The Haryana Veterinarian (June, 2025) **64(1),** 30-33 ISSN 0033-4359 (P) **Research Article**

POST-THAW QUALITY OF VITRIFIED IMMATURE OOCYTES FROM SHEEP OVARIES STORED AT VARIOUS TEMPERATURES AND DURATIONS

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ABSTRACT

The current investigation was conducted to evaluate the effect of ovarian storage temperature and duration on post thaw quality of vitrified immature oocytes in sheep. The work consisted of immature oocyte collection from ovaries of abattoir sheep stored at various temperatures (0, 4 and 25° C) and duration (for 0 h, 6 h, 12 h and 24 h) and evaluation of post thaw quality of immature oocytes. Vitrification was carried out in 30% vitrification solution (Ethylene Glycol + DMSO) with post vitrification evaluation after 1-week storage in liquid nitrogen. Fresh ovaries (at 0 h storage) showed highest yield of post thaw morphologically normal oocytes (88.3%) at all the temperature ranges (0, 4 and 25° C). Taken the storage duration variable into consideration, the percentage of post thaw morphologically normal oocytes were significantly higher at 6h of storage as 68%, 74% and 87.1% respectively at 0, 4 and 25° C compared to 12 h (45.8%, 69.9% and 74.6%) and 24 h storage. On the other hand, the effect of storage temperature on yield of post thaw morphologically normal oocytes was observed to be most favourable at 25° C for 6 h storage (87.1%) and 12 h storage (74.6%) followed by 4° C and then 0° C. However, ovaries stored for 24 h gave higher yield (68.9%) at 4° C temperature followed by 25° C and then 0° C. The highest abnormality in terms of shape change (41.1%) in control and zona pellucida breakage at all storage periods at 0° C (25%; 6 h, 41%; 12 h and 29.7%; 24 h). However, ovarian storage at 4° C and 25° C, the highest abnormality observed was the cumulus cell loss in all the storage periods of 6 h (69.2 and 49.4%), 12 h (31.7 and 58.8%) and 24 h (50 and 38.8%), respectively. In conclusion, with increase in storage duration irrespective of storage temperature, the yield of morphologically normal oocytes showed decreasing trend with acceptable post thaw quality at 4° C and 25° C compared to at 0° C. The optimum yield of normal oocytes post thawing was observed after the ovarian storage for 6h at 25° C temperature.

Keywords: Immature oocytes, Quality, Storage duration, Storage temperature, Vitrification

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Cryopreservation of oocytes has advanced significantly over the past few decades, given its importance in various assisted reproductive technologies. Cryopreserving immature oocytes enables immediate storage after follicular aspiration, removing the need for *in vitro* fertilization (IVF) laboratories at the site of collection. However, preserving female germplasm through vitrification of immature oocytes often involves using ovaries from deceased animals or slaughterhouse-derived ovaries. Unfortunately, oocytes from these sources typically exhibit reduced developmental potential compared to those collected from live animals (Zhang *et al.*, 2014).

Additionally, the locations where animals are slaughtered or die, such as in the case of wild animals, are often remote and far from research facilities. This necessitates prolonged storage of ovaries before oocyte retrieval, which can compromise oocyte viability.

The quality of oocytes is a key determinant of an embryo's developmental potential after fertilization, making it essential to preserve oocyte integrity from the moment ovaries are collected until processing. After death, the cessation of blood flow deprives the ovaries of oxygen and energy supplies, triggering rapid tissue deterioration due to their high metabolic demands (King, 2007). Under ischemic conditions, acute hypoxia leads to ATP depletion, reduced energy efficiency, and a drop in intracellular pH, all of which contribute to cellular dysfunction (Tellado *et al.*, 2014). Studies in sheep have demonstrated that oocyte maturation rates decline as post-slaughter storage time increases, particularly at temperatures of 4, 22 and 37° C (Moodie and Graham, 1989). For example, after 7 hours of storage, oocyte quality and developmental potential are significantly compromised, with increased DNA fragmentation, higher numbers of dead oocytes, and fewer viable, matured, and fertilized oocytes (Sharma *et al.*, 2010). Similarly, when ovaries were stored for 8 hours at 37° C, cleavage and blastocyst formation rates following *in vitro* culture and fertilization were notably reduced (Martin-Maestro *et al.*, 2020).

