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IMPACT OF DIFFERENT CRYOPROTECTANTS OR THEIR COMBINATIONS ON VITRIFIED BOVINE OOCYTES MORPHOLOGY

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ABSTRACT

The present study was conducted to investigate the impact of different cryoprotectant like Ethylene glycol (EG), Propanediol (PROH) and Dimethyl sulfoxide (DMSO) alone or their combinations on morphology of bovine oocytes using vitrification. The vitrification of bovine oocytes was performed using 10, 20 and 40% concentration of EG and 10, 20 and 40% DMSO, 2.5, 5 and 10% PROH alone or combinations of 20%EG + 20%DMSO, 5% PROH + 20% DMSO, 5% PROH + 20% EG or 5% PROH + 20% EG +20% DMSO. The results showed that combinations of the different cryoprotectant proved to be more efficient than using Propanediol (PROH) or Dimethyl sulfoxide (DMSO) alone except Ethylene glycol (EG). The morphological damages of bovine oocytes were found less (9.8%) using combination of 20% EG + 20% DMSO (34.14%) compared with 5% PROH or 20% DMSO to be (27.27%). A combination of 20% EG and 20% DMSO resulted in the highest percentage of the normal morphologically oocytes (82.81%) compared with 65.5 and 72.73% with 5% PROH and 20% DMSO, respectively. Furthermore, the combinations of PROH and DMSO when used alone were found to induce adverse effects on the morphology of bovine oocytes. However, it can be concluded that combinations of 20% EG + 20% DMSO were found better to maintain the integrity and internal structure of bovine oocytes by vitrification.

Keywords:

Bovine, Oocytes, Ethylene glycol, Propanediol, Dimethyl sulfoxide, Vitrification

INTRODUCTION

The cryopreservation of mammalian oocytes has a more recent and far less successful history than spermatozoa and embryos. The vitrification technique involves rapid cooling, warming rates, and the use of high concentrations of cryoprotectant solutions (at high viscosity and small volumes) to bring about a transition into a glass-like physical state without ice crystallization.

Vitrification is rapid, inexpensive, and has been used to cryopreserve embryos (from several mammalian species) at various stages of development **(*Vajta and Nagy, 2006*).** The strategy of cryoprotectant (CP) association is commonly used to reduce the 'solution effect which causes toxicity and osmotic injury to the oocytes. This mechanism is induced by high concentrations of CP. Among these methods, the use of non-penetrating CP is very useful for two reasons: 1) the shrinkage of the oocyte and consequently the amount of water inside the cell that may crystallize during rapid cooling and warming is lower and 2) the reduction of the amount of the CP that penetrates the cell thus reducing the possible toxic effect **(Arav, 2014**). The cryoprotectants commonly used for oocytes vitrification are classified as penetrating or non-penetrating. Some examples of penetrating are EG, propylene glycol (PG), glycerol (GLY) and DMSO that are characterized by small molecules which easily cross cell membrane. These compounds act by forming hydrogen bonds with water molecules thus preventing ice crystallization **(Jain and Paulson, 2006).** Vitrification requires high concentrations of CP. It is therefore important to minimize the damage caused to cells by the osmotic stress or chemical toxicity **(Arav, 2014).** Survival was shown to be better with Propanediol than EG for mouse embryos, indicating possible species differences **(Emiliani *et al.,* 2000 and Friedler *et al.,* 1988)** showed that successful cryopreservation requires the addition of cryoprotectants, such as dimethyl sulfoxide (DMSO), sucrose or l, 2- Propanediol, to protect cells from damage during freezing and thawing. However, it is now clear from the results of studies on mouse oocytes that, the addition of cryoprotectants can result in cytoskeletal disruption of both the meiotic spindle, with adverse effects on chromosome distribution **(Pickering and Johnson, 1987)**, and the sub cortical microfilament mesh **(Vincent *et al.,* 1990b)**. Disruption of the latter can lead to cortical granule release, a premature zona reaction and a block to fertilization **(Vincent *et al.,* 1990a).** The present study was conducted to investigate the impact of different cryoprotectants like Ethylene glycol (EG), Propanediol (PROH) and Dimethyl sulfoxide (DMSO) alone or their combinations on morphology of bovine oocytes using vitrification.

MATERIAL AND METHODS

The present study was carried out at the Laboratory of Artificial Insemination and Embryo Transfer belonging to Animal Reproduction Research Institute, Ministry of Agriculture, and Giza, Egypt.

