The Effects of Flunixin Meglumine and Meloxicam on the Mucosal Immunity of the Uterus in Postpartum Cattle

Running title: Effects of Flunixin and Meloxicam on Uterine Immunity

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Absract

During the transition period, dairy cows experience significant inflammatory immune responses that can act as erosive agents to the uterus. The immune response modulation and reducing the negative effects of systemic inflammation after parturition, may be achaived by postpartum administration of non-steroidal anti-inflammatory drugs (NSAIDs). The aim of this study was to investigate the effects of flunixin meglumine and meloxicam on local humoral immunity in the uterus of postpartum cows.

The study involved 60 cows that had experienced their second to fourth parturition, which were randomly divided into three groups. Treatment groups one and two received intravenous (IV) flunixin meglumine and meloxicam, respectively, on the 3rd and 8th days following delivery. The third group, serving as the control, received an IV injection of sterilized normal saline. Cervical mucus samples were collected on the 4th, 9th, and 15th days post-delivery from the treated animals. The uterine mucosal antibody titers against two primary pathogens, *T. pyogenes* and *E. coli*, were evaluated using an in-house enzyme-linked immunosorbent assay (ELISA).

Additionally, serum antibacterial activity against these bacteria was assessed using the microdilution method.

The antibacterial activity and mucosal antibody value against *E. coli* revealed no statistically significant differences among the analyzed groups. The specific mucosal antibody titers against *T. pyogenes* elevated during study period in the control and flunixin groups. The mucosal antibody titers against *T. pyogenes* demonstrated significant differences between the meloxicam and flunixin groups on days 4 and 9. In conclusion, these data suggest no adverse effects of the treatments on the mucosal immunity of the uterus and differing effects of NSAID treatment on antibody titers against various pathogens. These differences may be attributed to the induction of immune responses against target pathogens and variations in bacterial concentration in the environment. Veterinarians should consider farm management practices when administering flunixin and meloxicam.

Keywords: Cattle, parturition, flunixin meglumine, meloxicam, mucosal immunity

1. Introduction

The dairy cows undergo significant physiological, and immunological changes during the transition period, that are critical for delivery, secration of colostrum, and milk (1). These situation, along with with decreased dry matter intake, lead to a negative energy balance and immunosuppression. Consequently, in the periparturient period, dairy cows are particularly susceptible to infections in the reproductive system (2). Usually, uterine infection occurred following parturition in dairy cows due to the immunecompressed states and perecense of bacteria. Two bacteria, *Trueperella pyogenes* and *Escherichia coli*, play significant roles in uterine inflammation and infection. For this reason, *E. coli* lipopolysaccharide (LPS) has been widely used to investigate the response of genital epithelial cells to bacterial contamination.

A variety of peptides and proteins secreted from the epithelial cells of the genital tract possess non-specific antimicrobial properties and function as regulators of inflammation. The precise regulatory mechanisms by which endometrial epithelial cells secrete antimicrobial peptides remain unclear, but they likely involve cytokine stimulation. Immunoglobulins play a crucial role in preventing local infections by neutralizing invading bacteria, promoting phagocytosis, and stimulating complement activation pathways. The sequential appearance of IgM, IgA, and IgG has been reported in cervico-vaginal secretions following the challenge of the bovine uterus with *Campylobacter fetus* (3). There is evidence to suggest that the concentrations of different classes of immunoglobulins may vary in different regions of the genital tract. In cows with abnormal puerperium, both IgA and IgG concentrations rise rapidly in uterine fluids as endometritis develops (4).

Administration of Non-steroidal anti-inflammatory drugs (NSAIDs) could balance the immune response after postpartum and reduce the negative effects of inflammation after childbirth (5).

NSAIDs function by inhibiting the cyclooxygenase (COX) enzyme. The inducible form of this enzyme, COX-2, is responsible for the production of prostaglandins involved in inflammation, fever, and pain. Consequently, NSAIDs are utilized for their antipyretic, analgesic, and anti-inflammatory properties (6). Shwartz et al. (2009), evaluated the effects of treating with flunixin after calving, and suggested that the uterine trauma and subsequent inflammation resulting from normal parturition could be alleviated through NSAID treatment (7).

