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Institute of Genetics and Animal Biotechnology
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Effect of different oocyte retrieval and culture methods on *in vitro* maturation of bovine oocytes derived from vitrified ovarian tissue slices

Hassan Nazari[1], Azita Afzali[2], Pezhman Mirshokraei[3]*

[1] Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran

[2] Clinical Embryologist, Shahrekord University of Medical Sciences, Shahrekord, Iran

[3] Department of Clinical Sciences, Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad (FUM), Mashhad, Iran

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In a successful method of ovarian tissue cryopreservation, there are always a number of antral follicles remain intact, as a result of which the methods of retrieving and culturing oocytes from them becomes very important. Therefore, this study aimed to obtain an efficient method for oocyte retrieving and maturation *in vitro* from vitrified/warmed ovarian tissue slices. For this purpose, slaughterhouse-derived bovine ovaries were sliced and prepared in six replicates for each segment of each experiment and vitrified at 4°C and room temperature (RT) by 1.8 ml cryovial. After warming, the oocyte-cumulus complexes (COCs) were retrieved by two methods (aspiration and slicing), and matured *in vitro* in two conditions (in the presence or absence of oviductal epithelial cells (OECs) as feeder cells) for 24-48h, and finally, the nuclear maturation of oocytes was evaluated as the statistical analysis showed the higher degeneration rates of oocytes derived from vitrified ovarian tissue in aspiration groups (P<0.05) and the more M-II stage oocytes were obtained in the slicing groups (P<0.05), although the aspiration method was better to approach for oocyte retrieval from fresh ovarian tissue than slicing method (P<0.05). Also, the exposure temperature in the vitrification procedure of ovarian slices did not affect the percentage of M-II oocytes in the evaluated groups. It can be concluded that the slicing method is a more proper approach for oocyte retrieval from vitrified ovarian tissue than an aspiration, and the use of co-culturing of these immature oocytes with OECs during IVM is not required.

*Corresponding author: mirshokraei@um.ac.ir; https://orcid.org/0000-0001-6136-4260

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Recent advances in cancer diagnosis and treatment (radiotherapy, chemotherapy, or surgery) have increased the chance of cure or prolonged survival in young women. Nevertheless, these treatments may compromise their future fertility [Salam *et al.* 2016]. Cryopreservation of oocytes or embryos are alternatives for young adult women that require time for ovarian stimulation and oocyte collection and cryopreservation of antral follicles of ovarian tissue slices are options for the preservation of the fertility of the patients that require immediate treatment [Donnez and Dolmans 2015].

In addition to all the advances in cryopreservation methods, what is important is achieving a proper method for retrieving oocytes from vitrified ovarian tissues and efficiently culturing delivered oocytes *in vitro*, which can be done by modeling cattle species due to the accessibility of its ovaries with no ethical restrictions, and similarity of them to the human ovaries in the physiology and reproductive cycle dynamics [Jorssen *et al*. 2015].

The oocyte retrieval method can play an essential role in maximizing the total number of oocytes recovered per ovary and obtaining good-quality cumulus-oocyte complexes (COCs) with evenly granulated cytoplasm and several layers of compacted cumulus cells. Cumulus cells are metabolically coupled to the oocytes via heterologous gap junctions [Suzuki and Saito 2006] and have a modulatory role in the effect of hormones and growth factors during *in vitro* maturation (IVM) of bovine oocytes [Shimada 2012]. It has been proven that the presence of a few layers of compact cumulus cells efficiently influences the process of oocyte maturation [Shimada 2012], which in turn can be affected by the method of oocyte retrieval, especially from vitrified/warmed ovarian tissue.

