Original Article https://doi.org/10.47748/tjvr.1162968

TJVR 2023; 7 (1): 19-25

Turkish Journal of Veterinary Research

https://dergipark.org.tr/tr/pub/tjvr

e-ISSN: 2602-3695

Histopathological evaluation of uterine structure and immunohistochemical examination of MMP-1 and TIMP-1 in cows recovered from metritis

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Received: 18.08.2022 Accepted: 01.11.2022

ABSTRACT

Objective: The aim of this study is to compare the results of histopathological examination and immunohistochemical examination including matrix metalloproteinases 1 (MMP-1) and tissue inhibitor of matrix metalloproteinases (TIMP-1) of the uterus of healthy cows and the uterus of cows that have recovered from metritis.

Materials and Methods: The study materials obtained from the slaughterhouse were divided into two groups: the uterus of healthy cows (no metritis; Group N; n=10) and uterus of cows that had recovered from metritis about 45 days ago (metritis; Group M; n=10). Sections were taken from the uterus of both groups and were stained with hematoxylin and eosin (H&E) for pathological comparison. In addition, the sections were stained immunohistochemically for the examination of MMP-1 and TIMP-1 levels.

Results: The uterus showed pathological condition in Group M than in Group N. MMP1 immunopositivity was higher in the luminal epithelium ($p<0.01$), endometrial stroma, uterine gland ($p<0.05$) and myometrium sections ($p<0.01$) of the uterus in Group M compared to Group N. TIMP-1 immunopositivity of endometrial stroma and uterine gland sections decreased in Group M compared to Group N ($p<0.01$), However, TIMP-1 immunopositivity of myometrium sections was higher in Group M than Group N ($p<0.01$).

Conclusion: In conclusion, it was observed that the negative effects of metritis on the uterus persisted histologically even approximately 45 days after clinical recovery. However, this needs to be investigated extensively because time elapsed between the clinical recovery of the cows and the study may have been insufficient for the uterus to complete histological healing.

Keywords: *Cow, Metritis, MMP-1, TIMP-1, Uterus*

INTRODUCTION

It is known that one of the most important factors affecting the profitability of dairy animals is reproductive performance (LeBlanc, 2008; Almughlliq et al., 2017), which is expected to be reduced through uterine diseases (Baez et al., 2016). Uterine infections have an incidence of up to 50% in the postpartum period in dairy cows (Manimaran et al., 2016). It has also been reported that the most common uterine disease is metritis in dairy cows (Kurt et al., 2019), with an approximately 20% incidence (Pérez-Báez et al., 2021). Moreover, uterine diseases can occur in the form of endometritis or metritis. Endometritis is known as inflammation of the endometrium in the uterus and rarely causes systemic symptoms. Metritis is defined as inflammation of the uterine wall caused

by various pathogens and is a disease characterized by systemic findings such as fever, watery red-brown and foul-smelling uterine discharge, loss of appetite, and high heart rate (Genís et al., 2018). Metritis result in negative outcomes such as prolongation of the calving to conception interval, delayed resumption of ovarian cyclicity, decrease in conception rates, and milk yield and increase in culling rate in farm animals (Manimaran et al., 2016; Kurt et al., 2019; Pérez-Báez et al., 2021). Because uterine infections directly disrupt the uterine environment (Sheldon et al., 2006) within connective tissues and endometrial cells, histological recovery of the uterus can take time. Unless histological improvement of uterine occurs, the uterus cannot be considered to be ready for a new pregnancy. So, it is critical for uterine health that connective tissues and endometrial cells can regain their previous functions. As consequence, we think that the histological recovery of the uterus in cows suffering from metritis should be investigated comprehensively. In animals suffering from metritis, the histological status of the uterus can be monitored with Matrix metalloproteinases 1 (MMP-1) and the tissue inhibitor of matrix metalloproteinases (TIMP-1). Nikolov et al. (2020) reported that MMPs are known as zinc-dependent endopeptidases and have the ability to bind to components of the extracellular matrix. MMPs have different groups including collagenases (MMP-1 and MMP-13), gelatinases (such as MMP-2 and MMP-9), stromelysins (such as MMP-3), and membrane MMPs. Moreover, MMPs are involved in the remodeling of various tissues and organs, in many physiological and pathological processes (Chen et al., 2017; Li et al., 2017). It has also been stated that some of the MMP family can play a role in the remodeling of endometrial tissue (Chen et al., 2017). Activated macrophages in the wall of arterial vessels secrete MMPs. On the other hand, MMPs have the ability to change tissues, which is important for normal and pathological physiology (Nikolov et al., 2020). Therefore, it is understood that MMP-1 secretion increases in pathological conditions such as uterine infection. In this case, level of TIMP secretion, which is an inhibitor of MMPs, can be expected to change. Considering the above information, it is thought both MMP-1 and TIMP-1 levels can be related to the damage caused by the infection in the tissue.

