

## Vitrification of bovine preantral follicles with dimethylsulfoxide and sucrose plus $\alpha$ -tocopherol<sup>1</sup>

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**ABSTRACT.-** Jimenez C.R., Penitente-Filho J.M., Torres C.A.A., Medeiros A.M. & Silva L.S. 2016. **Vitrification of bovine preantral follicles with dimethylsulfoxide and sucrose plus  $\alpha$ -tocopherol.** *Pesquisa Veterinária Brasileira* 36(3):209-215. Laboratório de Nutrição Animal, Centro de Energia Nuclear a Agricultura, Universidade de São Paulo, Av. Centenário 303, Cx. Postal 96, Piracicaba, SP 13400-970, Brazil. E-mail: [zoocaro@hotmail.com](mailto:zoocaro@hotmail.com)

The objective of this study was to evaluate the vitrification of bovine preantral follicles with dimethylsulfoxide (D) and sucrose (S) plus  $\alpha$ -tocopherol 5mmol/L (T5) or 10mmol/L (T10) and, evaluate the thawed with minimal essential medium (m) with or without sucrose (s). Ovaries of cows were collected from slaughterhouse for the experiment I (n=66) and II (n=51). In the laboratory ovarian fragments were randomly assigned either to fresh control and 8 vitrification treatments (Controle and Dm; Dms, DSm; DSms; DST5m; DST5ms; DST10m; DST10ms). Ovarian fragments were placed in vitrification solution (5 min) and immersed in liquid nitrogen (-196°C), after a week, the fragments were thawed and analyzed. In the experiments I, preantral follicles were morphologically observed for histological evaluation, (normal; degenerated and developing of stage). In the experiment II, preantral follicles were mechanically isolated from ovarian tissue and examined with trypan blue, where dead and live corresponded to stained or non-stained. The treatments DSm, DSms and DST10m were effective in preserving the morphology *in situ*. However, the viability of isolated preantral follicles after vitrification remained high only in treatment DST10m. Thus, DST10m preserves survival rates and morphological integrity during vitrification of bovine preantral follicles.

INDEX TERMS: Antioxidants, dimethylsulfoxide, cryopreservation, cryoprotectant, oocyte, granulosa cells.

**RESUMO.- [Vitrificação de folículos pré-antrais bovinos com dimetilxulfoxido e sacarose adicionado de  $\alpha$ -tocoferol.]** Os objetivos deste estudo foram avaliar a vitrificação de folículos pré-antrais bovinos com dimetilsulfóxido (D) e sacarose (S) adicionando  $\alpha$ -tocoferol 5mmol/L (T5) ou 10mmol/L (T10) e, avaliar o aquecimento com meio essencial mínimo (m) com ou sem sacarose (s). Ovários

de fêmeas bovinas foram coletados de abatedouro, para o experimento I (n= 66) e II (n= 51). No laboratório fragmentos ovarianos foram distribuídos aleatoriamente para o controle fresco e 8 tratamentos de vitrificação (Controle e Dm; Dms, a DSm; DSms; DST5m; DST5ms; DST10m; DST10ms). Os fragmentos ovarianos foram colocados na solução de vitrificação (5 min) e imersos em nitrogênio líquido (-196°C). Após uma semana os fragmentos foram aquecidos e analisados. No experimento I, folículos pré-antrais foram observados morfologicamente para avaliação histológica (normal, degenerados e estágio de desenvolvimento). No experimento II, folículos pré-antrais foram mecanicamente isolados do tecido ovariano e examinados com o azul de trypan, observando mortos e vivos corados e não corados respectivamente. Os tratamentos a DSm, DSms e DST10m foram eficazes na preservação da morfologia *in situ*. No entanto, a viabilidade de folículos pré-antrais isolados após a vitrificação manteve-se elevada apenas no tra-

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tamento DST10m. Assim, DST10m preservou as taxas de sobrevivência e integridade morfológica durante a vitrificação de folicúlos pré-antrais bovinos.

TERMOS DE INDEXAÇÃO: Antioxidantes, dimetilsulfóxido, criopreservação, crioprotetor, oócito, células granulosas.

## INTRODUCTION

The main problem of cryopreservation of oocytes is associated to the movement of water and intra or extracellular cryoprotectant during cryopreservation. Alternatively, the preantral follicles surface area in relation to the volume is large, providing excellent exchange of substances. Besides being smaller than the mature oocytes, these oocytes are less differentiated, possess fewer organelles, lack pellucida zone and cortical granules, besides being less metabolically active (Campebell et al. 1999). All these features are potentially beneficial to cryopreservation (Oktay et al. 1998) and thus preantral follicles are regarded as an important target of cryopreservation programs.

