IN VITRO FERTILIZATION OF VITRIFIED IMMATURE OR MATURE OOCYTES AS AFFECTED BY MECHANICAL OR NATURAL DENUDATION

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SUMMARY

The current study aimed to evaluate the role of cumulus-corona radiate complex of immature or *in vitro* matured bovine oocytes on their vitrification, *in vitro* fertilization and embryo development. Bovine ovaries were collected from an abattoir and follicular oocytes (3-8 mm in diameter) were aspirated. Oocytes were examined and classified into cumulus oocytes–complexes (COCs), natural denuded oocytes (NDOs) and mechanically denuded oocytes (MDOs). Natural COCs were mechanically denuded by repeated pipetting in phosphate buffer solution (PBS). All types of oocytes were cryopreserved by vitrification as immature or *in vitro* matured for 24 h prior to vitrification by open-pulled straw cryodevice. After at least 2 weeks of vitrification, all types of vitrified oocytes were evaluated for survival and viability (Normal and abnormal oocytes). Morphologically normal immature oocytes were matured and fertilized *in vitro*, while morphologically normal mature oocytes were directly fertilized *in vitro* for determination of cleavage rate (CR). After co-culture of cleaved oocytes for 7 days, rate of morula (MPR) and/or blastocysts (BPR) production was recorded. Results show that survival (SR) and normality (NR) rates were the highest for CCOs (84.47 and 81.73%), followed by MDOs (73.28 and 62.0%), and the lowest for NDOs (60.71 and 54.39%), respectively ($P<0.05$). SR was higher ($P<0.05$) for mature than immature oocytes (78.03 vs. 67.60%), while NR was nearly similar for both. CR, MPR and BPR were higher ($P<0.05$) for CCOs (45.28, 18.05 and 15.28%) than those of MDOs (29.72, 6.06 and 3.03%) and NDOs (25.24, 7.69 and 7.69%), respectively. CR was higher ($P<0.05$) in mature than in immature oocytes (41.93 vs. 29.36%), while MPR and BPR were nearly similar in both. Based on the obtained results, cumulus cell surrounding cumulus oocytes–complexes play a vital role for survival of bovine oocytes during vitrification and successful *in vitro* maturation and fertilization as well as embryonic development to morula and blastocyst stages.

Keywords: *Buffalo, oocyte, fertilization, mechanical, natural denudation.*

INTRODUCTION

The cryopreservation of mammalian embryos and oocytes has become an integral part of methods to control animal reproduction as preservation and manangement of genetic resources (El-Shahat and Hammam, 2014; Babaei *et al.,* 2006). Cryopreservation of oocytes from slaughtered animals has great value in increasing the availability of materials for basic research and their subsequent utilization for embryo production may provide an opportunity to replenish the endangered species gene banking and the genetic improvement of the livestock species (Nucharin Sripunya, 2011). Recent advances in freezing techniques like vitrification procedure with modifications in equilibration time, concentration of cryoprotectants (CPAs), volume and dilution protocol resulted in a higher survival of different stages of oocytes to maturation and fertilization *in vitro* (Rienzi *et al.,* 2010). Vitrification is used to avoid chilling injury and ice crystal formation in the cryopreservation of tissue, oocytes and embryos (Abrishami *et al.*, 2010, Zhou *et al*., 2010). Although vitrification does not require a sophisticated and expensive programmable cell freezer, and is a fairly quick procedure, it requires skill and experience (Prentice-Biensch *et al.* 2012). The open pulled straw (OPS) method, which uses higher concentrations of CPAs, has resulted in successful cryopreservation of oocytes in variety of species (Vieira *et al*., 2002).

A higher number of competent oocytes for *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) to obtain superior transferable bovine embryos coupled with development of freezing technique through vitrification will entail more productivity from the non-descript animals (Dutta et al., 2013). Oocytes can be cryopreserved at immature or mature metaphase II (M-II) stage (Prentice and Anzar, 2011; Fernández-Reyez et al., 2012). Stage of development of oocytes and the presence or absence of cumulus cells are considered variables affect the outcome of vitrification of oocytes (Purohit et *al*., 2012; Karima *et al*.,

