANE, C. A. ANGELL, H. T. MERYMAN (1984) Vitrification as an approach to cryopreservation, *Cryobiology*, **21**(4), 407–426.
- [14] RUFFING N. A., P. L. STEPONKUS, R. E. PITT, J. E. PARKS (1993) Osmometric behavior, hydraulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages, *Cryobiology*, **30**(6), 562–580.
- [15] GUO X. F., X. L. YU, F. ZHANG, H. WU, X. Z. PEI et al. (2017) Effect of liquid helium vitrification on cytoskeleton of immature cattle oocytes, *Anim. Reprod. Sci.*, **187**, 91–99.
- [16] VINCENT C., V. GARNIER, Y. HEYMAN, J. P. RENARD (1989) Solvent effects on cytoskeletal organization and in-vivo survival after freezing of rabbit oocytes, *J. Reprod. Fertil.*, **87**(2), 809–820.
- [17] YOUNIS A. I., M. TONER, D. F. ALBERTINI, J. D. BIGGERS (1996) Cryobiology of non-human primate oocytes, *Hum. Reprod. (Oxford, England)*, **11**(1), 156–165.
- [18] DIEZ C., P. DUQUE, E. GÓMEZ, C. O. HIDALGO, C. TAMARGO et al. (2005) Bovine oocyte vitrification before or after meiotic arrest: effects on ultrastructure and developmental ability, *Theriogenology*, **64**(2), 317–333.
- [19] FORDER J., A. SRIABINE, H. RASMUSSEN (1985) Plasma membrane calcium flux, protein kinase C activation and smooth muscle contraction, *J. Pharmacol. Exp. Ther.*, **235**(2), 267–273.
- [20] MAHMOUD K. G., M. M. EL-SOKARY, M. M. KANDIEL, M. E. ABOU EL-ROOS, G. M. SOSA (2016) Effects of cysteamine during in vitro maturation on viability and meiotic competence of vitrified buffalo oocytes, *Iran. J. Vet. Res.*, **17**(3), 165–170.

College of Animal Science and Technology
Henan University of Science and Technology
Luoyang, Henan 471023, China
263 Kaiyuan St, Luoyang, China
and
Henan Provincial Key Laboratory
for Grass-Feeding Animals
Luoyang, 471023, China
e-mails: 1736427910@qq.com
1808397962@qq.com
wxj15139918259@163.com
liu15037826771@126.com
xxcphzs@126.com
9906187@haust.edu.cn
Liyingshua2008@yeah.net
yxl4282333@126.com