

Impact of Cryopreservation Techniques on Embryos Viability and Normality in Awassi Sheeps

Raed Kawkab Al-Muhja

College of Agriculture, University of Al-Qadisiyah, Iraq.

Corresponding author E-mail: raed.abudulhussain@qu.edu.iq

Received : 9/5/2022

Acceptance : 30/5/2022

Available online: 1/6/2022

Abstract. This study was conducted to show the effect of three different techniques of embryo freezing on the vitality and percentage of normal embryos. This research was carried out Between December 2020 and June 2021, in the Institute for Infertility Diagnosis and Assisted Reproduction at Al-Nahrain University . Ovaries were obtained from ewes that were slaughtered in a Shulla local slaughterhouse. 75 ovaries were obtained and randomly divided into three groups in this study. Oocytes were extracted from the ovaries using an aspiration procedure, followed by in vitro maturation, fertilization, early embryonic development, then vitrification, Rapid cryopreservation, slow cryopreservation, and finally vital tests and natural embryos. The nappy vitrification process was determined to be the best for cryopreservation of embryos in this investigation.

Keywords. Cryopreservation, Techniques, Viability, Vitrification.

1. Introduction

Slow freezing reduces the chance of intracellular ice formation by causing extracellular ice crystals (seeding) to form at a low temperature below zero (e.g., 6 to -9 oC). The rate of freezing determines the rate at which extracellular ice crystals grow, Extracellular ice pulls water from the cell, leaving a small amount of free water and forming one small ice crystal [1]. When the freezing rate is balanced between the rate of water loss from the cell (dehydration) and the rate of water incorporation in extracellular ice crystals, the best cryopreservation results are attained. It's vital to remember that the formation of ice crystals is a natural element of the freezing process. [2].

rapid freezing: High concentrations with penetrating cryoprotectants (1 to 1.6 M of DMSO, EC or glycerol is used in this method. Since high cryoprotectants concentrations have a high toxicity. The speed of freezing is very high (-1500 ° C per minute), which causes freezing without ice crystals formation [3]. While, the rapid drop in temperature during the period of transition is likely to the reduces amount of damage caused by cooling and decreasing the stress of thermal in cells [4].

Has two important points: the selection of cryoprotectants, and the appropriate concentration. Vitrification solutions often contain permeable cryoprotectants (such as glycerol, ethylene glycol, 1, 2 propanediol), small disaccharides (such as sucrose, trehalose, glucose) and macromolecules (propylene glycol, follicle 70, bovine serum albumin) [5].

The goal of this study was to see how three different embryo freezing procedures affected the vitality and percentage of normal embryos.

2. Materials Method

The ovaries of sheep slain in the local butcher of AL-Shualla were used in this investigation. The research was conducted in the laboratories of AL-Nahrain University's Institute of Embryo Research and Infertility Treatment.

2.1. Solution Preparation for Vitrification and Thawing

SMART medium was used in the equilibration solutions (ES), containing 10% bovine Serum Albumin (BSA) with 7.5% (v/v) from glycerol with 0.25M sucrose.

Vitrification solutions (VS) consist of SMART medium containing 10% bovine Serum Albumin (BSA) with 15% (v/v) from glycerol with 0.5M sucrose.

Sucrose-containing thawing solutions (TS) were made at three concentrations (0.5M/l, 0.25M/l and zero M/l) and mixed with 10% BSA-containing CM. With a few tweaks, these solutions were created in accordance with [6,7].

2.2. Solution Preparation for Rapid Cryopreservation

Equilibration solutions (ES) containing 7.5 percent (v/v) glycerol and 7.5 percent (v/v) EG were made by adding the appropriate volume of CPA to culture medium containing 10% BSA. 15 percent (v/v) EG and 15 percent (v/v) glycerol In quick cryopreservation solutions, they were added to CM supplemented with 10% BSA..

2.3. Solutions for Defrosting

Solution 1:

0.5 M sucrose in SMART medium

Solution 2:

0.25 M sucrose in SMART medium

Solution 3:

As a washing solution, use SMART medium..

2.4. Preparation of Slow Cryopreservation Solution

The slow cryopreservation solution was prepared by adding 7.5% (v/v) of glycerol to the culture medium.

2.5. Preparation of Trypan Blue Dye

Trypan blue is the stain according to method [8].

