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Using Of Simple And Novel Vitrification Tool For Sheep Oocytes And Embryos Cryopreservation.

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ABSTRACT

Thepresent study was aimed to cryopreserve mature, immature oocytes and *in vitro* produced embryos in Iraqi sheep through Ultra-rapid cryopreservation (vitrification technique) using local, simple, cost effective and novel vitrification tool. This tool was modified straws named Vitricareinvented, designed and used for the first time. Immature oocytes were aspirated from ovaries of slaughtered ewes and subjected into *in vitro* maturationand *in vitro* fertilization programs .The mature, immature oocytes and embryos were vitrified, then thawed and assessed for the morphology and viability at two periods: post thawing and 2hours post thawing.The results observed non-significant effect ($P>0.05$) for time in the viability and normal morphology of vitrified immature and mature oocytes for post-thawing and 2 hours post-thawing. Highly significant differences ($P<0.01$)were found in the viability of 1 cell embryo post-thawing and two hours post-thawing which were 72.22 % and 55.56 % , respectively, while no significant difference in the normal morphology at two periods. The results observed significant reduction ($P<0.05$)in the viability and normal morphology of 2 cell embryo for the time post-thawing and two hours post-thawing which were 71.43 %, 64.29 % for viability and 78.57 %, 71.43 % for morphology, respectively.The results showed significant differences($P<0.05$)in the post-thawing viability and normal morphology among immatureoocytes, mature oocytes, 1 cell and 2 cell embryos which were 84.47 %, 83.61 % , 72.22 % and 71.43%, for viability and 86.41 % , 88.52%, 72.22% and 78.57% for normal morphology,respectively. It was concluded from this study, successful vitrification of oocytes and embryos using this novel, simple and cost effective vitrification tools involving Vitricare.

Keywords: vitrification, viability, oocytes, embryos, sheep. The research is a part of Ph.D Dissertation to the first author.

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INTRODUCTION

Cryopreservation of mammalian oocytes and embryos are a mainbranch of assisted reproductive techniques (ARTs) because it allows for using of these embryos in the next future.When there are supernumeraryof embryos or an embryo transfer cannot be achieved,cryopreservation is needed (Herring, 2008). There are two main techniques were commonly used for oocytes and embryos cryopreservation, called slow freezing (SF) and ultra-rapid cryopreservation(vitrification) (Kuwayama *et al*., 2005b).

Cryopreservation of oocytes or embryos using slow freezing technique has a main disadvantages, these are includes the need for acostly programmable freezing equipment and the procedure of work take long period. Some studies have been observed that slow freezing method results in low survival rates and low implantation rates, and can cause spindle abnormalities (Oktay and Bang, 2006 and Bromfield *et al*., 2009). Thus, vitrification techniqueof embryos and oocytes may introduce a solution for this problem (Kuwayama *et al*., 2005a).

Vitrification is anewmethodof cryopreservation uses high cooling rate, avoiding the useof programmable freezing instruments. Moreover, the vitrification technique uses a high concentration of cryoprotectants agents (CPAs)which avoids water precipitation and eliminating the formation of ice crystals (Pereira and Marques, 2008).Therefore, the purpose of this study was to cryopreserve oocytes and embryos using novel, simple and cost effective vitrification tool.

MATERIALS AND METHODS

Collection of ovaries and oocytes

From all visible follicles with 2-8 mm diameteron the ovarian surface, oocytes were collected using aspiration method. Oocytes with follicular fluid were aspirated using 23-gauge hypodermic needle attached with a sterile disposable 5 mL syringe contain 0.5 ml of culture medium supplemented with 20 IU/ml heparin (Pan pharma Co. Egypt) to prevent clotting in follicular fluid. After oocyte retrieval, contents of each syringe were placed inside petri dish containingoocytes under dissecting microscope, the oocytes were collected using micropipette and washed for three times using RPMI-1640 culture medium (Sigma , Germany)(DeSmedt*et al*., 1992).

