Effect of two types and two concentrations of cryoprotectants on ovine oocytes morphology and viability post-vitrification

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Abstract:

Background:
Oocyte vitrification is a promising technique. The choice of appropriate types and concentrations of cryoprotectants is essential for the success of the oocytes vitrification.

Objective:
This study aimed to investigate the effect of vitrification on viability and morphology of oocytes and to compare the effect of several cryoprotectants on the viability and morphology of oocytes during vitrification and post-thawing.

Methods:
The sheep ovaries were collected from the local abattoir. Only normal and viable oocytes were included in this study. By using cryotop, immature oocytes that were viable with normal morphology were vitrified with 15% DMSO and 15% EG supplemented with 0.0 M, 0.25M, or 0.5 M of either sucrose or trehalose as control and treated groups. Oocytes viability and morphology were assessed post-aspiration and post-thawing.

Results:
From the results of the present study, the percentage of post-thawing normal and viable oocyte reported with the use of 0.5M trehalose and EG in vitrification solution (VS) significantly ($P<0.05$) higher than the percentage of post-thawing normal and viable oocyte reported with use of 0.25 M trehalose and EG.

Conclusion:
Vitrification is simple technique and easy to perform but it needs some experience to prevent any oocyte loss during vitrification and thawing processing. The use of 0.5 M of either sucrose or trehalose in vitrification solution improves the percentage of post-thawing viable and normal oocytes.

Key words: Cryopreservation, Vitrification, Oocyte, Cryoprotectant, Cryotop.

Introduction

Preservation at low temperatures is a practice that has found several applications in medicine and biotechnology. Several types of cells and tissues can be stored virtually indefinitely in liquid nitrogen (–196°C) and returned to physiological temperatures (1).

Oocyte cryopreservation has significant contribution in assisted reproductive technology (ART) programs (2). This technique can solve the legal and ethical problems associated with the cryopreservation of embryos in patients undergoing *in vitro* fertilization (IVF) (3), offer alternatives for infertile patients who are subject to ovarian hyper-stimulation syndrome, those who are poor responders to ovarian stimulation or women who are undergoing premature ovarian failure (4), also it can aid to preserve female fertility in cancer patients (5).

Oocytes can be cryopreserved at immature or at mature metaphase II (MII) stage (6). The main problem associated with cryopreservation of MII oocytes is the sensitivity of microtubule spindles to low temperatures and cryoprotectants (7). So that immature oocytes cryopreservation has been emerged as an alternative to the freezing of mature oocytes because immature oocytes are believed to be more resistant to the freezing process by being more undifferentiated, by the absence of a spindle and by having chromosomes protected by a nuclear membrane (8).

There are two major techniques for oocyte cryopreservation: slow freezing and ultrarapid (vitrification) (9). Both approaches are highly dependent upon cryoprotectant agents (CPAs) that protect oocytes from damage during the freezing process (10). CPAs are divided into two groups: permeable and non-permeable to the cell membrane (11).

Vitrification is gradually replacing slow freezing due to a better survival rate after thawing and less cellular damage (12). The physical definition of vitrification is the solidification of a solution at low temperature, not by ice crystallization but by extreme elevation in viscosity during cooling (13). The vitreous state can be achieved through the use of high concentrations of CPAs that can draw water out of the cytoplasm combined with high cooling and warming rates (14). Since particularly high cryoprotectant concentrations are required for the vitrification approach, the avoidance of damage due to cryoprotectant toxicity is a major problem (15). . However, many measures have been adopted to decrease the specific toxicity of cryoprotectants, like the use of mixtures of two or three cryoprotectants (16), stepwise addition of cryoprotectants (16), (17), and the application of minimum sample volume techniques that increase the cooling rates and reduce the required CPA levels to reduce the adverse effects of high CPA concentrations needed for vitrification (18).

This study aimed to investigate the effect of vitrification on viability and morphology of sheep oocytes and to compare the effect of two cryoprotectants on the viability and morphology of oocytes during vitrification and post-thawing.

