PAULA PICCOLO MAITAN

PHYSIOLOGICAL AND BIOCHEMICAL EVENTS RELATED TO STALLION SPERM CAPACITATION IN *IN VITRO* CONDITIONS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*

Orientador: José Domingos Guimarães

Coorientador: Heiko Henning

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RESUMO

MAITAN, Paula Piccolo, D.Sc., Universidade Federal de Viçosa, julho de 2020. **Eventos fisiológicos e bioquímicos relacionados a capacitação espermática de garanhões em condições *in vitro***. Orientador: José Domingos Guimarães. Coorientador: Heiko Henning.

Apenas dois potros foram nascidos de FIV (fertilização *in vitro*) convencional em equinos, ambos no início dos anos 90. O principal motivo do insucesso da técnica é direcionado ao espermatozoide do garanhão, que não é capaz de penetrar a zona pelúcida e fertilizar o oócito provavelmente devido a sua incompleta ativação (capacitação) em condições *in vitro*. Capacitação pode ser definida como o conjunto de mudanças físicas e biológicas que acontecem no espermatozoide o tornando apto a fertilizar o oócito em condições *in vivo* ou *in vitro*. Para que esse processo ocorra, o espermatozoide deve estar submetido a um ambiente que contenha bicarbonato (HCO_3^-), cálcio (Ca^{2+}) e albumina. Essas substâncias causarão mudanças nas concentrações de adenosina monofosfato cíclico (AMPc), pH e Ca^{2+} intracelular, além alteração no potencial de membrana causando reorganização da membrana plasmática e depleção do colesterol, levando ao espermatozoide à reação acrossomal e fertilização do oócito. Infelizmente, nos equinos várias etapas da capacitação ainda não estão bem elucidadas. O objetivo desse trabalho foi demonstrar, por meio do uso de citometria de fluxo, microscopia de fluorescência, crio-eletromicroscopia e imunohistoquímica, algumas etapas da capacitação espermática de garanhões na presença de moléculas que podem estimular esse processo, além de compreender qual adenilato ciclase (solúvel ou transmembrana) está envolvida em algumas das etapas desse evento. O uso da probe Annexina-V em condições capacitantes demonstrou a ocorrência da exposição da fosfatidilserina (PS) em espermatozoides de garanhão durante o processo de capacitação, porém em uma pequena porcentagem da população ($p \leq 0,05$). Além disso, o uso de cafeína e dibutiril-AMPc indicam a relevância da via sAC/pkA para esse processo ($p \leq 0,05$). A observação de três diferentes padrões de fluorescência da Annexina-V no espermatozoide viável de garanhão sugere etapas sequenciais

na remodelação da membrana plasmática. Em relação aos efeitos na fluidez de membrana e reação acrossomal induzida, diferentes concentrações de Ca^{2+} não interferiram na população espermática viva, merocianina e FITC-PNA 647 positiva ($p \geq 0,05$). O cálcio deve estar presente no meio capacitante uma vez que, na presença de seu quelante EGTA, essas etapas da capacitação foram bloqueadas. Porém o cálcio não deve estar obrigatoriamente presente em elevadas concentrações para iniciar a capacitação em espermatozoides de garanhão como é necessário em outras espécies. Diferentes concentrações de progesterona (P4) e albumina sérica bovina (BSA) também não foram capazes de aumentar a fluidez de membrana ou induzir reação acrossomal nos espermatozoides de garanhão ($p \geq 0,05$). Em relação as adenilato ciclases, a adenilato ciclase solúvel (sAC) no espermatozoide de garanhão demonstrou ser responsável pelo aumento da fluidez de membrana na presença de bicarbonato. Esse resultado foi demonstrado pelo uso do LRE1, um inibidor específico da sAC que demonstrou não causar efeitos colaterais em outros eventos fisiológicos espermáticos como alteração do potencial mitocondrial ($p \geq 0,05$). A imunohistoquímica da sAC em espermatozoide de garanhões demonstrou diferente localização comparada à sAC de suínos. No espermatozoide de garanhão essa enzima está presente formando uma linha pontilhada como um cordão na parte distal do acrosoma enquanto no suíno essa enzima está presente na parte apical da região acrossomal e na porção inicial da peça intermediária. A ação da forskolina indicou que a adenilato ciclase transmembrana (tmAC) possivelmente está presente no espermatozoide de garanhão e pode desempenhar um papel na reação acrossomal. Contudo, o fato da necessidade de uma alta concentração (500 µM) necessária para demonstrar seu efeito comparada a outras espécies indica que mais estudos como o uso de inibidores específicos (como o ddAdo) ou imunohistoquímica da tmAC (AC1-9) devem ser realizados no intuito de corroborar e validar os resultados encontrados. Em conclusão os resultados obtidos no presente trabalho elucidam diversos questionamentos na capacitação espermática de garanhões como exposição da PS, papel de diferentes concentrações Ca^{2+}, P4 e BSA nesse evento além do papel

de diferentes adenilato ciclases em alguns estágios da capacitação demonstrando que a capacitação é um processo complexo que varia entre as espécies.

Palavras-chave: Adenilato ciclases. Capacitação. Espermatozoide de garanhão. Reorganização de membrana.

ABSTRACT

MAITAN, Paula Piccolo, D.Sc., Universidade Federal de Viçosa, July, 2020. **Physiological and biochemical events related to stallion sperm capacitation in *in vitro* conditions**. Adviser: José Domingos Guimarães. Co-adviser: Heiko Henning.

Only two foals have ever been born from conventional equine IVF (*in vitro* fertilization), both in the early 1990's. The main reason for the conventional IVF failure is thought to be the stallion spermatozoa, which are not able to penetrate the zona pellucida, most likely due to incomplete activation (capacitation) under *in vitro* conditions. Capacitation is considered as a consecutive activation of different signaling pathways inducing physiological and biochemical modifications which primes the sperm for fertilization *in vitro*. To have the capacitation occur, the sperm must be under an environment (*in vivo* or *in vitro*) that contains bicarbonate (HCO_3^-), calcium (Ca^{2+}) and albumin. These elements will besides changes in membrane potential, provide changes in cyclic adenosine monophosphate (cAMP) levels, intracellular pH and intracellular Ca^{2+} causing plasma membrane reorganization and cholesterol depletion leading the sperm to undergo the acrosome reaction and fertilize the oocyte. The aim of this work was to show by flow cytometry, fluorescence microscopy, cryo-electron microscopy and immunohistochemistry some capacitation steps in stallion spermatozoa in the presence of molecules that can trigger this process and also understand which molecules may be involve in some capacitation steps. The use of the probe Annexin-V in capacitating conditions showed a significant increase in the live, Annexin-V positive sperm population indicating a phosphatidylserine (PS) exposure in stallion sperm during this process ($p \leq 0.05$), but it was only in a small percentage of viable sperm. Stimulation of the sAC/cAMP/PKA pathway by caffeine and dibutyryl-cAMP indicated the relevance of this pathway for PS exposure. The observation of three different staining patterns for Annexin-V in live stallion sperm may warrant further investigation with respect to whether these represent sequential steps in membrane remodeling. Regarding the effects in membrane fluidity and induced acrosome reaction different concentrations of calcium did not interfere in the population live merocyanine and PNA-positive

spermatozoa ($p>0.05$). Also, different concentrations of progesterone (P4) and bovine serum albumin (BSA) were not able to increase membrane fluidity or induced acrosome reaction ($p\leq0.05$). However, calcium should be present in the medium since EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; 2 mM) was able to block all this capacitation steps, but there is no need for higher concentrations (2 mM) to start capacitation in stallion sperm as is needed in other species. Regarding the adenilato cyclases (ACs), the presence of sAC (soluble) in the stallion sperm was shown to be responsible for the increase in membrane fluidity in the presence of bicarbonate. This finding was possible to demonstrate with the use of LRE1, a sAC specific inhibitor that showed no effect in other sperm physiological events ($p>0.05$). The immunostaining of sAC in stallion sperm indicated different localization compared to boar. In stallions, the enzyme was present as a dotted line (diadem-like pattern) distal from the acrosomal area, while in boars, the sAC was present in the acrosomal area and in the neck. The action of forskolin indicated that tmAC (transmembrane) may be present in the stallion sperm and may play a role in the acrosome reaction. However, the fact that a relatively high concentration (500 µM) was needed to show this effect compared to other reports, indicates that more studies like use of specific inhibitors of tmAC (as ddAdo) or immunostaining of the AC1 to 9 must be performed in stallion sperm to confirm the findings. In conclusion, the findings in this work elucidate several questions in stallion capacitation as PS exposure, role of different concentrations of calcium, progesterone and BSA in this process and the role of adenylyl cyclases in some stallion capacitation events showing that capacitation is a complex process that differs among species.

Keywords: Adenylyl cyclases. Capacitation. Membrane reorganization. Stallion sperm

SUMÁRIO

INTRODUCTION

After spermatogenesis, the sperm cell cannot synthesize proteins, phospholipids, cholesterol and other components but the cell is still not yet fully matured. The spermatozoon completes its maturation when reaching the cauda of the epididymis (Eddy et al., 1994). The mature sperm cell has three highly specialized regions: (I) the sperm head including the acrosome which is involved in the sperm-oocyte interaction; (II) the midpiece with the mitochondria involved in energy production; (III) the flagellum involved in motility (Flesch and Gadella, 2000).

The sperm plasma membrane is a very complex, heterogenic and dynamic bilayer structure. These two membrane compartments have different physiological functions and also differ in composition (Dunina-Barkovsaya, 1998). The sperm plasma membrane composition and lateral organization is also responsible for the affinity for adhesion factors, solutes permeability, cell signaling and fusion events (Flesch and Gadella, 2000). This membrane is the main interface between the extracellular environment and the cytoplasm being a thin, flexible lipid bilayer with selected permeability to polar solutes with integral and peripheral proteins in between. It is a fluid model due to the high proportion of long chain polyunsaturated fatty acids (PUFA) of the main phospholipids (Singer and Nicholson, 1972).

The glycocalyx with its lateral polarized nature is also relevant for the lateral organization of the plasma membrane molecules since the carbohydrate network via integral membrane proteins and glycolipids is in direct contact with the sperm plasma membrane (Flesch and Gadella, 2000).

The lipids are asymmetrically distributed over the lipid bilayer and phospholipids (70%), neutral lipids (25%) and glycolipids (5%) are the most abundant lipids found in the sperm plasma membrane (Mann et al., 1981). Phospholipids and cholesterol comprise around 90% of the total lipids in stallion sperm cell (Komarek et al., 1965). Phosphatidylcholine, phosphatidylethanolamine (PE) and sphingomyelin are the phospholipids with higher abundance percentage, respectively (Parks and Lynch, 1992).

Neutral or positive lipids like phosphatidylcholine, sphingomyelin and glycosphingolipids are preferentially in the external leaflet of the plasma membrane (Gadella et al., 1999) while anionic phospholipids as phosphatidylethanolamine, phosphatidylserine (PS) and inositol phospholipid are in the inner leaflet of the membrane. Differences in the length of the fatty acids, cholesterol interactions and coupling proteins (for transmembrane channels our receptor-ligand complexes) affect the interaction between the two layers (Cardullo and Wolf, 1990).

To fertilize an oocyte, the spermatozoa must undergo a process called capacitation that begins when spermatozoa reaches the female reproductive tract or when it is in a presence of a specific media in *in vitro* conditions. In this process alterations in the sperm plasma membrane occur partly due to the removal of glycocalyx components (Saxena et al., 1986) and adsorption of new components from the *in vivo* or *in vitro* environment. Also an enzymatic modification of glycocalyx components occurs (Mahmoud and Parrish, 1996; Revah et al., 2000). Another event in the capacitation process is a lateral reorganization of the transmembrane proteins (Aguas et al., 1989) resulting in the increased affinity of sperm for the zona pellucida (Yanagimachi, 1994)

Capacitation was originally defined as physiological membrane changes that enable the spermatozoa to acquire the fertilizing capacity that takes place in the female reproductive tract (Chang 1951; Austin 1952). Since new techniques like IVF (*in vitro* fertilization) have been developed, capacitation is now considered as a consecutive activation of different signaling pathways inducing physiological and biochemical modifications which prime the sperm for fertilization *in vitro* (Gervasi and Visconti, 2016). However, in *in vitro* conditions, capacitation presents varying degrees of success for each species (Bailey et al., 2010). The changes during capacitation make the sperm able to (i) bind the oocyte extracellular matrix, the zona pellucida (ZP) and to undergo acrosome reaction (Saling et al., 1978, 1979; Topper et al., 1999); (ii) acquire hyperactivation (flagellar motion required to penetrate the egg) (Ho and Suarez, 2001); and (iii) fuse with the oocyte (Evans and Florman, 2002).

For capacitation to occur, the sperm must be under an environment (*in vivo* or *in vitro*) that contains bicarbonate (HCO_3^-), calcium (Ca^{2+}) and albumin. These three factors are known to induce sperm changes required for the acquisition of the fertilizing potential in many species (mice: Visconti et al., 1995 a,b; man: Osheroff et al., 1999; pig: Flesch and Gadella, 2000). These elements will, besides changes in membrane potential (Vm), provide changes in cyclic adenosine monophosphate (cAMP) levels, intracellular pH and intracellular Ca^{2+} (Gervasi and Visconti, 2016). The capacitation environment will lead to the removal of decapacitation factors from the surface of the sperm plasma membrane leading to a reorganization of the plasma membrane and activation of several intracellular pathways. Besides these three main elements, some species-specific factors for this process to occur have been already identified. In cattle for example, heparin-like molecules such as glycosaminoglycans are essential for triggering capacitation (Parrish et al., 1988). However, no specific molecule has been yet defined for triggering capacitation in stallion sperm (Leemans et al., 2019).

A capacitating environment leads in the sperm cells of most species to an influx of HCO_3^- which in turn leads to an activation of a soluble adenylyl cyclase (sAC). The activation of the sAC increases the production of the second messenger cAMP which activates the protein kinase A (PKA) cascade trigging an increase in membrane fluidity (within 10 minutes). Later, a rise in intracellular Ca^{2+} and changes (such as exposure of phosphatidylserine – PS, and phosphatidylethanolamine - PE) in the plasma membrane occur (Flesch and Gadella, 2000). In the presence of albumin other changes such as cholesterol depletion occur, followed by the formation of microdomains that contain protein complex that have more affinity to the zona pellucida (van Gestel et al., 2007) and factors as N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) which play an important role in the fusion of the plasma membrane with the outer acrosomal membrane, resulting in acrosome reaction, an exocytotic event that releases lytic enzymes to locally digest the zona pellucida (Tsai et al., 2010). Besides these events, the activation of PKA is required to phosphorylate tyrosine residues, that especially in the sperm tail, lead to a Ca^{2+} dependent, hyperactivated motility mode necessary to cross the zona

pellucida of the oocyte (Nassar et al., 1999). In addition, the tyrosine phosphorylation increases the affinity for the zona pellucida (Pukazhenthi et al., 1998) and acts in the acrosome reaction (Benoff ,1998). Only capacitated sperm bind to the zona pellucida in a species specific manner. (Yanagimachi, 1994; Harayama et al., 2012) (Figure 1).

After binding, the fusion of the sperm plasma membrane and the oolemma occurs and, subsequently, the sperm cell is incorporated in the oocyte (Flesch and Gadella, 2000).

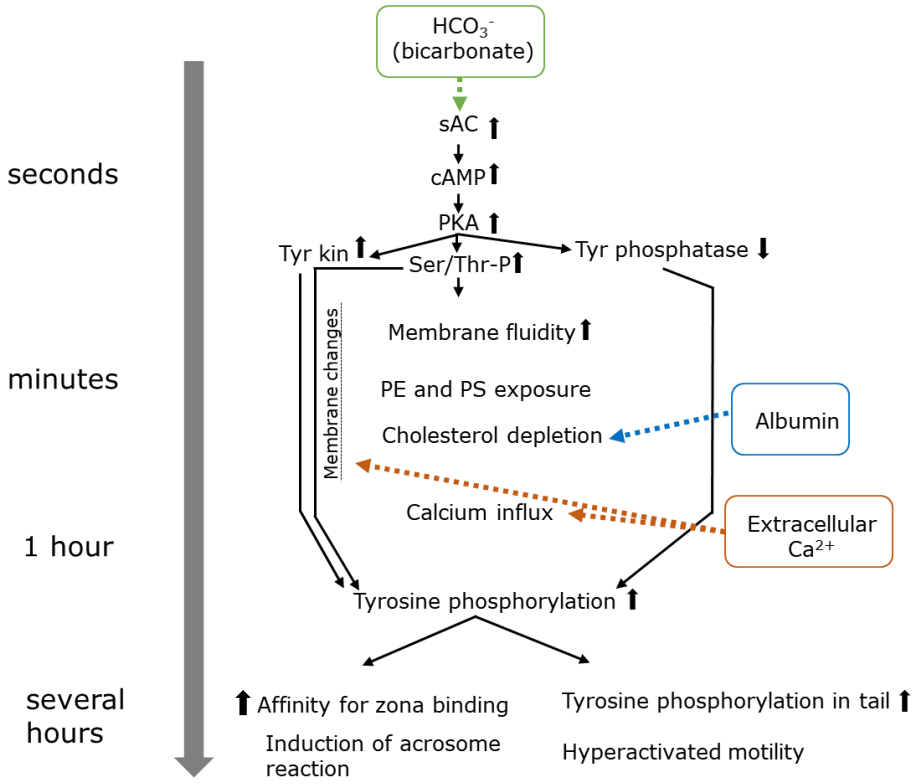

Figure 1. Capacitation pathway in mammalian sperm cell. The predominant *in vitro* capacitation factors are indicated in green, blue and orange and their role in the capacitation-induced responses is indicated with arrows at the same color. Abbreviations used: Bic = bicarbonate; sAC = soluble adenylate cyclase; cAMP = cyclic adenosine monophosphate PKA = protein kinase A; Ser/Thr-P = serine/threonine phosphorylation; PS = phosphatidylserine; PE = phosphatidylethanolamine. Black arrows indicate how the capacitation events are linked to each other. (Modified from Boerke et al., 2008).

In the cauda epididymis the HCO_3^- concentration is very low (< 1mM) and when spermatozoa reach the fertilization site in the female genital tract this

concentration rises to higher levels (> 15 mM) (Harrison, 1996). HCO_3^- can reach the intracellular environment via ion channels in the plasma membrane like Cl^-/HCO_3^- and Na^+/HCO_3^- exchangers (Ain et al., 1999; Jensen et al., 1999). Another way for the bicarbonate getting into the cell is by gas diffusion through the sperm plasma membrane once CO_2/HCO_3^- are in equilibrium in the intra and extracellular compartments (Harrison, 1996). The molecule involved in maintain high levels of intracellular HCO_3^- in the sperm cell by conversion of diffused CO_2 can be carbonic anhydrase, present in the sperm head (Parkkila et al., 1991). This event explains why in *in vitro* conditions, the capacitating media should be maintained inside the incubator with controlled CO_2 (5%) atmosphere.

In vitro, additionally to the key elements of capacitation the medium must also contain a limited supply of electrolytes like sodium (Na^+), potassium (K^+), chloride (Cl^-), magnesium (Mg^{2+}) and phosphate (PO_4^3) and energy metabolites such as glucose, pyruvate and lactate for the high consumption of ATP needed for motility and other process (Gervasi and Visconti, 2016).

Unfortunately, in equids the capacitation does not appear to occur that way. The increase in the intracellular pH (Leemans et al., 2014), protein tyrosine phosphorylation in sperm tail (Leemans et al., 2014, 2015) and hyperactivated motility (Leemans et al., 2015), important capacitation parameters were already identified in the stallion sperm cell using an oviduct epithelial explant model. However other parameters still need to be proven in *in vitro* conditions like membrane lipids rearrangement, cholesterol depletion, membrane reorganization and induced acrosome reaction.

In the current study, in order to clarify to what extent stallion sperm progress in capacitation, in the presence of bicarbonate, calcium and albumin, several assessments were carried out. The first step was to verify a re-organization of the plasma membrane after initiation of sperm capacitation. To this end, PS exposure in viable sperm was monitored with flow cytometry and with live imaging. Also, conformational changes of the plasma and outer acrosomal membrane were visualized with cryo electron microscopy. Experiments were also carried out to determine an optimal extracellular calcium concentration in a chemically defined

medium that allows acrosome reaction induced by progesterone. Moreover, different BSA concentration were tested whether they support induction of acrosome reaction in a higher proportion of sperm. Finally, pharmacological assays were performed to characterize which AC is involved in the increase of membrane fluidity in stallion sperm under in *in vitro* conditions and in the acrosome reaction. To this end, direct and indirect inhibitors of sAC (KH7 and LRE1) and a specific stimulator of tmAC (forskolin) were used to decipher the sources of cAMP in these capacitation steps.

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CHAPTER 1 – PLASMA MEMBRANE REORGANIZATION IN STALLION SPERMATOZOA DURING IN VITRO CAPACITATION

INTRODUCTION

The use of artificial reproduction techniques (ARTs) as *in vitro* fertilization (IVF) demand efforts to develop methods to select a sperm population *in vitro* with high fertilizing potential, i.e. with full activation and complete capacitation (Grunewald et al., 2006; Sakkas, 2013). This becomes more important in the horse conventional IVF since only two foals have ever resulted from this technique, both born in France in the early 1990s (Palmer et al., 1991). In the conventional IVF a capacitated sperm penetrates a mature oocyte. The fact that *in vitro* matured oocytes transferred to the oviduct of an inseminated mare yielded a similar percentage of embryos to spontaneous ovulation (Hinrichs et al., 2002) and that *in vitro* treated sperm failed to penetrate *in vivo* and *in vitro* matured oocytes (Tremoleda et al., 2003) suggests that the deficit in equine IVF may reside in the inability to adequately induce capacitation of the stallion sperm in *in vitro* conditions.

During capacitation, several steps are required to lead the sperm to acrosome reaction and the ability to fertilize the oocyte. The majority of these events are changes in the architecture of the sperm plasma membrane which make it less stable and more fusible (Harrison, 1996). One of the early steps are the activation of the sAC/cAMP/PKA pathway leading to an increase in membrane fluidity and reorganization of the lipids in the plasma membrane with the translocation of phosphatidylserine (PS) and phosphatidylethanolamine (PE) to the outer leaflet of the sperm plasma membrane (Flesch and Gadella, 2000; Gadella and Harrison, 2002).

In non-capacitating conditions, the phospholipids PS and PE are preferentially located in the inner leaflet of the membrane. During capacitation, PS and PE exposure on live sperm cells has been considered a step related to fertilization ability (Tavalee et al., 2014). The presence of the capacitating element bicarbonate (HCO_3^-) in the medium already results in an activation of the scramblase that translocates phospholipids species back and forth across the lipid bilayer's membrane leading to an externalization of PS (Bevers et al., 1999). The asymmetry of the phospholipids in the plasma membrane is maintained by an amino phospholipid translocase that transports PS and PE from the outer to the inner

membrane leaflets (Sims and Wiedmer, 2001) thus the exposure of PS and PE is a result from a balance between amino phospholipid translocase and scramblase activities (Diaz and Schroit, 1996).

