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Uterus histomorphometry of alpacas (*Vicugna pacos*) in induced luteal phase, with GnRH or copulation

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ABSTRACT

Background: Alpacas have reproductive traits such as induced ovulation and a higher gestation rate in the left uterine horn (LUH). Additionally, low fertility and high embryonic mortality are significant challenges in alpaca breeding. To address these issues, it is essential to study the histological changes occurring in the uterine wall during day 8 of the luteal phase (LP).

Aim: This research aims to describe these changes in alpacas with induced ovulation using GnRH or copulation.

Methods: The study was conducted on 8 sexually mature fertile alpacas with a preovulatory follicle of ≥ 7 mm. Ovulation was induced with GnRH (G1, $n = 3$) and natural mating' (G2, $n = 5$), confirmed by ultrasound showing the disappearance of the follicle and formation of the corpus luteum (CL). On day 8 of the LP, samples of the uterine wall from the right and LUHs were obtained through a midline laparotomy. A uterine lavage with PBS was also performed, and no embryo-ova was found. The tissues were fixed, processed, and stained with Hematoxylin and Eosin. Measurements of the myometrium and endometrium, the area of the superficial and deep endometrial glands, the height of the gland cells, and the height of the endometrial mucosal cells were performed using microscopy and software. The measurements were taken in microns (um), from 12 different photographs per animal.

Results: In G2 (copulation), greater thickness of the endometrium and myometrium, as well as a larger area of the superficial glands and cell height, were observed ($p < 0.005$). Additionally, the area of the superficial glands in the left horn was greater compared to the right.

Conclusion: The thickness of the myometrium and endometrium, and the area of the superficial glands of the LUH in alpacas during the LP (day 8), induced by copulation (G2), show better development. This research provides new insights into changes in the histomorphometry of the alpacas' uterus during this stage and is the first report on this species.

Keywords: Alpaca, Copulation, GnRH, Induced ovulation, Uterus.

Introduction

South American camelids, such as alpacas (*Vicugna pacos*) and llamas (*Lama glama*), exhibit distinct reproductive peculiarities compared to other species. These include induced ovulation, predominantly left uterine horn (LUH) gestation (Fernández-Baca *et al*., 1979), and early gestational losses exceeding 50% (Fernández-Baca *et al*., 1970), showing a rate of embryonic mortality higher than in small ruminants (Diskin and Morris, 2008) and in mating, intrauterine intromission of the penis (Brennan *et al*., 2024).

It should be noted that a favorable uterine environment is crucial for maintaining embryonic viability (Pigiòka-Vjaèeslavova *et al*., 2023). Progesterone (P4) is vital in inducing modifications in the endometrium, such as endometrial glandular hyperplasia and increased wall thickness, which are essential for ensuring the production of vital substrates needed by the embryo.

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On the other hand, β-NGF, present in seminal plasma (Ratto *et al*., 2012), appears to play an important role in the successful achievement of pregnancy stimulating the expression of the gene vascular endothelial growth factor (VEGFA) (Ratto *et al*., 2020), promoting angiogenesis, and creating favorable conditions for implantation in the LUH, due to the larger caliber of blood vessels (Rondón, 2017) and increased irrigation (Fernández-Baca *et al*., 1979; Vaughan *et al*., 2013) creating greater blood flow in the uterine lining (Urra *et al*., 2019), and facilitating vasculogenesis of the embryonic circulatory system (Valderrama *et al*., 2019).

The interaction between the embryo and the substances secreted by the endometrial glands into the uterine lumen is a critical step that influences embryonic survival, growth, development, and implantation (Bazer *et al*., 2011; Song *et al*., 2022; Pigiòka-Vjaèeslavova *et al*., 2023). Therefore, histomorphometric studies of the uterine wall (myometrium and endometrium) during the luteal phase (LP) (induced by GnRH application or copulation) will provide insight into the conditions of the uterine wall layers on day 8 post-ovulation, an important day within the period (8 to 12 days) where maternal recognition of pregnancy occurs (Aba *et al*., 2000; Bianchi *et al*., 2022).