Chemicals:

All the chemicals used in this study were purchased from Sigma Aldrich (St. Louis, Mo, USA) unless otherwise indicated. The disposable plastics were used in this procedures were obtained from Nunclon, Denmark. All the stock solutions and media were prepared in sterile triple distilled Milli-Q water.

Oocytes recovery and selection:

Immediately after slaughter, one hundred pairs of bovine ovaries were collected from slaughter- house, and washed twice with sterile normal saline supplemented with antibiotics at 32-37ºC. The ovaries were then transported to the laboratory. The ovaries were rinsed three times with warm phosphate- buffered saline (PBS) containing antibiotics (100.000 IU/ml penicillin, 100 mg/ml streptomycin). Oocytes were collected by aspiration of surface follicles (2-8mm diameter) with a 18-gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM-199 + 10 % FBS). The oocytes were examined under Zoom Stereomicroscope at around 20X magnification.

***In vitro* maturation (IVM) of oocytes:**

The maturation medium used was Charles Rosenkran1 containing amino acids (CR1aa) **(Sagirkaya *et al.,* 2006)** supplemented with 10% FCS, 10µg LH, 5µG FSH and 1µg estraduol-17β. *In vitro*, maturation of oocytes was carried out in Petri dishes (35mm diameter), previously equilibrated for at least 2 h, at 38.5 ºC in a moist atmosphere and 5% CO_2 in air. Cumulus Oocyte Complex (COCs) with multilayered cumulus cells and homogenous exoplasmic granulation were chosen. The oocytes were then shifted to 35 mm cell culture Petri dishes containing the washing medium (TCM-199+10%FBS+0.81mM sodium pyruvate). About 10-20 oocytes per droplet (100 ml) were allocated by Pasture pipette and cultured. Petri dish was incubated for about 24 h, in Co_2 incubator (5%) at 38.5^0C and high humidity (90-95 %) for maturation. Oocytes were prepared for vitrification by being exposed sequentially and very briefly to three cryoprotectants solutions. These solutions were prepared in TCM-199 medium supplemented with 10% FBS. Vitrification of oocyte was performed using 10, 20 and 40% concentrations of EG and 10, 20 and 40% of DMSO, 2.5, 5 and 10% of PROH alone and in combinations of 20% EG + 20% DMSO, 5% PROH + 20% DMSO, 5% PROH + 20% EG, 5% PROH +20% DMSO +20% DMSO, after exposure to the three CPA solutions,

Equilibration and vitrification of mature oocytes:

The basic medium used for equilibration, vitrification, and rehydration, was phosphate buffer saline (PBS) supplemented with 0.4% of bovine serum albumin (BSA)**.** For equilibration, oocytes of each treatment were kept in half concentration of equilibration solution (ES) for 10 min. After the end of the equilibration time, oocytes were transferred to complete concentration of vitrification solution (VS) for few seconds (30-40 sec). During this time, oocytes were quickly loaded into the straws and placed directly into liquid nitrogen (-196ºC).

Thawing and rehydration:

The vitrified oocytes were thawed after ten days of storage in all treatments and rehydrated (step wise rehydration) by immersing the straws in a water bath at 25-30°C for 30 sec. After immersion in the water bath, oocytes were rehydrated in sucrose solutions 0.125, 0.25 and 0.5M sucrose solution, respectively and held for 5 min in each concentration for step wise rehydration, according to **Wessel and Ball (2004) and Yamada *et al.* (2007).** The oocytes were then transferred to fresh washing medium 4 times.

Morphological evaluation of vitrified oocytes:

Vitrified /warmed oocytes were examined under stereo microscope (20X) for evaluation of normal and abnormal oocytes. According to **Sharma *et al.* (2010)**. The oocytes with spherical and symmetrical shape and no signs of lyses/degeneration were considered normal whereas oocytes with ruptured zona pellucida, fragmented cytoplasm, or degenerative signs were classified as abnormal and discard.

