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Follicular Status and Embryo Production in Ouled Djellal (Algeria) Ewes Breed Pretreated with a GnRH Agonist

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ABSTRACT

Variability in ovulation rate and number of embryos in response to superovulation is the main limiting factor in small ruminants transfer programs. The objective of this study was to evaluate the ovarian follicular status and *in vivo* embryo production in Ouled djellal ewes following a pre-treatment with a Gonadotropin Releasing Hormone (GnRH) agonist. Twenty (n = 20) cycling ewes were allotted into two groups, the first (n = 10) received subcutaneously a daily injection of 40 micrograms buserelin for 14 days prior to superovulatory treatment (pretreated group) while the second group (n = 10) did not receive GnRH agonist before superovulatory treatment (control group). Before batching, the ovarian follicular population was assessed by laparoscopy numbering of the ovarian follicles. In the pretreated ewes, a significant increase in small follicles (8.50±1.64 vs. 15.50±2.74, $p<0.01$) and a suppression of large follicles (≥4 mm) (4, 3±0.76 vs. 0.0, $p<0.001$) was observed after treatment with Buserelin. In addition to the pretreated group, the number of small follicles prior to porcine Follicle-Stimulating Hormone (pFSH) treatment was higher and the number of large follicles smaller than the control group. The ovulatory response and the number of transferable embryos per ewe treated was significantly higher in ewes pretreated than in control ewes (ovulatory response: 17,8±1,56 vs. 9,1±1,11; $p<0.001$) (transferable embryos: 10.2±1.87 vs. 4.1±0.40, $p\leq0.01$). Compared to the pretreated group a higher percentage of degenerated embryos was recorded in the control group (control: 20.40 vs. 7.27 pretreated, $p<0.05$). The pre-treatment with a GnRHa to the superovulation protocol improve embryo production in Ouled Djellal ewes, in allowing the terminal follicular growth inhibition and increasing of small follicles number at the start of p FSH treatment.

Key words: GnRH agonist, sheep, ouled djellal, superovulation, follicular status, embryos

INTRODUCTION

In Algeria, among the main local breeds with well defined standard (Chellig, 1992; ITELV, 2002), the Ouled Djellal, is the predominant sheep breed (Madani *et al*., 2009). Its total number of approximately about 11.340.000 heads represents more than 60% of the national sheep flock (BRG, 2003). However, nomadism and the traditional breeding techniques, based on a limited

knowledge and an anarchy in crossings between breeds, favors the disappearance of the genetic potential of the Ouled Djellal breed (BRG, 2003; Mamine, 2010).

Facing this zoo-genetics resources degradation (BRG, 2003), the application of biotechnologies reproduction methods, particularly, the superovulation and the embryonic transfer would be unavoidable for the implementation of the sheep's breed preservation programs. However, the variation of the ovarian response after superovulation gonadotrope induction treatment lives one of the factors limiting the embryo production yields (Rahman *et al*., 2008; Bartlewski *et al*., 2008). In sheep, improvement of these yields has been reported when at ovary level, large dominant follicles are absent and small gonadotrope sensitive follicles are present in large numbers early in the beginning of FSH treatment (Brebion *et al*., 1990; Gonzalez-Bulnes *et al*., 2002a, 2005; Veiga-Lopez *et al*., 2005). Recruitment and small antral follicles growth are under the main FSH monitoring while the large follicles dominance is established and maintained in the presence of high concentrations of LH (Campbell *et al*., 1995; Adams, 1999).

Production improvement of embryos would lead to low concentrations of gonadotropins by the use of antagonists (Cognie, 1999; Cognie *et al*., 2003; Oussaid *et al*., 1999; Gonzalez-Bulnes *et al*., 2003; Lopez-Alonzo *et al*., 2005a, b) or GnRH agonists in sheep (Brebion *et al*., 1992; Baril *et al*., 2004; Archa *et al*., 2005) inhibiting the terminal growth of ovarian follicles (Dufour *et al*., 2000) and resulting in the absence of large follicles (Campbell *et al*., 1998) and the accumulation of small follicles prior to administration of exogenous FSH.

