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IMPACT OF DIFFERENT CRYOPRESERVATION TECHNIQUES AND CRYOPROTECTENTS ON *IN VITRO* MATURATION OF XENOTRANSPLANTED OOCYTES IN SHEEP

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ABSTRACT
The present study was conducted to investigate the impact of sheep ovarian tissues cryopreservation using different cryoprotectants Ethylene glycol (EG), Propanediol (PrOH) and glycerol (GLY) supplemented with Sucrose or mannitol and techniques (vitrification or rapid cryopreservation) on ability of oocytes for *in vitro* maturation (IVM) post-xenotransplantation to mice bodies. In this study, 371 ewe ovaries were collected and divided randomly into eight groups, control group (without freezing), vitrification groups (EG + 0. 5M Sucrose, EG + 0**.** 5M Mannitol, PrOH + 0**.** 5M Sucrose, PrOH +0**.** 5M Mannitol, Glycerol+0. 5M Sucrose and Glycerol+ 0. 5M mannitol) and rapid cryopreservation groups were xenotransplanted to female mice for 1 week. After that, the oocytes were collected then assessed the ability of oocytes for IVM%, post- xenotransplantation. In this study, percentage of normal oocyte morphology was significantly increased ($P< 0.05$) for control group (G1) as compared with the other groups. However, significant decrease ($P< 0.05$) in the percentage of normal oocyte morphology was noticed for G8 when using rapid cryopreservation as compared with the other groups. Significant increased ($P< 0.05$) was appeared in IVM % in control group as compared with the other groups. In contrast, significant decline ($P< 0.05$) was shown in the percentage of IVM in G8 as compared with the other groups**.** From the results of the present study it was concluded that the, vitrification technique with cryoprotactents EG and PrOH supplemented with sucrose recorded improvement in the ability of oocytes to IVM post-xenotransplantation.

KEYWORDS: cryoprotectants, xenotransplantation, vitrification technique, IVM.

INTRODUCTION

During development of the mammalian fetus, the primordial germ cells (PGCs) are initially formed in the endoderm of the yolk sac and migrate in the ovary then develop into oocytes which then become part of primordial follicles. Female's ovaries have a limited number of primordial follicles that develop in the folliculogenesis process[1]. Folliculogenesis is a physiological process occurs via a series of steps which the continuation of the follicles development and maturation, the propulsion of the follicle to steroid hormone production and ovulation[2]. Although there is a deterioration risk of the cell during rapid cryopreservation by the high concentration of CPAs, intracellular ice formation is to prevent and the cryoprotectant exposure shortened compared with slow cooling. Samples can be heated and removed cryoprotectants similar to the procedure of conventional[3]. Different the vitrification process advantages is the low cost, practicality, since is not necessary for the acquisition of a programmable freezer, and the entire process can be completed in few minutes. On the other hand, during the vitrification, it is not possible to control the temperature [4]. During the slow freezing, the cells are confronted many physical constraints (such as the ice crystals formation also changes in the permeability of the membrane and the volume of cells)[5]. In this method can be started with a low cryoprotectant concentration, and permeable cryoprotectants are used with a concentration of about 1.5 M (such as DMSO, propanediol and EG)[5]. The cryoprotectants are typically added at room temperature; then, the temperature is gradually lowered to -7 °C with a velocity of -2 °C per minute. Meanwhile, the solution remains liquid. However, there is a formation possibility of small ice crystals at that temperature. The solutions usually remain at that temperature for about 10 to 30 minutes to be balanced[6]. After this period, the temperature decreases rapidly at a rate of -0.3°C per minute, and the temperature reached -40°C, because growth of ice crystals causes a progressive increase in the salt concentration in the remaining solution and then osmotic stress and mechanical damage. Then, the temperature is lowered to -100 °C, and finally immersed in LN_2, and the remaining solution becomes solid glass[7]. This process takes about three hours[8,9]. The major problem with this approach is the duration of exposure of tissues to cryoprotectant, which is a very long time. The rate of cooling, in fact, must be slow enough to allow sufficient intracellular dehydration and freezing; and also must be fast enough to avoid the cryoprotectant effecting of toxic. Freezing in Slow and rapid thawing offer less causes less damage and intracellular ice formation[10]. However, the objective of this study was to investigate the effect of sheep ovarian tissues cryopreservation on *in vitro* maturation, post-xenotransplantation to female mice bodies.