In vitro maturation of oocytes

Oocytes were washed three times in RBMI-1640 culture medium containing 5% BSA (BDH, England), and then 5-7 immature oocytes were directlyplaced in overnight incubated droplet (0.5mL) of culture medium.Maturation medium was supplemented with 10 IU/mL hCG (Intervet , Holland), 5 IU/mL eCG (Intervet , Holland),10 µl/ml penicillin - streptomycin antibiotic (Thermo scientific , Denmark) and cultured in four well Petri dish , covered with paraffin oil and incubated for 24 h in CO_2 incubator (5% CO_2) at 38.5°C with high humidity (95%) (De Feliciand Siracusa, 1982).

Sperm preparation of for *in vitro* fertilization

The testis from slaughtered adult rams (age equal to one year and above) were collected directly after slaughtering, placed in a thermosand transported to the laboratory and used as source for sperm. The tunica albuginea was removed and the testis was washed thoroughly with warmed (37 °C) PBS (Phosphate Buffered Saline). The cauda were cleaned with 70% ethanol. Then the cauda was incised deeply with blade and the gushing fluid, rich in sperms was flushed into a Petri dish containing RPMI medium for sperm washing.

Sperm were prepared according to technique byDeSmedt*et al* (1992) 1mL of RPMI medium added to 1 mL of collectedsperm then centrifuged at 700RPM for 7 minutes at room temperature (25^{o}C) for two times. Thesupernatant was discarded and add 1 ml of culture medium to the pellet. Thirty min later at 37^{o}C, sperms toned swim to the upper layer and aspirated the top 0.5 mL containing the sperm with high activity and used for *in vitro* fertilization.

In vitro fertilization

The mature acolytes were washed twice in fertilization medium and transported to 4-well culture plates containing 0.5 mL of the RPMI-1460. The motile spermatozoa were added to the acolytes at the concentration of approximately 5×10^4 sperm/oocyte. Culture medium containing sperm and acolytes was covered with paraffin oil and incubated at 38.5 °C in 5 % CO_2 incubator with high humidity (95%) for 24h.

Viability test:

All oocytes were examined for viability using the trypan blue (HI media , India) exclusion test. Unstained oocytes were classified as live and fully stained oocytes as dead.The viability test was done post-aspiration and immediately post-thawing(Abd-Allah, 2010).

Vitrification and thawing solution

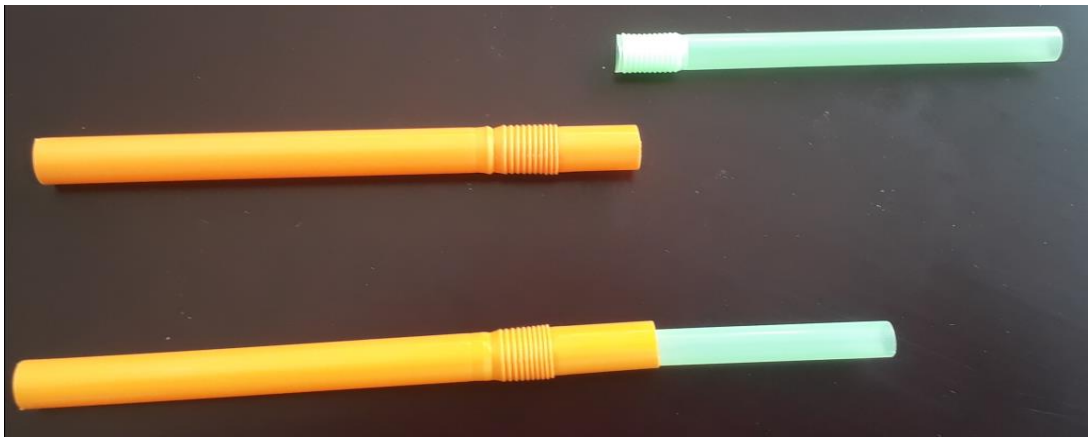

Plate 1: Vitrification tool: Vitripeace. A: Straw, B: Straw cover, C: Straw inside the cover after loading.

