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Abstract

Today breeding through artificial insemination (AI) with cryopreserved semen is frequently used in several domestic species, but its use is more problematic in horses, partly because of reduced sperm quality and partly because the timing of insemination relative to ovulation is more crucial for frozen semen than for fresh. Therefore, improvements in the freezing technique are needed. In this study, the effect of adding pooled seminal plasma (SP) from stallions with good freezability (Good freezer stallion; GF) and bad freezability (Bad freezer stallion; BF) to spermatozoa selected by single layer centrifugation from GF or BF stallions prior to cryopreservation was investigated. Of particular interest was whether SP can have a positive impact on sperm acrosome status, thereby enhancing fertility.

Frozen semen from 12 stallions was available. Aliquots of 5 ml from each ejaculate through normal centrifugation and then frozen were used as controls (C). The remaining parts of the ejaculates were processed by single layer centrifugation (SLC) to remove SP and divided into three aliquots; sample without SP addition (S), sample with SP from GF (S-GF) and sample with SP from BF (S-BF). After thawing, the proportion of live or dead spermatozoa in the sample and the proportion of spermatozoa with an intact or reacted acrosome were evaluated by flow cytometry.

The results indicate that the source of the SP (GF or BF) and the origin of the spermatozoa (GF or BF) were important. There was a positive effect of adding SP from GF to SLC-selected spermatozoa ($p = 0.0017$) compared to the control sample. If the sperm samples were divided according to whether they came from good freezer stallions or bad freezer stallions, the effect of adding SP was most marked when the spermatozoa came from a BF stallion ($P = 0.04$). In other words, the effect of adding SP to sperm samples is due to both the SP and the spermatozoa themselves. No statistical differences were seen between the different treatments (S, S-GF and S-BF) of SLC-selected samples. To draw more reliable conclusions more studies are needed. The study also confirms SLC as a reliable processing method for increasing sperm survival during freezing.

Keywords: acrosome, cryopreservation, artificial insemination, seminal plasma, spermatozoa, equine, stallion, “good freezer”, “bad freezer”, acrosome status, capacitation

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Abbreviations

AI	Artificial insemination
BF	Bad freezer stallion
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CL	Corpus luteum
DAI	Dead acrosome intact
DAR	Dead acrosome reacted
DNA	Deoxyribonucleic acid
FITC-PNA	Fluorescein isothiocyanate conjugated peanut agglutinin
FSH	Follicle stimulating hormone
GF	Good freezer stallion
GnRH	Gonadotropin-releasing hormone
HCO_3^-	Bicarbonate
IP_3	Inositol triphosphate
LAI	Live acrosome intact
LAR	Live acrosome reacted
LH	Luteinizing hormone
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
SACY	Soluble adenylyl cyclase
S-BF	Sample processed by SLC with addition of SP from BF
S-GF	Sample processed by SLC with addition of SP from GF
SLC	Single layer centrifugation
SNARE	N-ethylenmaleimide-sensitive factor activating protein receptor
SP	Seminal plasma
True LAR	True live acrosome reacted

1. Introduction

Breeding through artificial insemination (AI) with frozen semen is frequently used in several livestock species. However, there are some difficulties with using frozen semen for AI in equine breeding. As described by Al-Essawe *et al.* (2018), the stallions are not chosen on the basis of quality of semen nor the ability of the spermatozoa to survive cryopreservation. Instead they are often selected based on their performance in competition. Cryopreservation has a detrimental effect on stallion sperm quality, which is further exacerbated by differences between individual stallions (Vidament *et al.* 1997). In addition, when breeding with thawed semen the insemination must take place closer to ovulation due to shorter life span of the spermatozoon (Samper 2001), which can be problematic to achieve.

According to Loomis (2001) frozen semen can replace the use of cooled semen if the breeders can be confident that they can obtain the same fertility at the same costs as when using cooled semen. They also point out some of the major advantages with frozen semen, which are that it is easier to transport semen than live animals, including to other countries, and the availability of the semen is ensured even when the stallion is not available due, for example, to traveling away to take part in competition, or because of illness, injuries or death.

In an earlier project, the potential impact of seminal plasma (SP) on the ability of stallion spermatozoa to survive cryopreservation was studied (Al-Essawe *et al.* 2018). They added SP from stallions whose spermatozoa survived freezing well, i.e. having good motility after thawing, (good freezer stallion; GF), or seminal plasma from stallions whose spermatozoa showed bad motility after freezing and thawing (bad freezer stallion; BF). They concluded that addition of SP did not have an impact on the ability of the spermatozoa to survive cryopreservation. However, the acrosome status of the spermatozoa was not evaluated in their study. The acrosome reaction is a crucial part of the fertilization process. The reaction is essential to enable the spermatozoa to bind to the oocyte. During the acrosome reaction the membrane of the spermatozoa fuses with the underlying acrosomal membrane (Gadella *et al.* 2001). The enzyme-rich content of the acrosome is then released causing a dissolving of the zona pellucida allowing the spermatozoon to enter the oocyte.

This study is a sequel to the one made by Al-Essawe *et al.* (2018), using the same batches of frozen semen, which had been stored in liquid nitrogen. The objective of this study was to evaluate if seminal plasma can have a positive impact on the acrosome status of cryopreserved stallion spermatozoa.

2. Literature review

2.1. Equine history

The horse belongs to the family *Equidae.* The evolution of the horse has happened over millions of years. The earliest generally recognized ancestor, known as *Eohippus* existed approximately 65 million years ago during the Eocene Epoch and originates from North America. The *Eohippus* was small, about the size of a fox (MacFadden 1994, cited by Goodwin 2007), it had an arched back, raised hind-quarters, four toes on the front feet and three on the hind limbs which ended in padded feet (Encyclopedia Britannica 2020b). Based on the size and shape of the cranium, the *Eohippus* had a much smaller and less complex brain than the modern horse.

Later during the Oligocene Epoch, the *Eohippus* evolved to the *Mesohippus* and the *Miohippus*; they were the size of a large dog and ran on three toes (MacFadden 1994, cited by Goodwin 2007). By the time of the Miocene period the *Parahippus* appeared. The *Parahippus* had feet and teeth adapted for a life as a grazer on the plains. Its lateral digits still had pads, but only touched the ground when traveling on soft earth. When evolving to the *Pliohippus* the lateral digits were lost completely, the metacarpals was left as long thin remnants and later in its descendents, the *Equus*, they were reduced to only short splint bones by the sides of the third metacarpal bone.

During evolution, the horse has thus increased in size, lost its footpads, the independent bones of the lower legs have fused and the brain has increased in size and complexity to what we today call the modern horse (Encyclopedia Britannica 2020b).

Throughout history, the horse has been used for many different purposes such as food, agriculture, transportation, warfare, etc. The horse is an effective mover and can run shortly after eating, in contrast to, for example, the cow that has to rest, lie down, regurgitate and remasticate the food.

2.2. The reproduction of the horse

The horse is a seasonal polyestrous animal which means that during the breeding season they have multiple recurring estrous cycles if not pregnant (Brinsko & Blanchard 2011). The estrous cycle lasts approximately 21 days. The cycle begins with a follicular phase (estrus). During this phase the mare accepts mounting by the stallion, the genital tract allows spermatozoa to enter, and ovulation takes place. The spermatozoa can then reach the oviducts. The estrus phase is followed by a luteal phase, diestrus, leading to regression of the corpus luteum, which initiates a new follicular phase.

