

# Development of a method for preparing alpaca semen by colloid centrifugation

## Utveckling av en metod för att förbereda alpackaspermier genom kolloidcentrifugering



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*Uppsala  
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## **SUMMARY**

Alpacas belong to the South American Camelids and they share many characteristics with other camelids. The camelids have unique reproductive characteristics; one example of that is the high viscosity of their semen. The latter has caused problems for many years, since it causes difficulties in collecting, handling and processing the semen for artificial insemination. Several methods for handling alpaca semen have been attempted; One that has been doing particularly well in different studies is Single layer Centrifugation (SLC), which has shown promising results in species such as stallions, boar, goat, donkey, llama and camel. The aim of this study was to examine whether the semen could be liquefied in Tris-citrate-fructose to enable the spermatozoa to swim freely and whether SLC could be beneficial in processing alpaca semen as it has been in other animal species.

This study took place at San Marcos University in Lima; a total of 9 ejaculates from 4 different alpaca males were assessed. The ejaculates were liquefied with tris-citrate-fructose, prepared with SLC and thereafter evaluated for volume, concentration, motility, membrane integrity, vitality and morphology. The assessments took place both directly after collection as well as after 24 hours of storage in 4 degrees Celsius. The evaluation were made manually with a light microscope. The results were compared to control samples.

The results showed that it was possible to liquefy alpaca semen in Tris-citrate-fructose because all the samples had spermatozoa that swam freely after being subjected to Tris-citrate-fructose. Significantly better values ( $p < .05$ ) in motility, in some aspects of vitality and abnormal tails, but not membrane integrity were shown for spermatozoa that had been processed with SLC. These results demonstrate that SLC might have a beneficial effect on the quality of alpaca spermatozoa, but to draw more reliable conclusions there is need for further studies.



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## Abbreviations

AI = Artificial insemination

AV= Artificial vagina

SLC = Single Layer Centrifugation





## INTRODUCTION

The alpaca belongs to the Camelidae family and is native to south America (Wheeler, 1995). Alpacas are mainly held for their wool, but are also used for meat (Vaughan *et al.*, 2003). Because of the unique reproductive characteristics in camelids, the development of methods for artificial insemination is more complicated and progress is slower than for other domestic species. Different techniques have been tried for collection and handling of the viscous camelid semen, but a well-functioning general method has not yet been developed (Abraham *et al.*, 2017).

Artificial insemination allows a more efficient use of genetically superior males, reduces the need for transferring the males to the female alpacas for mating (“mobile matings”), improves animal welfare and reduces the risk of disease spread (Vaughan *et al.*, 2003). Therefore, the interest in artificial insemination is growing and its development should be considered as a priority both in native countries for poverty alleviation and in other countries where the species is gaining popularity (Abraham *et al.*, 2017). In order to develop artificial insemination in alpacas there is still a need for further research before these technologies can be applied to full commercial AI and semen storage (Morton *et al.*, 2008).

Single layer centrifugation (SLC) has been tested on various species and a recent study on dromedary camel semen showed promising results, with both higher sperm motility and more functional membrane integrity of the spermatozoa in the sample that was subjected to SLC (Malo *et al.*, 2017). In my study I will transfer these techniques to alpacas to evaluate if similar results can be accomplished with alpaca semen.

The main aims in my study are to investigate if the semen can be liquified by repeated pipetting after adding Tris-Citrate-Fructose, and if Single Layer Centrifugation will improve alpaca sperm quality. I will also examine if sperm quality is maintained after storage at 5 °C for 24 hours. My expectation with this study is to take the first steps towards finding a method that allows the use of alpaca semen for artificial insemination.

## LITERATURE REVIEW

### Alpaca history

The family Camelidae originated from western North America approximately 9-11 million years ago. At the end of Tertiary period - around 3 million years ago- one branch of the family Camelidae (*Camelus*) migrated and gave rise to the present day camels of Central Asia and Africa. Another branch moved south and came to South America about 2 million years ago and gave rise to the *Lama* species that we know today (Wheeler, 1995).

The family Camelidae consist of two genera, the Old World camelids and the New World camelids (Sumar, 1985). They are collectively known as lamoids (Bravo *et al.*, 2000b). The Old World camelids consist of dromedary *Camelus dromedaries* and Bactrian camels *Camelus bactrianus* (Wheeler, 1995). The New World camelid has one specie, the llama, with four subspecies (Sumar, 1985). The four subspecies of the lama that exists today are: the llama *L. glama*, alpaca *L. pacos*, guanaco *L. guanicoe*, and vicuna *Vicugna vicugna* (Wheeler, 1995). The llama and the alpaca are domesticated whereas the vicuna and guanaco are wild species (Bravo *et al.*, 2000b). The first signs of camelid domestication was 6000-7000 years ago in the Andean Mountains in Peru (Wheeler, 1995).

The alpaca is smaller than the llama and is, in general, bred for fiber in contrast to the llama that is more commonly used as a pack animal (Wheeler, 1995). During the Inca empire, llama pack trains worked as a link throughout the provinces, and the fiber from camelids was used both for utilitarian purposes and in ritual processes. The empire had huge herds that provided alpaca fiber for textile production and also high quality animals of pure color for ritual sacrifice (Wheeler *et al.*, 1995).

The Spanish conquest in 1532 had a disastrous effect on the alpaca and llama population. Massive mortality was the outcome when alpaca herds moved from the coast to the Andean Puna in extreme high altitudes (Wheeler, 1995). Roughly 90% of the alpacas and llamas are estimated to have disappeared during this conquest (Wheeler *et al.*, 1995). At present the South American camelids mostly inhabit these high altitudes in the Andean. One phenotype (Suri – see next paragraph) is not as well adapted to the high altitudes, which has led to high mortality in new-born and decreased efficiency in breeding. These reasons have led to a decline in the Suri population, from 30% to under 5% of the total alpaca population (Brown, 2000).

### Alpaca fiber

The alpaca is native to south America and over 90% of the world's population live in Peru and Bolivia (Brown, 2000). Alpacas are used as fiber producers; however, they also have an important role as a producer of meat. The fiber of the alpaca is soft, fine and light (Vaughan *et al.*, 2003). Of the farmers who keep alpacas, approximately 85% are smallholder and have less than 50 animals each (Roberto *et al.*, 2008).

There are two types of alpaca; the suri and the huacaya. The Huacaya has short, curly hair and is preferred by weavers (Sumar, 1985). Huacaya's fiber is compact, soft and crimped and resembles the fiber from Merino sheep (Allain & Renieri, 2010). The suri on the other hand has

long, straight hair (Sumar, 1985) and the fibers are similar to the Angora goats (Allain & Renieri, 2010). Approximately 90% of alpacas are huacayas. Both suri and huacaya have coats with colors ranging from white, black and brown with all different shades in between. Alpacas have more commonly a uniform coloration, in contrast to the llama (Wheeler, 1995).

