

## THE EFFECT OF VITRIFICATION DEVICE AND EMBRYONIC STAGE ON SURVIVAL AND DEVELOPMENTAL COMPETENCE OF GABALI RABBIT EMBRYOS

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### Abstract

The current study aimed to evaluate the effect of vitrification device (straw or cryotop) and embryonic stage (4-8 cell or morula stages) on survivability, normality and *in vitro* embryo development rate into blastocysts/hatched blastocysts. Total of 24 mature rabbit does of Gabali breed (5-6 months of age, 3-3.5 kg live body weight) were used in this study as embryo donors. Rabbit does were superovulated by PMSG and hCG. Embryos at 4-8 cell stage were recovered by flushing oviducts 32-43h post-mating, while, embryos at morula stage were recovered by flushing oviducts 64-66 h post-mating. Embryos were vitrified by straw or cryotop. Survival and normality rates were determined. The vitrified embryos were *in vitro* cultured for 3-5 days to record blastocyst and hatching blastocyst formation rates. Results showed insignificant differences in survival and normality rates of vitrified embryos as affected by embryonic stage, although there were a tendency of higher rates yielded from embryos at morula than at 4-8 cell stage (87.7 and 89.2% vs. 85.9 and 85.2%, respectively). Survival and normality rates were higher ( $P < 0.05$ ) using cryotop than straw, being 92.1 and 93.1% using cryotop versus 81.7 and 81.0% using straw, respectively. Expansion and hatching rates were higher ( $P < 0.05$ ) for vitrified embryos at morula than at 4-8 cell stage (80.0 and 71.5% vs. 66.0 and 54.0%, respectively) and using cryotop than straw (81.6 and 75.3 vs. 63.1 and 48.2%, respectively). The current study could be conclude that using the cryotop method to vitrify rabbit embryos rather than straw method at various developmental stages, particularly at morula stage.

**Keywords:** Rabbit embryo, vitrification, cryotop, survival, blastocyst.

## INTRODUCTION

Cryopreservation of mammalian oocytes and embryos has been used for several purposes such as to preserve the genes of elite livestock, increase the efficiency of animal breeding, preserve valuable genetically modified animals and endangered species, and use germ cells effectively in assisted reproductive technology in animals and humans (Yasuyuki *et al.*, 2011). In certain cases optimization of cryopreservation protocols for more sensitive embryos is relevant (Chrenek *et al.*, 2014). Vitrification is routinely being used in the artificial reproductive technologies and has become increasingly used than slow freezing method with no or little damage to provide the chance to maintain scientifically important stocks, strains, and lines. Also, vitrification methods have been made possible to store embryos for extended periods and allowed the import and export of embryos and gametes (Habibi *et al.*, 2013).

In rabbits, vitrification technique was used for embryo cryopreservation with improved general efficiency of embryo survival (Chrenek *et al.*, 2014). Several factors were reported on the relative efficiency of cryopreservation, such as embryo culture medium, type and concentration of cryoprotectant and cryopreservation container. Several devices have been developed to produce a small volume of vitrification solution, such as electron microscope grid, open pulled straw, cryoloop and cryotop. Such devices have improved the vitrification and led to the successful production of offspring from vitrified-warmed embryos (Lin *et al.*, 2010).

A cryotop is an alternative device that consists of a thin strip of plastic film (0.4 mm wide, 20 mm long, 0.1 mm thin) attach to a hard plastic holder. In the cryotop protocol, embryos are loaded with the use of a glass capillary under the control of stereomicroscope in small volume less than 0.1  $\mu$ l of vitrification medium (Kuwayama *et al.*, 2005a).

According to the changes in cryological properties, e.g. membrane permeability and surface area to volume ratio during embryonic development, vitrification device should be optimized for embryos at various developmental stages (Lin *et al.*, 2011). Cryopreservation of rabbit embryos was reported at morula (Naik *et al.*, 2005), and blastocyst (Lopez-Bejar and Lopez-Gatius, 2002) stages, but higher rates of development were achieved when blastocyst (but not morula) stage embryos were used.

In rabbit, the vitrification of rabbit embryos at pronuclear stage with cryotop yields a higher rate of post-warming survival than either the gel-loading tip or cryoloop. Higher survival rate (88%) and development (52%) was obtained post-

warming embryos vitrified at morula to blastocyst stages using a modified open pulled straw method (Lopez-Bejar and Lopez-Gatius, 2002). While, a 91% survival rate *in vitro* and 29% term development (kits/total embryos transferred) after transfer of vitrified embryos at morula stage (Naik *et al.*, 2005).

There were limited reports of vitrification on rabbit embryos at earlier developmental stages (pronuclear to cleavage) and results were generally unsuccessful, except that one study with 94% *in vitro* survival and 36% birth rates was reported on pronuclear stage embryos vitrified by cryoloop (Hochi *et al.*, 2004).

