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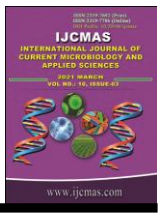

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Effect of L-proline Supplementation in Vitrification Media on Post Thaw Survival and Embryonic Development of Sheep Oocytes

D. Reena[1], G. Lakshmi[2], S. Kurunchi Malar[2] and S. Manokaran[3*]

[1]Department of Clinics, Madras Veterinary College, TANUVAS, Chennai, India
[2]Department of Biotechnology, Sree Sastha Institute of Engineering and Technology, Chembarambakkam, Chennai, India
[3]Kangayam Cattle Research Station, TANUVAS, Uppupallam, Baguthampalayam, Sathyamangalam, India

**Corresponding author*

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A B S T R A C T

The study was conducted to explore the cryoprotective effect of L-proline on sheep oocyte vitrification. *In vitro* matured sheep oocytes (n=836) were vitrified by open pulled straw method in vitrification solution consisting of 15 per cent (v/v) DMSO+ 15 per cent (v/v) EG + sucrose (0.5M) + 20 per cent FBS in TCMh (Group I) and 7.5 per cent (v/v) DMSO + 10 per cent (v/v) EG + sucrose (0.5M) + 20 per cent FBS in TCMh supplemented with 2M L-proline (Group II). The oocytes were warmed and observed for viability by tryphan blue staining. The mean ± SE live oocyte percentage was significantly higher in group II than in group I (59.80±1.02 vs. 44.99±1.34). The vitrified thawed oocytes were activated parthenogenitically using ionomycin and 6-DMAP and developmental stages of embryos were analysed. In parthenogenetically activated embryos, there was no significant difference was observed in cleavage rate (47.56±2.09 vs/ 42.45±3.44) and morula (14.90±1.68 vs. 12.95±2.25) percentage between L-proline and control group. From the study it is concluded that L-proline could be used as a cryoprotectant with high efficiency in sheep oocyte vitrification.

Introduction

Cryopreservation of mammalian and human oocyte has been significantly improved by the refined slow freezing methods and new vitrification technique (Michelmann and Nayudu, 2006). Slow freezing method using programmable freezer is traditionally employed for the cryopreservation of oocyte and causes damages to oocyte due to high intracellular ice crystal formation and also this method takes several hours. Generally, oocytes are very sensitive to freezing due to low surface-to-volume ratio (Arav, 2014) and also permeability of cryoprotectants into oocytes via simple diffusion was less than blastocysts due to lack of aquaporin expression which increase the movement of cryoprotectants (Ruffing *et al.*, 1990). Vitrification is widely using oocyte

cryopreservation technique because it is a simple and efficiently used for several species and transforms liquid into a glass like state without ice crystal formation (Rall and Fahy, 1985) within few minutes.

Cryoprotective agent is a substance used to protect biological tissue from freezing damage i.e. intracellular ice crystal formation. There are two types of cryoprotectants: (a) membrane permeating which can freely diffuse the membrane such as glycerol (G), ethylene glycol (EG) and dimethyl sulfoxide (DMSO), (b) non-membrane permeating which cannot permeate the cell membrane such as sugars (Pedro *et al.,* 2005). The combination of cryoprotective agents such as EG and DMSO showed greater effectiveness in the vitrification of matured oocytes (Cha *et al.,* 2011).

However, there are some disadvantages associated with currently used cryoprotective agents. Although DMSO is the most effective cryoprotective agent, it is highly cytotoxic and changes intracellular calcium stores by permeabilizing mitochondria and/or the endoplasmic reticulum (Gardner *et al.,* 2007). In addition, DMSO can influence microtubule polymerization, which may potentially affect spindle functions and subsequent embryo development (Vajta and Kuvayama, 2006).

