Use of purified FSH and LH for embryo production, cryopreservation by conventional freezing or vitrification and transfer of embryos in dairy ewes

Angela Gabriella D’Alessandro, Giovanni Martemucci
Dipartimento di Progettazione e Gestione dei Sistemi Agro-Zootecnici e Forestali.
Università di Bari, Italy

Corresponding author: Prof. Angela Gabriella D’Alessandro. Dipartimento di Progettazione e Gestione dei Sistemi Agro-Zootecnici e Forestali. Facoltà di Agraria, Università di Bari. Via G. Amendola 165/A, 70126 Bari, Italy - Tel. +39 080 5442825 – Fax: +39 080 5442828 – Email: dalex@agr.uniba.it

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ABSTRACT

Three experiments were carried out with the aim of evaluating the efficiency of techniques of in vivo production, storage and transfer of embryos in dairy sheep. Experiment I - For embryo production, thirty-one ewes were synchronized with FGA (vaginal sponges, 40 mg, 9 d) and PGF2α (ICI; 50 µg, 7th d), and subdivided into three groups corresponding to the following superovulatory treatments over 3 days with purified gonadotrophic preparations: A) control, FSH/LH ratio = 1 (250 IU p-FSH : 250 UI p-LH); B) FSH/LH ratio = 2 (250 IU p-FSH : 125 IU p-LH) and daily FSH/LH ratio of 3.4 – 1.7 – 0.8 in the 3 days of treatment, respectively; C) FSH/LH ratio = 2 (250 IU p-FSH : 125 IU p-LH) and daily FSH/LH ratio of 5.0 – 1.0 – 0.3. On the 7th day after oestrus and mating, ovarian response and embryo production were evaluated. Experiment II – Three freezing methods were evaluated based upon post-thaw embryo quality: CF) conventional slow freezing by 1.5 M ethylene glycol (EG); V-1) one-step vitrification based on exposure of the embryos to one solution (EG 7.15 M + ficoll 2.5 mM); V-3) vitrification in three steps, corresponding to three solutions at increasing concentration of glycerol (GLY) and EG (GLY 1.4 M; GLY 3.4 M + EG 1.4 M; GLY 4.6 M + EG 3.4 M). V-1) and V-3) frozen embryos were directly plunged in liquid nitrogen. At thawing, embryo viability was evaluated on the basis of morphological features. Experiment III – For embryo transfer, a total of 26 recipient ewes were synchronized with donors. On the 7th d from oestrus, 11 recipient ewes received fresh embryos (Group FE – control) and 15 recipients received vitrified-thawed embryos (Group VTE). Superovulatory treatment B) significantly advanced the onset of oestrus compared to the control (27.3 vs 34.7 h; P<0.05). Ovulation rate did not differ among the groups (6.5 to 10.8). Transferable embryos in Group B) (7.2) resulted similar to Group A) (5.3) and significantly (P<0.05) different when compared to Group C) (3.2). V3-method resulted in the highest (P<0.01) transferable embryos (74.5%) compared to CF- and V1-methods. After transfer, in FE and VTE recipient ewes were comparable in fertility rates (72.7 vs 73.3%; P>0.05) and embryo survival (63.6 vs 56.7%; P>0.05). In conclusion, the results demonstrated that treatments B) and C) did not improve superovulatory response compared to A); for embryo cryopreservation the V3 method can successfully be used for embryo transfer in ewes.

Key words: Ewes, In vivo embryo production, Embryo freezing, Embryo transfer.
D’ALESSANDRO - MARTEMUCCI

RIASSUNTO

IMPIEGO DI FSH ED LH PURIFICATI PER LA PRODUZIONE DI EMBRIONI, CONGELAMENTO CONVENZIONALE O PER VITRIFICAZIONE E TRAPIANTO DEGLI EMBRIONI NELLA PECORA DA LATTE

