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FACTORS AFFECTING VIABILITY OF DEEP FROZEN BUFFALO-EMBRYOS

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Abstract

Fifty buffalo embryos were recovered non-surgically on day 5-6 of the estrous cycle. The collected embryos were exposed to an increasing concentration of glycerol in phosphate buffer saline (PBS), either by a three step addition or six step addition. In the final concentration (1.5M glycerol), each embryo was aspirated into 0.25 ml straw. Two freezing procedures were used. Procedure (A): conventional freezing (slow cooling) in which the freezing rate was 2°C/min. from room temperature to -7°C and cooled at 0.3^{o}C/min to -35°C, then, to -80°C at cooling rate 10°C/min before being plunged into liquid nitrogen (LN). Procedure (B): Rapid cooling or vitrification, the embryos were directly plunged into LN after addition of cryoprotectant solution. Two techniques of thawing were applied on embryos frozen, either by rapid or slow methods. The first technique included plunging the straws into a water bath at 37°C for 30 seconds, while the second one included holding the straws in air at room temperature for 2 minutes. Cryoprotectant was removed and embryos were washed. The viability was assessed using morphological evaluation and by staining the embryos with fluorescein diacetate (FDA). Embryos were examined under a reflected-ligh fluorescence microscope, and according to the fluorescence observed the embryos were classified as bright and non-bright (fluorescence was not detected).

Twenty-one of them were frozen by rapid cooling method (vitrification), 10 of them were frozen after adding glycerol in 3 steps. On staining, the post-thawed embryos by FDA stain for evaluation 6 (60%) and 4 (40%) embryos were bright and non-bright, respectively. When embryos were frozen after adding glycerol in 6 steps (n=11), the respective values were 9 (81.8%) and 2 (18.2%). Differences among the 2 regimens of glycerol addition (3 and 6 steps) were significant ($P<0.01$). On the other side, 22 embryos were frozen by slow cooling. On adding glycerol in steps, 7 (77.8%) and 2 (22.2%), embryos were bright and non-bright, respectively. Six steps glycerol additions revealed that 10 (76.9%) and (23.1%) embryos were bright and non-bright, respectively. No significant differences were noted between the two regimens of glycerol addition. Irrespective of the regimen of glycerol addition, the comparison between rapid and slow methods of freezing illustrated that, on using rapid freezing, 15 (71.4%) and 6 (28.6%), embryos were bright and non-bright, respectively. The respective values on using slow method were 17 (77.3%) and 5 (22.7%). Differences between the two methods of freezing were non-significant. Across both methods of freezing, 14 blastocysts (87.5%) and 18 morulae (66.7%) were bright, the differences between the 2 stages were highly significant ($P<0.01$). Seven out 22 embryos (31.8%) developed damaged ZP on using slow method of freezing, while, only 4 out of 21 (19.1%) embryous showed similar lesions on using rapid freezing, the difference between them was significant ($P<0.05$). Embryos thawed in water bath resulted in recovery rate of 82.8%, while, those thawed in air at room temperature resulted in 90.5% recovery rate. The difference between the methods of thawing was non-significant.

INTRODUCTION

Embryo deep freezing could play an important role in a national breeding program for bull-mothers selection. It also opens up possibilities for the trade both locally and internationally with embryos. In this way, breeds with desirable genetic traits could be introduced into new areas as embryos (Wilmut *et al.*, 1991). The successful presevation of embryos depends on one or a combination of the principle variables of cryobiology which were stated by Leibo (1981). The first principle variable of cryobiology is the type and concentration of cryoprotective compound in which the embryos are suspended for freezing. The second is ice formation; the freezing point of a cryoprotectant solution depends on the number of molecules of solvent present and the volume being cooled. The third variable, is the cooling rate and temperature at which the frozen embryos are stored. The fourth principle variable is warming rate, and the final variable is the rate and temperature at which the embryo is diluted out of the solution in which it was originally frozen.

Therefore, the present investigation was designed to study the effect of cryoprotectant rate and its steps of addition, cooling rate and freezing type on the viability of buffalo-embryos, as well, as, different methods of embryos thawing employed to spot the most appropriate embryo freezing and thawing techniques in buffalo species.

