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Effects of Two Types of Estrogen on the Follicular Wave for *in Vivo* Oocyte Collection in Brown Swiss Cows

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ABSTRACT

The manipulation of follicular waves through hormonal treatments, such as estrogen administration, plays a crucial role in optimizing *in-vivo* oocyte collection for assisted reproductive technologies. The present study aimed to evaluate the effect of two specific types of estrogen on follicular wave dynamics and their impact on *in-vivo* oocyte collection in Brown Swiss cows. Fourteen cows, in their first lactation, weighing approximately 340 kg were randomly assigned to one of two treatments including T1 (estradiol cypionate) and T2 (estradiol benzoate). Both treatments were administered at 1.2 mg of estrogen, at day 0 of the experiment. All Brown Swiss cows were provided with a diet entirely consisting of alfalfa grazing. On day 7, follicular wave dynamics were assessed using a DP-50 vet ultrasound device equipped with a 7.5 MHz transducer for transvaginal follicular aspiration guidance. Follicle counts were categorized into three size ranges including 2-4 mm (small), 4-8 mm (medium), and greater than 8 mm (large). Additionally, the quantity and quality (viable oocytes) of the collected oocytes were evaluated by the Ovum Pick Up (OPU) team for oocyte viability on day 7. The study assessed the follicular dynamics (number of follicles) and efficiency of oocyte collection (viable oocytes) in cows treated with Estradiol Cypionate (T1) and Estradiol Benzoate (T2). The average number of small, medium-sized, and large follicles size were 6.048 ± 6.037, 3.16 ± 2.01, and 0.53 ± 0.67 respectively. The total number of follicles was 9.59 ± 3.56. The mean number of viable oocytes recovered was 3.024 ± 1.66, while the mean number of non-viable oocytes was 1.47 ± 1.01. The results indicated no significant differences between treatments in the size of small, medium, and large follicles, nor in the total number of follicles and viable oocytes recovered. However, a significant difference was observed in the number of non-viable oocytes recovered, with a higher mean in T2 (1.86) compared to T1 (1.09). The results indicated an adequate follicular response and viable oocyte recovery in both treatment groups (estradiol cypionate and estradiol benzoate). However, variations in oocyte viability were observed, with estradiol cypionate showing a slight advantage.

Keywords: Follicle count, Follicular wave dynamic, Oocyte collection, Transvaginal follicular aspiration

INTRODUCTION

The efficient and sustainable production of cattle is a key objective in the global agricultural industry. In the central region of Peru, dairy farming primarily revolves around the efforts of local producers, who are at the heart of this practice (Estremadoyro et al., 2024). Within this framework, reproductive biotechnology emerges as an essential tool to enhance herds' productivity and genetic quality (Dahlen et al., 2014). *In-vivo* oocyte collection followed by *in-vitro* fertilization has facilitated significant advancements in assisted reproduction, optimizing the selection and proliferation of desirable genetic traits (Lonergan and Fair, 2014). Specifically, the manipulation of the follicular wave through estrogen administration, including the use of different types of estradiol such as Estradiol Cypionate and Estradiol Benzoate, has become a common practice to maximize the efficiency of oocyte collection. These types of estradiol differ in their effects on follicular development, with variations in dosage, timing, and duration influencing follicle size progression, oocyte maturation, and overall follicular dynamics (Lima et al., 2011). Additionally, cloning has made significant strides, allowing the creation of uniform herds with high productivity and disease resistance (Gray et al., 2020). Genomic selection has revolutionized genetic improvement in livestock by enabling the identification and selection of animals with superior genetic traits from an early age (Gutierrez-Reinoso et al., 2021). Genetic editing, using technologies, such as CRISPR-Cas9, allows precise modifications in animal DNA to enhance specific traits, including increased muscle growth, improved milk production, and enhanced fertility. It also targets disease resistance, particularly against common bovine ailments like mastitis, bovine respiratory disease (BRD), and foot-and-mouth disease, and improves feed efficiency by optimizing nutrient absorption and reducing methane emissions (Perisse et al., 2021).

