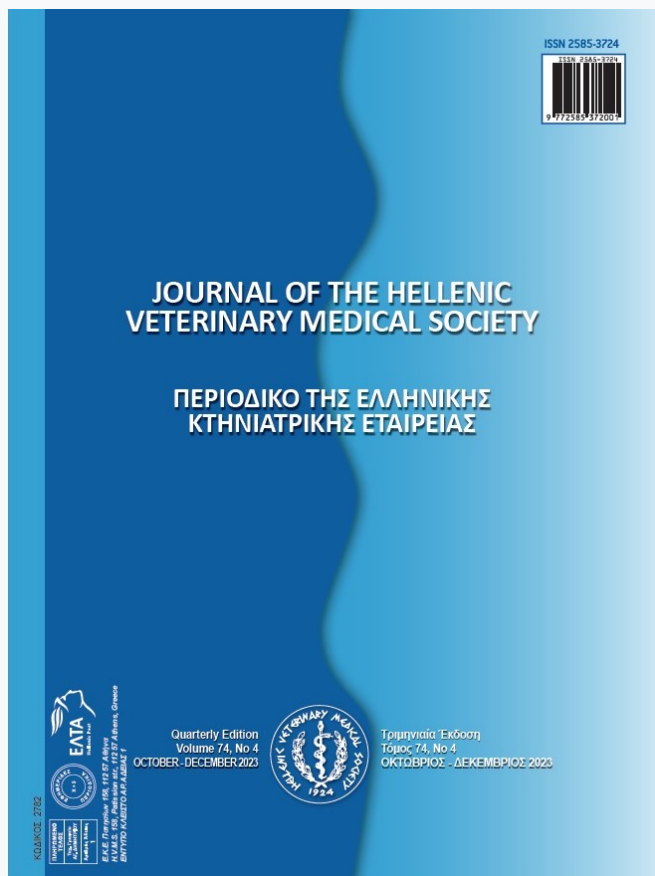


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Immunohistochemical Demonstration of Mast Cells in Bovine Papillomatous Digital Dermatitis of Holstein-Friesian Dairy Cattle

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ABSTRACT: Mast cells (MC) are unique members of immune system; their location and functions are of great importance in health and disease status. This study aims to evaluate MCs distribution and heterogeneity in healthy and digital dermatitis lesions of dairy cattle for the first time. A total of 50 skin samples, 25 healthy and 25 with digital dermatitis (DD) lesions were sampled in Holstein-Friesian dairy cattle. All samples were stained with hematoxylin-eosin (H&E) for microscopic examination and also stained with Toluidine blue for MCs demonstration. Epidermal acanthosis and hyperkeratosis with dermal inflammatory cell infiltrations were in consistent with digital dermatitis lesions. Spirochetal agents were successfully demonstrated with Warthin-Starry staining in 22 out of 25 DD samples. Increased number of mast cell was observed in digital dermatitis samples when compared with healthy skin samples. The average number of intact MCs was 4.6 ± 2 and 9.3 ± 1 in healthy and digital dermatitis samples, respectively. Two-fold increase in the number of intact MCs in digital dermatitis samples was observed. Degranulated mast cell numbers in tissue sections were also higher in digital dermatitis samples and 4.25-fold increase was recorded in affected skin samples. Immunophenotype of MCs in skin samples were identified by immunohistochemical staining with anti-tryptase and chymase antibodies. Only tryptase positive MCs were observed in healthy and DD samples. In the statistical analysis, differences in the mean intact and degranulated MCs were found to be significant (mean intact: $p < 0.05$, mean degranulated: $p < 0.01$). Our results possibly suggest that MCs may have important roles in the pathogenesis of bovine digital dermatitis.

Keywords: Bovine papillomatous Digital dermatitis; Holstein-Friesian cattle; Immunohistochemistry; Mast cells

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INTRODUCTION

Bovine digital dermatitis (BDD) is one of the most common infectious diseases of the foot skin in dairy cattle and causes serious economic losses worldwide. BDD was first described in Italy in 1974 as a disease affecting 70% of adult dairy cattle. The prevalence of the disease in the herd can reach up to 74% (Cheli and Mortellaro, 1974; Orsel et al., 2017). Similarly, there are data showing that the prevalence among herds can vary between 0% and 83% (Holzhauer et al., 2006). Serious financial losses due to decreased milk production, treatment costs and animal culling are major drawbacks of BDD. Annual cost of BDD has been reported to be around 190 million dollars in the United States (Losinger, 2006).

