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AN OVERVIEW ON CRYOPRESERVATION AND THAWING METHODS OF RABBIT EMBRYOS USED IN EMBRYO TRANSFERE.

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ABSTRACT

To defined the differences in ability of NZW rabbit embryos to survive with different freezing methods (vitrification, step wise and direct methods), donor does were i.m. injected with 75 unit from PMSG 72 h before mating and embryos were collected from slaughtered does 72 h after natural mating. Each preservation method was followed by special thawing method, or by one method (thawing of direct method). After thawing, embryos were *in vitro* cultured in TCM-199 for three days and embryo characteristics were measured. Results revealed that during cryostorage, mucin coat (MC) was thicker (P<0.05) for vitrification and direct methods than that for step wise method (111 and 114 vs. 104.8 µm, respectively). Thickness of zona pellucid (ZP) was thicker (P<0.05) for step wise and direct methods than that for vitrification method (23.9 and 23.9 vs. 21.8 µm, respectively). However, diameter of intrazonal (IZ) and total embryo (TE) was not affected significantly by preservation method. Only**,** thickness of MC and ZP reduced (P<0.05) post- than pre-preservation. Post thaw embryo recovery rate was higher (P<0.05) in direct method than vitrification and step wise methods (100% vs. 91.3 and 90%, respectively). Up to the 2nd day of culture, direct method resulted in the highest (P<0.05) viability rate, followed by step wise, while vitrification showed the lowest values. At the 3rd day of culture, step wise method showed the best (P<0.05) viability rate; followed by direct method, while vitrification still to be the lowest. Viability rate of embryos after preservation by three methods, thawed by one method, and *in vitro* cultured at three successive days show that, direct preservation method resulted in significantly (P<0.05) the highest viability rate (100%), followed by step wise (71.4%), while vitrification showed the lowest values (57.1%). No viable embryos were obtained on the 3rd day of culture with vitrification method and 85.7% of thawed embryos were examined in hatched blastocyst stage with direct method on the 3rd culture day versus 28.6% with step wise method. As affected by culture day after thawing, thickness of MC and diameter of IZ and TE increased (P<0.05) and thickness of ZP decreased (P<0.05) by progressing culture day.

In conclusion, cryostorage of embryos recovered from superovulated rabbits after 72 of mating could be carried out successfully by direct method without harmful effects on embryo characteristics, yielded the highest number of good quality embryos on thawing and supported the highest *in vitro* development of embryos to hatched blastocyst stage.

Keywords: Rabbit, embryo characteristics, vitrification, step wise, direct method, viability rate.

INTRODUCTION

Cryopreservation of mammalian embryos for different times prior to culture or transfer to recipient females is commonly carried out. The efficiency

of embryo cryopreservation is affected by a drop in viability after thawing. Also, embryo developmental stage is considered to be a critical factor for the viability of the embryo after freezing and thawing. This fact has stimulated the development of several techniques and freezing protocols in the recent decades (Cocero *et al*., 1996 and Leibo *et al*., 1996).

Conventional slow freezing and vitrification methods are the commonly used for long-term storage of pre-implantation mammalian embryos. The conventional slow freezing method was successfully used in a variety of species including mouse, cattle, sheep, goat, pig, horse, rat, rabbit, cat, monkey and human (Rall, 1992). This method suffers from several limitations such as chilling injury, physical damage due to external ice, zona damage, need for elaborate and expensive equipment and tedious freezing protocols. However, embryos cryopreserved by vitrification may still be injured by toxicity of cryoprotectants, extra cellular ice fracture and adverse osmotic effects (Kasai *et al*. 1996). In addition, vitrification may be suited better for cryopreservation of certain pre-implantation stages of rabbit embryos than other species (Somrag *et al*., 1989, Kobayshi *et al*., 1990 and Dobrinsky *et al*., 1990).

In cattle (Leibo *et al*., 1996) and goats (Begin *et al*., 2003), survival rates of cleavage stage embryos are lower than rates of embryos at blastocyst stage. This decrease in viability may be caused by a higher sensitivity to freezing of these earlier stages or by an inadequacy of the *in vitro* systems to the blastocyst stage (Wilmut, 1985). Therefore, the current study aimed to compare the efficiency of three methods of cryopreservation of rabbit embryos at morula stage as indicated by different post thaw methods and *in vitro* development of thawed embryos to hatched blastocyst stage.

