TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2014) 38: 707-711
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doi:10.3906/vet-1404-43

Survivability of caprine oocytes vitrified in conventional and open pulled straws

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Received: 10.04.2014 • **Accepted:** 09.07.2014 • **Published Online:** 24.10.2014 • **Printed:** 21.11.2014

Abstract: The aim of this study was to compare the efficacy of conventional straw vitrification with open pulled straw vitrification in terms of cryosurvivability and damages caused to the oocytes. Two hundred immature goat cumulus oocyte complexes (COCs) were vitrified in a solution of ethylene glycol, dimethyl sulfoxide, and sucrose using either conventional straws (100 COCs) or open pulled straws (100 COCs). The COCs were warmed and observed for morphological damages and viability after 7 days of preservation in liquid nitrogen. Among the 100 COCs cryopreserved in each case, only 83 COCs were recovered after warming in conventional straw vitrification as compared to 94 COCs in open pulled straw vitrification. In terms of morphological survivability, the percentage of morphologically normal oocytes was greater ($P < 0.01$) in the case of open pulled straw vitrification (86.2%) as compared to conventional straw vitrification (59.0%). Viability percentage of live oocytes was greater ($P < 0.01$) in the case of open pulled straw vitrification (90.4%) as compared to conventional straw vitrification (66.3%). The results indicate that open pulled straw vitrification is better than conventional straw vitrification for rapid freezing of immature goat COCs in terms of both morphological survival and viability.

Key words: Vitrification, oocytes, caprine, conventional straw, open pulled straw

1. Introduction

Vitrification refers to the physical phenomenon describing the solidification of water or water-based solutions into a glass-like amorphous liquid state (called the vitreous state), due to extreme elevation in viscosity during cooling, without the formation of ice crystals (1). Mammalian oocyte vitrification was first performed by Sherman and Lin in 1958 (2); since then, many researchers have used different devices to vitrify the oocytes of different species such as mouse (3–5), cow (6–8), buffalo (9–11), sheep (12,13), pig (14,15), and human (16–18).

Goat oocytes have been vitrified using different techniques such as conventional straw vitrification (19), solid surface vitrification (20), and other methods like open pulled straw, hemistraw, cryoloop, and cryotop methods (21). Despite all these efforts, the results of vitrification in caprine species are far from being comparable to those for other domestic species. There is an acute need of developing a safe and results-oriented method of vitrification along with a proper combination of vitrifying solution for cryopreserving female germplasm in this species. Thus, the present study was designed to compare the efficacy of 2 different vitrification techniques in terms of cryosurvivability and damages caused by vitrification to goat oocytes.

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2. Materials and methods

2.1. Chemicals and media

All the chemicals and media were purchased from Sigma Chemical Co. (USA) and plastic ware from Nunc (Denmark), unless otherwise indicated. All the media used in present study were supplemented with penicillin (100 IU/mL) and streptomycin (0.1 mg/mL) prior to use.

2.1.1. Holding media

Holding media (HM) were used for preparation of vitrification and warming solutions. Medium 199 with HEPES was supplemented with 20% fetal bovine serum for preparation of HM.

2.1.2. Vitrification solutions

Two vitrification solutions were prepared: vitrification solution-I (VS-I) for equilibration by adding 10% ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO) to the HM, and vitrification solution-II (VS-II) for final dilution adding 20% EG, 20% DMSO, and 0.6 M sucrose to the HM.

2.1.3. Warming solutions

Similar to the vitrification solutions, 2 warming solutions were prepared. Warming solution-I (WS-I) contained 10% EG, 10% DMSO, and 0.3 M sucrose and warming solution-II (WS-II) contained WS-I and HM in the ratio of 1:3 v/v.

2.2. Source of ovaries and oocyte collection

Goat ovaries were obtained from a municipal slaughter house in Jammu and transported to the laboratory in a thermos containing DBPS at 37 °C within 1 h of slaughter. In the laboratory each ovary was separated from the surrounding tissue and overlying bursa. The ovaries were rinsed in physiological saline and 70% alcohol followed by 3 washings in DBPS with antibiotics. Oocytes were collected by aspiration of visible surface follicles of 2–8 mm as per the method described by Wani et al. (22). The cumulus oocyte complexes (COCs) were graded under a stereo-zoom-microscope and only those having homogeneous cytoplasm surrounded by more than 3 layers of compact cumulus cells were selected for vitrification.

