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Cryopreservation of in vitro-produced ovine embryos

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Abstract

The purpose of this study was to evaluate different cryopreservation protocols for in vitro-produced ovine embryos and assess the survival rate after cryopreservation. The experiment was also designed to examine whether this technique is feasible to apply in large-scale operations. In a first experiment, ovine embryos cryopreserved in ethylene glycol using 3 steps (E-3S) showed a higher hatching rate and nuclei number than freezing with glycerol in 3 steps (G-3S) (40.0% versus 20.0% and 135.4 ± 20.7 versus 118.5 ± 19.5, respectively). In a second experiment, vitrification with ethylene glycol + Ficoll 70 + sucrose (EFS) recorded a higher hatching rate and nuclei numbers than vitrification with propylene glycol + glycerol (Pg + Gly) (51.1% versus 31.1% and 133.8 ± 36.8 versus 113.5 ± 22.0, respectively). In a third phase, vitrification of embryos with ethylene glycol + glycerol (Eg + Gly) resulted in higher development and hatching rates and nuclei number than EFS (87.3% versus 65.4%; 76.4% versus 54.5% and 135.7 ± 34.5 versus 113.1 ± 14.1, respectively). In a fourth experiment, fresh in vivo embryos produced a higher lambing rate (67.8%) than the other methods, and E-3S in vitro resulted in lower lambing rates (23.0%). E-3S in vivo, Eg + Gly in vivo, fresh in vitro and Eg + Gly in vitro recorded similar lambing rates (42.8, 37.5, 37.5 and 26.6%, respectively). Ewes receiving in vitro-produced ovine embryos resulted in higher assisted births and perinatal losses than those receiving in vivo-produced embryos (3.6% versus 16.9% and 1.2% versus 10.1%, respectively). Results demonstrate that the transfer of in vitro-produced embryos vitrified with Eg + Gly could have wide application, if the negative side effects of the produced offspring can be managed.

Keywords: In vitro survival; Transfer; Lambing

1. Introduction

In vitro embryo production and cryopreservation are important tools for controlling and manipulating mammalian reproduction. Over the past few years the efficiency and simplification of in vitro embryo production has been greatly improved, and many authors have reported offspring born following the transfer of fresh embryos in farm animal species. This technology has consequently become widely used in commercial embryo transfer programs (Massip et al., 1995; Thompson, 1997).

The cryopreservation of in vitro-produced embryos is more complex than that of embryos developed in

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vivo (Massip et al., 1995). It has been suggested that the lower viability of in vitro-produced embryos following freezing and thawing derives from physiological, morphological and biochemical differences that embryos develop in vivo (Greve et al., 1993; Massip et al., 1995). However, there have been significant advances in the cryopreservation of in vitro-produced embryos, particularly in bovine (Dinnyes et al., 1999; Sommerfeld and Niemann, 1999; Kaidi et al., 1998).

Many studies have reported lambs born after the transfer of fresh in vitro-produced ovine embryos (Cheng et al., 1986; Crozet et al., 1987; Cognie et al., 1991; Thompson et al., 1995; Holm et al., 1996; Catt et al., 1997; Brown and Radziewic, 1998; Ledda et al., 1999), however, data regarding the cryopreservation of ovine embryos are relatively scarce. These studies generally use conventional slow freezing (Earl et al., 1996; Songsasen et al., 1996) or vitrification methods, and, in all cases, two or more embryos were transferred to recipients (Ptak et al., 1999; Dattena et al., 2000; Papadopoulos et al., 2002).

Reports regarding survival rates after the transfer of cryopreserved in vivo and in vitro-produced ovine embryos are sparse. The purpose of this study was to evaluate the different cryopreservation protocols for in vitro-produced ovine embryos and the survival rate after cryopreservation, and the feasibility of using this technique in a large-scale embryo transfer program.

