A new method of ram frozen semen in form of pellets using the cold surface of cattle fat compared to the conventional methods

M. M. AWAD

Dept. Animal Production
Faculty of Agriculture
Suez Canal University Ismailia, Egypt

Abstract

Two extenders were used for semen extension, EYTGFE Vs EYTSFE. Three methods of freezing were used. Straws as a control, pellets using the cold surface of paraffin wax and pellets using the cold surface of cattle fat. Five pooled semen samples showed > 70% motility of spermatozoa. The same extension rate (1:8) and equilibration period (2 hours) were used in the three methods of freezing.

The overall means of post-thaw motility and acrosome integrity of frozen thawed ram spermatozoa using straws, paraffin wax pellets and cattle fat pellets were 49.00, 42.00, & 54.00% and 49.50, 39.20, & 48.30 respectively. The differences between those methods of freezing were highly significant ($p < 0.01$). Freezing of ram semen on the cold surface of cattle fat had a higher and significant differences ($P<0.01$) in post thaw motility and acrosome integrity compared to that obtained by straws or paraffin wax pellets.

The overall means of post-thaw motility and acrosome integrity were 46.00 & 50.67 and 42.27 & 49.13 for ram semen cryoprotected by glycerol and sucrose respectively. The differences between two types of cryoprotectants were highly significant ($P<0.01$).

In conclusion, freezing of ram semen as pellets on the cold surface of cattle fat could be used successfully more than straws or paraffin wax pellets. Sucrose (62.50 mM) was used as a cryoprotectant better than glycerol.

Key words: Ram Semen, Freezing Methods. Paraffin wax, Cattle fat, Sucrose

Introduction

One of the major limiting factors for the poor fertilizability of frozen-thawed spermatozoa is the reduction of post-thaw motility and viability of spermatozoa. To overcome these problems, it would be suitable to find easy method of freezing to improve the post-thaw sperm characteristics and fertilizability. The best preservation techniques to date of post-thaw survival is restricted to about 50% of the sperm population (Watson and Martin, 1975). The final cryopreservation goal of semen is not only to maintain the initial motility but also to survive in the female reproductive tract at the time of fertilization and to prevent any damage which reduce life span of spermatozoa and its fertilizability.

The cryoprotectant is necessary to reduce or protect the cells from consequences of ice crystal formation and changes in the composition of the medium, as well as the effects of the changes in temperature. Kind and concentration of the cryoprotectant have different results on sperm survival after freezing and thawing. The presence of glycerol lowers the quality of unfrozen as well as frozen semen in some animal species including sheep (Lightfoot and Salamon, 1969). Therefore, a reduction of glycerol content of the extended semen is beneficial for the survival of frozen-

thawed spermatozoa. In addition, the cryoprotective action of glycerol in ram semen is married by its detrimental effects on post-thaw viability and fertility.

Sugars have the capacity to act as nonpenetrating cryoprotective agents by direct interaction with the membranes. Sugars can prevent membrane damage through not only the extreme dehydration but also lowering temperature transition of phospholipids. These literatures may lead to study sucrose as noncryoprotective agent for freezing ram spermatozoa.

The conventional recommended methods to freeze semen of farm animals are straws using liquid nitrogen vapor and pellets of dry ice. In developing countries such as Egypt, sometimes there is deficiency in straws or dry ice in addition to the expensive cost of these materials. The present work aimed to improve the previous method of pellets using the cold surface of paraffin wax (Awad, 1989). By using the cold surface of cattle fat as a surface of pellets instead of paraffin wax. Also to examine the effect of sucrose as nonpenetrating cryoprotectant compared to glycerol as standard penetrating cryoprotectant on the post-thaw motility and acrosome integrity of ram spermatozoa.

Materials and Methods

Five different pooled ejaculates of ram semen were collected using the artificial vagina technique at Faculty of Agriculture Farm, Suez Canal University, Ismailia, Egypt. Each ejaculate was immediately evaluated for progressive motility. Three ejaculates of 70 % or more initial progressive motility were pooled together and considered as one sample. Then each pooled semen sample was extended.

Semen Extension: Two extenders were used in this experiment. The first one is Egg-Yolk-Tris-Glycerol-Fructose-Extender (EYTGFE) and the second is Egg-Yolk-Tris-Sucrose-Fructose-Extender (EYTSFE) using two-step to reach 1:8 extension rate. The first step was by adding 4 volumes of diluent A to one volume of the semen sample at 37°C. The second step was addition of 4 volumes of diluent B after 30 minutes of incubation at room temperature. The compositions of diluent A & B of
Both extenders are illustrated in Table 1.