MATERIALS AND METHODS

Embryo recovery and morphological evaluation

Recovery of buffalo-embryos was performed non-surgically on day 5-6 of the oestrous cycle using the method described by NewComb *et al.* (1978). Morphological evaluation has been used to delineate embryo quality; the evaluation was applied according to Takeda (1986). It included shape, colour, number and compactness of blastomere cells, size of perivitelline space, number of extruded and degenerated cells, number and size of vesicles and embryo age in relation to stage of the donor estrous cycle. Considering this, the embryos were classified as excellent, good, fair and poor, Only embryos of excellent and good quality were used in the freezing process. Stages of embryonic development ranged from morula to blastocyst.

Preparation of cryoprotectant stock solution (3.0 M glycerol)

Glycerol (1.5 M) was used as a cryoprotectant for embryo freezing.

1. To add 7.4 ml of modified Dulbecco's phosphate buffer saline (according to

Mohammed 1991) supplemented with 10-20% bovine serum albumin (BSA) to 2.1 ml of pure glycerol.

2. The prepared solution was then passed through a 0.2 um filter into 20 ml screw cap bottle, then stored in a freezer at-20°C for use.
3. Dilution of 3.0 M stock solution can be done by the following formula (Actual concentration of stock solution) - (Desired concentration) / Desired concentration (Takeda 1984).

This formula determines the number of part of PBS that should be added to one part of stock solution to obtain the desired concentration of the solution.

Embryo freezing

The collected embryos were exposed to an increasing concentration of glycerol in PSB at room temperature, either, by a three step addition (0.05 M, 1.0M then 1.5M), with 10,10 and 15 minute, respectively for equilibration, or six step addition (0.25 M, 0.5, 0.75M, 1.0M then 1.5M) with 10,10,10,10,10 and 15 minute for equilibration, respectively. In the final concentration (1.5 M glycerol), each embryo was aspirated into 0.25 ml straw. Two freezing procedves were used. Proceduse (A): Programmable freezer, conventional freezing rate (slow cooling) in which the freezing rate was 2^{O}C/min. from room temperature to-7°C. After 5 min of equilibration at that temperature, ice crystal formation was induced (seeding) by touching the wall of each straw with forceps previously embedded in liquid nitrogen (LN). Subsequently, samples were equilibrated for an additional 10 min at seeding temperature and cooled at 0.3^{O}C/min to-35°C, then, to-80°C at cooling rate 10°C/min before being plunged into LN (according to Mohammed 1995). In second freezing procedure (type B: Rapid cooling or vitrification), the embryos were directly plunged into LN (according to Ishimori *et al.*, 1992).

Embryo thawing

After 2-5 months of embryos storage in LN, the straws were thawed. Two techniques of thawing were applied on embryos frozen either by rapid or slow methods. The first technique, included plunging the straws into a water bath at 37°C for 30 seconds, while, the second one included holding the straws at room temperature for 2 minutes. The content of each straw was emptied into a sterile holding dish containing 1.5M glcerol-phosphat buffer saline solution freshly prepared. Cryoprotectant was removed by three step-wise (1.5M, 1.0M and 0.5M), or six step-dilution 0.25 M to 1.5M degradations with 5-10 min equilibration at each step.

The embryo was washed twice in a holding medium to remove all cryoprotectant, and the viability was assessed again using morphological evaluation and fluorescent stain.

