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Short communication. *In vitro* embryo production can be modified by the previous ovarian response to a superovulatory treatment in sheep

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Abstract

Thirty-two ewes were used to study how the ovarian response to a superovulatory treatment determines quality of oocytes recovered from ovaries after embryo collection, and their developmental capacity after *in vitro* maturation (IVM) and fertilization (IVF). Ewes were superovulated, and seven days after oestrus, embryos were collected and ewes divided into three groups: (+ +), n = 19, ewes responding to the treatment with embryos collected after flushing; (+ –), n = 8, ewes responding, but only oocytes were found; and (– –), n = 5, ewes not responding to the treatment and no embryos collected. Ovaries were recovered and oocytes collected from the three groups. A significant effect of the response to the treatment was observed for oocyte quality, so that (– –) ewes presented the higher number of oocytes per ewe ($p < 0.001$). Total number of oocytes selected for IVM and IVF was significantly higher in the same group, in comparison with (+ +) and (+ –) ($p < 0.001$). Group (+ –) ewes presented the lowest maturation ($p < 0.001$), fertilization ($p < 0.05$) and cleavage rates ($p < 0.001$). In conclusion, the ovarian response to a superovulatory treatment determines the number and quality of the oocytes recovered 7 days after the oestrus induced by the hormonal treatment. *In vitro* techniques could be an important tool to increase embryo production by particular ewes when they are not able to produce a significant amount of *in vivo* embryos.

Additional key words: ovary; oocyte; follicle-stimulating hormone.

Superovulation hormonal treatments and the subsequent embryo collection techniques from donor ewes have become one of the most efficient tools to improve genetic programs and recover endangered livestock breeds. Several attempts to optimize these procedures have focused on easing hormone administration protocols and increasing the number of transferable embryos per donor ewe. Forcada *et al*. (2000) developed a system to obtain the maximum number of embryos of high genetic value involving identification of the best females for a given character, and their use as embryo donors when they are candidates for culling for non-reproductive reasons. This particular treatment involves the superovulation up to three times at 60-day intervals before euthanasia. Later on, a simplified protocol to administer porcine follicle-stimulating hormone (pFSH) (one single injection of pFSH and equine chorionic gonadotropin [eCG] *vs.* six decreasing doses of pFSH) was developed with comparable results (Simmonetti *et al*., 2008; Forcada *et al*., 2011a). This protocol has also been tested using intrauterine insemination (Forcada *et al*., 2012). The efficiency of *in vivo* embryo production decreases significantly after the first superovulatory treatment (Forcada *et al*., 2011a). Apparently, changes in the ovulatory response after repeated superovulation with FSH have not been reported (Forcada *et al*., 2006), although Forcada *et al*. (2000) observed a significant decrease in ovulation

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Abbreviations used: CL (corpora lutea); eCG (equine chorionic gonadotropin); FGA (fluorogestone acetate); IU (international units); IVF (*in vitro* fertilization); IVM (*in vitro* maturation); pFSH (porcine follicle-stimulating hormone).

rate after the third FSH treatment. The formation of post-operative adhesions might impair the number of embryos recovered or sperm transport, which might increase the number of unfertilized ova (Forcada *et al*., 2006). Due to the drop of embryo production from the first to the third superovulation protocol, a combination of repeated superovulation treatments (*in vivo* embryo production) and laboratorial techniques (*in vitro* fertilization) was designed, becoming a new step to maximise the number of embryos recovered from a single donor. Thus, after the third embryo collection, ewes are euthanized and their ovaries collected to recover their oocytes to be maturated and fertilized *in vitro*. Forcada *et al*. (2011b) obtained about 16 embryos per ewe when combining *in vivo* and *in vitro* techniques. However, it was observed that the competence of the oocytes recovered at day 7 after oestrus, immediately after embryo flushing from previously superovulated ewes, was lower in those treated with a single dose of eCG plus pFSH than in those ewes treated with the standard pFSH treatment.

The objective of this work was to study how the ovarian response to a superovulatory treatment determines the number and quality of the oocytes recovered from the treated ewes after the *in vivo* embryo collection, and their capacity to become embryos after an *in vitro* maturation and fertilization techniques.

