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CRYOPRESERVATION OF IMMATURE BUFFALO OOCYTES BY VITRIFICATION

(With 6 Tables)

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تجميد بويضات الجاموس الغير ناضجة بالتزجج

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تعتبر عملية تجميد بويضات الحيوانات الغير ناضجة واستخدامها بعد الإسالة لإنتاج أجنة صالحة للنقل للام المستقبلة من أهم الوسائل الحديثة للحفاظ علي الصفات الو راثية الممتازة. وبالرغم من ذلك مازالت طرق تجميد البويضات الغير ناضجة تعطى نتائج ضعيفة مقارنة بطرق تجميد الأجنة. ولهذا تهدف الدراسة الحالية إلي دراسة بعض العوامل التي قد تؤثر في نجاح عملية تجميد البويضات مثل تحديد احسن تركيز من الاثيلين جليكول (١٠, ٢٠ ,٤٠%) في محلول الاتزان قبل وضع البويضات في محلول التجميد النهائي (محلول التزجج) وكذلك انسب وقت لتحضين البويضات في محلول الاتزان (٣, ٥, ١٠ دقيقة) قبل وضعها في محلول التزجج النهائي وكذالك دراسة تأثير إضافة السكروز والتريهالوز والميثيل سلفوكسيد إلى محلول التزجج لدراسة تأثير هذة العوامل علي الشكل الظاهري للبويضات بعد الإسالة وقدرتها علي النضوج والإخصاب والنمو معمليا. ولقد أوضحت نتائج الدراسة الحالية أن محلول الاتزان ووقت الاتزان يؤثران تأثيرا معنويا كبيرا علي الشكل الظاهري للبويضات بعد التجميد والإسالة. فلقد تبين من نتائج الدراسة الحالية أن استخدام تركيز ٢٠% من الاثيلين جليكول في محلول الاتزان وتحضين البويضات في محلول الاتزان لمدة ٥ دقائق مع إضافة السكروز إلى محلول التزجج أدى إلي استخلاص أعلى معدل للبويضات السليمة ظاهريا (٩٣,٧٣%) واقل معدل للبويضات الغير سليمة من الناحية الظاهرية (٦,١١%). كما أوضحت نتائج الدراسة الحالية أن تزجج بويضات الجاموس الغير ناضجة في محلول التزجج الذي يحتوي علي السكروز أو التريهالوز أو الميثيل سلفوكسيد نتج عنة انخفاض معنوي كبير في معدل النضوج (٤٧,٢٧ ,٢٦,٩٢, ٤٢,٠٣ % علي التوالي) وإخصاب البويضات (٣٠,٣٦ , ١٣,٢١ , ٢٣,٠٨% علي التوالي) وانقسام الأجنة معمليا (٢٣,٩١ , ٦,٦٧ , ٢١,٤٣% علي التوالي) ومعدل نمو الأجنة إلى الطور التوتي (٤,٣٥, ٠,٠٠ , ٢,٣٨ % علي التوالي) مقارنة بالمجموعة الضابطة (٧٨,٦٧ , ٥٨,٣٦ , ٤٤,٦٤ , ١٩,٦٤ % علي التوالي). كذلك أوضحت نتائج الدراسة الحالية أن جميع البويضات المزججة لم تنمو إلى طور البلاستوست مقارنة بالمجموعة الضابطة (١٠,٧١%). ولهذا يمكن أن نستنتج من هذه الدراسة أن بويضات الجاموس الغير ناضجة يمكن أن تحفظ بطريقة التزجج ويتضح ذلك من المحافظة علي الشكل الظاهري للبويضات وقدرتها علي النمو معمليا. كما يتضح من الدراسة الحالية أن أحسن طريقة لتزجج بويضات الجاموس الغير ناضجة هي وضع البويضات الغير ناضجة

في محلول الاتزان الذي يحتوي علي ٢٠% ايثلين جليكول لمدة ٥ دقائق ومحلول التزجج ٢٠% ايثلين جليكول و٠,٥ مول سكروز. ولكن لا يزال مطلوب اجراء العديد من الدراسات لتحسين معدلات نمو الأجنة معمليا من البويضات المجمدة.

