Effect of Addition Vitamin C and Zinc Chloride in Vitrification Medium on Viability, in vitro Maturation and Ultrastructure Changes of Vitrified Immature Bovine Oocytes

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ABSTRACT

This study aimed to evaluate the impact of adding vitamin C and zinc chloride to vitrification medium on viability in vitro maturation and ultrastructural changes of vitrified immature Baladi cow oocytes. Compact cumulus oocytes (COCs) (n=1370) were obtained from slaughtered bovine ovaries. Then the morphology of oocytes was examined using a stereomicroscope. Staining with trypan, the in vitro maturation and ultrastructural changes were studied. The results revealed significant (P<0.05) increase of total and normal survival rate of bovine oocytes vitrified with zinc chloride (90.28 and 81.11%) than in vitamin C media (82.5 and 65.65%) or control medium (74.44 and 54.72%). Recovery rate of abnormal bovine oocytes showed significantly an opposite trend (9.17 vs. 16.94 and 19.72%). Proportion of oocytes with viable cytoplasm and viable cumulus (VOC) was increased significantly (93.75%, P<0.05) in fresh (control) than in medium supplemented with vitamin C and untreated medium (74.55 and 68.63%), respectively. There were non-significant differences among zinc chloride, control and vitamin C media in oocytes with viable cytoplasm and unviable cumulus (VOC). Supplementation of the vitrification medium with zinc chloride and vitamin C significantly (P<0.05) improved maturation rates (MII) of recovered cumulus oocyte complexes (COCs) than medium without supplementation. The percentage of ultrastructural alterations in most organelles bovine oocytes significantly (P<0.05) increased in oocytes vitrified without supplementation followed by vitamin C, then zinc chloride medium. Conclusion, supplementation of vitamin C or zinc chloride to the vitrification medium improved survival rate, morphology and ultrastructural, as well as maturation rate of bovine oocytes

Keywords: Antioxidants, vitrification, bovine oocytes, ultrastructure

INTRODUCTION

Low fertilization rates in cryopreserved oocytes have been related to freeze damage, including solidification of zona pellucida as a result of releasing of premature cortical granules, spindle disorganization and microtubule loss or agglutination (Carroll et al., 1990; Hwang and Hochi, 2014). Furthermore, reactive oxygen species (ROS) such as superoxide (O2•−), hydroxyl radical (OH•), hydrogen peroxide (H2O2) may disrupt the cellular function of oocytes and embryos that survive cryopreservation (Vajta et al., 1997; Dobrinsky, 2002). Oocytes preserved via slow freezing suffer from osmotic shock and intracellular ice crystallization as reported by Ledda et al. (2006) and those undergone vitrification are exposed to toxic concentrations of the cryoprotectant agents (Sripunya et al., 2010).

Vitrification is still remaining an effective protocol for cryopreservation of gametes and embryos. In this regard, several trials have been conducted to reduce its side effects on the quality and developmental potential of oocytes (Wani et al., 2004; Sripunya et al., 2010; Mostagir et al., 2019).

Several studies showed that increasing ROS and H2O2 levels and decreasing glutathione (GSH) content following oocyte vitrification (Somfai et al., 2007; Gupta et al., 2010). The cryopreservation lead to lowering the rate of in vitro oocyte maturation, fertilization and production of embryo might be due to damaging the cytoskeleton of mature oocytes through disrupting the sub-cortical actin network, microtubule depolymerization, abnormal spindle configuration and chromosome scattering (Prentice and Anzar, 2011; Brambillasca et al., 2013; Dutta et al., 2013; Hwang and Hochi, 2014).

The beneficial effects of L-carnitine (LC) which can be included in the vitrification media of bovine oocytes and embryos to protect against harmful effects of vitrification Were reported by Badr et al. (2020). Mostagir et al. (2019) conducted a study to determine the efficiency of different vitrifying solutions. Dimethyl sulphoxide (DMSO) and ethylene glycol (EG) with or without supplementation of three concentrations of LC (5, 10 and 15 mM) on post-thawing viability of vitrified immature buffalo oocytes. They found that using EG or a mixture of EG and DMSO supplemented with LC at 5 and 10 mM increased oocyte recovery and survival rates.

Ascorbic acid (AA) addition also seemed to protect embryos against oxidative stress during in vitro culture (IVC) and improve development of embryos competence after either in vitro fertilization (Hossein et al. 2007). Supplementation with AA during IVC improves the quality
of blastocysts in terms of cell numbers and increases the survival rate after vitrification (Hu et al., 2012; Castillo-Martín et al., 2014).

