ZONA-FREE HAMSTER EGG PENETRATION BIOASSAY TO ASSESS FERTILITY OF BOVINE SEMEN

Brahmkshtri Balkrushna Pratapchandji
(ID No. DPV 91003)

Thesis submitted in part fulfilment
of the requirement for the degree of
DOCTOR OF PHILOSOPHY
in
ANIMAL GENETICS AND BREEDING
to
TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY

DEPARTMENT OF ANIMAL GENETICS
MADRAS VETERINARY COLLEGE
TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY
MADRAS
1995

Tamil Nadu Veterinary and Animal Sciences University

University Research Farm,
Madhavaram,
Madras-600 051.

CERTIFICATE

This is to certify that the thesis entitled **"ZONA-FREE HAMSTER EGG PENETRATION BIOASSAY TO ASSESS FERTILITY OF BOVINE SEMEN"** submitted in part fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL GENETICS AND BREEDING** to the Tamil Nadu Veterinary and Animal Sciences University, Madras, is a record of bonafide research work carried out by **BRAHMKSHTRI BALKRUSHNA PRATAPCHANDJI**, under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

Place : Madras-51
Date : 16.02.1995

(M.JOHN EDWIN)
Chairman

Approved

Chairman : **(M. JOHN EDWIN)** 27/7/95

Members : 1. **(A.R.KRISHNAN)** 27/7/95

2. **(A. MAHALINGA NAINAR)**

3. **(MATHEW C. JOHN)** 27/7/95

External Examiner : K.P. Ramesha 27/7/95

CURRICULUM VITAE

Name of the Candidate	:	**BRAHMKSHTRI BALKRUSHNA PRATAPCHANDJI**
Date of Birth	:	20.09.1962
Place of Birth	:	Deheli (Gujarat)
Major field of specialization	:	Animal Genetics and Breeding
Educational Status	:	Completed B.V.Sc. & A.H. in 1985 at College of Veterinary Science and Animal Husbandry, Anand (Gujarat). Completed M.V.Sc. (Animal Genetics and Breeding) in 1989 at College of Veterinary Science and Animal Husbandry. Anand (Gujarat) Joined Ph.D. Programme (full time) in February 1992.
Professional Experience	:	Worked as Senior Research Assistant in Gujarat Agricultural University, Anand from September 1985 to August 1989. Working as Assistant Research Scientist at Department of Animal Genetics and Breeding, College of Veterinary Science, Gujarat Agricultural University, Anand since September 1989.
Marital Status	:	Married
Permanent Address	:	At - Rudha, Po - Rajpara TA - Valia, Dist - Bharuch, Gujarat
Publications Made	:	One Research Article
Awards	:	GAU Merit scholarship, 1980. Senior Research Fellowship, CSIR, 1992.
Member ship of Professional Society	:	Gujarat Veterinary Council

Dedicated to

My

Brother – **Mohanbhai**

- A force behind my study

ACKNOWLEDGEMENT

I am greatly indebted to **Dr.M.John Edwin**, Chairman, Advisory Committee and Professor, University Research Farm, Madhavaram, Madras - 51 for immaculate guidance, encouragement and deep interest for the present study.

I am extremely thankful to **Dr.A.R.Krishnan**, Professor and Head, Department of Animal Genetics, Madras Veterinary college and member of the Advisory Committee for critical suggestions and inspiration during course of the study.

I am grateful to other members of the Advisory Committee, **Dr.A.Mahalinga Nainar**, Professor, Department of Animal Biotechnology and **Dr.Mathew C.John,** Professor and Head, Department of Wild Life Science, Madras Veterinary College for valuable advise on the subject.

I extend my sincere thank to **Dr.P.Thangaraju,** Technical Officer to Vice Chancellor, Tamil Nadu Veterinary and Animal Sciences University for his constructive suggestions during the study.

I convey my gratitude to **Dr.K.S.Raman, Dr.K.Venkoba Rao,** Professors, and **Dr.P.Kanakaraj, Dr.J.Kalatharan, Dr.P.S.Rahumathulla**, Associate Professors, Department of Animal Genetics for their cooperation for the period of this study.

I am highly thankful to **Dr.S.N.Sivaselvam** and **Dr.V.Venkatramanujam**, Associate Professors, Department of Laboratory Animal Medicine, Madras Veterinary College for their valuable help. I am also thankful to **Dr.A.Subramaniam**, Assistant Professor, Department of Animal Biotechnology and **Dr.T.Sivakumar**, Assistant Professor, Department of Livestock Production on account of their help for stastical analyses.

I convey my thanks to staff members of the Department of Animal Genetics for their help and others whose even little contribution matters for me.

I am greatful **Dr.S.L.Goswami,** Senior Scientist, N.D.R.I., Karnal and **Dr.K.P.Ramesha,** Scientist, N.D.R.I., Bangalore for their technical advise on the subject.

I thank **Dr.Kathiresan**, Associate Professor and **Dr.Palanisamy,** Assistant Professor for their help during the study.

Special thanks are also due to **Dr.C.G.Joshi, Dr.V.B.Kharadi, Dr.D.N.Rank** and **Dr.P.H.Tank** for their warm company and whole-hearted contribution. I thank **Dr.Tilak** and **Dr.Balasubramaniam** for their help.

I keep on record the help rendered by Department of Laboratory Animal Medicine, Madras Veterinary College, Laboratory Animal Information Service Centre, N.I.N., Hyderabad - 7 and Biopharmaceutical Corporation Limited, Bombay - 12 for supply of hamsters. I am highly thankful to Sabarmati Ashram Gaushala, Bidaj an undertaking of National Dairy Development

Board, Anand, Gujarat for providing semen samples with fertility records for the present study.

I thank the Council of Scientific and Industrial Research, Delhi for providing me the senior research fellowship during the course of the study. Moreover, I am also thankful to Gujarat Agricultural University for granting me deputation to undergo this study.

As a personal remark, I express my deep sense of gratitude for the blessings of my parents and encouragement by family members. Responsibility shared by **Anjoo** and patience shown by **Mudra** and **Viren** need not be shaped into the words.

Above all the blessing of almighty can not be expressed.

ABSTRACT

ZONA-FREE HAMSTER EGG PENETRATION BIOASSAY TO ASSESS FERTILITY OF BOVINE SEMEN

Name	:	**BRAHMKSHTRI BALKRUSHNA PRATAPCHANDJI**
Degree for which submitted	:	Ph.D (Animal Genetics and Breeding)
Chairman	:	Dr.M.John Edwin, Ph.D.
Department	:	Animal Genetics
College	:	Madras Veterinary College
University	:	Tamil Nadu Veterinary and Animal Sciences University
Year	:	1995

The various dosages of PMSG/HCG administered to golden hamsters resulted in significantly ($p<0.05$) highest recovery of (40.60 ± 2.57) oocytes per animal at 50/75 I.U. The interval (56 or 98 hrs) between their administration had no significant effect. Frozen semen of crossbred cattle and Murrah bulls were used in the Sperm Penetration Bioassay (SPB) study.

Swim up technique yielded significantly higher per cent of sperm with intact acrosome compared to swim down technique and initial sperm concentration of $5x10^7$ resulted in significantly ($p<0.05$) higher per cent of individual motility and livability.

Among the different capacitation agents attempted for inducing acrosome reaction of crossbred and Murrah buffalo spermatozoa CaI was found

to be rapidly acting in crossbred and Murrah buffalo sperm. A significant effect of CaI was recorded on most of the sperm characteristics studied except livability. The drastic effect of CaI was observed on decreasing individual motility and increasing completely acrosome reacted sperm in crossbred bull compared to buffalo sperm. Among the levels of pH tried 8.0 was found to influence significantly ($p<0.01$) the per cent completely acrosome reacted sperm and individual motility in both the species. The hypertonic medium of 400 mosmol/kg was found to be effective in increasing per cent completely reacted sperm of Murrah bulls, whereas 300 mosmol/kg was recorded to be good in case of crossbred bull sperm.

The gas phase, CO_2 vs air, had no significant ($p>0.05$) difference on SPB outcome whereas, sperm concentration in the insemination medium significantly ($p<0.01$) influenced Fertilization Index (FI).

Maximum hypo-osmotic swelling per cent was recorded after 15 minutes of incubation of Murrah bull's sperm in triple glass distilled water.

Results on SPB revealed mean ± S.E. of Fertilization Percentage (FP) and FI values for crossbred bull sperm as 94.91 ± 1.23 and 2.15 ± 0.29 and for Murrah bull sperm as 92.15 ± 2.71 and 1.97 ± 0.14. The FP had significant correlation with conception rate in crossbred (0.723, $p<0.01$) and Murrah bull sperm (0.588, $p<0.05$). The FI was found to be highly correlated (0.660) with conception rate in Murrah bull sperm. FP and FI were highly significantly ($p<0.01$) correlated with crossbred (0.804) and Murrah (0.665) bull sperm. The post thaw individual motility and post thaw livability per cent were not correlated with conception rate in crossbred and Murrah buffalo sperm. Significant correlation (0.558) was observed between the HOST and conception rate in case of Murrah buffalo sperm.

CONTENTS

LIST OF TABLES

LIST FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

The widespread use of Artificial Insemination (A.I) in livestock breeding initiated scientists to persistently develop laboratory tests that would accurately predict the fertility of sires. Further, the ushering in of frozen semen technology had removed barriers of time and distance in A.I. As a result the sires potential is being exploited to the maximum extent. It is imperative that such sires are genetically superior as well as have high fertilizing capacity.

Many reports have been published concerning the relationship among sperm morphology, per cent motile sperm, sperm metabolism, sperm enzymes and sperm chemical constituents with fertility of bull sperm. However, most tests were poorly correlated with fertility (Graham *et al.*, 1980). These tests when performed by experienced technicians have been effective in controlling the quality of bovine frozen semen used for A.I. but cannot be relied upon to accurately predict fertility. For many years, in most of the semen banks the motility of bull spermatozoa after thawing has served as the essential parameter for judging the prospective fertility of deep frozen sperm.

Since 1970, acrosome morphology has been progressively integrated into the evaluation of deep frozen bull sperm. The acrosome morphology assumed significance following observance of the role of acrosome in fertilization. Freshly ejaculated sperm with intact acrosome are unable to fertilize eggs immediately even if they are brought directly into contact with them. Investigations proved capacitation and acrosome reaction to be essential preliminaries to normal fertilization of all mammalian species (Bedford, 1983).

When the need for evaluating capacitation and acrosome reaction capacity of spermatozoa was assuming importance, Yanagimachi *et al.* (1976) came out with their spectacular discovery that sperm from fertile men, following capacitation can penetrate zona free hamster eggs (ZFE). Since then heterologus sperm penetration studies have been successfully used in fertility evaluations. In fact zona free eggs of most mammalian species retain very strong or fairly strong species specificity rejecting entry of spermatozoa of most other species. The golden hamster (*Mesocricetus auratus*) is exceptional and permits sperm entry of wide variety of other species provided the sperm have completed capacitation and acrosome reaction (Yanagimachi, 1984).

This sperm penetration bioassay (SPB) has been widely used in human semen evaluations and World Health Organisation has also approved this test in the year 1982 (Yanagimachi, 1984). Sperm from many other livestock species were also reported to penetrate ZFE (Imai *et al.*, 1977; Lorton and First, 1979; Takahashi *et al.*, 1989). Various workers have reported positive correlation between the bioassay measures and fertility (Bousquet and Bracket, 1982; Ramesha, 1991).

With the advent of Assisted Reproductive Technology in human and livestock practice, procedure like *In Vitro* Fertilization and Embryo Transfer are becoming practical propositions. In such programmes SPB could be used in screening and selection of donors with high fertility.

This investigation was undertaken with the main objectives of standardising the protocol of SPB with reference to levels of gonadotrophins, time of administration and role of various agents

on inducing acrosome reactions. Following standardization of the technique, frozen semen samples of crossbred cattle and Murrah buffalo bulls (of known fertility status) were evaluated with SPB.

The correlation between SPB and semen parameters, fertility rates were analysed. The results of statistical analysis are discussed. This being the second investigation undertaken in India the results of this study will be useful in implementing this assay, especially in buffaloes to monitor the fertility status of bulls used in large scale A.I. operations.

Review of Literature

2. REVIEW OF LITERATURE

The fertilizing potential of spermatozoa is indirectly assessed on the basis of the evaluation of the standard semen parameters like sperm number, sperm motility and sperm morphology (Logue and Greig, 1987). However, there are controversial reports regarding the validity of these tests in prognosing male fertility (Stewart *et al.*, 1972; Ansbacher, 1978; Aitken *et al.*, 1982; Hafez, 1993).

With the advances in artificial insemination technique in farm animals, attention has been drawn towards development of more sensitive tests to evaluate male fertility potential (Rogers *et al.*, 1979; Bousquet and Brackett, 1982; Graham *et al.*, 1987; Takahashi *et al.*, 1989).

One of the most sensitive method developed so far is the test to judge the capacity of human spermatozoa to interact with zona free hamster ova *in vitro* (Yanagimachi *et al.*, 1976). Reliability of this test was assessed in several species (Bousquet and Brackett, 1982; Aitken *et al.*, 1985; Graham *et al.*, 1987; Takahashi *et al.*, 1989; Berger *et al.*, 1994). Since SPB involves sperm-egg interaction, it is envisaged that reviewing the information available on mammalian sperm fertilization will be beneficial.

2.1 FERTILIZATION IN MAMMALS

Fertilization is the process whereby haploid male and female gametes unite to form a diploid zygote with the potential to produce a complete

individual. The sperm and ovum are unusual because of their halved genetic content and they are designed to fuse together to accomplish their task.

2.1.1 FERTILIZATION EVENTS

The key events regulating fertilization are sperm capacitation and acrosome reaction, two interrelated phenomena which assist the transit of male gamete through the egg investments. In mammals, sperms undergo series of subtle changes collectively known as capacitation to acquire capacity to fertilize eggs (Chang, 1951). The completion of this process is mandatory before a physiological acrosome reaction could take place (Bedford, 1970).

The normal motility pattern is modified as a result of capacitation to a mode termed as hyperactivated motility. Hyperactivated motility is characterized by a highly flexuous flagellum rhythmically assuming contorted positions.

Following the acrosome reaction and interaction with the external surface of the zona, the sperm penetrates this acellular structure by combination of enzymatic and mechanical forces (Bedford, 1983). The release of acrosomal enzymes like hyaluronidase is believed to depolymerize the cumulus matrix and assist the passage of spermatozoa through the cumulus oophorus. Some other enzymes like acrosin are believed to dissolve zona material making entry of sperm possible through zona (Fraser, 1984; Meizel, 1985).

Once the sperm passes through the zona, it fuses with plasma membrane. As a result of the fusion the properties of egg plasma membrane change, resulting in plasma membrane block. This triggers the release of cortical granule material and is responsible for the block preventing polyspermy. This modification of zona pellucida, is termed as zona reaction (Johnson, 1975). The mechanism involved for the block to polyspermy varies with species. The eggs of golden hamsters depend totally on zona reaction. Parallel to these events, egg activation takes place, eventually resulting into the formation of pronucleus in 1-2 hours as evidenced by *in vitro* experiments (Xu *et al.*, 1987).

Activation of egg includes a resumption of second meiotic division resulting in extrusion of the second polar body and the formation of the female pronucleus. Decondensation and pronucleus formation from sperm head further consumes 2-6 hours (Barnes *et al.*, 1987). After DNA synthesis and migration of pronuclei to the centre of the egg, their nuclear envelopes disintegrate allowing direct mingling of their chromosomes for the first cleavage. This syngamy denotes the end of fertilization (Fraser and Ahuja, 1988). The time required between sperm entry and first cleavage varies with species, being more than 12 hours in most mammals.

2.2 SEMEN EVALUATION

The real test for the functional integrity of a semen sample is its capacity to fertilize the ovum and to sustain embryogenesis. For accurate assessment of fertility of semen sample many inseminations are required. The fertility parameter also involves female component which may influence the

results. Moreover, male animals differ in their inherent fertility and these differences may be compounded by differences in the ability of their semen to withstand freezing (Stewart *et al.*, 1974; Beatty *et al.*, 1976). It will be proper to describe various tests adopted to judge semen quality in relation to fertility.

2.2.1 MOTILITY

Motility of the spermatozoa is important for the transport through the female reproductive tract (Lightfoot and Restall, 1971). The assessment of motility involves visual appraisal of the per cent motile spermatozoa. Motility estimates immediately after freezing and thawing or following one or two hours of incubation at 37°C have been shown to be positively correlated with fertility but of low predictive value (Linford *et al.*, 1976). Jasco *et al.* (1992) reported approximately 20 per cent variation in fertility due to motility characters. Similarly no correlation between motility of bull sperm and fertility was recorded by Soderquist *et al.* (1991), although Roussel *et al.* (1963) reported better correlation (r=0.79) after post thaw incubation of bull semen for 5 hours at 38°C. Post thaw motility was found to be significantly correlated with fertility as reported by Hafez (1987) and Kjaestad *et al.* (1993). However, a contradictory opinion has been reported by Linford *et al.* (1976) indicating that the motility of bull spermatozoa after freezing and thawing has a poorer correlation with fertility than that of fresh diluted spermatozoa.

This review suggests that, although motility is normally a prerequisite for fertility, changes in the quality of motility are not necessarily be associated with differences in fertility. These various observations suggest that it should

be desirable to consider some other parameter(s) more closely correlated with fertility.

2.2.2 LIVABILITY

To differentiate live and dead spermatozoa, a stain mixture of eosin and opal blue was reported by Lasely *et al.* (1942). The supravital stain is taken up by a cell with a damaged plasma membrane whereas a functional membrane resists permeation. Several stain mixtures (Hackett and Macpherson, 1965; Entwistle, 1972) have been used to estimate the percentage of live spermatozoa at the time of staining. Difficulty arises in interpretation of results because of tendency for some cells to stain partially.

Results of differential staining under controlled conditions have been correlated with fertility (Hulet *et al.*, 1964; Linford *et al.*, 1976) but the predictive value is low.

2.2.3 MORPHOLOGY

Differential and morphological stains (Hackett and Macpherson, 1965) reveal structural abnormalities of spermatozoa. Gross structural changes are not generally caused by preservation, however certain cell organelles, like acrosome, may show structural changes. Light microscopy of Giemsa stained semen smears reveal disruption of the acrosome in frozen bull spermatozoa (Hancock, 1952; Watson, 1975). Furthermore, Saacke and Marshall (1968) using differential phase interference microscopy of unfixed bull spermatozoa

recorded changes in acrosome similar to those seen with Giemsa stain. Such alterations revealed by light microscopy must be of relatively major degree.

A significant correlation between morphologically normal sperm in ejaculates and the per cycle fertility estimates were recorded for stallion sperm (Jasko, *et al.* 1992). Out of three different types of acrosomal aberrations studied in bull spermatozoa by Andersson *et al.* (1990), swollen apical segment was found to be associated with fertility. Hafez (1987) and Soderquist *et al.* (1991) had reported negative and significant correlation between per cent abnormal sperm and fertility.

Changes in acrosome inducing acrosome reaction with chondroitin sulfate or heparin was reported to be correlated with fertility (Ax *et al.*, 1985; Lenz *et al.*, 1988; Takahashi *et al.*, 1993). Similarly significant correlation ($r = 0.81$, $p < 0.01$), was obtained between the fertility of bull predicted based on acrosome reaction and non return rate (NRR) by Whitefield and Parkinson (1992). While, Berger *et al.* (1994) reported no correlation between ability of sperm to undergo acrosome reaction and fertility.

2.2.4 SPERM CONCENTRATION

Sperm concentration is estimated by hemocytometer method (Hafez, 1993). The linear effect of number of spermatozoa in the inseminate has been reported with fertility (Allison and Robinson, 1971). Conception rate in cattle was beneficially increased by 7.3 per cent by increasing concentration of spermatozoa from 0.5 million per insemination to 2.5 million per insemination. Longford and Marcus (1982) have reported drop in lambing percentage when

inseminated below 100 million spermatozoa per insemination against 68% at 200 to 400 million spermatozoa per insemination. Dose dependent effect of semen used for artificial insemination was reported by Gerald and Humblot (1991) for bull spermatozoa.

2.2.5 HYPO-OSMOTIC SWELLING TEST (HOST)

This is a simple and economical test used as a tool in evaluating fertilizing ability of human spermatozoa (Jeyendran, *et al.*, 1984). The dilution rate of 1 : 10 and incubation time of 15 minutes for bull sperm was found to be optimum to get best curling percentage as reported by Solanilla *et al.* (1993b). The percentage of spermatozoa with positive response in HOST tended to increase with increasing time of incubation in bull spermatozoa (Solanilla *et al.*, 1993a) and functional integrity of the sperm membrane.

Sivaramalingam (1994) reported good agreement in tail curling percentage and conception rates in a preliminary trial using bovine frozen semen. This test highly correlates with the ability of human spermatozoa to become capacitated and penetrate zona free oocytes (Jeyendran *et al.*, 1984). On the contrary, no correlation between HOST and SPB was recorded by Chan *et al.* (1987,1988) and Takahashi *et al.* (1990).

Takahashi *et al.* (1990) have recorded weak correlation between HOST with sperm concentration and motility, while positive correlation with per cent live acrosome reacted spermatozoa. In contrast, highly significant correlation was recorded between hypo-osmotic swelling and motility (Jeyendran *et al.*, 1984; Kumi-Diaka, 1993; Kumi-Diaka and Badtram, 1994). Similarly high

correlation between hypo-osmotic swelling with acrosome reaction and intact acrosome was reported by Kumi-Diaka and Badtram (1994) and Sivaramalingam (1994).

2.2.6 SPERM PENETRATION BIOASSAY

The evaluation of sperm fertility is based on the penetrability of the sperm into zona free hamster ova. This technique has been widely used to evaluate the fertility of human spermatozoa used for *in vitro* fertilization (Barros, *et al.*, 1978; Rogers *et al.*, 1979). This test involves number of steps including the preparation of zona free oocytes from golden hamster, preparation of sperm, sperm-egg interaction and the evaluation of penetration of the ova.

SPB is reported to be positively correlated with fertility potential in human (Rogers *et al.*, 1979; Cohen *et al.*, 1982), bovine (Bousquet and Brackette, 1982; Ramesha, 1991) and several other species like ram (Tateno *et al.*, 1990), boar (Ivanova and Mollova, 1993) and buck (Berger *et al.*, 1994). However, some contradictory results have also been reported by Eaglesome and Miller (1989) and Binor *et al.* (1982). Hafez *et al.* (1993) stressed further need to examine the reliability of the test in predicting fertility.

2.3 PREPARATION OF OOCYTES

2.3.1 EXPERIMENTAL ANIMAL

It is evident from the literature that species specificity exists at zona pellucida and vitelline membrane during the process of fertilization. This hinders cross fertilization between the species. However, Yanagimachi *et al.*

(1976) demonstrated that the golden hamster (Mesocricetus auratus) is an exception. Its zona free eggs (ZFE) permit entry of wide variety of spermatozoa of other species provided the spermatozoa have completed the process of capacitation and acrosome reaction (Table 2.1, Yanagimachi, 1984). This quality of the ZFE can be beneficially utilized to study fertilizing capacity of spermatozoa of various species. This approach was tried initially in human and subsequently it has been attempted in several other species like boar (Imai *et al.*, 1977), bull (Bousquet and Brackett, 1982), stallion (Brackett *et al.*, 1982) and ram (Pavlok and Flechon, 1985).

Most of the investigators have used adult female hamsters (Rogers *et al.*, 1979; Aitken *et al.*, 1982; Graham *et al.*, 1986; 1991; Berger, 1994) while others have tried younger animals (Samper *et al.*, 1989; Ramesha, 1991) for the purpose of the study.

2.3.2 SUPEROVULATION

Superovulation in animals is intended to increase the number of recoverable oocytes. To have optimum response, it is important to know the reproductive physiology of the hamster.

2.3.2.1 Development of hamster oocyte

The germ cells reach resting stage eight days after birth. The resting phase is followed by growth phase in which development of follicles occurs upto sixteen days (Chiras and Greenwald, 1977). Development pattern observed in the hamster consists of initial growth of small follicle possessing one or more

Table 2.1 **Penetration of zona-free eggs by spermatozoa of homologous and heterologous species (Yanagimachi, 1984)**

Spermatozoa	Zona-free eggs from					
	Golden hamster	Chinese hamster	Mouse	Rat	Guinea pig	Rabbit
Golden hamster	Yes	No	Yes/No	Yes/No	Yes	No
Chinese hamster	-	Yes	-	-	-	-
Mouse	Yes	-	Yes	Yes	Yes	Yes
Deer mouse	Yes	-	No	No	-	-
Rat	Yes	-	Yes/No	Yes	-	Yes
Guinea pig	Yes	-	No	No	Yes	No
Bat	Yes	-	-	-	-	-
Rabbit	Yes	-	-	-	-	Yes
Dog	No	-	-	-	-	-
Dolphin	Yes	-	-	-	-	-
Pig	Yes	-	-	-	-	-
Bull	Yes	-	-	-	-	-
Goat	Yes	-	-	-	-	-
Horse	Yes	-	-	-	-	-
Marmoset monkey	Yes	-	-	-	-	-
Human	Yes	No	No	No	No	-
Sheep**	Yes	-	-	-	-	-
Buffalo*	Yes	-	-	-	-	-

* Takahashi *et al.*, (1989)

**Pavlok and Elechon (1985)

layers of granulosa cells followed by formation of the larger preantral follicles which subsequently results in antral or preovulatory follicles (Richards, 1978). Oocytes are surrounded by eight or more layers of granulosa cells on day one of the four day oestrus cycle and on day two antra begin to appear. During last two days of the cycle about five to six follicles in each ovary become vesicular, hyperemic and very large. Equal number of large follicles go atretic which can be halted by either unilateral ovariectomy resulting in compensatory ovarian hypertrophy or by superovulation with hormones (Richards, 1978). Further 5-30 i.u. pregnant mare's serum gonadotrophin (PMSG) prevents atresia and a dose dependent effect of recruitment was observed at higher doses of PMSG. Hormone injection not only interrupts the normal follicular stimulating hormone pattern, it seems to recruit next set of antral follicles which ovulate ahead by one cycle (Greenwald, 1978).

2.3.2.2 Gonadotrophins

Yanagimachi *et al.* (1976) used PMSG and HCG for stimulating growth of follicles and for ovulation respectively in golden hamster. Since then most of the investigators have used PMSG and HCG for superovulation of hamster (Table 2.2).

2.3.2.2.1 Dosage

Most workers have used 25-50 i.u. of PMSG and HCG for inducing superovulation in golden hamster (Table 2.2). However, its administration varied from 10/20 i.u. (Rogers *et al.*, 1979; Bird *et al.*, 1989; Bird and Houghton, 1989; Horan *et al.*, 1992) to 100 i.u.(Slavik, 1987). Intraperitoneal

Table 2.2 Experimental protocols for superovulation in golden hamsters

Dosage (I.U.)		Duration (hour)		Digestion of cumulus/ zona		Handling medium	Reference(s)
PMSG (route)	HCG (route)	PMSG to HCG	HCG to sacrifice	Hyaluronidase conc.(%)	Trypsin (%)		
25	25	48-51	14-16	0.10	0.05 (pronase)	-	Hanada & Chang (1972)
20 (I/P)	10 (I/P)	52-56	15-16	0.10	0.10	BWW	Rogers *et al.* (1979)
25 (I/P)	25 (I/P)	48-52	14-16	0.10	0.10	KRB	Imai *et al.* (1979)
30 (I/P)	30	48	15-17	0.10	0.10	BWW	Binor *et al.* (1980)
25 (I/P)	25	56-58	14-16	0.05	0.03	TMPA	Hall (1981)
30 (I/P)	25 (I/P)	48	15-17	0.10	0.05 (pronase)	BWW	Hanada & Nagase (1981)
40 (I/P)	40	48?	17-18	0.10	0.10	BWW	Aitken *et al.* (1982)
30 (I/P)	20-25	48-54	15-16	0.10	0.10	TMPA	Cohen *et al.* (1982)
25-30 (I/P)	25 (I/P)	60?	15-17	0.10	0.10	BWW	Martin (1983)
40	50	48-56	16	0.10	0.30	mBWW	Urry *et al.* (1983)
25 (I/P)	25 (I/P)	60	16	0.10	0.10	TMPA or M-2	Barros *et al.* (1984, 1988)
100	100	72-78	16-18	190 TRU/ml	0.02 (α-chymo)	CM	Pavlok & Flechon (1985) and Slavik (1987)
25-40 (I/M)	25-40	58	17	0.10	0.10	BWW	Kamiguchi & Mikamo (1986)

Dosage (I.U.)		Duration (hour)		Digestion of cumulus/ zona		Handling medium	Reference(s)
PMSG (route)	HCG (route)	PMSG to HCG	HCG to sacrifice	Hyaluronidase conc.(%)	Trypsin (%)		
25	20	49-53	-	0.10	0.10	TALP	Graham *et al.* (1986,1991), Graham & Foote, (1987b), Davies *et al.* (1987), Eaglesome & Miller (1989), Williams *et al.*, (1991)
25-40 (I/M)	25-40 (I/M)	58	17-18	0.10	0.10	mBWW or TYH	Tateno & Mikamo (1987) and Tateno *et al.* (1990)
25	25	49-53	15	0.10	0.10	TALP	Graham *et al.* (1987), Graham & Foote (1987a)
40 (I/P)	50 (I/P)	98	14-16	0.10	0.10	mBWW	Creighton & Houghton (1987)
25	25	54-72	16-17.5	-	-	TBM	Berger & Horton (1988)
30 (I/P)	30	55-60	15-18	0.10	0.10	mBWW	Osser *et al.* (1988)
20 (I/M)	20 (I/P)	72	17	0.15	0.15	BWW	Bird & Houghton (1989)
40 (I/P)	25	55	18	0.10	0.30	TCF	Samper *et al.* (1989)
40	40	55	18-20	0.10	0.03	CFT	Ijaz & Hunter (1989a,b), Ijaz *et al.* (1989)
30 (I/P)	30	54	15-16	0.10	0.05	mKRB	Takahashi *et al.* (1989)
20 (S/C)	20 (I/P)	72	15-17	0.15	0.15	BWW	Bird *et al.* (1989) and Horan *et al.* (1992)
30 (I/P)	30	58	15-17	-	acidic pH	mTALP	Richards *et al.* (1990)
25 (I/P)	25 (I/P)	58	16	0.1	(α-chymo)	BWW	Sharma and Das (1992)
40	40	49-53	-	0.10	0.05	TALP	El-Gaafary *et al.* (1993)
50 (I/P)	50 (I/P)	98	17¼	0.10	0.10	mBWW	Ramesha & Goswami (1994a,b)

(I/P) route as portal of entry has been preferred over other routes like intramuscular (I/M), (Kamiguchi and Mikamo, 1986; Bird and Houghton, 1989; Tateno *et al.*, 1990) or subcutaneous route (S/C), (Bird *et al.*, 1989; Horan *et al.*, 1992).

2.3.2.2.2 Duration between PMSG and HCG administration

PMSG is administered to accelerate the follicular growth which is followed by HCG injection to induce rupture of the follicles. Minimum interval required between these two treatments was observed to be 48 hours (Table 2.2). HCG has also been administered as late as 98 hours following PMSG (Creighton & Houghton, 1987; Ramesha, 1991).

2.3.2.2.3 Duration between HCG injection to oocyte recovery

In normally cycling hamster, ovulation generally occurs after 14 hours of lutenizing hormone surge. Hence, most experiments were designed to recover oocytes after 15 hours of HCG treatment. Summary of the reviewed literature under Table 2.2 indicates optimum recovery of oocytes between 15 to 17 hours by most investigators. Oocytes have also been collected as early as 14 hours following HCG treatment (Imai *et al.*, 1979; Creighton and Houghton, 1987). Ijaz and Hunter (1989a) and Ijaz *et al.*, (1989) reported penetratable oocyte recovery till 20 hours post-HCG administration. Oocytes recovered 20-22 hours after HCG treatment is less penetratable as reported by Berger (1983).

2.3.2.2.4 Oocyte recovery

Wide variation in recovery of oocytes after superovulation was observed ranging from 30 to 60 oocytes per animal in golden hamster. Yanagimachi *et al.*(1976) reported average 200 ova from 6-7 females. Martin (1983), Pryor (1986), Kamiguchi and Mikamo (1986) and Ramesha (1991) recovered 40-60 oocytes per animal.

2.3.3 TREATMENT OF OOCYTES

Unfertilized oocytes recovered in the form of cumulus mass from oviducts are processed further to yield ZFE.

2.3.3.1 Handling medium

As simple as BSA-saline was reported to be used for the handling of the oocytes (Ijaz and Hunter, 1989a; Ijaz *et al.*, 1989). The most commonly used media as revealed from the Table 2.2 are either Biggers, Whitten and Whittingham medium (BWW) or Tyrode's solution (TAMP/TMPA/TALP) with minor modifications. The handling medium employed have been supplemented with bovine serum albumin (BSA) and used in the pH range of 7.2-7.5 by most investigators. The same medium was also used for the preparation of hyaluronidase and trypsin solutions required for the digestion of the cumulus and zona respectively.