SUMMARY

Cryopreservation of collected oocytes from slaughtered animals of high genetic value, for production of embryos may provide an opportunity to replenish the valuable germplasm lost. The aim of this study was to cryopreserve the immature buffalo oocytes by vitrification. Experiments were conducted to study the effect of using different ethylene glycol concentrations (10, 20 and 40%) in the equilibration solution, equilibration periods (3, 5 and 10 min) and vitrification solutions (ethylene glycol with 0.5 M sucrose, 0.3 M trehalose and 20% dimethyl sulfoxide) with a non vitrified group served as control on morphological survival, in vitro maturation and embryo development of vitrified-warmed immature buffalo oocytes. The selected cumulus oocyte complexes with compacted cumulus cells and evenly granulated ooplasm were vitrified. The present results revealed that, equilibration solution and equilibration time significantly ($P < 0.01$) decreased the proportion of morphologically normal oocytes. Using sucrose in the vitrification solution, an equilibration time of 5 min and an equilibration solution with 20% ethylene glycol (EG) yielded the highest proportion of morphologically normal oocytes (93.73%) and the lowest proportion of morphologically abnormal oocytes (6.11%). Moreover, vitrification of buffalo oocytes with different vitrification solutions, sucrose, trehalose or DMSO, significantly ($P < 0.5$) decreased the in vitro maturation rate (47.27, 26.92 and 42.03%, respectively), in vitro fertilization rate (30.36, 13.21 and 23.08%, respectively), cleavage rate (23.91, 6.67 and 21.43%, respectively) and morula development (4.35, 0.00 and 2.38%, respectively) compared to the control treatment (78.67, 58.73, 44.64 and 19.64%, respectively). The current results also showed that, all the oocytes that vitrified in different vitrification solutions failed to develop to the blastocyst stage compared to the control treatment (10.71%). Therefore, from the current results we can conclude that immature buffalo oocytes could be frozen by vitrification technique. Acceptable vitrification protocol of immature buffalo oocytes was observed when immature buffalo oocytes were vitrified using 20% EG in the equilibration solution, an equilibration time of 5 min, and a vitrification solution containing 20% EG and 0.5 M sucrose. However, further

studies are needed to improve the in vitro embryo development of the vitrified buffalo oocytes.

Key words: *Cryopreservation, buffalo, oocytes vitrification*

INTRODUCTION

In vitro embryo production technology may represent the best tool to improve genetic progress in buffalo, due to limitation of multiple ovulation and embryo transfer programs (Zicarelli, 1997). The main inadequacy of the embryo production technology in buffalo is represented by the low number of oocytes recoverable (Gasparrini, 2002). In this scenario, oocyte cryopreservation in buffalo is fundamental to increase the availability of female gametes for both research purposes and future commercial use (Gasparrini *et al.,* 2006). Oocyte cryopreservation is still an open challenge in most mammalian species, due to the extreme sensitivity of gametes to chilling injuries. However, vitrification has been used successfully to cryopreserve bovine (Vieira *et al.,* 2002), mouse (Wood *et al.,* 1993), equine (Maclellan *et al.,* 2002) and human oocytes (Bankowski *et al.,* 2005). Vitrification is a procedure that shortens the period of exposure to cryoprotectant solutions through the immediate plunging of material into liquid nitrogen (Vajta, 2000). This process results in the solidification of the material without formation of ice crystals, minimizing injuries to the oocyte cytoskeleton (Coticchio *et al.,* 2004). With the vitrification procedure, the exposure time of oocytes to cryoprotectant solutions must be short due to toxic effect of high cryoprotectant concentrations (Papis *et al.,* 2000). However, if the exposure is to short, the penetration of the cryoprotectant will be inadequate and intracellular ice could form, even in the absence of extracellar ice (Otoi *et al.,* 1998). Different cryoprotectants are used for vitrification of of mammalian oocytes and embryos from various mammalian oocytes and embryos (Vieira *et al.,* 2004). The most widely used vitrification cryoprotectants are ethylene glycol (EG), propylene glycol, glycerol, and dimethyl sulphoxide (DMSO), employed in different combinations and concentrations. Several studies demonstrated that ethylene glycol would be the ideal cryoprotectant (Shaw *et al.,* 1997), because it penetrates membranes faster than glycerol (Cha *et al.,* 2000) and is less toxic than other permeable cryoprotectants (Martino *et al.,* 1996 and Dinnyes *et al.,* 2000). Moreover, freezing solutions containing permeable (usually ethylene glycol) and non-permeable cryoprotectants, seem to be more advantageous than solutions containing just a permeable cryoprotectant

