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Author contribution Statement

MG: designed and performed the study and wrote the manuscript

CB: collaborated in the study design, performed the hormone assays and revised the manuscript

EZ: collaborated in the experiment 'in the field' and revised the manuscript.

MB: collaborated in the experiment 'in the field' and revised the manuscript

MA: critically revised the manuscript

DN: critically revised the manuscript

MM: collaborated in data analysis and critically revised the manuscript

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Abstract

A high embryo loss rate has been reported in llamas. As strategies that lead to an increase in plasma progesterone (P4) concentration might improve fertility, the aim of the present study was to evaluate if the administration of hCG on Day 7 post-mating is useful to develop an accessory corpus luteum (CL), increasing plasma P4 concentration. Twenty (n= 20) female llamas, ranging between 5 and 10 years of age and four (n= 4) males of proven fertility, ranging between 8 and 10 years of age were included in the study. Accessory CL developed in all treated llamas after hCG administration and plasma P4 concentration was significantly greater in treated than in control females (P<0.0001). The diameter and vascularization of the original CL were not affected by treatment in pregnant llamas. However, in treated non-pregnant llamas, corpus luteum diameter was greater than in the control group from Day 14 post-mating until

the end of the study (P<0.001). In treated llamas, the accessory CL was detected throughout the study in pregnant and non-pregnant females, but its vascularization started to decrease around Day 16 post-mating in non-pregnant animals (P<0.05). In conclusion, hCG treatment on Day 7 post mating was useful to induce an accessory CL and increase plasma P4 concentration in llamas. Thus, this treatment could be considered as a useful strategy to improve pregnancy rates in llama herds.

Keywords: hCG, progesterone, corpus luteum, pregnancy, llama.

1. Introduction

Llamas are induced ovulators requiring a stimulus, in presence of a mature follicle (≥7mm), to trigger the ovulatory process [1]. Mating is one of the stimuli to induce ovulation, leading to the development of a corpus luteum (CL). In non-pregnant llamas, CL formation is associated with an increase in plasma progesterone (P4) concentration, which starts to rise around Day 4 post-mating, peaks at Day 8 and starts to decrease on Day 9; reaching basal concentrations between days 10-12 post-mating [2,3]. In mated pregnant llamas, CL´s lifespan is prolonged and plasma P4 concentration remains elevated beyond Day 12 post-mating [2, 4]. However, it has been reported that in pregnant llamas a transient decrease in plasma P4 concentration occurs between days 8 and 18 post mating, which is coincident with a pulsatile release of prostaglandin F2 Alpha (PGF2α) [2,3] and a transient decrease in the CL´s vascularization [5]. These transitory changes that occur during early pregnancy could be related to the high incidence of embryo mortality registered in this species [6, 7].

In cattle, low plasma P4 concentration during early pregnancy might lead to embryo loss, while a high concentration of this hormone is associated with greater pregnancy rates [8, 9, 10]. Therefore, the development of strategies that lead to an increase in plasma P4 concentration would improve fertility [11].

The human chorionic gonadotrophin (hCG) is a hormone that shows a potent LH-like effect and thus exerts a luteotrophic action. In cattle, hCG administration between days 5 and 7 post-estrus or post-artificial insemination induced the ovulation of the mature follicle present at that time, leading to an accessory CL development and an increase in plasma P4 concentration [12, 13, 14, 15]. Moreover, some authors have reported an improvement in pregnancy rates after this treatment [15, 16, 17]. Similarly, in sheep, the administration of hCG some days post-mating induced the development of an accessory CL accompanied by an increase in plasma P4 concentration [18, 19, 20]. In llamas, the development of an accessory CL has been achieved by the administration of a GnRH analog on day 7 post-mating [21]. However, these authors have not found an increase in plasma P4 concentration on Day 14 post-mating nor an improvement in pregnancy rates. To the author´s knowledge, there are no reports on the use of hCG to induce an accessory CL in llamas. Therefore, the aim of this study was to evaluate if the administration of hCG on Day 7 post-mating is useful to induce the ovulation of the largest follicle present at that moment leading to the development of an accessory CL and an increase in plasma P4 concentration.

2. Materials and Methods

2.1. Population under study

This study was approved by the ethics committee of the School of Veterinary Sciences of Buenos Aires University (CICUAL Nº 2020/06). The experimental procedures were performed from August to November at Buenos Aires University, Argentina (34°36′ S, 58°22′ W, at sea level). Twenty (n= 20) non-pregnant, non-lactating female llamas, ranging between 5 and 10 years of age and with an average body weight of 110 ± 15 kg and four (n= 4) males of proven fertility, ranging between 8 and 10 years of age and with an average body weight of 136 ± 14 kg were included in the study.