2014). Immature oocytes were usually treated as an object better suitable for cryopreservation as having less number of vulnerable structures, such as condensed chromosomes and with genetic material protected by nuclear envelope (Luciano *et al.,* 2009; Prentice and Anzar, 2011), but mature oocytes have not yet been effectively cryopreserved, due to their structural sensitivity to cooling and freezing and to the exposure to CPAs (Modina *et al.,* 2004). Removing the cumulus cell prior to IVM or vitrification have shown to have a detrimental effect on oocyte morphology for immature vitrified bovine (Zhou et al., 2010), buffalo (Gasparrini *et al.,* 2007), goat (Purohit et al., 2012), equine (Tharasanit *et al.,* 2009) and mouse (Suo *et al.,* 2009) oocytes. In goat, Purohit *et al.* (2012) found that cumulus compact oocytes (COCs) are less vulnerable to cryo-injuries compared to their denuded counterparts. Few studies point out severe damage to *in vitro* matured oocytes when vitrified and subsequently fertilized *in vitro* (Taru Sharma et al., 2006). The immature oocyte should be considered as a one functional unit with, cumulus cell communicating each other via cellular projections penetrating across a zona pellucida. Undisturbed COCs communication and co-operation seem essential for adequate maturation process (Gilchrist, 2011), so an optimum cryopreservation method should provide protection for oocyte, cumulus cells and their intercommunication system.

Although immature oocytes vitrified without cumulus were matured and fertilized *in vitro* with acceptable efficiency (Luciano et al. 2009; Zhou et al. 2010), their further development as embryos was compromised (Modina et al., 2004). However, less information is known about the efficiency and the consequences of cryopreservation on immature and *in vitro* matured oocytes (Fausta *et al.,* 2013). In addition, efficiency of immature oocyte cryopreservation is rather low, regardless of method used (Papis *et al.,* 2013). Therefore, the present study aimed to evaluate the effect of developmental stage of vitrified oocytes (immature or mature) and the presence or absence of cumulus cell layer (cumulus compact, natural denuded or mechanically denuded oocytes) on *in vitro* fertilization and developmental competence of bovine oocytes.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center (ILMTC), belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture.

Oocytes collection

Bovine ovaries were collected from an abattoir and transported within 2-3 h to the laboratory in normal saline (0.9% NaCl) supplemented with (50 µg/ml) at -27-30°C. Follicular oocytes (3-8 mm in diameter) were aspired using 18-gauge needle attached to a 5 ml syringe containing 2 ml of (DPBS) with 20% fetal calve serum (FCS, sigma) and antibiotics (50 µg/ml gentamicin) as harvesting medium. Oocytes were examined under stereomicroscopy and classified according to their compaction, number of cumulus cell layers and homogeneity of ooplasm according to Ravindranatha *et al.* (2003) into 4 categories namely cumulus oocytes–complexes (COCs), expanded cumulus cells oocytes, natural denuded oocytes (NDOs), partial denuded oocytes.

Only, COCs and NDOs were used in this study.

Mechanical denudation of oocytes

Natural COCs were mechanically denuded (MDOs) by repeated pipetting in PBS supplemented 0.025% hyaluronidase solution (Sigma, St. Louis, MO) till the complete separation of the cluster of cumulus cells according to Papis *et al.* (2013). Therefore, vitrified immature or *in vitro* matured oocytes at three developmental stages, namely COCs, NDOs and MDOs were used in this study.

Vitrification of oocytes

All types of immature oocytes were cryopreserved by vitrification using open-pulled straw cryodevice (OPS) and then matured *in vitro* for 24 h. However, all types of *in vitro* matured oocytes were also vitrified by OPS.

The vitrification procedures employed throughout this experiment were based on the methods originally designed by (Shayegh and Barati, 2011) with minor modifications. TCM-199 medium (Sigma) supplemented with 20% (v: v) of FCS as a basic medium (BM) as well as ethylin glycol (EG) and dimethyl sulfoxide (DMSO) as cryoprotectants were used. Different types of immature or mature oocytes were vitrified by placing them in the first vitrification solution (V1, 10% EG+10% DMSO in BM) for 5

min, then they were transferred into the second vitrification solution (VS2: 20% DMSO, 20% EG, and 0.5M sucrose in BM) for 30 s, instantly oocytes were loaded in OPS and plunged in liquid nitrogen (LN2).

Thawing and evaluation of oocyte viability

After storage for at least 2 weeks in LN2, all types of vitrified oocytes(immature or mature) were warmed by holding the OPS for 6 s in air and then agitating them in water bath at 20 °C for at least 10 s. The contents of OPS were expelled into Petri dish. To remove of intracellular cryoprotectants effects, oocytes were transferred in BM plus 0.25M sucrose for 5 min and then transferred to buffer solution (BS) plus 0.125M sucrose solution for 5 min and finally, the oocytes were washed twice in BS without sucrose for 5 min according to Hajarian *et al.* (2011) with minor modifications.