Lo studio, articolato in tre esperimenti, è stato effettuato al fine di valutare in pecore da latte l’efficienza del trattamento di superovulazione per la produzione in vivo degli embrioni, del metodo di crioconservazione (congelamento convenzionale o per vitrificazione) e del trasferimento degli embrioni crioconservati in pecore riceventi. Esperimento I – Per la produzione di embrioni, 31 pecore adulte e in asciutta sono state sottoposte a trattamento di sincronizzazione degli estri mediante progestageno (FGA, in pessari vaginali, 40 mg, 9 giorni) e prostaglandina F2α (PGF2α; ICI, 50 µg al 7° giorno). Gli animali sono stati suddivisi in tre lotti sperimentali corrispondenti ai seguenti trattamenti di superovulazione, effettuati con preparati porcini purificati (FSH e LH) in 3 giorni, 6 dosi, ad intervallo di 12 ore, con inizio al 7° giorno del trattamento progestativo: Gruppo A) – controllo – rapporto FSH/LH del preparato gonadotropico pari a 1:1 (250 UI p-FSH : 250 UI p-LH), mantenuto costante per tutto il trattamento; Gruppo B) rapporto FSH/LH del preparato gonadotropico pari a 2:1 (250 UI p-FSH : 125 UI p-LH), somministrato secondo un regime con rapporto giornaliero FSH/LH di 3,4 – 1,7 – 0,8 nei tre giorni di trattamento; Gruppo C) rapporto FSH/LH del preparato pari a 2:1 (250 UI p-FSH : 125 UI p-LH) e rapporto giornaliero FSH/LH, nei tre giorni, di 5,0 – 1,0 – 0,3. Le pecore sono state controllate per rilevare la comparsa dell’estro ed accoppiate. Al 7° giorno dall’accoppimento è stata valutata la risposta ovarica (corpi lutei, follicoli preovulatori > 4 mm) e la produzione di embrioni. Esperimento II – E’ stata valutata in vitro l’efficienza di 3 metodi di congelamento (gonadotropici e per vitrificazione) e del trasferimento degli embrioni crioconservati in pecore riceventi. Allo scongelamento è stata rilevata la vitalità embrionale sulla base delle caratteristiche morfologiche. Esperimento III – E’ stata valutata la sopravvivenza in vivo degli embrioni vitrificati secondo la procedura V3. Ventisei pecore riceventi adulte sono state sincronizzate con le donatrici > 4 mm) e la produzione di embrioni. Esperimento II – E’ stata valutata in vitro l’efficienza di 3 metodi di congelamento (gonadotropici e per vitrificazione) e del trasferimento degli embrioni crioconservati in pecore riceventi. Allo scongelamento è stata rilevata la vitalità embrionale sulla base delle caratteristiche morfologiche. Esperimento III – E’ stata valutata la sopravvivenza in vivo degli embrioni vitrificati secondo la procedura V3.

Parole chiave: Pecora, Produzione in vivo embrioni, Congelamento di embrioni, Trapianto di embrioni.

Introduction

Although artificial insemination is of great importance for genetic improvement, it is still inadequate for the increase in productivity of dairy sheep. Improvement of the techniques of production, cryopreservation and transfer of embryos will contribute to a more rapid genetic progress through the increase in the number of offspring of the females with the best genotype. The use of gonadotrophic pituitary extracts for ovariain hyperstimulation enhances embryo production (Martemucci et al., 1988; Jabbour and Evans, 1991). Taking into account the physiological hormonal pattern from luteal regression and preovulatory peak in LH (Cahill et al., 1981), several studies have demonstrated that the daily FSH/LH ratio during treatment appears to be important in the superovulatory response in small ruminants for in vivo embryo production (Chupin et al., 1987; Baril et al., 1989; Martemucci et al., 1996; D’Alessandro et al., 1997).

Cryopreservation of embryos can be considered an integral part of embryo transfer biotechnology. It allows the transport of germ plasm resources as embryos, reducing risk of disease transmission,
and allows recipients well adapted to the local conditions to obtain offspring.