Materials and Methods

This study was done using oocyte collected from ovarian follicles of slaughtered ewes in AL-Shu'alla local abattoir. This study was carried out in the laboratories of The Higher Institute of Infertility Diagnosis and Assisted Reproductive Technologies at AL-Nahrain University during the period from June to October 2011.

1. Preparation of culture medium and additives:

Earl's culture medium (CM) was prepared by dissolving Earl's salt (0.0884 gm), Ampiciline (0.008 gm) (Troge Medical GMBH, Germany), Na-pyruvate (0.001gm), Na-HCO (30.21 gm) in 100mL Distilled water (DW). After preparation, the pH was adjusted to 7.2–7.4, then filtered through Millipore filter (0.22µm), kept in refrigerator till use.

2. Preparation of vitrification and thawing solutions:

The equilibration solution (ES) consisted of 7.5% (v/v) dimethyl sulphoxide (DMSO) (Scharlau, Spain) with 7.5% (v/v) ethylene glycol (EG) (Sigma- Aldrich. UK) was prepared by adding the corresponding volume of CPA to culture medium containing 10% Human Serum Albumin (HSA) (Global, USA).

Vitrification solutions (VS) consisting of 15% (v/v) DMSO with 15% (v/v) EG were added to CM supplemented with 10% HSA. This composition was used as VS for control groups. While VS used for treated groups contains either sucrose or trehalose in two concentrations 0.25M or 0.5 M.

Thawing solutions (TS) or (warming) solutions (WS) contain either sucrose or trehalose were prepared in three different concentrations (0.5M/l, 0.25M/l, 0.125M/l) which were added to CM containing10% HSA. It is important to shake the solution well to dissolve the sugars cryoprotectants. The final pH of the above solutions was adjusted to 7.2–7.4, then filtered through Millipore filter (0.22µm), sterilized by ultraviolet light for 30 min, kept in refrigerator till use.

3. Collection of Ovaries and Oocytes:

Ovine ovaries were obtained from a local slaughterhouse and placed into thermos at 33–35°C in 0.9% saline (Haidylena. Egypt) containing 100IU/

mL Ampiciline (Troge Medical GMBH, Germany) and 100µg/mL streptomycin (Troge Medical GMBH, Germany). Ovaries were transported to laboratory within less than 2hrs. In the laboratory ovaries were washed with warmed (38.5°C) normal saline solution to remove the clotted blood and reduce contamination on the ovarian surfaces (19). Oocytes were collected from all the visible follicles on the ovarian surface with >2mm diameter by aspiration technique. Oocytes with follicular fluid were aspirated using 20-gauge hypodermic needle attached to a sterile disposable syringe containing 0.5 ml of CM supplemented with 20 IU/mL heparin (Panpharma, Egypt) to prevent clotting in follicular fluid. After oocyte retrieval, content of each syringe was poured into a Petri dish. Then this content examined under dissecting microscope for oocytes collection using modified pasture pipette and washed for three times with CM (20).

4. Classification of oocytes:

After oocytes collection, they were classified under dissecting microscope into immature, mature and atretic oocytes according to morphological features of each class, such as the presence or absence of 1st polar body and/or the cumulus cell appearance (compacted or expanded) (21) (22). Only normal immature oocytes were included in this study.

5. Viability Test:

All oocytes were examined for viability using the trypan blue exclusion test. Immature oocytes were categorized on the basis of the degree of dye exclusion. Unstained oocytes were classified as live and fully stained oocytes as dead (23). The viability test was done post-aspiration and immediately post-thawing.