2. Materials and methods

2.1. Reagents

2.1.1. For in vitro embryo production

Purified ovine LH was obtained from the Immuno Chemical Products Laboratories (Auckland, New Zealand). Porcine FSH (pFSH) was supplied by Schering Laboratories (Omaha, NE, USA). Mineral oil was purchased from Laboratorios Pharmos (Buenos Aires, Argentina) and the TCM 199, heparin, antibiotics, fraction V BSA, FCS and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Reagents used were all of cell culture tested quality.

2.1.2. In vivo embryo production, cryopreservation and embryo transfer

Glycerol, ethylene glycol, propylene glycol, bovine serum albumin (BSA), sucrose and Ficoll 70 were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Reagents were all cell culture tested by the supplier. Dulbecco's phosphate buffered saline solution (PBS) and antibiotic–antimycotic (AA) were supplied by Gibco Laboratories (Grand Island, New York, USA) and the porcine FSH (pFSH) and fetal calf serum (FCS) were obtained from Serono Veterinary Laboratories (Buenos Aires, Argentina). Medroxyprogesterone acetate (MAP) was purchased from Gador Laboratories (Buenos Aires, Argentina) and the pregnant mare serum gonadotrophin (PMSG) from Syntex Laboratories (Buenos Aires, Argentina).

2.2. In vitro embryo production

Two hundred and nine adult (4–6-year old) Australian Merino × Corriedale ewes (mean body weight 56.4 ± 5.0 kg) were used as oocyte donors. The oocytes were aspirated by folliculocentesis (Baldassarre et al., 1996). The collection medium was TCM 199, supplemented with 0.05 mg/ml heparin, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 0.05 mg/ml kanamycin and 0.5% (w/v) fraction V BSA. The oocytes were transferred to TCM 199 buffered with 15 mM HEPES and supplemented with 10% FCS (holding medium). For in vitro maturation (IVM) the oocytes were washed twice in the holding medium and twice in the TCM 199 buffered with bicarbonate and containing 10% FCS, 10 μg/ml purified ovine LH (NIH-oLH-S1), 1 μg/ml pFSH and 1 μg/ml estradiol 17β (IVM medium). Cumulus–oocyte-complexes (COC's) were cultured for 26 h for IVM, in 50-μl drops of IVM medium under mineral oil at 39 °C in a humidified incubator gassed with 5% CO_2, 7% O_2 and 88% N_2. After maturation, the COC's were deposited for 22 h in 50-μl drops in vitro fertilization medium under mineral oil, containing 1×10^6 spermatozoa, selected by the Percoll technique (Parrish et al., 1986). The presumptive zygotes were transferred to modified SOF medium (Tervit et al., 1972; Gardner et al., 1994) for 7 days (Day 0, day insemination). The culture medium was changed at 48-h intervals during the 7-day culture period.

2.3. In vivo embryo production

Forty-eight adult (4–6-year old) Australian Merino × Corriedale ewes (mean body weight 56.4 ±

5.0 kg) were used as embryo donors. Estrus was synchronized using intravaginal progestagen sponges containing 60 mg MAP, for 14 days (day 0, sponge insertion). The donors were superovulated with a total dose of 250 IU pFSH in six decreasing doses (Naitana et al., 1995), administered at 12-h intervals, starting 48 h before sponge withdrawal. The ewes were exposed to a ram of proven fertility at the time of sponge withdrawal.

Six days after induced estrus, embryos were collected surgically (Baldassarre et al., 1992). The animals were anesthetized with 0.3 ml, 1% atropine i.m. and 0.7 ml, 2% xilazine i.m. and, 5 min later, 1.5 ml ketamine chlorhidrate (50 mg/ml) i.v. Both uterine horns were exposed and flushed with pre-warmed PBS, supplemented with 1% FCS and 1% AA (30 ml/horn). The embryos were located and immediately placed in a holding medium, consisting of PBS supplemented with 10% FCS and 1% AA.

