Investigating the effects of calcium on the capacitation and acrosome reaction of ram spermatozoa

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Abstract

There is ambiguity surrounding the mechanisms and stimulants of capacitation, particularly regarding the necessity and role of calcium (Ca^{2+}), and the species-specific requirements of ram spermatozoa. In this study, the complication of this process is further evidenced as the effects of different capacitation stimulants are investigated. Two experiments were conducted to better understand the effects and functions of calcium upon ram sperm capacitation and the acrosome reaction. Ram semen was collected, prepared and incubated in a non-capacitating basal media (Tyrode's Albumin Lactate Pyruvate, TALP) with or without calcium (Experiment 1), or a media that is thought to facilitate ram sperm capacitation (TALP plus caffeine, theophylline, dibutryl-cyclic adenosine monophosphate (db-cAMP), also known as CAPSTIM) with or without calcium (Experiment 2). The spermatozoa were assessed at two time points for motility, kinematics, acrosome integrity, viability, apoptotic rate and membrane stability, in order to provide insight into the capacitation and acrosome reaction status of the spermatozoa. In the current study, the addition of calcium did not induce changes indicative of successful capacitation. These results may indicate a dosage-related effect of calcium, or a heavier reliance of ram spermatozoa on calcium independent pathways, however further studies are required to define and deepen the understanding pertaining to the roles of different chemicals upon capacitation.

1. Introduction

The use of domestic animal species to investigate reproductive phenomena has great value for the comprehension of the physiology behind conception. Spermatozoa are highly complex single cells that undergo extensive changes as they evolve from spherical spermatogonia in the testis of the male, to morphologically mature spermatozoa through mitosis, meiosis and epididymal maturation (de Laminande *et al.,* 1997; Reid *et al.* 2011). A curiously unique trait

of spermatozoa, however, is that they do not achieve their functional ability to fertilise an oocyte until they have become “capacitated” in the oviduct of the female (Austin, 1951; Chang, 1951).

Capacitation occurs as spermatozoa migrate the female tract after ejaculation. The process includes the loss of proteins from the plasma membrane, which increases membrane fluidity and permeability, and allows parallel surface changes, as well as the influx of ions responsible for protein tyrosine phosphorylation, and the acquisition of forward- then hyper-motility (Fraser, 1984; de Lamirande *et al.,* 1997; Caballero *et al.*, 2009). The spermatozoa are then able to undergo the acrosome reaction, which is an exocytotic event that enables the spermatozoa to penetrate the zona pellucida of the ova and fuse with the oolema (Ickowicz *et al.,* 2012; Bakalczuk *et al*., 2016). Consequently, after ejaculation, difficulties arise when using spermatozoa for advanced reproductive techniques, such as *in vitro* fertilization (*IVF*). Without the incubation period in the female tract, spermatozoa must be stimulated to complete the final stages of maturation using a synthetic media that replicates the chemical composition of the female tract.

In synthesising an appropriate media to support capacitation and the acrosome reaction, a deep understanding of the physiological processes behind these events is both required and gained. There is extensive research and knowledge pertaining to the chemical stimuli of capacitation and the acrosome reaction in human and bovine spermatozoa (Yanagimachi, 2011; Perry, 2013; Leemans *et al*., 2016); however, species differences found in equine and ovine spermatozoa, for example, have presented challenges for the *in vitro* maturation of their spermatozoa (Huneau *et al*., 1994; Osheroff *et al*., 1999; Visconti *et al*., 1999a,b; Li *et al*., 2006; McPartlin *et al.,* 2008; Colas *et al*., 2008; Leahy *et al*., 2016a,b). Generally, the main

283 factors found to stimulate mammalian spermatozoa maturation in the female tract are known
284 to be bicarbonate, albumin and calcium (Xia & Ren, 2009; Fraser, 2010; de Lamirande *et al.*,
285 1997). These ions are combined with biological electolytes ($MgCl_2$, NaCl, KCl, NaH_2PO_4)
286 and energy sources (pyruvate and lactate) to create Tyrode's Albumin Lactate Pyruvate
287 (TALP) media, the base media for capacitation of mammalian spermatozoa.
288
289 The importance of the main components for *in vivo* capacitation became evident after studies
290 on the female reproductive tract displayed high concentrations of bicarbonate, albumin and
291 calcium in the oviductal milieu, in which spermatozoa are incubated as they undergo
292 capacitation and the acrosome reaction (Visconti *et al*., 1995a,b Harrison, 1996; van Gestel *et*
293 *al*, 2005; Fraser, 2010; Bailey, 2010). It is thought that calcium and bicarbonate play parallel
294 roles in capacitation, as they are concurrently involved in several mechanisms of capacitation
295 (Litvin *et al.,* 2003; Harrison & Gadella, 2005; Colas *et al.,* 2008). It is therefore difficult to
296 separate and define the individual roles of bicarbonate and calcium. Often both bicarbonate
297 and calcium are included in capacitating media, with the expectation that they work together
298 to stimulate spermatozoa to undergo several capacitation processes, although further
299 investigations are required to understand their exact roles in the process (Litvin *et al.,* 2003;
300 Harrison & Gadella, 2005; Colas *et al.,* 2008).
301
302 A further challenge in defining the roles of different stimulants of capacitation is the
303 differences found between species. In the ram, capacitation and the subsequent acrosome
304 reaction cannot be achieved in standard calcium, albumin and bicarbonate media, and instead
305 requires exogenous stimuli (Colas *et al.,* 2008; Leahy *et al.,* 2016a). These are commonly
306 caffeine, theophylline and dibutyryl-cyclic adenosine monophosphate (db-cAMP), which
307 have been shown to upregulate cyclic adenosine monophosphate (cAMP) necessary for

driving capacitation related changes in spermatozoa (Wani, 2002; Li *et al*., 2006; Colas *et al*., 2008; Leahy *et al.,* 2016a). The reasons behind the additional requirements of ram sperm maturation are not well understood, which demonstrates the importance of further research to uncover the underlying interactions and mechanisms that set sheep apart.

Therefore, the present study aimed to investigate the effects of calcium under different capacitating conditions. These conditions included basic Tyrode's Albumin Lactate Pyruvate (TALP) media, with and without cAMP upregulators (a combination of caffeine, theophylline, db-cAMP, also known as CAPSTIM). The spermatozoa were incubated in their respective treatments, and underwent comprehensive *in vivo* assessment at two different time points for functional and capacitation related parameters.

2. Materials and Methods

2.1. Experimental design

Each experiment was replicated three times using one ejaculate each from three Merino rams of proven fertility. Samples were assessed immediately after dilution and after 3 h of incubation. All chemicals were sourced from Sigma-Aldrich unless otherwise stated. The base medium used for Experiments 1 and 2 was modified Tyrode's medium supplemented with albumin, lactate and pyruvate (TALP), which was devoid of calcium and consisted of 10 mM HEPES, 0.4 mM $MgCl_2$, 100 mM NaCl, 3 mM KCl, 0.3 mM NaH_2PO_4, 15 mM $NaHCO_3$, 2 mM $C_3H_3NaO_3$, 5 mM glucose, 21.6 mM Sodium DL-lactate, and 3 mg/ml BSA. The addition of calcium was achieved through the inclusion of calcium stock to a concentration of 2 mM. The CAPSTIM used in Experiment 2 consisted of db-cAMP, caffeine and theophylline, and the addition of CAPSTIM was achieved through the inclusion of a

CAPSTIM stock to the concentration of 2 mM. 1 mM PEN was also included in each treatment to prevent agglutination of spermatozoa (Leahy *et al.,* 2016a).

2.1.1. *Experiment 1*

Experiment 1 was designed to test the effects of TALP plus calcium in comparison to TALP devoid of calcium. Spermatozoa were diluted in either TALP minus calcium or TALP plus calcium, and then spermatozoa were assessed for function, motility and kinematics at two timepoints (0 h and 3 h).

2.1.2. *Experiment 2*

Experiment 2 was designed to test the effects of calcium in conjunction with CAPSTIM. Spermatozoa were either diluted in TALP minus calcium plus CAPSTIM, or TALP plus 2 mM calcium plus CAPSTIM. Spermatozoa were then assessed for function, motility and kinematics at two timepoints (0 h and 3 h).

2.2. Semen collection and preparation

Semen was collected by artificial vagina from 3 mature rams with project approval from the University of Sydney's Animal Ethics Committee (Project No: 2016/1106). The rams were kept in animal housing at the Faculty of Veterinary Science, University of Sydney, Camperdown, NSW, Australia. Only ejaculates with a wave motion score of 4 (out of 5) or higher were used in the study. All semen sampled was evaluated immediately for percentage of motile spermatozoa. Samples with motility not less than 60% were used. Raw semen was diluted to 80 x 10^6 spermatozoa/ml in TALP media minus calcium, and then extended to 40 x 10^6 spermatozoa/ml in its respective treatment.

2.3. Evaluation of sperm

2.3.1. *Flow cytometric analysis of sperm function*

Samples were analysed using a CytoFLEX Flow Cytometer (Beckman-Coulter, calibrated daily with CytoFLEX Daily Q. Fluorospheres). Associated CytExpert software (2.0) was used for analysis. All stains were sourced from Life Technologies.

