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Vitrification of immature oocytes of goats in Bangladesh

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Abstract

Cryopreservation of oocytes and embryos by vitrification can have advantages in assisted reproductive technologies (ARTs) in mammals. The aim of this study was to establish an effective vitrification procedure and cryodevice for goat's oocytes in Bangladesh. Cumulus oocyte complexes (COCs) were collected from ovaries from slaughterhouse. COCs with more than 3 layers of cumulus cells were selected. COCs were vitrified by two-step procedure using 7.5% and 15% dimethyl sulphoxide (DMSO) as cryoprotective agent (CPA), loaded on Cryotop or French mini-straw, then directly plunged into liquid nitrogen (LN2). Then the COCs containing Cryotop or French mini-straws were warmed in 0.25 M sucrose and 20% FBS-supplemented tissue culture medium (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 h at 39°C with 5% CO_2 in humidified air. After maturation culture, oocytes were denuded and examined under inverted microscope for presence of polar body as the indication of maturation. The *in vitro* maturation rate of goat's oocytes after vitrification and warming was 39.3 ± 6.8%, 31.3 ± 9.4%, 61.6 ± 14.2% when using Cryotop (cryodevice), French mini-straws and without vitrification (control), respectively. Maturation rate was significantly higher ($P<0.05$) without vitrification. It is suggested that both Cryotop and French mini-straw are efficient cryodevices for vitrification of goat's oocytes and further investigation is required to optimize the protocol for vitrification and warming procedure for the satisfactory survival of goat's oocytes. (*Bangl. vet*. 2018. Vol. 35, No. 1&2, 7 – 12)

Introduction

The fertility of an animal can be increased by assisted reproductive technologies (ARTs) such as *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of embryos followed by transfer. For production of embryos using ARTs, cryopreservation of oocytes and embryos have great advantages.

Vitrification is a process of cryopreservation during which solidification of a solution occurs without the formation of ice crystals (Chian *et al*., 2004). Successful vitrification of mammalian oocytes may increase the success of ARTs. Vitrification has a practical importance in IVM, IVF, IVC, nuclear transfer and embryo preservation. After the technique was first used in embryology by Rall and Fahy (1985) on mouse embryos,

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considerable advances have been made and offspring have been produced using frozen-thawed oocytes in cows (Vieira *et al*., 2002), horses (Maclellan *et al*., 2002), goats (Purohit *et al*., 2012) and humans (Chian *et al*., 2009). However, no attempt on vitrification of oocytes or embryos has been made in Bangladesh.

A wide variety of approaches are used to improve methods of oocyte vitrification. The majority of studies examined the effects of various cryoprotective compounds, and compared different cooling and warming conditions (McGrath, 1997; Horvath *et al*., 2008). However, cryodevice used for vitrification is important for successful outcome of cryopreservation. In oocyte vitrification, open pulled straws (OPS; Vajta and Nagy, 2006), solid surface vitrification (SSV; Dinnyes *et al*., 2000) and Cryotops (Chian *et al*., 2004) have been reported as successful cryodevices. However, no precise report on cryoprotectant and cryodevice is available in Bangladesh. Therefore, the present study was conducted to establish an effective vitrification procedure of goat's oocytes in Bangladesh and to determine an effective cryodevice.

Materials and Methods

Chemicals and reagents

Chemicals, reagents and media constituents were purchased from Sigma-Aldrich Chemicals, St Louis, USA and Sigma-Aldrich Chemic Gmbh, Steinheim, Germany. Media were prepared under standard protocol following aseptic technique. The media used for IVM and vitrification of oocytes were filtered using a disposable filter (0.22 µm pore size membrane filter, Durapure membrane filter, Ireland). The IVM media were routinely equilibrated at 37°C with 5% CO_2 in humidified air for at least 1 h before use.

Oocyte recovery and culture

Ovaries of goats were collected from the local slaughterhouse and transported to the laboratory in a vacuum flask containing warm saline (0.9%, w/v, sodium chloride, at 35-37°C) within 2 to 3 h of slaughtering. In the laboratory, ovaries were rinsed 3 times in saline at 37°C before aspiration of follicles. Follicles of 2-8 mm diameter were aspirated using an 18-gauge needle attached to a 10-mL disposable plastic syringe (Sterio pack Disposable Syringe, Opso Saline Ltd., Dhaka, Bangladesh). The follicular fluid was transferred in a 60 mm petri dish (Bio Basic Inc., Markham, Canada) and left for 5 min for sedimentation. The retrieved follicular aspirate was diluted with HEPES-buffered TCM 199 supplemented with bovine serum albumin (BSA) (washing medium). Oocytes were selected under a stereomicroscope (Labomed Inc, USA). The cumulus-oocyte-complexes (COCs) with more than three compact cumulus cell layers and homogenous ooplasm were selected for maturation. The COCs were washed three times in washing medium.

Vitrification of oocytes

The COCs were washed twice in holding medium (HM) (Hepes-buffered TCM supplemented with 20% fetal bovine serum (FBS; Gibco®, Invitrogen, NY, USA) 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. Vitrification was done using two-step procedure using either 0.25 ml French mini-straw or Cryotop (Kitazato Supply Co, Fujinomiya, Japan) as cryodevice. By using mouth-controlled pipette, 2 oocytes were loaded on the film strip of a Cryotop from vitrification medium 2 under a stereomicroscope. Before vitrification, almost all solution was removed to leave only a thin layer covering the oocytes on Cryotop. The Cryotop was immediately submerged into liquid nitrogen vertically. During loading COCs into the straws, warming solution – 1 (WS - 1; HM supplemented with 0.25 M sucrose) was taken into the straw followed by filling with air. Then, 3-5 COCs was loaded into the straw with least volume of vitrification medium 2. Then air was taken again followed by filling with warming solution -1. Finally, small amount of air was taken into the straw and submerged into the LN_2 for vitrification.