The embryos were classified according to the stage of development and morphological appearance (Winterberger-Torres and Sevellec, 1987). Only excellent to good quality blastocysts and expanded blastocysts were used in the experiments.

2.4. Cryopreservation methods

2.4.1. Glycerol 3 step (G-3S) technique

The protocol as described by Prather et al. (1987) with modifications was used. Glycerol was added in 3 steps (0.5, 1 and 1.4 M) in PBS, supplemented with 20% FCS and 1% AA (10 min/step). Freezing was accomplished by cooling from 20 to −6 °C at a rate of −1 °C/min; seeding; holding at −6 °C for 10 min; cooling to −30 °C at a rate of −0.3 °C/min and then to −33 °C at a rate of −0.1 °C/min holding for 2 min at −33 °C and then plunging into liquid nitrogen (LN_2) for storage. Thawing was performed at room temperature for 10 s and then in a water bath at 27 °C for 20 s. The cryoprotectant was removed in 3 steps with decreasing concentrations (1, 0.5 and 0 M) of PBS supplemented with 0.5 M sucrose, 20% FCS and 1% AA (5 min/step).

2.4.2. Ethylene glycol 3 step (E-3S) technique

The protocol described by Tervit and Gold (1984) was used with modifications. Ethylene glycol was added in 3 steps (0.5, 1 and 1.5 M) in PBS, supplemented with 20% FCS and 1% AA (10 min/step). Freezing was performed by cooling from 20 to −7 °C at a rate of −1 °C/min; seeding; holding at −7 °C for 10 min; cooling to −35 °C at a rate of −0.3 °C/min and then to −38 °C at a rate of −0.1 °C/min holding for 2 min at −38 °C; and the plunging into LN_2 for storage. Thawing was performed at room temperature for 10 s, then in a water bath at 27 °C for 20 s. The cryoprotectant was removed in 3 steps with decreasing concentration (1, 0.5 and 0 M) of PBS supplemented with 0.5 M sucrose, 20% FCS and 1% AA (10 min/step).

2.4.3. Vitrification with propylene glycol + glycerol (Pg + Gly)

The protocol as described by Scheffen et al. (1986) and Massip et al. (1986) with modifications was used. The embryos were equilibrated in a solution of 10% glycerol + 20% propylene glycol in PBS supplemented with 20% FCS and 1% AA (10 min, at room temperature) and vitrified in a solution of 25% glycerol + 25% propylene glycol in PBS, supplemented with 20% FCS and 1% AA (30 s, at 4–12 °C). After storage in LN_2 the straws were warmed in a water bath at 20 °C. The embryos were expelled into a Petri dish in a mixture composed of equal volumes of vitrification solution and 1 M sucrose solution in PBS supplemented with 20% FCS and 1% AA. Then in a 1 M sucrose solution in PBS supplemented with 20% FCS and 1% AA (5 min each step, at room temperature), and finally washed in PBS supplemented with 20% FCS and 1% AA.

2.4.4. Vitrification with ethylene glycol + Ficoll 70 + sucrose (EFS)

The protocol by Kasai et al. (1990) and modified by Zhu et al. (1993) were used. The embryos were equilibrated in a solution of 20% ethylene glycol in PBS supplemented with 20% FCS and 1% AA (5 min, at room temperature) and vitrified in a solution of 40% ethylene glycol + 18% Ficoll 70 + 0.3 M sucrose in PBS supplemented with 20% FCS and 1% AA (30 s, at room temperature). After storage in LN_2 the straws were warmed in a water bath at 25 °C. The embryos were expelled in PBS supplemented with 0.5 M sucrose, 20% FCS and 1% AA (5 min, at room temperature) and finally washed in PBS supplemented with 20% FCS and 1% AA.

