The Role of Bovine Seminal Plasma in Fertility

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Abstract

Bovine seminal plasma (SP) is known to have an effect on spermatozoa and the female reproductive tract. The differences in fertility among individuals could be due to variations in SP composition and its effect on both spermatozoa and the inseminated female. Single Layer Centrifugation (SLC) has been shown to select the most robust spermatozoa from the ejaculate and can be used to separate spermatozoa from SP. The purpose of this thesis was to study the various effects of bovine SP. The thesis was divided into 4 studies: Study I was to determine the effects of SLC on post-thaw sperm quality. Study II investigated the effect of adding SP back to SP-free sperm samples. Study III investigated the effect of bovine SP on bovine endometrial epithelial cells (bEEC) in culture. Study IV determined the effect of season and SLC on sperm quality. Our results show that bull spermatozoa selected by SLC had a higher proportion with high mitochondrial membrane potential (MMP) and a higher superoxide production than controls. The SLC-selected samples had a higher proportion of spermatozoa with normal morphology and a lower proportion with bent tails than controls; they had better kinematics than controls. However, sperm viability and chromatin integrity were not different between treatments. Adding 5% bovine SP had a beneficial effect on sperm velocity. Moreover, there was a beneficial effect of adding 5% heterologous SP from high fertility bulls on sperm velocity but a deleterious effect on chromatin integrity. Total cell number and viability of bEEC after challenge with 1% SP from either high or low fertility bulls did not differ from controls. In contrast, challenge with 4% SP from high or low fertility bulls (300H or 300L) negatively affected bEEC cell number and viability. Challenge with 300L had a greater adverse effect than 300H. There were differences in semen characteristics and sperm morphology among seasons. However, sperm kinematics, viability, chromatin integrity and MMP were not different between seasons. In conclusion, these results indicated that SLC can be used to enhance bull sperm quality. Moreover, adding bovine SP prior to cryopreservation affected sperm quality depending on the proportion of SP and the fertility of the bull from which it came. Bovine SP had a negative effect on bEEC in both a dose-dependent and fertility–dependent manner. Season had a slight effect on sperm morphology. Further studies are needed to investigate the factors involved in the interaction between SP and spermatozoa or bEEC, such as differences in SP composition between low and high fertility bulls, and breed and age of bull, as well as their effects on fertility.

Keywords: bovine SP, fertility, proportion of SP, Uterus cell, sperm quality, Climate, frozen semen

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Abstract

Det har visats att bovin seminalplasma (SP) påverkar spermier och de honliga reproduktionsorganen. Skillnaden i fertilitet mellan individer kan bero på variationer både i sammansättningen hos SP och i dess effekter på såväl spermier som den inseminerade honan. Single Layer Centrifugation (SLC) har visats selektera fram de mest robusta spermierna från ejakulatet, och kan användas för att separera spermier från SP. Syftet med denna avhandling var att studera de olika effekterna av bovin SP. Avhandlingen är uppdelad i fyra delstudier; i Studie I fastställdes effekterna av SLC på spermiekvaliteten efter upptining, Studie II undersökte effekten av åter tillsätta SP till SP-fria spermieprover. Studie III undersökte effekten av bovin SP på cellkulturer av bovina epitelceller från endometriet (bEEC). Studie IV fastställde effekten av årstid och SLC på spermiekvaliteten hos tjurspermier i Thailand. Våra resultat visar att tjurspermier som selekterats med SLC hade en högre andel med hög mitokondriemembranpotential (MMP) och en högre superoxidproduktion än kontroller. De SLC-selekterade proverna hade en högre andel spermier med normal morfologi och en lägre andel med svansböjning än kontroller, dessutom hade de bättre kinematik än kontrollerna. Dock fanns det ingen skillnad i membranintegritet och kromatinintegritet mellan behandlingarna. Tillsats av 5% bovin SP hade en fördelaktig effekt på spermiernas hastighet. Dessutom fanns det en fördelaktig effekt av att tillsätta 5% heterolog SP från tjurar med hög fertilitet på spermiernas hastighet, men detta försämrade samtidigt kromatinintegriteten. Totalt cellantal och viabilitet hos bEEC efter exponering för 1% SP skilde sig inte från kontroller, oavsett om SP från hög- eller lågfertila tjurar användes. I motsats till detta gav exponering med 4% SP från hög- eller lågfertila tjurar (300H eller 300L) en negativ effekt på cellantal och viabilitet hos bEEC. Exponering med 300L gav en större negativ effekt än 300H. Det fanns skillnader mellan årstider i spermans karakteristika och spermiemorfologin. Dock skilde sig inte spermiernas kinematik, membranintegritet, kromatinintegritet eller MMP mellan årstiderna. Sammanfattningsvis indikerar dessa resultat att SLC kan användas för att förbättra kvaliteten hos prover av tjurspermier. Dessutom visades att en tillsats av SP innan nedfrysning påverkade spermiekvaliteten beroende på andelen SP som tillsattes och fertiliteten hos donatortjuren. Bovin SP hade en negativ effekt på bEEC som var både dos- och fertilitetsberoende. Årstiden hade en mindre effekt på spermiemorfologin. Ytterligare studier behövs för att undersöka de faktorer som är inblandade i samspelet mellan SP och spermier eller bEEC, såsom skillnader i sammansättningen hos SP mellan låg- och högfertila tjurar, ras och ålder hos tjurarna samt faktorernas effekter på fertiliteten.

Nyckelord: bovin SP, fertilitet, andel SP, livmodercell, spermiekvalitet, klimat, fryst sperma

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Dedication

To my family, my teachers and the animals.

“Strength and Honor”
Gladiator

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nongbua, T., Johannisson, A., Edman, A. & Morrell, J.M.* (2017). Effects of single layer centrifugation (SLC) on bull spermatozoa prior to freezing on post-thaw semen characteristics. Reproduction in Domestic Animals, 52, pp. 596-602.

II Nongbua, T.*, Al-Essawe, E., Johannisson, A., Edman, A. & Morrell, J.M. Effect of adding bovine seminal plasma prior to cryopreservation on bull sperm quality after thawing: part I and part II (Submitted)

III Nongbua, T.*, Guo, Y., Edman, A., Humblot, P. & Morrell, J.M. Effect of bovine seminal plasma on bovine endometrial epithelial cells in culture (Accepted)

IV Nongbua, T.*, Utta, A., Am-in, N., Suwimonteerabutr, J., Johannisson, A. & Morrell, J.M. Effects of season and Single Layer Centrifugation on bull sperm quality in Thailand (Submitted)

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The contribution of Thanapol Nongbua to the papers included in this thesis was as follows:

I Contributed to the conception and design of the study, sample analysis, data analysis, statistical analysis, and reviewing of the manuscript.

II Contributed to the conception and design of the study, sample preparation, sample analysis, data collection, data analysis and interpretation, statistical analysis, drafting the manuscript, and critical revision of the manuscript. Corresponded with the journal.

III Contributed to the conception and design of the study, sample preparation, sample analysis, data collection, data analysis and interpretation, statistical analysis, drafting the manuscript, and critical revision of the manuscript. Corresponded with the journal.

IV Contributed to the conception and design of the study, sample preparation, sample analysis, data collection, data analysis and interpretation, statistical analysis, drafting the manuscript, and critical revision of the manuscript. Corresponded with the journal.

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Abbreviations

aSFP	Acidic seminal fluid protein
AB	American Bhaman
AI	Artificial insemination
ALH	Amplitude of Lateral Head Displacement
BCF	Beat Cross Frequency
BCS	Body condition score
bEEC	Bovine endometrial epithelial cell
BSP	Binder of sperm protein
CASA	Computer assisted sperm analysis
C-Heterologous	Control-Heterologous
C-Homologous	Control-Homologous
DCAC	Dead cells after challenging
DCFDA	2',7'-dichlorodihydrofluorescein diacetate
DLD	Department of Livestock Development
DFI	DNA Fragmentation Index
DGC	Density Gradient Centrifugation
DLD	Department of Livestock Development
FITC	Fluorescein isothiocyanate
HE	Hydroethidine
HO	Hoechst
IM	Initial motility
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide
IL	Interleukin
IVF	*in vitro* fertilization
LCAC	Total number of live bEEC after challenge
LIN	Linearity
LSMEAN ± SEM	Least Square Means ± Standard Error of Mean
MA	Mass activity

MI	Plasma membrane integrity
MMP	Mitochondrial membrane potential
MOT	Total motility
NRR	Non-return rate
ns	Not significant
PI	Propidium iodide
PG	Prostaglandin
PMQ	Progressive motility (QualiSperm®)
PRO	Progressive motility
PTP	Protein Tyrosine Phosphorylation
ROS	Reactive oxygen species
SCSA	Sperm Chromatin Structure Assay
SLC	Single Layer Centrifugation
SLOW	Slow motility
SMO	Subjective sperm motility before processing
SP	Seminal plasma
SPC	Sperm concentration per mL
SPCE	Sperm concentration per ejaculate
STAT	Static motility
STR	Straightness
SW	Sahiwal
TAC	Total cells after challenging
TBC	Total cells before challenging
TGF	Transforming growth factor
TMQ	Total motility (QualiSperm®)
TNE	Tris-sodium chloride-EDTA
VAP	Velocity Average Path
VCL	Curvilinear Velocity
VOL	Ejaculate volume
VOLD	Total volume per day
VSL	Velocity Straight Line
v/v	Volume/volume
WOB	Wobble

1 Introduction

1.1 Background

Fertility in inseminated dairy cattle is thought to have declined over the last few decades (López-Gatius, 2003). Although this decline is most apparent in high yielding cows, milk production alone does not explain all of the decreased fertility (García-Ispierto *et al.*, 2007). Differences in sperm handling procedures and sperm numbers in insemination doses have changed over the years and may contribute to the decreased fertility (López-Gatius, 2013). Sperm structure and function depend on the male and his ability to produce spermatozoa, and to maintain the potential fertility of spermatozoa during storage before transfer to the female reproductive tract (Maxwell *et al.*, 2007a). Semen has two major compartments: spermatozoa and the fluid content known as seminal plasma (SP). It contains proteins, minerals, electrolytes, hormones and enzymes (Poiani, 2006) and has been shown to have an effect on spermatozoa (Garner *et al.*, 2001; Maxwell *et al.*, 1996; Graham, 1994). Bovine SP has a major function in stimulating and supporting spermatozoa, by providing nutrients and a protective environment, and by enhancing sperm motility in the female reproductive tract. (Maxwell *et al.*, 2007a; Poiani, 2006). Furthermore, bovine SP is implicated in the sperm de-capacitation mechanism and fertilization process (Rodriguez-Martinez *et al.*, 2011; Maxwell *et al.*, 2007a; Poiani, 2006). Therefore, the differences in fertility among individuals with apparently similar sperm quality in ejaculated semen could be due to variations in SP composition and its effect on both spermatozoa and the inseminated female.

1.2 Bovine SP and its effect

Seminal plasma (SP) is a fluid that promotes sperm motility and serves as a medium for transporting the spermatozoa into the female genital tract (Bromfield, 2016; Maxwell *et al.*, 2007a; Poiani, 2006). It is predominantly produced by the accessory sex glands and also contains small amounts of testicular and epididymal fluid (Bromfield, 2016). The major components of bovine SP include peptidase proteins, cytokines, enzymes, antioxidants, hormones, ions, sugar and lipid (Juyena & Stelletta, 2012). In *Bos taurus,* SP is a complex medium including peptidase, heparin-binding proteins, spermadhesins, bovine SP proteins, acidic seminal fluid protein (aSFP), glycosidase, exopeptidases, phospholipases, phospholipases A-2, sugar (Poiani, 2006) as well as inhibitors of tryptic enzyme activities (Gurupriya *et al.*, 2014; Manjunath *et al.*, 1994). The proteomic characterization of bovine SP indicates that 80.1% have a molecular weight < 25 kDa. The predominant specific proteins in bovine SP are binder of sperm protein 1 and 3 (BSP1 and BSP3), and seminal plasma protein BSP-30 kDa (BSP5) (Druart *et al.*, 2013).

Bovine SP enhances sperm motility (Juyena & Stelletta, 2012; Poiani, 2006) and protects spermatozoa against immune attack in the female reproductive tract (Poiani, 2006; Robertson, 2005). It contributes to the sperm capacitation process and also to the fertilization process by promoting sperm movement, regulating pH, supporting nutrition, and inhibiting the acrosome reaction (Rodriguez-Martinez *et al.*, 2011; Maxwell *et al.*, 2007a; Poiani, 2006). Thus, bovine SP can both inhibit and stimulate sperm function and fertility (Maxwell *et al.*, 2007a). Furthermore, SP from individual bulls can affect epididymal sperm motility (Graham, 1994). Active moieties in the seminal fluid stimulate endometrial epithelial cells to synthesis pro-inflammatory cytokines which modulate the inflammatory response, and facilitate embryo tolerance, expansion and implantation in mammals (Robertson, 2005; Robertson & Sharkey, 2001). However, the mechanisms by which these processes occur are unclear. The specific effects of bovine SP on bovine endometrial cells are not known.

1.3 Sperm assessment and fertility

Sperm quality evaluated in the laboratory is assessed by a combination of tests for structural and functional parameters such as morphology, motility, plasma membrane integrity, acrosomal membrane integrity, mitochondrial function, chromatin structure, capacitation and fertilization (Morrell *et al.*, 2009c; Graham & Moce, 2005). This is done by visual analyses of unstained and stained spermatozoa, or by automated sperm analyses using computer assisted

sperm analysis (CASA), flow cytometry and *in vitro* fertilization (IVF) (Morrell & Rodriguez-Martinez, 2011; Graham & Moce, 2005). These assays represent attempts to evaluate various aspects of sperm function as an indicator of sperm fertilizing potential.

In vivo fertility outcomes are a combination of both male and female fertility, which is one reason why assessments of sperm function alone are not totally predictive of fertility. Field fertility can be measured using breeding database records, including, for example, data on fertility rate, non-return rate, conception rate or pregnancy rate. Traditionally, it is calculated from non-returns to service (non-return rate, NRR) at 28, 56 and 128 days post-insemination (Rodriguez-Martinez, 2003), or on a weekly basis after 4–5 weeks and later at 2 months and up to 5 months after insemination (Foote, 2003). Many extraneous factors influence the fertility rate such as the inseminator, time of insemination, category of female, season, farm, geographical area etc.; these factors should be taken into consideration to achieve a reasonably accurate assessment of fertility (Rodriguez-Martinez, 2003).

Correlations between sperm quality and field fertility are reported (Rodriguez-Martinez, 2003; Graham, 2001) although there is little consensus on which parameter of sperm quality is indicative of field fertility. In various studies, different parameters of sperm quality were correlated with field fertility, e.g. sperm motility (Januskauskas *et al.*, 2003; Christensen *et al.*, 1999; Zhang *et al.*, 1998), viability (Nongbua *et al.*, 2014; Januskauskas *et al.*, 2003) and chromatin integrity (Nongbua *et al.*, 2014; Januskauskas *et al.*, 2003; Januskauskas *et al.*, 2001). Furthermore, a combination of sperm viability, hydrogen peroxide negative spermatozoa and DNA fragmentation index (%DFI) could distinguish between below-average and above-average fertility bulls (Kumaresan *et al.*, 2017).

