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Original Research Article

Effects of Sheep Ovarian Tissue Vitrification and Transplantation on *In Vitro* Fertilization and Early Embryonic Development of Oocytes

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A b s t r a c t

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 and mannitol and techniques (vitrification or rapid cryopreservation) on ability of oocytes for *in vitro* fertilization (IVF) and early embryonic development. In this study with post-xenotransplantation to mice bodies, 371 ewe ovaries were collected and divided randomly into eight groups, control group (without freezing), vitrification groups (EG + 0. 5M Sucrose, EG + 0. 5MMannitol, PrOH + 0. 5M Sucrose, PrOH +0.5M Mannitol, Glycerol+0. 5M Sucrose and Glycerol+ 0. 5M mannitol) and rapid cryopreservation there groups were xenotransplanted to female mice for 1 week. After that, the oocytes were collected then assessed the ability of oocytes to *in vitro* fertilization (IVF) and early embryonic development. In post-xenotransplantation study, significant increase ($p<0.05$) was observed in the percentages of IVF were noted for G1 (control group) as compared with the other groups. However, lowest IVF percentage was observed for G8 using rapid cryopreservation. 1-2 cell embryo stage recorded higher percentage in G8 using rapid cryopreservation. Highly significant ($p<0.01$) was observed in the percentage of 3-4 cell embryo stage for GLY+ sucrose as compared with the other groups. From the results of the present study it was concluded that the vitrification technique with cryoprotectants EG and PrOH supplemented with sucrose recorded improvement in the ability of oocytes for IVF and early embryonic development post-xenotransplantation.

K e y w o r d s

Cryopreservation
Cryoprotectants
In vitro fertilization
Oocyte development
Xenotransplantation

Introduction

The ovary consists of a medulla and cortex. The central part called medulla, which contains loose connective tissue, nerves, blood vessels and lymphatics. Ovarian cortex is the outer part of the ovary consists of ovarian follicles embedded in a stroma. Small ovarian follicles are at a depth of about1-2 mm in the ovarian cortex. However, the follicles considered the functional units of the ovary and are each composed of an oocyte and surrounding by granulosa cells with or without the theca cells, depending on the stage of development of the follicle. Granulosa cells was separated by a basement membrane, called the lamina propria from the stromal / tissue thecal and granulosa cells are devoid of vascular supply (Milan, 2011).The important points in thecryopreservation of ovarian tissues include: rates of freezing, the concentration and type of cryoprotectant, apoptosis inhibitors, the frozen buffer power of hydrogen (pH), the rate of thawing, temperature of storage, and the addition / cryoprotectants removing conditions (Brockbank and Taylor, 2007; Bastings et al., 2014).

There are two main different procedures of cryopreservation, vitrification and slow freezing. Both procedures comprise four common stapes: exposure of the samples to the CPA, freezing storage temperature (-196°C), thawing and the CPA elimination. The terms "freeze-thaw" refer to the slow cooling process while "cooling and warming" are correct with respect to the vitrification process (Shaw and Jones, 2003; Brockbank et al., 2014).

For the ovarian tissue cryopreservation purposes, it is necessary to determine the best concentration of cryoprotectant. Histology can be used to quickly assess the quality of the ovary (Lee, 2007). However, the morphology alone is not enough to evaluate the process of freeze-thaw (Schotanus et al., 1997; Van den Hurk et al., 1998; Martinez-Madrid et al., 2004). The morphological assessment only facilitate identification of primary signs of atresia visible microscopically (cytoplasmic damage, nuclear pyknosis, the oocyte granulosa cells detachment and the the basement membrane irregularity (Jorio et al., 1991; Hulshof et al., 1995; Demirci et al., 2002). The damage of Follicles by cryopreservation cannot always be seen immediately, which requires some *in vitro* culture of hours before the analysis of viability. The restoration of a normal cellular metabolism, which can be detected by enzyme activity and integrity of membrane, ensure the follicular viability (Schotanus et al., 1997; Van den Hurk et al., 1998; Santos, 2007). However, the objective of this study was to investigate the effect of sheep ovarian tissues cryopreservation on *in vitro* fertilization (IVF) and early embryonic development, postxenotrans-plantation to female mice bodies.

Materials and 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 Higher 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 (Fakhrildin and Flayyih, 2011) medium containing 10% Human Serum Albumin (HSA).

