

Veterinary and Comparative Biomedical Research

ORIGINAL ARTICLE

Relationship between Follicle Wall Diameter, Secretary Activity, and Histopathological Characteristics of Cyst-like Structures in Bovine Ovaries: An Abattoir Survey

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Online ISSN: 3060-7663

<https://doi.org/10.22103/VCBR.2025.25685.1087>

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Article History

Received: 3 August 2025

Revised: 3 September 2025

Accepted: 12 October 2025

Published: 16 October 2025

Keywords

Cow
Histopathology
Ovarian Cysts
Progesterone
Ultrasonography

Running Title

Bovine Ovarian Cyst Findings

Abstract

Ovarian cystic follicles are one of the most important reproductive problems in cows and animal husbandry. The aim of this study was to evaluate the relationship between follicle wall diameter and secretary and histopathological characteristics of cyst-like structure in the diagnosis of the type of ovarian cysts in slaughtered cows. In this study, 28 follicles larger than 25 mm in diameter and eight samples of follicles smaller than 20 mm in diameter were examined. Based on the criteria of follicular wall diameter, the samples were classified into three groups: control (non-cystic), follicular, and luteal groups. Pathological sections were prepared, and the levels of estrogen and progesterone in cystic fluids were measured using the ELISA method. The ultrasound evaluations revealed a statistically significant difference in follicle diameter among the control group and the luteal and follicular cystic groups. A significant increase was also seen in the mean follicular wall diameter, luteal, control and follicular cystic groups. Moreover, a significant difference in progesterone concentration was also observed between the control and the luteal cystic groups ($p < 0.05$). During imaging, the cystic wall diameters were 2.5 mm, and they must be considered as follicular cysts (the wall diameter of < 3 mm criterion); surprisingly, they were classified as luteal cysts in the histopathological evaluation. In conclusion, according to the pathological examination, 3 mm of wall thickness is not a good criterion for the diagnosis of ovarian cyst type, because luteal cysts may have a wall diameter of less than 3 mm.

How to cite this article: Ali Mohammad Alamdar Niaki, Arash Kheradmand, Omid Dezfoulian, Masoud Alirezaei. Relationship between Follicle Wall Diameter, Secretary Activity, and Histopathological Characteristics of Cyst-like Structures in Bovine Ovaries: An Abattoir Survey. *Veterinary and Comparative Biomedical Research*, 2026, 3(1): 29 – 36. <https://doi.org/10.22103/VCBR.2025.25685.1087>



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Introduction

During the past decades, the incidence of cystic ovarian degeneration has been estimated to be 15% in cows, ranging from 9.5% to 25% (1). A better diet and herd management, together with genetic selection, have resulted in a considerable increase in milk yield per dairy cow over the past few decades. Metabolic disorders, stress, uterine infections, lameness, and cystic ovarian degeneration are other problems affecting dairy production (2). In parallel with the selection for the production characteristics, the fertility of dairy cows has significantly decreased in recent years (3). It has been reported that cystic ovaries may occur at any point during lactation, but they tend to occur more often between 31-60, 40-150, and 120-210 days after delivery. In dairy cattle, cystic ovarian disease is a common condition that affects fertility (4). One of the latest definitions describes ovarian cysts as follicle-like structures with a diameter of at least 20 mm, present on one or both ovaries in the absence of any active luteal tissue, and clearly interfering with normal ovarian cyclicity. Macroscopically, cysts are classified into two types, follicular and luteal, which represent different forms of the same condition. Timely diagnosis and differentiation of these two types of cysts are the most important challenge concerning ovarian cysts (1).

It is well known that ovarian cysts are diagnosed based on behavioral symptoms, rectal palpation, ultrasonographic examination of the ovaries and reproductive organs, progesterone concentrations in plasma or milk or a combination of these methods (5). Based on the diameter of the follicle and its wall, ultrasound imaging is a diagnostic tool that can be used to distinguish between cyst types. Using ultrasound, follicular cysts are defined as follicular structures with a diameter of 20-25 mm and a wall thickness of less than 3 mm, and an echogenic space filled with echo follicular fluids. However, luteal cysts are defined as follicular structures with a diameter of 25-20 mm and a wall thickness of more than 3 mm. In this type of cyst, the echogenic space appears gray and has a network-like structure (6). The combined use of diagnostic methods such as ultrasonography, rectal palpation, and progesterone concentration assessment greatly improves the diagnostic accuracy for ovarian cysts (3).

