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Innovative biotechnologies of reproduction on sheep management

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Abstract. The aim of this paper is to review applicability of some reproductive biotechnologies in sheep towards a more sustainable management of sheep genetic resources under ordinary farming conditions. The concepts are based upon research being carried out in Sardinia (Italy). Artificial insemination without use of hormones to synchronise oestrus was undertaken in organic farming. Ewes were induced to ovulate with the ram effect and oestrous was daily checked 4 times by vasectomised rams. Lambing rates reached 54.1% when fresh semen at 15°C was used, 30% with chilled semen at 4°C for 24 hours and 23.8% for frozen semen. The results are similar to those currently obtained after hormonal treatments. Simplification of embryo transfer protocols aim at reduction of the use of hormones, the vitrification technique to freeze embryos together with the direct transfer of the embryos with OPS-catheter avoiding thus the need of microscope and costly expertise technician. Lambing rates of approximately 60% with either fresh or vitrified embryos may open a new chance to wide spread this sophisticated technique, particularly where geographical and economical aspect may limit the application of MOET.

Keywords. Artificial insemination – Embryo transfer – Ram effect – Vitrification.

Biotechnologies de la reproduction innovantes chez les ovins

Résumé. *L'objectif de ce papier et de discuter l'applicabilité de certaines biotechnologies de la reproduction en vue d'une gestion durable des ressources génétiques ovines sous des conditions de production ordinaires. Les concepts développés sont basés sur une recherche menée en Sardaigne (Italie). L'insémination artificielle sans le recours aux hormones pour la synchronisation des brebis a été testée en conditions d'élevage biologique. L'ovulation et l'œstrus ont été induits par effet bélier et l'œstrus a été contrôlé 4 fois par des béliers vasectomisés. Les taux d'agnelage ont atteint 54,1% lorsque du sperme frais conservé à 15℃ a été utilisé, 30% avec du sperme refroi di pour 24 heures à 4℃ et 23,8% pour le sperme congelé. Les résultats sont comparables à ceux obtenus suite à une synchronisation hormonale des œstrus. La simplification des protocoles de transfert des embryons vise une réduction de l'usage des hormones, la vitrification pour la congélation des embryons et le transfert direct des embryons moyennant un cathéter OPS qui évite le recours au microscope et à l'expertise technique coûteuse. Les taux d'agnelage obtenus d'environ 60% avec des embryons frais ou vitrifiés peuvent rendre cette technique plus accessible dans des zones géographiques présentant des contraintes économiques.*

Mots-clés. *Insémination artificielle – Transfert d'embryons – Effet bélier – vitrification.*

I – Introduction

Demand for animal products in the Mediterranean region will be determined by the population growth, urbanisation, increasing purchasing power and special requirements of consumers. Research for the future animal production in the Mediterranean system should also focus on an efficient utilisation of local genetic resource, innovation and improvement of traditional processing technologies.

Application of Reproductive Technologies such artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) for genetic improvement and small ruminant management has been mostly applied in advanced farm conditions. However, in our days the application of these tools might be taken into consideration for a wider kind of situations: (i) difficult farm conditions

from geographical and economical point of view, (ii) organic farming, (iii) preservation of genetic resources and (iv) endangered livestock.

AI is the most important technique that has been applied in assisted reproductive programmes to enhance the genetic improvement in animal production systems. Traditionally AI programmes in sheep have been carried out under hormonal treatments to induce and synchronise the oestrus cycles. In the last a few years new perspectives have been claimed for the reduction of the use of hormones and cost.

MOET is an important technology mainly for genetic improvement and preservation of endangered species, but it need to be re-considered from different points of view among which: (i) reduction of the use of hormones; (ii) simplification of embryo transfer technique; and (iii) reduction of cost. The aim of this report is to present some alternative procedures in AI and MOET programmes on sheep management looking at more sustainable conditions.

II – Materials and methods

1. Artificial Insemination

A. Natural oestrus without synchronisation of flock

During breeding season in natural conditions about 6-8% of females daily comes in oestrus spontaneously thus vasectomised rams were introduced in a flock of 250 ewes to select animals on heat. Ratio: 1 ram per 20 sheep. Oestrus detection was performed 4 times daily: 8:00 am, 12:00 pm, 16:00 pm and 20:00 pm. Among all the oestrus detection times the first detection (8:00 am) was not considered for AI. AI was carried out "on heat" 24h after the onset of oestrus with 3 different semen preservation techniques: (i) fresh semen (n=155) at 15°C and used within 7 hours after preparation (Colas *et al.*, 1968); (ii) chilled semen (n= 20) at 4°C for 24 hours after preparation (Mara *et al.*, 2005); and (iii) frozen semen (n =21) at -196 °C in liquid nitrogen (Cappai *et al.*, 1989).