Meloxicam, as an NSAID, inhibits the action of cyclooxygenase-2 (COX-2) and has been suggested as an effective method for alleviating inflammation (8); additionally meloxicam was mentioned as an analgesic drug (9) and have benefit effects on treatment of the uterine prolapse (10). The meloxicam adminstration in combination with antibiotics for treatment of mild to moderate clinical mastitis resulted in a higher treatment of infections, achieving elevation in first-time pregnancy after artificial insemination, reduced inoculation rates, and a more quantity of pregnant cows. Newby et al. (2017) reported that cows treated with flunixin meglumine, a potent anti-inflammatory drug, after calving experienced a higher incidence of retained placenta and, consequently, a greater occurrence of clinical metritis and postpartum fever. Additionally, these cattle exhibited decreased milk yield and an increased risk of stillbirth, retained placenta (RFM), and metritis (11). The current study designed to evaluates the effects of flunixin meglumine and meloxicam on the local immunity of the uterus in postpartum cows.

2. Materials and Methods

The evaluated samples was collected from August to September 2023. The Friesian Holstein cows from a dairy farm in Esfahan province, Iran were included in current study. A total of 60 cows that had undergone their second, third, and fourth parturitions were included into three studed groups. The animals were omitted from study if they experience the ocurrance of dystocia, gave birth to twins, mastitis, lameness, retained placenta, or other clinical conditions. In alignment with objectives, the initial inflammatory responses within the uterus are crucial for creating an immune responses and stablishing the subsequent repairment following parturition. Selection of the primary injection three days after parturition, was guided by these considerations and the presence of the necessary inflammatory phase to promote uterine repair. In Treatment Group 1, an intravenous (IV) injection of flunixin meglumine 2.2 mg/kg was injected on the 3rd and 8th days post-delivery (11). Treatment Group 2 received an IV injection of meloxicam at a dosage of 0.5 mg/kg on the same days after delivery (Pascottini et al. 2020). As the control group, Treatment Group 3 received an IV injection of sterilized normal saline.

2.1. Sampling

Considering the half-life of the drugs, which is less than 24 hours, the sampling time was selected to be 24 hours after treatment. The cervical mucus samples of the treated animals was collected on the fourth day, ninth day and the Day15 after delivery. The mucous membrane of the external opening of the cervix was removed using a plastic uterine pipette that was connected to a syringe

to create suction. The collected samples was poured in 2mL microtubes and was homogenized with the same volume of phosphate buffered saline (PBS). The suspension was mixed by vortexing for 3 minutes. After centrifugation 20 minutes at 10,000 rpm, the supernatant was removed by and stored at -70 ºC (12).

2.2. Preparation of bacterial antigens

The used *E. coli* and *T. pyogenes* bacteria were prepared from previously isolated stains at Microbiology Department of the Faculty of Veterinary Medicine, Shahid Chmran University of Ahvaz, Iran. These strains had been characterized through biochemical properties. The isolated strains were cultured individually on blood agar medium enriched with 5% sheep blood for 24 to 48 hours in an incubator set at 37°C. Suspensions of *E. coli* and *T. pyogenes* were prepared simultaneously, achieving concentrations of approximately 12×10^8 CFU/mL. Bacterial inactivation was achieved by incubating the bacterial strains in a 1% formalin solution for 24 hours at room temperature. After the elimination of formalin by centrifugation, the bacterial inactivation was confirmed by culture of the formalin-treated bacteria. The antigen solutions were sonicated by ten cycles, each lasting for 15 seconds. The prepared antigens were then stored in a -20°C.