1.4 The effects of season on sperm quality

Many factors, including genetic and environmental factors, can affect sperm quality (Snoj *et al.*, 2013). Season is one of these factors, as reported in many studies (Sabés-Alsina *et al.*, 2017; Valeanu *et al.*, 2015; Bhakat *et al.*, 2011). Season has an impact on reproductive performance through macro- and micro-climatic factors such as temperature, humidity, rainfall and photo-period (Bhakat *et al.*, 2009; Mandal *et al.*, 2000) and may depend on geographic location. However, the reported effects of season are contradictory for different sperm quality parameters. Sperm quality of bulls housed in northern Spain was affected by season, being better during spring than in other seasons (Sabés-

Alsina *et al.*, 2017). In contrast, a study in Brazil showed that ambient temperature, humidity and season did not affect sperm production and semen quality (Brito *et al.*, 2002). Breed can also affect the relationship between sperm quality and season. Studies in *Bos taurus* showed that ejaculate volume and total sperm count differed among seasons in different breeds (Snoj *et al.*, 2013) whereas a study in southeastern Brazil showed that ejaculate volume, sperm concentration, gross-motility, progressive sperm motility, vigor and morphological sperm defects were significantly affected by season and genotype between *Bos indicus* and *Bos taurus* (Koivisto *et al.*, 2009). A seasonal effect on sperm quality has been reported in swamp buffalo (Koonjaenak *et al.*, 2007a; Koonjaenak *et al.*, 2007b; Koonjaenak *et al.*, 2007c; Koonjaenak *et al.*, 2007d) and boar (Suriyasomboon *et al.*, 2006; Suriyasomboon *et al.*, 2005; Suriyasomboon *et al.*, 2004) in Thailand, which has a tropical climate, but there are no reports in the bull.

1.5 Single Layer Centrifugation (SLC)

Colloid centrifugation of semen has been used for several decades to select certain sub-populations of spermatozoa; the most motile spermatozoa, and those that are morphologically normal and with intact chromatin are the ones that are wanted for fertilization (Morrell *et al.*, 2009b). Density gradient centrifugation (DGC) is one of the methods recommended by the World Health Organisation for preparing human spermatozoa for assisted reproduction (World-Health-Organization, 2010). This method has been suggested as a potential means of improving the quality of animal sperm preparations (Morrell & Rodriguez-Martinez, 2009; Rodriguez-Martinez *et al.*, 1997) but the original methods used to prepare small quantities of semen in the laboratory were not practical for large-scale use in the field. Selection of the best spermatozoa from sub-standard bull ejaculates was achieved with DGC (Rodriguez-Martinez *et al.*, 1997); the method was also used for bovine spermatozoa after high speed sorting (Underwood *et al.*, 2010; Hollinshead *et al.*, 2004), or consecutive freezing and thawing (Maxwell *et al.*, 2007b). However, widespread application in the preparation of bovine semen for artificial insemination (AI) has been hampered by the time-consuming protocol for DGC utilising small volumes of semen, and a lack of species-specific colloid formulations (Thys *et al.*, 2009). A modification of density gradient centrifugation, known as Single Layer Centrifugation (SLC) through a species-specific colloid (Single Layer Centrifugation with species-specific formulations of Androcoll), was reported for fresh stallion semen (Morrell *et al.*, 2008a) and later for thawed bull semen (Thys *et al.*, 2009). Furthermore,

SLC can be used to separate spermatozoa from SP. Thus the effect of removing SP can be studied, as well as the effect on spermatozoa of adding back SP, either from the same male or from a different male. With SLC it was possible to scale-up the procedure to process large volumes of extended semen in 50 mL tubes, 100 mL tubes or even 500 mL tubes (Morrell *et al.*, 2011). More recently, the preparation of bovine semen, cooled and transported overnight before processing, was shown to select spermatozoa with intact chromatin and a high mitochondrial membrane potential (Goodla *et al.*, 2014). The effect of sperm selection using silane-coated silica colloids immediately after semen collection on post-thaw sperm quality has not been investigated. Gloria et al. (2016) were unable to show a beneficial effect when preparing "normal" quality bull semen on iodixanol, either as a density gradient (several layers of different densities) or as a single layer, although they could show an improvement in quality from poor quality ejaculates (Gloria *et al.*, 2016).

2 Aims

The overall aim of this project was to study the effects of bovine SP on spermatozoa and on bovine endometrial epithelial cells in culture. Specific aims were as follows:

Study I: To evaluate the effects of SLC of normal ejaculates, performed immediately after collection, i.e. prior to freezing, on post-thaw sperm quality.

Study II: To investigate whether the addition of small quantities of seminal plasma from bulls of "high" or "low" fertility to bull spermatozoa selected by SLC will improve sperm cryosurvival and fertility.

Study III: To determine the effect of SP from bulls of different fertility on number and viability of bEEC in culture.

Study IV: To assess the effect of season and SLC on bull sperm quality in Thailand.

3 Materials and Methods

3.1 Ethical permission

Ethical approval was not required for the experiments conducted in Sweden or Thailand at the time of the experiment. The semen was collected from bulls at commercial or government semen collection stations using an artificial vagina; this is a standard husbandry technique that does not compromise the animal′s welfare. The uterine tissue was obtained from a slaughterhouse in Sweden.

3.2 Study design

The contributions of bull spermatozoa and SP to dairy cattle fertility were studied by investigating the effect of SP on spermatozoa and on the female reproductive tract.

Four studies were performed:

Study I: To evaluate the effects of SLC performed immediately after collection of normal ejaculates, i.e. prior to freezing, on post-thaw sperm quality. Sperm viability, production of reactive oxygen species, chromatin integrity, mitochondrial membrane potential and protein tyrosine phosphorylation were evaluated in control and SLC samples after thawing. The experimental design is shown in Figure 1.

Study II: To investigate whether the addition of small quantities of SP from bulls of low or high fertility to bull spermatozoa selected by SLC will improve sperm cryosurvival and fertility. Sperm kinematics, sperm viability, production of reactive oxygen species, chromatin integrity, mitochondrial membrane potential and protein tyrosine phosphorylation were evaluated after thawing. The effects of adding SP from high and low fertility bulls were investigated (part I), as well as the addition of heterologous and homologous SP (part II), by evaluating sperm quality after freezing and thawing (Figure 2).

Study III: To determine the effect of SP on endometrial cells in culture, SP from high and low fertility bulls was added to cultured endometrial epithelial cells and their responses observed in terms of cell number and cell viability. The effect of SP from bulls of different fertility (high or low fertility) on the number and viability on bEEC in culture was investigated. The effect of concentration (1% and 4%) of bovine SP was also studied (Figure 3).

Study IV: To assess the effect of season and SLC, and the interaction between them, sperm quality (sperm motility, morphology, sperm viability, chromatin integrity and mitochondrial membrane potential) was evaluated. Semen was prepared by SLC in different seasons; control and SLC samples were then frozen and the straws were kept in liquid nitrogen until evaluation (Figure 4).

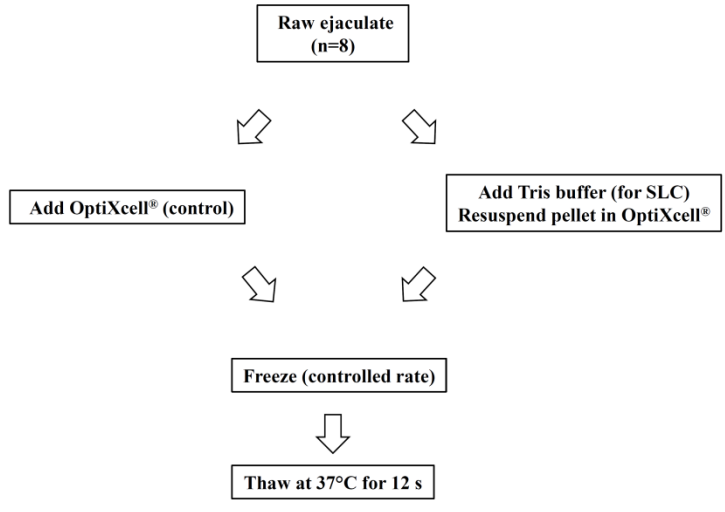

Figure 1. Flow chart showing the experimental design of Study I. Note: SLC, Single layer centrifugation

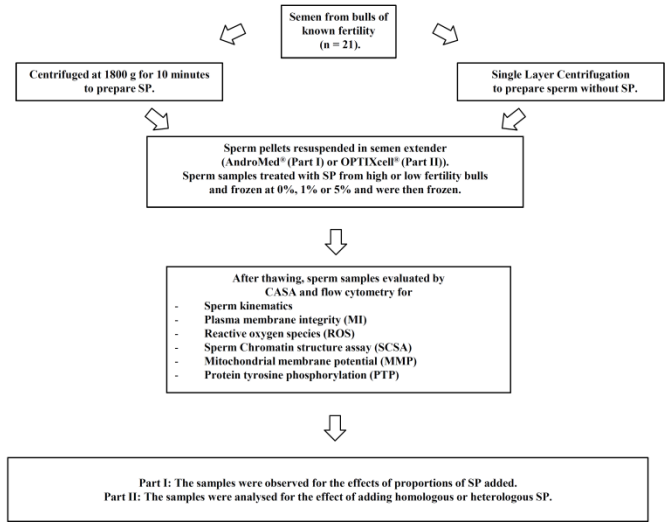

Figure 2. Experimental design of Study II. In part I, the effect of adding 1% or 5% SP was tested. In part II, the effect of adding homologous or heterologous SP was tested.

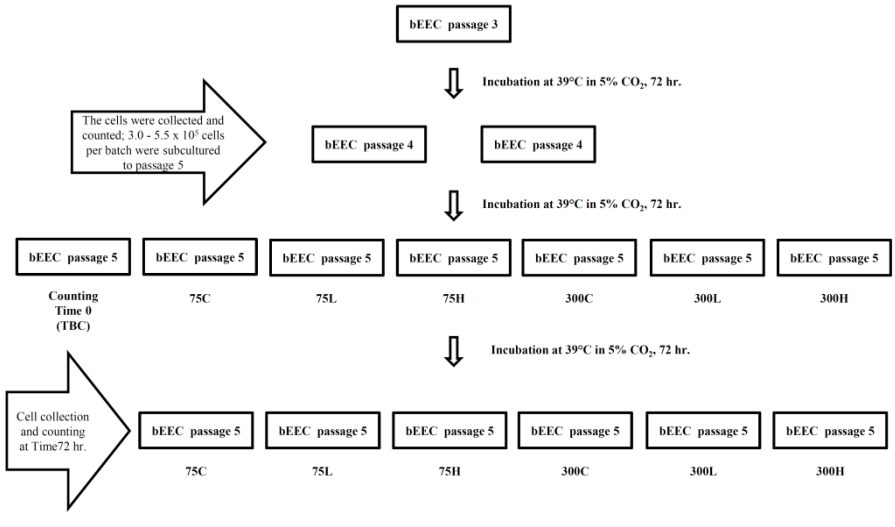

Figure 3. Experimental design of Study III. TBC = Total cells before challenging (number of cells before challenge are the same for each group), 75C = 75 µL of PBS (control), 75L = 75 µL of low fertility bovine SP, 75H = 75 µL of high fertility bovine SP, 300C = 300 µL of PBS (control), 300L = 300 µL of low fertility bovine SP, 300H = 300 µL of high fertility bovine SP.

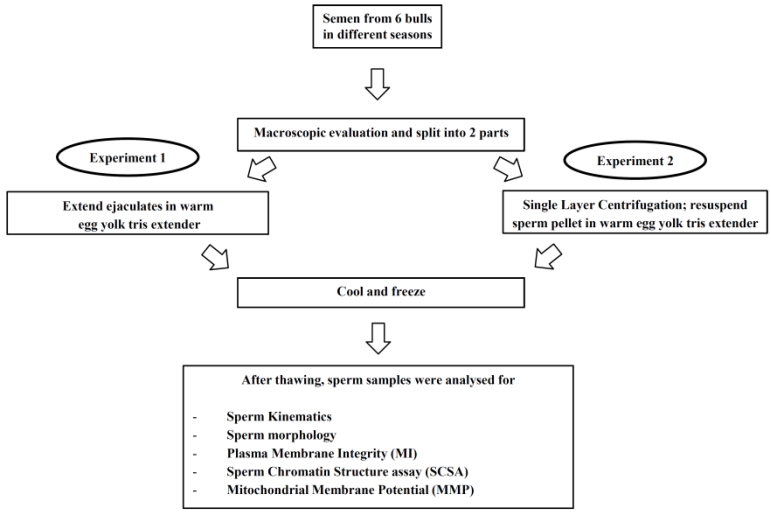

Figure 4. Experimental design of Study IV.

3.3 Animals, animal husbandry and tissue

Study I, II and IV: Semen samples were collected from bulls of known fertility using an artificial vagina (Viking Genetics, Skara, Sweden for Study I and II; North Eastern bull center, Department of Livestock Development (DLD), Khon Kaen, Thailand for Study IV). Only ejaculates meeting the internal standards of the bull station were included in the experiment.

Study II and III: Ejaculates were available from bulls of known fertility according to a fertility index used by the breeding company (Viking Genetics, Skara, Sweden). The semen was divided into two parts: the first part was used for the preparation of bovine SP by centrifuging at 1800 × g for 10 minutes to pellet the spermatozoa. The supernatant was removed and checked by light microscopy for the absence of spermatozoa. The second part was used for Single Layer Centrifugation (SLC) to select and separate spermatozoa from SP.

Study III: On each occasion, a uterus from a cow slaughtered at a local abattoir was packed into a sterile box on ice and was transferred to the laboratory at the Swedish University of Agricultural Sciences. Herd records were available, including the reason for culling. The uterus was examined macroscopically and microscopically for evidence of endometrial disorders; only normal organs from healthy animals were used.

Study IV: Semen collection took place in three seasons: summer (May – June 2014), rainy season (September – October 2015) and winter (January – February 2016). Data on the ambient temperature (°C), humidity (%), and

rainfall (mm) for the study period were accessed from the North Eastern Meteorological center (Upper Part), Khon Kaen, Thailand, which is located adjacent to the bull center (Koonjaenak *et al.*, 2007a).

3.4 Sample collection and preparation

3.4.1 Semen collection

Study I: The semen was collected from eight bulls (six Swedish Red and two Holstein; age range 1.5–8.5 years, median age 27.5 months). The ejaculate was immediately extended in warm (37°C) egg yolk Tris extender, consisting of 20 mL egg yolk, Tris 2.422 g, citric acid 1.36 g, glucose 1 g, de-ionized water 80 mL, to achieve a sperm concentration of 50×10^6/mL; chemicals were purchased from VWR, Stockholm, Sweden; the extended semen was used for Single Layer Centrifugation (see section 3.2). The sperm pellet was resuspended in OptiXcell® to a sperm concentration of 69×10^6/mL and was frozen. An aliquot of raw semen was extended in OptiXcell® at a sperm concentration of 69×10^6/mL and frozen, to serve as an unselected control.
Note: it was necessary to extend the semen in the egg yolk Tris extender prior to centrifugation because of poor survival if OptiXcell® was used at this stage. The sperm concentration of 69×10^6/mL is the standard used at VikingGenetics for frozen semen, designed to deliver an insemination dose of 15×10^6 spermatozoa per straw post-thawing.
Study II: The semen was collected from two breeds of bull (Swedish Red and Holstein). Ejaculates were available from 21 bulls of known fertility. In Study II, Part I, the effect of adding bovine SP to SP-free sperm samples was investigated. Semen from nine bulls was used for SLC and the sperm pellets were resuspended in AndroMed®; bovine SP from seven bulls was added (Figure 2). In Study II, Part II, the effect of adding either homologous or heterologous SP was investigated. Semen from 12 bulls was processed by SLC, the sperm pellets were resuspended in OptiXcell® and SP from the same bulls (high fertility bovine SP; n = 5, low fertility bovine SP; n = 7) was added (Figure 2).
Study III: Ejaculates were available from 5 bulls, one ejaculate per bull. Bulls with a fertility index score of > 104 (n = 3) were considered to be high fertility bulls whereas those with a fertility index score ≤ 92 (n = 2) were classified as low fertility bulls. The sperm quality was analyzed as follow: sperm motility (subjective), sperm concentration and viability (assessed by flow cytometry). The total protein concentration was analyzed using a Protein Assay kit (Pierce

Biotechnology, Thermoscientific, IL). These ejaculates were used to prepare SP.