MATERIALS & METHODS

This study was performed using ewe's ovaries which were slaughtered in AL-Shualla local abattoir. This study was

carried out in the laboratories of the High. Institute for Infertility Diagnosis and Assisted Reproductive Technologies at AL-Nahrain University during the period from December 2012 to June 2014.

Preparation of vitrification and thawing solutions

The equilibration solutions (ES) consist of 7.5% (v/v) from ethylene glycol (EG), Propanediol (PrOH) or glycerol with 0.25M of sucrose or Mannitol were prepared by adding the corresponding volume of CPA to SMART[11] medium containing 10% Human Serum Albumin (HSA). Vitrification solutions (VS) consist of 15% (v/v) from either from ethylene glycol (EG), PrOH (Propanediol) or glycerol with 0.5M sucrose or Mannitol were prepared by adding the corresponding volume of CPA to culture medium containing 10% Human Serum Albumin (HSA). Thawing solutions (TS) or (warming) solutions (WS) contain either sucrose was prepared in three different concentrations (0.5M/l, 0.25M/l and 0M/L) which were added to CM containing10% HSA. These solutions were prepared with some modifications according to[12,13]. It is important to shake the solution well to dissolve the sugars used as cryoprotectant. The final pH of the above solutions was adjusted to 7.2–7.4, then filtered through Millipore filter (0.22µm), kept in refrigerator till use.

Preparation of Rapid cryopreservation solutions

The equilibration solutions (ES) consisted of 7.5% (v/v) DMSO with 7.5% (v/v)ethylene glycol (EG) were prepared by adding the corresponding volume of CPA to culture medium containing 10% HSA.

Rapid cryopreservation solutions consisting of 15% (v/v) EG with 15% (v/v) DMSO were added to CM supplemented with 10% HSA.

Thawing solutions

Solution one: SMART medium supplemented with 0.5M sucrose.

Solution two: SMART medium supplemented with 0.25M sucrose.

Solution three: SMART medium as washing solution.

Ovaries collection

The sheep ovaries were collected from local abattoir in Baghdad. Both ovaries were collected from each ewe, immediately after slaughtering and placed into glass tubes contained normal saline solution (0.9% NaCl) supplemented with antibiotics (100IU/mL penicillin, 100 µg/mL streptomycin and 100µg/mL Metronidazole), and placed it into thermos at 30-35°C. Ovaries were transported to the laboratory at of High Institute for Infertility Diagnosis and Assisted Reproductive Technologies within less than 2h.

Vitrification Procedure for Ovarian cortex fragments

Ovarian cortex fragments were placed in 2 mL of ES at room temperature for 15 minutes. After that, they were placed for 1 minute into 2 mL of VS. Then 2-4 ovarian cortex fragments were placed on the modified simple Cryoleafe (Fakhrildin Cryoleafe) strip within a small amount of VS and the modified simple Fakhrildin Cryoleafe into LN_2 directly. Then, the strip was covered with the plastic tube inside LN_2 to protect it during storage. After 2-3 weeks, thawing process was done.

Ovarian cortex fragments rapid cryopreservation procedure

Ovarian cortex fragments (5-7fragments) were equilibrated in 2 mL of ES at room temperature for 15 minutes. After that, they were placed into 2 mL of rapid cryopreservation solution for1minute. Then there were placed in the Cryovial containing 1.5 ml of rapid cryopreservation and placed on the LN2 vapour for 5minuteS then the Cryovial immersed into LN2. After 2-3 weeks thawing process was done.

