EFFECT OF POLYVINYL ALCOHOL OR FETAL CALF SERUM AND WARMING TEMPERATURE ON POST-THAWING SURVIVAL RATE AND *IN VITRO* MATURATION OF VETRIFIED BUFFALO OOCYTES

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SUMMARY

The present study was designed to study the influence of two levels (2 and 4 mg/ml) of Polyvinyl alcohol (PVA), 20% fetal calf serum (FCS) and no additive in vitrification solution (VS) as well as two thawing temperature degrees (20 and 25 °C) on in vitro maturation of buffalo oocytes. Oocytes were recovered from ovarian follicles with diameter of 3-7 mm by aspiration from ovaries collected from slaughterhouse. Oocytes enclosed in a compact cumulus cells with evenly granulated cytoplasm were selected. Results show that post-thawing survival rate (SR) of all oocytes increased (P<0.05), to 77.4 and 79.6% with two levels of PVA (2 and 4 mg/ml of VS), respectively, and to 87.1% with 20% FCS vs. 62.4% for VS with no additives. The post-thawing survival rate of normal oocytes showed the same trend, being 54.0, 59 and 68.8% vs.35.4%, respectively. The effect of post-thawing warming temperature on survival rates was not significant, although these rates tended to be higher with 25 than 20°C. Maturation rate in term of percentage of oocytes at M II-stage was higher (P<0.05) for fresh than vitrified oocytes (67.6 vs. 21.2-34.0%). The percentage of oocytes at M II-stage was 34% for oocytes vitrified with VS and FCS, 26.3% and 28.7% with 2 and 4 mg/ml PVA, respectively, and 21.2% with VS with no additive (P<0.05). In conclusion supplementing VS with PVA (2 or 4 mg) or FCS (20%) improves maturation rate of vitrified buffalo oocytes.

Keyword: buffalo, in vitro, oocyte maturation, vetrification

INTRODUCTION

In a comparative study of Shamiah (1997), recovery rate of acceptable oocytes was lower in buffalo compared to bovine (5.8 *vs.*11.9%). Freezing oocytes was adopted for genetic resources conservation and to safe breeds in dangerous. Several investigators have attempted to freeze mammalian oocytes (Hernandez-Ledezma *et al.,* 1989 & Parks and Ruffing, 1987) but limited ones gave attention to buffaloes (Wani *et al.*, 2004 and Yadav *et al.,* 2008). Different protocols considering types and concentrations of cryoprotectants, numbers of equilibration steps, types of cryopreservation device, times of exposure and numbers of dilution steps at warming

(Vajta *et al.,* 1998; Massip *et al.,* 1987 and Dattena *et al.,* 2000) were executed through the last 15 years.

Number of studies were conducted to replace biological proteins in vitrification solution (VS) for rodent oocytes (Fuku *et al.*, 1995 and Nowshari and Brem 2000). However, VS for *in vitro* matured bovine oocytes are usually supplemented with fetal calf serum (FCS) and bovine serum albumin (BSA) as a macromolecular source of protein (Grill *et al.*, 1980). The macromolecules polyvinyl alcohol (PVA) and polyvinyl pyrrollidone (PVP) have also been used to substitute serum in the culture medium of bovine oocytes (Pugh *et al.,* 2000) with advantage of PVA due to its lower toxicity (Fuku *et al.,* 1995).

Critical warming rates needed to avoid ice crystal formation during warming were found to have many orders of magnitude higher than critical cooling rates needed to avoid ice crystal formation during cooling (Baudot and Odagescu, 2004).

In the light of lacking information concerning buffaloes oocyte cryopreservation, the current study was planned to investigate the effect of two factors, levels of PVA and warming temperature degrees during thawing on rehydration on *in vitro* maturation of buffalo oocytes.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center, Sakha, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture during the period from July 2006 to April 2008. All chemicals used in this study were purchased from Sigma (Madrid, Spain), unless otherwise indicated.

Oocyte recovery:

Ovaries collected from slaughtered buffaloes were placed in NaCl solution (9 mg/ml) containing antibiotics (penicillin, 100 UI/ml and streptomycin sulphate, 100 µg/ml) and maintained at 25-30 °C until oocyte recovery. The collected ovaries were washed twice in distilled water and once in freshly prepared saline. Ovarian follicles of 3-7 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 (DPBS) medium.

Vitrification and warming:

The basal solution used for vitrification was DPBS (Gibco) supplemented with polyvinyl alcohol (PVA) (average molecular weight 30,000-70,000; Sigma) at levels of 2 and 4 mg/ml to be compared with FCS (20% v: v, Sigma) and non-supplemented DPBS.