2.6. Collection of Ovaries

A local abattoir in Baghdad provided the sheep ovaries (Al shullah). Following protocol, both ovaries of each animal were taken immediately after slaughter and placed in glass tubes containing normal saline solution (0.85 percent NaCl). according to technique [9].

2.7. Oocytes Collection

Oocytes were collected from the ovaries using the aspiration technique [10].

2.8. In Vitro Maturation

About 5-10 oocytes per droplet (1mL) from culture SMART with PMSG (10 IU/mL), estradiol (1g/mL), and hCG (5 IU/mL) were cultured on a four-well Petri dish and covered with liquid paraffin and incubated for about 24 h in a CO₂ incubator (5 % CO₂) at 38.5°C with high relative humidity (100 %) [11].

2.9. In Vitro Fertilization Technique

Before being put to 4-well culture plates (5-10 oocytes/well) with 1mL of fertilization SMART media and motile spermatozoa (5104 sperm/oocyte), mature oocytes were washed twice in fertilization medium. Sperm and oocytes were incubated at 37°C for 24 hours after being coated with liquid paraffin in a humidified atmosphere containing 5% CO₂ and 95% humidity (CO₂ incubator) [12].

2.10. *In Vitro Early Embryonic Development*

Embryos were washed three times with SMART media SMART and cultured in 1mL of semi medium on 4-well culture plates (5zygotes/well) and covered with liquid paraffin for 24 hours at 37°C in a moist atmosphere of 5% CO₂ and 95% humidity in a CO₂ incubator [12].

2.11. *Vitrification and Thawing*

The vitrification process was carried out by immersing the embryos in a drop of equilibrium solution for 3 minutes, then transferred to a drop of the vitrification solution for one minute, then the embryo was carried on the modified cryotop with as little vitrification solution as possible, then the cryotop was immersed in liquid nitrogen. As for thawing, it is done by dropping the embryos with a drop of soluble solutions for a minute drop for each of the solutions.

2.12. *Rapid Cryopreservation and Thawing*

The rapid cryopreservation process was carried out by immersing the embryos in a drop of equilibrium solution for 3 minutes, then transferred to a drop of Rapid cryopreservation solution for one minute, then the embryo was carried on the cryovial with 0.25 ml from Rapid cryopreservation solution, then the cryovials was immersed in liquid nitrogen. As for thawing, it is done by dropping the embryos with a drop of soluble solutions for a one minute drop for each of the solutions.

2.13. *Slow Cryopreservation and Thawing*

Slow cryopreservation was carried out by placing the embryos 1/4 ml of the slow freezing solution in a seminal tube, then placed in the refrigerator for 2 hours, then placed on liquid nitrogen vapor for five minutes, then immersed in liquid nitrogen. Thawing is done by shaking the tube in the incubator at a temperature of 37°C for 30 minutes. Then the stalk is emptied into a petri dish and the embryos are washed using the culture medium.

2.14. *Viability Test*

The viability test of the embryos was carried out using trypan blue dye by placing the embryos with a drop of trypan blue dye after three minutes. Embryos are examined under the inverted microscope. Embryos that are stained in dark blue color are dead, and those that do not have cytoplasmic staining are viable, and the results are recorded. [13].

2.15. *Normal Morphology Test*

Normal morphology test The normal morphology was examined by examining the embryos under the inverted microscope and observing the external shape of the embryos in terms of regular cytoplasm granulation and the shape of the zona pellucida, regular, circular and unbroken, and the results were recorded as a percentage. [14].

2.16. *Statistical Analysis*

Statistical analysis of the results was carried out using the chi-square test to compare between the transactions. The spss software was used.

3. Results and Discussion

The percentage of viable 1-2 cell embryos increased significantly (P 0.05) using vitrification technique rapid cryopreservation while there were significant decrease (P ≤ 0.05) in the viable 1-2 cell embryo percentage (figure 1).

Figure (2) There was a significant increase (P 0.05) in the viable 3-4 cell embryo percentage using vitrification technique rapid cryopreservation, while there was a significant decrease (P 0.05) in the viable 3-4 cell embryo percentage fast cryopreservation using vitrification technology.

A significant enhancing (P ≤ 0.05) was noticed in the normal 1-2 cell embryo percentage using vitrification technique rapid cryopreservation while there were significant decrease (P ≤ 0.05) in the normal 1-2 cell embryo percentage (figure 3).