BDD is characterized macroscopically by papillomatous tissue growths on the plantar surface of the foot over the coronary bands. (Cheli and Mortellaro, 1974; Evans et al., 2016). In histopathological examination, acanthosis, parakeratosis, hyperkeratosis, intraepidermal pustules, infiltrations of inflammatory cells in dermis are major lesions. Although neutrophil leukocytes constitute big portion of these inflammatory cells (Görgül et al., 2002; Demirkan et al., 2018) other mononuclear leukocytes such as lymphocytes, plasma cells and macrophages are also present in dermis. BDD is of multifactorial etiology; large number of anaerobic bacteria and spiral shaped *Treponema* species are commonly isolated from clinical cases (Evans et al., 2009; Klitgaard et al., 2013; Demirkan et al., 2018; Caddeyand De Buck, 2021). The exact pathogenesis of BDD is still obscure. Hypoxic changes (Gomez et al., 2012) and various bacteria are major actors in the etiology of BDD. Recently, some studies have been conducted on the interaction between causative spirochete species and the immune system to understand the pathogenesis of the disease (Elliott and Alt, 2009; Moe et al., 2010; Refaai et al., 2013). In a study, inflammatory responses of foot skin fibroblasts and keratinocytes against to treponeme whole-cell sonicates have been investigated in the context of RANTES/CCL5, MMP-12, TNF- α , TGF- β , and TIMP3 molecules (Evans et al., 2014). They speculated that significantly upregulated expression of target molecules (RANTES/CCL5, MMP-12, TNF- α , TGF- β , and TIMP3) in dermal fibroblasts may involve BDD lesion formation. In another study, it was documented that neutrophils that encounter causative agents at inflammation area synthesize more cytokines and chemokines for modulation of inflammation and tissue regeneration (Refaai et al., 2013). In

the same study, T and B lymphocyte distributions and strong upregulation of IL-8 in keratinocytes have been identified in clinical BDD cases. Until now; however, presence, activity and immunophenotypes of mast cells have been omitted in the understanding of the pathogenesis of clinical BDD cases. The present study was designed to reveal the presence, activity, and immunophenotypes of mast cells in clinical BDD cases to fill the aforementioned gap.

Mast cells (MCs) are found in large numbers in certain body parts such as respiratory tract mucosa, gastrointestinal mucosa and skin which are in close contact with the external environment. Therefore, MCs are basically divided into two main subgroups as mucosal and cutaneous mast cells. These cells can also be classified according to their cytoplasmic enzyme contents; chymase (+), tryptase (+) and both tryptase and chymase (+) mast cells. Localizations of bovine MC send their enzyme contents in various organs have been documented, but there is no data regarding their presence in the foot skin. MCs are principal members in allergic reactions; moreover, very recent studies demonstrate they play active roles in the pathogenesis of some infectious diseases. MCs with phagocytic properties engulf infectious disease agents, so it should be kept in mind that mast cells may have local phagocytic functions against infectious agents responsible for the formation of digital dermatitis. On the other aspect, mast cells are known to induce proliferation of various cell types such as endothelial, smooth muscle, mesenchymal and epithelial cells (Marks et al., 1986; Cairns et al., 1996).

The role of MCs in BDD pathogenesis is still obscure. We believe that presence of MCs in bovine foot skin which is very vulnerable area due to constant contact with external environment (feces and urine) may be important in the pathogenesis of BDD. The proliferative effects of MCs granules on various cell types are known, they may contribute to formation of proliferative papillomatous skin lesions which are cardinal clinical and histopathological findings in BDD. According to the authors' knowledge, there is no study on the presence of mast cells in BDD lesions. Since the significance of MCs and their immunophenotypes are not yet clear in bovine foot, in the present study, demonstration, number, distribution and immunophenotyping of mast cells and their activation in healthy and digital dermatitis lesions of dairy cattle was aimed.