MATERIALS AND METHODS

This study was carried out at the International Livestock Management Training Center (ILMTC), Sakha, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture on 16 adult Newzeland white doe rabbits fed commercial pelleted diet during the period from November to March 2008.

Embryo collection:

The donor does were i.m. injected with 75 unit from PMSG (pregnant mares serum gonadotrophin) 72 h before mating and slaughtered 72 after neutral mating. Thereafter, embryos were collected after 72 hours post-coitum. Postmortem, the reproductive tract of each doe was removed and embryos were recovered by flushing from oviduct with Dulbecco·s phosphate buffer solution (DPBS) at room temperature (20-25^{o}C).

Recovered morphologically normal embryos (morulae with both intact and regular mucin coat, zona pellucida, and homogenous cell mass) from each donor doe were washed twice in fresh DPBS supplemented with 0.002 g, ml^{-1} bovine serum albumin (BSA), then embryo characteristics including,

thickness of mucin coat and zona pellucida as well as diameter of intrazonal and total embryo were measured before preservation.

Embryo preservation methods:

The collected embryos were preserved appropriate thawing method for each preservation method in liquid nitrogen (-196 ^{o}C) for 6 weeks.

Vitrification method:

Twenty three embryos at morulae stage were vitrified with this method. Cryoprotective solution consisting of 1:1:2 (vol/ vol/ vol) of dimethyl-sulfoxide (3.5 M, DMSO), ethylene glycol (4.4 M, EG) and DPBS supplemented with 0.002 g/ml BSA **(Vicent *et al*., 1999).**

Vitrification was carried out in two steps. In the 1st step, normal embryos were pipetted into 0.2 ml of DPBS medium and placed in a glass culture dish, thereafter 0.2 ml of the cryoprotective solution was added and diluted quickly to exposure embryos for 2 min in this medium. In the 2nd step, 0.6 ml of the cryoprotective solution was added and quickly diluted to yield a final concentration of 2.8 M. DMSO and 3.5 M. EG in DPBS with a BSA supplement in vetrification medium. Then the embryos were loaded into plastic mini-straw (IMV, L, Aigle, France) contained three sections separated by air bubbles, the first consisted of PBS in the cotton plug, the second section suspended in the vitrification medium and placed in the middle, and the third section consisted of PBS. Finally, the straws were sealed with heat and plunged directly into liquid nitrogen. Exposure time of the embryos to the final vitrification solution did not exceed 1 min. Both vitrification steps were carried out at 20 ^{o}C.

Thawing procedures of vitrified embryos:

Dvitrification was performed by immersing the second and third sections of the straws in water bath at 20 ^{o}C for 10-15 sec. Then cryoprotective solution was removed from the embryos in a two-step dilution procedure at room temperature (20-25 ^{o}C). The embryos suspended in the final the vitrification solution were released into a glass dish containing 1 ml of 0.33 M sucrose in DPBS medium after 2 min, and the embryos were washed twice in fresh DPBS medium and scored morphologically before incubation. Only embryos with a homogenous cell mass and intact mucin coat and zona pellucida were used for embryo culture.

Step wise method (programmable freezing):

Programmable freezing was carried out by equilibrating the embryos in cryoprotectant medium A (containing DPBS, 20% FCS and 3% glycerol) for 5 min and in cryoprotectant medium B (containing DPBS, 20% FCS and 6% glycerol) for 5 min at room temperature. Thereafter, the embryos were loaded into 0.25 ml French straw mini straws and placed in programmable liquid nitrogen (LN2) freezer at the rate of -0.3 ^{o}C/min from room temperature up to -7 ^{o}C, followed by seeding (employing a super cooled forceps to initiate crystallization) and holding embryos at –7 ^{o}C for 5 min, thereafter from -7 ^{o}C to – 32^{o}C (at a rate of -0.3 ^{o}C/min), followed by plunging of the embryos in LN2 after attaining temperature of –35 C^{0}.

Thawing procedures for step wise method:

Straws containing programmable frozen embryos were thawed in air temperature for 30 sec and exposed to thawing media to remove the cryoprotectants in step wise decrement of glycerol concentration with 5 min for each step. The thawing medium contained PBS+10% FCS with glycerol concentration of 6, 3 and 0%, respectively. Thereafter, the embryos were assessed for morphologic alterations (abnormal shape, cellular extrusions, degenerative changes, zona cracking, and rupture of vitelline membrane) under stereo zoom microscope.

