J. Product. & Dev., 12(2): 509 – 523(2007)

IN VITRO DEVELOPMENT OF VITRIFIED RABBIT EMBRYOS: EFFECT OF DOE HORMONAL TREATMENTS AND EMBRYO DEVELOPMENTAL STAGES

Samia Z. Meshreky, Salama O. A. and Eitedal H. EL-Sayed
Animal Production Research Institutes, Agriculture Research center, Dokki, Geza, Egypt.

ABSTRACT

The effects of donors hormonal treatment and embryo developmental stages on the survival of vitrified rabbit embryos were examined. Thirty six virgin New Zealand White rabbit does aging 6-7 months were divided in two groups. Does in the 1st group (n=24) were treated with intramuscular injection of 20 µg GnRH/doe for induction of ovulation and artificially inseminated. Does in the 2nd group (n=12) were treated with subcutaneous injection of 80 IU PMSG/doe and 68 h later with intravenous injection of 50 IU hCG/doe for the induction of superovulation and artificially inseminated following hCG administration. Eight and four does from the 1st and 2nd groups respectively were slaughtered in each of 24-26, 48-50 and 72-74 h after insemination for embryo collection. Embryos at various developmental stages were vitrified. Upon de-vitrification, embryos were evaluated for viability as indicated by the morphological appearance and subsequent development in culture medium for 48 h.

The percentage of normal embryos vitrified and embryos post-thawing with morphologically normal appearance recovered from GnRH treated does were higher than that obtained from superovulated donors (93.05 vs. 77.43% and 75.86 vs. 67.43%, respectively). Only 63 (36%) of 175 superovulated embryo vitrified was cleaved in vitro post-vitrification compared to 89 (51.15%) of 174 control embryos. No significant differences were observed among embryo developmental stages in the percentage of vitrified normal embryos. The percentages of embryos recovered post-vitrification with morphologically normal appearance were significantly (P<0.05) higher in later developmental stages compared to earlier stages. The best percentage of embryos developed in vitro have been obtained after de-vitrification at the early blastocyst stage, followed by that at compacted morulae and morulae stages, whereas the lower percentage were recorded with embryos vitrified at 2- and 4- cell stages. Markedly high in vitro development rates (84.85-92.31%) were obtained in morulae, compacted morulae and early blastocyst stages in control embryos compared to 63.33-81.48% in embryos produced through superovulation. These results show that treating donor with GnRH achieve the best embryos for cryopreservation and also rabbit embryos at morulae, compacted morulae and early blastocyst stages are appear to be proper candidate stages for cryopreservation.

Key words: Rabbits, superovulation, embryos developmental stages, vitrification.

INTRODUCTION

Cryopreservation enables banking of embryos for future use in medicine and in animal breeding. It also enables protection of germ plasm of endangered species and unique strains or lanes of laboratory animals (Papis *et al.,* 2005). Superovulation is considered to be an efficient economic method for producing additional embryos or oocytes from females of high genetic merit. For most domestic animals, the responses to superovulation treatments are not controlled as a consequence of the lack of knowledge on exogenous gonadotrophins effects on the ovarian function. Furthermore, the response to superovulation treatments is highly variable. This variability has several origins like genetic, age, breeding, parity, physiological status of the animals (Takagi *et al*., 2001) and the hormonal preparations used are very important too (Salvetti *et al.,* 2007). Few comparative studies have been carried out on the hormonal treatment used for ovulation induction in relation to embryo recovery and its *in vitro* and *in vivo* survival rates in rabbits (García-Ximénez and Vicente, 1992, Viudes-de-Castro *et al*., 1995, García *et al.*, 2000 and Vicente *et al.*, 2003).