The objective of the present study was to evaluate the effect of pretreatment with a GnRH agonist (Buserelin) on the follicular status and *in vivo* embryo production in Ouled Djellal ewes.

MATERIALS AND METHODS

Animals and experiment setting: This study was conducted during the breeding season from October to November (2009).Twenty cycled ewes of Ouled Djellal breed (03 primiparous and 17 multiparous) from 2 to 5 years old, with a live weight between 40 and 62 kg and having given birth from at least 90 days.

The animals were reared at the experimental farm of Blida University, located 50 km from Algiers (36°28'N Latitude and Longitude of 2°49'E) in a semi-intensive system (sheepfold) under a natural lighting and received water and hay ad libitum supplemented with 500 g of concentrate/animal/day. No female has previously received a superovulation treatment.

Oestrus synchronization: The oestrus synchronization was obtained by the insertion of an intravaginal progestagen sponge impregnated with 40 mg fluorogestone acetate (FGA, Chronogest®, Intervet, France) during 14 days to all ewes.

Superovulation treatments: From the ovarian status evaluated by counting large and small follicles with laparoscopy method, ewes were divided into two groups: a control group (n = 10) receiving the only FSHp and a pretreated group (n = 10) receiving an analogue of GnRH then FSHp.

The preparation of the ovary for the induction of the superovulation was realized by daily injection (subcutaneously) of 40 μg of GnRH agonist (Suprefact ® Busséréline 1 mg mL^{-1}; Aventis pharma, Frankfurt) during 14 days to the ewes of the pretreated group.

The superovulation had been induced by administration of a total dose of 200 and 320 μg of porcine FSH (Reprobiol-Liege University, Belgium), respectively to the ewes of control and

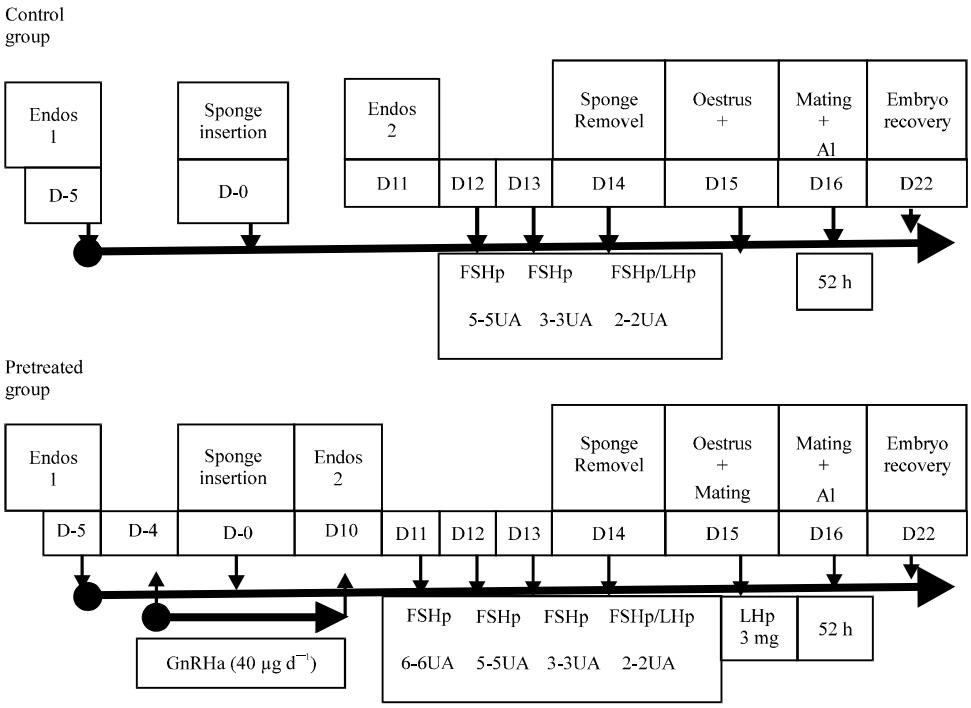

Fig. 1: Treatment protocols (control group vs. pretreated group) used for the embryo production in Ouled Djellal ewes breed. (Endos: Endoscopy, AI: Artificial insemination)

pretreated groups, divided in 6 and 8 twice-daily injections (12 h of interval) and decreasing, during the last three and four days of progestagen treatment, with addition of 60 and 90 µg of porcine LH, during the two last injections (Fig. 1).

