CRYOPRESERVATION OF SHEEP OOCYTES AND EMBRYOS USING LOCAL, MODIFIEID VITRIFICATION TOOL*

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ABSTRACT

The present study was aimed to cryopreserve mature, immature oocytes and *in vitro* produced embryos in Iraqi sheep using vitrification technique by local, simple and cost effective vitrification tool. This tool is an innovative straw called vitripeace invented, designed and used for the first time. Immature oocytes were aspirated from ovaries of slaughtered ewes and subjected to *in vitro* maturation and *in vitro* fertilization programs.The immature, mature oocytes and embryos were vitrified using Vitripeace tools, then thawed and assessed for the morphology and viability. The results revealed non-significant effect of time on viability (%) and normal morphology (%) of vitrified immature and mature oocytes for post-thawing and 2 hours post-thawing. The results showed significant (P<0.05) reduction in the viability (%) of 2 cell embryo namely 88.89% and 77.78 % for post-thawing and two hours post-thawing respectively. The results revealed a significant (P<0.05) reduction on normal morphology of 1 cell embryo namely 88.24 % and 76.47 % for post-thawing and two hours post-thawing respectively. Significant (P<0.05) differences in the percentage of normal morphology were found at post-thawing period for all stages of embryo development which were 90.74%, 88.31, 88.24 and 83.33 for immature , mature oocytes, 1 cell and 2 cell embryos, respectively while no significant differences in the viability at post-thawing period among all stages of embryo development. It was concluded that, successful vitrification of oocytes and embryos was resulted using Vitripeace which was novel, simple and cost effective vitrification tool.

Key words : vitrification, viability, oocytes, embryos, sheep.

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الحفظ بالتجميد لبويضات وأجنة الاغنام باستعمال أداة تزجيج محلية محورة

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المستخلص

هدفت الدراسة الحالية الى تجميد البويضات الناضجة وغير الناضجة والاجنة المنتجة مختبريا في الاغنام العراقية بطريقة التزجيج باستعمال أداة تزجيج محلية وبسيطة وغير مكلفة اقتصادياً. هذه الأداة عبارة عن قصبة سميت Vitripeace تم اختراعها وتصميمها واستخدامها لأول مرة. جمعت البويضات غير الناضجة من مبايض الاغنام المذبوحة في المجزرة وخضعت لبرنامجي الإنضاج ألمختبري والإخصاب الخارجي. البويضات غير الناضجة والناضجة والأجنة الناتجة جمدت باستخدام تقانة التزجيج وبعدها أذيبت وقيمت على الشكلياء والحيوية. أوضحت النتائج عدم وجود اختلافات معنوية في نسبة الحيوية والشكلياء للبويضات غير الناضجة والناضجة عند وقت الاذابة وبعد الاذابة بساعتين. أظهرت النتائج انخفاضا معنويا (P<0.05) في الحيوية (%) عند الاذابة وبعد ساعتين من الاذابة لمرحلة 2 خلية للأجنة حيث بلغت 88.89% و 77.78% على التوالي. أظهرت النتائج انخفاضا معنويا (P<0.05) في الشكلياء (%) عند الاذابة وبعد ساعتين من الاذابة لمرحلة 1 خلية للأجنة حيث بلغت 76.47% و77.78% على التوالي. بينت النتائج وجود اختلافات معنوية (P<0.05) في الشكل الطبيعي (%) عند الاذابة بين البويضات غير الناضجة والناضجة ومراحل 1 خلية و 2 خلية للأجنة عند استخدام اداة التزجيج Vitripeace حيث بلغت 90.74% و88.31% و88.24% و83.33% على التوالي في حين لم توجد فروقات في نسبة الحيوية لنفس الاداة ولجميع المراحل. يمكن الاستنتاج من هذه الدراسة نجاح تزجيج البويضات والاجنة باستخدام هذه الاداة البسيطة والمبتكرة وغير المكلفة اقتصاديا.

