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Follicular dynamics and *in vivo* embryo production in Santa Inês ewes treated with smaller doses of pFSH

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ABSTRACT

To evaluate follicular dynamics, there was assessment of superovulatory response and *in vivo* embryo production in ewes treated with relatively smaller doses of exogenous pFSH than typically used in combination with a dose of eCG at the beginning of the gonadotropin treatment period. Santa Inês ewes ($n = 24$) were randomly divided into three groups, based on mg dose of pFSH administered: G200 ($n = 8$), G133 ($n = 8$) and G100 ($n = 8$) in eight decreasing doses at 12 -h intervals. All ewes were treated with 300 IU of eCG concomitantly starting with first pFSH administration. Ovulatory follicular dynamics and follicular wall vascularization (FWV) were evaluated using a B-mode and color Doppler ultrasonic machine, respectively. Superovulatory response and embryo production were evaluated 6 days after estrous detection. In the G200 group, the preovulatory follicle size (PFS) were less ($P < 0.05$), ovulation time later ($P < 0.05$), and PFS rate greater ($P < 0.05$); while in the G100 group ovulation rate, and number and percentage of unfertilized eggs were greater ($P < 0.05$) than in the G133 group ($P < 0.05$). Number and percentage of viable embryos were greater in the G200 and G100 compared to G133 group ($P < 0.05$). The dose of 100 mg of FSH was as efficacious as the traditional dose of 200 mg, in combination with a dose of eCG, for superovulatory response and viable embryo production but there was a greater percentage of unfertilized eggs with this treatment.

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1. Introduction

Santa Inês ewes come from low latitude regions, have estrous cycles throughout the year in tropical conditions and generally have three parities in 2 years (Oliveira et al., 2014c). Litter size, however, is small (Barros et al., 2005), which indicates the importance of reproductive biotechniques, such as multiple ovulation and embryo transfer, to increase the reproductive efficiency of females.

Multiple ovulation and embryo transfer (MOET) is a biotechnology that can be used to increase the number of viable embryos produced per reproductive cycle, enabling major advances in the multiplication of animals with valuable genetics (Oliveira, 2011a). The superovulation treatment is an important component of MOET, which is based on hormonal preparations which stimulate a greater number of preovulatory follicle development than what typically occurs and, consequently there is a greater ovulation number (Blanco et al., 2003).

With use of the superovulatory process, there is great animal to animal variability in superovulatory response, oocyte fertilization, number and quality of embryos recovered and furthermore, it has been considered the most important aspect of *in vivo* embryo production in sheep programs (Amiridis and Cseh, 2013; Bartlewski et al., 2016). The gonadotropin treatment used for superstimulation of follicular development has been associated with the heterogeneous results, depending on the gonadotropin used, commercial product, manufacturing lot, FSH (follicle-stimulating hormone) to LH (luteinizing hormone) ratio, dose and application protocol (Quan et al., 2011).

Superstimulatory treatment for follicular development with the 256 mg dose of porcine FSH (pFSH, Folltropin®) is commonly used (Oliveira et al., 2012). The 175–200 mg pFSH doses have also been used with optimal results (ovulation rate and viable embryo production; Oliveira et al., 2014a). Large exogenous FSH doses, in addition to being costly to producers when used, can result in altered ovarian endocrine dynamics, changes in frequency of LH pulses and occurrence of early luteal regression (Bevers et al., 1989; Kafi and Mcgowan, 1997; Loiola Filho et al., 2015).

Larger doses also may induce a prolonged stimulus of anovulatory follicles, which continue to produce estrogen, leading to the persistence of relatively greater hormonal concentrations during initial luteal phase than would occur without treatment with the larger doses, resulting in early release of $PGF_2\alpha$ (prostaglandin F2α) followed by luteal regression and this endocrine milieu resulting from treatment with these larger doses of gonadotropin has been associated with a decrease in the quality and number of recovered embryos (Okada et al., 2000).

It is hypothesized that smaller doses of exogenous pFSH (133 and 100 mg) in ewes submitted to gonadotropin treatment can result in a satisfactory ovulatory follicular response, superovulatory responses and production of viable embryos equivalent or superior to what occurs with the traditionally used dose of 200 mg. the present randomized clinical trial, therefore, was conducted to study the follicular dynamics, follicular wall vascularization, superovulatory response and *in vivo* production of embryos in ewes treated with relatively smaller doses of pFSH as compared with these responses when there is treatment with the standard dose used commercially for induction of superovulatory responses in ewes.

