The continued development of artificial insemination technologies in alpacas

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Abstract
Artificial insemination is a tool which is widely used in animal breeding programs. At present the technology is not well developed in camelid species, but there is considerable interest in its application. Previous research aimed at improvement of artificial insemination (AI) in alpacas has had limited success owing to the vastly different reproductive physiology of camelids compared with other domestic species. Factors such as mating length and semen characteristics, particularly the low volume, low sperm concentration and viscous seminal plasma, have precluded the development of viable insemination and semen preservation methods. The Rural Industries Research and Development Council (RIRDC) has funded research into the development of AI in alpacas. This paper will discuss the importance of AI and our plans to improve this technology, including results from experiments where applicable.

Key Words
Artificial insemination, spermatozoa, cryopreservation

Introduction
The first successful attempt at artificial insemination (AI) was conducted in 1776 by Italian physiologist Spallanzani who inseminated a bitch producing three pups. The first lamb born in Australia from AI was in 1936 (Gunn 1936). Both the puppies and lamb were born from AI with fresh semen; since these early attempts there has been considerable effort in the development of semen preservation and AI technology. At present, fresh, chilled and frozen-thawed semen is used extensively for AI in animal breeding and production in Australia and throughout the world.

AI is available for most domestic animal species and some wildlife/companion animals. Breeders have the choice of inseminating females at a natural or synchronised oestrus with fresh, chilled and frozen-thawed semen. Fresh semen is normally extended (to increase the number of AI 'doses') and then inseminated within 1 - 3 hours of collection. Chilled semen is normally extended, cooled to 21, 15, or 4 °C (depending on the species) and kept for 1 – 7 days at this temperature prior to insemination. For freezing, semen is diluted with a cryodiluent (designed specifically to protect sperm during freezing and thawing), frozen and stored in liquid nitrogen until required. Frozen semen is then thawed and inseminated.

The type of oestrus and semen utilised are determined by the species, conditions and the breeder's preference. In species such as cattle and pigs where oestrus detection is easy and fairly reliable a natural oestrus may suffice. However for species such as alpacas who are induced ovulators, a synchronised oestrus would be more appropriate. The type of semen used also depends on the species. Ram and bull sperm are easy to freeze and are relatively tolerant of the freeze-thawing procedure; therefore sheep and cattle AI is based mainly on frozen-thawed sperm. Conversely, boar sperm is not as tolerant to freezing and thawing but remains viable for up to a week when chilled to 15 °C; thus the pig industry is based on AI with chilled semen.

Regardless of the type of AI performed there are a number of benefits including (modified from Evans and Maxwell, 1987):

- ***Increased rate of genetic gain*** **-** By rapid dissemination of desirable genetics as AI increases the number of offspring an elite male produces
- ***Easy transport of genetic material –*** It is cheaper to transport (or import) semen than the whole animal.
- ***Long-term storage of semen*** – Semen banks can be established and if frozen, semen can be used long after the death of a male.

- ***Increased efficiency of breeding*** – Subfertile males can be rapidly identified and eliminated from breeding programs.
- ***Reduction of elimination of the need to maintain males of farm*** – The cost and inconvenience of maintaining males is eliminated which is of benefit to small producers.
- ***Prevention and control of disease*** – AI eliminates male-female contact and therefore controls/prevents the spread of venereal and other diseases.
- ***Use of incapacitated males*** – Males can become injured/infirm which prevents them from mating females. However, AI can overcome this and allow his continued use for breeding.
- ***Use of other technology*** – AI is the foundation and allows incorporation of other technologies such as sperm sexing.

While the advantages of AI considerable there are also the potential for some disadvantages:

- ***Potential inbreeding*** – This may occur when the selection intensity is high particularly in small closed herds. Although AI also has the potential to increase the number of unrelated sires used (as frozen semen can be imported).
- ***Inaccurate breeding*** – This can occur when semen samples are not labeled correctly, accidental errors in inseminations may occur etc.
- ***Spread of disease*** - If sires have not been properly tested for venereal disease AI can potentially spread diseases faster than natural mating.
- ***Reduced fertility*** - AI can often result in lower pregnancy rates than natural mating particularly when oestrus is not properly synchronised or semen is handled incorrectly.
- ***Cost*** - There are costs associated with purchasing semen, hormone preparations, skilled labour and equipment.

At present AI technology is not commercially available for any camelid species. There is considerable interest in the development of this technology from camel and alpaca/llama breeders. There are a number of reasons why AI technology has not been developed in camelids. Camelid reproductive physiology is poorly understood and vastly different from that of other domestic, which has prevented the adaptation of existing methods from other domestic animal species. There have been several reviews of the reproductive physiology of male camelids (Brown 2000; Sumar 1996; Tibary and Vaughan, 2006). Rather than re-iterate the information contained in these publications, this paper will focus on the differences in reproductive physiology which are pertinant to the development of semen preservation and AI technology.