The choice of the FSH p doses used is justified by the fact that there is an effect dose-response of the FSHp (Baril *et al.*, 1993; Gordon, 1997), with effective doses included between 160 and 210 mg (Brebion *et al.*, 1992; Baril *et al.*, 1993; Gonzalez *et al.*, 2002) and that high FSHp doses provoked a decrease of the embryo production (Smith, 1984; Torres *et al.*, 1987; Lucero and Rutelle, 1990; Naqvi and Gulyani, 1998) in control group on one hand and a depressive effect on FSH secretion related to the pretreatment; imposing the use of high FSHp doses (Brebion *et al.*, 1992; Briois *et al.*, 1992; Dufour *et al.*, 2000; Cognie *et al.*, 2003) in ewes pretreated with GnRH agonist on the other hand.

Because of pretreatment suppress endogenous LH peak (Picton *et al.*, 1990; Briois *et al.*, 1992; Cognie *et al.*, 2003), the ovulation was induced by intravenous injection of 3 mg porcine LH (Reprobiol-Liege University, Belgium) 32 h after the withdrawal of vaginal sponge in pretreated ewes.

Assessment of ovarian follicular status: The follicular status was assessed before treatment GnRH and FSH, by counting ovarian follicles by laparoscopy method according to the method described by Brebion and Cognie (1989), Noel *et al.* (1993) and Bister *et al.* (1999). Follicles were classified according to their size: small (2-3 mm) and large [medium-follicle (4-5 mm) and largest-follicle (≥6 mm)].

Semen collection and preservation: Semen was collected using an artificial vagina, the day of intrauterine insemination in four adult rams Ouled Djellal breed and having known fertility. After qualitative and quantitative assessment (volume, mass and individual motility, concentration) of ejaculate, mixed in pool and diluted with Ovixcell (IMV Technologies, L'Aigle, France), the semen obtained was cooled and maintained at 15°C for less than 5 h.

Oestrus detection and fertilization: Detection of oestrus was performed using whole rams equipped with an apron every four hours, from the 12th to the 96th h after the withdrawal of the vaginal sponge. Immobilization of the female when mounted by the male was considered to be a sign of occurrence of oestrus (Baril *et al.*, 1993; Olfaz *et al.*, 2010).

Fertilization was obtained by twice hand mating, 32 and 44 h after removal of vaginal sponges, by reproductive rams at a rate of one male for five ewes, followed by in utero insemination under endoscopic control with 100×10^6 spermatozoa, 52 h after removal of vaginal sponges according to the technique described by Baril *et al.* (1993).

Corpus luteum numbering and embryos harvesting: The ovarian response was assessed, under laparoscopy method the 7th day after the oestrus onset, by enumeration of the corpora lutea and embryos were harvested by abdominal laparotomy.

Laparoscopy and laparotomy were performed under general anesthesia through intramuscular administration of xylazine (Rompun® Bayer Ag, Leverkusen, Germany, 6 mg) and Ketamine (Imalgène1000®, Merial, Lyon, France, 130 mg).

The embryo harvesting conducted according to retrograde way (Baril *et al.*, 1993) by successive washes of both horns with 40 mL PBS (phosphate-buffered saline IMV Technologies, L'Aigle, France) supplemented with 4‰ of BSA (bovine serum albumin; IMV Technologies, L'Aigle, France).

The search and ranking of embryos, using the criteria described by Muwalla *et al.* (1988) and Robertson and Nelson (1999) were carried out under binocular microscope and inverted microscope (X20 to 400). Classes of 1-3 embryos were considered as transferable.

Statistical analysis: For comparison of means, we used the Student's test and variance analysis ANOVA (SYSTAT Software, Version 10) and Chi-square test for comparing percentages. The relationship between the follicles number of different size categories and superovulation yield was investigated by rank correlation analysis of Spearman. The statistical significance was accepted from $p<0.05$.

