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How origin of ovaries influences the vitrification outcome of bovine ovarian tissue: effects of side of ovaries and corpus luteum

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Abstract

Although cryopreservation of ovarian tissue has advanced greatly, it remains a challenge, and protocols should be optimized to handle the heterogeneous nature of ovarian samples. In an effort to address this factor, the present study evaluated the effects of corpus luteum (CL) and side of ovaries (right versus left) on cellular morphology and viability of vitrified bovine ovarian fragments in a closed system. The ovaries were categorized according to whether they had a CL and which side they were on, and then divided into six groups: 1) CL+ (with CL) group; 2) CL- (without CL) group; 3) right ovaries group; 4) left ovaries group; 5) fresh control group (ovaries without vitrification or culture that were not selected for CL or ovarian side) and 6) *In vitro* culture medium control group (non-vitrified ovaries that were not selected for the presence or absence of CL or side of the ovaries). The current study shows that the CL- and right groups had the greatest percentage of follicles with normal morphology compared to other vitrified-warmed groups. Furthermore, the levels of necrosis and tissue damage of the right cultured group were the lowest compared to other groups. It was shown that bovine ovarian tissues derived from right ovaries and ovaries without a corpus luteum can be functionally and morphologically preserved after vitrification. For the first time, the present study suggests that bovine ovarian tissue vitrification can be improved by considering the origin of the ovaries.

Introduction

Ovarian tissue cryopreservation has become an accepted clinical practice following reports of the recovery of ovarian function[1] in domestic animals,[2] and endangered species.[3] Even though cryopreservation of ovarian tissue has advanced significantly, it still poses a challenge, and protocols must be adjusted to accommodate its diversity of cell types and structures (oocyte, granulosa and endothelial cells, extracellular matrix)[4] and biological diversity among species.[5]

It has been demonstrated that dairy cows display asymmetrical reproductive function due to different ovarian functions and possibly due to physiological differences in the tubular structures of reproductive organs as a result of the side of the previous pregnancy.[6,7] The development of corpus luteum (CL) may cause asymmetry in the function of dairy cows' reproductive organs based on its relationship with follicle development.[8] Researchers found that follicles from the ovaries with CL of a previous gestation were smaller in diameter than those from the ovaries without CL,[9] and others found that the number of follicles growing to full size decreased significantly in the ovary ipsilateral to the CL.[10]

The unequal activity of the sides of the reproductive system may be another factor associated with different success rates of cryopreservation of ovarian tissue. This unequal activity may be related to the differences in ovulation response between ovaries from the left and the right, the differences between left and right uterine horn environment, or the differences in the developmental potential of oocytes originating from these ovaries.[11,12]

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According to our knowledge, this is the first study attempting to examine the impacts of CL and side of ovaries (right versus left) on cellular morphology and viability of bovine ovarian tissue vitrified in a closed system.

Materials and Methods

The Ethics Committee for Animal Use at the University of Tabriz approved this experiment and it was performed according to their guidelines (D-39-1423/2015.10.15). Sigma Chemical Co. (St. Louis, USA) provided all the chemicals, except were indicated otherwise.

Collection of ovaries and experimental groups. At a local slaughterhouse, five pairs of ovaries (with diestrus CLs) were collected from five adult non-pregnant cross-bred cows.[11] These ovaries were categorized based on the presence or absence of a CL and the side of ovaries and divided into six pools: 1) CL+ (with CL) group; 2) CL- (without CL) group; 3) right ovaries group; 4) left ovaries group; 5) fresh control group (ovaries which were not selected for the presence or absence of CL or side of ovaries and without vitrification and culture); and 6) *In vitro* culture (IVC) medium control group (ovaries with no selection for CL, side of ovaries, and also not vitrified). Sixty fragments (~3.00 × 3.00 × 0.50 mm) were recovered from each ovarian pair. For each pair, control samples included 10 fresh control fragments and 10 fragments from an IVC control group (without vitrification). The remaining 40 fragments were distributed between four vitrification and culture groups (CL+, CL-, right ovaries, and left ovaries groups). For the IVC control group, fresh fragments were cultured for 1 day (D1) or 5 days (D5). For the vitrified groups, they were vitrified and then cultured for D1 or D5; histological analysis was performed for two samples in each group, and the other eight samples were cultured *in vitro* for D1 or D5. Viability tests and immunolocalization of Aquaporin 3 (AQP3), connexin 43 and 37 (Cx43 and Cx37) were performed for the fresh and vitrified-warmed groups after 5 days of culture.

