Animal Production (Group Scarabaeus)

Breeding strategies for improving animal performance

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Introduction

The low level of performance of the animal population in the Tropics and Subtropics and the necessity to improve the performance lead to considerations on transferring genes of European and American breeds to the Tropics and Subtropics.

Within the range of this topic the following points are to be discussed:

- Problems of import of high performance animals into the Tropics and Subtropics for pure breeding.
- Possible ways of applying such high performance animals in cross breeding.
- Selection within the local breeds and eventually applying imported breeds from areas of environmental conditions similar to the local conditions and
- The importance of genotype x environment-interactions for determining the breeding programme and the role of research for the decision.

Problems of import of high performance animals in the Tropics and Subtropics for pure breeding

The possible advantage of applying animals from temperate zones is the intensification of the performance, but it is only possible under optimal environmental conditions. These methods are not only very expensive, but lead to negative results as shown in many experiments. The factors limiting the rearing of animals from temperate zones in the Tropics can be summarised as follows:

- Very little acclimatisation to adverse climate conditions. They have little tolerance of heat as compared to native breeds.
- Their exposure to numerous diseases in the Tropics and Subtropics and
- the high demand of good feeding and rearing conditions.

These environmental factors and the interactions between them lead to the fact that the imported breeds in the Tropics and Tropics do not produce the expected results.

The possibility of keeping pure imported breeds in warm climates is limited to allocation of no infectious diseases and of course where sufficient feeding for these highly efficient animals is available. Even the few good results of high efficiency of temperate breeds in the Tropics have been due to favourable rearing conditions and good management. The results of these experiments are on no account to be applied to indigenous farmers where such favourable conditions are more or less impossible.
Over the last few years there has been intensive farming near the big cities where good marketing conditions are available for animal products. These farms are in the

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position to feed European breeds intensively and obtain good results. These farms produce well but they wholly depend on the import of both animal fodder and the animals themselves. In the light of this, such farms could not be classified under breeding to improve animal husbandry in their area. An example for that is the intensive import of poultry hybrids in many areas. These reasons lead more or less to joint breeding programmes.

Possible ways of applying high performance animals in the Tropics and Subtropics in crossbreeding

The purpose of crossing between high performance animals and the local breeds is to combine the high potential efficiency of the imported animals with the adaptation ability of the local breed to their rough environmental conditions. With the foregoing observations in mind, the next step is to consider some means whereby breeding programmes may be implemented. Recommendations are based on the premise that for any system to be effective some governmental participation and research work will be required - especially in guidance of programmes.

In numerous countries, individual breeders have initiated successful programmes of genetic improvement. They have not, however, been as judicious in consideration of their peers resulting often in dispersion of rather inferior stocks. Individual breeders frequently emphasise traits that may or many not be of the highest economic significance or contribute to the improvement in the general population. Furthermore, for a programme to be effective it should be closely allied to a research programme, which is another important reason for involvement of universities and research institutes.

In order to raise a new population which can adapt itself to the local environment there must be series of crossing procedures with different shares of genes of imported breeds measured on the environmental conditions.

The illustration in Fig. 1 is for grading up local population commonly practised in many tropical areas, frequently with disappointing results. This involves the importation of sires, semen or even groups of males and females, which are employed in a grading up scheme on local stocks. The major disadvantage as normally applied has been the same numbers of animals or semen imported. Nevertheless, this system has met in some places with reasonable success. In this system the local stock is gradually in independence in the environment replaced, but unless the environment is good the first generation cross may be the only group that performs satisfactorily. Disappointing late generations may result in a loss of confidence by villagers - and confidence is a critical feature in livestock improvement. This system should, therefore, be recommended on a broad scale only when managers understand all factors involved.

Fig. 2 illustrates a seemingly more appropriate approach than the previous one for widescale use. In this plan, the government agency initiates the programme by establishing a herd or flock of indigenous females, which are crossed with imported males to produce F_1 males and females. The herd could be carried on either by continuous replenishment of the local type females from the general population or by inter se rating of the crosses coupled with selection. Both methods have advantages. This system is more suitable in many respects than the one in Fig. 1 in that the first generation village animal would contain theoretically only 25 % imported blood and 75% of its native breeding. In subsequent generations, the infusions of imported stock would be

12 and 6%, respectively. Grading up could proceed for 4 to 5 generations before approaching the 50% level. By the time this stage is reached, either the stocks and managers should be good enough to go on with the scheme or the managers should be encouraged to take up some other type of enterprise. This system also has the advantage that if anywhere along the way some of the producers are capable of handling better quality stock, a direct cross can be made with the imported types. It also has flexibility and affords an opportunity to broaden the sampling of combinations of imported and local types.

Selection within the local breed

Although the selection within the local breed is slow, it is however a surer way to improve the animal material. This way has the advantage that because of natural and artificial selection the animals are adapted themselves to the raising conditions in their area.

The importation of breeding material from other tropical or subtropical areas similar to environmental conditions could be a possible method of improving the animals under the condition that there are no specific diseases in the area of breeding. The scheme in Fig. 3 shows an example for the selection within the local breed.

The simplest and least expensive system would be for the government or its agencies to initiate a recording system whereby representatives would go periodically to the village or individual farm and measure milk yield, in the case of dairy production, or weigh calves or lambs as a means of identifying females giving the best performance. The better producing females identified through these records would be earmarked so that their sons would be saved. The sons would be brought to a central location and reared in a common environment, where information could be obtained on rate of growth and development for use in making further selections among the males. Males selected from these groups could then be redistributed for natural service or artificial breeding (AI) for use among the general population of females. This system is illustrated in Fig. 3. The plan shows that the procedure would be repeated periodically, preferably on an annual basis, with the intent of genetic improvement. This would give primary emphasis to the use of superior dams. If used for milk yield in cattle, it would permit up to 33% of the total opportunity for genetic gain (Robertson and Rendel, 1950). In later years the rate of genetic improvement could be enhanced by progeny test information becoming available in the sires distributed in earlier years.

A second system, illustrated in Fig. 4, provides for the establishment of a breeding research institute as a seedstock herd or flock under intensive selection. Selected males could be distributed according to the previous plan for use among the general population. This procedure has often been applied but without very satisfactory results. The inadequacy has resulted from too few animals and too low selection differentials, mainly because the institute confined its base population to an original group of animals chosen principally on a phenotypic basis in one period of the year. The males, and indirectly the females, selected in this rather inefficient fashion have a large influence on later generations. This system could be effective if a selected herd or flock represented the upper 30% of the general population. A more efficient system would be to select a group of animals from the general population and assemble them at the research institute or a commercial farm, where they could be observed through one production cycle - e.g. lactation or lambing.

Following the first „production period“, 50% or more of the females should be discarded. The procedure of female selection should be repeated for several years - at least three and preferably five. The „selected“ herd could be developed as illustrated in Fig. 5. If the selection differential after arrival in the seedstock herd is 50% or higher, the basic group will be of much higher quality than a group produced by one selection period. This is not an expensive procedure as rental or condition of sale could be a part of the arrangements with the initial owners.

The plans described by no means represent all the possibilities. They are set forth to illustrate some possible systems, alone with their basic requirements and advantages. Independent of the breeding system there are important points for a successful breeding programme:

- Good co-operation between the breeding centre, the research institute and the farmers.
- Performance control and recording system as a basis for the selection of the breeding animals.
- Continuous systematic selection of the best animals for the breeding.

Importance of genotype x environment-interactions for the breeding programme and the role of research for the decision

In the last illustrations proposing possible ways of production of new breeds show bluntly that the addition of genetic materials is in many ways possible. The question here is whether the relative efficiency of breeding populations in the Tropics and Subtropics with different genes` shares of imported breeds remains the same even under different environments. From that point of view it remains to be decided which breeding population fits a particular environment and whether they possess special adaptable capacity for this environment.

Moreover questions of whether special breeding methods should be developed for extreme environmental conditions could be answered through information about the effects of interaction between genotype and environment.

The most important economic characteristics of animals which the breeders try to improve are the quantitative characteristics which generally are modified by environmental influences. The ability of a particular genotype for developing of a character is thus of no pronounced size, but just because of the environmental conditions under which it depends on:

Phenotypic characteristics value = genotypic characteristics value + environmental effect.