RESULTS

Ovarian follicular status: Ewes having divided into groups according to their ovarian status, the follicular population (≥2 mm) presents at the beginning of experiment did not differ among ewes of both groups (12.8±1.59 vs. 11.4±1.53). The use of GnRH resulted in a significant increase in the mean number of small folhcles (8.50±1.64 vs. 15.50±2.74, $p<0.01$) and a suppression of large follicles (≥4 mm) (4,3±0,76 vs. 0,0, $p<0,001$) (Table 1). In contrast, in control ewes, no significant changes were observed between 1st and 2nd endoscopy for the mean number of small (8.2±1.4 vs. 9.0±1.35) and large follicles (3.2±0.2 vs. 3.6±0.45). Consequently, prior to FSH treatment, a significant difference was observed for the follicular population between the two batches of sheep [small follicles (control group 9±1.35 vs.15.5±2.74 pretreated group, $p<0,05$), large follicles (control group 3.6±0.45 vs. 0.0 pretreated group, $p<0.001$).

Table 1: Effect of pretreatment with GnRH agonist (Buserelin) on the population follicular status in Ouled Djellal ewes breed

			Pretreatment		
Groups	Follicles [1]		Before	After	p
Control group (n = 10)	Small	2-3 mm	8.20±1.40	9.0±1.35[a]	Ns
	Large	≥4 mm	3.20±0.20	3.6±0.45[c]	Ns
Pretreated group (n = 10)	Small	2-3 mm	8.50±1.64	15.50±2.74[b]	p<0.01
	Large	≥4 mm	4.30±0.76	0.0[d]	p<0.001

(a vs. b) p<0, 05; (c vs. d) p<0.001; Ns: Not significant; [1]Mean±SD

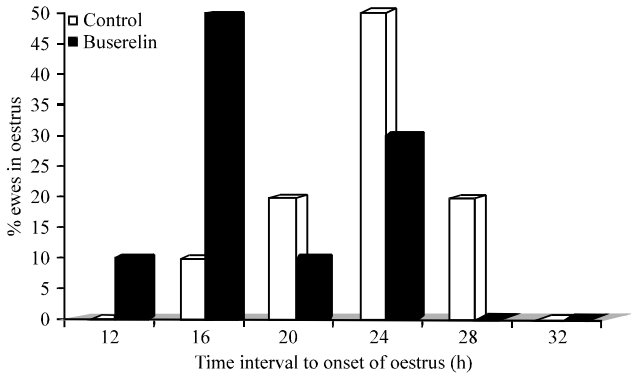

Fig. 2: Distribution of occurrence of oestrus in Ouled Djellal ewes after superovulatory treatments [control group vs. pretreated (Buserelin) group]

Onset and duration of estrus: The oestrus behavior characterized by the male overlap acceptance was detected in all ewes in 28 h (12-28 h) following the withdrawal of the vagina sponge (Fig. 2). The onset of oestrus was significantly earlier in pretreated ewes with Buserelin compared to that ones of control ewes (18.4 h±1.36 vs. 23.2 h±1.16, p≤0. 01). In addition, the mean duration of oestrus was significantly shorter in superovulated ewes in the absence of pretreatment (35.2±1.55 h vs. 48.4 h±2.63, p<0.001).

In all sheep, the total number of present small follicles at the start of FSHp treatment was positively correlated with the oestrus duration (r = 0.561, p = 0.009) and tended to be positively correlated with the onset of oestrus (r = 0.396, p = 0.08).

Yields of the superovulation: The induction results of superovulation and embryo production are reported in Table 2. The ovulatory response determined by the number of corpora lutea was significantly higher in ewes pretreated batch that ewes in the control group (17.8±1.56 vs. 9.1±1.11, p<0.001). For the whole superovulated ewes, the corpus luteum mean number obtained after ovarian stimulation was positively correlated with the small folhicles mean number present at the treatment FSH start (r = 0.729, p<0.001).