After achieving a high number of good quality oocytes, applying a proper culture system for their *in vitro* maturation is very important. Application of cell-based co-culture systems such as oviduct, cumulus and granulosa cells is a desirable approach for the improvement of IVM systems to obtain higher yields of better quality oocytes [Lin *et al.* 2009, Lee *et al*. 2018b]. Feeder cells (co-culture) have been applied in a culture of mammalian preimplantation embryos and occasionally maturation of oocytes *in vitro* [Nematollahi-Mahani *et al*. 2009, Shirazi *et al.* 2009, Lee *et al*. 2018b]. It has been suggested that the feeder cells can provide many specific growth factors, stimulators, and cytokines and also act as medium detoxifiers [Heidari *et al.* 2018], which can be supported by the lower quality oocytes [Lee *et al*. 2019], such as retrieving oocytes from vitrified/warmed ovarian tissue.

The motivic and embryotrophic properties of oviductal epithelial cells (OECs) by production and release of specific growth factors have been thoroughly defined in mammalian oocytes and embryos [Orsi and Reischl 2007, Lee *et al*. 2018b]. OECs have been cultured *in vitro* in many systems, as monolayers, in suspension, in perfusion chambers, in polarized or in 3D systems (reviewed by Maillo *et al.* 2016) [Maillo *et al.* 2016]. The simple OECs *in vitro*, cultured as a monolayer, might provide a good starting point to study oocyte and embryo signals through differential expression of

genes co-culturing early embryos on a bovine OECs monolayer [Schmaltz-Panneau *et al*. 2014], and early cross-talk mediated by bone morphogenetic protein (BMP) signaling bovine embryo-oviduct interaction *in vitro* [García *et al.* 2017]. Co-incubation of bovine oocytes with oviductal fluid showed that some oviductal proteins are in association with the zona pellucida [Gonçalves *et al*. 2008], and secreted cytokines and growth factors from OECs improve meiotic maturation and subsequent embryo development [Lee *et al.* 2018a].

In view of the controversial reports on the advantages of co-culture systems compared with a chemically defined medium in the maturation of the mammalian oocyte and the lack of information about how to retrieve the oocyte from the vitrified ovarian tissue, this study was aimed to obtain an efficient oocyte retrieval method (aspiration or slicing) from vitrified/warmed bovine ovarian tissue and subsequently, a suitable culture method (with/without co-culturing with OECs) for *in vitro* maturation of retrieved oocytes.

Material and methods

Collection and preparation of ovarian tissue

The ovaries of adult non-pregnant cows were collected and transferred to the laboratory in normal saline within 2-3 hours at 20-25°C and after removing the residual tissue, the ovaries were washed in 70% alcohol for 10 s followed by two washes in sterile physiological serum supplemented with 100 g/mL penicillin and 100 g/mL streptomycin.

Vitrification and warming protocol

The ovarian slices vitrification involved the equilibrium of the samples in two vitrification solutions with different concentrations of cryoprotectants. Equilibration solution (ES) consisting of TCM199 supplemented with 10% FBS (Fetal bovine serum, Gibco 10270), 0.5M sucrose, 7.5% Ethylene glycol, and 7.5% DMSO. Vitrification solution (VS) was composed of TCM199 supplemented with 10% FBS, 0.5M sucrose, 15% Ethylene glycol, and 15% DMSO. Ovarian slices were immersed in ES for 20min and then in VS for 10min. After that, the slices were placed in a 1.8 mL sterile cryovial (TPP, Swiss) and stored in liquid nitrogen [Kagawa *et al.* 2009].

After 1-2 weeks, the vitrified samples were rehydrated with three warming solutions, including warming solutions 1, 2, and 3 (WS1, WS2, and WS3), which consisted of TCM199 supplemented with 10% FBS, and 1, 0.5, and 0M sucrose, respectively. In this procedure, the ovarian slices were immersed to room temperature (about 25ºC) for the 30s and then transferred to the WS1 at 37ºC for 2 min. Slices were then kept in WS2 for 5min and then in WS3 for 5min, twice.

For the toxicity test, all processes, including exposure of ovarian slices to ES and VS solutions at all times applied for vitrification and warming, were performed at 25°C except plunging in liquid nitrogen.

