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Original article

Influence of maternal environment on the number of transferable embryos obtained in response to superovulatory FSH treatments in ewes

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Abstract — In a first experiment, embryo viability was estimated after recovery in the uterus or the oviduct of 70 Manchega ewes following a treatment of superovulation with decreasing doses of OVAGEN™. Fewer viable embryos (5.6 ±0.9 vs. 8.3 ±0.8, $P < 0.05$) and more degenerative embryos (31.3% vs. 6.8%, $P < 0.005$) were obtained from the uterus than from the oviduct respectively. In a second experiment performed on 14 ewes, embryo viability was analyzed in relation to the follicular population estimated by ultrasonography (follicles ≥ 2 mm) at the first FSH administration. Progesterone (P4) and oestradiol 17β (E2) concentrations were also determined from the beginning of the superovulation treatment to the recovery of the embryos. The number of viable embryos (4.3 ±1.4) was positively correlated ($r = 0.824$) with of 2–4 mm diameter follicles ($P < 0.05$), and with E2 concentrations at –12 h ($r = 0.891$, $P < 0.01$) , 0 h ($r = 0.943$, $P < 0.0001$) and +24 h ($r = 0.948$, $P < 0.05$) from estrus detection. Prolonged high levels of E2 up to 72 h with low levels of P4 on days 3 and 4 after estrus had a negative ($P < 0.05$) effect on embryo viability. These results indicate that ovarian response to superovulatory protocols is related to the individual variations in the number of follicles of 2–4 mm at the start of FSH treatment, and that embryo viability is conditioned by the steroid patterns during the time spent in the genital tract of the super-ovulated ewes.

embryo viability / ewe / follicular status / super-ovulation / steroid

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1. INTRODUCTION

In sheep embryo transfer protocols, the superovulatory response of a female is related to the number of gonadotrophin-responsive follicles stimulated to grow until preovulatory stages by the administration of high exogenous FSH doses [1]. Both the follicular status and the gonadotrophin preparation used, as well as the possible interactions between them, seem to be the factors responsible for the high variability on the ovulation rates obtained in response to superovulatory treatments. If it is known that the administration of superovulatory treatments induces a high increase in the number of follicles growing to preovulatory stages, it is also well-known that superovulation disturbs the process of final follicular development [2] when follicles reach full estrogenic competence [3]. The supply of large amounts of exogenous gonadotrophins necessary to achieve a superovulatory response exerts, at the same time, detrimental effects on follicular development and, then, on oocyte maturation and/or on ovulatory mechanisms [2]. Furthermore, some of the follicles stimulated to grow by the exogenous FSH would be in the early stages of atresia [4]. The administration of the superovulatory treatment allows the arrest of early atresia and the growth of these follicles [5]. However, although atretic follicles can be promoted to grow, their development is inadequate, leading to ovulatory fails [6] which can account for the variability observed in the ovulation rate.

Even more variable and disappointing than the number of corpora lutea is the final number of transferable embryos obtained after the superovulatory treatment [7]. The number of viable embryos can be affected by the inadequate follicular development and ovulation [6], which can result in abnormalities of the developmental competence of the oocytes or in disturbances of the normal processes of fertilization and early embryo development [2] and may be taken into account for the drop in the viability of embryos. However, the incidence of embryo degeneration during the early stages of FSH-stimulated cycles is high and variable regardless of the follicular status prior to the treatment. This fact seems to indicate that the appearance of embryo degeneration may be more related to detrimental effects of the superovulatory treatment per se. These effects would be exerted not only straight on the developmental competence of the embryo, but also through a negative influence on the oviduct/uterine environment. In this way, superovulatory treatments cause alterations of the normal reproductive physiology of the donor animal, deviating endocrine patterns from the normal [8]. Some hormones like progesterone and oestradiol have a key role in the maturation of the oviduct and uterine epithelium [9, 10]. Changes in the maturation and nutritive capacity of the endometrium within the first week of the cycle can be the cause of a wide variety of embryo developmental abnormalities [9], affecting the final number of transferable embryos.