Study IV: Semen was collected from 6 *Bos indicus* bulls: American Bhaman (AB; n = 4) and Sahiwal (SW; n = 2) used for routine semen collection. Their body condition score (BCS) on a scale of 1–5 was 3.50 – 3.75. The age of bulls at the start of semen collection was 8.0 ± 2.60 years (mean ± SD, range 4 – 11 years). The bulls were fed on grass (*Panicum maximum* and *Brachiaria ruziziensis*), commercial concentrate and minerals supplement. They were housed in an open barn (Koonjaenak *et al.*, 2007a). The effect of season was studied in Experiment 1 whereas the effect of SLC and season, and their interaction, on sperm quality were studied in Experiment 2 (Figure 4).

3.4.2 Single layer centrifugation (SLC) (Study I, II and IV)

A silane-coated silica colloid (Bovicoll, previously known as Androcoll-B) was used. In brief, after equilibrating the colloid to room temperature, 15 mL was poured into a 50 mL centrifuge tube and 20 mL of extended semen at a sperm concentration of 50×10^6 spermatozoa/mL were carefully pipetted on top. The tube was centrifuged at 300 × g for 20 min, and the supernatant (extender, seminal plasma and most of the colloid) was removed. The sperm pellet was aspirated into a clean tube and resuspended in semen extender. The sperm concentration was adjusted to 69×10^6/mL (Study I and II) or 80×10^6/mL (Study IV), depending on the standard freezing protocol of the semen collection station (VikingGenetics, Sweden for Study I and II; North Eastern bull center, Thailand, Study IV), followed by packing into 0.25-mL straws for cooling and freezing. The straws were kept in liquid nitrogen until evaluation. One straw of each sample was thawed at 37 °C for 12 seconds and used for the analyses.

3.4.3 Bovine SP preparation

Bovine SP from bulls of known fertility was prepared as follows (Study II and III): an aliquot of one ejaculate per bull was centrifuged at 1800 × g for 10 minutes to pellet the spermatozoa. The supernatant was removed and checked microscopically for the presence of spermatozoa. The centrifugation was repeated until the SP was free of spermatozoa; it was then frozen in 1 mL aliquots and stored at -20°C.

3.4.4 The bEEC preparation, Isolation and culture

Cell preparation was performed within 1 hour of receiving the uterus at the slaughterhouse. The endometrial tissue was prepared and isolated for bEEC using published techniques (Charpigny *et al.*, 1999; Zhang *et al.*, 1995). The epithelial cells were incubated at 39°C in 5% CO_2 for 5 days until cell confluence was reached (approximately 90% of flask). The purity of epithelial cells in culture from passage 3 (> 98%) were confirmed by anti-Cytokeratin 18 antibody (Abcam, UK) and anti-Vimentin V9 antibody (Abcam, UK) immunofluorescence staining and flow cytometer analysis (Becton Dickinson, San Jose, CA).

3.4.5 Addition of bovine SP to SP-free sperm samples (Study II)

Bovine SP was added at 1 % or 5 % of the final volume (v/v). Bovine SP from the low or high fertility bulls was added to SLC-sperm samples from high or low fertility bulls in a cross-over design (Figure 2). In addition, further aliquots of the sperm suspensions received homologous or heterologous SP. Finally, the sperm samples were cooled to 4°C and frozen in a controlled rate freezer according to the company's routine practice.

3.4.6 Challenge of bEEC with bovine SP (Study III)

The bEEC from passage 3 were cultured in flasks with 25 cm^2 surface area (BD Falcon) and incubated at 39°C in 5% CO_2 (Figure 3). Cells were sub-cultured to produce sufficient numbers for the experiment. Cell numbers per batch varied from 3.0 to 5.5 × 10^5. In each batch, the cells were split equally among 7 flasks for subculture. At 72 hours, the attached cells in one flask were collected and counted to give the total cells before challenge (TBC). After changing the medium in the remaining 6 flasks, cells were subsequently challenged with either 75 µL (1% of total volume medium in flask, Group 1), or 300 µL (4% of total volume medium in flask, Group 2) bovine SP or with PBS (control) (Gibco, Invitrogen, CA) (n = 13 per each challenge). The bEEC were incubated for a further 72 hours before harvesting, counting and evaluating the cells.

3.5 Semen evaluation

3.5.1 Sperm morphology

After thawing, an aliquot of each sample was fixed in formal saline and a further aliquot was used for the preparation of an air-dried smear. All samples were taken to the laboratory at the Swedish University of Agricultural Sciences and evaluated by skilled personnel at the Swedish Sperm Reference Laboratory. At least 200 spermatozoa were evaluated per fixed sample using a phase-contrast microscope (Leica Microsystems, Wetzlar, Germany) at 1000 × magnification, and 500 spermatozoa were evaluated from the air-dried smear stained with Williams´ stain (Morrell *et al.*, 2008b) (Study IV).

3.5.2 Sperm motility with Computer assisted sperm analysis (CASA)

Briefly, motility analysis was performed using a QualiSperm® (Biophos, AG, Switzerland) (Study II) and SpermVision® analyzer (Minitüb GmbH, Tiefenbach, Germany) (Study II) or CEROS II® Version 1.7 (Beverly MA, USA) (Study IV). In Study II, samples were analysed with the QualiSperm® (Biophos, AG, Switzerland). The following measurements were made: total motility (TMQ, %), progressive motility (PMQ, %), Velocity (μm/s), Velocity class A (%; rapid progressive > 50 μm/s), Velocity class B Velocity (%; slow progressive 50 < B > 15 μm/s), Velocity class C (%; slow progressive < 15 μm/s), Velocity class D (%; non-progressive 0 μm/s). Furthermore, the following sperm kinematics were evaluated using SpermVision® analyzer (Study II) or CEROS II® (Study IV): total motility (a spermatozoon that moves more than its head length from its original position during the acquisition; MOT, %), progressive motility (PRO, %), slow motility (SLOW, %), static motility (STAT, %), Velocity Average Path (VAP, μm/s), Curvilinear Velocity (VCL, μm/s), Straight Line Velocity (VSL, μm/s), Amplitude of Lateral Head Displacement (ALH, μm), Beat Cross Frequency (BCF; Hz), Linearity (LIN, VSL/VCL), Straightness (STR, VSL/VAP), Wobble (WOB, VAP/VCL) and Hyperactivity (Hyper, %).

3.5.3 Plasma Membrane Integrity (MI)

Plasma membrane integrity was evaluated by flow cytometry using SYBR14 and propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR), according to published procedures (Johannisson *et al.*, 2009), with slight adaption for bull samples (Goodla *et al.*, 2014). Briefly, the stained

samples were analyzed with a BD LSR flow cytometer (Becton Dickinson, San Jose, CA) (Study I and II) or FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) (Study IV). The spermatozoa were classified according to the integrity of the plasma membrane: living (%) (SYBR14-positive/PI-negative), dead (%) (SYBR14-negative/PI-positive), and dying (%) (SYBR14-positive/PI-positive).

3.5.4 Reactive Oxygen Species (ROS)

The method of ROS evaluation described by Guthrie & Welch (2006) was adapted for bull semen (Goodla *et al.*, 2014). Briefly, the samples were stained with Hoechst 33258 (HO; Sigma, Stockholm, Sweden), hydroethidine (HE; Invitrogen, Eugene, OR), and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen, Eugene, OR). Menadione (Sigma, St. Louis, MO) was added to a duplicate sample to stimulate ROS production. The use of HO permitted the simultaneous differentiation of living and dead cells; HE and DCFDA stains were used to detect superoxide anion and hydrogen peroxide (H_2O_2), respectively. The samples were analyzed with a BD LSR flow cytometer (Becton Dickinson, San Jose, CA). The sperm cells were classified as follows: live, superoxide-negative (R2); live, superoxide-positive (R3); dead, superoxide-positive (R4); live, H_2O_2-negative (R5); live, H_2O_2-positive (R6); dead, H_2O_2-negative (R7); and dead, H_2O_2-positive (R8) (Study I and II).

3.5.5 Sperm Chromatin Structure (SCSA)

Sperm chromatin integrity was evaluated according to a published method (Evenson *et al.*, 2002) with slight adaptations (Goodla *et al.*, 2014). Briefly, partial DNA denaturation in situ was performed by mixing with low pH detergent solution containing 0.17% Triton X-100 followed by staining with acridine orange. Finally, the stained samples were analyzed with a BD LSR flow cytometer (Becton Dickinson, San Jose, CA) (Study I and II) or FC500 flow cytometer (Becton Dickinson, San Jose, CA) (Study IV). The fluorescence from the stained spermatozoa was evaluated and the DNA fragmentation index (%DFI) was calculated as the ratio of the percentage of cells with denatured, single-stranded DNA to total cells acquired (both with stable, double-stranded DNA, and denatured single-stranded DNA); all results were calculated using FCS Express version 2 (De Novo software, Glendale CA, USA).

3.5.6 Mitochondrial Membrane Potential (MMP)

Briefly, the mitochondrial membrane potential (MMP) of sperm cells was analyzed following published procedures (Cossarizza *et al.*, 1993) with slight modifications (Goodla *et al.*, 2014), using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Invitrogen, Eugene, OR). The stained samples were evaluated with a BD LSR flow cytometer (Study I and II) or FC500 flow cytometer (Study IV) (Becton Dickinson, San Jose, CA). The spermatozoa were analysed and classified as follows: sperm cells with high respiratory activity (%) (Orange fluorescence) and with low respiratory activity (%) (Green fluorescence) (CellQuest, version 3.3; Beckon Dickinson).

3.5.7 Global Protein Tyrosine Phosphorylation (PTP)

Bull sperm PTP was evaluated using flow cytometry according to published procedures (Piehler *et al.*, 2006; Sidhu *et al.*, 2004) with modifications (Goodla *et al.*, 2014). Briefly, the diluted samples were stained with PI (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR) and processed. Then the samples were divided into 2 aliquots. Aliquot 1 was stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-phosphotyrosine antibody produced in mouse clone PT-66 (Sigma, St. Louis, MO). Aliquot 2 (control) was stained with antibody, IgG1-FITC isotype control from murine myeloma, clone MOPC21 (Sigma, St. Louis, MO). The stained samples were evaluated with a BD LSR flow cytometer. The fluorescence was measured using Cell Quest 3.3 software (Becton Dickinson). The cells were evaluated as the proportion of phosphorylated cells (%) and the mean fluorescence intensity (MFI) (Study I and II).

3.6 Cell evaluation

3.6.1 Cell trypsinization, counting and evaluation

After challenge, bEEC were detached using trypsin express (Gibco, Invitrogen, CA). Aliquots of the detached cell suspension were stained with trypan blue (Sigma, St. Louis, MO) and counted in a Bürker hemocytometer. Cell viability was classified by counting uncompromised cells (live cells, unstained) and compromised cells (dead cells, both floating and detached cells, stained blue) (Louis & Siegel, 2011). The evaluator did not know the identity of the treatment groups during the evaluation.

3.6.2 Cell viability

Proportion of cell viability (% of live cells) was calculated (Louis & Siegel, 2011) as;

$$x = \frac{\text{Total number of live bEEC after challenge (LCAC) - Total number of live bEEC before challenge}}{\text{Total number of live bEEC before challenge}} \times 100\%$$

3.7 Statistical analysis

In all response variables, residuals were examined for normality and homoscedasticity using diagnostic plots. A p value < 0.05 was considered to be statistically significant.

Study I: The treatment means were compared for all analyses. Mean values of SLC and control samples were analysed using ANOVA (SAS® 9.3, Inc., Cary, NC, USA). All values are reported as Means ± Standard Derivation (MEAN ± SD).

Study II: The treatment means were compared for all analyses. The comparisons between treatments were performed using mixed statistical models (Olsson, 2011; Littell, 2006) (Proc Mixed, SAS® 9.3, Cary, NC, USA). Study II, Part I: the proportion of SP (0%, 1% and 5%), fertility of bull (high or low fertility) and the interaction between both factors were used for the fixed part of the model to observe the response variable of the samples. The bull was considered to be a random factor. Study II, Part II: the treatment of adding homologous or heterologous SP samples was used for the fixed part of the model. The bull was used as random factor. Post-hoc comparisons were adjusted for multiplicity using Tukey's method. All values are reported as Least Squares Means ± Standard Error of Mean (LSMEAN ± SEM).

Study III: The sperm quality, total protein concentration and the effects of seminal plasma on bEEC cell number and viability were analyzed by ANOVA (Proc GLM, SAS® 9.3, Inc., Cary, NC, USA). The models included the effect of concentration of bovine SP (0, 75, 300), bull fertility (low, high) and time (before and after challenge). All main factors (concentration of SP, bull fertility and time) and second order interactions were initially introduced in the models. All values are reported as LSMEAN ± SEM. In the case of multiple comparisons, the Scheffe option and the Contrast option were used to analyze individual differences between groups or differences between several groups.

Study IV: The data analysis was performed using the mixed model (Olsson, 2011; Littell, 2006) in SAS® (Proc Mixed, SAS® 9.3, Cary, NC, USA). The

effects of meteorological data were analysed as follows: the fixed part of the model was of type y = seasons, x = time between season as a random factor, where y is the response variable meteorological data (Temperature, Humidity and Rain fall). The effects of season on sperm characteristics and sperm quality after thawing were analysed as follows; the fixed part of the model was of type y = seasons, x = bull and interaction between season as a random factor, where y is the response variable (sperm characteristic, sperm morphology after thawing and sperm quality after thawing). The effects of SLC in different seasons on sperm quality after thawing were analysed as follows; the fixed part of the model was of type y = seasons, SLC and interaction between them x = bull and interaction between season as a random factor, where y is the response variable (sperm morphology after thawing and sperm quality after thawing). Post-hoc comparisons were adjusted for multiplicity using Tukey's method. All values are presented as LSMEAN ± SEM.

4 Results

The results in Study I-IV are summarized as follows.

4.1 Effect of single layer centrifugation (SLC) of bull spermatozoa prior to freezing on post-thaw semen characteristics (Study I)

4.1.1 Effect of SLC on ROS production

The SLC-treated samples had a higher proportion of live, superoxide-positive spermatozoa than non-treated samples (Figure 5).

4.1.2 Effect of SLC on mitochondrial membrane potential

The SLC-samples had a higher proportion of spermatozoa with high MMP than non-treated samples ($p < 0.05$) and a lower proportion of spermatozoa with low MMP than non-treated samples ($p < 0.05$) (Figure 6).

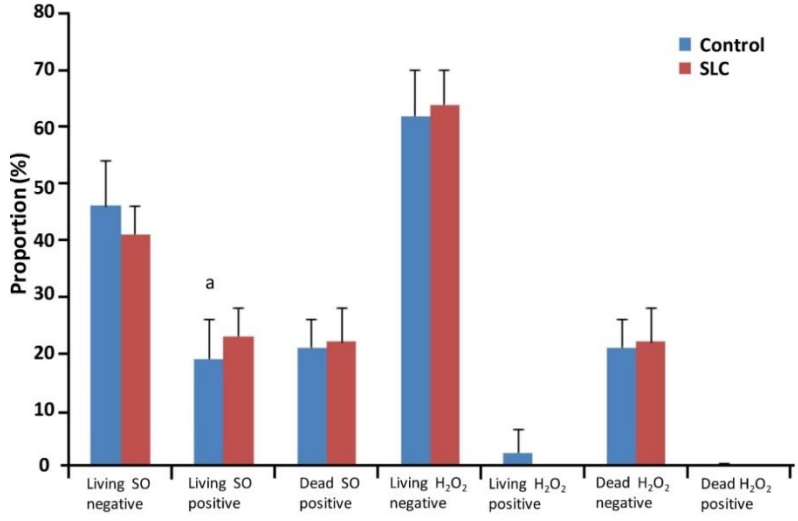

Figure 5. Reactive oxygen species in control and SLC-selected bull spermatozoa post-thaw, means ± SD (n = 8). Note: SLC, Single-layer centrifugation; SO, superoxide, H_2O_2, hydrogen peroxide. a = $p < 0.05$.