For thawing of vitrified ovarian cortex fragments, the protective cover was removed from the Fakhrildin Cryoleafe while it is still submerged in LN_2. Stepwise removal of the cryoprotectant was done by transferring the ovarian cortex fragments through a descending concentration of thawing solution at room temperature. The strip was immersed directly into the thawing solution of either 0.5M sucrose solution for 3 minute, depending on the sugar concentration of the vitrification solution. Then, the thawed ovarian cortex fragments were transferred to 0.25M sucrose solution for 3 minutes and then washed twice with SMART culture medium (14).

Xenotransplantation

The mice were given from Animal House Unit at High Institute for Infertility Diagnosis and Assisted Reproductive Technologies. For, the xenotransplantation animals were anesthesized using Inhaled Ether, than a small median slit was made in the abdomen and peritoneum of the animals, and sheep ovarian cortex strips were fixed to the inner side of the peritoneum with. The abdominal wall was then closed by 1 or 2 stitches[15]. To immunity inhibition every muse was orally administered Prisolone (Predisolone) (0.178µg) daily and for stimulation follicular development injected intera peritoneum 15 IU PMSG daily for 5 day and in last 2 day injected 15 IU PMSG and 10 IU HCG. After 1 week the sheep ovarian cortex were taken and washed three times in SMART culture medium containing 5% HSA, than oocytes were collected by slicing ovarian cortex fragment.

***In vitro* Maturation**

Oocytes were washed three times in SMART medium containing 5% HSA, than, about 5-10 oocytes per droplet (1mL) from culture SMART with supplied with hCG (5 IU/mL), PMSG (10 IU/mL) and Estradiol (1µg/mL) and cultured within four well Petri dish and covered their by liquid paraffin was incubated for about 24 h in CO_2 incubator (5% CO_2) at 38.5°C with high humidity (95%) [16]. The percentages of IVM were recorded in every well

IVM % = mature oocytes / cultured oocytes *100

Statistical analysis

The data were analyzed statistically using SPSS/PC version 18 software (SPSS, Chicago). The oocytes parameters were compared using Chi squire test. Significant level (0.05)

RESULTS & DISCUSSION

In the present study, the percentages of recovered oocyte when using different cryoprotectants and techniques were showed In the figure (1). Non significant differences in the percentages of recovered oocyte were observed among all groups. Highest and lowest percentages of recovered oocyte were noted in groups G8 and G7 respectively.