(Shaw *et al.*, 2000). The addition of a sugar (sucrose, glucose, fructose, sorbitol, saccharose, trehalose, or raffinose) to an EG-based vitrification solution influenced the overall properties of the solution (Kuleshova *et al.*, 1999). The disaccharides, sucrose and trehalose, are the most common non-permeable cryoprotectants used for oocyte cryopreservation. Sucrose acts as a stabilizer, minimizing the effects of high concentrations of ethylene glycol (Rayos *et al.*, 1994). Trehalose seems to act directly on lipids and proteins of the membrane, altering their behavior (Holt, 2000), and replacing water molecules on the membrane surface, thus inhibiting denaturation and aggregation of proteins during dehydration (Puhlev *et al.*, 2001). Therefor, the aim of the present study was to determine, the effect of equilibration solution, equilibration time, and the addition of two disacchrides in the vitrification solution in combination with ethylene glycol on immature buffalo oocytes vitrification and subsequent development in vitro.

MATERIALS and METHODS

Collection of cumulus-oocyte complexes (COCs)

Ovaries were obtained from buffalos at a local slaughterhouse. Immediately after slaughter and evisceration, ovaries were removed and placed in thermo flasks containing physiological saline, supplemented with antibiotic and antimycotic at 38°C. At the laboratory, oocytes were harvested from ovaries by aspirating the follicles (≤ 8 mm) using an 18 gauge needle attached to a 5 ml syringe. Only oocytes with at least three layers of compact cumulus cells and a homogeneous ooplasm were selected and used in the present experiments.

Experimental design

The experimental design was set up to study the effect of ethylene glycol (EG) concentrations (10, 20 and 40%) in the equilibration solutions (ES), equilibration time (3, 5 and 10 min) on the morphological characteristics of the vitrified buffalo oocytes and the effect of addition of a sugar (0.5 M sucrose and 0.3 M trehalose) to an EG-based vitrification solutions on the in vitro maturaion, fertilization and subsequent development of the vitrified buffalo oocytes. The control treatment had fresh oocytes that, immediately after selection, were submitted to the in vitro maturation (IVM) procedure.

Cryopreservation of COCs:

Equilibration and vitrification

The basic holding medium used for manipulation, equilibration, vitrification, and rehydration, was phosphate buffer saline supplemented

with 0.4% of bovine serum albumin (Sigma). For equilibration, oocytes of each treatment were kept in an ES for 3, 5 or 10 min. After the end of the equilibration time, oocytes were transferred to VS. Oocytes of all treatments were maintained in the vitrification solution (VS), for one minute. During this time, oocytes were loaded into 0.25 mL straws in the following order: a column of VS, an air bubble, a column of VS containing six to ten oocytes, an air bubble, and a column of VS. The straws were then sealed and plunged directly into liquid nitrogen at the end of one minute. Each straw was stored in liquid nitrogen for a minimum of 10 days according to Martins *et al.* (2005).

