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Buffalo Oocytes Maturation *In Vitro* as Affected by Vitrification of Whole Ovaries or Oocytes

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ABSTRACT

Aim of this paper is to find the possibility of *in vitro* maturation of buffalo oocytes recovered from vitrified whole ovaries. Ovaries from slaughtered buffaloes (n=400) were collected; out of these ovaries, 150 were fresh and 250 buffalo ovaries were vitrified and thawed. Number of all visible follicles was recorded on fresh ovaries and on each ovarian surface pre- and post vitrification, then oocytes recovery rate was calculated in fresh or vitrified ovaries. Oocytes were recovered by aspiration. From the recovered oocytes from fresh ovaries, COCs were vitrified by straw cryodevice. Post-thawing, morphologically normal oocytes from vitrified or fresh ovaries were vitrified. Results showed that numbers of total and normal follicles, and total and normal oocytes per ovary were significantly higher in fresh than in vitrified ovaries. Total number of abnormal follicles showed significantly ($P<0.01$) an opposite trend, while, the difference in number of abnormal oocytes/ovary was not significant. Oocytes recovered from fresh ovaries showed significantly higher recovery and normality rates than those recovered from vitrified ovaries. Percentage of compact and expanded oocytes was significantly higher, while percentage of denuded and partial denuded oocytes was significantly lower when oocytes were recovered from fresh than from vitrified ovaries. Maturation rate (MII-oocytes percent) was higher ($P<0.05$) when oocytes were recovered from fresh than from vitrified ovaries and those vitrified after recovery (62.50% vs. 35.90 and 27.50%, respectively). In conclusion, vitrification of the whole buffalo ovaries is a positive tool for genetic sources cryopreservation in term of beneficial effects on *in vitro* maturation of oocytes when compare with those directly vitrified after recovery from fresh ovaries.

Keywords: Buffalo, vitrification, whole ovary, oocyte, *in vitro* maturation.

INTRODUCTION

The ovary is a complex structure composed of several different types of cells. Cryopreservation of ovarian tissue is more difficult because different cell types have different requirements for optimal survival (Segino *et al.,* 2005). The ovarian follicles as basic structural and functional units of the mammalian ovaries provide the microenvironment necessary for oocyte growth and maturation (Saber, 2009). Cryopreservation of the whole ovaries in different species has become more important not as a tool of genetic resource and biodiversity conservation as well as a biomedical application (Van Hanh *et al.,* 2016).

Slow freezing is performed using a programmable freezer that undergoes a controlled temperature change by a computer program. However, vitrification is a rapid cooling method in which the tissue is directly plunged into liquid nitrogen after equilibration in cryoprotectants with high concentrations (Motohashi and Ishibashi, 2016). Vitrification is a good method for cryopreservation of the ovarian follicles, oocytes and embryos, in term of survival and development rates (Cha *et al.,* 2011; Mochida *et al.,* 2013), but its application to whole ovaries has been considered to be difficult (Migishima *et al.,* 2003).

In general, the vitrified follicles and oocytes have been found to mature somewhat more slowly than fresh samples (Haidari *et al.,* 2008; Desai *et al.,* 2011). However, the further development of vitrified follicles compared with non-vitrified ones is controversial (Segino *et al.,* 2005; Abedelahi *et al.,* 2010). Several alterations in the physical and chemical properties of cells occur during cryopreservation associated with osmotic forces created during dehydration, cooling, rehydration, and warming (Mojdeh *et al.,* 2013), including cell membrane and cyto-skeletal integrity loss, mitochondrial depolarization, and increased reactive oxygen species (ROS) production (Demant *et al.,* 2012; Liang *et al.,* 2012).

