Cryobiology 75 (2017) 106–116

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Effect of different cryo-devices on *in vitro* maturation and development of vitrified-warmed immature buffalo oocytes

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ARTICLE INFO

Article history:
Received 25 October 2016
Received in revised form
12 December 2016
Accepted 12 January 2017
Available online 16 January 2017

Keywords:
Buffalo
Cryo-devices
Immature oocytes
Gene expression
Oocyte secreted factors
Vitrification

ABSTRACT

The aim of the study was to identify a cryo-device that would be best suited for the vitrification of buffalo immature cumulus-oocyte complexes (COCs) as judged by viability and meiotic competence of the vitrified-warmed oocytes and their development ability following *in vitro* fertilization (IVF). The expression of oocyte secreting factors and their receptors (*GDF9, BMP15, BMPR2, TGFBR1*) and apoptosis related genes (*BCL2, BAX, P53, C-MYC*) were compared in vitrified-warmed oocytes after *in vitro* maturation. COCs from the ovaries of slaughtered buffaloes were vitrified in a combination of dimethyl sulfoxide, ethylene glycol, and sucrose using either a conventional straw (CS), open pulled straw (OPS), cryoloop (CL), hemistraw (HS) or cryotop (CT). The fresh COCs were exposed to vitrification and warming solutions as in other vitrification methods without plunging in to liquid nitrogen (EC). The viability of vitrified-warmed COCs, 2 h post warming in HS and CT was similar to fresh and EC groups but significantly higher than CS and OPS methods. The proportions of oocytes with first polar body after 24 h *in vitro* maturation were significantly higher in HS and CT methods than in CS, OPS and CL methods. The development ability of these vitrified-warmed oocytes to blastocyst stage following IVF in all vitrified groups was significantly lower than control and EC groups. Among the vitrified groups, the blastocyst rate in HS, CT and CL groups was significantly higher than in OPS and CS groups. It was also observed that the expression levels of *GDF9, BMP15, BMPR2, TGFBR1, BCL2, BAX, P53 and C-MYC* genes in vitrified-warmed COCs in CT, HS and CL groups were similar to control. The results indicated that HS, CT and CL are more suitable cryo-devices for vitrification of buffalo immature oocytes.

1. Introduction

India has approximately 105 million buffaloes comprising 56.7% of the total world buffalo population [12]. Buffalo positively influences the agricultural economy of the nation by providing milk, meat and draught power [84]. The abundance of the buffalo is attributed to its ability to survive in harsh climatic conditions and its ability to resist several tropical diseases [84]. Despite these attributes the reproductive performance of this species is low and affected due to delayed onset of puberty, silent estrus, seasonal anoestrus, poor conception rates and prolonged inter calving intervals [29]. Thus attempts have been made to improve their reproductive efficiency using multiple ovulation and embryo transfer (MOET) technology [19,84]. MOET posed problems and the success rate was low [59] primarily due to the low yield of transferable embryos (less than one) [51]. However, this problem of low yield of embryos could be overcome by collecting oocytes by ovum pick up method, maturing them *in vitro* (IVM) followed by fertilization (IVF) [26,52]. As an extension of this approach it should be possible to scale up the production of buffalo embryos from the oocytes derived from slaughter house ovaries. Further, cryopreservation of such embryos may provide an opportunity to improve the genetic potential of the species [23]. However, in the case of valuable/endangered animals the approach would be to retrieve immature oocytes from the animals at postmortem and use them for the generation of embryos immediately or cryopreserve them for future use [44]. Methods for cryopreservation of semen and embryos have been better studied and standardized compared to cryopreservation of oocytes. The efficiency of cryopreservation of oocytes is low and varies from species to species due to different physiological characteristics and biochemical properties of the

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http://dx.doi.org/10.1016/j.cryobiol.2017.01.004
0011-2240/

oocyte [13]. Slow freezing is successful with embryos of various species but the method was not as successful with oocytes due to formation of intra-cellular ice crystals [3,65,70]. Vitrification is an alternative method to slow freezing in which the oocytes with vitrification solutions were exposed directly to liquid nitrogen (LN_2) to avoid ice crystal formation as in slow freezing [32,78]. Different methods have been developed to achieve rapid cooling by minimizing the volume (0.1–1.0 μl) of vitrification solution containing oocytes and embryos by using glass capillaries [68], electron microscope grids [53], open pulled straws (OPS) [78], cryoloops (CL) [43], hemi straws (HS) [80], cryotops (CT) [41], solid surface vitrification (SSV) [45] and minidrop [87].

