The Efficency of Cryotop in Vitrification of In Vitro Produced Egyptian Buffalo (Bubalus Bubalis) Embryos

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ABSTRACT
Embryo cryopreservation becomes a pivotal side in the shape of assisted reproductive technologies. It plays an essential role in several cases such as, embryo storing during transportation, embryo preservation for future use, and establishment of cryobanks for endangered species and rare breeds. The present experiment was conducted to evaluate the cryotolerance of in vitro produced buffalo embryos at blastocyst stage to vitrification process using cryotop. Embryos were produced using conventional in vitro fertilisation technique and had been divided into two categories: non-vitrified group, which was cultured in vitro till hatched blastocyst stage and vitrified group, which was collected at day 7 for cryopreservation. A stepwise vitrification and thawing procedures were performed using Cryotop. Survival rate of embryos was observed within 4 hours of thawing, then the ability to expand and hatch was recorded. The expansion rate was higher in control group compared to vitrified group (91.66 vs. 86.36%, respectively) with no significant differences. In both groups, all expanded blastocysts reached the hatching stage normally. Therefore, our results suggested that Cryotop is an effective tool for cryopreservation of in vitro produced Egyptian buffalo blastocyst.

Keywords: Buffalo; Embryo; Vitrification; Cryotop; In vitro fertilisation.

INTRODUCTION
Water buffalo is considered to be major milk and meat producing livestock in many developing counties, especially Egypt. Although buffalo can adapt to difficult environmental conditions, it has a low reproductive efficiency. Water buffalo has not received the same concern from genetic improvement and applying reproductive technologies, as cattle. Many of these modern techniques, which have been successfully applied on bovine, could effectively help in overcoming the low reproductive efficiency of buffalo that compromises its performance.

The use of in vitro embryo production technology could significantly enhance the efficiency and logistics of embryo production in water buffalo (Drost, 2007). For example, taking the advantage of using in vitro embryo production techniques will help in exploiting wasted genetically valuable oocytes of slaughtered females. In buffalo, there were limited successful uses of in vitro embryo production techniques with low yield of high quality transferable embryos. Obviously, the system of laboratory production of buffalo embryos still sub-optimal and needs more considerable improvements (Nandi et al., 2002).

Superovulation and embryo transfer in buffalo have poor results (Drost, 2007). However, cryopreservation could be a beneficial way to preserve embryos and other germ cells, as a genetic resource, till a significant development would be achieved in these techniques. Unfortunately, there is a lack in research experiments conducted on buffalo embryos to evaluate different methods of cryopreservation. Therefore, it’s difficult to choose the proper technique that leads to the best survival, pregnancy and live calves production rate.

Since 1984, when Fahy and his colleagues published the first description of their vitrification approach as a new method for cryopreservation, vitrification has increasingly and rapidly used and proposed to become the most suitable method for cryopreservation of any cells or tissues in the near future (Fahy and Rall, 2007).

Vitrification, a type of cryopreservation techniques, is a process of glass solidification of a liquid by increasing its viscosity to become in vitreous structure (Craig and Turner, 2013). There are two main cryopreservation methods: the slow freezing method, which needs sophisticated instrument; takes long time and could not eliminate cryoinjuries completely, and the vitrification method a cheap, simple and rapid technique, and appeared to be the effective alternative (Parnpaet al., 2016).

Vitrification depends on using high concentration of cryoprotective agent (CPA) and sharply cooling and warming rate. Cryoprotectant agent is a chemical compound used to protect the cell from cryoinjuries by regulating water dynamics during cooling. Cryoprotectants are grouped into two major categories, working together: penetrating agents, which can cross cell membrane and replace the intracellular water content and lowering the freezing temperature to prevent ice crystal formation, and non-penetrating agents, which has a large mass to diffuse into cells so they work on cellular dehydration by increasing osmolarity of the extracellular space. The most common penetrating CPAs are a mixture of Dimethyl sulfoxide (DMSO) and Ethylene glycol (EG) with different concentration, which minimise the cytotoxicity effect of the CPA, and sucrose is considered to be the most frequent used non-penetrating CPAs (Swain and Smith, 2010).

The Cryotop was invented specifically in order to improve the cryopreservation of human embryos. This method, which is considered a minimum-volume cooling procedure, consists of loading the embryos on a very fine polypropylene strip using an extremely small volume less than 0.01µl of vitrification solution and immediately plungethem into liquid nitrogen. In the presence of the CPA, The reduction of the amount of vitrification medium surrounding the embryos allowsthem to rapidly pass the critical temperature zone. Those changesare the main factors responsible for the

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best results obtained with this approach for several species (Morimoto, 2010 and Leme, 2016).

Therefore, the objective of this study was to evaluate the cryotolerance of in vitro produced buffalo embryo at blastocyst stage to vitrification technique using Cryotop.

**MATERIALS AND METHODS**

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

**Experimental Design:**

A total number of 48 buffalo blastocysts (day 7) were produced in vitro from ten replicates and randomly distributed into two equal groups. The first group (24 blastocysts) was vitrified using Cryotop device and thawed, while the second group (24 blastocysts) was cultured in vitro as fresh embryos (control group). Both groups continued culture in vitro till hatching.