P = G + U

The validity of this equation is to be based on one of the most silent conditions which says:

There is no interaction between genotype and environment, i.e. their effectiveness behaves independently of one another. It is, however, conceivable that the possible reactions of individual genotypes could be interpreted in different ways. This means

that Genotype x Environmental Interaction exists. In the equation an additional changing effect between Genotype and Environment (P = G + E + GE). According to Pirchner (1979) there exists Genotype x Environmental Interaction, when the efficiency of a particular genotype in a particular environment does not show any explanation of the average value of both factors but deviate from that expected data.

To clarify a summarised meaning of a genotype x environment relationship in animal breeding, the real changing effects could be limited as illustrated in Fig. 6.

The genotypes could be imported breeds, local breeds or crossbreeds with different genetical shares from the imported and local genes. The environmental conditions could depend on location of the farm, feeding or management even within the Tropics and Subtropics.

In case 1 there is a balanced change of efficiency in the genotype through the bad environmental conditions Here there is no change in order of sequence of the genotypes and no noticeable change in the variation between the genotypes depending on the environment.

Case 2 shows clearly that a change in environment causes different effects in the genotypes. The genotypes A and B experience a decrease whereas genotypes D and E achieve an increase in valuable efficiency. That leads to a decrease of the genetic variations in the environment (Y) although the order of sequence remains unchanged.

In case 3 change in the order of sequence of the genotypes takes place in which some genotypes improve their order of sequence in the second environment (Y). However, there are others who do not improve but deteriorate. In this case, there is a clear genotype x environment-interaction whereby the variation between the genotypes remains the same.

In the last case, like in case 3, a change in the order of sequence occurs in the genotype, but leads additionally the change in efficiency to a decrease in variation between the genotypes in the environment (Y).

Like the illustration 6 shows the genotype x environment-interaction can be due to two reasons:

1. Populations show variations in different environments.
2. A change in the order of sequence of genotypes occurs in different environments.

Naturally the shifting order of sequence makes difficulties in the practical breeding work. This is very important for the selection of a population for a certain environment or even for the selection of breeding animals within the population.

This means that the order of sequence of sires in an environment is not transferable to other places. Genotype x environment-interaction has an influence on the selection possibilities and has an effect on successful selection in the environment affected.

There are many methods for research into genotype x environment-interaction. The first condition for research is that animals examined for the planned breeding are animals within a population which are surveyed under their environment. For any environment the suitable breeding groups should be sorted.

The necessity of examining the availability of genotype x environment-interaction

There is literature that is available on genotype x environment-interaction for meat and milk production. This examination has mostly to do with intro-origin genotypes (sires within origin) or with environments within the temperate zones.

No unpronounced genotype x environment-interaction has been taken notice of. The environmental conditions under which the genotypes have been examined are not so extreme as in the Tropics and Subtropics.

The genotype x environment-interaction may, however, be clearly expected depending on the difference between the genotypes and environments within the area.

These are no tests about the productivity of different breeds and crosses in the Tropics and Subtropics under different environmental levels. This makes it difficult to determine the suitable population for a certain environment. Here is the important role of the research institutes to find out the suitable breeds to the local environments.

Of interest are the tests of MADSEN and VINTHER (1975) in Thailand (Table 1 and 2) in which they tested many different crossing levels and the effect of different gene shares of Bos taurus regarding not only the milk production but the calving interval and the mortality rate. The results showed that under the good management at the Danish-Thai Farming and Training Centre, the increase in milk production was due to the increase in percent of the Bos taurus.

The problem of this test was that fertility rate (measured on the calving interval) had a significant effect on the annual milk yield. Cross breeding with high percentage of Bos taurus genes showed a high mortality rate, especially between the age of 6 months to maturity (23,7% mortality). Due to these results the breeding of 50% Sindhi/Sahiwal) and 50% Red Danish were considered suitable for the environmental condition in the area.

Unfortunately, there were no comparisons between the crossing methods carried under minimum conditions as shown with native breeding animals. By the judgement of the breeding groups, it must be clear that the crossing between different breeds could have crossing effects which overestimate the genetic efficiency of the F_1 generation. Moreover, each distant generation is reduced to half. This means that a sizeable genetic material would not remain within but lost, a fact that always been neglected.

The genetic ratio between the imported Bos taurus breed and each local breed is determined by the environmental conditions. Areas of different production levels (where the climatic effects, the food deficiency and poor management have great effects on productivity on local farms and where the local breeds show specific adaptation properties) enjoy the presence of genotype x environmental interaction to a great extent. In the light of these cases, it can be concluded that a gene transfer for these production conditions is not recommendable.

Data on 2422 and 27756 Friesian cows in Egypt and Germany, respectively, were used to estimate genetic and non-genetic effects on initial milk yield in 70 days (IM), 305-day milk yield (305-dMY), lactation period (LP) and calving interval (I). Data was collected in the period from 1987 to 1992 in Egypt and from 1979 to 1993 in Germany. Least squares analysis shows the significant effect of season and year of calving and parity on all traits.

In Table 3 are the results of the performance of animals in Egypt compared with the data in Germany.

Table 3: Means, standard deviations (SD) and coefficients of variability (CV) of unadjusted records of traits, initial milk yield (IMY), 305-day milk yield (305-dMY, lactation period (LP), and calving interval (CI) of Friesian cows in Egypt and Germany

	Egypt			Germany		
Trait	Mean	SD	CV	Mean	SD	CV
IMY, kg	966	319	33	1552	318	21
305 dMY, kg	4736	1097	23	6641	1484	22
LP, day	298	62	21	301	10	3
CI, day	379	72	19	396	58	15

Coefficients of variation computed from residual mean squares divided by the overall least squares means of a given by (Harvey Programme, 1990)
Numbers of records were 2422 in Egypt and 27756 in Germany

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Table 4 shows the ranking from high to low of certain groups of cattle for characteristics indicative of suitability to a hot environment under either advantageous or disadvantageous management conditions. The results of that test show according to the clear interaction between genotypes and environment the importance of the research to find out the suitable breed for that environment.

Table 4: Ranking from high to low of certain groups of cattle for characteristics indicative of suitability to hot environment under either advantageous or disadvantageous management conditions, breed groups in the same column with a common superscript (1 or 2) are not significantly different, but do differ from those not having the same superscript (P < 0.5)

Methods of measuring adaptation

Rise in body temperature	**growth rate**	**milk yield**
Environment „A“ adequate nutrition, no serious disease problems, good management[A)]		
Zebu	Brown Swiss	Holstein
Zebu-European X	Charolais	Brown Swiss
Santa Gertrudis	Holstein	Jersey
Brown Swiss[1]	Zebu X[1]	Zebu X[1]
Jersey[1]	Santa Gertrudis	Zebu 2
Charolais[1]	Hereford[1]	
Angus[1]	Angus[1]	
Hereford[1]	Jersey[1]	
Holstein[1]	Zebu[1]	
Environment „B“ low nutrition, disease problems, poor management B)		
Zebu	Zebu X	Zebu X
Zebu	Zebu	Jersey
Brown Swiss[1]	Brown Swiss[1]	Brown Swiss[1]
Jersey[1]	Hereford	Holstein[1]
Angus[1]	Charolais[1]	Zebu[2]
Charolais[2]	Holstein[1]	
Holstein	Jersey[1]	
Hereford[2]	Angus[1]	

A) Assumes ample quantity and quality of feed throughout the year, good disease control measures and experienced personnel for management
B) Assumes poor quality feed with serious seasonal fluctuations, little effort made to control disease, and inexperienced personnel

Summary

1. The use of pure breeds from temperate zones in the Tropics or Subtropics where the environment is severe, seems to be impossible.
2. The use of such imported breeds in a crossing programme with the local breeds is possible in different ways under the consideration of the local environmental factors.
3. The selection within the local breeds is a long but sure way to improve the performances
4. The role of research is very significant to find out the suitable programme for a certain environment
5. Independent of the breeding system there are important points for a successful breeding programme:
   - Good co-operation between the breeding centre, the research institute and the farmers
   - performance control and recording system as a basis for the selection of the breeding animals
   - continuous systematic selection of the best animals for breeding

Effect of semen extender components on rabbit sperm motility

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Abstract

Three different rabbit semen extenders based on Tris-egg yolk extender were used in this experiment. To examine the effects of 1% glycerol or 6% dimethylsulfoxide (DMSO) and control (free of glycerol and DMSO) on rabbit sperm motility and acrosomal changes % over a period of three days of storage at 5 °C. Semen samples from White New Zealand (WNZ) bucks were evaluated for initial concentration, pH value and volume in relation to sperm motility and acrosome loss % . The obtained results showed that, there were significant differences ($p < 0.05$) according to extender type used. Where sperm motility % were 38.0 ± 4.5, 47.3 ± 3.8, and 37.9 ± 4.1 % as for control, 1 % glycerol and 6 % DMSO extender, respectively. The correlated studied characters of acrosomal loss % for the same extenders differed significantly ($p < 0.05$) and were 8.5 ± 0.63, 4.5 ± 0.33 and 7.6 ± 0.68 % respectively.