Oocyte retrieval

Aspiration technique. Antral follicles with 2 to 4 mm diameter on the bovine ovary or vitrified/warmed slices were selected for the recovery of oocytes by aspiration technique. The competent cumulus oocytes complexes (COCs) with at least 3 layers of cumulus cells and homogenous cytoplasm were retrieved using an 18-gauge needle attached to a 10-ml disposable syringe that was loaded with an aspiration medium consisting of HEPES-TCM199 (H-TCM) supplemented with 10% FBS and 50IU/ml heparin and collected for *in vitro* maturation.

Slicing technique. The vitrified/warmed slices of ovaries were sliced into small pieces in a glass Petri dish containing the aspiration media with a surgical blade. After discarding the sliced stromal tissues, the content of the Petri dish was then explored under a stereomicroscope and the competent COCs were selected for *in vitro* maturation.

***In vitro* maturation of oocytes**

The COCs were rapidly washed four times in H-TCM199 containing 10% FBS and were cultured in two following methods at 38.5°C under an atmosphere of 5% CO_2 with a maximum humidity (99%) for 24 h.

Oocyte culture without any co-culture. *In vitro* maturation (IVM) of bovine oocytes was carried out according to Shirazi et al. method [Shirazi *et al.* 2009] with some modifications. In brief, the selected COCs were *in vitro* matured in a maturation medium consisting of TCM199 supplemented with 10% FBS and 0.1IU/ml FSH. Ten to 15 COCs were transferred in 50µl of the maturation medium in a 60mm Petri dish (Falcon 3004; Becton & Dickinson, Franklin Lakes, NJ), covered with sterile mineral oil.

Oocyte culture with OECs monolayer. In this study, the ewe oviduct was used for OEC cultivation. After transporting the ewe oviducts in phosphate-buffered saline (PBS) containing penicillin/streptomycin from a slaughterhouse to the laboratory within 3h, the oviduct was cleaned from the residual tissue washed with PBS, and its surface was sterile with 70% ethanol. They were placed in a Petri dish and 1cm of the upper and lower portions of oviducts was separated. Enzymatic digestion of the inner surface of the oviduct lumen was performed by trypsin-EDTA (0.5% trypsin, 0.25% EDTA in PBS) by injection into the lumen from the ampulla toward the isthmus, and after 5min, the lumen was squeezed with tissue forceps vice versa from the isthmus. The obtained suspension containing the cell and trypsin was washed with TCM199+ 10% FBS and centrifuged at 500g for 5min. The cells were cultured in 50µl droplets of TCM199 + 10% FBS at 39°C and 5% CO_2 to reach the 60-70% confluency and 2h before use, the medium was changed for maturation medium.

Evaluation of nuclear maturation of the oocyte

Twenty four hours after the onset of IVM, oocytes were denuded by vortexing in H-TCM-199 supplemented with 10% FCS and 0.3mg/ml hyaluronidase. Then, the

denuded oocytes were transferred in ice-cold ethanol containing 10µg/ml Hoechst 33342 for 15 min. The oocytes were directly mounted into the small droplet of glycerol on a glass slide and the nuclear status was evaluated under an epifluorescent microscope (IX71; Olympus, Tokyo, Japan). using standard measures like germinal vesicle (GV), germinal vesicle breakdown (GVBD), chromatin condensation and chromosomes organization (Metaphase I; M-I) and appearance of the first polar body (Metaphase II; M-II) as confirmation of nuclear maturation

Experimental design

Experiment I: Recovery and in vitro maturation of oocytes of vitrified ovarian slices

In the laboratory, the bovine ovarian cortex was cut into 10×10×4 mm slices and randomly distributed in control and vitrification groups that included fresh and vitrified ovarian slices, respectively. The oocyte retrieval and then culture from both fresh and vitrified ovarian slices were performed at room temperature (25°C) by aspiration or slicing, and *in vitro* maturation was performed in co-culture with oviductal epithelial cells (OECs) monolayer or without any co-culture system, respectively. Nuclear maturation at 22-24h after IVM was assessed. Six replicates were performed for each treatment (Fig. 1).