Statistical analysis:

Data were statistically subjected to ANOVA one way using SPSS for Windows version 18.0, statistical software. Comparison of means was carried out by Duncan's Multiple Range Test **(Duncan's, 1955).**

RESULTS

Effect of different concentrations of Ethylene glycol (EG) on bovine oocytes morphology concentrations. The total numbers of oocytes vitrified were 72, 76 and 82 using above concentrations respectively. (Table 1) shows the morphological evaluations immediately after warming of vitrified bovine oocytes using different concentrations of EG. The proportion of bovine oocytes found to be morphologically normal was significantly ($P<0.05$) lower in 10%EG (70.83) compared with the other concentrations (82.89 and 86.58%), respectively.

Furthermore, the proportion of damaged oocytes was significantly (P<0.05) higher in 10% EG (29.16%) than those in 20% (17.10%) and 40% (13.41%). Various morphological abnormalities observed in oocytes after vitrification thawing included cracking of zona pellucida and shrinkage of cytoplasm but with significant difference among various concentrations of EG. Among the damaged oocytes, shrinkage of cytoplasm was the most frequent abnormality observed.

Table (1): Effect of different concentrations of Ethylene glycol (EG) on bovine oocytes morphology

Ethylene glycol (%)	No. of oocytes	Normal oocytes No.% (Mean±SE)	Abnormal oocytes		Total abnormal No. % (Mean±SE)
			SC No.% (Mean±SE)	BZ No. % (Mean±SE)	
10	72	51 (70.83±2.64)[a]	12 (6.67±2.81)[a]	9 (12.50±0.39)[a]	21 (29.16±2.64)[a]
20	76	63 (82.89±1.68)[b]	7 (9.21±1.47)[b]	6 (7.89±0.18)[b]	3 (17.10±1.68)[b]
40	82	71 (86.58±2.26)[b]	6 (7.32±2.26)[b]	5 (6.09±0.15)[b]	11 (13.41±2.26)[b]

a-b Values with different superscript letters in the same columns are significantly different (P<0.05).
SC (Shrinkage cytoplasm), BZ (Brocken zona pellucida).

Effect of different concentrations of Dimethyl sulfoxide (DMSO) on bovine oocytes morphology:

The morphologic normal oocytes were 67. 24, 72, 73 and 33.33% using different concentrations of DMSO (10, 20 and 40%), respectively. The highest proportion of morphologically normal oocytes was seen in 20% DMSO and the lowest with 40% DMSO. There is a significant(P<0.05) differences were seen between the normal morphologically oocytes among 10 20 and 40% DMSO, on the other hand the highest frequency distribution of morphologically abnormal oocytes were observed with 40% DMSO (66.67%) and the lowest with 20% DMSO (27.27%).

Table (2): Effect of different concentrations of Dimethyl sulfoxide (DMSO) on bovine

oocytes morphology

Dimethyl Sulfoxide (%)	No. of oocytes	Normal oocytes No. % (Mean±SE)	Abnormal oocytes		Total, Abnormal No. % (Mean±SE)
			SC No.% (Mean±SE)	BZ No. % (Mean±SE)	
10	58	39 (67.24±2.99)^{a,b}	11 (18.96±2.77)^{a,b}	8 (13.79±0.43)^{a,b}	19 (32.75±2.99)^{a,b}
20	44	32 (72.73±2.24)^{a}	7 (15.90±2.23)^{b}	5 (11.36±0.29)^{a,b}	12 (27.27±2.24)^{b}
40	42	14 (33.33±2.33)^{b}	17 (40.47±2.40)^{a}	11 (26.19±0.48)^{a}	28 (66.67±2.33)^{a}

a-b Values with different superscript letter in the same column are significantly different (P<0.05).

SC (Shrinkage cytoplasm), BZ (Brocken zona pellucida).

There was a significant difference (P<0.05) among oocytes morphologically abnormal (broken zona pellucida) 26.19, 13.79 and 11.36% also shrinkage of cytoplasm 40.47, 18.96 and 15.90% with 40, 10 and 20% DMSO, respectively.