MATERIALS AND METHODS

Tissue Sampling and Histopathological Examination

Skin samples were obtained from local slaughterhouses in Bursa, Turkey. A total of 50 skin samples, 25 healthy and 25 with BDD specific lesions were sampled in Holstein-Friesian dairy cattle. The samples were placed in 10% buffered formalin solution and fixed for 48 hours at room temperature. Following fixation, tissues were routinely processed, and 4 micrometer serial sections were cut and adhered to poly-L-lysine-coated slides. All samples were stained with hematoxylin-eosin for microscopic examination and stained with Toluidine blue for demonstration of Mast cells. Tissue samples displaying papillomatous digital dermatitis lesions were also stained with Warthin-Starry silver stain to demonstrate the causative agents (Warthin and Chronister, 1920). BDD was confirmed by gross and histopathological findings.

Warthin-Starry Staining for Spirochetes

Sections from healthy and digital dermatitis samples were stained with Warthin-Starry method to demonstrate spirochetal agents. Commercially available Warthin-Starry staining kit from Bio-Optica (04-040903, Italy) was used according to the instructions of manufacturer.

Demonstration of Mast Cells with Toluidine Blue Staining

Tissue sections were stained with toluidine blue to identify mast cells. Briefly, toluidine blue solution was prepared as follows; 0.5 g of toluidine blue (198161, Sigma Aldrich, St. Louis, MO, USA) was dissolved in 70% ethanol, 5 ml of this solution was taken and mixed with 45 ml of 1% sodium chloride solution, and the sections were incubated in toluidine blue for 30 minutes at room temperature. After washing with tap water, the sections were dehydrated through graded alcohol solutions and cleared in xylene. Images obtained from 10 randomly selected areas at x400 magnification. Intact and degranulated mast cell numbers in healthy and bovine digital dermatitis samples were recorded. Results were expressed as the average number of mast cells.

Immunohistochemical Staining

To investigate mast cells in tissue samples according to their enzyme content, tissues were stained with commercially available anti-chymase (sc-59586, Santa Cruz, USA) and anti-tryptase (sc-59587, Santa

Cruz, USA) antibodies, which are known to work in bovine tissues. Deparaffinized tissues were boiled in citrate buffer (pH:6) at 600 watts for 3 times for 2 minutes to reveal antigens. When the temperature of the solution was equalized with room temperature, 3% hydrogen peroxide solution was added on the slides and endogenous peroxidase activity was suppressed. Then, protein block solution was placed on the slides and incubated for 10 minutes. After decanting the protein block solution without any washing, primary antibodies were applied to the tissues and incubated overnight at +4°C. At the end of the period, the tissues were washed 3 times in phosphate buffer solution (PBS) for 5 minutes and secondary antibody (TL015-HDJ, Thermo Scientific, USA) was applied on the tissues and incubated for 20 minutes. At the end of the period, streptavidin biotin solution (TL015-HDJ, Thermo Scientific, USA) was applied to the tissues washed in the same way with PBS and waited for 15 minutes. After a similar washing process, the reaction was completed by applying diaminobenzidine (DAB) on the tissues for 2 minutes. All tissues were counter stained with Harris' hematoxylin solution (HHS16, Sigma Aldrich, USA). During immunohistochemical staining, bovine udder tissue is used as positive control since all phenotypes are present.

Statistical analysis

Mean intact and degranulated mast cell counts obtained from healthy and digital dermatitis samples were compared with T-test using SPSS program.

RESULTS

Macroscopic Findings

Severe proliferative skin lesions were noted in the sampled tissues. Just above the heels, nodular, cauliflower-like, vegetative appearance hyperplastic papillomatous tissue growths with an average size of 3.5 cm \pm 0.4 cm were noted. In the cross-sections applied to the tissues, it was seen that the border of epidermis and dermis could be easily distinguished. Irregular growths in the epidermis were also evident (Figure-1 and 2).

Histopathological Findings

In light microscopic examination, severe hyperkeratosis (25 cases), parakeratotic hyperkeratosis (21 cases), sub corneal pustular epidermitis (19 cases), balloon-like degeneration of epithelial cells (25 cases), dermal neutrophil leukocyte infiltrates (25 cases), lymphocytic plasmocytic mononuclear cell infil-



Figure 1. Epithelial overgrowths (arrow) and wart formation in the digital skin of affected cattle.

