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Effects of new synthetic cryoprotectant agents on histological characteristics of various classes of vitrified bovine pre-antral follicles

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Abstract

Previous studies have reported many discrepancies about the best type and concentration of cryoprotective agents (CPAs) and biological variability among various pre-antral follicle classes after cryopreservation of ovarian tissue. The aim of this study was to investigate the impacts of some synthetic polymers on histological characteristics of different types of pre-antral follicles after bovine ovarian tissue vitrification. From each bovine ovarian pair, fragments were recovered and immediately fixed for analysis (fresh control group) or submitted to vitrification (sucrose, X-1000, Z-1000 and polyvinylpyrrolidone groups), either followed by *in vitro* culture for 1 or 5 days. In this case, although, the addition of X-1000 resulted in greater percentages of normal follicles for almost all pre-antral follicle classes compared to those of other groups, there are some exceptions. These results indicate that the inclusion of polyvinylpyrrolidone in the freezing media can improve the morphology of the post-warmed transitional follicles and cultured primordial follicles on day five more than other CPAs. According to the results of this study, it can be concluded that although ovarian tissue cryopreservation is often performed to preserve the primordial follicles, by choosing the best combination of permeating and non-permeating CPAs (synthetic polymers), more advanced stages of bovine pre-antral follicles, transitional, primary and secondary follicles, may also survive the cryopreservation process.

اثرات عوامل جدید سنتتیک محافظت‌کننده در برابر انجماد بر ویژگی‌های بافت‌شناسی انواع دسته‌جات فولیکول‌های پیش حفره‌دار گاوی شیشه‌سازی شده

چکیده

مطالعات گذشته اختلافات فراوانی در مورد بهترین نوع و غلظت عوامل محافظت‌کننده در برابر انجماد و تنوع زیستی بین دسته جات مختلف فولیکول های پیش حفره‌دار بعد از فرآیند محافظت در برابر انجماد بافت تخمدانی گزارشاتی را ارائه نموده اند. هدف مطالعه حاضر، بررسی آثارپلیمرهای سنتتیک جدید بر ویژگی های بافت شناسی انواع مختلف فولیکول های پیش حفره‌دار پس از انجماد به روش شیشه سازی بافت تخمدان گاوی بود. قطعات تخمدانی از هر جفت تخمدان گاوی به دست آمدند و به سرعت جهت ارزیابی مورد تثبیت قرار گرفتند (گروه شاهد تازه) یا تحت فرآیند شیشه سازی (گروه های سوکروز، X-1000، Z-1000 و پلی وینیل پیرولیدون) واقع شدند و هر دو گروه به مدت یک یا پنج روز به صورت برون تنی کشت داده شدند. در این رابطه اگرچه اضافه نمودن X-1000 موجب درصد های بالاتری از فولیکول های طبیعی تقریباً برای تمام انواع فولیکول پیش حفره‌دار در مقایسه با دیگر گروه ها گردید، اما استثناهایی نیز وجود داشت. این نتایج بیانگر آن است که اضافه نمودن پلیمر پلی وینیل پیرولیدون در محیط های انجماد می تواند نسبت به دیگر عوامل محافظت کننده در برابر انجماد موجب بهبود ریخت شناسی فولیکول های انتقالی در مرحله پس از گرم‌سازی و فولیکول های مقدماتی کشت داده شده در روز پنجم گردد. بر اساس نتایج حاصل از این مطالعه، این گونه می‌توان نتیجه گرفت که با وجود آنکه اغلب هدف از فرآیند محافظت در برابر انجماد بافت تخمدانی، حفظ فولیکول های مقدماتی می باشد، اما با انتخاب بهترین ترکیب از عوامل محافظت کننده در برابر انجماد نفوذ کننده و غیرنفوذ کننده (پلیمرهای سنتتیک) به داخل سلول، مراحل پیشرفته‌تر فولیکول های پیش حفره‌دار گاوی مانند فولیکول های انتقالی، اولیه و ثانویه نیز ممکن است پس از فرآیند محافظت در برابر انجماد حفظ گردند.