Generally, the cryopreservation protocols comprise the steps of exposure to the cryoprotectant, cooling, storage, thawing and removal of the cryoprotectants. Exposure to the cryoprotectants, also known as equilibrium period, is the time required for of cryoprotectants penetration or infusion into cells and, or, tissues (Ernst et al. 2010). This is a very important stage and is strongly influenced by cryoprotectants concentration, time and temperature which is performed the exposure (Rodrigues et al. 2010). The vitrification cooling rate is extremely rapid, ranging around 20000 to 40000°C/min (Lin et al. 2008), and as a result, the water goes from liquid to a vitreous state without the formation of intracellular ice crystals (Yeoman et al. 2005). Finished cooling, the sample is stored, usually in liquid nitrogen (-196°C) until the moment from the thawing. During thawing, undesirable changes may occur in the cells, due to chemical (oxidation) or physical (recrystallization, volume changes) (Hui et al. 2004). Thus, the thawing time, the substances and concentrations used for the removal of cryoprotectants are important steps in the process of cryopreservation.

Different cryoprotectants have been used in combination, with the possibility of optimizing protocols. Rodrigues et al. (2014) demonstrated that ovarian tissue can be frozen using intracellular cryoprotectant as ethyleneglycol, propandiol or dimethylsulfoxide, and can be combined with intra or extracellular cryoprotectant as sucrose in protocols of female gametes, acting as a buffer against osmotic stress during removal of the intracellular cryoprotectant (Demirci et al. 2003). Moreover, in the process of cryopreservation may occur increased of the reactive oxygen species (ROS) that can affect the balance between pro- and anti-oxidant factors in a biological system, leading to lipidic peroxidation (Romero et al. 1998) and, consequently, cellular death. The  $\alpha$ -tocopherol reduces generation of free radicals and removes them when formed (Souza et al. 1999). The most important biological function of  $\alpha$ -tocopherol is to protect cell membrane from lipidic peroxidation, avoiding irreversible damages and cellular death (Wang et al. 2002). Thus, since oxidative stress can be one of the factors involved in

apoptosis and, or, follicular atresia, the use of antioxidants may contribute for the success of vitrification of preantral follicles.

Therefore, the purpose of this study was (1) to evaluate the vitrification technique in bovine ovarian tissue, testing the effects of dimethylsulfoxide, sucrose and  $\alpha$ -tocopherol and (2) to evaluate the effect the thawing with minimal essential medium with or without sucrose.

## MATERIALS AND METHODS

**Collection of ovaries.** Ovaries (n=117) were collected in a slaughterhouse. In the experiment I, 66 ovaries were used to study the morphology of preantral follicles *in situ*. For Experiment II, 51 ovaries were used to evaluate the viability of isolated preantral follicles. All collected ovaries were washed in 70% alcohol for 10s and then rinsed twice in saline solution (NaCl 0.9%; Sigma/vetec® Brazil), supplemented with streptomycin sulfate (50mg/L; Sigma/Vetec® Brazil). Thereafter, ovaries were transported to the laboratory at 4°C, cut into fragments of approximately 9mm<sup>3</sup> and assigned to nine treatments: a fresh control and eight vitrification treatments (Table 1).

**Vitrification and thawing.** Ovarian fragments were placed in cryotubes with 1.8 mL of vitrification solution, followed by an equilibration period of 5 min at room temperature, time sufficient for ovarian fragments are kept in touch with all components of diluent without causing toxicity. Then the tubes were immersed vertically in liquid nitrogen (-196°C) and stored for one week. After of one cryostorage week, all cryotubes were removed, exposed to room temperature (25°C) for 1 min, and then immersed in a water bath (37°C) until the vitrification solution was completely thawed. The cryoprotectant was removed from the ovarian cortex fragments in three washings with MEM or MEM with decreasing sucrose concentrations (0.50, 0.25, 0.0mol/L of sucrose), for 5 min each. Then, the fragments for histology were fixated in 4 % paraformaldehyde for 12 h.

**Histology.** After fixation, the fragments were dehydrated in increasing concentrations of alcohol, diaphanized in xylene, embedded in paraffin and cut into sections (5µm thickness). The slides were stained with hematoxylin and eosin (Martinez-Madrid et al. 2004) and a total of 50 preantral follicles per slide were examined under an optical microscope (400x; Olympus BX60, Japan) in 30 replications (1500 preantral follicles per treatment).