Effect of different concentrations of Propanediol (PROH) on bovine oocytes morphology

Data presented in (Table 3), indicated that, the total numbers of oocytes vitrified were 54, 41 and 39, respectively. However, (Table 3) illustrates the morphological evaluations immediately after warming of vitrified cow oocytes using different concentrations of PROH cryoprotectant. The proportion of cow oocytes found to be morphologically normal was significantly (P<0.05) lower in 10% PROH compared with the other concentrations 35.89 vs. 55.56 and 65.85%, for 2.5, 5%, respectively. The best significant (P<0.05) percentages of normal morphologically oocytes in 5% PROH (65.85%), Furthermore, the proportion of damaged oocytes was significantly (P<0.05) higher in 10% PROH (64.10%) than those in 2.5% PROH (44.45%) and 5% PROH (34.14%). Significant differences were assessed for normal oocytes morphology and total abnormal morphology oocytes for 2.5, 5 and 10% of PROH (55.56, 65.85 and 35.89%), (44.45, 34.14 and 64.10%), respectively. Various morphological abnormalities observed in oocytes after vitrification worming included cracking of zona pellucida and shrinkage of cytoplasm with significant differences (P<0.05) among various concentrations of PROH. Among the damaged oocytes, shrinkage of

cytoplasm was the most frequent abnormality observed.

Table (3): Effects of different concentrations of Propanediol (PROH) on bovine oocytes morphology

Propanediol (%)	No. of oocytes	Normal oocytes No. % (Mean±SE)	Abnormal oocytes		Total, abnormal No .% (Mean±SE)
			BZ	SC	
			No.% (Mean±SE)	No.% (Mean±SE)	
2.5	54	30 (55.56±2.48)^{a,b}	14 (25.92±1.57)^{a,b}	10 (18.52±4.05)^{b}	24 (44.45±)^{a,b}
5	41	27 (65.85±0.75)^{a}	6 (14.63±1.28)^{b}	8 (19.51±0.53)^{b}	14 (34.14±)^{b}
10	39	14 (35.89±2.30)^{b}	16 (41.02±1.10)^{a}	9 (23.07±1.19)^{b}	25 (64.10±2.30)^{a}

a-b Values with different superscript letters in the same columns are significantly different (P<0.05).

SC (shrinkage cytoplasm), BZ (Brocken zona pellucida).

Effect of different combinations of cryoprotectants on bovine oocytes morphology.

Results of oocytes morphology after vitrification, normal oocytes are shown in (Table 4). Rates of normal oocytes significantly (P<0.05) higher in 20%EG + 20%DMSO compared with the other concentrations (82.81 *vs.* 64.38%) with 5% PROH + 20%EG + 20% DMSO. Similarly, oocytes morphology percentages were 60.37% were added with 5% PROH + 20%EG compared to 54.84 with 5% PROH + 20% DMSO. Furthermore, the proportion of damaged oocytes was higher in 5% PROH + 20% DMSO (45.16%) than those in the other groups (Table 4). Among the damaged oocytes, broken zona pellucida was the most frequent abnormality observed.

Table (4): Effect of different combinations of cryoprotectants on bovine oocytes morphology.

Combinations (%)	No. of oocytes	Normal oocytes No. % (Mean±SE)	Abnormal oocytes		Total abnormal No. % (Mean±SE)
			SC No. % (Mean±SE)	BZ No. % (Mean±SE)	
5PROH+20EG	53	32 (60.37±1.60)^{b}	10 (18.86±1.62)^{b}	11 (20.75±0.27)^{b}	21 (39.62±1.60)^{b}
5PROH+20DMSO	62	34 (54.84±1.99)^{a,b}	16 (25.81±2.60)^{b}	12 (19.35±0.49)^{b}	28 (45.16±1.99)^{a,b}
20EG+20DMSO	64	53 (82.81±2.67)^{a}	5 (7.81±2.68)^{a}	6 (9.38±0.32)^{a}	11 (17.18±2.67)^{a}
5PROH+20EG+20DMSO	73	47 (64.38±2.85)^{a,b}	17 (23.28±2.71)^{b}	9 (12.33±0.23)^{b}	26 (35.61±2.85)^{a,b}

a-b Values with different superscript letters in the same columns are significantly different (P<0.05).

SC (shrinkage cytoplasm), BZ (Brocken zona pellucida).

