Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science

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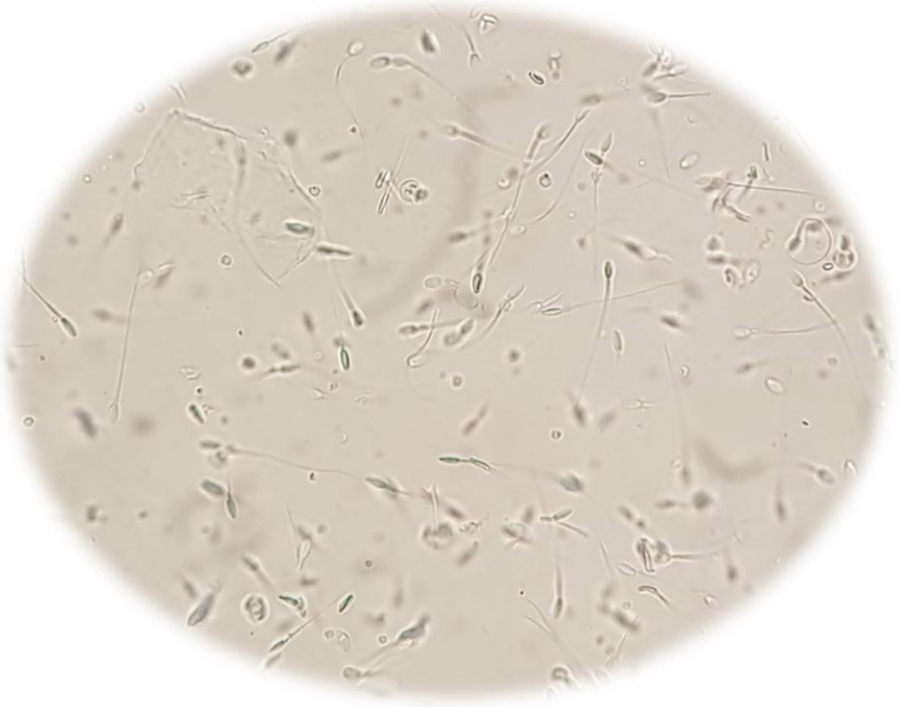

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SUMMARY

The aim of this study was to evaluate if sperm quality could be maintained after the freezing and thawing procedure by adding a mixture of Tris-citrate-fructose and by using SLC (Single Layer centrifugation) with a colloid formerly known as Androcoll-E. This technique has been tried out successfully in dromedary camel semen and the purpose was to transfer these techniques to alpaca semen.

Semen was collected from four alpaca males located at San Marcos University in Lima; in total nine semen samples, were analyzed. Each semen sample was divided into two parts, one part was layered over colloid before centrifugation and the other served as a control (centrifugation only). Both groups were treated in the same way using the same speed and time for centrifugation and the same temperature and time for both freezing and thawing. The samples were frozen the same day as they were collected, and were thawed, in general, 48 h hours, after freezing.

Fresh semen was evaluated for volume, concentration, viscosity and motility. Parameters evaluated before and after freezing included motility, membrane functionality (HOST-test), membrane integrity (vitality) and sperm morphology. All tests were evaluated manually using a light microscope.

The results showed a significantly higher ($p<0.05$) motility, HOST and vitality for the samples pre-freezing compared to the samples post-thawing in both groups. These results suggest that the sperm quality is highly affected by the freezing-thawing procedure, which corresponds to earlier studies. A significant difference ($p<0.05$) was seen regarding motility and the proportion of abnormal tails between the group treated with colloid and the control group pre-freezing. Single layer centrifugation might be able to select for spermatozoa with greater motility and fewer abnormal heads. Post-thawing, the proportion of abnormal heads was significantly lower ($p<0.05$) and the proportion of spermatozoa with normal morphology was significantly higher ($p<0.05$) for the group treated with colloid compared to the control. However, no improvement was observed regarding other sperm quality parameters for samples treated with SLC. These results suggests that single layer centrifugation might have a positive impact in selecting for spermatozoa with normal morphology.

CONTENT

INTRODUCTION

The breeding of domestic camelids (alpacas and llamas) has an important role in the socioeconomic development of the Andean population in Peru. Alpacas are adapted to difficult environmental conditions and are able to live and breed at heights of more than 4,000 meters above sea level. Today Peru has more than 3 million alpacas that are used for the production of good quality fiber (hair) and also for their meat. However, unscheduled mating of alpacas and llamas have contributed to diminish the quality of the animals, causing a loss in the quantity and quality of fiber (Huanca *et al*., 2007).

Artificial insemination has been used for many years in other species as an effective reproductive technology for selective genetic improvement (Santiani *et al*., 2005). There have been some trials of development of a technique for artificial insemination in alpacas which indicated a pregnancy rate of 2.4 % and 38 % but these rates are still poor (Fernandez- Baca *et al*., 1968, Leyva *et al*., 1977; presented in Bravo *et al*., 1997a). The technique of artificial insemination has not been widely applied to camelids and there is still a need for knowledge about the reproductive physiology of South American Camelids (SACs) (Adams *et al*., 2009). Several factors in alpacas differ from other domestic animals including special anatomical, behavioural and physical characteristics (Abraham *et al*., 2017). Some of the most important factors limiting reproduction in alpacas are their low fertility rate, which is due both to low sperm production in the males and a high incidence of pseudo pregnancy in females (San-Martin *et al*., 1968), and the physiological capacity of a female that can usually have up to four offspring throughout their reproductive life (Huanca *et al*., 2007).

The optimization of reproductive technologies has been challenging because of several physiological characteristics of alpacas. The viscous character of the semen is one of the limiting factors that aggravates its handling in the laboratory (Tibary and Vaughan, 2006). Several procedures have been tested for extracting the spermatozoa by reducing the viscosity of the seminal plasma but there is still a need for further research before these techniques can be applied for full commercial artificial insemination and semen storage (Morton-Maxwell, 2018).

The aim of this study is to evaluate if the sperm quality can be maintained after freezing and thawing by physical manipulation of the semen in Tris-citrate-fructose buffer and Single layer centrifugation (SLC) through a colloid formerly known as Androcoll-E, supplied by Dr. Morrell (patent applied for). This technique has been developed successfully in dromedary camel semen and the purpose of this project is to transfer these techniques to alpaca semen.

LITERATURE REVIEW

Lama pacos

The alpacas (*Lama pacos*) belong to the group of South American Camelidae (SACs), or New World camelids, which also includes the domestic llama (*Lama glama*) and two wild species, guanaco (*Lama guanicoe*) and Vicuna (*Vicugna vicugna)*. Excavations in Peru have placed the origin of the alpacas at 6000 to 7000 years ago and the distribution of the animals extends from a latitude of approximately 8°S to 20°S (Wheeler, 1995; Wheeler *et al*., 1995). Alpaca females are traditionally called hembras and the males are called machos. The newborn alpacas are called crias (Österlen alpacka, 2018).

There are two different phenotypes of alpacas which are defined by differences in their fiber (hair), but these do not breed true. The *huacaya* has short, dense and crimped fiber while the *suri* has long, straight fibers, organized in waves. In general alpacas are bred for fiber production but are also used for their meat. Approximately 75% of all alpacas are kept by traditional herders in South America and both inbreeding and hybridization between llamas and alpacas commonly occur because they are not consistently bred for selected phenotypes (Wheeler, 1995; Wheeler *et al*., 1995).

The hybridization between llamas and alpacas has been an ongoing process since the Spanish conquest and today it is hard to define hybrid animals precisely based upon phenotype. During the process of hybridization, the fine fiber-producing alpaca and llama have disappeared. The fleece of the llama is uneven, with wide variation in fiber diameter and a strong tendency to hairiness. The increased hairiness of the llama is a result of a lack of controlled breeding and for this reason the primary value of the llama currently lies in its use as a pack animal rather than as a fiber producer. To assure the preservation of alpacas and to improve the quality of the fiber production there is an urgent need to identify pure populations of alpacas. The loss of the animals with good quality fiber occurred because of the increased movement of both wild and domestic camelids throughout the Andes and the beginning of exportation of animals with superior genetics in 1983 (Wheeler, 1995).

