STUDY OF FACTORS AFFECTING SURVIVAL AND FERTILITY OF

RAM SPERMATOZOA

BY

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DECLARATION

I hereby declare that the work in this thesis was carried out by me in the Laboratory of the Small Ruminant Research Programme, National Animal Production Research Institute, Ahmadu Bello Univerrsity, Zaria, under the supervision of Prof. O.A. Osinowo and Dr. N.I. Dim.

No part of this thesis has been presented in any previous application for a higher degree. All sources of information are acknowledged by means of references.

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CERTIFICATION

This thesis entitled "Study of Factors Affecting Survival and Fertility of Chilled Ram Spermatozoa", by Peter Barje Pano, meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria. It is approved for its literary presentation and contribution to scientific knowledge.

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DEDICATION

This work is dedicated to my children, Reuben and Anita Muraba, and to the memory of my beloved parents, Mr. Barje Pano, and Mrs. Amina Barje.

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PREFACE

The experimental work reported in this thesis was carried out under the supervision of Prof. O.A. Osinowo, Small Ruminant Research Programme, N.A.P.R.I. (A.B.U) Shika-Zaria and Dr. N.I. Dim, Department of Animal Science, A.B.U., Zaria. The work was carried out in the Laboratory and Sheep Project farm of the Small Ruminant Research Programme, N.A.P.R.I., Shika. Two types of experiments were carried out during the course of this study as shown below:

1. *In vitro* Experiments.

These were designed to determine the survival of ram spermatozoa during chilled storage in various diluents. Six experimemts were carried out in all.

2. Fertility Experiment.

A comparative study of the effects of inseminating ewes with chilled ram spermatozoa at the cervix (cervical insemination), and deep into the cervix (deep cervical insemination) was carried out. In addition, the effects of length of storage and type of diluent on the fertility of chilled ram semen was also examined.

The thesis is presented in the following order:

Part I

A. Introduction (Chapter 1).

B. Review of Literature (Chapter 2).

Section 2:1 Survival of ram spermatozoa during chilled storage.

Section 2:2 Morphological and Biochemical changes in ram spermatozoa during storage.

Section 2:3 Insemination techniques in sheep.

Section 2:4 Fertility of chilled ram spermatozoa.

Part II: (Experimental).

Materials and Methods (Chapter 3).

Results (Chapter 4).

Discussion (Chapter 5).

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ABSTRACT

Seven factorial experiments were conducted to study factors affecting survival and fertility of ram spermatozoa during chilled storage.

Spermatozoa motility during storage was best preserved by Tris diluent followed by Egg yolk-glucose-citrate and Milk diluents. Addition of glucose or fructose to Milk diluent improved its performance, with glucose giving better results. The optimum level of glucose in milk diluent was 27.75 mM. Tris-Milk diluent containing 50% milk and 50% Tris diluents was intermediate between Tris and Milk diluents in preserving sperm motility during storage. Sperm survival was improved in Tris diluent by the inclusion of ethylene diamine tetra acetic acid di-sodium salt (EDTA) but was depressed in milk diluent. The optimum level of EDTA in Tris diluent was 10 mM. In all experiments, sperm motility declined ($P<0.001$) with increase in storage period. There was also significant interaction between type of diluent and length of storage.

There was no significant difference ($P>0.05$) in non-return rates between Tris and Milk diluent (32.5 v 41%). Increase in length of storage resulted in significant ($P<0.001$) decline non return rate (47.5 v 29.5% for 0 and 1 day storage respectively). It was also significantly higher ($P<0.001$) following deep cervical than when cervical insemination was carried out (45.8 v 28.3% respectively).

Lambing rate was significantly higher ($P<0.001$)

following deep cervical insemination compared to normal cervical insemination (40.0 v 24.2%). It was also higher ($P<0.001$) for fresh diluted semen than chilled semen stored for 24 hours at 5°C (41.7 v 22.5%). There was no significant difference ($P>0.05$) in lambing rates between Tris and Tris-Milk diluents (27.5 v 36.5% respectively).

PART I

Chapter 1: Introduction

Chapter 2: Literature Review

Chapter 1

INTRODUCTION

A major objective of animal breeding is to achieve improvement in their productivity. This can be done in various ways including the adoption of good husbandry practices such as the provision of good housing, health care, and nutrition. However, the productivity of an animal is not determined by its environment alone . Its genetic make up is also a determining factor. Therefore, to sustain any improvement in livestock productivity, genetic improvement is essential, since it is of a more permanent nature. Artificial selection of animals with good production records as sires is used to improve the productivity of animals. However, the extent to which this can be carried out is often limited by a number of factors. These include low heritability of some of the production traits, and the frequently insufficient number of animals from which the selection can be made.

Some techniques in reproductive physiology have been applied to animal breeding for the achievement of faster genetic improvement. Such techniques include artificial insemination, oestrus synchronization, induction of multiple ovulation, anti-steroid immunization, long term storage of gametes and embryos transfer (Osinowo, 1977).

Artificial insemination is a powerful tool by means of which genetic improvement of livestock can be achieved, if fully integrated with the breeding programme. The advantages of this method of breeding are that it:

1. Enhances quick determination of genetic merits of male animals through progeny testing of a large number of contemporary offsprings, thus facilitating selection.
2. Increases the usefulness of genetically superior sires that for one reason or the other have become incapable of performing natural service.
3. Facilitates the mating of animals of different sizes.
4. Saves time and space in the breeding programme.
5. Reduces cost especially that caused by adaptation and management problems associated with expensively imported sires for use in cross-breeding programmes (Dettmers, 1972).

Artificial insemination has been successfully integrated with sheep breeding programmes in most advanced countries such as U.S.S.R., France and Australia. Of these, U.S.S.R. appears to have the most advanced sheep artificial insemination programme. Milovanov (1945) and Ozin (1954) described this development in their reviews. In other countries, though much has been done in the area, the practice is still limited by environmental, cultural, and

socio-economic factors. These and the peculiar nature of ram semen, which requires careful handling, have restricted the practice to governmental and institutional flocks. There is a need for the practice to be extended to the local farmers' flocks. However, in Nigeria, this is further limited by illiteracy among the local herdsmen who own the largest proportion of the animal population in the country, and poor transportation system. Semen to be used for artificial insemination has to be transported from the processing centres, but because of the poor transportation system, this could take hours or even days.

The greatest advances in artificial insemination programmes have resulted from the development of techniques for the dilution and storage of semen which offer the best protection to the spermatozoa. Several methods of preserving mammalian semen for use in artificial insemination has been developed. The methods commonly used for the preservation of ram semen include ambient temperature storage, chilled storage and deep frozen storage. Others being experimented with are formaldehyde treatment, freeze drying and storage by flow dialysis. Prior to the development of other methods of preservation, ram semen used for artificial insemination of sheep was usually stored by the chilled method. Despite its earlier adoption and wide usage, insemination results with chilled ram semen has remained low. It has been shown that several factors influence the survival and fertility of ram

spermatozoa during chilled storage. Among these factors are type and composition of diluents, rates of dilution and cooling the semen, length of storage and insemination technique. Much investigative work on the extent to which these factors influence semen quality has been carried out.

The present study was designed to investigate the effects of three factors, length of storage, type of diluent and depth of insemination, on the survival and fertility of chilled Yankasa ram spermatozoa. The main objectives of the study were:

1. To investigate the influence of length of storage on the survival and fertility of chilled ram spermatozoa.
2. To investigate the effects of inseminating ewes at various depths on the fertility of chilled ram spermatozoa.
3. To develop improved methods of storage and insemination of chilled ram semen.

Chapter 2

LITERATURE REVIEW

Section 1: Survival of chilled ram spermatozoa.

Section 2: Morphological and Biochemical changes in ram spermatozoa during storage.

Section 3: Insemination techniques in sheep.

Section 4: Fertility of chilled ram semen.

Section 2:1: SURVIVAL OF CHILLED RAM SPERMATOZOA

Introduction:

Preservation of ram spermatozoa over a long period *in vitro* without losing their viability forms an important aspect in artificial insemination in sheep breeding. Different methods have been adopted for the preservation of mammalian spermatozoa, but those widely used for ram spermatozoa are the chilled storage, deep freezing and ambient temperature storage methods. Survival rate of ram spermatozoa during storage has continued to remain low despite its earlier development and adoption. Several factors have been reported to be responsible for this. These include the effects of type and composition of diluents, rates of cooling and dilution of semen and length of storage period. The extent to which these factors influence the survival of ram spermatozoa during chilled storage is reviewed in this section. The literature is reviewed under 3 major headings: diluents, dilution and cooling and cooling rate.

2:1:1 DILUENTS

The development of semen diluents has been reviewed by Salisbury and VanDemark (1961), Melrose (1962), Foote (1974) and Evans (1988). The choice of semen diluent is crucial to

the viability of the spermatozoa during storage. The basic requirement for a semen diluent is for it to provide a suitable environment for the survival of the spermatozoa with the least damage to their fertilising ability. Characteristics of good semen diluents have been described by Anderson (1945), Salisbury and VanDemark (1961) and Foote (1962). These include proper osmotic pressure and pH, correct electrolyte (cation and anion) balance, stability of the diluent even after prolonged storage, supply of energy to support the metabolic activities of the spermatozoa during storage, good buffering capacity against toxic end products of cell metabolism, bacterial control and non toxicity or freedom from substances that may be harmful to the spermatozoa, or impair their fertilising ability and/or affect implantation and subsequent foetal development.

The preservation of spermatozoa motility during storage has been used as the main criterion for assessing the suitability of semen diluents. Most of the diluents so far adopted for storage of ram semen were adapted from bull semen extenders. These have been used with some modifications in their basic compositions. The egg yolk based diluents appear to have been most favoured by most earlier investigators. Of these, egg yolk-citrate and egg yolk-phosphate were considered to be standard diluents for semen of most farm animal species (Foote, 1975). However, other types have since been developed with specific species in mind.

Lardy and Philips (1939) first described the egg yolk-phosphate diluent. This consisted of equal volumes of fresh egg yolk and sterilised phosphate buffer (0.2g di-Potassium hydrogen orthophosphate (K_2HPO_4), and 2.0g disodium hydrogen orthosphate dodecahydrate ($Na_2HPO_4.12H_2O$), per hundred millilitres of distilled water. In this form, the diluent was found to be satisfactory for ram semen (Terrill, 1963). The diluent was, however, observed to have a serious disadvantage in that owing to the presence of large fat globules, which render it opaque, it did not allow proper observation of individual spermatozoa motility under the microscope (Bishop and Salisbury, 1955). However, its depressing effect on spermatozoa metabolism and good buffering capacity against changes in pH during storage made it suitable for semen dilution especially under aerobic conditions. The depression of motility was observed to be due to the action of phosphate ions which reduce the oxygen uptake and lactic acid formation by the spermatozoa. It was suspected that because of the drepressing effect of the egg yolk-phosphate on not only the spermatozoa motility but also their livability, it could adversely affect the fertility of the diluted semen (Salisbury, 1957). Campbell and Edwards (1955) reported that without the addition of antibiotics to both egg yolk-phosphate and egg yolk-citrate diluents, the phosphate diluent offered the best protection to the spermatozoa which accounted for the better conception rates

obtained with the former. However, the situation was reversed when antibiotics were added. Kharche *et al* (1976) reported a similar result which showed that the egg yolk-phosphate offers the best protection to the spermatozoa than did other diluents including egg yolk-citrate. The egg-yolk-citrate diluent was said to offer a clear field which allow individual spermatozoa studies. This might have been one of the reasons for it wide use.

The composition of the egg yolk-citrate diluent was first described by Salisbury *et al*.(1941). They showed that by replacing the phosphate in the egg yolk-phosphate diluent with 3.6 or 3.9% solution of hydrated sodium citrate ($Na_3C_6H_5O_7.2H_2O$), the new diluent was equally good as the phosphate diluent. In addition it had the advantage of offering a clear field which allowed microscopic assessment of the spermatozoa motility. It had no depressing effect on the spermatozoa metabolic activities. Studies on the effects of storing ram semen in this diluent on the motility and fertility of the spermatozoa at 2-4°C gave poor results (Dauzier, 1956). Dauzier *et al*. (1954) also tested various egg yolk based diluents. Result of the study showed that ram spermatozoa motility was preserved longest in a 3-part 3% citrate solution added to 1 part egg yolk. Filimon *et al*. (1956) also reported that in egg yolk-citrate diluent, 80% of the spermatozoa remained viable after 48 hours of storage and 60% after 73 hours. Kuznecor (1950) found that by adding

fructose or glucose to both the egg yolk-phosphate or egg yolk-cirate diluents, the survival rate of the spermatozoa was significantly improved. This was accompanied by increase in fertility of about 4.2 and 5.3-8.5% when fructose and glucose were used in the diluent respectively. Sakala (1957) tested 19 different diluents and also found similar increases in the survival of ram spermatozoa when either glucose or fructose was added to 25% egg yolk-citrate diluent. The best survival rates were obtained when the semen characteristics were scored at dilution rates of 1:3 to 1:10 (v/v, semen:diluent). Inclusion of glycine in the yolk diluent singly or in addition to other compounds was also said to have improved the results. Roy *et al.* (1956) found that at high dilution rates of 1:10 to 1:100, the overall survival time of ram spermatozoa was much better in glycine-egg yolk than in egg yolk-citrate diluent. Schindler and Amir (1961) also reported finding addition of glycine to the egg yolk-citrate diluent to be beneficial in maintaining ram spermatozoa motility for a longer period than did yolk-phosphate or yolk-citrate. In the egg yolk-citrate diluent, 50% yolk shortened the life span of the spermatozoa significantly, compared to when 25% egg yolk was used. The latter gave equal results to 18% egg yolk diluent, with regards to total spermatozoa survival time. However, the decline in percentage motile sperm cells was slower in the yolk-fructose-phosphate diluent as shown by the half life

period. The addition of different proportions of egg yolk supernatants did not materially affect spermatozoa longevity. Joshi and Singh (1968a) compared the efficiency of egg yolk-citrate based diluents (egg yolk-citrate-glucose-fructose, egg yolk-citrate-fructose, egg yolk-citrate-fructose-glycine, egg yolk-citrate-glucose, and egg yolk-citrate-glucose-glycine) against egg yolk-citrate control. The results show that after 48 hours of storage, motility declined sharply in egg yolk-glucose-fructose diluent. The decline was least in diluents containing glycine. After 96 hours of storage there was a further decline in spermatozoa motility in all diluents. The highest motility score was maintained in egg yolk-citrate-fructose-glycine diluent up to the 144th hour of storage. The authors attributed the superiority of the glycine containing diluents to the additional buffering capacity of glycine in these diluents, and its nutritional value to the spermatozoa. This together with both fructose and glucose are utilized as sources of energy for the spermatozoa.

Wiggan and Clark (1967) modified the "Minnesota GO" diluent first described by Rajamannan *et al.* (1964). They substituted fresh egg yolk for spray dried egg yolk in the basic buffer containing various polyhydric alcohols, inositol, dextrose, levulose, citric acid and sodium citrate. This was allowed to separate into two fractions. The lower

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clear fraction was used as the semen diluent after adding glycine and antibiotics. This was found to be satisfactory when used to store ram spermatozoa at 5°C for up to 36 hours. Martin (1962) reported finding diluents containing egg yolk-sodium citrate, lactose and casein satisfactory for storing ram semen at 5 or 37°C. However, the motility of twice washed spermatozoa stored at 5 Or 37°C was depressed by diluents containing high proportions of sodium citrate.

Zlatarev (1976a) formulated a diluent specially for the preservation of ram semen based on sodium citrate with 20% egg yolk. Compared to a glucose-phosphate extender, sperm motility scores in the tested diluent were significantly higher (75.4 and 75.1% v 68.2 and 14.8%) after 24 and 48 hours of storage respectively.

After series of systematic investigations Igboeli (1970) developed a Tris (hydroxy methyl amino ethane) based diluent for liquid storage of boar semen. Petruzzi *et al.* (1976) carried out a comparative evaluation of the effects of 6 diluents on the survival of ram spermatozoa during chilled storage. They reported that Tris-based diluents performed significantly better at protecting the spermatozoa than the others after 96 hours of storage . Schleicher and Bruckner (1973) reported that the motility of ram spermatozoa on the third day of storage was better in semen extended with Tris-based diluents than that extended with glucose-phosphate diluents (58 v 37%). The best results

reported by Abdelhakean *et al.* (1978) were obtained with Tris-based diluents containing 40% egg yolk and 7% glycerol which maintained motility over a 3-day period better than the best of glucose based diluents (72 v 65%). Similar good survival results were earlier obtained with bull semen stored in Tris-based diluents than in Cornell University Extender (CUE) and yolk-citrate-glycerol diluents (Davis *et al.*, 1963). The reported good spermatozoa motility recovery following storage of semen in the Tris-based diluents seems to be due its good buffering capacity : being a Zwitterion it is capable of maintaining the pH of the diluent about the neutral point.

The discovery that the addition of certain proteins in diluents for ram or bull semen could buffer the solution against changes in pH and partially protect the spermatozoa during dilution (Blackshaw, 1953) and cooling (Choong and Wales, 1962), may be the possible reason for the success of milk as a diluent for semen of these species. Thacker and Almquist (1953) found fresh cow milk to be toxic to bull spermatozoa. Thacker *et al.* (1954) defined the toxic factor in fresh milk as being inactivated by heat at 92°C, non-dialysable, and present in the serum protein fraction of milk. Flipse *et al* (1954) confirmed opinions that lactenin, the anti-streptococcal factor in milk, first described by Jones and Simms (1930) was responsible for the toxicity of milk to spermatozoa. Lactenin is irreversibily inactivated by

the addition of compounds with free sulphydryl groups (Wilson and Rosenblum, 1952 a,b). These groups are present in lactoglobulin. When milk is heated above pasteurisation temperature (Hutton and Patton, 1952), it is reasoned that the heat activates the sulphydryl group of the *B*-lactoglobulin which renders lactenin non toxic to the spermatozoa. When the toxicity of milk is reduced in this way, both bull and ram spermatozoa can survive equally well in fresh milk, most especially if the milk is heated for 10 minutes at 92°C. Blackshaw (1960) found that for ram semen, heating the milk at 85°C for 5 minutes was sufficient to detoxify pasteurised milk. Albright *et al*. (1960) also reported that diluents composed solely of milk constituents performed favourably at 5°C and poorly at 37°C especially if the storage period is prolonged. This finding was also confirmed by Martin (1962). He reported finding milk diluents to be good for deep frozen storage of semen but not suitable for liquid storage at 5 or 37°C compared to egg yolk-citrate diluent. But Salamon and Robinson (1962 b) reported that milk remained inferior to other diluents used for storage of ram semen at 0°C or 5°C, especially egg yolk-glucose-citrate diluent only in the absence of antibiotics. That the situation was changed when antibiotics (1000 i.u. of Penicillin per millilitre of diluent) was added to both diluents. Both diluents gave equally good results. Modifications of the milk diluents have also been attempted

with much improvement in their semen preservation qualities. Forinstance, a commercial milk diluent, "Laciphos 123", was added at a rate of 10% to a 20% egg yolk-diluent and used for diluting ram semen, it was found to be better than a variety of other yolk diluents (Szumowski *et al.*, 1956). Hill *et al.* (1958) reported finding skim milk to be much better as an extender for ram semen than yolk-citrate. Their report was based on motility observation after storage at 4°C for 15 days. Salamon and Robinson (1962 a) reported that dilution of ram semen in cow milk either whole or skim adversely affected the spermatozoa viability. Ewe milk was, however, found to be unsuitable for ram semen dilution. Almquist amd Wickersham (1962) found that addition of 10 or 13% glycerol to skim milk diluent, by gradual addition to partially diluted semen at 5°C improved spermatozoa livability during 14 days storage at this temperature. Increasing the levels of glycerol to about 16-25% did not significantly improve spermatozoa livability over semen diluted without glycerol. Saxena and Singh (1967) added egg yolk to the skim milk diluent for ram semen and reported that percentage motility and live spermatozoa remained significantly higher in skim milk-yolk diluents after 72, 120 and 168 hours of storage than in any other diluent tested. Joshi and Singh (1968 b) also tested the livability of ram spermatozoa in skim milk diluent series. The result of the test showed that there was a gradual decline in motility from 0 to 144 hours of storage in skim

milk-yolk-glucose, and skim milk-glucose-fructose diluents. The decline was sharp up to 48 hours and much below 50% after storage in egg yolk-citrate, skim milk-yolk, skim milk-glucose-glycine, and skim milk-yolk-glucose-bicarbonate. The authors suggested that the superiority of skim milk diluents over egg yolk-citrate may be ascribed to the presence of glucose and fructose in the milk diluents, as sources of energy for the spermatozoa. Carmenate and Gamicik (1984) tested 3 milk diluents namely: powdered milk-glucose-lactose, glucose-powdered milk-sodium citrate, and powdered milk alone, against egg yolk-citrate-glucose diluent. They observed that after 12 hours of storage at 5°C, the motility of ram spermatozoa stored in egg yolk-citrate-glucose diluent was poor compared to that stored in the milk diluents. This seems to support the findings of Joshi and Singh (1968b), who found the presence of exogenous sources of energy such as glucose or fructose, in milk diluent to improve the survival of the spermatozoa.

Other compounds have been tried as diluents for chilled storage of ram semen. These include Glucose-phosphate (GPS) solution (Chang, 1943), and tomato juice (Joshi and Singh, 1968c). The tomato juice was reported to have maintained spermatozoa motility over 3 storage periods of 48, 96 and 144 hours, better than egg yolk-citrate. *In vitro* studies have shown that the addition of fractions of extracts from egg yolk to the glucose-phosphate solution and used for the

storage of ram semen improved the survival of the spermatozoa chilled to 5°C (Watson and Martin, 1975a). Martin and Richardson (1979) showed that ram spermatozoa survived better during incubation at 37 or 5°C after dilution if glucose was replaced by an equimolar amount of inositol in the diluent. In addition, some organic buffers have been tested as substitutes for phosphate buffer usually included in diluents. The response of ram spermatozoa to these compounds closely followed that of bull (Jones and Foote, 1972). The compound 2-(N-morpholino)-ethane sulphonic acid (MES) was selected as a better pH buffer than phosphate for ram semen diluents. Lapwood and Martin (1972) used synthetic diluents consisting of various levels of buffers, inorganic and organic sodium salts, and sodium salts of volatile acids.

2:1:1a. Diluent Additives

The inclusion of certain compounds in semen diluents have been observed to improve the survival of ram spermatozoa during storage. These compounds include cryoprotective agents, sugars, electrolytes and antibiotics.

For low temperature storage of ram semen, the use of cryoprotective agents have been found to enhance the survival of the spermatozoa. Several compounds have been used as cryoprotectants. Lardy and Phillips (1939) first reported the cryoprotective properties of egg yolk. Since then egg yolk

has become a major component of most semen diluents. Attempts have been made at isolating the factor in the egg yolk that is responsible for the protection accorded spermatozoa during storage. It was suspected that the factor may be either lecithin or a similar phospholipid which may occur freely or in combination with other proteins (Blackshaw,1954; Mayer and Lasley, 1945). But it has been shown that both the lipoprotein and phospholipid fractions derived from egg yolk are capable of this action. Smith *et al*. (1956) reported that there were other factors in the egg yolk which can support spermatozoa metabolism such as glucose. The authors stated that the physical protective action of egg yolk on the spermatozoa was due to lecithin, but that the factors responsible for spermatozoa survival were associated with cholesterol. Watson and Martin (1973) observed that egg yolk had two effects on the spermatozoa. It was said to contain a certain substance which preserves the motility of the spermatozoa during both chilled and deep frozen preservation. This substance was observed to be a regular component of the hen's egg, non-dialysable and remained stable up to 28 days after laying. The response of the ram spermatozoa to this substance increased with increase in the level of the egg yolk added to the diluent. However, this was to a limited extent, because at higher egg yolk levels, a second factor which, hitherto, had been inactive was activated and had a detrimental effect on the acrosomal membrane. In solutions

containing about 15 mg/ml of soluble egg yolk solids, more than 50% of the spermatozoa survived 72 hours of storage at 5°C. The protective action of egg york was later attributed to the activity of low density lipoproteins present in the egg yolk (Watson and Martin, 1975 a).

Watson (1981) investigated the role of lipids and proteins in protecting ram spermatozoa at 5°C. He reported that both low density lipoprotein fractions of egg yolk and lecithin liposomes provided an equal measure of protection for ram spermatozoa subjected to cold shock. The low density lipoproteins gave greater protection than lecithin during low temperature storage especially at 5°C. These protective properties were not modified by subfractionation procedures that did not alter the molecular organisation of the lipoprotein. Removal of the protein from the surface of the lipoprotein fraction altered the lipid:protein ratio which resulted in particles that were not good at protecting the spermatozoa. The author also showed that the absolute lipid content in the lipid:protein complex was more important than the ratio of lipid:protein as far as spermatozoa protection was concerned. He concluded that the phospholipids of low density lipoprotein fraction provided protection to the spermatozoa membrane, and that the proteins dissolved the lipid and were bound to the cell membrane.