DISCUSSION

The percentage of oocytes recovered morphologically normal in the present study compared with other reports in which mouse oocytes were vitrified either in DMSO alone **(Wood *et al.,* 1993)** or in a mixture of DMSO, acetamide, propylene glycol (PG) and polyethylene glycol **(Shaw *et al.,* 1991)** or when bovine oocytes were vitrified in DMSO, acetamide and PG **(Hamano *et al*., 1992)**. This is substantially higher than the survival rate of 36-39% when mouse oocytes were vitrified in a mixture of EG, ficoll-70 and sucrose **(Miyake *et al*., 1993).** Ethylene glycol (EG) has however been successfully used as the alone cryoprotectant in vitrification solutions used for various other species (mice, rabbits, horse, cattle, marsupials, **(Kasai, 1997)** and buffaloes **(Hufana- Duran *et al.,* 2004).**The present findings showed better performance of ethylene glycol as a vitrification cryoprotectant, compared to DMSO and the optimum concentration of both cryoprotectants being 40%.The present study suggested that there was a limit to the concentration of the cryoprotectant to be used beyond which it may exert suboptimal effects. This is in part similar to previous findings of **(Wani *et al.,* 2004)**. These workers observed that the highest IVM of buffalo oocytes was

observed for oocytes vitrified in 7 M solution of all cryoprotectants and that EG was better compared to glycerol. Ethylene glycol has been found to be an effective cryoprotectant for the vitrification of mouse **(Miyake *et al*., 1993)**, cattle **(Delval *et al.,* 1998 and Saha *et al., 1996*)** and buffalo **(Dhali *et al.,* 2000a and b and Wani *et al.,* 2004)** oocytes, since it offers advantages over other cryoprotectants in terms of higher permeation into oocytes/embryos for vitrification, and, faster removal during dilution, as its molecular weight is lower than that of glycerol **(Dhali *et al*., 2000a)**. Ethylene glycol has been found to be less toxic than glycerol and propylene glycol to mouse embryos **(Kasai *et al.,* 1990)** and the post-vitrification survival of bovine embryos has been found to be much higher in ethylene glycol than in either a combination of DMSO, PG and polyethylene glycol or a combination of glycerol and PG **(Mahmoudzadeh *et al.,* 1993).** The combination of cryoprotectants induce less toxic effects and proved more to be suitable than independent cryoprotectants. From study it is proved that DMSO and PROH together or alone are not effective cryoprotectants. It was observed that EG and DMSO provides 85% and 86% morphologically normal oocyte. However, when using cryoprotectants (EG and DMSO) in combinations the morphologically normal oocytes percentage was recovered 92%. The present results were similar to those recorded previously (81.4%-95%) in vitrified goat oocyte **(Garg and Purohit, 2007).** Earlier study showed that, the caprine oocyte and embryos vitrified by solid surface vitrification had significantly low survival rate than controls. In conclusion, combinations of 20% EG + 20% DMSO was found better to maintain the integrity and internal structure of bovine oocytes by vitrification.

REFERENCES

Arav, A. (2014): Cryopreservation of oocytes and embryos**.** Theriogenol, 81:96 -102.

Delval, A.; Ectors, F.J.; Beckers, J.F.; Ectors, F. and de Loos, F. (1998): Vitrification of day-6 in vitro produced bovine embryos as donors for cloning by nuclear transfer. Theriogenol, 49:165-172.

Dhali, A.; Manik, R.S.; Das, S.K.; Singla, S.K. and Palta, P. (2000a): Vitrification of buffalo (*Bubalus bubalis*) oocytes. Theriogenol, 53:1295-1303.

Dhali, A.; Manik, R.S.; Singla, S.K. and Palta, P. (2000b): Post-vitrification survival and in vitro maturation rate of buffalo (*Bubalus bubalis*) oocytes: effect of ethylene glycol concentration and exposure time. Anim. Reprod., Sci., 63:159-165.

Duncan, D.B. (1955): Multiple ranges and multiple F test. Biometrics, 11:1- 42.

Emiliani, S.; Van den Bergh, M.; Vannin, AS.; Biramane, J. and Englert, Y. (2000): Comparison of ethylene glycol, 1, 2-propanediol and glycerol for cryopreservation of slow-cooled mouse zygotes, 4-cell embryos and blastocysts. Hum. Reprod., 15:905-910.

Friedler, S.; Giudice, L.C. and Lamb, E.J. (1988): Cryopreservation of embryos and ova**.** Fertile. Steril., 49:743-764.

Garg, N. and Purohit, G.N. (2007): Effect of different cryoprotectant concentrations for ultrarapid freezing of immature goat follicular oocytes on their subsequent maturation and fertilization in vitro. Theriogenol, 68:1455-1463.