Therefore, the current study was carried out to evaluate the effect of vitrification device (straw or cryotop) and embryonic stage (pronuclear or morula embryos) on survivability, normality and embryo development rate into blastocysts/hatched blastocysts.

## MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center (ILMTC), belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture.

### **Embryos collection:**

Total of 24 mature rabbit does of Gabali breed (5-6 months of age, 3-3.5 kg live body weight) were used in this study as embryo donors. Rabbit does were superovulated using injection of 20 IU PMSG/kg live body weight (Foligon, Intervet International B.V., Boxmeer), followed by 40 IU hCG/kg live body weight (Pregnyl, Organon, Nile Co., Egypt), 48 h later on day of natural mating with fertile bucks belonging to the same breed.

Reproductive tracts of the donors were flushed to embryo collection. The embryos at 4-8cell stages were recovered 32–43 h post-mating (Lin *et al.*, 2010) and pooled as one group, while, embryos at morula stage were recovered 64-66 h post-mating.

Embryos were recovered using flushing medium in Petri dish. The composition of the flushing medium was phosphate buffer saline (PBS) supplemented with 0.2% (w/v) of bovine serum albumin (BSA) and antibiotics (100 IU penicillin and 100 µg/ml streptomycin, Sigma Chemical Co., St. Louis, Mo, USA).

The recovered embryos were counted and evaluated morphologically under inverted microscope and washed twice in PBS supplemented with 0.2% BSA and antibiotics. All recovered embryos at 4-8 cell or morula stages were cryopreserved by vitrification method (cryotop or straw).

Total of 416 rabbit embryos were used 105 and 100 at 4-8 cell stages as well as 108 and 103 at morula stage for straw and cryotop, respectively.

#### **Embryo vitrification:**

A basal medium (BM) containing PBS supplemented with 20 % fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) was used for vitrification. Vitrification was carried out in two steps: in the 1<sup>st</sup> step, embryos were pipetted into vitrification solution 1 (VS1) containing BM + 12.5% (v:v) ethylene glycol (EG) + 12.5% (v:v) dimethyl-sulfoxide (DMSO) + 0.5 M sucrose) in disposable sterile Petri dish for 2 min. In the 2<sup>nd</sup> step, the embryos were pipette into VS2 (BM + 20% (v:v) EG+20% (v:v) DMSO + 0.5 M sucrose) for 30 s. Then, three embryos were loaded into cryotop with <0.1 µl VS2, and immediately submerged in liquid nitrogen (Habibi *et al.*, 2013). Also, eight embryos were suspended in VS2, loaded in 0.25 ml plastic straws (IMV, L'Aigle, France), sealed and plunged directly into liquid nitrogen for at least 15 days.

#### **Thawing of vitrified embryos:**

Vitrified embryos were thawed by plunging the straw or cryotop into one ml of thawing solution (PBS + 4% BSA + 0.33 M sucrose) for 1 min, followed by serial dilutions in PBS + 0.175 M sucrose solution for 2 min to remove the intracellular cryoprotectants, then embryos were washed three times in PBS solution for 5 min per time to remove cryoprotectants at 25°C.

#### **Evaluation of post-thawing embryos:**

Survival rate was calculated based on number of post-thawing recovered embryos, while normal and abnormal number of vitrified embryos (normality rate) was morphologically evaluated based on abnormalities in mucin coat, intact zona pellucida, blastomeres, and refractive cytoplasm.

#### **Embryo culture *in vitro*:**

The survived viable embryos were *in vitro* cultured in 100 µl drops of tissue culture medium-199 (TCM 199, Sigma) supplemented with 4 mg/ml BSA and 50 µg/ml of Gentamicin sulphate under mineral oil in CO<sub>2</sub> incubator at 38°C, 95% humidity and 5% CO<sub>2</sub> in air to develop into the blastocyst stage. Embryos were assessed to record blastocyst and hatching blastocyst formation rates for 3-5 days.

#### **Statistical analysis**

Data obtained from this study were statistically analyzed using a software package (SAS, 2004). Mean differences in survival and normality rates, and development of vitrified embryos at different stages, were performed using factorial design. The significant differences among means were tested using Duncan's Multiple Range Test.

## RESULTS AND DISCUSSION

### Post-thawing survival and normality rates:

Data presented in Table 1 showed insignificant differences in survival and normality rates of vitrified embryos as affected by embryonic stage, although there were a tendency of higher rates yielded from embryos at morula than 4-8 cell stage (87.7 and 89.2% vs. 85.9 and 85.2%, respectively). However, significant effect of cryodevice was obtained on survival and normality rates, being significantly ( $P < 0.05$ ) higher using cryotop than straw. The survival and normality rates were 92.1 and 93.1% using cryotop versus 81.7 and 81.0% using straw, respectively.