2.3. Vitrification of COCs

Vitrification of COCs was carried out in 2 steps. Initially, COCs were equilibrated in VS-I for 2–5 min followed by vitrification in VS-II for 30 s before being loaded into either conventional straws or open pulled straws.

2.3.1. Conventional straw (CS) method

COCs were vitrified using French mini straws (IMV, France, Figure 1A) according to the method described by Naik et al. (23). After the proper exposure to VS-II, 5 COCs were loaded into 0.25-mL straws sequentially as follows: VS-II, air bubble, VS-II containing COCS, air bubble, and VS-II, and then the open end of the straw was closed by heat sealing. Immediately after loading straws were plunged into liquid nitrogen (LN_2) and stored for 1 week.

2.3.2. Open pulled straw (OPS) method

OPS vitrification was carried out as described by Rao et al. (24). Open pulled straws were prepared by slightly melting French mini straws over a flame and then hand pulling them to achieve a diameter that was half of their original diameter. The straws were then held in air for a few seconds prior to cutting at the narrowest point of the pulled portion (Figure 1B). After the proper exposure to the VS-II, 5 COCs were loaded into the straws by capillary action. Immediately after loading straws were plunged into LN_2 and stored for 1 week.

2.4. Warming of vitrified COCs

Warming of vitrified COCs was carried out in 2 steps. Initially, COCs were warmed in WS-I for 1 min and subsequently transferred into WS-II for 5 min. Warmed COCs were then washed twice in HM before being examined. For the CS method, straws were exposed to air at room temperature until the frozen solution liquefied. Sealed ends were cut and the contents were poured into 35-mm culture dishes, and COCs were transferred into WS-I followed by transfer into WS-II and washings. For the OPS method, straws were taken out of the LN_2 and the open end was immersed vertically in WS-I solution. The vitrification medium liquefied in 2–4 s and the COCs were released into the WS-I followed by transfer into WS-II and washings.

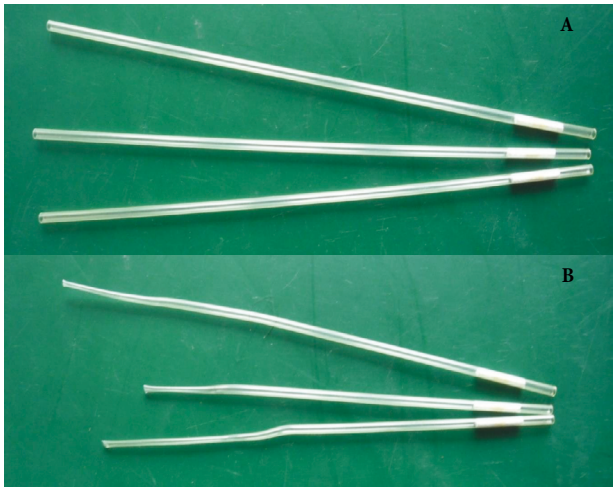

Figure 1. Types of cryodevices used in the study: **A)** conventional (French mini) straw and **B)** open pulled straw to achieve a diameter that was half of the original diameter.

2.5. Morphological assessment

The vitrified and warmed COCs were evaluated for morphological damage within 30 min of warming as per the method described by Garg and Purohit (19). Oocytes were considered abnormal when there was change in shape, breakage of zona pellucida, cumulus cell loss, or oocytes split into 2 halves. The morphologic survival percentage was calculated as the proportion of COCs seen to be normal against the total number vitrified and recovered.

2.6. Evaluation of oocyte viability

The viability of vitrified and warmed COCs was evaluated by the method of Gupta et al. (25). One drop of trypan blue (0.4%) was added to a drop of holding medium. Five oocytes were added to this solution and allowed to settle for 5 min, after which they were transferred to HM and examined under inverted phase contrast microscopy. Both cumulus cells and ooplasm took up a blue stain in the case of dead oocytes, whereas live oocytes remained unstained.

2.7. Statistical analysis

Data were analyzed by the chi-square test (2 × 2 contingency tables).

3. Results

In the present study, 83 out of the 100 oocytes vitrified were recovered in the case of the CS method, whereas 94 oocytes were recovered in the OPS method (Table 1). The percentage of morphologically normal and live oocytes was greater ($P < 0.01$) in OPS vitrification (86.2% and 90.4%, respectively) as compared to CS vitrification (59.0% and 66.3%, respectively).

Table 1. Effect of 2 vitrification techniques on morphology and viability of caprine COCs.