This study aimed to evaluate the effect of addition of vitamin C or zinc chloride to vitrification medium on viability, ultrastructural changes and in vitro maturation of vitrified immature bovine oocytes.

MATERIALS AND METHODS

The present study was done at the lab of Physiology and Biotechnology, Department of Animal production, Faculty of Agriculture, Mansoura University during the period of April 2018 to March 2020.

Ovaries collection:
In this study, the ovaries were collected immediately, after slaughtering, from the local Balidi cows in Elbagor slaughterhouse, Minufia government. The collected ovaries were placed into thermos (35 °C) which contains saline solution (0.9% NaCl), penicillin G (100 IU/mL) and streptomycin sulfate (100 µg/mL) and transported to the lab within 3-5 h.

Oocyte recovery:
An eighteen-gauge needle joined to a 10 ml syringe were used for aspiration of cumulus oocyte complexes (COCs) from the follicles that have 2-8 mm diameter. Only e high quality immature oocytes that characterized by the homogeneous distribution of the cytoplasmic granules and enclitic by compact layers of cumulus cells were used for vitrification.

Experimental design:
This experiment aimed to study the effect of cryopreservation of Baladi cow oocytes by vitrification method (supplemented with vitamin C 1mg or zinc chloride 1.5µg/mL vitrification solution) on survival; changes in ultrastructure of oocytes and in vitro maturation rates (IVM) as well as compared with fresh oocytes.

Vitrification of oocytes:
The HEPES-buffered TCM 199 plus 20% (v/v) fetal bovine serum was used as a holding medium (HM) for handling oocytes during vitrification and warming.

All operations are performed on a heating plate at 37°C in a warm room at 25°C to 27°C. Except for the heating solution used at 37°C, all media are used at room temperature. The straw method is used for vitrification and thawing of immature oocytes. The vitrification process includes two main steps: equilibrium and vitrification. First, the immature COCs is placed on the middle surface of the equilibrium solution, which is obtained by adding both of ethylene glycol (EG) and dimethyl sulfoxide (DMSO) at a concentration of 7.5% (v:v) to the HM. After five minutes of equilibration, the shrunken oocytes restore its original size, which refers to the time for vitrification. Ten oocytes were transferred to a vitrification solution prepared using both of EG, DMSO at a concentration of 15% (v:v) and 0.5-M sucrose dissolved in the HM. Within one minute, the oocytes must be thoroughly rinsed with vitrification solution, loaded on the straw with a volume of less than 0.01 mL, and finally immersed in liquid nitrogen.

Warming of oocytes and assessment of oocyte viability:
Two weeks post storing, the oocytes warms process was done by placing the straws in the air for 6 second and then stirring in a 38°C water bath for at least 20 second. The content of each straw was discharged into a petri dish, and the oocytes were transferred to three diluent solutions. The HM solution contained 1 M sucrose for 1 minute, 0.5 M sucrose for 3 minutes, and then washed twice in HM for 5 minutes respectively. The viability of oocytes was investigated morphologically by using the stereomicroscope according to its integrity of oolemma, zona pellucida and the loss of membrane (lysis).

The criteria that were used to evaluate the morphology of vitrified warmed oocytes after thawing were as follows: (a) Normal oocytes with spherical and symmetrical shapes without signs of lysis, (b) Abnormal oocytes having fissure of the zona pellucida, disintegration or leakage of cell contents, changes in the shape and shrunken of the oocytes.

Evaluation of oocytes viability:
The trypan blue staining was used to evaluate the viability of oocytes according to Abd Allah et al. (2008).

In Vitro maturation:
Prepare maturation medium:
The main tissue culture medium (TCM –199) used in this study contained 10% (v/v) FCS and 50 µg/mL Gentamycin and supplemented with 10 µg/mL epidermal growth factor.

The pH value of the medium was adjusted to 7.2–7.3, and the osmotic pressure was 280–300 mOsm/kg. It was filtered through a 0.22 µM Millipore filter. About 50 µL of the prepared culture medium were put into a sterile disposable plastic petri dish (3.5 cm in diameter) and covered with sterile mineral oil. Before using the Petri dishes must be incubated in a CO₂ incubator with 38.5°C, 5% CO₂ and 95% relative humidity for at least one hour for equilibration.