Few other domestic species can graze and reproduce as successfully as the alpacas in the local farming community in the Andes with difficult environmental conditions including high altitudes and grass with low nutritional content. They are therefore a very important source of income (Huanca *et al*., 2007). The low fertility rate (50-60%) of the alpacas is one of the most important factors limiting their production (Fernandez-Baca *et al*., 1970b). The low fertility rate is due to both low sperm production in the males and high incidence of pseudo pregnancy in females (San-Martin *et al*., 1968).

Male anatomy and reproduction

The testes of the camelidae are found in the scrotum in a perineal position, as in the dog and pig, and they have an ovoid shape. The testes might not be in the scrotum at birth but descend by 6 months of age; when the animal are mature the testes weigh between 18 and 24g. The epididymis can be divided macroscopically into head, body and tail regions and is located on the anterior edge of the testis in alpacas. The vas deferens measures 35-40 cm and has two

portions, the proper vas deferens and a fusiform dilation, the ampulla. (Bravo *et al*., 2000b). Measurements show that the circumference of the testes is highly correlated with total sperm output in bulls and the firmness of the testes is correlated with the fertility, which means that a non-invasive testis evaluation provides a valuable estimate of a male's reproductive potential (Coulter and Foote, 1979). Camelid testes are relatively small and sperm production is low; presumably the weight and size of the testes have a high impact on the daily sperm output and fertility rate (Tibary *et al*., 1999, Galloway 2000; see Vaughan *et al*., 2003).

The penis of the alpacas is fibro elastic and at its end it has a curved hook to the right, with a small 'urethral process' of 1 cm in length. When the penis is erect, it has a length of 35 to 40 cm; there is a pre-scrotal sigmoid flexure. The glans penis is completely attached to the prepuce in alpacas from birth and detachment is related to the secretion of testosterone during puberty; by the age of 34 months, in 94.5% of males the penis is free from any attachment to the prepuce. Because of this special feature of the penis it is advisable to wait until three years of age to use the male for reproduction. Alpacas do not have any seminal vesicles but they have an H-shaped prostate lying dorsally and laterally above the neck of the urinary bladder. They also have bulbourethral glands that are oval, almond-shaped and located at the sides of the urethra in the pelvic outlet (Sumar, 1985).

Female anatomy and reproduction

Anatomy

The length of the vagina is approximately 13.37±2cm and has a diameter around 3.35 cm. The uterus is bipartite with two uterine horns separated by a septum; the left uterine horn is slightly greater than the right. It is impossible to flush liquid under pressure from the uterus to the oviducts because the isthmus of the oviducts acts as well-defined sphincter. The ovaries of the alpaca have a globular irregular shape and the left ovary is heavier than the right. Normally the size of the follicles varies from five to 12 mm; anything bigger than this is considered pathological (Sumar, 1985).

Ovulation and puberty

Most female alpacas show sexual interest at 12 months of age but nutritional levels influence the commencement of puberty. Ovarian activity begins at 10 months of age and at two years of age most of the female alpacas in Peru are bred, reflecting inadequate nutrition and management (Sumar, 1985). Alpacas do not have a regular oestrus cycle although they do exhibit waves of follicular development. Ovulation is induced by stimulation during copulation if there is a follicle of an appropriate size. Oestrus is hard to define precisely and any changes in behavior might not be related to physiological changes. During the breeding season, oestrus can last for 21 to 36 days. Females can still accept the male up to 10 days after copulation; probably in these animals oestrus continued even after ovulation. Females in heat can show a male behavior by mounting other females and also assume a prone position close to a copulating couple (San-Martin *et al*., 1968; Sumar, 1985)

Gestation

The gestation period is considered to be approximately 342 to 345 days (San-Martin *et al*., 1968). Even though both ovaries ovulate to an equal degree, almost all fetuses are found to be implanted in the left uterine horn. The reason for this migration to the left horn is still a mystery (Sumar, 1985).

As in other camelids, alpacas have a diffuse, epitheliochorial placenta. The corpus luteum acts as an endocrine gland and is a major source of progesterone throughout the whole pregnancy. It reaches its fully size around day 8-9. (Fernandez-Baca *et al*., 1970a; Steven *et al*., 1980).

In practice, pregnancy diagnoses are made by the herdsman around month eight of pregnancy by external palpation. However, pregnancy can be detected by rectal abdominal palpation after day 30 of gestation. It is common that the pregnant females reject the males by spitting at him ("spit-off) which can be used as an indicator of pregnancy, although this is a doubtful tool because it is shown that some spitting females may not be pregnant (Sumar, 1985)

San-Martin *et al*. (1968) estimated that about one third of the females can be pseudo pregnant during a breeding season. This pseudo pregnancy possibly lasts for a long time and the animal might not come in to oestrus again during the whole breeding season. This is considered to be an important contributing factor to the low fertility rate in alpacas (San-Martin *et al*., 1968). Another important factor contributing to a low reproductive rate is the failure to ovulate; approximately 8 % of females fail to ovulate (Sumar, 1985).

Females come into heat 24 to 48 hours after parturition but are not fertile if mated at this time (San-Martin *et al*., 1968). It takes around 20 days for the uterus to completely involute, and it is recommended to check the reproductive organs of the female within 15-20 days after parturition to obtain good fertility results (Sumar, 1985).

Mating behavior

The breeding season begins in December and lasts until March, which are the warmest months of the year (Sumar, 1996). When the animals are ready for mating, the male starts to run behind the female that is in heat and tries to force her down by mounting and putting pressure on her pelvis. If the female is in heat she adopts a seated position with her legs tucked beneath the body to allow the male to mount. During copulation the male extend his forelimbs on each side of the female with the front feet infrequently reaching the ground. The male moves his pelvis close to the pelvis of the female performing short pelvic thrusts and at the same time making a loud guttural sound. The females are, however, very quiet during the whole copulation (Tibary and Vaughan, 2006). Sometimes a second female that is also in heat sits against the mating couple as a sign that she is ready to accept a male (Sumar, 1985). In a study with natural mating it was observed that the duration of copulation frequently lasted for 25 to 35 minutes (San-Martin *et al*., 1968). In the beginning of the breeding season males show intense sexual activity and can copulate up to 18 times a day (Sumar, 1985).

Figure 1. *Semen collection at the Faculty of Veterinary Medicine - UNMSM (Universidad Nacional Mayor de San Marcos).*

Picture: Emma Norrestam 2018.

Semen collection

Semen collection is quite complicated because of the position of the mating couple and long duration of copulation, see Fig. 1 (Bravo *et al*., 2000b). Semen can be collected by different methods including an artificial vagina, vaginal pessaries or sponges and electro-ejaculation (Bravo *et al*., 1997a).

The different methods each have several advantages and disadvantages. The benefits of using an artificial vagina for semen collection including its reliability and that it does not require general anaesthesia, as is required for semen collection by electro-ejaculator. An artificial vagina is the most common way for semen collection and is the preferred method of obtaining semen that is suitable for laboratory manipulation and artificial insemination trials in alpacas and llamas (Bravo *et al*., 2013). Collection of semen by artificial vagina is more natural and reliable than the other methods (Sumar and Leyva, 1981; Moscoso, 1996; see Bravo *et al*., 2000b).

Ziapour *et al*. (2014) found that collecting semen using a phantom instead of a live female animal is easy and enables a sample to be collected without contamination. The male's behavior was similar between the phantom and live female (Ziapour *et al*., 2014). Collection of semen from the reproductive tract of a mated female results in samples that are contaminated by other secretion or cells and may be stressful for the female (Abraham *et al*., 2017)

Guilano *et al*. (2008) investigated the difference between artificial vagina and electro-ejaculator in llamas; the results show that the proportion of successful semen collections was greater when using electro-ejaculator than an artificial vagina. Ejaculates were obtained with a greater volume, sperm motility and membrane integrity (Giuliano *et al*., 2008). However, the method may not be suitable for repeated use on the same males in the field and requires anesthesia because the intense muscular contractions produced can be painful (Adams *et al*., 2009).

Semen characteristics

The semen of the alpaca is highly viscous forming a coagulum after copulation. The spermatozoa are retained within this gelatinous mass, which is produced at the end of copulation, making it difficult to separate the spermatozoa from the seminal plasma and to estimate concentration by conventional means. This might be an adaption in the alpaca to retain the spermatozoa in the female´s reproductive tract until ovulation, which occurs approximately

36h after copulation. Sperm motility in this gelatinous mass is oscillatory rather than progressive (Bravo *et al*., 1997a).