Several procedures for embryo freezing have been developed. The conventional method is based on the use of cryoprotectant agents at low concentrations (1 to 2 M) and a slow controlled freezing program (Rall, 1992). Recent interest has been focused on the vitrification method for the cryopreservation of embryos (see review: Massip, 1989; Niemann, 1991; Rall, 1992; Kasay, 1996). This procedure relies on the use of high concentration (greater than 6 M) of a mixture of permeating cryoprotectant agents and direct submersion into liquid nitrogen, without the occurrence crystallization. It prevents extracellular ice formation, which is the major cause of injury to cells (Rall and Fay, 1985). The cryoprotectant agents can be added to the embryos according to a one-step procedure (Schiewe et al., 1991; Ali and Shelton, 1993) or by using solutions at increasing concentrations (McGinnis and Youngs, 1990; Martinez and Matkovic, 1998). The first one has the advantage of reducing time consumed while the other tends to reduce the toxicity of the high concentration of cryoprotectants.

Compared to the conventional method of cryopreservation, vitrification is cheaper because it does not require expensive equipment and results more rapid because it needs only a few seconds to complete cooling.

Efficiency of embryo transfer is variable in relation to transferring of fresh or frozen embryos (Brebion et al., 1992; McMillan and Hall, 1994).

The study, constituted in three experiments, had the aims to evaluate: the efficiency of in vivo embryo production using purified gonadotrophins for superovulation; the effects of different methods for embryo freezing; the efficiency of transfer of frozen-thawed embryos.

Material and methods

The study was carried out in Southern Italy (41°N latitude) on a total of 57 non-lactating adult ewes of the Leccese breed.

Experiment I
This experiment was designed to evaluate embryo production of ewes treated for superovulation with purified porcine pituitary gonadotrophic extracts (p-FSH and p-LH), combined to obtain different FSH/LH ratios.

During the breeding season, thirty-one ewes were synchronized with fluorogestone acetate (FGA, 40 mg, in vaginal sponges; Intervet, Milan, Italy) for 9 days, and prostaglandin F_2α (50 µg; Cloprostenol, Estrumate, Schering-Plough) on the seventh day (D’Alessandro et al., 1995). The ewes were subdivided into 3 homogeneous groups corresponding to the experimental superovulatory treatments. Each ewe received a total of 250 IU purified p-FSH (Laboratorios Calier; Spain) supplemented with 250 IU (Group A, control) and 125 IU (Groups B and C) purified p-LH (Laboratorios Calier) (Table 1). The gonadotrophins were administered i.m. at 12-h intervals for 3 days, starting 48 h before sponge removal. During the three days of superovulatory treatment, the FSH/LH ratio of gonadotrophic preparation was: 1.0 in Group A (Control); 3.4 (1st d) -1.7 (2nd d) -0.8 (3rd d) in Group B; 5.0-1.0-0.3, respectively in the three days, in Group C (Table 1). Twenty hours after sponge removal, the ewes were checked for oestrus every 6 hours and were hand-mated. The evaluation of ovarian response (corpora lutea and preovulatory follicles > 4 mm) and embryo production was performed by laparotomy seven days after oestrus (Martemucci et al., 1988), under general anesthesia. Ova were flushed from uterine horns in modified PBS medium (m-PBS; Whittingham, 1971) and examined under a phase contrast microscopy (225 X), taking cleavage as evidence for fertilization. Embryos of good quality, referred as transferable embryos, were established on the basis of the development stage in relation to oestrus time and to their morphological features (Martemucci et al., 1988).

Experiment II
In order to evaluate the efficiency of embryo freezing by two methods of vitrification in comparison to a conventional method, 121 transferable quality embryos at the blastocyst stage were considered.

Source of embryos
Leccese breed ewes were used as embryo
donors for Experiment II and Experiment III (described below). The embryos were produced following both the A) superovulatory treatment and the procedures described for Experiment I. The recovered embryos at the blastocyst stage that scored as excellent- to good-quality embryos (Martemucci et al., 1988) were used.