The present study hypothesized that the negative effects of metritis on the uterus could persist even after clinical recovery. Therefore, the aim of this study is to compare the results of histopathological examination and immunohistochemical examination including MMP-1 and TIMP-1 of the uterus of healthy cows and the uterus of cows that have recovered from metritis.

MATERIALS and METHODS

Ethical approval

Ethical approval for this study was obtained from the Local Ethics Committee of Ceyhan Veterinary Faculty, Cukurova University, Adana, Turkey (Approval number: 08/01, date: 16.06.2021).

Animals and Groups

The present study was carried out on the uterus of a total 20 Holstein dairy cows obtained from the slaughterhouse. Study materials were divided into two groups: the uterus of healthy cows (no metritis; Group N; n=10) and uterus of cows that had recovered from metritis approximately 45 days ago (Group M; n=10). In addition, the cows in both groups were between about 3.5-5 years old, and did not have any clinical problems at the time of study. The previous health and disease information of the cows was obtained from the records, and metritis was characterized as previously described, taking into account findings such as fever, and fetid watery red-brown uterine discharge (Genís et al., 2018; Kurt et al., 2019). In addition, animals in two groups were included in the study according to the results of microbiological examination.

Microbiological examination

Swab samples were taken from the uterus under aseptic conditions for microbiological examinations immediately after slaughter in both groups. Gram-negative and Gram-positive bacteria isolation was performed in all swab samples taken. All processes were done according to standard procedures previously described (Markey et al., 2013). In Group N and Group M, animals that any bacteria could not be isolated in their intrauterine environment were included in the study.

Tissue collection, preparation and staining

Uteri of both groups were harvested immediately after slaughter. Then, the uteri were randomly divided into small pieces from their middle regions and immediately fixed in 10% neutral buffered formalin solution. These uteri were dehydrated with increasing series of alcohols and clarified using xylene. After these procedures, all fixed uteri were embedded in paraffin blocks, and serial sections of 5 μm thick were taken using a Rotary microtome.

Sections were stained with hematoxylin and eosin (H&E) for histopathological comparison (Feldman and Wolfe, 2014). In addition, MMP-1 and TIMP-1 immunohistochemistry were applied to these sections.

Immunohistochemistry and obtaining quantitative data

The prepared sections were stained for immunohistochemical examination of MMP-1 and TIMP-1 levels (Nagel et al., 2004; Naruse et al., 2009). The samples were washed in PBS (Phosphate-buffered saline without calcium and magnesium) and antigen retrieval was applied in citrate buffer. Then the samples were incubated in 3% H_2O_2. MMP-1 (Bioss Antibodies, MA, USA) and TIMP-1 (Biorbyt, Cambridge, UK) antibodies diluted 1:300 were dripped onto the samples, followed by overnight incubation. In order to prevent non-specific binding, the blocking solution was used before the antibody application, and the subsequent steps were performed with a ready-to-use kit (Thermo Scientific, Waltham, MA, USA). The chromogen reaction was performed with DAB (3,3 Diaminobenzidine) using a commercial kit (Thermo Scientific, Waltham, MA, USA). Samples that developed a reaction were counterstained with hematoxylin and covered with entellan and examined under a light microscope at 10x and 20x magnification (Zeiss Axio Imager 2).

