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Developmental Potential and Apoptosis Incidence of *In Vitro* Produced Buffalo Embryos Vitrified by Solid Surface Technique

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ABSTRACT

Under the present study the post-thawed developmental potential and apoptotic incidence of *in vitro*-produced (IVP) buffalo embryos vitrified by solid surface technique were assessed. The abattoir derived oocytes were *in vitro* matured in maturation media and fertilized with capacitated epididymal sperm. The fertilized embryos were cultured in commercial Research vitro cleave media for *in vitro* embryo development. Embryos at morula stages were vitrified using vitrification media composed of 35% Ethylene glycol. The vitrified morulae were thawed and allowed to grow in culture media similar to non-vitrified control embryos up to the blastocyst stage. The post-thawed developmental competence of vitrified embryos was recorded to be significantly lower in terms of compact morula (57±1.22% vs 81±1.87%), blastocyst (32±1.22% vs 58±1.22%) and blastomere count (129.0±1.22 vs 159.2±1.31) as compared to non-vitrified control. Furthermore, Annexin-V and PI staining dye were used for staining the embryos for apoptotic study under confocal microscope. The apoptotic study of embryos observed by confocal microscopy revealed that rate of apoptosis was significantly higher in vitrified group as compared to control (53.33±2.13% vs 26.67±3.43%). Our study concluded that the vitrification increased the rate of apoptosis which significantly affected the development potential of vitrified-thawed embryos.

Keywords: Buffalo, *In vitro* fertilization, Embryo, Vitrification, Apoptosis.

For the enhancement of the productivity of buffalo and rapid dissemination of elite germplasm, the cutting edge reproductive biotechnological tools like ovum-pick up, *in vitro* fertilization and embryo transfer are extensively used (Madan *et al.,* 1996). Unlike other livestock, success rate of embryo production and embryo transfer is much lower in buffaloes due to their inherent lower fertility, poor super ovulatory response and weak estrus signs (Drost, 2007).

Cryopreservation accelerates the efficacy of assisted reproduction by widespread dissemination of embryos or germplasm in to the distant herds within short period as well as it enables the storage of the embryos for extended period (Alminana and Cuello, 2015). Vitrification, a rapid method of cryopreservation with high concentrations of salts or cryoprotectants and ultra-rapid freezing velocity, is gaining popularity due to its high success rate (Pereira and Marques, 2008). Further, the technique was modified as solid surface vitrification (SSV) which involves metal surface precooled to -180^{o}C, over which the embryos encapsulated with micro drop of vitrification solution are instantly vitrified (Dinnyes *et al*., 2000). The freezing and thawing process probably causes a risk for intracellular ice formation, uncontrolled dehydration, increased viscosity as well as post-thawed re-crystallization and osmotic shock may exert lethal effects leading to reduced embryo survival rates (Coticchio *et al.,* 2004; Elnahas *et al.,* 2010). These physical and chemical damages trigger a cell stress response that activates the apoptotic cascade which reduces the embryo viability and ultimately leads to necrosis. Annexin-V is widely used in conjunction with Propidium iodide (PI) to determine if cells are viable, apoptotic or necrotic through differences in plasma membrane integrity and permeability (Vermes *et al.,* 2000). Annexin-V, serves as an apoptosis cell surface marker by translocating from

the inner to the outer leaflet of the plasma membrane. The fluorochromes conjugate to annexin-V can stain the cells before loss of membrane integrity, which accompanies till last cell death stages of apoptotic and necrotic processes (Diana *et al*., 2012) whereas, PI does not stain live or early apoptotic cells due to the presence of an intact plasma membrane (Vermes, *et al.*, 2000; Darzynkiewicz *et al.,* 1992). Propidium iodide is stable and a good indicator of cell viability due to its ability to enter a cell depending upon the permeability of the membrane thus it has a capacity to exclude dye in living cells (Bacso *et al.,* 2000).

In view of the above facts the present study was planned to determine the developmental potential of vitrified-thawed buffalo embryos and to assess the apoptosis incidence due to cryo-stress by confocal microscopy.