Freezing of embryos

Conventional freezing method (CF)

The protocol described by Voelkel and Hu (1992) was used, with some modifications using n. 19 blastocysts. All cryoprotectant solutions were prepared in m-PBS. For equilibration embryos were held for 20 min at room temperature in a 1.5 M ethylene glycol (EG) solution. A drop of 1.5 M EG solution containing embryos was aspirated into the 0.25 mL plastic straw (IMV, l'Aigle, France) and it was separated bilaterally by an air space from two columns of 1.5 M EG solution. The straw was sealed with polyvinyl alcohol (PVA) powder and placed into a programmable liquid nitrogen freezer (Planer, Biomed MVE Cryogenics). Embryos were cooled at –2 °C/ min to –7 °C and held for 1-2 min. The straws were sealed by touching the upper column of the medium with a metal rod pre-cooled in liquid nitrogen. After seeding, the temperature was held at –7 °C for 5 min, then it was decreased at –0.5 °C/ min to –35 °C and maintained for 15 min before plunging into liquid nitrogen at ~196 °C. For thawing, the straws were submerged in a 30 °C water bath for 30 sec and the embryos were transferred into m-PBS and equilibrated for 10 min to remove cryoprotectant.

Vitrification methods.

Only excellent and good embryos at the blastocyst to expanded blastocyst stage (n. 102) were frozen by vitrification.

Two procedures of vitrification were considered: the one-step procedure by Mahmoudzadeh et al. (1993) and the procedure by Naitana et al. (1995, 1997).

The one-step procedure by Mahmoudzadeh et al. (1993) (V1-method), used originally for vitrification of bovine embryos, was slightly modified. Briefly, the base solution for freezing and thawing was m-PBS. Forty four embryos were exposed in vitrification solution, constituted by EG 7.15 M, ficoll 2.5 mM and sucrose 0.3 M, at room temperature for 1 min. The embryos were aspirated into the middle of straw (IMV) and separated bilaterally, by two air spaces, from two columns of sucrose 0.5 M solution. Two embryos were placed into each straw. After sealing the straw, the equilibration of

| Table 1. Schedule of superovulatory treatment in six decreasing doses over three days for embryo production (Experiment I). |
|---------------------------------------------------------------|
| Treatment group     | Treated ewes n. | Administration of gonadotrophins |
|                    |                | Total  | 1st day* | 2nd day* | 3rd day |
| A – Control         | 10 IU FSH      | 250    | 71.5     | 35.7     | 17.8    |
|                    | 10 IU LH       | 250    | 71.5     | 35.7     | 17.8    |
|                    | FSH / LH       | 1.0    | 1.0      | 1.0      | 1.0     |
| B                    | 11 IU FSH      | 250    | 72.1     | 36.0     | 17.0    |
|                    | 11 IU LH       | 125    | 21.2     | 21.2     | 21.2    |
|                    | FSH / LH       | 2.0    | 3.4      | 1.7      | 0.8     |
| C                    | 10 IU FSH      | 250    | 100.0    | 20.0     | 6.0     |
|                    | 10 IU LH       | 125    | 20.0     | 20.0     | 20.0    |
|                    | FSH / LH       | 2.0    | 5.0      | 1.0      | 0.3     |

*2 administrations per day, 12 h apart.
Embryos in the vitrification solution was completed for a total of 2 min and the straw was plunged rapidly into liquid nitrogen. After thawing (water bath at 25 °C, 10 sec), the embryos were expelled into a Petri dish containing 0.5 M sucrose solution, in 1 min washed in the same solution and equilibrated for 4 min. Then, the embryos were washed in m-PBS and assessed for in vitro viability.

As the second vitrification procedure, the EG + glycerol (GLY) in three-step protocol described by Naitana et al. (1995, 1997) (V3-method) was followed using n. 58 blastocyst. Briefly, the base solution was m-PBS supplemented with 0.1% PVA. After equilibration at room temperature for 5-10 min in the base solution, the embryos were transferred into 1.4 M GLY solution (5 min, at room temperature) and then in 5 min in 3.4 M GLY and 1.4 M EG (5 min, at room temperature). Finally, the embryos were aspirated in a 25 mL drop of vitrification solution (3.4 M GLY and 4.6 M EG) in straw (IMV). Each straw contained 2 embryos and was filled with sucrose 0.5 M solution at the ends of the straw, a first column of vitrification solution followed by a second column of vitrification solution containing the embryos. The different columns were separated by air spaces. The straw was rapidly sealed, plunged into liquid nitrogen and stocked until use. For thawing, the straws were warmed rapidly in 25 °C water bath and emptied into a Petri dish. To facilitate mixing of the sucrose and vitrification solutions, the dish was stirred gently. The collected embryos were transferred into 0.25 M sucrose solution to remove the cryoprotectants. After 5 min the embryos were washed in the base solution.

