



Effects of GnRH administration on ovulation and fertility in ewes subjected to estrous synchronization

Amanda dos Santos Cavalcanti¹ Felipe Zandonadi Brandão¹, Luiz Altamiro Garcia Nogueira¹, Jeferson Ferreira da Fonseca²

¹ Programa de Pós-Graduação em Medicina Veterinária - Faculdade de Veterinária, Universidade Federal Fluminense. Rua Vital Brasil Filho, 64, Niterói, RJ, Brasil, 24230-340.

² Embrapa Caprinos e Ovinos, Sobral, CE, Brasil.

ABSTRACT - The objective of this study was to verify the effects of GnRH on ovulation and pregnancy of ewes subjected to a short-term synchronization of estrus. Santa Inês and crossbred Santa Inês/Dorper ewes received 60 mg MAP sponges during 6 days plus 300 IU eCG and 30 µg d-cloprostenol 24 h prior to sponge withdrawal (SW). Ewes were assigned to receive 0.9% NaCl solution (T_{control} ; n = 32) or 25 µg GnRH (lucerelin, T_{GnRH} ; n = 34) 24 hours after SW. Each group was assigned to intrauterine insemination by laparoscopy (n = 25) or to natural mating (n = 41). Artificial insemination was performed with a single dose of fresh semen. For controlled mating, females were exposed to males 12, 24, 36 and 48 hours after SW. Ten females per treatment were subjected to transrectal ultrasound examination at 12-hour intervals (SW to 60 hours after). Estrous response (100.0% vs 95.2%), interval from SW to estrus (32.9±7.4 vs 29.8±6.9 hours), estrous length (37.4±9.0 vs 31.5±10.4 hours), pregnancy rates (57.0% vs 41.0%), ovulation rate (100.0% vs 90.0%), number of ovulations/ewe (1.1±0.3 vs 1.2±0.4), maximum follicular diameter (6.4±0.7 vs 6.1±0.6 mm), interval from SW to ovulation (59.1±3.5 vs 58.4±3.5 hours) did not differ between T_{control} and T_{GnRH} , respectively. Administration of GnRH 24 hours after SW does not improve ovulation or pregnancy rate in estrous synchronization in ewes.

Key Words: artificial insemination, intravaginal progesterone insert, ovulation, pregnancy rate, sheep

Introduction

The use of reproductive biotechnologies associated to induction of a synchronized estrus is a valuable tool that favors productivity, offers homogeneous groups of animals and consequently allows the constancy of products offered irrespective to season. Ovarian response of sheep to estrous synchronization varies according to the type of intravaginal device, kind of progestagen, nutritional status, presence of stress, environmental aspects, male effect (Hill et al., 1997; Amarantidis et al., 2006; Kleemann et al., 2006) and breed (Boscos et al., 2002), although Donovan et al. (2004) found no breed effect.

The Gonadotropin-releasing hormone (GnRH) is the key neuro-peptide controlling reproductive function in all vertebrate species. In females of spontaneously ovulating species, including sheep, ovarian steroids secreted by maturing ovarian follicles control a pulsatile pattern of GnRH release from the hypothalamus that, in turn, stimulates a preovulatory secretion of luteinizing hormone (LH) by the anterior pituitary gland (Bakker & Baum, 2000). The GnRH treatment induces an LH peak

within 1–4 h of treatment and reduces the period over which ovulation occurs (Eppleston et al., 1991).

Many authors have successfully used GnRH treatment in combination with progestagens, gonadotropins and prostaglandin (Husein & Kridli, 2003; Reyna et al., 2007; Rubianes et al., 2007; Beilby et al., 2009) or prostaglandin only (Murdoch & Van Kirk, 1998; Deligiannis et al., 2005); and it is well known that all preovulatory events and ovulation can be induced in seasonally anovular ewes if they are treated with multiple injections of GnRH. Besides, it has been demonstrated that exogenous GnRH treatment immediately after artificial insemination increases the multiple birth rate in synchronized ewes (Türk et al., 2008).

Fertility was related positively to concentrations of progesterone during exogenous treatment (Johnson et al., 1996). A short-term treatment provides exposure to higher average concentrations of progestagen during the treatment period. Such treatments (5 to 8 d) have been effective in anestrous ewes (Knights et al., 2001). The efficiency in synchronizing oestrus of short progestagen protocol is given by the fact that progestagen prevents new formation of corpora lutea (Martemucci & D'Alessandro, 1999;

Wildeus, 2000) and when given with prostaglandins (PG) and gonadotropins, progestagen ensures the luteolysis 5 days later and eCG improves the synchronization.

