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In vitro Maturation, Fertilization and Embryo Development of Immature Buffalo Oocytes Vitrified by Different Cryodevice Types

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ABSTRACT

This study aimed to compare the efficiency of three cryodevices (Conventional Straws (CS), Hemi-Straws (HS) and spatula (SP)) in vitrification of buffalo oocytes. Oocytes were recovered from ovaries of slaughtered buffaloes. They were vitrified and thawed according to each cryodevice used. Then they were *in vitro* matured, fertilized and cultured to blastocyst stage. Survival and quality, *in vitro* maturation, fertilization rate and blastocyst production rates of vitrified immature buffalo oocytes were assessed. Results showed that total survival rate of the vitrified oocytes was higher ($p<0.05$) for SP and HS (96 and 95%) than CS (80%). Recovery rate of normal oocytes relative to vitrified or survival oocytes was the highest ($p<0.05$) for SP compared to other cryodevices. *In vitro* maturation rate (oocytes at metaphase II stage) was lower ($p<0.05$) for all vitrified oocytes than fresh (61.1 vs. 27.8-44%). SP showed higher ($p<0.05$) maturation rate (44%) than HS and CS (34.4 and 27.8%, respectively). Cleavage rate was lower ($p<0.05$) for all vitrified oocytes than fresh oocytes (37.7-58.9% vs. 70.8), being higher ($p<0.05$) for SP and HS (55.8 and 58.9%, respectively) than for CS (37.7%). Blastocyst production rate relative to cleaved oocytes was lower ($p<0.05$) for vitrified than fresh oocytes (5.9-14.3% vs. 31.3) but the differences among vitrification cryodevice were not significant. In conclusion, immature buffalo oocytes can vitrified successfully by spatula, conventional straws and Hemi-straws. Spatula device is a reliable alternative, inexpensive and easy to assemble for vitrification of immature buffalo oocytes. Also, vitrification spatula has the largest effective holding capacity.

Key words: Buffaloes, immature oocyte, vitrification, cryodevice, maturation, cleavage, *in vitro*

INTRODUCTION

Freezing mammalian oocytes was investigated by several authors (Parks and Ruffing, 1987; Hernandez-Ledezma and Wright, 1989) but limited studies gave attention to buffaloes (Wani *et al*., 2004; Yadav *et al*., 2008; Shamiah *et al*., 2008). Vitrification provides an alternative method for oocyte cryopreservation that minimizes cellular damage caused by ice crystal formation (Vajta, 2000). The extent of damage in oocytes or embryos caused by vitrification may depend on the cryopreservation procedures used (Chen *et al*., 2003).

Different protocols considering type and concentration of cryoprotectants, number of equilibration steps, type of cryopreservation device, time of exposure and numbers of dilution steps

at warming were executed through the last 15 years (Massip *et al*., 1987; Vajta *et al*., 1998; Dattena *et al*., 2000). To reduce the chances of intracellular ice crystal formation and chilling injury during oocyte cryopreservation, the cooling rate must be increase by introducing a small volume of vitrification solution into a device cooled by Liquid Nitrogen (LN) or by direct plunge into LN using available techniques such as Solid Surface Vitrification (SSV) (Dinnyes *et al*., 2000), cryoloop (Lane *et al*., 1999), cryotop (CT) (Kuwayama and Kato, 2000), microdrop (Papis *et al*., 2000) and electron microscope grid (Martino *et al*., 1996). One of the most successful ultra-rapid vitrification techniques is the cryotop method that has resulted in excellent survival and developmental rates with human and bovine oocytes (Kuwayama *et al*., 2005). Both cryotop (Muenthaisong *et al*., 2007; Attanasio *et al*., 2010) and SSV (Boonkusol *et al*., 2007; Gasparrini *et al*., 2007; Mahmoud *et al*., 2013) have been successfully applied to cryopreserve buffalo oocytes.