Key Words: Sperm, Rabbit, Glycerol, Dimethylsulfoxide, *Cryoprotectants*

Introduction

Many research articles were conducted to study the effects of glycerol addition in rabbit semen extenders on both sperm motility and acrosome integrity (Weitze, 1977 and Weitze *et al.,* 1982). The most popular theory for the beneficial effect of glycerol addition in semen extenders is protection impact of glycerol through salt-buffering mechanism (Lovelock, 1953; Polge and Soltys, 1957). Others reported that glycerol can penetrate sperm cell membrane and concentrate in the posterior part of the sperm head (Pickett and Merilan, 1957).

The recent studies tried to prove that some cryoprotective agents like glycerol have toxic effect on rabbit sperm survival through its content of hydroxyl groups (Hanada and Nagase, 1980). Other authors stated that cryoprotectants containing amides or methyl groups like DMSO have been recommended and used successfully for rabbit sperm preservation (Arriola, 1982; Chen and Foote, 1988).

The objectives of this study were to investigate the effect of cryoprotectants like glycerol and DMSO on rabbit sperm motility and acrosome changes in relation to essential semen characteristics in cooled preserved rabbit semen at 5 °C.

Materials and Methods

Bucks and semen collection:
Three New Zealand White (NZW) bucks were used in this experiment with an average body weight 2.5 – 3.5 kg and 12 months of age. The bucks were housed in a wire cages and the doe was transmitted to buck's cage for semen collection. Semen samples were collected by using a glass artificial vagina, supported with a graduated collecting tube and the added water was adjusted on 45 °C at the time of semen collection (Morrel, 1995).

Semen sample evaluation:
The collected semen samples were put in a water bath adjusted at 38 °C. The samples were subjected to the following examinations: general appearance, sample volume, sperm concentration / ml, pH value and initial motility. Sperm motility was estimated by using a light microscope at 100x magnification on a stage warmed at 38 °C. Semen samples less than 60 % motility were discarded and not included in the experiment.

Semen extender components:
Tris-egg yolk extender was prepared according to Fischer and Odenkirchen, 1988. Tris-egg yolk extender contained 360 mM Tris, 33.3 mM glucose and 113.7 mM citric acid for control treatment. The second treatment contained 1 % glycerol in the Tris-egg yolk extender. While the third treatment contained 6 % (vol. / vol.) dimethylsulfoxide (DMSO) in Tris-egg yolk extender. For all treatments egg-yolk, penicillin and streptomycin were added to give a final concentration of 5 % (vol. / vol.), 0.01 % (wt / vol.) and 0.05 % (wt / vol.) respectively.

Semen extension and storage:
The evaluated semen samples were diluted (1 : 5) with three different treatments as mentioned previously at room temperature. The diluted semen samples were put in a closed test tubes (5 ml) in a refrigerator at 5 °C. The storage period of the diluted semen samples was 3 days. To achieve required cooling temperature the tubes were put in a water bath provided with a thermometer to check the required cooling temperature (5 °C) in the water bath every day of the storage period.

Sperm motility and acrosomal changes assessment:
Initial motility was recorded for both raw collected semen and diluted samples directly after semen collection and during three successive days of storage period at 5 °C by using light microscope with 100x magnification. As for acrosome changes determination, two smeared slides of diluted semen samples were prepared directly after dilution and daily during the storage period. The dried smeared slides were examined directly without staining for acrosomal changes according to Watson and Martin, 1972 by using oil lens of light microscope to obtain 1000x magnification. A total count of one hundred sperm cells was examined for the acrosomal change % for each slide. The average of two slides was recorded for each determination.

Statistical analysis:
Analysis of variance (ANOVA) was carried out using starting spss/pc program (1993) to study the effects of semen extender ingredients and storage period on both sperm motility % and percentage of acrosomal loss of different semen samples. Correlation was estimated between raw semen characteristics and each of sperm motility % and acrosomal changes % and between sperm motility % and acrosome changes %.

Results and Discussion

Sperm motility examination:

Results presented in Table 1 showed that, the sperm motility % was superior and differed significantly ($P < 0.05$) as for Tris-egg yolk extender contained 1 % glycerol which was 47.4 ± 3.8 % for the entire storage period of three days. Whereas, sperm motility % of control treatment (Tris-egg yolk free of glycerol and DMSO) and Tris-egg yolk extender contained 6 % DMSO were 38.0 ± 4.5 and 37.9 ± 4.1 %, respectively for the same storage period. These significant differences may be due to the effect of glycerol in avoiding or controlling cold shock when sperm cells are preserved at 5 °C. It is obvious that rabbit sperm is sensitive and may be died by chilling at temperatures above freezing points as declared by Walton, 1957 and for other animal species by Kumar et al., 1994; Singh et al., 1994; Katila, 1997. The beneficial effect of glycerol which was achieved at this added concentration (1 %) may be associated with the non-toxic effect of glycerol to rabbit sperm especially at this level. The useful effect of glycerol addition was not achieved in the case of DMSO, which can demonstrate the unnecessary to add DMSO alone as a cryoprotective agent in rabbit semen extenders for cooling preservation. Other authors found that cryoprotectants containing amides or methyl groups have been recommended and used successfully for frozen semen (Hanada and Nagase, 1980; Arriola, 1982). Sperm motility percent varied significantly ($P < 0.05$) according to storage period for different treatments (Table 1). The addition of 1 % glycerol in Tris-egg yolk extender maintained sperm motility significantly differed as compared to control or DMSO treatments (Table 1). It is obvious that both of glycerol and DMSO addition maintained sperm motility in a relatively good condition comparing to control treatment during storage period especially glycerol treatment at third day of storage period (Table 1). Also there was a sharp decrease in sperm motility after first day of storage period for all treatments (Table 1) especially control and DMSO treatments. The explanation of this phenomena may be due to high sensitivity of rabbit sperm to face cooling preservation.

Acrosomal changes assessment:

Studying the acrosomal loss percentage showed that, there were significant differences ($P < 0.05$) among different treatments. The acrosomal changes % were 8.5, 4.5 and 7.6 for control, 1 % glycerol and 6 % DMSO extenders respectively for complete storage period at 5 °C (Table 2). The addition of 1 % glycerol to Tris-egg yolk extender was significantly increased ($P < 0.05$) preservation of acrosomal integrity across storage period as compared to the other treatments during three successive days of cooling as shown in Table 2. There were different stages of acrosomal loss. The observed acrosomal status was recorded to be five different stages as shown in Figure 1. To distinguish between different stages of acrosomal loss, shape No. of sperm cell considered to be intact acrosome for the typical complete or non-changed acrosome, while shapes from No. 2 until No. 5 considered to be acrosomal changes (Fig. 1). There are many changes can occur to sperm cell at acrosomal level during cooling preservation. One of this alterations is the spatial arrangements of the internal structure and outer covering of the cells. Beside a contraction of protoplasm may occur and can happen at different rates depending on the chemical components of the various structures (Slisbury, 1978) including acrosomal structures. The beneficial effect of glycerol at this level in this experiment (1%) may be come from the ability of glycerol to reduce mechanical destructiveness to rabbit sperm cells at acrosomal level.

