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Genotoxicity of Cryoprotectants on Buffalo Oocytes

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Abstract: The possible chemical toxicity of cryoprotectants to buffalo oocytes was examined by evaluation of oocytes survival and meiotic chromosomes after exposure to cryoprotectants. Oocytes were collected from ovaries of slaughtered buffalo. *In experiment 1*, Oocytes were exposed to different types of cryoprotectants, the meiotic chromosome and rate of aneuploidy were determined and compared with untreated oocytes (control). The aneuploidy percentages were higher in oocytes exposed to cryoprotectants compared with the control. However, among the treated oocytes, the highest aneuploidy percentages was seen in oocytes exposed to dimethyl sulfoxide (DMSO) than ethylene glycol (EG) and glycerol groups. *In experiment 2*, oocytes were exposed for 30 seconds to different concentrations (0.75, 1.5 and 3M) of DMSO, EG and glycerol. The maturation rate was significantly ($p<0.01$) higher for glycerol than DMSO and EG groups. *In experiment 3*, oocytes were exposed to 0.75M of DMSO, EG and glycerol for different exposure times (30 seconds, 1 minute and 5 minutes). The maturation rate was lowered at 5 minutes exposure than 1 minute and 30 seconds. *In experiment 4,* it was like experiment 2 except that the oocytes were washed with 0.5 M galactose after exposure. The maturation rate of oocytes in the galactose groups was significantly ($p<0.01$) higher than that without galactose for glycerol, DMSO and EG groups. *In experiment 5*, oocytes were exposed to different combinations of DMSO, EG and glycerol. The maturation rate was highest in EG + DMSO group followed by EG + glycerol group. The lowest maturation rate was observed in DMSO + glycerol group. In conclusion, the maturation rate and aneuploidy percentage were severely affected after exposure of oocytes to different cryoprotectants. The use of mixtures of cryoprotectant and addition of galactose has the potential to reduce the solutions toxicity to oocytes.

Key words: Cryoprotectant · Buffalo oocytes · Meiotic maturation · Aneuploidy

INTRODUCTION

Cryopreservation of oocytes and embryos is of a great importance for preservation of animal genetic resource [1,2]. Embryos and oocytes were first successfully cryopreserved more than 30 years ago. This procedure has come to be an important, almost essential component in the practice of assisted reproduction in animals and humans. Literally millions of animals of more than 20 species and undoubtedly hundreds of thousands of children have been born from frozen embryos. Nevertheless, there still remain shortcomings with methods used to cryopreserve oocytes and embryos [3]. Many compounds act as cryoprotectants (CPAs) that protect cells against freezing damage. Cryoprotectants are divided into two types: membrane-permeating which can freely permeate the cell (e.g. dimethyl sulphoxide (DMSO), ethylene glycol (EG), glycerol) and membrane non-permeating which cannot cross the lipid bilayer (e.g. saccharides and other large molecular weight compounds). Permeating cryoprotectants have low molecular weight, permeate the cell membrane and exert intracellular cryoprotective action [4,5]. Sugars, as non-permeating cryoprotectants, have been used in the vitrification of oocytes or embryos of many species. The protective action of sugar is very complex and they could preserve the structural and functional integrity of the membrane at low water activities [6].

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Injury from CPA is not limited to those which occur during freezing. Exposing cells to solutions containing CPA prior to cooling can also be damaging due to an osmotic effect. Almost all of the commonly used permeating CPA has lower plasma membrane permeability coefficients compared to that of water. This relationship results in cells experiencing osmotically-driven volume excursions during cryoprotectant addition to and removal from the cell during the course of a cryopreservation procedure. It has been shown in numerous cell types that damage to cells can occur as a result of volume excursions alone [7- 9].

The effect on mammalian oocytes of exposure to some permeating CPA has also been investigated. The adverse effect on oocytes was reported on the MII spindle [10], maturation [11], developmental potential [12,13] and oocyte chromosomes [14,15]. Chilling injuries and toxic effects of the cryoprotectants are the major adverse consequences following cryoprocedures [11, 16]. One problem with vitrification procedures is that the high concentrations of the cryoprotectants required can be toxic under some conditions. Since cryoprotectants are cytotoxic [17], the selection of concentration and time of exposure is very important to decrease their toxicity. For this reason, this study was carried out to examine the genotoxic effect of different types of cryoprotectants on immature buffalo oocytes.

