Capacitation and acrosome reaction in stallion spermatozoa: Functional and clinical aspects

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The work described in this thesis was financially supported by the Graduate School of Animal Health, Utrecht, The Netherlands.

The studies described in this thesis were performed at the Department of Equine Sciences, Department of Farm Animal Health, and the Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

Printing of this thesis was financially supported by:
Graduate School of Animal Health
Department of Equine Sciences

ISBN: 90-393-1941-3

Lay-out : Harry Otter and Rahul Rathi
Front Cover : Rahul Rathi

Printed by : ADDIX, Wijk bij Duurstede

Capacitation and acrosome reaction in stallion spermatozoa: Functional and clinical aspects

Capacitatie en acrosoom reactie in hengstenspermatozoa:
Functionele en klinische aspecten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op donderdag 6 december 2001 des middags te 12.45 uur

door

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In the name of

Neem Karoli Baba Maharaj ji

I dedicate my PhD and this thesis

to my

Pitaji and Babaji

Contents

Chapter 1

General introduction

R. Rathi

Antoni van Leeuwenhoek, a Dutch tradesman from Delft, made the first documented report of the existence of spermatozoa in the late 17th century [1]. Since then much more has been learnt about the detailed structure of the mammalian male gamete and its function during the process of fertilisation. Fertilisation in mammals is a complex process and much current research is directed towards understanding the role played by the male gamete in this process and the changes that must occur in this cell between its genesis and its eventual interaction with the oocyte. The studies presented in this thesis focus on the two main physiological changes that must take place in a spermatozoon if it is to be capable of fertilisation; namely, capacitation and acrosome reaction.

Sperm structure

A mammalian spermatozoon comprises three major components, the *head*, the *midpiece* and the *tail* or flagellum (Fig 1 A). All three of these components are surrounded by a continuous plasma membrane, a mosaic of proteins (intrinsic and extrinsic) and lipids (mainly phospholipids and cholesterol). The phospholipids are oriented with their hydrophilic heads toward the membrane's surface and their hydrophobic tails towards the membrane's interior (Fig 2). The sperm head is made up almost exclusively of a nucleus and an acrosome with only a small amount of intervening cytoplasm, and, as a result of the two meiotic divisions that occur during spermatogenesis [2], the nucleus contains only a haploid set of chromosomes with the chromatin in a highly condensed state. The sperm nucleus is enclosed by a nuclear envelope which is rather different from that in typical somatic cells as it contains very few nuclear pores and its outer and inner membranes are only 7-10nm apart (ca. 60nm in other cells) [for review see 2]. The acrosome covers the nucleus in a cap-like fashion, with its inner membrane (inner acrosomal membrane) opposing the nuclear envelope and its outer membrane (outer acrosomal membrane) lying just below the plasma membrane (Fig 1 A). The acrosome contains a variety of enzymes, most notably proacrosin and hyaluronidase [for review see 2]. The sperm midpiece is the connecting point between the head and the tail and contains mitochondria that generate the energy necessary for tail movement. Finally, the tail provides the motive force necessary for the sperm to reach and penetrate the zona pellucida of the oocyte.

Sperm capacitation

When they leave the testes, spermatozoa are still both immature and immotile [3]. Thereafter, they undergo a series of maturational changes within the epididymis during which they become both structurally mature and motile. Nevertheless, at the time of ejaculation, sperm are still not capable of fertilising an ovum. This latter ability is acquired only after a period of further maturation within the female reproductive tract [3] and the changes that a spermatozoon undergoes during this time are collectively known as “capacitation”. Although the term capacitation was coined by Austin in 1952 [4], this

concept was first described simultaneously by both Austin and Chang in 1951 [5,6]. Of course, physiological capacitation takes place within the female reproductive tract, however it is also possible to capacitate spermatozoa *in vitro* using defined incubation media [7].

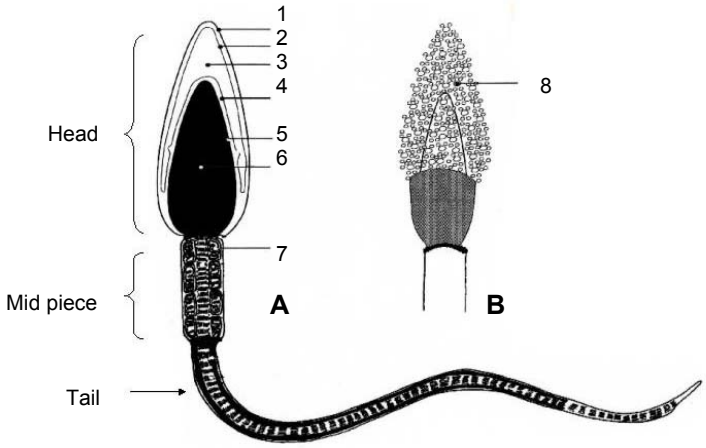

Fig. 1. Schematic presentation of a stallion spermatozoa. A: a sectional view of the spermatozoon; the sperm head consists of: Plasma membrane (1), Outer acrosomal membrane (2), Acrosome (3), Inner acrosomal membrane (4), Nuclear envelope (5), and Nucleus (6). The mid piece mainly consists of mitochondria (7). B: The sperm acrosomal exocytosis: during acrosome reaction the plasma membrane and the outer acrosomal membrane fuse and fenestrate forming vesicles (8).

Such capacitation media typically include energy substrates (pyruvate, lactate and glucose), a protein component (usually bovine serum albumin), sodium bicarbonate and calcium [8]. However, while the ability to induce capacitation *in vitro* has enabled scientists to study the changes that take place within the sperm cell during this process, it is quite likely that some of these changes differ to those that occur in the *in vivo* situation. In general however, it appears that capacitation involves modifications of the plasma membrane and changes in intracellular metabolism [9]. Certain materials coating the surface of ejaculated sperm are removed or altered during capacitation, and these include the so-called decapacitating factors [3,10] and other proteins and glycoproteins [3,11] while the cholesterol content of the membrane is reduced during this process [3,8]. Capacitation also involves an important increase in membrane lipid fluidity which results in redistribution of previously asymmetrically distributed lipids both along and across the two phospholipid layers that make up the sperm head plasma membrane. Furthermore, it is this lipid redistribution which enables the subsequent depletion of cholesterol [12].

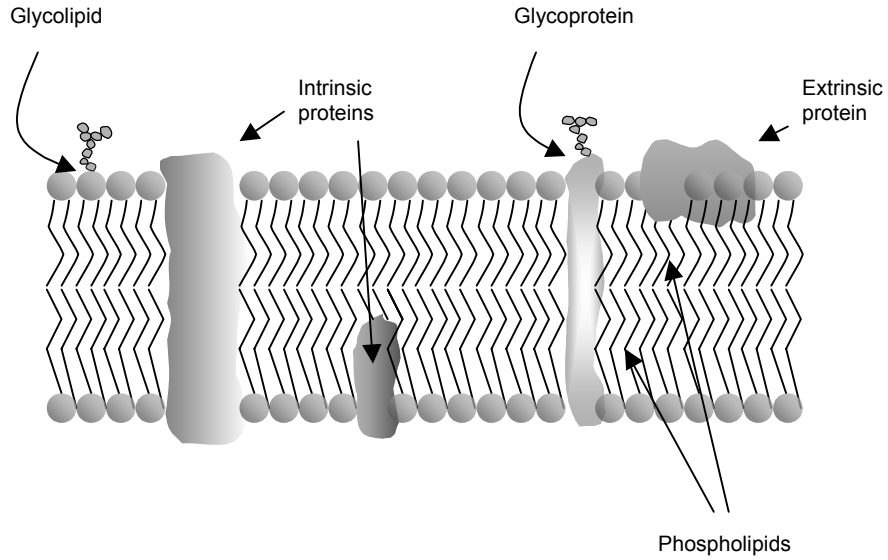

Fig. 2. A two dimensional diagrammatic representation of a typical plasma membrane. The phospholipids form the membrane bilayer and the proteins are embedded therein (intrinsic or extrinsic). Carbohydrates are found in the membranes usually attached to the lipids (glycolipids) or to the proteins (glycoproteins). Plasma membranes are dynamic structures, serving not only as an envelope for the cell but also as a selective barrier for regulating the passage of substances into and out of the cell.

It has also been noted that a modest and gradual rise in intracellular calcium occurs during capacitation [10]. However, the importance of extracellular calcium concentration in the initiation and/or regulation of capacitation is controversial [8]; not in the least because externally added calcium is not required for lipid redistribution and cholesterol efflux [12]. Capacitation also involves changes in sperm motility, which have become known collectively as hyperactivation [3] and are characterised by vigorous flagella movements, marked lateral displacement of the sperm head and a non-progressive trajectory [13]. The particular form of motility is believed to aid sperm progression up the oviduct and to provide the motile thrust needed for zona pellucida penetration [14, 15].

Despite the documentation of these various changes, the molecular basis of capacitation is not yet fully understood and moreover, the results of discrimination between capacitated and non-capacitated sperm remains very much dependent on the method and criteria applied. The definitive indication that a sperm has undergone capacitation is of course the successful fusion of a spermatozoon with the egg. However, the ability of spermatozoa to undergo the acrosome reaction in response to physiological inducers such as a zona pellucida [8] or progesterone [16], is usually taken as indicating completed capacitation.

Furthermore, chlortetracycline staining has widely been considered as the assay of choice for assessing the capacitation state of the spermatozoa [17-20] because it distinguishes three different stages of sperm maturation: pre-capacitation, capacitated acrosome-intact, and acrosome-reacted. Unfortunately, the way in which chlortetracycline interacts with the sperm surface at the molecular level is not clearly understood and the molecular significance of this calcium dependent method remains unclear.

Two specific components of capacitation media, serum albumin and bicarbonate, have been shown to play a key role in regulating sperm capacitation *in vitro*. However, the significance of a third proposed inducer of capacitation, calcium, is less clear [8]. Mechanistically, serum albumin is believed to act as a cholesterol scavenger and to help remove this component from the sperm plasma membrane [21,22], The removal of cholesterol leads to a decrease in the cholesterol/phospholipid ratio and is thought to influence transmembrane signalling and cellular function [8]. The requirement of bicarbonate for sperm capacitation has been established for several different species [23,24,25] and it is interesting to note that while the bicarbonate concentration in the epididymis is low, that in the oviduct is high [25]. Furthermore, it has been demonstrated that bicarbonate stimulates adenylate cyclase activity in spermatozoa, thereby resulting in an increase in cAMP concentrations and activation of protein kinase A [26,27].

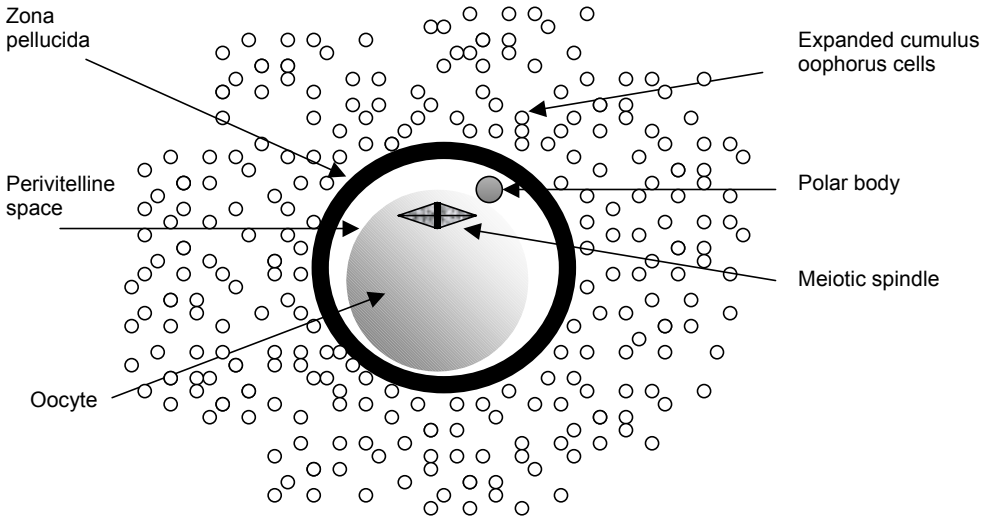

Fig. 3. Schematic diagram of a mammalian ovum and its extracellular layers. The mammalian egg is surrounded by zona pellucida and cumulus oophorus cells in an expanded form.

Sperm acrosome reaction

The oocyte of all eutherian mammals is surrounded by a thick glycoprotein coat, the zona-pellucida, which at the time of ovulation, itself is surrounded by the granulosa cells of the freshly expanded cumulus oophorus [3] (Fig 3). Before it can fertilise an egg, a spermatozoon must pass through the zona pellucida and, for this to happen, the spermatozoon undergoes the acrosome reaction soon after binding to the zona. The acrosome reaction is an irreversible, exocytotic event, characterised by a sudden increase in intracellular calcium concentrations and fusion of the outer acrosomal membrane with the overlying plasma membrane. This fusion results in fenestration of the double membrane and release of the acrosomal contents (Fig 1 B) which include numerous hydrolysing enzymes. These enzymes lyse part of the zona pellucida and the sperm (usually one per oocyte under physiological conditions), with its nucleus covered only by the inner acrosomal membrane, passes through the zona pellucida and across the perivitelline space to fuse with the oolemma of the egg (Fig 4).

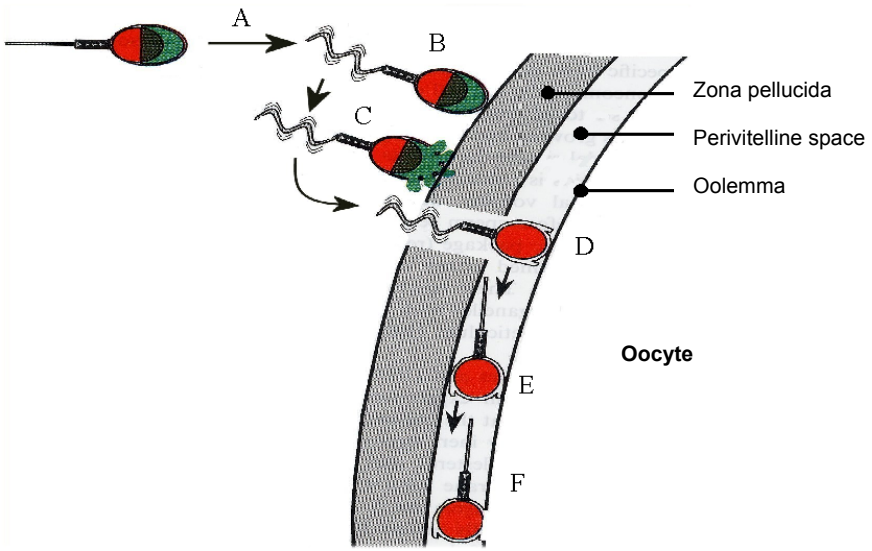

Fig. 4. Sequence of mammalian fertilisation. The spermatozoa when inside the female genital tract undergo capacitation (A), and get hyperactivated (B). The hyperactive motility enables spermatozoa to move away from the oviductal epithelium and provide the motile thrust needed for penetration of the zona pellucida. The sperm binds to the zona pellucida (B) and gets acrosome reacted (C) releasing hydrolytic enzymes which lyse the zona pellucida. Subsequently the sperm makes its way across the perivitelline space and binds to the oolemma initially with the apical tip (D) and later sideways (E). The sperm cell then fuses with (F) and gets incorporated in the oocyte. [Adapted from 48]

A number of techniques are available for studying the AR, and these include the application of electron microscopy, phase contrast microscopy and fluorescence microscopy and may employ immunogold labelling, fluorescent lectin labelling and immunofluorescent antibodies for visualisation of the membranes [3].

In vitro, the sperm acrosome reaction can be induced in capacitated spermatozoa by incubating them with solubilised zona pellucida [8] calcium-ionophore A23187 [28], or specific ligands such as progesterone [29], platelet activating factor [30], epidermal growth factor [31], atrial natriuretic peptide [32] and heparin [33]. The ensuing signal transduction cascade has not been fully described but appears to involve several protein kinases and, perhaps, the observed increase in cytosolic calcium concentrations [34].

Progesterone and the acrosome reaction

Progesterone is a major component of the fluid within the preovulatory follicle [29] and is also secreted by the cumulus oophorus cells surrounding the ovum [35]. Progesterone is also thought to contribute to physiological induction of the acrosome reaction [29] by causing a rapid influx of calcium into, and efflux of chloride from, the sperm cell and by and phosphorylating sperm proteins [36]. Progesterone has been shown to exert its acrosome reaction inducing effects via a non-genomic receptor located on the sperm plasma membrane [37] and, because not all spermatozoa in the ejaculate have exposed progesterone-receptors, only a limited number of spermatozoa undergo the acrosome reaction in response to progesterone [37,38,39]. Furthermore, progesterone and the sperm plasma membrane progesterone receptor are increasingly accepted as playing an important role in male fertility. For example, Meyers et al. [40] correlated the inability of stallion spermatozoa to undergo the acrosome reaction in response to progesterone treatment with subfertility, and Tesarik and Mendoza [41] attributed subfertility in human males to either an absolute lack of or to non-functionality of sperm plasma membrane progesterone receptors. Progesterone may also affect capacitation since the ability of sperm to bind to the zona pellucida is enhanced in a dose dependent fashion by progesterone [29].

Various pathways involving both membrane-associated and cytoplasmic protein kinases have been proposed to elicit the signalling cascade initiated by progesterone that leads to acrosome reaction. Proof for this includes the suppression of the progesterone mediated acrosome reaction by tyrosine kinase inhibitors [42,43] and phosphorylation of tyrosine residues in sperm plasma membrane proteins by progesterone stimulation [44]. In addition, protein kinases A and C have been reported to participate in the signal transduction pathway between progesterone stimulation and AR [45,46,47]. Despite a great deal of research, however, there is not yet a clear consensus on how progesterone induces acrosomal exocytosis.

Scope of the thesis

The aim of this thesis is to examine in stallion sperm, how capacitation and the AR, two processes critical to fertilisation in mammals, are initiated and controlled and how we can best monitor the progression of these important events.

In Chapter 2, the events that occur during sperm capacitation are reviewed and our current understanding of the relationship between the dynamics of sperm plasma membrane organisation and the physiology of capacitation and the acrosome reaction are outlined.

Chapter 3 describes the *ex vivo* techniques available for evaluating capacitation and the acrosome reaction and introduces novel flow cytometric techniques which better discriminate these physiological phenomena.

Chapter 4 investigates the possible contribution of various signal transduction pathways to induction of the acrosome reaction by progesterone.

In Chapter 5, the practical relevance of the role of knowledge of progesterone and its sperm plasma membrane receptors are addressed by investigating the relationship between the percentage of spermatozoa with exposed progesterone receptors and stallion fertility.

Finally, the results of these studies and their possible implications for further research are summarised in Chapter 6.

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Chapter 2

Capacitation and acrosome reaction in equine spermatozoa

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Animal Reproduction Science (2001)
in press

Abstract

During sexual reproduction, the sperm and oocyte must fuse before the production of a diploid zygote can proceed. In mammals such as equids, this fusion depends critically on complex changes in the plasma membrane of the sperm and, not surprisingly, this membrane differs markedly from that of somatic cells. After leaving the testes, sperm cease to synthesise plasma membrane lipids or proteins, and vesicle-mediated transport stops. When the sperm reaches the female reproductive tract, it is activated by so-called capacitation factors that initiate a delicate reorientation and modification of molecules within the plasma membrane. These surface changes enable the sperm to bind to the extracellular matrix of the egg (zona pellucida) and the zona then primes the sperm to initiate the acrosome reaction, an exocytotic event required for the sperm to penetrate the zona. This paper will review the processes that occur at the sperm plasma membrane before and during successful penetration of the equine zona pellucida. It is noted that while several methods have been described for detecting changes that occur during capacitation and the acrosome reaction in bovine and porcine sperm, relatively little has been documented for equine sperm. Special attention will therefore be dedicated to recent attempts to develop and implement new assays for the detection of the capacitation status of live, acrosome-intact and motile equine sperm.

Introduction

During insemination, enormous numbers of sperm are deposited in the mare's genital tract. However, in the normal situation only one sperm will eventually fertilise the egg and, to ensure that this is the case, sperm-egg interaction and fertilisation are highly regulated processes. Moreover, the sperm plasma membrane plays a critical role in regulating sperm-egg interaction and, for this reason, it is an extremely dynamic structure. During spermatogenesis [for review see 1], the sperm plasma membrane and many of its other structures are tailored for their roles during transport through the female genital tract and interaction with the oocyte. However, due to the concurrent loss of most of the cell organelles and the cessation of DNA-transcription, spermatozoa are unable to produce proteins or maintain vesicular transport and, therefore, components of the plasma membrane cannot be newly synthesised. Nevertheless, the plasma membrane of sperm entering the epididymis is not yet fully " mature" [for review see 2] and during the passage of sperm through the epididymis the plasma membrane changes significantly by the release, modification and adsorption of proteins and lipids. The role of these surface alterations is not fully understood, although some adsorbed proteins are involved in sperm-oocyte binding and, certainly, sperm only reach full maturity after entering the cauda epididymidis .

