Effect of vitrification on the morphology and viability of early embryos of sheep

Ali.A. Al-sadoon* Mohammed- B.M-R. Fakhrildin Mohamad A.Ishak *****
University of Al-Muthanna / college of Agriculture*
University of al-Nahrain / High Institute of Infertility Diagnosis and Assisted Reproductive Technology**
University of Baghdad / college of Agriculture***

Abstract:

This experiments was conducted in the laboratory of High Institute of Infertility Diagnosis and Assisted Reproductive Technologies in Bagdad, to investigate the effects of ultra rapid cryopreservation (vitrification) on the normal morphology and viability of ewes early stage embryos . The sheep ovaries were collected from the slaughters ewes immediately after slaughtering ,the immature oocytes were subjected into *in vitro* maturation(IVM) and *in vitro* fertilization(IVF) programs .The early embryonic stages were cryopreserved using the vitrification technique, then thawed and assessed from the morphology and viability at two periods : post thawing and 2hr.post thawing. The morphology and viability of early embryonic stages, showed no significant differences in two pronuclear (2PN) at post thawing and 2hr.post thawing periods namely 88.88 and 83.33% respectively. While the 1-2cell showed a higher percentage of normal morphology (93.33%) at the post thawing and 2hr.post thawing periods,also showed a high percentage of normal morphology 3- 4cell embryos at post-thawing and 2hr.post-thawing (86.66%). A high percentage of viable 3-4 cells embryos were obtained at post-thawing (93.33%),which were decreased to 86.67% at 2hr.post-thawing .These results described for the first time in Iraq, using the vitrification technique with 2PN,1-2cells,3-4cells embryonic stages. This will participates in improving the reproductive efficiency of sheep via producing large numbers of embryos which could be used for application of reproductive technologies .

The research is a part of PhD Dissertation to the first author.
Key words : vitrification, morphology, viability, embryos, sheep.

تأثير التزجيج في شكلياء وحيوية الأجنة المبكرة للأغنام

علي عبدالله زعيري االسعدون* محمد باقر محمد رشاد فخرالدين ** محمد علي اسحق ***

جامعة المثنى/كلية الزراعة * جامعة بغداد / كلية الزراعة***

جامعة النهرين/المعهد العالي لتشخيص العقم والتقنيات المساعدة على الإنجاب****

المستخلص

أجريت هذه الدراسة في مختبرات المعهد العالي لتشخيص العقم والتقنيات المساعدة على الإنجاب في بغداد لمعرفة تأثير التجميد فائق السرعة (التزجيج) في شكلياء (الشكل المورفولوجي) وحيوية المراحل الجنينية المبكرة

للأغنام. جمعت مبايض الأغنام من النعاج المذبوحة بعد الذبح مباشرة . البويضات الغير ناضجة (المستحصلة من المبايض) أخضعت لبرنامجي الإنضاج ألمختبري والإخصاب الخارجي .الأجنة الناتجة جمدت باستخدام تقانة التزجيج وبعدها أذيبت وقيمت على الشكلياء والحيوية ولفترتين: بعد الإذابة مباشرة وبعدها بساعتين . وأظهرت نتائج التقييم عدم وجود فروق معنوية في حيوية وشكلياء الأجنة في مرحلة (2PN) بين فترتي التقييم .وكانت النسب 88.88% و83.33% على التوالي .فيما أظهرت نتائج تقييم الأجنة في مرحلة (1–2 خلية) وجود نسبة عالية من الأجنة ذات الشكلياء الطبيعية وكانت 93.33% لفترتي التقييم ، كذلك تم الحصول على نسبة عالية من الأجنة ذات الشكلياء الطبيعية للمرحلة (3–4خلية) لفترتي التقييم وكانت النسبة 86.66% ، وتم الحصول على نسبة عالية من الأجنة الحية لهذه المرحلة الجنينية في الفترة بعد الإذابة وكانت 93.33% وانخفضت إلى 86.67% بعد ساعتين من الإذابة . هذه النتائج سجلت لأول مرة في العراق . وهذا يمكن أن يساهم في تحسين الكفاءة التناسلية للأغنام عبر إنتاج وحفظ أعداد كبيرة من الأجنة والتي يمكن أن تستخدم في تطبيق التقاتات التناسلية .