Materials and Methods

This study was conducted at the Kotosh Production Center of the National University Hermilio Valdizán in Huánuco, Peru. Eight alpacas clinically healthy, aged between 2 and 3 years, with an average weight of 34,875 ± 1.27 kg and a body condition of 3.19 ± 024 were used, nor mounted or exposed to the males for mating and arranged into two experimental groups (G1 and G2). The ovarian activity of animals was evaluated by ultrasonography every other day to detect preovulatory follicles, which was considered ideal with a diameter of 7 mm (Ascencio *et al*., 2019).

Ovulation induction

Upon detection of the preovulatory follicle, ovulation was induced in Group 1 (G1, n = 3) by a single intravenous dose of 0.008 mg of Buserelin acetate (GnRH, Conceptase®, Agrovet Market Peru, Lima, Peru), whereas in Group 2 (G2, n = 5), ovulation was induced by copulation (natural mating) (Fig. 1).

Collection of uterine wall samples

The samples were obtained via median laparotomy after a 24-hours fasting period. The procedure followed a protocol involving xylazine, ketamine, and lidocaine infiltration at the incision site. The uterine horns were positioned extraperitoneally using a Hook's retractor. A 0.5 cm sample of the uterine wall was excised from the middle third of both horns (Rondon, 2017).

Additionally, in G2, during surgery, the uterine horns were exteriorized to perform uterine lavage with PBS using a Foley catheter. During this intervention, no embryo-ova was found after searching with a stereoscope.

The samples were stored in vials containing 10% buffered formalin for 24 hours before being transported to the Animal Health Laboratory of the National Agricultural Health Service (SENASA - Peru) in Lima, Peru.

Using an automatic tissue processor (PF16), the samples were fixed, dehydrated, cleared, and finally embedded in paraffin. Paraffin blocks were sectioned into 5 µm slices using a microtome and subsequently stained with Hematoxylin and Eosin (Luna, 1968).

Morphometry of the uterine wall

Histological slides were observed using a microscope (Leica DM 1000 LED) equipped with a camera (Leica MC 190 HD). Measurements (µm) were taken for the following structures: myometrium thickness, endometrium thickness, and the height of epithelial cells in deep glands, superficial glands, and mucosa (Fig. 2), using Leica Application Suite software (Version 3.4.0). The glandular area (µm²) was determined using ImageJ software from the National Institutes of Health, Bethesda, MD (Fig. 2).

For each animal in both the GnRH and Copulation groups, twelve photographs were taken—six from the LUH and six from the right. These photographs were used to measure all the structures specified in the study, including myometrium thickness, endometrium thickness, glandular area (deep and superficial), and the height of epithelial cells in deep glands, superficial glands, and endometrial mucosa. This resulted in a total of 36 and 60 photographs for the GnRH and Copulation groups, respectively (Fig. 1).

Statistical analysis

Data tabulation and creation of comparative tables were conducted using Microsoft Excel. Statistical tests were performed using IBM SPSS Statistics 27. The normality criteria were tested using Kolmogorov Smirnov´s. Variables were compared using Z or Student´s t-test for independent samples with a confidence level of 95%.

Ethical approval

All animal procedures performed in this study were done according to the protocol for handling animals for research approved by the Bioethics Committee of Veterinary Medicine and Zootechnics faculty of UNHEVAL, Peru (N°52-2021-UNHEVAL-FMVZ).

Results

Morphometry of the uterine endometrium and myometrium

Table 1 shows the average thickness of the endometrium in alpacas with GnRH-induced ovulation (G1) and natural mating (G2). The average endometrial thickness was lower in G1 (3,138.323 µm) compared to G2 (4,146.734 µm) (p = 0.001). Similarly, the mean myometrial thickness in G1 was lower compared to G2, with means of 2,153.543 µm and 2430.566 µm (p = 0.002), respectively.