The exposure of PS is a process that is similarly occurring during capacitation and apoptosis (de Vries et al., 2003). In apoptosis, PS exposure can be considered an early event (Vance and Steenbergen 2005). However, PS externalization is also considered a capacitation marker in sperm since the incubated cells in capacitating conditions with exposed PS, did not show any signs of caspases or degeneration of mitochondria or DNA (human: de Vries et al., 2003; boar: Gadella and Harrison, 2002). Exposed PS also showed to be a capacitation event in human sperm (Kotwicka et al., 2001), and, the presence of PS externalized in mouse viable sperm showed to be important for sperm/egg fusion since the oocyte has receptors to recognize this the externalized PS (Rival et al., 2019).

The exposure of PS on the sperm surface can be visualized by the use of the Annexin-V. Annexins are a calcium dependent, lipid membrane binding protein family which have an affinity for the negatively charged phospholipids (Klee, 1988; Burgoyne and Geisow,1989; Rescher and Gerke, 2004). Annexin-V, a 35-36 kDa protein, has a high calcium-dependent binding affinity for the negatively charged PS (Tai et al., 1989).

Most events during capacitation depend on the activation of a protein kinase A (PKA) which occurs after an increase of cyclic adenosine monophosphate (cAMP) levels, produced by soluble adenylyl cyclase (sAC) activity (Visconti et al., 1995a). Phosphodiesterase (PDE) are enzymes that metabolize cAMP to 5'-AMP (Nelson and Cox, 2004) and it was shown that the addition of phosphodiesterase inhibitors, e.g. caffeine, can maintain high levels of cAMP in the cell promoting capacitation and spontaneous acrosome reaction with consequently increase in sperm motility (Stephens et al., 2013). Another way to raise intracellular cAMP levels is by adding its cell-permeable analogues in the medium. A chemically defined compound called dibutyryl cyclic adenosine monophosphate (db-cAMP) is an analog of the cAMP that penetrates the cell due to its increased lipid solubility (Smetatina et al., 2019) and

can support or enhance capacitation in some species including mouse and human (Fraser, 1981; Visconti et al., 1995b; Flaherty et al., 2004).

In order to elucidate to what extent stallion sperm progress beyond the very early stage of sperm capacitation and are prepared for the acrosome reaction, an assessment of intermediate stages in the process of membrane remodeling was carried out in the current study. The aim of this study was to investigate to what extent viable stallion sperm undergo a progressive re-organization of the plasma membrane after initiation of sperm capacitation. To this end PS exposure in viable sperm was monitored with flow cytometry and a live imaging approach under basic capacitating conditions, i.e. in the presence of bicarbonate, and after additional stimulation of the sAC/cAMP/PKA axis with caffeine and/or db-cAMP. Comparisons were made to membrane changes observed in a Tris-based medium which is employed for porcine IVF. The coincidence of PS exposure and occurrence of a spontaneous acrosome reaction in viable sperm was monitored. Finally, conformational changes in the spatial organization of the plasma and outer acrosomal membrane under capacitating conditions were visualized with cryo electron microscopy.

MATERIAL AND METHODS

1. Media

1.1. Tyrode's based media

The basic variant of Tyrode's medium (TyrControl) consisted of 111 mM NaCl, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 100 µg/mL gentamycin sulfate, 1.0 mM sodium pyruvate, 21.7 mM sodium DL-lactate. In the bicarbonate containing variant (TyrodeBic) a defined amount of NaCl was replaced by 30 mM of $NaHCO_3$. Both media contained 1 mg/mL of bovine serum albumin (BSA- A6002-25G – Sigma-Aldrich) and 2 mM of Ca^{2+} supplemented as $CaCl_2$. 1 mM of caffeine (Fluka – 27600) and/or 1mM of db-cAMP (D0260-25MG) were added to TyrControl and TyrBic for experimentation where indicated. The pH of all media was adjusted to 7.40 ± 0.05 at room temperature with NaOH or HCl and

the osmolality was adjusted to 300 ± 5 mOsmol/kg. All media were passed through a syringe filter (PES membrane, pore size 0.22 µm; Merck Millipore, Amsterdam, the Netherlands) for sterile filtration. The bicarbonate containing medium (TyrodeBic) and its variants were kept in an incubator with 5% CO_2 and 100% humidity at 37°C for at least 24 hours equilibration prior to experimentation. Incubations of spermatozoa in bicarbonate containing media took place in the same incubator used for equilibration. Incubations of spermatozoa in control medium (TyrControl) were carried out in a metal heating block at 37°C.

1.2. Porcine IVF medium

The porcine IVF medium consisted of 113 mM NaCl, 3 mM KCl, 11 mM glucose, 5 mM Na-pyruvate, 20 mM TRIS, 7.5 mM CaCl2, 1mg/mL BSA, 100 µg/mL penicillin/streptomycin and 1 mM caffeine. Osmolality was adjusted at 290 ± 5 mOsmol/kg. The IVF medium was passed through a syringe filter (PES membrane, pore size 0.22 µm; Merck Millipore, Amsterdam, the Netherlands) for sterile filtration and incubated for at least 18 hours in an incubator with 5% CO_2 and 100% humidity at 37°C in order to stabilize the pH at 7.6 prior to experimentation.

2. Semen collection and dilution

Semen was collected with an artificial vagina (Hanover model) from stallions attending the Faculty of Veterinary Medicine at Utrecht University for breeding soundness examination or from stallion located at nearby horse farms (Stal Schep and Stal Van Vliet). After collection, semen was filtered through gauze to remove the gel fraction and large debris. A smear of raw semen with Aniline Blue-Eosin was prepared for sperm morphology assessment. Concentration of the sample was determined by means of a Bürker Türk haemocytometer chamber and sperm was diluted in INRA96® (IMV, l'Aigle, France) to obtain a concentration of 30 x 10^6 spermatozoa/mL. Motility was objectively checked with a CASA (Computer-assisted sperm analysis) system (SpermVision 3.5, Minitüb, Tiefenbach, Germany) as described in Brogan et al. (2015). Only samples with more or equal than 70% motile sperm where used for experimentation. Diluted semen was kept at room temperature

until further processing took place. For each experiment semen from three to six different stallions was used.

2.1. Semen preparation for experimentation

Density gradient centrifugation was performed to separate the spermatozoa from the semen extender and seminal plasma prior to experimentation. Diluted semen (6 mL) was layered on top of a discontinuous gradient consisting of 2 mL of 70% Percoll®-saline solution and 4 mL of 35% Percoll®-saline in a 15-mL centrifugation tube as described by Harrison et al. (1993). Tubes were centrifuged for 20 minutes at room temperature: 10 minutes at 300 *g* followed by 10 min at 750 *g* without stopping in between. After centrifugation the supernatant was removed. The remaining pellet was resuspended in 1 mL of TyrControl without Calcium and BSA to obtain the concentration of the pellet with a Bürker Türk haemocytometer. After this, the dilution was completed until a concentration of 30 x 10^6 sperm/mL was obtained. The sperm suspension was used within 30 min for experimentation.

Experimental design

A total of nine media were evaluated at three different 3 times (15, 30 and 60 minutes). The list of media was as follows:

1. TyrControl
2. TyrControl + 1mM caffeine
3. TyrControl + 1mM db-cAMP
4. TyrControl + 1mM caffeine + 1mM db-cAMP
5. TyrBic
6. TyrBic + 1mM caffeine
7. TyrBic + 1mM db-cAMP
8. TyrBic + 1mM caffeine + 1mM db-cAMP
9. Porcine IVF medium

3. Evaluation of Annexin-V signal in different media in flow cytometry

Bicarbonate is supposed to directly stimulate soluble adenylyl cyclase (sAC) and thus have its effect on phosphatidylserine (PS) exposure by stimulating the sAC/cAMP/PKA pathway (Gadella and Harrison 2002). The aim was to investigate the effect of sAC/cAMP/PKA pathway stimulators, namely dibutyryl-cAMP (db-cAMP) and caffeine (caff), on PS exposure in live spermatozoa. Dibutyryl-cAMP is a membrane permeable cAMP analogue directly increasing intracellular cAMP. Caffeine is a phosphodiesterase inhibitor, preventing degradation of endogenous cAMP, and thus increases cAMP in an indirect manner.

For this purpose, 10 µl of the Percoll-washed sperm was added to pre-incubated FACS tubes containing 500 µl Tyrode's media (TyrControl or TyrBic) with either 1 mM of caffeine, or 1mM of db-cAMP or both. TyrControl and TyrBic free of caff or db-cAMP were used as a control media to monitor the effect of the basic compounds. The porcine IVF medium already contained caffeine and was used as is.

All media contained 2 µl each of Hoechst 33258 (Sigma-Aldrich 94403, Zwijndrecht, the Netherlands) (stock solution: 0.1 mg/mL in aqua dest) and PNA-AlexaFluor™647 (PNA-AF647) (ThermoFisher Scientific C10634, Waltham, USA) (stock solution: 0.25 mg/mL in aqua dest). Nine tubes were prepared for each time point (see section on experimental design).

Fifteen minutes before measurements took place, 100 µL of a tube's content was transferred to each of two new prewarmed tubes and 2 µl of Annexin-V FLUOS (11828681001- Merck, Darmstadt, Germany) was added. The Annexin V staining was either carried out in presence of 0.2 µL of M540 (Molecular Probes M24571, Eugene, OR, USA) (stock solution: 750 mM in DMSO) or in absence of M540. Samples were assessed after 15 min, 30 min and 60 min of incubation.

Before analysis on a FACS Canto IICalibur flow cytometer (BD Biosciences, Breda, The Netherlands), samples were briefly vortexed. Samples were kept on 37°C and under CO_2 atmosphere (TyrBic and porcine IVF medium) during transport from the incubator to the flow cytometer (less than 30 seconds).

Excitation of Hoechst 33258 was achieved by a 405 nm laser and fluorescence was collected by a 450 nm BP filter. For PNA-AlexaFluor™647 a 633 nm laser was used and fluorescence was captured with a 661 nm BP filter. Excitation of M540 was achieved by using a 488 nm laser and fluorescence was collected by a 585 BP filter. For excitation of Annexin-V a 488 nm laser was used and fluorescence was captured with a 515 nm BP filter.

For each sample, data from 10,000 individual spermatozoa were acquired at medium speed (35 µl ±5 µl/min). Compensation of spectral overlap between dyes was compensated post acquisition. Data were analyzed using FCS Express (version 3, De Novo Software, Glendale, CA, USA).

First, a forward and side scatter plot was used to the determine the gate for the single sperm population. For further evaluation, only data from gated population were considered (Figure 1). Thresholds for quadrants that distinguish between Hoechst 33258 positive (dead) sperm and Hoechst 33258 negative (live) sperm with either: low PNA-AlexaFluor™647 signal (acrosome intact) or high PNA-AlexaFluor™647 signal (acrosome defect/reacted); low M540 signal (M540low; low membrane fluidity) or high M540 signal (M540High; high membrane fluidity) and low Annexin-V signal or high Annexin-V signal (PS exposure) fluorescence intensity were set.

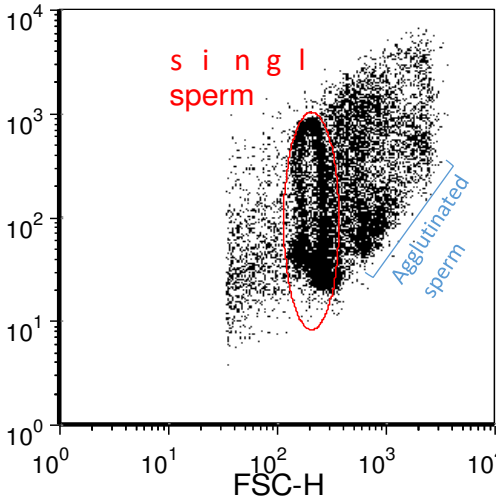


Figure 1: A general overview of the gating strategy on flow cytometer. Forward scatter (FSC) and side scatter (SSC) are shown in the figure and gated population represents the single sperm population which were used for further analysis.

The percentage of live, acrosome intact spermatozoa positive for PS (Hoechst 33258 and Alexa-Fluor 647 negative, Annexin-V positive in the absence of M540 compared to Hoechst 33258 and Alexa-Fluor 647 negative, Annexin-V positive in the presence of M540) were evaluated to check the influence of the sAC/cAMP/PKA pathway stimulators and the porcine IVF medium on the PS exposure during stallion sperm capacitation. Also the M540 interference in the detection of Annexin-V positive sperm was evaluated.

Another evaluation focused on the effect of intracellular cAMP upregulation on the subpopulations of Annexin-V and/or PNA positive sperm inside the cells that were viable and M540 positive. All medium variants based on TyrControl were excluded from this analysis since the main goal was to check the behavior of the subpopulation that already underwent an early capacitation step, i.e. were M540 positive, in capacitating conditions, i.e. in TyrBic-based media and a porcine IVF medium. After gating the population of interest (Figure 2 – blue gate), another graph at FCS Express was made comparing the Annexin-V and PNA-FITC signal for this subset of sperm (Figure 3) in the different media and time.

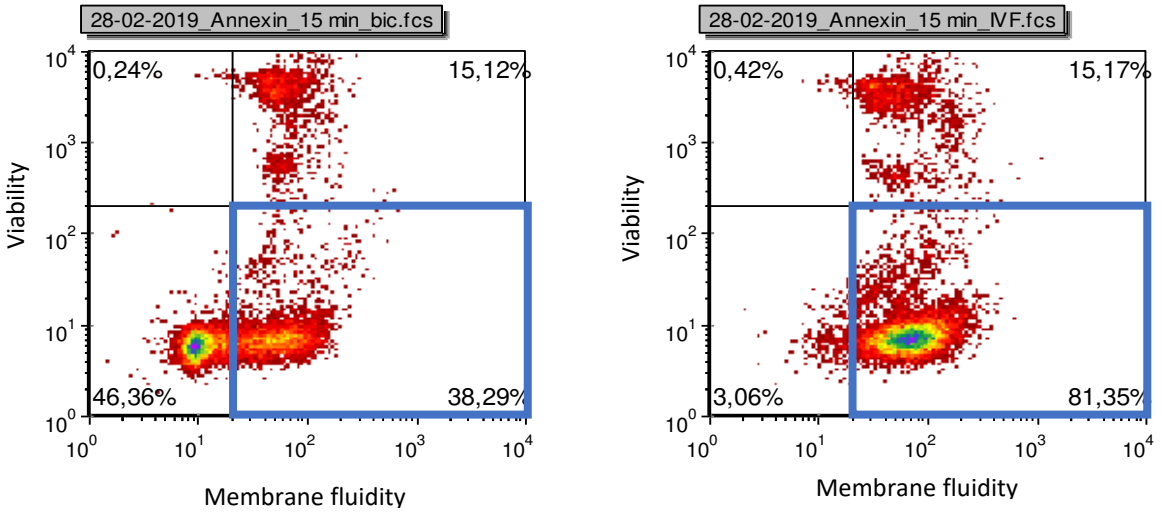


Figure 2: Exemplary dot plot graphic from flow cytometry showing in the blue gate the population of single sperm identified as being viable with high membrane fluidity in TyrBic (left side) and a porcine IVF medium (right side).

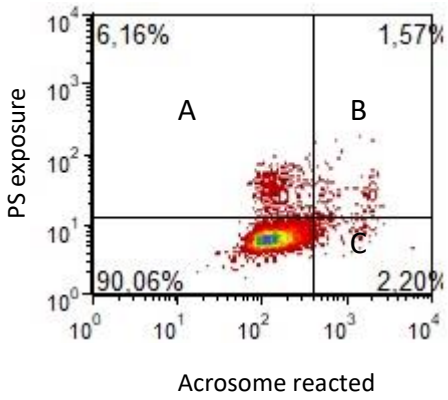


Figure 3: Exemplary dot plot graphic from flow cytometry showing three different sperm populations that progressed beyond the stage of having a high membrane fluidity: (A) only Annexin-V positive; (B) Annexin-V and PNA-Alexa Fluor 647 positive and (C) only PNA Alexa Fluor 647 positive sperm.

4. Annexin-V staining pattern

Flow cytometry is only able to indicate whether or not a fluorescent staining is present in/on a cell. The exact location of a signal, such as where Annexin V binds and thus PS residues are exposed is not possible to determine with flow cytometry. Therefore, a live imaging approach was used to demonstrate the Annexin-V staining pattern in stallion sperm under capacitating conditions. Three animals were used for this experiment.

For this purpose, 10 µL of Percoll-washed sperm ($120x10^6$ sperm/mL) was added to pre-incubated FACS tubes containing 500 µL of either TyrBic, TyrBic + caffeine and porcine IVF media. The chosen media for this purpose was because of the higher Annexin-V intensity in the previous experiments. All media contained 2 µl of each Hoechst 33258 (stock solution: 0.1 mg/mL in aqua dest.) and PNA-AlexaFluor™647 (stock solution: 0.25 mg/mL in aqua dest). Samples were evaluated after a total incubation time of 30 minutes and 60 minutes, respectively. 15 minutes before measurements took place, 100 µL of the tube content was transferred to a new one and 2 µl of Annexin-V FLUOS was added.

Live imaging was performed on a NIKON STORM/A1Rsi/TIRF microscope, with preheated desk at 37 °C. After incubation, the samples were centrifuged for 2 minutes at 1000 xg and then resuspended in 10 µl of the respective medium. Next,

a 1 µl droplet was placed in a FluoroDish Cell Culture Dish (FD35-100, World Precision Instruments, Friedberg, Germany) and covered with a round coverslip (8 mm ø). Mineral oil was put around the coverslip to prevent the sample from drying.

The autofocus (range of 20) of the microscope was performed and the software general image settings in ND acquisition were: 40x objective, lasers 405 nm, 488 nm, 647 nm, channels (Hoechst, Alx488/Alx647/DIC); scan speed ½; size image of 1024x1024 and zoom 1. A large scan was performed in a 2x2 panel series (at least 5 per sample) and the pinhole was 5.0. In the Nis Element Viewer software (Nikon, NY, USA), the first step of the image analysis was an automated detection of the different fluorescent stainings in each cell. Subsequently, the staining pattern were visually validated and evaluated (Figure 4). At least 200 viable cells were evaluated in each treatment.

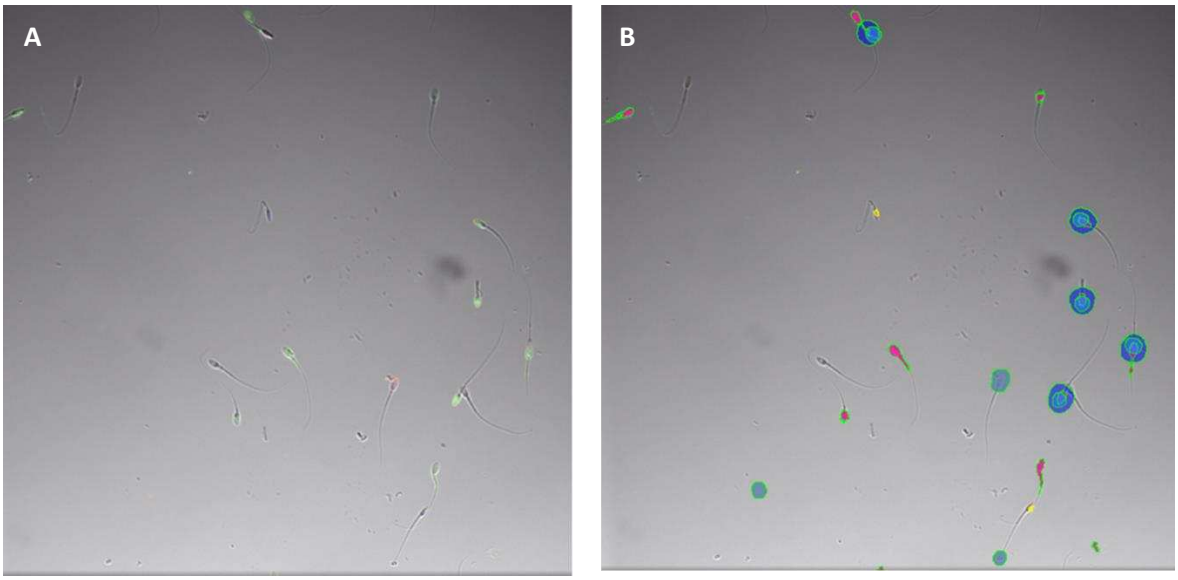


Figure 4: Image captured from a NIKON STORM/A1Rsi/TIRF microscope in Nis Element Viewer software. First image (A) shows the raw overlay image of sperms with different fluorescent labels. The second image (B) shows the sperms already tagged by an automated equation which identifies which fluorescent signals (Hoechst 33258 (yellow tag), Annexin V Fluos (pink tag), PNA Alexa Fluor 647 (light blue) are present in each cell. Dark blue indicates the presence of more than one staining in the cell. Non marked cells represent viable cells without any fluorescence.

5. Sorting process for Cryo-EM

For population sorting, a FACS Influx was used (Becton Dickinson, San Jose, Canada). A total of $4x10^7$ spermatozoa were incubated in either 0.5 mL TyrControl or TyrBic supplemented with 1 mg/mL BSA and 2 mM Ca^{2+} for 60 minutes with 2 µl of each Hoechst 33258 (0.1mg/mL) and PNA-Alexa Fluor 647 (0.25 mg/mL). 2 µl of M540 (750 mM) was added 15 min before sorting.

The system was triggered on the forward light scatter signal (FSC). Hoechst 33258 was excited with a 405nm Laser. Emission was captured with a 460/50 nm filter. M540 was excited with a 561 nm laser, and emission was captured with a 585/42 nm filter. Sperm was analyzed at a rate between 8,000 and 10,000 events per second. FSC and sideward light scatter (SSC) were recorded and only sperm cell specific events, were positively gated for further analysis.

During sorting the sample-input tube on the FACS was kept at 38°C to maintain incubation temperature during the complete sorting procedure. Sperm cells were run through the machine using PBS as a sheath fluid. Two subpopulations were sorted: (1) sperm cell events that were not stained with Hoechst 33258 (viable) showing low M540 fluorescence from the control medium; and (2) viable cells with high M540 fluorescence from the bicarbonate medium.

The sorting process time per tube was from 8-15 minutes. The concentration of the sorted sample was approximately 250.000 cells per tube. Immediately after this process, the sorted cells were then centrifuged at 11,000 g for 10 minutes. The supernatant was discarded and the pellet resuspended with PBS to a concentration of ~3 x 10^6 cells/mL.

5.1. Cryo-Electron Tomography Sample Preparation

Sorted sperm from three stallions were diluted to ~3 x 10^6 cells/mL in PBS. Approximately 3 µl of cell suspension was applied to glow-discharged Quantifoil R 2/1 200-mesh holey carbon grids. Approximately 1 µl of BSA-gold (Aurion) was added, after which grids were blotted manually from the back for 4-6 s and immediately plunged into a 37% liquid ethane/propane mix cooled to liquid nitrogen temperature. Grids were stored under liquid nitrogen until imaging.