It is important for breeders to select superior males for mating to achieve offspring with high quality fiber. If superior males could be used to a greater extent via AI, it would give faster decrease in fiber diameter, higher fiber density for each animal and better control over the fiber colors. Producers would achieve better quality, as well as a higher quantity of the alpaca fiber (Vaughan *et al.*, 2003).

### **Reproduction in alpacas**

Reproduction is a key part of all livestock production (Sumar, 1985). Alpacas and llamas have unique reproductive characteristics that are a disadvantage compared to other domestic species. For example, both the female and the male reach puberty later than other domestic livestock and thus are older at the time of first breeding, the gestation period is long and usually only one offspring is born (Brown, 2000). The gestation length is about 11.5 months (Vaughan *et al.*, 2003), which is long compared to other small ruminants and that, combined with that the fact that males reach sexually maturity late, gives long generation intervals (Vaughan *et al.*, 2003). Therefore the rate of genetic gain is slow in conventional breeding (Brown, 2000).

Puberty is reached over a range of ages depending on genetics, climate, season of birth and nutrition. Male alpacas can show mounting behaviors early, but until the penis is detached from its preputial adhesions, complete erection and copulation is impossible. Preputial adhesions are lost in 10% of the males at 1 year of age, 70% are lost in two-year-olds and 100% of the adhesions are lost in three-year-old animals (Sumar & Garcia, 1984).

Alpacas have follicular waves similar to those seen in llamas and camels. Eight to ten follicles are included in a wave but only one follicle will grow and become the dominant follicle while the other follicles will regress (Vaughan *et al.*, 2004). The dominant follicle remains viable for several days until ovulation is induced (Ratto *et al.*, 2013). All camelids have induced ovulation, which occurs 26 hours after copulation (Fernandez-Baca, 1970) and is stimulated by copulation (El Allali *et al.*, 2017). In camelids an intramuscular or intrauterine injection of seminal plasma will induce LH followed by ovulation (El Allali *et al.*, 2017).

### **Male physiology**

The scrotum is located high in the perineal region, the testicles are directed caudo-dorsally and are relatively small compared to other animals. It appears that the season does not affect the size of the testes or scrotum in alpacas (Tibary & Vaughan, 2006). The testes of Camelidae are ovoid (Bravo *et al.*, 2000b). In alpacas the width of the testes are 2.5-3 cm and the length 4-5 cm (Smith *et al.*, 1994); there is an age-related size variation (Tibary & Vaughan, 2006).

In mature alpacas, the testis weigh around 18 gram, which is about 0.02-0.03% of their total body weight (Bravo *et al.*, 2000b). In comparison with other animals, the camelid testes are

relatively small, for example rams have testes that account for 1.4% of their bodyweight and bulls 0.18% of their body weight. Testicular size and sperm production are correlated in alpacas (Vaughan *et al.*, 2003) and concomitant with the small size of alpacas testes, they also have low sperm production (Vaughan *et al.*, 2003).

The epididymis has three parts, the caput (head), corpus (body) and cauda (tail). The epididymis is on the dorsal side of the testis, with the head on the cranial aspect of the testis. The prepuce is adherent to the glans penis until the alpaca is two or three years old. The camelids' penis is fibroelastic and in the absence of an erection the penis is retracted via a pre-scrotal sigmoid flexure. The penis is between 35-45 cm long and cylindrical in form, with the base at the ischial arch. The penis has a root, a body, the free end and a glans penis. The glans penis is 9-12 cm long and the distal tip, which is curved in a clockwise direction, consists of cartilage. The curve of the glans penis allows penetration of the cervical rings and the semen can therefore be deposited intrauterine (Tibary & Vaughan, 2006).

The alpaca does not have any seminal vesicles, but they have a H-shaped prostate and two bulbourethral glands (Tibary & Vaughan, 2006).

## **Mating**

Mating behavior of the female alpaca is essentially submissive behavior. She will lie down in sternal recumbency if a male approaches, mounts or pushes her. Estrous females might seek the male in pasture or lie beside a mating couple. On the other hand, if the female is non-receptive she will run away from the male, spit and/or scream if cornered or pushed down by a male (Smith *et al.*, 1994).

The males' mating behavior consist of running behind the females, and an aggressive male might continue chasing the female who rejects him until she lies down. When the female lies down, the male mounts her and crawls forward on her back (Smith *et al.*, 1994). Copulation occurs with the female in sternal recumbency and the male squatting with the hocks flexed and the forelimbs on each side of the female. The glans penis makes rotational movements inside the female during copulation (corkscrew) (Tibary & Vaughan, 2006). During copulation the male alpaca emits a sound "orgling" with air expired and the cheeks inflated (Tibary & Vaughan, 2006). Semen is deposited in the uterine horns, and most likely at the utero-tubule-junction (Tibary & Vaughan, 2006).

## **Semen collection**

Semen collection in alpacas is complicated by the positioning of the male during copulation, the long copulation time (5-50 min) and the fact that the semen is naturally deposited into the uterus (Bravo *et al.*, 2000b). Several methods have been attempted for semen collection in alpacas. For example; intravaginal condoms, fistulation of the urethra, electroejaculation, vaginal sponges, post-copulatory vaginal aspiration and artificial vaginas (Bravo *et al.*, 2000b). Vaginal sacs and sponges placed inside the vagina did not give good quality samples and therefore these methods are not used anymore (Bravo *et al.*, 2013). Electroejaculation was

introduced in 1968, but was not used a great deal after that, because the sample was contaminated with urine and it was painful for the male (Bravo *et al.*, 2013).

Collection by an AV inside a dummy or by allowing the male to mount a female and deflecting the penis into the AV is more dependable and natural than other methods to collect semen. The problem is that the male alpacas have to be trained with the AV and dummy, but after acceptance ejaculation is comparable to natural mating (Bravo *et al.*, 2000b). Collection with an AV will give an ejaculate that has a volume between 0.2-7.9 ml (Giuliano *et al.*, 2008). Collection with an AV has many advantages, amongst them ease and reliability once the males have been trained, and that it does not require general anesthesia. An AV is the most common way to collect semen from alpacas in AI trials (Bravo *et al.*, 2013).

When using an AV for collection it is also possible to do an assessment of the male's sexual behavior, which is an advantage compared to, for example, electroejaculation. A receptive female stimulates the male to mount and ejaculate, and a receptive female alongside a surrogate gives a fast intromission into the AV. The AV is a preferable method over postcoital vaginal aspiration, because the sample is not contaminated with cells and secretions from the female reproductive tract (Lichtenwalner *et al.*, 1996). Semen collection with AV has increased our knowledge about semen physiology in alpacas (Urquieta *et al.*, 2005).