Holt and North (1988) also investigated the role of membrane active lipids in the protection of ram spermatozoa

during storage or cooling. The results showed that liposomes containing various preparations of phosphatidyl choline (lecithin) and cholesterol enhanced spermatozoa viability during storage at 5°C, assessed in terms of both motility and acrosomal integrity. A membrane fluidising agent, A_2C, was slightly deleterious in this respect. Cholesterol containing liposomes were not more effective than those prepared without cholesterol. Thus, stabilisation of the plasma membrane by cholesterol may be unimportant. Using sodium vanadate as a functional probe for membrane integrity, the authors found that despite increased plasma membrane permeability, the cryoprotective effect of the lipids was apparent. Incubation of spermatozoa with positively charged liposome containing stearylamine caused considerable loss of motility and acrosomal damage coupled with cellular aggregation. The presence of calcium lessened the effectiveness of liposomes in protecting the spermatozoa against damage during cooling.

Other compounds that have been used as cryoprotective agents include glycerol (Polge *et al.* 1949), Dimethyl sulphoxide (DMSO) (Bishop, 1959), ethylene glycol (Platov, 1965), sugars and macromolecules such as lactose and polyvinyl pyrollidone (PVP) (Doebbler, 1966), as well as phospholipids, lipoproteins and proteins (Jones and Martin, 1965; Fisher *et al.*, 1981). Of these, glycerol remains the best and most widely used cryoprotectant in most semen diluents. During chilled storage, addition of glycerol to

certain semen diluents have been reported to improve the survival of the spermatozoa of some species. Holt (1953) reported that a 4.3% increase in 3-month non-return rates was achieved when bull semen was chilled in a diluent containing glycerol. However, with ram semen it seems the results of chilled storage with glycerol-containing diluents have not been reported.

Emmens and Blackshaw (1950) found that the addition of sugars, particularly the pentoses improved the survival of mammalian spermatozoa after deep freezing. Similarly, viability and fertility studies of chilled bull, ram and cock semen have revealed that the addition of sugar improved the survival of the spermatozoa. The most effective sugar was, however, said to vary with both the storage temperature and animal species. For instance, glucose was found to be beneficial in improving the motility of cock spermatozoa after storage in hypertonic, isotonic and hypotonic diluents at room temperature (Wales and White, 1958a). Fructose added to phosphate diluents improved both motility and fertility of the spermatozoa when stored at 10°C. Habibulin (1963) reported that ram semen diluted in citric-yolk-arabinose had better spermatozoa motility than when diluted in glucose -yolk-citrate diluents and stored at 7°C for 4 days. Under some conditions, replacement of electrolytes with sugar in semen diluents led to improved survival rates of the spermatozoa. Martin (1960) showed that by replacing part or

all of the sodium chloride (123 mM) with lactose in a phosphate buffered saline diluent improved the survival of ram spermatozoa stored at 37 or 5°C was improved. Further studies showed that there was no difference in spermatozoa survival rates in diluents containing 185 mM of either fructose, glucose, lactose or sucrose at 5°C. Lapwood and Martin (1966) reported that at 37°C the use of maltose, lactose , sucrose or raffinose in diluents was equally beneficial in improving the survival of ram spermatozoa. But of these, and for storing semen at 5°C, the sugars of choice were ribose, arabinose, xylose and galactose. The use of glucose and manose was said to reduce the survival rate of the spermatozoa at this temperature.

Salisbury *et al.* (1939) first drew attention to the risk of transmitting pathogenic bacteria in semen, and the need for the control of these in view of their possible effect on the spermatozoa during storage. The advent of egg yolk diluents which permitted semen to be stored for longer periods also provided suitable media for bacterial survival and growth. These gave rise to many publications on the need for the use of bacteriocides/bacteriostatic agents in semen diluents. The inclusion of chemotherapeutic agents such as sulphanilamide (usually at a rate of 300 mg/100ml) resulted in increased spermatozoa survival (Salisbury and Knodt, 1947; Foote and Salisbury, 1948; and Branton *et al.*, 1949). However, Knodt and Salisbury (1946) doubted whether these

increases were due to anti-bacterial action alone, since with sufficient sulphanilamide included in the diluent to keep bacterial multiplication at a low level over 72 hours there was also a stimulation of glycolysis and reduction in oxygen uptake. Foote and Salisbury (1948b) also found that only sodium sulphamezathine and carboxyl-sulphathiazole were superior to sulphanilamide in prolonging spermatozoa motility. Further use of these and other bacteriostatic agents was limited by the fact that they were not so effective in controlling bacterial growth. Attention was turned towards the use of antibiotics, especially penicilin (Foote and Branton, 1950b), and streptomycin (Almquist, 1951; Foote and Branton, 1950 b). Other antibiotics tried were more or less toxic to the spermatozoa. The risk of toxic effects with penicillin, though slight, was however, reported. Foote and Salisbury (1948b) reported that occasionally penicillin treated samples may become toxic. Knodt and Salisbury (1946), and Sykes and Mixner (1951) found that at 1000 i.u. per millilitre of diluent the toxicity of penicillin to spermatozoa stored at 5°C was less. The beneficial effect of penicillin on conception was observed with levels up to the 1000 i.u./ml of diluent. Thus penicillin has become the most widely used antibiotic in semen diluents. For most practical purposes, 1000 ug of streptomycin per millilitre of semen diluent appears to be widely used.

There are extensive literature on the use of combinations of antibiotics in semen diluents (Almquist *et al*., 1948; Campbell and Edwards, 1955; Willet and Ohms, 1955). These reports are often contradictory. Commonly reported are the use of combination of streptomycin and penicillin. Streptomycin is used at a concentration of 1000 *u*g/ml and penicillin at 1000 i.u./ml. Other antibiotics that have been tried include oxytetracycline (Stallcup and McCartney, 1953). chlorotetracycline (Foote and Branton, 1950), Chloromycetin (Easterbrooks, 1951) and neomycin (Diliello *et al*., 1957).

The use of gases as components of semen diluents have been reported. These have been reported to improve the survival of the spermatozoa during storage. The most commonly used gases are nitrogen, oxygen and carbon dioxide. VanDemark and Couturier (1958) reported gassing semen samples with nitrogen and oxygen through dialysis in the supply flask at a rate of 15 ml per minute over a period of 2 hours before the samples were stored at 5 or 26°C for 7 days. These gases were found to be detrimental to spermatozoa motility. But carbon dioxide was beneficial under the same conditions. Salisbury and VanDemark (1961) also reported finding the bubbling of carbon dioxide in an egg yolk-citrate diluent beneficial during the storage of bull semen. Leon Crespo (1966) diluted ram semen in yolk-citrate-glycol, with or without the bubbling of carbon dioxide. The result showed that

spermatozoa survival rates as indicated by percentage motility was slightly improved by carbon dioxide. However, the reported increase was not significant.

2:1:1b pH and Osmotic Pressure of the Diluent

Most investigators seem to agree that pH and osmotic pressure of diluents interact. This relationship and its effect on the survival of the spermatozoa is further complicated by their single or combined interaction with other factors such as temperature and cation concentration of the diluent. The effects of diluent pH and osmotic pressure on the survival and metabolism of spermatozoa during chilled or deep frozen storage has been studied in most species of farm animals (Johnson *et al.*, 1956; Cragle and Salisbury, 1959; Steinbach and Foote, 1967; Salamon and Visser, 1972; Fisher *et al.*, 1984). Blackshaw and Emmens (1951) reported that provided diluents were buffered and isotonic to the semen, the spermatozoa motility was not affected by changes in the levels of glucose and sodium chloride in the diluent. However, spermatozoa survival rate was greatest in isotonic diluents and least affected by hypertonicity than hypotonicity. But this depended on the rate of dilution of the original semen sample. Johnson *et al* (1956) did not find any significant difference in the survival of spermatozoa when diluents with freezing point depression (FPD) of -0.50

or -0.70°C, and pH of 6.2, 6.6, or 7.0 were used for liquid storage of bovine semen. Cragle and Salisbury (1959), on the other hand reported that a diluent having a low pH and high potassium concentration had an inhibitory effect on the metabolism of bull spermatozoa during liquid storage.

Quinn and White (1969) reported the adverse effect of hypotonicity on the motility of ram spermatozoa. This occurred when the spermatozoa were exposed to rapid changes in osmotic pressure. Good motility scores were however, maintained at low tonicity when care was taken to avoid osmotic shock during semen dilution. The best results were obtained when the pH of the diluent was 7.0. Fisher *et al* (1984) observed that during deep freezing, ram spermatozoa motility improved with increasing osmolarity of the freezing solution. Diluents of 600 and 750 mOsm gave the highest motility scores immediately after thawing. Inclusion of sugars in these diluents was found to be beneficial to spermatozoa survival. However, glucose proved to be more suitable than fructose or raffinose. The amount of glucose required varied with the tonicity of the diluent. In a hypertonic Tris diluent, relatively low concentrations (27.75 mM) of glucose are required while in hypotonic media a higher glucose level (150 mM) improved sperm cell recovery (Salamon and Visser, 1972).

The response of mammalian cells to the tonicity of storage media is not unique to spermatozoa alone. Human red

blood cells in culture and Chinese hamster ovary cells (Griffith, 1978) exhibited relatively greater tolerance to hypertonic solutions than hypotonic ones. Human embryonic epithelial cells exhibited improved survival rates after freezing in a hypertonic medium (Tenchini *et al.*, 1980). The cells were found to be well adapted to the medium, and could be conditioned to survive at higher tonicity after initial exposure to moderate or lower hypertonic medium (Bolognani *et al.*, 1976; Dal Pra *et al*, 1978).

2:1:2 DILUTION

The extent to which mammalian semen can be diluted during artificial insemination has been shown to be critical to the survival of the spermatozoa. This dilution effect, seen mostly as an inactivation of the spermatozoa at high dilution, occurs in ram semen as in that of other species. The effect occurs irrespective of the nature of the diluent, unless it contains protective agents (Koger, 1951; Blackshaw, 1953; Dauzier, 1956; Roy *et al.*,1956; Schindler and Amir, 1961). Factors associated with semen dilution that affect the viability of spermatozoa during storage include holding time (period between collection and dilution), dilution temperature, dilution method and dilution rate.

There seems to be no established theoretical basis for holding freshly collected semen for sometime before dilution. Many investigators such as Graham *et al.* (1971, Wilmut and Polge (1972) and Pursel and Johnson (1974) have investigated the effect of holding time on the survival of spermatozoa especially during deep freezing of bull and boar semen. These authors observed that holding time did not affect motility or release of the enzyme glutamic oxaloacetic transaminase. Graham and Crabo (1972) suggested that holding boar spermatozoa in its own seminal plasma might result in a protective protein coat being formed around the spermatozoa which improves their survival. From the literature, this

added advantage of holding semen for sometime before dilution may not be applicable to ram spermatozoa. Morozov (1954) reported not finding any difference in spermatozoa viability when the semen was diluted less or more than 1 hour after collection. Blackshaw (1953) had shown that the dilution effect could be partially prevented by suspending the spermatozoa in cell free supernatant from the same species, for example the accessory secretions of a vasectomised ram. Jones and Holt (1974) observed that leaving bull and ram spermatozoa in epididymal plasma had a slight initial stimulatory effect on motility, but had no effect on spermatozoa survival. The same initial stimulatory effect on motility was observed when seminal plasma was used. But exposure of ram spermatozoa in seminal plasma for even 15 minutes reduced the spermatozoa survival rate. Osinowo (1975) also observed that holding boar semen for a length of time after collection before dilution had no beneficial effect on the post thawing survival of the spermatozoa after deep freezing.

Various temperatures at which semen dillution can be done have been adapted. There seems to be three approaches to the dilution of ram semen. These are dilution at near body temperature (30 - 37°C), at room temperature (14 - 25°C) or at 5°C. In most cases dilution at 30 - 37°C is widely used. Dilution at 5°C involves a 2-step dilution. The first is at 30 - 37°C during which a cryoprotective agent is added, and

cooling to 5°C before the second dilution (Polge and Rowson, 1952a; Miller and VanDemark, 1954). Dilution at 30-37°C has been widely used for ram semen especially when chilled storage is to be employed. The 2-step dilution method is mostly used during deep freezing (Salamon, 1968; Colas, 1975).

Semen dilution is either carried out on a volume to volume (semen:diluent) basis, or to a fixed sperm concentration. In a systematic study of the effect of dilution rate on the survival of ram spermatozoa, Koger (1951) reported that there was a negative correlation between rate of dilution of semen and the length of time motility is maintained. Salamon (1968) reported a highly significant interaction between type of diluent and dilution rate on the survival of ram spermatozoa. Roy *et al* (1956) had reported successful storage of ram semen for a long time in glycine-yolk diluent at 1:10 to 1:100 dilution rates. This was comparable to the results obtained when the dilution rate was 1:10 to 1:50 in yolk-citrate. Wiggan and Clark (1967) used a dilution rate of 1:5 with relatively good success. Tewari *et al*. (1968) showed that there was a direct relationship between spermatozoa survival and dilution rate. Motility was best maintained in a 1:10 dilution rate and worst at 1:1.

Hess *et al* (1960) showed that fresh semen may be diluted to a fixed cell concentration provided the total

number of spermatozoa does not fall below a certain limit generally accepted as 50 x 10^6/ml (Koger, 1951; Morozov, 1954; Emmens and Robinson, 1962). Salamon and Robinson (1962a) stated that semen may be diluted at least 1:4 without impairing both the survival and fertility of the spermatozoa. A general experience of most workers has been that storage results in a decline in spermatozoa viability, at a dilution rate of 1:4 moderately good results were reported with boar semen after 24 hours of storage. Almquist and Wickersham (1962) also reported that diluting ram semen to provide about 15 million motile spermatozoa per millilitre, resulted in successful inseminations. While Foote (1970) used a higher dilution rate to give a final concentration of 8 million motile spermatozoa per millilitre. There seems to be a general consensus among investigators that semen could be diluted to a certain level ($50x10^6$ spermatozoa/ml, or 1:4v/v,semen:diluent) without any adverse effect on the viability of the spermatozoa. But at higher dilution rates, say 20 to 0.06 x 10^6 spermatozoa/ml, the spermatozoa motility is greatly reduced or lost completely (Blackshaw, 1953).

2:1:3: COOLING AND COOLING RATE

Ram spermatozoa behave in the same way as any other tissue in their response to temperature changes. When exposed to sudden fall in temperatures, they suffer an irreversible loss of viability generally refered to as cold shock. Associated with this phenomenon of 'cold shock' is a reduction in the rate of glycolysis and dehydrogenase activity which may be responsible for the loss of motility of the spermatozoa (Blackshaw, 1958a). Although egg yolk and cryoprotectants greatly reduce the risk of cold shock, rapid cooling of diluted semen adversely affect spermatozoa motility. Several methods of cooling ram semen to 5°C prior to storage have been adapted. However, the methods varied from one investigator to the other. Almquist and Wickersham (1962) reported that after diluting the semen to about 15 million motile spermatozoa per millilitre, the samples were cooled to 5°C over a period of hours. Salamon (1970) used 1.5 hours, Salamon and Visser (1972) and Colas (1975) used 2 hours, while Visser and Salamon (1973) used 1 hour. The most widely adopted method is placing the ejaculate in test tubes and standing them in a water bath at either room temperature or between 30-37°C before transfering them into a refrigerator. This allows several hours to elapse before the cooling is completed, thus avoiding the adverse effects of rapid cooling on the spermatozoa (Emmens and Robinson, 1962).

SECTION 2.2: MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN RAM SPERMATOZOA AFTER STORAGE

Introduction

The viability of spermatozoa measured before, during and after storage has been the basis for the development of methods of semen preservation in research laboratories. Man's efficiency in developing successful methods of semen preservation is dependent on the relationship between test of semen quality and fertility after such preservation (Saacke, 1982b). Estimation of spermatozoa motility or percentage motile cells before and after storage has been used as criterion for predicting the fertility of the semen. These have become inadequate in explaining the often large fertilization failures encountered with ram semen stored beyond 24 hours. It has been shown that ram spermatozoa lost their fertility long before any visible loss of motility is observed (Baudet *et al.*, 1956; Schindler and Amir, 1961). Thus attention has been focused on the morphological and biochemical changes that may occur in the ram spermatozoa during storage for possible explanation. The correlation between these changes and the fertilising ability of ram spermatozoa has not been clearly established.

This review is carried out in 2 sections. The first section deals with morphological changes and covers topics such as acrosomal damage, membrane rupture and the relationship between the altered structures and the

functional integrity of the spermatozoa. The second section deals with biochemical changes including changes in enzyme, DNA, phospholipid, fatty acids structure and concentration and changes in cation concentration. Closely related observations made in spermatozoa of other species have also been included for comparison.

2:2:1 MORPHOLOGICAL CHANGES

Austin and Bishop (1958), and Saacke and Marshall (1968) gave detailed descriptions of the morphology of the mammalian spermatozoa. Damages to the spermatozoa structure can occur at the various stages of semen processing prior to storage, and during storage. The most vulnerable structures have been observed to be the acrosome and plasma membrane (Milovanov *et al.*, 1970). Tasserson *et al.* (1977) stated that about 40% of the cummulative damages to these structures can be detected by ordinary light microscopy, and about 70% by electron microscopy.

The acrosome has been given more attention in morphological studies of spermatozoa, probably because it exhibits the most visible changes. Damages to both the acrosomes and plasma membrane of ram spermatozoa have been characterised by moderate protrusion, swelling and/or disintegration. The percentage of spermatozoa with these abnormalities were observed to increase with time of storage (Smagulov and Martynov, 1968; Schleicher and Bruckner, 1973).

Jones and Martin (1973) observed some vacuolation of the acrosomes in a small proportion of ram spermatozoaa. In addition there was a general loss of cellular materials (enzymes such as acrosin), which leaked out of the damage cells. Slight decrease in the length and breadth of ram spermatozoa head following storage have been reported (Mukherjee and Dott, 1960; Rathore and Mukherjee, 1961; Kharche *et al.*, 1976). Rathore and Mukherjee (1961a) also reported observing stored ram spermatozoa having roughly shrunken acrosome with some degree of vacuolation. The decrease in the size of the spermatozoa head may be attributed to the shrinkage of the acrosome as result of loss of cellular materials associated with the damaged spermatozoa structures.

Although the acrosome and the plasma membrane are observed to suffer most from preservation damages, other structures have been shown to suffer similar damages though to a lesser extent. Quinn *et al* (1969) found that structural changes occurred in the midpieces especially in the matrix of the mitochondria which makes up the mitochondrial sheath of ram spermatozoa. Jones and Martin (1973) also reported a significant proportion of spermatozoa in diluted ram semen had damaged acrosome, while the mitochondria lost materials after storage at 35°C for 2 hours. The mitochondria were scored as pale in 80.5% of the spermatozoa compared to only 13.5% in the control samples. Both the acrosome and

midpieces were affected by cooling at 5°C and storage at this temperature for up to 72 hours. The midpieces showed condensation of the mitochondria and loss of material.

Similar structural changes observed with preserved ram spermatozoa have been reported in other species. Somade *et al.* (1974) reported changes in bull spermatozoa ranging from loosening of the plasma membranes to complete loss of acrosomes following storage. Kunze *et al.* (1973) carried out detailed investigation on the structural changes that occurred in boar spermatozoa after freezing and reported that apart from slight swelling and rupture of the plasma membrane, no other cell damage was found prior to freezing. However, there were considerable ultra-structural changes in the head region of the spermatozoa especially the acrosome. Visser and Salamon (1974) observed that the degree of damage to the acrosome of boar spermatozoa following freezing also followed the same pattern with that of bull and ram, ranging from slight to severe damage and complete loss.

Comparative studies have shown that there is species variation in resistance of the spermatozoa to structural changes following storage. Varnaskii (1970) reported that similar structural damage was observed in both the acrosomes of ram and bull spermatozoa, but the proportion of damaged acrosomes was about twice as high for ram as for bull spermatozoa. Watson and Martin (1972) also reported that the acrosome in ram spermatozoa appeared to be more severely

damaged than bull's. Similarly, Healey (1969) found that both boar and ram spermatozoa were more susceptible to freeze damage than either bull or stallion spermatozoa. Watson and Martin (1972) compared acrosomal changes that occurred in deep frozen ram and bull spermatozoa. They reported that a highly significant deterioration of the acrosome occurred in 48.3% of ram spermatozoa compared to 22.7% of bull spermatozoa. Jones (1973c) also showed that the plasma membrane of bull spermatozoa was more resistant to breakage than those of either the boar or ram when stressed by dilution or fixation.

The proportion of spermatozoa with damaged structures, and the type and extent of the damage could be influenced by a number of factors associated with processing. Rathore and Mukherjee (1961) observed that the preservation of ram semen for 72 hours in egg yolk-phosphate or egg yolk-boric acid-sodium bicarbonate diluent decreased the size of the spermatozoa head. Similar decreases in the spermatozoa head were observed in egg yolk-citrate diluent. But Schleicher and Bruckner (1973) observed that there were considerably fewer structurally altered spermatozoa after 72 hours of storage in Tris diluents than after 24 hours in either egg yolk-citrate or egg yolk-phosphate. The same type of morphologically altered spermatozoa heads were found in all 3 diluents. Tasserson *et al.* (1977) investigated acrosome damage in ram spermatozoa during dilution, cooling and

freezing, and reported that about 50% of the damage occurred during dilution and cooling alone. Jones and Martin (1973) stated that swelling of the acrosome was caused by cooling to 5°C. It was further observed that acrosomes and midpieces were most affected by cooling to 5°C and storage at this temperature for up to 72 hours. Freezing and thawing processes are also responsible for the disruption of the acrosome and damage done to the midpieces. According to Jones and Stewart (1979) any further loss in viability of bull spermatozoa that could be attributed to semen processing probably occurred during the freezing and thawing process. Wiggins and Almquist (1974) investigated the effects of equilibration time and thawing rate on the acrosomal damage of bull spermatozoa. The mean percentage spermatozoa with normal acrosomes increased for each increase in thawing rate. There was, however, an interaction between thawing rate and equilibration time. Shorter equilibration periods coupled with rapid thawing resulted in a lower percentage of spermatozoa with damaged acrosome. Pursel *et al.* (1974) reported that the changes in acrosome morphology of boar spermatozoa during liquid storage is influenced by type of diluent and temperature. Jones (1973) found that cooling of boar spermatozoa to 5°C in diluents containing glycerol increased the incidence of spermatozoa showing various stages of vesiculation. These findings were similar to those reported by Jones and Martin (1973) with ram spermatozoa.

Lindemann *et al.* (1982) discussed in details freezing injury to cells and methods for its prevention. It has not been clearly established whether the changes in cell membranes are results of a selective effect of the freezing process on the cell membrane alone or are a single manifestation of generalised damage to the entire cell structure. Although it is possible to demonstrate that specific structures are damaged during preservation, it is usually not possible to determine which cell structures are left unaffected.

The relationship between the morphological damages and the lowered fertilising ability of stored ram spermatozoa has not been fully established. Gunn *et al.* (1942) suggested that the occurrence of even a 1% abnormal spermatozoa in the semen will reduce fertility from 80-100 to 60%, and more than 10% abnormal spermatozoa was associated with severe infertility. Rathore and Mukherjee (1961b) stated that although the decrease in the size of the spermatozoa head due to preservation is associated with decrease in the fertilising ability, the shrinkage may not be responsible for the loss of fertility. Better conception results were obtained with semen stored in diluents that prevent (or present minimum) shrinkage of the spermatozoa head (Gokhale, 1958). Jones and Martin (1973) observed a relationship between structure and function of ram spermatozoa, which is useful in evaluating semen quality. However, factors at the molecular level must also be considered since ultrastructural changes do not

explain the reduced fertility due to processing and storage of ram semen. Visible acrosome deterioration during liquid storage rendered boar spermatozoa incapable of initiating fertilization and markedly reduced the capacity of the spermatozoa to attack the zona pellucida of the ovum (Pursel *et al.*, 1972). Both ram and bull spermatozoa suffered the same type of structural damage to varying degrees. This damage was not enough to constitute the sole factor responsible for the observed difference in fertility following insemination (Watson and Martin, 1972). Martin and Richardson (1976) stated that the capacity of ram spermatozoa to fertilize appeared to have been damaged before defects in morphology could be detected. Once the spermatozoa with structurally damaged acrosomes and midpieces enter the fluid of the genital tract of the ewe, their rate of transportation may be greatly hampered. Baker and Degen (1972) have observed that both dead or damaged spermatozoa may be transported within the reproductive tract of gilts, but the rate is slower. Krzanowska (1974) indicated that in the mouse, the utero-tubal junction may serve as a barrier against morphologically abnormal spermatozoa especially the severely damaged. More information is needed on the structures of a spermatozoon capable of fertilising an ovum, and the relationship between damage to these structures which may be associated with the observed reduced fertilising capacity of structurally damaged spermatozoa.