The objective of this study was to evaluate reproductive parameters and GnRH influence on ovulation and pregnancy rate in ewes subjected to estrous induction and synchronization.

Material and Methods

This experiment was conducted in Cachoeiras de Macacu, Rio de Janeiro, southeast Brazil (tropical region), during the summer. The farm is located at 22°27'S, 43°39'W.

A total of 66 ewes during breeding season were studied, 54 of Santa Inês breed and 12 crossbreed Santa Inês/Dorper ewes. The ewes had been maintained isolated from any ram for at least one month prior to the experiment. Body condition score (BCS, ranging from 1 to 5) was evaluated by palpation of the lumbar and sternal region. The mean BCS and weight for all ewes were 3.2 ± 0.5 and 41.8 ± 9.6 kg, respectively. Animals were kept in a semi-intensive system on *Brachiaria sp* paddocks, and also received concentrate once a day. Water and mineral salt were offered *ad libitum*. Two Santa Inês and one Dorper rams were used for semen collection and natural mating. The rams (aged between 1.5 and 3 years) were sexually active and tested for fertility, and blocked between treatments.

Ewes were equally divided into two treatment groups: control (T_{Control} , $n = 32$, 3.2 ± 0.5 and 41.5 ± 10.6 kg) and treated (T_{GnRH} , $n = 34$, 3.2 ± 0.5 and 41.8 ± 9.6 kg) according to the BCS and weight. Estrus was induced using vaginal sponges impregnated with 60 mg Medroxyprogesterone Acetate (Progespon®, Tecnopec, SP, Brazil) for 6 days. A dose of 300 IU eCG i.m. (Novormon 5000®, Tecnopec, SP, Brazil) and 30 µg d-cloprostenol (Prolise®, ARSA S.R.L., Buenos Aires, Argentina) via the intra-vulvo-submucosal route was administered 24 h prior to sponge removal. One day after sponge removal, T_{Control} received 1.0 mL NaCl 0.9% solution and T_{GnRH} received 25 µg i.m. licerelin, a GnRH analogue (Gestran®, Tecnopec, SP, Brazil).

Ten animals per group were subjected to real-time ultrasound (Aloka SSD 500®- Aloka Co., Tokyo, Japan) equipped with 5.0 MHz linear transducer, adapted with a plastic support to be manipulated externally in the rectum. Exams were performed at 12, 24, 36, 48 and 60 hours after sponge removal, by the same operator. The number, relative position and size of all follicles with a diameter of 3mm were mapped. The timing of ovulation, total numbers of follicles ovulating and mean diameter of the largest and second largest follicle were recorded. The day

of ovulation was defined as the day when the largest follicle was no longer seen.

Blood samples were collected by jugular venipuncture using EDTA venous blood vacuum collection tubes (Becton & Dickinson Vacutainer® Systems, Americana, São Paulo) at the time of sponge insertion (D0), on the fourth day of progestagen treatment (D4), at eCG and d-cloprostenol administration (D5), at sponge removal (D6) and one day later (D7). Samples were immediately placed and maintained on ice until centrifugation at 1000 X g for 15 min. Plasma was stored in duplicate at -20 °C until hormone analyses. Progesterone (ng/mL) was determined by the direct solid-phase radioimmunoassay kits (RIA; Coat-a-Count®, Diagnostic Products Corporation, Los Angeles, CA). The mean intra- and inter-assay coefficient of variation were 8.8% and 9.7%, respectively. Animals were assigned again to be laparoscopic intrauterine inseminated ($n = 25$) or naturally mated ($n = 41$). All the ewes were exposed in groups of five animals for 30 min every 12 hours from 12 to 60 h following the sponge withdrawal with the aid of the rams, when estrous was detected and recorded. The onset of oestrus was confirmed by the passive ability of the ewes to stimulate the interest of the ram, and was usually quantified by measuring his behavior. The end of oestrus was determined by the refusal of immobility in front of the ram by ewe.

Semen from rams was collected with artificial vagina. Each ejaculate was immediately evaluated to determine the motility of the semen. All the ejaculates with motility higher than 70% were diluted in a Tris-Fructose extender (Evans, Maxwell, 1987) to contain 100x106 viable sperm per 0.25 mL straw. The diluted semen was kept at 37 °C in a water bath until insemination and semen motility was assessed no more than 30 minutes prior to insemination. Each ewe was intrauterine-inseminated at 48 hours after SW with a single dose of fresh semen. Half the semen dose was deposited within each uterine horn by laparoscopy. Ewes were further evaluated for pregnancy status 45 days after artificial insemination/mating by real-time ultrasonography.