6. Vitrification and thawing:

The vitrification and warming procedures were performed according to Kuwayama *et al*. (24), Kuwayama (25) with some modifications. Normal and viable immature oocytes (three oocytes) were equilibrated in 0.5 mL of ES at room temperature for 15 minutes. After that, they were placed into 0.5 mL of VS. Then oocytes were placed on the Cryotop strip (Kitazato Supply Co. , Fujinomiya, Japan) in a small drop of VS and the Cryotop immersed into LN2. The transfer of oocytes into the vitrification solution and the vitrification process were performed within 1minute. Then, the strip was covered with the plastic tube in LN2 to protect it during storage. After 2-3 weeks thawing process was done.

For thawing, the protective cover was removed from the Cryotop while it is still submerged in LN2. Stepwise removal of the cryoprotectant was done by transferring the oocytes through a descending concentration of thawing solution at room temperature. The strip was immersed directly into the thawing solution of either 0.5M or 0.25M (sucrose or trehalose) solution for 3 minute, depending on the sugar concentration of the vitrification solution. Then, the thawed oocytes were transferred to 0.25M and 0.125M (sucrose or trehalose) solutions for 3 minutes and then washed twice with culture medium.

The thawed oocytes were considered abnormal when there was a change in shape, breakage of zona pellucida, uneven granulation or leakage of oocyte contents (26).

7. Experimental design: The oocytes were subdivided randomly into control and treated groups and vitrified with DMSO and EG using two different types and two concentrations of sugars cryoprotectants.

8. Statistical analysis: Statistical analysis was performed by using SPSS (Statistical Package of Social Science; version 15.0 LED Technology, USA). The results were expressed as mean ± standard error of the mean (SEM). The Independent Samples T-Test was applied by use SPSS statistical program to compare between the means of percentages of post-aspiration and post-vitrification oocytes viability and morphology for the same group as well as between the treated and control groups. The differences between the values were considered statistically significant if the P value was less than 0.05 (P> 0.05).

Results

Post-thawing oocytes viability and morphology using DMSO+EG.

1. Supplied with either 0.25M or 0.5 M sucrose:

The observed percentage of viable and normal oocytes of 0.25 M sucrose treated group (76.02± 4.06) was not significantly differed (P>0.05) from the observed percentage of viable and normal oocytes (63.16± 3.01) of its control group. Similarly, there was no significant difference (P>0.05) between the percentage of viable and abnormal oocytes of the 0.25 M sucrose treated group (15.31± 4.06) as compared to that of its control group (7.44± 4.05) (Table 1).

Regarding the comparison of 0.5 M sucrose treated group with its control group, no significant differences (P>0.05) were observed in the percentages of normal and viable oocytes (Table4.9). Similarly, no significant differences (P>0.05) were observed in the percentages of abnormal and viable oocytes of 0.5 M sucrose treated group (3.32± 3.32) and its control group (7.50± 3.27), (Table1). The percentages of abnormal and non viable oocyte for 0.25 M

(8.66± 3.21) and 0.5 M (4.00± 3.33) treated groups were not significantly different (P>0.05) from their control groups, (21.67± 2.03) and (29.40 ±4.05), respectively (Table1).

Not Significant differences (P>0.05) were observed in the percentages of (normal and viable, abnormal and viable) oocytes of 0.25 M sucrose treated group and percentages of 0.5 M sucrose treated group. Regarding the comparison between their control groups, no significant differences (P>0.05) were obtained in the percentages of post-vitrification (normal and viable, abnormal and viable) oocytes (Figure 1).

2. Supplied with either 0.25M or 0.5 M trehalose:

The percentage of viable and normal oocyte morphology obtained from treatment with 0.25 M trehalose (76.50± 1.00) was not significantly different (P>0.05) from its control group (64.17± 3.06). Similarly, the difference was not significant (P>0.05) between the 0.25 M trehalose treated group and its control group respecting the percentage of post-thawing viable and abnormal oocyte (14.78± 3.12) and (8.00± 3.90) respectively, (Table 2). From the same table, the percentage of non-viable and abnormal oocyte of 0.25 M trehalose treated group (8.72± 2.57) was significantly (P<0.05) differed from its control group (27.83 ±3.90).