Survival of cryopreserved rabbit embryos depends on the embryonic stage of development and on the cryoprotective additives; although 32% of 774 rabbit oocytes frozen in PG+sucrose appeared morphologically normal after freezing and thawing, only 14% cleaved to the 2- to 8-cell stages after *in vitro* fertilization (Al-Hasani *et al*., 1989). The cytoskeleton and meiotic spindle, oocytes are even more sensitive to cryopreservation (Vincent *et al*., 1989). In addition, Hochi *et al.* (2001) demonstrated that pronuclear stage rabbit zygotes could be successfully cryopreserved in a solution of EG plus sucrose. Only 18% of rabbit zygotes vitrified in a mixture of EG+Ficoll+sucrose (EFS) solution developed into blastocysts. With 2-cell stage rabbit embryos, *in vitro* survival has ranged from 58% developing to blastocysts for 101 embryos vitrified in EFS (Smorag and Gajda, 1998), to 89% developing into morulae for 54 embryos frozen in PG (Renard *et al*., 1982). Only 40% of 176 morulae vitrified in EG alone or in EG+DMSO, developed into blastocysts (Vicente and Garcia-Ximenez, 1994); 72% of 281 rabbit morulae frozen by equilibrium cooling in DMSO, developed into blastocysts *in vitro* (Kojima *et al.*, 1987); however, 89% developed into blastocysts of 235 morulae vitrified in EFS (Kasai *et al*., 1992). Thus, it is clear that rabbit zygotes are rather difficult to be cryopreserved compared to later stage embryos.

The objectives of this study were to examine the effects of hormonal treatment of does on the morphological appearance and *in vitro* development of rabbit embryos vitrified in dimethyl-sulphoxide (DMSO) and ethylene glycol (EG) at different preimplantation stages.

MATERIALS AND METHODS

This experimental work was carried out at the laboratory of Reproduction and Biotechnology, Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt.

Animals

Thirty six nulliparous New Zealand White female rabbits aging 6-7 months old were used as donors. Animals were housed in individual metal cages for 3 weeks perior to hormonal treatments to eliminate the chances of pseudopregnancy. Five rabbit bucks of proven fertility of the same breed were also utilized. The animals were kept under controlled 16h light: 8h dark photoperiod and fed *ad-libitum* with a commercial pelleted diet contained 18.4 % crude protein, 14 .0% crude fibers and 2.4 % fat. Fresh potable water was made available all times through stainless nipples.

Embryo recovery

Experimental does were divided into two groups. Does in the first group (24 does) were treated with an intramuscular injection of 20 µg GnRH (Fertagyl, Intervet, International B. V. Boxineer-Holland) followed by artificial insemination (AI) with fresh semen. Does in the second group (12 does) were administered subcutaneously with 80 IU PMSG/doe (Folligon, Intervet, International B. V. Boxineer-Holland) followed 68 h later with intravenous injection (iv) of 50 IU hCG/doe (Pregnyl, Organon, Nile Co., Egypt). Does were inseminated at the time of hCG injection. Eight does from 1st group and four does from 2nd group (superovulation) were slaughtered at 24-26, 48-50 and 72-74 h after insemination. The reproductive tracts (oviducts and uterine horns) were removed and embryos stages were recovered by flushing twice with 5 mL of Dulbecco´s Phosphate Buffered Saline (®DPBS, (PBS: Gibco, Cat. No 21300-017, UK) supplemented with $CaCl_2$ (0.132 g/L), 0.2% of bovine serum albumin (®BSA, Sigma Chemical Co., St. Louis, Mo, USA) and antibiotics (10,000 IU Penicillin G potassium+ 10 mg streptomycin sulfate/ml, Sigma) at room temperature (20-25 ºC). After recovery, embryos and oocytes were washed twice in fresh DPBS supplemented with 10 % FCS and antibiotics, counted and morphologically evaluated under stereoscopic microscope. Embryos with no abnormalities in mucin coat, *zona pellucida* and with homogenous blastomeres were scored as freezable embryos (grade 1 or 2) according to International Embryo Transfer Society classification. The other embryos were considered as grade 3 embryos (non-freezable). Morphologically normal embryos (grade 1 or 2) from each donor doe were washed twice in fresh DPBS and kept within 15-30 min. at ambient temperature, away of light, until vitrification

Vitrification procedure

Grade 1 and 2 embryos collected from treated does at different developmental stages were vitrified and devitrified using the method described by Vicente *et al.* (1999). The cryoprotective solution was a 1:1:2 solution (v/v/v) of dimethyl-sulfoxide (3.5 M DMSO, Sigma), ethylene glycol (4.4 M EG, Sigma), in DPBSCa (DPBS supplemented with 0.132 g $CaCl_2$/L) supplemented with 0.2 (w/v) bovine serum albumin (BSA; Sigma) per liter of cryoprotective solution.