The mature sperm has three highly specialised regions (Fig 1A): (i) the DNA containing sperm head, which is vital to sperm-oocyte interaction; (ii) the midpiece, which contains

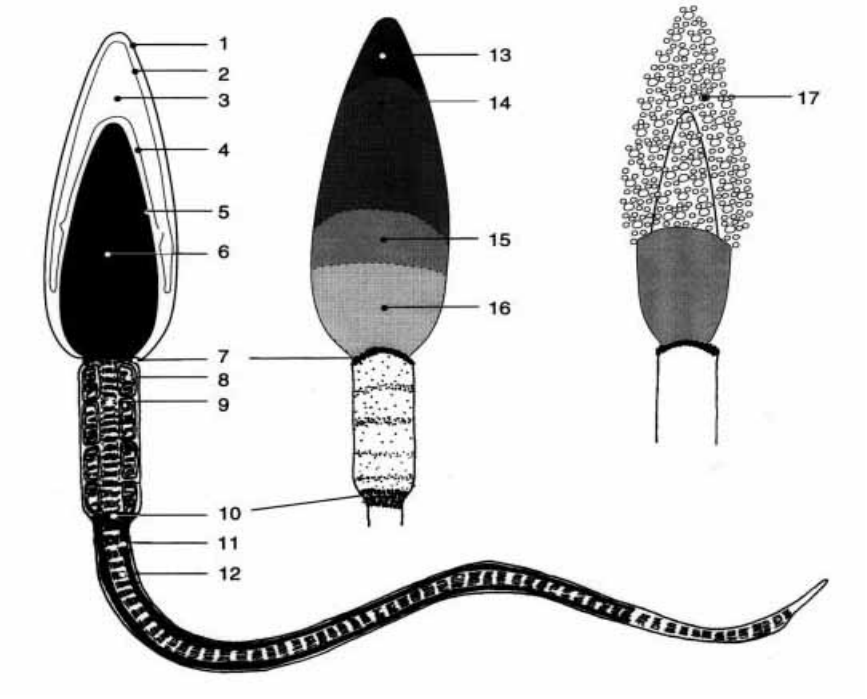

Fig.1. Schematic presentation of a stallion spermatozoon: Panel A: a sectional view of the spermatozoon. Panel B: plasma membrane subdomains of the sperm head (a surface view). Panel C: the acrosome reaction. Solid lines represent membrane bilayers. 1. Plasma membrane; 2. Outer-acrosomal membrane; 3. Acrosome fluid; 4. Inner-acrosomal membrane; 5. Nuclear envelope; 6. Nucleus; 7. Nuclear ring; 8. Mitochondria; 9. Proximal part of the flagellum (mid-piece); 10. Annular ring; 11. Distal part of the flagellum (principal and end pieces); 12. Fibrous sheath; 13. Apical subdomain; 14. Pre-equatorial subdomain; 15. Equatorial subdomain; Post-equatorial subdomain; 17. Mixed vesicles formed during the acrosome reaction via fusions of the plasma membrane with the outer acrosomal membrane.

mitochondria and is involved in energy production; (iii) the flagellum, which is involved in motility. Other than the nucleus, the sperm head contains little except a small amount of cytoplasm and, at its apical extreme, the acrosome (Fig 1B), a large vesicle containing the hydrolytic enzymes necessary for penetration of the zona pellucida (ZP) [3]. The boundary between the plasma membrane that overlies the acrosome and that caudal to the acrosome is known as the equatorial segment. Sperm-oocyte interaction can be subdivided into a sequence of events (Fig 2) [for review see 4]. First, ejaculated sperm must be activated in the female genital tract in a process called capacitation because only capacitated sperm can bind to the ZP (Fig 2; process 1). This binding of sperm to oocyte in turn induces changes in the sperm

collectively termed the acrosome reaction (Fig 1C, 2). In particular, zona-binding triggers a Ca^{2+}-influx that causes the plasma membrane to fuse, at multiple sites, with the outer acrosomal membrane to form "mixed" vesicles that disperse into the ZP and release hydrolytic enzymes to digest the ZP (Fig 2; process 2-3). This loss of the outer two membranes leaves the inner acrosomal membrane as the new outer cell membrane and it becomes continuous with the plasma membrane of the post equatorial segment via an inflexion that resembles a hairpin [5]. Consequently, the inner acrosomal membrane is exposed to, and binds to, the ZP (secondary ZP-binding) and the sperm that have reacted properly are able to penetrate the ZP and enter the perivitelline space (Fig 2; process 4). Here the sperm binds to the egg plasma membrane (oolemma), firstly via its tip and subsequently via its equatorial region and, in particular, via the hairpin membrane structure. After this lateral binding, the sperm plasma membrane and the oolemma fuse and the sperm is incorporated into the oocyte that, in turn, is activated and initiates an effective block to polyspermy (Fig 2; processes 5 and 6).

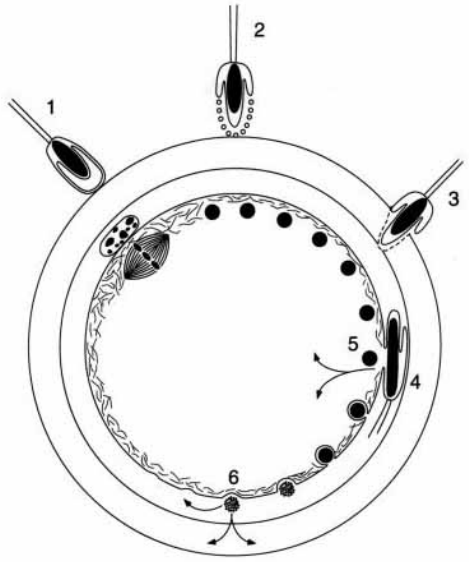

Fig. 2. Schematic representation of the sequence of interactions between the male and female gamete required for fertilisation. 1. Sperm binding to the zona pellucida with its apical subdomain; 2. The acrosome reaction, a multiple fusion event of the outer-acrosomal membrane with the apical and pre-equatorial plasma membrane; 3. The penetration of the sperm cell through the zona pellucida; note that the equatorial membrane hairpin structure remains intact. 4. Sperm binding and fusion with the oolemma (fertilisation) are both exclusive events for the equatorial plasma membrane; 5. Activation of the oocyte by cytosolic factors and fast poly-spermy block; 6. Secretion of cortical granules (cortical reaction) causing a definitive (slow) poly-spermy block. Note that, although the apical subdomain is exclusively involved in primary zona binding, the bound sperm appears to have a more flattened position towards the zona surface as indicated in this cartoon. Consequently the zona penetration curve is rather diagonal than radial towards the oolemma.

It is only relatively recently that the heterogeneity of the sperm plasma membrane has become clear. However, the dramatic differences in different regions clearly relate to physiological specializations of the plasma membrane. Moreover, the composition and lateral organization of the plasma membrane regulate its affinity for adhesion factors, its permeability to hydrophilic solutes and its role in cell signalling and cell fusion events.

This review will compare events that occur during the capacitation of stallion sperm with those reported in other mammalian species. Furthermore, we will review the current understanding of the relationship between the dynamics of sperm plasma membrane organization and the physiology of capacitation and the acrosome reaction.

Capacitation induced alterations in sperm lipid organisation and their role in sperm-zona interaction

The mature sperm lacks the major organelles involved in lipid synthesis and breakdown. Furthermore, its surface membrane is not in contact with intracellular membranes because vesicle- mediated membrane transport has ceased and will only briefly resume when the apical plasma membrane fuses with the underlying outer acrosomal membrane during the acrosome reaction (Fig 1). The unusual composition and organization of lipids in the sperm plasma membrane are almost certainly reflections of these limitations.

Lipid composition

There is considerable variation in the lipid composition of the sperm plasma membrane in different mammalian species. However, there is a general similarity. For example, the plasma membrane of boar sperm contains approximately 67 % phospholipids, 25 % neutral lipids and 8% glycolipids [6] while that of a stallion sperm contains approximately 57 % phospholipids, 37 % cholesterol and 6 % glycolipids, such that the stallion sperm differs primarily with regard to its relatively high cholesterol content.

Phospholipids can be divided into phosphoglycerolipids and sphingomyelin and the phospholipid class composition of sperm is generally similar to that of somatic cells. More specifically, stallion sperm contain approximately 48% phosphocholineglycerides (PC), 16% phosphoethanolamineglycerides (PE), 13% sphingomyelin (SM), 15% phosphatidylserine /cardiolipin, 5% phosphatidylglycerol and approximately 3% phosphatidylinositol. Moreover, not only do the PC and PE fractions of mammalian sperm have fairly unique structural characteristics, but stallion sperm differ from those of the other mammalian species that we have investigated so far, by having a surprisingly very high content of 22:5 fatty acids in their PC and PE fractions and relatively few 22:6 fatty acids [Gadella and Brouwers; unpublished data]. In sperm from other mammals species the situation was just reversed in that the PE and PC fractions contained a profound amount of 22:6 and much less 22:5 PC fatty acids [7,8]. Stallion and other mammalian sperm share the property that PE and PC contain almost

exclusively 22:5 or 22:6 fatty acids which is fairly unique for sperm (with the exception of retina cells; [6]).

Major variations in the neutral lipid composition of sperm membranes are found not only between different species but also between different males within a species and even between different ejaculates from a single male. The major variable is the amount of cholesterol in the sperm plasma membrane. For instance, human sperm contain much higher amounts of cholesterol (>40 % of total lipids) than boar sperm (24 %) while equine sperm have relatively high cholesterol content (37 %). Furthermore, the cholesterol content seems to be related to the rate of capacitation (Yanagimachi, 1984) possibly because cholesterol must be depleted from the plasma membrane during this process.

The remaining class of lipids, the glycolipids, are found in sperm almost exclusively in the form of seminolipid, a structurally unique glycolipid found only in mammalian sperm and Schwann cells [for review see 6]. Stallion sperm contains similar amounts of seminolipid as sperm from other mammalian species (approximately 6.8 mol % of total lipid in equids versus approx. 8 mol % of total lipids in man, bull, mouse, rat, boar; [9]). Both seminolipid and its desulfated counterpart are believed to participate in certain fertilisation processes [for review see 4] and to this end accessory sex gland secretions contains low amounts of arylsulfatase. However, it has yet to be shown whether seminolipid can be desulfated in equine sperm after ejaculation, as it can in human and porcine sperm.

Lateral membrane topology

The functional division of the sperm head surface into lateral domains and subdomains, based on the topology of the surface molecules, was first recognized in the 1970's and the domains are summarized in Fig 1. This lateral heterogeneity has been clearly demonstrated by lectin binding patterns and freeze-fracture studies and revealed that integral membrane proteins are unevenly distributed among different regions of the sperm head and other parts of the sperm [10, 2]. Furthermore, this delicate sperm surface organization alters during capacitation as a result, at least in part, of decoating (i.e. removal of glycocalyx components [11]), adsorption of new components from female genital fluids and enzymatic modification of glycocalyx components [12,13,]. In addition, a lateral reorganization of transmembrane proteins occurs across the sperm head [14, 15,]. These phenomena have been shown to occur in stallion sperm [16, 17], although it is difficult to understand how the lateral heterogeneity is maintained and altered specifically when there are few obvious structural means to divide areas within the sperm head. It has been variously proposed that the sperm head cytoskeleton [18] or differential electrostatic interactions help to maintain the regional differences [4]. More recently, it has been found that the lipids within the sperm head plasma membrane are also organized into distinct domains [19, 20, 21, 22, 23, 24,] This uneven distribution of lipids within the equine sperm plasma membrane was first detected using probes that complex with unesterified sterols (e.g. filipin, a fluorescent molecule that emits blue light when exited by UV light; [25]). Filipin/cholesterol complexes can be visualized on freeze-fracture analysis (by electron

microscopy) or by UV fluorescence confocal microscopy. In freshly ejaculated sperm, filipin complexes are distributed over the entire sperm head, but a lower density is apparent in the post-equatorial segment. Following capacitation, the post-equatorial region becomes devoid of filipin complexes while the amount in the apical region increases slightly. Although, it has been suggested that lipid segregation and reorientation in the sperm plasma membrane can be explained by lipid phase transitions (from the fluid to the gel phase), such transitions are only observed after cooling of sperm [24]. It has alternatively been suggested that, the organization of the glycocalyx is important for the lateral lipid heterogeneity of the equine sperm head plasma membrane and that capacitation dependent reorganization of lipids is most likely to reflect changes in the glycocalyx and a collapse of the phospholipid asymmetry (see below).

Membrane bilayer topology

As in somatic cells, the lipids of the sperm plasma membrane are distributed asymmetrically across the lipid bilayer. More specifically, choline phospholipids, SM and, to a lesser extent, PC are found predominantly in the outer lipid leaflet [26] while the aminophospholipids PE and, in particular, PS are located in the inner lipid leaflet. The inner leaflet concentration of PE and PS is probably due to active inward transport by an aminophospholipid translocase. Recently, we performed a set of experiments to determine whether lipid asymmetry in stallion sperm is affected by capacitation in vitro [27] and, indeed, a partial scrambling of lipid asymmetry was observed. After 5 h in capacitating conditions, sperm showed a marked increase of PC and SM levels in the inner leaflet and the normal inward movement of PE and PS was considerably slowed down (up to 10 times). These changes probably result from bicarbonate mediated activation of a non-specific bidirectional phospholipid scramblase during capacitation and, certainly, this would explain the increased inward translocation of PC and SM and also the decrease in net PS and PE translocation. Indeed, PS and PE are only found in the outer leaflet of the plasma membrane of equine, or other mammalian, sperm during capacitation (see also Figure 3). That scrambling of phospholipids is controlled by a bicarbonate mediated signalling pathway is demonstrated most clearly by the dose dependent increase in phospholipid scrambling that accompanies increased bicarbonate levels. In the absence of bicarbonate, scrambling can be induced by phosphodiesterase inhibitors (inhibit cAMP breakdown), protein kinase A (PKA) activators, protein phosphatase 1 and 2a inhibitors and by the addition of cAMP analogues [28]. From a practical point of view, this lipid scrambling can be monitored using the fluorescent probe merocyanine-540, a marker for disordered lipid packing of membranes [29]. Non-capacitated sperm with maximal lipid asymmetry show no fluorescence when stained with merocyanine, whereas scrambling allows intercalation of the dye into the membrane and, therefore, results in high fluorescence. In fact, the merocyanine response mirrors the scrambling response, because it is dependent on the same bicarbonate induced signalling cascade [28]. Indeed the merocyanine-540 response in stallion sperm correlates well with the number of cells that have either a capacitated or an acrosome-reacted CTC labelling pattern [27].

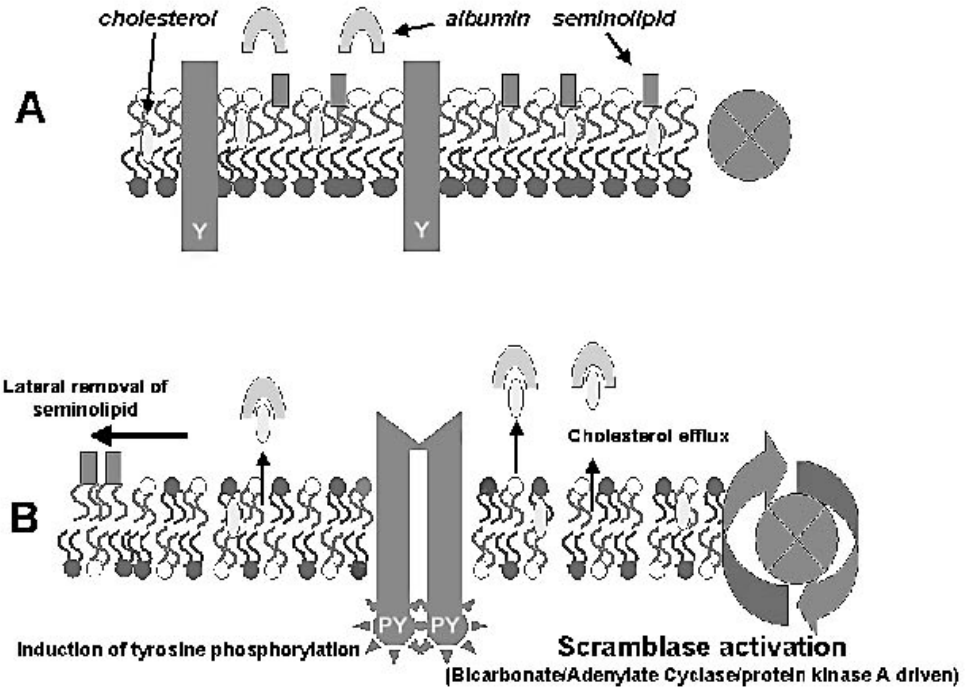

Fig. 3. Lipid reordering in capacitating stallion sperm cells. Panel A: The lipid ordering of the apical plasma membrane of freshly ejaculated sperm cells. Note the asymmetric bilayer distribution of phospholipids and the lack of phospholipid scramblase activity. Relatively high levels of cholesterol are present in the phospholipid bilayer and this cholesterol cannot be depleted by albumin. The sperm specific glycolipid seminolipid is concentrated in the apical plasma membrane (outer leaflet) and trans membrane proteins are not phosphorylated at tyrosine residues (Y). Panel B: Changes in lipid ordering during capacitation in Tyrode's media containing albumin, bicarbonate and calcium. Scramblase is activated via a bicarbonate adenylate cyclase protein kinase A driven signalling pathway, as a result the phospholipid asymmetry collapses. This phospholipid asymmetry collapse permits albumin-mediated depletion of cholesterol, the lateral membrane removal of seminolipid, phosphorylation of tyrosine residues (PY) in membrane proteins (inducing conformational changes and dimerisation of such proteins).

Lipoprotein mediated cholesterol transport

The group of Langlais found that inclusion of albumin in the in vitro fertilisation (IVF) media developed by them improved embryo production rates and they therefore proposed a model for capacitation and acrosome reaction based on the hypothesis that lipoproteins extract sterols from the sperm plasma membrane [30]. Certainly, albumin decreases markedly (up to 40%) the cholesterol content of sperm of various mammalian species [31] including stallion sperm

capacitated in vitro (Gadella and Brouwers, unpublished results). However, this albumin-mediated decrease in cholesterol content occurs only when sperm are incubated in the presence of bicarbonate and the extraction of cholesterol is very specific since it does not affect the phospholipid content of the membranes. The decrease in cholesterol content only takes place in bicarbonate-stimulated sperm. In fact, only merocyanine positive subpopulations of sperm allow albumin mediated cholesterol depletion. Therefore, it appears that albumin mediated cholesterol depletion from sperm membranes is a later event in sperm capacitation than the bicarbonate mediated changes in membrane fluidity and the induction of a collapse of phospholipid asymmetry (see Fig 3).

Cholesterol can also be extracted from the sperm plasma membrane in the absence of bicarbonate (i.e. in non-capacitating conditions) using cyclodextrins [32, 33]. Both albumin and cyclodextrin-treated sperm incubated under capacitating conditions showed a very rapid and pronounced activation of PKA and protein tyrosine kinase (PTK) is observed [33] (see Figure 3), thereby demonstrating that, at low cholesterol concentrations, capacitation and the acrosome reaction proceed rapidly. It is, therefore, possible that the rate of sperm capacitation relates to the rate of cholesterol efflux from the plasma membrane and, in this respect, sperm with high cholesterol contents (eg. from man and bull) are slow to capacitate (respectively 8 and 6 h) whereas those with lower cholesterol contents (boar and ram) are much faster (1 and 2 h, respectively) [3]. Probably, stallion sperm capacitate slowly due to their relatively high content in cholesterol (see section *Lipid composition*). It is also worth noting that seminal plasma of men, stallions and perhaps other mammals contains cholesterol rich vesicles secreted by the prostate (prostasomes) [34, 35], which block cholesterol efflux from sperm and probably help to delay capacitation until the appropriate time.

Lipid peroxidation

Because of their high polyunsaturated fatty acid content and relatively poor antioxidant defences [36, 37], mammalian sperm are somewhat prone to oxidative stress. Of course sperm will contact free radicals during their passage towards the oocyte and, for example, neutrophils and leucocytes in the seminal plasma secrete reactive oxygen species (ROS) [38, 39] and sperm themselves secrete intracellular ROS as a result of flagellar activity [40, 41]. The resultant oxidative attack is believed to regulate sperm function in two ways (i) Beneficial; mild peroxidation may promote capacitation by activating the sperm specific PKA [42, 43,] and switching on tyrosine kinases [42], while superoxide anions induce sperm hypermotility and increase their affinity for the ZP. (ii) Detrimental; on the other hand excessive peroxidation results in sperm deterioration [44]. Unfortunately, while the detrimental effects of lipid peroxidation for sperm have been extensively described [45] the importance of peroxidation for normal fertilisation remains to be investigated.

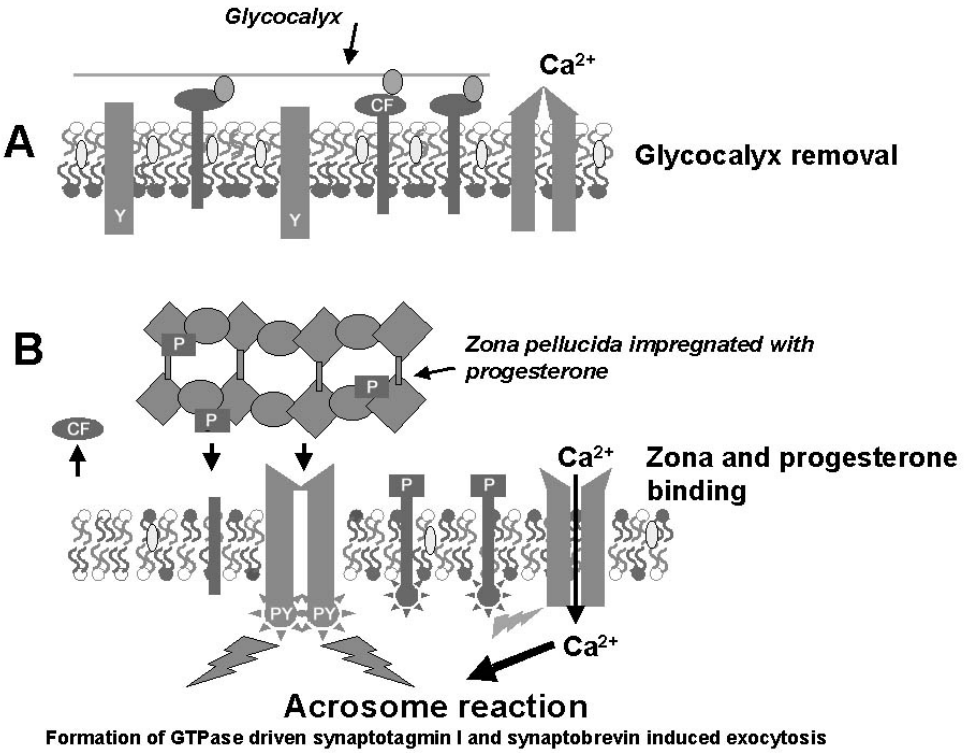

Fig. 4. The role of the glycocalyx in zona induced acrosome reaction. Panel A: In freshly ejaculated sperm cells a highly complex extracellular matrix is attached to the sperm plasma membrane (note the lipid ordering of the freshly ejaculated sperm cell; see also Fig. 3A). This glycocalyx prevents sperm zona binding and progesterone binding to the sperm surface receptor (CF = coating factor). Panel B: In capacitated sperm cells large parts of the glycocalyx are released (and lipids are reordered as detailed in Fig. 3B). As a result the progesterone receptor is unmasked now can bind and progesterone (P). The conformation changes in tyrosine phosphorylated (PY) transmembrane proteins enable binding of the zona pellucida (ZP3). Progesterone coupling to its receptor is coupled to the opening of Ca^{2+} channels and therefore invovled in Ca^{2+} influx. This Ca^{2+} synergistically with the activated zona receptor induces the GTPase driven synaptotagmin I , synaptobrevin like induced exocytosis (acrosome reaction).

