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Original

Comparison of Two Techniques for Antral Follicle Counts (<4 mm) in Slaughtered Heifers

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ABSTRACT

Aim. To compare the efficiency of ultrasound and direct visual techniques for antral follicle counts, and the morphological characteristics of the ovaries of slaughtered heifers. **Methods:** A number of 102 ovary pairs from crossbred Holstein heifers collected postmortem were used. The study utilized the ultrasound and direct visual techniques to determine the size and weight of the ovaries, corpus luteum, and ≤4 mm >4 mm antral follicles. A simple analysis of variance and the Tukey test at 5% were performed to compare the means of the variables pooled by phase of the estrus cycle and follicular wave. A two-way analysis of variance was performed to compare the two techniques and location of the ovarian structures (RO and LO). Additionally, the Pearson test was conducted to establish the correlation between the techniques. **Results:** The study found that the right ovary (RO) weighed 3.21 g more than the left ovary (LO). Besides, 86.27% of animals presented corpus luteum (CL) with a mean weight of 3.38 g. The size of ovaries, (CL), and > 4 mm antral follicle counts (AFC) performed through direct visual (Vis) and ultrasound (US) techniques were similar (P>0.05), with a high and significant correlation (r=0.75; P<0.001). However, the ≤4 mm AFC showed statistically significant differences (P<0.05) between the techniques, whereas the correlation was low and non-significant. **Conclusion:** Ultrasonography is a highly efficient tool to assess > 4 mm ovarian structures. However, the correct determination of smaller structures will depend on other additional factors.

Keywords: Echography, ovaries, follicles, corpus luteum, cattle (*Source: DeCS*)

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INTRODUCTION

Reproductive biotechnologies like superovulation (SOV), ultrasound-assisted ovum pick up (OPU), and the production of *in vitro* embryos (PIVE) have shown significant progress in recent years, and therefore, are alternatives that contribute to a quick genetic breeding of cattle (Bó y Mapletoft, 2014). However, these techniques are influenced by individual physiological ovarian features of female cattle (Ireland *et al*., 2007), such as antral follicle counts (AFC), which because of its high variability among donors can compromise the efficacy of these biotechniques (Ireland *et al*., 2011). It has been claimed that the size of the ovary, the presence of the corpus luteum, and AFC, might be good markers for the selection of donors with a higher potential to generate embryos with the proper response to these reproductive biotechnologies (Alward and Bohlen, 2020).

Today, transrectal echography is used to identify and select donors with a high AFC within the frame of MOET (multiple ovulation embryo transfer) programs of assisted breeding in bovines (Singh *et al*., 2004). Nevertheless, this technique requires trained personnel, costly equipment, and adequate facilities to run detailed evaluation of imagery (Perry and Cushman, 2016). Besides, it is necessary to conduct echographic explorations during the estrus cycle that help determine AFC accurately (Ginther, 2016) that entails complicated animal management, which depending on the idiosyncrasy of the region, is sometimes impossible (Requelme and Bonifaz, 2012)**.**

Ultrasonography is a reliable tool to assess structures like the corpus luteum and antral follicles (Ginther, 2014), compared to transrectal palpation (Zduńczyk *et al*., 2009); however, technician expertise is fundamental for proper interpretation of images, reaching up to 70.3% confidence when performed by inexperienced technicians, and 97% otherwise (Gómez *et al*., 2017).

Besides, the resolution of the echography is essential for proper interpretation of small structures. A 3 mm resolution device, for instance, helps distinguish two separate interfaces. However, if the distance separating the two interfaces is less than 2 mm, it will be spotted as a single structure (Díez Bru, 1992). Hence, the correct determination of less than 3 mm follicles is associated with the resolution of the echograph (Rico *et al*., 2011) and the field level, as the echographs used do not have a very high resolution.

Accordingly, comparing the efficiencies of the ultrasound and direct visual techniques for antral follicle counts, and the morphological characteristics of the ovaries of slaughtered heifers has a clear importance.

