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Effect of different types of cryoprotectants on developmental capacity of vitrified-thawed immature buffalo oocytes

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SUMMARY

The study aimed to determine the effects of different types of cryoprotectants on developmental capacity of vitrified-thawed immature buffalo oocytes. The vitrification solution (VS) consisted of Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 M sucrose, 0.4% bovine serum albumin (BSA) and different types of molar (M) concentrations of the cryoprotectants which composed of either glycerol (G) , ethylene glycol (EG) or dimesthyl sulfoxide (DMSO) in order to determine the best type of vitrification cryoprotectants. The concentrations tested were 4 M, 7 M and 7M concentration of G, EG and DMSO, respectively. Cumulus oocyte complexes (COCs) were obtained from slaughterhouse ovaries. Oocytes were vitrified immediately after collection .The COCs were pre-equilibrated in 50% of the VS for 3–5 min, then kept in VS for 1 min and loaded in pre-sterilized 0.25 ml straws for 7–10 days of storage in liquid nitrogen. The straws were thawed in warm water at 37°C for 10 seconds and COCs were evaluated for morphological damage. Morphologically normal COCs were cultured in vitro and evaluated for maturation. Oocytes were fertilized with frozen-thawed semen capacitated in Brackett and Oliphant (BO) medium contained heparin and caffeine and evaluated for cleavage and embryonic development. The results revealed that the proportion of buffalo oocytes found to be morphologically normal was significantly ($p<0.05$) higher in EG and DMSO than those obtained in G (85.0 and 83.33 vs 65.0%, respectively). Among the damaged oocytes, cracking of zona pellucida was the most frequent abnormality observed. A significantly higher ($p<0.05$) percentage of maturation and cleavage rates derived from vitrified -thawed immature oocyte in EG and

DMSO than those obtained in G (47.05, 46.67%; 28.57, 25.71 vs 30.76% and 10.0%, respectively). A similar trend was observed in blastocyst stage produced *In vitro*. However, in vitro developmental competence was higher for vitrified-thawed fresh oocytes (control) than those obtained from all groups of cryoprotectants. In conclusion, that 7M solution of EG or DMSO could be used for vitrification of immature buffalo oocytes for their subsequent utilization in the in *vitro* maturation, fertilization and embryo production.

INTRODUCTION

In Buffaloes, embryo transfer has had a limited success as compared to other livestock species. There is, therefore, an increasing interest for multiplication of the superior germplasm on the large –scale basis (Wani, *et al.*, 2004). Recovery of acceptable oocytes is also low in buffaloes compared to other livestock species. The low productive capacity of the buffalo animal as evidence by less number of follicles in the ovary (Agrawal and Tommer, 1998) and high percentage of atretic follicles (Totey *et al.*, 1992) are of great concern. A large number of buffaloes are being slaughtered daily in the slaughterhouse. Cryopreservation of oocytes collected from ovaries of such animals is of practical importance to the development of reproductive technologies such as nuclear transfer, cryobanking of animal species and the routine or commercial application of breed improvement like embryo transfer (Libo *et al.*, 1996; Wani, 2002). Several cryopreservation methods such as conventional (slow), equilibrium rapid freezing (vitrification) and ultra rapid freezing have been used to preserve embryos and oocytes of many animal species resulting in the birth of live offspring. Vitrification which is a relatively recent approach is defined as a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without formation of ice crystals (Niemann, 1991). It offers several advantages over conventional equilibrium methods e.g. faster and simplified freezing and thawing procedures, high oocyte / embryo survival and no need for a freezing machine (Dhali *et al.*, 2000). Vitrification has been successfully applied for cryopreservation of bovine oocytes and embryos at various developmental stages (Le Gal and Massip, 1999).Different cryoprotectants like DMSO, EG, propylene (PROP) and glycerol have been used in different combinations for vitrification of mammalian oocytes and embryos (Palasz and Mapletoft, 1996). However, the cryopreservation protocols need substantial improvement .Therefore, the present study was undertaken to investigate the comparative effect of glycerol (G), dimesthyl sulfoxide (DMSO) and ethylene

glycol (EG) on the developmental capacity of vitrified- thawed immature buffalo oocytes.