**Classification of preantral follicles.** Ovarian fragments (fresh control and vitrification treatments) were analyzed and classified as primordial follicles with spherical or ovoid oocytes completely surrounded by a single layer of flattened and some cuboid-shaped granulosa cells (GCs); the primary follicles were described as spherical oocytes with a single layer of cuboid-shaped GCs; and the secondary follicles as spherical oocytes with two or more layers of cuboid-shaped GCs. The qualitative evaluation of the follicles was based on the integrity of the oocyte and the GCs. The follicles were classified morphologically as: normal - follicles containing intact oocytes and GCs; type I degenerated follicles - follicles with cytoplasmic shrinka-

**Table 1. Designation of treatments, fresh control and vitrified and thawed fragments**

Treatment*	Vitrification solution			Thawing solution	
	Intracellular cryoprotectant	Extracellular cryoprotectant	Antioxidant	Base medium	Extracellular cryoprotectant
	5mol/L	0.5mol/L	5 mmol/L 10mmol/L	mL	0.50, 0.25, 0.0mol/L
Control					
Dm	Dimethylsulfoxide	-	-	MEM	
Dms	Dimethylsulfoxide	-	-	MEM	Sucrose
DSm	Dimethylsulfoxide	Sucrose	-	MEM	
DSms	Dimethylsulfoxide	Sucrose	-	MEM	Sucrose
DST5m	Dimethylsulfoxide	Sucrose	$\alpha$ -tocopherol 5	MEM	
DST5ms	Dimethylsulfoxide	Sucrose	$\alpha$ -tocopherol 5	MEM	Sucrose
DST10m	Dimethylsulfoxide	Sucrose	$\alpha$ -tocopherol 10	MEM	
DST10ms	Dimethylsulfoxide	Sucrose	$\alpha$ -tocopherol 10	MEM	Sucrose

\* Vitrification solution in uppercase. Thawed solution in lowercase. Dm = Dimethylsulfoxide thawed with minimal essential medium (MEM); Dms = Dimethylsulfoxide thawed with MEM + sucrose; DSm = Dimethylsulfoxide + sucrose thawed with MEM; DSms = Dimethylsulfoxide + sucrose thawed with MEM + sucrose; DST5m = Dimethylsulfoxide + sucrose +  $\alpha$ -tocopherol 5mmol/L thawed with MEM; DST5ms = Dimethylsulfoxide + sucrose +  $\alpha$ -tocopherol 5mmol/L thawed with MEM + sucrose; DST10m = Dimethylsulfoxide + sucrose +  $\alpha$ -tocopherol 10mmol/L thawed with MEM; DST10ms = Dimethylsulfoxide + sucrose +  $\alpha$ -tocopherol 10mmol/L thawed with MEM + sucrose.

ge and/or a pyknotic oocyte nucleus; type II degenerated follicles - follicles with oocyte degeneration accompanied by GCs disorganization (Basso & Esper 2002).

**Mechanical isolation and viability analysis.** Ovarian fragments were finely cut with a scalpel blade and placed in 50mL tubes with 10mL of MEM plus 5% bovine serum albumin and mechanically dissociated by repeated pipetting (1000 and 500 $\mu$ L). Thereafter, the suspension was filtered twice through nylon mesh (300 and 106 $\mu$ m, respectively). In each 100 $\mu$ L of suspension medium containing isolated preantral follicles 5 $\mu$ L of trypan blue (0.4%; Sigma/Vetec®, Brazil) was added. Follicles were classified as viable or non-viable, when non-stained or blue-stained, respectively, under an inverted microscope (400x; Olympus, Japan). Fifty preantral follicles were observed per treatment with 30 replicates (1,500 preantral follicles per treatment).

**Statistical analysis.** The data of the morphological (normal, type I and II degenerated) of preantral follicles were subjected to analysis of variance (f test,  $P < 0.05$ ), and means were compared by Tukey's test (SAS/2002). The effects of vitrification on the morphological of each developmental stage (primordial, primary and secondary) and the viability of isolated preantral follicles were arranged in contingency tables and analyzed by chi-square test (df 1 = 3.84;  $P = 0.05$ ).