Fluorescence method for embryo evaluation

In order to assess the viability of the post-thawed embryos, more accurate and rapid, fluorescence tests assay were applied. This easy test was used for embryo viability determination. The living blastomere cells of the frozen-thawed embryos were detected by staining the embryos with fluorescein diacetate (FDA), (Sigma Chemical Co., USA) in concentration of 1:400 000 according to Schilling and Dopke (1978). A stock solution of 5 mg FDA/ml of acetone was prepared and stored at-20°C, and just before use a solution of 0.5 ml stock/ml PBS was prepared giving a final concentration of 1:400 000. Embryos were incubated in FDA solution for 3-5 minutes at room temperature before examination. The embryos were then transferred onto a sterile glass depression slide with small amount of FDA-free PDS to reduce distracting background fluorescence. Embryos were observed under a reflected-light fluorescence microscope at a magnification of X200. The ligh source was a mercury lamp with a series of excitors and barriers which restricted light reaching to the specimen to 530 nm. Each embryo was exposed to ultraviolet (UV) and the fluorescence observed appeared green, and was classified as:

1) Completely bright (more than 75% of the embryo size appeared fluorescing).

2) Partial bright (fluorescence not completely covering the embryo, or only dim fluorescence).

3) No fluorescence detected, or only one to few blastomeres appeared bright. All statictical analysis were done according to Snedecor and Cochran (1980).

Table 1. Influence of rapid cooling on post-thawing buffalo embryos quality.

Regime of glycerol addition	Stages of embryos development (no.)	post thawing quality using fluorsecence stain			
		Bright			Non-bright (%)
		Complete (%)	Partial (%)	Total (%)	
Three steps	Blastocyst (3)	0 (0.0)	2 (75.0)	2 (75.0)	1 (25.0)
	Morula (7)	2(28.6)	2(28.6)	4(57.1)	3(42.9)
Total	10	2(20.0)	4(40.0)	6(60.0)	4(40.0)
Six steps	Blastocyst (4)	2 (50.0)	2 (50.0)	4(100.0)	0(00.0)
	Morula (7)	2(28.6)	3(42.8)	5(71.4)	2(28.6)
Total		4(36.4)#	5(45.5)	9(81.8)#	2(18.2)#

Chi square between percentage of total values in the same column.

significant at (P<0.01) ¯ non-significant.

Table 2. Influence of slow cooling on post-thawing buffalo embryos quality.

Regime of glycerol addition	Stages of embryos development (no.)	post-thawing quality using fluorscence stain			
		Bright			Non-bright (%)
		Complete (%)	Partial (%)	Total (%)	
Three steps	Blastocyst (4)	1 (25.0)	2(50.0)	3(75.0)	1 (25.0)
	Morula (5)	1(20.0)	3(60.0)	4(80.0)	1(20.9)
Total	9	2(22.2)	5(55.6)	7(77.8)	2(22.2)
Six steps	Blastocyst (5)	2 (40.0)	3(60.0)	5(100.0)	0(00.0)
	Morula (8)	2(25.0)	3(37.5)	5(62.5)	3(37.5)
Total		4(30.8)	6(46.2)	10(76.9)	3(32.1) ⁻

Chi square between percentage of totol values in the same column.
⁻ non-significant.

Table 3. Comparison between rapid and slow methods of freezing.

Regime of glycerol addition	Stages of embryos development (no.)	post thawing quality using fluorscence stain			
		Bright			Non-bright (%)
		Complete (%)	Partial (%)	Total (%)	
Three steps	Blastocyst (7)	2 (28.6)	4(57.1)	6(85.7)	1 (14.3)
	Morula (14)	4(28.6)	5(35.7)	9(64.3)	5(35.7)
Total	21	6(28.6)	9(42.9)	15(71.4)	6(28.6)
Six steps	Blastocyst (9)	3(33.3)	5(55.6)	8(88.9)	1(11.1)
	Morula (13)	3(23.1)	6(46.2)	9(69.2)	4(30.8)
Total		6(27.3)	11(50.0)	17(77.3)	5(22.7)⁻

Chi square between percentage of totol values in the same column.
⁻ non-significant.

RESULTS

The influence of rapid cooling method of freezing (vitrification) on the embryo quality is presented in Table 1. Out of 10 embryos frozen by adding glycerol in 3 steps, 6 (60%) and 4 (40%), emberyos were bright and non-bright, respectively on staining the post thawing embryos by FDA stain. When the embroys frozen by adding blycerol in 6 steps, the repective values were 9 (81%) bright and 2 (18.2%) non-bright. Differences between the 2 regimens of adding glycerol (3 and 6 steps) were significant ($p<0.01$) with bright {completely bright, (Fig. 1) and partial bright. (Fig 2} and not bright (Fig. 3)post-thawing embryos.