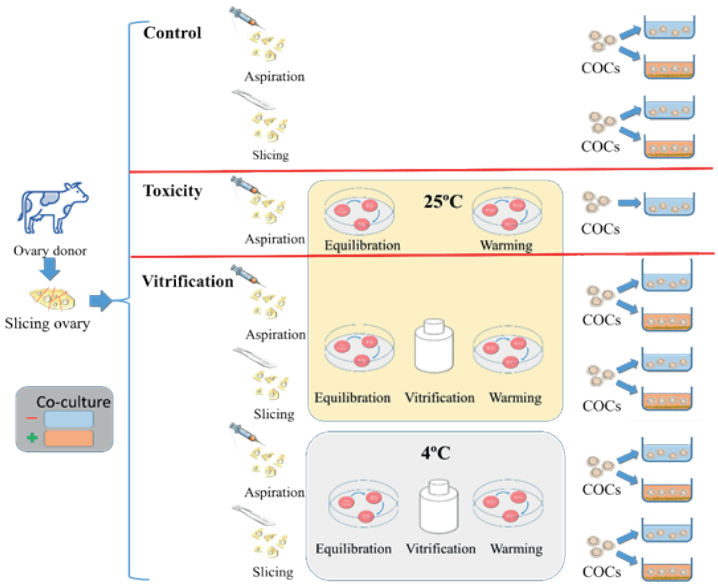

Fig. 1. A schematic illustration of the experiments. In experiment 1, bovine ovaries were sliced and the COCs were recovered by two methods (aspiration and slicing) and then cultured in two culture systems (with or without oviductal epithelial cells (OECs) co-culture) in control and vitrification groups. In experiment 2, the vitrification group was repeated in 4ºC. The toxicity test was also performed in this study.

Experiment II: Vitrification of ovarian slices at different temperatures

Because a lower temperature decreases the deleterious toxic effects of cryoprotectants (CPAs) in immersed tissue [Fahy *et al*. 1987, Mouttham and Comizzoli 2016], the exposure of ovarian slices to equilibration and vitrification solution in the vitrification procedure was also performed at 4°C. The rest of this experiment was carried out as experiment I, in six replicates (Fig. 1).

Statistical analysis

The difference in oocyte maturation between experimental groups was analyzed using one-way analysis of variance (ANOVA) followed by LSD (Least Significance Difference) post hoc test after arcsin transformation. The statistical analysis were only preformed between subclasses with more than one observation. When the normality test failed, the Kruskal Wallis was applied and followed by Mann–Whitney test. Data were expressed as mean and SEM. All analyses were conducted with SPSS Version 25 (SPSS Inc., Chicago, IL, USA) and $P<0.05$ was considered as significant.

Results and discussion

In this paper, due to the possible change in nature of the attachment of the cumulus-oocyte complex (COC) to the internal lining of the follicle and COC structure and the function itself after ovarian tissue freezing, different methods of COC retrieval culture were examined for the first time.

As shown in Table 1, the aspiration method was a better approach for oocyte retrieval from fresh ovarian tissue (control groups) than the slicing method regardless of the culture methods of bovine oocytes (the presence or absence of OECs monolayer), because the statistical differences were found in M-II rate of *in vitro* matured bovine oocyte between the aspiration method and the slicing method among the control groups ($P<0.05$). The vitrification had the deleterious effects on oocyte survival and maturity regardless of the oocyte retrieving method so that the percentage of M-II stage oocytes recovered from vitrified ovarian slices was significantly lower than their counterparts delivered from the fresh ovarian slices ($P<0.05$), and in oocyte degeneration rates, the significant differences were only observed between the aspiration method in vitrified groups and all other groups ($P<0.05$).

The toxicity test showed that the used CPAs decreased the percentage of M-II oocytes (61.77±1.89%) compared to the control group (77.52±2.17%) ($P=0.001$), whereas an increase in all other groups (GV-, GVBD- and MI-stage oocytes) was observed.

In vitrified groups, the highest oocyte degeneration rates were observed in aspiration groups either with (11.29±4.34%) or without a co-culture system (16.02±7.21%). The highest arrested oocytes in the GV stage were also observed in oocytes that recovered by slicing approach followed by the co-culture system (13.87±1.96%), so that had a significant difference from those recovered by the aspiration method followed by

the co-culture system (0.84±0.84%) (P=0.001). The highest rate of M-II stage oocytes was obtained in the group of slicing regardless of the co-culture system (16.15±3.17%), in comparison to the other vitrification groups.