Semen Equilibration: Extended semen samples were kept in a water bath in refrigerator to cool gradually to 5°C within 2 hrs. Then kept at this temperature for another 2 hrs as equilibration period.

Freezing Techniques Three freezing methods were evaluated for motility and acrosome integrity after freezing and thawing. Straws as a control, pellets on the cold surface of paraffin wax and pellets on cold surface of cattle fat as recent pellets methods of freezing. Salamon (1971) technique for freezing ram semen in 0.25-ml straws was used. Awad (1989) technique for freezing ram semen in pellet form on cold surface of paraffin wax was used.

A new technique of pellets on the cold surface of cattle fat was used as the following:

Pellets on the cold surface of cattle fat: Some cattle fat was melted in a bored box of aluminum foil (5 cm high x7cm width x15cm length) to make a layer of 1 cm depth of cattle fat. Some holes were engraved in the surface before left it to reach room temperature. Cattle fat blocks were cooled by immersion in liquid nitrogen for 30 seconds then placed horizontally and lowered into liquid nitrogen vapor 2-3 cm above the surface of liquid nitrogen. About 200 *ul* of semen after equilibration were pipetted into each hole. After 10 minutes on the vapor of liquid nitrogen, pellets were immersed in liquid nitrogen (-196°C), followed by careful packaging into small goblets of appropriate size and transferred into liquid nitrogen storage container.

Thawing of frozen semen: Straws were thawed in a water bath at 37°C for 1 minute, then the surface of each straw was cleaned and dried. Pellets of paraffin wax or cattle fat were thawed by placing the pellets in clean test tube containing 0.2 ml/pellet sodium citrate medium (2.9%) in a water bath at 37°C.

Examination of Frozen Semen Thawed spermatozoa from straws and both methods of pellets were microscopically tested for motility after thawing according to Salamon (1976). However, acrosome integrity after thawing was determined.

Statistical Analysis*:* Factorial design (3 x 2) was applied to analyze the data by using SPSSWIN (Version 3) computer program. The statistical model was the following:

Y ij = u + Fi + Ej + FEij + eij

Such as: Fi: The effect of freezing method
Ej: The effect extender type
FEij: Interaction between freezing method and extender type
eij: Error

Results and Discussion

Results of this experiment are shown in Table 2 and Figure 1 & 2. The overall means of post-thaw motility and acrosome integrity of frozen thawed spermatozoa using straws, paraffin wax pellets and cattle fat wre pellets 49.00, 42.00, & 54.00% and 49.50, 39.20, & 48.30 respectively. The differences between these methods of freezing were highly significant ($p < 0.01$).

Frozen ram semen in pellets (Lightfoot and Salamon, 1969) and in straws produced lower fertility than fresh semen. 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. Ram semen cryopreserved in straws has resulted in fertility higher than that of semen frozen by dry ice pellet procedure. In Egypt, comparison between straws, and pellets frozen on paraffin wax surface, Awad (1989) found that post thaw motility were 37.2 and 33.9 % and conception rates were 50 and 53 % for straws and pellets respectively. However, the fertility of ewes was 73% in case of pellets and 67% in case of straws (Fiser *et al.,* 1987).

The differences between the three method of freezing may be related to cold damage during freezing. These changes may be less in spermatozoa frozen by cattle fat pellets method than that obtained by the other methods of freezing.

Frozen ram semen is used in artificial breeding with acceptable results only for intrauterine insemination, with conception rate of 60-75%. Frozen semen is not suitable for cervical insemination because spermatozoal motility is poor, resulting in low conception rate (25-45%). This limitation of artificial insemination can be overcome by improvement in the freezing procedure. A new procedure of freezing of this experiment using the cold surface of cattle fat is suitable to improve the post-thaw motility and acrosome integrity compared to straws. These results may be improving the freezing technique of farm animals especially ram spermatozoa which have poor results of sperm characteristics and fertility after freezing and thawing.

The poorer performance of pellet semen is explained by some authors. Paquignon (1985) illustrated that the pellets spherical configuration causes uncontrolled temperature variations which leads to disturbance in internal freezing rate and crystallization patterns. On the other hand freezing straws in liquid nitrogen vapor resulted in a constantly changing rate of cooling as the internal temperature decreases (Robbins *et al*., 1976). These 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 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. Awad (1989) stated that it could be expected that the number of injured spermatozoa by cold damage is higher in paraffin wax pellets than in straws. But the good results of cattle fat pellets in this experiment may be due to lipid molecules which act to protect sperm plasma membrane against cold damage during freezing by reducing the ultrastructural, biochemical and functional damage to a significant proportion of spermatozoa. These changes are usually accompanied by a reduction in motility. This reduction may be minimized during freezing by cattle fat pellets than that obtained by straws or paraffin wax pellets.

