J. Animal and Poultry Prod., Mansoura Univ., Vol. 6(8): 555 - 566 ,2015.

MORPHOLOGICAL CHARACTERIZATION AND *In vitro* DEVELOPMENT OF VITRIFIED EMBRYOS FROM BALADI AND APRI RABBIT .

Fahim, H.N. ; M.A Shitifa. and Samia Z. Meshreky
Animal Production Research Institutes, Agriculture Research center, Dokki, Giza, Egypt,
hanoysliman@yahoo.com

ABSTRACT

A total of 185 morphologicaly normal embryos were collected from 32 superovulated multiparous baladi black (BB) and APRI donor rabbits. Embryos collected were divided into three groups. The first group (n=63) was non-vitrified (used as fresh control) group and the second group (n=60) was vitrified by one step ((DMSO alone), the third group (n=62) was vitrified by two step (DMSO+EG). Embryos vitrified by one and two step methods storage in liquid nitrogen at 15 day post thawing. Results showed that the two-steps vitrification procedure gave insignificantly better results of post-thawing morphologically normal embryos grade excellent (38.71%) compared to the one-step vitrification procedure (31.67%). The total morphological normal embryo recovered post- thawing in excellent and good grades as affected by vitrification procedure and genotype (breeds) showed that BB was high insignificantly compared with APRI vitrified by the two steps vitrification method (77.42 *vs*. 74.19%) or one step (72.41 *vs*. 70.97%). The fresh APRI embryos gave significantly ($P<0.05$) total embryo diameter thick compared with the same embryo breed vitrified by one or two step methods (424.8±3.8 *vs* 365.8±3.6 and 360.5±3.5 µm). The BB rabbit have the smallest total embryo diameter vitrified by two or one step methods (351.7±4.0 and 357.1±4.0µm) compared to BB intact embryo (409.4±4.1 µm). Only 19/60 (31.67%) vitrified embryos by one step method reached hatching blastocyst stages in culture medium post thawing compared to 28/62 (45.16%) vitrified embryo by two steps, the difference was significant. The highest percentages of embryos cleaved *in vitro* reached hatching blastocysts stage have been obtained from APRI (75.00%) and BB (74.19%) un-vitrified embryos, followed by BB (48.39%) and APRI (41.93%) embryos vitrified-thawing by two steps vitrification method, whereas the lower percentages were recorded with embryos vitrified-thawing by one step procedure in APRI (32.26%) and BB (31.03%) embryos. The present study demonstrate that the two step vitrification protocol (DMSO+EG) provides superior embryo survival rates over the one step (DMSO alone) vitrification protocol for morulae stage rabbit embryos for Baladi black or ABRI rabbits.

Keyword: Vitrification, one step, two step, rabbit breed.

INTRODUCTION

Cryopreservation is an important tool for creation of embryo banks for future use in animal breeding, veterinary and human medicine. In certain cases optimization of cryopreservation protocols for more sensitive embryos is relevant. The quick freezing in liquid nitrogen (vitrification) is an important tool for preservation of mammalian embryos. At this temperature, all biochemical activities, which can lead to cell death, are effectively stopted (Özkavukcu and Erdemli, 2002). Vitrification technique, introduced for rabbit embryo cryopreservation by Kobayashi *et al.* (1990), improved general

efficiency of embryo survival. Rabbit embryos have been successfully cryopreserved by classical one-step or two-step vitrification (Kasai *et al*., 1992; Kauffman *et al*., 1998*;* Silvestre *et al.,* 2003; Naik *et al*., 2005), open pulled straw (OPS) vitrification (Naik *et al.,* 2005) or modified (sealed) OPS procedure (López-Béjar and López-Gatius, 2000).

Despite intensive research, procedures of cryopreservation still cause biochemical and morphological changes, which may result in loss of embryo viability and even induce cell death. Analysis of the viability and quality of vitrified/devitrified embryos is of great importance (Popelková *et al*., 2005).

Embryo measurement are considered marker of embryo quality after warming. Thickness of the zona pellucida, as well as diameter of intrazonal and embryo with its coverings ranged between 19.3-27.4, 87.5-158.8 and 290.0-392 µm in different rabbit breeds treated with 0.2 or 0.4 ml GnRH (El-Keraby *et al*., 1991).

