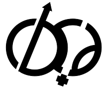

DOI: 10.21451/1984-3143-AR843

Anim. Reprod., v.14, n.2, p.392-399, Apr./Jun. 2017

A new simple and reliable vitrification device based on Hollow Fiber Vitrification (HFV) method evaluated using IVP bovine embryos

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Abstract

A new vitrification device based on hollow fiber vitrification (HFV) was constructed using a glass capillary, which lead to simplified construction process and increased practicality of the device. The hollow fiber was attached to heat-pulled tip of the glass capillary using forceps. A protective sheath fitted on the capillary provided protection for the cellulose triacetate hollow fiber with loaded embryos and allowed safe storage in liquid nitrogen for long periods of time (2-12 month), transfer between tanks with liquid nitrogen and transportation within these tanks. No embryos were lost in the process. The device was tested using seven-day-old and eight-day-old IVP bovine blastocysts and expanded blastocysts as a model. Obtained survival (90% at 24 h post warming) and hatching rates (62% at 72 h post warming) of day 7 blastocysts and expanded blastocysts were comparable to those gained using various vitrification carriers. Vitrified embryos did not show an increase in the number of cells with damaged membrane or a decrease in total cell number per embryos in comparison to their non-vitrified counterparts. Day 7 and 8 expanded blastocysts did not differ significantly in terms of survival at 24 (97.01 *vs*. 97.50%) and 48 h post warming (95.52 *vs*. 95%), but showed significantly higher survival and hatching rates than day 7 and 8 blastocysts. These results indicated that high and repeatable survival rates can be obtained by selection of IVP bovine embryos at the developmental stage of expanded blastocyst for HFV. Further modification of the method may be required to achieve high and stable results with different developmental stages of IVP bovine embryo. The vitrification device presented in the current article may contribute to wider application of HFV method in livestock production.

Keywords: embryo cryopreservation, hollow fiber vitrification, glass capillary device, IVP bovine embryo.

Introduction

Vitrification is a perspective method of cryopreservation of mammalian embryos and gametes. This method tends to be more effective than slow freezing for cryopreservation of *in vitro* produced (IVP) mammalian embryos, which are known to be more cryosensitive than embryos obtained *in vivo* (Nedambale *et al.*, 2004; Saragusty and Arav, 2011). Introduction of the minimal volume cooling (MVC) principle further improved the survival rates after vitrification and led to the emergence of various open-type or surface method carriers based on the principle (Saragusty and Arav, 2011). Yet, vitrification is not routinely used in the livestock production. Despite simplicity of the method, vitrification does require significant experience level of the operator to achieve proper results (Do *et al.,* 2016). Most open-type carriers are not suited for successful cryopreservation of multiple mammalian oocytes or embryos (Kuwayama *et al.*, 2005; Kim *et al.*, 2012) and those that are still require handling of each oocyte or embryo as single object in the vitrification/warming solutions (Park *et al.*, 1999; Kim *et al.*, 2012).

A novel method for simultaneous vitrification of a group of mammalian embryos in cellulose triacetate hollow fibers (hollow fiber vitrification, HFV) was introduced by Matsunari *et al.* (2012). The method was highly effective for cryopreservation of such cryosensitive objects as *in vivo* and *in vitro* produced porcine morulae. Subsequent embryo transfer of these morulae led to the birth of viable offspring (Matsunari *et al.*, 2012; Maehara *et al.*, 2012).

HFV method was initially considered for embryo cryopreservation in polytocous species, as a large number of embryos needed for a single embryo transfer procedure can be vitrified and warmed simultaneously. However, HFV may be also advantageous for preservation of a single embryo of monotocous species such as cow. The handling of an embryo within the hollow fiber in vitrification/warming solutions is significantly simpler and more standardized in comparison to treatment procedures in case of many open-type carriers. HFV method may be also beneficial for groups of IVP bovine embryos as, for instance, several recipients may be prepared simultaneously for practical reasons. HFV may also be used for cryopreservation of bovine oocytes and zona-free embryos produced during handmade cloning, both of which still require a safe and reliable cryopreservation method (Zhou and Li, 2013; Taylor-Robinson *et al.* 2014).

