

Calcium influx and the progesterone-induced acrosome reaction in stallion spermatozoa

Master Thesis

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Abstract

In vitro fertilization has been successfully applied in humans, rodents and dairy cattle but remains unsuccessful in the horse. One of the main proposed reasons is that the sperm cells fail to penetrate the zona pellucida on their own, most likely due to incomplete capacitation of spermatozoa because of inadequate activation or an unsuitable fertilization media. The main components in medium to induce *in vitro* capacitation in mammalian spermatozoa have been identified as bicarbonate, calcium and albumin. In other mammalian species, but the horse, sperm incubated in a medium with the three main components showed capacitation-related characteristics as high membrane fluidity, protein tyrosine phosphorylation, hyperactivated motility and acrosome reaction. We investigated whether bicarbonate (HCO_3^-), extracellular calcium and progesterone modulate membrane fluidity and acrosome reaction in stallion spermatozoa. Semen was initially diluted in INRA96 (30×10^6 spermatozoa/mL). After Percoll® washing, spermatozoa were incubated at 37°C in Tyrode's medium without ($\text{Tyr}_{\text{Control}}$) or with 30 mM bicarbonate (Tyr_{Bic}). The media were either calcium free, i.e. contained ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), or an increasing amount of calcium (0.1 mM - 2 mM). Simultaneous staining for viability (Hoechst 33258), acrosome integrity (PNA-AlexaFluor™647), membrane fluidity (merocyanine 540) was evaluated by flow cytometry after 15, 30, 60 and 120 minutes of incubation of the equine spermatozoa in the first experiment and after 30, 60 and 120 minutes in the second experiment. The first experiment showed that bicarbonate and calcium are necessary to induce early steps of capacitation, such as high membrane fluidity. An amount of calcium ranging from 0.1 mM to 2 mM had the same effect on the percentage of spermatozoa with high membrane fluidity. The aim of the second experiment was to determine the amount of progesterone needed to induce an acrosome reaction. However, in the current experiment it appeared that there was no significant difference between the three progesterone concentrations (10 ng, 100 ng and 1000 ng) and the control group with only ethanol ($p > 0.05$, ANOVA for repeated measures). In conclusion, progesterone appears not to be the relevant molecule to induce the acrosome reaction in stallion spermatozoa. Further research is needed to elucidate this finding, which is in contradiction to earlier published research.

Introduction

In vitro fertilization has been successfully applied in humans, rodents and dairy cattle (Betteridge, 2006; Galli et al., 2003; Perry, 2014) but remains unsuccessful in the horse (Leemans et al., 2016). One of the main proposed reasons is that the sperm cells fail to penetrate the zona pellucida on their own (Leemans et al., 2016). Tremoleda confirmed this speculation by showing that sperm failed to penetrate both *in vivo* and *in vitro* matured oocytes. Sperm were able to bind to the zona pellucida, but did not acrosome react (Tremoleda, Stout, Gadella, & Colenbrander, 2004). Gathering knowledge on the regulation of the acrosome reaction in stallion spermatozoa is important to develop a chemically-defined medium which supports the acrosome reaction in stallion spermatozoa *in vitro* and subsequently *in vitro* fertilization.

The inability of equine spermatozoa to penetrate the zona pellucida *in vitro* is most likely due to incomplete capacitation of spermatozoa because of inadequate activation due to unsuitable fertilization media (Leemans et al., 2016). The spermatozoa need to undergo some physiological changes in the female reproductive tract to gain the ability to fertilize (Chang, 1951). This process is called capacitation. Capacitation is nowadays considered a consecutive activation of different signalling pathways inducing physiological and biochemical modifications which primes the sperm for fertilization *in vitro*. It enables the sperm to recognize and bind to the zona pellucida (Gervasi & Visconti, 2016). Subsequently, the sperm undergoes an acrosome reaction, which is necessary to penetrate and fertilize the oocyte. The ability for an acrosome reaction is an important and critical feature which determines a sperm's fertilization capacity.

Capacitation is a series of molecular reorganization process in the plasma membrane that result in cholesterol depletion in order to enable the spermatozoa for the acrosome reaction. The main medium components to induce *in vitro* capacitation in mammalian spermatozoa are bicarbonate, calcium and albumin (Frits M. Flesch & Gadella, 2000). In other mammalian species, but the horse, sperm incubated in a medium with the three main components showed capacitation-related characteristics as high membrane fluidity, protein tyrosine phosphorylation and hyperactivated motility (Tremoleda et al., 2004).