All animals were sexually mature and clinically and reproductively healthy. Llamas are routinely subjected to clinical and reproductive evaluations, including blood tests and a general and particular examination, to ascertain that they continue in good health. Llamas were in good nutritional status with a mean body condition score of 3 (body condition score from 1 = thin to 5 = obese) [22] and were fed bales of hay and water ad libitum.

2.2. Experimental design

The experimental design is described in Figure 1. Llamas were examined daily by rectal palpation and B-mode transrectal ultrasonography to asses ovarian status (MyLab 30 Vet Gold ESAOTE Pie medical attached to a 5.0 MHz lineal-array electronic transducer) until a growing follicle with a diameter ≥7 mm, considered ovulatory in this species [23], was observed. At that moment, females were mated with a male (Day 0). Occurrence of ovulation was assessed on Day 2, based on ultrasonographic visualization of the disappearance of the dominant follicle and further confirmed by plasma P4 concentration on the following days. On Day 7 after mating, llamas were randomly divided into two groups: Treated group (n=11) received an intramuscular injection of 750 IU of human chorionic gonadotrophin (Ovusyn, Zoetis, Argentina) and Control group (n= 9) received an intramuscular injection of 1.5 ml of saline solution.

All females were examined by transrectal doppler ultrasonography, every other day from Day 6 until Day 18 post-mating; in order to assess the ovulation of the largest follicle after treatment, the development of an accessory CL and the diameter and vascularization of both CLs (the original CL and the accessory CL). The vascularization of both CLs was evaluated as previously described [5, 24, 25]. Briefly, the transducer was placed over the ovary and three seconds video-clips of the CL vascularization were registered and downloaded. The video-clips were examined frame by frame to select six images that showed the maximum vascular signal over the maximum cross-sectional area of the CL (Adobe Premiere Pro CS6). Images were saved in TIFF format and analysed by ImageJ 1.52a software (National Institute of Health, USA). The degree of vascularization was estimated by measuring the area of the vascular flow signals (Doppler mode) over the CL area (B-mode) [5, 26, 27]. Thus, percent area of vascularization was calculated by the following equation: percent area of vascularization = (CL vascular area/ CL total area) × 100. The average of the six selected images was considered the final value for each animal [28].

Additionally, blood samples were collected by jugular venipuncture for the evaluation of plasma P4 concentration. These samples were collected before hCG administration (Day 6) and after hCG administration (daily, from Day 8 until Day 12 and then, every other day, until Day 18 post-mating). Blood samples were collected in tubes with heparin and centrifuged immediately after collection. Plasma was stored at −20 °C until the assay was performed.

Plasma P4 concentration > 1 ng/ml on Day 12 post-mating was considered indicative of pregnancy only in the control group [29], as hCG could affect this hormone's concentration. Pregnancy was further confirmed by visualization of the embryonic vesicle from Day 15 until the end of the study in both groups [5, 30, 31].

2.3. Progesterone determinations

Plasma P4 concentration was measured using a RIA kit (IM 1188, Beckman Coulter, Immunotech, Czech Republic) previously validated for its use with llama plasma [32]. All samples were measured in duplicates. The sensitivity of the assay was 0.15 ng/mL and the intra-assay coefficient of variation was less than 4% for concentrations between 0.15 and 0.55 ng/mL.

2.4. Statistical analysis

Plasma P4 concentration and CLs diameter and vascularization along the luteal phase and between groups (treatment vs. control) were analysed by repeated measures one-way ANOVA or one-way ANOVA, respectively; followed by Bonferroni test. Fisher´s exact test was used to compare pregnancy rates between groups (Graph Pad 5, USA). Values are expressed as mean ± SEM. Differences were considered significant when *P* values were < 0.05.

3. Results

3.1. Ovulation, CLs development, and pregnancy rates

Occurrence of ovulation and CL development after mating was registered in all animals (100%).