Thawing and rehydration

The oocytes of all treatments were thawed by immersion of straws in a water bath at 37°C for 30 sec. After immersion in the water bath, oocytes were rehydrated in sucrose or trehalose solutions. Oocytes were expelled into the holding medium with 0.5 M sucrose or0.3 M trehalose or sucrose and held for 5 min for one step rehydration. The oocytes were then transferred to fresh washing medium and were washed 4 times. The recovery rate was definied as the number of oocytes counted after the end of rehydration, in relation to the total of oocytes vitrified. The morphological appearance of oocytes after warming was evaluated under an inverted microscope. The oocytes with spherical and symmetrical shape and no signs of degeneration were considered normal and submitted to in vitro maturation, whereas oocytes with ruptured zona pellucida, ruptured vitelline membrane or having fragmented cytoplasm with degenerative signs were classified as abnormal and discarded.

In vitro maturation of the vitrified-thawed oocytes

The maturation medium used was TCM 199 (Sigma) supplemented with 10% FCS. In vitro maturation was carried out in Petri dishes (35mm diameter), previously equilibrated for at least 2 h at 38.5^{o}C in a moist atmosphere of 5% CO_2 in air. Oocytes were cultured in these conditions for 24 h. After this period, maturation rate was evaluated according to Dhali *et al.* (2000).

Evaluation of maturation status

After 24 h of culture, cumulus cells were removed from the COCs by repeated pipetting. Clean glass slides were prepared by laying two parallel thin rails of vaseline paraffin along with the breadth of the slide in its center. Five denuded oocytes were then placed on a slide with minimum medium. Cover slip was placed on the vaseline paraffin rails and pushed down until fluid contacted the coverslip. Oocytes were

watched carefully while pressing further, under a stereo-microscope, so as to get a good squash without breaking the zona pellucida of the oocytes. The small space left between the slide and coverslip was flushed with maturation medium. Vaseline paraffin was put on the edges of the coverslip and slide was immersed in acid–methanol for 24 h. After fixation, the slides were gently removed from the jar, the excess fixative was dried and the oocytes were stained with 1% (w/v) orcein stain for 5 min. The slides were examined under phase contrast microscope to access the state of nuclear maturation according to Costa *et al.* (1997).

In vitro fertilization (IVF) and in vitro culture (IVC)

Frozen-thawed sperm were treated by swim-up procedure in S-TALP medium for 1 h. The pellet obtained after centrifugation of supernatant was resuspended in the fertilization medium supplemented with 20µg heparin for in vitro sperm capacitation and a final concentration of 2×10^6 sperm cell/ml were used to in vitro fertilization. Insemination was performed in 50 µl drops of IVF medium under mineral oil over a 24 h period at 38.5 ^oC under humidified 5% CO_2 in air. Approximately 24 h after IVF, putative zygotes were washed twice in a Hepes-buffered TCM-199 and cultured in TCM-199 medium with Hepes modification for 7- days at 38.5 ^oC in an atmosphere of 5% CO_2 in air with maximum humidity. The proportional of cleaved oocytes was recorded 48 hour after insemination (day 0 = day of insemination), the uncleaved oocytes were discarded and embryos were transferred into fresh medium. On day 5- 7, the percentage of morula and blastocysts was recorded according to Lim *et al.* (1999).

Statistical analysis:

Data of recovery rate and morphological characteristics of the vitrified oocytes were analyzed by using Costat Computer Program, Version 3.03 copyright (1986), and were compared by the least significant difference least (LSD) at 1% and 5% levels of probability. The results were expressed as means ± S.E.M. Differences in the maturation rate, fertilization rate, cleavage, morula and blastocyst development among different treatment groups were initially compared using Chi-square (χ^2) analysis.

