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EXTRACT OF *GELIDIELLA ACEROSA* (S-ACT-1) AS A CAPACITATION AGENT FOR *IN VITRO* FERTILISATION IN GOAT

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By

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DEDICATION

This thesis is exclusively dedicated to the memory of my late father

Mr. A.D. Gunatissa

and

my beloved mother

Mrs . R.A.D.C. Rupasinghe

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AR	Acrosome reaction
BOEC	Bovine oviductal epithelial cell
BSA	Bovine serum albumin
BWW	Biggers, Whitten and Whittingham
CAIS	Central Artificial Insemination Station
COC	Cumulus ophorus complexes
COECM	Caprine oviductal epithelial cell monolayer
DA-	Dead sperm with detached acrosome
DA+	Dead sperm with intact acrosome
DAP&H	Department of Animal Production and Health
ESS	Oestrus sheep serum
ET	Embryo transfer
FAR	False acrosome reaction
FCS	Foetal calf serum
FSG	Fucose sulphated glycoconjugate
FSH	Follicular stimulating hormone
GAG	Glycoseaminoglycans
GV	Germinal vesicle
GVBD	Germinal vesicle break down
hCG	Human chorionic gonadotrophin

IU	International units
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
LA-	Live sperm with detached acrosome
LA+	Live sperm with intact acrosome
LH	Luteinising hormone
MOET	Multiple ovulation and embryo transfer
MRI	Medical Reserach Institute
PBS	Phosphate buffered saline
PHE	Penicillamine, hypotaurine and epinephrine
PMSG	Pregnant mare serum gonadotrophin
POD	Post ovulatory discharge
S-ACT-1	Sperm activating factor 1
SOF	Synthetic oviductal fluid
SS	Sheep serum
TAR	True acrosome reaction
TCM 199	Tissue culture medium 199
UPM	Universiti Pertanian Malaysia
VRI	Veterinary Research Institute
ZHOPT	Zona-free hamster oocyte penetration test
ZP	Zona pellucida

Abstract of dissertation submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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By

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March, 1997

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Faculty: Veterinary Medicine and Animal Science

A study was conducted to determine if goat ovaries obtained from a slaughterhouse could be used for *in vitro* fertilisation (IVF). Three experiments were conducted to ascertain if a substance named S-ACT-1, extracted from a marine algae, (*Gelidiella acerosa)*, with a chemical structure similar to heparin could be used for sperm capacitation.

The acrosome reaction was tested by a dual staining technique (Experiment 1) and the zona free hamster penetration test (Experiment 2). *In vitro* maturation of goat oocytes and fertilisation were assessed by the number of zygotes cleaving beyond the 2-cell stage (Experiment 3).

Freshly ejaculated semen from two fertile bucks were used for sperm capacitation using two defined media (DM-H and DM-Ca). Heparin and S-ACT-1 were tested at concentrations of 10, 20, 50 and 100 μg /ml. After incubating for 15 min. at 39° C with 5% CO_2 in air under maximum humidity, samples were stained with trypan blue and Giemsa stain (dual stain). The results indicated that the sperm treated with heparin and S-ACT-1 showed similar staining characteristics.

Concentration of the capacitation agent had a significant effect on the number of spermatozoa undergoing true acrosome reaction. The number of true acrosome reacted spermatozoa was significantly (F= 6.69) different between the treatment and control groups.

The zona free hamster ova penetration test showed that the sperm penetration rate was higher with heparin than S-ACT-1. Based on the results of Experiments 1 and 2, S-ACT-1 can be used as a sperm capacitation agent for IVF in goat at a concentration of 20 μg/ml and 3 h of incubation. A total of 865 oocytes obtained from slaughtered goats were incubated for 27 h at 39° C and 5% CO_2 under maximum humidity. The maturation rate was 89.75%. Matured oocytes were inseminated with the goat spermatozoa capacitated with 10, 20, 50, 100 and 200 μg /ml of heparin or S-ACT-1 and incubated for 24 h. The maximum cleavage rate was 42.2% (38 out of 90) for 10 μg /ml heparin and 33.8% (23 out of 68) for 20 μg /ml S-ACT-1. Two celled embryos were cultured either in synthetic oviductal fluid (SOF) medium or caprine oviductal epithelial cell monolayer (COECM) cultures up to the blastocyst stage. Higher survival rates were observed in the embryos fertilised with the sperm capacitated with 10 μg/ml of heparin (21.1% or 4 out of 19) or 20 μg/ml of S-ACT-1 (25% or 3 out of 12).