The variables recorded were: interval from sponge removal to artificial insemination (hours); ovulation rate (%): (number of animals that ovulated/number of ultrasound evaluated animals) × 100; number of ovulations: average number of ovulations per ewe; follicle diameter (mm); percentage of animals in estrus (%): (number of females in estrus/number of females treated) × 100; pregnancy rate (%): (number of pregnant females/number of females exposed) × 100; interval from sponge removal to ovulation (h); interval to estrus: interval (h) from sponge removal to time of first estrous identification (onset of estrus); duration

of estrus: interval (h) from the time of the first to the last estrous identification for each animal. The intervals were calculated considering the difference of time between the first and the last identification.

Statistical analysis was performed for a 95% confidence interval. Non-parametric variables expressed in percentage (%) were compared between treatments by using the Chi-Square Test (χ^2). Parametric variables were expressed as mean \pm standart deviation and submitted to one-way analysis of variance and compared by the Tukey Test using the software SAEG (System for Statistical Analysis, version 8.1).

Results and Discussion

The administration of 25 μ g GnRH 24 h after sponge removal had no significant effect on fertility or time of estrus. Ovulation rate, follicular diameter and average interval to ovulation did not differ ($P>0.05$) between treatment groups (Table 1).

The total ovulation rate of 95% (19/21) indicates the efficiency of the protocol employed. The number of ovulations per ewe, the average follicular diameter and interval to ovulation did not differ between groups and were similar to other studies (Pinna et al., 2012). Other studies with short progestagen protocols showed similar efficiency. Reyna et al. (2007) confirmed ovulation in 100% of Merino ewes during the breeding season using sponges impregnated with 30 mg Flugestone Acetate for 12 days plus an intramuscular injection of 400 IU pregnant mare serumgonadotrophin (PMSG) at SW and 40 μ g of a synthetic GnRH given at 36 h after SW. From the standpoint of mean follicle size (6.09 \pm 1.16 vs 6.09 \pm 1.16 mm) and synchronization of ovulation (3.8 vs 4.4 days) these authors did not observe difference between GnRH-treated or non-treated groups. Luther et al. (2007) also tested GnRH in Hampshire and Montadale ewes during the breeding season. All ewes were implanted with norgestomet plus 400 IU eCG and 25 μ g GnRH 36 h after progestin removal. The GnRH treatment did not affect the efficiency of estrous synchronization (progestin with GnRH, 85.1% versus progestin without GnRH, 90.3%) and pregnancy rates after laparoscopic

artificial insemination (progestin with GnRH, 53.7% versus progestin without GnRH, 61.3%). However, Takada et al. (2012) reported 58.0% of Suffolk ewes ovulating, using 60 mg MAP-impregnated sponges for 4 days associated to estradiol benzoate, d-cloprostenol and 50 μ g GnRH (gonadorelin) 48 hours after sponge removal.

In regard to number of ovulation per ewe, our results are similar to those presented for other protocols. Takada et al. (2012) reported 1.17 \pm 0.17 in Suffolk ewes using 60 mg MAP-impregnated sponges for 4 days associated to estradiol benzoate, d-cloprostenol and 50 μ g of GnRH (gonadorelin) 48 h after sponge removal. However, Knights et al. (2001) induced estrous of 275 ewes (Suffolk and Dorset predominance) with controlled internal drug release device (CIDR) for 5 days in combination with follicle-stimulating hormone (FSH) and observed an average of 1.95 \pm 0.1 ovulations per ewe by transrectal ultrasonography.

In accordance with the present results, Bartlewski et al. (1999) and Evans et al. (2000) reported the maximum follicular diameter in ewes as 5–7 mm. The former examined Western White-faced and Finnish Landrace ewes daily by transrectal ultrasonography during one estrous cycle in the middle of the breeding season and the latter used Texel and Suffolk-Cross ewes by transrectal ultrasonography and follicle dissection after synchronization using a progestagen-releasing intravaginal device.