Hamano, S.; Koikeda, A.; Kuwayama, M. and Nagai, T. (1992): Full term development of *in vitro* matured vitrified and fertilized bovine oocytes. Theriogenol, 38:1085-1090**.**

Hufana-Duran, D.; Pedro Prudencio, B.; Venturina- Hernando, V.; Hufana-Rogelio, D.; Salazar- Apolinario, L.; Duran-Peregrino, G. and Cruz- Libertado, C. (2004): Post- warming hatching and birth of live calves following transfer of in vitro-derived vitrified water buffalo (*Bubalus bubalis*) embryos. Theriogenol, 61:1429-1439.

Jain, J.K. and Paulson, R.J. (2006): Oocyte cryopreservation. Fertility and Sterility, 86:4.1037-1046.

Kasai, M. (1997): Cryopreservation of mammalian embryos. Molecular Biotechnology, 7:173-179.

Kasai, M.; Komi, H.; Takakamo, A.; Tsudera, H.; Sakurai, T. and Machida, T. (1990): A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J. Reprod. Fert, 89:91-97.

Mahmoudzadeh, A. R.; Van Soom, A.; Van Vlaenderen, I. and De Kruif, A. (1993): A comparative study of the effect of one-step addition of different vitrification solutions on in vitro survival of vitrified bovine embryos. Theriogenol, 39:1291-1302.

Miyake, T.; Kasai, M.; Zhu, S.F.; Sakurai, T. and Machida, T. (1993): Vitrification of mouse oocytes and embryos at various stages of development in an ethylene glycol based solution by a simple method. Theriogenol, 40:121-134.

Pickering, S.J. and Johnson, M.H. (1987): The effect of dimethylsulfoxide on the micro tubular system of the mouse oocytes. Development 100:313-324.

Saha, S.; Boediono, A.; Sumantri, E.; Murakani, M.; Kikkawa, Y. and Suzuki, T. (1996): Vitrification of bovine in Garg and Purohit. Vitrification of goat oocytes. J. Anim. Reprod., 4:114-118.

Sagirkaya, H.; Misirlioglu, M.; Kaya, A.; First, N. L.; Parrish, J. J. and Memili, E. (2006): Developmental and molecular correlates of bovine preimplantation embryos. Reprod., 131:5.895-904.

Shaw, P.W.; Fuller, B.J.; Bernard, A. and Shaw, R.W. (1991): Vitrification of mouse oocytes:

improved rates of survival, fertilization and development. Mol. Reprod., Dev., 29:373- 378.

Sharma, G.T.; Dubey, P.K. and Chandra, V. (2010): Morphological changes, DNA damage and developmental competence of in vitro matured, vitrified-thawed buffalo (*Bubalus bubalis*) oocytes: A comparative study of two cryoprotectants and two cryodevices. Cryobiol., 60: 315-321.

Vajta, G. and Nagy, Z.P. (2006): Are programable freezers still needed in the embryo laboratory? Review on vitrification. Reprod. Biomed. Online, 12:16.779 -796.

Vincent, C.; Pickering, S. J.; Johnson, M.H. and Quick, S.J. (1990a): Dimethyl sulfoxide affects the organization of microfilaments in the mouse oocyte. Mol. Reprod. Dev., 26:227-235.

Vincent, C.; Pickering, S.J. and Johnson, M.H. (1990b): The hardening effect of dimethyl sulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present. J. Reprod. Fer., 89: 253-259.

Wani, N. A.; Maurya, S. N.; Misra, A. K.; Saxena, V. B. and Lakhchaura, B. D. **(2004):** Effect of cryoprotectants and their concentration on *in vitro* development of vitrified warmed immature oocytes in buffalo (*Bubalis bubalis*). Theriogenol, 61:831-842.

Wessel, M.T. and Ball, B.A. (2004): Step-wise dilution for removal of glycerol from fresh and cryopreserved equine spermatozoa. Anim. Reprod., Sci. 84:147-156.

Wood, M.J.; Barros, C.; Candy, C.J.; Carroll, J.; Melendez, J. and Whittingham, D.G. (1993): High rates of survival and fertilization of mouse and hamster oocytes after vitrification in dimethyl sulphoxide. Biol. Reprod., 49:489-495.

Yamada, C.; Caetano, H.V.A.; Simões, R.; Nicacio, A.C.; Feitosa, W.B.; Assumpção, M.E.O.D.Á. and Visintin, J.A. (2007): Immature bovine oocyte cryopreservation: comparison of different associations with ethylene glycol, glycerol and dimethyl sulfoxide. Anim. Reprod., Sci. 99:384-388.