Direct method:

Embryos were selected for conventional freezing with EG as a cryoprotectant. All cryoprotective solutions were prepared in PBS (PBS plus 20% FCS, fetal calf serum, Sigma). Embryos were firstly equilibrated in 0.75 M of EG at 32 ^{o}C for 10 min and then placed for a further 10 min in 1.5 M of EG under the same conditions. Thereafter, embryos were loaded in groups (2-4 embryos) into 0.25 ml straw (IMV, L, Aigle, France) and placed in-between two control columns of cryoprotectant-PBS separated by air bubbles. The slow freezing program of straws was carried out according to that described by Willadsen *et al*. (1976) by reducing temperature to 7 ^{o}C (at a rate of 1 ^{o}C/min), seeding, to -32 ^{o}C (at a rate of 0.3 ^{o}C/min) and to -35 ^{o}C (at a rate of 0.1 ^{o}C/min), then straws were plunged into liquid nitrogen and stored for one month at -196 ^{o}C.

***Thawing procedures for direct method*:**

Thawing of straws was performed with the protocols commonly used by Cocero *et al*. (2002) and Cocero *et al*. (1996). Briefly, straws were immersed in water at 32 ^{o}C for 30 sec, and then embryos were transferred to PBS solution with 10 % FCS for 10 min and washed three times in PBS for 5 min.

Thawing procedures for all cryopreserved methods:

Straw containing cryopreserved embryos by different methods were removed from the storage container of liquid nitrogen, then exposed quickly to water bath at 37 ^{o}C for 5 sec, then washed in fresh PBS only for 10 sec.

***In vitro* culture of cryopreserved embryos:**

Buffered tissue culture medium 199 (TCM 199) supplemented with 10% FCS and 5 mg/ml of Gentamycin sulphat and stored at 4 ^{o}C in disposable 10 ml syringe with 0.22 µm filter attached for up to 1 week were used for *in vitro* embryo culture. On the day of use, the culture media was filtered directly into four-wall tissue culture plates and wormed in a carbon dioxide incubator for a minimum of 1 h prior to culture of embryos.

The recovered embryos after cryopreservation and thawing by different methods or by one method for all cryopreservation methods were examined under a stereo zoom microscope, and embryos with uniform blastomeres and intact zona pellucida were regarded as good embryos suitable for culture. Damaged embryos exhibiting broken zona pellucida and

or lysed blastomeres were discarded. About 4-5 good embryos were placed in 1 ml of culture medium and incubated at 39 ^{o}C a humidified atmosphere of 5% CO_2 for 72 h to hatch blastocyst stage at the end of culture period.

At the 1st, 2nd and 3rd day of embryo culture, thickness (µm) of mucin coat and zona pellucida as well as diameter (µm) of intrazonal and total embryo with its coverings was measured.

Statistical analysis:

The statistical analysis was performed, as a 3x2 factorial design (3 cryopreservation methods and 2 preservation times) for embryo preservation and as 3x3 factorial design (3 cryopreservation methods and 3 culture days) for embryo culture, using computer programme of **SAS (1987)** according to **Snedecor and Cochran (1982).** The significance differences were carried out using **Multiple Range Test Duncan, (1955)**. Data of embryo viability were statistically analyzed using Student Chi-square test. All significant differences were set at a level of $P<0.05$.

RESULTS

Embryo characteristics pre- and post-preservation:

As affected by preservation method, thickness of mucin coat and zona pellucida was affected significantly ($P<0.05$) by preservation method. However, diameter of intrazonal and total embryo was not affected significantly by preservation method. Mucin coat was thicker ($P<0.05$) for vitrification and direct methods than that for step wise method. However, zona pellucida was thicker ($P<0.05$) for step wise and direct methods than that for vitrification method (Table 1).

Data in Table (1) show that all embryo characteristics including thickness of mucin coat and zona pellucida as well as diameter of intrazonal and total embryo reduced post- than pre-preservation, but the differences were not significant only for diameter of intrazonal. It is of interest to note that the observed reduction in total diameter of embryos was associated with decreasing thickness of mucin and zona pellucida as well as diameter of intrazonal. Thickness of mucin coat was mostly affected and zona pellucida showed the lowest effect.

The interaction of preservation method with time (pre- and post-preservation) was not significant reflecting similar trend of decrease in thickness of mucin coat and zona pellucida as well as diameter of intrazonal post- than pre-preservation with all methods.