The interval to ovulation in both control (59 h) and GnRH ewes (58 h) was shorter than in other studies that did not use GnRH and assessed each ovary using a transrectal ultrasonographic procedure (Cardwel et al., 1998; Cline et al., 2001). Interestingly Martemucci & D'Alessandro (2011) conducted experiments in dry ewes with different protocols using GnRH. As a result, they observed an earlier ovulation time (53.0 h) in ewes treated with FGA during 5 days, plus PGF(2 α) on day 5 and GnRH 30 h after SW than in ewes that received an extra dose of eCG on day 5 (61.6 h). In the present trial, induction of early peak of LH and faster response from the largest follicle enhancing follicular steroidogenesis and induction of ovulation were expected. In cattle, exogenous progesterone-progestagen treatments combined with the use of exogenous oestradiol or GnRH

Table 1 - Ultrasonography parameters (mean \pm standart deviation) of ewes treated with MAP sponges for 6 days with (T_{GnRH}) or without (T_{Control}) administration of GnRH 24 hours after sponge removal and subjected to artificial insemination or natural mating

	T _{Control} (n)	T _{GnRH} (n)	Total (n)
Ovulation rate (%)	100.0 (10/10)	90.0 (9/10)	95.0 (19/20)
Number of ovulations	1.1 \pm 0.3 (9)	1.2 \pm 0.4 (9)	1.2 \pm 0.4 (18)
Follicular diameter (mm)	6.4 \pm 0.7 (9)	6.2 \pm 0.6 (9)	6.3 \pm 0.6 (18)
IOV (hours)	59.1 \pm 3.5 (9)	58.4 \pm 3.5 (9)	58.7 \pm 3.4 (18)

No differences detected between treatments ($P>0.05$; Tukey test).

IOV - interval from sponge removal to ovulation; SD - standart deviation.

are used to control new follicle wave emergence and to shorten the life span of the corpus luteum (Diskin et al., 2002), improving synchronization of ovulation. Moreover, follicles acquired ovulatory capacity at about 10 mm, corresponding to about 1 day after the start of follicular deviation, but they required a greater LH dose to induce ovulation compared with larger follicles (Sartori et al., 2001). In sheep, there is broad agreement that the ovulatory follicle(s) exerts dominance over other follicles during the follicular phase as seen by a decrease in the numbers and emergence of non-ovulatory follicles; however, other data would suggest that clear dominance does not occur in sheep (Evans et al., 2003a). Given the capacity of subordinate follicle to acquire ovulatory capacity, one can infer that the GnRH and subsequent LH surge could stimulate ovulation. However, only three of nineteen animals had double ovulation (data not shown). Ovulatory response in cattle may involve an increased expression of LH receptors on granulosa cells of the dominant follicle and this change may also be important for further growth of the dominant follicle (Sartori et al., 2001). The ovulatory response to GnRH injection might depend on the time of treatment and size of ovulatory follicle. Further research is worthwhile to measure in vivo ovulatory capacity and the mechanisms involved in follicle growth near the critical time of diameter deviation in ewes.

Progesterone declined progressively to subleutal levels on day 5, one day after cloprostenol administration and remained basal up to day 7 (Figure 1). As observed by plasma progesterone concentration along the treatment, few animals (4/20) presented less than 1 ng/mL in all measurements and were considered non-cyclic. All other animals had higher dosages at some point. According to Sasa et al. (2002) and Traldi (2000), the cyclic activity of Santa Inês ewes in this region of the country does not cease during the year. Considering this, animals were considered as cycling.

Estrous response, interval to estrus and duration of estrus were similar ($P>0.05$) for T_{Control} and T_{GnRH} (Table 2).

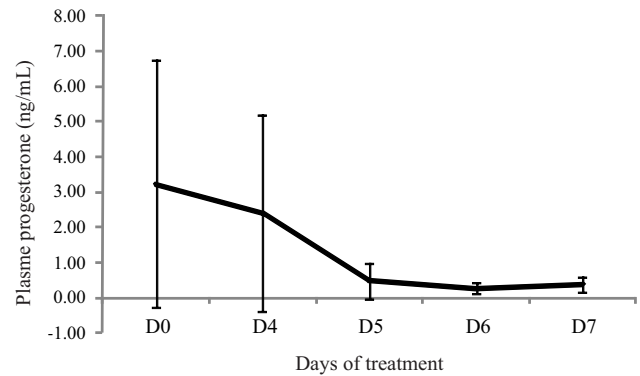


Figure 1 - Plasma progesterone concentration (ng/mL) in estrous-synchronized ewes receiving 60 mg MAP sponges for six days plus 300 IU eCG and 30 µg d-cloprostenol 24 hours before sponge removal.

