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# Effect of the exposure to methyl-β-cyclodextrin prior to chilling or vitrification on the viability of bovine immature oocytes ☆

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ABSTRACT

The present study aimed to evaluate the effect of methyl-β-cyclodextrin (MβCD) as a cholesterol loader to change oocyte plasma membrane and increase its tolerance toward cryopreservation. The first and second experiments were conducted to investigate if MβCD could improve nuclear and cytoplasmic maturation after oocyte exposure to cold stress for 10 or 30 min, respectively. No differences ($P > 0.05$) in either experiment in the metaphase II (MII) rate of oocytes exposed to MβCD and cold stress; but these oocytes presented lower maturation rates than control groups. In the second experiment, a lower percentage of oocytes showed degenerated chromatin ($P < 0.05$) after exposure to 2 mg/mL of MβCD compared to the group exposed to 0 mg/mL. However, no differences among treatments were observed in cytoplasmic maturation. Groups exposed to cold stress demonstrated a lower ($P < 0.05$) capacity for embryonic development compared to the control groups. In the third experiment immature oocytes were exposed to MβCD and then, vitrified (cryotop). After warming, we observed that the ability to reach MII and chromatin degeneration were altered ($P < 0.05$) by MβCD. The blastocysts rate ($P < 0.05$) on D7 was higher in the 2 mg/mL MβCD group, but an identical finding was not observed on D8 ($P > 0.05$). Chromatin degeneration was higher in the vitrification groups. We conclude that MβCD improved nuclear maturation by reducing oocyte degeneration after cold stress or vitrification; however, more studies are required to clarify the usefulness of MβCD use in oocyte cryopreservation.



## Introduction

The ability to preserve the female gamete is becoming an integral part of assisted reproductive techniques (ARTs) as it increases the availability of oocytes for use in such techniques. Successful cryopreservation of the oocyte would allow for the preservation of genetic resources of farm and wild animals as well as the preservation of gametes of women with premature loss of ovarian function. However, because of their large size and marked sensitivity to cooling, the cryopreservation of oocytes is very difficult in most mammals.

Up to now, the standard method used to cryopreserve mammalian oocytes has been slow freezing, which includes slow cooling rates and low concentrations of cryoprotectants agents. Vitrification, which uses rapid cooling rates and a very high concentration of cryoprotectants to prevent the formation of ice crystals, usually replaces slow freezing. This method has been utilized in several species of domestic animals, such as sheep [7], horses [34], cats [16] and cattle [21,33]. However, the overall success of the oocytes in developing to the blastocyst stage is still very low.

Multiple attempts have been made for improving the efficiency of oocyte vitrification by maximizing the cooling rate and minimizing the cryoprotectant concentration. One approach for achieving a rapid cooling rate is to reduce the volume of the vitrification solution. In this regard, various methods have been proposed, initially MDS was developed by Arav in 1992 [28], and then many other devices were developed such as Open Pulled Straw (OPS) [35], cryoloop [13], hemi-straw [37] and cryotop [12]. Among these methods, cryotop uses a very small amount of vitrification solution and is reportedly more practical and efficient for cryopreserving bovine oocytes [21,22]. Even with the advantages of the cryotop method compared to others, the results obtained with vitrification of bovine oocytes remain unsatisfactory [5,19,21,22,42].

The cell damage that occurs during cryopreservation is caused by several factors, such as osmotic stress, toxicity of the

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cryoprotectants used, formation of ice crystals with consequent damage to cellular organelles [29] and direct chilling injury (DCI). Chilling injuries trigger the destabilization of cell membranes during the thermotropic phase transition from the fluid phase to the gel phase during the cooling process, which is considered one of the major obstacles to the success of cryopreservation of oocytes [3,27].

Irreversible damage to membrane integrity caused by chilling during the lipid phase transition is directly related to the quantity of lipids present [3]. Cholesterol is a major structural lipid constituent of the membrane and regulates its function. Therefore, the cholesterol/phospholipid ratio is a vital determinant of plasma membrane fluidity and stability during cryopreservation [10]. Membranes with high concentrations of cholesterol are more fluid at low temperatures and consequently more resistant to damage during cooling [40,41]. To increase membrane fluidity and permeability at low temperatures, cholesterol can be added to the plasma membrane, thereby providing an alternative method for increasing oocyte tolerance for cryopreservation.