The alpaca ejaculates in a fractionated manner constantly during the whole copulation and sometimes only a gelatinous seminal plasma is ejaculated (Bravo *et al*., 2002; Lichtenwalner *et al*., 1996a). Garnica *et al*. (1993) reported that the volume of the ejaculate varies from 0.4 ml to 4.3 ml and the average seminal plasma volume was 1.5±0.1ml. The predominant color of semen is opalescent-milky white which depends on the concentration of spermatozoa (Garnica *et al*., 1993; Urquieta *et al*., 2005). Sperm concentration can vary from approximately 62-750 x 10^6 spermatozoa/ml with an average motility of 85% (Bravo *et al*., 2000b; Bravo *et al*., 1997a). Thus, sperm motility is affected by the viscosity of the samples and low mass motility appears to be the normal pattern in alpacas (Garnica *et al*., 1993). Bravo *et al*. (2000a) reported that the mean motility in alpaca semen samples was 68.2%. The study of Bravo *et al*. (1997a) on semen characteristics and abnormalities of the spermatozoa revealed that there were more abnormalities affecting the tail, including double and bent tails, than the head (Bravo *et al*., 1997a).

Seminal plasma

The reason for the high viscosity of the alpaca semen is the presence of the highly viscous seminal plasma. Alpacas lack vesicular glands and the seminal plasma is therefore derived from the bulbourethral glands (Kershaw-Young and Maxwell, 2012). Approximately 88% of the total volume of the semen consists of seminal plasma, with the rest being mainly sperm cells (Garnica *et al*., 1993). The gelatinous attribute of the seminal plasma is suggested to be a result of the presence of mucopolysaccharides (glycosaminoglycans); these are made up of 95% long chain polysaccharides and 5% protein (Morton and Maxwell, 2018). Liquefaction of alpaca semen does not occur for at least 24 hours (Garnica *et al*., 1993).

Approximately 10% of seminal plasma maintains the acrosome integrity of the spermatozoa and reduces the proportion of non-viable sperm cells. The viscous nature of the seminal plasma is the major limitation to the development of frozen storage and it might therefore be necessary to remove all the seminal plasma before freezing the spermatozoa. However, it is not known how dilution or removal of seminal plasma before freezing will affect the function of alpaca spermatozoa. It is suggested that instead of seminal plasma, protective factors should be added to the cryopreservation medium to maintain the viability and acrosome integrity of the sperm cell (Kershaw-Young and Maxwell, 2011). It is essential to increase an understanding of the components of the seminal plasma of the alpaca semen, including those involved in viscosity, to develop effective semen handling and artificial insemination protocols. It is also important to determine the role that these components play in reproductive physiology (Morton *et al*., 2012).

Sperm quality

To maintain good fertility, the male must produce spermatozoa of decent quality. Sperm quality can be evaluated using a number of different assays, such as concentration, motility, progressive motility, live-dead sperm ratio, membrane integrity, mitochondrial activity, acrosomal status and morphology of the sperm (Tanghe *et al*., 2002).

Assessment of sperm quality

A variety of different investigation techniques can be used to evaluate sperm quality. When evaluating motility, an aliquot of the sperm sample can be placed in a disposable chamber slide and analyzed using an objective method, Computer Assisted Sperm Analysis or CASA; this technique evaluates total and progressive motility, and the motility patterns (kinematics) (Malo *et al*., 2018). Traditionally motility has been evaluated subjectively by visual assessment using a light microscope. Sperm concentration in an ejaculate can be determined by putting 10 times diluted sperm in a counting chamber evaluated manually by light microscopy (Tanghe *et al*., 2002). Sperm concentration can also be determined using photometry which measures the degree of light scatter that a semen sample causes compared to a standard curve. In other species, sperm concentration is used to determine how many insemination doses can be produced from each ejaculate (Graham, 1996). However, in alpacas, the number of spermatozoa required to produce pregnancy after insemination is not known (Brown, 2000).

Live-dead sperm ratio can be determined by placing a small drop of semen on a glass slide and thereafter adding an eosin drop about double the size of the semen drop, and finally adding a drop of the nigrosin solution about twice the size of the eosin drop on the slide. The cells which were dead at the moment of mixing will have a distinct red colour whereas the cells that were living will remain uncoloured (Blom, 1950). Another way to assess the vitality of the spermatozoa is to use a fluorescent stain or probe that only penetrates damaged membranes and binds to the DNA of the spermatozoa (Love 2016).

The same smear as used for live-dead evaluation can be used to evaluate acrosome integrity (Bamba, 1988). The spermatozoon must have an intact acrosome until it encounters the oocyte, when it must acrosome react in order to bind to the zona pellucida. Premature acrosome reaction results in sperm death (Graham, 1996).

The sperm plasma membrane consists of three different compartments; different viability assays are used to assess the integrity of these compartments. To assess the integrity of the plasma membrane covering the principal piece the hypo-osmotic swelling test can be used which involves mixing the semen with a hypo-osmotic solution. Spermatozoa with an intact membrane show a characteristic coiling of the tail (see Figure 2) (Jeyendran *et al*., 1984).

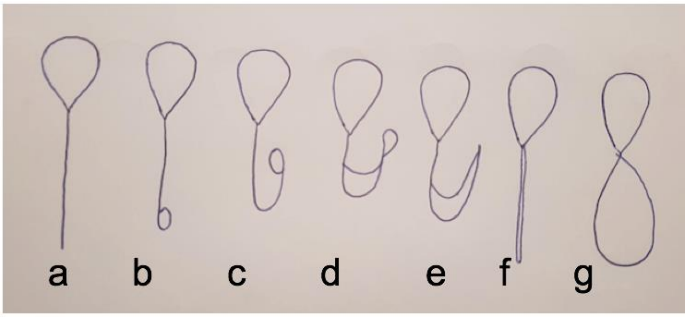

Figure 2. *Schematic presentation of typical morphological changes of spermatozoa subjected to hypo-osmotic stress: a= no stress, b-g=various types of tail changes.*

Picture: Emma Norrestam 2018, adapted from Jeyendran et al. (1984).

The spermatozoa of some species possess an acrosomal ridge and this can be used to assess the integrity of the plasma membrane covering the acrosome by differential interference phase contrast microscopy. In other species acrosome integrity is assessed by the integrity of the outer

acrosomal membrane. Mitochondrial function can be assessed indirectly using sperm motility measurements, but several fluorescent probes have been used to assess the function of the mitochondria and to determine the amount of mitochondrial activity (Mocé and Graham, 2008).

Morphology can be assessed visually or using automated morphometry estimation (Mocé and Graham, 2008). When using a light microscope for analysing the morphology, both wet mount preparations and staining (e.g. eosin-nigrosin) can be used; staining is essential if only brightfield microscopy is available (Graham, 1996).

The morphological abnormalities reported for alpaca spermatozoa vary between different studies; one study showed that between 70.6% and 84.1% of spermatozoa were morphologically normal, with 6.7% abnormal heads, 12.3% abnormal tails and 3.8% with cytoplasmic droplets. However, despite several studies, a full description of the prevalence and occurrence of the abnormalities in the spermatozoa remains to be elucidated for the alpaca (Bravo *et al*., 1997a).

Methods to increase the quality of the spermatozoa

One of the main factors to increase sperm quality is to reduce the viscosity before using it for insemination. Different hydrolytic enzymes have been tested for liquefaction of the semen without damaging the spermatozoa. Bravo *et al*. (2000a) reported that collagenase was effective in eliminating the viscosity in llama and alpaca semen without having a detrimental effect on motility, live spermatozoa and acrosome integrity. However, the use of enzymes is controversial since they reduce the viscosity of the semen but may damage the spermatozoa (Morton *et al*., 2012).

The findings of Bravo *et al*. (2000a) using collagenase for reducing the viscosity of the semen included a birth rate of 26% in inseminated females. These findings highlight the advantage of reducing seminal plasma viscosity prior to cryopreservation to be able to develop a working artificial insemination protocol.