Bicarbonate initiates the capacitation process. It is taken up via a bicarbonate-sodium co-transporter. Intracellular bicarbonate targets a soluble adenylyl cyclase which becomes activated once bicarbonate has bound (Okamura, Tajima, Soejima, Masuda, & Sugita, 1985). This results in an increase of intracellular cAMP levels, leading to protein kinase A activation, which induces membrane changes (Gadella & Van Gestel, 2004). One of these membrane changes, i.e. an increase in membrane fluidity, can be detected by an increased merocyanine 540 stainability (Steckler, Stout, Durandt, & Nöthling, 2015).

Albumin is thought to play an important role in the capacitation process of the spermatozoa. It seems to remove oxysterol and sterol from the surface of the plasma membrane (Boerke et al., 2012). Addition of albumin to capacitating media causes cholesterol efflux in bicarbonate responsive cells (F M Flesch et al., 2001).

Another key component in the *in vitro* capacitation process is extracellular calcium (Ca^{2+}). Capacitation of mammalian sperm is only achieved in the presence of high Ca^{2+} levels (Stival et al., 2016). Calcium is involved in several capacitation induced events, of which tyrosine phosphorylation, the acrosome reaction and hyperactivated motility are the most important ones (Tardif, Dubé, & Bailey, 2004). The acrosome reaction is of special importance for the penetration of the cumulus-oocyte complex (Talbot, 1985). Progesterone is supposed to be a

physiological inducer of the acrosome reaction in stallion spermatozoa (F.-P. Cheng et al., 1998; Meyers, Overstreet, Liu, & Drobnis, 1995; Rathi, Colenbrander, Stout, Bevers, & Gadella, 2003). In the oviduct, progesterone works as a chemoattractant for mammalian spermatozoa (Teves et al., 2006). Spermatozoa are more likely to migrate to the ipsilateral oviduct, where progesterone levels are higher than in the contralateral oviduct (Ballester et al., 2014). The high local concentrations of progesterone in the oviductal tissue and fluids of the ipsilateral oviduct may be the result of four different mechanisms: (1) a peritoneal route (2) a local counter-current system (3) local synthesis in the oviduct and (4) by follicular cells which are shed in the oviduct at ovulation (paracrine contribution) (Nelis et al., 2016). Progesterone binds to a receptor in the plasma membrane of the sperm head (F.-P. Cheng et al., 1998). Subsequently, an influx of calcium from the medium initiates the acrosome reaction in spermatozoa. A similar mechanism has been hypothesized for stallion spermatozoa (McPartlin et al. 2011), but clear experimental evidence for a progesterone-mediated calcium influx which leads to an acrosome reaction in stallion sperm is missing (Coutinho da Silva, Seidel, Squires, Graham, & Carnevale, 2014, McPartlijn et al. 2011). However, a progesterone-induced increase in the viable, acrosome-reacted spermatozoa has been reported (F. P. Cheng et al., 1998; Meyers et al., 1995). Bicarbonate is supposed to initiate the capacitation process in spermatozoa. Strikingly, a high degree of live, spontaneously acrosome-reacted equine spermatozoa has been reported after incubation of media with 2 mM extracellular calcium in the absence of bicarbonate (Rathi et al., 2003). Although, identical observation in absence of bicarbonate were not made in a recent research project, a high extracellular calcium concentration (2 mM) was the critical factor to observe a live-acrosome-reacted sperm population in the presence of bicarbonate (Hoogendijk HP thesis 2018). In order to further substantiate the findings from Hoogendijk (2018), the aim of the current 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.

Material and methods

Experimental strategy

The aim of the first experiment was to identify an extracellular calcium concentration that is most likely to support capacitation in equine spermatozoa in presence of bicarbonate. At the same time the extracellular calcium concentration should not induce a spontaneous acrosome reaction in the absence of bicarbonate. Calcium concentrations between 0.1 mM and 2 mM were tested. Flow cytometry was used to determine which calcium concentration supported the induction of a live spermatozoa with high membrane fluidity, while keeping the percentage of live, acrosome-reacted spermatozoa low.

The condition with the best results regarding the early hallmarks of sperm capacitation, e.g. an increase in membrane fluidity, from the first experiment was used for experiment two, where different progesterone concentrations were added to induce the acrosome reaction. Staining patterns were compared in the non-capacitating and capacitating medium to visualize changes in fluorescence intensity with flow cytometry.

Chemicals and fluorescent probes

The fluorescent probes that were used were merocyanine 540 (M540), Hoechst 33258 and PNA-AlexaFluor™647.