**Collection, Transportation and Preparation of ovaries:**

Buffalo ovaries were collected from slaughtered buffaloes from three different local abattoirs and transported to the lab within 2 hrs in a thermos containing sterilised physiological saline (0.9% NaCl) supplemented with antibiotic (100 µg/ml streptomycin sulphate and 100 IU/ml penicillin) and maintained at a temperature of 30-35°C. The collected ovaries were washed once in sterilised physiological saline, then once in 70% ethanol alcohol and twice in sterilised physiological saline again.

**Oocytes recovery:**

Cumulus oocyte complexes (COCs) were aspirated from 2-8 mm diameter follicles using 18-gauge needle attached to a 10 ml syringe. COCs received in pre-warmed Hepes-buffered medium 199 (Gibco, UK). Using stereomicroscope, COCs with homogeneous cytoplasm and surrounded by at least three layers of cumulus cells were selected as good quality oocytes.

**In vitro maturation:**

Selected COCs were washed three times by gently pipetting in pre-warmed maturation media (modified Parker medium supplemented with 12% inactivated foetal bovine serum and 10 µg/ml FSH). Maturation was performed in 4-well plate (Nunc, Denmark), where 30 COCs were incubated in 400 µl of maturation media over lied with 400 µl of mineral oil for 18 hours at 38.5°C in a humidified atmosphere containing 5% CO₂.

**Semen preparation:**

Frozen 0.25 ml semen straw was thawed in water bath at 37°C for 30 seconds, and then wiped with 70% ethanol alcohol before being opened. Sperm washed three times by centrifugation at 500 xg for 5 minutes, twice in sperm washing medium (modified Ca²⁺ free TALP medium) and the last one in fertilisation medium (modified TALP medium). The final pellet was re-suspended in fertilisation medium and the final concentration of sperm was adjusted to be 2x10⁶/ml.

**In vitro fertilisation:**

Conventional IVF was performed in 4-well plates, where each 30 matured oocytes were co-cultured with the prepared sperm in 400 µl of fertilisation medium over lied with 400 µl of mineral oil for 18 hours at 38.5°C in a humidified atmosphere with 5% CO₂.

**In vitro culture:**

Presumed zygotes were transferred to embryo washing medium (modified Hepes-SOF medium), and mechanically denuded by repeated pipetting. Denuded zygotes were cultured, using 4-well plates, in culture medium (modified SOFAA medium) for 7 days at 38.5°C in a humidified atmosphere with 5% CO₂, 5% O₂, and 90% N₂. Culture medium was half renewed every 48 hours.

**Vitrification:**

Blastocysts at day 7 were vitrified using Cryotop (Kitazato, Japan). Groups of three blastocysts were equilibrated in holding medium for 5 minutes (Hepes-buffered medium 199 supplemented with 20% FBS and 50 g/ml, 1 M DMSO and 7.5% EG). Equilibrated blastocysts were transferred to the vitrification solution (Hepes-buffered medium 199 supplemented with 20% FBS and 50 g/ml, 0.5 M sucrose, 2 M DMSO and 15% EG) for 30 seconds. All vitrification procedures were performed at room temperature. Each group was then loaded on a Cryotop with less than 0.1 µl of vitrification solution and plunged into liquid nitrogen (LN₂) and stored for 6 months.

**Thawing/Warming:**

Cryotop was directly inserted into 1 ml of warming medium (Hepes-buffered medium 199 supplemented with 20% FBS and 0.5 M sucrose) at 37°C for 1 minute. Blastocysts were then sequentially rehydrated in the warming medium at 37°C in stepwise serial dilution of 0.25, 0.1 and 0 M sucrose for 5, 10 and 10 minutes, respectively. Post-warming, all thawed blastocysts were subjected to in vitro culture.

**Statistical analysis:**

Data were analysed using the Statistical Analysis System (SAS), University Edition (SAS Institute Inc., Cary, NC, USA). Differences in mean values were tested using analysis of variance followed by t-test for two independent means. Differences of P ≤ 0.05 were considered to be significant.

**RESULTS AND DISCUSSION**

The objective of this study was to evaluate the cryotolerance of in vitro produced buffalo blastocyst to vitrification technique using Cryotop. Embryos were produced using conventional in vitro fertilisation technique. In general, cleavage rate was 61.32% and the average blastocyst rate (Day 7) (Fig. 2), calculated based on the cleaved embryos, was 27.27%. Only embryos in the blastocyst stage were used for vitrification. After thawing, embryos with cryoinjuries like zona cracking and shrinkage were considered to be non-survived (Fig. 1). Blastocyst expansion and hatching rates were calculated for both groups. Those blastocysts that re-expanded were considered alive. In the vitrified group, only 2 blastocysts out of the 24 were appeared morphologically abnormal after the vitrification process and considered as non-survived embryos. The remaining blastocysts were survived and continued development (Fig. 2). The expansion rate was
higher in control group compared to vitrified group (91.66% vs. 86.36%, respectively) with no significant differences (Table 1). In both groups, all expanded blastocysts reached the hatching stage normally.