Quantification of Immunohistochemistry Analyses

In order to examine MMP-1 and TIMP-1 immunoreactivities in stained sections, a scoring was done in three different areas determined in each section. The immunopositivity of MMP-1 and TIMP-1 was determined using H scoring. H-score classification was done with a scoring grade of 0 to 3 as previously described (Nakopoulou et al., 2003; Nagel et al., 2004) and detailed below:

- Grade 0: Absence of immunoreactivity
- Grade 1: Low level of immunopositive
- Grade 2: Increased level of immunopositive
- Grade 3: Very densely immunopositive

Finally, the obtained scores were analyzed statistically.

Statistical analysis

Obtained MMP-1 and TIMP-1 immunopositivity levels were analyzed statistically (IBM SPSS Statistics 24). For this purpose, independent samples t-test was used. Results were shown as mean ± standard deviation (mean ± SD) and $p<0.05$ was considered statistically significant.

RESULTS

Histopathology

In Group N, the presence of normal histological structures was observed in H&E sections. It was found that the luminal epithelium had a characteristic structure, the stroma was wide and regular, and the uterine glands were normally distributed. In addition, it was observed that the myometrium muscle tissue was thick and the vascular structures were in normal histological view between the fiber bundles.

In Group M, it was determined that epithelial vacuolization was intensely increased in the luminal epithelium. There was a deformative appearance in the uterine glands in the stroma, and an increase in mononuclear cell infiltration was observed in the stromal area. It was determined that the hyalinization image was intensified in the endometrium and myometrial transition region (Figure 1).

Table 1. Results of MMP1 and TIMP1 immunopositivity scoring in Group N and Group M.

		Group N	Group M	p value
MMP-1	Luminal Epithelium	1.20±0.61	2.27±0.69	<0.01
	Endometrial Stroma	0.90±0.48	1.23±0.57	<0.05
	Uterine Gland	0.93±0.58	1.30±0.65	<0.05
	Myometrium	0.57±0.50	2.07±0.87	<0.01
TIMP-1	Luminal Epithelium	0.57±0.63	0.40±0.56	>0.05
	Endometrial Stroma	1.27±0.58	0.57±0.63	<0.01
	Uterine Gland	1.40±0.56	0.50±0.63	<0.01
	Myometrium	0.27±0.45	0.70±0.70	<0.01

Immunohistochemistry

In the uterine sections of the two groups, luminal epithelial stroma and endometrial gland epithelium showed varying levels of TIMP and MMP positivity (Figure 2). On the other hand, although TIMP-1 immunopositivity was generally low in the myometrium of Group M, it was highly concentrated in the transition regions (Figure 3). MMP-1 immunopositivity was found to be significantly higher in the luminal epithelium ($p<0.01$), endometrial stroma, uterine gland ($p<0.05$) and myometrium sections ($p<0.01$) of the uterus in Group M compared to Group N. No significant difference was observed in the Luminal Epithelium of Group N and Group M in terms of TIMP-1 immunopositivity ($p>0.05$). TIMP-1 immunopositivity of endometrial stroma and uterine gland sections decreased in Group M compared to Group N ($p<0.01$), However, TIMP-1 immunopositivity of myometrium sections was higher in Group M than Group N ($p<0.01$). MMP-1 and TIMP-1 immunopositivity results are presented in detail in Table 1.

