Reproductive biotechnologies for genetic improvement in sheep

Biotecnologias reprodutivas para melhoria genética em ovelhas

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Abstract

Artificial insemination is considered a rapid and appropriate reproductive biotechnology for disseminating characteristics of high genetic value rams. We describe different methods of estrus synchronization for the use in several artificial insemination protocols, thus giving the possibility to achieve greater participation of these reproductive biotechnologies in ovine genetic improvement. Another reproductive assisted technology for genetic improvement in sheep is multiple ovulation for embryo transfer (ET). Briefly, we present our advances to obtein a considerable number of offspring per donor sheep at reduced cost, and the development of a simple vitrification technique for sheep embryo cryoconservation, that will allow a greater use of ET as a tool in genetic improvement programs.

Keywords: artificial insemination, embryo transfer, oestrus synchronization, vitrification.

Resumo

Inseminação artificial é considerada uma biotecnia de reprodução rápida e apropriada para a disseminação de características de alto valor genético em carneiros. Descrevemos diferentes métodos de sincronização de estro para uso em diversos protocolos de inseminação artificial, dando assim a possiblidade de alcançar maior participação destas biotecnologias de reprodução em melhoria de genética ovina. Outra tecnologia de reprodução assistida para melhoria genética em ovelhas é a ovulação múltipla para transferência de embrião (TE). Brevemente apresentamos nossos avanços para obter um número considerável de prole por ovelha doadora a custo reduzido e o desenvolvimento de uma técnica simples de vitrificação para criopreservação de embrião de ovelha, o que permitirá um uso maior de TE como ferramenta em programas de melhoria genética.

Palavras-chave*: inseminação artificial, transferência de embrião, sincronização de copulação, vitrificação.*

The possibility of preserving sheep semen has been a priority topic of research, since artificial insemination (AI) was considered a rapid and appropriate reproductive biotechnology for disseminating characteristics of high genetic value rams. Studies for seminal preservation have successfully extended the fertile life of sperm for long periods, using methods that include the reduction of cellular metabolism and/or the use of cryoprotectants (Salamon and Maxwell, 2000). In turn, AI has facilitated regional and international diffusion of genes of genetically superior males, avoiding transport, thus reducing stress and physical and sanitary risk.

The estrus synchronization methods are a very useful tool for AI programs. In Argentina, two hormonal treatments are the most frequently used: synthetic prostaglandins or progestagen intravaginal sponges (MAP, 60 mg). Prostaglandins can be used only during the breeding season. They provoke luteal regression from day 4 to day 14 of the sheep estrous cycle. Currently, we recommend the use of two doses of prostaglandins (Cloprostenol, 125 ug/animal), separated 14 days from each other; with this treatment, it is possible to obtain a 90% concentration of estrus within 2 days (Cueto and Gibbons, 2008). The second treatment involves the use of progestagen sponges which are placed in the vagina for 12-14 days; the application of eCG (equine chorionic gonadotrophin) at the end of the progestagen treatment, induces a synchronized ovulation (Maxwell, 1986). In general, we recommend a dose of 200 IU of eCG in Merino ewes and 300 IU for Corriedale and Texel breeds. In the Merino breed, between 36 and 60 hours after sponge removal and 200 IU eCG application, 85-95% of ewes exhibited heat, reaching the highest concentration of estrus between 36 and 48 hours after the end of the progestagen treatment. In most sheep, ovulation occur around 60 hours post-sponge removal (Walker et al., 1989b). However, when estrus synchronization treatments are carried out outside the breeding season, an increase in the dose of eCG (250-400 IU), according to breed and production system, is recommended (Catalano et al., 1997). Always, the eCG application should be set at a minimum dose according to each breed, ewe body condition and reproductive season. High doses of eCG may cause multiple ovulation and pregnancies, potentially leading to loss of animals by toxemia of pregnancy and perinatal mortality. It can also alter oocyte maturation (Murray et al., 1994), which would reduce pregnancy rates.