RESULTS

Data presented in Tables 1, 2 and 3 revealed that, equilibration solutions, equilibration times and vitrification solutions did not influence the oocytes recovery rate. However, the equilibration solutions and equilibration times influence significantly ($P < 0.01$) the proportion of

morphologically normal oocytes. Using sucrose in the vitrification solution, (Table 1), an equilibration time of 5 min and an equilibration solution with 20% ethylene glycol (EG) yielded the highest proportion of morphologically normal oocytes (93.73%) and the lowest proportion of morphologically abnormal oocytes (6.11%). Similarly, the results presented in Table 2 demonstrated that, using trehalose in the vitrification solution, an equilibration time of 5 min and an equilibration solution with 20% EG yielded the highest proportion of morphologically normal oocytes (83.68%) and the lowest proportion of morphologically abnormal oocytes (16.32%). Wheras, using DMSO in the vitrification solution, (Table 3) an equilibration time of 3 min and an equilibration solution with 20% EG yielded the highest proportion of morphologically normal oocytes (91.46%) and the lowest proportion of morphologically abnormal oocytes (8.54%). Moreover, the present results showed that, increased the concentration of equilibration solution (40% EG) and equilibration time (10 min) with all the vitrification solutions used in the currant study, yielded the lowest proportion of morphologically normal oocytes (59.46, 58.43 and 68.49%, respectively) and the highest proportion of morphologically abnormal oocytes (40.54, 41.57 and 31.51%, respectively).

Table 1: Effect of equilibration solution and equilibration time on the morphology of buffalo oocytes that vitrified with solution containing 0.5 M sucrose.

Treatments		No. Of vitrified oocytes	Recovery rate	Morphological normal oocytes	Morphological abnormal oocytes
ES	ET				
EG 10%	3	62	93.62±1.22^{a}	89.80±2.50^{a}	9.88±2.35^{d}
EG 10%	5	65	93.79±1.91^{a}	91.64±1.91^{a}	8.36±1.91^{d}
EG 10%	10	56	92.91±0.61^{a}	83.33±4.17^{ab}	16.67±4.17^{bc}
Overall			**93.44±0.63^{A}**	**88.32±1.95^{A}**	**11.63±1.96^{B}**
EG 20%	3	63	93.33±2.22^{a}	91.78±2.75^{a}	7.99±2.84^{d}
EG 20%	5	66	93.79±1.71^{a}	93.73±0.88^{a}	6.11±0.94^{d}
EG 20%	10	59	94.19±3.34^{a}	70.83±4.17^{bcd}	29.17±4.17^{abc}
Overall			**93.77±1.26^{A}**	**85.47±1.62^{A}**	**14.42±3.98^{B}**
EG 40%	3	58	90.87±2.46^{a}	73.72±9.34^{bc}	26.28±9.34^{bc}
EG 40%	5	61	91.94±1.25^{a}	63.99±3.26^{bcd}	36.01±3.26^{ab}
EG 40%	10	67	92.21±1.98^{a}	59.46±2.46^{d}	40.54±2.46 ^{a}
Overall			**91.67±1.73^{A}**	**65.73±3.61^{B}**	**34.28±3.61^{A}**
Overall all			92.96±0.58	79.84±0.52	20.11±2.69

Values with different superscrit in the same column are significantly different at least ($P < 0.5$)
ES: Equilibration solution ET: Equilibration time VS: Vitrification solution EG: Ethylen glycol

Table 2: Effect of equilibration solution and equilibration time on the morphology of buffalo oocytes that vitrified with solution containing 0.3 M trehalose.

