Journal of Applied Animal Research

ISSN: 0971-2119 (Print) 0974-1844 (Online) Journal homepage: http://www.tandfonline.com/loi/taar20

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To cite this article: F. Forcada , A. Buffoni , J.A. Abecia , B. Asenjo , I. Palacín , M.I. Vázquez , J.C. Rodríguez-Castillo , L. Sánchez-Prieto & A. Casao (2011) Maximising embryo production in endangered sheep breeds: *in vitro* procedures that complement *in vivo* techniques, Journal of Applied Animal Research, 39:4, 412-417, DOI: 10.1080/09712119.2011.622929

To link to this article: https://doi.org/10.1080/09712119.2011.622929

Accepted author version posted online: 03 Oct 2011.
Published online: 28 Nov 2011.

Journal of Applied Animal Research,
Vol. 39, No. 4, December 2011, 412–417

Taylor & Francis
Taylor & Francis Group

Maximising embryo production in endangered sheep breeds: *in vitro* procedures that complement *in vivo* techniques

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(*Received 16 February 2011; final version received 19 April 2011*)

This study investigated the use of previously superovulated ovaries as a source of oocytes, assessing the competence of them for *in vitro* embryo production. Two superovulatory treatments were performed: equine Chorionic Gonadotrophin (eCG) plus porcine Follicle-Stimulating Hormone (pFSH) in a single dose or the conventional protocol of six decreasing doses of pFSH. Thirty donor ewes of the endangered Ojalada breed were given either the simplified (group S; $n = 15$) or the decreasing-dose (group D; $n = 15$) treatments three times at intervals of ≥ 50 days. Ovaries were recovered on day 7 after the oestrus following the third treatment, just after embryo flushing, and the oocytes were collected to assess *in vitro* maturation, fertilisation and development to the blastocyst stage. The two superovulatory treatments did not differ in the mean number of oocytes selected for maturation (7.1 ± 1.2 and 8.5 ± 1.5 per ewe in the D and S groups, respectively). The oocytes recovered from ewes in Group D (87.5%) had a significantly ($p < 0.05$) higher maturation rate than did those recovered from ewes in group S (75%), but no differences were found in fertilisation rate (94% and 94.6% in the D and S groups, respectively); both groups did not differ in their blastocyst rates and the total number of cells in *in vitro*-produced blastocysts. In the two experimental groups, 1.7 (D) and 1.8 (S) *in vitro*-produced blastocysts were generated per ewe, which indicate that it is feasible to combine *in vivo* and *in vitro* techniques to maximise embryo production in endangered sheep breeds.

Keywords: sheep; superovulation; *in vitro* embryo production

1. Introduction

In vitro embryo production (IVEP) in sheep provides an excellent source of low-cost embryos for basic research in developmental biology and physiology. In addition, IVEP might provide a means of improving genetic gain by reducing the generation interval and helping in the rescue of some endangered species or breeds (Cognié et al. 2003; Mishra et al. 2010). Currently, however, the efficiency of IVEP is lower than that of *in vivo* embryo production, and collecting embryos from a donor female is the most efficient way to obtain high-quality embryos at a given stage of development. In fact, and although no statistics on current sheep embryo production are available, recent data from the European Embryo Transfer Association show that only 5.8% of bovine transferred embryos were *in vitro* produced (Merton 2010; data from 23 countries).

Ojalada Soriana is a Spanish breed of sheep of which there are 8531 and 209 registered ewes and rams, respectively; therefore, the Spanish government considers the breed 'in danger of extinction' and eligible to benefit from several programmes for the production and cryoconservation of gametes and embryos. Embryo banks seem to be the most practical means of maintaining genetic diversity and improving the genetic diversity of such local endangered sheep genotypes that have few individuals but are well adapted to specific breeding systems. However and in order to progressively transfer the technology to the breeders associations, the in vivo embryo production can be the first technology to be implemented (Forcada et al. 2000).

A new means of maximising the number of embryos recovered from a single donor ewe might be to combine *in vivo* and *in vitro* embryo production, which might be feasible for preservation of endangered genotypes within a very small population and, therefore, from a limited source of ewes as embryo donors. In recent years, we have examined the effects

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ISSN 0971-2119 print/ISSN 0974-1844 online

http://dx.doi.org/10.1080/09712119.2011.622929
http://www.tandfonline.com

of repeated superovulation for *in vivo* embryo production in donor ewes at the end of their reproductive lives as a means of increasing the progeny of these valuable females (Forcada et al. 2000, 2006, Forcada et al. 2011); however, we have not examined the prospects of maximising embryo production from ewes by combining *in vitro* and *in vivo* techniques. Thus, the objective of our study was to investigate the use of previously superovulated ovaries as a source for oocyte production in mature Ojalada Soriana ewes by assessing the effect of repeated ($3\times$) superovulations on the number of oocytes recovered from ovaries collected 7 days after oestrus following the third superovulatory treatment, just after embryo flushing. The competence of the oocytes for IVEP was also assessed.