Semen samples characteristics:

Values of semen sample evaluation as regard volume, pH value and initial concentration presented in Table 1. There was positive correlation between these characters and sperm motility as shown in Table 3. In addition there were negative relations between sperm motility % and value of pH as shown in Table 3. In addition there was negative relationship between sperm motility and acrosomal changes % especially for first two days of cooling preservation as for glycerol and control treatments as shown in Table 4. But this relation did not persist as for third day of storage where there was a positive relationship between sperm motility and acrosome changes %. These relations may explain the importance of initial semen evaluation for the samples that will process for refrigeration storage.

It could be concluded, that rabbit sperm is sensitive to great extent to cooling preservation. The extenders, which are used, must contain a cryoprotectant agent like glycerol but in a low concentration to avoid toxicity of rabbit sperm (1 % glycerol could be recommended). Also to obtain a moderate rate of rabbit sperm motility, it is useful to store rabbit semen for only one day at 5 °C. Beside, the technique of acrosomal loss % determination using oil lens of light microscope without any kind of staining is a rapid and simple method for assessing acrosomal changes during storage.

Table 2. Mean ±SE of sperm acrosomal changes percent of different semen extenders during storage period at 5 °C.

Sample No.	Sperm Acrosomal Loss %											
	Control				Glycerol				DMSO			
	A.D	1st	2nd	3rd Day	A.D	1st	2nd	3rd Day	A.D	1st	2nd	3rd Day
1	3	8	12	12	2	5	5	7	2	5	8	13
2	4	6	10	10	4	3	5	6	4	4	8	12
3	2	7	9	10	2	3	7	8	3	6	9	14
4	4	8	10	11	3	4	4	5	4	5	7	12
5	5	7	11	13	4	3	4	6	4	6	10	11
6	4	8	12	14	2	4	6	7	5	5	9	14
7	6	9	11	13	4	2	5	7	4	6	10	12
Mean ±SE	4 .49	8 A .37	11 D . 42	12 G .59	3 .38	3 B .37	5 E .4	7 H .37	4 .36	5 C .29	9 F .42	13 G .43
Overall Mean ±SE	8.5 A 0.63				4.5 B 0.33				7.6 A 0.68			

Means with the same letters showed no significant differences using Duncan multiple range test.

Table 3. Correlation among raw semen characters, sperm motility & sperm acrosomal loss%

Correlation	Raw Sperm Conc.			Value of pH			Sample Volume		
1st day sperm motility (Control) (Glycerol) (DMSO)	0.56	0.64	0.63	-0.66	-0.47	-0.7(	0.83**	0.66	0.66
2nd day sperm motility (Control) (Glycerol) (DMSO)	0.90**	0.90**	0.77*	-0.68	-0.58	-0.4	0.80*	0.55	0.5
3rd day sperm motility (Control) (Glycerol) (DMSO)	0.91**	0.86**	0.63	-0.46	-0.37	0.0	0.42	0.47	0.0
1st day acrosomal loss (Control) (Glycerol) (DMSO)	0.30	-0.37	0.50	0.34	0.76*	-0.2	-0.25	-0.73	0.2
2nd day sperm motility (Control) (Glycerol) (DMSO)	0.12	0.67	0.67	0.29	-0.37	-0.(	-0.53	0.71	0.4
3rd day sperm motility (Control) (Glycerol) (DMSO)	0.5	0.68	0.55	-0.09	-0.29	-0.(	-0.28	0.63	0.3

**P < 0.01

* P < 0.05

Table 4. Correlation between sperm motility and acrosmal changes % during storage period.

Correlation	Acrosomal changes %
1st day sperm motility (Control) (Glycerol) (DMSO)	-0.46 -0.23 -0.5
2nd day sperm motility (Control) (Glycerol) (DMSO)	-0.27 0.78 0.35
3rd day sperm motility (Control) (Glycerol) (DMSO)	0.44 0.82** 0.72

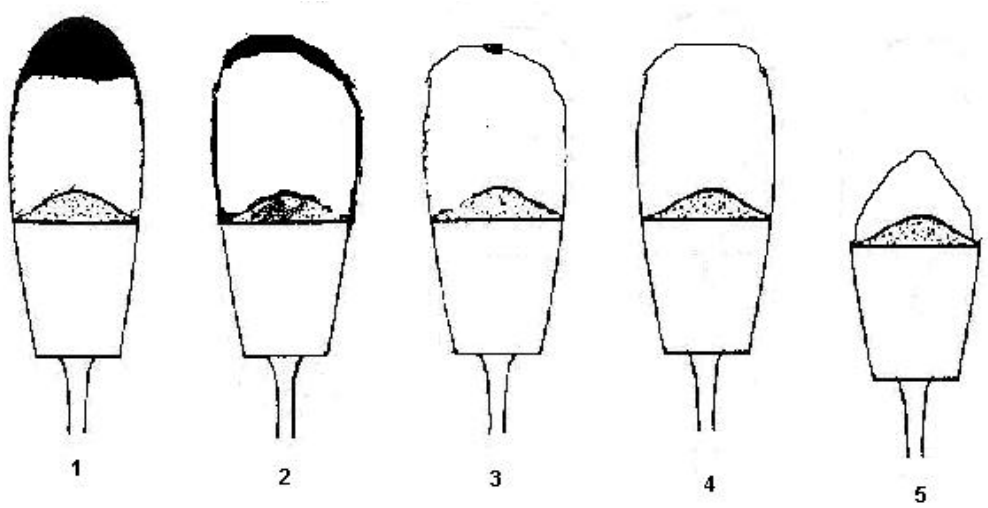

Figure1. Different stages of acrosomal changes of rabbit sperm during cooling storage. No. 1 considered normal acrosome while No. 2 to No. 5 are changed acrosome when examined by using oil lens of light microscope(1000x magnification).

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The role of cow's raw milk in transmission of brucellosis

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Summary

A total of 150 individual samples of blood and raw milk of cows collected from El-Behera Governorate were examined for detection of brucellosis. The samples were tested for SAT. The milk samples were subjected to MRT and isolation of brucella organisms, while the whey was examined for WRBPT and WRiv.T. The results showed that MRT was found to be reliable and sensitive for diagnosis of brucellosis in milk, as it gave positive results in 8 % of samples, as compared with the results of SAT (10%) for serum and WRBPT (4.7%), and WRiv.T (4.0 %) for milk whey. Brucella organism could be recovered from one (0.7%) sample which identified as *Br. melitensis* biovar 3.

The results of experimental study reveal that *Br. melitensis* biovar 3 survived for 8 days in refrigerated raw milk (4 ±1°C) and 2 days in raw milk kept at room temperature (22 ±2°C). While, it also survived for 12 days in yoghurt cream and 8 days in yoghurt body. The organism cannot withstand pH4.7 in raw milk and 4.2 in yoghurt.

The public health significance, epizootiological importance as well as the necessary measures recommended to control brucellosis were discussed.

Introduction

Brucellosis is an important milkborne zoonotic disease that it could be, under natural conditions, transmitted from animals to man. This hazard practically spread allover the world, only 17 countries of the whole world have been declared free from animal brucellosis (OIE, 1985). Brucellosis is still reported in some people of these countries, where the disease is usually contracted during travel to endemic areas. The disease in man can be caused by any of the *Brucella* species, *abortus, melitensis* or *suis.* The disease due to *Br. melitensis* is more severe and virulent than that caused by the other *Brucella* species.

The infected animals (sheep, goat, cow, buffaloe and camel) excrete brucella organisms in their milk sporadically throughout the entire period of lactation in counts varied from a few to up to 15000 cells / ml milk (Robertson, 1961; Sdiwerifeger, 1963; Ismail, 1971; Awad et al., 1975 and El-Gibaly et al., 1981). The brucella content of milk depends on the stage of lactation, as usually, the largest number of brucellae are in the milk at the onset of lactation periods, but both the occurrence and numbers of brucella excreted at any time can vary (Elberg, 1986). Hence, raw milk and its products from infected dairy animals play a significant role in the transmission of the disease to man. It was recorded that two-thirds of brucellosis in human cases at California, USA, arose through consumption of raw dairy products (Wynns, 1944).