Treatments		No. Of vitrified oocytes	Recovery rate	Morphological normal oocytes	Morphological abnormal oocytes
ES	**ET**				
EG 10%	3	61	90.07±2.97^{a}	82.40±1.31^{a}	17.60±1.31^{c}
EG 10%	5	67	93.92±3.08^{a}	80.47±2.95^{a}	19.53±2.95^{c}
EG 10%	10	69	92.79±1.37^{a}	76.62±2.19^{ab}	23.38±2.19^{bc}
Overall			93.59±1.31^{A}	79.83±1.41^{A}	20.17±1.41^{B}
EG 20%	3	69	92.87±1.10^{a}	82.99±3.84^{a}	17.00±3.84^{c}
EG 20%	5	66	92.35±1.66^{a}	83.68±0.95^{a}	16.32±0.95^{c}
EG 20%	10	63	92.13±1.44^{a}	74.21±2.59^{ab}	26.18±2.25^{bc}
Overall			92.45±0.72^{A}	80.29±2.05^{A}	19.84±2.06B
EG 40%	3	71	91.45±2.28^{a}	66.83±6.27^{bc}	33.17±6.27^{ab}
EG 40%	5	68	91.10±0.58^{a}	66.71±5.79^{bc}	33.29±5.79^{ab}
EG 40%	10	67	92.65±1.12^{a}	58.43±2.16^{c}	41.57±2.15^{a}
Overall			91.74±0.79^{A}	63.71±2.93^{B}	36.01±2.89^{A}
Overall all			92.59±0.56	75.89±2.08	25.34±1.92

Values with different superscrit in the same column are significantly different at least ($P < 0.5$)
ES: Equilibration solution ET: Equilibration time VS: Vitrification solution EG: Ethylen glycol

Table 3: Effect of equilibration solution and equilibration time on the morphology of buffalo oocytes that vitrified with solution containing 20% DMSO.

Treatments		No. Of vitrified oocytes	Recovery rate	Morphological normal oocytes	Morphological abnormal oocytes
ES	**ET**				
EG 10%	3	78	94.93±1.05^{a}	87.76±0.69^{ab}	12.24±1.05^{bc}
EG 10%	5	73	93.16±5.04^{a}	85.32±0.87^{ab}	14.68±0.87^{bc}
EG 10%	10	74	93.32±1.17^{a}	84.04±1.53^{b}	15.96±1.17^{b}
Overall			93.80±1.55^{A}	85.71±0.77^{B}	14.29±0.77^{B}
EG 20%	3	75	94.96±3.41^{a}	91.46±0.61^{a}	8.54±0.61^{c}
EG 20%	5	77	91.95±2.71^{a}	89.77±2.39^{ab}	10.23±2.39^{bc}
EG 20%	10	69	92.89±1.06^{a}	87.29±2.10^{ab}	12.71±2.10^{bc}
Overall			93.27±1.37^{A}	89.51±1.11^{A}	10.49±1.11^{C}
EG 40%	3	73	90.62±0.87^{a}	74.76±1.99^{c}	25.24±1.99^{a}
EG 40%	5	79	92.39±0.25^{a}	70.04±2.49^{c}	29.96±2.49^{a}
EG 40%	10	82	92.67±2.23^{a}	60.66±1.13^{c}	39.34±1.13^{a}
Overall			91.89±0.76^{A}	68.49±2.29^{C}	31.51±2.29^{A}
Overall all			92.98±0.72	81.23±1.98	17.77±1.98

Values with different superscrit in the same column are significantly different at least ($P < 0.5$)
ES: Equilibration solution ET: Equilibration time VS: Vitrification solution EG: Ethylen glycol

Effect of oocytes vitrification on the in vitro maturation rate:

The results presented in table 4, showed that, vitrification of buffalo oocytes with different vitrification solutions, sucrose, trehalose or DMSO, significantly ($P < 0.5$) decreased the in vitro maturation rate (47.27, 26.92 and 42.03%, respectively) compared to the control treatnent (78.67%). Data presented in Table 4, also revealed that among the different vitrification solutions, using sucrose or DMSO in the vitrification solution significantly increased ($P < 0.5$) the in vitro oocyte maturation rate (47.27 and 42.03%, respectively) compared to trehalose (26.92%).

Table 4: Effect of equilibration solution, equilibration time and vitrification solutions on the in vitro oocytes maturation rate.