Figure 1. Collection with an AV and a receptive female. Photo: Sofia Karlsson Warring, 2018.

## **Semen characteristics**

Ejaculation in alpacas is a continuous process and semen quality is similar during the whole copulation (Sumar, 1996). Alpaca semen has low volume, low sperm concentration and highly viscous seminal plasma (Morton *et al.*, 2008) (Kershaw-Young & Maxwell, 2012). The viscous nature of the alpaca semen makes it difficult to handle the sample (Bravo *et al.*, 2000b). The semen contains spermatozoa from the testes and seminal plasma from the accessory sex glands, testes and epididymides (Kershaw-Young & Maxwell, 2012). Spermatozoa constitute 12% of the total ejaculate. There does not seem to be any variation in the biochemical components between first-time breeders and adults (Bravo & Johnson, 1994). The seminal plasma represents around 85% of the ejaculate (Bravo *et al.*, 1997). In camelids there is very little information known about what the seminal plasma contains and what role it plays in sperm function (Kershaw-Young & Maxwell, 2012). In a study from 2011 it was concluded that if alpaca semen was diluted to a final concentration of 10% seminal plasma it would prolong motility, preserve acrosome integrity and maintain viability of the spermatozoa (Kershaw-Young & Maxwell, 2011).

In a study from 2008 on llamas, it was shown that the season affects semen quality. Ejaculates had lower sperm concentration as well as more tail abnormalities in the summer than in the winter (Giuliano *et al.*, 2008).

## **Methods for handling of alpaca semen**

### ***Enzymes***

Various hydrolytic enzymes have been used for liquification of the viscous alpaca semen (Bravo *et al.*, 2000b). Bravo *et al.* in 2000 compared the effect of 4 different enzymes; collagenase, fibrinolysin, hyaluronidase and trypsin. The semen was analyzed for viscosity, motility, vitality and acrosome integrity in both alpacas and llamas. All enzymes reduced the viscosity of the semen, but collagenase was the most effective in reducing viscosity and had less effect on sperm motility, vitality and acrosome integrity than the other enzymes (Bravo *et al.*, 2000a). On the other hand, in another study from 2008, 3 enzymes (papain, trypsin and collagenase) were tested to see if the viscosity of alpaca semen could be removed. All of the enzymes managed to reduce the viscosity of the semen, but the addition of collagenase killed all of the spermatozoa within 5 minutes. For trypsin and papain the results were better and motility declined at the same pace as the control (Morton *et al.*, 2008).

### ***Single layer centrifugation***

In single layer centrifugation a high density colloid layer is poured into a centrifuge tube and extended semen is pipetted gently on top. The preparation is thereafter centrifugated at 300 x g for 20 min (Morrell & Rodriguez-Martinez, 2009). Single layer centrifugation selects viable spermatozoa (Malo *et al.*, 2017) and has been successfully used in different species including stallions (Morrell *et al.*, 2009), boar (Morrell & Wallgren, 2011), goat (Jiménez-Rabadán *et al.*, 2012), donkey (Ortiz, 2013), llama (Santa Cruz *et al.*, 2016) and camel (Malo *et al.*, 2017). In a study made by Malo *et al.* 2017 using SLC in camel semen, it was shown that both total and

progressive motility were higher with SLC than the controls, as well as more positive membrane-intact spermatozoa being identified by HOST (Malo *et al.*, 2017).

## **Sperm quality**

There are different opinions between authors on what sperm quality is. For the authors in a study in 2002, sperm quality parameters include sperm concentration, motility, live-dead sperm ratio, morphology, membrane integrity, mitochondrial activity and acrosomal status (Tanghe *et al.*, 2002). Sperm quality does not always correlate with sperm fertility, because of extraneous factors not connected with the sperm sample or the laboratory analyzes and which cannot be controlled. Even though sperm quality is not always associated with fertility, it is still important to assess sperm quality parameters to exclude low quality semen (Mocé & Graham, 2008).

## **Semen evaluation**

Light microscopy was previously the main way to evaluate sperm concentration, motility and morphology. However, this method gives a subjective view and can vary between observers depending on their experience, as well as the microscope's quality. To evaluate each sample manually takes time, and these disadvantages were the main reasons for developing objective computer measured devices, for example; computer-assisted sperm analysis systems (CASA). CASA will quickly provide an objective view of numerous sperm parameters and can analyze a high numbers of spermatozoa per sample, which is practical for daily use. The downside to CASA include the high purchase price and the need for calibration and validation of the system for each species before use. Furthermore, CASA also needs to be standardized for frozen and chilled semen, since settings for fresh semen are not optimal for stored semen (Rijsselaere *et al.*, 2012).

The volume of the ejaculate ranges between 0.8 and 3.1 ml (Bravo *et al.*, 1997), and the normal sperm concentration varies from 350,000 to 600,000 spermatozoa/mL (Bravo & Johnson, 1994). The spermatozoa oscillate rather than show progressive forward motility in the presence of seminal plasma (Bravo *et al.*, 1997).

In a study on alpaca sperm morphology made by Evangelista *et al.*, it was shown that 49% of the spermatozoa had a morphological normal head, 18% had a long head, short 2%, pyriform 12%, round 9%, large 6% and small 4% (Evangelista-Vargas *et al.*, 2016). In a recent study made by Meza *et al.* (2018), sperm motility in the alpaca ranged from 30.4-50.9% with a mean value of 41.7% (Meza *et al.*, 2018).

In a study from Giuliano *et al.* 2008 it was showed that semen volume, sperm motility, sperm membrane integrity, normal vs. sperm with head or tail abnormalities, differ greatly between different males (Giuliano *et al.*, 2008). Also physiological factors such as age, nutrition status and season might affect quantity and/or quality of semen. The biggest problem with semen analysis in camelids is the fact that there are no standard methods yet, either for collection or for evaluation (Tibary & Vaughan, 2006).

## **Artificial insemination**

The first successful artificial insemination (AI) was on a dog, made in 1776 by an Italian physiologist by the name of Spallanzani (Morton *et al.*, 2008). The first attempt of AI made in South American Camelids was done in the 1960s (Fernandez-Baca, 1968).

Artificial insemination is one of the most important ways to develop genetic progress in animals, and the advantages include genetic improvement, economic benefits and as a way to control venereal diseases (Ax, 2016). Moreover, AI will reduce the need for “mobile-mating” and thereby decrease injuries and diseases that are associated with transportation, as well as other diseases (Vaughan *et al.*, 2003).