MATERIALS AND METHODS

Oocyte Recovery and Selection: Buffalo ovaries were transported from a slaughter-house to the laboratory in a thermocontainer at 24-39°C. Oocytes were collected in a 50 ml conical tube by aspiration of antral follicles (2-5 mm in diameter) using a 18-gauge needle and a syringe. After being washed three times in Dulbecco's phosphate buffer saline, oocytes were washed three times in maturation medium. Oocytes with intact complement of cumulus cells and a homogenous appearing ooplasm were selected for exposure to cryoprotectants according to experimental design.

Experimental Design for Oocytes Exposure: Serial of experiments were conducted to study the effect of DMSO, EG and glycerol on maturation of buffalo oocytes *in vitro*. Oocytes were exposed at room temperature to different cryoprotectants prepared in tissue culture media-199 as a based media then the exposed oocytes were matured for 24 - 26 hours. A group of untreated oocytes was left as a control.

Experiment 1: Effect of different types of cryoprotectant. Oocytes were exposed to different types of cryoprotectants DMSO, EG and glycerol for 30 seconds. The meiotic stages and rate of aneuploidy were determined.

Experiment 2: Effect of cryoprotectant concentrations (one step exposure). Oocytes were exposed for 30 seconds to different concentrations (0.75, 1.5 and 3M) of DMSO, EG and glycerol.

Experiment 3: Effect of exposure time (one step exposure). Oocytes were exposed to 0.75M of DMSO, EG and glycerol for different periods of time (for 30 seconds, 1 minute and 5 minutes).

Experiment 4: Effect of addition of galactose (one step exposure). Oocytes were exposed as experiment 2 then were washed with 0.5 M galactose for 5 minutes before washing with maturation medium.

Experiment 5: Effect of different combinations of cryoprotectants (two steps exposure). Oocytes were exposed to different combinations of DMSO, EG and glycerol in tissue culture medium. Oocytes were exposed to two cryoprotectants solutions; the first one was half the concentration of the second solution. Cp1: 1.5M EG + 1.5 M DMSO, 1.5 M EG + 1.5 M glycerol and 1.5 M DMSO + 1.5 M glycerol for 45 seconds (step one). After initial exposure time, oocytes were transferred to Cp2: 3M EG + 3 M DMSO, 3 M EG + 3 M glycerol and 3 M DMSO + 3 M glycerol for 25 seconds (step two). The oocytes were washed with 0.5 M galactose for 5 min before washing with maturation media.

Viability Evaluation of Oocytes after Exposure: After exposure, the oocytes of all experiments were washed three to four times in maturation medium. 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. Oocytes with morphologically normal appearance were cultured for 24-26 h.

Oocyte Maturation: The control and exposed oocytes were washed 3 times in TCM-199 with Earl's salts and 25 mM HEPES supplemented with 10% fetal calf serum and 50 μg/ml gentamycin sulfate [18]. Oocytes were cultured in Petri-dishes containing medium, prepared 24 hrs before culturing of oocytes. Each drop of media contains about

10-15 oocytes/100 µl of medium covered with a layer of mineral oil. oocytes were incubated for 24-26 hrs at 38.5°C in 5% CO_2 in air and 95% humidity.

Chromosomes Preparation from Oocytes: At the end of the culture period, slides of chromosomes were prepared according to the procedure described by Tarkowski [19]. Briefly, cumulus cells were removed mechanically by gentle pipetting. Each oocyte was transferred to 1% hypotonic sodium citrate solution for 10 min and then placed on a microscope slide. Fixative (methanol: acetic acid, 3:1) were dropped onto the oocytes. Subsequently, the fixed material was stained with 1% orcein. The state of nuclear maturation was determined as described earlier by [20, 21]. Oocytes that reached telophase I or metaphase II stages were considered matured. The Oocytes with less or more than 25 chromosomes were scored as hypohaploid and hyperhaploid, respectively. The Oocytes with 50 chromosomes were scored as diploid oocytes. The rates of chromosomal abnormalities were calculated by summing the number of aneuploid (hypohaploid + hyperhaploid) and diploid oocytes [22].

Statistical Analysis: Data in all experiment were subjected to ANOVA using SPSS for Windows version 13.0, statistical software. Comparison of means was carried out by least significant difference (LSD) test. Differences were considered significant $p < 0.05$.

