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Influence of the *FecX*^{R} Allele in Heterozygous Ewes on Follicular Population and Outcomes of IVP and ET using LOPU-Derived Oocytes

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Contents

Ewes heterozygous for the *FecX*^{R} allele (R+) in the *bone morphogenetic protein 15* (*BMP15*) gene display increased ovulation rate and prolificacy. Besides this phenotypic advantage, the influence of the *FecX*^{R} allele on follicle number and size, oocyte competence and *in vitro* production (IVP) remains undefined. With these aims, 8 R+ and 8 wild-type (++) ewes were subjected to 2 laparoscopic ovum pick-up (LOPU) trials (four sessions per trial; two with and two without FSH) and subsequent IVP and fresh transfer. All follicles >3 mm were punctured (n = 1673). Genotype did not significantly affect the number of punctured follicles per ewe and session (10.4 and 10.2 in R+ and ++ untreated ewes, 17.4 and 14.3 in R+ and ++ FSH-treated ewes, respectively), but follicular diameter of R+ ewes was significantly reduced compared with ++ ewes (−0.2 mm in untreated and −0.8 mm in FSH-treated ewes; $p < 0.01$). R+ ewes showed higher recovery rate and increased numbers of total and suitable cumulus–oocyte complexes for *in vitro* maturation (IVM). Similar rates of day 8 blastocysts were observed in R+ (36.1%, 147/407) and ++ (32.6%, 100/307) ewes, but the final output of day 8 blastocysts per ewe and session was higher in R+ ewes (+0.75; $p < 0.005$), without differences in survival rate at birth of the transferred embryos (40.4%, 21/52 vs 40.0%, 16/44; NS). In conclusion, a higher number of oocytes proven to be competent for *in vitro* development and embryo survival after transfer are recovered from R+ ewes, despite the lower mean size of their follicles at puncture.

Introduction

In sheep, several breeds have been identified with different mutations in the *bone morphogenetic protein 15* (*BMP15*) gene, which display a higher ovulation rate in heterozygous ewes when compared with wild-type animals (Galloway et al. 2000; Hanrahan et al. 2004; Bodin et al. 2007; Martínez-Royo et al. 2008). One of these polymorphisms corresponds to the *FecX*^{R} allele in the Rasa Aragonesa breed and was discovered in 2008 (Martínez-Royo et al. 2008). In a previous study, we described phenotypic effects of 0.63 extra ovulations and 0.35 extra lambs per *FecX*^{R} heterozygous (R+) lambing ewe (Lahoz et al. 2011). However, the influence of the *FecX*^{R} allele on follicle number and size, oocyte competence and how it could further affect *in vitro* embryo production and viability remains to be assessed.

The exact mechanism by which these mutations increase ovulation rate is still unclear, although reduced activity of the BMP signalling system in heterozygous ewes may cause smaller antral follicles with fewer granulosa cells and altered sensitivity to gonadotropins (revised by Fabre et al. 2006). Thereby, folliculogenesis in heterozygous ewes is expected to be altered, which potentially affects the proportion of developmentally competent oocytes, that is, with the ability to mature, be fertilized and give rise to normal and fertile offspring after normal gestation (Duranthon and Renard 2001). The proportion of competent oocytes is widely accepted to increase along with follicular size (Mermillod et al. 2008); thus, follicle size undoubtedly affects the overall efficiency of ovum pick-up, and increased productivity is associated with large and medium follicles (Rodríguez et al. 2006).