واژه های کلیدی: ریخت شناسی، فولیکول پیش حفره‌دار، محافظت در برابر انجماد، محیط های انجماد

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Introduction

Cryopreservation of ovarian tissue has been applied in clinical practice since the reports indicated ovarian function recovery in human,[1] domestic animals[2,3] and endangered species.[4] Cryopreservation of ovarian tissue offers many advantages over mature oocyte or embryo to preserve female germline of endangered animals. Firstly, the ovary contains a large pool of oocytes enclosed in primordial follicles. Secondly, ovarian tissue can be collected from animals at almost all developmental ages (adult, pre-puber and foetus) and statuses (alive or dead).[5] Vitrification has become an effective alternative for the preservation of oocytes and embryos[6,7] and has proven to be a very attractive method for the cryopreservation of primordial follicles within ovarian tissue.[8,9] Although ovarian tissue cryopreservation is often performed to preserve the primordial follicles,[10,11] more advanced stages of pre-antral follicles, i.e., primary and secondary follicles, may also survive following cryopreservation process.[12-14] It is important to mention that the outermost layer of the ovary contains primordial and most of the primary follicles and can be used for future transplantation, whereas the second layer of the ovarian cortex typically contains some primary and most secondary follicles and can be used for follicle isolation and *in vitro* maturation.[15]

However, compared to the more advanced follicular stages, primordial follicles are more effectively cryopreserved because their oocytes have a relatively inactive metabolism, lack of metaphase spindle, zona pellucida and cortical granules and low amounts of lipids.[12,16] Studies in the last decade have demonstrated the feasibility of vitrification of the ovarian cortex containing pre-antral follicles. For example, vitrification of ovarian tissue has resulted in preserved follicular morphology and viability[17] and stromal density.[18]

Despite all advances of cryopreservation of ovarian tissue, this strategy still is challenging and protocols should be optimized to handle the diversity of cell types and components of this tissue including oocyte, granulosa cells, endothelial cells, and extracellular matrix,[13] dissimilarities between different types of pre-antral follicles[16] and the biological variability among species.[19] According to Amorim *et al*., many factors influence the likelihood of a successful vitrification outcome such as the type and concentration of cryoprotective agents (CPAs), the temperature of exposure to vitrification solution, stepwise addition of vitrification solution, sample size, carrier system, quality of samples and technical expertise.[20] The challenge in formulating successful vitrification solutions is to use CPAs at concentrations that are non-toxic but allow for vitrification at realistic cooling and warming rates.[21] Nature has evolved elegant solutions to mitigate the effects of ice recrystallization by the production of anti-freeze glycoproteins and anti-freeze proteins (AF(G)Ps) identified and isolated from polar fish, plant and insects, commonly.[22] As an alternative, synthetic polymers were investigated as AF(G)P mimics.[22] Some polymers are easy to access on a large scale, are highly tunable in terms of composition and architecture and are widely used in personal care and pharmaceutical industries making them appealing additives.[23] Fahy *et al*. have developed several synthetic polymers including a copolymer of polyvinyl alcohol (PVA; super cool X-1000), polyvinylpyrrolidone (PVP) K12 and polyglycerol (super cool Z-1000).[24] Super cool X-1000 is an additive that enhances the performance of vitrification solutions and super cool Z-1000 is an additive that suppresses ice formation to enhance the performance of vitrification solutions. One or more of these polymers have recently been used to supplement vitrification solutions in a variety of different living systems such as rabbit tissues[24], embryos and oocytes,[25,26] and rat tissues.[27] Unlike conventional cryoprotectants that inhibit freezing by interacting with water, such synthetic ice blockers are believed to act by direct molecular recognition of ice nucleators[28] or perhaps ice itself.[29,30]

The aim of the present study was to investigate the impacts of some new synthetic polymers on the morphology of different classes of pre-antral follicles (primordial, transitional, primary and secondary follicles) after bovine ovarian tissue vitrification.

Materials and Methods

This experiment was approved and performed under the guidelines of the Ethics Committee for Animal Use of the State University of Ceará in Fortaleza, Ceará, Brazil. Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, USA).