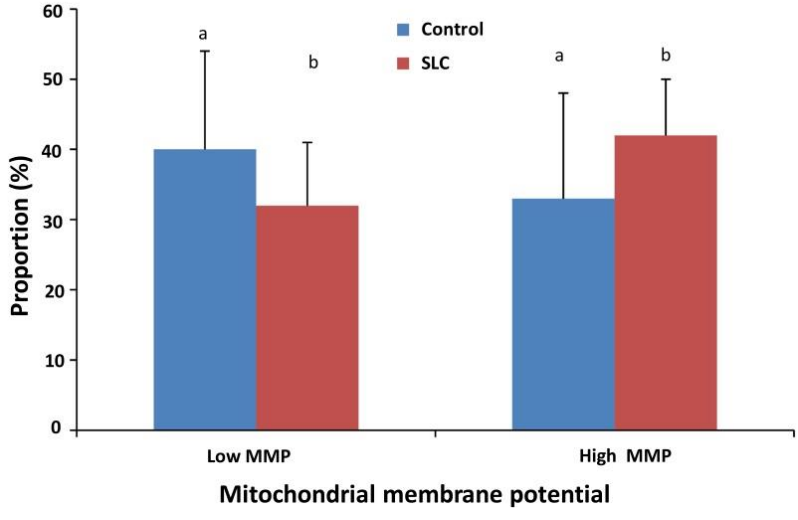

Figure 6. Mitochondrial membrane potential in control and SLC-selected bull spermatozoa post-thaw (n = 8). Note: Similar letters denote statistical significance ($p < 0.05$). SLC, Single layer centrifugation; MMP, mitochondrial membrane potential

4.2 Effect of adding bovine seminal plasma prior to cryopreservation on bull sperm quality after thawing (Study II)

4.2.1 Effect of bovine SP on sperm kinematics

Study II, Part I: The kinematics for the treatment groups are shown in Table 1. The proportion of SP added had a significant effect on VAP ($p < 0.05$), VCL ($p < 0.01$), LIN ($p < 0.05$), WOB ($p < 0.01$) and ALH ($p < 0.05$) but there was no interaction with bull fertility. The VAP was lowest for 5% SP, which was different to control ($p < 0.05$) but was not significantly different to 1% SP. The VCL and ALH were lowest for treatment 5% SP which was different to 1% SP ($p < 0.05$) and control ($p < 0.05$). Furthermore, LIN was highest for treatment 5% SP which was different to 1% SP ($p < 0.05$) but was not different to control. The WOB was highest for treatment 5% SP which was different to 1% SP ($p < 0.01$) and control ($p < 0.01$). In addition, bull fertility had a significant effect on VSL (53.5 ± 2.3 versus 60.2 ± 2.0; $p < 0.05$); VAP (68.3 ± 2.4 versus 75.2 ± 2.2; $p < 0.05$); and VCL (122.8 ± 5.1 versus 138.0 ± 4.5; $p < 0.05$). However, there were no differences between proportion of SP and bull fertility, or their interaction, for the other sperm kinematics.

Study II, Part II: The proportion of spermatozoa with velocity class A was highest for treatments 5H-Heterologous and differed from 5L-Homologous *($p < 0.01$),* 5L-Heterologous *($p < 0.05$),* 1H-Heterologous, C-Homologous *($p < 0.01$* for each*)* and C-Heterologous *($p < 0.05$)* (Table 3). The VSL was significantly different in treatment 5H-Homologous compared to treatments C-Homologous and C-Heterologous *($p < 0.05$* for both*)*. Furthermore, treatment with 1H-Heterologous had the highest values of VCL, ALH and HYP. The 1H-Heterologous differed from 5L-Homologous *($p < 0.05$)* in VCL, and differed from 5L-Heterologous in ALH and HYP *($p < 0.05$* for both*)*. Treatment 5H-Heterologous had a tendency to higher MOT and lower proportion of spermatozoa in velocity class D than control. The 5L-Homologous had a tendency to higher VAP than control (Table 2).

4.2.2 Effect of bovine SP on Reactive Oxygen Species (ROS)

Study II Part I: No significant differences in ROS production were seen for different proportions of SP, fertility of bull, and the interaction of both factors (Table 3). Study II, Part II: Treatment 1H-Heterologous had the lowest proportion of dead, superoxide-positive spermatozoa (R4) and differed from C-Homologous ($p < 0.05$) and C-Heterologous ($p < 0.05$). Furthermore,

treatment 1H-Heterologous had the lowest proportion of dead, H_2O_2-negative spermatozoa (R7) and differed from C-Heterologous ($p < 0.05$). The 1H-Heterologous treatment group had a tendency to a higher proportion of live, superoxide-negative (R2) and proportion of live, H_2O_2-negative (R5) than the other treatments (Table 2).

4.2.3 Effect of bovine SP on Sperm Chromatin Structure (SCSA)

Study II, Part I: No significant differences in %DFI were seen between proportion of SP, bull fertility and their interaction (Table 3). Study II, Part II: There were significant differences in %DFI between treatments with homologous and heterologous SP ($p < 0.05$) (Table 2), with treatment 5H-Heterologous having the highest %DFI compared to treatment with 5H-Homologous ($p < 0.05$), 1L-Homologous ($p < 0.05$), C-Homologous ($p < 0.05$) and C-Heterologous ($p < 0.05$).

Table 1. *Sperm kinematics following addition of different proportions of bovine SP; control (n = 14), 1% (1SP; n = 17) and 5% (5SP; n = 14).*

Sperm Parameter	Control	1SP	5SP
MOT (%)	51.6 ± 5.5	55.5 ± 5.2	47.5 ± 5.7
PRO (%)	44.6 ± 5.3	52.1 ± 5.0	44.0 ± 5.5
VSL (μm/s)	60.5 ± 2.7	57.0 ± 2.5	52.9 ± 2.7
VAP (μm/s)	77.6 ± 2.9^{a}	73.6 ± 2.6^{ab}	65.5 ± 2.9^{b}
VCL (μm/s)	140.8 ± 6.0^{a}	135.9 ± 5.4^{a}	114.5 ± 6.0^{b}
LIN	0.4 ± 0.0^{ab}	0.4 ± 0.0^{a}	0.5 ± 0.0^{b}
STR	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
WOB	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.6 ± 0.0^{b}
ALH (μm)	5.0 ± 0.2^{a}	5.0 ± 0.3^{a}	4.0 ± 0.2^{b}
BCF (Hz)	27.2 ± 0.7	26.5 ± 0.7	26.9 ± 0.7
Hyper (%)	10.5 ± 2.8	13.6 ± 2.6	9.1 ± 3.0
TMQ (%)	55.9 ± 7.7	61.1 ± 7.5	67.7 ± 7.9
PMQ (%)	45.0 ± 8.7	50.6 ± 8.4	53.2 ± 9.1
Velocity (μm/s)	38.1 ± 2.9	37.1 ± 2.6	35.9 ± 3.0
Velocity class A (%)	16.3 ± 3.1	17.0 ± 2.9	18.6 ± 3.2
Velocity class B (%)	29.8 ± 7.5	34.6 ± 7.1	38.0 ± 7.8
Velocity class C (%)	10.7 ± 1.5	10.6 ± 1.4	13.2 ± 1.6
Velocity class D (%)	44.1 ± 7.7	38.9 ± 7.5	32.3 ± 7.9

Different superscript letters within a row indicate significant difference ($p < 0.05$). MOT (total motility), PRO (progressive motility), VSL (Velocity Straight Line), VAP (Velocity Average Path), VCL (Curvilinear Velocity), LIN (linearity), STR (Straightness) WOB (Wobble), ALH (Amplitude of Lateral Head Displacement), BCF (Beat Cross Frequency), Hyper (Hyperactive), TMQ (total motility, QualiSperm®) and PMQ (progressive motility, QualiSperm®).

Table 2. *Effect of adding homologous or heterologous bovine seminal plasma on sperm kinematics, reactive oxygen species and sperm chromatin structure assay for sperm samples extended in OptiXcell®; C-Homologous (n = 9), C-Heterologous (n = 6), 1H-Homologous (n = 5), 1H-Heterologous (n = 3), 1L-Homologous (n = 6), 1L-Heterologous (n = 4), 5H-Homologous (n = 4), 5H-Heterologous (n = 5), 5L-Homologous (n = 8) and 5L-Heterologous (n = 4).*

Sperm Parameter	C-Homologous	C-Heterologous	1H-Homologous	1H-Heterologous	1L-Homologous	1L-Heterologous	5H-Homologous	5H-Heterologous	5L-Homologous	5L-Heterologous
MOT (%)	76.38 ± 2.74	75.01 ± 3.16	85.96 ± 4.23	86.13 ± 5.43	87.99 ± 3.86	72.82 ± 4.72	87.82 ± 4.72	90.42 ± 4.22	78.93 ± 3.35	83.54 ± 4.72
A (%)	19.33 ± 1.97^{b}	19.34 ± 2.21^{b}	20.95 ± 2.84^{ab}	15.15 ± 3.50^{b}	22.23 ± 2.61^{ab}	17.46 ± 3.10^{ab}	23.12 ± 3.13^{ab}	31.37 ± 2.80^{a}	18.74 ± 2.32^{b}	17.22 ± 3.13^{b}
D (%)	23.63 ± 2.74	24.99 ± 3.16	14.04 ± 4.23	13.87 ± 5.43	12.01 ± 3.86	27.18 ± 4.72	12.18 ± 4.72	9.58 ± 4.22	21.07 ± 3.35	16.46 ± 4.72
VSL (µm/s)	48.21 ± 1.44^{b}	48.33 ± 1.54^{b}	46.93 ± 1.8^{ab}	48.40 ± 2.15^{ab}	45.93 ± 1.73^{ab}	48.53 ± 1.97^{ab}	45.70 ± 1.99^{a}	44.82 ± 1.81^{ab}	42.93 ± 1.59^{ab}	44.48 ± 1.99^{ab}
VAP (µm/s)	60.57 ± 1.89	60.70 ± 2.01	59.56 ± 2.37	63.40 ± 2.74	59.45 ± 2.23	60.26 ± 2.52	58.33 ± 2.54	56.45 ± 2.33	55.24 ± 2.07	54.85 ± 2.54
VCL (µm/s)	104.1 ± 3.63^{ab}	104.9 ± 3.81^{ab}	101.9 ± 4.36^{b}	110.3 ± 4.92^{a}	102.4 ± 4.15^{ab}	103.2 ± 4.58^{ab}	100.2 ± 4.63^{ab}	98.73± 4.29^{ab}	95.08 ± 3.90^{b}	96.59 ± 4.63^{ab}
ALH (µm)	3.92 ± 0.16^{ab}	3.95 ± 0.18^{ab}	3.90 ± 0.21^{ab}	4.44 ± 0.25^{a}	4.08 ± 0.20^{ab}	3.84 ± 0.23^{ab}	3.84 ± 0.23^{ab}	3.73 ± 0.21^{b}	3.70 ± 0.18^{ab}	3.24 ± 0.23^{ab}
HYP (%)	5.47 ± 1.22^{ab}	5.70 ± 1.35^{ab}	5.35 ± 1.73^{ab}	11.33 ± 2.12^{a}	7.45 ± 1.60^{ab}	4.29 ± 1.89^{ab}	4.13 ± 1.91^{ab}	3.46 ± 1.70^{b}	4.53 ± 1.42^{ab}	1.33 ± 1.91^{ab}
R2 (%)	46.50 ± 2.31	45.96 ± 2.59	51.23 ± 3.15	56.71 ± 3.81	50.07 ± 2.91	52.18 ± 3.42	55.02 ± 3.45	49.85 ± 3.10	45.48 ± 2.60	52.91 ± 3.45
R4 (%)	23.81 ± 2.03^{b}	24.30 ± 2.17^{b}	24.81 ± 2.46^{ab}	15.71 ± 2.81^{a}	19.60 ± 2.33^{ab}	21.59 ± 2.60^{ab}	20.18 ± 2.62^{ab}	22.88 ± 2.42^{ab}	23.85 ± 2.17^{ab}	22.03 ± 2.62^{ab}
R5 (%)	76.11 ± 2.05	75.71 ± 2.21	73.81 ± 2.55	84.30 ± 2.95	80.55 ± 2.40	77.87 ± 2.72	78.39 ± 2.74	76.78 ± 2.51	75.15 ± 2.22	77.55 ± 2.74
R7 (%)	23.73 ± 2.02^{ab}	24.35 ± 2.15^{b}	24.07 ± 2.43^{ab}	15.83 ± 2.76^{a}	19.61 ± 2.30^{ab}	21.46 ± 2.56^{ab}	20.20 ± 2.58^{ab}	22.69 ± 2.39^{ab}	23.03 ± 2.15^{ab}	22.07 ± 2.58^{ab}
%DFI (%)	2.71 ± 0.33^{b}	2.67 ± 0.34^{b}	2.68 ± 0.36^{ab}	2.85 ± 0.39^{ab}	2.71 ± 0.35^{b}	2.60 ± 0.37^{ab}	2.52 ± 0.37^{b}	3.48 ± 0.36^{a}	2.88 ± 0.34^{ab}	2.76 ± 0.37^{ab}

Different superscript letters indicate statistical difference within a row ($p < 0.05$). MOT (total motility), A (Velocity class A; rapid progressive > 50 µm/sec), D (Velocity class D; non-progressive 0 µm/sec), VSL (Velocity Straight Line), VAP (Velocity Average Path), VCL (Curvilinear Velocity), ALH (Amplitude of Lateral Head Displacement), HYP (Hyperactive), R2 (live, superoxide-negative), R4 (dead, superoxide-positive), R5 (live, H_2O_2-negative), R7 (dead, H_2O_2-negative) and %DFI (DNA fragmentation index).

Table 3. *Sperm quality of sperm samples at different proportion of SP; control (n = 14), 1% (1SP; n = 17) and 5% (5SP; n = 14).*

Sperm Parameter	Control	1SP	5SP
Living spermatozoa (%)	54.9 ± 3.3	52.0 ± 3.2	49.3 ± 3.4
Dead spermatozoa (%)	40.1 ± 3.0	41.8 ± 2.8	43.9 ± 3.0
R2 (%)	35.8 ± 3.7	39.0 ± 3.3	37.6 ± 3.7
R3 (%)	19.4 ± 2.0	20.5 ± 1.8	20.3 ± 2.1
R4 (%)	44.2 ± 4.7	39.7 ± 4.2	41.9 ± 4.7
R5 (%)	53.3 ± 4.9	58.5 ± 4.4	56.2 ± 4.9
R6 (%)	0.6 ± 0.2	0.3 ± 0.2	0.4 ± 0.2
R7 (%)	44.6 ± 4.9	40.6 ± 4.3	42.9 ± 4.9
R8 (%)	0.9 ± 0.4	0.3 ± 0.5	0.4 ± 0.5
%DFI (%)	2.7 ± 0.4	2.2 ± 0.4	2.5 ± 0.5
High MMP (%)	36.5 ± 5.7[a]	33.3 ± 5.6[a]	23.0 ± 5.9[b]
Phosphorylated cell (%)	27.3 ± 6.4	40.7 ± 6.1	32.5 ± 6.7

Different superscript letters within a row indicate significant difference ($p < 0.05$). R2 (live, superoxide-negative), R3 (live, superoxide-positive), R4 (dead, superoxide-positive), R5 (live, H_2O_2-negative), R6 (live, H_2O_2-positive), R7 (dead, H_2O_2-negative), R8 (dead, H_2O_2-positive), %DFI (% DNA Fragmentation Index), High MMP (High Mitochondrial Membrane Potential).