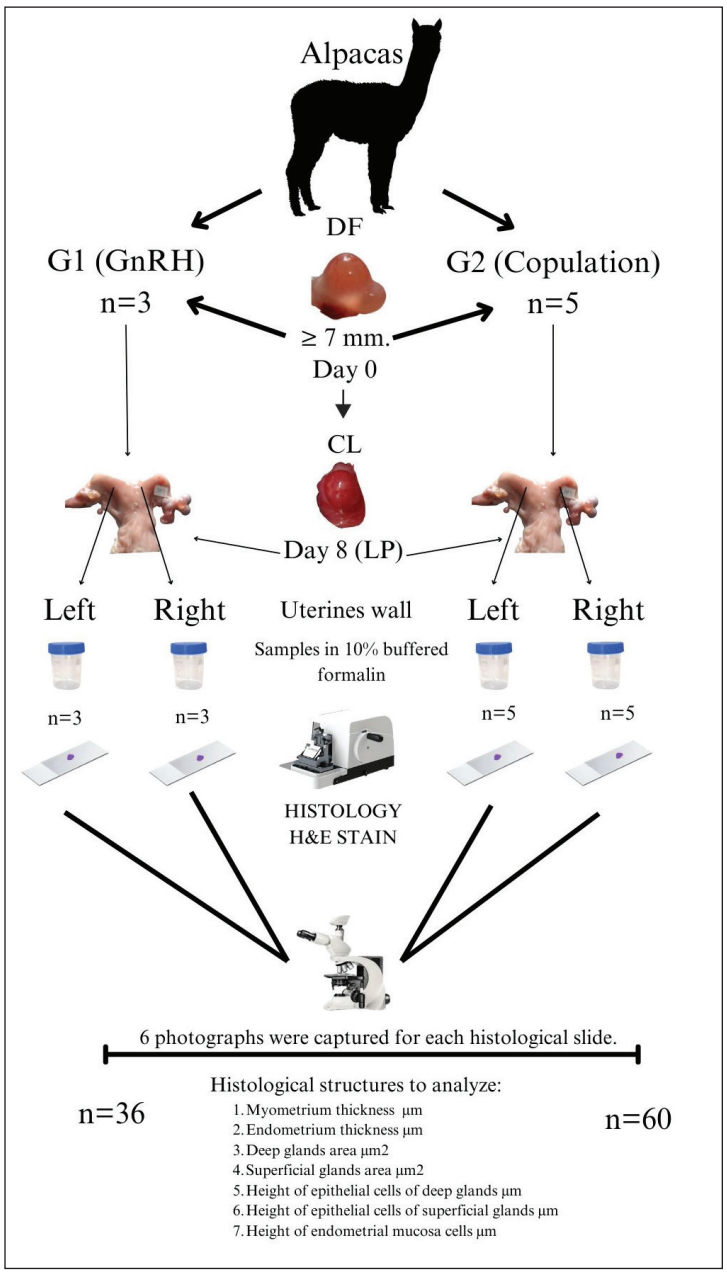

Fig. 1. Scheme simplified of the experimental procedure: after detection by ultrasonography of a dominant follicle (DF), ovulation was induced with GnRH (G1) or copulation (G2). The day 8 in the LP, uterine wall samples were taken after confirming the presence of a CL, subsequently the samples were processed, observed under the microscope and analyzed.

Morphometry of uterine endometrial glands and epithelial cells

Table 2 the average area of deep endometrial glands in alpacas induced to ovulate with GnRH (G1) was 512.057 µm², showing a statistically significant difference compared to alpacas induced by copulation (G2) with 637.066 µm² (p = 0.001). Additionally, the average area of superficial endometrial glands was greater in G2 (1,771.506 µm²) compared to G1 (1,585.575 µm²) (p = 0.04).

The mean height of deep glandular cells in alpacas ovulated with GnRH (G1) was 19.265 µm, whereas in those induced with copulation (G2) was 19.017 µm (p = 0.727). The average height of superficial glandular cells treated with GnRH was 20.881 µm, which was lower compared to alpacas in G2 (22.995 µm,