Table (1): Characteristics of NZW embryos recovered after 72 hours of mating as affected by cryopreservation method and preservation time.

Item	n	Thickness (µm)		Diameter (µm)	
		Mucin coat	Zona pellucida	Intrazonal	Total embryo
Cryopreservation method:					
Vitrification	46	111.0±2.3[a]	21.8±0.66[b]	115.8±2.4	381.2±7.2
Step wise	40	104.8±2.2[b]	23.9±0.58[a]	118.3±2.1	385.8±5.6
Direct	44	114.0±2.0[a]	23.5±0.49[a]	115.9±1.7	387.4±4.1
Preservation time:					
Pre	65	114.2±1.8[a]	24.0±0.48[a]	118.1±1.7	394.5±4.4[a]
Post	65	106.0±1.7[b]	22.0±0.47[b]	115.1±1.7	374.9±4.8[b]

[a and b]: **Means denoted within the same column having different superscripts are significantly different at (P<0.05).**

However, total diameter of embryo showed pronounced decrease with direct method, moderate with vitrification and slight with step wise method (Table 2).

Table (2): Change in characteristics of NZW embryos recovered after 72 hours of mating pre- and post-preservation by different methods.

Embryo characteristics	Vitrification		Step wise		Direct	
	Pre-	Post-	Pre-	Post-	Pre-	Post-
Thickness (µm) of:						
Mucin coat	115.5	106.6	108.7	100.8	117.9	110.1
Zona pellucida	22.7	20.8	25.0	22.9	24.5	22.4
Diameter (µm) of:						
Intrazonal	117.4	114.3	119.9	116.7	117.3	114.6
Total embryo	391.0	371.3	393.1	378.5	399.2	375.6

pre- and post-preservation

Embryo *in vitro* culture:

Viability rate:

Data of freezing-thawing procedures for each method (Table 3) revealed that post thaw embryo recovery rate was significantly (P<0.05) higher in direct method than vitrification and step wise methods (100% vs. 91.3 and 90%, respectively). While, the differences in recovery rate of vitrification and step wise methods were not significant. On all culture days, direct method of freezing-thawing resulted in the highest viability rate, followed by step wise, while vitrification showed the lowest values.

It is of interest to note that viability rate gradually decreased by progressing culture day. The lowest reduction rate in viability rate was observed for step wise method at the 3rd culture day (Table 3).

Table (3): Viability rate of embryos preserved and thawed by three methods and *in vitro* cultured in TCM medium for three successive days.

Preservation method	N	Day after thawing 1st day Morula stage		2nd day Blastocyst		3rd day Hatched blastocyst	
		n	%	n	%	n	%
Vitrification	23	21	91.3[b]	19	82.6[b]	17	73.9[b]
Step wise	20	18	90.0[b]	17	85.0[ab]	16	80.0[ab]
Direct	22	22	100[a]	21	95.5[a]	19	86.4[a]

[a and b]: Means denoted within the same column having different superscripts are significantly different at (P<0.05). N: Number of thawed embryos

Data regard to viability rate of embryos that were frozen by three methods, thawed by one method (thawing of direct method), and *in vitro* cultured at three successive days in TCM-199 culture medium (Table 4) show that embryos frozen by direct method had significantly (P<0.05) the highest viability rate, followed by step wise, while vitrification showed the lowest values at all.

Table (4): Viability rate of embryos preserved by three methods, thawed by one method and *in vitro* cultured in TCM medium for three successive days.

Method	N	Day after thawing 1st		2nd		3rd		Hatched blastocyst		
		n	%	n	%	n	%	n	%*	%**
Vitrification	7	4	57.1[c]	2	28.6[c]	-	-	-	-	-
Step wise	7	5	71.4[b]	5	71.4[b]	3	42.9[b]	2	28.6[b]	66.7[b]
Direct	7	7	100[a]	7	100[a]	6	85.7[a]	6	85.7[a]	100[a]

[a and b]: Means denoted within the same column having different superscripts are significantly different at (P<0.05). N: Number of thawed embryos.
* As a percentage of total number of thawed embryos.
** As a percentage of developed embryos at the 3rd culture day.

It is of interest to note that no viable embryos were obtained on the 3rd day of culture with vitrification method and 85.7% of thawed embryos were examined in hatched blastocyst stage with direct method on the 3rd culture day versus 28.6% with step wise method (Table 4).