5.2. Cryo-Electron Tomography

Imaging was performed on a Talos Arctica (ThermoFisher) operated at 200 kV and equipped with a post-column energy filter (Gatan) in zero-loss imaging mode with a 20-eV energy-selecting slit. All images were recorded on a ~ 4k X 4k K2 Summit direct electron detector (Gatan) in counting mode with dose-fractionation.

Tilt series were collected with SerialEM, using a grouped dose-symmetric tilt scheme covering an angular range of ± 56° in 2° increments. Tilt series were acquired with a Volta phase plate (VPP) at a target defocus of -0.75 µm and with a pixel size of 3.514 Å. The total dose was limited to < 100 e-/Å2.

5.3. Tomogram Reconstruction

Frames were aligned using Motioncor2 1.2.1. Tomograms were reconstructed in IMOD 4.10.25 using weighted back-projection. As tilt series were acquired close to focus with the VPP, CTF correction was not performed. For segmentation and presentation, 6X-binned tomograms were reconstructed with a SIRT-like filter corresponding to 20 iterations.

STATISTICAL ANALYSIS

Statistical analysis was performed, using SPSS statistics, version 25 (IBM analytics, Amsterdam, The Netherlands). Data were tested for normal distribution with a Shapiro-Wilk test and compared with a multi-factorial ANOVA for repeated measures. Considered factors differed according to the experiment. They included media (TyrControl, TyrBic) and the presence of caffeine and dybutyryl-cAMP. Paired Student's t-tests for dependent samples were performed to investigate differences between individual medium variants. Unless otherwise stated, data are presented as mean ± standard deviation (SD). Differences were considered to be significant at a significance level of 5% ($p \leq 0.05$).

RESULTS

1. Influence of bicarbonate and cAMP upregulation on plasma membrane fluidity

In TyrControl there was an increase in the population of live, M540 positive sperm in the presence of caffeine alone or in combination with db-cAMP. There was no increase with dbcAMP alone. A distinct increase in membrane fluidity comparing TyrBic and TyrControl (both no additions), especially after 15 minutes incubation, indicates the importance of bicarbonate in this early capacitation event. Caffeine and db-cAMP were alone or in combination with each other able to further increase the percentage of the viable M540 positive population in TyrBic compared to only bicarbonate in all times. (Table 1).

Table 1: Percentage live, M540 positive sperm in different base media either without bicarbonate (TyrControl) or with 30 mM bicarbonate (TyrBic). Media where supplemented with either 1 mM db-cAMP, 1mM caffeine, or both to increase intracellular cAMP levels.

Media/Time (min)	15	30	60
TyrControl			
Control	5.0 ± 1.0^{cB}	5.3 ± 0.6^{cB}	5.9 ± 0.6^{dB}
db-cAMP	5.2 ± 0.5^{cB}	6.7 ± 0.8^{cB}	10.0 ± 1.5^{cB}
Caffeine	34.8 ± 12.8^{bB}	28.7 ± 6.8^{bB}	25.6 ± 3.8^{bB}
Caff+db-cAMP	41.0 ± 12.5^{aB}	$55.3 \pm 3.^{5aA}$	53.2 ± 2.6^{aA}
TyrBic			
Bic	45.7 ± 6.0^{cA}	33.5 ± 4.1^{cA}	27.4 ± 4.6^{bA}
db-cAMP	$52.2 \pm 5.0b^{bA}$	44.2 ± 3.3^{bA}	35.1 ± 2.7^{aA}
Caffeine	76.3 ± 1.8^{aA}	54.4 ± 2.9^{aA}	38.2 ± 2.0^{aA}
Caff+db-cAMP	76.5 ± 2.1^{aA}	55.1 ± 2.7^{aA}	39.1 ± 2.3^{aB}

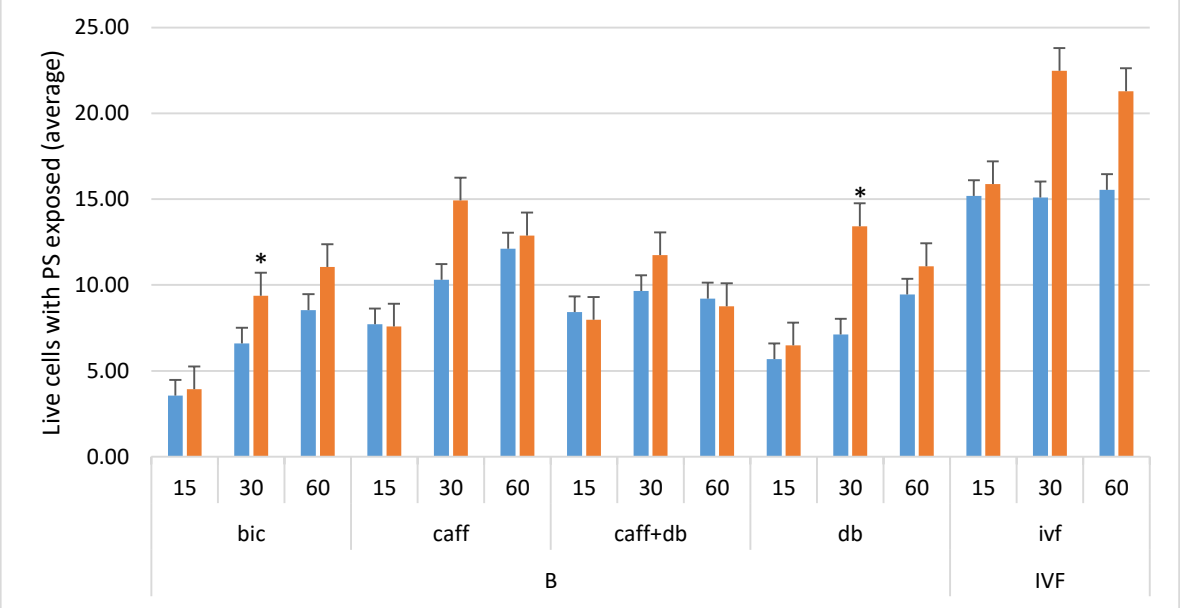
Different small letters indicate effects of the supplements in a given medium and at a given time. Different capital letters indicate difference between the same treatment in TyrControl and TyrBic at a given time point ($p<0.05$). All values are mean ± SD

2. Influence of M540 staining on the Annexin V staining

In all variants of control group (TyrControl without or with caff and/or db-cAMP) the presence of M540 did not interfere in the Annexin-V signal.

In major media of TyrBic without and with caffeine and/or db-cAMP, M540 did not interfere in the signal of Annexin-V. The only difference was that, without M540, the signal of Annexin-V was higher in the TyrBic without any stimulator in time 30 minutes and in TyrBic+db-cAMP at the same time. The presence or not of M540 showed no difference in the porcine IVF medium (Graphic 1).

This leads to the conclusion that the fluorescence intensity of M540 does not interfere in the Annexin-V signal making possible the use of both dyes together in flow cytometer trials.

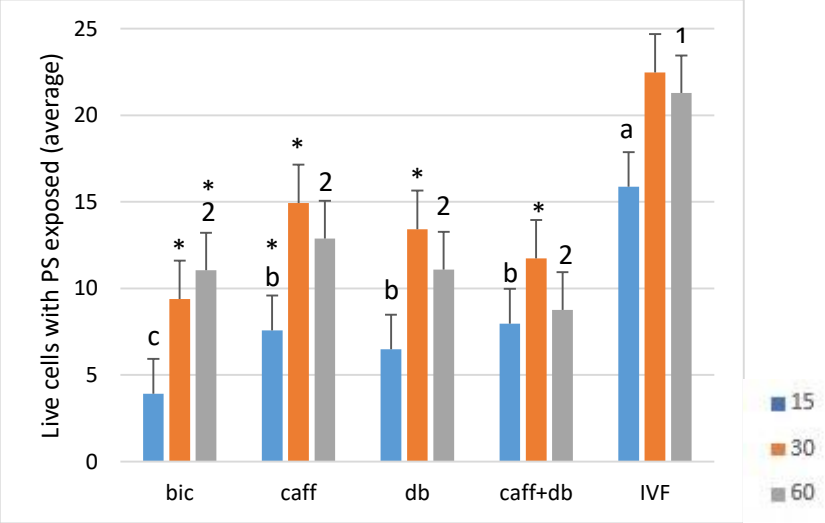


Graphic 1: Population of live, Annexin-V positive spermatozoa (with PS exposed) in TyrBic (in the presence or not of Caffeine and db-cAMP) and porcine IVF media after 15, 30 and 60 min of incubation and in the presence (blue bars) or absence (orange bars) of merocyanine (M540). An asterisk indicates significant differences between both medium variants ($p<0.05$; mean ± SD).

3. Impact of bicarbonate and cAMP upregulation on PS exposure

For this purpose, the experiment without M540 was considered. In graphic 2 is shown the percentage of live, Annexin-V positive cells in the TyrBic media (with all the variants) and in the porcine IVF medium in different time of incubation.

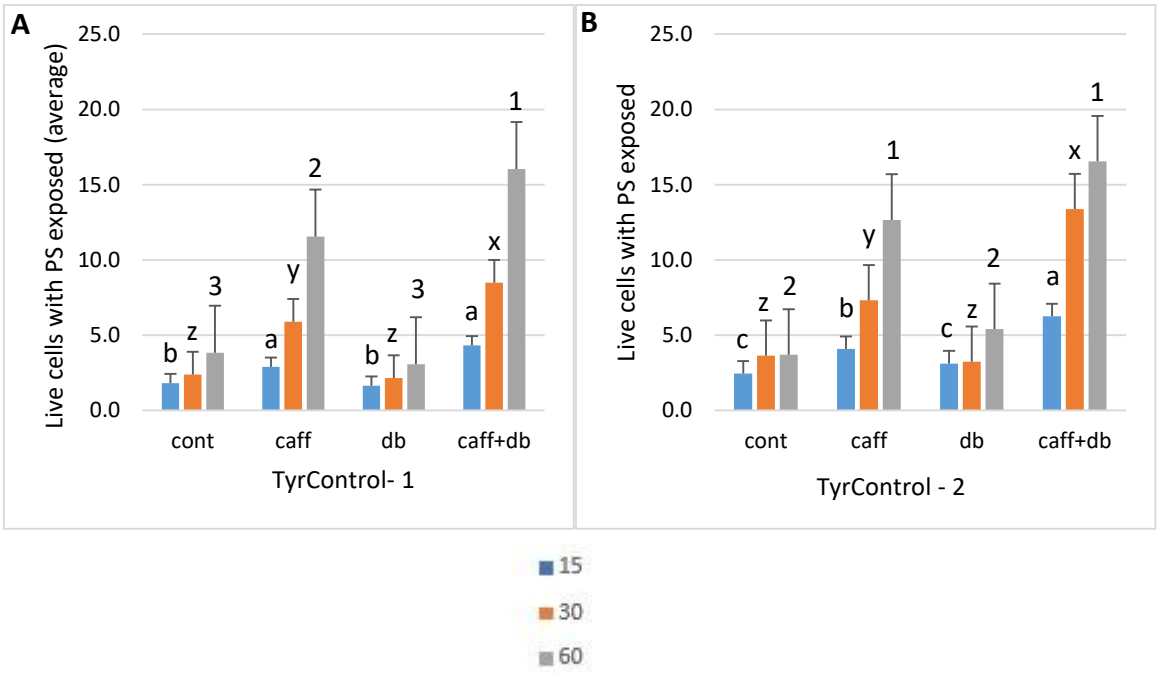
The presence of the sAC/cAMP/PKA pathway stimulators increased the Annexin-V signal in the first 15 minutes of incubation only. After 30 and 60 minutes of incubation the percentage of live, Annexin-V positive cells are the same as in the TyrBic medium without caffeine and db-cAMP. The stars indicate the difference between the TyrBic media end the TyrControl media indicating the importance of the bicarbonate for the increase of the population with phosphatidylserine exposure. The porcine IVF medium had a higher percentage of sperms with PS exposure in times 15 and 60 minutes compared to all TyrBic variants.



Graphic 2: Population of live, Annexin-V positive spermatozoa (with PS exposed) in TyrBic (in the presence or not of caffeine and db-cAMP) and porcine IVF medium after 15, 30 and 60 min of incubation. Letters and numbers indicate the difference between the media in the same time and stars indicate the difference between the TyrBic and TyrControl media (graphic 3) ($p<0.05$; mean ± SD).

In both experiments (with or without M540) the signal intensity of Annexin-V was higher in the presence of caffeine associated or not with db-cAMP in TyrControl at 60 minutes of incubation, probably because of the stress caused in the cells leading them to apoptosis. There was no difference between the different incubation

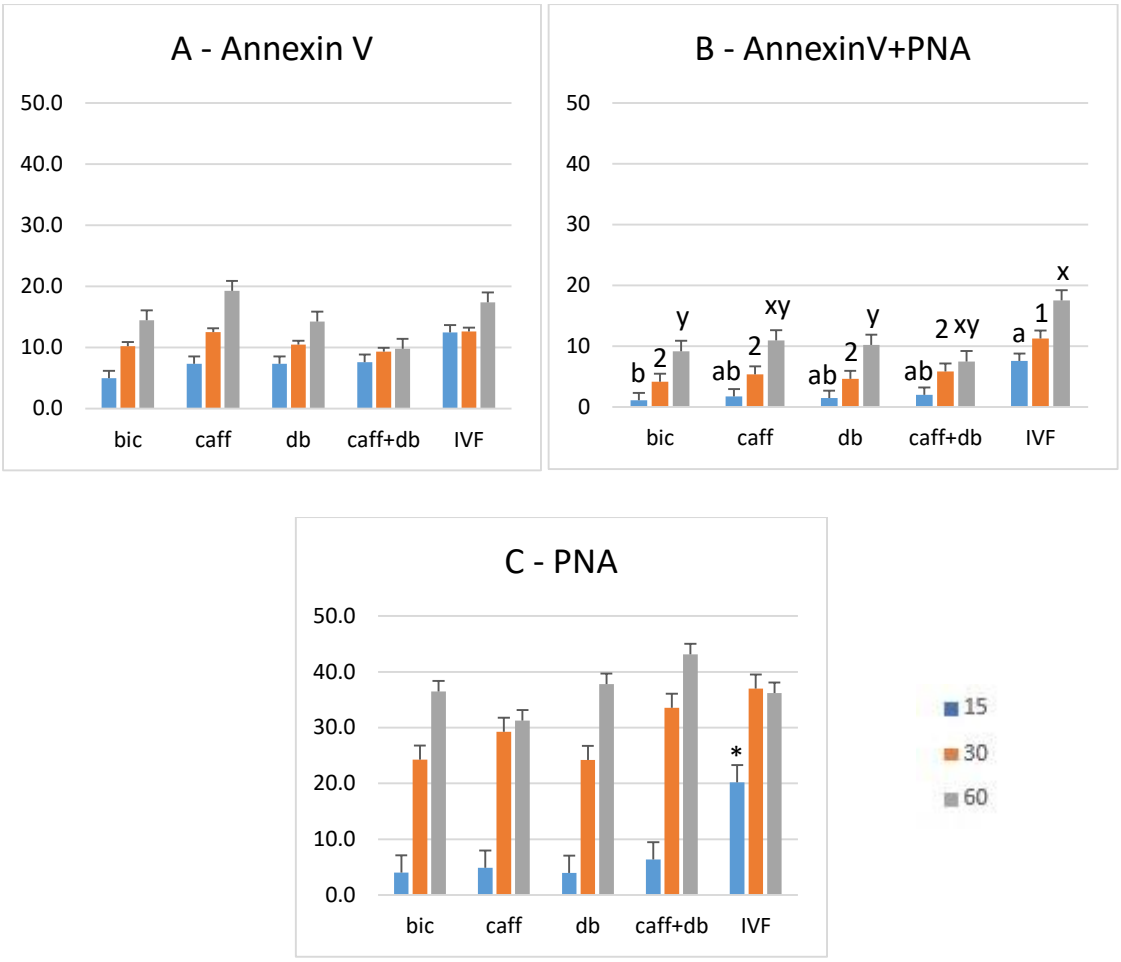
time in the TyrControl medium without caffeine or db-cAMP showing that only in the presence of the sAC/PKA pathway was possible to raise the Annexin-V signal in control group (graphic 3).



Graphic 3: Population of live, Annexin-V positive spermatozoa (with PS exposed) in TyrControl (in the presence or not of Caffeine and/or db-cAMP) with (A) and without (B) M540 after 15, 30, and 60 min of incubation. Letters and numbers indicate the difference between the media in the same time ($p<0.05$; mean ± SD).

4. Coincidence of high plasma membrane fluidity, PS exposure and a spontaneous acrosome reaction in viable stallion sperm during capacitation

The presence of caffeine and/or db-cAMP did not increase more than bicarbonate, the expression of the live, Annexin-V population indicating that the sAC/cAMP/PKA pathway stimulators have no additive effect on the PS exposure (Graphic 4 A). Considering only the M540 positive sperm with only PS exposure, results for the porcine IVF medium were not different when compared to a basic TyrBic or variants with caffeine or db-cAMP. In contrast, a higher percentage of Annexin-V and Alexa Fluor-PNA 647 positive sperm was found in some time point when compared to a basic Tyrode's medium, (graphic 4, B).



Graphic 4: Population of live, M540 positive sperm was further dissected for being either only Annexin-V positive (A), Annexin-V + PNA-Alexa Fluor-647 positive (B) or only PNA-Alexa Fluor-647 (C) positive after 15, 30 and 60 min of incubation in TyrBic with either no addition (none), 1mM caffeine (caff), 1 mM db-cAMP (db) or both (caff+db) and a porcine IVF medium. Different letters or numbers indicate significant differences between the media in the same time and star in (C) indicate the difference between 15 minutes incubation in IVF ccompared to all TyrBic variants ($p<0.05$; mean ± SD).

5. Annexin V staining patterns in viable sperm

The fluorescent pattern of Annexin-V in viable stallion sperm under capacitating conditions, i.e. in TyrBic, TyrBic+caff and porcine IVF medium, was showing three different patterns described as staining (1) in the acrosome region; (2) in whole area of the sperm head and (3) in the head and in the midpiece (Figures 6, 7 and 8 and graphic 5). Most cells that had a full head staining pattern also had

fluorescence for PNA-Alexa Fluor-647 in the acrosome region (Figure 7) indicating that those cells were already acrosome reacted. The staining pattern in dead sperm cell had all probes showing its fluorescence or only Hoechst 33258 (blue).

The predominant staining pattern in TyrBic was a staining in the acrosome region followed by the entire head and head + midpiece respectively. In contrast, all three staining patterns for Annexin-V in viable sperm were almost equally abundant in the porcine IVF medium.

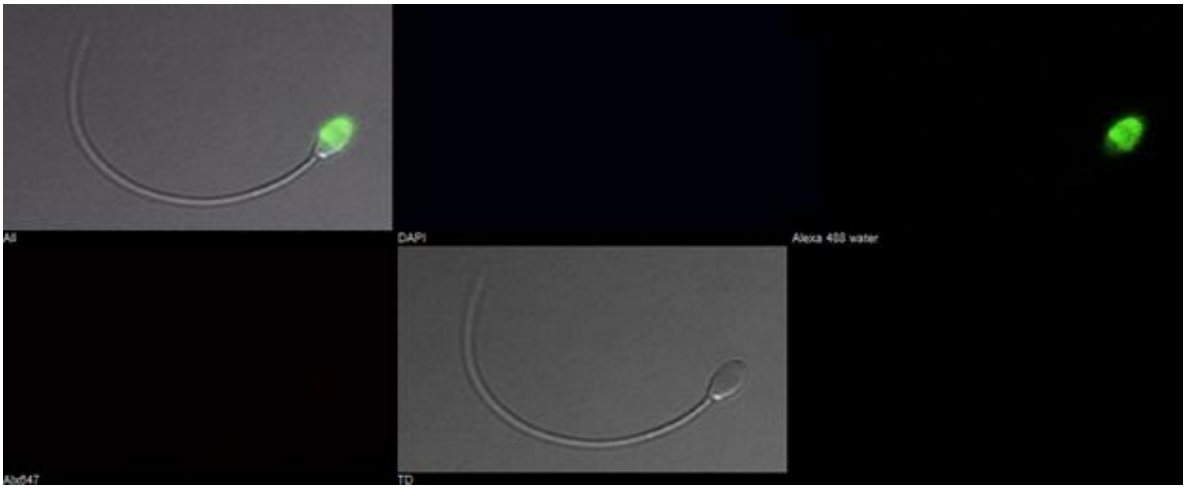


Figure 6: Staining pattern of Annexin-V in a viable stallion spermatozoon in the acrosome region. 40x objective; channels: DAPI (Hoechst 33258); Alexa488 (Annexin-V Fluos); Alx647 (PNA-Alexa Fluor 647) and TD (Differential interference contrast). Size image of 1024x1024 pixel.

Figure 7: Staining pattern of Annexin-V in stallion spermatozoa filling all the head. Also the presence of the staining of Alexa Fluor 647 in the acrosome region. 40x objective; channels: DAPI (Hoechst 33258); Alexa488 (Annexin-V Fluos); Alx647 (PNA-Alexa Fluor 647) and TD (Differential interference contrast). Size image of 1024x1024 pixel.