Then, the main causes of the decrease in the viability of young embryos from superovulated ewes can be related either to the alterations in follicular estrogenic competence, to changes in the periovulatory and preimplantational patterns of progesterone and oestradiol, to decreases in the developmental capacity of the embryos or to negative influences from the uterine environment. The influence of follicular and endocrine patterns have been explored for many years in cattle, for a review see Greve et al. [8], but to a lesser extent in ewes. The current paper reports results from two experiments designed to discern the role of these factors on the quality of sheep embryos. The effects of follicular status and endocrine patterns were examined by assessing the follicular population and plasma progesterone and oestradiol levels in a group of ewes in which embryos were thereafter recovered from the uterus. The influence of a developmental competence of the embryos and the effects

from the genital tract of the donor ewe were determined by examining the differences in the viability of the embryos recovered on different days of the cycle, either from oviduct or uterus.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

Two experiments were conducted in a total of 84 Manchega ewes, 4 to 7 years old, maintained outdoors with access to indoor facilities at the experimental farm of the INIA Animal Reproduction Department in Madrid, Spain, latitude 40º N.

Oestrus was synchronized in all the animals during the breeding season, with the insertion of one intravaginal progestagen impregnated sponge (40 mg fluorogestone acetate, FGA, Chronogest®, Intervet International, Boxmeer, The Netherlands) on Day 0, changed by a new one that was maintained from Day 7 to Day 14. The ewes were also injected with a single dose of 125 μg i.m., cloprostenol (Estrumate, Mallinckrodt Vet GmbH, Friesoythe, Germany) coincidentally with the first FSH injection. The superovulatory treatment consisted in eight decreasing doses (1.5 mL × 3, 1.25 mL × 2, and 1 mL × 3) of FSH (OVAGEN™, ICP, Auckland, New Zealand), in two daily i.m. injections starting 60 h before sponge removal and finishing 24 h after progestagen withdrawal. Progestagen removal was coincident with the 6th FSH injection and oestrus detection was performed with adult rams at 24 h after sponge withdrawal. The matings were repeated at 36 and 48 h after sponge withdrawal and one ram per 6 ewes remained with the sheep for 2 additional days following the second mating.

The first study, involving a group of 70 females, was conducted in November 1999 with the aim to determine if decreases in viability are related to the developmental competence of the embryos per se or affected by negative factors derived from the genital tract of the donor. In this way, the viability of the embryos recovered either from the oviduct (n = 34 ewes) or uterus (n = 36 ewes) was compared. The second experiment, conducted in 14 females in October 2000, was designed from the results of the prior trial with the aim to determine the possible influence of the follicular status just prior to the superovulatory treatment and the possible effects of endocrine patterns during the preovulatory and preimplantational periods on the quality of the embryos obtained from the uterus. In this way, the number and largest diameter of all the follicles ≥ 2 mm were evaluated by transrectal ultrasonography coinciding with the first FSH injection. Plasma progesterone and oestradiol levels were determined from samples of 10 mL of jugular blood, collected every 12 h from the day of the first FSH injection to the day of the embryo recovery, by using vacuum blood evacuation tubes with heparin (Vacutainer® Systems Europe, Becton Dickinson, Meylan Cedex, France).

2.2. Ultrasonic equipment and procedure

Ultrasonographies were realized using a 7.5 MHz linear-array probe fitted to an Aloka SSD 500 (Ecotron, Madrid, Spain), as described by Schrick et al. [11] and validated in our laboratory by Gonzalez-Bulnes et al. [12].

2.3. Hormonal assays

Blood samples were centrifuged at 3500 r.p.m. (1500 g) for 15 min. Thereafter, the plasma was stored at –20 ºC until being assayed for hormone concentrations. Progesterone concentration was determined in individual plasma by radioimmunoassay as

described by Lopez-Sebastian et al. [13]. Sensitivity was 0.06 ng·mL^{-1} and the inter- and intra-assay variation coefficients were 13.6 and 10.4%, respectively. The concentrations of oestradiol were measured using the Spectria radioimmunoassay kit (Orion Diagnostic Corp.), as described by Romeu et al. [14]. Sensitivity was 0.5 pg·mL^{-1} and the inter- and intra-assay variation coefficients were 6.1 and 3.5%, respectively.