Accordingly, the slicing method was overall the better method than an aspiration for vitrified ovarian tissue; however, the aspiration method is the most commonly used technique in retrieving bovine COCs from fresh slaughterhouse collected ovaries [Gordon 2003, Shirazi *et al.* 2012]. The slicing method is efficient for the recovery of plenty amount of COCs per ovary [Vajta *et al.* 1996, Wani *et al*. 2000, Singh *et al*. 2018]. Kątska [1984] demonstrated that retrieving the lower oocytes number (30-60%) from the punctured follicles is one of the complications of the aspiration method [Kątska 1984]. This emphasizes the advantage of this method in retrieving a large number of oocytes, due to those that are located in more internal follicles. Nevertheless, the number of blastocysts obtained was higher from oocytes taken by aspiration alone than by the other methods such as mincing [Takagi *et al*. 1992] or slicing [Ulloa *et al*. 2015].

In a study, the ultrastructural evaluation of oocytes indicated that cryopreservation disrupted the intercellular communication between the cumulus cells and the oocyte [Fuku *et al*. 1995]. This damage or interruption was intensified by the method of oocyte retrieval. In confirmation, many studies compared the effect of two oocytes retrieving protocols, slicing and aspiration, and showed that slicing provided higher-quality oocytes percentage than the aspiration method because aspiration could lead to greater disruption of surrounding cumulus cells and a possibility of retention because of cumulus cells being firmly attached to the stratum granulosum. They preferred the slicing technique as a superior method

Table 1. Effect of oocyte retrieval and culture methods on *in vitro* maturation of bovine oocytes derived from ovarian tissue vitrified in 25ºC

Groups		Oocyte No.	Oocyte maturation stage No. (mean±SEM)				
oocyte retrieval	co-culture		degenerated	GV	GVBD	M-I	M-II
Toxicity	aspiration no	73	0	0	12 (16.72±4.44)^{a}	16 (21.51±2.97)^{acd}	45 (61.77±1.89)^{a}
Control	aspiration no	149	0	2 (1.27±0.85)^{a}	6 (3.75±1.91)^{b}	26 (17.20±2.59)^{a}	115 (77.52±2.17)^{b}
	aspiration yes	81	0	0	5 (5.87±2.82)^{b}	17 (18.94±5.41)^{a}	59 (75.19±7.58)^{b}
	slicing no	85	0	0	5 (7.68±0.64)^{ab}	29 (33.93±6.27)^{bd}	51 (59.67±6.68)^{a}
	slicing yes	111	0	1 (1.04±1.04)	9 (8.19±0.95)^{ab}	40 (36.33±2.39)^{bc}	61 (54.44±2.01)^{a}
Vitrified	aspiration no	97	13 (16.02±7.21)^{a}	4 (5.82±3.25)^{a}	56 (53.84±8.14)^{e}	17 (17.85±4.53)^{ac}	7 (6.46±2.05)^{e}
	aspiration yes	108	14 (11.29±4.34)^{a}	1 (0.84±0.84)	45 (42.10±3.60)^{cd}	45 (43.03±3.49)^{b}	3 (2.75±1.95)^{e}
	slicing no	81	0	4 (6.07±1.99)^{a}	26 (32.05±5.15)^{d}	34 (39.67±7.02)^{b}	13 (16.15±3.17)^{d}
	slicing yes	68	3 (3.47±2.59)^{b}	10 (13.87±1.96)^{b}	35 (51.20±5.41)^{e}	16 (26.33±4.60)^{acde}	4 (5.13±2.56)^{e}

^{abcd}Mean values followed by different superscript letters in the same column differ statistically among the different groups (P<0.05). Data are expressed as mean±standard error of the mean (SEM).

for higher recovery of oocytes with a good supporting amount of cumulus cells [Pawshe *et al*. 1994, Wani *et al*. 2000, Shirazi *et al.* 2005, Wang *et al.* 2007, Zarcula *et al.* 2012]. This indicates that the maturation competency of oocytes varies with the type of retrieving method, and as the current study showed that oocytes derived from vitrified ovarian tissue by slicing had a higher M-II rate than an aspiration, the presence of another intervention such as vitrification could exacerbate this effect, in addition to the type of retrieving method.