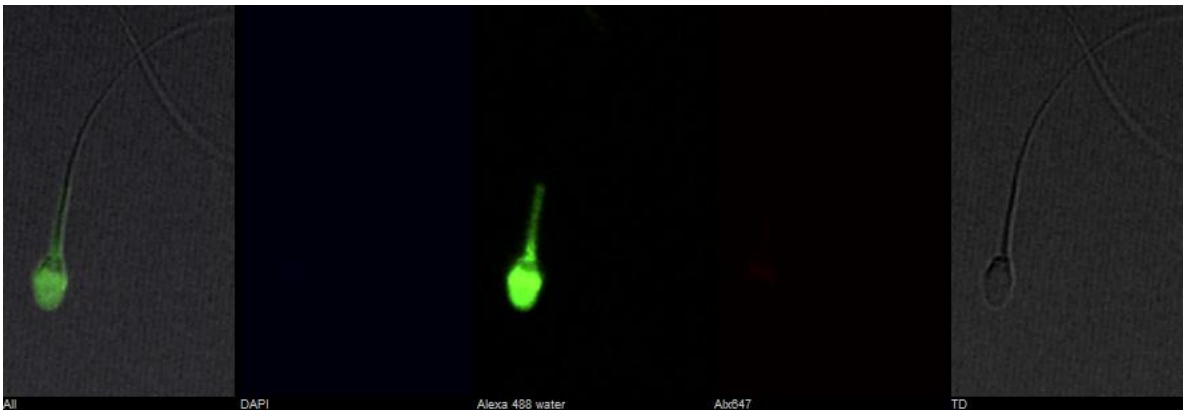
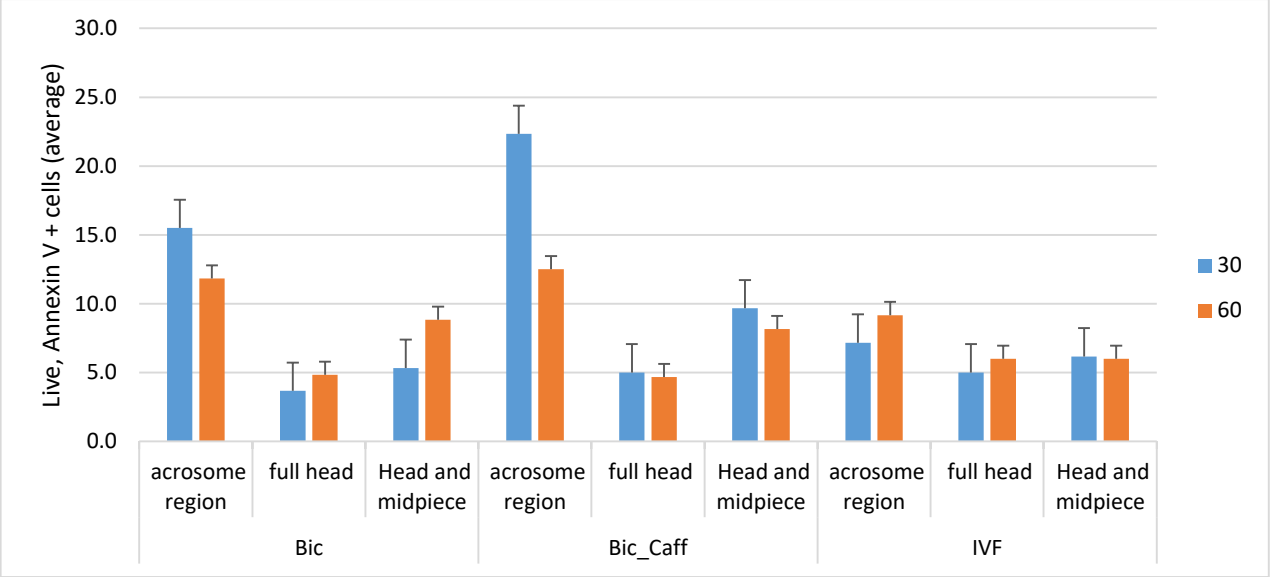


Figure 8: Staining pattern of Annexin-V in stallion spermatozoa in the head and midpiece. 40x objective; channels: DAPI (Hoechst 33258); Alexa488 (Annexin-V Fluos); Alx647 (PNA-Alexa Fluor 647) and TD (Differential interference contrast). Size image of 1024x1024 pixel.



Graphic 5: Staining pattern of Annexin-V in the viable sperm population after 30 min and 60 min of incubation in a basic bicarbonate-containing Tyrode's medium with 1mM caffeine (TyrBic+Caff) or without (TyrBic) and a porcine IVF medium. Data are means ± SD for 200 viable sperm.

6. Cryo-EM

In TyrControl media it was possible to measure the plasma membrane (PM) and the outer acrosomal membrane (OAM) thickness and the distance between the PM and the OAM. In TyrBic, there was a considerable decrease in the distance between the PM and the OAM indicating some rearrangement of the intermembrane space in the presence of bicarbonate. OAM was not possible to have a reliable measurement due difficulties in distinguish its borders.

Table 2: *Measurements of the stallion sperm regions in nanometers (nm)*

	PM thickness	OAM thickness	Intermembrane distance (PM – OAM)
TyrControl	5.3± 0,51	8.4 ± 1,71	10.2 ± 2,18[a]
TyrBic	5.3± 0,45	-	5.8 ± 1,14[b]

Measurements of the PM and OAM thickness and intermembrane distance between PM and OAM (nm; Mean ± SD).

Despite not being able to measure the thickness of the OAM in TyrBic medium some tomograms indicate that the space between PM and OAM is changing in the stallion sperm membrane in capacitating conditions (Figures 9 and 10).

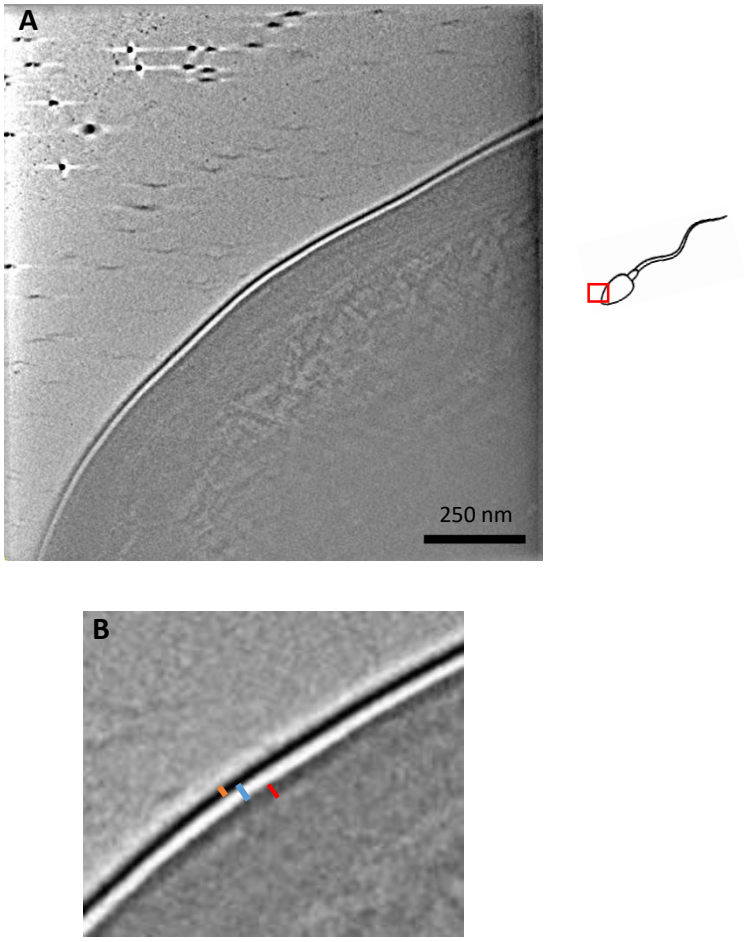


Figure 9: Still image from tomogram of a sperm cell in TyrControl medium showing the intact PM (B: orange line); the space between the PM and OAM (B: blue line) and the OAM (B: red line) demonstrating a homogenous organization of the structures. The red box indicates the region of the sperm head that is shown in the tomogram.

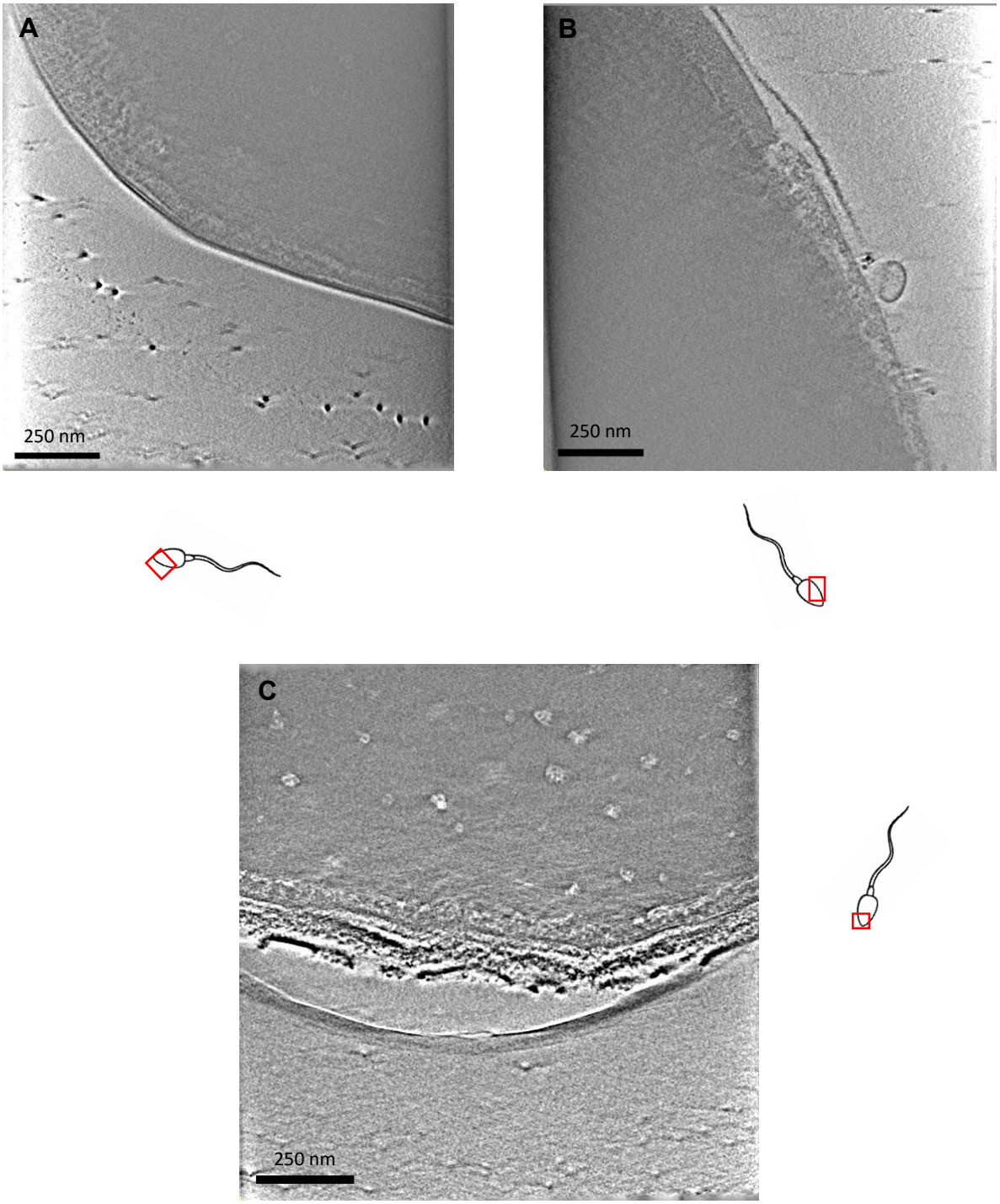


Figure 10: Tomogram of sperm cells in TyrBic medium showing (A) the reduce in the space between the PM and the OAM; (B) loss of the PM in some areas and vesiculation of the membranes; and (C) twisting membranes for possible vesiculation formation for acrosome reaction. The red box indicates the region of the sperm head that is shown in the tomogram.

DISCUSSION

In the fertilization process the sperm must undergo a process called the acrosome reaction (Yanagimachi, 1994), but to get to this stage, the cell must pass through capacitation steps which bring the plasma membrane in a metastable fusible state (Gadella and Harrison, 2002). The process can be achieved in capacitating conditions *in vitro* where bicarbonate is a key element.

An early alteration induced by bicarbonate is an increase in membrane phospholipid packing disorder detected by the fluorescent amphiphilic probe merocyanine 540 (Harrison et al., 1996). The increase in the live, M540 positive sperm population in TyrBic in our study (table 1) and the sperm surface seen in figure 10A indicates that this capacitation step is easy achieved in a large number of stallion sperm in the presence of bicarbonate. It is even more stimulated when there is an increase in the intracellular cAMP levels. This confirms earlier reports in which this step of capacitation has been demonstrated in stallion sperm and sperm from other species (pig: Harrison et al., 1996, stallion: Rathi et al., 2001; dog: Steckler et al., 2015).

The increase in membrane phospholipid packing disorder leads to an activation of phospholipid scramblase which in turn is collapsing the lipid asymmetry in the plasma membrane lipid bilayer. As a result, the exposure of PE and PS at the outer surface of the spermatozoa can be detected (Williamson and Schlegel, 1994; Harrison and Gadella, 2002). Gadella and Harrison (2002) incubating boar sperm for 2 h in a bicarbonate containing medium showed that the presence of 15 mM of HCO_3^- induced exposure of PS in a substantial subpopulation of intact cells (around 48.5 ± 17.2%) reaching a maximum of exposure at 60 minutes of incubation. Incubating human sperm with bicarbonate (15 or 24 mM) for 4h de Vries et al. (2003) reached a steady-state labeling for PS only at 90 min of incubation. However, at 30 min of incubation the percentage of the population with PS exposed was around 25% while at 4h was 40.6 ± 15.7%. In our experiment, after 60 min of incubation in a bicarbonate medium, exposure of PS is induced in only 12% of the stallion sperm population and in 23.1% in porcine IVF medium (graphic 2). However, Kurz et al. (2005), incubating boar sperm in capacitating conditions (20 mM of bicarbonate) for

1h showed that only 6 ± 5% of the whole sperm population bound exclusively to Annexin-V, whereas a large proportion of the cells (56 ± 11%) was found to be viable and labeled by neither FITC-Annexin-V nor propidium iodide (PI). They conclude that PS does not become exposed on the outer surface of the viable cells in capacitating conditions.

Despite contradictory results in boar sperm, to our knowledge this is the first experiment to show the PS exposure in stallion sperm in capacitating conditions, although the percentage if Annexin V positive sperm is lower compared to other species. In the stallion sperm cell, the PS exposure must have taken place at the external surface of the plasma membrane after 30 min of incubation with bicarbonate and 15 min of incubation in porcine IVF medium (graphic 2) and must have stemmed from a collapse (or scrambling) of the plasma membrane's phospholipid asymmetry. This is also confirmed when looking at graphic 3, where in TyrControl medium without caffeine or db-cAMP there was no difference in the population that is live, Annexin-V positive between the times (15, 30 and 60 minutes). Only in the presence of sAC/cAMP/PKA pathway stimulators this was possible in control group and in the TyrBic media with or without caffeine or db-cAMP there was already difference in the percentage of this population indicating the importance of the HCO_3^- in this event.

Changes in the transbilayer movements of phospholipids are controlled through a cAMP-dependent phosphorylation pathway in the same way as increases in merocyanine stainability (Gadella and Harrison, 2000; Harrison and Miller, 2000). The presence of caffeine associated or not with db-cAMP in TyrControl media increased the percentage of live, M540 and Annexin-V positive population (table 1 and graphic 3). In the bicarbonate group both molecules only increased the percentage of the population with PS exposed in the firsts 15 minutes of incubation being equal to only bicarbonate at 30 and 60 minutes of incubation (graphic 2). Regarding the live, M540 positive cells, caffeine and db-cAMP associated or not made the increase on this population in the presence of bicarbonate (table 1).

Gadella and Harrison (2002) incubating boar sperm for 2h in a Hepes-buffered bicarbonate-free Tyrode medium (HBT) with phosphodiesterase inhibitor papaverine resulted in PS exposure similar to that induced in HBT-Bic (50.6 ± 18.3

x 48.5 ±17.2, respectively) and similar results were found when the incubation was with cAMP analogue 8Br-cAMP (44.1± 17.5%). Similar results were found in the experiment performed in human sperm by de Vries et al. (2003). In our experiment the increase in PS exposure was higher only at the firsts minutes of incubation with caffeine or db-cAMP. Even though, these findings again indicated that bicarbonate in TyrBic media is the element that exerted its effect increasing cAMP levels which are leading to a phospholipid rearrangement. The sAC/cAMP/PKA pathway stimulators can accelerate, but not increase the process in TyrBic media. Caffeine was already used with success in *in vitro* conditions to increase motility in mouse sperm enhancing fertilization (Fraser, 1979; Nabavi et al., 2013) and induce ram sperm hyperactivation (Colás et al., 2010; Spaleková et al., 2011). The use of caffeine alone or associated with db-cAMP in macaque sperm also increased the number of sperm bound to the zona pellucida and db-cAMP alone or with caffeine resulted in a higher percentage of acrosome-reacted sperm. Also sperm hyperactivation was achieved only when both molecules were used together (Vandevoort et al., 1994). It is believed that caffeine activates a calcium permeable cation channels in the plasma membrane (Schoppe et al., 1997) enabling the entrance of Ca^{2+} inside the cell promoting the hyperactivated status. Tyrosine phosphorylation of bull (Galatino-Homer et al., 1997), stallion (Pommer et al., 2003) and ram sperm (Colas et al., 2008) is also increased in the presence of caffeine and db-cAMP. Thus, the supported idea is that these elements enhance others steps of stallion sperm capacitation, like increase in membrane fluidity (table 1), instead of the PS exposure. These results might indicate that others phosphodiesterases inhinitors as used in Gadella and Harrison (2002) and de Vries et al. (2003) should also be tested in stallion sperm to check the effect in the PS exposure during capacitating conditions.

The porcine IVF medium could maintain higher percentage of PS exposure since the beginning of incubation. This can be because of the stable pH generate by the equilibration of the medium in CO_2. Also, the presence of caffeine (1 mM) and specially a higher concentration of calcium (7.5 mM) compared to TyrBic medium could help obtain this result. It was already shown that Annexin-V is a Ca^{2+}

dependent probe and described that raises in intracellular Ca^{2+} can help the phospholipids scrambling (Flesch and Gadella, 2000) which could lead to an increase in the percentage of live, Annexin-V positive population.

With respect to the staining pattern, the fluorescence only in the acrosome region was more prevalent than the others in the media containing bicarbonate. Harrison and Gadella (2002) using a confocal microscope found in boar sperm a staining pattern in live cells only over the anterior acrosomal region of their head plasma membrane. They showed that when the Annexin-V labeled the midpiece of the sperm the cell was dead, i.e. propidium iodide positive. De Vries et al. (2003) inducing capacitation in human sperm with bicarbonate also had the same result. Even saying that PS is not a capacitation step in boar sperm, Kurz et al. (2005) also found a acrosomal region labeling of Annexin-V in the low percentage of capacitated sperm. The three staining pattern of Annexin-V found in our experiment suggest that we might have discovered in stallion sperm a sequential evolution of PS exposure. First step in remodeling being the labelling in the acrosomal region (figure 6).

Most of the cells that had the full head stained for Annexin-V also labeled for Alexa Fluor-PNA 647. This indicate that these cells are already in a late event of capacitation where the fusion of the plasma and outer acrosomal membrane led to an acrosome reaction. This could be the second step in PS exposure evolution (figure 7).

Like the previous staining pattern, the labeling in the head and midpiece was never described in any specie in live cells in capacitating conditions. However, when the head and the midpiece was labelled in boar and human sperm in the head and midpiece the cell was already dead (Gadella and Harrison 2002; de Vries et al, 2003). By this, the last staining pattern in live stallion sperm can indicate that this cell is in its final evolution step of PS exposure which will guide to cell death (figure 8).

The comparison between the results that a high percentage of viable cells become M540 positive in the presence of bicarbonate (table 1) with the percentage of viable sperm that has PS exposed (graphic 2) introduces the question: Is PS exposure in stallion sperm really required for full capacitation, becasue

rearrangements of the membranes (figure 9 compared to figure 10) and the acrosome reaction (graphic 4, C) could also happen without a high percentage of viable sperm population with PS exposed, or this might be the main question why conventional IVF does not work in horses? Is there more than just cAMP that controls the molecules that are responsible for PS exposure?

Regarding all findings, must be considered the fact that all cited works used confocal microscopy or fixed cells to check the staining patterns while we used for the first time life imaging for this purpose, indicating that this can be a new methodology for further analysis in capacitation process. Further addition with times series in live imaging may help to figure out whether there is a sequential occurrence of the Annexin-V staining patterns and the PNA staining patterns.

CONCLUSION

In conclusion, a large sperm population shows changes in phospholipid packing disorder while only a relatively smaller population of viable stallion spermatozoa exposes phosphatidylserine residues in bicarbonate containing media. Stimulation of the sAC/cAMP/PKA pathway by caffeine and dibutyryl-cAMP indicated the relevance of this pathway for PS exposure. The observation of three different staining patterns for Annexin-V in live stallion sperm cell may warrant further investigation with respect to whether these represent sequential steps in membrane remodeling. Finally, the largely independent occurrence of an acrosome reaction from PS exposure poses the question whether both events are indeed necessarily required for providing a spermatozoon with the ability to penetrate the zona pellucida of an equine oocyte.

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CHAPTER 2 – CALCIUM, BOVINE SERUM ALBUMIN (BSA) AND PROGESTERONE EFFECTS IN STALLION SPERM IN *IN VITRO* CONDITIONS

INTRODUCTION

In several species as human, cattle, pigs and mice, the conventional *in vitro* fertilization (IVF) is a standard procedure for *in vitro* embryo production (Steptoe and Edwards, 1978; Galli et al., 2003; Betteridge 2006; Perry, 2013). However, when equine gametes are the basis, this is not a successful technique. The co-incubation of mature oocytes and capacitated sperm in *in vitro* conditions to produce embryos in horses can support the binding of stallion sperm to the oocyte's zona pellucida, but fertilization rates are not good (Tremoleda et al., 2003). Despite several attempts, no reliable conventional IVF protocol has yet been established for equine gametes (Choi et al., 1994; Dell'Aquila et al., 1997a,b; Alm et al., 2001; Hinrichs et al., 2002; Mugnier et al., 2009). Low fertilization rates were already reported when calcium ionophore A23187, heparin (Li et al., 1995; Alm et al., 2001), zona pellucida proteins, caffeine and lysophospholipids (Graham, 1996) were used to trigger sperm activation. Despite this, few experiments report a good fertilization rate co-incubating oocytes with sperm treated with procaine to induce hyperactivated motility (McPartlin et al., 2009; Ambruosi et al., 2013) but no birth of a foal has been reported since the early 1990's (Palmer et al., 1991). This emphasizes the need to explore the effect of chemical capacitation triggers on stallion sperm capacitation and consequently fertilization.

At the molecular level, sperm capacitation involves, due to an increase in intracellular bicarbonate concentrations: (A) activation of a sAC/cAMP/PKA pathway (Harrison, 2004; Krapf et al., 2010); (B) increase in intracellular pH (Zeng et al., 1996); (C) increase in intracellular calcium (Ca^{2+}) concentrations (Ruknudin and Silver, 1990); (D) increase in membrane fluidity leading to its rearrangement (Zeng et al., 1995; Escoffier et al., 2012) like (E) lipid modifications (Gadella and Harrison, 2000) and loss of cholesterol by albumin (Davis et al., 1980; Cross,1996); and (F) increase in protein tyrosine phosphorylation (Visconti et al., 1995; Krapf et al., 2010). Ultimately, these modifications raise the affinity of the spermatozoon to bind the zona pellucida and undergo acrosome reaction (Benoff, 1998).

In all cell types, Ca^{2+} plays essential roles as second messenger in controlling several cellular processes. In sperm, this ion plays a major role in capacitation-

related events such as hyperactivated motility and tyrosine phosphorylation that lead to acrosome reaction and fertilization (Navarette et al., 2015). Ca^{2+} has two transport systems in the sperm cell already identified, the Ca^{2+} channel complex Catsper (Qi et al., 2007) and Ca^{2+} extrusion systems composed by the Na^{+}/Ca^{2+} Exchanger and the Plasma Membrane Ca^{2+} ATPase (PMCA) protein families (Okunade et al., 2004). In relation to its target, calcium dependent enzymes can be directly activated by the ion or indirectly by Ca^{2+}/Calmodulin (CaM) interaction (Wasco et al., 1989; Wu and Means, 2000; Ignotz and Suarez, 2005; Marin-Briggiler et al., 2005).