The effect of interaction between embryonic stage and cryodevice on survival and normality rates was insignificant, reflecting the highest survival and normality rates of vitrified embryos at morula stage using cryotop device (93.2 and 94.9%, respectively, Table 1).

The present results are higher than those reported by Koprdoová *et al.* (2009) who found that viability of rabbit embryos after short-term storage in liquid nitrogen by straw was 46.16% and after long-term storage it was 44.1%. Survival of rabbit embryos after devitrification, described in the study of Makarevich *et al.* (2008) was 58.8%. On the other hand, higher survival rate of mouse embryos than that obtained in the current study was recorded by Zhang *et al.* (2009), who found that the post-vitrification survival rates of vitrified mouse embryos at 2-cell, 4-cell and 8-cell stages were 96, 97 and 97%, respectively. Also, Yasuyuki *et al.* (2011) reported that the viability of the canine embryos at 4-cell to 16-cell, morula, and blastocyst stages was 90, 50 and 40%, respectively.

In accordance with the present results, Hochi *et al.* (2004) found higher post-thawing survival of rabbit embryos vitrified by cryotop than gel-loading tip and cryoloop. In this respect, Hochi *et al.*, (2004) suggested that cryotop is a better alternative cryodevice than open pulled straw. Kuwayama *et al.* (2005b) reported that use of a cryotop container improves the survival rate of vitrified-warmed embryos and has advantages over conventional vitrification procedures.

The advantages of vitrification of embryos by cryotop was attributed to using very small volumes of embryo suspension (less than 1  $\mu$ l). Minimizing the volume of the solution with which embryos are vitrified might result in higher viability of the embryos post-vitrification (Yasuyuki *et al.*, 2011). Also, the vitrification by cryotop prevents chilling injury to sensitive cells (Kuwayama *et al.*, 2005c). Generally, problems with viability of embryos after vitrification can be caused by permeable cryoprotectants presented in vitrification solution (Shaw and Jones, 2003).

**Embryo culture *in vitro*:**

Results shown in Table 2 revealed significant differences in expansion and hatching rates of vitrified embryos as affected by embryonic stage, cryodevice and their interaction. Expansion and hatching rates were significantly ( $P < 0.05$ ) higher for vitrified embryos at morula than 4-8 cell stage (80.0 and 71.5% vs. 66.0 and 54.0%, respectively) and using cryotop than straw (81.6 and 75.3 vs. 63.1 and 48.2%, respectively).

The effect of interaction between embryonic stage and cryodevice on expansion and hatching rates was insignificant, reflecting the highest expansion and hatching rates of vitrified embryos at morula stage using cryotop device (91.3 and 84.7%, respectively, Table 2).

The present trend of increasing expansion and hatching rate of embryos at morula than 4-8 cell stages was reported by Lin *et al.* (2011), who found that the *in vitro* development of rabbit embryos at 8-cell and morula stages post-vitrification and warming to expanding blastocyst was 70.1 vs. 91.7% and hatching blastocyst was 63.7 vs. 86.2%, respectively.

In rabbit, the vitrification of rabbit embryos at pronuclear stage with cryotop yields a higher rate of post-warming survival than either the gel-loading tip or cryoloop. Higher survival rate (88%) and development (52%) was obtained post-warming embryos vitrified at morula to blastocyst stages using a modified open pulled straw method (Lopez-Bejar and Lopez-Gatius, 2002). While, a 91% survival rate *in vitro* and 29% term development (kits/total embryos transferred) after transfer of vitrified embryos at morula stage (Naik *et al.*, 2005).

In mouse, Zhang *et al.* (2009) found that the blastocyst formation rate of the 2-cell embryos was significantly lower than of vitrified 4 cell and 8-cell embryos, suggesting the higher sensitivity of younger embryos. The developmental rates of vitrified 2-cell embryos to the blastocyst and hatched blastocyst stages were 69.4 and 52.6%, respectively, versus corresponding values of 93.3 and 60% for vitrified-warmed 4-cell embryos, 91.1 and 78.4% for 8-cell embryos, respectively.

Generally, embryos at morula stage appear to be the most optimal stage for embryo vitrification by cryotop. Therefore, the current study could be conclude that using the cryotop method to vitrify rabbit embryos better than straw method at various developmental stages, particularly at morula stage.

Table 1. Post-warming survival and normality rates of vitrified rabbit embryos as affected by embryonic stage, vitrification cryodevice and their interaction.

