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Effect of natural mating or laparoscopic artificial insemination in superovulated Santa Inês ewes on superovulatory response, fertility and embryo viability

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Abstract. This study evaluated the effect of two mating methods (G_{NM}: natural mating or G_{AI}: laparoscopic artificial insemination) on superovulatory response, fertility and embryo yield in superovulated ewes. Fifteen non-pregnant Santa Inês ewes were superovulated and either mated by G_{NM} or G_{AI} in a crossover design. Oestrus was synchronised using intravaginal progestagen sponges for 6 days and on Day 5, 300 IU eCG and 0.0375 mg d-cloprostenol were given. Twelve hours after sponge removal, 0.025 mg gonadotropin-releasing hormone was administered. Superovulation started 48 h after gonadotropin-releasing hormone treatment, using 5 IU/kg follicle-stimulating hormone (pFSH). At the first pFSH dose, new sponges were inserted. At the fifth dose, 0.0375 mg cloprostenol was administered and the sponges were removed. The G_{NM} was mated with rams every 12 h, until the end of oestrus. The ewes of G_{AI} were laparoscopic inseminated with frozen–thawed semen 36 and 48 h after sponge removal. Ultrasonography was performed every 24 h from the beginning of oestrus synchronisation treatment and every 12 h from the second sponge removal to 2 days after the last pFSH dose. Six to seven days after mating, the number of corpora lutea (CL) was evaluated by laparoscopy and the females with > 4 CL were subjected to embryo collection. The interval from sponge removal to ovulation was shorter ($P < 0.05$) in the G_{NM}. The overall superovulatory response was 63.3% (19/30), with 60.0% and 66.7% in G_{NM} and G_{AI}, respectively ($P > 0.05$). The number of recovered structures (6.4 ± 2.4 vs 4.5 ± 3.0), recovery rate (74.0 ± 16.0 vs 52.3 ± 26.5%), number of transferable embryos (3.0 ± 2.9 vs 3.6 ± 2.0) and viability rate (47.2 ± 45.3 vs 77.4 ± 37.1%) did not differ between G_{AI} and G_{NM} ($P > 0.05$). However, the G_{AI} group showed a higher ($P < 0.05$) number of unfertilised oocytes (3.1 ± 3.1) and a higher non-fertilisation rate (47.1 ± 45.3%) than the G_{NM} (0.9 ± 2.1 and 11.5 ± 21.5%). The mating method did not affect the superovulatory response, and production of viable embryos although the non-fertilisation rate has been inferior for the AI group.

Additional keywords: artificial insemination, estrus synchronisation, sheep, superovulation.

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Introduction

The multiple ovulation and embryo transfer technologies (MOET) has the potential to promote the genetic improvement of sheep flocks (Gordon 1997; Bari *et al.* 2000) obtaining multiple embryos of genetically superior ewes (Haresign 1992; Cognié *et al.* 2003). In this context, superovulation is a very important tool. Advances in superovulation protocols aiming the reduction of variations on ovulation and embryo recovery rates can improve the commercial sheep herd productivity and profitability. The treatment using follicle-stimulating hormone (FSH) was considered a better choice for superovulation induction in ewes (Armstrong and Evans 1983), resulting in higher ovulation and fertilisation rate and in recovery of greater quality embryos (Cognié 1999).

Among the major superovulation regimens, the 'Day 0 protocol' was developed from the current available research about the follicular dynamic of small ruminants and is considered the most efficient and natural formulation for follicular wave synchronisation (Rubianes and Menchaca 2006; Menchaca *et al.* 2007). Therefore, on the 'Day 0 protocol' the FSH treatment starts after the ultrasonographic detected ovulation or from the time when it is expected to occur (Day 0) (Rubianes and Menchaca 2006). The ovulation can occur 36 h after the onset of oestrus or 48–72 h after the administration of certain short-term protocols for oestrus synchronisation (Rubianes and Menchaca 2006; Cavalcanti *et al.* 2012).

