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The Effect of Cryopreservation Temperature and Time on Viability and *In Vitro* Maturation of Sheep Oocytes

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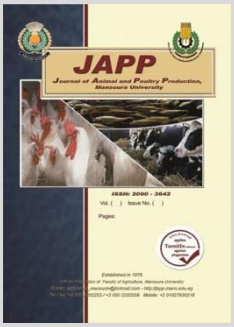

ABSTRACT

This study aim to compare the effects of freezing techniques on viability and in vitro maturation of sheep oocytes. This experiment was carried out in Animal Production Research Institute. Sheep ovaries were obtained from slaughterhouse. A total of 695 oocytes were used in this study, 114 oocytes as control and 581 were exposed to LN2 vapor then divided into 3 groups: G1(190); directly plunged into LN2, G2 (198): cryopreserved in -80 ºC freezer and, G3 (193), plunged into LN_2 for 10 min and then transferred to –80 ºC freezer. Oocytes in each group were cryopreserved for (24h, and 7days). There were high significantly differences (P<0.01) between the percentage of damage for cryopreserved oocytes, where zona dissolution was the main damage for (G1and G3) oocytes. Whereas, zona rupture was the highest damage occurred in (G2) oocytes. Survival rate of G1 oocyte was higher significantly (P<0.05) than G3 and G2, the same results were obtained pattern, either after 24h or 7 days storage. Highly significant differences (P<0.01) between maturation rate achieved with the control oocytes (63%) compared with the other groups. After 24 h of storage, G1 recorded higher MII rate followed by G3 and G2 (47, 37 and 28%, respectively), MII % decreased at 7 days storage in G1, G3 and G2 (44%, 34% and 31%, respectively). In conclusion, Cryopreservation of sheep oocytes (G3) in –80 °C freezer achieved acceptable survival and in vitro maturation rates. The –80°C freezer provide a simple and cheap tool to maintain frozen sheep oocytes.

Keywords: Ewes - Oocyte - cryopreservation- storage time– IVM.

INTRODUCTION

Gamete cryopreservation has achieved a great progress during the last five decades. It provides valuable sperm and oocytes and accelerates the dissemination of genetic materials and ensure providing continuous source of oocytes and embryos for many applications in genetic improvement programs; *in vitro* embryo production, embryo transfer (Dhali *et al*., 2019).

Sperm cryo-storage attempts were achieved earlier than oocytes and embryos, and a huge number of intact and viable spermatozoa was obtained from each sample. Whereas, oocytes are more valuable and fragile, it took long time for cryopreservation techniques to develop considerably for their efficient suspension, cryopreservation of embryo is the traditional first-choice technique for fertility preservation.

Preservation of cells or even whole tissues are carried out by exposure to ultra cool temperature in LN_2 (-196 °C) (Woods *et al*., 2004). At this cooling condition, vitality is functionally blocked, and the cells can be conserved for a long time (Mazur, 1970). However, many physical damage the cells with cryopreservation. Intracellular ice formation is the main reason to cell death; therefore, cryopreservation protocols use a combination of dehydration, freezing point depression, super-cooling, and intracellular vitrification in an attempt to avoid cell damage (Wolfe and Bryant, 2001).

There are real attempts to apply cryopreservation of gametes to as low as -80ºC, because storage at -80ºC has several advantages versus to LN_2; dedicated space/room is not required, it is cheaper than a LN_2 tank with constant refills and it just needs electrical support. Moreover, -80ºC freezer engaging area is easy to be provided in any laboratories (Raspa, *et al*., 2018).

Freezing mouse spermatozoa at -80 ºC, without liquid nitrogen, and subsequent long-term exposure to -80 ºC did not affect the potency of frozen spermatozoa, regardless of the cryoprotective agent used (Raspa *et al*., 2018). The kind and concentration of the cryoprotectant agents for germplasm cryostorage, have to be considered for all cryopreservation protocol. Ethylene glycol is one of the permeable cryoprotectants that can enter cells, can be used to induce the vitrification of intracellular environment before ice crystal formation, thus protecting excessive loss of cell volume (Chen, *et al*., 2000).

The importance of this approach is providing many advantages; preserving of large number of samples, shortening sample processing time and reducing the economical cost, more easier to find any sample because of the well-organized system in the freezer, and it is very suitable in placed where LN_2 is difficult to obtain. Therefore, this study aims to compare the effects of the freezing protocol using LN_2 or -80 ºC freezer on viability and *in vitro* maturation of frozen-thawed sheep oocytes.