RESULTS

Effect of Different Types of Cryoprotectant: Aneuploidy percentages of buffalo oocytes after 30 seconds exposure to different types of cryoprotectants as glycerol, DMSO and EG are summarized in Table 1. The aneuploidy percentages were higher in oocytes exposed to cryoprotectants compared with the control. However, among the treated oocytes, the highest aneuploidy percentages was seen in oocytes exposed to DMSO (19.5 ± 1.8) than EG and glycerol groups.

Effects of Different Concentrations of Cryoprotectants: As shown in Table 2, the survival rate of immature buffalo oocytes after short time exposure (30 seconds) to 0.75 , 1.5 and 3 M of cryoprotectants was similar to each other. The highest survival rate was observed in the glycerol group, but it was not significantly different from the other concentrations of EG and DMSO groups. The maturation rates of immature buffalo oocytes were significantly ($p<0.01$) higher for glycerol than DMSO and EG groups (Table 3). With increasing the concentration of cryoprotectants the percentages of oocytes reaching Telophase I and Metaphase II stages was lowered for all types of cryoprotectants. Among the 3 cryoprotectants, DMSO was the less suitable at the 3 concentrations than EG and glycerol for the maturation of germinal vesicle buffalo oocytes.

Effect of Exposure Time: The survival rate of immature buffalo oocytes after 30 seconds, 1 minute and 5 minutes exposure to 0.75 concentrations of DMSO, EG and glycerol is illustrated in Table 4. The percentage of survival rates did not differ among the three groups of exposure time. The maturation rate was severely affected in DMSO group followed by EG and glycerol groups at different exposure time; 30 seconds, 1 minute and 5 minutes (Table 5). The maturation rate decreased at 5 minutes exposure than 1 minute and 30 seconds. Oocytes exposed in all groups with glycerol had an overall higher maturation rate.

Effect of Galactose Addition: The effect of galactose on the maturation rates of buffalo oocytes after 30 sec exposure was studied. The lowest rate was obtained in 3M exposure of cryoprotectants than 1.5 and 0.75M in the presence of galactose. The exposure of freshly collected COCS to the different concentrations of the cryoprotectants followed by the removal of cryoprotectants in galactose is presented in Table 6. The maturation rates of immature buffalo oocytes were significantly ($P<0.01$) higher for glycerol than DMSO and EG groups with the addition of galactose. With increasing the concentration of cryoprotectants the percentages of oocytes reaching Telophase I and Metaphase II stages was lowered for all types of cryoprotectants. As shown in Tables 3 and 6, the maturation rate of oocytes in the galactose groups was significantly higher ($p<0.0001$) than the groups without galactose for DMSO, EG and glycerol at different concentrations of 0.75 , 1.5 and 3 M.

Effect of Different Combinations of Cryoprotectants: In this experiment a combinations of cryoprotectants with galactose addition were used to overcome the toxicity associated with high concentrations. The toxicity of high concentrations of cryoprotectants was reduced, but not eliminated, by combination (Table 7). Statistical analysis showed there was a significance difference ($p < 0.01$) among the maturation rates in the three groups (EG + DMSO, EG + glycerol and DMSO + glycerol). The highest maturation rate was obtained after exposure to EG + DMSO followed by EG + glycerol group, the lowest maturation rate was observed in DMSO + glycerol group. It seems that toxicity of DMSO can be reduced by mixing with other cryoprotectants.

Table 1: Effect of different types of cryoprotectants on aneuploidy percentages of buffalo oocytes after 30 sec exposure (% ± S.E).

Cryoprotectants (0.75 M)	No. of exposed oocytes	Total No.of metaphases	Undefined metaphases	GVBD	MI	AI	TI	Haploid MII	Diploid MII	Hypo-haploidy	Hyper-haploidy	Aneuploidy%(hypo + hyperploidy) NO	Aneuploidy%(hypo + hyperploidy) %
DMSO	90	50	6	5	1	4	3	20	1	8	2	10	19.5 ± 1.8[a]
EG	67	49	10	6	2	2	3	19	0	7	0	7	14.3 ± 1.3[b]
Glycerol	87	61	11	4	3	2	3	30	0	7	1	8	13.6 ± 1.3[b]
Control	110	89	8	3	3	1	2	67	1	3	1	4	4.6 ± 0.8[c]

DMSO, dimethyl sulfoxide; EG, ethylene glycol.