Post-thaw embryo evaluation

After thawing, the percentage of post-thaw recovered embryos and viability were estimated. The embryos were classified under an inverted microscopy (225 X) on the basis of morphological features to evaluate the post-thaw morphological damages. The embryos were evaluated as follows: Grade 0 (embryos without any defects); Grade 1 (embryos with full cell mass, uniform cytoplasm and only few extruded blastomeres); Grade 2 (embryos with few extruded and/or damaged blastomeres, few vesicles); Grade 3 (embryos with non-uniform cells, fragmentation, grainy cytoplasm breakdown of some blastomeres and zona pellucida); Grade 4 (embryos with severe defects). Only Grade 0 – 1 embryos were considered transferable.

Experiment III

On the basis of more satisfactory results obtained in Experiment II using the three-step vitrification method, this experiment was designed to evaluate in vivo survival of embryos frozen by this vitrification protocol in comparison to fresh embryos.

Twenty-six recipient ewes were synchronized with donors by FGA (40 mg, in vaginal sponge, 9 d) + PGF₂α (Cloprostenol, 50 µg, 7th d) + PMSG (400 IU, 7th d). Oestrus was detected by teaser rams starting 20 h after sponge removal, every 6 – 8 h. On the seventh day after oestrus, n. 15 recipients were transferred with vitrified-thawed embryos (Group VTE) and n. 11 recipients received fresh embryos as control (Group FE).

A total of thirty re-expanded blastocysts were transferred on VTE recipients Group (two embryos per each recipient) while a total of 22 fresh embryos morphologically classified as transferable (Martemucci et al., 1988) were transferred into FE recipient group (two embryos per each recipient) less than two hours after recovery.

Transfer of the embryos into the recipient ewes was performed using a surgical approach (Martemucci et al., 1988). The embryos were aspirated in a drop of m-PBS into a catheter (IMV) and placed at the tip of the uterine horn ipsilateral to the ovulation.

Fertility (lambing ewes / transferred ewes) and embryo survival, defined as the proportion of embryos transferred which resulted in lambs at terms, were recorded.

Statistical analysis

The effect of superovulatory treatment on onset of oestrus, ovulation rate, global ovulatory response, embryo production and transferable embryos was analyzed by one-way analysis of variance using the GLM procedure of the SAS (SAS, 1999/2000). Least squares means were compared by t test with the predicted difference (pdiff) option of GLM. Data of percentages were analyzed by the chi-square test (SAS, 1999/2000).
Results

Experiment I
Oestral and ovarian responses and embryo production are reported in Table 2.
Superovulatory treatment performed with different FSH/LH ratios significantly influenced (P<0.05) the onset of oestrus. In control ewes treated with a daily FSH/LH ratio of 1.0 – 1.0 – 1.0 (Group A), the occurrence of oestrus resulted significantly (P<0.05) delayed (34.7 h) compared to Group B and C (27.3 and 28.6 h).
The ovulation rate, as well as total ovarian response, did not differ among the groups (range 6.5 to 10.8 per ewe). Treatment with total FSH/LH = 2:1 and daily FSH/LH ratio of 3.4 – 1.7 – 0.8 (Group B) gave mean productions of embryos and transferable embryos (8.4 and 7.2) similar to Group A (6.2 and 5.3) but significantly higher (P<0.05) compared to Group C (3.4 and 3.2).

Experiment II
The results of this experiment are presented in Table 3.
The comparison among the conventional and the two-vitrification methods (V1 and V3) did not show significant differences in percentages of post-thaw recovered blastocysts. However, a higher value was observed in V3 frozen embryos (87.9%). Also corresponding to the V3 vitrification method was the highest percentage of transferable embryos (74.5%) with significant differences (P<0.01) compared to both V1-method (17.9%) and CF-method (30.0%).