Cyclodextrins can act as carrier molecules for the incorporation of cholesterol into plasma membranes [1,10,25]. Cyclodextrins are water-soluble cyclic oligosaccharides consisting of glucose units (α-D-glucopyranoside) joined by connections typeα-1,4 that contain a hydrophobic center capable of integrating lipids. Due to its structure, free cyclodextrin can selectively deplete cholesterol from isolated or intact membranes from a variety of cells, including spermatozoa and oocytes [23], whereas cyclodextrins preloaded with cholesterol deliver cholesterol to the plasma membrane. Therefore, this simple approach can be used prior to cryopreservation to change the membrane composition and minimize membrane damage.

Methyl-β-cyclodextrin (MβCD) is the most potent cyclodextrin family member with respect to its affinity for cholesterol binding. Moreover, it was showed that cholesterol improve bovine [1,25] and equine [20] sperm viability after cryopreservation [23].

One study demonstrated that cholesterol carried by cyclodextrin entered cumulus cells and oocytes, which improved the survival of vitrified mature bovine oocytes [10]. No further studies have investigated this simple approach to reduce oocyte cold sensitivity.

In the present study, we used MβCD to load cholesterol from fetal calf serum (FCS) and deliver it to the oocyte plasma membrane. The purpose of this study was to investigate the effect of MβCD exposure on the in vitro maturation rates and developmental ability of cold-stressed as well as vitrified immature bovine oocytes.

## Materials and methods

### *Chemicals and supplies*

Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO, USA). Cryotop devices were purchased from Ingámed (Maringá, PR, Brazil).

### *Oocyte recovery*

Ovaries from crossbred cows (*Bos indicus* × *Bos taurus*) were collected immediately after slaughter and transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (100 g/mL) at 35 °C. Cumulus oocyte complexes (COCs) were aspirated from 3- to 8-mm diameter follicles with an 18-gauge needle and pooled in a 15-mL conical tube. After 10 min, COCs were recovered and selected in holding medium consisted by HEPES-buffered TCM-199 (GIBCO® BRL) supplemented with 10% FCS. Only COCs with homogenous cytoplasm and at least three layers of cumulus cells were used in the experiments.

### *Cholesterol-loaded methyl-β-cyclodextrin (MβCD) preparation*

In a glass tube, a stock solution (SS) with 1 g of methyl-β-cyclodextrin was dissolved in 2 mL of methanol and stored at −20 °C [10]. To load cholesterol from FCS, the SS was diluted with different concentrations (1, 2 or 3 mg) of MβCD in 1 mL of HEPES-buffered TCM-199 (GIBCO® BRL) supplemented with 20% FCS. The solution was incubated overnight at 38.5 °C.

### *Vitrification and warming*

Oocyte vitrification was performed as previously described [12] with slight modifications. The holding medium (HM), which was used to handle oocytes during vitrification and warming, was composed of HEPES-buffered TCM-199 (GIBCO® BRL) supplemented with 20% FCS. For vitrification, groups were first washed three times in an equilibrium solution composed of 7.5% ethylene glycol and 7.5% dimethylsulfoxide ($Me_2SO$) dissolved in HM for a total of 9 min. Oocytes were transferred to a vitrification solution of 15% ethylene glycol, 15% $Me_2SO$ and 0.5 M of sucrose in HM where they were incubated for 45–60 s. Next, the oocytes were placed into the cryotop device in sets of 3–5 under a stereomicroscope. Before vitrification, most of the solution that was transferred with the oocytes was removed from the device, and only a thin layer (<0.1 μl) remained to cover the oocytes. Subsequently, the cryotop device was immediately submerged into liquid nitrogen. Warming was performed immediately after vitrification by immersing the cryotop end into a drop of HM supplemented with 1 M of sucrose for 1 min pre-warmed at 37 °C. The oocytes were transferred to HM medium supplemented with 0.5 M of sucrose for 3 min, respectively, and finally to the original holding medium. Afterwards, the oocytes were placed in the culture dishes to mature or were fixed for maturational stage evaluation.