Collection of ovaries and experimental groups. Ovaries (n = 10) were collected from five adults cross-bred cows at a local abattoir located in Fortaleza, Ceará, Brazil. Ovaries from pregnant females (pregnancy was diagnosed after slaughtering by visual examination of fetal membranes, amniotic vesicle, cotyledons, fetus or changes in uterine tone and shape) and those with pathological lesions such as cystic follicles were not included in the current study.

Only animals with a diestrus corpus luteum (CL) were included in the study; the cows in diestrus had a CL on either right or left side. Immediately postmortem, ovaries were washed once in 70.00% (v v^{-1}) ethanol and then washed twice in hydroxyethyl piperazineethanesulfonic acid (HEPES)-buffered minimum essential medium (MEM) supplemented with 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The ovaries were then transported to the laboratory in a MEM at 4 ˚C within 1 hr. At the laboratory, ovaries were stripped of surrounding fat and fibrous tissue and the ovarian cortex from each ovarian pair was cut into

60 small fragments (~ 3 × 3 × 1 mm) using a tissue slicer (Thomas Stadie-Riggs Tissue Slicer/Blades, Thomas Scientific, Swedesboro, USA) under sterile conditions. For each animal, 10 fragments were used as fresh control samples and 10 fragments were used as fresh cultured samples (without vitrification) followed by *in vitro* culture (IVC) for 1 (D1) or 5 (D5) days. The remaining 40 fragments were randomly distributed across the following vitrified + cultured groups (n = 10 in each group): 1) Vitrified sucrose, 2) Vitrified X-1000, 3) Vitrified Z-1000 and 4) Vitrified PVP followed by IVC (D1 or D5). Fresh control samples were immediately intended for histological analysis. Fragments from fresh cultured samples were subjected to the same analyses after 1 or 5 days of culture. After warming, two fragments from each cryopreserved group were immediately submitted to analysis and the remained fragments (n = 8) were cultured *in vitro* for 1 or 5 days (D1 or D5).

Vitrification and warming procedures. All procedures for exposure to CPAs and vitrification were performed by using the new ovarian tissue cryosystem, as previously described.[31,32] Shortly, equilibrium of the samples in the vitrification solution (VS) was performed gradually in two steps: i) VS1 contained MEM supplemented with 10 mg mL^{-1} bovine serum albumin (BSA), 0.25 M sucrose (only in vitrified sucrose group), 10% ethylene glycol (EG) (–Dinâmica, Química, Diadema, Brazil) and 10% dimethyl sulfoxide (DMSO;–Dinâmica, Química, Diadema, Brazil) with or without polymers (0.20% [v v^{-1}] super cool X-1000, 0.40% super cool Z-1000 or 0.20% PVP K-12; (21st Century Medicine, Fontana, USA).[15] Similarly, ii) VS2 was composed of MEM supplemented with 10 mg mL^{-1} BSA, 0.25 M sucrose (only in vitrified sucrose group), 20.00% EG and 20.00% DMSO with or without polymers. The fragments were initially exposed to VS1 for 4 min at 38 ˚C. Subsequently, they were exposed to VS2 for 1 min at 38 ˚C. After 1 week of storage in liquid nitrogen, the samples were warmed at room temperature (25 ˚C) for 1min. The CPA removal was handled by washing the fragments in three solutions (5 min each) described below: 1) αMEM +3.00 mg mL^{-1} BSA + 0.50 M sucrose, 2) αMEM + 3.00 mg mL^{-1} BSA + 0.25 M sucrose, and 3) αMEM + 3.00 mg mL^{-1} BSA.[32]