Sperm samples were diluted 3:1 in a combination of stains, with all samples counter-stained with 1 μg/ml Hoechst for gating purposes, allowing only spermatozoa to be assessed. Membrane lipid order and viability were assessed through dual fluorescent staining for 10 minutes at 37°C with 0.83 mM merocyanine 540 (M540) and 25 nM Yo-Pro. Viability and acrosome integrity were determined by dual fluorescence staining for 10 min at 37°C with 6 mM propidium iodide (PI) and 0.4 mg/ml fluorescein isothiocyanate-peanut agglutinin (FITC-PNA).

The cytometer recorded events at a flow rate of 30 μl/ minute, and a total of 10,000 events gated as spermatozoa were recorded. The cytometer excited the various fluorophores using three lasers (50 mW 488 nm; 50 mW 638 nm; 80 mW 405 nm), and three different filters were used to detect the fluorescence from the various stains (450/45 bp for Hoechst 33342; 525/40 bp for FITC and YoPro; 585/42 bp for PI and M540).

2.3.2. *Sperm motility and kinematics*

Sperm motility and kinematics were measured objectively using computer-assisted sperm analysis (CASA) (Hamilton-Thorne CASA IVOS II (Animal Breeder) Version 1.8; Hamilton-Thorne, Beverly, MA, USA) using factory CASA ram settings. Semen samples

were placed on 37°C prewarmed slides (Cell Vu; Millenium Sciences, Mulgrave, Vic., Australia) and enclosed using a 22x22 mm coverslip before immediate transfer to the CASA. A minimum of 200 spermatozoa were observed from at least five randomly selected fields. Recorded parameters were: total motile spermatozoa (MOT, %), progressively motile spermatozoa (PROG MOT, %), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), average-path velocity (VAP, μm/s), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), linearity (LIN, %) and straightness (STR, %).

2.4. Statistical analysis

Statistical analysis was performed using linear mixed models regression in GENSTAT (Version 16; VSN International, Hemel Hempstead, UK). Interactions between treatment effects and time were assessed with ejaculate and ram incorporated into the blocking structure. Data are presented as the model-derived mean ± standard error of the means (SEM), backtransformed as appropriate. Results are expressed as significant ($P < 0.05$) and highly significant ($P < 0.001$) compared to control samples.

3. Results

3.1. Experiment 1

3.1.1. *The addition of calcium to TALP media had no significant effects upon the kinematics of spermatozoa.*

No differences were found between spermatozoa incubated in TALP minus calcium and TALP plus 2 mM calcium. However, there were significant differences between the two time points for several kinematic parameters. BCF decreased at a highly significant rate ($P <$

0.001) between 0 and 3 hours. Both MOT and PROG MOT decreased significantly between 0 and 3 hours ($P = 0.007$ and $P = 0.008$, respectively). LIN increased significantly between 0 and 3 hours ($P = 0.003$) (Table 1).

3.1.2. *The addition of calcium to TALP media had no significant effects upon the percentage of acrosome reacted spermatozoa or viability of spermatozoa.*

Neither treatment nor timepoint had a significant effect upon the viability of spermatozoa. However, there was a significant effect of timepoint on the percentage of acrosome reacted spermatozoa, with significantly more acrosome reacted spermatozoa after 3 hours of incubation compared to 0 hours ($P = 0.014$) (Figure 1).

3.1.3. *The addition of calcium to TALP media had no significant effects upon the apoptotic rate or membrane lipid order of spermatozoa.*

The rate of apoptosis in the sperm population was not significantly affected by treatment nor timepoint. However, viable median PE-A decreased at a significant rate over the two time points ($P = 0.009$), indicating increasing membrane lipid order (Figure 2).

3.2. Experiment 2

3.2.1. *The addition of calcium to TALP media with CAPSTIM significantly affected several kinematic parameters of spermatozoa*.

Significant differences in several kinematic parameters were evident between spermatozoa incubated in TALP plus CAPSTIM plus calcium and spermatozoa incubated in TALP plus CAPSTIM only. The addition of calcium significantly decreased ALH over the two time points ($P = 0.003$), while increasing VCL over the 3 hours at a significant rate ($P = 0.001$). The addition of calcium increased VAP and VSL significantly ($P = 0.010$ and $P = 0.021$,

418 respectively), while time point decreased these characteristics highly significantly ($P < 0.001$
419 for both VAP and VSL) (Table 2).

420 3.2.2. *The addition of calcium to TALP media with CAPSTIM had no significant*
421 *effects upon the percentage of acrosome reacted spermatozoa or viability of*
422 *spermatozoa.*

423 The percentage of acrosome reacted spermatozoa was not affected by treatment nor time
424 point. Time point did, however, have an effect upon the viability of spermatozoa, with the
425 percentage of live spermatozoa decreasing highly significantly between 0 hours and 3 hours
426 ($P <0.001$) (Figure 3)

427 3.2.3. *The addition of calcium to TALP media with CAPSTIM had no significant*
428 *effects upon the apoptotic rate or membrane lipid order of spermatozoa.*

429 No significant effects of treatment were found on the proportion of apoptotic spermaotozoa,
430 or viable median PE-A. However, time point had an effect upon the viable median PE-A,
431 which increased significantly between 0 hours and 3 hours ($P = 0.007$), indicating a decrease
432 in membrane lipid order of spermatozoa (Figure 4).

433 ## 3.3. Comparisons of Experiment 1 and 2

434 3.3.1. *The addition of CAPSTIM to TALP media with and without calcium*
435 *significantly affected several kinematic parameters of spermatozoa.*

436 Significant differences in kinematic parameters between spermatozoa incubated in media
437 with and without CAPSTIM were evident. The addition of CAPSTIM decreased ALH at a
438 significant rate ($P = 0.001$). CAPSTIM also decreased the VCL ($P < 0.001$) and BCF ($P <$
439 0.001) at a highly significant rate. Furthermore, the addition of CAPSTIM significantly

decreased the MOT ($P = 0.013$) and PROG MOT (P = 0.017) of spermatozoa in comparison to the control treatment. VSL ($P < 0.001$) and VAP ($P < 0.001$) decreased at a highly significant rate with the addition of CAPSTIM, whereas LIN increased significantly ($P = 0.001$) (Table 3).

3.3.2. *The addition of CAPSTIM to TALP media with and without calcium had a significant effect upon the viability of spermatozoa*

There were no significant treatment effects upon the percentage of acrosome reacted spermatozoa, however, there was a significant increase in acrosome reacted spermatozoa over the two time points ($P = 0.001$) (Figure 5). There were highly significantly less live spermatozoa at 3 hours compared to 0 hours ($P < 0.001$) and significantly more live spermatozoa with the addition of calcium ($P = 0.023$) (Figure 6).

3.2.3. *The addition of CAPSTIM to TALP media with and without calcium significantly effected the membrane lipid order of spermatozoa.*

There were no significant treatment effects upon the apoptotic rate of spermatozoa, however viable median PE-A increased at a highly significant rate ($P < 0.001$) with the addition of CAPSTIM, indicating decreasing membrane lipid order (Figure 7).

4. Discussion

This work has shown that the addition of calcium does not cause definitive capacitation-related changes to the motility patterns, viability, membrane lipid order, acrosome reaction status or apoptotic rate of ram spermatozoa. These results exemplify the complex mechanisms underlying the process of capacitation, and demonstrate the need for further research to elucidate them. Although artificial reproduction is undertaken regularly under

clinical human settings, and also commercially in domestic animals, the exact requirements for spermatozoa to become reproductively functional, otherwise known as capacitated, and the delineation of this process are not fully understood in species such as the sheep (de Laminade *et al.,* 1997; Naz & Rajesh, 2004; Colas *et al.,* 2008). This has led to analogous capacitation media for all species, with alterations occurring as further experimentation exposes species differences, such as the requirement for cAMP upregulators (CAPSTIM) in the ram (Xia & Ren, 2009). Generally, this media will include a cholesterol acceptor or 'sink' (commonly albumin), energy substrates (such as lactate, glucose or pyruvate), bicarbonate and calcium (Leahy *et al.,* 2016a), although not all of these may be necessary for capacitation (Grasa *et al*., 2006; Xia & Ren, 2009; Colas *et al.,* 2010), as demonstrated by the current study.

In this study, two experiments were designed to gain a deeper understanding of the role that calcium plays in ram sperm capacitation and acrosome reaction. Experiment 1 investigated the effects of calcium when added to basic TALP media. Ram spermatozoa does not commonly capacitate in TALP media, so this experiment sought to study the ability of calcium to stimulate capacitation without cAMP upregulation. The results of Experiment 1 showed no treatment effects upon motility characteristics of ram spermatozoa when assessed over 3 hours. Motility characteristics are often used to indicate the viability and capacitation status of spermatozoa (Mortimer & Maxwell, 1999; Rota *et al*., 1999; Colas *et al.,* 2010; Partyka *et al*., 2012), therefore, a lack of significant differences between spermatozoa incubated in TALP and spermatozoa incubated in TALP with calcium demonstrated that at a concentration of 2 mM, calcium does not solely initiate capacitation-related changes to ram spermatozoa at the studied timepoints. This is not surprising, as it is widely accepted that ram spermatozoa require additional stimulants of capacitation (Wani, 2002; Li *et al*., 2006; Colas