In livestock production embryos are transferred mainly at the age of 7 days after IVF (Pontes *et al.,* 2010) with the highest pregnancy rates obtained after transfer of day 7 expanded blastocysts (Hasler *et al.,* 1997). Thus, ability of day 7 IVP bovine embryos at different developmental stages to produce steady survival rates after vitrification in hollow fibers holds special interest in regard to the practical application. However, information about effectiveness of bovine embryo vitrification in hollow fibers is limited to

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Received: June 16, 2016
Accepted: February 3, 2017

several abstracts (Beck *et al.*, 2013; Saucedo *et al.*, 2015a,b), and an article concerning cryopreservation of cleavage or morula stage embryos (Uchikura *et al.*, 2016).

Unfortunately, cellulose triacetate hollow fibers have a tendency to become very fragile at low temperatures. To protect hollow fibers from mechanical damage during storage in liquid nitrogen, Matsunari *et al.* (2012) developed a modified vitrification device. However, authors pointed out that a possible drawback of such device is its' complex structure and complicated construction process. In the following works involving HFV method hollow fibers with vitrified embryos were either warmed immediately (Beck *et al.*, 2013; Saucedo *et al.*, 2015a, b; Uchikura *et al.*, 2016) or placed individually into the cryotubes for storage (Maechara *et al.*, 2012). Simple and safe long-term storage of the samples remained a challenge.

To solve these applicational problems and to make HFV method more practical, the main goal of this article was to introduce a new simple and reliable device based on HFV that allows long term storage of vitrified embryos in liquid nitrogen and can be easily constructed both manually and industrially. The efficiency of the vitrification device was evaluated using day 7 and 8 IVP bovine embryos as a model with special attention to the developmental stage of an embryo.

Materials and Methods

Construction of the vitrification device

Present vitrification device was composed of a 2 cm long piece of a cellulose triacetate hollow fiber (inner diameter 200 µm, outer diameter 230 µm, pore size 7 nm; Nipro, Japan), a 7 cm long glass capillary (outer diameter 1.2 mm; IBI RAS, Russia) with a heat pulled tip and a protective sheath. Outer diameter of the tip varied between 150 and 180 µm. The piece of hollow fiber was pulled over to the tip of glass capillary for 4-5 mm using forceps (Fig. 1A).

The protective sheath was constructed as follows. A 3.5 cm long piece of a 0.25 ml cryostraw (Minitube, Germany) was partially inserted (for 2.5 cm) into a 5.5 cm long piece of a 0.5 ml cryostraw. A 0.05 cm thick, 0.2 cm wide and 2.5 cm long polyethylene strip was inserted between contiguous walls of the cryostraws to secure the construction. The piece of 0.25 ml cryostraw was flattened near the open end (Fig. 1B) with a heat sealer. The protective sheath was placed directly over the glass capillary and could be easily moved into “closed” (Fig. 1C) or “open” (Fig. 1D) positions under liquid nitrogen. Additional containers for transfer and identification purposes were constructed from 13 cm long pieces of a plastic drinking straws (diameter 0.5 cm) of different colors heat sealed at one end. On the other end a segment of 3-4 cm in length was cut diagonally.

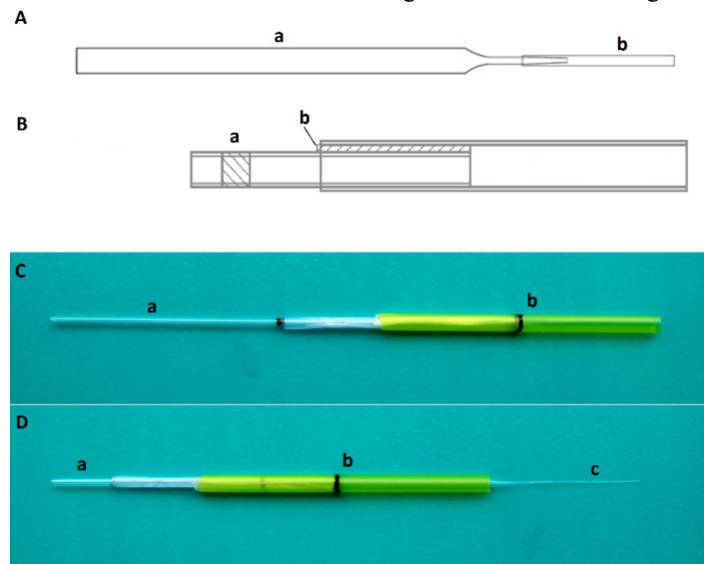

Figure 1. The vitrification device based on cellulose triacetate hollow fiber and glass capillary. A) General scheme of the vitrification device: a) glass capillary with heat-pulled conical tip, b) cellulose triacetate hollow fiber; B) General scheme of the protective sheath made from a piece of 0.25 ml cryostraw and a piece of 0.50 ml cryostraw: a) heat-flattened region of 0.25 ml cryostraw, b) polyethylene strip 0.05 cm thick; C) Assembled vitrification device in “closed” position: a) glass capillary, b) protective sheath; D) Assembled vitrification device in “open” position: a) glass capillary, b) protectve sheath, c) cellulose triacetate hollow fiber.