Vitrification was carried out in two steps. First, normal embryos were pipetted into 0.2 ml of PBS medium and placed in a culture dish and then 0.2 ml of the cryoprotective solution was added and agitated. Embryos were kept in this medium for 2 minutes. In the second step, 0.6 ml of the cryoprotective solution was added and quickly agitated. Then, embryos suspended in the final vitrification solution were loaded into 0.25 ml plastic straws (IMV, L'Aigle, France), sealed with polyvinyl-alcohol sealing powder and plunged directly into liquid nitrogen. The exposure time of embryos to the final vitrification solution did not exceed 1 minute. The two vitrification steps were carried out at 20 ºC. The straws contained three sections separated by air bubbles. The first consisted of PBS in the cotton plug, the second section contained the embryos suspended in vitrification medium (0.1 ml) and the third section consisted of PBS. The straws were sealed and identified. Each straw held between 5 to 8 normal embryos.

Devitrification was performed by immersing the second and third sections of the straws in a water bath at 20 ºC for 10-15 sec. The cryoprotective solution was removed from the embryos in a two step dilution procedure at room temperature (20-25 ºC). Embryos suspended in the final vitrification solution were released into a culture dish containing 1 ml of 0.33 M sucrose in PBS medium. After 5 minutes, embryos were washed twice in fresh PBS medium and morphologically scored before culture. Only embryos with homogenous cell mass and intact zonae pellucida were *in vitro* cultured.

Post-vitrification in vitro development

Embryos were cultured for 48 h in 50 µl microdrops of Ham's F10 medium+ 20% FBS (Sigma) under mineral oil (Sigma) at 38.5 °C in 5% CO_2 and saturated humidity. The *in vitro* cleaved and development ability of devitrified embryo and quality were assessed and recorded for analysis.

Statistical Analysis

A Chi-square test was used to compare the effect of hormonally treated donors and embryo stage on the pre and post-vitrification quality and *in vitro* development of embryos.

RESULTS AND DISCUSSION

1. Effect of hormonal treatment

Results in Table (1) showed that the percentage of verifiable embryos recovered from the GnRH treated does (control group) was higher than that obtained from PMSG+hCG treated donors (superovulated group, 93.05 *vs*. 77.43%), difference was not statistically significant. This may due to the effect of the superovulatory treatment as several authors have observed negative effect of superovulatory treatment on embryo production (García-Ximénez and Vicente, 1990), embryo recovery rates (Schmidt *et al*., 1992 and Meshreky and Salama, 2005) and on the survival rate of rabbit embryos (Maertens *et al.*, 1995 and Rebollar *et al.*, 2000). Moreover, superovulation treatment cause the ovulation of a high number of abnormal haemorrhagic and cystic follicles (García-Ximénez and Vicente, 1990) and defaults of ovulation (Salvetti *et al.,* 2007). Several authors observed that the administration of exogenous gonadotrophins decreases the frequency and the amplitude of the pulsatile endogenous LH secretion linked with an increase of estradiol and progesterone plasmatic concentrations (Ben Jeraba *et al.* 1994 and Gosselin *et al*., 2000). As the estrogen concentration increases, the LH concentration decreases and would become inadequate to activate the positive feedback of the estradiol on the LH secretion by the hypothalamus during the preovulatory period. Further, the absence of estrogens' positive feedback was underlined by Ramirez and Beyer (1998) and Bakker and Baum (2000) to explain the absence of spontaneous ovulation in induced ovulators. Thus, the larger amount of estradiol could inhibit the preovulatory pulse of LH and the ovulation mechanism. Mehaisen *et al*. (2005) observed that the number of ovulation sites tended to be higher with a dose of 200 IU eCG/doe in superovulated does, it did not improve the number of normal embryos recovered and had a negative effect on the *in vitro* development of embryos after thawing when compared to 50 IU eCG/doe. In addition, Salvetti *et al.* (2007) reported that superovulation treatments were associated with a significant increase of the number of corpora lutea present on the ovaries, hemorrhagic follicles, the number of embryos recovered, good quality embryos, bad quality embryos and of "non-freezable" eggs per female donor in comparison with the control group. Confirming Kennelly and Foote (1965) observations, the gonadotrophins stimulations of the ovaries which led to an increase of the number of hemorrhagic follicles may be attributable to the supra-physiological amount of gonadotrophins administrated, involving the precocious apoptosis of the granula's cells and leading to the follicles atresia.

