Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Structural Changes in Cattle Immature Oocytes Subjected to Slow Freezing and Vitrification

H. Wahid*, M. Thein[1], E.A. El-Hafez[2], M.O. Abas[3], K. Mohd Azam[4], O. Fauziah[5], Y. Rosnina and H. Hajarian[6]

Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; [1]Department of Surgery and Reproduction, Faculty of Veterinary Science, Yezin, Nay Pyi Taw, Myanmar; [2]Faculty of Veterinary Medicine, Assiut University, Egypt; [3]Agro-Biotechnology Institute, Ministry of Science, Technology and Innovation, Aras 3-7, Blok C4, Parcel C, 62662 Putrajaya, Malaysia; [4]Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Karung Berkunci 36, Pengkalan Chepa, 16100 Kota Bharu, Kelantan, Malaysia; [5]Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; [6]Department of Animal Science, Faculty of Agriculture, Razi University, Iran.
*Corresponding author: wahid@vet.upm.edu.my

ARTICLE HISTORY

Received: July 16, 2011
Revised: August 27, 2011
Accepted: September 13, 2011

Key words:
Bovine
Cryopreservation
Oocyte
Ultrastructure
Vitrification

ABSTRACT

This study was conducted to evaluate the effect of different cryopreservation methods (slow-freezing and vitrification) on structural changes of bovine immature oocytes. Bovine ovaries were collected from local abattoirs. Cumulus-oocyte-complexes (COCs) were retrieved using aspiration method from 2-6 mm follicles. In Experiment 1, selected oocytes were randomly divided into 4 treatment groups namely freezing solution-exposed, frozen-thawed, vitrification solution-exposed and vitrified-thawed and then oocytes abnormalities were examined under a stereomicroscope. In Experiment 2, oocytes were randomly allocated to the same grouping as experiment 1 plus control group. Following freezing or vitrification, all oocytes were fixed in glutaraldehyde and processed for transmission electron microscopy. In experiment 1, there was a higher incidence of abnormalities in the frozen-thawed and vitrified-warmed oocytes compared to those in freezing solution and vitrification solution-exposed groups ($P<0.05$). In experiment 2, there were marked alterations in the perivitelline space, microvilli and vesicles of frozen-thawed and vitrified-warmed oocytes characterized by loss of elasticity and integrity of cytoplasmic processes and microvilli following cooling and warming. In conclusion, ethylene glycol-based freezing and vitrification solutions are suitable choices for cryopreservation of immature oocytes and most organelles are able to retain their normal morphology following cryopreservation and thawing processes.

To Cite This Article: Wahid H, M Thein, EA El-Hafez, MO Abas, KM Azam, O Fauziah, Y Rosnina and H Hajarian, 2012. Structural changes in cattle immature oocytes subjected to slow freezing and vitrification. Pak Vet J, 32(2): 188-192.

INTRODUCTION

Morphological characteristics of immature bovine oocytes and their ability to mature *in vitro* were first documented in 1979 (Leibfried and First, 1979). Since then assessment schemes for immature bovine oocytes have been developed based mainly on the morphological features of cumulus attachment and ooplasm (Younis *et al*., 1989; de Loos *et al*., 1991). It is now generally accepted that assessment of bovine oocytes based on microscopic appearances of cumulus-oocyte-complexes is reliable for predicting oocyte viability. Besides, cryopreservation of immature oocytes is going to be the main interest of researchers due to its clinical and research applications (Isachenko *et al*., 2006; Gupta *et al*., 2007; Hadi *et al*., 2010).

Several studies have shown that freezing and vitrification procedures currently used are detrimental to immature bovine oocytes. These procedures induce the cytoplasm of cryopreserved-thawed immature oocyte to become irregular and light in color, reducing the maturation rate and compromising the developmental competence (Otoi *et al*., 1992; Fuku *et al*., 1995b; Kim *et al*., 2007). Others reported that oocytes frozen to -196°C showed various degrees of cryoinjuries including the separation of cumulus cells from cumulus-oocyte complex and fracture of the zona pellucida (Wu *et al*., 2006). Although ultrastructural alterations of bovine oocytes

caused by dimethyl sulfoxide (DMSO)-based cryoprotective solutions and vitrification procedures have been reported (Fuku *et al*., 1995a), there is a paucity of information on the ultrastructural alterations of immature oocytes induced by other cryoprotective agents (e.g. ethylene glycol) and cryopreservation methods. It has been shown that long time exposure of oocytes to DMSO induces spontaneous parthenogenesis (Isachenko *et al*., 2006). Therefore, this study was undertaken to describe the microscopic changes in immature cattle oocytes following freezing and vitrification procedures. The study was further extended to include ultrastructural alterations in immature oocytes after exposure to different molarities of ethylene glycol-based cryoprotective solutions and after cryopreservation-thawing.