Vitrification solutions (VS) consist of 15% (v/v) from either from ethylene glycol (EG), PrOH or glycerol with 0.5M sucrose or Mannitol were prepared by adding the corresponding volume of CPA to culture medium containing 10% 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 Kuwayama et al. (2005) Kuwayama (2007).

It is important to shake the solution well to dissolve the sugars used as cryoprotectants. 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 1: SMART medium supplemented with 0.5M sucrose.
Solution 2: SMART medium supplemented with 0.25M sucrose.
Solution 2: 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 2 h.

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 5 min. 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 min. and then washed twice with SMART culture medium (Yadav et al., 2008).

Xenotransplantation

The mice were given from Animal House Unit at High Institute for Infertility Diagnosis and Assisted Reproductive Technologies. For, the xeno-transplantation animals were anesthetized 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 (Marie-Madeleine et al., 2010). To immunity inhibition every muse was orally administered Prisolone (Predisolone) (0.178µg) daily and for stimulation follicular development injected intraperitoneum 15 IU PMSG daily for 5 days and in last 2 days 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%) (De Felici and Siracusa, 1982). The percentages of IVM were recorded in every well.

Sperm preparation for *in vitro* fertilization

Before sperm preparation the cryoprserved thawed semen samples were evaluated to record sperm parameters pre–activation. Sperms were prepared by DeSmedt et al. (1992), as the 0.25mL from cryopreserved semen was reduced with 1 mL of culture medium (SMART), centrifuged at 1000rpm for 8 min. and the temperature at 25^{o}C for two times, the pelleted accumulated sperm was obtained and the remaining supernatant was reflected and 1 mL of SMART medium was added. Then sperm was left to swim to the top (Swim-up) for 1 h at 37^{o}C in an incubator, the top 0.5 ml containing the sperm floating with dynamic high was pulled and estimated using a light microscope with an estimate of sperm concentration and recorded the results of sperm.

In vitro fertilization technique

The mature oocytes were washed twice in SMART fertilization medium and were transported to 4-well culture plates (5-10 oocytes/well) containing 1mL of the SMART fertilization medium. The motile spermatozoa were added to the oocytes at the concentration of approximately 5×10^4 sperm/oocyte. Sperm and oocytes were covered in liquid paraffin and incubated at 37°C in a moist atmosphere 5% $CO_2$with high humidity (95%) for 24h in CO_2 incubator. The percentages of IVF were recorded in every well.

IVF % = fertilized oocytes / mature oocytes × 100

In vitro early embryonic development

Embryos were washed for three times by using SMART mediumSMART and were cultured in 1mL of the semi medium in 4-well each 5zygotes/well culture plates and was covered by liquid paraffin 37°C in a moist atmosphere of 5% CO_2 and 95% humidity in the CO_2 incubator for 24 h, the embryonic cleavage was recorded to for cell stage. The percentages of ED were recorded in every well.

ED % = embryonic stage/ fertilized oocytes × 100

Statistical analyses

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

Results and discussion

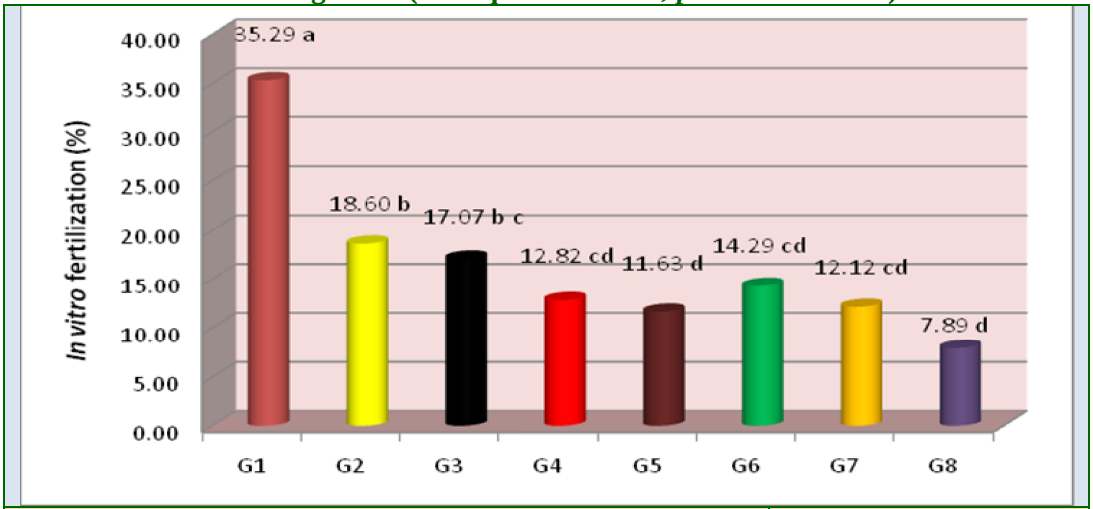
The percentages of *in vitro* fertilization (IVF) when using different cryoprotectants and techniques are shown in Fig. 1. Significant increment (p<0.05) was observed in the percentages of IVF for G1 (control group) when compared to the other groups. However, lowest percentage was observed for G8 when using rapid cryopreservation. However, no significant differences were assessed for IVF (%) between G2 and G3 groups, with same result between G3 and G4 group. Also, non significant differences were noted among G4, G5, G6 and G7 groups. Finally, there was no significant difference between G7 and G8 groups.