Currently, AI is being used in most domestic animal species and also in some companion animal species. Breeders use females that are either in oestrus naturally or synchronized, and inseminate fresh, chilled or frozen-thawed semen (Morton *et al.*, 2008). The synchronized oestrus is achieved artificially with administration of hormones (e.g. hCG and GnRH) (Gigli *et al.*, 2006).

The type of oestrus and semen utilized depends on the species and conditions, and possibly also on the breeders’ preference. With fresh semen, insemination usually takes place 1-3 hours after collection and the semen is extended to create more AI-doses and to provide a suitable environment for the spermatozoa to survive. Normally, chilled semen is extended and cooled to 21, 15 or 4 °C. It is kept at that temperature for 1-7 days (depending on the species) before insemination. Frozen semen has been diluted with a cryodiluent and is thereafter frozen and stored in liquid nitrogen until it is time to thaw the semen for the insemination (Morton *et al.*, 2008). In a study from 2009 by Pacheco *et al.*, the highest fertility in alpacas was achieved if semen was deposit in the uterine horn 30 hours after induction of ovulation (Pacheco *et al.*, 2009).

At this moment, there is no commercially available AI technology for the camelids but there is great interest among camelid breeders to develop this kind of technology (Morton *et al.*, 2008). In other breeds used for fiber production, for example Merino sheep, artificial insemination is already being used to improve the quality of the wool faster than is possible by natural mating (Vaughan *et al.*, 2003).

## **MATERIAL AND METHODS**

### **Animals**

Four male alpacas was used in this experiment, all held at the faculty of Veterinary Medicine, San Marcos university. The males were kept separately from the females and were between 4 and 12 years of age. They were accustomed to semen collection with an artificial vagina and during this study were used for collection 2 times a week. The males were allowed to mount a receptive female for semen collection.



## Semen preparation

Semen was collected during a period of 3 weeks (15th of October – 3rd of November) with the help of a hand-held artificial vagina (figure 2) and a receptive female. The artificial vagina was prepared with hot water to reach an inner temperature at approximately 45 °C and air was put inside to simulate the pressure from a natural vagina. The semen was collected in 50 ml falcon tubes at one end of the AV. The collections took between 5 and 10 minutes.



Figure 2. An artificial vagina. Photo: Sofia Karlsson Warring, 2018.

The volume, viscosity, concentration and motility were determined directly after collection. The volume of the ejaculate was measured in a graduated falcon tube. The viscosity/filament was measured by placing a drop of semen on a glass slide and measuring the distance between the base of the sheet and the rupture of the drop of semen when lifting it (thread test). The concentration and motility were determined as described under sperm assessment.

The semen was extended 1:3 with a diluent containing 10 ml fraction A ( 2.71 g Tris (N-Tris hydroxymethyl aminomethane), 1.4 g anhydrous citric acid, 1 g Fructose, 1 g Glycine, 100 mg gentamycin and 50 ml water), 18 ml water and 2 ml egg yolk. The sample was incubated at 37 °C for 30 minutes and was aspirated repeatedly with a pasteur pipette every 5 minutes to help liquefy the sample. After 30 minutes the sample was evaluated by microscopy to confirm that the spermatozoa could move freely, so that they could pass into the colloid during centrifugation. Once liquefied, the sample was divided between 15 ml falcon tubes -one with the colloid and one control sample without colloid. In this experiment Colloid One (JM Morrell, patent applied for) was mixed with buffer 1:1. Depending on the amount of semen we collected, we put the same amount of colloid/buffer mix (colloid : semen, 1:1) in the tube and added the semen sample slowly at the edge of the tube to form two separate layers.

Both samples were centrifuged at 300 g for 20 minutes. After the centrifugation the supernatant was eliminated with a glass Pasteur pipette and the pellet was kept in the tube. The pellet was resuspended with fraction A. The colloid sample and the control were checked for motility, membrane integrity and morphology.

Thereafter the samples was transferred into glass tubes and put in a beaker containing water at 37 °C and placed in a refrigerator, which gradually cooled the sample down to 5 °C over 2 hours. The samples were kept in the refrigerator for 24 hours. After 24 hour the sample was put in 37 °C for 5 minutes before repeating the sperm quality evaluations.

### **Sperm assessment methods**

Macro- and microscopic analyses of the samples were made at 37 °C. For example, the motility was evaluated on a 37 °C warm plate and the laboratory equipment was kept at 37 °C before placing in contact with the samples to avoid thermal shock to the spermatozoa.

The macroscopic and microscopic studies performed were as follows: volume, viscosity, sperm concentration, sperm motility, hypo-osmotic swelling test (HOST) for membrane integrity, and morphology. Ejaculates without spermatozoa as well as ejaculates with a motility lower than 10% were discarded (in this study, two samples).

**Motility:** Motility was assessed subjectively; 10 µl semen was put on a pre-warmed slide and covered with a cover slip on a warm plate (37 °C). The sample was observed using a light microscope at 40X and motility was expressed as a percentage estimated from 10 evaluated fields. In the case of few spermatozoa (less than 25 spermatozoa/field), the motility of the spermatozoa was counted manually in 10 different fields.

**Concentration** was determined with a Marienfeld Neubauer-improved counting chamber loaded with a mixture of 0.9 ml water mixed with 0.1 ml semen. After allowing time for the sample to settle so that the spermatozoa remained stationary, they were viewed at 40X magnification and the number of spermatozoa in 5 squares (corners and middle square) was counted. This number was multiplied by 500 000 to get the concentration in spermatozoa/ml.

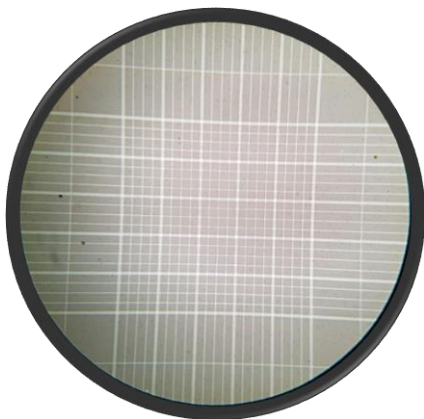


Figure 3. *A microscopic view of a counting chamber.*  
*Photo: Sofia Karlsson Warring, 2018*

**Eosin-nigrosin staining:** Eosin-nigrosin staining was used to assess membrane integrity (vitality) and also morphology (Bamba, 1988). A total of 200 spermatozoa were assessed per sample.

Eosin/nigrosin stain solution in the amount of ten  $\mu\text{l}$  was mixed with an equal volume of semen sample and a smear was made on a glass slide. After air drying, the smears were evaluated by light microscope at 100X. Sperm that acquire color inside the sperm head are considered dead. The number of vital spermatozoa were expressed as a percentage dividing with the total number of spermatozoa, multiplied by 100.