487 *et al*., 2008; Leahy *et al.,* 2016a), evidencing the existence of calcium-independent
488 capacitation pathways, which are of more physiological consequence than those that are
489 calcium-mediated.
490
491 The addition of calcium to basic TALP media resulted in no significant effects upon the
492 viability of ram spermatozoa. It has recently been reported that increased calcium reduces
493 viability of spermatozoa, as it facilitates motility and therefore results in increased metabolic
494 usage of spermatozoa, which contradicts the findings in the current study (Scott *et al.,* 2016).
495 There was also no significant effect of calcium upon the percentage of acrosome reacted
496 spermatozoa incubated in TALP. Although calcium is known to be necessary for the
497 induction of the acrosome reaction (Jin *et al.* 2011, Yanagimachi 2011; Parodi, 2013; Leahy
498 *et al.,* 2016a), the acrosome reaction can only occur after successful capacitation, which was
499 not expected to have occurred in basic TALP media, even with the addition of calcium.
500 Experiment 1 did, however, result in acrosome reacted spermatozoa, with the percentage of
501 acrosome reacted spermatozoa increasing significantly from 0 hours to 3 hours. Since
502 capacitation was not quantified at these timepoints, it could be hypothesized that spermatozoa
503 may have capacitated in the time between measurements, or more likely is that the loss of
504 acrosomal membrane integrity over time may have resulted in the spontaneous acrosome
505 reaction of spermatozoa (Kheradmand *et al.,* 2009). Although the acrosome reaction is
506 defined as a process that follows successful capacitation, a proportion of spermatozoa have
507 been shown to undergo the acrosome reaction in the absence of capacitation inducing agents,
508 however these spontaneously acrosome reacted spermatozoa do not possess the ability to
509 fertilise an oocyte (Watson *et al*., 1991; Huang *et al*., 2005, 2007). To determine whether the
510 acrosome reacted spermatozoa are also capacitated, further characteristics of physiological

sperm function, such as the successful fertilisation of an oocyte through *IVF*, could be undertaken to determine the biological success of spermatozoa.

The apoptotic rate and membrane lipid order of spermatozoa was assessed, and the results of flow cytometry showed no significant effects of calcium upon these parameters in Experiment 1. Membrane lipid order is an indicator of membrane fluidity, which increases at the initiation of capacitation in order to facilitate the influx of ions, such as calcium and bicarbonate, into the sperm cell (Harrison, 1996; Leahy & Gadella, 2011; Reid *et al.,* 2011). The loss of cholesterol has been shown to decrease membrane lipid order, and therefore increase membrane fluidity and permeability (Harrison, 1996; Leahy & Gadella, 2011; Reid *et al.,* 2011), so it is not surprising that calcium did not significantly affect this calcium independent characteristic. This is further supported by studies of membrane lipid order in bull spermatozoa, which show changes in membrane lipid order to be bicarbonate-induced and independent of exogenous calcium (Harrison, 1996; Harrison *et al.*, 1996; Flesch *et al.,* 2001).

To ensure the capacitation of ram spermatozoa, several additional chemicals have been found to complement the basic capacitation media. Caffeine, Theophylline and db-cAMP are a common combination of cAMP upregulators included to stimulate capacitation in ram spermatozoa, and are referred to as CAPSTIM. The addition of CAPSTIM was utilised in Experiment 2 to provide a positive control, which would produce capacitated spermatozoa. The ability of CAPSTIM to capacitate ram spermatozoa was supported in this study. CAPSTIM significantly altered many motility parameters, and had a highly significant effect on lipid order, which is a strong indicator of capacitation. The addition of CAPSTIM also

caused significant differences in the viability of spermatozoa and the percentage of acrosome reacted spermatozoa.

In terms of kinematic characteristics, CAPSTIM significantly decreased motility of ram spermatozoa, and significantly decreased several kinematic parameters expected to increase in capacitated spermatozoa (VCL and BCF). CAPSTIM also increased linearity, which is commonly seen in non-capacitated spermatozoa. Although this appears to contradict the ability of CAPSTIM to capacitate ram spermatozoa, it is more likely to indicate the discrepancy that can be found between motility characteristics and capacitation. It has been shown that the vigour of flagellar movement corresponds to oocyte penetration, however the motility charactersitics measured using CASA are related to sperm head movement, a secondary measure of flagellar movement (Morales *et al.,* 1988; Mortimer *et al*., 1997). Furthermore, under artificial breeding conditions such as intracytoplasmic sperm injectection (ICSI), hyperactive motility is rendered negligible for biological sperm function (fertilisation), as the viscous mucous of the oviduct is not present (Yeung & Cooper, 2010). More correlated measures of capacitation, such as fluorescence detected using the chlortetracycline (CTC) technique, would provide a more accurate depiction of capacitation than kinematic parameters indicating hyperactivation (Ward & Storey, 1984; Storey & Kopf, 2013). Also, the time at which hyperactivation can be detected may not have corresponded to the time points measured, as it has been demonstrated that capacitation can occur at a different time course to hyperactivation (or independent of hyperactivation altogether) (Olds-Clarke, 1989; Storey & Kopf, 2013). Therefore, assessing spermatozoa at more frequent time intervals could provide more detailed information regarding the changes in spermatozoa motility patterns over time.

The other capacitation-related parameters measured at 0 and 3 hours indicated that capacitation was significantly increased by CAPSTIM. For example, the addition of CAPSTIM significantly decreased sperm viability. This could indicate higher metabolic activity of spermatozoa incubated in CAPSTIM, which is often linked to activities that occur during capacitation, such as hyperactive motility (Parodi, 2013; Scott *et al.,* 2016), however no motility characteristics measured supported this. Membrane lipid order also decreased at a highly significant rate with the addition of CAPSTIM, which is characteristic of capacitation. This result contradicts the motility data, as it provides evidence of successful capacitation induced by CAPSTIM. A potentially related phenomenon has been documented in several studies, in which the spermatozoa of some species (including the sheep) capacitated *in vitro* without displaying characteristics of hyperactivated motility (Boatman & Robbins, 1991; Ho & Suarez, 2001a,b; Colas *et al.,* 2010). However, further measures of capacitation would be necessary to conduct in order to provide an answer as to whether capacitation occurred due to the addition of CAPSTIM. A potential assay to include could be a Western blot visualization of samples incubated with and without CAPSTIM. Western blots establish presence and amounts of proteins in a sample, which could provide insight into the capacitation status of spermatozoa, as protein tyrosine phosphorylation is a hallmark of capacitation (Roberts *et al.,* 2003).

Regardless of the success of CAPSTIM to stimulate capacitation, this treatment provided another environment to test the effects of calcium. The results of Experiment 2 slightly differed from Experiment 1, indicating interactions between the chemicals present that influence ram sperm capacitation. The addition of calcium to media containing TALP and CAPSTIM caused significant differences in several motility characteristics compared to the calcium free control. While VCL significantly increased, other velocity measures (VAP,

585 VSL, ALH) significantly decreased, indicating a faster pace of spermatozoa, but less distance
586 covered over time. While these measures combined do not directly correspond to
587 capacitation-related changes, they are potentially indicative of hyperactive swimming
588 patterns of spermatozoa, which occurs during capacitation. This was not seen in Experiment
589 1, with the addition of calcium to TALP media alone; thus, these results signify a change in
590 the role of calcium when cAMP upregulators are present.
591
592 Similarly to Experiment 1, the addition of calcium did not significantly affect viability,
593 percentage of acrosome reacted spermatozoa, rate of apoptosis or membrane lipid order of
594 ram spermatozoa. Viability and membrane lipid order were significantly affected by time,
595 with viability decreasing at a highly significant rate, which is commonly seen in an aging
596 sperm population (Appell & Evans, 1977). Membrane lipid order decreased at a significant
597 rate between 0 and 3 hours, indicating increased fluidity of the sperm plasma membrane,
598 which is associated with capacitation (Harrison, 1996; Leahy & Gadella, 2011; Reid *et al.,*
599 2011). Calcium lacks significance regarding these measurements in both Experiment 1 and 2,
600 which could indicate a heavier reliance upon calcium-independent capacitation pathways, or
601 alternatively, sufficient stores of calcium may be present within the sperm cell to render
602 exogenous calcium redundant. Alongside the discovery of intercellular calcium stores in
603 spermatozoa (Berruti & Franchi, 1986; Watson & Plummer, 1986; Breitbart, 2002), there is
604 also evidence that exogenous calcium is not necessary for capacitation and protein tyrosine
605 phosphorylation in human (Leclerc *et al.,* 1998) and ram (Grasa *et al.,* 2006) sperm
606 capacitation, supporting this theory (Huang *et al.,* 2009).
607
608 The results of this study are antithetical, indicating interactions that have not yet been
609 explained. There are further conflicting accounts in the literature regarding the inclusion of

610 calcium in *in vitro* sperm preparation protocols, with this conflict in itself supporting the
611 complexity of this topic. In the mouse, it was shown that tyrosine phosphorylation only
612 occurred when calcium was added to the media, with maximal expression at 1 mM
613 concentration (Visconti *et al.,* 1995a). Other studies in the mouse corroborated these findings,
614 giving the impression that calcium was required for *in vitro* induction of capacitation and the
615 acrosome reaction (Storey & Kopf, 1991; Bailey & Storey, 1994). However, it was then
616 found that the calcium requirement for mouse sperm capacitation could be overcome when
617 biologically active cAMP analogues were included in media devoid of calcium (measured
618 using the CTC assay; Visconti *et al.,* 1995b). Although this is not what was found when
619 adding cAMP upregulating agents in the current study, this could be due to the types or
620 dosages of these cAMP upregulators. This provides further reasoning for the lack of
621 significance associated with the addition of calcium. Furthermore, in the horse and the
622 human, the addition of extracellular calcium has been demonstrated to potently inhibit
623 tyrosine phosphorylation, whereas in the bull and boar, this is not the case (Kalab *et al*.,
624 1998; Luconi *et al*., 1996; Marin-Briggiler *et al*., 2003; Galantino-Homer *et al.*, 2004;
625 Macías-García *et al*., 2016). It is possible that calcium has a similar inhibitory effect upon
626 ram spermatozoa, however it would be necessary to include a Western Blot assay in order to
627 measure the effects of calcium upon protein tyrosine phosphorylation of ram spermatozoa.
628
629 A 2006 study by Grasa *et al* displayed similar results to those attained in the current study,
630 finding that the proportion of capacitated ram spermatozoa (measured by the CTC technique)
631 was not significantly affected by the addition of calcium to the capacitation media. Grasa (*et*
632 *al.,* 2006) also investigated the viability and tyrosine phosphorylation of spermatozoa, and
633 demonstrated that the presence of calcium in the capacitation media did not result in any
634 differences in these parameters either (Grasa *et al.,* 2006). However, it was shown that there