The design of the cryodevices affects cooling/warming rates. High cooling rate is an important factor in improving the effectiveness of oocyte vitrification to allow reduction of the concentration of the cryoprotectants used and in this way reduce their toxicity. Also, a reduction in the amount of cryoprotectants required decreases their toxic and osmotic effects. The volume of the vitrification solution is minimized by using special carriers during the vitrification process. These carriers include: Open Pulled Straw (OPS, Vajta *et al*., 1998; Oberstein *et al*., 2001), Flexipet-Denuding Pipette (FDP; Liebermann and Tucker, 2002), electron microscope copper grids (Park *et al*., 2000), hemi-straw system (Vandervorst *et al*., 2001), nylon mesh (Matsumoto *et al*., 2001), solid-surface microdrop (Bagis *et al*., 2005), Gel-Loading Tip (Tominaga and Hamada, 2001) Stripper Tip® (Walker *et al*., 2004), cryotop (Kuwayama, 2007) and cryoloop (Oberstein *et al*., 2001). These carriers or vessels have all been used to achieve higher cooling rates. Extremely high warming rates are also important to prevent damage at the time of warming (Isachenko *et al*., 2005; Seki and Mazur, 2008).

Egyptian buffaloes are important livestock animals because of their draft power, hide and milk and meat production. Buffalo oocytes vitrified at the mature stage cleaved and developed into morula and blastocyst stage after thawing at higher rates than those vitrified at the immature stage (Hammam *et al*., 2005; Mahmoud *et al*., 2010). Cryopreservation of immature oocytes would significantly advance basic research and commercial applications (Schroeder *et al*., 1990; Candy *et al*., 1994), as previously reported for mice (Eroglu *et al*., 1998), cattle (Suzuki *et al*., 1996), buffalo (Wani *et al*., 2004), pigs (Isachenko *et al*., 1998) and humans (Tucker *et al*., 1998).

Cryopreservation of oocytes from slaughtered animals may enable a flexible utilization in time and space of live oocytes for research and animal production purposes. Likewise, subsequent use of cryopreserved oocytes for embryo production may provide an opportunity to replenish gene banks of endangered species and contribute to the genetic improvement of domestic animals. Therefore, *in vitro* maturation and fertilization of slaughterhouse-derived oocytes, large-scale production of buffalo embryos, is gathering increasing interest for the production and faster multiplication of superior germplasm.

To date few reports on the effects of vitrification by different cryodevice types on *in vitro* maturation of immature oocytes of Egyptian buffaloes. Therefore, this study aimed to compare the impact of Conventional Straws (CS), Hemi-Straws (HS) and spatula (SP) as vitrification cryodevices on survival and quality, *in vitro* maturation and fertilization rates and blastocyst production rate of vitrified immature buffalo oocytes.

MATERIALS AND METHODS

This study was carried out at the Laboratory of Physiology and Biotechnology, Animal Production Department, Faculty of Agriculture, Mansoura University, in cooperation with Animal Production Department, Faculty of Agriculture, Tanta University.

All chemicals used in this study were purchased from Sigma, unless otherwise indicated.

Oocyte recovery: Ovaries collected from slaughtered buffaloes were placed in NaCl solution (9 mg mL^{-1}) containing antibiotics (penicillin, 100 UI mL^{-1} and streptomycin sulphate, 100 μg mL^{-1}) and maintained at 30°C until oocyte recovery. The collected ovaries were washed in freshly prepared saline. Ovarian follicles of 2-8 mm in diameter were aspirated using 18-gauge needle connected to a syringe. Follicular fluids were placed in Petri dishes for oocyte collection and then oocytes were examined under stereomicroscope. Oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were selected and washed three times in Dulbecco's phosphate buffer solution medium.

Vitrification: The basal solution used for vitrification was TCM-199 medium supplemented with Fetal Calf Serum (FCS, 20% v/v), penicillin (100 UI mL^{-1}) and streptomycin sulphate (100 μg mL^{-1}). The vitrification procedures employed throughout this experiment were according to (Shamiah *et al*., 2008). The 1st vitrification solution (VS1) contained 10% (v/v) Ethylene Glycol (EG), 10% (v/v) dimethyl sulfoxide (DMSO) and 0.5 M sucrose. The 2nd Vitrification Solution (VS2) contained 20% (v/v) EG, 20% (v/v) DMSO and 0.5 M sucrose in basal medium.

Cryodevice types

Straw: Five Compact Oocytes Cumulus Cells (COCs) were loading into the center between two air bubbles in 0.25 mL plastic insemination straws (IVM L' Aigle, France) using a fine glass capillary pipette. After heat-sealing the straws were plunged immediately into liquid nitrogen within 30 sec at room temperature.

Hemi straw: About five oocytes were deposited using an attenuated pipette to the tip of the trough of the hemi straw 0.25 mL which was then instantly plunged into a Dewar of LN2 and with the aid of forceps inserted in a larger pre-cooled 0.5 mL straw (IVM L' Aigle, France) before closing it.