The equilibration solutions (ES) consisted of 15% (v/v) dimethyl sulphoxide (DMSO) (Scharlau, Spain.)with 15% (v/v) ethylene glycol(EG) (Scharlau, Spain) were prepared by adding the corresponding volume of CPA to culture medium containing 10% BSA.Vitrification solutions (VS) consisting of 30% (v/v) DMSO with 30% (v/v) EG which were added to culture medium supplemented with 10% BSA. For thawing,

Vitrification and thawing techniques

The vitrification and warming procedures were performedaccording to (Al-Hasani*et al.,* 2007). Normal and viable acolytes (immature and mature) or embryos weretransferred to 0.5 mL of the vitrification solution 1 (VS1) at room temperature to equilibrate for 15 min for acolytes and 8 min for embryos. Then after, acolytes or embryos were placed into 0.5 mLof vitrification solution 2 (VS2) for 1 min. Then the acolytes orembryos loaded on the straw strip and directly immersed into LN_2. Then, the strip was covered with the plastic tube in LN_2 to protect it during storage.

For thawing, the straws weretaken out from the LN_2 after two months and immersed inthawing solution 1 (TS1) at37 ºC for 1 min. Then acolytes and embryos were transferred into thawing solution 2 (TS2) at room temperature for 3 min and thawing solution 3 (TS3)at room temperature for 3 min,and then washed twice with RBMI 1640 medium.

Statistical analysis

The Statistical Analysis System- SAS (10) used to compare between studied groups in different traits. Chi-square test was used to compare the significant differencesbetween different percentages.

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RESULTS

The results indicated that no significant decrease (P<0.05) in the viability (%) of immature and mature acolytes for the time post-thawing and two hours post-thawing which were 84.47 % (87/103), 81.55 % (84/103) for immature and 83.61 % (51/61) , 81.97 % (50/103) for mature acolytes respectively (Figure1). On the other hand, the results observed no significant effect (P<0.05) in the normal morphology (%) of immature and mature acolytes for the time post-thawing and two hours post-thawing which were 86.41 % (89/103), 83.50 % (86/103) for immature and 88.52 % (54/61), 85.25 % (52/61) for mature acolytes respectively (Figure 2).

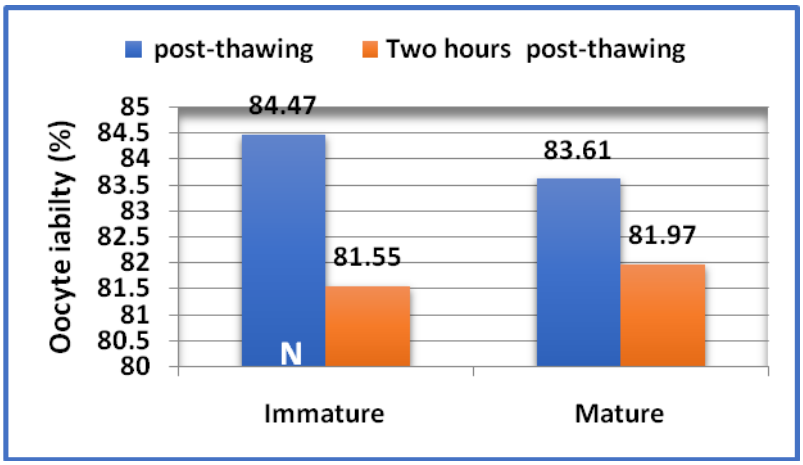

Figure1: Effect oftime post-thawing and 2 hours post-thawing on the viability (%) of sheep immature and mature oocytesusing Vitricare tool.

NS: Non-Significant

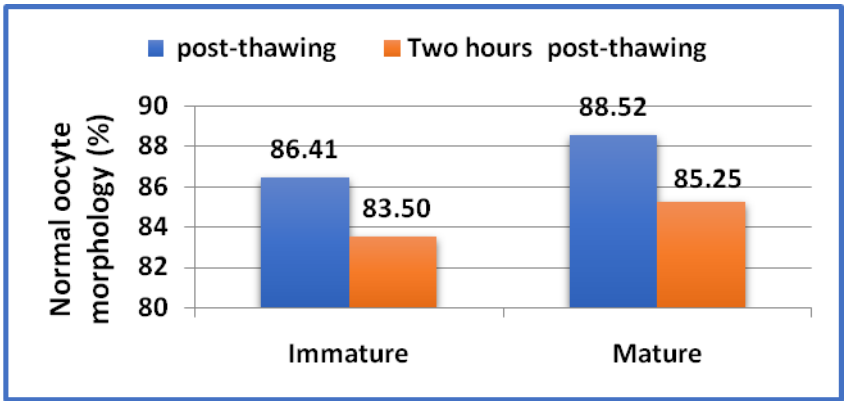

Figure2: Effect time post-thawing and 2 hours post-thawing on the normai morphology (%)of sheep immature and mature oocytesusing Vitricare tool.

