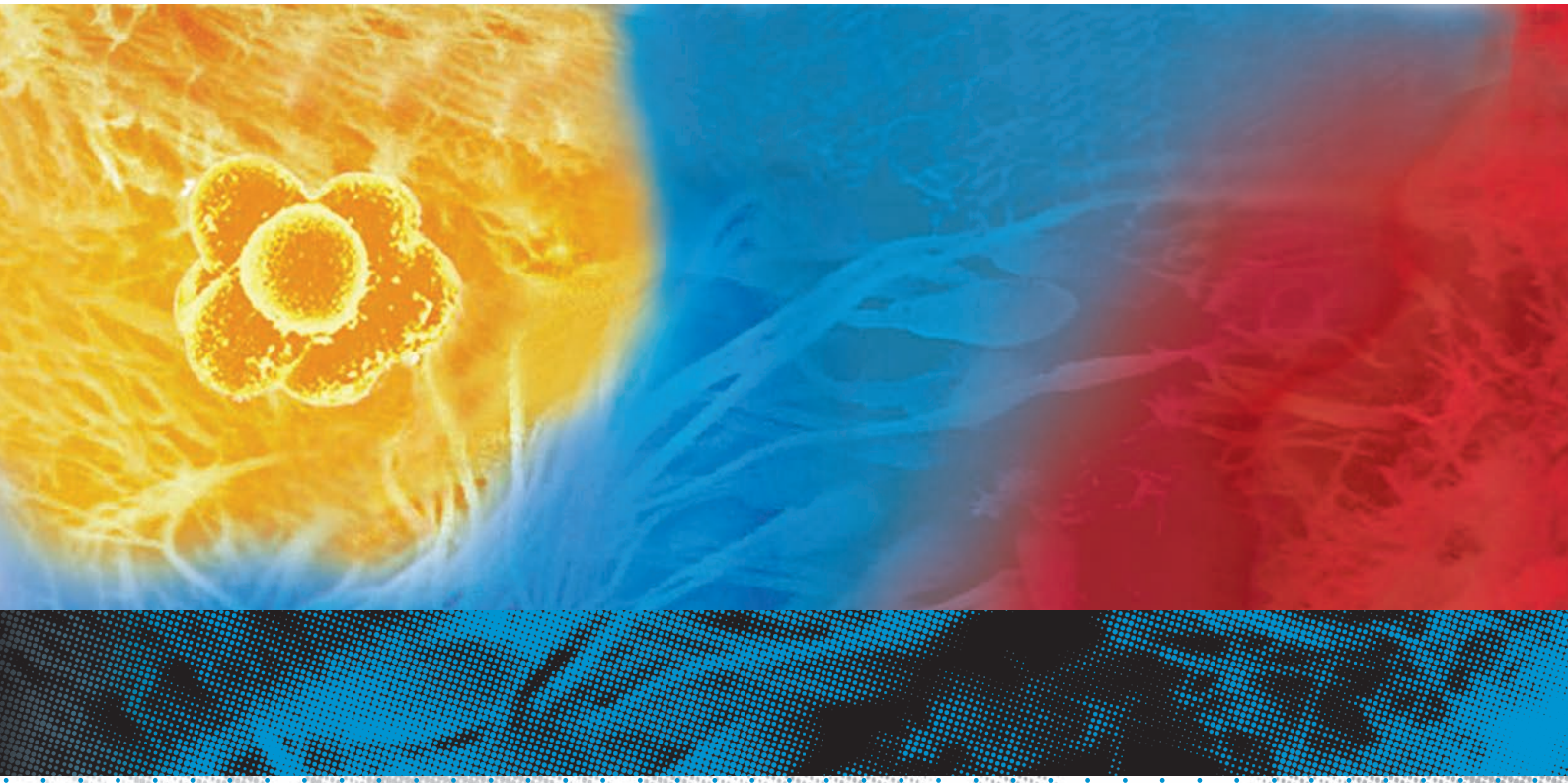

The international AI news from Minitube

SpermNotes®

Special edition: Modern assisted reproduction techniques in small ruminants

Minitube International AG
Hauptstrasse 41
84184 Tiefenbach – Germany
Phone: +49 (0) 8709 9229 0
Fax: +49 (0) 8709 9229 39
E-Mail: minitube@minitube.de

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Modern assisted reproduction techniques in small ruminants

Dr. Ana Kassens, Minitube

Introduction

Looking at the worldwide agricultural production, the most important factor for success seems to be higher efficiency. Nevertheless, efficiency in animal breeding is not only based on optimised care and feeding, but also on improved genetics. As international trade of all kinds of goods enables breeders to get animals and semen from all over the world, genetic improvement has never been easier. In order to take full advantage of these possibilities, modern breeding techniques and equipment have to be utilised. An overview of these techniques with a focus on the latest innovations is given in this issue of the Minitube Sperm Notes.