***In vitro* culture.** For the IVC, the cortex tissue samples were transferred to 24-well culture dishes containing 1 mL of the culture medium per well. The culture was performed at 38 ˚C in 5.00% CO_2 in a humidified incubator. Fresh media were incubated for 1 hr prior to use and every alternate day, half of the culture media was exchanged with fresh culture media. The culture medium consisted of the McCoy medium as a recommended medium for the culture of vitrified bovine ovarian tissue[33] with bicarbonate supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; Invitrogen Ltd., Cheshire, UK), glutamine (3.00 mM; Invitrogen), BSA (Fraction V 0.10%), penicillin G (0.10 mg mL^{-1}), streptomycin (0.10 mg mL^{-1}), transferrin (2.50 mg mL^{-1}), selenium (4 ng mL^{-1}), insulin (10 ng mL^{-1}) and ascorbic acid (50 mg mL^{-1}). Fragments were cultured for 1 (D1) or 5 (D5) days.[33,34] The reason behind the including two culture time in the present study was to evaluate the effects of these polymers on different follicles during the culture. Because the culture condition is not usually that perfect to preserve all follicles morphology and in most cases the percentage of morphologically normal follicles is decreased during the culture. On the other hand, there is no way to distinguish which follicle was developed to which one during the culture (it is only possible in follicle isolation technique, Ting *et al.*,[15] thus we have to compare between the same class during different days of culture or with fresh control group to determine the effects of polymer on follicle culture similar to methods published previously.[15,33]

Histological analysis. Samples were fixed in Millonig's solution (phosphate-buffered 40% v v^{-1} formaldehyde in water) for 2 hr, dehydrated in a graded series of ethanol, clarified with xylene, embedded in paraffin wax and serially sectioned into 7 µm sections. Every fifth section was mounted on a glass slide, stained with periodic acid–Schiff and evaluated using a light microscope (Nikon, Tokyo, Japan) at a magnification of 400×. In each treatment, a total of 150 pre-antral follicles (30 per animal) were examined, which were classified as follicles without an antrum and with an oocyte, surrounded by one layer of flattened (primordial follicle), flattened and cuboidal (transitional follicle) or cuboidal granulosa cells (primary follicle) or with an oocyte surrounded by two or more layers of cuboidal granulosa cells (secondary follicle; Fig. 1).[16] The quality of the pre-antral follicles was classified according to the parameters previously described.[35]

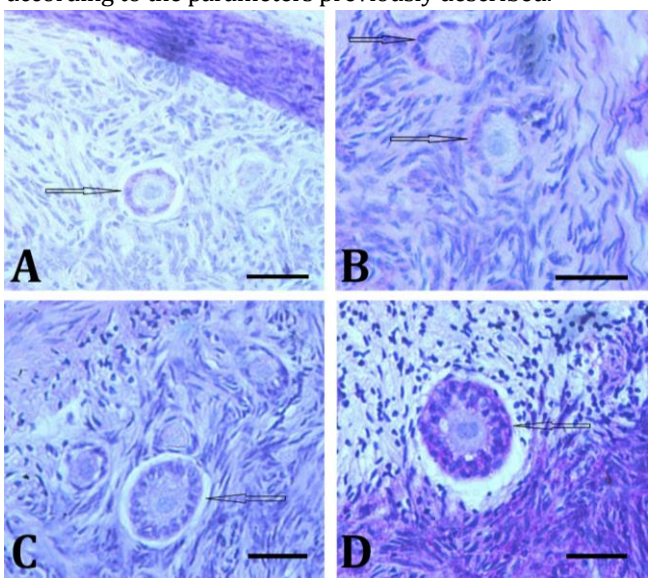

Fig. 1. Photomicrographs of bovine ovarian cortex sections. Arrows show **A)** primordial; **B)** transitional; **C)** primary; and **D)** secondary follicle (periodic acid–Schiff, Bar = 100 µm).