Figure (4) noting results of normal Embryo with 3-4 cells percentage when using various cryopreservation techniques there were a substantial increase ($P \leq 0.05$) was noticed in the viable Embryo with 3-4 cells percentage using vitrification technique rapid cryopreservation while there was significant decrease ($P \leq 0.05$) In the typical 3-4 cell embryo percentage. Has two important points: the selection of cryoprotectants, and the appropriate concentration. Vitrification solutions often contain permeable cryoprotectants (such as glycerol, ethylene glycol, 1, 2 propanediol), small disaccharides (such as sucrose, trehalose, glucose) and macromolecules (propylene glycol, follicle 70, bovine serum albumin) [5]. The rate of freezing determines the rate at which extracellular ice crystals grow. The extracellular ice sucks water out of the cell, leaving a small amount of free water and forming one small ice crystal [1]. The optimum result this method of cryopreservation is attained whenever The rate of water loss from the cell (dehydration) and the rate of water incorporation in extracellular ice crystals must be in balance. It is important to note that the production of ice crystals is part of the gradual freezing process. [2]. This study concludes that nappy vitrification technique was best for embryos cryopreservation.

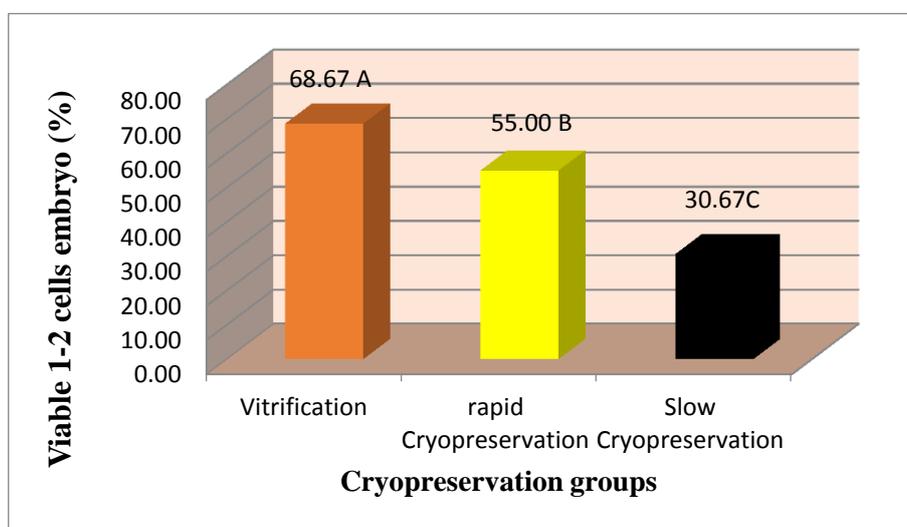


Figure 1. Effect of cryopreservation technique on viable 1-2 cell embryo percentage Chi square= 33.192 P value= 0.002**