Capacitation induced alterations in sperm glycocalyx and its role in sperm-zona interactions

On the extracellular side of the sperm plasma membrane, there are a number of carbohydrate structures that are bound to plasma membrane proteins (glycoproteins) or specific lipids (see Fig 4) and are known collectively as the glycocalyx. The glycocalyx has been proposed to play an important role in sperm-oocyte interaction because it is the part of the sperm that first comes

into direct contact with the oocyte. Furthermore, the glycocalyx may help to organize the sperm plasma membrane with which it is in direct contact via the transmembrane proteins and glycolipids.

During capacitation the glycocalyx, or at least its lectin binding properties, change markedly [reviewed by 3]. However, whether these changes in lectin binding reflect modification of carbohydrate structures, (un)covering of carbohydrate structures, or repositioning of glycoproteins or glycolipids is not yet known. Certainly, glycolipid repositioning during capacitation (Fig 3) [20, 21] is thought to involve the active removal of decapacitation factors [46, 47] and other factors bound to the sperm plasma membrane [48] and this almost certainly alters the glycocalyx (Figure 4).

Glycocalyx and tyrosine phosphorylation

During capacitation, tyrosine phosphorylation of proteins occurs in the plasma membrane of the flagellum and of the sperm head and it is thought that conformational changes in these proteins may alter their properties and contribute to the increased ZP affinity [49] and hyperactivity [50] and the induction of the acrosome reaction [51] that are characteristic of capacitated sperm. Indeed, a large number of proteins are tyrosine phosphorylated during in vitro capacitation of sperm from several mammalian species including man, mouse, boar, and bull [see 4]. It is currently believed that removal of the glycocalyx allows oligomerisation of transmembrane proteins, which is due to their conformational changes, and they become active tyrosine kinases. While, these data are not yet available for stallion sperm, preliminary experiments in our lab show clearly that bicarbonate and albumin are also involved in the induction of tyrosine phosphorylation in capacitating stallion sperm.

Glycocalyx and progesterone binding

Progesterone, like other steroid hormones, classically exerts its biological effects on protein expression via intracellular receptors [52]. However, it now appears that in several mammalian cell types biologically active progesterone (and other steroid hormone) receptors can also be found on the plasma membrane [53]. In this respect, the presence of progesterone binding sites/receptors on the plasma membrane of mammalian sperm heads has been shown using both FITC labelled BSA conjugated to progesterone (PBF) and the monoclonal antibody C-262, raised against the human intracellular progesterone receptor [17, 54, 55]. Although the specificity of both these labelling techniques has been questioned, isolation and identification of the putative progesterone receptor(s) may prelude the discovery of a new family of plasma membrane hormone receptors with low affinity for steroids but an ability to elicit rapid physiological changes. Isolating the progesterone receptor must be a major goal of studies to further detail the signalling events that lead to the acrosome reaction. Certainly, progesterone will induce the acrosome reaction in the sperm of several mammalian species such as human, boar or stallion [17]. However, the progesterone-binding site seems to have a rather low affinity for progesterone compared to the cytosolic progesterone receptor of somatic cells (μM versus

pM respectively [17, 56, 57, 58, 59]. Nevertheless, progesterone stimulation of the acrosome reaction may be physiologically relevant because the follicular fluid surrounding an ovulated oocyte contains enormous quantities of progesterone (horse: ~0.5 μM in follicular fluid vs < 6 nM in simultaneously harvested serum [60]. Mechanistically, progesterone induces a rise in the intracellular Ca^{2+} of capacitated sperm [61]. However, it is not sure how this Ca^{2+}-influx is triggered. Recent evidence favours mediation via the γ-aminobutyricacid$_A$ ($GABA_A$) receptor /Cl^--channel complex [62]. It also appears that progesterone primes the sperm plasma membrane to acrosome react more readily in response to ZP binding [60].

Finally, it appears that progesterone receptor exposure is regulated by accessory sex gland secretion because dog epididymal sperm, which expose the progesterone receptor are coated by prostate fluid during ejaculation and cease to bind progesterone [63]. Similarly, freshly ejaculated sperm from other mammalian species (including horses) will not bind progesterone but, during capacitation in vitro, they regain their affinity for progesterone and will acrosome react in response to progesterone exposure [17, 55, 60] These results suggest that some factor picked up from seminal plasma is removed during capacitation, thereby leading to the uncovering of the progesterone receptors and enabling the cells to respond to the hormone (Fig 4).

Sperm zona binding and acrosome reaction

The acrosome reaction is an exocytic event initiated immediately after primary binding of a sperm to the oocyte. The sperm plasma membrane fuses to the underlying acrosomal membrane at multiple sites and the acrosome contents are released. (Fig 1 and 2). The hydrolytic enzymes released are required to dissolve the ZP-matrix around the penetrating sperm and allow that sperm to enter the perivitelline space. However, if the acrosome reaction is initiated too early (i.e. prior to ZP-binding) the enzymes will be lost and the sperm will no longer be capable of zona penetration or, therefore, fertilisation.

Induction of the acrosome reaction

Several chemical or physical agents have been reported to induce the acrosome reaction in vitro [4]. However, it should be remembered that capacitated sperm have a destabilised plasma membrane, which is sensitive to even small environmental stresses. Thus exposure to calcium ionophore in the presence of Ca^{2+} will induce fusion of the plasma membrane to the underlying acrosome membrane of capacitated, but not of uncapacitated sperm, because the former have such fragile plasma membranes. The fragility of the plasma membranes of capacitated sperm is clearly demonstrated by the fact that they will undergo spontaneous acrosome reaction as a result of simply cooling them from 38°C to 30°C. Therefore, if we are to study physiological induction of the acrosome reaction, sperm must be kept at oviductal temperature. Furthermore,

it should be noted that calcium ionophore induced membrane fusion bypasses much of the signal-transduction pathway involved in the ZP-induced, physiological acrosome reaction. Thus, dramatic differences exist between the ZP-induced (physiological), spontaneous (detrimental) and calcium ionophore-induced acrosome reactions [64, 65] and if research concentrates on how the acrosome reaction proceeds after the forced introduction of Ca^{2+} into the sperm we will probably miss numerous changes that may be critical to normal fertilisation.

G-proteins

Guanine nucleotide binding proteins (G-proteins) are involved in signal transduction in nearly all mammalian cells and plasma membrane receptors frequently exert their effect on cells via G-protein coupling. Not surprisingly then, G-proteins appear to play a role in the signal transduction which leads to the acrosome reaction. Sperm from men, mice and bulls all possess G-proteins in the plasma membranes of their acrosomal and equatorial segments [66, 67] and, G-proteins have been implicated in the Ca^{2+}-influx that occurs during the acrosome reaction [61].

Ion channels

The acrosome reaction is a membrane fusion event that requires rather high (mM-range) cytosolic Ca^{2+}-levels. In capacitated sperm, the intracellular Ca^{2+}-concentration is very much lower (μM-range) and, since sperm do not possess an intracellular pool of mobilisable Ca^{2+}, extracellular Ca^{2+} has to pass through the plasma membrane prior to the initiation of the acrosome reaction [for review see 68]. In this respect, it appears that sperm voltage-dependent Ca^{2+}-channels are activated by ZP-binding, although it is also thought that a secondary Ca^{2+}-response of separate origin is necessary to induce membrane fusion [69].

Fusion proteins

Recently, studies of the proteins involved in the acrosomal fusion machinery have been reported in the literature, and they have likened the acrosome reaction to synaptic vesicle exocytosis. The reports suggest that an increase in intracellular Ca^{2+} is a priming step that enables a conformational shift in the proteins that make up the membrane fusion complex (i.e. that span between the plasma membrane and the acrosomal outer membrane). These proteins include the soluble *N* -ethylmalmeide-sensitive factor attachment protein receptors (SNARE) proteins [70] and are controlled by the activation of small GTPases [71] (see also Fig 4). The control of the fusion machinery in the secretory-pathway has been reviewed in detail by Harter and Reinhard [72].

Detection of the acrosome reaction

Over the years, a variety of assays have been used to detect occurrence the acrosome reaction [see 73]. However, it is now most common to monitor acrosome integrity in living sperm using

lectins with acrosome specific binding characteristics. For example, peanut agglutinin-fluorescein conjugates (PNA-FITC) bind specifically to the outer acrosome membrane of stallion sperm [74]. Thus, incubated sperm samples can be stained with FITC-PNA, and counterstained with a supravital stain such as propidium iodide, and the still living cells can then be monitored using an inverted confocal microscope equipped with a life cell chamber (equilibrated at 38°C and 5% CO_2) or monitored by flow cytometry [27]. This life cell staining method differs from the commonly used method in which sperm are first subjected to membrane solubilisation before FITC-PNA staining [28]. Solublisation procedures as well as alternative staining methods such as Triple stain [75] or CTC [76] are less reliable because the staining can only be detected on fixed cells after washing off the unbound dye. Such fixation and preparation is likely to induce cell deterioration, especially in the fragile capacitated sperm.

Conclusions

This paper gives an overview of the dynamic changes that occur in the plasma membrane of an equine sperm during capacitation and the acrosome reaction. These changes in this highly organized membrane are vital to subsequent membrane adhesion and fusion processes. Unfortunately, the fragility of the sperm plasma membrane after capacitation makes it extremely difficult to study, and this may explain some of the conflicting data on the roles, and indeed existence of, surface molecules. Artefacts are easily introduced when primed cells are studied under non-physiological conditions. However, at least three of the intracellular signalling pathways that have been described to occur in capacitating or acrosome reacting sperm deserve more detailed study: (i) Bicarbonate activation of adenylate cyclase. The subsequent induction of PKA seems to cross-talk with tyrosine kinase and is involved in the collapse of the normal phospholipid asymmetry in the apical plasma membrane. These effects seem to be important for both sperm binding and the acrosome reaction. (ii) Cholesterol efflux from the sperm plasma membrane. This process appears to sensitise sperm to bicarbonate induced capacitation. However, it is not yet known how cholesterol efflux is regulated or what proteins are involved in this process. (iii) The cell surface progesterone receptor. Sperm contain a specific cell-surface progesterone receptor that enables Ca^{2+} influx by opening voltage dependent Ca^{2+}-channels. This receptor may belong to an entirely new family of steroid receptors.

Sperm-ZP binding is divided into more than one phase, each apparently involving different proteins at different locations of the sperm plasma membrane. Furthermore, the various regional plasma membrane specializations, and reorganizations thereof, may be important not only for sperm adhesion and acrosome fusion but also for intracellular signalling. However, because zona binding is initiated by sperm plasma membrane proteins, whereas secondary binding is mediated by acrosomal matrix proteins, it may be sensible to isolate the sperm plasma membrane from the acrosome before attempting to study its role in primary zona binding. A similar membrane isolation approach may also help to distinguish primary sperm

tip binding to the oolemma, which is probably initiated by an inner acrosomal membrane protein, from secondary oolemma binding which is mediated by a plasma membrane protein located in the equatorial region of the sperm head. Similarly, it may be better to study sperm-zona binding using intact ZP rather then solubilized zona proteins.

If there is still much to learn about sperm-zona binding, even less is known about how the sperm plasma membrane fuses with the outer acrosomal membrane and what proteins are involved in this fusion; this may be a useful area for future research.

In conclusion, a better understanding of how the sperm plasma membrane is organized and of the relevance of rearrangements in this organization to the signalling events that lead to sperm capacitation, zona binding and the physiological acrosome reaction will help greatly in understanding how the sperm and oocyte normally interact prior to fertilisation and how this can be optimised in the in vitro situation or how we can protect against functionally disastrous damage during semen preservation and storage.

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Chapter 3

Evaluation of *in vitro* capacitation of stallion spermatozoa

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Biology of Reproduction 65, 462-470 (2001)

Abstract

The primary aim of this study was to establish a flow cytometric technique for determining the capacitation status of stallion spermatozoa. To this end, a flow cytometric technique that demonstrates changes in the plasma membrane fluidity, namely merocyanine 540 staining, was compared with the more conventional Ca^{2+} dependent fluorescence microscopic technique for assessing capacitation status, chlortetracycline (CTC) staining. In addition, the effect of bicarbonate/CO_2 on the progress of capacitation and the acrosome reaction (AR) and on temporal changes in sperm motility with particular regard to hyperactivation, was analysed. For the study, fresh semen was washed and then incubated for 5 h in bicarbonate-containing or bicarbonate-free medium, with or without Ca^{2+}-ionophore to induce the AR, and at intervals during incubation aliquots were taken and analysed with respect to capacitation and acrosome status. The AR was assessed using both the CTC and FITC-PNA staining techniques with similar results. In brief it was found that merocyanine 540 detects capacitation related changes much earlier than CTC (0.5 h versus ~3 h) and that flow cytometry for evaluation of capacitation and AR was a quicker (10 sec per sample) and more accurate (10,000 cells counted) technique than fluorescence microscopy. Furthermore, it was observed that Ca^{2+}-ionophore could not induce the AR in the absence of bicarbonate, but that the ionophore synergies the bicarbonate-mediated induction of the AR as detected by CTC (although not significant when evaluated using FITC-PNA). The percentage of hyperactive sperm in each sample was not affected by time of incubation under the experimental conditions studied. In conclusion, merocyanine 540 staining is a better method for evaluating the early events of capacitation, for stallion spermatozoa incubated in vitro, than CTC staining. Furthermore, bicarbonate sperm activation clearly plays a vital role in the induction of the AR in stallion spermatozoa.

Introduction

"Capacitation" is a collective term for the changes that a spermatozoon undergoes when it comes into contact with the female reproductive tract. These changes include reorganisation of membrane proteins, metabolism of membrane phospholipids, a reduction in membrane cholesterol levels, and hyperactivation [1]. These changes, together with the subsequently induced acrosome reaction (AR), an irreversible exocytotic event, are essential if a sperm is to bind to and penetrate the zona pellucida and thereafter to fuse with the oocyte plasma membrane [1]. Capacitation is, thus, a critical event in the process of fertilisation. However, differentiating capacitated from non-capacitated spermatozoa remains inexact science, despite almost half a century having past since Chang [2] and Austin [3] first described the phenomenon of capacitation, and it is frustrating that a straightforward, validated and easy to interpret method for assessing capacitation is still not in common use.

On the other hand, chlortetracycline (CTC) staining has been used to assess the capacitation state of spermatozoa [4 -7] and it is currently the assay of choice because it distinguishes

three different stages of sperm activation, viz. non-capacitated, capacitated acrosome-intact and capacitated acrosome-reacted. However, a clear understanding of how CTC interacts with the sperm surface at the molecular level is lacking and unfortunately the evaluation of CTC staining is performed on fixed sperm cells.

Merocyanine-540 staining is another technique that may be useful for assessing the capacitation status of spermatozoa. Merocyanine-540 is a hydrophobic dye that has been shown to stain cell membranes more intensely if their lipid components are in a higher state of disorder [8,9], as is the case for capacitated spermatozoa. In this latter respect, merocyanine-540 has recently been used to monitor alterations in the lipid architecture of the boar sperm plasma membrane during capacitation [10], a process that appears to be due to bicarbonate induced transbilayer scrambling of phospholipids [11]. One major advantage of merocyanine 540 over CTC is its suitability as a probe for assessing capacitation flow-cytometrically, since this latter technique should allow for more objective analysis of larger numbers of unfixed (and therefore relatively undamaged) sperm samples. Furthermore, using the flow cytometer, merocyanine-540 staining can be combined with the membrane impermeable DNA binding probe Yo-Pro-1 to allow coincident analysis of membrane lipid status and cell viability, while the acrosome reaction of living stallion spermatozoa, similarly can be assessed flow cytometrically using fluorescein isothiocyanate (FITC) conjugated peanut (Arachis hypogea) agglutinin (PNA) as a label [12,13].

Capacitation also involves changes in sperm motility, known as hyperactivation [1], which are thought to aid sperm progression up the oviduct by enabling spermatozoa to move away from the oviductal epithelium [14], and to provide the motive thrust needed for penetration of the zona pellucida [15].

The primary aim of this study was to establish a flow cytometric technique for assessing capacitation of stallion spermatozoa using a reporter probe with known sperm-binding characteristics rather than CTC which, though is empirically accepted but is laborious and its working mechanism is scientifically unexplained. To this end, we investigated whether CTC identification of capacitation and the AR in fixed sperm preparations correlated with flow cytometric detection of the same processes in unfixed sperm samples, where capacitation was indicated by changes in membrane lipid fluidity, as demonstrated by merocyanine 540 staining, and the AR was detected by FITC-PNA staining. In both cases capacitation was induced using a modified Tyrode's medium, as described by Harrison et al.[10], in the presence of 15 mM bicarbonate/ 5% CO_2 (Tyr+bic) and the results were compared to control samples incubated in bicarbonate/ CO_2–free Tyrode's medium (Tyr). Furthermore, Ca^{2+}-ionophore was added to aliquots of both Tyr+bic and Tyr incubated sperm samples in an attempt to induce the AR. For all treatments, the alterations in CTC, merocyanine-540 and FITC-PNA labelling patterns were monitored over time and in addition, temporal changes in sperm motility and hyperactivation were assessed using a computer assisted sperm analysis (CASA) system.

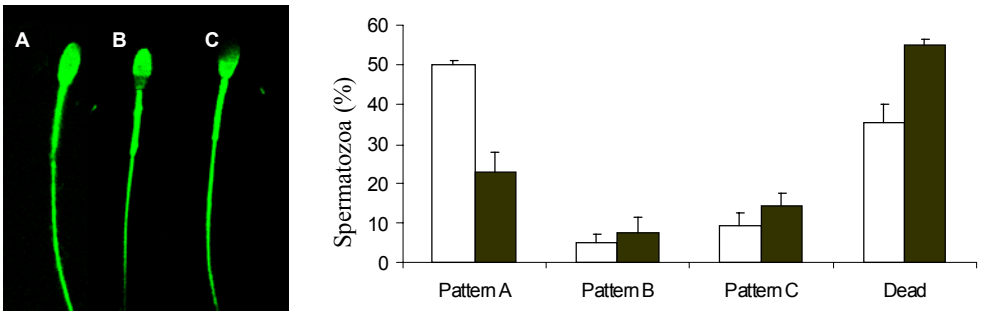

Fig. 1. The three patterns obtained for CTC-stained viable spermatozoa. A) Pattern A: whole sperm head shows bright fluorescence, with or without a brighter equatorial band; this is indicative of noncapacitated spermatozoa. B) Pattern B: the acrosomal region of the sperm head fluoresces brightly but the postacrosomal region does not; this denotes capacitated, acrosome-intact spermatozoa. C) Pattern C: the acrosomal region of the sperm head is nonfluorescent, with or without a fluorescent, postacrosomal region; this indicates capacitated, acrosome-reacted spermatozoa. Sperm head length ~7.0 µm. D) A bar chart demonstrating the mean (± sd) percentage of viable sperm fluorescing in the various CTC staining patterns after 0 h (unshaded) and 5 h (shaded) of incubation.

Materials and methods

Materials

CTC and Ca^{2+} ionophore A23187 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Merocyanine 540, ethidium homodimer (EthD-1), Yo-Pro-1 and propidium iodide (PI) were obtained from Molecular Probes Inc. (Eugene, OR, USA). FITC-PNA was purchased from EY Laboratories Inc. (San Mateo, CA, USA).

Media

A modified Tyrode's medium was used for incubating sperm in "capacitating" conditions (Tyr+bic). The complete Tyrode's medium contained 96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 50 µg kanamycin/ml, 20 mM Hepes, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 15 mM $NaHCO_3$ and 7 mg/ml BSA [16]. For incubating sperm in the control "non-capacitating" conditions, a modified Tyrode's medium without bicarbonate (Tyr) was prepared. In both cases stock solutions minus the $CaCl_2$, BSA and pyruvate were prepared, filtered through a 0.2 µm filter and stored at 4°C [11]. The remaining three ingredients were added 20-24 h prior to the experiment and the medium was maintained in equilibrium with 5% CO_2 in air at 37°C until the time of the experiment. The pH and osmolality of both media were maintained at 7.4 and 300 mOsm/kg, respectively.

Semen collection and preparation

During the breeding season, semen was collected from three adult (4-10 years old) stallions once a day for three consecutive days (total: 3 ejaculates per stallion). All 9 ejaculates had a gel-free sperm concentration of >150 x 10^6 cells/ml and a progressive sperm motility of >65%. The semen was collected using an artificial vagina, filtered through gauze to remove the gel and any large particles of debris and immediately processed for use. Ejaculates were processed separately. Two ml semen was transferred to a pre-warmed 15-ml tube, mixed with 6 ml Tyr and centrifuged at 900g for 10 min, to allow removal of seminal plasma. After removal of the supernatant, the pellet was resuspended with Tyr to a final volume of 2 ml (equal to the volume of semen used) and incubated at 37°C for 30 min for equilibration and subsequently divided into 2 equal portions: One of which was diluted with Tyr+bic and the other with Tyr, to final sperm concentrations of 25 x 10^6/ml. These samples were divided further into two portions, one of which was supplemented with Ca^{2+}-ionophore to a final concentration of 1µM [17]. All 4 samples were then incubated at 37°C [4] for 5 h. At 0, 0.5, 2, 3.5 and 5 h, aliquots of each sample were recovered and the state of capacitation and/or acrosome reaction was assessed using all three staining methods, as described below, and sperm motility was analysed with respect to the incidence of hyperactivation.

CTC/ EthD-1 staining

CTC staining was made freshly by dissolving CTC and L-cysteine in a chilled 20 mM Tris buffer supplemented with 130 mM NaCl to produce final concentrations of 0.75 mM CTC and 5 mM L-cysteine, respectively. The pH of the final solution was adjusted to 7.8 and it was kept in the dark at 4°C until it was used. For staining, a 100 µl aliquot of sperm suspension was mixed with 100 µl of a 2 mM solution of EthD-1 (a supra vital stain) in PBS and this mixture was incubated for 5 min before 100 µl of the CTC stain was added. Thereafter, the sample was fixed with 30 µl of 12.5% glutaraldehyde in 1 M Tris (pH = 7.0) to produce a final concentration of 1.1% fixative, and a 10 µl drop of the fixed sperm suspension was mixed with 5 µl of antifade on a glass microscope slide. Next, the droplet was covered with a cover-slip, and the slide was gently but firmly pressed under two folds of a tissue paper to absorb any excess fluid. The prepared slide was then stored in the dark until it was analysed, within 1 h of preparation. For analysis of the CTC staining an Epi-fluorescence microscope (BH-2; Olympus, Tokyo, Japan) equipped with a 458 ± 15 nm wavelength band-pass excitation filter, a 470 nm dichroic mirror and a 500 nm long-pass emission filter was used to asses at least 100 spermatozoa at a magnification of 400 x. This combination of filters enabled simultaneous identification of dead cells (EthD-1 positive) versus live cells (EthD-1 negative) and CTC fluorescence patterns. For more detailed visualisation CTC stained sperm were examined with a spectral confocal microscope (Leica TCS SP, Leica GmbH, Germany). The CTC and EthD-1 stains were excited with the 458 nm Argon laser line (emission selected at 495-535 nm) and resolution was optimised by using extended focus made up of 3 optical Z-sections (0.25 µm step size) (average of three X-Y scans per section) and recombined into one image.