Spatula: To assemble a vitrification spatula, the tip of an autoclaved microcapillary tip (Cat. no. LW-1100; Alpha laboratories Ltd, UK) was crushed with a pair of fine forceps that had been gently heated with a Bunsen burner to generate a petal-like plate of 1 mm. The distal edge was heat-sealed to avoid liquid infiltration. The other end of the microcapillary tip was removed to shorten the spatula stalk.

The oocytes are then loaded onto one side of the vitrification spatula with a fine glass capillary pipette. The tip is immediately dipped into liquid nitrogen and inserted in the cryopreservation vial with the cap tightly screwed. The vial is then transferred to the liquid nitrogen for storage.

Warming

Straw: After storage for two weeks, the oocytes were warmed by holding the straws for 6 sec in air and then agitating them in water bath at 25°C for at least 15 sec. The contents of each straw were emptied into Petri dish and oocytes were transferred to three diluents solution, 0.25 M and 0.125

M sucrose in TCM-199 supplemented with 10% Bovine Serum Albumin (BSA), then in TCM-199 supplemented with 10% BSA for 3 min per solution, to remove of intracellular cryoprotectant (Asada *et al*., 2002).

Hemi straw: A Dewar of LN2 containing the HS was placed close to the microscope. Under LN2 and with forceps, the HS were pulled out of the larger straw and the tip of the straw holding the oocytes was immediately immersed into a Petri dish containing 3 mL of 0.25 M and 0.125 M sucrose in TCM-199 supplemented with 10% BSA), then in TCM-199 supplemented with 10% BSA for 3 min per solution, to remove of intracellular cryoprotectant.

Spatula: The tip of the spatula containing oocytes is dipped into the Petri dish containing 3 mL of 0.25 M and 0.125 M sucrose in TCM-199 supplemented with 10% BSA), then in TCM-199 supplemented with 10% BSA for 3 min per solution, to remove of intracellular cryoprotectant.

Evaluation of oocyte viability: Oocyte viability was evaluated morphologically based on the integrity of the oolemma and zona pellucida loss of membrane integrity was obvious upon visual inspection as the sharp demarcation of the membrane disappeared and the appearance of the cytoplasm changed.

The criteria used for assessing the post-thaw morphology of vitrified-warmed oocytes were as follows: Normal oocytes with spherical and symmetrical shape with no signs of lysis and abnormal damage oocytes, crack in zona pellucida, oocytes split in two halves, change in shape of oocytes and leakage of oocytes contents (Dhali *et al.,* 2000).

***In vitro* oocyte maturation:** Complex tissue culture medium (TCM-199, powder) was dissolved in deionized double distilled water. On the day of maturation, TCM-199 medium stock was supplemented with 10% FCS, 20 IU mL^{-1} hCG (Pregnyl, Nile, CO. for Pharm. Cairo, Egypt), 5 μg mL^{-1} FSH, 1μg mL^{-1} estradiol β_{17}, 20 mmol final concentration of pyruvate, 50 μg mL^{-1} gentamicin. The pH value of the medium was adjusted at 7.3-7.4. The medium was filtrated by 0.22-μm millipore filter (Shamiah, 2004).

Each of 500 μL from prepared maturation medium was placed into a four well dishes and covered by sterile mineral oil. Before placing oocytes into culture dishes, the medium was incubated in CO_2 incubator (5% CO_2, at 38.5°C and high humidity) for 60 min. After warming, oocytes were washed three times in each of PBS plus 3% BSA and once in TCM-199. Thereafter, fresh and vitrified oocytes were placed in the medium and incubated for 24 h at 38.5°C, 5% CO2 and high humidity.

Fixation, staining and examination of oocytes: After 24 h as a maturation period, oocytes were washed using PBS containing 1 mg mL^{-1} hyaluranidase to remove the cumulus cells. Then, oocytes were washed two times in PBS supplemented with 3% BSA and loaded on clean slide. Slides were placed into fixation solution (3 ethanol: 1 glacial acetic acid) overnight. Thereafter, oocytes were stained with 1% orcein in 45% acetic acid and examined for maturation under phase-contrast microscopy as the following: Matured oocytes: Chromosome at metaphase II (MII) and polar body exhausted in previtilline space, oocytes with Germinal Vesicle (GV): chromosomal in disk in cytoplasmic with intact membrane of nuclei, oocytes with Germinal Vesicle Breakdown (GVBD): chromosomal in disk in cytoplasmic but intact membrane of nuclei is breakdown, oocytes at

metaphase I (MI): The chromosomes are arranged in the metaphase plate and the diploid sets of chromosomes (2n) are fully condensed with absence of polar body component of chromatin mass and degenerated oocytes: Ocytes were vaculated or cytoplasmic shrinked or chromatin condensed (Shamiah, 2004).