Considering treatment with 0.5 M trehalose, the observed percentages of viable oocyte, both normal (91.01± 3.57) and abnormal (3.32± 2.00), were not significantly differed (P>0.05) from its control group (80.50 ±1.180) and (3.66± 2.00) respectively, (Table2). Similarly, no significant difference (P>0.05) was obtained by comparing the percentage of non-viable and abnormal oocyte morphology of 0.5 M trehalose treated group to its control group as presented in (Table 2).

The percentage of viable and normal oocyte morphology observed in 0.5 M trehalose treated group was significantly higher (P<0.05) than 0.25M trehalose treated group. Whereas no significant difference (P>0.05) was noticed in the percentage of viable and abnormal oocyte observed from treatment with 0.25 M trehalose compared to that obtained with 0.5 M trehalose treated group. Regarding their control groups, there were not significant differences (P>0.05) between the percentages of viable and normal oocytes as well as between the viable and abnormal oocyte morphology percentage (Figure 2).

3. Comparison between sucrose and trehalose:

The percentages of viable normal and abnormal oocyte morphology observed in 0.25 M sucrose treated group were not significantly differed (P>0.05) from that of 0.25 M trehalose treated group, (Figure 3). No significant differences (P>0.05) were noticed in the percentage of viable normal and abnormal oocytes morphology between 0.5 M sucrose treated and 0.5 M trehalose treated groups, (Figure 3). Similarly, the differences were not significant (P>0.05) between their control groups concerning the percentages of viable normal and abnormal oocytes morphology (Figure 4).

Table (1): Percentage of post-thawing oocytes viability and morphology using DMSO+ EG with either 0.25 M or 0.5 M sucrose for VS. (mean ± SEM).

Groups		Post thawing			
		Viable (%)		Non-viable (%)	
Sucrose 0.25M	Control*	63.16A ±3.01	7.44B ±4.05	–	29.40 C ±4.05
	Treated	76.02 A ±4.06	15.32 B ±4.06	–	8.66 C ±3.21
Sucrose 0.5M	Control*	70.83 D ±4.17	7.50 E ±3.27	–	21.67F ±2.03
	Treated	92.68 D ±4.22	3.32 E ±3.32	–	4.00 F ±3.33

*VS contains 15% DMSO and 15%EG for control group.
Means with the same capital letters are not significantly different (P≥0.05).

Table (2): Percentage of post-thawing oocytes viability and morphology using DMSO +EG with either 0.25 M or 0.5 M trehalose for VS. (mean ± SEM).

Groups		Post thawing			
		Viable (%)		Non-viable (%)	
Sucrose 0.25M	Control*	Normal	Abnormal	Normal	Abnormal
		64.17A ±3.06	64.17A ±3.06	–	27.83b= ±3.90
	Treated	76.50 A ±1.00	76.50 A ±1.00	–	8.72 a ±2.57
Sucrose 0.5M	Control*	80.50 C ±1.18	80.50 C ±1.18	–	15.84 F ±3.12
	Treated	91.01 C ±3.57	91.01 C ±3.57	–	5.67 F ±2.00

*VS contains 15% DMSO and 15%EG for control group. Means with the different small letters are significantly different (P<0.05) ; means with the same capital letters are not significantly different (P≥0.05).

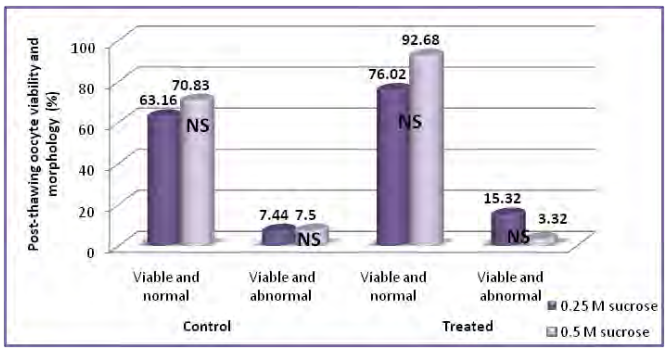

Figure (1): Percentages of post-thawing viable normal and abnormal oocytes vitrified with EG for (0.25 M, 0.5 M) sucrose treated and their control groups.