MATERIALS AND METHODS

Collection of cumulus-oocytes complexes

The experiment was carried out at the Reproduction and Biotechnology Laboratory, Animal Production Research Institute. Sheep ovaries were obtained from the

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local abattoir and transported to the laboratory in 0.9% (w/v) saline solution at 35ºC. The cumulus-oocytes complexes (COCs) were obtained by aspiration of 2 to 8mm follicles using a syringe needle (18-gauge). The follicles content were collected in modified PBS medium supplemented with gentamycin sulphate (50 µg/mL). After that oocytes were selected and classified morphologically. Only oocytes with at least 3 layers of cumulus cells and homogeneous cytoplasm were selected for experiment execution (Madison and Fraser, 1992).

Experimental design

A total of 695 COCs were used in this study, 114 oocytes were cultured directly for *in vitro* maturation(IVM) as control (without cryopreservation process). Whereas, 581 immature oocytes were divided into three experimental groups (for cryopreservation process) and then incubated for IVM.

Oocytes vitrification and thawing method

Vitrification protocol:

Ethylene glycol (EG) was used as a cryoprotectant agent for oocytes vitrification (Chen *et al*., 2000) in this study for vitrification protocol.

The first vitrification solution (VS1) was composed of TCM-199 as a base medium (BM) with 15% ethylene glycol (EG) (Carl Roth, Denmark) and 0.5% (w/v) bovine albumin, the second vitrification solution (VS2) was composed of BM with 30% EG and 0.5% (w/v) bovine albumin. All solutions were kept at room temperature.

Cumulus Oocytes Complex (COC) were loaded into modified straws described by Madboly (2018), that called "Modified- Filter Standard Straws" (MFSS), these straws were designed for oocytes vitrification. Each straw is loaded with 5 COCs in 50 µLBM, by negative pressure using 1 mL syringe allowing the oocytes to attach with the filter of the MFSS, and separated by an air bubble, then 50µLof VS1was loaded by negative pressure too. During this step COCs were submitted to VS1 for 2 min. Then, submitted to VS2 for 45 sec. followed by negative pressure to exclude the surrounding VS2. After sealing with polyvinyl alcohol powder, straws were exposure to LN2 vapor for 15 min. After that, straws with vitrified oocytes were divided to three different groups;

G1: oocytes (n= 190) were directly plunged into LN2.

G2: oocytes (n= 198) were cryopreserved in -80 ºC freezer.

G3: oocytes (n=193) were directly plunged into LN_2 for 10 min and then transferred to -80 ºC freezer.

Oocytes in each group were divided into 2 subgroups according to the cryo-storage period; the first one was cryo-preserved for 24h, and the second for 7 days, to evaluate their viability by the percentage of oocytes reached second metaphase (MII) stage, after *in vitro* oocytes maturation.

Thawing and rehydration

After 24h and 7 days of cryopreservation, the loaded COCs in MFSS were melted in air for 10 sec, followed by plunging in a water bath at 37 °C for 1 min, then loading BM at 37ºC into MFSS by negative pressure for 10 seconds. Thereafter, cryoprotectant agents were removed gradually by loading descending concentrations of sucrose (0.5, 0.25, and 0.125 M) for 60s each, at 37 °C, then COCs exposed twice to BM for 1 min each. Final solution with COCs of each straw was evacuated into Petri dish for evaluation. Oocytes with zona pellucida injury and/or shrinkage of cytoplasm were excluded. The surviving oocytes were submitted to IVM.

***In vitro* maturation (IVM)**

Tissue culture medium-199 (TCM-199) was used for oocytes maturation, supplemented with 10% fetal calf serum (FCS), 10 ng /mL of FSH, 10 IU/mL LH, 1 µg/mL estradiol 17β, 0.25 mg /mL Na pyruvate and 50 µg/mL gentamycin sulphate (Wani and Wernery, 2010). *In vitro* oocytes maturation was carried out in 35 mm Petri dishes, containing maturation medium droplets with additives and covered with mineral oil, and incubated previously for 2 h at 38.5 °C, Relative humidity 90% and 5% CO_2. Oocytes incubation time was 24h, after this period, maturation rates were evaluated.