The cycle is controlled by a delicate balance between hormones (Brinsko & Blanchard 2011) and is stimulated by daylight. During the spring and summer more light hits the retina of the eye signaling to the pineal gland to produce less melatonin. The hypothalamus then increases its production of gonadotropin-releasing hormone (GnRH), thereby stimulating the pituitary gland to release follicle stimulating hormone (FSH) and luteinizing hormone (LH) which in turn stimulate follicular recruitment, maturation, ovulation and luteinization.

During the growth process, the follicles produces inhibin and estrogen (Brinsko & Blanchard 2011). Inhibin has a negative feedback on FSH, whereas increased concentrations of estrogen result in behavioral estrus. Usually only one follicle, the largest, ovulates and initiate the luteal phase, which consist of the formation of a corpus luteum (CL) in the ovary and thereby the production of progesterone.

As described by Sendel (2010) the CL is functional for approximately 12-14 days, if the oocyte is not fertilized. During this time the endometrium will release prostaglandin, which has a luteolytic effect. If the oocyte becomes fertilized, the migration of the growing embryo will stimulate pregnancy recognition and inhibit the release of prostaglandin. The CL will then remain functional for 25 to 30 days before regression starts. In the meantime, a girdle-like band of special cells forms around the fetal sac, the endometrial cups, which produce a hormone called equine chorionic gonadotropin. This hormone stimulates a secondary follicular development and luteinization via the bloodstream, forming a second corpus luteum, which is active for 130-150 days. Thereafter, special areas of the uterus and the fetal membranes produce progesterone to maintain the pregnancy. The pregnancy lasts approximately 340 ± 20 days post-breeding.

2.3. Artificial insemination

The Italian physiologist Lazzaro Spallanzani was the first person to develop a method to artificially inseminate a dog in 1780 (Encyclopedia Britannica 2020a). The technique was later refined during the 1930s in Russia.

Artificial insemination is the process whereby semen is collected from the male animal, diluted and stored, often deep-frozen or cooled until it is needed for transfer to a female (Encyclopedia Britannica 2020a). When stored deep-frozen it can

maintain its fertility for long periods of time. By virtue of artificial insemination, threatened or endangered animals can be impregnated.

Stallions used for breeding through artificial insemination are not selected based on the quality of the semen, nor the ability of the spermatozoa to survive cryopreservation. Often they are selected based only on their performance in competition (Al-Essawe *et al.* 2018). Therefore, the quality of frozen semen samples can vary widely between ejaculates or between individuals.

2.4. Cryopreservation

Unprotected freezing of intact living cells is normally lethal (Pegg 2007). A way to preserve structurally intact living cells and tissues is by cryopreservation where very low temperatures are used in combination with the addition of cryoprotectants.

When cells are frozen in a solution, the concentration of the extracellular solution increases, causing a disruption of the chemical potential of the water inside and outside the cells (Duncan 2020). Two mechanisms of cell injury occur: intracellular fluid streams out of the cell by osmosis, causing it to die of dehydration, or intracellular ice crystals form, which injure the cell. Both of these causes of injury can lead to cell death during cryopreservation.

The formation of intracellular ice crystals can be prevented by adding a cryoprotectant (Thompson 2012). There are two main classes of cryoprotectants, intracellular agents and extracellular agents. The first class penetrates the cell and prevents the formation of ice crystals and thereby prevents rupture of the cell membrane. Example of intracellular agents are dimethyl sulfoxide, glycerol and polyethylene glycol. The second class of cryoprotectants improves the osmotic imbalance which occurs during freezing. Example of extracellular agents are sucrose, trehalose and dextrose. Noteworthy though is that most cryoprotectants, for example glycerol, are toxic to the cells in high concentration (Duncan 2020).

Vitrification could be an alternative method (Youngs 2011). It is a method where the solution is cooled ultra-rapidly to prevent the formation of intracellular ice crystals. The solution passes from a liquid directly to a 'glassy' state. O'Neill *et al.* (2019) studied a special protocol for vitrification and compared it with conventional freezing of human spermatozoa. Their results showed that the vitrification method significantly improved the quality of the spermatozoa regarding motility, progressive motility, DNA integrity and gave a lower fragmentation index. However more studies are needed; the technique required extensive training in management of the vitrification device, and each operator can only handle one sample at the time. It remains to be seen whether the technique can be transferred to other species as well.

Although there are several major advantages of AI, there is one major disadvantage with using frozen semen compared to cooled or fresh semen, which is reduced fertility (Moore *et al.* 2005).

2.5. The formation of the spermatozoa

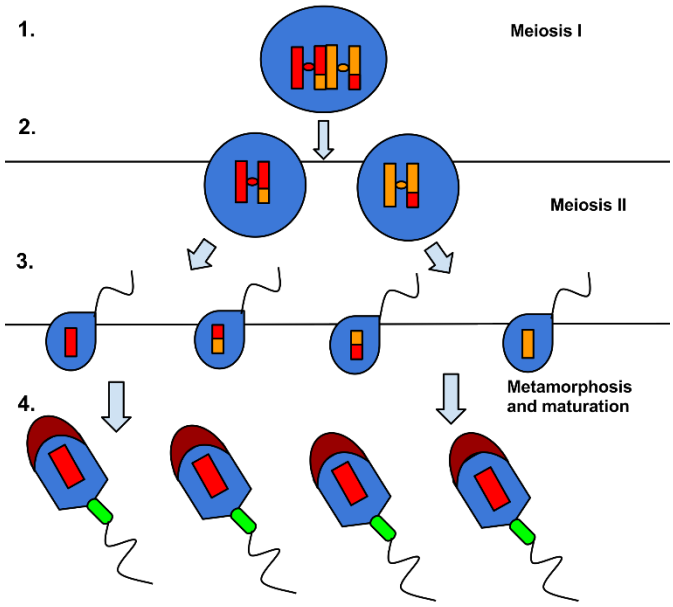

Figure 1: "File:Spermatogenesis.svg" by Anchor207 is licensed under CC BY-SA 3.0.

2.5.1. Spermatogenesis

Production of male spermatozoa (*see Figure 1*) occurs in the testis through a complex process called spermatogenesis (de Kretser *et al.* 1998). This process can be divided into three steps (Fayrer-Hosken 1997; de Kretser *et al.* 1998). In the first step, some of the spermatogonia enter the luminal compartment of the testis and multiply through a process called mitosis to form primary spermatocytes, B-spermatocytes; some of these will revert back to stem cells to maintain a reservoir of progenitor cells. In the second step, the B-spermatocyte undergoes meiotic division, reduces its chromosome number from diploid to haploid, and the daughter cells become secondary spermatocytes. They continue to divide and differentiate to form haploid, round spermatids. The third and last phase is the differentiation phase, spermiogenesis. Defects in any of these three steps can results in defects in, or absence of, spermatozoa (de Kretser *et al.* 1998).

2.5.2. Spermiogenesis

The round spermatids differentiate into spermatozoa during this phase (de Kretser *et al.* 1998). The nucleus moves to the periphery of the cell and the deoxyribonucleic acid (DNA) becomes condensed. Moreover, the modified lysosome, also known as the acrosome, forms and attaches to the surface of the nucleus and cell membrane. The flagella forms from one of the centrioles of the round spermatid; this includes the development of a core of microtubules. Eventually, the spermatid loses most of its cytoplasm and the remnant forms a cytoplasmic drop on the midpiece. The spermatozoon now has a head with an acrosome, a midpiece con-

taining a mitochondrial helix, and a tail with microtubules, and is released to the luminal content (Fayrer-Hosken 1997).