MATERIALS AND METHODS

Collection of ovaries and recovery of oocytes: Cattle ovaries were collected from slaughtered adult females regardless of their reproductive cycle and transported to the laboratory within 3h after slaughter in phosphate buffered saline (PBS, Sigma) supplemented with 100 IU mL^{-1} penicillin and 100 µL mL^{-1} streptomycin and held at 33 to 35°C. At the laboratory, oocytes were collected from 2 to 6 mm follicles using an 18-G needle attached to a 10 mL disposable syringe (Terumo, Japan) previously primed with 2 mL of tyrode's albumin lactate pyruvate solution (TALP-HEPES) containing 3 mg mL^{-1} bovine serum albumin (BSA; A-3311, Sigma) and 50 µL mL^{-1} gentamycin (G-1264, Sigma). Cumulus-oocyte-complexes with moderate to slightly dark, homogeneously pigmented ooplasm were washed 3 times in TALP-HEPES.

Exposure test: In Group 1, oocytes were only exposed to freezing solution containing 1.8M ethylene glycol (1.8M-EG), 0.1M sucrose, 10% calf serum, 0.3 mM sodium pyruvate, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin in Dulbecco's buffered saline (DPBS) for 10 min. Then, the cryoprotectants were removed by 3 changes of maturation medium (Takahashi *et al*., 1996) for 10 min each. In Group 3, the oocytes were exposed to EG-based vitrification solutions 1 and 2 for 2 min and 45sec, respectively. The EG-based vitrification solution 1 (VS1) contained 3.5M ethylene glycol, 0.18M sucrose, and EG-based VS2 contained 7.0M ethylene glycol, 0.35M sucrose. Both VS1 and VS2 contained 20% calf serum, 0.3 mM sodium pyruvate, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin in DPBS.

Slow freezing: Oocytes were frozen using EG-based freezing solution (1.8M-EG) at initial cooling temperature of 30°C. To freeze, the oocytes were loaded into 0.25 mL straws. The straws were placed in a computer-controlled freezer (CL-863, Cryologic, Australia) maintained at 30°C initial cooling temperature and then cooled down to -7°C at a rate of 1°C min^{-1}. The straws were manually seeded at -7°C with a precooled cotton swab and held for another 10 min at -7°C. The temperature was further reduced to -32°C at a rate of 0.3°C min^{-1} and finally the straws were plunged into liquid nitrogen. The straws were frozen-stored for at least 72 hours before thawing. Before use, the oocytes were initially waved in air for 5 seconds and then thawed in a water-bath at 37°C for 20 seconds.

Vitrification: Oocytes were equilibrated in VS1 for 2 min and then in VS2 for 45 sec. Oocytes were loaded in open-pulled straws (OPSs) and directly plunged into liquid nitrogen (LN_2) as previously described (Vajta *et al*., 1998). After 1 to 3-day storage in LN_2, the OPSs were warmed and cryoprotectants were removed serially in sucrose diluting solutions (Vajta *et al*., 1998).

Microscopic evaluation: Oocytes from each group were washed 3 times in TALP-HEPES solution and the morphology of oocytes was assessed under a stereomicroscope (Olympus SZ 40, Japan) and using an inverted microscope (10-40 X) (Leitz Labovert, Germany).

Transmission electron microscopy: Oocytes from treatment groups were fixed in 2.5% buffered glutaraldehyde (pH 7.2) at room temperature for 5 min, kept at 4°C for an additional 1h and then post-fixed using 1% OsO_4 for 1h at 4°C. Sample dehydration was performed in ascending grades of acetone (35%, 50%, 75%, 95%, and 100%) each for 10 minutes. A mixture of resin and acetone was used for sample infiltration and subsequently samples were polymerized in 100% resin at 60ºC for 20 hours. Blocks were sectioned using an ultra microtome (Leica Ultracut, UCT), ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate and then washed in double distilled water. Samples were viewed under a transmission electron microscope (LEO 912AB EFTEM, Omega Filtering System, Germany) at 80 kV.