The vitrification procedures employed throughout this experiment were according to Asada *et al.* (2002) with some modification. Vitrification solution (VS) containing 40% (V: V) ethylene glycol (EG) and 0.5 M sucrose in basal medium was used. Ten 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 (LN2) within 30 sec at room temperature.

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 20 or 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, 0.5 M and 0.25 M sucrose in DPBS supplemented with 10% BSA), then in DPBS supplemented with 10% BSA for 3 min per solution, to remove of intracellular cryoprotectant (Asada *et al.,* 2002).

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 sings of lysis and damage oocytes, crack in zona pellucida, oocytes split in two halves, change in shape of oocytes and leakage of oocytes contents (Figs. 1-5, Dhali *et al.*, 2000).

Oocyte maturation:

Complex tissue culture medium (TCM-199, powder) was dissolved in deionized double distilled water and 50 µg/ml gentamicin was added to the medium. On the day of maturation, TCM-199 medium stock was supplemented with 10% FCS, 0.5 µg/ml FSH, 10 µg/ml LH (Pregnyl, Nile, CO. for Pharm. Cairo, Egypt), 1 µg/ml estradiol β_{17}, 20 mmol final concentration of pyruvate, 50 µg/ml gentamicin. The pH value of the medium was adjusted at 7.3-7.4 and osmolarity at 280-300 mOsmol/kg. 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 39°C and high humidity) for 60 minutes.

After warming, oocytes were washed three times in each of PBS plus 3% BSA and once in TCM-199. Thereafter, fresh or vitrified oocytes were placed in the medium and incubated for 24 h at 39°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 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 1 M1 and metaphase MII and degenerated oocytes: Oocytes were vaculated or cytoplasmic shrinked or chromatin condensed (Shamiah, 2004).

Statistical analysis:

The experiment was replicated 5 times for both survival rates (Factorial design) and maturation rates (one way). The data were statistically analyzed by analysis of variance (ANOVA) after arcsine transformation. Dunacn's Multiple Range Test was followed for test the significant differences among treatments or storage times (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of type of additives to vetrification solution (VS):

Adding either PVA or FCS to vertification solution (DPBS) increased ($P<0.05$) post-thawing survival rate of buffalo oocytes by about 15-25% in term of total oocytes and by about 20-34% in term of normal oocytes. However, post-thawing survival rate of abnormal oocytes insignificantly decreased from 27.1 to 18.3-23.4% by adding both levels of PVA or FCS. Adding FCS improved oocyte survival rates compared to two PVA levels, however, the differences were non-significant (Table 1).

Table 1. Post-thawing survival rate (%) of immature buffalo oocytes vitrified in DPBS medium as affected by type of additives to vetrification solution

Type of Additive	N	Post-thawing survival					
		Total oocytes		Normal oocytes		Abnormal oocytes	
		n	%	n	%	n	%
No additive	229	143	62.4[b]	81	35.4[b]	62	27.1
PVA (2 mg/ml)	252	195	77.4[a]	136	54.0[a]	59	23.4
PVA (4 mg/ml)	221	176	79.6[a]	131	59.3[a]	45	20.4
FCS (20%)	224	190	87.1[a]	147	68.8[a]	43	18.3

[a and b]: Percentages in the same column with different superscripts are significantly different at $P<0.05$.
N: Total number of vitrified oocytes.

Obtained post-thawing survival rates for recovered oocytes are lower than that recorded in buffaloes by Dhali *et al.* (2000) (92.3%) and in bovine by Asada *et al.* (2002) and Hamano *et al.* (1993) (49.5 to 87.5%). Improvement in survival rate in buffalo oocytes by adding PVA or FCS to vetrification medium comes in agreement with the results of Naitana *et al.* (1997) in sheep and Sommerfeld and Niemann (1999) in bovine. This may be due to decrease toxicity of cryoprotectans and change the physical properties of the solution (Shaw *et al.,* 2000 and Woods *et al.,* 2004). Moreover, it may protect cell membranes during freezing process (Naitana *et al.,* 1997).

Effect of warming temperature (thawing):

Post-thawing warming temperature did not affect significantly survival rates of total, normal and abnormal recovered buffalo oocytes, although survival rates of total and normal oocytes tended to be higher with 25 than 20^{o}C (Table 2).

Fig. 1. Compact oocytes cumulus cells (COCs)

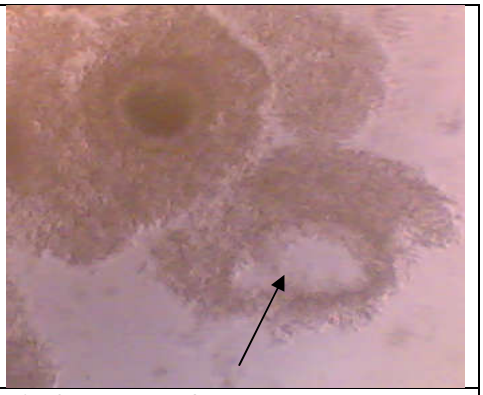

Fig. 2. Leakage of oocytes contents.