Table 2 depicting the effect of effect of using slow cooling method of freezing (automatic freezer) on post thawing embryo quality as illustrated by using fluores cent stain. On adding glycerol in 3 steps, 7 (77.8%) and 2 (22.2%) embryos were bright and non-bright, respectively. Six steps glycerol addition revealed that, 10 (76.9%) and 3(23.1%) embryos were bright and non-bright, respectively. Statistical analysis showed non-significant differences between the two regimens (3 and 6 steps) of glycerol addition.

Irrespective of the regimen of adding glycerol during freezing (3 and 6 steps), the comparison between rapid and slow method of freezing (Table 3) illustrated that, on using rapid method, 15 (71.4%) and 6(28.6) embryoss were bright and non-bright, respectively. The rspective values on using slow method were 17 (77.3%) and 5 (22.7%). Differences between rapid and slow method of freezing were statistically non-significant.

Amongat all buffalo embryos (n=43) that were frozen in this experimentm, 16 (37.2%) were blastocysts, whereas, 27 (62.8%) were morulae (Table 4). Across both methods of freezing (rapid and slow) 14 blastocysts (87.5%) and 18 morulae (66.7%) were bright when stained with fluorescent stain after thawing.On the other hand, 2 blastocys (12.5%) and 9 morulae (33.3%) were non-bright. Statistical, analysis revealed highly significant diffrences ($p<0.01$) between the two stages. Irrespective of the embryo stage, 32 embryos (74.4%) kept their vialitity after freezing, whereas, 11 emboryos (25.6%) did not survive post-freezing. Statistically, the difference between viable and dead embryos due to frezing was significant ($p<0.01$).

Table 4. The influence of embryo stage on the post-thawing embryos quality.

embryo stages	Bright (%)	Not bright(%)	Total (%)
Blastocyst	14 (87.5)	2 (12.5)	16 (37.2)
Morula	18 (66.7)	9 (33.3)	27 (62.8)
Total	32(74.4)	11(25.0)	43

Table 5 presents the influence of freezing method on the dmage or of zona pellucida of post - thawing embroyos. Seven out of 22 embryos (931.8%) developed dmaged ZP on using slow method of freezing. However, only 4(19.1%) out of 21 embryos showed similar lesions on using the rapid method of freezing. Statistical analysis revealed significant differences ($p < 0.05$) between the values of damaged zona in both methods of freezing.

Table 5. The influence of freezing method zone on pellucide of embryos.

Freezing methods	Total embryo	embryos with damaged ZP(%)
I. Slow	22	7(31.8)*
II. Rapid	21	4(19.1)
Total	43	11(25.6)

*Significant ($P < 0.05$)

Table 6. The influence of thawing method on of embryo recovery.

Methods of embryos thawing	Total thawed embryo	Embryos recovery after thawing (%)
I.Water beth thaw (37 ^{o}C/30Sec.)	29	24(82.8)
II. Room Temperature (2 min.)	21	19(90.5)
Total	50	43(86)

⁻ non-Signifcent

The influence of thawing method on the embryo recovery rate is shown in Table 6. Thawing in water bath resulted in recovery of 24 (82.8%) embryos from 29 straw, each containing one embryo. However, by thawing straws at room temperature, 19 (90.5%) embryos were recovered from 21 straws each containing one embryo. Difference between the recovery rate of both methods were statistically non-significant temperature. 19 (9.5%) 190.

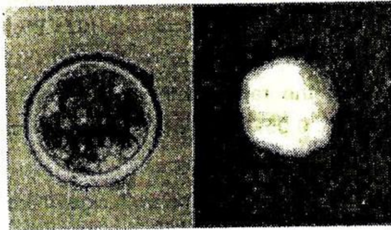

Figure 1. Completely bright embryo.