To date, diagnostic ultrasonography of ovarian cysts still faces challenges, such as the 20 mm cutoff between cystic and normal follicles, and the 3 mm wall thickness criterion used to distinguish follicular from luteal cysts. This study was conducted to evaluate the relationship between follicle wall diameter and secretory activity with histopathological characteristics of cyst-like structures in the ovaries of slaughtered cows.

Materials and Methods

In total, 648 ovaries from 324 crossbred (native × Holstein) cows were evaluated from November 2017 to January 2018 at Golshan Industrial Slaughterhouse, Khorramabad, Iran. Twenty-eight ovaries with fluid-filled structures greater than 25 mm in diameter were transferred to the Veterinary Hospital of Khorramabad, Iran, in order to distinguish the type of cysts. In addition, eight ovaries with follicles smaller than 20 mm in diameter were isolated as non-cystic samples or controls.

Separation of Samples Based on Ultrasonography

The samples were differentiated using a portable ultrasound device (V9, EMP, China) with a rectal probe frequency of 7.5 MHz. The diagnostic ultrasound method used in this study was water bath-based ultrasonography (8). In this method, the fluid-filled structures smaller than 25 mm in diameter were classified into the non-cystic, and those greater than 25 mm were classified as cystic (9). In the cystic group, fluid-filled structures with a wall diameter of less than 3 mm were classified as follicular cyst, whereas those with a wall diameter greater than 3 mm were classified as the luteal cyst (10). Therefore, the three main sample groups in this study (i.e., non-cystic follicles (n=8), follicular cystic group (n=21), and luteal cystic group (n=7)) were separated based on ultrasound findings.

The follicular fluid of each sample was collected using an insulin syringe and poured into a 1.5 mL microtube labeled with the sample name and number. The microtubes were kept at -70°C until measurement of estrogen and progesterone concentrations.

Measurement of Estrogen and Progesterone Concentrations

The 17-beta-estradiol concentration of follicular fluid was measured through the competitive ELISA method using the DiaMetra kit (Foligno (PN), Italy) (11), and progesterone concentration was measured using the Monobind kit (California, USA) (11). The sensitivity and specificity of this test were 95% and 100%, respectively.

Protein Assay

Follicular fluid protein concentration was measured through the Bradford method using a Kiazist kit (Hamedan, Iran). The Bradford protein assay is a well-known protein measurement method with high sensitivity. This method is fast and provides accurate protein measurements (12).

Histopathology

Initially, the follicular fluid was completely drained from each sample inside the microtubes, and then the samples were transferred to the specialized pathology laboratory of the Faculty of Veterinary Medicine. Afterward, longitudinal and transverse incisions were made on each follicle so that the follicular content was completely drained and formalin could penetrate the tissue for fixation. Each sample was then fixed in formalin. To prepare histopathological sections, several slices were taken from the follicle wall. The samples were stained with hematoxylin and eosin (H&E stain). For the final grouping, the wall diameter of each sample was measured using a microscope with a special lens according to the presence or absence of luteal cells in the corresponding follicle.

Statistical Analysis

The descriptive data were presented as mean ± standard error. The normality of the data was assessed using the Shapiro-Wilk test and Kolmogorov–Smirnov test before data analysis. The data were analyzed using SPSS software (version 22) through one-way analysis of variance (ANOVA), Tukey’s test as a post hoc, and Pearson’s correlation test. The significance level was set at $P < 0.05$.

Results

Ultrasonographic Examination

The samples with a mean follicular diameter of 27.65 mm and a wall thickness of more than 3.0 mm were classified into the luteal cystic group. The samples with a mean follicular diameter of 26.50 mm and a wall thickness of less than 3.0 mm were classified into the follicular cystic group, and the samples with a mean follicular diameter of 17.31 mm were classified into the control group (non-cystic), based on ultrasound findings. Ultrasonographic examination of follicular ovaries showed that eight follicles

were classified as non-cystic, 21 as follicular cystic, and seven as luteal cystic (Table 1).

The ultrasound imaging revealed a significant difference in the mean of follicle and its wall thickness among the groups. Moreover, a significant difference was observed in the progesterone concentration between the control and luteal cystic groups ($p < 0.05$). However, no significant difference was observed in mean follicle diameter between the follicular cystic group and the luteal cystic group, or in mean wall thickness between the control and the follicular cystic groups. There were also no significant differences in progesterone levels between the control and the follicular cystic groups, between the follicular and luteal cystic groups, or in estradiol concentrations among the three groups ($p > 0.05$).