MATERIALS AND METHODS

All the chemicals and media were purchased from Sigma Chemicals Co. (St Louis, MO) and disposable plastic were from Nunc (Roskilde, Denmark), unless stated otherwise.

In vitro maturation (IVM) of oocytes

The buffalo ovaries were collected from government large animal abattoir within 1-2 h of slaughter and brought to the laboratory in sterile normal saline at 37°C temperature. The ovaries were trimmed and washed with normal saline and Dulbecco's phosphate buffer saline (DPBS) aseptically. The cumulus oocyte complexes (COCs) were aspirated from medium sized ovarian follicles (2-8 mm) by follicular aspiration technique using 18 gauge needle with syringe in 1-2 ml DPBS. The COC's having more than 3 cumulus layers and homogenous ooplasm were selected for IVM and kept in groups of 20–25 numbers into 50µl droplets of maturation medium consisted of TCM-199, 7.5% (v/v) fetal bovine serum, 2.5 unit/ml luteinizing hormone, 2.5 unit/ml follicle stimulating hormone 1 µg/ml estradiol, 0.8 mM/ml sodium pyruvate and 50 µg/ml gentamicin under humidified atmosphere of incubator having 5% CO_2 at 38.5°C for 24 h. The degree of oocytes maturation was assessed on the basis of cumulus expansions under stereo microscope (Fig. 1A).

In vitro fertilization and embryo culture

In the present study the sperms were harvested from bull epididymis which was obtained from abattoir and processed as described for ovaries. The sperm were harvested by a sharp incision on epididymis which were used for *in vitro* fertilization with matured oocytes similar to the protocol described by Kumar *et al*. (2016) with little modifications. Briefly, the epididymal sperm were collected in 10 ml of pre-equilibrated Bracket and Oliphant (BO) media supplemented with 1mM caffeine sodium benzoate. The sperm suspension was centrifuged at 1,000 rpm for 8 min. The pellet was re-suspended in fresh BO media and re-centrifuged. The pellet was washed in 5 ml fertilization BO media consisted of 1% Bovine serum albumin (BSA) and 50 µg/ml heparin by centrifugation at 1,000 rpm for 5 min. The sperm pellet was suspended with 1 ml of fertilization media and centrifuged at 1,000 rpm for 1 min. The pellet was loosened with a fine closed tip of Pasteur pipette and the tube was kept at 45° angle under humidified atmosphere at 38.5°C for 30 min to facilitate the sperm movement upward or swim -up. In the meantime, *in vitro* matured oocytes were washed and transferred into 50 µl droplets of pre-equilibrated fertilization media @ 20-25 oocytes per drop for 30 min. From the top layer of sperm suspension, aliquots of sperm were added into the fertilization droplets to achieve a final concentration of 10^6 sperm/ml. The sperms and oocytes were co-incubated for 18 h under humidified atmosphere of 5% CO_2 at 38.5°C (Fig. 1B). After 18 h the co-incubated oocytes were denuded by 0.1% hyaluronidase and the presumptive zygotes were cultured in Research vitro cleave media (RVCL, COOK®, Australia) upto 7th days or till blastocysts.

Vitrification and thawing of IVF embryos

The morula stage embryos were subjected to two steps solid surface vitrification (SSV) by using equilibration media (4% Ethylene Glycol + TCM-199 + 20% FBS) for 15 min and subsequently with vitrification media (35% Ethylene Glycol + TCM-199 + 0.5M sucrose + 0.5% Polyvinyl pyrollidone + 20% FBS) for 45 second. In this method 2-3 numbers of embryos were vitrified with minimal amount (0.5-0.7µl) of vitrification medium by dropping on solid metallic surface, partially immersed into liquid nitrogen (LN_2). Finally, the vitrified embryos were collected into pre-cooled cryovials and stored into LN_2 container.