[a,b,c] Values with different superscripts differ (P<0.0001).

Table 2: Survival rates of immature oocytes after 30 sec exposure to different concentrations of cryoprotectants (% ± S.E).

	Concentrations								
	0.75 M			1.5 M			3 M		
Cryoprotectants	NO of exposed oocytes	NO of survived oocytes	% of survived oocytes	NO of exposed oocytes	NO of survived oocytes	% of survived oocytes	NO of exposed oocytes	NO of survived oocytes	% of survived oocytes
DMSO	64	51	80.1± 2.4	80	62	76.8 ± 2.7	92	67	73.5 ± 3.6
EG	88	69	79.7 ± 3.6	90	69	76.6 ± 2.9	74	57	76.77 ± 1.30
Glycerol	64	55	82.9 ± 0.7	64	52	80.8 ± 3.0	77	62	80.70 ± 1.36

DMSO, dimethyl sulfoxide; EG, ethylene glycol.

Table 3: Maturation rates (TI + MII) of immature oocytes after 30 sec exposure to different concentrations of cryoprotectants (% ± S.E).

	Concentrations								
	0.75 M			1.5 M			3 M		
Cryoprotectants	NO of exposed oocytes	NO of matured oocytes	% of matured oocytes (TI + MII)	NO of exposed oocytes	NO of survived oocytes	% of matured oocytes (TI + MII)	NO of exposed oocytes	NO of matured oocytes	% of matured oocytes (TI + MII)
DMSO	48	19	39.9 ± 1.8[a]	58	23	39.4 ± 2.6[a]	54	19	34.7 ± 3.1
EG	68	31	44.9 ± 2.6[a]	62	25	39.5 ± 2.1[a]	60	22	36.64 ± 0.9
Glycerol	43	23	53.4 ± 2.3[b]	46	21	48. 7 ± 2.8[b]	54	23	41.6 ± 3.1

[a,b] Values with different superscripts differ (P<0.01).

Table 4: Survival rates of immature oocytes after different exposure times to 0.75 M concentrations of cryoprotectants (% ± S.E).

	Exposure times								
	30 sec			1 min			5 min		
Cryoprotectants(0.75 M)	NO of exposed oocytes	NO of survived oocytes	% of survived oocytes	NO of exposed oocytes	NO of survived oocytes	% of survived oocytes	NO of exposed oocytes	NO of survived oocytes	% of survived oocytes
DMSO	64	51	80.1 ± 2.4	99	76	77.3 ± 1.5	97	67	69.7 ± 1.9
EG	88	69	79.7 ± 3.6	112	87	77.9 ± 2.9	126	104	77.8 ± 2.9
Glycerol	108	84	77.8 ± 2.9	64	55	72.9 ± 0.7	98	70	71.0 ± 2.4

Table 5: Maturation rates of immature oocytes after different exposure times to 0.75 M concentrations of cryoprotectants (% ± S.E).

	Concentrations								
	30 sec			1 min			5 min		
Cryoprotectants(0.75 M)	NO of exposed oocytes	NO of matured oocytes	% of matured oocytes (TI + MII)	NO of exposed oocytes	NO of survived oocytes	% of matured oocytes (TI + MII)	NO of exposed oocytes	NO of matured oocytes	% of matured oocytes (TI + MII)
DMSO	48	19	39.9 ± 1.7[a]	66	24	35.3 ± 1.2[a]	67	14	21.3 ± 1. 9[a]
EG	68	31	44.9 ± 2.55[a]	84	35	41.6 ± 1.6[b]	86	21	25.4 ± 1.3[a]
Glycerol	43	23	53.4 ± 1.8[b]	83	42	50.6 ± 1.4[c]	67	32	45.6 ± 0.6[b]

[a,b,c] Values with different superscripts differ (P<0.01- P< 0.0001).

Table 6: Maturation rates of immature oocytes after 30 sec exposure to different concentrations of cryoprotectants with galactose addition (% ± S.E).