Key words: buffaloes, oocyte, vitrification, cryoprotectant, *in vitro* maturation, fertilization.

MATERIALS AND METHODS

Collection of oocytes

Buffalo ovaries were collected from an abattoir and were transported within 2 h to the laboratory in 0.9% normal saline supplemented with antibiotics (100 ug/ml streptomycin and 100 iu/ml penicillin) at 37°C. Follicular oocytes (3-8 mm in diameter) were aspirated using 20-gauge needle attached to a 5 ml syringe. The aspiration medium consisted of M-PBS (modified phosphate buffer saline) supplemented with 10% FCS (Fetal calf serum. Sigma, USA). All the aspirated cumulus – oocyte complexes (COCs) with homogenous cytoplasm were used in the study. The oocytes were washed three times in Tissue Culture medium (TCM-199, HEPES modification with Earle's salt and L-glutamine , Sigma, USA) plus 10% FCS and 1% antibiotic – antimycotic (Gibco, Switzerland).

Vitrification of oocytes

Freshly collected oocytes were cryopreserved by ultrarapid cooling as described previously by Das (1997) with some modifications. The vitrification solution (VS) comprised Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5 M sucrose, 0.4% bovine serum albumin (BSA) and different types of molar (M) concentrations of the cryoprotectants which are either glycerol (G) , ethylene glycol (EG) or dimesthyl sulfoxide (DMSO) in order to determine the best type of vitrification cryoprotectants. The concentrations tested were 4 M, 7 M and 7M concentration for G, EG and DMSO, respectively (Yadav et al., 2008). The oocytes were pre-equilibrated in 50% of the vitrification solution (prepared by dilution of VS in DPBS) for 3–5 min and then kept in VS for 1 min and loaded (4–5 oocytes per straw) in pre-sterilized 0.25 ml straws (IMV, France).The straws were heat-sealed and pre-cooled by keeping the straws over liquid nitrogen level (LN_2) vapour for 2 min at the height of about 5 cm from LN_2. The straws were then plunged in LN_2 and stored for 7–10 days.

Thawing and evaluation

Frozen straws containing the oocytes were thawed in a water bath at 37°C for 10 seconds .The content of the straw was emptied in a 35 mm Petri dish and the oocytes were morphological evaluated. Oocyte viability was evaluated as mentioned previously (Dhali *et al.*, 2000) by their post-thaw morphological appearance under stereo microscope. Normal oocytes have spherical and symmetrical shape with no signs of lysis, membrane damage, swelling, degeneration or leakage of the cellular content; but abnormal-

oocytes show ruptured zona pellucida or having fragmented cytoplasm with signs of degeneration. The survival percentage was calculated as the proportion of normal oocytes against the total number vitrified. The cryoprotectant was removed by placing oocytes in 50% VS and then transferred to DPBS. The morphologically normal oocytes were matured and fertilized in vitro. Freshly collected oocytes were simultaneously matured in vitro and kept as control.

In Vitro maturation **(IVM):**

The morphologically normal oocytes were cultured in 50 µl of TCM-199 + 10% FCS and 1% antibiotic – antimycotic covered with mineral all (Sigma, USA) in Four-well culture plate (10 to 15 oocytes per droplet) for 24 h in a Co_2 incubator (5% Co_2 and 95% relative humidity) at 39°C. In addition, a number of COCs was run as a control group in each of vitro maturation trial. Maturation was assessed by expansion of cumulus cell mass (Schellander *et al.*, 1989).