Contrarily, the aspiration method is rapid, easier and provides a greater visual evaluation of follicles as well as better selection and assortment for quality oocytes than the slicing method. Moreover, the slicing method produces more debris which might interfere with the searching of oocytes under the microscope and also require more washing when compared to aspiration [Gordon 2003]. As a result, some COCs were denuded from cumulus cells due to repeated washing and ultimately resulted in a lower number of normal COCs when compared to aspiration at the final observation.

It has been confirmed that reliable and successful oocyte maturation would positively influence the competence of embryonic development [Sagirkaya *et al*. 2007]. To evaluate the competency of *in vitro* maturation of oocytes taken from vitrified ovarian slices, in this assessment, we cultured them after two recovery methods in the presence or absence of OECs monolayer. The result showed the simple culture of oocytes in a maturation medium without co-culture with OECs was considerably better than in co-culture with these cells, especially when the oocytes were retrieved by slicing method (16.15±3.17% vs 5.13±2.56%, respectively).

The direct effects of OECs on the preimplantation developmental rate and quality of *in vitro* produced embryos were frequently evaluated in several species [Shirazi *et al*. 2009, Schmaltz-Panneau *et al.* 2014, Carvalho *et al*. 2017, Lee *et al*. 2018b]. As known, co-culture with other somatic cells improves embryo development through the secretion of bioactive factors and glucose reduction caused by their metabolism [Lange-Consiglio *et al*. 2012]. Furthermore, the trophic effects of these bioactive factors have been shown in the follicular growth and *in vitro* oocyte maturation in mammals, rather than removing toxic products from the culture medium [Ling *et al.* 2008, Lin *et al*. 2009, Asgharzadeh *et al*. 2015, Lee *et al*. 2019]. Although many of these studies have shown the positive effects of co-culture on *in vitro* maturation of oocytes, Trounson *et al*. [1994] showed in a few cases that the co-culture of human oocytes with mature granulosa cells did not improve the M-II rates [Trounson *et al.* 1994]. Similarly, Heng *et al*. [2004] showed granulosa cell co-culture could not improve the outcome of *in vitro* maturation of mice oocytes.

In our evaluation, co-culture with OECs could not overcome the adverse effects of ovarian tissue vitrification on *in vitro* development of retrieved oocytes to M-II stage. As mentioned, the freezing had very deleterious effects on the percentage of M-II stage oocytes recovered from vitrified ovarian slices in comparison to those delivered from the fresh ovarian slices. The toxicity test also showed that the used CPAs decreased the M-II rate (61.77±1.89% vs. 77.52±2.17% in the control group).

The adverse effects of vitrification might be via the cumulus cells. It is generally accepted that cumulus-oocyte communication via an intact corona radiata is necessary for meiosis resumption in immature oocytes and attain full cytoplasmic maturation and developmental competence [Vanderhyden and Armstrong 1989, Tanghe *et al.* 2003], which is probably mediated by the gap junction cytoplasmic connections to the ooplasm that are on short distance communications or direct transfer type via extensions of corona radiata cells through the zona pellucida [Marchais e*t al*. 2022], as well as by the cumulus mass exerting a modulatory effect on the surrounding microenvironment of oocytes [Duque *et al*. 2002, Lonergan *et al*. 2003] It has been determined that damage to the these communications plays a critical role in compromised developmental competence, since vitrification and warming damages cumulus and corona radiata cells surrounding mouse [Ruppert-Lingham *et al.* 2003] and equine [Hochi *et al.* 1996, Tharasanit *et al*. 2009] oocytes. In vitrification, exposure to cryoprotectants causes losses of microvilli and, consequently, disrupts the close coupling between the oocyte and its cumulus cells, and gap junction integrity [Fuku *et al*. 1995, Diez *et al*. 2005]. In this study, the oocytes derived from vitrified ovarian pieces had reduced developmental competence. Since decoupling of cumulus cells occurs, premature breakdown likely eliminates the trophic communication essential to maturation. Therefore, we can infer that produced growth and bioactive factors with somatic cells couldn't use by the co-cultured oocytes and in addition, the essential nutrients available in culture media were consumed by OECs. It was probably the reason for the lower M-II rate of oocytes co-cultured with OECs than simple cultured oocytes. Interestingly, the resumption of meiotic divisions (total number of oocytes in the GVBD, M-I and M-II stages) had not significantly different between oocytes cultured in two different conditions and the meiotic arrest has occurred during IVM.