Figure 4. *Non-vital (left) and vital (right) spermatozoa.*  
*Photo: Sofia Karlsson Warring, 2018*

**Abnormalities:** The abnormal sperm were identified from the Eosin-Nigrosin stained smear with the help of a microscope, at a magnification of 100X. Abnormal spermatozoa were classified according to the location of the abnormality (tail or head). Morphology was assessed using the parameters; normal, abnormal head (narrow, narrow base, pear-shaped, abnormal contour) or abnormal tail (simple, coiled, double folded). Distal and proximal droplets were not calculated because an experienced lab technician advised against it, due to the poor quality of the slides.

The Eosin-nigrosin stained slides smears were brought to Sweden to assess vitality and morphology in the laboratory here, due to the better quality microscopes at SLU.

**HOST** (hypo-osmotic swelling test) was assessed to evaluate the functional integrity of the sperm membrane. 100  $\mu\text{l}$  of semen sample were mixed with a mixture of 500  $\mu\text{l}$  Citrate-Na adjusted to 150 mOsm (Solution E 100Ormm/kg). The mixture was incubated at 37 °C for 25 minutes and thereafter mixed with Formel 4% (fijador 4% formel + PBS) to stop the reaction. 10  $\mu\text{l}$  of the mixture were put on a slide, covered with an cover slip and then evaluated for swelling characterized by coiled tails (positive test), that indicates an intact sperm membrane. A total of 200 spermatozoa was assessed per sample. Any spermatozoa where the tail in full could not be assessed were excluded. The percentage was calculated by taking the number of coiled tails divided by the number of spermatozoa counted and multiplying by 100.



Figure 5. *Positive membrane-integrity (upper row). Negative membrane integrity (lower row).*  
*Photo: Sofia Karlsson Warring, 2018*

### **Statistical analysis**

Variables are given as percentages. Mean values  $\pm$  standard deviation were calculated. A paired T- test were performed to compare the different groups. Statistical significance was set at  $p < 0.05$ . Analyses were accomplished using SPSS Statistic 23 for Mac.

## RESULTS

Initial semen quality is shown in Table 1.

Table 1. *Information about the semen collection and initial values. Samples in bold are included in this study*

	ID	Time for collection	Volume (ml)	Consistency/viscosity	Motility prior centrifugation	Concentration (millions/ml)
Sample 1	Antonio	9 min	2.25	1 cm	60.5	150
Sample 2	Messi	8 min	1	4 cm	34.5	23.2
Sample 3	Pirata	2 min	0.5	1.5 cm	27.5	24.8
Sample 4	Antonio	10 min	1.5	1 cm	-	2.8
<b>Sample 5</b>	Messi	7 min	0.5	1.5 cm	75	40.5
<b>Sample 6</b>	Rayito	10 min	1.5	3 cm	75	148
<b>Sample 7</b>	Pirata	5 min	0.5	1.5 cm	55	351
<b>Sample 8</b>	Antonio	10 min	3.5	1 cm	52	47.5
Sample 9	Messi	10 min	1	2.5 cm	60	89.5
<b>Sample 10</b>	Antonio	10 min	2	2 cm	33.75	39.5
<b>Sample 11</b>	Rayito	10 min	1	3 cm	50	59
<b>Sample 12</b>	Pirata	7 min	1	1 cm	54	101
Sample 13	Messi	8 min	0.5	1 cm	5	34
<b>Sample 14</b>	Antonio	7 min	2.5	3 cm	60.5	38.5
<b>Sample 15</b>	Rayito	5 min	1.5	4.5 cm	51.5	146.5

In sample 1-3 we did not manage to create a pellet from the centrifugated colloid samples and the samples were therefore discarded. Sample number 4 contained too few spermatozoa for continued evaluation and was therefore rejected.

As shown in table 1, the times for collection differed between 2-10 minutes, consistency/viscosity was between 1-4.5 cm, motility prior to centrifugation was 5-75% and the concentration was 2.8 - 150 millions/ml.

Sperm motility, HOST, membrane integrity and morphology at 0h are shown in Table 2 and at 24 h are shown in Table 3.

Table 2. *Spermatozoa evaluation values prior to storage (n=9); A = controls, B = colloid samples*

	Motility after centrifugation (%)	HOST (%+)	Vitality (%)	Normal morphology	Abnormal heads (n/nb/p/ac)**	Abnormal tails (s/c/df)***
Sample 5A	62.5	51	60.5	62	24(5/11/5.5/2.5)	14(3/10/1)
Sample 5B	77.5	50	69	67	19.5(2.5/6/5.5/5.5)	13.5(3.5/9/1)
Sample 6A	58	41	63	65	22(3/14/3/2)	13(2/10/1)
Sample 6B	85	40.5	80	72	22(4/12/3/3)	6(1/4/1)
Sample 7A	50	56	50.5	40.5	41(5.5/12.5/19.5/3.5)	18.5(4.5/13/1)
Sample 7B	62	61.5	54.5	41	40.5(7.5/16/7/9.5)	19(6.5/12/0.5)
Sample 8A	50	46	52	72	15(2/6.5/4/2.5)	13(6/6/1)
Sample 8B	80	35	37.5	42	6.5(0.5/3.5/1/1.5)	2.5(1.5/1/0)
Sample 10A	50	30	45	73	11(0/2/1/8)	16(8/5/3)
Sample 10B	85	36*	68	89	9(1/1/1/6)	2(1/1/0)
Sample 11A	64.9	25.5	53	68	18(7/4/1/6)	14(2/11/1)
Sample 11B	74	42	70	81	9(0/8/0/1)	10(3/7/0)
Sample 12A	58	28.5	46	41	43(0/13/25/5)	16(3/13/0)
Sample 12B	82	33	72	50	40(2/11/23/4)	10(4/6/0)
Sample 14A	66.7	24	67	79	17(1/3/9/4)	4(1/3/0)
Sample 14B	77.3	43	51	68	27(1/9/4/13)	5(1/4/0)
Sample 15A	51.5	49	52	58	20(1/16/2/1)	22(4/18/0)
Sample 15B	72.8	54	64.5	66	27.5 (4/12/7/4.5)	6.5(0.5/5/1)

\* Merely 100 spermatozoa counted due to low concentration

\*\* n= narrow, nb= narrow base p= pear ac= abnormal contour

\*\*\* s= simple, c= coiled, df = double folded

Table 3. Sperm evaluation values after 24 hours of storage in refrigerator at 4 degrees Celsius (n=9)