Results in Table (1) and Plate (1) revealed that the percentage of embryos recovered from superovulated does with post-thawing morphologically normal appearance was lower than that recovered from GnRH treated does (67.43 *vs*. 75.86%). Structurally intact embryos and high morphological quality grade may be essential components for successful rabbit embryo cryopreservation. Rebollar *et al.* (2000) reported that

cryopreservation of rabbit embryos led to a higher rate of morphological damage when recovered from superovulated does compared with untreated one. Mehaisen *et al*. (2006) also reported that the primary treatments with eCG or FSH increased the number of normal embryos recovered per a donor doe, but these embryos are more sensitive to vitrification protocols. However, superovulation may cause the cytogenesis defects and chromosomal alterations of recovered embryos (Carney and Foote, 1990; Chrenek *et al*., 1998 and Kauffman *et al*., 1998). In addition, Yaakub *et al.* (1998) observed in heifers that a higher superovulatory response could produce an inferior embryo quality.

Table 1. Effect of hormonal treatment on pre and post vitrification embryo quality.

Donor hormonal treatments	No. embryos recovered	Embryos vitrified *n* (%)[1]	Morphologically normal embryos post-thawing *n* (%)[2]	Embryos cleaved and developed *in vitro* *n* (%)[3]
GnRH	187	174 (93.05)	132 (75.86)	89 (67.42)
PMSG+hCG	226	175 (77.43)	118 (67.43)	63 (53.39)

[1] Percentage based on the number of embryos recovered.
[2] Percentage based on the number of embryos vitrified.
[3] Percentage based on the number of morphologically normal embryos post-thawing.

Embryos produced by superovulation were inferior as to *in vitro* post vitrification development compared to those from the control group (Table 1 & Plate 1). Only 63 (36%) of 175 superovulation produced vitrified embryos did cleave *in vitro* compared to 89 (51.15%) of 174 control embryos. Mehaisen *et al*. (2005) also found that 200 IU eCG in superovulated does did not improve the number of normal embryos recovered and had a negative effect on the *in vitro* development of embryos after thawing when compared to 50 IU eCG. In addition, Mehaisen *et al.* (2006) obtained successful results of embryo viability only in the control group (without superovulation treatment), 67.9% *in vitro* development to hatched blastocyst stage after vitrification. Moreover, Parvex (1982) found that superovulation treatment affected the development of embryos *in vitro* and *in vivo* and resulted in abnormality and death of embryos after transfer. However, Salvetti *et al.* (2007) reported that no significant differences were observed between superovulated does and control group for *in vitro* development of embryos thawed after cryopreservation reached expanded blastocyst stage. All of these reports with our results indicate that embryos recovered from superovulated donors, although may have normal morphological

