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Comparison between Two Cryo-devices for Vitrification of Immature Oocytes of Indigenous Zebu Cows in Bangladesh

Sk Mohiuddin Choudhury[1], Mohammad Musharraf Uddin Bhuiyan[1,†], Mohammad Moshiur Rahman[1], Md. Masudur Rahman[2], Md. Newaz Sharif[1], Jayonta Bhattacharjee[1], Farida Yeasmin Bari[1] and Nasrin Sultana Juyena[1]

[1]*Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh*
[2]*Veterinary Surgeon, Department of Livestock Services, Dhaka, Bangladesh*

ABSTRACT

Cryopreservation of oocytes by vitrification technique may contribute a lot in the field of reproductive biotechnology. The objectives of the present study were to evaluate the effectiveness of two cryo-devices for vitrification of immature oocytes of indigenous zebu cows. Slaughter house derived immature cumulus-oocyte-complexes (COCs) of cows were vitrified using 15% dimethyl sulphoxide (DMSO) as cryoprotective agent (CPA) with 0.5 mol sucrose in TCM 199 supplemented with 20% FBS. Vitrification of COCs was completed after immediate plunging of COCs loaded cryotop or French mini straw into the liquid nitrogen (LN_2). Then the COCs containing cryotop or French mini straws were warmed in 0.25 mol sucrose and 20% FBS supplemented TCM 199 followed by *in vitro* culture in 50 µl droplets of bicarbonate buffered TCM 199 supplemented with 10% FBS, pyruvate, FSH and oestradiol for 24 hrs at 39°C with 5% CO2 in humidified air. After maturation culture, oocytes were denuded and examined under inverted microscope for presence of polar body as the indication of maturation. Denuded oocytes were also stained by whole mount technique using 1% orcein to examine the maturation by presence of MII chromosomes. The *in vitro* maturation rate was significantly ($p<0.05$) higher in oocytes vitrified and warmed using crytop (47.1±6.9%) than that of French mini straw (15.9±12.5%). Moreover, in vitro maturation rate was significantly ($p<0.05$) highe r in control oocytes (not vitrified) (84.5±14.2%) than that of vitrified oocytes. In conclusion, cryotop is better than French mini straw as cryo-device for vitrification of bovine immature oocytes

(Key Words : Cryo-device, immature oocytes, vitrification, zebu cows)

INTRODUCTION

Cattle are important livestock species in Bangladesh. It provides milk, meat, manure, draught power and leather to the vast majority of the people. It plays an important role in the agricultural production sphere in Bangladesh. Statistics show that about 2.9% of national GDP is contributed by the livestock sector in which majority portion comes from cattle (http://en.banglapedia.org/index.php?title=Livestock). Livestock population in Bangladesh is currently estimated to comprise 25.7 million cattle (http://en.banglapedia.org/index.php?title= Livestock). However, the milk and meat production in Bangladesh is not sufficient due to poor yielding of native cattle due to poor genetic merit. The Department of Livestock Services (DLS) of Bangladesh has been introduced artificial insemination (AI) since sixties using semen of exotic breed for genetic up gradation of our native cattle (Ahmed and Islam, 1993). By AI programme, although a good number of crossbreed cattle with improved genetic merit has been produced in Bangladesh, the rate of genetic gain is slower than stake holders expectation. Therefore, application of assisted reproductive technologies (ARTs) such as, multiple ovulation and embryo transfer (MOET), *in vitro* maturation (IVM), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT), *in vitro* culture (IVC) and transfer of embryos may contribute to speed up the production of a population of cattle with desired traits.

† Correspondence: Mohammad Musharraf Uddin Bhuiyan
Phone: +880-1715-020254
E-mail: mmubhuiyan@gmail.com

Like semen, in any ARTs, successful cryo preservation of oocytes and embryos is desired if it requires preserving oocytes or embryos long time in absence of recipient animals. Cryo preservation of oocytes is possible by either slow freezing or vitrification technique. Vitrification is the solidification of a solution at low temperature, not by icecrystallization but by extreme elevation in its viscosity using high cooling rates of 15,000 to 30,000℃ / min (Rama Raju *et al*., 2006). It has become a satisfactory option for cryo preservation of cells and organs in the field of medical science (Rall and Fahy, 1985) and also in the field of veterinary medical science (Hadi *et al.,* 2011).