كلمات مفتاحية: تزجيج، حيوية، بويضات، اجنة، اغنام

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INTRODUCTION

Cryopreservation of mammalian oocytes and embryos are a main branch of Assisted Reproductive Techniques (ARTs) because it allows for using of these embryos in the next future. When there are supernumerary of embryos or an embryo transfer cannot be achieved, cryopreservation is needed (1).There are two main techniques were commonly used for oocytes and embryos cryopreservation, called slow freezing (SF) and ultra-rapid cryopreservation (vitrification). Cryopreservation of oocytes or embryos using slow freezing technique has a main disadvantages, these are including the need for a costly 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 (2, 3). Thus, vitrification technique of embryos and oocytes may introduce a solution for this problem (4). Vitrification is a new method of cryopreservation uses high cooling rate, avoiding the use of 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 (5).Therefore, the purpose of this study was to cryopreserve oocytes and embryos using novel, simple and cost effective vitrification tool which called Vitripeace

MATERIAL AND METHODS

Collection of ovaries and oocytes

From all visible follicles with 2-8 mm diameter on the ovary 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 containing oocytes under dissecting microscope, the oocytes were collected using micropipette and washed for three times using RPMI-1640 culture medium (Sigma , Germany) (6).

In vitro maturation of oocytes

Oocytes were washed three times in RPMI-1640 culture medium containing 5% BSA (BDH, England), and then 5-7 immature oocytes were directly placed 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%) (7).

Sperm preparation 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 thermos and 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) Phosphate Buffered Saline (PBS). 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 of DeSmedt *et al* (6) 1mL of RPMI medium added to 1 mL of collected sperm then centrifuged at 700 RPM for 7 minutes at room temperature (25^{o}C) for two times. The supernatant was discarded and add 1 ml of culture medium to the pellet. After 30 min 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 oocytes 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 oocytes at the concentration of approximately 5×10^4 sperm/oocyte. Culture medium containing sperm and oocytes was covered with paraffin oil and incubated at 38.5 °C in 5 % CO_2 incubator with high humidity (95%) for 24h.

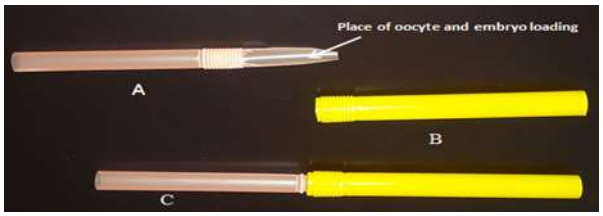
Viability test

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 immediately post-thawing and 2 hrs. Post-thawing (8).

Vitrification and thawing solution

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.



Picture1.Vitrification tool: Vitripeace. A: Straw, B: Straw cover, C: Straw inside the cover after loading.

Vitrification and thawing techniques

The vitrification and thawing procedures were performed according to (9). Normal and viable oocytes (immature and mature) or embryos were transferred to 0.5 mL of the vitrification solution 1 (VS1) at room temperature to equilibrate for 15 min for oocytes and 8 min for emb ryos. Then after, oocytes or embryos were placed into 0.5 mL of vitrification solution 2 (VS2) for 1 min. Then the oocytes or embryos loaded on the straw (Picture1) 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 were taken out from the LN_2 after two months and immersed in thawing solution 1 (TS1) at 37 ºC for 1 min. Then oocytes 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 RPMI 1640 medium.

Statistical analysis

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

RESULTS AND DISCUSSION

Results of the current study showed no significant (P<0.05) differences in the oocyte viability (%) for the time post- thawing and two hours post- thawing which were 89.81% (97/108), 85.19 % (92/108) for immature oocytes and 88.31% (68/77) , 84.42 % (65/77) for mature oocytes respectively (Figure1). Regarding normal oocyte morphology (%), results observed non-significant (P<0.05) effect for the time post- thawing and two hours post- thawing in the normal morphology of immature and mature oocytes which were 90.74% (98/108), 85.19 % (92/108) for immature oocytes and 88.31% (68/77), 85.71 % (66/77) for mature oocytes respectively (Figure 2).