2.4.5. Vitrification with ethylene glycol + glycerol (Eg + Gly)

This protocol, described by Yang et al. (1992) was modified as in Pg + Gly. The embryos were equilibrated in a solution of 10% glycerol in PBS supplemented with 20% FCS and 1% AA (5 min at room temperature) and then in a solution of 10% glycerol + 20% ethylene glycol in PBS supplemented with 20% FCS and 1% AA (5 min, at room temperature). Finally, embryos were vitrified in a solution of 25% ethylene glycol + 25% glycerol in PBS, supplemented with 20% FCS and 1% AA (30 s, at room temperature). After storage in LN_2 the straws were warmed in a water bath at 37 °C. The embryos were expelled into a Petri dish in a mixture composed of equal volumes of vitrification solution and 1 M sucrose solution in PBS, supplemented with 20% FCS and 1% AA. Then in a 0.5 M sucrose solution, and in a 0.25 M sucrose solution (5 min each step, at room temperature). The embryos were washed in PBS supplemented with 20% FCS and 1% AA.

2.5. Embryo culture after thawing

Evaluation of the survival rate after cryoprotectant removal was performed by culturing the embryos in 50-μl drops of SOF medium, under mineral oil (five to seven embryos per drop) at 39 °C in a controlled atmosphere (7% O_2–5% CO_2–88% N_2). Development (as assessed by blastocele re-expansion) and hatching were monitored every 24 h for a 72 h period to estimate embryo viability.

Finally, those blastocysts that survived after 72-h of culture, were evaluated by mounting and staining with the fluorescent DNA-specific dye (Hoechst 33342), under an epifluorescence microscope (Nikon, Optiphot).

2.6. Embryo transfer after thawing

Three hundred and ninety healthy adult 4–6-year old Australian Merino × Corriedale ewes were used as recipients (mean body weight 47.0 ± 5.0 kg). The animals were synchronized using intravaginal progestagen sponges (60 mg MAP) for 14 days, followed by 300 IU PMSG at the time of sponge removal. Six day after the induced estrus, the embryos were transferred surgically (one embryo per recipient) under anesthesia (Section 2.3) into the uterine horn ipsilateral to the corpus luteum, using a tom-cat catheter connected to a 1 ml syringe. After transfer, all ewes received an intravaginal 60 mg of MAP sponge that remained in place for 14 days.

Pregnancy was diagnosed 30 and 60 days after transfer by abdominal ultrasonography, using an Aloka 500 scanner (Aloka, Tokyo, Japan) with a 5.0-MHz linear-array transducer. Pregnancies were allowed to go to term. Litter size, date of birth, birth weight and sex of lambs were recorded as well as the incidence of assisted births and perinatal deaths. During the lambing period the flock was checked twice daily to record lambing difficulties.

The pregnancy rate was defined as ewes pregnant/recipient ratio and lambing rate as the proportion of lambs born/recipient ewe. Sex ratio was calculated as the proportion of male-to-female offspring and, length of gestation as the time interval between the lambing date and transfer date plus 6 days (embryo age).

2.7. Experimental design

In Experiments 1–3 embryo development, hatching rate and nuclei number were recorded.

In Experiment 1, one hundred in vitro-produced embryos (50 per treatment) were used to compare the G-3S versus E-3S freezing methods. In Experiment 2, ninety in vitro-produced embryos were used to compare the vitrification solutions Pg + Gly versus EFS. In Experiment 3, one hundred and ten in vitro-produced embryos were used to compare the vitrification solutions EFS versus Eg + Gly, and in Experiment 4, one hundred and twenty in vivo-produced embryos and two hundred and fifty two in vitro-produced embryos were transferred either fresh or as frozen-thawed embryos vitrified with Eg + Gly or frozen with E-3S. Pregnancy and lambing rates, length of gestation, birthweight of the lambs and sex ratio were recorded.