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Brown Swiss cows, known for their high milk production and adaptability to various environmental conditions, represent an excellent candidate for studies in reproductive biotechnology (Baykan and Özcan, 2017). However, the physiological response of these cows to different types of estrogens concerning follicular dynamics and *in-vivo* oocyte collection efficiency is not yet fully understood (Mikkola et al., 2020). Understanding these aspects will not only provide valuable insights into best practices for estrogen administration to improve reproductive efficiency but also contribute to the development of more effective and sustainable reproductive management strategies in bovine livestock (Boneya, 2021).

Estrogen plays a crucial role in oocyte collection by influencing follicular wave dynamics, Estradiol Cypionate, with its longer half-life, provides a sustained release of estrogen, which leads to more prolonged and controlled synchronization of follicular waves (Abdulghani, 2022). Estrogen administration improves synchronization and stimulates follicular development in the ovary, promoting the selection, and growth of dominant follicles and thereby increasing the number of mature oocytes available for collection. Additionally, estrogen can enhance oocyte quality and optimize the hormonal environment, which is essential for efficient and effective *in-vivo* oocyte collection. Therefore, the present study aimed to evaluate the effect of two specific types of estrogen on follicular wave dynamics and their impact on *in-vivo* oocyte collection in Brown Swiss cows.

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Committee of the Dirección Regional de Agricultura Junín, Perú (LETTER N° 008-GRJ-DRA-AAC-PERÚ-2023) approved all animal handling procedures employed in this study.

Study area

The study was carried out at the "Estación Experimental el Mantaro", Mantaro, Jauja, Junin of Peru (Figure 1). Positioned at an elevation of 3,320 meters above sea level, the station experiences average temperatures between 4°C and 8°C and receives an annual rainfall of approximately 749 mm (Senamhi, 2023). Due to its strategic placement in the Junín region, this station is pivotal for research, offering an in-depth understanding of follicular dynamics in local cattle populations.