2. Materials and methods

The study was conducted between September 2008 and March 2009 at the experimental farm of the University of Zaragoza, Spain (latitude 41°40′N). All procedures were approved by the in-house Ethics Committee for Animal Experiments, University of Zaragoza. The care and use of the animals adhered to the Spanish Policy for Animal Protection RD1201/05, which meets the requirements of the European Union Directive 86/609 on the Protection of Animals used for Experimental and Other Scientific Purposes.

2.1. Animals

Thirty mature Ojalada Soriana ewes (6.7 ± 0.5 year of age and 5.2 ± 0.4 previous lambings) from the Spanish Association of Ojalada Soriana Breeders were brought to the experimental farm, where they were housed in communal yards that had uncovered areas and fed a concentrate ration, lucerne hay and barley straw at rates designed to provide 1.2 times their maintenance requirements. Water was available at all times.

2.2. Experimental design

Oestrus was synchronised using intravaginal sponges that contained 30 mg fluorogestone acetate (Sincropart®; Ceva Salud Animal S.A., Barcelona, Spain), which were inserted for 14 d. Two superovulatory protocols were performed: ewes in the Simplified group (S; $n=15$) received 250 IU of pFSH (Folltropin®; Bioniche Animal Health, Dublin, Ireland) and 500 IU eCG (Sincropart®; Ceva Salud Animal S.A.) in a single intramuscular (i.m.) administration 48 h before the intravaginal sponge was removed. Ewes in the Decreasing group (D; $n=15$) received 280 IU of pFSH in six i.m. administrations that contained decreasing dosages (2, 1.5 mL, 1.25 mL $\times$ 2 and 1 mL $\times$ 2) at 12-h intervals, which began 48 h before the intravaginal sponge was removed. Fertile rams were placed with the ewes at the time of pessary withdrawal, and ewes were examined for evidence of oestrus every 8 h.

Embryos from superovulatory treatments were collected through a mid-ventral laparotomy 7 days after the onset of oestrus. For surgical procedures, ewes were anesthetised using an i.m. administration of 0.4 mL 2% xylazine and, 5 min later, an intravenous (i.v.) injection of 10 mL sodium thiopental (20 mg/mL) (Thiobarbital, Braun Medical, Jaen, Spain). Throughout the experiment (September through March), the ewes were given the same superovulatory treatment as many as three times at intervals of at least 50 days.

2.3. Collection and in vitro maturation (IVM) of oocytes

After embryos were collected following the third superovulatory treatment, 7 days after oestrus, ewes were euthanised using an i.v. injection of sodium thiopental (T-61®; Intervet, Salamanca, Spain). Ovaries were recovered and placed in Phosphate Buffered Saline (PBS) supplemented with 100 IU/mL of penicillin G and 100 μg/mL of streptomycin sulphate and stored at 39°C until they were processed, not later than half an hour after ovariectomy. Except where otherwise indicated, the reagents were from Sigma-Aldrich Co., St Louis, MO, USA. A combination of puncture and slicing techniques (Wani et al. 1999) were used to collect oocytes in a Petri dish that contained a handling medium (Hepes-buffered TCM-199 supplemented with 0.1% polyvinyl alcohol, 0.04% sodium bicarbonate, 25 IU/mL of heparin, 100 IU/mL of penicillin G and 100 μg/mL of streptomycin sulphate). Following Wani et al. (2000), oocytes were classified based on their cumulus cells and cytoplasm morphology, and only the oocytes that had several layers of cumulus cells and a uniform cytoplasm (healthy oocytes) were selected for IVM. Oocytes were transferred into a maturation medium that contained bicarbonate-buffered TCM-199 supplemented with 10% (v/v) oestrous sheep serum, 0.1 μg/mL each of FSH and Luteinizing Hormone (LH), 100 μM of cysteamine, 0.3 μM of sodium pyruvate, 100 IU/mL of penicillin G and 100 μg/mL of streptomycin sulphate, which was

covered with mineral oil and incubated at 39°C under 5% CO_2 and saturated humidity for 24 h.