2. Material and methods

This study was conducted at the School of Agricultural and Veterinary Sciences, (FCAV, Unesp), located in the municipality of Jaboticabal/São Paulo/Brazil (21° 15′18″South and 48° 19′ 19″ West), after approval by the Institutional Ethical Animal use committee (protocol nº 12062/14). Pluriparous (n = 24) Santa Inês ewes (2–3 years of age) were used that were not related to each other, and that were non-pregnant, non-lactating with a body weight of 45.17 ± 5.76 kg, and deemed to be healthy after general inspection and reproductive ultrasonic evaluation by research personnel.

The animals were maintained in an enclosed area with easy access to sheds and were exposed to natural light and temperature, with an intensive management system, with *ad libitum* access to mineral salt and water, with corn silage and balanced grain concentrate (200 g/animal/day) offered twice a day.

Estrous synchronization treatments were initiated on a random day of estrous cycle (Day 0) with application of an intravaginal device containing 0.3 g of progesterone (Eazy Breed CIDR® - Controlled Internal Drug Release, Pfizer, New Zealand) until Day 8. On Day 0 and 8, the ewes were administered intramuscularly 125 μg of a synthetic analog of $PGF_2\alpha$ (Cloprostenol Sodium - Sincrocio ®, Ouro Fino, Brazil).

Gonadotropic treatment started 48 h before intravaginal progesterone device withdrawal (Day 6), and at this time ewes were randomly divided into three experimental treatment groups based on the doses of exogenous pFSH treatments (Folltropin®-V, Bioniche Animal Health, Belleville, ON, Canada). The ewes in the G200 group (n = 8) were treated with a dose of 200 mg by intramuscular administration; and in the G133 (n = 8) group with 133 and G100 group (n = 8) with 100 mg at 12 h intervals for 4 days (eight applications) (20%; 20%; 15%; 15%; 10%; 10%; 5% and 5% of pFSH dose). On day 6 the ewes of all groups were also treated with 300 IU of eCG (equine chorionic gonadotrophin, Novormon®, Syntex, Buenos Aires, Argentina). There was validation of the efficacy of the superovulation protocol used in the G200 group in previous studies by the research group conducting the present study (Oliveira, 2011a) and this protocol is based on initiation FSH treatment near the time of follicular wave emergence during the treatment period (Oliveira et al., 2016a).

Ultrasonographic evaluations (color Doppler and B-mode) were performed with an ultrasonic device Mylab Vet 30 (ESAOTE, Italy) connected to a variable frequency (6–8 MHz) linear-array transducer and were performed by one experienced operator. This assessment was performed every 24 h, starting on Day 5 until withdrawal of the intravaginal progesterone device (Day 8) to determine the pattern of follicular growth during the wave of follicular development and area of follicular wall vascularization. Subsequently, an ultrasonic assessment was performed every 8 h for 72 h to ascertain time of ovulation. Ovarian localization videos

and images of the ultrasonic assessments using the B-mode and color Doppler devices were subsequently recorded for off-line evaluation of vascularization areas and follicular dynamic analysis.

With the B-mode assessment of ovarian follicles, there were measurements using electronic calipers and the number, diameter and position of all antral follicles ≥ 2 mm (average of two dimensions: vertical and horizontal) were sketched on ovarian charts, allowing for assessment of the pattern of daily changes in follicular dynamics. Ovulations were defined as the absence of a large follicle(s) recorded at the time of the previous ultrasonographic examination and confirmed by the detection of the corpus luteum on the day of embryo collection. The time of ovulation was defined as having occurred at the half way point of the interval between the time of last visualization of the preovulatory follicle and first time of assessment when it was no longer visible. The dynamics of wave of follicular development that resulted in ovulation was determined as described by Oliveira et al. (2016b): ovulatory diameter (mm); day of follicular wave emergence; maximum diameter (mm); day of maximum diameter; ovulation time (h); growth rate (mm/day); duration of follicle growth (h); reduction of preovulatory follicle size (mm) and preovulatory follicle size reduction rate (mm/day).