For successful vitrification of oocytes, among others, cryo-device and cryo protectant may play an important role (Rama Raju *et al*., 2006). As cryo-device, cryotop has been shown to be superior to open pulled straw (OPS) for vitrification of immature pig (Liu *et al.,* 2008), human (Kuwayama *et al.,* 2005) and matured bovine ocytes elsewhere (Morato *et al.,* 2008). However, still there is no report on vitrification of immature bovine ocytes in Bangladesh. Therefore, it is essential to establish technique of vitrification of oocytes and embryos in Bangladesh resulting in wide application of ARTs in the field of reproduction. Considering the above mentioned facts, the aim of the present study was to compare the efficiency of two cryo-devices namely cryotop and French mini straw for vitrification of immature bovine oocytes.

MATERIALS AND METHODS

The study was carried out at the Community based Dairy Veterinary Foundation (CDVF) Laboratory, Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh during the period from January to May, 2016.

1. Chemicals and media

All the media constituents, reagents and chemicals were purchased from Sigma-Aldrich Inc., St Louis, USA. Media and reagents were prepared using standard protocol and under aseptic condition. All media were filtered using 0.22μm pore size filter (Durapure® membrane filter, Carrigtwohill, Ireland) and culture medium was routinely equilibrated in incubator (VS-9000C, Vision Scientific Co. Ltd. South Korea) at 39°C with 5% CO_2 in humidified air for at least 2hrs before use.

2. Collection of ovaries

The ovaries of indigenous zebu cows were collected from local slaughter house within 2 hrs after slaughtering and carried to the laboratory in a thermoflask containing warm normal saline (37°C, 0.9%, w/v, sodium chloride solution).

3. Collection of oocytes

In the laboratory, the ovaries were washed three times with warm (37°C) normal saline. The follicular fluid of 2 to 8 mm diameter follicles was aspirated using an 18 gauge needle (TERUMO®, Beijing, China) fitted with a 10 ml disposable plastic syringe (JMI Syringes and Medical Devices Ltd ®, Chauddagram, Comilla, Bangladesh).

4. Selection of occytes for culture

The aspirated follicular fluid was transferred in a 60 mm petridish (Greiner bio-one, Frickenhausen, Germany) and left for 5 minutes for sedimentation. The retrieved follicular aspirate was diluted with HEPES-buffered tissue culture medium (TCM) 199 supplemented with bovine serum albumin (BSA) (washing medium). Oocytes were selected under a stereo-microscope (LABOMED, USA). The cumulus-oocyte-complexes (COCs) with more than 3 compact cumulus cell layers and homogenous ooplasm (Fig.1a) were selected for maturation. The COCs were washed three times in washing medium.

5. Vitrification and warming

Recovered oocytes were washed twice in holding medium (HM, HEPES-buffered TCM 199 medium) supplemented with 20% fetal bovine serum (FBS), and kept there for about 15 min. The COCs were kept in the vitrification medium 1 (HM supplemented with 7.5% DMSO) for 35-40 sec and then transferred to the vitrification medium 2 (HM supplemented with 15% DMSO and 0.5M sucrose) for a further 25 sec. Then single COC was loaded on cryotops (Kitazato Supply Co, Fujinomiya, Japan) and 4-5 oocytes were loaded on French mini straw with minimum vitrification medium 2 followed by submerging into Liquid Nitrogen (LN_2) immediately for vitrification. After keeping the cryo devices (loaded with COCs) under LN_2 for 5 to 10 min, these were submerged in warming medium 1 (HM supplemented with 0.25 M sucrose) for 5 min followed by transferring COCs in warming medium 2 (HM supplemented

with 0.125 M sucrose) for 5 min before culturing for *in vitro* maturation. Finally, the COCs were washed twice in HM for 5 min each followed by washing once in maturation medium before culture in vitro.

6. In vitro culture of oocytes for maturation

Vitrified-warmed COCs or not vitrified control COCs were cultured in 50 µl droplets of bicarbonate buffered TCM 199 supplemented with 0.25 mM pyruvate, 10% FBS, 0.05 µg / ml FSH and 1.00 µg / ml oestradiol under mineral oil. For *in vitro* maturation, 5-10 COCs were cultured in each drop of medium for 24 hrs at 39°C with 5% CO_2 in humidified air.

7. Evaluation of oocytes for maturation

The culture drops were examined under the stereo microscope for cumulus expansion after 24 hrs of culture in the maturation medium. Presumptive maturation was indicated by the degree of cumulus expansion (Fig.1b). To examine the maturation of oocytes, the COCs were denuded by using 3% sodium citrate (w/v) in HEPES buffered TCM 199 medium and pipetting. After pipetting, the denuded oocytes were kept in 10µl droplets of HEPES buffered TCM 199 and examined for presence of polar body (Fig.1c) under inverted microscope (Leica DM IRE2, Germany) with the help of a mouth controlled pipette. Under microscope, observing extrusion of first polar body under zona pellucid was regarded as maturation of oocyte.

