EFFECT OF CRYOPROTECTANTS AND CRYOPRESERVATION METHODS ON THE QUALITY OF BUFFALO OOCYTES

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ABSTRACT

This study was conducted to evaluate the effect of two types of cryoprotectants and two cryopreservation methods on viability and normal morphology of the immature and *in vitro* mature of post-thawed buffalo oocytes. The *in vitro* mature and immature oocytes were kept in the vitrification solution (VS1) for 5 min and the vitrification solution (VS2) for 15 sec. The results revealed that the proportion of buffalo oocytes found to be viable and normal morphology were significantly (P< 0.01) higher in using glycerol which is 76.14% and 55.1%; respectively. We conclude from the present study that the viability and normal morphology of post-thawed buffalo vitrified *in vitro* matured oocytes were significantly (P < 0.01) higher in glycerol than those obtained in dimethyl sulfoxide (DMSO) and in vitrification technique than rapid technique.

Keywords: glycerol, DMSO, oocyte viability, normal morphology of oocyte, buffalo.

تأثير مواد الحماية من التجميد وطرق الحفظ بالتجميد على نوعية البويضات لدى الجاموس.

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المستخلص

أجريت هذه الدراسة لتقييم تأثير نوعين من المواد الواقية من التجمد وطريقتين للحفظ بالتجميد على الحيوية والشكل الطبيعي لبويضات الجاموس غير الناضجة والناضجة في المختبر بعد الإذابة. وتم معاملة البويضات الناضجة في المختبر وغير الناضجة في محلول التزجيج (VS1) لمدة 5 دقائق ومحلول التزجيج (VS2) لمدة 15 ثانية. أوضحت النتائج أن نسبة الحيوية والشكل الطبيعي لبويضات الجاموس كانت أعلى معنوياً (P <0.01) عند استخدام الجلسرين والتي كانت 76.14% و55.18%; على التوالي. نستنتج من الدراسة الحالية أن الحيوية والشكل الطبيعي لبويضات الجاموس الناضجة في المختبر بعد الاذابة كان أعلى بشكل ملحوظ (P <0.01) عند المعاملة بالجلسرين من تلك التي تم الحصول عليها من DMSO، وكذلك كانت أعلى في تقنية التزجيج مقارنة بتقنية التجميد السريع.

الكلمات المفتاحية: الكليسرول، دايمثيل سالفو اكسيد، حيوية البويضات، الشكل الطبيعي للبويضات، الجاموس.

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INTRODUCTION

One of the most important challenges facing Iraqi agriculture is protecting and improving national agricultural resources, including buffalo, to achieve sustainable food security (2). Buffaloes have low reproductive efficiency, which is affected by late maturity, poor expression of estrous symptoms particularly during summer, irregular estrous cycle, silent heat, poor pregnancy rate, early embryonic mortality, and prolonged inter-calving interval (14). Reproductive traits are important in minimizing cost and maximizing the net return from the dairy enterprise (15). Reproductive efficiency is an important attribute even where most of the emphasis is on the milk (1). Genetic improvement of buffalo can be achieved by the application of assisted reproductive technologies, however, in buffaloes, those methods were not as efficient as in bovine (22). Cryopreservation of oocytes and embryos is the key to preserving genetic resources for future protection and managing species population integrity and heterozygosity (22), and it is helpful to enhance the better handling of buffalo gametes for both research and commercial purposes (3). Vitrification is a process that induces the glass-like solidification of living cells in high concentrations of cryoprotectant agents (CPAs) and rapid freezing at -196°C, minimizing the ice crystal formation that causes cryoinjury (14,19), The vitrification technique of oocytes may introduce a solution to low survival and low implantation rates problem in slow freezing method (6). Oocyte vitrification is now considered an established assisted reproductive technology for fertility preservation, (18). Rapid freezing (vapor phase) can be considered a midway technique between slow freezing and vitrification, it is quicker than the slow-freezing technique, does not involve the use of programmable machines, and requires lower concentrations of cryoprotectant agents (CPA) than those used in vitrification (28,10). A cryoprotectant is a substance used to protect biological tissue from freezing damage, is simply by increasing the total concentration of all solutes in the system, reducing the amount of ice formed at any given temperature; but to be biologically acceptable they must be able to penetrate the cells and have low toxicity and prevents lipid peroxidation of the plasma Membrane. (14,24). Many compounds act as cryoprotectants and are used for the protection of cells against freezing, there are two types of cryoprotectants: (a) Penetrating cryoprotectants are small molecules able to cross cell membranes, such as glycerol (G), ethylene glycol (EG), and dimethyl sulfoxide (DMSO); (b) Non-penetrating cryoprotectants were large molecules, such as Polyethylene glycol (PEG) and polyvinylpyrrolid- one (PVP) (13). To improve the effectiveness of vitrification and rapid freezing of buffalo oocytes, the present study evaluated the comparative effect of cryoprotectants (glycerol and dimethyl sulfoxide) on the viability and normal morphology of vitrified-thawed *in vitro* mature and immature buffalo oocytes.