**RESULTS**

**Experiment I: In situ morphology of bovine preantral follicles after vitrification**

A total of 13,500 preantral follicles were analyzed (1,500 follicles per treatment), following the distribution of normal, type I and II degenerated, respectively, in primordial follicles (56.8%, 28.1% and 15.1%), primary follicles (39.8%, 35.4% and 24.8 %) and secondary follicles (30.4%, 49.6% and 20%) (Fig.1). There were no differences ( $P > 0.05$ ) between control and DSm, DSms and DST10m treatments

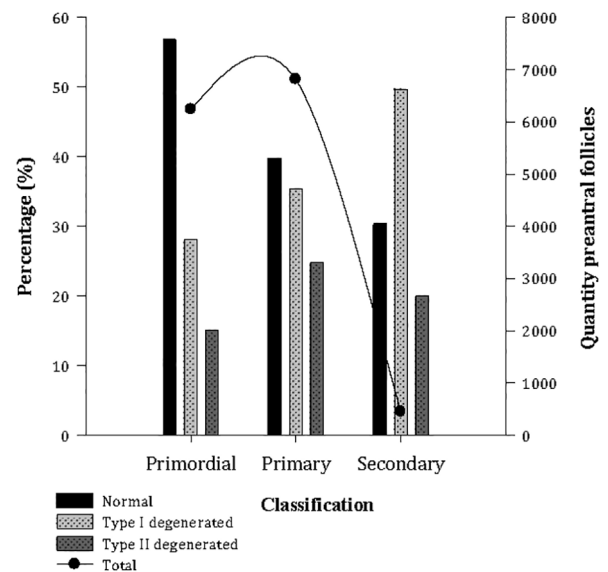


Fig.1. Classification of preantral follicles (a) per treatments and (b) developmental stage. Vitrification solution in uppercase; D = Dimethylsulfoxide; S = Sucrose; T5 =  $\alpha$ -tocopherol 5mmol/L; T10 =  $\alpha$ -tocopherol 10mmol/L. Thawed solution in lowercase; m = MEM; ms = MEM+sucrose.

with regard to the morphologically normal follicles (Table 2). Type I degenerative follicles did not differ from control and vitrification of treatments ( $P > 0.05$ ). The fresh control, DSm and DSms, had the lowest percentage of type II degenerative follicles ( $P > 0.05$ ) and between the vitrification treatments there were no differences in most treatments.

**Primordial follicles**

A total of 6,238 primordial follicles were analyzed and classified into normal, type I and II degenerated follicles (3,544; 1,750 and 944, respectively). For the follicles with normal morphology there were no differences ( $P > 0.05$ )

**Table 2. Morphological classification of preantral follicles in normal and degenerate (type I and II) with different vitrification treatments**

Quality (%)	Treatments								
	Control	Dm	Dms	DSm	DSms	DST5m	DST5ms	DST10m	DST10ms
Normal	56.0 <sup>a</sup>	45.2 <sup>bc</sup>	43.6 <sup>bc</sup>	49.3 <sup>ab</sup>	55.9 <sup>a</sup>	43.0 <sup>bc</sup>	44.1 <sup>bc</sup>	49.6 <sup>ab</sup>	39.4 <sup>c</sup>
Type I degenerated	30.7 <sup>b</sup>	31.3 <sup>ab</sup>	32.9 <sup>ab</sup>	31.8 <sup>ab</sup>	30.0 <sup>b</sup>	34.8 <sup>ab</sup>	34.2 <sup>ab</sup>	29.8 <sup>b</sup>	37.1 <sup>ab</sup>
Type II degenerated	13.3 <sup>c</sup>	23.5 <sup>a</sup>	23.5 <sup>ab</sup>	18.9 <sup>abc</sup>	14.1 <sup>bc</sup>	22.2 <sup>ab</sup>	21.7 <sup>ab</sup>	20.6 <sup>ab</sup>	23.5 <sup>a</sup>

<sup>abc</sup> Different letters in the same row differ (P<0.05) based on ANOVA; Based on mean comparisons of the Tukey's studentized range. Vitrification solution in uppercase; D = Dimethylsulfoxide; S = Sucrose; T5 = α-tocopherol 5mmol/L; T10 = α-tocopherol 10mmol/L. Thawed solution in lowercase; m = MEM; ms = MEM+sucrose.