635 was a significantly higher proportion of acrosome reacted spermatozoa in the treatment
636 containing 4 mM calcium (Grasa *et al.,* 2006). This was not found in the current study, which
637 used a lesser concentration of calcium (2 mM), indicating a potential dosage linked effect of
638 calcium. Further studies involving the incubation of ram spermatozoa in different
639 concentrations of calcium would help to clarify this.
640
641 The results from the current study indicate that 2 mM calcium did not increase the occurrence
642 of capacitation-like changes in ram spermatozoa over a 3 hour time period when added to
643 TALP media. When 2 mM calcium was added to TALP plus CAPSTIM, calcium was shown
644 to induce several changes to the motility of ram spermatozoa, some of which indicated
645 changes associated with capacitation. CAPSTIM was shown to capacitate ram spermatozoa,
646 however there were some kinematic parameters that indicated a lack of hyperactivation. In
647 order to understand and define the functions of calcium or CAPSTIM upon ram sperm
648 capacitation, further investigations are required. The information acquired from studying the
649 effects of different dosages of these chemicals, as well as incorporating experimental assays
650 such as western blots for protein tyrosine phosphorylation, CTC fluorescence to define the
651 stage of capacitation/acrosome reaction, or *IVF* as a biological test of sperm capacitation,
652 would assist in elucidating the complex underlying mechanisms and interactions of
653 capacitation.

654 Declaration of Interest

655 The authors declare that there is no conflict of interest that could prejudice the impartiality of
656 this research.

Funding

This work was supported by the Australian Wool Education Trust (SC1830).

Acknowledgements

The author wishes to thank Dr Tamara Leahy and Associate Professor Simon de Graaf for their roles as supervisors of this project. Also, the excellent technical assistance provided by Emmah Tumeth, Taylor Pini, Kiri Farmer, Dr Jessica Rickard, Kelsey Poole and Naomi Bernecic is greatly appreciated. Lastly, the Australian Wool Education Trust Scholarship has played an integral part in facilitating this research and is highly valued by the author.

References

- **Appell RA & Evans PR** 1977 Effect of temperature on sperm motility and vitality. *Fertility and Sterility***28** 1329-1332.
- **Austin CR** 1951 Observations on the penetration of the sperm into the mammalian egg. *Australian Journal of Scientific Research***B4** 581-96.
- **Bailey JL & Storey BT** 1994 Calcium influx into mouse spermatozoaactivated by solubilized mouse ZP, monitored with the calcium fluorescentindicator, fluo-3. Inhibition of the influx by three inhibitors of the zonapellucida-induced acrosome reaction: tyrphostin A48, pertussis toxin, and 3-quinuclidinyl benzilate. *Molecular Reproduction and Development***39** 297-308.
- **Bailey JL** 2010 Factors regulating sperm capacitation. *Systems Biology in Reproductive Medicine***56** 334–348.
- **Bakalczuk G, Wdowiak A, Lewicka M, Sulima M, Perovic S, Anton J & Kielak MA** 2016 Induction effectiveness of acrosome reaction in prepared human

679 spermatozoa assessed using the CD46 surface antigen. *European Journal of Medical*
680 *Technologies***1** 44-53.

- 681 **Berruti G & Franchi M** 1986 Calcium and polyphosphoinositides: their distribution
682 in relation to the membrane changes occurring in the head of boar spermatozoa. *Eur.*
683 *Journal of Cell Biology***41** 238–245.
- 684 **Boatman DE & Robbins RS** 1991 Bicarbonate: carbon-dioxide regulation of sperm
685 capacitation, hyperactivated motility, and acrosome reactions. *Biol Reprod***44** 806-13.
- 686 **Breitbart H** 2002 Intracellular calcium regulation in sperm capacitation and
687 acrosomal reaction. *Molecular and Cellular Endocrinology***187** 139–144.
- 688 **Caballero I, Vasquez JM, Mayor GM, Almiñana C, Calvete JJ, Sans L, Roca J**
689 **& Martinez EA** 2009 PSP-I/PSP-II spermadhesin exert a decapacitation effect on
690 highly extended boar spermatozoa. *International Journal of Andrology***32** 505-513.
- 691 **Chang MC** 1951 Fertilizing capacity of spermatozoa deposited into the Fallopian
692 tubes. *Nature (London)***168** 697-8.
- 693 **Colas C, James P, Howes L, Jones R, Cebrián-Pérez JÁ & Muiño-Blanco T** 2008
694 Cyclic-AMP initiates protein tyrosine phosphorylation independent of cholesterol
695 efflux during ram sperm capacitation. *Reproduction Fertility and Development***20**
696 649–658.
- 697 **Colas C, Cebrián-Pérez JÁ & Muiño-Blanco T** 2010 Caffeine induces ram sperm
698 hyperactivation independent of cAMP-dependent protein kinase. *International*
699 *Journal of Andrology***33** 187–197.
- 700 **De Lamirande E, Leclerc P & Gagnon C** 1997 Capacitation as a regulatory event
701 that primes spermatozoa for the acrosome reaction and fertilisation. *Molecular*
702 *Human Reproduction***3** 175-194.

- **Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B & Gadella BM** 2001 Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *Journal of Cell Science***114** 3543-3555.
- **Fraser LR** 1984 Mouse sperm capacitation *in-vitro* involves a loss of a surface-associated inhibitory component. *Journal of Reproduction and Fertility***72** 373-384
- **Fraser LR** 2010 The ‘‘Switching on’’ of Mammalian Spermatozoa: Molecular Events Involved in Promotion and Regulation of Capacitation. *Molecular Reproduction & Development***77** 197–208.
- **Galantino-Homer HL, Florman HM, Storey BT, Dobrinski I & Kopf GS** 2004 Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalinization on capacitation-associated protein tyrosine phosphorylation. *Molecular Reproduction and Development***67** 487– 500.
- **Grasa P, Cebrián-Pérez JÁ & Muiño-Blanco T** 2006 Signal transduction mechanisms involved in in vitro ram sperm capacitation. *Reproduction***132** 721–732.
- **Harrison RAP** 1996 Capacitation mechanisms and the role of capacitation as seen in eutherian mammals. *Reproductive Fertility Development* 8: 581-594.
- **Harrison RA, Ashworth PJ & Miller NG** 1996 Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Molecular Reproduction and Development***45** 378-391.
- **Harrison RAP & Gadella BM** 2005 Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology***63** 342–351.
- **Ho HC & Suarez SS** 2001a An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca(2+) store is involved in regulating sperm hyperactivated motility. *Biology of Reproduction***65** 1606–1615.

728 • **Ho HC & Suarez SS** 2001b Hyperactivation of mammalian spermatozoa: function
729 and regulation. *Reproduction***122** 519–526.
730 • **Huang YH, Chen YH, Lin CM, Ciou YY, Kuo SP, Chen CT, Shih CM & Chang**
731 **EE** 2007 Suppression effect of seminal vesicle autoantigen on platelet-activating
732 factor-induced mouse sperm capacitation. *Journal of Cellular Biochemistry***100** 941-
733 951.
734 • **Huang YH, Kuo SP, Lin MH, Shih CM, Chu ST, Wei CC, Wu TJ & Chen YH**
735 2005 Signals of seminal vesicle autoantigen suppresses bovine serum albumin-
736 induced capacitation in mouse sperm. *Biochemical and Biophysical Research*
737 *Communications***338** 1564-1571.
738 • **Huang J, Wang G & Kong L** 2009 Effects of Ca2+ and HCO3- on Capacitation,
739 Hyperactivation and Protein Tyrosine Phosphorylation in Guinea Pig Spermatozoa.
740 *Asian-Australian Journal of Animal Science***22** 181–186.
741 • **Huneau D, Crozet N & Ahmed-Ali M** 1994 Estrous sheep serum as a potent agent
742 for ovine IVF: effect on cholesterol efflux from spermatozoa and the acrosome
743 reaction. *Theriogenology***22** 1017–1028.
744 • **Ickowicz D, Finkelstein M & Breitbart H** 2012 Mechanism of sperm capacitation
745 and the acrosome reaction: role of protein kinases. *Asian Journal of Andrology***14**
746 816-821.
747 • **Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba S, Chiba K &**
748 **Hirohashi N** 2011 Most fertilizing mouse spermatozoa begin their acrosome reaction
749 before contact with the zona pellucida during in vitro fertilization. *Proceedings of the*
750 *National Academy of Sciences in the United States of America***108** 4892–4896.

