

**EFFECTS OF DIFFERENT VITRIFICATION
TEMPERATURES AND CRYOPROTECTIVE AGENT
CONCENTRATIONS ON ULTRASTRUCTURE AND
APOPTOSIS RELATED PROTEINS IN BOVINE GV STAGE
OOCYTES**

**Jia-Qi Liu, Shi-Yu Zhao, Xiao-Jing Wang,
Yi-Heng Liu, Xiao-Xia Li, Ying-Hua Li,
Zhi-Qian Xu, Xue-Li Yu[#]**

Received on December 15, 2022

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

Abstract

The effects of different vitrification temperatures (VTs) and cryoprotective agent concentrations (CPAs) on the viability, ultrastructure, and apoptosis related proteins Bcl-2, Bax, and TP53 of bovine oocytes at the GV (germinal vesicle) stage were analyzed in this study. Cumulus oocyte complexes (COCs) were randomized into five groups: fresh (control), LHe 5.6 M (liquid helium, -269°C , cryoprotectant concentration of 5.6 M), LHe 6.6 M (liquid helium, -269°C , cryoprotectant concentration of 6.6 M), LN 5.6 M (liquid nitrogen, -196°C , cryoprotectant concentration of 5.6 M), LN 6.6 M (liquid nitrogen, -196°C , cryoprotectant concentration of 6.6 M). Statistically, in the vitrification groups, LN 5.6 M group showed the lowest rate of morphologically normal, mature, and cleavage (58.1%; 32.9%; 12.9%). LHe 5.6 M showed the highest cleavage rate (39.6%), as well as effectively ameliorated ultrastructural damage, significantly reduced Bax protein expression, effectively increased Bcl-2 protein expression, reduced TP53 protein expression, in general, it increased the anti-apoptotic ability of the oocytes after vitrification. It was concluded that lower

[#]Corresponding author.

DOI:10.7546/CRABS.2023.05.18

vitrification temperature and decreased cryoprotectant concentrations upregulate the expression of Bcl-2 protein and downregulate the expression of Bax and TP53 protein in oocytes, and enhance the anti-apoptotic capacity of oocytes, ameliorated the damage of the ultrastructure of the oocytes, perhaps improved the developmental capacity of bovine GV stage oocytes after vitrification can be partially attributed to the above changes.

Key words: oocytes, vitrification temperatures, cryoprotective agent concentrations, ultrastructure, apoptosis

Introduction. Vitrification of bovine GV stage oocytes is important for both livestock breeding and germplasm resource protection, but cryoinjury and the toxic stress of cryoprotectants severely affect freezing efficacy, and freezing efficiency remains to be improved. The main factors of vitrification include VT and CPAs. Liquid helium (LHe, -269°C) has been used as a cryogen, instead of liquid nitrogen (LN, -196°C), for vitrification of bovine GV stage oocytes in our lab, which showed a significant improvement in maturation rate and cleavage rate [1]. To reduce ice crystal damage, a high CPA needs to be added during vitrification [2], but cryoprotectants impair the vitrification effect because of severe toxicity. HUANG et al. [3] demonstrated that EG (ethylene glycol) has low toxicity and that it is often used in combination with DMSO (dimethyl sulphoxide) and sucrose. In addition, CPAs directly affect the permeability of the cytoplasmic membrane, currently the combination of 20% EG and 20% DMSO is most effective which is commonly used for bovine oocytes vitrification [4].

The organelles of oocytes play important roles during maturation and development. The zona pellucida is a bridge for the exchange of material between oocytes and cumulus granulosa cells by the abundance of gap junctions, whereas microvilli function to enhance the material exchange function of the zona pellucida. The zona pellucida was sclerotic and microvilli disappeared which affected fertilization and development of oocytes [5]. The cortical granule can block polyspermy into oocytes, which vitrification causes the release of cortical granules. Mitochondria provide energy for various stages of oocytes development, enlarged mitochondria with unclear mitochondrial cristae affect oocytes energy supply. In addition, a series of ultrastructural damages, such as cell shrinkage and increased lipid droplet number, can affect oocytes developmental competence [6,7].