In MOET programs, the donors can be subjected to natural mating (NM) or artificial insemination (AI). The male presence and the mechanical copulation stimulus ('copulation effect') induces neuroendocrine changes in the hypothalamic-pituitary-axis (Delgadillo *et al.* 2009) that can hasten the luteinising

hormone (LH) pre-ovulatory peak, stimulating follicular development and ovulation (Lucidi *et al.* 2001). The onset and duration of oestrus are also affected by ram exposure (Romano *et al.* 2000). However, failures in fertilisation are common in breeding programs after NM. The multiple rings interlocking finger-like projections of the ewe cervix, the cervical high viscous mucus and the synchronisation and superovulation protocols can affect the sperm transport through the cervix, resulting in low fertilisation rates (Evans and Armstrong 1984; Bari *et al.* 2000; Simonetti *et al.* 2008). Regarding cervical AI, a vaginal stimulus during routine procedures due to the use of a speculum promotes a reflex release of oxytocin, which could affect the ewe's fertility (Houdeau *et al.* 2002). The laparoscopic AI allows semen deposition in the uterine horns, closer to the fertilisation site, which bypasses sperm transport through the cervix, increasing fertilisation rate (Bari *et al.* 2000; Azawi and Al-Mola 2011). However, frequently high fertilisation failure rates following superovulation were reported in small ruminants due to asynchrony between ovulation and AI (Menchaca *et al.* 2010).

The aim of this study was to assess the effect of two mating methods (NM and AI by laparoscopy) on superovulatory response, fertility and viable embryo yield of Santa Inês ewes subjected to a superovulation treatment at the onset of the first follicular wave.

Materials and methods

Location and experimental conditions

This study was approved by the Animal Care Committee of Fluminense Federal University and it was conducted in the rural area of Cachoeiras de Macacu located in the state of Rio de Janeiro (latitude 22°27′S, longitude 43°39′W). The minimum and maximum average temperatures at the location are 18°C and 23°C, respectively; the average annual rainfall ranges from 2.000 to 2.600 mm^3.

Animals, oestrus synchronisation and superovulatory treatment

Fifteen healthy non-pregnant Santa Inês ewes, aged from 2 to 4 years, weighing 47.8 ± 6.3 kg and with a body condition score of 3.3 ± 0.4 were used as embryo donors. The ewes were twice superovulated in a crossover design to be mated by either NM or intrauterine AI with eight and seven ewes per group each time. The interval time between both superovulations was ~60 days. Oestrus was synchronised according to Arashiro *et al.* (2009). Briefly, the follicular wave was synchronised using a short-term treatment with an intravaginal progestagen device containing 60 mg medroxyprogesterone acetate (MAP, Progespon, Schering Plough, São Paulo, Brazil), and maintained for 6 days. At 24 h before sponge removal, 300 IU eCG (Novormon, Schering Plough) and 0.0375 mg d-cloprostenol (Prolise, Tecnopec, São Paulo, Brazil) were administered i.m. Twelve hours after the sponge removal ewes received 0.025 mg licerelin i.m., a gonadotropin-releasing hormone (GnRH) analogue (Gestran Plus, Tecnopec). The superovulation treatment started 60 h after device removal and consisted in 5 IU/kg pFSH i.m. (Pluset, Hertape Calier, Juatuba, Brazil) in six decreasing doses at 12-h intervals (25–25–15–15–10–10%). At the first pFSH dose a new MAP was inserted in all ewes. At the fifth pFSH dose, 0.0375 mg d-cloprostenol was administered i.m and the second sponge was removed (Menchaca *et al.* 2010).

Oestrus detection and mating

Oestrus detection and mating began after the second sponge removal and every 12 h until the end of oestrus. The AI ewes were exposed to a teaser with the penis diverted for oestrus detection and the NM ewes were exposed to two rams of proven fertility for oestrus detection and mating. The ewes from the NM group were randomly allocated into two groups, using one ram for each group. The teaser and the rams remained with the females during the night. At this moment, the interval (h) from second sponge removal to onset of oestrus (SRE) and the interval (h) from first to last mount acceptance (DE) were both recorded.