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In general, there are several methods for the use of non-frozen semen in sheep. The semen collected by artificial vagina can be used immediately (fresh semen) or preserved for short periods of 8 hours (semen chilled at 15°C) or 12 hours (semen cooled at 5°C). Insemination doses when using cooled, chilled or fresh semen must present a minimum sperm concentration of 300, 150 and 100 million spermatozoa per inseminated ewe, respectively. We have obtained pregnancy rates of 40% (Naim et al., 2009), 55% (Gibbons et al., 2008) and 65% (Cueto and Gibbons, 2010), by performing fixed-timed artificial insemination (FTAI, 52-56 hours after the end of the progestagen treatment), via vagina, with cooled, chilled or fresh semen, respectively. When AI with fresh semen is performed at estrus detection, mean pregnancy rates of 70% are achieved (Table 1).

The use of frozen semen has facilitated worldwide transport of genetic material, allowing national and international dissemination of superior genes. The intrauterine insemination by laparoscopy with frozen semen enables the placement of the semen inside the lumen of the uterine horns. In Argentine, during 10 years, we used intrauterine AI with frozen semen for the Genetic Evaluation of Merino Breed (Cardellino and Mueller, 2009). A total of 6015 ewes was synchronized with progestagen sponges (60 mg MAP) plus 200 IU eCG treatment and inseminated with frozen semen of 71 rams. Mean pregnancy rates obtained were 50% (FTAI performed at 60 ± 2 hours after hormonal treatment) and 65% (AI performed 12 hours after estrus detection) (Cueto and Gibbons, 2004). This technique gave us the possibility of evaluating rams from Argentine and foreign origins, even when not contemporaneous, and avoiding male transport.

In our experience, laparoscopic AI with frozen semen is more efficient when it is done 12 hours after estrus detection (Cueto and Gibbons, 2005). Values of reproductive efficiency for different alternatives of estrus synchronization and AI protocols with fresh or frozen semen are presented in Table 1.

Table 1. Reproductive efficiency of vaginal insemination with fresh semen and laparoscopic insemination with frozen semen for different estrus synchronization protocols in Merino sheep.

Estrus synchronization protocol	AI method	Estrus concentration (%)	Labor time (days)	Vaginal AI Fresh semen		Laparoscopic AI Frozen semen	
				Pregnancy rate (%)	Global efficiency (%)[1]	Pregnancy rate (%)	Global efficiency (%)[1]
PF2 alpha x 2	Detected estrus	91	2	71	65	--	--
PF2 alpha x 2	FTAI*	--	0.5	60-70	60-70	--	--
MAP sponge + 200 IU eCG	Detected estrus	90	1.5	70	63	65	59
MAP Sponge + 200 IU eCG	FTAI**	--	0.5	65	65	51	51

[1]Number of pregnant ewes/number of hormonally synchronized ewes x 100.
*Fresh semen: AI 52-56 hours after the 2nd application of PF2 alpha.
**Fresh semen: AI 52-56 hours after sponge removal. Frozen semen: 58-62 hours after sponge removal.

The FTAI with fresh semen, using synchronized estrus with intravaginal sponges or double dose of prostaglandins (Gibbons et al., 2010a), yields an acceptable overall reproductive efficiency; besides, daily estrus detection is avoided and insemination labor time is reduced. In relation to laparoscopic AI with frozen semen, it is clear that fertility achieved after estrus detection is higher than that obtained after FTAI, indicating the importance of time of frozen semen deposition with regards to time of ovulation. Differences in fertility rates for FTAI with fresh and frozen semen could be due to the reduced viability of frozen semen, which begins to decline from 6 to 12 hours post insemination (Walker et al., 1989a).

Although global efficiency with frozen semen after FTAI (51%) is similar to that observed for AI with heat detection (59%), it should be borne in mind that with the former technique, a greater number of semen doses of high cost is used, because every sheep and not only those detected in estrus, are inseminated. In general, when genetic improvement programs need to apply AI with frozen semen, we recommend performing AI after estrus detection, because it enables better utilization of expensive frozen semen and because non-measured variables (time of semen deposition with regards to time of ovulation, estrus induction and synchronization rates) can reduce pregnancy percentages when applying FTAI.

In conclusion, only by setting up different estrus synchronization and artificial insemination protocols according to each production system, will it be possible to achieve greater participation of these reproductive biotechnologies in ovine genetic improvement.