These results suggest that the acrosomal membranes of ram spermatozoa were more injured in the straw or in paraffin wax pellets than in cattle fat pellets. The spermatozoa injury described 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. Frozen spermatozoa may be injured during thawing by rewarming, due to recrystalization of microscopic ice crystal to form larger ice crystals that are widely recognized to be damaging. Abdalla (1983) on goat semen found that the extracellular enzyme contents were significantly increased after freezing and thawing due to the damage of sperm membrane which allowed the intracellular components to go out. He also reported negative correlation coefficient between extracellular enzyme contents and physical characteristics of ram semen. On the other hand, Lindemann et. al. (1982) reported that the dead spermatozoa have a negative toxic effect on the remaining normal sperm population.

The overall means of post-thaw motility and acrosome integrity were 46.00 & 50.67 and 42.27 & 49.13 for ram semen cryoprotected by glycerol and sucrose respectively. The differences between two types of cryoprotectants were highly significant ($P<0.01$). These results showed that sucrose used as noncryoprotectant is better than glycerol used as penetrating cryoprotectant for both post-thaw motility and acrosome integrity of ram spermatozoa.

Glycerol is one of penetrating cryoprotectant agents that is used successfully to prevent ice crystal formation during freezing bull spermatozoa. To fully prevent ice crystallization, the addition of more than 30% glycerol would be necessary. However, a percentage of 6% glycerol turned out to yield satisfactory cell survival. Glycerol must enter the cell before it can exert its effect and the cell might be injured by the addition or removal of glycerol. Injuries observed are usually attributed to osmotic shock, rather than to chemical toxicity. The presence of glycerol lowers the quality of unfrozen as well as frozen semen in some animal species including sheep (Lightfoot and Salamon, 1969). Therefore, at least a reduction of glycerol concentration of the extender might be beneficial for the survival of frozen semen. The cryoprotective action of glycerol in ram semen is marred by its detrimental effects on post-thaw viability and fertility. Glycerol proved to be a necessary cryoprotective agent in cryopreservation of Boer goat spermatozoa and the optimum concentration was 5%.

Sucrose has the capacity to act as nonpenetrating cryoprotective agents by direct interaction with the membranes. This interaction involves hydrogen bonding of sugar hydroxyl groups with the phosphate groups of membrane phospholipids by replacing the water around the phospholipid head group. Sucrose can also prevent membrane damage caused by extreme dehydration and lower the transition tem-

perature of phospholipids by increasing the head group spacing and thereby decreasing the Van der Waal`s interactions between the phospholipid acyl chains.

Generally, disaccharides are more effective in stabilizing bilayer than monosaccharides. Sucrose is the most regularity used protective disaccharides, in some reports to prevent freeze-thaw bilayer destabilization. On the other hand, sucrose yielded higher percentages of intact cells after freezing and thawing than equal amounts of trehalose. Sucrose concentration of 62.5 mM was being superior to 125 mM. The sucrose containing extender was better significantly than the standard extender, which had 6% glycerol for both motility and acrosome integrity after freezing and thawing. These results because sucrose cannot penetrate the cell, the effect is to provide a high external osmotic counterforce to avoid water movement into the cell and because the extracellular fluid does not contain the permeating cryprotectant, the cryoprotectant is free to diffuse out of the cell.

In conclusion, freezing of ram semen as pellets on the cold surface of cattle fat could be used successfully. Sucrose (62.50 mM) was used as a cryoprotectant better than glycerol.

Tables and Figures

Table 1: *The components of* Egg *Yolk-Tris-Fructose-Glycerol (EYTFGE) and Egg-Yolk-Tris-Fructose-Sucrose (EYTFSE) extenders.*

Components	**Extenders**			
	EYTFGE		**EYTFSE**	
	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)	----	12.00	----	----
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.