	Motility (%)	HOST (%+)	Vitality (%)	Normal morphology (%)	Abnormal head (%) (n/nb/p/ac)**	Abnormal tail (%) (s/c/df)***
Sample 5A	40	55	39	79.5	7(1/2/3/0)	13.5(5.5/8/0)
Sample 5B	45	56.5	47	75.5	12.5(1/9/2.5/0)	12(2/10/0)
Sample 6A	51.6	46.5	44	69.5	27(2/16.5/5.5/3)	3.5(1/6.5/1)
Sample 6B	60	57.5	64	72	24.5(9/8.5/6/1)	3.5(1/1.5/1)
Sample 7A	25	29	25	54	28(3/13/11/1)	18(7.5/10.5/0)
Sample 7B	35	33.5	38	55	23(3/9/10/0)	22(10.5/10.5/1)
Sample 8A	51	32	46	57.5	31(2/12.5/11/4.5)	11.5(5/5/1.5)
Sample 8B	71	55*	67	71	17(1/2/2/12)	12(6.5/2.5/3)
Sample 10A	33.7	38.5	56	66	23(1/5/2/15)	11(3/7/1)
Sample 10B	55.8	33	51	64.5	27.5(2/6.5/3/16)	8(4/2/2)
Sample 11A	13.6	23	62	65.5	20.5(2.5/3/3/12)	14(4/7/3)
Sample 11B	21.9	26	36	60.5	30(5/14/4/7)	9.5(3/6.5/0)
Sample 12A	21	29	18	36.5	40(1/13.5/21.5/4)	23.5(9/14.5/0)
Sample 12B	44	29	34	40	45(0/16/24.5/4.5)	15(6/9/0)
Sample 14A	18.2	21.5	20	72	19(1/16/0/2)	9(4/4/1)
Sample 14B	50	21	48	75	19.5(1/9.5/4/5)	5.5(3.5/1/1)
Sample 15A	48	33.5	49	79.5	9.5(2/5.5/1/1)	11(3/8/0)
Sample 15B	72.6	44	75	85.5	11(1/5/1/4)	3.5(3.5/0/0)

\* Merely 100 spermatozoa counted due to low concentration.

\*\* n= narrow, nb= narrow base p= pear ac= abnormal contour

\*\*\* s= simple, c= coiled, df = double folded

Samples before and after colloid centrifugation and both before storage after storage, were compared. In total 24 different analyses have been made.

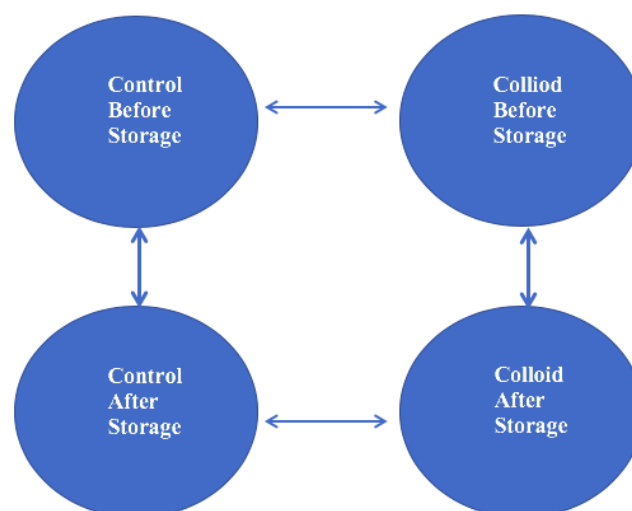


Figure 6. Illustration of the comparisons that have been made.

Table 4. Mean, standard deviation (SD) and p-value (n=9)

Parameter	0 h	After 24 hours in 4 °C	P-value
Motility Control	56.84 ± 6.76	33.57 ± 14.77	<b>0.006</b>
Motility Colloid	77.29 ± 7.19	50.59 ± 16.42	<b>0.001</b>
Membrane integrity Control	39.00 ± 12.17	34.22 ± 10.89	0.26
Membrane integrity Colloid	43.89 ± 9.52	39.50 ± 14.08	0.45
Vitality Control	54.33 ± 7.56	39.89 ± 15.74	<b>0.05</b>
Vitality Colloid	62.94 ± 12.98	51.11 ± 14.62	0.13
Normal Morphology Control	62.06 ± 13.57	64.44 ± 13.59	0.58
Normal Morphology Colloid	64.00 ± 16.64	66.56 ± 13.44	0.68
Abnormal Head Control	23.44 ± 11.19	22.78 ± 10.33	0.86
Abnormal Head Colloid	22.33 ± 12.77	23.33 ± 10.35	0.84
Abnormal Tails Control	14.50 ± 4.90	12.78 ± 5.62	0.42
Abnormal Tails Colloid	8.28 ± 5.48	10.11 ± 5.98	0.24

Statistical significant values are marked in red.

Table 5. Comparison control and colloid

Comparison colloid and control	P-value at 0 h	P-value at 24 h
Motility control – colloid	<b>&lt;0.001</b>	<b>0.001</b>
Membrane integrity control – colloid	0.14	0.1
Vitality control – colloid	0.13	0.09
Normal Morphology control - colloid	0.69	0.29
Abnormal head control - colloid	0.62	0.82
Abnormal Tails control - colloid	<b>0.016</b>	0.08

Statistically significant values are marked in red.

There are six pairs of parameters that showed statistically significant values ( $p < 0.05$ ), marked in red in tables 4 and 5. As seen in table 4, motility for both control and colloid as well as vitality for the control sample were significantly higher before storage compared to after storage.

As seen in table 5, motility in the colloid-treated samples are significantly better compared with the control, both at 0 h and at 24 h. We can thereby reject the null hypothesis that there is no difference between the two groups.



Neither membrane integrity, normal morphology nor abnormal heads were different in any comparison. However, there was a significant difference for abnormal tails between control and colloid at 0 h ( $p < 0.05$ ) but not at 24h.

The statistical results from this study must be interpreted with great caution because of the low number of animals, low number of samples and because it is not evaluated if the samples are normally distributed or not. Since the samples are collected from four different animals, the different samples from the same animal cannot be considered to be independent of each other and T-test is therefore not the optimal statistical analysis for this study.

## **DISCUSSION**

The aim with this study was twofold. First of all, we were to investigate whether the semen sample could be liquefied by gentle pipetting in tris-citrate-fructose. This method seems to work well with alpaca semen, because after 30 minutes of pipetting every 5 minutes, the spermatozoa swam freely in every sample.