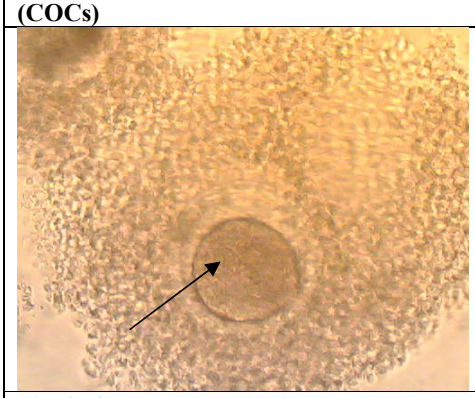

Fig. 3. Cytoplasm shrinking

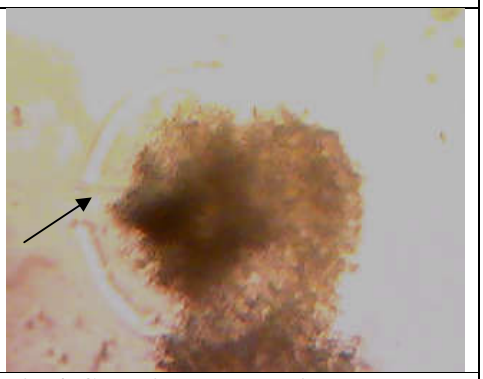

Fig. 4. Crack in zona pellucida

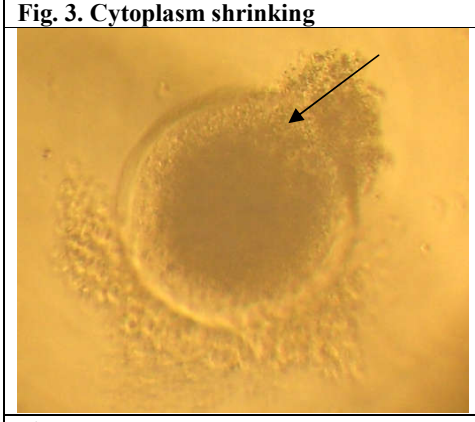

Fig. 5. Rupture oocytes

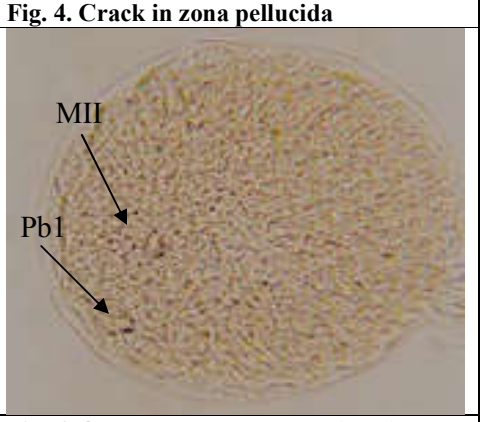

Fig. 6. Oocyte at metaphase II (M II)

Table 2. Post-thawing survival rate (%) of immature buffalo oocytes vitrified in DPBS medium as affected by warming temperature

Warming temperature (^{o}C)	N	Post-thawing survival					
		Total oocytes		Normal oocytes		Abnormal oocytes	
		n	%	n	%	n	%
20	477	357	74.8	248	52	109	22.9
25	449	347	77.3	247	55	100	22.3

N: Total number of vitrified oocytes.

Whittingham *et al*. (1972) reported that rapid warming of mammalian cells after cryopreservation was always better because cells had shorter times to recrystalize and were exposed for less time to cryoprotectants. Generally, warming temperature was reported to interacte with cooling temperature on post-thawing survival rates of oocytes (Orief *et al*., 2005).

In vitro maturation:

Present results cleared that the maturation rate in term of percentage of oocytes reached M II-stage was higher ($P<0.05$) for fresh than vitrified oocytes (Table 3 and Fig. 6). Among vitrified oocytes, the percentage of matured oocytes was higher (34%) for those vitrified in FCS, followed by 2 and 4 mg/ml PVA compared to those with no additive.

It is of interest to note that the lower percentage of oocytes at M II-stage for vitrified than fresh oocytes was attributed to degeneration of oocytes during vitrification.