After follicular evaluation using the B-mode device, color Doppler assessments occurred by assessing follicular wall vascularization. Briefly, images containing the cross-sectional area of the ovary with the greatest color Doppler signal were analyzed using Image J® (USA) software to calculate total follicle vascularization area, as the sum of the follicle wall vascularization area divided by total ovarian area x 100.

Estrous detection and mating was with rams that were assessed and determined to be fertile. Ewes were re-located to a pen with rams fitted with crayon marking harnesses for 3 days after progesterone device withdrawal (rams to ewe ratio of 1:5). The estrous detection was performed three times a day and it was ensured that each female was mated at least two times during estrus. The estrous onset (hours) was determined in relation to progesterone device withdrawal (Day 8).

At 6 days after estrous onset, the animals were submitted to videolaparoscopy to evaluate ovarian response, quantifying the number of follicles with diameter greater than 5 mm (anovulatory follicles) and the corpora lutea (CL). These data were used to calculate superovulatory response index for each ewe. The ovulation rate was calculated by dividing the number of CL observed using laparoscopy by the sum of the number of CL and preovulatory follicles observed with follicular dynamics assessments x 100. The number of anovulatory follicles was calculated by subtracting the number of preovulatory follicles as determined with follicular dynamic assessments from the number of corpora lutea observed using laparotomy approaches (Oliveira, 2011b). The rate of anovulatory failure was determined by dividing the total number of anovulatory follicles by the number of corpora lutea plus anovulatory follicles x 100. The superovulatory response of the ewes was also classified using three scores (Oliveira et al., 2012): 0 - ewes that did not respond to superovulatory treatment; 1 - ewes having four or fewer ovulations; 2 - ewes that responded to gonadotropin treatment with the number of corpora lutea being between 5 and 10; and 3 - large superovulatory response to gonadotropin treatment with number of corpora lutea equal or greater than 11.

In addition to evaluating the efficacy of the protocols, embryo collections were performed surgically (laparotomy) immediately after video-laparoscopic evaluation (ie., 6 days after onset of estrus). After flushing uterine horns, the recovered liquid was placed in Petri dishes and there was morphological evaluations using a stereomicroscope (20 to 50X). Cleavage was used as evidence for oocyte fertilization. Embryos were classified according to developmental stage. Embryos in the compact morula, initial blastocyst, blastocyst and expanded blastocyst developmental stages were further classified as having quality characteristics using the International Embryo Transfer Society criteria (IETS, 1998) for embryo viability as Grades I to III. Embryos with a delayed development and/or classified as being Grade IV were grouped into a degenerated category.

Viable embryos were also evaluated for the apoptotic cell percentage using the Caspase staining technique, which was performed using an inverted optical epifluorescence microscopy. The number of cells with DNA fragmentation (positive - red fluorescence inside the nucleus) and number of embryonic cells (with blue stained nuclei as a result of staining with Hoechst 33342) were quantified using Image J® software.

Statistical analyses were performed using R® software (R Foundation for Statistical Computing, Vienna, Austria). This experiment was considered a completely randomized design (CRD). Only the data for the variable area of follicular vascularization were normally distributed, consequently this variable was compared between treatments and times using an ANOVA in CRD with time-subdivided plots, when the results (treatment, time or interaction) were significant, the means were compared using the Tukey *posthoc*-test. The other response variables were non-parametric; therefore, there was use of the Kruskal Wallis test to determine if there were differences between treatments. The number of viable embryos recovered was blocked by group based on the presence or absence of a dominant follicle and there was comparisons among treatments using the Friedman test. If significant differences were identified between treatments, the Dunns *posthoc*-test was conducted. The percentages were compared among treatment groups using the Chi-square test and a correlation analysis (Pearson) was performed to ascertain associations between follicle numbers, vascularization area, follicle vascularization area, superovulatory response variables and embryo production. The significance level used for the statistical tests was $P < 0.5$.