On the other hand, BMP15 is an important oocyte-secreted factor (OSF), which plays key roles in folliculogenesis, follicular development and ovulation, as well as in oocyte quality, in mammalian ovaries. Recent studies highlight the importance of OSFs as regulators of cumulus cell function and the quality of the oocyte (Gilchrist et al. 2008). Supplementing *in vitro* oocyte maturation media with exogenous OSFs [BMP15 or growth differentiation factor (GDF) 9] enhances oocyte developmental competence in cattle (Hussein et al. 2006), and the lower developmental competence of calf oocytes compared with those of adult cows has been proposed to be due to different expression of BMP15 and/or GDF9 in oocytes and cumulus cells between calves and cows (Hosoe et al. 2011). In humans, oocytes retrieved from follicles with a significantly higher follicular fluid level of BMP15 had a higher fertilization rate and superior cleavage and embryo quality (Wu et al. 2007). A very recent study in Booroola ewes has confirmed lower oocyte-derived BMP15 mRNA levels in ewes homozygous for a mutation in BMPR-1B (Crawford et al. 2011), and lower overall concentration or biological activity of BMP15 has been proposed in Inverdale (*FecX*^{I}) or Hana (*FecX*^{H}) ewes heterozygous for a mutation in *BMP15* (Galloway et al. 2000). Because analogous reductions are expected in R+ ewes, a hypothetical deleterious effect on oocyte competence should be discarded in this genotype.

Moreover, the overall efficiency of certain embryo biotechnologies in ovines, such as laparoscopic ovum pick-up (LOPU)–*in vitro* production (IVP) programmes, is still low. Therefore, protocols including exogenous gonadotropins are commonly used to increase oocyte number. Considering that gonadotropins may affect R+ ewes differently, and in the present study, we aimed to investigate the suitability of R+ ewes in embryo biotechnologies. To our knowledge, the only reported studies on IVP of embryos in *Fec*-mutation carrier ewes involve the Booroola mutation (Cognié et al. 1998), but there are no reports on the results of LOPU procedures in any of these genotypes. It must be taken into account that the *FecB*^{B} allele is located in a different gene, corresponding to a single mutation in the coding sequence of the bone morphogenetic protein receptor type 1B (revised by Fabre et al. 2006), a receptor of

BMP15 as well as other BMPs and other members of the transforming growth factor-beta (TGF-β) superfamily, such as anti-Müllerian hormone (Kaivo-oja et al. 2006).

Thus, the aims of the present study were to evaluate the influence of the *FecX*^{R} allele, with or without *in vivo* FSH treatment, on (i) the quantity and size of follicles available for aspiration in a LOPU-IVP programme and (ii) the competence of LOPU-derived oocytes subjected to *in vitro* production of embryos and their further ability to sustain pregnancy to term.

Materials and Methods

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research. All chemicals used for IVP were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise specified. All FSH used in this study was highly purified porcine FSH provided by J.F. Beckers (Laboratory of Endocrinology of the Faculty of Veterinary Medicine, University of Liege, Belgium). Quantities are expressed in mg of the NIH-FSH-P1 standard (1 mg NIH-FSH-P1 = 0.038 mg NIADDK-oFSH-17).

Animals and experimental design

The experiment was performed in January and November 2010 (breeding season) at the facilities of the research centre (CITA). The live donors included 16 healthy adult Rasa Aragonesa ewes, similar in weight and body condition score. Half the ewes (n = 8) were *FecX*^{R} heterozygous (R+) and the other half (n = 8) were wild-type ewes (++). Two LOPU-IVP trials, 10 months apart, were performed. In each trial, four LOPU sessions were conducted at 7-day intervals, the first two without and the last two with FSH treatment. Collected cumulus–oocyte complexes (COCs) were subjected to *in vitro* maturation, fertilization and culture procedures. day 7 and 8 blastocysts were fresh-transferred to assess their viability by their ability to sustain pregnancy to term.

Hormonal treatment

Prior to each LOPU trial, all animals received two doses of 125 μg cloprostenol (Estrumate®; Schering-Plough Animal Health, Madrid, Spain) 8 days apart, and 5 days after the second injection, a 30-mg fluorogestone acetate (FGA) sponge (Sincropart® 30 mg; CEVA Animal Health SA, Barcelona, Spain) was inserted and replaced by a new one in each LOPU session. The first LOPU session was performed 12 days after the insertion of the sponge and the second session 7 days later. For the third and fourth LOPU sessions, ewes received a total of 32 mg FSH administered in decreasing doses: 8 mg (60 h), 8 mg (48 h), 6 mg (36 h), 6 mg (24 h) and 4 mg (12 h) prior to each LOPU session. Coinciding with the first injection of FSH, 125 μg cloprostenol was administered.