Mean diameter of the largest follicle at Day 6 post-mating was 0.65±0.02 cm (range 0.55-0.75 cm) and 0.69±0.02 cm (range 0.6-0.76 cm), in the treated and control group, respectively. In the treated group, after hCG administration, the largest follicle reached a mean diameter of 0.82±0.02 cm on Day 8. Afterwards, ovulation was confirmed on Day 10 post-mating, based on the disappearance of the mentioned follicle. All females developed one accessory CL, except for one llama that developed two. Accessory CLs were first visualized on Day 12 post-mating. In only three llamas accessory CL developed in the contralateral ovary to the original CL, while in the others it developed in the same ovary (Fig 2) (in the llama that developed 2 accessory CLs, they were visualized one in each ovary).

Pregnancy was diagnosed in 8/11 llamas (73%) of the treated group and in 6/9 (67%) llamas of the control group. No significant differences were found in pregnancy rates between treatment and control groups.

3.2. Plasma P4 concentration

Plasma P4 concentration showed significant differences between treated and control groups ($P<0.0001$). In treated pregnant llamas, P4 concentration was greater on Days 12, 14, 16 and 18 ($P<0.001$) than in control pregnant llamas; while in treated non-pregnant females, plasma progesterone concentration was greater on Days 12, 14 and 16 ($P<0.05$) than in control non-pregnant animals (Fig. 3 a,b).

Plasma P4 concentration showed significant differences between pregnant and non-pregnant llamas of the treated group ($P<0.0001$). In pregnant llamas, plasma P4 concentration increased towards Day 12 and was maintained until the end of the study (P<0.0001). In non-pregnant llamas, plasma P4 concentration started to rise towards Day 8 and decreased around Day 10 and 11. Then it started to rise again towards Day 14 and decreased at the end of the study (Day 18) (P<0.05) (Fig. 3a,b).

3.3. Diameter and vascularization of the original and accessory CL

The diameter of the CL did not show significant differences between the treated and the control groups in pregnant llamas (Fig 4a). However, in non-pregnant llamas, CL diameter was greater in treated than in control females on days 14, 16 and 18 (P<0.001) (Fig. 4b). In the control group, the CL diameter of non-pregnant llamas started to significantly decrease from Day 12 in all animals. In this group, CL could no longer be detected on Day 18 post-mating, but it was still visualized in non-pregnant treated llamas.

In the treated group, original CL diameter increased from Day 6 towards Day 10 post mating (P<0.05) and then the diameter was maintained during the study in both pregnant and non-pregnant llamas, without significant differences among the studied days (Fig. 4a,b).

The development of an accessory CL was only registered in treated animals. In pregnant llamas, accessory CL diameter increased from Day 12 to Day 14 post-mating and remained unchanged until the end of the study (P<0.05) while in the non-pregnant llamas, the accessory CL diameter did not show significant differences among the different days (*P*= 0.57) (Table 1).

No significant differences were observed in the vascularization of the original CL of pregnant llamas between treated and control groups (Fig. 5a). On the contrary, CL vascularization in non-pregnant llamas was significantly different between both groups ($P<0.0001$). In control non-pregnant animals, CL vascularization started to decrease after Day 8 post-mating and Doppler signal was not observed after Day 14. However, in treated non-pregnant llamas, CL vascularization was observed until the end of the study, with this variable being significantly greater than in the control group on Days 14, 16 and 18 ($P<0.05$) (Fig. 5b).

In the treated group, vascularization of the original CL did not show significant differences over time neither in pregnant nor in non-pregnant llamas.

Accessory CL vascularization in pregnant llamas did not show significant differences over the study, but differences were observed in the non-pregnant group ($P= 0.003$). In this group, CL vascularization was significantly greater on Days 12 and 14 than on Days 16 and 18 ($P<0.05$) (Table 1).

4. Discussion

To our knowledge, this is the first study to demonstrate that the injection of hCG on Day 7 after natural mating is capable of inducing the ovulation of the largest follicle present at that time, the development of an accessory CL and an increase plasma P4 concentration in llamas. Similarly, in different species, hCG administration during early luteal phase is capable of developing an accessory CL, leading to an increase in plasma P4 concentration [goat: 33, cattle: 15, sheep: 20, 34]). Thus, this treatment could be considered a useful strategy to improve fertility because the increase in plasma P4 concentration in early pregnancy is proposed to play a major role in reducing embryo mortality [10].