4.3 Effect of bovine seminal plasma on bovine endometrial epithelial cells in culture (Study III)

4.3.1 Effect of SP on bEEC number after challenge

The bEEC after challenge at time 72 hours, and total cell numbers before and after challenge with bovine SP, are shown in Figures 7 and 8. There were significant differences between the total cell numbers before and after challenge for the following treatments: 75 µL bovine SP (75SP), 75 µL PBS as control (75C) and 300 µL of PBS as control (300C) ($p < 0.0001$). However, there were no differences in total cell number before and after challenge for the treatments with 300 µL SP from either high or low fertility bulls (300H and 300L, respectively). Challenge with 75 µL SP had less effect on total bEEC numbers than challenge with 300 µL SP (300SP) ($p < 0.0001$), although the

overall trend was the same, i.e. the cell number for 75 µL SP from low fertility bulls (75L) was less than the cell number for 75 µL SP from high fertility bulls (75H) which was less than control). The total bEEC numbers after challenge with 75L were similar to those after challenge with 75H and 75C ($p > 0.05$). After challenging with 300SP the total bEEC numbers declined compared to pre-challenge numbers, irrespective of whether the SP came from high or low fertility bulls. The lowest total cell numbers occurred in bEEC challenged with 300L ($p < 0.0001$). Challenge with 300H resulted in a higher total cell number than challenge with 300L ($p < 0.05$) but the total cell number was still less than the control treatment with 300C ($p < 0.0001$). The live bEEC numbers after challenge with 75L were similar to those after challenge with 75H, 75C and 300C ($p > 0.05$). The live bEEC after challenging with 300SP showed the same trends as for the total cell number. The lowest live bEEC numbers occurred after challenge with 300L and 300H ($p < 0.0001$). The dead bEEC after challenge with 300L was lowest ($p < 0.05$) whereas numbers of dead cells were similar for 75C, 75L, 75H, 300C and 300H.

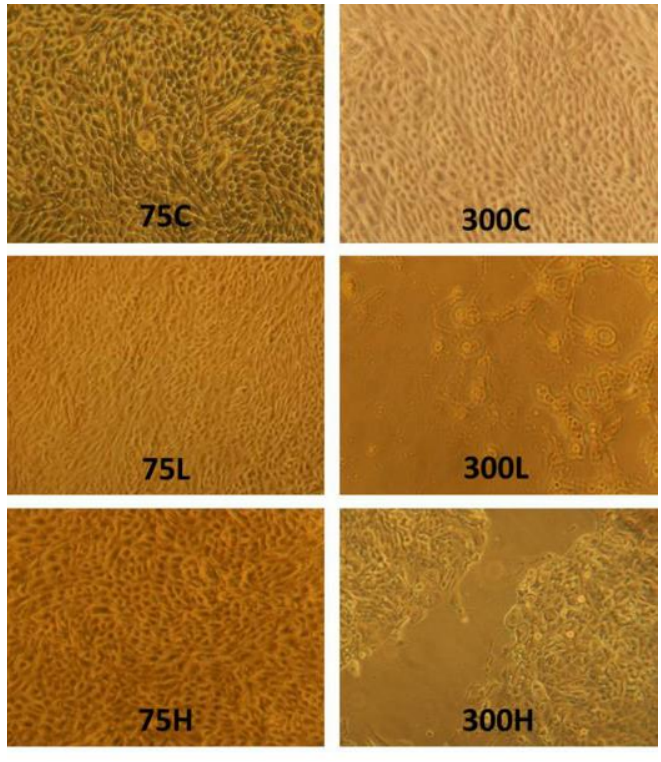

Figure 7. The bEEC after challenge with SP at time 72h. 75C = 75 µL of PBS (control), 75L = 75 µL of low fertility bovine SP, 75H = 75 µL of high fertility bovine SP, 300C = 300 µL of PBS (control), 300L = 300 µL of low fertility bovine SP, 300H = 300 µL of high fertility bovine SP.

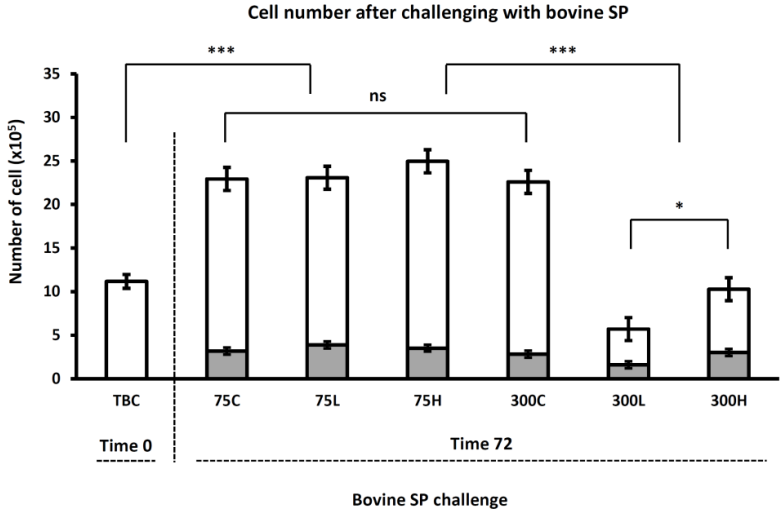

Figure 8. Total bEEC numbers (LSMEAN ± SEM) after challenging with bovine SP (n = 13 per each group). TBC = Total cells before challenging, 75C = 75 µL of PBS (control), 75L = 75 µL of low fertility bovine SP, 75H = 75 µL of high fertility bovine SP, 300C = 300 µL of PBS (control), 300L = 300 µL of low fertility bovine SP, 300H = 300 µL of high fertility bovine SP, LCAC = Live cells after challenging (White bar), DCAC = Dead cells after challenging (Grey bar), SP = Seminal Plasma. *** = $p < 0.0001$, * = $p < 0.05$ and ns = not significant.

4.3.2 Effect of SP on bEEC viability after challenge

Cell viability (%) for different treatments is shown in Figure 9. Bull fertility and concentration of SP had a strong significant effect on viability ($p < 0.0001$ for both). The interaction of bull fertility and the concentration of bovine SP had a highly significant effect on cell viability ($p < 0.0001$). The effect of bull fertility on bEEC viability was different between groups. The highest bEEC viability was found in control, differing from low and high fertility bulls ($p < 0.0001$ for both). The lowest cell viability was seen after challenge with SP from low fertility bulls, differing from challenge with SP from high fertility bulls ($p < 0.05$). The effect of concentration of bovine SP on viability differed between challenge with 75 µL and 300 µL ($p < 0.0001$). The interaction between bull fertility and the concentration of bovine SP on viability of bEEC is shown in Figure 9; the viability of bEEC challenged with 75L or 75H was not different to the control ($p > 0.05$). Challenge with 300L and 300H produced the lowest viability ($p < 0.0001$). In particular, 300L caused the most severe loss of viability and differed from 300H in the contrast-and-estimate option models (29.55 ± 13.20) ($p < 0.05$), and differed from control ($p < 0.0001$). The highest viability was seen in the control group and differed from 300H ($p < 0.0001$).

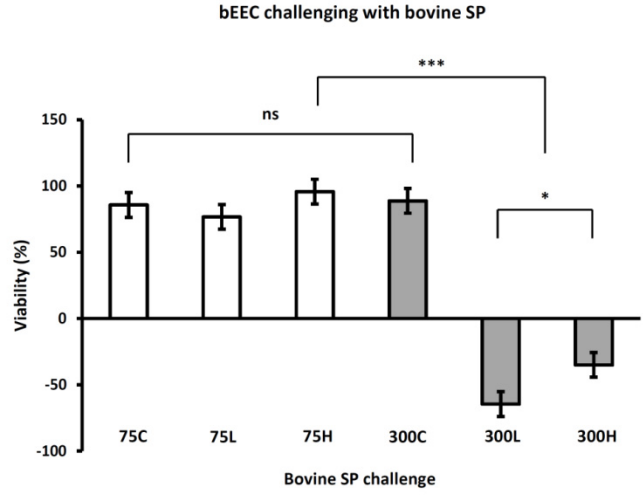

Figure 9. The interaction between fertility and concentrations of bovine SP on viability after challenging 72 hr. (LSMEAN ± SEM) (n = 13 per each group). LFG = Low fertility group, HFG = High fertility group, SP = Seminal Plasma, 75C = 75 µL of PBS (control), 75L = 75 µL of low fertility bovine SP, 75H = 75 µL of high fertility bovine SP, 300C = 300 µL of PBS (control), 300L = 300 µL of low fertility bovine SP, 300H = 300 µL of high fertility bovine SP. *** = $p < 0.0001$, * = $p < 0.05$ and ns = not significant.

4.4 Effects of season and Single Layer Centrifugation on bull sperm quality in Thailand (Study IV)

4.4.1 Meteorological data

Temperature was significantly different between the seasons ($p < 0.001$). The highest temperature occurred in summer (29.7 ± 0.5) and differed from the rainy season (27.6 ± 0.5) ($p < 0.05$) and winter (25.4 ± 0.5) ($p < 0.001$). The temperature was higher in the rainy season than in winter ($p < 0.05$). The humidity also differed significantly between seasons ($p < 0.05$). The rainy season (80.9 ± 1.8) had the highest humidity and differed significantly from winter (73.6 ± 2.4) ($p < 0.05$) but there was no significant difference between the rainy season and summer (78.3 ± 2.9). Furthermore, there was a trend for rainfall to be higher in the rainy season (151.4 ± 34.3) than in winter (17.4 ± 34.3) ($p = 0.05$) but there was no significant difference in rainfall between summer (79.4 ± 34.3) and winter.

4.4.2 Effect of Season on Semen characteristics

The effect of season on semen characteristics is shown in Table 4. There was a significant difference in pH among seasons ($p < 0.0001$), being lower in winter than in the rainy season ($p < 0.05$) and summer ($p < 0.0001$). The pH was higher in summer than in the rainy season ($p < 0.01$). There were no significant differences in volume, concentration or subjective sperm motility among seasons.

4.4.3 Effect of season on Sperm morphology

The effect of season on sperm morphology is shown in Table 4. A higher proportion of normal spermatozoa was seen in winter compared to summer ($p < 0.05$) and rainy season ($p < 0.01$). Furthermore, acrosome defects were lower in winter than in the rainy season ($p < 0.05$), proximal droplets were lower in winter than in summer ($p < 0.01$) and bent tails were lower in winter than in the rainy season ($p < 0.05$).

4.4.4 Effect of Season on Sperm kinematics, Plasma Membrane Integrity (MI), DNA fragmentation (%DFI) and Mitochondrial Membrane Potential (MMP)

There were no significant differences in sperm kinematics, MI, DNA fragmentation (%DFI) and MMP among seasons (Table 5).

4.4.5 Effect of SLC and interaction between seasons on sperm morphology

There were significant differences between treatments (Table 6), with the SLC samples having higher normal morphology ($p < 0.05$) and lower bent tails than controls ($p < 0.05$). There were no significant differences in other sperm abnormalities between SLC and control ($p > 0.05$), nor was there an interaction between treatment and seasons.

4.4.6 Effect of SLC and interaction between seasons on Sperm kinematics.

Significant differences occurred between treatments, as follows: the SLC samples showed greater values for PRO ($p < 0.001$), BCF ($p < 0.0001$), LIN ($p < 0.0001$), STR ($p < 0.001$) and WOB ($p < 0.001$) than control, whereas they showed lower values for SLOW ($p < 0.01$), VAP ($p < 0.0001$), VCL ($p < 0.0001$) and ALH ($p < 0.0001$) than control (Figure 10). There were no

significant differences in MOT and STAT between SLC and control ($p > 0.05$). The interaction between the season and treatment was significant for PRO, VAP and WOB ($p < 0.05$ for each). The SLC-samples had a greater PRO than control in the rainy season ($p < 0.05$) and winter ($p < 0.0001$) but were not different from control in summer ($p > 0.05$). The SLC had a lower VAP than control in summer and the rainy season ($p < 0.05$) but there was no effect of treatment in winter. The SLC samples had higher WOB than control in summer and winter, although there was no significant effect on WOB in the rainy season (Figure 11).

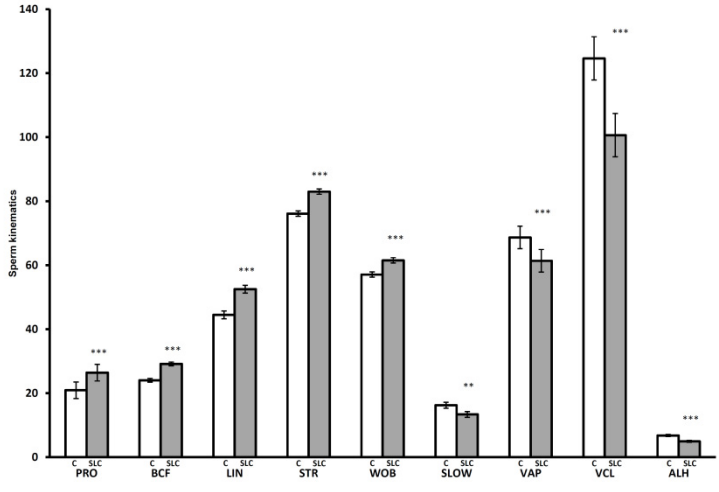

Figure 10. Sperm kinematics after thawing for control (C; white bar) and SLC (gray bar) (LSMEAN ± SEM) (n = 79 each for control and SLC; summer; n = 19, rainy season; n = 29 and winter; n = 31). Progressive motility (PRO, %), BCF (beat cross frequency; Hz), Linearity (LIN, VSL/VCL, %), Straightness (STR, VSL/VAP, %), Wobble (WOB, VAP/VCL, %), Slow motility (SLOW, %), Velocity Average Path (VAP, µm/s), Curvilinear Velocity (VCL, µm/s) and Amplitude of Lateral Head Displacement (ALH, µm). ** $p < 0.01$ and *** $p < 0.001$.

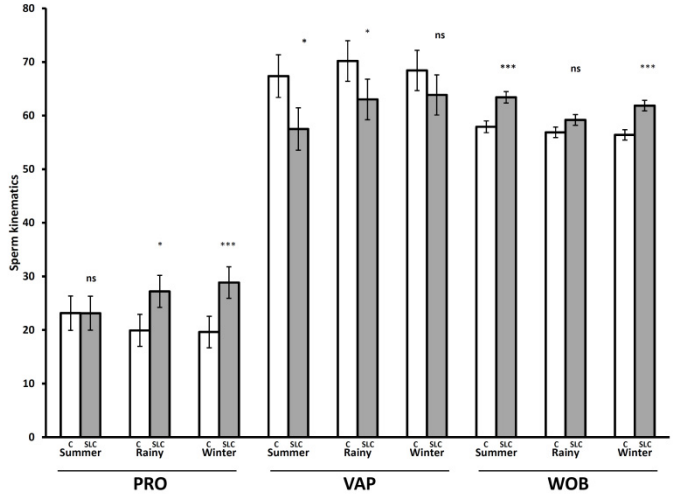

Figure 11. Post-thaw Sperm kinematics for control (C; white bar) and SLC (gray bar) in different seasons (LSMEAN ± SEM) (n = 79 each for control and SLC; summer; n = 19, rainy season; n = 29 and winter; n = 31). Progressive motility (PRO, %), Velocity Average Path (VAP, µm/s) and Wobble (WOB, VAP/VCL, %). ns; no significant difference, * $p < 0.05$ and *** $p < 0.001$.