Evaluation of maturation status

After maturation period, the cumulus cells of COCs were removed by negative pressure of pipette or by vortexing. The cumulus-free and homogeneous oocytes were then fixed in acetic acid: ethanol (1: 3 v/v) in small culture dishes (35 mm) for at least 48 h at 4 °C. Fixed oocytes were transferred to glass slides; silicone gel was used to maintain a cover slip in contact with the oocytes. The slides were immersed in 1% aceto-orcein stain for 30 min. Then, slides were washed 3 times in increasing concentrations of ethanol to remove the surplus orcein dye as follows: 5 sec in 70% ethanol, then 1 and 3 min in 100% ethanol (Rao *et al*., 2002).The slides were examined at 1000X magnification to evaluate the stage of maturation. Oocytes were categorized according to their nuclear development as second metaphase (MII), first anaphase (AI), first metaphase (MI), Germinal vesicle (GV), or degenerated (Deg). Oocytes were considered mature in MII stage.

Statistical analysis

IVM proportions were compared by Chi-square test; when the difference was ($P \leq 0.05$) considered statistically significant. In case of significant differences, pair wise comparisons were carried out by applying normal Z-test (Snedecor and Cochran, 1992).

RESULTS AND DISCUSSION

Results

Sheep oocytes cryopreservation either under LN_2 or in -80ºC freezer followed by subsequent thawing affected considerably on the morphological status of the immature oocytes in this experiment. Three main changes were found and consider as not survived (damaged) oocytes; zona rupture, zona dissolution, and shrinkage of cytoplasm (Fig. 1).

There were high significantly differences ($P<0.01$) between the proportion of ewes' oocytes damage as a result of cryopreservation protocol after 24h and 7 days. The obtained results revealed that zona dissolution was the main damage for oocytes stored in LN_2 (G1), and oocytes stored in LN_2, (10 min) and then at (-80°C) (G3). Whereas, zona rupture was the highest damage occurred in oocytes stored at (-80°C) freezer (G2). While squeezing of cytoplasm recorded the less percentage of damage for all groups(Fig. 2).

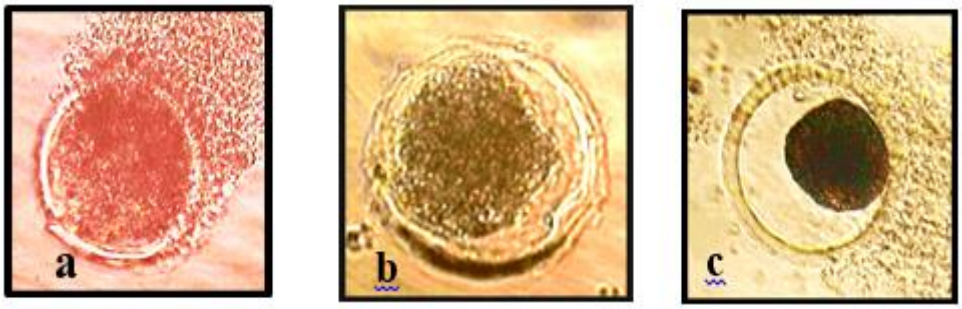

Fig. 1. Effect of cryopreservation process on sheep immature oocyte's zona pellucida and cytoplasm morphology
(a: zona rupture, b: zona dissolution, c: shrinkage of cytoplasm)

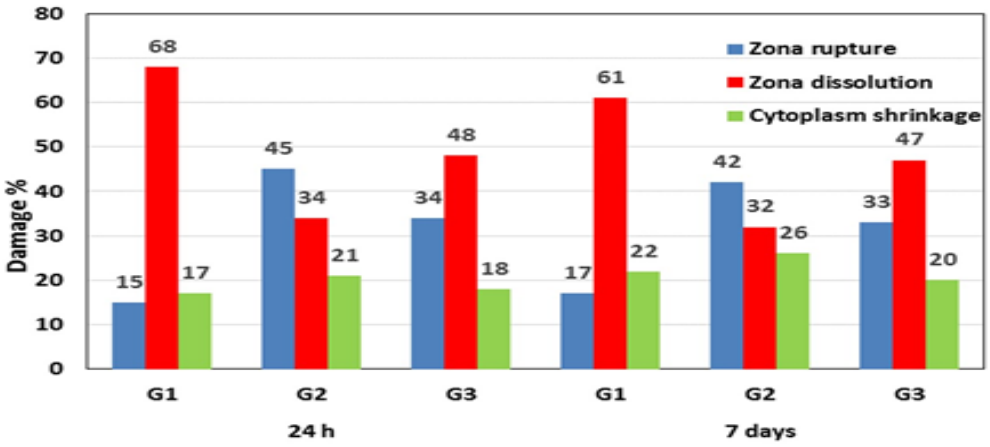

Fig. 2. Percentage of damage type classification in ewes' oocytes as a result of cryopreservation protocol after 24h and 7 days.(Cryopreservation protocol; G1: Oocytes stored in LN_2, G2: oocytes stored at -80ºC and G3: oocytes stored in LN_2, (10 min) and then at -80ºC.