Recovery of oocytes by LOPU

Donor ewes were fasted for 12 h prior to each LOPU session. Ewes were anaesthetized with 3 mg/kg propofol (Propofol ®Lipuro 1%; Braun, Barcelona, Spain) intravenously (i.v.) followed by inhalation maintenance anaesthesia with isofluorane (IsoFlo®; Veterinary Esteve, Barcelona–,Spain). Follicular puncture was performed by a modified LOPU procedure based on the technique previously described by Alberio et al. (2002). An endoscope was inserted through a 1-cm incision, approximately 10 cm cranial to the udder, into the abdominal cavity, and an atraumatic grasping forces was introduced through a contralateral incision to immobilize the ovary. By a third 1-cm incision adjacent to the midline, a 23-G needle connected to a vacuum pump (V-MAR 5100; Cook Ltd, Australia). adjusted to 25 mmHg was introduced to aspirate all ovarian follicles >3 mm in diameter. The number and diameter of punctured follicles in each donor and session were recorded, using a 2-mm scale located on the needle. COCs were collected into 15-ml falcon tubes, which contained 2 ml collection medium (TCM) 199 with 10 mM/ml Hepes supplemented with 100 IU/ml heparin, 4 μl/ml gentamicin and 0.2% bovine serum albumin (BSA). Falcon tubes were maintained in a water bath at 35°C until processing. After oocyte collection, the ovaries were flushed with sterile physiological saline solution (0.9% NaCl) supplemented with 5 UI/ml heparin. The incisions were closed with staples and sprayed with topical chlortetracycline hydrochloride (Pederol® spray; Syva, León, Spain), and 200 mg/10 kg oxytetracycline dihydrate (Oxycen-200 L.A., s.p.® veterinaria, s.a., Tarragona, Spain) was administered by intramuscular injection to each ewe. During each session, ewes of both genotypes were subjected to LOPU in alternate order and the practitioner was blinded to the genotype.

In vitro maturation (IVM)

The recovered COCs were washed 4 times in TCM 199 + 10 mM HEPES + 0.04 mg/ml gentamicin and morphologically scored into five categories (I to V) as previously described by Stangl et al. (1999). Only those surrounded by continuous multilayer unexpanded cumulus cells and showing a homogeneous cytoplasm (categories I, II and III) were used for IVM. Until placed in the maturation medium, all the COCs remained in TCM 199 + 0.04 mg/ml gentamicin. For maturation, 30–40 COCs were placed in 500 μl maturation medium, containing TCM 199 + 132 μg/ml pFSH (equivalent to 5 μg/ml NIADDK-oFSH-17) + 10% (v/v) follicular fluid + 40 μg/ml gentamicin sulphate, using 4-well plates (Nunclon®; Fisher Bioblock, Madrid, Spain). Follicular fluid was prepared as described previously (Cocero et al. 2011). Plates were incubated at 38.5°C in 5% CO_2 in humidified air for 24 h.

In vitro fertilization (IVF)

Oocytes were denuded by gentle pipetting with a small-bore pipette and washed three times in fertilization medium [synthetic oviductal fluid (SOF) + 4 μl/ml gentamicin + 20% heat-inactivated oestrous ewe serum]. Ten to 15 oocytes were placed into 49-μl droplets of IVF

medium on polystyrene Petri dishes under mineral oil. Fresh semen from a single Rasa Aragonesa ram of proved *in vitro* fertility was used in all sessions. For capacitation, semen was maintained at room temperature in the dark for 2 h after collection, subsequently diluted 1:10 (v/v) in SOF + 0.3% BSA + 40 μg/ml gentamicin sulphate and then centrifuged at 200 ***g*** for 5 min. After counting and evaluating progressive individual subjective motility, the pellet was diluted with fertilization medium, and 1 μl was introduced into each 49-μl oocyte-containing drop, yielding a final concentration in the drop of 1×10^6 spz/ml. Incubation was carried out for 20–24 h at 38.5°C in 5% CO_2 humidified air.