Viability of embryos post thawing depends not only on the type of hormone and the method of cryopreservation, but also differs among rabbit breeds (Bolet *et al*., 2000). From a genetic point of view, in term of re-establishing a line or strain, the number of donors with offspring and the offspring identification are important. Because of this, it is necessary to ensure embryo recovery from the greatest number of donor does and to define both optimal cryopreservation and transfer methods. On the contrary, current research methods related to embryo cryopreservation have focused on technical factors which affect the process efficiency, but very few comparative studies have been done on the interactive relationship between technical factors and animal genotype (Maurer and Haseman, 1976; Vicente and Garcia-Ximénez, 1993) in rabbits.

Therefore, the main objective of the present study was to assess quality and viability of rabbit embryos post-thawing vitrified by two procedures in two genetic resources (Baladi Black rabbits as a local breed and APRI as a synthetic line).

MATERIALS AND METHODS

Thirty-two multiparous rabbit does (16 APRI and 16 Baladi black) with an average live body weights (2.8-3.5 kg) were used as embryo donors in the present work, and five fertile bucks from each breed were used as semen donors does were housed in individual metal cages for 12 weeks prior to hormonal treatments to eliminate the chances of pseudopregnancy. Animals were kept under controlled 16h light: 8h dark photoperiod and fed ad-libitum with a commercial pelleted diet contained 17.5 % crude protein, 13.6% crude fibers and 2.5 % fat. Fresh potable water was made available all times through stainless nipples.

Embryo recovery and evaluation:

Does from each breed were intramuscularlly injected with 150 IU PMSG (Folligon, Intervet, International B.V. Boxineer-Holland), followed by 75 IU of HCG (Pregnyl, Organon-oss, Holland) in ear vein at the time of natural mating (after 72 h of PMSG injection). Does were mated with fertile

bucks fom the same breed. All does were slaughtered at 72-74 h after mating. The reproductive tract (oviduct and uterine horns) was removed and embryos were recovered by flushing twice with 5 mL of Dulbecco´s Phosphate Buffered Saline (®DPBS, (PBS: Gibco, Cat. No 21300-017, UK) supplemented with CaCl2 (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 were washed twice in fresh DPBS supplemented with 10% FCS (Fetal Calf Serum) and antibiotics, counted and evaluated for their stage of development and morphological appearance using an inverted microscope fitted with a calibrated eye piece micrometer reflected on stereomicroscope. Embryos with no abnormalities in mucin coat, zona pellucida and with homogenous blastomeres were scored as excellent and good embryos (excellent- ideal embryo: spherical, symmetrical with cells of uniform size, and texture; 2. Good-trivial imperfections: a few extruded blastomeres with irregular shapes and few vesicles) according to International Embryo Transfer Society classification.

Vitrification and devitrification:

Morphological normal embryos from each breed were divided into three groups, the 1st group was saved as control (fresh), whereas the 2nd and the 3rd groups were cryopreserved by one of the vitrification methods (one-step or two-step).

In one step vitrification method, embryos were placed for 60 seconds in a vitrification solution contain 20% (v/v) dimethyl-sulfoxide (3.5M DMSO, Sigma) in DPBS supplemented with 0.2% (w/v) of FCS then loaded in straws and plunged in liquid nitrogen at -196°C.

In two steps vitrification method, described by Vicente *et al. (1999*), the cryoprotective solution was a 1:1:2 solution (v/v/v) of (3.5 M DMSO, Sigma), ethylene glycol (4.4 M EG, Sigma), in DPBSCa (DPBS supplemented with 0.132 g CaCl2/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 4 to 5 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 s. 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 zona pellucida were *in vitro* cultured.

Embryo measurements (thickness of mucin coat and zona pellucida, diameter of intrazonal and embryo with its coverings) were determined from the same images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a ×40 objective and ×10 eyepieces.

Post-vitrification in vitro development

The non-vitrified (fresh) and warmed embryos were cultured in a standard *in vitro* culture condition for 72 h in 50 µl microdrops of Ham's F10 medium+ + 10% FCS (Sigma) under mineral oil (Sigma) at 38.5 °C in an incubator containing 5% CO2 and humidified air. The in vitro cleaved and development ability of devitrified embryo were assessed and recorded for analysis.