The potential value of xenograft ovarian tissue for rare and endangered species was recognized at its fair value rescue female gametes of these species is not yet established. Snow et al. (2001) was able to generate live young ovarian tissue xenografted but this required IVF and embryo transfer, and it could therefore be a problem for any species in which hormonal stimulation, culture *in vitro*, IVF and embryo transfer protocols are not well established.

In addition, this work was carried out in a consistent pattern xenogeneic (sheep to mouse) and it is not clear whether this success can be booked on larger xenogeneic barriers. On a more positive note, ovarian xenograft technology allows ovary, be divided into several small fragments usable. However, this is the most valuable species that form single, dominant, antral follicles per cycle. Ovarian xenograft has already proven to be an interesting model system that allowed full egg size and antral follicles to develop for a wide range of species including wallabies, cats and humans (Gook et al., 2001; Mattiske et al., 2002; Fassbender et al., 2007).

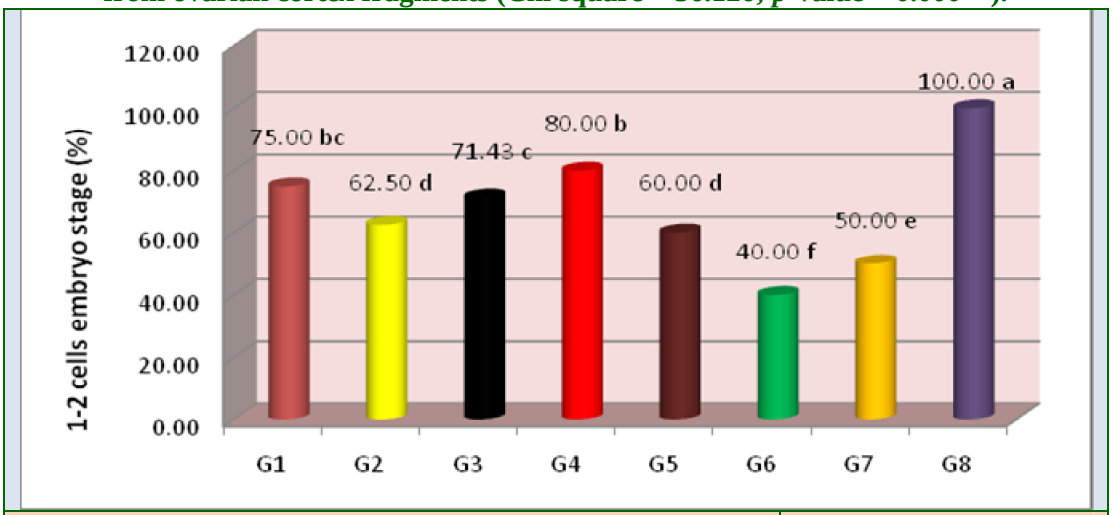
In regard to 2-cell embryo stage, higher percentage was recorded in G8 when using rapid cryopreservation as compared to the control and other groups. In contrast using Glycerol and mannitol: G5 had the lowest significantly (p<0.05) percentage of 2-cell embryo stage. Non significant differences for 2-cell embryo stage percentage were assessed between G1 and G3 groups. Similar result was achieved between G1 and G4 groups. Finally, there was non-significant difference between G2 and G5 groups (Fig. 2).