In vitro culture (IVC)

After IVF, presumptive zygotes were washed four times in culture medium, containing SOF + 0.3% BSA (fatty acid free) + 40 μg/ml gentamicin sulphate. Once spermatozoa were removed, presumptive zygotes were transferred in 30-μl droplets containing culture medium, overlaid with mineral oil (10–15 presumptive zygotes/droplet) in Petri dishes and incubated for 8 days at 38.5°C in a 5% CO_2/5% O_2/90% N_2 humidified atmosphere. At days 3 and 5 after IVF, half of the media (15 μl) was replaced with 15-μl SOF supplemented with 20% and 10% (v/v) foetal calf serum (FCS), respectively, to obtain from day 3 onwards a FCS concentration of 10% (v/v). Cleavage and blastocyst rates were assessed at 48 h and 7 and 8 days after IVF.

Embryo transfer

Blastocysts produced *in vitro* were functionally assessed by their ability to sustain pregnancy to term. For this purpose, a random sample of either day 7 or 8 blastocysts showing good morphology, from all the experimental groups and LOPU-IVP trials, were directly transferred. A total of 48 adult wild-type Rasa Aragonesa ewes were used as recipients. Prior to the embryo transfer, ewes received 2 doses of 125 μg cloprostenol 7 days apart, and 4 days after the second injection, a 30-mg FGA sponge was inserted for 12 days. At sponge withdrawal, 400 IU eCG (Sincropart® PMSG 6000 IU; CEVA Animal Health SA) was administered. Eight or 9 days after withdrawal, depending on whether day 7 or 8 blastocysts were transferred, respectively, ovulation rate was assessed by laparoscopy and embryo transfer was carried out.

Sedation was performed using propofol (8 ml/ewe i.v.). Two blastocysts were placed preferably into the uterine horn ipsilateral to the ovary showing a morphologically healthy corpus luteum using a tomcat catheter.

In vivo embryo viability

For early pregnancy diagnosis, a blood sample was collected from each recipient by jugular puncture 25 days after embryo transfer using 5-ml vacuum tubes containing lithium heparin for plasmatic pregnancy-associated glycoprotein (PAG) determination. Ewes with a plasma PAG concentration ≥ 1.3 ng/ml were considered pregnant (Alabart et al. 2010). Additionally, abdominal ultrasound pregnancy diagnosis was performed 30 days after embryo transfer. One week before lambing, ewes were placed in individual pens and the offspring were immediately identified at lambing.

Statistical analysis

The effects of genotype and FSH treatment on the following LOPU-related variables, which were measured per ewe and session, were analysed by repeated-measures analysis of variance (ANOVA) using the MIXED procedure of SAS Institute Inc., 2004 (SAS OnlineDoc® 9.1.3; SAS Institute Inc., Cary, NC, USA): the number of punctured follicles of each size, the mean follicle size, the number of recovered COCs of each quality score and individual recovery rate. The following fixed effects were included in the models: genotype, treatment and session, as well as all the possible interactions among them. Treatment and session within each treatment were treated as repeated factors. Normality of residuals was tested by the Jarque–Bera test, applying a correction for finite samples (Lawford 2005). A Box–Cox transformation was applied to variables failing to accomplish the requirement of residual normality.

The effects of genotype, FSH treatment and their interaction on the variables related either to *in vitro* embryo production, survival of the transferred embryos, or fertility of the recipient ewes were analysed by ANOVA for categorical variables using the categorical modelling procedure (PROC CATMOD) of SAS (SAS Institute Inc. 2004).