Oocyte viability (survival) was evaluated morphologically based on the integrity of the oolemma and zona pellucida; loss of membrane integrity (lysis) 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 sing of lysis, and damaged oocytes (abnormal) with crack in zona pellucida, split in two halves, change in shape and leakage of contents.

The survival rate was calculated as the proportion of normal morphology oocytes against the total number of vitrified oocytes. Thereafter, morphologically normal immature oocytes were matured and fertilized *in vitro, while* morphologically normal mature oocytes were fertilized *in vitro.*

In vitro maturation

All types of fresh or post-thawing vitrified immature oocytes (morphologically normal) were cultured in 500 µl of TCM-199+20% FCS+1 µg/ml oestradiol-17β (Sigma) and 50 µg/ml gentamicin covered with mineral oil (Sigma) in four-well culture plates (8-10 oocytes per droplet) for 24 h in a CO_2 incubator (5% CO_2 and in humidified air at 38°C). Oocytes were washed using DPBS containing 1 mg /ml hyaluronidase to remove the cumulus cells. Then, oocytes were washed two times with PBS supplemented with 2% bovine serum albumin (BSA), and loaded on clean slide. Slides were placed into fixation solution (3 ethanol: 1 glacial acetic acid) for 24 h and stained with 1% orcein in 45% glacial acetic acid. The nuclear status of oocytes was evaluated under a microscope and considered to be matured if they were at metaphase II stage with reduced number of chromatin, metaphase plate and extrusion of the 1st polar body (Purohit *et al.,* 2012).

Sperm preparation

Spermatozoa were recovered from frozen semen by swim-up separation in Tyrode's Albumin Lactate Pyruvate medium (TALP) previously described by (Parrish *et al.,* 1988) with some modifications. One straw of frozen Holstein bull (0.25 ml) was thawed in a water bath at 37.5 ^{o}C for 1 min. The contents were washed twice in 5 ml of sperm-TALP supplemented by 6 mg/ml BSA (fraction V, A- sigma) by centrifugation at room temperature for 5 min to remove extender and cryoprotectants. The sediment of spermatozoa was resuspended in 5 ml of IVF-TALP containing 10 µg/ml heparin and then centrifuged for 5 min. The supernatant was removed leaving 0.25-0.5 ml of IVF-TALP and sperm pellet. The concentration of sperm was adjusted by adding IVF-TALP medium to reach 1x10^{6} sperm/ml as tested by a haemocytometer.

In vitro fertilization process

All types of *in vitro* matured/vitrified oocytes were washed two times in sperm-TALP, followed by final washing in fertilization medium (IVF-TALP). In vitro matured oocytes were transferred into Petri dish containing 100 µl droplets of fertilization medium at the rates of (10 oocytes per drop). Aliquots of the sperm suspension (8 µl) were added to each droplet containing matured oocytes. The oocytes and spermatozoa were co-cultured in a CO2 incubator at 38.5˚C in 5% CO2 in air, with saturated humidity for 24 h.

In vitro culture and embryo development

After 24 h co-incubation of spermatozoa and oocytes from each type, the presumptive embryos were washed in sperm-TALP medium .The final washing was done in a culture medium consisting of TCM-199 supplemented with 3 mg/ml BSA, 20 µg/ml Na-pyruvate and 50 µg/ml gentamycin.

After co-incubation, presumptive embryos were placed in petri dish in the culture medium covered with mineral oil and incubated at 38.5^{o}C under 5% CO_2 in humidified air for 7 days. The frequency of

morula and/or blastocyst was recorded. Medium was replaced with fresh medium after every 48 h of culture (Dutta *et al.,* 2013).

Statistical analysis

Statistical analysis for the obtained data were analyzed using factorial design according to the following model:

$Yijk = U + Ai + Bj + ABij + eijk.$

Where: Yijk = Observed values U = Overall mean A_i = Types of oocyte B_j = Meiotic stages AB_{ij} = Interaction due to types of oocyte x meiotic stages e_{ijk} = Random error

While differences among the treatment means were performed using Duncan's Multiple Range Test (Duncan's, 1955).