Abstrak dissertasi yang dikemukakan kepada Senat Universiti Pertanian Malaysia
bagi memenuhi syarat-syarat untuk Ijazah Doktor Falsafah

Akstrak *Gelidiella acerosa* (S-ACT-1) SEBAGAI AGEN KAPASITASI UNTUK PERSENYAWAAN *IN VITRO* PADA KAMBING

oleh

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Satu kajian telah dijalankan untuk mengenalpasti samada ovari kambing yang diambil dari rumah sembelih boleh digunakan dalam persenyawaan *in vitro* (IVF). Tiga eksperimen dikendalikan untuk menentukan jika bahan yang dikenali sebagai S-ACT-I, diakstrak dari alga laut, *(Gelidiella acerosa),* mempunyai struktur kimia menyerupai heparin boleh digunakan dalam kapasitasi sperma.

Reaksi akrosom diuji dengan teknik pewarnaan berkembar (Eksperimen 1) dan ujian penembusan ova hamster tanpa zona (Eksperimen 2). Pematangan *in vitro* oosit kambing dan persenyawaannya dinilai melalui bilangan zaigot yang membelah melebihi peringkat 2-sel (Eksperimen 3).

Semen yang baru diejakulat dari dua kambing jantan subur digunakan untuk kapasitasi dengan menggunakan dua media (DM-H and DM-Ca). Heparin dan S-ACT-1 diuji pada kepekatan 10, 20, 50 dan 100μg /ml. Selepas diincubat selama 15 min. pada 39°C dengan 5% $C0_2$ dalam udara di bawah humiditi maksimum, kesemua sampel diwarnakan dengan pewarna tryphan biru dan Giemsa (pewarnaan berkembar). Keputusan menunjukkan sperma yang dirawat dengan heparin dan S-

ACT-1 memberikan ciri-ciri pewarnaan yang serupa. Kepekatan agen kapasitasi mempunyai kesan yang ketara terhadap bilangan spermatozoa yang mengalami reaksi akrosom sebenar. Bilangan spermatozoa yang mengalami reaksi akrosom sebenar mempunyai perbezaaan yang ketara (F= 6.69) di antara kumpulan rawatan dan kawalan.

Ujian penembusan ova hamster tanpa zona menunjukkan kadar penembusan sperma yang tinggi dengan heparin berbanding S-ACT-1. Berdasarkan Eksperimen 1 dan 2, S-ACT-1 boleh digunakan sebagai agen kapasitasi yang berkesan untuk IVF kambing pada kepekatan 20μg/ml dan 3 jam inkubasi.

Sejumlah 865 oosit didapati dari kambing yang disembelih dan diinkubat selama 27 jam pada 39°C dan 5% $C0_2$ di bawah humiditi maksimum. Kadar pematangan adalah 89.75%. Oosit matang diinseminasi dengan spermatozoa kambing yang dikapasitasi dengan 10, 20, 50, 100 dan 200μg/ml heparin atau S-ACT-1 dan diinkubat selama 24 jam. Kadar penembusan maksima adalah 42.2% (38 dari pada 90) untuk 10μg/ml heparin dan 33.8% (23 dari pada 68) untuk 20 μg/ml S-ACT-1.

Ova yang ditembusi dikultur dalam media cecair oviduk sintetik (SOF) dan monolayer sel epitelium oviduk kambing (COECM) sehingga ke peringkat blastosista. Kadar hidup yang tinggi diperhatikan pada embrio yang disenyawa dengan 10μg/ml of heparin (21.1% atau 4 dari pada 19) atau 20μg/ml S-ACT- 1 (25.0% atau 3 dari pada 12).