Table 2. Effects of superovulatory treatment with gonadotrophic preparations having different FSH/LH ratios during the three days of treatment on oestrus, ovarian response and embryo production (X ± ES).

| Daily FSH/LH | Group A (Control) | Group B | Group C |
|-------------|-------------------|---------|---------|
| Treated ewes | n.                | 10      | 11      | 10      |
| Onset of oestrus | h | 34.7 ± 2.2a | 27.3 ± 2.2b | 28.6 ± 2.2b |
| Ovulations | 6.5 ± 2.1         | 10.8 ± 2.1 | 7.8 ± 2.1 |
| Global ovarian response | 7.8 ± 2.0 | 12.0 ± 2.0 | 9.8 ± 2.0 |
| Embryo production | 6.2 ± 1.5a | 8.4 ± 1.5a | 3.4 ± 1.5a |
| Transferable embryos | 5.3 ± 1.4a | 7.2 ± 1.4a | 3.2 ± 1.4a |

a, b: P<0.05.

Table 3. Effects of freezing system (conventional, CF, one-step, V-1, and three-step vitrification, V-3), on percentages of recovered and transferable embryos.

| Freezing System | Recovered Embryos | Transferable Embryos |
|-----------------|-------------------|----------------------|
| CF              | n.                | 14/19                | 4/14                 |
|                 | %                 | 73.6                 | 30.0%                |
| V-1 Method      | n.                | 28/44                | 5/28                 |
|                 | %                 | 63.6                 | 17.9%                |
| V-3 Method      | n.                | 51/58                | 38/51                |
|                 | %                 | 87.9                 | 74.5%                |

A, B: P<0.01.
Experiment III

Reproductive performance in the two groups of recipients transferred with vitrified-thawed embryos (Group VTE) and fresh embryos (Group FE) are reported in Table 4. Eleven of the 15 recipients (73.3%) transferred with frozen embryos gave birth to living lambs. Overall, the total number of lambs born was 17, resulting in an embryo survival rate of 56.7%. The mean number of lambs born per ewe was 1.5. Fertility rate and embryo survival were similar to those obtained with fresh embryos. In FE-recipient groups fertility was 72.7%, producing a total of 14 living lambs while embryo survival rate was 63.3%, with a mean number of lambs per ewe of 1.7.

Discussion

In a previous study (D’Alessandro et al., 1997), using a total FSH amount of 525 IU for superovulation, a comparison of different gonadotropic preparations having global FSH/LH ratio of 1.0, 2.0 and 4.0 and daily FSH/LH administration regimen constant or decreasing was carried out during the anestrous period. The results indicated the superiority of treatment characterized by total FSH/LH = 2.0 and daily FSH/LH of 3.4 – 1.7 – 0.8 in the three days of treatment. On the basis of these positive results, we tested this treatment using a total dose of 250 UI (D’Alessandro et al., 1996) and adopting FSH/LH ratio = 1.0 kept constant as control. Moreover, we wanted to evaluate the possibility of improving ovarian response and embryo production by modifying the daily FSH/LH ratio. Thus, considering the FSH/LH ratio of 2:1, FSH amount was increased on the first day of the hormonal treatment (FSH/LH = 5), FSH and LH amounts on the second day were equal (FSH/LH = 1) and LH was increased on the third day (FSH/LH = 0.3).

The results show that the ovarian response did not differ among the experimental treatments. This finding demonstrate that during the breeding season in order to stimulate multiple ovulations it is not very important to decrease the total amount of pLH from 250 IU (Group A, control) to 125 IU (Groups B and C). Again, administration regimen of gonadotrophins (FSH/LH=2:1 and daily FSH/LH= 3.4 – 1.7 – 0.8) provides ova production and transferable embryos comparable to the control treatment (FSH/LH=1:1, kept constant throughout the treatment). This lack of improvement in superovulatory response is in disagreement with results obtained in other studies which have shown the positive effect of the adding of pLH in the last injections (FSH/LH =0.3-0.4) during the non breeding season (Chupin et al., 1987; D’Alessandro et al., 1997). The season and/or the total FSH amount of treatment may explain this difference.