2:2:2 BIOCHEMICAL CHANGES

Several authors have observed that associated with morphological damages are biochemical changes that occur in stored mammalian spermatozoa. These changes in composition of the spermatozoa have been studied with the aim of establishing a relationship between them and the loss in fertility of the spermatozoa. In some instances, the loss of extracellular materials has been used to assess the extent of cell membrane damage during *in vitro* storage of spermatozoa (Crabo *et al.*, 1970; Bower *et al.*, 1973; Foulkes and Watson, 1975). Commonly observed biochemical changes in spermatozoa of most species have been reported as changes in enzymic activities, deoxyribonucleic acid (DNA), phospholipids and fatty acid content of the sperm cells.

The biochemical changes that occur in ram spermatozoa during storage have been shown to depend largely on the properties of the diluents, the degree of dilution, temperature and light (Crabo *et al.*, 1970). These authors reported that dilution of semen caused a release of the enzyme glutamic oxalacetic transaminase (GOT) from spermatozoa, although the amount released varied with the type of buffer used. High glycerol concentration was observed to increase the rate of GOT loss (Bower *et al.*, 1973). Graham *et al.* (1970) also observed a relationship between cell damage and the loss of intracellular enzymes into the extracellular medium during storage of spermatozoa. There

were large differences in the amount lost between samples subjected to minimal or maximal cellular damage. The authors reported that there were increases in the activities of GOT, lactic dehydrogenase (LDH) hydluronidase, acid phosphatase and alkaline phosphatase in the extracellular fluids of bull, boar, stallion and turkey semen, subjected to maximal cellular damage than those with minimal damage. Kurilov (1972) further observed that the extracellular activities of glucose-phosphate isomerase (GPI) and alkaline and acid phosphatase in boar semen did not increase following freezing, although aldolase increased more than 2-fold. Osinowo (1981) observed similar significant losses in glucose phosphate isomerase, as well as lactic dehydrogenase from ram and bull spermatozoa. This, the author stated followed the washing of the spermatozoa . Resuspension of the washed bull spermatozoa caused an immediate release of enzymes from the cells. Pre-incubation of ram spermatozoa with 0.025% formaldehyde rather decreased LDH than GPI levels in the intracellular fluid. He suggested that enzyme release from spermatozoa may occur in the absence of apparent cellular damage.

Osinowo (1981) speculated that the leakage of intracellular materials could be a mechanism for eliminating osmotic differences between the intracellular and extracellular fluids, in order to control cell volume. Similar assertion had been made earlier by Kharche *et al.*

(1976). These authors observed that although ram spermatozoa head length and breadth are reduced in stored semen than fresh samples, the decrease was less in samples diluted with diluents containing potassium ions, where there was a free shifting of potassium ions between the sperm cells and the diluting medium. This confirmed the reports of Blackshaw (1953) and White (1959). These authors had earlier observed that potassium ions maintained the internal osmotic pressure and the integrity of spermatozoa. Fisher *et al.* (1984) found that spermatozoa survived longest in isotonic solutions than in hypotonic solutions. The poor survival rates in the hypotonic solutions could be associated with the leakage of materials from the cells to make up for osmotic imbalance between the two media (intracellular and extracellular solutions).

There appears to be no established correlation between the leakage of materials from the spermatozoa during processing and subsequent storage and the loss of fertilising ability of the spermatozoa. However, Graham *et al.* (1970) proposed that the amount of enzymes left in the cells after such procedural manipulations could be very important to the potential fertilising ability of the spermatozoa. A sequential study showed that the amount of hyaluronidase released and amount GOT left in boar spermatozoa were poorly correlated with motility or acrosome damage (Crabo and Graham, 1972). There were, however, high

correlations between intracellular hyaluronidase and extracellular GOT (r= 0.68), percentage of spermatozoa with normal acrosomes and hyaluronidase (r= 0.63), and between motility and intracellular hyaluronidase (r= 0.56).

Changes in deoxyribonucleic acid (DNA) content of bull spermatozoa during storage has been more intensively studied than any other species in relation to the loss of fertilising capacity. Quinn (1968) suggested that the loss of DNA materials from ram spermatozoa may be due to the action of the enzyme deoxyribonuclease present in ram seminal plasma. Degradation of DNA into dialysable components occurs at a slow rate in senescent ram semen. But the process is accelerated in semen that has been cold-shocked or frozen (Quinn and White, 1969). Quinn *et al.* (1969) however, reported that there was no loss of DNA material from cold-shocked or frozen ram spermatozoa during 30 minutes of incubation, but on incubation for 6 hours at 37°C, loss of material was observed.

A loss of about 30% of the initial DNA material in bull spermatozoa, following storage at 5°C or deep freezing has been reported by Salisbury *et al.* (1961). At ultralow temperatures of about -192°C, Shikima (1965) reported no structural changes in the double helix of the DNA of bull spermatozoa. But Hanada *et al.* (1968) reported non-significant changes caused by dilution, equilibration and freezing. The authors also found no correlation between DNA

content and post- thawing motility or fertility of the spermatozoa. Significant decrease in the DNA content of boar spermatozoa was reported by Anard *et al.* (1967) after storing the samples for 1 day at 37°C. This was related to the sub-fertility of aged boar spermatozoa, and increased incidence of embryonic mortality. With ram spermatozoa, Martynov (1970) reported that storage for 12, 24 and 48 hours, resulted in a loss in DNA content of about 1.5, 8.8 and 12.2% respectively, compared to the content in fresh semen samples. Conception rates after insemination with the semen samples were 61.6, 46.5 and 30.6% respectively. The authors suggested that the variation in the conception rates could be attributed to loss in DNA materials.

Platov (1970) carried out a study of the variation in the adenosine polyphosphate content of bull and ram semen following storage at 0 and 5°C. It was found that both ADP (adenosine diphosphate) and ATP (adenosine triphosphate) contents of the spermatozoa decreased at a faster rate following storage than did the motility of the spermatozoa. There was no correlation between the ADP and ATP content in the spermatozoa and spermatozoa motility. The relationship between the two cellular components was affected by the nature of the diluent and degree of spermatozoa activities. Iljinskaya and Kazantseva (1972) found that 50-60% of the ATP content of boar spermatozoa was destroyed by freezing and thawing.

Poulos *et al.* (1973), and Darin-Bennett *et al.* (1974) observed that the susceptibility of spermatozoa to cold shock was affected by poly-unsaturated and saturated fatty acid ratio of the phospholipids content of the spermatozoa. Cholesterol has been shown to play an important role in the low temperature tolerance of most cells (Rotten *et al.*, 1973). The role of this lipid in promoting close packing of other phospholipids especially those with unsaturated fatty acid chains has been demonstrated (Benedetti and Emmelot, 1968; Philips, 1972). The structure of the spermatozoa membrane and its subsequent ability to withstand stress such as cold shock seems to be correlated to the ratio of cholesterol to phospholipids, and poly unsaturated to saturated fatty acids. The amount of cholesterol in cold shocked semen samples has been found to increase significantly compared to that in control fresh samples (Bonadonna and Roychoudhurry, 1969). The different interaction of cholesterol with both saturated and unsaturated fatty acids has been well documented in model membrane systems (Philips, 1972; Hauser and Barrat, 1973; Jason *et al.*, 1973). Relatively large amounts of saturated fatty acids in the spermatozoa result in a more rigid structure than is the case with unsaturated acids (Benedetti and Emmelot, 1968). The nature of the fatty acids also affects the ability of cholesterol to decrease membrane permeability (Daniel *et al.*, 1968).

SECTION 2:3 INSEMINATION TECHNIQUES IN SHEEP

Introduction

Success in artificial insemination of sheep has depended largely on the proper use of fertile semen at the time of insemination for maximum fertility. This has been made possible by the development of effective methods of semen processing. In addition, successful development of effective methods of detecting ewes in oestrus so that they can be inseminated at the right time, and proper techniques of handling and inseminating ewes, have also made valuable contributions in this regard.

In this section, various techniques of artificial insemination in sheep are reviewed. Emmens and Blackshaw (1956) reviewed literature on semen collection and processing, methods of restraining ewes, equipment for introducing semen, spermatozoa number and volume of inseminate, time of insemination and number of inseminations during an oestrus, oestrus synchronization, heterospermic insemination and site of semen deposition. The present review covers only volume of inseminate and number of spermatozoa, timing and number of inseminations during an oestrus, and the site of semen deposition.

2:3:1: Timing and number of inseminations during an oestrus

The optimum period during which satisfactory conception rates can be expected following the onset of oestrus is of utmost importance in the artificial insemination of sheep. Thibault (1967) and Terrill (1975) gave estimates of the fertile life span of ovine ova as 12-24 hours, and that of the spermatozoa within the ewe's reproductive tract as about 24-48 hours. These are very important factors in fertilisation following insemination. Maximum fertility will result if viable healthy spermatozoa are found in sufficient numbers at the proper site in the female reproductive tract, and at the optimum time in relation to ovulation. This, however, depends on the time of insemination and the rate of transportation of the spermatozoa within the female reproductive tract - estimated to take from a few minutes to about 8 hours (Killeen and Moore, 1970a; Mattner and Braden, 1969). The rate of transportation of the spermatozoa is said to diminish in late oestrus. Thus it is apparent that insemination of ewes should be done when both the spermatozoa and ova are at their optimum fertile life and when the transportation of the spermatozoa within the ewe's reproductive tract is at its most efficient. Questions have been raised as regards the time within the oestrus cycle which is right for the insemination to take place. Several reports of trial inseminations of ewes at various periods during oestrus have been made. Most authors tend to agree

that for maximum conception rates, inseminations carried out mid-oestrus have the greatest probability of success, with the least probability in late oestrus. Duran Del Campo (1960) could not make any conclusive inference on the result of inseminating ewes at various time during the heat period. He however stated that inseminations were less likely to result in pregnancies if done very early or after 24 hours in the heat period. In a study of the optimum conditions for spermatozoa delivery in the ewes Salamon (1972) observed that the percentage of ewes lambing was better when the spermatozoa were inseminated 15-17 hours after detection. Inseminations carried out in early or late oestrus often resulted in lowered fertility. It appears from the reports of most authors that inseminations carried out 12-18 hours after the onset of oestrus have higher probabilities of resulting into pregnancies. Those done at about 24 hours have reduced chances, while beyond 24 hours into the oestrus, the inseminations may not result into any pregnancy. In most cases, the general practice is to inseminate the ewes between 18-24 hours after drafting for high conception rates (Moss *et al.*, 1979).

To increase the conception rates several authors have suggested that multiple inseminations be carried out within an oestrus. Early workers such as Peregon (1936) reported that repeated inseminations during one heat period was beneficial and yielded good results which were significantly

above the flock average. They however noted that in itself, multiple insemination could not be regarded as the only reason for the increase in conception rates. Other factors such as genetic and physiological state of the animal, and environmental factors may affect the process. Aamdal and Hogset (1955), Lopyrin *et al.* (1957) and Salamon and Robinson (1962a) did not find any significant difference in conception rates between single and double inseminations carried out within one oestrus. Kazakov (1963) reported that inseminations with semen diluted and used fresh gave 58.3% conception rate out of 49 ewes inseminated once, while double insemination at 24 hour interval on 250 and 400 ewes gave 64.3 and 94.4% onception rates respectively. Aamdal and Anderson (1968) in a double insemination experiment with frozen semen obtained a non-return rate of between 61.0 and 62.5%. In 2 separate tests, Salamon (1971) found that insemination of ewes 1-2 hours or 15-17 hours after heat detection resulted in 42.9 and 51.3%, and 69.1 and 75.7%, of the ewes lambing respectively. If the ewes were inseminated again 10 hours later, then the average increases in lambing rates were 13-15 and 6.2% if inseminated early or late in the oestrus period respectively.

Both Salamon (1971), and Visser and Salamon (1973) observed that two inseminations carried out within one oestrus always gave slightly higher levels of fertility than single insemination. This is so because the time between the

onset of oestrus and ovulation is more variable in ewes in natural oestrus and between breeds, thus the chances of contact between male and female gametes are improved by increasing the number of inseminations within an oestrus. Firth (1976) conclusively attributed the increase in the lambing rates following multiple inseminations within an oestrus to a better timed insemination rather than larger number of spermatozoa as a result of double insemination. However, the difference in fertility between single and double inseminations within one oestrus is most often small that it may not justify the extra effort involved in carrying out the second insemination.

Most of these suggestions and conclusions were drawn based on ewes observed in natural oestrus. With the advent of oestrus induction through the use of exogenous hormones, these conclusions may not hold true. In a series of experiments carried out on ewes that were treated with Repromap (Upjohn) vaginal sponges for 13 days, Gemert and Hoogenkamp (1975) reported that conception rate of ewes inseminated at first oestrus following withdrawal of the sponges was 55.3% compared to 66.0% obtained following inseminations carried out during the second oestrus. In a separate experiment, the ewes were inseminated at 3 periods, 40, 65 and 90 hours after sponge withdrawal, the conception rates ranged between 40.8, 55.0 and 76.7% respectively. Aguer and Provost (1976) recommended that ewes in which

oestrus synchronisation has been carried out should be mated or inseminated at predetermined intervals after pessary withdrawals: Double inseminations should be carried out 50 and 60 hours after pessary removal in the case of lactating ewes, and a single insemination 55 hours after pessary withdrawal is recommended for dry ewes. Deas *et al* (1979) recommended double inseminations carried out at 50 and 64 hours after pessary withdrawal, or a single insemination at about 57 hours. They pointed out that conception rates following a single insemination were generally not more than 7% lower than the double insemination, which may not warrant the extra cost involved in carrying out the second insemination.

2:3:2. Volume of inseminate and number of spermatozoa

Studies have been carried out on the minimum and maximum number of spermatozoa required per insemination to achieve good conception rates. Koger (1951) found a linear relationship between the number of spermatozoa introduced and conception rates. The author observed that for good conception rates of more than 50% the spermatozoa number should not fall below 50 x 10^6 spermatozoa per insemination dose. Terrill (1960) reported successful conceptions with spermatozoa number as low as 5 x 10^6. Emmens and Robinson (1962) asserted that a dose of 50 to 150 x 10^6 spermatozoa

in either diluted or undiluted semen should yield conception rates in the range of 55 to 60%. In a preliminary work with Awassi sheep, Schindler and Amir (1973) obtained about equal conception rates with 120, 80 or 40 million spermatozoa diluted in reconstituted milk. Similarly, in a 2-year study, Zlatarev (1961) inseminated a total of 6517 ewes with 0.2 ml doses of semen diluted in egg yolk-citrate, stored at 0-3°C. Each semen dose contained either 40, 60, 80 or 100 million spermatozoa. The conception rate was highest (67.0%) for the 80 million spermatozoa dose.

Lapwood *et al.* (1972) showed that with a single dose of 50 x 10^6 spermatozoa per ewe inseminated, the fertility of ram semen was improved by centrifugation of the diluted semen to return the concentration to that in the original semen sample. Entwinstle and Martin (1972) used the technique of centrifugation and reconcentration of ram semen diluted 10-fold to prepare doses of semen containing 50- or 100 x 10^6 spermatozoa in volumes of 50 or 200 *ul*. There was no significant difference in fertility rates between treatments or between diluted and undiluted semen. Martin and Watson (1976) also tested the fertility of ram semen diluted and centrifuged to produce insemination doses of between 25 and 100 x 10^6 spermatozoa per volume (50 and 250 *ul*). They found that the initial dilution rate and the use of spermatozoa number of about 25 x 10^6 significantly lowered the conception rates. El-Gaafrey *et al.* (1987) observed that it was not

advisable to use less than 200 and 400 million spermatozoa in the insemination dose for either fresh (or short term stored), and frozen semen respectively.

It has been reported (Quinlivan and Robinson 1969; Hawk and Conley, 1975) that hormone treatment of ewes often resulted in lowered fertility especially after artificial insemination. This was attributed to the lowered rate of spermatozoa transportation in the reproductive tract of the treated ewes. Thus it is often necessary to inseminate progestagen treated ewes with large numbers of spermatozoa to overcome this problem. Since it has been shown that there is no advantage in inseminating more than once at the optimal time (Salamon, 1971; Visser and Salamon, 1973; Firth, 1976; Colas, 1979; and Langford *et al*, 1982), the desire to determine the smallest number of spermatozoa which could be used in a single insemination of progestagen treated ewes without compromising fertility has been stressed. Gemert and Hoogenkamp (1975) used a 500 x 10^6 spermatozoa number in an insemination dose and reported that the average conception rate was 81.8%. Colas (1979) reported that insemination of 300- to 400 x 10^6 spermatozoa stored for less than 5 hours in a skim milk-citrate diluent resulted in a 65% conception rate in progestagen treated ewes on pasture. Langford and Marcus (1982) reported that the fertility (number of ewes lambing) after insemination with 400- Or 200 x 10^6 spermatozoa was (68%) was similar to that obtained after natural service at

progestagen induced oestrus. When less or equal to 100 x 10^6 spermatozoa were inseminated, the fertility fell markedly and the number of lambs born per ewe inseminated also decreased. The authors further observed that insemination of 200 x 10^6 spermatozoa, i.e. less than 10% of the number in a single ram ejaculate, allowed normal conception rates in progestagen treated ewes. The smallest number of spermatozoa that gave the maximum fertility in such treated ewes (200 x 10^6) was more than twice the number reported to provide maximal fertility in ewes mated at natural oestrus (Salamon, 1962; Zlatarev, 1976; Entwinstle and Martin, 1972).

When a similar number of total live sperm cells is used, insemination volume is relatively of less importance. According to Sinclair (1957), there seems to be no added advantage in introducing more than 0.1 ml of semen into the first fold of the cervix. Doses as low as 0.05 ml have been reported to give satisfactory results if the semen is of good quality. Mies Filho and De Almeida Ramos (1955) found that there was no difference in fertility when volumes of semen ranging from 0.05 ml to 0.1 ml were used for inseminating ewes with semen diluted in milk or undiluted. The 0.1 ml volume has been the most widely used in most studies with fresh undiluted or diluted semen. However, Tjupic (1959) reported that lambing rates of 47 to 56% were obtained with vaginal inseminations of between 0.5 and 1.0 ml of semen diluted 1:10 or 1:20, and 70 to 72% when 1.0 ml dose of semen

diluted 1:2 or 1:4 were used. Reconcentration of the spermatozoa has allowed the use of very low volumes of semen during inseminations. Entwinstle and Martin (1972), and Martin and Watson (1976) used ultra-low volumes in the range of 50 or 200 *u*l and reported obtaining no significant difference in the fertility of ram semen. Martin and Richardson (1979) also reported that the volume of the insemination dose had no effect on the fertility of stored ram semen when the sample were of good quality. From these reports, it appears that there is no standard insemination dose volume for stored ram semen. It could be concluded that since under normal storage conditions the number of motile spermatozoa reduces due to cell death, a large volume of inseminate is required to carry the same number of spermatozoa needed for good conception rates. However, centrifugation of the diluted semen samples to reconcentrate spermatozoa number close to that in the initial semen sample, can be used to reduce the volume of the inseminate.

2:3:3. Site of semen deposition.

The reduced viability of stored ram spermatozoa within the ewe's genital tract stimulated research on the optimum site for depositing semen during artificial insemination, to maximise fertility. Generally, insemination should be done as close as possible to the site of fertilization, or at a site

that will allow both spermatozoa and ova to meet when they are at their most fertile life. In cattle, Salisbury and VanDemark (1951), and Stewart and Melrose (1952) obtained very good conceptions with cow inseminated at the cervix, uterine body and uterine horns. Though the uterine inseminations gave the best results. Since then the common practice has been to deposit the semen in cow into the uterus. Access to the uterus is gained by means of rectal palpation to locate the uterine end of the cervix, and the catheter is slowly manipulated into the uterus through the cervix.

The rectum in the ewe is so small that that it does not allow the recto-uterine method of insemination to be carried out. Thus, the usual site of semen deposition during artificial insemination of ewes has been at the cervix, usually to a depth of not more than 5 mm. Koger (1951), and Mies Filho and De Almeida Ramos (1955) found no difference in conception rates when insemination were carried out at the cervix or in the vagina. But Tjupic (1959) stated that although vaginal insemination was quite successful, most studies showed the cervical method to be considerably superior. The efficacy of the cervical insemination however depends on the efficiency of finding the cervical orifice, and the form of the os cervix. The form of the os cervix varies from animal to animal and with the age of the animal (Inskeep, 1974). Cervical insemination remained popular

until it was observed that deposition of the semen deep into the cervix often resulted in increases in conception rates. According to Boyarskii (1986), even within the cervix, when semen was deposited further at a depth of 2.5 cm as against 2.0 cm mostly used, there was marked increase in the conception rate. The cervix constitutes an initial barrier to the ascent of the spermatozoa, such that only a relatively small proportion of the deposited spermatozoa reach the uterus to effect fertilization (Lightfoot and Salamon, 19/0a). Thus, attempts have been made to by-pass the cervix and place the semen directly into the uterus. One of the methods developed is the intra-uterine technique. Two types of intra-uterine insemination have been tried. These are deposition of the semen in the uterine horns after laparotomy (Lightfoot and Salamon, 1970a), or a forceful entry into the uterus through the cervix (Anderson *et al.*, 1973; Fukui and Roberts, 1976).

It was assumed that gaining access to the ewe uterus was almost impossible because of the small nature of the cervical wall and the variation in length of the cervical canal from one animal to the other. These, together with the numerous annular folds in the cervix, make it difficult to penetrate the cervix. But Anderson *et al.* (1973) descibed a technique of non-surgical intra-uterine insemination of ewes. Inskeep (1974) also showed that with a concious effort, it is possible to reach the uterus in some ewes with a tapered

inseminating tube, or with 12-guage metal inseminating tube 36 cm long. Fukui and Roberts (1976) carried out a study of the non-surgical intra-uterine insemination techniques similar to that described by Anderson *et al.*(1973). They reported that insemination with frozen semen resulted in comparable fertilization and lambing rates similar to the use of fresh undiluted semen. A further study of the technique and its effects on the transportation of spermatozoa in the ewe's genital tract (Anderson *et al.*, 1976) revealed that the technique significantly increased the number of spermatozoa recovered from the oviduct. Moreover, far more of the spermatozoa found in the oviduct were intact (i.e. not tailless) after uterine insemination, compared to cervically inseminated spermatozoa. It was concluded that the deposition of semen into the uterus by non-surgical methods increased the chances of the spermatozoa passing into the oviduct without damage or loss of fertility. This might have accounted for the high fertility recorded with the method.

The repeatability of penetrating into the uterus via the cervical canal by the non-surgical intra-uterine technique of insemination in the ewe was reported by Fukui and Roberts (1977) to be 0.745. The current techniques of non-surgical intra-uterine insemination of ewes are time consuming as penetration of the whole length of the cervical canal must be completed. The success also depends on the nature of the cervical orifice (Fukui and Roberts, 1978). The complexity of

the cervical canal was observed to be greater in older ewes than maidens (Dun,1955). Fukui and Roberts (1978) applied carbon dioxide gas pressure to the intra-cervical walls of freshly slaughtered ewes to improve the ease of penetration with relatively good success. However, attempts at this approach with live animals seems not to have been reported.

Similar improvement in fertility of ram semen following surgical intra-uterine inseminations have been reported. Salamon *et al.* (1977) reported that after surgical insemination the egg fertilisation rates for 2- and 4- day old semen were high and similar to that of fresh semen, but decreased for semen stored beyond 4 days. When normal cervical insemination was performed, the fertilisation rates for fresh and 2-day old semen were indistinguishable. No fertilised eggs were recorded using semen stored for 4-days. Killeen and Caffrey (1982) described another technique of intra-uterine insemination of ewes by means of laparoscopy. Preliminary results of insemination by this method indicated that high levels (69%) of egg fertilisation could be obtained in ewes following intra-uterine insemination with fresh or frozen semen via laparoscopy (Killeen *et al.*, 1982).

Studies of inseminations at various sites have been carried out. Tervit *et al.* (1984) reported that in ewes inseminated at the 2nd oestrus after synchronisation, recovery of fresh or frozen semen deposited once or twice into the cervix, vagina, onto the ovaries, or into the

uterine horns was undertaken. Very poor results occurred after ovarian insemination. The ranking of the insemination position (best to worst) was uterine, cervix and vagina. A single vaginal insemination of fresh semen gave a lambing rate of 64% which was just slightly lower than for cervical insemination (69%), and the intra-uterine technique gave good results with both fresh and frozen semen. Kerton *et al.* (1984) also compared fertility following insemination by cervical, vaginal or intra-uterine techniques. Their results showed that conception rate for vaginal insemination were significantly lower than for the other 2 methods which were different from each other. Conception rates to uterine, cervical and vaginal inseminations were 56, 53 and 31% respectively. Maxwell (1986) also reported significant difference in conception rates following vaginal, cervical and intra-uterine inseminations. The intra-uterine method was superior to the cervical, which was better than the vaginal method. Haresign *et al.* (1986) also reported the same observations. They further compared deposition of semen at the top, middle or bottom of the uterine horn. The percentage of ewes lambing following insemination at the various sites were 43.6, 52.8 and 42.4% respectively.