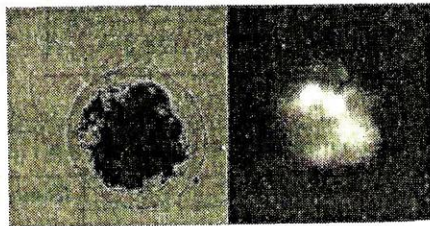

Figure 2. Partial bright embryo.

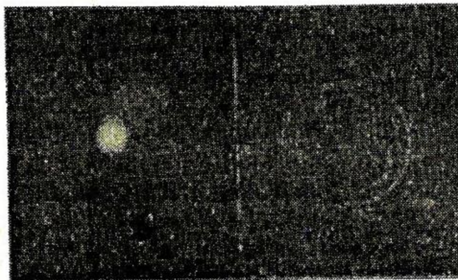

Figure 3. Non-bright embryo.

DISCUSSION

The cryoprotective compounds are usually divided into two categories, those which freely permeate most cells as glycerol, dimethyl sulphoxide and ethylene glycol, and those which do not permeate as sucrose, polyvinyl pyrrolidone, hydroxy ethyl starch, dextrans and albumin (Leibo, 1981).

Two methods of freezing have been demonstrated for embryo freezing, the slow (conventional), and rapid (vitrification) methods. The method of slow cooling embryos was based on the need to avoid intracellular ice formation by progressive dehydration of cells during the ice formation phase. Specific training, equipment and liological freezing machines were mecessary for the application of this method (Elsden *et al.*,1982). The rapid method included freezing in one step by directly plunging into liquid nitrogen (Rall *et al.*, 1986). Rapid freezing techique had the advantage of avoiding the damage caused by intracellular ice formation and osmotic effects caused by extracellular ice formation. It also offered considerable promise for simplifying and improving the cryopreservation associated with the formation of ice in the suspension not was eliminated (Rall, 1987). In our study, no significant differences of in the percentage of positive fluoresced embryos were noted between the rapid and slow methods of freezing buffalo embryos. Positive fluoresced embryos were accounted to be 71.4% and 77.3%, respectively. However, addition of glycerol in 6 steps during the rapid method of freezing developed bettr results than recorded when glycerol was added in 3 steps. These results were in agreement with those reported by Franks *et al.* (1986). Also, Takeda, *et al.* (1984) reported a high survival rate reaching to 88% for bovine embryos frozen by vitrificantion method.

Irrespective of the method of embryo freezing used in this investigation, blastocysts had higher survival rate than morulae (87.5 vs 66.7%; P<0.01) as evaluated after thawing by FDA florescent stain. This means that the developmental stage of the embryo is a factor affecting its tolerance to cryopreservation. The same concept was given by Urano *et al.* (1986). Similarly, Seidel *et al.* (1983) reported that bovine blastocysts survived freezing better that morulae. Moreover, Lade *et al.* (1985) observed a marked effect of the developmental stage on post thawing viabillty of equine embryos. They reported that early blastocysts of frozen thawed equine embryos survived better (62%) than blastocysts (10%). Recently, Ishimori *et al.* (1992) found that the rate of development of vitrified mouse morulae and blastocysts were 34% and 50%, respectively. On the contrary to our findings, Vldez *et al.* (1990) reported that, after frezing by vetrification method, mouse blastocysts had

post-thawing survival rate lower than that of morulae. However, the reason that, frozen thawed buffalo blstocysts were superior to morulae are unknown, but it was well documented that embryonic stages differed in their sensitivity to various cryoprotective concentration. Differences in permeability were likely to exist between morulae and blastocysts. Blastocysts with peripheral trophoblast cells and a distinct blastocoel responded to osmotic change completely different than morulae with their compacted blastomeres (Niemann, 1985). Maurer and Haseman (1976) reported that, sensitivity to cooling perocedures could be influenced by blastomere size and that procedure (freezing and cryoprotectant concentration) should therefore be varied to accommodate these changes in the blastomeres size associated to embryo stage. Expanded and hatched blastocysts did not appear to be as viable after cryopreservatation (Niemann *et al.*, 1987).