Oocyte collection and in vitro *maturation*

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (USA) and were of embryo tested or cell culture tested quality. All used media were supplemented with 40 µg/ml gentamycin. Procedures involving oocytes and embryos outside the incubator were carried out at room temperature (22-24ºC).

Bovine ovaries were obtained at a local abattoir and transported to the laboratory in physiological saline solution at 26-30ºC within 3-5 h. Cumulus-oocyte

complexes (COCs) were obtained from individual antral follicles (2-8 mm in diameter) using a dissection device with a single blade (Malenko, 1999). Oocytes with evenly granulated ooplasm and compact or slightly expanded multilayered cumulus were selected for *in vitro* maturation. Maturation was carried out in TCM-199 (PanEco, Russia) supplemented with 0.66 mM of sodium pyruvate, 1 mM L-glutamine, 0.6 mM L-cysteine, 5 IU/ml chorionic gonadotropin (Moscow endocrine plant FPUE, Russia), 5 µg/ml follicle stimulating hormone (FSH-super, Agrobiomed LLC, Russia), 1 µg/ml of estradiol-17β and 10% of fetal calf serum (FCS, HyClone, USA). 30-40 COCs were placed into a well of 4-well plate (Nunc, Denmark) containing 0.5 ml of maturation medium and incubated for 22-23 h in the CO_2-incubator at 38.5ºC and 7% CO_2 in the atmosphere.

In vitro *fertilization*

Frozen-thawed bull sperm was used for *in vitro* fertilization. Spermatozoa were selected by swim-up in TALP-HEPES medium (Bavister and Yanagimachi, 1977; TH). TALP-Fert medium (Parrish *et al*., 1988; TF) supplemented with 2 µg/ml heparin (Moscow endocrine plant FPUE, Russia) was used for *in vitro* fertilization. COCs were washed sequentially in TH for 5 min and in TF and transferred into a wells of 4-well plate (30-40 COCs per 0.4 ml of TF covered with mineral oil). Then 4 µl of PHE solution (20 µm D-penicillamine, 10 µm hypotaurine, 1 µm epinephrine) and bull sperm (0.5 x 10^6 spermatozoa/ml) were added into each well. Oocytes and spermatozoa were incubated for 18-20 h in the CO_2-incubator at 38.5ºC and 7% CO_2 in the atmosphere. The day of IVF was considered day 0.

In vitro *culture*

Modified SOF medium (Tervit *et al*., 1972) without BSA or glucose and supplemented with MEM essential and non-essential amino acids and 5% FCS was used for embryo culture. Presumptive zygotes were denuded of cumulus cells and spermatozoa by vortexing in hyaluronidase solution (1.1 mg/ml) for 3 min. Then they were washed in modified SOF and placed into wells of 4-well plate, containing 0.4 ml of modified SOF covered with mineral oil. *In vitro* culture was carried out in the atmosphere containing 5.5% CO_2, 6% O_2, and 88.5% N_2 at 38.5ºC. Developmental stage of the embryos was evaluated on day 7.

To test the device, morphologically normal day 7 embryos that reached a developmental stage of at least a blastocyst were selected for vitrification and non-vitrification groups. Experiments were repeated at least eight times.

To evaluate the effects of embryo development stage on the outcome of vitrification in hollow fibers morphologically normal day 7 or 8 embryos were selected as follows. On day 7 embryos that reached a developmental stage of at least a blastocyst were removed from the culture. Blastocysts with thinned zona pellucida (about 1/3 of original thickness), significantly larger diameter and compact inner cell mass were considered expanded and selected into one group. The remaining blastocysts were selected into other experimental group. Both groups were vitrified.

The remaining embryos were cultured for another 24 h. Blastocysts and expanded blastocysts that appeared on day 8 were classified as described above and selected into two experimental groups according to their developmental stage. Both groups were vitrified.