Statistical Analysis

Chi-squared test was used with data in percentage . Data for embryo diameters were expressed as mean values ±S.E. and assessed by analysis of variance using GLM procedure of SAS ® Program (1998). Differences between groups at $P<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Embryo quality post-thawing:

Results in Table 1 showed that the two-steps vitrification procedure (DMSO+EG) gave insignificantly better results of post-thawing morphologically normal embryos (excellent, 38.71%) compared to the one-step vitrification procedure (DMSO alone, 31.67%). These results are in agreement with Salvetti *et al. (2007),* who found that DMSO and EG mixtures of cryoprotectants may have some advantages over solutions containing only one solute. Also, the mixture of EG and DMSO improved post-warming survival of oocytes and early embryos at various stages *(*Cai *et al., 2005).* Furthermore the use of high concentrations of cryoprotectant in vitrification dramatically reduces the risk of intracellular ice formation *(*Valdez *et al., 1992)*. Only 43 embryos from 60 (71.67%) vitrified by the one-steps vitrification procedure appeared morphologically normal (in excellent and good grades) after thawing compared to 47/62 (75.81%) vitrified by the two-steps vitrification method (Table 1). These results are in accordance with Meshreky *et al. (2012a)* who found 67.0% post-thaw morphologically normal embryos vitrified by two-step vitrification procedure with EG+DMSO as compared to 70.8% vitrified by one-step vitrification procedure with EG+Ficoll+sucrose. The composition of the vitrification solution (permeating and non-permeating macromolecules or saccharides) is among the factors influencing the cryosurvival of embryos. Also Vicente *et al. (1999)* using the same vitrification procedure used in this study, observed an embryo survival of 70-71%.

Results in Table 1 showed that the percentage of verifiable embryos post-thawing in excellent grade recovered from APRI does was insignificantly higher (37.10%) than that obtained from Baladi black (BB) donors (33.33%, Table 1). Though the total morphological normal embryos recovered post-thawing in excellent and good grades as affected by genotype (breed) showed that BB were high insignificantly compared with APRI (75.00 vs. 72.58%).

In regards to the embryo quality in two steps vitrification method, 41.93% (13/31) of embryos recovered from APRI does appeared morphologically normal in excellent grade after thawing vs. 35.48% (11/31) of embryos collected from BB does (Table 1). Whereas, the total morphological normal embryo recovered post-thawing in excellent and good grades as affected by interaction between vitrification procedure and genotype (breeds) showed BB were insignificantly higher than APRI with the two steps vitrification method (77.42 vs. 74.19%) or one step (72.41 vs. 70.97%). In addition, results showed that percentage of embryos in poor grade vitrified by one step vitrification procedure with increased APRI donors (19.35%), followed by two step vitrification procedure with the same breed (16.13%) compared to embryos vitrified by two (12.90%) or one step (10.34%) vitrification procedures with BB does rabbits (Table 1). Only 62.1-67.4% of vitrified-thawed NZW rabbit embryos appear undamaged *(*Meshreky *et al. (2012b).* These phenomenon may be explain by Fabian *et al., (2005)* who observed that vitrification lead to morphologic changes and DNA fragmentation in pig embryos and *(*Fair *et al., 2001)* a negative effect on the ultrastructure of cattle embryos.

Table 1. Morphologically normal embryos and quality recovered post-thawing as affected by vitrification methods, source of genetic (breed) and their interaction.

Factors	Total number of normal embryos vitrified	Embryo quality post-thawing† No. (%)			
		Excellent	Good	Fair	Poor
Vitrification methods:					
One-Step (DMSO)	60	19 (31.67)	24 (40.00)	8 (13.33)	9 (15.00)
Two-Steps (DMSO+EG)	62	24 (38.71)	23 (37.10)	6 (9.68)	9 (14.52)
Breed:					
APRI	62	23 (37.10)	22 (35.48)	6 (9.68)	11 (17.74)
Baladi Black	60	20 (33.33)	25 (41.67)	8 (13.33)	7 (11.67)
Vitrification methods with breed:					
One-Step x APRI	31	10 (32.26)	12 (38.71)	3 (9.68)	6 (19.35)
One-Step x BB	29	9 (31.03)	12 (41.38)	5 (17.24)	3 (10.34)
Two-Step x APRI	31	13 (41.93)	10 (32.26)	3 (9.68)	5 (16.13)
Two-Step x BB	31	11 (35.48)	13 (41.94)	3 (9.68)	4 (12.90)

† Percentage based on the total number of morphologically normal embryos vitrified /number of embryo after thawing %.