2.4. Embryo recovery

The embryos were obtained by surgical access to the genital tract, through a prepubian laparotomy under general anaesthesia with xylazine (Rompun, Bayer Ag, Leverkusen, Germany, 6 mg i.m.) and ketamine (Imalgène 1000, Merial, Lyon, France, 130 mg i.m.). The ovarian response in terms of CL number was determined by laparoscopy just before the laparotomy, avoiding surgery in non responding females and possible haemorrhage caused by exteriorization of the ovaries in responding ewes. In brief, uterine recoveries were performed on Day 7 after progestagen withdrawal by flushing both uterine horns with 0.2% PBS (Dulbecco phosphate buffered saline, Sigma Chemical Co., St. Louis, MO, USA) supplemented with BSA (bovine serum albumin, Fraction V, Sigma Chemical Co.) from a blunt needle inserted at the major curvature of the horn, kept fixed with forceps to avoid backflow of the flushing medium, towards a catheter provided with a blunt needle and kept fixed with silk at the uterotubal junction. Oviductal retrievals were performed on Day 4 after sponge removal by flushing both oviducts with BSA-PBS from the catheter with the blunt needle at the utero-tubal junction, kept fixed with silk to avoid backflow of the flushing medium, towards another catheter without a needle introduced into the fimbria and also kept fixed by silk.

2.5. Assessment of embryo viability

Viability of the embryos from the uterus was evaluated just after recovery by morphological criteria [15] validated in our laboratory by preliminary fresh embryo transfers. Viability of the embryos from the oviduct was determined by assessing its development to expanding or hatching blastocysts in co-culture with the oviduct epithelial cell monolayer [16]. The coculture of embryos from oviduct recoveries with oviduct epithelial cell was chosen to avoid the block to in vitro embryo development in a conditioned medium described at the 8–16 cell stage [17], when the control of the embryo development changes from the maternal genome to the embryonic genome [18]. In brief, preparation of the oviduct cells was performed from sheep oviducts in the luteal phase of the oestrous cycle. Oviducts were collected from the abattoir, were washed with PBS containing a gentamicin solution (10 mg·mL^{-1}, Sigma Chemical Co.) and were dissected free from adjacent connective tissue. Two small portions (1–1.5 cm) of the oviduct were selected (one close to the utero-tubal junction and another one close to the fimbria), slit lengthways and slightly scraped at its luminal surface with a scalpel blade. Oviduct scrapes were washed in M199 (Sigma Chemical Co.) supplemented with L-glutamine (Sigma Chemical Co.), FCS (fetal calf serum, Sigma Chemical Co.) and antibiotic antimycotic solution (Sigma Chemical Co.) at 37 ºC. Clusters of the cells were disaggregated by expelling with a syringe through a 21 G needle until formation of a cell suspension. The cells were stained with 4% trypan blue to assess their viability and the concentration of the viable cells was counted with a haemocytometer. Then, the concentration of the viable cells was adjusted to 5×10^5 mL^{-1} by dilution in a culture media, pipetted into multidish 4 wells (Nunclon^{TM}, Nunc International, Roskilde, Denmark) and cultured at 38.5 ºC in a humidified environment of 5% CO_2 for 48 h,

when the embryos were recovered from the oviduct and incorporated to the oviduct cell monolayer for 5 days, changing the culture media every 2 days.

2.6. Indexes of the superovulatory response

The number of corpora lutea (CL), total number of recovered oocytes/embryos (RE), number of viable embryos (VE, grade 1 and 2), number of non-fertilized oocytes (NF) and number of degenerated embryos (DE, grades 3 and 4) were recorded for each ewe. The rate of recovery (RR) was obtained by dividing, in every sheep, the total number of RE by the total number of CL. Rates of viability (VR), non-fertilization (NFR) and degeneration (DR) were obtained by dividing the number of VE, NF or DE by the total number of RE, respectively. All rates are expressed as percentages for clarity of the manuscript.

2.7. Statistical analysis

Statistical treatment of the results expressed as percentages (RR, VR, NFR and DR) was performed after transformation of the values for each individual percentage to the arcsine. In the first experiment, statistical analysis determined, by analysis of variance (ANOVA) and the Kruskar-Wallis non-parametric test, possible differences between the oviduct and uterine recoveries on the superovulatory indexes in response to the treatment. Data from the ovarian status when the FSH administration started in the second trial were summarized to characterize its effect on the superovulatory yields. Firstly, all follicles recorded by ultrasonography were classified by its largest diameter. The Pearson correlation analysis and linear and Stepwise regression procedures were carried out to correlate the number of follicles of various size categories with the superovulatory indexes recorded. Second, data from the hormonal assays from the time of the first FSH dose to the day of embryo retrieval were used to assess the possible influences of follicular status on plasma oestradiol and progesterone patterns during the period of study, relationships between oestradiol and progesterone levels and the effects of oestradiol and progesterone on the superovulatory response by using ANOVA, Kruskar-Wallis and Pearson analysis. All the results are expressed as mean ± S.E.M. and significance was taken at a level of $P < 0.05$.