Feed and water were restricted from the AI ewes 24 and 12 h before the laparoscopic AI, respectively. Fifteen minutes before the procedure, the ewes received the pre-anaesthetic treatment using 0.1 mg/kg acepromazine i.v. (Acepran, Vetnil, São Paulo, Brazil), 0.5 mg/kg diazepam i.v. (Diazepam, Teuto, Goiás, Brazil) and 0.4 mg/kg morphine i.m. (Dolo Moff, São Paulo, Brazil). After this procedure, the ewes were positioned using the *Trendelenburg* position and received local anaesthetic at the incision site, using 2% lidocaine s.c. (maximum dose of 7.0 mg/kg) for the insertion of the trocars. The females were inseminated 36 and 48 h after the second sponge removal with commercial frozen–thawed semen (35°C for 30 s – electronic defroster, Fertilize, Uberaba, Brazil) by laparoscopy using a 0.25-mL straw, with half of the straw deposited in each uterine horn.

Ultrasonography

Ovarian follicular development was evaluated by real-time transrectal ultrasound equipped with a 6.0- or 8.0-MHz linear transducer (Pie Medical, Áquila Vet, Nutricell, Brazil) adapted with a slightly arched plastic tube to facilitate manipulation. Ultrasound exams were performed by the same operator every 24 h from the beginning of the oestrus synchronisation treatment and every 12 h from the second sponge removal to 2 days after the last pFSH dose. Ovaries were located as previously described (Ginther and Kot 1994), and the number, diameter, and position of ovarian follicles ≥3 mm were recorded. The moment of first ovulation was defined when one large follicle, previously identified, was no longer detected. The interval (h) from second sponge removal to ovulation (SRO) was recorded and the percentage of ovulated ewes within the first 48 h (Ov ≤ 48 h) or greater than 48 h (Ov ≥ 48 h) after the second sponge removal were calculated.

Laparoscopy before embryo recovery

Ovarian response was determined by laparoscopy 6 or 7 days after mating and the number of corpora lutea (CL) and anovulatory follicles were recorded. Donor ewes showing an ovulation rate lesser than 4 CL were considered non-responsive to superovulation and, thus, they were not subjected to embryo recovery.

Embryo recovery

Embryos were surgically recovered via longitudinal ventral laparotomy; sedation was induced using i.v. treatment with propofol, at a maximum dose of 4 mg/kg (Profolen, Balusiegel, Cotia, Brazil) and 0.1 mg/kg diazepam. General anaesthesia was induced and maintained by inhalation with isoflurane (Forane, Abbott Laboratórios, São Paulo, Brazil). Each uterine horn was flushed with 40 mL of a 37°C DMPBS solution (modified Dulbecco's phosphate-buffered saline) from Dulbecco and Vogt, modified by Whittingham (1971) using an 18-gauge i.v. catheter inserted near the utero-tubal junction. The embryos were recovered in a Petri dish using a Foley catheter inserted at the external bifurcation of the uterine horns. During this procedure the genital tract was constantly washed with heparinised saline solution (4 IU/ mL) (Liquemine, Roche, Rio de Janeiro, Brazil) at 37°C.

Embryos were morphologically evaluated under a stereomicroscope (Nikon, Tokyo, Japan) using a magnification from ×20 to ×40 and classified according to the criteria recommended by the International Society of Embryo Transfer (Stringfellow and Seidel 1998), with adaptations. The number of viable, degenerated, unfertilised and non-viable structures was recorded and the rates of recovery (embryos recovered/total CL counted at laparoscopy × 100), viability (viable embryos/total structures × 100), non-fertilisation (unfertilised structures/ total structures × 100) and degeneration (degenerated embryos/ total structures × 100) were calculated.

Statistical analyses

The data were analysed using the Statistical Analysis System for Windows SAS software. The quantitative variables related to SRE, DE, SRO, and recovery, viability, non-fertilisation and degeneration rates were expressed as mean ± s.d. and submitted to variance analysis using the GLM system. The means were compared using Student's *t* test at 5% of significance. The qualitative variables expressed as categorical responses (ovulatory periods after the sponge removal, ovulatory response score and the mean number of CL at each score) were compared using the Kruskal–Wallis test at 5% of significance.