The spermatozoa undergo further maturation in the epididymis, becoming fully mature as they progress along this organ, but are still not fully ready to fertilize the oocyte directly after ejaculation (Yanagimachi & Knobil 1995, cited by Breitbart 2002a). They require some time in the female reproductive tract before acquiring the ability to fertilize the oocyte, which is termed capacitation.

2.6. The capacitation process

The capacitation takes place in the female genital tract (Gadella *et al.* 2001). It is a series of cellular and molecular changes the spermatozoa have to undergo before the spermatozoa becomes fertile.

Spermatozoa stored in the epididymis are immotile; when in contact with seminal fluid the flagellum starts to beat vigorously (Visconti 2009). The seminal fluid is an isotonic solution containing bicarbonate (HCO_3^-) and calcium (Ca^{2+}) in high concentrations. The first part of the capacitation process starts when HCO_3^- and Ca^{2+} start to move across the membrane leading to an increased intracellular pH and stimulation of a unique type of soluble adenylyl cyclase (SACY), which regulates Cyclic adenosine monophosphate (cAMP) metabolism. In turn, the increased levels of cAMP leads to an activation of Protein kinase A (PKA), which is responsible for the phosphorylation of several target proteins and initiates several signaling pathways leading to hyperactivated spermatozoa. Simultaneously the increased levels of cAMP, intracellular HCO_3^- and Ca^{2+} will lead to depletion of cholesterol, a surface-adhered decapacitation factor, from the plasma membrane of the spermatozoa, removal of seminal plasma and activation of intracellular pathways (Leemans *et al.* 2016). This is followed by aggregation of lipid-ordered microdomains at the apical part of the sperm head, which will function as a zona pellucida binding protein complex.

The capacitation process is essential for the spermatozoon to detach from the epithelial cell in the oviduct and to leave the oviductal reservoir, for transport through the oviduct lumen and for the ability to enter the oocyte (Leemans *et al.* 2016). As Visconti (2009) points out, some parts of the capacitation process require longer incubation periods than others, but the process is not yet fully understood.

Tremoleda *et al.* (2003) observed that stallion spermatozoa incubated under typical IVF conditions can bind to the zona pellucida but cannot penetrate the mature oocyte, either *in vitro* or *in vivo.* They suggest that a failure in the capacitation process results in an inability to undergo the acrosome reaction. Exposure to progesterone and prostaglandin E_1 does not give rise to increasing levels of Ca^{2+} and hyperactivation as it does in other mammalian species (Leemans *et al.* 2016).

2.7. The acrosome reaction

The acrosome forms a cap over the anterior part of the spermatozoa (*see figure 1*) and is considered to consist of an inner and an outer membrane (Allen & Green 1997). Before the spermatozoon can penetrate the zona pellucida it has to undergo the acrosome reaction. During this process, the outer acrosomal membrane and the overlying plasma membrane of the spermatozoon fuse (Gadella *et al.* 2001; Breitbart 2002a), the fusion is calcium-dependent and regulated through the formation of soluble N-ethylenmaleimide-sensitive factor activating protein receptor (SNARE) (Bernecic *et al.* 2019). In turn, hydrolytic enzymes are released to digest the zona pellucida (Allen & Green 1997, cited by Breitbart 2002b). This creates a groove about the size of the head of the spermatozoa.

According to Breitbart (2002b), the concentration of calcium is regulated through the plasma membrane, intracellular Ca^{2+} stores and the acrosome. Only spermatozoa that have undergone capacitation are able to perform the acrosome reaction. During the capacitation process, protein kinase A (PKA) is activated which also opens a calcium channel in the outer acrosomal membrane. The increased levels of cytosolic calcium activate phospholipase C which is coupled with the second tyrosine kinase receptor. As a result of the phospholipase C activity, diacylglycerol and inositol triphosphate (IP_3) are produced leading to activation of protein kinase C (PKC) and IP_3-receptor which will open calcium channels in the plasma membrane and the outer acrosomal membrane. The increased levels of cytosolic calcium will also activate a store-operated Ca^{2+} channel within the acrosome. The rapidly increasing levels of cytosolic calcium will lead to membrane fusion and thereby complete the acrosome reaction.

A spermatozoon that has acrosome-reacted properly will penetrate the zona pellucida and enter the perivitelline space, bind to the egg plasma membrane (oolemma), and become incorporated into the oocyte (Gadella *et al.* 2001). The oocyte will, in turn, activate and initiate an effective block to prevent polyspermy.

To mimic the in *vivo* situation in *vitro*, the acrosome reaction can be induced with different chemicals. Alm (1999) compared the penetration and fertilization rate of spermatozoa treated with calcium-ionophore and heparin and saw a slightly higher penetration and fertilization rate when using calcium-ionophore. However, treatment after cryopreservation did not lead to any penetration or fertilization at all. In addition, Alm (1999) saw that frozen/thawed samples showed an increase in the proportion of acrosome reacted spermatozoa after treatment with Calcium-ionophore.

Capacitated spermatozoa have a destabilized plasma membrane which is sensitive to environmental stresses (Gadella *et al.* 2001). Exposure to calcium-ionophore in the presence of Ca^{2+} will therefore induce the fusion of the plasma membrane with the underlying acrosome membrane of capacitated spermatozoa.

2.8. Seminal plasma

Seminal plasma (SP) contains several proteins, enzymes, electrolytes and trace elements (Kareskoski & Katila 2008) and is thought to have both positive and negative effects on spermatozoa (Akcay *et al.* 2006). It contains substances that are beneficial for the transport and survival of the spermatozoa in the female reproductive tract but also contains factors that affect sperm motility negatively and increase sperm death during storage. Therefore, some people advocate removing most of the seminal plasma from stallion spermatozoa for cooled storage. When freezing spermatozoa, most of the SP is removed to decrease the volume and thereby increase sperm concentration. The SP is removed by centrifugation, with up to 5% remaining (Moore *et al.* 2005).

The stallion ejaculate typically consists of six to nine sequential fractions of semen with 70 % of the spermatozoa being in the first three fractions (Kareskoski & Katila 2008). The sex glands release their content in a specific order leading to a high variation in the composition of the SP between the fractions.

Colloid centrifugation (Single Layer Centrifugation, SLC) is a method where the extended semen is layered on top of a colloid in a centrifuge tube and then subjected to gentle centrifugation (Morrell *et al.* 2010). The sperm pellet, containing the most robust spermatozoa, is then transferred to a clean centrifuge tube. In contrast, centrifugation without a colloid typically uses a higher centrifugation force and can damage the spermatozoa. Al-Essawe *et al.* (2018) showed that preparation of the sperm sample by SLC improved sperm chromatin integrity, gave a higher mitochondrial membrane potential, and a reduction in hydrogen peroxide production after thawing. This method enables robust spermatozoa to be selected, as well as separating them from seminal plasma. The selected spermatozoa survive cryopreservation better than non-selected spermatozoa.

Aurich *et al.* (1996) observed that membrane integrity and progressive motility were improved significantly when SP from stallions with high post-thaw motility was added to semen from stallions with low post-thaw motility before cryopreservation, and decreased when the conditions were the opposite. However, Moore *et al.* (2005) found that SP did not have an effect on motility and viability if stallion spermatozoa were frozen directly after processing, but it had a detrimental effect if the spermatozoa were incubated for longer periods before freezing in the presence of high levels of SP.