M540 was purchased from Molecular Probes (Eugene, OR, USA). Hoechst 33258 from Sigma-Aldrich (Zwijndrecht, the Netherlands) and PNA-AlexaFluor™647s from Thermo Fischer Scientific (Waltham, USA). The commercial milk-based semen extender (INRA96) contains gentamycin and penicillin, and was purchased from IMV technologies (l'Aigle, France). Gentamycin was from SERVA Electrophoresis GmbH (Heidelberg, Germany). The progesterone was purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) and Ethanol absolute (EssentQ ref.: ET00021000) from Scharlau (Barcelona, Spain).

Semen collection and dilution

Semen was collected from stallions presented for a breeding soundness examination to the Clinic for Horses of Utrecht University or from stallions housed in the near proximity of Utrecht. Semen was collected from stallions on a phantom using an artificial vagina (Hanover model). After removing the gel fraction and large debris by filtering the semen through gauze, the semen was diluted to approximately 200×10^6 spermatozoa/mL in INRA96 and motility was assessed by computer assisted sperm motility assessment (CASA). Only samples with a motility of 60% or more were further processed. If the motility was above 60%, the concentration was determined with a Bürker-Türk haemocytometer chamber and then the semen was further diluted to a concentration of 30×10^6 spermatozoa/mL in INRA96. Diluted semen was kept at room temperature until further processing.

Sample preparation for flow cytometry

First the semen had to be separated from the extender and the seminal plasma. Therefore, density gradient centrifugation was performed. In a 15 mL tube first 2 mL of 70% Percoll®-saline solution and 4 mL of 35% Percoll®-saline solution were layered. On top of this discontinuous gradient 6 mL of the diluted semen was placed without mixing the layers. Tubes were centrifuged for 20 minutes at 21°C. The first 10 minutes at 300g followed by 10 minutes at 750g. After centrifugation the supernatant was removed and the remaining pellet was taken out and resuspended in 1 mL of Tyrode's medium without calcium and BSA. The concentration of the pellet was determined with a Bürker-Türk haemocytometer chamber and, if necessary, further diluted to a concentration of 60×10^6 sperm/mL.

Incubation media for equine spermatozoa

The non-capacitating Tyrode's medium (Tyr_{Control}) consisted of 111 mM NaCl, 3.1 mM KCl, 0.4 mM MgSO₄, 5 mM glucose, 0.3 mM KH₂PO₄, 20 mM of HEPES, 100 µg/mL gentamycin sulfate, 1.0 mM Na-pyruvate, 21.7 mM Na-lactate. In the bicarbonate containing solution (Tyr_{BIC}) 30 mM NaHCO₃ was added and the final concentration of NaCl lowered to 96 mM. The pH was adjusted to 7.40 ± 0.05 at room temperature with NaOH and the osmolality was adjusted to 300 ± 5 mOsmol with NaCl. Media were passed through a syringe filter (PES

membrane, pore size 0.22 μm ; Merck Millipore, Amsterdam, the Netherlands) for sterile filtration. In the first experiment ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; calcium free), 0.1 mM, 0.5 mM, 1 mM or 2 mM of calcium was added to the non capacitating medium (Tyr_{Control}) and the bicarbonate-containing medium (Tyr_{Bic}). In the second experiment only media with 0.1 mM and 2 mM of calcium were used, because we did not see a significant difference between the four calcium concentrations and therefore used the two extremes. The bicarbonate containing media were kept in an incubator with 5% CO₂ and 100% humidity at 37°C for equilibration prior to experimentation. Incubations of spermatozoa in non capacitating media were carried out in a metal heating block at 37°C.

Progesterone was only added in the second experiment. Concentrations of 10 ng/mL, 100 ng/mL and 1000 ng/mL were tested in both capacitating and non-capacitating Tyrode's media. 100 ng/ml was found by Nelis et al. 2016 in the oviduct of the horse and was therefore chosen as initial concentration (Nelis et al., 2016). To cover a wider range we chose a ten times higher and lower concentration of progesterone for our second experiment.

Flow cytometry

Flow cytometry was used to assess membrane changes. 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 of each Hoechst 33258 and PNA-AlexaFluor™647. One tube was prepared for each measurement. In the first experiment, when a concentration series of calcium was tested, samples were assessed after 15 min, 30 min, 60 min and 120 min of incubation. In the second experiment, when also progesterone was added, samples were assessed after 30 min, 60 min and 120 min. Two microliter of M540 (stock solution: 750 mM in DMSO) was added 15 min before measurements took place in both experiments. Two microliter progesterone was added 30 min before measurement in experiment 2. Before analysis on a BD FACSCanto™ (BD Biosciences, Breda, The Netherlands), samples were briefly vortexed. Samples were kept on 37 °C and under CO₂ atmosphere (Tyr_{bic}) 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 analysed 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 for forward scatter versus side scatter. For further evaluation, only cells from this gate were considered. Thresholds for quadrants that distinguish between Hoechst 33258 positive (dead) sperm and Hoechst33258 negative (live) sperm with either low PNA 647 signal (acrosome intact) or high PNA 647 signal (acrosome defect/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.