CHAPTER I

INTRODUCTION

Manipulation of reproduction has been accepted as the major tool of optimizing the productivity of farm animals. Since the 1940s, artificial insemination (AI) has made a significant contribution to the genetic improvement of farm animals. This is possible because a few highly selected males produce enough spermatozoa to inseminate thousands of females.

On the contrary, despite the presence of several thousands of oocytes in the ovary, only a few offspring are born during the lifetime of a female. Since the 1970s, efforts have been made to increase the number of offspring from superior animals through superovulation, multiple conceptions recovery and transfer of embryos into the recipients who are genetically less distinguished. This technique known as multiple ovulation and embryo transfer (MOET) can be regarded as the female counterpart of artificial insemination.

Several commercial companies for embryo transfer (ET) in farm animals are found worldwide. However, a major problem confronting ET is the lack of a cheap and reliable technique of inducing multiple ovulation. In recent years, researchers have turned their attention to seek ways of harvesting immature oocytes from developing follicles, promoting their maturation, fertilisation and embryo culture *in vitro* to obtain a large number of embryos for embryo transfer.

These i*n vitro* techniques are gaining importance for several other reasons: to understand the process of fertilisation, extend the value of ovaries which contain thousands of oocytes which would otherwise be wasted under natural circumstances, and allow gene manipulation through which desirable characteristics could be amplified or new characteristics introduced.

In vitro techniques for sperm capacitation, oocyte maturation (IVM), fertilisation (IVF) and culture (IVC) systems could be used to produce a large numbers of embryos at the same stage of development. Reliable techniques for producing developmentally competent goat embryos *in vitro* using slaughter house ovaries would solve the major problem of non-availability of genetically superior breeding materials.

Though *in vitro* techniques have been practised for over two decades, they need refinement of sperm capacitation agents, their optimum concentration, and the incubation periods required to achieve maximum oocyte penetration rate. Suitable *in vitro* culture media are also lacking for embryos to develop to the blastocyst stage.

Among the sperm capacitation agents, heparin is most widely used. It is a synthetic compound and belongs to a group called glycoseaminoglycans (GAG). Recently, a natural substance isolated from marine red algae found in coastal areas of Sri Lanka has been proved to possess human sperm motility activation property. This substance, named as S-ACT-1, has a structure quite similar to that of heparin. Whether this motility activating property could also induce capacitation of sperm is not known.

Therefore a series of experiments was conducted in Kandy, Sri Lanka to understand the process of sperm capacitation, the acrosome reaction and *in vitro* maturation, fertilisation and culture of oocytes. S-ACT-1 was tested against heparin as the capacitation agent. The goat was selected as the model animal mainly because of the availability of goat ovaries from the local slaughterhouse.

The objectives of this study were

1. to compare heparin and S-ACT-1 as agents for capacitation of goat sperm
2. to develop the IVF technique for production of embryos from oocytes recovered from goat ovaries collected at slaughterhouse.

The following experiments were conducted:

Experiment 1 Capacitation and acrosome reaction of goat spermatozoa (Chapter III)

Experiment 2 Assessment of sperm capacitation in goat (Chapter IV)

Experiment 3 *In vitro* oocyte maturation, fertilisation and culture of goat embryos (Chapter V)

CHAPTER II

LITERATURE REVIEW

Introduction

This review is divided into two parts. The first part reviews some fundamental process on the male and female gametes, their development and events leading to fertilisation *in vivo* and embryonic development. The second part reviews studies on *in vitro* fertilisation (IVF) in farm animals.

In Vivo Fertilisation

Understanding how spermatozoa achieve fertilisation *in vivo* is of utmost importance to solve many problems associated with interaction between oocytes and spermatozoa *in vitro*. Fertilisation in mammals requires three critical events: sperm migration through cumulus cells, sperm attachment and migration through the ZP and sperm fusion with the ooplasm (Hafez, 1983).

Spermatogenesis

The seminiferous epithelium of the testis contains a series of developing germ cells which will be finally converted to spermatozoa (Hafez, 1993).