2.8. Statistical analysis

Differences in development, hatching, pregnancy and lambing rates and sex ratio were analyzed by Fisher's Exact test. Differences in nuclei number were analyzed using the Mann–Whitney test or Kruskal–Wallys test, as appropriate. Differences in length of gestation and birthweight were analyzed by

two-way ANOVA. All tests were done using Statistica for Windows. A confidence level of $P < 0.05$ was considered significant (Stat Soft Inc., 1999).

3. Results

A total of 1774 oocytes from live ewes were processed, obtaining a cleavage rate of 79.3% ($n = 1407$) and a blastocyst yield of 39.3% ($n = 553$).

In Experiment 1 there were no significant differences in development rates of embryos using different cryoprotectants. However, the hatching rate and nuclei number was higher in the E-3S than the G-3S group ($P < 0.01$) (Table 1). In Experiment 2 there were no differences in the embryonic development rates between the cryoprotectants solutions used. However, EFS resulted in higher hatching rates and nuclei numbers than the Pg + Gly treatment ($P < 0.05$ and <0.01, respectively) (Table 2). In Experiment 3 embryos frozen with Eg + Gly had a level of in vitro development, hatching rate and nuclei numbers significantly higher than blastocysts frozen with EFS ($P < 0.05$, <0.05 and <0.01, respectively) (Table 2). In Experiment 4 the pregnancy rate at 30 and 60 days of gestation and lambing rates of in vivo-produced embryos transferred fresh were significantly higher than those subjected to cryopreservation ($P < 0.05$). Moreover, the other methods used recorded similar performances for these parameters, except the E-3S using in vitro-produced embryos, which showed significantly lower pregnancy rates than the other techniques ($P < 0.05$) (Table 3).

There were no significant differences in gestation length and sex ratio between treatments. However, oversized offspring resulted from the in vitro-produced embryos, the difference in birth weight being significant ($P < 0.01$) between lambs born from in vivo and in vitro-produced embryos (Table 4). A significant ($P < 0.001$) interaction was recorded between the source of the embryos and the treatment used when the gestation length was analyzed. No interactions were recorded in the other parameters.

Table 1
Effect of cryopreservation methods on in vitro development, hatching rates and mean (±S.D.) nuclei number of in vitro-produced ovine embryos

Parameters	E-3S	G-3S
Blastocoel re-expansion (%)	25/50 (50.0)	27/50 (54.0)
Hatching (%)	20/50 (40.0)[a]	10/50 (20.0)[b]
Nuclei number	135.4 ± 20.7[a]	118.5 ± 19.5[b]

Values with different superscripts (a,b) within rows differ significantly ($P < 0.05$).

Table 2
In vitro development, hatching rates and mean (±S.D.) nuclei number after 72-h culture of in vitro-produced ovine embryos vitrified with Pg + Gly and EFS (Experiment 2) and with EFS and Eg + Gly (Experiment 3)

	Experiment 2		Experiment 3	
Parameters	Pg + Gly	EFS	EFS	Eg + Gly
Blastocele re-expansion (%)	23/45 (51.1)	29/45 (64.4)	36/55 (65.4)[a]	48/55 (87.3)[b]
Hatching (%)	13/45 (31.1)[a]	23/45 (51.1)[b]	30/55 (54.5)[a]	42/55 (76.4)[b]
Nuclei number	113.5 ± 22.0[a]	133.8 ± 36.8[b]	113.1 ± 14.1[a]	135.7 ± 34.5[b]

Values with different superscripts (a,b) within rows differ significantly ($P < 0.05$).