3. RESULTS

3.1. The effects of time in the genital tract of the donor ewe on embryo viability

The estimation of the CL number in the first experiment showed a mean ovulation rate around 11.5 CL, being similar in ewes with uterine or oviductal recoveries (Tab. I). There were no significant differences in the recovery rates between both procedures, since the mean number of total recovered oocytes/embryos (RE) was only 0.8 higher in the oviductal than in the uterine recoveries. A mean of 0.3–0.4 of these RE were non-fertilized oocytes, which indicates that the fertilization rate was not different between the groups. If the results from both techniques were comparable because of a similar ovulatory response and fertilization success, data from Table I highlight a strong influence of degeneration processes in the final number of transferable embryos obtained after uterine flushing. In this way, the degeneration rate was around 24.5% higher in uterine retrieval than in the oviductal recoveries ($P < 0.005$).

Table I. Superovulatory yields in Manchega sheep with oviduct or uterine embryo recoveries after treatment with FSH.

	Embryo recovery		
	1st trial		2nd trial
	Oviduct	Uterus	Uterus
Number of animals	34	36	14
Ovulation rate	11.2 ± 0.8	12.0 ± 1.1	11.1 ± 1.1
Number of recovered embryos	9.1 ± 0.8 [a]	8.3 ± 1.1 [a,b]	5.9 ± 1.2 [b]
Recovery rate (%)	77.9 ± 3.7 [a]	65.2 ± 5.3 [a,b]	56.3 ± 10.1 [b]
Number of viable embryos	8.3 ± 0.8 [a]	5.6 ± 0.9 [b]	4.3 ± 1.4 [b]
Viability rate (%)	88.6 ± 3.5 [e]	62.9 ± 5.5 [f]	65.1 ± 10.3 [f]
Number of unfertilized oocytes	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.2
Non-fertilization rate (%)	5.9 ± 3.0	6.3 ± 3.4	12.8 ± 8.0
Number of degenerated embryos	0.6 ± 0.2 [a,c]	2.5 ± 0.5 [b,d]	1.2 ± 0.3 [b]
Degeneration rate (%)	6.8 ± 3.0 [e]	31.3 ± 5.0 [f]	22.5 ± 7.2 [f]

Different superscripts are statistically different: [a] vs. [b] $P < 0.05$; [c]vs. [d] $P < 0.01$; [e] vs. [f] $P < 0.005$.

3.2. Effects of follicular status and preovulatory oestradiol pattern on the ovulation rate and number and viability of the embryos

The superovulatory response to FSH obtained in the second experiment reached a mean of 11.1 ± 1.1 corpora lutea, 5.9 ± 1.2 recovered embryos, 4.3 ± 1.4 viable embryos, 1.2 ± 0.3 degenerated embryos and 0.4 ± 0.2 non-fertilized oocytes. There were no significant differences with the indexes of superovulatory response found in the uterine recoveries of the first experiment; however, the number of recovered embryos was lower than that obtained in the oviduct retrievals ($P < 0.05$). We also found a decrease in the final number of transferable embryos obtained by uterine flushing from the second trial when compared to the oviduct recoveries in the first trial ($P < 0.05$), due to a higher degeneration rate ($P < 0.005$), thus confirming data from a comparison made in the first experiment.

The analysis of ultrasonographic data from the second experiment showed that the total number of follicles 2 to 4 mm in diameter present in the ovary at the beginning of the treatment (9.1 ± 0.7) was positively correlated with the ovulation rate ($P < 0.05$, $r = 0.591$), and with the number of recovered ($P < 0.05$, $r = 0.565$) and viable embryos ($P < 0.05$, $r = 0.632$). However, Stepwise regression procedures showed that the indexes of superovulatory response were mainly influenced by the more limited category of follicles 3 mm in size.