### *Oocyte maturation and assessment of meiotic progression*

After warming, COCs were washed and transferred (groups of 25–30) to a 200 μL drop of maturation medium under silicone oil and incubated for 22 h at 39 °C in 5% $CO_2$ in air. The maturation medium was TCM-199 supplemented with 10% FCS (v/v), 10 mg/mL of FSH and antibiotics (100 IU/mL of penicillin and 50 mg/mL of streptomycin). CCOs were distributed into 4 groups, each group represented one maturation period. The first one was fixed immediately after selection, before IVM; the second group was fixed with 8 h of IVM; the third was fixed 22 h of IVM and the fourth group completed IVM period and was fixed with 24 h of IVM.

For meiotic progression evaluation, oocytes were denuded and fixed for at least 48 h with acetic alcohol (1:3). On the day of the evaluation, these oocytes were placed on a slide, covered with a coverslip and were stained with 1% lacmoid in 45% glacial acetic acid. The maturational stage of each oocyte was determined using phase contrast microscopy. Oocytes were classified as follows: immature – did not reach metaphase II; mature – showed metaphase II plate; abnormal – any chromosomal aberrations (diploid, abnormal metaphase II, multidirectional spindle, chromosomal dispersion); degenerate – diffuse or undefined chromatin.

### *In vitro fertilization (IVF) and embryo culture (IVC)*

Following maturation, COCs (groups of 25–30) were transferred to a 200-μL drop of fertilization medium. For fertilization, frozen semen from a Nelore bull previously tested in the lab for IVF was used. Motile spermatozoa were obtained by the Percoll method

[18] and were added to droplets containing COCs at a final concentration of $1 \times 10^6$ spermatozoa $mL^{-1}$. The fertilization medium was TALP [24] supplemented with penicillamine (2 mM), hypotaurine (1 mM), epinephrine (250 mM) and heparin (10 $\mu g/mL^{-1}$). Spermatozoa and oocytes were co-incubated for 18 h at 39 °C with 5% $CO_2$ in air, and the day of in vitro insemination was considered as day 0.

Eighteen hours post insemination (pi), presumptive zygotes were washed, transferred to 200-μmL drops of synthetic oviduct fluid medium with amino acids, citrate and inositol (SOFaaci; [9] supplemented with 5% FCS. This medium was incubated at 39 °C with 5% $CO_2$ in air.

Embryos were evaluated on day 2 pi for cleavage and on days 6, 7 and 8 pi for blastocyst rates.

*Assessment of fertilization rate*

To evaluate the fertilization rate, oocytes were removed from culture 18 h pi, fixed with acetic acid: alcohol (1:3), and stained with a 1% solution of lacmoid in 45% glacial acetic acid. Cells were examined under a phase contrast microscope (Nikon Eclipse E200, 1000×) and classified as either (a) non fertilized – presence of female and absence of male chromatin; (b) fertilized – presence of female and sperm chromatin in the cytoplasm, decondensed sperm head, pronuclei or cleaved; (c) degenerated; or (d) abnormal.

*Experimental design*

*Experiment 1.* The effects of different MβCD concentrations on the in vitro maturation and development of immature bovine oocytes submitted to cold stress for 10 min.

In this experiment, a total of 1452 COCs were distributed into six treatments (T) as follows: (T1) control: untreated COCs; (T2) 0 MβCD: COCs were incubated for 1 h without MβCD and exposed to 4 °C for 10 min; (T3) 1 MβCD: COCs were incubated for 1 h in the presence of 1 mg/mL of MβCD and exposed to 4 °C for 10 min; (T4) 2 MβCD: COCs were incubated for 1 h in the presence of 2 mg/mL of MβCD and exposed to 4 °C for 10 min; (T5) 3 MβCD: COCs were incubated for 1 h in the presence of 3 mg/mL of MβCD and exposed to 4 °C for 10 min; (T6) bench control: COCs remained at room temperature for the same amount of time as the treated groups. Following all treatments, oocytes were transferred to maturation medium.