There are two main pathways of apoptosis in mammals: the intrinsic pathway and the extrinsic pathway, the former is mainly carried out in mitochondria that involved pro-apoptotic protein Bax, TP53 and anti-apoptotic Bcl-2 [8]. After vitrification, the Bcl-2 is integrated into the mitochondrial membrane to close the mitochondrial channel, inhibits Ca^{2+} transmembrane flow and reduces oxidative radical production and lipid peroxide formation, thereby resisting apoptosis. The protein Bax mainly functions by forming dimers with Bcl-2, when Bax expression is higher, it promotes apoptosis. So Bcl-2/Bax ratio index represents the anti-apoptotic intensity of cells [9,10]. Vitrification causes TP53 overexpression,

thereby arresting cells in G1 phase. Also, it will increase the transcriptional synthesis of pro-apoptotic proteins Bax, Bak, inhibit the production of Bcl-2, and finally induce cell apoptosis [11].

Therefore, the effects of different VTs and CPAs on the developmental competence, ultrastructure, and the expression of apoptosis related proteins Bcl-2, Bax, and TP53 of bovine oocytes at GV stage is analyzed in this study, thereby providing a theoretical basis for improving vitrified oocytes developmental competence.

Materials and methods. Chemicals. Unless otherwise stated, the reagents were purchased from Sigma-Chemical Corporation (St. Louis, MO, USA).

Collection and vitrification of bovine GV stage oocytes. The bovine ovaries were transported back from the slaughterhouse, the COCs were aspirated from follicles of the ovaries and randomized into five groups: fresh (control), LHe 5.6 M (-269°C , cryoprotectant concentration of 5.6 M), LHe 6.6 M (-269°C , cryoprotectant concentration of 6.6 M), LN 5.6 M (-196°C , cryoprotectant concentration of 5.6 M), LN 6.6 M (-196°C , cryoprotectant concentration of 6.6 M).

Vitrification was realized following VAJTA's method [12], COCs were kept in HM (Holding Medium), VS1 (Vitrified Solution 1), VS2 (Vitrified Solution 2), siphoned into the OPS (Open pulled Straw) using the suction method, and immediately plunged into the LN or LHe. For thawing, the thin of the OPS were immediately immersed in W1 (Warming Solution 1), and COCs were blown out and sequentially passed through W1, W2 (Warming Solution 2), HM, selected the morphologically normal oocytes for the following 3 experiments.

1. Evaluation of developmental competence. The process of oocytes IVM (in vitro maturation), IVF (in vitro fertilization) and IVC (in vitro culture) experiments is the same as in ZHANG et al. [1]. After vitrification and thawing, morphologically normal oocytes were selected and placed in each 50 μl droplet of IVM medium, covered with mineral oil, and put into the oxidation incubator for cultivation for 22–24 h, then the maturation rate was calculated. The cultured oocytes were blown with a pipette gun to separate the surrounding cumulus granulosa cells. Then selected the mature oocytes (with first polar body) to sperm suspension for IVF, after 22–24 h, presumptive zygotes were washed in IVC medium and transferred to cumulus cell droplets to grow. Then assessed the cleavage rates after 48 h. Statistical analyses were performed using SPSS. Comparisons of means among groups were performed by one way ANOVA followed by the Tukey's post hoc test. $P < 0.05$ was the threshold for statistical significance.

2. Transmission electron microscopy (TEM). Oocytes without cumulus granulosa cells were washed with PBS (Phosphate Buffer Solution) after IVM, and were fixed with 3% glutaraldehyde and 1% OsO_4 at 4°C . The samples were dehydrated step by step with acetone and then embedded them with Epon812. Ultrathin slicing mechanism (UC7rt, LEICA) was used to prepare ultrathin slices

of about 60 ~ 90 nm, expand the slices, salvage them to the copper mesh, stained with uranyl acetate for 10~15 min and lead citrate for 1 ~ 2 min at RT (Room Temperature), and then examined and took photos by TEM (Transmission electron microscope, JEM-1400FLASH, Japan).

3. Western blot analysis. One hundred oocytes from each group after IVM were lysed in 50 µl RIPA (Radio Immunoprecipitation Assay, Beyotime, China), denatured proteins were separated by 12% SDS-PAGE gel (Sodium dodecyl sulphate polyacrylamide gel), and transferred onto 0.22 µm PVDF (polyvinylidene fluoride) membranes with transfer buffer, then blocking with 5% skim milk in TBST (Tris Buffered Saline with Tween) for 4 h at RT. Immersion the membranes were incubated overnight at 4 °C with primary antibody: Bcl-2 (Santa Cruz, 1:500); anti-TP53 (Santa Cruz, 1:500); anti-BAX (Proteintech Group Inc., 1:1000); GAPDH (internal reference protein, Beyotime, 1:1000). After washing, the membranes were incubated for 2 h at RT with HRP-conjugated goat Anti-rabbit IgG (H+L), HRP-conjugated goat Anti-mouse IgG(H+L), (Beyotime, 1:1000), then the membranes were exposed on a chemiluminescence imaging system with BeyoECL Star. Bands were extracted for gray scale values with ImageJ and the statistics graphed with GraphPad Prism 8.