Cryopreservation and warming methods. The Ovarian Tissue Cryosystem (OTC) was used to expose the samples to cryoprotectant agents (CPAs) and to vitrify them, as previously described by our group.[13,14] To achieve equilibrium between the tissues and the vitrification solution (VS), two steps were employed: (i) VS1 was a blend of Minimum Essential Media (MEM) supplemented with 10.00 mg mL^{-1} bovine serum albumin (BSA), sucrose (0.25 M), 10.00% dimethyl sulfoxide (DMSO; Dinâmica Química, Diadema, Brazi) and 10.00% ethylene glycol (EG; Dinâmica). Additionally, VS2 consisted of MEM supplemented with 10.00 mg mL^{-1} BSA, 0.25 M sucrose, 20.00% DMSO and 20.00% EG. Initially, CPA exposure (VS1) was performed at 20.00 ˚C for 4 min followed by an exposition to the CPA cocktail (VS2) at 20.00 ˚C for 1 min. A week after being stored in liquid nitrogen, a 1min exposure to room temperature (25.00 ˚C) was used to rewarm the vitrified ovarian tissues. The vitrified-rewarmed samples were washed in three solutions for 5 min each to remove the CPAs as follows: (1) α Minimum Essential Media (αMEM) supplemented with 3.00 mg mL^{-1} BSA and 0.50 M sucrose; (2) αMEM supplemented with 3.00 mg mL^{-1} BSA and 0.25 M sucrose; (3) αMEM supplemented with 3.00 mg mL^{-1} BSA.[14]

***In vitro* culture medium.** Culture of vitrified ovarian fragments was conducted in McCoy medium supplemented with 50.00 mg mL^{-1} ascorbic acid , 20.00 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10.00 ng mL^{-1} insulin, 3.00 mM glutamine (Invitrogen Ltd., Paisley, UK), 4.00 ng mL^{-1} selenium, 0.10% BSA (Fraction V), 2.50 mg mL^{-1} transferrin, 0.10 mg mL^{-1} penicillin G, and 0.10 mg mL^{-1} streptomycin for 1 (D1) or 5 days (D5) in a humidified incubator (Memmert GmbH, Büchenbach, Gremany) to provide 38.50 ˚C and 5.00 % CO_2 condition.[15]

Histological assessment. Fragments were used for classic histology at different stages of the experiment. After mounting ovarian sections on a glass slide and staining with Periodic acid-Schiff, a light microscope (Nikon, Tokyo, Japan) was employed to examine the morphology of follicles. In brief, a normal ovarian follicle contains intact granulosa cells surrounding an oocyte, while the main characteristics of degenerated follicles are the presence of a pyknotic nucleus in oocytes, shrunken ooplasma, and/or detached and disorganized granulosa cell layers from the basement membrane.[13]

Viability assessment of vitrified ovarian tissue by fluorescence microscopy: reactive oxygen species (ROS) levels. To determine the amount of ROS, ovarian fragments from each treatment were incubated in 2′,7′ dichlorodihydrofluorescein diacetate (DCF; H2DCFDA) for 30 min.[16] Subsequently, the ovarian fragments were fixed with 4.00% paraformaldehyde for 15 min and stored in Phosphate buffered saline (PBS) solution at 4.00 ˚C away from light until examination. The fluorescence intensity of three randomly selected fields (1024 × 1024 pixels) was measured for each sample, and three-dimensional distribution analysis was performed using a z-stack of 10 optical series (step size of 10.00 µm).[16] The DCF probe (emission 519; excitation 495 nm) was assessed using an Argon ion laser ray at 488 nm, and the program Zen converts each sample's fluorescence intensity into numbers, which were statistically analyzed and reported as mean fluorescence for each probe.[17,18]