After maturation, oocytes were either fixed for evaluation of nuclear staining or fertilized in vitro for culturing until the blastocyst stage. For all treatments embryos were evaluated on D2, D6, D7 and D8 pi to determine cleavage and blastocyst rates.

*Experiment 2.* The effects of MβCD on the response of bovine immature oocytes to longer durations of cold stress.

We found in experiment 1 that there were no differences among the groups treated with the various concentrations of MβCD. Thus, we decided to expose oocytes to 2 mg/mL of MβCD for longer stints of cold stress. A total of 966 COCs were distributed into three treatments as follows: (T1) control: after selection, COCs were immediately washed; (T2) 0 MβCD: COCs were incubated for 1 h without MβCD and exposed to 4 °C for 30 min; (T3) 2 MβCD: COCs were incubated for 1 h in the presence of 2 mg/mL of MβCD and exposed to 4 °C for 30 min. Following all treatments, oocytes were transferred to maturation medium.

After maturation, oocytes were either fixed for evaluation of nuclear staining or fertilized in vitro for culturing until the blastocyst stage. For all treatments, embryos were evaluated on D2, D6, D7 and D8 pi to determine cleavage and blastocyst rates.

*Experiment 3.* Developmental capacity of vitrified immature oocytes exposed to MβCD prior to vitrification

To evaluate the effect of MβCD exposure prior to vitrification in immature bovine oocytes, COCs were distributed into four treatments as follows: (T1) control group: after selection, COCs were immediately washed; (T2) vitrified exposed to MβCD: COCs were incubated for 1 h in the presence of 2 mg/mL of MβCD, vitrified and warmed; (T3) vitrified not exposed to MβCD: COCs were incubated for 1 h without MβCD, vitrified and warmed; (T4) bench control: COCs remained at room temperature during the time COC from T2 and T3 were manipulated. Following all treatments, oocytes were transferred to maturation medium.

After maturation, oocytes were either fixed for evaluation of nuclear staining or fertilized in vitro for culturing until the blastocyst stage. For all treatments, embryos were evaluated on D2, D6, D7 and D8 pi to determine cleavage and blastocyst rates.

To evaluate fertilization rates, a group of oocytes were removed from culture at 18 h pi, fixed, stained and examined by phase contrast microscopy.

*Statistical analysis*

Data were analyzed by Chi-square testing with a significance level of 5% ($P < 0.05$).

## Results

*Experiment 1*

Table 1 shows nuclear maturation rates of bovine immature oocytes exposed to different concentrations of MβCD and submitted to cold stress for 10 min. A lower percentage ($P < 0.05$) of oocytes (all groups) exposed to cold stress reached MII after 24 h of maturation compared to control and bench control groups. The oocytes that remained on the bench while the groups were submitted to cold stress showed a similar nuclear maturation rate ($P > 0.05$) relative to the control group but had a higher percentage of abnormal chromatin ($P > 0.05$). Although cold stress increased the percentage of oocytes with degenerated chromatin, exposure to MβCD protected oocytes from degeneration ($P > 0.05$) (Table 1).

Embryo development, on D7 and D8, showed no difference ($P > 0.05$) between oocytes in the control and bench control group (Table 2). Both percentages were higher ($P < 0.05$) than those observed for the groups exposed to cold stress and different concentrations of MβCD.

*Experiment 2*

To evaluate the protective effect of MβCD, the time of the cold stress was increased from 10 to 30 min, after the treatment with 2 mg $mL^{-1}$. Only one concentration of MβCD was used. Data on nuclear maturation and embryo development are presented in Tables 3 and 4. No differences ($P > 0.05$) in the percentages of immature oocytes were observed among groups. However, a higher percentage of oocytes reached MII in the control group ($P < 0.05$) relative to the treated groups. The exposure of oocytes to MβCD decreased the percentage of oocytes that degenerated due to cold stress. Regardless, oocytes exposed to MβCD and submitted to cold stress for 30 min had lower ($P < 0.05$) cleavage and blastocyst rates than the control group.