The fundamental differences between other domestic animals and alpacas are:

- ***Lower sperm production by camelids -***
- ***Mating length -*** alpacas also mate for a longer length of time in sternal recumbancy
- ***Ejaculate characteristics*** - low volume, low sperm concentration and high viscosity.

Sperm production is a function of testis size and sperm production per gram of testicular tissue. Comparatively, Llama testes are small, accounting for 0.01 % of body weight, compared with an average of 0.02 – 0.5 % of body weight (Setchell, 1978). Testes size and therefore sperm production rates can be increased to some extent through nutrition. The feeding of lupins to rams increases testes weight independent of body weight, and every extra gram of testes produces 20 x 10^6 sperm per day (Evans and Maxwell 1987). Considerable research is required to elucidate the effects of nutrition on testis size, sperm production and other reproductive parameters.

The problems association with increased mating length in sternal recumbancy have been largely overcome. There have been a considerable number of studies examining the collection of semen from alpacas (reviewed by Vaughan et al., 2003). The majority of these studies have concluded that semen collection with an artificial vagina (AV) fitted inside a wooden mannequin covered in a tanned alpaca hide is the most suitable method. The AV and collecting container are kept warm by wrapping in a small electric blanket.

Ejaculates of low volume/sperm concentration do not pose much of a problem when protocols are established (as would be the case in commercial practice). Commercial ejaculates are diluted with 4 parts extender for every part of semen. However, during the development of semen preservation

protocols it is often necessary to perform large experiments (which can detect synergistic effects of two or more factors) and an ejaculate of low volume cannot be split into enough aliquots to conduct such large experiments. Furthermore, several large experiments are cheaper and faster to perform than numerous small experiments. The concentration of sperm in an ejaculate limits the number of AI doses that can be divided from each ejaculate, which is an important consideration for any AI program. While it is theoretically possible to manipulate the number of sperm a male produces (through nutrition etc) the research has not been conducted in alpacas.

While these factors can all be taken into consideration in commercial practice, the biggest hindrance to the development of semen preservation technology is the mucoid nature of the ejaculate. The viscosity makes semen samples hard to divide into aliquots, smear on slides (to assess integrity and viability) and dilute with extenders (heterogeneous mixing results in poor post-thawing motility). The seminal plasma also interferes with staining of the sperm making assessment of integrity and viability difficult. The viscous nature of the seminal plasma is a result of the presence of mucopolysaccharides (now referred to as glycosaminoglycans; GAGs) which are made up of 95 % long chain polysaccharides and 5 % protein. Histological staining of semen samples at our laboratory has confirmed that GAGs are present in the seminal plasma (unpublished data).

Given these constraints, our approach to the development of semen preservation and AI technology in alpacas was to divide the project into a number of stages (Table 1).

Table 1. The six stages, timeframe and methodologies used to develop semen preservation and AI in alpacas.

Stage	Timeframe	Methodologies
1. Obtaining and training of males for semen collection	July 2005 – January 2006	-Train males to mate with mannequin.
2. Improving the semen collection procedure	January 2006 – July 2006	-Determine the optimal collection procedure by investigating the different AV configurations, the presence of females and an extender during semen collection.
3. Developing protocols for general handling and laboratory assessment of alpaca sperm	January 2006 – July 2006	- Investigate enzymatic and mechanical methods for the removal of seminal plasma viscosity. - Determine the optimal centrifugation speed/time for washing and separation of sperm. - Develop stains for the determination of integrity and viability of alpaca sperm.
4. Developing liquid storage procedures for alpaca sperm	August 2006 – November 2006	-Determine the optimal extender, temperature and dilution rate for the storage of alpaca sperm -Investigate the effects of novel addatives (such as antioxidants) during the chilled storage of alpaca sperm.
5. Developing frozen storage procedures for alpaca sperm	November 2006 – March 2007	Determine the optimal frozen storage of alpaca sperm by investigating the optimal extender, cryoprotectant, freezing rate and packaging. Investigate the use of novel cryoprotectants in the freezing of alpaca sperm.
6. Optimising the artificial insemination procedure in alpacas.	March 2007 – July 2006	Determine optimal artificial insemination procedure by investigating sperm dose and sperm deposition site.

