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VIABILITY OF BISECTED RABBIT EMBRYOS AFTER CRYOPRESERVATION USING VITRIFICATION

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ABSTRACT

To study the effects of developmental capacity after in vitro culture of intact and demi-embryos of rabbit in fresh case or after cryopreservation using vitrification technique, total 89 embryos at morula and blastocyst stages recovered from 16 does. Compaction rabbit embryos maintained intact (control) were compared with bisected embryos using a simplified splitting protocol at fresh or vitrified thawed embryos. The survivability of intact embryos and demi-embryos were evaluated after 24 hours of in vitro culture by development ability and morphological assessment. The percentage of development after 24 h of in vitro culture was higher (P<0.05) in fresh intact embryos than in fresh bisected embryos, either at morula (80 vs. 56%) or blastocyst (84.6 vs. 63.6%) stages. In case of intact or bisected embryos, percentage of development after 24 h of in vitro culture was insignificantly higher in embryos at blastocyst than at morula stages. The percentage of development after 24 h of in vitro culture was higher (P<0.05) for vitrified intact embryos than for vitrified bisected embryos, either at morula (61.5 vs. 45.0%) or blastocyst (75.0 vs. 42.1%) stages. In case of intact embryos, percentage of development after 24 h of in vitro culture was higher (P<0.05) for embryos at blastocyst than at morula stages. However, the percentage of development after 24 h of in vitro culture was nearly similar in vitrified bisected embryos, either at morula or at blastocyst stages.

***In conclusion**, bisection and vitrification can be used for rabbit embryos, but embryonic stage and cryopreservation are limiting factors in the success of these procedures.*

Keywords: Rabbit, embryo, bisection, developmental capacity, *in vitro* culture.

INTRODUCTION

Mammalian embryo splitting has successfully been established in farm animals. Embryo splitting is safely and efficiently used for assisted reproduction in several livestock species. Splitting embryos could increase the number of embryos available for transfer. Human embryo splitting has been reported recently (Karl and Mike, 2010) and embryo splitting may be more realistic approach to creating pairs of genetically identical monkeys (Schramm and Paprocki, 2004).

It is known that the pregnancy rate resulting after transfer of bisected embryos is lower than after transfer of whole embryos. The main reason is the reduced cell number in the demi-embryo which is less than half of that in the intact embryo. The commonly used method for obtaining monozygous twins in mammals is embryo splitting (Willadsen and Godke., 1984 and Baker, 1985) applied to embryos at post compaction, i.e.; the morula and blastocyst stages. Usually embryo splitting is performed with a metal micro-blade or a glass micro-needle (Mertes and Bondioli, 1985).

Since a number of blastomeres are damaged as a result of the procedure of splitting, the cell losses depend mostly on the stage (Skrzyszowska and Smorag, 1989) and quality (Brem *et al.*, 1984) of the embryo and precision of the microsurgery. The destruction of cells during splitting could be one of the main reasons for the lower pregnancy rate seen following the transfer of bisected embryos. The development of an alternative method reducing cell losses during bisection could improve the demi-embryo transfer technology.

Since the first reports of embryo bisection in cattle (Willadsen *et al.*, 1981 and Ozil *et al.*, 1982), the technique has been used in this species to produce identical twins (Willadsen and Polge, 1981; Willadsen and Godke., 1984 and Bredbacka, 1996), facilitate embryo sexing (Picard *et al.*, 1985 and Bredbacka *et al.*, 1994), and increase the number of transferable embryos (Leibo and Pall, 1987; Gray *et al.*, 1991 and Kippax *et al.*, 1991), as has been reviewed (Picard and Betteridge 1989; Willadsen, 1982).