Embryo measures post-thawing:

Results in Table 2 revealed that embryo measures as additional marker of embryo quality after warming showed significant ($P<0.05$) differences between the vitrified and intact fresh embryos. The diameter (µm) of interzonal of the recovered embryos was significantly($P<0.05$) thicker than one and two step vitrification methods (126.3 vs. 109.7 and 106.9 µm), respectively. On the other hand zona pellucida thickness was significantly($P<0.05$) thicker in fresh embryos than one and two step methods (25.4 vs 22.8 and 22.7 µm) respectively. The total diameter embryo was significantly ($P<0.05$) lower in embryo groups vitrified by one or two step vitrification methods (361.4 and 358.1 µm) than that recorded in intact embryos group (417.1 µm) . There were no significant differences between the two vitrification methods in embryo measurement, These results are inagreement with Meshreky *et al. (2012b)* who observed significance difference in interzonal rabbit embryo diameter between vitrified groups and control group (123-124 vs. 129 µm). Also, Chrenek *et al. (2014)* found that interzonal rabbit embryo diameter was 123.2±7.2 after cryostorage and devitrification µm compared to 129.85±10.9 µm in the intact embryos (un-vitrified).

The obtained results was for two breeds (APRI and BB) were insignificant for interzonal diameter and zona pellucida thickness in the normal morphologically embryo post-thawing (Table 2). while, results recorded significant difference ($P<0.05$) in the normal morphologically total embryos diameter post-thawing between APRI and BB rabbit (383.7 µm vs 372.6 µm).

The effect of interaction between vitrification methods with rabbit breed. Indicated that fresh APRI and fresh BB embryos gave significantly ($P<0.05$) higher total embryo diameter than that of vitrified by one and two step methods for each breed (Table 2). The fresh APRI embryos gave significantly ($P<0.05$) total embryo diameter higher than that vitrified by one or two step methods (424.8±3.8 vs 365.8±3.6 and 360.5±3.5 µm). The BB rabbit have the smallest total embryo diameter vitrified by two or one step methods (351.7±4.0 and 357.1±4.0µm) as compared to intact embryos (409.4±4.1 µm). These results indicated that cryoprotectant concentrations and vitrification procedure affected embryo diameter. Also, Kasai *et al. (1992)* observed differences between vitrification media with respect to maximum swelling and suggested that the EG medium has a lower capacity to permeate embryo cells than the EG+ DMSO medium. Moreover, the high degree of swelling of the embryo cell mass caused interzonal volumes increase of about 105%, despite the presence of zona pellucida *(*Vicente and Garcia-Ximenes*., 1994).* Kasai *et al. (1994)* reported that attempt during the addition process, the final cryoprotectant concentrations are usually reached in two steps, although it is possible to achieve it by one step, reducing the possible toxic effect of cryoprotectants.

Table 2. Embryo measurements post-thawing as affected by vitrification methods, source of genetic (breed) and their interaction.

Factor	Interzonal layer diameter (μm)	Zona pellucida thickness (μm)	Total diameter embryo (μm)
Vitrification methods:			
Fresh	126.3a±2.1	25.4a±0.43	417.1a±2.8
One-Step (DMSO)	109.7b±2.0	22.8b±0.41	361.4b±2.7
Two-Steps (DMSO+EG)	106.9b±2.0	22.7b±0.40	358.1b±2.7
Breed:			
APRI	114.3±1.5	23.6±0.32	383.7a±2.12
Baladi Black	113.1±1.7	23.3±0.35	372.6b±2.32
Vitrification methods with breed:			
Fresh x APRI	126.4±2.9	25.6±0.58	424.8a±3.8
Fresh x Baladi Black	126.2±3.1	25.3±0.62	409.4b±4.1
One-Step x APRI	108.6±2.7	22.6±0.56	365.8c±3.6
One-Step x Baladi Black	110.8±3.0	23.0±0.61	357.1cd±4.0
Two-Step x APRI	107.8±2.6	22.7±0.53	360.5cd±3.5
Two-Step x Baladi Black	105.9±3.0	22.6±0.61	351.7d±4.0

[a, b] **Values with different superscripts in the same column for each factor, differ significantly (P<0.05).**

In vitro embryos development post-thawing:

Results in table 3 showed that in vitro embryos developmental stages post-thawing were significantly (P<0.05) affected by vitrification procedure. *In vitro* embryos development to blastocysts, expanded blastocysts and hatching blastocysts stages were 95.24, 85.71 and 74.60%, respectively in unvitrified embryo group (fresh), being better than that vitrified-thawing by two or one step vitrification methods. No significant difference was found in developmental potent to expanded blastocysts stage of warmed embryos vitrified by either one or two steps. Only 19/60 (31.67%) vitrified embryos by one step method reached hatching blastocyst stages in culture medium post thawing compared to 28/62 (45.16%) vitrified embryo by two steps, the difference was significant (P<0.05). Our observation indicates that rabbit embryos after freezing have altered their viability and quality. Earlier reports *(*Fabian *et al., 2005 and* Popelkova *et al., 2005)* documented that post-thaw embryo survival can be a valid tool to evaluate the efficiency of cryopreservation technique. Our results are in close agreement with Cristina Cuello *et al. (2004),* who found that the hatching rate of fresh morula and the early blastocyst stage embryos was higher than their vitrified counterparts. Meshreky *et al. (2012b)* found that blastocyst and hatching rates ranged between 51.7-58.9 and 39.8-47.4% in vitrified-thawed embryos groups as compared to 94.7 and 90.4% in control group (unvitrified) . After cryostorage and devitrification almost 73 % of embryos survived and developed to advanced blastocyst stage versus 96 % in the intact control (unvitrified) .*(*Chrenek *et al., 2014)*. This difference between vitrified and intact embryos can be explained by their different dynamics of the development: embryos following devitrification need some time to recover from the deep freezing and, therefore, have the delayed proliferation and the lower cell number

compared to the intact embryos. The improved results of post-thawing quality and developmental potent of rabbit embryos vitrified by two step method (DMSO+EG) compared to one step method (DMSO alone) in this work may be due to EG and may have the advantage of lower toxicity *(*Kasai *et al., 1996).* EG has a lower molecular weight than DMSO, which facilitates a rapid permeation into the cell during short-term exposure and a rapid removal from the cell after warming. These properties prevent both toxic and osmotic injuries *(*Massip, *2001).* In two-step freezing, Hochi *et al. (2004*) observed that 52% of pronuclear-stage rabbit zygotes developed beyond early blastocysts during 120 h of post-thaw culture. However, Meshreky *et al. (2012a)* found that 44.9% (74/165) of embryos recovered from does treated with eCG/hCG reached hatching blastocysts stage vitrified by one-step vitrification method (EG+Ficoll+sucrose) vs. 37.5% (63/168) vitrified by two-step vitrification procedure with EG+DMSO.

Results in Table 3 showed that the in vitro embryos development post-thawing was not affected by genotype (rabbit breed). The BB embryos vitrified-thawing had insignificantly higher percentages to develop to blastocyst, expanded blastocyst and hatching blastocyst stages compared to APRI breed. There was a significant increase in the development of rabbit embryos after thawing in the R line than in the V line *(*Vicente*, 2003).* The lower development post-vitrification of V line embryos in comparison with the R line embryos may be explained by differences in sensitivity of morulae to vitrification procedure between selection lines. Moreover, García *et al. (2000)* observed differences in the survival rate at birth between vitrified embryos belonging to lines V and R (43% vs. 22%, respectively).

Results of interaction between vitrification methods with genotype (breed) indicated that the highest percentages of embryos cleaved in vitro reached hatching blastocysts stage have been insignificantly obtained from APRI (75.00%) and BB (74.19%) un-vitrified embryos, followed by BB (48.39%) and APRI (41.93%) embryos vitrified-thawing by two steps vitrification method, whereas the lower percentages were recorded with embryos vitrified-thawing by one step procedure in APRI (32.26%) and BB (31.03%) embryos (Table 3). These results are in agreement with Maria Ribese *(2014),* who found that embryo cryopreservation with slow freezing or vitrification decrease rabbit embryo survival rate between 20-50%. This percentage depends on the genetic stock and the procedure followed. Also, Vicente *(2003)* mentioned that the differences between lines in transferable embryos and cryopreservation efficiency (live-born at birth/vitrified or frozen embryos) were in accordance with the results from previous studies carried out to define the optimal freezing procedure *(*Vicente *and* García-Ximénez*, 1994;* García *et al., 2000).* Schmid*t et al. (1987)* observed differences in the tolerance to cryoprotectants and freezing procedures in accordance with the studies in a mouse line.

It can be concluded that the present study demonstrates that the two step vitrification protocol(DMSO+EG)provides superior emb ryo survival rates over the one step vitrification protocol (DMSO alone) Baladi black rabbit embryos at morula stage

Table 3. *In vitro* developmental stages post-thawing as affected by vitrification methods, source of genetic (breed) and their interaction.