2.3. Preparation of specific antibodies against *E. coli* and *T. pyogenes*

Polyclonal specific IgGs were purified from the rabbit´s hyperimmune serum. Briefly, the concentration of the bacterial antigens was adjusted to a turbidity of 4 McFarland. A suspension of 0.5 mL antigen was mixed with an equal volume of complete Freund's adjuvant and intramuscularly injected to the thigh muscle of two rabbits. In addition, four booster were injected at 10-day intervals, containing 0.5 mL of bacterial suspensions adjusted to a 2 McFarland concentration, along with with incomplete Freund's adjuvant 0.5 mL. An indirect ELISA was used for confirmation of the appropriate antibody titers; after that, blood samples were collected and the hyperimmune sera were collected. The purification of IgG from hyperimmune serum was achieved according to Khosravi et al. (2021) using the ion exchange chromatography method (13). Briefly, the hyperimmune sera were precipitated with a 33% saturated ammonium sulfate. After centrifugation, the sediments were dialyzed against a phosphate buffer (0.02 M, pH 7.2) for 48 hours. The ion exchange chromatography was conducted on a DEAE-cellulose column. The resin powder was soaked in a 0.02 M phosphate buffer solution (pH 7.2) for 24 hours and then transferred to the column. The dialyzed solution was added to the column and incubated for 2 hours. The IgG fraction was eluted by adding 0.02 M phosphate buffer solution (pH 7.2). The purity and reactivity of the isolated antibodies were evaluated through respectively, dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the ELISA, as described in the next section.

2.4. Evaluation of total antibody titer against *E. coli* and *T. pyogenes*

The mucosal antibody titer against *E. coli* and *T. pyogenes* were detected by using an in-house indirect competitive ELISA. Initially, the concentrations and volumes of the reagents were optimized to facilitate competition between cattle antibodies in mucus samples and the specific

antibodies from rabbits. Also, in addition to the negative samples, serial dilutions of the specific rabbit antibodies, without mucus samples, were used as controls for the tests. The prepared sonicated bacterial antigens concentration were adjusted to 10 µg/mL in carbonate-bicarbonate coating buffer (pH=9.6) and 100 µL were poured into the microplate of ELISA. After 24 hours incubation at 4°C, three steps of washing were conducted using PBS contain 0.05% Tween 20 solution (PBS-T). The blocking step was conducted by adding 250 µL of skimmed milk 4% to each well. In a separate plate, 10 µL of the supernatant from the mucus samples were diluted with 90 µL of PBS in each well and then simultaneously poured into wells. After that, rabbit anti-*E. coli* IgG or rabbit anti-T. pyogenes IgG 150 µg/mL in volume of 20 µL was added to each well and the plate was incubated for 1 hour. After washing, 100 µL of horseradish peroxidase-conjugated anti-rabbit antibody was added to each well and incubated at room temperature for an additional hour. Subsequent to five washes step, 75 µL of TMB solution was added to each well. After 10 minutes, the stop solution 2M sulfuric acid 75 µL was poured in each well. The optical densities were then assessed using an spectrophotometer (AccuReader, Taiwan) at a wavelength of 450 nm. The prepared samples containing rabbit anti-*E. coli* IgG or rabbit anti-*T. pyogenes* IgG 32.5-300 µg/mL was used as positive controls. Protein concentrations in the analyzed samples were determined using the Bradford method. The mucosal specimen 20 µL, distilled water 80 µL, and Bradford buffer 100 µL were mixed in a 96-well microplate wells. Additionally, six dilutions of bovine serum albumin (BSA) were prepared to serve as standards. Finally, the optical density (OD) values were measured using a spectrophotometer at a wavelength of 600 nm. There was a inverse relationship between the optical densities obtained in the competitive ELISA and the antibody titers; so, the results were normalized by detract from 1 and dividing by their corresponding protein volume.

2.5. Serum antibacterial effects

The microdilution method in a sterile 96-well microplate was utilized for assessment of the antibacterial effects of cattle sera. The *T. pyogenes* and *E. coli* strains were cultivated and a single colony from each strain was transferred into nutrient broth media at 37˚C until it achieved a turbidity corresponding to the 0.5 McFarland standard. Each sample equal to 50 µL, was combined with the same volume of the nutrient broth medium in the specified wells of the microplate. Subsequently, 50 µL of the bacterial suspension was poured into the wells. The optical densities were measured at 600 nm using a spectrophotometer, after which the plates were incubated for 24 hours at 37˚C, and the optical densities were recorded as previously outlined. The interpretation of results involved calculating the bacterial growth in both the test and control wells (14). Because of the inverse relationship between the obtained optical densities and the antibacterial activity of the mucus samples, the results were normalized by detract from 1 and divided by their corresponding protein volume.