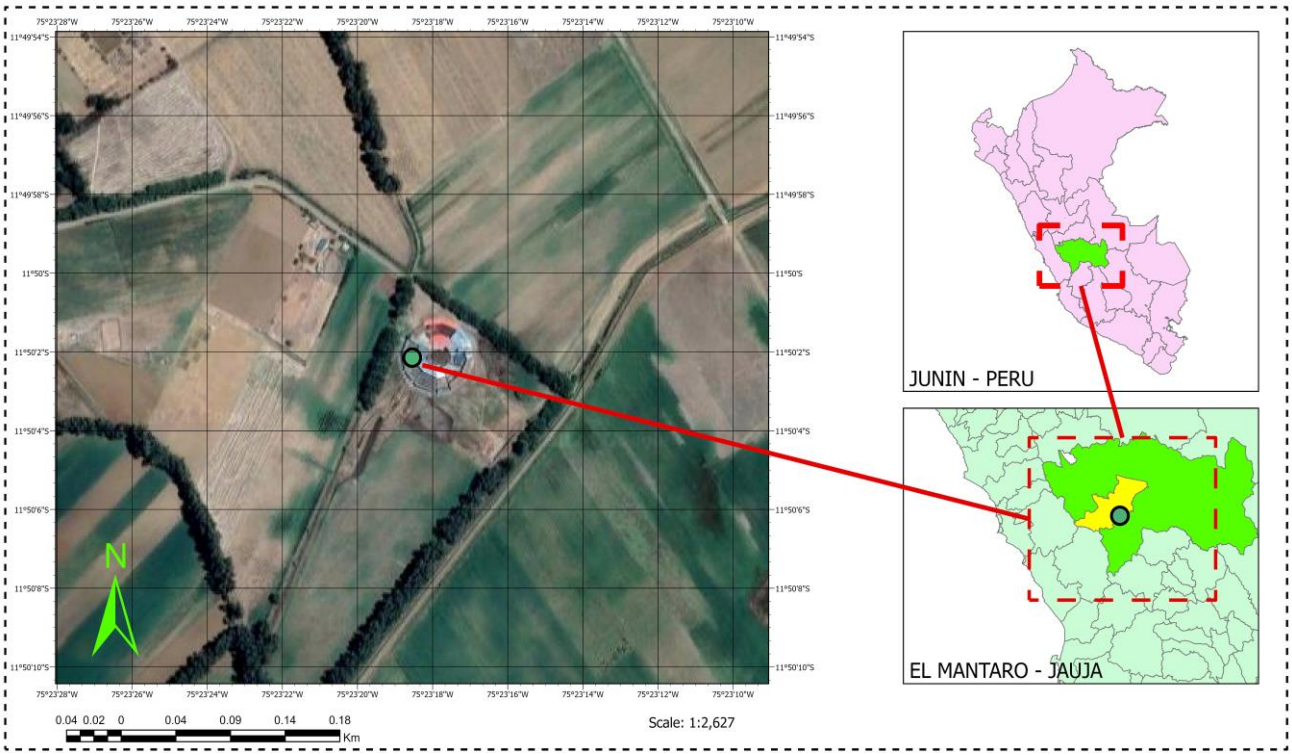

Figure 1. The location of study in country of Peru. Junín region (green color), district of El Mantaro (yellow), place of execution

Animal and distribution

A total of 14 cows (7 for each group) of an approximate weight of 350 kg and a body condition of 3 (Figure 2a), in the first third of lactation, were selected and randomly distributed among the treatments (T1: estradiol cypionate and T2 : estradiol benzoate). All of the Brown Swiss breeds were fed 100% alfalfa grazing.

Data collection

Follicular wave

For both experimental groups, an intravaginal DIB (Bovine intravaginal device), was purchased from Argentina (Figure 2b). The first group (T1) received 1.2 mg of Estradiol Benzoate (Estrovet, Montana, Peru) and 0.524 mg of Cloprostenol Sodium, was implemented on day "0", intramuscular route. The second group (T2) received 1.2 mg of Estradiol Cypionate and 0.524 mg of Cloprostenol Sodium (Figure 2c), which was implemented on day "0", one month after calving. In both groups, intravaginal ultrasonography was performed on days 0 and 7 to evaluate follicular dynamics and follicle growth. For imaging, a DP-50 vet ultrasound of Peru device equipped with a 7.5 MHz transducer for transvaginal follicular aspiration guidance (WTA) was used (Figure 2d). Follicle counts were categorized into three size ranges including 2-4 mm (small), 4-8 mm (medium), and greater than 8 mm (large, Figure 2e) (Haadsma et al., 2007). Additionally, the quantity and quality of the collected oocytes were evaluated (Figure 2f).

Oocyte collection by ovum pick-up

Epidural anesthesia with Xylazine (0.05 mg/kg, LIDOCAINA OVER, PERU) was administered to facilitate the manipulation of the ovaries (Lima et al., 2011). Subsequently, the rectum was manually emptied, followed by the cleaning and disinfection of the vulva and perineal area (Simões et al., 2021). A transducer was introduced into the vagina using a sanitary latex cover. The Ovum Pick-Up (OPU) handle was held in the right hand. Visualization was achieved using an ultrasound machine fitted with a transvaginal probe and a 60 cm OPU handle (Figure 2e). A disposable puncture needle (18 G, 0.9 x 70 mm) attached to a sterile 50 ml collection tube was then inserted through the guide. The OPU setup was completed with a foot-pedal-operated vacuum pump, applying a constant aspiration of 75 mm Hg. To observe the follicle sizes, the ovaries were positioned rectally in front of the probe. Before starting the puncture session, the system was flushed by aspirating a small amount of collection medium. After aspirating 3-4 follicles, the follicular fluid in the aspiration (Figure 2e) needle and collection system was thoroughly washed with washing and collection medium (PBS supplemented with sodium heparin, 2.2 IU/ml, and fetal bovine serum, 1%) (Landeo et al., 2022).