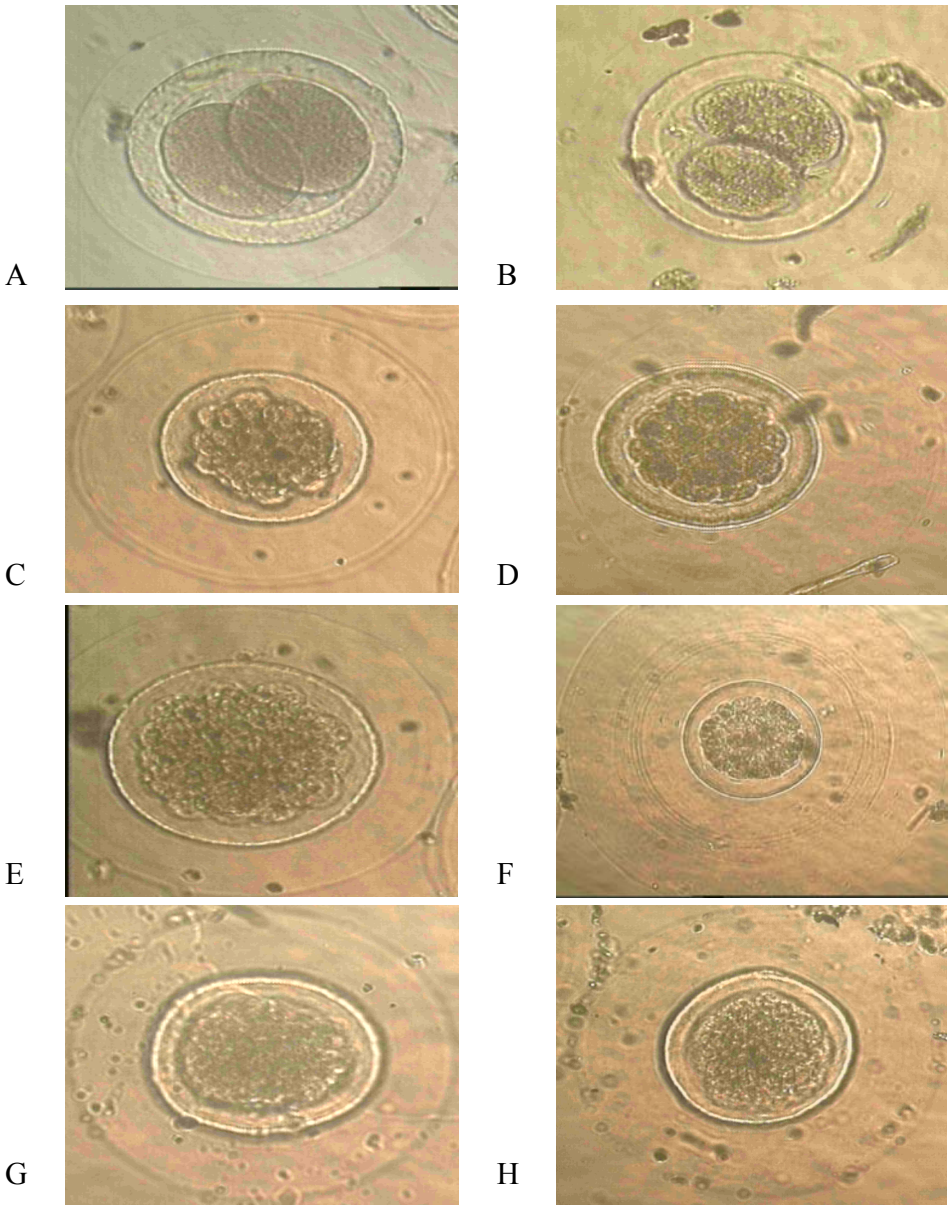

Plate 1. Pre and post vitrification rabbit embryos: (A & B) 2-cell stage (superovulated does). (C & D) 16-cell (superovulated does). (E & F) Compacted morula (GnRH treated does). (G & H) Early blastocyst developed from vitrified superovulated rabbit morula,

appearance, exhibit lower developmental potential than embryos recovered from non-superovulated donors (Kauffman *et al*., 1998). The higher sensitivity of embryos recovered from superovulated does to low temperatures leads to a decrease in their subsequent potential capacity for development after vitrification (Leoni *et al*., 2001). Vajta (2000) explained the lower viability rate after vitrification based on the number of trophoblastic cells and inner cell mass per embryo. The surviving blastomeres after vitrification procedures may be insufficient in number to permit the re-expansion of the blastocoelic cavity and continue the physiological development.