Survival of vitrified ovarian tissues in rabbit and monkey after transplantation into rat uteri (Kagabu and Umezu, 2000) and live offspring have been born from vitrified mouse ovarian follicles matured *in vitro* (de la Pena *et al.,* 2002) was reported. Also, oocytes at metaphase II after *in vitro* maturation were obtained from isolated follicles from vitrified ovarian tissue in sheep (Al-Aghbari and Menino, 2002; Courbiere *et al.,* 2006) and rabbits (Meherz *et al.,* 2017). Moreover, birth of four lambs after auto-transplantation of vitrified warmed ovarian cortex into ewes was reported (Bordes *et al.,* 2005). Recently,

Although ovarian tissue cryopreservation was successful with *in vitro* maturation, until now there were no reports on successful *in vitro* maturation of oocytes recovered from whole buffalo ovaries. Therefore, the aim of this study is to find the possibility of *in vitro* maturation of buffalo oocytes recovered from vitrified whole ovaries.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center (ILMTC), belonging to the Animal Production Research Institute, Agriculture Research Center, Ministry of Agriculture. All chemicals used in this study were purchased from Sigma (Sant Luis, MO, USA).

Collection of ovaries:

Ovaries (n=400) were collected from unknown 200 buffaloes slaughtered in El-Batanoun slaughter house, Menofeya Governorate. Ovaries were transported to the laboratory within 3-4 hours after slaughtering in thermos in

normal saline (0.9% Nacl) containing 50 µg/ml of gentamicin at temperature of 25-30°C. In the laboratory, removal of extraneous ovarian tissues and ovaries washing in phosphate buffer saline (PBS) with pH value of 7.3 (3 times) were carried out.

Vitrification and thawing process of ovaries:

Out of 400 collected ovaries, 250 buffalo ovaries were vitrified in tissue culture medium (TCM-199) as a basic medium (BM). This medium contained fetal calf serum (FCS, 20% *v: v*) and cryoprotectants, namely ethylin glycol (EG) and dimethylsulfoxide (DMSO). Ovaries were placed in BM with 7.5% EG and 7.5% DMSO as a 1st vitrification solution (VS1) for 15 min at room temperature, then transferred into BM with 15% DMSO, 15% EG and 0.5 M sucrose as a 2nd vitrification solution (VS2) for 5 min at 4°C, instantly ovaries were loaded in large tube and plunged in liquid nitrogen (LN2).

The vitrified ovaries stored in LN2, for 4 weeks at least, were thawed by holding the large tube in air for 30 s and in water bath (20°C) for 30 s at least. Ovaries were placed into BM containing 20% FCS and 0.5 M sucrose, then in BM with 0.25 M sucrose, BM with 0.125 M sucrose and finally in BM without sucrose, for 5 min/solution to remove intracellular cryoprotectants from vitrification ovaries.

Oocyte collection and evaluation:

Normal follicle number on the surface of the fresh ovaries (n=150) and pre- or post- vitrification of the vitrified ovaries (n=250) was recorded to calculate oocyte recovery rate of fresh or vitrified ovaries. Oocytes were recovered by aspiration using 18-gauge needle attached by syringe (5 ml) containing 1 ml PBS supplemented with FCS (20%) and gentamicin (50 µg/ml) in the Petri dishes and kept undisturbed for 5 min, allowing the oocytes to settle down. Under stereomicroscopy, oocytes were examined, then transferred into a searching dish containing PBS for and evaluated according to Hammad *et al.* (2015) for compaction, cumulus cell layer number and ooplasm homogeneity into four categories: cumulus oocytes-complexes (COCs) expanded (ECs), denuded (DOs) and partial denuded (PDOs) oocytes.

Vitrification, thawing and evaluation of oocyte viability:

From the recovered oocytes from fresh ovaries, COCs were vitrified by straw (0.25 ml plastic, IMV, L'Aigle, France) cryodevice according to the procedures of Shayegh and Barati (2011) with minor modifications. TCM-199 supplemented with 20% of FCS (*v: v*) was used as a basic medium (BM) as well as EG and DMSO. The COCs were vitrified by placing them in BM with 10% EG and 10% DMSO as V1 for 5 min, then transferred into BM with 20% DMSO, 20% EG and 0.5 M sucroseas V2 for 30 s. Instantly, oocytes were loaded in straw and plunged in LN2.