Statistics

SPSS statistics 25 (IBM Corporation, Armonk, New York, USA) was used to perform the statistical analysis of the collected data. Data were tested for normal distribution and compared with a multi-factorial ANOVA for repeated measurements. In the first experiment the factors included time and calcium concentration (EGTA, 0.1 mM, 0.5 mM, 1 mM and 2 mM) and in the second experiment the factors included were time, calcium concentration, progesterone concentrations and presence of bicarbonate. Unless otherwise stated, data are presented as mean \pm standard deviation (SD). Differences were considered to be significant at a significance level of 5 % ($p \leq 0.05$).

Results

Experiment 1

Bicarbonate and calcium are necessary to induce the first steps of capacitation.

In the bicarbonate containing media (Tyrbic), the percentage of live, merocyanine positive spermatozoa increased drastically (Figure 1). This response occurred as early as in 15 minutes after exposing spermatozoa to bicarbonate irrespectively of the calcium level in the medium. This was seen as a clear shift from a live, M540_{low} to a live M540_{high} state indicated. The merocyanine positive population differed extremely in the non-capacitating and capacitating Tyrode's media (Figure 1 and 2). In the non-capacitating media the live sperm population was \pm 83.7% , of which \pm 79.3% were merocyanine negative spermatozoa and only \pm 3.4% were merocyanine positive. In the capacitating media the live population was \pm 72.4%, of which only \pm 9.5% were merocyanine negative spermatozoa and \pm 62.9% were merocyanine positive spermatozoa (Figure 2). Bicarbonate was necessary to induce a high membrane fluidity in the majority of the viable sperm population ($p < 0.05$).

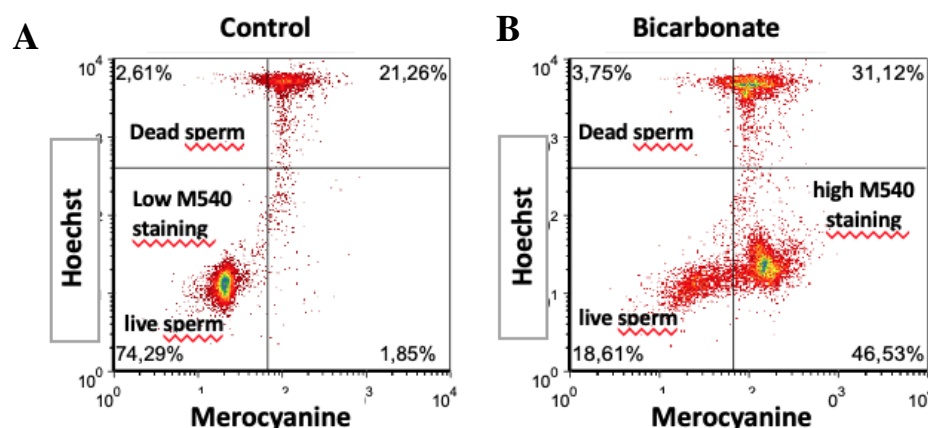


Figure 1: Dotplot on forward scatter versus side scatter. Spermatozoa were incubated in a non capacitating Tyrode's medium (A; Tyr_{Control}) or with 30 mM of bicarbonate (B; Tyr_{BIC}) for 60 minutes.