Triggering acrosome reaction, calcium influx participates in the increase in tyrosine phosphorylation leading to actin polymerization with the formation of actin filaments bound to the plasma membrane (PM) and outer acrosomal membrane (OAM). This is followed by phospholipases C (PLC_{γ} and $PLC_{\beta1}$) attachment to this membrane bound actin after its phosphorylation (Spungin et al., 1995). The PLCs cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) forming inositol triphosphate (IP_3) and diacylglycerol (DAG). $PLC_{\beta1}$ has been identified in the acrosomal region of mammalian sperm while IP_3 has been shown to induce release of Ca^{2+} probably from the acrosome (Walensky and Snyder, 1995). In contrast, DAG activates protein kinase C (PKC) to open Ca^{2+} channels in the PM initiating its depolarization (Breitbart, 2002). This increase in cytosolic calcium concentrations ($[Ca^{2+}]_i$) causes a rise in protein phosphorylation inducing PM and OAM close apposition by the formation of stable docks of the SNARE[1] family proteins (syntaxin 1B, SNAP 23 and VAMP 3). The consequence is fusion of the membranes leading to exocytosis of acrosomal content (Tsai et al., 2010), showing that capacitation and acrosome reaction (AR) is a process that is only completely achieved when Ca^{2+} is present (Stival et al., 2016).

The AR involves the fusion and vesiculation of the PM and OAM resulting in the exocytosis of hydrolytic enzymes (Yanagimachi, 1994) as the trypsin-like acrosin (Honda et al., 2002) with exposure of new membrane domains known to be essential for fertilization (Breitbart and Spungin, 1997). After one hour of incubation of sperm-oocytes complexes only low incidence of AR is observed in stallion sperm (Ellington

[1] Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment protein Receptor (SNARE) family

et al., 1993; Cheng et al., 1996; Meyers et al., 1996) implying that *in vivo* additional physiological components may be involved in the induction of AR in horses.

It has been already described that at the time of fertilization, the oviduct environment has elements that play a role in sperm chemotaxis. Capacitated sperm are attracted towards follicular fluid (human: Ralt et al., 1994; mice: Oliveira et al., 1999; rabbit: Fabro et al., 2002). Progesterone (P4), present in this environment in the cumulus oophorus cells and also in the pre-ovulatory follicular fluid, is thought to be one of this elements (Teves et al., 2006). The concentration of this molecule in the oviduct ipsilateral to the site of ovulation is around 100 ng/mL (Nelis et al., 2016).

A progesterone receptor has been already identified in the stallion sperm plasma membrane (Cheng et al., 1998) and the proportion of stallion's spermatozoa that have exposed P4-receptors correlates well with its fertility (Rathi et al., 2000). Progesterone seems to act by increasing the AR in stallion sperm in an independent PKA-pathway since PKA blockers failure in inhibit progesterone-mediated induction of AR. It has been proposed that progesterone bind to its receptor (exposed during capacitation) in the sperm plasma membrane activating a protein tyrosine kinase (PTK) receptor coupled directly or via G protein to PLC leading to the activation of a PKC and increase in Ca^{2+} concentration (Rathi et al., 2003). Acting on equine AR there is also a family of cAMP-activated guanine nucleotide exchange factors (RAPGEF3/RAPGEF4) but the ion channels affected by its activation is still not clear (Holz et al., 2006; McPartlin et al., 2011).

Despite progesterone-induced acrosome reaction has been reported in equine sperm (Meyers et al., 1995; Cheng et al., 1998; Rathi et al., 2003; McPartlin et al., 2008) there is a lack in clear experimental evidences for a progesterone-mediated calcium influx leading to acrosome reaction in stallion sperm (McPartlin et al., 2011; Silva et al., 2014). In human sperm P4 mediates a several fold transient increase in calcium concentration within seconds of addition (Blackmore et al., 1990).

To undergo AR, the sperm must fuse the PM to the OAM, which is possible when the PM is in an unstable fusible state. To get to this fusible membrane status, the sperm cell must rearrange its surface to get rid of molecules that make the cell

stay in a stable form. Cholesterol, a non-integral membrane lipid, is a molecule considered to be a plasma membrane stabilizer preventing the membrane fusion controlling the responsiveness to inducers of the AR (Cross, 1998) and its efflux form the PM is one essential capacitation step (Davis et al., 1980; Cross,1996). This molecule increases the orientation order of the membrane lipid hydrocarbon chains, retaining the capacity of the membrane to undergo conformational changes making it less fluid (Travis and Kopf 2002; Hsieh et al., 2013).

Evidences that sperm membrane sterol levels decline following exposure to albumin as the observation that other cholesterol acceptors like HDL and β-cyclodextrins effectively support capacitation, suggests that the primary action of serum albumin is in mediating cholesterol efflux (mouse: Visconti et al., 1999 a, b). Albumin, present in the oviduct and in the follicular fluid (Ehrenwald et al., 1990) can decrease the cholesterol content of the sperm and acts in synergy with bicarbonate and calcium (boar: Gadella et al., 2001). Albumin mediates the efflux of sterols from the sperm PM, especially of cholesterol (Boerke et al., 2008; Browers et al., 2011), in a lipid specific way since only sterols are lost in capacitating medium and phospholipids remain at constant levels (Flesch et al., 2001). It is believed that albumin acts providing a relatively hydrophobic environment in the PM facilitating the otherwise inefficient diffusion of cholesterol into an aqueous medium (Travis and Kopf, 2002). The cholesterol depletion appears to be critical for the activation of tyrosine kinases which in turn leads to changes in the protein conformation of the sperm plasma membrane contributing for the increase in the zona pellucida affinity, hypermotility and induction of AR (Neild et al., 2005).

Due the fact that several elements can activate or increase acrosome reaction, the aim of this study was to determine the optimal extracellular calcium concentrations in a chemically defined medium that minimizes the degree of a spontaneous acrosome reaction and still allows the induction of a progesterone-induced acrosome reaction in stallion spermatozoa and also check the BSA concentration that supports a higher induction of acrosome reaction mediated by progesterone.

MATERIAL AND METHODS

1. Media

The basic variant of Tyrode's medium (TyrControl) consisted of 111 mM NaCl, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 100 µg/mL gentamycin sulfate, 1.0 mM sodium pyruvate, 21.7 mM sodium DL-lactate. In the bicarbonate containing variant (TyrodeBic) a defined amount of NaCl was replaced by 30 mM of $NaHCO_3$. Both media contained 1 mg/mL of bovine serum albumin (BSA- A6002-25G – Sigma-Aldrich) and 2 mM of Ca^{2+} supplemented as $CaCl_2$, unless otherwise stated. The pH of both medium was adjusted to 7.40 ± 0.05 at room temperature with NaOH or HCl and the osmolality was adjusted to 300 ± 5 mOsmol/kg. All media were passed through a syringe filter (PES membrane, pore size 0.22 µm; Merck Millipore, Amsterdam, the Netherlands) for sterile filtration. The bicarbonate containing medium (TyrodeBic) was kept in an incubator with 5% CO_2 and 100% humidity at 37°C for at least 24 hours equilibration prior to experimentation. Incubations of spermatozoa in bicarbonate containing media took place in the same incubator used for equilibration. Incubations of spermatozoa in control medium (TyrControl) were carried out in a metal heating block at 37°C. Variants of the media with different inhibitors/activators and their solvents were prepared as stated in the description of the respective experiments.

2. Semen collection and dilution

Semen was collected with an artificial vagina (Hanover model) from stallions attending the Faculty of Veterinary Medicine at Utrecht University for breeding soundness examination or from stallion located at nearby horse farms (Stal Schep and Stal Van Vliet). After collection, semen was filtered through gauze to remove the gel fraction and large debris. A smear of raw semen with Aniline Blue-Eosin was prepared for sperm morphology assessment. Concentration of the sample was determined by means of a Bürker Türk haemocytometer chamber and sperm was diluted in INRA96® (IMV, l'Aigle, France) to obtain a concentration of 30 x 10^6 spermatozoa/mL. Motility was objectively checked with a CASA (Computer-assisted

sperm analysis) system (SpermVision 3.5, Minitüb, Tiefenbach, Germany) (as described in Brogan et al., 2015). Only samples with more or equal than 70% motile sperm where used for experimentation. Diluted semen was kept at room temperature until further processing took place. For each experiment semen from three to six different stallions was used.

2.1. Semen preparation for experimentation

Density gradient centrifugation was performed to separate the spermatozoa from the semen extender and seminal plasma prior to experimentation. Diluted semen (6 mL) was layered on top of a discontinuous gradient consisting of 2 mL of 70% Percoll®-saline solution and 4 mL of 35% Percoll®-saline in a 15-mL centrifugation tube (described by Harrison et al., 1993). Tubes were centrifuged for 20 minutes at room temperature, 10 minutes at 300 *g* followed by 10 min at 750 *g* without stopping in between. After centrifugation the supernatant was removed. The remaining pellet was resuspended in 1 mL of TyrControl without Calcium and BSA to obtain the concentration of the pellet with a Bürker Türk haemocytometer. After this the dilution was completed until a concentration of 30 x 10^6 sperm/mL was obtained. The sperm suspension was used within 30 min for experimentation.

3. Flow cytometry

To assess membrane changes flow cytometry was used. Ten microliter of the Percoll-washed sperm was added to pre-incubated FACS tubes containing 500 µl Tyrode's medium (all variants). All media contained 2 µl of each Hoechst 33258 (Sigma-Aldrich 94403, Zwijndrecht, the Netherlands) (stock solution: 0.1 mg/mL in aqua dest) and PNA-AlexaFluor™647 (ThermoFisher Scientific C10634, Waltham, USA) (stock solution: 0.25 mg/mL in aqua dest). 2 µl of M540 (Molecular Probes M24571, Eugene, OR, USA) (stock solution: 750 mM in DMSO) was added 15 min before measurements took place. Before analysis on a BD FACSCanto™ (BD Biosciences, Breda, The Netherlands), samples were briefly vortexed. Samples were kept on 37°C and under CO_2 atmosphere (TyrBic) during transport from the incubator to the flow cytometer.

Excitation of Hoechst 33258 was achieved by a 405 nm laser (30 mW) and fluorescence was captured with a 450/50 filter. Excitation of M540 was achieved by using a 488 nm laser (20 mW) with a 530/30 filter. For PNA-AlexaFluor™647 a 633 nm laser (17 MW) with a 660/20 filter was used. Data from 10,000 individual spermatozoa were acquired at medium speed. Data were analyzed using FCS Express (version 3, De Novo Software, Glendale, CA, USA).

First of all, the population of single spermatozoa was determined. A gate was placed in the dotplot on forward scatter versus side scatter (Figure 1 – Chapter 1). For further evaluation, only cells from this gate were considered.

Thresholds for quadrants that distinguish between Hoechst 33258 FITC- PNA 647 signal (acrosome intact) or high FITC-PNA signal (acrosome reacted) fluorescence intensity were set in the control medium. Similarly, the quadrants that distinguish between viable sperm with low M540 signal (M540 low; low membrane fluidity) and high M540 signal (M540 High; high membrane fluidity) were set.

3.1. Use of different concentrations of calcium

For this experiment, TyrControl and TyrBic were adjusted to four different calcium concentrations (0.1 mM, 0.5 mM, 1 mM and 2 mM of Ca^{2+}) to check the effect on capacitation steps such as membrane fluidity and spontaneous acrosome reaction. EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; 2 mM), a calcium chelator, was used to obtain a medium that was essentially calcium-free. Samples were assessed after 15, 30, 60 and 120 minutes of incubation.

3.2. Use of different concentrations of progesterone (P4)

For this experiment the amount of calcium in TyrControl and TyrBic was adjusted to two different concentrations (0.1 mM and 2 mM of $Ca2^{+}$). Three different concentration of progesterone (Sigma-Aldrich, P0130-25G, Zwijndrecht, the Netherlands) (10, 100 and 1000 ng/mL) were added 30 minutes before reading the tubes on the flow cytometer to check the effect the effect of progesterone on the

acrosome reaction. Ethanol was used as solvent for P4 and solvent controls were included in the experiment Samples were assessed after 30, 60 and 120 minutes.

3.3. Use of different concentrations of BSA

For this experiment, TyrControl and TyrBic media were used with two different concentrations of BSA (1 and 7 mg/mL). For this experiment, in both media, Ca^{2+} concentration was 2 mM. Again three different concentration of progesterone (10, 100 and 1000 ng/mL) were added 30 minutes before reading the samples. Both molecules were for checking the effect of BSA and P4 on the membrane fluidity and acrosome reaction. DMSO (dimethyl sulfoxide) was used as P4 solvent and control. Samples of flow cytometer were assessed after 30, 60 and 120 minutes.

For all three substance tested (calcium, P4 and BSA) the population of live (Hoechst33258 negative) and PNA-AlexaFluor™647 positive (acrosome reacted) sperm was evaluated. Positive staining of sperm with M540 was evaluated as an internal control step for early stages of capacitation, i.e. an increase in membrane fluidity, occurring in TyrBic media.

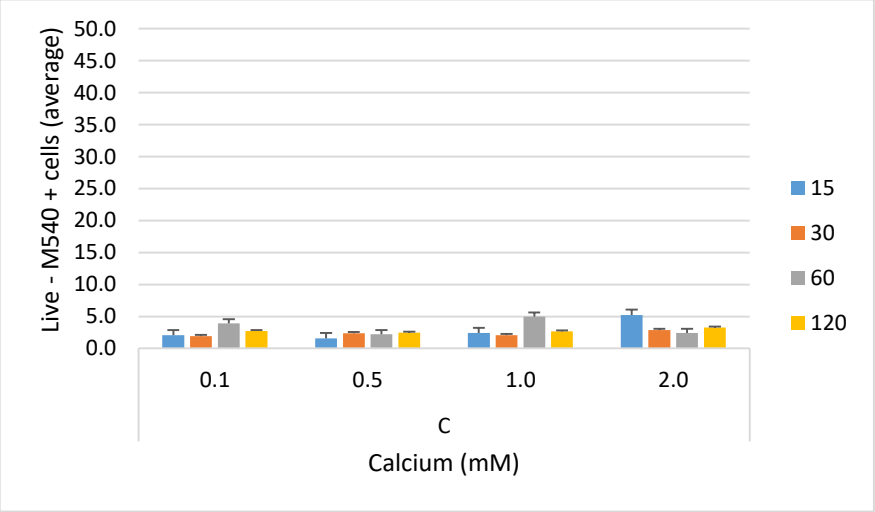
STATISTICAL ANALYSIS

Statistical analysis was performed, using SPSS statistics, version 25 (IBM analytics, Amsterdam, The Netherlands) and SAS University (Statistical Analysis Software- Cary, U.S.A.). Data were tested for normal distribution with a Shapiro-Wilk test and compared with a multi-factorial ANOVA for repeated measures. Considered factors differed according to the experiment. They included time, media (TyrControl, TyrBic) and the presence of different concentrations of molecules (calcium, BSA or progesterone). Paired Student's t-tests for dependent samples were performed to investigate differences between individual medium variants. Data are presented as mean ± standard deviation (SD) or mean ± standard error of mean (SEM). Differences were considered at a significance level of 5 % ($p \leq 0.05$).

RESULTS

1. Effect of different concentrations of calcium on the live, M540 positive and acrosome reacted sperm population

In relation to the sperm population that is live, M540 positive, the observation that the extracellular calcium concentration has no impact on the viable, M540+ sperm population indicates that a little bit of calcium (0.1 mM) is sufficient to maintain the high membrane fluidity. In general, the percentage of population live, M540 positive decreased while incubation time increased (table 1). In graphic 1 shows that no difference was found with different concentrations of calcium in the sperm population live, M540+ also in TyrControl media and the cell population of interest was, as expected, very low due the absence of bicarbonate.



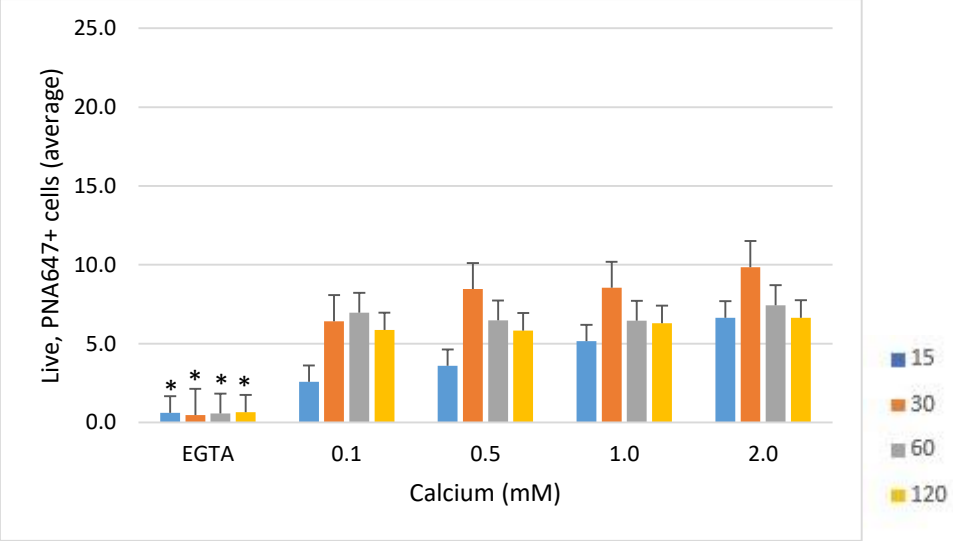
Graphic 1: Percentage of the population live, M540 positive spermatozoa in TyrControl media (with four different concentrations of calcium) after 15, 30, 60 and 120 min of incubation (Mean ± SD).

Table 1. Percentage of the live, M540 positive sperm population in TyrBic media with different concentrations of Ca^{2+} and EGTA

[Ca^{2+}] (mM) /Time	15	30	60	120
EGTA	71.8 ± 3.07 ^{Aa}	57.9 ± 9.01 ^{Aa}	14.7 ± 2.41 ^{Bb}	5.3 ± 1.20 ^{Bc}
0.1	61.9 ± 3.78 ^{ABa}	49.2 ± 4.46 ^{Ab}	33.4 ± 6.03 ^{Ac}	36.7 ± 8.29 ^{Abc}
0.5	57.7 ± 4.14 ^{Ba}	45.5 ± 5.55 ^{Ab}	38.7 ± 6.75 ^{Abc}	31.3 ± 7.20 ^{Ac}
1.0	55.5 ± 3.46 ^{Ba}	46.4 ± 4.80 ^{Ab}	29.2 ± 4.59 ^{Ac}	33.0 ± 7.85 ^{Ac}
2.0	50.6 ± 3.85 ^{Ba}	36.5 ± 9.01 ^{Aab}	33.1 ± 2.41 ^{Ab}	26.2 ± 6.95 ^{Ab}

Capital letters indicate the difference between the Ca^{2+} -containing variants and EGTA-containing TyrBic medium at the same time. Small letters indicate differences between the same medium at different times ($p \leq 0.05$) (Mean ± SEM).

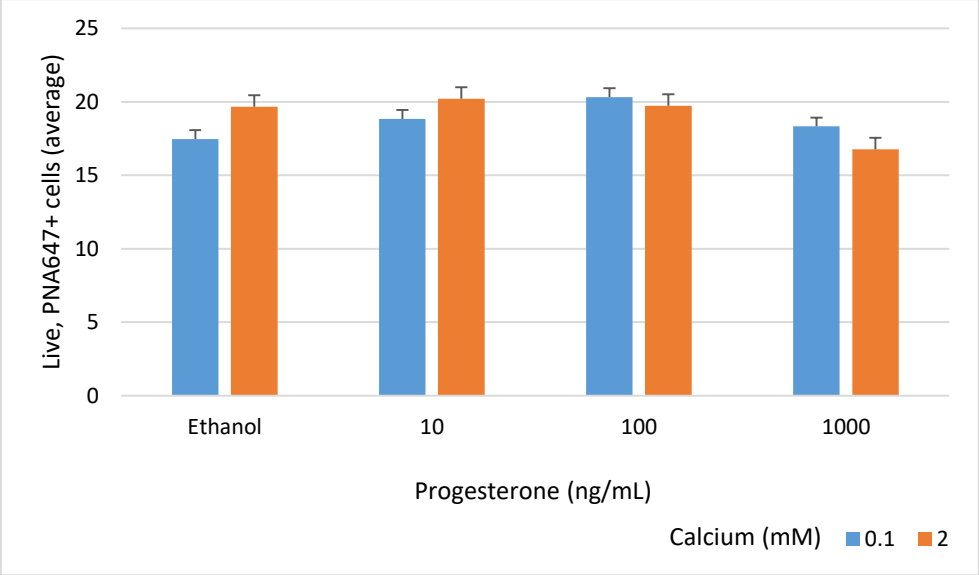
In TyrBic-based media there was no difference in the population of live, PNA-AlexaFluor647 positive sperm between the different calcium concentrations, but this population was always higher than in the medium with EGTA (graphic 1).



Graphic 2: Percentage of live, Alexa Fluor-PNA-647 positive spermatozoa in TyrBic media with EGTA or four different concentrations of calcium after 15, 30, 60 and 120 min of incubation. Stars indicate the difference from EGTA with all other treatments ($p \leq 0.05$) (Mean ± SD).

2. Effect of different concentrations of P4 (diluted in ethanol) in the live, acrosome reacted sperm population

Both extreme calcium concentration (0.1 and 2 mM) were selected for the experiment with the different concentrations of P4. There was no difference in the percentage of live, acrosome reacted sperm population among the different concentration of P4 and calcium in relation to the control (only ethanol) (graphic 2) in the different times of incubation.



Graphic 3: Percentage of the population live FITC-PNA positive spermatozoa after 30 min of incubation in TyrBic media with three different concentrations of progesterone (ng/mL) or ethanol as a control and different concentration of Calcium (0.1 and 2 mM) (Mean ± SD).

3. Effect of different concentrations of BSA and P4 (diluted in DMSO) in the live, M540 positive and acrosome reacted sperm population

Regarding the live, M540 positive population, 1 or 7 mg/mL of BSA and the different concentrations of progesterone did not interfere in the membrane fluidity of the population in TyrBic media. As expected, only time of incubation had significant difference, where the population live, M540 positive decrease when time incubation increased (table 2 and 3).