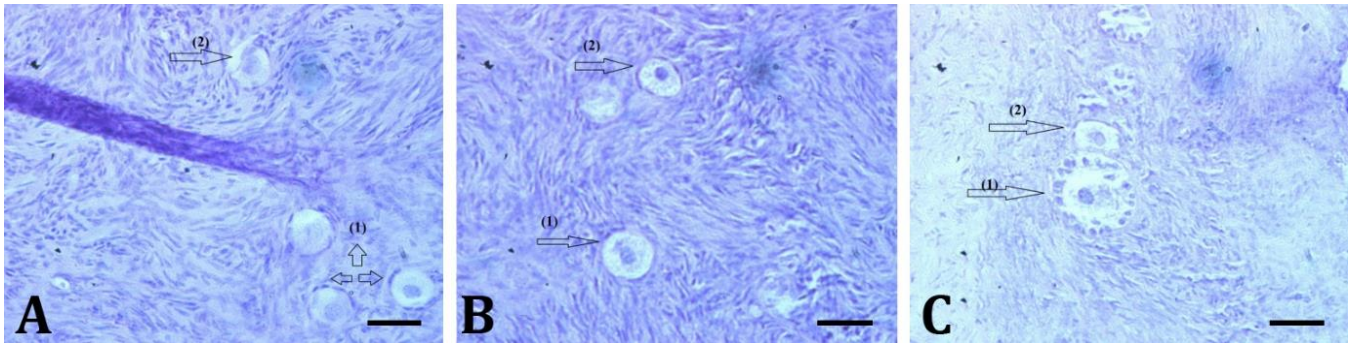

Fig. 2. Photomicrographs of bovine ovarian cortex sections after warming at day 1 and day 5 of culture. **A)** Warming: Three normal primordial follicles (1) and degenerated primordial follicle with misshape ooplasm and shrinkage (2); **B)** Day 1: Normal primordial follicle (1) and degenerated primordial follicle with pyknotic nucleus; **C)** Day 5: Degenerated primary follicle with ooplasm shrinkage and/or granulosa cell disorganization (1) and normal primordial follicle (2), (periodic acid–Schiff, Bar = 100 μm).

Briefly, morphologically normal follicles contained an intact oocyte and granulosa cells, whereas degenerated follicles contained an oocyte with a pyknotic nucleus, ooplasm shrinkage and/or granulosa cell layers that had disorganized and detached from the basement membrane (Fig. 2). To avoid evaluating and counting the same follicle more than once, pre-antral follicles were analyzed only in the sections in which an oocyte nucleus was observed.[31]

Statistical analysis. In this study, data that were not normally distributed (Kolmogorov-Smirnov test) were submitted to logarithmic transformation. Comparisons of means (morphologically normal follicles) were analyzed by Kruskal-Wallis test and Mann-Whitney test, when appropriate. All statistical tests were performed using Sigma Plot 11 (Systat Software Inc., San Jose, USA). Differences were considered significant when $p < 0.05$.

Results

Tables 1 and 2 show primordial, transitional, primary and secondary follicles percentages in ovarian tissue samples after warming and IVC. The corresponding values for fresh control samples including primordial and developing follicles were 52.40 ± 2.50, 32.80 ± 2.40, 11.90 ± 1.80, and 2.70 ± 0.80, respectively.

Table 1. Mean ± SEM percentage of morphologically normal primordial (Po), transitional (Tr), primary (Pr) and secondary (Se) follicles after warming, (PVP: Polyvinylpyrrolidone).

Groups	Po	Tr	Pr	Se
Control	-	-	-	-
Sucrose	56.60 ± 3.60^{aAB}	31.30 ± 3.30^{bB}	10.20 ± 2.40^{aAC}	1.10 ± 0.80^{abA}
X-1000	59.00 ± 4.60^{aAB}	$22.70 \pm 3.90^{bC*}$	17.10 ± 3.90^{abA}	1.00 ± 0.70^{aA}
Z-1000	$64.90 \pm 3.80^{aA*}$	31.90 ± 3.70^{bAB}	$3.20 \pm 1.40^{aB*}$	$0.30 \pm 0.30^{aA*}$
PVP	52.50 ± 3.80^{aB}	$41.80 \pm 3.70^{aA*}$	$5.40 \pm 1.50^{aBC*}$	$0.10 \pm 0.10^{bA*}$

^{abc} Lowercase letters indicate significant differences within a row and the same follicular class ($p < 0.05$).

^{ABC} Uppercase letters indicate significant differences within a column ($p < 0.05$).

* Asterisk indicates significant differences between fresh control group and the same follicular class ($p < 0.05$).

After warming. Immediately after warming (non-cultured tissue), the primordial follicles percentages in Z-1000 samples (64.90%) did not differ from that of fresh control (52.40%). Furthermore, PVP samples resulted in a lower percentage of primordial follicles compared to Z-1000 group (52.50% and 64.90%, respectively). Although the percentages of transitional follicles in X-1000 samples (22.70%) were lower than those of fresh control (32.80%), PVP samples (41.80%) had a greater percentage of transitional follicles than that of fresh control (32.80%).