Killeen and Caffrey (1982) described another technique for intra-uterine insemination in ewes via laparoscopy. Preliminary results of insemination by the technique showed that high conception rates in ewes could be obtained with

both stored and fresh semen. Maxwell *et al.* (1984) reported increase in conception rate with time of insemination, using intra-uterine insemination with the aid of laparoscopy. El-Gaafary *et al.* (1987) reviewed the technique of intra-uterine insemination by laparoscopy and observed that the technique resulted in a considerably higher rate of fertilization than any other method (cervical or vaginal). In addition the method was considered more efficient in that relatively small doses of semen (as low as 0.02 ml) containing 20 x 10^6 spermatozoa can be used with great success.

SECTION 2:4. FERTILITY OF CHILLED RAM SPERMATOZOA

Introduction

The ultimate measure of the functional integrity of stored ram semen is the fertilising capacity of the spermatozoa. Motility estimation and analysis of the biophysical and/or biochemical changes may serve as useful tools for laboratory evaluation of the spermatozoa. They however have some direct or indirect bearing on the final fertility of the spermatozoa. The literature in this section is reviewed under 2 sub-headings: fertility results with chilled ram semen and factors that influence the fertility of the chilled spermatozoa.

2:4:1 Results of insemination with chilled ram semen

Results of artificial insemination of sheep has been reviewed by Emmens and Robinson (1962). Most initial fertility tests conducted by early investigators were not satisfactory and only few reported obtaining pregnancies. For instance, Gunn (1942) reported obtaining 11 pregnancies out of 129 ewes (about 8.5%) inseminated. However, with further development in techniques of semen processing, storage and insemination of ewes, claims of higher fertility results were made. A further break-through was made with the development of suitable diluents for extending the semen. Studies on the storage and fertility of ram semen in various diluents at

2-5°C have revealed that conception rates with semen stored for 12-24 hours varied between 6 to 25% in citrate, egg yolk-citrate or egg yolk-phosphate diluents. It was up to 51% when storage was done in heated cow milk without added antibiotics rising to about 65% when antibiotics were added (Dauzier, 1956). Albright *et al.* (1960) also reported that ram semen extended in diluents composed solely of milk constituents performed favourably at 5°C and poorly with prolonged storage at 37°C. Salamon and Robinson (1962b) compared an egg yolk-citrate-glucose and heated cow milk at a dilution rate of 1:2 with or without the addition of 1000 *i.u.* of sodium penicillin per millilitre. After 72 hours of storage at 5°C, the milk diluent without antibiotic was inferior to the egg yolk-citrate diluent. But there was no difference between the two diluents when antibiotics were added to the milk diluent. Fertility declined with length of storage from 60% with fresh semen to 50% at 24 hours, 30% at 48 hours and 20% at 72 hours. These values were based on 80 ewes per group. Wiggan and Clark (1967) reported that for 3 groups of 18 ewes each, inseminated with semen stored in a modified "Minnesota Go" diluent for 12, 36 and 60 hours at 5°C, conception rates to first service were 83, 67, and 22% respectively. Firth (1976) reported not finding any significant difference between ewes inseminated with either fresh, frozen or chilled semen. Lambing rates were 62.3, 55.7 and 52.6% respectively. Much better results were reported by

Zlatarev (1976). He obtained lambing rates of 87.1 and 85.6% v 62.1 and 67.2% after storage at 24 and 48 hours respectively in 2 successive years respectively. Carmenate and Gamicik (1984) reported obtaining 57.8, 61.7 63.8, and 51.4% conception rates after inseminating ewes with ram semen diluted in 1) glucose-lactose-powdered milk, 2) glucose-powdered milk-sodium citrate, 3) powdered milk, or 4) glucose-sodium citrate-egg yolk, and stored at 5°C for 12 hours respectively.

Garcia Del Alba Reyes (1987) inseminated 288 ewes with semen containing 400 x 10^6 spermatozoa per dose. Of the ewes inseminated with fresh or refrigerated semen diluted in egg yolk-lactose, 51.5 and 33.3% respectively lambed, compared to 40.2 and 40.0% for ewes inseminated with fresh or refrigerated semen diluted in milk diluents and 63.6% obtained with naturally mated ewes. White España Garces (1987), reported that insemination of ewes with fresh or refrigerated semen stored 8-12 hours containing 15 x 10^6 resulted in fairly high conception rates. In four groups of ewes inseminated pregnancies rates, detected by means of ultrasonic test 60 days after insemination were 77, 50, 33 and 51%. A summary of fertility results with chilled ram semen is given in Table 1.

There is a further possibility of improving the fertility of chilled ram spermatozoa with the advent of methods of insemination that involve cervical by-pass. The

techniques have been shown to improve the fertility of both fresh and frozen ram semen. Killen and Caffrey (1982) described the technique for intra-uterine insemination of ewes by laparoscopy. Results of trial inseminations have shown that high levels of egg fertilisation are possible with the methods. Lightfoot and Salamon (1970a) described another method of depositing semen into the uterus via surgical incision. While Anderson *et al.* (1973) passed a catheter through the cervix into the uterus. Fertilisation rates ranged from 89 to 90% compared with 45 to 48% for uterine and cervical inseminations respectively.

Higher conception rates have been reported when ewes are inseminated more than once during the heat period. Keast and Morley (1949) reported that out of 62 ewes inseminated twice and one three times at 24-hour interval during the heat period, 37% of these conceived compared to 39% for a comparable group of ewes inseminated only once. But in a series of experiments involving single and multiple inseminations, Salamon (1971), and Visser and Salamon (1973) both stated that double insemination of ewes within one oestrus period always give slightly higher levels of fertility than single inseminations. This was attributed to the fact that the time between the onset of oestrus and ovulation is more variable in ewes especially when in natural oestrus. Thus the chances of contact between the male and female gametes are improved with multiple inseminations.

Firth (1976) conclusively attributed the increase in the lambing rates of ewes inseminated twice within an oestrus to a better timed insemination rather than larger number of spermatozoa occurring at the site of fertilisation, or double insemination itself. However, the limitation of the practice is that the difference in fertility between double and single inseminations is often so small that it may not justify the extra energy expended in carrying out the second insemination.

Table 1: Summary of fertility tests with chilled ram spermatozoa

S/No. Reference	Diluent(s)	Length of Storage	No. of ewes inseminated	Conception rates(%)	Lambing rates (%)	Remarks
1. Albornoz Bustmante (1952)			800	80		
2. Mies Filho and De Almeida Ramos (1954)	Skim milk	12-24 hrs	2063	61.7		Inseminatios were carried out on several farms with semen diluted 1:5 or 1:10
3. Aamdal and Hogset (1955)	Egg yolk citrate	40 days	64	34.4		Conception rates to first inseminations.
	Egg yolk	40 days	19	0		
4. Dauzier (1956)	Citrate, yolk-citrate or yolk-phosphate	12-24 hrs		6-25		
	Skimmed cow milk heated to 95°C for 10 mi.	12 hours		51.0		without antibiotics.
				65		with antibiotics.
5. Istvan (1956)	Boiled cow milk		535	75.5		
			2652	83.4		This was comaparable 83.1% obtained with fresh semen.
6. Tjupic (1959)	Yolk-citrate-glycol-penicillin streptomycin				47-56	Vaginal insemination
					75.0	Cervical Insemination
7. Ozkoca (1961)	Skim milk-arabinose					Range for semen stored in both diluents. Ewes were slaughtered at 3-6 weeks of pregnancy
	Yolk-citrate	1.5-2 hr		61-71.4		
8. Roberts and Houlaham (1962)		24±2 hrs		25		Number of foetuses recovered as % of ewes inseminated were 32%

9. Dzumurov (1962)	Glucose-phosphate		1592	91.2		
	Skimmed and boiled cow milk		1032	92.96		
10. Dzabrailov and Sel'kon (1962)	Glucose-yolk saturated with CO_2		over 9000		98.5	Number of services done were not stated.
11. Linge and Holberg (1963)	Yolk-citrate-glucose.				56	Low lambing rate was attributed to the small of ewes used (9)
	Pasteurized cow milk	10 hours		90		
12. (1964a)	Pasteurized skim milk		27	40.7		These were conceptions to first inseminations
		4-6 hrs				
Ozkoca	Yolk-citrate glucose		23	73.9		
(1964b)	Yolk-citrate glucose			71.8		
13. Ozkoca (1966)	Illinois variable temperature	24 hrs		48.3		
	diluent	3 hrs		66.6	49	
14. Smagulov and Martynov (1965)	Yolk-glucose-citrate	12 hrs	100	49.0	49	
		48 hrs	100	29.0	33	
15. Smagulov and Martynov (1972)	Yolk-glucose-citrate	12 hrs	404	46.5	61.4	The results are as % ewes lambs/100 inseminated.
		48 hrs	500	30.6	39.4	

16. Wiggan and Clark (1967)	Modified Minnesota "GO" diluent	12 hrs 36 hrs			94 93	These were results of inseminations carried out in 2 oestrus
17. Ozkoca (1967)	Cornell University Extender(CUE)				57.1-57.9	
	Yolk-glucose citrate				47.8	
18. Pavlovic and Varadin (1968)	Diluents containing 10% skim milk	3-6 hrs	387	65		Conception rates to first insemination
19. Colas *et al.*	Skim milk: With catalase Without catalase				 74.6 76.0	
	CUE with catalase Without catalase				69.4 62.9	
20. Lopatko (1971)	Diluent containing Tris, 1.25 glucose per 100 ml	1 hr	115	73		
	Glucose-yolk citrate		99	61.6		
21. Zlatarev (1976)	Yolk-citrate		6219	67		This was the result of a year study using 0.2 ml semen dose containing $80x10^6$ spermatozoa
22. Zlatarev (1976)	Diluent based on sodium citrate, with 20% yolk	24 hr 48 hour			87 & 85.6 62.1 & 67.7	Lambing rates for 2 successive years.
23. Firth (1976)	Heated cow milk or Tris based diluent		97		55.7	
24. Salamon *et al.* (1977)	Tris based diluents with 14%yolk	2 days 4 days 6 days 8 days		94.4 91.3 75.0 52.9		Being % fertilised eggs recovered following surgicla insemination of ewes.

25. Martins and Richardson (1979)	Yolk-glucose Yolk-inositol Yolk-NaCl Without glucose		78 82 78 80	46 52 50 58		Semen diluted 20- to 30 fold with phosphate present in all diluent
26. Ozkoca (1980)	Yolk-citrate-glucose+penicillin +streptomycin	4-6 hrs		81.2-91.3		
27. Zamfirescus *et al.*(1983)			74	65	48.1	Inseminations were carried out at 3 oetrus periods
28. Carmenate and Gamcik (1984)	Glucose-lactose-powdered milk Glucose-powdered-milk-citrate Powdered milk Glucose-citrate-yolk			57.8 61.7 63.8 51.5		
29. Zlatarev (1984)	Golden fleece diluent+yolk	24 hrs	13860	73.8		Number of lambs born per ewe lambing was 1.77
30. Garcia De Alba Reyes (1987)	Yolk-lactose Milk diluent		288 288	33.3 40.2		
31. Espana Garces (1987)		8-12 hrs	154	77, 50 33 & 51		Pregnancies were detected by ultrasonic means 60 days after insemination in 4 groups of ewes.
32. Gokcen *et al.* (1987)	Dried milk with or without added PGF_{2a}		250	36-48		
33. Cordvasoto (1988)	Sodium citrate Milk diluent		137 137	73.5 68.9		

2:4:2. Factors affecting the fertility of chilled ram spermatozoa

Attempts at extending and store ram semen for periods beyond 24 hours have met with little success due to the peculiar nature of ram semen which has been shown to loss fertility rapidly with increase in storage period. Laboratory studies of the motility and morphology of chilled ram semen have not revealed any changes in the spermatozoa that could be specifically associated with the rapid loss in fertility of the spermatozoa. The capacity to fertilise appears to have be lost long before any defect in morphology and motility can usually be detected (Martin and Richardson, 1979). Several factors, other than biophysical changes in the spermatozoa during storage have been reported to greatly influence the fertility of chilled ram semen. These include length of storage dilution, dilution rate, type of diluent and technique of inseminating the ewes viz site of semen deposition, timing of of insemination, volume of inseminate and number of spermatozoa per insemination dose. The results of studies carried out on the extent of the influence of these factors on the fertility of the spermatozoa are reviewed in this section.

2:4:2 a. Effect of length of storage

Under normal liquid storage conditions, ram semen cannot last longer than 1-2 days after collection without showing loss in fertilising capacity. Generally conception

rates obtained with ram semen stored for over 24 hours have been unsatisfactory. Fertility of the semen generally declines with storage although good motility may be maintained. However, the extent to which the fertility is lost depends on the type of diluent. Yoshioka *et al.* (1951) reported that in an "RH" solution, and boric acid-sodium bicarbonate diluent, ram semen maintained its fertility for up to 168 and 144 hours respectively. Investigations carried out using egg yolk diluents have also shown similar trends of decline in fertility of the spermatozoa with storage. Koger (1951) reported that storage of semen in either egg yolk-citrate or egg yolk-phosphate diluents for 24 hours resulted in a significant drop (11%) in fertility per day over a period of 5 days (from 53 to 0%). On the other hand Aamdal and Hogset (1955) reported that ram semen stored for 24 hours at 4°C in yolk-citrate diluent gave 22 pregnancies out of 64 ewes inseminated. However, the inclusion of glycine in this diluent reduced the conception rate to no pregnancy in 19 ewes. Filimon *et al.* (1956) reported a large decline in fertility with increase in storage period (20-30% in 24 hours), when either yolk-citrate or milk diluents were used. Dauzier (1956) obtained low conception rates varying from 6-25% after inseminating ewes with semen stored for 12-24 hours at 2°C-4°C in either egg yolk-citrate or egg yolk-phosphate diluents. By adding antibiotics and glucose to the egg yolk-citrate and saturating with carbon dioxide,

Beljakov (1959) obtained no decline in fertility of ram spermatozoa following storage for up to 30 days. Ozkoca (1961) reported that the conception rate to first insemination ranged between 65.1 and 71.4% where both penicillin and streptomycin were added to the egg yolk-citrate-glucose diluent. Salamon and Robinson (1962b) obtained a linear regression of percentage fertility of ram spermatozoa on duration of storage in both egg yolk-citrate and cow milk diluents. These authors concluded that the maximum duration of storage of ram semen in either of the 2 diluent was 24 hours, and that even this would probably result in about 15% loss of fertilising ability of the spermatozoa.

2:4:2b. Effects of dilution and dilution rates

The effects of dilution on the fertility of semen have been studied most extensively most farm animal species. The dilution effect in bull semen is most often seen as an inactivation of the spermatozoa, especially when the semen is diluted at higher rates. This effect has also been reported in ram semen and occurs irrespective of the diluent used (Blackshaw, 1953; Emmens and Blackshaw, 1956). The rapid inactivation of the spermatozoa during dilution has been associated with leaking out of materials from the sperm cells. There appears to be little evidence linking this to the reduced fertility of diluted semen. However, Graham *et al.* (1970) proposed that the amount of intracellular

materials, especially enzymes, left in the cells after such losses could be very important to the potential fertilising ability of boar spermatozoa. Gokhale (1958) asserted that better conception results could be obtained with semen extended in diluents that caused minimum leakage of the intracellular materials.

A number of reports have inferred that ram semen may be diluted without loss of fertilising ability. However, comparison of insemination results with diluted and undiluted semen suggests that dilution causes a significant loss of fertility. The effect is further compounded when the semen is cooled and stored at 5°C. Keast and Morley (1961) reported that dilution caused about 13% decline in fertility. Jones *et al.* (1962) also reported that lambing rate following inseminations with undiluted semen was 46.2%, but that after dilution, this was lowered to about 26.2%. A similar drop in fertility of ram semen occurred when cooled to 5°C. Storage at this temperature further lowered the fertility (Martin and Richardson, 1979).

While it is accepted that dilution of ram semen results in the lowering of its fertilising ability, reports have shown that the rate of dilution is very important in determining the extent to which dilution affects the fertilty of spermatozoa. Most reports seem to agree that a 1:1 or 1:2 (v/v, semen:diluent) dilution rate is the most that ram semen can tolerate without a severe drop in

fertility. But some authors have claimed to have obtained satisfactory results with higher dilution rates. Forinstance, Kardymowicz (1953) reported inseminating an average of 389 ewes with one ejaculate, and a maximum of 615. The mean conception rate was 51%. While Lunca (1964) reported that dilution rates of up to 1:6 resulted in conception rates to single inseminations ranging from 50-90%. Salamon and Robinson (1962a) had earlier reported that dilution of ram semen 1:4 did not affect the fertility (this gave similar results to undiluted semen). Pavlovic and Varadin (1968) reported that a dilution rate of 1:10 in diluents containing 10% skim milk gave the best results compared to when other diluents were used. Lopyrin and Manilov (1967) claimed that all ewes inseminated with ram semen diluted 1000x with glucose-phosphate or sodium chloride, conceived. However, Watson and Martin (1976) reported a significant decline in non-return rates following dilution of ram semen 10- to 30-fold in a buffered glucose-saline solution containing 1.5 or 6% v/v egg yolk. This resulted in a significant decline in non-return rates.

Some authors have associated the loss of fertility of diluted ram semen with reduced number of spermatozoa in the inseminate. Koger (1951) reported that for a conception rate of over 50%, the spermatozoa number in the inseminate should not fall below 50 million. Similarly, Salamon and Robinson (1962a) reported that provided the number of the spermatozoa

did not fall below 50 million per inseminate, a 1:4 dilution rate did not have any effect on the fertility of ram semen. Lopyrin and Manilov (1967) obtained conception rates of 88.9% with 150 million spermatozoa per inseminate in glucose-phosphate compared to 75% in a control diluent. However, there was a significant lowering of the conception rates in both diluents when the spermatozoa number was reduced to about 30 million per insemination volume. Further poor results were recorded when spermatozoa doses of about 15 million were used. From these reports it appears that for good conception rates (over 50%) to be obtained, the number of spermatozoa inseminated should not fall below the 50 million level given by Koger (1951) and Salamon and Robinson (1962a). Also, the effect of dilution on the fertility of ram semen depends largely on the initial number of the spermatozoa in the ejaculate before the dilution. It has been suggested that this initial spermatozoa concentration should be taken into account during dilution to avoid the adverse effect of dilution processes on the fertility of the spermatozoa.

2:4:2 c. <u>The influence of time of insemination</u>

The importance of accuracy in the timing of insemination for successful conception of ewes has been stressed (Salamon, 1972; Moss *et al.*, 1979). Maximum fertility will be realised when viable spermatozoa are found in sufficient numbers at the proper site in the female tract, and at the optimum time

in relation to ovulation. This optimum time is critical because of the poor viability of the ovine egg after ovulation. Estimates of its fertile life span range from 12-24 hours (Dauzier and Wintenberger, 1952; Thibault, 1967). The ovum rapidly loses its fertility upon reaching the isthmus, and is completely infertile after reaching the uterus (Terrill, 1975). Fertility failures following inseminations is partly due to improper timing.

Establishment of sufficient numbers of spermatozoa in the fallopian tube before the arrival of the ovum is important. However, this depends on the rate of transportation of the spermatozoa in the ewe's reproductive tract. Estimates of the time taken by the deposited spermatozoa to reach the middle of the fallopian tube vary from a few minutes to 8 hours (Robinson, 1959). The efficiency of spermatozoa transportation diminishes in late oestrus. Insemination late in the oestrus resulted in poor lambing rates (Sinclair, 1957; Dunn and Restall, 1961), due to poor establishment of sufficient spermatozoa in the cervix, uterus and fallopian tubes. Increased resistance of the cervical mucus to spermatozoa penetration during oestrus could be responsible for these effects (Mattner and Braden, 1969). Robinson (1973) also observed that penetration of the cervix by spermatozoa and establishment of a cervical spermatozoa populaton was related to the nature of the cervical mucus. It is not certain whether the mucus score

gives a time scale of oestrus or whether ewes display for a considerable period a particular type of mucus or possibly certain mucus types that may be correlated with a receptive female reproductive tract (Restall, 1961). However, there seems to be a relationship between fertility and mucus score in the reproductive tract of the female. Keast and Morley (1961) observed 3 types of vaginal mucus in ewes - copious clear glairy, thick creamy (said to be characteristic of termination of oestrus), and sparse sticky mucus - with little lubricating values (associated with off season or early stages of oestrus). Out of 21 ewes inseminated at the second stage (creamy mucus stage) only 5 concieved, while out of 10 ewes mated to rams 4 conceived. These results suggest that insemination of ewes showing the creamy type of mucus was less effective than of ewes showing the clear glairy mucus type. Although it has been shown that ovulation occurs about the termination of oestrus, it is possible that this type of mucus occurs well after ovulation so that the chances of fertilisation may further be reduced.

Mattner (1968) suggested possible association of the poor results obtained following late insemination of female farm animals with the loss of spermatozoa through phagocytosis by leucocytes. The number of leucocytes was observed to increase in the reproductive tract during late oestrus.

2:4:2 d. Effects of oestrus synchronisation

Fertility following artificial insemination of ewes in which oestrus has been synchronised by means of progestagen treatment are generally lower than that obtained with untreated ewes. This is more so when diluted semen is used. The initial use of intravaginal sponges resulted in apparently normal fertility following insemination with 0.2 ml of undiluted semen, or with natural mating (Robinson, 1965). But other subsequent investigations have shown that while fertilisation rates measured in oestrus synchronised ewes after insemination with undiluted semen have remained within a fairly high range (Allison and Robinson, 1971), insemination with diluted semen on the other hand, have resulted in very low conception rates, and subsequent lambings (Moore *et al.*, 1967; Robinson amd Moore, 1967).

Hancock (1962) postulated that an alteration in the pattern of distribution of spermatozoa in the female reproductive tract, hence the number available for fertilisation, might be responsible for the lowered fertility often observed in progestagen treated sheep and cattle. Experimental evidence for this was presented by Quinlivan and Robinson (1967). They observed that the pattern of sperm recovery from the fallopian tubes of the ewes was altered at the post withdrawal oestrus following sychronisation with intravaginal sponges impregnated with a synthetic fluoro-progestagen (17a-acetoxy-9a-thioro-11B-hydroxypregn-4-

ene-3,20-dione, Cronolone, Searl). This means that the relatively poor conception rates of progestagen treated ewes inseminated may be attributed to the reduced chances of large number of spermatozoa entering the cervix. These group of ewes (progestagen treated) were said to have an intrinsic problem of spermatozoa transportation and survival (Quinlivan and Robinson, 1967). Preliminary observations by Smith and Allison (1971) indicated that the difference in the pattern of production of cervical mucus might be implicated in the poor transportation of the spermatozoa in the genital tract of the progestagen treated ewes. There is an earlier peak production of the cervical mucus in such ewes compared to normal animals. Davies and Dun (1957), and Robinson (1958) had earlier suggested that this might be due to an early onset of oestrus relative to the time of ovulation.

Moore *et al.* (1967), and Robinson and Moore (1967) reported that dilution rate and number of spermatozoa in an inseminate were more critical for ewes in which oestrus was controlled than ewes in natural oestrus. Allison and Robinson (1971) reported observing a linear relationship between increasing spermatozoa concentration per inseminate and increase conception rates in progestagen treated ewes. Emmens and Robinson (1962) and Salamon and Robinson (1962) also reported obtaining good conception rates in treated ewes when semen was diluted 1:4 (v/v, semen:diluent) and spermatozoa concentration of between 50-150 x 10^6 were used. The

observations of these investigators seem to suggest that to obtain goog conception rates in progestagen treated ewes, low dilution rates and high spermatozoa concentrations per insemination dose should be used.

2:4:2e. Effects of site of semen deposition

The fertility of stored semen is significantly influenced by the site of its deposition during insemination. The cervix has remained the usual site for semen deposition because the small size and complexity of the cervical canal rarely makes it possible for a normal insemination pipette to be passed through the cervix of the ewe (Inskeep, 1974). The cervix constitutes an initial barrier to the ascent of the spermatozoa into the uterus. Thus few spermatozoa normally succeed in entering the uterus to effect any fertilisation, hence the low fertility. Attempts at deeper penetration of the cervix have resulted in increased fertility of stored semen, while deposition of semen directly into the uterus has given even higher conception rates. Within the cervix, depositing the semen at a site 0.5 cm deeper than normal resulted in a significant increase in the conception rate (Boyarskii, 1986). Koger (1951) observed no difference in conception rates between inseminations carried either in the vagina or at the cervix. Tjupic (1959) reported that though vaginal insemination resulted in good conception rates, the cervical insemination was superior. Kerton *et al.* (1984) reported conception rates of 56%, 53% and 31% following

inseminations at the uterus, cervix or vagina. Tervit *et al.* (1984) reported obtaining similar differences in conception rates following inseminations at the uterus, cervix or vagina. Direct inseminations onto the ovaries resulted in very poor conception rates. Tervit (1985) observed that the percentage of ewes lambing was greater after intra-uterine insemination followed by deep cervical and cervical inseminations in that order.