Given that both the temperature and duration of ovarian storage prior to oocyte retrieval critically affect oocyte quality and developmental competence, this study aimed to examine the effects of varying storage temperatures and durations on the post-thaw quality of vitrified immature sheep oocytes.

MATERIALS AND METHODS

The investigation consisted of immature oocyte collection from abattoir sheep ovaries preserved at various temperature (0, 4 and 25° C) and durations (0 h, 6 h, 12 h and 24 h) and post thaw quality evaluation of immature

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oocytes vitrified in open pulled straw using ethylene glycol and DMSO as cryoprotectants.

Chemicals and media

All the chemicals/media and reagents were procured from Sigma-Aldrich (USA) and plastic wares from Nunc (Denmark) and Axiva Sichem Biotech (India). Media used in present study was supplemented with gentamicin (50 µg/ml) prior to use.

Storage of ovaries and oocyte collection

Sheep ovaries were collected from local butchers near the Veterinary Clinical Complex (VCC), F.V.Sc & A.H, SKUAST-K, Srinagar, India. Immediately after slaughter, the ovaries were transported to the laboratory in a thermos containing Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with gentamicin (50 µg/ml) at 37° C. Ovaries were trimmed off from the surrounding tissue, rinsed in distilled water and washed in 70% ethanol followed by 2 washings in DBPS with gentamicin. After that, ovaries were stored at temperatures of 0° C, 4° C and 25° C for 6 h, 12 h and 24 h before oocytes were collected. For storage at 0º C, ovaries were kept in sterile polythene bags and kept in between ice cubes for 6 h (n=6), 12 h (n=6) and 24 h (n=6) before oocytes were collected and for storage at 4° C, ovaries were kept in sterile sample containers containing DBPS with gentamicin and kept in refrigerator for 6 h (n=6), 12 h (n=7) and 24 h (n=7) before oocytes were collected. Similarly, in case of ovarian storage at 25° C, ovaries were kept in sterile sample containers containing DBPS with gentamicin and kept in room maintained at 25° C for 6 h (n=8), 12 h (n=6), and 24 h (n=6) before oocytes were collected. The oocytes collected from ovaries (n=8) immediately (0 h) in the lab served as control.

Collection of oocytes from ovaries stored at various temperature-duration combinations was done in 35 mm petri dish containing collection media (DPBS+ 50 µg/ml Gentamicin + 0.3% BSA) from visible follicles of >2 mm diameter by puncture technique. Oocytes were searched under stereo zoom microscope and good (Completely surrounded by multiple layers (>3) of cumulus cells with uniformly granulated cytoplasm) and fair (Completely surrounded by multiple layers (<3) of cumulus cells with uniformly granulated cytoplasm) oocytes were transferred to fresh warm collection media for further processing.