Treatments			No. of oocytes	Maturation rate	
ES	ET	VS		No.	(%)
EG 20%	5	Sucrose	55	26	(47.27) b
EG 20%	5	Trehalose	52	14	(26.92) c
EG 20%	3	DMSO	69	29	(42.03) b
Control			75	59	(78.67) a

Values with different superscrit in the same column are significantly different at least ($P < 0.5$)
ES: Equilibration solution ET: Equilibration time VS: Vitrification solution EG: Ethylen glycol

Effect of oocytes vitrification on the in vitro fertilization rate:

The results presented in table 5, revealed that, vitrification of buffalo oocytes with different vitrification solutions, sucrose, trehalose or DMSO, significantly ($P < 0.5$) decreased the in vitro fertilization rate (30.36, 13.21 and 23.08%, respectively) compared to the control treatnent (58.73%). Using sucrose in the vitrification solution significantly increased ($P < 0.5$) the in vitro fertilization rate (30.36%) compared to trehalose (13.21%).

Table 5: Effect of equilibration solution, equilibration time and vitrification solutions on the in vitro oocytes fertilization rate.

Treatments			No. of oocytes	Penetration rate		Fertilization rate	
ES	ET	VS		No.	(%)	No.	(%)
EG 20%	5	Sucrose	56	24	(42.86)b	17	(30.36) b
EG 20%	5	Trehalose	53	13	(24.53)c	7	(13.21) c
EG 20%	3	DMSO	52	21	(40.38)bc	12	(23.08) bc
Control			63	43	(68.25) a	37	(58.73) a

Values with different superscrit in the same column are significantly different at least ($P < 0.5$)
ES: Equilibration solution ET: Equilibration time VS: Vitrification solution EG: Ethylen glycol

Effect of oocytes vitrification on the in vitro embryo development:

The results presented in Table 6, showed that, vitrification of buffalo oocytes with different vitrification solutions, sucrose, trehalose or DMSO, significantly ($P < 0.5$) decreased the cleavage rate (23.91, 6.67 and 21.43%, respectively) and the morula development (4.35, 0.00 and 2.38%, respectively) compared to the control treatment (44.64 and 19.64%, respectively). The current results also showed that, all the oocytes that vitrified in differant vitrification solutions failed to develop to the blastocyst stage compared to the control treatment (10.71%). Among the different vitrification solutions, using sucrose in the vitrification solution yielded the highest cleavage rate and the morula stage development (23.91 and 4.35%, respectively). Meanwhile, using trehalose in the vitrification solution yielded the lowest percentage of cleavage rate (6.67%) and all the vitrified oocytes in this solution failed to develop to the morula or the blastocyst stages.

Table 6: Effect of equilibration solution, equilibration time and vitrification solutions on the in vitro buffalo embryo development**.**

Treatments			No. of oocytes	Cleavage rate No. (%)	Morula No. (%)	Blastocyst No. (%)
ES	ET	VS				
EG20%	5	Sucrose	46	11 (23.91)b	2 (4.35)b	0 (0.00)b
EG20%	5	Trehalose	45	3 (6.67)c	0 (0.00)c	0 (0.00)b
EG20%	3	DMSO	42	9 (21.43)b	1 2.38)bc	0 (0.00)b
Control			56	25 (44.64)a	11 (19.64)a	6 (10.71)a

Values with different superscrit in the same column are significantly different at least ($P < 0.5$)
ES: Equilibration solution ET: Equilibration time VS: Vitrification solution EG: Ethylen glycol

DISCUSSION

The cryopreservation of immature oocytes would facilitate the application of assisted reproductive procedures, such as in vitro fertilization, cloning, and stem cell biology. Improvement of the vitrification method, optimization of vitrification solution contents, exposure time to vitrification solution and/or warming method may facilitate development to the blastocyst stage after vitrification of buffalo oocytes. The exposure period of the immature buffalo oocytes to cryoprotectants is of critical importance. Oocytes are generally equilibrated in a solution containing low concentration of cryoprotectants before very short exposure to vitrification solution (Wood *et al.*, 1993). The present results revealed that, the recovery rate