Stage I: Obtaining and training of males for semen collection

Our first step was to approach a number of alpaca breeders to donate males for semen collection. By December 2005, 16 males were housed at The University of Sydney's Animal Reproduction Unit at Cobbitty. After four weeks of training (n= 98 attempted collections), three males show consistent interest in the mannequin and will mate, two others will mate periodically and the remainder

demonstrated little or no interest in the mannequin or real females. With continued training, all but one male have been trained to mate with the mannequin. We observed that some males take readily to mating with the mannequin while others require patient training. The latter males were placed in with a real female and allowed to mate. The mannequin was then introduced and slowly, over time, the males accepted the mannequin. During the training period we observed considerable variation between the males in semen volume and sperm concentration (Figure 1a and 1b respectively). This variation between males and within males (ejaculate variation) has been reported previously for alpacas (Vaughan et al., 2003).

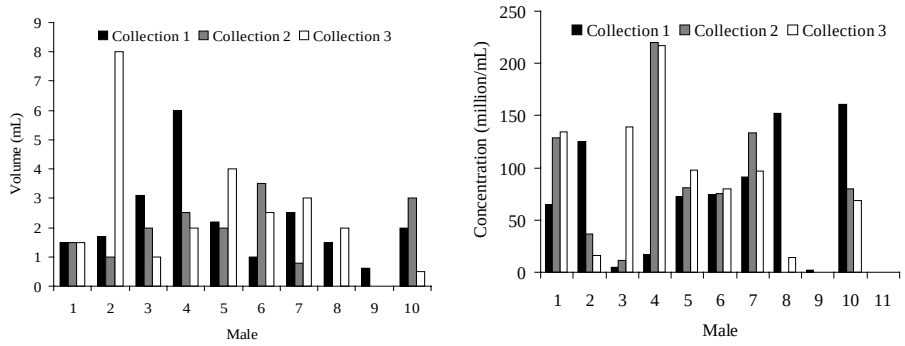

Figure 1a and b. Variation between ejaculate volume and sperm concentration for three sequential ejaculates collected from 10 male alpacas.

Stage II: Improving the semen collection procedure

In this stage, experiments were devised to investigate the effects of re-designing the mannequin and the AV liners to mimic more closely the female reproductive tract, with the aim of increasing the quality and reducing the variability of collected semen. The specifications of the new mannequin were designed to reflect the external dimensions of live females (n=5), and to result in a posture similar to that at natural mating. The new mannequin also contained additional insulation to ensure that the temperature of the AV did not decrease during the collection process thereby permitting indoor and outdoor collections. The new mannequin was tested at the University's Reproduction Unit. Some males found the mannequin too high and too wide. However, the double insulation helped maintain the temperature of the AV during the collection period. Smaller versions of this mannequin have been constructed and are now used routinely.

Given the length of copulation, and the perceived effect of the design of the AV on the subsequent quality of semen, we decided to re-design the AV liner to include a vagina, cervix and uterus. The length of the liner was also increased from 40 cm to 51 cm and a three-ring cervix was included. Three designs for liners were created. The first was the straight liner used by Dr. Vaughan. The second was similar to the design of the first liner, but with the addition of a cervix. The third liner had a variable diameter and a cervix (Figure 2), taking into account the descriptions and dimensions of the female reproductive tract.

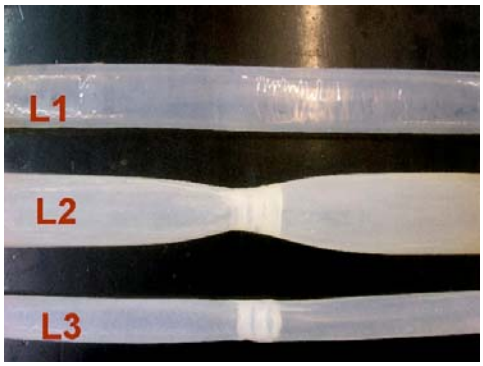

Figure 2. Three AV liner designs tested at The University of Sydney. All liners were made from silicone using an aluminum mould.

Initially liners were made from latex. However, the males would not continue mating with latex liners for more than 1-2 minutes and, in order to avoid any potential problems with the toxicity of latex liners, the decision was made to switch to silicone. To avoid potential toxicity problems owing to residues from the production process, medical grade silicone was used (Prosil 8®; Barnes Products Pty. Ltd.; Bankstown, NSW, Australia) and the liners were produced "in house" at The University of Sydney.

When the collections were made with the first liner (constant diameter, no cervix) the penis could be seen hitting the collection glass. When using the second liner (with the constant diameter and cervix) males appeared to mate without problems but no sperm were found in four of four collections. Males mating with the third liner showed discomfort and retrieved their penises from the AV, suggesting that the cervix did not adequately mimic that of a real female. The decision was then made to re-test the straight liner with a cervix fitted into the AV.