Additionally, the percentage of transitional follicles was the highest in the PVP samples (41.80%), lower in the sucrose group (31.30%) and the lowest in X-1000 group (22.70%). For primary follicles, Z-1000 (3.20%) and PVP groups (5.40%) resulted in a lower percentage of primary follicles compared to fresh control group (11.90%). Moreover, the percentages of primary follicles in the X-1000 group (17.10%) were greater than Z-1000 (3.20%) and PVP samples (5.40%). On the other hand, Z-1000 samples also resulted in a lower percentage of primary follicles than sucrose ones (3.20% and 10.20%, respectively). Percentages of secondary follicles in Z-1000 and PVP samples showed a similar trend to that of primary follicles in which these two polymers resulted in a lower percentage of secondary follicles compared to fresh control group.

After 1 day of IVC. On day 1, freshly culture ovarian tissue (cultured control) resulted in a lower percentage of primary follicles compared to fresh control group (44.20% and 52.40%, respectively). For transitional follicles, freshly culture ovarian tissue (42.80%) and Z-1000 (42.80%) groups resulted in a greater percentage of transitional follicles compared to fresh control group (32.80%).

After 5 days of IVC. On day 5, the percentage of primordial follicles in all treatments was lower than fresh control samples. Sucrose (38.70%) and PVP samples (39.80%) had a greater percentage of primordial follicles than that of X-1000 (24.50%). Except for the PVP group,

Table 2. Mean (± SEM) percentage of morphologically normal primordial (Po), transitional (Tr), primary (Pr) and secondary (Se) follicles after *in vitro* culture, (PVP: Polyvinylpyrrolidone).

Groups	Day 1				Day 5			
	Po	Tr	Pr	Se	Po	Tr	Pr	Se
Control	44.20 ± 3.30^{aA*}	42.80 ± 3.30^{aA*}	10.30 ± 2.10^{bA}	2.50 ± 1.10^{aA}	31.10 ± 3.50^{bAB*}	46.90 ± 3.60^{aAB*}	16.60 ± 2.70^{aA}	5.30 ± 2.10^{aA}
Sucrose	47.50 ± 3.80^{abA}	38.30 ± 3.60^{bA}	9.80 ± 2.20^{aA}	4.30 ± 1.60^{aA}	38.70 ± 3.90^{bA*}	54.70 ± 3.90^{aA*}	6.20 ± 1.90^{aB*}	0.20 ± 0.20^{bB*}
X-1000	47.40 ± 3.50^{bA}	40.40 ± 3.50^{aA}	8.40 ± 2.20^{bA}	3.50 ± 1.40^{aA}	24.50 ± 5.10^{cB*}	48.30 ± 6.00^{aAB*}	20.20 ± 4.90^{aA}	6.90 ± 2.90^{aA}
Z-1000	46.50 ± 4.50^{bA}	42.80 ± 4.40^{bA*}	9.70 ± 2.70^{aA}	0.70 ± 0.50^{aA}	38.10 ± 6.00^{bAB*}	57.50 ± 6.00^{aA*}	4.40 ± 2.60^{aB*}	-
PVP	48.50 ± 3.90^{abA}	38.40 ± 3.80^{aA}	9.50 ± 2.30^{abA}	3.50 ± 1.50^{aA}	39.80 ± 5.50^{bA*}	40.30 ± 5.60^{aB}	17.80 ± 4.80^{bA}	1.90 ± 1.60^{abAB}

^{abc} Lowercase letters indicate significant differences within a row and the same follicular class ($p < 0.05$).
^{ABC} Uppercase letters indicate significant differences within a column ($p < 0.05$).
* Asterisk indicates significant differences between fresh control group and the same follicular class ($p < 0.05$).

all treatments that were cultured for 5 days showed a greater percentage of transitional follicles compared to the fresh control. Furthermore, the transitional follicles percentage in PVP samples (40.30%) was lower than that of sucrose (54.70%) and Z-1000 (57.50%) groups.

For primary follicles, sucrose (6.20%) and Z-1000 samples (4.40%) had lower percentages than those of fresh control (11.90%). The percentage of primary follicles in Z-1000 samples (4.40%) was lower than that of X-1000 (20.20%) and PVP ones (17.80%). Although there was no significant difference in the percentage of primary follicles among Z-1000 and sucrose samples, all other treatments showed greater percentages of primary follicles compared to sucrose groups.