According to Table 2, those oocytes obtained by slicing method and matured without the presence of OECs were significantly less degenerated and arrested than oocytes obtained by aspiration method and matured in co-culture with OECs (0% vs. 15.60±5.00%; P<0.001 and 1.88±1.28% vs. 13.77±4.40%; P=0.001, respectively). The best M-II rate of vitrified ovarian tissue derived-oocytes was obtained from those oocytes that were recovered by slicing approach and cultured simply (without the presence of OECs) (19.95±0.87%), so this group had a significant difference from the other compared groups.

To better understand the effect of the temperature of the vitrification procedure on oocyte competency retrieved from vitrified ovarian slices, the most important criteria include the rates of degenerated (Fig. 2A), arrested (Fig. 2B), and matured (Fig. 2C) oocytes were re-evaluated. Reduction of exposure temperature from 25°C to 4°C, decreased the percentage of degenerated oocytes in an aspiration/without co-culture group (16.02±7.21 vs. 8.87±2.87, respectively; P=0.043)(Fig 2A), increased the rate of arrested oocytes (GV oocyte number) in aspiration/with co-culture group (0.84±0.84 vs. 13.77±4.40, respectively; P=0.001), and decreased the rate of arrested oocytes in slicing/with co-culture group (13.87±1.96 vs. 6.15±1.45, respectively; P=0.050)

(Fig. 2B). Alteration of the temperature of vitrification media to 4°C did not affect M-II rates in evaluated groups (Fig. 2C). These results are consistent with some studies that the exposure temperature of CPAs has not affected the survival rate of oocytes after vitrification/warming [Szurek and Eroglu 2011, Mouttham and Comizzoli 2016]. Contrarily, the dominant hypothesis states that a lower temperature decreases the deleterious toxic effects of CPAs in immersed tissue compared to a higher exposure temperature for the same amount of time [Fahy *et al*. 1987, Mouttham and Comizzoli 2016]. Successful vitrification of ovarian tissue has been attained following CPA exposure at 4°C in some species such as cattle [Mouttham *et al*. 2015], sheep [Fathi *et al.* 2011], humans [Huang *et al*. 2008, Amorim *et al*. 2011], mice [Fatehi Ebrahimi *et al*. 2014], cat [Mouttham and Comizzoli 2016], rhesus macaque [Ting *et al*. 2011], and baboons [Lu *et al*. 2014]. In a study, isolated secondary bovine follicles were immersed to a vitrification medium containing permeable CPAs, EG and DMSO for either 1 or 30 min at room temperature or 30 min at 4°C; fewer abnormal follicles were observed in exposure at 4°C compared to exposure at room temperature (80% vs. 100% abnormal follicles, respectively) [Bao *et al*. 2010]. The lack of effect of exposure temperature of CPAs can be due to the recovery of oocytes from follicles immediately after vitrification/warming without any culture period for follicle and its oocyte healing. In almost all cases, vitrification leads to a decrease in fairly normal morphology and viability of follicles immediately following warming [Mouttham and Comizzoli 2016]. This suggests two types of cryoinjury: an immediate effect, likely caused by osmotic shock despite the gradual increase in CPAs concentration and the presence of sucrose [Pegg 2005], and a short- to mid-term effect, likely caused by the cytotoxic effect of EG and DMSO, which mostly manifests itself after a period post-warming culture [Keros *et al*. 2009]. In this study, culture of the follicles following warming was not performed, so only the first type of cryoinjury could be traced which cannot in related to the temperature of exposure to CPAs wherein lower temperature caused lower toxicity.