Semen collection and processing

Semen collection and processing is an essential technique for any kind of modern breeding. As it is the first step towards achieving a pregnancy via artificial insemination (AI), high quality standards have to be achieved regarding hygiene and sperm quality. To guarantee these standards, various aspects have to be taken into account. This starts with concepts for facilities and equipment when building a new semen collection centre and ends up with well-educated, motivated staff and standardised working procedures.
The construction of a stud building is the basis for producing high quality semen doses as it provides the framework for an optimised workflow under high hygienic aspects.

An example of a construction plan for a sheep semen collection facility is given in figure 1.

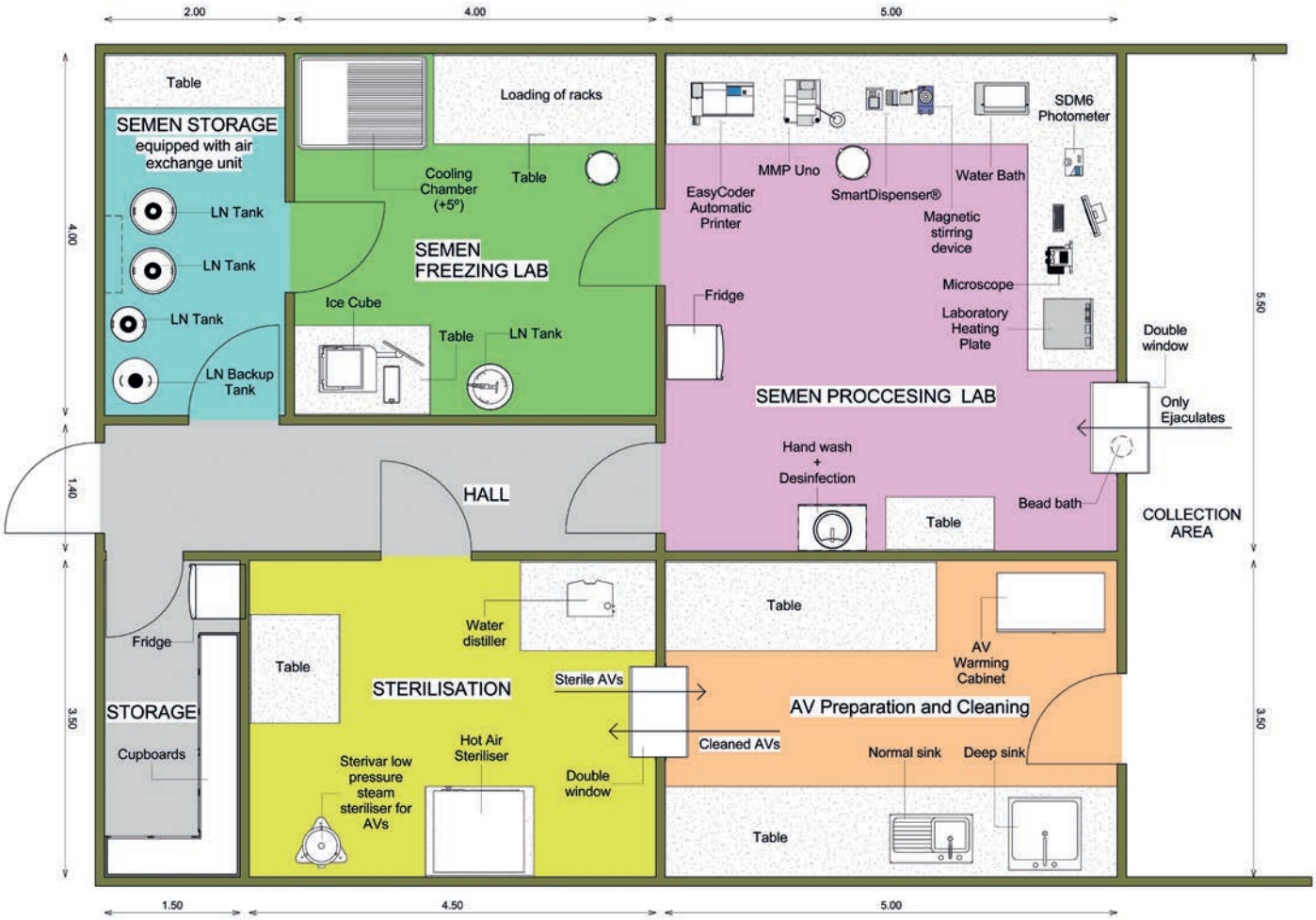

Figure 1: Semen processing facility for small ruminants

Nevertheless, producing and providing a good product is not the only issue a semen collection centre has to deal with. In times of a highly competitive market, documentation of quality control and its demonstration to the customer is also (if not even more) important. In the case of frozen semen, it is nearly impossible for the client to test its quality in a daily routine. Therefore he has to rely on quality control and quality standards implemented in the semen processing lab. One method to give the client the proof of having bought a premium product is an automated system for semen quality control. CASA (computer assisted sperm analysis) systems like AndroVision® provide an objective analysis which is documented and can be delivered to the clients at any time needed. Information on AndroVision® is shown in the excursion below.

Excursion: AndroVision® - The optimal system for quantitative and qualitative analyses of semen

AndroVision® (Figure a) is the ideal system for quick, precise and objective determination of sperm concentration and initial motility during routine work.
Automatic documentation and therefore a quick overview of the collection and quality control history of a male animal under minimal effort is one of the big benefits of a CASA system. Furthermore, with AndroVision® customized sample and quality control reports can be prepared with a few clicks to be handed out to the customer.

Fig. a: AndroVision®, the modular system for semen analysis.