B. Oestrus synchronisation of flock with "ram effect"

"Ram Effect" was used to synchronise 47 sheep of organic farm during transition season (May). Vasectomised rams were introduced in a flock at a ratio of 1 ram per 10 ewes. The rams were isolated from ewes the 30th March for 5-6 weeks. The day of rams re-introduction (8th May) was named Day 0. The rams were left in the flock during all period until the starting of presumptive onset of oestrus (Day 17). From this moment oestrus was detected every day (4 times in a day) with the vasectomised rams. AI with fresh semen was carried out 24 h after oestrus detection.

2. In vivo embryo production

A. Superovulation protocol

Twenty Sarda ewes were selected as donors and divided into 2 groups: without sponge (WS) (n=10) and single sponge (SS) (n=10) (control group). The group WS was selected throughout oestrus detection (Day 0) with the introduction of a vasectomized ram in the flock. The ewes were considered to be on oestrus when they show oestrus behaviour and were mated by the vasectomized ram. In the case of SS group, the animals were selected at random and the oestrus cycles were synchronised by insertion of a intravaginal sponge containing 40 mg fluorogestone acetate for 12 days.

Superovulatory treatment consisted of 350 IU of porcine FSH administered in eight decreasing doses at every 12 h starting on day 4 after oestrus detection (Day 0) in the WS group and 48 h before sponge removal in the SS group. A single dose of 125 µg cloprostenol was injected on day 6 after oestrus detection in the WS group to induce luteolysis. All ewes were naturally mated 24 h after cloprostenol injection or sponge removal.

B. Recovery of embryos

Seven days after mating an inguinal mini-laparotomy of 5 cm was performed and the reproductive tract was exteriorised with minimal manipulations to assess the number of corpora lutea (CL). Embryos were recovered by flushing each uterine horn with 20-40 ml flushing media (HTCM 199 + BSA 0.4%), at flushing the recovered embryos were evaluated morphologically using a stereomicroscope and their quality was scored on a scale of 1 to 3 (Niemann *et al.*, 1981); only embryos with quality 1-2 were kept less than 30 min at room temperature in HTCM 199 + BSA 0.4% and considered for to be transferred or vitrified. Data on corpora lutea (CL), embryos recovered (ER), embryos fertilized (EF) and high quality embryos (EQ1) per ewe were analysed by ANOVA, while, recovery (RR), fertility (FR) and embryo high quality (Q1R) rates per treatment by Chi square analysis.

C. Vitrification of blastocysts

The vitrification procedure employed was based on the method originally designed by Yang *et al.* (1992) and Vajta *et al.* (1998) for cow embryos and modified for ovine embryos by Dattena *et al.* (2000). Briefly, all vitrification solutions were prepared using Dulbecco's PBS supplemented with 0.3 mM sodium pyruvate, 3.3 mM glucose and 20% FBS. Expanded blastocysts were exposed at room temperature to equilibration solution (V1) 10 % ethylene glycol (EG) and 10% dimethylsulfoxide (DMSO) for 4-5 min, then to the vitrification solution (V2) 20% EG; 20% DMSO and 0.5 M sucrose for ≤ 45 s, then were loaded into open pulled straws (OPS) and immediately plunged into liquid N_2 (2 blastocysts per straw).

3. Embryo transfer

Due to the reduction of experimental animals only embryos of WS group were considered for embryo transfer. Among all recovered embryos (N=72) after flushing, 52 were vitrified but only 22 of these were utilised for transfer with the help of OPS-catheter, while, 20 embryos were transferred as fresh. Embryos were transferred surgically into 21 naturally D7 synchronised recipients ewes (D7: day 7 after natural onset of oestrus). At the moment of transfer an inguinal mini-laparotomy 5 cm was performed and the number and quality of corpora lutea (CL) was assessed, after this procedure 2 fresh embryos per recipient (n=10) were transferred into the top of the uterine horn ipsilateral to the ovary showing at least one functional CL using a TomCatTM catheter connected to a 1 ml syringe, while, in the vitrified-embryo transfer, the OPS containing the vitrified embryos were warmed by holding for 6 s in air and then dipped into a falcon tube contain HTCM 199+ 20% serum+ 0.5 M sucrose in a water bath at 37°C for aprox. 15 s. After this procedure 2 vitrified - thawed embryos per recipient (n=11) were transferred into the top of the uterine horn ipsilateral to the ovary showing at least one functional CL using the OPS as a catheter connected to a Tom Cat TM catheter then connected to a 1 ml syringe (Isachenko *et al.*, 2003). Pregnancy diagnose was performed 45 days after transfer by abdominal ultrasonography. Results in terms of pregnancy and lambing rates were analysed by a Chi Square analysis.