The second aim was to investigate if SLC would have any beneficial effect on the semen quality. From the results I gathered, it seems the sperm quality improves with SLC, mainly sperm motility, but also morphology in some cases. Due to the fact that there were only 4 males in this study and a total of 9 ejaculates, the reliability of the study is low. To draw better conclusions, the study should be repeated with a larger sample size. However, this study shows that SLC seems to have a positive effect on alpaca spermatozoa, as has been shown in other species. Even though semen quality is not always related to high fertility, it is an encouraging result that SLC seems to be having a positive effect on sperm quality, which is one step forward towards being able to develop artificial insemination in alpacas.

In my study no visible pellet was obtained after centrifuging the colloid sample. I tried with firstly only colloid, thereafter I tried by diluting the colloid with buffer in different ratios. In all of the samples included in this study, the dilution was 1:1. There was still no apparent pellet, but when I discarded the supernatant and only kept the bottom layer of colloid, there were a few, highly motile spermatozoa. These spermatozoa always had higher motility compared to the sperm cells in the control.

The reason for the lack of a pellet in the colloid sample is noteworthy. If compared with the study on dromedary camel semen by Malo *et al.* (2017), there are some things that are not performed in the same way. One example is the different sizes of tubes centrifuged. In Malo's study, they worked with 50 ml tubes, but because the centrifuge that was available in the laboratory in Lima could not centrifuge 50 ml falcon tubes, we had to work with 15 ml falcon tubes instead. Maybe this difference in tube size could affect the formation of the pellet.

Another reason for the lack of pellet could be that somehow the density of the semen differs between species and the alpaca spermatozoa therefore had more trouble getting through the colloid. Another possible reason could be due to the low volume of ejaculate compared to camels – there could be too few good quality spermatozoa to be able to form a pellet. The SLC

is supposed to select viable spermatozoa, and if there are too few of them, they do not form a visible pellet.

In my study, 200 spermatozoa were calculated for most assays, with a few exceptions when only 100 was calculated. Due to the low sperm concentration and poor quality microscope, it took a long time to evaluate membrane integrity, sometimes up to 3 hours for 100 spermatozoa. On days when we collected semen from more than one male and also the 24 hours evaluation was due for samples collected on the previous day, the time available just was not enough to evaluate 200 spermatozoa. This of course gives the study lower reliability, and in further studies this should be taken into consideration when designing the study.

### **Sources of error**

After centrifugation of the samples, the control sample always had a pellet and you could easily distinguish the pellet from the supernatant that was going to be discarded. But because there was no visible pellet in the colloid sample, a different proportion of the bottom layer was kept between different samples. This could be a source of error in my results.

Two people prepared fraction A during my study. This means that even though the formula is the same, small variations could be done depending on the different routines and experience of the people fabricating Fraction A. It would have been better if all fraction A came from the same preparation or at least better if the same person prepared fraction A on the two occasions it was prepared.

Two people evaluated membrane integrity as well as the Eosin-Nigrosin calculation at 0 h. This means that the interpretation could be a little different between us and the results could be affected. The hypo-osmotic-swelling test was calculated by 2 people due to lack of time, and the Eosin-Nigrosin assay was also evaluated by two persons. This, however, obviously gives the study less security due to different interpretations between observers. However, we both have the same experience and have been taught by the same people and have asked each other as well as more experienced coworkers for help, and we anticipate that our interpretations should be similar.

### **What could have been done differently**

There are some aspects that need to be considered and also perhaps adapted if this study were to be repeated. First of all, the study should be bigger and include both additional male alpacas and a larger number of ejaculates to be able to make more reliable conclusions. To dispense with the subjective part of the study, maybe CASA should be used to evaluate the semen. If the study were to be made again manually, the samples should be evaluated blind to avoid bias.

It would have been interesting to analyze the concentration of spermatozoa in colloid verses control after centrifugation. When calculating both HOST and Eosin-Nigrosin as well as motility it became clear that in the sample with colloid there were far fewer spermatozoa. But due to low volume of semen we received, we were not able to calculate concentration in the different samples after centrifugation. In further research this would be an interesting result to see.

There is further testing that can be done to evaluate sperm quality. Examples are chromatin testing and acrosome integrity:

Sperm Chromatin Dispersion (SCD) test is a test to assess sperm DNA fragmentation, which has been documented as an essential cause of infertility. The test is relatively simple method, but both a fluorescence microscope and a brightfield microscope are necessary to read the results (Fernández *et al.*, 2003). It is also possible to use a flow cytometer to determine the results from Sperm Chromatin Structure Assay (SCSA) (Malo *et al.*, 2018). In our laboratory in Lima we did not have access to either fluorescence, brightfield microscopy or a flow cytometer, and it was therefore not possible to evaluate the sperm DNA fragmentation.

Acrosome integrity can be evaluated by Eosin-Nigrosin staining (Bamba, 1988), which we had access to and also performed in Peru. But unfortunately the smears were poorly stained with a lot of clumps and lack of focus, which made it hard to evaluate in the first place. To evaluate acrosome integrity a good quality smear is needed to properly evaluate if the spermatozoa have an intact apical ridge. This was not possible to assess in the smears we made.

In future studies more analyses could be included to gain more results, for example chromatin integrity and acrosome integrity. The statistical analysis should also be changed in future, similar studies. An ANOVA model should be used instead of a paired T-test, due to the fact that samples from the same male cannot be considered independent from each other. With an ANOVA model consideration would be taken to the different individuals and also the small number of animals and ejaculate in this study.

### **Comparison to similar studies**

When I compare my results with the results on dromedary camel in Malo *et al.* study from 2017, the similarity in the results include the significant differences in total motility. We both showed higher motility in the semen sample that was prepared with SLC, compared with our controls. Malo *et al.* also found significantly higher Membrane functionality, which I did not get. But I observed a significant value for vitality, which they did not achieve. To sum up, it is clear that the study's results are similar, but not identical. Malo *et al.* had a total of 12 ejaculates and I only had 9, which means that her study should be more reliable. They also used CASA to evaluate total motility, progressive motility and kinematic parameters whereas I used manually assessment with a light microscope; CASA gives a less subjective view than manual evaluation. On the other hand, they only assessed 100 spermatozoa per sample for membrane integrity, whereas I compared 200 spermatozoa per sample. I would judge that the reliability of the studies is similar, and so are our results.

### **CONCLUSIONS**

To summarize, there is need for further studies to draw more reliable conclusions concerning SLC and alpaca semen. It appears that colloid has a positive effect on the quality of the spermatozoa, but few results were statistically significant in this study, probably due to the low number of ejaculates analyzed.

## POPULÄRVETENSKAPLIG SAMMANFATTNING

Hos samtliga djurslag är det viktigt med genetisk utveckling. "Survival of the fittest" är ett gammalt Darwin-uttryck som de flesta känner igen. Survival of the fittest handlar just om genetisk utveckling. Hos vilda djur innebär den genetiska utvecklingen att de snabbaste eller mest intelligenta djuren överlever. Nuförtiden när vi pratar om genetisk utveckling syftar vi dock ofta till våra produktionsdjur.