Even small particles (from egg yolk or milk) present in most extenders do not disturb the identification (Figure b).

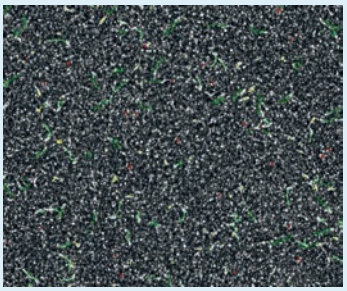

Fig. b: Bull sperm in milk extender

AndroVision allows the motility analysis of a semen sample regarding percentage of sperm showing movement, direction and speed of movement.

The interactive Morphology and Morphometry software module assists the user with a thorough and complete analysis of morphological abnormalities of sperm.

Further advanced analyses via fluorescent stains are available:

1. **Viability / Membrane integrity (Figures c and d)**

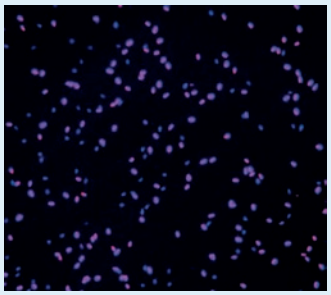

Fig. c: Viability stain: Hoechst 33342/PI

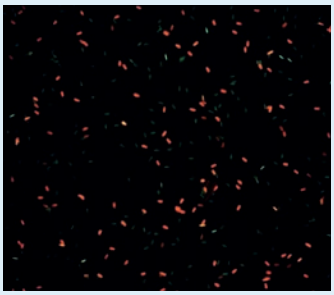

Fig. d: Viability stain with SYBR/PI

2. **Acrosome integrity**

3. **Mitochondrial activity**

The basic principle of those modules is the staining with two dyes: one marks all cells, the other is specific for the criteria used for analysis. As the stains emit light of different frequencies, the different colours can be used to differentiate.
Successfully addressing the challenges of computer assisted semen analysis in the lab or on the production line, AndroVision® proves to be the perfect tool not just for quantitative and qualitative analyses of semen but also for documentation.

The semen collection protocol for sheep and goat is similar to those in the bovine. Semen is usually collected once or twice a week with an artificial vagina with a companion animal used to be mounted. Also likewise to the bull, the small ruminant male reacts with an interruption of mating behaviour on a contact to the penile or preputial mucosa as well as the introduction of the penis into the artificial vagina by the collector.
The ejaculate is then brought into the lab for further evaluation (macroscopic and microscopic evaluation, including density measurement) and processing (dilution and eventually cryopreservation).

Artificial insemination

There are different techniques for artificial insemination (AI) in small ruminants. In very simple terms: the higher the viability of the semen (it is lowered by procedures like cryopreservation or sexing), the less invasive the technique and the less precise the timing for the insemination. Optimising efficiency, most animals are synchronised for artificial insemination using progestogen sponges. Additionally, an injection of PMSG at sponge withdrawal induces ovulation reliably.