**Table 3. Morphological classification (normal, type I and II degenerated) of preantral follicles in each developmental stage (primordial, primary and secondary) in different vitrification treatments**

Development stage	Treatments								
	Control	Dm	Dms	DSm	DSms	DST5m	DST5ms	DST10m	DST10ms
Follicles classified as normal (%)									
Primordial	69.0 <sup>a1</sup>	51.4 <sup>c1</sup>	50.0 <sup>c1</sup>	60.0 <sup>ab1</sup>	66.4 <sup>a1</sup>	49.0 <sup>c1</sup>	53.9 <sup>c1</sup>	60.4 <sup>ab1</sup>	52.8 <sup>c1</sup>
Primary	46.3 <sup>a2</sup>	39.2 <sup>cd2</sup>	39.6 <sup>cd2</sup>	42.9 <sup>abc2</sup>	44.7 <sup>ab2</sup>	37.8 <sup>d2</sup>	37.3 <sup>d2</sup>	41.9 <sup>bc2</sup>	31.8 <sup>e2</sup>
Secondary	26.9 <sup>ab3</sup>	18.0 <sup>b3</sup>	25.0 <sup>ab2</sup>	42.6 <sup>a2</sup>	38.1 <sup>ab2</sup>	22.2 <sup>bc3</sup>	26.7 <sup>ab2</sup>	36.7 <sup>ab2</sup>	35.9 <sup>ac2</sup>
Follicles classified type I degenerated (%)									
Primordial	22.4 <sup>c3</sup>	30.3 <sup>b2</sup>	30.7 <sup>b2</sup>	29.2 <sup>b2</sup>	22.6 <sup>c2</sup>	31.0 <sup>ab3</sup>	30.8 <sup>ab3</sup>	24.2 <sup>c3</sup>	30.1 <sup>b2</sup>
Primary	36.2 <sup>abc2</sup>	31.5 <sup>c2</sup>	34.0 <sup>bc12</sup>	32.8 <sup>bc12</sup>	37.2 <sup>ab1</sup>	37.5 <sup>ab2</sup>	35.8 <sup>abc2</sup>	38.3 <sup>abc2</sup>	40.2 <sup>a1</sup>
Secondary	55.2 <sup>a1</sup>	48.7 <sup>ab1</sup>	50.0 <sup>ab1</sup>	42.6 <sup>ab1</sup>	57.1 <sup>ab1</sup>	55.6 <sup>a1</sup>	53.3 <sup>ab1</sup>	57.1 <sup>a1</sup>	35.9 <sup>b12</sup>
Follicles classified type II degenerated (%)									
Primordial	8.6 <sup>c2</sup>	18.3 <sup>a2</sup>	19.4 <sup>a2</sup>	10.8 <sup>c2</sup>	11.0 <sup>c2</sup>	20.0 <sup>a2</sup>	15.3 <sup>b2</sup>	15.4 <sup>b2</sup>	17.1 <sup>b2</sup>
Primary	17.5 <sup>b1</sup>	29.3 <sup>a1</sup>	26.4 <sup>a1</sup>	24.3 <sup>a1</sup>	18.1 <sup>b1</sup>	24.7 <sup>a1</sup>	26.9 <sup>a1</sup>	19.8 <sup>b1</sup>	28.0 <sup>a1</sup>
Secondary	17.9 <sup>abcd1</sup>	33.3 <sup>a1</sup>	25.0 <sup>abc12</sup>	14.8 <sup>bcd12</sup>	4.8 <sup>cd12</sup>	22.2 <sup>abc12</sup>	20.0 <sup>abc12</sup>	6.2 <sup>d2</sup>	28.2 <sup>ab1</sup>

<sup>abcde</sup> Different letters in the same row differ by the χ<sup>2</sup> test (P<0.05); Different numbers in the column in each developmental stage differ by the χ<sup>2</sup> test (P<0.05). Vitrification solution in uppercase; D = Dimethylsulfoxide; S = Sucrose; T5 = α-tocopherol 5mmol/L; T10 = α-tocopherol 10mmol/L. Thawed solution in lowercase; m = MEM; ms = MEM+sucrose.

among the control (69%), DSms (66.4%), DSm (60 %) and DST10m (60.4%). In the type I degenerated preantral follicles, the treatments DSms (22.6%) and DST10m (24.2 %) did not differ (P>0.05) from the control (22.4%), and the other vitrification treatments differed from control with larger percentages of degenerated follicles (P<0.05). For the preantral follicles with type II degenerated, the treatments Dm (18.3%), Dms (19.4%) and DST5m (20.0%), the cell damage or follicular degeneration was higher (P<0.05) than in the control (8.6%), DSm (10.8%) and DSms (11.0%) (Table 3).