In the current research, two regimens of thawing the emmbryos were applied. Embryo recovery rate was 82.8% when embryos were thawed in a water both at 37°C for 30 seconds. This value was lower than that obtained (90.5%) when embryos were thawed in air at room temperature for 2 min. Similar results were obtained by Bielanaski *et al.* (1986) on thawing embryos in water bath. In fact, the low recovery rate obtained by using water bath method might, presumably, be due to the the sudden increase in the temperature, and consequently, increasing the size of air bubbles inside the straw which led to increasing the pressure inside straws resulting in embryo rupture. This suggestion might be of little effect in thawing method due to the gradual increase in the temperature and pressure inside the straws.

With regard to the function of zona pellucida (ZP) during embryo preservation, it is well known that an intact ZP is essential for the survival of bovine embryos during freezing and thawing (Lehn-Jensen and Rall, 1983). Niemann (1985) stated that, although damaged embryos developed well in culture, none developed into fetuses when transferred into recipients. Moreover, Hoppe and Bavister (1983) reported that, despite the fact that ZP is not required for normal development of bovine embryos in vitro and vivo, damage or breakage of ZP during freezing and thawing could seriously limit the use of such embryo. However, Willadsen, *et al.* (1978) reported that transferring embryos with damaged ZP have developed 30% pregnancy rate. Our results revealed that, amongst embryos thawed by either of the two methods of thawing, it was clear that those frozen by slow method exhibited zona damage more than those frozen by the rapid method (31.8% vs 19.15). Similarly, Elsden *et al.* (1982) reported that 30% of the embryos with damaged ZP resulted on using slow method of freeging. Also, Niemann (1985) found that the percentage of embryos with

damaged ZP (26.6%) which originated on freezing with 1.4M glycerol was reduced to 13.2% when glycerol concentration was decreased to 1.0M. However, earlier reports suggested 2 possible mechanisms for zona fracture in cryopreserved embryos. Landa (1982) proposed that, mechanical stress when ice crystals surrounding embryos melt unevenly during thawing and moved in opposite directions and produced shearing forces causing zona fracture, cryomicroscopic evidence did not subscribe to this view. The second proposed mechanism (Rall *et al.*, 1984) suggested that, embryo suspensions were susceptible to thermally induced mechanical stress when liquid channels completely were solidified at temperature below-110°C. Subsequent rapid change in temperature while thawing in water bath resulted in a very high level of mechanical stress causing zona damage. Cryomicroscopic studies of embryo suspensions provided direct evidence to this view. Furthermore, it was also reported that the zona pellucida was predisposed for damage owing to burst at heat produced by ice formed on the surface releasing latent heat of freezing during thawing (Rall and Mayer, 1989).

REFERENCES

1. Bielanshi, A., U. Schneider, V. Pawlyshyn, and R. Mapletoft. 1986. Factors affecting survival of deep frozen bovine embryos in vitro: The effect of freezing container and method of removing cryoprotectant. Theriogenology, 25: (3): 429-437.

2. Elsden, R.P., Jr. Seidel, T. Takeda,. and G.D. Farrand. 1982. Field experiments with frozen-thawed bovine embryos transferred non-surgically". Theriogenology, 17: 10.

3. FDA, Sigma Chemical Co., USA.

4. Franks, G. Coley, S., B.Betterbed, and R. Page. 1986. The effect of freezing type, cryoprotectant, and processing methods on viability of frozen embryo. Theriogenology, 26 (2): 135-144.

5. Hoppe, R.W. and b.D. Bavister. 1983. Effect of removing the zona pellucida on development of hamster and bovine embryos in vitro and vivo. Theriogenology, 19: 391-404.

6. Ishimori, H.,Y Miki, M.Kishi, K., N. Saeki, Seike, and H. Kainuma. 1992. Vitrification of bovine embryos. Theriogenology, 37 (1): 228 (Abstr.).

7. Landa, V. 1982. Protection of non-nuclear investments of rabbit morula storage at 196°C. Folia Biologica. 33 : 350-358.