of morphologically normal oocytes differed significantly according to the ethylene glycol concentrations in the equilibration solutions and the equilibration times. The present results also demonstrated that, high concentration of ethylene glycol (40%) combined to equilibration time (10 min) with the vitrification solutions were detrimental to the morphological characteristics of oocytes. This effect is in consistent with Stachecki *et al.* (1998), Wani *et al.* (2004) and Martins *et al.* (2005) who found that, the toxicity of cryoprotectants varies with concentration and exposure time; the lower the concentration and the shorter the exposure time, the less toxic they are to embryo development. This may be due to biochemical (inactivation of enzymes needed for meiotic progression) and/or biophysical events (lipid elution from membranes) instead of osmotic stress (Im *et al.*, 1997). Moreover, the ethylene glycol and DMSO, commonly used cryoprotectants in vitrification protocols, cause transient increases in intracellular calcium in the matured mouse oocytes (Larman *et al.* 2006). Elevated and sustained increases in intracellular calcium can lead to inappropriate activation of proteases and phospholipases, degeneration and apoptosis (Orrenius *et al.*, 2003; Takahashi *et al.*, 2004) and consequantly a decrease in oocyte viability. The oocyte is a large single cell, with a small surface/volume ratio, and surrounded by several cumulus cell layers, that reduces the cryoprotectants entrance into the cell (Massip, 2003). Factors that influence the passage velocity of cryoprotectants through the cell membrane and entrance into the ooplasm are important in oocyte vitrification. The molecular weight (MW) of ethylene glycol, sucrose, trehalose and DMSO and their concentrations in the vitrification solutions are likely to have influenced the differences in the recovery rate of mophologically normal oocytes.

The present results revealed that, in spite of high recovery rate of morphologically normal oocytes, the rates of in vitro maturation, fertilization and embryo development were significantly lower in vitrified-warmed oocytes compared to that of control. These results are in consistent with Lim *et al.* (1999), Saxena and Maurya, (1999), Dhali *et al.* (2000), Succu *et al.* (2005) and Kelly *et al.* (2005). One of the factors that limit the maturation rate of the vitrified oocytes is the occurrence of cold injury during the process. Cold injury results in irreversible damage of the plasma membrane during short exposures to low temperatures prior to freezing (Arav *et al.*, 1993). Moreover, vitrification would lead to expansion of cumulus cells and irregular distribution of cortical granules probably due to damage of the gap

junctions between cumulus and oocytes (Arav *et al.,* 1993 and Hurt *et al.* 2000). Furthermore, the greater lipid content present in the buffalo oocytes may also be one of the factors responsible for low maturation rates, as it has been reported that the more lipid content present in the oocytes makes them more sensitive to chilling injury (Ledda *et al.,* 2001). In addition, there are also other multifactorial causes for the reduced maturation rates of the GV stage oocytes, including toxic effects of cryoprotectants, ultrastructure damage to the oocytes and deleterius effects on chromosomes and other cytoplasmic structures (Martino *et al.,* 1996 and Park *et al.,* 1997).

Moreover, the decreased in vitro fertilization and embryo development of the vitrified immature oocytes observed in the present study may be attributed to the vitrification critically damaged plasma membranes, or induced zona hardening of the vitrified oocytes and decrease the ability of fertilization (Larman *et al.,* 2006). It is possible that the poor embryo development of buffalo oocytes following vitrification is due to their higher lipid content (Boni *et al.,* 1992) that has been linked to an increased sensitivity to chilling injuries.

Therefore, from the current results we can conclude that immature buffalo oocytes could be frozen by vitrification technique as demonstrated by their ability to cleave and to develop in vitro. Acceptable vitrification protochol of buffalo oocytes was observed when immature buffalo oocytes were vitrified using 20% EG in the ES, an equilibration time of 5 min, and a VS containing 20% EG and 0.5 M sucrose. However, extensive studies are needed to improve the efficiency of oocyte vitrification in buffalo species with the prospect to utilize vitrified oocytes as a source of gametes for the in vitro embryo production and other reproductive technologies.

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