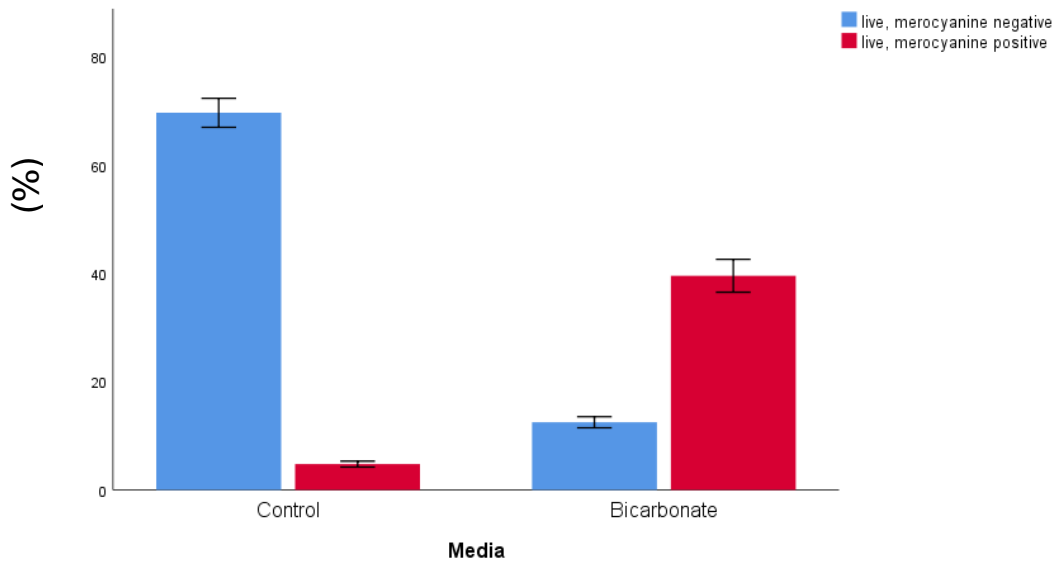


Figure 2: Population live spermatozoa in non-capacitating Tyrode's medium and capacitating Tyrode's medium treated with merocyanine after 60 minutes (n= 6 stallions).

In the bicarbonate containing media high membrane fluidity could be induced in the majority of live spermatozoa after all different times in the presence of all four calcium concentrations and in the absence of calcium. In contrast to the findings in the calcium containing media one can see that the live, merocyanine positive sperm population in the EGTA containing medium decreases over time (Figure 3). EGTA binds the available calcium so it cannot be used by the spermatozoa. With no calcium no high membrane fluidity can be demonstrated.

The actual calcium concentration had no significant impact on the percentage of merocyanine positive spermatozoa ($p > 0.05$) and also the time of incubation had no significant influence ($p > 0.05$).

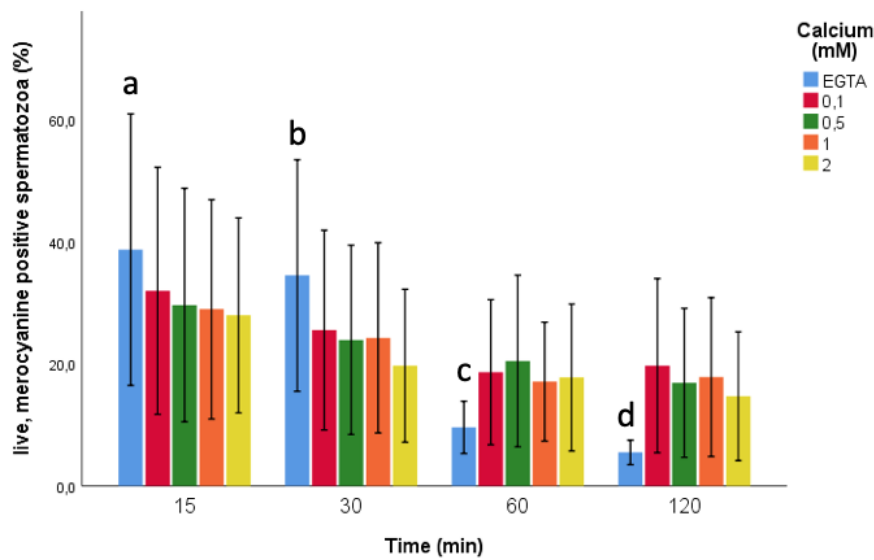


Figure 3: Population of live, merocyanine positive spermatozoa after 15, 30, 60 and 120 min in Tyrode's capacitating medium with four different concentrations of extracellular calcium ($n=6$ stallions) (a, b, c, d = different small letters indicate significant differences over time within a given medium ($p<0.05$)).

Furthermore, there was only a low percentage of PNA 647 positive cells under the same conditions (Figure 4). Time had no significant influence ($p>0.05$). On the contrary, the percentage of dead spermatozoa increased from x percent after 15 minutes to y percent after 120 minutes ($p<0.05$).