	Concentrations								
	0.75 M			1.5 M			3 M		
Cryoprotectants	NO of exposed oocytes	NO of matured oocytes	% of matured oocytes (TI + MII)	NO of exposed oocytes	NO of survived oocytes	% of matured oocytes (TI + MII)	NO of exposed oocytes	NO of matured oocytes	% of matured oocytes (TI + MII)
DMSO	66	33	50.6 ± 1.4[a]	45	21	47.6 ± 1.2[a]	50	20	40.3 ± 1.4[a]
EG	51	28	53.1 ± 1.8[a]	64	32	50.8 ± 1.6[a]	73	33	44.3 ± 2.5[a]
Glycerol	71	43	61.2 ± 1.6[b]	56	32	56.6 ± 1.5[b]	56	29	51.9 ± 1.1[b]

[a,b] Values with different superscripts differ (P<0.01).

Table 7: Maturation rates of immature oocytes after two step exposures to different combination of cryoprotectants with galactose addition (% ± S.E).

		Maturation rates (TI + MII)	
Cryoprotectants	NO of exposed oocytes	No	%
3 M EG + 3 M DMSO	64	32	47.2 ± 2.8[a]
3 M EG + 3 M glycerol	75	30	39.9 ± 0.4[b]
3 M DMSO + 3 M glycerol	72	24	33.9 ± 1.8[c]

[a,b,c] Values with different superscripts differ (P<0.01).

DISCUSSION

A wide variety of approaches has been used to select the suitable cryoprotectants for optimization the cryopreservation methods.

The observation of the oocytes chromosomes at MII stage is more reliable mean for defining the *in vitro* maturation progress [23,24]. The frequency of aneuploidy noticed in the control (4.6 %) falls into the range of results published for various mammalian species: cattle, 5.8% [25] and 7.1 % [26] ; horse, 5.5% [27] and mouse, 2.7% [28]. In our study the aneuploidy percentage of buffalo oocytes exposed to different cryoprotectants was higher compared to the control. It seems that the cryoprotectants as a chemical substances have cytotoxic effect on the buffalo oocytes matured *in vitro*. Moreover, aneuploidy may be affected by *in vitro* maturation and *in vitro* fertilization media [28]. With respect to the type of cryoprotectants the highest aneuploidy percentage was seen in oocytes exposed to DMSO than EG and glycerol groups. In this respect, [10] showed that brief exposure of oocytes to DMSO (Me2SO) resulted in the emergence of microtubular asters and longer exposure to DMSO resulted in disassembly of the spindle and chromosome dispersal. Vincent *et al*. [29] also found that Me2SO affects microtubules and spindles in oocytes. However, the disturbance in microtubules and spindles in oocytes lead to aneuploidy. Moreover, CPA has been shown to alter cytoskeletal components in mammalian oocytes, particularly the filamentous actin network and meiotic spindle [30]. Repolymerization after treatments is common, but the particular organization of the polymers often does not represent those of untreated oocytes. Also, Rajaei *et al.*[31] reported a significant increase in the DNA-fragmented nuclei occurred in the porcine blastocysts exposed to cryoprotectants compared to control blastocysts.

The germinal vesical buffalo oocytes were exposed to different concentrations (0.75 , 1.5 and 3 M) of glycerol, EG and DMSO for short exposure time (30 seconds). The survival rates were similar for all three types of the cryoprotectants at the 3 concentrations. Similarly, Wani *et al.*[32] reported that the exposure of immature buffalo oocytes to EG, DMSO, PROH (1,2-propanediol) and glycerol followed by removal of cryoprotectants in descending concentrations of sucrose didn't affect the survival rate of oocytes compared with the control. Regarding the concentrations of the cryoprotectants the maturation rates of immature buffalo oocytes were significantly higher for glycerol than EG and DMSO groups in the absence of galactose. DMSO was less suitable at the three concentrations. In this respect, Schellander *et al.* [33] tested different concentrations (1 M, 1.5 M) of various cryoprotectants (DMSO, PROH and glycerol) on cumulus cell-enclosed bovine oocytes in germinal vesicle and in Metaphase II stages. They found that, DMSO was less suitable at 1 M and 1.5 M concentrations than PROH and glycerol for oocytes development. The glycerol and PROH yielded significantly better cleavage and 4-cell rate compared to

DMSO . Glycerol has been widely used for freezing bovine embryos because of its low toxicity. This may be due to the fact that glycerol is a large molecule and move slowly through the cell membranes of the oocytes [4]. The smaller molecules of EG and DMSO allow them to pass through the cell membranes more rapidly causing more detrimental effect than glycerol. Increasing the concentrations of cryoprotectants, the percentage of oocytes reaching Telophase I and Metaphase II stages decreased for all types of cryoprotectants. The higher concentrations of cryoprotectants the more damage done to the cell by the cryoprotectants itself and the harder to remove as no galactose was used in this experiment. Moreover, Rall [34] reported that the high concentrations of cryoprotectants cause cell damage because of osmotic and cytotoxic injuries.