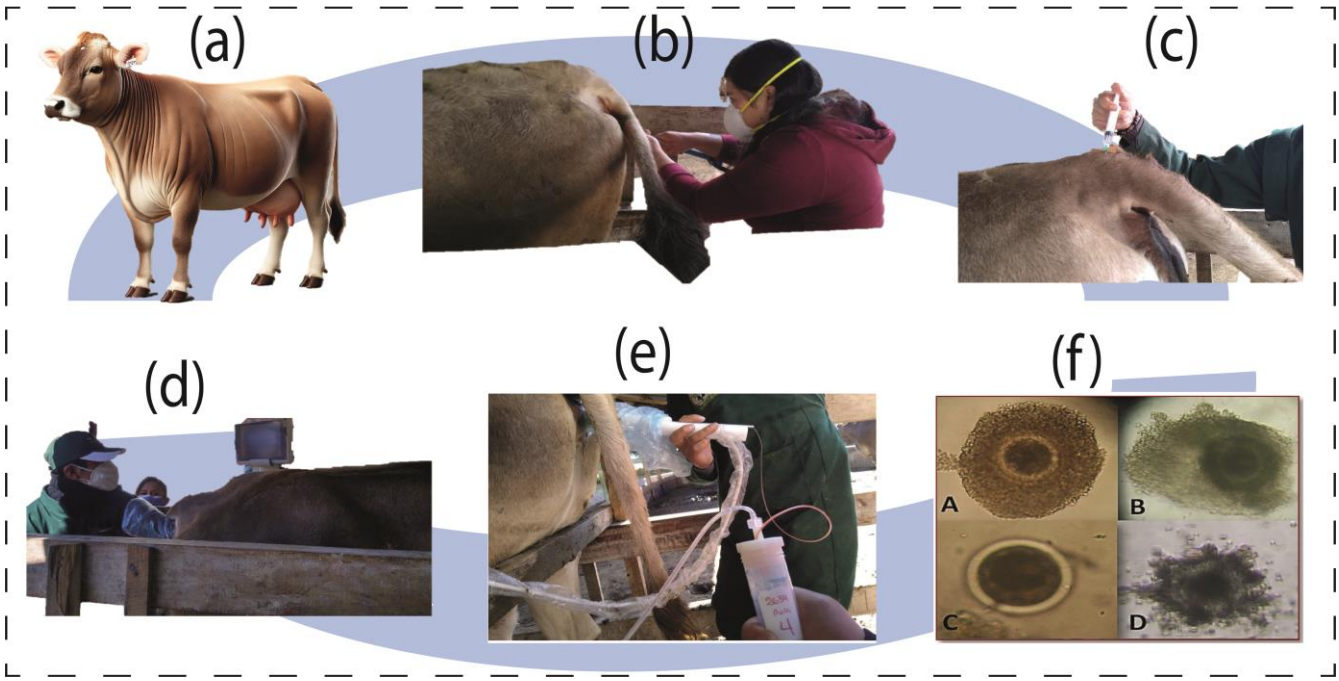

Figure 2. Oocyte collection procedure for Brown Swiss cows. Sequence of steps; **a**: Swiss brown cattle, **b**: Application of the synchronization protocol (estradiol cypionate and estradiol benzoate) to the study cows Brown Swiss, **c**: Application of anesthetic via epidural to the cow, **d**: Oocyte collection using the Ovum Pick Up technique, **e**: Oocyte aspiration guide and oocyte collection tube, **f**: Oocyte qualities evaluation (viable oocytes)

Oocyte search and sorting

After collection, the conical tubes were transported to the laboratory, maintaining the appropriate temperature throughout (20 °). The process began using a 100 µm diameter filter to separate the oocytes and cumulus cells from other cellular debris and blood. The filtered liquid was transferred to a stereoscopic microscope (LSM-B10 Labtron, Peru) at 20X magnification to visualize the cumulus-oocyte complexes (COCs). The COCs were observed at 40X magnification and evaluated based on their morphology to classify them into four categories including A (surrounded by ≥ 3 layers of

cumulus cells with homogeneous cytoplasm), B (oocytes partially surrounded by cumulus cells and irregular cytoplasm), C (denuded oocytes), and D (oocytes surrounded by fibrin). Oocytes of grades A and B were defined as viable, while those of grades C and D were considered non-viable (Figure 2f).

Statistical analysis

Normality and homogeneity tests (Shapiro-Wilk) were conducted on the data. Variables that followed a normal distribution included follicle size of 2-4 mm, total follicles, and total recovered oocytes; for these variables, T-tests (T-Student) were performed. However, the variables follicle size of 4-8 mm, size greater than 8 mm, viable oocytes, and non-viable oocytes did not meet the normality assumptions, so Mann-Whitney tests were applied. All analyses at a confidence level of 95% ($p < 0.05$), were conducted using R-Studio (Team et al., 2018) using version 4.3.0.