RESULTS AND DISCUSSION

Post-thawing survival and normality rates

Effect of presence or absence of cumulus cells

Data in Table (1) showed that the rate of survival and normality was affected significantly ($P<0.05$) by presence of *cumulus cells, being the* greatest for COCs (84.47 and 81.73%), followed by MDOs (73.28 and 62.00%), while NDOs showed the smallest rates (60.71 and 54.39 %), respectively. However, proportion of morphological damaged oocytes showed significantly ($P<0.05$) an opposite trend (18.27, 38.00 and 45.61%).

Table (1): Effect of *presence or absence of cumulus cells* of vitrified oocytes on post-thawing survival and normality rates.

Item	No. Oocytes vitrified	Survival rate (%)	Morphologically oocytes (%)	
			Normal	Damaged
CCOs	200	84.47[a]	81.73[a]	18.27[c]
MDOs	180	73.28[b]	62.00[b]	38.00[b]
NDOs	200	60.71[c]	54.39[c]	45.61[a]
Std. Error		±1.38	±1.13	±1.13

In accordance with the present results, Prentice-Biensch *et al.* (2012) reported higher survival rate of bovine oocytes vitrified with enclosed cumulus cells than partially denuded cells. They showed that the survival rate and normal morphologically immature oocytes of goats was significantly higher for COCs than MDOs (86.73 and 89.22% *vs.* 80.31 and 94.12%, respectively. Also, Zhou et al. (2010) reported that the survival rate of vitrified bovine oocytes was significantly higher for COCs than partially-denuded vitrified and control oocytes. Moreover, Babaei *et al.* (2006) reported a high proportion of morphologically normal bovine oocytes (90%) were recovered after vitrification-warming using glass capillary micropipette.

In buffalo, Karima *et al*. (2004) found significant ($P<0.01$) difference between COCs and NDOs (91.6 *vs.* 78.0%). However, percentage of morphological normal oocytes (COCs) after vitrifying, was non-significantly differing with partially Dos. Also, Mahmoud *et al.* (2013) reported that the rate of morphologically intact oocytes following vitrification/warming was high, ranging from 87.7% in straws to 90.8% in cryotops using a mixture of 3 M DMSO + 3 M EG. In mice, Nikseresht *et al.* (2015) showed that the survival rate of oocytes in the stepwise cumulus oocytes complexes and denuded oocytes were significantly higher than those for the single-step NDOs. On the other hand, Chian *et al.* (2004) reported that bovine oocytes survival rates following vitrification were not affected by the presence or absence of cumulus cells. Also, Zhang *et al*. (2009) observed no difference in the survival rate of vitrified mature ovine oocytes with or without cumulus cells. The difficulty, in obtaining acceptable rates of survival and functionality for oocytes after cryopreservation, is due to the size of this cell and its unique morphologic characteristics (Martins *et al.,* 2005).

It was suggested that the presence of the cumulus cells can reduce the adverse effects of DMSO on the oocytes (Johnson and Packering, 1987). Also, the presence of cumulus cells can minimize the release

of cortical granules and premature zona reaction for zona hardening resulting in low fertilization rates (Vincent *et al.,* 1990).

Effect of developmental stage

Regardless of presence or absence of cumulus cells, data presented in Table (2) indicate that the percentage of survival rates of vitrified–thawed mature oocytes was higher (P< 0.05) than immature oocytes post thawing (78.03 vs. 67.60 %, respectively). However, the percentage of post thawing morphologically oocytes was not affected significantly by meiotic stages, regardless of presence or absence of cumulus cells.

Table (2): Effect of developmental stage of vitrified oocytes on Survival and normality rate post-thawing.

Item	No. Oocytes	Recovery Rate (%)	Morphologically Oocytes	
			Normal (%)	damage (%)
Vitrified immature oocytes	340	67.60±1.06 [b]	63.53±0.87	36.47±0.87
Mature vitrified oocytes	240	78.03±1.19 [a]	68.56±0.97	31.44±0.97

Survival rates was higher (P<0.05) for mature than immature oocytes (78.03 vs. 67.60%), while normality rate was not affected by type of oocyte.

Hammam and El-Shahat (2005) observed that the survival rates of Morphological normal and abnormal of vitrified–thawed buffalo oocytes was (70.0 and 30.00% versus 72.23 and 27.77) for immature and mature groups, respectively.