Certainly, the natural reproductive potential is a limiting factor for genetic progress in sheep. In this way, another reproductive assisted technology is the multiple ovulation embryo transfer (MOET), which multiplies the reproductive potential of superior females, by using low genetic ewes as recipients of genetically superior embryos. It is a tool for a rapid improvement in the genetic level of different breeds or cross-breeds and

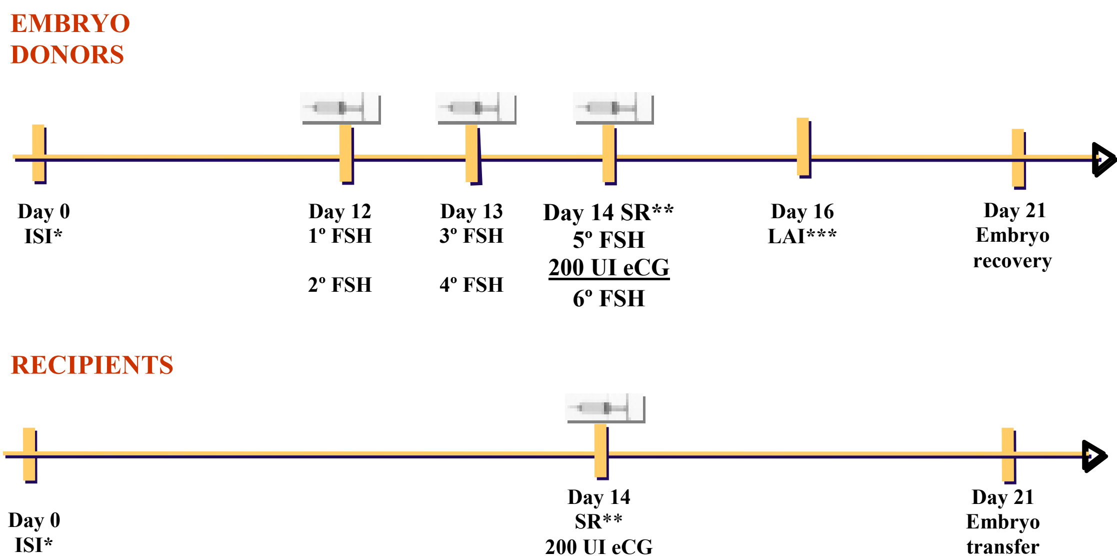
can improve the production of meat, milk or wool. The donors must be selected taking into account their genetic value and on the basis of adequate criteria to improve productivity of each breed. The MOET requires maximizing embryo production and survival in order to obtain various offspring of high genetic value, by taking advantage of the great oocytes reserve in the ovary. In addition, genetically superior ewes can be used in MOET programs more than once. The AI and MOET, together, constitute excellent tools for genetically improving flocks and herds isolated from suppliers of male improvers.

The following points must be carefully considered before undertaking a MOET program: Factors involved in the response to multiple ovulation. Induction of multiple ovulation in recipients and estrus synchronization between donor and recipient. Fertilization of donors. Embryo recovery techniques. Assessment of embryo quality. Embryo transfer techniques. Protocols for embryo preservation (See each point in Baril et al., 1995). General factors such as reproductive condition, sanitary and nutritional aspects are also taken into consideration for MOET programs.

The intrinsic factor of each animal plays a primary role in the ***response to multiple ovulation treatment.*** Individual variability in hormonal response to multiple ovulation is conditioned by extrinsic factors (source, purity of gonadotropins and protocol of administration) as well as intrinsic ones (breed, nutrition, age, reproductive status; Cognie et al., 2003; Gonzalez-Bulnes et al., 2004; Ammoun et al., 2006). At present, there are several not well-known factors that control folliculogenesis, follicle growth, oocyte maturation, ovulation and fertilization. Further advances in understanding their functions and interrelations will determine greater efficiency in hormonal multiple ovulation treatments, and lead to reduced costs and greater benefits derived from this technique.