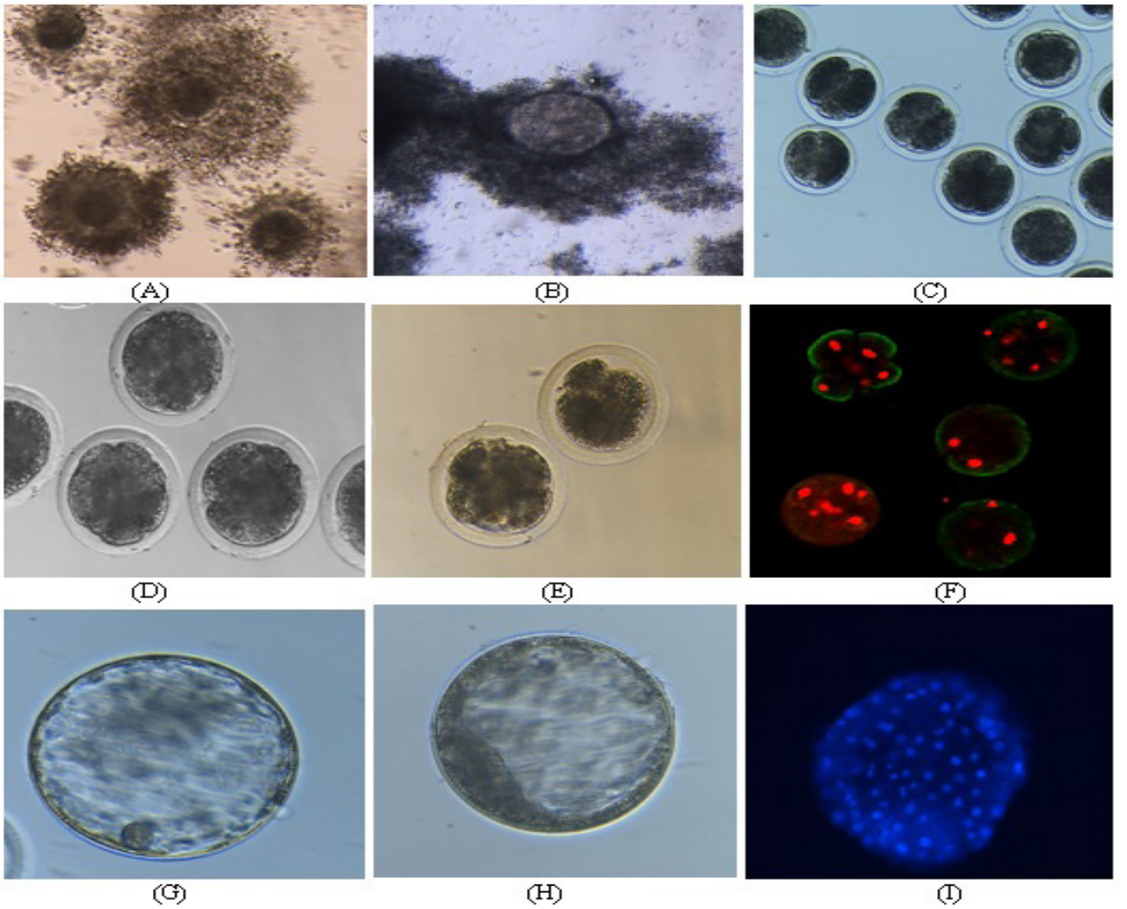

Fig. 1: *In vitro* maturation, fertilization and embryo development

(A) *In vitro* matured oocytes from abattoir derived ovaries (100X)**;** (B) Co-incubation of matured oocyte with capacitated sperm (100X)**;** **(**C) Early cleaved embryos (100X); **(**D) Morulae from fresh control group (100X); (E) Vitrified-thawed morulae (F) Apoptotic stained morula embryos under confocal microscope; (G) Expanded blastocyst from fresh control (100X); (H) Vitrified-thawed blastocysts (100X); (I) Hoechst stained blastocysts (100X).