8. Lehn-Jensen, H. and W.F. Rall. 1983. Cryomicrscopic observations of cattle embryos during freezing and thawing. Theriogenology, 19:263-277.

9. Leibo, S.P. 1981. Preservation of ova and embryos by freezing. In: New technologies in animal breeding. Academic Press, PP. 127:139.

10. Maurer, R.R. and J.K. Haseman. 1976. Freezing morula stage rabbit embryos. Biol. Reprod. 14: 256-263.

11. Mohammed, K.M.E. 1991. Embryo recovery and evaluation in Egyptian buffaloes. M.V.Sc., Alexandria University.

12. Mohammed, K.M. 1995. Studies on embryo transfer in buffalo and Friesian cows. Ph.D. D., Cairo University.

13. NewComb, R., W.R. Christie, and L.E. Rowson. 1978. Nonsurgical recovery of bovine embryos. Vet., Rec., 13:414-417.

14. Niemann, H. 1985. Freezing of bovine embryos. Effect of a one step addition of 1.4M glycerol. Theriogenology, 23 (2): 369-379.

15. Niemann, H., W.W., Lampeter, B. Sacher, and B. Kruff. 1987. Comparison of survival rates of day 7 and day 8 bovine embryos after fast freezing and thawing. Theriogenology, 18: 445-452.

16. Rall, W.F. 1987. Factors affecting the survival of vitrified mouse embryos Cryobiology, 24: 387-402.

17. Rall, W.F. and T.K. Mayer. 1989. Zona fracture damage and its avoidance during cryopreservation of mammalian embryos. Theriogenology, 31 (31): 683-692.

18. Rall, W.F., T.K. Mayer, and S.P. Leibo, . 1986. Effect of warming conditions on the survival of mouse embryos crypreserved by a one step straw procedure. Theriogenology, 25:186.

19. Rall, W.F., D.S. Reid and C. Polge. 1984. Analysis of slow warming injury of mouse embryos by cryomicroscopical and physiochemical methods. Cryobiology, 21:106-121.

20. Schilling, E. and H.H. Dopke. 1978. A rapid diagnostic test for the viability of early cattle and rabbit embryos usinf Diacetyl Fluorescein. Naturwissenschafter, 65, 658.

21. Seidel, G.E., R.P. Elsden, T. Takeda and G.D. Farrand. 1983. Field trials with cryopreservation bovine embryos. In: Fertilization of the human egg in vitro. PP. 343-352.

22. Slade, N.P., E.L. Takeda Squires, R.P. Elsden, and G.E. Seidel. 1985. A new procedure for the cryopreservation of equine embryos. Theriogenology, 24 (1) 45-58.

23. Snedecar, G.W. and W.G. Cochran. 1980. Statistical methods. 7th Ed., The Iowa State Univ., Press. Amer. Iowa, USA.

24. Takeda, T. 1984. Equipment and techniques for thawing of embryos. Proc. No. 70, Bovine embryo transfer workshop, Univ. of Sydney, Australia, PP. 153.

25. Takeda, T. 1986. Identification and evaluation of embryos in bovine embryo transfer. Workshop for Veterinarians, Feb., 13-14, Gainsiuille, USA.

26. Urano, K., Y. Tahahashi, and H.Kanagawa. 1986. Effect of various cryoprotectec-tants on the survival of frozen thawed mouse embryos. Jpn. Anim. Reprod, 32: 130-133.

27. Valdez, C.A., O., Abas,Y., Mazni, M. Hishinuma, and H. Kanagawa. 1990. Effects of equiliberation time, precooling and developmental stage on the survival of mouse embyos cryopreserved by vitrification. Theriogenology, 33: 627-636.

28. Willadsen, S.M., C. Polge, and L.E. Rowson. 1978. The viability of deep frozen cow embryos. J. Reprod. Fertil., 52: 391-393.

29. Wilmut, I., M.L. Hooper, and J.P. Simons. 1991. Genetic manipuolation of mammalians and its application in reproductive biology. J. Reprod. Fertil., 9 (2) 245.