Table 2. Percentage of the sperm population live, M540 positive in TyrBic media with different concentrations BSA

[BSA mg/mL]/Time	30	60	120
1	65.3 ± 4.22 ^{A}	52.7 ± 3.02 ^{B}	46.8 ± 4.38 ^{B}
7	57.4 ± 3.34 ^{A}	48.4 ± 7.80 ^{B}	42.2 ± 5.70 ^{B}

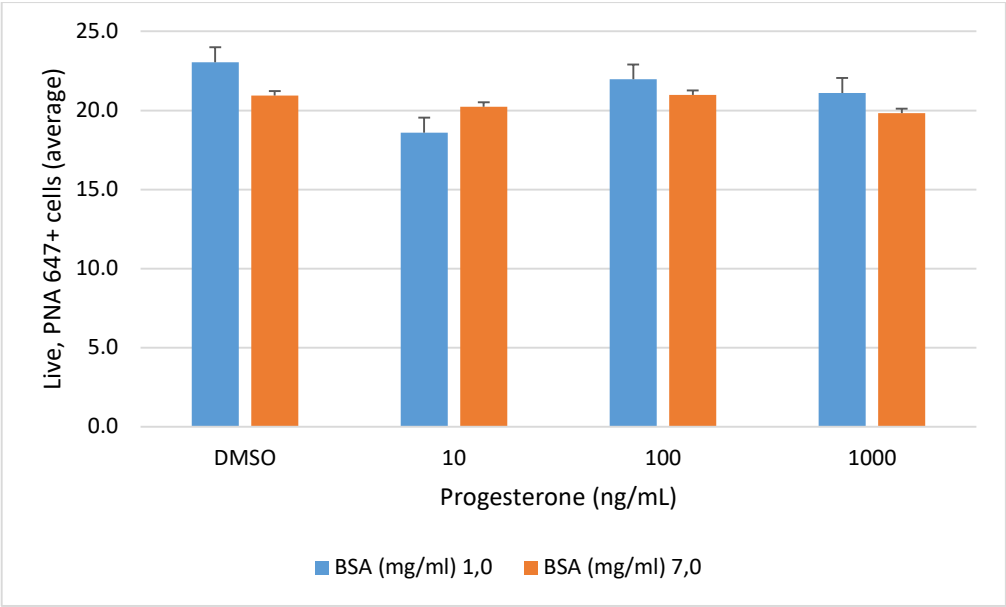
Capital Letters indicate difference between time in the same medium within a column. Mean ± SEM ($p \leq 0.05$).

Table 3. Percentage of the sperm population live, M540 positive in TyrBic media with different concentrations P4 and DMSO as a control

[P4] (ng/mL)/Time	30	60	120
DMSO	60.6 ± 3.57 ^{A}	50.2 ± 4.50 ^{B}	45.8 ± 3.59 ^{C}
10	62.3 ± 2.54 ^{A}	49.8 ± 3.24 ^{B}	40.2 ± 2.09 ^{C}
100	60.6 ± 4.05 ^{A}	56.6 ± 3.36 ^{B}	46.3 ± 4.88 ^{C}
1000	61.8 ± 1.84 ^{A}	50.6 ± 2.76 ^{B}	41.2 ± 3.52 ^{C}

Upper Letters indicate difference between times in the same medium. Mean ± SEM ($p \leq 0.05$).

In relation to the Alexa Fluor PNA-647 positive sperm population, to make sure that the effect in the live, acrosome reacted ones was not made by the diluter (ethanol) in the previous experiment, another diluter (DMSO) was tested for P4. Again, no difference was found between the control (DMSO) and the different concentrations of P4 and the different times of incubation (graphic 3). The same happened with the different concentrations of BSA (1 and 7 mg/mL).



Graphic 4: Population live PNA-647 positive spermatozoa after 30 min of TyrBic medium with three different concentrations of progesterone (ng/mL) or DMSO as a control and two different concentrations of BSA (1 and 7 mg/mL) (Mean ± SD).

4. Difference in the percentage of the population live, PNA-647 positive between the experiments

Higher percentage of the population live with acrosome reacted was seen in the experiments with progesterone and its vehicles compared to the experiment with different Ca^{2+} concentrations (table 4).

Table 4. Percentage of the sperm population live, Alexa-PNA647 positive in the presence of different molecules (none, ethanol or DMSO) in TyrBic media and with different [Ca^{2+}] at 30, 60 and 120 minutes of incubation

Vehicle	[Ca^{2+}] (mM)	30	60	120
-	2	9.9 ± 3.3 ^{Ba}	7.4 ± 1.8 ^{Ba}	6.6 ± 0.9 ^{Ba}
Ethanol	2	19.7 ± 3.3 ^{Aa}	16.2 ± 4.0 ^{Aa}	15.6 ± 3.2 ^{Aa}
DMSO	2	22.8 ± 0.7 ^{Aa}	18.8 ± 1.4 ^{Aab}	16.0 ± 0.9 ^{Ab}
-	0.1	5.2 ± 1.4 ^{Ba}	4.4 ± 1.8 ^{Ba}	4.7 ± 1.0 ^{Aa}
Ethanol	0.1	17.5 ≠ 4.7 ^{Aa}	15.3 ≠ 2.0 ^{Aab}	9.6 ≠ 1.5 ^{Ab}

Upper letters indicate the difference between the vehicle used in the Ca^{2+} and P4 experiments in TyrBic media at the same time and down letters indicate difference between the same variant at different time (Mean ± SEM) ($p \leq 0.05$).

DISCUSSION

The ability to fertilize an oocyte is acquired by the sperm in the female reproductive tract or in adequate *in vitro* conditions where the sperm gets fully matured by passing throughout capacitation. Many intracellular changes are known to occur in this capacitation process including increases in membrane fluidity, cholesterol efflux, alteration in intracellular Ca^{2+} and cAMP concentrations, protein tyrosine phosphorylation, hypermotility induction and acrosome reaction (Breitbart et al., 2002).

Several capacitation steps are Ca^{2+} dependent and its presence, associated with bicarbonate and albumin are important sperm surface alterations (Yanaginamachi, 1994). They together cause a synergic activation of PKA in an irreversible way (Gadella and Visconti, 2006; Boerke et al., 2008). In the present study, the different concentrations of calcium did not interfere in the concentration of the population live, M540 positive. However, the use of EGTA cause a significant decrease in this population indicating the importance of calcium presence in *in vitro* conditions at the firsts steps of capacitation in stallion sperm, as increase in

membrane fluidity (table 1). Initially, Ca^{2+} was considered to be required by sperm only at the end of capacitation (for hyperactivation acquisition acrosome reaction) (Yanagimachi and Usui, 1974). However, cells incubated with Ca^{2+} continuously showed to be more fertile (Fraser 1982; Yanagimachi, 1982) indicating that Ca^{2+} is required in all events for completing this process. It has been already demonstrated that Ca^{2+} might act in parallel with bicarbonate through stimulation of adenylyl cyclase(s) (Litvin et al., 2003) and that bicarbonate opens Ca^{2+} ion channels in mouse sperm (Wennemuth et al., 2003). Also, a rise in intracellular Ca^{2+} induced scrambling in red blood cells (Bevers et al., 1998).

Regarding AR, calcium was also found to be essential for this late event of capacitation (graphic 2). Like other membrane fusion events the acrosome reaction in the sperm cell is dependent of an increase in the levels of intracellular Ca^{2+} (Bailey et al., 1994; Brewis et al., 1996) and this calcium-dependent release of acrosomal contents is an essential step in mammalian fertilization because it facilitates the penetration through the acellular glycoprotein coat of the oocyte and oolema fusion (Yanaginamachi, 1994). This makes calcium the key messenger in the interaction between sperm and egg (Darszon et al., 2006). The importance of calcium in the medium for fertilization is an old statement demonstrated already in invertebrates (Loeb, 1915) and mammalian (Iwamatsu and Chang, 1971).

It has been already shown that quite low concentrations of Ca^{2+} (90 µM) promote capacitation in mouse sperm, but to acquire maximum fertilization status i.e. complete capacitation, a minimum concentration of 1.8 mM Ca^{2+} is needed (Fraser, 1987; Fraser, 1992). Experiments with this species has a calcium concentration in the capacitating medium varying between 1.7 to 2.7 mM (Fraser, 1984, 1987, 1992; Visconti et al., 1995; Si and Olds-Clark, 2000; Baek 2017; Olabarrieta et al., 2020). Based on the mouse sperm studies, in stallion capacitation ones that attempt to accomplish conventional IVF, have the Ca^{2+} concentration in the medium varying between 1.8 to 2.0 mM (Dell'Aquila et al., 1997a,b; Rathi et al., 2003; Tremoleda et al., 2003; McPartlin et al., 2008, 2009, 2011; Lange-Consiglio and Cremonesi, 2011). In our experiment, from the lowest (0.1 mM) to the highest (2 mM) Ca^{2+} concentration no difference was found in the membrane fluidity or

spontaneous acrosome reaction (table 1; graphic 2) which indicates that, to start those capacitation process in stallion sperm, there is no need to have high amounts of calcium in the medium.

However, even not having influence in the spontaneous acrosome reacted population with different concentrations of calcium, the percentage of these cells is still low in stallion sperm in *in vitro* conditions (graphic 2). The options for increasing the percentage of the live, M540 and especially PNA-positive population are the use of a higher amount of calcium or addition of molecules in the medium that can stimulate this process. Since in most species the capacitation is acquired with concentrations of calcium not higher than 2 mM the addition of molecules present in the moment of fertilization in the oviduct, that can stimulate AR, seems more reasonable to get to the desirable result. Marin-Briggiler et al. (2003) showed that human sperm previous incubated with 0.58 mM Ca^{2+} was able to demonstrate maximum AR induced when human follicular fluid (FF) was added in the medium.

Equine semen under *in vitro* conditions already showed to have an increase in acrosome reaction in addition of progesterone (P4) in the medium compared to only bicarbonate (Cheng et al., 1998; Rathi et al., 2003; McPartlin et al., 2008). In all these experiments the concentration of calcium present in the medium was 2 mM. Since the percentage of the population with spontaneous acrosome reaction was the same comparing the different concentration of calcium, the lowest (0.1 mM) and the highest (2 mM) [Ca^{2+}] were tested with different [P4] to check the interference of calcium in the progesterone-mediated acrosome reaction in stallion sperm.

Physiologically, the induction of AR by P4 leads to a rapid increase in intracellular calcium concentrations which are enhanced by a Ca^{2+} influx from the external medium (Gonzalez-Martinez et al., 2002). In human sperm, P4 has an extra genomic action characterized by the rapid induction of a calcium transition followed by a plateau phase during which [Ca^{2+}], remains significantly above baseline (Aitken and McLaughilin, 2007). P4 treatment of capacitated human sperm resulted in a wave-like increase in sperm head $[Ca^{2+}]_i$. The progesterone-mediated Ca^{2+}/increase in the sperm head was strongly inhibited by picrotoxin (an inhibitor of the progesterone-initiated AR of human sperm) (Meizel et al., 1997). Unfortunately, our

experiment was not able to repeat P4 effect in stallion sperm as in previous reports since the three different concentrations of P4 (10, 100 and 1000 ng/mL) had no significant difference in percentage of acrosome reacted population compared to ethanol or DMSO as a control not being able to prove the interference of calcium concentrations in the P4-mediated acrosome reaction (graphic 3 and 4). If comparing to experiments where only calcium effect were tested (table 4), P4 experiments showed a higher population live, PNA-647 positive however the effect cannot be directed to P4 since there was no difference between [P4] and the controls (ethanol or DMSO).

Despite this difference between the experiments, the percentage of population live, acrosome reacted in the presence of progesterone or its vehicles is still low compared to other experiments conducted in stallion sperm (55.6 ± 3.4% (150 ng/mL P4) Cheng et al., 1998; 39.0 ± 2.0% (1 µg/mL P4) Rathi et al., 2003; 44.6% (3.2 µM P4) McPartlin et al., 2008). In these experiments the sperm was incubated in the medium for a longer time (4-6 hours) and the progesterone, as in our experiment, added 20-30 minutes before reading the samples. However, Tremoleda et al. (2003) treating stallion sperm with 150 ng/mL of P4 showed that despite biding the zona pellucida nor fresh or frozen semen acrosome-reacted penetrated any oocyte having the same conclusion as Moros-Nicolás et al (2019), that showed that sperm pre-incubation with progesterone did not significantly influence the fertilization rate when fresh or frozen stallion sperm was used showing contradictory effects of P4 in stallion sperm in capacitation in *in vitro* conditions. Also, Lukoseviciute et al. (2004) demonstrated in bovine sperm that P4 had no significant effect on plasma membrane lipid stability or induction of acrosome reaction.

Since 120 minutes of incubation significantly increased dead cells in our experiment (data not shown) not making able the raise in our incubation time, another difference observed was that, the studies that showed induced acrosome reaction with P4, had a higher BSA concentration in the medium than what is often used in TyrBic media (1 mg/mL) (10 mg/mL Cheng et al., 1998; 7mg/mL Rathi et al., 2003; 7mg/mL McPartlin et al., 2008). The capacitating agent BSA, besides being the receptor for cholesterol (Visconti et al., 1999a,b) also induces Ca^{2+} influx in

sperm via CATSPER channel-dependent (Xia et al., 2009) which may act in synergism with the progesterone-mediated AR. However, increase in BSA in our experiment also did not interfere in the population live, M540 and specially FITC-PNA-647 positive (table 2, graphic 4). Stewart-Savage (1993) proposed that hamster capacitation has a BSA-independent (first hour) and BSA-dependent phase. Maybe this can also happen in stallion sperm in the first 2 hours of incubation in capacitating medium. The lack of BSA response (with 1 mg/mL or 7 mg/mL) regarding acrosome reaction in our experiment can be due to incubation time (120 minutes) compared to 4-6 hours in by Cheng et al. (1998), Rathi et al. (2003) and McPartlin et al. (2008) experiments. By this, changes in the used protocol could be done to maintain higher percentage of live cells for longer period to check the effect of different progesterone and BSA concentrations.

CONCLUSION

The unsuccessful conventional IFV in horses has been directed mostly to the failure of sperm to capacitate in *in vitro* conditions. In the stallion capacitation events we were able to show that extracellular calcium is necessarily required to maintain the population live with high membrane fluidity and enable a spontaneous acrosome reaction. There was no indication that there is a need for higher calcium concentrations to start capacitation as it is needed in other species. Different concentrations of progesterone and BSA were not able to increase the number of sperm that underwent an acrosome reaction indicating that other molecules present in the follicular fluid or that can lead to capacitation in other species should be tested in *in vitro* conditions to trigger a successful acrosome reaction in stallion sperm.

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CHAPTER 3 – ROLE OF SOLUBLE AND TRANSMEMBRANE ADENYLYL CYCLASES IN STALLION SPERM CAPACITATION

INTRODUCTION

Fertilization in mammalian species relies on the proper activation, i.e. capacitation, of the spermatozoa (Visconti et al., 1998). In this context, a range of essential events have been already linked to changes in the intracellular concentration of cAMP, e.g. motility (Lardy et al., 1971; Esposito et al., 2004), membrane lipid remodeling (Gadella et al., 2000; Flesch et al., 2001) and acrosome reaction (Branham et al., 2006; 2009; Wertheimer et al., 2013); hyperpolarization of the sperm plasma membrane (Demarco et al., 2003); increase in intracellular pH and calcium (Zeng et al., 1996; Wang et al., 2003; 2007) and tyrosine phosphorylation (Visconti et al., 1995); and gamete fusion (de Lamirande et al., 1997).

Adenylyl cyclases (ACs) are enzymes which are considered to be the major producer of cAMP (cyclic adenosine 3′,5′-monophosphate), an important second messenger discovered in 1958 (Rall and Sutherland, 1958; Sutherland and Rall 1958). Since then, ACs and cAMP have been implicated in several cellular signaling pathways in various cell types. The discovery of cAMP led to the finding that this second messenger signaling occurs independently and in a highly regulated fashion in intracellular compartments or microdomains (Dessauer, 2009; Houslay, 2010; Zaccolo, 2011). The ACs synthetize cAMP by catalyzing adenosine triphosphate (ATP) releasing a pyrophosphate. The cellular effects of the resulting cAMP are mediated by the effector protein kinase A (PKA), exchange proteins activated by cAMP (EPACs), and cyclic nucleotide regulated ion channels (Gancedo, 2013). Phosphodiesterases (PDEs) are responsible for the degradation of cAMP, thus, regulating the cAMP levels in the cellular compartment (Houslay, 2010; Jager et al., 2012). Besides its contribution to physiological process in somatic mammalian species, cAMP can also participate in virulence mechanism in bacteria and fungi (McDonough and Rodriguez, 2012).

There are two distinct families of AC already described in mammalians cells, the soluble adenylyl cyclase (sAC) and transmembrane-associated adenylyl cyclase (tmAC) (Kamenetsky et al., 2006). In spermatozoa, the sAC is present in two isoforms characterized by a single, alternatively spliced gene (AC10); a long and a

short one (Buck et al., 1999). Regarding the tmAC family, nine genes encode nine different isoforms (AC1-9) which have been already identified in somatic cells. The isoforms differ in structure and function, as well in their expression pattern in various tissues (Hanoune and Defer, 2001).

The two families of ACs share similar mechanisms of action (Kamenetsky et al., 2006), but they differ in their subcellular localization and regulation. sACs are found in the cytoplasm (Zipping et al., 2004), inside the mitochondria (Zippin et al., 2003; Acin-Perez et al., 2009) and nuclei (Zippin et al., 2003, 2004, 2010) while tmACs are present in the plasma membrane (Calebiro et al., 2009; Ferrandon et al., 2009). Consequently, tmACs consist of two transmembrane domains with six predicted helices each, and two cytosolic domains, referred to as C1 and C2, respectively (Sunahara et al., 2002). The tmACs are mainly regulated by heterotrimeric G proteins upon activation of G-protein-coupled receptors (GPCRs), due the action of extracellular hormones) and neurotransmitters (Sunahara et al., 2002). In contrast, sAC activity is modulated by bicarbonate (Chen et al., 2000) and calcium ions (Jaiswal and Conti, 2003; Litvin et al., 2003). Soluble adenylyl cyclase activity is also sensitive to variations in intracellular ATP concentrations (Litvin et al., 2003; Zippin et al., 2013), but proteins which might be involved in its regulation have not been identified, yet.

In spermatozoa, expression of sAC and tmAC has only been confirmed in human and mouse sperm demonstrated by immunofluorescence, immunoblotting (Spehr et al., 2004; Baxendale et al., 2013); RT-PCR (Wertheimer et al., 2013) and suggested in cAMP immunoassays (Uguz et al., 1994; Lefievre et al., 2000; Harrison et al., 2000; Tardif et al., 2004). It has been demonstrated that tmACs are present in mouse sperm and the localization of tmAC by immunostaining indicates that it can play a role in acrosome reaction, since this molecule is present is only in the plasma membrane of the head and the immunoreactivity was lost in acrosome-reacted sperm (Wertheimer et al., 2013; Buffone et al., 2014). This theory is supported by the notion that the acrosome reaction involves a putative G-Protein linked sperm receptor (Leclerc and Kopf, 1999). On the other hand, the sAC is supposed to be involved in motility activation in mouse, boar, bovine and hamster sperm activation

and motility (Hess et al., 2005; Okamura et al., 1985; Garti et al., 1988; Visconti et al., 1990 respectively), as well as in the capacitation-related rearrangement of lipids of human sperm (de Vries et al., 2013) and tyrosine phosphorylation (Wertheimer et al., 2013).

In stallion sperm, only few data are yet available regarding the nature of the expressed ACs and their involvement in specifics steps during capacitation, the acrosome reaction and hyperactivated motility. It is believed that bicarbonate and thus bicarbonate-mediated activation of the sAC is essential for cAMP upregulation and consequently initiating capacitation in stallion sperm (Bromfield et al. 2014). However, only recently the contribution of AC generated cAMP to the hyperactivation in stallion spermatozoa has been questioned as no cAMP upregulation was detected in what was assumed to be capacitating conditions (Leemans et al. 2019). Based on these controversial observations it is thus of vital interest to understand to what extent ACs are indeed contributing to capacitation-related phenomena in stallion sperm.

The differentiation which type of AC (sAc or tmAC) is contributing to the regulation of individual cAMP signaling pathways can be accomplished by the use of molecules that can specifically inhibit or stimulate only one family of AC.

For sAC stimulation, there is no specific pharmacological compound to activate it. This enzyme is directly regulated by HCO_3^-, ATP and Ca^{2+} (Chen et al., 2000; Jaiswal & Conti, 2003; Litvin et al., 2003) and because sAC's function as pH, calcium and ATP sensor make it almost impossible to stimulate its activity without causing a plethora of side effects (Wiggins et al., 2018).

A range of sAC inhibitors with differing specificity and affinity has been recently reviewed by Bitterman et al. (2013). The most widely used pharmacological tool for elucidating sAC function is KH7 ((E)-2- (1H-Benzo[d]imidazol-2-ylthio)-N′-(5-bromo-2-hydroxybenzylidene) propanehydrazide)) (Hess et al., 2005, Bitterman et al., 2013) with use also in stallion sperm (McPartlin et al., 2011). Another molecule that can be used for this purpose is 6-chloro-N4-cyclopropyl-N4-((thiophen-3-yl) methyl)pyrimidine-2,4-diamine (LRE1). LRE1 binds allosteric to sAC's regulatory bicarbonate binding site similar to bithionol, being competitive with bicarbonate but

more efficient than bithinol (Ramos-Espiritu et al., 2016; Jakobsen et al., 2018; Wiggins et al., 2018).

The tmACs are bicarbonate insensitive but can be stimulated by forskolin, while sAC is insensitive for this molecule (Buck et al., 1999; Sunahara and Taussig, 2002). This molecule comes from the Indian plant *Coleus forskohlii* (Laurenza et al., 1989). Forskolin directly stimulates tmAC activity independent of hormonal regulation (Tesmer et al., 1999). It interacts with the catalytical site of the enzyme and increases the basal activity manifold (Seamon et al., 1981). Forskolin-dependent activation appears to involve dimer stabilization and smaller active site rearrangements (Tesmer et al., 1997).

For tmAC inhibition, molecules as SQ 22,536 [9-(tetrahydro-2- furanyl)-9H-purin-6-amine, also known as THFA or 9-THF-Ade] can be used (Haslam et al., 1978) but they have often low potency (Onda et al., 2001). More potent alternatives are P-site inhibitors such as [9-β-D-arabinosyladenine (vidarabine)] (AraAde) or 2′5′-dideoxyadenosine (ddAdo) (Ramos et al., 2008; Bitterman et al., 2013). Because the former can exhibit cytotoxic effects (Seifert et al., 2012), ddAdo is the most specific compound for tmAC inhibition.

The aim of the study was to pharmacologically characterize which AC is involved in the initiation of sperm capacitation in stallion sperm under in *in vitro* conditions and in the acrosome reaction. To this end, direct and indirect inhibitors of sAC (KH7 and LRE1) and a specific stimulator of tmAC (forskolin) were used to decipher the sources of cAMP governing either an early event of sperm capacitation, i.e. an increase in plasma membrane fluidity, or an acrosome reaction in viable spermatozoa.