Embryo bisection has, therefore, found commercial application in livestock production (Gray *et al.*, 1991 and Bredbacka, 1996), particularly after simplification of the technique (Utsumi and Iritani, 1990 and Bredbacka, 1996). Attempts to improve the bisection technique have included the use of surrogate zonae pellucidae (Voelkel *et al.*, 1984; Warfield *et al.*, 1986), modification of the micromanipulation medium (Suzuki and Shimohira, 1986; Herr et al 1988 and Szell and Hudson, 1991), and selection of embryos at particular ages and developmental stage (Williams *et al.*, 1984 and McEvoy and Sreenan, 1990).

Therefore, the objectives of the present study were to examine the effects of developmental stage and cryopreservation on the *in vitro* survival of intact and demi-embryos

MATERIALS AND METHODS

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

Animals:

Sixteen mature New Zealand White (NZW) rabbit does having 1–2 years of age were used as embryo donors in this study. All does were fed commercial pelleted diet and kept under the same environmental conditions. They were individually caged for 3–5 weeks prior to start of the experiment to avoid pseudo-pregnancy.

Media:

The medium used for embryo culture was serum-free M-199 containing Earle's salts, 2.5 mM Na pyruvate, 1 mM L-glutamine, 0.5% penicillin-streptomycin, and 0.1 mg/mL polyvinyl alcohol (MW 30,000 to 70,000; Sigma), the pH was adjusted to 7.4 and the osmolality to 280 mOsm/kg. Further manipulation of embryos was performed in drops of serum-free Dulbecco's phosphate buffered saline (DPBS) so that embryos adhered to the bottom of the Petri dishes.

Conditioned M-199 medium prepared by the method outlined by (Mermillod *et al.* 1993) was used to culture the demi -embryos. The culture was maintained at 38.5°C in a humidified atmosphere of 5% CO_2 in air. The cells grew to confluence; the confluent mono-layers were washed 3 times with serum-free M-199 and then cultured in the same medium until recovering after 48 h of conditioning.

Superovulation and embryo recovery:

Rabbit does were super-stimulated with a single injection of 75 IU of PMSG (Folligon, Intervet, Holand). After 72 h of PMSG injection, ovulation was induced by 100 IU of HCG (Pregnyl) injected into ear vein followed by mating with fertile buck (two consecutive inseminations). Total of 98 embryos were flushed from the oviduct and uterus of slaughtered does with Dulbecco's phosphate-buffered saline solution (PBS, Sigma) supplemented with 5% heat-inactivated fetal calf serum (FCS, Sigma) and

50 µg/ml^{-1} of gentamicin sulphate. Embryos at morula and blastocyst stages were recovered after 48 h and 72 h post-coitus.

The recovered embryos were classified according to their developmental stage and morphological appearance under a microscope, good quality embryos, according to the criteria established by Lindner and Wright (1984) for embryos with presence of a normal mucin coat. Embryos at morula stage with uniform sized blastomeres and blastocyst were considered suitable for splitting or freezing.

Bisection and classification of embryos:

Bisection was performed in 60-µL drops of DPBS at room temperature using a micromanipulator with an attached microsurgical steel blade but without a holding pipette (Figure 1). For this, the embryos were washed twice in DPBS and transferred to the micromanipulation drop. Only embryos at morula and blastocyst stages were selected and bisected by slowly lowering the micro-blade and then gently moving it to embryo. Bisection method is illustrated in Figure 1, started with intact embryo (phase 1) to bisected embryo (phase 6). In the case of blastocysts, care was taken to orientate the embryos, so that the inner cell mass (ICM) was divided as evenly as possible. After completing the bisection, DPBS containing 10% fetal calf serum was added to the micromanipulation drop to allow the demi-embryos to float free of the floor of the dish. After 24 h of culture in conditioned M-199 medium, the demi-embryos that redeveloped into three categories. Divided embryos, without a zona pellucida, were washed 3 times with conditioned M-199 and cultured in 10 µL of the same medium under mineral oil. The quality of demi-embryos was morphologically evaluated 24 h after bisection.