Recent studies has shown that L-proline is a membrane permeable non-toxic cryoprotectant to protect cells from injuries caused by freezing and thawing in many species including plant, ram sperms and human endothelial cells (Kostal *et al.,* 2012). L-proline can reduce the excessive reactive oxygen species (ROS) to prevent oocytes from oxidative stress damage and consequently stabilize protein, DNA and membrane to maintain normal meiotic spindle morphology, avoid apoptosis and protect mitochondrial membrane (Anjum *et al.,* 2000). Increasing the concentration of L-proline and reducing the concentration of cryoprotectants increase the quality of oocyte similar to the fresh oocyte and it does not affect the embryonic development in *in vivo* and *in vitro* studies (Zhang *et al.,* 2016). Considering the above, the study was conducted with the objective of studying the effect of natural antioxidant (L-proline) as cryoprotectant in vitrification of sheep oocytes.

Materials and Methods

All the culture media, chemical, growth factors and hormones were purchased from Sigma Aldrich (St. Louis, MO, USA) and fetal bovine serum (FBS) from GIBCO (Invitrogen, USA).

Sheep ovaries were collected from slaughterhouse and placed in normal saline supplemented with Penicillin (100 IU/ml) and Streptomycin (50 mg/ml) at 30-35ºC in a thermos flask and transported to Centralized Embryo Biotechnology Laboratory (CEBU). The ovaries were transported and processed within 2 hrs of slaughter.

The adherent tissues and ligaments around the ovaries were trimmed and washed thoroughly. The cumulus oocyte complexes (COCs) were retrieved in oocyte collection medium by slicing method. Oocytes were screened under a stereo zoom microscope and graded based on the cumulus cells investment and ooplasm homogeneity as detailed.

Grade A: Oocytes with more than 3 layers of cumulus cells and homogenous evenly granular ooplasm.

Grade B: Oocytes with 2-3 layers of cumulus cells and homogenous evenly granular cytoplasm.

Grade C: Oocytes with either partially denuded or with 1-2 layers of cumulus cells and with an irregular and dark cytoplasm.

Grade D: Oocytes completely devoid of cumulus mass and having irregular and dark ooplasm.

The A, B and C grade oocytes were utilized for *in vitro* maturation (IVM). The oocytes were washed three times in TCM 199 supplemented with 10 per cent FBS and finally washed in *in vitro* maturation (IVM) medium.

The IVM medium composed of TCM 199 supplemented with 10 per cent FBS, 1µg/ml of follicle stimulating hormone (FSH), 0.02 IU/ml of luteinizing hormone (LH), 1 µg/ml of estradiol, 10 ng/ml of epidermal growth factor (EGF). In a 35 mm petridish, 50 µl droplets of maturation medium was made, covered with mineral oil and pre-equilibrated in the CO_2 incubator for 2 hrs at 38.5º C and 5 per cent CO_2. Ten to fifteen COCs were transferred to maturation droplets and matured *in vitro* for 24 hrs.

After 24 hrs, the maturation rate was assessed based on the degree of cumulus expansion as detailed.

Degree 2: Cumulus cells were homogenously spread and clustered cells were no longer present (Full cumulus expansion)

Degree 1: Cumulus cells were slightly expanded and clustered cells were still observed (Moderate cumulus cell expansion)

Degree 0: No morphological change compared with fresh COCs (Slight or no expansion).

The oocytes classified as degree 2 and 1 were considered as matured.

Experimental design

Study 1: It was conducted to study the effect of the exposure of the matured sheep oocytes to the vitrification solution containing with or without L-proline.

Matured oocytes were exposed to 7.5 per cent (v/v) DMSO + 7.5 per cent (v/v) EG + 20 per cent FBS in Hepes buffered TCM199 TCM (TCMh) medium for 3 min for equilibration. Then the oocytes were exposed to vitrification solution consisting of 15 per cent (v/v) DMSO+ 15 per cent (v/v) EG + sucrose (0.5M) + 20 per cent FBS in TCMh (Group I, control) and 7.5 per cent (v/v) DMSO + 10 per cent (v/v) EG + sucrose (0.5M) + 20 per cent FBS in TCMh supplemented with 2M L-proline (Group II, L-proline) for 30-45 sec. The oocytes were loaded into OPS device and directly plunged into liquid nitrogen and stored for 7 days. Twelve replicates were carried out in each of the trial groups.