MATERIAL AND METHODS

1. Media

The basic variant of Tyrode's medium (TyrControl) consisted of 111 mM NaCl, 20 mM HEPES, 5 mM glucose, 3.1 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 100 µg/mL gentamycin sulfate, 1.0 mM sodium pyruvate, 21.7 mM sodium DL-lactate. In

the bicarbonate containing variant (TyrodeBic) a defined amount of NaCl was replaced by 30 mM of $NaHCO_3$. Both media contained 1 mg/mL of bovine serum albumin (BSA- A6002-25G – Sigma-Aldrich) and 2 mM of Ca^{2+} supplemented as $CaCl_2$. The pH of both medium was adjusted to 7.40 ± 0.05 at room temperature with NaOH or HCl and the osmolality was adjusted to 300 ± 5 mOsmol/kg. All media were passed through a syringe filter (PES membrane, pore size 0.22 µm; Merck Millipore, Amsterdam, the Netherlands) for sterile filtration. The bicarbonate containing medium (TyrodeBic) was kept in an incubator with 5% CO_2 and 100% humidity at 37°C for at least 24 hours equilibration prior to experimentation. Incubations of spermatozoa in bicarbonate containing media took place in the same incubator used for equilibration. Incubations of spermatozoa in control medium (TyrControl) were carried out in a metal heating block at 37°C. Variants of the media with different inhibitors/activators and their solvents were prepared as stated in the description of the respective experiments.

2. Semen collection and dilution

Semen was collected with an artificial vagina (Hanover model) from stallions attending the Faculty of Veterinary Medicine at Utrecht University for breeding soundness examination or from stallion located at nearby horse farms (Stal Schep and Stal Van Vliet). After collection, semen was filtered through gauze to remove the gel fraction and large debris. A smear of raw semen with Aniline Blue-Eosin was prepared for sperm morphology assessment. Concentration of the sample was determined by means of a Bürker Türk haemocytometer chamber and sperm was diluted in INRA96® (IMV, l'Aigle, France) to obtain a concentration of 30 x 10^6 spermatozoa/mL. Motility was objectively checked with a CASA (Computer-assisted sperm analysis) system (SpermVision 3.5, Minitüb, Tiefenbach, Germany) (as described in Brogan et al., 2015). Only samples with more or equal than 70% motile sperm where used for experimentation. Diluted semen was kept at room temperature until further processing took place. For each experiment semen from three to six different stallions was used.

2.1. Semen preparation for experimentation

Density gradient centrifugation was performed to separate the spermatozoa from the semen extender and seminal plasma prior to experimentation. Diluted semen (6 mL) was layered on top of a discontinuous gradient consisting of 2 mL of 70% Percoll®-saline solution and 4 mL of 35% Percoll®-saline in a 15-mL centrifugation tube (described by Harrison et al., 1993). Tubes were centrifuged for 20 minutes at room temperature, 10 minutes at 300 *g* followed by 10 min at 750 *g* without stopping in between. After centrifugation the supernatant was removed. The remaining pellet was resuspended in 1 mL of TyrControl without Calcium and BSA to obtain the concentration of the pellet with a Bürker Türk haemocytometer. After this the dilution was completed until a concentration of 30 x 10^6 sperm/mL was obtained. The sperm suspension was used within 30 min for experimentation.

3. Flow cytometry

To assess membrane changes flow cytometry was used. Ten microliter of the Percoll-washed sperm was added to pre-incubated FACS tubes containing 500 µL Tyrode's medium. All media contained 2 µL each of Hoechst 33258 (Sigma-Aldrich 94403, Zwijndrecht, The Netherlands) (stock solution: 0.1 mg/mL in aqua dest) and PNA-AlexaFluor™647 (ThermoFisher Scientific C10634, Waltham, USA) (stock solution: 0.25 mg/mL in aqua dest). 2 µL of merocyanine (M540) (Molecular Probes M24571, Eugene, OR, USA) (stock solution: 750 mM in DMSO) or 2 µL of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) (Sigma-Aldrich, T4069-5MG, The Netherlands) (stock solution: 250 µM) was added 15 min before measurements took place. Before analysis on a BD FACSCanto™ (BD Biosciences, Breda, The Netherlands), samples were briefly vortexed. Samples were kept on 37 °C and under CO_2 atmosphere (TyrBic) during transport from the incubator to the flow cytometer.

Excitation of Hoechst 33258 was achieved by a 405 nm laser (30 mW) and fluorescence was captured with a 450/50 filter. Excitation of M540 and JC-1 were achieved by using a 488 nm laser (20 mW) with a 530/30 filter. For PNA-AlexaFluor™647 a 633 nm laser (17 MW) with a 660/20 filter was used. Data from

10,000 individual spermatozoa were acquired at medium speed. Data were analyzed using FCS Express (version 3, De Novo Software, Glendale, CA, USA).

First of all, the population of single spermatozoa was determined. A gate was placed in the dot plot on forward scatter versus side scatter (Figure 1 – Chapter 1). For further evaluation, only cells from this gate were considered. Thresholds for quadrants that distinguish between Hoechst 33258 positive (dead) sperm and Hoechst 33258 negative (live) sperm with either low PNA-AlexaFluor 647 signal (acrosome intact) or high PNA-AlexaFluor 647 signal (acrosome damaged or reacted) fluorescence intensity were set in the control medium. Similarly, the quadrants that distinguish between viable sperm with low M540 signal (low -plasma membrane fluidity) and high M540 signal (high plasma membrane fluidity) and high JC-1 signal (high mitochondrial potential) and low JC-1 signal (low mitochondrial potential) were set.

3.1. KH7 and LRE1

For this experiment KH7 (Sanbio, 13243-10, The Netherlands) and LRE-1 (Sigma-Aldrich, SML1857, Zwijndrecht, The Netherlands) were used to check whether an early step of capacitation, i.e. an increase in membrane fluidity would be blocked by the inhibition of the sAC. It was also checked with the use of the probe JC-1 whether the molecules influenced the mitochondrial transmembrane potential as it has been reported for KH7 (Jakobsen et al., 2018).

Three different concentrations of either KH7 or LRE-1 (final concentration: 10 µM, 60 µM and 120 µM) were tested in TyrControl and TyrBic. Concentrations were based on previous published work on mouse and stallion sperm (Hess et al., 2005; McPartlin et al., 2011). The molecules were added to the percoll washed sperm 30 minutes before experimentation.

Samples were assessed after 15, 30 and 60 minutes in the flow cytometer. DMSO was used as solvent control. The main populations of interest were viable sperm with high membrane fluidity, and viable sperm with high mitochondrial potential

3.2. Activation of tmACs with forskolin

For this experiment forskolin (MCE, 66575-29-9, USA) was used to check whether the number of acrosome-reacted spermatozoa can be increased by this specific stimulator of tmACs.

Four different concentrations of forskolin (final concentration: 10 µM, 50 µM, 100 µM and 500 µM) (10, 50 and 100 µM based on mouse sperm as described in Baxendale et al., 2003; Bitterman et al., 2013; Wertheimer et al., 2013) were added to TyrControl and TyrBic and samples were assessed after 15, 60 and 120 minutes in the flow cytometer. DMSO was used as vehicle control and the population of viable, acrosome reacted spermatozoa was evaluated.

4. Immunofluorescent detection of sAC

For this experiment, stallion semen and boar semen (from AIM Varkens KI Netherlands) were used to compare the localization of sAC in the sperm cell. Boar semen was used as a control, because these sperm are reported to have a high sAC activity (Leemans et al. 2019). This experiment used one ejaculate from each three different animals from each species.

For both species, the spermatozoa were separated from the semen extender and seminal plasma by density gradient centrifugation. Diluted semen (9 mL) was layered on top of 3 mL of 35% Percoll®-saline in a 15-mL centrifugation tube and centrifuged for 10 minutes at room temperature at 750 x *g*. A less selective procedure of density gradient centrifugation was chosen to obtain a representative image of the original samples. After centrifugation, the supernatant was removed. The remaining pellet was resuspended in DPBS (Sigma D8537) and adjusted to a concentration of $100x10^6$ sperm/mL. 500 µl of the semen were incubated with 500 µl of 4% paraformaldehyde for 15 minutes for fixation. After fixation, the samples were centrifuged for 5 minutes at 600 x g. The supernatant was removed, the pellet diluted in DPBS and another centrifugation was performed. After this, the samples were resuspended in a blocking solution (PBS with 5% of normal goat serum and 0.1% of Triton X-100). For the sample preparation a Superfrost™ (Thermo Fischer)

slide was used to attach the cells in the slide. 8 µl droplet of the sample was put above a coverslip then the coverslip was turned back and placed on the slide.

After 30 minutes of incubation the slides were washed twice with DPBS and after the wash, the primary antibody (ADCY10 Polyclonal Antibody,bs-3916R, Bioss Antibodies Inc., Woburn, Massachusetts, U.S.A) was placed in the sample in an 8 µl droplet. The samples were incubated overnight and then washed twice with DPBS. Next, the secondary antibody (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, A-11008, Life Technologies, Bleiswijk, the Netherlands) was placed in an 8 µl droplet on the slide and incubated for 2 hours. Also Hoechst 33342 and AlexaFluor™647 were included to stain the nuclei and the acrosome of the sperm, respectively. After the incubation, the slide was washed with PBS and later covered with 5 µl of Vectashield (Vector Laboratories, California, USA) and a coverslip that was sealed with nail polish. Vectashield was added to prevent the fluorescence from bleaching.

For cell imaging, a fluorescence microscope (Olympus BX60, Olympus Nederland BV, Leiderdorp, The Netherlands) and a laser scanning confocal microscope (LEICA SPE II DMI 4000, Leica Microsystems, Wetzlar, Germany) were used. On the LEICA SPE II DMI 4000, Hoechst33342 which labels all DNA was excited with the 405 nm laser and the secondary antibody (conjugated to Alexa Fluor 488) for detection of the ADCY10, was excited with the 488 nm laser. For one image, PNA-AlexaFluor™647 was excited in a 633 nm laser for acrosome detection.

STATYSTICAL ANALYSIS

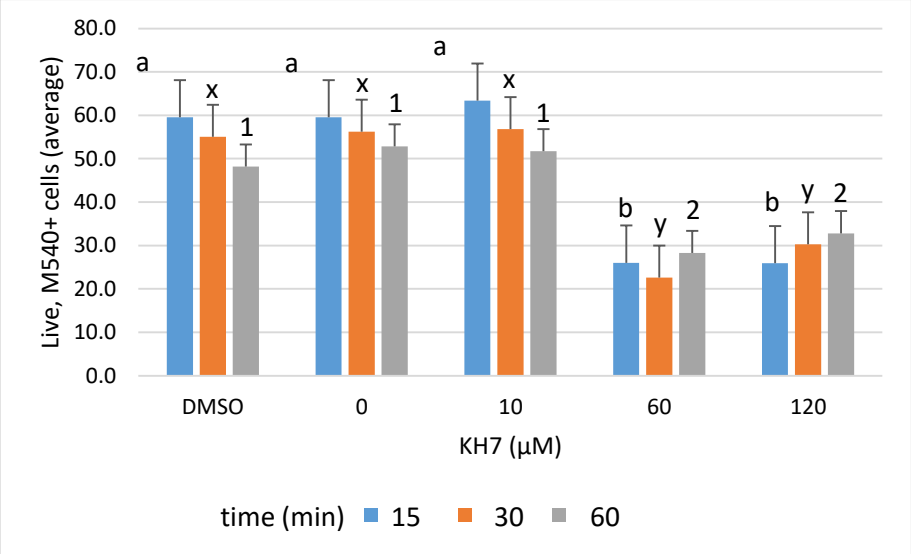
Statistical analysis was performed using SPSS statistics version 25 (IBM analytics, Amsterdam, The Netherlands). Data were tested for normal distribution with a Shapiro-Wilk test and compared with a multi-factorial ANOVA for repeated measures. Considered factors differed according to the experiment. They included time, medium (TyrControl, TyrBic) and the different concentration of the tested molecules (KH7, LRE1, and forskolin). Paired Student’s t-tests for dependent samples were performed to investigate differences between individual medium

variants. Unless otherwise stated, data are presented as mean ± standard deviation (SD). Differences were considered at a significance level of 5% (p≤0.05).

RESULTS

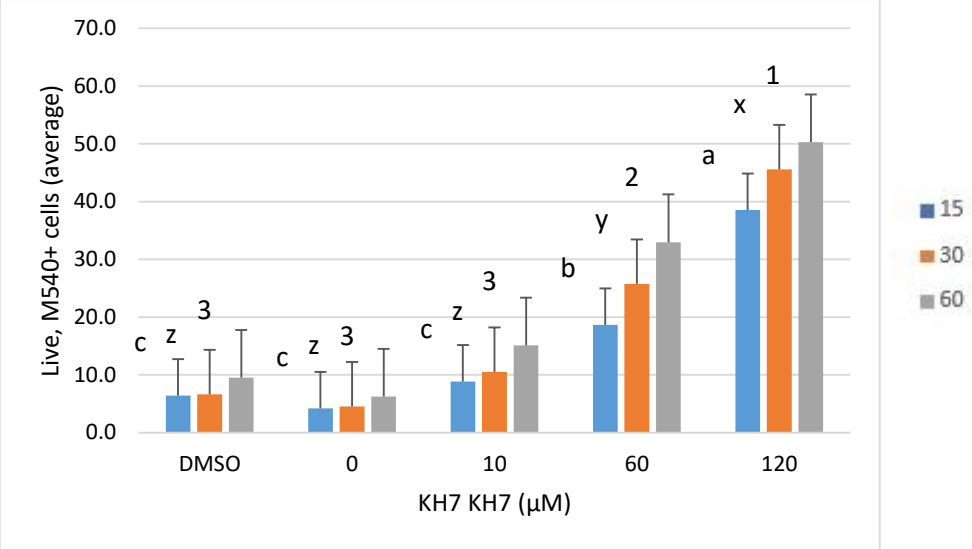
1. Inhibition of sAC in stallion sperm by KH7

In graphic 1 it is shown that KH7 at concentrations of 60 and 120 µM, different from 10 µM, was efficient in reducing at all times the population that was live with high membrane fluidity indicating that sAC is responsible for the cAMP production in this capacitation step in stallion sperm.



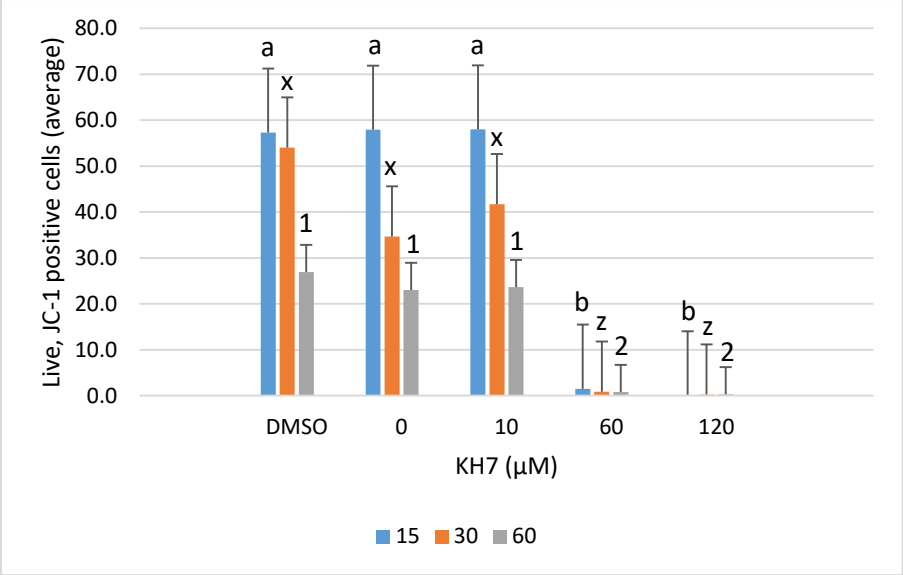
Graphic 1: Population of live, merocyanine positive spermatozoa in TyrBic media with four different concentrations of KH7 (0; 10; 60 and 120 µM) and DMSO as control after 15, 30 and 60 min of incubation. Letters and numbers indicate the difference between the media in the same time (Mean ± SD; p≤0.05).

However, when the TyrControl media was evaluated, the concentrations of 60 and 120 µM of KH7 increased the membrane fluidity in the live sperm population in all times (graphic 2).



Graphic 2: Population of live, merocyanine positive (high membrane fluidity) spermatozoa in TyrControl media with four different concentrations of KH7 (0; 10; 60 and 120 µM) and DMSO as control after 15, 30 and 60 min of incubation. Letters and numbers indicate the difference between the media in the same time (Mean ± SD; p≤0.05).

The observations in Tyr Control stimulated an experiment to check possible side effects of KH7 on other physiological events in the sperm cell like the mitochondrial function. It has been suggested that KH7 may inhibit sAC partly by decreasing mitochondrial ATP production through working as an uncoupler of mitochondrial respiration and ATP production (Di Benedetto et al., 2013; Ramus-Espiritu et al., 2016). From Figure 3 it is obvious that the use of KH7 is completely abolishing a high mitochondrial transmembrane potential in viable sperm at the same concentrations that caused a reduction in the membrane fluidity in TyrBic in stallion sperm. The reduction in the mitochondrial membrane potential was also seen in the TyrControl medium (data not shown).

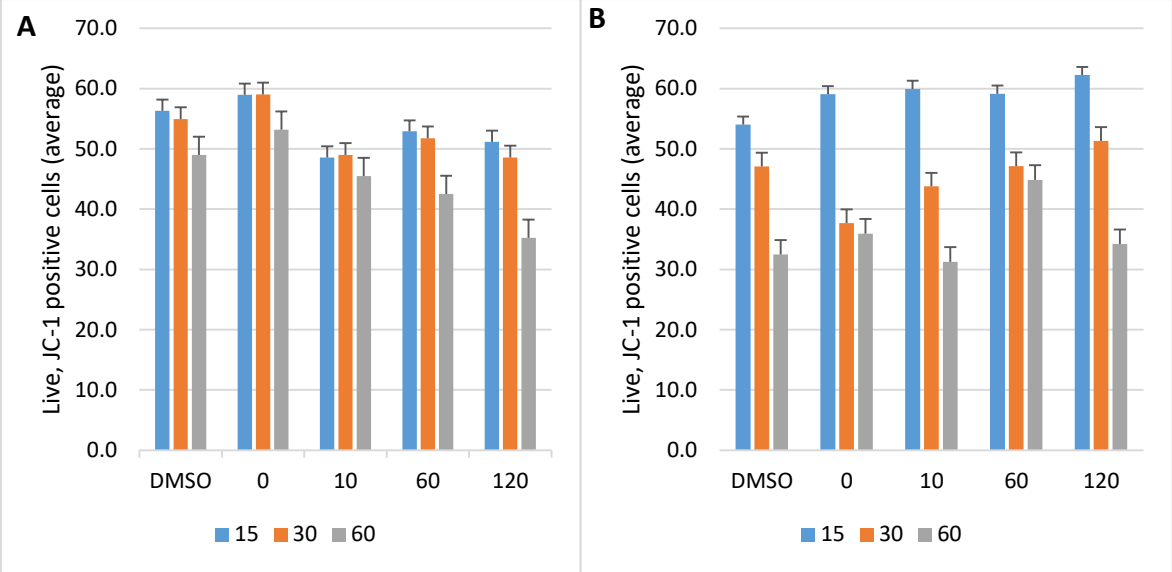


Graphic 3: Population of live, JC-1 positive (high mitochondrial potential) spermatozoa in TyrBic media with four different concentrations of KH7 (0; 10; 60 and 120 µM) and DMSO as control after 15, 30 and 60 min of incubation (n = 3 stallions). Letters and numbers indicate the difference between the media in the same time (Mean ± SD; p≤0.05).

2. Inhibition of sAC in stallion sperm by LRE1

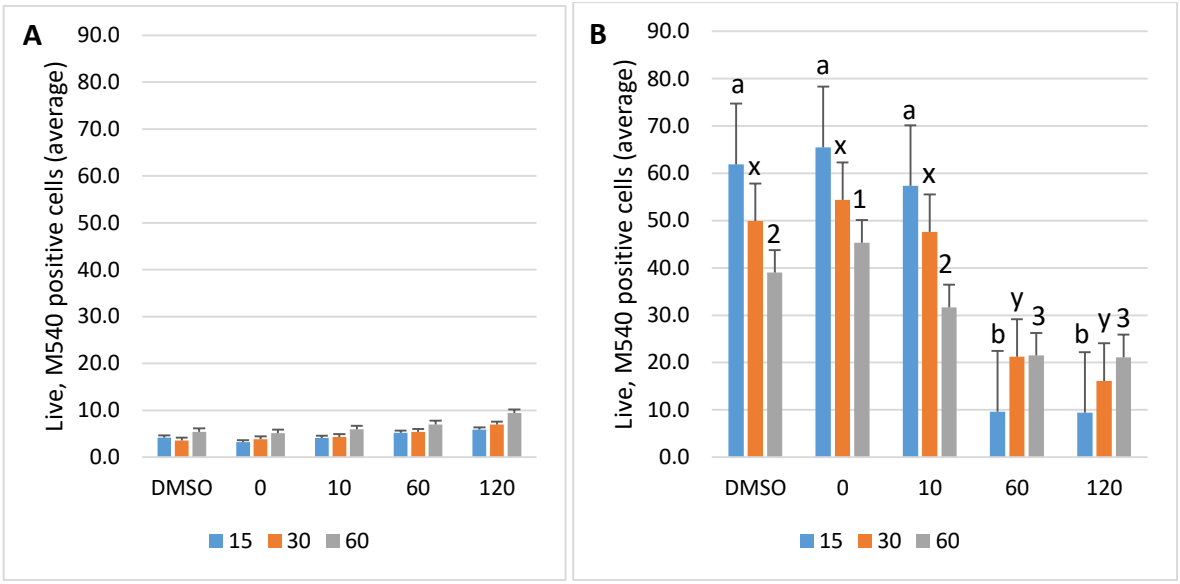
Since KH7 blocked the mitochondrial potential in the stallion sperm, LRE1 was tested to check whether this is a suitable alternative molecule for sAC inhibition during capacitation in stallion sperm. Because LRE1 is a relatively new molecule (Ramos-Espiritu et al., 2016) which has not been used in sperm before, the same concentrations as for KH7 were tested.

Graphic 4 shows that LRE1, unlike KH7, does not interfere with the mitochondrial transmembrane potential.



Graphic 4: Population of live, JC-1 positive (high mitochondrial potential) spermatozoa in TyrControl (A) and TyrBic (B) media with four different concentrations of LRE1 (0; 10; 60 and 120 µM) and DMSO as control after 15, 30 and 60 min of incubation (n = 3 stallions). Letters and numbers indicate the difference between the media in the same time (Mean ± SD; p≤0.05).

Regarding membrane fluidity, graphic 5 shows that LRE1 does not raise the membrane fluidity in viable sperm in TyrControl ($P>0.05$), but LRE1 efficiently decreased the live, M540 positive sperm population in TyrBic at concentrations of 60 µM and 120 µM ($P<0.05$). The inhibitory effect of LRE1 was the strongest after 15 minutes incubation and lessened with ongoing incubation time.



Graphic 5: Population of live, M540 positive (high membrane fluidity) spermatozoa in TyrControl (A) and TyrBic (B) media with four different concentrations of LRE1 (0; 10; 60 and 120 µM) and DMSO as control after 15, 30 and 60 min of incubation. Letters and numbers indicate the difference between the media in the same time (Mean ± SD; p≤0.05).