Vitrification and warming

Vitrification and warming were conducted according to Kuwayama *et al.* (2005) and Matsunari *et al.* (2012) with minor modifications. In brief, TH medium (without BSA) supplemented with 20% FCS (TH20) was used as a base solution. All solutions were at room temperature (22-24ºC). A group of 5-10 embryos was washed twice in TH20 and transferred into equilibration solution containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO). An aspiration tube was connected to the glass capillary of the vitrification device. The embryos in equilibration solution were aspirated into a hollow fiber. The solution within the hollow fiber containing embryos was separated from the rest of the medium by two air plugs (Fig. 2A). The device was disconnected from the aspiration tube and the capillary was used as a handle for transfer of the hollow fiber between solutions. After incubation in the equilibration solution for 5 min, the hollow fiber with loaded embryos was transferred into vitrification solution containing 15% EG, 15% DMSO and 0.5 M sucrose for 60 sec (Fig. 2B) and then was immersed into liquid nitrogen. The protective sheath was “closed” and the entire device was immersed into liquid nitrogen. The devices were either kept under liquid nitrogen for 1 min before warming or placed into an additional container and inserted into dewar for long-time storage.

Before warming the sheath was “opened”. Then the device was removed from liquid nitrogen and the hollow fiber was immediately immersed into warming solution containing 1M sucrose for 1 min. The tip of the glass capillary was broken off and used as a makeshift “handle” during transfer of the hollow fiber between solutions. The hollow fiber was transferred using forceps into dilution solution with 0.5 M sucrose and incubated for 5 min. Then it was washed twice in TH20 for 5 min, where the embryos were unloaded from the hollow fiber. The embryos were washed in modified SOF medium and cultured for 48 h (day 8 embryos) or 72 h (day 7 embryos). Survival and hatching rates of the embryos were evaluated at 24, 48 and 72 h post warming. Survival rate at 24 h post warming was determined as a percent of reexpanded embryos with normal morphology from the total number of vitrified embryos. Survival rates at 48 and 72 h post warming were determined as a percent of nondegraded embryos with normal morphology from the total number of vitrified embryos. Hatching rates were calculated as a percentage from total number of

vitrified/warmed or non-vitrified embryos.

Cell count

Average number of cells with damaged membrane per blastocyst was counted as follows. After 24 h of culture day 7 vitrified/warmed and non-vitrifed embryos were transferred into 0.5 ml of TH containing 10 µg/ml of propidium iodide and incubated for 5 min at room temperature in the darkness. Then embryos were transferred into a drop of fresh medium and examined under the fluorescent microscope Nikon Eclipse Ti-U (Nikon, Japan) with corresponding filter (EX 540/25, DM 565, BA 605/55). Number of nuclei with fluorescent signal was counted for each embryo.

Total number of cells was counted as described by Ushijima *et al.* (2009) with minor modifications. Embryos were placed into hypotonic solution for 5-10 min. Then embryos were fixed in the first fixative (ethanol, acetic acid and distilled water, 3 : 2 : 1) for 1 minute, and transferred onto a glass slide in the minimum volume drop. A small drop of the second fixative (ethanol and acetic acid, 3 : 1) was added to each specimen. Samples were left to dry for 1 h at room temperature and then were stained with 5% Giemsa for 1 h at room temperature. Total number of nuclei in the blastocysts was counted under a microscope at 200X or 400X magnification.

Statistical analysis

Survival and hatching rates are presented as corresponding percentages. Results for total cell count in the blastocysts are presented as mean ± SD. Data was analyzed using chi-square or Student's t-test as appropriate. Significance was set at $P < 0.05$.

Results

Vitrification device

A new device based on HFV was developed. Glass capillary served as a base for the whole device and as a supporting frame for a protective sheath. Conical shape of the capillary tip allowed fixation of the hollow fiber without additional measures. Hollow fibers with loaded embryos remained intact within the device during vitrification, storage for 2 to 12 months, transfer between tanks with liquid nitrogen and transportation of these tanks. No embryos were lost in the process. Calculated total volume of the medium containing embryos within the hollow fiber in vitrification solution varied between 0.02-0.04 µl (Fig. 2B). Embryos were easily unloaded from the hollow fibers after warming.