Morrell *et al.* (2012) studied the effect of adding homologous SP back to fresh or cooled spermatozoa selected by SLC. They observed that when adding SP to fresh SLC-selected spermatozoa the progressive motility was significantly improved but the effect was lost if the samples were first stored for 24-36 hours. Adding SP to fresh SLC-selected samples also restored the sperm velocity to its original level from decreased levels in SLC samples. However, if the SLC samples were first stored before adding the SP, velocity was not affected. Furthermore, adding SP to SLC samples led to increased chromatin damage, although the DNA fragmentation index was still lower than in the uncentrifuged samples.

3. Material and methods

3.1. Experimental design

Samples of semen from twelve freezer stallions from a former experiment conducted by Al-Essawe *et al.* (2018) were used.

3.1.1. Collection and preparation of samples in earlier projects

For more detailed information regarding the husbandry of the stallions, selection of stallions, collection of semen and preparation before cryopreservation see the article "Improved cryosurvival of stallion spermatozoa after colloid centrifugation is independent of the addition of seminal plasma*"* (Al-Essawe *et al.* 2018).

Classification based on motility

A week after freezing, the total and progressive motility were evaluated using the AndroVision (Al-Essawe *et al.* 2018). The semen was classified as "good freezers" (GF) or "bad freezers" (BF) based on the motility after thawing.

Table 1: Classification of stallions as good or bad freezers according to sperm motility.

	Total motility	**Progressive motility**
"Good freezers"	≥60%	≥40
"Bad freezers"	≤50%	≤30

Description of the samples

Al-Essawe *et al.* (2018) confirmed six of the twelve stallions as "good freezers stallions" (GF) and six as "bad freezers stallion" (BF). They collected semen samples twice a week from January to February in 2015, 3-4 ejaculates per stallion.

From each ejaculate, an aliquot was removed as a control (C) and was prepared for freezing using the stud´s standard protocol. The rest of the ejaculate was centrifuged through a colloid (SLC), the sperm pellet was resuspended in cryoextender and was divided into three aliquots. To the first aliquot (S-GF), a small portion of pooled seminal plasma was added from GF, and to the second aliquot (S-BF) seminal plasma from BF was added. The final seminal plasma concentration was 5%. The

third aliquot (S) was frozen without seminal plasma. The samples were then frozen and stored in liquid nitrogen.

Additional ejaculates were used to prepare seminal plasma. The ejaculate was centrifuged without a colloid, using high centrifugation force to enable seminal plasma free of spermatozoa to be harvested. The seminal plasma was then frozen and stored in liquid nitrogen.

Table 2: Sample description.

Sample	Treatments
C	Semen prepared according to the stud´s usual protocol
S	SLC-selected spermatozoa without SP
S-BF	SLC-selected spermatozoa mixed with 5 % pooled SP from six stallions classified as BF
S-GF	SLC-selected spermatozoa mixed with 5 % pooled SP from six stallions classified as GF

3.1.2. Preparation and evaluation of samples in this project

Preparation of medium

Modified Whitten´s medium (MW) was prepared (see table below), divided into aliquots of 4 mL, and then stored frozen until used.

Table 3: Modified Whitten´s medium.

Component	MW g/mol	mM	Amount/100mL	In g
KCl	74.56	4.7	35.04 mg	0.035
$MgCl_2.6H_2O$	203.3	1.2	24.39 mg	0.024
Glucose (anhydrous)	180.2	5.5	99.11 mg	0.099
NaCl	58.44	100.0	584.4 mg	0.584
Pyruvatic acid	110.0	1.0	11 mg	0.011
Sodium lactate	112.1	2.4	26.89 mg	0.027 (45 µl)
HEPES (free acid)	238.3	22.0	524.26 mg	0.524
$CaCl_2.2H_20$	147.02	2.4	35.28 mg	0.035
Distilled water			100 ml	

At the time of analysis, the medium was thawed and 7 mg/mL of bovine serum albumin (BSA) was added at room temperature. The osmolarity was measured using a Micro-Osmometer (Labex, Helsingborg, Sweden) and adjusted to 290-310 mOsm/kg by adding NaCl. Thereafter 2.1 mg/mL of $NaHCO_3$ was added and the pH was adjusted to be approximately 7.1.

Thawing

The different sperm samples, C, S, S-GF and S-BF, from the same ejaculate were thawed in a 37 °C water bath for 30 seconds. A mixture of 300 µL of the medium, 1 µL of Calcium- Ionophore (5 mg/mL) and 3 µL of each sample were prepared in double setup, in total eight test tubes per stallion and ejaculate.

Staining

One set of non-stimulated samples was stained with 5 µL of Hoechst 33342 (5 µg/mL stock solution) (ThermoFisher, Waltham, Massachusetts), 5 µL of fluorescein isothiocyanate-peanut agglutinin (FITC-PNA) (1 mg/mL) (Sigma-Aldrich, St. Louise, Missouri), and 2.5 µL of Propidium iodide (PI) (2.4 mM, ThermoFisher, Waltham, Massachusetts and thereafter incubated at 37 °C in dark with 5 % CO_2 for 30 minutes and evaluated by flow cytometry.

Hoechst 33342 can permeate the cell membrane and thereby stain all cells (Chazotte 2011). It is excited by ultraviolet or violet light with peak excitation at 350 nm and emits blue fluorescence light, with peak emission at 461 nm. Acrosomal leakage can be identified by using fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) as a marker (Rathi *et al.* 2001). It has peak excitation at 490 nm and provides a green-fluorescent emission at 525 nm. Propidium iodide (PI) can be used as a marker for identifying non-viable cells i.e. dead cells (Rathi *et al.* 2001). It has an excitation max at 535 nm and max emission is at 617 nm.

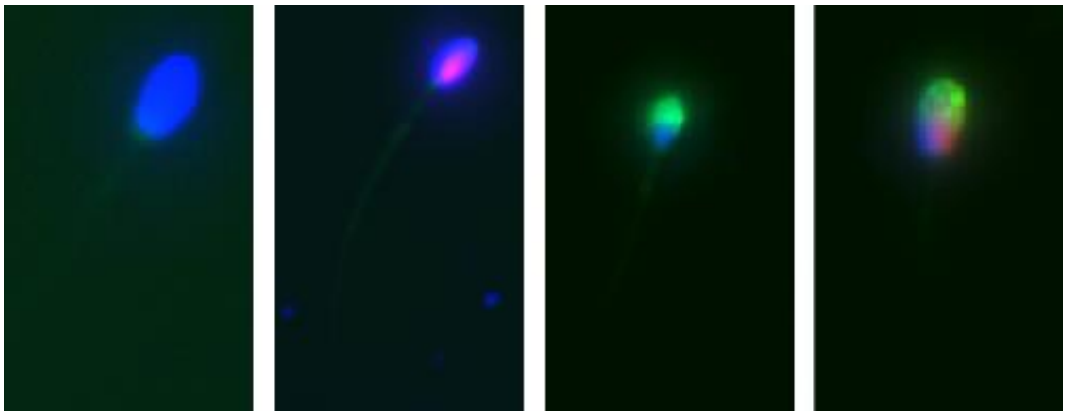

Figure 2: From left to right are as follow: a live spermatozoon with intact acrosome (LAI), a dead spermatozoon with intact acrosome (DAI), a live spermatozoon with a reacted acrosome (LAR) and, a dead spermatozoon with a reacted acrosome (DAR).

The second set of tubes (stimulated samples) was first incubated for 45 minutes at 37 °C in the dark with 5 % CO_2 and then stained in the same way as the first set of tubes and incubated for 30 minutes before being evaluated by flow cytometry.