Study design: In the first experiment, a total of 397 immature bovine oocytes were randomly divided into 4 treatment groups: Group 1 (freezing solution-exposed), Group 2 (frozen-thawed), Group 3 (vitrification solution-exposed), and Group 4 (vitrified-thawed) and then the effects of freezing solution and cryopreservation methods were studied regarding abnormalities after using treatments. In the second experiment, similar treatment and control groups were used for 50 immature oocytes. Ultrastructural alterations in zona pellucida, perivitelline space, microvilli, mitochondria, cortical granules, cytoplasmic vesicles, lipid droplets and germinal vesicle were examined, interpreted and assessed according to the scheme described previously (Fuku *et al*., 1995a; Fuku *et al*., 1995b).

Statistical analysis: Data were statistically analyzed using analysis of variance (ANOVA), followed by Duncan's test for abnormalities and Chi-square (χ^2) test for ultrastructure alterations. The statistical program used was the Statistical Analysis System (SAS, Release 6.12).

RESULTS

In Experiment 1, the total number of oocytes studied in four treatment groups and the proportion of abnormal oocytes observed after respective treatments are presented in Table 1. The abnormalities observed in the cryo-protective solution-exposed oocytes were changes in

transparency of cellular matrix (ooplasm) and cumulus attachment. In both cryopreserved-thawed groups, dark clusters were found in about 25% of abnormal oocytes (7/28 in frozen-thawed and 8/26 in vitrified-warmed groups). The most common abnormality in all groups was transparent and heterogeneous ooplasm. Higher incidence of this abnormality was observed in frozen-thawed and vitrified-warmed groups than freezing solution and vitrification solution-exposed groups ($P<0.05$). Percentages of abnormality were non significantly different ($P>0.05$), not only between the two cryoprotective solution-exposure treatments but also between the two cryopreservation techniques. Zona abnormality was not observed.

In Experiment 2, the results obtained from control, FS-exposed, frozen-thawed, VS-exposed and vitrified-warmed groups are summarized in Table 2. Significant ultrastructural alterations were found in PVS, microvilli/ oolema, and vesicles in frozen/thawed as well as vitrified/ warmed treatment groups (Table 2). In control (Fig. 1) and all treated groups, most of the cumulus cells surrounding the immature oocytes appeared normal, with very few cumulus cells showing cytoplasmic vacuoles. Numerous cytoplasmic processes (also known as cumulus cells projections, CCPs) and their distended ends were found to traverse the zona pellucida to make a close contact with microvilli from oolemma (Fig. 2). Zona cracking was absent in all oocytes.

Table 1: Proportion of abnormal cryopreserved-thawed immature bovine oocytes

Treatment	Numbers of oocytes recovered	% Abnormal oocytes (n)
FS-exposed	82	3.3 ± 2.0^{a} (3/82)
Frozen-thawed	102	27.7 ± 2.7^{b} (28/102)
VS-exposed	88	5.6 ± 2.1^{a} (5/88)
Vitrified-thawed	125	21.0 ± 3.6^{b} (26/125)

The values for abnormal oocytes are expressed as mean ± S.D; Data were accumulated from 4 replicates; ^{a,b}-Values with different superscripts within column are significantly different ($P<0.05$).

Table 2: Oocytes exhibiting ultrastructural abnormalities following freezing and vitrification

Ultrastructure	Oocytes with ultrastructure abnormalities (%)				
	Control (n = 10)	FS-exposed (n = 10)	Freezing (n = 10)	VS-exposed (n = 10)	Vitrification (n = 10)
Zona pellucida	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Perivitelline space	1(10)^{a}	2 (20)^{a}	10 (100)^{b}	3 (30)^{a}	10 (100)^{b}
Microvilli/oolema	1 (10)^{a}	2 (20)^{a}	10 (100)^{b}	3 (30)^{a}	10 (100)^{b}
Mitochondria	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cortical granules	1 (10)	1 (10)	2 (20)	1 (10)	1 (10)
Vesicles	3 (30)^{a}	2 (20)^{a}	7 (70)^{b}	3 (30)^{a}	7 (70)^{b}
Lipid droplets	0 (0)	2 (20)	3 (30)	3 (30)	3 (30)
Germinal vesicle	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