3. Results and discussion

Different dosages of pFSH affected the values for variables related to follicular dynamics as evidenced by the effects on time of ovulation relative to CIDR removal, preovulatory follicle size and the decrease in size of the preovulatory follicle s preceding the time of ovulation (Table 1). The ovulation occurred later (hours in relation to the CIDR® withdrawal) in the ewes of G200 group ($P < 0.05$) than in ewes treated with smaller FSH doses (Table 1). Similar findings were reported by, Oliveira et al. (2012) using 256 mg of FSH with great variability among times of ovulation from different follicles in the same ewe with most ovulations occurring 48 h after progesterone device withdrawal. Almeida (2013), using 200 mg of FSH also reported there were ovulations from 70% of the follicles

Table 1
Mean values (± SD) of ovulatory follicular dynamic variables of Santa Inês ewes subjected to superovulation protocols with different doses of exogenous pFSH.

Variables	Treatments			*P* value
	G100	G133	G200	
Day of emergence†	6.27 ± 0.90	6.07 ± 0.79	6.14 ± 1.01	0.339
Maximum Diameter (mm)	5.84 ± 0.85	6.01 ± 0.92	6.06 ± 0.81	0.0523
Day of maximum diameter†	9.37 ± 0.60	9.26 ± 0.52	9.46 ± 0.59	0.252
Ovulatory diameter (mm)	5.71 ± 0.76	5.78 ± 0.84	5.80 ± 0.77	0.617
Ovulation time (h‡)	40.39 ± 12.19[b]	39.61 ± 11.11[b]	45.36 ± 11.46[a]	0.0037*
Growth length (h)	76.57 ± 18.05	79.77 ± 16.68	80.17 ± 19.49	0.262
Growth rate (mm/day)	1.07 ± 0.29	0.99 ± 0.24	1.10 ± 0.34	0.0942
Preovulatory follicle size reduction (mm)	0.13 ± 0.30[b]	0.23 ± 0.39[ab]	0.26 ± 0.38[a]	0.0027*
Preovulatory follicle size reduction rate (mm/day)	0.25 ± 0.59[b]	0.46 ± 0.83[ab]	0.52 ± 0.77[a]	0.0024*

*Significance level at 5%; †Day 0 = CIDR insertion, beginning of the protocol; ‡hours after CIDR® removal; [ab]Lowercase superscript letters on the same line indicate differences between treatments $P < 0.05$.

within 48 h after CIDR® withdrawal. This variable is very important for artificial insemination (AI) because it is important that the timing of AI be different with the different doses of pFSH as a result of the time of ovulation being associated with size of pFSH dose and considering the duration of oocyte and spermatozoa viability in the reproductive tract subsequent to AI.

There were a lesser preovulatory follicle size and greater decrease (i.e., “shrinkage”) in preovulatory follicle size ($P < 0.05$) for ewes in the G200 than G100 group, though the values for these variables were similar in ewes of the G133 and G200 groups. The follicle "shrinkage" phenomenon before ovulation has already been described during the period of changes in follicular dynamics preceding ovulation in sheep (Oliveira et al., 2016b), however, its physiological importance and the mechanisms of action involved are still unknown. Also, it is not known if this “shrinkage” is harmful or not to the oocyte.

The data for follicular dynamic assessments for the emergence of the superstimulated wave of follicular development in the present study indicate the emergence occurred on about day 6 of the treatment protocol (Table 1). It is noteworthy that the basic estrous synchronization protocol used in the present study was developed and assessed previously and the beginning of gonadotropic treatment was considered to occur near the time of ovulatory follicular wave emergence (Oliveira et al., 2016a). The use of the protocol does not have a direct effect on elimination of dominant follicles from the previous wave of follicular development. The majority of animals (16/24) had at least one large follicle (≥ 5 mm) besides the small antral follicle population at the beginning of superovulatory treatment (Day 6), which is not considered ideal, because the presence of the dominant follicle could impair hormonal treatment responses. Control with effective elimination of follicles with a diameter of greater than 5 mm remains a great strategy to be achieved because the presence of these follicles is considered to be associated with a suppressed t response to superovulatory treatment. There are changes in follicular development, such as increased follicular atresia; late recruitment and slow follicular growth and ovulation from smaller follicles, which leads to reduction of oocyte quality and capacity for meiosis resumption during oocyte maturation (Veiga-Lopez et al., 2008). These changes appear to have effects in reducing the number of ovulations and embryo quality and quantity. It is not possible to ascertain if the presence of dominant follicles at the beginning of the treatment with pFSH in the present study had an effect on the superovulatory response and embryo production, however, the methodology used ensured there was the same effect on all experimental groups.