Results

The ewes from the AI and NM groups showed oestrus up to 2 days (Table 1) after the second progestagen sponge removal. However, only 46.7% (7/15) showed signs of oestrus in the AI group whereas 86.7% (13/15) from the NM group did. Both SRE and DE did not differ between AI and NM groups (Table 1). The time from SRO was shorter in the ewes from NM than the AI group (Table 1). According to the ovulation period distribution, most of the females of the AI group ovulated greater than 48 h after the second sponge removal and most of the NM group ovulated within the first 48 h after the second sponge removal (Table 1).

Table 1. Interval (h) from sponge removal to onset of oestrus (SRE); duration of oestrus (DE); sponge removal to first ovulation detected (SRO), and ovulation time after the second sponge removal within the first 48 h (Ov ≤ 48 h) or greater than 48 h (Ov ≥ 48 h) in superovulated Santa Inês ewes (mean ± s.d.)

a, b: means within a row with different letters are different ($P < 0.05$)

Variables	G_{AI}	G_{NM}
SRE	31.8 ± 5.9 (7)a	25.3 ± 10.4 (13)a
DE	29.9 ± 11.6 (7)a	26.7 ± 8.7 (13)a
SRO	56.5 ± 15.4 (13)a	31.9 ± 12.2 (11)b
Ov ≤ 48 h %	15.9 (2/13)a	90.9 (10/11)b
Ov ≥ 48 h %	84.6 (11/13)a	9.1 (1/11)b

At the beginning of the superovulatory treatment, a total of 165 pre-ovulatory follicles were detected by ultrasound. At the laparoscopy, after the superovulatory treatment, it was observed that 6.7% (11/165) of the follicles evaluated by ultrasonography, resulted in anovulatory follicles at the laparoscopic evaluation.

Within 30 repetitions, 11 (36.7%) ewes did not respond to superovulation treatment and were not subjected to embryo recovery. Interestingly, 3 of 11 ewes that did not respond presented a follicle >4 mm of diameter at the beginning of the superovulatory treatment. Thus, the overall superovulatory response was 63.3% (19/30). Out of these 19, four ewes were not collected as they presented low response (4 CL), and anovulatory follicles were detected in the laparoscopic evaluation. Three of these four ewes presented a follicle >4 mm of diameter at the beginning of the superovulatory treatment. Therefore, a total of 15 ewes were subjected to embryo collection. Considering only the responding ewes, in terms of number of CL, the superovulatory response was similar between treatments for females presenting 4–10 CL [80.0% (8/10) for AI, and 77.8% (7/9) for NM group] or more than 10 CL [20.0% (2/10) for AI, and 22.2% (2/9) for the NM group]. Therefore, the mean ovarian response was 5.9 ± 4.0 CL in the AI group and 5.6 ± 3.9 CL in the NM group.

In the AI group, the higher number of non-viable structures could be attributed to the higher number of unfertilised ova. Consequently, there was a higher ($P < 0.05$) non-fertilisation rate in ewes of AI group (Table 2). The ewes recovery rate of both groups was 44.4%, 63.6% and 67.3%, in ewes with 4–5 (2/15), 6–10 (9/15) and more than 10 (4/15) CL, respectively.

Discussion

In the present study the AI ewes were laparoscopic inseminated 36 and 48 h after the second sponge removal. Before the inseminations, the onset of oestrus was not observed in seven ewes from this group until the end of oestrus detection. Possibly, the stress condition determined by the laparoscopic AI procedure can explain the absence of oestrus in these females. Stress caused by feed and water restrictions, and excessive handling of the animals for procedures such as laparoscopic intrauterine insemination can affect sheep sexual behaviour (Gordon 1997). It has been demonstrated that the acute or chronic increase in plasmatic cortisol levels in stressful situations, during critical phases of the oestrous cycle, affect the pre-ovulatory oestradiol increase induced by the follicular growth. This phenomenon modifies the oestradiol positive feedback on the induction of the LH surge and on the sexual receptivity (Wagenmaker *et al.* 2009; Papargiris *et al.* 2011).