Correlation coefficient examination based on ultrasound findings showed no significant correlation between follicle diameter or wall thickness and the estrogen or progesterone levels in the control and luteal groups ($p > 0.05$). In the follicular cystic group, a significant correlation was observed only between follicle diameter and wall thickness ($p < 0.05$). However, no significant correlation was observed between follicle diameter or wall thickness and estrogen or progesterone levels ($p > 0.05$, Table 2).

Histopathology

In this study, the presence or absence of luteal cells was considered as a criterion for diagnosing the cyst type. Based on histopathological findings, there were 4, 14, and 16 samples in the control (non-cystic), follicular cystic, and luteal cystic groups, respectively.

The presence of luteal cells without any granulosa cells was considered as luteal cystic ovaries. Although according to the ultrasound images, the cystic wall diameters were 2.5 mm, which would classify them as follicular cysts (the wall diameter of < 3 mm criterion), surprisingly, they were classified as luteal cysts in histopathological evaluation (Figure 1A).

Table 1. Mean ± SEM of follicles and cystic structures, follicular wall diameter, follicular fluid progesterone, and estrogen concentration in the different groups.

Group	Follicle diameter (mm)	Wall diameter (mm)	P4 (ng/mg protein)	E2 (pg/mg protein)
Non-cyst (n=8)	17.31 ± 1.07 ^b	1.40 ± 0.05 ^a	5.14 ± 0.51 ^a	185.80 ± 39.89 ^a
Follicular cyst (n=21)	26.50 ± 1.49 ^a	1.76 ± 0.09 ^a	6.68 ± 0.55 ^a	162.99 ± 32.18 ^a
Luteal cyst (n=7)	27.65 ± 2.60 ^a	3.21 ± 0.09 ^b	8.69 ± 1.19 ^b	229.02 ± 30.66 ^a

P4: Progesterone; E2: 17-beta-estradiol, means in each column with different superscripts differ significantly ($p < 0.05$).

It is well established that follicular cysts are characterized by cyst walls lined with granulosa cells. Amazingly, in our results, some cysts based on the diameter index in ultrasonographic images were classified as luteal cysts (3.2mm of rim diameter). Therefore, based on

histopathological assessment the classification was reconsidered (Figure 1 B).

The presence of lipid-laden luteal cells around the luminal surfaces characteristic of luteal cysts. Cysts that measured more than 25mm and 3 mm in body diameter and

rim thickness in ultrasound, respectively, were classified as luteal cysts according to histological evaluation (Figure 1 C). The covering of the luminal cyst by granulosa cells in

histological examination, in which the wall diameters were also <3mm (≈ 2.5 mm) in sonographic evaluation, conformed to a follicular type (Figure 1 D).

Table 2. Correlation between the different parameters of ultrasound based on the study groups

Groups		Non-cystic		Follicular cystic		Luteal cystic	
		p-value	R	p-value	P-value	p-value	R
Follicle diameter (mm)	Wall diameter (mm)	0.99	-0.22	0.006	0.57*	0.06	0.72
Follicle diameter (mm)	Estrogen (pg/mg protein)	0.54	-0.25	0.62	-0.12	0.99	-0.05
Follicle diameter (mm)	Progesterone (ng/mg protein)	0.82	0.09	0.08	0.63	0.12	0.63
Wall diameter (mm)	Estrogen (pg/mg protein)	0.16	-0.54	0.46	0.17	0.39	-0.38
Wall diameter (mm)	Progesterone (ng/mg protein)	0.63	0.20	0.46	0.17	0.06	0.72

R: The correlation of coefficient, *p<0.05 is considered significant level.