2. Effect of embryo developmental stages

No significant differences were observed among embryo developmental stages in the percentage of embryos vitrified (Table 2). However, Lopez-Bejar and Lopez-Gatius (2002) reported that blastocyst stages yielded best results compared to earlier stages of development for the cryopreservation of rabbit embryos.

The percentages of embryos with post-vitrification morphologically normal appearance were significantly ($P<0.05$) higher in later developmental stages compared to earlier stages (Table 2). These may be related to exposure of embryos to high levels of cryoprotective solutions which induce deleterious effects on the embryo development after devitrification (Gajda and Smorag, 1993; Kasai *et al.,* 1996; Leoni *et al*., 2001 & 2003), especially at the early fetal development (Mocé *et al*., 2004).

Table 2. Effect of embryo developmental stage on pre and post vitrification embryo quality and development.

Developmental stages	No. embryos recovered	Embryos vitrified *n* (%)[1]	Morphologically normal embryos post-thawing *n* (%)[2]	Embryos cleaved and developed *in vitro* *n* (%)[3]
2-cell	65	56 (86.15)	29 (51.79)[c]	7 (24.14)[c]
4-cell	68	57 (83.82)	37 (64.91)[bc]	11 (29.73)[c]
16-cell	68	61 (89.71)	47 (77.05)[ab]	24 (51.06)[b]
Morulae	97	77 (79.38)	59 (76.62)[ab]	44 (74.58)[a]
Compact Morula	88	74 (84.09)	60 (81.08)[a]	50 (83.33)[a]
Early blastocyst	27	24 (88.89)	18 (75.00)[ab]	16 (88.89)[a]

[1] Percentage based on the number of number of embryos recovered.
[2] Percentage based on the number of embryos vitrified.
[3] Percentage based on the number of morphologically normal embryos post-thawing.
[a,b,c] Values with different superscripts in the same column differ significantly ($P<0.05$).

The highest percentage of embryos cleaved *in vitro* have been obtained after devitrification at early blastocyst stage, flowed by that at compacted morulae and morulae stages, whereas the lower percentage were recorded with embryos vitrified at 2- and 4-cell stages (Table 2). The results agree with those repored by Lopez-Bejar *et al.* (1994) who found that all embryonic stages (2-cell, 8 to 16-cell, compacted morula and early blastocyst stages of rabbit embryos studied developed *in vitro* after cooling procedure; however, the best results were obtained at compacted morula and blastocyst stages. Whereas, Lopez-Bejar and Lopez-Gatius (2002) observed lower rates of *in vitro* development when the embryos were cryopreserved at the morula stage and higher rates achieved using rabbit embryos at blastocyst stage. However, Kobayashi *et al*. (1990) found the higher embryo survival rates after de-vitrification in the rabbit at the morula stage, indicating that morulae seem to tolerate osmotic changes and the toxicity of cryoprotectants and this embryo stage allows the conventional freezing procedures to be substituted efficiently by a simple method of vitrification. Ogawa and Tomoda (1976) also recorded that 69% morulae stage developed *in vitro* after frozen-thawing compared with 51% only of 16-cell rabbit embryo stage. The previous reports and our results confirm that the development stage of the cryopreserved embryo significantly affects the viability rates of rabbit morulae and blastocysts. This effect is generally observed when rabbit embryos are cryopreverved at earlier stages (2- to 4-cell embryos) (Smorag *et al*., 1989). The ratio of cell surface to volume is one of the major factors determining survival following cryopreservation (Mazur *et al*., 1984). Thus, the factors associated blastomere size and membrane permeability to water and cryoprotectants have been related to the reduced viability of embryos cryopreserved at initial stages of development (Schneider and Mazur, 1984 and Gajda and Smorag, 1993). In addition, Pollard and Leibo (1994) and (Nagashima *et a*l., 1995) mentioned that both cell size and lipid content of zygotes are factors thought to influence their sensitivity to cryoprotective additives (CPAs) and to cryopreservation.