Single layer centrifugation

Single layer centrifugation is a tool for selecting viable spermatozoa and to improve sperm quality and, consequently, cryosurvival. A specific feature of this colloid centrifugation technique is that only a single layer of colloid is needed to select live spermatozoa, compared with the two or more colloids of different densities required for a density gradient. By using SLC, an enriched population of motile dromedary spermatozoa with improved total and progressive motility, positive HOST and in vitro penetration of oocytes can be selected (Malo *et al*., 2017).

Single layer centrifugation has been used prior to freezing of dromedary camel spermatozoa and resulted in improved post-thaw sperm variables. This selection technique improves motility and mitigates cryodamage, which probably improves the ability of spermatozoa to interact with oocytes in vitro compared with non-selected counterparts (Malo *et al*., 2018). These results indicate that the functions of SLC can be either to select spermatozoa of better quality or to allow for more access of cryoprotectants by eliminating viscous seminal plasma, or both (Malo *et al*., 2018).

Successful trials of SLC have been done for llamas with the usage of a colloid developed for stallion spermatozoa (Androcoll-E). The results revealed that the colloid selected good quality spermatozoa e.g. the proportion of spermatozoa with functional membranes was significantly higher in the group treated with colloid (Trasorras *et al*., 2012). Santa Cruz *et al*. (2016) reported similar results, particularly that the colloid selected for motile and morphologically normal spermatozoa.

Cryopreservation

Cryopreservation is a method for long-term preservation of mammalian spermatozoa by storing it at very low temperatures, usually −196°C. At low temperatures cell metabolism decreases, allowing long-term preservation of germ cells, embryos and tissues. Sperm function is strongly impaired by the freezing and thawing process particularly due to cryodamage from the phase change of intracellular and extracellular water (Gao and Critser, 2000).

There is considerable individual variability in sperm resilience to cryodamage. For each sperm cell there is a cooling rate that is optimal to avoid intracellular ice formations and to reduce cryoinjury. The temperature range between −15°C and −60°C is the main challenge during freezing and thawing because of the lethality to the spermatozoa. At −5°C the cells and extracellular matrix remain unfrozen, but at temperatures between −5°C and −15°C the intracellular contents of the cells remain unfrozen although the surrounding media becomes frozen (see Figure 3) (Gao and Critser, 2000). To avoid the formation of ice crystals inside the cells during freezing at these temperatures, a protocol with slow freezing is used, although even with these freezing protocols ice crystal formation is not completely prevented (Yeste, 2016).

Santiani *et al*. (2005) reported the best viability results after thawing in alpaca semen samples diluted with skim milk, egg yolk and fructose. However most other studies with freezing of camelid semen have used an extender containing TRIS (tris(hydroxymethyl)aminomethane), citrate, glucose and egg yolk (Santiani *et al*., 2005).

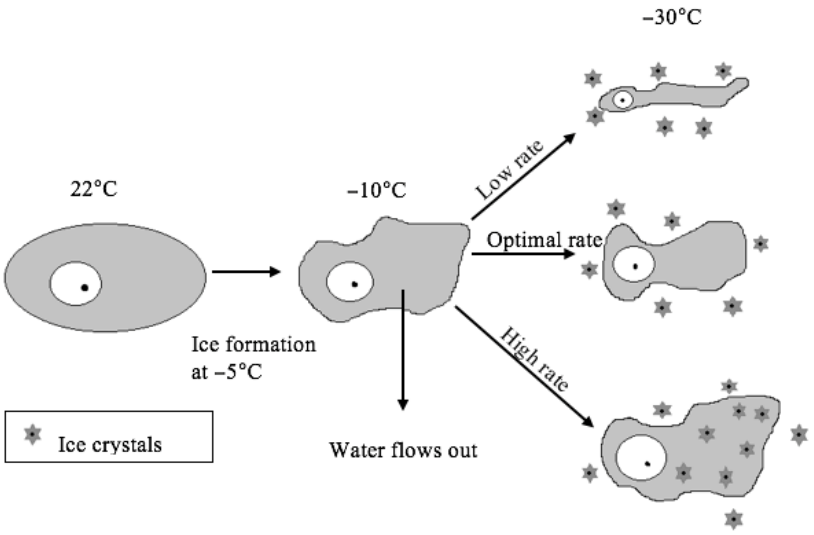

Figure 3. *An illustration of what happens with the cells during freezing.*

Picture: Emma Norrestam 2018, adapted from (Gao and Critser, 2000).

Impact on the sperm quality

Abnormal sperm function can be observed after cryopreservation, because of lipid peroxidation and antioxidant imbalance during the freezing process (Partyka *et al*., 2012). Partyka *et al*. (2012) showed that production of ROS (reactive oxygen species) increased during the freezing-thawing process as a result of electron leakage from uncoupled mitochondria oxidative phosphorrylation.

Sperm motility can be decreased further after freezing and thawing which indicates that the spermatozoa of camelids suffer considerable damage during cryopreservation (Santiani *et al*., 2005).

Cryoprotectants

Several substances have been tested in freezing protocols to protect the spermatozoa from damage during the freezing-thawing process (Yeste, 2016). Cryoprotectants such as glycerol and ethylene glycol have been used to counteract cryodamage in alpaca spermatozoa, which seem to promote the survival of more sperm cells with intact acrosomes. Many of the techniques of freezing protocols for alpacas, including the amount and concentration of cryoprotectants, have been adapted from other species; therefore it is important to take into account that alpaca spermatozoa probably require a specific concentration of cryoprotectant to obtain decent post-thaw motility and viability rates (Santiani *et al*., 2005).

Artificial insemination

Artificial insemination is an effective reproductive technology, enabling rapid genetic progress, and additionally is economic and hygienic compared to natural mating (Ziapour *et al*., 2014). However, the development of this technique in alpacas has been very slow and it is still in its infancy (Bravo, 2014).

The use of artificial insemination has several benefits; increased rate of genetic gain**,** easy transport of genetic material, long-term storage of semen, increased efficiency of breeding, elimination of the need to maintain males on farms, and prevention and control of disease (Morton and Maxwell, 2018).

Although artificial insemination has many benefits there are potentially some disadvantages including the possibility of inbreeding when the selection intensity is high in small closed herds, inaccurate breeding when semen samples are not labelled correctly, spread of disease if sires have not been properly tested for venereal disease, reduced fertility and high costs. Still, the development of artificial insemination in camelids is restricted due to lack of understanding of their reproductive physiology. The latter is vastly different from that of other domestic animals, which has prevented the adaptation of existing methods of artificial insemination from other domestic animal species (Morton and Maxwell, 2018).

The first attempt at artificial insemination in alpacas was made in 1968 by Fernandez-Baca *et al*. (1968) in 42 alpacas using fresh, non-extended semen. Only one of the 42 females gave birth. Subsequently studies revealed that the pregnancy rate after artificial insemination with fresh raw or extended semen varied from 39 to 68% (Adams *et al*., 2009). Bravo *et al*. (1997a)

obtained a conception rate of 68 % in alpacas using raw semen. During natural mating, semen deposition is intrauterine which is an important factor to be taken into account during artificial insemination (Bravo, 2000b).

Both in their native countries and in other countries where alpacas are gaining popularity, the development of artificial insemination should be considered as a priority. Use of an artificial vagina is the only practical method for regular semen collection in the field; therefore, efforts should be made to disseminate information about this technique (Abraham *et al*., 2017).

Specific objectives

The object of this study was to assess if the colloid formerly known as Androcoll-E can be used successfully in the processing of alpaca semen to improve cryosurvival of alpaca spermatozoa.

MATERIAL AND METHODS

Semen collection

Semen was collected from four alpaca males from the faculty of veterinary medicine- UNMSM (National University of San Marcos) in Lima during a three-week period in October 2018, using an artificial vagina after the male had mounted a receptive alpaca female. The artificial vagina was prepared in the same way every time using hot water at 45°C (see Figure 4). After collection, the semen was transported directly to the laboratory and placed in an incubator at 37°C in approximately two minutes. In total nine different semen samples were collected from the four males during this period. All of the males (male 1-3) gave two samples each except male four which gave three samples.

Figure 4. *Artificial vagina used for semen collection, filled with 45℃ water. Picture: Emma Norrestam 2018.*

Dilution of semen

After collecting the semen, the volume of each sample was measured and the motility and concentration of the sample were evaluated. Samples with a motility less than 50 % were discarded.