8. Staining of oocyte

Some oocytes with polar body extrusion after vitrification – warming and maturation culture were stained for confirmation of metaphase II chromosome (Fig. 1d). For examination of chromosomes, denuded oocytes were stained by whole mount technique using orcein. Briefly, the oocytes were mounted on glass slide under coverslip using paraffin-vaseline supports to prevent oocyte rupture and fixed in ethanol : acetic acid (3:1) for 24 hrs. The oocytes were stained with 1% orcein (w/v)

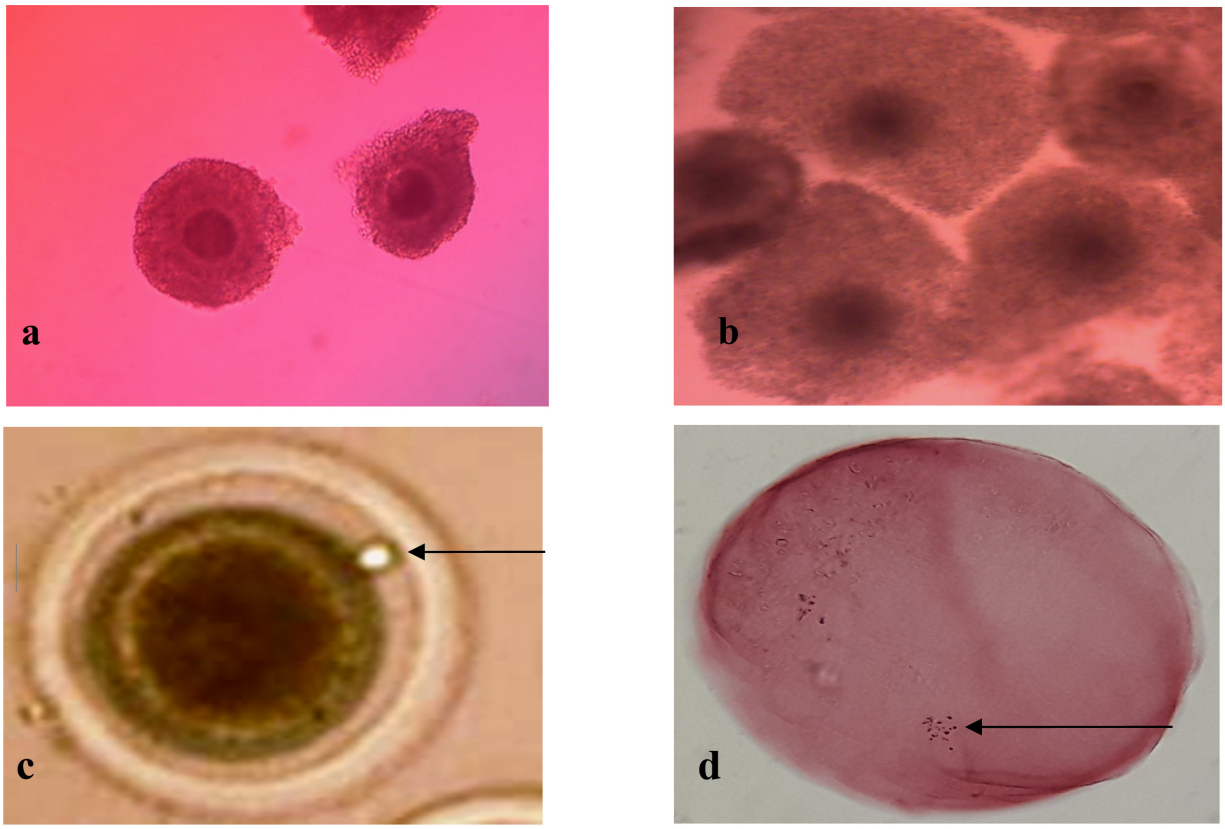

Fig 1. a) Immature cumulus oocytescomplex (COC) of a zebucow ; b) ExpandedCOCsafter maturation culture after vitrification and warming; c) Extrusion of 1st polar body in a matured oocyte cultured after vitrification and warming (arrow indicates polar body); d) Metaphase II chromosome in a matured oocyte cultured after vitrification and warming (black arrow indicates chromosomes)

in 45% acetic acid (v/v) for 20 min followed by cleaning of oocytes using aceto-glycerol (20% glycerol (v/v) and 20% acetic acid). The stained oocytes under cover slip were sealed with nail polish before examination under microscope. Presence of metaphase II chromosome in the oocytes was regarded as maturation of oocytes.