Results

Follicular puncture and recovery of LOPU-derived oocytes

A total of 1673 follicles were aspirated in 8 LOPU sessions, yielding 995 oocytes (mean collection rate, 59.5%). Genotype did not affect the total number of aspirated follicles per ewe and session (10.4 and 10.2 in R+ and ++ untreated ewes, 17.4 and 14.3 in R+ and ++ FSH-treated ewes, respectively) but significantly affected mean follicular size (Table 1). The *FecX*^{R} heterozygous ewes showed smaller follicular diameter, lower number of >6 mm follicles ($p < 0.01$, both with and without FSH treatment) and higher number of 4-mm follicles ($p < 0.02$, only with FSH treatment), compared with ++ ewes. Genotype affected the recovery rate significantly, favouring R+ over ++ ewes. The number of collected COCs (+3.8; $p < 0.01$) as well as the number of suitable COCs for IVM (+3.6; $p < 0.01$) was higher in R+ than in ++ treated ewes. The number of quality I COCs was similar in R+ and ++ ewes, but the number of quality II–III COCs was higher in R+ ewes when treated with FSH (7.0 in R+ vs. 3.3 in ++; $p < 0.01$).

Independent of genotype, FSH treatment increased all the LOPU-related variables, except the number of 3-mm follicles, significantly (Table 1). FSH treatment affected

Table 1. Follicle population and COC recovery depending on genotype and treatment

	Follicles						COCs					RR
	3 mm	4 mm	5 mm	6 mm	>6 mm	Total	MFS	Total	MIV	I	II+III	IV+V	%
Untreated													
R+	5.8 ± 0.7	2.0 ± 0.3	1.2 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	10.4 ± 1.1	4.1 ± 0.1	5.9 ± 0.6	5.0 ± 0.5	2.8 ± 0.4	2.3 ± 0.3	0.9 ± 0.2	59.2 ± 3.7
++	4.9 ± 0.7	2.2 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	1.3 ± 0.2	10.2 ± 1.1	4.3 ± 0.1	5.4 ± 0.6	4.7 ± 0.5	2.6 ± 0.4	2.0 ± 0.3	0.7 ± 0.2	52.0 ± 3.7
FSH treated													
R+	5.6 ± 0.6	5.3 ± 0.7	3.8 ± 0.4	1.7 ± 0.3	1.0 ± 0.2	17.4 ± 1.3	4.3 ± 0.1	11.8 ± 0.8	11.0 ± 0.7	4.0 ± 0.7	7.0 ± 0.6	0.8 ± 0.3	68.2 ± 2.5
++	3.5 ± 0.6	2.7 ± 0.4	3.2 ± 0.4	1.9 ± 0.3	3.0 ± 0.4	14.3 ± 1.3	5.1 ± 0.1	8.0 ± 0.9	7.4 ± 0.7	4.1 ± 0.7	3.3 ± 0.6	0.6 ± 0.3	56.5 ± 2.6
Significance (p<)													
Treatment (T)	0.23	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.03**	**0.01**	0.76	**0.03**
Genotype (G)	0.06	**0.02**	0.17	0.47	**0.01**	0.23	**0.01**	**0.01**	**0.01**	0.95	**0.01**	0.45	**0.01**
T × G	0.19	**0.01**	0.62	0.97	0.13	0.16	0.31	**0.01**	**0.01**	0.95	**0.01**	0.94	0.47

Least squares means per ewe and LOPU session ± SEM. R+: heterozygous *FecX*^{R} ewes. ++: wild-type ewes. Follicles: number of follicles of each diameter (mm). MFS: mean follicle size (mm). COCs: cumulus–oocyte complexes. MIV: COCs of quality I, II and III according to the classification of Stangl et al. (1999). RR: recovery rate (100 × COCs total/follicles total) on a per ewe basis.

R+ ewes differently from ++ ewes with regard to the numbers of 4-mm follicles, total COCs, IVM COCs, and quality II–III COCs ($p < 0.01$ for all) and the proportion of quality I COCs over IVM COCs ($p < 0.02$), as shown by the significant interactions found between genotype and treatment.