4.4.7 Effect of SLC on Plasma Membrane Integrity (MI), DNA fragmentation (%DFI) and Mitochondrial Membrane Potential (MMP).

There were no significant differences between SLC and control respectively in MI (Living spermatozoa: 31.7 ± 4.2, 33.4 ± 4.2), %DFI (6.5 ± 1.2, 5.8 ± 1.2) or High MMP (36.1 ± 4.4, 36.1 ± 4.7). Furthermore, there were no effects of treatment, and no interaction between season and treatment for MI (Living spermatozoa, summer: 23.4 ± 5.1, 30.3 ± 5.1; rainy: 34.9 ± 5.2, 33.3 ± 5.2; winter: 36.7 ± 5.0, 36.6 ± 5.0, for SLC and control respectively), %DFI (summer: 6.9 ± 1.8, 6.0 ± 1.8; rainy: 5.5 ± 1.9, 5.7 ± 1.9; winter: 7.0 ± 1.8, 6.0 ± 1.8, for SLC and control respectively) or MMP (High MMP; summer: 30.5 ± 4.5, 34.9 ± 4.5, rainy: 36.6 ± 4.5, 34.4 ± 4.5, winter: 41.2 ± 4.4, 39.1 ± 4.4, for SLC and control, respectively).

Table 4. *Semen characteristics before freezing (n = 91; summer; n = 26, rainy season; n = 33 and winter; n = 32) and morphology (after thawing) (n = 54; summer; n = 18, rainy season; n = 18 and winter; n = 18) in 3 seasons in Thailand (LSMEAN ± SEM).*

Sperm Parameter	Summer	Rainy season	Winter
Volume (mL)	7.5 ± 0.7	6.7 ± 0.8	6.1 ± 0.8
Concentration (10^9/mL)	1.2 ± 0.2	1.4 ± 0.1	1.1 ± 0.1
pH	6.2 ± 0.1[a]	5.8 ± 0.1[b]	5.5 ± 0.1[c]
SMOT (%)	54.8 ± 4.7	51.2 ± 5.4	39.0 ± 5.3
Normal Morphology (%)	60.7 ± 4.9[a]	57.4 ± 4.9[a]	70.1 ± 4.9[b]
Acrosome defects (%)	3.4 ± 1.9	3.6 ± 1.9[a]	0.6 ± 1.9[b]
Abnormal acrosomes (%)	15.6 ± 2.9	18.8 ± 2.9	16.3 ± 2.9
Proximal droplet (%)	13.8 ± 4.5[a]	10.5 ± 4.5	3.4 ± 4.5[b]
Detached head (%)	3.7 ± 1.5	3.4 ± 1.5	3.8 ± 1.5
Bent tail (%)	5.0 ± 2.9	7.9 ± 2.9[a]	3.6 ± 2.9[b]

Different superscript letters within a row indicate significant difference ($p < 0.05$). Subjective motility (SMOT), acrosome defects and proximal droplet were treated with log transformation.

Table 5. *Comparison of post-thaw sperm kinematics (n = 91; summer; n = 26, rainy season; n = 33 and winter; n = 32), viability, DNA fragmentation index and plasma membrane integrity (n = 72 per test, summer; n = 24, rainy season; n = 26 and winter; n = 22) in bull semen collected during different seasons in Thailand (LSMEAN ± SEM).*

Sperm Parameter	Summer	Rainy season	Winter
MOT (%)	59.9 ± 4.6	56.6 ± 4.4	59.9 ± 4.4
PRO (%)	22.7 ± 2.9	19.8 ± 2.8	19.5 ± 3.0
SLOW (%)	15.3 ± 1.5	14.9 ± 1.5	17.0 ± 1.5
STAT (%)	40.1 ± 4.6	43.4 ± 4.4	40.0 ± 4.4
VAP (µm/s)	68.7 ± 4.8	70.7 ± 4.7	68.9 ± 4.7
VCL (µm/s)	121.7 ± 9.6	129.1 ± 9.4	127.7 ± 9.4
VSL (µm/s)	53.2 ± 3.7	53.9 ± 3.6	51.5 ± 3.6
ALH (µm)	6.9 ± 0.5	6.7 ± 0.5	6.9 ± 0.5
BCF (Hz)	23.8 ± 0.8	24.6 ± 0.8	24.3 ± 0.8
LIN (%)	45.8 ± 1.6	44.7 ± 1.6	43.2 ± 1.6
STR (%)	77.1 ± 1.3	76.5 ± 1.3	74.7 ± 1.3
WOB (%)	58.0 ± 1.1	56.9 ± 1.1	56.3 ± 1.1
Viability (%)	30.9 ± 5.8	33.7 ± 5.8	39.1 ± 5.8
%DFI (%)	5.6 ± 1.5	5.5 ± 1.5	5.2 ± 1.2
High MMP (%)	35.5 ± 5.1	34.9 ± 5.1	40.8 ± 5.2

Total motility (MOT), Progressive motility (PRO), Slow motility (SLOW), Statistic motility (STAT), Velocity Average Path (VAP), Curvilinear Velocity (VCL), Velocity Straight Line (VSL), Amplitude of Lateral Head Displacement (ALH), BCF (beat cross frequency), Linearity (LIN), Straightness (STR) and Wobble (WOB), High mitochondria membrane potential (MMP) and DNA Fragmentation Index (%DFI).

Table 6. *Comparison of post-thaw sperm morphology in bull semen between control and SLC-selected sperm in Thailand (LSMEAN ± SEM) (n = 54).*

Sperm Parameter	Control	SLC
Normal (%)	65.2 ± 3.3[a]	68.6 ± 3.3[b]
Acrosome defects (%)	2.6 ± 1.5	2.6 ± 1.5
Abnormal acrosomes (%)	16.9 ± 1.4	15.9 ± 1.4
Proximal droplet (%)	9.3 ± 3.8	8.3 ± 4.5
Detached head (%)	3.6 ± 1.0	3.9 ± 1.0
Bent tail (%)	5.5 ± 2.2[a]	3.6 ± 2.2[b]

Different superscript letters within a row indicate significant difference ($p < 0.05$).

5 Discussion

5.1 Effect of SLC (Study I and IV)

Bull spermatozoa selected by SLC in Study I had a higher MMP and a higher superoxide production than uncentrifuged controls, although membrane integrity, chromatin integrity and capacitation were not different between treatments. These results are in partial agreement with a previous study on stallion spermatozoa selected by SLC (Morrell *et al.*, 2016), in which the SLC-selected spermatozoa were shown to have higher superoxide production than controls, although the MMP was not different between SLC and controls in the case of stallions. In both of these studies, the two assays were carried out on different aliquots of the sperm samples; therefore, it is not clear whether spermatozoa with high MMP were the ones producing more superoxide. In order to carry out simultaneous measurements of MMP and ROS on the same aliquots, a different fluorochrome would be needed; this possibility is currently being investigated. In contrast, Gloria et al. (2016) were unable to detect a change in MMP levels between selected and unselected sperm samples using iodixanol as the separation medium.

Some authors have suggested a link between MMP and fertility (Garner & Thomas, 1999) whereas others have not found such a link using a different fluorochrome to evaluate MMP (Ericsson *et al.*, 1993). Rhodamine 123 was used to evaluate MMP in the study by Ericsson et al. (1993), whereas JC-1 was used in the present study and also in the study by Garner and Thomas (1999). Thus the increased proportion of SLC-selected spermatozoa with high MMP compared to unselected controls seen in Study I might indicate better fertility of the selected sperm samples than the controls. A fertility trial in horses showed that, indeed, SLC-selected samples resulted in a higher pregnancy rate than controls after artificial insemination (AI) (Morrell *et al.*, 2014). The

present results are interesting observations in SLC-selected bull spermatozoa and warrant a fertility trial in cattle.

Tyrosine phosphorylation was not different between controls and SLC samples in Study I. This result is similar to previous studies (Bucci *et al.*, 2013) with boar spermatozoa, although these authors did detect some differences between controls and SLC depending on the assay used. Bergqvist et al. (2011) did not observe a difference in the capacitation status of stallion spermatozoa after SLC. However, Goodla et al. (2014) found an increased degree of capacitation in bull SLC samples, although in their study the spermatozoa were stored for 24h in a different extender to the present study and were subjected to two centrifugations. Previous studies have investigated the effect of SLC on fresh bull semen (Goodla *et al.*, 2014), or the effect of SLC carried out post-thawing (Abraham *et al.*, 2016; Thys *et al.*, 2009). In Study I, fresh semen samples were prepared by SLC immediately after semen collection and the resulting selected sperm samples were cryopreserved. Although the improvement in sperm quality seen following SLC of bull spermatozoa is not as dramatic as seen with stallion spermatozoa (Morrell *et al.*, 2009a; Morrell *et al.*, 2009b), partly because sperm quality was high anyway in the original unselected samples, SLC-selected bull spermatozoa did show improvements in some parameters of sperm quality. One particularly interesting aspect is the apparent increased production of superoxide, without a concomitant increase in hydrogen peroxide. Superoxide is considered to be necessary for sperm capacitation (de Lamirande & O'Flaherty, 2008), although excess ROS in general are detrimental to sperm survival (Guthrie & Welch, 2012). Conventional thinking is that superoxide is rapidly converted to hydrogen peroxide, which is known to be toxic to spermatozoa. However, it was observed that SLC-selected stallion spermatozoa had a higher production of superoxide but significantly reduced hydrogen peroxide production than unselected sperm samples, suggesting that either superoxide is not converted to hydrogen peroxide or that the SLC-selected spermatozoa metabolise differently to unselected spermatozoa (Morrell *et al.*, 2016). The same increased superoxide production was seen in the SLC-selected bull spermatozoa in Study I. In the study by Goodla et al. (2014) in which the spermatozoa were cooled for 24h before SLC, the SLC-selected samples showed an increased in superoxide production but there was also an increase in hydrogen peroxide production, albeit at a low level.

In Study IV (experiment 2), SLC-selected samples showed higher normal morphology and a lower proportion with bent tails than uncentrifuged controls. Spermatozoa selected by SLC had higher values of the kinematics PRO, BCF, LIN, STR, WOB and lower values of SLOW, VAP, VCL and ALH, than

uncentrifuged controls. In addition, SLC had a favorable effect on PRO, VAP and WOB in certain seasons. The sperm morphology results are in partial agreement with previous studies; SLC resulted in an increased proportion of normal stallion spermatozoa and removed head and tail defects (Morrell *et al.*, 2009b; Morrell *et al.*, 2008b) Sperm morphological defects are related to fertility potential (Al-Makhzoomi *et al.*, 2008). Since SLC-selected bull samples had better morphology than controls, we could speculate that they may also have better fertility.

Our results (Study IV) are in partial agreement with a previous study on bull sperm kinematics (Yulnawati *et al.*, 2014), in which SLC-selected spermatozoa were shown to have higher STR, LIN and BCF than controls but lower VAP, VCL and ALH, although the total and progressive motility were not different. Sperm kinematics might be indicative of fertility *in vivo*, although opinions differ as to which kinematics are predictive of fertility (Michos *et al.*, 2013; Kathiravan *et al.*, 2011; Farrell *et al.*, 1998). Total motility, progressive motility or BCF were thought to be predictive of high fertility, whereas VCL was linked with low fertility (Oliveira *et al.*, 2012). Increased VCL and ALH of bull spermatozoa are indicative of hyperactivity; since hyperactivity is related with capacitation, it is not desirable in sperm doses for insemination but could be beneficial for IVF (Suarez & Ho, 2003). Therefore, it is likely that SLC-selected bull sperm samples might have better fertility than controls, as observed for stallion sperm samples (Morrell *et al.*, 2014) although this possibility was not investigated in our study. Furthermore, in Study IV, SLC had a positive effect on sperm morphology and sperm kinematics, which could be expected to influence fertility. Our results suggest that SLC could be used prior to cryopreservation in different seasons to enhance normal sperm morphology and sperm kinematics of thawed bull sperm samples without adversely affecting other parameters of sperm quality.

In Study IV, sperm viability, chromatin integrity and MMP were not different between treatments, which is in partial agreement with results reported previously for bulls in Sweden (Study I). In the previous study by Goodla et al. (2014) sperm samples were transported in Andromed® for 24h before SLC, and the preparations were centrifuged twice. In contrast, in Study I, SLC was performed immediately after semen collection and the semen was extended in an egg yolk extender, only one centrifugation was used, and the samples were subsequently frozen in OptiXcell®. In Study IV, the preparation was the same as for Study I except that the samples were frozen in an egg yolk extender instead of in OptiXcell®. Antioxidants in seminal plasma are believed to limit damage to spermatozoa from ROS; therefore, it is likely that the antioxidative capacity of the semen extender may also play a role in protecting

spermatozoa from ROS-induced damage. The exact composition of the commercial extenders used in these studies is not known but it is likely that they contain at least some substances with anti-oxidative properties, such as zwitterion buffers (Ermilov *et al.*, 1999). In study I, the ejaculate was extended in a Tris-egg yolk extender prior to SLC because our previous studies using the commercial extenders that do not contain material of animal origin (e.g. OptiXcell® or Andromed®) showed that it is not optimal to use them prior to SLC, although they can be used to freeze the sperm pellet after SLC without any adverse effects. Since the original extender remains on top of the colloid during centrifugation, the sperm pellet can then be frozen in the absence of material of animal origin.

It has been shown in several studies that SLC selects spermatozoa with intact chromatin (Johannisson *et al.*, 2009; Morrell *et al.*, 2009a); chromatin integrity has been linked with continued development of the embryo and establishment of pregnancy. In Study I and IV, we did not see an improvement in chromatin quality after SLC, in contrast to a previous study with bull spermatozoa (Goodla *et al.*, 2014). However, the number of bulls in Study I and IV was small and chromatin damage was relatively low in the unselected samples (%DFI values of 2-7%); these levels would not be associated with fertility issues in bulls (Evenson, 2016). Nevertheless, the ability to select for spermatozoa with intact chromatin could be an advantage, particularly where the chromatin damage in the unselected ejaculate is higher than in the bulls in our studies. Chromatin instability of bull spermatozoa has been shown to be affected by sperm handling procedures and to adversely affect fertility of inseminated heifers (Lymberopoulos & Khalifa, 2010). Furthermore, chromatin stability of bull spermatozoa varies according to the semen extender used (Bollwein *et al.*, 2008). The chromatin of some stallions might be more susceptible to damage during storage than of others (Love *et al.*, 2002); this may also be the case for bulls (Love *et al.*, 2002).

5.2 Effect of bovine SP on spermatozoa (Study II)

Study II; Part I; The proportion of bovine SP included in commercial insemination doses varies between ejaculates, depending on sperm concentration, number of spermatozoa per straw, and sometimes on the fertility of the bull (Bromfield, 2016). Spermatozoa account for a volume of approximately 1.0 to 1.5 mL in raw semen (depending on sperm concentration) as observed from the pellet obtained after centrifugation. Using this rough estimation, commercial semen doses contained approximately 0.8 to 12% (v/v) SP, in agreement with other studies (Bromfield, 2016; Hering *et al.*, 2014). The

choice of 1% and 5% SP in Study II was based on the results of a preliminary study in which SP was added at 0%, 1%, 5%, 10%, 20 % (v/v), and sperm kinematics analysed. The results indicated that adding 1% or 5% SP to SLC-selected bull sperm samples tended to increase sperm kinematics.