Vitrification of oocytes

Immature oocytes (good and fair) collected from ovaries stored at various temperature-duration combinations were subjected to vitrification separately using 30% concentration of cryoprotectants (Ethylene glycol and DMSO) in open pulled straw and stored in LN2 using standard protocol. Open - pulled straws (OPS) were prepared by gently softening French mini straws over a hot plate, followed by hand-pulling to reduce their thickness and diameter to approximately half of the original size. The modified straws were then allowed to cool in air for a few seconds before being cut at the narrowest point of the pulled section using a sharp razor blade. Usable oocytes (good and fair) were washed three times in holding medium (TCM-199+ 20% FBS) and finally transferred to a drop of holding medium (HM) before being subjected to vitrification. Vitrification was performed using a 30% vitrification solution, with Ethylene Glycol and DMSO (v/v) as cryoprotectants in an equal ratio within the holding medium. Vitrification solution-I (VS-I) consisted of half the concentration of vitrification solution-II (VS-II). VS-II was also added with 0.5 M sucrose. After equilibration, oocytes were washed two times in VS-I and then transferred to a droplet of vitrification solution-I for 5 min. After 5 min. in VS-I, oocytes were washed two times in VS-II, transferred to a droplet of vitrification solution-II for 30 seconds and then 3-5 oocytes were loaded in open pulled straw in a minimum volume (<1 µl). Open pulled straw with oocytes was immediately plunged in LN2 and stored in LN2 in the modified canisters with a lid to prevent outflow of straws especially during refilling with fresh liquid nitrogen.

Evaluation of morphology after vitrification

Post-vitrification evaluation was conducted one week of post preservation in liquid nitrogen. Thawing was initiated by exposing the OPS straw to air for 3 seconds, followed by immersing its narrow end into Thawing Media (TCM-199 incorporatedwith 20% FCSand 0.5 Msucrose) maintained at 37°C. Oocyteswerekeptinthismediafor 5 minutes. Oocytes were transferred to HM containing 0.25 M sucrose and then to HM without sucrose for 5 minute each. Finally, the oocytes were washed 2-3 times in HM andtransferred to HM for 30 minutes followed by examination under microscope. Oocytes were classified as normal if they exhibited a spherical and symmetrical shape with no signs of lysis or degeneration. In contrast, those displaying a ruptured zona pellucida, shape irregularities, cumulus cell loss, DNA fragmentation, or other degenerative signs were categorized as abnormal, following the criteria established by Yang *et al.* (1990).

Statistical analysis

Data was analyzed by Chi square test with P value <0.01 considered as significant.

RESULT AND DISCUSSION

In this study, the post-thaw morphology of vitrified immature oocytes, collected from abattoir-derived sheep ovaries stored at different temperatures (0° C, 4° C, and 25° C) and durations (0 h, 6 h, 12 h and 24 h), was assessed to evaluate the impact of storage conditions on oocyte quality prior to collection. At storage temperature of 0° C and 25° C, the present study (Table 1, 2) revealed significantly higher percentage of post thaw morphologically normal oocytes from the ovaries stored for 6 h (68% and 87.1%) compared to that for 12 h (45.8% and 74.6%) and 24 h

(33.3% and 64%). However, at 4° C storage temperature, non-significantly higher percentage of post thaw morphologically normal oocytes were observed from ovaries under storage for 6 h (74%) compared to that for 12 h (69.9%) and 24 h (68.9%), respectively. Similarly, from ovaries under storage duration for 6h at 0° C, 4° C and 25° C, significantly higher percentage of post thaw morphologically normal oocytes were retrieved from ovaries at 25° C (87.1%) and followed by 4° C (74%) compared to that at 0° C (68%). At storage duration for 12 h at 0° C, 4° C and 25° C, significantly higher percentage of post thaw morphologically normal oocytes were obtained from ovaries at 25° C (74.6%) and 4° C (69.9%) compared to that at 0° C (45.8%). However, ovarian storage for 24 h at 0° C, 4° C and 25° C, post thaw morphologically normal oocytes were highest at 4° C (68.9%) followed by 25° C (64%) and both were significantly higher than at 0° C (33.3%). Further, the percentage of post thaw morphologically normal oocytes at 6 h were significantly lower at all storage temperatures of 0° C (68%), 4° C (74%) and 25° C (87.1%) compared to the control (88.3%). Studies in sheep have shown that oocyte maturation rates decline as post-slaughter storage time increases at 4° C, 22° C, and 37° C. However, ovaries stored at 22° C exhibited higher maturation rates compared to those stored at the other temperatures (Moodie and Graham, 1989). After 7 hours of storage, increased DNA fragmentation and lower rates of viable, matured, and fertilized oocytes were observed, leading to compromised oocyte quality and developmental potential (Sharma *et al.*, 2010).