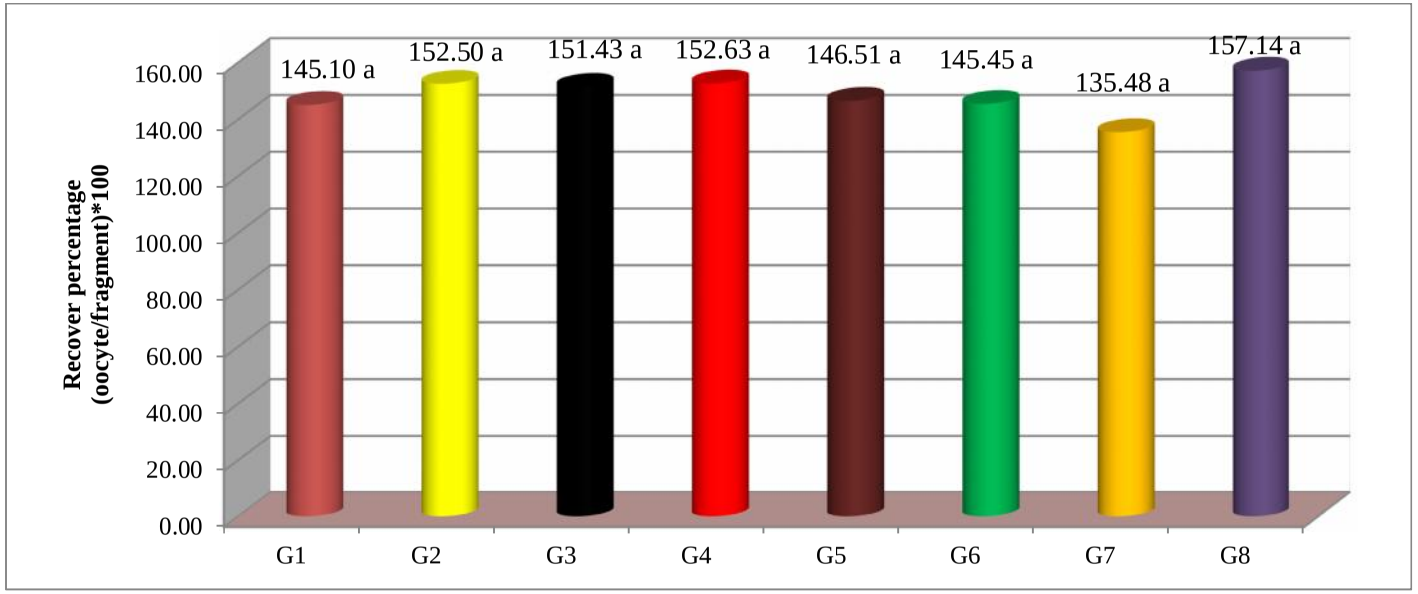

FIGURE 1: Effect of cryopreservation technique and cryoprotactents on percentage recovered of oocytes from ovarian cortex fragments
Chi square= 2.081 P value= 0.955 ns

Treatments		No. of fragments	No. of oocytes
G1: Control		51	74
Uitra rapid (vitrification)	G2: Ethylene glycol + sucrose	40	61
	G3: Ethylene glycol + mannitol	35	53
	G4: glycerol+ sucrose	38	58
	G5: Glycerol + mannitol	43	63
	G6: Propanedaiol + sucrose	33	48
	G7: Propanedaiol + mannitol	31	42
G8: Rapid cryopreservation		42	66