The eggs rate harvested was not significantly different depending upon the batch of sheep (control group 54 vs. 63% lot pretreated). Moreover, for the whole ewes, a highly significant correlation was observed between the total number of harvested eggs and the total number of small follicles present on the ovaries before stimulation (r = 0.530, p = 0.01). Positive correlation was observed between the total number of cleaved embryos and that of small follicles present on the ovaries before stimulation (r = 0.52, p = 0.01). Moreover, a very close relationship was found

Table 2: Effect of pretreatment with GnRH agonist (Buserelin) on the *in vivo* embryos production in Ouled Djellal ewes breed ([1]Mean±SEM)

	Treatments (groups)	
Parameter	FSHp (Control group)	GnRHa/FSHp (Pretreated group)
Number of Ewes	10	10
Onset oestrus[1] (h)	23.2±1.16[a,**]	18.4±1.36[b,**]
Duration of oestrus[1] (h)	35.2±1.55[c,***]	48.4±2.63[d,***]
Corpora lutea[1]	9.1±1.11[e,***]	17.8±1.56[f,***]
Collected eggs/Corpora lutea (%)	54	63
Unfertilized eggs/Collected eggs (%)	0	1.78
Transferable embryos/Collected eggs (%)	79.6[g,*]	91.07[h,*]
Degenerated embryos/Cleaved eggs (%)	20.4[i,*]	7.27[j,*]
Transferable embryos/treated ewe[1]	4.1±0.40[k,**]	10.2±1.87[l,**]

Values in the same rows with different superscripts differ significantly (g vs. h), (i vs. j) *p<0.05; (a vs. b), (k vs. l) **p<0.01; (c vs. d), (e vs. f) ***p<0.001

between the total number of embryos cleaved and the ovulatory response (r = 0, 806, p<0.01). When harvesting embryos at day 7, the rate and the mean number of transferable embryos were significantly higher in the batch of pretreated ewes [transferable embryos rates (control group 79.6 vs. 91.07% pretreated group, p = 0.05), mean number of transferable embryos (control 4.1±0.40 vs. 10.2±1.87 pretreated, p = 0.01)] and the rate of degenerated embryos was significantly higher in ewes superovulted in the absence of pretreatment (control group 20.40 vs. 7.27% pretreated batch, p<0.05). For all of superovulated ewes, the number of transferable embryos was correlated positively with the number of small follicles present before stimulation (r = 0.434, p = 0.05).

DISCUSSION

In Ouled Djellal ewes, the use of GnRH agonist pretreatment (Buserelin) allowed to double the ovarian response to FSH treatment by modification of the follicular status, that is to say by inhibition of terminal growth (suppression of large follicles) and increased number of small follicles (2-3 mm). Our results confirm that pretreatment with an agoniste of GnRH improve embryo production. These results thus support the findings of Cognie (1999) and Cognie *et al.* (2003) in sheep, where FSH/GnRHa treated ewes recorded a significant higher ovulation rate (19.2±4.1) than those treated with FSH alone (13.2±5.5).

In fact, pituitary desensitization to GnRH with an agonist can increase the number of follicles in class's size which precedes the dependence of these follicles towards gonadotrophin (2-3 mm), by inhibiting the endogenous LH and FSH secretion and by significantly decreasing the follicular atresia rate in this class size (Brebion *et al.*, 1992).

The long-lasting treatment with GnRH agonist induces modifications in pituitary secretion in small ruminants (Cognie, 1999; Bister *et al.*, 2004; Gonzalez-Bulnes *et al.*, 2004; Monniaux *et al.*, 2009).

Indeed, the low induced plasma FSH and LH levels, involves suppress large ovarian follicles growth (>3 mm), increasing of small emergent folhicles permanently from the basal folhicular growth (Brebion *et al.*, 1990; Campbell *et al.*, 1999; Lopez-Alonzo *et al.*, 2005a; Gonzalez-Bulnes *et al.*, 2002c, 2004, 2005; Veiga-Lopez *et al.*, 2005) and the accumulation of these last ones on the surface of the ovary (Bister *et al.*, 2004).