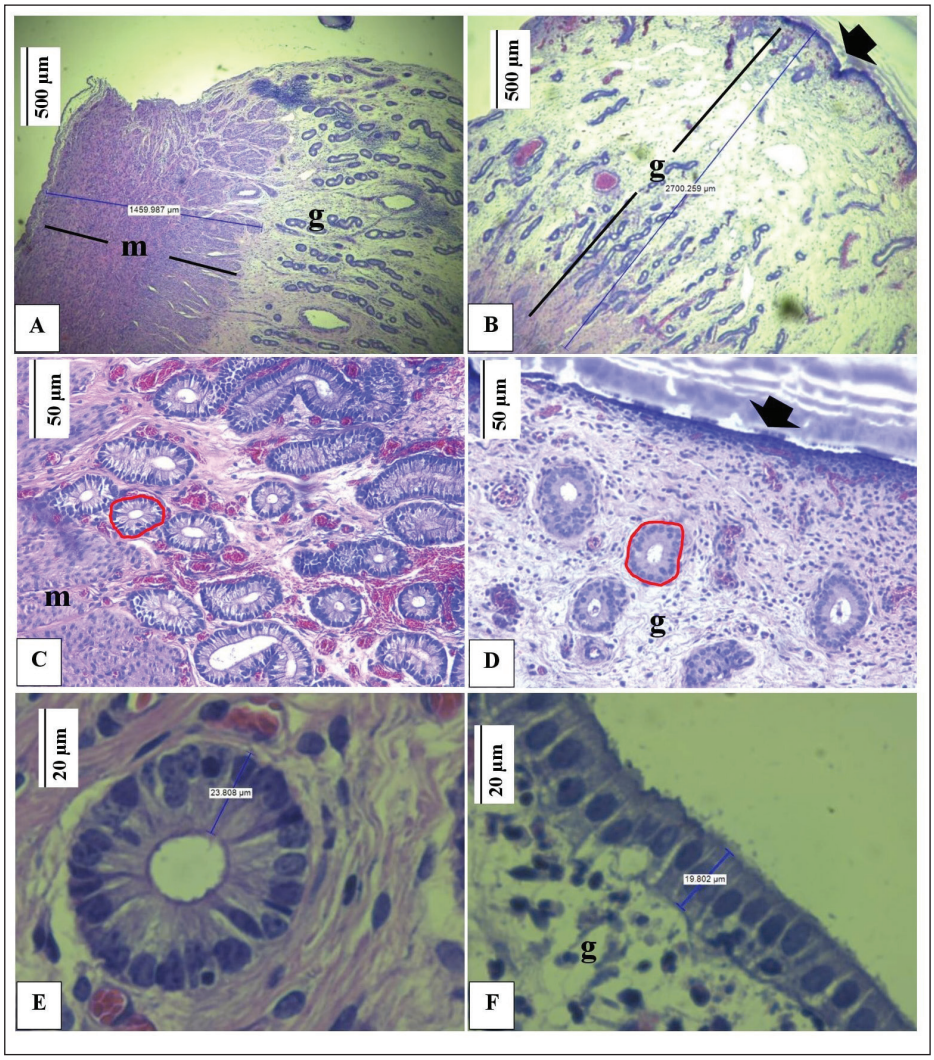

Fig. 2. Microphotographs of measurements of the uterine wall in alpacas in the LP (day 8) induced, with GnRH or copula. (A) Measurement of myometrial thickness, (g) Endometrium, (m) myometrium, (H&E 4x, bar 500 µm). (B) Measurement of (g) endometrial thickness, (Arrowhead) endometrial mucosa, (H&E 4x, bar 500 µm). (C) Measurement of the area of deep endometrial glands, (m) myometrium, (H&E 40x, bar 50 µm). (D) Measurement of the area of superficial endometrial glands, (Arrowhead) endometrial mucosa, (g) endometrium (H&E 40x, bar 50 µm). (E) Measurement of glandular cell height, (H&E 100x, bar 20 µm). (F) Height of uterine mucosal cells, (g) endometrium, (H&E 100x, bar 20 µm).

$p = 0.003$). There were no differences in the height of mucosal epithelial cells between both groups (Fig. 3), with means of 15.428 (G1) and 15.006 µm (G2) ($p = 0.515$).

Morphometry of endometrium and myometrium of both uterine horns during the LP in Alpacas with GnRH-induced ovulation and copulation

Table 3 presents the average thickness of the myometrium and endometrium of the uterine horns (right and left) of alpacas that were ovulated using GnRH (G1) or copulation (G2). G1 shows no differences in the uterine horns for these structures ($p \geq 0.05$). In contrast, alpacas induced with copulation (G2) exhibited a greater thickness of the myometrium in the LUH (2,550.789 µm) compared to the right (2,310.344 µm). A similar pattern was observed in the endometrium, with a greater thickness in the LUH (4,513.001 µm) compared to the right (3,780.468 µm) ($p < 0.05$).

Morphometry of endometrial glands and epithelial cells of the uterine horn (right and left) during the LP, with ovulation induced by GnRH or copulation

Table 4 shows the average area of endometrial glands and the height of their cells, as well as the endometrial

Table 1. Mean ± SD of endometrial and myometrial thickness in alpacas during (day 8) the LP: comparison between GnRH-induced ovulation or copulation (natural mating).