البحث مستل من أطروحة دكتوراه للباحث الأول

الكلمات المفتاحية: التزجيج ، شكلياء ،حيوية، اجنة ، اغنام

Introduction:

Sheep is considered as the most important animals in Iraq, the productivity of Awassi sheep as well as other Iraqi breeds is rather low and likely to cause low efficiency of meat production (7). The sheep industry has not been able to utilize many of the assisted reproductive technologies (ART) in general and artificial insemination(AI) in particular or multiple ovulation and embryo transfer (MOET), that widely adopted into normal breeding programs in dairy cattle (12).

Certainly the IVF protocol requires achievement of the following major steps : oocytes collection from slaughtered or live animals, *in vitro* maturation (IVM) in culture media, preparation of semen for *in vitro* fertilization (IVF) ,*in vitro* development assurance (IVD) and preservation of oocytes and zygote obtained (11). Therefore, the purposes of the present study were as follows:

Application of vitrification technique in the cryopreservation of early embryonic stages in sheep .

Materials and Methods:

***In vitro* production of sheep embryos.**

A normal viable immature oocyte was collected from sheep ovaries and subjected into IVM program in CO_2 incubator 24 hours, washed and tested of maturation under inverted microscope .

The mature oocytes resulted from IVM program were subjected into IVF program using cryo-semen for 24 hours. Next oocytes were washed and immediately assessed for fertilization under inverted microscope .

The IVF program was produced forty five 2pronuclear,fifteen 1-2 call and fifteen 3-4 cell , all this early embryonic stages were vitrified using cryotop devices .After 3 months , all cryopreserved embryos were thawed and assessed after thawing and 2hrs after thawing from morphology and viability.

Vitrification process:

The vitrification and warming procedures were performed according to works were done by (9) and (8). Normal and viable embryos were transferred to 0.5 ml of the equilibrium solution (ES) without cryoprotectant at room temperature for 15 min. Thereafter, they were placed into 0.5 ml of vitrification solutions (VS1,VS2) contain 0.25M and 0.5 M sucrose + ethylene glycol for 1minute. The embryos were loaded on the cryotop strip in microdroplete of VS and then cryotop immersed into liquid nitrogen(LN_2) directly. The strip was covered with the plastic tube in LN_2 to protect it during storage. Three months later, thawing process was carried out. For thawing, the protective cover was removed from the cryotop while it is still submerged in LN_2. Stepwise removal of the cryoprotectant was done by transferring the embryos 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 solution for 3 minute, depending on the original sugar concentration of the vitrification solution, and then washed twice with culture medium.The thawed embryos were considered abnormal according to the morphological characteristics of embryos , embryo recovery rate, embryo survival rate and embryonic development were investigated using trypan-blue assay according to (18).

Results and Discussion :

1. Effect of vitrification on the morphology and viability of 2PN .

The assessment of morphology and viability of 2PN (plate 1) showed no significant difference ($P<0.05$) in the normal morphology at post-thawing and 2hr.post thawing (88.88 and 83.33%respectively) (Figure.1).Similar results were showed in the percentage of viability (83.33 %) at two periods (Figure.2). The results also showed no significant differences in the norma and viable 2PN at post- thawing and 2hr. post- thawing period which were 72.22% and 66.66%; respectively(Figure. 3) .

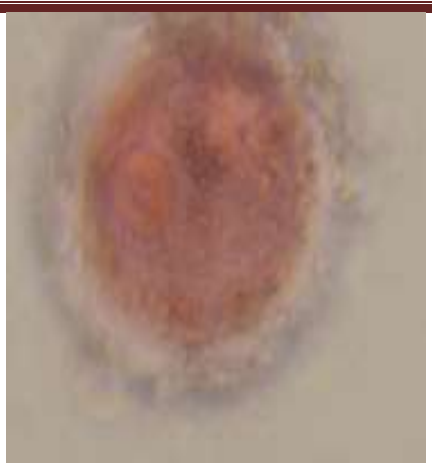

Plate 7.Viable sheep 2PN.