The final experiment in this phase will be to test the benefits or otherwise of including extender in the semen collection container. During mating, which may take up to 20 minutes, the semen is exposed to air and additional oxygen due to frothing in the collection vessel. This is potentially detrimental to sperm survival owing to the development of reactive oxygen species causing lipid peroxidation and membrane damage. There has been some suggestion that the addition of extender, possibly containing antioxidants, to the collection container may improve semen quality and prolong sperm viability.

Stage III: Develop protocols for general handling and laboratory assessment of alpaca semen

Basic laboratory handling procedures and staining protocols are paramount to all experimental work and are not well established for alpaca semen. We have commenced research to determine the appropriate handling medium for alpaca sperm, and to modify protocols for staining alpaca sperm. Our current data suggests that Androhep® (Minitube, Germany) is a suitable medium for the general dilution and handling of alpaca sperm. Androhep is commercially available, cheap ($13 per litre) and has been used at the University for the processing and preservation of sheep, cattle, and pig semen.

Density gradient separation of the motile from the immotile sperm is another fundamental research technique. Camelid sperm do not progress through the standard 94/45 % Percoll®/ Puresperm® gradients, and we have determined that a 45/22.5% gradient is suitable for the separation of motile and immotile alpaca sperm. Further research is continuing to determine the optimal speed and time required for centrifugation of alpaca sperm.

Previous studies have used non-fluorescent stains (such as Hancock's stain) to examine sperm membrane integrity. FITC-PNA staining protocols (used to examine sperm membrane integrity) are currently being modified for use with alpaca sperm. Preliminary data suggests that the gelatinous seminal plasma interferes with the penetration of the stain into the sperm membranes. However, this stain is well suited to epididymal sperm. The determination of membrane integrity in ejaculated sperm is done using a Giemsa stain which is not affected by the presence of the seminal plasma.

The first of our viscosity removal experiments has been completed. The experiment investigated the addition of three enzymes (papain, trypsin and collagenase) at four concentrations (0.5, 1.0, 2.0 and 4.0 mg/ml) to the ejaculated semen. The motility of sperm after the addition of papain, collagenase and trypsin at four concentrations are presented in Figure 2. All enzymes reduced the viscosity of the seminal plasma. However, collagenase killed all sperm within 5 minutes of addition. Importantly, for the other enzymes, the sperm displayed strong forward progressive motility, rather than the usual oscillatory motility, within 2 - 3 minutes of their addition.

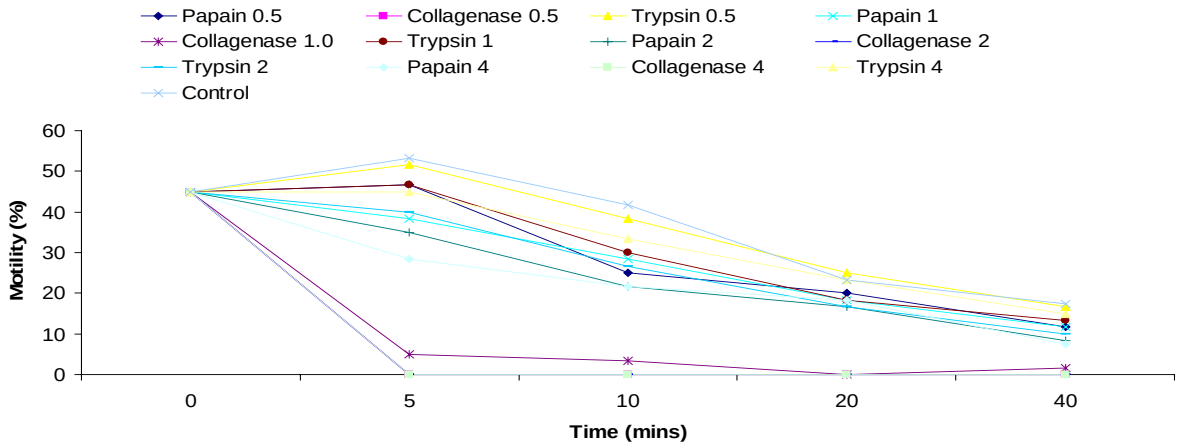

Figure 2. Motility of alpaca sperm 5, 10, 20 and 40 minutes after the addition of papain, trypsin and collagenase at 0.5, 1.0, 2.0 and 4.0 mg/mL.