NS: Non-Significant

The results noticed highly significant differences (P<0.01)in the viability (%) of 1 cell embryo post-thawing and two hours post-thawing which was 72.22 % (13/18) and 55.56 % (10/18) respectively (Figure 3). Also the results observed significant reduction (P<0.05)in the viability (%) for the time post-thawing and two hours post-thawing of 2 cell embryo which was 71.43 % (10/14) and 64.29 % (9/14) respectively.No significant decrease in the normal morphology (%) of 1 cell embryo which was 72.22 % (13/18) and 66.67 % (12/18)post-thawing and two hours post-thawing respectively(Figure 4).It was found significant difference (P<0.05)in the normal morphology of 2 cell embryo which was 78.57 % (11/14) and 71.43 % (10/14)post-thawing and two hours post-thawing respectively(Figure 4).

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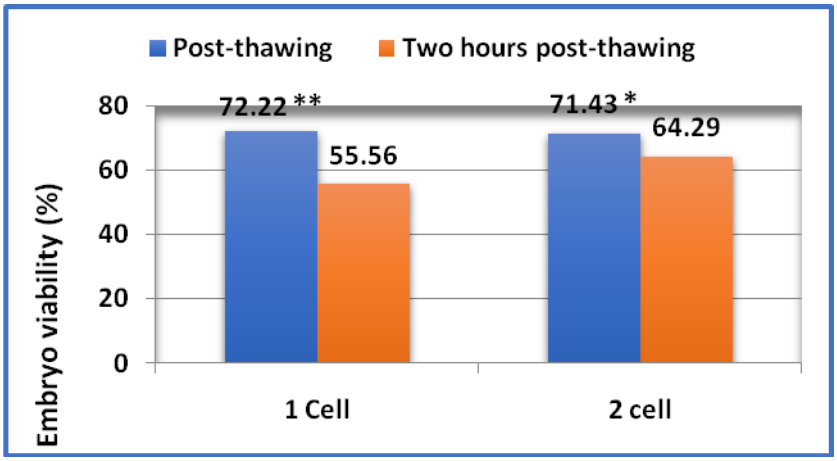

Figure 3: Effect time post-thawing and 2 hours post -thawing on the viability (%) of sheep 1 cell and 2 cell embryosusing Vitricare tool. (P<0.01),* (P<0.05).**

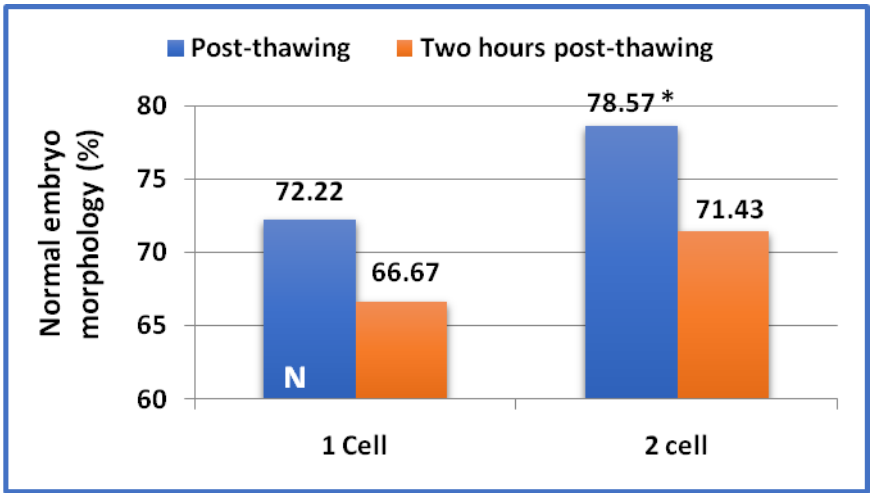

Figure4: Effect time post-thawing and 2 hours post -thawing on the normal morphology (%) of sheep 1 cell and 2 cell embryosusing Vitricare tool.

NS: Non-Significant,* (P<0.05).