All procedures were approved by the Ethics Committee for Animal Experiments from the University of Zaragoza. Thirty-two mature Ojalada de Soria ewes (mean age 8.23 ± 0.4 years; mean number of previous lambings 5.3 ± 0.4; mean liveweight 58.8 ± 1.7 kg) were used. In late August, before culling, ewes were housed in communal yards (41°N) that had uncovered areas and fed with a concentrate ration, lucerne hay, and barley straw. During the breeding season, ewes were superovulated and natural mated three times at 50-60 days intervals with 210 international units (IU) of pFSH (Folltropin; Bioniche Animal Health, Dublin, Ireland) and 500 IU eCG (Folligon, MSD Salud Animal, Madrid, Spain) in a single i.m. administration 48 h before the intravaginal sponge (fluorogestone acetate, FGA, 14 day-treatment; Folligon, MSD Salud Animal, Madrid, Spain) was removed. After every treatment, embryos were collected through a mid-ventral laparotomy 7 days after the onset of oestrus. Results of the reproductive performances have been previously published (Forcada *et al*., 2012). Immediately afterwards the third embryo collection, ewes were euthanized using an i.v. injection of sodium thiopental (80 mg kg^{-1} liveweight, T-61, MSD, Salamanca, Spain). Ovaries were recovered and stored at 39°C until they were processed, not later than half an hour after ovariectomy. Ewes were divided in three groups according the ovarian response to the superovulation treatment and their embryo collecting performances: Group (+ +), n = 19, ewes which responded positively to the treatment (ovaries with more than three healthy corpora lutea [CL]), those of which embryos were collected after uterine flushing; Group (+ –), n = 8, ewes which responded positively to the treatment, but only oocytes were found after uterus flushing; and Group (– –), n = 5, ewes which did not respond to the treatment (ovaries with corpora albicans or without signals of ovulation) and consequently, no embryos were collected. A combination of puncture and slicing techniques were used to collect oocytes, which were classified based on their cumulus cells and cytoplasm morphology, as: good (all oocytes with a lot of complete layers of granulose cells and homogeneous cytoplasm), fair (all oocytes with few or incomplete layers of granulose cells and homogeneous cytoplasm) or poor (oocytes with few or absence of granulose cells and non-homogeneous cytoplasm). Only good and fair oocytes (healthy oocytes) were selected for *in vitro* maturation (IVM). At the end of IVM, the oocytes were denuded from the cumulus cells and transferred to the fertilization medium. On the same day of fertilization, the semen collected from four Ojalada rams was pooled, diluted 1:10 in a saline medium and kept at 15°C until *in vitro* fertilization (IVF). Highly motile spermatozoa were selected by swim-up technique and added to the fertilization medium that contained the oocytes at a final concentration of $1 \cdot 10^6$ spermatozoa mL^{-1}, covered with mineral oil and incubated for 24 h at 39°C in an atmosphere of 5% CO_2 and saturated humidity. After 24 and 36 h, presumptive zygotes were assessed for cleavage. Non cleaved oocytes were observed to assess their maturation stage. Oocytes showing the first polar body were considered matured, and oocytes with two polar bodies were considered fertilized but not cleaved. After fertilisation, cleaved embryos were placed in a culture medium for 8 days. Media used for oocyte collection and IVM, IVF and embryo culture have been described by Forcada *et al*. (2011b).

Total number and classification of oocytes recovered and number of healthy oocytes selected for

Table 1. Number (mean ± S.E.M.) of good, fair or poor oocytes per ewe recovered from ovaries of Ojalada ewes: which responded positively to the previous superovulatory treatment and embryos were collected after uterine flushing (Group + +); which responded positively to the treatment, but only oocytes were found after uterus flushing (Group + –); and which did not respond to the treatment and no embryos were collected (Group – –)

Oocyte quality	Group		
	(+ +) (n = 19)	(+ –) (n = 8)	(– –) (n = 5)
Total	5.74±0.84[a]	8.00±0.96[a]	18.40±4.4[b]
Good	2.21±0.27[a]	2.59±0.57[a]	9.20±2.92[b]
Fair	1.11±0.28	1.38±0.26	2.00±0.71
Poor	2.42±0.54[c]	4.13±0.61[c]	7.20±1.72[d]
Healthy (Good + Fair)	3.32±0.45[a]	3.88±0.72[a]	11.20±2.91[b]

Different superscripts in the same row mean significant differences between groups ([a, b]: $p < 0.001$; [c, d]: $p < 0.05$).

Table 2. Results of the *in vitro* maturation (IVM) and fertilization (IVF) of oocytes recovered from ovaries of Ojalada ewes: which responded positively to the previous superovulatory treatment and embryos were collected after uterine flushing (Group + +); which responded positively to the treatment, but only oocytes were found after uterus flushing (Group + –); and which did not respond to the treatment and no embryos were collected (Group – –)

Group	IVM	IVF	Cleavage rate 36 h	Blastocysts rate
(+ +)	48/63[a]	47/48[c]	46/63[a]	23/46
(n = 19)	(76.2%)	(97.9%)	(73.0%)	(50.0%)
(+ –)	10/30[b]	7/10[d]	7/30[b]	1/7
(n = 8)	(33.3%)	(70.0%)	(23.3%)	(14.3%)
(– –)	45/56[a]	43/45[c]	43/56[a]	23/43
(n = 5)	(80.4%)	(95.6%)	(76.8%)	(53.5%)

Different superscripts in the same column mean significant differences between groups ([a, b]: $p < 0.001$; [c, d]: $p < 0.05$).

maturation were compared using a one-way ANOVA. The rate of matured and fertilised oocytes, cleaved embryos and blastocysts were expressed as percentage 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. They were evaluated statistically using chi-square or Fisher Exact tests as appropriate. The probability level for statistical significance was set to $p < 0.05$.