As intra or pericervical (intravaginal) insemination is very simple and efficient, up to now mainly fresh semen is used for this insemination technique of small ruminants. An insemination dose of minimum 240 x 10^6 up to 400 x 10^6 sperm (fresh semen) is recommended for intra- and pericervical insemination (Faigl et al 2012 [review]). As in the bovine, insemination with fresh semen should be performed half a day after oestrus is detected (if oestrus detection is realised twice daily). Target for the cervical insemination with fresh semen is 70 % for sheep (Sargison 2008), those intended for goats are similar. While the use of fresh semen limits the benefits of AI as the local variety of rams is limited, the full advantage of AI can be employed by the use of frozen semen.
However, in order to achieve high pregnancy rates with frozen-thawed semen, it has to be deposited directly into the uterus (nearer to the site of fertilisation), as its viability is decreased by the cryopreservation process. Unfortunately, due to anatomical reasons the cervical passage especially in ewes is rarely successful. Therefore, the intrauterine insemination has to be done surgically. To minimise invasion, the technique of laparoscopic AI has been invented in the early 1980ies (Killeen and Caffery, 1982).

In sheep, laparoscopic (as well as cervical) artificial insemination is usually performed at 50-56 hours after sponge removal (dependent on animals being superovulated or not and inseminated with fresh or frozen-thawed semen). In goats, laparoscopic AI with fresh semen is done between 20 to 24 hours after the onset of estrus (Vallet and Baril 1990). Artificial insemination with frozen-thawed semen is performed 43-46 hours after sponge removal. The recommended dose for laparoscopic insemination is 20 x 10^6 spermatozoa (Cseh et al., 2012).

The intended pregnancy rates for laparoscopic intrauterine insemination in sheep are 75 % and 65 %, with fresh and frozen-thawed semen, respectively (Sargison 2008). For goats, success rates range between 60 and 80 % (Shipley et al., 2007, Parkinson, 2009), so rates similar to those in sheep should be targeted.

Previous to laparoscopic AI, a feed and water deprivation is recommended by many experts. The procedure itself is done under sedation. The animal is then fixed in a dorsal position with the head slightly downwards (a cradle is very useful, see figure 2). This helps to keep away any pressure from the intestine.

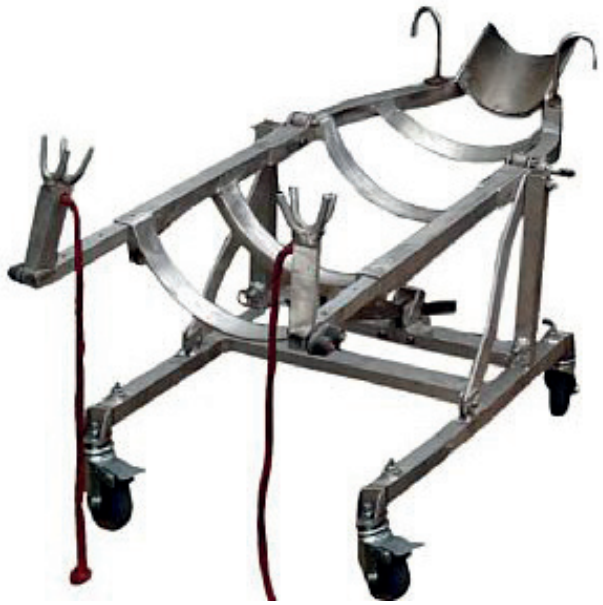

Figure 2: Cradle for fixation of small ruminants (Minitube)

Two incisions for accesses are made cranial of the udder to get an optimal view on the uterus. The trocars are entered into the abdomen and it is inflated with air. A general overview is obtained and ovaries are examined for the preovulatory follicles.
Meanwhile, the Lap AI gun (Figure 3) has to be prepared.

Figure 3: Lap AI gun (Minitube)

Two types of Robertson pipettes are available for laparoscopic AI with a different length of the pipettes needle part. If animals are superovulated and therefore have a thicker uterine wall, the needle part of the pipette has to be longer (for comparison of needles see figure 4).

Figure 4: Different needle length of different type of Robertson pipettes (Minitube)

The apex of the Lap AI gun is brought through the trocar sleeve and semen is injected into the uterine horn ipsilateral to the preovulatory follicle(s). An easy release of the semen ensures the intraluminal position of the pipette`s needle. Additionally, the Semen can be coloured with PBS blue. When the semen is applied into the uterus wall the blue colour will make this visible. The Lap AI gun is taken out, air is deflated and incisions are treated. The animal is brought into a quiet surrounding to recover.