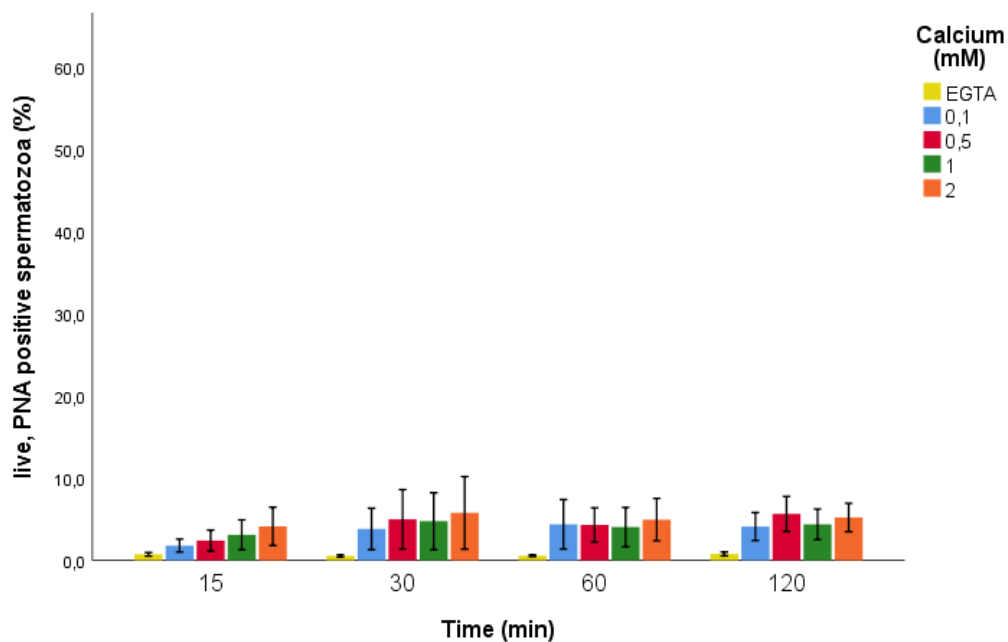


Figure 4: Population of live, PNA 647 positive spermatozoa after 15, 30, 60 and 120 min in Tyrode's capacitating (Tyr_{BIC}) media with four different concentrations of Calcium (n=6 stallions).

Experiment 2

In the series of concentrations that were used in the experiment, the amount of calcium did not matter.

Time had an influence ($p < 0.05$) on the membrane fluidity of the spermatozoa (Figure 5). Regarding the membrane fluidity, after 30 minutes there were the most live, merocyanine positive spermatozoa and after 120 minutes there were the least merocyanine positive spermatozoa ($p < 0.05$). However, there was no significant difference in merocyanine staining when the two different concentrations of calcium (0.1 mM or 2 mM) were compared ($p > 0.05$) in the same incubation time (Figure 5).

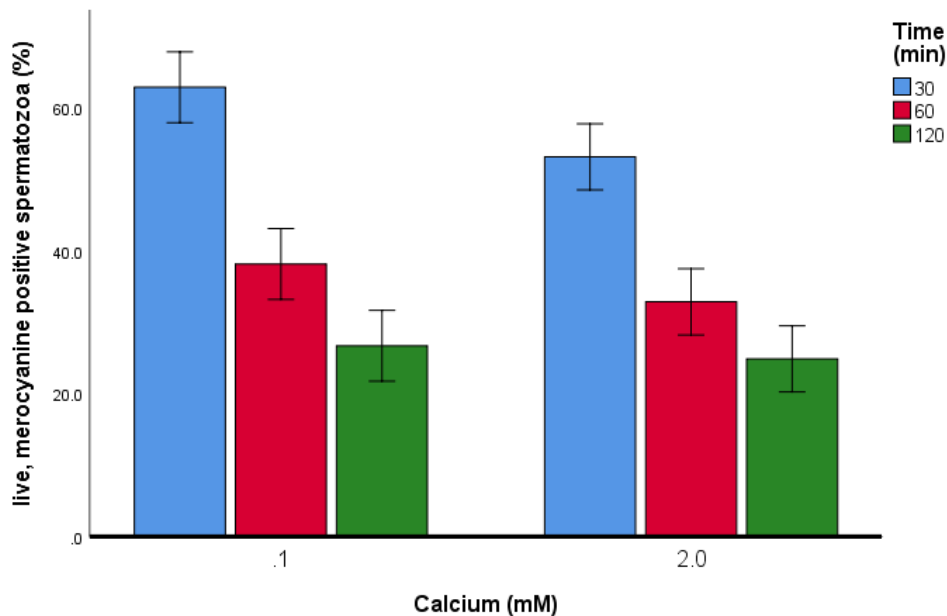


Figure 5: Population of live, merocyanine positive spermatozoa in Tyrode's capacitating media with two different concentrations of Calcium (0.1 mM and 2 mM) after 30, 60 and 120 min of incubation (n = 6 stallions).

Effect of progesterone on the acrosome reaction in viable spermatozoa in media containing different concentrations of calcium.

Furthermore, the aim of the second experiment was to determine the amount of progesterone needed to induce an acrosome reaction. However, in the current experiment it appeared that there was no significant difference between the three progesterone concentrations and the control group with only ethanol ($p > 0.05$) (Figure 6). There is no significant difference between the percentage of live, PNA 647 positive cells after 30, 60 or

120 minutes in medium variant with 0.1 mM and 2 mM extracellular calcium after progesterone addition ($p>0.05$; Figure 6).