2.6. Data analysis

The statistical analysis was conducted using version 23 of SPSS software by two-way ANOVA. GraphPad Prism software version 8 was utilized for plotting the results.

3. Results

The purification of IgG from the hyperimmune serum of rabbits immunized against *T. pyogenes* and *E. coli* was performed using ion exchange chromatography. The results obtained from SDS-PAGE revealed distinct bands in each lane of the gel, indicating the effective separation of IgG (Figure 1A). The fractions containing the antibodies were pooled, and the final concentrations were standardized to 300 µg/mL. Additionally, the ELISA result indicated hyperimmunization of the treated rabbits (Figure 1B).

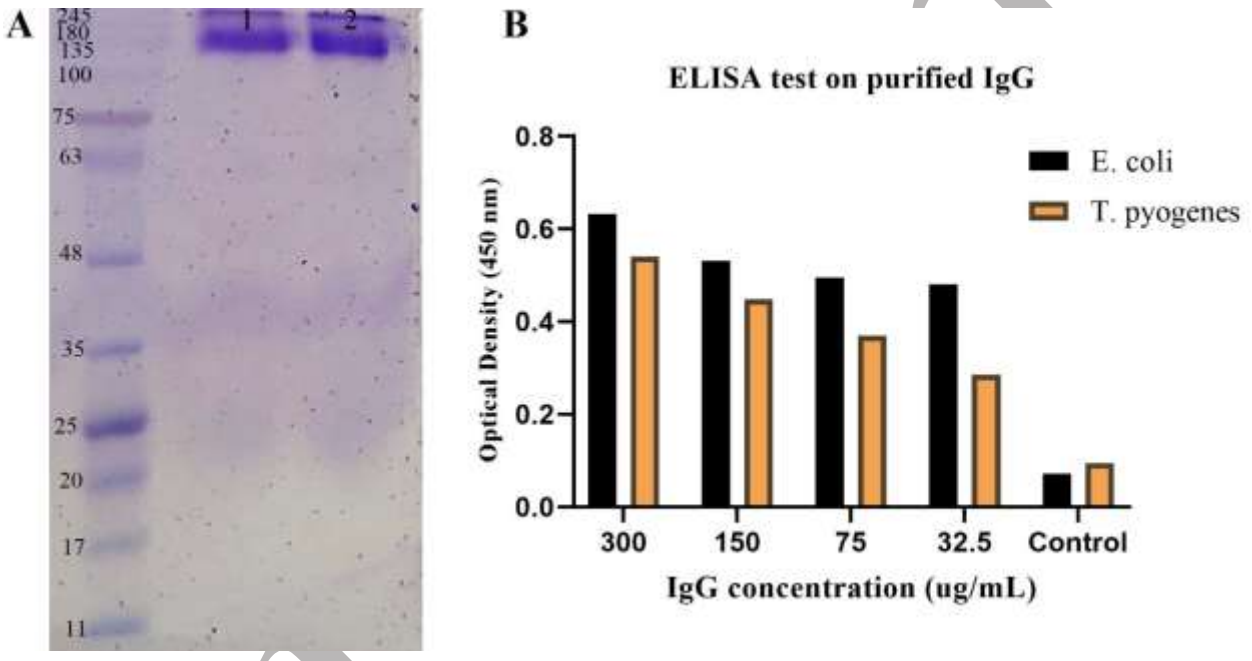

Figure 1. Evaluation of the purity and reactivity of the extracted IgG from rabbits' hyperimmune serum against *T. pyogenes* and *E. coli*. The standard molecular weight proteins are indicated on the gel. A) 1. Purified IgG against *T. pyogenes*; 2. Purified rabbit IgG against *E. coli*. B) The optical density obtained from the ELISA test at four concentrations of the purified IgG against *T. pyogenes* and *E. coli*.