MATERIALS AND METHODS

The buffalo ovaries from the normal reproductive tract of adult buffaloes of unknown breeding history were collected from a local abattoir in Al-Najaf Al-Ashraf Province from January 2021 to April 2021. Both ovaries were collected directly from each buffalo after being slaughtered and kept in a thermos flask containing warm normal saline (0.9% NaCl) inserted with antibiotics (100IU/mL penicillin and 100 µg/ml streptomycin). Ovaries were transported to a well-equipped laboratory Unit at the College of Medicine, Jabir Ibn Hayyan Medical University, Al-Najaf Al-Ashraf province within 2 hours. In the laboratory, ovaries were washed with warmed normal saline solution (37.5 C°) three times to remove the clotted blood and reduce contamination on the ovarian surfaces (6,21).

Preparing cryopreservation solution

The vitrification solution (VS1) consisted of a SMART culture medium supplemented with 10% bovine serum albumin (BSA) and two types of cryoprotectants, glycerol (G) (15%), and dimethyl sulfoxide (DMSO) (10%), also the vitrification solution (VS2) consisted of SMART culture medium supplemented with 0.5 M sucrose, 10% bovine serum albumin (BSA) and two types, glycerol (G) (15%), and dimethyl sulfoxide (DMSO) (10%), and they consist of rapid technique solution is: =====G

A- 7.5 mL Glycerol (15%) with 42.5 mL SMART (85%). G B- 5.0 mL DMSO (10 %) with 45.0 mL SMART (90 %). (SMART culture medium up to 10ml with 10% bovine serum albumin), and the thawing solution consisted of 1 M of sucrose (3.42 g) and SMART culture medium up to 10 mL with 10% bovine serum albumin.

Cryopreservation of oocytes:

Normal and viable oocytes (immature and *in vitro* mature) were transferred to 0.5 mL of the vitrification solution (VS1) at room temperature to equilibrate for 5 minutes for oocytes. Then, oocytes were placed into 0.5 mL of vitrification solution 2 (VS2) for 15 seconds, then the oocytes were loaded on the straw of VS2 and directly immersed into LN2. Then, the straw is covered with the plastic tube in the LN2 to protect it during storage. The samples were pipetted into cryovials, the cryovials were left at room temperature for 10 minutes, and then frozen by static-phase vapor cooling and plunged into liquid nitrogen vapor (10 cm above the level of liquid nitrogen; – 80 C°) for 15 minutes, The samples were then plunged into liquid nitrogen (–196C°) and stored until required, thawing was carried out at room temperature (9).

Thawing of oocytes

For thawing, the straws and cryovials were taken out from the LN2 after two months and immersed in the thawing solution at 37 ºC for 1 min, and then washed twice with SMART medium and tested for viability and morphology directly post-thawing. The thawed oocytes were considered abnormal when some changes occurred in shapes such as breakage of zona pellucida, uneven granulation, and leakage of oocyte contents (22).