Merocyanine540/Yo-Pro-1 staining

For flow cytometric analysis of capacitation status, sperm cells were incubated in Tyr+bic or Tyr containing 2.7 µM merocyanine-540 (a reporter probe for phospholipid scrambling; [10]), 25 nM Yo-Pro-1 (a membrane impermeable nucleic acid stain; [10]), 0.5mg/ml polyvinyl alcohol (PVA) and 0.5 mg/ml polyvinyl- pyrolidone (PVP). In vitro capacitation was performed in airtight sealed 5ml flow cytometer tubes (Becton Dickinson, San Jose, CA, USA) containing 3 ml medium. The tubes were flushed with air containing 5% CO_2 before closing, and incubated for approximately half an hour in a shaking water-bath at 37□C before flow cytometric analyses. Sperm cell analysis was performed using a flow cytometer (FACS Vantage SE; Becton Dickinson, San Jose, CA, USA). The system was triggered by the forward light scatter signal (FSC). The Yo-Pro-1 and merocyanine 540 probes were both excited by an argon ion laser (Coherent Innova, Palo Alto, CA) with 200 mW laser power at a wavelength of 488 nm. Fluorescence of the Yo-Pro-1 probe was then measured using a 520 ± 15 nm band pass filter (fluorescence detector equipped with a photo multiplier tube) while merocyanine 540 emission was deflected with a 560 nm short pass dichroic mirror in the emission pathway and measured using a 575±15 nm band pass filter. Sperm cells were analysed at a rate of between 8,000 and 10,000 per second using PBS as sheath fluid, and for each sample 10,000 events were stored in the computer for further analysis with Cell-Quest software (Becton Dickinson, San Jose, CA). Sideward light scatter (SSC) and FSC were recorded so that only sperm cell specific events, which appeared in a typical L-shaped scatter profile, were selected for further analysis. During measurement, the sample input tube in the FACS Vantage SE was kept at 37°C and 5% CO_2 using a controlled temperature bath/circulator, to maintain constant incubation conditions during the whole analysis using a controlled temperature bath/circulator.

For visualisation of individual sperm cells, samples were placed in a life chamber at 37°C in which the bicarbonate/CO_2 equilibrium was maintained by continuous infusion of humidified air containing 5% CO_2, thus maintained under physiological conditions. The sperm were examined using an inverted spectral confocal microscope (Leica TCS SP, Leica GmbH, Germany) fitted with a 488 nm Argon laser line for exciting fluorescent probes. Yo-Pro-1 emission was detected using photo multiplier tube 1 which selected emissions with in the wavelength range of 500-550 nm while merocyanine 540 emission was detected using photo multiplier tube 2 (580-620 nm). Single scans were made to record the labelling patterns of motile sperm cells.

FITC-PNA/PI staining

The acrosomal status of the sperm was examined by staining the incubated samples with 5µg/ml FITC-PNA (as a marker for acrosomal leakage, [18]) and 1 µM PI (as marker for cell death [19]), and analysing the labelled sperm on a flow cytometer (FACScan; Becton Dickinson, San Jose, CA) as described before [18,20].

For visualisation of individual sperm cells, samples were placed in a life chamber at 37°C in which the bicarbonate/CO_2 equilibrium was maintained by continuous infusion of humidified air containing 5% CO_2 in a spectral confocal microscope (Leica TCS SP, Leica GmbH, Germany) and excited with the 488 nm Argon laser line. The emission of PNA-FITC was detected using photo multiplier tube 1, focused for emissions in the 500-550 nm wavelength range, and the emission of PI was detected using photo multiplier tube 2 (600-700 nm). Single scans were made to capture the labelling patterns of (hyper) motile sperm cells.

Comparison of CTC, Merocyanine 540 and FITC-PNA staining

In a separate experiment, the proportion of viable cells with positive FITC-PNA staining were evaluated for 3 stallions (3 ejaculates per stallion analysed). The FITC-PNA staining was analysed under bicarbonate enriched (15 mM); and bicarbonate depleted (0 mM) conditions using either a flow cytometer or a confocal microscope (as described above). The labelled sperm samples were analysed in unfixed state as well as after 2% (w/v) paraformaldehyde fixation. The results obtained were compared with microscopic evaluations of CTC staining of the same incubated sperm samples (i.e. after fixation with 1.1 % w/v glutaraldehyde) and both stainings were also compared with merocyanine 540 staining (unfixed cells only).

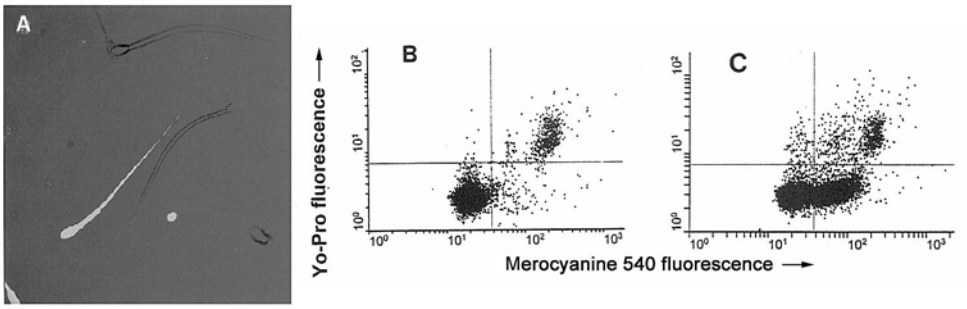

Fig. 2. The two patterns obtained for viable sperm stained with merocyanine 540. A) The highly fluorescent sperm heads demonstrate capacitated spermatozoa, and the poorly fluorescent heads demonstrate non-capacitated spermatozoa. Sperm head length ~7.0µm. Scatter plots for the amount of fluorescence detected flow cytometrically for sperm from a single ejaculate stained with merocyanine-540 and Yo-Pro after 0 h (B) and 5 h (C) of incubation in Tyr+bic medium. The X-axis depicts the amount of fluorescence emitted by merocyanine-540 probe that was bound to individual sperm cells as measured in arbitrary units by the FL-1 detector, and the Y-axis depicts the amount of fluorescence in arbitrary units emitted by Yo-Pro-1 probe that was bound to individual sperm cells by the FL-3 detector. In both plots, sperm cells that fluoresced more intensely with Yo-Pro than the superimposed horizontal line were considered to be non-viable, whereas the viable sperm cells with higher merocyanine-540-depended fluorescence than the superimposed vertical line were considered to be capacitated.

Hyperactivation

In order to examine the motility pattern of incubated spermatozoa, samples from each treatment and time point were examined using a Hamilton Thorne Research Motility Analyzer (HTM IVOS; model 8020, version 8.1, Beverly, MA). The Hamilton Thorne CASA system was programmed to the following settings: frames acquired = 20; frame rate = 30/sec; minimum contrast = 8; minimum size = 6; Low/High size = 0.5-1.8; Low/High intensity gates = 0.5-1.8; non-motile head size = 13; non-motile intensity = 25; Medium VAP value = 25; Low VAP value = 9; Slow cells motile = No; Threshold STR = 80. These values were selected on the basis of previous experience and their suitability was confirmed using the PLAYBACK option of the HTM. A 20 μm counting chamber (Cell Vision, Anthos Labtec BV, The Netherlands) maintained at 37°C was used for analysis, and samples were tested within 1 min of being taken out of the incubator. It took less than 5 minutes to analyse a sample on the HTM machine and particular attention was paid to the classic parameters of sperm hyperactivation, namely curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) [21,22]. A spermatozoon was designated as being hyperactive if it had a VCL □ 180 μm/sec and an ALH □ 12 μm.

Statistical analysis

Analysis was centred around identifying decreases in the percentages of non-capacitated or acrosome-intact spermatozoa. Statistical analysis was carried out using repeated measures ANOVA (n=3) to examine the effects of staining method, time and stallion. Since the sample size was only 3, normality could not be properly assessed and the data were therefore analysed untransformed. As an additional check the data were reanalysed after a normalising sine transformation and since this did not alter the outcome the assumption of normality was strengthened. In the case of the hyperactivation data, a one-way ANOVA was used to compare the motility of the 6 ejaculates. The statistical package used was SPSS 8.0 (SPSS for windows 1996, SPSS Inc. Chicago). Differences were taken to be statistical significant when $P < 0.05$.

Results

CTC staining patterns

CTC-stained viable spermatozoa (EthD-1 negative) showed 3 major fluorescence patterns. In the first of these, pattern-A, the whole of sperm head showed bright fluorescence, with or without a brighter equatorial band; this pattern was representative of non-capacitated spermatozoa (Fig 1 A). In pattern-B, the acrosomal region of the sperm head fluoresced brightly while the post-acrosomal segment was non-fluorescent; this pattern was indicative of capacitated but acrosome-intact spermatozoa (Fig 1 B). Finally, in pattern-C; the acrosomal region did not fluoresce while the post-acrosomal segment could be fluorescent

or not; this pattern indicated capacitated and acrosome-reacted spermatozoa (Fig 1 C). With regard to the effect of time, incubation in Tyr+bic medium for 5 h resulted in a significant decrease in the average percentage of spermatozoa showing pattern-A (that is viable, non-capacitated sperm); 50.2±0.8% at 0 h of incubation and 22.8+4.9% at 5 h, and an increase in the percentage of spermatozoa displaying pattern-C (i.e. viable, capacitated and acrosome-reacted sperm); 9.5±3.2% at 0 h and 14.6±2.85% at 5 h.

Merocyanine staining pattern

Merocyanine-540 staining gave rise to two basic fluorescence patterns (Fig 2 A) in viable (Yo-Pro-1 negative) sperm cells, one of which was characterised by poorly and the other by relatively higher fluorescent sperm cells. Sperm cells with poorly fluorescent heads were considered to be non-capacitated while a highly fluorescent sperm head was considered to be a characteristic of a capacitated spermatozoon. These patterns were analysed flow cytometrically and the resulting data were presented in quadrants (Figs 2 B, C), where the X-axis represents increasing merocyanine-540 fluorescence and the Y-axis, shows increasing Yo-Pro-1 fluorescence. When incubated in Tyr+bic medium, 49.6±2.9% (mean ± SD) of the spermatozoa showed low merocyanine-540 fluorescence at 0 h. After the 5 h incubation, the proportion of poorly fluorescent sperm had decreased to 8.7±2.8% (Figs 2 B,C), thereby indicating a significant time-dependent decrease in the percentage of non-capacitated spermatozoa. Conversely, the percentage of spermatozoa with high merocyanine 540 fluorescence increased from 6.4% at 0 h to 25.6% at 5 h), demonstrating a corresponding increase in the percentage of capacitated live spermatozoa (Figs 2 B, C). The remaining sperm cells were labelled with Yo-Pro-1 and therefore non-viable.

FITC-PNA staining pattern

FITC-PNA staining differentiate the viable (PI negative) sperm cells into two distinct groups (Fig 3 A). The sperm were either not labelled with FITC-PNA, thereby demonstrating that their acrosomes were intact, or showed acrosomal FITC-PNA staining, which indicated that their acrosome was either reacting or reacted. Absence or presence of FITC-PNA labelling was also analysed flow cytometrically and the resulting data presented in quadrants (Figs 2 B, C), where the X-axis shows increasing FITC-PNA fluorescence and the Y-axis shows increasing PI fluorescence (Figs 3 B, C). At the onset of incubation in Tyr+Bic medium (0 h) 53.8±3.2% (mean ± SD) of the spermatozoa were not labelled with FITC-PNA. After 5 h of incubation, the proportion of unlabeled sperm cells had decreased to 38.9±4.1%, indicating a time dependent decrease in the percentage of acrosome-intact spermatozoa. On the other hand, the increase in the percentage of viable spermatozoa that were labelled with FITC-PNA between 0 h (2.2%) and 5 h (4.0%), demonstrated that only a slight increase in the percentage of acrosome-reacted live spermatozoa occurred. The remaining cells were labelled with PI and thus shown to be non-viable.

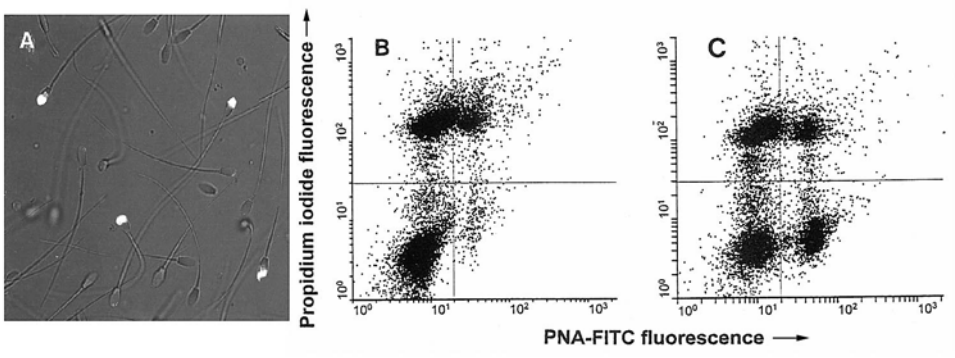

Fig. 3. The two different patterns obtained for viable sperm stained with FITC-PNA. (A) A green fluorescent sperm head denotes an acrosome-reacted/ reacting sperm a non-fluorescent sperm head indicates an acrosome-intact sperm. Sperm head length ~7.0µm. Scatter plots that depict the amount of fluorescence detected flow cyto-metrically per sperm for sperm cells stained with FITC-PNA and propidium iodide after incubation in Tyr+Bic for 0 h (B) and 5 h (C). The amount of fluorescence emitted by the FITC-PNA label, as detected by the FL-1 detector, is indicated in arbitrary units on the X-axis while the amount of fluorescence (in arbitrary units) emitted by the propidium iodide vital stain and detected by the FL-3 detector is indicated on the Y-axis. All sperm that had a PI dependent fluorescence value higher than the superimposed horizontal line were regarded as non-viable while the viable sperm cells with higher FITC-PNA dependent fluorescence than the superimposed vertical line were classified as acrosome reacted.

Comparison between capacitation state detected with merocyanine and CTC

At the onset of incubation in Tyr+bic medium (0 h), merocyanine 540 staining demonstrated that 49.6±2.9% of the spermatozoa had not undergone capacitation. Thereafter, the percentage of non-capacitated spermatozoa decreased initially rapidly to reach 21.5±5.0% after 0.5 h and then more gradually to reach a final value of 8.7±2.8% after 5 h of incubation (Fig 4 B). On the other hand, when capacitation status was monitored using CTC staining a less dramatic and more gradual decrease in the number of non-capacitated sperm, from 50.2±0.8% at 0h to 22.8±4.9% at 5 h, was observed (Fig 4 A).

Thus, although the proportion of non-capacitated sperm detected at the onset of incubation did not differ between the two techniques, the apparent rate of loss of non-capacitated sperm differed significantly ($P<0.05$). Therefore, if it is accepted that merocyanine-540 and CTC staining both follow changes in capacitation status then it is clear that CTC staining is very much slower to recognise these changes.

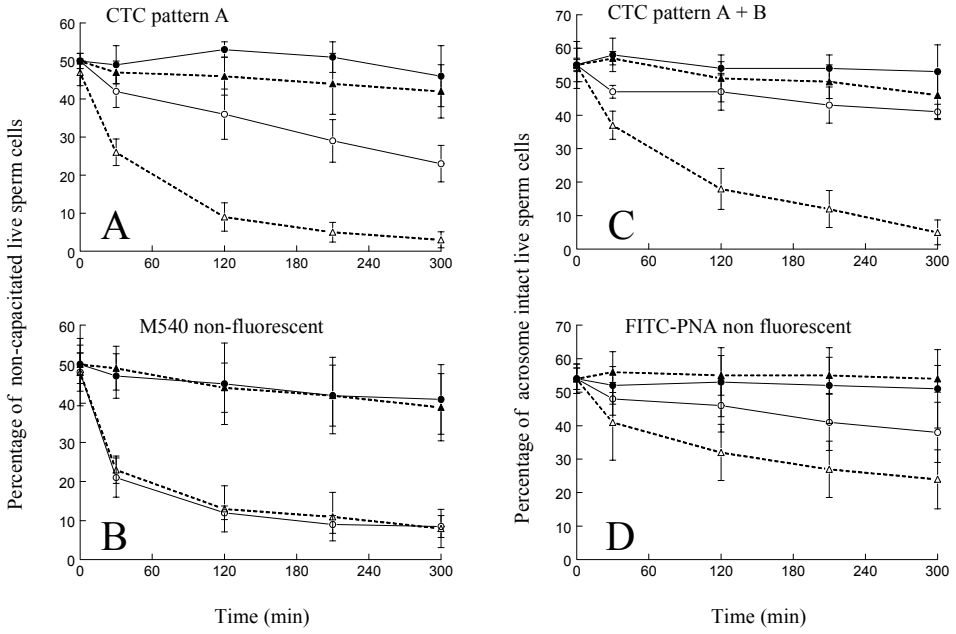

Fig. 4. The mean (± sd) percentages of sperm cells that remained viable and demonstrated the indicated responses in the four incubation conditions tested, for nine ejaculates collected from three stallions. Percentages of sperm cells that remained viable and not capacitated, as detected by CTC (A) and merocyanine 540 (B) respectively. Percentages of total sperm cells that remained viable with intact acrosome as detected by CTC (C) and FITC-PNA (D), respectively. Solid line (□), Tyrode's medium; solid line (□), Tyrode's medium with 15 mM bicarbonate; broken line (□), Tyrode's medium with 1 μM Ca^{2+}-ionophore; broken line(□), Tyrode's medium with 15 mM bicarbonate and 1 μM Ca^{2+}-ionophore.

In the presence of Ca^{2+}-ionophore and bicarbonate, a similar decrease in the percentage of non-capacitated spermatozoa, from 48.2±5.0% at time 0 h to 22.7±3.5% at 0.5 h time-point and 7.7±4.9% at 5 h, was observed with merocyanine-540 staining (Fig 4 B). In these latter conditions, CTC staining gave similar results to merocyanine-540 for the rate of decrease in the percentage of non-capacitated sperm since the values went from 45.2±3.5% at time 0 h, to 25.8±4.9% at 0.5 h and to 2.4±2.1% at 5 h, (Fig 4 A). Repeated measures analysis of variance did not show any significant difference between these two staining methods for the proportion of non-capacitated sperm detected at the various time points, when both bicarbonate and Ca^{2+}-ionophore were included in the incubation medium.

In bicarbonate-free Tyrode's medium (Tyr), only a very slight decrease in the percentage of non-capacitated sperm over time was observed with merocyanine-540 (51.5±2.9 % at 0 h to

42.2±8.9% at 5 h; Fig 4 B) and CTC (52.3±3.1% at 0 h and 45.6±7.5 % at 5 h; Fig 4 A) staining. Furthermore, incubation with Ca^{2+}ionophore had no apparent effect on the capacitation status of sperm, as detected with the merocyanine-540 and CTC stain (Fig 4 A, B). In contrast, the change in capacitation status of sperm was only fully (i.e. comparable to the effects monitored by the merocyanine-540 staining) detectable in presence of Ca^{2+}-ionophore with the CTC stain (Fig. 4 A).

Treatment		CTC		merocyanine		FITC-PNA	
Fixation	bicarbonate	CLSM-Cap	CLSM-AR	CLSM	FACS	CLSM	FACS
Unfixed	0 mM	ND	ND	40±9	41±9	50±8	52±11
Unfixed	15 mM	ND	ND	12±4	11±5	37±9	41±8
Fixed	0 mM	46±7	45±6	ND	ND	46±8	47±6
Fixed	15 mM	24±4	31±4	ND	ND	23±5	26±4

Table1: A comparison of the results obtained when stallion sperm were incubated for 5 h in Tyrode's medium (with 15mM bicarbonate) before sperm plasma membrane integrity, capacitation status and acrosome integrity were analysed simultaneously using flow cytometry (FACS) or confocal microscopy (CLSM) on cells in a life chamber. The values in the table are the mean (± standard deviation) percentages of the viable (plasma membrane intact) cells which had either not undergone capacitation (CTC Cap and merocyanine) or were recorded to have an intact acrosome (CTC-AR and FITC-PNA), for nine ejaculates collected from three stallions. For each ejaculate CLSM counts were performed on 300 cells while FACS data were recorded for 10,000 sperm specific events. FACS analyses of CTC staining was not possible. ND: not detectable;

Comparison of CTC and FITC-PNA staining for the detection of acrosomal integrity

The percentage of acrosome-intact cells as determined by CTC staining was taken to be the sum of the percentages of non-capacitated and capacitated acrosome-intact sperm (see Figs. 1 A, B). At the onset of incubation in Tyr+Bic medium, the percentage of acrosome-intact cells as detected by CTC staining was 55.2±1.6 %. During incubation the proportion of acrosome intact sperm cells decreased gradually, to 30.4±2.7 % after 5 h (Fig 4 C). These two values were similar to those observed with FITC-PNA staining for which the percentage of acrosome-intact cells dropped from 53.8±3.2 % at 0 h to 38.9±4.1 % after 5 h of incubation.

The addition of Ca^{2+}-ionophore to the Tyr+bic incubation medium resulted in a more rapid and pronounced decrease of acrosome-intact sperm cells, as detected by CTC and, to a lesser extent, by FITC-PNA stainings (Fig 4 C, D), thereby indicating that Ca^{2+}-ionophore induces the acrosome reaction in stallion sperm incubated in a bicarbonate containing medium. Although the decrease in the proportion of acrosome-intact cells was more prominent in CTC stained (55.6± 2.0% at 0 h to 5.6±3.7% at 5 h) than in FITC-PNA stained samples (53.3±4.4% at 0 h to 24.6±8.8% at 5 h) there was no significant difference between the data.

In the absence of bicarbonate, however, there was no time-dependent decrease in the percentage of acrosome-intact spermatozoa as detected by either CTC or FITC-PNA staining (Fig 4 C, D) and even addition of Ca^{2+}-ionophore to the Tyr medium did not induce AR in incubated spermatozoa (Fig 4 C, D).