Milk ring test (MRT) and blood serological tests are mainly used for diagnosing the disease among cattle. The evaluation of these tests was carried out by many workers (Nicoletti and Muraschi, 1966 and Katz et al., 1976). They stated that neither single milk nor blood serological tests were sufficient to give conclusive diagnosis of positive cases.

Tests for detection of brucella antibodies in milk are considered the principal methods for detecting infected herds and for diagnosing brucellosis in an individual cattle, because it is difficult to recover brucella from infected animals. The ideal diagnostic test for brucellosis should be easy, simple, and rapid test that will detect infected animals as early as possible during the course of the disease. Furthermore, this test should not be influenced by presence of non-specific antibodies arising from other Gram-negative bacteria (Morgan, 1977). Morgan et al. (1978) and Alton et al. (1988) described more specific tests for detecting brucella antibodies in milk such as milk ring test (MRT), whey buffered acidified plate antigen test (WBAPAT), whey rose bengal plate test (WRBPT), whey Rivanol test (WRiv.T), whey ELISA (WELISA) and ELISA milk tests. They were divided classically into screening and confirmatory tests. The screening tests include the MRT, WRBPT and WBAPAT, while the cofimatory tests involve WRiv.T, WELISA and ELISA milk tests.

Therefore, the present work attempts to study the prevalence of brucellosis in cows milk, to select the best screening and confirmatory test or tests suitable for accurate diagnosis of brucellosis, and to study the viability of *Br. melitensis* biovar 3 in raw milk and yoghurt.

Material and Methods

A total of 150 individual samples of blood and cow's milk of different breeds were collected from different localities in El-Behera Governorate. The milk samples were examined to be free from subclinical mastitis using Schalm test (A.P.H.A., 1985), and lactation period of 1 to 5 months to avoid factors affecting false-positive results of MRT. Each milk sample was divided into two parts, the milk and the milk whey prepared according to Morgan et al. (1978). Each sample was subjected to different diagnostic tests as recorded in the Table.

Samples and diagnostic tests of brucellosis

Samples	Tests	References
- Serum	- SAT	-Alton and Jones (1967)
- Milk	-MRT -Isolation of brucellae	-Alton et al. (1988) -Alton et al.(1975)
- Whey	-WRBPT -WRiv.T	-Alton et al. (1988) -Nat. Vet. Serv. Lab. (1984)

The titre of MRT, WRBPT and WRiv.T of positive samples of milk and the milk whey were detected using double-serial dilutions of normal milk (non-mastitis, fresh and brucella-free milk), or its whey. Isolation of brucella organisms were carried out by inoculation of albini brucella agar containing antibiotics with the sediment-cream mixture of milk. Simultaneously, enrichment technique using tryptose broth with antibiotics was run parallel. The plates were incubated at 10% carbon dioxide tension. Cultured plates were examined for brucella growth at the 4^{th} day and daily up to 14^{th} day. Suspected brucella colonies were identified according to Alton et al. (1975).

Experimental technique

Preparation of culture

The isolated local field strain of *Br. melitensis* biovar 3, the prevalent biovar among animals and man in Egypt, was used for the experiment. A two-days old culture was suspended in a sterile saline to be used for inoculation of milk and yoghurt.

Viability of *Br. melitensis* in raw milk

Freshly drawn cow's milk, free from brucella, in a clean and sterile stoppered-bottle was inoculated with the prepared culture of the organism to produce an initial inocula of $6x10^4$ cells / ml. The control and the inoculated milk were distributed aseptically into two sterile stoppered-bottles (one litre capacity). The first was placed in refrigerator ($4 \pm 1°C$), while the second was kept at room temperature ($22 \pm 2°C$). The cream layer as well as milk were examined daily for presence of the organism according to Alton et al. (1975), and pH by using Jeway pH meter.

Viability of *Br. melitensis* in yoghurt

Two lots of yoghurt were prepared from milk free from brucella organisms for test and control. The milk were inoculated with *Br. melitensis* biovar 3 at 42°C immediately after the starter to provide an initial inocula of $6x10^4$ cells / ml. Addition of starter cultures was done according to Lampert (1975). The control and infected yoghurt after being prepared were kept in refrigerator ($4 \pm 1°C$). Fat layer and curd of yoghurt were examined daily for presence of *Br. melitensis* and pH value.

Results

Results of the study are presented in Tables 1, 2, 3 & 4

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Discussion

The incidence of brucellosis in dairy animals becomes high with the importation of cattle, this was due in fact to the open door policy where a marked increase in the numbers of intensive breeding farms was recorded following the importation of large numbers of foreign breeds of animals from different countries (Adawy, 1985).

Serological examination of blood serum using SAT showed a higher reactors percentage (10.0 %) than of the milk or milk whey (Table 1). These findings substantiate the results of Alton (1963); El-Gibaly et al. (1990); El-Gibaly et al. (1991) and Hosein and El-Kholy (1993). This result can be attributed to the high sensitivity of this test to detect both IgG and IgM fractions (Salem et al., 1987).

Examination of cow's milk by MRT reveal that 12 (8.0%) of 150 samples gave positive results (Table 1). More or less nearly similar results were recorded by Hamdy (1992), who found 10 % of cow's milk samples were positive. Higher incidence (38%) was recorded by Saaed and Salem (1980), 82.4% by El-Gibaly et al. (1990), 29.2% by El-Sheery (1993) and 66.6% by Hamdy (1997), while lower incidence (4 %) was recorded by Hosein and El-Kholy (1993). The lower reactors detected by MRT, in comparison with blood serological test, may be ascribed to the stage of infection, or to the irregularity in the filteration of the agglutinins from the blood to the milk (Lembke et al., 1950). Moreover, it may be due to the level of the agglutinins in the blood not enough to be excreted in the milk (Pat and Panigahi, 1965).

Boer (1981) considered MRT as unreliable for individual diagnostic test, while other investigators referred to the test as simple, accurate, time saving, highly sensitive, reliable and usefull for detecting brucella agglutinins in milk of individual cows or herds (Ferguson and Robertson, 1954; Nicoletti and Burch, 1969; Morgan et al., 1978; Salem et al., 1987; El-Gibaly et al., 1991 and Hamdy (1992). MRT is known for its sensitivity for IgA (Collin, 1976 and Sutra et al., 1986). It also gave some false-positive cases, as in late lactation period and shortly after parturition, milk from cows with hormonal disorders and those with lower clustering power (Bercovich and Moearman, 1979 and Corbel et al., 1984), which are avoided in this study.

Shifting to the results of the whey agglutination test, it was clear that the over all results revealed low incidence of brucellosis ranging from 4.0 % for WRiv.T to 4.7%. for WRBPT (Table 1). This finding substantiate what have been recorded by Morgan et al. (1978); El-Gibaly (1990) and Hamdy (1997), who found that the whey tests are less sensitive, but less influenced by non-specific factors than MRT. The lower sensitivity of whey tests may be attributed to that the defattening process may deprive milk whey from some immunoglobulins mainly IgA adsorbed to the fat surfaces (Sutra et al., 1986). The removal of the solid parts by rennin, the change in the pH of the whey by the addition of rennet and the changes in molecular weight of immunoglobulins are the other additional factors that may led to low sensitivity of the whey agglutination tests (Sutra et al., 1986 and Hamdy, 1997).

Regarding the sensitivity of the diagnostic tests, results in Table 2 indicate that cows having a high positive serum titres showed positive MRT reaction when their milk diluted up to 1/128 with negative milk, followed by WRBPT (dilution 1/16), and WRiv.T (dilution 1/4). It was also observed that agglutinins titres of milk and whey correspondingly increased with those of blood serum, this may be attributed to the fact that brucella agglutinins in milk originate from the blood stream (Martin and Frank, 1970). These findings are coincident with the results of El-Gibaly et al. (1990); El-Gibaly et al. (1991); Hamdy (1997) and Roepk and Stiles (1970). This result refers to the reliability and sensitivity of MRT in picking up the infected cases than injuring animals for collecting blood serum.