NS: no significant difference (P≥0.05).

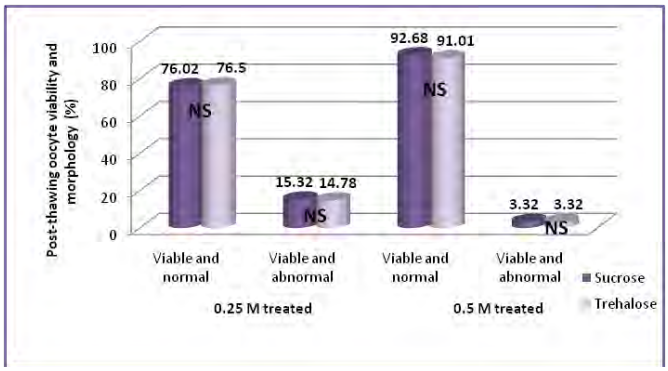

Figure (2): Percentages of post-thawing viable normal and abnormal oocytes vitrified with EG for 0.25 M or 0.5 M trehalose treated and control groups.

NS: no significant difference (P≥0.05), *Means are significantly different (P<0.05).

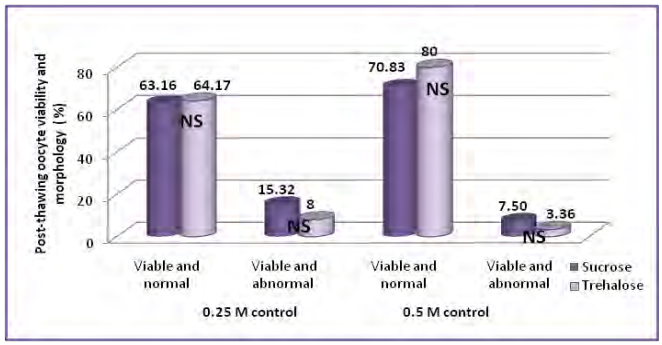

Figure (3): Percentages of post-thawing viable (normal and abnormal) oocytes vitrified with EG in the treated groups of (0.25 M, 0.5 M) sucrose and/or trehalose. NS: no significant difference (P≥0.05).

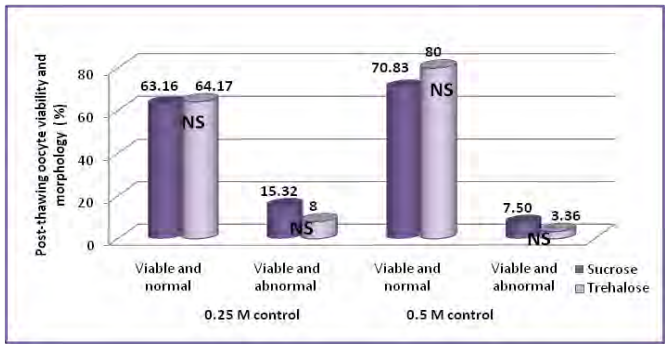

Figure (4): Percentages of post-thawing viable (normal and abnormal) oocytes vitrified with EG in the control groups of (0.25 M, 0.5 M) sucrose and/or trehalose. NS: no significant difference (P≥0.05).

Discussion:

In the present study DMSO and EG were employed combined with 0.25 M or 0.5 M of either sucrose or trehalose. The presence of cryoprotectant in the vitrification solution decreases the probability of intracellular crystallization which is considered to cause most damage when very rapid cooling takes place, but the high concentration of the cryoprotectant required is toxic and causes osmotic injury to the oocytes (27).