Vitrification technique	Total no. of COCs vitrified	No. of COCs recovered	No. of morphologically normal COCs	No. of damaged COCs	No. of live COCs	No. of dead COCs
Conventional straw (CS) vitrification	100	83	49 (59.0)[a]	34 (41.0)[a]	55 (66.3)[a]	28 (33.7)[a]
Open pulled straw (OPS) vitrification	100	94	81 (86.2)[b]	13 (13.8)[b]	85 (90.4)[b]	9 (9.6)[b]

Means with different superscripts within a column vary significantly ($P < 0.01$). Values in parentheses indicate percentages.

The most common abnormalities found in the present study were cumulus cell loss, abnormal shape, and cracking of zona (Table 2; Figure 2). However, among the cumulus cell loss, partial loss was greater ($P < 0.05$) in OPS vitrification (38.5%) as compared to CS vitrification (17.6%), and conversely complete loss was greater ($P < 0.05$) in CS vitrification (35.3%) as compared to OPS vitrification (23.1%). In addition, cracking of zona was greater ($P < 0.05$) in CS vitrification (26.5%) as compared to OPS vitrification (15.4%).

4. Discussion

The higher recovery rate in the OPS method may be because of the low volume of vitrification solution used for preservation of COCS, whereas chances of oocyte loss increased with the increase in vitrification medium as was the case with the 0.25-mL French mini straws used in the CS method. The results for morphological survival confirm the findings of Sharma and Purohit (10) for OPS vitrification; they recorded an 85.6% morphological survival rate. However, the present findings for CS vitrification are much lower than their observations (88.8%), which may be because of the different combinations and concentrations of cryoprotectants used. The difference between the 2 methods seems to be due to the difference in the microtubular structure leading to chilling injury as well as the volume and the surface ratio influencing the penetration of cryoprotectants. Very high cooling and warming rates (theoretical rate of over 20,000 °C/min) and short contact with concentrated cryoprotective additives (less than 30 s at over –180 °C) suggest OPS as a possibility for circumventing chilling injury and decreasing toxic and osmotic damages (26).

The superiority of the OPS vitrification as compared to CS vitrification is evident from the results of the present study as the percentage of live oocytes was greater ($P < 0.01$) in OPS vitrification as compared to CS vitrification. Similar results were recorded by Rao et al. (21); however, El-Sokary et al. (27) recorded a survivability rate of 75.3% using conventional straws, which is slightly higher than the present findings. This difference may be because of the difference in the combinations and concentrations of cryoprotectants used. The higher rate of survivability in the OPS method is again justified in terms of the small volume of vitrification solution used, which achieves faster cooling and warming rates than conventional straws. Moreover, oocytes in vitrification solution (1–2 µL) in OPS are directly expelled in the warming solution (within 2–4 s) and immediately diluted. That reduces exposure to inappropriate temperatures and concentrated cryoprotectants. In contrast, the conventional straw is warmed in air and then cut with scissors. The oocytes in vitrification medium (65–70 µL) are expelled into the culture dishes and then placed into warming solution. It takes more time to pass through the unsuitable conditions (28). These effects may explain why vitrification of oocytes using OPS preserves viability better than that using CS.

The postvitrification abnormalities observed in the present study are in close correlation with the earlier

Table 2. Morphological damages caused by 2 vitrification techniques to caprine COCs.

Vitrification technique	No. of COCs recovered	No. of damaged COCs	Types of morphological damages				
			Partial cumulus loss	Complete cumulus loss	Abnormal shape	Cracked zona	Split into 2 halves
Conventional straw (CS) vitrification	83	34	6 (17.6)[a]	12 (35.3)[a]	5 (14.7)[a]	9 (26.5)[a]	2 (5.9)[a]
Open pulled straw (OPS) vitrification	94	13	5 (38.5)[b]	3 (23.1)[b]	2 (15.4)[a]	2 (15.4)[b]	1 (7.7)[a]

Means with different superscripts within a column vary significantly ($P < 0.05$). Values in parentheses indicate percentages.