Fatalities are possible, when the abdominal aorta is pierced with an uncontrolled and brutal insertion of the first trocar. It is possible to pierce a full rumen as well as a full bladder. Operators should be well trained (Dovenski et al., 2013).

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Multiple ovulation and embryo transfer

Multiple ovulation and embryo transfer (MOET) is the most widely used procedure to increase the number of offspring of donors with high genetic value. This results in the enhancement of genetic improvement, which enables to adapt more quickly to external requirements (market, changing climate etc.).
Dependent on breed, climate, technique of flushing and various other factors, recovery rates (embryos/corpora lutea) are between 60% and 70%. This is in accordance with rates achieved via surgical recovery in cattle (Betteridge 1977). The number of ovulations and therefore recovered embryos is - besides a high individual variability - very much dependent on breed (Baril and Guignot 2010).

While the technique is widespread in the bovine, its use in sheep and goat is limited. One reason is the higher effort of the procedure in small ruminants, another might be the limited access to frozen semen, which limits the efficiency regarding genetic improvement (see section above). Furthermore, the (financial) value of the individual is not as high as in the bovine.

Nevertheless, according to the International Embryo Transfer Society (IETS) statistics, from 2007 to 2013 a total number of 146.039 and 14.681 embryos have been produced in sheep and goat, respectively (December newsletters of IETS 2008-2014). So regardless of effects of the worldwide financial crisis a few years ago, the demand for animals with extraordinary genetic potential is high.
An example for a facility for embryo production is shown in figure 5. The requirements are the same as for the semen collection facility (easy to clean and disinfect, no or minor light reflections…).

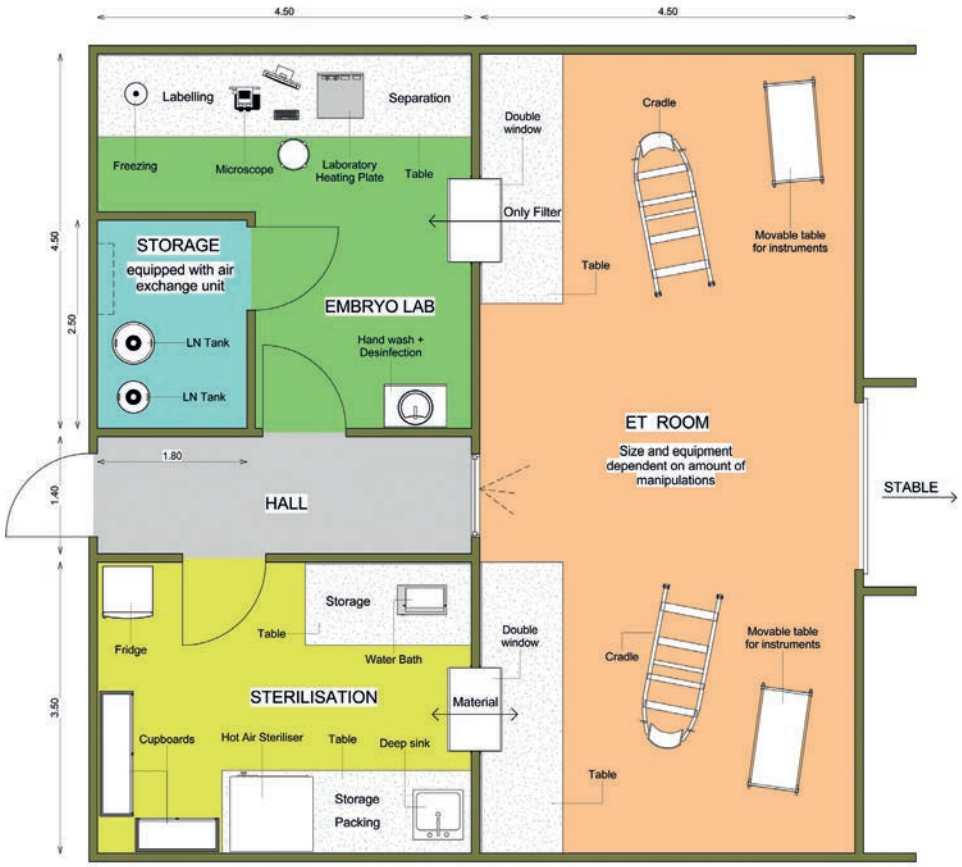

Figure 5: Example for an embryo transfer facility for small ruminants

An embryo transfer program in small ruminants includes the steps listed below.