Ethidium homodimer-1 (EthD-1) coloration. By binding to nucleic acids, EthD-1 increases fluorescence 40-fold in cells whose membranes have been damaged, causing dead cells to emit a bright red fluorescence (excitation/emission ~ 495 nm / ~ 635 nm). It does not penetrate intact cell membranes of live cells.[19] Incubation at 37.00 ˚C for 15 min was carried out on 5-day cultured

ovarian tissues in 1,000 mL of medium containing 100 µM EthD-1drops (Molecular Probes, Karlsruhe, Germany). For image analysis, all ovarian tissues were fixed in 4.00% paraformaldehyde for 15 min and stored in PBS solution (4.00 ˚C) away from light. A fluorescence microscope (Eclipse 80i; Nikon) was used to examine the follicles after they had been washed in MEM HEPES. The fluorescence signals generated by EthD-1 were monitored at 568 nm, respectively, and program Zen was used to convert the fluorescence intensity of each fragment into numbers. These values were statistically analyzed and reported as mean fluorescence for each probe.[20]

Statistical analysis. Sigma Plot (version 11.0; Systat Software Inc., Palo Alto, USA) was used for all statistical analysis. The Mann-Whitney test was used to compare two groups, and GLM procedure for (ANOVA) was used for normal traits with more than two categories. Least square means were used for comparisons of means, and logarithmic transformation was used for the traits that were not normal. The non-parametric Kruskal-Wallis test was used for traits whose residual distribution was not normal. Results were considered different when the *p*-value was less than 0.05 and presented as means (± standard error of the mean). Probability values between 0.05 and 0.1 indicated that a difference approached significance (two-sided).

Results

Histological findings. Classical histological analysis was used to evaluate a total of 3934 follicles. In non-cultured samples, the percentage of morphologically normal follicles in fresh control (408/576; 69.20 ± 2.70%) was higher ($p < 0.05$) than that in CL+ samples (43.70%) and left samples (29.40%; Table 1). Furthermore, in post-warmed groups, the percentage of normal follicles was the highest in CL- and right samples (72.40% and 65.70%, respectively), lower in the CL+ samples (43.70%) and the lowest in left samples (29.40%; $p < 0.05$). After IVC for 5 days, the same percentage of normal follicles was observed in right cultured samples (75.90%) and in the fresh control (69.20%). On the other hand, cultured samples (1 day) had fewer ($p < 0.05$) normal follicles in all groups than post-warmed ones. Right and left cultured samples on D5 were the only vitrified-warmed samples that kept an equal percentage of normal follicles as in the post-warmed ones (Right: 75.90% compared to 65.70%; Left: 24.40% compared to 29.40%; respectively; Table 1). Furthermore, in groups which were cultured for 1 day, the normal follicles percentage was the highest in the IVC control samples (56.00%), lower in the CL- and right samples (42.80% and 40.90%, respectively) and the lowest in CL+ and left samples (27.30% and 18.10%, respectively). On day 5, the normal follicles percentage was the highest in the right culture ovarian tissue and the lowest in CL+ and left samples. However, the same percentages of normal follicles were observed on day 5 in IVC control samples and CL- samples (52.00% and 51.80%, respectively).