Assessing sperm concentration was done by diluting 0.1 ml of fresh semen sample with 0.9 ml water, and loading some of the mixture into a counting chamber (Neubauer improved bright-line) for manual counting under the microscope.

To reduce the viscosity, the semen sample was extended 1:3 (v:v) with a diluent of 10 ml Fraction A (2.71 g TRIS (N-tris hydroxymethyl aminomethane), 1.40 g anhydrous citric acid, 1.0 g fructose, 1.0 g glycine, 100 mg gentamicin and 50 ml water), 18 ml water and 2 ml egg yolk. The sample was placed in a 37°C incubator and manually mixed with a disposable plastic pipette every 5 minutes until the sample was liquefied, approximately 30 minutes in total. The samples had to be completely liquefied so that the spermatozoa did not become trapped at the colloid interface in the next step. When the sample had a liquid consistency it was examined under the microscope to confirm that the sperm cells could move freely, and the motility of the sample was evaluated once again.

Single layer centrifugation

The diluted semen was then divided into two. To separate the spermatozoa from the seminal plasma, one portion of liquefied extended semen was subjected to Single Layer Centrifugation, using a method modified from Morrell *et al*. (2008). Colloid was mixed with buffer 1:1 (v:v). An equal volume of the semen sample was added to the colloid by the following process; the colloid was placed in a 15 ml falcon tube, the sample was added by slowly dropping it down

the wall of the tube to form a separate layer over the colloid. The preparation was then centrifuged at 300g in 20 minutes using slow acceleration and no brake. At the same time, the other portion of liquefied extended semen was placed in a 15 ml Falcon tube and centrifuged at the same speed, to provide a control. Following centrifugation, the supernatants (semen extender, seminal plasma and colloid) were removed with a glass pipette and the pellet was resuspended with fraction A for analysis of motility, membrane integrity, sperm membrane functionality and morphological abnormalities.

Freezing

To prepare the sample for freezing and to prevent cryodamage, fraction B, containing 5 ml of fraction A, 0.76g trehalose, 0.6 ml glycerol, 9 ml water and 1 ml egg yolk, was added to the samples 1:1 (v:v). The two samples (one prepared with colloid and one without) were then placed in a water bath at 37°C in the refrigerator for 2 hours. When the temperature of the water bath had reached 5°C the samples were loaded into 0.5 ml straws and cooled stepwise as follows; the straws were placed in a basket 20 cm above liquid nitrogen for 10 minutes, 15 cm above for 10 minutes, and 5cm above for 5 minutes, before plunging into liquid nitrogen for storage.

Thawing

The semen samples were kept in liquid nitrogen until evaluation, approximately 2 days later. When thawing the samples, the straws were dipped into a water bath at 37°C for 45 seconds and then kept in an incubator at the same temperature for 5 minutes before evaluation. Post-thawing, the evaluation parameters included motility, membrane integrity, sperm membrane functionality and morphological abnormalities.

Sperm evaluation

The parameters used to assess the spermatozoa in this experiment were motility, membrane integrity, membrane functionality and morphological abnormalities. Evaluation of motility and membrane functionality was performed at a magnification of 40×, and evaluation of membrane integrity and morphological abnormalities was performed at a magnification of 100×.

Motility

To evaluate motility, a small drop of the semen samples was placed on a glass slide on a warm plate at 37°C, which was then covered with a cover glass and examined under the microscope. Ten different fields were evaluated and the average motility calculated.

Membrane integrity – vitality (VIT)

Membrane integrity was evaluated using the eosin-nigrosin stain. Ten µl of each semen sample were diluted 1:1 (v:v) with stain solution, rested for 20 seconds and then smeared on a glass slide which then was dried on a warm plate at 37°C. If the stain had not penetrated the spermatozoa, they remained uncolored and the membrane was considered to be intact. If the spermatozoa had a permeable membrane they were colored red and therefore counted as non-functional (see Figure 5). For all smears made pre-freezing, 200 spermatozoa were assessed per

sample and the percentage of intact acrosomes and live spermatozoa were calculated. For the samples made post-thawing, 100 spermatozoa were assessed per sample.

Membrane functionality (HOST)

Sperm membrane functionality was assessed by the hypo-osmotic swelling test (HOST). Aliquots, (500 µl) of a hypo-osmotic solution (containing sodiumcitrate 150mOsm) were mixed with 100 µl of semen and incubated at 37°C for 25 minutes. After 25 minutes, 130 µl of 4% formalin was added to the sample which was then evaluated under the microscope. In total 200 spermatozoa were assessed per sample; a coiled tail indicated that the spermatozoon was swollen and therefore the plasma membrane was intact, whereas the tail of spermatozoa with a non-functional membrane remained uncoiled (see Figure 6).

Morphological abnormalities

Abnormalities were evaluated using eosin-nigrosin staining. The spermatozoa were evaluated by assessing the head and the tail by light microscopy. If the head had an abnormal appearance (narrow, narrow base, pear shape, abnormal contour) it was counted as an abnormal head. If the tail was coiled or folded it was counted as an abnormal tail. A presentation of different morphological abnormalities can be seen in Figure 5. The spermatozoa which lacked any of the forgoing abnormalities were considered as normal. In total 200 spermatozoa were counted in the evaluation pre-freezing and 100 spermatozoa were counted post-thawing due to differences in concentration.

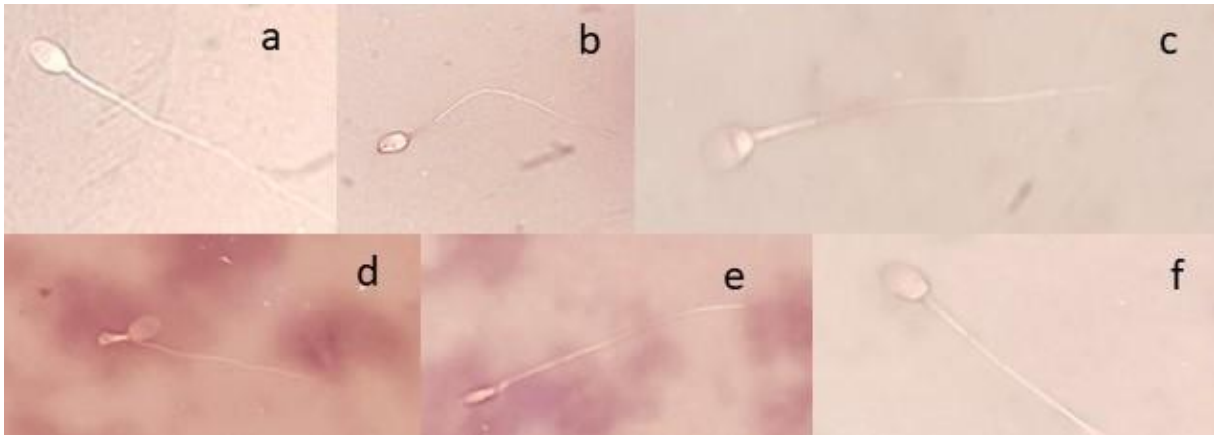

Figure 5. *Examples of different morphological abnormalities. Pictures taken in Eosin-Nigrosin stain. (a) narrow base, (b) abnormal contour of the head, (c) normal morphology, (d) simple coiled tail, (e) narrow head (f) simple coiled tail at the tip. Picture: Emma Norrestam 2018.*

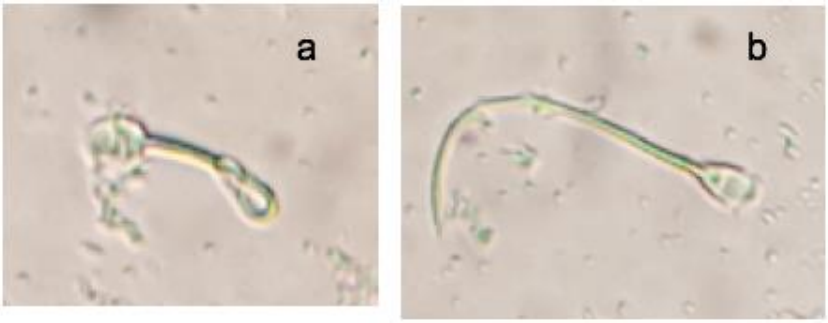

Figure 6. *Examples of sperm reaction in hypo-osmotic solution: (a) coiled tail, indicating a reaction in the hypo-osmotic solution (b) no reaction, uncoiled tail.*

Picture: Emma Norrestam 2018.