For thawing, vitrified embryos were gradually exposed to thawing medium-I (TCM-199 + 0.4M sucrose solution + 20% FBS) for 1 min and subsequently to thawing medium-II (TCM-199 + 0.2M sucrose + 20% FBS) for 3 minutes. The thawed embryos were washed and kept in holding medium (TCM 199 + 20% FBS) for 15 min. and finally cultured in RVCL media till blastocyst development as described for fresh embryo group. The quality of blastocysts of each embryos group were determined by number of blastomere count using Hoechst staining under fluorescence microscope as per protocol described by Kumar *et al.* (2014). In brief, the blastocysts of each group were washed 5-6 times in 50µl drops of PBS supplemented with 1 mg/ml polyvinylpyrolidone and transferred in to a 50µl droplet of Hoechst 33342 stain (10 µg/ml in PBS) for 15 min in a dark compartment. Finally, the embryos were transferred on a clean glass slide with cover slip for microscopic examination under fluorescence.

Assessment of apoptosis in vitrified and fresh IVF embryos by confocal microscopy

The vitrified thawed and non-vitrified embryos were subjected to apoptotic assay using ApoDETECT^{TM}

ANNEXINV-FITC Kit (Invitrogen™, Cat- 33-1200) as per manufacturer's instructions. Briefly, the embryos of each group were washed in ice cold PBS (pH-7.4) and suspended in 500µl of 1X binding buffer. Embryos were then transferred into 10µl of Annexin-V FITC +190µl 1x binding buffer and incubated for 10 min at room temperature in dark chamber. These embryos were washed with 1X binding buffer and finally resuspended in 10µl propidium iodide (20 µg/ml) + 190 µl of binding buffer. The embryos were kept on micro confocal dish (Ibidi, GmbH, Germany) and apoptosis were assessed by fluorescence emitted by Annexin-V (FITC, 495-519 nm) and propidium iodide (PI, 537-619 nm) under confocal microscope (FV10i, Olympus, Japan). Annexin-V showed green signal indicating early apoptosis while red fluorescent light emitted by propidium iodide is indicator of late apoptosis or necrosis (Fig. 1F).

Statistical analysis

The post-thawed developmental competence and apoptosis incidence of vitrified-thawed embryos as compared to non-vitrified control were statistically analysed using t test at $P \leq 0.05$ level of significance.

RESULTS AND DISCUSSION

In vitro maturation and fertilization

During 10 experimental trials, a total of 822 oocytes were aspirated from 296 abattoir derived buffalo ovaries. Out of total aspirated COCs, 522 (63.50%) COC's having more than 3 cumulus layers, were subjected to maturation and only 465 (89.08%) matured oocyte were fertilized *in vitro*. After fertilization, a total of 385 (82.79±0.40%) oocytes were cleaved and subsequently developed into 8-16 cells stage (72.50±0.86%) and 16-32 stage early morula (65.36±13%) (Fig. 1C, D). Further, 100 early morula were randomly selected for vitrification and embryo culture (Table 1).

Comparative assessment of embryonic developmental competence of vitrified-thawed and non-vitrified embryo control

A total ten experimental trials comprised of five replications each for vitrified-thawed and non-vitrified morula were set for assessment of developmental potential up to blastocyst stages. In each replication consisting of 20 early morulae, the viability and developmental competence of vitrified-thawed embryos were assessed against fresh embryo control. Our results as revealed in Table 2 showed that post-thawed developments of compact morula stage embryos (57±1.22%) were significantly lower *($P \leq 0.05$)* than control (81±1.87%) (Fig. 1E). Likewise, there was a significantly lower ($P \leq 0.05$) development rate of blastocyst (32±1.22%) along with the blastomere count (129.0±1.22) of vitrified-thawed group as compare to non-vitrified control blastocyst (58±1.22%) and blastomere counts (159.2±1.31) (Fig. 1 G,H,I).