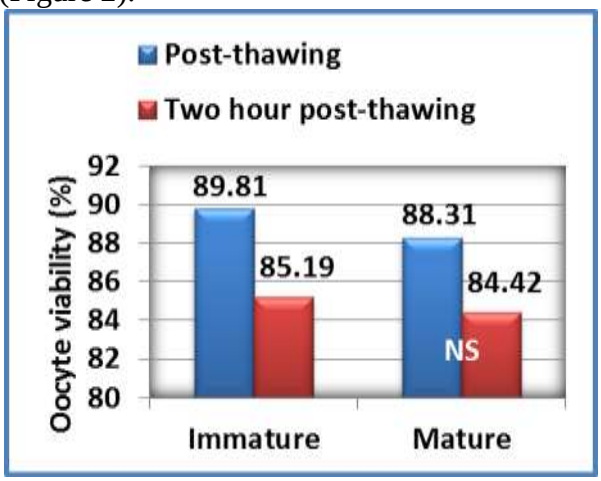


Figure1. Effect of vitrification on the viability of immature and mature oocytes in the time post-thawing and 2 hrs. post-thawing using Vitripeace tool. NS: Non significant

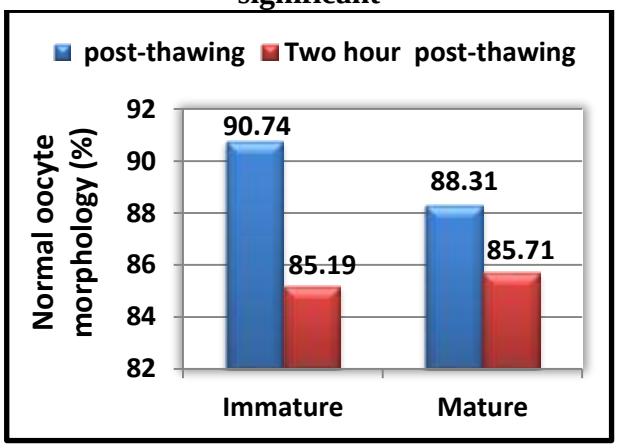


Figure 2. Effect of vitrification on the normal morphology of immature and mature oocytes in the time post-thawing and 2 hrs. post-thawing using Vitripeace tool. NS: Non significant

It was found that no significant differences in the viability of 1 cell embryo which was 88.24% (15/17) and 82.35 % (14/17) post-thawing and two hours post-thaw respectively (Figure 3). The present results observed significant (P<0.05) reduction in the viability (%) of 2 cell embryo which were 88.89% (16/18) and 77.78 % (14/18) post-thawing and two hours post-thawing respectively (Figure 4). On the other hand, the results revealed significant (P<0.05) reduction in the normal morphology of 1 cell embryo for the time which was 88.24 % (15/17) and 76.47 % (13/17) post-thawing and two hours post-thawing respectively. No significant effect was found for time in the normal morphology of 2 cell embryo which was 83.33 % (15/18) and 77.78 % (14/18) post-thawing and two hours post-thawing respectively.

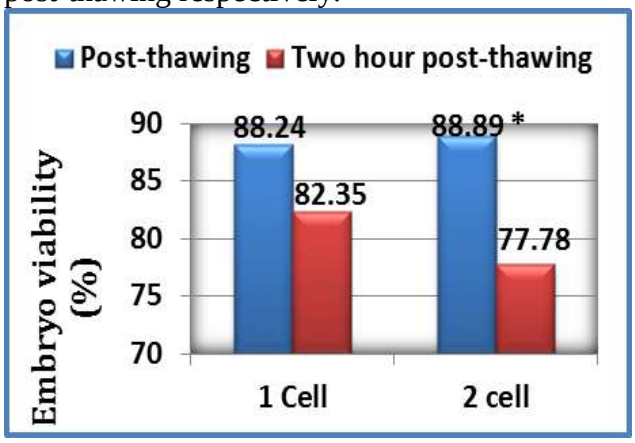


Figure 3. Effect of vitrification on the viability of 1 cell and 2 cell embryos in the time post-thawing and 2 hrs. post -thawing on using Vitripeace tool
*** (P<0.05), NS: Non-Significant.**