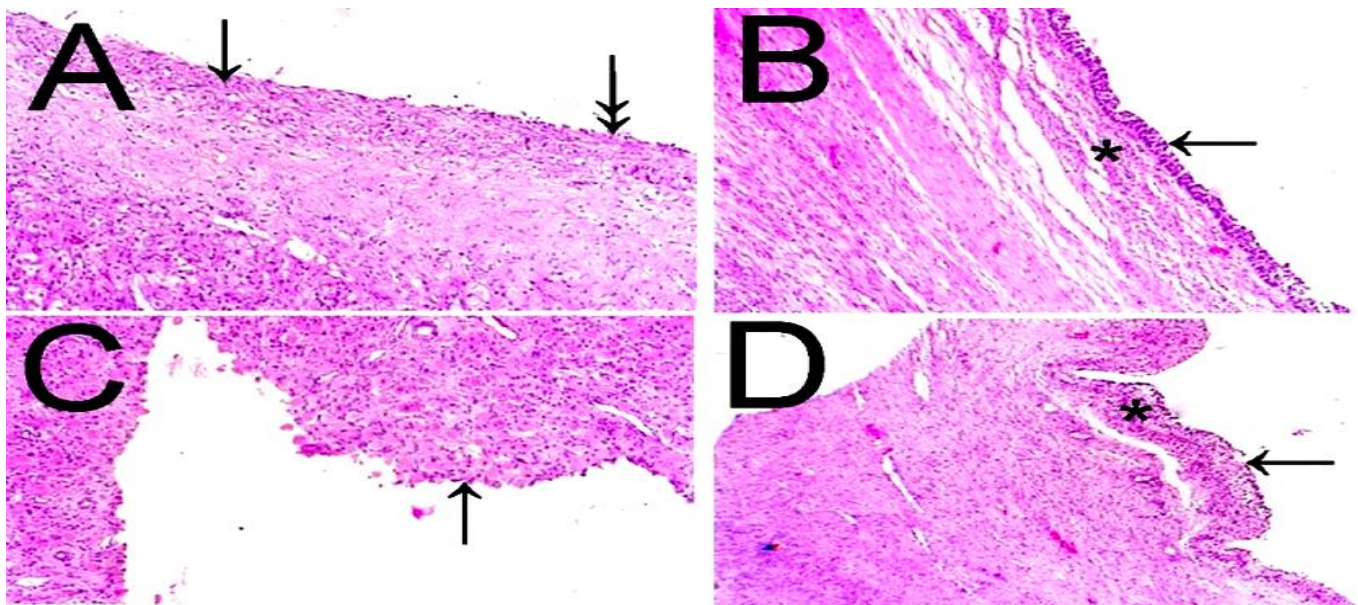


Figure 1. A) Cyst lining composed of an inner layer of luteinized granulosa cells and an outer layer of theca cells (arrow). Very few granulosa cells are attached to the wall (double arrows). B) A follicular cyst wall is lined by an intact inner granulosa layer (arrow) with a little evidence of an outer theca interna cell layer (asterisk). Peripheral ovarian stroma is composed of plumped fibroblastic spindle cells. C) Luteal cyst with broadly luteal cells around the lumen without evidence of granulosa luteinized cells (arrow). D) A benign cyst lined by an inner layer of granulosa cells (arrow) with an outer layer of theca cells (asterisk).

In Table 3, the findings for follicle diameter, wall diameter, progesterone, and estrogen levels according to histopathological assay are depicted. Based on pathological evaluation, there was a significant difference in the mean follicle diameter between the control and follicular cystic groups, and between the control and luteal cystic groups. Moreover, a significant difference was also observed in mean wall diameter between the control and follicular cystic

groups ($p \leq 0.05$). However, no significant difference was seen between the follicular and luteal cystic groups in mean follicle diameter and between the control, follicular, and luteal cystic groups in terms of progesterone and estradiol concentrations ($p > 0.05$).

Table 3. Mean ± SEM of follicle diameter, wall diameter, progesterone and estrogen according to histopathological assay.

Group	Follicle diameter (mm)	Wall diameter (mm)	P4 (ng/mg protein)	E2 (pg/mg protein)
Non-cyst(n=8)	17.50 ± 1.17 ^b	1.44 ± 0.06 ^a	4.85 ± 0.19 ^a	182.31 ± 26.62 ^a
Follicular cyst(n=21)	25.68 ± 1.31 ^a	2.18 ± 0.18 ^b	6.73 ± 0.61 ^a	147.99 ± 36.00 ^a
Luteal cyst(n=7)	25.65 ± 2.65 ^a	1.92 ± 0.18 ^a	7.42 ± 0.86 ^a	243.56 ± 37.28 ^a

P4: Progesterone; E2: 17-beta-estradiol, means in each column with different superscripts differ significantly (p<0.05).