Table 1. Usable post-thaw vitrified oocytes from ovaries stored at different temperature-durations

Rx	Hours (n)	Usable Oocytes Vitrified	Oocytes after Thawing	Oocytes lost
Control	0 h (8)	157	145	12
0° C	6h (6)	107	100	7
	12h (6)	85	72	13
	24h (6)	60	56	4
4° C	6h (6)	58	50	8
	12h (7)	145	136	9
	24h (7)	126	122	4
25° C	6h (8)	94	85	9
	12h (6)	72	67	5
	24h (6)	54	50	4

Table 2. Post thaw quality of vitrified immature oocytes from ovaries stored at different temperature-durations

Hrs	0° C		4° C		25° C	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Control	128*(88.3%)	17 (11.7%)	128* (88.3%)	17(11.7%)	128* (88.3%)	17 (11.7%)
6 hr	68^{a1}* (68%)	32 (32%)	37^{a12}* (74%)	13 (26%)	74^{a2}* (87.1%)	11 (12.9%)
12 hr	33^{b1} (45.8%)	39 (54.2%)	95^{a2} (69.9%)	41 (31%)	50^{b2} (74.6%)	17 (25.4%)
24 hr	19^{b1} (33.3%)	37 (67.1%)	84^{a2} (68.9%)	38 (31.1%)	32^{b2} (64%)	18 (36%)

Values within the same column with different superscripts (alphabets) differ significantly at P value <0.01.
Values within the same row with different superscripts (Numbers) differ significantly at P value <0.01.
Values with sign asterisk (*) differ significantly at P value <0.01.

Table 3. Different morphological abnormalities* of oocytes observed in post thaw vitrified immature oocytes from ovaries stored at different temperature-durations

Temp.	Hours	CCL (%)	ZPB (%)	SC (%)	SO (%)	HC (%)	SDLC (%)	BE (%)	WPVS (%)	Total
Control	0 h	2 (11.7)	1(5.8)	7(41.1)	1(5.8)	1(5.8)	2(11.7)	-	3(17.6)	17
0° C	6 h	5(15.6)	8(25.0)	3(9.3)	-	6(18.7)	7(21.8)	0	3(9.3)	32
	12 h	6(15.3)	16(41.0)	2(5.1)	-	9(23.0)	3(7.6)	0	3(7.6)	39
	24 h	4(10.8)	11(29.7)	6(16.2)	1(2.7)	4(10.8)	3(8.1)	2(5.4)	6(16.2)	37
4° C	6 h	9(69.2)	-	1(7.6)	-	1(7.6)	1(7.6)	-	1(7.6)	13
	12 h	13(31.7)	10(24.3)	2(4.8)	3(7.3)	4(9.7)	2(4.8)	2(4.8)	5(12.1)	41
	24 h	19(50.0)	3(7.8)	-	-	5(13.1)	4(10.5)	-	7(18.4)	38
25° C	6 h	5(45.4)	3(27.2)	-	-	-	1(9.0)	-	2(18.1)	11
	12 h	10(58.8)	-	2(11.7)	-	2(11.7)	1(5.8)	-	2(11.7)	17
	24 h	7(38.8)	4(22.2)	2(11.1)	1(5.5)	-	1(5.5)	-	3(16.6)	18

*CCL: Cumulus Cell loss, ZPB: Zona Pellucida break, SC: shape change, SO: split oocytes, HC: Heterogenous cytoplasm, SDLC: Shrinked darkless cytoplasm, BE: Bulls eye and WPVS: Wide peri-vitelline space.