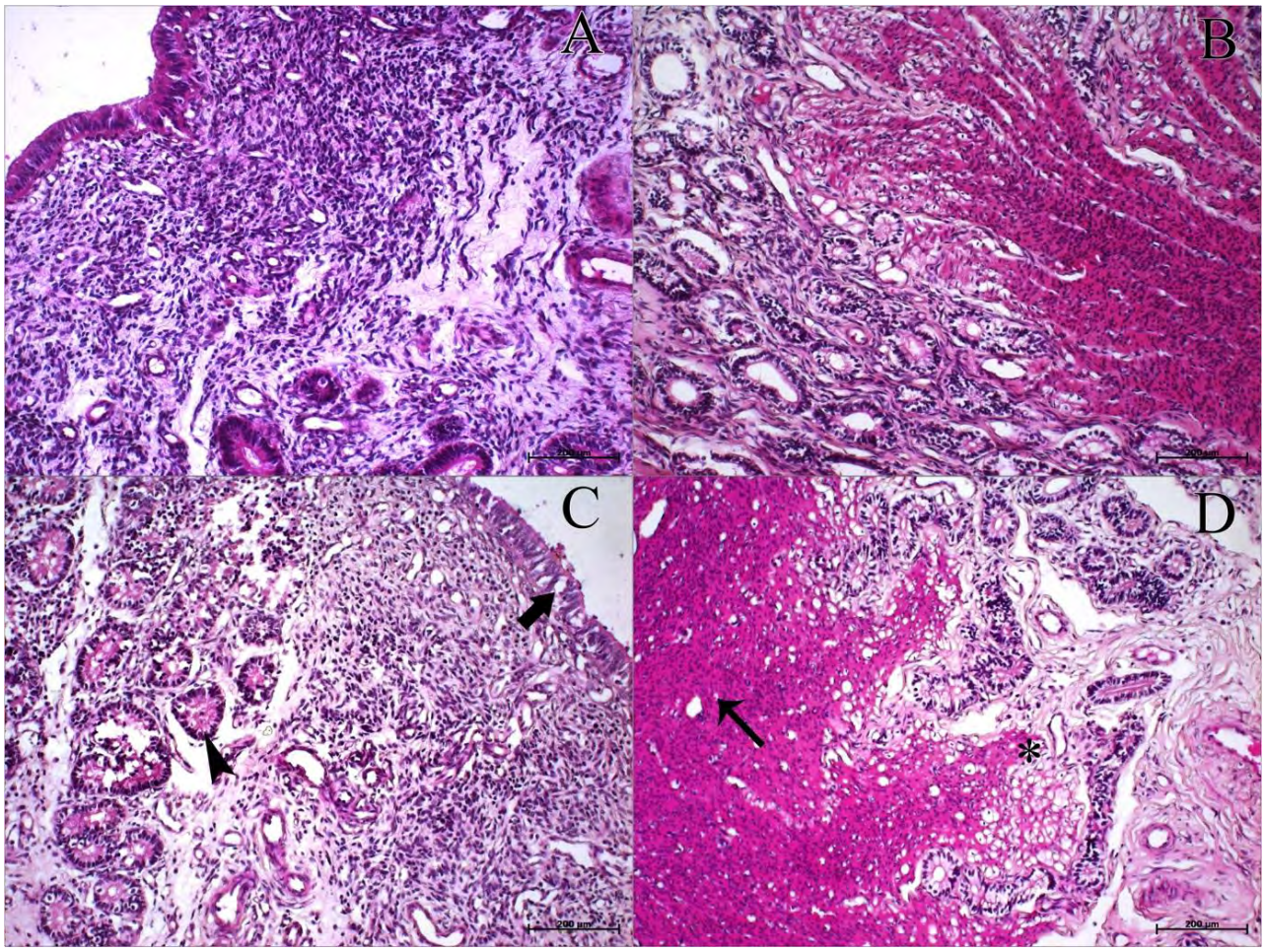

Figure 1. Light microscope examination of uterus sections in Group N (A, B) and Group M (C, D). Vacuolized epithelium image in uterine luminal epithelium (thick arrow), irregular image of the uterine glands (arrowhead), an increase in the appearance of hyalinization in the transition zone between the endometrial stroma and the myometrium (*) and vacuole and edema in the muscular layer of the myometrium (arrow). Staining: H&E, Bar: 200 µm.