Table 2. Embryo recovery and quanti-qualitative structures yield of the recovered structures of superovulated Santa Inês ewes mated by artificial insemination (AI) or natural mating (NM) (mean ± s.d.)

a, b: means within a row with different letters are different ($P < 0.05$)

Structures quality/rates	G_{AI} ($n = 7$)			G_{NM} ($n = 8$)		
	Mean	Min.	Max.	Mean	Min.	Max.
Total	6.4 ± 2.4a	4	11	4.5 ± 3.0a	0	9
Viable	3.0 ± 2.9a	0	8	3.6 ± 2.0a	0	6
Degenerated	0.3 ± 0.5a	0	1	0.0 ± 0.0a	0	0
Unfertilised	3.1 ± 3.1a	0	7	0.9 ± 2.1b	0	5
Non-viable	3.4 ± 2.9a	0	7	0.9 ± 2.1b	0	5
Recovery rate %	74.0 ± 16.0a	55.6	100.0	52.3 ± 26.5a	0.0	85.7
Viability rate %	47.2 ± 45.3a	0.0	100.0	77.4 ± 37.1a	0.0	100.0
Non-fertilisation rate %	47.1 ± 45.3a	0.0	100.0	11.5 ± 21.5b	0.0	55.6
Degeneration rate %	5.7 ± 9.8a	0.0	20.0	0.0 ± 0.0a	0.0	0.0

The mechanical stimulation induced by the contact of the penis with the vagina fornix triggers a neuroendocrine mechanism on the hypothalamic-pituitary-axis, shortening oestrus without affecting ovulation time in goats (Romano and Abella 1997). Interestingly, in the present study, the ovulation time was possibly affected by the service in the naturally mated ewes, which had the ovulation time hastened when comparing with the laparoscopic AI ewes. Besides the absence of the service, another condition that may have affected the ovulation time of the AI ewes was the sedation treatment before the laparoscopic inseminations. It is well documented that neuroleptic drugs can affect the LH surge, blocking or delaying ovulation (Radford 1966; McKelvey *et al.* 1985). The second intrauterine insemination was 48 h after the second sponge removal and, as it may be observed in Table 1, until this moment, 11 ewes have not yet ovulated. Therefore, stress related to AI by laparoscopy, affecting the LH surge (Martin *et al.* 1981; Papargiris *et al.* 2011), probably also contributed to delayed ovulation time.

The superovulatory response in AI and NM groups was similar to that reported by Cordeiro *et al.* (2003) and lower than Oliveira *et al.* (2012). In those previous studies 66.7% and 100.0% of the Santa Inês ewes responded to superovulation with an ovulation rate of 10.6 ± 1.0 and 10.5 ± 1.2 CL, respectively. The data in the present experiment compared with the data from the literature confirm that in both groups (AI and NM), the superovulatory response was reasonably satisfactory.

In regard to the ewes' superovulatory response in the present study (according to the numbers of CL) and to the classification described by Cordeiro *et al.* (2003) most of the ewes presented a regular superovulatory response. Possibly, the low superovulatory response of some ewes in this study is related to the unresponsiveness to wave emergence synchronisation or to the fact that some ewes did not reach ovulation at the moment of first FSH administration due to early GnRH administration. Previous studies reported that the synchronisation protocol allows the beginning of superstimulatory treatment on 'Day 0', in which FSH treatment is initiated at the emergence of a follicular wave, after ovulation (i.e. 72 h after sponge removal), resulting in the growth and ovulation of multiple follicles (Menchaca *et al.* 2009; Varago *et al.* 2009). In the present study the FSH treatment was initiated earlier (i.e. 48 h after sponge removal) and it is possible that some of the females did not reach ovulation at this moment, which could have negatively affected the superovulatory response by the presence of a large dominant follicle.