The noticeable increases in fertilization rates following insemination at greater depths in the ewe's reproductive tract may be attributed to the establishment of a larger spermatozoa number at the site of fertilization. Despite the advantages of inseminating ewes using the intra-uterine techniques, widespread application of the method is limited by its complexity.

SECTION 2:5 STUDIES ON RAM SEMEN PRESERVATION IN NIGERIA

2:5:1 Survival of stored ram spermatozoa

Much of the research work reviewed in the previous sections were carried out mainly on the exotic breeds such as the Merino and Karakul. Little concerted effort has been made on the artificial insemination of the four main breeds of sheep in Nigeria (Yankasa, Balami, Uda and West African Dwarf sheep). The Yankasa breed seems to have been given more attention in this regard than the other three. In a preliminary trial, Osinowo (1982) compared artificial insemination and natural mating using fresh semen diluted in Tris-glucose-yolk diluent. Overall, natural mating gave a significantly higher lambing rate than artificial insemination (88.9 v 59.4%). In a similar study involving the use of heated cow milk as diluent, Odaibo (1990) reported that dilution of ram semen in cow milk resulted in significantly higher conception rates than when Tris diluent was used (76.7 v 46.7% respectively). The lambing rates also followed the same pattern with the milk diluent giving 46.7% compared to 26.7% for Tris diluent.

Studies involving various methods of semen preservation have yielded contradictory results. In a study on ambient temperature storage of ram semen Pano (1983) observed that based on motility of the spermatozoa after storage, Illinois Variable Temperature diluent (IVT) offered better protection than Tris diluent. Better spermatozoa survival was also

observed when Carbon dioxide gas was bubbled in both diluents. There was no consistent pattern of response of sperm to dilution rates. In the IVT diluent, there was a decrease in motility with increase in dilution rate up to 1:6 (v/v, semen:diluent), while in Tris-glucose diluent, motility remained high even at higher dilution rates. Addition of either glucose or fructose as sources of energy in Tris diluent resulted in significant increases in spermatozoa survival with glucose giving the best results (34.8 v 29.3% for Tris-glucose and Tris-fructose diluents respectively). Under these conditions, the author observed that ram semen could last for only 2 days. In a more recent study on ambient temperature storage of ram semen, Butswat (1989) observed that ram spermatozoa were better preserved under a flow dialysis method than in McCartney bottles over a storage period of 10 days (45 v 31.4%) with the best results obtained over a 4-day storage period (73 v 66%). With both continuous and discontinuous flow dialysis techniques, the use of low semen volume in the dialysis bags (0.5 and 1.0 ml) resulted in better sperm survival rates.

Formaldehyde treatment of semen as a method of spermatozoa preservation has been experimented with. Osinowo *et al.* (1982) observed that apart from the reversible inhibition of spermatozoa motility and metabolism, formal-saline had an added advantage of stabilizing and maintaining the integrity of the spermatozoa membranes. The authors

reported that recovery rate decreased with increased dilution when 0.01% formaldehyde was used. While the pre-wash incubation temperature had no significant effect on the motility of the spermatozoa, it had a significant effect on the proportion of eosinophilic spermatozoa. Osinowo *et al.* (1988) reported that of the two methods of washing formaldehyde treated ram semen - Centrifugation, aspiration of supernatant and resuspension of the sperm pellet in a simple medium and Centrifugation through a dense medium to wash the spermatozoa free of seminal plasma - the ficoll washing method caused loss of sperm motility although it still maintained the plasma membrane integrity and viability after resuspension in diluents containing egg yolk or phospholipids. In a similar study Onipe (1989) reported that Tris diluent was a better post-wash diluent than Phosphate Buffered Saline (PBS) and Citrate diluents. Inclusion of glucose in the pre-wash diluent resulted in superior motility recovery after 6 hours of storage.

In their review, Osinowo *et al.* (1988) reported that repeated washing of ram semen is associated with biochemical changes similar to those due to dilution effect. The severity of the cellular damages increased with the efficiency of washing regardless of the type of diluent used - although hypertonic media resulted in less cell damage compared with hypotonic media. Enzymes are generally lost during washing and there is also agglutination, increased eosin uptake and

depressed motility as well as ultr-structural alteration as a result of leakage of proteins and other low molecular weight substances from the sperm cells. Osinowo (1981) had earlier reported that the effect of temperature on enzyme leakage patterns may be due to its effect on diffusion processes generally and membrane structure.

Except for the work of Ahmed (1989) there is no reported work on deep freezing of ram semen in Nigeria. In his study involving a comparative evaluation of cryoprotective compounds for deep freezing ram semen, the author reported that the viability of spermatozoa during freezing was influenced by the concentration of the cryoprotective compound. The optimum concentration being 0.5 M at a dilution rate of 1:4 (v/v, semen:diluent). The best cryoprotectants were glycerol, erythritol and ethylene glycol. The author also reported that the maintenance of acrosomal membrane integrity was influenced by some cryoprotectants. Damage to the plasma membrane appeared uniform irrespective of the cryoprotective agent used. With chilled method of semen preservation, there seems to be no reported investigation in the country.

2:5:2 Artificial insemination results in sheep

For most of the reported insemination trials in Nigeria, the cervix has remained the usual site of semen deposition. Odaibo (1990) speculated that an insemination dose of 150 x

10^6 fresh spermatozoa in 0.25 ml of milk diluent deposited in the cervix can result in conception rates of about 80% and lambing rates of 70%. Deniran (1990) compared vaginal and cervical inseminations. The author reported that deep cervical insemination resulted in significantly higher conception and lambing rates than did vaginal insemination. Ahmed (1989) also compared cervical and intra-uterine insemination by laparotomy. The results showed that intra-uterine insemination was better than cervical insemination.

Reported results of sheep artificial insemination in Nigeria are scanty and varied. Artificial insemination involving the use of fresh ram semen gave fairly good results (Osinowo, 1982; Odaibo, 1990) although this seems to depend on the type of diluent used. A further improvement in both conception and lambing rates was achieved following deep cervical insemination compared to vaginal insemination (Deniran, 1990). Following ambient temperature storage, the fertility of ram semen declined significantly over the storage period: 55.6, 44.4 and 27.8% for 0, 1 and 2 days of storage (Butswat, 1989). Insemination doses of either 250 or 500 x 10^6 spermatozoa in 0.25 ml of inseminate did not differ significantly in terms of conception rates (44.4 v 40.7%). The lambing rates also followed the same pattern as the conception rates. Ahmed (1989) obtained a generally poor result following insemination with frozen ram semen.

considerably good conception and lambing results, the need to transport the semen from the processing centres to the farm and the needs for short, medium and even long-term storage have given rise to studies in the methods of preservation. Some of the methods of preservation experimented with in this country, e.g. the ambient temperature preservation by flow dialysis techniques (Butswat, 1989) and deep freezing (Ahmed, 1989) have resulted in good spermatozoa survival. But large scale adoption of the methods has been limited by the high cost of the equipment involved. Thus the need for other cheaper and less complex methods has been stressed. The present study was designed to investigate the possibility of improving chilled storage as an alternative to the other methods so far examined.

PART II

EXPERIMENTAL WORK

Chapter 3: Materials and Methods

Chapter 4: Results

Chapter 5: Discussion

CHAPTER 3

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

Semen

Semen was collected by means of artificial vagina from yearling Yankasa rams. The number of rams used varied with the experiment and is stated under each experiment below.

Chemicals used

All chemicals used in the preparation of the diluents in this study were analytical grade except where otherwise stated. These are given in Table 1 below.

Table 2 : List of chemicals used in diluent preparation

Name of chemical	Mol. Wt.	Manufacturer
D-Glucose	180.16	BDH Ltd Poole, England
Tris(Hydroxymethylaminoethane)	121.14	Fisher Sc. Co USA
Tri-Sodium citrate	294.10	BDH Ltd Poole, England
Citric acid monohydrate	210.14	BDH Ltd Poole, England
Fructose	180.16	BDH Ltd Poole, England
Ethylene diamine tetra acetate, di-sodium salt	372.24	May-Baker Ltd England
Sodium penicillin G	---	Galenika, Yugoslavia
Streptomycin sulphate	---	Remed. S.A (Bruxelles)

Diluents

Four (4) types of diluents were used in this study. These are Tris (hydroxymethylaminoethane) based diluent (Salamon and Robinson 1972), Milk diluent (Salamon and Robinson, 1962a), Egg yolk-glucose-citrate (Salamon and Robinson, 1962b) and Tris-EDTA-fructose diluent (Visser and Salamon 1974).

All the diluents were made up in 100 ml volumetric flask without warming. The diluents were sealed in polythene bags and kept in a freezer at -18°C until needed. The original compositions of the diluents given by the above investigators are listed in Table 3 a,b,c and d. The original compositions were altered where necessary for experimental purposes.

Table 3: Composition of semen diluents

a) *Tris diluent (Salamon and Robinson, 1972)*

Component	Concentration / 100 ml
Tris (hydroxymethylaminoethane)	300 mM
Citric acid	94.7 mM
D-Glucose	27.75 mM
Egg yolk	15%,v/v
Sodium penicillin G	100,000 i.u
Streptomycin Sulphate	0.1 g
Distilled water	made up to 100 ml

b) Egg yolk-glucose-citrate diluent (Salamon and Robinson, 1962 b)

Component	Concentration / 100 ml
Tri- Sodium citrate	2.9%
D-Glucose	27.75 mM
Egg yolk	15%, v/v
Sodium penicillin G	100,000 i.u
Streptomycin Sulphate	0.1g
Distilled water	made up to 100 ml

c) Milk diluent (Salamon and Robinson, 1962 a)

Component	Composition / 100 ml
Egg yolk	15%, v/v
Sodium penicillin G	100,000 i.u
Streptomycin Sulphate	0.1g
Fresh heated cow milk	made up to 100 ml

d) Tris-EDTA-fructose diluent (Visser and Salamon, 1974)

Component	Composition / 100 ml
Tris	250 mM
Fructose	111 mm
EDTA	15 mM
Citric acid	79.5 mM
Sodium penicillin G	100,000 i.u
Streptomycin Sulphate	0.1g
Egg yolk	15%, v/v
Distilled water	made up to 100 ml

Semen processing and storage

Semen samples were analysed for initial spermatozoa motility and concentration. The motility was estimated by observing a drop of semen on a glass slide with cover slip under a microscope at 400 x magnification. Only samples with 85-95% motility were used for the experiments. The spermatozoa concentration was determined by photoelectric method using a spectrophotometer (Beckmann, Model C-400), calibrated against haemocytometer counts at 600 nM wavelength. Average spermatozoa concentration in the semen samples used was in the range of 2.5-3.0 x 10^9 / ml.

Semen dilution and storage was done in coded glass test tubes placed in a water bath at 30°C. A dilution rate of 1:4 (v/v, semen:diluent) was used in all the experiments. The diluted semen samples were transfered into beakers containing some of the water from the water bath and stored in a refrigerator set at 5°C. At the end of each period, replicates of the stored semen were taken out and warmed in a water bath at 37°C for 30 minutes before examining them for post storage motility estimation.

Oestrus detection

Ewes between the ages of 2 and 5 years were checked twice daily for oestrus at 0800-0900 and 1500-1600 hours using vasectomized rams. A total of 240 ewes were used in this study.

Insemination

Inseminations were carried out 24 hours after oestrus detection. Two (2) types of insemination pipettes were used:

1. Normal straw insemination pipette.
2 A modified version of the straw pipette tapered at one end and fitted with a micro-pipette tip for easy penetration of the cervix.

The cervix was located during insemination by means of a self illuminating vaginoscope. Each ewe was inseminated with 0.2 ml of either fresh or stored semen containing approximately 250 x 10^6 spermatozoa.

Non-return to oestrus check

Inseminated ewes were checked for non-return (NR) to oestrus using vasectomized rams from 12-21 days after insemination. Ewes not returning to oestrus during this period were assumed to be pregnant.

Lambing rate check

Lambing rates were determined by observing the actual number of ewes lambing about 150 days after insemination.

Experiments

Experiment 1

This was a 3 x 3 x 5 factorial experiment which compared the effects of 3 types of diluents (Tris v egg yolk-glucose-citrate v Milk) and storing for 5 periods (0 v 2

v 4 v 6 v 8 days) on the survival of ram spermatozoa during chilled storage. Semen was collected from 3 Yankasa rams and used as replicates.

Experiment 2

This was of a 2 x 2 x 3 x 4 factorial design. The effects of adding 2 types of sugar (glucose v fructose) at 2 levels (0 v 27.75 mM) as sources of energy in milk-yolk diluent and storing for four periods (o v 2 v 4 v 6 days) on the survival of chilled ram spermatozoa were compared. Replicates of the treatments were made up from semen collected from 3 yearling Yankasa rams.

Experiment 3

This was a 3 x 4 x 5 factorial experiment. The effects of varying the levels of glucose (0 v 13.875 v 27.75 v 41.625 v 55.5 mM) in milk-yolk-glucose diluent and storing for four (4) periods (0 v 2 v 4 v 6 days) on the survival of ram spermatozoa during chilled storage were compared. Semen collected from 3 yearling Yankasa rams was used as replicates.

Experiment 4

In this experiment (3 x 4 x 5 factorial) the effects of 3 types of diluents (Tris v Milk v Tris-Milk) and storing for 4 periods (0 v 2 v 4 v 6 days) on the survival of ram spermatozoa during chilled storage were examined. Semen

collected from 5 yearling Yankasa rams were used as replicates for the treatments.

Experiment 5

In this 4 x 4 x 5 factorial experiment the effects of adding 5 levels of ethylene diamine tetra acetic acid di-sodium salt (EDTA) (0 v 5 v 10 v 15 v 20 mM) to Tris-glucose diluent and storing for 4 periods (0 2 v 4 v 6 days) on the survival of ram spermatozoa during chilled storage were examined. Semen was collected from 4 yearling Yankasa rams.

Experiment 6

This was also a 4 x 4 x 5 factorial experiment that examined the effects of adding 5 levels of EDTA (0 v 5 v 10 v 15 v 20 mM) to Milk diluent and storing for 4 periods (0 v 2 v 4 v 6 days) on the survival of ram spermatozoa during chilled storage. Semen was collected from 4 yearling Yankasa rams.

Insemination experiment

This was a 2 x 2 x 2 factorial. The effects of the following factors on the fertility of chilled ram semen were examined: Type of diluent (Tris v Tris-Milk), storage period (0 v 2 days) and depth of insemination (cervical v deep cervical). A total of 240 ewes divided into 8 groups of 30

each were used as replicates for the treatments. Each ewe was inseminated with 0.2 ml fresh diluted or stored semen containing approximately 250 x 10^6 spermatozoa.

Data analysis

Data from the *in vitro* experiments (experiments 1-6) were analysed using Standard Analysis of Variance following angular transformation of the data. In the insemination experiment Chi-Square analysis were used to test the relevant main effects.

CHAPTER 4

RESULTS

Chapter 4

RESULTS

Experiment 1

The result of this experiment is summarized in Tables 4 and 5. Type of diluent significantly ($P<0.001$) affected the survival of ram spermatozoa during storage. Spermatozoa were better preserved by Tris diluent than by Egg yolk-glucose-citrate and Milk diluents (43.0 v 37.7 v 27.3% respectively). Spermatozoa motility rapidly declined with increase in storage period (81.1 v 57.8 v 27. 8 v 8.9 v 4.5% for 0, 2, 4, 6 and 8 days of storage respectively). There was a significant ($P<0.001$) interaction between type of diluent and storage period.

Table 4: Summary of analysis of variance: Effects of type of diluent and length of storage on the survival of chilled ram spermatozoa (Experiment 1)

source	D F	M S
Diluent (D)	2	951.67***
Storage period (SP)	4	9692.22***
D X SP	8	198.89***
Error	30	20.56

*** $P<0.001$

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Table 5: Effects of type of diluent and length of storage on the survival of chilled ram spermatozoa (Experiment 1)

	Storage Period (days)					
Diluent	0	2	4	6	8	Mean
Tris	81.7±3.3*	61.7±3.3	48.3±1.7	15.0±2.9	8.3±1.7	43.0
EYGC	81.7±3.3	60.0±2.9	30.0±2.9	11.7±1.7	5.0±0	37.0
Milk	80.0±5.0	51.7±3.3	5.0±0	0	0	27.8
Mean	81.1±3.9	57.8±3.2	27.7±2.3	8.9±2.3	4.3±1.7	

* % motile spermatozoa (Mean±S.E)

Experiment 2

The result of this experiment is summarized in Tables 6 and 7. The addition of either glucose or fructose to the Milk diluent significantly (P<0.001) improved the survival of of ram spermatozoa during chilled storage. Addition of glucose gave better results compared to fructose. Overall mean percentage motile spermatozoa were 45.4 v 36.3% for glucose and fructose diluents respectively compared to 31.3% for milk diluent without any sugar. There was a general decline in percentage motile spermatozoa with increase in storage period (86.3 v 38.3 v 10.8 v 8.8% for 0, 2, 4 and 6 days respectively). There were significant interactions between sugar and level, and between level of sugar and length of storage (P<0.001).

Table 6: Summary of analysis of variance: Effects of type and level of sugar and length of storage on the survival of chilled ram spermatozoa (Experiment 2)

Source	D F	M S
Sugar (S)	1	119.42***
Level (L)	1	682.90***
Storage period (SP)	3	6923.49***
Sugar X Level	1	127.84***
S X SP	3	12.29ns
L X SP	3	64.16***
S X L X SP	3	8.94ns
Error	32	6.01

*** P<0.001, ns = Non-significant.

Table 7: Effects of type and level of sugar and length of storage on the survival of chilled ram spermatozoa (Experiment 2)

Sugar	Level (mM)	Storage Period (days) 0	2	4	6	Mean
Glucose	0	86.7±1.7*	28.3±3.3	5.0±0	5.0±0	31.3
	27.75	90.0±2.9	58.3±1.7	18.3±1.7	15.0±0	45.4
Fructose	0	85±0	28.3±1.7	6.7±1.7	5.0±0	31.3
	27.75	83.3±3.3	38.3±1.7	13.3±1.7	10.0±0	36.3

* % motile spermatozoa (Mean±S.E)

Experiment 3

The result of this experiment is summarized in Tables 8 and 9. There was significant increase (P<0.001) in spermatozoa survival with increase in the level of glucose used up to 27.75 mM but beyond this, there was a significant

decline in sperm motility (30.9 v 35.9 v 40.8 v 37.9 v 39.4% for 0, 13.875, 27.75, 41.625, and 55.5 mM respectively). Increase in the length of storage also resulted in a significant ($P<0.001$) decline in spermatozoa survival rates (82.4 v 45.3 v 5.7% for 0, 2, 4 and 6 days storage respectively). There was a significant ($P<0.05$) interaction between type of diluent and length of storage.

Table 8: Summary of analysis of variance: Effects of levels of glucose in milk diluent and length of storage on the survival of chilled ram spermatozoa (Experiment 3)

Source	D F	M S
Glucose level (GL)	4	156.55***
Storage Period (SP)	3	8222.62***
GL X SP	12	21.91*
Error	40	8.35

* $P<0.05$, *** $P<0.001$

Table 9: Effects of level of glucose in milk diluent and length of storage on the survival of chilled ram spermatozoa (Experiment 3)

Glucose level (mM)	Storage Period (days) 0	2	4	6	Mean
0	81.7±1.7	35.0±2.9	5.0±0	1.7±1.7**	30.9
13.875	81.7±1.7	43.3±1.7	11.7±1.7	6.7±1.7	35.9
27.75	85.0±0	48.3±1.7	20.0±2.9	10.0±2.9	40.8
41.625	81.7±1.7	50.0±2.9	15.0±0	5.0±0	37.9
55.5	81.7±1.7	51.7±1.7	20.0±2.9	5.0±0	39.4
Mean	82.4	45.7	14.3	5.7	

** % motile spermatozoa (Mean± S.E)

Experiment 4

A summary of the analysis of the result of this experiment is given Tables 10 and 11. There was a significant (P<0.001) effect of type of diluent on the survival of ram spermatozoa during chilled storage. Tris-Milk diluent containing 50% Tris and 50% Milk diluents was intermediate between Tris and Milk diluents in preserving the spermatozoa during storage. Mean percentage motile spermatozoa were 53.0 v 46.3 v 38.3% for Tris, Tris-Milk and Milk diluents respectively. Increase in the length of storage also resulted in a significant decline in motility (85.0 v 52.7 v 30.0 v 15.3% for 0, 2, 4 and 6 days storage respectively). Significant (P<0.001) interaction between type of diluent and length storage was also observed.

Table 10: Summary of analysis of variance: Effects of type of diluent (Tris v Tris-Milk v Milk) and length of storage on the survival of chilled ram spermatozoa (experiment 4)

Source	D F	M S
Diluent (D)	2	662.42 ***
Storage Period (SP)	3	5962.89 ***
D X SP	6	106.97 ***
Error	48	15.37

*** P<0.001

Table 11: Effects of type of diluent (Tris v Tris-Milk v Milk) and length of storage on the survival of chilled ram spermatozoa (*Experiment 4*).

	Storage Period (days)				
Diluent	0	2	4	6	Mean
Tris	86.0±2.5**	57.0±2.6	42.0±2.0	27.0±1.2	53.0
Milk	83.0±1.2	48.0±2.6	18.0±2.0	4.0±1.9	38.3
Tris-Milk	86.0±2.5	53.0±2.0	31.0±1.9	15.0±1.6	46.3
Mean	85.0	52.7	30.0	15.3	

** % motile spermatozoa (Mean± S.E)

Experiment 5

Summaries of the analysis of the result of this experiment are given in Tables 12 and 13. There was a significant ($P<0.001$) increase in spermatozoa survival rates with increase in the level of EDTA included in the diluent up to 10 mM but beyond this, EDTA was detrimental to the spermatozoa. Overall mean percentage motile spermatozoa were 45.4 v 49.1.0 v 52.8 v 52.2 v 41.3% for 0, 5, 10, 15 and 20 mM of EDTA respectively. The optimum level of EDTA was 10 mM. Increase in the length of storage period resulted in significant ($P<0.001$) decline in survival rates (87.0 v 54.4 v 33.3 v 17.8% for 0, 2, 4 and 6 days storage respectively). There was a significant ($P<0.05$) interaction between type of diluent and length of storage.

Table 12: Summary of analysis of variance: Effects of level of EDTA in Tris-EDTA-glucose diluent and length of storage on the survival of chilled ram spermatozoa (Experiment 5).

Source	D F	M S
EDTA Level (EL)	4	176.31***
Storage Period (SP)	3	7375.64***
EL X SP	12	26.59*
Error	60	12.75

* P<0.05, *** P<0.001

Table 13: Effects of level of EDTA in Tris-EDTA-glucose diluent and length of storage on the survival of chilled ram spermatozoa (Experiment 5)

Level of EDAT (mM)	Storage Period (days) 0	2	4	6	Mean
0	90.0±2.0*	48.8±2.4	31.3±2.4	11.3±2.4	45.4
5	83.8±1.3	57.5±3.2	33.8±2.4	21.3±2.4	49.1
10	87.5±3.2	62.5±3.2	37.5±1.4	23.8±1.3	52.8
15	90.0±2.9	58.8±2.4	37.5±3.2	22.5±1.4	52.2
20	82.8±2.4	42.5±2.5	28.8±2.4	10.0±2.0	41.3
Mean	87.0	54.0	33.8	17.8	

* % motile spermatozoa (Mean ± S.E)

Experiment 6

Tables 14 and 15 summarize the results of the analysis of this experiment. EDTA was highly (P<0.001) detrimental to spermatozoa survival when added to milk diluent (43.8 v 35.3 v 32.2 v 29.1 v 24.1% for 0, 5, 10, 15 and 20 mM of EDTA

respectively). Also, motility declined significantly (P<0.001) with increase in storage period(90.3 v 33.5 v 6.8 v 1.0% for 0, 2, 4 and 6 days storage respectively). There was a significant interaction between level of EDTA and storage period (P<0.001).

Table 14: *Summary of analysis of variance*: Effects of level of EDTA in milk diluent and length of storage on the survival of chilled ram spermatozoa (Experiment 6)

Source	DF	M S
EDTA Level (EL)	4	764.21 ***
Storage Period (SP)	3	19525.16 ***
EL X SP	12	138.28 ***
Error	60	20.47

*** P<0.001

Table 15: Effects of level of EDTA in milk diluent and length of storage period on the survival of chilled ram spermatozoa.