*Experiment 3*

The results are depicted in Tables 5–7. Vitrification and exposure to MβCD altered the percentage of oocytes that reached MII and the percentage of degenerated oocytes after in vitro maturation (Table 5). Oocytes vitrified after exposing to 2 mg of MβCD showed higher percentages ($P < 0.05$) of MII oocytes and lower ($P < 0.05$) rates of degeneration compared to unexposed cells

**Table 1**
Effect of methyl-β-cyclodextrin (MβCD) on the nuclear maturation of bovine immature oocytes exposed to cold stress (CS) for 10 min at 4°C.

| Oocyte treatment | *N* | Stages of meiosis | | | |
|---|---|---|---|---|---|
| | | Immature *n* (%) | Metaphase II *n* (%) | Abnormal *n* (%) | Degenerate *n* (%) |
| Control | 102 | 12 (11.7)[b] | 78 (76.4)[a] | 5 (4.9)[a] | 7 (6.8)[a] |
| 0 mg/ml MβCD + CS | 114 | 2 (1.7)[a] | 33 (28.9)[b] | 18 (15.7)[b,c] | 61 (53.5)[c] |
| 1 mg/ml MβCD + CS | 98 | 7 (7.1)[a,b] | 35 (35.7)[b] | 23 (23.4)[b,c,d] | 33 (33.6)[b] |
| 2 mg/ml MβCD + CS | 97 | 5 (5.1)[a] | 37 (38.1)[b] | 25 (25.7)[c,d] | 30 (30.9)[b] |
| 3 mg/ml MβCD + CS | 92 | 6 (6.5)[a,b] | 27 (29.3)[b] | 25 (27.1)[d] | 34 (36.9)[b] |
| Bench control | 91 | 7 (7.6)[b] | 58 (63.7)[a] | 13 (14.2)[b] | 13 (14.2)[a] |

[a,b,c,d] Values with different superscripts in the same column are significantly different ($P < 0.05$).

**Table 2**
Cleavage and blastocyst rates for bovine immature oocytes exposed to different concentrations of methyl-β-cyclodextrin (MβCD) and submitted to cold stress (CS) for 10 min at 4 °C.

| Oocyte treatment | *N* | Cleaved, D2 *n* (% ± S.D.) | Blastocyst, D7 *n* (% ± S.D.) | Blastocyst, D8 *n* (% ± S.D.) |
|---|---|---|---|---|
| Control | 181 | 142 (78.4 ± 13.6)[a] | 63 (34.8 ± 6.1)[a] | 70 (38.7 ± 7.7)[a] |
| 0 mg MβCD + CS | 113 | 61 (53.9 ± 6.1)[b] | 15 (13.3 ± 2.0)[b] | 16 (14.2 ± 1.9)[b] |
| 1 mg MβCD + CS | 133 | 67 (50.4 ± 8.1)[b] | 12 (9.0 ± 2.1)[b,c] | 13 (9.8 ± 2.8)[b,c] |
| 2 mg MβCD + CS | 116 | 62 (53.4 ± 5.0)[b] | 14 (12.1 ± 1.4)[b] | 14 (12.1 ± 1.1)[b,c] |
| 3 mg MβCD + CS | 135 | 65 (48.1 ± 8.2)[b] | 13 (9.6 ± 1.5)[b,c] | 16 (11.9 ± 1.9)[b,c] |
| Bench control | 126 | 92 (73.0 ± 2.9)[a] | 44 (34.9 ± 3.1)[a] | 46 (36.5 ± 3.0)[a] |

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).

**Table 3**
Effect of methyl-β-cyclodextrin (MβCD) on the nuclear maturation of bovine immature oocytes exposed to cold stress (CS) for 30 min at 4 °C.

| Oocyte treatment | *N* | Stages of meiosis | | | |
|---|---|---|---|---|---|
| | | Immature *n* (%) | Metaphase II *n* (%) | Abnormal *n* (%) | Degenerate *n* (%) |
| Control | 108 | 18 (16.6)[a] | 79 (73.1)[a] | 3 (2.7)[a] | 8 (7.4)[a] |
| 0 mg/ml MβCD + CS | 101 | 10 (9.9)[a] | 30 (29.7)[b] | 15 (14.8)[b] | 46 (45.5)[c] |
| 2 mg/ml MβCD + CS | 103 | 14 (13.5)[a] | 40 (38.8)[b] | 21 (20.3)[b] | 28 (27.1)[b] |

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).