Table 2 presents the mean percentages of primordial and developing follicles. The mean ± SEM percentage of primordial and developing follicles in fresh control group were 59.00 ± 3.70 and 41.00 ± 3.70, respectively. In almost all groups, the proportion of primordial follicles on D5 was lower than that of post-warmed tissues ($p < 0.05$); how-ever, the percentages of morphologically normal follicles in CL- cultured samples on D5 tended to decrease when compared to those of post-warmed CL- tissues ($p = 0.06$). Throughout all groups, a similar proportion of primordial or developing follicles was maintained from D1 to D5. Although CL+ and right treatment groups maintained a similar percentage of primordial or developing follicles from post-warming to D1, other cultured samples (CL- and left) experienced a decrease on day 1 in the percentages of normal follicles when compared to those of post-warmed tissues ($p < 0.05$). Furthermore, the proportion of primordial or developing follicles of post-warmed samples did not differ among groups ($p > 0.05$). However, this amount on day 1 in CL- samples (39.30 %) was lower ($p < 0.05$) than IVC control (57.30%), CL+ (57.60%) and fresh control samples (59.00%). Additionally, in either right or left samples, the percentage of follicles with primordial or developing characteristics did not differ significantly on day 1 ($p > 0.05$). On D5, no difference was observed between treatment groups in the proportion of primordial or developing follicles ($p > 0.05$). However, the percentages of primordial or developing follicles on day 5 in CL- (37.30%) and left samples (39.8%) were lower ($p < 0.05$) than that of fresh control samples (59.00 %; Table 2).

Ethidium homodimer-1. In comparison with the fresh control group, CL- cultured samples on D5 exhibited higher ($p < 0.05$) tissue damage rates (Table 3). Compared to ovarian fresh control tissues, the right culture ovarian tissue and IVC control group showed lower levels of tissue damage on D5. However, the levels of necrosis and tissue damage of the IVC control group on D5 were higher compared to the right samples. Other groups showed higher levels of tissue damage than the IVC control group. Furthermore, the levels of necrosis and tissue damage were the highest in CL- cultured samples (46.60%), lower in the CL+ (27.10%), and left cultured samples (27.90%) and the lowest in right samples (14.60%) on day 5.

Reactive oxygen species. There was a higher level of ROS ($p < 0.05$) in the CL- cultured group (D5) compared to fresh control samples (Table 3). Furthermore, ROS levels of the CL- cultured samples on D5 were higher ($p < 0.05$) compared to other treatment groups.

Table 1. Average ± standard error of the mean (SEM) percentage of morphologically normal follicles. For ovarian fragments that were non-vitrified (fresh control), *in vitro* cultured control (IVC control), or vitrified and cultured CL-, CL+, right and left groups (*in vitro* culture day 5).

Group	Post-warming		*In vitro* culture			
			Day 1		Day 5	
	Counted follicles	Percentage of normal follicles (n)	Counted follicles	Percentage of normal follicles (n)	Counted follicles	Percentage of normal follicles (n)
IVC control	-	-	191	56.00 ± 4.10 (109)^{aA*}	209	52.00 ± 4.00 (119)^{aB*}
CL-	221	72.40 ± 3.50 (158)^{aA}	244	42.80 ± 3.50 (117)^{bB*}	200	51.80 ± 4.90 (102)^{bB*}
CL+	307	43.70 ± 4.10 (127)^{aB*}	231	27.3 ± 3.50 (62)^{bC*}	299	25.20 ± 3.40 (64)^{bC*}
Right	207	65.70 ± 4.20 (137)^{aA}	155	40.90 ± 4.80 (60)^{bB*}	203	75.90 ± 3.20 (154)^{aA}
Left	235	29.40 ± 3.40 (93)^{aC*}	298	18.10 ± 3.00 (52)^{bC*}	258	24.40 ± 2.90 (60)^{abC*}

^{ab} indicate significant differences within a row ($p < 0.05$).
^{ABC} indicate significant differences within a column ($p < 0.05$).
* indicates significant differences compared to the fresh control group ($p < 0.05$).