Brucella organisms could be recovered from one (0.7%) milk sample of SAT, MRT, WRBPT and WRiv.T positive, and this isolate was typed as *Br. melitensis* biovar 3, the more prevalent biovar among animals and man in Egypt (Hamdy, 1992). Isolation of *Br. melitensis* from cattle, as non-original host, was firstly recoverd in Malta by Shaw (1906), who found that two cows shedding *Br. melitensis* in their milk. Isolation of such organism from cows milk in Egypt was recorded by El-Gibaly (1969); El-Gibaly et al. (1975); Abdel-Aal (1985); El-Sheery (1993); Salem et al. (1987); Hamdy (1989); Hamdy (1992); Hamouda (1989) and Hosein and El-Kholy (1993) by variable incidence ranging from 0.9% to 1.6% . The low rate of recovery of brucella organisms from milk may be ascribed to that these organisms were secreted intermittently in milk (Elberg, 1986) .

Br. melitensis is endemic in the mediterranean countries. The concept that *Br. melitensis* infects only sheep and goats is nothing , but a hypothesis paradox. When *Br. melitensis* is endemic in sheep and goats, the disease can be easily transmitted to cattle and buffaloes leading to human infection (Verger, 1985). Recovering of *Br. melitensis* from cow's milk represents both epidemioloical and zoonotic importance, as such organism is most virulent and pathogenic than other *Brucella* species in man and animals (Elberg, 1986). Moreover, this strain does not lose its pathogenicity in cattle (Ivanov and Kolmakin , 1959 and Hamdy, 1989) .

Results presented in Table 3 show that *Br.melitensis* biovar 3 was survived for 8 days in refrigerated raw milk (4±1°C) and for 2 days in raw milk kept at room temprature (22± 2°C). Nearly similar survival periods were recorded by Awad et al . (1975), who found that the organism survived for 5-9 days in raw milk. Also, Hamdy (1992) recorded survival periods of 5 days in refrigerated raw milk and only one day in raw milk kept at room temperature. The difference in survival periods may be due to the bacterial population of raw milk, as well as the initial inocula used in the experiment. The survival periods of such organism in cream layer exeeds those in milk column. This finding run parallel to those recorded by Hamdy (1992). This might be due to that cream is usually more heavily infected than milk , as the organism tend to adhere to the surface of the fat globules forming a complex and the protective effect of the high fat content of cream layer (Champnyz, 1953). The killing effect of milk on the brucella organisms may be due to the acidity developed by the lactic acid bacteria .

Table 4 illustrates the viability of *Br. melitensis* in yoghurt, as the organism was viable longer in fat layer of yoghurt (12 days) than in yoghurt curd (8 days). This may be due to the that the organism tends to be carried up to the top by the fat globules protecting it from yoghurt acidity. Nearely similar results were reported by Hamdy (1992).

Storage temperature obviously affects the survival rate, as brucellae survived longer at refrigerated temperature. These findings are coincident tothose obtained by Nour et al. (1975); Abdel-Hakiem et al.(1994) and Hamdy and Abdel-Hakiem (1994). This may be due to the different degrees of acidity developed in raw milk or yoghurt stored at different storage temperatures.

It was found that the pH was determinative for the brucella organisms. When pH reached 4.7 in raw milk and 4.2 in yoghurt, it was impossible to recover *Br. melitensis* (Tables 3 & 4). As the storage period advanced, the acidity percentage increased and consequently affects the survival of the organism. This observation agree with that of Kudaz and Morse (1954); Ghoniem (1972); Hamdy (1992) and Hamdy and Abdel-Hakiem (1994).

It can be concluded that raw milk and its products may be considered a significant vehicle for transmission of brucellosis to man, bearing in mind that *Br. melitensis* is the most virulent biovar to man. MRT proved to be sensitive test and useful in

diagnosing of infected individual cows. As *Br. melitensis* can survived in raw milk and yoghurt, so the prevention of the disease in man depends mainly on the eradication of disease in animals as well as heat-treatment of milk to safeguard the consumers, as effecient pasteurization was enough to destroy brucella organisms in milk.

Table 1: Prevalence of brucellosis according to different diagnostic tests

No. of samples	TAT		MRT		WRBPT		WRiv.T		Culture	
	No.	%	No.	%	No.	%	No.	%	No.	%
150	15	10	12	8.0	7	4.7	6	4.0	1	0.7

Table 2: Sensitivity of diagnostic tests (blood serum, milk and milk whey) of brucella positive samples.

Positive samples	End titre at which positive reaction occurs			
	TAT	MRT	WRBPT	WRiv. T.
1	1/ 80	¼	-	-
2	1/80	½	-	-
3	1/ 320	1/128	1/16	1/4
4	1/320	1/64	1/4	1/2
5	1/160	1/8	-	-
6	1/160	1/16	-	-
7	1/80	1/2	-	-
8	1/80	1/4	-	-
9	1/320	1/64	1/2	-
10	1/80	1/16	-	-
11	1/80	1/2	-	-
12	1/320	1/128	1/8	1/2
13	1/40	-	-	-
14	1/80	-	-	-
15	1/40	-	-	-

Table 3: Survival of *Br. melitensis* biovar 3 in raw milk.

Survival periods (days)	At refrigerator (4 ±°1C)			At room temperature(22 ± 2°C)		
	Cream layer	Milk column	pH	Cream layer	Milk column	pH
1	+	+	5.9	+	+	5.1
2	+	+	5.6	+	-	4.8
3	+	+	5.5	-	-	-
4	+	+	5.4	-	-	-
5	+	+	5.4	-	-	-
6	+	-	5.3	-	-	-
7	+	-	5.1	-	-	-
8	+	-	4.9			
9	-	-	4.7			

Table 4: Survival periods (days) of *Br. melitensis* biovar 3 in yoghurt.

	Survival periods (days)											
	1	2	3	4	5	6	7	8	9	10	11	12
Fat layer	+	+	+	+	+	+	+	+	+	+	+	+
Curd	+	+	+	+	+	+	+	+	-	-	-	-
pH	4.7	4.7	4.6	4.6	4.5	4.5	4.5	4.4	4.3	4.2	4.2	4.2

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Brucellosis in man and animals in the Middle East Region

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1. Introduction

Brucellosis remains a serious zoonotic disease in most countries of the region. The disease is reported in animals in all countries of the region, except in Cyprus where it has been eradicated since 1932 in cattle and 1984 in sheep and goats. Most countries of the region depend on importation of animals, particularly cattle both for slaughter and breeding from outside the region. With the intensification of the importation of animals and the establishment of big farms in the last few years, the incidence of brucellosis rose sharply in many countries., both in man and animals. A high incidence rate of brucellosis was reported particularly from several modern commercial dairy farms. The infection was found to be caused by Br. abortus in cattle, buffaloes and camels and by Br. melitensis, in sheep and goats. The incidence of reactors in those newly established farms of cattle reached in some countries to more than 30%. This alarming situation led to the enforcement of control policy, mostly in the form of test and slaughter policy . The extensive application of S19 vaccination of young females either with the full dose or the reduced dose together with slaughtering of positive animals has resulted in the reduction of the overall reactors rate drastically. On the other hand , brucellosis in sheep and goats , although reported since many years in almost all countries of the region, the incidence is still high and little is done to control the disease . This situation has resulted in the transmission of Br. melitensis to cattle, and that is why Br. melitensis is now the predominant cause of brucellosis in animals and humans in most countries.

2. Situation of brucellosis in animals and man in different countries of the region

2.1. Brucellosis in animals

2.1.1. In Egypt, brucellosis in animals was reported for the first time in 1939. However , intensive surveillance programmes were initiated after the approval of the National Brucellosis Control Programme in 1981, adopting the test and slaughter policy and vaccination of young female calves with the reduced dose Br. abortus S19 vaccine. The average incidence rate of reactors has dropped drastically in 1997 to 0.85% in cattle and 0.3% in buffaloes. In sheep , the incidence is 1.78 and in goats it is 8.17%. Brucellosis has been reported also in camels, swine and dogs. The bacteriological studies revealed the predominancy of Br. melitensis biovar 3 in cattle. This organism was isolated also from dogs.