**Table 4**
Cleavage and blastocyst rates for bovine immature oocytes exposed to different concentrations of methyl-β-cyclodextrin (MβCD) and submitted to cold stress (CS) for 30 min at 4 °C.

| Oocyte treatment | *N* | Cleaved, D2 *n* (% ± S.D.) | Blastocysts, D7 *n* (% ± S.D.) | Blastocyst, D8 *n* (% ± S.D.) |
|---|---|---|---|---|
| Control | 226 | 190 (84.1 ± 10.7)[a] | 84 (37.2 ± 4.3)[a] | 97 (42.9 ± 4.2)[a] |
| 0 mg/ml MβCD + CS | 205 | 71 (34.6 ± 6.9)[b] | 13 (6.3 ± 2.5)[b] | 16 (7.8 ± 2.8)[b] |
| 2 mg/ml MβCD + CS | 223 | 74 (33.2 ± 6.8)[b] | 20 (9.0 ± 4.1)[b] | 20 (8.9 ± 3.7)[b] |

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).

**Table 5**
Effect of MβCD on the nuclear maturation of bovine immature oocytes exposed to methyl-β-cyclodextrin (MβCD) and vitrified (Vit).

| Oocyte treatment | *N* | Stages of meiosis | | | |
|---|---|---|---|---|---|
| | | Immature *n* (%) | Metaphase II *n* (%) | Abnormal *n* (%) | Degenerate *n* (%) |
| Control | 92 | 2 (2.1)[a] | 72 (78.2)[a] | 10 (10.8)[a] | 8 (8.6)[a] |
| 0 mg/ml MβCD + Vit | 78 | 4 (5.1)[a,b] | 23 (29.4)[c] | 13 (16.6)[a] | 38 (48.7)[c] |
| 2 mg/ml of MβCD + Vit | 74 | 2 (2.7)[a,b] | 34 (45.9)[b] | 14 (18.9)[a] | 24 (32.4)[b] |
| Bench control | 87 | 8 (9.1)[b] | 57 (65.5)[a] | 10 (11.4)[a] | 12 (13.7)[a] |

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).

**Table 6**
Cleavage and blastocyst rates for bovine immature oocytes exposed to methyl-β-cyclodextrin (MβCD) and vitrified (Vit).

| Oocyte treatment | *N* | Cleaved, D2 *n* (% ± S.D.) | Blastocysts, D7 *n* (% ± S.D.) | Blastocyst, D8 *n* (% ± S.D.) |
|---|---|---|---|---|
| Control | 181 | 147 (81.2 ± 1.4)[a] | 63 (34.8 ± 0.8)[a] | 68 (37.5 ± 0.8)[a] |
| 0 mg/ml MβCD + Vit | 121 | 27 (22.3 ± 2.0)[b] | 1 (0.8 ± 0.4)[c] | 3 (2.4 ± 0.8)[b] |
| 2 mg/ml MβCD + Vit | 131 | 32 (24.4 ± 2.2)[b] | 7 (5.3 ± 0.8)[b] | 7 (5.3 ± 0.8)[b] |
| Bench control | 183 | 137 (74.8 ± 2.5)[a] | 52 (28.4 ± 1.4)[a] | 59 (32.2 ± 1.8)[a] |

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).