Superovulation and embryo recovery in donors:

1. **Superovulation**
   The growth stimulation of multiple follicles in sheep and goat is mostly achieved by the application of equine chorionic gonadotropin (eCG) or follicle stimulating hormone (FSH). While eCG is given in a single dose, follicle stimulating hormone is injected twice per day (morning and evening) in a decreasing dose (Grazul-Bilska et al., 2001). Another option is the simultaneous injection of eCG and FSH in a lowered dose. This superstimulation is applied during the last days of a progesterone exposure of 12-17 days. Additionally, gonadotropin releasing hormone (GnRH) analogues can be used. On one hand it can be used to ablate a dominant follicle (disturbing the ovarian response on superstimulation), on the other hand it can be utilised to reduce the interval from first to last ovulation. The latter is important especially when frozen-thawed semen is used.

2. **Insemination**
   As details on insemination have already been mentioned above, only MOET-specific facts are brought up in this section.
   Due to the fact that sperm transport is lowered by superovulation (Evans and Armstrong 1984), superovulated animals should be inseminated into the uterus. Furthermore, fertilisation rate of oocytes in multi-ovulated females is lower compared to that in non-treated control animals (Moore and Eppleston 1979; Armstrong and Evans 1984). Unfortunately, this cannot be compensated by more inseminations or by increasing sperm concentration in the seminal dose (Vallet et al. 1992; Brebion et al. 1992).
   So to achieve optimised results, superovulated females should be inseminated with semen doses of highly fertile males.

3. **Uterine flushing for embryos**
   Embryo recovery is usually performed surgically 6-7 days following insemination. Therefore recovered embryonic stages are morulae and blastocysts.
   In goats, a transcervical uterine flushing can be performed in 60-100 % of the females. Unfortunately, it is associated with lower recovery rates than laparoscopic procedures (Flores-Foxworth, 1992 [abstract]) and is therefore not commonly used. In sheep, a surgical recovery is essential due to the formation of the cervix, which prevents a passage of a catheter.
   The surgical recovery is the most critical step of the superovulation as it may lead to adhesions after this procedure and as a consequence reduce the number of flushings obtainable from one donor (Nellenschulte and Niemann 1992). Due to these consequences of laparotomic uterine flushing, attempts are made to establish laparoscopic flushing procedures. This is associated with lower recovery rates for embryos, but recovery rates will stay stable in repeated flushings (Vallet et al., 1987 [abstract]).
   The surgical uterine embryo flushing is performed under general anaesthesia in a supine position. The uterus is exteriorized and both horns are flushed separately. The flushing media is brought into the uterus near the horn tips via an injection catheter and lead away into a filter through an 8 CH flushing catheter (positioned close to the uterine bifurcation). For hygienic reasons, a tubing between the flushing catheter and the filter should be used to locate the filter out of the operation field. For an overview see figure 6.

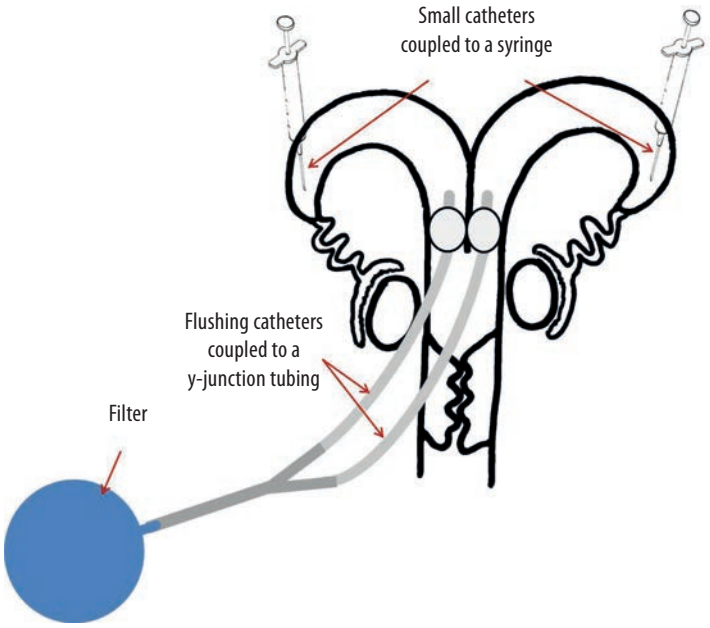

Figure 6: Overview of a surgical uterine flushing for embryos in sheep using 2 catheters simultaneously.

When the flushing procedure is finished, embryos are separated from the flushing medium and classified according to their morphology. This allows a gross prediction for the pregnancy rate which can be achieved (see table 1).