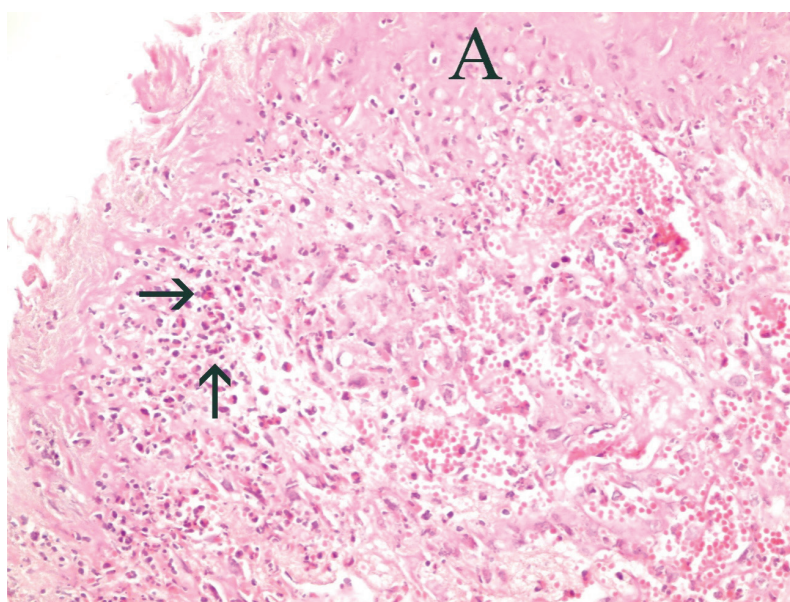


Figure 2. Intraepidermal pustular changes, infiltration of polymorphonuclear leukocytes (arrow), acanthosis and hyperkeratosis (A), hematoxylin-eosin staining, X200 magnification.

trations were observed in tissue samples (25 cases). Hyperemia in dermal blood vessels and endothelial hypertrophy were evident. Increased amount of collagenous matrix and fibroblastic proliferation were noted in the dermis (Figure-2). In healthy animals, normal architecture of the skin was preserved. Keratinized stratified squamous epithelial cells and the dermis rich in collagen tissue were observed within normal limits. None of the skin samples in healthy animals displayed prominent inflammatory changes.

Warthin-Starry Staining

Twenty-two out of 25 (88%) digital dermatitis

samples were positively stained with the kit. Spiral-shaped black bacteria on a yellowish background were demonstrated. Most of the agents were located within deeper acanthotic epidermal layer, in some cases (3 out of 22, 13%) spirochetal bacteria were also observed just beneath the stratum basale of epidermis.

Toluidine Staining

In animals in the control and digital dermatitis groups, mast cells with granules showing metachromasia in toluidine staining were found just below the epidermis and around the blood vessels located in the dermis (Figure-3 and 4). It was observed that the

mean number of mast cells in skin samples with digital dermatitis was higher than in the control groups. An average of 4.6 ± 2 mast cells were found in each objective area in the foot skin sections of the healthy animals, while this number was 9.3 ± 1 in digital dermatitis. When the number of mast cells in healthy samples was normalized to 1, digital dermatitis samples were displayed 2.02-fold increase in number of mast cells. Average degranulated mast cell numbers in healthy and digital dermatitis samples were 1.2 and 5.1, respectively. Again, when we normalized the number of degranulated mast cells in healthy samples to 1, digital dermatitis samples had 4.2-fold increase

in the number of degranulated mast cells. In the statistical analysis, differences in the mean intact and degranulated mast cells were found to be significant (mean intact: $p < 0.05$, mean degranulated: $p < 0.01$).