Analysis by flow cytometry

The spermatozoa were assessed using a blue laser (488 nm) to induce excitation. A fluorescence channel (FL) 1 bandpass filter (527/32 nm) detected green fluorescence from FITC-PNA and a FL 3 band-pass filter (700/54 nm) was used to detect red fluorescence from PI. Blue Hoechst 33342 fluorescence (FL5) was excited with a violet 405 nm laser and collected by using a 528/45 nm band-pass filter. In total

100 000 cells were assessed and classified as described below (Kumaresan *et al.* 2017).

The cells were then divided into four types of cell populations/categories:

1. Living, acrosome intact (LAI)
2. Living, acrosome reacted (LAR)
3. Dead, acrosome intact (DAI)
4. Dead, acrosome reacted (DAR)

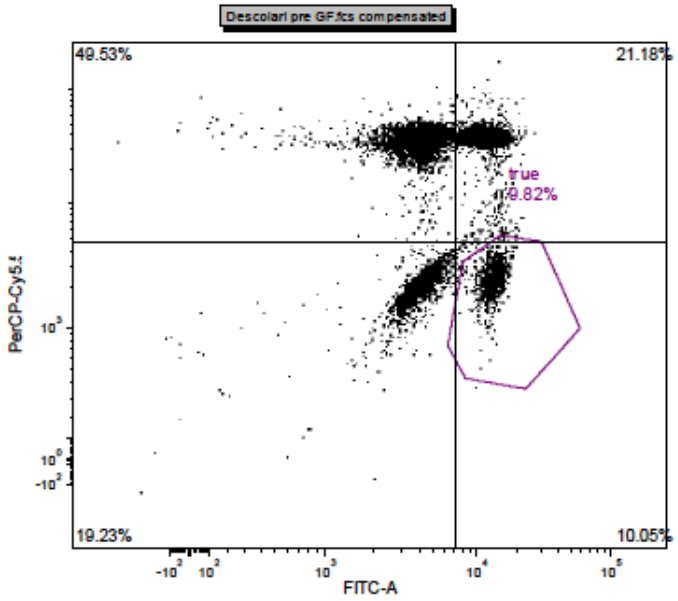

Figure 3: Example of a dot-plot generated by flow cytometry. Represented on the x-axis is green fluorescence from FITC-PNA and on the y-axis, red fluorescence from PI. The sample was gated to include only spermatozoa and cells with blue fluorescence. On the left DAI (dead, acrosome intact, upper quadrant) and LAI (living, acrosome intact, lower quadrant). On the right DAR (dead, acrosome reacted, upper quadrant) and LAR (living, acrosome reacted, lower quadrant). The violet region shows true LAR, an attempt to better define the live acrosome-reacted spermatozoa by a polygon gate.

3.2. Statistical analyses

All statistical analyses were performed using SAS® studio software version 9.4. The data were first tested for normality with Shapiro-Wilks test. Variables with non-normal distribution were subjected to logarithmic transformation to achieve normal or close to normal distribution. Levine´s test was used to evaluate the homogeneity of variances. Means were compared by Mixed Model ANOVA. The effect of incubation with calcium ionophore (stimulated) and freezability were tested by Mixed Model ANOVA.

The statistical model included the fixed effects of treatments (C, S, S-BF and S-GF) and freezability (BF and GF); in addition, treatment *freezability interaction and the random effect of individual stallion (n = 12) were included. The effects of pre-incubation (stimulated) and freezability were tested by Mixed Model ANOVA included the fixed effects of treatments (C, S, S-BF, S-GF), the stimulated status (pre-incubated and non-incubated) and treatment *stimulated interaction, the random effects of individual stallions were also included. Tukey Post-Hoc adjustment was applied. A P-value ≤ 0.05 was considered significant. A P-value between 0.05 and 0.1 was considered as showing a tendency to be different.

4. Results

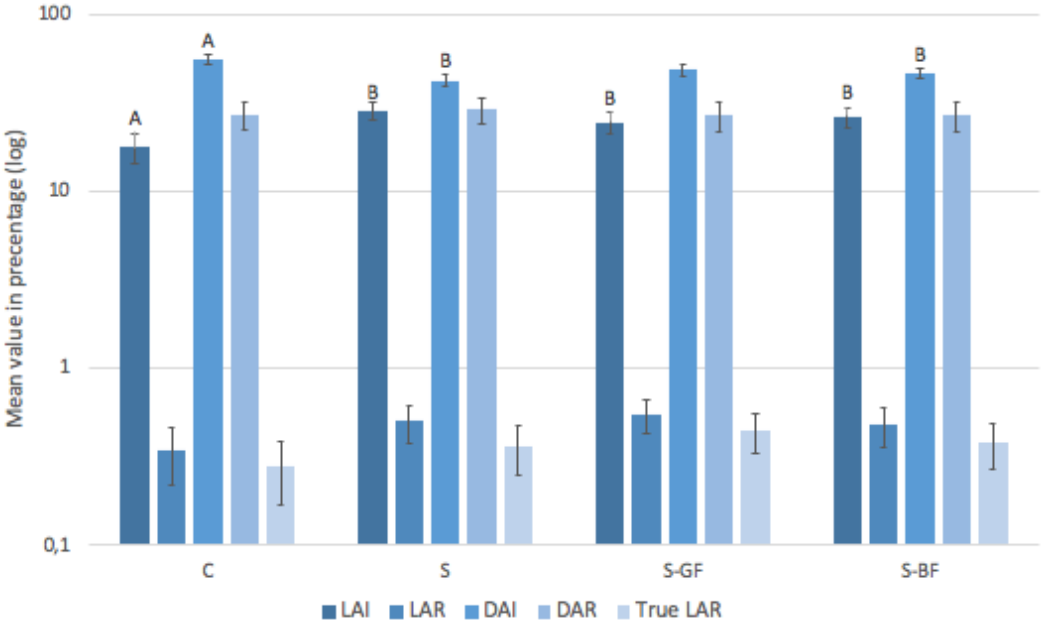

Figure 4: The effects of adding 5 % seminal plasma from bad or good freezer stallion prior to cryopreservation on acrosome integrity after thawing (non-stimulated samples). Mean values ± SEM, n = 12 stallions. Different capital letters (A/B) within the same category indicate significant differences.

For the non-stimulated samples there was a significant difference between C and S, S-BF or S-GF. The C samples had fewer live cells with intact acrosome (LAI) than S (P = 0.0014), S-GF (P = 0.0186) and S-BF (P = 0.0065). In addition, C had significantly more dead cells with intact acrosome (DAI) than S (P = 0.0001), and S-BF (P = 0.01), with a tendency to have more DAI than the S-GF sample (P = 0.065). Furthermore, S had a tendency to have fewer DAI than S-GF (P = 0.08) and a tendency to have more live cells with acrosome reacted (LAR) than C (P = 0.06). There were no significant differences between treatments for the categories true live acrosome reacted (True LAR) or dead acrosome reacted (DAR).