3. Effect of donor hormonal treatment and embryo developmental stage

Superovulation produced embryos yielded lower values as to the percentage of normal verifiable embryos compared to those in the GnRH treated does especially at early developmental stages (Table 3 and Plate 1). Only 78.38% of 37 superovulation produced embryos recovered were vitrified compared to 96.43% of 28 control embryos at 2-cell stage, whereas 77.78 and 80.00% superovulation produced embryos at compacted morulae and early blastocyst stages were vitrified compared to 90.70 and 94.12% in GnRH produced embryo at the same developmental stages. In addition, it is indicated that the percentages of the devitrified embryos with morphologically normal appearance ranged from 44.83 to 85.0% including all developmental stages with different hormonal treatment with an average of 71.63%. However, the developmental ability of 2- and 4-cell embryos was low than other developmental stages (Table 3). *In vitro* survival rates of

later stage embryos were significantly higher than earlier stages, especially with superovulation produced embryos. Very high *in vitro* cleavage and development rates (84.85-92.31%) were obtained with morulae, compacted morulae and early blastocyst in control group embryos compared to 63.33-81.48% in superovulation produced embryos.

In this experiment, the overall numbers of collected embryos were 413 out of which 349 embryos were considered for vitrification (84.5%), although 60.53% (250/413) appeared morphologically normal after thawing, only 36.80% (152/413) continued to develop when cultured *in vitro*. These results showed that treating rabbit donors with GnRH yielded better embryos for cryopreservation and that embryos at the 2-cell to early blastocyst stages could soundly be vitrified, and that the morulae, compacted morulae and early blastocyst stages are potentially suitable candidates for vitrification.

Table 3. Rates of morphologically normal rabbit embryos cryopreserved at different developmental stages as affected by donor hormonal treatments.

Embryos developmental stages	Donor treatments	No. embryos recovered	Embryos vitrified *n* (%)[1]	Morphologically normal embryos post-thawing *n* (%)[2]	Embryos developed *in vitro* *n* (%)[3]
2-cell	**GnRH**	28	27 (96.43)[a]	16 (59.26)[a]	5 (31.25)[a]
	PMSG+hCG	37	29 (78.38)[b]	13 (44.83)[b]	2 (15.38)[b]
4-cell	**GnRH**	39	36 (92.31)[a]	24 (66.67)[a]	8 (33.33)[a]
	PMSG+hCG	29	21 (72.41)[b]	13 (61.91)[a]	3 (23.08)[a]
16-cell	**GnRH**	20	20 (100.0)[a]	17 (85.00)[a]	11 (64.71)[a]
	PMSG+hCG	48	41 (85.42)[a]	30 (73.17)[a]	13 (43.33)[b]
Morulae	**GnRH**	40	36 (90.00)[a]	29 (80.56)[a]	25 (86.21)[a]
	PMSG+hCG	57	41 (71.93)[b]	30 (73.17)[a]	19 (63.33)[b]
C. Morulae	**GnRH**	43	39 (90.70)[a]	33 (84.62)[a]	28 (84.85)[a]
	PMSG+hCG	45	35 (77.78)[a]	27 (77.14)[a]	22 (81.48)[a]
E. blastocyst	**GnRH**	17	16 (94.12)[a]	13 (81.25)[a]	12 (92.31)[a]
	PMSG+hCG	10	8 (80.00)[a]	5 (62.50)[b]	4 (80.00)[a]
Total		413	349 (84.50)	250 (71.63)	152 (60.80)

[1] Percentage based on the number of number of embryos recovered.
[2] Percentage based on the number of embryos vitrified.
[3] Percentage based on the number of morphologically normal embryos post-thawing.
C. Morulae= Compacted morulae and E. blastocyst= Early blastocyst.
[a,b] Values with different superscripts in the same column within embryos developmental stage differed significantly ($P<0.05$).

CONCLUSION

It could be concluded that when treating rabbit donors with GnRH prior to embryo cryopreservation programs is remarkably effective towards the production and recovery of morphologically good quality embryos and that although embryos at 2-cell to early blastocyst stages could be vitrified, yet, embryos at morula to blastocyst stages exhibited higher resistance to the negative effects of vitrification on viability when cultured *in vitro*.