Fig. 1: Effect of cryopreservation technique and cryoprotectants on IVF (in percentage) from ovarian cortex fragments (Chi square= 32.222; *p*-value= 0.000).**



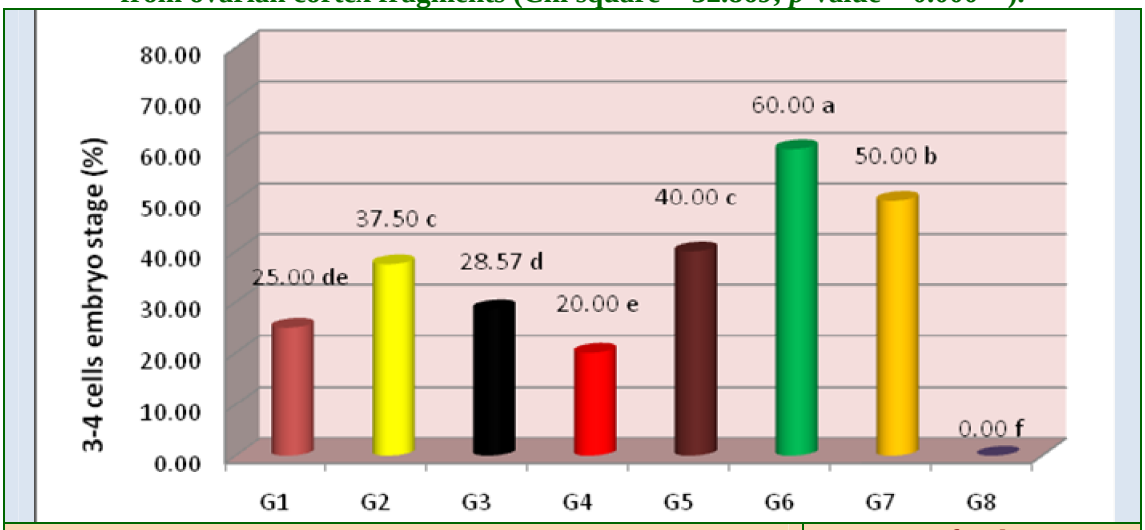
Treatments		No. of oocytes
G1: Control		24
Ultra rapid (vitrification)	G2: Ethylene glycol + sucrose	8
	G3: Ethylene glycol + mannitol	7
	G4: glycerol+ sucrose	5
	G5: Glycerol + mannitol	5
	G6: Propanediol + sucrose	5
	G7: Propanediol + mannitol	4
G8: Rapid cryopreservation		3

Fig. 2: Effect of cryopreservation technique and cryoprotectants on 2-cell embryo stage (in percentage) from ovarian cortex fragments (Chi square = 36.126; *p*-value = 0.000).**



Treatments		No. of embryo
G1: Control		18
Ultra rapid (vitrification)	G2: Ethylene glycol + sucrose	5
	G3: Ethylene glycol + mannitol	5
	G4: glycerol+ sucrose	4
	G5: Glycerol + mannitol	3
	G6: Propanediol + sucrose	2
	G7: Propanediol + mannitol	2
G8: Rapid cryopreservation		3

Fig. 3: Effect of cryopreservation technique and cryoprotectants on 3-4 cell embryo stage (in percentage) from ovarian cortex fragments (Chi square = 32.869; *p*-value = 0.000).**



Treatments		No. of embryo
G1: Control		6
Ultra rapid (vitrification)	G2: Ethylene glycol + sucrose	3
	G3: Ethylene glycol + mannitol	2
	G4: glycerol+ sucrose	1
	G5: Glycerol + mannitol	2
	G6: Propanediol + sucrose	3
	G7: Propanediol + mannitol	2
G8: Rapid cryopreservation		0

Fig. 3 shows the results of 3-4 cell embryo stage percent when using different cryopreservation techniques and cryoprotectants. Highly significant increment was observed ($p<0.01$) for the percentage of 3-4 cell embryo stage was observed for G6 as compared to the control group and the other groups. However, significant reduction ($p<0.05$) was noticed for G8 group when using rapid cryopreservation (it was zero). Non significant differences were assessed for 3-4 cell embryo stage percent between G1 and G3, so between G4 and G4.

From the results of the present study it was concluded that the vitrification technique with cryoprotectants EG and PrOH supplemented with sucrose recorded improvement in the ability of oocytes for IVF and early embryonic development post-xenotransplantation.

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