Bisected embryos were classified subjectively for quality using the criteria previously described in analogous studies in pigs (Nagashima *et al.*, 1989, Reichelt and Niemann, 1994). Category I: excellent embryos had a clearly defined blastocoel, very few degenerated cells (<10%), and a clearly visible ICM. Category II: fair embryos were also blastocysts but 10 to 20% of their cells appeared to be degenerated or the ICM was not clearly visible. Category III: degenerated embryos showed no visible ICM and blastocoel, and contained a large number of degenerated cells (>20%). For the purposes of the present study, embryos classified into categories I and II were deemed viable and transferable.

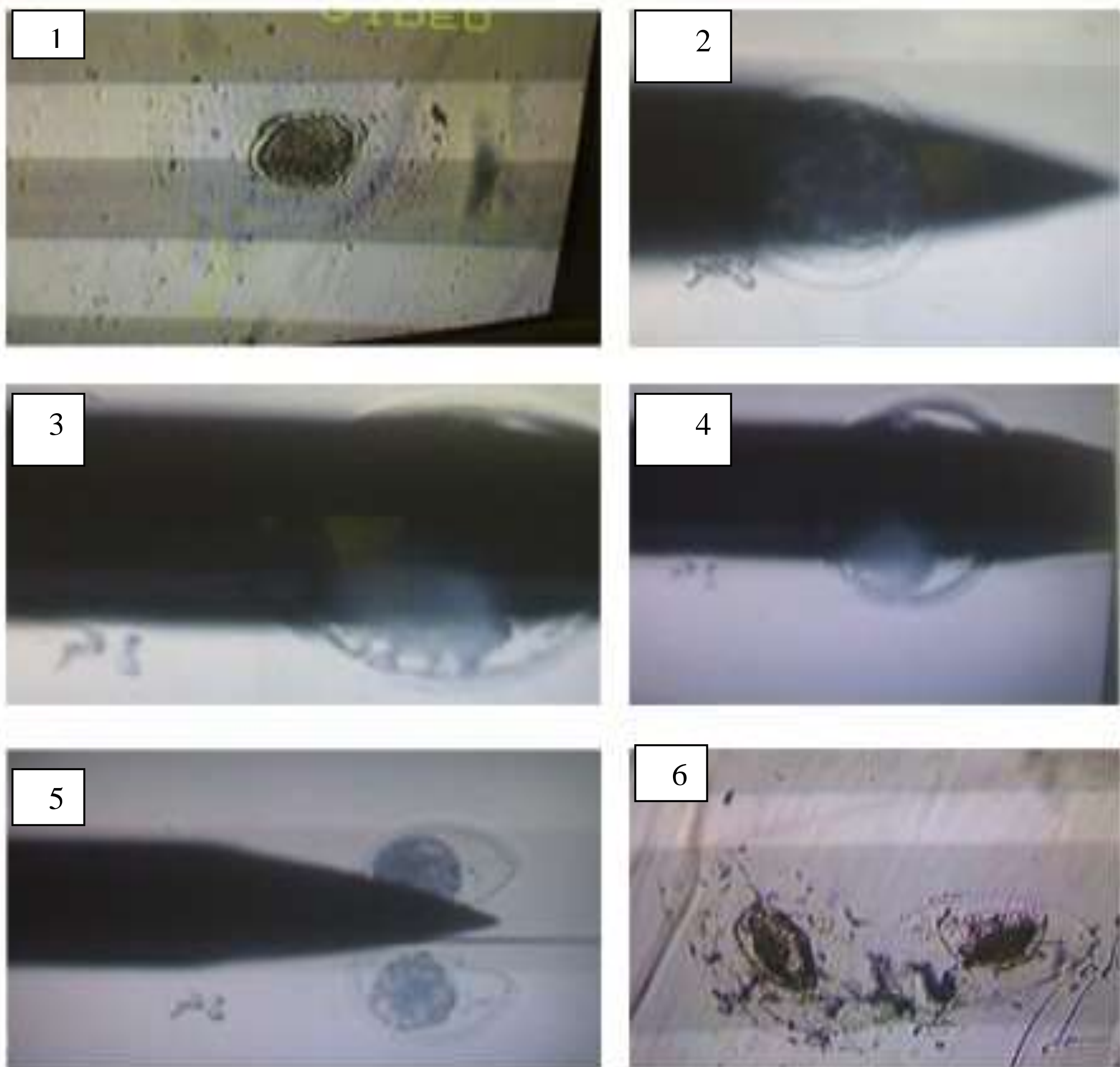

Figure 1: Steps of embryo splitting technique using micromanipulator with metal blade (X 200).