^{a,b}Values with different superscripts within rows are significantly different ($P<0.05$; Chi square)

Enlarged perivitelline space and few microvilli were the major abnormalities in the vitrified-warmed and frozen-thawed oocytes (Figs. 4 and 5). No marked changes were observed in the cortical granule organization or mitochondria in all groups (Figs. 3 and 5). Majority of Golgi complexes were compactly stacked and only a few (1 to 3) were observed swollen. It was also noted that a few vesicles and lipid droplets had migrated slightly towards oocyte periphery in all groups. Some lipid droplets (Fig. 5) and a few large vesicles (Fig. 3) were also apparent in some treated oocytes. However, germinal vesicle of oocytes in all groups was intact.

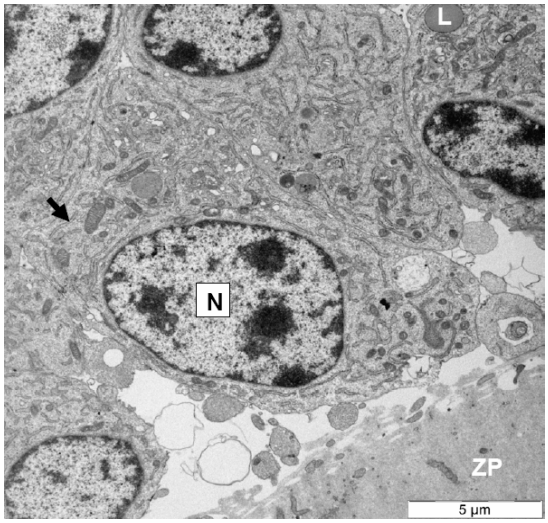

Fig. 1: Electron micrograph of cumulus cells surrounding immature bovine oocyte from control group. N, Nucleus. ZP, zona pellucida. L, lipid droplet. Rough endoplasmic reticulum (arrow). 6,000X.

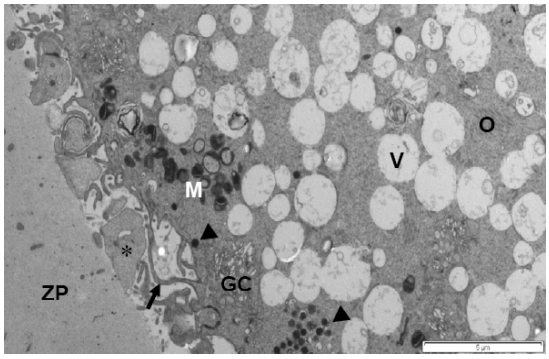

Fig. 2: Electron micrograph of immature bovine oocyte from control group. ZP, zona pellucida. O, ooplasm matrix. M, hooded shape mitochondria. V, vesicle. GC, Golgi complexes. *, extended end of cumulus cell projections at perivitelline space. Cortical granules scattered peripherally (arrowhead). Microvilli (arrow). 4,600X.

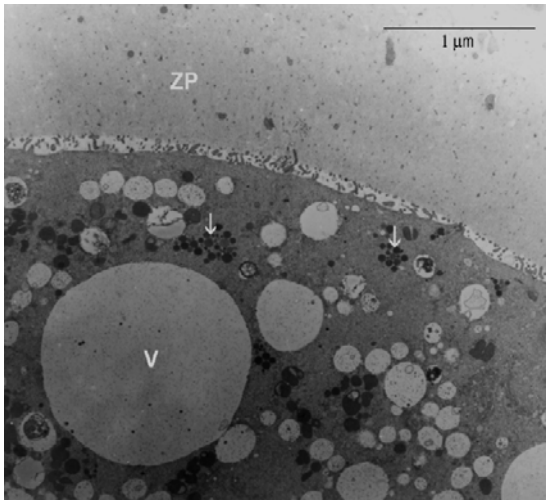

Fig. 3: A VS-exposed immature oocyte. Few microvilli are apparent at the perivitelline space. Almost there is no visible distended end of cumulus cell projections at perivitelline space. Normal clusters of cortical granules (arrows) and an abnormally big vesicle (V). 3,150X.