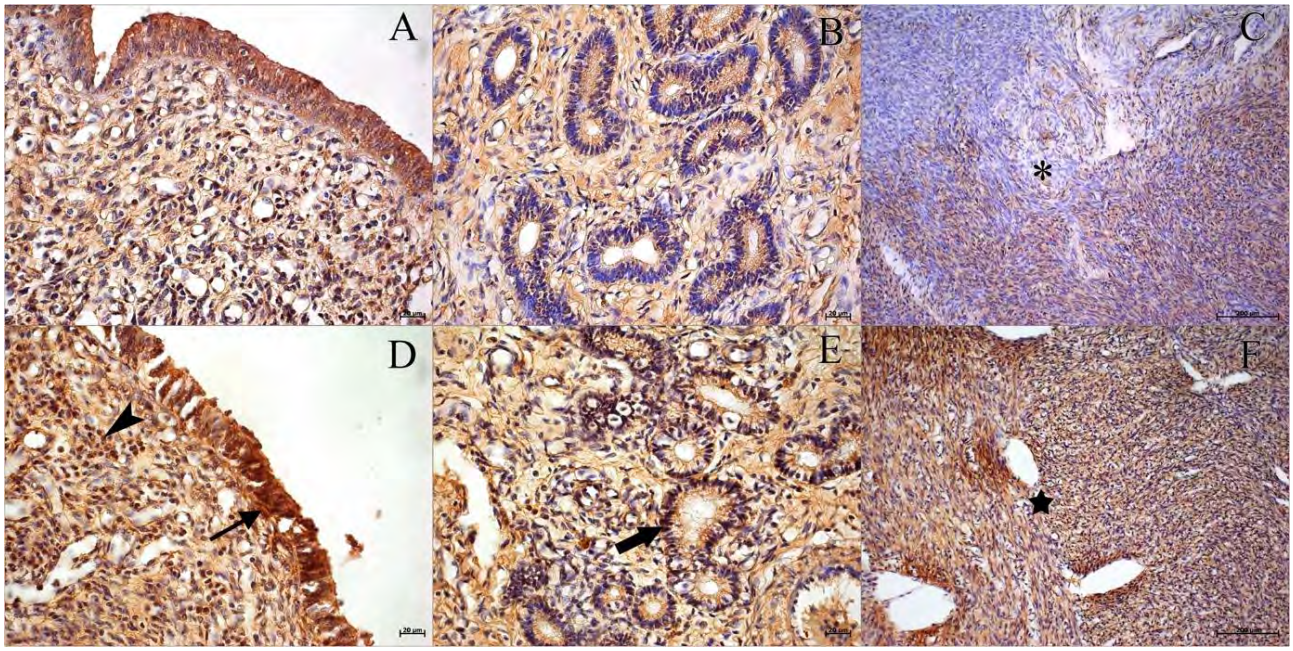

Figure 2. MMP-1 immunohistochemistry in Group N (A, B, C) and Group M (D, E, F). Presence of condensation of MMP-1 immunopositivity in the lumial epithelium (arrow) and stroma (arrowhead). Similar immunopositivity in uterine gland epithelium in Group N and Group M. Density difference in terms of MMP-1 between inner and outer muscle fiber bundles of myometrium (star). Concentration of MMP-1 immunopositivity in the myometrium (star). Staining: MMP-1 immunohistochemistry, Bar: 20 μm (A, B, D, E) 200 μm (C, F).