III – Results

1. Natural oestrus without synchronisation of flock

Table 1. Pregnancy and lambing rate with three different semen preparation techniques without synchronisation of flock

Treatment (semen)	Animals	Pregnancy rates (45 days)	Lambing rates	Lambs born/ewe lambing
Fresh (7h 15°C)	155	60.0% (93/155)	54.1% (84/155)	1.3 (110/83)
Chilled (24h 4°C)	20	30.0% (6/20)	30.0% (6/20)	1.5 (9/6)
Frozen (Liquid N_2)	21	23.8% (5/21)	23.8% (5/21)	1.2 (6/5)

2. Oestrus synchronisation of flock with "ram effect"

Out of 47 ewes of the organic farm, 37 came on heat (78.7%) between 19 and 24 day after re-introduction of rams. Unfortunately only 12 animals were inseminated with fresh semen and 9 become pregnant (75%), with a rate of lamb born for ewe lambed of 1.22. The rest of 25 ewes were on heat during the weekend and it was not possible to do the AI (no production of semen in the laboratory during the weekend), thus controlled natural mating was performed for this group with 80% of pregnancy and 1.3 lamb born per ewe lambed.

3. MOET

Among all the variables analysed statistical differences were found only in the mean number of CL/ewe (10.7±3.4 *vs* 7.0±3.2) and FR% (100 *vs* 80) between WS and SS groups respectively (Table 2).

Table 2. Length and cost of the treatments, number of corpora lutea (CL), embryo recovery (ER), embryo fertilized (EF), embryo quality (EQ1) per ewe, recovery (RR), fertility (FR) and embryo quality (EQ1) rates in natural oestrus (WS) and single sponge (SS) groups

Group	(n)	Days	Cost/ewe	CL/ewe	ER/ewe	EF/ewe	EQ1/ewe	RR	FR	Q1R
WS	10	14	€ 101.8	10.7±3.4a	7.2±3.9	7.2±3.9	6.2±3.8	67	100a	86
SS	10	20	€ 103.8	7.0±3.2b	5.6±3.2	4.5±3.5	4.0±3.0	80	80b	88

Different superscripts indicates treatments with significant differences ($p<0.05$).

Pregnancies and lambing rates after transfer were similar for vitrified and fresh WS embryos (Table3).

Table 3. Pregnancy and embryo lambing rates following transfer of fresh and vitrified embryos

Source of embryos	Recipients	Embryos transferred (n)	Pregnancy rate (45 days)	Lambing rate
Fresh	10	20	70	60
Vitrified	11	22	82	59

IV – Discussion and conclusion

Artificial Insemination with fresh, chilled and frozen semen performed 24 h after onset of natural oestrus detected by the vasectomised ram reach similar results when compared to conventional sponge treatments.

These results represent the first step towards the possibility to change the AI technique avoiding the use of sponges. This opportunity might be of interest for small farms where geographical or economical conditions might make difficult to introduce genetic improvement or more controlled reproductive management with the use of hormones. If this opportunity is combined with the "ram effect" at the starting of the breading season a proper synchronisation of the flock can be reached and more farmers might considered the utilisation of this kind of reproductive management. Indeed in the last a few years the increasing request from the consumers for organic products (milk, cheese and meat) gave the opportunity to several farmers to start organic management of the flock. It is well known that this kind of flock management imply no-use of hormones, thus no-use of AI. On the contrary the re-introduction of natural methods such as "ram effect" might gave again the opportunity to these farmers to introduce AI.

In the paper of Paulenz *et al.*, (2007) the natural system for AI called by the author "do it your self" is strongly recommended for several different farm conditions with the use of frozen semen. Unfortunately in our experimental conditions the pregnancy rate obtained by the sheep fertilised with frozen semen was not as good as the results obtained by Paulenz *et al.* (2007). Genetic characteristics of the sheep and of the semen are considered to be the main factors affecting the success of this kind of AI programme (Fair *et al.*, 2007). More study need to be done to overcome the factors that still limit the use of the AI carried out with frozen semen.

The good results obtained by the superovulation and embryo transfer programme in this experiment introducing alternatives such the reduction of the use of hormones, the use of vitrification technique together with the direct transfer of the embryos with OPS-catheter avoiding thus the need of microscope and costly expertise technician in addition with the inguinal mini laparotomy may open a new chance to wide spread this sophisticate technique, particularly where geographical and economical aspects may limit the application of MOET.

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