***In vitro* fertilization:** Fresh semen collected by artificial vagina from fertile buffalo bulls was swim-up separated as described by Parrish *et al.* (1986) and diluted to 25×10^6 sperm mL^{-1}. Swim up separated sperm diluted with IVF-TALP medium were co-incubated with 20 μg mL^{-1} heparin sodium for 30 min in CO_2 incubator at 38.5°C, 5% CO_2 in air and humidified atmosphere.

Matured oocytes were washed three times in TL- HEPES medium (Parrish *et al.*, 1989) and twice in fertilization medium (IVF-TALP). Oocytes were inseminated with capacitated semen (1.5×10^6 sperm mL^{-1}). Oocytes and spermatozoa were co-cultured in fertilization medium and incubated for 22 h at 38.5°C, 5% CO_2 in air with maximum humidity.

Embryo development: After co-incubation, presumptive zygotes were stripped of cumulus cells, washed three times in embryo culture medium, (TCM-199 medium supplemented with 20 mMol Na-pyruvate, 3 mg mL^{-1} BSA, and 50 μL mL^{-1} Gentamycin sulphate) and cultured in pre-equilibrated embryo culture medium in 4 well Petri dishes and overlaid with sterile mineral oil, then incubated at 38.5°C, 5% CO_2 with maximum humidity (Eyestone and First, 1989). The medium was replaced every 48 h. The number of cleaved embryos (divided into four cells or more) was recorded on day 3 (fertilization was day 0) and blastocysts were counted on day 8.

Statistical analysis: The experiment was replicated 5 times for survival, maturation and development rates. Data were statistically analyzed by analysis of variance (ANOVA, one way analysis) after arcsine transformation. Dunca's Multiple Range Test was followed for test the significant differences among treatments (Duncan, 1955).

RESULTS

Survival rate and quality of vitrified oocytes: Results presented in Table 1 showed that survival rate of vitrified oocytes was significantly ($p<0.05$) higher using spatula and hemi-straws

Table 1: Effect of cryodevice on post-thaw survival rate and quality of vitrified immature buffalo oocytes

	Cryodevice		
	Straw	Hemi-straw	Spatula
Vitrified oocytes (n)	131	106	95
Total survival oocytes			
n	105	101	91
%[(1)]	80±2.33[b]	95±2.85[a]	96±2.10[a]
Normal oocytes			
n	67	72	78
%[(2)]	51±6.13[c]	68±3.91[b]	82±4.87[a]
%[(3)]	64±7.28[b]	71±3.43[ab]	86±4.53[a]
Abnormal oocytes			
n	38	29	13
%[(2)]	29±5.65[a]	27±3.38[a]	14±4.44[b]
%[(3)]	36±7.28[b]	29±3.43[ab]	14±4.53[a]

[a and b]Means denoted within the same row with different superecripts are significantly different at $p<0.05$, [(1)]Survival rate of oocytes, [(2)]Relative to total vitrified oocytes, [(3)]Relative to post-vitrification survival oocytes

Table 2: Effect of cryodevice on maturation rate of vitrified immature buffalo oocytes

	Cryodevice			
Meiotic stage of vitrified oocytes	Straw	Hemi Straw	Spatula	Fresh
Total oocytes	108	96	93	113
Germinal visciles (G.V.) stage				
n	12	9	8	6
%[(1)]	11.1±2.43	9.4±2.34	8.6±1.30	5.3±2.08
Germinal Vesicles Breakdown (G.V.B.) stage				
n	14	13	10	9
%[(1)]	13.0±1.09	13.5±1.44	10.8±3.22	8.0±1.50
Metaphase I (M-I) stage				
n	16	16	21	19
%[(1)]	14.8±2.74	16.7±2.91	22.6±2.74	16.8±4.32
Metaphase II (M-II) stage				
n	30	33	41	69
%[(1)]	27.8±3.37^{c}	34.4±4.85^{cb}	44.0±4.33^{b}	61.1±6.11^{a}
Degenerated oocytes (DO)				
n	36	25	13	10
%[(1)]	33.3±2.47^{a}	26.0±1.72^{a}	14.0±4.04^{b}	8.8±2.44^{b}

$^{a, b \text{ and } c}$Means denoted within the same row with different superecripts are significantly different at $p<0.05$. [(1)]Relative to total vitrified oocytes

(96 and 95%) than conventional straws cryodevice (80%). Using spatula device yielded the highest ($p<0.05$) recovery rate of normal oocytes and the lowest ($p<0.05$) recovery rate of abnormal oocytes relative to vitrified or total survival oocytes as compared to other cryodevices. Generally, spatula as a vitrification device showed significantly ($p<0.05$) the highest post-thaw survival rate and the best quality of vitrified immature oocytes.