2.3.3.2 Digestion of cumulus mass

Cumulus mass contains large number of oocytes which are released free by treating cumulus mass with glycolytic enzyme hyaluronidase. Hyaluronidase takes five to ten minutes to digest cumulus depending on its concentration and releases oocytes containing zona pellucida. Most adopted concentration of hyaluronidase is 0.1 per cent (Table 2.2).

2.3.3.3 Digestion of zona pellucida

Trypsin, being proteolytic in nature, have been used by most workers to digest zona pellucida at concentration of 0.1 per cent (Table 2.2). It takes 30-60 seconds to remove zona pellucida (Fig.2.2). Effect of the trypsin can be nullified by transferring and washing ZFE in 2-3 drops of the medium. Zona pellucida have also been digested by other proteolytic enzymes like pronase, alpha chymotrypsin, diapase, mercaptoethanol, dithiothreitol as reported by Hoshi *et al.*, (1982) and by treatment with high acidic pH (Richords *et al.*, 1990).

2.4 CAPACITATION OF SPERMATOZOA

The ejaculated spermatozoa immediately after release from male reproductive tract are unable to fertilize oocytes despite being mature morphologically as well as motile. The spermatozoa must undergo a post-release maturation phase termed capacitation which is obligatory for mammalian spermatozoa to attain full fertilization potential (Chang, 1951). Capacitation of the spermatozoa can be characterized by change in morphology

of acrosome because of vesicual formation as a result of fusion of outer acrosomal membrane with the overlying plasma membrane. Capacitation results in a decrease in the net negative charge, an efflux of membrane cholesterol and influx of calcium between the plasma and outer acrosomal sperm membranes (Langlais and Roberts, 1985). Upon completion of capacitation the spermatozoa attain the capacity to undergo acrosome reaction, resulting in the release of lytic enzymes and membrane alterations necessary for sperm egg fusion.

The enzymes released by acrosome reaction include hyaluronidase and the trypsin like protease acrosin which are thought to aid sperm passage through the zona pellucida which surround the egg (Yanagimachi, 1981; Harrison, 1982; Miezel, 1985). The acrosome reaction is believed to occur in the immediate vicinity of the egg, however there is no concensus on its exact site. The cumulus cell mass and zona pellucida are probable sites for the acrosome reaction as evident from *in vivo* and *in vitro* studies (Bedford, 1983; Miezel, 1985).

2.4.1 MEDIUM

Various media were tried by different investigators for the handling of spermatozoa (Table 2.3). However, BWW and TAMP with suitable modifications have been more commonly used. The pH used for the medium ranges from 7.2-7.5. The medium has been suitably adjusted to give tonicity of 290-315 mosmol/kg (Table 2.3).

Table 2.3 Capacitation of spermatozoa

Species	Medium			Capacitation agent	Preincubation time	Reference(s)
	Type	Osmolarity (mosmol/kg)	pH			
Boar	KRB	-	-	Oviduct or uterus	4 to 10 h	Imai *et al.* (1977, 1979)
Boar	TCM 199	300	7.45	Liposome (20 µm for 7 min)	0	Graham *et al.* (1986) 1987
Boar	mBWW	-	7.4	Bovine follicular fluid	4-6 h	Creighton & Houghton (1987)
Boar	mBWW	310	7.35	A23187 (0.5 µm for 1 min)	-	Bird & Houghton (1989)
Boar	BWW	312	7.3-7.4	A23187 (0.5 µm for 1 min)	-	Horan *et al.* (1992)
Boar	TBM	-	7.7	Ionic pH	4-5 h	Ivanova & Mollova (1993)
Bull	DM	-	-	Hypertonic (380-390) mosmol/kg for 5 min)	30-60 min	Bousquet & Brackett (1982)
Bull	KRB	-	-	Hypertonic (370-380 mosmol/kg for 10 min.)	-	Bondioli & Wright Jr. (1983)
Bull	mTALP	-	-	Heparin(10 µg/ml) chondroitin sulphate (10 µg/ml)	6 h	Parrish *et al.* (1985)
Bull	TALP	-	7.4	Liposome (20 µm for 7 min)	0	Graham *et al.* (1986) 1987
Bull	mBWW	-	7.2	A23187 (10-15 µm for 10 min)	3-3.5 h	Tateno & Mikamo (1987)
Bull	mBWW	312	7.35	A23187 (0.5 µm for 1 min.)	-	Bird *et al.* (1989)
Bull	BSA-saline	-	7.2 or 7.6	Test-yolk buffer	to 48 h at 4°C	Ijaz & Hunter (1989a)
Bull	BSA-saline	-	7.2-8.4	Ionic pH (7.2 to 8.4)	0 to 8 h	Ijaz & Hunter (1989b)
Bull	mTALP	-	-	Liposome (20 µm for 7 min)	-	Eaglesome & Miller (1989)
Buffalo	mKRB	-	-	A23187 (0.5 to 2.0 min)	-	Takahashi *et al.* (1989)

Species	Medium			Capacitation agent	Preincubation time	Reference(s)
	Type	Osmolarity (mosmol/kg)	pH			
"	mBWW	290-300	7.4	A23187 (5 to 15 µm for 8 min)	3 h	Ramesha (1991)
Goat	mKRB	-	7.4	Storage	4 h	Anand *et al.* (1989)
Goat	TBM	335	7.85	Ionic pH	1-12 h	Berger *et al.* (1994)
Hamster	TALP	-	-	Storage	0-7 h	Barros *et al.* (1984)
Human	BWW	305	-	Hypertonic (410 mosmol/kg, for 5 min).	-	Aitken *et al.* (1985)
Ram	-	304	7.4	A23187	2 h	Shams-Bohran & Harrison (1981)
Ram	CM	-	-	Storage	15 - 20 h	Pavlok & Flechon (1985)
Ram	mBWW	308	8.0	Hypertonic medium (360 mosmol/kg, 5 min)	12 h	Thompson & Cummins (1985)
Ram	TALP	300	7.45	Liposome (20 µm for 7 min)	0	Graham *et al.* (1987, 1991)
Ram	mTCM 199	-	7.2-7.5	Storage	to 10 h	Slavik (1987)
Ram	TYH	-	7.2	A23187 (10-15 µm for 10 min)	1.5 - 2.0	Tateno *et al.* (1990)
Ram	mTALP	-	-	A23187 (0.5 µg/ml heparin Na (10µg/ml)	-	Williams *et al.* (1991)
Ram	BWW	-	-	Storage	to 24 h	Garde *et al.* (1993)
Stallion	BWW	300	7.45	Liposome (20 µm for 7 min)	0	Graham *et al.* (1987)
Stallion	TCF	300-310	8.0	Ionic pH (8.0 pH)	to 10 h	Samper *et al.* (1989)
Stallion	TALP	300	7.45	A23187 (0.1 µm for 10 min)	0 to 2h	Zhang *et al.* (1990)

Capacitation medium employed by most investigators was found to be supplemented with BSA (Table 2.3) at varying levels. BSA at 3.5 per cent has been commonly used in the medium (Overstreet *et al.*, 1980; Yanagimachi 1984; Tateno and Mikamo, 1987; Tateno *et al.*, 1990; Ramesha, 1991).

2.4.2 WASHING OF SPERMATOZOA

The first and important step adopted by various workers to induce acrosome reaction is removal of seminal plasma to get rid of decapacitation factor present in seminal plasma (Oliphant *et al.*, 1985). Under *in vivo* conditions seminal plasma gets removed during passage through mucus secretion of the female genital tract. *In vitro* this has been achieved either by sperm storage at 38°C (Barros *et al.*, 1984; Samper *et al.*, 1989; Zhang *et al.*, 1990) or treatment of various capacitation agents in order to accelerate acrosome reaction (Imai *et al.*, 1977; 1979; Bousquet and Brackett, 1982; Tateno and Mikamo, 1987; Bird *et al.*, 1989; Table 2.3).

2.4.3 PROTEIN SUPPLEMENTATION OF MEDIUM

Most widely used material as protein source is BSA fraction V. The capacitation medium prepared after supplementation with addition of BSA contained concentrations of BSA from 0.3 per cent (Takahashi *et al.*, 1989) to 3.5 per cent (Kamiguchi and Mikamo, 1986; Tateno and Mikamo, 1987; Ramesha, 1991, Table 2.4).

2.4.4 RECOVERY OF MOTILE SPERMATOZOA

In vivo, the potentially functional spermatozoa population is separated from liquified semen by migration into cervical mucus (Montimer, 1983). Consequently, sperm preparation technique commonly involves self migration step. There are several methods in vogue including 'swim up' and 'swim down' from post-thawed washed spermatozoa.

After centrifugation of motile sperm suspension, the sperm will begin to move upwards from sediment into the supernatant in swim up technique (Drevius, 1971). This principle was utilised to recover highly motile sperm (Tateno *et al.*, 1990; Ramesha, 1991).

Alternatively, gravity causes sperm head to turn downwards after which the oriented spermatozoa continue to move down their own tail movements in swim down technique (Makler *et al.*, 1993). Gonzales and Zapana (1992) successfully increased progressive motility from 42 ± 2 to 77 ± 6 per cent using swim down method. Gonzales and Pella (1993) recovered motile sperm employing swim down technique using pure serum and recorded higher sperm recovery and motility rate in comparison to swim up technique.

2.4.5 CAPACITATION AGENTS

Various capacitation procedures have been used in different laboratories and are summarized in Table 2.3. As simple a method, as storage or incubation of spermatozoa after washing with medium have been tried (Imai *et al.*, 1979; Barros *et al.*, 1984; Slavik, 1987; Zhang *et al.*, 1990) to perform SPB. This

method has disadvantage as it requires longer preincubation period for preparation of spermatozoa. Preincubation period have been shortened either by use of hypertonic medium (400 mosmol/kg, Bondioli and Wright Jr., 1983) or medium with alkaline pH (around 8.0 pH, Ijaz and Hunter, 1989b). Various chemical agents like heparin (Parrish *et al.*, 1985), dilaurophosphatidylcholine (Graham *et al.*, 1986) and calcium ionophore (CaI), (Tateno and Mikamo, 1987, Table 2.3) have also been tried to accelerate capacitation of the spermatozoa.

2.4.5.1 High pH medium

The pH of medium affects the ionization of substances within it including the proteins. Under *in vivo* condition capacitation is reported to occur in the female genital tract. Studies have shown that the pH of the bovine oestral fluid to be 7.91 (Gupta, 1962) and that of rabbit oviductal fluid 7.8 (Hamner and Williams, 1965). However, most *in vitro* procedures are performed between the pH range of 7.2 and 7.6 (Rogers, 1978).

Cheng (1985) and Behnke (1987) increased the pH of calcium free tyrode's (TCF) solution to 8.5 to capacitate bull sperm and obtained higher fertilization rates of bovine oocytes than that achieved using other systems (Irritani *et al.*, 1981; Brackett *et al.*, 1982). Murphy and Yanagimachi (1984) have reported induction of acrosome reaction at pH 8.2-8.4 in guinea pig spermatozoa incubated either in TCF or in medium containing calcium. Peak acrosome reaction was recorded at pH 8.0 in rabbit (Oliphant *et al.*, 1977). Ijaz and Hunter (1989b) studied effect of pH of the medium on acrosome reaction and penetration of ZFE using bovine spermatozoa and recorded increase in acrosome reacted sperm with increase in pH of the medium.

2.4.5.2 Hypertonic medium

Washing sperm in buffered hypertonic salt solution prior to incubation has been demonstrated to accelerate capacitation *in vitro* in mouse (Oliphant and Brackett, 1973) and rabbit (Brackett and Oliphant, 1975; Brackett *et al.*, 1978). Hosoi *et al.*, (1981) reported isotonic medium to be equally good in capacitating rabbit epididymal sperm.

Bovine spermatozoa have been capacitated *in vitro* using hypertonic or high ionic strength medium by Brackett *et al.*, 1980; 1982). Bondioli and Wright Jr., (1983) have reported that standard medium is equally effective as hypertonic medium in inducing *in vitro* capacitation of the bovine spermatozoa. Bousquet and Brackett (1982) have reported use of hypertonic medium for the preparation of spermatozoa for SPB for bulls. Thompson and Cummins (1985) have reported increased acrosome reaction percentage by treating ram spermatozoa with hypertonic medium (Table 2.3).

2.4.4.3 CaI A23187

The acrosome reaction which follows capacitation (Bedford, 1970) has been shown to be calcium dependent. It has been shown that acrosome reaction could be induced *in vitro* in several species, by treating the spermatozoa with calcium in the presence of bivalent cation A23187 (Reed and Lardy, 1972; Reyes *et al.*, 1978; Shams-Bohran and Harrison, 1981; Cooper, 1984; Tateno and Mikamo, 1987; Takahashi *et al.*, 1989; Table 2.3).

Shams-Bohran and Harrison (1981) have tried 0.01, 0.1, 1.0 and 10 µM concentration of CaI to induce acrosome reaction of ram spermatozoa and found dose dependent increase in reacted spermatozoa at different time intervals. Acrosome reaction has been induced using 10-15 µM concentration of CaI in bovine sperm (Tateno and Mikamo, 1987).

Bird and Houghton (1989) tried various concentration of CaI (0.01, 0.5, 1.0, 2.5 and 5.0 µM) for 1 min, in boar spermatozoa to induce acrosome reaction. They have reported 0.5 µM treatment as the optimum concentration to get maximum sperm penetration.

Stallion spermatozoa were reported to respond to the CaI at concentration of 0.1 µM by Zhang *et al.*(1990).

Bird *et al.* (1989) have treated bovine spermatozoa with CaI to induce acrosome reaction using a low concentration of 0.1 to 2.0 µM for one minute and reported dose dependent effect.

In a preliminary trial with buffalo spermatozoa, Takahashi *et al.*, (1989) used 0.01, 0.25, 0.5 and 1.0 µM of CaI for 0.5, 1.0 and 2.0 minutes and reported highest penetration rate at 0.25 and 0.50 µM concentration of CaI.

A higher concentration of CaI (5-15 µM) was used in an experiment involving human sperm chromosome study by Kamiguchi and Mikamo (1986).

The effects of various concentrations of CaI viz 0, 1.25, 2.5, 5.0, 10 and 15 µM and exposure times of 5, 8 and 10 minutes were investigated by

Ramesha (1991) in bovines. Both the treatments had significant effects on individual motility and acrosomal integrity.

Sarkadi *et al.* (1976) and Shams-Bohran & Harrison (1981) have reported role of albumin to bind with ionophore, thereby reducing the effective concentration associated with reduction in acrosome reaction.

2.4.5.4 Effect of capacitation agents on sperm characters

2.4.5.4.1 Individual motility

Individual motility of the spermatozoa is of interest because of its possible role during process of fertilization. Loss of motility is generally been observed with the ageing of the spermatozoa. Ijaz and Hunter (1989a) reported loss of motility of bovine spermatozoa over a period of time on incubation at 37°C. Similar observation has also been made with spermatozoa of other species like ram (Pavlok and Flechon, 1985; Williams *et al.*, 1991) and buck (Berger *et al.*, 1994).

Cooper (1984) recorded promotion in hyperactivated motility by increasing ionic strength of the medium using electrolyte. Ijaz and Hunter (1989) have observed gradual decline in motility of mouse at high ionic strength. Significantly fewer motile sperm cells with higher velocities were recorded following post-incubation period in hypertonic medium of 410 mosmol/kg as recorded by Aitken *et al* (1985). Ijaz and Hunter (1989b) have observed gradual decline in motility by increasing the pH of the medium from 7.2 to 8.4.

Bird *et al.* (1989) and Bird and Houghton (1989) reported adverse effect of CaI on sperm motility with bull and boar spermatozoa respectively. They preferred lower CaI concentration of 0.4-0.5 μM for 1 minute to induce acrosome reaction without drastic effect on individual motility. Dose dependent effect of CaI has been reported over the period of time on sperm motility by Takahashi *et al.* (1989) on buffalo spermatozoa. Shams-Bohran & Harrison (1981) have reported decline in motility with increase in CaI concentration and on incubation of ram spermatozoa after CaI treatment. They have reported beneficial effect of BSA for maintenance of motility.

Tateno and Mikamo (1987) and Tateno *et al.*, (1990) studied motility pattern of spermatozoa following treatment with CaI in bull and ram spermatozoa respectively. They have reported that the sperm head become sticky on adequate capacitation and 2-3 spermatozoa move together with head-head association.

Sperm motility was impaired by the increasing ionophore concentration and prolonging treatment time in goat sperm (Shorgan & Hanada, 1985) and bull sperm (Hanada & Nagase, 1981).

Although, addition of albumin is thought to sequester the CaI and also known to remove ionophore from plasma membrane, thus allowing extracellular calcium level and return of the sperm flagellar movements. Beneficial effect of presence of BSA in insemination medium was recorded on motility by Hanada & Nagase (1981) for bull spermatozoa.

During the process of acrosome reaction the spermatozoa are reported to exhibit hyperactivated motility. Hyperactivated motility is characterised by a highly flexous flagellum rhythmically assuming contorted positions that have prompted the descriptive terms such as "bobbling" (Gwatkin and Anderson, 1969), "serpentine" (Yanagimachi, 1972) or "whiplash" (Fraser, 1977) motility. Photographs or diagrams of hyperactivated spermatozoa from the hamster (Yanagimachi, 1970), guinea pig (Yanagimachi and Usui, 1974), mouse (Fraser, 1977) and ram (Cummins 1982) have been reported. All these studies revealed large amplitude bends in the flagellum to be their most recognizable feature. Change in the motility pattern in many mammalian species was observed both *in vivo* and *in vitro*. The biological role of such hyperactivated motility is the penetration of zona pellucida of eggs in mice (Fraser, 1981; 1982) and guinea pig (Fleming and Yanagimachi, 1982). Similar pattern of motility was also reported for the bull (Tateno and Mikamo, 1987) and ram (Tateno *et al.*, 1990) spermatozoa.

2.4.5.4.2 Acrosome reaction

Acrosome reaction which follows capacitation is characterized by the release of hydrolytic enzymes, the exposure of the inner acrosomal membrane and the modification of the sperm segment (Fraser and Ahuja, 1988).

Most of the investigators have characterized and recorded acrosome reaction by the loss of the apical ridge or acrosome (Whitefield and Parkinson, 1992; Edwin, 1985; Didion and Graves, 1986; Steinholt *et al.*, 1991). However, Shams-Bohran and Harrison (1981), Bousquet and Brackett (1982) and

Arvindakshan (1992) have recorded status of acrosome as intact, partially reacted and completely reacted.

Acrosome integrity was found to reduce in general, with the period of incubation irrespective of the capacitation agents. Efficiency of the capacitation agent can be evaluated by the time taken to induce complete acrosome reaction without much compromising on other traits like individual motility and livability.

Storage of spermatozoa after washing in an isotonic medium has resulted in increased acrosomal reaction (Anand *et al.*, 1989; Williams *et al.*, 1991; Varner *et al.*,1993; Berger *et al.*, 1994). Ijaz and Hunter (1989b) have reported increase in per cent acrosome reacted spermatozoa at different ionic pH of the capacitation medium. They have also reported marked increase in acrosome reaction percentage in bovine spermatozoa at higher ionic pH at different intervals.

Brackett and Oliphant (1975) reported increased incidence of the acrosome reaction by washing rabbit spermatozoa in hypertonic medium. Similar findings have also been reported for ram (Thompson and Cummins, 1985) and bull (Bousquet and Brackett, 1981; 1982) spermatozoa.

Most investigators reported rapid induction of acrosome reaction by CaI A23187 in several species. Acrosome reaction percentage were found to increase with the increase in CaI concentration for boar (Bird and Houghton, 1989) bull (Bird *et al.*, 1989) and ram (Shams-Bohran and Harrison, 1981)

spermatozoa. Percentage of reacted spermatozoa were also reported to increase with increase of duration after the treatment.

2.4.5.4.3 Livability

Viability of the spermatozoa is essential to achieve maximum sperm egg interaction. About fifty per cent of spermatozoa dies during freezing and thawing (Salisbury *et al.*, 1978). Therefore, quality of post thaw frozen semen has been improved by various techniques such as swim up (Tateno & Mikamo, 1987; Ramesha, 1991) or swim down (Gonzales and Pella, 1993).

Live sperm percentage of spermatozoa significantly reduced over the storage in incubator for bull (Parrish *et al.*, 1985), ram (Thompson and Cummins, 1985), dog (Kawakami *et al.*, 1993), stallion (Varner *et al.*, 1993) and buck (Berger *et al.*, 1994).

Parrish *et al.* (1985) recorded viability in the ejaculated bull spermatozoa kept at 39°C at different intervals. Significant loss of viability was recorded by them after 6 and 9.5 hours. Ram spermatozoa lose viability over the time in hypertonic media as recorded by Thompson and Cummins (1985). Loss of livability recorded for equine spermatozoa by Varner *et al.* (1993) in 4 hours was 10 per cent whereas, Kawakami *et al.* (1993) recorded loss of 7.7 per cent in 3 hours for canine spermatozoa.

2.5 SPERM-EGG INTERACTION

The sperm-drops consisting of the capacitated spermatozoa readily kept in incubator under light liquid paraffin have been inoculated immediately

with ZFE (Imai, *et al.*, 1977; Rogers *et al.*, 1979; Graham *et al.*, 1987; Bird *et al.*, 1989). Contrarily, ZFE were inoculated after pre-incubation of sperm for an additional 1.5 - 3.5 hours following CaI treatment (Tateno and Mikamo, 1987; Tateno *et al.*, 1990; Ramesha, 1991)

2.5.1 MEDIUM

A most commonly used medium for SPB is either Kreb Ringer's solution (e.g. BWW) or Tyrode's medium (e.g. TAMP/TMPA/TALP) containing energy sources and albumin (Table 2.4). The standard medium with osmolarity of 290-310 mosmol/kg and pH ranging from 7.2-7.5 have been widely used (Table 2.3). The BSA concentration in the medium varied from 0.3% to 3.5% (Table 2.4). The concentration of albumin is important as higher concentration induced acrosome reaction of spermatozoa faster resulting in higher sperm penetration.

2.5.2 COINCUBATION PERIOD

Immediately following penetration of spermatozoa, decondensation process begins. Sperm tails disintegrate and become undetectable by light microscopy after several hours of penetration (Yanagimachi, 1984). The coincubation of sperm and ZFE varied from 1 hour (Imai *et al.*, 1979) to 16/18 hours (Slavik, 1980; Pavlok and Flechon, 1985; Ivanova and Mollova, 1993). However, most commonly adopted period of coincubation is 3 hours for several species (Eaglesome and Miller, 1989; Bird *et al.*, 1989;1991; Williams *et al.*, 1991, Table 2.4.).

Table 2.4 Summary of experimental conditions for sperm penetration bioassay

Species	Medium	BSA concentration (%)	Gas phase	Sperm concentration x 10^6/ml	Coincubation (hour)	Scoring		Reference(s)
						FP	FI	
Boar	BWW	0.4	5% CO_2	0.67-13.9	7	0-100	-	Hanada & Nagase (1981)
"	mKRB	-	5% CO_2	0.2-11.1	1-8.5	0-100	-	Imai *et al.* (1977, 1979)
"	mBWW	1.0	5% CO_2	25	3	61.5-88.6	-	Creighton & Houghton (1987)
"	mBWW	1.0	5% CO_2	20	3	-	-	Horan *et al.* (1992)
"	TBM	0.5	5% CO_2	1-8	4.5-7.5	0-93	1.1-9.0	Berger & Horton (1988)
"	TBM	0.5	5% CO_2	2-4	16-18	25-63	-	Ivanova & Mollova (1993)
Bovine	mTALP	0.3	5% CO_2	15	1	18-69	-	Richords *et al.* (1990)
Bull	DM	-	5% CO_2	1-2	6	68.2-94.5	-	Bousquet & Brackett (1982)
"	mKRB	0	1% CO_2	10	-	-	-	Koehler *et al.* (1984)
"	TALP	0.6	5% CO_2	3-20	3	6-98	1.2-2.3	Graham *et al.* (1986, 1987)
"	mBWW	3.5	5% CO_2	0.01-0.1	2	72.6-92.1	-	Tateno & Mikamo (1987)
"	mBWW	1.0	5% CO_2	12.5	3	3.6-40.6	-	Bird *et al.* (1989)
"	mTALP	-	5% CO_2	6	3	0-100	1.0-3.4	Eaglesome & Miller (1989)
"	BWW	0.4	5% CO_2	0.67-13.9	7	0-100	-	Hanada & Nagase (1981)
"	mBWW	3.5	5% CO_2	5	2.5	59.1-82.6	0.61-0.84	Ramesha & Goswami (1994)

Species	Medium	BSA concentration (%)	Gas phase	Sperm concentration x 10^6/ml	Coincubation (hour)	Scoring		Reference(s)
						FP	FI	
Buffalo	mKRB	1.0	5% CO_2	5	3.5-4	0-50	-	Takahashi *et al.* (1989)
"	mBWW	3.5	5% CO_2	5	2.0	40-95.5	0.4-1.48	Ramesha (1991)
Goat	mTALP/BO	0.3	5% CO_2	1.12-14.4	3	0-94.4	0-2.53	Shorgan & Hanada (1985)
"	TBM	0.5	Air	10	-	0-100	0-7.0	Berger *et al.* (1994)
Hamster	mTALP	1.5	Air	1.5	2	-	-	Barros *et al.* (1984)
Human	BWW	0.3	Air	10	2	14-100	-	Rogers *et al.* (1979)
"	mBWW	3.5	Air	0.4-18	4	5-100		Overstreet *et al.* (1980)
"	mTMPA	1.0	5% CO_2	0.5-1.5	3	20-100	-	Hall (1981)
"	BWW	1.8	5% CO_2	10	3	14-90	-	Aitken *et al.* (1982)
"	TMPA	3.0	Air	2-4	2-2.5	0-100	-	Cohen *et al.* (1982)
Rabbit	mKRB	0	1% CO_2	10	-	-	-	Koehler *et al.* (1984)
Ram	mTCM 199	0.5	Air	0.2-0.8	16	0-100	0.5	Pavlok & Flechon (1985), Slavik (1987)
"	TALP	0.6	5% CO2	5	3	0-52	0.4-2.2	Graham *et al.* (1987, 1991)
"	TYH	3.5	5% CO_2	0.01-0.1	2	54.7-84.4	-	Tateno *et al.* (1990)
"	mTALP	0.6	5% CO_2	4.5	3	0-50	-	Williams *et al.* (1990)
Stallion	mKRB	0	1% CO_2	10	-	-	-	Koehler *et al.* (1984)
"	BWW/TALP	0.6	5% CO_2	1.5-5.0	3	0-58	1.0-4.6	Graham *et al.* (1987)
"	TCF	0.3	5% CO_2	10-50	3	0-87	0-7.8	Bird & Houghton (1989)
"	TALP	0.3	5% CO_2	0.25-2.5	3	56-68	1.5-3.1	Zhang *et al.* (1990)

2.5.3 GAS PHASE

Two different atmospheres used for the SPB are pure air or 5% CO_2 in air incubation. Most experiments were conducted using 5% CO_2 in air gas phase for the bioassay in livestock species (Table 2.4, Brackett *et al.*, 1982; Tateno & Mikamo, 1987; Bird *et al.*, 1989; Ramesha, 1991). However, coincubation in pure air incubator also have been reported for human (Rogers *et al.*, 1979; Cohen *et al.*, 1982; Urry *et al.*, 1983), ram (Pavlok and Flechon, 1985) and buck (Berger *et al.*, 1994) as revealed from the Table 2.4.

The pH of the medium was 7.5-7.6 when the preparation was incubated under 5% CO_2 while it was increased to 8.1.-8.3 under pure air incubation. Tyler *et al.* (1981) reported faster sperm penetration under pure air than under 5% CO_2 in air during 18 hours of preincubation of sperm in BWW medium.

On longer storage a gradual rise in pH is inevitable but covering sperm preincubation medium with mineral oil may slow down the rise in pH. The pH of sodium bicarbonate buffered media BWW or TAMP which have been most commonly used can be kept constant in 5% CO_2 in air incubator (Yanagimachi, 1984).

2.5.4 SPERM CONCENTRATION

The variation in sperm concentration of the insemination medium considerably affects bioassay outcome. Relatively higher sperm concentration was preferred (Table 2.4) in the insemination medium to which ZFE were introduced in most experiments involving SPB. Varying sperm concentration

of 10^3-10^4 (Tateno and Mikamo, 1987; Samper *et al.*, 1989; Tateno *et al.*, 1990) to 5 - 12.5 x 10^7 per ml. (Creighton and Houghton, 1987; Bird *et al.*, 1989) per ml of the medium have been tried (Table 2.4).

Consistently high penetration rates have been reported at sperm concentration of 5 x 10^6 (Binor *et al.*, 1980; Rogers *et al.*, 1983; Shorgan & Hanada, 1985; Ramesha, 1991) and at 10^6 per ml (Bousquet and Brackett, 1982; Bird and Houghton, 1989; El-Gaafary *et al.*, 1993; Garde *et al.*, 1993). The optimum sperm concentration has been reported between 10^6 - 10^7 per ml (Tyler *et al.*, 1981; Roger *et al.*, 1983). According to Tablot and Chacon (1981) only 10-20 per cent of the entire sperm population is physiologically acrosome reacted after 2-8 hours of preincubation. It may be higher or lower in other animals but if it is assumed that only 1 per cent of the spermatozoa are acrosome reacted among 10^6-10^7 spermatozoa, the total number of acrosome reacted spermatozoa would be rather high, although only a fraction of bovine spermatozoa bind to and penetrate ZFE. Some species specificity seems to operate on or in the plasma membrane of the hamster egg regarding attachment and penetration by spermatozoa of various species.

2.5.5 BSA CONCENTRATION

Protein is an essential component of any media used for *in vitro* capacitation and fertilization. In certain species such as mouse and hamster (Lui *et al.*, 1977; Bavister, 1981) albumin from bovine serum and follicular fluid has been identified as a major factor influencing the motility. Since then commercially prepared BSA has been extensively used for sperm incubation.

Most media used for the hamster test (SPB) contain energy sources and albumin at varying concentrations ranging from 0.3 to 3.5 per cent (Table 2.4). Concentration of BSA significantly affects capacitation of spermatozoa (Stewart-Sawage, 1993) and thereby the penetration. Ramesha (1991) and Stewart-Sawage (1993) also confirmed the effect of source of BSA on SPB. Yanagimachi (1984) has reported marked variation in quality of BSA of different batches under same catalogue and indicated trial use of new batch of BSA before its routine use.

Thompson and Cummins (1985) reported no difference in percentage acrosome reacted spermatozoa between the various protein supplementations although serum promoted better survival in ram spermatozoa. Tajik *et al.* (1993) have reported concentration dependent increase in sperm penetration or polyspermy with BSA, foetal calf serum (FCS) and calf serum (CS) supplementation using bovine oocytes. Notably higher penetration was reported with FCS than with or without BSA. The growth promoting effect of protein sources on mouse pronuclear stage embryos was reported to be due to its antioxidative property (Natsuyama *et al.*, 1991).

2.6 SCORING

Either four wax spots (Graham *et al.*, 1986) or two wax strips (Pavlok and Flechoń, 1985; Slavík, 1987) were prepared on clean, grease free glass slide by help of vaseline: paraffin mixture (10:1) filled in a syringe attached with a needle, so that drop of medium containing ZFE remained in the centre of the slide.

The slides prepared thus were fixed in methanol : acetic acid (3:1) (Graham *et al.*, 1986; Eaglesome & Miller, 1989) or ethanol : acetic acid (3:1) (Rogers *et al.*, 1979; Bird *et al.*, 1989) for overnight. The slides were stained with 0.5-2.0% aceto-orcein (Bird *et al.*, 1989; Eaglesome & Miller 1989) or 0.025-1.00 aceto - lacmoid stain (Rogers *et al.*, 1979; Graham *et al.*, 1986; Takahashi *et al.*, 1989). Later on scoring is done either by fertilization percentage and fertilization index or penetration percentage and penetration index discussed below.

2.6.1 FERTILIZATION PERCENTAGE (FP)

Takahashi *et al.* (1989) reported increase in FP with CaI treatment upto 0.5 µM and thereafter declined in FP at higher concentration of CaI in buffalo spermatozoa. Similar trend in FP was also reported by Bird *et al.* (1989) for bull spermatozoa and Bird and Houghton (1989) for the boar spermatozoa. Graham *et al.* (1986) have reported increase in FP upto 20 µM concentration of liposome followed by drop at higher concentration.

Ijaz *et al.* (1989) have reported 1.0 to 36.4 per cent penetration at varying storage periods of spermatozoa at 4°C for zero to 48 hours. Ijaz & Hunter (1989a) recorded FP of zero to 37.5% and zero to 31.6% after single and triple washing of bovine spermatozoa respectively. They have also reported zero to 28.9 per cent penetration for varying periods of incubation of bovine spermatozoa.

FP ranging from 89.3 to 100 and zero to 85.7 have been reported by Pavlok and Flechon (1985) after preincubation of ram spermatozoa for 1 to 6

hours and varying storage period at 22-24°C. They have also reported FP of 32-90.9 and 53.1-89.6 for fresh and for 120 hours stored spermatozoa respectively. Rams scored significantly different from each other with regards to FP when either stored or frozen thawed semen. Tateno *et al.* (1990) have reported FP for four rams ranging from 54.7 to 84.4.