Level of EDTA (mM)	Storage Period (days) 0	2	4	6	Mean
0	93.8±1.3*	60.0±2.0	18.8±2.4	2.5±1.4	43.8
5	87.5±4.3	40.0±2.9	11.3±2.4	2.5±1.4	35.3
10	91.3±2.4	35.0±3.5	2.5±1.4	0	32.2
15	91.3±2.4	23.8±1.3	1.3±1.3	0	29.1
20	87.5±1.4	8.8±1.3	0	0	24.1
Mean	90.3	33.5	6.8	1.0	

* % motile spermatozoa. (Mean ± S.E)

Insemination Experiment

Non-return rates

The result is shown in figure 1. There was no significant effect (X^2=0.142, df=1, P>0.05) of type of diluent on non-return rates (32.5 v 41.7% for Tris and Tris-milk diluents respectively). Increase in storage period however resulted in a significant (X^2=7.876, df=1, P<0.001) decline in the number of ewes not returning to oestrus (47.5 v 29.5 % for 0 and 1 day storage respectively). However, inseminations carried out deep into the cervix resulted in higher (X^2=6.447, df=1, P<0.001) non-return rates than those carried out at the cervix (45.8 v 28.3% respectively).

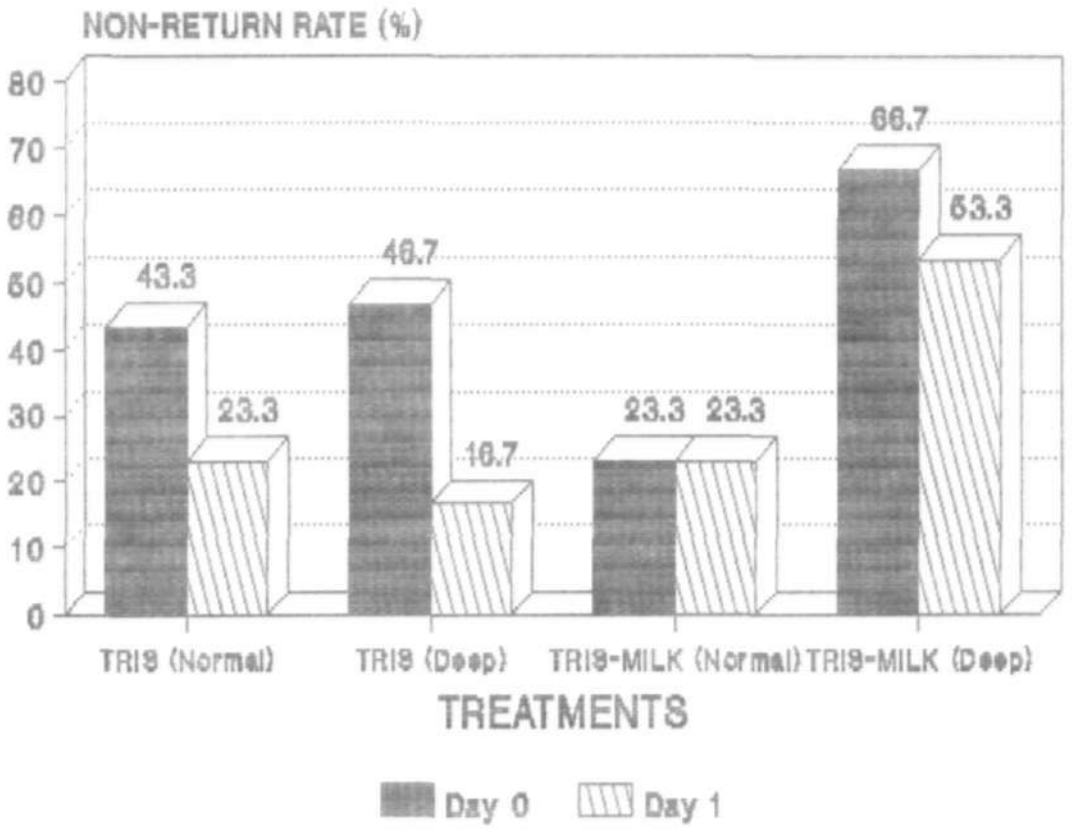


Fig 1. Effect of type of diluent, depth of insemination and storage of chilled ram semen on non-return rates

Lambing rates

The result is summarized in figure 2. The lambing rates also followed the same pattern as the non-return rates. Lambing rates were significantly higher (X^2= 6.35, d.f=1, P<0.001) following deep cervical insemination compared to cervical inseminations (40.0 v 24.2% respectively). They were also significantly higher (X^2=8.32, d.f= 1 , P<0.001) following insemination with fresh diluted semen than with semen stored for 24 hours after dilution (41.7 v 22.5% respectively). There was no significant effect (X^2=1.53, d.f= 1, P>0.05) of type of diluent on lambing rates (36.5 v 27.5% for Tris+Milk and Tris diluents respectively).

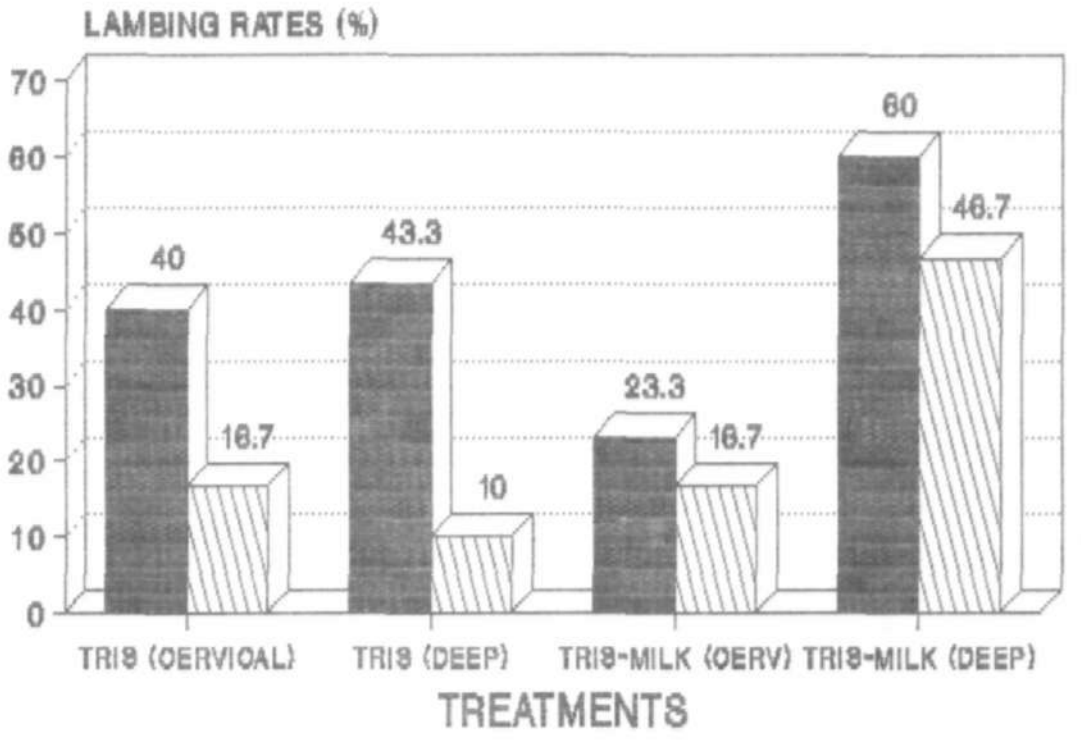


Fig.2. Effects of diluent type, depth of insemination and storage length of chilled ram semen on lambing rates

CHAPTER 5

DISCUSSION

DISCUSSION

The results of these experiments show that for chilled storage of ram semen at 5°C type of diluent and length of storage significantly influenced the survival of the spermatozoa. Fertility of the spermatozoa was also influenced by the length of storage and depth of insemination.

In experiment 1, the observed inferiority of Milk diluent to other diluents especially Tris diluent for storage of ram semen at 5°C agreed with the findings of Salamon and Robinson (1962a) and Carmenate and Gamicik (1984). The interaction of diluent and length of storage was due to the poor performance of the Milk diluent in which spermatozoa survived for only 24 hours of storage. The poor performance of the Milk diluent could be attributed to the absence of suitable sources of energy in it to support the spermatozoa. This assertion was confirmed in experiment 2 in which the addition of either glucose or fructose to the milk diluent resulted in a significant improvement in spermatozoa survival. The better survival of spermatozoa in the glucose-Milk diluent than the fructose containing diluent could be as a result of the preferential utilization of glucose as an exogenous source of energy. Foote (1969) and Salamom and Visser (1973) reported similar findings although fructose is the natural sugar in the seminal plasma of most farm animals including rams. The reason has been attributed

to the greater affinity of glucose to the spermatozoa enzyme hexokinase which is responsible for the phosphorylation of both glucose and fructose (Mann and Mann, 1981).

In experiment 3, the relatively poor spermatozoa survival observed at low glucose concentrations could be attributed to insufficient amounts in the diluent to support the spermatozoa for longer periods. Its detrimental effect at higher levels may be a result of altered physio-chemical properties of the diluent such as osmotic pressure beyond optimal levels. The optimum level of glucose (27.75 mM) obtained in this study is similar to that reported by Foote (1969) and Salamon and Visser (1973).

In experiment 4, it is possible that the better buffering capacity of Tris and the glucose added to Milk diluent were the factors responsible for the observed improved spermatozoa survival compared to Milk diluent. Lapwood and Martin (1972) listed Tris as one of the buffers that had a depressing or sometimes toxic effect on spermatozoa in the presence of milk solids. This effect of Tris was not observed in the present study although percentage motile spermatozoa in the Tris-Milk diluent were lower than in Tris diluent (53.0 v 46.3%) but the difference was not significant.

The beneficial effect of EDTA on the viability of spermatozoa during chilled storage was evident in experiment 5. Visser and Salamon (1974), Babicheva and Morgov (1971) and

Macmillan *et al.* (1972) reported observing a similar beneficial effect of adding EDTA to semen diluent on the survival of the spermatozoa. Macmillan *et al.* (1972) alledged that EDTA protects the spermatozoa against the toxic effects of hydrogen peroxide formed by the oxidation of some amino acids in the semen extender. For deep freezing of ram semen Visser and Salamon (1974) gave the optimum level of EDTA as 15 mM but in the present study involving chilled storage the optimum concentration of EDTA was 10 mM. The beneficial role of EDTA in protecting spermatozoa during storage was not observed when it was included in Milk diluent. Salamon *et al.* (1973) and Visser and Salamon (1974) reported that the protection afforded spermatozoa by EDTA depends on the type and composition of the diluent used: the effect being more pronounced in Tris diluents than other types of diluents.

The significant interaction between type of diluent and length of storage in experiments 1 and 4 were due to the rapid decline in spermatozoa motility in Milk diluent after 1 day of storage compared to other diluents. Similarly the rapid decline in motility at higher glucose and EDTA levels (experiments 3 and 5) accounts for the observed interaction between levels of glucose and EDTA and length of storage period.

Fertility of chilled ram semen, indicated by the rate of non-return of ewes to oestrus and lambing rates obtained in the present study was not significantly affected by type

of diluent. This conflicts with the reports of Schindler and Amir (1973), Zlatarev (1976), Ozkoca (1980) and Espana Garcia (1987). It seems the mixing of 50% Tris and 50% Milk diluents *in this study did not materially affect the fertility of* the spermatozoa to bring about any significant difference in conception rates following insemination compared to when Tris diluent was used. The effect of storage on conception rates observed in the present study agrees with the findings of Salamon and Robinson (1962b), Wiggan and Clark (1967), Zlatarev (1976) and Firth (1976). Fertility of the spermatozoa seems to be lost somewhere between processing and storage of the semen. It is possible that the reported morphological and biochemical changes that occur in the spermatozoa during storage (Gokhale, 1958; Graham *et al.*, 1970) may in one way or the other contribute to the decline in fertility of stored ram spermatozoa. Gokhale (1958) reported observing fertility of semen to be least affected where there was a minimum leaking of materials especially from the spermatozoa into the surrounding fluid. The difference in conception and lambing rates following insemination at the cervix or deep into the cervix agrees with the findings of Tervit *et al.* (1984), Kerton *et al.* (1984) and Boyarskii (1986). These increases in fertility could be attributed to the possibility of more viable spermatozoa reaching the site of fertilization. The cervix has been reported to constitute an initial barrier to the

movement of the spermatozoa into the uterus as a result of which only a few spermatozoa reach the fertilization site. By depositing the semen deep into the cervix, the chances of viable spermatozoa reaching the ovum and fertilising it are increased, resulting in the observed high conception and subsequent lambing rates.

From the results of the present study, it can be concluded that for chilled storage of ram semen, Tris diluent is the best diluent. Its performance is further improved by the addition of 10 mM of EDTA. The performance of Milk diluent for chilled storage of ram semen is improved by the inclusion of either glucose or fructose, glucose being the sugar of choice. The optimum level of glucose that can be added is 27.75 mM. Under storage conditions as provided in this study, chilled ram spermatozoa can last up to 2 days of storage after collection. However, for good fertility the inseminations should be carried out within 24 hours of semen collection and dilution. Further increase in fertility of chilled ram semen can be obtained if inseminations are carried out deep into the cervix.

REFERENCES

REFERENCES

Aamdal,J. and Hogset, I. 1955. Insemination in Sheep:Preliminary Communication *Nord. Vet. Med. Journal.7: 309-314.*

Aamdal, J. and Anderson, K. 1968. Freezing of ram semen in straws. *6th Inter. Congr. Anim. Reprod. and A.I. 2: 971-980.*

Abdelhakean, A., Tony,S.M., Yassen, A.M., and El-Alamy,M.A. 1978. Ram sperm motility aged in glucose and Tris buffered extenders.*Alexandria J. Agric. Res. 26: 301-308.*

Adler, H.C. and Rasbach, N.O 1953. The effect of antibiotics on the fertility of bull semen.4,500*ug* neomycin/ml diluent with special reference to its use in the semen of bulls transmitting *Vibrio foetus. Nord. Vet. Med. 8: 351-356.*

Aguer, D. and Provost, F. Le. 1976. Induction and sychronisation of oestrus in ewes during seasonal induction with FGA impregnated pessaries: Natural mating or artificial insemination. *In: 8th Inter. Congr. Anim. Reprod. and A.I. Krakov (1976) Proceed. 1: Communication Abstract 6.*

Ahmed, M.S. 1989. Comparative evaluation of cryoprotective compounds for deep freezing of ram spermatozoa in Tris diluent. *Ph. D Thesis. Dept. Vet. Surgery and Medicine, Fac. Vet. Med. A.B.U. Zaria.*

Albright, J.L., Erb, R.E., and Ehler, M.H. 1960. Influence of yolk-citrate, yolk-glycine, whole milk and skim milk extenders containing glycerol on motility of bovine sperm at 37°C and 5°C. *J. Dairy Science 43: 250-255.*

Albornoz Bustmante, A. 1952. Analysis of 3 years' experience of artificial insemination of Karakul sheep. *Zootech. Vet. 7:373-376.*

Allison, A.J., and Robison, T.J. 1971. The effets of dose level of intravaginal progestagen on sperm transport, fertilisation and lambing in cyclic Merino ewes. *J. Reprod. Fert. 22: 515.*

Almquist, J.O. 1948. The effect of penicillin upon the fertility of semen of relatively infertile bulls. *J. Dairy Science. 31: 681-682.*

Almquist, J.O. 1951. A comparison of penicillin, streptomicin and sulfanilamide for improving the fertility of semen from bull of low fertility. *J. Dairy Science. 34:819-822*

Almquist, J.O. and Wickersham, E.W. 1962. Diluents for bovine semen XI: Effect of glycerol on fertility and motility of spermatozoa in homogenised milk and skim milk. *J. Dairy Science 45: 911-916.*

Anard, A.S., Hoekstra, W.G. and First, N.L. 1967. Effect of ageing boar spermatozoa on cellular loss of DNA. *J. Animal Science. 26 : 171.*

Anderson, J. 1945. The semen of animals and its use for artificial insemination. *Tech. Commun. Bur. Anim. Breed. Genet. (Edinburgh), No.6: viii and 151.*

Anderson, K.A., Aamdal, J. amd Fouger, J.A. 9173. Uterine and Deep Cervical insemination with frozen semen in sheep. *Zuchthygiene 8: 113-116.*

Austin, C.R. and Bishop, M.W.H. 1958. Capacitation of mammalian spermatozoa. *Nature (London): 181, 185.*

Babicheva, L.Ye. and Mozgov, G.M. 1971. Comparative estimation of the protection afforded boar semen by disodium ethylene diamine tetra acetate (EDTA). *Dokl. vses Aked. Sel'-khoz Nauk. VI. Lemina (1971): 9: 37-38.*

Baker, R.D. and Degen, A.A. 1972. Transport of live and dead boar spermatozoa within the reproductive tract of gilts. *J. Reprod. Fert. 28: 369.*

Balashov, N.G. and Evysukov, M.E. 1971. Influence of different sugars added to diluents on the survival of bull spermatozoa stored at temperatures above 0°C. *Trudy Gosudarstven'nogo Nauchokontro'nogo Instituta Veterinarynykh preparatov (1971). 17: 343-346.*

Beljakov, S.P. 1959. The storage of bull and ram semen at high atmospheric temperatures. *Veterinarija 36(1): 78-81.*

Benedetti, E.L. and Emmelot, P. 1968. Structure and function of plasma membrane isolated from liver. *In: The Membrane. (Eds. Dalton, A.J. and Hagnanan, F) Academic Press.N.Y. and London: 33-100.*

Bishop, M.W.H. and Salisbury, G.W. 1955a. Effect of dilution with saline and phosphate solutions on oxygen uptake of bull semen. *Amer. J. Physiol. 181: 114-118.*

Bishop, M.W.H. 1959. Spermatozoa motility. *Physiology Review. 43: 1-59.*

Blackshaw, A.W. 1953. The motility of ram and bull spermatozoa in dilute suspension. *J. Gen. Physiol. 36: 449-462.*

Blackshaw, A.W. 1954. The prevention of temperature shock of bull and ram semen. *Austr. J. Biol. Science. 7: 573-582.*

Blackshaw, A.W. 1958a. The effects of incubation temperature and cold shock on the metabolism of ram spermatozoa. *Austr. J. Biol. Science. 14: 581-588.*

Blackshaw, A.W. 1960. The effects of pH, tonicity and temperature of glycerolisation on the revival of ram and bull spermatozoa after freezing at -79°C. *Austr. Vet. Journal. 36: 376.*

Blackshaw, A.W. and Emmens, C.W. 1951. The interactions of pH, osmotic pressure and electrolyte concentration on the motility of ram, bull, and hen spermatozoa. *J. Physiology. 114: 16-26.*

Blackshaw, A.W. and Salisbury, G.W. 1972. The effect of storage *in vitro* on the DNA content of bull spermatozoa. *Austr. J. Biol. Science. 25: 175.*

Bonadonna, T. and Roychoudhurry, P.N. 1969. Studies on the chemical constituents of bull semen.IV: Effect of cold shock on the total cholestrol content. *Zentbl. Vet. Med. Reih A. 16: 176-179.*

Bolognani, L., Bologa-Fautin, A.M., Fuhrmanconti, A.M. and Omodeo-Sale, M.F. 1976. Studies on polar lipids in human cultured cells adopted to hypertonic medium. *Biochem. Exp. Biol. 12: 167-173.*

Bower, R.E., Crabo,B.G., Pace,M.M. and Graham, E.F. 1973. Discrepancies in anlysis of DNA in rabbit spermatozoa involving Feulgen staining (Feulgen DNA), and ultraviolet light absorption (UV- DNA) measurement. *J. Reprod. Fert. 14: 355*

Boyarskii, V.M. 1986. The effect of deep insemination of frozen semen on lambing results. *Referativnyi Zhurnal. 5: 58.*

Branton,C., Bratton, R.W. and Salisbury, G.W. 1949. Semen production and fertility of dairy bulls fed rations containing protein of plants and animal origin. *J. Dairy Science 32: 292-300.*

Butswat, I.S. 1989. Ram semen preservation at ambient temperature by flow dialysis techniques. *M.Sc Thesis. Dept of Animal Sc. A.B.U. Zaria.*

Campbell, R.C. and Edwards, J. 1955. The effect on onception rates of semen diluents containing citrate or phosphate buffer with all combinations of sulphanilamide, streptomycin and Penicillin. *J. Agric. Science. 46: 44-55.*

Carbonero, P.C. and Gamicik, P. 1984. Artificial insemination in Karakul sheep. *An. Vet. Zootec. (Madr.). 2: 273-276.*

Carmenate, P.C. and Gamicik, P. 1984. Different diluents for ram semen stored at room temperature or for 12 hours at 5°C, and their effects on fertility. *Folia veterinaria Bratislava. 26(1); 53-64.*

Chang, M.C. 1943. A study of the physiology of ram spermatozoa. *Abstr. Dis. University of Camb. (1941-42) 12:13.*

Choong, C.H. and Wales, R.G. 1962. The effect of cold shock on spermatozoa. *Austr. J. Biol. Science. 15: 543-551.*

Colas, G., Dauzier, L., Courot, M., Ortavant, R. and Signovet, J.P. 1968. Results obtained while investigating some important factors in artificial insemination of the sheep. *Annals of Zootech. 17: 47-57.*

Colas, G. 1975. Effects of initial freezing temperature, addition of glycerol and dilution on the survival and fertilising ability of deep frozen ram semen. *J. Reprod. Fert. 42: 277-285.*

Colas, G. 1979. Fertility in the ewe after artificial insemination with fresh and frozen semen at induced oestrus, and influence of the photoperiod on the semen quality of the ram. *Livestock Production Science. 6: 153-166.*

Cordvasoto, M.I. 1987. A comparison of different diluents for artificial insemination of ewes. *Veterinaria, Mexico (1987). 18(4): 376.*

Crabo, B.G., Bower,R.E., Brown,K.F., Graham,E.F. and Pace, M.M. 1970. Extracellular glumatic oxaloacetic transaminase as a measure of membrane injury in spermatozoa during treatment. *In: Current Problems in Infertility.(Eds. A.Ingelmansumberg and N.O. Lundell) Plenum N.York. pp 33-38.*

Crabo, B.G. and Graham, E.F. 1972. Correlation between some laboratory methods of evaluation of boar semen after freezing. *In: Proceed. 7th Intern. Congr. Anim. Reprod. and A.I. (Munchin). 2: 1641.*

Cragle, R.G. and Salisbury, G.W. 1959. Factors influencing metabolic activities of bull spermatozoa. IV: pH, Osmotic pressure, and the cations sodium, potassium, and calcium. *J. Dairy Science. 42: 1304.*

Dal Pra,L., Fuhrmanoconti, A.M., Sacchi, N. and Decarti, L. 1978. Adaptation to hypertonicity and selection of cell hybrids. *Biochem. Exp. Biol. 14: 119-123.*

Daniel, R.A., Kinsky, S.C., Kinsky, C.B. and Van Deenen, L.L.M. 1968. Effects of temperature and cholesterol on the glucose permeability of liposomes prepared with natural and synthetic lecithin. *Biochem. Biophy. ACTA. 150: 655-665.*

Darin-Bennette, A., Poulous, A. and White, I.G. 1974. The phoshpolipids and phospholipid bond fatty acids and aldehydes of dog and fowl spermatozoa. *J. Reprod. Fert. 14: 471-474.*

Dauzier, L. and Wintenberger, S. 1952. Mammalian fertility: Spermatozoa transport in the genital tract of ewes. *C. R. Soc. Biol. (Paris). 146: 67-70.*

Dauzier, L., Thibault, C. and Wintenberger, S. 1954. Presrvation of ram semen after dilution and maintenance of its fertility. *Ann. Endocr. (Paris). 15: 431-350.*

Dauzier, L. 1956. Some results obtained from artificial insemination of ewes and goats in France. *Paper in 3rd Intern. Congr. Anim. Reprod. (Camb.). Section 3: 12-14.*

Davies, H.L. and Dun, R.B. 1957. A note on the fertility of ewes treated with multiple injections of progesterone followed by PMSG. *Austr. Vet. Journal. 33: 92-94.*

Davis, I.S., Bratton, R.W. and Foote, R.H. 1963. Livability of bovine spermatozoa at 5°C in Tris buffered and citrate buffered yolk-glycerol extenders. *J. Dairy Science. 46: 57-60.*

Deas, D.M., Laing, J.A., Melrose, D.R., Reed. H.C.B. and Vandeplassche, M. 1979. The management of ovarian function. *In: Fertility and Infertility in Domestic Animals. (Edr. J.A. Laing) ELBS 3rd Edn. Baillere and Tindall. pp 114.*

Deniran, A. I. 1990. Effect of depth of cervical insemination on lambing rate of Yankasa ewes. *B. Agric. Project. Fac. of Agric. A.B.U. Zaria.*

Dettmers Almut, A. 1972. Genetics in livestock improvement. *Proceed. Genetic Society of Nigeria. pp 29-47.*