Given the significant coefficients in the control and the follicular groups (Table 4), there was no significant correlation between follicle diameter or wall diameter and the level of estrogen or progesterone hormones (p>0.05). In contrast, in the luteal group, significant correlations were observed between the follicle diameter and wall diameter

(r=0.78), as well as follicle diameter with progesterone level (r=0.79) and wall diameter with progesterone concentration (r=0.72). However, no significant correlation was observed between follicle diameter or wall diameter and estrogen levels in this group.

Table 4. Correlation between the different parameters of histopathology based on the study groups

Groups		Non-cystic		Follicular cystic		Luteal cystic	
		p-value	R	p-value	R	p-value	R
Follicle diameter (mm)	Wall diameter (mm)	0.36	-0.37	0.22	0.31	0.003	0.78*
Follicle diameter (mm)	Estrogen (pg/mg protein)	0.25	0.45	0.20	-0.33	0.97	0.01
Follicle diameter (mm)	Progesterone (ng/mg protein)	0.82	0.09	0.78	0.07	0.001	0.79*
Wall diameter (mm)	Estrogen (pg/mg protein)	0.39	-0.35	0.43	-0.20	0.50	0.19
Wall diameter (mm)	Progesterone (ng/mg protein)	0.16	-0.54	0.49	0.18	0.003	0.72*

R: The correlation of coefficient, *p<0.05 is considered significant level.

Discussion

In this study, we compared the ultrasonography, biochemistry, and histopathology parameters to diagnose their accuracy in the farm examinations. Based on the results, follicle diameter can be related to luteal cystic and follicular cystic ovaries, and there were significant differences in wall diameter between the luteal and follicular groups. Moreover, the follicular fluid progesterone level was significantly different between the control group and the luteal cystic group. In addition, the pathological results revealed that cysts with a wall diameter of <3 mm can also be classified as luteal cysts. Therefore, a wall diameter of 3 mm cannot be considered an accurate criterion for differentiating follicular cysts from luteal cysts. It is well known that the follicle diameter is a criterion for distinguishing non-cystic follicles from cystic follicles on ultrasonography (13). In other studies, different follicular diameters have been regarded as diagnostic landmarks to distinguish between these two types of follicles. In this regard, Youngquist and Threlfall reported that follicular structures of 2.5 cm or larger are a differentiating criterion in the absence of a corpus luteum (14). Based on ultrasound measurements, ovulating follicles in cows have a diameter of 16-19 mm (15). Accordingly, they stated that follicles larger than 20 mm in diameter can also be cystic (16). In contrast, Mimounet al. (2021) and Dhara and Sharma (2019) defined ovarian cysts as structures with diameters of

at least 17 mm (17, 18). The results obtained from the present study showed that follicles with diameters of 17.8, 18.7, and 19.2 mm were detected in the pathological examination as luteal cysts, indicating the inaccuracy of setting 20 mm follicle size (in ultrasound images) as the diagnostic criterion.

A previous study reported that ovarian cysts are larger than non-cystic follicles and that follicles that develop into cysts grow in a manner similar to Graafian follicles (19). Khalil et al. (2019) also reported that the diameter of cystic follicles was greater than that of non-cystic follicles (13). The increase in diameter of ovarian follicular size was indicated by ultrasonography between non-cystic and follicular cystic groups and by pathology between non-cystic, follicular cystic, and luteal cystic groups. Therefore, the mean diameter of follicular cysts was less than the mean diameter of luteal cysts in ultrasonography method, but this amount differed prominently after pathological classification. This difference also revealed that ultrasound is probably not a precise tool for differentiating these two types of cysts.

According to previous studies, cysts with a wall diameter >3 mm are classified as luteal cysts, and those with a wall diameter <3 mm are classified as the follicular cysts. Therefore, it is possible to discriminate follicular from luteal cystic follicles based on the wall diameter (17, 20-22).

In the present study, prior to histopathological evaluation, all cysts according to their wall thickness (less

than or more than 3mm) were divided into follicular and luteal cysts, respectively, by ultrasound. However, after a histopathological study to confirm this accuracy, according to the literature, it was found that using ultrasound alone resulted in misclassification. It was determined that even though some cysts had wall thicknesses greater than 3 mm in diameter and were classified as luteal cysts by ultrasound, they were actually lined with intact or partial granulosa cells, placing them in the follicular cyst category. Conversely, cysts with wall diameters less than 3mm were considered follicular cysts; however, histological results unveiled counter-factual. For instance, some cysts were defined as follicular cysts with less than 3mm wall thickness, but histological examination showed no granulosa cells, and their luminal surfaces were covered by lipid-laden luteal cells.