Bousquet and Brackett (1982) recorded 68.2 vs 94.5 FP for two bulls with varying fertility potential as evident from field trials. Ivanova and Mollova (1993) reported penetration percents of 25.08 vs 66.03 for subfertile vs fertile boars respectively. Eaglesome and Miller (1989) have reported 91 vs 56 per cent FP using frozen bull semen and 60 vs 89 penetration with fresh semen for high vs low fertile bulls respectively.

Imai *et al.* (1977; 1979) reported zero to 100% penetration for various capacitation agents, different preincubation and coincubation periods with boar spermatozoa. The FP recorded by various investigators showed great variation as revealed from the Table 2.4. However, Rogers (1985) reported score range of 15-100 per cent can be considered as normal fertility range for human spermatozoa. This can be attributed to the different experimental conditions like composition of the medium used, capacitation agents, protein source, sperm concentration and gas phase etc.

2.6.2 FERTILIZATION INDEX

Fertilization index (FI) as a parameter for the scoring has less commonly been used than FP. The results of various experiments scored in form of FI is summarized in Table 2.4.

Eaglesome and Miller (1989) have reported FI of 2.7 vs 1.4 and 1.6 vs 3.0 for high vs low fertile bulls using frozen and fresh semen respectively. FI of 1.2 to 3.3 was recorded at varying concentrations of dilaurylphosphatydilcholine (liposome) by Graham *et al.* (1986) for bull spermatozoa. Bird *et al.* (1989) reported FI of zero to 1.5 at varying concentrations of CaI for boar spermatozoa. FI found to increase till 0.5 µM concentration (7.08 $\pm$ 1.9) from zero which gradually declined with the further increase in the concentration with boar spermatozoa.

Slavik (1987) recorded FI of 1.57 to 2.02 with increase in glycerol concentration from zero to 10 per cent in the medium. He also reported FI of 1.16 to 1.82, 0 to 2.22 and 1.14 to 2.57 over different exposure time to glycerol, preincubation and coincubation periods respectively. Pavlok and Flechon (1985) reported FI of 0.11 to 3.37, 0 to 2.02, 1.0 to 2.15, 1.07 to 1.6, 1.0 to 5.0 and 0 to 2.36 for different preincubation times, varying storage periods at 22-24°C, various coincubation periods for fresh vs stored spermatozoa, different rams using semen stored for 20 hours and frozen thawed semen respectively.

Berger *et al.* (1994) have reported FI ranging from zero to 7 averaging 1.51 and zero to 2.92 with mean of 0.54 after 1 hour and 12 hours of capacitation of buck spermatozoa.

2.7 CORRELATION

2.7.1 SEMEN PARAMETER WITH SPB

Several investigators attempted to study possible correlation of semen parameters like individual motility, acrosome status, livability, sperm

concentration and hormonal parameters with fertilization outcome *in vitro*. Aitken *et al.* (1982) have reported non-significant correlation between fertilizing capacity and semen parameters like motility, density, morphology and volume. No significant correlation between movement characteristics of human spermatozoa with that of SPB was reported by Aitken *et al.* (1985). Similarly, poor correlation of SPB with conventional seminal and hormonal parameters have been recorded by Osser *et al.* (1988). The correlation coefficient with sperm concentration and motile spermatozoa was reported to be 0.204 and 0.316 respectively for human sperm. Similarly non-significant correlation have been reported by Hall (1981) between sperm concentration, progressively motile spermatozoa and normal spermatozoa with *in vitro* fertilization rates using ZFE and donor human spermatozoa, whereas, it was significant ($p<0.01$) with that of sperm concentration and progressively motile spermatozoa for the patient group.

Berger *et al.* (1994) reported correlation coefficient of results of SPB with semen characteristics for cryopreserved goat spermatozoa. Sperm parameters like motility, acrosomal status and livability were not correlated significantly with differences in sperm fertilization potential and R^2 value was recorded to be 0.59, 0.12 and 0.49 respectively.

2.7.2 FERTILITY WITH SPB

In a review, Yanagimachi (1984) opined that fertility is high in men if the egg penetration rate is greater than 10-15% in the hamster test (SPB). The review also indicated that negative test appears to predict infertility of men with accuracy.

Bousquet and Brackett (1982) have compared results of the SPB of two bulls and found good agreement with the 60 day non-return rates using frozen semen. Similar findings were also reported by Eaglesome and Miller (1989) using frozen bovine semen but not so with fresh semen.

An experiment involving fertile and subfertile groups of boar indicated significantly high ($p<0.001$; 66.53%) number of oocytes found to be penetrated by fertile boars against subfertile boars (25.08 per cent, Ivanova and Mollova, 1993). Berger *et al.* (1994) reported correlation coefficients for both the FP (R^2 = 0.78) and FI (R^2 = 0.75) with that of relative fertility. Similarly high correlation of heterospermic SPB with non return rate (NRR) was reported by Davies *et al.* (1987) and Graham and Foote (1987) (R^2=0.75-0.93). These values were statistically significant indicating possible role of SPB in evaluating male fertility potential in the livestock species. Similar findings have also been recorded for bovine spermatozoa by Ramesha (1991).

2.8 SPERM CHROMOSOME STUDY

The chromosome complement of spermatozoa is packed in a highly condensed and inert state within the sperm head and direct cytogenetic analysis is not possible. Decondensation of sperm occurs only after penetration of an ovum, when factors and mechanisms within the egg cytoplasm decondense the sperm chromatin and allow the male and female pronuclei to prepare for the first cleavage division.

For the first time a technique for direct visualisation of huamn sperm chromosome was reported by Rudak *et al.* in 1978. This method involved the

in vitro penetration of ZFE by capacitated human spermatozoa and then subsequent fixation of pronuclear chromosomes. The technique originally devised by Rudak *et al.* (1978) was subsequently modified for the sperm chromosome study of the other livestock species viz. bull (Tateno and Mikamo, 1987), pig (Creighton and Houghton, 1989), ram (Tateno *et al.*, 1980) and buffalo (Ramesha 1991).

Materials and Methods

3. MATERIALS AND METHODS

The study on SPB was carried out at the Semen Bank, Department of Animal Genetics, Madras Veterinary College, Madras-600 007 during the period 1992-95.

3.1 CLEANING AND STERILIZATION

Glassware were soaked overnight in 10% chromic acid solution and rinsed in tap water followed by soaking in soap solution (Labklin, SD's) overnight. Next day glassware were washed thoroughly in tap water before rinsing them with triple glass distilled water (TGDW) for five times. Packing was done using aluminium foil after proper drying and then glassware were sterilized in hot air oven at 160°C for one hour.

Surgical instruments, vial corks, rubber corks and filter assembly were kept in soap solution overnight and cleaned same way as glassware. Sterilization was done (after packing with aluminium foil) by autoclaving them at 15 lbs per sq. inch pressure (121°C) for 15 minutes.

Medium mBWW was sterilized by filteration using membrane filter of 0.2 µm pore size (cellulose nitrate, Sartorius, Germany).

3.2 PREPARATION OF OOCYTES

3.2.1 ANIMALS

Female golden hamsters (*Mesocricetus auratus*; Plate 3.1) of 6-12 weeks old and 80-120 g body weight were procured from the Department of

Plate 3.1 **Female golden hamster (Mesocricetus auratus)**

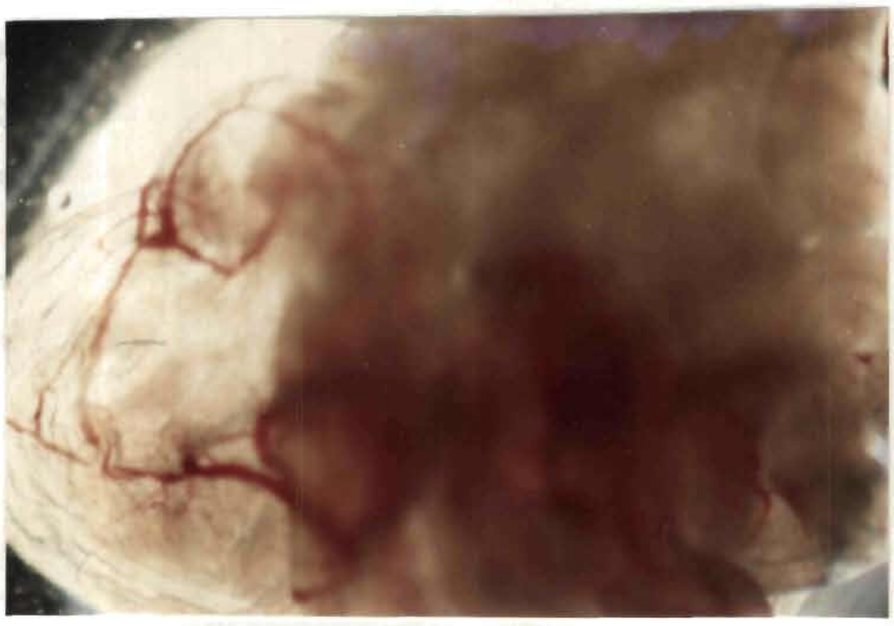


Plate 3.2 **Oviduct of the golden hamster (30X)**

Laboratory Animal Medicine, Madras Veterinary College, Madras-7, Laboratory Animal Information Service Centre, National Institute of Nutrition, Hyderabad-7 and Haffkine Biopharmaceutical Corporation Limited, Bombay-12 and utilized in the present study. The animals were kept in the department under natural dark-light cycle and given high energy high protein diet. They were maintained between 25° to 30°C temperature and provided with *ad libitum* water. A total of 190 animals were utilized for this study.

3.2.2 GONADOTROPHINS

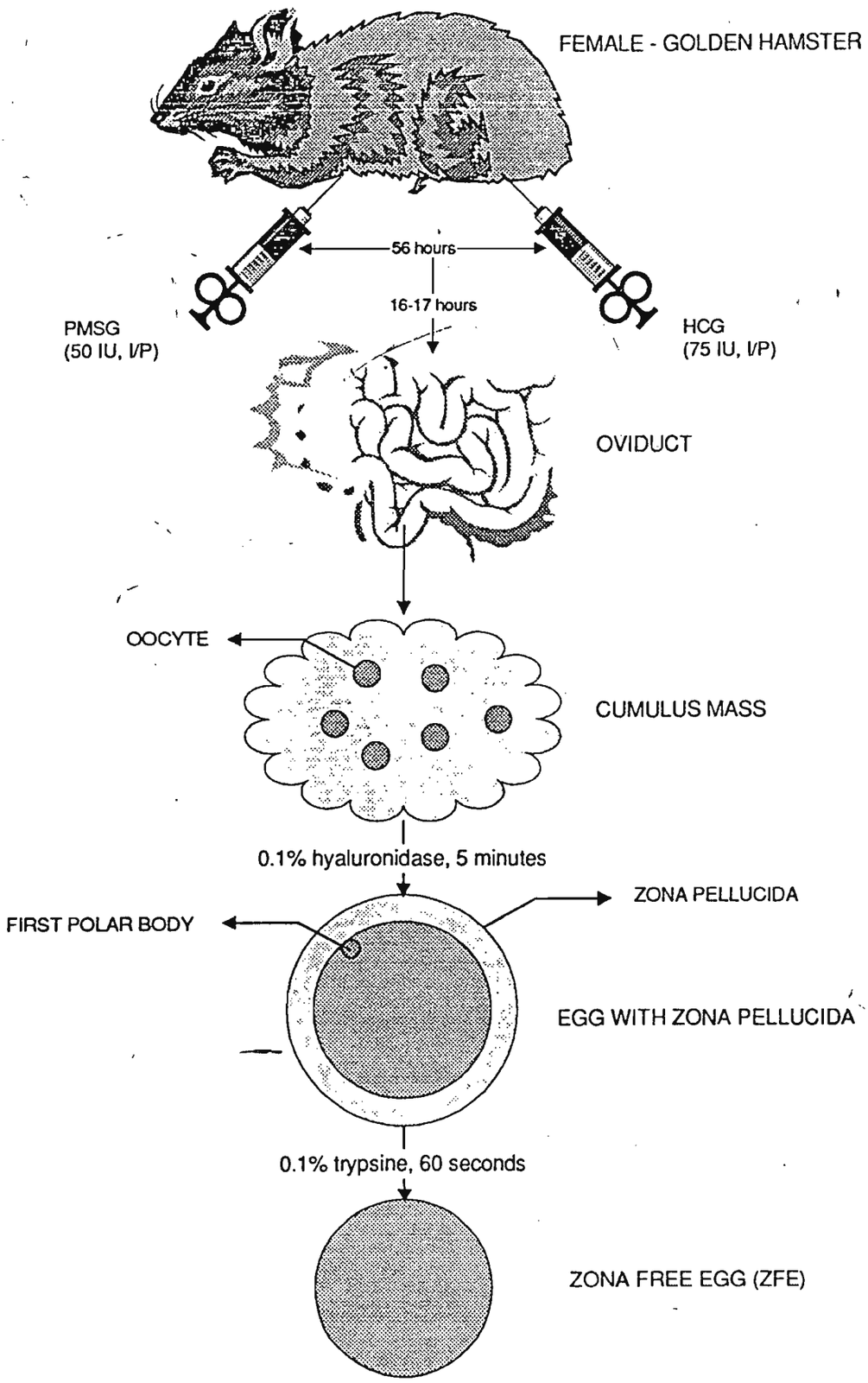
Female golden hamsters normally ovulate 8-12 oocytes after 14 hours of luteinising hormone surge (Mandelbaum *et al.*, 1982). The number of oocytes ovulated can be increased by superovulation through the use of gonadotrophins like PMSG and HCG.

3.2.2.1 PMSG

1000 I.U. PMSG (Folligon, Intervet, Holland) was dissolved in 20 ml of sterile TGDW. Aliquotes containing 1 ml solution in 5 ml glass culture vials (Borosil) were stored at -20°C till use.

Two dosages 30 I.U. and 50 I.U. of PMSG were tried in adult golden hamsters for superovulation by intraperitoneal (I/P) administration (Fig.3.1).

FIGURE 3.1 PREPARATION OF ZONA FREE HAMSTER EGGS



3.2.2.2 HCG

A vial containing 1500 I.U. HCG (Chorulon, Intervet) was dissolved in 30 ml TGDW. Aliquotes containing 1 or 1.5 ml solution in 5 ml glass culture vials were stored at -20°C.

Three different dosages of 30 I.U., 50 I.U. and 75 I.U. were tried intraperitoneally following PMSG treatment at varying intervals of time for superovulation.

3.2.2.3 Effect of interval between gonadotrophin administration

In order to identify the optimal interval between PMSG and HCG administration, two different intervals of time were tried. Injection of PMSG was followed by injection of HCG at 56 or 98 hours of interval.

3.2.3 MEDIUM FOR HANDLING OOCYTES

Modified Biggers, Whitten and Whittingham (mBWW) medium (Biggers *et al.*, 1971) containing 10 mM HEPES (Appendix I) was used for handling of the oocytes. The tonicity of the medium was adjusted to 290-300 mosmol/kg by changing the concentration of sodium chloride. The osmolarity of the medium was checked by osmometer (Gonotech, Germany). The pH of the medium was adjusted to 7.2-7.3 after equilibriation in 5% CO_2 in air incubator (Nuaire, U.S.A.) overnight.

3.2.4 RECOVERY OF CUMULUS MASS

After 16-17 hours of HCG injection female hamsters were sacrificed by toxic dose of chloroform anaesthesia. Following sterile precautions, abdomen was opened by midventral incision. The oviducts (Plate 3.2) were removed after cutting from the uterine and ovarian end by a curved scissors. The oviducts were transferred to 35 x 10 mm petri dish (Tarsons) containing 0.5 ml of the medium.

After switching off all the external lights to avoid activation of the oocytes, oviducts were located under stereo microscope at 30 x under red light and cumulus mass was removed by puncturing infundibulum region.

3.2.5 DIGESTION OF CUMULUS MASS

Freshly prepared 0.1% solution of hyaluronidase (H-3506, Sigma Chemical Co., U.S.A.) in mBWW medium was used for digestion of the cumulus mass. The cumulus mass was transferred to a drop of hyaluronidase solution in 60 x 15 mm petri dish (Laxbro) for 5-10 minutes This was followed by washing of eggs with zona by successively transferring them to 2-3 drops of the medium. Washing was done three times.

3.2.6 DIGESTION OF ZONA PELLUCIDA

Zona pellucida was digested using freshly prepared 0.1% trypsin (T-8253, Sigma) in mBWW medium. Oocytes were transferred to a trypsin drop in 60 x 15 mm petri dish for 30-60 seconds and were taken out immediately after zona removal. Action of the trypsin was determined by distortion in shape

of the zona. The dissolution of zona was constantly monitored under stereo microscope to avoid excessive digestion. Zona free eggs (ZFE) obtained were washed thrice by transferring them to drops of the mBWW medium containing 3.5% BSA (A-4503, Sigma) in 60 x 15 mm petri dish. ZFE of normal size and shape were immediately inoculated into the sperm drops.

Before preparation of the ZFE, capacitated spermatozoa were kept ready for the bioassay. Spermatozoa could be capacitated using a variety of agents *in vitro*. In this study high ionic pH, hypertonic medium and CaI A23187 were tried at different levels for the preparation of the spermatozoa.

3.3 INDUCTION OF ACROSOME REACTION

3.3.1 SEMEN

Semen was collected from the bulls maintained at the Semen Bank, Department of Animal Genetics, Madras Veterinary College by artificial vagina method. The spermatozoa were extended in egg-yolk-tris-glycerol, frozen and stored at -196°C.

Frozen semen samples of crossbred cattle and Murrah buffalo bulls were also procured from Sabarmati Ashram Gaushala, Bidaj farm, Gujarat. Upon rapid thawing in a water bath at 37°C for 30 seconds spermatozoa were used for experimentation.

3.3.2 MEDIUM FOR SPERM HANDLING

Medium utilized for washing, swim up and treatment with capacitating agents was same as mentioned in 3.2.3 for handling of the oocytes.

Capacitation medium was prepared by addition of 35 mg/ml BSA (A-4503 , Sigma) to the mBWW medium and it was filter sterilized using syringe filter (Minisart, Sartorius, Germany).

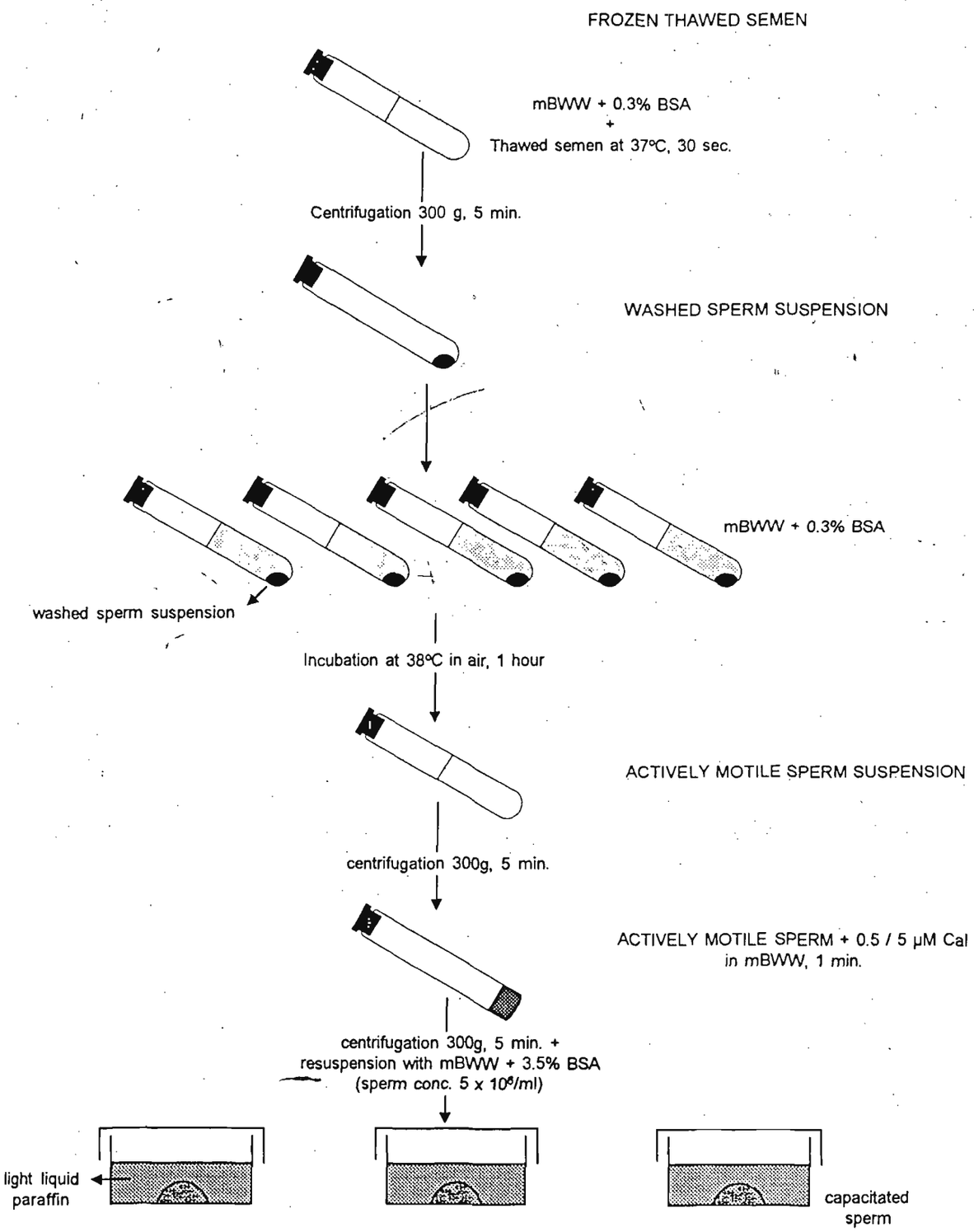
3.3.3 RECOVERY OF MOTILE SPERMATOZOA

Stress of freezing causes loss of motile spermatozoa and it results in 40-50% revival rate. In order to increase number of motile spermatozoa in the semen samples to be used for the SPB, swim up and swim down methods were tried.

3.3.3.1 Swim up

Before use, 4-5 semen straws of 0.25 ml capacity were thawed quickly in water bath at 37°C. The thawed semen was diluted with 5 ml mBWW and centrifuged at 300g for 5 minutes to remove cryoprotective medium. Sperm pellet was resuspended in 0.5 ml mBWW medium and sperm concentration was determined using the hemocytometer method. After adjusting the desired sperm concentration, 100 µl of the sperm suspension was layered at the bottom of 4-5 centrifuge tubes containing 2 ml of the medium (Fig.3.2). These centrifuge tubes were kept at 38°C in an air incubator for one hour in slanting position to allow motile spermatozoa to swim upwards. For the experiment on-gas phase the centrifuge tubes were incubated either in 5% CO_2 in air and pure air incubator. The supernatant suspension of active spermatozoa was centrifuged and the pellet was resuspended in the medium. Various sperm parameters like individual motility, sperm recovery, livability and acrosome integrity were assessed.

Fig.3.2 PREPARATION OF CAPACITATED SPERM



3.3.3.2 Swim down

1 ml of the bovine calf serum was taken in the centrifuge tubes and 100 µl of the washed sperm suspension with desired sperm concentration was placed on the top of the column and incubated at 38°C for 5 minutes in an air incubator in slanting position. After incubation, most of the serum from the bottom of the tube containing motile spermatozoa was carefully aspirated (Gonzales and Pella, 1993). The sperm suspension was diluted with the medium and centrifuged twice to get rid of serum. Different sperm parameters as indicated for swim up method were studied.

3.3.4 HIGH IONIC pH

High ionic pH medium was used to induce acrosome reaction as per method of Ijaz and Hunter (1989b) with modifications in the following manner.

3.3.4.1 Preparation of high ionic pH solutions

The medium mBWW was used by adjusting ionic pH with 0.1M NaOH to 7.4, 7.7 and 8.0. Osmolarity of the medium was kept at 290-300 mosmol/kg.

3.3.4.2 Sperm treatment

Supernatant sperm suspension recovered after swim up was equally distributed to three centrifuge tubes and were then centrifuged at 300 g for 5 minutes. Sperm pellets were resuspended in media with different ionic pH and kept in air incubator at 38°C. Various sperm parameters like individual

motility and intact, partially reacted or completely reacted acrosome were studied upto 5 hours at hourly interval. Livability was recorded at 0 and 5 hour of incubation.

3.3.5 HYPERTONIC MEDIUM

Hypertonic medium was successfully used to accelerate acrosome reaction in bovine adopting the method of Bousquet and Brackett (1982).

3.3.5.1 Preparation of hypertonic media

Isotonic medium (mBWW) with 290-300 mosmol/kg osmolarity was supplemented with sodium chloride to elevate the osmolarity to 340-350 or 390-400 mosmol/kg. The pH of the medium was adjusted to 7.3-7.4.

3.3.5.2 Sperm treatment

Actively motile sperm rich supernatant suspension was pooled equally into three centrifuge tubes following swim up procedure. After centrifugation at 300 g for 5 minutes, sperm pellets were resuspended in various media having different osmolarities for 5 minutes. After stipulated exposure, the isotonic medium (mBWW) was added to increase the volume by 5 times and centrifuged. The sperm pellets were resuspended in the medium and kept at 38°C in air incubator. Different sperm parameters were recorded.

3.3.6 CaI A23187

Acrosome reaction was induced using CaI (Bird *et al.*, 1989; Takahashi *et al.*, 1989), adopting the procedure detailed below.

3.3.6.1 Preparation of CaI solution

The ionophore stock solution was prepared by diluting calcium ionophore A23187 (C-7522, Sigma) to 100 µM in dimethyl sulphoxide (DMSO) and stored at -20°C. Aliquotes containing 50 µl each were further diluted with the medium to give desired concentration of 0.0, 0.1, 0.5, 1.0, 2.5 and 5.0 µM taking care at all times to protect the sample from direct light.

3.3.6.2 Sperm treatment

Following swim up and washing of motile spermatozoa, sperm pellet was diluted with medium containing desired concentration of CaI for one minute (Fig.3.2). The treated spermatozoa were further diluted with the medium and then centrifuged to remove CaI. The sperm pellet so obtained was diluted with capacitation medium to give final sperm concentration of 5×10^6 sperm per ml.

3.3.7 EFFECTS OF CAPACITATION AGENTS

3.3.7.1 Individual motility

Effect of various capacitating agents on individual progressive motility was recorded at hourly intervals for five hours by light microscopy. The initial post thaw motility of the semen samples were also recorded. A

small drop of the diluted semen was placed on a clean slide and covered with a cover glass. Minimum of three fields were scanned under phase contrast microscope to assess the proportion of motile spermatozoa. Percentage scales from 0 to 100 in multiples of 5 were used for the assessment.

3.3.7.2 Livability

Eosin-nigrosine stain mixture was prepared as reported by Swanson and Bearden (1951). A drop of semen was placed on a clean glass slide and this was mixed with two drops of eosin-nigrosine mixture. Thin smears were drawn and allowed to air dry before evaluating at 1000 x magnification. Spermatozoa stained with eosin were recorded as dead (Plate 3.3). At least hundred spermatozoa were counted and livability per cent was recorded for the semen sample at the beginning as well as at the end of five hours of sperm treatment with capacitating agents.

3.3.7.3 Acrosome reaction

Acrosome integrity of spermatozoa for post thawed semen and following treatment with capacitating agents was determined by Giemsa staining (Watson, 1975).

Semen smears were prepared, air dried and fixed with 5% formal saline for 30 minutes. Then the slides were washed in running tap water and again air dried. Freshly prepared buffered Giemsa solution (Appendix II) was used and fixed smears were immersed overnight in Giemsa solution. Later, slides were washed and air dried. At least 100 spermatozoa were counted at

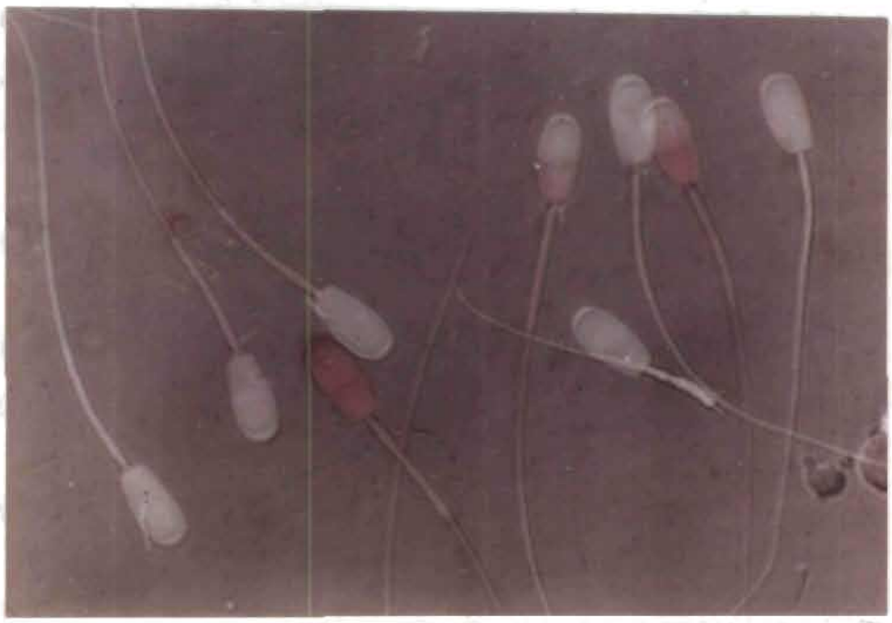


Plate 3.3 **Live and dead spermatozoa of crossbred cattle (Eosin-Nigrosine, 1250X)**

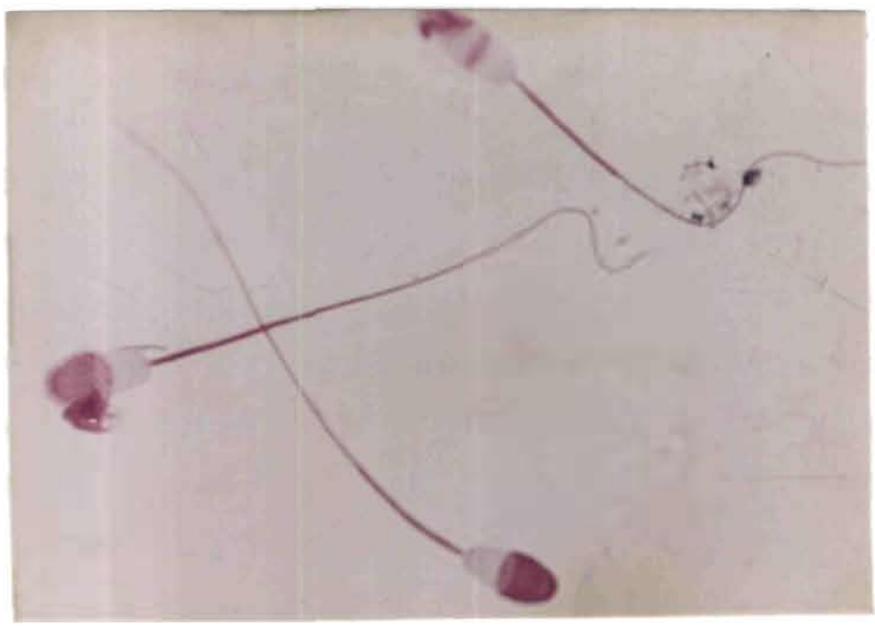


Plate 3.4 **Intact, partially and completely acrosome reacted spermatozoa of crossbred bull (Giemsa, 1250X)**

a magnification of 1000 x for acrosome integrity and categorized as intact acrosome, partially reacted or completely acrosome reacted spermatozoa (Plate 3.4).

3.3.8 SPERM CONCENTRATION

Sperm concentration was determined by Hemocytometer method (Hafez, 1993).

3.3.9 PREPARATION OF SPERM DROPS

Following sperm treatment with CaI A23187 at desired concentration and diluting it with capacitation medium, three sperm drops of each 100 µl were prepared in 35 x 10 mm disposable petri dish individually (Fig.3.2, Plate-3.5). The sperm drops were layered with 2-3 ml of sterile light liquid paraffin and kept at 38°C in air incubator. Sperm drops prepared were also kept in 5% CO_2 in air incubator for gas phase study.

3.4 SPERM-EGG INTERACTION

ZFE were inoculated to capacitated bovine sperm drops within one hour of preparation of the sperm drops in an air incubator at 38°C. Capacitation of the spermatozoa was determined by observing head to head association of 2-3 spermatozoa (Tateno and Mikamo, 1987) before inoculation of oocytes. The sperm-egg interaction were allowed for 3 hours at 38°C before scoring (Fig.3.3).