Table 2. Average (± SEM) percentage of primordial and developing follicles. Bovine ovarian samples were non-vitrified (fresh control), *in vitro* cultured control (IVC control), or vitrified and cultured CL-, CL+, right and left groups (*In vitro* culture Day 5).

Group	Post-warming		*In vitro* culture			
			Day 1		Day 5	
	Primordial	Developing	Primordial	Developing	Primordial	Developing
IVC control	-	-	57.30 ± 5.30^{aA}	42.70 ± 5.30	44.50 ± 5.20^{aA}	55.50 ± 5.20
CL-	53.60 ± 4.70^{aA}	46.40 ± 4.70	39.30 ± 5.00^{bB*}	60.70 ± 5.00	37.30 ± 6.20 $^{\dagger A*}$	62.70 ± 6.20
CL+	66.40 ± 5.20^{aA}	33.60 ± 5.20	57.60 ± 6.60^{abA}	42.40 ± 6.60	47.00 ± 6.60^{bA}	53.00 ± 6.60
Right	63.00 ± 5.60^{aA}	37.00 ± 5.60	50.00 ± 7.20^{abAB}	50.00 ± 7.20	46.60 ± 4.40^{bA}	53.40 ± 4.40
Left	67.50 ± 5.80^{aA}	32.50 ± 5.80	46.70 ± 7.90^{bAB}	53.30 ± 7.90	39.80 ± 6.70^{bA*}	60.20 ± 6.70

^{ab} indicate significant differences within a row ($p < 0.05$).
^{AB} indicate significant differences within a column ($p < 0.05$).
* indicates significant differences compared to the fresh control group ($p < 0.05$).
† indicates tendency to difference compared to CL- post-warming group ($p = 0.06$)

Table 3. Percentage of fluorescence intensity (pixels) of follicle degeneration and assessment of ROS levels (mean ± SEM) on bovine ovarian tissues submitted to treatments. Bovine ovarian fragments were non-vitrified (fresh control), *in vitro* cultured control (IVC control), or vitrified and cultured CL-, CL+, right and left groups (*in vitro* culture Day 5).

Treatment	Follicle degenerating	ROS levels
Fresh control	30.80 ± 1.20	42.40 ± 1.70
	In vitro culture - Day 5	
IVC control	20.40 ± 0.40^{b*}	44.10 ± 0.40^{a}
CL-	46.60 ± 0.50^{d*}	69.40 ± 3.10^{b*}
CL+	27.10 ± 0.50^{c}	40.60 ± 2.30^{a}
Right	14.60 ± 1.80^{a*}	46.80 ± 3.30^{a}
Left	27.90 ± 2.10^{c}	46.40 ± 2.90^{a}

Bovine ovarian fragments were non-vitrified (fresh control), cultured control (IVC control), or vitrified and cultured CL-, CL+, right and left groups (*In vitro* culture Day 5).
^{abcd} indicate significant differences within a column ($p < 0.05$).
* indicates significant differences compared to the fresh control group ($p < 0.05$).

Discussion

Despite all the success of ovarian tissue cryo-preservation, some issues still remain, and protocols must be optimized to deal with the heterogeneity of ovarian samples.[21] The present study indicates that morphological features of vitrified bovine preantral follicles are significantly affected by ovarian side and CL. Compared to other vitrified-warmed groups, the CL- and right groups showed the highest percentage of morphologically normal follicles. These results are in consistent with those of previous studies in cows, which demonstrated that oocytes from right ovaries[22] or ovaries without CLs had greater developmental potential than those of left and CL-bearing ovaries, respectively. Basic scientific findings have revealed that both sexes of human fetuses have right gonads that are bigger, heavier, and contain more deoxyribonucleic acid (DNA) compared to their left counterparts.[23] Furthermore, it has been demonstrated that several physical and physiological differences exist between them in terms of venous drainage, anatomical relation, and physiological control.[13]