Statistical analyses

All statistical analyses were performed using Welch two sample t-test using R studio version 3.5.1 for windows. Variables are given as percentages. P-values <0.05 was considered significant.

RESULTS

Initial measurements (see Table 1)

Table 1. *Sperm quality parameters evaluated immediately after collection. Values shown are means ±SD*

Parameters	Mean	Range
Volume (ml)	1.4±0.9	0.5-3.5
Thread (cm)	2.3±1.1	1-4.5
Concentration (10^6)	113.5±92.9	38.5-351
Motility (%)	59.8±9	50-75

Quality parameters pre-freezing (see Table 2)

A significant difference ($p<0.05$) was seen for motility and abnormal tails between the group treated with colloid and the control group pre- freezing.

Table 2. *Pre-freezing sperm quality parameters for two different treatments (A= without colloid, B= with colloid). Values shown are means ±SD*[1]

Parameters	A	B	P-value
Motility (%)	56.4±6.9	71.8±12.3	0.008723
HOST (%)	39.1±9.7	42±6.3	0.4927
Vitality (%)	53.8±8	63.8±12	0.06806
Abnormal head (%)	24.9±9.6	25.1±10	0.9645
Abnormal tail (%)	14.4±4.6	9.5±4.4	0.04636
Normal morphology (%)	60.7±12.2	65.3±12.6	0.4675

[1] The numbers in red are statistically significant ($p<0.05$).

Quality parameters pre-freezing compared to post-thawing

As seen in Table 3, motility, HOST and vitality were significantly lower in the A^2 group representing semen samples post-thawing compared to the A^1 group representing semen samples pre-freezing. Abnormal tails were significantly higher in the A^2 group compared to the A^1 group.

Table 3. *Sperm quality parameters for two different groups without treatment with colloid (A^1= pre-freezing, A^2= post-thawing). Values shown are means ±SD[1]*

Parameters	**A^1**	**A^2**	**P-value**
Motility (%)	56.4±6.9	12.3±5.6	p<0.001
HOST (%)	39.1±9.7	18.7±5.8	p<0.001
Vitality (%)	53.8±8	16.3±5.3	p<0.001
Abnormal head (%)	24.9±9.6	23.4±8.2	0.7509
Abnormal tail (%)	14.4±4.6	20.7±3.6	0.0080
Normal morphology (%)	60.7±12.2	55.9±10.9	0.4150

[1] The numbers in red are statistically significant (p<0.05).

As seen in Table 4 the parameters motility, HOST and vitality were significantly higher in the B^1 group representing samples pre-freezing, compared to the B^2 group representing samples post-thawing.

Table 4. *Sperm quality parameters for two different groups, both treated with treated with colloid (A^1= pre-freezing, A^2= post thawing). Values shown are means ±SD[1]*

Parameters	**B^1**	**B^2**	**P-value**
Motility (%)	71.8±12.3	16.7±7.4	p<0.001
HOST (%)	42±6.3	19.8±5.2	p<0.001
Vitality (%)	63.8±12	20.3±6.8	p<0.001
Abnormal head (%)	25.1±10	21.1±9.6	0.4267
Abnormal tail (%)	9.5±4.4	12.3±3.1	0.1613
Normal morphology (%)	65.3±12.6	66.6±9.6	0.7597

[1] The numbers in red are statistically significant (p<0.05).

Quality parameters post-thawing

Post-thawing the percentage of abnormal tails were significantly lower (p<0.05) in the group treated with colloid compared to the control. For samples treated with colloid there was a significantly higher proportion (p<0.05) of spermatozoa with normal morphology (see Table 5).

Table 5. *Sperm quality parameters evaluated post-thawing in two groups (A= control, B= treated with colloid). Values shown are means ±SD*[1]

Parameters	**A**	**B**	**P-value**
Motility (%)	12.3±5.6	16.7±7.4	0.1968
HOST (%)	18.7±5.8	19.8±5.2	0.6915
Vitality (%)	16.3±5.3	20.3±6.8	0.2092
Abnormal head (%)	23.4±8.2	21.1±9.6	0.6083
Abnormal tail (%)	20.7±3.6	12.3±3.1	0.0001
Normal morphology (%)	55.9±10.9	66.6±9.6	0.0484

[1] The numbers in red are statistically significant ($p<0.05$).

DISCUSSION

The aim of this study was to evaluate if treatment with Single layer centrifugation with the colloid formerly known as Androcoll-E could improve post-thaw sperm quality. The technique has been successfully used for dromedary camel semen and because of the similarities in the semen characteristics between the species, the expectation of this study was to obtain good results also for alpaca semen.

The main focus in this study was to evaluate the spermatozoa after freezing to determine if selection of "robust" spermatozoa with colloid could have a beneficial impact on post-thaw sperm quality. Apart from the evaluation post-thawing, the samples were evaluated and compared before and after freezing with the aim of showing how sperm quality is affected in general by the freezing and thawing procedure.

A significant difference ($p<0.05$) was seen regarding motility and the proportion of abnormal tails between the group treated with colloid and the control group pre- freezing. There were no significant differences ($p>0.05$) between SLC and control group pre-freezing regarding membrane intact sperm, vitality, abnormal heads or spermatozoa with normal morphology. The results suggest that the colloid could be used for selection of spermatozoa with greater motility and fewer abnormal tails because the immature, immotile or damaged spermatozoa are not able to pass through the colloid. Results of this study are consistent with a previous study of Malo *et al*. (2017) where improved motility, membrane functionality and *in vitro* fertilization (IVF) ability were seen in dromedary camel semen treated with colloid. Trasorras *et al*. (2012) published a study using single layer centrifugation in llamas which, as in the study of Malo *et al*. (2017), revealed good results in selecting good quality spermatozoa. Fernández-Baca, (1993) points out that the basic physiological reproductive events of alpacas and llamas have many similarities and comparable results might be expected between the species. However, Malo *et al*. (2017) revealed a significant increase regarding both total and progressive motility, and membrane intact spermatozoa in the samples treated with SLC. This is the first trial of using SLC in alpaca semen and the differences of the results compared to earlier studies on dromedary camels and llamas suggests differences of the spermatozoa between the species. However, it is important to keep in mind that the colloid used for alpaca semen was a dilution of the colloid used for camel or llama semen, and that different evaluation techniques were used in the various trials.

In the present study, significant difference ($p<0.05$) were seen regarding motility, HOST and vitality, when values pre-freezing were compared to those post-thawing. These results correspond with results achieved earlier with alpaca semen indicating that sperm motility decreased further after freezing and thawing (Santiani *et al*., 2005), along with studies in other species indicating the same results (Adams *et al*., 2009; Partyka *et al*., 2012). Maintaining good quality frozen thawed spermatozoa is still a huge challenge. Previous studies showed that the proportion of motile spermatozoa decreases after cryopreservation (Santiani *et al*., 2005) which correlates with the results in this study.

The most commonly used semen extenders for camelids today are based on TRIS, citrate, glucose or egg yolk. Santiani *et al*. (2005) reported that the best viability results after thawing

were observed in the groups extended with skim milk, egg yolk and fructose. A study on freezability after dilution of camel semen in different extenders indicated that Tris – lactose gave the highest post-thaw motility and the highest survival rate (El Bahrawy *et al*., 2006). The type of extender that was used in this study contained TRIS, anhydrous citric acid, fructose, glycine, gentamicin and egg yolk, despite the findings of Santiani *et al*. (2005) that the motility and viability with this kind of extender (TRIS, citric acid, glucose and egg yolk) were lower than 10 % post-thawing. The main reason for using this extender in the present study was due to the availability of solutions and previous good results at the University of Lima.

Cryoprotectants play an important role in the protocol of cryopreservation. Commonly glycerol is used as a cryoprotectant agent for cryopreservation of camelid semen and that is the component included in the protocol in the present study (fraction B included Trehalose, and glycerol). Ethylene glycol is another cryoprotectant that can be used for cryopreservation which has showed a better cryoprotectant effect than glycerol (Santiani *et al*., 2005); further studies might be of value to compare these two methods in conjunction with single layer centrifugation. There is still a lack of an optimized adapted protocol for cryopreservation of alpaca semen.