Considering that the total FSH and LH amounts were equal in both B and C treatment...
groups, we can argue that the increase in the pFSH dose at the start of gonadotrophic treatment (Group C) is not effective in improving ovulation rate. Between these two experimental FSH/LH daily regimens (Groups B and C) the difference became significant for the embryo production. The lower yield of transferable embryos recorded in ewes treated with daily FSH/LH ratio of 5.0 – 1.0 – 0.3 may have negatively influenced hormonal pattern and/or the physiological events connected to the embryo production.

However, the mean number of transferable embryos produced in this experiment following the 3.4 – 1.7 – 0.8 FSH/LH treatment was equivalent to that reported in other breeds treated with ovine-FSH (Dingwall et al., 1993; Barli et al., 1999) but higher than for other hormonal treatments (Armstrong and Evans, 1983; Martemucci et al., 1988; Wierczos et al., 1992; Scudamore et al., 1993; Boland et al., 1995).

To test embryo survival after freezing, in this study we used post-thaw morphological assessment of embryos which makes it possible to evaluate the major damage to embryonic structures. Although this criterion does not give an reliable indication on embryo capability to continue its in vivo development, it has the advantage of permitting a quick selection of embryos and it is followed before transfer (Rao et al., 1988; Sakul et al., 1993).

Considering the effects of the freezing procedures, the three-step vitrification was the more efficient method positively affecting post-thaw recovery of embryos and transferable rate.

The V1 vitrification protocol provided unsatisfactory results compared to in vitro development assessed in bovine embryos (Mahmoudzadeh et al., 1993). The difference in the findings could be due to the species, because embryo characteristics such as size, shape, membrane properties and sensitivity to cryoprotectant change between species (Kasai, 1996).

In this study, post-thaw recovery rate was lower than that reported in other research (Seidel et al., 1990; D'Alessandro et al., 1997; Lane et al., 1998). In particular, the lowest recovery rate, although without significant differences compared to the other methods, corresponds to V1 vitrification method, and is probably connected with the difficulty in handling embryos, due to sticking and floating. This freezing method affects the lowest embryo viability and this could be attributed to interaction between cryoprotectant sucrose and macromolecules. In mice, Gutierrez et al. (1993) showed that embryos frozen in media with ficoll were more difficult to handle and to recover than those with PVA and suggested that interaction between cryoprotectant sucrose and macromolecules can affect embryo viability, since solutes such as sucrose and macromolecules influence the rate of cryoprotectant permeation.

Fertility of recipient ewes transferred with V3 vitrified/thawed embryos was similar to that obtained with transfer of fresh embryos, confirming the results of other studies (Barili et al., 2001). Again, these findings demonstrate that the vitrification method used in this experiment, as reported by other researcher (Naitana et al., 1995), is able to provide good results. The results concerning embryo survival rate, although based on a limited number of transfers, were comparable to or even higher than those reported for embryos frozen by conventional slow freezing methods (Tervit, 1984, Tervit and Goold 1984; Tervit et al., 1986; Heymann et al., 1987; Sakul et al., 1993) or by other vitrification methods (Ali and Shelton, 1993; Szell and Windsor, 1994; Traldi et al., 1998; Barili et al., 2001).

Conclusions

Our findings show that in superovulatory treatment both the increase of total FSH/LH ratio from 1:1 to 2:1 and, for the latter gonadotrophic ratio, the modifying of daily FSH/LH from 3.4 – 1.7 – 0.8 to 5.0 – 1.0 – 0.3 do not improve ovarian response and embryo production.

Vitrification of embryos by the three-step procedure yielded good results as estimated in vitro and in vivo by lambing rate and survival rate of embryos and it might be favorable when considered for use in embryo transfer techniques in sheep.

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EMBRYO YIELD, FREEZING AND TRANSFER IN EWES

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