2.1.2. In Libya, brucellosis in animals was reported to be particularly widespread among sheep and goat flocks, especially in the west mountain area and west coastal strip. Br. melitensis biovar 1 and 2 were isolated from sheep, goats, cows and camels.

2.1.3. In Tunisia, the year 1991 was the year of expolsive outbreaks of brucellosis in sheep and goats. The first outbreak was reported in Gafsa, where the seropositivity reached 61% in goats and 30% in sheep flocks. In positive flocks, 15-20% of females aborted. Outbreaks were reported in 23 governorates. Br. melitensis biovar 3 was isolated from infected animals. In bovines, the percentage of herds infected was found to be 13.7%. Surveys carried out in 1992 showed that the percentages of reactors were 1.5%, 4% and 18% in bovines, ovines and caprines, respectively.

2.1.4. In Algeria, the examination of sheep and goats in 1986-1989 revealed an overall seropositivity of 2.18% in sheep and 12.0% in goats. The percentages of infected flocks were however 43.5% and 42% in sheep and goat flocks, respectively.

2.1.5. In Morocco, the overall infection rate among sheep and goats flocks was 14.28%. The highest rate of infection was reported in the central region, followed by the north-eastern region. The percentages of infection in flocks were 15% in 1980-83, 7.4% in 1984-87 and 21.34% in 1988-1991.

2.1.6. In Sudan, brucellosis in cattle, sheep and goats is endemic throughout the country. According to recent surveys prevalnce of 14.2% and 16.7% were reported in Khartoum and Central States, respectively.

2.1.7. In Jordan, Br. melitensis infection is probably the most serious zoonosis. The first reported focus of infection in goats was identified in 1971 among an imported herd. Between 1971 and 1973, 7% of goats in the country were diagnosed serologically positive. The rate of positive reactors increased continuously so that it reached 22.8% in sheep, 21.0% in goats and 8.7% in cattle. Br. melitensis biovar 3 was predominantly isolated from positive animals.

2.1.8. In Israel, Br. abortus has been eliminated from dairy and beef herds. Br. melitensis represents the major challenge to the livestock industry as it is endemic in cattle , small ruminants and man. The survey of intensive sheep and goat flocks by solid phase ELISA in 1993-95 revealed a reactor rate of 8.2%. In about 10% of the flocks the percentage of reactors ranged between 19 to 37%. The field strain of Br. melitensis was isolated from the internal organs and milk , while Rev.1 was isolated only from milk of reactor animals..

2.1.9. In Lebanon, brucellosis in sheep and goats is very frequent. A mini-serological survey done by the Ministry of Agriculture in some private farms showed that almost all of the farms had infected animals. Br. melitensis is the main agent of infection in all ruminanats. The last screening revealed an incidence of 18% in cattle and 9.2 in sheep and goats. In a last report in 1998, it was mentioned that about 800 cases are reported annually to the Ministry of health.

2.1.10. In Syria, the prevalence of brucellosis in 1988 was reported to be 2.5% in cows and 1.8% in sheep.

2.1.11. In Turkey, a national survey carried out in 1989 estimated the overall rate as 1.26% in sheep and 3.56% in cattle. In 1990 it was 2.08% in sheep and 1.2% in cattle, in 1991 it was 1.83% in sheep and 1.01% in cattle and in 1992 it was 1.48% in sheep and 0.6 in cattle. Data concerning the incidence of brucellosis in the years 1995-97 revealed the occurrence of 7,5 and 7 outbreaks of Br. abortus and 58,53 and 26 out-

breaks of Br. melitensis in the years 1995,1996 and 1997, respectively. Br. melitensis biovar 2 in sheep and Br. abortus biovar 3 in cattle were most predominant. Other biovars determined were 1,2,4 and 6 for Br. abortus and biovar 1 for Br. melitensis.

2.1.12. In Iran, the prevalence of brucellosis reached 44% in 1956 and dropped to 5% following control programme that started in 1958. Because of relactancy in control, the reactor rate increased again to 17.4% in 1977. A control programme started again in 1983 with consequent decrease of the prevalence to 1.25% in 1987. In 1991, the prevalence rate was 0.85%. Similarly, the prevalence rate in sheep and goats went up and down. It was 13.7% in 1970, 6.4% in 1980 and 10.18% in 1991.

2.1.13. In Iraq, the disease was reported in sheep (15%) due to Br. melitensis and in cattle (3%) due to Br. abortus.

2.1.14. In Saudi Arabia, the incidence of brucellosis increased in the years 1986-1988 from 5.7% to 26.0% in sheep and goats and from 0.7% to 7.0% in cattle. During the last 10 years, all Brucella species isolated from sheep, goats, cattle and camels were Br. melitensis biovar 2.

2.1.15. In Kuwait, the percentages of reactors in cattle increased from 3.0% in 1984 to 5.2% in 1989 . In sheep and goats the incidence was 11.1% in 1986 and 6.6% in 1989. In camels, seropositivity rate was reported to be 14.6% in 1985, 14.8% in 1988 and 7.7% in 1989. Serological test done on a flock of sheep in 1993 showed a seropositivity of 9.4%. In 1994, serum samples collected from animals suspected to have brucellosis revealed a positive test in 14% in sheep and 7% in goats. Br. melitensis was isolated from sheep, goats and cows.

2.1.16. In Oman, the serological studies undertaken in 1989 showed that the percentages of reactors were 0-8% in camels, 0.3-6.4% in goats and 0.9-3.3% in cattle.

2.1.17. In the United Arab Emirat, a survey done in 1989 revealed an average incidence of 6.4% in goats, 5.4% in sheep, 14.4% in cattle and 1.5% in camels. A survey conducted in 1990 showed prevalence rates of 3.4%., 2.0%, 1.3% and 02% in goats, sheep, cattle and camels, respectively.

2.2. Brucellosis in man

Although brucellosis is a notifiable disease in some countries of the region, it is often unrecognized and unreported. In many countries, the awareness of medical specialists in relation to brucellosis is very weak and in most of the cases , public health laboratories are not carrying out diagnostic tests. Cases of brucellosis very often remain unrecognized and are treated as other diseases. They are often labeled " Fever of unknown causes". For these reasons, the actual number of cases of brucellosis is unknown and is believed to be far more than the officially reported figures.
The age distribution of reported brucellosis cases from several countries of the region indicates that children are particularly at risk. The incidence has a seasonal pattern with a maximum number of cases during the spring and early summer period.
Infection is transmitted from infected animals by ingestion of raw milk or dairy products, especially cheese made from raw or lightly heated milk. Transmission also occurs through contact with farmers and veterinarian coming in contact with infected animals and frequent infection has been reported in laboratory personnel dealing with

diagnostic works, as in most laboratories, Brucella diagnostic is done in the general laboratory and not in a separate one. The incidence of brucellosis in man in different countries according to the available date is as follows:

2.2.1. In Egypt, human brucellosis has received little attention until the WHO strenghthened the Zoonosis Centre in Imbaba Fever Hospital in 1990. Before this date only few cases of brucellosis were recorded although the disease is notifiable, e.g. in 1988 only 45 cases were reported. In 1991, a survey was done in 4 governorates with a total population of 6.34 million. The serological examination of 2720 serum samples revealed positive reactors in 10.5% of the samples. The examination of serum samples from 747 cases admitted to the Imbaba Fever Hospital and diagnosed as cases of fever of unknown causes were positive for brucellosis in 323 cases (43.23%). In 1994, 309 cases were confirmed by isolation in Imbaba hospital.

2.2.2. In Libya, brucellosis was diagnosed in man in 150 cases in Nalut Hospital in the mountains area in 1988. In the following year, more than 200 cases were reported out of a population of about 30 000. Br. melitensis biovar 2 was isolated from 2 cases.

2.2.3. In Tunisia, the first case of brucellosis was diagnosed in 1909. The official cases of brucellosis were 59 in 1989, 55 in 1990 and 344 in 1991. On October 1991, an explosive outbreak was reported in the southern governorates, particularly in Gafsa, where 407 cases were diagnosed and 85% of the patients had a history of consuming raw milk. The highest rate of infection was registered in May, June and July. The age group mostly affected was 25-34 years, particularly in males.