After storage of 3-4 weeks, straws of COCs were warmed for 6 s in air and in water bath (20°C) for at least 10 s, and then COCs with straws contents were expelled into Petri dishes. Thereafter, oocytes were transferred in BM plus 0.25 M sucrose for 5 min and in buffer solution (BS) plus 0.125 M sucrose for 5 min. Finally, COCs were washed (2 times) in BS free from sucrose for 5 min to remove the intracellular cryoprotectants effects (Hajarian *et al.,* 2011),

***In vitro* maturation:**

In vitro maturation of post-thawed morphologically normal oocytes from vitrified or fresh ovaries was conducted in 100 µl of TCM-199 supplemented with FCS (20%), oestradiol-17β (1 µg/ml) and gentamicin (50 µg/ml) in four-well culture plates (10 oocytes /droplet) in Petri dishes covered with mineral oil. The duration of oocyte maturation was done for 24 h at 38°C in a CO2 incubator (5% CO2, humidified air). After maturation period, oocytes were washed in PBS containing hyaluronidase (1 mg /ml) to remove the cumulus cells, then washed twice with PBS supplemented with 2% bovine serum albumin, and loaded on clean slide. Slides were placed for 24 h into fixation solution (3 ethanol: 1 glacial acetic acid) and stained (1% orcein in 45% glacial acetic acid). Oocytes were evaluated under a microscope and only percentage of metaphase-II oocytes were considered to be matured (reduced number of chromatin, metaphase plate and extrusion of the 1st polar body according to Purohit *et al.* (2012).

Statistical analysis:

Data of the comparison between fresh and vitrified ovaries were analyzed by independent T-test using SAS (2002) software. However, data of *in vitro* maturation of oocytes were analyzed using Chi-Square test.

RESULTS AND DISCUSSION

Yield of follicles and oocytes:

Numbers of total and normal follicles, and total and normal oocytes per ovary were significantly higher in fresh than in vitrified ovaries. However, total number of abnormal follicles showed significantly (P<0.01) an opposite trend, while, the difference in number of abnormal oocytes/ovary was not significant. On the same line, oocytes recovered from fresh ovaries showed significantly higher recovery and normality rates than those recovered from vitrified ovaries (Table 1).

Table 1. Number of follicles and oocytes on surface of fresh and vitrified ovaries.

Item	Fresh ovaries	Vitrified ovaries	T-Value	P-Value
Total number of ovaries	150	250	-	-
Number of ovarian follicles:				
Total	710	850	-	-
Total/ovary	4.73±0.40	3.40±0.20	2.95	0.05*
Total normal	550	350	-	-
Total normal/ovary	3.67±0.13	1.40±0.13	5.17	0.001***
Total abnormal	160	500		
Total abnormal/ovary	1.06±0.07	2.00±0.13	4.78	0.01**
Number of recovered oocytes:				
Total	412	220	-	-
Total/ovary	2.75±0.23	0.88±0.03	7.98	0.01**
Normal/ovary	130.00±10.50	43.75±3.88	7.70	0.01**
Abnormal/ovary	7.33±2.19	11.25±2.43	1.20	NS
Recovery rate	58.37±4.18	26.09±2.04	6.93	0.01**
Normality rate	75.08±4.66	63.82±2.84	2.07	0.05*

NS: Not significant. * Significant at P<0.05. ** Significant at P<0.01. * Significant at P<0.001**

In accordance with the present results in buffaloes, Meherz *et al.* (2017) found the same trend in rabbit ovaries. Also, Babaei *et al.* (2007) found that the proportion of atretic follicles in non-vitrified and vitrified mouse ovaries differed significantly. The number of pre-antral follicles was significantly higher from fresh than those from frozen/thawed ovaries (Miwa *et al.,* 2005). In rat, Sugimoto *et al.* (2000) observed that ovarian follicles survived after vitrification and transplantation with a decrease in the number of healthy antral follicles. In rat, the frozen-thawed ovaries contained significantly fewer follicles than the fresh ovaries (Migishima *et al.,* 2003). Moreover, Baird *et al.* (1999) found that about 28% of primordial follicles survived after transplantation of frozen/thawed ovarian tissue.