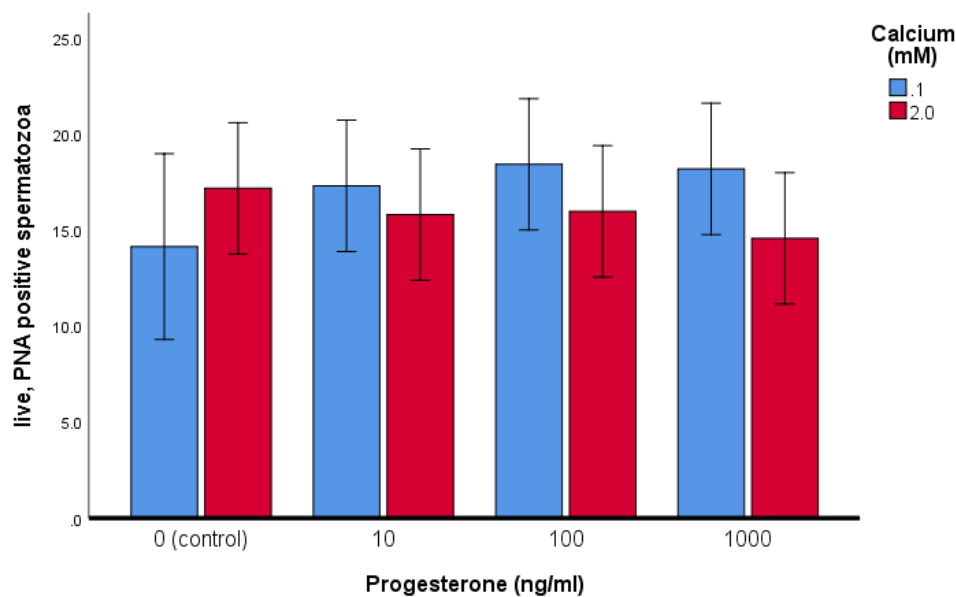


Figure 6: Population live PNA-AlexaFluor 647 positive spermatozoa after 30 min of incubation in capacitating Tyrode's medium (Tyr_{BIC}) with three different concentrations of progesterone or ethanol as a control

Discussion

The aim of the current 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.

Induction of sperm capacitation.

It has been stated that bicarbonate plays an essential role for equine sperm capacitation (Rathi, Colenbrander, Bevers, & Gadella, 2001; P E Visconti & Kopf, 1998). Also in this study only the bicarbonate containing medium induced an increase in membrane fluidity, an early hallmark of capacitation, in the live spermatozoa. Already after 15 minutes a shift can be seen from live merocyanine negative sperm cells to live merocyanine positive sperm cells. This suggests that the conditions that have been used in our study were sufficient to induce the capacitation process. However, it was not checked whether later events in the capacitation process such as hyperactivation, or tyrosine phosphorylation were supported by the experimental conditions.

Impact of extracellular calcium on induction of capacitation

When looking at figure 4 one can see that the sperm cells did not maintain early hallmarks of capacitation when EGTA was added to the Tyrode's medium. EGTA has a high affinity for calcium and binds it in the solution it is brought into, so that the spermatozoa are not able to

use any calcium ions from their environment anymore. Considering this mechanism and seeing that there are only few merocyanine positive spermatozoa in the Tyrode's medium with EGTA after 60 minutes of incubation it can be said that calcium is definitely necessary for sperm cells to undergo capacitation. Furthermore the amount of calcium did not matter in our experiment. The shift from live cells with low membrane fluidity to high membrane fluidity was not significant higher in conditions with much more extracellular calcium (2.0 mM). In most experiments higher concentrations (Breininger, Cetica, & Beconi, 2010; Pablo E. Visconti et al., 1999) are used and the question is, whether this concentrations could harm the cells. The amount of 2 mM of calcium is based on an experiment in mouse spermatozoa and apparently this amount is not necessary in horse spermatozoa (P E Visconti et al., 1995).

Spontaneous acrosome reaction

Strikingly, a high degree of live, spontaneously acrosome-reacted spermatozoa has been reported after incubation of media with 2 mM extracellular calcium in the absence of bicarbonate (Rathi et al., 2003) or in the presence of bicarbonate (25 mM of bicarbonate; McPartlin et al., 2008).

As seen in figure 2 we did not have spontaneous acrosome reaction in the non-capacitating Tyrode's medium (=without bicarbonate). The main difference between the experiment by Rathi et al. (2003) and our experiment was the incubation time. We tested 15 minutes to 120 minutes, whereas he used an incubation time of up to 6 hours.