För olika djurslag är utveckling av olika egenskaper önskvärda. Tar man våra produktionsdjur som exempel så vill man på mjölkkor avla på stor mjölkkvalitet, på kött djuren vill man generera stor muskelmassa på djuren och hos får vill man ha bra kvalitet på ullen. För våra produktionsdjur är genetiska framsteg en stor och viktig del när det kommer till vårt avelsarbete.

För att uppnå genetisk framgång avlar man på de djur som har de egenskaper man önskar. I stor utsträckning görs detta med artificiell insemination, då detta är den snabbaste vägen till genetisk utveckling. Det innebär att de genetiskt önskvärda hanarnas sperma samlas, processas och insemineras sedan i honorna. I många djurslag är detta en relativt enkel process, där man samlar sperman, behandlar den och sedan sprider denna till olika individer på olika gårdar och ibland till och med i olika länder. Hos alpackor däremot är denna process lite mer komplicerad.

Alpackan är ett kameldjur som lever i Sydamerika och ingår i samma familj som laman, guanaco och vicuña. Alpackan och lamorna är domesticerade medan guanaco och vicuña lever vilt. Alpackan blev domesticerad för 6000-7000 år och lever idag på extremt höga höjder (ca 4000 meter över havet) i bergskedjan Anderna. 90 % av alpackapopulationen återfinns i dagsläget i Peru. Alpackan är mindre i storlek än laman.

Precis som andra kameldjur har alpackorna mycket unika reproduktiva egenskaper. De är relativt gamla innan de blir könsmogna, de har lång dräktighetsperiod (11,5 månader) och föder vanligtvis endast en avkomma. Detta leder till att ett generationsintervall tar långt tid. Kameldjurens sperma har dessutom mycket hög viskositet som är svår att hantera och få till vätskeform. Trots forskning har det ännu inte hittats några bra metoder som fungerar för att standardisera samling och hantering av alpackans sperma, och detta är anledningen till att artificiell insemination ännu inte används på alpackor.

Men vad är anledningen till att man vill använda artificiell insemination hos alpackor? Alpackor hålls idag framför allt för deras ull. Deras ull har samma struktur som ull från merinofår eller angora getter, men är ännu tunnare och finare. Det man framför allt vill uppnå med artificiell insemination är avkommor med förbättrad kvalitet på ullen, vilket skulle gynna uppfödarna. Skulle man kunna nyttja artificiell insemination skulle man även kunna sprida bra genetisk material längre sträckor, och inte bara så långt som en hane kan förflyttas.

Min studie gick ut på att utveckla en metod som skulle kunna användas för artificiell insemination hos alpackor. Jag använde mig av en metod som visat lovande resultat på flertalet djurslag, bland annat hästar, grisar, getter, åsnor, lamor och kameler. Denna metod kallas för "Single layer Centrifugation", och förkortas SLC. Sperman från alpackorna samlades in med

hjälp av en artificiell vagina. Den insamlade sperman analyserades angående volym, viskositet, koncentration och motilitet (alltså hur många av spermerna som rörde på sig). Därefter försökte jag få sperman att anta vätskeform genom att addera ett ämne som skulle bryta upp viskositeten. För detta förflyttades vätskan mellan pipett och provrör upprepade gånger under 30 minuter. Efter detta hölls en vätska med hög densitet - en så kallad kolloid - i botten på ett provrör och spermablandningen hölls försiktigt ovanpå kolloiden, vilket gjorde att två olika lager bildades - en med kolloiden och ett lager med sperma-blandningen. Jag gjorde även ett provrör med endast spermablandning som fungerade som kontroll till min studie.

Dessa två provrör centrifugerades sedan i 20 minuter, vilket innebar att spermerna rent mekaniskt trycktes ned i botten av provröret. I provröret med kolloid var tanken att endast de spermerna som var levande kunde passera igenom kolloiden och hamna i botten på provröret, medan de skadade och/eller döda spermerna skulle fastna ovanför, eller i kolloiden.

Efter centrifugeringen skulle det ha bildats en liten ”pellet” i botten av provröret, vilket ser ut som en liten klump av celler. Denna lämnades kvar i provröret medan jag tog bort all vätska ovanför. Sedan tillsatte jag återigen vätskan som användes för att bryta viskositeten och blandar. Efter detta gjorde jag en del analyser på mina prover. Jag analyserade motiliteten av spermerna. Jag blandade färg med spermier och gjorde ett utstryk av detta på ett objektglas för att utvärdera hur många av spermerna som levde respektive var döda (vitalitet) samt hur många som hade normal morfologi/utseende respektive någon typ av missbildning, till exempel en böjd svans eller ett onormalt format huvud. Jag testade även spermernas membranintegritet (HOST), vilket är viktigt för att kunna transportera viktiga ämnen genom membranet. HOST kan ge en god bild av spermernas kvalitet genom att se om spermerna har ett intakt membran eller ej. Motiliteten, vitalitet och HOST räknades manuellt med hjälp av ett mikroskop. De resultat jag fick från kolloid-provet respektive kontrollprovet jämfördes för att se ifall SLC hade en positiv effekt på spermerna.

Mina prover sattes sedan in i ett kylskåp (4 grader Celsius) under 24 timmar och jag re-evaluerade sedan de tester som gjordes innan kylskåpsförvaringen. Anledningen till att man är intresserad av hur spermiekvalitén påverkas av kylförvaring är ifall man vill transportera sperma för artificiell insemination i kyld form, vill man veta hur kylskåpsförvaringen påverkar spermernas kvalitet. De resultat jag får efter 24 timmar jämförs med resultaten innan kylskåpsförvaringen. Jag gör en statistisk analys på mina resultat för att se om mina värden har statistisk signifikans.

Resultaten från min studie tyder på att de prover som hade utsatts för SLC med kolloid hade bättre motilitet, färre missbildade svansar och större antal levande spermier. De statistiska resultaten från denna studie måste dock tolkas med försiktighet på grund av det låga antalet provet, det låga antalet djur inkluderat i studien och eftersom det ej är utvärderad ifall proverna är normalfördelade eller ej. Då proverna är samlade från 4 olika djur kan proverna från samma djur ej anses vara oberoende av varandra och därför är T-test ej den optimala statistiska analysen för denna studie.

För att kunna dra några säkra slutsatser angående SLC och alpacka spermier bör studien upprepas i en större skala. Dock tyder mina resultat på att SLC med kolloid har potential att fungera och vi är därmed ett steg närmare att utveckla artificiell insemination hos alpackor.

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