2.4. In vitro fertilisation and embryo culture

At the end of IVM, the oocytes were denuded from the cumulus cells and transferred to the fertilisation medium, which consisted of synthetic oviductal fluid (SOF) without glucose (Tervit and Whittingham 1972) and was supplemented with 2% (v:v) of oestrous sheep serum, 10 μg/mL of heparin and 1 μg/mL of hypotaurine. On the same day of fertilisation, the semen collected from four Ojalada rams was pooled, diluted 1:10 in a saline medium that contained 0.25 mol/L of sucrose, 10 mmol/L of Hepes, 2 mmol/L of potassium hydroxide, 5 mmol/L of glucose, 0.5 mol/L of sodium phosphate monobasic and 100 mmol/L of ethylene glycol tetra-acetic acid, and kept at 15°C until fertilisation. Highly motile spermatozoa were selected based on the swim-up technique (Wani et al. 2000; Luther et al. 2005), added to the fertilisation medium that contained the oocytes at a final concentration of 1×10^{-6} spermatozoa/mL, which was covered with mineral oil and incubated for 24 h at 39°C in an atmosphere of 5% CO_2. At 24 and 36 h after fertilisation, cleaved embryos were placed in a culture medium that contained SOF supplemented with essential and non-essential amino acids at oviductal concentrations (Walker et al. 1996), 0.4% bovine serum albumin (wt/vol), 1 mM of l-glutamine, 100 IU/mL of penicillin G and 100 μg/mL of streptomycin sulphate, and covered with mineral oil and kept at 39°C in a maximally humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 8 days until the blastocyst stage.

2.5. Peroxide status evaluation

High intracellular concentrations of hydrogen peroxide in blastocysts can cause cellular damage and apoptosis (Yang et al. 1998; Sakatani et al. 2008). The concentrations of peroxides in hatched *in vitro*-produced embryos ($n = 8$ and $n = 7$ from ewes in the D and S groups, respectively) were quantified on day 8 after fertilisation using dichlorodihydrofluorescein diacetate (DCHFDA) (after Hashimoto et al. 2000). Blastocysts were transferred to a culture medium that contained 10 μM DCHFDA and, after 20 min, the embryo nuclei were counterstained using HOECHST 33342 (10 μg/mL) for 10 min. Embryos were fixed using 1.5% of glutaraldehyde for 15 min, washed in PBS and then mounted on glass slides using CC/mount® mounting medium. The fluorescent emissions from the embryos were recorded under a fluorescent microscope equipped with Fluorescein Isothiocyanate (FITC) filters (excitation, 405–435 nm; emission, 515 nm). To minimise subjective observation errors, the coded samples were evaluated by a blinded operator. A bright green fluorescence indicated that a cell was DCHFDA-positive, and the HOECHST stain facilitated the counting of cells.

2.6. Statistical analysis

The following information was recorded for each animal in both experimental groups: number of oocytes recovered, number of healthy oocytes selected for maturation and number of non-healthy oocytes. All of them were expressed as mean ± SEM. The numbers of matured oocytes, fertilised embryos, cleaved embryos and blastocysts were expressed as totals for each group. Maturation and cleavage rates were calculated over the number of healthy oocytes, fertilisation rate was based on the number of matured oocytes, and blastocyst rates were based on the number of cleaved embryos. The significance of differences in maturation, fertilisation, cleavage and blastocyst rates were evaluated statistically using Chi-square tests. Differences among the two experimental groups in the number of oocytes selected for maturation, total number of cells and the number of cells that were DCHFDA-positive (i.e., higher hydrogen peroxide concentrations) per blastocyst were compared using a one-way ANOVA. Within each superovulatory treatment, the relationship between *in vivo* embryo production and the number of oocytes recovered per ewe was analysed using a correlation matrix. The probability level for statistical significance was set to $p < 0.05$.

3. Results

The number of oocytes recovered per ewe and selected for maturation from previously superovulated Ojalada Soriana ewes did not differ significantly between the D (7.1 ± 1.2) and the S treatments (8.5 ± 1.5). In both groups, the mean numbers of non-selected oocytes were 4.1 ± 0.7 and 5.1 ± 0.9, respectively. In the D group, neither the total number of oocytes recovered nor the number of oocytes selected per ewe was significantly correlated with *in vivo* embryo production parameters (ovulation rate, numbers of ova and embryos recovered and the number of viable or freezable embryos) in the first recovery or in the three consecutive recoveries combined (Table 1). In the S group, however, oocyte production was negatively correlated with the total number of viable and freezable embryos produced after the three consecutive recoveries, which indicated that high embryo production, in response to repeated

Table 1. Pearson correlation coefficients between the number of oocytes recovered *post-mortem* (R) or the number of oocytes selected for *in vitro* maturation (M) per ewe and parameters associated with *in vivo* embryo production from Ojalada Soriana ewes treated with either 210 IU of pFSH plus 500 IU of eCG administered as a single dose (S) or 280 IU of pFSH administered in six incrementally decreasing doses (D). The *in vivo* embryo production data were from the first (F) or the combined totals (T) of three consecutive embryo recoveries.