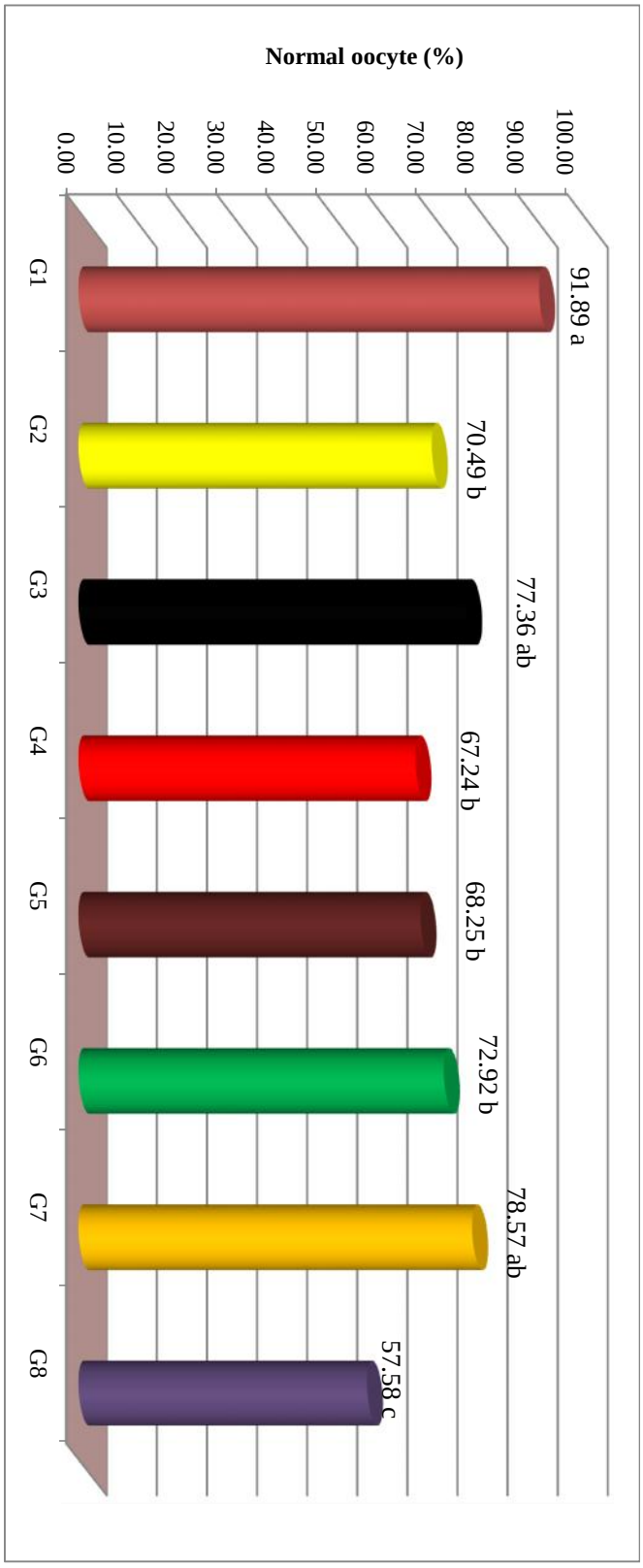

FIGURE 2: Effect of cryopreservation technique and cryoprotactents on normal oocytes percentage from ovarian cortex fragments. Chi square= 9.517 P value= 0.0218 *

Treatments	No. of oocytes
G1: Control	68
Uitra rapid (vitrification) G2: Ethylene glycol + sucrose	43
G3: Ethylene glycol + mannitol	41
G4: glycerol+ sucrose	39
G5: Glycerol + mannitol	43
G6: Propanedaiol + sucrose	35
G7: Propanedaiol + mannitol	33
G8: Rapid cryopreservation	38

FIGURE 3: Effect of cryopreservation technique and cryoprotactents on IVM percentage from ovarian cortex fragments

Chi square= 25.123 P value= 0.001**

Treatments	No. of oocytes
G1: Control	43
Uitra rapid (vitrification) G2: Ethylene glycol + sucrose	21
G3: Ethylene glycol + mannitol	19
G4: glycerol+ sucrose	16
G5: Glycerol + mannitol	14
G6: Propanedaiol + sucrose	12
G7: Propanedaiol + mannitol	10
G8: Rapid cryopreservation	8

Figure (2) shows the percentages of normal oocyte morphology when using different cryoprotectants and techniques. In the current work, best percentage was observed for control group (G1). However, significant decrease ($P< 0.05$) was noticed in the percentage for normal oocyte morphology for G8 when using rapid cryopreservation. Non significant differences were assessed for normal oocyte morphology (%) among G1, G3 and G7. Also, non significant differences were noted among G2, G4, G5 and G6. In sheep, the oocytes were successfully cryopreserved by vitrification of ovarian tissue, and presented a similar maturation rate in the vitrified and nonvitrified oocytes[17]. More recently, bovine oocytes recovered from vitrified ovarian tissue showed lower oocytes maturation rate compared with fresh tissue, although they have developed into the blastocyst stage after IVF with a similar rate [18,19]. In this work, significant increase ($P< 0.05$) was appeared in IVM percentage for control group as compared to the other treated groups. In contrast, significant decline ($P< 0.05$) was shown in the percentage of IVM for G8 as compared to control and other treated groups. Non significant differences were assessed for IVM (%) among remain groups (table 3). In cats, *in vitro* induction of chromatin compaction has been shown to be beneficial for the survival and competence of oocytes after vitrification at the stage of GV[20]. Therefore, actin microfilaments and microtubules are strictly involved in the process of maturation of the oocyte, and their integrity is essential for proper progression through meiosis [21, 22]. In fact, tubulin is responsible for the formation of the spindle of meiosis. In conclusion, using vitrification technique with protactents EG+ sucrose and PrOH+ sucrose recorded improvement in the ability of oocytes to in IVM post- xenotransplantation.

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