The exposure time is a very important parameter in the selection of cryoprotectants. So the strategy to avoid toxicity of cryoprotectants was to shorten the exposure time. Therefore optimal exposure time for successful vitrification must be compromised between preventing toxic injury and preventing intracellular ice formation [35]. In the present result, the morphological examination for the oocytes after exposure showed that the survival rate of the immature buffalo oocytes did not differ among the three exposure time (30 sec, 1 min, 5 min) for glycerol, EG and DMSO. In this respect, Wood *et al.* [36] reported a high proportion (> 70%) of mouse and hamster oocytes exposed for 3-5 min to 1.5 M DMSO appeared morphologically normal on recovery. In the present study, the maturation rate was severely affected in DMSO group followed by EG and glycerol groups at the three exposure time. The maturation rate decreased at 5 min exposure than 1 min and 30 sec. Martins *et al.*[37] reported that a high concentration (40%) of EG in addition to a long equilibration time (5 or 15 min) was detrimental to bovine oocytes maturation.

The maturation rate of buffalo oocytes was significantly higher in groups of galactose addition than groups without galactose addition for all types and concentrations of cryoprotectants. The protective action of sugars is very complex, attributable to a number of their special properties [38]. Due to their large molecular size, sugars could cause an osmotic gradient across the cell membrane, which enhance dehydration of the cell before freezing of extracellular water. Kuleshova, *et al.*[39] reported that penetrating cryoprotectants concentration can be lowered, while the solution's vitrification properties are maintained, providing that they are replaced instead by polymers or sugars. Clearly such modifications should have the potential to reduce the solution's toxicity to the cells. Toth *et al.* [40] also showed that the maturation rate of frozen human immature oocytes was improved when sugars were added. In a study of common carp embryos, it was clearly suggested that sugars addition lowered the toxicity of permeating cryoprotectants [41]. Regardless to the galactose addition, the maturation rate decreased in higher concentration (3 M) than lower concentrations (1.5 and 0.75 M). Also, the maturation rates of immature buffalo oocytes were significantly higher for glycerol than DMSO and EG groups either with or without galactose. Rall [34] reported that, the high concentrations of cryoprotectants cause cell damage because of osmotic and cytotoxic injuries.

A common practice to reduce the toxicity of the cryoprotectants, but not its effectiveness, is to place the cells first in a solution of lower strength cryoprotectants to partially load the cells with it, before transferring them to the full-strength cryoprotectants. In our experiment, we tried to reduce the toxicity of higher concentration of cryoprotectants by combination and two step exposures to oocytes. The highest maturation rate was obtained in EG + DMSO followed by EG + glycerol group, the lowest maturation rate was observed in DMSO + glycerol group. Inspite of the toxicity of DMSO alone was increased in our work than EG and glycerol, it was reduced by mixing with other cryoprotectants. Many authors reported that the mixture of cryoprotectants may have some advantages over solutions containing only one solute [42,43] and the combination of DMSO and EG was reported to be the mixture of choice [11]. In contrast, Albarracyn *et al.*[44] reported that oocytes exposed only to 20% EG and 20% DMSO showed a similar appearance to the control. Moreover, Wani *et al.*[32] reported that the exposure of immature buffalo oocytes to 7 M cryoprotectants (DMSO, EG, PROH and glycerol) followed by the removal of cryoprotectants in descending concentrations of sucrose didn't affect the survival and the maturation rate of the oocytes compared with the control. Also, Yadav *et al.* [45] had found no benefit of combining DMSO with EG for oocyte vitrification.

In conclusion, the severity of toxicity resulting from exposure to a permeating cryoprotectant depends on many factors, including the type of a cryoprotectant, the length of exposure and the cryoprotectant concentrations. The toxicity of the cryoprotectants was reduced by mixing of different cryoprotectants together and the adding of sugar.

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