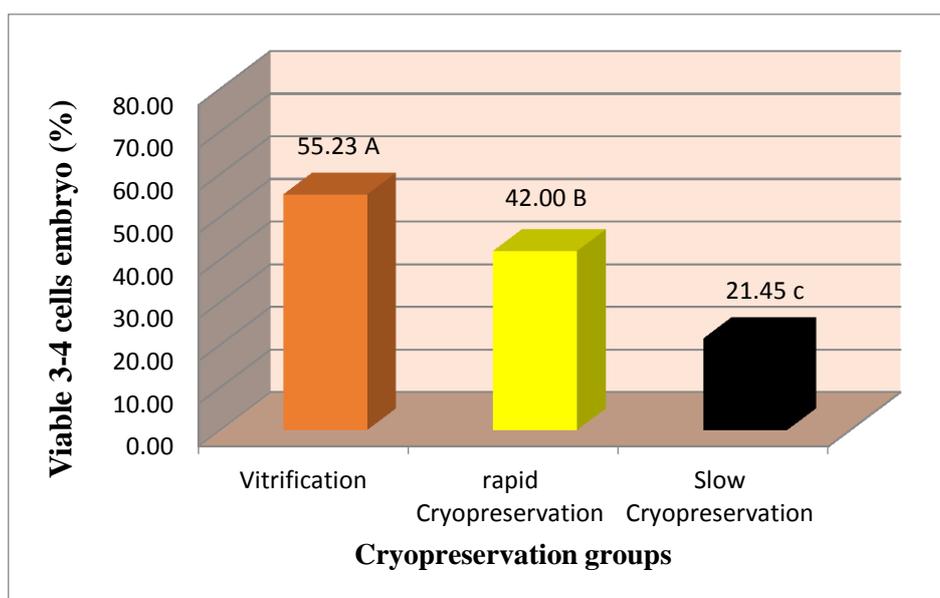


Figure 2. Effect of cryopreservation technique on viable 3-4 cell embryo percentage Chi square= 64.133 P value= 0.0005**

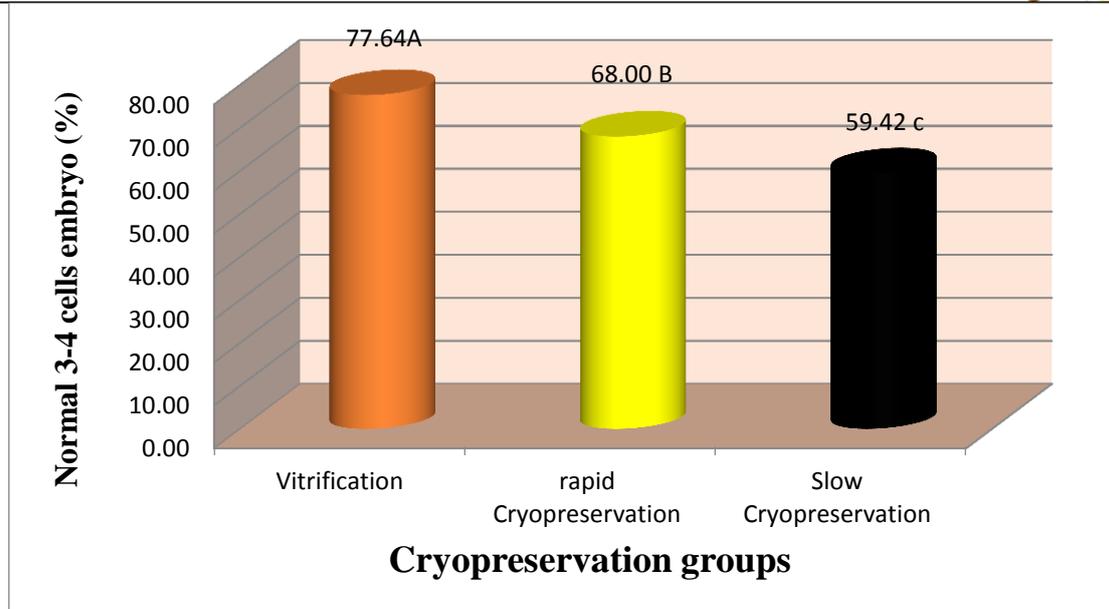


Figure 3. Effect of cryopreservation technique on normal 1-2 cell embryo percentage Chi square= 28.419 P value= 0.03*