MATERIALS AND METHODS

Location and experimental unit

The experiment was conducted between January and July 2021 at the biotechnology breeding laboratory, the University of Cuenca, Ecuador. A total of 102 slaughtered crossbred Holstein heifers in Cuenca (EMURPLAG), were studied. The body condition of the animals was greater than 3 in a 1-5 scale, according to the description of Song *et al*. (2019), and diagnosed as healthy

by the sanitary technician of the facility. After slaughtering, the two ovaries were collected by separate from all the animals.

Experimental design

The experiment was based on transversal cut, with a first evaluation of the ovary, corpus luteum, and antral follicle counts (≤4 mm and >4 mm) in the two ovaries, through ultrasonography. Then direct visual determination of weight, ovary size, and corpus luteum was performed, along with antral follicle counts (≤4 mm and >4 mm). Finally, the ultrasound technique was compared to direct visualization. Then it was grouped depending on the estrus cycle phase and the follicular wave phase of the animals at slaughtering.

Methodology

The selection of animals was done in the yard (pre-slaughtering). Later, the two ovaries from every animal were removed and placed in sterile containers with a physiological solution at 35 °C, separately, in a cooler for transportation to the biotechnology laboratory of the University of Cuenca. At the site, the ovary adjacent tissue was removed, and the ovaries were washed with a physiological solution at 35 °C.

Ultrasonography evaluation of the ovaries:

First, the right and then the left ovaries were placed in a scanning water container using an Aloka ProSound 2®, (Tokyo-Japan) scanner, with a linear 7.5 MHz transducer. The size of the ovary was determined by the width x length/2 formula. Then, the presence or absence and size of the corpus luteum (CL) was determined using the above formula. Finally, the same technician performed antral follicle counts (≤4 mm and >4 mm) using the method described by Ayala *et al*. (2019) through dorsal-ventral and lateral-medial scanning of each ovary.

Visual evaluation of the ovary:

First the right and then left ovaries were transported to a different room, where another technician who was unaware of the results of the echography, made a direct evaluation of the ovaries. At first, the number of antral follicles (≤4 mm and >4 mm) was evaluated. Then the whole ovary was weighed in a FS-400C (g) precision analytical balance, and the width and length were measured using a caliper gauge (200 mm). Later, the presence of CL was determined, and the nuclei were removed in order to set the weight and size of the structure.

Determination of the estrus cycle phase:

Two criteria were used to establish the physiological phase of the CL when slaughtering (forming metaestrus, functional diestrus, and regression proestrus). One was based on the size of the CL (<15 mm metaestrus or proestrus, and >15 mm diestrus, as determined by Ayala *et al*. (2017). The other consisted in CL color (hemorrhagic metaestrus, dark brown diestrus, yellow diestrus, according to Adams and Singh (2014).

Follicular wave phase

The follicular wave phase (recruitment, selection, or dominance) was determined according to two criteria stated by (Ginther, 2016), about the size of antral follicles present in the two ovaries. Hence, the size of most antral follicles was ≤4 mm, as considered in the recruiting phase. However, if most follicles had been 5-8 mm, they would have been classified as animals in the selection phase. Lastly, if the largest follicle was 10 mm, and no <4 mm follicles were observed, it was considered in the dominance phase.

Statistics

The data were processed using SPSS, version 25. Principal statigraphs were established for all the variables analyzed in the ovaries and their structures. The Kolmogorov-Smirnov test was used to determine data normality. The analysis of variables ovary size and number of antral follicles (≤4 mm and >4 mm) using the two techniques (ultrasonography and direct visualization) relied on a two-way analysis of variance (ANOVA); a simple analysis of variance (ANOVA) was performed to evaluate the characteristics of the corpus luteum, whereas the Tukey test 5% was used comparison of means. Additionally, the Pearson test was conducted to establish the correlation between the counts of two variables through the two techniques.