**Primary follicles**

A total of 6,812 primary follicles were analyzed and classified into normal, type I and II degenerated (2,714; 2,411 and 1,687), respectively. For preantral follicles with normal morphology, there were no differences (P>0.05) among the treatments control (46.3%), DSm (42.9%) and DSms (44.7%). Though in vitrification treatments, were observed no differences among DSm or DSms and DST10m, Dm and Dms (P>0.05). The treatments that showed lower percentage of type II degeneration follicles were DSms and DST10ms without differences with the control (P>0.05) and differed the other vitrification treatments (P<0.05; Table 3).

**Secondary follicles**

A total of 450 secondary follicles was analyzed and classified into normal, type I and II degenerated (137; 223 and

90, respectively). The morphological classes did not differ between control and vitrification treatments (P>0.05; Table 3).

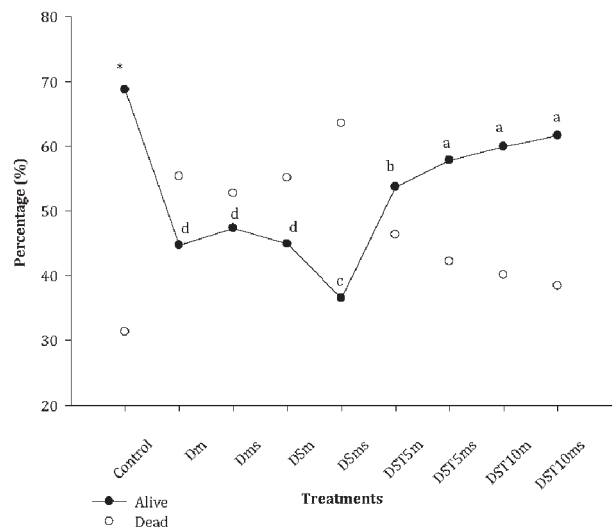


Fig.2. Viability of bovine preantral follicles. \*Difference with the control; a,b,c,d Letters indicate differences between treatments by the χ<sup>2</sup> test (P<0.05). Vitrification solution in uppercase; D = Dimethylsulfoxide; S = Sucrose; T5 = α-tocopherol 5mmol/L; T10 = α-tocopherol 10mmol/L. Thawed solution in lowercase; m = MEM; ms = MEM+sucrose.

### Experiment II: Viability of bovine preantral follicles after vitrification

The percentage of live preantral follicles in fresh control (68.7%) was higher than in the vitrification treatments ( $P<0.05$ ). Notwithstanding among the vitrification solutions, DST10s and DST10ms, was most effective in maintaining the viability of preantral follicles after vitrification (live follicles 59.9% and 61.6%, respectively; Fig.2). The