If there are a large number of small antral follicles at the beginning of FSH treatment, this is a positive effect on the superovulatory response. This small antral follicular population is potentially responsive to FSH and these follicles are capable of growing ostensibly to ovulatory sizes (Bartlewski et al., 2016). Although more studies are needed to clarify the relationships between this follicular population and the superovulatory response, this is one of the reasons to start the superovulatory treatment near the time of follicular wave emergence. Menezes (2014) used a superovulatory protocol that was initiated on Day 0 of a pre-synchronized estrous cycle and found that the number of follicles at the beginning of superovulatory treatment did not differ for the varying pFSH doses evaluated (200 mg, 133 mg and 80 mg), and percentage of follicles smaller than 4 mm in diameter was greater than 85% of all follicles that were present at the beginning of pFSH treatments for all experimental animals. This allowed for this synchronization protocol to be effective for enhancing the number of follicles potentially responsive to exogenous pFSH, aiming for satisfactory superovulatory and embryonic outcomes. Consistent with these previous results, in the present study most of the ewes also had a large number of small follicles (mean value of 11 follicles < 4 mm, per ewe) at the beginning of superovulatory treatment period. Although many ewes also had dominant follicles, the ovarian status among ewes was similar for conducting the present study with the different doses of pFSH.

Data for ovarian responses in the ewes of the present study are summarized in Table 2. The ovulation rate and the percentage of ovulatory follicles differed among groups ($P < 0.05$), with there being a greater ovulation rate and lesser percentage of ovulatory failures/anovulatory follicles in the G100 compared to G133 and G200 groups. These results provide evidence that the use of 133 mg of pFSH should not be recommended for superovulatory protocols, while the other two treatments (100 mg and 200 mg) resulted in similar responses. It, therefore, is possible to confirm that the gonadotropin dose is a factor that affects the ovarian responses, although there are innumerable other factors, associated or not, that can also affect the follicular response to pFSH treatments. The possibility of using the smaller dose of pFSH (100 mg) for sheep superovulation allows for a reduction of cost for hormonal preparations and may favorably affect use of the MOET biotechnology in sheep.

Table 2
Ovarian responses of Santa Inês ewes submitted to superovulation protocols with different pFSH doses (mean ± SD).

Variables	Treatments			*P* value
	G100	G133	G200	
Number of ovulations	13.50 ± 5.71	9.00 ± 3.66	14.88 ± 6.94	0.1226
Ovulation rate (%)	96.85 ± 3.53[a]	86.30 ± 21.75[b]	93.74 ± 6.85[ab]	0.016[*]
Number of anovulatory follicles	0.50 ± 0.53	1.25 ± 1.75	1.12 ± 1.13	0.5522
Anovulatory Follicles(%)	3.13 ± 3.51[b]	13.70 ± 21.75[a]	6.26 ± 6.85[ab]	0.016[*]

[*]5% Significance level; [ab]Superscript letters in the same line indicate a difference in percentage values $P < 0.05$.

The use of FSH for sheep superovulation is associated with greater ovulation rates and a lesser incidence of anovulatory follicles with eCG treatments (Armstrong and Evans, 1983), but the large amount of variability in superovulatory responses also occurs with the FSH treatment (Bartlewski et al., 2016). In the present study, there was a large and problematic variation in the number of corpora lutea (Table 2). The different pFSH doses used induced satisfactory superovulatory responses (95.83% of the animals, 23/24), considering that 20%–30% of ewes do not have or have a lesser superovulatory response to superstimulation of follicular development to induce multiple ovulations and for conducting embryo transfer programs (Brebion et al., 1992; González-Bulnes et al., 2000). Only one animal (G133) in the present study did not respond to superstimulation with multiple ovulations with this ewe having only three ovulations. It is emphasized that superovulatory response was considered to have occurred when there were at least five corpora lutea, because there can be 1–4 ovulations of follicles during typical estrous cycles when there is not superstimulation of follicular development (Deshpande et al., 1999; Evans, 2003).Consistent with results of the present study, Loiola Filho et al. (2015) evaluated two doses of pFSH (200 and 128 mg) and there were satisfactory superovulatory responses of 90% (128 mg) and 95% (200 mg) while in another study (Oliveira et al., 2012) using a dose of 256 mg, there was a 100% superovulatory response. Besides the ovarian structural status at the beginning of the gonadotropin treatment, factors such as genetics, breed, season of the year, nutrition, mating method, type of gonadotropin treatment used for follicular superstimulation (gonadotropin, commercial product, manufacturing lot, FSH:LH ratio of the preparation and application protocol) affect superovulatory response; (Bari et al., 2000; González-Bulnes et al., 2004; Quan et al., 2011; Gusmão et al., 2013).