Factors	Number of normal embryos vitrified	*In vitro* developmental stages post-thawing† No. (%)		
		Blastocyst	Expanded blastocyst	Hatching blastocyst
Vitrification methods:				
Fresh	63	60 (95.24a)	54 (85.71a)	47 (74.60a)
One-Step (DMSO)	60	37 (61.67b)	27 (45.00b)	19 (31.67c)
Two-Step (DMSO+EG)	62	42 (67.74b)	34 (54.84b)	28 (45.16b)
Breed:				
APRI	94	70 (74.47)	57 (60.64)	47 (50.00)
Baladi Black	91	69 (75.82)	58 (63.74)	47 (51.65)
Vitrification methods with breed:				
Fresh x APRI	32	31 (96.87a)	27 (84.37a)	24 (75.00a)
Fresh x BB	31	29 (93.55a)	27 (87.10a)	23 (74.19a)
One-Step x APRI	31	19 (61.29b)	14 (45.16c)	10 (32.26c)
One-Step x BB	29	18 (62.07b)	13 (44.83c)	9 (31.03c)
Two-Step x APRI	31	20 (64.52b)	16 (51.62bc)	13 (41.93bc)
Two-Step x BB	31	22 (70.97b)	18 (58.06b)	15 (48.39b)

† Percentage based on the total number of morphologically normal embryos vitrified /number of embryo after thawing %.

[a, b, c] Values with different superscripts in the same column for each factor, differ significantly (P<0.05).

REFERENCES

Bolet, G., J.M. Brun, M. Monnerot, F.. Viudes de Castro, J.S. Virag and J.M. immermann (2000). Evaluation and conservation of European rabbit (Oryctolagus cuniculus) genetic resources. First results and inferences, Proceedings of 7th World Rabbit Congress, vol. A Valencia, Spain., 281–315.

Cristina Cuello, A; M. Antonia Gila, Inmaculada Parrillaa, Jose Tornela, Juan M. Vázqueza, Jordi Rocaa, Françoise Berthelotb, Françoise Martinat-Bottéb and Emilio A. Martíneza (2004). In vitro development following one-step dilution of OPS-vitrified porcine blastocysts. Theriogenology Vol, 62, Issue 6 , p.p 1144-1152 .

Cai, X.Y.; Chen, G.A.; Lian,Y.; Zheng,X.Y. and Peng, H.M.(2005). Cryoloop vitrification of rabbit oocytes. Hum. Reprod , 20: 1969 – 1974.

Chrenek, P., A.V. Makarevich and E. Kubovičová (2014). Developmental potential of vitrified rabbit embryos. The 2nd International Scientific Conference, Animal Biotechnology, Slovak J. Anim. Sci., 47, (4): 198-201.

El-Keraby, F.; Tork M.Dorra; A.I. Abd El-Ghani and S.A. Fawzy (1991). Effect of GnRH administration rate and embryo recovery in some rabbit breeds. J. Agric. Sci., Mansoura Univ.,16 (8);1749-1757.

Fabian D, Gjorret J.O, Berthelot F., Martinat-Botte F., Maddox-Hyttel P. (2005). Ultrastructure and cell death of in vivo derived and vitrified porcine blastocysts. Mol Reprod Dev ;70:155–165.

Fair T., Lonergan P., Dinnyes A., Cottell D.C., Hyttel P, Ward F.A., Boland M.P (2001). Ultrastructure of bovine blastocysts following cryopreservation: effect of method of blastocyst production. Mol Reprod Dev ;58:186–195.

García, M.L.; O. Blumetto; G. Capra ; J.S. Vicente and M. Baselga (2000). Vitrified embryos transfer of two selected Spanish rabbit lines to Uruguay, Proceedings of 7th World Rabbit Congress,. Valencia, Spain3., 139–142

Hochi, S; T. Teraoa; M. Kameib; M. Katoc; D.M. Hirabayashid; M. Hiraob(2004). Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. Theriogenology 61: 267–275 .

Kasai, M.; Y., Hamaguchi; S.E. Zhu; T. Miyake; T, Sakurai and T. Machida (1992). High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. Biol Reprod .,46:1042–1046.

Kasai,M; T.Tominaga; T.Mori. T.Aono and M.Hirio (1994). Cryopreservation of mammalian embryos by vitrification. Perspectives on Assisted Reproduction. Ares-Serono Symposia Publications, Roma., 481–487.

Kasai M., Zhu S.E., Pedro P.B., Nakamura K., Sakurai T., Edashige K. (1996). Fracture damage of embryos and its prevention.