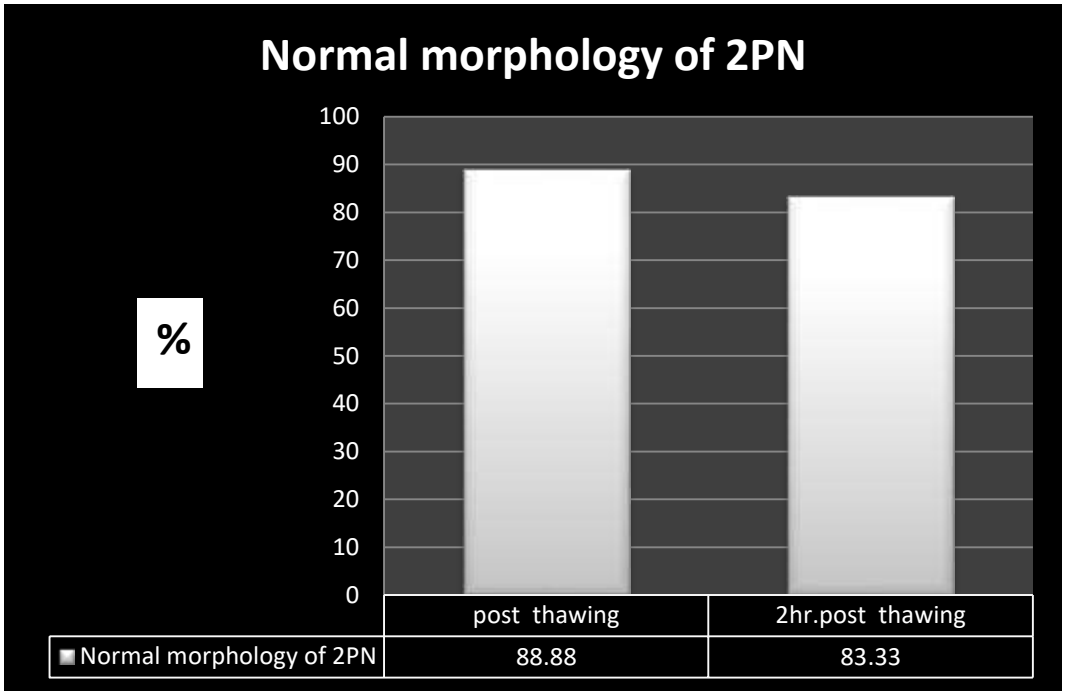

Figure 1: Effect of vitrification on the morphology of sheep 2PN.

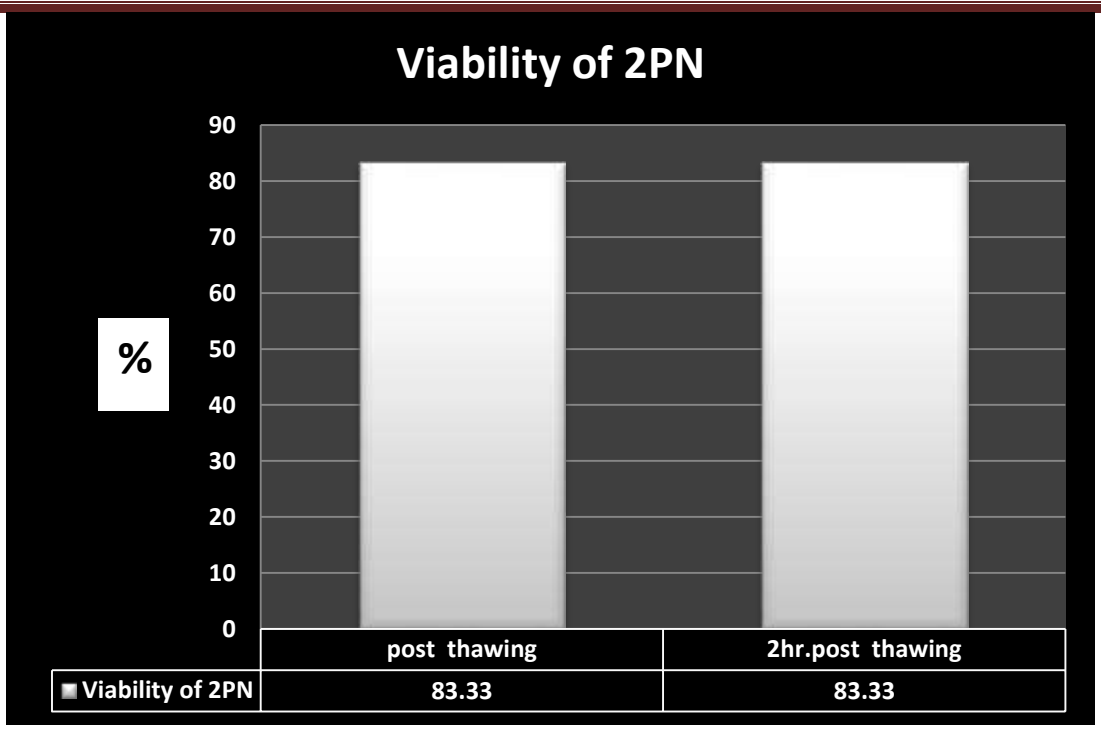

Figure 2: Effect of vitrification on the viability of sheep 2PN.