RESULTS AND DISCUSSION

General results of ovary weights

The study found that the right ovary weighed 3.21 g more than the left ovary (Table 1). Besides, in 86.27% of the 102 animals studied one of the ovaries had corpus lutea at different stages of development, with an average weight of 3.38 g. The fact that the right ovaries were heavier than the left ovaries is in keeping with the data published by González, De la Rosa, and Mendoza, (2017), who described mean values of 9.7 g in the right ovary, whereas it was 7.8 g in the left ovary. These results confirm greater activity of the right ovary compared to the left ovary in cattle (Condo Plaza *et al*., 2015).

Table 1. Average ovary and corpus luteum weights

Variable	N	$\bar{X}$	SE	Values	
				Minimum	Maximum
Weight of right ovary (g)	102	8.23	0.38	2.40	21.90
Weight of left ovary (g)	102	5.02	0.24	1.10	13.30
Weight of corpus luteum (g)	88	3.38	0.22	0.20	9.20

N=number of cases X=value mean SE=standard error

Evaluation of ovary size and corpus luteum *through ultrasonography and direct visualization*

The evaluation of ovary size using direct visualization showed that the right ovary was larger (22.9 ± 0.42 mm) than the left ovary (20.96 ± 0.33 mm), having a statistical difference ($P<0.05$). On the other hand, ultrasonography determined that the right ovary was larger (23.4 ± 0.38 mm) than the left one (21.4 ± 0.35 mm) (Fig. 1). These results reinforce the criterion of larger right

ovaries than left ovaries, expressed by), which is justified by a greater level of functionality of the right ovary (Alba *et al*., 2006).

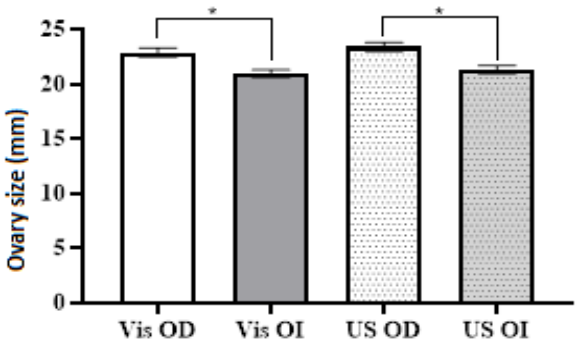

Figure 1. Mean right and left ovary sizes, evaluated through direct visualization (Vis) and ultrasound tests (US). * = statistical difference (P<0.05) Pearson correlation (r=0.75; P<0.001) between direct visualization and ultrasonography

However, the comparison of the mean ovary values found in this study using the two techniques (ultrasonography and direct visualization) determined that there was no statistical difference between them (P>0.05). Besides, the correlation of the mean values found through these techniques is high and significant (r=0.75; P<0.001). Similar results were found upon evaluation of the size of the corpus luteum through ultrasonography and direct visualization, with similar values (15.3 ± 0.42 mm and 14.8 ± 0.44 mm, respectively, Fig. 2A), with a high and significant ratio between the techniques (r=0.86; P<0.001). Therefore, the utilization of ultrasonography as a tool to determine the sizes of the ovary and corpus luteum is highly reliable in cattle breeding (Crane and Muirhead, 2020).