Kauffman R.D., Schmidt P.M., Rall W.F., Hoeg J.M.(1998). Superovulation of rabbits with FSH alters in vivo development of vitrified morulae. Theriogenology, 50, 1081–1092.

Kobayashi, K; H. Nagashima; H.Yamakawa; Y. Kato and S. Ogawa. (1990). The survival of whole and bisected rabbit morulae after cryopreservation by the vitrification method. Theriogenology 33: 777-788

López-Béjar, M. and F. López-Gatius (2000). In vitro and in vivo survival of vitrified rabbit embryos. Theriogenology., 53: 25958: 1541–1552.

Maria, D.S. Ribes (2014). Study of the effect of cryopreservation on rabbit embryo gene expression. Thesis submitted to the Polytechnic University of Valencia.

Maurer RR, Haseman JK. (1976). Freezing morula stage rabbit embryos. Biol Reprod, 14: 256–263.

Massip A.(2001). Cryopreservation of embryos of farm animals. Reproduction in Domestic Animals 36, 49-55.

Meshreky, Samia Z. , Abdel-Aal E.S.1., Fahim H.N.1., Ashmawy N. A. (2012a) Effect of eCG Dose and Vitrification Methods on inVitro Survival Rate of Rabbit Embryos Proceedings 10 th World Rabbit Congress – September 3 - 6, 2012– Sharm El- Sheikh –Egypt, 297 – 301.

Meshreky, Samia Z., Fahim H.N., Abdel-Aal E.S., Ibrahim S.E.(2012b) Effect of Vitrification and Cryostorage length on Viability of Rabbit Embryos After thawing. World Rabbit Science Association Proceedings 10 th World Rabbit Congress – September 3 - 6, Sharm El- Sheikh –Egypt, 303 – 30.

Naik B.R., Rao B.S., Vagdevi R., Gnanprakash M., Amarnath D., Rao V.H.(2005). Conventional slow freezing, vitrification and open pulled straw (OPS) itrification of rabbit embryos. Anim. Reprod. Sci.;86 (3-4):329-38.

Özkavukcu, S. and Rdemli, E. (2002). Cryopreservation: Basic knowledge and biophysical effects. *J. Ankara Med. School, vol. 4, p. 187 – 196.*

Popelkova, M., Chrenek, P., Pivko, J. Makarevic, A.V.- Kubovicova,E, Kacmarik, J.(2005). Survival and ultrastructure of gene-microinjected rabbit embryos after vitrification. Zygote, Vol. 13, 2005, p. 283-293

SAS (1998). SAS/STAT User's Guide (Release 6.03). SAS Inst. Inc., Cary NC, USA.

Schmidt MP., Schiewe MC., Wildt DE.(1987). The genotypic response of mouse embryos to multiple freezing variables. Biol. Reprod., 37:1121–1128

Silvestre,M.,A.MSaeed.,M.J.Escribá and F. García-Ximénez (2003) Vitrification of in vitro cultured rabbit morulae . Animal Reproduction Science., Volume 76,Pages 113-124

Salvetti P, Theau-Clément M., Beckers J.F., Hurtaud J., Guérin P., Neto V., Falières J., Joly, T. (2007). Effect of the luteinizing hormone on embryo production in superovulated rabbit does. Theriogenology 67,1185-1193.

Vicente, J.S.; M.P. Viudes-de-Castro and M.L. Garcia (1999). In vivo survival rate of rabbit morulae after vitrification in a medium without serum protein. Reprod. Nutr. Dev., 39 : 657–662.

Vicente and F. Garcia-Ximenes (1994) Laboratory of reproduction, department of animal science polytecnical university of Valencia. Theriogenology 42: 1205-1215.

Valdez, C.A.; Abas Mazni, O.; Takahashi, Y.; Fujikawa, S. and Kanagawa, H. (1992). Successful cryopreservation of mouse blastocysts using a new vitrification solution. J. Reprod. Fertil., 96:793–802.

Vicente et al., (2003) J.S. Vicente, M.P. Viudes-de-Castro, M.L. García and M. Baselga, Effect of rabbit line on a program of cryopreserved embryos by vitrification, Reprod. Nutr. Dev. 43, pp. 137–143.

Vicente JS, Garcia-Ximénez F. (1993). Effects of two cryoprotective agents used in slow or rapid standard freeze-thaw procedure on the in vivo survival of morulae from three rabbit strains. INIA, 8: 247–256.