FIG.3.3 SPERM PENETRATION BIOASSAY

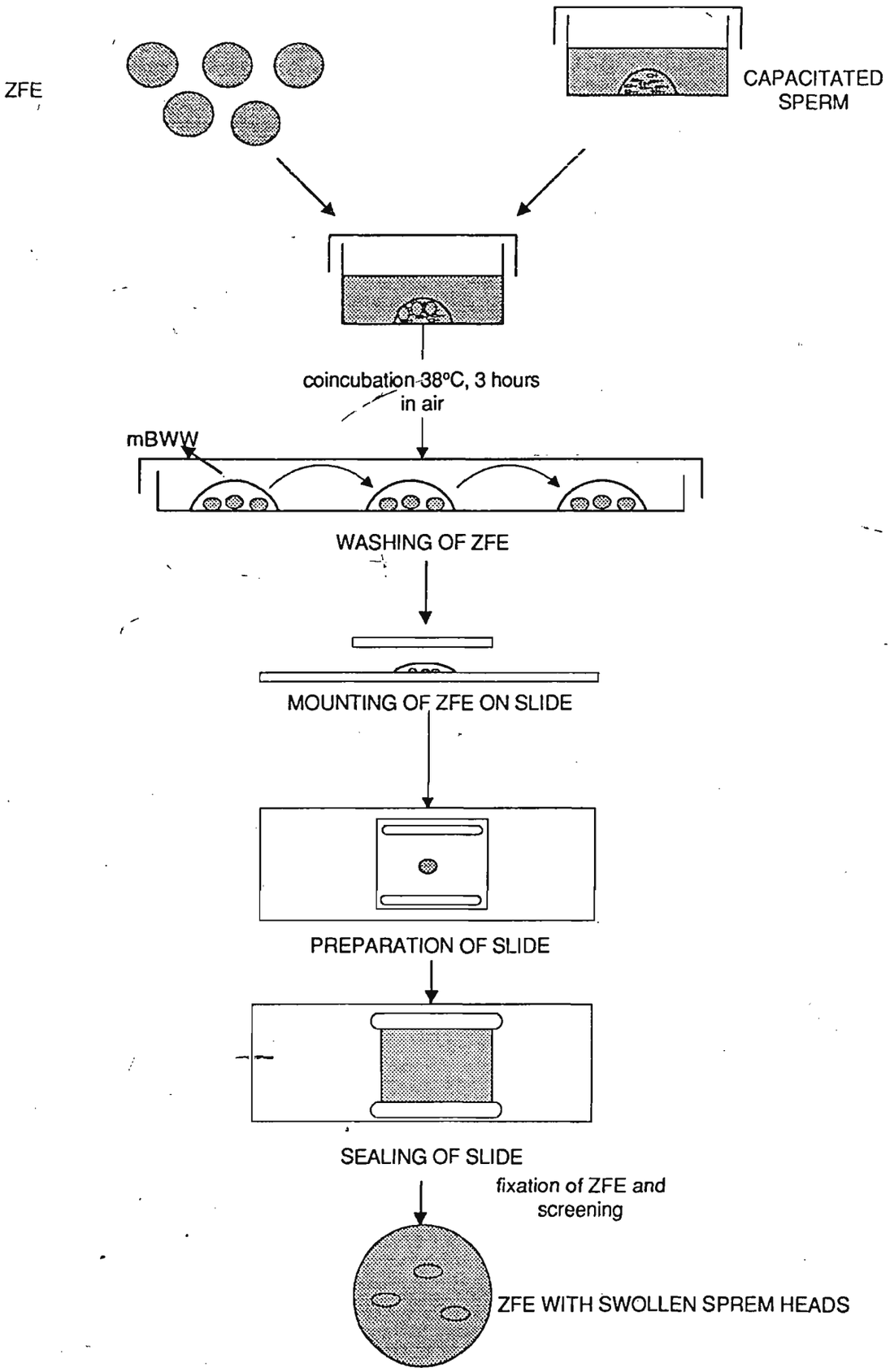


Plate 3.5 Preparation of sperm drops layered with light liquid paraffine

3.4.1 PREPARATION OF OOCYTE SLIDES

On completion of coincubation period, ZFE were transferred to a petri dish containing medium and were washed thrice to remove loosely attached spermatozoa. Oocytes were then transferred to clean and dry slides in small drops. A vaseline-paraffin (10:1) mixture filled in a syringe was stripped parallel to the length of the slide in order to hold the cover glass leaving space between glass slide and cover slip. The cover glass was gently pressed observing ZFE under the microscope to avoid damage to the oocytes (Fig.3.3).

3.4.2 FIXATION OF OOCYTES

Oocyte slides prepared were sealed lengthwise using vaselin-paraffin mixture to avoid possible loss of the oocytes. The sealed slides were fixed in a staining jar containing methanol : acetic acid (3:1) overnight.

3.4.3 SPERM CHROMOSOME STUDY

3.4.3.1 Post insemination culture

Post insemination culture was carried out as per methodology described by Martin (1983) after modification as follows. After coincubation ZFE were washed by transferring 2 - 3 drops of Medium 199 (M-5017 Sigma) containing 15% FCS (Sigma) and transferred to 0.2 ml drop of Medium 199 with FCS under paraffin oil in 35 x 10 mm petridishes, and incubated overnight for 12 to 12.5 hours at 38°C in 5% CO_2 in air and 95% humidity. The next morning majority of eggs were transferred directly to 0.2 ml drop of Medium 199 with FCS containing 0.5 µg/ml colcemid (15212-012 Gibco) under

paraffin oil in 35 x 10 mm petri dish and incubated for a further 4 to 7 hours, whereas rest of eggs were allowed for further incubation without colcemid to study cell division upto 24 hours.

3.4.3.2 Slide Preparation

The eggs were fixed on glass slides after a minimum of 5 hours of incubation in colcemid. Then eggs were transferred to the hypotonic solution (1% sodium citrate) in a 35 x 10 mm petri dish at room temperature for 5 - 7 minutes. Three to five eggs were transferred in a small drop of hypotonic solution to the centre of a clean glass slide taking care to avoid bursting. The fixation was done by adding a drop of fixative (3 : 1, ethanol : acetic acid) directly on the eggs. Second drop of fixative was added from a height of approximately 1 cm when egg began to flatten but before drying of fixative completely. Totally four drops were added in this manner for fixation. Staining was done using 4% Giemsa stain for 30 minutes.

3.4.4 STAINING OF OOCYTES

Oocytes were stained with 1.0% aceto-orcein stain. This stain was prepared by dissolving 1 g of orcein in 100 ml of 45% formaldehyde solution. A drop of the stain was placed on the open side of cover glass allowing the stain to enter by capillary action to stain the oocytes.

3.5 SCORING FOR SPERM-EGG INTERACTION

Different semen samples were scored in the following ways for ability of the spermatozoa to penetrate ZFE (Rogers, 1983).

1. Fertilization Percentage (FP) = $\frac{\text{No.of eggs penetrated}}{\text{No.of eggs inseminated}} \times 100$

2. Fertilization Index (FI) = $\frac{\text{Total No.of swollen sperm heads}}{\textit{Total No.of eggs}}$

3.6 HYPO-OSMOTIC SWELLING TEST (HOST)

HOST was performed as per procedure of Sivaramalingam (1994). 0.1 ml of frozen thawed semen was mixed with 1.0 ml of TGDW and incubated for 15 minutes at 38°C in an air incubator. After incubation, smears were prepared and stained with 3% Rose Bengal stain for 15-20 minutes and tail curling percentage was recorded under the phase contrast microscope. In case of buffalo semen incubation period of 15, 30 and 45 minutes were attempted.

3.7 FERTILITY RECORDS

Fertility records of the crossbred cattle and Murrah buffalo bulls studied were generously provided by Sabarmati Ashram Gaushala, Bidaj Farm of National Diary Development Board, Gujarat. The conception rates were based on pregnancy diagnosis averaging 247.5 and 761.6 for each crossbred cattle and Murrah bulls respectively.

3.8 PHOTOMICROGRAPHY

Different stages of hamster oocytes, spermatozoa and sperm egg interaction were photographed by Carl Zeiss photomicroscope III using Kodak

colour film of 100 ASA. The photographs for oocytes were taken under phase contrast.

3.9 STATISTICAL ANALYSIS

The results were analysed by analysis of variance. Per cent values were transformed to arcsin for the analysis. The data for correlation between sperm parameters, HOST, sperm egg interaction and conception rates records were subjected to the correlation analysis (Snedecor and Cochran, 1967).

Results

4. RESULTS

4.1 SUPEROVULATORY RESPONSE IN GOLDEN HAMSTER

The effect of administration of PMSG and HCG, at different dosages, on the response of oocytes recovered in the golden hamsters were studied and the results are presented in Table 4.1. The influence of time interval between the administration of PMSG and HCG on the superovulatory response are also presented in Table 4.1 and the analysis of variance on the above parameters are presented in Table 4.2.

4.1.1 GONADOTROPHIN DOSAGE

The different dosage combinations of gonadotrophins (PMSG/HCG) tried at 30/30, 50/50 and 50/75 I.U. resulted in the recovery of 29.90 ± 1.64, 32.35 ± 1.91 and 40.60 ± 2.57 oocytes respectively. The analyis of variance revealed significant ($p < 0.05$) dose dependent effect of gonadotrophins on the superovulatory response.

4.1.2 DURATION BETWEEN PMSG to HCG ADMINISTRATION

The administration of HCG after PMSG at 56 and 98 hours of interval resulted in recovery of 33.37 ± 1.92 and 35.20 ± 1.83 oocytes respectively. However, the analysis of variance showed no significant difference ($p > 0.05$) between the two treatments.

Table 4.1 Effect of Gonadotrophin levels and the intervals between their administration on the recovery of oocytes in Golden Hamsters

Treatment	Gonadotrophins PMSG/HCG	Number of oocytes harvested Mean ± S.E.
Dosage of Gonadotrophin (I.U.) (n=20)	30/30	$29.90^{p} \pm 1.64$
	50/50	$32.35^{p} \pm 1.91$
	50/75	$40.60^{q} \pm 2.57$
Interval between PMSG & HCG Gonadotrophin administration (hour) (n=30)	56	33.37 ± 1.92
	98	35.20 ± 1.83

Means bearing different superscripts among treatments differ significantly.

Table 4.2 Analysis of variance on the effect of Gonadotrophin levels and intervals between administration on recovery of oocytes in Golden Hamsters

Source of variation	df	Mean squares
Between dosages	2	628.52*
Between intervals	1	50.42
Dosage x Interval	2	11.52
Error	54	89.55

* $P < 0.05$

4.1.3 RELEASING OOCYTES FROM CUMULUS MASS

The gradual release of oocytes from the cumulus mass by the action of hyaluronidase is presented in plate 4.1, 4.2 and 4.3.

4.1.4 DIGESTION OF ZONA PELLUCIDA

The different stages of trypsin digestion of zona resulting finally in ZFE are presented in plate 4.4, 4.5 and 4.6.

4.2 RECOVERY OF MOTILE SPERMATOZOA

The efficiency of two techniques viz. swim up and siwm down in recovering motile spermatozoa was evaluated and the results are presented in Table 4.3 and the analysis of variance in Table 4.4.

4.2.1 SWIM UP

The swim up technique has resulted in enhancing individual motility to 82.55 ± 0.06 per cent from the initial post thaw motility percent of 52.50 ± 1.79. However, the sperm recovery per cent in the swim up sample was 20.70 ± 0.16 only. The post-swim up sample had 36.90 ± 0.07 per cent intact acrosome while the pre treated sample had 44.17 ± 3.83 per cent intact acrosome.

4.2.2 SWIM DOWN

The swim down technique also had a similar effect of enhancing the individual motility to 82.80 ± 0.04 per cent with a sperm recovery rate of

Table 4.3 Sperm characteristics of crossbred bulls subjected to swim up and swim down techniques at different sperm concentrations (Mean ± S.E.)

Treatment		Characteristics (percent)					
		Sperm recovery	Acrosome reaction			Individual motility	Livability
			Intact acrosome	Partially reacted	Completely reacted		
Technique (n=12)	Swim up	20.70 ± 0.16	36.90^{p} ± 0.07	47.30_{q} ± 0.06	14.00 ± 0.12	82.55 ± 0.06	89.50 ± 0.08
	Swim down	25.80 ± 0.95	13.00^{q} ± 0.14	65.70^{p} ± 0.27	20.20 ± 0.13	82.80 ± 0.04	88.20 ± 0.10
Sperm concentration (sperm/ml) (n=12)	1×10^{7}	27.65^{a} ± 0.09	27.65 ± 0.23	51.50 ± 0.15	19.40 ± 0.07	79.80^{b} ± 0.04	85.10^{b} ± 0.08
	5×10^{7}	19.00^{b} ± 0.12	20.30 ± 0.31	61.70 ± 0.27	14.70 ± 0.19	85.30^{a} ± 0.04	92.20^{a} ± 0.05

Means bearing different superscripts among treatment differ significantly.

Table 4.4 Analyses of variance on the sperm characteristics of crossbred bulls subjected to swim up and swim down techniques at various sperm concentrations

Source of Variation	df	Mean squares					
		Sperm recovery	Acrosome reaction			Individual motility	Livability
			Intact acrosome	Partially reacted	Completely reacted		
Between techniques	1	71.83	1592.19**	688.44**	135.95	0.20	8.40
Between sperm concentrations	1	206.74*	145.93	210.04	76.90	103.25*	254.93**
Technique x sperm concentration	1	7.15	2.27	1.64	0.01	19.08	11.56
Error	20	818.28	37.26	59.66	50.17	17.15	26.27

* $P < 0.05$; ** $P < 0.01$

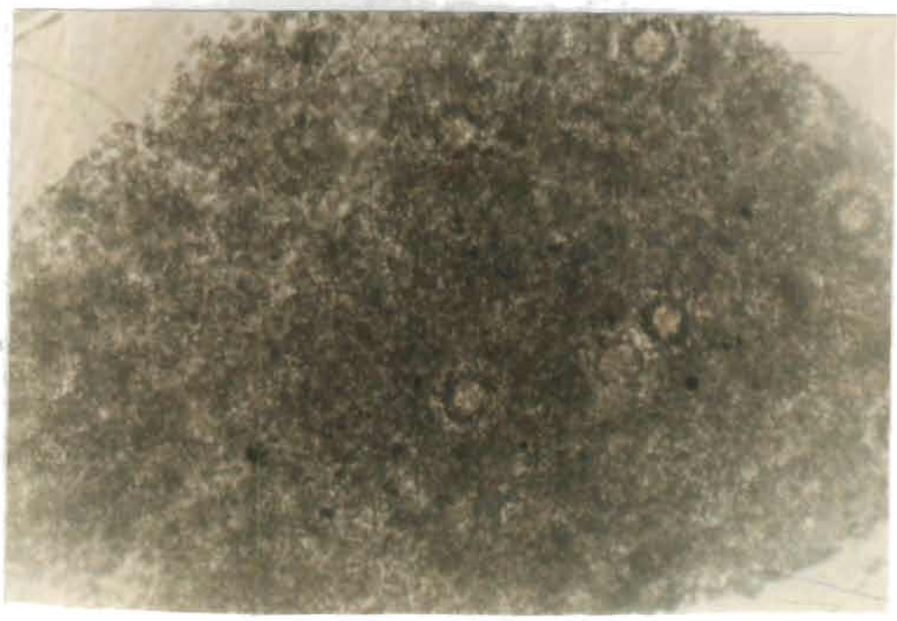


Plate 4.1 Cumulus mass containing oocytes (85X)

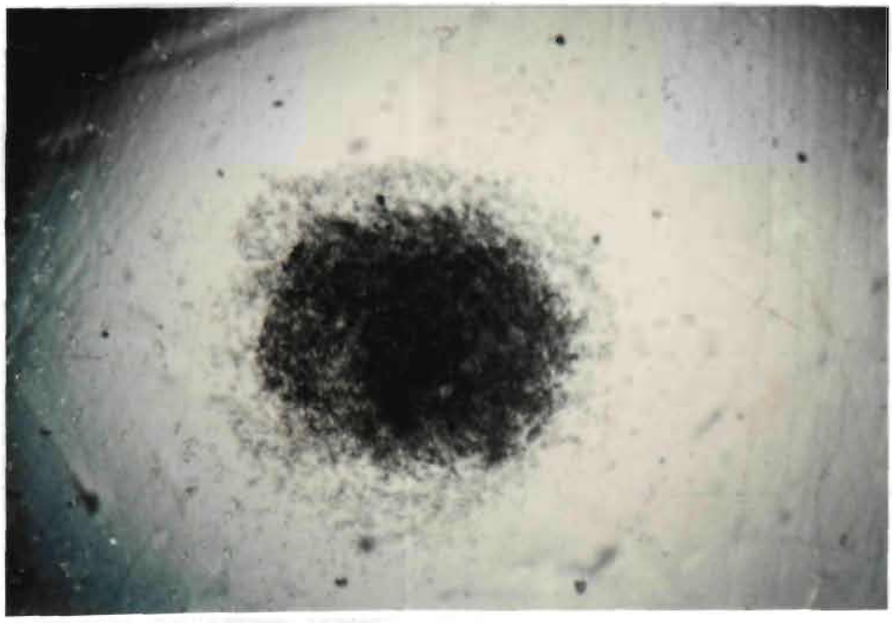


Plate 4.2 Digestion of cumulus with hyaluronidase (30X)

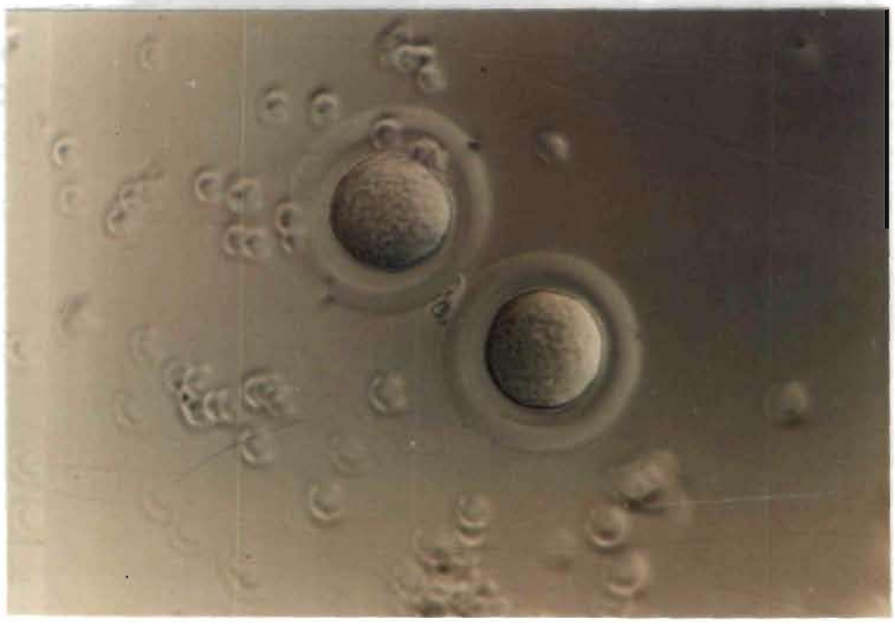


Plate 4.3 **Oocyte with zona released from cumulus mass (Phase contrast, 300X)**

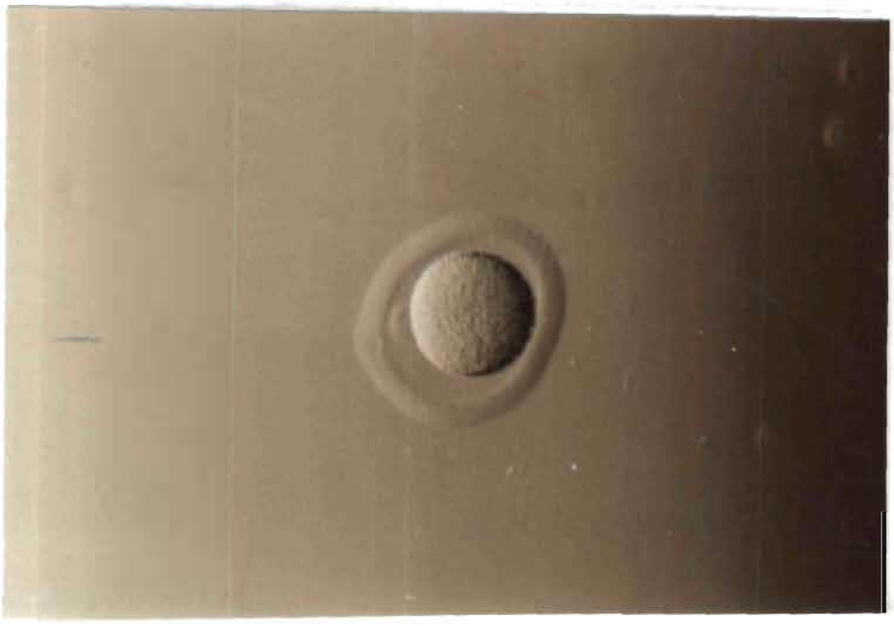


Plate 4.4 **Digestion of zona with trypsin (Phase contrast, 300X)**

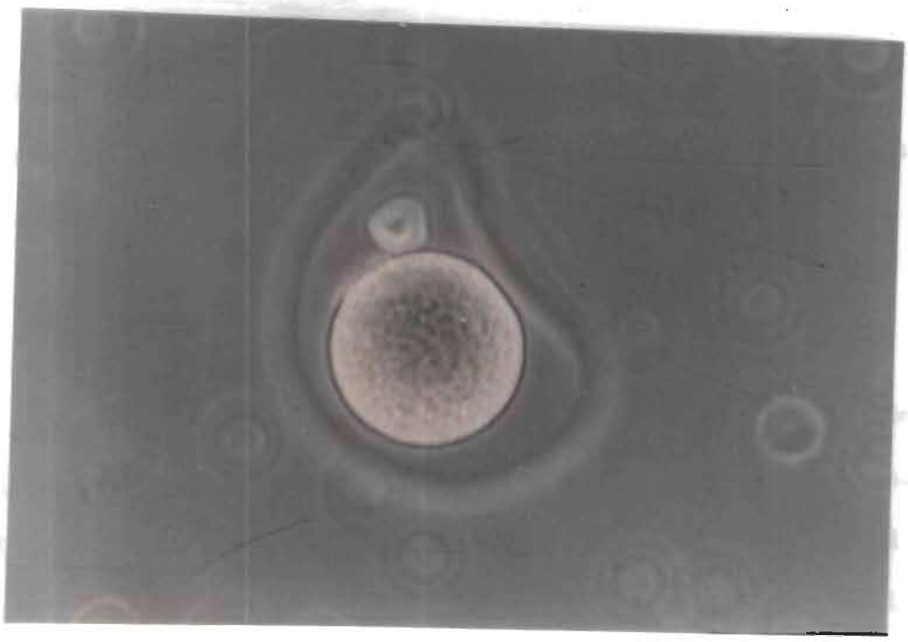


Plate 4.5 **Digestion of zona with trypsin (Phase contrast, 300X)**

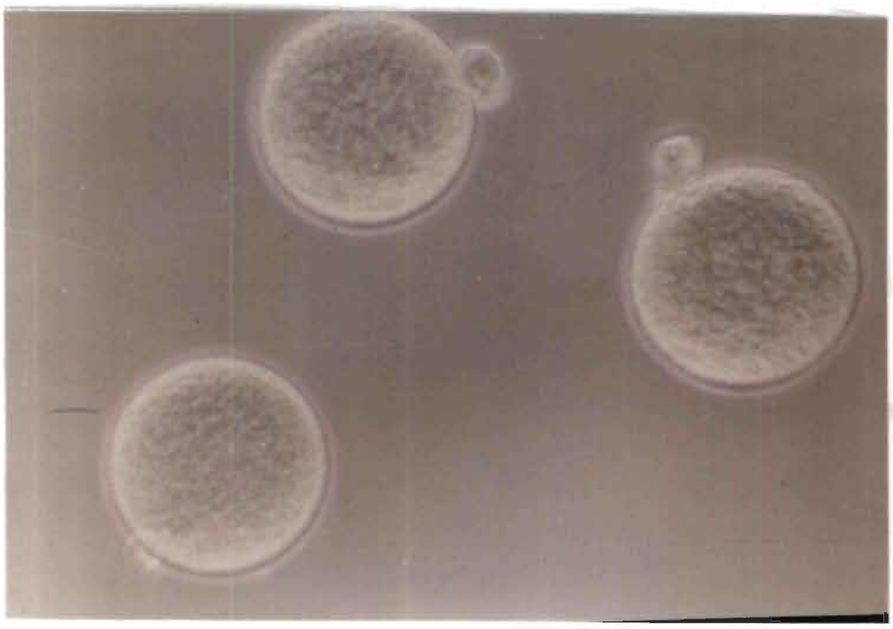


Plate 4.6 **Zona free eggs (ZFE) on digestion of zona (Phase contrast, 300X)**

25.80 ± 0.95 per cent. However, swim down technique had a significantly ($p < 0.01$) lower intact acrosome (13.00 ± 0.14 per cent) in comparison to swimup technique (36.90 ± 0.67 per cent).

4.2.3 SPERM CONCENTRATION

The influence of sperm concentration of the post thaw washed sample on swim up and swim down techniques was studied and the results are presented in Table 4.3. The sperm concentration at 1 x 10^7 per ml had a recovery per cent of 27.65 ± 0.09 as against 19.00 ± 0.12 per cent for the sample with 5 x 10^7 sperm per ml. However, the individual motility was significantly ($p < 0.05$) higher (85.30 ± 0.04) with 5 x 10^7 sperm per ml concentration in contrast to 79.80 ± 0.04 per cent in 1 x 10^7 sperm ml. Sperm concentration at 5 x 10^7 had significantly higher ($p < 0.01$) live sperm per cent of 92.20 ± 0.05.

4.3 CAPACITATION OF SPERMATOZOA

4.3.1 CROSSBRED CATTLE

4.3.1.1 *Effect of pH of mBWW medium*

The effect of three levels of pH of mBWW medium and five incubation periods on acrosome reaction and other sperm characteristics of crossbred bulls and Murrah buffalo bulls were studied and the results are recorded in Table 4.5 and the analysis of variance in Table 4.6.

The crossbred bull spermatozoa incubated for acrosome reaction at pH of 7.4, 7.7 and 8.0 resulted in a mean intact acrosome per cent of 50.50 ±

Table 4.5 : Effect of pH of mBWW medium and incubation periods on acrosome reaction and other sperm characteristics of crossbred cattle and Murrah buffalo bulls (Mean ± S.E.)

Treatment		Characteristics (per cent)									
		Crossbred cattle					Murrah				
		Acrosome reaction			Individual motility	Livability	Acrosome reaction			Individual motility	Livability
		Intact acrosome	Partially reacted	Completely reacted			Intact acrosome	Partially reacted	Completely reacted		
pH mBWW medium (n=36)	7.4	50.50^{p} ± 0.11	36.60 ± 0.06	11.20^{q} ± 0.04	48.78^{p} ± 0.15	78.67 ± 0.17	35.92^{p} ± 0.08	50.56 ± 0.04	11.24^{r} ± 0.03	40.88^{p} ± 0.28	79.59 ± 0.24
	7.7	48.08^{pq} ± 0.12	36.93 ± 0.08	13.12^{pq} ± 0.04	39.46^{q} ± 0.34	73.75 ± 0.27	33.11^{q} ± 0.07	51.27 ± 0.03	14.69^{q} ± 0.03	32.92^{q} ± 0.38	78.60 ± 0.19
	8.0	42.00^{q} ± 0.13	40.33 ± 0.08	14.79^{p} ± 0.15	33.78^{r} ± 0.38	74.13 ± 0.22	28.20^{q} ± 0.07	50.62 ± 0.02	19.57^{p} ± 0.05	25.83^{r} ± 0.40	73.53 ± 0.26
Period (hour) (n=18)	0	70.18^{a} ± 0.11	22.38^{d} ± 0.10	5.99^{e} ± 0.04	75.97^{a} ± 0.04	86.60^{a} ± 0.02	55.60^{a} ± 0.03	38.72^{c} ± 0.04	5.51^{e} ± 0.01	75.73^{a} ± 0.05	86.94^{a} ± 0.04
	1	63.07^{a} ± 0.09	29.50^{cd} ± 0.10	6.32^{e} ± 0.02	62.32^{b} ± 0.09		47.22^{b} ± 0.02	43.08^{c} ± 0.02	9.50^{d} ± 0.02	61.37^{b} ± 0.07	-
	2	51.83^{a} ± 0.06	36.45^{bc} ± 0.06	11.20^{d} ± 0.02	42.42^{c} ± 0.11		33.10^{c} ± 0.01	51.68^{b} ± 0.02	15.00^{c} ± 0.01	44.30^{c} ± 0.07	-
	3	38.75^{b} ± 0.10	44.32^{ab} ± 0.09	15.21^{bc} ± 0.03	33.41^{c} ± 0.11		27.64^{d} ± 0.03	53.27^{b} ± 0.03	18.33^{b} ± 0.04	29.71^{d} ± 0.08	-
	4	32.60^{bc} ± 0.08	46.60^{ab} ± 0.09	19.64^{b} ± 0.04	23.85^{d} ± 0.13		18.90^{e} ± 0.03	59.38^{a} ± 0.03	20.70^{b} ± 0.03	8.50^{e} ± 0.34	-
	5	25.64^{c} ± 0.14	49.98^{a} ± 0.11	23.56^{a} ± 0.03	8.05^{e} ± 0.19	52.20^{b} ± 0.04	16.29^{e} ± 0.01	57.60^{a} ± 0.02	25.10^{a} ± 0.04	1.42^{f} ± 0.23	65.89^{b} ± 0.07

Means bearing different superscripts among treatment differ significantly.