Sucrose samples showed a lower percentage of secondary follicles compared to fresh control group (0.20% and 2.70%, respectively). Interestingly, there was no secondary follicle in Z-1000 samples. Sucrose samples (0.20%) resulted in a lower percentage of secondary follicles compared to cultured control (5.30%) and X-1000 samples (6.90%).

Comparing periods of IVC. Cultured control samples on day 5 experienced a decrease in the percentages of primordial follicles compared to those of cultured control samples on day 1 (31.10% and 44.20%, respectively). On the other hand, cultured control samples on day 5 had greater percentages of primary follicles than those of cultured control samples on day 1 (16.60% and 10.30%, respectively). Sucrose treatment group maintained a similar percentage of morphologically normal primordial, transitional, primary and secondary follicles from post-warming to day 1 of culture ($p > 0.05$). However, on day 5 (54.70%) there was an increase in the percentages of transitional follicles compared to those of post-warmed (31.30%) and samples on day 1 (38.30%). Secondary follicles showed a different trend to that of transitional follicles in which sucrose samples on day 5 had lower percentages of secondary follicles than those of day 1 (0.20% and 4.30%, respectively).

For X-1000 group, the percentage of primary follicles was the highest in the post-warmed X-1000 samples (59.00%), lower in cultured samples on day 1 (47.40%) and the lowest on day 5 (24.50%). On the other hand, the transitional follicle percentages in all X-1000 cultured samples (D1 and D5; 40.40% and 48.30%, respectively) were increased in comparison with post-warmed ones (22.70%). The X-1000 samples on day 5 showed a greater percentage of primary follicles compared to samples on day 1. Furthermore, X-1000 group maintained a similar percentage of secondary follicles from post-warming to day 5 of culture.

For the Z-1000 group, the percentages of primordial follicles in all cultured samples (D1 and D5; 46.50% and 38.10%, respectively) were decreased in comparison with post-warmed ones (64.90%). Samples on day 5 (57.50%) experienced an increase in the percentages of transitional follicles compared to those of post-warmed (31.90%) and cultured samples on day 1 (42.80%). The Z-1000 group maintained a similar percentage of primary and secondary follicles from post-warming to day 5 of culture.

For PVP group, cultured samples on day 5 (39.80%) showed a lower percentage of primary follicles compared to those of post-warmed ones. Furthermore, PVP treatment group maintained a similar percentage of transitional follicles from post-warming to day 5 of culture. Samples on day 5 (17.80%) showed greater percentages of primary follicles compared to those of post-warmed PVP (5.40%). For secondary follicles, samples on day 1 (3.50%) experienced an increase in the secondary follicle percentages compared to those of post-warmed PVP samples (0.10%).

Discussion

A huge amount of conflicting data exists regarding the best type and concentration of CPAs and biological variability among various pre-antral follicle classes after cryopreservation of ovarian tissue.[16,19] In an attempt to address these questions, the present study was conducted to evaluate the impacts of synthetic polymers on histological characteristics of different classes of pre-antral follicles after bovine ovarian tissue vitrification.

Our results indicated that for almost all pre-antral follicle classes, post-warmed and cultured samples on day 1 of X-1000 and sucrose groups resulted in a similar percentage of normal follicles compared to fresh control group. These results are consistent with the results of Wowk *et al.*, in which PVA was tested in the form of a