The results obtained in the present study show that the onset and duration of behavioral oestrus were significantly different between batches. The early onset of behavioral oestrus in pretreated ewes seems to be related to the presence, before FSH treatment start, to a high number of small follicles (2-3 mm) and at the high rate of ovulation as reported by Torres *et al.* (1987), Martemucci *et al.* (1988) and Baril *et al.* (1993). Indeed, Gonzalez-Bulnes *et al.* (2004), Lopez-Alonzo *et al.* (2005b) and Veiga-Lopez *et al.* (2005) observed while the first injection of FSH, that a greater growth of follicles gonadotropin-dependent (2-3 mm) at the stage of large follicles (≥4 mm), was positively correlated with early onset of estrus, the LH peak and a high ovulation rate.

In present study, the number of corpora lutea obtained after super ovulation has been positively correlated with the number of small follicles (2-3 mm) present on the ovaries during hyperstimulation beginning treatment. These results are similar to those previously reported in other sheep breeds after treatment using a GnRH agonist or antagonist for the preparation of the ovary to the superovulation induction (Cognie *et al.*, 2003; Veiga-Lopez *et al.*, 2005). Indeed ovarian status and the follicular dynamics are the main factors responsible for variability of ovarian response (Bartlewski *et al.*, 1999; Cognie, 1999; Cognie *et al.*, 2003; Veiga-Lopez *et al.*, 2006).

The low ovulation rate obtained in control ewes may be explained by the presence of large follicles at the start of FSH treatment as it has been already observed (Rubianes *et al.*, 1995, 1997; Cognie *et al.*, 2003; Gonzalez-Bulnes *et al.*, 2004).

The mean number of collected eggs obtained in control ewes, low compared to pretreated ewes, appears to be related to a negative effect of the presence of large follicles at the start of FSH stimulation as reported by Gonzalez-Bulnes *et al.* (2002b). The established influence because of the presence of large follicles during superovulation on the rate of harvesting or loss of embryos in the uterus may be due to failures in the process of ovulation or to an alteration in the transport of embryos from the oviduct to the uterus (Veiga-Lopez *et al.*, 2005). Indeed, when used in combination of progestagens treatments with FSH, these effects may be exacerbated and high levels of circulating progesterone might damage induced embryos transport through genital tract (Crisman *et al.*, 1980) and explain the differences between the rates of recovery from the oviduct and uterus (Gonzalez-Bulnes *et al.*, 2003).

In our experiment, the induction of superovulation with FSH in the presence or absence of large follicles had no deleterious effect on the cleavage rate. The high fertilization rate achieved in superovulated ewes with or without pretreatment could be related to the combined use at, the optimal time of mating and intrauterine insemination (Bari *et al.*, 2000) and to the good ovulation synchronization in the case of GnRH pretreated batch as reported by the work of Cognie (1999) and Cognie *et al.* (2003). In addition, deposition of semen in the uterine horns, under laparoscopic control, reduces alterations of the survival and transport of spermatozoa during their passage through the cervix in superovulated ewes (Evans and Armstrong, 1984) and thus increases the percentage of viable embryos (Brebion *et al.*, 1992; Cognie *et al.*, 2000).

The results of our study show that treatment with Buserelin, significantly increases the mean number of transferable embryos per ewe treated, without affecting the quality of embryos collected as described previously (Cognie, 1999; Ben Said *et al.*, 2004). This may result from the complete abolition of early discharge of LH-induced follicle phase. Indeed, in the absence of pretreatment preovulatory early discharge may negatively affect the terminal maturation of oocytes (Ben Said *et al.*, 2004).

The percentage of degenerated embryos obtained in this study, was significantly higher with superovulated ewes in the absence of pretreatment, contrary to comments of Gonzalez-Bulnes *et al.* (2003), this rate is not correlated in our study with the presence of large follicles before superovulation treatment.

CONCLUSION

Buserelin treatment, for ovarian preparation toward superovulation induction of has increased significantly the mean number of transferable embryos per ewe treated. This improvement is mainly due to an increase in ovulatory response to gonadotropin treatment and higher quality embryos. The application of pretreatment with GnRH agonist is appropriate for the implementation of the production, preservation and transfer of embryos to maintain the Ouled Djellal breed.

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