Table 3
Pregnancy and lambing rates (%) after transfer of in vivo and in vitro-produced ovine embryos, either fresh or cryopreserved

Treatment	Source of embryos	Pregnancy rate at 30 days	Pregnancy rate at 60 days	Lambing rate
Fresh	In vivo	40/56 (71.4)[a]	39/56 (69.6)[a]	38/56 (67.8)[a]
E-3S	In vivo	26/56 (46.4)[bc]	26/56 (46.4)[b]	24/56 (42.8)[b]
Eg + Gly	In vivo	23/56 (41.1)[bc]	23/56 (41.0)[bc]	21/56 (37.5)[bc]
Fresh	In vitro	32/64 (50.0)[bc]	27/64 (42.1)[bc]	24/64 (37.5)[bc]
E-3S	In vitro	20/65 (30.8)[c]	18/65 (27.6)[c]	15/65 (23.0)[c]
Eg + Gly	In vitro	32/75 (42.7)[bc]	24/75 (32.0)[bc]	20/75 (26.6)[bc]

Values with different superscripts (a,b,c) within columns differ significantly ($P < 0.05$).

Table 4
Mean (±S.D.) gestation length and birth weight and sex ratio after transfer of in vivo and in vitro-produced ovine embryos, either fresh or cryopreserved

Method	Source of embryos	Gestation length (days)	Birth weight (kg)	Sex ratio (female:male)
Fresh	In vivo	153.7 ± 2.2	3.2 ± 0.6[a]	20:18
E-3S	In vivo	154.2 ± 2.5	3.1 ± 0.6[a]	12:12
Eg + Gly	In vivo	152.3 ± 2.5	3.3 ± 0.6[a]	10:11
Fresh	In vitro	155.9 ± 2.3	5.5 ± 0.5[b]	11:13
E-3S	In vitro	152.5 ± 2.5	4.9 ± 0.4[b]	7:8
Eg + Gly	In vitro	153.4 ± 2.2	5.4 ± 0.5[b]	10:10

Values with different superscripts (a,b) within columns differ significantly ($P < 0.05$).

Table 5
Assisted births and perinatal death rates of lambs after transfer of in vivo and in vitro-produced ovine embryos

	In vivo-produced	In vitro-produced
Assisted birth (%)	3/83 (3.6)[a]	10/59 (16.9)[b]
Perinatal death (%)	1/83 (1.2)[a]	6/59 (10.1)[b]

Values with different superscripts (a,b) within columns differ significantly ($P < 0.05$).

There were significant ($P < 0.05$) differences in the incidence of assisted births and perinatal deaths between the in vivo and in vitro-produced embryos ($P < 0.05$) (Table 5).

4. Discussion

The different cryopreservation protocols evaluated in terms of embryo survival in vitro, pregnancy and lambing rates, were in general with acceptable results. In contrast with previous work (Earl et al., 1996; Songsasen et al., 1996; Ptak et al., 1999; Dattena et al., 2000; Papadopoulos et al., 2002; Leon et al., 2002) where either the conventional slow freezing or vitrification was used, or using two or more embryos per recipient ewes, in the present study slow freezing as vitrification were used, transferring one embryo to a recipient.

According to the results obtained in Experiment 1, ethylene glycol was superior to glycerol as a cryoprotectant for the freezing of in vitro-produced ovine embryos. Széll et al. (1989) and Songsasen et al. (1995), found enhanced permeability to ethylene glycol compared to propylene glycol, glycerol and DMSO. In addition, an improvement in post-thaw survival in embryos treated with ethylene glycol were recorded. Results obtained support these earlier findings. Similar results were found with in vitro-produced embryos (Leibo and Loskutoff, 1993) and in vivo-produced embryos (Cocero et al., 2002).

According to Experiment 2, EFS was superior to Pg + Gly, regarding the hatching rate and nuclei number. This fact could be explained by the higher permeability to ethylene glycol by the blastocysts, compared to propylene glycol and glycerol (Palasz and Mapletoft, 1996). Moreover, sucrose and Ficoll 70 (non-permeable cryoprotectants) presumably confer an additional protective effect on EFS, whereas Pg + Gly lacks non-permeable cryoprotectants in its formulation. Sucrose causes embryos to dehydrate, thereby reducing the likelihood of intracellular ice formation and concentrating macromolecules in the cytoplasm thus facilitating intracellular vitrification (Rall, 1987). The use of a macromolecule such as Ficoll 70 facilitates vitrification and prevents extracellular ice formation during vitrification and warming (Fahy et al., 1984; Kasai et al., 1990).