Ovulation	Endometrial thickness (µm)	Myometrial thickness (µm)
GnRH (G1)	3138.323 ± 554.125	2153.543 ± 415.058
Copulation (G2)	4146.734 ± 1303.893	2430.566 ± 399.176
p	0.001	0.002

Table 2. Mean ± SD of the area of the endometrial glands (superficial and deep) and height of the endometrial epithelial cells in alpacas during (day 8) the LP, with ovulation induced by GnRH or copulation.

Ovulation	Deep endometrial glands (µm²)	Superficial endometrial glands (µm²)	Cell height of deep glands (µm)	Cell height of superficial glands (µm)	Cell height of the mucosal ephitelium (µm)
GnRH (G1)	512,057 ± 97,698	1585,575 ± 265,704	19.265 ± 3.813	20.881 ± 4.199	15.428 ± 3.549
Copulation (G2)	637,066 ± 93,214	1771,506 ± 498,215	19.017 ± 3.064	22.995 ± 2.558	15.006 ± 2.744
p	0.001	0.04	0.727	0.003	0.515

Table 3. Mean ± SD of the thickness of the uterine wall (myometrium and endometrium), corresponding to the right and left uterine horns in alpacas during (day 8) the LP, with ovulation induced by GnRH or copulation.

Ovulation	Uterine horn	Myometrial thickness (µm)	*p*	Endometrial thickness (µm)	*p*
GnRH (G1)	Right	2124.088 ± 502.665	0.677	3057.427 ± 195.224	0.389
	Left	2182.997 ± 316.498		3219.219 ± 761.709	
Copulation (G2)	Right	2310.344 ± 414.082	0.018	3780.468 ± 1398. 209	0.028
	Left	2550.789 ± 350.442		4513.001 ± 1107.410	

Table 4. Mean ± SD of the area of the endometrial glands (superficial and deep) and height of the endometrial epithelial cells in relation to the uterine horns (right and left) in alpacas during (day 8) the LP, with induced ovulation by GnRH or copulation.

Ovulation	Uterine horn	Deep endometrial glands (µm²)	Superficial endometrial glands (µm²)	Cell height of deep glands (µm)	Cell height of superficial glands (µm)	Cell height of the mucosal ephitelium (µm)
GnRH (G1)	Right	524.101 ±108.895	1567.289 ± 282.837	19.101 ± 3.635	21.709 ± 3.923	16.558 ± 3.729
	Left	500.013 ± 86.520	1603.861 ± 254.255	19.430 ± 4.082	20.053 ± 4.411	14.299 ± 3.053
p		0.467	0.686	0.800	0.242	0.055
Copulation (G2)	Right	651.584 ± 102.941	1490.705± 265.963	19.679 ± 3.249	22.797 ± 2.715	14.868 ± 2.990
	Left	622.548 ± 81.512	2052.307± 520.695	18.355 ± 2.764	23.193 ± 2.421	15.143 ± 2.517
p		0.231	0.001	0.095	0.553	0.701

Table 5. Mean ± SD of the myometrial and endometrial thickness between the same uterine horns (G1 right *vs.* G2 right and G1 left *vs.* G2 left) in alpacas during (day 8) the LP, with ovulation induced by GnRH or copula.

Ovulation	Uterine horn	Myometrial thickness (µm)	p	Endometrial thickness (µm)	p
GnRH (G1)	Right	2124.088 ± 502.665	0.171	3057.427 ± 195.224	0.035
Copulation (G2)	Right	2310.344 ± 414.082		3780.468± 1107.410	
GnRH (G1)	Left	2182.997 ± 316.498	0.001	3219.219 ± 761.709	0.001
Copulation (G2)	Left	2550.789 ± 350.442		4513.001 ± 1107.410	

Table 6. Mean ± SD of the area of the endometrial glands (superficial and deep) and the height of the endometrial epithelial cells, between the same uterine horns (G1 right *vs.* G2 right and G1 left *vs.* G2 left) in alpacas during (day 8) the LP, with ovulation induced by GnRH and copula.