When matured oocytes are used for vitrification better membrane stability during chilling may occur (Chaves *et al.,* 2014). However, exposure to low temperature induces damage determining changes in the meiotic spindle, which in turn may result in chromosomal aberrations, increasing in polyploidy and problems of fertilization (Chen *et al.,* 2003; Tharasanit *et al.,* 2006; Dike, 2009). On the other hand, chromosomes of immature oocytes would not be directly affected by the meiotic spindle of MII, since at GV stage; the genetic material remains uncondensed and confined within the nucleus (Bogliolo *et al.,* 2007). However, there are still few studies that assess the effects of oocyte vitrification comparing the stages of maturation, especially in sheep and goats (Moawad *et al.,* 2011). The vitrification of immature ovine oocytes induced significant changes in spindle and chromatin configuration after IVM (Bogliolo *et al.,* 2007). These results agree with Moawad *et al.* (2012) who mentioned that this form of vitrification negatively affected the IVM rate, resulting in meiotic resumption failure in 63% of oocytes. Shirazi *et al.* (2012) found that despite the lack of difference in survival rate of ovine oocytes vitrified at MII or GV, matured oocytes showed a higher resistance to vitrification with cleavage rate of 53%, higher than the rate obtained for immature oocytes (37%). Mo *et al.* (2014) also demonstrated that the meiotic status determines the ability of sheep oocytes to survive vitrification while MII oocytes showed the highest survival rates and developmental competence after vitrification. Quan *et al.* (2014) compared the vitrification of immature with MII goat oocytes and described that the rate of vitrified/thawed MII oocytes with normal morphology and cleavage rate after parthenogenetic activation were significantly higher than 431vitrified/thawed GV oocytes. These authors suggest that the tolerance of MII oocytes to vitrification and thawing may be more than that of the GV oocytes, so MII oocytes may be more suitable for vitrification.

Interaction between cumulus cells and developmental stages

Effect of Interaction between oocyte with or without cumulus cells (MDOs, NDOs and CCOs) and meiotic stages (Immature and mature oocytes) on Survival and normality rate post-thawing is illustrated in Fig. (1). The survivability of CCOs and NDOs found to be recovered post-vitrification were significantly (P<0.05) higher for mature oocytes than vitrified thawed immature oocyte (89.94 and 78.75% *vs.* 79.00 and 67.80%, respectively). While, the survivability of MDOs were not affected significantly between immature and mature of post-vitrification.

On the other hand, the percentage of morphological normal CCOs was significantly (P<0.05) higher for mature oocytes than vitrified thawed immature oocyte (87.46 *vs.* 76.00 %). While, the proportions of morphological normal NDOs and MDOs were non-significantly between them. This results agreement with Purohit *et al.* (2012) who found that the survival rate was lower for immature cumulus compact and

immature mechanically denuded compared to mature cumulus compact and mature mechanically denuded (88.23 and 81.65 % versus 92.13 and 91.81%, respectively).

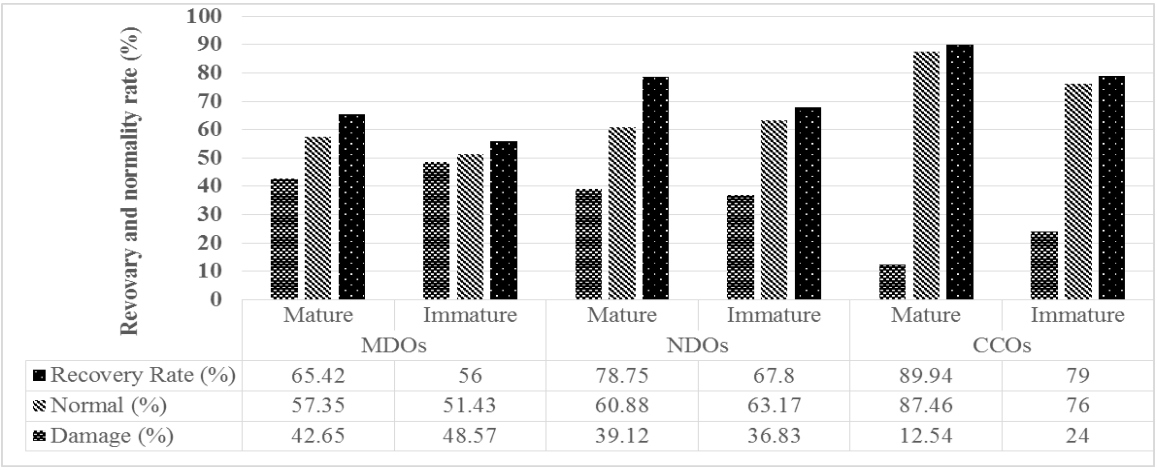


Fig. (1): Effect of Interaction between cumulus cells and meiotic stages on Survival and normality rate post-thawing.