- **Kalab P, Peknicova J, Geussova G & Moos J** 1998 Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Molecular Reproduction and Development***51** 304–314.
- **Kheradmand A, Sookhtezary A, Moosavi SM & Joorabi S** 2009 The effect of platelet activating factor on the motility and acrosome reaction of ram spermatozoa. *Iranian Journal of Veterinary Research***10** 315-322.
- **Leahy T, Rickard JP, Aitken RJ & de Graaf SP** 2016a Penicillamine prevents ram sperm agglutination in media that support capacitation. *Reproduction***151** 167–177.
- **Leahy T, Rickard JP, Aitken RJ & de Graaf SP** 2016b d-penicillamine prevents ram sperm agglutination by reducing the disulphide bonds of a copper-binding sperm protein. *Reproduction***151** 491–500.
- **Leemans B, Gadella BM, Stout TA, De Schauwer C, Nelis H, Hoogewijs M & Van Soom A** 2016 Why doesn't conventional IVF work in the horse? The equine oviduct as a microenvironment for capacitation/ fertilization. *Reproduction***152** 233–245.
- **Li F, Pi WH, Zhu HZ, Zhang SS, Liu SR & Xue JL** 2006 The effect of estrous ewe serum and heparin on in vitro fertilization and subsequent embryonic development in sheep, *Small Ruminant Research***63** 226–232.
- **Litvin TN, Kamenetsky M, Zarifyan A, Buck J & Levin LR** 2003 Kinetic Properties of "Soluble" Adenylyl Cyclase: synergism between calcium and bicarbonate. *The Journal Of Biological Chemistry***278** 15922–15926.
- **Luconi M, Krausz C, Forti G & Baldi E** 1996 Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine activity during capacitation of human spermatozoa. *Biology of Reproduction***55** 207-215.

775 • **Macías-García B, Rocha A & González-Fernández L** 2016 Extracellular Calcium
776 Regulates Protein Tyrosine Phosphorylation Through Calcium-Sensing Receptor
777 (CaSR) in Stallion Sperm. *Molecular Reproduction & Development***83** 236–245.
778 • **Marin-Briggiler CI, Gonzalez-Echeverria F, Buffone M, Calamera JC, Tezon JG**
779 **& Vazquez-Levin MH** 2003 Calcium requirements for human sperm function in
780 vitro. *Fertility and Sterility***79** 1396 –1403.
781 • **Mcpartlin LA, Littell J, Mark E, Nelson JL, Travis AJ & Bedford-Guaus SJ**
782 2008 A defined medium supports changes consistent with capacitation in stallion
783 sperm, as evidenced by increases in protein tyrosine phosphorylation and high rates of
784 acrosomal exocytosis. *Theriogenology***69** 639–650.
785 • **Morales P, Overstreet JW & Kats DF** 1988 Changes in human sperm motion
786 during capacitation in vitro. *Journal of Reproduction and Fertility***83** 119-128.
787 • **Mortimer ST & Maxwell WM** 1999 Kinematic definition of ram sperm
788 hyperactivation. *Reproduction, Fertility and Development***11** 25-30.
789 • **Mortimer ST, Schoëvaërt D, Swan MA & Mortimer D** Quantitative observations
790 of flagellar motility of capacitating human spermatozoa. *Human Reproduction***12**
791 1006–1012.
792 • **Naz RK & Rajesh PB** 2004 Role of tyrosine phosphorylation in sperm capacitation /
793 acrosome reaction. *Reproductive Biology and Endocrinology***2** 75.
794 • **Olds-Clarke P** 1989 Sperm from tw32/+ mice: capacitation is normal, but
795 hyperactivation is premature and nonhyperactivated sperm are slow. *Developmental*
796 *Biology***131** 475–482.
797 • **Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J & Kopf GS** 1999
798 Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal

799 transduction pathway leading to protein kinase A-mediated up-regulation of protein
800 tyrosine phosphorylation. *Molecular Human Reproduction***5** 1017–26.
801 • **Parodi J** 2013 Motility, viability, and calcium in the sperm cells. *Systems Biology in*
802 *Reproductive Medicine***60** 65-71.
803 • **Partyka A, Niżański W & Ochota M** 2012 Methods of assessment of cryopreserved
804 semen. *Current Frontiers in Cryobiology* 565- 574.
805 • **Perry G** 2013 2012 statistics of embryo collection and transfer in domestic farm
806 animals. *Embryo Transfer Newsletter***31** 1–67.
807 • **Reid AT, Redgrove K, Aitken RJ & Nixon B** 2011 Cellular mechanisms regulating
808 sperm-zona pellucida interaction. *Asian Journal of Andrology***13** 88-96.
809 • **Roberts KP, Wamstad JA, Ensrud KM & Hamilton DW** 2003 Inhibition of
810 Capacitation-Associated Tyrosine Phosphorylation Signaling in Rat Sperm by
811 Epididymal Protein Crisp-1. *Biology of Reproduction***69** 572–581.
812 • **Rota A, Penzo N, Vincenti L, & Mantovani R** 1999 Hypoosmotic swelling (HOS)
813 as a screening assay for testing in vitro fertility of bovine spermatozoa.
814 *Theriogenology***53** 1415-1420.
815 • **Scott I, Ramirez-Reveco A & Parodi J** 2016 The Restraint of Bovine Sperm Cell
816 Motility Increases Survival: Role of Extracellular Calcium in the Phenomena. *Journal*
817 *of Veterinary Science and Technology***7** 359.
818 • **Storey BT & Kopf GS** 1991 Fertilization in the Mouse: II. Spermatozoa. *A*
819 *Comparative Overview of Mammalian Fertilization* (edited by Dunbar B & O'Rand
820 M) 167-216.
821 • **Storey BT & Kopf GS** 2013 *A Comparative Overview of Mammalian Fertilization*.
822 (edited by Bonnie S. Dunbar, M.G. O'Rand) chapter 10 179.

- **Van Gestel RA, Brewis IA, Ashton PR, Helms JB, Brouwers JF & Gadella BM** 2005 Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. *Molecular Human Reproduction***11** 583-590.
- **Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS** 1995a Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development***121** 1129–1137.
- **Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P & Kopf GS** 1995b Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP dependent pathway. *Development***121** 1139–1150.
- **Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP & Jorgez CJ** 1999a Cholesterol efflux-mediated signal transduction in mammalian sperm. Beta-Cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *Journal of Biological Chemistry***274** 3235–3242.
- **Visconti PE, Stewart-Savage J, Blasco A, Battaglia L, Miranda P & Kopf GS** 1999b Roles of bicarbonate, camp, and protein tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. *Biology of Reproduction***61** 76–84.
- **Wani NA** 2002 In vitro maturation and in vitro fertilization of sheep oocytes. *Small Ruminant Research* **44** 89-95.
- **Ward C & Storey BT** 1984 Determination of the Time Course of Capacitation in Mouse Spermatozoa Using a Chlortetracycline Fluorescence Assay. *Developmental Biology***104** 287-296.

- **Watson PF, Jones PS & Plummer JM** (1991) A quantitative comparison of the spontaneous and ionophore-induced acrosome reaction in ejaculated ram spermatozoa: the effects of temperature, time and individual. *Animal Reproduction Science***24** 93-108.
- **Watson PF & Plummer JM** 1986 Relationship between calcium binding sites and membrane fusion during the acrosome reaction induced by ionophore in ram spermatozoa. *Journal of Experimental Zool*ogy**238** 113 – 118.
- **Xia J & Ren D** 2009 The BSA-induced Ca(2+) influx during sperm capacitation is CATSPER channel-dependent. *Reproductive Biology and Endocrinology***7**.
- **Yanagimachi R** 2011 Mammalian Sperm Acrosome Reaction: Where Does It Begin Before Fertilization? *Biology Of Reproduction***85**: 4–5.
- **Yeung CH & Cooper TG** 2010 Sperm Quality and Function Tests. *Andrology: Male Reproductive Health and Dysfunction* (edited by Eberhard Nieschlag, Hermann M. Behre, Susan Nieschlag)**1** 141.

Figure legends

Figure 1: Percentage of Acrosome Reacted spermatozoa (total AR) over 3 hours of ram spermatozoa incubated in TALP minus Ca^{2+} versus TALP plus Ca^{2+}. Between 0 and 3 hours there was a significant increase in the percentage of acrosome reacted spermatozoa (P = 0.014), with no significant difference between treatments (TALP minus Ca^{2+} and TALP plus Ca^{2+}) at either time points (0 hours and 3 hours). Data correspond to model derived mean ± SEM. Values with different subscripts are statistically different (*abcd* indicates $P < 0.05$).