The warming solution (WS) was TCMh containing various concentrations of sucrose. Stepwise warming was performed by placing the cryo device directly into 0.5M sucrose for 5 min. Then the oocytes were rinsed with other solutions of 0.25M sucrose and handling media, each for 5 min. All warming procedures were performed at 38.5ºC. Vitrified oocytes were evaluated for viability by trypan blue exclusion test. Oocytes were incubated for 30 min in a CO_2 incubator and then quickly washed three times in m-PBS solution. Finally, the oocytes were cultured in m-PBS containing 0.05 per cent trypan blue for 2 min at 38.5ºC and then washed three times in m-PBS solution before examination of oocytes viability under a microscope. Oocytes viability was categorized on the basis of the degree of dye exclusion. Unstained oocytes were classified as live and fully stained oocytes as dead.

Study 2: It is conducted to examine the influence of L-proline on early embryonic development of vitrified partenogenetically activated oocytes. Viable oocytes were activated with 5 µM ionomycin in TCMh for 5 min, followed by culture in 2 mM 6-DMAP for 4 hrs (Reena *et al.,* 2012).

Activated oocytes were cultured *in vitro* synthetic oviduct fluid (SOF) media. The activated oocytes were placed in pre-equilibrated 50µl SOF media droplets (10-15/droplet) and *in vitro* cultured at 38.5°C in 5 per cent CO_2 in CO_2 incubator. Cleavage rate was assessed after 24 hrs and subsequent developmental stages were assessed every 24hrs.The collected data were analyzed statistically using student't' test.

Results and Discussion

In the present study a total of 934 good quality sheep COCs were used for *in vitro* maturation. Among the 934 oocytes, 836 oocytes were matured and the mean maturation rate was 89.5 ± 0.84 per cent. The mean ± SE viability percentage of sheep oocytes vitrified by OPS technique is given in Table 1. In Group I, 411 matured oocytes were vitrified using combination of 15 per cent (EG+DMSO) with 0.5M of sucrose and 20 per cent FBS as vitrification media (control). In group II, 425 matured oocytes were vitrified in 10 per cent EG, 7.5 per cent DMSO, 0.5 M sucrose and 20 per cent FBS with 2M L-proline media (L-proline group).

The vitrified matured sheep oocytes from both groups were warmed and the viability was assessed on tryphan blue staining. The viability of oocytes was analysed and compared with control group. The mean ± SE live oocyte percentage was significantly higher in L-proline group (59.80±1.02) than the control group (44.99±1.34). Similar to this study, Zhang *et al.,* (2016) reported that vitrification with L-proline group showed significantly higher survival rates (93.8 per cent) than the control group (88.4 per cent) in mouse oocytes. They also observed that osmotic pressure of a mixture of 2M L-proline and 15 per cent DMSO + 15 per cent EG were close to that of the without L-proline group (15 per cent DMSO +15 per cent EG) and declared that an addition of 2M L-proline with low concentrations of DMSO and EG was considered to be a suitable cryopreservation solution for mouse oocyte vitrification. Sun *et al.,* (2012) demonstrated that L-proline is the most effective CPA, and it improves the efficiency of recultivation of human endothelial cells by more than 100 per cent in the presence of a low concentration of DMSO. In the present study, EG (10 per cent), DMSO (7.5 per cent), sucrose (0.5M) and FBS (20 per cent) with L-proline (2M) media was used as vitrification media in treatment group and OPS technique was used as vitrification device. Rao *et al.,* (2012) reported that oocytes vitrified with OPS technique showed lower survival rate which might be due to larger volume of vitrification solution. Succu *et al.,* (2007) proved that the cryotop method could promote the cleavage of vitrified oocytes compared with the OPS (42.86 vs 11.58 per cent). Further they observed that COCs vitrified using cryotop showed an increase in the potential to complete meiotic maturation postwarming, inferred by higher proportion of polar body extrusion and visualization of the metaphase II plate.