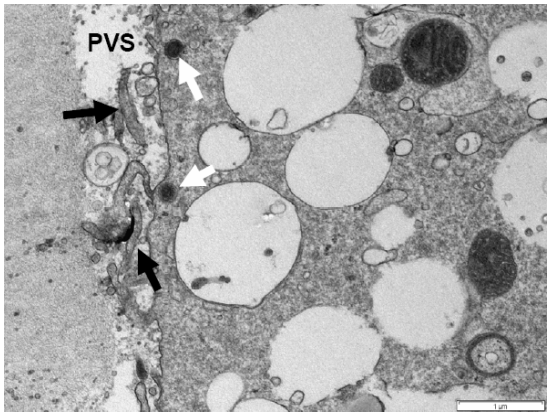

Fig. 4: A vitrified-warmed immature bovine oocyte showing a group of cortical granules with different electron densities (white arrow), moderately enlarged perivitelline space (PVS) and microvilli (black arrow). 25,000X.

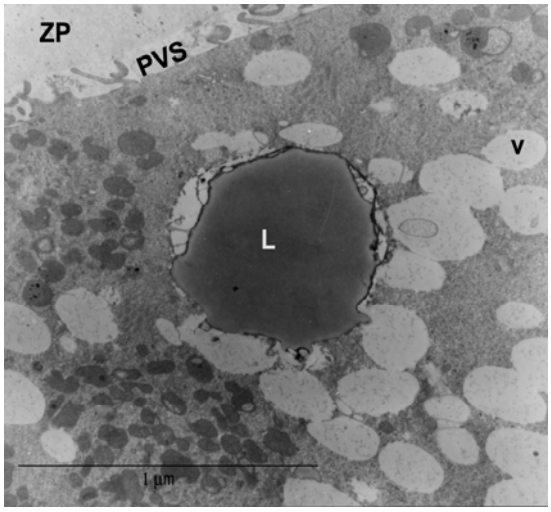

Fig. 5: A frozen-thawed immature bovine oocyte showing an enlarged lipid droplet (L). Zona pellucida (ZP), perivitelline space (PVS) and vesicles (V) are shown for reference. 6,300X.

DISCUSSION

The immature oocytes after undergoing freezing and vitrification procedures showed color and transparency changes in the ooplasm during equilibration. These changes may be due to dehydration and rehydration processes. The darker ooplasm indicated that the oocyte was dehydrated and lighter color indicated that the oocyte has been rehydrated. The darker oocytes may also be associated with shrinkage, while lighter oocytes may be associated with swelling. Damage to the cumulus attachment may be caused by frequent pipetting and serial washing during the procedure. It seems that cryoprotective solution and cryopreservation techniques are not responsible for the detachment of cumulus mass from the oocyte. However, in the present study, a few oocytes were lighter in color or more transparent after equilibration and washing in media.

This study suggests that more morphological abnormalities occur in the frozen-thawed and vitrified-warmed oocytes than in freezing solution-exposed and vitrification solution-exposed oocytes. Although morphological alterations in the bovine oocytes were more obvious following freezing and vitrification procedures, it may have been the cooling and warming that is mainly responsible for these abnormal microscopic features in the bovine oocytes. This was apparent when the study showed that microscopic abnormalities were much more obvious following cooling and warming than exposure to freezing or vitrification solutions. Oocytes have to pass the process of volume changes twice when they undergo cryopreservation; first during equilibrating/ cooling and second during thawing. Some of the manifestations of oocyte damage include vesicular exclusions indicating cytoskeleton damage and dark foci indicating degenerative cellular changes (Elsden, 1984). One factor that can damage oocytes is osmotic stress, which can cause volume changes and adversely affect the viability of oocytes (Shaw *et al*., 2000). Intracellular ice formation also can potentially damage oocytes by injuring intracellular organelles.