Sperm capacitation and *in vitro* fertilization of frozen-thawed buffalo oocytes (IVF):

Fertilization was performed with frozen-thawed semen which was capacitated in BO medium (Brackett and Oliphant, 1975) containing 3.88/ml sodium caffeine benzoate (Sigma, Chemical Co. USA) and 0.02 mg/ml heparin (Sigma, USA). The spermatozoa were washed twice by centrifugation at 1850 r.p.m. for 5 minutes each time. Then the sperm suspension was diluted 1:1 with BO medium containing 20mg/ml BSA (bovine serum, albumin, fraction V. Fluka, Switzerland). The concentration of sperm for fertilization was 5 to 8 x 10^6 cells /ml of BO medium (Niawa *et al.*, 1991). After 24 hours, matured oocytes were washed 3 times in BSA-containing BO medium, and were co-cultured for 5 hours under the same condition in Co_2 incubator. After insemination, the oocytes were washed in TCM-199+10% FCS and 1% antibiotic-antimycotic 3 times and incubated for 5-6 days in Co_2 incubator. The frequency of morula and / or blastocyst was recorded.

Statistical Analysis: The experiment was replicated 5 times. The data were analyzed using Chi-square analysis according to Snedecor and Cochran (1976).

RESULTS

Following vitrification-thawing, the percentage of oocytes recovered morphologically normal or damaged is shown in Table 1. A significantly ($p<0.05$) higher proportion of morphologically normal oocytes was recovered after vitrification-thawing using EG and DMSO than those obtained in G (85.0 and 83.33 vs 65.0%, respectively). Furthermore, the proportion of damaged oocytes was significantly ($p<0.05$) higher in G (35.0%) than those in EG (15.0%) and DMSO (16.66%). Various morphological abnormalities observed in oocytes after

vitrification –thawing included cracking of zona pellucida , leakage of cellular content and shrinkage of cytoplasm but without significant difference among various cryoprotectants of buffalo oocytes (Table 2). Among the damaged oocytes, cracking of zona pellucida was the most frequent abnormality observed.

Table 1: Percentagee of immature buffalo oocytes recovered morphology normal after vitrification-thawing among different cryoprotectants.

Types of cryoprotectant	Number of oocytes vitrified	Number of morphological normal oocytes (%)	Number of damaged oocytes (%)
EG	100	85(85.00)[a]	15(15.00)[a]
DMSO	90	75(83.33)[a]	15 (16.66)[a]
G	100	65(65.00)[b]	35(35.00)[b]

Within the same column, values with different superscripts (a, b) are significantly different at least at ($p<0.05$).

Table 2: Kinds of damages observed in buffalo oocytes after vitrification-thawing

Types of cryoprotectant	Number of oocytes damaged	Kiinds of damage		
		Cracking of zona pellucida (%)	Shrinkage of cytoplasm (%)	Leakage of cellular content (%)
EG	15	8 (53.34)	3 (20.0)	4 (26.67)
DMSO	15	9 (60.00)	2 (13.34)	4 (26.67)
G	35	20 (57.14)	5 (14.28)	10 (28.57)

The maturation rates of Buffalo oocytes after thawing and removal of cryoprotectant are illustrated in Table 3. The percentage of matured oocytes was significantly ($p<0.05$) lower in oocytes cryopreserved in EG, DMSO and G as compared to control. However, among the vitrified group, the higher maturation rates were observed in EG and

DMSO (47.05 and 46.67%, $p<0.05$, G(30.76%). respectively) than those obtained in

Table 3: Maturation rates of buffalo oocytes vitrified in different cryoprotectants.

Type of cryoprotectant	Number of oocytes cultured	Number of oocyte matured (%)
EG	85	40(47.05)^{b}
DMSO	75	35 (46.67)^{b}
G	65	20(30.76)^{c}
control	100	65(65.00)^{a}

Within the same column, values with different superscripts (a, b, c) are significantly different at least at ($p<0.05$).

As shown in Table 4, a significantly higher proportion of oocytes were cleaved in EG and DMSO compared to G (28.57 and 25.71 vs 10.0%; $p<0.05$, respectively). A similar trend was observed in blastocyst produced *in vitro* while, the percentage of morula stage did not significantly vary among the different groups. On the other hand, the in vitro cleavage and blastocyst stage were significantly ($p<0.05$) lower in oocytes cryopreserved in EG, DMSO and G as compared to control.

Table 4: Cleavage and development rates of buffalo oocytes vitrified in different cryoprotectants.