In the present study, the vitrified oocytes were warmed, parthenogenetically activated and the developmental competence was analysed. The mean ± SE cleavage rate was 42.45 ± 3.44 and 47.56 ± 2.09 per cent and morula development was 12.95 ± 2.25 and 14.90 ± 1.68 per cent in group I and II, respectively (Table 1). The results showed that the cleavage rate and morula development was

slightly higher in L-proline group than control group but statistically no significant difference was observed.

Generally, cryopreserved oocytes showed low fertilization efficiency due to premature cortical granule exocytosis which led to zona pellucida hardening (Gardner *et al.,* 2007). Similarly, Zhang *et al.,* (2016) observed after ICSI, the two pro-nuclei rates of the proline-containing groups (94.7 vs 88.4) and morula percentage (93.6 vs 92.4) were equivalent to that of the proline free group and the fresh group ($P > 0.05$). The combination of calcium ionophores and 6-DMAP induced high rates of activation, pro-nucleus formation and development to blastocyst stage in sheep (Loi *et al.,* 1998). The cleavage rate of activated oocytes and their potential for further embryonic development depends on several factors like species, source and quality of oocytes, IVM conditions, type and composition of culture media and activating agent (Cevik *et al.,* 2009). Liang *et al.,* (2014) reported that a reduction in global genomic methylation due to vitrification of metaphase II (M II) oocytes might compromise the *in vitro* developmental potential of early mouse embryos. Similarly, Lee *et al.,* (2019) found that the low developmental efficiency of cloned mouse embryos using cryopreserved oocytes might be due to increased apoptosis and altered gene expression resulting from cryo-injury. They also suggested that this decreased developmental competence should be alleviated by the addition of antioxidant and anti-apoptotic agents such as melatonin in *in vitro* culture media.

Table.1 Effect of L-proline as cryoprotectant on the viability of matured sheep oocytes and embryo development on parthenogenetic activation

Groups	No. of trials	No. of matured oocytes vitrified	Viability of oocytes after vitrification				Embryo development rate on parthenogenetic activation			
			Live		Dead		Cleavage		Morula	
			No.	Mean % ± SE	No.	Mean % ± SE	No.	Mean % ± SE	No.	Mean % ± SE
Group I (Control)	12	411	185	44.99^{a}± 1.34	226	55.01^{a}±1.34	78	42.45±3.44	23	12.95± 2.25
Group II (With L-proline)	12	425	254	59.80^{b}± 1.02	171	40.20^{b}±1.02	121	47.56±2.09	38	14.90± 1.68

Values with different superscripts (a,b) within the same column differ significantly ($p< 0.01$)

L-proline is a membrane permeable cryoprotectant that can penetrate the cell membrane and stabilize it to confer freezing tolerance (Rudolph and Crowe, 1985) and increasing the survival rate of oocytes. The current study explored the effect of L-proline as cryoprotectant on sheep oocyte vitrification. Zhang *et al.,* (2016) reported that L-proline improved the mitochondrial function and has no adverse effects on the spindle configuration and the *in vitro* and *in vivo* embryonic development. L-proline has neutral pH and having high osmotic pressure classified as an osmoprotectant. This indicates that L-proline is a useful cryoprotectant for sheep oocyte vitrification.

Ogawa *et al.,* (2012) reported that L-proline does not affect gene expression in cryopreserved cells. Further studies are required the effects of L-proline on methylation pattern and epigenetic modifications of vitrified oocytes.

From this study it was concluded that

L-proline can be used as a cryoprotectant in vitrification of sheep oocytes

The supplementation of L-proline in the vitrification media increases the postwarming survival and embryo development in sheep oocytes

The vitrification solution containing EG (10 per cent), DMSO (7.5 per cent), sucrose (0.5M) and FBS (20 per cent) in TCMh supplemented with L-proline (2M) showed high postwarming survival rate and embryo development rate on parthenogenetic activation in sheep oocytes.

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