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في هذه الدراسة تم استخدام خمسين جنينا جاموسي اشتملت علي الآتي:

التجميد السريع للأجنة (التزجج)

في هذا النوع من التجميد تم تجميد ٢١ جنينا ، لعشرة منها أضيف الجليسرول علي ثلاثة مراحل تمهيدا لتجميدها بينما تم اضافة الجليسرول للأحد عشر الباقية علي ٦ مراحل متتالية. وبعد الانتهاء من اضافة الجليسرول وضعت الأجنة في قصيبات ثم وضعت في النتروجين السائل . تم اسالة الأجنة المجمدة لدراسة مدي تأثير التجميد علي حيوية الأجنة وذلك باستخدام صبغة الفلوري والميكروسكوب الفلوري. وقد أظهرت النتائج أن ٦ أجنة (٦٠٪) تشع اللون الفلوري للصبغة بينما كانت ٤ أجنة (٤٠٪) غير مشعة للصبغة. هذا بالنسبة للأجنة التي أضيف لها الجليسرول علي ثلاث مراحل أما الأجنة التي أضيف لها الجليسرول علي ٦ مراحل فكان ٩ منها (٨١,٨٪) مشع واثنان (١٨,٢٪) غير مشعة.

التجميد بالطريقة التقليدية (البطيئة)

استخدم في هذا النوع من التجميد ٢٢ جنينا. وباضافة الجليسرول علي ثلاث مراحل ل ٩ منها تمهيدا للتجميد كان عدد الأجنة التي أظهرت اشعاعا لصبغة الفلوري ٧ (٧٧,٨٪) بينما ٢ فقط (٢٢,٢٪) لم يظهرا الاشعاع المميز للصبغة. في حين أن ١٠ أجنة (٧٦,٩٪) من ١٣ (أضيف لها الجليسرول علي ٦ مراحل) أظهرت اللون المميز للصبغة بينما الثلاث الباقية (٢٣,١٪) لم يظهر عليها الاشعاع. أيضا أظهرت النتائج (بصرف النظر عن نظام اضافة الجليسرول) أن ٧١,٤٪ من الأجنة التي تم تجميدها بالطريقة السريعة كانت مشعة بينما ٢٨,٦٪ لم يظهر عليها هذا الاشعاع. أما باستخدام الطريقة البطيئة (التقليدية) في التجميد كان عدد الأجنة المشعة ١٧ (٧٧,٣٪) والغير مشعة ٥ (٢٢,٧٪) وأظهرت النتائج أن الاختلاف بين النظامين (التجميد بالتزجج والتجميد التقليدي) في التجميد لم يكن له تأثير معنوي علي حيوية الأجنة.

دراسة تأثير طريقة التجميد علي الغلاف الشفاف للجنين

تبين من الدراسة أنه عند استخدام النظام التقليدي (البطئ) للتجميد كان عدد الأجنة التي أظهرت تمزق في غلافها الشفاف ٧ (٣١,٨٪) بينما ٤ فقط (١٩,١٪) أصابها الضرر في الغلاف الشفافي. وعموما فان الفرق بين الاثنين معنوي.

دراسة تأثير التجميد علي المرحلة الجنينية

بصرف النظر عن نظام التجميد وطريقة اضافة الجليسرول وجد أن ١٤ جنينا (٨٧,٥٪) في مرحلة البلاستوسيست قد أظهرت صبغة الفلوري بينما ١٨ جنينا (٦٦,٧٪) في الطور التوتي أظهرت الاشعاع المميز للصبغة ووجد أن الفرق بين مرحلتي النمو كان معنويا بالنسبة لاشعاع الصبغة.

دراسة تأثير طريقة الاسالة علي عدد الأجنة المستخلصة

عند اسالة الأجنة المجمدة في حمام مائي درجة حرارته ٣٧ درجة مئوية لمدة نصف دقيقة كانت نسبة الأجنة المستخلصة ٨٢,٨٪. وارتفعت هذه النسبة الي ٩٠,٥٪ عندما تركت قصيبات الأجنة المجمدة في الهواء عند درجة حرارة الغرفة لمدة دقيقتين.