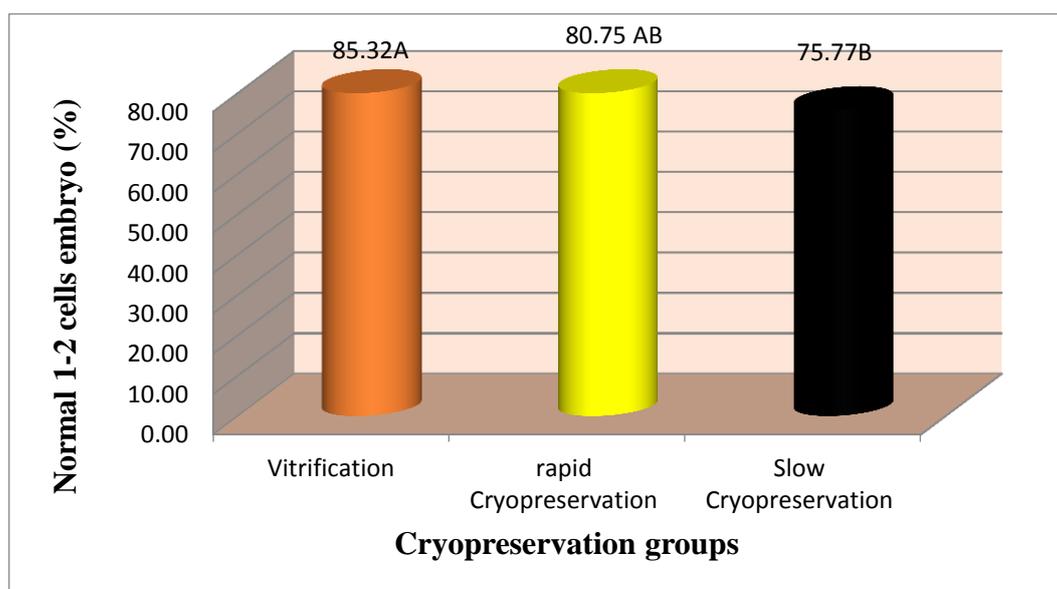


Figure 4. Effect of cryopreservation technique on viable 3-4 cell embryo percentage Chi square= 13.579 P value= 0.04*

References

- [1] Shaw, J.M., Cox, S.L., Trounson, A.O. and Jenkin, G. 2000 . Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications. *Mol Cell Endocrinol*, 161:103-110.
- [2] Ayalew, E. and Lemma, A. 2010. Evaluation of cryopreservation methods and extender types for storage of donkey semen. As part of DVM thesis, Faculty of Veterinary Medicine, Addis Ababa University.
- [3] Ataya, K. and Moghissi, K. 1989. Chemotherapy-induced premature ovarian failure: mechanisms and prevention. *Steroids*, 54:607–26.

- [4] Hsieh, Y.Y., Tsai, H.D., Chang, C.C., Chang, C.C., Lo, H.Y. and Lai, A.C. 1999. Ultrarapid cryopreservation of human embryos: experience with 1,582 embryos. *Fertil. Steril*, 72: 253–6.
- [5] Elnahas, A., Alcolak, E., Marar, E.A., Elnahas, T., Elnahas, K., Palapelas, V., Diedrich, K. and Al-Hasani S.2010. Vitriification of human oocytes and different development stages of embryos: an overview *Middle East Fertil Soc J*, 15: 2–9.
- [6] Kuwayama, M., Vajta, G., Kato, O and Leibo, S.P. 2005. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 11: 300–308.
- [7] Kuwayama M. 2007. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology*, 67(1): 73-80.
- [8] Fakhridin, M.B.M.R. and Al-Moussawi, R. H. A. 2013. Effect of two types and two concentrations of cryoprotectants on ovine oocytes morphology and viability post-vitrification *Iraqi J. Embryos and Infertility Researches* 3:64-75.
- [9] Rezk, W. A. K. 2009. Studies on In Vitro fertilization in Camels (*Camelus dromedaries*) Faculty of Agriculture Animal Production Department Mansoura University (PhD thesis).
- [10] Wang, Z.G.; Xu, Z.R. and Yu, S.D. 2007. Effects of oocyte collection techniques and maturation media on in vitro maturation and subsequent embryo development in Boer goat *Anim. Sci.*, 52, (1): 21–25.
- [11] De Felici, M. and Siracusa, G. 1982: Spontaneous hardening of the zona pellucida of mouse oocytes during in vitro culture. *Gamete. Res.*, 6: 107-113.
- [12] Crozet, N., Huneau, D., Desmedt, V., Théron, M.-C., Szöllösi, D., Torrès, S. and Sévellec, C. 1987. In vitro fertilization with normal development in the sheep. *Gamete Res.*, 16: 159–170.
- [13] Alberto, R., Stefano, C., Andrea, C., Claudia, F., Carlotta, P., Gianluca, G., Luisa, D. P. and Chiara, B. 2019. Impact of the addition of Early Embryo Viability Assessment to morphological evaluation on the accuracy of embryo selection on day 3 or day 5: a retrospective analysis *Journal of Ovarian Research* volume 12, Article number: 73 .
- [14] Jihui, Ai, Lei, Jin, Y.u. Zheng, Peiwen Yang, Bo Huang and Xiyuan, D. 2021. The Morphology of Inner Cell Mass Is the Strongest Predictor of Live Birth After a Frozen-Thawed Single Embryo Transfer *Front. Endocrinol.*, 24 February | <https://doi.org/10.3389/fendo.2021.621221>.