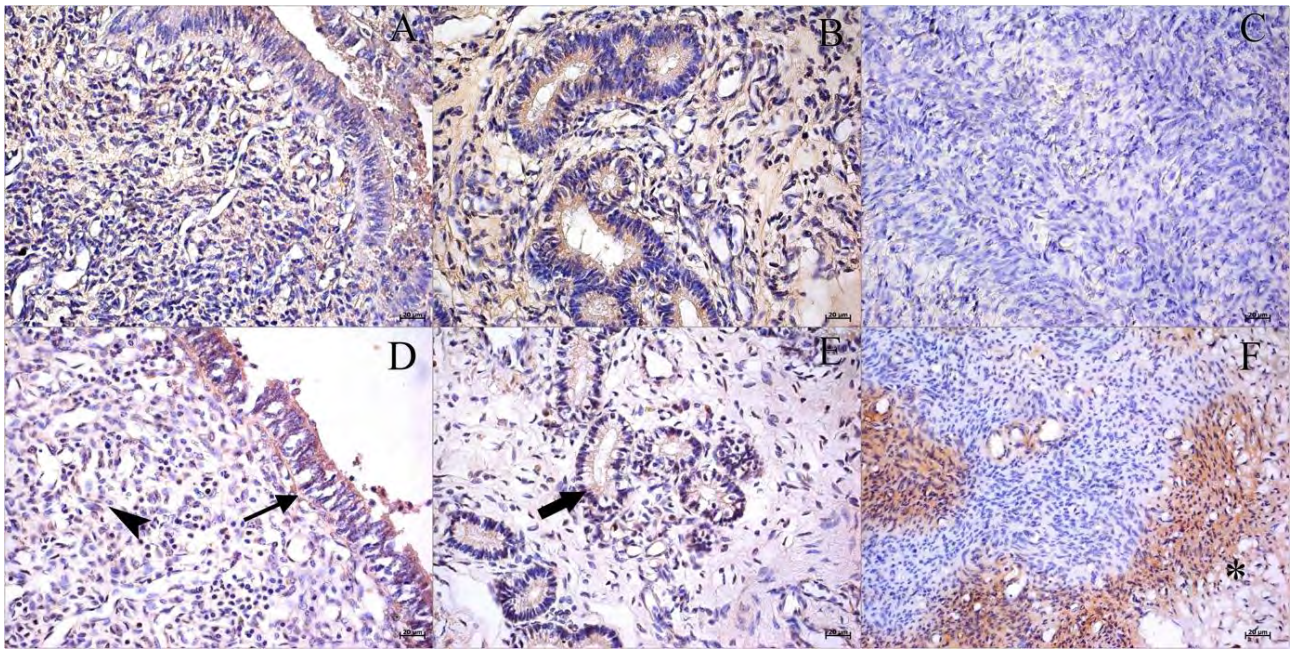

Figure 3. TIMP-1 immunohistochemistry in Group N (A, B, C) and Group M (D, E). In Group M, TIMP-1 condensation in the basal lamina of the luminal epithelium (arrow) and decreased TIMP-1 immunopositivity in the stroma (arrowhead). Low level of immunopositivity at the apical pole of the uterine gland epithelium (thick arrow). Although negative TIMP-1 areas are observed in the myometrium of Group N and Group M, areas of immunopositivity at different densities in the myometrium, especially in the edometrio-myometrial transition region, in Group M (star). Staining: TIMP-1 immunohistochemistry, Bar: 20 μm.

DISCUSSION

The present study investigated the histopathological effect of metritis on the uterus in cows recovered from metritis. For this purpose, the uteri of healthy cows (no metritis) and uteri of cows that have previously recovered from metritis were compared in terms of MMP-1 and TIMP-1 immunopositivity.