Table 1. Effect of cryopreservation protocol and storage period on the percentage of Survival *vs.* damage immature ewes oocytes

Treatment	No. Oocytes used		Cryo-Storage period	No. Oocytes Survived(%)	Damage of Oocytes (%)
	Group	Sub Group			
Control	114	-	-	114 (100%)	0
G1:LN_2 vapor + LN_2 storage	190	94	24h	70 (75%)	24 (25%)
		96	7 days	75 (78%)	21 (22%)
G2:LN_2 vapor + (-80ºC) freezer	198	101	24h	60 (59%)	41 (41%)
		97	7 days	59 (61%)	38 (39%)
G3:LN_2 vapor + LN_2 (10 min) + (-80ºC) freezer	193	98	24h	68 (69%)	30 (31%)
		95	7 days	65 (68%)	30 (32%)

Cryopreservation process of immature ewes oocytes include; exposure to cryoprotectant, vitrification, LN_2 vapor, plunged in LN_2 or storing in -80ºC freezer, thawing and rehydration. This process significantly affected with varying degree of damage on the ewes immature oocytes zona pellucida and cytoplasm morphology. The occurred damage differed significantly according to cryopreservation protocol and storage period compared to the control oocytes (fresh oocytes that had not been subjected to cryopreservation/thawing process).

The obtained result revealed that the survival rate of G1 oocyte was higher significantly ($P<0.05$) than G3 and G2 (average 72.5 vs. 68.5 and 60%, respectively) regardless the storage period (Table 1). Survival rate of immature ewes oocytes followed the same pattern either cryo-storage was 24h or 7 days, but it tend to be better after 24h compared to 7 days, except in G3 the survival rate increased a little bit.

After cryopreservation process oocytes with rupture or dissolution zona pellucida and squeezing cytoplasm, were discarded, and the remaining oocytes from all cryopreserved groups and fresh immature oocytes were submitted to IVM. Results in (table 2) cleared highly significant differences ($P<0.01$) between maturation rate (MII %) achieved with the fresh sheep immature oocytes (control) (63%) compared with the other groups. After 24 h of cryo-storage, oocyte in G1 recorded higher maturation rate followed by G3 and G2 (47, 37 and 28%, respectively). The maturation rate decreased by extending the storage period to 7 days, following the same previous pattern of 24 h storage, where oocytes reached MII stage in G1 recoded were 44%, followed by G3 (34%), and finally G2 recorded the less percentage (31%). Differences in maturation rate between the storage periods within each group were not significant. High percentage of the cryopreserved oocytes in the present study was remained in the Germinal vesicle (GV) stage.

Table 2. Effect of cryopreservation protocol and storage period on *in vitro* maturation of immature ewes oocytes

Treatment	Cryo- Storage period	No. Survived oocytes	Stage of Nuclear Maturation				
			GV (%)	MI (%)	AI (%)	MII (%)	Deg. (%)
Control	-	114	13(11%)^{c}	9(8 %)^{b}	16(14 %)^{a}	72 (63%)^{a}	4(4 %)^{c}
G1: LN_2 vapor +	24h	70	13(18%)^{c}	12(17%)^{a}	6(9%)^{b}	33(47%)^{b}	6(9%)^{b}
LN_2 storage	7 days	75	15(20%)^{b}	11(15%)^{a}	9(12%)^{a}	33(44%)^{b}	7(9%)^{b}
G2: LN_2 vapor +	24h	60	20 (33%)^{a}	7 (12%)^{b}	4 (7%)^{b}	17(28%)^{c}	12(20%)^{a}
(-80ºC) freezer	7 days	59	17 (29%)^{a}	5 (8%)^{b}	6 (10%)^{b}	18 (31%)^{c}	13(22%)^{a}
G3: LN_2 vapor +	24h	68	16 (24%)^{b}	10 (14%)^{a}	8 (12%)^{a}	25(37%)^{bc}	9 (13%)^{b}
LN_2 (10 min) +(-80ºC) freezer	7 days	65	18 (28%)^{a}	8 (12%)^{b}	6 (9%)^{b}	22(34%)^{bc}	11(17%)^{b}

Values in columns with different superscripts (a – c) differ significantly (P<0.05).