Step (1) : Shows intact embryo at morula stage before bisection.

Steps(2-4): Show microsurgical steal blade of the micromanipulator upper an intact embryo immediately before bisection.

Step (5): Shows the final stage of embryo bisection.

Step(6): Shows two embryos produced by bisecting an intact embryo at morula stage.

Vitrification by modified Open Pulled Straw (mOPS) technique:

Cryopreservation was done using mOPS described by Lopez-Bejar and Lopez-Jatius. (2002). Steps and solutions were done according to Naik *et al.* (2005). Sucrose stock solution was prepared by dissolving 10.27 g of sucrose in 20 ml of HEPES buffered TCM-199 (TCM 199H). This medium was stored in 50 ml plastic tubes at 4°C till used. Holding medium for vitrification was prepared by supplementing TCM-199H with 20% fetal calf serum (FCS, v/v) and 50 µg/ml−1 of gentamicin sulphate.

Two vitrification solutions (VS) were prepared, the 1st solution (VS-I) contained 10% ethylene glycol (EG) and 10% dimecyle sulfoxide (DMSO) was prepared, on day prior to use, by mixing EG, DMSO and the holding medium at a ratio of 1:1:8 and stored at 4°C. The 2nd one (VS-II) contained 20% EG, 20% DMSO and 0.6 M sucrose was prepared on the day prior to use by mixing EG, DMSO, FCS and sucrose stock solution at a ratio of 1:1:1:2 and stored at 4°C.

Also, two cryoprotective diluents were prepared, the 1st diluent (CPD-I) had the same composition as VS-I but contained 0.3 M sucrose in addition. This was prepared by mixing equal volumes of VS-II and the holding medium. The 2nd one (CPD-2) was prepared by mixing CPD-I and holding medium at a ratio of 1:3.

Good quality embryos at morula and blastocyst stages were equilibrated for 5 min in the holding medium prior to OPS vitrification. In order to vitrify 3–5 embryos, they were initially placed in VS-I for 2 min. Subsequently, the embryos were consecutively transferred into three droplets of VS-II for 10 s each. During the last 10 s of exposure to VS-II, open end of the pulled straw was placed on the surface of the third droplet. The embryos entered into the straw by capillary action. Immediately after loading, the straws were plunged vertically into liquid nitrogen (LN) and stored for up to 2 months.

Thawing method:

Straws containing embryos were taken out of LN and the open end of straw was immersed vertically in 1.2 ml of VS-CPD-I solution. The vitrification medium became liquid within 2–4 seconds and the contents of the straw were then released into the well by gentle blowing using a mouth pipette. One minute after, they were transferred into VS-CPD-II solution for 5 min. Finally, they were washed twice in holding medium for 5 min each.

In vitro culture of cryopreserved embryos:

For *in vitro* culture of rabbit embryos, bicarbonate buffered tissue culture medium (TCM 199B) was supplemented with 15% heat inactivated

FCS and 50 μg/ml−1 of gentamicin sulphate and stored at 4°C for up to one week. It was warmed in a carbon dioxide incubator for a minimum of one h prior to culture of embryos. After removal of the cryoprotectants, morphology under a microscope, embryos with uniform blastomeres were regarded as good embryos suitable for culture. Damaged embryos exhibiting lysed blastomeres were discarded.