Results. Effects of different VTs and CPAs on the developmental competence of bovine GV oocytes (mean ± SD). As shown in Table 1, LN 5.6 M showed the lowest rate of morphologically normal, mature, and cleavage (58.1%; 32.9%; 12.9%), and LHe 5.6 M showed the highest cleavage rate (39.6%) in the vitrification groups ($p < 0.05$).

T a b l e 1

Effects of different VTs and CPAs on the developmental competence of bovine GV oocytes (mean±SD)

Groups	Repli- cates	Oocytes	N/(%) of morphologically normal oocytes	N/(%) of maturation	N/(%) of cleavage
Control	6	310	308(99.3±0.94) ^a	231(74.5±2.99) ^a	158(51.0±1.11) ^a
LHe 5.6 M	6	323	289(89.5±2.40) ^b	185(57.3±1.34) ^b	128(39.6±0.82) ^b
LHe 6.6 M	6	306	268(87.6±1.03) ^b	168(54.9±2.24) ^b	113(36.9±1.60) ^c
LN 5.6 M	6	310	180(58.1±1.11) ^c	102(32.9±3.26) ^c	40(12.9±0.38) ^d
LN 6.6 M	6	302	224(74.2±1.64) ^d	159(52.6±1.70) ^b	99(32.8±1.69) ^e

Values in the same column with different letters are significantly different ($p < 0.05$)

Effects of different VTs and CPAs on ultrastructure of bovine GV oocytes. As shown in Fig. 1, in the fresh and LHe 5.6 M groups (Fig. 1A, B), the structure and localization of the cells were intact, the cortical granules were arranged in an aggregate, and the mitochondria of the fresh group oocytes were mostly regularly oval with clear mitochondrial cristae. The LHe 5.6 M group

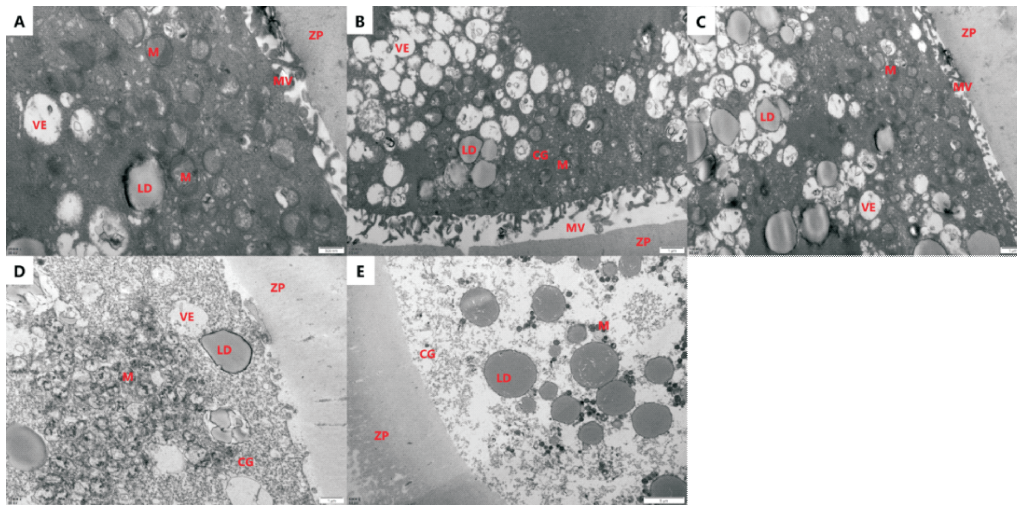


Fig. 1. Effects of different VTs and CPAs on the ultrastructure of bovine GV oocytes. Ultrastructure of bovine oocytes fixed at 24 h of IVM: Fresh control oocytes (A), LHe 5.6 M group (B), LHe 6.6 M group (C), LN 5.6 M (D), LN 6.6 M (E). CG – cortical granules; LD – lipid droplets; M – mitochondria; MV – microvilli; ZP – Zona pellucida; VE – vesicle