Table 1: Survival rate of embryos of different quality grades in Scottish Blackface breed ewes (Bari et al. 2003)

Embryo grade	Transferred embryos (n)	Embryo survival (%)
Grade 1	825	75.6[a]
Grade 2	550	73.6[a]
Grade 3	114	61.4[b]
Grade 4	24	37.5[b]

Different letters indicate statistical differences (χ^2 test; $P<0.05$)

To achieve good pregnancy rates, the handling of the embryos is quite essential. Temperature should be constant at nearly body

temperature and stress like light should be avoided as far as possible. Additionally, a holding media for storage until transfer should be used to prevent vitality loss of embryos. Alternatively to fresh transfer, embryos can be cryopreserved for a later transfer (to spare out the synchronisation of recipients or for trade reasons).

Synchronisation of recipients:

1. **Oestrus synchronisation**

To ensure optimised pregnancy rates after embryo transfer, the difference between cycle stage of recipient and donor should not exceed/ undercut one day.

The oestrus synchronisation of recipients during the breeding season can be achieved: by prolonging the cycles with progesterone or shortening the cycles with prostaglandin 2α ($PGF_{2\alpha}$) application. Both approaches reveal similar success rates.

Progesterone application mimics a functional corpus luteum (CL) and therefore extends the oestrus cycle. To ensure the absence of a functional CL after withdrawal of progesterone, the animal should be supplemented at minimum the lifespan of a natural CL. If a shorter supplementation is realised, animals should be additionally treated with $PGF_{2\alpha}$. Following the withdrawal, LH increases and oestrus and ovulation will be synchronised. For a higher degree of synchronisation some practitioners recommend the application of eCG at the end of progesterone supplementation. There are several progestogens available on the market, which are supplemented by feeding, implantation under the skin, or insertion into the vagina as sponges or plastic vehicles.

$PGF_{2\alpha}$ application induces luteolysis and therefore terminates the so called progesterone block which inhibits a rise in LH. In a natural cycle it is released at day 11 or 12 after estrus. Luteolysis is followed be a rise in LH, estrus and ovulation. It is important to take into account, that there is a refractory period of about 5 days after estrus in which the CL will not luteolyse. To work irrespective of stage of cycle and for a higher level of synchronisation, $PGF_{2\alpha}$ is provided twice 9-11 days apart.

The rate of females coming into heat 36 to 96 hours after synchronisation will be between 80 and 90 %, or even more (Bowridge et al., 2013, Silva et al., 2010).

An oestrus induction and synchronisation outside of the breeding season is not recommended. Different approaches such as a combination of progestogen treatment and ram introduction (with or without additional $PGF_{2\alpha}$ application) or melatonin supplementation have been tried with limited success.

2. **Embryo Transfer**

The actual transfer of embryos is done utilising the laparoscopic technique. In goats a cervical transfer is possible in most animals, but is still fairly used.

As the laparoscopic procedure for insemination is similar and already mentioned above, only embryo transfer (ET) specific facts will be discussed here. In summary, during the laparoscopic procedure the animal is checked for CLs (gross appearance and number). During this, manipulation of genital tract should be as minimum as possible. Following CL check, embryos are transferred into the ipsilateral horn(s) of the CL. One to three embryos are used for ET per recipient. Nevertheless, overall efficiency regarding lambs produced/transferred embryos is higher when only one embryo is transferred (Cseh and Seregi, 1993).

The stage of cycle should be nearly synchronous (+/- 1 day is generally accepted), as close synchronization is a prerequisite for normal development of transferred embryos (Rowson and Moor, 1966). Pregnancy rates are reported to be between 60-70 % with a few percent less when cryopreserved embryos are transferred (Gibbons and Cueto 2011).