***In vitro* maturation:** Data in Table 2 showed that the effect of cryodevice on *in vitro* maturation rate of vitrified immature buffalo oocytes was significant ($p<0.05$). Percentage of oocytes arrested at metaphase II (M-II) stage for all cryodevices was significantly ($p<0.05$) lower than that of the control fresh oocytes (61.1 vs. 27.8-44%). In comparing maturation rate of vitrified immature oocytes, spatula showed significantly ($p<0.05$) higher maturation rate (44%) than that of hemi-straws and conventional straws cryodevices (34.4 and 27.8%, respectively). On the other hand, percentage of degenerated oocytes showed an opposite trend to that of those at M-II.

Such results indicated the best results of spatula as cryodevice on maturation of vitrified immature oocytes as previously proved on their survival and quality.

Cleavage rate and embryo development: Results shown in Table 3 cleared that the effect of cryodevice on cleavage rate and percentage of embryos reaching blastocyst stage relative to inseminated or cleaved oocytes was significant ($p<0.05$). Cleavage rate and percentage of embryos reaching blastocyst stage relative to inseminated or cleaved oocytes were significantly ($p<0.05$) lower for all cryodevices than that of the control fresh oocytes (37.7-58.9% vs. 70.8). In comparing the vitrification cryodevices, both hemi-straws and spatula showed significantly ($p<0.05$) higher cleavage rates (55.8 and 58.9%) and percentage of embryos at blastocyst stage relative to inseminated oocytes (6.5 and 7.7%) or cleaved oocytes (11.3 and 14.3%) than that of conventional straws cryodevice (2.1 and 5.9%, respectively).

Table 3: Effect of cryodevice on cleavage and blastocyst rate of vitrified immature buffalo oocytes

	Cryodevice			
	Straw	Hemi Straw	Spatula	Fresh
Inseminated oocytes, n	90	102	103	91
Cleaved oocytes				
n	34	53	56	64
%[1]	37.7±2.6[c]	58.9±12.73[b]	55.8±3.64[b]	70.8±6.41[a]
Embryos at blastocyst stage				
n	2	6	8	20
%[2]	2.1±1.30[b]	6.5±1.40[b]	7.7±1.09[b]	22.0±4.96[a]
%[3]	5.9±3.15[b]	11.3±1.98[b]	14.3±2.15[b]	31.3±5.95[a]

[a and b]Means denoted within the same row with different superecripts are significantly different at $p<0.05$, [1]Cleavage rate, [2]Relative to inseminated oocytes, [3]Relative to cleaved oocytes

These findings indicated similar results of both hemi-straws and spatula cryodevices on embryo development, being significantly ($p<0.05$) higher than that of conventional straws as cryodevice but these results still to be significantly ($p<0.05$) lower than that of fresh control oocytes.

DISCUSSION

Buffalo oocyte vitrification has yet to produce convincing results capable of widespread application. It is critical that researchers achieve more consistent results to further elucidate the species-specific mechanisms influencing poor survivability following vitrification and to establish a "universal" protocol that can be applied for the cryopreservation of oocytes at different developmental stages which can be used later on the commercial scale for different applications (Mahmoud and El-Sokary, 2013). The success of vitrification is based on procedures that minimize the formation of intra-cellular ice crystals when an embryo and the surrounding vitrification solution are glassified. The reduced exposure time of the embryos to osmotic stress and toxic cryoprotectant leads to a high revival rate of the stored mouse embryos (Tsang and Chow, 2009). The present results indicated pronounced impact of using spatula device for immature buffalo oocyte vitrification in term of improving survival rate and normality of vitrified oocytes as compared to conventional straws device but did not differ significantly from hemi-straws. Also, immature buffalo oocytes vitrified by spatula showed the highest rates of maturation and blastocyst production. In comparing different cryodevices of vitrified buffalo oocytes, Sharma and Purohit (2008) mentioned that the developmental capacity of oocytes could be improved by using Open Pulled Straws (OPS) as compared to the conventional straws method. However, Liang *et al.* (2012) found that the cryotop method performs better than the solid surface vitrification method in terms of viable immture oocyte yield after vitrification and subsequent *in vitro* maturation. In bovine, Hadi *et al.* (2011) mentioned that vitrification of immature bovine oocytes with the cryotop method also resulted in higher survival and nuclear maturation rates. In this respect, Sharma *et al.* (2010) reported that OPS is a cryodevice which permitted high cooling/warming rates with less cryo injuries and is suitable for cryopreservation of matured buffalo oocytes to allow better embryo development up to the blastocyst stage as compared to French mini straw. In accordance with the present results, Tsang and Chow (2009) reported that spatula device that can be readily homemade and has a large holding capacity to vitrify pre-implantation mammalian embryos in a micro-drop employing ultra-rapid cooling in Liquid Nitrogen (LN_2). Vitrified embryos have high survival rates after thawing and the fertility of the derived progeny is comparable to that of the control un-vitrified group.