(Fig. 1B) presented more vacuoles and individual mitochondria were enlarged compared with the fresh group. Microvilli were reduced in the LHe 6.6 M group (Fig. 1C), and swollen mitochondria with an increased number of lipid droplets were present. The cytoplasm became uneven, the zona pellucida was sclerotized, microvilli disappeared in LN 5.6 M (Fig. 1D) and LN 6.6 M groups (Fig. 1E), droplet size and number increased in LN 6.6 M group, most mitochondria were enlarged, granule cells were sharply reduced and arranged diffusely, organelles in LN 5.6 M group were severely damaged, mitochondria were swollen and deformed, mitochondrial cristae were not obvious, and vacuolar shape was also irregular.

Effects of different VTs and CPAs on the expression of apoptosis related proteins in bovine GV oocytes (mean $\pm$ SEM). As shown in Fig. 2, in the vitrification groups, the protein expression of Bcl-2 was significantly increased by LHe vitrification, and the expression of Bax was lowest in LHe 5.6 M group, the expression of TP53 was significantly lower in the LHe 5.6 M group than in LN vitrification groups. In addition, Bcl-2/Bax ratio showed that oocytes of LHe 5.6 M group had superior anti-apoptotic ability.

Discussion. Vitrification can affect morphology, ultrastructure of oocytes, and changes in cellular gene and protein expression, thereby limiting oocytes maturation and viability [13]. In our study, the rates of normal morphology, maturation, and cleavage were significantly increased in LHe 5.6 M group compared with LN 5.6 M group, inferred that a lower VT could improve developmental ability of oocytes. The significantly higher cleavage rate of LHe 5.6 M group compared with LHe 6.6 M group, and the significantly lower cleavage rate of LN 5.6 M

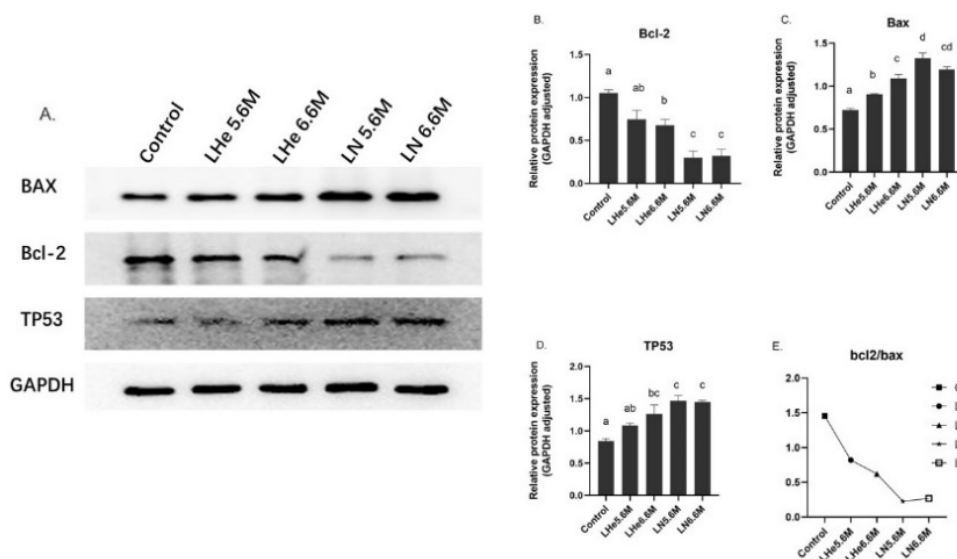


Fig. 2. Effects of different VTs and CPAs on the expression of apoptosis related proteins (Bcl-2, Bax, TP53) in bovine oocytes. Protein expression of Bcl-2, Bax and TP53 was detected by western blot. After vitrification, the expression of Bcl-2 was upregulated in the LHe groups (B), protein expression level of Bax was downregulated in the LHe 5.6 M group (C), protein expression level of TP53 was downregulated in the LHe 5.6 M group compared with LN groups (D). Results for Bcl-2/Bax ratio indicate anti-apoptotic capacity of bovine oocytes, the fresh group had the highest anti-apoptotic ability of bovine oocytes, and the LHe 5.6 M group had the highest anti-apoptotic ability of bovine oocytes after vitrification (E)

group compared to LN 6.6 M group, proved that a lower VT decreased CPAs effectively improved the developmental capacity of bovine GV stage oocytes after vitrification.