Discussion

Cryopreservation of immature sheep oocytes is an important challenge. The present findings for immature oocytes show high sensitiveness to both ultra-low temperature and vitrification solutions (either on morphological or cytological levels), Explaining that the capability to recover injuries is limited by the effective applications of cryopreservation processes.

In this study, the impact of exposure to vitrification solutions and cryopreservation process on viability and developmental ability of immature sheep oocytes were studied, through the signs of damage such as; cytoplasm shrinkage, zona rupture and zona dissolution.

There is a difference between the oocytes tolerance to vitrification, cryo-storage, either in LN_2, -80ºC freezer or LN_2 for a short period (10 min) and then -80ºC freezer. This difference is refer to freezing method and thawing process that make vitrified oocytes were affected morphologically and cytologically. These results are the same with Succu *et al*. (2007) who reported that exposing ovine oocytes to CPs-induced changes in the arrangement of microtubule, where, the morphology of spindle configuration was strongly affected after vitrification, but not only after exposition to vitrification solution. In the same context, the cytoskeleton of immature oocyte is more sensitive to damage by CPs than mature oocytes (Men, *et al*., 2002).

The results showed that surviving rate of oocytes plunged directly in LN_2(G1) was higher significantly than G2 and G3. This results could be explained by the finding of Shinsuke and Mazur (2011), where they found that small non-fatal amount of intracellular ice created through the cryopreservation at -80ºC, and these intracellular crystals increased to a harmful size by extending the storage period to 3 months.

In G3, it seems that subjected oocytes to LN_2 vapor and plunged them in LN_2 for 10 min (rapid cooling rate), clearly affected positively the survival rate of oocytes compared with G2 oocytes which are not plunged into LN_2, this means exposing oocytes to LN_2 vapor is not enough to pass the intracellular ice formation threshold. Paynter (2005) mentioned that the fast cooling rate of vitrification process permits frosting of intracellular and extracellular water into a glass-like status crossing over the formation of ice crystals. Where, ice crystals is the major source of cryo-injury.

Keisuke (2017) mentioned that the sensitivity of oocytes to chilling, cryoprotectant toxicity and permeability of water and cryoprotectants to the plasma membrane of oocytes and embryos, is the main characteristic which is markedly concerning to prime reasons of cell damage.

The results revealed that the percentage of oocytes in (G1) reached second metaphase of the nuclear maturation was (47%) and lower than fresh *in vitro* matured oocytes (63%). This decrease is due to cryopreservation and keeping under LN_2 for 24 h, whereas extending the storage period to 7 days, percentage of MII oocytes decreased to (44%). This finding could be explained by Succu *et al*. (2007), they mentioned that the microtubular structure of oocyte spindle is very sensitive to changes, physically (cooling - warming) or chemically (exposure to cryoprotectants) which may trigger tubulin depolymerization and microtubular dissociation with deleterious effects on chromosomal regulation. Different works reported depolymerization of spindle microtubules and microfilaments after cryopreservation (Chen, *et al*., 2003)

Decreasing the percentage of oocytes in G2 and G3, that reached MII, accompanied with increasing of degenerated oocytes (Deg), compared with oocytes in G1. This deleterious effect could be refer to the formation of non-lethal quantities of intracellular ice crystals during freezing at -80 ºC, that may hurt the cellular cytoskeleton and cell components. In mammals, mitochondria are the most copious organelles in the oocyte cytoplasm, and they are the only source providing energy, so abnormalities or dysfunction in mitochondria are affecting negatively on the development of oocytes and embryos. The drooping in ATP production by mitochondria is related to the developmental arrest of oocytes and embryos (Brevini *et al*., 2005). The loss of chromosomal configuration (CC) is generally considered as an indicator of degeneration (Fagundes, 2002). According to Stojkovic *et al*. (1999), CC is a fundamental biochemical mechanism in the cell cycle that undergoes in the pre-metaphase I. In the present experiment, cryopreservation might have stopped the activity of the meiotic regulators that arrested the maturation development (Wu *et al*., 1999). The increased sensitivity of oocytes matured *in vitro* was not only due to the vitrification procedures, but also to a greater extent to oocyte quality (Mahmoud zadeh *et al*., 1995).