Experiment 3 showed that embryos cryopreserved with EFS showed less development, and lower hatching rates and nuclei number than Eg + Gly were recorded. This difference appears to be related to the number of equilibration steps and/or cryoprotectant concentrations, rather than the combination of the cryoprotectants. This hypothesis is supported by previous research, where it was found that an increase in the number of equilibration steps before final immersion in vitrification solution resulted in higher embryo survival rates after warming (Kuwayama et al., 1992; Darvedil et al., 1994; Mahmoudzadeh et al., 1995; Ohboshi et al., 1997).

The differences in nuclei numbers are consistent with the differences in hatching rates. The methods

that produced the highest hatching rates, also showed a higher mean nuclei number. This association between embryo survival and nuclei number would be useful to predict the embryo implantation ability for a given technique. This hypothesis is currently being investigated.

These results prompted the transfer of embryos after freezing with E-3S or after vitrification with Eg + Gly.

Pregnancy and birth rates after transfer of fresh, in vivo-produced embryos gave remarkably higher rates than those obtained with the other treatments which was expected, as the embryos are more robust than their in vitro-matured counterparts, and are not subjected to cryopreservation. This agrees with previous work where different susceptibilities between embryos were obtained in vivo and in vitro (both fresh and cryopreserved) (Greve et al., 1993; Pollard and Leibo, 1994; Massip et al., 1995).

E-3S with in vitro-produced embryos had the lowest pregnancy and birth rates, showing that the embryos obtained in vitro are particularly sensitive to this freezing method. This was not observed with Eg + Gly, or when in vivo-produced embryos were frozen with E-3S. These results agree with previous studies in bovine embryos (Tachikawa et al., 1993; Massip et al., 1995).

The significant interaction between the source of the embryos and treatment that was found when gestation length was analyzed, is difficult to explain and further research using a larger number of embryos is essential to elucidate this phenomenon.

In all treatments, pregnancy losses, as recorded by the difference between pregnancy rate at 30 days of gestation and birth rate, were recorded. Despite similar implantation rates between treatments, the largest losses were observed in the in vitro-produced embryos (7.8–16.1%), whereas embryos obtained in vivo showed a 3.6% pregnancy loss, regardless of the cryoprotectants used. This agrees work on bovines, where not only lower pregnancy rates, but also higher fetal losses were observed when in vitro-produced embryos were used when compared to in vivo-produced embryos (Greve et al., 1993; Hasler et al., 1995; Massip et al., 1995; Wright and Ellington, 1995).

The birth weight from in vitro-produced embryos was greater than that from in vivo-produced embryos an effect that is not attributable to differences in the genetic make-up of the ewes used. The increase in birth weight led to a subsequent significant increase in the occurrence of assisted births and perinatal deaths. This fact has been previously reported in ovine and bovine, and has been related to the use of serum in the culture medium, or some other factors still unknown, related to the process of the in vitro production (Holm et al., 1996; Walker et al., 1996; Farin and Farin, 1995; Thompson, 1997).

There were no differences in the sex ratio of the lambs born. These results agree with the sex ratio reported by Catt et al. (1997), who despite finding a higher proportion of males among in vitro-produced ovine embryos that reach the earlier blastocyst stage, no differences were recorded at birth.

5. Conclusion

Results demonstrate that the transfer of in vitro-produced embryos vitrified with Eg + Gly would be a feasible procedure for large-scale commercial programs, if dystocia due to fetal size could be overcome. Obtaining unusually big lambs from in vitro-produced embryos could be a limiting factor for the use of this type of embryo in commercial programs. This would be particularly critical in areas that require extensive management of the flocks.

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