Diliello, L.R., Poelman, L. J. and Faber, J.E. 1957. Effect of antibiotics on *Vibrio foetus* and bovine spermatozoa. *Amer. J. Vet Research 18: 86-92.*

Doebbler, G. F. 1966. *Cryobiology 3:2 (cited by Ahmed, 1989)*

Dun, R.B. and Restall, B. J. 1961. Artificial insemination of Australian sheep. *Austr. Vet. Journal. 37: 145-149*

Duran Del Campo, A. 1960. Artificial insemination in sheep: Effect on fertility of time of heat at which insemination takes place. *Agron. J. Vet (B. Aire) 10(103): 5-11,32.*

Dzabrailov, D.M. and Sel'kon, I.I. 1962. Gaseation of ram semen diluent. *Ovcevodstov 8(9) 29: 125-129.*

Dzumurov, N. 1962. Cow's milk as a diluent for ram semen. *Veterinaria (Sarajero) 10: 495-498.*

Easterbrooks, H.L. 1951. Antibiotic treatment of diluted bull semen used in artificial. *Fertility and Sterility. 2: 430-443.*

Efendieva, T. F. 1963. The efffect of the rate of dilution of ram semen with various diluents on conception. *Abstr. Referat. Z. Zivotn. Vet (1964). 10: 126.*

El-Gaafary, M.N., Axford, R.F.E. and Chamberlain, A.G 1987. Artificial Insemination. *In: New Techniques in Sheep production.London U.K. Butterworth. pp 91-107.*

Emmens, C.W. and Blackshaw, A.W. 1950. Low temperature storage of ram, bull and rabbit spermatozoa. *Austr. Vet. Journal. 26: 226-228.*

Emmens, C.W. and Blackshaw, A.W. 1956. Artificial Insemination. *Physiology Review. 36: 277-306.*

Emmens, C.W. and Robinson, T.J. 1962. Artificial insemination in sheep. *In: The Semen of Animals and A.I. (Edr. J.P. Maule). CommonW. Agric. Bureau, Edinburg. pp 205.*

Entwistle, K.W. and Martin, I.C.A. 1972. Effects of the number of spermatozoa and volume of diluted semen on fertility in the ewe. *Austr. J. Agric. Res. 23: 467-472.*

Espana Garces ,M.S. 1987. Evaluation of fertility in ewes inseminated with fresh or refrigerated diluted semen at 2 sperm concentrations *Veterinaria, Mexico 18(1): 91.*

Evans, C. 1988. Current topics in artificial insemination of sheep. *Austr. J. Biol. Science. 41: 103-116.*

Filimon, S., Lunca, N., Bratescu, I. and Otel, V. 1956. The dilution and storage of ram and bull semen. *Anal. Inst. Cerc. Zootec. 14: 231-241.*

Firth, J.H. 1976. Studies in artificial insemination of sheep in Western Australia. *In: Sheep Breeding. Proceed. 1976 Intern. Congr. (Muresk and Perth) W. Australia. pp 475-481.*

Fisher, P.S., Ainsworth, L. and Langford, G.A. 1981. Effects of osmolarity of skim milk diluents and thawing rates on cryosurvival of ram spermatozoa. *Cryobiology. 18: 399-403.*

Fisher, P.S., Ainsworth, L. and Fairfull, R.W. 1984. Cryosurvival of ram spermatozoa in hypertonic and isotonic diluents. *Canadian J. Anim. Science. 62: 425-428.*

Flipse, R.J., Patton, S. and Almquist, J.O. 1954. Diluters for bovine semen. III: Effects of lectenin and of lactoperoxidase upon spermatzoa livability. *J. Dairy Science. 37: 1205-1211.*

Foote, R.H. and Salisbury, G.W. 1948. The effect of sulphonamides upon the livability of spermatozoa and upon the control of bacteria in diluted bull semen. *J. Dairy Science. 31. 769-778.*

Foote, R.H. and Bratton, R.W. 1950a. Motility of spermatozoa and control of bacteria in bovine semen extenders comtaining sulphanilamide, polymixin and aureomycin. *J. Dairy Science. 33: 531-543.*

Foote, R.H. and Bratton, R.W. 1950b. The fertility of bovine semen in extenders containing sulphanilamide, penicillin, streptomycin and polymixin. *J. Dairy Science. 33: 544-547.*

Foote, R.H. 1962. Artificial insemination. *In: Reproduction in Farm Animals. (edr. E.S.E. Hafez) Lea and Gebiher, 3rd edn. pp 257-264.*

Foote, R.H. 1960. *In: Reproduction in Domestic Animals,(edrs. H.H. Cole and P.T. Cupps) 2nd edn. (Academic Press. N. York). pp 313.*

Foote, R.H. 1970. Fertility of bull semen at high extension rates in tris-buffered extenders. *J. Dairy Science. 53: 1475-1477.*

Foote, W.D. 1974. Cattle. *In: Reproduction in Farm Animals. (edr. E.S.E. Hafez). Lea and Febiger. 3rd edn. pp 257-264.*

Foote, R.H. 1975. Semen quality from bull to the freezer. An assessment. *Theriogenology. 3: 219-235.*

Foulkes, J.A. and Watson, P.A. 1975. Hyaluronidase activity in seminal plasma as a method of assessing bull sperm integrity. *J. Reprod. Fert. 43: 349-353.*

Fukui, Y., and Roberts, E.M. 1976. Fertility of non-surgical intra-uterine insemination with frozen pelleted semen in ewes treated with prostaglandin F_{2a}. *Proceed. Inter. Congr. Sheep Breed. (Muresk),(edrs. G.T. Tomes, De Roberton, and R.J. Lightfoot). W. Austr. Inst. Tech. pp 482-494.*

Fukui, Y. and Roberts, E.M. 1977. Repeatability of non-surgical intra-uterine Technique for artificial insemination in the ewe. *Theriogenology. 8(2-3): 77-81.*

Fukui, Y. and Roberts, E.M. 1978. Further studies on non-surgical intra-uterine techniques for artificial insemination in the ewe. *Theriogenology. 10(5): 381-393.*

Garcia Del Alba Reyes, A. 1987. A comparison of ferility using fresh and refrigerated ram semen in two diluents. *Veterinaria. (Mexico). 18(2): 171.*

Gemert, W. Van. and Hoogenkamp, H. 1975. Artificial in sheep in combination with oestrus synchronisation. *Tjdschrift voor Diergecoskunde. 100(9): 489-497.*

Gokcen, H., Asti, R., Cekgul,E. and Sener, E. 1987. The relation between the number of live sperm per insemination dose of diluted ram semen and conception rate. *Veteriner Fakultesi Dergisi Uludag Universitesis. 4(1-3): 77-82.*

Gokhale, D.R. 1958. Glucose-sodium bicarbonate and sulphamezathine buffer as a diluent of buffallow semen. *Indian Vet. Journal. 35: 573-578.*

Graffith, J.B. 1978. Effect of hypertonic stress on mammalian cell lines and its relevance to freeze-thaw injury. *Cryobiology 15: 517-529.*

Graham, E.F., Schmehl, M.K.L. and Makilaurila, M. (1970). Some physical and chemical methods of evaluating semen. *In: Proceed. 3rd Technic. Conf. Anim. Reprod. and A.I. (N.A.A.B): 1-6.*

Graham, E.F., Rajamannan, A.H.J., Schmehl, M.K.L., Makilaurila, M. and Bower, R.E. 1971. Preliminary report on procedures and rational for freezing boar semen. *Artificial Insemination Digest. 19(1): 12.*

Graham, E.F. and Crabo, P.G. 1972. Some factors influencing the freezing of boar spermatozoa. *In: Proceed. 7th Intern. Congr. Anim. Reprod. A.I. (Munich). 2: 1627-1634.*

Gunn, R.M.C., Sanders, R.N. and Granger, W. 1942. Studies in ferility in sheep. II: Seminal changes affecting fertility in rams. *Bull. Comm. Sc. Industr. Res. Austr. Nº.94: 116.*

Habibulin, I.H. 1963. The preservation of ram semen at temperatures above freezing point. *Ovcevodstvo. 9(1): 28-29.*

Hanada, A., Nagase, H., Yamashika, S. and Irie, S. 1968. DNA content of bull spermatozoa after freezing and thawing and its relation with fertility rate. *Bull. Nation. Inst. Anim. Industr. Nº.18. Chiba, Japan*

Hancock, J.L. 1962. Fertilization in farm animals (a review). *Animal Breeding Abstracts. 30: 285-310.*

Haresign, W.M., Curmock, R.M. and Reed, H.C.B. 1986. Conception rates following cervical and intra-uterine insemination in the ewe. *In: Brit. Soc. Anim. Prod. Winter Meeting (1986). Paper number 65. pp 2.*

Hauser, H. and Barrat, M. D. 1973. Effect of chain length on stability of lecithin bilayer *Biochemical and Biophysics Research Communication. 53: 399-405.*

Hawk, H.W. and Conley, H.H. 1975. Involvement of cervix in sperm transport failure in the reporductive tract of the ewes. *Biology of Reproduction. 13: 322-328.*

Healey, P. 1969. Effect of freezing on the ultra structure of the spermatozoa of some domestic animals. *J. Reprod. Fert. 18: 21.*

Hennaux, L., Dimitropoulous, E. and Cordiez, E. 1947. The effect of streptomycin on the vitality of bull semen. *C.R. Soc. Biol. (Paris). 141: 1272-1274.*

Hess, E.A., Ludwick, T.M. and Teague, H.S. 1960. Motility of boar spermatozoa as influenced by semen freezing procedures. *J. Anim. Science. 19: 936.*

Hill, J.R.(jr.)., Hurst, V. and Godley, W.C. 1958. A comparison of reconstituted skim milk and egg yolk-sodium citrate as extender for ram semen. *Amer. J. Vet. Res. 19: 132-134.*

Holt, A.F. 1953. The effect of glycerolisation of bull semen on fertility. I and II. *Vet. Rec. 65: 624-625.*

Holt, W.V. and North, R.D. 1988. The role of membrane active lipids in the protection of ram spermatozoa during cooling and storage. *Gamete Research. 19(1): 77-89.*

Hutton, J.T. and Patton, S. 1952. *J. Dairy Science. 35, 699 (Cited by Jones, 1969).*

Igboeli, G. 1970. A tris-buffered egg yolk extender. *J. Animal Science. 30: 569.*

Ijinskay, T.P. and Kazantseva, G.M. 1972. Evaluation of the freezing resistance of boar spermatozoa. *Proceed. 7th Inter. Congr. Anim. Reprod. and A. I. (Munich). 2: 1664.*

Inskeep, E.K. 1974. Artificial insemination and preservation of ram semen. *In: Artificial Insemination of sheep. Bulletin 629 (1974), W. Virginia Univ. Agric. Exp. Stat. pp 5-23.*

Istvan, S. 1956. The effectiveness of using milk as a diluent for ram semen. *Ovcevodstvo. 4: 33-37.*

Jason, J.M., Kunst, M., Rip, A. and Bordewijk, P. 1973. Interaction between phosphatidylcholine and cholesterol in Benzene. *Chemistry and Physics of Lipids. 10: 114-126.*

Johnson, D.E., Flipse, R.J. and Almquist, J.O. 1956. Diluters for bovine semen. VIII: The effect of alterations of some physical factors of a milk diluter on the livability of bull spermatozoa. *J. Dairy Science. 39: 180.*

Jones, F.S. and Simms, H.S. 1930. *J. Exp. Med. 51: 321. (Cited by Jones, 1969).*

Jones, R.C. and Martin, I.C.A. 1965. Deep freezing ram spermatozoa: The effects of milk-yolk-citrate and synthetic diluents containing sugars. *J. Reprod. Fert. 10: 413-423.*

Jones, R.C. 1969. Studies on the suitability of preservations of ewes and cow milk for storing ram spermatozoa at 37°C, 5°C and -79°C. *Austr. J. Biol. Science. 22: 983-994.*

Jones, R.C. and Foote, R.H. 1972. Effects of osmolality and phosphate Tris, 'TES', 'MES', 'HEPES' and hydrogen ion buffers on motility of bull spermatozoa stored at 37°C or 5°C. *Austr. J. Biol. Science. 25: 1047-1055.*

Jones, R.C. and Holt, W.V. 1974. The effects of washing on the ultra structure and cytochemistry of ram spermatozoa. *J. Repr. Fert. 41: 159-167.*

Jones, R.C. and Martin, I.C.A. 1973. The effects of dilution, egg yolk and cooling to 5°C on the ultra structure of ram spermatozoa *J. Reprod. Fert. 35: 311.*

Jones, R.C. 1973c. The plasma membrane of ram, boar, and bull spermatozoa. *J. Reprod. Fert. 33: 179.*

Jones, R.C. and Stewart, D.L. 1979. The effects of cooling to 5°C and freezing and thawing on the ultra structure of bull spermatozoa *J. Reprod. Fert. 56: 233-238.*

Joshi, J.D. and Singh, G. 1968a. Studies on ram semen diluents. I: Effect of egg yolk-citrate series diluents on the livability and morphology of ram spermatozoa. *Indian J. Vet. Sc. Anim. Husb. 38: 574-582.*

Joshi, J.D. and Singh, G. 1968b. Studies on ram semen diluents. II: Effect of skim milk series diluents on the livability and morphology of ram spermatozoa. *Indian J. Vet. Sc. Anim. Husb. 38: 583-590.*

Joshi,J.D. and Singh, G. 1968c. Studies on ram semen diluents. III: Effect of tomato juice series diluents on the livability and morphology or ram spermatozoa. *Indian J. Vet. Sc. Anim. Husb. 38: 591-598.*

Kardymovic, M., Marsakova, A. and Pavljucuk, V. 1934. Insemination of sheep at different times during oestrus. *Probl. Zivotn. 5: 110-115.*

Kardymovic, M. 1953. Artficial Insemination of sheep. *Med. Wet. 9: 402-404.*

Kazakov, V.W. 1963. The best method of inseminating ewes with cooled semen. *Ovcevodstvo. 9(1): 20-22.*

Keast, J.C. and Morley, F.H.W. 1961. Some observations on artificial insemination of sheep. *Austr. Vet. Journal. 25: 281-287.*

Kerton, D.I., Mcphee, S.R. Davis, I.F., White, M.B., Banfield, J.C. and Cahill, L.P. 1984. A comparison of insemination techniques in Corriedale ewes. *In: Proceed. Austr. Soc. Anim. Prod. 15: 701.*

Kharche, K.G., Pangawkar, G.R. and Mittal, V.P. 1976. Effect of certain dilutors on the morphology of ram spermatozoa on preservation at 5°C. *Indian Vet. J. 53(9): 694-698.*

Killeen, I.D. and Moore, N.W. 1970. Fertilization and survival of fertilized eggs in the ewe following surgical insemination at various times after onset of oetrus. *Austr. J. Biol. Science. 23: 1279.*

Killeen,I.D. and Caffrey, G.J. 1982. Uterine insemination of ewes with the aid of a laparoscope. *Austr. Vet. Journal. 59: 95-105.*

Killeen, I.D., Caffrey, G.J. and Holt, N. 1982. Fertility of ewes following intra-uterine insemination with the aid of a laparoscope. *Proceed. Austr. Soc. Reprod. Biol. Abstr. 15.*

Knodt, C.B. and Salisbury, G.W. 1946. The effect of sulphanilamide upon the livability and metabolism of bovine spermatozoa. *J. Dairy Sc. 29: 285-291.*

Koger, M. 1951. Storage, dilution and use of ram semen in artificial breeding of sheep.*Bull. N. Mex. Agric. Exp. Station. No. 366, pp 12.*

Koks, J.C.N. 1953. Some factors influencing the longevity of bull sperm cells *in vitro. In: Mammalian germ cells (London) J and A Churchill Ltd. pp 82-85.*

Krzanowska, H. 1974. The passage of abnormal spermatozoa through the uterotubal junction of the mouse. *J. Reprod. Fert. 38: 81.*

Kunze, C., Schmidt, D. and Bierwolf, D. 1973. Morphological changes in boar sperm heads following deep freezing. *Animal Breeding Abstract. 41: No.506.*

Kurilov, J.G. 1972. The influence of the silution and freezing of boar semen on the activity of some enzymes of Carbohydrate-phosphate metabolism and sperm survival. *Proceed. 7th Inter. Congr. Anim. Reprod. and A. I. (Munchen). 2: 1668.*

Kuznecor, M.P. 1950. Causes of return to oestrus in Karakul sheep and methods of their elimination. *Karakullevodstvot Zverovodstvo. 5: 7-13 (Eng. Summ.).*

Langford, G.A., Ainsworth, L. and Wolynetz, M.S. 1982. Reproductive reponse of progestagen treated sheep in confinement to a single and double insmeination. *J. Animal. Science. 54: 12-17.*

Langford, G.A. and Marcus, G.J. 1982. Influence of sperm number and seminal plasma on fertility of progestagen treated sheep in confinement. *J. Reprod. Fert. 65: 325-329.*

Lapwood, K.R. and Martin, I.C.A. 1966. The use of monosaccharides, dissacharides and trissaccharides in synthetic diluents for storage of ram spermatozoa at 37°C and 5°C. *Austr. J. Biol. Science. 19: 655.*

Lapwood, K.R. and Martin, I.C.A. 1972. Effects of some buffers and inorganic and organic sodium salts in synthetic diluents for storage of ram, spermatozoa at 37°C and 5°C. *Austr. J. Biol. Science. 25: 365.*

Lapwood, K.R., Martin, I.C.A. and Entwistle, K.W. 1972. The fertility of Merino ewes artificially inseminated with semen diluted in solutions based on skim milk-glucose or ribose. *Austr. J. Agric. Res 23: 457*

Lardy, H.A. and Phillip, P. H. 1939. Preservation of spermatozoa. *Amer. Soc. Anim. Prod. Proceed. 32:19-221.(Cited by Salisbury and VanDemark, 1961).*

Lardy, H.A. and Phillip, P.H. 1943. Effect of pH and certain electrolytes on the metabolism of ejaculated spermatozoa. *Amer. J. Physiology. 138: 741-746.*

Leon Crespo, L. 1966. Use of Carbon dioxide in ram semen diluents. *Revta Patvo Biol. Anim. 10(1): 67-78.*

Lightfoot, R.J. and Salamon, S. 1969. Freezing ram spermatzoa by the pellet method. *Austr. J. Biol. Science. 22: 1561.*

Lightfoot, R.J. and Salamon, S. 1970. Fertility of ram spermatozoa frozen by pellet method. *J. Reprod. Fert. 22: 385.*

Lindemann, C.B., Fisher, M. and Lipton, M. 1982. A comparative study of the effects of freezing and storage on intact annd demembraned bull spermatozoa. *Cryobiology. 19: 20-28.*

Linge, F.O. and Holmberg, O. 1963. Field trials with artificial insemination. *Farskotsel Svenska Faravels foren Tidsk. 43: 155-160.*

Lopatko, M.F. 1971. Tris-phosphate diluent for ram semen. *Visnik Sil'skogospodars' koii Nauk. 14: 89-94.*

Lopyrin, A.I., Donskaja, V.I. and Kastornova, E.K. 1957. Fertility of ewes with regards to various methods of double insemination. *Ovtsevodstvo. 3: 19-21.*

Lopyrin, A.I. and Mamilov, I.M. 1967. The insemination of ewes with dose of semen. *Ovtsevodstvo. 13: 34-36.*

Lunca, N. 1964. Artificial insemination in sheep and goat on the international plane. *In. Proceed. 6th Inter. Congr. Anim. Reprod. and A.I. 4: 118.*

Macmillan, K.L., Tiku, J.L. and Hart, N.L. 1972. Effects of aromatic amino acids on the livability of bull spermatozoa. *Austr. J. Biol. Sc. 25:1039.*

Mann, T. and Mann, C.L. 1981. Male Reproductive Function and Semen. *(Themes and Trends in Physiology, Biochemistry and Investigative Andrology)* Springer- Verleg, Berlin Heidelberg New York. pp 198

Martin, I.C.A. 1960. Diluents for the preservation of ram spermatzoa. I: Diluents used at 37°C and 5°C containing casein. *Austr. J. Biol. Science. 19: 645.*

Martin, I.C.A. 1962. The viability of diluted ram spermatozoa at 37°C, 5°C and -79°C. *In: Artificial Breeding of sheep in Australia. 2nd edn. (edrs. G.L. Tomes, D.E. Robertson, and R.J.Lightfoot), Butterworth London Boston. pp. 143-148.*

Martin, I.C.A. and Watson, P.F. 1976. Artificial insemination of sheep: Effects on fertility of number of spermatozoa inseminated and of storage of diluted semen for up to 18 hours at 5°C. *Theriogenology. 5:29-35.*

Martin, I.C.A. and Richardson, B. A. 1979. Factors affecting the fertility of diluted ram semen. *In: Sheep Breeding. 2nd edn. (edrs. G.L. Tomes, D.E. Robertson, and R.J. Lightfoot). Butterwotths, London, Boston. pp 513-520.*

Martynov, Yu. F. 1970. Changes in the nucleic acids of stored and irradiated ram spermatozoa in relation to their fetiliising ability. *Izv. Akad. Nauk-Kazakh. SSR NSer. Biol. 8: 81-83.*

Mattner, P.E. and Braden, A.W.H,. 1969. Effect of time of insemination on the distribution of spermatozoa in the genital tract in ewes. *Austr. J. Biol. Science. 22, 1283.*

Mattner, P.E. 1968. The duration of spermatozoa and leucocytes in the female genital tracts of goats and cattle. *J. Reprod. Fert. 17: 253-261.*

Maxwell, W.M.C., Wilson, H.R. and Butler, L.G. 1984. Fertility of ewes after intra-uterine insemination with frozen semen. *In: Proceed. of the Austr. Soc. of Animal Produc. 15: 448-451.*

Maxwell, W.M.C. 1986. Artificial insemination of ewes with frozen thawed semen at a synchronised oestrus. II: Effect of dose ofspermatozoa and site of intra-uterine insemination on fertility. *Animal Reproduction Science. 10(4): 309-316.*

Mayer, D.T. and Lasley, J.T. 1945. The factor in egg yolk affecting the resistance storage potentialities and fertilising capacity of mammalian spermatozoa. *J. Animal Science. 4: 261-269.*

Melrose, D.R. 1962. Artificial insemination in cattle. *In: The semen of animals and artificial insemination. (edr. J.P. Maules) Commw. Agric. Bur. pp. 1-181.*

Mies Filho, A.S. and De Almeida Ramos, A. (1954). Artificial, insemination in sheep - Results obtained with stored semen subjected to different techniques. *Bol. Insem. Artif. (Rio de J.). 6: 28-34.*

Mies Filho, A. and De Almeida Ramos., A. 1955. The efficacy of different artificial insemination techniques in sheep. *Bol. Insem. Artif. (Rio de J.). 7: 28-36.*

Miller, W. J. and Van Demark, N. L. 1954. The influence of glycerol level, various temperature aspects, and certain other factors on the survival of bull spermatozoa at sub-zero temperatures. *J. Dairy Science. 37:45-51.*

Molovanov, V.K. 1945. Artificial insemination of livestock. *Practical Handbook (Moscow Linnigrad). Seljhozgiz. pp 96.*

Milovanov, V.K., Varnavskii, A.N. and Nauk, V.A. 1970. The nature of freezing damage of ram spermatozoa. *Vest. Sel-khoz Nauk. Mosk. 15:86-93.*

Moore, N.W., Quinlan, T.D., Robinson, T.J. and Smith, J.F. 1976. The fertilisation of ova following natural mating or artificial insemination after withdrawal of SC-9880 impregnated intra-vaginal sponges from cyclic Merino ewes. *In: The Control of the Ovarian cycle in sheep. (edr. T. J. Robinson), Sydney Univ. Press. pp. 169.*

Morozov, V.A. 1954. Artificial insemination of ewes in Dagestan. *Zivotnodstvo (1954). 9: 105-109.*

Moss, J.A., Melrose, D.R., Reed, H.C.B. and VanDeplassche, M. 1979. Spermatozoa, semen, and Artificial Insemination. *In: Fertility and Infertility in Domestic Animals.3rd edn. (edr. J.A. Laing). ELBS and Bailliere Tindall. pp. 86-90.*

Mukherjee, D.F. and Dott, H.M. 1960. Effects of egg yolk-citrate and egg yolk-glycine diluters on the morphology of bovien spermatozoa. *J. Agric. Science. 55 225-228.*

Odaibo, H. B. 1990. Effects of diluent type and insemination dose with fresh semen on lambing rate of Yankasa ewes. *B. Agric. Project Fac. of Agric. A.B.U. Zaria.*

Onipe, A. Y. 1989. Formaldehyde preservation of ram spermatozoa. *B. Agric. Project. Fac. of Agric. A.B.U. Zaria.*

Osinowo, O.A. 1975. Studies on deep freezing boar spermatozoa. *M.Sc. Thesis University of Sydney Australia.*

Osinowo, O.A. 1977. Research notes on applied reproductivie physiology and genetic improvement in of farm animals. *Nigerian J. Genetic. 1: 99-101.*