The number of oocytes recovered after puncture and slicing of the ovaries is shown in Table 1. A significant effect of the previous response to the superovulatory treatment was observed for the three oocyte categories, so that ewes which did not respond to the treatment (Group – –) presented the higher number of total oocytes per ewe ($p < 0.001$). Thus, the total number of oocytes selected for IVM was significantly higher in this group, in comparison with (+ +) and (+ –) groups ($p < 0.001$). Oocytes recovered from ewes of the group (+ –) presented a low competence in the early stages of the in vitro embryo production procedure (Table 2). In particular, it is stressed the extremely low maturation rate showed by the group (+ –) significantly different from those of the other two experimental groups ($p < 0.05$). Significant differences were also observed for IVF and cleavage rates; again, group (+ –) presented the lowest values for fertilisation and cleavage rate ($p < 0.05$ and $p < 0.001$ respectively) (Table 2). Consequently, the blastocysts rate produced after 8 days of culture was lower in the (+ –) group

(14.3%) in comparison with (+ +) (50%) and (– –) (53.5%) groups. However, these differences were not significant, perhaps due to the initial low number of cleaved embryos obtained from oocytes of the (+ –) group. The final response to the *in vitro* embryo production procedures was dependent on the response to the superovulation. Ewes that did not respond to the hormonal treatment group (– –) had the best response (4.6 blastocysts per ewe), mainly due to the high number of oocytes recovered; however, group (+ –) showing *in vivo* fertilization problems, only produced 0.12 blastocysts per ewe. Finally, ewes with a "normal" response after superovulation (+ +) produced 1.2 blastocyst per ewe, a response similar to that obtained previously with the same breed and similar experimental conditions (Forcada *et al*., 2011a).

The total number of oocytes recovered was slightly lower than that presented previously by our group using the same protocol and breed (Forcada *et al*., 2011a), where the simplified superovulatory treatment used in that experiment was compared with the conventional protocol of six decreasing doses of pFSH. Thus, those ewes comparable with the present experiment presented 13.7 oocytes per ewe and 62.5% selected for IVM, higher than the present values (8.3 and 57%, respectively). In Rasa Aragonesa ewes superovulated with FSH, Alberio *et al*. (2002) obtained 3.7-6.2 oocytes per ewe and 85% to fertilize, and Hammami (2008) reported 6.6-11.1 oocytes per ewe and 90% selected for fertilization. In this context, results obtained in the present study by those ewes which did not respond to the superovulatory treatment are especially remarkable, since 20-30% of the animals present a lack of response to FSH-based superovulatory protocols (Forcada *et al*., 2000, 2011b, 2012). In fact, early luteal regression is assumed as a failed response to superovulation and has also been reported by our group in both Rasa Aragonesa and Ojalada breeds (Forcada *et al*., 2000, 2006, 2011a), although always associated with multiple FSH injection protocols, not including eCG (Simonetti *et al*., 2008; Forcada *et al*., 2011b). This regression after superovulatory treatments seems to be related with a lack of progesterone priming before treatment, so that its incidence should be higher during anestrus (Ryan *et al*. 1991). Some authors have indicated that a simplified superovulatory FSH protocol produced the higher percentage of premature CL regression (Riesenberg *et al*., 2001) with no eCG in the treatment. However, a reduced eCG dose in combination with FSH could be enough to support follicular growth and avoid premature CL regression. Thus, previous results of our group applying the same treatment in Corriedale (Simonetti *et al*., 2008) or Ojalada ewes (Forcada *et al*. 2011a) presented a low percentage of CL regression. The low maturation and fertilization rates exhibited by the oocytes recovered from ewes that only produced oocytes after superovulation and natural mating, clearly show that the ewes superovulated with fertilization problems *in vivo* cannot be used as oocyte donors. It is difficult to determine the causes of such infertility, although two possibilities have been set out. Our group has previously demonstrated that repeated superovulation treatments with this protocol can induce high concentrations of anti-eCG antibodies in a variable percentage of ewes (Forcada *et al*., 2011a); such immunological response has been associated with a lack or delay in oestrus and preovulatory surge of LH and subsequent impaired after natural mating (Forcada *et al*., 2011a). On the other hand, it has been demonstrated that high FSH doses at the beginning of the superovulatory treatment are detrimental on oocyte competence (Berlinguer *et al*., 2004); those high first doses can induce a rapid abnormal follicular development and asynchrony between growing follicles and follicular status (Blondin *et al*., 1996), because a plateau phase is necessary to complete oocyte capacitation (Sirard *et al*., 1999). Although in the present experiment oocytes were not recovered in the oestrus induced by the superovulatory treatments but 7 days later, it is possible that the detrimental effects of high FSH levels at the beginning of the superovulatory treatment can also affect the subsequent follicular phase.

In conclusion, results of the present study show that the ovarian response to a superovulatory treatment determines the number and quality of the oocytes recovered from the ovary 7 days after the oestrus induced by the hormonal treatment, and therefore the response to the *in vitro* embryo production procedures. Although further research is needed, the combination of the *in vivo* and *in vitro* techniques could be an important tool to increase embryo production from particular ewes of high genetic value or from endangered genotypes.

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