Oocytes maturation:
After warming, the high-quality of vitrified oocytes and non-vitrified oocytes (control), were washed 3 times in washing medium and twice in the prepared maturation medium to remove the preventing substances maturation. Finally, the oocytes were incubated for 24 hours in the medium in the previously prepared Petri dish at 38.5°C, 5% CO₂ and high humidity.

Fixation and staining of oocytes:
At the end of the maturation, the compact cumulus oocytes were taken from the maturation medium and the oocytes were isolated from the cumulus cells by using 2.9% sodium citrate for five minutes and then repeat pipetting. Seven to ten oocytes in a drop of the medium were pipetted and mounted on a glass slide. The coverslip with inert paraffin spots on each of its four corners was placed directly above the center of the droplet of the oocyte-containing medium. After that, the oocytes were investigated under microscope magnification. The coverslip was pressed down on the oocyte until it was firmly fixed in place.

Fixation of oocytes was done by putting the slides in a freshly prepared mixture of acetic acid and ethanol (1:3) overnight. The slides were stained with 1% aceto orcein (1% orcein in 40% acetic acid w/v) for a few minutes and washed with acetyl-glycerol (3:1). The low power and oil immersion were used for detailed examination.

Criteria of maturation:
After maturation, oocytes (vitrified and control) were categorized into 5 groups as follows:
a - Germinal vesicle (GV): Interphase chromosomes enclosed within a nuclear membrane.

b - Germinal vesicle breakdown (GVBD): An absence of a visible nuclear membrane and chromatin condensation characterized by a cluster of DNA material without individual chromosomes.

c - Oocytes at metaphase I stage (MI): Chromosomes were condensed in pairs and without detected polar body (immature oocytes).

d - Oocytes at metaphase II stage (MII): One large group of chromosome formed an equatorial plate and the remaining chromosomes are highly condensed or had extruded a polar body (oocytes mature).

e - Degenerated oocytes (Deg): Oocytes were vacuolated or had scattered.

Electron microscopy

Twenty-four oocytes in each group were fixed and subjected to TEM treatment as described by Nottola et al. (2009). The oocytes were fixed in 1.5% glutaraldehyde at 4°C for 2-5 days, embedded in 1% gelose, and exposed to 1% osmium tetroxide. Then, the samples were dehydrated in increasingly higher concentrations of ethanol, immersed in propylene oxide for solvent replacement, and then respectively embedded in Epon 812. Then the oocytes were ultrathin sectioned (60-80 nm), stained with uranyl acetate and lead citrate, examined and photographed with a TEM.

Statistical analysis

Statistical analysis of the data was carried out through analysis of variance (ANOVA) after arcsine transformation. Duncan’s multi-range test was subsequently used to test for significant differences among treatments (Duncan, 1955). Chi-square test (χ2) tests the changes in ultrastructure. The statistical procedure used is the Statistical Analysis System (SAS, 2004).

RESULTS AND DISCUSSION

Survival and viability rates (proportion of normal and abnormal) of bovine oocytes vitrified in medium supplemented with vitamin C or Zinc chloride are showed in Table 1. Data in Table 1 showed significant (P<0.05) increase of survival and normal percentages of bovine oocytes cryopreserved using vitrification solution supplementation with Zinc chloride (90.28 and 81.11%) as compared to vitamin C (82.5 and 65.65%) and free (74.44 and 54.72%) media. However, the survival rate of abnormalities bovine oocytes showed significantly an opposite trend (9.17 vs. 16.94 and 19.72%, Table 1).

<table>
<thead>
<tr>
<th>Vitrification medium</th>
<th>Total number of oocytes</th>
<th>Survival</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh medium</td>
<td>360</td>
<td>268</td>
<td>97</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>360</td>
<td>297</td>
<td>82.5</td>
</tr>
<tr>
<td>Zinc chloride</td>
<td>360</td>
<td>325</td>
<td>90.28</td>
</tr>
</tbody>
</table>

Results showed significant differences between the groups (P<0.05).

Selection of oocytes for in vitro culture based only on the morphological features is not enough to obtain better in vitro maturation (IVM) and in vitro fertilization (IVF) (Blanco et al., 2011). Unknown factors seem to affect the 1st step of embryo development accumulated throughout the oogenesis period. The in vitro production of cryopreserved oocyte-derived embryos is also important in a commercial environment, as an aid to embryo transfer programs and as a guarantee for future reproductive biotechnology (Pomp and Critser, 1988).