RESULTS AND DISCUSSION

According to Table 1, the following data were reported, the follicle size of 2-4 mm (small) showed an average of 6.048 ± 6.037 units, with a maximum of 13 follicles; the follicle size of 4-8 mm (medium) presented an average of 3.16 ± 2.01 units, with a maximum of 8 follicles; and the follicle size greater than 8 mm (large) had an average of 0.53 ± 0.67 units, with a maximum of 2 follicles. The average total number of follicles was 9.59 ± 3.56 units, with a maximum of 15. For viable recovered oocytes, an average of 3.024 ± 1.66 units was observed, with a minimum of 0 and a maximum of 8. Non-viable oocytes had an average of 1.47 ± 1.01 units, ranging from 0 to 4. Finally, the total average number of recovered oocytes was 4.5 ± 1.98, with a minimum of 1 and a maximum of 9 (Viable oocyte refers to the oocyte that is suitable, oocyte retrieved refers to the number of oocytes retrieved).

According to Table 2, there are no significant statistical differences between the treatments (T1 and T2). For follicle sizes of 2-4 mm, the averages were 5.67 for T1 (Estradiol Cypionate) and 6.43 for T2 (Estradiol Benzoate), showing similar results ($p > 0.05$). Similarly, for follicles sized 4-8 mm, the averages were 3.52 for T1 and 2.81 for T2, with no significant statistical differences ($p > 0.05$). For follicles larger than 8 mm, no significant differences were evident ($p > 0.05$), with averages of 0.66 for T1 and 0.38 for T2. For the total number of follicles, no significant differences were found ($p > 0.05$), with averages of 9.81 for T1 and 9.38 for T2. Similarly, no significant differences were found in viable oocytes recovered or the total oocytes recovered ($p > 0.05$). However, the variable of non-viable oocytes recovered caused statistically significant differences, with averages of 1.09 units for T1 and 1.86 units for T2.

Table 1. The mean of follicle sizes and oocyte recovery in the follicular collection of Brown Swiss cows (estradiol cypionate and estradiol benzoate)

Variable	Mean	SD	min	max	median
2-4 mm size	6.048	6.037	0	13	5.5
4-8 mm size	3.16	2.01	0	8	3
8 mm (Larger size)	0.53	0.67	0	2	0.0
Total follicles	9.59	3.56	2	15	10
Viable oocytes recovered	3.024	1.66	0	8	3
Non-viable oocytes recovered	1.47	1.01	0	4	1
Total oocytes recovered	4.5	1.98	1	9	4

SD: Standard deviation, Median: Mean value of the data

Table 2. The comparison of the two treatments means' in follicle sizes and oocyte recovery of follicular collection in Brown Swiss cows (estradiol cypionate and estradiol benzoate).

Variable	T1	T2	P-value
Size 2-4 mm	$5.67^{a} \pm 2.99$	$6.43^{a} \pm 3.75$	0.471^{T}
Size 4-8 mm	$3.52^{a} \pm 2.14$	$2.81^{a} \pm 1.87$	0.302^{M}
Larger size 8 mm	$0.66^{a} \pm 0.73$	$0.38^{a} \pm 0.58$	0.184^{M}
Total follicles	$9.81^{a} \pm 3.41$	$9.38^{a} \pm 3.79$	0.702^{T}
Viable Oocytes Recovered	$3.47^{a} \pm 1.94$	$2.57^{a} \pm 1.20$	0.132^{M}
Non-viable Oocytes Recovered	$1.09^{a} \pm 0.94$	$1.86^{b} \pm 0.96$	0.008^{M}
Total Oocytes Recovered	$4.57^{a} \pm 2.08$	$4.43^{a} \pm 1.91$	0.818^{T}

[a, b] Similar letters in the same row indicate similarity ($p > 0.05$) and different letters imply statistical differences ($p < 0.05$). [T], implies a T-Student test, due to its normal distribution. [M], implies a Mann-Whitney test, because it does not meet the assumptions of normality; T1: Estradiol cypionate group, T: Estradiol benzoate group