الخصائص الفسيولوجيه مع تطورالاجنه المجمده معمليا لسلالات الارانب البلدي الاسود و الأبري

هاني نبيل فهيم ، محمد عبد الغني شطيفه و ساميه زكريا مشرقي
معهد بحوث الإنتاج الحيواني ـ مركز البحوث الزراعية ـ وزارة الزراعة ـ الدقي ـ جيزة.

تم أجراء التجربه علي عدد ٣٢أنثي ارانب متعددة الولادات (١٦ أنثي بلدي اسود+ ١٦ أنثي من سلاله الابري) وكان عدد الاجنه المتحصل عليه من هذه الامهات يصل الي ١٨٥ جنين و التي تم جمعها عن طريق معاملات الامهات هرمونيا لاجراء تعدد التبويض حـ يث تم تقسيم الاجنه الي ٣ مجاميع . الاولي و تسمي بمجموعه الكونترول و الثانيه التجميد بخطوه واحده (استخدام داي ميثيل سلفو اكسيد فقط) و الثالثه التجميد بخطوتين (استخدام داي ميثيل سلفو اكسيد+ ايثيلين جليكول) و كان عدد الاجنه ٦٣ في مجموعه الكونترول و يصل الي ٦٠و٦٢ ف المجاميع الثانيه و الثالثه علي التولي. ثم وضع الاجنه المجمده للمجموعتين ٢و٣ في النتروجين السائل لمده ١٥ يوم وبعده يتم اساله الاجنه لدراسه خصائصها مع دراسه تطوره بعد الاساله لمده ٧٢ ساعه. و كانت ابرز النتائج المتحصل عليها كما يلي:-

١- التجميد بخطوتين أعطي زيادة غير معنويه للأجنه الطبيعيه المتحصل عليها بعد الاسالة و الحاصلة علي تقييم ممتاز في جودتها بنسبه ٣٨.٧١% مقارنه بالتجميد بخطوه واحده (٣١.٦٧%).

٢- أوضحت نتائج البحث ان اجراء التجميد بخطوتين (استخدام داي ميثيل سلفو اكسيد+ ايثيلين جليكول تعطي افضل النتائج مقارنه باستخدام التجميد بخطوه واحده (استخدام داي ميثيل سلفو اكسيد فقط) خلال مرحله الموريوللا لأجنه الأرانب المتحصل عليها من سلالات البلدي الاسود و الابري.

٣- النسبه المئويه لأجمالي عدد للاجنه و التي تم فحصها ظاهريا و الحاصله علي تقييم ممتاز و جيد و المتأثره بطريقه التجميد مع نوع السلاله كانت أعلي بصوره غير معنويه مع سلاله البلدي الاسود مقارنه مع سلاله الابري عند أجراء التجميد بخطوتين (٧٧,٤٢ مقابل ٧٤,١٩%) أو بخطوه واحده (٧٢,٤١ مقابل ٧٠,٩٧%).

٤- كانت هناك زياده معنويه واضحه لصفه سمك القطر الكلي للجنين لسلاله الابري الغير معامله بأي من طرق التجميد (الكونترول) مقارنه بأجنه نفس السلاله المجمده بطريقه خطوه او خطوتين (٤٢٤,٠٨ مقابل ٣٦٥,٠٨ و٣٦٠,٥٠ملليميكرون). لوحظ ان أجنه سلاله البلدي الاسود اعطت اقل سمك في القطر الكلي للجنين و التي تم تجميدها بطريقه خطوتين او خطوه واحده (٣٥١,٧ و ٣٥٧,١ ملليميكرون) مقارنه بالاجنه الغير معامله و التي تصل ابعادها الي ٤٠٩,٤ ملليميكرون.

٥- وجدت فروق معنويه واضحه عند دراسه النسبه المئويه لتطور الاجنه في المعمل فكانت٣١,٦٧% للوصول لمرحله البلاستوسيت عند اجراء التجميد بخطوه واحده مقارنه نسبه ٤٥,١٦% عند أجراء التجميد بخطوتين لنفس المرحله. و كانت اعلي نسبه مئويه في أجنه الابري و يليها سلاله البلدي الاسود والتي لم يتم معاملتها بأي من طرق التجميد بنسبه ٧٥ و ٧٤,١٩ % علي التوالي و يليهم الاجنه المتحصل من التجميد بخطوتين مع البلدي الاسود و الابري بنسبه ٤٨,٣٩ و ٤١,٩٣ % علي التوالي.