Vitrification procedures of immature buffalo oocytes by hemi-straws or spatula in the current study yielded mature oocytes reached to embryos at blastocysts after *in vitro* fertilization but at lower rates than that of fresh oocytes. In buffalo oocytes, combination of meiotic arrest and vitrification under the conditions of this study is responsible for serious damages at the cellular level, making several oocytes unable to undergo *in vitro* maturation and subsequent embryonic development to blastocyst stage as compared to fresh oocytes. Thus, vitrification of immature buffalo oocytes using spatula device in this study may improve cryopreservation procedures by minimizing chilling injuries and finding less toxic cryoprotectants, as well as probably a specialized culture system to cryopreserved oocytes (Diez *et al*., 2005). These findings may suggest that the mature buffalo oocytes are an appropriate to progress in research in oocyte vitrification as compared to immature oocytes. Similarly, mature M-II goat oocytes were more suitable for vitrification compared with immature GV oocytes (Quan *et al*., 2014), although hemi-straws and cryotop are the best for vitrification of goat immature oocytes (Rao *et al*., 2012). Moreover, Vieira *et al*. (2002) reported a small proportion of immature bovine oocytes vitrified with OPS were fully competent to produce blastocysts. Moreover, blastocysts could be produced from immature buffalo oocytes subjected to slow freezing in 1.5 M DMSO (Gautam *et al*., 2008). Accordingly, immature cumulus compact buffalo oocytes had tolerate cryopreservation stress by vitrification in term of fertilization rate and blastocyst rate using hemi-straws or spatula devices. In goats, similar results were obtained by Purohit *et al*. (2012). In the present study, immature buffalo oocytes vitrified by different cryodevices (conventional straws, hemi-straws and spatula), had ability to fertilize and develop to reach blastocyst stage. Therefore, vitrification is a successful method for cryopreservation of immature buffalo oocytes, in particular, using spatula cryodevice. However, blastocyst rate obtained in the current study is still lower than fresh oocytes. Similar results were obtained by El-Sokary *et al*. (2013) on vitrification of matured buffalo oocytes. In the same line, immature ovine oocytes vitrified by Solid Surface Vitrification (SSV) as a simple and rapid procedure can survive and subsequently be matured, fertilized and cultured *in vitro* up to the blastocyst stage, although the frequency of development is low (Moawad *et al*., 2012).

Despite the great improvement of survival and cleavage rate of vitrified immature buffalo oocytes, blastocyst development still needs to be increased, Attanasio *et al*. (2010) stated that further investigations are required in order to optimize the efficiency of immature oocyte cryopreservation. Also, more research is needed into prevention of cryo injuries, presumably using lower concentrations of cryoprotectants and increased cooling and warming rates, thereby reducing exposure of oocytes to cryoprotectants (De Leon *et al*., 2012).

Generally, the reduction in the amount of cryoprotectants required in hemi-straw and spatula decreases their toxic and osmotic effects. Using both cryodevice as carriers may minimize the volume of the vitrification solution during the vitrification process. These carriers or vessels had all been used to achieve higher cooling rates, particularly spatula device and Hemi-straw system (Vandervorst *et al*., 2001).

CONCLUSION

Spatula device allows an ultra-rapid cooling during vitrification. It is a reliable alternative, inexpensive and easy to assemble for vitrification of immature buffalo oocytes. Also, vitrification spatula has the largest effective holding capacity.

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