Type of cryoprotectant	Number of fertilized oocytes	Cleavage rate Number (%)	Morula stage Number (%)	Blastocyst stage Number (%)
EG	40	10 (28.57)^{b}	2 (20.00)^{a}	1 (10.00)^{b}
DMSO	35	9 (25.71)^{b}	2 (22.23)^{a}	1 (11.12)^{b}
G	20	5 (10.00)^{c}	1 (20.00)^{a}	0 (0.00)^{c}
control	65	30 (46.15)^{a}	8 (26.67)^{a}	8 (26.67)^{a}

Within the same column, values with different superscripts (a, b, c) are significantly different at least at ($p<0.05$).

DISCUSSION

The results of the present study indicated that a high proportion of oocytes retain their normal morphology after a short exposure to high concentrations of different cryoprotectants except for 4M glycerol at which a high proportion of oocytes were damaged. These findings corroborate well with previous findings of Dhali *et al.* (2000); Wani *et al.* (2004) and Yadav *et al.* (2008) in buffaloes. The damage of oocytes during cryopreservation may be due to the large lipid like material found in oocytes of many species, since lipid removal or lipid polarization reduces chill and cryo-injury (Otoi *et al.*, 1997).In spite of the present high recovery rate of morphologically normal oocytes in all types of cryoprotectant, the maturation rate was lower in vitrified-thawed oocytes than those in control. Many publications concerning the problems of mammalian oocyte cryopreservation contain information regarding the negative effects of low temperature including the cytoskeleton depolymerization effects of permeable cryoprotectants (Yoon *et al.*, 2000). The freeze-thaw process is known to induce an alteration in the physic-chemical properties of intracellular lipids (Kim *et al.*, 2001) and such damages may render the oocyte incapable of retaining its developmental competence. Despite the protective effects of cryoprotectants during freezing they may impose concentration, time and temperature dependant toxicity (Fahy *et al.*,1984).On other hand, higher maturation rate of the oocytes vitrified in 7M concentration of EG and DMSO as compared to 4 M concentration of G (47.05 and 46.67% vs. 30.76, respectively). Similar findings were recorded by Wani et al. (2004) have obtained 40-44% maturation rates in buffaloes. However, the maturation rates of the oocytes vitrified in 4M glycerol (30.76%) were similar to those reported by Dhali et al. (2000) and higher than those reported by Wani *et al.* (2004). Ethylene glycol has been found to be an effective cryoprotectant for the vitrification of cattle (Delval *et al.*, 1996) and buffalo (Wani *et al.*, 2004; Yadav *et al.*, 2008) oocytes, since it offers advantages over other cryoprotectants in terms of higher permeation into oocytes/embryos for vitrification and faster removal during dilution, as its molecular weight is lower than that of glycerol (Dhali et al., 2000).Moreover, ethylene glycol has been found to be less toxic than glycerol and propylene glycol to mouse embryos (Kasai *et al.*, 1990). Furthermore, the post-vitrification survival of bovine embryos has been found to be much higher in ethylene glycol than in a combination of either DMSO, polyethylene glycol (PG) or glycerol and PG (Mahmoudzadeh *et al.*, 1993). Data presented here also indicated that buffalo oocytes vitrified in EG or DMSO cleaved and developed into morula and blastocyst stage