From the results of the present study, the percentage of post-thawing normal and viable oocytes were reported by the use of EG the used concentration of either sugar (sucrose, or trehalose) which are close to the survival rate reported by others. By using cryotop with 15% of DMSO+EG +0.5 M sucrose, survival rate of 91.8% and 89.7% were reported for vitrified *in vitro* matured bovine oocytes, MII human oocytes respectively (28), (29). Zhou (30) has vitrified cumulus-enclosed and partially-denuded GV bovine oocytes in 15% EG+15% DMSO+0.5M sucrose in two steps and reported a survival rate of 93.8% and 81.3%, respectively. Nedambale (31), reported a survival rate of 82% for bovine oocytes vitrified with 35% EG+0.4 M trehalose+5% PVP, Dike (32) reported a survival rate with 5.5 M EG +1 M sucrose (89.8%). Whereas Fujiwara *et al* (33) obtained a survival rate of 98.3% of rat mature oocyte vitrified with 15%DMSOand EG +0.5 M sucrose using Cryotop as vitrification device.

In the present study, sucrose and trehalose were used in two concentrations (0.25 M and 0.5 M) for both. The use of sugars assist in the formation of stable glass at low temperatures, control the rate of permeation by the cryoprotectant resulting in the prevention of excessive swelling during vitrification and warming, and increase the viscosity of the solution while lowering the concentration of the permeable cryoprotectant, thereby lowering the coefficient of toxicity (34).

The disaccharides sucrose and trehalose are membrane impermeant and of low toxicity to oocytes and embryos used to promote dehydration before, during and/or after cryopreservation (35) and can help to stabilize membranes (36) (37). In typical cryopreservation protocols by vitrification, the probability of ice crystal formation is reduced by increasing viscosity through an increase in the concentration of the cryoprotectants (38).

The above mentioned roles during vitrification process are evident from the reported higher survival rates of all treated groups compared to their control groups, though the differences were not significant (P>0.05). Additionally, it was reported that the use of 0.25 M trehalose cause a significant decrease (P<0.05) in the percentage of post-thawing non viable oocytes. Despite the differences were not significant (P>0.05) between the 0.25 M and 0.5 M of either sugar the percentage of viable and normal oocyte morphology reported with 0.5 M trehalose treated group was significantly higher (P<0.05) than the reported percentage with 0.25M trehalose treated group, it is also evident that increasing the

concentration of sugar in vitrification solution help to improve the percentage of post-thawing normal and viable oocyte.

This observation is in accordance with other research groups. A significantly better post-thaw survival of slowly freezed human oocytes in was observed in the presence of 0.2 mol/l sucrose rather than 0.1 mol/l (39) and the presence of 0.3 mol/l sucrose has resulted in greater post-thaw survival compared with 0.1 or 0.2 mol/l sucrose (40). Similar observation was reported by Eroglu (41) who found that in the presence of intracellular trehalose, increasing extracellular trehalose concentrations provide excellent cryosurvival rates of vitrified mouse oocytes.

In the present study, non significant differences (P>0.05) were observed in the percentage of post-thawing normal and viable oocyte between the two sugar for both concentrations. These results are in accordance with Abe *et al*. (42) who obtained similar results with either trehalose or sucrose during bovine GV oocyte vitrification using a stepwise protocol and Rayos *et al*. (43) who found that survival and fertilization rates did not differ between the use of either sucrose or trehalose with EG. On the other hand, Kim *et al*. (44) reported an improvement in the viability of mouse morula frozen ultra-rapidly in the presence of trehalose and found that trehalose is superior to sucrose during freezing. Similarly, Arav *et al*. (27) found that exposure of bovine oocytes to trehalose was less harmful than exposure to sucrose, and high normospermic fertilization (70%) was achieved after exposure to 0.25 mol trehalose despite the reported non significant differences in the osmotic response of vitrified bovine oocytes to (0.25 M, 0.5 M, 1 M) of either sucrose or trehalose.

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