The survival rate of day 7 embryos was 90% at 24 h after warming and gradually dropped during next 48 h of culture. Hatching rates at 24, 48 and 72 h post warming were significantly lower in vitrification than in non-vitrification group (Table 1). Average number of cells in the blastocyst did not differ significantly ($P = 0.54$) between vitrified (168 ± 60 cells, n = 27) and non-vitrified (178 ± 65 cells, n = 30) groups after 24 h of culture. No significant difference was found between average numbers of cells with damaged membranes either (3.43 *vs*. 2.86 cells per blastocyst, correspondingly).

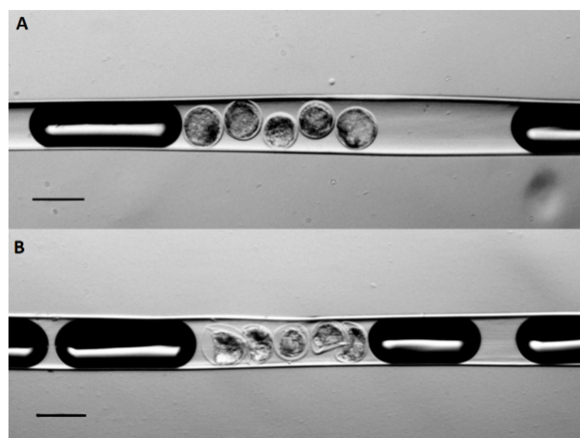

Figure 2. IVP bovine blastocysts loaded into a cellulose triacetate hollow fiber. A) Loaded blastocysts in the equilibration solution containing 7.5% of EG and 7.5% DMSO; B) Loaded blastocysts transferred within the hollow fiber into vitrification solution containing 0.5 M sucrose, 15% of EG and 15% DMSO. Scale is equal 200 µm.

Table 1. Survival and hatching rates of day 7 IVP bovine embryos vitrified in cellulose triacetate hollow fibers.

Group	No. of embryos	Hours post warming (h)					
		24		48		72	
		Survived (%)	Hatched (%)	Survived (%)	Hatched (%)	Survived (%)	Hatched (%)
Vitrification	150	135 (90)	24 (16[a])	125 (83.33)	76 (50.67[a])	115 (76.67)	93 (62[a])
Non-vitrification	81	81 (100)	33 (40.74[b])	81 (100)	56 (71.60[b])	81 (100)	69 (85.19[b])

[a,b]percentages with different superscripts within the same column differ significantly ($P < 0.05$).

Vitrification of IVP bovine blastocysts and expanded blastocysts

Average diameter of blastocysts and expanded blastocysts prior to vitrification was 168 and 187 µm, correspondingly. Survival rate of vitrified day 7 expanded blastocysts was 97.01% at 24 h post warming. Hatching rate at 72 h was 94.54%. Survival and hatching rates of expanded blastocysts were significantly higher at all time points than corresponding rates of blastocysts (Table 2). Day 7 expanded blastocysts had on average more cells in total (192 ± 56 cells) and less cells with damaged membrane (3.42) at 24 h post warming than day 7 blastocysts (144 ± 56 and 5.17, correspondingly). However, the results did not differ significantly.

The survival rates of vitrified day 8 expanded blastocysts at 24 (97.50%) and 48 h post warming (95%) were significantly higher than those obtained for day 8 blastocysts (52.78 and 41.67%, correspondingly). Same tendency was observed for corresponding hatching rates (Table 2).

Table 2. Survival and hatching rates of day 7 and 8 IVP bovine blastocysts and expanded blastocysts vitrified in cellulose triacetate hollow fibers.

Age of embryos	Developmental stage	No. of embryos	Hours post warming (h)					
			24		48		72	
			Survived (%)	Hatched (%)	Survived (%)	Hatched (%)	Survived (%)	Hatched (%)
day 7	Blastocyst	70	50 (71.43[a])	5 (7.14[a])	49 (70[a,c])	29(41.43[a,c])	47 (67.14[a])	40 (57.14[a])
	Expanded blastocyst	67	65 (97.01[b])	28 (41.79[b])	64 (95.52[b])	50 (74.63[b])	64 (95.52[b])	62(94.54[b])
day 8	Blastocyst	36	19 (52.78[a])	1 (2.78[a])	15 (41.67[a,d])	5 (13.89[a,d])	-	-
	Expanded blastocyst	40	39 (97.50[b])	15 (37.50[b])	38 (95.00[b])	27 (67.50[b])	-	-

[a,b]percentages with different supperscripts within the same column and the same day differ significantly ($P < 0.05$).
[c,d]percentages with different supperscripts within the same column differ significantly ($P < 0.05$).