Interval to estrus varied from 22.5 to 32.5 hours, except for one Santa Inês ewe of T_{Control} , which showed estrous at 56.5 hours after SW and low plasma progesterone (0.42 ng/mL) on day 0 and two Santa Inês ewes of T_{GnRH} that showed estrous earlier, at 8.5 h after SW. Duration of estrus ranged from 12 to 40 hours. Although the expected is from 24 to 30 hours (Robertson, 1977), estrous synchronization protocol resulted in great synchrony of estrus, since 70.7% (29/41) of the animals were in estrus at 24 hours (Figure 2) after sponge removal, except for one ewe treated with GnRH which did not display signs of behavioral estrus (2.4% - 1/41).

The average interval to estrus in the present study (31.4 ± 7.3 hours) indicates a highly acceptable estrous synchrony of animals. Previous studies (Boscos et al., 2002; Luther et al., 2007) reported similar interval to estrus using short (6 days) or long (14 days) progestagen protocols, with or without GnRH. The interval to estrus (29.85 ± 6.98 h for T_{GnRH} and 32.9 ± 7.5 h for T_{Control}) was shorter than those reported previously (Pierson et al., 2003). However, maximum and minimum values, 13.5 and 65.1h, respectively, were similar to others studies (Husein & Kridli, 1998;

Table 2 - Interval to estrous (h) and duration of estrous (hours; mean±standart deviation) of ewes treated with MAP sponges for 6 days with (T_{GnRH}) or without (T_{Control}) administration of GnRH 24 hours after sponge removal

	T_{Control} (n)	T_{GnRH} (n)	Total (n)
Interval to estrus (hours)			
Santa Inês/Dorper crossbred	29.5±0.4 (6)	29.5±0.4 (7)	29.5±0.0 (13)
Santa Inês	34.4±8.6 (14)	30.0±8.8 (13)	32.3±8.8 (27)
Total	32.9±7.0 (20)	29.8±7.0 (20)	31.4±7.3 (40)
Estrous duration (hours)			
Santa Inês/Dorper crossbred	36.0±7.6 (6)	34.3±4.5 (7)	35.1±5.9 (13)
Santa Inês	38.1±9.7 (14)	30.1±12.5 (13)	34.2±11.7 (27)
Total	37.4±9.0 (20)	31.5±10.4 (20)	34.5±10.1 (40)

No differences between treatments ($P>0.05$; Tukey Test); SD - standard deviation.

Barret et al., 2004). In cyclic ewes, eCG given after progestagen treatment reduces the interval to onset of estrus, when compared with ewes given only progestagen (Husein & Kridli, 1998; Dias et al., 2000). Gonadotropin enhances estrogen concentration and induces estrous and LH surge.

As expected, GnRH did not influence estrous duration (ED), once eCG is more important in determining estrous duration because it stimulates follicle development enhancing the recruitment of small follicles (Evans et al., 2003). The mean estrous duration for all animals (34.5 ± 10.1 hours) is compatible with physiological reports. According to Mobini et al. (2002) estrous could vary between 15 and 45 hours, with an average of 30 hours. A classical report by Robertson (1977) considered that behavioral estrus in ewe normally lasts 24 – 30 hours. Besides that, the findings agree with results of Ekiz et al. (2009), who reported a longer estrous duration (34.80 ± 2.15 hours) when estrous was synchronized with fluorgestone acetate intravaginal sponges left for 14 days, plus an injection of 600 IU PMSG at the time of sponge removal and ewes were exposed to ram twice a day than when remaining with the ram permanently (15.60 ± 1.83 hours). Turk et al. (2008) reported 29.9 h after progestagen, PMSG and prostaglandin treatment.

Estrous was recorded during mating. Most animals 70.7% (29/41) showed estrus within 24 h after SW (Figure 2). Regardless of the GnRH treatment, when short-term progestagen protocol is used, there is a little dispersion of estrus.

The interval from sponge removal to artificial insemination was similar ($P > 0.05$) for T_{Control} (53.7 ± 0.9 hours) and T_{GnRH} (53.6 ± 1.3 hours), at around 6 h before ovulation detection. The minimum and maximum values recorded were 51.7 and 56.5 hours. The resultant conception rates were similar between treatment and mating groups (Table 3).

In spite of a greater conception rate for T_{Control} , there was no statistical difference between treatment groups (57.0% for T_{Control} and 41.0% for T_{GnRH}). Reyna et al. (2007) showed that GnRH injection 36 hours after sponge removal improved ovulation synchronization, despite having no effect on conception rates using laparoscopic artificial insemination at 42 hours (20.20%) or 48 hours (19.26%) after progestagen treatment.