CONCLUSION

In the present study, cryopreservation of sheep oocytes (G3) in -80ºC freezer achieved acceptable survival rate and *in vitro* maturation rate when the oocytes plunged into LN_2 for 10 min before transferring to -80ºC freezer, comparing with oocytes in G2. Cryo-storage of immature oocytes in -80ºC freezer may produce small non-fatal amounts of intracellular ice during the cooling and that intracellular crystals increased to a harmful size by extending the storage period. The -80ºC freezer offers a simple and inexpensive way to maintain frozen sheep oocytes for a short period. Cryopreservation of oocytes is still challenging due to oocyte's complex structure and sensibility to chilling. However, further investigations to minimize the harmful effect of vitrification procedures and to maximize sheep oocyte maturation rate *in vitro* would be required.

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"تأثير درجة الحرارة ووقت الحفظ بالتجميد على حيوية ونضوج بويضات الأغنام في المختبر "

إيهاب صلاح عبد العال

معهد بحوث الإنتاج الحيواني – مركز البحوث الزراعية –الدقي –الجيزة – مصر

تهدف الدراسة الحالية إلى مقارنة تأثير تقنيات الحفظ بالتجميد باستخدامالنيتروجين السائل أو الفريزر ٨٠ درجة مئوية تحت الصفر على السلامة والنضج المعملي لبويضات الأغنام المجمدة والمذابة.أجريت التجربة الحالية في معمل التكاثر والتكنولوجيا الحيوية بمعهد بحوث الإنتاج الحيواني. تم الحصول على مبايض الأغنام من المجزر في محلول ملحي دافئ. تم استخدام عدد ٦٩٥ بويضة في هذه الدراسة، ١١٤ بويضة كمجموعة تحكم و ٥٨١ بويضة تعرضت لبخار النتروجين السائل، ثم قسمت إلى ٣ مجموعات: مجموعة ١ (١٩٠بويضة) تمغمر هامباشرة في النتروجين السائل، مجموعة ٢ (١٩٨ بويضة) تم حفظها بالتجميد في فريزر - ٨٠ درجة مئوية، و مجموعة ٣ (١٩٣ بويضة) تم غمر هامباشرة في النتروجين السائل لمدة ١٠ دقائق ثم نقلها إلى الفريزر -٨٠ درجة مئوية. تم حفظ البويضات في كل مجموعة بالتجميدلمدة (٢٤ ساعة و ٧ أيام). كانت هناك فروق معنوية عالية ($P<0.01$) بين نسبة تلف البويضات نتيجة الحفظ بالتجميدحيث كان الانحلال النطاقي هو الضرر الرئيسي لبويضات المجموعة ١ و ٣. بينما كان تمزق غلاف البويضة هو أعلى ضرر حدث في بويضات المجموعة ٢. كان معدل سلامة البويضات بالمجموعة ١ أعلى بكثير ($P<0.05$) من المجموعة ٢ و ٣ سواءاً بعد ٢٤ ساعة أو ٧ أيام من الحفظ بالتجميد. وجد فروق ذات دلالة إحصائية عالية ($P<0.01$) بين معدل النضج (نسبة الطور الأستوائي الثاني) التي تحققت مع البويضات (مجموعة التحكم) (٦٣٪) مقارنة بالمجموعات الأخرى بعد ٢٤ ساعة من الحفظ بالتجميد، حيث سجلت بويضات المجموعة ١ معدل نضج معملي أعلى يليه بويضات المجموعة الثالثة ثم الثانية (٤٧ و ٣٧ و ٢٨ ٪ على التوالي) ، وبينما انخفض معدل نضج البويضات عند التخزين لمدة ٧ أيام في المجموعة ١ و٣ و٢ (٤٤ و ٣٤ و ٣١٪ على التوالي). في الختام، حقق الحفظ بالتجميدلبويضات الأغنام بالمجموعة ٣ في الفريزر -٨٠ درجة مئوية معدل سلامة ونضج معملي مقبول. يوفر استخدام الفريزر -٨٠ درجة مئوية طريقة بسيطة وغير مكلفة للحفاظ على بويضات الأغنام المجمدة لفترة قصيرة.