Ovulation	Uterine horn	Deep endometrial glands (µm²)	Superficial endometrial glands (µm²)	Cell height of deep glands (µm)	Cell height of superficial glands (µm)	Cell height of the mucosal ephitelium (µm)
GnRH (G1)	Right	524,101 ± 108,895	1567,289 ± 282,837	19.10078 ± 3.635	21.70861 ±3.923	16.55800 ± 3.729
Copulation (G2)	Right	651,584 ± 102,941	1490,705 ± 265,963	19.67890 ± 3.249	22.79717 ± 2.715	14.86841 ± 2.990
p		0.001	0.350	0.571	0.262	0.091
GnRH (G1)	Left	500,013 ± 86,520	1603,861 ± 254,255	19.42956 ± 4.082	20.05318 ± 4.411	14.29867 ± 3.05
Copulation (G2)	Left	622,548 ± 81,512	2052,307 ± 520,695	18.35537 ± 2.764	23.19347 ± 2.421	15.14347 ± 2.517
p		0.001	0.001	0.282	0.003	0.304

mucosa of the uterine horns (right and left) in alpacas in the LP induced to ovulate.

Alpacas induced with GnRH (G1) did not show a difference between uterine horns in the area of deep endometrial glands (p = 0.467) (Fig. 3), with the right horn measuring 524.101 µm² and the left horn measuring 500.013 µm². Similarly, no difference was observed in the area of superficial endometrial glands, with the right horn averaging 1,567.289 µm² and the left horn averaging 1,603.861 µm² (p = 0.686).

Conversely, in G2, a significant difference was found, with a larger area of superficial endometrial glands in the LUH (2,052.307 µm²) compared to the right horn (1,490.075 µm²) (p = 0.001). In contrast, G2 did not show differences in the area of deep endometrial glands between uterine horns ($p \geq 0.05$).

Regarding the height of the deep and superficial glandular cells and the endometrial mucosa, no differences were found between the uterine horns in G1 ($p \geq 0.05$), and similarly in G2 (Fig. 3).

Morphometry of uterine horns on the same side (right vs. right and left vs. left) of Alpacas in the LP with different methods of ovulation

Table 5 shows the averages ± SD of the myometrial and endometrial thickness of both uterine horns in alpacas with ovulation induced by GnRH (G1) or copula (G2). The G2 group exhibited greater thickness in the LUH for both the myometrium (2,550.789 ± 350.442 µm) and the endometrium (4,513.001 ± 1,107.410 µm) compared to the contralateral horn (myometrium: 2,310.344 ± 414.082 µm; endometrium: 3,780.468 ± 1,107.410 µm) (p = 0.001) in copula-induced ovulation. Similarly, the thickness was also greater than in the myometrium (2,124.088 ± 502.66 µm) and endometrium (3,057.427 ± 195.224 µm) of the right uterine horn (RUH) and the LUH (myometrium: 2,182.997 ± 316.498 µm; endometrium: 3,219.219 ± 761.709 µm) (p = 0.001) in GnRH-induced ovulation.

Table 6 shows the results for the area of deep and superficial endometrial glands, as well as the height of cells in these glands and the endometrial mucosa cells of both uterine horns for groups G1 and G2 (Fig. 3).

A significant difference was found in the area of deep endometrial glands, with it being larger in alpacas induced by copulation (p = 0.001). Similarly, a difference was found in the area of superficial endometrial glands between LUHs, with G2 having a larger area (2,052.307 ± 520.695 µm²) compared to G1 (1,603.861 ± 254.255 µm²) (p = 0.001). Likewise, the height of superficial glandular cells was greater in G2 (23.193 ± 2.421 µm) compared to G1 (20.053 ± 4.411 µm) (p = 0.003).

No differences were found in the height of deep glandular cells, endometrial mucosa cells, or superficial cells of the RUHs ($p \geq 0.05$) (Fig. 3).

Discussion

During day 8 of the LP induced with GnRH (G1) and copulation (G2), the uterine wall in alpacas, similar to other species, undergoes variations in size and development. Supported by P4, the endometrium prepares for embryo implantation (Pawlina and Ross, 2020). In the present study, comparing the thickness of the endometrium and myometrium of the uterine horns between both groups revealed a greater thickness in those ovulated by copulation.