Cleavage and developmental rates

Effect of presence or absence of cumulus cells of vitrified oocytes

Data presented in Table (3) showed that cleavage rate was significantly (P<0.05) higher for COCs than those of MDOs and NDOs (45.47 *vs.* 29.72and 25.24%, respectively), regardless of meiotic stages. Also, production rate of embryos at morula and blastocyst stages was significantly (P<0.05) higher for COCs than those of MDOs and NDOs (18.05 and 15.28 *vs.* 6.06, 3.03, 7.69 and 7.69 %, respectively). But embryos at 2-16 cells was significantly (P<0.05) higher for MDOs and NDOs (90.91 and 84.62 %) as compared to COCs (66.67%), regardless of meiotic stages.

Table (3): Effect of type of vitrified oocytes on cleavage and development rates.

Item	No oocytes vitrified	Cleavage		Embryonic stage					
		n	%	2-16 cell		Morula		Blastocyst	
				n	%	n	%	n	%
CCOs	159	72	45.28^{a}	48	66.67^{b}	13	18.05^{a}	11	15.28^{a}
MDOs	111	33	29.72^{b}	30	90.91^{a}	2	6.06^{b}	1	3.03^{b}
NDOs	103	26	25.24^{b}	22	84.62^{a}	2	7.69^{b}	2	7.69^{b}
Std. Error			±0.98		±5.29		±4.83		±3.98

Cleavage rate and morula and blastocyst production rates were higher (P<0.05) for CCOs (45.28, 18.05 and 15.28%) than those of MDOs (29.72, 6.06 and 3.03%) and NDOs (25.24, 7.69 and 7.69%), respectively.

In accordance with the present results, Purohit *et al.* (2012) showed significantly (P<0.05) higher proportion of fertilized oocytes for immature COCs of goat than immature MDOs (31.7 *vs.* 25.0%, respectively).

Also, Modina *et al.* (2004) observed a lower percentage of fresh NDOs reached the blastocyst stage in comparison with intact COCs (23.9 *vs.* 35.4, P<0.05). The blastocyst rate was 4.3%, being lower than those reported in the current study (6.25-9.37%). In fact, this is one of the evidences of successful oocyte cryopreservation for which, to date, only controversial and sporadic data are available for the bovine species (Hochi, 2003). Moreover, Zhou *et al.* (2014) achieved cleavage (63.5%) and blastocyst development (20.0%) after parthenogenetic activation of vitrified-warmed bovine oocytes similar to that from oocytes vitrified by the open-pulled straw method (57.0%) cleavage and 23.0% blastocyst development, respectively (Hou *et al.,* 2005). Low blastocyst development rates (less than 10%) was reported by Martins *et al.* (2005). In buffalo, Hammam and El-Shahat (2005) found that oocytes vitrified

at the immature cleaved and developed into morula and blastocyst stage after thawing were 20.0, 3.3 and 2.0%, respectively).The developmental capacity of the vitrified-thawed immature buffalo oocytes was significantly lower compared to control (El-Shahat and Hammam, 2014; Yadav *et al.*, 2008). Furthermore, Mahmoudi *et al.* (2005) reported that intact mouse oocytes had a higher developmental competence than denuded oocytes. In this respect, Hochi *et al.* (1998) vitrified immature bovine oocytes in straws by using a mixture of 40% EG, ficoll and sucrose as a vitrification medium. They reported 47.5% fertilization rate from the vitrified bovine oocytes. Abe et al. (2005) reported developmental rates to blastocyst of bovine GV-COCs, using Nylon-Mesh and exposer with stepwise cryoprotctant, being significantly higher than with the single-step vitrification.

In this respect, Zhou *et al.* (2010) reported that cleavage and blastocyst rates of cumulus-enclosed vitrified bovine oocytes of GV were significantly higher than those of partially-denuded vitrified and control oocytes.

Effect of developmental stage

Regardless of presence or absence of cumulus cells, data presented in Table (4) showed that cleavage rate was significantly (P<0.05) higher for vitrified thawed mature oocytes than vitrified thawed immature oocyte (41.93 *vs.* 29.36 %, respectively), however, production rate of embryos at morula and blastocyst stages was not affected significantly by meiotic stages. But embryos at 2-16 cells was significantly (P<0.05) higher for vitrified thawed immature oocytes as compared to vitrified thawed mature oocytes (85.51*vs.* 66.13%).

Table (4): Effect of developmental stage of vitrified oocytes on cleavage and development rates.