Warming and in vitro maturation (IVM) of oocytes

After removal of the COCs containing cryodevice (Cryotop/French mini-straw) from LN_2, COCs were inserted directly into warming solution - 1 for 5 to 10 min. Then the oocytes were transferred into warming solution - 2 (WS - 2; HM supplemented with 0.125 M sucrose) for 5 min. Finally, the COCs were washed twice in HM followed by washing once in maturation medium before culture *in vitro*. The basic medium for oocyte maturation was bicarbonate-buffered TCM 199 supplemented with 0.25 mM pyruvate, 10% FBS, 0.05 µg/ml FSH and 1.00 µg/mL oestradiol. Fifty µL droplets of maturation medium were prepared in a 35 mm petri dish (Greiner Bio_One, Frickenhausen, Germany) and covered with mineral oil. For *in vitro* maturation, 5 to 10 COCs were cultured in each drop of medium for 24 hrs at 39°C with 5% CO_2 in humidified air.

Evaluation of maturation of oocytes

After 24 h of maturation culture, oocytes were denuded using 3% (w/v) sodium citrate in Hepes-buffered TCM 199 under stereomicroscope. The cumulus-free oocytes were examined for polar body extrusion under inverted microscope (Leica DM IRB) with the help of mouth-controlled pipette. The oocytes were regarded as matured when extrusion of 1st polar body was observed under zona pellucida.

Experimental approaches and statistical analysis

To determine an effective cryodevice for vitrification of immature oocytes of goats, COCs were vitrified using either Cryotop or French mini straw. The immature COCs without vitrification were used as control. The data on rate of oocyte maturation were expressed as mean ± SD. The data were analysed by using non-parametric one-way

ANOVA followed by Bartlett's test for comparison using Graphpad PRISM software version 5.0. The variation was considered significant when the P value was less than 0.05.

Results and Discussion

This study was done to establish an effective vitrification procedure of goat's oocytes and to determine an effective cryodevice. The efficacy of vitrification was determined by evaluation of post-vitrification maturation on the basis of presence of extrusion of first polar body in the oocytes.

Table 1 shows that the IVM rate of oocytes of goats after vitrification and warming was 39.3 ± 6.8 when Cryotop was used as cryodevice and 31.3 ± 9.4% with French mini-straw. The difference was not significant. The IVM rate was 61.6 ± 14.2% when culture was done without vitrification (control). The difference between vitrified and control groups was significant (P<0.5).

Table 1: Comparis on of maturation rate of immature oocytes between two cryodevices after vitrification and warming

Cryodevice	No. of oocytes vitrified	No. of oocytes matured	Maturation rate (%)
Cryotop	40 (n = 5)	15	39.3 ± 6.8[b]
Straw	51 (n = 4)	16	31.3 ± 9.4[b]
No vitrification (control)	92 (n = 9)	57	61.6 ± 14.2[a]

Percentage values are mean ± SD. n = number of replicates.
[a,b] Values with superscripts within same column differed significantly from each other P<0.05).

The IVM rate of oocytes of goats after vitrification and warming was lower than without vitrification (control) (31.3 to 39.3 % vs. 61.6%, respectively). Similarly, lower rate of IVM of goat oocytes after vitrification and warming was reported in India than non-vitrified control (Purohit *et al.,* 2012). Moreover, lower rates of IVM 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 that may reduce success of vitrification are ice crystal formation and chilling injury to the oocytes (Vajta and Kuwayama, 2006). Chilling injury mostly affects the cytoskeleton and cell membranes of oocytes, resulting in lower maturation rate than non-vitrified control (Morato *et al.*, 2008). This indicates that the protocol for vitrification of oocytes of goats and cows needs to be improved.

In vitro maturation rate of goat's oocytes without vitrification is similar to that reported in Bangladesh (Khatun *et al.,* 2011) and India (Purohit *et al.,* 2012). Contrasting to the present finding, higher rates of *in vitro* maturation (71.4 to 81.4%) were reported in goat oocytes when cultured without vitrification elsewhere (Izquierdo *et al.,* 2002; Anguita *et al.,* 2007). The variation in maturation rates in

oocytes of goats may be due to variations in culture period. Goat's oocytes were cultured for 24 h compared to 27-28 h by other investigators. Higher rates of IVM of goat oocytes have been reported when oocytes were cultured for 27 h than for 24 h (Rho *et al*., 2001). However, Agrawal *et al.* (1995) and Samake *et al*. (1999) suggested that goat's oocytes can be successfully matured *in vitro* by culturing for 24 to 27 h.

Contrasting to the present finding, Cryotop has been reported superior to other cryodevices when IVM of bovine oocytes was done using open pulled straw (OPS), Cryotop and electro microscopic grid (Hajarian *et al.,* 2011). No precise reports in goats are available to compare the efficacy of cryodevices for vitrification of immature oocytes in Bangladesh. Moreover no vitrified oocytes in buffaloes matured when Cryotop was used as cryodevice in Bangladesh (Akter, 2015). This means, the present investigation for vitrification of oocytes of goats in Bangladesh was encouraging. Further studies are required to optimize the protocol for vitrification of immature goats' oocytes. In conclusion, both Cryotop and French mini-straw are efficient as cryodevices for vitrification of goat oocytes. Further investigation is required to optimize the protocol for vitrification and warming of goat oocytes.

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