This could be due to the luteotrophic support of the ovulation-inducing factor (OIF) present in seminal plasma, now known as nerve growth factor (B-NGF) (Ratto *et al*., 2016). Ulloa *et al*. (2014) reported that plasma P4 concentration is higher in the OIF/B-NGF group compared to the group induced with GnRH. Thus, it is probable that copulation indirectly favors a higher concentration of circulating P4, leading to more significant changes in the endometrium, particularly in the LUH, since this hormone modifies the size, height, morphology, density, and function of cells (Rondón,

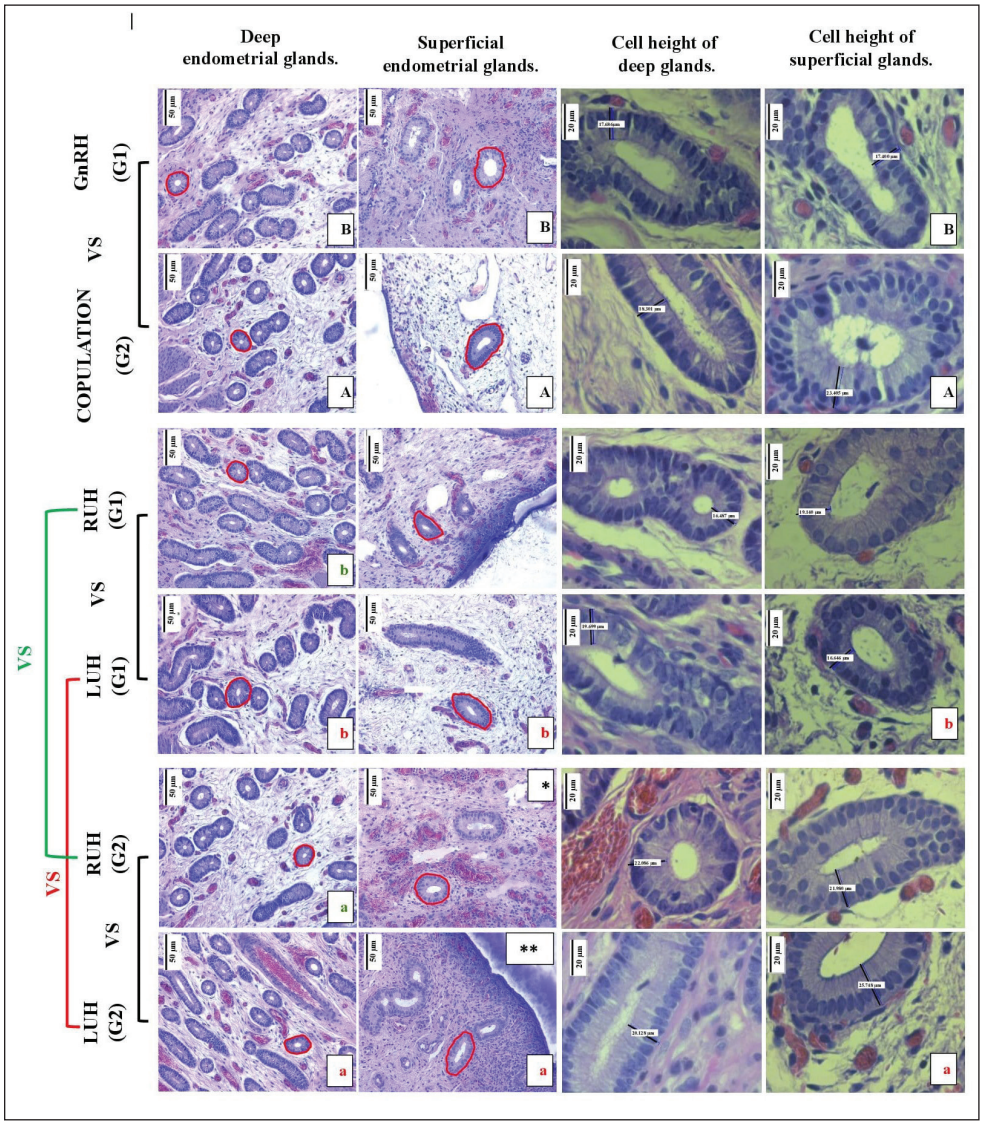

Fig. 3. Microphotographs of endometrial glands and the height of their cells in alpacas during (day 8) the LP induced with GnRH (G1) or copula (G2). Different uppercase letters (A: higher, B: lower) indicate significant differences between copula and GnRH ($p < 0.05$). Asterisks (** higher, * lower) indicate significant differences ($p < 0.05$) between the RUH and the LUH. Different lowercase letters (a: higher, b: lower) indicate significant differences ($p < 0.05$) between the same horns and different ovulation methods (G1 LUH *vs.* G2 LUH) (G1 RUH *vs.* G2 RUH).

