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FREEZING RABBIT SEMEN IN PELLET FORM USING COLD SURFACE OF PARAFFIN WAX COMPARED TO STRAWS

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ABSTRACT

This experiment was carried out to evaluate a simple method for freezing rabbit spermatozoa in pellet form on the cooled surface of paraffin wax compared to traditional straws method. Semen was collected from ten New Zealand White bucks by means of an artificial vagina. Ejaculates exhibiting active progressive motility and high concentration were pooled, diluted with Tris-based extender containing sucrose or glycerol as cryoprotectant, cooled to 5°C over 2 h and stored at this temperature for another 2 hours. The overall means of post-thaw motility were 40.75 and 37.50 for straws and paraffin wax pellets respectively. The percentages of spermatozoa with integral acrosomes were 46.35 and 43.30% for straws and paraffin wax pellets respectively. The results showed that addition of sucrose to semen extender improved significantly both motility and acrosomal integrity compared to glycerol (41.50% of progressive motile spermatozoa Vs 36.75% and 47.50% of integral acrosome Vs 42.13% for sucrose and glycerol respectively). Freezing rabbit spermatozoa in straws could produce high number of does pregnant, pregnancy rate and number of young born using sucrose or glycerol as cryoprotectant compared to paraffin wax pellets. Mean litter size per pregnant doe was similar for does inseminated with frozen semen in straws and paraffin wax pellets. The pregnancies rates were highest when rabbit spermatozoa frozen in extender containing sucrose (62.5 mM) as cryoprotectant compared to glycerol (3%). In conclusion freezing rabbit semen in pellets using the cold surface of paraffin wax could be used successfully for artificial insemination programs (AI).

INTRODUCTION

The new intensive methods for rabbit breeding have recently achieved particular interest in the storage of rabbit semen at very low temperature. It has been impossible to freeze rabbit spermatozoa without reducing their fertilising ability. Theau-Clement and Roustan (1982), used deep frozen rabbit semen, obtained a poor pregnancy rate (39%) without any effect of dilution. Thus, it is very important to improve fertility rate and develop a reproductive performance, which optimise the entire AI procedure (Castellini and Lattaioli, 1999). Using the frozen-thawed semen in artificial insemination programs allows the perfectly use of genetically superior rabbit bucks even in the absent of those males. The most conventional methods for cryopreservation at very low temperature are straws using liquid nitrogen vapour and pellets using dry ice. These materials are very expensive, especially in developing countries such as Egypt. The present work aimed to compare a new technique of cryopreservation in pellet form on cooled surface of paraffin wax and straws. Rabbit spermatozoa can be frozen successfully, but sperm from rabbit species are relatively sensitive to cryoprotectant containing hydroxyl group such as glycerol (Maurer *et al*., 1976; Hanada and Nagase, 1980). The aim of the present work is also to evaluate sucrose as non-penetrating cryoprotectant compared to glycerol as penetrating cryoprotectant.

MATERIALS AND METHODS

Animals: Bucks and does were sexually mature WNZ rabbits New Zealand animals raised in Faculty of Agriculture Farm in Ismailia, Egypt. The experiment was conducted

during the period September – December 1999. The temperature inside the rabbitry was 25°C. Rabbits were fed *ad libtum* and free access of water by nipple was provided

Semen Collecting and handling: Semen was collected from each of 6 mature New Zealand bucks by glass artificial vagina. The ejaculates were pooled after motility examination and determining that each ejaculate contained at least 75% motile spermatozoa. This percentage was estimated subjectively by examining spermatozoa on a microscope slide warmed at 37°C. Two types of extenders were used: Tris-Egg Yolk-Glycerol and Tris-Egg yolk-Sucrose. Approximately one part of semen was mixed with six part of the extender using two-step extension procedure. One part of semen was added to three parts of diluent A, which containing no glycerol or sucrose at 37°C. After 30 minutes at room temperature, three parts of diluent B, that containing 6% glycerol or 125 mM sucrose was added. The components of two extenders (diluent A and B) are shown in Table 1. Extended semen was placed in small sterile conical tubes in a water bath at 37°C. Then the extended semen was packaged in plastic straws containing 0.25 ml. Straws were sealed with thawed paraffin wax. Liquid semen and straws were held in a water bath and cooled gradually to 5°C within 2 hrs. Then kept at this temperature for another 2 hrs as equilibration period.

Table 1: The components of Glycerol and Sucrose extenders.