IVM, IVF and IVC

Cleavage rate ranged from 84.0% to 87.8%, with no differences between oocytes originating from R+ or ++ ewes, either with or without FSH treatment (Table 2). No significant differences were observed in day 7 and 8 blastocysts, as well as the *in vitro* final yield, between R+ and ++ groups (average differences, 1.6, 3.9 and 3.6 percentage points, respectively). The effect of R+ genotype on day 7 and 8 blastocysts, and on *in vitro* final yield, was slightly higher in untreated ewes, although the interaction between genotype and treatment was not significant.

The percentage of day 7 and 8 blastocysts, as well as *in vitro* final yield, was significantly higher in both R+ and ++ groups treated with FSH when compared with untreated groups (average increases, 8.3, 8.2 and 7.6 percentage points, respectively). The increases in day 7 and 8 blastocysts due to FSH treatment were slightly higher in ++ than in R+ ewes, although significance was not achieved. In fact, there was no significant interaction between treatment and genotype in any of the studied variables.

As the number of recovered and suitable COCs for IVM was higher in R+ donors, and no significant differences in blastocyst rate were observed between genotypes, R+ ewes demonstrated a better yielding of competent oocytes than ++ ewes in the whole. Thereby, R+ ewes produced 0.75 extra day 8 blastocysts per session compared with ++ ewes, and FSH-treated ewes produced 1.6 extra day 8 blastocysts compared with untreated ewes (average increases). Therefore, the global output of IVP blastocysts obtained at day 8 per ewe and session was significantly affected by both genotype and FSH treatment ($p < 0.0001$ and $p < 0.005$, respectively; Table 2).

Embryo transfer and viability

Fertility of recipients and survival rate of transferred embryos are shown in Table 3. No differences were observed in any of these variables, neither between genotypes nor between treatment groups. Overall fertility was 77.1% (37/48 recipients) at 25 days post-transfer determined by PAG, 66.7% (32/48 recipients) at 30 days determined by ultrasound diagnosis and 56.3% (27/48) at lambing. Overall *in vivo* survival rate of transferred embryos was 38.5% (37/96).

Discussion

In the present study, the *FecX*^{R} allele did not affect the number of follicles ≥ 3 mm, but reduced the mean follicle size in R+ ewes. In particular, R+ showed a decreased number of follicles larger than 6 mm in diameter when compared with ++ ewes. These results

Table 2. Embryo IVP depending on genotype and treatment

	Cleavage rate (%)	Day 7 blastocyst rate (%)	Day 8 blastocyst rate (%)	*In vitro* final yield (%)	Day 8 blastocysts per ewe and session[a]
Untreated					
R+	84.0 (126/150)	27.0 (34/126)	32.5 (41/126)	27.3 (41/150)	1.3 ± 0.3
++	85.4 (117/137)	21.4 (25/117)	25.6 (30/117)	21.9 (30/137)	0.9 ± 0.3
FSH treated					
R+	87.8 (281/320)	31.3 (88/281)	37.7 (106/281)	33.1 (106/320)	3.3 ± 0.1
++	84.8 (190/224)	33.7 (64/190)	36.8 (70/190)	31.3 (70/224)	2.2 ± 0.2
Significance (p<)					
Treatment (T)	0.53	**0.02**	**0.03**	**0.02**	**0.0001**
Genotype (G)	0.76	0.64	0.29	0.26	**0.005**
T × G	0.40	0.26	0.41	0.58	0.13

R+: heterozygous $FecX^R$ ewes. ++: wild-type ewes.
[a]Means ± SEM per ewe and LOPU session.