3. Localization of sAC in stallion and boar sperm

The results from the immunofluorescent staining showed that the sAC has a different localization in freshly ejaculated boar and stallion spermatozoa (Figure 1 and 2). In boar sperm, the strongest signal for the sAC is localized mainly in the acrosomal area and in the neck. A less bright, dotted pattern was observed in the midpiece and principal piece. In stallion sperm, the sAC was localized primarily in the post acrosomal area, forming a dotted signal in the shape of a necklace around all the head of the sperm (Figure 2 and 3). A slightly weaker signal was detected in the neck of the stallion sperm (Figure 3).

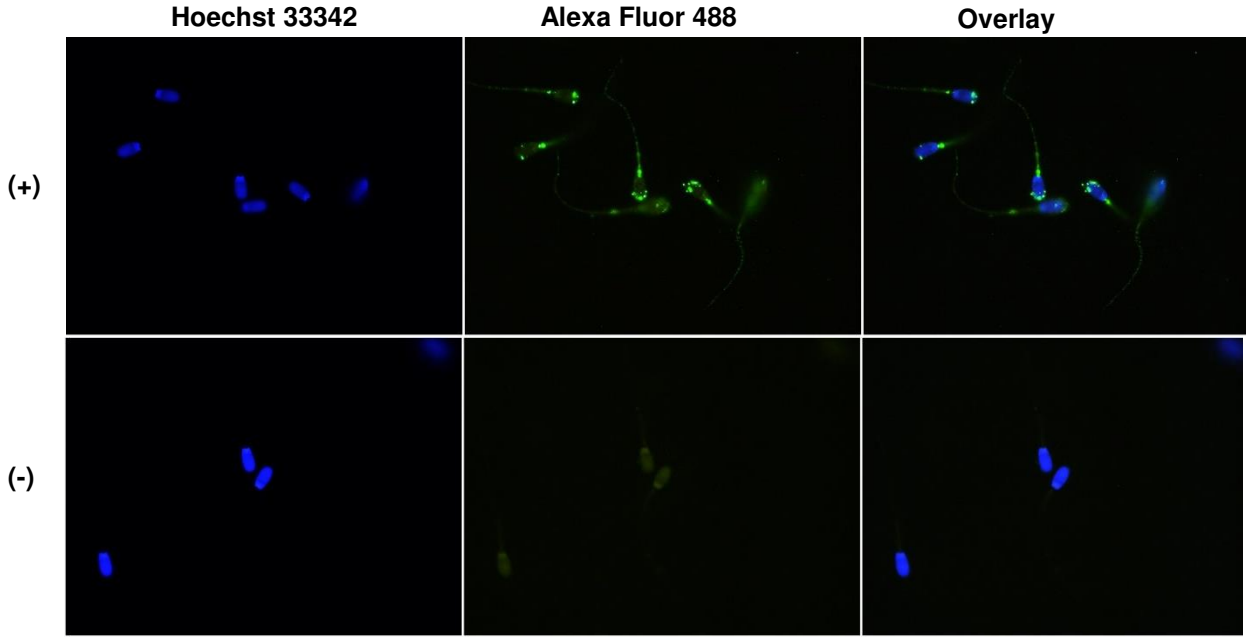


Figure 1: Confocal image of immunofluorescence staining demonstrating the cellular localization of sAC in boar sperm (upper row; 1000x). The negative control (lower row) was processed without the primary antibody. Images are representative results for sperm from three different boars (n = 3). Differences in signal intensity in individual sperm are due to different optimal focus levels.

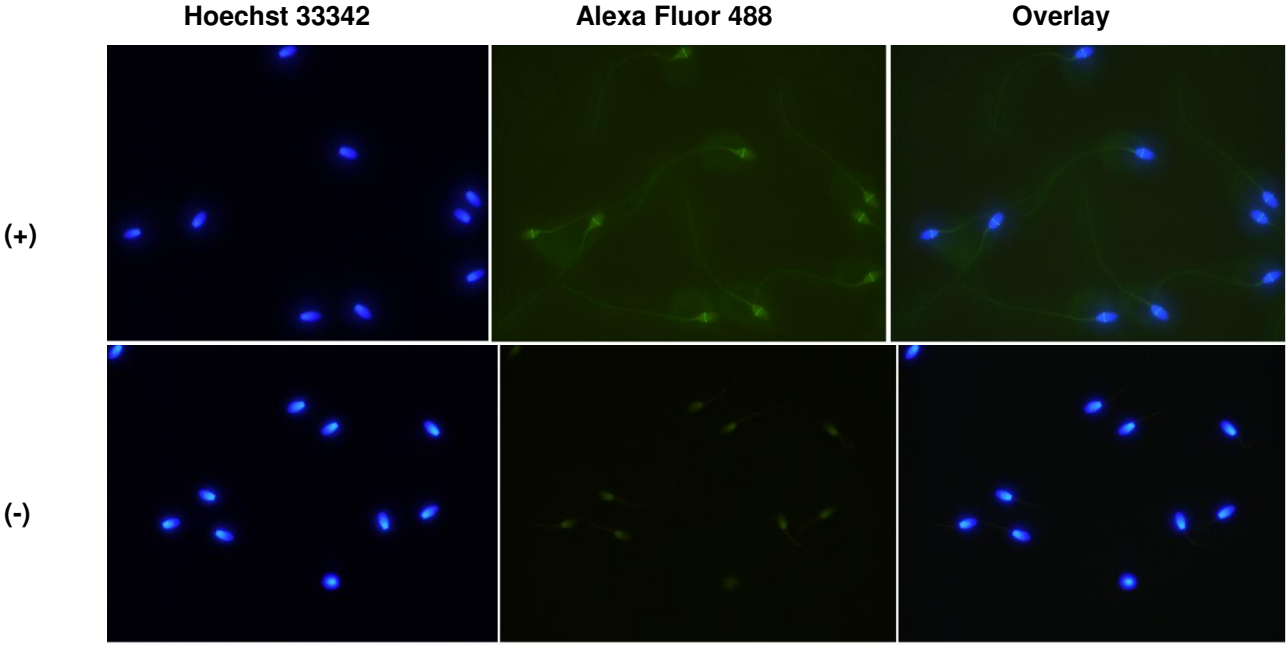


Figure 2: Confocal image of immunofluorescence staining demonstrating the cellular localization of sAC in stallion sperm (upper row; 1000x). The negative control (lower row) was processed without

the primary antibody. Images are representative results for sperm from three different boars (n = 3). Differences in signal intensity in individual sperm are due to different optimal focus levels.

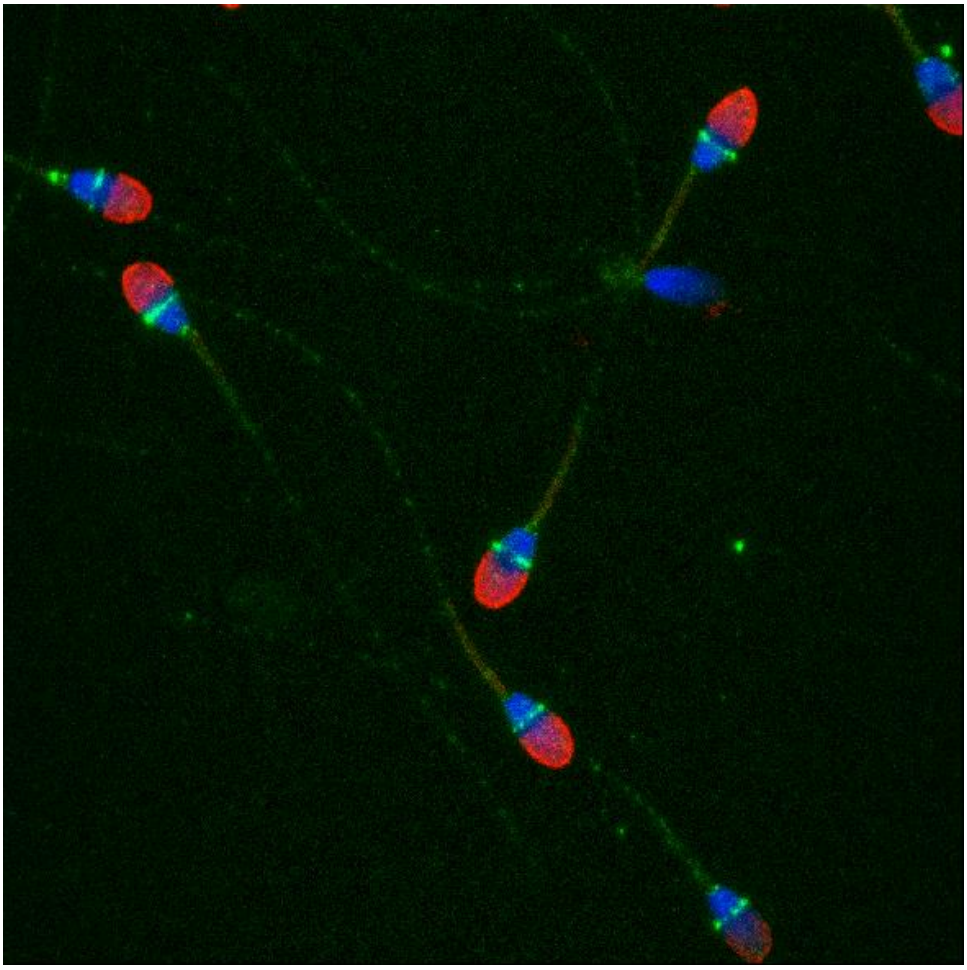
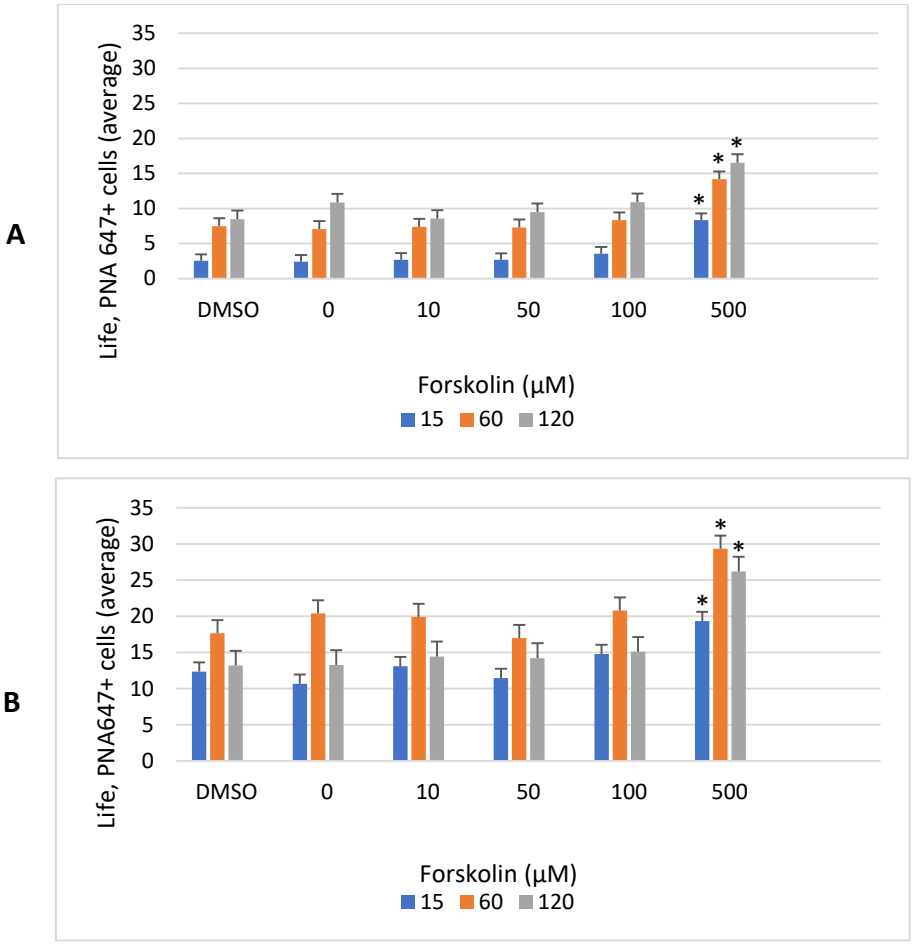


Figure 3: Confocal image of immunofluorescence staining demonstrating the cellular localization of sAC in stallion sperm (1000x). sAC is presented in green (Alexa 488), while nuclei and acrosome are presented in blue (Hoechst 33342) and red (Alexa 647) respectively.

4. Stimulation of tmAC in stallion sperm by forskolin

The findings of this experiment showed that forskolin stimulates the acrosome reaction in the population that is live only in the concentration of 500 µl in TyrControl and in TyrBic media (graphic 6). These findings may suggest that the tmAC can be present in the stallion sperm head playing a role in the acrosome reaction.



Graphic 6: Population of live, Alexa Fluor PNA-647 positive spermatozoa in TyrControl (A) and TyrBic (B) media with five different concentrations of forskolin (0; 10; 50; 100 and 500 µM) and DMSO as control after 15, 60 and 120 min of incubation. Letters and numbers indicate the difference between the media in the same time (Mean ± SD; p≤0.05).

DISCUSSION

Two different types of AC seem to be present in sperm cell with different localization, regulatory properties and distinct functions besides having the same product (cAMP): A soluble adenylyl cylclase (sAC) (Chen et al., 2000) and a transmembrane adenylyl cyclase (Sunahara et al., 2002). Therefore, the aim of the study was to pharmacologically characterize which AC is involved in the initiation of sperm capacitation in stallion sperm under in in vitro conditions and which AC is involved in the acrosome reaction.

Pharmacological experiments provide meaning to study the acute effects of a modulation in a signaling pathway. The addition of a molecule that inhibits or stimulates can rapidly turn off or increase its target indicating which pathway it is involved. Regarding cAMP production, to understand which AC is involved, this pharmacological way becomes preferential than the use of knockout models for example (Bitterman et al., 2013). Performing different pharmacological experiments, our results strongly suggest that in stallion sperm there is also two sources of cAMP, the sAC and the tmAC. During mouse sperm capacitation, two different sources of cAMP orchestrate signaling pathways in the cell. The presence of HCO_3^- and Ca^{2+} increase the levels of cAMP by stimulation of a sAC. The raised cAMP acts on a PKA as well as on cyclic nucleotide-gated channels (CNG) and NHE (Na+/H+ exchangers) channels. These molecules have a direct impact on the tyrosine phosphorylation pathway that correlates to sperm capacitation. By the use of LRE1 in our experiment, (graphic 5) the same AC (sAC) is also responsible for the increase in membrane fluidity in stallion sperm. In the sperm head the levels of cAMP are increased by the stimulation of a tmAC which acts primarily on EPAC leading to the preparation to undergo the acrosome reaction (Buffone et al., 2014). Our results possibly indicate also the presence of a tmAC in the stallion sperm (graphic 6).

To determine the efficacy of KH7 in mouse sperm, Hess et al. (2005) measured the levels of cAMP in non-capacitating and capacitating conditions in the presence or not of different concentrations of KH7 (10 and 50 µM). In non-capacitating conditions only 50 µM was able to reduce the levels of cAMP while in capacitating conditions, 10 µM was already sufficient to block a cAMP-dependent capacitation induction. The findings concluded that sAC represents the predominant source of cAMP in mouse sperm generated in capacitation conditions.

Capacitation-related tyrosine phosphorylation in stallion sperm was also reduced to levels similar to non-capacitating conditions with 60 µM of KH7 (McPartlin et al., 2011). The amount needed for stallion sperm was twice the concentration needed to do the same effect in mouse sperm (30 µM). Lower concentrations of KH7 (15 and 30 µM) were not sufficient to conduct this effect in stallion sperm. Since increase in membrane fluidity is an early capacitation step dependent in the raise

levels of cAMP, and McPartlin et al. (2011) showed that stallion sperm needed higher concentration of KH7 than mouse sperm to show its effect. By this, these experiments helped choose the concentration of KH7 used in our experiment (10, 60 and 120 µM).

KH7 in membrane fluidity inhibition was used to prove if this capacitation step uses cAMP generated from a sAC. Our results show that only when higher concentrations of KH7 were used (60 and 120 µM) the goal was achieved (graphic 1). However, the use of KH7 also affected the population live, M540-positive sperm in TyrControl (graphic 2) and the mitochondrial function in this experiment (graphic 3). KH7 has a chemical structure that includes weakly acid phenols and other properties suggesting that it can work as an uncoupler (Terada, 1990). Jakobsen et al. (2018) working with preparation of mouse brain cortex mitochondria showed that in the concentration of 30 and 50 µM the ATP production dropped drastically and at 30 µM the mitochondrial potential had also decreased confirming that KH7 works as an uncoupler, a non sAC-mediated effect on mitochondrial function. KH7 seems to inhibit ATP production by reducing respiration at lower concentrations and perturbing membrane potential at higher concentrations.

In contrast, the structure of LRE1 suggest that this molecule may not work as a strong uncoupler of mitochondria respiration since it does not have an acidic dissociable group (Jakobsen et al., 2018). LRE1 inhibits basal sAC activity by altering the conformation of the active site disturbing the proper arrangement of the bound ATP for turnover and it is non-toxic to a variety of cell types (Ramos-Espiritu., 2016) being a preferred sAC specific inhibitor to use for long term cellular assays (Wiggins et al., 2018).

Since it is a new molecule and not widely studied in the sperm capacitation field, the chosen concentrations for LRE1 (10, 60 and 120 µM) were based on KH7 studies and to compare it to our KH7 experiment. As seeing in graphic 4 the use of LRE1 did not interfere at the mitochondrial potential in TyrControl and TyrBic indicating that this molecule, in sperm assays, also does not work as a mitochondrial uncoupler being a more suitable inhibitor than KH7.

Regarding the membrane fluidity, LRE1 was able to reduce it in TyrBic in the concentrations of 60 and 120 µM with no alteration in TyrControl (graphic 5 A and B) strongly suggesting that the cAMP produced for this capacitation step in stallion sperm comes from an activation of a sAC. Ramos-Espiritu et al. (2016) working with mouse sperm used LRE1 at concentrations of 30 and 50 µM were able to block, in a concentration dependent manner, the sAC PKA-dependent phosphorylation of its substrates and tyrosine phosphorylation.

In cell culture assays, sAC was already found by immunocytochemistry in many compartments as cytoplasm, inside the mitochondria and nuclei (Zippin et al., 2003, 2004, 2010). This technique was also used to immunolocalizate different tmACs isoforms in mouse and human spermatozoa (Baxendale et al., 2003; Spehr et al., 2004 respectively). Regarding the sAC in the sperm cell, its presence has been already demonstrated in mouse sperm by RT-PCR (Wertheimer et al., 2013) and especially through cAMP immunoassays (Uguz et al., 1994; Lefievre et al., 2000; Harrison et al., 2000; Tardif et al., 2004). Liguori et al. (2004) working with mouse sperm electron microscopy showed that sAC activity in HTF bicarbonate containing medium was present in the head region as fine granules that were localized on the plasma membrane and in the small residual cytoplasmic area. In the midpiece and in the tail products of reaction were observed also as fine granules in the mitochondria and inside the tail showing different localization of sAC. In our experiment, the immunohistochemistry results of sAC localization were different between stallion and boar sperm. Boar sperm showed an acrosomal and neck staining more related to the one already found in mouse (Liguori et al., 2004) while stallion sperm sAC formed a necklace in the post acrosomal area (Figures 1 and 2). The localization of sAC in stallion sperm is intriguing when is consider that the membrane fluidity changes happens in the entire cell. However, this is an interesting finding that confirm one of the differences from the same cell (sperm) from different species.

The presence and role of the tmACs in the sperm cell have controversial studies. Many studies concluded that this enzyme is present in mammalian sperm (Monks et al., 1986; Fraser and Duncan, 1993; Fraser and Dudley 1999; Lecler and

Kopf, 1999; Livera et al., 2005) and different methodologies including enzymatic assays (Baxendale et al., 2003), mass spectrometry (Spehr et al., 2004) and mouse knock-out studies (Livera et al., 2005) have been used for this purpose. However, also a large amount of studies could not find evidence of the presence of tmACs in the sperm cell (Cheng and Boettcher, 1979; Forte et al., 1983; Strunker et al., 2011; Brenker et al., 2012). Due to this subject of controversy we investigated the role of forskolin in the stallion sperm during capacitation to understand if tmAC can be present and play a role in capacitation in this cell.

Leclerc et al. (1996) working with human sperm in capacitating medium (10 mM of HCO_3^-) showed that 100 µM of forskolin induced capacitation (which was measured by the ability of the spermatozoa to undergo acrosome reaction upon exposure to lysophosphatidylcholine).

The concentration of 50 µM of forskolin significantly enhanced cAMP production and capacitation (acrosome reaction) in permeabilized mouse spermatozoa, while inclusion of 2',5'- dideoxyadenosine (ddAdo) in this treatment significantly blocked these responses (Baxendale et al., 2003). The same concentration of forskolin used in Bitterman et al. (2013) experiment in mouse parental HEK293 cells that contain the AC4-7 and AC9 tmAC isoforms also stimulate the activity of these enzymes. Wertheimer et al. (2013) demonstrated the increase in cAMP levels in Adcy10-null mice sperm using forskolin (10, 30, 100 µM) in a dependent concentration manner indicating the presence of the tmAC in this species. They also demonstrated its role in acrosome reaction, since Gαs protein is localized in the anterior head and this capacitation step was also increased in the presence of forskolin (50 µM).

Based on these studies and the lack of work of this molecule in stallion sperm, the concentrations used in our experiment were selected and, the higher concentration (500 µM), was chosen because in sAC previous experiments was already demonstrated that stallion sperm needed higher concentrations of molecules to have the same response as mouse sperm (McPartlin et al., 2011).

Acrosome reaction was increased in TyrControl media but more evident in TyrBic media in the presence of 500 µM of forskolin in all incubation times. The

increase in the population live, acrosome reacted in the control group indicate the non-dependence for bicarbonate from the tmACs corroborating with all other studies that says that this is a G protein regulated enzyme (Sunahara et al., 2002; Baxendale et al., 2003; Wertheimer et al., 2013; Buffone et al., 2014). The effect was more evident in TyrBic due the presence of bicarbonate, which triggers the early steps of capacitation leading to a higher population with complete activation.

Due the fact that tmACs may have overlapping functions in many cell types, it is difficult to point the specific isoform involved in a given process but our results indicate that tmACs are present in stallion sperm. Inhibition studies with the use of ddAdo for example and immunohistochemistry of the tmACs different isoforms should be performed to strongly affirm our findings.

CONCLUSION

The presence of sAC in the stallion sperm is responsible for the increase in membrane fluidity in the presence of bicarbonate. But the immunostaining of sAC in stallion sperm indicated different localization compared to boar. The action of forskolin indicate that tmAC may be present in the stallion sperm and may play a role in the acrosome reaction in a subset of responsive sperm. Further research might be directed to why there is such discrepancy in sperm that show early activation, but only few where AR can be induced.

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