Studies revealed that in llama spermatozoa the proportion of abnormalities can vary between 20.9 and 96.1% (Lichtenwalner *et al*., 1996b). Morphologically, between 70.6% and 84.1% of alpaca spermatozoa are normal with 6.7% abnormal heads, 12.3% abnormal tails and 3.8% with cytoplasmic droplets (Bravo *et al*., 1997a). The results from this study are not in agreement with previous results. The abnormalities varied, especially regarding abnormalities of the head which had standard deviation of 9.6% (control) vs 10% (treated with SLC) in the samples pre-freezing respectively 8.2% (control) vs 9.6% (treated with SLC) in the samples post-thawing. No significant difference regarding abnormal heads could be seen between colloid and controls post-thawing ($p>0.05$). On further investigation the samples with many abnormal heads were collected from the same male which was 7-8 years old, whereas the other three males were younger. Bravo *et al*. (1997b) reported that the fertility of the male alpaca is affected by consecutive mating and there is a variation in fertility between individual males and days. Buendía *et al*. (2002) also reported that morphometric values differ between single ejaculates from different males. The preferred age of alpaca males for breeding seems to be around 3 years of age; the age might as well be a contributing factor for the reduction of sperm quality (Brown, 2000). However, alpacas are bred at older ages than most domestic animals due to the late onset of puberty. Knowledge of physiological and endocrine changes is therefore of importance for breeding and requires further research to be fully understood.

Malo *et al*. (2018) indicated that SLC in dromedary camel semen improves motility and mitigates cryodamage, which probably improves the ability of spermatozoa to interact with oocytes *in vitro* compared with non-selected counterparts. Their study revealed significantly greater motility, viability and acrosome ridge integrity in the group treated with SLC. In comparison, the only parameters with significant difference post-thawing compared to values pre-freezing in this study included spermatozoa with abnormal heads and normal morphology, which was not evaluated in the study by Malo *et al*. (2018). However, the study by Malo *et al*. (2018) was conducted on dromedary camel semen and the differences between the species should be taken into account.

In conclusion the results of this study indicate that the processes of freezing and thawing have a high impact on alpaca sperm quality. The usage of SLC prior to cryopreservation might be preferable for selecting spermatozoa with fewer morphological abnormalities.

The statistic results must be interpreted with great caution due to the low number of samples and the low number of animals. It is not known whether the samples are normally distributed and it cannot be excluded that the samples are not independent of each other; a t-test as used in this study might not provide a suitable statistical analysis in this case.

This study was performed in the field in a laboratory with some limitations. The equipment available made it possible to evaluate motility, concentration and HOST manually. The evaluation of vitality and morphological abnormalities was made approximately one week after the stains were made using a light microscope at the Swedish University of Agricultural Sciences. Thus the evaluation was not optimal and might have had an impact on the results.

The main reason for using different techniques for sperm evaluation is to achieve a reliable fertility estimation. The sperm cell is one of the most important component of the semen, as its role is to pass through the female reproductive tract and penetrate the oocyte. Because of this the evaluations focus on examining different parts of the spermatozoa (Foote, 2003).

In this study 3 different techniques were used for examination of the spermatozoa (motility, vitality and morphological abnormalities). A further important point is to evaluate the impact of sperm quality on fertility rate, but this was not the focus of this trial. A correlation between sperm motility and morphological features of breeding stallions has been studied by Love, (2011) in which morphologically normal sperm were positively correlated with most sperm motility measures. Identifying different abnormalities could help to explain why a sample has reduced motility and poor quality motion. Motile spermatozoa might have a fertility-limiting abnormality such as an abnormal head (Love, 2011). Foote (2003) likewise indicates that all of the parameters assessed in this study (motility, vitality and morphological abnormalities) have been correlated with fertility rate in other species.

Love (2011) stated that total and progressive motility are essential parameters to evaluate the overall quality of spermatozoa. Love (2016) suggests that ideally sperm motility should be tested using a phase-contrast microscope; light microscopy should be avoided because it lacks clear visualization. In this study, a light microscope was used in Peru; due to several practical reasons examination of both total and progressive motility were impracticable and the results might therefore be doubtful. Nowadays Computer Assisted Sperm Analyzers (CASA) are available which provide a variety of motility values, including total motility, velocity values, and compound measures such as progressive motility, straightness, and linearity (Malo *et al*., 2018). Greater objectivity is achieved with CASA and this might therefore be useful in further studies of alpaca semen to produce a result that includes more metrics.

In present study the eosin-nigrosin stain (EN) was used both for evaluation of the vitality of the spermatozoa and morphological abnormalities. This is a type of background stain which requires only a light microscope and low magnification. However, the EN technique can induce artifactual morphological changes (eg. bent tails, detached heads) owing to composition

changes in the stain or preparation mistakes. The EN stain produces an image of lesser quality which may result in the clinician missing subtle but important fertility-limiting abnormalities such as abnormal midpieces. Another technique to evaluate morphology is to use buffered formol saline, examined either by phase-contrast or differential interference microscopy. In comparison with EN stain evaluated by light microscope, the buffered formol saline stain provides an enhanced resolution (Love, 2016). Usage of buffered formol saline might be a more reliable technique. Neither of these techniques is time-dependent; the samples can be fixed at a remote location and then evaluated at a later time, as occurred in this study.

Other parameters including integrity of the plasma membrane covering the acrosome (acrosome ridge integrity), mitochondrial function, capacitation and the acrosome reaction, can be used to evaluate sperm quality (Mocé and Graham, 2008). Due to lack of access to a fluorescence microscopy or a flow cytometer in this study, there was no possibility of evaluating these parameters.

Because of the low volume of the ejaculate (in this study the volume varied between 0.5 ml and 3.5 ml with a mean value of 1.4 ml) there was a limit to the number of evaluations that could be performed. Bravo *et al*. (2000b) reported that the volume of the alpaca ejaculate varies between 0.8 to 3.1 ml which means that in some cases there may be very small volumes. Evaluation of the concentration requires 0.1 ml of the semen sample and because of this the concentration was only measured once. After centrifugation and removal of the supernatant, the concentration of the sample was not measured and is unknown. A measurement of the concentration after centrifugation could have been preferable to be able to see the difference between the sample treated with colloid and the control group to evaluate to what extent the colloid selects away non-viable spermatozoa.

Four males were involved in this study and only nine different samples were analyzed; this is a small number of samples which makes the reliability doubtful. All the examinations of the spermatozoa were made manually using a light microscope. In all HOST, vitality and morphology evaluations, 200 spermatozoa were counted pre-freezing but when evaluating vitality and morphological abnormalities in the samples post-thawing, only 100 spermatozoa were counted. The difference in counted spermatozoa depended on the differences in the concentration. Although it was not optimal to evaluate different numbers in samples before and after freezing, it would have impractical to evaluate more spermatozoa because of the number of different assays performed at the same time. However, Varner (2008) suggests that only 100 spermatozoa could be evaluated for evidence of morphological defects.

Human error is an important parameter to consider in this study. All the microscopic assessment was made by two persons and the risk for inter-observer variation is therefore important to remember. The analyses were performed in both Peru and Sweden using different microscopes with varying quality; this could affect the outcome of the results. To minimize the bias a blinded study would have been preferable.

CONCLUSIONS

In conclusion, the results of this study indicate an encouraging impact when using SLC regarding post-thaw sperm qualities including selecting for spermatozoa with normal morphology. However, further studies are needed to improve the performance of the study, optimize the conditions and involve a larger number of animals to obtain a more reliable result.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Aldrig tidigare har studier med målet att få fram en metod för att välja ut spermier av god kvalité genomförts på djurslaget alpacka, vilket denna studie fokuserar på. Genom att centrifugera alpackasperma på ett speciellt sätt, så kallad single layer centrifugation (SLC) fanns förhoppningen att välja ut spermier av god kvalité. SLC har tidigare testats på spermier från andra djurslag så som hästar och kameler, men aldrig tidigare har liknande studier gjorts på alpackaspermier. Denna metod innebär att spermierna hälls i ett rör fyllt med en speciell lösning så kallad kolloid, spermierna lägger sig då som ett skikt ovanför denna lösning på grund av dess olika densitet. Röret med spermier och kolloiden centrifugeras sedan. Efter centrifugering ska de spermier som är av dålig kvalité, t.ex. har en skadad svans eller simmar dåligt, stanna ovanpå kolloiden och de spermier med god kvalité som kan simma bra tar sig igenom lösningen och hamnar på botten av röret.