In the present study, the injection of hCG on day 7 post-mating induced ovulation and the development of an accessory CL in 100% of the treated animals. In pregnant llamas of the treated group, plasma P4 concentration increased progressively, to reach its maximum concentration on Day 14 post mating and then was maintained above 12 ng/ml until the end of the study. Interestingly, P4 reached this peak around the time when the accessory CL is expected to produce the highest P4 concentration (Day 7 after accessory CL induction). Plasma P4 concentration in pregnant llamas of the control group was similar to that previously reported [2, 5]; being lower than in the treated group. These results indicate that hCG administration is useful for increasing plasma P4 during early pregnancy. Similar results were observed in cows treated with hCG on Day 7 after mating, which showed significantly higher plasma P4 concentration than the non-treated animals [35].

In non-pregnant llamas of the treated group, plasma P4 concentration showed a two-peaks profile. This pattern might reflect the alternating function of both CLs (original and accessory). First, plasma progesterone produced by the original CL, increased towards Day 8 and in absence of an embryo, decreased between Days 11 and 12 post-mating; similarly to that previously reported in other studies in this species [2,36]. Thereafter, the accessory CL commanded progesterone production, which reached its maximum concentration around Day 14 post-mating (Day 7 after its induction) and then decreased to reach basal concentrations around Day 18 post-mating, i.e. 11 days after hCG injection. On the contrary, in non-pregnant llamas of the control group, plasma P4 reached basal concentrations around Days 11-12 post mating, as described in previous reports [2,5].

The P4 profiles observed in both pregnant and non-pregnant treated females may suggest that hCG did not exert a luteotrophic effect on the original CL, since plasma P4 concentration was greater only once the accessory CL was detected. Thus, it could be assumed that the increase in plasma P4 concentration after Day 12 is a consequence of the development of an accessory CL and its P4 secretion. These results are in agreement with the study of Fonseca et al. [33] who reported an increase in plasma P4 concentration in goats 8 days after hCG treatment, in coincidence with the period of maximum P4 production by the accessory CL. On the contrary, in cattle and sheep a luteotrophic effect of hCG on the original CL has been described, since P4 concentrations increased also before accessory CL had developed [sheep: 20, 37; cattle:15]. The presence or lack of a luteotrophic effect of hCG on the original CL might be related to species-specific characteristics that remain to be studied.

A previous study performed in llamas has reported that the injection of a GnRH analogue on Day 7 after natural mating was useful to induce an accessory CL formation [21]. However, plasma P4 concentration, which was evaluated only on Days 7 and 14 post-mating, did not show significant differences between treated and control groups. Differences between this study and the present one might be related to the protocol used, since hCG shows a more long-lasting effect than LH [17, 38] and leads to a greater increase of plasma P4 than GnRH analogs [17].

The diameter and vascularization of the original CL could be determined throughout the experimental period in all treated llamas (pregnant and non-pregnant). The hCG administration did not modify any of these variables in pregnant animals. This observation reinforces the idea that hCG is not exerting a luteotrophic effect on the original CL. In contrast, the vascularization and diameter of the original CL in non-pregnant treated llamas were significantly greater than in the control group; and remained unchanged until the end of the study. However, non-pregnant treated llamas showed a decrease in plasma P4 concentration around Days 10-11 post-mating. Thus, it is probable that in this group the original CL maintained its morphology but not its function; maybe due to a hemodynamic effect related to the development of the accessory CL (it must be considered that 2/3 non-pregnant llamas showed both original and accessory CL in the same ovary) (data not shown). In sheep, CL diameter and vascularization were greater in hCG treated females compared to the control group, both in pregnant and non-pregnant animals, suggesting a luteotrophic effect on the original CL [34]. On the contrary, no significant differences were found in CL diameter between hCG treated and control cows, although a differentiation between pregnant and non-pregnant animals was not included in the experimental design of this study [15].

The accessory CL was first observed on Day 12 post mating (Day 5 after induction) and its diameter and vascularization could be determined until the end of the study in all treated females. In pregnant llamas, these variables remained relatively constant during the studied period, suggesting that the CL remained active. In non-pregnant llamas, accessory CL vascularization started to decrease around Day 16 post-mating in coincidence with plasma P4 concentration decline; suggesting that the CL was losing its function.