**Table 7**
Fertilization rates for immature oocytes exposed to methyl-β-cyclodextrin (MβCD) and vitrified (Vit).

| Oocyte treatment | *N* | Non Fertilized *n* (%) | Fertilized *n* (%) | Degenerated *n* (%) | Abnormal *n* (%) |
|---|---|---|---|---|---|
| Control | 91 | 14 (15.3)[b] | 70 (76.9)[a] | 3 (3.2)[a] | 4 (4.3)[a] |
| 0 mg/ml MβCD + Vit | 88 | 7 (7.9)[b] | 19 (21.5)[c] | 45 (51.1)[c] | 17 (19.3)[b] |
| 2 mg/ml MβCD + Vit | 85 | 1 (1.1)[a] | 28 (32.9)[c] | 44 (51.7[c] | 12 (14.1)[b] |
| Bench control | 95 | 16 (16.8)[b] | 58 (61.0)[b] | 16 (16.8)[b] | 5 (5.2)[a] |

[a,b,c] Values with different superscripts in the same column are significantly different ($P < 0.05$).

(Table 5). The vitrification process was also detrimental to oocyte fertilization and development in vitro (Tables 6 and 7). Regardless of MβCD concentration, vitrified oocytes exhibited lower ($P < 0.05$) cleavage and blastocyst rates than controls. Although at D8 the blastocyst rate was similar for both groups with vitrified stress, an increase in the blastocyst rate at D7 was observed in vitrified oocytes that were exposed to MβCD prior to vitrification (Table 6).

When the fertilization capacity was evaluated in vitrified oocytes, it was observed that the group not exposed to MβCD showed the lowest percentage ($P < 0.05$) of non-fertilized oocytes at 18 h pi. Both vitrified groups had lower rates ($P < 0.05$) of fertilization and higher ($P < 0.05$) percentages of degenerate and abnormal chromatin oocytes relative to the control groups (Table 7). Compared to control, it was observed that the bench group presented lower fertilization rates ($P < 0.05$) and higher percentages ($P < 0.05$) of degenerated oocytes (Table 7).

## Discussion

The main limiting factor for achieving optimal cryopreservation of oocytes is their high sensitivity to cooling injuries. Among cellular components, the plasma membrane is usually described as one of the most affected structures during the cryopreservation process [3,40]. This sensitivity to cooling is determined by the membrane phospholipid composition and membrane cholesterol: phospholipid ratio [3,10,30,31,40,41]. When cholesterol is added to the cell membrane, fluidity is more easily achieved [3], which leads to higher resistance to cold stress. In fact, the manipulation of the plasma membrane lipid profile by increasing the cholesterol concentration in sperm [1,25,26,41] and oocyte [10,30,41] membranes has resulted in a reduction of cell damage after cryopreservation.

In the present study, we attempted to add cholesterol to the oocyte plasma membrane using MβCD as a vehicle. We aimed to increase the cholesterol: phospholipid rate to improve oocyte vitrification results. For our approach, we loaded MβCD with cholesterol removed from FCS by incubating it overnight in a medium enriched with serum. After incubation, MβCD loaded with cholesterol was added to medium containing the immature bovine oocytes, which were then exposed to cold treatments and assessed for cytoplasmic as well as nuclear viability.

In the first experiment, different concentrations of MβCD were tested to determine if it could protect oocytes during their exposure to a 4 °C cold stress for 10 min. It was very clear that this duration of exposure was sufficient to affect oocyte viability and cause a subsequent decrease in nuclear and cytoplasmic maturation as well as an increase in degenerated oocytes. These results were similar to those observed by Wu et al. Wu et al. [39] who demonstrated that storing bovine immature oocytes at 4 °C for 10 min substantially reduced their maturation and cleavage rates. Our results also showed that short MβCD exposure did not effectively protect oocytes against cold stress, as different concentrations did not increase the percentage of oocytes that reached MII by the end of the maturation period. However, it is worth mentioning that the MβCD-treated groups displayed a reduction in oocytes with degenerated chromatin. These results indicate that cyclodextrin might positively affect oocytes. Similar to nuclear maturation, the exposure to MβCD treatments did not improve either the cleavage rate or blastocyst production.

To analyze whether the time of exposure to cold stress in the first experiment was insufficient to detect the effect of MβCD, a second experiment was designed that increased the exposure time to cold stress from 10 to 30 min. Because no differences were observed with the different concentrations of MβCD, an intermediate concentration of 2 mg/mL was used. The amount of time of cold stress exposure in oocytes did not seem to be the main cause of the cold-related damage, as increasing the time did not alter the rate of MII oocytes after IVM. Oocyte maturation was still significantly affected, regardless of the presence of MβCD, when the temperature was reduced to 4 °C even for a short period of time. Treatment with MβCD did not protect oocytes nor improve the maturation rates of the nucleus or cytoplasm.