9. Experimental approaches and statistical analysis

To determine an effective cryo-device for vitrification of immature oocytes of zebu cows, COCs were vitrified using either crytop or French mini straw as cryo-device. The immature COCs without vitification were used as control. The experiment was repeated 5 times across the days. The data on rate of oocyte maturation were expressed as mean ± SD. The data were analyzed by using nonparametric one-way ANOVA followed by Bartlett's test for comparison using Graph pad PRISM, software version 5.0 a (Inc, USA). The variation in maturation rate was considered significant when the P value was less than 0.05.

RESULTS

A total of 291 oocytes were collected from 191 ovaries and the mean number of oocytes collection from each ovary was 1.52. The maturation rate was calculated on the basis of presence of extrusion of first polar body in the oocytes (Fig.1c). The maturation of oocytes was also confirmed by staining of chromosome using whole mount technique (Fig. 1d).

The *in vitro* maturation rate after vitrification and warming is shown in Table 1. The *in vitro* maturation rate of oocytes of zebu cows after vitrification and warming was 15.9±12.5% when French mini straw was used as cryo-device, 47.1±6.9% when cryotop was used as cryo- device and 84.5±14.2% when culture was done without vitrification (control). The difference in maturation rate was statistically significant between two cryo-devices and between vitrified and without vitrified oocytes ($P<0.05$).

DISCUSSION

The objectives of this study were to establish the technique for vitification of immature bovine oocytes in Bangladesh andto select an effective cryo-device for vitrification. We evaluated the outcome of vitrification of immature oocytes after *in vitro* maturation determined on the basis of presence of extrusion of first polar body in the oocytes. The maturation of oocytes was also confirmed by staining of chromosome using whole mount technique.

Vitrification procedures circumvent two of the major limiting factors for achieving optimal cryopreservation such as chilling injury (Vajta*et al*., 2006) and ice formation (Mazur *et al*., 1984). Chilling injury mainly affects the cytoskeleton (Pickering *et al*., 1990) and cell membranes (Ghetler *et al*., 2005) and can be minimized during vitrification by the use of high cooling rates (Liebermann *et al*., 2003). Cooling speed is dependent on the vitrification solution volume such that the smaller the sample volume, the higher the coolingrate. In addition, direct contact with liquid nitrogen also increases the cooling rate. To avoid ice formation, the vitrification technique uses high cryoprotectant (CPA) concentrations, which have been described as toxic to cells (Fuller *et al*., 2004). However, nature of CPA may mitigate the toxic and osmoticconsequences of highly concentrated CPAs in vitrification medium (Vajta *et al*., 2006). Thus, a mixtureof CPAs can decrease individual specific toxicity. The mostcommon mixture used is ethylene glycol, DMSO and sucrose in base medium (Vajta *et al*., 2006). To optimize results, besides an appropriate selection of CPAs, these agents should be used at as low a concentration as possible to achieve successful vitrification of oocytes or embryos. By dramatically increasing thecooling rate, the CPA

Table1. Comparison of maturation rate of immature oocytes after vitrification and warming between two cryo devices

Cryo devices	No. of oocytes vitrified	No. of oocytes Matured	Maturation%
Straw	73	10	15.9±12.5[c]
Cryotop	33	16	47.1±6.9[b]
No vitrification(Control)	43	36	84.5±14.2[a]

Number of replicates is 5. Proportion values are mean ± *SD*.
[a,b,c] Values with superscripts within same column differed significantly from each other ($p<0.05$).

concentration can be reduced, preserving the cells at non-toxic concentrations of cryoprotectant.

For any vitrification procedure, cryo-device or oocyte holding device plays an important role for its successful outcome. In the present study, it was tried to select an effective cryo-device for successful vitrification of bovine oocytes as the first attempt to establish vitrification technique in Bangladesh. For selection of better cryo-device, I compared the *in vitro* maturation of bovine oocytes after vitrification and warming using cryotop and French mini straw as cryo-device. In the present study, the *in vitro* maturation rate of oocytes of zebu cows after vitrification and warming was significantly (P<0.05) higher when cryotop (47.1%) was used as cryo-device than that of French mini straw (15.9%). Moreover, 84.5% oocytes became matured when cultured as control oocytes without vitrification. The present finding is in agreement with the earlier studies where lower rates of *in vitro* maturation of bovine oocytes after vitrification and warming were reported in Turkey by Cetin and Bastan (2006) and in Malaysia by Hajarian *et al.* (2011). Two major obstacles which may negatively affect success of vitrification procedure are ice crystal formation and chilling injury to the oocytes (Vajta and Kuwayama, 2006). Chilling injury mostly affects the cytoskeleton and cell membranes including oolema and membrane of organelles of oocytes resulting in lower maturation rate than non vitrified control group (Morato *et al*, 2008).