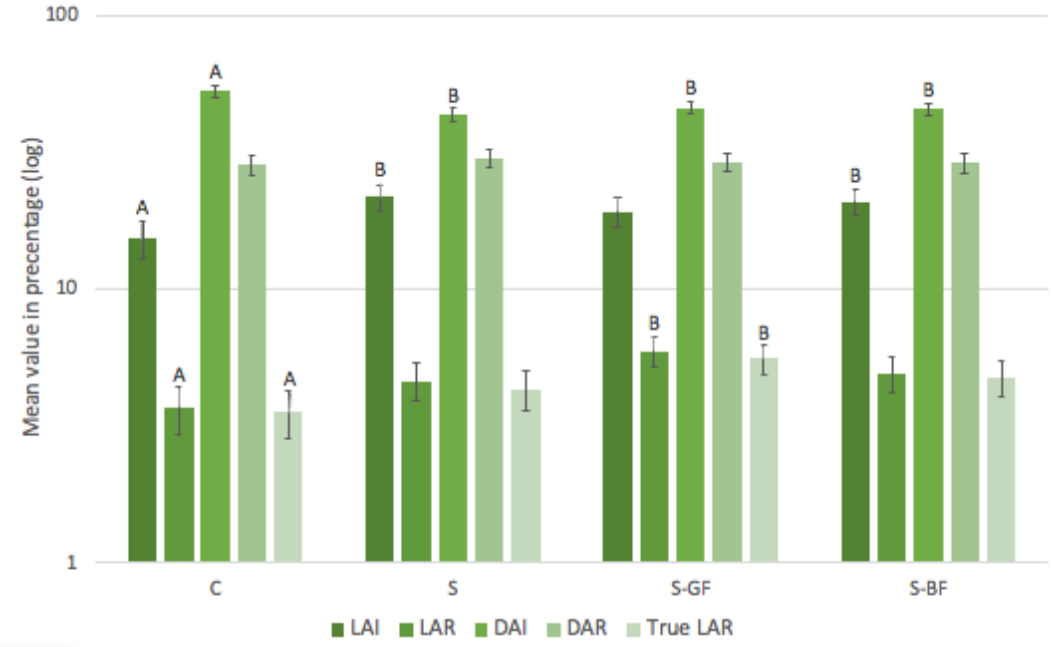

Figure 5: The effects of post thaw incubation with calcium ionophore (stimulated samples) divided based on treatment (C, S, S-BF, S-GF). Mean values ± SEM, n = 12 stallions. Different capital letters (A/B) within the same category indicate significant differences.

There was a significant difference between the stimulated samples. The C samples had significant fewer LAI than S (P = 0.007), or S-BF (P = 0.01), and a tendency to fewer LAI than S-GF (P = 0.09). In addition, C had significantly more DAI than S (P = 0.0009), S-BF (P = 0.01) and S-GF (P = 0.016) and there were fewer LAR for C than S-GF (P = 0.017) as well as for the category True LAR (P = 0.018). No significant differences were found between treatments in the category DAR.

Table 4: The effects on post thaw acrosome integrity (non-stimulated samples). The stallions are classified according to their freezability (bad or good) and treatment (C, S, S-GF, S-BF). Mean values ± SEM, n = 12 stallions.

	Bad freezer stallion					Good freezer stallion				
	LAI	**LAR**	**DAI**	**DAR**	**True LAR**	**LAI**	**LAR**	**DAI**	**DAR**	**True LAR**
C	16.13 ± 4.65[A]	0.33 ± 0.18	53.53 ± 4.75[B]	30.52 ± 4.08	0.25 ± 0.15	19.16 ± 4.65	0.35 ± 0.18	56.57 ± 4.75[C]	23.92 ± 4.08	0.30 ± 0.15
S	27.59 ± 4.65[A]	0.49 ± 0.18	41.76 ± 4.75[B]	30.16 ± 4.08	0.36 ± 0.15	29.52 ± 4.65	0.51 ± 0.18	42.57 ± 4.75[C]	27.40 ± 4.08	0.37 ± 0.15
S-GF	22.23 ± 4.65	0.47 ± 0.18	48.69 ± 4.75	28.59 ± 4.08	0.42 ± 0.15	26.52 ± 4.65	0.62 ± 0.18	48.15 ± 4.75	24.71 ± 4.08	0.47 ± 0.15
S-BF	24.79 ± 4.65	0.41 ± 0.18	45.42 ± 4.75	29.39 ± 4.08	0.33 ± 0.15	27.79 ± 4.65	0.56 ± 0.18	47.43 ± 4.75	24.21 ± 4.08	0.43 ± 0.15

Same capital letters within columns indicate significant differences as follow: [A] P = 0.024; [B] P = 0.048; [C] P = 0.01.

Differences were also apparent when sperm samples were classified according to whether they were from a good or bad freezer stallion (table 3). For sperm samples from bad freezer stallions, C had significantly fewer LAI than S (P = 0.024); for LAI there was also a tendency for C to be lower compared to S-BF (P = 0.07). In addition, C had significantly more DAI than S for both bad freezer stallions (P = 0.048) and for good freezer stallions (P = 0.01). For the other categories there were no significant differences.

Table 5: The effects on post thaw incubation (stimulated samples). The stallions are classified according to their freezability (bad or good) and treatment (C, S, S-GF, S-BF). Mean values ± SEM, n = 12 stallions.

	Bad freezer stallion					Good freezer stallion				
	LAI	**LAR**	**DAI**	**DAR**	**True LAR**	**LAI**	**LAR**	**DAI**	**DAR**	**True LAR**
C	12.41 ± 3.32[A]	3.15 ± 1.05[B]	50.29 ± 3.34[C]	34.15 ± 3.11*	2.86 ± 1.01[D]	18.05 ± 3.32	4.24 ± 1.05	55.10 ± 3.34	22.61 ± 3.11*	4.23 ± 1.01
S	20.07 ± 3.32	4.70 ± 1.05	40.96 ± 3.34[C]	34.27 ± 3.11	4.33 ± 1.01	23.37 ± 3.32	4.54 ± 1.05	46.08 ± 3.34	26.05 ± 3.11	4.26 ± 1.01
S-GF	17.58 ± 3.32	6.65 ± 1.05[B]	44.27 ± 3.34	31.50 ± 3.11	6.30 ± 1.01[D]	20.74 ± 3.32	5.22 ± 1.05	47.55 ± 3.34	26.50 ± 3.11	4.86 ± 1.01
S-BF	19.91 ± 3.32[A]	5.33 ± 1.05	43.07 ± 3.34	31.69 ± 3.11	5.20 ± 1.01	21.88 ± 3.32	4.48 ± 1.05	47.65 ± 3.34	25.99 ± 3.11	4.34 ± 1.01

Same superscript letters within columns indicate significant differences as follow: [A] P = 0.047; [B] P = 0.04; [C] P=0.05; [D] P = 0.025. An asterisk (*) shows a difference between good and bad stallions.

Between the stimulated samples, which was incubated with calcium-ionophore for 45 minutes before staining, C from bad freezer stallions tended to have fewer LAI compared to S (P = 0.07), and lower LAI than S-BF (P = 0.047), and had more DAI (P = 0.05). Furthermore, C had fewer LAR compared to S-GF (P = 0.04), and also for the category true LAR (P = 0.025). No significant differences were found among bad freezer stallions for the other categories, or in any categories for good freezer stallions.

For the stimulated samples, when comparing the sperm samples from bad freezer stallions with the corresponding sample from good freezer stallions, the good freezer stallions tended to have fewer DAR than the bad freezer stallions (P = 0.05). No significant differences were found for the other categories.

Table 6: The effects of post thaw incubation (stimulated samples) based on treatment compared with un-stimulated portion on stallion acrosome integrity (non-stimulated samples). Mean values ± SEM, n = 12 stallions.