Finally, we tested the effect of MβCD on oocytes prior to vitrification. Oocytes incubated with or without cholesterol-loaded MβCD were vitrified and subsequently matured, fertilized and cultured in vitro. In this experiment, MβCD lowered the percentage of oocytes that underwent degeneration, while a higher percentage of oocytes reached MII stage. This beneficial effect was not observed in our previous experiment when oocytes were exposed to 4 °C temperature but not vitrification. It is possible that very rapid cooling, such as that performed with the cryotop method, minimizes the time spent at the critical temperature and circumvents the deleterious effect of cold stress. Thus, the beneficial effect of cell membrane stabilization by MβCD could protect the oocytes' structures, which allows them to reach metaphase II.

As expected [12,21,22,38,42], vitrification negatively affected the developmental ability of oocytes, and no effect was observed after the MβCD treatment in terms of cleavage and blastocyst rates. Although Horvarth and Seidel [10] found significant differences in cleavage and eight cell embryos when loaded MβCD was used, these variations gradually disappeared by the blastocyst stage.

While day 8 blastocyst rates were similar among vitrified oocytes, higher blastocyst rates at D7 were observed in oocytes exposed to MβCD. It is well established that the speed of development is related to embryo quality; thus, it is possible that the quality of embryos was better. Since there was no significant difference in D8 blastocysts rates, developmental delay indicates a lower embryonic viability [15]. One approach to confirm the quality of the embryos would be to perform other evaluations, such as embryo cell counting [10], differential staining and gene expression assays [2,7,32].

While the nuclear maturation of vitrified oocytes was improved by MβCD, there was no change in blastocyst rate. It is difficult to understand the full impact of this data because there is scarce precedent in the available literature on MβCD pretreatment. However, rationales can be constructed to explain the lack of a beneficial effect. One possibility is that we used a alternate approach for loading MβCD with cholesterol by incubating it with FCS, while previous groups used MβCD that was already loaded with cholesterol [10]. Potentially, our FCS incubation did not effectively load MβCD with cholesterol; thus, no cholesterol was incorporated into the membrane. The direct isolation of cholesterol incorporation

sites in oocytes could answer these questions. An alternative explanation is that MβCD decreased damage to the plasma membrane, possibly supported by the lower degeneration rate, but did not prevent damage to other regions that have a higher impact on oocyte viability.

During oocyte maturation, cytoplasmic organelles undergo various remodeling and redistribution processes [8,36]. Vitrification has been reported to affect some of those events. Among organelles, cortical granules are seriously affected [11,21]. Normally after IVM, cortical granules exhibit a peripheral distribution, while vitrified oocytes display a clustered distribution. This alteration could impair fertilization and compromise embryonic development. In addition, studies show that cryopreservation of mouse oocytes can cause zone hardening [14], which can also impair fertilization. To ensure that low cleavage rates were not due to difficulties in sperm penetration, we evaluated the fertilization process. The results showed that MβCD pretreatment did not benefit vitrified oocytes compared to vitrified oocytes without MβCD pretreatment. A majority of the oocytes were already degenerated by the time fertilization occurred. These results suggested that besides plasma membrane other sites also important for oocyte viability can be affected by this technique. Potential sites of damage include regions related to nuclear maturation and retention of the polar body [17], chromosomal aberrations [6], multidirectional or meiotic spindle disorganization [4,16,34], mitochondrial and cortical granules distribution, and alterations in gene expression [2,6,7].

The results presented here suggest that more research is required to clarify whether MβCD is beneficial to the oocyte plasma membrane as well as to determine its optimal dose and time of exposure prior to cryopreservation. This information is vital for optimizing the use of this procedure to improve oocyte viability after vitrification because it can be used in association with other substances or procedures that would protect other cell structures from cold-related damage.

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