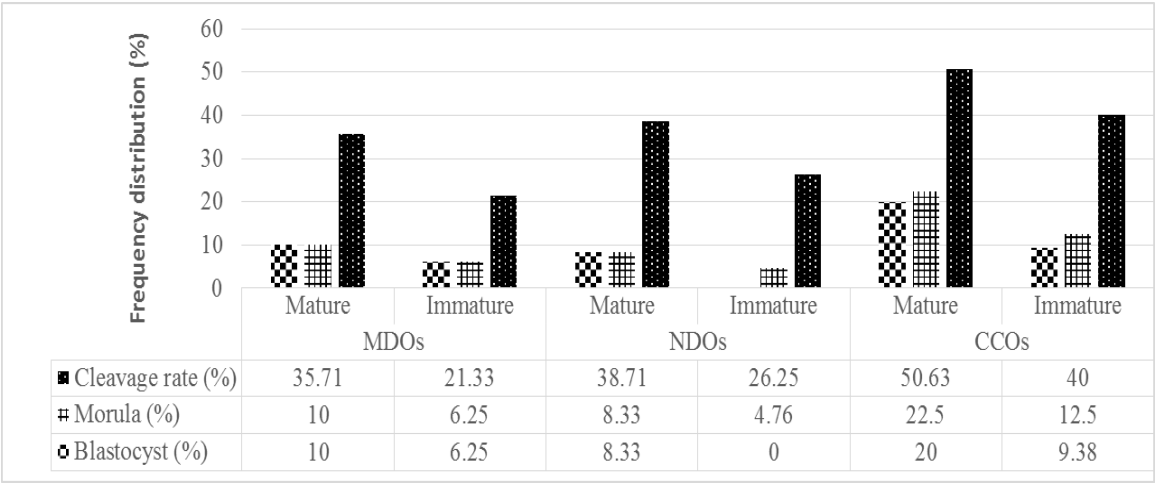
Item	No. Oocytes vitrified	Cleavage rate		Embryonic stage					
				2-16 cell		Morula stage		Blastocyst stage	
		n	%	n	%	n	%	n	%
Vitrified/ immature oocytes	235	69	29.36[b]	59	85.51[a]	6	8.69	4	5.80
Mature/ Vitrified oocytes	138	62	41.93[a]	41	66.13[b]	11	17.74	10	16.13
Std. Error			±0.80		±4.32		±3.95		±3.25

Cleavage rate was higher (P<0.05) in mature than in immature oocytes (41.93 vs. 29.36%), while morula and blastocyst production rates were not affected by type of oocyte.

Hammam and El-Shahat (2005) found that the 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 (37.50%; 8.34%; 6.67% VS 20.0%; 3.34% ; 2.0%, respectively). These finding which come in accordance with the previous study in buffalo (Abdallah, 2003). This reduction in developmental ability of vitrified immature oocyte could be due to a possible multifactorial cause, including toxic effect of cryoprotectants, ultra structural damage to the oocytes, and deleterious effects on chromosomes and other cytoplasmic structures (Dobrinsky, 1996). In addition, the freezability of immature oocyte has been reported to be low, and increases as development proceeds to the blastocyst stage after fertilization (Kasai et al., 1979 and Schroeder et al., 1990).The normal fertilization rate of goat oocytes can be increased by supplementation of media by cysteamine (Rodríguez-González et al., 2003 ; Urdaneta *et al.*, 2003). The reduced in vitro fertilization ability of vitrified oocytes compared to fresh oocytes could be due to the toxic effects of cryoprotectants and osmotic injuries.

Interaction between cumulus cells and developmental stages

Effect of Interaction between oocyte with or without cumulus cells (MDOs, NDOs and CCOs) and meiotic stages (Immature and mature oocytes) on Cleavage and development rates is illustrated in Fig. (2). The cleavage rate was significantly (P<0.05) higher for vitrified thawed mature CCOs, NDOs and MDOs than vitrified thawed immature CCOs, NDOs and MDOs (50.63, 38.71and 35.71 vs. 40.00, 26.25 and 21.33 %, respectively), however, production rate of embryos at morula and blastocyst stages were significantly (P<0.05) higher for only mature CCOs than vitrified thawed immature CCOs, (22.50 and 20.00 *vs.* 12.50 and 9.38% respectively). But embryos at morula and blastocyst stages cells was not significantly for vitrified thawed immature oocytes as compared to vitrified thawed mature oocytes. However, proportion of embryos at 2-16 cells showed an opposite trend.