The obtained results provide information on follicular dynamics and the efficiency of oocyte collection in cows (Brown Swiss) treated with different types of estrogens. The follicle size of 2-4 mm (small) showed an average of 6.048 ± 6.037, with a maximum of 13 follicles. This follicle size range is crucial, as it represents follicles in the initial growth phase, this range of follicle sizes is critical because it reflects the early growth phase, during which follicles are most responsive to hormonal stimulation (Ferst et al., 2020). For follicles sized 4-8 mm (medium), an average of 3.16 ± 2.01 was observed, with a maximum of 8 follicles. This size range is important because follicles within these dimensions are at a more advanced stage of development. At this point, they are nearing the final stages of maturation and are therefore more likely to be selected for ovulation. This makes them crucial targets in reproductive protocols, as they have a higher potential for yielding viable oocytes, which are essential for successful fertilization and subsequent embryo development. Understanding the development of follicles in this size range helps optimize the timing and efficiency of oocyte collection procedures (Richard et al., 2024). The variability in follicle size may be influenced by factors such as estrous cycle synchronization and the administration of exogenous hormones (Quirino et al., 2020). For follicles larger than 8 mm, the average was 0.53 ± 0.67 units, with a maximum of 2 follicles. These results are comparable to those reported by Gomez-Leon et al. (2023), who found that dominant follicles are usually few but essential for ovulation and subsequent fertilization. The lower number of follicles in this category suggests that most follicles are in earlier stages of development during the collection process. The average total number of follicles was 9.59 ± 3.56, with a maximum of 15, indicating a good response to hormonal treatments in terms of follicular recruitment. This finding is consistent with Reineri et al. (2023), who observed a similar follicular response in estrogen synchronization protocols in beef cattle.

Regarding viable recovered oocytes, an average of 3.024 ± 1.66 was observed, with a range of 0 to 8. This result is important as viable oocytes are crucial for the success of *in vitro* fertilization (IVF). Compared to other studies, this average is slightly lower than that reported by Zago et al. (2023), who observed higher viable oocyte recovery in superovulation protocols in eight Flemish and eight Holstein females. The observed difference may stem from variations in study design, including differences in hormone dosages, timing of oocyte collection, and the specific breed of cattle used. For non-viable oocytes, the average was 1.47 ± 1.01, with a range of 0 to 4. The proportion of non-viable oocytes could be influenced by factors such as the precision of handling during collection and the hormonal conditions within the follicular environment, which can significantly impact oocyte quality and viability (Sartori et al., 2023). Finally, the average number of oocytes recovered was 4.5 ± 1.98, with a range from 1 to 9. This result is in line with previous studies that have reported similar recovery rates in bovine follicular collection protocols, suggesting that the methods used in this study are effective and consistent with established practices. The consistency of these findings with existing literature reinforces the reliability of the oocyte collection techniques employed and underscores their applicability in routine reproductive procedures (Souza-Fabjan et al., 2023).

CONCLUSION

The obtained results demonstrated a similar follicular response and viable oocyte recovery in both treatment groups (estradiol cypionate and estradiol benzoate). However, variations in oocyte viability were observed, with estradiol cypionate showing a slight advantage. It is suggested that while both estrogens can be effectively used in follicular wave programs, estradiol cypionate may offer a marginal improvement in the recovery of viable oocytes. Although the type of estrogen used does not appear to significantly affect follicular dynamics in terms of follicle size and number, but it may influence oocyte viability, with the use of estradiol cypionate being slightly superior. The findings underscore the importance of carefully selecting hormonal agents, considering the specific objectives of the reproductive protocol and the individual characteristics of the cattle. Precise selection of hormonal agents can enhance the quality of recovered oocytes, thereby increasing the efficiency and success of oocyte collection programs, further studies are suggested to explore the long-term effects of different types of estrogens on subsequent fertility and embryo development after oocyte retrieval, as well as possible interactions with other reproductive hormones.

DECLARATIONS

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authors' contributions

Ide Unchupaico Payano supervised the research, Carlos Quispe Eulogio executed the research, Edith Ancco Gómez did laboratory analysis, Jordan Ninahuanca Carhuas did statistical analysis, Fernando Arauco Villar did animal non-monitoring, Jorge Unchupaico Fermín collected data, and Noemí Mayorga Sánchez did the laboratory analysis. All authors confirmed the last edition of the manuscript before submission to the journal.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The authors confirm that the manuscript has been reviewed and submitted to this journal for the first time and all content of article have been checked via a well-known plagiarism checker software before submission.

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