	Groups			
	D		S	
Parameters	M	R	M	R
Ovulation rate F	0.03	0.03	0.09	0.23
Ovulation rate T	0.08	0.12	−0.12	−0.12
Recovered ova and embryos F	0.15	0.10	0.23	0.29
Recovered ova and embryos T	−0.15	−0.12	0.06	0.11
Viable embryos F	0.20	0.14	–0.15	−0.12
Viable embryos T	−0.01	−0.30	–0.46**	−0.46**
Freezable embryos F	0.24	0.16	–0.16	−0.14
Freezable embryos T	0.02	0.10	−0.40*	−0.38*

*$p < 0.1$; **$p < 0.05$.

applications of a single dose of eCG + FSH, can impair oocyte yield and quality from these superovulated ovaries.

In the early stages of the IVEP procedure, the oocytes recovered from the ewes that received the pFSH treatment (D group) seemed to have a higher competence (significantly higher maturation and cleavage rates) than did those recovered from ewes that received the eCG + FSH treatment (S group) (Table 2). However, both experimental groups did not differ significantly in blastocyst rates (Table 2) or in the development during embryo culture when data were analysed by culture day and blastocyst type (Table 3).

Superovulatory treatments did not differ significantly in the mean number of cells (HOECHST staining) (160 ± 24 and 188 ± 28 cells in the D and S groups, respectively), and the number of cells that were DCHFDA-positive (higher hydrogen peroxide concentrations) per embryo (13.7 ± 3.5 and 19.1 ± 4.1 cells in the D and S groups, respectively).

4. Discussion

In the present study, the production and quality of oocytes from previously superovulated ovaries that were subjected to three consecutive superovulatory treatments were similar in the two superovulatory experimental groups (11.2 and 13.7 oocytes recovered per ewe with a 63% and 62% of selected oocytes in group D and S, respectively). Recently, Vázquez et al. (2010) have reported recovery of 16.1 oocytes and a 60% of selected oocytes after a commercial treatment with 400 IU of eCG also during the seasonal anoestrus. Concerning ovum pick-up recovery through laparotomy or laparoscopy, the mean number of selected oocytes per ewe varied between 3.1 and 10.0 among ewes subjected to different FSH superovulatory regimes (Baldassarre et al. 1996 in Merino × Corriedale; Ptak et al. 1999 in Sarda; Alberio et al. 2002 and Hammami 2008 in Rasa Aragonesa; Veiga-López et al. 2008 in Manchega).

The weak, but statistically significant negative correlation between oocyte production and *in vivo* embryo recovery after three consecutive treatments among Ojalada Soriana ewes in the S group was unexpected. It is possible that mature, previously superovulated Ojalada Soriana ewes respond to repeated administrations of a single dose of pFSH + eCG for superovulation by creating anti-eCG antibodies (Forcada et al. 2011). In those ewes, plasma anti-eCG antibody concentrations increased from day 5 after eGC administration (Roy et al. 1999). In general, high levels of anti-eCG antibodies are associated with alterations of follicular dynamics, especially, delays in follicular growth (Roy et al. 1999) and, therefore, with alterations in the corresponding follicular wave. Given that *in vivo* embryo production after three consecutive superovulatory treatments was very similar in the two treatment groups, the relationship between high embryo production and the impairment of oocyte yield and quality among ewes that received repeated administrations of the simplified treatment might reflect the negative effects of high concentrations of anti-eCG plasma antibodies.

Oocytes recovered from superovulated ovaries can have impaired maturation rates (Berlinguer et al.

Table 2. *In vitro* maturation, fertilisation, cleavage and blastocyst rates for oocytes recovered *post-mortem* from Ojalada Soriana ewes that had previously received three consecutive superovulatory treatments for *in vivo* embryo production. The superovulatory treatments were either 210 IU of pFSH plus 500 IU of eCG administered as a single (S) or 280 IU of pFSH administered in six incrementally decreasing doses (D).