Table 4.6 : Analyses of variance on the effect of pH of mBWW medium and incubation periods on acrosome reaction and other sperm characteristics of crossbred cattle and Murrah buffalo bulls

Source of variation	df	Mean squares												
		Crossbred cattle						Murrah						
		Acrosome reaction			Individual motility	df	Livability	Acrosome reaction				Individual motility	df	Livability
		Intact acrosome	Partially reacted	Completely reacted				df	Intact acrosome	Partially reacted	Completely reacted			
Between pH	2	228.41*	51.13	85.07**	994.02**	2	40.65	2	207.54**	4.71	401.66**	761.84**	2	57.79
Between periods	5	1949.17**	758.25**	674.76**	4569.22*	1	2399.37**	5	1677.95**	397.56**	669.90**	7526.32**	1	1907.65**
pH x period	10	16.45	10.93	16.21	34.47	32	18.32	10	11.54	23.49	12.20	52.51	2	3.06
Error	90	58.14	59.50	16.08	50.01	35	14.75	90	9.71	14.87	7.38	59.60	32	36.31

* $P < 0.05$; ** $P < 0.01$

FIG.4.1 EFFECT OF CAPACITATION AGENTS ON ACROSOME INTEGRITY OF CROSSBRED CATTLE

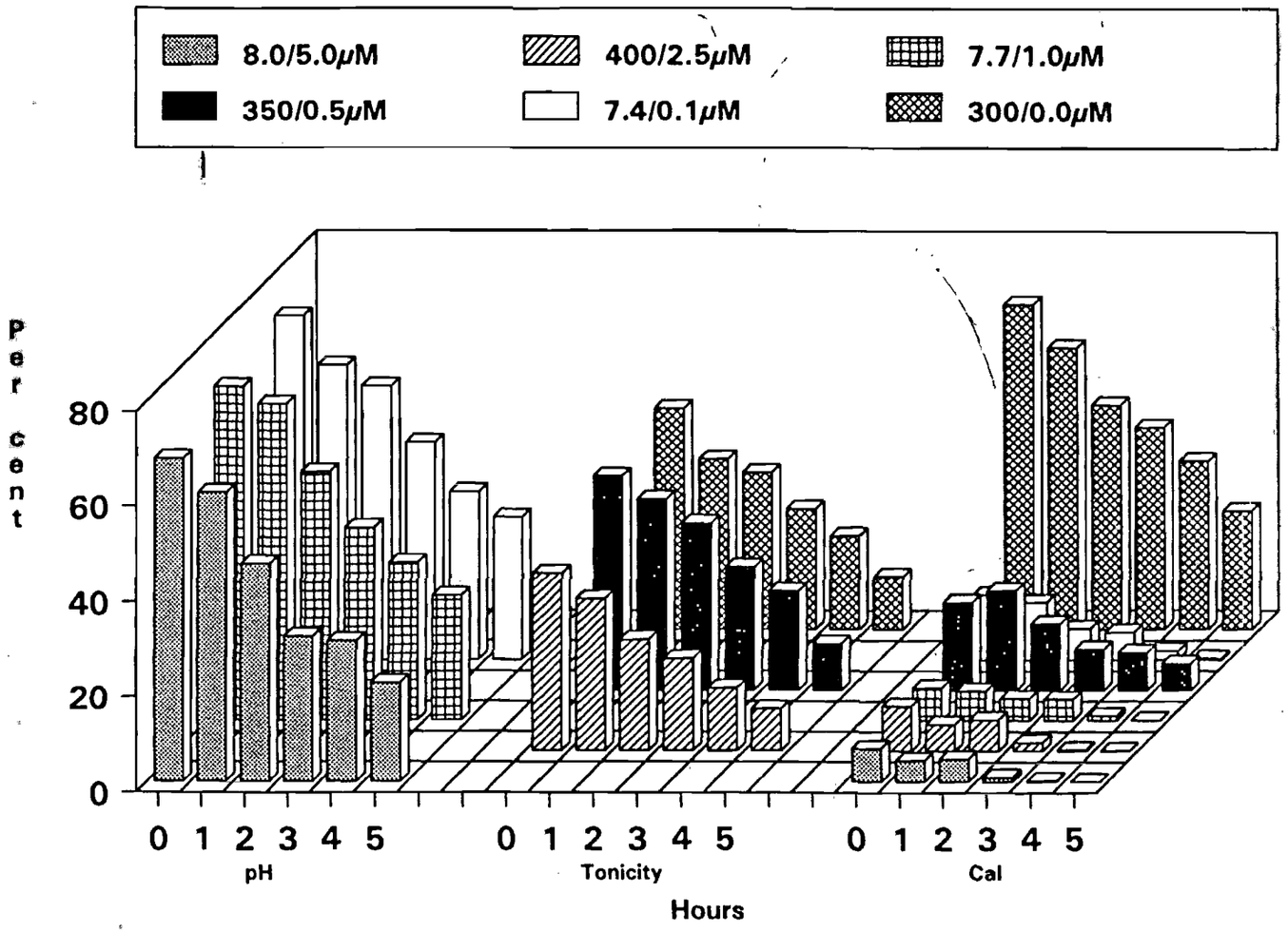


FIG.4.2 EFFECT OF CAPACITATION AGENTS ON COMPLETELY ACROSOME REACTED SPERM OF CROSSBRED CATTLE

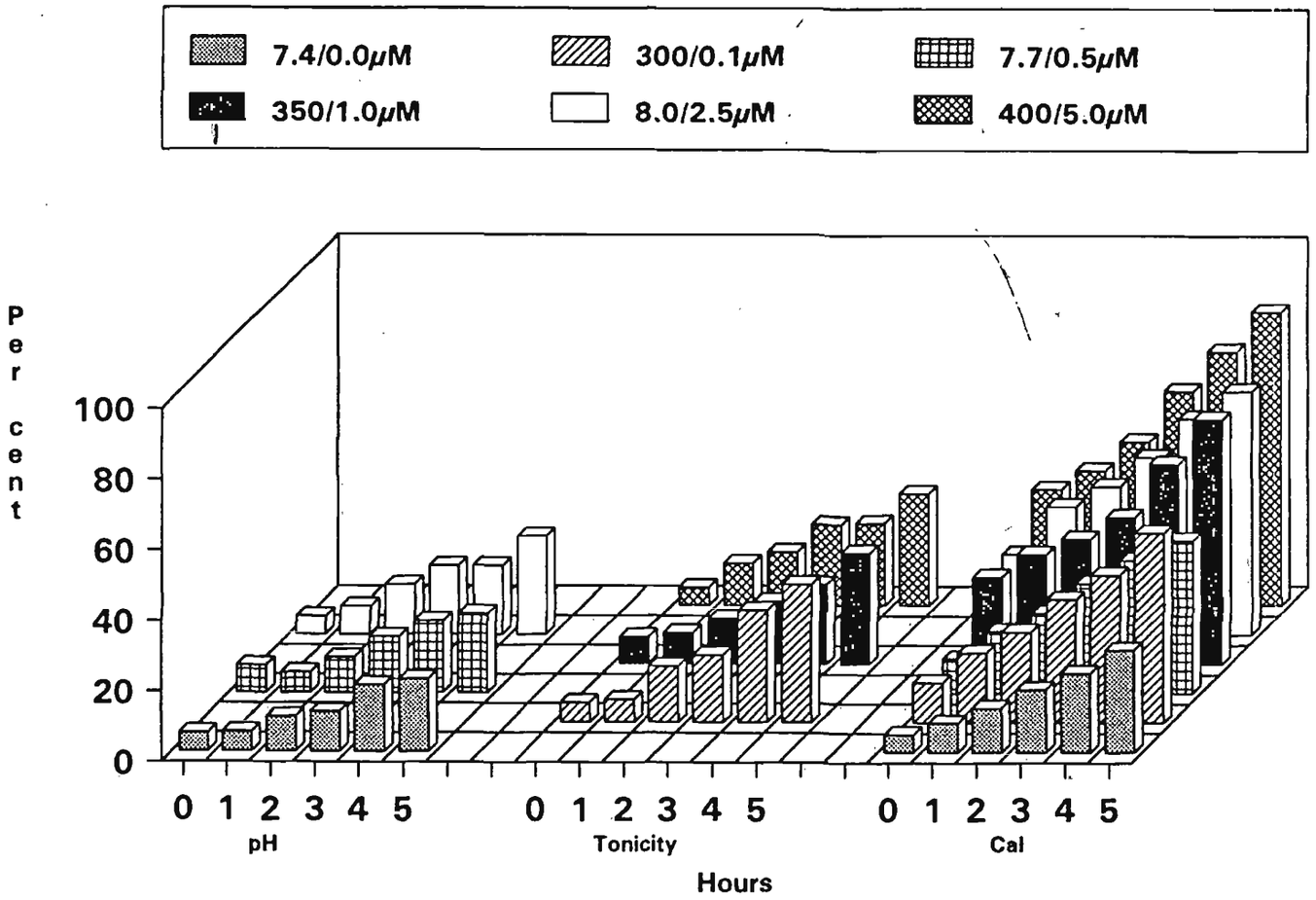
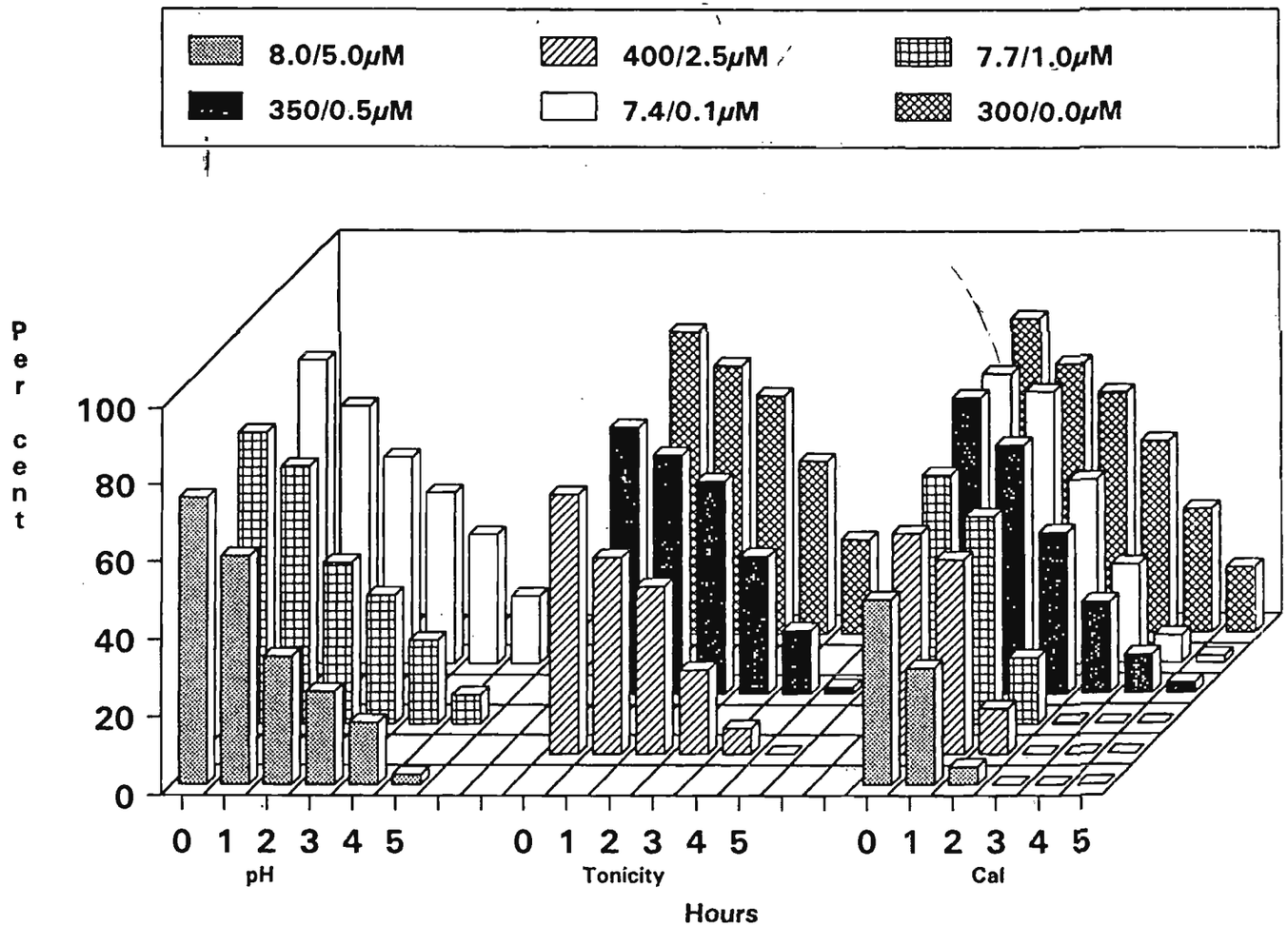


FIG.4.3 EFFECT OF CAPACITATION AGENTS ON INDIVIDUAL MOTILITY OF CROSSBRED CATTLE



0.11, 48.08 ± 0.12 and 42.00 ± 0.13 respectively (Fig.4.1). The intact acrosome percentage at pH 8.0 was highly significantly ($p < 0.05$) different from that at pH of 7.4. Similarly the completely acrosome reacted sperm per cent (14.79 ± 0.15) was highest with pH 8.0 compared with that at pH 7.4 (11.20 ± 0.04, Fig.4.2) the difference being highly significant ($p < 0.01$). In contrast a reverse trend of the effect of pH on individual motility was recorded (Fig.4.3). The pH 7.4 had highest motility of 48.78 ± 0.15 while pH 8.0 exhibited lowest motility of 33.78 ± 0.38 per cent.

The analysis showed that the incubation periods had a highly significant ($p < 0.01$) influence on all the parameters studied (Fig.4.1 to 4.3). The individual motility declined from 75.97 ± 0.04 at zero hour to 8.05 ± 0.19 per cent after five hours of incubation. Similarly the livability of sperm declined from 86.60 ± 0.02 to 52.20 ± 0.04 per cent and intact acrosome from 70.18 ± 0.11 to 25.64 ± 0.14 per cent. As expected, completely acrosome reacted sperm increased from 5.99 ± 0.04 at zero hour to 23.56 ± 0.03 per cent at end of 5 hour incubation period.

4.3.1.2 Effect of tonicity of mBWW medium

The results of the investigation on the effect of pretreatment of hypertonic medium on acrosomal reaction and other sperm characteristics of crossbred and Murrah buffalo bull are presented in Table 4.7 and the analyses of variance in Table 4.8.

The results of the study in crossbred bull revealed that as the tonicity of the medium increased (300, 350 and 400 mosmol/kg) the individual

Table 4.7 : Effect of tonicities of mBWW medium and incubation periods on acrosome reaction and other sperm characteristics of crossbred cattle and murrah buffalo bulls (Mean ± S.E.)

Treatment		Characteristics (per cent)									
		Crossbred cattle					Murrah				
		Acrosome reaction			Individual motility	Livability	Acrosome reaction			Individual motility	Livability
		Intact acrosome	Partially reacted	Completely reacted			Intact acrosome	Partially reacted	Completely reacted		
Tonicity of mBWW medium (mosomol/kg) (n=36)	300	27.80^{p} ± 0.09	51.93 ± 0.04	17.39 ± 0.08	46.75^{p} ± 0.28	76.87 ± 0.29	29.58^{p} ± 0.12	56.83 ± 0.06	12.23^{q} ± 0.02	44.00^{p} ± 0.31	83.03 ± 0.14
	350	28.55^{p} ± 0.11	51.85 ± 0.05	16.09 ± 0.08	37.50^{p} ± 0.33	73.84 ± 0.39	24.53^{pq} ± 0.13	58.62 ± 0.06	13.15^{q} ± 0.05	30.69^{q} ± 0.04	79.44 ± 0.13
	400	21.48^{q} ± 0.10	55.62 ± 0.06	18.02 ± 0.16	25.96^{q} ± 0.37	70.82 ± 0.39	23.19^{q} ± 0.10	56.53 ± 0.04	16.80^{p} ± 0.03	26.25^{q} ± 0.38	77.70 ± 0.20
Period (hour) (n=18)	0	42.95^{a} ± 0.11	49.62 ± 0.14	6.07^{e} ± 0.08	73.21^{a} ± 0.10	86.14^{a} ± 0.07	49.76^{a} ± 0.07	44.85^{d} ± 0.07	5.09^{d} ± 0.03	73.79^{a} ± 0.21	87.61^{a} ± 0.04
	1	36.92^{ab} ± 0.11	54.83 ± 0.11	8.92^{de} ± 0.06	60.83^{ab} ± 0.18	-	37.33^{b} ± 0.07	50.63^{cd} ± 0.07	10.82^{c} ± 0.03	62.01^{a} ± 0.12	-
	2	30.32^{bc} ± 0.09	52.35 ± 0.06	14.61^{cd} ± 0.05	53.33^{b} ± 0.21	-	30.69^{b} ± 0.10	55.64^{bc} ± 0.07	13.40^{c} ± 0.03	47.72^{b} ± 0.10	-
	3	23.44^{cd} ± 0.19	54.08 ± 0.15	19.86^{bc} ± 0.16	33.50^{c} ± 0.20	-	21.04^{c} ± 0.09	60.88^{ab} ± 0.07	17.22^{bc} ± 0.03	28.60^{c} ± 0.12	-
	4	17.81^{de} ± 0.12	54.13 ± 0.09	25.81^{ab} ± 0.15	14.92^{d} ± 0.17	-	13.11^{cd} ± 0.13	65.45^{ab} ± 0.07	18.83^{ab} ± 0.02	8.71^{d} ± 0.31	-
	5	9.95^{e} ± 0.07	53.83 ± 0.06	34.09^{a} ± 0.07	1.95^{e} ± 0.16	59.32^{b} ± 0.12	9.67^{d} ± 0.06	66.35^{a} ± 0.09	21.93^{a} ± 0.04	2.79^{e} ± 0.23	71.37^{b} ± 0.05

Means bearing different superscripts among treatment differ significantly.

Table 4.8 : Analyses of variance on the effect of tonicities of mBWW medium and incubation periods on acrosome reaction and other sperm characteristics of crossbred cattle and Murrah buffalo bulls

Source of variation	df	Mean squares											
		Crossbred cattle						Murrah					
		Acrosome reaction			Individual Motility	df	Livability	df	Acrosome reaction			Individual motility	df	Livability
		Intact acrosome	Partially reacted	Completely reacted					Intact acrosome	Partially reacted	Completely reacted			
Between tonicities	2	238.43*	55.14	20.42	1416.86**	2	46.80	2	172.96*	15.28	139.07**	1114.88**	2	47.09
Beetween periods	5	1214.11**	21.71	1173.14**	6577.43**	1	2842.67**	5	1861.12**	451.03**	516.06**	7266.84**	1	1241.62**
Tonicity x Period	10	8.76	25.39	35.66	17.82	2	20.10	10	7.24	23.02	13.65	35.18	2	18.22
Error	90	69.74	64.95	58.63	80.12	30	61.66	90	53.83	46.07	13.24	92.19	32	25.12

* $P < 0.05$; ** $P < 0.01$

motility declined significantly ($p < 0.01$ at 300 mosmol/kg, 46.75 ± 0.28%) to 400 mosmol/kg (25.96 ± 0.37 per cent). Similarly the highest intact acrosome of 27.80 ± 0.09 per cent was recorded in 300 mosmol/kg, while the lowest of 21.48 ± 0.10 per cent in 400 mosmol/kg.

The results also revealed that as the incubation period increased there are highly significant ($p < 0.01$) changes in all the parameters studied excepting the partially acrosome reacted spermatozoa. On incubation there was a drastic reduction of individual motility from 73.21 ± 0.10 to 1.95 ± 0.16 per cent, 86.14 ± 0.07 to 59.32 ± 0.12 per cent in case of livability and 42.95 ± 0.11 to 9.95 ± 0.07 per cent on intact acrosome, while the completely acrosome reacted sperm increased from 6.07 ± 0.08 to 34.09 ± 0.07 per cent.

4.3.1.3 Effect of CaI

The data on the effect of different levels of CaI concentrations on acrosome reaction and other sperm characteristics of crossbred and Murrah buffalo bull are presented in Table 4.9, the analysis of variance in Table 4.10.

The individual motility of sperm, acrosome reacted with different levels of calcium ionophore (0.1, 0.5, 1.0, 2.5 and 5 µm) ranged from 6.56 ± 0.28 to 34.10 ± 0.43 per cent as against the control value of 52.25 ± 0.19 per cent. The intact acrosome per cent of 51.45 ± 0.09 of control declined to the lowest level of 2.83 ± 0.04 at 2.5 µm concentration of calcium ionophore in the medium. The completely acrosome reacted sperm percentage increased from 30.23 ± 0.13 at 0.1 µm to 56.23 ± 0.16 at 5 µm concentration of CaI. The level

Table 4.9 : Effect of calcium ionophore concentrations and incubation periods on acrosome reaction and other sperm characteristics of crossbred cattle and Murrah buffalo bulls (Mean ± S.E.)

Treatment		Characteristics (per cent)									
		Crossbred cattle					Murrah				
		Acrosome reaction			Individual motility	Livability	Acrosome Reaction			Individual motility	Livability
		Intact acrosome	Partially reacted	Completely reacted			Intact acrosome	Partially reacted	Completely reacted		
CaI (μM) (n=36)	0	$51.45^{p} \pm 0.09$	$37.40^{t} \pm 0.04$	$14.76^{a} \pm 0.04$	$52.25^{p} \pm 0.19$	85.45 ± 0.28	$35.30^{p} \pm 0.10$	$51.98^{r} \pm 0.06$	$11.07^{s} \pm 0.03$	$47.72^{p} \pm 0.20$	76.21 ± 0.32
	0.1	$5.62^{r} \pm 0.06$	$61.15^{p} \pm 0.06$	$30.23^{r} \pm 0.13$	$34.10^{q} \pm 0.43$	85.91 ± 0.30	$31.30^{pq} \pm 0.10$	$52.13^{r} \pm 0.04$	$14.70^{r} \pm 0.03$	$40.00^{pq} \pm 0.26$	73.63 ± 0.31
	0.5	$12.06^{q} \pm 0.04$	$59.56^{pq} \pm 0.05$	$26.13^{r} \pm 0.07$	$33.32^{q} \pm 0.37$	83.60 ± 0.18	$29.63^{qr} \pm 0.08$	$51.38^{r} \pm 0.03$	$17.68^{q} \pm 0.03$	$37.32^{qr} \pm 0.27$	71.84 ± 0.30
	1.0	$3.21^{s} \pm 0.03$	$52.92^{qr} \pm 0.10$	$42.33^{q} \pm 0.23$	$13.19^{r} \pm 0.45$	82.43 ± 0.24	$26.14^{r} \pm 0.10$	$53.96^{qr} \pm 0.03$	$18.07^{q} \pm 0.04$	$30.78^{rs} \pm 0.21$	72.12 ± 0.32
	2.5	$2.83^{s} \pm 0.04$	$47.63^{rs} \pm 0.11$	$47.62^{q} \pm 0.15$	$10.58^{rs} \pm 0.41$	82.67 ± 0.41	$20.40^{s} \pm 0.08$	$57.18^{pq} \pm 0.04$	$20.02^{pq} \pm 0.06$	$24.00^{st} \pm 0.35$	73.38 ± 0.36
	5.0	$3.05^{s} \pm 0.04$	$40.33^{st} \pm 0.13$	$56.23^{p} \pm 0.16$	$6.56^{s} \pm 0.28$	81.95 ± 0.21	$13.71^{t} \pm 0.08$	$61.08^{p} \pm 0.04$	$21.76^{p} \pm 0.08$	$19.13^{t} \pm 0.39$	73.70 ± 0.42
Period (hour) (n=36)	0	$18.18^{a} \pm 0.19$	$61.32^{a} \pm 0.12$	$16.56^{e} \pm 0.08$	$67.72^{a} \pm 0.07$	$92.47^{a} \pm 0.03$	$49.60^{a} \pm 0.05$	$43.54^{c} \pm 0.06$	$6.18^{f} \pm 0.01$	$68.60^{a} \pm 0.08$	$88.45^{a} \pm 0.02$
	1	$15.32^{a} \pm 0.17$	$56.10^{a} \pm 0.09$	$24.22^{d} \pm 0.01$	$56.48^{b} \pm 0.10$		$38.68^{b} \pm 0.06$	$50.43^{b} \pm 0.04$	$10.91^{e} \pm 0.01$	$59.68^{b} \pm 0.03$	-
	2	$11.77^{b} \pm 0.12$	$53.96^{a} \pm 0.09$	$30.02^{d} \pm 0.13$	$28.25^{c} \pm 0.22$		$27.01^{c} \pm 0.04$	$55.57^{ab} \pm 0.04$	$16.09^{d} \pm 0.01$	$42.78^{c} \pm 0.06$	-
	3	$7.78^{c} \pm 0.14$	$49.25^{ab} \pm 0.06$	$38.86^{c} \pm 0.10$	$9.84^{d} \pm 0.30$		$21.39^{d} \pm 0.04$	$57.23^{a} \pm 0.02$	$20.17^{c} \pm 0.02$	$27.69^{d} \pm 0.08$	-
	4	$4.32^{d} \pm 0.14$	$42.75^{bc} \pm 0.06$	$48.47^{b} \pm 0.12$	$3.88^{e} \pm 0.22$		$14.31^{e} \pm 0.03$	$60.65^{a} \pm 0.02$	$24.64^{b} \pm 0.03$	$11.23^{e} \pm 0.13$	-
	5	$2.12^{e} \pm 0.11$	$35.53^{c} \pm 0.09$	$58.85^{a} \pm 0.14$	$1.45^{e} \pm 0.11$	$72.40^{b} \pm 0.04$	$10.01^{f} \pm 0.03$	$60.08^{a} \pm 0.03$	28.90 ± 0.03^{a}	3.55 ± 0.14^{f}	$56.55^{b} \pm 0.02$

Means bearing different superscripts among treatment differ significantly.

Table 4.10 : Analyses of variance on the effect of CaI concentrations and incubation periods on acrosome reaction and other sperm characteristics of crossbred cattle and Murrah buffalo bulls

Source of variation	df	Mean squares											
		Crossbred cattle						Murrah					
		Acrosome reaction			Individual Motility	df	Livability	df	Acrosome reaction			Individual Motility	df	Livability
		Intact acrosome	Partially reacted	Completely reacted					Intact acrosome	Partially reacted	Completely reacted			
Between CaI concentrations	5	6235.86**	1157.36**	3237.82**	5354.91**	5	20.11	5	1039.29**	73.93*	326.78**	1557.68**	5	12.33
Beetween periods	5	1531.23**	1070.90**	3334.75**	14559.21**	1	4469.22**	5	3527.15**	512.83**	1625.51**	11125.56**	1	7574.10**
CaI conc. x period	25	29.73	164.61**	24.82	192.56**	5	11.75	25	13.04	64.68*	17.21	43.82	5	8.78
Error	180	23.19	60.83	61.74	59.24	60	42.54	180	27.62	32.56	13.13	69.96	60	19.73

* $P < 0.05$; ** $P < 0.01$

FIG.4.4 EFFECT OF CAPACITATION AGENTS ON ACROSOME INTEGRITY OF MURRAH BUFFALO

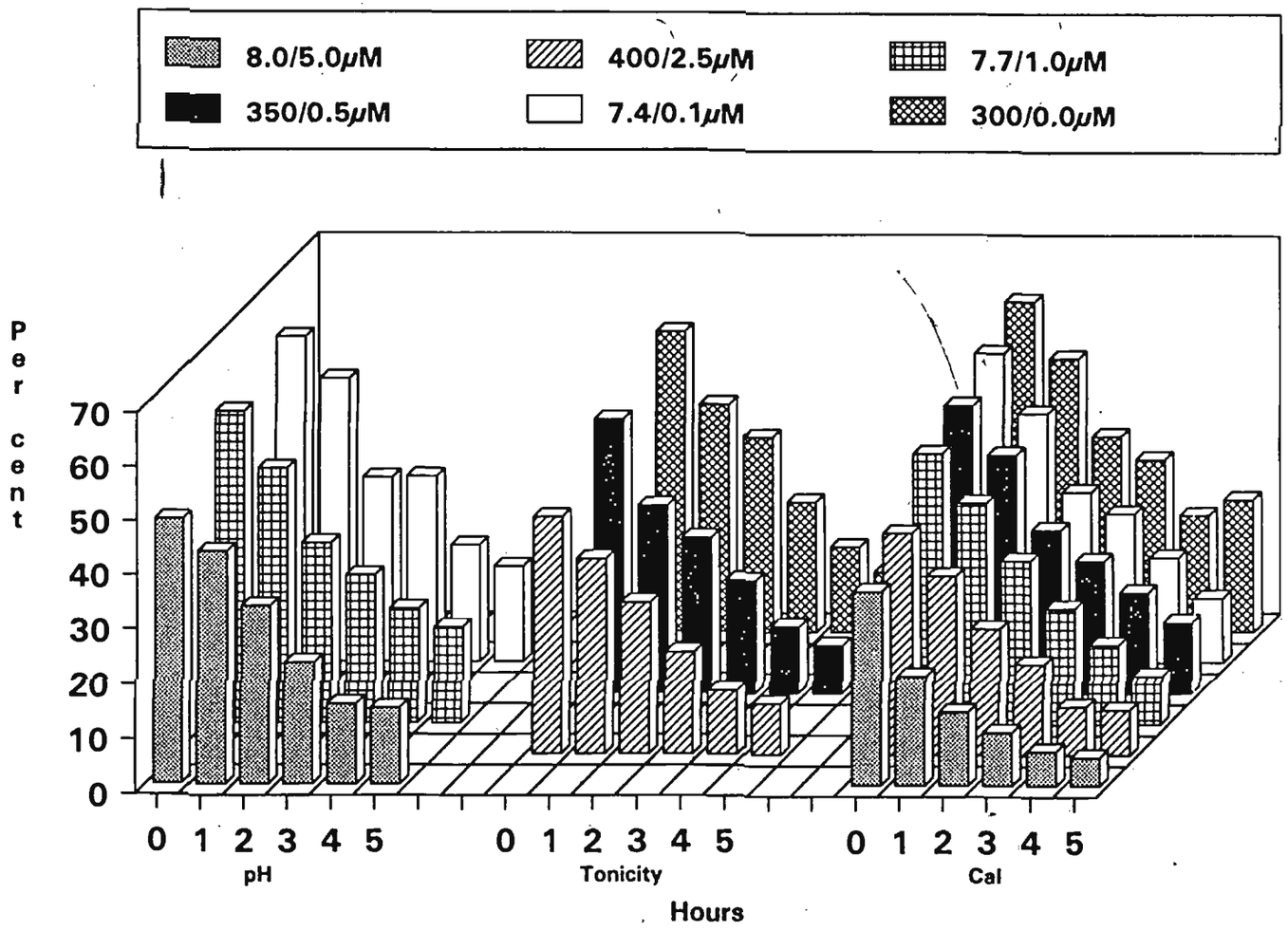


FIG.4.5 EFFECT OF CAPACITATION AGENTS ON COMPLETELY ACROSOME REACTED SPERM OF MURRAH BUFFALO

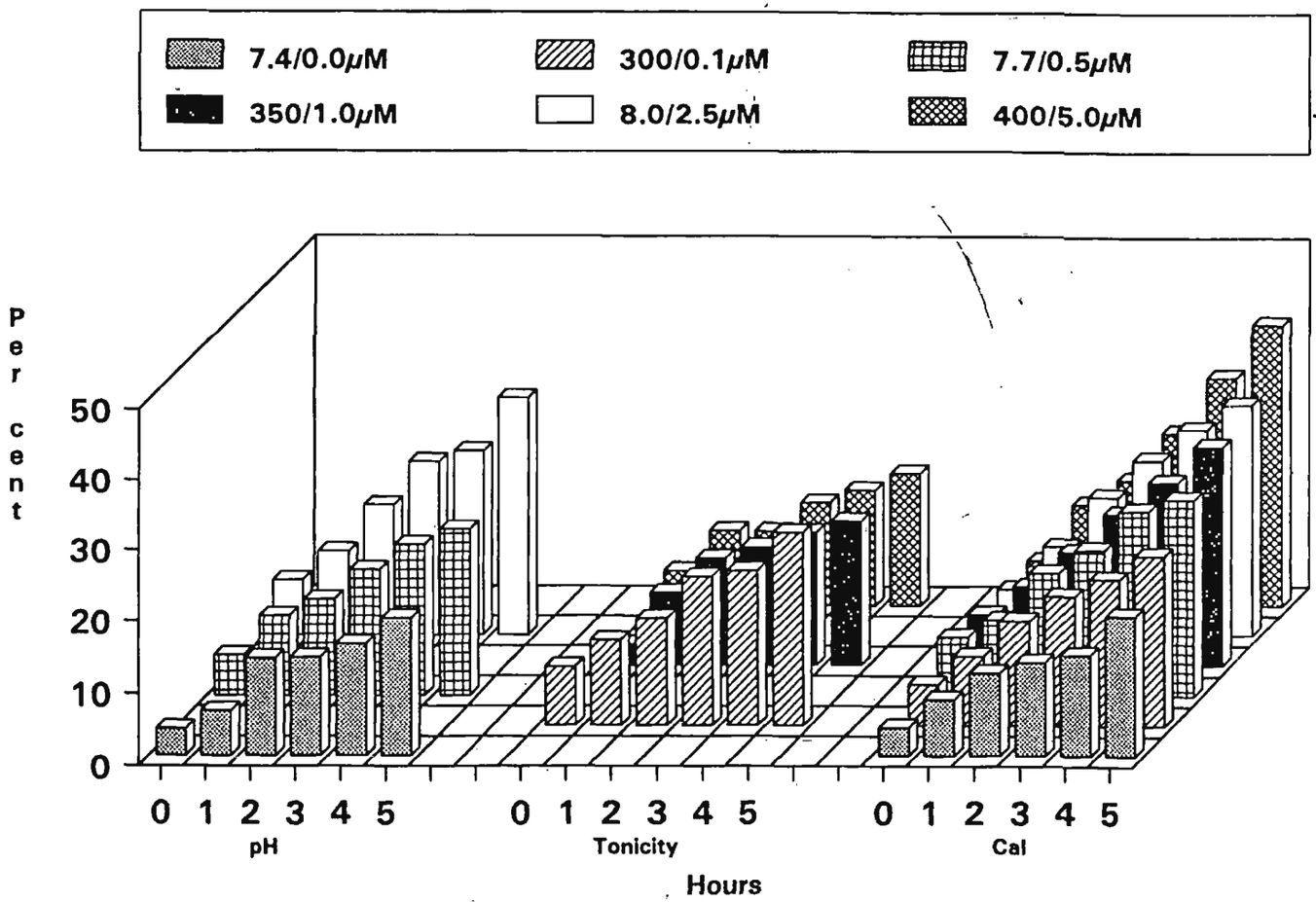
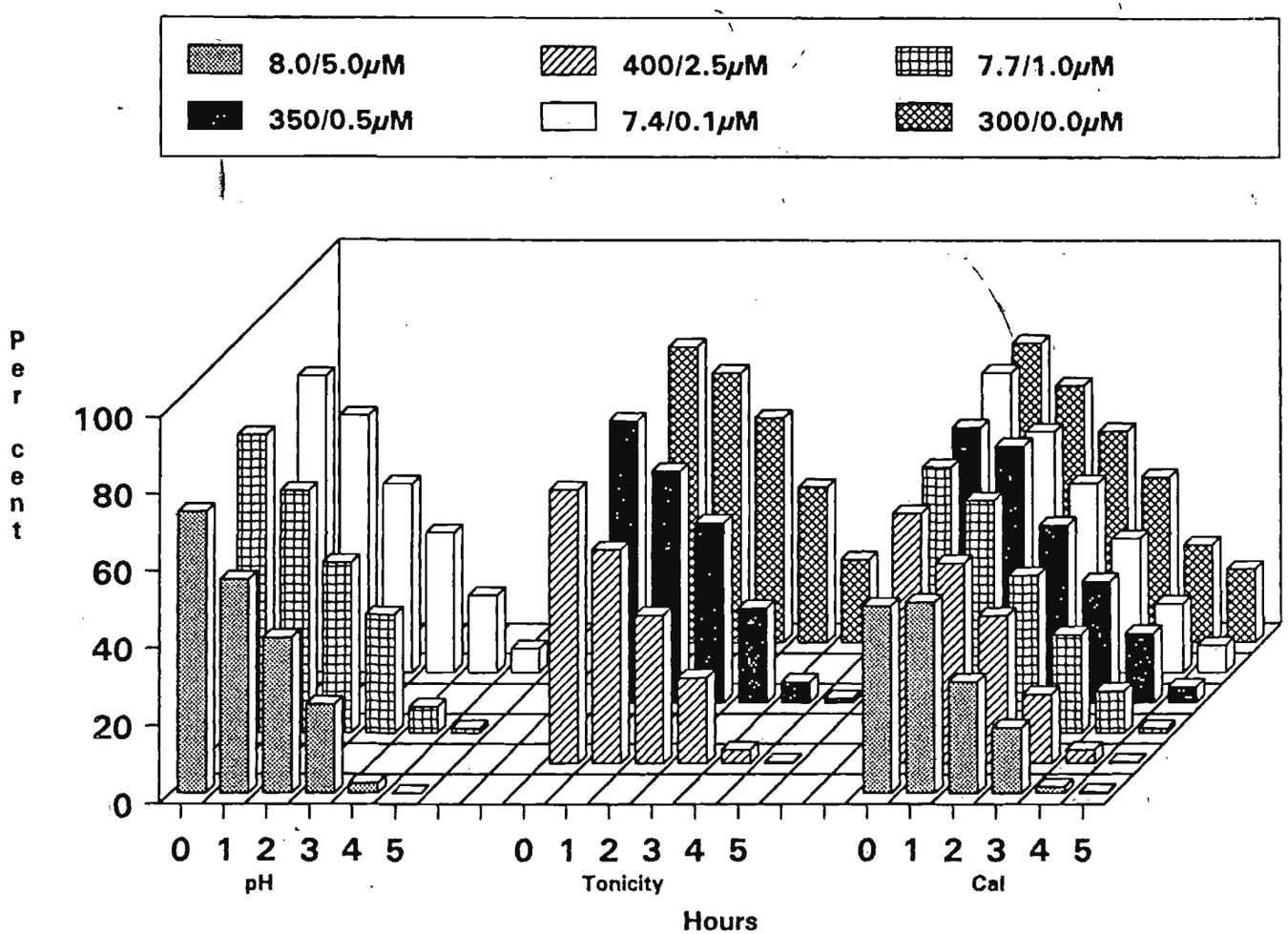


FIG.4.6 EFFECT OF CAPACITATION AGENTS ON INDIVIDUAL MOTILITY OF MURRAH BUFFALO



of CaI had a highly signficant ($p < 0.01$) influence on all the sperm characteristics studied except livability.

