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 *in 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°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% CO₂ 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 10⁶ sperm/ml. The sperms and oocytes were co-incubated for 18 h under humidified atmosphere of 5% CO₂ 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 (LN₂). Finally, the vitrified embryos were collected into pre-cooled cryovials and stored into LN₂ container.
Effect of vitrification on post-thawed embryonic development and apoptosis

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 polyvinylpyrrolidone 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™...
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 $P\leq0.05$ 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 ($P\leq0.05$) than control (81±1.87%) (Fig. 1E). Likewise, there was a significantly lower ($P\leq0.05$) 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 cryoinjuries (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 trials by confocal microscopy for both the vitrified-thawed and non-vitrified embryos group. Our findings revealed that vitrified thawed morula embryos showed significantly
Effect of vitrification on post-thawed embryonic development and apoptosis

Table 1: Fertilization and early development of IVF buffalo embryos

<table>
<thead>
<tr>
<th>Total no. of trials</th>
<th>Total no. of Ovaries</th>
<th>Total no. of grade oocyte A and B</th>
<th>Matured oocyte set for IVF (2-4 cell)</th>
<th>Total no. of fertilized oocytes</th>
<th>Total 8-16 cell developed</th>
<th>Total 16-32 cell early morula</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>296</td>
<td>522</td>
<td>(63.50%)</td>
<td>465 (89.08%)</td>
<td>338</td>
<td>(72.50±0.86%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(65.36±13%)</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>No of trial</th>
<th>Total no. of Morula taken</th>
<th>Total Compact Morula developed</th>
<th>Total Blastocysts developed</th>
<th>Average no of Blastomere count in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified</td>
<td>5</td>
<td>100</td>
<td>57 (57±1.22%)</td>
<td>32(32±1.22%)</td>
</tr>
<tr>
<td>Non-Vitrified (Control)</td>
<td>5</td>
<td>100</td>
<td>81(81±1.87%)</td>
<td>58(58±1.22 %)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Total embryos stained</th>
<th>Apoptotic embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified</td>
<td>30</td>
</tr>
<tr>
<td>Non-Vitrified (Control)</td>
<td>30</td>
</tr>
</tbody>
</table>

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


Byrne, A.T.J., Southgate, D.R. Brison and H.J. Leese. 1999. The early detection of apoptosis may provide an access to improve the overall culture condition for embryonic development.

Bubalus bubalis