In the present study, the follicular wall vascularization area was similar for all treatment groups and varied only in relation to time subsequent to initiation of gonadotropin treatments, increasing gradually, with a peak between 72 and 88 h, and later decreasing ($P < 0.001$). Furthermore, there was no correlation between the vascularization area and superovulatory responses and embryo yield ($P > 0.05$). Unlike responses in humans and cows, there is not a response is ewes in the present study where amount of blood flow was associated with greater ovarian responses in the way that has been previously reported (Van Blerkom et al., 1997; Van Blerkom, 1998; Clark and Stokes, 2011) as well as for oocyte and embryonic quality (Siddiqui et al., 2009). Follicular blood flow and intrafollicular oxygen content appear to be important determinants of oocyte competence in some species (Van Blerkom, 1998). Oliveira et al. (2014a,b,Oliveira et al., 2014c reported there was a positive correlation between follicular blood flow (at the time of the last two pFSH of a total of eight treatments) and the number and percentage of unfertilized oocytes in ewes treated for superovulation with 200 mg of pFSH for relatively short- and long-term periods. It is suggested that other studies need to conducted to enhance understanding of the vascular mechanisms in relation to the superovulatory responses in ewes, because the results from previous studies are not very conclusive.

The follicular vascularization area increased ($P < 0.05$) near the time of ovulation (72–88 hours after pFSH treatment) indicating blood flow in the follicular wall may be related to preovulatory development and the ovulatory process. In cows, Acosta et al. (2003) observed that there were complex structural, secretory and functional changes in the ovary before ovulation that were closely associated with increased blood flow in the preovulatory follicle wall. In bitches, increased blood flow and velocity of flow are associated with the ovulatory process, and there are no apparent differences in the values for these two variables when there is spontaneous and induced ovulation (Barbosa et al., 2013). In these studies, there was an increase in follicular vascularization immediately before ovulation consistent with findings in the present study.

All females in the present study had behavioral estrous symptoms after the CIDR withdrawal indicating the estrous synchronization protocol was effective. The variable "onset of estrus" did not differ between experimental groups in the present study ($P > 0.05$; Table 3).

The *in vivo* embryo production was affected by the exogenous pFSH doses in the present study. Although the number and percentage of ovarian structures were similar between the experimental groups, there were differences for the number and percentage of

Table 3
Estrous behavior of Santa Inês sheep submitted to superovulation protocols with different FSH doses (mean values ± SD).

Variables	Treatments			*P* value
	G100	G133	G200	
Onset of estrus (h†)	21.90 ± 7.63	17.49 ± 9.0	23.63 ± 5.97	0.0655

[*]5% significance level; [ab]Lowercase letters with these superscripts on the same line differ indicate a difference $P < 0.05$.

Table 4
Unfertilized oocytes and embryos produced by Santa Inês ewes submitted to superovulation protocols with different pFSH doses (mean ± SD).

Variables	Treatments			*P* values
	G100	G133	G200	
Number of recovered structures	6.0 ± 3.7	4.13 ± 4.02	6.25 ± 4.33	0.069
Number of unfertilized structures	2.12 ± 2.8[a]	1.0 ± 2.45[ab]	0.88 ± 1.73[b]	0.008*
Number of viable embryos	2.63 ± 2.92[ab]	1.5 ± 2.51[b]	3.88 ± 3.48[a]	0.038*
Number of degenerated embryos (Grade IV)	1.25 ± 1.83	1.63 ± 2.07	1.5 ± 1.31	0.089
Recovery rate (%)	48.29 ± 27.04	48.1 ± 40.8	48 ± 36.5	0.864
Unfertilized structures rate (%)	39.3 ± 43.2[a]	20.0 ± 38.1[ab]	12.72 ± 21.61[b]	0.0477*
Viability rate (%)	42.3 ± 34.4[ab]	35.8 ± 33.3[b]	62.0 ± 28.8[a]	0.0493*
Degenerated embryos (%)	18.41 ± 20.12	44.2 ± 34.1	25.32 ± 13.65	0.154
Apoptotic embryonic cells (%)	5.00 ± 1.96[a]	3.24 ± 2.29[b]	3.54 ± 2.33[b]	0.012*