The decrease in the viability of vitrified-thawed embryos might be due to fact that vitrification exert some cellular, structural and biochemical changes resulting from cryo-injuries (Liu *et al.,* 2011 and Zhao *et al.,* 2012). Manjunatha *et al.* (2009) reported that chemical activities of different cryoprotectants used in various combinations and exposure time might be affected to the post-thaw development of IVP buffalo embryos. In the present study, ethylene glycol was used alone as cryoprotectant however, many scientists proposed the use of a combinations of cryoprotectants like ethylene glycol, Dimethylsulphoxide, Glycerol, Propelyne glycol etc. (Manjunatha *et al.,* 2008). Similar to our study, Hufana-Duran *et al*. (2004) vitrified the buffalo's embryos by ethylene glycol and reported higher rates of post-thawed hatched blastocyst (83.10%) after 72h of *in vitro* culture. Many other scientists reported the better cryosurvival rates of embryos using ethylene glycol alone (Cetin and Bastan, 2006; Cha *et al.,* 2000; Magnusson *et al.,* 2008; Hurtt *et al.,* 2000). Intracellular ice formation and osmotic injuries are the most important factors for cellular damages during cryopreservation, which primarily depend on the concentration of cryoprotectants, adequate equilibration and freezing time (Wani *et al.,* 2004).

Assessment of apoptosis in vitrified and fresh IVF embryos by confocal microscopy

In the present study the incidence of apoptosis was evaluated on thirty morula embryo under five experimental trails by confocal microscopy for both the vitrified-thawed and non-vitrified embryos group. Our findings revealed that vitrified thawed morula embryos showed significantly

Table 1: Fertilization and early development of IVF buffalo embryos

Total no. of trail	Total no. of Ovaries	Total no. of oocytes A and B grade oocyte	Matured oocyte set for IVF	Total no. of fertilized oocytes (2-4 cell)	Total 8-16 cell developed	Total 16-32 cell early morula
10	296	522 (63.50%)	465 (89.08%)	385 (82.79±0.40%)	338 (72.50±0.86%)	304 (65.36±13%)

Table 2: Comparative developmental rates of vitrified-thawed and non-vitrified IVF buffalo embryos

	No of trial	Total no. of Morula taken	Total Compact Morula developed	Total Blastocysts developed	Average no of Blastomere count in blastocysts
Vitrified	5	100	57 (57±1.22%)[a]	32(32±1.22%)[a]	129.0±1.22 [a]
Non-Vitrified (Control)	5	100	81(81±1.87%)[b]	58(58±1.22 %)[b]	159.2±1.31 [b]

Note: Values within parenthesis indicate the percentage in each group. Averages having different superscript within columns are significantly different *(P≤0.05)*.

(P<0.05) higher apoptotic incidence 16(53.33±2.13%) as compared to non-vitrified control 8(26.67±3.43%) (Table 3).

Table 3: Incidence of apoptosis in morula stage embryo

	Total embryos stained	Apoptotic embryos
Vitrified	30	16(53.33±2.13%)[a]
Non-Vitrified Control)	30	8(26.67±3.43%)[b]

Note: Values within parenthesis indicate the percentage in each group. Averages having different superscript within columns are significantly different (*P≤0.05*).

The differences in embryo vitrification outcome might be the consequence of chemical toxicity and osmotic shock due to high concentration of cryoprotectants (Fahy *et al.,* 2004; Szurek and Eroglu, 2011), cellular damage (Dobrinsky *et al.,* 2000), DNA damage (Park *et al.,* 2006; Kopeika *et al.,* 2015) and zona hardening (Matson *et al.,* 1997; Wiesak *et al.,* 2017) and these factors might have affected embryonic developmental potency by apoptotic process. Byrne *et al.* (1999) reported that the higher incidence of apoptosis and DNA fragmentations are associated with lower developmental competence of embryos.

In the present study, annexin-V and propidium iodide were used to detect phosphatidylserine (PS) on the outer leaflet of the bilayer plasma membrane as it is the first event of the apoptosis process. Most of the previous studies had used TUNEL assay which stained only fragmented DNA and detect the last stage of the apoptosis process. However, the literature for comparing the findings related to apoptosis in vitrified embryos could not be traced in buffaloes. Park *et al.* (2006) in bovine studied the TUNEL assay that resulted in a significantly higher apoptotic index (11.9% by conventional straw vitrification and 11.0% by minimum volume cooling vitrification methods) than in non-frozen embryos (3.0%).