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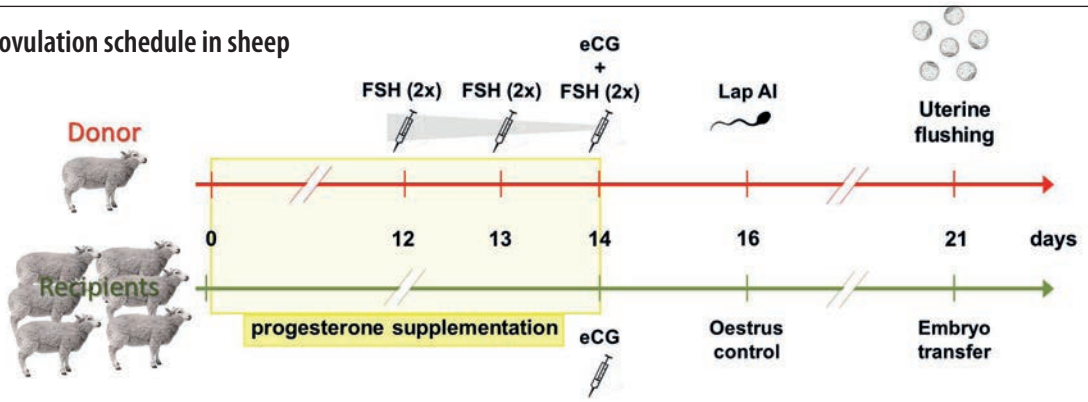
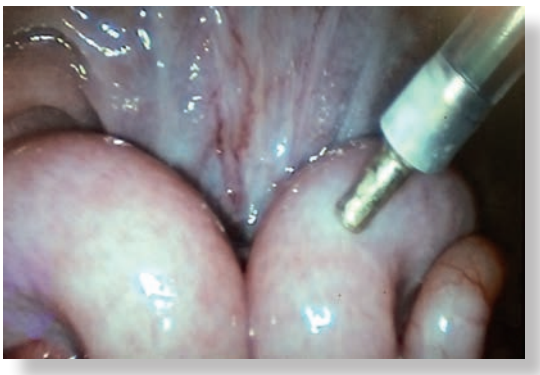
Example for superovulation schedule in sheep

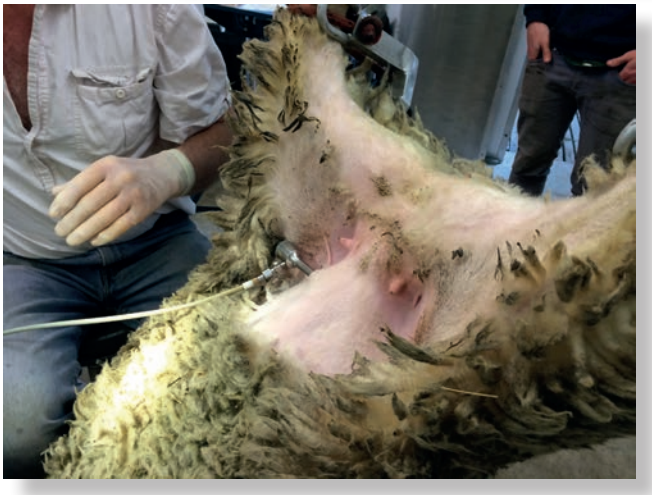


Minitube training courses in ovine and caprine reproduction techniques

Minitube offers a variety of seminars and training opportunities in ovine/caprine reproduction techniques. These are also applicable for deer. The seminars begin with a get-together meeting to discuss each participant's experience and practical expertise.

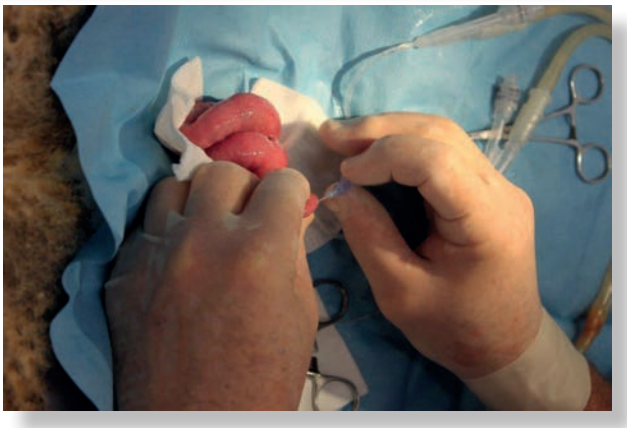
A comprehensive theory presentation is followed by hands-on lessons during which the trainees repeatedly perform all steps under the supervision of the trainer. Minitube Training sessions always offer many useful troubleshooting measures. The participants benefit greatly from a "learning by doing" approach.





Seminars

- Laparoscopic artificial insemination (3-5 days)
- Embryo flushing and transfer (3-5 days)
- Semen collection, evaluation and freezing (2 days)
- Embryology



Lecturers

Our trainers are very experienced in practical laparoscopic AI techniques and embryo transfer in small ruminants. They have trained veterinarians and technicians in these techniques for more than 30 years.

Organisational Information

Training locations: locations provided for customers upon demand

Maximum number of participants per seminar: 5

We organise guest house / hotel accommodation upon demand.

If you are interested in a seminar please contact your local sales representative or send an e-mail to seminar@minitube.de.

Please see the complete seminar information at www.minitube.de/DE_eng/Products-Services/Small-Ruminants.

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