	MDOs		NDOs		CCOs	
Cleavage rate (%)	35.71	21.33	38.71	26.25	50.63	40
Morula (%)	10	6.25	8.33	4.76	22.5	12.5
Blastocyst (%)	10	6.25	8.33	0	20	9.38

Fig. (2): Effect of Interaction between cumulus cells and meiotic stages on cleavage and development rates.

The immature cumulus compact oocyte (31.70%) group evidenced significantly higher ($p < 0.05$) fertilization compared to mature cumulus compact (19.44 %) and mature denuded (16.86%) oocytes (Purohit et al., 2012).

CONCLUSION

Cumulus cell surrounding cumulus oocytes–complexes play a vital role for survival of bovine oocytes during vitrification and successful in vitro maturation and fertilization as well as embryonic development to morula and blastocyst stages.

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الإخصاب المعملى للبويضات الغير ناضجة أو الناضجة المحفوظة بالتجميد والمتأثرة بالتعرية الميكانيكية أو الطبيعية

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- أجريت هذه الدراسة بهدف تقييم دور الخلايا الركامية للبويضات الغير ناضجة او الناضجة معمليا ثم تجميدها بالتزجيج على الإخصاب المعملي ومعدل تطور الأجنة فى الأبقار . في الدراسة تم تجميع مبايض الأبقار من المجازر ثم تم تجميع البويضات منها بطرقة الشفط من الحويصلات ذات قطر 3-8 ملل . تم فحص البويضات ميكروسكوبيا وتقييمها وتصنيفها إلى COCs وهى بويضات محاطة بشكل كامل بالخلايا الركامية و NDOs وهى بويضات معراة أو خالية من الخلايا الركامية بشكل طبيعي و MDOs وهى بويضات معراة أو خالية من الخلايا الركامية بطريقة ميكانيكية . مع ملاحظه أن الخلايا المعراة بطريقة ميكانيكية تم الحصول عليها بسحبها بالمصة وطردها بشكل متكرر في محلول منظم . كل أنواع البويضات تم تجميدها بطريقة التزجيج لمدة أسبوعين على الأقل سواء بويضات غير ناضجة أو بويضات تم إنضاجها معمليا . بعد التجميد لفترة الأسبوعين تم تقييم معدل الاسترداد ومدى حيوية البويضات من الأنواع المختلفة (طبيعية وغير طبيعية) . البويضات الطبيعية تم إنضاجها وإخصابها معمليا والبويضات الطبيعية التي سبق إنضاجها تم عمل إخصاب معملي لها ثم تم تقدير معدل انقسام الأجنة . بعد عمل استزراع معملي للاجنه المنقسمة لمدة 7 أيام تم حساب معدل الأجنة التي وصلت لمرحلة التوتية ولمرحلة الكيسات الأريمية وتشير النتائج إلى إن معدل الاسترداد و معدل البويضات الطبيعية كان أعلى في حالة البويضات المغطاة بشكل كامل بالخلايا الركامية 84.47 و81.73 % متبوعا بالبويضات المعراة بطريقة ميكانيكية 73.28و62.0 % وكان اقل معدل في حالة البويضات المعراة طبيعيا 60.71 و 54.39 % .

- كان معدل الاسترداد أعلى في حالة البويضات الناضجة عن البويضات الغير ناضجة 78.03 % بالمقارنة 67.60 % بينما كان معدل الخلايا الطبيعية متقارب لكليهما . أما معدل انقسام الأجنة وكذلك معدل التوترية ومعدل الكيسات الأريمية كانت أعلى في حالة البويضات المغطاة بشكل كامل بالخلايا الركامية 45.28 ,18.05 و 15.28 % عنها فى حالة البويضات المعراة بطريقة ميكانيكية 29.72, 6.06و 3.03 % ثم الخلايا المعراة طبيعيا 25.24 ,7.69 و 7.69% على الترتيب . معدل انقسام الأجنة كان أعلى في حالة البويضات الناضجة عنه في حالة البويضات الغير ناضجة 41.93 مقابل 29.36 . بينما كان معدل التوتية والاكياس الاريمية كان متقاربا لكلاهما.

- وبناء على النتائج السابقة فان وجود الخلايا الركامية لبويضات الأبقار يلعب دور هام في عملية التجميد وكذلك في عمل إنضاج وإخصاب معملي بشكل ناجح وأيضا مدى انقسام الأجنة ووصلها لمرحلة التوتية والأكياس الاريمية .