However, in our staining method by annexin-V and PI the early apoptosis stages could also be successfully detected by green fluorescence ring, hence these early apoptosis changes can be control in subsequent experiments by improving the culture conditions.

CONCLUSION

In our experiment, the vitrified-thawed embryos showed significantly lower developmental competence as compared to non-vitrified embryos. Further, the lower developmental rates were correlated to higher apoptosis incidence in vitrified-thawed embryos. Our study

described a staining technique based on annexin-V and propidium iodide for detection of early apoptosis in buffaloes developing embryos by confocal microscopy. The early detection of apoptosis may provide an access to improve the overall culture condition for embryonic development.

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REFERENCES

Alminana, C. and Cuello, C. 2015. What is new in the cryopreservation of embryos? *Anim. Reprod.,* **12**(3): 418-427.

Bacso, Z., Everson, R.B. and Eliason, J.F. 2000. The DNA of Annexin V-binding apoptotic cells is highly fragmented. *Cancer Res.,* **60**: 4623-4628.

Byrne, A.T.J., Southgate, D.R. Brison and H.J. Leese. 1999. Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. *J. Reprod. Fertil*., **117**: 97-105.

Cetin, Y. and Bastan, A. 2006. Cryopreservation of immature bovine oocytes by vitrification in straws. *Anim. Reprod. Sci.,* **92:** 29-36.

Cha, K., Chung, H. and Lim, J. 2000. Freezing immature oocytes. *Mol. Cell End.,* **169:**43-47.

Coticchio, G., Bonu, M.A., Borini, A. and Flamigni, C. 2004. Oocyte cryopreservation: a biological perspective. *Eur. J. Obstet. Gynecol. Reprod. Biol.,* **115**(1): S2-S7.

Darzynkiewicz, Z., Bruno, S., Del, Bino, G., Gorczyca, W., Hotz, M.A., Lassota, P. and Traganos, F. 1992. Features of apoptotic cells measured by flow cytometry. *Cytometry*, **13**: 795-808.

Diana, V., Maria, E.P., Cristobal, A.S.M., Ana, C.C., Antonio, P. and Carlos, S. 2012. Comparison of Cryotip vs. Cryotop for mouse and human blastomere vitrification. *Fertil. Steril.,* **97**: 1.

Dinnyes, A., Dai, Y.P., Jiang, S. and Yang, X.Z. 2000. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization and somatic cell nuclear transfer. *Biol. Reprod.,* **63**: 513-518.

Dobrinsky, J.R., Pursel, V.G., Long, C.R. and Johnson, L.A. 2000. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. *Bio. Reprod.*, **62**: 564-570.

Drost, M. 2007. Advanced reproductive technology in the water buffalo. *Theriogenology*, **68**(3): 450-453.

Elnahas, A., Alcolak, E., Marar, E.A., Elnahas, T., Elnahas, K. and Palapelas, V. 2010. Vitrification of human oocytes and different development stages of embryos: an overview. *Middle East Fertil. Soc. J.,* **15**(1): 2-9.

Fahy, G.M., Wowk, B., Wu, J. and Paynter, S. 2004. Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology,* **48**: 22-35.

Hufana-Duran, D., Pedro, P.B., Venturina, H.V., Hufana, R.D., Salazar, A.L., Duran, P.G. and Cruz, L.C. 2004. Post-warming hatching and birth of live calves following transfer of in vitro-derived vitrified water buffalo (*Bubalus bubalis*) embryos. *Theriogenology,* **61:** 1429-1439.

Hurtt, A.E., Squire, E.L. and Seidel, J.R. 2000. Vitrification of equine and bovine oocytes in an ethylene glycol, ficoll and sucrose use open pulled straws. *Theriogenology,* **51:** 166.

Kopeika, J., Thornhill, A. and Khalaf, Y. 2015. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. *Hum. Reprod. Update,* **21**(2): 209-227.