Change in embryo characteristics:

Results in Table (5) show that all embryo characteristics including thickness of mucin coat and zona pellucida as well as diameter of intrazonal and total embryo was affected as found in Table (1) indicating the effect of preservation method rather than the thawing method on embryo characteristics during *in vitro* culture.

Table (5): Effect of preservation method on characteristics of NZW embryos recovered after 72 hours of mating and cultured for three days.

Method	n	Thickness (µm)		Diameter (µm)	
		Mucin coat	Zona pellucida	Intrazonal	Total embryo
Cryopreservation method:					
Vitrification	69	112.3±1.9[a]	18.8± 0.55[b]	120.2±1.9	382.9±5.8
Step wise	60	105.9±1.8[b]	21.2± 0.50[a]	122.5± 1.8	389.6± 4.5
Direct	66	115.3±1.7[a]	20.4±0.43[a]	120.4±1.5	392.8±3.4
Culture day after thawing:					
1st	65	106.0±1.7[b]	22.0±0.47[a]	115.1±1.4[c]	374.9±4.8[c]
2nd	65	112.1±1.8[a]	20.1±0.48[b]	121.0±1.7[b]	387.9±4.4[b]
3rd	65	116.1±1.8[a]	18.1±0.49[c]	126.8±1.7[a]	402.2±4.4[a]

[a] and [b]: **Means denoted within the same column having different superscripts are significantly different at (P<0.05).**

As affected by culture day after thawing, thickness of mucin coat and diameter of intrazonal and total embryo significantly (P<0.05) increased and thickness of zona pellucida significantly (P<0.05) decreased by advancing culture day (Table 5).

The interaction of preservation method with culture day was not significant reflecting similar increase in thickness of mucin coat and diameter of intrazonal and total embryo and reduction in thickness of zona pellucida with increasing culture day (Table 6).

Table (6): Characteristics of NZW embryos recovered after 72 hours of mating as affected by preservation method and culture day.

Method	Culture day	Thickness (µm)		Diameter (µm)	
		Mucin coat	Zona pellucid	Intrazonal	Total embryo
Vitrification	1st	106.6	20.8	114.3	371.3
	2nd	113.2	18.8	120.3	381.6
	3rd	117.2	16.7	125.9	396.1
Stepwise	1st	100.8	22.9	116.7	378.5
	2nd	106.4	21.3	122.5	387.8
	3rd	110.7	19.3	128.3	402.6
Direct	1st	110.1	22.4	114.6	375.6
	2nd	116.1	20.4	120.4	394.5
	3rd	119.8	18.3	126.3	408.3

DISCUSION

Three different methods for cryopreservation of rabbit embryos were investigated for their ability to support post thaw *in vitro* viability. Regarding embryo characteristics on different culture days, it is worthy noting that the significant differences in thickness of mucin coat and zona pellucida was mainly related to these measures in pre-preserved embryos for different

methods and the nearly similarity in trend of change pre- and post-preservation. The effect of interaction between preservation and time of measure (pre- and post-preservation) was not significant, revealing similar trend of change in thickness of mucin coat and zona pellucida with all preservation methods. These findings may indicate similar effect of different cryoprotectants on embryo osmolarity during preservation. The trend of change in all embryo characteristics pre- as compared to post-preservation by all methods is similar to that obtained by **Fahim (2008)** on superovulated rabbit embryos collected after 72 h of mating. The author found that thickness of mucin coat significantly decreased and thickness of zona pellucida significantly increased after preservation of embryos with vitrification and slow freezing methods as compared to fresh embryos.