Immunohistochemical Staining

To demonstrate the phenotype of mast cells in healthy and digital dermatitis samples we used commercially available primary anti-chymase and anti-tryptase antibodies. Staining with anti-chymase and anti-tryptase antibodies showed only tryptase (+) mast cells were positively reacted with antibody (Figure-5 and 6). No chymase (+) mast cells were iden-

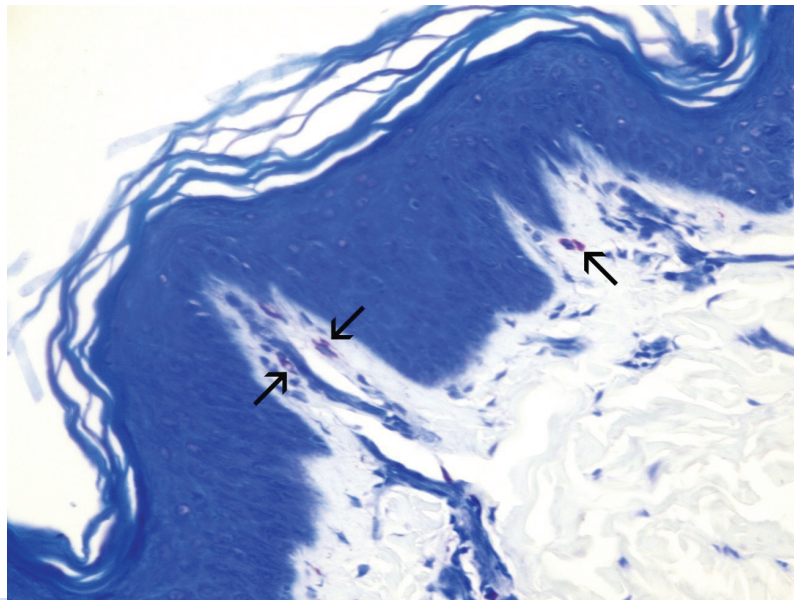


Figure 3. Mast cells with metachromatic granules (arrows) in healthy skin samples, Toluidine blue staining, X400 magnification.

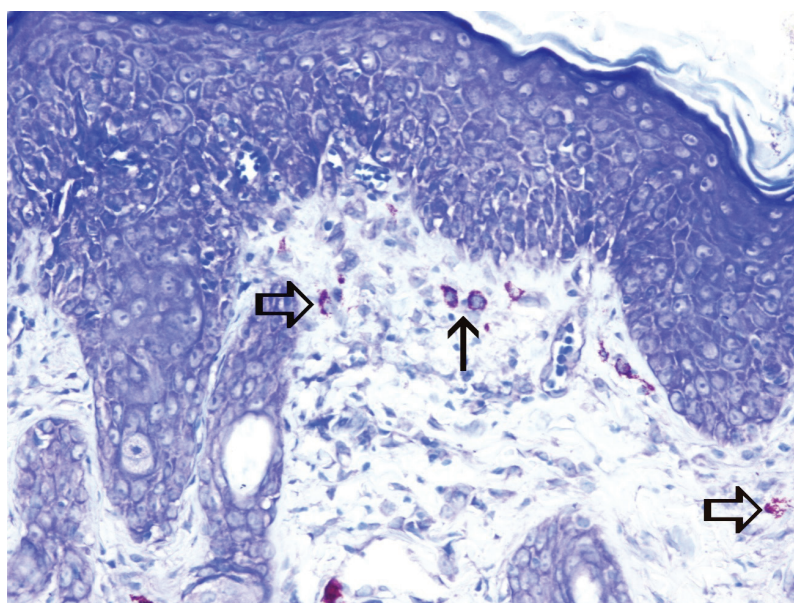


Figure 4. Intact mast cells with metachromatic granules in their cytoplasm (arrows) and degranulated mast cells (open arrows) in skin sample with digital dermatitis, Toluidine blue staining, X400 magnification.

tified. Chymase (+) mast cells were demonstrated in bovine mammary tissue samples which were previously known to have both chymase (+) and tryptase (+) phenotype MCs.

DISCUSSION

In this study, presence of mast cells and their immunophenotypes in the foot skin of dairy cattle with digital dermatitis were investigated for the first time. Dairy cattle breeding is extremely important for the economy and human health. Foot diseases are serious

welfare and health problems, and they are resulted with production losses in dairy cattle. BDD is indisputably one of the most important foot diseases of cattle due to the severity of lesions and higher prevalence in herds. The foot skin is constantly subjected to external environmental stimuli such as physical trauma, urine, and feces, and depending on the stage of BDD, ulcerative wounds and/or papillomatous growths is formed on the heels. Determination of the presence and immunophenotype of cutaneous mast cells of foot skin may provide useful data for BDD.