Groups of 3–5 good embryos were placed in one ml of embryo culture medium under mineral oil (Sigma Aldrich Chemie, Steninheim, Germany) and incubated at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Checks for developmental stage were done after 24 h. The demi-embryos were classified according to morphological criteria (number of degenerated cells, development of blastocoel and inner cell mass), good quality embryos at the end of culture period was considered suitable for transfer and indicator of success of splitting, freezing and thawing of embryos.

Statistical analysis:

Chi-square test was used according to Snedecor and Cochran (1982) to determine differences between rates of survival and development of whole and bisected embryos at different stages.

RESULTS AND DISCUSSIONS

In vitro culture of whole or bisected fresh embryos:

Results in Table (1) show that the percentage of development after 24 h of *in vitro* culture was significantly ($P<0.05$) higher in fresh intact embryos than in fresh bisected embryos, either at morula (80 vs. 56%) or blastocyst (84.6 vs. 63.6%) stages.

In case of intact or bisected embryos, percentage of development after 24 h of *in vitro* culture was insignificantly ($P>0.05$) higher in embryos at blastocyst than at morula stages. Generally, the intact embryos presented greater percentage of alive cells than cultivated hemi-embryos.

Table 1: Development in culture of fresh whole or bisected embryos at morula and blastocyst stages.

Embryo stage	Type of embryo	Number of embryos	Development in culture	
			n	%
Morula	Whole	15	12	80.0^{a}
	Bisected	25	14	56.0^{b}
Blastocyst	Whole	13	11	84.6^{a}
	Bisected	22	14	63.6^{b}

[a and b]: Means denoting with different superscripts within the same column are significantly ($P<0.05$) different.

Similar results were obtained in rabbits by Celestinos and Gatica (2008), who found that the corresponding percentages were 96% for control intact embryos versus 74% for fresh bisected embryos, and all the observed differences were highly significant (P<0.001). The present results indicated difference in developmental capacity between bisected fresh embryos and intact fresh ones following *in vitro* culture.

The percentage of development obtained with fresh bisected embryos at morula or blastocyst stages, being 56.0 and 63.6%, respectively, is in the range reported following *in vitro* culture of demi-embryos (Bredbacka *et al.,* 1994). In bovine embryos, Bredbacka (1996) found higher proportion of viable cells in bisected morulae compared with bisected blastocysts (75.5 vs. 70.0%).

In vitro culture of whole or bisected vitrified embryos:

Results in Table (2) show that the percentage of development after 24 h of *in vitro* culture was significantly (P<0.05) higher in vitrified intact embryos than in vitrified bisected embryos, either at morula (61.5 vs. 45.0%) or blastocyst (75.0 vs. 42.1%) stages. In case of intact embryos, percentage of development after 24 h of *in vitro* culture was significantly (P<0.05) higher in embryos at blastocyst than at morula stages. However, the percentage of development after 24 h of *in vitro* culture was nearly similar in vitrified bisected embryos, either at morula or at blastocyst stages. Similarly, Celestinos and Gatica (2008) found that the percentages of development after 24 h of *in vitro* culture was significantly (P<0.001) higher in vitrified intact (36%) than in vitrified bisected (10%) embryos. The obtained results indicated higher developmental capacity for fresh (Table 1) than vitrified (Table 2) and intact ones following *in vitro* culture, either at morula or blastocyst stages.

Table 2: Development in culture of whole or bisected embryos at morula and blastocyst stages post-vitrification.

Embryo stage	Type of embryo	Number of vitrified embryos	Number of post-thawed embryos	Development in culture	
				n	%
Morula	**Whole**	13	13	8	61.5[b]
	Bisected	21	20	9	45.0[c]
Blastocyst	**Whole**	12	12	9	75.0[a]
	Bisected	22	19	8	42.1[c]

[a and b]: Means denoting with different superscripts within the same column are significantly (P<0.05) different.

