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The Effeciency 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 usesand establishment ofcryobanks for endangered species and rare breeds. The presentexperiment was conducted to evaluate the cryotoleranceof*in vitro* produced buffalo embryosat 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 thatCryotop is aneffective 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 geneticallyvaluable 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 achievedin these techniques. Unfortunately, there is a lake 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 became 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,andthe vitrification method a cheap, simple and rapid technique, and appeared to be the effective alternative (Parnpai*et al*., 2016).

Vitrification depends on using high concentration of cryoprotective agent (CPA) and sharply cooling and warming rate. Cryoprotectent agent is a chemical compoundused to protect the cell from cryoinjuries by regulating water dynamics during cooling. Cryoprotectents 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 veryfine 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 ofthe amount ofvitrification medium surrounding the embryos allowsthem to rapidly pass the critical temperature zone. Those changesare the main factors responsible for the

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

Therefore, the objective of this study was to evaluate the cryotoleranceof *in vitro* produced buffalo embryosat 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 twoequal 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 buffalos fromthree 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 2-8mm 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 10µg/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% CO_2.

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 Ca^{2+} 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 $2x10^6$/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 400µl of fertilisation medium over lied with 400µl of mineral oil for 18 hours at 38.5°C in a humidified atmosphere with 5% CO_2.

***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% CO_2, 5% O_2, and 90% N_2. 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 0.1µl of vitrification solution and plunged into liquid nitrogen (LN_2) 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 mediumat 37°C in stepwise serial dilution of 0.25, 0.1 and 0 M sucrose for 5, 5 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 $P \leq 0.05$ 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 theaverage blastocyst rate (Day 7) (Fig. 2), calculated based on the cleaved embryos,was 27.27%. Only embryos in the blastocyst stage were usedfor vitrification. After thawing, embryos with cryoinjuries like zona cracking and shrinkagewere 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.

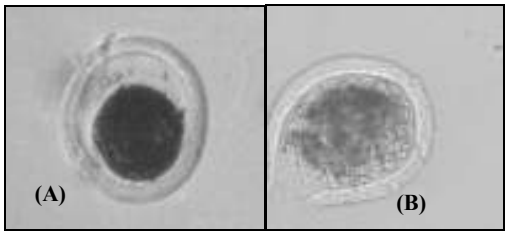

Figure 1. Non-survived blastocyst after warming: (A) shrinkage and (B) zona cracking.

In an experiment conducted in China(Yang *et al*., 2012) to evaluate the cryotolerance of IVF produced blastocysts using open-pulled straw (OPS) and slaw freezing in 0.25 ml French straw, the survival rate of embryos vitrified in three different concentrations of cryoprotectent (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%.

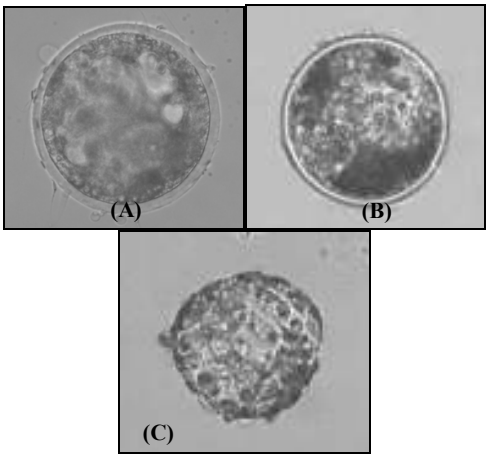

Figure 2. Blastocyst morphology before and after warming: (A) day 7 blastocyst which was used in vitrification, (B) re-expanded blastocyst after 24 hours of warming and (C) hatched blastocyst after 48 hours of warming.

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

Groups	Immature oocytes	Cleavage	Blastocyst (day 7)No.	Survival rate No. (%)	Expansion rate (24 hours) No. (%)	Hatching rate (48 hours) No. (%)
Vitrified	150	89	24	22(91.66)	19(86.36)	19 (86.36)
Control	150	87	24	24(100)	22 (91.66)	22 (91.66)

The effect of vitrification of *in vitro* derived blastocyst using 0.25 ml straw has been studied by (Hufana-Duran*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 cryopreservedembryos at different stages (day 5, 6 and 7) and the survival ratesof the vitrified embryos were 96.5%, 87.5% and 94.2%, and hatched embryos rates were 32.7, 52.4% and 67.3%, respectively(Kelly*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 cryoprotectents 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 Cryotopallows acceptable survival and development rates of *in vitro* produced blastocyst in Egyptian buffalo. These results showed promising information for usingCryotop in vitrification method for preservation of *in vitro* produced embryos in buffalo.

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كفاءة إستخدام الـ Cryotop في تجميد أجنة الجاموس المصري المنتج معمليًا.

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أصبح تجميد الأجنة جزء محوري في تكوين التقنيات التناسلية المساعدة. حيث تلعب دور أساسي في حالات كثيرة منها حفظ الأجنة خلال عملية النقل والإستخدامات المستقبلية ، وإنشاء بنوك الأجنة للحيوانات المهددة بالإنقراض والسلالات النادرة. أجريت هذه التجربة لتقييم مدى حساسية أجنة الجاموس المصري المنتجة معمليًا - في مرحلة البلاستوسيست - لعملية التجميد بإستخدام الـ Cryotop. تم إنتاج الأجنة بإستخدام تقنية الإخصاب المعملي ثم قسمت الأجنة الناتجة لمجموعتين: الأولى لم يتم تجميدها ولكن تُركت لتكمل نموها حتى مرحلة الفقص ، أما المجموعة الثانية فتم جمع الأجنة خلال اليوم السابع لإجراء التجميد عليها. إستخدمت طريقة التجميد والإذابة ذات التركيزات المتعددة المتتالية. تم إختبار والتاكد من معدل النجاة والقدرة على التمدد والفقص. كان معدل التمدد أعلى في المجموعة الغير معاملة عن المجموعة التي تم تجميدها (٩١.٦٦% مقابل ٨٦.٣٦%) بدون أي إختلافات معنوية. في المجموعتين كل الأجنة التي تمددت مرة بمرحلة الفقص بشكل طبيعي. لذلك فالنتائج تشير إلى أن الـ Cryotop يعتبر أداة فعالة لتجميد أجنة الجاموس المصري المنتجة معمليًا.

الكلمات المفتاحية: الجاموس ، الأجنة ، التجميد ، Cryotop ، الإخصاب المعملي.