At the end of superovulatory treatment a low percentage of anovulatory follicles was observed. This result can be attributed to the use of FSH in the superovulation protocol, as reported by Armstrong and Evans (1983), Cognié (1999) and Oliveira (2011), which associated the low incidence of anovulatory follicles to the use of FSH. However, besides the low incidence of anovulatory follicles, some of the ewes showed a low superovulatory response. It has been reported that the ewes' ovulatory response to FSH treatment was positively correlated with the number of recruited follicles and negatively correlated with the number of large follicles at the beginning of the superovulatory treatment (Cognié 1999; González-Bulnes *et al.* 2002*a*, 2002*b*). For this reason, the large follicles observed at the beginning of the superovulatory treatment may explain the low ovulatory response of those ewes. This result is in accordance with other studies, which have reported that the presence of dominant follicles, at this stage, affected the ewes' ovulatory response (Rubianes *et al.* 1995; Rubianes *et al.* 1997).

It is known that follicular dominance, as well as its influence on the results of superovulation and embryo production in sheep are not yet fully elucidated (Evans *et al.* 2000; Bartlewski *et al.* 2011). It is possible to suggest that other factors may have influenced the superovulatory response of the females in this study. Greater results on fertility are determined by the synchrony between ovulation time and the moment of artificial insemination (Cognié 1999; Veiga-Lopez *et al.* 2008). Currently, in MOET programs using frozen–thawed semen the intrauterine insemination time recommended is 48 and 60 h (Baldassare 2008) or 48 and 72 h (Simplício *et al.* 2007) after the sponge removal. The AI ewes' embryo recovery rate was greater than reported by Oliveira *et al.* (2012) (51.7 ± 10.2%) in superovulated ewes inseminated by laparoscopy, using frozen–thawed semen, 42 and 48 h after the sponge removal. Nevertheless the viability rate was lower than observed by the previous author (47% vs 64%). Cognié (1999) also reported greater fertility rates and low degeneration rates in superovulated ewes artificially inseminated by laparoscopy 48 h after sponge removal, concluding that the intrinsic ability of oocytes to be fertilised was not affected when

FSH is used for superovulation treatment. Based on the previous results reported by Cognié (1999) and Oliveira *et al*. (2012) and on the low degeneration rate observed, it was expected, in the present study, higher fertility rates for laparoscopic AI ewes. Possibly, the artificial insemination time of the AI group was advanced when regarding the ovulation time. Although the recovery rate of the AI group was higher than the NM group (not significant) the viability rate was low due to the higher non-fertilisation rate. Thus, the lower frozen–thawed semen viability associated with the asynchrony between insemination time and ovulation time can possibly explain the lower fertilisation rate when comparing with the NM group. Even though commercial frozen–thawed semen has been used and evaluated before AI, sperm quality could have been responsible for the fertilisation rate.

Bari *et al*. (2000) observed that superovulated ewes mated by NM presented a lower fertilisation rate (75%) than the ewes mated by laparocopic insemination (82%), different from the fertility results observed in the present study. Bari *et al*. (2000) also observed that the low fertilisation rate of ewes mated by NM was due to a failure in the sperm migration through the cervix and uterus, also reported by other research (Naqvi *et al.* 2001). This phenomenon does not occur when females are mated by laparocopic AI, as this technique bypasses sperm transport through the vagina and cervix (McKelvey *et al.* 1985; Haresign 1992; Bari *et al.* 2000), which are responsible for retrograde loss and retention of part of the sperm by the environment's mucus (Morello and Chemineau 2008). Therefore, it was expected a greater fertilisation rate of the ewes from the AI group than those from NM group. Even though the non-fertilisation rates were higher for AI group, the mating method did not affect viable embryo yield as the number of transferable embryos and embryo viability rate did not differ between AI and NM ewes. The higher fertilisation rate of the NM ewes demonstrated that the NM program used in the present study was efficient and resulted in satisfactory fertilisation rates in this group. This result is in agreement with Cordeiro *et al*. (2003) and Baldassare (2008) that reported great fertility results when ewes were naturally mated with rams, in a 12-h interval, between 48 and 72 h after sponge removal.

Conclusions

The mating method directly affected oestrous behaviour and ovulation time with laparoscopic AI females presenting delayed ovulation. This fact promoted an asynchrony between the laparoscopic insemination time and ovulation, resulting in low fertilisation rates, but sperm quality also could have affected it. However, the mating method does not affect viable embryo yield. This study also indicates that the use of the NM in the MOET program does not affect the fertilisation rate of Santa Inês ewes.

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