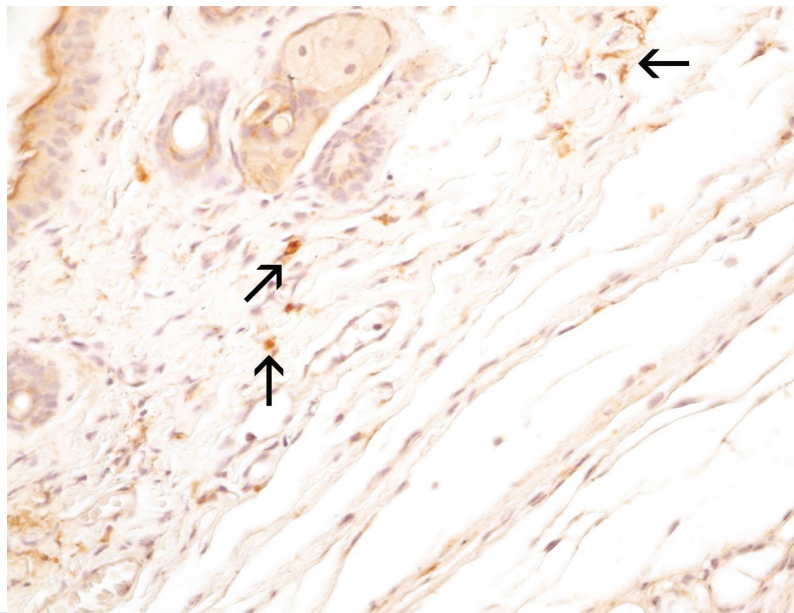


Figure 5. Tryptase positive mast cells (arrows) in the dermis of healthy skin samples. Streptavidin-biotin peroxidase, DAB chromogen, X400 magnification.

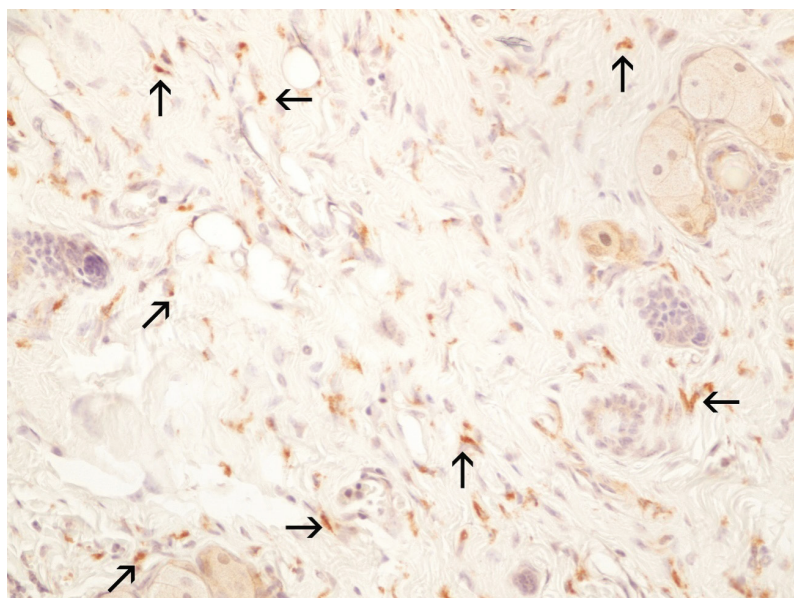


Figure 6. Tryptase positive mast cells (arrows) in the dermis of skin samples with digital dermatitis. Streptavidin-biotin peroxidase, DAB chromogen, X400 magnification.

Histopathologically, severe acanthosis, infiltrations of large numbers of polymorphonuclear (especially neutrophils) and mononuclear leukocytes in skin layers have been reported, but no data were found regarding the presence of MCs among those inflammatory cells. To fill this gap and shed light on future studies on the pathogenesis and treatment options of the disease, mast cells and their immunophenotypes in bovine foot skin were investigated in the submitted study. For the confirmation of BDD, in addition to unique gross findings, Warthin-Starry staining procedure was followed to demonstrate causative agents on suspected tissue samples. Most of digital dermatitis samples (22 out of 25) were stained positively; black spiral bacteria on a yellowish background were evident. The rest three samples did not show any positive reaction with Warthin-Starry staining; however, those samples were having characteristic gross and microscopic changes with no hesitation.