Table 3. *In vitro* maturation of buffalo oocytes vitrified in vetrification solution with different additives as compared to fresh oocytes

Type of oocyte	N	Maturation stage								Degenerated oocytes	
		G.V		G.V.B		M I		M II			
		N	%	n	%	n	%	n	%	N	%
Vitrified with no additive	85	9	10.6	14	16.5	4	4.7	18	21.2^{d}	40	47.0^{a}
Vitrified with PVA (2 mg/ml)	95	14	14.7	13	13.7	5	5.3	25	26.3^{c}	38	40.0^{a}
Vitrified with PVA (4 mg/ml)	101	11	10.9	8	7.9	7	6.9	29	28.7^{c}	46	45.6^{a}
Vitrified with FCS (20 %)	100	8	8.0	7	7.0	9	9.0	34	34.0^{b}	42	42.0^{a}
Fresh oocytes	105	4	3.8	5	4.8	10	9.5	71	67.6^{a}	15	14.3^{b}

$^{a \text{ and } b}$: Percentages in the same column with different superscripts are significantly different at $P<0.05$. N : Total number of oocytes GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, M I: Metaphase I and M II: Metaphase II.

In this respect, many authors found that the developmental ability of oocytes frozen at germinal vesicle (GV) stage was lower than that *in vivo* or *in vitro* matured oocytes recovered from buffaloes (Parkway et al., 2007 and Abdoon et al., 2002) and cattle (Lim *et al.,* 1992). The ultrastructural damages to the oocytes deleterious effects on chromosome and other cytoplasmic structure during cryopreservation of

mouse (Van der Elst *et al.,* 1992) and human oocytes (Park *et al.,* 1997) may provide an rational explanation for the lower development rates of verified oocytes. Moreover, exposure to cryoprotectant or lower temperature may cause a damage in microtublar spindle formation of M II as observed in mouse and human oocytes (Pickering *et al.,* 1990 and Van der Elst *et al.,* 1988).

In conclusion supplementing VS with PVA (2 or 4 mg) or FCS (20%) improves maturation rate of vitrified buffalo oocytes.

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تأثير كحول البولى فينيل أو سيرم العجول الصغيرة و درجة حرارة الإسالة على الحيوية والإنضاج المعملي لبويضات الجاموس المزججة.

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أجريت الدراسة الحالية بغرض معرفة تأثير كل من ١- إضافة مستويين من كحول البولي فينيل (٢ و ٤ ملجم) أو ٢٠ % من سيرم العجول الصغيرة ، ٢- درجة حرارة الإسالة (٢٠ أو ٢٥ م°) على معدل حيوية بويضات الجاموس المجمدة بعد الإسالة وكذلك على معدل إنضاجها معمليا. تم جمع المبايض من المجازر المحلية في ترمس ونقلت إلى المعمل ثم جمعت البويضات من الحويصلات ذات أقطار من ٣-٧ مم . وكانت البيئة المستخدمة في التزجج لهذه البويضات (بيئة محلول الفوسفات المنظم مضاف إليها ٤٠ % من الاثلين جليكول). بعد الإسالة تم إنضاج البويضات الجيدة مورفولوجيا معمليا.

وكانت النتائج المتحصل عليها كالتالي:

١- جميع الإضافات إلى محلول التزجج أدت إلى زيادة معنوية ($p<0.05$) في معدل الاسترداد للبويضات السليمة مورفولوجيا محسوبة على أساس العدد الكلى للبويضات المزججة.

وكانت معتدلة في المستويين ٢,٤ مجم PVA (٧٧.٤ و٧٩.٦%)على التوالي وعالية مع ٢٠ % سيرم العجول الصغيرة ٨٧.١ % وكنت منخفضة في محلول التزجج بدون إضافة ٦٢.٤ %.

٢- جميع الإضافات إلى محلول التزجج أدت إلى زيادة معنوية ($p<0.05$) في معدل الاسترداد المحسوب على أساس العدد الكلى للبويضات أو على أساس عدد البويضات المسترد بعد الإسالة.

٣- لم يكن هناك اثر معنوي لدرجة حرارة الإسالة للبويضات بعد التزجج.

٤- نسبة البويضات التي وصلت إلى مرحلة الإنضاج MII (معدل الإنضاج) كانت عالية بمعنوية ($P<0.05$) في البويضات الطازجة ٦٧.٦ مقارنة بالمزججة من ٢١.٣ إلى ٣٤ %).

٥- بين البويضات المزججة كان معدل النضج للبويضات المزججة مع سيرم العجول الصغيرة كان أعلي وكان معتدل ٢٨.٧ و٢٦.٣ % مع إضافة ٢ و ٤ مجم PVA وكانت منخفضة ٢١.٣ % في البيئة المقارنة.

الخلاصة أنة يمكن استخدام PVA أو سيرم العجول الرضيعة لتحسين معدل إنضاج البويضات