Concerning the effect of different cryopreservation methods on viability rate of embryos on three successive days of culture after thawing, direct method showed the best method, since it not only yielded the highest number of good quality embryos on thawing but it also supported the highest *in vitro* development of embryos to hatched blastocyst stage. To defined the differences in ability of embryos to survive with different freezing methods, direct method showed the optimal freezing procedure, indicating differences in the tolerance of rabbit embryos to cryoprotectants and freezing procedures as indicated in studies of Schmidt *et al*. (1987) on mouse line. Post thaw recovery rate of rabbit embryos obtained on the 1st day after thawing in our study was 91.3% in vitrification, 90.0% in step wise and 100% in direct method. This is much higher than a rate of 85.4% reported by Vicente *et al*. (2003) on different rabbit lines using vitrification method. However, nearly similar post thaw recovery rate (90%) was recorded for rabbit embryos line V21 preserved by vitrification. Although step wise method suffered from several limitations as reported by Rall (1992), it was successfully used in this study to cryopreserve rabbit embryos with recovery rate of 90%. However, lower post thaw recovery rate (81%) than that obtained in our study was obtained by Naik *et al*. (2005) using programmed method (step wise) for cryopreservation of rabbit embryo as compared to 91% for Open pulled straw (OPS) method. Thus, it appears that direct method rather than vitrification and step wise methods is better for the cryopreservation of rabbit embryos at morula stage. The superiority of direct method observed in our study may entirely be due to procedures of freezing and/or due to the composition of cryoprotective solutions. Naik *et al*. (2005) found that when rabbit morulae were cryopreserved by conventional vitrification and OPS using the same OPS solutions survival of rabbit morulae was better with OPS vitrification. Also, different results concening survival rate were obtained by Lopez-Bejar and Lopez-Gatius (2002) using galactose in the cryoprotectant dilution while sucrose as used by Naik *et al*. (2005). The differences in post thaw viability of embryos as affected by preservation methods may attributed to concentration of cryoprotective solutions used in each method during preservation. Kasia (2002) reported that the concentration of cryoprotectants for slow freezing methods is limited to 1-2 mol/l, and the toxicity is relatively low. In vitrification method, the concentration can be as higher as 8 mol/l and the selection of low toxicity agent is more important. On the other hand, Kasia (1997)

indicated higher survival of embryos preserved by vitrification. He indicated that vitrification not only reduce the cooling stage duration to minimize, but also eliminate any injuries caused by extracellular ice, which is the major cause of cell injury. The obtained low post thaw survival rate in vitrification method in our study may be associated with exposure time of embryos to cryoprotective solution immediately pre plunging straws in liquid nitrogen. Exposure time of the embryos to the final vitrification solution did not exceed 1 min (Kasia (1997).

The highest viability rate of direct method as compared to the other methods on the 2nd and 3rd culture day indicated the highest ability of rabbit embryos to tolerate cryoprotectants used in direct method and cold shock during storage by this method as well as it also supported the highest *in vitro* development of embryos to hatched blastocyst stage. Cooling rate seems to be key factor in embryo cryopreservation. An increase in cooling rate decreases chilling injury (Shaw *et al*., 1995) and may permit a reduction of cryoprotectant concentration. The slow freezing program of straws in direct method was carried out according to Willadsen *et al*. (1976) by reducing temperature to -7 ^{o}C (at a rate of 1 ^{o}C/min), seeding, to -32 ^{o}C (at a rate of 0.3 ^{o}C/min) and to -35 ^{o}C (at a rate of 0.1 ^{o}C/min), then straws were plunged into liquid nitrogen and stored at -196 ^{o}C. This regimen was appropriate for rabbit embryos as compared to that applied in this study according to Vicent *et al*. (1999) in vitrification method and that applied in step wise method (See Materials and Methods). In addition, the present results indicated that thawing method of embryos should be appropriate for each preservation method. This was proven in our study when one thawing method (thawing of direct method) to was carried out for all preservation methods, whereas, this method showed again the highest post thaw recovery rate of embryos with direct method (100%) than those with vitrification (57.1%) and step wise (71.4%) methods. When thawing method for vitrification and step wise methods were carried out, post thaw viability rate increased to 91.3 and 90.0%, respectively.

On different days of *in vitro* culture of embryos, findings in term of the significant increase in thickness of mucin coat and in diameter of intrazonal and total embryo as well as increasing thickness of zona pellucida were indicated with embryos preserved by all methods because the effect of interaction between preservation method and culture day on all embryo characteristics was not significant. These findings may indicate similar osmolarity of different cryoprotectants on embryo characteristics during preservation. The trend of increase in mucin coat thickness was mainly related to culture medium. However, decreasing and zona pellucida thickness, which was associated with increasing diameter of intrazonal and the observed increase in total diameter of embryos was mainly attributed to development of embryos from morula up to blastocyst or hatched blastocyst stages by progressing culture day. Similar findings were reported by Fahim (2008) on superovulated rabbit embryos collected after 72 h of mating, preserved by vitrification and slow freezing methods.