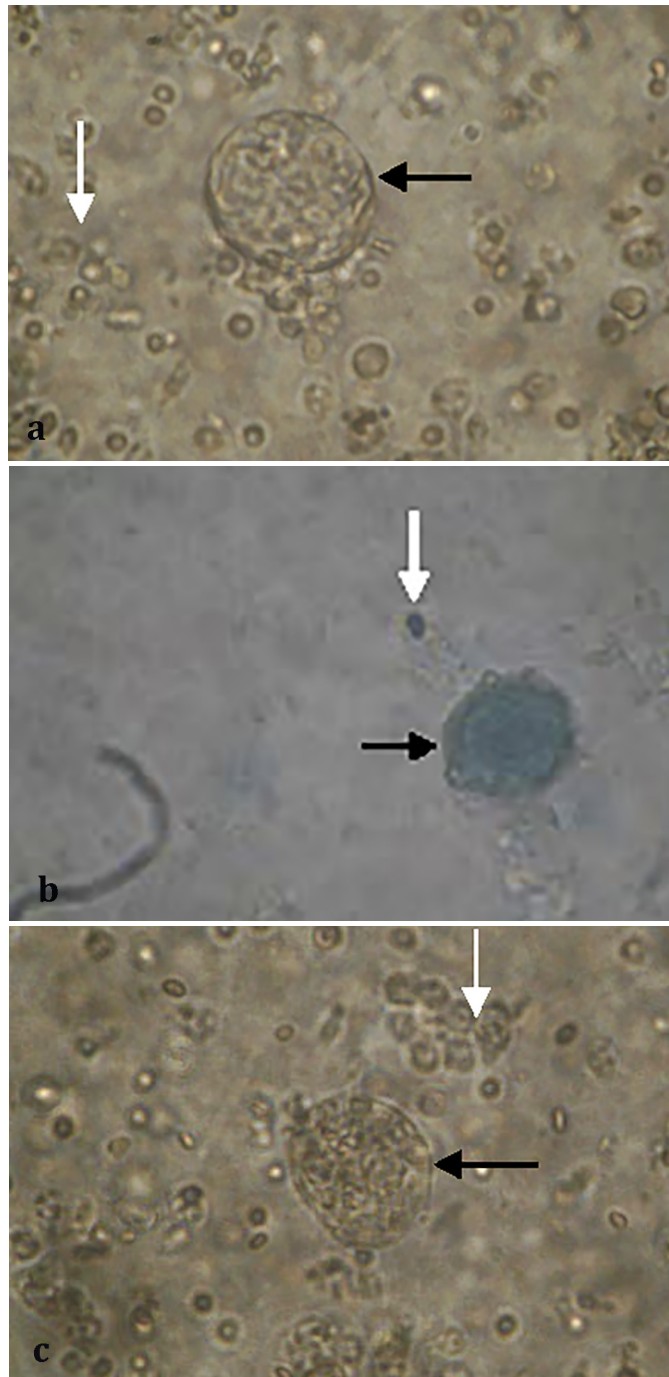


Fig.3. Isolated bovine preantral follicles stained with trypan blue. (a) Control; secondary preantral follicles - viable, (b) DSms; secondary preantral follicles - non-viable, and decreased ovarian stromal cells (c) DST10m; secondary preantral follicles - viable. Preantral follicles (black arrow) ovarian stromal cells (white arrow). Photographic camera Sony; obj.40x.

DSms treatment (36.5%) was less effective in the percentages of viable follicles, differing from the other vitrification treatments ( $P<0.05$ ; Fig.3).

## DISCUSSION

### Experiment I

This study showed that bovine preantral follicles can be cryopreserved in the presence of: dimethylsulfoxide 5mol/L, sucrose 0.5mol/L added or not of  $\alpha$ -tocopherol 10mmol/L, helping in maintaining the morphological quality of preantral follicles after vitrification. Several authors have studied the dimethylsulfoxide for cryopreservation protocols. Luna & Munhoz (2008) successfully vitrified bovine ovarian tissue fragments, using dimethylsulfoxide (2.6 M). In caprine preantral follicles cryopreserved with dimethylsulfoxide, remained good conditions the granulosa and nucleus cells (Rodrigues et al. 2004). Castro et al. (2011) used dimethylsulfoxide as intracellular cryoprotectant agent and demonstrated its effectiveness in preserving caprine ovarian tissue. Similarly, different dimethylsulfoxide concentrations have been successfully used with ovarian tissue fragments of sheep, 3M (Santos et al. 2006b), 2.6 M (Castro et al. 2011) and 5M (Melo et al. 2008). Lucci et al. (2004) evaluated several cryoprotectants: ethylene glycol, propanediol and dimethylsulfoxide (1.5 or 3mol/L) and found more pyknotic nuclear changes in bovine ovarian tissue fragments when using ethyleneglycol than with dimethylsulfoxide. In this study, the concentration of 5mol/L of dimethylsulfoxide showed up effective as intracellular cryoprotectant in the process of vitrification of bovine preantral follicles.

The extracellular cryoprotectants such as sucrose in the cryopreservation medium have been used in several species, with variable results. This study found that adding of sucrose 0.5mol/L to the vitrification solutions of bovine preantral follicles confers good morphology in granulosa cells and in oocytes. It was reported that sucrose resulted in water retention in the extracellular medium, preventing the entry of water quicker than the output of the intracellular cryoprotectant, reducing osmotic damage (Bacter et al. 2006). Thus, it is suggested that the beneficial effect of sucrose consists in its action as an osmotic buffer against cell stress during the addition and removal of permeable cryoprotectant agents (Newton et al. 1998). According to Salehnia et al. (2002), sucrose can be successfully used as an extracellular cryoprotectant of murine preantral follicles. Likewise, Santos et al. (2006b) reported the effectiveness of goat ovarian tissue cryopreservation with sucrose 0.5mol/L. Melo et al. (2011) concluded that sheep preantral follicles can be successfully vitrified using dimethylsulfoxide 5mol/L when combined with 0.5mol/L sucrose. However, some authors believe that sucrose is not essential in the vitrification solution. Carvalho et al. (2011) tested the concentration of sucrose 0.5mol/L and concluded that there was an increase in the number of follicles in the presence of cytoplasmic retraction. Baird (1984) analyzed several aspects and concluded that the follicular cell, association of cryoprotectants, cryopreservation methods, as well as species, breed, and physiological stage should be taken into account in the cryopreservation procedure.