product called super cool X-1000 and even small concentrations of PVA were sufficient to inhibit ice formation in vitrification solutions.[36] Furthermore, the results of the present study confirmed the observations of Santos *et al*., who reported that inclusion of sucrose in the freezing media generally improves the post-thaw morphology of the ovine pre-antral follicles.[16] On the other hand, although, sucrose cultured samples on day five experienced a decrease in the percentages of primary and secondary follicles compared to those of fresh control group, X-1000 cultured samples on day five resulted in similar percentages of primary and secondary follicles compared to those of fresh control group. These conflicting results are consistent with the results of Santos *et al*. who reported that contrary to primordial and primary follicles, the addition of sucrose did not result in more viable oocytes and granulosa cells in secondary follicles.[16] The X-1000 ice blocker is a copolymer of PVA of approximately 2000 molecular weight with a 20.00% vinyl acetate content to reduce the viscosity and self-association tendencies of the molecule.[36] It significantly suppresses ice formation in vitrification solutions, even at concentrations as low as one part per 10^6. [37] The X-1000 has been shown to reduce the activity of several specific ice nucleators,[28] although its mechanism of action seems to be more general than a just affinity for specific nucleators. Some evidence suggests that direct interaction with ice may be involved.[30] The mechanism of action of this compound is not clear, but based on visual observations and calorimetry, Wowk *et al*. have suggested that PVA blocks ice primarily by inhibition of heterogeneous nucleation.[36] Such compounds might therefore be classified as anti-nucleating agents, as opposed to ice blockers that bind in some way to an ice nucleus and prevent or slow its growth into a damaging ice crystal.

The results of the present study showed that each CPA has a distinctive impact on the histological characteristics of different types of pre-antral follicles. In this case, although, the addition of X-1000 resulted in greater percentages of normal follicles for almost all pre-antral follicle classes compared to those of other groups, there are some exceptions. These results indicated that the inclusion of PVP K-12 in the freezing media could improve the morphology of the post-warmed transitional follicles and cultured primordial follicles on day 5 more than other CPAs. These results are in accordance with the observations reported by Hashimoto *et al.*, who stated that a CPA containing a combination of EG and PVP supports follicle morphology of monkey ovarian tissues after ultra-rapid vitrification compared to a CPA containing a combination of EG and DMSO.[38]

The PVP has been shown to contribute as effectively as EG to the properties of vitrification solutions, [39] and to increase the cryoprotective properties of EG solutions.[40] One study used PVP as the only component in the cryoprotectant solution,[41,42] but it is more commonly used as an additive at concentrations up to 20.00%.[43] The PVP has a very specific role in glass formation, because it shows increasingly high viscosity at lower temperatures, preventing water molecules from fusing in growing ice crystals.[44] The PVP also has the advantage of low toxicity levels.[45] Indeed, the lower percentages of post-warmed transitional and primordial follicles on day 5 of X-1000 group than those of other groups support the hypothesis about the existence of toxicity of this product at least for these two types of follicles.[26]

Isachenko *et al*. have tested different vitrification solutions supplemented with super cool X-1000 to vitrify human ovarian tissue.[46] They have reported that long exposure to this synthetic ice blocker could dramatically reduce the viability of ovarian tissue after warming, possibly due to the toxicity of this product.[46] In two further studies, Isachenko's group has compared a vitrification solution containing super cool X-1000 with a freezing solution and has showed that after xenografting of human ovarian tissue, the extent of necrosis does not differ between treatments,[47] while the number of apoptotic cells is higher in vitrified tissue.[48] Similarly, Marco-Jimenez *et al*. have suggested that synthetic polymers disrupt the embryonic implantation rate reflecting the offspring rate reduction.[26] According to these observations, it seems that mixtures of X-1000 and PVP may be more effective in ice formation inhibition than either agent alone, suggesting that X-1000 and PVP complement each other by inhibiting different sources of ice nucleation during the cryo-preservation process of different pre-antral follicles classes.[20,44] These findings are in agreement with the results of Ting *et al*. who reported that a combination of non-permeating polymers such as PVP, a specific copolymer of PVA and polyglycerol, improved the outcome for vitrified macaque ovarian tissue.[15]

In conclusion, the results of this study clearly demonstrated that the addition of X-1000 resulted in a greater percentage of normal follicles for almost all pre-antral follicle classes, while PVP K-12 is more effective for transitional follicles after warming and cultured primordial follicles on day five. According to these findings, it can be concluded that although ovarian tissue cryopreservation is often performed aimed at preserving the primordial follicles, by choosing the best combination of permeating and non-permeating CPAs, more advanced stages of bovine pre-antral follicles, i.e., transitional, primary and secondary follicles, may also survive the cryopreservation process.

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Conflict of interest

None of the authors of the present paper have any conflict of interest to declare.

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