Discussion

A new simple and practical vitrification device based on HFV is presented in the current article. The construction of the device is based on 1.2 mm thick glass capillary with a heat-pulled tip. One of the advantages of the glass capillary is that it is available, practical and easily disposable material. The tip of the capillary can be easily prepared both industrially for mass production and manually in the laboratory for experimental purposes. Conical shape of the tip allows secure fixation of the hollow fiber without any additional measures (Matsunari *et al.*, 2012), which significantly simplifies construction process. Glass capillary also serves as a supporting frame for a protective sheath, which can be easily "closed" or "opened" under liquid nitrogen. The sheath protected hollow fiber with loaded embryos during storage in liquid nitrogen and no fibers were lost in the process despite their fragility. Due to relative compactness of the device, it can be placed easily into additional container such as conventional goblet or a suggested above container, which allows relocation of individual devices between tanks with liquid nitrogen and easier identification. Placement and storage of the device required no additional precaution and attention from the operator unlike the situation when hollow fibers were stored in the standard cryotubes (Maehara *et al.*, 2012; Matsunari *et al.*, 2012).

The device was tested using day 7 bovine embryos as a model. The survival rate (90%) in the experimental group at 24 h post warming was comparable to those obtained after group vitrification of IVP bovine embryos using hollow fibers (Beck *et al.*, 2013) and various open-type carriers (Abdalla *et al.*, 2010; Kim *et al.*, 2012). The highest survival rates of the bovine embryos (100%) were obtained using McGill Cryoleaf (Huang *et al.*, 2007) and Cryotech (Gutnisky *et al.*, 2013).

The survival rate of the vitrified/warmed embryos declined during 72 h of culture, and hatching rates were significantly lower compared to non-vitrified embryos. However, a total of 93 out of 115 surviving blastocysts (80.85%) hatched after 72 h of culture (Table 1). Total cell count per blastocyst did not differ significantly between the groups (168 ± 60 *vs*. 178 ± 65 cells for vitrified and non-vitrified embryos, respectively). Unlike data shown in some of the works (Shirazi *et al.*, 2009), there was also no significant increase in the average number of cells with damaged membrane per blastocyst in the vitrification group (3.43 cells) compared to the non-vitrification group (2.86). Obtained results indicate that the surviving blastocysts may retain their viability after vitrification in hollow fibers.

When day 7 embryos were divided before vitrification according to their developmental stage, expanded blastocysts showed significantly higher survival rate (97.01%) than blastocysts (71.43%). The same tendency was observed for embryos that developed into expanded blastocysts or blastocysts on day 8 (97.50 *vs*. 52.78%, correspondingly). Hatching rates of the expanded blastocysts were also significantly higher for both day 7 and 8 embryos. Day 7 vitrified blastocysts had a tendency to have more cells with damaged membrane (5.17) in comparison to vitrified

expanded blastocysts (3.42). However, the results did not differ significantly.

Obtained results are in agreement with data shown in literature (Han *et al.*, 1994; Hasler *et al.*, 1997; Dinnyes *et al.*, 1999; Rios *et al.*, 2010), as it is generally expected for morphologically normal and more developmentally advanced embryos on the given day to be of higher quality and have higher viability after cryopreservation. It was also shown that day 7 blastocysts and expanded blastocysts survived cryopreservation better than their slower developing day 8 and 9 counterparts (Han *et al.*, 1994; Saha *et al.*, 1996; Dinnyes *et al.*, 1999; Gomez *et al.*, 2008). Contrary to this, in our experiment there was no significant difference in survival and hatching rates found between day 7 and 8 expanded blastocysts at 24 and 48 h post warming. These results are in agreement with Abdalla *et al.* (2010). Moreover, in our case the survival rate of day 7 and 8 expanded blastocysts shows no significant decline during culture unlike tendency shown by day 7 and 8 blastocysts. These results may indicate that expanded blastocysts have morphological and physiological traits that allow them to survive cryopreservation better than earlier developmental stages as suggested by Dinnyes *et al.* (1999). Stage dependent ability to ensure cryopreservation is indirectly confirmed by survival rate of day 9 hatched bovine blastocysts vitrified in hollow fibers (23/23 embryos survived 24 h post warming; Malenko et al., 2016, CEERB FASO, Russia; unpublished data). The effect may be correlated with general lower quality of IVP bovine morulae and blastocysts compared to *in vivo* produced ones due to abundance of lipid droplets in the cytoplasm and less developed junctional complexes between trophoblast cells (Abe *et al.*, 1999). At the same time, concentration of lipid droplets was shown to decline after morula stage in both bovine and porcine *in vivo* produced embryos (Abe *et al.*, 1999; Romek *et al.,* 2009). This tendency may be present in IVP embryos during further development making later stages such as expanded or hatched blastocyst less sensitive to suboptimal cryopreservation conditions. There are, however, also data showing that expanded bovine blastocysts produced both *in vitro* (Shirazi *et al.*, 2009) and *in vivo* (Hasler, 2011), are more susceptible to cryoinjury than early blastocysts, which correlates with the trait observed in other mammalian species (Vanderzwalmen *et al.*, 2002; Mukaida *et al.*, 2003; Li *et al.,* 2012).