Besides the intra or extracellular cryoprotectant, this study showed that the addition of  $\alpha$ -tocopherol 10mmol/L in the vitrification solution provided optimal morphological preservation of preantral follicles. Thus can be seen that  $\alpha$ -tocopherol performs its function as an antioxidant and protective membrane on preantral follicles. Ferreira & Matsubar (1997) affirmed that  $\alpha$ -tocopherol is highly effective because it removes reactive oxygen species before they cause injury. Different studies have successfully tested protective antioxidants; Carrascal et al. (2012) tested dimethylsulfoxide and ethyleneglycol with the antioxidant ascorbic acid and reported the best percentage of cells with morphological integrity after vitrification. On the other hand, in studies with males,  $\alpha$ -tocopherol added to the freezing solution of boar semen reduced the oxidative damage, improved sperm motility, preserved the integrity of the plasma and mitochondrial membranes (Satorre et al. 2007). Penitente-Filho et al. (2014) observed no differences in parameters of motility, vigor and hypoosmotic test in the thawed goat semen with  $\alpha$ -tocopherol. However, it is important to note that in studies using  $\alpha$ -tocopherol or other antioxidants these effects on preantral follicles cryopreservation are reduced. Therefore, further research on the effect of antioxidants is required to prove their effectiveness.

Santos et al. (2008) emphasized that cell damage is not only observed during vitrification, but also during the thawing process, when the cell metabolism is being re-established in the presence of toxic agents such as cryoprotectants. According to El-Naggar et al. (2006) the cryopreserved tissue should be thawed quickly in three successive washes, Although one washing is enough for thawing of embryos, isolated or *in situ* preantral follicles, three washing are recommended to remove traces of cryoprotective agents (Lima-Verde et al. 2009). Thus, in this study was performed rapid thawing and three washing in minimal essential medium or minimal essential medium with decreasing concentrations of sucrose (0.50, 0.25, 0.0mol/L). Nevertheless, the results showed generally no significant differences among the treatments when using minimal essential medium or minimal essential medium with sucrose as thawing medium. The data analysis of this study found that greater morphological changes were attributed to the vitrification solutions and not to thawing solutions.

The resistance of the different follicular stages (primordial, primary and secondary) on cryopreservation is still fairly debated and requires further elucidation (Luna & Munhoz 2008). According to Santos et al. (2006a), primordial follicles are more resistant the process of cryopreservation. Confirming this assumption, in this study, primordial follicles showed a higher percentage of normal morphologically follicles than degenerates morphologically (I and II). Similarly, Carrascal et al. (2012) found that primordial and transitional follicles are more resistant than primary and secondary follicles to cooling procedures after vitrification. The result disagrees with Lucci et al. (2004), who found no differences in cryopreserved ovarian follicles of cattle in the primordial, primary and secondary classes. This greater tolerance of primordial follicles can be explained by

several features that make them less vulnerable to cryoinjuries, such as: a small oocyte size, low metabolic rate, stage of the cell cycle, absence of pellucid zone and peripheral cortical granules, and a small amount of intra cytoplasmic lipid droplets (Shaw et al. 2000).

## Experiment II

Cryopreservation can induces a disruption of the cell membrane, imperceptible by histological examination, resulting in the death of the follicles after thawing (Martinez-Madrid et al. 2004). Thus, cells with an apparently intact morphology may be non-viable and non-functional (Santos et al. 2006a). Therefore in experiment I, treatment DSms showed efficient morphologically in vitrification of preantral follicles, however, in experiment II it had the lowest follicular viability in comparison with other treatments of vitrification. Thus, in this study the percentage of viable follicles after vitrification, thawing and mechanical isolation of bovine ovarian tissue was highest in the treatment with DST10m (61.6%). Similar results were obtained by Carrascal et al. (2012) with dimethylsulfoxide, ethyleneglycol and acid ascorbic (66%) in the percentage of viable isolated bovine follicles. Luna et al. (2011) studied the cryoprotectants dimethylsulfoxide, ethyleneglycol and sucrose and she concluded that the percentage of follicle viability after exposure to cryoprotectants (64%) was not different than after vitrification (66%). In other species, follicle viability was 65% after vitrification and isolation of sheep ovarian tissue with cryoprotectants ethyleneglycol, sucrose and fetal bovine serum (Lunardi et al. 2012).

## CONCLUSION

The association of dimethylsulfoxide, sucrose and  $\alpha$ -tocopherol 10mmol/L thawed with minimal essential medium provides high survival rates and preserves morphological integrity during vitrification of bovine preantral follicles.

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