Kumar, D., Gopalakrishna, R., Singh, A.P., Ranjan, R., Pandey, S.K. and Sarkhel, B.C. 2014. Developmental potency of pre-implant parthenogenetic goat embryos: effect of activation protocols and culture media. *In Vitro Cell Dev-An.,* **50**(1): 1–6.

Kumar, D., Jain, S.K. and Sarkhel, B.C. 2016. A comparison of *in vitro* fertilization potential of sperm obtained from refrigerated (4°C) epididymis and fresh ejaculation of buck under different embryo culture media. *Indian J. Anim. Sci.,* **86**(8): 883-886.

Liu, W.X., Lu, H., Luo, M.J. and Xu, L.Z. 2011. Effects of different cryoprotectants and cryopreservation protocols on the development of 2-4 cell mouse embryos. *CryoLetters*, **32**(3): 240-7.

Madan, M.L., Das, S.K. and Palta, P. 1996. Application of reproductive technology to buffaloes. *Anim. Reprod. Sci.,* **42:** 299-306.

Magnusson, V., Feitosa, W.B., Goissis, M.D., Yamada, C., Tavares, L.M., D'Avila, A.M.E. and Visintin, J.A. 2008. Bovine oocyte vitrification: Effect of ethylene glycol concentrations and meiotic stages. *Anim. Reprod. Sci.,* **106:** 265-273.

Manjunatha, B.M., Gupta, P.S., Ravindra, J.P., Devaraj, M. and Nandi, S. 2008. *In vitro* embryo development and blastocyst

hatching rates following vitrification of river buffalo embryos produced from oocytes recovered from slaughterhouse ovaries or live animals by ovum pick-up. *Anim. Reprod. Sci.,* **104:** 419-26.

Manjunatha, B.M., Gupta, P.S., Ravindra, J.P., Devaraj, M. and Nandi, S. 2009. Effect of vitrification medium composition and exposure time on post-thaw development of buffalo embryos produced *in vitro. Vet. J.,* **179**(2): 287-91.

Matson, P.L., Graefling, J., Junk, S.M., Yovich, J.L. and Edirisinghe, R.W. 1997. Cryopreservation of oocytes and embryos: use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an *in vitro* fertilization programme. *Hum. Reprod.,* **12:** 1550-1553.

Park, S.Y., Kim, E.Y., Cui, X.S., Tae, J.C., Lee, W.D., Kim, N.H., Park, S.P. and Lim, J.H. 2006. Increase in DNA fragmentation and apoptosis-related gene expression in frozen-thawed bovine blastocysts. *Zygote,* **2**: 125-31.

Pereira, R.M. and Marques, C.C. 2008. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank.,* **9:** 267-277.

Szurek, E.A. and Eroglu, A. 2011. Comparison and avoidance of toxicity of penetrating cryoprotectants. *PLoS One,* **6**(11): e27604.

Vermes, I., Haanen, C. and Reutelingsperger, C. 2000. Flow cytometry of apoptotic cell death. *J. Immunol. Methods*, **243**: 167-190.

Wani, N.A., Maurya, S.N., Misra, A.K., Saxena, V.B. and Lakhchaura, B.D. 2004. Effect of cryoprotectants and their concentration on *in vitro* development of vitrified-wanned immature oocytes in buffalo *(Bubalus bubalis). Theriogenology,* **61**:831-842.

Wiesak, T., Wasielak, M., Zlotkowska, A. and Milewski, R. 2017. Effect of vitrification on the zona pellucida hardening and follistatin and cathepsin B genes expression and developmental competence of *in vitro* matured bovine oocytes. *Cryobiology,* **76:** 18-23.

Zhao, X.M., Du, W.H., Hao, H.S., Wang, D., Qin, T., Liu, Y. and Zhu, H.B. 2012. Effect of vitrification on promoter methylation and the expression of pluripotency and differentiation genes in mouse blastocysts. *Mol. Reprod. Dev.,* **79**(7)**:** 445-50.