Several attempts have been made to cryopreserve buffalo oocytes by vitrificatioin but based on survival, maturation and fertilization rates and embryo development of vitrified buffalo oocytes, a standard method for vitrification is yet to be established. This may be due to presence of high lipid content in the oocyte, presence of high cholesterol and unsaturated fatty acids in the plasma membrane and cytoskeletal damage during freezing [62]. Several vitrification protocols using different cryoprotective agents (CPAs) alone or in combination, different regimes of vitrification and different cryo-devices have been tested for vitrification of buffalo immature oocytes [1,17,18,24,37,45,49,82,83] and *in vitro* matured oocytes [6,8,27,28,46,50]. Studies indicated that vitrification of buffalo matured oocytes was better than immature ones [27] but embryo development competence following IVF was lower than fresh oocytes [71]. Over the years buffalo blastocysts from vitrified oocytes were generated using different protocols involving ethylene glycol (EG), dimethyl sulfoxide (Me2SO), propylene glycol (PROH), glycerol alone [82], mixture of EG and trehalose [1], and EG plus Me2SO [24,37]. Vitrification of buffalo immature oocytes was attempted using traditional French mini straw (0.25 ml) [1,24,83], CT and SSV [24,45] and HS and spatula [37]. In these studies the blastocyst development rate of vitrified buffalo oocytes following IVF was quite variable and was most likely related to type of cryoprotectant and cryo-devices used for vitrification [62]. Exposure of oocytes to high concentration of CPAs and rapid cooling and warming rates and osmotic stress during vitrification may contribute to altered expression of genes that are important for oocyte survivability, maturation and development [75]. With this in view the temporal expression of number of genes involved in the above processes was studied. For instance, the expression of growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) which are essential for oocyte development and maturation, cumulus cell proliferation and differentiation and control of ovulation [55,56] was studied. In addition, these genes prevent the apoptosis of cumulus cells by maintaining a localized gradient of anti-apoptotic factors within COC [35]. *GDF9* and *BMP15* generate signals through their specific receptors activin receptor-like kinase 5 and 6 (*ALK5* or *TGFBR1*, *ALK6*) respectively, and a common receptor, bone morphogenetic protein receptor II (*BMPR2*). Both receptors undergo expression in the oocytes, granulosa and theca cells [36,85]. Vitrification of oocytes influences DNA damage in cumulus oocyte complexes (COCs) [58] and thus affects oocyte developmental competence [75]. Since apoptosis is one of the factors that contribute to cellular damage and death in vitrified oocytes [73], the expression of *BCL2* and *BAX* genes which modulate apoptosis was also monitored. *BCL2* promotes germ cell survival in females by first modulating the mitochondrial release of cytochrome C causing the interaction of *APAF1* with *CASPASE9* and secondly through binding to *BAX* and finally blocking apoptosis induced by *C-MYC* [34,39]. *BAX* is a *BCL2* antagonist whose cytoplasmic elevation is sufficient to cause apoptosis in oocytes [60]. The stress response gene *P53* is induced by DNA damage leading to the activation of downstream effectors genes (including *BAX*). The expression of OSF and apoptosis related genes have been studied in vitrified oocytes of sheep [21,22], goat [67], bovine [4] and dog [76]. It is in this context, the present study was aimed to compare the effect of different cryodevices such as conventional straw, open pulled straw, cryoloop, hemi straw and cryotop on *in vitro* maturation and fertilization of vitrified immature buffalo oocytes. Relative expression levels of developmental and apoptotic related genes in the *in vitro* matured oocytes were also studied.

2. Materials and methods

The procedures/methods used for preparation of cryo-devices, processing of ovaries, collection of oocytes, vitrification, warming, *in vitro* maturation and fertilization and culture of fertilized oocytes in this study were followed as described earlier in our laboratory [48,66,67].

2.1. Chemicals and media

The chemicals, hormones, media, and serum used in the study were procured from Sigma Chemical Co. USA and plastic ware were from Thermo scientific Waltham, MA USA, unless otherwise stated. All media used for processing of ovaries, manipulation of oocytes and vitrification, warming, *in vitro* maturation and fertilization of oocytes and culture of fertilized oocytes were supplemented with penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). HEPES buffered tissue culture medium 199 (TCM199H) supplemented with 10% fetal bovine serum (FBS) and 25 IU/ml heparin was used for collection of oocytes (Collection medium) and collection medium without heparin (Handling medium) was used for handling and washing of oocytes. The solutions for vitrification and warming of oocytes were prepared as shown in Table 1. TCM199H supplemented with 20% FBS was used as holding medium (HM). *In vitro* maturation (IVM) medium was prepared by supplementing bicarbonate buffered tissue culture medium 199 with 10% FBS, 0.22 mM sodium pyruvate, 10 μg/ml of follicle stimulating hormone, 6 IU/ml luteinizing hormone, 1.0 μg/ml 17-β estradiol and 5 μg/ml Insulin, transferrin and Selenium. Collection, handling, maturation, fertilization and embryo culture medium were filter (0.22 μm) sterilized and equilibrated for at least 2 h before use. All the oocyte manipulations for vitrification and warming was carried out at 39 °C on a heating stage in a room maintained at 25 °C. Equilibration of media, *in vitro* maturation and fertilization of oocytes and culture of fertilized oocytes were carried out in an incubator containing 5% carbon dioxide, in humidified atmosphere at 38.5 °C.

2.2. Preparation of cryo-devices

Open pulled straws were prepared by pulling French mini straws (IMV Technologies, L'Aigle, France) over a flame to reduce diameter to half its original diameter. Hemi straw was prepared by cutting the open end of French mini straw at an angle with a surgical blade. A fine and thin strip (15 mm long, 1.5 mm wide and 0.1 mm thick) of polypropylene film was glued to a pointed end of plastic tooth pick for preparation of cryotop. The cutting end of the HS and the plastic strip of the CT were covered with a French medium straw to protect the COCs during storage of devices in liquid nitrogen (LN_2). Cryoloops were prepared by twisting a loop of steel wire (0.3 mm thickness) around an 18 G needle. The free end of each loop was fixed to the cap of a 1.5 ml cryovial such that the loop hangs clearly above the bottom of the vial.

2.3. Collection of ovaries and oocytes

Ovaries were collected from a local slaughter house and