The most morphological abnormalities noticed in the vitrified warmed oocytes in this study (19.72, 16.94 and 9.17%) could be attributed to cooling and warming that damage cytoskeleton and inducing degenerative cellular changes (Elsden, 1988). Also, osmotic stress is consider another factor that can induce damage in oocytes and causing changes in its volume and adversely affect their viability (Shaw et al., 2000).

Categories of oocytes viability using trypan blue after cryopreservation immature bovine oocytes supplementation with vitamin C and Zinc chloride are illustrated in Table 2. The proportion of viable cytoplasm and viable cumulus (VOVC) oocytes was significantly (P<0.05) higher significant (93.75%, P<0.05) in fresh group than in those vitrified with medium supplemented with vitamin C and untreated medium (74.55 and 68.63%), respectively. However, no significant differences between Zinc chloride and fresh group (Table 2).

Table 2. Viability of cytoplasm and cumulus of different types of fresh and vitrified cow oocytes as affected by treatments using trypan blue staining.

<table>
<thead>
<tr>
<th>Vitrification medium</th>
<th>Post-thawing survival oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh oocytes (control)</td>
<td>64</td>
</tr>
<tr>
<td>Free medium</td>
<td>51</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>55</td>
</tr>
<tr>
<td>Zinc chloride</td>
<td>85</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>Post-thawing survival oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOVOC</td>
<td>VOUC</td>
</tr>
<tr>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>64</td>
<td>93.75%</td>
</tr>
<tr>
<td>51</td>
<td>68.63%</td>
</tr>
<tr>
<td>55</td>
<td>74.55%</td>
</tr>
<tr>
<td>85</td>
<td>85.88%</td>
</tr>
</tbody>
</table>

Statistically analysis showed insignificant (P>0.05) difference among treatments in the proportion of VOUC and UOVC oocytes, being nearly similar for vitamin C and free media, and also between zinc chloride and control medium. The proportion of UOUC were significantly (P<0.05) lower in fresh group (1.56%) than in other treatments, followed by zinc chloride medium. It was observed that the percentage oocytes with viable cumulus cells was higher in all groups as compared to in oocytes with viable ooplasm, that may be refer to smaller sizes of cumulus cells and lead to easily and quickly inlet and outlet cryoprotectants from cells during (freezing/thawing).

Vital dyes are chemical compounds bind to tissues or cells and widely used in basic and applied research to evaluate specific characteristics of a cell or tissue. It can be used as an alternative method for quality assessment of female gametes in mammalians (Rodrigues et al., 2009). Trypan blue staining has previously been used to assess quality and the viability of oocytes. The dead oocytes show a dark blue egg mass with translucent cumulus cells (Jewgenow and Görüt, 1995; Abd Allah et al., 2008).
Maturation rates of vitrified/thawed bovine oocytes

Supplementation of the vitrification medium with zinc chloride or vitamin C significantly (P<0.05) improved maturation rates (MII) of recovered COCs than without supplementation as depicted in Table 3. Fresh COCs (Fig. 1) achieved the highest significant (P<0.05) maturation rates (71.15%), while the lowest maturation rate was obtained for COCs matured in vitrification medium without supplementation (39.34%). The moderate maturation rate was obtained by supplementation of vitamin C (49.41%) and zinc chloride (52.17%) in vitrification medium of immature bovine oocytes without significant differences between them.

The reduction of maturation rate of oocyte in vitrification medium without supplementation (untreated) than fresh COCs may be due to the harmful effect of cryopreservation such as cytoskeleton damage (Hwang and Hochi, 2014). This may be also attributed to oxidative stress, higher levels of hydrogen peroxide and lower concentrations of glutathione as a result of vitrification process (Kelly et al., 2005). The improvement in maturation rate after supplementation of antioxidants like vitamin C and zinc may be effective in protecting the oocytes from harmful ROS (Kere et al., 2012).

In addition, Jeon et al. (2014) and Geravandi et al. (2017) reported that supplementation with adequate zinc concentrations to vitrified medium regulates the intracellular GSH concentration, the ROS level, transcription factor expression and meiosis during maturation of oocytes. In this context, Jeon et al. (2015) revealed that supplementation with zinc during IVC improved the viability of embryos might be due to increasing PCNA, POU5F1, and Bcl2 gene expression. Furthermore the supplementation with vitamin C promotes the growth of vitrified-thawed bovine oocytes through inhibiting lipid peroxidation and biosynthesis of collagen (Geesin et al., 1991; Sonowal et al., 2017).