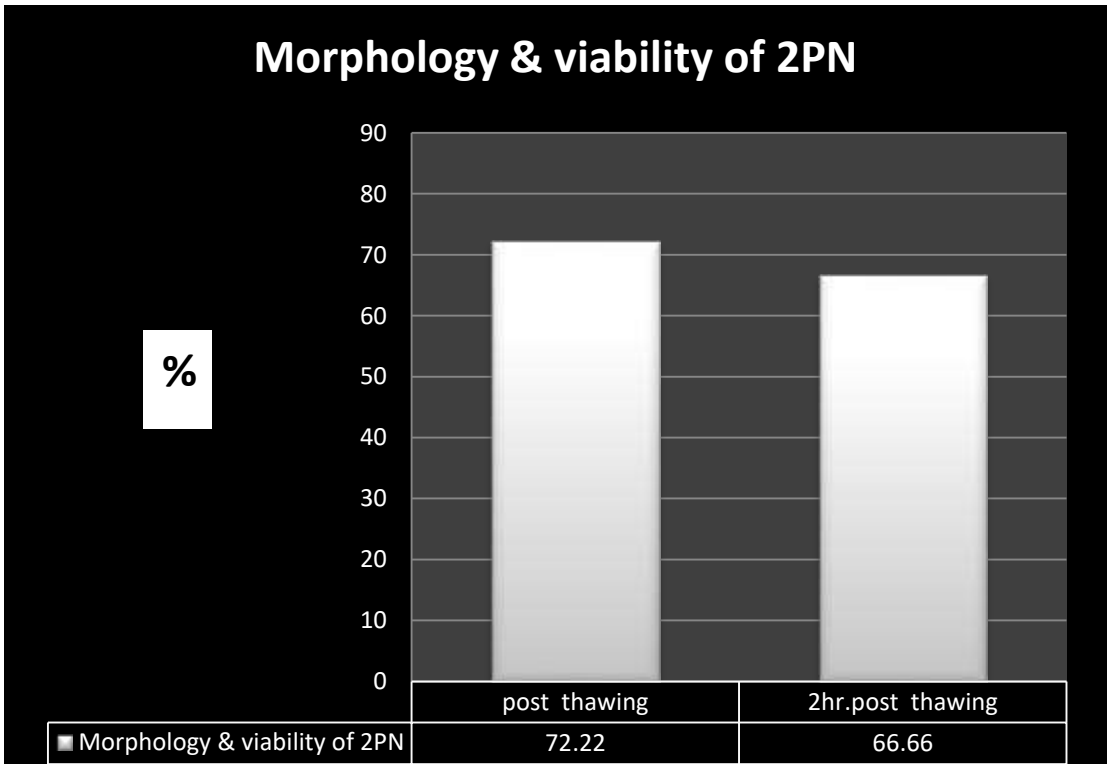

Figure 3:Effect of vitrification on the morphology and viability of sheep 2PN.

2. Effect of vitrification on the morphology and viability of 1-2 cells embryonic stages.

The assessment results of vitrified 1- 2cell morphology and viability, showed a high percentage of normal morphology (93.33%) at post- thawing and 2hr. post- thawing

period .Also the viability percent is high (86.67%) at the two periods. The percentage of normal morphology and viable vitrified 1- 2cells was 80.00% as well as at post-thawing and 2hr. post-thawing period (Table.1) .

Table 1: Effect of vitrification on the morphology and viability of 1- 2cells sheep embryonic stage.

Embryo parameters	Total No.	Post thawing		2hr .post thawing		Chi-square value
		No	%	No	%	
Normal morpholog	15	14	93.33	14	93.33	0.207NS
Viable	15	13	86.67	13	86.67	0.219NS
Normal and viable	15	12	80.00	12	80.00	0.00NS
NS:Non-significant						

3.Effect of vitrification on the morphology and viability of 3-4cell embryos. Results of vitrification showed a high percentage of normal morphology 3- 4cell embryos (plate 2) at post-thawing and 2hr.post-thawing (86.66%). A high percentage of viable 3-4 cells embryos were obtained at post-thawing(93.33%),which were de-creased to 86.67% at 2hr.post-thawing . The normal and viable 3-4cell embryos were 80% at post-thawing and 73.33% at 2hr. post-thawing.(Table.2).