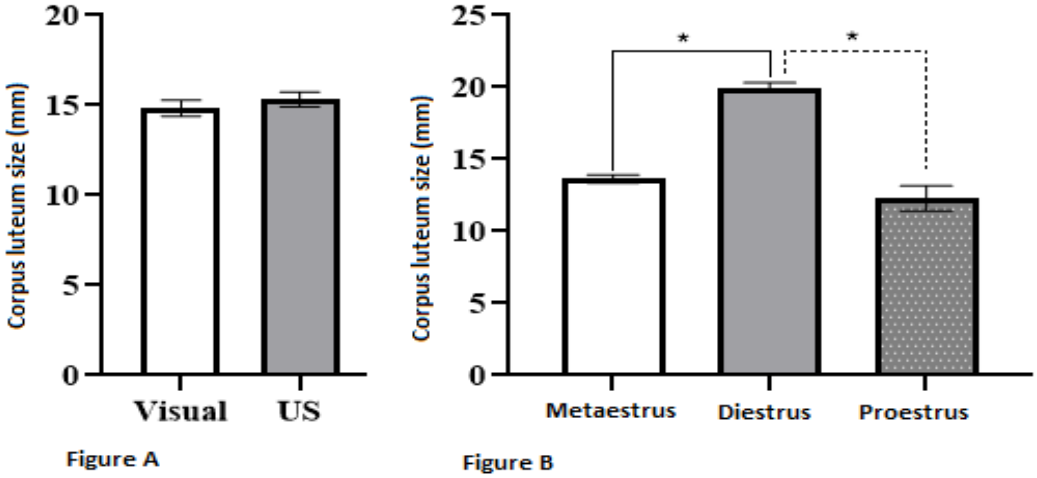

Figure 2A. Mean size of the corpus luteum (LB), evaluated through direct visualization (Vis) and ultrasonography (US) Figure 2B. Mean size of the corpus luteum (LB) in the estrus cycle phases (metaestrus, diestrus, and proestrus). * = statistical difference, according to Tukey (P<0.05).

The evaluation of the corpus luteum in the growth phase (metaestrus = 13.6±0.28 mm), static (diestrus = 19.9 ± 0.33 mm), and regression (proestrus = 12.3 ± 0.88 mm; Fig. 2B), showed similar values to the ones described by Perea *et al*. (1998) in crossbred heifers (Holstein and Swiss Brown with Brahman), set the maximum CL diameter on the fourth day (growth phase) in 11 ± 0.2 mm, and the tenth day (static phase) in 19 ± 0.3 mm. These results match the reports made by Ayala *et al*. (2019) in relation to CL size in the growth phase (14.4 ± 0.23 mm), static (19.63 ± 1.06 mm), and regression (12.3 ± 0.25 mm) in crossbred Holstein heifers.

Results of mean antral follicle counts (≤4 mm and >4 mm) from the total ovaries evaluated through ultrasonography and direct visualization.

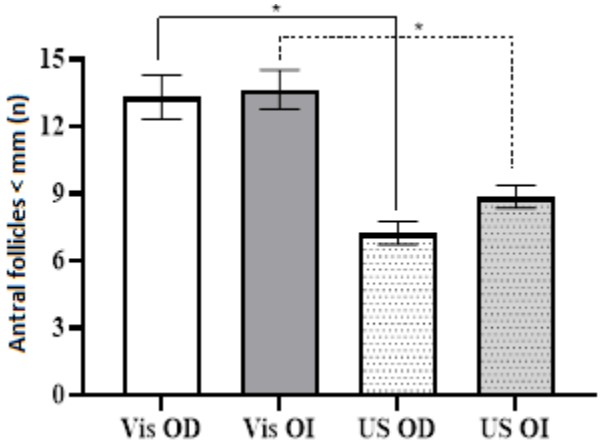

Figure 3. Mean antral follicle count (≤4 mm) in the right and left ovaries, evaluated through direct visualization (Vis) and ultrasonography (US). * = statistical difference (P<0.05)

The mean number of antral follicles (≤4mm) observed through direct visualization in the right ovary (13.3 ± 0.99 follicles) were similar (P>0.05). The same result was found in follicle count using ultrasonography in the right (7.2 ± 0.51 follicles) and left (8.9 ± 0.50 follicles; P>0.05).