Components	**Extenders**			
	Glycerol Extender		**Sucrose Extender**	
	Diluent A	**Diluent B**	**Diluent A**	**Diluent B**
Tris (g)	3.785	3.785	3.785	3.785
Citric acid (g)	2.115	2.115	2.115	2.115
Fructose(g)	1.00	1.00	1.00	1.00
Sucrose (mM)	----	----	----	125.00
Glycerol (mL)	----	6.00	----	----
Egg yolk (mL)	20.00	20.00	20.00	20.00
Antibiotic (mL)*	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Distilled water	To 100 ml	To 100 ml	To 100 ml	To 100 ***ml***

*Each 1.0 ml of the antibiotic contained 30,000 IU penicillin and 50,000 micrograms streptomycin.

Freezing procedures: Two freezing methods were used: Straws as a control and a new method of rabbit sperm cryopreservation in pellets form on the cold surface of paraffin wax:

a- Straws: The straws were placed horizontally in a rack approximately 3 cm above a reservoir of liquid nitrogen (LN_2) as described by Foote (1987). After 10 minutes in this position, straws were then plunged directly into LN_2 unit.

b- Paraffin wax pellets: Some paraffin wax was melted in a bored box of aluminium foil (5 cm depth x7 cm width x15 cm length) to make a layer of 1 cm depth containing small holes and kept to reach room temperature. Paraffin wax block was cooled by plunged it into liquid nitrogen for 15 seconds. Then, it placed horizontally and lowered into liquid nitrogen vapour 2-3 cm above the surface of liquid nitrogen. About 200 *ul* of equilibrated semen were pipetted into each hole. After 9 minutes on the vapour of liquid nitrogen, the frozen pellets with paraffin wax block were plunged together into liquid nitrogen (-196°C). Pellets were collected under the surface of liquid nitrogen and packaged into small goblets of appropriate size and transferred into liquid nitrogen storage container.

Thawing of semen: Straws were thawed in a water bath at 37°C for 30 second. Then, the surface of each straw was dried .The pellets were thawed by placing it into a clean

dry test tube in a water bath at 37°C for one minute.

Examination of Frozen Semen: Rabbit thawed semen was resuspended in 2-ml sodium citrate (2.9%). The percentage of motile spermatozoa was estimated microscopically at a magnification of X400 at 37°C. immediately after thawing. Acrosome integrity was determined according to Weitze (1977).

Artificial Insemination: Two **e**pididymalectomized bucks were used to induce ovulation before insemination. Does were inseminated at the same day of parturition. The number of spermatozoa per mL of frozen semen was calculated to deliver 5 x 10^6 motile sperm per doe. Each inseminate contained 0.5-mL thawed semen.

Statistical Analysis*:* This experiment was carried out to evaluate two freezing methods (Straws vs. paraffin wax pellets) and two extenders (Glycerol vs. Sucrose). SPSSWIN program computer was used for the analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Results of this experiment are shown in Table 2 and 3. The overall means of post-thaw motility were 40.75 and 37.50 for straws and paraffin wax pellets respectively. The differences between these two methods of freezing were significant (P<0.05). The Arriola (1982) procedure resulted in 43% post-thaw progressively motile spermatozoa, which was consistently higher than the average of 32% progressively motile spermatozoa resulting by using of the Weitze (1977) procedure. Some authors explain the poorer performance of pellet semen. Paquignon (1985) pointed out that the pellets spherical configuration causes uncontrolled temperature variations which leads to disturbance in internal freezing rate and crystallisation patterns. On the other hand freezing straws in liquid nitrogen vapour resulted in a constantly changing in the rate of cooling as the internal temperature decreases (Robbins *et al*., 1976). This phenomena may cause more damage to sperm cells in paraffin wax pellets compared to those in straws. It seems reasonable that semen within straws may be cooled uniformly than semen frozen in paraffin wax pellets. In other words, paraffin wax pellets offer less protection to spermatozoa during cooling and freezing to some extent because of the direct contact of pellets with the surface of cold paraffin wax. This also causes faster and less gradual changes in deep freezing temperatures in paraffin wax pellets than in straws.

Table 2: Motility and acrosome integrity of frozen-thawed rabbit spermatozoa as affected by method of freezing and type of extender.

	Spermatozoan Motility %			**Acrosome Integrity%**		
Freezing Method	Glycerol Extender	Sucrose Extender	Mean	Glycerol Extender	Sucrose Extender	Mean
Straws	38.50	43.00	40.75*	42.80	49.90	46.35**
Paraffin Wax Pellets	35.00	40.00	37.50*	41.50	41.50	43.30**
Mean	36.75**	41.50**		42.15**	42.15**	