In the present study, although hCG treatment increased plasma P4 concentration it did not improve pregnancy rates. It could be possible that the improvement in pregnancy rates was not observed because the number of animals included in the study was not sufficient to demonstrate this effect. The fact that hCG lead to an increase in plasma P4 concentration suggests that this treatment would be a useful strategy to improve pregnancy rates in this species. This treatment could possibly show even better results in animals that live in extreme conditions of the Andean steppes by increasing the low pregnancy rates registered in camelids after mating in their natural environment, where a greater embryo loss was has been reported between Day 12 and 27 post mating [39]. In addition, this treatment could be useful after embryo transfer, since pregnancy rates in recipient females are around 50% [1, 40]. In fact, in cows and mares, treatment with hCG in embryo recipients at the time of transfer, increased plasma P4 concentration and pregnancy rates [41, 42, 43]. Further studies in a greater number of animals or "in-field" conditions are needed to elucidate this. In other species, the effect of hCG treatment on pregnancy rates has shown variable results [17, 44]. Some of them have referred development of an accessory CL and improvement in plasma P4 and pregnancy rates [cattle: 13, 16; sheep: 34], while others have not reported an improvement in fertility [cattle: 15, 45; goats: 33; sheep: 20, 46]. However, it must be considered that reports that have evidenced fertility improvement are large-scale studies and that the referred increment in pregnancy rates was around 5-10% [13, 47, 48].

5. Conclusion

In conclusion, hCG treatment on Day 7 post mating was useful to induce development of an accessory CL and to increase plasma P4 concentration during early pregnancy in llamas, indicating that this treatment might be considered as a possible strategy to increase pregnancy rates in this species.

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Author contribution Statement

MG: designed and performed the study and wrote the manuscript

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Conflict of interest

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

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Figure Legends

Fig 1. Experimental protocol. U: ultrasonography, BC: Blood collection, hCG: administration of hCG to the treatment group. Day 0 is the day of mating, while the others refer to days "post-mating".

Fig. 2. Original (right) and accessory (left) CL at 14 days post-mating in in the same ovary of one llama. Luteal vascularization can be observed in the periphery of both CLs. Depth scale can be observed at the right side. Distance between lines is 1 centimeter. Color scale bar can be observed at the left side.

Fig. 3. Plasma progesterone concentration in pregnant (a) and non-pregnant (b) llamas of the treatment and control groups. * Indicates significant differences between treatment and control groups.

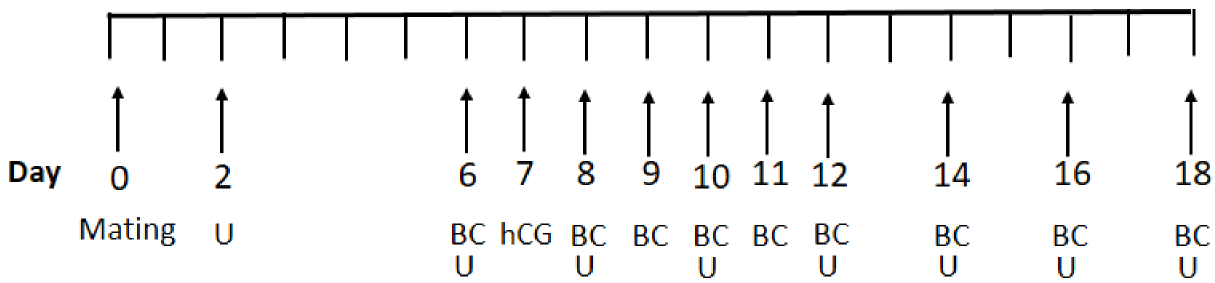
Fig. 4. Original CL diameter in pregnant (a) and non-pregnant (b) llamas of the treatment and control groups. * Indicates significant differences between treatment and control groups.