Motility of sperm for all treatments (including the control) declined over time, as would be expected. The results of this experiment are particularly promising, as they provide a means of removing the gelatinous nature of the ejaculate while maintaining sperm motility. In the near future we will also be investigating several mechanical techniques such as centrifugation, spinning and passing semen through a needle for the removal of viscosity.

Stage IV: Develop protocols for liquid storage of alpaca semen

Several liquid storage experiments are planned to investigate the benefits of different diluents, storage temperatures, dilution rates, gaseous atmospheres and novel additives such as antioxidants. We have an experiment planned which will begin in a few weeks to compare five diluents as potential storage media (Androhep, Triladyl, Biladyl, Salamon's buffer and a lactose-based extender) and two different storage temperatures (4 and 15 °C).

Our preliminary data suggests that the transport of alpaca sperm in the seminal plasma at 37 ^{o}C is detrimental to their survival (0 % motile four hours post-collection). Transport at room temperature produced slightly better results (10 % motility at 12 hours post-collection) and dilution of alpaca sperm with Androhep® and storage at 15 °C increased longevity to around 24 hours.

Stage V: Develop protocols for frozen storage of alpaca semen

Several experiments will be conducted using both ejaculated and epididymal sperm to determine the optimal diluent (extender), cryoprotectant, freezing rate, and packaging for freezing alpaca sperm.

Epididymal sperm respond to freezing and thawing in the same manner as ejaculated sperm but they are not entrapped within the seminal plasma. For this reason they are an ideal model for ejaculated alpaca sperm. Before using epididymal sperm, we established a method of transporting the testes which would allow the sperm to remain viable. A number of trials were conducted using camel and alpaca testes and we now have a method that allows the recovery of sperm that are 60% motile 24 hours after castration.

A number of freezing experiments have been conducted using epididymal sperm. In the first experiment three freezing diluents were compared. Both the citrate and lactose diluents have been used previously for the freezing of alpaca and camel sperm, respectively. The Salamon's buffer is a Tris-based medium which is widely used for the freezing of ram semen. Best survival before freezing and post-thaw was obtained for sperm frozen in the lactose medium (Figure 3).

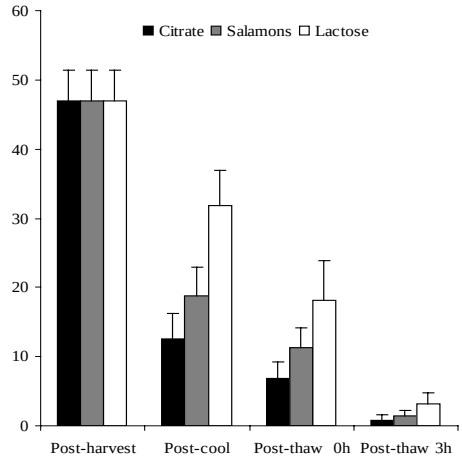

Figure 3. Motility of epididymal sperm at post-harvest, post-cool, post-thaw (0 h) and post-thaw (3h) after dilution in a citrate, Salamon's buffer or lactose diluent.

For importing and exporting sperm straws are required. However for sperm to be used within Australia freezing in pellets is permitted. In a second experiment with epididymal sperm, the freezing of sperm in pellets or straws was investigated. By far the best post-thaw recovery of sperm was obtained after freezing in pellets (Figure 4).

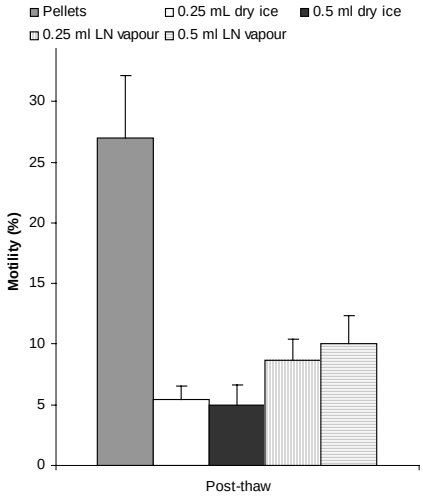

Figure 4. Motility of epididymal sperm post-thaw, after freezing in pellets, 0.25 or 0.5 mL straws on dry ice or over liquid nitrogen vapour.

The results from our experiments so far have demonstrated that epididymal sperm can be transported to for 24 h prior to freezing and still yield sperm which are 30 % motile after thawing.

Conclusion

Camelids have a vastly different reproductive physiology from other domestic and wildlife species. This precludes the adaptation of existing reproductive technologies to alpacas and other camelids. While the development of artificial reproductive technologies is underway in alpacas and there has been success in the preliminary development of a number of procedures, there is still considerable future research required before these technologies can be applied to full commercial AI and semen storage.

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