*The differences between treatments differ, P<0.05

** The differences between treatments differ, P<0.01

The percentages of spermatozoa with integral acrosomes were 46.35 and 43.30% for straws and paraffin wax pellets respectively. The differences between these method of freezing were highly significant (P<0.01). These results demonstrated that the proportion of spermatozoa with integral acrosomes was reduced by freezing and

thawing in paraffin wax pellets than in straws, because freezing at very low temperature affected acrosomal membranes. Awad (1989) stated that it could be expected that the number of injured ram spermatozoa by cold damage is higher in paraffin wax pellets than in straws. These results found also that the acrosomal membranes of rabbit spermatozoa were more injured in the in paraffin wax pellets than in straws. It is well known that spermatozoa had no acrosome is not capable to fertilise ova. The injury found in this study ranged from slight swelling of acrosome to the total removal of the acrosomal sperm membranes. The poorer characteristics of spermatozoa after freezing and thawing are not only due to freezing method. But also from rewarming, and this is due to recrystalization of microscopic ice crystal to form larger ice crystals, which are widely recognised to be damaging.

Table3: Pregnancy results and mean of litter size per pregnant doe following insemination with frozen-thawed rabbit spermatozoa.

Freezing Method	Extender	No. of does pregnant	Pregnancy rate %	Mean litter size per pregnant doe
Straws	**Glycerol**	3/10	30.00	3.30
	Sucrose	5/11	45.50	4.00
	Mean	8/21	37.80	3.70
Paraffin Wax Pellets	**Glycerol**	2/8	25.00	3.50
	Sucrose	4/9	44.40	4.30
	Mean	6/17	34.70	3.90

The results showed that addition of sucrose improved significantly both motility and acrosomal integrity compared to glycerol addition (41.50% of progressive motile spermatozoa Vs 36.75% and 47.50% of integral acrosome Vs 42.13% for sucrose and glycerol respectively). Glycerol is one of penetrating cryoprotectant agents that is used successfully to prevent ice crystal formation during freezing bull spermatozoa (Morris and Farrant, 1972). To fully prevent ice crystallisation, the addition of more than 30% glycerol would necessary (De Leeuw *et al.* 1993). However, a percentage of 6% glycerol turned out to yield satisfactory cell survival (Hammerstedt 1990). Glycerol must enter the cell before it can exert its effect and the cell being injured by the addition or removal of glycerol are usually attributed to osmotic shock, rather than to chemical toxicity (Frim and Mazur, 1983). The presence of glycerol lowers the quality of unfrozen as well as frozen semen in some animal species including sheep (Lighfoot and Salamon,1970). Therefore, at least a reduction of glycerol concentration to 3% of the extender might be beneficial for the survival of frozen rabbit semen. Glycerol proved to be a necessary cryoprotective agent in cryopreservation of Boer goat spermatozoa and the optimum concentration was 5% (Abbas, 1994). Sucrose has the capacity to act as nonpenetrating cryoprotective agents by direct interaction with the membranes (Dee Leeuw *et al.*.,1993). Sucrose is the most regularity used protective disaccharide's, in some reports to prevent freeze-thaw bilayer destabilisation (Strauss *et al.*, 1986). The concentration of 62.5 mM. being superior to 125 mM (Abbas, 1994).

Studying the numbers of pregnancies and young born following insemination of does with frozen-thawed spermatozoa in straws or in paraffin wax pellets are summarised in Table 3. Freezing rabbit spermatozoa in straws could produce high number of does

pregnant, pregnancy rate and number of young born using sucrose or glycerol as cryoprotectant compared to paraffin wax pellets. Mean litter size per pregnant doe was similar for does inseminated with frozen semen in straws or paraffin wax pellets. These results were consistent with results by Graham *et al*. (1978), they found that ram semen cryopreserved in straws has resulted in fertility higher than that of semen frozen by dry ice pellet procedure. Awad (1989) found that the conception rates were 50 and 53 % for straws and paraffin wax pellets respectively. However, Fiser *et al*. (1987) found that the fertility of ewes with frozen thawed semen in 0.5 ml straws or as pellets on dry ice were 73 and 80% respectively, compared to 93% fertility obtained with fresh semen.

The pregnancy rate was highest when rabbit spermatozoa frozen in extender containing sucrose (62.5 mM) as cryoprotectant compared to glycerol (3%). The cryoprotective action of glycerol in ram semen is marred by its detrimental effects on post-thaw viability and fertility (Abdelhakeam *et al*., 1991). In generally, The lower fertility of this experiment was not only due to spermatozoa after thawing but also for environmental factors and the physiological status of female especially at the time of insemination, that is, its sexual receptivity and lactation stage (Theau-Clement and Roustan, 1992).

It is concluded that rabbit spermatozoa frozen in pellets form using the cold surface of paraffin wax was fertile as frozen spermatozoa using straws. Extender of rabbit spermatozoa containing sucrose (62.50 mM) was used as a cryoprotectant better than glycerol for freezing rabbit spermatozoa.

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