There are many treatments for the ***induction of multiple ovulations*** in sheep. In Merino breed and during the breeding season, we have proved it is possible to reduce the total dose per ewe from 200 to 80 mg of NIH-FSH-P1, distributed in 6 applications every 12 hours of 18, 18, 14, 14, 8 and 8 mg, starting on the morning of day 12 after sponge insertion. The 5th application coincides with the withdrawal of the intravaginal sponge together with a 200 IU eCG im (Novormon 5000, Syntex, Argentina; Gibbons et al., 2010b). In this way, although there is a lower ovulation rate (13 vs. 17.5 corpora lutea) due to low-dose treatment, a similar number and quality of embryos are obtained (6 vs. 6.5 embryos and 5 vs 5.5 good quality embryos per ewe donor). With this treatment, more than 80% of sheep donors show estrus 36 hours following pessary removal. Reducing the high cost of FSH to nearly one third implies an economic benefit in commercial ET programs. It must be emphasized that it will always be necessary to determine the minimum dose of FSH, adjusted by breed, time of year, production system, etc. If the option is to carry out successive embryo recoveries in the same ewes, the source of FSH used for multiple ovulation treatments should be considered. Baril et al. (1992) proved that using porcine FSH for successive hormonal treatments is less effective than employing ovine or caprine FSH. This is attributed to the development of heterospecific anti-gonadotrophin antibodies in each treatment.

The synchronization of estrus in recipients by means of progestagen treatment is performed at the same time as for female donors (Fig. 1). The purpose is that both recipients and donors present the same day of the estrous cycle at the time of embryo recovery and transfer.



*ISI: Intravaginal sponge insertion.
**SR: Sponge removal.
***LAI: Laparoscopic artificial insemination with frozen semen

Figure 1. Hormonal treatment schedule for multiple ovulation in Merino ewe donors and embryo recipients for embryo transfer.

The ***fertilization in multiovulated females*** has shown great variability according to fertilization technique employed, time of AI and individual ovulatory response to hormonal treatments. Currently, mating in corral is performed every 12 hours from the onset until the end of estrus. If AI is used, either with fresh or frozen semen, laparoscopic procedure is recommended: in this way, semen is deposited in the uterine horns and close to the fertilization site. This ensures an increase in fertilization rate as well as a reduction of the insemination dose required. Seminal doses for laparoscopic AI with fresh semen are 80 million spermatozoa (Brebion et al., 1992). A recovery rate of 87% viable embryos is obtained when laparoscopic FTAI with *fresh semen* is performed 32 hours after the onset of estrus (Brebion et al., 1992).

Our experience indicates that FTAI with frozen semen (100 million spermatozoa) can be carried out 42 or 55 hours after progestagen withdrawal, applying GnRH analog (8 ug Buserelin, Receptal) at 36 hours after sponge removal (Wolff et al., 1994). Fertilization efficiency of FTAI using frozen semen has presented variable results. Although Armstrong and Evans (1984) reported 50% fertilization rate, in our experience, the use of GnRH led to higher fertilization percentages of 70-80% (Wolff et al., 1994). However FTAI programs using laparoscopy and frozen semen in MOET programs are very risky: they must only be used when distribution of estrus for a specific population is known, considering the hormonal regime and time of the year. Taking this into account, we recommend performing laparoscopic AI after estrus detection. Ewes detected in heat at 24 hours after sponge removal must be inseminated 24 hours following heat detection; those showing heat 36 or 48 hours after sponge withdrawal, are inseminated 12 hours following estrus detection. In this manner, we achieve fertilization rates close to 90% (Cueto et al., 2010).

Embryo recovery techniques in small ruminants can be performed by surgical or non-surgical procedures and are usually carried out in days 7 or 8 after pessary withdrawal. Surgical procedures are performed under general anesthesia. A combination of Xylazine (2 mg/10 kg, Xylazine 2%) and Ketamine (25 mg/10 kg, Ketamine Clorhidrate), both administered intramuscularly, and local anesthesia (1 cc, Lidocaine 2%) in the surgical field, are applied. Ovulatory response is determined (corpora lutea count), either by exteriorization of the ovaries or by laparoscopic observation.