2017). Additionally, it provides better conditions for the development and survival of the conceptus (Pigiòka-Vjaèeslavova *et al.*, 2023).

The superficial endometrial glands in G2 showed a larger area compared to G1, as well as a greater height of their cells. This effect could be influenced by P4, which promotes endometrial secretory activity (Bernabé *et al.*, 2008) and higher secretory activity of epithelial cells, as observed in bovines (Benbia *et al.*, 2017), an essential condition for implantation since it is a critical site for embryonic implantation (Aba *et al.*, 1995). However, the greater development of the endometrium, myometrium, and endometrial glands in alpacas induced by copulation could be due to the intrusion and penile friction exerted on the reproductive tract, causing mucosal injury from the vagina to the uterus (Brennan *et al.*, 2024), through which B-NGF contained in the seminal plasma reaches the bloodstream and exerts its luteotrophic effect. This mechanical effect cannot be achieved with GnRH application.

Furthermore, there is evidence of macroscopic morphometry showing size differences in the uterine horns in South American camelids and Old World

camelids, where the left horn is noticeably larger than the right, even in fetuses (Arthur *et al*., 1985; Sato and Montoya, 1990; Mendoza *et al*., 2014). However, the characteristics of microscopic (histological) morphometry have not been reported.

In this study, differences were found in the thickness of the myometrium and endometrium, being greater in the LUH of alpacas in G2, similar to camels, where endometrial thickness is also greater in this horn, regardless of the estrous cycle phase (Porjoosh *et al*., 2010).

Gestation in South American camelids occurs in the LUH (Fernández-Baca *et al*., 1979) independent of the side of ovulation. During day 8 of the LP, the differentiation of the uterine wall of each uterine horn according to the method of ovulation induction reveals significant implications for understanding the reproductive physiology of alpacas. The use of GnRH as an ovulation inducer does not show significant differences in the development of the myometrium, endometrium, endometrial glands, and the cells that make them up between both horns. However, inducing ovulation through copulation shows greater development in the uterine wall of the left horn, including the endometrium, myometrium, and superficial glands. This finding may suggest that the mechanical action and bioactive compounds present in seminal plasma, released during copulation, have a significant impact on uterine development.

The β-NGF protein present in seminal plasma could be a key factor in this differentiation. Previous studies have identified the presence of β-NGF and TrkA receptors in the endometrium of the uterine horns, particularly in the luminal, glandular, and vascular epithelium of the alpaca uterus (Barraza *et al*., 2021). These receptors are found in greater quantity in the LUH, which could explain the greater development observed in this horn when ovulation is induced by copulation.

The unequal distribution of β-NGF and TrkA receptors in the uterine horns may also influence the right horn's ability to maintain pregnancy. The superficial glandular activity in the right horn does not guarantee successful implantation, as it seems less suitable for maintaining pregnancy (Brown, 2000; Powell *et al*., 2007).

This suggests that copulation not only induces ovulation but also optimizes the uterine environment for implantation and embryonic development. In contrast, although GnRH induction can stimulate ovulation, it does not seem to replicate the complete benefits of copulation, especially in terms of the development and preparation of the uterus for gestation.

It appears that there are other intimate factors involved in the embryo's survival and development in the LUH (Ferradas *et al*., 2016). Therefore, the combination of mechanical and biochemical factors present during copulation provides a more favorable environment for reproduction in alpacas.

Conclusion

The substantial modifications in the endometrium and myometrium concerning thickness, the diameter of deep endometrial glands, and the height of epithelial cells of the endometrial mucosa suggest that the LUH of alpacas ovulated through natural mating presents a better uterine environment for embryo implantation and development compared to those alpacas induced with GnRH.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Authors' contributions

JGV, WRJ, and MRF participated in the design and conception of the study. WRJ, FAP, and JGV performed the surgery and collected the required data. RAG and WRJ processed the histological samples, MSA and WRJ performed the tissue and cell measurements, FAP, RAG, and WRJ analyzed the collected data and JGV, WRJ, EMP, MSJ, MRF, and XVL wrote the manuscript. All authors reviewed and approved the final manuscript.

Data availability

All data supporting the findings of this study are available in the manuscript.

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