2.2.4. In Algeria, human brucellosis was discovered as early as 1895 in the Pasteur Institute d' Algeria. Recently, attention has been given to the disease following the serious epidemic reported in 1984 in Ghardaia, where 600 cases were diagnosed. The disease was then reported in other regions. The analysis of data obtained in the years 1988-1990 revealed that the infection rate varied from 0.36-0.67 per 100 000.The highest rate was recorded in May and August which corresponds to the period of parturition and lactation of sheep and goats..

2.2.5. In Morocco, although the first case of human brucellosis was reported in 1916, there no available data on human cases in the recent years.

2.2.6. In Jordan, the infection in man was rarely diagnosed before 1984. During 1984-1985, 69 cases were reported. The number of cases during 1986-1991 in the various districts were 730. 42% of the cases were reported in the capitol region, 9% in Irbid and 7% in the southern regions. On the other hand, the Mafraq area (nomadic) delivered 13% of the cases. 60% of the patients were under the age of 24 years. The peak of infection was in the Spring, which corresponds to the peak of lambing, the maximum production and consumption of fresh cheese products and period of maximum flock movement toward open range.

2.2.7. In Israel, most human cases of brucellosis are reported to be among livestock owners of extensively raised small ruminants.

2.2.8. In Lebanon, human brucellosis is undoubtedly frequent and it occurs throughout the year. During the years 1984-1986, the limited survey done in the country indicates a prevalence rate of 69.6/100 000.

2.2.9. In Syria, human brucellosis is found in most of the provinces. About 220 cases are reported every year, although the estimated number is around 1000. Consumption of fresh cheese is considered the main source of infection.

2.2.10. In Turkey, brucellosis was reported for the first time in 1915. A survey carried out among workers at slaughterhouses in Ankara in 1947 indicated that 10% were infected. Various serological surveys conducted by the Faculty of Medicine in Ankara revealed positive reactor rates between 5.5 and 7%. The largest survey covering different regions of the country was carried out between 1984-1987, where 70 000 serum samples were tested. The prevalence varied between 1.8 - 6%. It was calculated that about 1,75 million person had contracted brucellosis in Turkey. The number of outbreaks increased from 3145 in 1989 (5.48/100 000) to 8383 in 1994 (13.88/100 000). Both Br. abortus and Br, melitensis were isolated from human cases, but Br. melitensis was predominant.

2.2.11. In Iran, human brucellosis is endemic in all parts of the country. Patients recorded in 1988 were 71 051 (132.4/100 000).

2.2.12. In Iraq, the incidence is high in the northern governorates. In 1985, 369 cases (2.3/100 000) and in 1988, 1187 cases (7.2/100 000) were diagnosed.

2.2.13. In Saudi Arabia, the human brucellosis cases increased sharply during the period 1985-1990 from 4.9-69.5/100 000. The highest rate was recorded in 1988 (79.6/100 000).The infection was reported all over the kingdom, but with marked increase at Al-Jouf, Aser and Qasiem. The highest incidence was seen in the Spring and Summer.

2.2.14. In Kuwait, there was an epidemic increase in brucellosis beginning in 1983 with an annual infection rate of 26.8/100 000 which reached its peak in 1985, with a rate of 68.9/100 000. After that the rate began to fall, where it reached 20.1/100 000. Epidemiological investigations of the reported cases have repeatedly confirmed that the traditional habit of drinking raw milk and the consumption of raw dairy products were the main means of transmission. This was especially true among Bedouins where the infection rate among them reached 545.7/100 000.

2.2.15. In Oman, brucellosis is considered, after rabies, the most serious disease.Most of the cases were reported among livestock owners, their families and veterinarians.In 1985, 260 cases were reported, in 1986, 186 cases, in 1987 and 1988 the number increased to 292, in 1989 there were 224 and in 1990 only 184 cases were reported.

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

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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**	

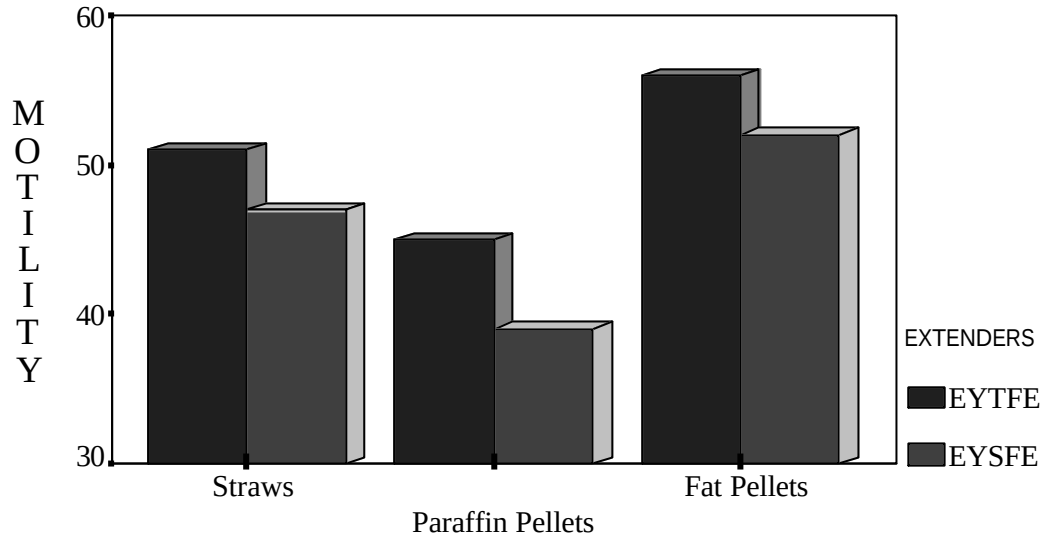

Figure 1: ***Effect of freezing methods and type of cryoprotectant on Post-thaw motility of ram spermatozoa.***

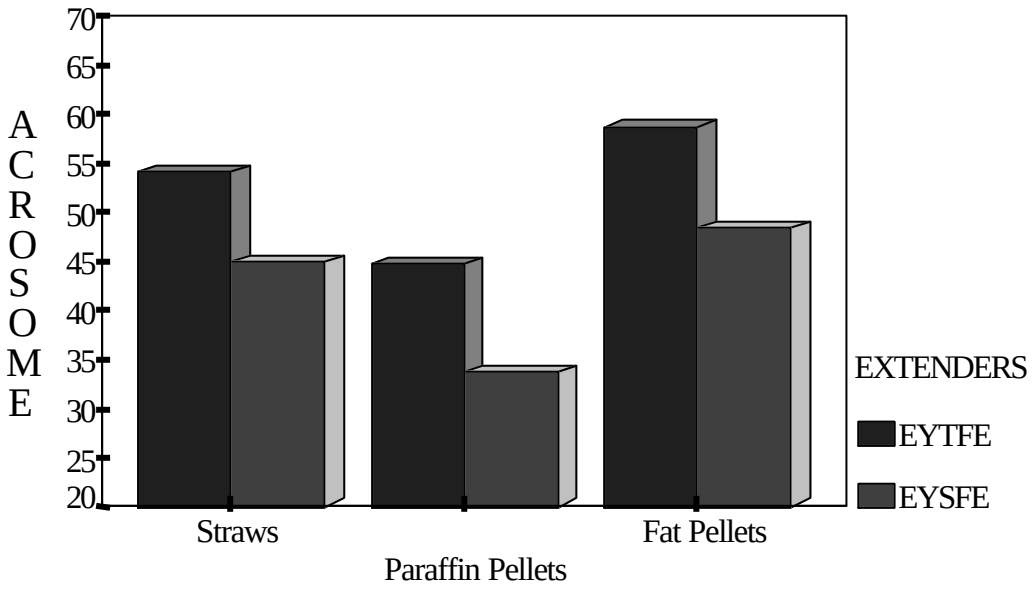

Figure 2: ***Effect of freezing methods and type of cryoprotectant on acrosome integrity ram spermatozoa.***

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