Alla spermaprover som samlades in delades upp i två olika grupper där den ena behandlades med SLC och den andra fungerade som en kontroll och då ej behandlades med SLC. I denna studie visades att behandling med SLC gjorde att fler spermier med bättre rörelse och färre spermier med trasiga svansar valdes ut i jämförelse med de spermaprover som ej behandlats med SLC. När spermieproverna sen frystes in och tinades upp någon dag senare kunde man se att i de prover som hade behandlats med SLC fanns fler spermier med normalt utseende och färre spermier med skadade svansar än i de prov som ej behandlats med SLC. Slutsatsen som kan dras i denna studie är att genom SLC behandling av spermier från alpackahanar kan man få ut fler spermier av god kvalité.

Djuren som användes i denna studie var fyra alpackahanar i olika åldrar från fakulteten för veterinärmedicin UNMSM (National University of San Marcos) i Lima, Peru. Sammanlagt samlades nio olika spermaprover från de fyra hanarna. Antalet djur och även antalet prover i denna studie är väldigt lågt. Detta innebär att tillförlitligheten hos testerna inte är särskilt hög. För att få en bättre tillförlitlighet och på så vis ett bättre resultat krävs ett större antal djur och prover vilket är viktigt att tänka på om vidare studier genomförs inom ämnet. Två personer hjälptes åt att analysera samtliga prover. Viktigt att även belysa är att vissa analyser utfördes i Peru och andra i Sverige, vilket innebar att proverna transporterades en avsevärd sträcka och att olika ljusmikroskop användes. För att få ett så bra provresultat som möjligt vore det bästa om alla proverna analyserats av en och samma person och att denna person inte hade haft kännedom om vilket prov som kom från vilket djur eller vilka prover som var behandlade med SLC.

Intresset för alpackor har under de senaste åren vuxit mycket i Sverige, men de är framförallt otroligt populära i Sydamerika och speciellt i Peru där alpackapopulationen idag ligger på över tre miljoner. Framförallt hålls alpackorna för den goda kvalitén på ullen som används för att tillverka flertalet olika textilier som klädesplagg, vilka i sin tur är en stor inkomstkälla för många människor i Peru. Många personer har lätt för att förväxla djurslaget alpacka med lama och har därmed svårt att urskilja deras olikheter. Denna förväxling mellan de två djurslagen finns sedan en lång tid tillbaka även i Peru. Förväxlingen har gjort att alpackor och lamor har parats med varandra och på så vis förekommer det många avkommor som är halvt alpacka, halvt lama. Att lama-alpacka gener blandas kan kanske ses som något positivt men detta har

påverkat aveln negativt. Denna djurslagsförvirring medför en förlust av den goda kvalitén på ullen då lamornas ull inte lever upp till en lika hög standard som alpackornas. Genom att genomföra denna studie finns en förhoppning om att göra det enklare att föra de gener från alpackor som står för god ullkvalité vidare.

Artificiell insemination har länge varit viktigt inom aveln på andra husdjur så som mjölkkor, grisar och hästar. Detta är en metod som används istället för naturlig parning. Artificiell insemination innebär att sperma samlas från ett handjur som sedan förs in ett hondjur på konstgjord väg i samband med att hondjuret har ägglossning. Det finns väldigt många fördelar med att använda sig av artificiell insemination istället för naturlig parning. Genom att kunna styra vilka djur som ”paras” med varandra kan man styra vilka gener som förs vidare och då utrota de gener som står för vissa kvalitéer, så som sämre ull kvalité. Det finns även en möjlighet att minska sjukdomsspridningen, underlätta transport av genetiskt material i form av spermier, öka effektiviteten av aveln och minska behovet av att ha en alpackahane på varenda enskild gård.

Alpackors reproduktionsfysiologi skiljer sig mycket från våra vanligaste husdjur och på grund av dessa skillnader är avelsarbetet inom djurslaget alpacka inte lika utvecklat. Alpackahonorna visar sexuellt intresse vid 1 års ålder men brukar inte avlas på förrän vid 2 års ålder. Honorna har inte en regelbunden ägglossning. Ägglossning sker i samband med att de parar sig. Alpackor är dräktiga i ca 11 månader och vanligtvis får de bara 4 avkommor under en livstid. De flesta alpackahanar är könsmogna när de är ca 3 år men detta kan skilja sig väldigt mycket mellan olika djur. Rekommendationen är att inte använda alpackahanar i avel förrän vid tre års ålder. En hona som är redo för att para sig lägger sig ner på marken för att hanen ska kunna stiga upp på henne, under tiden som de parar sig gör hanen ett typisk gurglande ljud medan honan oftast är helt tyst. Parningen kan hålla på i 35 minuter och det är framförallt under de varmaste och regnigaste månaderna (december- mars) som de har sin parningsperiod.

För att kunna använda sig av artificiell insemination krävs det att sperma samlas ifrån hanen, något som försvåras av den position alpackorna intar när de parar sig samt även att parningen pågår under en mycket lång tid. Det finns olika metoder för att samla sperman bl.a. en konstgjord vagina, elektrisk stimulering av hanen eller kondomliknande grej som förs in i vaginan på honan och på så vis samlar upp spermierna. Ytterligare en viktig aspekt som försvårar spermainsamling och artificiell insemination är att sperman hos alpackor är extremt trögflytande, vilket innebär att den är svår att hantera och analysera. Spermierna som är fast i denna trögflytande geléklump rör sig inte särskilt mycket framåt utan ligger snarare och rör sig på ett och samma ställe. Alpackahanar producerar väldigt få spermier vilka kan variera väldigt mycket i kvalité. För att lyckas med artificiell insemination krävs det att spermierna har tillräckligt hög kvalité och koncentration att möjlighet finns att befrukta ett ägg i honan.

I detta försök samlades spermier med hjälp av en konstgjord vagina som träddes på penis i samband med att alpackahanen besteg en alpackahona som var i liggande ställning och fungerade som stimulering. För att späda ut den trögflytande sperman, underlätta hantering samt ha möjlighet till att göra olika analyser av spermierna, användes en lösning med funktionen att bryta upp den geleaktiga vätskan i sperman. På så vis gjordes sperman mer

vattning och inte lika tråddragande. Denna lösning innehåller även ämnen som ska skydda spermierna mot frysskador vid frysning.

För att kontrollera att spermierna är tillräckligt bra finns det en del olika analyser som utvärderar deras kvalité och även deras förmåga till befruktning. I detta försök användes tre olika analyser för att utvärdera spermiekvalitén. Valet av analyser berodde på de rutiner som fanns på laboratoriet i Peru och även tillgängligheten till olika lösningar, färger och mikroskop som krävdes för de olika analyserna. I Peru fanns tillgång till ett vanligt ljusmikroskop och därför valdes analyser som kunde utföras med hjälp av detta mikroskop. Analyserna innefattade utvärdering av spermiernas rörelse, andelen levande och döda spermier i ett prov samt spermiernas morfologi. Två av analyserna gjordes i Sverige (andelen levande och döda samt spermiernas morfologi) på grund av tillgängligheten till bättre mikroskop i Sverige. Detta innebar att proverna i form av utstryk på objektglas transporterades från Peru till Sverige och analyserades någon vecka efter att de samlats in.

Förhoppningen med denna studie är att komma ett steg längre i utvecklingen av artificiell insemination på alpackor. Ännu finns mycket forskning kvar att göra då mycket av reproduktionsfysiologin är okänd eller oförstådd gällande alpackor. Slutsatsen som kan dras av denna studie är att alpackaspermier i hög grad påverkas negativt vid frysning. Genom att genomföra behandling med SLC av spermierna innan frysning kan fler spermier med normal morfologi och färre spermier med skadade svansar överleva frysningsprocessen i jämförelse med om spermierna inte behandlats med SLC.

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