The analyses indicated the existance of highly significant ($p < 0.01$) effect of periods of incubation on all the sperm characteristics studied. The individual motility, the livability, the intact acrosome and partially acrosome racted sperm per cent declined as the incubation period increased. In contrast the completely acrosome reacted sperm per cent increased from 16.56 ± 0.08 to 58.85 ± 0.14 per cent as the incubation period increased to five hours.

4.3.2 MURRAH BUFFALO

4.3.2.1 Effect of pH

The buffalo spermatozoa incubated in the medium at different pH viz 7.4, 7.7 and 8.0 exhibited individual motility of 40.88 ± 0.28, 32.92 ± 0.38 and 25.83 ± 0.40 respectively (Table 4.5). The intact acrosome per cent decreased from 35.92 ± 0.08 to 28.20 ± 0.07 as the pH increased from 7.4 to 8.0 (Fig.4.4). While completely acrosome reacted sperm increased from 11.24 ± 0.03 to 19.57 ± 0.04 per cent with the increase in pH from 7.4 to 8.0 (Fig.4.5).

The incubation periods had a highly significant ($p < 0.01$, Table 4.6) influence on all the sperm characteristics. The individual motility, livability and intact acrosome per cent decreased while partially reacted and completely reacted spermatozoa increased as incubation period increased from zero to five hours (Fig.4.4 to 4.6).

4.3.2.2 Effect of tonicity of mBWW medium

The results (Table 4.8) revealed highly significant ($p < 0.01$) effect of tonicity on individual motility and completely acrosome reacted spermatozoa.

The tonicity at 300 mosmol/kg had a motility of 44.00 ± 0.31 per cent while it declined to 26.25 ± 0.38 per cent when the tonicity increased to 400 mosmol/kg (Fig.4.6). The completely reacted sperm also increased from 12.23 ± 0.02 to 16.80 ± 0.03 per cent as the tonicity increased from 300 to 400 mosmol/kg.

The incubation periods had highly significant ($p < 0.01$) influence on all the sperm parameters studied. The individual motility, livability and intact acrosome per cent declined whereas partially reacted sperm per cent increased as incubation periods increased.

4.3.2.3 Effect of CaI

The calcium ionophore concentration had highly significant ($p < 0.01$) effect on individual motility (Fig.4.4), intact acrosome and completely acrosome reacted sperm. The individual motility declined from 47.72 ± 0.20 to 19.93 ± 0.39 per cent whereas the completely reacted sperm increased from 11.07 ± 0.03 at control to 21.76 ± 0.08 per cent in 5 µm CaI concentration.

The influence of incubation period was found to be highly significant ($p < 0.01$) on all the sperm characteristics studied. The individual motility, livability and intact acrosome per cent decreased while per cent

increase in partially reacted and completely reacted spermatozoa was recorded as incubation periods increased (Fig.4.4 to 4.6).

4.4 SPB

4.4.1 EFFECT OF GAS PHASE AND SPERM CONCENTRATION

The suitability of 5% CO_2 in air and pure air as gas phase for conducting SPB was evaluated along with the influence of sperm concentration and the results are presented by Table 4.11.

4.4.1.1 Gas phase

The results of the study showed FP of 98.40 ± 0.67 and 98.55 ± 0.55 in 5% CO_2 in air and pure air (Table 4.11). However the analysis of variance presented in Table 4.12 revealed no significant ($p > 0.05$) difference between the two treatments. Similarly the differences in FI between 5% CO_2 in air (1.90 ± 0.27 per cent) and pure air (2.04 ± 0.31 per cent) was non-significant ($p > 0.05$).

4.5.1.2 Sperm concentration

Mean FP of 95.90 ± 1.60, 99.46 ± 0.53 and 100.00 ± 0.00 was recorded for SPB containing 10^6, 5 x 10^6 and 10^7 sperm/ml respectively in sperm drop. Interestingly the differences were non significant ($p > 0.05$). However, the sperm concentration in insemination medium had highly significant ($p < 0.01$) effect on FI. The FI was highest (4.72 ± 0.53) for 10^7 sperm/ml compared to 2.00 ± 0.36 with 10^6 sperm/ml.

Table 4.11 Effect of gas phases and concentrations of bull sperm on SPB

Treatment		Mean ± S.E.	
		FP	FI
Gas phase (n=5)	5% CO_2 in air	98.40 ± 0.67	1.90 ± 0.27
	Pure air	98.55 ± 0.55	2.04 ± 0.31
Sperm concentration (sperm/ml) (n=4)	10^6	95.90 ± 1.60	2.00^b ± 0.36
	5×10^6	99.46 ± 0.53	2.91^b ± 0.47
	10^7	100.00 ± 0.00	4.72^a ± 0.53

Means bearing different superscripts among treatment differ significantly.

Table 4.12 Analyses of variance on the effect of gas phases and sperm concentrations on SPB

Source of variation	df	Mean squares				
		Gas phase		df	Sperm concentration	
		FP	FI		FP	FI
Between treatments	1	0.246	0.048	2	140.20	7.63**
Error	8	100.945	0.426	9	93.80	0.83

** $P < 0.01$

4.4.2 CULTURE OF ZFE AFTER SPB

4.4.2.1 Cell division study

The culture of ZFE in TC 199 medium containing 15% FCS resulted in the cell division (plate 4.14) of 49.28 ± 2.64 per cent of total ZFE on examination after 24 hours of coincubation.

4.4.2.2 Sperm Chromosome Study

Chromosome complements of hamster oocyte bearing chromosomes of buffalo sperm have been presented in plate 4.15. The microphotograph indicates the haploid complement of the hamster oocyte on right side in group whereas buffalo sperm haploid scattered complement on the left.

4.5 EVALUATION OF CROSSBRED BULL SEMEN

The details of FP, FI, conception rates post thaw motility and HOST for the crossbred bull semen utilized for SPB are presented in Table 4.13 and Fig.4.7.

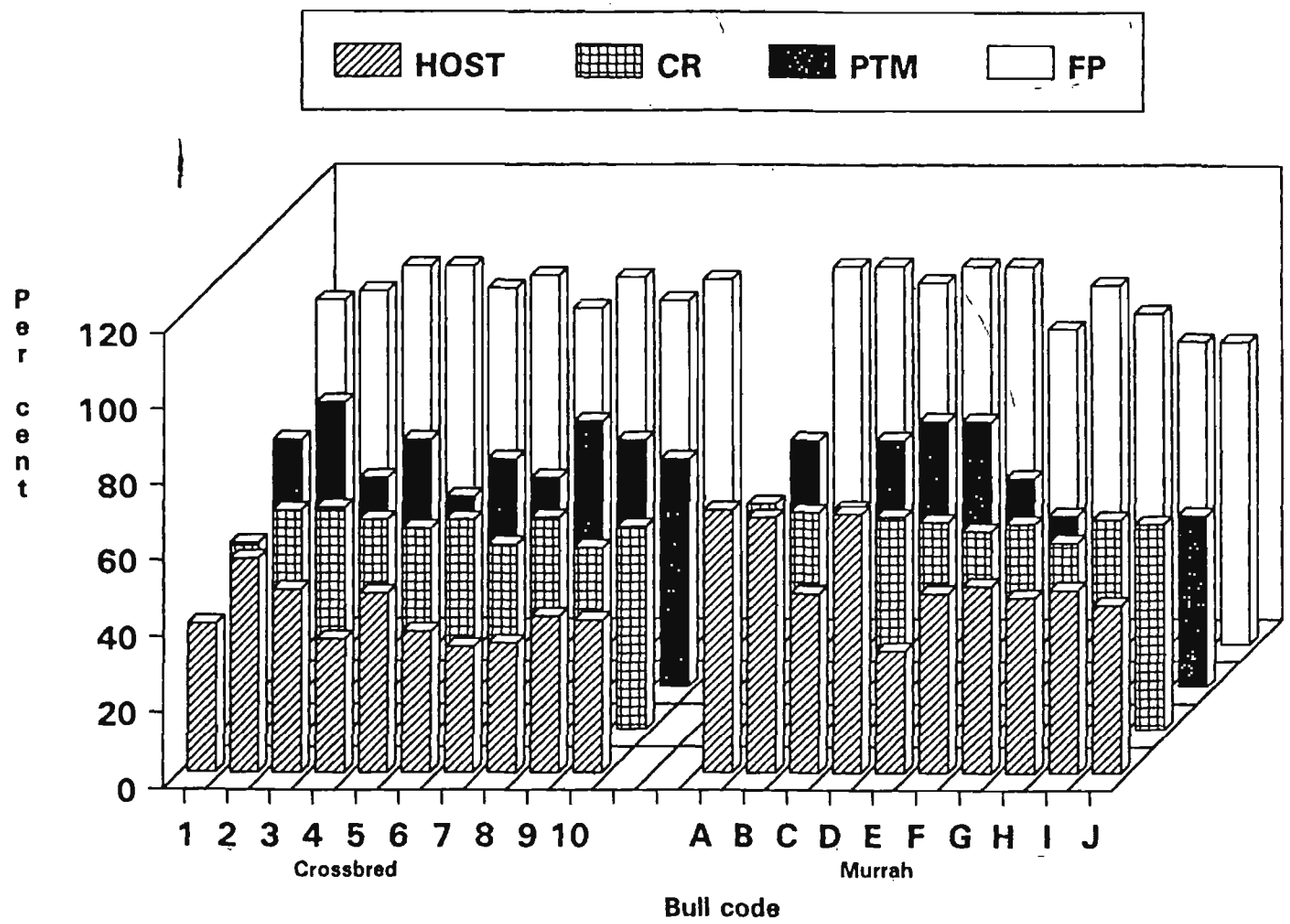
In the present investigation the number of eggs inseminated for each semen sample ranged from 28 to 45 with a mean of 37.90. The eggs penetrated ranged from 27 to 45. Out of these total number of sperm heads or pronuclei (plate 4.10 & 4.11) seen in ZFE ranged from 49 to 195. Among the samples studied highest and lowest FP of 100.00 and 88.89 were recorded. The FI ranged from 1.50 to 4.33. Conception rates based on pregnancy diagnosis for these samples ranged from 47.60 to 58.30 per cent.

Table 4.13 Fertility status of crossbred bulls and measures of SPB, conception rates, post thaw motility and HOST

Bull code	ZFE examined	ZFE penetrated	Total sperm heads in ZFE	FP	FI	Concepti on rate (per cent)	Post thaw motility (per cent)	HOST (per cent)
1.	33.00	30.00	57.00	90.90	1.73	48.80	65.00	39.00
2.	45.00	42.00	94.00	93.33	2.09	57.60	75.00	56.00
3.	40.00	40.00	130.00	100.00	3.25	58.30	55.00	48.00
4.	45.00	45.00	195.00	100.00	4.33	55.10	65.00	35.00
5.	34.00	32.00	54.00	94.11	1.59	52.70	50.00	47.00
6.	40.00	39.00	69.00	97.50	1.72	55.10	60.00	37.00
7.	36.00	32.00	69.00	88.89	1.92	48.50	55.00	33.00
8.	34.00	33.00	56.00	97.06	1.65	55.70	70.00	34.00
9.	44.00	40.00	66.00	90.91	1.50	47.60	65.00	41.00
10.	28.00	27.00	49.00	96.43	1.75	53.20	60.00	40.00
Mean ± SE	37.90 ± 1.83	36.00 ± 1.87	83.90 ± 14.51	94.91 ± 1.23	2.15 ± 0.29	53.26 ± 1.21	62.00 ± 2.38	41.00 ± 2.31
11*	173.00	161.00	315.00	93.06	1.82	*	55.00	53.00

* Used for standardization.

FIG.4.7 SPERM CHARACTERISTICS OF CROSSBRED CATTLE AND MURRAH BUFFALO



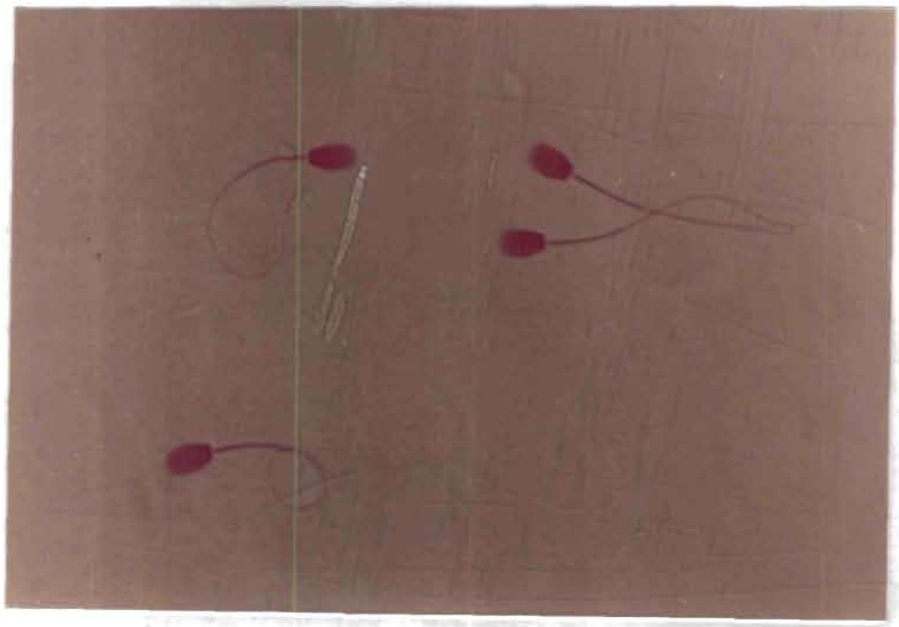


Plate 4.7 **Hypo-osmatic swelling of Murrah buffalo spermatozoa (Rose Bengal, 800X)**

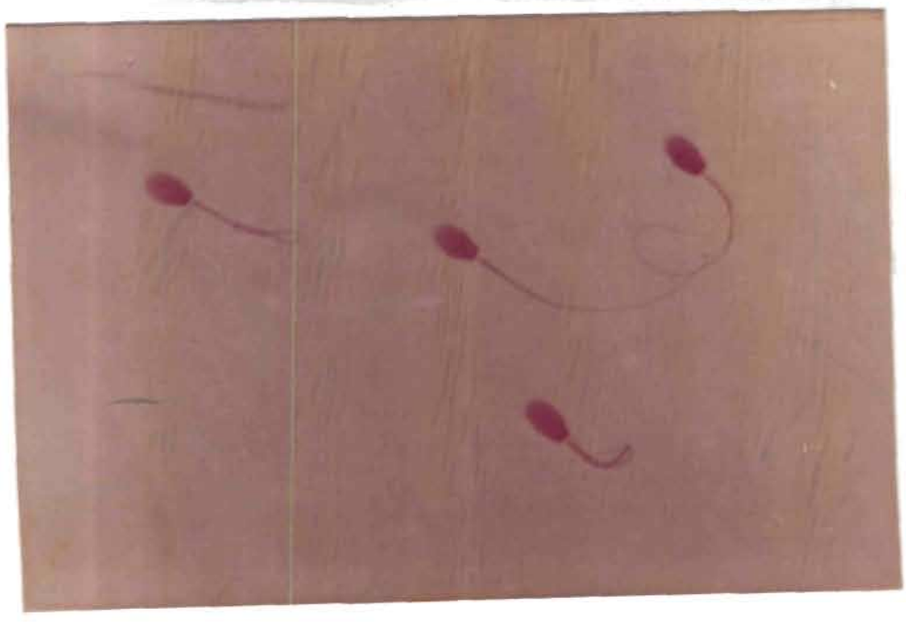


Plate 4.8 **Hypo-osmatic swelling of Murrah buffalo spermatozoa (Rose Bengal, 800X)**

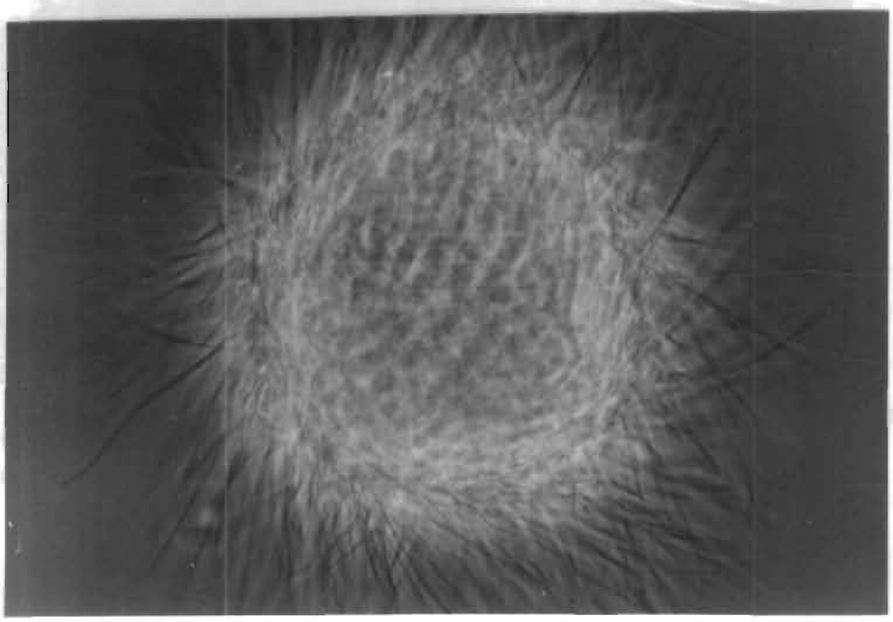


Plate 4.9 **Microphotograph showing heavy attachment of Murrah buffalo spermatozoa (Phase contrast, 500X)**

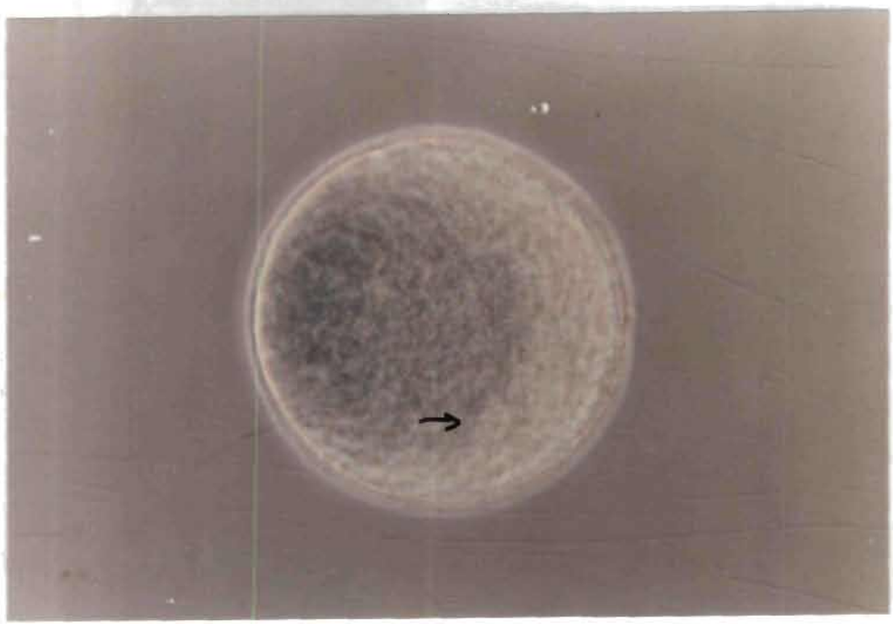


Plate 4.10 ZFE showing swollen sperm head on coincubation with crossbred cattle (Aceto-orcein, Phase contrast, 500X)

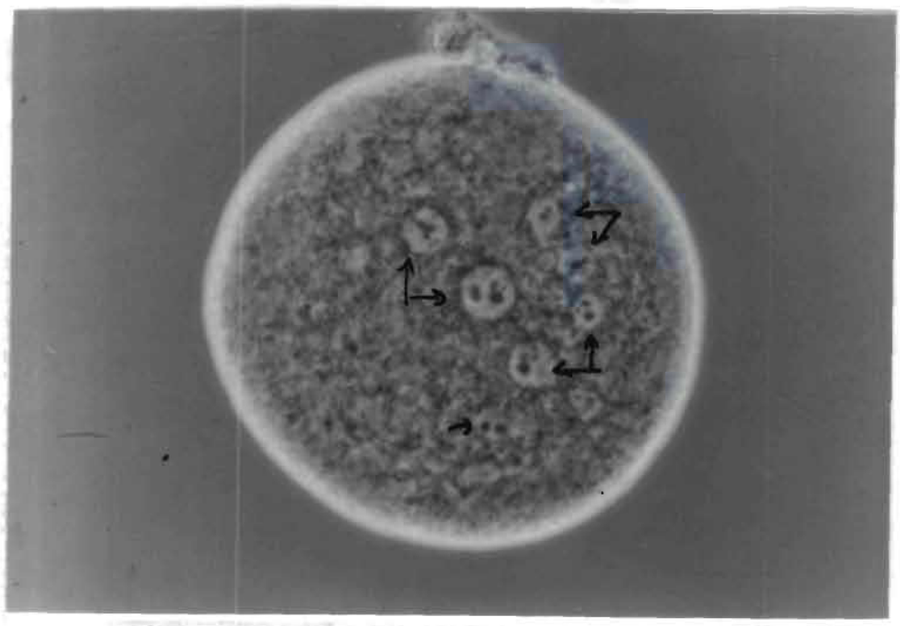


Plate 4.11 ZFE showing pronuclei on coincubation with crossbred spermatozoa (Phase contrast, 500X)

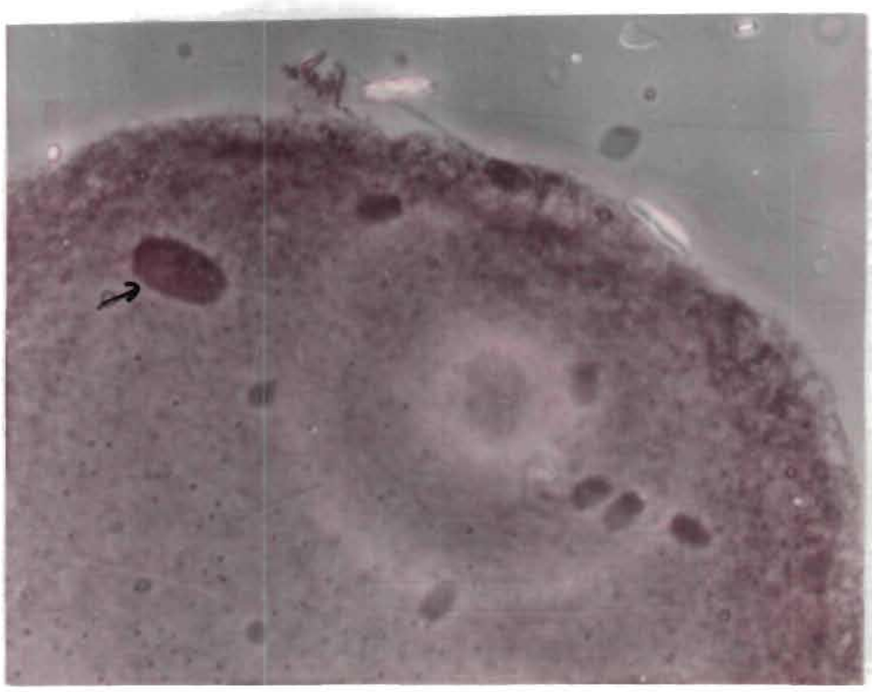


Plate 4.12 **ZFE showing swollen sperm head on coincubation with Murrah buffalo spermatozoa (Aceto-orcein, Phase contrast, 500X)**

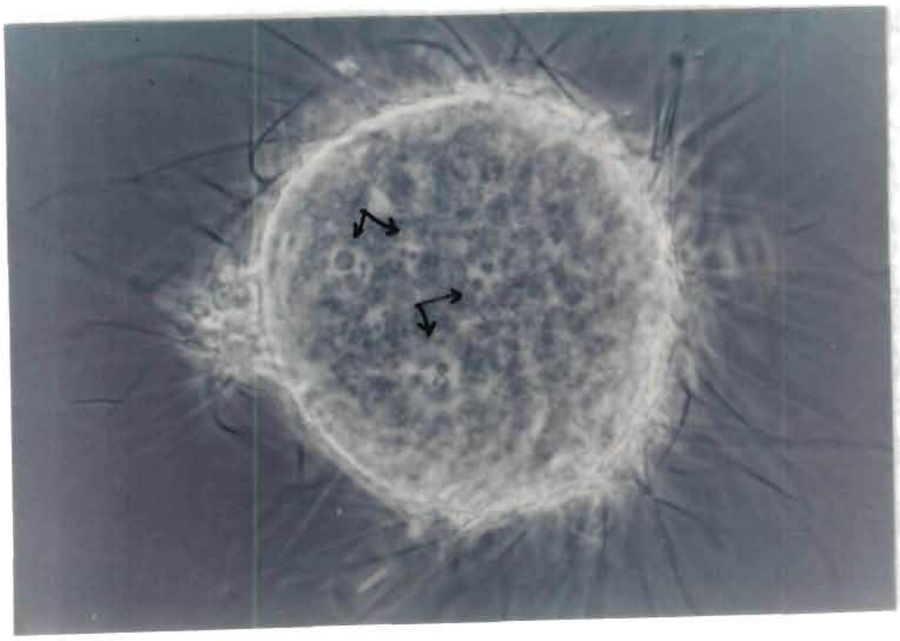


Plate 4.13 **ZFE showing pronuclei on coincubation with Murrah buffalo spermatozoa (Phase contrast, 500X)**

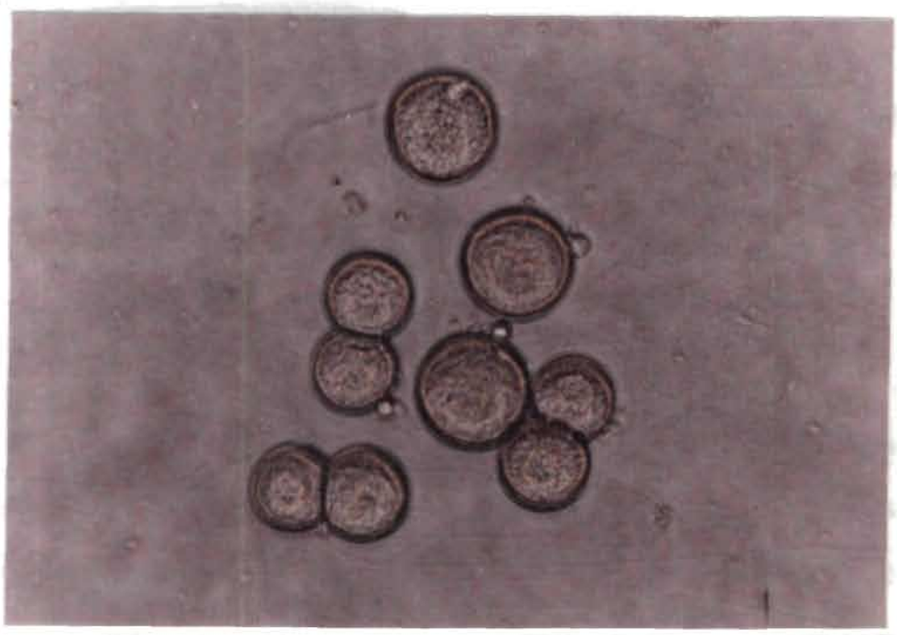


Plate 4.14 Cell division after coincubation of ZFE for 24 hours (200X)

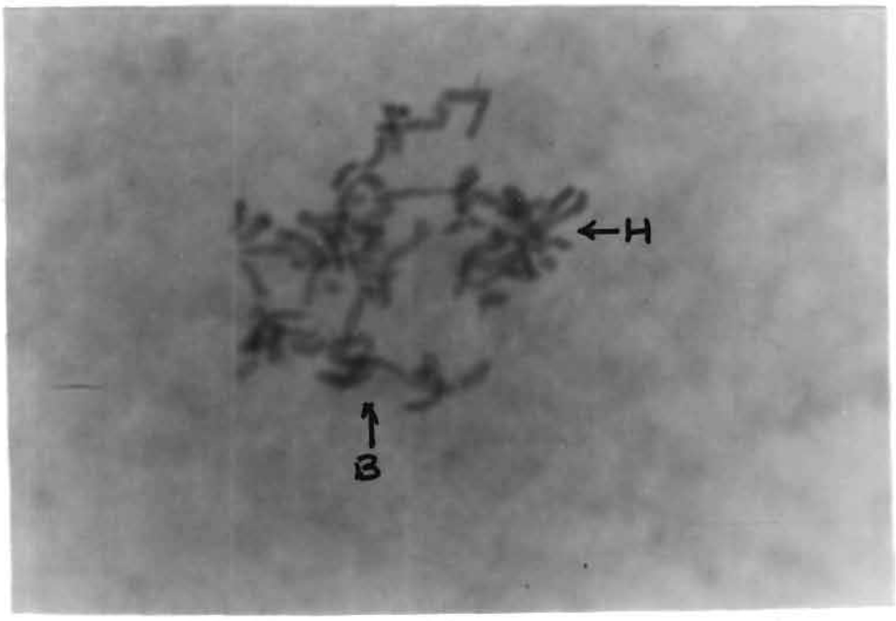


Plate 4.15 Chromosome preparation of ZFE penetrated buffalo spermatozoa (Giemsa, 800X)

4.5.1 CORRELATION COEFFICIENTS

The correlation coefficients between semen characteristics, parameters and conception rates of crossbred bull semen are presente Table 4.17.

4.5.1.1 Semen parameters

Surprisingly the results of the study indicated that conception had no significant ($p > 0.05$) bearing on the semen parameters studied. individual motility after zero hour of CaI treatment was found to be negati correlated (-0.645, $p < 0.01$) with completely acrosome reacted spermatozc zero hour and at one hour (-0.823, $p < 0.01$) of CaI treatment. Post t motility had highly significant ($p < 0.01$) correlation (0.892) with post t livability.

4.5.1.2 SPB and semen paramters

The FP had a high and positive correlation of 0.723 with concep rate. The FI was found to have highly significant correlation (0.804) with

4.6 HOST IN POST-THAW BUFFALO SEMEN

The results of different incubation times of buffalo sperm for H and the analysis of variance are presented in Table 4.15 and 4.16 respecti

The different incubation times adopted (15, 20 and 30 minutes) no significant ($p < 0.05$) effect on curling percentage (Plate 4.7 & 4.8).

Table 4.15 HOST of Murrah buffalo spermatozoa after exposure to TDW for different periods of incubation

Treatment		Mean ± S.E. (per cent)
Exposure time (minute) (n=10)	15	51.70 ± 3.86
	30	56.90 ± 1.44
	45	57.40 ± 1.54

Means bearing different superscripts among treatment differ significantly.

Table 4.16 Analyses of variance on the HOST of Murrah buffalo spermatozoa after exposure to TDW for different periods of incubation

Source of Variation	df	Mean square
Between exposure times	2	99.63
Error	27	64.64

Table 4.17 Correlation coefficients among sperm characteristics, HOST, SPB parameters and conception rates of crossbred bulls

Characters (n=10)			Conception rate	Individual motility			Completely acrosome reacted sperm			Post thaw livability	HOST	FP
				Post thaw	After CaI traetment		Post thaw	After CaI traetment				
					0 Hour	1 Hour		0 Hour	1 Hour			
Individual motility	Post thaw		0.210									
	After CaI treatment	0 Hour	0.097	0.363								
		1 Hour	0.261	0.206	0.734**							
Completely acrosome reacted sperm	Post thaw		0.060	-0.482	-0.294	0.073						
	After CaI treatment	0 Hour	0.384	-0.610*	-0.645**	-0.390	0.338					
		1 Hour	0.490	-0.559*	-0.823**	-0.140	0.314	0.929**				
Post thaw livability			-0.021	0.892**	0.217	0.056	-0.201	-0.608*	-0.372			
HOST			0.435	0.136	0.234	0.257	-0.384	-0.208	-0.027	-0.044		
FP			0.723**	-0.079	0.227	0.376	0.288	0.423	0.479	-0.289	-0.003	
FI			0.449	0.088	0.523	0.516	0.074	0.023	0.163	-0.166	-0.023	0.804**

* $P < 0.05$; ** $P < 0.01$

4.7 EVALUATION OF MURRAH BUFFALO BULL SEMEN

Various samples of Murrah buffalo bull semen evaluated based on different sperm characteristics like SPB parameters (FP & FI), conception rates, post thaw motility and HOST are presented in Table 4.14 and Fig.4.7.