The deleterious effects of freezing and vitrification solutions on the viability of immature oocytes have been documented by several authors (Arav, 1992; Agca, 2000). It has been demonstrated that frozen-thawed and vitrified-warmed immature oocytes showed poor *in vitro* maturation and developmental competence (Le Gal, 1996; Suzuki *et al*., 1996; Bautista *et al*., 1998). Exposure to various cryoprotective solutions and cryopreservation methods also caused ultrastructural changes and low viability in oocytes of farm animals (Fuku *et al*., 1995a; Fuku *et al*., 1995b; Hochi *et al*., 1996). Vitrification and warming also resulted in immature oocytes showing 30-50% lysis within the cumulus cell, with necrosis of the nuclei, lysis of the cellular membrane and swollen mitochondrial crestae and endoplasmic reticulum (Diez *et al*., 2005). In contrast, our study showed that freezing and vitrification solutions did not induce ultrastructural alterations of immature bovine oocytes markedly. In this study, the morphological changes of the microvilli, mitochondria and vesicles were observed in 30, 0 and 30% of the oocytes respectively, when they were exposed to EG-based VS. It was indicated that exposure to DAP 213 vitrification solution (DMSO 2M, acetamide 1M, PROH 3M) for 1.5 min caused significant morphological changes in microvilli, mitochondria and vesicles formation (58, 75 and 83%, respectively) of immature bovine oocytes (Fuku *et al*., 1995b). Milder ultrastructural alterations in these structures occurred in this study, probably because the permeable cryoprotectant and equilibration/dilution methods used were physically and chemically less damaging.

Recently, it was reported that abnormal mitochondria were found in 40% of frozen-thawed oocytes using DMSO, whilst no abnormal mitochondrion was observed in treatments with EG, suggesting the EG to be a better intracellular cryoprotectant (Thein M *et al*., 2002). Others reported that the M-SER disorganization observed in oocytes was more probably related to the use of EG in a slow freezing protocol than to the cryo-damage itself (Nottola *et al*., 2008). However, it remains to be determined whether the lack of deleterious effect of EG on oocytes is because of better permeability, lesser toxicity or both. In our study, using the vitrification solution, stepwise equilibration and dilution may have reduced osmotic stress on the immature bovine oocytes.

In the frozen-thawed and vitrified-warmed oocytes, marked alterations in perivitelline space, microvilli and vesicles were noted in a significant number of oocytes. The majority of cytoplasmic processes and microvilli lost their elasticity and integrity following cooling and warming. The perivitelline space and microvilli are closely associated structures and thus osmotic stress exerted on the oocytes might have caused alterations in these structures simultaneously. Chilling alone can also disrupt the integrity of the cytoplasm. The oocyte and entire mass of surrounding cumulus cells are regarded as a structural and functional syncytium (Thibault *et al*., 1987). The maturation of bovine oocyte depends on the intact connection between the oocyte and surrounding cumulus. Any undue disturbance in this intimate connection from damaged and interrupted cytoplasmic processes may negatively influence intercellular communication between cumulus cells and oocyte and subsequently affect the developmental capacity (Gordon, 2003).

In conclusion, this study showed that using ethylene glycol-based freezing and vitrification solutions, most organelles are able to retain their normal morphology following cryopreservation and thawing.

Acknowledgements: We thank all staff of Shah Alam and Senawang abattoirs, Department of Veterinary Services, Malaysia for helping in the sample collection. We also thank staff of Electron Microscopy Unit, Universiti Putra Malaysia for their assistance.

REFERENCES

Agca Y, 2000. Cryopreservation of oocyte and ovarian tissue. ILAR J 41, 207. http://dels-old.nas.edu/ ilar_n/ilarjournal/41_4/Oocyte.shtml

Arav A, 1992. Vitrification of oocytes and embryos. Embryonic development and manipulation in animal production. Lauria, A. and Gandolfi, F (eds), Portland Press, London and Chapel Hill, pp: 255-264.

Bautista JAN, EC Dela Peña, S Katagiri, Y Takahashi and H Kanagawa, 1998. In vitro viability of mouse oocytes vitrified in an ethylene glycol-based solution. Jpn J Vet Res, 46: 13-18.

de Loos F, P Kastrop, PV Maurik, THV Beneden and TAM Kruip, 1991. Heterologous cell contacts and metabolic coupling in bovine cumulus oocyte complexes. Mol Reprod Dev, 28: 255-259.

Diez C, P Duque, E Gomez, CO Hidalgo, C Tamargo, A Rodriguez, L Fernandez, S de la Varga, A Fernandez, N Facal and M Carbajo, 2005. Bovine oocyte vitrification before or after meiotic arrest: effects on ultrastructure and developmental ability. Theriogenology, 64: 317-333.

Elsden R, 1984. Classification of embryos after thawing. Proceedings No. 70, Bovine Embryo Transfer Workshop, Sydney, Australia, pp: 159-163.