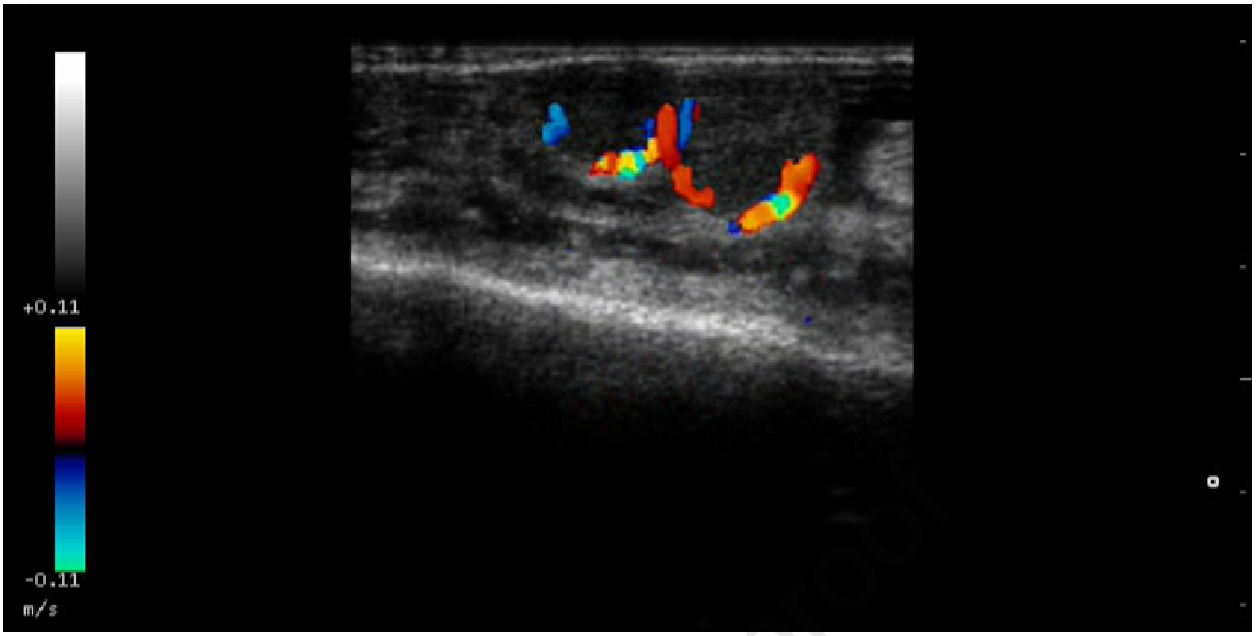
Fig. 5. Luteal vascularization area (%) in pregnant (a) and non-pregnant (b) llamas of the treatment and control groups. * Indicates significant differences between treatment and control groups.

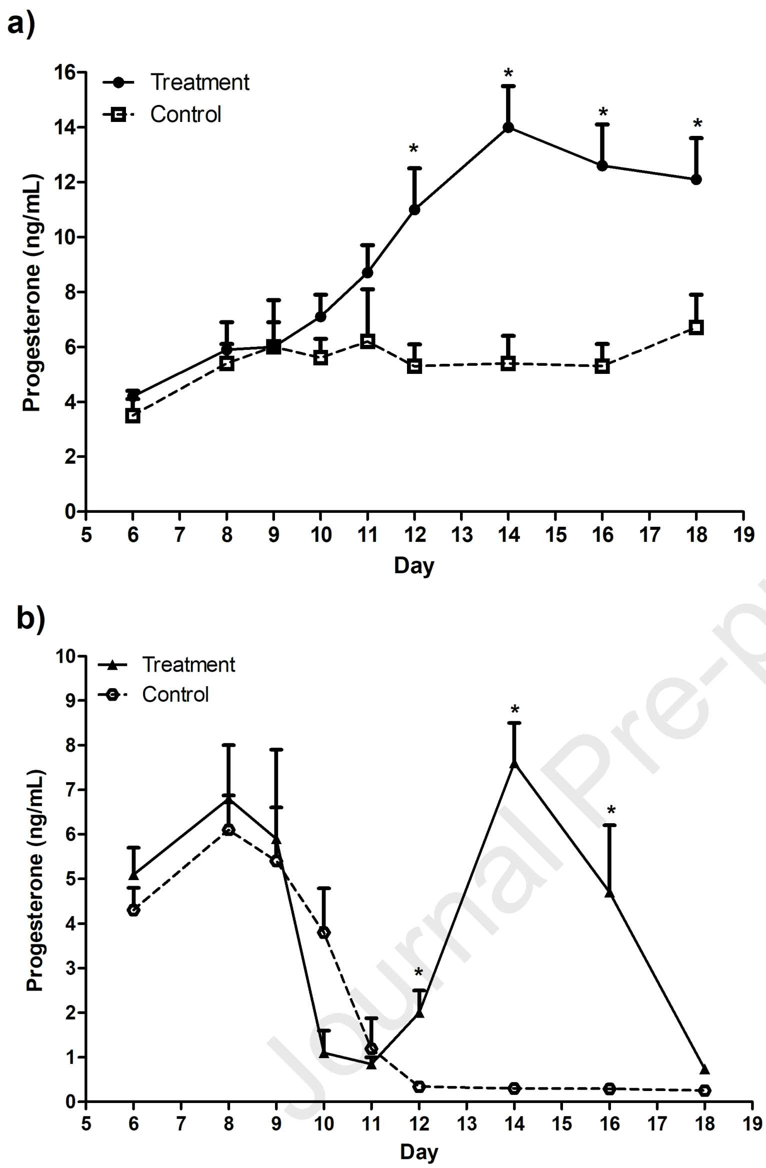
Table 1. Accessory CL diameter and luteal vascularization area in pregnant and non-pregnant llamas of the treatment group.