The details of the FP and FI scoring for Murrah buffalo bulls revealed penetration of 22 to 40 eggs out of 23 to 40 ZFE used for scoring. The total number of swollen sperm heads or pronuclei (Plate 4.12 & 4.13) found in ZFE ranged from 39 to 108. FP and FI was found to be 80 to 100 and 1.30 to 2.70 respectively. The conception rate for different buffalo bulls ranged from 49.10 to 59.50 per cent. The post thaw motility and tail curling percentage for HOST recorded was found to be 35.00 to 70.00 and 32.00 to 69.00 per cent respectively.

4.7.1 CORRELATION COEFFICIENTS

Table 4.18 presents correlation coefficients between SPB parameters, sperm characteristics and conception rates of Murrah buffalo bulls.

4.7.1.1 Semen paramters

Completely acrosome reacted sperm after one hour of CaI treatment had highly significant ($p < 0.01$) correlation with conception rate. HOST was found to be significantly ($p < 0.05$) correlated with conception rate. Post thaw motility and post thaw livability were highly significantly ($p < 0.01$) correlated (0.907). Individual motility studied (post thaw, 0 and 1 hour after

Table 4.14 Fertility status of Murrah buffalo bulls and measures of SPB, conception rates, post thaw motility and HOST

Bull code	ZFE examined	ZFE penetrated	Total sperm heads in ZFE	FP	FI	Conception rate (per cent)	Post thaw motility (per cent)	HOST (per cent)
A	40.00	40.00	108.00	100.00	2.70	59.50	65.00	69.00
B	36.00	36.00	84.00	100.00	2.33	57.20	40.00	67.00
C	23.00	22.00	50.00	95.65	2.17	58.00	65.00	47.00
D	24.00	24.00	42.00	100.00	1.75	55.90	70.00	68.00
E	33.00	33.00	70.00	100.00	2.12	54.40	70.00	32.00
F	36.00	30.00	54.00	83.33	1.50	52.10	55.00	47.00
G	40.00	38.00	92.00	95.00	2.30	54.00	45.00	49.00
H	32.00	28.00	50.00	87.50	1.56	49.10	40.00	46.00
I	30.00	24.00	39.00	80.00	1.30	55.20	35.00	48.00
J	40.00	32.00	80.00	80.00	2.00	54.00	45.00	44.00
Mean ± S.E.	33.40 ± 1.98	30.7 ± 1.97	66.90 ± 7.40	92.15 ± 2.71	1.97 ± 0.14	54.94 ± 0.45	53.00 ± 4.29	51.70 ± 3.86
K*	63.00	56.00	102.00	88.89	1.62	*	45.00	46.00

* Used for standardization.

Table 4.18 Correlation coefficients among sperm characteristics, HOST, SPB parameters and conception rates of Murrah buffalo bulls

Characters (n=10)			Conception rate	Individual motility			Completely acrosome reacted sperm			Post thaw livability	HOST	FP
				Post thaw	After CaI traetment		Post thaw	After CaI traetment				
					0 Hour	1 Hour		0 Hour	1 Hour			
Individual motility	Post thaw		0.148									
	After CaI treatment	0 Hour	-0.181	0.754**								
		1 Hour	-0.432	0.632**	0.940**							
Completely acrosome reacted sperm	Post thaw		0.079	0.222	0.044	0.128						
	After CaI treatment	0 Hour	0.176	-0.784**	-0.872**	-0.858**	0.020					
		1 Hour	0.770**	-0.763**	-0.820**	-0.814**	0.133	0.957**				
Post thaw livability			0.373	0.907**	0.631**	0.528	0.316	-0.679**	-0.686**			
HOST			0.558*	0.010	-0.448	-0.573*	-0.388	0.176	0.071	0.062		
FP			0.588*	0.542	0.156	0.068	0.258	-0.197	-0.182	0.743**	0.492	
FI			0.660**	0.233	0.068	0.042	0.342	-0.129	-0.110	0.346	0.348	0.665**

* $P < 0.05$; ** $P < 0.01$

CaI treatment) was found to be correlated with each other significantly ($p < 0.01$). As expected negative and highly significant ($p < 0.01$) correlation between individual motility after CaI treatment (0 and 1 hour) and per cent completely acrosome reacted spermatozoa after CaI (o and 1 hour) treatment was recorded in Murrah buffalo (-0.814 to -0.872). Similarly significant ($p < 0.01$) negative correlation was observed with completely acrosome reacted spermatozoa after CaI treatment at 0 and 1 hour with post thaw livability.

4.7.1.2 SPB and semen paramters

The SPB paramters viz. FP and FI were found to be significantly and positively correlated with conception rates. The correlation coefficients recorded for FP and FI with conception rate were 0.588 ($p < 0.05$) and 0.660 ($p < 0.01$) respectively. The FP and FI were also observed to be correlated significantly ($p < 0.01$). Moreover, FP had a highly significant ($p < 0.01$) correlation of 0.743 with post thaw livability of spermatozoa.

Discussion

5. DISCUSSION

5.1 SUPEROVULATORY RESPONSE IN GOLDEN HAMSTERS

The results (Table - 4.1) on the effect of dosages of PMSG/HCG and the interval between PMSG and HCG administration on the superovulatory response in Golden Hamsters are discussed below.

5.1.1 GONADOTROPHIN DOSAGE

In the present investigation the levels of PMSG/HCG (30/30, 50/50 and 50/75) had a significant ($p<0.05$) effect on the number of oocytes recoverd. The highest number of oocytes (40.60 ± 2.57) was recovered at 50/75 I.U. dosage while the lowest (29.90 ± 1.64) number of oocytes was harvested for 30/30 I.U.

Perusal of the literature indicates a wide range (10 to 100 I.U) of dosages being used for the superovulation of the golden hamsters (Rogers *et al.*, 1979 Pavlok and Flechon, 1985; Bird and Houghton, 1989; Bird *et al.*, 1989). However, majority of the workers have not specified the number of oocytes recovered per animal as superovulatory response in their results.

The maximum number of oocytes (40.60 ± 2.57) harvested in this study as dose response to 50/75 I.U. is in agreement with the reports of Ramesha (1991), Interestingly, Martin (1983) Pryor (1986) and Tateno and Mikamo (1987) were able to recover 40-60 oocytes per animal with a dose as low as 21-25 I.U.

The variation in superovulatory response might be due to the light/dark rhythm of the day in which the animals were maintained, potency of the gonadotrophin used and strain of the animals used. Considering the overall picture, the superovulatory response obtained in this study was satisfactory.

5.1.2 DURATION BETWEEN PMSG TO HCG ADMINISTRATION

The results of the present investigation reveal that administration of HCG after PMSG either at 56 or 98 hours had no significant ($p>0.05$) differential effect on the superovulatory response. The interval between PMSG and HCG administered in various studies have been reported as short as 48 (Binor *et al.*, 1980; Hanada and Nagase, 1981; Aitken *et al.*, 1982) to as long as 98 hours (Creighton and Houghton, 1987; Ramesha, 1991 Table 2.2). The earlier reports are based on random studies. A comparative study taking into account the number of oocytes collected for various durations between gonadotrophin administrations are not available for comparison. The results of this study suggest that a shorter duration of 56 hours can be optimally employed to obtain superovulatory response in Golden Hamsters. This also coincides with the normal estrous cycle duration of the hamsters.

5.2 RECOVERY OF MOTILE SPERMATOZOA

The comparative merits of swim up and swim down techniques using two different concentrations of spermatozoa in isolating motile spermatozoa were assessed (Table 4.3 and Table 4.4).

5.2.1 SWIM UP AND SWIM DOWN TECHNIQUE

In the present study there was no significant ($p>0.05$) difference between the two techniques in terms of sperm recovery, individual motility and livability per cent. In contrast, Gonzales and Pella (1983) working on human semen recorded higher sperm recovery and individual motility per cent with swim down technique compared with swim up. Makler *et al.*, (1993) hypothesized that gravity causes the sperm heads to turn downward after which the oriented spermatozoa continue to move down their own tail movements, causing accumulation of motile spermatozoa at the bottom. This might explain why in some recent studies swim down was superior to the swim up procedure during sperm seperation by self-migration.

Significantly ($p<0.01$) higher per cent of intact acrosome (36.90 ± 0.07) was recorded in the present study following swim up procedure as against 13.00 ± 0.14 per cent in swim down. Considering this fact swim up technique seems to be a better method of isolating motile and morphologically intact spermatozoa to be used for *invitro* fertilization studies.

5.2.2 SPERM CONCENTRATION

The sperm concentration of the initial semen sample utilised for swim up or swim down technique at 10^7 and $5X10^7$ sperm/ml has got a significant ($p<0.05$) effect on sperm recovery, individual motility and livability per cent. The sperm recovery per cent was lower with $5X10^7$ sperm/ml (19.00 ± 0.12) as against 27.65 ± 0.09 per cent with 10^7 sperm/ml. However, the individual motility (85.30 ± 0.04 vs 79.80 ± 0.04) and livability (92.20 ± 0.05 vs 85.10 ±

0.04) per cent favour $5X10^7$ sperm/ml for utilizing in the swim up and swim down procedures.

5.3 CAPACITATION OF SPERMATOZOA

5.3.1 EFFECT OF PH ON CROSSBRED AND MURRAH BUFFALO SPERM

The effect of pH of the medium on acrosome reaction in crossbred and buffalo bull sperm has been presented in Tables 4.5 and 4.6.

In crossbred bulls the levels of pH (7.4, 7.7 and 8.0) had a highly significant ($P<0.1$) effect on individual motility and completely acrosome reacted sperm. The individual motility declined from 48.78 ± 0.15 to 33.78 ± 0.38 per cent as the pH increased. This might be due to the fact that the pH of the medium determines many important aspects of the structure and function of biological micromolecules, including enzyme activity and determines behaviour of the cells (Ijaz and Hunter, 1989b).

However, a different trend was observed in the completely acrosome reacted sperm per cent which increased from 11.20 ± 0.04 to 14.79 ± 0.15 as the pH increases from 7.4 to 8.0. Similar reports on the effects of higher pH of the medium hastening the capacitation and acrosome reactions has been reported by earlier workers (Cheng, 1985; Behnke, 1987; Ijaz and Hunter, 1989b).

To penetrate ZFE the sperm must be both motile and capacitated. Therefore both these factors must be considered together. The present results suggest that a higher pH of 8.0 will be more suitable for initiating acrosome reaction in bull sperm.

Results of the study on the effect of incubation periods on acrosome reaction and sperm viability revealed highly significant ($p<0.01$) changes over the periods, there was highly significant ($p<0.01$) reduction in individual motility, viability, and intact acrosome whereas, the partially and completely acrosome reacted sperm increased. The results of this study concur with the findings of Parrish *et al.*, (1985) in bovines and Samper *et al.*, (1989) and Varner *et al.*, (1993) in equine semen.

The present investigation with buffalo spermatozoa had established significant ($p<0.01$) effect of levels of pH on per cent individual motility, intact acrosome and completely reacted sperm. The incubation periods had a highly significant ($p<0.01$) effect on all the sperm characteristics studied. From the foregoing analysis it can be stated that buffalo sperm more or less behaves like bull sperm in its response to the effect of change in pH on acrosome reaction.

5.3.2 EFFECT OF TONICITY OF mBWW ON CROSSBRED AND MURRAH BUFFALO SPERM

The effect of pre-treatment of sperms with different tonicities of the medium on the acrosome reaction in crossbred and Murrah buffalo sperm has been presented in Table 4.7 and 4.8.

Pretreatment of sperm with different tonicities of medium viz. 300, 350 and 400 mosmol/kg resulted in highly significant reduction in motility. Aitken *et al.*, (1985) investigating the effect of hypertonic medium (410 mosmol/kg) recorded a significant reduction in motility with human semen.

Observations on acrosome reaction indicated significant ($p<0.01$) reduction in intact acrosome per cent as tonicity increased. Interestingly the tonicity had no significant ($p>0.05$) effect on the completely acrosome reacted sperm. Similar views were expressed by Bondioli and Wright Jr. (1983) with bovine sperm subjected to hypertonic (370-380 mosmol/kg) medium. With a differing note, Thompson and Cummins (1985) reported that hypertonic (360 mosmol/kg) medium had a significant effect on increasing the acrosome reaction in ram spermatozoa.

On incubation, a highly significant ($p<0.01$) reduction in the individual motility, intact acrosome and livability per cent was recorded while the completely acrosome reacted sperm increased from 6.07 ± 0.08 to 34.09 ± 0.07 per cent at 5 hours. These results are in agreement with the findings of Thompson and Cummins (1985).

The response of the buffalo sperm to hypertonic treatment was different from that of bull. The completely acrosome reacted sperm per cent significantly ($p<0.01$) increased from 12.23 ± 0.02 to 16.80 ± 0.03 per cent. This indicates pretreatment of hypertonic medium (400 mosmol/kg) helps buffalo sperm in acrosome reaction. The species variation on response to hypertonic treatment in rams had already been recorded by Thompson and Cummins (1985).

The incubation periods had a highly significant ($p<0.01$) effect on all the sperm characteristics studied as with bull sperm.

5.3.3 EFFECT OF CaI ON CROSSBRED AND MURRAH BUFFALO SPERM

The levels of CaI studied (0.1 to to 5.0 µM) had significant ($p<0.01$) influence on the individual motility and acrosome reaction. The decline in motility was gradual upto 0.5 µM CaI concentration, thereafter there is a drastic reduction in motility from 33.32 ± 0.37 at 0.5 µM to 13.19 ± 0.45 per cent, at 1.0 µM CaI concentration. Similarly significant increase in complete acrosome reaction was observed when the concentration goes up from 0.5 to 1.0 µM CaI concentration. Eventhough there was a substantial increase in acrosome reaction beyond 1 µM CaI concentration, this was accompanied by a concomitant reduction in motility. Since both motility and acrosome reaction are essential for successful fertilization the level of 0.5 µM of CaI was preferred in this study which compromises on both motility and acrosome reaction.

Shams-Bohran and Harrison (1981) opined that treatment of ram spermatozoa with calcium and the ionophore A23187 followed by serum albumin, results in modifications very similar if not identical to those in spermatozoa which have undergone capacitation and the acrosome reaction *in vivo*.

The divalent cation ionophore A23187, introduced by Reed and Lardy (1972), has been used to induce artificially a calcium dependent acrosome reaction in guinea pig, rabbit, boar, human, ram, bull and buffalo spermatozoa.

Increasing concentration of ionophore induced the acrosome reaction more rapidly, although it was associated with reduced motility. The finding of

this study also confirms the results of Shorgan and Hanada (1985) and Bird *et al.*, (1989) in bovine spermatozoa and Shams-Bohran and Harrison (1981) in ram spermatozoa. Shams - Bohran and Harrison (1981) have reported maximum response of CaI for inducing acrosome reaction at 1 µM concentration or above.

The incubation periods has a highly significant ($p<0.01$) effect on all the sperm characteristics studied. Bird *et al.*, (1989) observed significant ($p<0.05$) increase in acrosome reacted spermatozoa with incubation periods upto three hours. A similar trend was recorded in ram spermatoza by Shams-Bohran and Harrison (1981). They have also recorded a drop in individual motility as the incubation period increased.

Incubation periods showed highly significant ($p<0.01$) effect on all the parameters studied. Beyond one hour there was a drastic reduction in the individual motility from 56.48 ± 0.10 to 28.25 ± 0.22 per cent. This suggests that it will be ideal to use the calcium ionophore treated sperm for SPB within one hour of its treatment.

In buffalo sperm the level of CaI had highly significant effect on per cent individual motility, intact acrosome and completely acrosome reacted sperm. A distinct refracted response was observed with buffalo sperm for all the characteristics studied. For example, the individual motility was reduced to 6.26 ± 0.28 per cent at 5.0 µM CaI concentration in cattle while it was 19.13 ± 0.39 per cent for the same strength in buffaloes. Similarly the complete acrosome reaction was also minimal in buffalo sperm. The complete acrosome

reaction at 5.0 µM CaI concentration was 21.76 ± 0.08 in buffalo as against 56.23 ± 0.16 in cattle.

On incubation, there was a decline in individual motility and increase in complete acrosome reaction per cent. Similar trend was also reported by Takahashi *et al.*, (1989).

5.4 SPB

5.4.1 GAS PHASE

The results of this study indicate that preincubation of sperm and its coincubation with ZFE either in pure air or 5% CO_2 in air had no significant ($p>0.05$) difference on FP and FI. Many investigators have preincubated spermatozoa under pure air atmosphere and some of them did not even cover the sperm preincubation medium with mineral oil (Yanagimachi, 1984). However, on prolonged incubation a gradual change in pH is likely to occur. In SPB, when shorter preincubation periods are employed the gas phase may not be a critical factor. Further, as in the present study whenever HEPES buffered medium is used the pH is maintained well under both CO_2 and air atmospheres. The preparations can be taken in and out of the incubator without much worry about pH drift and its subsequent effect on FP and FI.

5.4.2 SPERM CONCENTRATION

The concentration of sperm in the insemination medium at 10^6, $5X10^6$ and 10^7 sperm/ml had no significant ($p>0.05$) effect on FP. However, the FI increased significantly ($p<0.01$) as the sperm concentration increased. Review of literature reveal wide variations in the sperm concentration utilized for SPB

ranging from 10^4 (Tateno and Mikamo, 1987) to 1.25 X 10^8 (Bird *et al.*, 1989) for bull spermatozoa (Table 2.4). Yanagimachi (1984) offers a possible explanation for the wide variation in sperm concentration reported in literature as follows. The concentration of sperm mentioned does not mean the concentration of live sperm, it includes both live and dead sperms. The effective, live and acrosome reacted sperm available at the end of preincubation period in each study is not known.

5.4.3 CULTURE OF ZFE AFTER SPB

5.4.3.1 Cell division study

The culture of ZFE in 5% CO_2 in air incubator after SPB in TC 199 medium containing 15% FCS resulted in the cell division of 49.28 ± 2.64 per cent on examination after 24 hours of coincubation. This value seems to be lower than the values of 78 per cent cell division with TC 199 medium at two cell stage eggs, recorded by Kamiguchi and Mikamo (1986). The possible higher cell division reported by them could be due to the following factors. Their primary aim was to obtain more of monospermic fertilization in order to study the chromosome complement. This was achieved by reducing sperm concentration and by shortening the time of insemination. It is not possible to obtain good spreads with excessively polyspermic eggs, since male pronuclear development is arrested in such eggs (Kamiguchi and Mikamo, 1986). In this study since karyotyping of sperm was not the primary aim of this investigation we have used higher sperm concentration and longer time of insemination, which might have resulted in lowered cell division rate.

5.4.2.2 Sperm chromosome study

Martin (1983) had reported the technique of preparation of sperm chromosome as time consuming, but it can be standardized for routine study. The sperm chromosome studies were attempted in various livestock species (Tateno and Mikamo, 1987; Creighton and Houghton, 1989; Tateno *et al.*, 1990) but a report on buffalo sperm chromosome appeared only recently (Ramesha and Goswami 1994[b]). Although, the study on buffalo sperm chromosome is beyond the scope of the objectives of this investigation, limited trials were made. Result indicated the possibility of studying of buffalo sperm chromosome through SPB.

5.5 EVALUATION OF CROSSBRED BULL SEMEN

The FP recorded for crossbred bulls ranged from 88.89 to 100.00 per cent with a over all mean of 94.91 per cent. Perusal of literature reveals wide variation in FP ranging as low as 6 to 100 per cent (Graham *et al* ., 1986; Hanada and Nagase, 1981). The present FP of 94.91 ± 1.23 is comparatively a higher value indicating a higher fertility status of the bulls studied. This is supported by a higher overall conception rate of 53.26 ± 1.21 per cent recorded for the bulls evaluated in this study.

Ramesha and Goswami (1994[a]) in a similar study recorded mean FP of 71.49 ± 2.34 which is lower than the present report. This is understandable since the conception rate for the samples utilized by Ramesha and Goswami (1994[a]) was also lower (34.37 ± 2.81). Rogers (1985) in a review article on the usefulness of SPB in evaluating the fertility of human semen

suggested that a score in the range of 15-100 % is taken as normal and 0-14% as below normal. The results also confirms the validity of the test in reflecting the fertility status of the semen samples.

The FI (2.15 ± 0.29) obtained in this study falls within the range (0.73 to 3.00) reported in literature (Ramesha and Goswami, 1994[a]; Eaglesome and Miller, 1989).

The mean per cent hypo-osmotic swelling of 41.00 ± 2.31 recorded in the present study is lower than 65.60 ± 4.64 reported by Sivaramlingam (1994) using TDW as medium whereas, the results concur with the findings of Sollanilla *et al.*, (1993) and Correa and Zavos (1994) with hypo-osmotic solution as a medium.

5.5.1 CORRELATION COEFFICIENTS

A highly significant ($p<0.01$) correlation (0.723) between FP and conception rate was recorded in this investigation. The uniqueness of this test in evaluating the potentiality of the sperm to pass through the various events of fertilization *in vivo* has been well established. Yanagimachi (1984) reported that the SPB evaluates the ability of the sperm to complete a series of biochemical and biophysical changes collectively refered to as capacitation. The assay also indirectly monitors the presence of acrosome reacted sperm which could fuse with egg plasma membrane and form sperm pronuclei (and possibly participate in the first cleavage). Results of this study further substantiate the usefulness of this test in evaluating fertility status of bull *in vitro*. The results of this study concur with the earlier reports of Graham and Foote (1987; r =

0.97), Davies *et al.*, (1987; r = 0.87) and Ramesha (1991 ; r = 0.77) on SPB with conception rate.

The precise mechanism of block to polyspermy depends on the species. The eggs of Golden Hamster totally depend on zona reaction they have no distinct (at least no rapid) egg plasma membrane block to polyspermy. This is one of the unique and useful feature of the hamster egg. Hence both FP and FI are indicators of fertilization potential of the spermatozoa tested. The FI value recorded in this investigation is comparable with the earlier reports of Graham *et al.*, (1986) and Eaglesome and Miller (1989). A lower FI value of 0.73 was reported by Ramesha and Goswami (1994a), incidently the conception rates for the samples were also low (34.37 ± 2.81).

A highly significant ($p<0.01$) negative correlation between individual motility at zero hour after CaI treatment and completely acrosome reacted sperm after zero and 1 hour of CaI treatment was recorded. Though CaI induces acrosome rection, its negative effect on individual motility has been well documented (Bird *et al.*, 1989; Shams-Bohran and Harrison, 1981). The absences of significant ($p>0.05$) correlations between semen parameters (individual motility, and livability) and the SPB might be due to the fact that routine semen analysis and hamster test do not necessarily measure the same physiological properties of the spermatozoa.

In the present investigation there was no significant ($p>0.05$) correlation between HOST and SPB. Lack of literature on HOST with SPB in bovine semen necessiates comparison of earlier work done in human semen. A positive correlation between HOST and SPB in human semen has been recorded by

several workers (Jeyendran *et al.*, 1984, Liu *et al.*, 1988; Fuse *et al.*, 1993). Lack of significant association between HOST and SPB as observed in this study has also been reported by others (Chan *et al.*, 1987; 1988 Takahashi, *et al.*, 1990).

5.6 EVALUATION OF MURRAH BUFFALO BULL SEMEN

An interesting phenomenon regarding the attachment of spermatozoa of buffalo with hamster oocyte was observed in this study (Plate 4.9). At lower concentration of CaI (0.5 µM) for 1 minute the attachment of the sperm was very high resulting in interference with scoring of the eggs although, this level of 0.5 µM was found to be ideal with cattle sperm. Similar observation was also recorded by Takahashi *et al.*, (1989). In the present study, this problem was eliminated when the CaI level was increased to 5 µM for 1 minute. The FP (92.15 ± 2.71) and FI (1.97 ± 0.14) values of buffalo sperm were slightly lower compared to crossbred bull sperm. Similarly the post-thaw motility was also lower with buffalo sperm (53.00 ± 4.29 vs 62.00 ± 2.38 in cattle). In contrast HOST value was higher in buffalo sperm (51.70 ± 3.86) as against 41.00 ± 2.31 in cattle.

5.6.1 CORRELATION COEFFICIENTS

Conception rate had significant ($p<0.05$) correlations with FP (0.588) and highly significant ($p<0.01$) correlation with FI (0.660). Further, the FP and FI were also significantly ($p<0.01$) correlated ($r=0.665$). Literature of SPB on buffalo are scanty. These values are comparatively lower than that of the finding of Ramesha (1991) who recorded correlation coefficients of 0.91 and

0.73 for FP and FI respectively. The present finding suggest the suitability of using SPB in predicting the fertility status of buffalo semen samples.

The HOST was found to be correlated significantly ($p<0.05$) with conception rate ($r=0.558$). This result confirms the earlier report of Sivaramlingam (1994). HOST determines the functional integrity of sperms plasma membrane. Spermatozoa with chemically and physically intact membrane will show tail curling under hypo-osmotic conditions whereas, spermatozoa with an inactive membrane will not. The post thaw livability had a highly significant correlation (0.743, $P<0.01$) with FP indicating its value on semen evaluation. However, the individual motility has no significant correlation with conception rate, HOST, FP and FI. This result undermines the significance of motility in predicting the fertility status of sperm. This statement also reflects the views of several other workers (Linford *et al.*, 1976; Soderquist *et al.*, 1991; Jasko *et al.*, 1992).

5.7 HOST IN POST-THAW BUFFALO SEMEN

The results on the incubation periods of HOST with TDW revealed 15 minutes to be ideally suited for buffalo sperm. Literature on HOST with buffalo sperm are lacking. However, this finding concurs with the incubation timings recomended for bull semen (Sivaramalingam 1994).

Summary

6. SUMMARY

The study on SPB utilizing zona free oocytes of golden hamsters (Mesocricetus auratus) was carried out at the Semen Bank, Department of Animal Genetics, Madras Veterinary College, Madras during the period 1992-95 with the main objectives of

i) to standardise the technique of SPB and

ii) to correlate SPB of cryopreserved semen with fertility of the bulls.

The studies on different levels of PMSG/HCG in inducing superovulation in golden hamsters indicated that 50/75 I.U. as the superior dosage combination resulting in the maximum harvest of 40.60 ± 2.57 oocytes per hamster.

The intervals between the administration of PMSG and HCG at 56 or 98 hours had no significant ($p>0.05$) effect on the superovulatory response.

A comparative study on swim up and swim down techniques in isolating motile sperm revealed no significant ($p>0.05$) difference in terms of sperm recovery, individual motility, livability and completely acrosome reacted sperm per cent. However, swim up technique seems to be a better method for isolating motile and morphologically intact spermatozoa.

The sperm concentration of the initial semen sample utilised for swim up or swim down technique had significant ($p<0.05$) influence on the sperm recovery, individual motility and livability per cent and $5x10^7$ sperm/ml was more suitable than 10^7 sperm/ml.

The different levels (7.4, 7.7 and 8.0) of pH studied in initiating acrosome reaction revealed a higher pH of 8.0 to be suitable for both crossbred and buffalo spermatozoa.

The influence of tonicities of mBWW medium (300, 350 and 400 mosmol/kg for 5 minutes) had a highly significant ($p<0.01$) effect on the per cent individual motility and intact acrosome in crossbred bull sperm. However, there was no acceleration in the acrosome reaction at higher ionic strengths. In contrast, use of hypertonic medium (400 mosmol/kg) helps in inducing better acrosome reaction in buffalo sperm.

Trials on the CaI, at various concentrations (0.1, 0.5, 1.0, 2.5 and 5.0 µM for one minute) revealed the suitability of 0.5 µM in inducing acrosome reaction in post-thaw sperm of crossbred bulls. In contrast, buffalo sperm had a refractory response resulting in the suitability of 5 µM for one minute of CaI for obtaining optimum acrosome reaction required in the SPB.

The effect of gas phase (5% CO_2 in air vs pure air) for incubation had no significant ($p>0.05$) difference on the SPB outcome.

Post insemination culture of ZFE resulted in 49.28 ± 2.64 per cent of cell division based on total ZFE cultured for the cell division.

The present investigation with limited trials revealed the possibility of the study of buffalo sperm chromosomes through SPB.

The HOST of Murrah buffalo sperm exposed to TDW for 15 minutes was found to result in maximum tail curling per cent.

The SPB with crossbred bull sperm resulted in mean FP of 94.91 ± 1.23 and mean FI of 2.15 ± 0.29. The FP had a highly significant ($p<0.01$) correlation (0.723) with conception rate in crossbred bull sperm indicating the reliability of FP in predicting the fertility status of spermatozoa. The FP had a highly significant ($p<0.01$) correlation (0.804) with FI. The individual motility and livability had no significant ($p>0.05$) correlation with either conception rate or SPB.

The buffalo sperm evaluated in the present investigation had a mean FP of 92.15 ± 2.71 and FI of 1.97 ± 0.14. The conception rate was correlated with FI (0.660, $p<0.01$) and with FP (0.588, $p<0.05$). There was also a highly significant ($p<0.01$) correlation (0.665) between FI and FP in buffalo sperm.

HOST conducted with TDW on frozen thawed sperm had a significant ($p<0.05$) correlation (0.558) with conception rate in buffalo indicating its potentiality in semen evaluation.

CONCLUSIONS

i) The PMSG/HCG at 50/75 I.U. with 56 hours of interval between their administration and air incubation of sperm and oocytes results in optimal response in SPB in both cattle and buffalo.

ii) CaI at 0.5 µM and 5.0 µM levels for 1 minute result in inducing ideal acrosome reaction for SPB with frozen-thawed spermatozoa of cattle and buffaloes respectively.

iii) The SPB reliably predicts the fertility status of cryopreserved crossbred and buffalo bull sperm.

APPENDIX

I. COMPOSITION OF MODIFIED BIGGERS-WHITTEN-WHITTINGHAM (mBWW) MEDIUM

Stock Solution (already prepared and refrigerated)

Component	g/L TGDW
NaCl	5.540
KCl	0.356
$CaCl_2.2H_2O$	0.250
KH_2PO_4	0.162
$MgSO_4.7H_2O$	0.294
0.2% phenol red	1.0 ml

The following chemicals were added to 100 ml of the stock solution to prepare complete mBWW medium just before use.

$NaHCO_3$	150 mg
Glucose	100 mg
Na-pyruvate	3 mg
Albumin (Fraction V, A4503, Sigma)	300 mg
HEPES sodium	128 mg
HEPES free acid	120 mg
Na-lactate	0.37 mg
Strepto-penicillin (100X)	1 ml

II. PREPARATION OF GIEMSA STAIN SOLUTION

Giemsa Stain powder	3.8 gm
Methanol	375 ml
Glycerol	125 ml

The Giemsa stain was ground with absolute methanol in a glass mortar and pestle. Warm glycerol (60°C) was added and this stock solution was stored at 37°C for one week. During the period the stock stain solution was mixed well for a few minutes every day by-shaking the bottle.

3.0 ml of above Giemsa stock solution was diluted with 2.0 ml of Sorenson's M/15 phosphate buffer at pH 7.0 and 35 ml of TGDW was added and mixed well. The above staining mixture was prepared afresh at the time of use for staining.

References

REFERENCES

Aitken, R.J., F.S.M. Best, D.W. Richardson, O. Djahanbakhch, and M.M. Lees, 1982. The correlates of fertilizing capacity in normal fertile men. Fert. Steril. **38** : 68-76.

Aitken, R.J., M. Sutton, P. Warner, and D.W. Richardson, 1985. Relationship between the movement characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona-free hamster oocytes. J. Reprod. Fert. **73** : 441-449.

Allison, A.J. and T.J. Robinson, 1971. Fertility of progestagon - treated ewes in relation to the number and concentration of spermatozoa in the inseminate. Aust. J. Biol. Sci. **24** : 1001 -1008.

Anand, S.R. S.K. Atreja, M.S. Chauhan, and R. Belh, 1989. *In vitro* capacitation of goat spermatozoa, Indian J. Exp. Biol. **27** : 921-924.

Andersson, M.M., Vierula, M. and Alanko, 1990. Three types of acrosomal aberrations of bull spermatozoa and their relation to fertility. Acta Vet. Scand. **31** : 175-179.

Ansbacher, R., 1978. Artificial insemination with frozen spermatozoa. Fert. Steril. **29** : 375.

Aravindakshan, T.V., 1992. Effect of platelet activating factor on the motility and acrosome reaction of cattle and buffalo spermatozoa. M.V.Sc. Thesis submitted to N.D.R.I., Karnal.

Ax, R.L., K.Dickson and R.W.Lenz, 1985. Induction of acrosome reactions by chondroitin sulfates *in vitro* corresponds to nonreturn rates of dairy bulls. J. Diary Sci., **68** : 387-390.

Barnes, F.L., J.L. Parrish, Susko - Parrish and N.L. First, 1987. Morphological and molecular aspects of early development in bovine. Theriogenology, **27** : 210.

Barros, C., J.E. Ganzales, E. Herrera, E. Bustos-Obregon, 1978. Fertilizing capacity of human spermatozoa evaluated by actual penetration of foreign eggs. Contraception. **17** : 83.