In a separate experiment FITC-PNA staining was assessed in bicarbonate primed and control cells prior to and after 2% (w/v) paraformaldehyde fixation using flow cytometry and confocal laser scanning microscopy (Table 1). Flow cytometry and confocal laser scanning microscopy gave similar results. This was also the case for merocyanine-540 staining of the same sperm samples (only unfixed samples were analysed, Table 1). However, it was clear that fixation allowed FITC-PNA/PI binding to an extended sperm subpopulation (Table 1) both for control and bicarbonate stimulated samples. The CTC acrosome staining gave lower relative numbers of acrosome intact cells when compared with the FITC-PNA staining of unfixed sperm cells (Table 1). This effect may be attributed to fixation because FITC-PNA staining of paraformaldehyde fixed stallion sperm gave similar results as CTC staining of glutaraldehyde fixed cells (Table 1). Finally, the CTC capacitation staining gave lower relative amounts of capacitated cells when compared with merocyanine-540 staining (Table 1).

Assessing the viability of sperm cells

In this study the sperm cells stained with CTC, FITC-PNA or merocyanine-540 for the assessment of capacitation or acrosome status were counter–stained with EthD-1, PI and Yo-Pro-1, respectively for the simultaneous detection of sperm viability. At the onset of incubation in Tyr+bic, PI and Yo-Pro-1 staining identified approximately 43% of the cells as being dead whereas, EthD-1 staining recorded only 37% of the cells as dead, however, at all other times and for all treatments, the three viability stains gave nearly identical results for the proportions of dead cells. Thus, after 5 h of incubation, the percentage of non-viable cells as recorded by all three staining methods had increased to approximately 55% (the three methods gave non-significant differences), while the addition of Ca^{2+}-ionophore to the Tyr+Bic medium induced even greater cell death such that by 5 h of incubation more than 70% of the cells were non-viable.

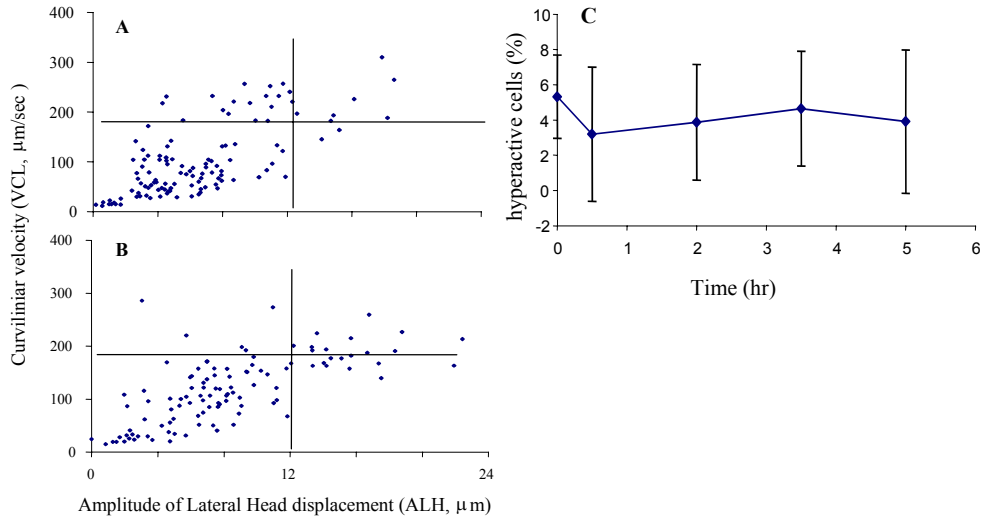

Fig. 5. The scatter plots used to detect hyperactivation of the motile spermatozoa for sperm from one ejaculate incubated for 0 h (A) or 5 h (B) in Tyr+Bic medium without Ca^{2+}-ionophore. The amplitude of lateral head displacement (ALH) is shown on the X-axis and the curvilinear velocity (VCL) is represented on the Y-axis. Spermatozoa with a VCL > 180 µm/s and an ALH > 12µm were considered to be hyperactive. (C) The percentages of spermatozoa incubated in Tyr+bic medium for 5 h that fulfilled the criteria for hyperactivation at various time points during incubation. The values represent the mean percentages (± sd) for six ejaculates (two stallions).

In absence of bicarbonate, however, minimal cell death was observed such that by the end of the 5 h incubation period only 45% of cells were non-viable, irrespective of the presence or absence Ca^{2+}-ionophore. This demonstrated that, in the absence of bicarbonate, sperm cells do not lose their membrane integrity, even in the presence of Ca^{2+}-ionophore. By contrast, when exposed to the capacitating agent bicarbonate, the sperm became vulnerable to membrane disruption and cell death during the 5 h incubation period and these processes were facilitated by Ca^{2+}-ionophore.

Sperm motility and hyperactivation

Sperm motility with specific regard to hyperactivation was analysed for the spermatozoa of two stallions (3 ejaculates each) incubated in Tyr+bic medium. The average motility at 0 h was approximately 60% in all cases and had decreased to around 30% after 5 h of incubation. The addition of Ca^{2+}-ionophore had a negative effect on sperm motility and no spermatozoa were scored as motile after 3.5 h of incubation, despite the fact that

approximately 35% of the cells were at this time point still viable as determined with counter-staining using EthD-1 as supra vital dye.

Hyperactivation was determined objectively by plotting the VCL and ALH of the motile cells on a two-dimensional scatter-graph [22]. Spermatozoa were considered to be hyperactive if their VCL was □180 and ALH □12 (i.e. they were in the upper right quadrant on figs 5 A, B). Using these parameters, 5.3 ± 2.4 % of the motile spermatozoa were considered to be hyperactive and the proportion of hyperactive cells did not change significantly with time (Fig 5 C). However, the presence of Ca^{2+}-ionophore in the incubation medium resulted in the loss of about all hyperactive spermatozoa by 2 h and an almost complete motility loss within 3.5 h of incubation. It was clear then, that at any given time, the percentage of hyperactivated spermatozoa was lower than the percentage of capacitated spermatozoa as detected by either merocyanine-540 or CTC staining.

While assessing sperm motility visually it was observed that hyperactivated sperm (identified visually using Yanagimachi's criteria [1]) showed a biphasic motility pattern. In short, hyperactive sperm tended to make vigorous non-progressive movements, but then become static for a while before moving again with the same vigorous non-progressive movement.

Discussion

Capacitation is a vital phenomenon that a spermatozoon must undergo before it can fertilise an oocyte. However, the lack of a reliable and easy method for assessing sperm capacitation has resulted in a rather incomplete understanding of this important process. At present, the only generally accepted capacitation assay is CTC staining where CTC is a fluorescent antibiotic that binds to the surface of sperm cells in a Ca^{2+} dependent manner [23]. In short, it appears that the CTC-Ca^{2+} complex binds preferentially to hydrophobic regions, such as the cell membrane [23,24], and capacitation induced changes in the sperm cell plasma membrane result in the changes of the CTC labelling pattern that are widely considered to reflect the attainment of the capacitated state [4,5,6,7]. However, the molecular basis of the interaction between CTC, Ca^{2+} and the sperm plasma membrane are far from clear and, moreover, CTC staining is a laborious technique, particularly because it cannot be analysed using a flow cytometer (Table 1). The reason for this flow cytometer incompatibility is that although the distribution of the CTC dye changes clearly (Fig 1), the total amount of CTC staining does not change between non-capacitated and capacitated cells, and it is this absolute change that a FACS machine would require for the differentiation of the cells. Furthermore, assessment of CTC staining implies fixation of cells and it is therefore important that the cells should be labelled with a membrane impermeable DNA stain for the discrimination of live and dead (fluorescent) cells before fixation, since this latter process introduces artefacts such as cellular and/or acrosomal degeneration (Table 1). A further drawback of CTC staining is its Ca^{2+} dependency which renders it useless for detecting

Ca^{2+} independent capacitation changes in sperm cells. This latter point was illustrated clearly in the present study by the fact that the capacitation induced changes detected by merocyanine-540 staining were only fully detectable by CTC staining when Ca^{2+}-ionophore was present (Fig 4). These drawbacks to the CTC technique underline the importance of developing flow cytometric assays to monitor capacitation dependent changes in, for example, membrane fluidity [11], and acrosome status [17,18] in live cells.

Flow cytometric detection of capacitation related changes in membrane architecture, using merocyanine-540 as a reporter probe, and acrosome status, using FITC-PNA staining, have some clear advantages over the all-compassing CTC staining technique. First, given the clear differences in the intensity of fluorescence between control and capacitated or acrosome reacted cells, flow cytometry allows for the very rapid and objective discrimination of the status of large numbers of sperm cells. For example, in the current study we analysed 10,000 sperm cells per data point in only a few seconds. Second, prior to analysis the sperm suspension requires only simultaneous addition of appropriate amounts of PI and FITC-PNA or Yo-Pro-1 and merocyanine-540, followed by a 10 min incubation for the completion of labelling. Third, the cells can be analysed in a flow cytometer in the unfixed state and under relatively physiological conditions (i.e. at 37°C and 5% CO_2). This ability to control the ambient conditions minimises the risk of cell deterioration, especially for the notoriously delicate capacitated sperm cells.

In the current study, we analysed the results in terms of the decrease in the percentage of viable non-capacitated or acrosome-intact spermatozoa, rather than by the increase in the percentage of viable capacitated or acrosome-reacted cells. It was considered that this represented a more realistic approach, because of the greater likelihood that the more delicate capacitated or acrosome-reacted cells would die for reasons other than incubation condition or treatment per se and thus be missed from the analysis.

The results of this study demonstrate clearly that CTC differs significantly to merocyanine-540 in terms of its ability to detect changes in the capacitation-state of spermatozoa incubated in Tyr+bic medium. Merocyanine-540, a marker for increased membrane fluidity, detected a rapid decrease in the number of non-capacitated sperm during the first 0.5 h of incubation, after which numbers tended to plateau. By contrast CTC detected the apparent loss of non-capacitated status much more slowly and a similar level of apparent capacitation was not seen until the end of the 3 h incubation.

It is proposed that these differences relate to the probability that the membrane fluidity related changes detected by merocyanine-540 precede the Ca^{2+} influx on which CTC binding depends. This conclusion is supported by the CTC and merocyanine-540 staining patterns in the presence of Ca^{2+}-ionophore. That is, a more pronounced and rapid decrease in the proportion of live non-capacitated sperm cells as detected by CTC staining but no change detected by merocyanine-540 staining when Ca^{2+}-ionophore was present. In summary, the ability of CTC staining to detect changes in capacitation status equalled that

of merocyanine-540 only when Ca^{2+}-ionophore was included in the incubation medium, otherwise CTC was very slow at detecting changes in sperm membrane status.

It was also interesting that, in the absence of bicarbonate, capacitation whether measured by CTC or merocyanine-540 staining was minimal. In total then, the data presented in this paper indicate that bicarbonate induces a change in the lipid packaging of the plasma-membrane of stallion sperm that can be monitored by merocyanine-540, a marker for lipid 'scrambling' [11]. A further experiment (data not shown), has demonstrated that only a sub-population of the merocyanine-540 responsive cells show the CTC capacitation response and this presumably indicates that the rise in intracellular Ca^{2+} concentration necessary for CTC binding occurs later in sperm capacitation than bicarbonate mediated lipid scrambling. Similar results obtained for two-staining methods when Ca^{2+}-ionophore was included in the incubation medium is presumably because the ionophore allows the merocyanine-540 responsive cells to take up Ca^{2+} in a fashion that allows appropriate CTC binding because the ionophore makes the plasma membrane permeable to Ca^{2+} (2mM).

The other parameter commonly assessed using CTC is acrosome intactness [7]. The AR is a Ca^{2+} dependent process [1] and high intracellular Ca^{2+} is required for fusion of the sperm plasma membrane to the outer acrosome membrane. Thus the AR can proceed only after the intracellular Ca^{2+}levels of capacitated sperm cells have increased and it is only after this secretory event has occurred that FITC-PNA is able to bind to the appropriate epitope on the outer acrosomal membrane [12,18]. Therefore, like CTC, FITC-PNA staining is Ca^{2+} dependent, albeit indirectly and this may explain why AR assessments were similar for the two techniques. Interestingly, a moderate induction of the AR was observed when sperm were incubated in Tyr+bic whereas the AR induction was not observed in the absence of bicarbonate. Addition of Ca^{2+}-ionophore resulted in an even more widespread induction of the AR in Tyr+bic incubated sperm suspensions (not significant for FITC-PNA stained sperm), probably by facilitating the rise in intracellular Ca^{2+} required for this secretory event. Thus it appears that Ca^{2+}-ionophore synergies the effect of bicarbonate regarding the AR induction and, whereas bicarbonate alone is sufficient to induce the AR, Ca^{2+}-ionophore alone is not. It must be presumed that the bicarbonate-mediated increase in plasma membrane fluidity allows a subsequent increase in the permeability of that membrane to Ca^{2+}.

With regard to sperm hyperactivation, it has been reported previously that this phenomenon occurs spontaneously in the majority of spermatozoa incubated under capacitating conditions in a medium that contains bicarbonate [25] and Ca^{2+} [see 16]. The suggestion was that bicarbonate activates adenylate cyclase, either directly or indirectly by causing Ca^{2+} influx (see [16]), and thereby elevates intracellular cAMP concentrations, which in turn induce sperm hyperactivation [26]. For this reason, we expected that in the current experiment the incubation of sperm in a medium containing bicarbonate and Ca^{2+} would lead to sperm hyperactivation, a phenomenon that is characterised by an increase in flagellar bending amplitude, that can be detected in a CASA system by an increase in ALH

and VCL (27,28). However, the results of our study indicated that only a small proportion of the spermatozoa demonstrated characteristics of hyperactivity at any given time and, while it is likely that the percentage of hyperactive spermatozoa was underestimated because of the typical stop-start biphasic motility pattern recorded for hyperactive cells it was clear that hyperactivity is not a suitable parameter for accurately estimating the percentage of capacitated cells.

In conclusion, we have compared several methods for detecting sperm capacitation, acrosome status, sperm viability and particular characteristics of motility, under conditions where either sperm membrane fluidity was affected or the acrosome reaction was induced. The major aim of the study was to relate the CTC staining patterns obtained with fixed sperm cells, and used for simultaneous assessment of capacitation and acrosome reaction status, to the results of novel flow cytometric assays that detect membrane changes in a Ca^{2+} independent manner [10,11,18]. It is concluded that the merocyanine-540 and FITC-PNA assays are preferable to CTC staining because flow cytometric assays are easier to perform, quicker, more objective and more accurate than fluorescence microscope assays (CTC), and, in addition, because the washing and fixation steps that may interfere with apparent cell viability and integrity are not required. Moreover, the mechanisms of the molecular interaction between merocyanine-540/ FITC-PNA and the biomembranes and their relationship to the occurrence of capacitation and the AR are clear, whereas, the same cannot be said for CTC. It is, of course, important to simultaneously detect sperm viability using a membrane impermeable DNA stain to ensure that detected membrane changes are biologically relevant. In this respect, all three stains examined in the current study gave equally satisfactory results.

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Chapter 4

Progesterone induces acrosome reaction in stallion spermatozoa via a protein tyrosine kinase dependent pathway

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In preparation

Abstract

Progesterone (P_4) is a physiological inducer of the acrosome reaction (AR) in stallion spermatozoa. However, the capacitation-dependent changes that enable progesterone binding, and the nature of the signalling cascade that is triggered by progesterone and results in induction of the AR, are poorly understood. The aim of the current study was, therefore, to investigate the protein kinase dependent signalling cascades involved in progesterone-mediated induction of the AR in stallion spermatozoa. In addition, we aimed to determine whether bicarbonate, an inducer of sperm capacitation, acted via the same pathway as P_4 or whether it otherwise synergised P_4-mediated induction of the AR. We examined the effect on AR progression of specific inhibitors and stimulators of protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG) and protein tyrosine kinase (PTK), in the presence or absence of 15mM bicarbonate and/or 1μg/ml progesterone. Progression of the AR was assessed by fluorescence microscopy after staining the acrosomal membranes with Pisum sativum agglutinin conjugated to fluorescein isothyocyanate (FITC-PSA). These experiments demonstrated that bicarbonate specifically activates a PKA-dependent signalling pathway, whereas the effect of P_4 is independent of PKA. Conversely, while P_4-mediated AR induction was dependent on PTK, the effects of bicarbonate were PTK-independent. Finally, although the AR inducing effects of both P_4 and bicarbonate were sensitive to staurosporin, a potent blocker of PKC activity at moderate (50nM) concentrations, the effect of P_4 was completely blocked at 50nM staurosporin whereas that of bicarbonate was only completely inhibited by much higher concentrations (2μM), at which staurosporin acts as a general protein kinase blocker and would, therefore, also have blocked PKA activity. In conclusion, P_4-mediated activation of the AR is dependent on a pathway that includes both PTK and PKC. And, while the effects of bicarbonate on the AR are mediated via a separate PKA-dependent signalling pathway, P_4 and bicarbonate have synergistic effects on the AR.

Introduction

Before they attain the ability to fertilise an ovum, ejaculated mammalian spermatozoa must undergo a process of physiological maturation known as “capacitation” [1]. Normally, capacitation takes place within the female reproductive tract, however very similar changes can be induced *in vitro* by incubating spermatozoa under defined conditions [1]. Capacitation is an obligatory precursor to the acrosome reaction (AR), an exocytotic event that involves fusion of the outer acrosomal membrane to the overlying plasma membrane. In turn, the AR results in the release of hydrolytic enzymes that enable a spermatozoon to partially lyse and thereby penetrate the zona pellucida (ZP; the extracellular matrix of the oocyte) so that it can contact and fertilise the oocyte [1]. Of the several proposed physiological inducers of the AR [2], the most extensively studied are ZP3 (a constituent glycoprotein of the ZP) and progesterone (P_4) which is present in cumulus oophorus cells [3] and the fluid of pre-ovulatory follicles [4. It has also been reported that the bicarbonate

ion is essential for sperm capacitation [5] and that it plays an important role in the preparation of a sperm for the AR [6].

Cheng et al. [7] reported the existence of a sperm plasma membrane P_4-receptor and subsequently we demonstrated that the proportion of a stallion's spermatozoa that have exposed P_4-receptors correlates well with its fertility [8]. However, our understanding of the signal transduction pathway that is initiated by the binding of P_4 to the sperm plasma membrane receptor and terminates in the AR, is far from complete. We do, however, know that numerous plasma membrane proteins are phosphorylated during the AR [9] and it has therefore been suggested that regulatory kinases such as protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG) and protein tyrosine kinase (PTK), are involved in the P_4-AR signal transduction pathway. Similarly, bicarbonate has been shown to act via a PKA-dependent pathway to induce the rapid changes in the lipid architecture of the sperm plasma membrane that probably represent the onset of capacitation [10] and that have been reported to play an important role in the AR in man [11]. In addition, both PKC [12,13] and an activated trans-membrane PTK [14,15,16] have been proposed to play roles in P_4-mediated induction of the AR.

The aim of the current study was to further categorise the P_4-initiated signal transduction pathway that leads to the AR in stallion sperm. In addition, we aimed to determine whether P_4 and bicarbonate exert their effects on capacitation and the AR via common pathways or independently/synergistically.

Materials and Methods

Materials

Progesterone (4-Pregnene-3,20-dione), 8Br-cAMP (8-bromoadenosine 3□:5□-cyclicmonophosphate), 8Br-cGMP (8-bromoguanosine 3□:5□-cyclicmonophosphate), Staurosporin, Bovine serum albumin (BSA), and DDA (2□,3□-dideoxy adenosine) were bought from Sigma (St. Louis, MO, USA). H89 (N-[2-(p-bromocinnamyl) ethyl]-5-isoquinoline-sulfonamide) was obtained through Omnilabo (Breda, The Netherlands). Erbstatin analogue (methyl 2,5-dihydroxycinnamate) designated as Erbstatin was bought from ICN Biomedicals (Ohio, USA). FITC-PSA (*Pisum sativum* agglutinin conjugated to fluorescein isothyocyanate) was bought from EY-Laboratories (San Mateo CA, USA) and Ethidium homodimer (EthD-1) from Molecular probes Inc (Eugene, OR, USA). The water used in all the experiments was purified using Milli-Q Ultrapure Water Systems from Millipore Corp (Bedford, MA, USA). Progesterone, H89, Erbstatin, Staurosporin and EthD-1 were dissolved in DMSO and further diluted in the base medium (described in sub-section: Medium) to the required concentration (final conc. of DMSO< 0.1%). 8Br-cAMP, and DDA were dissolved in water. FITC-PSA was diluted in water.

Medium

Modified Tyrode's medium [17] was used in the experiments. The stock solution of the medium, consisting of 96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 20 mM Hepes, 5 mM glucose and 21.7 mM sodium lactate was prepared in water, filtered through 0.2 µm filter and stored a 4°C. Sodium pyruvate (final conc.: 1mM) was added 14-16 hr prior to the experiment and the medium was then maintained at 38°C till the time of the experiment. The pH and osmolality of the medium were maintained at 7.4 and around 300 mOsm/kg, respectively. This served as the base medium and was used for washing. BSA (7mg/ml) was added to the base medium just before the experiment and this served as the incubation medium sans bicarbonate (Tyr). All incubations were initiated in this medium, and when bicarbonate was needed in the medium it was added later (final conc.: 15mM) by adding 20µl of 750-mM sodium bicarbonate freshly prepared in Milli Q water just before the experiment. This served as the bicarbonate-containing medium (Tyr+bic).

Semen handling and preparation

Fresh semen (2-4 ml) was obtained from 5 fertile Dutch Warm Blood horses (aged between 4-10yrs) during the breeding season. Immediately after collection the semen was diluted (1:1 conc.) in the base medium, and washed by centrifugation at 900g for 10 min. The supernatant was removed and the pellet resuspended in the same medium (final volume equal to the original volume of the semen) and kept in the incubator (38°C) for 15-20 min to allow swim-up of motile sperm. Thereafter, the top layer (1ml) of the sample, containing motile spermatozoa, was removed and placed in a 15-ml conical tube. The sample was further diluted with Tyr-medium to a sperm concentration of approximately 25×10^6 per ml.