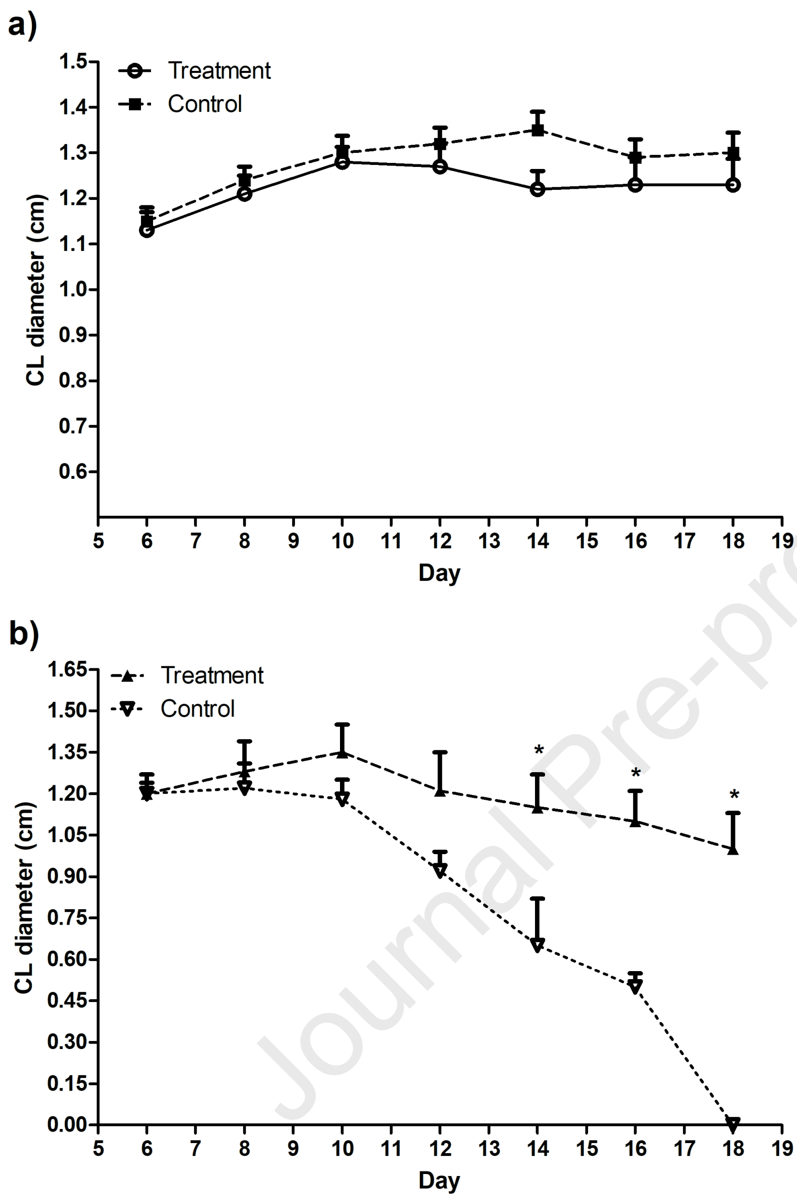
	Pregnant		Non-Pregnant	
Day	**Diameter (cm)**	**Vascularization (%)**	**Diameter (cm)**	**Vascularization (%)**
12	0.95±0.05[a]	0.18±0.02[a]	0.84±0.06[a]	0.17±0.04[a]
14	1.14±0.05[b]	0.20±0.02[a]	1.00±0.15[a]	0.16±0.03[a]
16	1.19±0.04[b]	0.19±0.01[a]	0.99±0.14[a]	0.09±0.01[b]
18	1.15±0.06[b]	0.24±0.01[a]	0.85±0.07[a]	0.10±0.03[b]