Mc Partlin et al. (2008) also showed that calcium is a main trigger for the sperm cells in a capacitating medium to undergo the acrosome reaction. In our experiment the spermatozoa did not acrosome react spontaneously in a capacitating medium with calcium, but in contrary to the experiment Mc Partlin the sperm cells were incubated for only 30, 60 and 120 minutes and not for 6 hours. After 6 hours of incubation Mc Partlin showed acrosome reacted spermatozoa, but one cannot see when exactly the acrosome reaction took place and whether the sperm cells were still alive and therefore able to penetrate the zona pellucida of an oocyte. In our experiment the population of dead sperm cells increased significantly after 120 minutes of incubation, probably due to stressful conditions for the spermatozoa. In our experiment less BSA was used than in Mc partlin et al. BSA stabilizes the spermatozoa which can be the reason for the extended life-span of the sperm cells in Mc Partlin's experiment (Bredderman & Foote, 1971).

Progesterone induced acrosome reaction

Progesterone has shown to be a natural inducer of acrosome reaction in stallion spermatozoa (F. P. Cheng et al., 1998; Meyers et al., 1995; Rathi et al., 2003). In our experiment this could not be proven. Three concentrations of progesterone had been tested and there was no significant difference in PNA staining of the spermatozoa in the media with only ethanol as vehicle and 10, 100 and 1000 ng/ml of progesterone. As we look at the staining pattern in the first experiment without ethanol or progesterone we see that we had less than 5% acrosome reacted spermatozoa. As soon as we add ethanol or ethanol with progesterone it rises above 14% but there is no significant difference in PNA staining between the media with or without progesterone, so we cannot conclude that progesterone was the inducer of the acrosome reactions. In the future another vehicle could be used for the avoidance of doubt that actually ethanol was the reason for this slightly higher

percentage in acrosome reacted cells. A water-soluble progesterone could be used instead. Furthermore a higher concentration of progesterone should be used to guarantee a sufficient stimulation.

In addition, a longer incubation time could be tested. Cheng et al. and Rathi et al. showed that 55% of their sperm cells acrosome reacted after an incubation time of 6 hours (F.-P. Cheng et al., 1998; Rathi et al., 2003).

Alternative strategies to induce the acrosome reaction

Next to progesterone, also other factors from the follicular fluids (or oviduct fluids) could be of interested for the activation and capacitation of stallion spermatozoa. Progesterone was identified as a key factor in the capacitation process of human spermatozoa (Harper, Barratt, & Publicover, 2004), whereas it is glycosaminoglycans in cattle (Boice, Geisert, Blair, & Verhage, 1990). Furthermore, heparin is an important factor for bovine sperm capacitation and possibly the acrosome reaction (An et al., 2017). Until now little is known about capacitation factors in follicular fluids of the mare.

Another suggestion made by Leemans et al. 2014 was that oviduct-sperm interactions are essential to induce capacitation of stallion spermatozoa. An optimized equine sperm-oviduct binding model could therefore be used. It was shown that incubation in capacitating Tyrode's medium was a poor stimulator for the spermatozoa to capacitate, whereas binding of sperm cells to oviduct explants caused a huge increase in the percentage of spermatozoa which underwent capacitation-related events like tail-associated protein tyrosine phosphorylation (Leemans et al., 2014). Also a direct membrane contact of oviduct cells and stallion sperm cells *in vitro* result in a higher motility of spermatozoa compared to cells incubated in the absence of oviduct cells (Ball, Brinsko, Thomas, Miller, & Ellington, 1993). To perfectly mimic the oviductal environment oviduct secreted factors should be in the focus of further research. Oestrogen-dependent oviduct specific glycoproteins (OSG) are oviduct factors that enhance sperm-oocyte binding and penetration through the zona pellucida. In the mare these proteins are not found yet, but that does not mean that they are truly absent and further research is necessary (Leemans et al., 2016).

Conclusion

This study showed that bicarbonate is necessary to induce early hallmarks of capacitation, i.e. an increase in membrane fluidity in viable acrosome intact spermatozoa within 15 minutes. It was also demonstrated that calcium is necessary for the achievement of a high plasma membrane fluidity and that lower concentrations than 2 mM (0.1 mM-1 mM) are already sufficient to accomplish this step. However, progesterone did not induce acrosome reaction in our experiment. In the future, a revision of successful IVF protocols of other species to compare induced capacitation hallmarks might be indicated. This might help to develop a medium that successfully supports equine *in vitro* fertilization.

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