Group	Maturation	Fertilisation	Cleavage	Blastocyst rate
D	84/96 (87.5%)[a]	79/84 (94%)[a]	79/96 (82.3%)[a]	25/79 (31.6%)[a]
S	93/124 (75%)[b]	88/93 (94.6%)[a]	87/124 (70.2%)[b]	27/87 (27%)[a]

Note: Within columns, different letters (a, b) indicate differences at $p < 0.05$.

Table 3. *In vitro* blastocyst production on days 6, 7 and 8 after oocyte fertilisation from oocytes recovered *post-mortem* from Ojalada Soriana ewes that had previously received three consecutive superovulatory treatments for *in vivo* embryo production. The superovulatory treatments were either 210 IU of pFSH plus 500 IU of eCG administered as a single dose (S) or 280 IU of pFSH administered in six incrementally decreasing doses (D).

Day after fertilisation	Group	Young blastocyst	Expanded blastocyst	Hatching/hatched blastocyst
6	D	6/79 (7.6%)	5/79 (6.3%)	
	S	4/87 (4.6%)	8/87 (9.2%)	
7	D	5/79 (6.3%)	11/79 (13.9%)	3/79 (3.8%)
	S	3/87 (3.4%)	15/87 (17.2%)	2/87 (2.3%)
8	D	6/79 (7.6%)	10/79 (12.7%)	9/79 (11.4%)
	S	6/87 (6.9%)	12/87 (13.8%)	9/87 (10.3%)

2004; Hammami 2008), particularly, after 'decreasing-dose' FSH protocols, in which the first high doses can induce rapid, abnormal follicular development and asynchrony between growing follicles and follicular status (Blondin et al. 1996), because a plateau phase (Sirard et al. 1999) is necessary to complete oocyte capacitation (Hyttel et al. 1997). In our experiment, oocytes were not recovered in the oestrus induced by the superovulatory treatments, but in the subsequent follicular wave. The maturation and cleavage rates exhibited by the oocytes recovered from ovaries of ewes superovulated using the simplified protocol were lower than the rates of the oocytes recovered from ovaries subjected to the decreasing-doses protocol; the high anti-eCG antibody concentrations in some of the ewes in the S group (Forcada et al. 2011) might have contributed to the difference in oocyte competence. In our experiment, blastocyst rates did not differ significantly between treatment groups. Thus, the *in vitro* culture of embryos did not indicate a negative effect of previous treatments with eCG on oocyte competence. In ewe lambs, Kelly et al. (2005) reported that oocytes following a pFSH-based superovulatory treatment that included eCG that were matured and fertilised *in vitro*, had higher blastocyst rates than did the oocytes recovered after a treatment without eCG (Kelly et al. 2005).

Embryos produced *in vivo* following a simplified pFSH + eCG superovulatory treatment can have fewer cells and, therefore, lower viability and tolerance to cryopreservation than do embryos derived from treatments based on repeated doses of pFSH (Leoni et al. 2001; Forcada et al. 2011). In our study, the numbers of total and DCHFDA-positive cells in blastocysts from *in vitro*-produced oocytes did not differ significantly between the two superovulatory treatment groups which suggests that the negative effect of the simplified protocol on embryo quality is not apparent in the follicular wave that follows the oestrus induced by the superovulatory treatment. However, the technical and commercial viability of the pFSH + eCG protocol require further study.

In conclusion, our study demonstrates that, in the early stages of IVEP, the competence of the oocytes recovered at day 7 after oestrus, just after embryo flushing, from mature, previously superovulated Ojalada Soriana ewes was lower in those treated with a single dose of eCG plus pFSH than it was in ewes treated with the standard pFSH treatment. Those differences disappeared after fertilisation, and blastocyst rates and the viability of blastocysts did not differ among experimental groups. A total of 25 and 27 *in vitro*-produced blastocysts were generated in the decreasing and simplified groups, corresponding to 1.7 and 1.8 blastocysts per ewe, respectively. Considering that the total number of *in vivo*-produced embryos per ewe that received the three superovulatory treatments was 14.1 (D group) and 13.7 (S group), the numbers of *in vivo*- and *in vitro*-produced embryos per donor ewe were 15.8 (D group) and 15.5 (S group), which indicates that, although the *in vitro* procedures are less efficient, the combination of both techniques could be useful to maximise embryo production in endangered sheep genotypes.

Acknowledgements

This study was supported by Grant RZ2008-0002 from INIA and Grant A26 from DGA (Spain).

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