On the contrary, the comparison of the average values found through direct visualization and ultrasonography in the right (13.3 ± 0.99 and 7.2 ± 0.51 follicles, respectively) and left (13.6 ± 0.88 and 8.9 ± 0.50, follicles, respectively) ovaries, showed a statistical difference between the techniques (Fig. 3). These results mean that ultrasonography permits the identification of 54.13% of follicles (≤4mm) in the ovaries, which is directly linked to the size of follicles and the resolution of the scanner, since, in theory, a a 3 mm resolution device, for instance, helps distinguish two interfaces apart by 3 mm as two different structures. However, if the distance separating the two interfaces is less than 2 mm, it will be spotted as a single structure (Díez Bru, 1992). Hence, follicles smaller than 2 mm are not identified as two independent structures using ultrasonography. One of the explanations is that because 45.87% of follicles of this category are not identified, though transrectal echography is a very useful tool for antral follicles counts (Singh *et al*., 2004).

Other factors that can influence antral follicles (≤4 mm) counts (AFC) is that the technique requires trained personnel, costly equipment, and appropriate facilities to conduct detailed

assessment of images (Perry and Cushman, 2016), since excessive movement can interfere with the interpretation of the image, so the animals should be isolated, as stated by Colazo and Kastelic, (2014). Besides, Ginther, (2016) said it was necessary to perform several echographic explorations in every estrus cycle to determine follicle counts accurately.

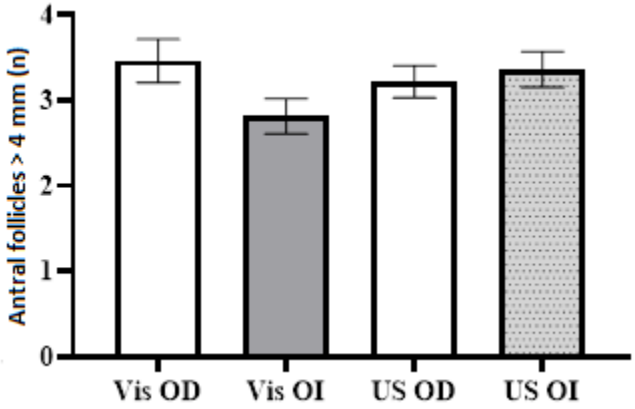

Figure 4. Mean antral follicle count (>4 mm) in the right and left ovaries, evaluated through direct visualization (Vis) and ultrasonography (US). * = statistical difference (P<0.05)

On the contrary, the comparison of AFC performed through direct visualization and ultrasonography of >4 mm follicles showed similar values in the right (3.46 ± 0.25 and 3.21 ± 0.18 follicles, respectively) and left (2.81 ± 0.20 and 3.36 ± 0.21, follicles, respectively) ovaries; Fig. 4), which could strengthen the theory that transrectal echography is a useful technique for antral follicles counts that is effective in cattle breeding.

However, Gómez *et al*. (2017) stated that the results are accurate when the ovary has few large follicles, but the accuracy diminishes when the ovary has a big quantity of small follicles. Besides, the presence of structures similar to follicles, like vessels, makes image interpretation even more complicated.

Cushman *et al*. (2009) described a large variation in the number of follicles among cows; however, they observed little difference in the number of follicles in the right and left ovaries in the same animal. These assumptions coincide with the results observed in the current study, where no significant difference was found in the mean follicle counts of the two ovaries, though the two techniques did show differences (Fig. 5).

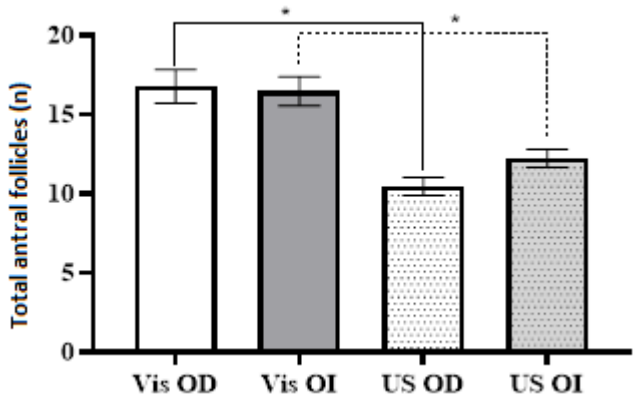

Figure 5. Total mean antral follicle count in the right and left ovaries, evaluated through direct visualization (Vis) and ultrasonography (US). * = statistical difference (P<0.05)

Upon grouping the number of ≤4 mm antral follicles `detected through direct visualization and ultrasonography, depending on the estrus cycle phase (metaestrus, diestrus, and proestrus), an AFC difference was observed between the techniques (31.8% and 48.6%, respectively). Nevertheless, when the same analysis was performed using >4 mm follicles, no difference was observed between the values, using the two techniques (P>0.05, Table 2).