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تطور أجنة الأرانب المجمدة خارج الجسم: تأثير معاملة الإناث هرمونيا و مراحل تطور الجنين

سامية زكريا مشرقي، عمر عبد الرحمن سلامة، أعتدال حسن السيد
معهد بحوث الإنتاج الحيواني، مركز البحوث الزراعية- وزارة الزراعة.

أستخدم في هذه الدراسة ٣٦ أنثى أرنب نيوزيلاندى أبيض عذراء عمر ٦-٧ شهور قسمت إلي مجموعتين. حقنت إناث المجموعة الأولى (٢٤ أنثى) ب ٢٠ ميكروجرام هرمون ال GnRH لكل أنثى في العضل للحث على التبويض الطبيعي ثم لقحت صناعيا. حقنت إناث المجموعة الثانية (١٢ أنثى) ب ٨٠ وحده دولية من هرمون ال PMSG لكل أنثى تحت الجلد ثم بعد ٦٨ ساعة حقنت في الوريد ب ٥٠ وحدة دولية من هرمون hCG لإحداث التبويض المتعدد ، لقحت صناعيا بعد المعاملة بهرمون hCG. تم ذبح عدد ٨ إناث من المجموعة الأولى و ٤ إناث من المجموعة الثانية بعد ٢٤-٢٦ و٤٨-٥٠ و٧٢-٧٤ من التلقيح وذلك لجمع الأجنة. تم تجميد الأجنة عند مراحل تطور مختلفة ثم تم تقييم حيويتها بعد الإسالة على أساس الشكل المورفولوجى و قابليتها للانقسام في بيئة الاستزراع.
أظهرت النتائج أن نسبة الأجنة المسترجعة الطبيعية التي تم تجميدها المتحصل عليها من الإناث المعاملة بهرمون ال GnRH أعلى من التي حصل عليها من الإناث المعاملة هرمونيا لإحداث التبويض المتعدد (٩٣.٠٥ مقابل ٧٧.٤٣%). كانت نسبة الأجنة المتحصل عليها من الإناث ذات التبويض المتعدد التي تبدو طبيعية مورفولوجيا بعد الإسالة أقل مقارنة بالمتحصل عليها من الإناث المعاملة بهرمون ال GnRH (٦٧.٤٣ مقابل ٧٥.٨٦%). ٦٣ (٣٦%) فقط من ١٧٥ جنين من الإناث ذات التبويض المتعدد المجمدة انقسمت خارج الجسم بعد الإسالة مقابل ٨٩ (٥١.١٥%) من ١٧٤ جنين متحصل عليها من إناث المجموعة الضابطة. لم يلاحظ فرق معنوي بين مراحل تطور الأجنة في نسبة الأجنة الطبيعية التي تم تجميدها . كانت نسبة الأجنة المسترجعة بعد الإسالة مع مظهر طبيعي مورفولوجي أعلى معنويا (على مستوى ٥%) في مراحل التطور المتأخرة مقارنة بالمراحل المبكرة أفضل نسبة أجنة انقسمت و تطورت خارج الجسم حصل عليها بعد الإسالة كانت عند مرحلة تطور جنيني البلاستوسيست المبكرة ، يتبعها مرحلة الجسم التوتي المدمج و مرحلة الجسم التوتي ، بينما أقل نسبة سجلت مع الأجنة المجمدة عند مرحلة ٢ و ٤ خلية . تم الحصول على نسبة عالية من الأجنة المنقسمة و المتطورة خارج الجسم (٨٤.٨٥ – ٩٢.٣١ %) في الأجنة المجمدة عند مرحلة الجسم التوتي ، الجسم التوتي المدمج و البلاستوسيست المبكرة المتحصل عليها من إناث المجموعة الضابطة مقارنة بـ ٦٣.٣٣ – ٨١.٤٨% تحصل عليها من إناث ذات التبويض المتعدد .
هذه النتائج تظهر أن معاملة الإناث بهرمون ال GnRH أعطت أفضل جنين للتجميد و أيضا مراحل جنين الأرنب عند الجسم التوتي و الجسم التوتي المدمج و البلاستوسيست المبكرة أفضل مراحل لتجميد الأجنة.