The mean mucosal antibody titers against *E. coli* and *T. pyogenes* are presented separately in Figure 2. The specific mucosal antibody titers against *E. coli* in the flunixin, meloxicam, and control groups at various time points showed no significant differences. Comparisons between groups at specific time points on days 4, 9, and 15 also indicated no significant differences (Figure 2A).

The specific mucosal antibody titer against *T. pyogenes* showed a non-significant elevation over time in both the control and flunixin groups at the time of calving. In contrast, the meloxicam group exhibited nearly constant titers on day 9, with a non-significant decrease in antibody titer observed on day 15. The specific mucosal antibody titers against *T. pyogenes* on days 4 and 9 demonstrated significant differences between the meloxicam and flunixin groups (P = 0.199 and P = 0.013, respectively) (Figure 2B). The antibacterial activity against *E. coli* revealed no statistically significant differences among the analyzed groups. However, the meloxicam group exhibited significantly higher antibacterial activity against *T. pyogenes* (P = 0.008) on day 9, with non-significantly higher activity on other days compared to the flunixin and control groups (Figures 2C and 2D).

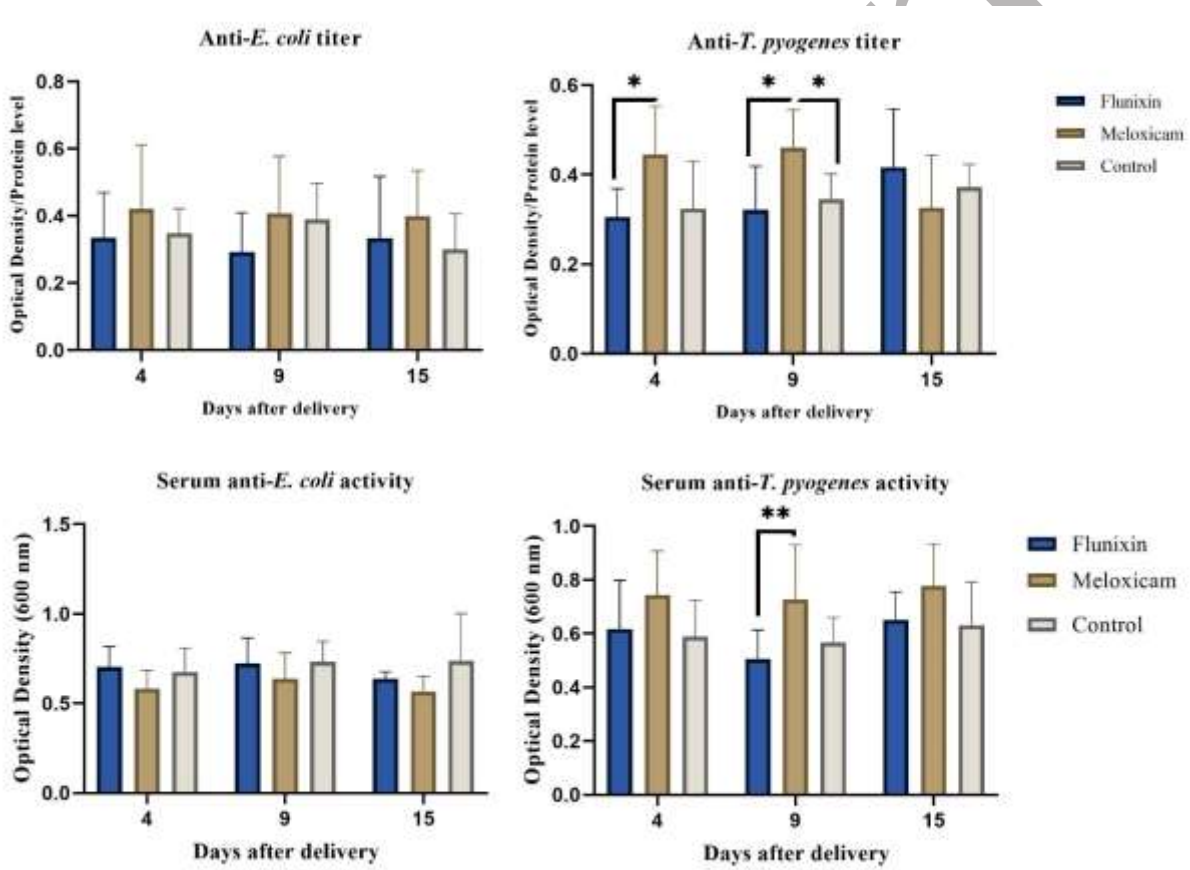

Figure 2. The cattle serum immunoglobulin (mean ± standard deviation) titer (A and B) and antibacterial activity (C and D) against *E. coli* and *T. pyogenes* in cows treated by flunixin, meloxicam and control groups. The single asterisk (*) denotes a significance level of p<0.033, two asterisks (**) indicate a significance level of p<0.002, and three asterisks (***) represent a significance level of p<0.001.

4. Discussion

The structure of the reproductive system serve as crucial mechanisms against infections that may occur during and after calving, thereby preventing pathogenic organisms from infiltrating the uterus. It is also noteworthy that all healthy animals typically harbor a significant bacterial population in the uterus following a normal delivery. Consequently, uterine contamination post-calving is unavoidable. *E. coli* may play a main role in initiating the infectious process and its detection several days after parturition in the uterus correlates with an elevation in metritis and endometritis. Additionally, *T. pyogenes* has been identified in the uterine environment of cows suffering from metritis (15). The treatment groups in the current study, while demonstrating an increase in antibody titer and antibacterial