Furthermore, the levels of tissue damage of the right cultured group on D5 were the lowest compared to other groups. There is evidence that several membrane proteins, including connexins (Cxs), were damaged by cryo-

preservation.[24] Oocytes and follicular cells interact through these proteins. Intercellular communication damage is usually detrimental to the development of follicles and maturation of oocytes, as it reduces molecule exchange, such as ribonucleic acid (RNA),[25] amino acids, nucleotides, growth factors and glucose.[26] The IVC also reduce the expression of Cx43 on follicles that are either isolated or surrounded by ovarian tissue.[18] The ovary contains a number of Cxs (Cx26, Cx30, Cx32, Cx37, Cx40, Cx43, and Cx45), but Cx37 and Cx43 are shown to exert a significant influence on folliculogenesis.[19] The Cxs are the main gap junction proteins expressed by granulosa cells and the Cumulus Oocyte Complex in bovine.[27] Immunohistochemical analysis revealed that all follicle classes presented strong staining for Cx43 in the right group (data no shown). Moreover, transitional follicles were strongly immunostained for Cx37 in right samples, whereas transitional follicles of the fresh control showed moderate labeling for Cx37.

This may also account for a greater proportion of normal follicles in vitrified-warmed ovarian fragments derived from the right ovaries and the lowest levels of tissue damage of the right cultured group in the present study. This contrasts with previous studies in mice[28] and felines,[29] where low Cx43 expression was associated with low follicular development in cryopreserved ovarian fragments. It has been determined that 55.00 - 65.00% of ovulations and pregnancies in cattle and sheep occur on the right side,[22,29] the opposite side of the rumen. According to McDonald,[29] the rumen reduces blood flow to the left side, which might affect gonadotropin levels in the left ovary. Furthermore, in humans, even though ovarian arteries directly from the aorta provide both ovaries with arterial blood supply, they have a different venous drainage; unlike the right ovarian vein, which drains directly to the inferior vena cava, the left drains first to the left renal vein, and later to the inferior vena cava.[23] Surgical denervation and pharmacological studies have demonstrated that ovarian innervation affects ovarian functions[30] and vagal innervation influences follicular counts in left and right ovaries differently in rats.[31] In addition, there might be some form of vascularization information between the ovaries[32] or intra-ovarian mechanisms.[33]

A number of Cxs have been shown to influence vascular function and angiogenesis in multiple adult organs, including Cx37, Cx40, Cx43, and Cx45.[34] Therefore, the strong presence of Cx37 and Cx43 in the right ovaries may be another explanation for the superior activity of the right side in previous reports. Accordingly, a greater proportion of morphologically normal follicles in vitrified-warmed ovarian fragments derived from right ovaries in the present study may be due to higher concentrations of luteinizing hormone (LH) and follicle stimulating hormone receptors in the right versus left ovaries.[35]

According to our results, the proportion of morphologically normal follicles was higher in the CL- than CL+ samples. It was found that the presence or absence of a CL affected the number, quality, and cryotolerance of ovine[36] and bovine[37] embryos. Many *in vitro* studies have reported local paracrine and autocrine effects exerted by CL and follicles.[12,38] Progesterone suppresses LH pulse frequency, which is vital for the production of estradiol[39] and the development of large follicles.[40] Some researchers have demonstrated that local inhibitory factors are secreted from the CL.[10] All of the follicles that have reached the recruitment phase, with the exception of the dominant ovarian follicle, die from local inhibin, which is released by the CL of goats and cattle.[16] Additionally, a previous study[41] suggested that follicular fluid derived from follicles in bovine ovaries without a CL is better suited for oocyte maturation than that derived from ovaries with a CL.

We have shown that bovine ovarian tissues derived from right ovaries and ovaries without a corpus luteum can be functionally and morphologically preserved after vitrification. In other words, this study demonstrates for the first time that the origin of ovarian tissue plays an important role in the success of bovine tissue vitrification.

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Conflict of interest

The author declares that there is no conflict of interest.

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