Evaluation of oocytes

Livability was assessed according to El-Sokary *et al*. (10) using trypan blue stain solution (0.05%, PH=7.0 2min) at room temperature, hence, exclusion of stain by oocytes is indicative of viability. Oocytes were categorized based on the degree of dye exclusion, Unstained oocytes were classified as live and fully stained oocytes as dead (Plate 1)(4).

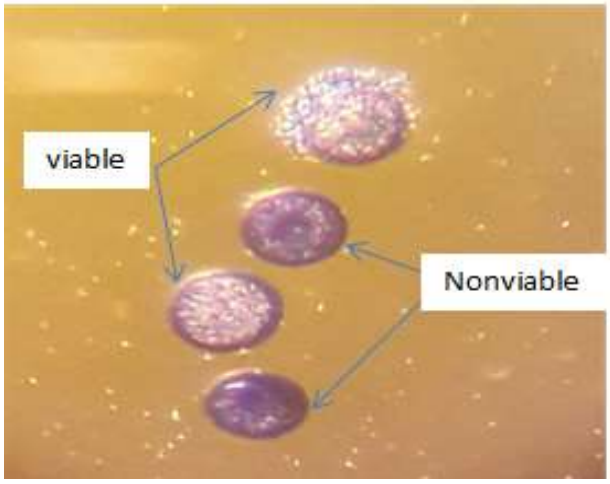

Plate 1. Viability and nonviable of buffalo oocyte (X=200).

Statistical analysis

The statistical analysis system (SAS) program (26) was used to determine the effect of two types of cryoprotectants in post-thawed oocytes. The analysis of variation (ANOVA) and Duncan's Multiple Range Test for viability were used to compare between means.

RESULTS AND DISCUSSION

In this study, several buffalo oocytes were collected (427), All the oocytes were viable and had normal morphology, it's observed Highly significant (P<0.01) differences were assessed in the percentage of viability (%) and normal morphology (%) of *vitro* matured comparison with immature of post-thawed buffalo cryopreserved oocytes using glycerol, which showed that the best result is achieved in *in vitro* matured oocytes with using glycerol, which is 76.14% for viability and 55.18 % for normal morphology (Table 1,2).

Table 1. Effect of the relationship between the types of cryoprotectant with the type of oocytes on the percentage of viability of post-thawed buffalo vitrified oocytes

Type of Cryoprotectants	Type of oocyte	
	Immature	IVM
Glycerol	72.96 ± 1.05 B	76.14 ± 0.98 A
DMSO	70.41 ± 1.23 C	72.00 ± 1.11 B

IVM: *in vitro* matured, DMSO: Dimethyl sulfoxide. Different letters in the table refer to significant (P < 0.01) differences

Table 2. Effect of the relationship between the types of cryoprotectant with the type of oocytes on the percentage of normal morphology of post-thawed buffalo vitrified oocytes

Type of Cryoprotectants	Type of oocyte	
	Immature	IVM
Glycerol	49.33 ± 1.09 B	55.18 ± 1.05 A
DMSO	47.30 ± 1.08 C	49.34 ± 1.08 B

IVM: *in vitro* matured, DMSO: Dimethyl sulfoxide. Different letters in the table refer to significant (P < 0.01) differences.

Table (3,4) shows the viability and normal morphology of cryopreserved oocytes, The oocyte cryopreserved through vitrification technique with glycerol significantly (P<0.01) increased in viability and normal morphology of the oocytes 78.11% and 58.14%; respectively, and the viability and normal morphology of the cryopreserved oocyte with rapid freezing is 67.38% and 50.05%; respectively.

Table 3. The effect of the relationship between the type of cryoprotectants, and oocyte on the percentage of viability of post-thawed buffalo vitrified oocytes

Type of Cryoprotectants	Type of Cryopreservation	
	Rapid tech.	Vitrification tech.
Glycerol	67.38 ± 1.01 C	78.11 ± 0.98 A
DMSO	64.90 ± 1.26 D	73.99 ± 1.07 B

DMSO: Dimethyl sulfoxide. Different letters in the table refer to significant (P < 0.01) differences

Table 4. The effect of the relationship between the type of cryoprotectants, and oocyte on the percentage of normal morphology of post-thawed buffalo vitrified oocytes

Type of Cryoprotectants	Type of Cryopreservation	
	Rapid tech.	Vitrification tech.
Glycerol	50.05 ± 1.15 C	58.14 ± 1.07 A
DMSO	45.44 ± 1.12 D	51.75 ± 1.03 B

DMSO: Dimethyl sulfoxide. Different letters in the table refer to significant (P < 0.01) differences.