after thawing at higher rates than those vitrified in G. These findings were in accordance with the previous study in buffaloes (Yadav *et al.*, 2008). However, the in vitro cleavage and developmental capacity of the vitrified thawed immature buffalo oocytes were significantly lower compared to control in the present study. This could be due to the use of immature rather than mature oocytes for vitrification, since the developmental ability of oocytes frozen at germinal vesicle vesicle-stage (immature) has been reported to be much lower than that of in vivo or in vitro matured oocytes in the mice (Schroeder *et al.*, 1990) and cattle (Lim *et al.*, 1992). The debate surrounding the appropriate cell stage for cryopreservation of oocytes revolves, primarily, around cytoskeleton elements at different maturation stages (Hurtt *et al.*, 2000). Matured oocytes are more resistant to cryopreservation than immature ones (Isachenko *et al.*, 1998). The cytoskeleton of the first meiotic division in immature oocytes is particularly susceptible to damage. Matured oocytes display a more flexible cytoskeleton, which may be one reason that they are less subjected to cryodamage (Allworth and Albertini, 1993). In our investigation, the developmental capacity of the oocytes vitrified in glycerol was lower compared to those in EG or DMSO. This is may due to glycerol induces osmotic damage to the cytoplasm owing to its low membrane permeability (Szell *et al.*, 1989). Osmotic stress produced by the cryoprotectants has deleterious effect on survival of mature bovine oocytes (Martino *et al.*, 1996). The low permeability of the cells to glycerol may increase the risk of osmotic stress during thawing and dilution as the water enters the cells more quickly than the glycerol is lost. This could explain the poor survival rate and thus maturation rates with subsequent embryonic development of oocytes vitrified in glycerol.

The present study concluded that 7M solution of EG or DMSO can be used for vitrification of immature buffalo oocyte for their utilization in the *vitro* maturation and fertilization as well as embryonic production.

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تأثيرالانواع المختلفة من مضادات أثارالتجمدعلى قدرة البويضات للجاموس الغير ناضجة المزججة بعد الاسالة على الانقسام والتطور

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تهدف الدراسة لقياس تاثير تأثيرالانواع المختلفة من مضادات أثارالتجمدعلى قدرة البويضات للجاموس الغير ناضجة المزججة بعد الاسالة على الانقسام والتطور. يتكون محلول التزجج من محلول الفوسفات مضاف الية ٠.٤% من السيرم ألبومين و٠.٥ ام من سكروز مع تركيزات مختلفة هى ٤ و٧و٧ أم من الجليسرول و الايثلين جليكول أو داى ميثيل سلفواوكسيد على التوالى لاختيار انسب محلول ضد اثار التزجج . تم سحب البويضات من المبايض. تم تجميد البويضات مباشرة بعد التجميع بطريقة التزجج وتم وضعها فى محاليل مختلفة التركيز المذكورة سابقا بتركيز ٥٠% لمدة ٣ دقائق ثم نقلت الى المحاليل المختلفة بتركيزها النهائى لمدة ١ دقيقة. تم وضعت فى القصيبات ربع ملى وخزنت فى محلول السائل النتروجين لمدة اسبوع. تم أسالت القصيبات فى حمام مائى عند درجة حرارة ٣٧ مئوية لمدة ١٠ ثوانى. تم فحصها لمعرفى الطبيعى من المشوهة. تم زرعت البويضات فى وسط لأ نضجها فى حضانة ثانى اوكسيد الكربون لمدة ٢٤ ساعة. تم تلقيح البويضات معمليا بسائل منوى مسال تم تحفيزة فى وسط براكت والفين الذى يحتوى على الهيبارين والكافين وتمت متابعة البويضات الملقحة لحساب معدل الانقسام وتكوين مرحلتى الموريولا والبلاستوست. دلت النتائج على ان معدل البويضات بعد الاسالة الطبيعى يكون اعلى فى محلولى الايثلين جليكول و داى ميثيل سلفواوكسيد عن الجليسرول. كما أن أكثر التشوهات للبويضات التى لوحظت هى ثقب فى جدار البلوسيدا فى المحاليل المختلفة. معدل انضاج البويضات المسالة التى تم تجميدها يكون اعلى معنويا فى الايثلين جليكول و داى ميثيل سلفواوكسيد عن الجليسرول ونفس الشئ بالنسبة لمعدل الانقسام وتكوين مرحلة البلاستوست الا ان معدلات الانضاج والانقسام للبويضات المزججة- المسالة فى المحاليل المختلفة تكون اقل من البويضات الغير مجمدة نستنج من هذة الدراسة ان محلولى الايثلين جليكول و داى ميثيل سلفواوكسيد عند تركيز ٧ أم أنسب محلول للتجميد بطريقة التزجج لبويضات الجاموس التى يمكن الاستفادة منها فى الانضاج والاخصاب المعملى وكذلك انتاج الاجنة