	Non-stimulated samples					Stimulated samples				
	LAI	**LAR**	**DAI**	**DAR**	**True LAR**	**LAI**	**LAR**	**DAI**	**DAR**	**True LAR**
C	17.76 ± 2.77	0.34 ± 0.52^{A}	55.05 ± 2.82	27.22 ± 2.64	0.28 ± 0.50^{B}	15.23 ± 2.77	3.69 ± 0.52^{A}	52.69 ± 2.82	28.38 ± 2.64	3.55 ± 0.50^{B}
S	28.55 ± 2.77	0.51 ± 0.52^{A}	42.16 ± 2.82	28.78 ± 2.64	0.36 ± 0.50^{B}	21.70 ± 2.77	4.62 ± 0.52^{A}	43.52 ± 2.82	30.16 ± 2.64	4.29 ± 0.50^{B}
S-GF	24.37 ± 2.77	0.55 ± 0.52^{A}	48.42 ± 2.82	26.65 ± 2.64	0.44 ± 0.50^{B}	19.16 ± 2.77	5.93 ± 0.52^{A}	45.91 ± 2.82	29.00 ± 2.64	5.58 ± 0.50^{B}
S-BF	26.29 ± 2.77	0.48 ± 0.52^{A}	46.43 ± 2.82	26.79 ± 2.64	0.38 ± 0.50^{B}	20.89 ±2.77	4.91 ± 0.52^{A}	45.36 ± 2.82	28.84 ± 2.64	4.77 ± 0.50^{B}

Same superscript letters across rows indicate significant differences as follow: $^{AB}P = 0.0001$.

There were significant differences for all treatments for LAR and true LAR with and without stimulation with calcium-ionophore.

5. Discussion

The aim with this study was to evaluate if adding 5 % seminal plasma (SP) from stallions with good or bad freezability prior to cryopreservation can improve the spermatozoon's ability to undergo the acrosome reaction and thereby increase the fertility rates when breeding with frozen semen.

Stimulation with calcium ionophore resulted in more live cells with reacted acrosome than in for the non-stimulated samples. The stimulated samples were in total exposed to calcium-ionophore for 75 minutes. Therefore, our findings do not agree with the review by Leemans *et al.* (2016) where it was indicated that calcium-ionophore will lead to complete loss of sperm motility and plasma membrane integrity within one hour.

In this study it was observed that samples processed by SLC had an increased number of living spermatozoa with intact acrosome compared to normal centrifugated samples (C). Addition of SP to SLC-selected samples did not improve acrosome status regardless of source. This observation was interesting because almost all SP is usually removed through normal centrifugation, as it was for sample C, prior to freezing, leaving approximately 5% SP.

Furthermore, in our study, stallions classified as bad freezer stallions had a tendency to have more dead cells with reacted acrosome if normal centrifugation was performed prior to cryopreservation compared to stallions classified as good freezer stallions. As described by Aurich *et al.* (1996) the composition of SP affects the resistance to freezing and thawing of the spermatozoa. They showed that SP from stallions with high post-thaw motility significantly improved the membrane integrity and progressive motility when added to spermatozoa from a stallion with low post-thaw motility. Increased membrane integrity results in higher survival rate.

In addition, there were no significant differences in acrosome status between samples regardless of whether SP was added or its source. However, adding 5% good freezer stallion SP had a beneficial effect on the proportion of live spermatozoa which acrosome reacted after stimulation with calcium ionophore compared to the control group. This effect was especially noticeable if the spermatozoa came from a bad freezer stallion.

These results might be due to that SLC appears to select live spermatozoa with an intact acrosome. Stallions classified as good stallions have semen with high freezability. The variation in freezability is affected by the composition of the SP

(Aurich *et al.* 1996), and the composition of the SP varies between stallions (Vidament *et al.* 1997). The SP contains both factors that are beneficial for the spermatozoa and detrimental (Akcay *et al.* 2006). By processing semen from bad stallions with SLC, the most robust spermatozoa are selected and the SP is completely removed, preventing it from impacting the spermatozoa negatively. However, by removing the SP, the beneficial factors in the SP are also removed. In addition, the SP also contains HCO_3^- and Ca^{2+} which are needed to initiate the capacitation process (Visconti 2009). Only spermatozoa which have undergone the capacitation process are able to perform the acrosome reaction (Breitbart 2002b). When the semen from stallions with bad freezability (BF) is first processed through SLC, more robust spermatozoa are selected and the SP with poor freezability containing detrimental factors is removed. Adding SP from GF which has higher freezability and probably contains more beneficial factors than SP from BF, seems to increase the freezability and the proportion of spermatozoa which can capacitate. Therefore, when stimulated, the portion of spermatozoa which will acrosome react is increased.

A previous report indicated that there is a positive correlation between high fertility and the proportion of acrosome reacted spermatozoa after stimulation (Tello-Mora *et al.* 2018). However, premature acrosome reaction is correlated with idiopathic infertility (Singh *et al.* 2016). In other words, it is beneficial to fertility if the sperm sample has a high proportion of live spermatozoa with an intact acrosome in the unstimulated state, but that these spermatozoa can undergo the acrosome reaction when stimulated.

These results are in agreement with the result from a previous study made by Al-Essawe *et al.* (2018), in that SLC prior to cryopreservation seems to improve post-thaw sperm characteristics. In this study, post-thaw acrosome status was not improved. However, a beneficial effect on acrosome status was seen when 5% good freezer stallion SP was added in comparison with C. In the previous study by Al-Essawe *et al.* (2018), no adverse effect was seen on DNA integrity regardless of whether SP from good freezer or bad freezer stallions was added.

More studies are needed to determine whether adding different concentrations of SP prior to cryopreservation can influence the ability of SLC-selected spermatozoa to acrosome react. In this study, samples with 5% of SP added prior to cryopreservation, were used because these samples were available from the previous study made by Al-Essawe *et al.* (2018). In that previous study 5 % SP was chosen because previous reports state that 0-5% SP remains after preparing the sperm sample for cryopreservation. As indicated by Al-Essawe *et al.* (2018), the SP in the study was pooled from several stallions and mixed with fresh sperm samples. They found that the effect of SP varies depending on the source. Therefore, in the present study, a beneficial effect from one stallion might be obscured by being diluted with SP from other stallions. Similarly, a detrimental effect from one stallion´s SP could be reduced by SP from other stallions.

It has been observed by Moore *et al.* (2005) that incubation for longer periods of time before freezing with high levels of SP has detrimental effects on the spermatozoa. This is in agreement with observation by Akcay *et al.* (2006). How-

ever, some components of SP are essential for the survival and transportation of the spermatozoa in the female genital tract. High levels of Cysteine-rich secretory-protein-3 (CRISP-3), a non-heparin binding protein have been reported to be associated with higher motility (Usuga *et al.* 2018), and fibronectin has been observed to improve freezability (Rungruangsak *et al.* 2018). In addition, lacto-ferrin, a heparin-binding protein SP, seems to have a beneficial effect by sup-pressing the mRNA expression of cytokines, such as tumor necrosis factor (TNF)-alpha (Fedorka *et al.* 2017). Other reports have associated high sperm quality with high levels of vitamin A, and high levels of vitamin E are associated with good motility (Usuga *et al*. 2017). Therefore, more studies are needed to examine the components in SP, to find out which have beneficial effects, and which have detrimental effects for frozen storage.

It is also worth mentioning is that in this study Whitten´s medium was used to pre-pare the sperm samples for analysis. This medium is not typically used to prepare thawed sperm samples. Additional studies with different types of semen extenders would be desirable, to see if the type of extender can affect sperm acrosome status.