Effect of PKA stimulators/inhibitors on AR

One ejaculate each from 5 stallions was analysed. Per ejaculate, two batches of four samples (1ml) in medium Tyr were prepared. From each batch one served as control, and to the remaining three 50 µl of H89 (a specific PKA inhibitor; final conc.: 50 nM)[10], DDA (a specific adenylate cyclase inhibitor; final conc.: 100 µM) [18], and 8Br-cAMP (a potent PKA stimulator; final conc.: 100 µM) [10], were added, respectively. The samples were incubated at 38°C. After 5 min of preincubation 20µl bicarbonate solution was added to one batch of samples (Tyr+bic), the other batch thus did not have bicarbonate (Tyr). Samples were further incubated at 38°C for 5 h in humidified air containing 5% CO_2. At the end of the incubation period 500µl of each sample was transferred into a corresponding tube containing 5µl P_4 (final conc.: 1µg/ml)[11]. All the sixteen samples per ejaculate (thus the 8 samples with P_4 and the 8 without) were further incubated for 20 min before the AR was assessed.

Effect of cGMP stimulator on AR

One ejaculate each from three stallions was analysed. Per ejaculate, three samples (1ml) in medium Tyr were prepared of which one served as control, and to the remaining two 50µl of 8Br-cAMP (final conc.: 100 µM) and 50 µl of 8Br-cGMP (a specific cGMP stimulator; final conc.: 100 µM) [10] were added, respectively. The samples were subsequently incubated at 38°C for 5 h. After incubation, 500µl of each sample that contained either 8Br-cAMP or 8Br-cGMP was transferred into a corresponding tube containing 5µl P_4 (final conc.: 1µg/ml). All five samples per ejaculate were further incubated for 20 min before the AR was assessed.

Effect of PTK and PKC inhibitors on AR

One ejaculate each from 5 stallions was analysed. Per ejaculate 5 semen samples (1ml) in medium Tyr were prepared. One sample served as control, and to the three out of remaining four, 50µl of Erbstatin (a PTK-inhibitor; final conc.: 10µM)[18], Staurosporin (final conc.: 50nM; at this conc. a potent inhibitor of PKC [19], and Staurosporin (final conc.: 2µM; at this conc.: inhibiting most serine/threonine kinases) [20] were added, respectively. These samples were pre-incubated for 5 min and subsequently 20µl bicarbonate solution was added to the four tubes other than the control. All the samples were further incubated at 38°C for 5 h in humidified air containing 5% CO_2. After the incubation period 500µl of the sample (except from the control) was transferred into a corresponding tube containing 5 µl P_4. The nine samples were further incubated for 20 min before the AR was assessed.

Acrosome reaction evaluation

From each sample 50 µl was transferred to a corresponding tube containing 50µl EthD-1 (final conc.: 2µM), and kept at room temperature in the dark for 3 min. Subsequently, the sample was diluted with 400µl PBS and centrifuged at 600g for 3 min to remove excess EthD-1. The supernatant was removed and the pellet resuspended in 400 µl PBS. The washing process was repeated twice. Then the pellet was suspended in 100 µl ethanol (96%) to fix and permeabilise the spermatozoa, and kept in the dark for 10 min. Thereafter, 10µl was transferred to a glass slide and was dried. Then 50 µl of FITC-PSA (100 µg/ml in PBS) was over-layered over the dried area and the slide kept in dark for 30 min. The slides were then washed with PBS to remove the excess FITC-PSA and kept in dark until evaluation, the following day. Just before evaluation a 5 µl drop of glycerol was put over the slides and covered with a cover-slip. The spermatozoa showing bright fluorescence over the head were considered as acrosome intact and those with dark head were considered to be acrosome reacted [21]. The red stained (EthD-1) spermatozoa were considered as dead and the unstained ones as alive [4]. In total 200 sperm cells per slide were evaluated.

Statistics and data presentation

The data was analysed with a presumption of normality. The statistical test used was a paired *t*-test, with significance at $P < 0.05$. The data are expressed in graphs as the mean ± standard deviation of the increase in the percentage of acrosome reacted spermatozoa relative to the control, which in all cases was the percentage of acrosome reacted spermatozoa in medium Tyr following an incubation period of 5h + 20 min.

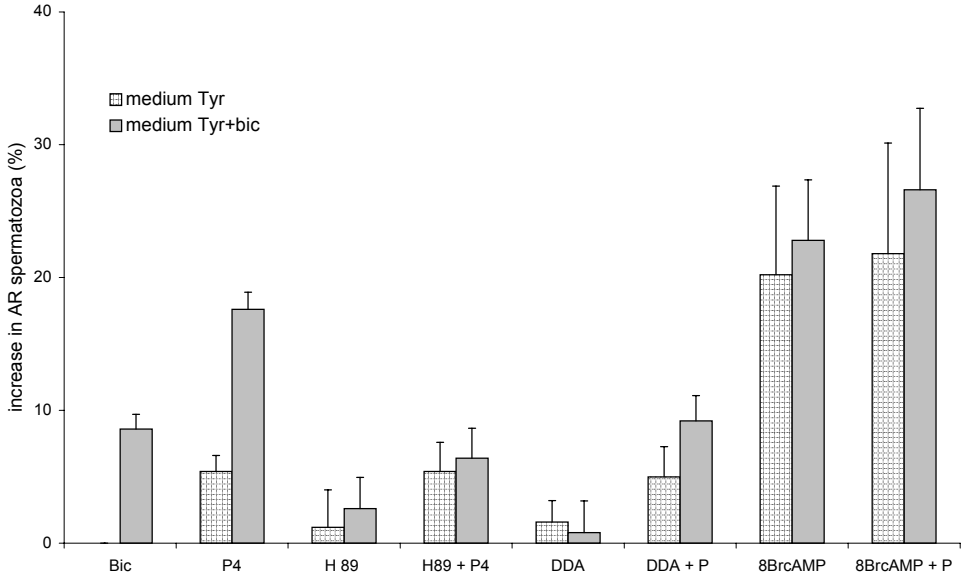

Fig. 1. Effect of H89 (a PKA-inhibitor), DDA (an adenylate cyclase inhibitor) and 8Br-cAMP (a cAMP analogue) on progesterone (P_4) induced acrosome reaction in spermatozoa incubated in the medium with bicarbonate (Tyr+bic) or without bicarbonate (Tyr). The percentage of acrosome-reacted cells in medium Tyr without any additives (36±4%) served as control, and the relative increase in percentage of acrosome-reacted sperm cells is depicted. Results are expressed as means ± SD, n = 5.

Results

P_4 induces AR in a PKA independent manner

After incubation in medium Tyr, 36 ± 4 % of the sperm cells underwent AR. In Tyr+bic medium the percentage of acrosome reacted cells increased significantly by 9% (Fig 1). Addition of P_4 in medium Tyr led to an increase by 5%, and in Tyr+bic the acrosome

reacted cells increased by 18% ($P < 0.05$). The effect of bicarbonate is clearly visible and attributed to the bicarbonate binding to soluble adenylate cyclase (sAC) [22] which switches on the PKA mediated protein phosphorylation. In presence of blockers of the PKA-pathway (H89 and DDA) in Tyr+bic medium a non-significant increase in percentage of acrosome reacted cells was seen (Fig 1), suggesting a complete inhibition of the stimulatory effect of bicarbonate. As expected, presence of these blockers had no significant effect in the Tyr medium (Fig 1). Addition of P_4 to either Tyr or Tyr+bic medium containing H89 or DDA led to a significant increase in percentage of acrosome reacted cells (Fig 1), suggesting that PKA-pathway blockers did not block the P_4 effect on spermatozoa. This effect of P_4 was, however, not as intense as in case of absence of the PKA-pathway blockers in the medium, further suggesting the inhibition of bicarbonate effect. Presence of 8Br- cAMP, in Tyr or in Tyr+bic medium, significantly increased the percentage of acrosome-reacted spermatozoa by around 20% (Fig 1).

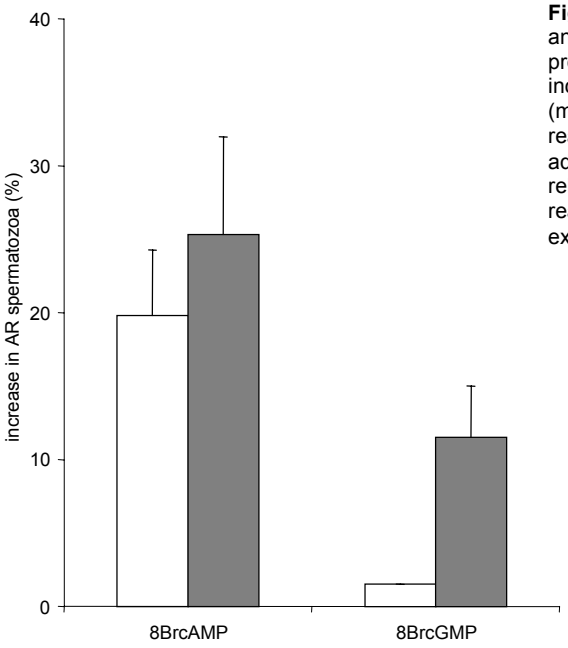

Fig. 2. Effect of 8Br-cAMP (a cAMP analogue) and 8Br-cGMP (a cGMP analogue) on progesterone (P_4) induced acrosome reaction incubated in medium devoid of bicarbonate (medium Tyr). The percentage of acrosome-reacted cells in medium Tyr without any additives (36±4%) served as control, and the relative increase in percentage of acrosome-reacted sperm cells is depicted. Results are expressed as means ± SD, n= 3.

Addition of P_4 to the medium with 8Br-cAMP (both Tyr and Tyr+bic) led to a slight but non-significant increase in the percentage of acrosome reacted cells. Taken together these results showed that the P_4 mediated AR is not dependent on the PKA-pathway, and is independent of the bicarbonate effect that in fact uses the PKA-pathway.

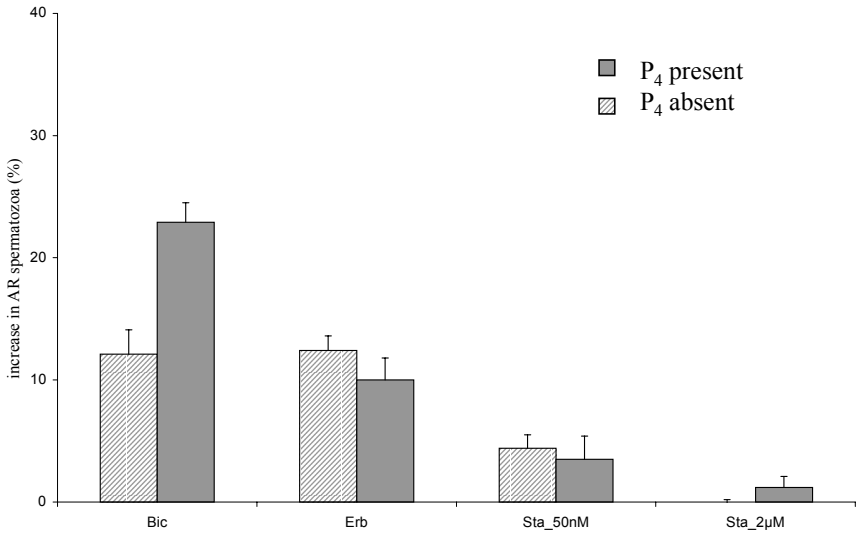

Fig. 3. Effect of erbstatin (Erb), a PTK-inhibitor and staurosporin on progesterone (P_4; shaded bars) induced acrosome reaction in spermatozoa incubated in medium containing bicarbonate (medium Tyr+bic). Staurosporin behaves as a potent PKC-inhibitor at a concentration of 50nM (Sta_50nM), and as a general protein kinase inhibitor at a concentration of 2µM (Sta_2µM). The percentage of acrosome-reacted cells in medium Tyr without any additives (39±5%) served as control, and the relative increase in percentage of acrosome-reacted sperm cells is depicted. Results are expressed as means ± SD, n = 5

Bicarbonate effect on AR is mediated via cAMP-dependent pathway

To further specify the cyclic nucleotide specificity of the bicarbonate-mediated AR the stimulatory effects of 8Br-cAMP and 8Br-cGMP were tested in absence of bicarbonate (Tyr medium). The percentage of acrosome reacted cells in the control medium (Tyr) was 36 ± 4 %. In presence of 8Br-cAMP in the medium a significant increase in the percentage of acrosome-reacted cells occurred (20%). Addition of P_4 to the medium containing 8Br-cAMP led to a further increase in percentage of acrosome-reacted cells (Fig 2). In contrast, 8Br-cGMP in the medium did not result in an increase in the percentage of acrosome-reacted cells (1.5%), and the addition of P_4 to this medium led to a significant increase by 11.5%. This indicates that the induction of AR is in part PKA-specific (pathway used by bicarbonate) and that P_4 induces, on top of this a cAMP/cGMP-independent AR.

Involvement of other kinases in P_4-induced AR

The results above indicated that P_4 elicits its effect independently from PKA. Therefore the involvement of PKC and PTK was tested, by incubating spermatozoa in presence and

absence of specific PK inhibitors in Tyr+bic medium. The control sample (medium Tyr) resulted in 39 ± 5% of acrosome reacted cells, and the presence of bicarbonate (Tyr+bic medium) increased this percentage significantly by 12%. Addition of P_4 to the medium resulted in a total increase of 23%. In presence of Erbstatin, the increase in percentage of acrosome-reacted spermatozoa was not different from that in the Tyr+bic medium, i.e. 12%, and on addition of P_4 to this medium, no further increase was observed (Fig 3). These results suggest that P_4 effect was blocked by erbstatin but the bicarbonate effect was not.

Low concentrations of staurosporin (50 nM) block PKC. These concentrations of staurosporin significantly reduced the increase in the percentage of acrosome-reacted cells in Tyr+bic medium (4% vs 12%, Fig 3), showing that bicarbonate effect was partially blocked by staurosporin at 50 nM conc. The addition of P_4, however, did not lead to any further increase in percentage of acrosome-reacted cells (Fig 3) indicating that P_4 response was staurosporin sensitive. This implies that P_4 induces a PKC-dependent AR. In presence of staurosporin at a higher concentration (final conc.: 2µM, i.e. when all the kinases are blocked), almost no increase in percentage of acrosome-reacted cells was seen, irrespective of addition of P_4 (Fig 3). This is indicative of the involvement of protein kinases in the bicarbonate as well as in the P_4-mediated AR.

Discussion

During capacitation of a mammalian sperm, changes take place in the sperm plasma membrane that subsequently enable that spermatozoon to bind specifically to the zona pellucida and then to acrosome react in response to the ZP binding stimulus [1]. Bicarbonate/CO_2 plays a major role in sperm capacitation *in vitro* [5] because it induces alterations in the sperm surface coatings and the lipid architecture of the apical plasma membrane [9], and because it increases the binding affinity of a sperm for the ZP [23]. Not only is the ZP an intrinsic AR inducer, but it is also impregnated with P_4, a hormone that is believed to contibute to AR induction *in vivo* [3,4] and that appears to act via a specific sperm surface receptor [7]. Although the activation of a number of protein kinases has been proposed to be instrumental to AR induction [2], there is little information about how these kinases contribute to the intracellular signalling cascades induced by P_4 or bicarbonate/CO_2. In the present study, we used a panel of drugs that either stimulate or inhibit the activity of the various types of protein kinase to determine which are activated during the induction of capacitation or the AR *in vitro*.

In the presence of bicarbonate, a significant proportion of incubated sperm acrosome-reacted. Furthermore, because this bicarbonate induced AR was blocked completely by the PKA inhibitors DDA and H89, PKA must be involved in bicarbonate mediated induction of the AR in stallion sperm. Further proof that PKA is involved in at least the bicarbonate-stimulated pathway leading to the AR was provided by the finding that, in the absence of

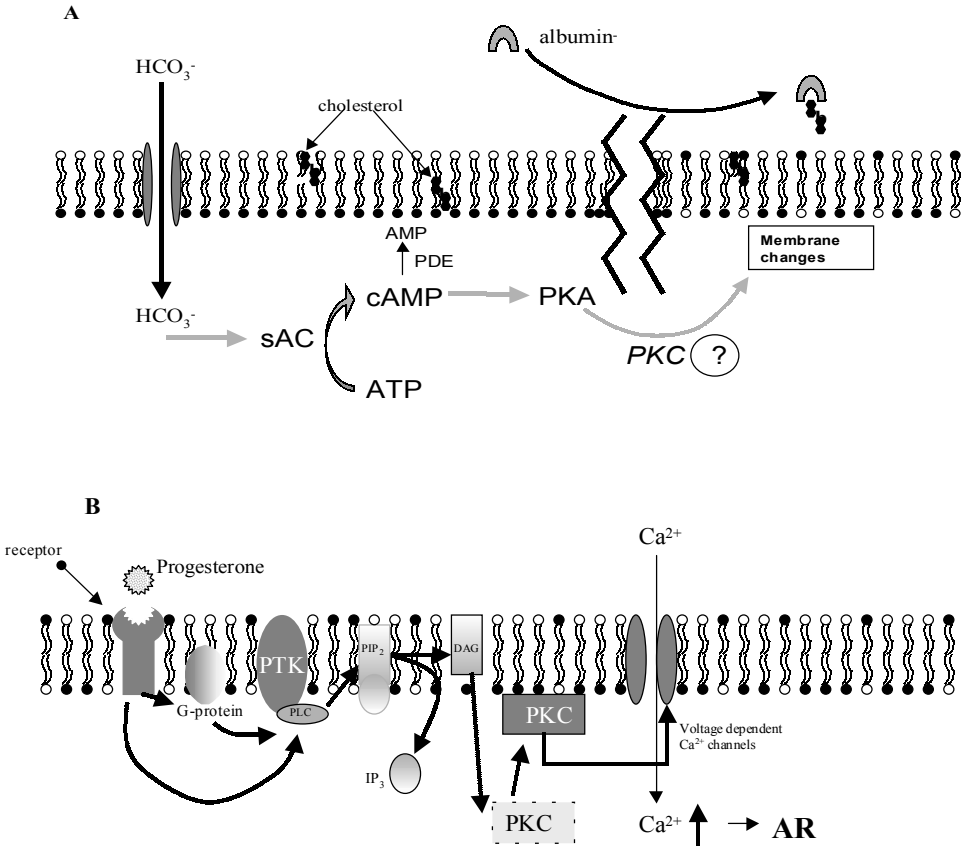

Fig. 4. Proposed signalling pathways involved in bicarbonate and progesterone induced sperm arosome reaction. (A) Bicarbonate, when present in high concentrations (~15mM) in the extracellular medium, enters the sperm cell by a bicarbonate-chloride exchanger and activates a soluble adenylate cyclase (sAC), which triggers production of cAMP and activates protein kinase A (PKA). Activation of PKA leads to plasma membrane changes leading to increased membrane fluidity and cholesterol efflux, provided a cholesterol acceptor is present in the medium. PKA also stimulates protein kinase C (PKC) which then leads to acrosomal exocytosis. The levels of cAMP are regulated also by phosphodiesterases (PDE) which convert it into AMP, thus reducing its level. (B) Progesterone acts on a plasma membrane bound putative receptor, which is exposed during capacitation. It activates a protein tyrosine kinase receptor coupled to phosoholipase C (PLC) directly or via a G protein. The activated PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG then activates the PKC and translocates it to the plasma membrane. PKC opens the voltage-dependent Ca^{2+} channels in the plasma membrane, leading to influx of extracellular Ca^{2+}. The increase in intracellular Ca^{2+} leads to acrosomal exocytosis.

bicarbonate, the AR could be induced using 8BrcAMP, a PKA stimulator, but not 8BrcGMP, an activator of related cyclic nucleotide dependent protein kinases (e.g. protein kinase G). It was also noted that 8BrcAMP induced the AR much more potently than bicarbonate which suggests that PKA was not fully stimulated by bicarbonate in the assay conditions we used. Furthermore, we were able to demonstrate that the other protein kinases tested (PKC and PTK) are not involved in the bicarbonate-mediated AR induction in stallion sperm, because: (i) erbstatin (a potent PTK inhibitor) did not inhibit bicarbonate stimulated AR. This is in line with the observation of Breitbart and Naor [2], that induction of the AR in bovine sperm using cAMP analogues (i.e. mimicking PKA stimulation) could not be suppressed by genistein, another PTK blocker. (ii) the bicarbonate induced AR was completely inhibited by high concentrations of staurosporin (i.e. at micromolar levels, when most PKs would be inhibited) [20] but only marginally affected by low staurosporin concentrations (i.e. at nanomolar levels, when staurosproin would inhibit mainly PKC) [19]. Flesch and Gadella [9] reported that, for boar sperm, enhanced tyrosine phosphorylation plays a role in the bicarbonate-activated AR down-stream to PKA. This contrasts with our current finding that tyrosine phosphorylation is not involved in bicarbonate induction of the AR in stallion sperm. The intracellular signalling pathway upon which we hypothesise that bicarbonate acts when initiating/inducing the AR is summarised in Fig 4A.

Unexpectedly, P_4 stimulated the AR irrespective of the presence or absence of bicarbonate in the incubation medium. Furthermore, bicarbonate and P_4 together induced the AR in a significantly greater proportion of stallion sperm than bicarbonate alone. These observations indicate that P_4 can induce the AR independently of bicarbonate and presumably via an alternative signalling pathway. Further proof that P_4 exerts its AR inducing effects via a different intracellular signalling pathway to bicarbonate was provided by the failure of the PKA-blockers, DDA and H89, to inhibit P_4-mediated induction of the AR. By contrast, Sabeur and Meizel [24] found that, for human sperm, induction of the AR by P_4 was low in the absence, but high in the presence, of bicarbonate. The likelihood that this effect acts via the PKA pathway was increased by their parallel finding that the role of bicarbonate (in allowing P_4 induced AR) could be substituted by cAMP analogues. More directly, Harrison et al. [11] showed that PKA inhibitors could block the effects of P_4 on the AR in human sperm. The major difference in our findings with horse sperm is thus that in this species P_4 does not appear to depend on bicarbonate or the PKA pathway for its induction of the AR. The third line of evidence for our hypothesis that P_4 induces the AR in stallion sperm independently of bicarbonate was that the effect of P_4, unlike that of bicarbonate, was suppressed by inhibitors of both the PTK and the PKC pathways. In particular, erbstatin (the PTK blocker) completely inhibited the P_4 induced AR. These findings with regard to the pathways stimulated by P_4 are consistent with the observations of Tesarik et al. [14] that P_4 induces an increase in tyrosone phosphorylation that can be inhibited by genistein and that the drug will inhibit P_4 induced AR *per se*. In contrast to the bicarbonate-induced AR, the P_4-induced AR was extremely sensitive to the effects of staurosporin, which at low concentrations is a specific PKC blocker. This suggests that the

binding of P_4 to receptors on the sperm membrane activates PKC in order to induce the AR in stallion sperm. It is also possible that the way in which P_4 induces the AR is similar to that employed by the ZP. In the latter instance, Breitbart and Naor [2] proposed that ZP3 activates a sperm PTK that is coupled to phospholipase C (PLC) which, in turn, stimulates a PKC by generating diacylglycerol (DAG) from phosphoinositol-bisphosphoate (PIP_2). Based on these amalgamated findings, we propose that the intracellular signalling pathway by which P_4 induces the AR in stallion sperm is as depicted in Fig 4B.