The concentration of bovine SP can affect sperm quality (Maxwell *et al.*, 1996). The addition of SP modulates the dilution effect in bovine semen, promoting sperm viability when semen is diluted extensively (Garner *et al.*, 2001). In Study II, adding bovine SP to SLC-selected samples affected sperm kinematics with VAP, VCL and ALH decreased by 5% SP, whereas WOB and LIN were increased compared to SP-free controls or 1% SP. Moreover, treatment with 5% SP reduced the proportion of spermatozoa with high MMP. The effects were not dependent on bull fertility. Sperm kinematics can be an indication of *in vivo* fertility, although opinions differ as to which kinematics are predictive of fertility (Michos *et al.*, 2013; Kathiravan *et al.*, 2011). In one study total motility, progressive motility or BCF were considered to predict fertility, whereas VCL was associated with low fertility (Oliveira *et al.*, 2012). In cattle, increased VCL and ALH are indicative of hyperactivity which may not be desirable in sperm doses for insemination but could be acceptable for IVF (Suarez & Ho, 2003).

Our results in Study II, Part I indicated that adding SP had a significant effect on sperm motility, which could suggest a beneficial effect on fertility. Equine sperm motility was maintained in Kenney extender or modified Kenney Tyrodes-type extender (Love *et al.*, 2005) if SP was removed, whereas DNA fragmentation increased in samples containing SP. However, adding 5% SP was shown to increase motility in seminal plasma-free stallion sperm samples (Morrell *et al.*, 2010). Our findings suggest that 5% bovine SP had a beneficial effect on sperm velocity, independent of bull fertility, and an unfavorable effect on high MMP. Further studies are needed to investigate other factors involved, e.g. breed of bull, SP concentrations, and different extenders, and to investigate fertility in manipulated samples. In future, it might be possible to optimize fertility in sperm doses for AI by adding SP to sperm samples that have had their own SP removed by colloid centrifugation.

Study II, Part II; Sperm quality was affected by the addition of SP but both the fertility of the bull from which the SP originated and whether the SP was homologous or heterologous contributed to this effect. In general, adding 5% SP tended to decrease some sperm kinematics (VCL, ALH and hyperactivity) compared to the SP-free controls and 1% SP. Sperm samples treated with SP from high fertility bulls had better sperm velocity parameters than those treated with SP from low fertility bulls. Membrane integrity, mitochondrial activity

and capacitation status were not affected by adding SP, regardless of its source, whereas chromatin integrity was adversely affected by 5% heterologous SP.

These results are in partial agreement with a previous study, in which washed bull spermatozoa exposed to high fertility SP showed greater penetration of zona-free bovine oocytes than spermatozoa mixed with low fertility SP (Henault & Killian, 1996). In another study, SP from bulls of known fertility was shown to increase the motility of epididymal sperm samples but did not affect their membrane integrity or mitochondrial membrane potential (Holden *et al.*, 2017), although the functionality of the treated spermatozoa was not affected in an IVF system. Using electroejaculates from Nellore bulls (Campanholi *et al.*, 2017), sperm velocity was improved if SP was removed from the samples prior to cryopreservation using either centrifugation or a filtration technique, and lower oxidative stress was observed. Plasma membrane integrity was reduced by SP removal in their study. Blastocyst production in IVF was adversely affected by centrifugation but not by filtration. However, the studies of Holden et al (2017) and Campanholi et al. (2017) did not compare the effects of heterologous and homologous SP on sperm quality, nor did they study the effect of SP-treated samples in artificial insemination. Fertility in IVF differs from *in vivo* fertility, being less dependent on spermatozoa being able to navigate their way through the female reproductive tract while still retaining functionality (Thys *et al.*, 2009). Therefore, the effects of adding SP on the functionality of bull spermatozoa in AI are still to be determined.

A previous study in stallions showed differences in effect between homologous and heterologous SP (Morrell & Johannisson, 2014). In the present study, treatment with 5% heterologous SP from high fertility bulls had a positive effect on spermatozoa velocity class A, ALH and HYP compared to 1% heterologous bovine SP, which is in agreement with results reported for stallion spermatozoa (Morrell & Johannisson, 2014). Samples treated with 1% heterologous SP from high fertility bulls showed reduced proportions of dead, superoxide-positive and dead, H_2O_2-negative spermatozoa compared to samples without SP. Furthermore, there was a tendency to higher proportions of live, superoxide-negative spermatozoa, and live, H_2O_2-negative spermatozoa than in the other samples. Hydrogen peroxide can be associated with a decrease in the proportion of capacitated spermatozoa (O'Flaherty *et al.*, 1997), and an excess of ROS in general are detrimental to sperm survival (Guthrie & Welch, 2006).

Nonetheless, our study determined that treatment with 5% heterologous SP from high fertility bulls had a negative effect on %DFI compared to treatment with 5% homologous SP from high fertility bulls or control. This result is

similar to the study of the effects of heterologous stallion SP, where the SP of most individuals caused an increase in %DFI in the spermatozoa of other stallions during storage, with one exception (Morrell & Johannisson, 2014). Our result are in partial agreement with those of Henault and Killian (1996) who showed that spermatozoa from a low fertility bull combined with high fertility SP had high penetrating ability compared with adding low fertility SP. The spermatozoa from a low fertility bull treated with his own SP had greater ability to penetrate oocytes than did spermatozoa from a bull of high fertility combined with low fertility SP (Henault & Killian, 1996). In contrast, our results suggest that the presence of 5% heterologous bovine SP had a negative effect on sperm motility and chromatin integrity. However, the fertility of the treated samples was not examined in our study.

The semen extender used can affect sperm quality. Some studies on the effect of extender showed that bovine SP protein can bind to the low-density fraction of egg yolk extender (Manjunath *et al.*, 2002). Therefore, bovine SP proteins can be either beneficial or detrimental to sperm quality depending on the concentration of SP and exposure time (Manjunath *et al.*, 2002; Thérien *et al.*, 1998). Furthermore, bull sperm quality can be affected by the concentration of SP in the extender and the collection medium (Maxwell *et al.*, 1996). The extenders in the current study were egg yolk-free media for bovine semen; either a soy lecithin-based extender (AndroMed®) (Aires *et al.*, 2003) or a liposome-based extender (OptiXcell®) (Röpke *et al.*, 2011). Nevertheless, a parallel study indicated that bull sperm quality of frozen semen in these two extenders, and the outcome of an AI trial, was similar, although the semen frozen in the liposome-based extender had higher mitochondrial membrane potential (Lima-Verde *et al.*, 2017). The exact formulation of these commercial extenders is not known. Nevertheless, it is likely that they contain some substances with buffering and anti-oxidative properties. The effects of adding SP to spermatozoa were slightly different for the two extenders. However, the extenders were not compared directly with the same samples in Study II. Moreover, breed of bull might be a confounding factor since sperm characteristics are different according to the breed of bull (Morrell *et al.*, 2017)

5.3 Effect of bovine SP on bEEC in culture (Study III)

The results from **Study III** clearly indicate a strong significant negative effect of 300 µL of bovine SP (4%) on cell number and viability whereas challenge with 75 µL of bovine SP had almost no effect on bEEC. Such unfavorable effects of SP on uterine cells are probably not found during natural mating since bovine semen is deposited in the vagina and spermatozoa migrate away

from the seminal plasma through the cervix into the uterus. However, in artificial insemination, semen can be deposited at the anterior os of the cervix, just inside the uterus or using a deep-uterine semen deposition technique (Bergeron *et al.*, 2004). This can result in the introduction of varying amounts of SP into the uterus, since the SP is not removed from the sample during processing – it is merely diluted by adding semen extender (Bromfield, 2016). The proportions of SP investigated here (75 µL and 300 µL) represent 1% and 4% of the cell culture medium; it is difficult to obtain a higher proportion in our cell culture system without depriving the cells of nutrients.

Our results (Study III) are in agreement with studies on the modulatory effects of bovine SP on the uterus (Aloé *et al.*, 2012). However, the effective components of bovine SP, and their actual mechanism of sperm protection, will have to be defined and the results of Study III verified in *in vivo*. In human cervical epithelium, the regulation of inflammatory cytokines after exposure to SP is involved in the regulation of the immune response and immunity against infectious agents introduced during mating (Sharkey *et al.*, 2007). Our results (Study III) suggest that there is a strong negative effect of bovine SP on bEEC that may be dose-dependent. They also show a relationship between the fertility of the bull and the effect of its SP on bEEC.

The results of Study III are in agreement with *in vitro* fertilization studies using spermatozoa from bulls of known field fertility, in which spermatozoa from high fertility bulls were more effective in penetrating artificial mucus and had an increased ability to fertilize oocytes *in vitro* than the spermatozoa from low fertility bulls (Al Naib *et al.*, 2011). Although bovine SP has been shown to affect spermatozoa (Leahy & de Graaf, 2012; Garner *et al.*, 2001; Maxwell *et al.*, 1996), their reports did not examine an impact of SP on field fertility. Components of bovine SP are related to sperm function and fertilization (Juyena & Stelletta, 2012; Maxwell *et al.*, 2007a). The viability and membrane integrity of spermatozoa *in vitro* could be improved when adding 10% seminal plasma in the collection medium for bull spermatozoa (Test buffer containing 2% (v/v) egg yolk) (Maxwell *et al.*, 1996). In addition, a beneficial effect of adding SP on sperm viability can be found when semen is diluted to a low cell number/dose (Garner *et al.*, 2001). However, their reports did not examine an impact of SP on field fertility. The composition of bovine SP varies in proteins including heparin, gelatin, choline phospholipids, glycosaminoglycans and lipoproteins (Leahy & de Graaf, 2012; Manjunath *et al.*, 2007) and this variation could be related to biological function (Leahy & de Graaf, 2012).

5.4 Effect of season on spermatozoa (Study IV)

During the semen collection period, temperature and humidity were different among seasons, and rainfall tended to be different. Since the bulls were kept in an open barn, the meteorological conditions in different seasons would be expected to have a direct effect on them. The results of Study IV (experiment 1) indicated that there were differences in semen characteristics and sperm morphology among seasons. There were higher proportions of normal spermatozoa and fewer abnormalities in winter than in the rainy season and summer. However, sperm kinematics, viability, %DFI and MMP were not different between seasons.

Our observations are in partial agreement with a study in Simmental bulls in Brazil in which a higher proportion of major sperm defects was seen during the summer than in winter; breed and season affected minor sperm defects, whereas season affected total defects (Nichi *et al.*, 2006). Another study in Brazil indicated that neither ambient temperature, humidity nor season affected sperm production and semen quality (Brito *et al.*, 2002). Nevertheless, the genotype or age of bulls can affect total number of spermatozoa and their viability, and the effect varies in different years (Brito *et al.*, 2002). Our results in Study IV are also in partial agreement with previous studies on swamp buffalo in Thailand, wherein ejaculate volume, pH, sperm concentration, total sperm number and initial sperm motility did not differ between seasons. Plasma membrane integrity and the proportion of morphologically normal spermatozoa were greatest in summer and lowest in winter (Koonjaenak *et al.*, 2007a), in contrast to our results (Study IV). Season affected the proportion of tail defects, being highest in the rainy season and lowest in summer but sperm morphology was also affected by buffalo age, week of collection and season (Koonjaenak *et al.*, 2007a); age and ejaculate affected the occurrence of proximal cytoplasmic droplets (Koonjaenak *et al.*, 2007b). However, the proportion of sperm with abnormal head shapes was low, with no significant differences between seasons (Koonjaenak *et al.*, 2007b). In a study in boars in Thailand, housed in either a conventional open air system or housing with an evaporative cooling system, there were minor differences in temperature and humidity between seasons, and differences in the seasonal pattern of sperm production between the two housing systems. High temperature and high humidity had unfavorable effects on sperm production (Suriyasomboon *et al.*, 2004) and on sperm morphology (Suriyasomboon *et al.*, 2005).

In contrast to our results (Study IV), a study in Sahiwal bulls found that ejaculate volume (VOL), total volume per day (VOLD), mass activity (MA), initial motility (IM), sperm concentration per mL (SPC), and sperm concentration per ejaculate (SPCE) were affected by age, season and period.

During the hot-humid season, VOL, VOLD, MA, IM, SPC, and SPCE were highest compared to summer and the cold season, but there was variation between individual bulls (Bhakat *et al.*, 2011). Sperm kinematics could be indicative of *in vivo* fertility, although opinions differ as to which kinematics are predictive of fertility (Michos *et al.*, 2013; Kathiravan *et al.*, 2011) (for more details, see discussion of Study II). In Holstein bulls in northern Spain, semen collected in spring had higher total and progressive motility and viable spermatozoa than semen collected in winter; VAP and STR were higher in spring than in summer. Sperm morphology and %DFI were not different among seasons (Sabés-Alsina *et al.*, 2017). A study on dairy bulls in Sweden showed that the proportion of viable spermatozoa varied between seasons, with the proportion of living spermatozoa being lowest in summer. The %DFI was lowest in spring and differed significantly from summer. There was a trend towards significance in motility parameters LIN and STR during spring and summer (Valeanu *et al.*, 2015). Furthermore, in various breeds of *Bos taurus,,* ejaculate volume and total sperm were different among seasons in Holstein, Brown Swiss, Limousin and Charolais breeds (Snoj *et al.*, 2013). The ejaculate volume and total sperm number were highest in summer followed by spring, autumn, and winter respectively, although sperm concentration was similar among seasons (Snoj *et al.*, 2013). In Thailand, effects of season on sperm quality were reported in different species. In swamp buffalo, %DFI varied among seasons and the year of collection, being lower in the rainy season than in winter or summer (Koonjaenak *et al.*, 2007c). Plasma membrane integrity and stability in Thailand were higher in winter than in the rainy season or summer. The VSL, VAP, and VCL were higher in the rainy season than in winter or summer, while ALH was higher in summer (Koonjaenak *et al.*, 2007d). In boars, a seasonal effect was seen in sperm morphology, sperm volume and total sperm production, with high temperatures and high humidity having negative effects on sperm quality (Suriyasomboon *et al.*, 2005; Suriyasomboon *et al.*, 2004).

Our study (Study IV) evaluated the seasonal effect in two *Bos indicus* breeds which were kept in an open barn; season had a marginal effect on sperm quality. Our findings (Study IV) suggest that season had an effect on sperm morphology, with ejaculates collected in winter having a higher proportion of normal spermatozoa than in the rainy season or summer. Nevertheless, no effect of season was found on sperm kinematics, sperm viability, %DFI or MMP. These results indicated that these bulls were well adapted to the local climate, with seasons having only a slight effect on sperm morphology. The other parameters of sperm quality were not different between seasons. However, there was considerable variation among individual bulls in %DFI,

mitochondrial membrane potential, and sperm viability. A seasonal effect on sperm quality varies among studies. Factors such as environment, housing, age and breed have been shown to influence sperm quality (Snoj *et al.*, 2013; Suriyasomboon *et al.*, 2004; Brito *et al.*, 2002). It is possible that different climatic factors influence sperm quality in specific cohorts of bulls, depending on the interaction with other factors such as genetics, husbandry, nutrition and semen collection regimens; such interactions should be studied in the future.