In Merino and Corriedale ewes, we have used the surgical technique. In brief, embryos are surgically recovered just after laparoscopy, through prepubian laparotomy. Both uterine horns are flushed with PBS plus 10% commercial adult bovine serum, using a blunt needle inserted at the major curvature of the horn, the flushing medium being directed towards a catheter attached with a vascular clamp to the utero-tubal junction. The average time required for embryo recovery per donor is around 15 minutes. Whichever technique is used for embryo recovery, and if it is necessary to make sure that donor females do not become pregnant because of non-recovered embryos, the administration of prostaglandins (125 ug Cloprostenol) after interventions, is recommended (see technique process in Baril et al., 1995).

Normally, the first surgical embryo recovery will produce future adherences thus reducing the efficiency of subsequent embryo recoveries. In Merino ewes, submitted to three successive superovulatory hormonal treatments, we obtained recovery yields of 66, 41 and 35% for the 1st, 2nd and 3rd surgical operations. The embryo recovery rate was higher in the first surgery with respect to the second and third. However, total and transferable embryos obtained (grades 1-2) were only reduced in the third recovery. In this way, successive surgical recovery of embryos yielded an average of 18.5 embryos per donor sheep over a period of 2.5 months (Cueto et al., 2010).

The ***assessment of embryo quality*** is carried out based on morphological aspects. The integrity of the zona pellucida and its sphericity must be observed. Embryo development must correspond to that determined by its date of collection; a 24 hour delay is tolerable. Cells must be clear and present regular boundaries; opacity, if present, indicates degeneration. Partial detachment of cells in the perivitelline space is tolerable if the remainder constitute a uniform cellular mass. This type of morphological examination does not provide an absolute viability test for embryos. However, significant embryo survival differences are found when only regular quality embryos are transferred compared with good or excellent quality embryos (Bari et al., 2003).

The ***embryo transfer*** must take place immediately after collection and, in no case, be more than 2 hours in the embryo preserving commercial medium. For frozen embryos, the time lapse between thawing and transfer is reduced to 20 or 30 minutes. Mostly, we have accomplished embryo transfer by a “semi-laparoscopic” technique whereby the uterine horn is visualized using laparoscopy. A small 1 cm incision is practiced in the abdominal half line and the uterine horn is exposed using a clamp, so as to carry out embryo transfer (semi-surgical embryo transfer). Results obtained in INTA Bariloche for Merino breed, by immediate semi-laparoscopic ET technique, were around 65% of pregnancy (Gibbons and Cueto, 2010). It is very important to consider the time interval between embryo recovery, identification and assessment of embryo quality until time of embryo transfer. The MOET programs must be very well organized and coordinated to ensure optimum results, because of the hard work and high cost involved in their implementation.

The ***embryo preservation*** permits the spread of high value genetic material at local or international scale. It has allowed world-wide diffusion of germoplasm with very low sanitary risk. Also, newborn lambs will acquire local immunological resistance. The vitrification technique, for the preservation of embryos at very low temperatures, is currently under development. The physical principle is based on submitting embryos to a high

cryoprotectant concentration in very low solution volumes, and thus avoiding formation of ice crystals. We have recently published an easy-handling vitrification technique for sheep embryos using plastic tips of micropipettes. Using this methodology *in vitro*, we obtained an embryo hatching rate of 50% for morulae and 81.6% for blastocysts (Gibbons et al., 2008). *In vivo*, we registered embryo survival rates of 42 (morulae) and 47% (blastocysts), and pregnancy rates of 50% for both embryonic stages (2 embryos/recipient; Gibbons et al., 2011).

The last decade has seen greater overall success in embryo survival following vitrification for many species. Advancement in cryopreservation of mammalian oocytes and embryos has almost exclusively been achieved with the new vitrification techniques. In the foreseeable future, technically improved and standardized vitrification methods may replace traditional slow-rate freezing in everyday practice and may offer new perspectives to commercial embryology (Vajta, 2006).

In conclusion, there is no doubt at present that ET is the safest method, when importing different high production biotypes. Increase in international commerce of genetic material through the use of ET, has proved the importance of this technique in providing sanitary guarantees against exotic diseases. Future advances in increasing the number of offspring per donor sheep at reduced cost and the development of new easy embryo conservation techniques, will allow greater use of ET as a tool in genetic improvement programs.

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