![Figure 1](image1.png)  
**Figure 1.** Non-survived blastocyst after warming: (A) shrinkage and (B) zona cracking.

In an experiment conducted in China (Yang et al., 2012) to evaluate the cryotolerance of IVF produced blastocysts using open-pulled straw (OPS) and slow freezing in 0.25 ml French straw, the survival rate of embryos vitrified in three different concentrations of cryoprotectant (40% EG, 25% EG + 25% DMSO and 20% EG + 20% DMSO) were 63.1%, 78.0% and 88.7%, respectively. On the other hand, the cryosurvival of slow freezing in two different concentrations (10% EG and 0.05 M trehalose dehydrate + 1.8% EG + 0.4% BSA) were 75.2% and 64.6%.

![Figure 2](image2.png)  
**Figure 2.** Blastocyst morphology before and after warming: (A) day 7 blastocyst which was used in vitrification, (B) re-expanded blastocyst after 24 hours of warming and (C) hatched blastocyst after 48 hours of warming.

**Table 1. Survival, expansion and hatching rates from vitrified and non-vitrified (control) blastocysts using the Cryotop method.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Immature oocytes</th>
<th>Cleavage</th>
<th>Blastocyst (day 7)No.</th>
<th>Survival rate No. (%)</th>
<th>Expansion rate (24 hours) No. (%)</th>
<th>Hatching rate (48 hours) No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified</td>
<td>150</td>
<td>89</td>
<td>24</td>
<td>22 (91.66)</td>
<td>19 (86.36)</td>
<td>19 (86.36)</td>
</tr>
<tr>
<td>Control</td>
<td>150</td>
<td>87</td>
<td>24</td>
<td>24 (100)</td>
<td>22 (91.66)</td>
<td>22 (91.66)</td>
</tr>
</tbody>
</table>

The effect of vitrification of *in vitro* derived blastocyst using 0.25 ml straw has been studied by (Hufana-Duranet et al., 2004) and found that hatched blastocyst rate after thawing was 75%. Another study showed that the blastocyst hatching rate after vitrification using 0.25 ml straw of *in vitro* derived blastocyst using oocytes aspirated from slaughterhouses ovaries was 40.2% versus 52.8% in oocytes were collected by OPU from live animals (Manjunatha et al., 2008).

In Bovine, most of studies that used Cryotop were conducted on oocytes, however, some studies used Cryotop to cryopreserved embryos at different stages (day 5, 6 and 7) and the survival rates of the vitrified embryos were 96.5%, 87.5% and 94.2%, and hatched embryos rates were 32.7, 52.4% and 67.3%, respectively (Kelyet et al., 2003). Leme et al., (2016) vitrified *in vitro* produced blastocyst (day between 6 and 6.5) and survival, re-expansion and expanded blastocyst rates were 87%, 58.7% and 28.4%, respectively. When Cryotop was compared with aluminium block and conventional cryopreservation of *in vitro* produced bovine blastocyst at day 7 the survival rates were 85.9%, 70.6% and 56.1%, respectively (Kruse, 2012). Inaba et al., (2011) investigated the effect of vitrification by Cryotop on re-expansion, hatching and hatched rate after thawing of cryopreserved day 6 blastocyst and they were 100%, 93.2% and 95.5%, respectively.

Generally, Cryopreservation of embryos would be affected by many factors adversely or supportively in different pitches. One of these factors which influence the cryosurvival of embryos is the CPA. Using of mixture of cryoprotectants such as EG and DMSO have some advantage over using only one type. The combining of various cryoprotectent enable the use of lower concentration of each compound in vitrification solution and resulting in alleviating the toxicity (Manjunatha et al., 2008). In addition, using CPAs at room temperature or less than 37°C could be a reason of decreasing their toxicity (Kuwayama et al., 2015). Furthermore, using stepwise addition and removal of cryoprotective agents upon warming/ thawing by gradually increasing and decreasing the CPA concentration help in minimising the osmotic stress (Swain and Smith, 2010).

Kuwayama et al., (2005) mentioned that when Cryotop was used, the cooling rate was 22800°C/minute, warming rate was 42100°C/minute and volume of solution was less than 0.1µl. Reducing the volume of the sample and increasing the cooling rate promotes vitrification by decreasing the amount of liquid which has to be cooled and declining the probability of ice crystal formation (Yavin and Arav, 2007).

By comparing the obtained results in the current study with the previous experiments in which vitrification method was used to cryopreserve *in vitro* produced embryos, it seems that Cryotop device has a superior efficiency more than other used tools. Regardless of using different CPA concentration and
exposure time, the successful of vitrification using Cryotop could be attributed to three main factors: using a moderate concentration of mixed CPAs, a minimal volume of vitrification solution and ultra-rapid cooling and warming rates.

**CONCLUSION**

In conclusion, this study demonstrated that vitrification method using Cryotop allows acceptable survival and development rates of *in vitro* produced blastocyst in Egyptian buffalo. These results showed promising information for using Cryotop in vitrification method for preservation of *in vitro* produced embryos in buffalo.

**REFERENCES**