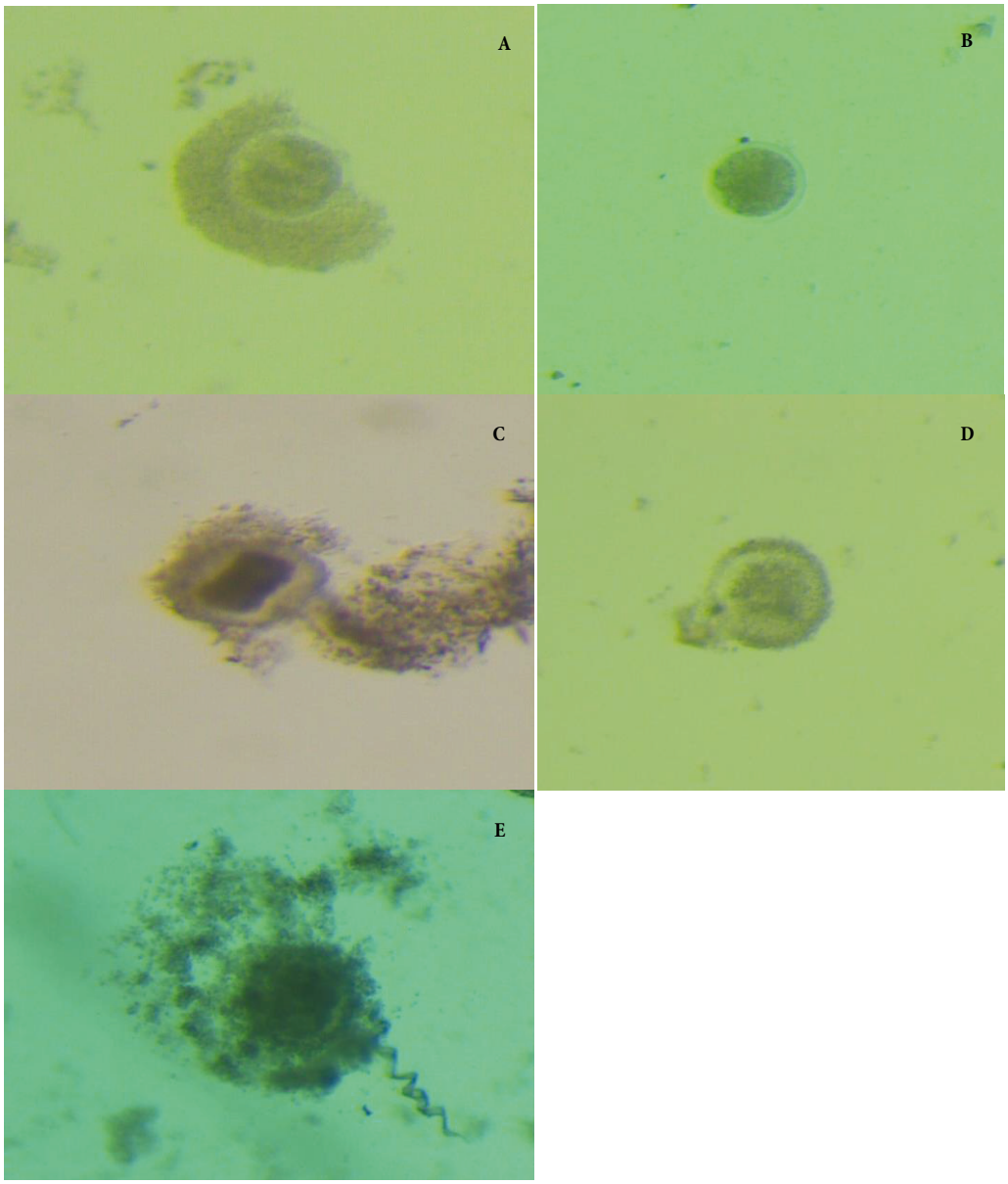

Figure 2. Types of cryodamages inflicted on oocytes by vitrification: **A)** partial cumulus loss, **B)** complete cumulus loss, **C)** abnormal shape, **D)** cracking of zona, and **E)** degenerated cytoplasm observed in oocytes after vitrification.

studies of Yadav et al. (29) and Purohit et al. (30). The major cryoinjuries were associated with CS vitrification (Table 2), which may explain the low survivability of COCs vitrified by the CS method as compared to the OPS method in the present study.

In conclusion, open pulled straw vitrification was superior to the conventional straw vitrification in terms of preventing cryoinjuries and increasing survivability of goat COCs. The use of minimum vitrification solution in open pulled straws achieved faster cooling and warming rates, which prevented major damage during the critical stages. Moreover, OPS vitrification minimized the time delay during warming, thus not exposing COCs to unsuitable conditions.

Acknowledgment

The first author is thankful to the University Grants Commission for providing financial assistance under the Maulana Azad National Fellowship for minority students during the study.

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