In summary, the results of this study demonstrate that, in stallion sperm, P_4 and bicarbonate induce the AR in an independent but partially additive manner. Bicarbonate directly activates sAC and thereby induces the AR via a PKA dependent pathway [22]. Moreover, PKA probably acts quite early in the pathway leading to the AR, or in more than one place, since it appears to be responsible for inducing capacitation-like changes in the plasma membrane of stallion sperm such as phospholipid scrambling [6] and subsequent cholesterol efflux both in boar and stallion sperm [23,25]. These changes probably help to enable the AR by making the plasma membrane more fusogenic [9] and, possibly, by allowing an influx of extracellular Ca^{2+} to stimulate the AR. In addition, bicarbonate induces the loss of lectin binding components from the glycocalyx of sperm cells [26] another capacitation-type phenomenon that may lead to the exposure of functional P_4-receptors. Importantly, we showed that in stallion sperm, induction of the AR by P_4 is additional and independent of the effects of bicarbonate. P_4 binding leads to the activation of both PTK and PKC, where the latter is likely to result from PTK activation. As yet, it is not clear whether ligand binding causes the P_4-receptor to become a PTK or whether the P_4-receptor complex recruits and activates a separate protein PTK. Answering this question will depend largely on a fuller investigation of the structure and mode of action of the sperm surface P_4 receptor.

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Chapter 5

Exposure of progesterone receptors on the plasma membranes of stallion spermatozoa as a parameter for prediction of fertility

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Journal of Reproduction and Fertility 56, 87-91 (2000)

Abstract

Subfertility in stallions is attributed to the inability of spermatozoa to undergo the acrosome reaction in response to progesterone. In the present study, it was assessed whether there is a correlation between stallion fertility, defined on the basis of first cycle foaling rate and first cycle 'non return rate', and the proportion of spermatozoa with exposed progesterone receptors on their plasma membranes. Semen from Dutch Warmblood (n=10) and Friesian (n=4) stallions was analysed. Progesterone 3-(o-carboxymethyl) oxime-BSA coupled with fluorescein isothiocyanate was used as a progesterone receptor probe and ethidium homodimer was used as a supravital stain. A high correlation was observed between the proportion of spermatozoa with exposed progesterone receptors and stallion fertility (r >0.70; p< 0.01). This result indicates that exposure of progesterone receptors is a potential parameter for predicting stallion fertility.

Introduction

Methods for predicting stallion fertility on the basis of semen characteristics require improvement. Conventional semen parameters such as ejaculate volume, sperm concentration, sperm morphology and sperm motility are moderately related to stallion fertility [1,2]. Although, foaling data are the ultimate proofs of fertility of a stallion [3], such data are only available by the end of the next breeding season and so other parameters, such as pregnancy rate per service, pregnancy rate per oestrous cycle and 'non-return-rate', which all provide insights into the fertility performance at an earlier stage, are also used. Nevertheless, it has to be considered that, apart from the stallion factors, all these parameters are also influenced by mare-related and management factors [4].

Several studies have investigated the relationship between male fertility and the capacity of the spermatozoa to undergo the acrosome reaction (AR) [5,6,7,8,9]. The sperm AR is an exocytotic event that is obligatory for mammalian fertilisation. It facilitates penetration of the zona pellucida (ZP) by the spermatozoon and subsequent fusion of the sperm plasma membrane with the oocyte plasma membrane. The ZP is considered to be the prime inducer of AR. However, only a low incidence of AR has been demonstrated in ZP-bound stallion spermatozoa *in vitro* [10,11,12]. Progesterone (P_4) in follicular fluid and secreted by cumulus oophori is considered to be the major inducer of the AR for stallion spermatozoa [9,13]. Moreover, the inability of stallion spermatozoa to undergo the AR in response to P_4 correlates with subfertility in the stallion [9] and Cheng et al. [14] have demonstrated that there are progesterone receptors [PRs] on the plasma membrane of stallion spermatozoa.

In the present study, the relationship between stallion fertility and the percentage of spermatozoa with exposed PRs on the plasma membrane was evaluated.

Materials and Methods

Stallions

Semen samples from Dutch Warmblood (n=10) and Friesian (n=4) stallions aged 4 - 25 years from three stud farms, were used to assess the presence of PRs on spermatozoa. Each stallion had been used to inseminate at least 29 mares per season. Each stud farm included in the study inseminated at least 100 mares in the breeding season.

Semen

Ejaculates were collected from the stallions during June and July 1997, using an artificial vagina (Hannover AV; Minitueb, Tiefenbach). After collection, each semen sample was filtered, mixed with an equal volume of an egg yolk or skimmed milk extender [15,16] and centrifuged at 900 g for 10 min, at ambient temperature. The supernatant was decanted and the sperm pellet resuspended with the same volume of extender. The centrifuged extended semen was transported to the laboratory at 4^0C, where it was processed within 3-4 h after collection of the ejaculate. One ejaculate from each stallion was analysed and to evaluate the influence of time on exposure of PR, four ejaculates collected on consecutive days were analysed from each of two additional stallions.

Stallion fertility

The fertility of the stallions was calculated on the basis of first cycle foaling rate and first cycle 'non-return rate' [17]. Each mare was considered to be undergoing her first cycle when she was detected as in oestrus and was inseminated for the first time. The percentage of mares that did not show signs of oestrus 10-28 days after the last insemination of the first cycle was defined as the 28 day 'non-return rate'.

Foaling rate data and 'non-return rate' data were available from nine stallions during the 1995 breeding season (FR_{95} ; NRR_{95}, respectively) when a total of 1004 mares were inseminated. Each stallion inseminated 40 to 291 mares. In the 1997 season, the 'non-return rate' was calculated for all 14 stallions examined (NRR_{97}). A total of 1480 mares were inseminated in this season and each stallion inseminated 29-190 mares.

Pre-incubation of spermatozoa and treatment with P_4

Spermatozoa were pre-incubated in modified Tyrode's medium [18] for 5 h and treated with progesterone 3-(o-carboxymethyl) oxime-BSA coupled with fluorescein-isothiocyanate conjugate (P-BSA-FITC; Sigma Chemical Co, St Louis, MO) as the PR probe and ethidium homodimer (EthD-1; Molecular Probes, Oregon) as the supra vital stain, as described by Cheng et al. [14], with the following minor modifications. After pre-incubation the spermatozoa were washed by transferring 500 µl of the sperm suspension to

an eppendorf tube and centrifuging it at 600g for 2 min. The supernatant was decanted and the sperm pellet resuspended in 500 µl modified Tyrode's medium and centrifuged again at 600g for 2 min. After decanting the supernatant, the sperm pellet was suspended in 150 µl modified Tyrode's medium to give a total volume of 160 µl. Subsequently, 20 µl of P-BSA-FITC conjugate solution (1mg/ml in PBS) and 20µl of EthD-1 (2µl/ml in PBS) were added to the suspension, resulting in final concentrations of 10 µM P_4 and 2µM EthD-1. The suspension was incubated for 3 min at 37°C and 10 µl sodium salt DNA (1mg/ml in PBS; Sigma Chemical Co) was added to compete for the unbound EthD-1, and incubation was continued for a further 2 min. PBS (200µl) containing 4% (v/v) paraformaldehyde (E. Merck, Darmstadt) and 1% (v/v) glutaraldehyde (E. Merck) was added to fix the spermatozoa. The fixed spermatozoa were washed twice with 1 ml PBS with centrifugation at 600g for 5 min. Spermatozoa (10 µl) and 5 µl antifade (Molecular Probes Inc, Eugene) were mixed on a glass slide and covered with a coverslip. The slides were examined using an epifluorescence microscope.

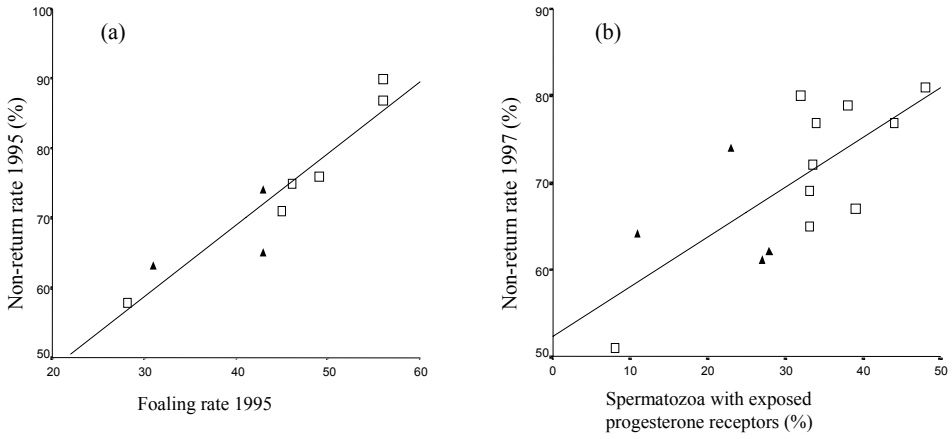

Fig. 1. (a) Scatter-plot of the fertility parameters foaling rate (%) and 'non-return rate' (%) in Warmblood (□; n=6) and Friesian (▲; n=3) stallions during the 1995 breeding season. (b) Scatter-plot of the proportion of stallion spermatozoa with exposed PRs(%) and the 'non-return rate' (%) in Warmblood (□; n=10) and Friesian (▲; n=4) stallions during the 1997 breeding season.

Statistical analysis

The association between the semen parameters of the single ejaculate and the fertility of the stallin was assessed by Pearson's rho *(r)* correlation coefficient, after the normality of the data was checked using a Kolmogorov-Smirnov test. All the statistical analyses were carried out using the SPSS statistical package [19].

Results

Different staining patterns were observed when the stallion spermatozoa were treated with P-BSA-FITC. Spermatozoa with a brightly stained acrosomal region of the head were considered to have exposed PRs, whereas those that stained faintly or not at all were considered to be either to have not exposed their PRs or not to possess PRs.

The percentage of spermatozoa with exposed PRs was 8-48 % (Fig 1). The FR_{95} value for the nine stallions surveyed in 1995 ranged from 28 to 56%, whereas the NRR_{95} and NRR_{97} values were 58-90% and 51-81 %, respectively (Fig 1a,b). The percentages of spermatozoa with exposed PRs were highly correlated with the FR_{95} ($r = 0.84$, $p < 0.005$), NRR_{95} ($r = 0.89$, $p < 0.005$) and the NRR_{97} ($r = 0.73$, $p < 0.005$) values (Fig 1b). Furthermore, there was a high correlation between the FR_{95} and NRR_{95} ($r = 0.94$, $p < 0.005$) (Fig 1a). The results of the experiment performed on the semen of two stallions to assess the repeatability of the measurement of the percentage of spermatozoa with exposed PRs over time is shown (Table 1).

Table 1. Percentages of spermatozoa with exposed PRs in four ejaculates collected on consecutive days from each of two stallions

	Proportion of stained spermatozoa (%)	
Ejaculate number	Stallion 1	Stallion 2
1	42.5	60.5
2	44.5	51.0
3	34.0	60.0
4	41.5	62.0
Mean proportion of stained spermatozoa	40.63	58.38
Standard deviation	4.59	4.99

Discussion

Cheng et al. [13] demonstrated that P_4 secreted by cumulus oophorus cells and present in follicular fluid enhances binding of spermatozoa to the ZP and induces the AR. Fazeli et al. [20] demonstrated that in horses there is a positive relationship between the number of spermatozoa that bind to the ZP *in vitro* and the fertility of the stallion. Tesarik and

Mendoza [21] proposed that sub-fertility in men might be attributable to a lack of PRs on spermatozoa. In the present study, it is demonstrated that there is a high positive correlation between the overall fertility of the stallions throughout a breeding season and the number of spermatozoa in a single ejaculate with PRs exposed on the plasma membranes. This result indicates that the higher the proportion of spermatozoa with exposed PRs after capacitation, the higher the proportion of spermatozoa that can bind to the ZP and, hence, the higher the fertility of the stallion. These findings support the hypothesis that exposure of the PR coincides with exposure of the receptors on the spermatozoa that are responsible for binding to the ZP [13]. However, it is also possible that binding of P_4 to the receptors on the plasma membrane triggers a physiological mechanism that results in exposure of other receptors leading to binding of spermatozoa to the ZP.

Reliable prediction of the fertility of a stallion is only possible if the stallion is used to inseminate a large number of mares during the breeding season [2]. In the present study, effects due to mares were reduced by only considering stallions that inseminated > 29 mares in one season. The theoretical mean number of mares inseminated per stallion was 110, but the actual number ranged from 29 to 291. The results of only first cycle inseminations were considered as this was thought to minimise any management effects on the mare, as her management would probably be adapted in subsequent cycles. Different laboratories use different definitions of stallion fertility and all definitions have drawbacks. In the present study, foaling rate and 'non return rate' [17] were used to define stallion fertility for the 1995 breeding season, but only 'non-return rate' was used for the 1997 season as foaling data were unavailable. A high correlation was observed between the first cycle 'non-return rate' and first cycle foaling rate for the nine stallions in 1995, which indicates that 'non-return rate' within 28 days is a good indicator of foaling rate. There was a high correlation between fertility in 1995 (FR_{95} and NRR_{95}) and the percentage of spermatozoa with exposed PRs measured in 1997. The stallions surveyed in the present study were resident on commercial breeding farms, which meant that only one ejaculate per stallion could be analysed. The data obtained by studying four ejaculates each of two stallions indicate that the percentage of spermatozoa with exposed PRs remained relatively constant over time, but it must be investigated whether the rate of exposure of PRs varies with season or between years.

In conclusion, the results of this study indicate that staining spermatozoa to determine the percentage of sperm cells with exposed PRs may be an effective method for predicting stallion fertility.

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Chapter 6

Summarising discussion

R. Rathi,

Male fertility requires completion of a sequence of elaborate processes including not only spermatogenesis, sperm transport, accessory gland secretion and sperm deposition in the female reproductive tract, but also two very important sperm related events, i.e. capacitation and acrosome reaction (AR). In absence of either no fertilisation in vivo can take place. The site of capacitation in vivo is the oviduct or the uterus, depending on the species [1], and that of the acrosome reaction is the zona pellucida (ZP)[2]. A ZP glycoprotein, ZP3 and progesterone (P_4) are the two most studied physiological inducers of acrosome reaction. Since the report of the presence of P_4 binding sperm proteins, conveniently termed progesterone receptors (PR), on the plasma membrane [3], P_4 induced sperm acrosome reaction has attracted a great deal of attention.

The work presented in this thesis concerns the membrane changes taking place in stallion spermatozoa during capacitation and acrosome reaction, and the signal transduction mechanism leading to the latter in response to P_4 stimulation. Novel flow cytometry-based techniques for evaluating capacitation and acrosome reaction have been described, and a new parameter to predict male fertility, based on exposure of P_4 receptor, has been proposed.

Membrane changes during capacitation

When inside the female reproductive tract, or in a defined medium *in vitro*, the spermatozoa undergo capacitation, and the plasma membrane, which is directly exposed to the capacitating environment, undergoes significant changes [1]. The sperm plasma membrane, like any other biological membrane, is a bilayer chiefly made up of lipids and proteins (Fig 1). A carbohydrate-rich coat, called glycocalyx, is present on the extracellular side of the plasma membrane and interacts with plasma membrane lipids (glycolipids) and proteins (glycoproteins). Similarly, a network of fibers, the cytoskeleton, is found on the cytoplasmic side of the cell [4]. The primary types of fibers comprising the cytoskeleton are microfilaments, microtubules, and intermediate filaments. The lipids mainly give a stable structure to the cell and enable the proteins, lying there in, to function. The proteins, on the other hand, function as receptors, ion channels, enzymes, transporters, etc. [4] and phosphorylation of various proteins leads to different cellular functions with certain lipids playing an important role therein [5]. In chapter 2 we have discussed the composition of the plasma membrane lipids, their organisation into lateral membrane domains, and their asymmetric distribution over the lipid bilayer. The lipids present in the sperm plasma membrane are mainly phospholipids and sterols (cholesterol), the major phospholipids being phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and shingomyelin (SM). The choline phospholipids (SM and PC) are found predominantly in the outer lipid leaflet, while the aminophospholipids (PE and PS) are found in the inner leaflet (probably as a result of being constantly transported by an aminophospholipid translocase) [6].

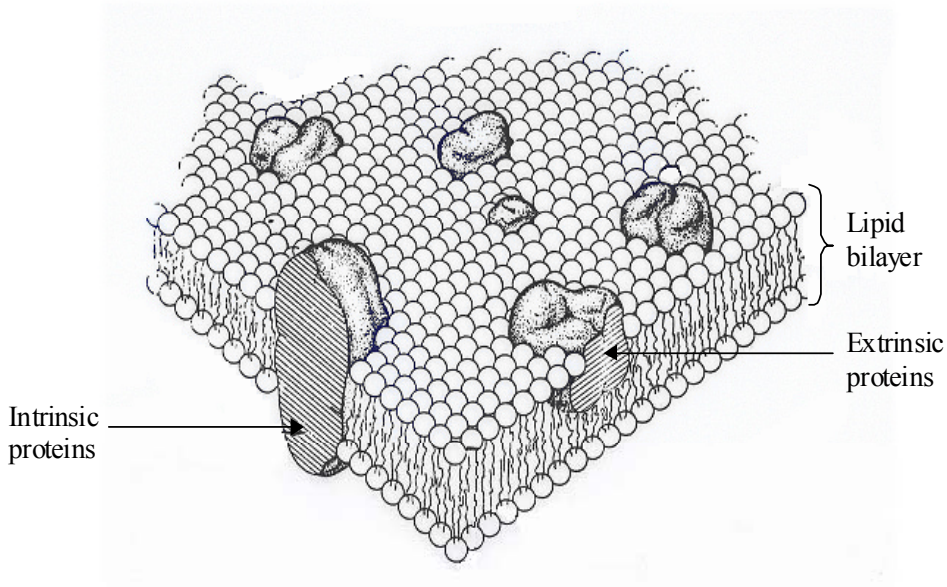

Fig. 1. Singer Nicolson's fluid mosaic model of a plasma membrane. The phospholipids form the membrane bilayer and the proteins are embedded therein (intrinsic or extrinsic). Plasma membranes are dynamic structures, serving not only as an envelope for the cell but also as a selective barrier for regulating the passage of substances into and out of the cell [7].

During capacitation there are specific alterations in the sperm head glycocalyx, like decoating and enzymatic modification of certain components, which probably play a role in reorganisation of plasma membrane proteins and lipids [6]. Also scrambling of lipid asymmetry occurs with a marked increase of PC and SM levels in the inner leaflet and a drop in the inward movement of PE and PS. This scrambling is attributed to the presence of bicarbonate in the external medium [8], which activates a soluble adenylate cyclase (sAC) and thereby switches on protein kinase A (PKA) [9], as shown in chapter 4. Most likely PKA then activates an enzyme (scramblase) resulting in altered lipid asymmetry in the capacitating and capacitated cells [6]. This altered lipid asymmetry leading to increased membrane fluidity can be monitored by merocyanine. Merocyanine stains cell membranes more intensely if their lipid components are in a higher state of disorder [10,11]. In chapter 3 we describe the use of merocyanine to evaluate capacitation of spermatozoa or in other words the scrambling of phospholipids, and compare it to the results from the existing technique for evaluation of capacitation, i.e. the chlortetracycline (CTC) staining [12]. It was observed that merocyanine detected the sperm capacitation within half an hour of incubation of the sperm cells. Comparatively, the similar level of capacitation was not detected by CTC staining until after a 3-h incubation period. Apparently, merocyanine detects the initial alterations taking place during capacitation, and hence the bilayer

scrambling of phospholipids is probably an early stage of in the process of sperm capacitation [6]. It was also observed that merocyanine was unable to give high fluorescence in the spermatozoa incubated in media without bicarbonate (suggesting absence of scrambling). Moreover, it was seen that the spermatozoa in bicarbonate-free medium did not undergo acrosome reaction, as detected by FITC-PNA staining, even in presence of Ca^{2+}-ionophore. This reiterates the significance of bicarbonate-induced membrane changes, which are suggested to be important in sperm-ZP interaction [13]. Bicarbonate stimulation of cells also leads to lateral redistribution [14] and an albumin-mediated cholesterol depletion from the plasma membrane, another important event during capacitation [15]. Thus the amount and redistribution of cholesterol in the sperm plasma membrane alters upon capacitation and these alterations play a role in modulating signalling pathways in the sperm cells [16,17]. It has been recently reported that bicarbonate induces its sAC-mediated sperm priming effects only in cells with a low cholesterol content in the plasma membrane [18] and this may explain why not all spermatozoa respond to merocyanine staining [chapter 4]. The stallion sperm, like the human sperm, have on an average a high concentration of cholesterol embedded in the plasma membrane, and hence they capacitate slowly [chapter 2], so an inverse relation of the concentration of cholesterol to the rate of capacitation is proposed.

Progesterone and sperm acrosome reaction

The freshly ovulated oocyte is surrounded by an expanded cumulus oophorus in which components of the follicular fluid (FF) are entrapped. Both the FF and cumulus oophorus cells have been shown to induce the AR of sperm [19,20], and one of the AR inducers in the FF has been identified as P_4 [19,21,22]. Progesterone has been shown to lead to sperm AR by facilitating Ca^{2+} influx [23] probably through voltage-dependent calcium channels involving $GABA_A$ receptor-like/chloride channel [24,25]. In chapter 4 we investigated the possible signal transduction pathway leading to the P_4-mediated AR, and concluded that in stallion spermatozoa P_4 induced the AR in a protein tyrosine kinase (PTK) and protein kinase C (PKC) dependent pathway (Fig 2). It was seen that P_4 exerts its effect even in absence of bicarbonate, and thus, probably, in absence of scrambling of the membrane. Hence, it is postulated that P_4 acts via its receptor that gets exposed not as a direct result of membrane scrambling but due to alterations in the glycocalyx.