Results of Table (5,6) illustrate that the viability and normal morphology of the *in vitro* matured type of cryopreserved oocytes, the oocyte cryopreserved through vitrification technique with glycerol significantly (P<0.01) increased produced in viability and normal morphology 79.78% and 58.17%; respectively. The viability and normal morphology of cryopreserved oocytes by rapid freezing with glycerol is 75.30% and 50.48%; respectively.

Table 5. The effect of the relationship between the type of cryoprotectants, cryopreservation, and oocyte on the percentage of viability of post-thawed buffalo cryopreserved oocytes

Type of Cryoprotectants	Type of Cryopreservation	Type of oocyte	
		Immature	IVM
Glycerol	Rapid tech.	69.41±1.48 D	75.30±1.44 B
	Vitrification tech.	71.47±1.36 C	79.78±1.33 A
DMSO	Rapid tech.	66.46±1.74 E	74.02±1.69 BC
	Vitrification tech.	69.48±1.83 D	75.97±1.34 B

IVM: in-vitro matured, DMSO: Dimethyl sulfoxide. Different letters in the table refer to significant differences. ** (P < 0.01).

Table 6. The effect of the relationship between the type of cryoprotectants, cryopreservation, and oocyte on the percentage of normal morphology of post-thawed buffalo cryopreserved oocytes

Type of Cryoprotectants	Type of Cryopreservation	Type of oocyte	
		Immature	IVM
Glycerol	Rapid tech.	47.73±1.53 E	50.84±1.55 C
	Vitrification tech.	52.63±1.73 B	58.17±1.27 A
DMSO	Rapid tech.	44.44±1.57 F	49.92±1.48 CE
	Vitrification tech.	46.54±1.60 EF	51.52±1.44 B

IVM: in-vitro matured, DMSO: Dimethyl sulfoxide. Different letters in the table refer to significant differences. ** (P < 0.01).

The current study constructs that the viability and normal morphology of post-thawed buffalo cryopreserved oocytes decreased in samples vitrified with DMSO than those vitrified with glycerol in all treatments and this reflects the dominance of glycerol in the protection of vitrified oocytes. These findings compare favorably with Wani *et al*.(29) illustrated that the high recovery rate of buffalo immature oocytes is cryopreserved of morphologically normal oocytes in all the concentrations of all cryoprotectants (Glycerol, EG, DMSO, and PROH). Also, Farshad *et al*.(11) showed when studying the effect of different concentrations of glycerol and DMSO on Makhoz goat spermatozoa, there was a significant difference in viability and motility by increasing glycerol concentration (P<0.05). Furthermore, Rasul *et al*.(25) which studied the effect of glycerol and DMSO during cryopreservation of buffalo semen, showed that within 6% glycerol treatment, post-thaw sperm motility is 47 ± 6.9% without DMSO, which reduced to 29 ± 3.1 and 21 ± 3.1% in the presence of 1.5 and 3% DMSO, respectively (P < 0.05). Likewise, post-thaw plasma membrane integrity was the highest in 6% glycerol without DMSO (47 %) than with DMSO (32 %, P<0.05). Glycerol demonstrated a strong affinity with the cells' phospholipid head groups during freezing (5). The cryoprotection of glycerol is due to its ability to buffer the salt at low temperatures, bind with metallic ions, dehydrate the cell, and reduce the ice expansion during water solidification (7, 17). The absence of glycerol in the extender led to a complete cessation of sperm movement, despite negligible protection to the plasma membrane and acrosome (25). Furthermore, glycerol acts by reducing the concentration of the electrolyte in the extracellular fluid and increasing the portion of the unfrozen solution at any temperature (16). In addition to its effect on preventing the formation of ice crystals and the dehydration of cells during the cryopreservation process (30). Results of the present study indicated a too-high percentage of viability (78.11 %) and normal morphology (58.14%) of post-thawed buffalo cryopreserved oocytes when using the vitrification technique and glycerol, but there were non-significantly (P>0.05) differences were assessed when using a rapid technique with glycerol or DMSO on viability characteristic, which is 67.38% and 64.90%; respectively. This result is agreed with Mahmoud *et al*.(29) indicated the percentages of viable post-thawed buffalo vitrified oocytes were (72.8, 27.0) % for viability and abnormality oocytes respectively, via a straw. From the results of the present study, it was revealed that the percentage of post-thawing morphologically normal and viability vitrified *in vitro* mature buffalo oocytes were significantly (p<0.01) higher than that of vitrified immature one in 15% glycerol.