The comparison between vitrified immature,mature,1 cell and 2 cell embryos in the viability and morphology post-thawing and 2 hours post-thawing were illustrated in table (1).The results revealed significant differences (P<0.05)in the viability among stages at post-thawing and also highly significant differences (P<0.01) in the viability were found at two hours post-thawing which were 81.55 % , 81.97 % , 55.56 % and 64.29 % for immature and mature acolytes, 1 cell and 2 cell embryos, respectively.

The results observed significant differences(P<0.05)in the normal morphology at post-thawing which were 86.41 % , 88.52 % , 72.22 % and 78.57 %for immature, mature, 1 cell and 2 cell embryo, respectively. Regarding the normal morphology at two hours post-thawing, the results were found highly significantdifferences (P<0.01) among stages were 83.50 %, 85.25 %, 66.67 % and 71.43 % for immature, mature, 1 cell and 2 cell embryo respectively (Table1)

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Table 1: Comparison between acolytes and embryos in the viability and morphology.

Type of cells	Viable (%)		Normal morphology (%)	
	Post thawing	2hr.post-thawing	Post thawing	2hr.post-thawing
Immature	84.47	81.55	86.41	83.50
Mature	83.61	81.97	88.52	85.25
1 cell embryo	72.22	55.56	72.22	66.67
2 cell embryo	71.43	64.29	78.57	71.43
Chi-square value	0.0744 *	0.0056 **	0.0298 *	0.0084 **
* (P<0.05), **(P<0.01),				

DISCUSSION

Several tools have been used for mammalian acolytes and embryos vitrification, some of these tools involve open-pulled straw (Vajta *et al*. 1997), hemi-straw (Vanderzwalmen *et al*., 2000), cryoloop (Yeoman *et al*., 2001), Cryotop (Kuwayama, 2007), solid-surface microdrop (Begin *et al*., 2003), Stripper Tip (Walker *et al*., 2004), and grid (Park *et al*., 1999). Most of these vitrification tools have rate of deletion and most costly, while in this study no any deletion in vitrified acolytes and embryos as well as cheap and easy to use tools.

The current study revealed that higher percentage of viability and normal morphology of vitrified immature and mature acolytes were yielded post-thawing and 2hr post-thawing as a result of several factors including presence of cumulus cell, optimal size of acolytes and components of culture media

Results of the current study showed non-significant effect in the viability and normal morphology of immature and mature acolytes for time post-thawing and 2 hours post-thawing. These results represent a good indicator of morphology and survival rate post vitrification-thawing process using Vitricare tool.

UsingCryotop with 15% of DMSO+EG +0.5 M sucrose,survival rate of 91.8% and 89.7% were reported forvitrified mature acolytes in bovine and human respectively (Chian*et al*., 2004 and Ubaldi*et al*., 2010). Zhou *et al*(2010) has vitrifiedcumulus-enclosed and partially-denuded GVbovine acolytes in 15% EG+15% DMSO+0.5M sucrose in two steps and reported a survival rate of 93.8% and 81.3%, respectively. Nedambale*et al* (2006)reported a survival rate of 82% for bovine oocytesvitrified with 35% EG+0.4 M trehalose+5% PVP, Dike (2009) reported a survival rate with 5.5 M EG +1 M sucrose (89.8%).

Significant differences were found in the viability (%) and normal morphology of 1 cell and 2 cell embryos in the time post-thawing and 2 hours post-thawing using Vitripeace. This differences in the viability and normal morphology observed in the present study can be attributed to several factors, such as thevitrification cryodevices, utilization of different cryoprotectants, using embryos in different developmental stages, keeping the embryos in equilibration and vitrification solutions for different time periods during the vitrification process, and the difference of the volume of the vitrification solution that the embryos were held in during the transfer of the embryos to the straws (Kose and Tekeli, 2016).

The vitrification protocol of the current study ensured a rapid thawing rate of vitrified embryos by directly placing the vitrified drop into sucrose solution at 37 ˚C .Therefore, we achieved high viabilityand morphology of the vitrified embryos, and this agrees with reported by Ghorbani *et al* (2012).It was concluded from this study, successful vitrification of acolytes and embryos using this novelty, simple and cost effective vitrification tool involving Vitricare.

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