Table 3. Fertility of recipients and embryo survival depending on genotype and treatment

	Fertility (%)[a]			Survival rate (%)[b]
	At day 25 (PAG)	At day 30 (ultrasound)	At lambing	At lambing
Untreated				
R+	83.3 (5/6)	66.7 (4/6)	66.7 (4/6)	41.7 (5/12)
++	66.7 (4/6)	50.0 (3/6)	50.0 (3/6)	50.0 (6/12)
FSH treated				
R+	75.0 (15/20)	70.0 (14/20)	60.0 (12/20)	40.0 (16/40)
++	81.2 (13/16)	68.7 (11/16)	50.0 (8/16)	31.3 (10/32)
Significance (p<)				
Treatment (T)	0.82	0.49	0.84	0.38
Genotype (G)	0.71	0.58	0.41	0.99
T × G	0.41	0.63	0.84	0.46

R+: heterozygous $FecX^R$ ewes. ++: wild-type ewes.
[a]Pregnant/transferred recipient.
[b]Lambs born/transferred embryos.

therefore concur with those previously reported in Belclare ewes carrying a similar mutation on *BMP15* gene (Reynaud et al. 1999), as well as in Booroola (*FecB*) ewes carrying a mutation in the BMP type IB receptor (Driancourt et al. 1985; McNatty et al. 1986). In both studies, ovulatory follicles from carrier ewes were also found to be smaller. Similar differences in follicular population were found between Inverdale ewes carrying a similar mutation in *BMP15* ($FecX^I$) and wild-type ewes, exhibiting more small follicles (1–2.5 mm) than ++ ewes, with no differences in larger follicles (Shackell et al. 1993). The increased ovulation rate and prolificacy observed in *FecX* and *FecB* carrier ewes are thought to be due to follicles having a lower number of granulosa cells, with precocious LH receptor expression, which results in a higher number of smaller follicles susceptible for selection and ovulation, which explains such differences in follicle size between genotypes (revised by Fabre et al. 2006).

FSH treatment increased the mean follicle diameter, the number of punctured follicles per ewe and session, the recovery rate, and the numbers of recovered COCs and suitable COCs for IVM per ewe and session in both genotypes. This fact highlights the usefulness of stimulation treatment with FSH to increase the performance of LOPU-IVP procedures in sheep. Regarding to follicle population, FSH treatment increased the numbers of follicles from 4 mm onwards in both genotypes. However, the observed increase in the number of 4-mm follicles due to FSH treatment was significantly higher (4.7 times) in R+ than in ++ ewes. Although the cause of this difference between genotypes could not be elucidated with the present work, it is reasonable to assume that R+ ewes could have had a greater proportion of non-atretic follicles smaller than 4 mm able to respond to gonadotropins the days before LOPU, when FSH was administered. If so, this would agree with recent results in Inverdale ewes ($FecX^I$), which yielded a greater proportion of non-atretic follicles >2.5 mm with granulosa cells able to respond to hCG (McNatty et al. 2009). Conversely, the increase in the number of follicles $\geq$6 mm due to FSH treatment was higher in ++ than in R+ ewes (4.25 times), although significance was not achieved. The reduced size of the preovulatory follicles is a characteristic of ewes carrying the $FecX^I$ (Inverdale), $FecB^B$ (Booroola) and $FecL^L$ (Lacaune) mutations, as well as in wild-type prolific Romanov breed, whose control of ovulation rate is polygenic (Drouilhet et al. 2010).

The FSH treatment also increased the proportion of grade II–III COCs over the suitable COCs for IVM in R+ but not in ++ ewes. In our opinion, this observation may be due to the fact that R+ ewes showed smaller follicle diameter and therefore their follicles are assumed to have fewer granulosa cell layers. As explained before, similar ovarian phenotypes with reduced number of granulosa cells were found in ewes carrying similar *FecX* mutations (Belclare: Reynaud et al. 1999; Inverdale: Shackell et al. 1993), as well as in $FecB^B$ mutated Booroola ewes (McNatty et al. 1986). As a result, the morphological evaluation method used in this study for assessing COC quality (Stangl et al. 1999), widely accepted and used by many groups, could have disadvantaged the $FecX^R$ heterozygous ewes when classifying their COCs. Nevertheless, it should be noted that, while follicles from R+ ewes were smaller and their COCs apparently had fewer granulosa cell layers, IVP did not show differences in developmental competence. In our opinion, this is an indication of advanced developmental competence for smaller follicle size in R+ ewes. Nevertheless, this should be further confirmed by comparing the

developmental competence of oocytes of both genotypes for each follicle size.