Affected by the vitrification injury, the oocyte's cortical granules undergo premature exocytosis, zona pellucida sclerosis, and temporary cryoinjury to the spindle, which can be repaired to some extent after IVM [5], whereas the intracellular spindles in GV stage oocytes have not been formed, and ultrastructure such as microfilament microtubules are more resistant to low temperature [14]. To be more objective for our study, IVM was performed after vitrification and thawing of GV stage oocytes, and ultrastructure was examined by TEM. The LHe 5.6 M group in comparison with the LN 5.6 M group, the integrity of organelle structure was better maintained, with microvilli and clear mitochondrial cristae by lower VT, that temperature reduction was confirmed to effectively reduce vitrification cryoinjury; LHe 5.6 M group compared with LHe 6.6 M group, oocytes cytoplasm was more uniform, there were more microvilli and fewer lipid droplets, but LN 5.6 M group had severely damaged organelles, ruptured vacuoles and severely deformed mitochondria compared with LN 6.6 M group, probably because cells need

higher CPAs at -196°C to resist ice crystallization damage, so we concluded that lower VT and low CPAs could better maintain the ultrastructure of oocytes.

Apoptosis is a programmed death influenced by genetic mechanisms [15], pro-apoptotic protein Bax would increase the permeability of mitochondrial membrane and promote the release of mitochondrial cytochrome C, thus significantly increased ROS (Reactive Oxygen Species) levels and then promote apoptosis [16]. On the contrary, the Bcl-2 protein will prevent the release of cytochrome C from the mitochondria, and then inhibit apoptosis, so ratio of Bcl-2/Bax is essential for cell survival [17]. TAGHAVI et al. [18] proved that survival of cells could be effectively improved by upregulating Bcl-2 protein expression and down-regulating Fas, Bax and TP53 protein expression. We showed that Bcl-2 protein expression was significantly upregulated and the protein expression of Bax and TP53 were significantly downregulated, meanwhile the rate of survival, maturation and cleavage of oocytes were increased in LHe 5.6 M group compared with LN 5.6 M group, which was consistent with the results of Taghavi, and it was inferred that lower VT effectively improved the anti-apoptotic ability of bovine GV stage oocytes. Protein expression of Bax was significantly downregulated and Bcl-2/Bax ratio was higher in LHe 5.6 M group compared with LHe 6.6 M group, but LN 5.6 M group only decreased ratio of Bcl-2/Bax compared with LN 6.6 M group, which proved that the concentration of LHe vitrification had a more pronounced effect on the apoptosis related protein Bax, and the anti-apoptotic ability of the oocytes was enhanced after reducing the CPAs. In summary, lower VT and decreased CPAs effectively improved the anti-apoptotic capacity of bovine GV stage oocytes.

It was concluded that lower vitrification temperature and decreased cryoprotectant concentrations upregulate the expression of Bcl-2 protein and downregulate the expression of Bax and TP53 protein in oocytes, and enhance the anti-apoptotic capacity of oocytes, ameliorated the damage to the ultrastructure of the oocytes, and the above changes may be partly responsible for the improved developmental capacity of bovine GV stage oocytes after vitrification.

REFERENCES

- [1] ZHANG F., Z. Y. ZHANG, M. D. CAI, X. X. LI, Y. H. LI et al. (2020) Effect of vitrification temperature and cryoprotectant concentrations on the mRNA transcriptome of bovine mature oocytes after vitrifying at immature stage, *Theriogenology*, **148**, 225–235.
- [2] KEROS V., B. J. FULLER (2021) Cryopreservation of mammalian oocytes: slow cooling and vitrification as successful methods for cryogenic storage, *Methods Mol. Biol.*, **2180**, 437–454.
- [3] HUANG J. Y. J., H. Y. CHEN, S. L. TAN, R. C. CHIAN (2006) Effects of osmotic stress and cryoprotectant toxicity on mouse oocyte fertilization and subsequent embryonic development in vitro, *Cell Preserv. Technol.*, **4**(3), 149–160.