Table 2: Means ± S.E of *post-thaw motility and acrosome integrity of frozen-thawed ram spermatozoa as affected by method of freezing and type of extender.*

Freezing Methods	**Extenders**		Overall Mean
	EYTFE	**EYSFE**	
Post-Thaw Motility (%)			
Straws	47.00	51.00	**49.00**
Paraffin Wax Pellets	39.00	45.00	**42.00**
Cattle Fat Pellets	52.00	56.00	**54.00**
Overall Mean	**46.00**	**50.67**	
Acrosome Integrity After Thawing (%)			
Straws	45.00	54.20	**49.50**
Paraffin Wax Pellets	33.60	44.80	**39.20**
Cattle Fat Pellets	48.20	48.40	**48.30**
Overall Mean	**42.27**	**49.13**	

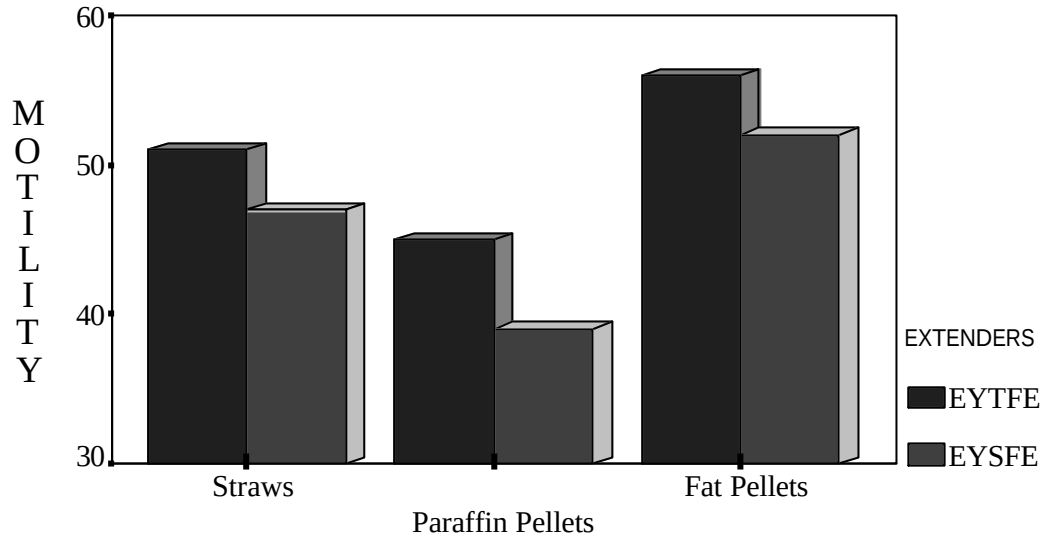

Figure 1: ***Effect of freezing methods and type of cryoprotectant on Post-thaw motility of ram spermatozoa.***

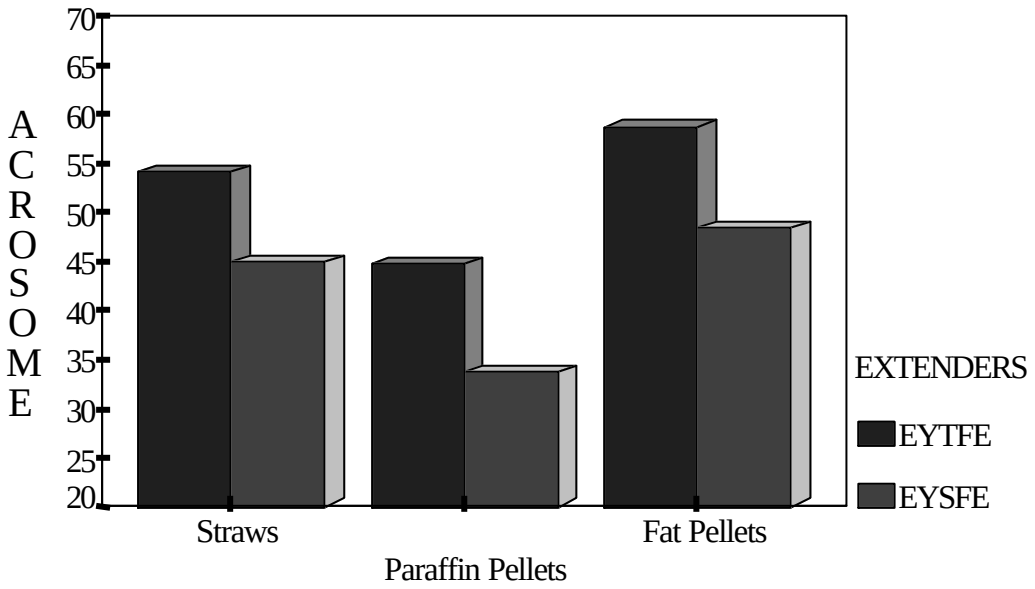

Figure 2: ***Effect of freezing methods and type of cryoprotectant on acrosome integrity ram spermatozoa.***

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