Spermatogenesis involves two processes: *spermatocytogenesis* and *spermiogenesis.* In *spermatocytogenesis,* spermatogonia situated at the periphery of the seminiferous epithelium divide several times to form spermatocytes which after undergoing meiosis to become spermatids. In *spermiogenesis,* spermatids contain haploid number of chromosomes and after a series of morphological and developmental changes, become spermatozoa, which are elongated in shape. All the developing germinal cells are closely associated with the Sertoli cells and with further development, sperm move from the basement membrane toward the lumen. The process of release of spermatozoa into the lumen is known as spermiation. Semen is the semi-gelatinous liquid containing sperm and secretions of the accessory organs of the male reproductive tract.

Maturation

In mammals, maturation of spermatozoon primarily occurs in the caput and corpus epididymis (Bedford and Hoskin, 1990). It is a complex and sequential process (Parks and Hammerstedt, 1985). Spermatozoon's maturation changes depend on the epididymal secretions and transport time through the epididymis. In the ram, the transport time in the epididymis is 16 days (Amann, 1981). At ejaculation, although spermatozoa show their normal morphologic characteristics and motility, they are still unable to penetrate and fertilise the ova (Burks and Saling, 1992).

The final stage of sperm maturation is completed in the female reproductive tract. It involves biochemical, physiological and some morphological changes just before fertilisation. The changes, which take place in the female reproductive tract will be reviewed in detail later.

During transit along the male tract, sperm will undergo some remodelling of membrane lipids (phospholipid) (Parks and Hammerstedt, 1985). Sperm cell proteins undergo changes during maturation. Modification of DNA protein complex, plasma membrane, mitochondria, fibrous and microtubular components and development of surface characteristics have been observed (Amann et al., 1993). Migration of some macromolecules along the cell surface has also been observed.

Maturation of spermatozoon is not completed until they acquire motility, fertilisability and viability. The spermatozoon attains progressive motility during passage through the epididymis (Bedford, 1975). With increase in progressive motility, the water content of the spermatozoon decreases and as a result the specific gravity increases (Hafez, 1983).

Morphology of the Spermatozoon

The general morphology of mammalian spermatozoon is similar (Hafez, 1993). But detailed sperm ultra-structure is considerably different from species to species. Basically, the spermatozoon consists of two main parts: the head and the tail. Average length of a mammalian spermatozoon is about 60 μm and the head is about 40 μm long, 4 μm wide and 0.5 μm thick. The nucleus which contains DNA and the tail which contains the apparatus necessary for cell motility mainly occupy the head. The tail can further be subdivided into the neck, the middle piece, the principal piece and the end piece. Shape of the head varies greatly from species to species. In most of mammals including goat, the head has a flattened pyriform shape. The nuclear chromatin is highly condensed and exhibits a rather uniform thickness except at the anterior end where it tapers gently and thickening slightly at the base.

The anterior two third of the head is covered with another structure known as the acrosomal cap. The acrosomal cap consists of three layers; the outer membrane, the inner membrane and a thicker, moderately electron-dense middle layer. The inner and outer acrosomal membranes are continuous with each other at the posterior margin of the cap forming an enclosed space. This space is filled with acrosomal material mainly proteolytic enzymes such as hyaluronidase, proacrosin, acrosin, proteases, esterases, and acid hydrolases. These enzymes are involved in the fertilisation process (Mann and Lutwak-Mann, 1981). The outer acrosomal membrane is closely associated with the plasmalemma or cell membrane, which covers the entire length of the sperm.

Along the anterior margin of the sperm head, the acrosomal cap is somewhat thickened and bulges out reaching it's maximum thickness at the apex. This structure is known as the apical ridge. This is the widest part of the acrosome. The acrosomal content is nearly uniform and has a moderate density. The posterior portion of the acrosome is termed as the equatorial segment. This structure is important because this is the part of the spermatozoon, which initially fuses with the oocyte during fertilisation.

The area posterior to the equatorial segment consists of the plasmalemma and the nuclear membrane and is known as the post nuclear cap or post acrosomal cap. Anterior end of the post-nuclear cap is attached to the outer membrane of the equatorial segment. At the base of the sperm head, the post nuclear cap is closely in contact with the plasmalemma. The two nuclear membranes fuse together at the base of the head and closely contact with the nuclear material.