It is well known that metritis causes inflammatory symptoms such as edema in all layers of the uterus, leukocyte infiltration, and myometrial degeneration (Sheldon et al., 2006). On the other hand, it is stated that ovarian function is impaired in cows with uterine infection (Sheldon and Owens, 2018). As a result, fertility decreases. The only way to reverse this situation is to treat the disease allowing the return of the physiological uterine function. It is known that the uterus of cows that have recovered from metritis is restored histologically (Sheldon et al., 2006; Hansen, 2013). However, it cannot be estimated exactly how long this period lasts. Sheldon et al. (2008) reported that although epithelial regeneration is completed approximately 25 days after parturition, a period of 6-8 weeks after calving can be required to restore deeper tissue layers. However, some cases of uterine infections are known to prolong this period (Koh et al., 2018). On the other hand, it is a matter of interest whether the uterus returns to its histologically healthy function in cows with metritis. We suppose that one way to assess this was to investigate the level of regeneration in uterine cells and connective tissue. For this purpose, we investigated the positivity of immunohistochemistry of MMP-1, which is involved in the remodeling of various tissues including endometrial tissue (Chen et al., 2017; Li et al., 2017), and tissue inhibitor of MMP (TIMP-1) in the uterine tissue. We found that the uterus was in a pathological state since the expression levels of MMP-1 and TIMP-1 differed in Group M compared to Group N. However, no significant difference was found between the groups in terms of TIMP-1 in the luminal epithelium of the uterus. In fact, a previous report stated that the luminal epithelium may be the most sensitive site in the rat uterus (Seker et al., 2020). Thus, with this sensitivity, we think that the uterine lumen epithelium may be the first site to provide histological recovery in cow uterus with metritis. We were focused on two assumptions as the reason for this situation. The first is that metritis causes damage to the uterus that is histologically difficult to reverse. The second assumption is that the uterus in Group M has not yet recovered histologically and more time is needed for complete histological recovering. The second assumption is more likely to be true because cows in Group M are known to have clinically recovered from metritis approximately 45 days ago. Therefore, we think that this period cannot be sufficient to complete the histological healing of the uterus. We think that the histopathological results of the study also support this. However, in order to clarify this assumption, the histological recovery period should be determined by taking repeated biopsy samples from the uterus of live cows using biopsy forceps. For this purpose, we think that a similar study should be conducted on both live cows and slaughtered cows at different time periods of clinical recovery. We also think that factors such as immune system function, metabolic conditions, care, feeding, management, individual differences or the type of bacteria that cause metritis can affect the histological recovery period in the uterus. Furthermore, it is noted that a normal endometrium is required for a successful breading (Sheldon and Owens, 2018). Similarly, Koh et al. (2018) reported that an abnormal uterine environment can affect normal embryo development and reproductive performance in a variety of ways. In the present study, results of histopathological MMP-1 and TIMP-1 show that the histological restoration of the uterus is not complete and they do not yet have a normal endometrium in Group M compared to Group N. Considering above information, it is estimated that cows with metritis may experience fertility problems even approximately 45 days after the disease has clinically resolved.

CONCLUSION

In conclusion, it was observed that MMP-1 immunopositivity increased in various parts of the uterus of cows recovered from metritis. However, TIMP-1 immunopositivity decreased in endometrial stroma and uterine gland sections and increased in myometrial sections in the uterus of cows recovered from metritis. This demonstrated that the negative effect of metritis on the uterus persisted histologically even approximately 45 days after clinical recovery. However, this needs to be investigated extensively because time elapsed between the clinical recovery of the cows and the study may have been insufficient for the uterus to complete histological healing. Therefore, whether the negative effect of metritis on the uterus is

permanent or temporary should be clarified with further studies.

ACKNOWLEDGMENTS

The authors would like to thank Assist. Prof. Dr. Ugur Seker for his kind support in histological studies, and Laboratory Veterinarian Şifa Karahan for her support in microbiological studies.

Conflict of Interests: The authors declared that there is no conflict of interests.

Financial Disclosure: The authors declared that this study has received no financial support.

Author's Contributions: SK principal investigator, planning the study, manuscript preparation. TW contribution to data evaluation and preparation of the manuscript. FE contribution to the planning of the study, field studies and literature research. All authors read and approved the final manuscript. SK: Serdal Kurt, TW: Thomas Wittek, FE: Funda Eşki. All authors have read and agreed to the published version of the manuscript.

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