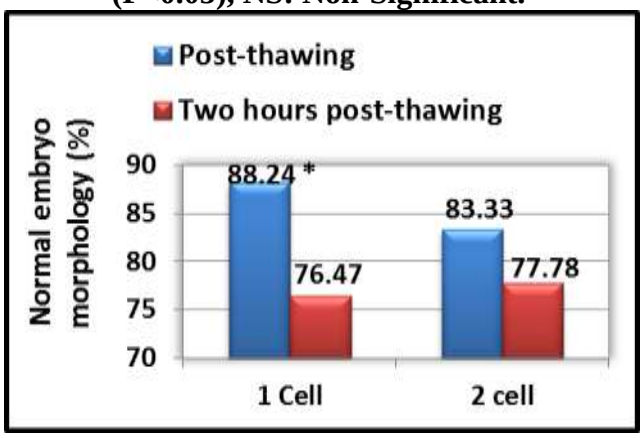


Figure 4. : Effect of vitrification on the normal morphology of 1 cell and 2 cell embryos in the time post-thawing and 2 hrs. post -thawing using Vitripeace tool.
*** (P<0.05), NS: Non-Significant**

Comparison between vitrified oocytes and embryos in the viability (%) and morphology (%)

Results of the present study noticed no significant (P>0.05) differences between vitrified immature and mature oocytes, 1 cell and 2cell embryos in the viability (%) post-thawing which were 89.81 %, 83.31 %, 88.24 %, 88.89% for immature, mature, 1 cell and 2cell embryos respectively (Table 1). While there was significant (P<0.05) differences in the viability at two hours post-thawing among stages above which were 85.19 % ,84.42 % and 82.35 % and 77.78 % for immature oocytes, mature oocytes, 1 cell and 2 cell embryo respectively. =Results obtain in Table 1 reveal significant differences (P<0.05) in the normal morphology (%) at post-thawing which were 90.74 %, 88.31 % , 88.24 % and 83.33 % for immature oocytes, mature oocytes, 1 cell and 2 cell embryo respectively. The results were also recorded a significant (P<0.05) differences among the stages in the percentages of normal morphology at two hours post-thawing which were 85.19 %, 85.71 % , 76.47% and 77.78 % for immature oocytes, mature oocytes, 1 cell and 2 cell embryo respectively (table 1).

Table 1. Comparison between vitrified oocytes and embryos in the viability (%) and morphology (%).

Type of cells	Viable (%)		Normal morphology (%)	
	Post thawing	2hr.post-thawing	Post thawing	2hr.post-thawing
Immature	89.81	85.19	90.74	85.19
Mature	88.31	84.42	88.31	85.71
1 cell embryo	88.24	82.35	88.24	76.47
2 cell embryo	88.89	77.78	83.33	77.78
Chi-square value	0.894 NS	0.046 *	0.049 *	0.045 *

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

The current study revealed high percentage of viability and normal morphology of vitrified immature and mature oocytes, 1 cell and 2 cell embryos post-thawing and 2hr post-thawing using Vitripeace tool. Results of the present study showed non-significant effect in the viability and normal morphology of immature and mature oocytes for time post-thawing and 2 hours post-thawing. This result represents a good indicator of morphology and survival

rate post vitrification-thawing process. The results of the present study suggest that immature cumulus compact sheep oocytes process more tolerability cryopreservation using vitrification technique. Our results agreed with Purohit *et al* (11). Another advantage of using Vitripeace tool is the clear observation at the time of loading the sample (oocytes or embryos) under microscope and also withdrawing the excessive volume of vitrification media on the tool which lead to direct touch with liquid nitrogen and sample and therefore reaching to better heat transfer which can lead to decreasing the damage crossing the critical zone at lesser time. 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 viability and morphology of the vitrified embryos, and this is agrees with reported by Ghorbani *et al* (12). Results of the present study were similar to those reported by Fakhrildin and Al-Moussawi (13), they mentioned that percentage of viability and normal morphology of vitrified sheep oocytes using Cryotop was 91.01±3.57.By using Cryotop with 15% of DMSO+EG +0.5 M sucrose, survival rate of 91.8% and 89.7% were reported for vitrified mature oocytes in bovine and human respectively (14, 15). Zhou *et al* (16) 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 *et al*. (17) reported a survival rate of 82% for bovine oocytes vitrified with 35% EG+0.4 M trehalose+5% PVP. Dike (18) reported a survival rate with 5.5 M EG +1 M sucrose (89.8%),whereas Fujiwara *et al* (19) 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.

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