Fuku E, J Liu and BR Downey, 1995a. In vitro viability and ultrastructural changes in bovine oocytes treated with a vitrification solution. Mol Reprod Dev, 40: 177-185.

Fuku E, L Xia and BR Downey, 1995b. Ultrastructural changes in bovine oocytes cryopreserved by vitrification. Cryobiology, 32: 139-156.

Gordon I, 2003. Laboratory Production of Cattle Embryos, 2nd Ed, CABI Publishing, London, UK.

Gupta MK, SJ Uhm and HT Lee, 2007. Cryopreservation of immature and in vitro matured porcine oocytes by solid surface vitrification. Theriogenology, 67: 238-248.

Hadi H, H Wahid, MOA Mazni, Y Rosnina, M Daliri, M Dashtizad, A Faizah, KC Yap and FJ Fahrul and A Fazly, 2010. Effect of equilibration temperature on *in vitro* viability and subsequent embryo development of vitrified-warmed immature bovine oocytes. Am J Anim Vet Sci, 5: 71-75.

Hochi S, M Kozawa, T Fujimoto, E Hondo, J Yamada and N Oguri, 1996. In vitro maturation and transmission electron microscopic observation of horse oocytes after vitrification. Cryobiology, 33: 300-310.

Isachenko V, M Montag, E Isachenko, S Dessole, F Nawroth and H van der Ven, 2006. Aseptic vitrification of human germinal vesicle oocytes using dimethyl sulfoxide as a cryoprotectant. Fertil Steril, 85: 741-747.

Kim DH, HS Park, SW Kim, IS Hwang, BC Yang, GS Im, HJ Chung, HW Seong, SJ Moon and BS Yang, 2007. Vitrification of immature bovine oocytes by the microdrop method. J Reprod Dev, 53: 843-851.

Le Gal F, 1996. In vitro maturation and fertilization of goat oocytes frozen at the germinal vesicle stage. Theriogenology, 45: 1177-1185.

Leibfried L and NL First, 1979. Characterization of bovine follicular oocytes and their ability to mature in vitro. J Anim Sci, 48: 76-86.

Nottola SA, G Coticchio, L De Santis, G Macchiarelli, M Maione, S Bianchi, M Iaccarino, C Flamigni and A Borini, 2008. Ultrastructure of human mature oocytes after slow cooling cryopreservation with ethylene glycol. Reprod Biomed Online, 17: 368-377.

Otoi T, S Tachikawa, S Kondo and T Suzuki, 1992. Developmental capacity of bovine oocytes cryopreserved after maturation in vitro and of frozen-thawed bovine embryos derived from frozen mature oocytes. Theriogenology, 38: 711-719.

Shaw JM, A Oranratnachai and AO Trounson, 2000. Fundamental cryobiology of mammalian oocytes and ovarian tissue. Theriogenology, 53: 59-72.

Suzuki T, A Boediono, M Takagi, S Saha and C Sumantri, 1996. Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by a one-step dilution method in vitro. Cryobiology, 33: 515-524.

Takahashi Y, M Hishinuma, M Matsui, H Tanaka and H Kanagawa, 1996. Development of in vitro matured/fertilized bovine embryos in a chemically defined medium: influence of oxygen concentration in the gas atmosphere. J Vet Med Sci, 58: 897.

Thein M, H Wahid, Y Rosnina, O Fauziah, RG Sianturi, AJ Azilah, OK Ho and MA Nooraini, 2002. Ultrastructural alterations in frozen-thawed immature bovine oocytes, 11th Scientific Conference of the Electron Microscopy Society of Malaysia, Johor Bahru, Malaysia, pp: 296-300.

Thibault C, D Szollosi and M Gérard, 1987. Mammalian oocyte maturation. Reprod Nutr Dev, 27: 865-896.

Vajta G, P Holm, M Kuwayama, P Booth, H Jacobsen, T Greve and H Callesen, 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev, 51: 53-58.

Wu C, R Rui, J Dai, C Zhang, S Ju, B Xie, X Lu and X Zheng, 2006. Effects of cryopreservation on the developmental competence, Ultrastructure and cytoskeletal structure of porcine oocytes. Mol Reprod Dev, 73: 1454-1462.

Younis AI, BG Brackett and RA Fayrer-Hosken, 1989. Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. Gamete Res, 23: 189-201.