activity against *T. pyogenes*, did not show a significant difference in antibody titer or antibacterial activity against *E. coli* when compared to the control group. indicating that NSAIDs treatment did not have adverse effect on the main humoral aspects of the innate and adaptive immune responses. However, the results revealed certain points and limitation that will be discussed in the following sections.

The presence of IgA-secreting cells in the vagina and the secretion of blood or endometrial IgG into the uterus constitute the primary humoral immunity of the reproductive system in cattle. These antibodies can directly neutralize uterine infectious agents or indirectly enhance the phagocytosis of microorganisms by opsonizing them or stimulating complement pathways (16). Evidence suggests a correlation between systemic or local immunization and its effect on the levels of specific antibody titers in the uterus or bloodstream (17). The induction of local specific antibody titers, including IgM, IgG, and IgA, following local challenges with *Campylobacter fetus* has been previously reported by Corbeil et al. (1974) (18). However, the antibody isotype may be influenced by the type of stimulating antigens (19). Therefore, in line with the results of the mucosal antibacterial and antibody titer against *T. pyogenes* in the current study, it is not surprising to observe the effects of systemic modifications of immune responses on the regulation of the mucosal immunity.

In agreement with higher non-significant titer against *E. coli* and significant elevation of the antibody titer against *T. pyogenes* in meloxicam treated group, an investigation revealed that weaned calves treated with meloxicam exhibited elevated antibody titers following vaccination against *bovine respiratory syncytial virus, bovine herpesvirus type 1*, *parainfluenza virus type 3*, and *coronavirus*, as well as increased serum bactericidal activity. However, these treated animals faced a higher risk of treatment complications (19). Duffield et al. (2009) administered flunixin at a dosage of 1.25 g intramuscularly (I.M.) for cows and 1.1 g for heifers. Treatment was provided approximately 2 hours after calving, with a repeat injection given approximately 24 hours later. No significant effects of treatment were observed on the risk of subsequent hypocalcemia, displaced abomasum, clinical ketosis, mastitis, or milk yield. However, cows treated with flunixin

were more likely to retain their fetal membranes and had an increased risk of developing metritis (20).