*5% Significance level; [ab]Superscript lowercase letters on the same line indicate differences $P < 0.05$.

unfertilized oocytes and viable embryos, and apoptotic embryonic cell percentage (Table 4). The number and percentage of unfertilized oocytes were greater in the G100 than G200 group ($P < 0.05$), while in the G133 group there were similar responses as in the G200 group. Unexpectedly, with the largest dose (200 mg) there was a greater fertilization outcome than with the smaller dose (100 mg of pFSH). It was suggested based on results in a previous study that the larger doses of pFSH may induce a prolonged stimulus of anovulatory follicles which continue to produce estrogen. This hormonal milieu may interfere negatively with development of a uterine environment, that is facilitative for oocyte transport through the fimbriae and sperm transport to the site of fertilization in the oviduct (Chagas e Silva et al., 2003), thus, impairing oocyte fertilization. Loiola Filho et al. (2015) reported that there was a lesser fertility in animals submitted to a superovulation protocol of 200 mg (number of unfertilized oocytes 3.5 ± 0.7 and percentage of unfertilized oocytes 37.6%) as compared to ewes treated with the smaller dose of 128 mg (number of unfertilized oocytes 1.1 ± 0.7 and percentage of unfertilized structures 16.4%). Gibbons et al. (2010) also reported that with smaller doses of exogenous pFSH there were fewer unfertilized oocytes (with 80 mg 9.7 ± 4.3%; and with 200 mg 25.1 ± 8.4%). The difference in the findings in the present study as compared with these in previous studies probably occurred due to other factors besides the FSH doses implying a need for additional studies to be conducted that elucidate the factors that contribute to these inconsistencies in results among studies.

The number and rate of viable embryo production were greater in the G200 compared to G133 ($P < 0.05$) and G100 ($P > 0.05$) group in the present study. These results indicate once again that the pFSH dose of 133 mg should not be recommended when there are conditions similar to those in the present study where with the smaller doses (100 mg) there was a similar response for percentage of viable embryos when compared to the response with larger doses. Loiola Filho et al. (2015) reported that number of viable embryos did not differ with administration of doses of 200 mg (4.4 ± 0.7) or 128 mg (5.1 ± 0.7), however, there was a difference in percentage of viable embryos (44.6% and 71.8% respectively) in Dorper ewes. Menezes (2014) evaluated different doses of pFSH (200, 133, and 80 mg) and reported there was no difference for number of viable embryos (1.54 ± 2.63; 3.86 ± 3.98; 0.33 ± 0.58, respectively) in Santa Inês ewes.

The percentage of the apoptotic cells in the embryos produced in ewes of the G100 group was greater as compared with that of ewes in the other groups, indicating that the smaller dose may have impaired the oocyte and, consequently, embryonic quality. Oliveira et al. (2014b) reported that there was 3.1 ± 1.6% apoptotic cells in embryos produced in ewes treated with 200 mg of pFSH; a value similar to that in the G200 group of the present study. The quantification of the apoptotic cells is indicative of embryonic quality, however, does not ensure the survival of the transferred embryo.

4. Conclusion

With treatment with the 100 mg dose of pFSH, there was a similar efficacy as with treatment with the 200 mg dose that is generally used for superovulatory treatments of ewes. The ovulation response and percentage of viable embryo production is associated with an increased impairment of embryonic quality (relatively greater percentage of apoptotic embryonic cells). The ovulation time relative to time of initiation of gonadotropin treatments is affected by the smaller pFSH dosage.

There continued to be a large variability of superovulatory response in superovulated females with the smaller doses of pFSH and is still a common and limiting factor for MOET in sheep. It would be interesting and justified to gain a greater understanding of the detrimental effects of the smaller doses of pFSH (100 mg) on oocyte fertilization and apoptosis of embryonic cells.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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