REFERENCES

1-Alkass J.E., H.N.Hermiz, and M.,I., Baper .2021. Some aspects of reproductive efficiency in awassi ewes: A review, Iraqi Journal of Agricultural Sciences,52(1):20-27.https://doi.org/10.36103/ijas.v52i1.1232

2-Al-Taiy H. K., A. T. H. Al-Salhi, and A. L. J. Al-mashadani. 2020. Development extension service to meeting the requirements of buffalo breeders in Iraq. Iraqi Journal of

Agricultural Sciences, 51(1):432-442. https://doi.org/10.36103/ijas.v51i1.942

3-Attanasio, L., L. Boccia, G. Vajta, M. Kuwayama, G. Campanile, L. Zicwerelli, G. Neglia, and B. Gasparrini, 2010. Cryotop vitrification of buffalo (*Bubalus bubalis*) in vitro matured oocytes: effects of cryoprotectants concentrations and warming procedures. Reprod. Dom. Anim. 45: 997-1002

4-Abd Allah S., A. Khalil, and K. Ali, 2008. The use of Trypan Blue Staining to Select the Developmentally Competent Dromedary Camel Oocytes and its Effect on in Vitro Maturation Rate. The Proc. 20th Annual Scientific Conference, 2-7

5-Anchordoguy TJ, AS. Rudolph, JF. Carpenter, and JH. Crowe, 1987. Modes of the interaction of cryoprotectants with membrane phospholipids during freezing. Cryobiology 1987;24:324–31

6-Atiyah S. S., A. A. Hobi, and M.B.M. Fakhrildin 2019. Cryopreservation of sheep oocytes and embryos using local, modified vitrification tool. Iraqi Journal of Agricultural Sciences–2019 50(Special Issue):216-221. https://doi.org/10.36103/ijas.v50iSpecial.192

7-Bredderman PJ, and RH. Foote, 1969. Volume of stressed bull spermatozoa and protoplasmic droplets, and the relationship of cell size to motility and fertility. J Dairy Sci 1969; 28: 496–501

8-Darvishnia H., N. Lakpour, M.S. Lahijani, H. Heidari-Vala, M.A. Akhondi, H. Zeraati, and M.R. Sadeghi, 2013. Effects of very rapid versus vapor phase freezing on human sperm parameters, Cell Tissue Bank, 14:679–685. DOI 10.1007/s10561-012-9351-z

9-Donnelly E. T., N. McClure, and S. E. M. Lewis, 2001a. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil. Steril. 76(5): 892–900

10-El-Sokary, M.M.M., M.M.M. Kandiel, K.G.H.M. Mahmoud, M.E.A. Abouel-Roos and G.A.M. Sosa, 2013. Evaluation of viability and nuclear status in vitrified mature buffalo oocytes. global Veterinaria, 10: 297-302

11-Farshad A., B. Khalili, and P. Fazli, 2009. The effect of different concentrations of glycerol and DMSO on the viability of markhoz goat spermatozoa during different freezing temperatures steps. Pakistan Journal of Biological Sciences 12(3): 239-245

12-Fuller, B., and S.Paynter, 2004. Fundamentals of cryobiology in reproductive medicine. Reproductive Biomedicine Online, 9, 680–691. DOI: https://doi.org/10.1016/S1472-6483(10)61780-4

13-Joshi A.J., 2016. A review and application of cryoprotectant: the science of cryonics, PharmaTutor; 2016; 4(1); 12-18.