Figure 2: M540 median fluorescence (viable median PE-A) over 3 hours for spermatozoa incubated in TALP minus Ca2+ versus TALP plus 2 mM Ca2+. Between 0 and 3 hours there

871 was a significant decrease in the viable median PE-A ($P = 0.009$), indicating an increase in
872 membrane lipid order, with no significant difference between treatments (TALP minus Ca^{2+}
873 and TALP plus Ca^{2+}) at either time points (0 hours and 3 hours). Data correspond to model
874 derived mean ± SEM. Values with different subscripts are statistically different (*abcd*
875 indicates $P < 0.05$).
876
877 **Figure 3**: Viability (total % of live spermatozoa) over 3 hours of spermatozoa incubated in
878 TALP plus CAPSTIM minus Ca^{2+} versus TALP plus CAPSTIM plus Ca^{2+}. Between 0 and 3
879 hours there was a highly significant decrease in the total % of live spermatozoa ($P <0.001$),
880 indicating a decrease in sperm viability, with no significant difference between treatments
881 (TALP plus CAPSTIM minus Ca^{2+} and TALP plus CAPSTIM plus Ca^{2+}) at either time
882 points (0 hours and 3 hours). Values with different subscripts are statistically different (*abcd*
883 indicates $P < 0.05$ and *ABCD* indicates $P < 0.001$).
884
885 **Figure 4**: M540 median fluorescence (viable median PE-A) over 3 hours of spermatozoa
886 incubated in TALP plus CAPSTIM minus Ca^{2+} versus TALP plus CAPSTIM plus Ca^{2+}.
887 Between 0 and 3 hours there was a significant increase in in the viable median PE-A ($P=$
888 0.007), indicating a decrease in membrane lipid order, with no significant difference between
889 treatments (TALP minus Ca^{2+} and TALP plus Ca^{2+}) at either time points (0 hours and 3
890 hours). Data correspond to model derived mean ± SEM. Values with different subscripts are
891 statistically different (*abcd* indicates $P < 0.05$ and *ABCD* indicates $P < 0.001$).
892
893 **Figure 5**: Percentage of Acrosome Reacted spermatozoa (total AR) over 3 hours of ram
894 spermatozoa incubated in TALP minus Ca^{2+}, TALP plus Ca^{2+}, TALP minus Ca^{2+}+ plus
895 CAPSTIM, and TALP plus Ca^{2+} plus CAPSTIM. Between 0 and 3 hours there was a highly

significant increase in the percentage of acrosome reacted spermatozoa ($P = 0.001$), with no significant difference between treatments (TALP minus Ca^{2+} and TALP plus Ca^{2+}) at either time points (0 hours and 3 hours). Data correspond to model derived mean ± SEM. Values with different subscripts are statistically different (*abcd* indicates $P < 0.05$).

Figure 6: Viability (total % of live spermatozoa) over 3 hours of spermatozoa incubated in TALP minus Ca^{2+}, TALP plus Ca^{2+}, TALP minus Ca^{2+}+ plus CAPSTIM, and TALP plus Ca^{2+} plus CAPSTIM. Between 0 and 3 hours there was a highly significant decrease in the total % of live spermatozoa ($P < 0.001$), indicating a decrease in sperm viability. There was also a significant difference between treatments with Ca^{2+} and those without. The addition of Ca^{2+} resulted in a significantly higher % of total live spermatozoa ($P = 0.023$). Data correspond to model derived mean ± SEM. Values with different subscripts are statistically different (*abcd* indicates $P < 0.05$ and *ABCD* indicates $P < 0.001$).

Figure 7: M540 median fluorescence (viable median PE-A) over 3 hours of spermatozoa incubated in TALP minus Ca^{2+}, TALP plus Ca^{2+}, TALP minus Ca^{2+} plus CAPSTIM, and TALP plus Ca^{2+} plus CAPSTIM. There was a highly significant increase in in the viable median PE-A ($P < 0.001$) with the addition of CAPSTIM, indicating a decrease in membrane lipid order. Data correspond to model derived mean ± SEM. Values with different subscripts are statistically different (*abcd* indicates $P < 0.05$ and *ABCD* indicates $P < 0.001$).

Tables

***Table 1**: Effect of incubation in Ca^{2+} on kinematic parameters of ram spermatozoa (mean ± SEM).*

	TALP minus Ca^{2+}		TALP plus 2mm Ca^{2+}	
Kinematic Parameter	0	3	0	3
ALH (μm)	4.64 ± 0.20	4.22 ± 0.32	4.80 ± 0.12	4.48 ± 0.21
BCF (Hz)	33.43 ± 1.38[a]	29.37 ± 1.04[b]	34.35 ± 1.60[a]	29.31 ± 1.04[b]
LIN (%)	77.13 ± 3.71[a]	82.68 ± 1.31[b]	78.43 ± 1.95[a]	84.05 ± 1.37[b]
STR (%)	93.78 ± 0.97	94.85 ± 0.49	94.41 ± 0.32	94.76 ± 0.47
VAP (μm/s)	137.97 ± 10.39	138.11 ± 9.44	145.76 ± 7.12	159.48 ± 8.13
VCL (μm/ s)	168.91 ± 6.70	159.0 ± 11.10	177.18 ± 6.67	179.69 ± 8.33
VSL (μm/s)	131.14 ± 10.76	132.39 ± 9.17	138.92 ± 7.05	153.27 ± 8.04
MOT (%)	62.58 ± 4.65[a]	58.14 ± 4.15[b]	61.77 ± 4.72[a]	45.74 ± 5.80[b]
PROG MOT (%)	51.59 ± 5.22[a]	45.79 ± 3.91[b]	53.40 ± 3.87[a]	37.41 ± 4.83[b]

ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; LIN: linearity; STR: straightness; VAP: average-path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; MOT: total motility; PROG MOT; progressive motility.

Values in the same row with different superscripts (abcd) are statistically different ($P < 0.05$).

935 ***Table 2**: Effect of incubation in Ca^{2+} on kinematic parameters of ram spermatozoa (mean ± SEM).*

	TALP plus CAPSTIM minus Ca^{2+}		TALP plus CAPSTIM plus 2 mM Ca^{2+}	
Kinematic Parameter	0	3	0	3
ALH (μm)	4.23 ± 0.16[a]	3.59 ± 0.31[b]	3.50 ± 0.21[c]	3.09 ± 0.15[d]
BCF (Hz)	25.05 ± 1.14	26.57 ± 1.50	25.51 ± 1.77	27.02 ± 1.31
LIN (%)	87.72 ± 1.07	85.60 ± 2.94	84.57 ± 1.86	84.04 ± 2.56
STR (%)	95.61 ± 0.97	96.20 ± 0.47	95.21 ± 0.63	94.87 ± 1.07
VAP (μm/s)	122.55 ± 7.07[a]	104.85 ± 10.09[b]	137.67 ± 7.03[c]	117.37 ± 11.27[d]
VCL (μm/ s)	134.02 ± 6.70[a]	159.0 ± 11.10[b]	177.18 ± 6.67[c]	179.69 ± 8.33[d]
VSL (μm/s)	119.17 ± 7.01[a]	101.16 ± 10.34[b]	132.81 ± 7.15[c]	112.31 ± 11.20[d]
MOT (%)	52.24 ± 5.77	48.13 ± 7.04	49.79 ± 7.04	46.18 ± 4.81
PROG MOT (%)	40.79 ± 5.08	34.58 ± 5.99	41.96 ± 6.91	33.26 ± 5.01

936 ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; LIN: linearity; STR:
937 straightness; VAP: average-path velocity; VCL: curvilinear velocity; VSL: straight-line velocity;
938 MOT: total motility; PROG MOT; progressive motility.
939 Values in the same row with different superscripts (abcd) are statistically different ($P < 0.05$).

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943 ***Table 3***: *Effect of incubation in Ca^{2+} or CAPSTIM on kinematic parameters of ram spermatozoa (mean ± SEM).*

	TALP minus Ca^{2+}		TALP plus 2mm Ca^{2+}		TALP plus CAPSTIM minus Ca^{2+}		TALP plus CAPSTIM plus 2 mM Ca^{2+}	
Kinematic Parameter	0	3	0	3	0	3	0	3
ALH (μm)	4.64 ± 0.20^{A}	4.22 ± 0.32^{A}	4.80 ± 0.12^{A}	4.48 ± 0.21^{A}	4.23 ± 0.16^{B}	3.59 ± 0.31^{B}	3.50 ± 0.21^{B}	3.09 ± 0.15^{B}
BCF (Hz)	33.43 ± 1.38^{A}	29.37 ± 1.04^{A}	34.35 ± 1.60^{A}	29.31 ± 1.04^{A}	25.05 ± 1.14^{B}	26.57 ± 1.50^{B}	25.51 ± 1.77^{B}	27.02 ± 1.31^{B}
LIN (%)	77.13 ± 3.71^{A}	82.68 ± 1.31^{A}	78.43 ± 1.95^{A}	84.05 ± 1.37^{A}	87.72 ± 1.07^{B}	85.60 ± 2.94^{B}	84.57 ± 1.86^{B}	84.04 ± 2.56^{B}
STR (%)	93.78 ± 0.97	94.85 ± 0.49	94.41 ± 0.32	94.76 ± 0.47	95.61 ± 0.97^{B}	96.20 ± 0.47^{B}	95.21 ± 0.63^{B}	94.87 ± 1.07^{B}
VAP (μm/s)	137.97 ± 10.39^{A}	138.11 ± 9.44^{A}	145.76 ± 7.12^{A}	159.48 ± 8.13^{A}	122.55 ± 7.07	104.85 ± 10.09	137.67 ± 7.03	117.37 ± 11.27
VCL (μm/ s)	168.91 ± 6.70^{A}	159.0 ± 11.10^{A}	177.18 ± 6.67^{A}	179.69 ± 8.33^{A}	134.02 ± 6.70^{B}	159.0 ± 11.10^{B}	177.18 ± 6.67^{B}	179.69 ± 8.33^{B}
VSL (μm/s)	131.14 ± 10.76^{A}	132.39 ± 9.17^{A}	138.92 ± 7.05^{A}	153.27 ± 8.04^{A}	119.17 ± 7.01^{B}	101.16 ± 10.34^{B}	132.81 ± 7.15^{B}	112.31 ± 11.20^{B}
MOT (%)	62.58 ± 4.65^{a}	58.14 ± 4.15^{a}	61.77 ± 4.72^{a}	45.74 ± 5.80^{a}	52.24 ± 5.77^{b}	48.13 ± 7.04^{b}	49.79 ± 7.04^{b}	46.18 ± 4.81^{b}
PROG MOT (%)	51.59 ± 5.22^{a}	45.79 ± 3.91^{a}	53.40 ± 3.87^{a}	37.41 ± 4.83^{a}	40.79 ± 5.08^{b}	34.58 ± 5.99^{b}	41.96 ± 6.91^{b}	33.26 ± 5.01^{b}