The analysis of hormonal assays showed that plasma oestradiol (E2) levels, depicted in Figure 1, increased from the first FSH injection (4.0 ± 0.7 pg·mL^{-1}) to the onset of

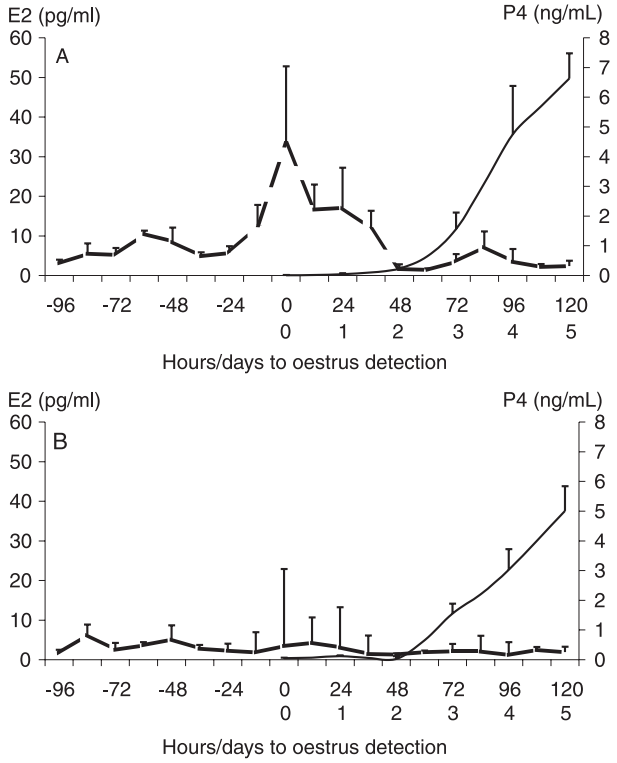

Figure 1. Plasma oestradiol concentrations (- - -, hours before and after oestrus detection) and plasma progesterone concentrations (—, days after oestrus detection) in superovulated ewes with plasma oestradiol levels above (**A**) and under (**B**) the mean value at oestrus detection.

oestrus behavior (20.8 ±10.6 pg·mL^{-1}). The E2 levels at –12 h, 0 h, 12 h, 24 h and 36 h from the detection of oestrus behavior were positively correlated with the total number of follicles 2–4 mm in diameter at the first FSH dose ($P < 0.05$, $r = 0.824$), and mainly with the number of 3–4 mm follicles ($P < 0.005$, $r = 0.900$). The ovulation rate obtained in response to the FSH treatment was related to the plasma E2 concentration at –12 h ($P < 0.05$, $r = 0.768$), 0 h ($P < 0.01$, $r = 0.830$), 24 h ($P < 0.01$, $r = 0.836$) and 36 h ($P < 0.05$, $r = 0.734$). Both the mean number of recovered and viable embryos were also correlated with the E2 levels at –12 h ($P < 0.01$, $r = 0.883$ and 0.891, respectively), 0 h ($P < 0.0001$, $r = 0.921$ and 0.943, respectively) and 24 h ($P < 0.0001$, $r = 0.927$ and 0.948, respectively). In this way, ewes with E2 above the mean value at oestrus ($n = 5$) showed a higher number of CL (16.0 ±0.4 vs. 10.2 ±0.9, $P < 0.05$), RE (13.0 ±0.6 vs. 4.6 ±0.7, $P < 0.01$) and VE (12.5 ±6.5 vs. 2.9 ±0.7, $P < 0.005$) than the ewes with below average E2 ($n = 9$).

3.3. Influence of preimplantational oestradiol and progesterone patterns on embryo viability

Plasma E2 levels from blood samples of the second experiment decreased after oestrus, reaching a mean value of 4.3 ±1.6 pg·mL^{-1} from 48 h to 120 h after oestrus detection (Fig. 1). The analysis indicated that high E2 levels at 72 h were related to a decrease in the viability rate of embryos

(VE/RE, $P < 0.05$, $r = 0.741$). The mean E2 value at 72 h was 5.47 ± 1.8 pg·mL^{-1}. The viability rate was higher ($P < 0.05$) in those ewes with E2 levels under the mean value at 72 h ($n = 9$, 76.1%) than in the ewes with E2 under the average ($n = 5$, 28.5%).

Plasma progesterone (P4) levels increased from basal values at the time of oestrus detection to a mean value of 5.3 ± 0.7 ng·mL^{-1} when the embryos were recovered from the uterus on Day 5 after oestrus behavior (Day 7 after progestagen withdrawal), as depicted in Figure 1. The analysis of the possible effects from follicular status and preovulatory oestradiol patterns on the P4 levels showed that P4 concentrations on Day 3 and 4 after oestrus were correlated ($P < 0.05$, $r = 0.655$ and 0.773, respectively) with the number of follicles 2–4 mm in size at the start of the superovulatory treatment. At the same time, P4 levels on Day 4 after oestrus were correlated with the E2 levels at oestrus detection and 24 h later ($P < 0.05$, $r = 0.771$ and 0.785, respectively).