Item	Main effects				Interaction			
	Embryonic stage (E)		Cryodevice (C)		4-8 cell stage		Morula stage	
	4-8 cell	Morula	Straw	Cryotop	Straw	Cryotop	Straw	Cryotop
Total embryos, n	205	211	213	203	105	100	108	103
Post-warming embryos, n	176	185	174	187	85	91	89	96
Survival rate, %	85.9± 0.74	87.7± 0.74	81.7± 0.74 <sup>b</sup>	92.1± 0.74 <sup>a</sup>	80.95±1.1	91.00±1.1	82.41±1.1	93.20±1.1
Normal embryos, n	150	165	141	174	67	83	74	91
Abnormal embryos,	26	20	33	13	18	8	15	5
Normality rate, %	85.2± 0.74	89.2± 0.74	81.0± 0.74 <sup>b</sup>	93.1± 0.74 <sup>a</sup>	78.82±1.1	91.21±1.1	83.15±1.1	94.79±1.1

a and b: Means denoted within the same row with different superscripts for each effect are significantly different at P<0.05.

Table 2. Developmental competence of rabbit embryos vitrified at different stages and cryodevice after thawing.

Item	Main effects				Interaction			
	Embryonic stage (E)		Cryodevice (C)		4-8 cell stage		Morula stage	
	4-8 cell	Morula	Straw	Cryotop	Straw	Cryotop	Straw	Cryotop
Cultured embryos, n	150	165	141	174	67	83	74	91
Expanded blastocysts, n	99	132	89	142	40	59	49	83
Expansion rate, %	66.0± 0.79 <sup>b</sup>	80.0± 0.79 <sup>a</sup>	63.1± 0.79 <sup>b</sup>	81.6± 0.79 <sup>a</sup>	59.70±1.1	71.08±1.1	66.22±1.1	91.21±1.1
Hatched blastocysts, n	81	118	68	131	27	54	41	77
Hatching rate, %	54.0±0.83 <sup>b</sup>	71.5±0.83 <sup>a</sup>	48.2±0.83 <sup>b</sup>	75.3±0.83 <sup>a</sup>	40.29±1.2	65.10±1.2	55.41±1.2	84.63±1.2

a and b: Means denoted within the same row with different superscripts for each effect are significantly different at P<0.05.

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## تأثير طرق التجميد بالترزج والمرحلة الجنينية على معدل الحيوية والكفاءة التنموية لأجنة الأرناب الجبلى

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تهدف هذه الدراسة إلى تقييم تأثير كلا من طرق التجميد مثل (straw أو cryotop) والمرحلة الجنينية (٤-٨ خلايا أو الموريولا) على معدل حيوية الأجنة وجودتها ومعدل تطورها إلى مرحلة الفقس. تم أخذ الأجنة من ٢٤ من أمهات الأرناب الجبلى ذات عمر من ٥-٦ شهور ووزن حي من ٣-٣,٥ كجم. تم معاملة الأمهات هرمونيا ب (hCG , PMSG) لإحداث عملية التبويض الفائق. وتم عمل غسيل لقناتي المبيض وجمع الأجنة في مرحلة ٤-٨ خلايا بعد (٣٢-٤٣ ساعة من التلقيح) بينما تم جمع الأجنة في مرحلة الموريولا بعد (٦٤-٦٦ ساعة من التلقيح). تم تحميل الأجنة وتجميدها بالترزج بواسطة straw أو cryotop. تم قياس معدل حيوية الأجنة وزراعة الأجنة المجمدة لمدة ٣-٥ أيام بعد عمل إسالة لها وقياس معدل قدرتها على التطور والوصول إلى مرحلتى البلاستوسيسست والفقس. أوضحت النتائج عدم وجود اختلافات معنوية في معدل حيوية الأجنة والأجنة الطبيعية بعد التجميد بالترزج والتي تأثرت بالمرحلة الجنينية على الرغم من وجود اتجاه لزيادة معدل حيوية الأجنة وجودتها في مرحلة الموريولا عن مرحلة ٤-٨ خلايا (٨٧,٧ و ٨٩,٢ % مقابل ٨٥,٩ و ٨٥,٢ %، على التوالي). بينما وجد أن معدل حيوية الأجنة وجودتها أعلى معنويا ( $P < 0.05$ ) باستخدام cryotop عن straw (٩٢,١ و ٩٣,١ % مقابل ٨١,٧ و ٨١,٠ %، على التوالي). وقد لوحظ أن معدل الأجنة التي وصلت إلى مرحلتى البلاستوسيسست والفقس للأجنة المجمدة كانت أعلى معنويا في مرحلة الموريولا عن مرحلة ٤-٨ خلايا (٨٠,٠ و ٧١,٥ % مقابل ٦٦,٠ و ٥٤,٠ %، على التوالي) واستخدام cryotop عن straw (٨١,٦ و ٧٥,٣ % مقابل ٦٣,١ و ٤٨,٢ % على التوالي). ونستخلص من هذه الدراسة جدوى استخدام cryotop بديلا عن straw كوسيلة في تجميد أجنة الأرناب بالترزج في مراحلها الجنينية المختلفة وخصوصا مرحلة الموريولا .