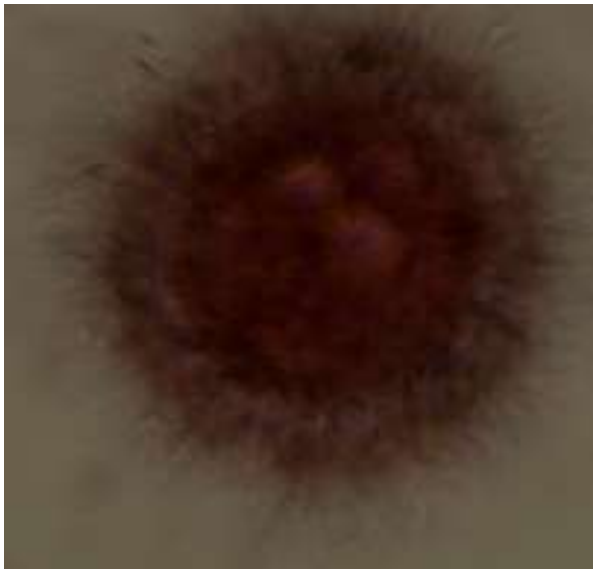

Plate 2.Viable 3-4 cell sheep embryo

Table 2: Effect of vitrification on the morphology and viability of 3-4cell sheep em-bryos.

Embryos Parameters	Total tal-No.	Post thawing		2h.post thawing		Chi-square value
		No	%	No	%	
Normal morphology	15	13	86.66	13	86.66	0.00NS
Viable	15	14	93.33	13	86.66	0.86NS
Normal and Viable	15	12	80.00	11	73.33	1.48NS
NS:Non-significant						

In the present study, a new technique of cryopreservation named vitrification or ultra rapid cryopreservation were used to cryopreserve sheep, early stage embryos (2PN,1-2cell,3-4cell).

According to our knowledge in Iraq, this is the first work dealing with vitrification of animal embryos by direct contact with LN2. Because of the necessity to store large numbers of sheep oocytes and embryos for biotechnology application and livestock industry improvement, needs to improve the techniques of cryopreservation (1) .The vitrification technique is simple, rapid and cost-effective method for cryopreservation of mammalian cells and embryos . Also the water molecules do not have enough time to form ice crystals which causes damage organelles within the cell(5) .To achieve vitrification state required to use a high concentration of cryoprotectant agents, and this lead to the cell may be exposed to determinal osmotic effect(3) . Three main factors should be considered during vitrification process including : cooling rate, medium viscosity and volume(16) .If any defect occurs in one or more of these factors, is affect will the efficiency and the outcome of vitrification process. Other important factor to obtain good results with the vitrification methods is the use of a mixture of cryoprotectants in order to decrease toxicity levels during vitrification(2).Using of ethylene glycol as one of the cryoprotectants is also important as with dimethyl sulphoxide, it prevents excessive osmotic stress and minimizes exposure to high concentrations of this substance as well as, being less toxic(14). Oocytes are very sensitive to volume excursions, which occur during cryopreservation as the cell is exposed to CPAs, freezing, warming and removal of CPAs(15).If the cell cannot withstand these volume changes, the cellular membranes often rupture and cell lysis will occure (13).In the present study, the relative high in vitro viability and normal morphology of vitrified embryos by the percentage post-thawing to 2hr. post-thawing was attributed to the maximum cooling and warming rate achieved by the direct contact of microdrops with LN2 and a warm diluting solution.

To avoiding fracture damage during vitrification pressure changes should not occurs (19) and implies minimal de- and re-hydration of cells. Container or carrier free vitrification method favors rapid heat exchange between the oocyte, embryo-containing

drop and LN2, thus preventing chilling injury. Another risk that may limiting the efficiency of vitrification process, nameley drop cracking which occurred instantaneously contacting with LN2 might partly contribute to the high survival rate of vitrified-thawed embryos(6).

Cracking, which leads to rupture of the zona pellucida or plasma membrane lyses, is generally caused by extreme temperature gradients between the outer layer and the core of the vitrified drop with the volume larger than 2 µl, therefore to avoid this risk ,the droplet contain the oocyte or embryo should be less than 2 µL volume (4). The period of exposure to CPAs before plunging into LN2 is critical and should not be expanded. High concentration of the cryoprotectant used for vitrification is toxic and causes osmotic injury to the vitrified cell(17).Therefore to avoid this toxicity, the time of exposing cells to the cryoprotectants should be reduced to the minimum time (less than one min.) (10).

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