Osinowo, O.A. 1981. Studies on the leakage of enzymes from washed bull and ram spermatozoa. *J. Reprod. Fert. 65: 549-554.*

Osinowo, O.A. 1982. Oestrus synchronization, A.I. and early re-breeding in Yankasa sheep. *Nig. J. Anim. Prod. 9(2): 107-111*

Osinowo, O.A., Bale, J.O., Oyedipe, E.O. and Eduvie, L.O. 1982 Motility and eosin uptake of formaldehyde treated ram spermatozoa. *J. Repr. Fert. 65: 387-394*

Osinowo, O.A., Ekpe, G., Eduvie, L.O., Ahmed, M.S. and Ogbona, J. 1988. Control of washing induced changes in ram spermatozoa. In: *11th Inter. Congr. Anim. Reprod. and A.I. Univ. Coll. Dublin Ireland June 26-30 1988, Vol. 3 (Brief Comm. paper No. 281, pp3*

Ozin, F.V. 1954. Artificial insemination in sheep. *(Moscow) Seljhozgiz. pp 94.*

Ozkoca, A. 1961. Insemination results with ram semen diluted in modified egg yolk-citrate diluent. *Lalahan Zootek. Arast. Enst. Derg. 1: 27-32.*

Ozkoca, A. 1964a. The fertility results obtained with diluted and stored ram semen. *Lalahan Zootek. Arast. Enst. Derg. 4: 55-63.*

Ozkoca, A. 1964b. Lambing results obtained with undiluted and stored ram semen . *In: 5th Inter. Congr. Anim. Reprod. A. I. 4: 236-240.*

Ozkoca, A. 1965. Studies in the dilution and storage of ram semen. VI. *Lalahan Zootek. Arast. Enst. Derg. 5: 101-106.*

Ozkoca, A. 1967. Studies on dilution and storage of ram semen. *Lalahan Zootek. Arast. Enst. Derg. 7. 131-136.*

Ozkoca, A. 1980. Lambing results obtained with diluted and stored ram semen. *In: 9th Inter,. Congr. Anim. Reprod. and A.I. III: Symposia (Free Communications)., Madrid, Spain Editorial Garsi (1980). pp 320.*

Pano, P.B. 1983. Ambient temperature storage of ram semen. *B. Sc. (Agric.) Project. Fac. of Agric. A.B.U. Zaria*

Pavlovic, A. and Varadin, M. 1968. Effects of some diluents on fertilising capacity of ram spermatozoa. *Veterinaria Saraj. 17: 445-449.*

Peregon, I. L. 1936. Experience with repeated insemination of sheep. *Probl. Zivoto. 10: 149.*

Petruzzi, V., Tarantini,S. and Riychoudhury, P.N. 1976. Effects of different semen diluents on survival of ram spermatozoa at 5°C. *Zentralblatt fur Vereinarmedizin, A. (1976. 23: 556-561.*

Philips, P.H. and Lardy, H.A. 1940. A yolk buffer pabulum for the preservation of bull spermatozoa. *J. Dairy Science. 23: 399-404.*

Philips, M.C. 1972. The physical state of phospholipids and cholesterols in monolayers and membranes. *Progr. Surf. Memb. Sc. 5: 139-221.*

Platov, E.M. 1965. Freezing of ram semen in egg yolk-lactose-ethylene glycol diluent. *Ostsevodstvo. 11: 11.*

Platov, E. M. 1970). Variation in the adenosine polyphosphate content in bull and ram semen on storing at 0°C and 5°C. *Dokl. Uses. Akad. Sel'khoz Nauk VI. Lenina. 1: 31-33.*

Polge, C., Smith,A.U. and Parkes, A.S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature (London). 164: 666.*

Polge, C. and Rowson, L.E.A. 1952a. Fertilising capacity of bull spermatozoa after freezing at -79°C. *Nature (London) 169: 626-627.*

Polge, C. and Rowson, L.E.A. 1952b. Results with bull semen stored at -79°C. *Vet. Rec. 64: 851.*

Poulos, A., Darin-Bennette, A. and White, I.G. 1973. The phospholipids bound fatty acids and aldehydes of mammalian spermatozoa. *Compar. Biochem. Physiol. 46: 541-546.*

Pursel, V.G., Johnson, L.A. and Rampack, G.B. 1972. Acrosome morphology of boar spermatozoa inubated before cold shock. *J. Anim. Science. 34: 278.*

Pursel, V.G. and Johnson, L.A 1974. Glutaradelhyde fixation of boar spermatozoa for acrosome evaluation. *Theriogenology. 1: 63.*

Pursel, V.G., Johnson, L.A. and Schulmen, L.L. 1974. Acrosome morphology of boar spermatozoa during *in vitro* ageing. *J. Anim. Science. 38: 113.*

Quinn, P.J. 1968. Deoxyriborudease activity in semen. *J. Reprod. Fert. 17: 35-39.*

Quinn, P.J., White, I.G. and Cleland, K.W. 1969. Chemical and ultra-structural changes in ram spermatozoa after washing, cold shock and freezing. *J. Reprod. Fert. 18: 109-120.*

Quinn, P.J. and White, I.G. 1969. Osmotic shock of ram spermatozoa. *J. Reprod. Fert. 18: 375-377.*

Quinla, J., Steyn, H.P. and Devos, D. 1941. Observation of artificial isemination of sheep with fresh and stored semen. *Onderstepoort. J. Vet. Sc. 16: 263-267.*

Quinlivan, T.D. and Robinson, T.J. 1967. The number of spermatozoa in the fallopian tubes of ewes at intervals after artificial insemination following withdrawal of SC-9880 impregnated intra-vaginal sponges. *In: The control of the ovarina cycle in sheep. (edr. T.J. Robinson). Sydney Univ. Press. pp. 177.*

Quinlivan, T.D. and Robinson, T.J. 1969. Number of spermatozoa in the genital tract after artificial insemination of progestagen treated ewes. *J. Reprod. Fert. 19: 73-86.0*

Rajamannan, A.H.J., Graham, E.F. and Smith, F. 1964. The effect of a new extender in corporating dried egg yolk on the fertility of bovine spermatozoa. *Proceed. 5th Congr. Anim. Reprod. (Trento). 4:392-397.*

Rathore, A.K. and Mukherjee, D.P. 1961. Effects of egg yolk-phosphate, milk, and egg yolk-boric acid-sodium bicarbonate on the morphology of ram semen. *Indian Vet. J. 38: 383-390.*

Restall, B. J 1961b. Artificial insemination of sheep. *Austr. Vet. J. 37: 70-73.*

Roberts, E.M. and Houlaham, P.M. 1962. A comparison of the ferility of stored and fresh sheep semen. *Austr. J. Exp. Agric. Anim. Husb. 1: 156-158.*

Robinson, T.J. 1958. Studies in controlled artificial insemination of Merino sheep. *Austr. J. Agric. Res. 9: 693-703.*

Robinson, T.J. 1959. The oestrus cycle of the ewe and the doe: Reproduction following Progesterone and PMSG treatment iin the cyclic ewes. *J. Agric. Sc. 57: 129-134.*

Robinson, T.J. 1965. Use of progestagen impregnated sponges inserted intra-vaginally and sub-cutaneously for the control of the oetrus cycle in the sheep. *Nature (London). 206: 39-41.*

Robinson, T.J. and Moore, N.W. 1967. The evaluation of progesterone and SC-9880 impregnated intra-vaginal sponges for the synchronisation of oestrus for large scale artificial of Merino ewes. *Paper 8, In: The control of the ovarian cycle in sheep. (edr. T.J. Robinson). Sydney Univ. Press. pp. 116-132.*

Robinson, T.J 1973. Factors involved in the failure of sperm transport in the female reproductive tract. *J. Reprod. Fert. (Suppl.) 8: 103-109.*

Rotten, S., Yashou, V.J., Ne'eman, Z. and Razin, S.1973. Cholesterol in mycoplasma membranes composition ultra structure, and biological properties of membranes from *Mycoplasma mycoides var. Capri* cells adapted to grow with low cholesterol concentrations. *Biochem. Biophys. ACTA. 323: 195-208.*

Rottenstan, K. 1954. Comparative studies on the addition of 500 and 1000 units of streptomycin per millilitre of diluents.*Beretn. Forsogslab (Kbh). Paper 7: 28.*

Roy,A., Gupta, H.C., Srivastava,R.K. and Pandey, M.D. 1956. Storage of ram spermatozoa *in vitro*. I: Preservation in glycine-egg yolk medium. *Indian Vet. J. 33: 18-20.*

Saacke, R.G. and Marshall, C.E. 1968. Observation on the acrosomal caps of fixed and unfixed bovine spermatozoa. *J. Reprod. Fert. 16: 511.*

Saacke, R.G 1982. Semen quality in relation to semen preservation. *J. Dairy Science. 66: 2635-2644.*

Sakala, J. 1957. The effect of antibiotics and sulphonamides on the fertility of bull semen. *Sborn CSL. Akad. Zemed. Ved; vet.Med. 2: 51-64.*

Salamon, S.1962. Studies on the artificial insemination of Merino sheep. *Austr. J. Agric. Res. 13: 1137-1150.*

Salamon, S. and Robinson, T.J. 1962a. Studies on the artificial insemination of Merino sheep. I: The effect of frequency and season of insemination, age of ewes and ram, and milk diluent on lambing performance. *Austr. J. Agric. Res. 13: 52-68.*

Salamon, S. and Robinson, T.J. 1962b. Studies on the artificial insemination of Merino sheep. II: The effects of semen diluents and storage on lambing performance. *Austr. J. Agric. Res. 13: 271-281.*

Salamon, S. 1968. Deep freezing of ram semen: Recovery of spermatozoa after pelleting and comparison with other methods of freezing. *Austr. J. Biol. Sciecne. 21: 351.*

Salamon, S. 1970. The survival of ram spermatozoa following pellet freezing below -79°C. *Austr. J. Biol. Science. 23: 459-464.*

Salamon, S. 1971. Fertility of ram spermatozoa following pellet freezing on dry ice at -79°C and -14°C. *Austr. J. Biol. Science. 24: 183-185.*

Salamon, S. and Visser, D. 1972. Effects of composition of tris-based diluent and thawing solution on the survival of ram spermatozoa frozen by the pellet method. *Austr. J. Biol. Science. 25: 605-618.*

Salamon, S. and Visser, D. 1973. Fertility of ram spermatozoa frozen in a tris-based diluent. *Austr. J. Biol. Science. 26: 513-516.*

Salamon, S., Maxwell, W.M.C. and Firth, J.H. 1977. The fertilising capacity of ram spermatozoa stored at 5°C. *Theriogenology. 8: 200.*

Salisbury, G.W., Willet., E.L. and Gunsalus, I.C. 1939. Some problems in bull semen storage. *Proceed. Amer. Soc. Anim. Prod. 32rd. Ann. Meet. 210-212.*

Salisbury, G.W., Fuller, H.K. and Willet, E.L. 1941. Preservationn of bovine spermatozoa in yolk-citrate diluents and field results from its use. *J. Dairy Science. 24: 905-910.*

Salisbury, G.W. and Knodt, C.B. 1947. The effect of sulphanilamide in the diluent upon fertility of bull semen. *J. Dairy Science. 30: 361-369.*

Salisbury, G.W. and VanDemark, N.L. 1951. The effect of cervical, uterine and cornual insemination on the fertility of the dairy cow. *J. Dairy Science. 34: 68-74.*

Salisbury, G.W. 1957. Recent development with bull semen diluents (a review). *Anim. Breed. Abstr. 25: 11-123.*

Salisbury, G. W. and VanDemark, N.L. 1961. Physiology of reproduction and artificial insemination of cattle. Freeman and Co. San Francisco. pp 400-402.

Salisbury, G. W., Birge, W.J., Dela Torre, L. and Lodge, J.R. 1961. Decrease in nuclear feulgen-positive material (DNA) upon ageing in *in vitro* storage of bovine spermatozoa. *The J. Biophys. Biochem. Cytol. 10: 353.*

Saxena, V.B. and Singh, G. 1967. Preservation of ram spermatozoa. I: Effect on motility, livability and morphology or ram spermatozoa in 4 semen diluents. *J. Anim. Morph. Phydiol. 14: 114-120.*

Schindler, H. and Amir, D. 1961. Longevity of ram sperm in various diluents at different dilution rates. *J. Agric. Science. 56: 183-189.*

Schindler, H., Eyal,____. and Amir, D. 1972. 4th Annual Report P.L. 480, Project.Israel Aro. Volcanic. *(Cited by Inskeep, 1974).*

Schindler, H. and Amir, D. 1973. The conception rates of ewes in rlation to sperm dose and time of insemination. *J. Reprod. Fert. 34: 191-196.*

Schleicher, J. and Bruckner, G. 1973. Ultra structural findings on ram spermatozoa stored in liquid form in different buffers. *Monatshefte Fur Veterinarmedizin. 28: 291-395.*

Shikima, K. 1965. Effects of freezing and thawing on the stability of double helix DNA. *Nature (London). 209: 529.*

Sinclair, A.N. 1957. Effects of variation of time of mating freguency, and semen dose rate on conception in Merino sheep. *Austr. Vet. J. 33: 88-91.*

Smagulov, S.B. and Martynov, Ju V. 1966. Dilution rate, storage time, and insemination results. *Ovtsevodstvo. 12: 30-31.*

Smagulov, S.B. and Martynov, Ju. F. 1968. Morphological variability of ram spermatozoa under various conditions of *in vitro* preservation. *SSR Ser. Biol. 6: 77-80.*

Smagulov, S.B. and Martynov, Ju F. 1972. The effects of semen storage duration on conception rates of ewes and production characters of their progenies. *Vestnik Sel'Sicokhosynistvennoi Nauk. Alma USSR. 15: 51-53.*

Smith, J.T., Mayer, D.T. and Merilan, C.P. (1956). Effect of egg yolk and its isolated constituents upon the dehydrogenase activity of bovine spermatozoa. *J. Dairy Science. 39: 552-560.*

Smith, J.F. and Allison, A.J. 1971. Effect of exogenous progestagen on the production of cervical mucus in the ewe. *J. Reprod. Fert. 24: 279.*

Snedneker, W.H. and Gaunya, W.S. 1970. Dimethyl sulfoxied as a cryoprotective agent for freezing bovine semen. *J. Anim. Science. 30: 953-956.*

Somade, B., Macpherson, J.N. and King, G.J. 1974. Physical damage to the head of bovine spermatozoa induced by processing and freezing. *J. Dairy Science. 57: 625.*

Stallcup, O.T. and McCartney, H.K. 1953. Toxicity to bull spermatozoa of tetramycin hydrochloride and its use as antibacterial agent in diluents. *J. Dairy Science. 36: 293-295.*

Steinbach, J. and Foote, R.H. 1967. Osmotic pressure and the pH effects on survival of frozen bovine spermatozoa. *J. Dairy Science. 50: 205-213.*

Stewart, D.L. and Melrose, D.R. 1952. The comparative efficiency of the intra-cervical and intra-uterine methods of insemination in the dairy cow. *Vet. Rec. 64: 303-308.*

Sykes, J.G. and Mixner, J. P. 1951. Toxicity to bull spermatozoa of various salts brands and lots of penicillin, streptomycin, aueromycin and chloromycetin. *J. Dairy Science. 34: 342-346.*

Szumowskii, P., Markovic, B. and Cano, A. 1956. Powdered skim milk for the dilution and freezing of ram semen. *Rec. Med. Vet. 132: 124-134.*

Tasserson, E., Amir, D. and Schindler, H. 1977. Acrosome damage of ram spermatozoa during cooling and freezing. *J. Reprod. Fert. 51:461*

Tenchini, K.L., Bologani, L. and Decarli, L. 1980. Effect of hpertonicity on the survival of unprotected human cultured cells following freezing and thawing. *Cryobiology. 17: 120.*

Terrill, C.E. 1960. Sheep and Goat. *In: The A.I. of Farm Animals. (edr. E.J. Perry), Rutgers, Univ. Press,. New Brunswick. pp. 178.*

Terrill, C.E. 1963. Sheep and Goat. *In: The A.I. of Farm Animals. (edr. E.J. Perry). Rutger Univ. Press. Brunswick. pp 178-203.*

Terrill, C.E. 1975. Sheep. *In: Reproduction in Farm Animals. (edr. E.S.E. Hafez). 3rd edn. Lea adn Febiger Philadelphia. pp. 269-270.*

Tervit,H.R., Goold, P.G., James,R.W. and Fraser, M.D.C. 1984. The insemination of shepp with fresh or frozen semen.*Proceed. New Zeal. Soc. Anim. Prod. 44: 11-13.*

Tervit, H.R. 1985. Artificial insemination of sheep. *In: New Zealand Min. Agric. Fisheries. Agric. Res. Division. Annual Report (1983-1984) Wellington. pp 46.*

Tewari, S.B., Shaarma, R.P. and Roy, A. 1968. Effect of dilution rate on the preservation of ram and goat spermatozoa viability. *Indian J. Vet. Science. 35: 561-572.*

Tewari, S.B., Srivastava, A.K. and Sahni, K. 1977. Some metabolic changes in ram semen stored in the milk diluent. *Indian Vet. J. 54: 111-115.*

Thacker, D.L. and Almquist, J.O. 1953. Diluents for bovine semen. I: Fertility and motility of bovine spermatozoa in boiled milk. *J. Dairy Science. 36: 173-180.*

Thacker, D.L., Flipse, R.J. and Almquist, J.O. 1954. Diluents for bovine semen. II: Effect of milk proteins upon spermatozoa livability. *J. Dairy Science. 37: 220-227.*

Thibault, C. 1967. A comparative analysis of fertilisation and its anomalies in the ewes and rabbits. *Annl. Biol. Biochem. Biophys. 7: 5.*

Tjupic, M.M. 1959. Artificial insemination by the vaginal method. *Ovcevodstvo. 5(10): 24-27.*

VanDemark, N.L. and Couturier, L.E. 1958. Flow dialysis as a means of preserving bovine semen at room temperature. *J. Dairy Science. 41: 530-535.*

Varnavskii, A.N. 1970. The effect of freezing method on motility and ultra-structure of bull and ram semen. *Zhivotnovod stvo. Mosk 32: 53-55.*

Vinha, N.A. and Coubrough, R.I. 1975. Gelatinised media for diluting ram semen. A Preliminary report concerning *in vitro* trials. *J. S. African Vet. Assoc. 43: 267-270.*

Visser, D. and Salamon, S. 1973. Fertility of ram spermatozoa frozen in a tris-based diluent. *Austr. J. Biol. Science. 26: 513-516.*

Visser, D. and Salamon, S. 1974. Fertility following insemination with frozen thawed reconcentrated and unconcentrated ram semen. *Austr. J. Biol. Science. 27: 423.*

Visser, D. and Salamon, S. 1974. Effect of composition of tris-based diluent on the survival of boar spermatozoa following deep freezing. *Austr. J. Biol. Science. 27: 485.*

Wales, R.G. and White, I. G. 1958. The interaxction of pH, tonicity, and electrolyte concentration on the motility of fowl spermatozoa. *Austr. J. Biol. Science. 11: 177.*

Watson, P.F. and Martin, I.C.A. 1972. A comparison of changes in the acrosomes of deep frozen ram and bull spematozoa. *J. Reprod. Fert. 28: 99-107.*

Watson, P.F.D. and Martin, I.C.A. 1973. The response of ram spermatozoa to preparations of egg yolk in semen diluents during storage at 5°C or -196°C. *Austr. J. Biol. Science. 26: 927-935.*

Watson, P.F. and Martin, I.C.A. 1975. The influence of some egg yolk fractions on the survival of ram spermatozoa at 5°C. *Austr. J. Biol. Science. 28: 145-152.*

Watson, P.F. 1981. The role of lipid and protein in the protection of ram spermatozoa at 5°C by egg yolk liprotein. *J. Reprod. Fert. 62: 483-492.*

Welch, R.A.S. and Shannon, P. 1970. Storage of ram semen at ambient temperature. *Proc. New Zealand Soc. Anim. Prod. 30: 51.*

White, I.G. 1959. Studies on the estimation of glycerol, fructose, and lactic acid with particular reference to semen. *Austr. J. Exp. Biol. Med. Science. 37: 441.*

Wiggan, L.S. and Clark., J.B.K. 1967. Observations on the fertility of diluted and stored ram semen used for artificial insemination. *Brit. Vet. J. 123: 447-457.*

Wiggins, H.B. and Almquist, J. O. 1974. Effects of glycerol equilibration time and thawing rate on acrosomal maintenance of bull sperm frozen in plastic straws. *J. Dairy Science. 57: 625.*

Wilmut, I. and Polge, C. 1972. Recent progress in the deep freeze preservation of boar semen. *J. Reprod. Fert. 25: 301.*

Willet, E.L. and Salisbury, G.W. 1942. The effect nof various diluents cooling rate temperature of storage and some other factors on the livability of spermatozoa in stored samples of bull semen. *Mem. Cornell Agric. Exp. Stat. No. 249. pp. 45.*

Willet, E.L. and Ohms, J.L. 1955. Field trials with semen containing several combinations of antibacterial agents. *J. Dairy Science. 38: 1360-1368.*

Wilson, A.T. and Rosenblum, H. 1952a. *J. Exp. Med. 95: 25. (Cited by Jones, 1969).*

Wilson, A.T. and Roseblum, H. 1952b. *J. Exp. Med. 95: 39. (Cited by Jones, 1969).*

Yoshioka, Z., Inudo, Y. and Torizuka, T. 1951. Experiment on the storage of semen on artificial insemination of the stored semen in sheep and goat. *Bull. Nat. Inst. Agric. Science. 1: 53-60.*

Zamfirescus, S., Barbutescu, I., Opera, R. and Pivoda, C. 1983. Improving the fertility of ewes artificially inseminated with frozen or chilled semen. *Revista de Cresterea Animalelor. 32: 22-24.*

Zlaterev, S.T. 1976. Studies on the artificial insemination of sheep with semen stored for 24 and 48 hours at 0°C to 3°C. *In: Proceed. 20th World Vet. Congr. (1975). Thessaloniki(Greece). 2: 988-989.*

Zlaterev, S.T. 1976. Optimal number of spermatozoa for artificial insemination of sheep with semen stored for 24 hours at 0°C to 3°C. *In: 8th Inter. Congr. Anim. Reprod. and A.I. Proceed. 1: pp. 9.*

Zlaterev, S.T. 1984. Organization of artificial insemination in large scale sheep farm and a new technology of sperm storage. *In: 10th Inter. Congr. Anim. Reprod. and A.I. Illinois U.S.A. 2: (Brief Commun.). Paper Number 218: 3.*

APPENDIX

APPENDIX 1

SEMEN EVALUATION BEFORE DILUTION

a) Experiment 1

Ram Tag No.	Sperm Concentration ($\times 10^9$)	Motility (%)
173	2.738	90
111	2.909	85
190	2.595	80

b) Experiment 2

Ram Tag No.	Sperm Concentration ($\times 10^9$)	Motility (%)
137	2.223	85
156	2.566	85
190	2.052	90

c) Experiment 3

Ram Tag No.	Sperm Concentration (x 10^9)	Motility (%)
1407	3.252	95
111	2.738	85
190	4.281	90

d) Experiment 4

Ram Tag No.	Sperm concentration (x 10^9)	Motility (%)
161	3.639	90
173	2.567	80
111	2.909	85
190	2.594	95
137	3.281	90

e) Experiment 5

Ram Tag No.	Sperm concentration (x 10^9)	Motility (%)
111	3.025	90
173	2.795	85
190	2.832	95
161	2.999	80

f) Experiment 6

Ram Tag No.	Sperm concentration ($\times 10^9$)	Motility (%)
161	2.738	85
173	3.081	80
111	3.595	85
190	3.252	90

g) Insemination experiment

Ram Tag No.	Sperm concentration ($\times 10^9$)	Motility (%)
190	2.738	90
1:07	3.081	95
111	3.281	85
161	2.233	95
173	2.909	85

Experiment 4

Factors: 3 x 4 x 5

1. Diluent: Tris v Tris+Milk-glucose v Milk
2. Storage period: 0 v 2 v 4 v 6 days
3. Replicates: 5 rams

Experiment 5

Factors: 5 x 4 x 4

1. Level of EDTA in Tris diluent: 0 v 5 v 10 v 15 v 20mM
2. Storage period: 0 v 2 v 4 v 6 days
3. Replicates: 4 rams

Experiment 6

Factors: 5 x 4 x 4

1. Level of EDTA in Milk diluent: 0 v 5 v 10 v 15 v 20mM
2. Storage: 0 v 2 v 4 v 6 days
3. Replicates: 4 rams

b) Factorial design for insemination experiment

Factors: 2 x 2 x 2

1. Diluent: Tris v Tris+Milk
2. Storage: 0 v 1 day
3. Depth of insemination: Cervical v Deep cervical

A total of 240 ewes were inseminated. These were divided into 8 groups of 30 each.

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