Mast cells are unique cells of the immune system and play important roles in allergic reactions and infectious diseases. MCs contain multifunctional cellular granules which are primarily responsible for modulation and regulation of inflammation. Their functions in allergy, asthma, anaphylaxis, and various immune responses are known in detail, but results of some studies on the function of mast cells direct attention into the infectious diseases (Siebenhaar et al., 2007; DiNardo et al., 2008; Nakamura et al., 2013; Zimmermann et al., 2019). In addition to classical functions of MCs, their phagocytic properties have been documented (Lima et al., 2013). Moreover, upon bacterial challenge, MCs quickly responds and secrete many inflammatory cytokines like TNF- α , IL-3, IL-4 and IL-13 to orchestrate other immune cells. Zuerner et al. (2007) have reported that innate immune functions of bovine macrophages were hampered by treponemal cell wall fragments *in vitro*. In their study, they exposed macrophages to treponemal cell sonicates and evaluated many inflammatory genes. According to their data, IL-1, -6, -11 genes were downregulated or unchanged and genes encoding proteins responsible for antigen presentation were downregulated. While mast cells are known with their undeniable functions in innate immunity and they serve as a pool of cytokines (Mukai et al., 2018), it is intriguing that their possible roles in BDD have not been investigated.

Albeit in small numbers, there are data on the localization of mast cells in various organs of cattle, (Küther et al., 1998; Özen et al., 2007; Ertuğrul and

Kurtdede, 2017). In a study, the presence of mast cells in urinary system tissues of cattle were demonstrated histochemically (Ertuğrul and Kurtdede, 2017) Özen et al. (2007) successfully demonstrated the presence of mast cells in the ovarian tissues of healthy cows. However, these studies did not provide any information on immunophenotypes of mast cells on various locations. Küther et al. (1998) showed that mast cells with different immunophenotypes were found in forestomach, duodenum, lung, lymph node, uterus and skin. According to their findings, mast cells were primarily located in the dermal layer of skin, only tryptase (+) mast cells were noticed immunophenotypically. In the submitted study, mast cells were successfully identified in the dermis of healthy and digital dermatitis samples. We noticed that MCs in dermis were only positive for tryptase phenotype both in healthy and digital dermatitis lesions. No positivity was found in staining with anti-chymase antibody. To confirm the specificity of the chymase antibody, we used bovine mammary tissue as positive control and chymase positive mast cells were successfully demonstrated in udder tissue.

The submitted study includes comparison of intact and degranulated mast cells and mast cell immunophenotypes in healthy and papillomatous digital dermatitis lesions in cattle. We observed 2.02 and 4.2-fold increase in number of intact and degranulated MCs in healthy and digital dermatitis samples, respectively. The presence of higher number of cutaneous intact and degranulated mast cells in animals with digital dermatitis when compared with healthy animals strongly suggest that MCs may play important roles in the pathogenesis of BDD. Increased amount of fibrotic tissue and acanthotic changes in keratinocytes were noticed in all digital dermatitis samples. Since mast cell tryptase is known as a potent mitogen for epithelial cells and fibroblasts, we believe that increased number of tryptase (+) mast cell in dermis may contribute to the proliferation of keratinocytes and fibroblasts in BDD. It is known that different immunophenotypes of mast cells are predominant-type in certain inflammatory reactions (Rozniecki et al., 1995; Knight et al., 2000; Huang et al., 2001; Caughey, 2007). Moreover, heterogeneous population of MCs have been reported in lung tissue even in different microanatomical positions (Bradding, 2009). Some *in vitro* studies showed that MCs with one phenotype can shift to another MC phenotype in special culture circumstances (Mierke et al., 2000; Hsieh et al., 2005).

CONCLUSION

We successfully demonstrated MCs in bovine digital dermatitis lesions for the first time. Presence of MCs in bovine foot and determination of their phenotypes may bring new perspectives into BDD management. There are many chemical compounds capable of inhibition and stabilization of MCs, these chemicals may be useful in the alleviation of hyperplastic epidermal lesions. Further clinical studies are strongly needed to test the action of MC stabilizers or

inhibitors in the treatment of BDD.

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CONFLICT OF INTEREST

None declared by the authors.

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