Conception rates by natural mating (55.00%) were similar in others studies (Viñoles et al., 2001; Dixon et al., 2006). However, results differed from studies with long and short progestagen protocols and variable dosages of eCG (Ungerfeld, Rubianes, 1999; Shahneh et al., 2006), which reported conception rates superior to 70%.

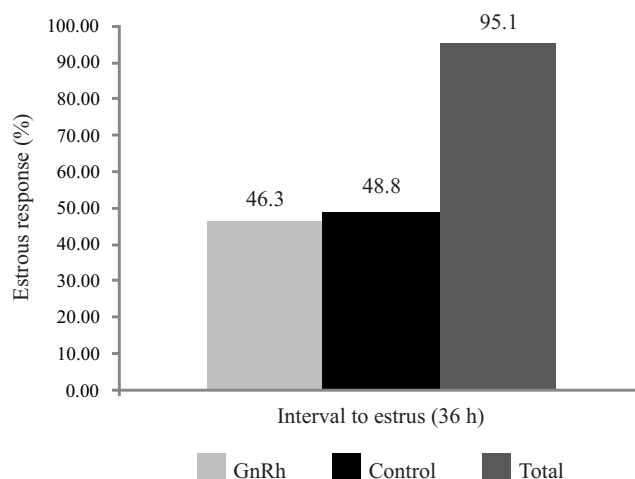


Figure 2 - Interval to estrous response in ewes receiving 60 mg MAP sponges for six days plus 300 IU eCG, 30 μ g d-cloprostenol 24 hours before and with or without GnRH 24 hours after sponge removal.

Nevertheless, the low conception rate observed when laparoscopic artificial insemination was used (37.5%) with fresh semen was an unexpected finding, since rates of 55.9% (Luther et al., 2007) and 53.3% (Lucidi et al., 2001) were reported. Contreras-Solis et al. (2009), using 12-day FGA treatment and artificial insemination at 55 h after SW showed 47.4% of fertility rate. In a Brazilian study, Dias et al. (2001) carried out an experiment on artificial insemination of hairy ewes. In their experiment, females were synchronized with sponges impregnated with 30 mg fluorogestone acetate and 200 IU eCG were given at the end of treatment. After laparoscopic artificial insemination with frozen semen, they achieved a 33.3% pregnancy rate.

Average interval to artificial insemination (53.6 ± 1.1 h) was very close to ovulation (58.7 ± 3.4 h) and fresh semen needs some interval in the female reproductive tract for sperm capacitation (Cohen et al., 2004; Breitbart et al., 2005). Therefore, when spermatozoa were able to fertilize, the oocyte was old, resulting in low fertility. Artificial insemination using fresh semen was recommended to be

Table 3 - Conception rate in ewes treated with MAP for 6 days with (T_{GnRH}) or without (T_{Control}) GnRH 24 h after sponge removal and subjected to laparoscopic artificial insemination (LAI) or natural mating (NM)

	T_{Control} (n)	T_{GnRH} (n)	Total (n)
NM	65.0% (13/20)	45.0% (9/20)	55.0% (22/40)
LAI	40.0% (4/10)	35.7% (5/14)	37.5% (9/24)
Total	57.0% (17/30)	41.0% (14/34)	48.0% (31/64)

No differences detected between treatments ($P > 0.05$; Chi-Square Test).

done 48 h after sponge removal and using frozen semen this interval is 58 h after sponge removal (Maxwell, 1986) This interval corresponds to the interval to ovulation in the present study (58.7±3.4 h). These data suggested that when GnRH is used, fixed time artificial insemination with fresh semen should be done earlier. Gillan et al. (1997) showed that 54% of freshly collected spermatozoa become capacitated with incubation (6 h at 37 °C). In contrast, frozen spermatozoa displayed a 65.9% capacitation rate. This demonstrates that cryopreservation may cause membrane changes in ram spermatozoa functionally equivalent to capacitation. This way, the interval to artificial insemination (53.6±1.1 hours) used in the present study should be more appropriate for frozen semen. Success of fertilization depends on artificial insemination or ovulation time and control of these factors could improve conception rate.

Conclusions

Six-day progesterone priming, in combination with GnRH, PGF₂α and eCG can be used successfully for ovulation and estrous induction/synchronization in Santa Inês and Santa Inês/Dorper crossbred ewes, but there is no effect on pregnancy rate in Santa Inês/Dorper crossbred ewes during the breeding season.

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