Vitrification is better than slow freezing for ovarian stroma preservation (Keros *et al.,* 2009). In this respect, Amorim *et al.* (2003) obtained high success rates of vitrified ovaries in mice, because they are smaller and less fibrous than those of larger mammals. They added that cryoprotectants efficiency to permeate whole mice ovaries was more into mice ovaries, which are more porous than large mammal ovaries such as cow, sheep and human.

The observed reduction in normality of follicles or oocytes of fresh and vitrified ovaries may be attributed to that cryopreservation causes alterations in the physical and chemical properties of cells, including loss of cell membrane and cytoskeletal integrity, mitochondrial depolarization, and increased production of reactive oxygen species (Demant *et al.,* 2012; Liang *et al .,* 2012). These alterations are associated with osmotic forces created during dehydration, cooling, rehydration, and warming and may affect mainly cytoplasmic activities such as mitochondrial function, metabolism, and intracellular signaling pathways (Mojdeh *et al.,* 2013).

Oocytes category:

Percentage of compact and expanded oocytes was significantly higher, while percentage of denuded and partial denuded oocytes was significantly lower when oocytes were recovered from fresh than from vitrified ovaries (Table 2).

It is of interest to note that percentage of compact oocyte showed the highest frequency distribution when oocytes were recovered from fresh or vitrified ovaries. Also, vitrification of the whole ovaries resulted in reduction in expanded oocytes and increasing denuded and partial denuded oocytes (Table 2). Nearly similar trend was obtained by Meherz *et al.* (2017) on vitrified rabbit ovaries. On the other hand, no significant differences were observed in the proportion of normal oocytes between fresh and vitrified mouse ovaries (Mojdeh *et al.,* 2013) and also between the fresh and vitrified transplanted ovaries in regards to the number of litters (Liu *et al.,* 2008).

Table 2. Frequency distribution of different oocyte categories recovered from fresh and vitrified ovaries.

Item	Fresh ovaries	Vitrified ovaries	T-Value	P-Value
Total normal oocytes (n)	390	175	-	-
Compact oocytes, n	230	78	-	-
Compact oocytes, %	58.97±4.40	44.57±1.55	12.23	0.001***
Expanded oocytes, n	95	17	-	-
Expanded oocytes, %	24.36±3.85	9.71±0.47	7.08	0.01**
Denuded oocytes, n	25	45	-	-
Denuded oocytes, %	6.41±1.20	25.71±1.49	6.52	0.01**
Partial denuded oocytes, n	40	35	-	-
Partial denuded oocytes, %	10.26± 2.90	20.01±1.11	4.47	0.025*

*** Significant at P<0.05. ** Significant at P<0.01.**
***** Significant at P<0.001.**

***In vitro* maturation:**

Percentage of oocytes reached to MII stage (Maturation rate) significantly (P<0.05) increased when oocytes were recovered from fresh than vitrified ovaries or that of vitrified oocytes (62.50% vs. 35.90 and 27.50%, respectively). It is of interest to note that maturation rate was higher for oocytes of vitrified ovaries than that of vitrified oocytes, but the difference was not significant. However, there is a wide variation in maturation rate of fresh-ovaries oocytes and those exposed to vitrification (ovaries or oocytes). The present results indicated that the whole ovary vitrification had impact on oocyte maturation rather than direct oocyte vitrification. This trend was associated with an opposite trend in percentage of degenerated oocytes (Table 3). In agreement with the obtained results, Mojdeh *et al.* (2013) found that maturation rate of oocytes was significantly lower in vitrified samples compared to control sample. In the present study, oocyte maturation rate of fresh- or vitrified-ovaries in buffaloes was lower than those obtained for ovaries of other species. In this respect, percentage of *in vitro* matured murine oocytes was 62.2%, when they were recovered from vitrified-warmed ovaries as compared 86.4% in the control group (Wang *et al.,* 2011) and was 77% of mice oocytes recovered from vitrified ovarian ovaries versus 84% for those recovered from slow-cooled ovaries or 83% for fresh oocytes ((Wang *et al.,* 2009).

Table 3. Maturation rate of oocytes recovered from fresh, vitrified ovaries and vitrified oocytes.