The previous studies and also the results obtained from the ultrasound-based segmentation in the present work showed that the mean diameter of the wall in the luteal group was greater than that in the follicular group (16, 23-25). However, in this study, according to the pathology-based classification, the mean diameter of the wall in the follicular cystic group was greater than that in the luteal cystic group. Since the wall diameter of 3.4 mm measured by ultrasonography was found to be more accurately determined by pathology (with a repeatability of 2.3 times), the accuracy of wall diameter was found to be higher in pathology compared to ultrasonography. It seems that ultrasound is not an accurate diagnostic tool for differentiating between the two types of cysts based on the wall diameter; however, it is suitable for differentiation of cystic and non-cystic ovarian fluid-filled structures.

Pirestani and Jamali (2018) and Samadi et al. (2013) reported that the formation of ovarian cysts was influenced by the follicular fluid compositions (26, 27). The results of studies conducted by Barański et al. (2021) and Gareis et al. (2020) demonstrated that the concentration of 17-beta estradiol in the follicular fluid of cysts was significantly lower than that in adult follicles (28, 29). The results of the present study showed that the concentration of 17-beta estradiol was not significantly different between the non-cystic, follicular cystic, and luteal cystic groups in both ultrasonography and pathology classifications. Notably, a lower but non-significant amount of estradiol was observed in the follicular cystic group compared to the non-cystic and luteal cystic groups. It was expected that, due to greater steroidogenic activity in granulosa cells in follicular cysts, the amount of estradiol would be higher. Due to the presence of aromatase inhibitors within the follicular cysts, estrogen production can be inhibited, and this is why estrogen did not differ among groups (30).

Mimoune et al. (2021) reported that the amount of progesterone in the follicular fluid of cystic follicles was higher than that in normal follicles (17). Another study indicated that the concentration of progesterone in follicular cysts was significantly lower than that in mature follicles (31). In addition, Rodríguez et al. (2022) stated that the amount of progesterone in luteal cysts was higher than that in follicular cysts (32). In line with previous reports, our results showed that the amount of progesterone in the luteal cystic group was higher than that in the non-cystic group. This increase between the non-cystic and luteal cystic groups was found to be statistically significant. Although an increase in progesterone levels was observed in the luteal group compared with the follicular group, this increment was not statistically significant. In the classification based on pathology, an increase in the amount of progesterone was observed between the non-cystic and the luteal groups; however, this increase was not statistically significant.

Previous studies showed that there was no statistical correlation between serum estradiol levels in follicular and luteal cysts with different sizes of cyst diameter and wall diameter (10, 33). In the current study, a statistical correlation was observed between progesterone levels in the follicular fluid and both cyst and wall diameters. Considering the positive correlation coefficient, we can conclude that with an increase in the diameter of the cyst and wall in the luteal cystic group, progesterone levels also increase. In addition, we observed no correlation between estradiol levels and either follicle or wall diameters in any of the study groups based on ultrasonographic or pathological findings.

Conclusion

For many years, veterinarians have attempted to accurately diagnose and differentiate ovarian cysts based on the criteria of follicle diameter of >25 mm and wall diameter of 3 mm in ultrasound images in order to adopt the appropriate treatment protocol. However, the results of the present study showed that a wall diameter of 3 mm might not be an accurate criterion for distinguishing follicular cysts from luteal cysts, since pathological findings showed that cysts with a wall diameter of less than 3 mm can also be luteal cysts.

Acknowledgments

The authors are very grateful to the Research Deputy of Lorestan University for financially supporting this study.

Authors' Contributions

Ali Mohammad Alamdar Niaki: Sample preparation, writing the original draft. **Arash Kheradmand:** Investigation, methodology, supervision, review & editing. **Omid Dezfoulian:** Preparation and interpretation of pathology slides. **Masoud Alirezaei:** Hormonal assay.

Data Availability

All data generated are included in this published article.

Ethical Approval

Owing to the nature of this research, adherence to medical ethical principles is not required

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for Publication

Not applicable.

Funding

This research was funded by a grant awarded by the Lorestan University, Khorram Abad, Iran.

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