6. Conclusion

There might be a positive effect of adding SP from GF to SLC-selected spermatozoa especially from when using spermatozoa from stallions classified as bad freezer stallions. Regarding the different treatments (no SP, SP from GF or SP from BF) of SLC-selected spermatozoa no differences between treatments could be found. Further studies are needed to investigate the impact on the acrosome status of different concentrations of SP. Larger samples sizes are needed and different extenders should be investigated. In addition, the study also confirms that SLC is a reliable processing method in comparison with normal centrifugation for selecting spermatozoa that can survive freezing.

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Popular science summary

Artificial insemination is frequently used in some livestock species. It is a method where the semen is collected from the male animal, stored and then inserted into the female genital tract. The semen is often stored cooled or frozen in liquid nitrogen (cryopreserved), until it is needed. This method has several advantages because it ensures the availability of the semen when it is needed, it provides a wider availability of genetic material than would be possible with fresh semen, and it can help conservation breeding of rare breeds or endangered species.

In the equine breeding industry, however, insemination of frozen semen can result in low fertility rates and the technique needs to be improved. Often the breeders choose to breed horses that have performed well in competition with no regard to sperm quality or how well the fertility of the sperm is preserved during storage.

The semen consists of the male germ cells, i.e. the spermatozoa (sperm), and the surrounding fluid, the seminal plasma, which is produced by the accessory sex glands. This fluid has been seen to have both good and bad impact on the sperm, especially during storage. The seminal plasma consists of different components, but their effect on the sperm or on the female reproductive tract is not fully understood. Most of the seminal plasma is usually removed during preparation of stallion semen for freezing.

The removal of seminal plasma can be done in different ways. In this study, two different methods were used. The first one is where the semen is centrifuged, the seminal plasma and extender are removed and the sperm pellets is collected. The second method is colloid centrifugation where the sperm pass through silica particles, enabling the collection of the most viable and robust sperm. In this study, the method of colloid centrifugation employed was single layer centrifugation, where only one layer of silica particles is used. The seminal plasma is retained above the layer of colloid.

Sperm from certain stallions maintain motility (and presumably fertilizing ability) better than others after freezing, i.e. they have better freezability. These stallions are referred to as good freezers. Stallions whose sperm do not survive freezing well are known as bad freezers.

Previously it was shown that sperm freezability can be improved by adding seminal plasma from a stallion with good freezability. In other studies however, no connection between the sperm survival rate and the addition of seminal plasma from stallions with good or bad freezability could be found.

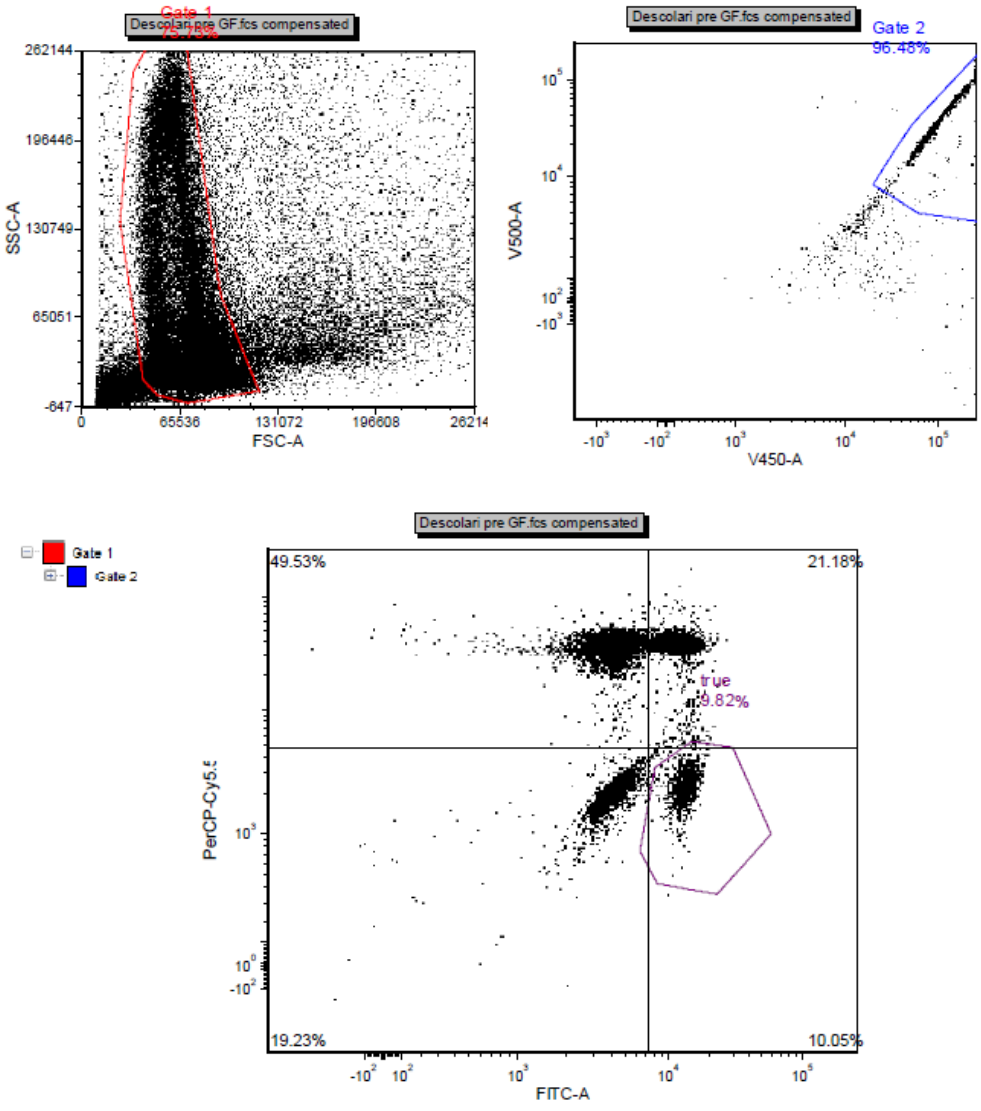
The acrosome reaction is an essential reaction that the sperm has to undergo before it is fertile and ready to merge with the female gamete, the oocyte. The acrosome is a cap located over the anterior part of the head of the sperm. The oocyte is protected by a layer called the zona pellucida. Before a sperm can penetrate the zona pellucida it has to undergo the acrosome reaction, releasing enzymes. These enzymes break down the zona pellucida adjacent to the bound sperm to enable it to penetrate and fertilize the oocyte, thus contributing its DNA.

This study evaluated whether adding seminal plasma from stallions with good or bad freezability to the sperm samples before cryopreservation could have a positive impact on acrosome status, thereby enhancing the fertility of the sperm sample.

From each stallion, four types of samples were prepared. Sample one, the control, was centrifuged to remove most of the seminal plasma. The remaining semen was prepared by Single Layer Centrifugation and was then divided into three parts. One of these parts did not receive any seminal plasma, one was treated with seminal plasma from stallions with good freezability, and one part was treated with seminal plasma from stallions with bad freezability. The samples were then frozen and stored in liquid nitrogen. On thawing, two sets of samples were prepared. The sperm acrosome status was evaluated by flow cytometry before and after incubation of the sperm samples with calcium ionophore for 45 minutes to stimulate the acrosome reaction. Spermatozoa were classified as living or dead, acrosome intact or acrosome reacted.

The results suggest that there might be a positive effect of adding seminal plasma from stallions with good freezability especially to sperm from stallions with bad freezability. However, these results should be confirmed in a larger study. In addition, the results confirm that sperm quality is better in samples after single layer centrifugation than for centrifugation without a colloid.

Appendix 1



Flow cytometry chart example: Descolari sample S-GF preincubated in 37 °C for 45 minutes before staining.