In addition, these differences in follicular diameters between genotypes could partially be responsible for the higher recovery rate found in R+ ewes. Some authors have reported in sheep a tendency for higher rates of recovery for 1- to 5-mm follicles compared with follicles >5 mm, as large follicles have increased density of follicular fluid and aspiration may be negatively affected by low flow rates and small needle diameters (Baldassarre et al. 1994). Others have found no influence of follicular diameter on recovery rate (Rodríguez et al. 2006). Discrepancy between authors exists regarding the effect of follicle size on recovery rate, although in our work, the potential influence of an advanced follicle structure on recovery rate should not be discarded.

In the present work, no differences were observed between genotypes for *in vitro* embryo production and *in vivo* survival rates regardless of FSH treatment, demonstrating their ability to yield healthy offspring after the LOPU-IVP process. However we would expect lower *in vitro* development of oocytes from R+ ewes as they are supposed to have lower BMP15 protein levels, and in our work, a greater number of grade II and III oocytes from R+ ewes were included in IVP. Therefore, based on our findings, it is reasonable to assume that oocytes from R+ ewes acquire competence at earlier stages of antral follicular development, as there was a higher number of suitable oocytes for IVM recovered from R+ ewes, which came from smaller follicles, but which displayed similar percentages of day 7 and 8 blastocysts when compared with those from ++ ewes. This would be possibly due to an altered folliculogenesis, resulting in a mismatch between follicular diameter and maturation status, when comparing R+ and ++ ewes. Although there are no similar works on *BMP15* mutated ewes, oocytes from heterozygous Booroola ewes are able to resume meiosis and develop to the blastocyst stage at a smaller size compared with wild-type oocytes (Cognié et al. 1998). These *in vitro* results in Rasa Aragonesa ewes concur with what is observed *in vivo*, as we did not find differences on fertility between R+ and ++ ewes using artificial insemination (Alabart et al. 2009). Likewise, oocytes from mature follicles in homozygous Booroola ($FecB^B$) ewes appear fully competent and produce viable offspring with no apparent differences in fertility or embryo viability among genotypes (Montgomery et al. 2001). Moreover, in short-term immunizations against BMP15 protein, which results in an increment in ovulation rate similar to that observed in *BMP15*-mutated ewes, no adverse affects on fertilization, embryo survival or ability of ewes to maintain their pregnancy were observed (McNatty et al. 2004).

Regarding FSH treatment of LOPU donor ewes, it enhanced day 7 and 8 blastocyst rates as well as *in vitro* final yield, independently of genotype, without affecting embryo quality. From a productive point of view, FSH treatment still plays an important role in increasing the output of embryo biotechnology programmes in sheep.

In conclusion, the $FecX^R$ allele did not affect the number of punctured follicles per ewe and session, but reduced follicular size. In addition, R+ ewes displayed higher performance in the number of competent oocytes available after LOPU, demonstrating to be as competent as those from wild-type ewes for IVP, with no differences between genotypes in survival rate at birth of the transferred embryos, despite their follicles being smaller. Collectively, the increased number of IVP blastocysts per R+ donor and session demonstrated the higher efficiency of R+ ewes as donors in a LOPU-IVP programme when compared with ++ ewes.

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Conflict of Interest Statement

None of the authors have any conflict of interest to declare.

Author Contribution

B. Lahoz, J.L. Alabart, J. Folch and M.J. Cocero carried out the experimental design. B. Lahoz, J. Folch and E. Echegoyen performed LOPU and embryo transfer. B. Lahoz, P. Sánchez and M.J. Cocero carried out *in vitro* embryo production. J.L. Alabart performed statistical analysis. B. Lahoz, J.L. Alabart, J. Folch and M.J. Cocero wrote the manuscript. All authors have read and approved the final version of the manuscript.

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