The highest concentrations of total secretory immunoglobulin A (sIgA) antibodies were found in the vaginal mucus of cows treated oronasally with lipopolysaccharide (LPS). This indicates that oronasal LPS was capable of inducing a short-lived IgA response in the vagina of dairy cows. Inducing an immune response in the genital tract of transition cows is crucial, as uterine infections are prevalent and can lead to infertility, making them the primary reason for the culling of dairy cows (21). Previously, the dose-dependent effects of flunixin and meloxicam were reported on leukocytes of calf (22). Furthermore, Bednarek et al. (2003) documented the immunosuppressive benefits of both medications in calves affected by enzootic bronchopneumonia. They also proposed a combined therpy using antibiotics and meloxicam for calves which resulted in an elevated level of interferon-gamma, although no changes in immunoglubolin concentration were detected four days post-treatment (23). Pascottini et al. (2020) noted that administering meloxicam once daily for four days, two weeks postpartum, reduced systemic inflammatory responses but did not affect the inflammatory status of the endometrium (8). The treatment of the animals three days after delivery allowed for the occurrence of initial inflammatory immune responses within the first three days post-delivery. This response could have beneficial effects on defenses against infectious microorganisms and the repair phases of injuries following parturition. Therefore, the insignificant effects observed in the bactericidal tests and antibody titers support the hypothesis of the current study.

The oral administration of meloxicam to beef cattle treated with LPS did not influence the neutralizing antibody titer following vaccination against respiratory pathogens, nor did it affect the concentration of acute-phase proteins or the level of TNF-α (24). In this regard, the current study found no significant difference in the uterine antibody titer against *E. coli*. Similarly, a previous research exhibited that the transdermal injection of flunixin meglumine had no effects on the antibody titers against *M. haemolytica* in beef heifers (25).

The current study evaluated the effects of systemic injections of flunixin and meloxicam on the modulation of local uterine immunity against two primary pathogenic bacteria. The results indicated no significant difference in the levels of specific antibodies and antibacterial activity against *E. coli* among the flunixin, meloxicam, and control groups at the same time point. This finding contrasts with some studies suggesting that NSAID administration is associated with immunosuppression effects. Also, the anti-*T. pyogenes* antibody titer was higher in meloxicam-treated animals just four days after calving (one day post-treatment) compared to flunixin group. In the treated group receiving meloxicam, local vaginal antibody titers may be neutralized due to increased exposure to *T. pyogenes*, resulting in decreased antibody titers on day 15. Furthermore, unlike the steady-state antibody titers observed in the meloxicam group on days 4 and 9, the induction of a humoral immune response against *T. pyogenes* during the 15 days following calving led to a non-significant elevation in antibody titers in the control and flunixin groups. These results suggest that, in addition to the humoral factors evaluated in the current study, the role of innate immune cells should also be assessed to determine the precise effects of meloxicam.

Overall, the data indicate no adverse effects of treatment with flunixin and meloxicam on the humoral elements of the mucosal immunity in uterus. However, differing effects of NSAIDs treatment were observed on antibody titers and antibacterial activity against *E. coli* and *T. pyogenes* pathogens. The observed differences can be ascribed to the activation of immune responses directed towards specific pathogens, as well as fluctuations in bacterial density within the environment. It is essential for veterinarians to take into account farm management strategies when prescribing flunixin and meloxicam.

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Author Contribution

Study concept and design: M.Kh and S.Gh. Investigation, acquisition of data, analysis and interpretation of data, writing original draft: M.Kh and A.R. Analysis and interpretation of data: M.Kh. Statistical analysis: M.Kh and M.M. Administrative, technical, and material support: M.Kh and S.Gh. Study supervision: M. Kh, S. Gh, M.M and M. N. Critical revision of the manuscript for important intellectual content: M.Kh. All authors review and edited the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data availability

All analyzed/raw data are available on request from the corresponding author.

Ethics approval

All tests on animals were carried out in accordance with animal protection laws and guidelines of research ethics committee of Shahid Chamran University of Ahvaz, Iran, with accepted code of IR.SCU.REC.1403.022.

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