Table 2. Effect of oocyte recovery and culture methods on *in vitro* maturation of bovine oocytes derived from ovarian tissue vitrified in 4°C

Groups		Oocyte No.	Oocyte maturation stage No. (mean±SEM)				
oocyte retrieval	co-culture		degenerated	GV	GVBD	M-I	M-II
Vitrified aspiration	no	78	78.87 (2.87)^{ab}	4 (6.17±3.53)^{ab}	44 (55.05±4.07)^{a}	14 (18.19±3.77)^{a}	9 (11.71±1.47)^{a}
aspiration	yes	98	16 (15.60±5.00)^{a}	16 (13.77±4.40)^{a}	35 (35.15±4.56)^{b}	24 (28.78±8.06)^{ab}	3 (3.53±1.76)^{a}
slicing	no	151	0	2 (1.88±1.28)^{b}	57 (43.52±3.89)^{ab}	62 (39.01±4.68)^{b}	30 (19.95±0.87)^{b}
slicing	yes	101	5 (4.18±1.42)^{b}	6 (6.15±1.45)^{ab}	54 (53.67±6.77)^{a}	28 (27.88±5.47)^{ab}	8 (8.11±1.06)^{a}

^{abc}Mean values followed by different superscript letters in the same column differ statistically among the different groups (P<0.05).
Data are expressed as mean and standard error of the mean (SEM).

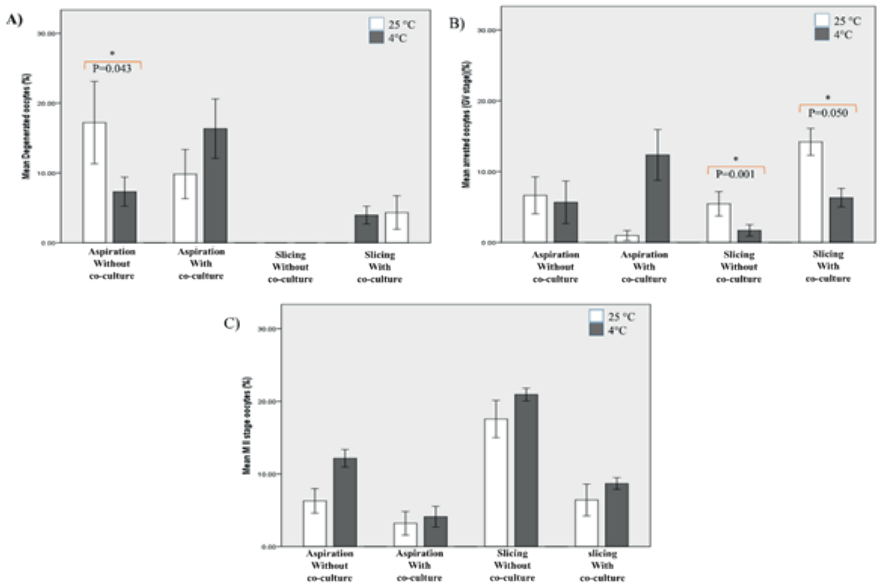

Fig. 2. Effect of the exposure temperature (4°C and 25°C) on the degeneration (A), arresting (B), and maturation (C) rates of bovine vitrified ovarian tissue derived- oocytes.
*The asterisk indicates significant differences between groups of each section.

In conclusion, it is better to retrieve the oocytes from vitrified ovarian tissue by slicing method and culture them in the maturation medium without co-culture with oviductal cells. Meanwhile, the exposure temperature in the vitrification procedure did not affect M-II rates of bovine oocytes.

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Conflict of interest statement
None of the authors have any conflict of interest to declare.
Data availability statement
Data are available on request from the authors.

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