Several studies have reported the existence of a plasma membrane bound PR in sperm [26,27,28,29,30]. Only those spermatozoa that expose PR are able to bind to the ZP in vitro, and are capable of undergoing the AR, either in response to the P_4 or to ZP3 [29]. The PR has been suggested to be an intrinsic sperm protein and not to have been acquired through proteinaceous secretions of accessory reproductive glands [31], and because the spermatozoa are transcriptionally and translationally inert, this putative PR must be present in the plasma membrane before the spermatozoa are released from the testes. However, spermatozoa obtained from the testis, caput and corpus epididymidis lack exposure of the

PR, though the exposure on cauda epididymidal sperm has been shown [32,33]. This could be attributed to subtle differences in the stage of sperm maturation; the spermatozoa are more mature in cauda epididymidis and are able to expose their receptors. During the passage through the epididymis the sperm plasma membrane undergoes distinct physical and chemical alterations [1], and some intrinsic proteins change their location in or on the plasma membrane during sperm maturation, others are altered, masked or replaced progressively by new proteins of epididymal origin. The PR proteins are coated during ejaculation with extra cellular components, probably secreted by accessory sex glands, which are removed during capacitation [34,35,36], as a result of the alterations in the glycocalyx. Cheng et al. [29] demonstrated that the stallion spermatozoa exposed their PR to a maximum after 5 h of *in vitro* incubation in modified Tyrode's medium.

The sperm plasma membrane PR and P_4 are increasingly accepted as playing an important role in male fertility. Tesarik and Mendoza [37] attributed subfertility in human males to either an absolute lack of or to non-functionality of sperm plasma membrane PRs. In chapter 5 we demonstrated a high positive correlation ($r = 0.73$) between the percentage of spermatozoa exposing PR and the fertility of stallions. This reiterates the significance of P_4 in male fertility and we, thus, proposed a new staining technique as an effective method for predicting stallion fertility. The PRs in our study were stained using P_4-bound BSA-FITC molecule and fertility was assessed by "non-return-rate" of the mares [38] and the foaling rate.

Research applications

Infertility is a prevalent condition in animal kingdom and in humans it also has a significant social and psychological impact. More than 30% of human infertility cases arise due to male related problems [39] and this could also be valid for other mammalian species too. Semen production in the "artificial-insemination" industry is highly commercialised and thus, not only the improvement in fertility rate but also prediction of the fertility status of the male is important. Our work has shed some light on the molecular basics of the changes taking place in spermatozoa during capacitation and AR, two very essential process during mammalian fertilisation. This added to the understanding of these physiological events that form the base of sperm-oocyte interaction. Most importantly flow cytometry enables a quick assessment of the sperm capacitation and AR. Moreover, this technique evaluates live sperm cells, unlike most other techniques using fixed cells. The description of signal transduction mechanisms leading to P_4-induced sperm AR increases our understanding of the mechanism of acrosome reaction, the event just before the fertilisation. This knowledge can help to improve the semen handling procedures for the breeding industry and might be used in developing procedure for male contraception as well. Fertility prediction based on exposure of P_4 receptor is a new parameter in addition to the existing ones like assessment of motility, morphology and viability.

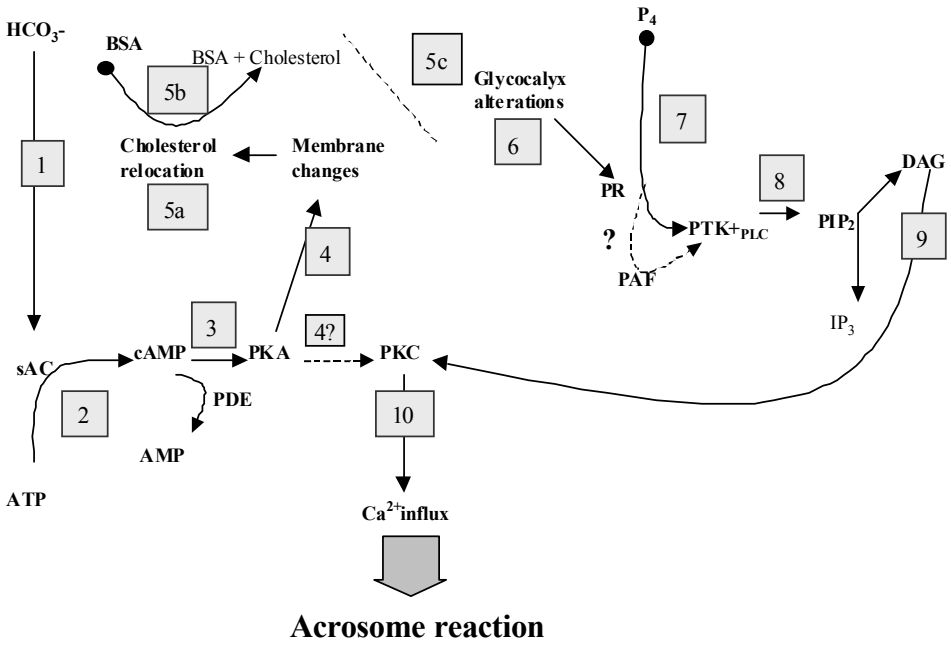

Fig. 2. Schematic model for the signalling pathways involved in bicarbonate (HCO_3^-) mediated plasma membrane changes, and progesterone (P_4) induced sperm acrosome reaction. Bicarbonate enters into the spermatozoa [1] stimulating a soluble adenylate cyclase (sAC), which triggers production of cAMP [2], thus activating [3] protein kinase A (PKA). In turn PKA leads to plasma membrane changes [4], scrambling the asymmetry of the membrane lipids. It is postulated that PKA also stimulates [4?] a protein kinase C (PKC) leading to Ca^{2+} influx and acrosome reaction (AR). The plasma membrane changes also lead to redistribution of [5a] of membrane cholesterol, which is taken away [5b] by an albumin molecule (BSA). Alteration in the glycocalyx [5c] accompany the membrane changes, which probably lead to [6] progesterone receptor (PR) exposure. The exposed PR is stimulated [7] by progesterone (P_4) and in turn, directly or via a G-protein, activates a protein tyrosine kinase coupled to phospholipase C ($PTK+_{PLC}$). The activated PLC cleaves [8] phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG then activates [9] the protein kinase C (PKC), which gets translocated to the plasma membrane. PKC opens the voltage-dependent Ca^{2+} channels [10] in the plasma membrane, leading to influx of extracellular Ca^{2+}. The increase in intracellular Ca^{2+} leads to acrosome reaction.

Concluding remarks

Sperm capacitation and acrosome reaction have been extensively studied. Nevertheless, the molecular bases of these processes are far from clear, and still a lot remains to be investigated. We have shown that bicarbonate leads to sperm capacitation via a cAMP/PKA

pathway, however, the exact mechanism by which this pathway is stimulated is still not fully clear. Moreover, the mechanism by which the stimulated PKA leads to capacitation related changes like activation of the putative scramblase resulting in scrambling of plasma membrane lipid asymmetry, and other substrates getting phosphorylated, needs to be researched . The nature of these substrates, and how their phosphorylation affects the major end-points of capacitation (hyperactivation and AR) need further investigation. We have shown a high positive correlation between the percentage of spermatozoa exposing PR and the stallion fertility, and a P_4-mediated signalling pathway leading to AR has been proposed. The role of P_4 in sperm functions has been well documented. Hence, the importance of identification and characterisation of the PR has become an obvious research topic, in order understand the signalling events leading to AR and the role played by P_4 during fertilisation. Many studies have tried to identify the putative PR, though without a clear consensus. More clinical trials are needed to confirm the use of the exposure of PR as a fertility predicting parameter.

Recently, P_4 has been reported to produce an increase in the synthesis and release of platelet activating factor (PAF), an endogenous component of spermatozoa that has been shown to play a role in the fertilisation process [39,40]. Studies to elucidate the exact role of PAF, also in relation to P_4 should be initiated to shed light into the complex process of male gamete function.

Overall, the signalling processes underlying sperm capacitation and acrosome reaction are quite unique and might differ between species. Hence a detailed species-specific investigation is warranted for. The knowledge of the signalling mechanism and the cross talk between various inducers of sperm capacitation, and its components like hyperactivation, and acrosome reaction would be very beneficial in finding solutions to fertility related problems. It will also help in improvement of sperm preservation techniques, an essential element in today's commercial breeding industry, and for the cryopreservation of gametes of endangered species.

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Samenvatting

Mannelijke fertiliteit vereist dat er een aantal ingewikkelde processen worden voltooid zoals de spermiogenese, de productie van spermacellen in de testis, het sperma transport naar de bijbal, de secretie van accessoir geslachtsvocht (o.a. vanuit de prostaat) en de uiteindelijke depositie van het sperma in het vrouwelijk geslachtskanaal (tijdens de ejaculatie). Daarnaast zijn ook twee zeer belangrijke sperma gerelateerde processen, te weten capacitatie en acrosoomreactie, van belang bij mannelijke fertiliteit. Wanneer een van beiden deficiënties vertoont zal een eventuele bevruchting ernstig belemmerd kunnen worden. Capacitatie vindt, afhankelijk van de diersoort, plaats in het oviduct, of de uterus, terwijl de acrosoomreactie door de zona pellucida (de extracellulaire matrix van de eicel) wordt geïnduceerd. Het ZP-3, het sperma bindende eiwit van de zona pellucida, en progesteron kunnen de acrosoomreactie induceren. Sinds het bekend worden van de aanwezigheid van progesteronbindende eiwitten op de plasmamembraan, is er relatief veel aandacht besteed aan de door progesteron geïnduceerde acrosoomreactie.

Het onderzoek dat beschreven staat in dit proefschrift was erop gericht om de membraan veranderingen in kaart te brengen, die optreden tijdens de capacitatie en de acrosoomreactie van hengstensperma. Daarin staat de door progesteron gestimuleerde signaaltransductie-route die leidt tot de acrosoomreactie centraal. Flow cytometrische bepalingen zijn ontwikkeld om sperma capacitatie en acrosoomreactie te kunnen volgen. Gebleken is dat het vermogen om de progesteronreceptor te exposeren aan het spermaoppervlak tijdens de in vitro sperma capacitatie een nieuwe parameter kan vormen voor het bepalen van de fertiliteit van de hengst.

Membraan veranderingen gedurende sperma capacitatie

Zowel in het vrouwelijk geslachtskanaal als in een gedefinieerd medium kan een spermacel capacitatie ondergaan. De plasmamembraan, in direct contact met de capacitatie-omgeving ondergaat significante veranderingen. De plasmamembraan van de spermacel ziet er uit als een dubbellaag van fosfolipiden. Aan de buitenkant van de membraan zijn er glycolipiden aanwezig, en in het hydrofobe membraangedeelte zijn sterolen en andere neutrale lipiden aanwezig. Dobberend in deze lipiden bilaag zijn er diverse membraaneiwitten te onderscheiden. Een netwerk van extracellulaire glycoproteinen, de glycocalyx, heeft een interactie met de membraan eiwitten en de glycolipiden in de membraan. In de cel bevindt zich het cytoskelet bestaande uit microfilamenten, microtubuli, en intermediaire filamenten. Dit cytoskelet ligt verankerd aan eiwitten of aan het fosfatidylserine aan de binnenkant van de plasmamembraan. De lipiden geven de cellen voornamelijk een stabiele structuur en maken het de eiwitten die erin liggen mogelijk om te functioneren. Op hun beurt functioneren eiwitten als hormoonreceptoren, ionkanalen, enzymen of als transporteurs Modificatie van eiwitten, bijvoorbeeld door fosforylering, kan leiden tot veranderingen in cellulaire functies. Eiwitfosforylering kan o. a. op gang worden gebracht door afbraak van bepaalde lipiden (bijvoorbeeld het fosfatidylinositolbifosfaat).

In **hoofdstuk 2** wordt de compositie van de plasmamembraan lipiden in hengstensperma bediscussieerd alsmede de laterale en bilaag organisatie van deze lipiden. De voornaamste lipiden zijn het seminolipide (een spermaspecifiek glycolipide) fosfolipiden (met name het fosfatidylcholine, fosfatidylethanolamine, sfingomyeline en fosfatidylserine) en cholesterol. Van de fosfolipiden zijn aan de buitenkant van de plasmamembraan sfingomyeline en fosfatidylcholine sterk verrijkt, terwijl fosfatidylserine en in mindere mate fosfatidylethanolamine een sterke voorkeur hebben voor de binnenkant van de plasmamembraan.

Gedurende capacitatie treden er veranderingen op in de glycocalyx van de spermacel, o.a. verwijdering en enzymatische modificaties van bepaalde componenten. Deze veranderingen spelen waarschijnlijk een rol in de reorganisaties van de plasmamembraaneiwitten en lipiden. Zo treedt er bij capacitatie een menging van fosfolipiden uit de lipidenmonolagen op hetgeen resulteert in verhoogd transport van fosfatidylcholine en sfingomyeline naar de binnenkant van de plasmamembraan en een verhoogd transport van fosfatidylserine en fosfatidylethanolamine naar de buitenkant van de plasmamembraan. Deze zogenaamde fosfolipiden-scrambling wordt veroorzaakt door bicarbonaat uit het externe medium. De bicarbonaat concentratie bedraagt ca. 15 mM in in vitro fertilisatie media, nog hogere concentraties zijn aanwezig in het oviduct, terwijl het seminale plasma minder dan 1 mM bicarbonaat bevat. Bicarbonaat activeert het adenylaat cyclase waardoor de concentratie cyclisch adenosine monofosfaat (cAMP) toeneemt en een cAMP gevoelige proteinkinase (PKA) wordt geactiveerd. Waarschijnlijk is de geactiveerde PKA betrokken bij de inductie van een scramblase hoewel de aanwezigheid en structuur van dit hypothetische eiwit nog moeten worden opgehelderd. De fosfolipiden scrambling gaat gepaard met een sterke verhoging van de vloeibaarheid van de plasmamembraan hetgeen gedetecteerd kan worden met de membraan probe merocyanine. Deze probe blijkt in spermacellen die scrambling vertonen een veel hoger fluorescentie signaal te geven. In **hoofdstuk 3** wordt beschreven dat de subpopulatie van cellen die na capacitatie een positieve merocyanine kleuring bezitten overeenkomt met de populatie die met de klassieke chloortetracycline kleuring als capaciterend kunnen worden getypeerd. De chloortetracycline kleuring is echter langzamer waardoor de verandering pas na circa 3 uur gedetecteerd kan worden terwijl de merocyanine kleuring dezelfde processen binnen een half uur vastlegt. Het lijkt er op dat de merocyanine kleuring, een weerspiegeling van het optreden van fosfolipiden scrambling, een vroege capacitatie verandering aan het spermaoppervlakte markeert. De merocyanine respons blijkt volledig afhankelijk te zijn van de capacitatie factor bicarbonaat. Tevens blijkt dat in afwezigheid van bicarbonaat de spermacellen geen acrosoomreacties vertonen in aanwezigheid van factoren die deze secretie stimuleren. Zelfs de aanwezigheid van Ca2+ ionofoor blijkt in afwezigheid van bicarbonaat niet afdoende om de acrosoomreactie te induceren. Deze waarnemingen bevestigen het belang van de bicarbonaat gemedieerde membraanveranderingen die cruciaal worden geacht voor een adequate spermabinding aan de zona pellucida. Bicarbonaat blijkt tijdens de capacitatie tevens de laterale verdeling van membraancomponenten te beïnvloeden. Een belangrijk lipide, het cholesterol, blijkt in aanwezigheid van bicarbonaat zich te concentreren in het apicale gedeelte van de

plasmamembraan in de spermakop. Alleen cellen die deze herverdeling vertonen blijken gevoelig te zijn voor een andere capacitatie factor; het albumine. Albumine blijkt in deze subpopulatie bicarbonaat gestimuleerde cellen het cholesterol te depleteren. De extracellulaire bicarbonaat concentratie staat dus centraal bij een aantal capacitatie gerelateerde veranderingen aan de plasmamembraan van de spermakop.

Progesteron en de sperma acrosoomreactie

Progesteron veroorzaakt een acrosoomreactie door de plasmamembraan permeabel te maken voor extracellulair calcium. Waarschijnlijk wordt dit bewerkstelligd door het openen van zogenaamde voltage dependent calcium kanalen en hier zijn GABA-receptorachtige chloride kanalen bij betrokken. In **hoofdstuk 4** is het signaaltransductiepad bestudeerd dat door progesteron wordt geïnitieerd en dat resulteert in de acrosoomreactie. Progesteron blijkt te opereren via een tyrosinekinase en proteinkinase C afhankelijk pad. Tevens blijkt dat progesteron opereert via een bicarbonaat onafhankelijk pad.

Een aantal studies hebben de aanwezigheid van plasmamembraan gebonden receptoren voor progesteron in de spermacel aangetoond. Alleen spermacellen die affiniteit hebben voor progesteron blijken in vitro in staat te zijn om aan de zona pellucida te binden en vervolgens de acrosoomreactie te initiëren.

In toenemende mate worden progesteron en de progesteronreceptor op de plasmamembraan van de spermacel van belang geacht voor de mannelijke fertiliteit. In **hoofdstuk 5** is aangetoond dat er bij hengsten een hoge correlatie ($r = 0.73$) bestaat tussen het percentage spermacellen dat na capacitatie functionele progesteronreceptoren exposeert en de fertiliteitresultaten bereikt met sperma van deze hengsten. De kleuring van gecapaciteerde spermamonsters met het progesteron-albumine-FITC complex, waarmee de expositie van progesteron receptoren kan worden gevisualiseerd, kan in de toekomst mogelijk worden toegepast om het fertiliserend vermogen van spermamonsters in te schatten.

Onderzoekstoepassingen

Infertiliteit is een belangrijk en veel voorkomend gezondheidsprobleem in het dierenrijk en bij de mens. Het heeft tevens een significant sociaal en psychologisch effect. Meer dan 30% van de menselijke infertiliteit wordt veroorzaakt door een mannelijke stoornis. Deze getallen zouden voor andere zoogdieren vergelijkbaar kunnen zijn. Spermaproductie in de kunstmatige inseminatie industrie is sterk commercieel geworden en daarom is niet alleen de verhoging van de kans op bevruchting van belang, maar tevens de voorspelling van deze kans. Het in dit proefschrift beschreven werk heeft nieuw inzicht opgeleverd ten aanzien van de moleculaire veranderingen die optreden gedurende de capacitatie en de acrosoomreactie bij hengstensperma, hetgeen de basis vormt voor de interactie van de spermacel met de eicel. Het gebruik van de flow cytometer blijkt bij dit onderzoek onontbeerlijk omdat met dit apparaat snel de relevante parameters ter beoordeling van de

progressie van capacitatie en acrosoomreactie bij individuele spermacellen kunnen worden vastgesteld. Voorts is het met deze techniek mogelijk gebleken om cellen in levende en zelfs gestimuleerde conditie te meten met eenvoudige kleuringprotocollen zodat er een minimum aan artefacten optreedt. De beschrijving van de progesteron geïnduceerde signaaltransductie die leidt tot de acrosoomreactie verhoogt het inzicht in de processen die zich vlak voor de bevruchting afspelen in de spermacel. Dit inzicht kan bijdragen aan de ontwikkeling van meer adequate spermabehandelingsprotocollen en de ontwikkeling van mannelijke contraceptiva Ten aanzien van de bestaande mannelijke fertiliteitstesten, kan de mate van expositie van de progesteronreceptor op capaciterend sperma benut worden als nieuwe kwaliteitsparameter Thans worden voornamelijk motiliteit, morfologie en vitaliteitsgegevens van spermacellen gebruikt als kwaliteitsparameters.

Concluderende opmerkingen

Sperma capacitatie en de acrosoomreactie zijn intensief bestudeerd. Desondanks zijn de moleculaire processen die er bij optreden nog onduidelijk en blijft er veel stof over voor nadere studie. In dit proefschrift staan experimenten beschreven waarbij aangetoond wordt dat bicarbonaat via een proteinkinase A gemedieerd pad sperma-capacitatie induceert. Echter de relatie tussen stimulering van PKA en het tegelijkertijd optreden van fosfolipiden scrambling is nog onbekend. De relatie tussen PKA stimulering en de eindpunten van de spermacapacitatie, het optreden van hypermotiliteit en de acrosoomreactie welke beiden noodzakelijk zijn voor de penetratie van de zona pellucida, is eveneens onduidelijk. In dit proefschrift is aangetoond dat er een hoge correlatie bestaat tussen het aantal spermacellen dat na capacitatie *in vitro* in staat is progesteron te binden, door de expositie van een progesteronreceptor, en de fertiliteit van de hengst. De zuivering, identificatie en karakterisering van de progesteronreceptor heeft daarom op dit moment hoge prioriteit. Er zijn, overigens zonder noemenswaardige resultaten, al pogingen hiertoe gedaan. Meer klinische experimenten zijn nodig om de bruikbaarheid van de progesteronreceptorbepaling op capaciterend sperma als fertiliteitparameter te bevestigen.

Recent is er aangetoond dat progesteron de synthese en afgifte van platelet activating factor (PAF) stimuleert. PAF is een component van spermacellen die een actieve rol speelt in het bevruchtingsproces. Verder onderzoek op dit gebied is nodig om de progesteron gemedieerde inductie van de acrosoomreactie beter te begrijpen.

Concluderend zijn de signaalprocessen die betrokken zijn bij de spermacapacitatie en de acrosoomreactie relatief uniek. De kennis van het signaaltransductie mechanisme en de samenhang van de diverse inductors van spermacapacitatie alsmede de daarbij optredende hypermotiliteit en acrosoomreactie zijn van belang bij het vinden van oplossingen voor fertiliteit gerelateerde problemen. Deze kennis kan ook van belang zijn voor het verbeteren van bewaartechnieken voor sperma, een essentieel element in de huidige fokkerij industrie, alsmede voor de opslag van gameten van bedreigde diersoorten.

Acknowledgement

I wish to express my gratitude to all who have contributed, in one way or the other, to the completion of my PhD degree. In particular I am grateful to my supervisors, who have nurtured me all throughout to make me a better scientist.

Curriculum Vitae

Rahul Rathi was born in Muzaffarnagar (India) on 22nd of October 1972, to Sneh and Yashpal Singh Rathi. Following his graduation from Campus School, Pantnagar in 1990 he got selected for the Bachelor's in Veterinary Science and Animal Husbandry programme at G.B. Pant University. After getting the degree of BVSc & AH in 1995, he worked in the capacity of Teaching Associate in the department of Veterinary Public Health in the same university, for a period of 7 months. In 1996 he received a scholarship from Nuffic and came to The Netherlands to pursue his Master's in Veterinary Epidemiology at Utrecht University. He was associated with the research in stallion fertility, and in 1998 after receiving the degree of Master's in Science-Veterinary Epidemiology, he continued with his research towards his PhD. During this period he worked on fundamental and clinical aspects of sperm capacitation and acrosome reaction with major emphasis on the action of progesterone on these two physiological events.