The present data showed that the proportion of GV oocytes were significantly (P<0.05) decreased after vitrification as shown in Table 3. These results are in agreement with the results obtained by Huang et al. (2018).

Table 3. Effect of vitamin C or Zinc chloride in vitrification medium of immature bovine oocytes on in vitro maturation rate.

<table>
<thead>
<tr>
<th>Vitrification</th>
<th>Total</th>
<th>GV</th>
<th>GVBD</th>
<th>M1</th>
<th>M11</th>
<th>DEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Fresh (control)</td>
<td>104</td>
<td>4</td>
<td>3.85%</td>
<td>8</td>
<td>7.69</td>
<td>11</td>
</tr>
<tr>
<td>Untreated</td>
<td>61</td>
<td>7</td>
<td>11.48%</td>
<td>5</td>
<td>8.2</td>
<td>12</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>85</td>
<td>8</td>
<td>9.41%</td>
<td>7</td>
<td>8.24</td>
<td>15</td>
</tr>
<tr>
<td>Zinc chloride</td>
<td>92</td>
<td>8</td>
<td>8.7%</td>
<td>9</td>
<td>9.78</td>
<td>14</td>
</tr>
</tbody>
</table>

a, b and c. in the same row, the values with different superscripts are significantly different (P<0.05).

Ultrastructural change after in vitro maturation of immature bovine oocytes.

Table 4 summarizes the ultrastructural changes of immature bovine oocytes supplemented with vitamin C and zinc chloride in vitrification medium after in vitro maturation. In the fresh control group (Figure 1) and all treatment groups, most of the cumulus cells around mature oocytes appeared to be normal, and very few cumulus cells showed cytoplasmic vacuoles. There are no zona cracks in all oocytes.
Vitamin C is the most important antioxidant in extracellular fluid (Warren et al., 2000). It has a positive effect on oocyte nuclear maturation (Tao et al., 2004). In addition, it can also protect embryos from oxidative stress during in vitro culture. Also addition of ascorbic acid to the vitrified and serum-supplemented warm medium can improve the embryo survival rate of pig blastocysts produced in vitro (Castillo-Martín et al., 2014).

The structural changes of lipid droplets in this study are consistent with previous reports in bovine and pig oocytes (Isachenko et al., 1998; Isachenko et al., 2001; Wu et al., 2006). In this respect, Wu et al. (2006) proposed that the increased small lipid droplets come from broken larger lipid droplets, which exist in the form of smaller droplets during the vitrification of porcine oocytes. Isachenko et al. (2001) also reported that the lipid droplets in pig oocytes changed in shape during the cooling process. They become spherical with transparent stripes. Ghetler et al. (2006) found that the cryopreservation process resulted in the loss of cortical particles in the cortex, and the appearance of vesicles in the cytoplasm of unfrozen and mature human cryopreserved oocytes, which may indicate structural damage during freezing and warming. Gualtieri et al. (2009) proposed that the mitochondria of freeze-thaw human oocytes have reduced matrix electron density or rupture of the outer and inner membranes. Mitochondria are the most abundant organelles in mammalian oocytes, and their disfunction or abnormalities would determine the oocyte and embryos developmental competence.

Conclusion, supplementation of vitamin C or Zinc chloride to the vitrification medium improved, morphologically and ultrastructural survival and viability rates as well as maturation rate of bovine oocytes.

REFERENCES

Hoda A. M. Yassen et al.


تأثير إضافة فيتامين ج وكلوريد الزنك في بيئة الترجم الابقار غير الفطري إلى التزجيج على الحيوية والنمو والتطورات في التركيب الدقيق للبيضات في المجموعة المضاف إلى بيئات الابقار.

نستخلص من هذه الدراسة أن إضافة كلوريد الزنك وفيتامين ج إلى البيئة النووية للبيضات في المجموعة المضاف إلى بيئات الابقار غير الفطري تمكنت من تعزيز النمو والتطورات في التركيب الدقيق للبيضات بالمقارنة مع البيئة النووية للبيضات في المجموعة، بينما لم يظهر الفروق بين المجموعتين في نسبة الترجم النجح.

كلمات الدراسات المقدمة: تزجيج البويضات، إضافة الزنك، فيتامين ج، البيئة النووية، معدل الترجم النجح.