Item	N	Oocyte stage									
		Germinal vesicles		Germinal vesicles breakdown		Metaphase-I		Metaphase-II		Degenerated oocytes	
		n	%	n	%	n	%	n	%	n	%
Fresh oocytes	80	5	6.25	6	7.50^{b}	10	12.50	50	62.50^{a}	9	11.25^{b}
Oocytes of vitrified ovaries	78	7	8.97	13	16.67^{ab}	15	19.23	28	35.90^{b}	15	19.23^{ab}
Vitrified oocytes	80	10	12.50	16	20.00^{a}	13	16.25	22	27.50^{b}	19	23.75^{a}

Means with different superscripts within the same column are significantly different at P<0.05. N: Total oocytes.

The noted differences in *in vitro* maturation of oocytes from vitrified ovaries may be attributed to variation in ovarian tissues of different species, cryoprotectants used and/or vitrification procedures.

These findings may provide a rational explanation for the lower development rates of verified oocytes. The observed reduction in *in vitro* maturation of oocytes recovered from vitrified ovaries or oocyte directly vitrified from fresh ovaries may be due to several reasons, including damages in the oocyte ultra-structure as well as chromosomal and cytoplasmic deleterious effects during cryopreservation as reported in mouse (Van der Elst *et al.,* 1993) and human (Park *et al.,* 1997) oocytes. Moreover, exposure of oocytes to cryoprotectants or lower temperature may cause damage in microtublar spindle formation of MII as observed in mouse oocytes (Pickering and Johnson, 1987).

CONCLUSION

Based on the obtained results, vitrification of the whole buffalo ovaries is a positive tool for genetic sources cryopreservation in term of beneficial effects on *in vitro* maturation of oocytes when compare with those directly vitrified after recovery from fresh ovaries.

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الإنضاج المعملي لبويضات الجاموس تحت تأثير التجميد بالتزجج للمبايض الكاملة أو البويضات

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أجريت هذه الدراسة لمعرفة مدى امكانية انضاج بويضات الجاموس المسترده من تجميد المبايض الكاملة . استخدم في هذه الدراسه 400 مبيض تم تجميع البويضات بعد الذبح مباشرة من مجزر محلي حيث تم تجميد 250 مبيض بالكامل وظل 150 مبيض طازج بدون تجميد. تم تسجيل عدد الحويصلات لكل من المبايض الطازجه والمجمده ثم حساب معدل إسترداد البويضات لكل من المبايض الطازجه والمجمده والتي تم تجميعها بواسط عملية الشفط . تم تجميد البويضات المسترده من المبايض الطازجه ثم اسالة المبايض المجمده وجمع البويضات من المبايض الطازجه والمجمده. وقد تم الحصول على النتائج التالية: لوحظ زيادة العدد الكلي للحويصلات والبويضات الطبيعيه المسترده من المبايض الطازجه لكل مبيض معنويا مقارنة بالحويصلات والبويضات المسترده من المبياض المجمده . كما لوحظ زيادة فى عدد الحويصلات والبويضات غير الطبيعيه في المبايض المجمده مقارنة بالمبايض الطازجه، ولكن بدرجه معنويه للحويصلات فقط. زاد معدل استرداد البويضات الكليه والطبيعيه المسترده من المبايض الطازجه معنويا مقارنة المبايض المجمده. كما لوحظ زيادة النسبه المؤيه للبويضات الجيدة والممتدة وانخفاض عدد البويضات المعراه كليا والمعراه جزئيا المسترده من المبايض الطازجه مقارنه بالمبايض المجمده. زاد معدل الانضاج المعملي للبويضات المسترده من المبايض الطازجه (62.5%) التي وصلت لمرحلة الطور الميتوزي الثاني عن البويضات المسترده من المبايض المجمده (35.9%) و البويضات المجمده (27.5%). نستخلص من هذه الدراسة ان التجميد الكلي للمبايض هو وسيله جيده لحفظ المصادر الوراثيه مقارنة بالتجميد المباشر للبويضات المستردة من المبايض الطازجه.