Barros, C., A. Jedlicki, I. Bize, and E. Aquirre, 1984. Relationship between the length of sperm preincubation and zona penetration in the golden hamster. A scanning electron microscopy study. Gamet Res., 31-43.

Barros, C., A. Jedlicki, I. Fuenzalida, E. Herrera, B. Arguello, P. Vigil, P.Villaseca and E. Leontic, 1988. Human sperm - cervical mucus interaction and the ability of spermatozoa to fuse with zona-free hamster oocytes. J. Reprod. Fert. **82** : 477-484.

Bavister, B.D., 1981. Analysis of culture media for *in vitro* fertilization and criteria for success. In : L. Mastroianna Jr. and Biggers. J.D. (eds.) Fertilization and Embryonic Development *in vitro*. Plenum, New York, p.41-60.

Beatty, R.A., D.L. Stewart, R.L. Spooner and J.L. Hancock, 1976. Evaluation by the heterospermic insemination technique of the differential effect of freezing at -196°C on fertility of individual bull semen. J. Reprod. Fert. **47** : 377-379.

Bedford, J.M., 1970. Sperm capacitation and fertilization in mammals. Biol. Reprod. (Suppl.) **2** : 128-158.

Bedford, J.M., 1983. Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biol. Reprod. **28** : 108-120.

Behenke, E.J., 1987. Bovine sperm capacitation and oocyte maturation *in vitro*, Ph.D. Thesis, Univ. Minnesota, St.Paul. (Ijaz and Hunter, 1989b, J.Dairy Sci., **72** : 2691-2699).

Berger, T., E.Z. Drobnis, L. Foley, J.K. Metzler, and M. Horton, 1994. Evaluation of relative fertility of cryopreserved goat semen. Theriogenology **41**: 711-717.

Berger, T. and M.B.Horton, 1988. Evaluation of assay conditions for the zona-free hamster ova bioassay of boar sperm fertility. Gamet Res. **19** : 101-111.

Berger, T., R.P. Marrs. H. Saito and D.A. Kletzky, 1983. Factors affecting human sperm penetration of zona-free hamster ova. Am. J. Obstet. Gynecol. **145** : 397-401.

Berger, T. and K.Parker, 1989. Modification of the zona-free hamster ova bioassay of boar sperm fertility and correlation with *in vivo* fertility. Gamet Res. **22** : 385-397.

Berger, R.E., W.D. Smith, C.W. Critchlow, M.A. Stenchever, D.E. Moore, L.R. Spadoni and K.K. Holmes, 1983. Improvement in the sperm penetration (hamster ova) assay (SPA) results after doxycycline treatment of infertile man. J. Androl. **4** : 126-130.

Biggers, J.D., W.K.Whitten and D.G.Whittingham, 1971. The culture of mouse embryos *in vitro*. In Methods in Mammalian Embryology (J.C.Daniel, Jr.Ed.), W.H. Freeman and Company, San Francisco, pp.86-116.

Binor, Z., R. Rao and A. Scommegna, 1982. Further studies on dysfunction human spermatozoa using the zona-free hamster oocyte penetration assay. Fert. Steril. **37** : 300.

Binor, Z., J.E. Sokoloski and D.P.Wolf, 1980. Penetration of the zona-free hamster egg by human sperm. Fert. Steril. **33** : 321-327.

Bird, J.M., S. Carey, and J.A. Houghton, 1989. Motility and acrosomal changes in ionophore treated bovine spermatozoa and their relatinoship with *in vitro* penetration of zona-free hamster oocytes. Theriogenology. **32** : 227-243.

Bird, J.M. and J.A. Houghton, 1989. Fertilization of zona-free hamster ova by ionophore treated boar sperm and the condensation of sperm chromosomes. Anim. Reprod. Sci. **21** : 125-140.

Bondioli, K.R. and R.W. Wright, Jr. 1983. *In vitro* fertilization of bovine oocytes by spermatozoa capacitated *in vitro*. J. Anim. Res. **57** : 1001-1005.

Bousquet, D. and B.G. Brackett, 1981. Penetration of zona-free hamster ova by bull sperm after frozen storage. Theriogenology **15** : 117.

Bousquet, D. and B.G. Brackett, 1982. Penetration of zona-free hamster ova as a test to-assess fertilizing ability of bull sperm after frozen storage. Theriogenology **17** : 199-213.

Brackett, B.G., M.A. Cofone, M.L. Boice and D. Bousquett, 1982. Use of zona-free hamster ova to assess sperm fertilizing ability of bull and stallion. Gamet Res. **5** : 217-227.

Brackett, B.G., J.L. Hall, and Y.K. Oh, 1978. *In vitro* fertilizing ability of testicular, epididymal and ejaculated rabbit spermatozoa. Fert. Steril. **29** : 571.

Brackett, B.G., Y.K. Oh, J.F. Evans, and W.J. Donawick, 1980. Fertilization and early development of cow ova. Biol. Reprod. **23** : 189-205.

Brackett, B.G. and G. Oliphant, 1975. Capacitation of rabbit spermatozoa *in vitro*. Biol-Reprod. **12** : 260-275.

Chan, S.Y.W., S.Q.Li and C. Wang, 1987. Test Yolk buffer storage increases the capacity of human sperm to penetrate hamster eggs *in vitro*. Int. J. Androl. **10** : 507-514.

Chan, S.Y.W., C. Wang, M. Ng, W.W.K. So and P.C. Ho, 1988. Multivariate discriminant analysis of the relationship between the hyposmotic swelling test and *in vitro* fertilizing capacity of human sperm. Int. J. Androl. **11** : 369-378.

Chang, M.C., 1951. Fertilizing capacity of spermatozoa deposited into fallopian tubes. Nature **168** : 697-698.

Cheng, W.T.K., 1985. *In vitro* fertilization of farm animal oocytes. Ph.D. Thesis. Anim. Res. Stn. Cambridge, England. (Ijaz and Hunter, 1989b, J.Dairy Sci. **72** : 2691-2699).

Chiras, D.D. and G.S. Greenwalds, 1977. An autoradiographic study of long term follicular development in the cyclic hamster. Anat. Rec. **188** : 331-338.

Cohen, J., R.F.A., Weber, J.C.M. van der Vijver and G.H. Zeilmaker, 1982. *In vitro* fertilizing capacity of human spermatozoa with the use of zona-free hamster ova : interassay variation and prognostic value. Fert. Steril. **37** : 565-572.

Cooper, T.G., 1984. The onset and maintenance of hyperactivated motility of spermatozoa from the mouse. Gamet Res. **9** : 55-74.

Correa, J.R. and P.M. Zavos, 1994. The hypoosmotic swelling test : its employment as an assay to evaluate the functional integrity of the frozen thawed sperm membrane. Theriogenology **42** : 351-360.

Creighton, P. and A. Houghton, 1987. Visualization of pig sperm chromosomes by *in-vitro* penetration of zona-free hamster ova. J. Reprod. Fert. **80** : 819-822.

Cummins, J.M. 1982. Hyperactivated motility patterns of ram spermatozoa recovered from the oviducts of mated ewes. Gamet Res. **6** : 53-63.

Davies, A.P., J.K.Graham, and R.H.Foote, 1987. Homospermic versus heterospermic insemination of zona free hamster eggs to assess fertility of fluorochrome-labeled acrosome-reacted bull spermatozoa. Gamet Res. **17** : 343-354.

Didion, B.A. and C.N. Graves, 1986. *In vivo* capacitation and acrosome reaction of bovine sperm in estrous and diestrous cows. J. Anim. Sci. **62**: 1029-1033.

Drevius, L. - O., 1971 The 'sperm-rise' test. J. Reprod. Fert. **24** : 427-429.

Eaglesome, M.D. and S.A. Miller, 1989. Prediction of fertility of bovine semen: preliminary studies with the hamster egg penetration test. Theriogenology : **31** : 643-651.

Edwin, M.J. 1985, Freezability of buffalo semen in relation to biochemical constituents and processing techniques. Ph.D. Thesis submitted to Tamil Nadu Agricultural University, Coimbatore.

El-Gaafary, M.N., C.N. Graves, and P.B. Goncalves, 1993. Re-activated bull spermatozoa : motility acrosome status and ability to penetrate cervical mucus and zona-free hamster oocytes. Anim. Reprod. Sci. **32** : 163-172.

Entwistle, K.W. 1972. Congored-Fast green FCS as a supra-vital stain for ram and bull spermatozoa. Aust. Vet. J. **48** : 55-59.

Fleming, A.D. and R. Yanagimachi, 1982. Fertile life of acrosome reacted guinea pig spermatozoa. J. Exp. zool. **220** : 109-115.

Fraser, L.R. 1977. Motility patterns in mouse spermatozoa before and after capacitation. J. Exp. Zool. **282** : 439-444.

Fraser, L.R. 1981. Dibutyrylcyclic AMP decreases capacitation time *in vitro* in mouse spermatozoa. J. Reprod. Fert. **62** : 63-72.

Fraser, L.R. 1982. Ca^{2+} is require for mouse sperm capacitation and fertilization *in vitro* J. Androl. **3** : 412-419.

Fraser, L.R. 1984. Mechanisms controlling mammalian fertilization : In J.R.Clarke (ed) "Oxford Reviews of Reproductive Biology". Oxford University Press, **6** : 174-225.

Fraser, L.R. and K.K. Ahuja, 1988. Metabolic and surface events in fertilization. Gamet Res. **20** : 491-519.

Fuse, H.,S.Ohatta, M.Sakumato, T.Kazama and T.Katayama, 1993. Hypo-osmotic swelling test with medium of distilled water. Arch. Androl. **30**:111-116

Garde, J., A., Gutie'rrez, C.G. Artiga, and I. Va'zquez. 1993. Influence of freezing process on *in vitro* capacitation of ram semen.

Gerald, O. and P. Humblot, 1991. Influence of interaction between semen extender and number of spermatozoa on nonreturn rate estimates of fertility for individual bulls. Theriogenology, **36** : 727 - 736.

Gonzales, G.F. and R.E. Pella, 1993. Swim-down : A rapid and easy method to select motile spermatozoa. Arch. Androl. **30** : 29-34.

Gonzales, G.F. and M.Zapana, 1992. Sperm motility should be assessed in fresh sperm and after sperm washing procedure. Arch. Androl. **28** : 83-89.

Graham, J.K. and R.H.Foote, 1987. Dilaurylphosphatyl-choline liposome effects on the acrossome reaction and *in vitro* penetration of zona-free hamster eggs by bull sperm : I, A fertility assay for fresh semen. Gamet Res. **16**: 133-146.

Graham, J.K. R.H. Foote, and S.R. Hough, 1987. Penetration of zona-free hamster eggs by liposome treated sperm from the bull, ram, stallion and boar. Biol. Reprod. **37** : 181-188.

Graham, J.K., R.M. Foote, and J.J. Parrish, 1986. Effect of Dilauroylphosphatidylcholine on the acrosome reaction and subsequent penetration of bull spermatozoa into zona-free hamster eggs. Biol. Reprod. **35** : 413-424.

Graham, J.K., J.P. Nolan, and R.H. Hammerstedt, 1991. Effect of dilauroylphophatidylcholine liposomes on motility induction of the acrosome reaction and subsequent egg penetration of ram epididymal sperm. Biol. Reprod. **44** : 1092-1099.

Graham, E.F., M.K. Schmehl, and D.S. Nelson, 1980. Problems with laboratory tests. Proc. 8th NAAB Tech. Conf. A.I. Reprod. pp.59-66.

Greenwald, G.S., 1974. Quantitative aspects of follicular development in untreated and PMS-treated cycle hamster, Anat. Rec. 178 : 139-144.

Greenwald, G.S. 1978. Follicular activity in the mammalis ovary. In the vertebrate ovary, pp. 638-689. Plenum Press, N.Y. and London.

Gupta, H.C. 1962. Biochemical and physiological properties of cervical and uterine fluids of the cow during estrus. Diss. Abstr. **28** : 803.

Gwatkin, R.B.L. and O.F. Andersen, 1969. Capacitation of hamster spermatozoa by bovine follicular fluid. Nature, Lond. **224** : 1111.

Hackett, A.J. and J.W. Macpherson, 1965. Some staining procedures for spermatozoa : a review. Can. Vet. J. **6** : 55-61.

Hafez, E.S.E., 1987. Semen Evaluation. In Reproduction in Farm Animals. E.S.E. Hafez (ed.), pp.455-480, Vth Edition, Lea & Febiger, Philadelpia,

Hafez, E.S.E., 1993. Semen Evaluation in Reproduction in Farm Animals. E.S.E. Hafez (ed.) pp.405-23. VIth Edition, Lea & Febiger, Philadelphia.

Hall, J.L. 1981. Relationship between semen quality and human sperm penetration of zona-free hamster ova. Fert. Steril. **35** : 457-463.

Hamner, C.E. and W.L. Williams, 1965. Composition of rabbit oviductal secretions. Fert. Steril. **16** : 170.

Hanada, A. and M.C. Chang, 1972. Penetration of zona-free eggs by spermatozoa of different species. Biol. Reprod. **6** : 300-309.

Hanada, A. and M.Nagase, 1981. Effects of sperm preincubation in rabbit uterus and of imidazole on the penetration of zona free hamster eggs by bull and boar spermatozoa *in vitro*. Japan. J. Anim. Reprod. **27** : 113-118.

Hancock, J., 1952. The morphology of spermatozoa. J. Exp. Biol. **29** : 445-453.

Harrison, R.A.P., 1982. The sperm, its hydrolases, and egg penetration. In : The Sperm Cell, J. Andre' (ed.), The Hague; Martinus Nijhoff, pp.259-273.

Horan, R., R. Powell, F. Gannon and J.A. and Houghton, 1992. The fate of foreign DNA associated with pig sperm following the *in vitro* fertilization of zona free hamster ova and zona intact pig ova. Arch. Androl. **29** : 199-206.

Hoshi, K., A. Saito, M. Suzuki, K. Hayashi and R. Yanagimachi, 1982. Effects of agents used for removal of zona pellucida on human sperm penetration into zona-free hamster egg, Acta. Obstet. Gynecol. Spn. **34**: 2229-2234.

Hosoi, Y., K. Niwa, S. Hatanaka, and A. Iritani, 1981. Fertilization *in vitro* of rabbit eggs by epididymal spermatozoa capacitated in a chemically defined medium. Biol. Reprod. **24** : 637.

Hulet, C.V., W.C. Foote, and R.L. Blackwell, 1964. Effects of natural and electrical ejaculation on predicting fertility in the ram. J. Anim. Sci. **23**: 418-424.

Ijaz, A. and A.G. Hunter, 1989a. Induction of bovine sperm capacitation by TEST-yolk semen extender. J. Dairy Sci. **72** : 2683 - 2990.

Ijaz, A. and A.G. Hunter 1989b. Impact of washing and capacitation media pH on bull sperm motility, acrosome integrity and ability to penetrate zona-free hamster oocytes. J. Dairy Sci. **72** : 2691-2699.

Ijaz, A., A.G. Hunter, and E.F. Graham, 1989. Identification of the capacitating agent for bovine sperm in egg yolk-TEST semen extender. J. Dairy Sci. **72** : 2700-2706.

Imai, H., K. Niwa, and A. Iritani, 1977. Penetration *in vitro* of zona-free hamster eggs by ejaculated boar spermatozoa. J. Reprod. Fert. **51** : 495-497.

Imai, H., K. Niwa, and A. Iritani, 1979. Time requirement for capacitation of boar spermatozoa assessed by their ability to penetrate the zona-free hamster ova. J. Reprod. Fert. **56** : 489-492.

Iritani, A. M., Kasai, K. Niwa, and H.B. Song, 1981. Fertilization *in vitro* of cattle follicular oocytes with ejaculated spermatozoa capacitated in a chemically defined medium. J. Reprod. Fert. **70** : 487.

Ivanova, M. and M. Mollova, 1993. Zona-penetration *in vitro* test for evaluating boar sperm fertility. Theriogenology **40** : 397-410.

Jasko, D.J., T.V.Little, D.H.Lein, R.H.Foote, 1992. Comparison of spermatozoal movement and semen characteristics with fertility in stallions : 64 cases (1987-88). J. Am. Vet. Med. Asso. **200** : 979-985.

Jeyendran, R.S., H.H. Van-der Ven, M. Perezpelaez, B.G. Crabo and L.J.D Zaneveld, 1984.Development of an assay to asses the functional integrity of human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fert. **70** : 219 - 228.

Johnson, M.H. 1975. The macromolecular organization of membranes and its bearing on events leading upto fertilization. J. Reprod. Fert. **44** : 167.

Kamiguchi, Y. and K. Mikamo, 1986. An improved, efficient method for analysing human sperm chromosomes using zona-free hamster ova. Am.J. Hum. Genet. **38** : 724-740.

Kawakami, E., C.A. Vandevcort, C.A. Mahi-Brown, and J.W. Overstreet, 1993. Induction of acrosome reactions of canine sperm by homologous zona pellucida. Biol. Reprod. **48** : 841-845.

Kjaestad, H., E.Ropstad, K.A.Berg, 1993. Evaluation of spermatological parameters used to predict the fertility of frozen bull semen Acta. Vet. Scand., **34** : 299-303.

Koehler, J.K., D. Smith, and L.K. Karp, 1984. The attachment of acrosome-intact sperm to the surface of zona-free hamster oocytes. Gamet Res. **9**: 197-205.

Kumi - Diaka, J., 1993. Subjecting canine semen to the hypo-osmatic test. Theriogenology **39** : 1279-1289.

Kumi - Diaka, J. and G. Badtram, 1994. Effect of storage of sperm membrane integrity and other functional chanracteristics of canine spermatozoa : *in vitro* bioassay for canine semen. Theriogenology **41** : 1355-1366.

Langlais, J. and K.D.O. Roberts, 1985. A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamet Res. **12** : 183-224.

Lasley, J.F., G.T. Easley, and F.I. McKenzie, 1942. A staining method for the differentiation of live and dead spermatozoa. I. Applicability to the staining of ram spermatozoa. Anat. Rec. **82** : 167-174.

Lenz, R.W., J.L. Martin, M.F.Bellin and R.L.Ax, 1988. Predicting fertility of dairy bulls by inducing acrosome reactions in sperm with chondroitin sulfates. J.Dairy Sci. **71** : 1073-1077.

Lightfoot, R.J. and B.J. Restall, 1971. Effects of site of insemination, sperm motility and genital tract contractions on transport of spermatozoa in the ewe. J. Reprod. Fert. **26** : 1-13.

Linford, E., F.A. Glover, C. Bishop, and D.L. Stewart, 1976. The relationship between semen evaluation methods and fertility in the bull. J. Reprod. Fert. **97** : 283-291.

Logue, D. and A. Greig, 1987. Infertility in the bull, ram and boar 3 : Collection and examination of semen. In Practice **9** : 167-170.

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

Lorton, S.P. and N.L.First, 1979. Hyaluronidase does not disperse the cumulus oophorus surrounding bovine ova. Biol. Reprod. **201** : 301-308.

Lui, C.W., L.E. Cornett, and S. Meizel, 1977. Identification of the bovine cellular fluid protein involved in the *in vitro* induction of the hamster sperm acrosome reaction. Biol. Reprod. **17** : 34-41.

Makler, A., J. Stoller, Z. Blumenfeld, P.D. Feigin and J.M. Brandles, 1993. Investigation in real time of the effect of gravitation on human spermatozoa and their tendency to swim-up and swim-dowm. Int. J. Androl. **16** : 251 - 257.

Mandelbaum, J.M. Plachot, I. Mowszowicz, I. and Dray, F. (1982), Follicular sex steroids and prostaglandins during oocyte maturation in hamsters. In follicular maturation and ovulation", pp.296-331. R. Rolland *et al.* (eds.). Excerpta Medica, Amsterdam.

Martin, R.H., 1983. A detailed method for obtaining preparations of human sperm chromosomes. Cytogenet. cell Genet. **35** : 252 - 256.

Meizel, S. 1985. Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. Am. J. Anat. **174** : 285-302.

Montimer, D., 1983. Sperm transport in the human female reproductive tract. In Oxford Review of Reproductive Biology, Vol.5 (Ed. C.A. Finn) pp.30-61, Oxford University Press, Oxford.

Murphy, S.B. and R. Yanagimachi, 1984. The pH dependence of motillity and acrosome reaction of guinea pig spermatozoa. Gamet. Res. **10** : 1.

Natsuyama, S., Y. Noda, K. Narimoto, and T. Mori, 1993. Role of protein supplements in the culture of mouse embryos. Theriogenology. **40** : 149-157.

Oliphant, G., C.L., Cabot, and C.A. Singhas, 1977. Nature of the rabbit acrosome reaction inducing activity of follicular fluid. J. Reprod. Fert. **50** : 245.

Oliphant, G. and B.G. Brackett, 1973. Capacitation of mouse spermatozoa in media with elevated ionic strength and reversible decapacitation with epididymal extracts. Fertil. Steril : 948-955.

Oliphant, G.,Al.B.Reynolds and Ted.S.Thomas, 1985. Sperm surface components involved in the control of acrosome reaction. Am. J.Anat. **174** : 269-283.

Osser, S., H. Wramsby, and P. Liedholm, 1988. A comparison between the hamster egg penetration test and the seminal parameters in men of infertile couples. Int. J. Fert. **83** : 207-211.

Overstreet, J.W., R. Yanagimachi, D.F. Katz, K. Hayashi and F.W. Hanson, 1980. Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg : A study of fertility donors and infertile patients. Fert. steril. **33** : 534 - 542.

Parrish J.J., J.L. Susko-Parrish and N.L. First, 1985. Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm *in vitro*. Theriogenology **24** : 537-549.

Pavlok, A. and J.E. Flechon, 1985. Some factors influencing the interaction of ram spermatozoa with zona-free hamster eggs. J. Reprod. Fert. **74** : 597-604.

Pryor, J.P., 1986. Clinical experience of the heterologous oocyte penetration test 1979-1985. Int. J. Androl (Suppl. 6) : 76 - 81.

Ramesha, K.P. 1991. Study of the sperm chromosomes in bovines. Ph.D. Thesis submitted to N.D.R.I. Karnal.

Ramesha, K.P. and S.L.Goswami, 1994a. Zona-free hamster egg penetration test for assessing fertility of Karan-Swiss and Karan fries bulls. Indian. Vet. J. **71** : 47-50.

Ramesha, K.P. and S.L.Goswami, 1994b. Chromosome analysis of Murrah buffalo (*Bubalus bubalis*) spermatozoa using zona-free hamster oocytes Theriogenology **42** : 297-305.

Reed, P.W. and H.A.Lardy, 1972. A23187 : A divalent ionophore, J.Biol. Chem. **247** : 6970-6977.

Reyes, A., B. Goicoechea and A. Rosado, 1978. Calcium ion requirement for rabbit spermatozoal capacitation and enhancement of fertilizing ability by ionophore A23187 and cyclic adenosine 3'-5' monophosphate. Fert. Steril. **29** : 451-455.

Richards, J.S., (1978). Hormonal control of follicular Growth and Matuaration in Mammals. In the Vertebrate Ovary' pp.337-360. plenum press, N.Y.

Richords, L.R., K.L. White and J.M. Wiltbank, 1990. Effect of microinjection and two types of electrical stimuli on bovine sperm hamster egg penetration. Mol. Reprod. Develop. **27** : 163 - 167.

Rogers, B.J. 1978. Mammalian sperm capacitation and fertilization *in vitro* : A critique of methodology. Gamet Res. **1** : 165.

Rogers, B.J., 1985. The sperm penetration assay : its usefulness reevaluated. Fert. steril. **43** : 821-839.

Rogers, B.J., H.Y. Campen, M., Ueno, H., Lambert, R. Bronson, and R. Hale, 1979. Analysis of human spermatozoal fertilizing ability using zona free ova. Fert. Steril. **32** : 664-670.

Rogers, B.J., S.D.Perreault, B.J. Bentwood, C.Maclarville, R.W.Hale and D.W.Soderdahl, 1983. Variability in the human-hamester *in vitro* assay for fertility evaluation, Fert. Steril., **39** : 204-211.

Rudak, E., P.A.Jacobs and R.Yanagimachi, 1978. Direct analysis of chromosome constitution of human spermatozoa. Nature **274** : 911-912.

Roussel, J.D., T.E. Patrick, H.C. Kellgren, and J.O. Shelwick, 1963. Influence of nitrogen and argon gases on post-thawing motility, laboratory stress tests and fertility of frozen bovine spermatozoa. J. Dairy Sci. **41** : 1278-1282.

Saacke, R.G. and C.E. Marshall, 1968. Observations on the acrosomal cap of fixed and unfixed bovine spermatozoa. J. Reprod. Fert. **16** : 511-514.

Salisbury, G.W., N.L.VanDemark and J.R.Lodge, 1978. Physiology of Reproduction and Artificial Insemination of cattle. 2nd Edition, W.H.Freeman & Co., San Francisco.

Samper, J.C., E.J., Behnke, A.P., Byers, A.G. Hunter, and B.G. Crabo, 1989. *In vitro* capacitation of stallion spermatozoa in calcium-free tyrode's medium and penetration of zona-free hamster eggs. Theriogenelogy **31**: 875-885.

Sarkadi, B., P. Szaszard G. Gardes, 1976. The use of ionophore for rapid loading of human red cells with radioactive cation pump studies. J. Membr. Biol. **26** : 357-370.

Shams-Bohran, G. and R.A.P. Harrison, 1981. Production characterization and use of ionophore-induced, calcium dependent acrosome reaction in ram spermatozoa, Gamet Res. **4** : 407-432.

Sharma, R.K. and R.P.Das, 1992. Effect of fertility regulating agents on motility and zona free hamster egg penetration by spermatozoa of bonet monkey. Indian J.Exp. Biol. **30** : 977-981.

Shorgan, B. and A.Hanada, 1985. Penetration of zona-free hamster eggs *in vitro* by ejaculated goat spermatozoa after treatment with ionophore A23187. Japan. J. Anim. Reprod. **31** : 115-121.

Sivaramalingam, K., 1994. Studies on hypo-osmotic swelling test (HOST) for evaluation of bull spermatozoa. M.V.Sc. Thesis submitted to Tamil Nadu Veterinary and Animal Sciences University, Madras.

Slavik, T. 1987. Effect of glycerol on the penetrating ability of fresh ram spermatozoa with zona-free hamster eggs. J. Reprod. Fert. **79** : 99-103.

Snedecor, G.W. and W.G. Conchran, 1967. Statistical Methods. (6th ed.). Iowa State Univ. Press, Iowa.

Soderquist, L., H. Rodriguez-Martinez, L. Janson, 1991. Post thaw motility, ATP content and cytochrome C oxidase activity of A.I.Bull spermatozoa in relation to fertility. J. Vet. Med. series A. **38** : 165-174.

Solanilla, E., E. Espinosa, A. Josa, L. Gil, A - del. Nino-Jesus, M.V. Falceto, A. Del - Nino-Jesus, A., 1993. Use of an endosmosis test (Mostest) for the evaluation of semen from a spanish Brown bull. Publicacoes do 50 simposio International de Reproducao Animal, Luso, Portugal, 30 de septembro a 2 de outubro, Vol. II pp. 195-199. (BEASTCD 1988-11/94, AN-A52395).

Solanilla, E., E. Espinosa, A. Josa, L. Gil, A - del. Nino-Jesus, M.V. Falceto, A. Del - Nino-Jesus, A., 1993. Use of an endosmosis test (Mostest) for the evaluation of semen from a spanish Brown bull. Publicacoes do 50 simposio International de Reproducao Animal, Luso, Portugal, 30 de septembro a 2 de outubro, Vol. II pp. 195-199. (BEASTCD 1988-11/94, AN-A52396).

Steinholt, H.C., J.E. Chandler, and V. Tirado, 1991. Evaluating acrosome reaction steps with bright field and differential interference contrast microscopy techniques. J. Dairy Sci. **74** : 3822-3826.

Stenchever, M.A., L.R. Spadoni, W.D. Smith, L.E. Karp, K.K. Shy, D.E. Moore, and R. Berger, 1982. Benefits of the sperm (hamster ova) penetration assay in the evaluation of the fertile couple. Am. J. Obstet. Gynecol. **143** : 91-96.

Stewart, D.L., C. O'Hogen, and F.A. Glover, 1972. The prediction of fertility of bull semen from laboratory tests. Proc. VII Intern. congr. Anim. Reprod. Artif. Insem. **11** : 1280-83.

Stewart, D.L., R.L. Spooner G.H., Bennett, R.A. Beatty, and J. Hancock, 1974. A second experiment with heterospermic insemination in cattle. J. Reprod. Fert. **36** : 107-116.

Stewart-Sawage, J. 1993. Effect of bovine serum albumin. Concentration and source on sperm capacitation in the golden hamster. Biol. Reprod. **49** : 74-81.

Swanson, E.W. and H.J. Bearden, 1951. An Eosin-Nigrosin stain for differentiating live and dead bovine spermatozoa. J. Anim. Sci. **10** : 981-987.

Tablot, P. and R.S.Cachon, 1981. A triple-stain technique for evaluating normal acrosome reactions of human sperm. J. Exp. Zool. **215** : 201-208.

Tajik, P., K. Niwa, and T. Murase, 1993. Effects of different protein supplements in fertilization medium on *in vitro* penetration of cumulus intact and cumulus-free bovine oocytes matured in culture. Theriogenology **40** : 949-958.

Takahashi, K. Allchida and M, Kitao, 1990. Hypoosmatic swelling test of sperm. Arch. Androl. **25** : 225-242.

Takahashi, Y., M. Nihayah, M., M. Hishinuma, M.R. Jainudeen, O.A. Mazni, Y. Mori and H. Kanagawa, 1989. Preliminary study of buffalo sperm penetration into zona free hamster eggs after treatment with calcium ionophore A23187. Jpn. J. Vet. Res. **37** : 161-166.

Takahashi, K., R.Sakoda, H.Yamasaki, A.Uchida, K.Yoshino and M.Kitao, 1993. Evaluatioon of sperm fertilizing capacity using the determination of acrosome reaction. Asia-oceania. J.Obstet. Gynaecol. **19** : 235-240.

Tateno H., M. Akaike, Y. Fukui, Y. Kamiguchi and K. Mikamo, 1990. A method for chromosome analysis of ram spermatozoa using zona-free hamster oocytes. Theriogenelogy **34** : 845-852.

Tateno, H. and K. Mikano, 1987. A chromosomal method to distinguish between X and Y bearing spermatozoa of the bull in zona-free hamster ova. J. Reprod. Fert. **81** : 119-125.

Thompson, J.G.E. and J.M. Cummins, 1985. The effect of washing and protein supplementation on the acrosome reaction of ram spermatozoa *in vitro*. Anim. Reprod. Sci. **9** : 75-84.

Tyler, J.P.P., J.P. Pryor, and W.P. Collins, 1981. Heterologous ovum penetration by human spermatozoa. J. Reprod. Fert. **63** : 499-508.

Urry, R.L., D.T. Carrell, D.B. Hull, R.G. Middleton, and M.C. Wiltbank, 1983. Penetration of zona free hamster ova and bovine cervical mucus by fresh and frozen human spermatozoa. Fert. Steril. **39** : 690-694.

Varner, D.D., J.A. Bowen, and L. Johnson, 1993. Effect of heparin on capacitation/acrosome reaction of equine sperm. Arch. Androl. **31** : 199-207.

Watson, P.F. 1975. Use of Giemsa stain to detect changes in the acrosomes of frozen ram spermatozoa. Vet. Rec. **97** : 12-15.

Whitfield, C.H. and T.J. Parkinson, 1992. Relationship between fertility of bovine semen and *in vitro* induction of acrosome reactions by heparin. Theriogenology **38** : 11-20.

Williams, R.M., J.K. Graham, and R.H. Hammerstedt, 1991. Determination of the capacity of ram epididymal and ejaculated sperm to undergo the acrosome reaction and penetrate ova. Biol. Reprod. **44** : 1080-1091.

Yanagimachi, R. 1970. The movement of golden hamster spermatozoa before and after capacitation. J. Reprod. Fert. **23** : 193-196.

Yanagimachi, R. 1972. Fertilization of guinea pig sperm *in vitro*. Anat. Rec. **174** : 9-20.

Yanagimachi, R. 1981. Machanisms of fertilization in mammals. In L. Mastroianni, Jr. and J.D. Biggers (eds.) Fertilization and Embryonic Development *in vitro* pp.181-182. Plenum Press, New York.

Yanagimachi, R. 1984. Zona free hamster eggs : Their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. Gamet Res. **10** : 187-232.

Yanagimachi, R. and N. Usui, 1974. Calcium dependence of acrosome reaction and activation of guinea pig spermatozoa. Exp. Cell. Res. **89** : 161-174.

Yanagimachi, R., H.Yanagimachi and B.J.Rogers, 1976. The use of zona-free animal ova as a test system for the assessment of the fertilizing capacity of human spermatozoa. Biol. Reprod. **15** : 471-476.

Xu, T., Greve, H., Calleben, P. Hiffel, 1987. Pregnancy resulting from cattle oocytes matured and fertilized *in vitro*. J. Reprod. Fert. **81(2)** : 1501-504.

Zhang, J., M.S., Boyle, C.A. Smith and H.D.M. Moore, 1990. Acrosome reaction of stallion spermatozoa evaluated with monoclonal antibody and zona-free hamster eggs. Mol. Reprod. Develop. **27** : 152-158.