Similarly, Zhang *et al.* (2014) examined the survival of ovarian oocytes from post-mortem ICR mice placed at different temperatures (25° C, 4° C, and 37° C) for varying durations (0-10 hours). Their findings indicated that as preservation temperature increased, the percentage of oocytes emitting the first polar body gradually decreased. Likewise, prolonged ovarian storage under warm conditions (30°-37° C) for 7-8 hours was associated with reduced

blastocyst formation rates in bovine oocytes following IVF (Martin-Maestro *et al.*, 2020). Further, Schernthaner *et al.* (1997) investigated the effects of storing bovine ovaries for 24 hours at 15° C, 18° C, and 21° C on embryonic developmental capacity, comparing them to a control group where follicle aspiration was initiated approximately 4 hours post-slaughter. The percentage of morulae and blastocysts in the experimental groups and in the control group did not differ significantly (P>0.05). He indicated that it is possible to store ovaries before maturation for more than 12 hours without losing in vitro developmental competence. Guignot *et al.* (1999) opined that both duration and temperature of ovarian preservation significantly impact oocyte quality. Likewise, Wongsrikeao *et al.* (2005) investigated the quality, nuclear maturation, and developmental potential of porcine oocytes retrieved from ovaries stored in physiological saline for 0, 3, 6, 9, and 12 hours at different temperatures (4, 15, 25 and 35° C). Their findings indicated that storing ovaries at 25-35° C for 6 hours effectively preserved oocyte developmental competence, although development rates were higher when ovaries were preserved at 35° C for only 3 hours.

Similarly, storage of equine ovaries at 25° C for 5-8 hours and at 35° C for 3-15 hours had no adverse effects on oocyte meiotic competence (Guignot *et al.*, 1999), whereas lower temperatures (4° C) proved detrimental compared to room temperature (Love *et al.*, 2003). However, contrasting findings were reported in feline oocytes, where ovaries stored at 4° C for up to 12 hours did not show an increase in granulosa cell apoptosis (Jewgenow *et al.*, 1997).

In the present study (Table 3), different morphological abnormalities of oocytes like cumulus cell loss (CCL), zona pellucida break (ZPB), shape change (SC), split oocyte (SO), heterogeneous cytoplasm (HC), shrinked darkless cytoplasm (SDLC), bull's eye (BE) and wide peri vitelline space (WPVS) were observed in post thaw vitrified immature oocytes at different storage temperature-time intervals. In control group, the highest abnormality observed was the SC (41.1%), whereas, at 0° C, the highest abnormality observed was the ZPB at all the storage periods of 6 h (25%), 12 h (41%) and 24 h (29.7%). However, at storage temperature of 4° C and 25° C, the highest abnormality observed was the CCL at all the storage periods of 6 h (69.2 and 45.4%), 12 h (31.7 and 58.8%) and 24 h (50 and 38.8%), respectively. Bulls eye/inclusion body was observed at 0° C (5.4%) and 4° C (4.8%). Further, split oocytes were seen at 24 h of storage time at 0° C (2.7%) and 25° C (5.5%) and at 12 h of storage time at 4° C (7.3%), respectively. El-Soudy *et al.* (2016) investigated several types of morphological abnormalities of camel oocytes and reported the most common abnormality like abnormal oocytes shape, zona pellucida, increase in peri vitelline space and heterogeneous cytoplasm. Shrinking of 66.6 and 43.7% of heterogeneous cytoplasm in the vitrified mature and immature oocytes, respectively was attributed to the high concentration of CPAs (20% DMSO, 20% EG) that penetrate intracellular to avoid ice crystal formation, which have toxic effects on cells and may cause osmotic injury.

CONCLUSIONS

With increase in storage time at various temperatures, yield of morphologically normal oocytes showed a decreasing trend at 0, 4 and 25° C with higher values at 4° C and 25° C compared to that at 0° C. However, acceptable post thaw quality of vitrified immature oocytes is maintained up to 24 h in ovaries stored at 4 and 25° C compared to at 0° C, thus may be used for storage of ovaries for oocyte collection for preservation through vitrification.

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