Table 2. Mean and standard error in the number of (≤4 mm and >4 mm), determined through direct visualization and ultrasonography in the three phases of the estrus cycle (metaestrus, diestrus, and proestrus)

Estrus cycle phase	Visual technique			Ultrasound technique			Significance		
	<4mm	>4mm	Total	<4mm	>4mm	Total	<4mm	>4mm	Total
Metaestrus	22.0±1.97	5.9±0.40	28.0±2.06	14.9±0.91	6.5±0.39	21.4±1.08	*	ns	*
Diestrus	32.4±3.65	6.3±0.99	38.7±3.76	18.9±2.69	6.6±0.68	25.6±2.91	*	ns	*
Proestrus	34.9±4.28	7.0±0.79	41.9±4.38	18.3±2.03	6.7±0.65	25.0±2.12	*	ns	*

*** = 5% statistical difference in every line for each variable between same size follicles, by comparing the direct visualization and ultrasound techniques. Ns = no difference between the groups.**

The size of the follicle is essential for efficient identification and quantification through ultrasonography. Table 3 shows AFC >4 mm in the three phases of the follicular wave: recruitment, selection, and dominance, as effective as direct visual counts (P>0.05). However, when the size of most follicles evaluated is ≤4 mm, as in recruitment and selection, ultrasonography is more effective (P<0.05, Table 3). This particularity is not observed in the dominance phase (P>0.05), when evaluating ≤4 mm follicles, because in this phase (dominance) the number of ≤4 mm follicles is reduced in 57.15% and 31.82% compared to the phases of recruitment and selection, respectively (Webb *et al*., 2004).

Table 3. Mean and standard error in the number of follicles (≤4 mm and >4 mm), determined through direct visualization and ultrasonography in the three phases of follicular wave (recruitment, selection, and dominance).

Follicular wave phase	Visual technique			Ultrasound technique			Significance		
	<4mm	>4mm	Total	<4mm	>4mm	Total	<4mm	>4mm	Total
Recruitment	35.3±2.64	6.5±0.53	41.8±2.66	24.1±1.39	7.9±0.97	32.0±1.83	*	ns	*
Selection	22.3±2.15	7.3±0.74	29.6±2.53	14.9±0.95	7.6±0.39	22.6±1.11	*	ns	*
Dominance	15.3±2.31	4.8±0.44	20.1±2.37	14.0±0.31	4.7±0.31	18.7±1.71	ns	ns	ns

*** = 5% statistical difference in every line for each variable between same size follicles, by comparing the direct visualization and ultrasound techniques. Ns = no difference between the groups.**

CONCLUSIONS

Ultrasonography is a valuable tool that permits the observation of ovarian structures greater than 4 mm very efficiently; however, follicles smaller than 4 mm are hard to identify properly using this technique in field conditions. Therefore, if the job is related to research with the purpose of identifying and assessing < 4 mm antral follicles, a high resolution scanning device is required, along with a trained technician, and adequate facilities for optimum animal management.

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AUTHOR CONTRIBUTION

Conception and design of research: IJLM, JJZ, ASJA, JBDS, LEAG; analysis and interpretation of data: IJLM, JJZ, ASJA, JBDS, LEAG; redaction of the manuscript: IJLM, JJZ, ASJA, JBDS, LEAG.

CONFLICT OF INTERESTS

The authors declare the existence of no conflicts of interests.