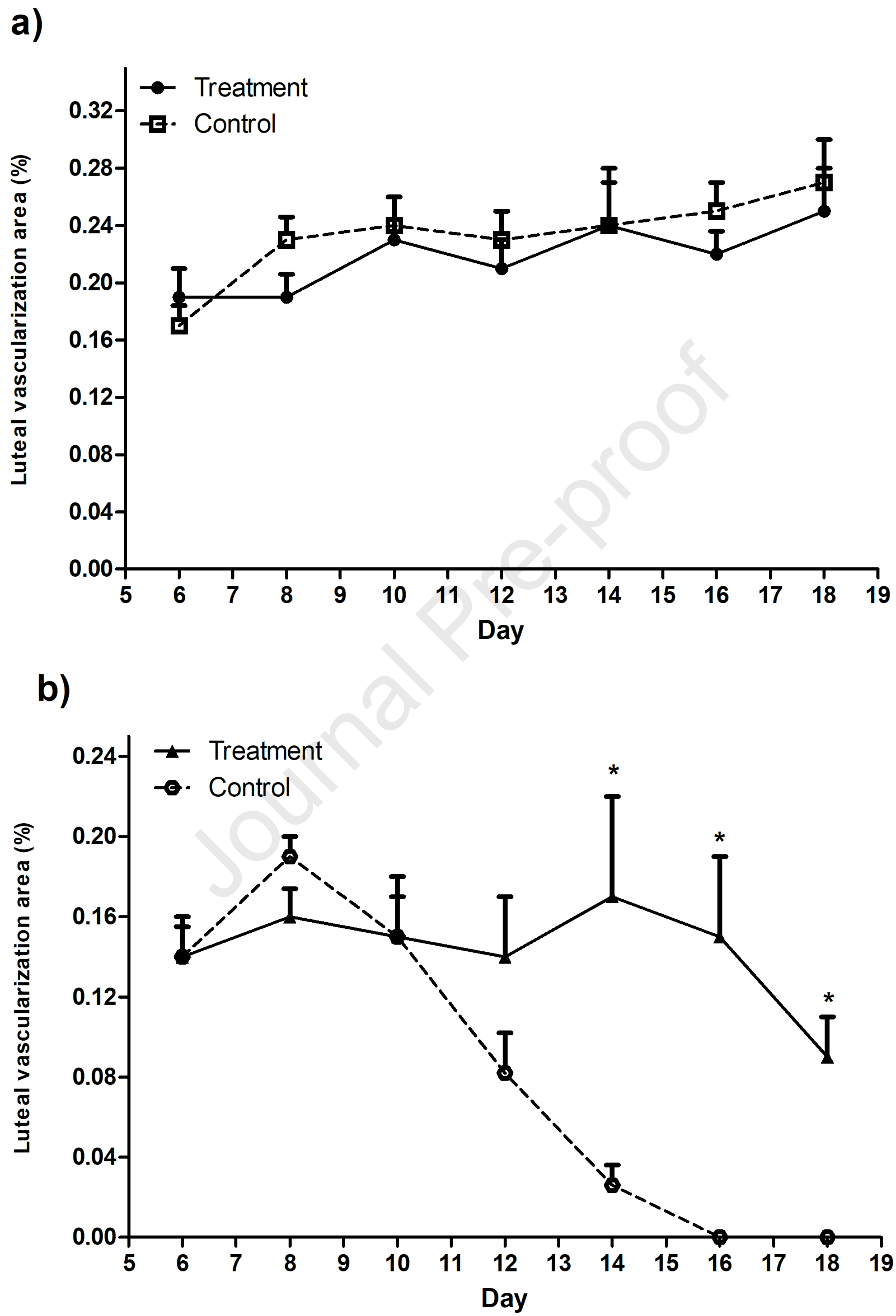
[a,b]Different letters within the same column indicate significant differences between days.


Day
0
Mating
2
U
6
BC
U
7
hCG
8
BC
U
9
BC
10
BC
U
11
BC
12
BC
U
14
BC
U
16
BC
U
18
BC
U


+0.11
-0.11
m/s


a)
Treatment
Control
Progesterone (ng/mL)
Day
b)
Treatment
Control
Progesterone (ng/mL)
Day


a)
Treatment
Control
CL diameter (cm)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day
b)
Treatment
Control
CL diameter (cm)
1.65
1.50
1.35
1.20
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0.00
*
*
*
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Day


a)
Treatment
Control
Luteal vascularization area (%)
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Day
b)
Treatment
Control
Luteal vascularization area (%)
0.24
0.20
0.16
0.12
0.08
0.04
0.00
*
*
*
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Day

Highlights

hCG treatment allowed the development of an accessory corpus luteum in llamas

Progesterone concentration increased after hCG treatment in llamas

A luteotrophic effect after hCG treatment was not observed in llamas

Accessory corpus luteum remained active during all the studied period