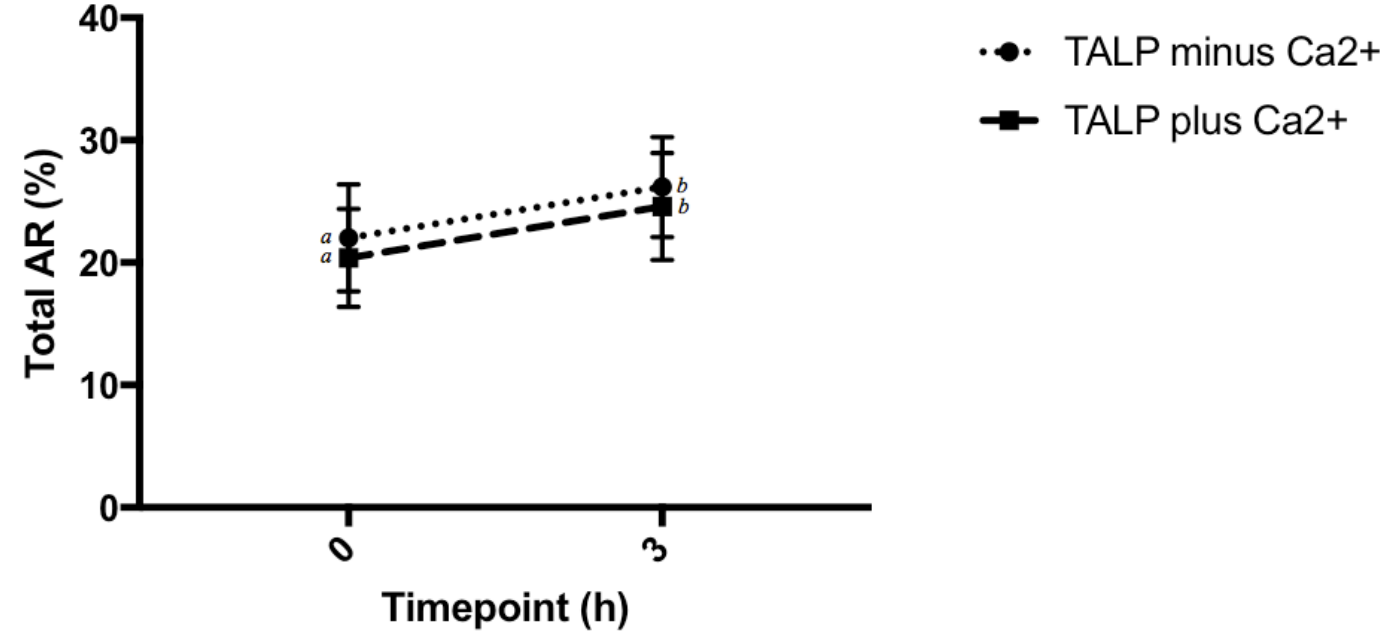
944 ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; LIN: linearity; STR: straightness; VAP: average-path velocity; VCL: curvilinear velocity;

945 VSL: straight-line velocity; MOT: total motility; PROG MOT; progressive motility.

946 Values in the same row with different subscripts are statistically different (*abcd* indicates $P < 0.05$ and *ABCD* indicates $P < 0.001$).

947 Figures

948 **Figure 1**



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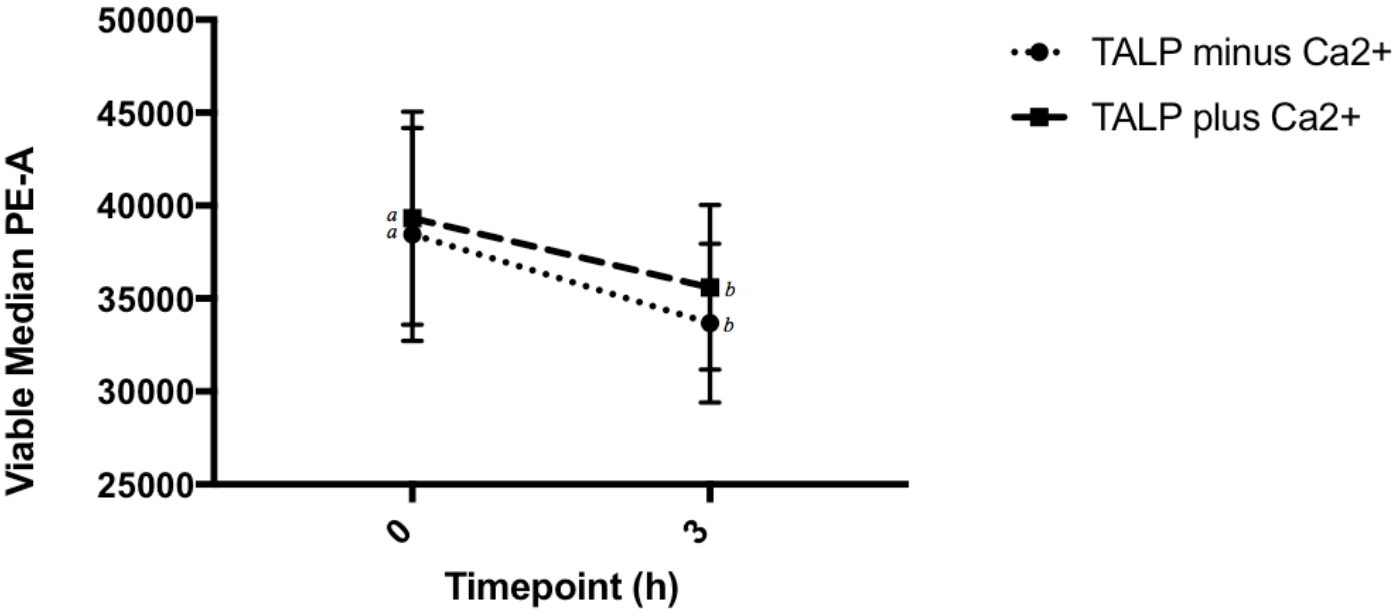
952 **Figure 2**



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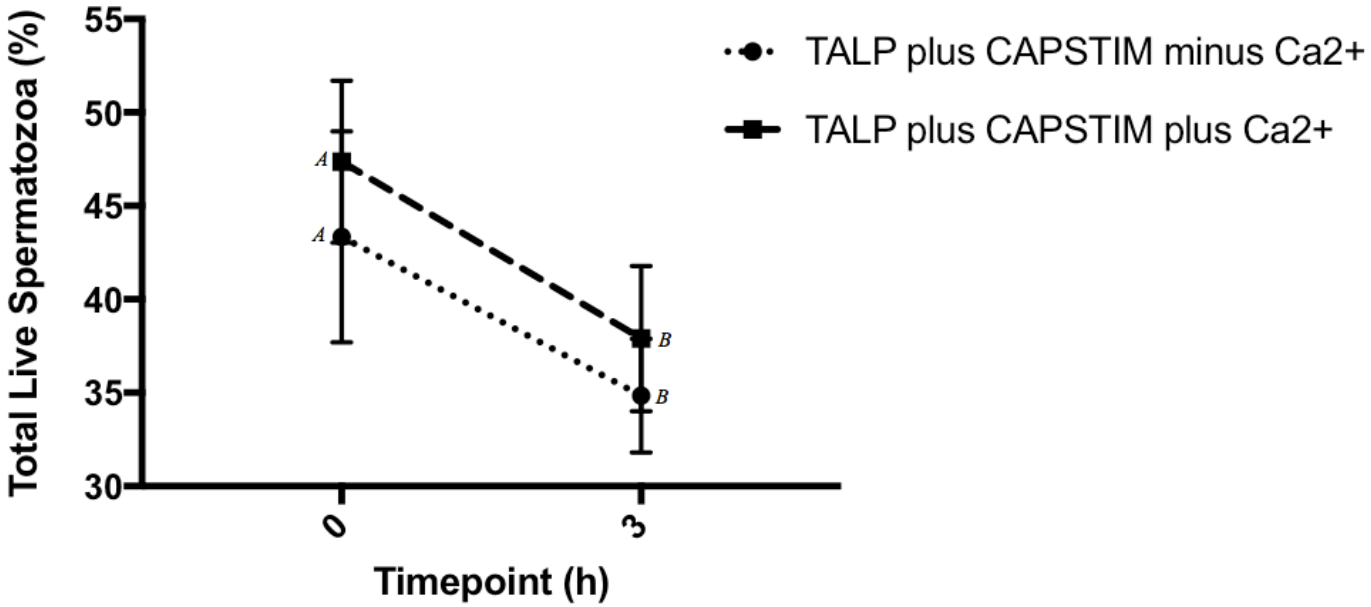