5.5 Bovine SP composition

Bovine SP composition, especially protein content, is related to sperm fertilizing ability (Juyena & Stelletta, 2012; Maxwell *et al.*, 2007a). The major components of bovine SP include peptidase proteins, cytokines, enzymes, antioxidants, hormones, ions, sugar and lipid (Juyena & Stelletta, 2012). Variations in specific protein content (Killian *et al.*, 1993) and protein expression (Moura *et al.*, 2006) between high and low fertility bulls have been reported. The initial quality of the ejaculates used for preparing bovine SP in Study III was similar between low fertility and high fertility bulls, although the only measure of post-thaw "sperm quality" was from the fertility index score. No differences in total protein concentration between low and high fertility bulls were found. Nonetheless, the fertility-associated proteins measured in a parallel study (Morrell *et al.*, 2015) indicated that the ratio of phosphorylcholine to heparin-binding proteins for the Holstein bulls was related to their fertility i.e. a low ratio (indicating that the proportion of heparin-binding proteins is increased) was seen in high fertility bulls and vice versa. Bovine SP is involved with sperm quality, sperm functions and fertility (Maxwell *et al.*, 2007a). In Study IV, season had a slight effect on sperm quality. Therefore, potential differences in the composition of bovine SP between seasons should be investigated.

Heparin-binding proteins are believed to be involved with the processes of sperm capacitation and acrosome reaction (Chandonnet *et al.*, 1990). In another study, a strong relationship was observed between calving rates and the concentration of heparin-binding proteins in the semen of beef bulls (Bellin *et al.*, 1994). Phosphorylcholine-binding proteins and heparin-binding proteins have a major function on spermatozoa at fertilization and are involved with immunoregulation in the female reproductive tract (Juyena & Stelletta, 2012; Moura *et al.*, 2007); the proportions of these specific proteins may determine the response of the cells. Heparin-binding proteins from boar SP can stimulate interleukin-6 (IL-6) release, while inhibiting the release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) by porcine endometrial and cervical cells.

Furthermore, they can stimulate IL-6 release but decrease the level of $PGF_{2\alpha}$ by bovine endometrial and cervical cells. These responses are involved in the inflammatory process (Madej *et al.*, 2013). In addition, effect of SP on cytokine production from bovine endometrial epithelial cells in culture following the Study III has been analysed. Higher concentrations of SP stimulated more cytokine production; 4% of bovine SP from low fertility bulls stimulated more transforming growth factor beta (TGF-β1, TGF-β2 and TGF-β3) and IL-8 production than SP from high fertility bulls (Nongbua *et al.*, in preparation).

5.6 Summary

Bovine SP can be separated from spermatozoa in the ejaculate by many methods (Morrell & Rodriguez-Martinez, 2011; Morrell & Rodriguez-Martinez, 2009). Bull sperm quality after colloid centrifugation (SLC) was better than controls, as shown in Study I and IV. The SLC could be useful for enhancing sperm quality and therefore help to reverse the decline in fertility that has been observed over recent decades. Furthermore, improvements in sperm quality after adding various proportions of bovine SP to selected spermatozoa have been reported (Garner *et al.*, 2001; Maxwell *et al.*, 1996). In addition, heterologous recombinant protein with decapacitating activity can enhance ram sperm quality after freezing (Zalazar *et al.*, 2016). Thus, it should be possible to improve sperm quality in frozen semen by manipulating the SP content before freezing: SP could be removed from some samples and added to others, or added from a different species, if necessary.

Moreover, our *in vitro* (Study III) model could be helpful to differentiate the effects of low and high fertility bulls in inseminations. These studies could provide useful information to enable fertility outcomes to be improved. There may be an effect on cell signalling, which is currently being investigated. However, Study II did not show an effect of adding SP from bulls of different fertility on spermatozoa. Other factors affecting bovine SP, such as breed of bull or components of SP such as enzymes, antioxidants and hormones, can affect sperm function; their relationship to field fertility should be investigated. Study III should be extended to investigate other factors e.g. different cattle breeds or species, different concentrations, a time series of exposure to bovine SP, an assessment of cellular and molecular changes and also the components of seminal plasma related to fertility.

6 Conclusions

Based on the results performed in Studies I-IV, the following conclusions can be drawn:

- SLC before cryopreservation selects the most metabolically active bull spermatozoa from the rest of the population in normal ejaculates. The pattern of ROS production may be different in selected spermatozoa than in unselected controls.
- Preparing semen by SLC prior to cryopreservation had a positive effect on post-thaw sperm morphology and sperm kinematics, without adversely affecting other parameters of sperm quality. Thus SLC could be expected to improve fertility.
- Adding 5% bovine SP had a beneficial effect on sperm velocity, independent of bull fertility, but an unfavorable effect on MMP. Moreover, there was a beneficial effect of 5% heterologous SP from high fertility bulls on sperm velocity but a deleterious effect on chromatin integrity.
- Bovine SP had a negative effect on bEEC, in terms of cell number and viability, in both a dose-dependent and fertility–dependent manner, with SP from low fertility bulls having a greater negative effect than SP from high fertility bulls.
- Season had an effect on sperm morphology, with ejaculates collected in winter having a higher proportion of normal spermatozoa than in the rainy season or summer. No effect of season was found on sperm kinematics, sperm viability, %DFI or MMP.

7 Future perspectives

The results from these studies indicated that preparing semen by SLC prior to cryopreservation can enhance bull sperm quality, and could be used for selecting spermatozoa that are free of SP. Adding SP has some effects on sperm quality but has more of an effect on uterine cells in culture, especially when the SP originates from bulls of low fertility. Furthermore, season has some effects on sperm quality but whether this is due to an effect on the spermatozoa themselves or on the composition of SP is unknown. These findings suggest several further lines of investigation.

- The interaction between SLC and season in Sweden should be investigated, as there seems to be such an interaction in Thailand. This needs to be studied if SLC is to be useful for processing bull semen. Moreover, a fertility trial should be conducted in cattle with SLC-selected spermatozoa to determine whether the fertility rates following AI reflect improved sperm quality in the insemination doses.
- The small effects of SP on spermatozoa found in this project may be due to the comparatively narrow range of fertility in the bulls studied and the fact that SP was collected in only one season in Sweden. Therefore, the effects of SP from a wider range of bulls should be evaluated. The relationship between season and SP composition on sperm quality in Thailand are currently under investigation, and this study should be extended to the SP collected in Sweden too. The effect of other factors, such as breed and age of bull, and different husbandry conditions, on the composition of SP between seasons should be studied.
- The effect of SP on bEEC was dramatic. More work needs to be done e.g. to examine the effects of SP on cytokine expression from bEEC in culture. The relevance to the *in vivo* situation should be clarified, given the fact that only a small volume of SP is inseminated in semen and the volume of the cow uterus is large. Our results showed that SP

had a negative effect on bEEC, especially from low fertility bulls. The proportion of SP that can be included in semen doses for AI should be examined, with a view to standardizing the proportion of SP.

- The differences between heterologous and homologous bovine SP are interesting and should be investigated further, particularly since some researchers are suggesting adding SP from other species to semen doses for AI to improve fertility. However, the differences in composition of bovine SP between high and low fertility bulls, such as proteins, enzymes, antioxidants and hormones, should be determined. Such information could be useful for determining the mechanism of action of SP on spermatozoa and in the female. In the future, it might be possible to synthesize the active components from SP to avoid adding unprocessed SP from other animals.
- Field fertility data such as a fertility index for each bull is very useful information for improving bull selecting program and can be used to increase genetic gain in the cattle industry. In Thailand, selection of breeding bulls is based on more limited criteria. A fertility index could be useful to develop the breeding database records in Thailand, to improve cattle production.

Popular science summary

Fertility in inseminated dairy cattle is thought to have declined over the last few decades. Although differences in sperm handling procedures and sperm numbers in insemination doses have changed during this time and may have contributed to the decreased fertility, other factors are likely to be involved. Semen has two major compartments; spermatozoa and a complex fluid component known as seminal plasma (SP). Bovine SP is composed of secretions from the accessory sex glands together with a small volume of fluid from the testis and epididymis. It contains proteins, minerals, electrolytes, hormones and enzymes and supports and stimulates spermatozoa. Furthermore, it is implicated in sperm de-capacitation and in fertilization. Since bovine SP plays a role in activating spermatozoa, the decrease in fertility in dairy cattle may be associated with a reduction in the SP content of semen doses for insemination, or differences in SP composition between high and low fertility bulls.

Centrifugation through colloids has been shown to select the most robust spermatozoa from the ejaculate in several species and to separate them from SP. Thus the effect of removing SP on sperm quality can be studied, as well as the effect of adding back SP, either from the same bull or from a different bull. A technique using only one layer of colloid, known as Single Layer Centrifugation, has been developed to process semen from several species, including bull. The purpose of this project was to study bovine SP from bulls of known fertility, to determine its effects on spermatozoa and on uterine epithelial cells in culture. The thesis is divided into 4 studies.

Study I: Effects of Single Layer Centrifugation (SLC) of bull spermatozoa prior to freezing on post thaw semen characteristics.

Study II: The effect of adding seminal plasma prior to cryopreservation on bull sperm quality after thawing.

Study III: The effect of bovine seminal plasma on bovine endometrial epithelial cells in culture.
Study IV: The effects of season and Single Layer Centrifugation on bull sperm quality in Thailand.

In Study I, we found that SLC had a beneficial on sperm quality, with SLC-selected samples showing higher metabolic activity than controls. Furthermore, in Study IV, the SLC-selected samples contained a higher proportion of spermatozoa with normal morphology and a lower proportion with bent tails than uncentrifuged controls and they showed better kinematics than uncentrifuged controls (Study IV, experiment II). However, sperm viability and chromatin integrity were not different between treatments. In Study II, we found that adding bovine SP had a beneficial effect on sperm velocity, independent of bull fertility, but an unfavorable effect on mitochondrial activity. These effects were dependent on the amount of SP added. Treatment with 5% heterologous bovine SP from high fertility bulls had a beneficial effect on sperm velocity compared to 1% heterologous bovine SP but had a negative effect on sperm DNA. When SP was added to uterine epithelial cells in culture (Study III), we saw that 4% SP from high or low fertility bulls negatively affected cell number and viability, although SP from low fertility bulls had a more adverse effect than SP from high fertility bulls. In Study IV, we found that there were differences in some semen characteristics and sperm morphology among seasons. However, sperm kinematics, viability, DNA damage and mitochondrial activity were not different between seasons. Therefore, the fertility of some bulls may be due to the effects of their SP on the uterus of the inseminated cow as well as on their spermatozoa.

These results suggested that SLC could be used prior to cryopreservation to enhance normal sperm morphology and sperm kinematics in thawed bull sperm samples and could be used to remove SP. Since sperm quality is linked with fertility, it may be possible to improve reproductive efficiency in cattle by processing the semen with SLC. Since the addition of SP affects both spermatozoa and uterine cells in culture, it might be possible to optimize fertility in sperm doses for artificial insemination by adding SP to sperm samples that have had their own SP removed by SLC. However, the effect on fertility *in vivo* remains to be determined. Further studies are also needed to investigate the factors affecting involved in SP composition, such as differences between low and high fertility bulls, the effect of season, the effect of breed and age of bull, and even the interaction of semen extender and SP.

Populärvetenskaplig sammanfattning

Fruktsamheten hos inseminerade mjölkkor anses ha minskat under de senaste årtiondena. Trots att hanteringen av spermieprover och antalet spermier i spermiedoser har förändrats, och detta kan ha bidragit till den försämrade fruktsamheten, är det troligt att andra orsaker är inblandade. Sperma har två huvudsakliga beståndsdelar, dels spermierna, dels en komplex vätska som kallas seminalplasma (SP). Tjurarnas SP består av sekret från accessoriska könskörtlar (exempelvis prostata) samt en mindre volym vätska från testikeln och bitestikeln. Den innehåller proteiner, mineraler, elektrolyter, hormoner och enzymer, och dess funktion är att försörja och stimulera spermierna. Dessutom är den inblandad i olika steg i samband med befruktningen. Eftersom tjurens SP har en roll i aktiveringen av spermierna, så skulle nedgången i fruktsamhet hos mjölkboskap kunna ha ett samband med minskat andel SP i doser för inseminering, eller skillnader i sammansättningen hos SP mellan tjurar med hög eller låg fruktsamhet.

Centrifugering genom kolloider har visats selektera fram de mest robusta spermierna från ejakulat från flera olika arter, dessutom separerar denna centrifugering spermierna från SP. På detta vis kan effekterna på spermiekvaliteten av att avlägsna SP studeras, liksom effekten av att åter tillsätta SP till SP-fria prover. SP kan då komma från samma tjur som spermierna, eller från en annan tjur. En teknik som använder endast ett lager av kolloid, och kallas Single Layer Centrifugation, har utvecklats för att behandla spermier från flera olika arter, inklusive tjur. Syftet med detta projekt var att använda SLC för att studera SP från tjurar med känd fruktsamhet, och bestämma dess effekter på spermier och kulturer av celler från livmoderns epitel. Avhandlingen är indelad i fyra delstudier.

Studie I: Vilka effekterna på spermiers karakteristika efter upptining blir av att använda Single Layer Centrifugation (SLC) på tjurspermier innan nedfrysning.

Studie II: Effekterna av att tillsätta SP innan nedfrysning på tjurspermiernas kvalitet efter upptining.

Studie III: Effekterna av tillsats av SP till kulturer av bovina epitelceller från endometriet.

Studie IV: Vilka effekterna av årstid och SLC är på kvaliteten hos tjurspermieprover från Thailand.

I Studie I fann vi att SLC hade en fördelaktig effekt på spermiekvalitet, på så vis att SLC-proverna visade en högre metabolisk aktivitet än kontrollerna. Dessutom visade vi i Studie IV att SLC-selekterade prover innehöll en högre andel spermier med normal morfologi, och en lägre andel med svansböjning än ocentrifugerade kontroller. Dessutom visade de ett bättre rörelsemönster än de ocentrifugerade kontrollerna (Studie IV, experiment II). Dock skilde sig inte andelen levande spermier eller andelen spermier med skadad arvsmassa mellan behandlingarna. I Studie II fann vi att tillsats av bovin SP hade en fördelaktig effekt på spermiernas hastighet, oberoende av tjurens fruktsamhet, men en ofördelaktig effekt på mitokondrieaktiviteten. Dessa effekter var beroende av mängden SP som tillsats. Behandling med 5% heterolog bovin SP från tjurar med hög fruktsamhet hade en fördelaktig effekt på spermiehastigheten jämfört med 1% heterolog bovin SP, men hade en negativ effekt på spermiernas arvsmassa. När SP tillsattes till kulturer av livmoderepitelceller (Studie III) såg vi att tillsats av 4% SP från tjurar med hög eller låg fruktsamhet hade en negativ effekt på antalet celler och deras överlevnad, dock gav SP från tjurar med låg fruktsamhet en starkare negativ effekt än SP från tjurar med hög fruktsamhet. I Studie IV fann vi att det fanns en del skillnader i spermans karakteristika och spermiernas morfologi mellan årstiderna. Dock fanns inga skillnader mellan årstiderna i spermiernas rörelsemönster, överlevnad, kvalitet hos arvsmassan eller mitokondrieaktivitet. Därför kan fruktsamheten hos en del tjurar bero på vilken effekt deras SP har på livmodern lika väl som på deras spermier.

Dessa resultat tydde på att SLC kan användas innan nedfrysning för att förbättra morfologin och rörelsemönstret hos tjurspermieprover efter upptining, och att SLC kan användas för att ta bort SP från prover. Eftersom spermiekvalitet är kopplat till fruktsamhet, kan det vara möjligt att höja reproduktionens effektivitet genom att behandla spermieproverna med SLC. Eftersom tillsats av SP påverkar både spermier och kulturer av livmoderceller, kan det vara möjligt att optimera fruktsamheten hos spermiedoser genom att tillsätta SP till spermieprover där egen SP tagits bort med SLC. Dock återstår

att fastställa fruktsamheten *in vivo*. Dessutom krävs framtida studier för att fastställa faktorerna som är inblandade i innehållet i SP, som skillnader mellan tjurar med låg och hög fruktsamhet, effekten av årstid, påverkan av ras och ålder hos tjuren, och till och med samspelet mellan spädningsvätska och SP.

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