The statistical analysis of possible correlations between the P4 levels and superovulatory yields showed a significant relationship between the number of non transferable embryos (1.1 ± 0.3) and the P4 levels on Day 3 (1.5 ± 0.2 ng·mL^{-1}, $P < 0.05$, $r = -0.730$), 4 (3.4 ± 0.6 ng·mL^{-1}, $P < 0.05$, $r = -0.795$) and 5 (5.3 ± 0.7 ng·mL^{-1}, $P < 0.01$, $r = -0.892$) after oestrus behaviour. The number of non viable embryos was higher in those ewes ($n = 6$) with P4 levels under the mean value at Day 3 (2.0 ± 0.5 vs. 1.0 ± 0.4, $P < 0.05$), 4 (1.8 ± 0.4 vs. 1.2 ± 0.5, $P < 0.05$) and 5 (2.5 ± 0.2 vs. 0.3 ± 0.2, $P < 0.01$). A second analysis was carried out to avoid the effect derived from the different number of CL present in the ovaries of each ewe on the P4 levels. Then, since the mean ovulation rate was 11.1 ± 1.1 CL, this analysis took into account only those ewes with 11 or 12 CL (11.4 ± 0.2, $n = 7$). The mean number of non transferable embryos (1.6 ± 0.7) tend to be lower with higher P4 values on Day 3 (1.7 ± 0.4 ng·mL^{-1}) and Day 4 after oestrus behavior (3.9 ± 0.8 ng·mL^{-1}), although statistical significance was only reached on Day 5 (6.0 ± 0.7 ng·mL^{-1}, $P < 0.01$, $r = -0.921$). The number of non transferable embryos increased, due to an increase in the degeneration rate, with delays in the day in which P4 reached a value ≥ 0.5 ng·mL^{-1}, considered as indicative of the presence of at least one functional corpus luteum (mean: 2.7 ± 0.2 days after oestrus behavior, $P < 0.05$). Then, at the same ovulation rate, the number of non-transferable embryos was higher when P4 was lower than 0.5 on Day 3 after oestrus detection (2.0 ± 1.0 vs. 0.0 ± 0.0, $P < 0.01$).

4. DISCUSSION

Protocols for sheep multiple ovulation and embryo transfer (MOET) usually include the administration of a superovulatory treatment during the last days of a progestagen sponge insertion and the recovery of embryos from the uterus on Day 6–7 after sponge withdrawal. The high variability in the ovulation rate and the variable and low number of transferable embryos recovered after superovulation, compared to the ovulation rates, are the main limiting factor in sheep embryo transfer programs [7].

The present results showed a very strong decrease in the viability of embryos during its time in the genital tract of the donor, which suggests that the low number of transferable embryos obtained from uterine flushing in MOET protocols can be related to the occurrence of embryo degeneration during the passage from the oviduct to the uterus of the donor. Data from the current study confirmed that the number of corpora lutea and embryos obtained in response to superovulatory FSH protocols are determined by individual variations in the number of gonadotrophin-responsive follicles present in both ovaries at the start of the gonadotrophin treatment [4]. In response to

exogenous FSH, these follicles were able to grow, reach full oestrogenic competence around the onset of estrus behavior (as indicated by plasma oestradiol levels), and ovulate. However, the viability of the embryos derived from these follicles decreased during their time in the sheep genital tract, which would be related to alterations in preovulatory and preimplantational progesterone and oestradiol patterns of the superovulated ewes.

The effect of the ovarian follicular population at the start of the gonadotrophin treatment on the superovulatory yields found in the current study confirmed data from previous studies using the same protocol with the same commercial preparation in the same breed [4]. This fact emphasizes the contribution of the large variation in the antral follicular population between animals to the variability in superovulatory response after FSH treatments [19]. The relationship between the number of follicles 2–4 mm in diameter and the oestradiol levels from 12 h before to 36 h after oestrus detection also confirms that the administration of high doses of exogenous gonadotrophins induces the growth of these follicles until the preovulatory stages. Thereafter, the relationship found between the oestradiol levels around estrus and the number of corpora lutea and total and viable embryos confirmed the results already published for cattle [20–22], indicating that oestradiol concentration around the onset of oestrus behavior can be considered as an indicator of the population of healthy preovulatory follicles, as in cattle [8].