The present study shows that acceptable results can be achieved following in vitro culture of rabbit embryos that have been bisected but that the survivability of such embryos seems to be reduced following vitrified-thawing. There were no difference in developmental capacity between biopsied embryos at both morula and blastocyst stages following in vitro culture. However the level of survival was overestimated using in vitro culture systems compared to survival after embryo transfer in agreement with other reports (Gustafsson *et al.*, 1994).

It is generally accepted that freezability of embryos is reduced following splitting. In this respect, Bredbacka *et al.,* (1994) reported that survival rates were lower for frozen-thawed demi-embryos (52%) compared with 60% for fresh embryos. Also, Vajta *et al.* (1997) report a survival rate of 86% for biopsied vitrified-thawed embryos versus 69% for vitrified-thawed biopsied embryos. The variation in developmental capacity between fresh and vitrified intact or bisected embryos is associated with loss in blastomeres during splitting and cryopreservation. A number of cells (around 10%) are shown to be damaged due to the splitting procedure.

The number of cells in an embryo had no effect on cell viability. Apparently the developmental stage effect can be contributed to morphological changes in the embryo rather than to the increase of cell associated with it (Bredbacka (1994). The developmental stage effect found in this study was expressed in a higher proportion of viable blastocysts as compared to morulae. This difference may be of minor relevance for practical purpose, as demi-blastocyst develop equally well or better (Williams and Godke., 1984) than demi-morulae following transfer, possible because blastocysts loss fewer cells (Bredbacka, 1994).

***In conclusion**, bisection and vitrification can be used for rabbit embryos, but embryonic stage or cryopreservation are limiting factors in the success of these procedures.*

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قابلية أجنة الأرانب للحياة بعد التقسيم والحفظ باستخدام طريقة التزجيج

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تم إجراء هذا البحث لدراسة تأثيرات نمو وتطور الأجنة معمليا سواء الطازجة أو بعد الحفظ باستخدام طريقة الزجزجة.تم استرداد ٨٩ جنين في مرحلتى المريولا والبلاستوسست من ١٦ أرنبة. تم مقارنة الأجنة الكاملة (مقارنة) بالأجنة المقسمة سواء الطازجة أو المجمدة. تم تقييم الأجنة بعد ٢٤ ساعة من الزراعة معمليا عن طريق الشكل المورفولجي والتطور.
كانت النسبة المئوية للتطور بعد ٢٤ ساعة من الزراعة معمليا أعلى معنويا عند مستوى ٥% فى الأجنة السليمة مقارنة بالأجنة المقسمة سواء عند مرحلة المريولا (٨٠% مقابل ٥٦%) أو عند مرحلة البلاستوسست (٨٤,٦ % مقابل ٦٣,٦ %). النسبة المئوية للتطور سواء فى الأجنة السليمة أو المقسمة بعد ٢٤- ساعة من الزراعة معمليا كان أعلى معنويا فى مرحلة البلاستوسست عن مرحلة المريولا. النسبة المئوية للتطور بعد ٢٤ ساعة من الزراعة معمليا كان أعلى معنويا عند مستوى ٥% فى الأجنة السليمة المحفوظة عن الأجنة المقسمة سواء فى مرحلة المريولا (٦١,٥ % مقابل ٤٥%) أو مرحلة البلاستوسست (٧٥% مقابل ٤٢,١ %). فى حالة الأجنة السليمة كانت النسبة المئوية للتطور بعد ٢٤ ساعة من الزراعة معمليا أعلى معنويا عند مستوى ٥% عند مرحلة البلاستوسست عن مرحلة المريولا. كانت النسبة المئوية للتطور بعد ٢٤ ساعة من الزراعة معمليا كانت متشابهة تقريبا من الأجنة المقسمة المحفوظة سواء عند مرحلة المريولا أو البلاستوسست.
التوصيـــــة: يمكن استخدام تقنية التقسيم والحفظ بالتجميد السريع لأجنة الأرانب لكن مرحلة التطور الجنينى وطريقة الحفظ تعتبر من العوامل المحددة فى ذلك.