There are numerous discrepancies in the protocols used by different laboratories making direct comparison of the results quite difficult. For example, the division of the embryos according to their developmental stage is highly subjective (Dinnyes *et al.*, 1999; Gomez *et al.*, 2008; Abdalla *et al.*, 2010). However, one of the major factors that may contribute to the differences in the survival rates of vitrified bovine embryos of different developmental stage and age seems to be the choice of the either surface type carriers based on MVC principle (Huang *et al.*, 2007; Abdalla *et al.*, 2010; Gutnisky *et al.*, 2013) or tubing carriers (Dinnyes *et al.*, 1999; Gomez *et al.*, 2008; Rios *et al.*, 2010). Thus, our preliminary data obtained using Cryotop carrier showed leveling of the difference in survival rates of day 7 IVP bovine blastocysts (17/18 embryos) and expanded blastocysts (15/15 embryos). No significant difference in the survival rates was also found between day 7 or 8 expanding (85 and 84%) and fully expanded blastocysts (88 and 89%) vitrified using Cryotop (Abdalla *et al.* (2010) and maximum survival rates of the mixed population of day 7 bovine blastocysts (100%) were obtained using close analogues of this carrier (Huang *et al.*, 2007; Gutnisky *et al.*, 2013).

Minimum volume cooling principle is also utilized in HFV method. In our case, volume of the solution within the hollow fiber containing 5-10 embryos varied between 0,02 and 0,04 µl after incubation in vitrificatin solution. However, in comparison to open-type carriers embryos are separated from vitrification and warming solutions by a cellullose triacetate film. The thickness of the fiber wall is comparable to thickness of zona pellucida (15 µm). Eventhough the pores in the hollow fibers are relatively large (7 nm), the rate of cryoprotectant redistribution between solutions in and out of the hollow fiber might be affected. This may lead to suboptimal vitrification/warming conditions crucial for the survival of more sensitive part of the embryo population. Thus, the HFV method would show lower survival rates for mixed population of day 7 IVP bovine embryos compared to Cryologic method (Saucedo *et al.*, 2015a, b). At the same time, the developmental rate of pig parthenogenetic morulae obtained using HFV method was significantly higher compared to the one obtained using the Cryotop method (Nagashima *et al.*, 2015). In the current state, high and repeatable survival rates after HFV can be obtained using expanded and possibly hatched IVP bovine blastocysts. The procedures, however, should be modified to accommodate embryos at the earlier stages of blastocoel expansion.

In conclusion, a new simple, compact and reliable device for HFV was constructed and tested using day 7 and 8 IVP bovine embryos as a model. The device was based on a glass capillary and supplied with a protective sheath. It was proven to be reliable and practical for vitrification and long term storage of the IVP bovine embryos in liquid nitrogen. Presented device may contribute to wider application of HFV method in livestock production for both politocous and monotocous species leading to significantly simplified and standardized vitrification and storage procedures. Mixed group of day 7 IVP bovine blastocysts and expanded blastocysts vitrified using the device showed survival rates comparable to those obtained using other vitrification carriers. Day 7 and 8 expanded blastocysts survived vitrification significantly better than blastocysts of the same age. Currently, high and repeatable results with HFV method can be obtained by selection of IVP bovine embryos at the developmental stage of expanded blastocyst. However, the ability of such embryos to successfully implant and produce viable offspring must be verified in further experiments.

Further modification of the method may be required to achieve high and stable results with different developmental stages of IVP bovine embryos.

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