14-Hassan M. S., S. M.Eidan, F. F. K. Ibrahim, and J. Yahya. 2021. Effect of swim-up and glass wool techniques, with adding antioxidants to tris extender on improving post-cryopreserved total sperm characteristics in straw and freez ability percentage for low semen quality of Holstein bulls. Iraqi Journal of Agricultural Sciences, 52(4): 552-563. https://doi.org/10.36103/ijas.v52i3.1342

15-Hermiz H.N., and G.M.A.Hadad. 2020. Factors affecting reproductive traits in several breeds of dairy cattle in Iraq. Iraqi Journal of Agricultural Sciences,51(2):629-636. https://doi.org/10.36103/ijas.v51i2.990

16-Hubalek, Z., 2003. Protectants used in the cryopreservation of microorganisms. Cryobiology. 46: 205–229

17-Gao DY, S. Lin, PF. Watson, and JK. Critser. 1995. Fracture phenomena in an isotonic salt solution during freezing and their elimination using glycerol. Cryobiology 1995;32:270–84

18-Lee J.H., J.K. Park, S.Y. Yoon, E.A. Park, J.H. Jun, H.J. Lim, J. Kim, and H. Song,. 2021. Advanced maternal age deteriorates the developmental competence of vitrified oocytes in mice, cells 2021, 10, 1563.

19-Liebermann, J., F. Nawroth, V. Isachenko, E. Isachenko, G. Rahimi, and M. Tucker, 2002. Potential importance of vitrification in reproductive medicine. Biology of Reproduction, 67, 1671–1680.

20-Mahmoud KGM, MMM. El-Sokary, MEA. Scholkamy Abou El-Roos, GAM. Sosa, and MF. Nawito,. 2013. The effect of cryodevice and cryoprotectant concentration on buffalo oocytes vitrified at MII stage. *anim reprod*, 10:689-696

21- Mostagir A.M., M.A. Elmetwally, A. Montaser, M. Samy, and S.M. Zaabel, 2019.

Effects of L-carnitine and cryoprotectants on viability rate of immature buffalo oocytes in vitro after vitrification, AJVS. 62(2): 45-52.

22-Parnpai R., Y. Liang, M. Ketudat-Cairns, T. Somfai, and T. Nagai, 2016. Vitrification of buffalo oocytes and embryos. Theriogenology, 86:214–220

23-Phogat JB, and AK. Pandey, 2016. Singh I. seasonality in buffaloes reproduction. International Journal of Plant, Animal, and Environmental Sciences. 6(2):46-54

24-Preciado J.A.,and B. Rubinsky, 2010. Isochoric preservation; A novel characterization method; Cryobiology; 60(1); 23-29

25-Rasul Z, N. Ahmed, and M. Anzar, 2007. Antagonist effect of DMSO on the cryoprotection ability of glycerol during cryopreservation of buffalo sperm. Theriogenology 68: 813–819

26-SAS.2012. Statistical Analysis System. User´s Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C., USA

27-Trigos. 2011. Effects of Cryopreservation on the Meiotic Spindle, Cortical Granule Distribution, and Development of Rabbit Oocytes. M.SC Thesis. University of Valencia .Italy. 31-32

28-Van den E. Abbeel, Van der J. Elst, van der M. Linden, and van A.C. Steirteghem, 1997b. High survival rate of one-cell mouse embryos cooled rapidly to -196 degrees C after exposure to a propylene glycol–dimethylsulfoxide –sucrose solution. Cryobiology 34, 1–12

29-Wani N.A., A.K. Misra, and S.N. Maurya, 2004. Maturation rates of vitrified-thawed immature buffalo (*Bubalus bubalis*) oocytes: effect of different types of cryoprotectants. Animal Reproduction Science 84 (2004) 327–335

30-Wowk, B., 2007. How cryoprotectants work. Cryonics. 3: 3-7.