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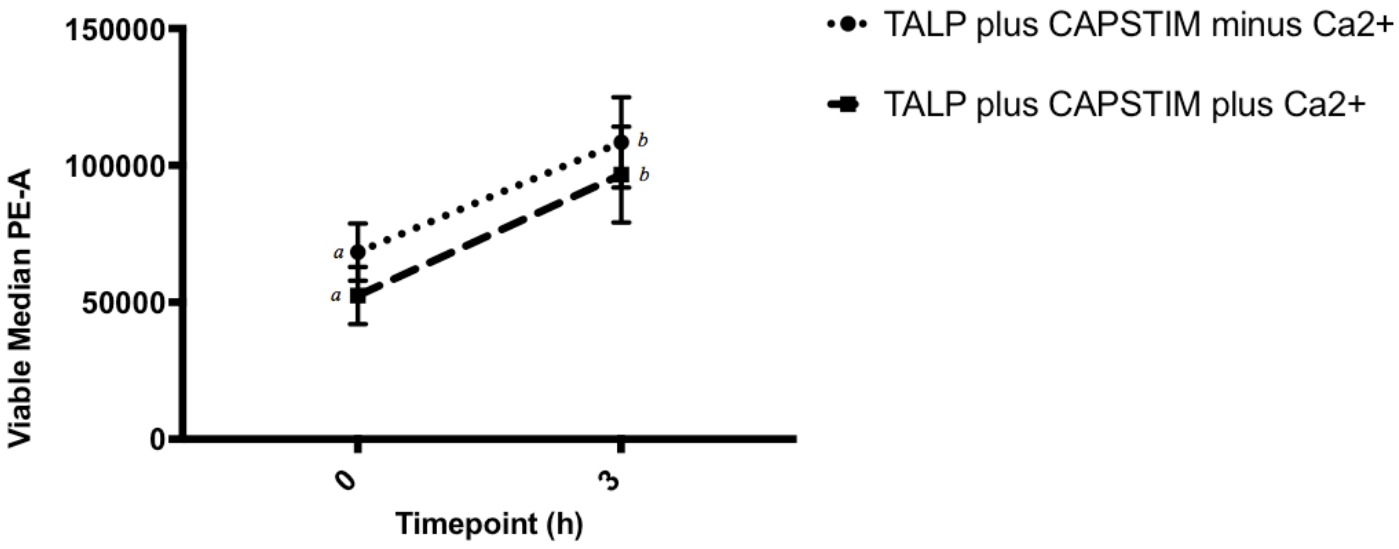

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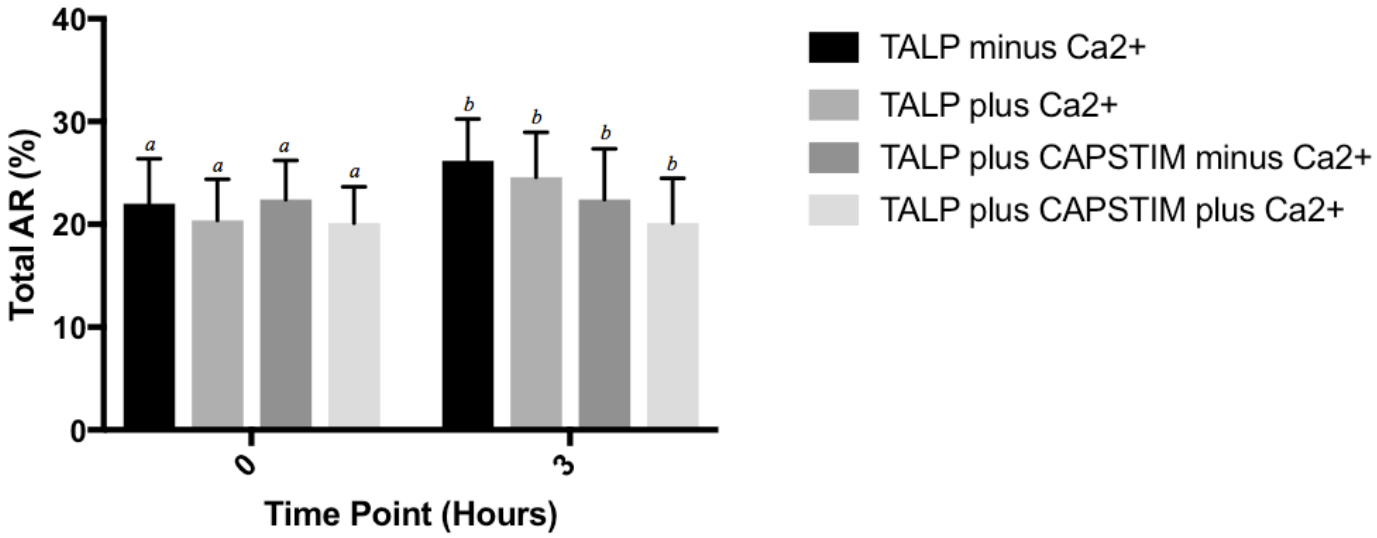
961 **Figure 5**



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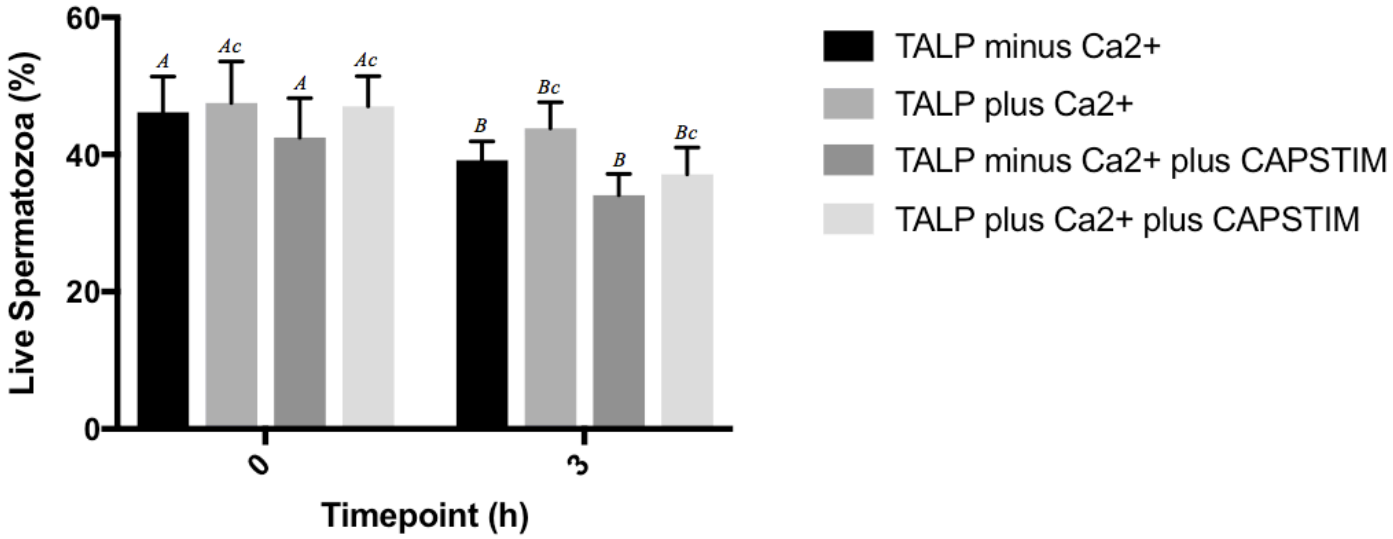
966 **Figure 6**



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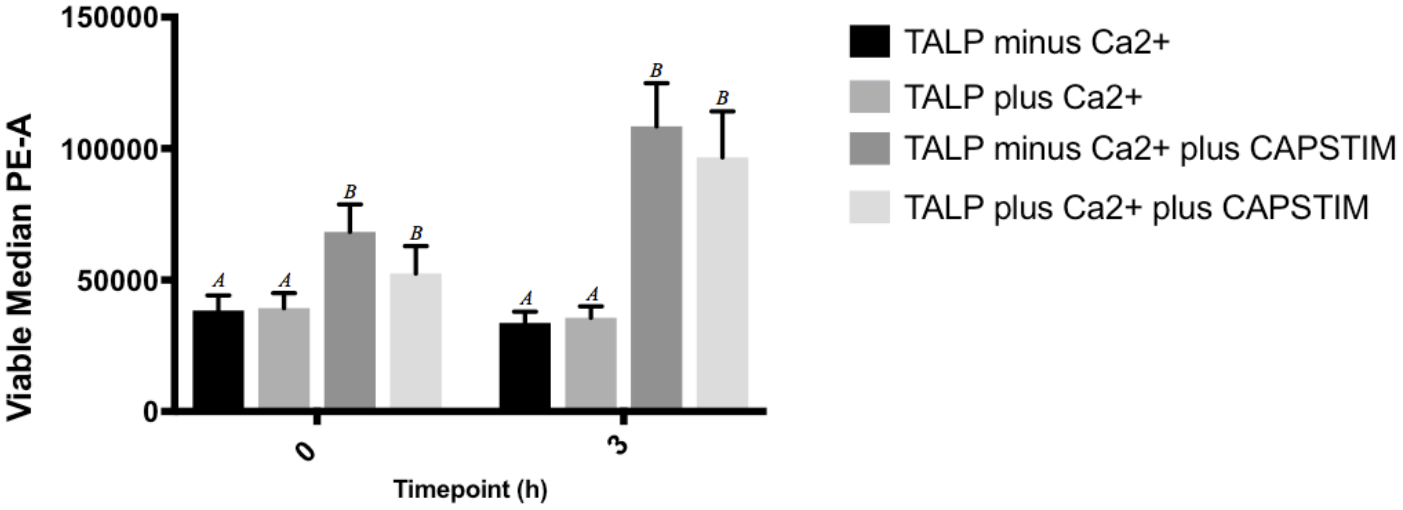
970 **Figure 7**



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