In conclusion, cryostorage of embryos recovered from superovulated rabbits after 72 hours of mating could be carried out successfully by direct method without harmful effects on embryo characteristics, yielded the highest

number of good quality embryos on thawing and supported the highest *in vitro* development of embryos to hatched blastocyst stage. The superiority of freezing-thawing of rabbit embryos by direct method may gave some advantages for this method from the economic point of view, whereas for thawing embryos preserved by direct method, straws were immersed only in water at 32 ^{o}C for 30 sec. From the commercial point of view, this may also facilitates transporting preserved embryos between countries for embryo transfere.

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دراسات على طرق الحفظ و الإسالة لأجنة الأرانب المستخدمة في نقل الأجنة

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للتعرف على الاختلافات في مقدرة أجنة أرانب النيوزيلندي على البقاء حيه باستخدام طرق مختلفة للتجميد (الزجزجه – التجميد التدريجي – التجميد المباشر). تم حقن الأرانب بـ٧٥ وحده دولية PMSG قبل التلقيح بـ ٧٢ ساعة وبعد مرور ٧٢ ساعة من التلقيح الطبيعي تم ذبح الأرانب وأجراء عملية جمع الأجنة. بعد التجميد تمت الإسالة وكان لكل طريقة حفظ طريقة إسالة خاصة وأيضا تمت الإسالة للطرق الثلاث بطريقة واحدة.

تمت زراعة الأجنة معمليا في بيئة TCM- 199 لمدة ثلاثة أيام وتم إجراء القياسات على الأجنة. أوضحت النتائج أثناء حفظ الأجنة في الحضان أن طبقة الميوسين كانت اسمك في طريقة الزجزجة والطريقة المباشرة عن طريقة التجميد التدريجي عند مستوى معنويه ٥% (١١١-١١٤,٨-١٠٤ ميكروميتر على التوالي) بينما كان سمك طبقة Zona Pelluceda اكبر في طريقة التجميد التدريجي والطريقة المباشرة عن طريقة الزجزجة عند مستوى معنوية ٥% (٢٣,٩-٢٣,٩-٢١,٨ ميكروميتر على التوالي). بينما لم يتأثر Interzonal وTotal Embryo معنويا بطريقة الحفظ. بينما انخفض فقط سمك طبقة الميوسين معنويا (٥%) وطبقة Zona Pelluceda بعد الإسالة. كان معدل الأجنة المتحصل عليها بعد الإسالة مقارنة بقبل الحفظ أعلى معنويا (٥%) في الطريقة المباشرة عن طريقة الزجزجة التجميد التدريجي (١٠٠% مقابل ٩١,٣ و ٩٠% على الترتيب).

حتى اليوم الثاني من الزراعة كان معدل الحيويه أحسن معنويا (٥%) في طريقة التجميد المباشر يتبعها طريقة التجميد التدريجي بينما أقل قيمه كانت في طريقة الزجزجة بينما كان معدل الحيويه عند اليوم الثالث من الزراعة أحسن في طريقة التجميد التدريجي (٥%) يليها الطريقة المباشرة وأقلها كانت في طريقة الزجزجة. كان معدل الحيويه للأجنة بعد الحفظ بـ الثلاث طرق والإسالة لهما بطريقه واحده والزرع في المعمل لمدة ثلاثة أيام الأفضل (٥%) (١٠٠%) بالطريقة المباشرة يليها طريقة التجميد التدريجي (٧١,٤ %) ثم طريقة الزجزجة (٥٧,١ %). في اليوم الثالث من الزراعة لم نحصل على أجنه حيه في طريقة الزجزجة بينما كانت الأجنة الحية بعد الإسالة في مرحلة Hatched Blastocyst (٨٥,٧ %) في طريقة التجميد المباشر مقابل ٢٨,٦ % في طريقة التجميد التدريجي. تأثير يوم الزراعة بعد الإسالة أدى إلى زيادة سمك طبقة الميوسين وقطر طبقة Internal Zona و Total Embryo وكانت الزيادة معنوية عند مستوى ٥% بينما قل سمك طبقة Zona Pelluceda معنويا (٥%) بزيادة يوم الزراعة.

الخلاصة أن معدل استرداد الأجنة المحفوظة والمخزنة الناتجة من الأرانب التي حدث لها تعدد تبويض والتي تم جمعها بعد ٧٢ ساعة من التلقيح وتم حفظها بطريقة التجميد المباشر هي الأفضل حيث لم تؤدى إلي أي أضرار لصفات الأجنة وكذلك في عدد الأجنة الجيدة المتحصل عليها بعد الإسالة والتطور في المعمل حتى مرحلة Hatched Blastocyst .

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