- [4] GAUTAM S. K., V. VERMA, P. PALTA, M. S. CHAUHAN, R. S. MANIK (2008) Effect of type of cryoprotectant on morphology and developmental competence of in vitro-matured buffalo (*Bubalus bubalis*) oocytes subjected to slow freezing or vitrification, *Reprod. Fertil. Dev.*, **20**(5), 490–496.
- [5] HYTTEL P., G. VAJTA, H. CALLESEN (2000) Vitrification of bovine oocytes with the open pulled straw method: ultrastructural consequences, *Mol. Reprod. Dev.*, **56**(1), 80–88.
- [6] TAGHIZABET N., M. A. KHALILI, F. ANBARI, A. AGHA-RAHIMI, S. A. NOTTOLA et al. (2018) Human cumulus cell sensitivity to vitrification, an ultrastructural study, *Zygote*, **26**(3), 224–231.
- [7] LIU M. H., W. H. ZHOU, D. P. CHU, L. FU, W. SHA et al. (2017) Ultrastructural Changes and Methylation of Human Oocytes Vitrified at the Germinal Vesicle Stage and Matured in vitro after Thawing, *Gynecol. Obstet. Invest.*, **82**(3), 252–261.
- [8] VINING L. M., L. J. ZAK, S. C. HARVEY, K. E. HARVEY (2021) The role of apoptosis in cryopreserved animal oocytes and embryos, *Theriogenology*, **173**, 93–101.
- [9] PEPPER C., T. HOY, D. P. BENTLEY (1997) Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance, *Br. J. Cancer*, **76**(7), 935–938.
- [10] SPITZ A. Z., E. GAVATHIOTIS (2022) Physiological and pharmacological modulation of BAX, *Trends Pharmacol. Sci.*, **43**(3), 206–220.
- [11] KAZEMI P., M. DASHTIZAD, M. SHAMSARA, F. MAHDAVINEZHAD, E. HASHEMI et al. (2016) Effect of blastocoel fluid reduction before vitrification on gene expression in mouse blastocysts, *Mol. Reprod. Dev.*, **83**(8), 735–742.
- [12] VAJTA G., P. HOLM, M. KUWAYAMA, P. J. BOOTH, H. JACOBSEN et al. (1998) Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos, *Mol. Reprod. Dev.*, **51**(1), 53–58.
- [13] CASILLAS F., Y. DUCOLOMB, A. LÓPEZ, M. BETANCOURT (2020) Effect of porcine immature oocyte vitrification on oocyte-cumulus cell gap junctional intercellular communication, *Porc. Health Manag.*, **6**(1), 37.
- [14] GALEATI G., M. SPINACI, C. VALLORANI (2011) Pig oocyte vitrification by Cryotop method: Effects on viability, spindle and chromosome configuration and in vitro fertilization, *Animal Reprod. Sci.*, **127**(1–2), 43–49.
- [15] LOBASCIO A. M., F. G. KLINGER, M. L. SCALDAFERRI, D. FARINI, M. DE FELICI (2007) Analysis of programmed cell death in mouse fetal oocytes, *Reproduction*, **134**(2), 241–252.
- [16] AGHAZ F., A. VAISI-RAYGANI, M. KHAZAEI, E. ARKAN (2021) The Anti-oxidative Effects of Encapsulated Cysteamine during Mice In Vitro Matured Oocyte/Morula-Compact Stage Embryo Culture Model: a Comparison of High-Efficiency Nanocarriers for Hydrophilic Drug Delivery – a Pilot Study, *Reprod. Sci.*, **28**(5), 1290–1306.
- [17] MICHURINA S. V., S. I. KOLESNIKOV, A. L. BOCHKAREVA, I. YU. ISHCHENKO, S. A. ARKHIPOV (2019) Expression of Apoptosis Regulator Proteins Bcl-2 and Bad in Rat Ovarian Follicular Apparatus during Recovery after Extreme Hypothermia, *Bull. Exp. Biol. Med.*, **168**(2), 205–209.
- [18] TAGHAVI S. A., M. R. VALOJERDI, M. F. MOGHADAM, B. EBRAHIMI (2015) Vitrification of mouse preantral follicles versus slow freezing: Morphological and apoptosis evaluation, *Anim. Sci. J.*, **86**(1), 37–44.

*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-mail: 18438592825@163.com
1736427910@qq.com
wxj15139918259@163.com
liu15037826771@126.com
xxcphzs@126.com
Liyinhua2008@yeah.net
9906187@haust.edu.cn
yxl4282333@126.com*