There is another finding to take in mind when comparing oestradiol levels around oestrus and superovulatory yields in response to the FSH treatment. Ewes with lower oestradiol levels had many fewer recovered (4.6) and viable embryos (2.9) with respect to the number of corpora lutea (10.2) when compared to ewes with higher oestradiol levels, in which the number of recovered (13.0) and viable embryos (12.5) were more similar to the number of corpora lutea (16.0). This similarity in the number of corpora lutea from ewes with high and low oestradiol levels suggests the presence, in ewes with lower oestradiol levels, of preovulatory follicles with a lower oestrogenic capacity rather than a lower number of preovulatory follicles. It is well-known that healthy antral follicles contain high oestradiol concentrations [23] while morphologically degenerated follicles contain low oestradiol concentrations [24] and are less abile to release oestradiol [25]. Then, although it is not possible to test this hypothesis with the conditions of the present study, the disparity in the number of both the total and viable embryos with not so different ovulation rates between ewes with high and low oestradiol suggests that low oestrogenic follicles would reach the ovulation but would be unable to release an oocyte with a full developmental competence. Firstly, alterations in the follicular function may be related to ovulatory abnormalities like the presence of anovulatory luteinized follicles [20] or deficiencies in the release of the oocyte [26, 27], which can account for the decrease observed in the number of recovered embryos. Second, many of the changes required by the oocyte to achieve developmental competence occur during the latter stages of follicular development [28, 29]. Alteration of stereidogenesis affects both the prematuration and maturation of the oocyte and, then, fertilization and early embryo development [8], which can account for the decrease observed in the number of viable embryos.

From the results reported in the first experiment, it would be possible to identify the degeneration rate observed in the embryos from oviduct recoveries (6.8%) as caused by decreases in the developmental competence of the oocytes/embryos by themselves. However, the high viability of the embryos from oviduct flushing and the high incidence of degeneration in the embryo recovered from uterine flushing (31.3

and 22.5%) indicate that the appearance of embryo degeneration is also strongly influenced by the detrimental effects of the oviduct/uterine environment. In this way, it is described that decreases in the viability found in the embryos between 2 and 11 days old may be related to alterations in the development of the endometrium or in its nutritive capacity, which expose the embryos to a hostile or non supportive environment [9]. Data obtained in the second experiment of the current paper showed that progesterone and oestradiol patterns had a key role in the appearance of embryo degeneration. Firstly, as described above, the number of viable embryos was related to the oestradiol concentrations around oestrus, but oestradiol is not only the signal of a healthy population of preovulatory follicles. Oestradiol induces maturation and differentiation of the secretory apparatus of the epithelial cells of the sheep ampulla oviduct [30]. In response to the oestradiol levels around the oestrus [31], these cells synthesize and secrete a specific oestrus-associated glycoprotein (oEGP) which regulates embryo development [32].

Conversely, the maintenance of increased oestradiol output afterwards may also contribute to reduced embryo development [8] which may be related to adverse effects of prolonged high oestradiol levels on maternal secretory patterns [33]. In this way, data from current study report a negative effect of prolonged high E2 levels on the viability of embryos recovered from the uterus. Moreover, delays or deficiencies in progesterone release also affected the viability of the recovered embryos. Prior authors have described the role of both hormones, oestradiol and progesterone, on the expression of several growth factors, mainly IGF on the oviduct and uterine secretions [34–37]. Although there are no studies on superovulated ewes, the presence of IGF and its binding proteins during the early embryo development, from 1 cell to the blastocyst, suggests an important role of these peptides [38–40]. There is an interrelationship of events again, since low levels of progesterone may be related to subnormal corpus luteum formation, which is caused by an inadequate follicular development preceding luteal gland formation [41, 42].

In conclusion, the number of corpora lutea and embryos obtained in response to standard superovulatory FSH protocols is determined by individual variations in the number of follicles present in both ovaries at the start of the gonadotrophin treatment. Afterwards, the viability of embryos derived from these follicles is decreased by an influence of the maternal environment during its very early developmental stages in the genital tract of the superovulated ewes, due to alterations in ovarian steroid patterns from oestrus to the day of embryo recovery.

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