Nevertheless, higher rate of *in vitro* maturation (58%) of bovine oocytes was achieved by earlier studies after vitrification than that of present study when crytop was used as cryo-device (Hajarian *et al.,* 2011). The variation in *in vitro* maturation rate of cow oocytes after vitrification and warming between two studies can be explained by the fact that the previous study used combination of Ethylene glycol and DMSO as cryoprotectic agent compared to using DMSO in the present study (Hajarian *et al.,* 2011). This indicates that the protocol for vitrification of oocytes of cows is not yet optimized which needs to be improved. Higher survival rate (94.5%) after vitrification and maturation culture than present study was also reported in bovine oocytes in Spain where combination of ethylene glycol and DMSO was used as CPA (Morato *et al.,* 2008).

In the present study, *in vitro* maturation rate (84.5%) of cow oocytes without vitrification was higher than that of earlier study reported in Bangladesh (Singha *et al.,* 2015). Additionally, lower maturation rate was reported by Morshed *et al.* (2014) (53.8%) and Das *et al*., (2006) (65.4%) in indigenous zebu cows in Bangladesh. The reasons for variation in maturation rate among studies might be due to variation in basic media and percentage of serum supplementation in it used for oocyte maturation. Moreover, grades of oocytes may influence the *in vitro* maturation rates of oocytes as variation in rate of maturation *in vitro* was demonstrated between good and poor grade oocytes (Goswami, 2002). All retrieved oocytes were cultured for maturation irrespective of grading which may contribute for obtaining lower maturation rate by Morshed *et al.*, (2014) than that of present study. In the present study, oocytes with at least 3 compact cumulus cell layers were used for maturation which might contribute to obtaining satisfactory rate of oocyte maturation *in vitro.*

The cryo-devices are various types of carrier or container to hold oocytes containing vitrification medium. The carrier tools which have been developed to minimize the volume and to submerge the oocytes or embryos quickly into LN_2 include electron microscopic grids (Martino *et al*., 1996), open pulled straw (OPS) (Vajta *et al*., 1998), cryoloop (Lane *et al*., 1999), cryotop (Kuwayama and Kato, 2000) and nylon mesh (Matsumoto *et al*., 2001). Very small amount of vitrification medium (2μl) used to reduce the risk of cry injury occurred instantaneously contacting with LN_2 might partly contribute to the high survival rate of vitrified-thawed oocytes or embryos. Cryoinjury which leads to rupture of the zona pellucida or plasma membrane lysis, is generally caused by extreme temperature gradients between the outer layer and the core of the vitrified drop with the volume larger than 2 μl (Dinnyes *et al*., 2000).

In the present study, cryotop proved to be better cryo-device compared to French mini straw. Similarly, cryotop has been reported to be superior to other cryo-devices when *in vitro* maturation of bovine oocytes was compared among open pulled straw (OPS), cryotop and electromicroscopic grid (Hajarian *et al*., 2011). The cryotop device designed by (Kuwayama *et al*., 2005) allows samples to be loaded in a very small volume of vitrification solution. Once the oocytes are placed on the cryotop, almost the entire loading solution is removed by aspiration before direct immersion in liquid nitrogen. The greatest advantage of this method is that an extremely fast cooling rate is achieved (23,000℃ / min) and chilling injury is avoided (Kuwayama *et al*., 2005). The extremely small volume alsohelps achieve a faster warming rate (42,000℃ / min), there by avoiding ice crystal formation

during warming. Contrasting to the present finding, no maturation of vitrified oocytes of buffaloes has been occurred when cryotop was used as cryo-device in Bangladesh (Akter, 2015). However, no study has been conducted in bovine to compare the efficacy of cryodevices for vitrification of immature oocytes in Bangladesh. This means, the present investigation is the first attempt for vitrification of oocytes of bovine in Bangladesh with encouraging success of maturation *in vitro*. Further studies are required to optimize the protocol for vitrification of immature bovine oocytes with greater success rate *in vitro.*

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