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).

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

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

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

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1. Introduction

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

٦٠ A variety of peptides and proteins secreted from the epithelial cells of the genital tract possess non-
٦١ specific antimicrobial properties and function as regulators of inflammation. The precise
٦٢ regulatory mechanisms by which endometrial epithelial cells secrete antimicrobial peptides remain
٦٣ unclear, but they likely involve cytokine stimulation. Immunoglobulins play a crucial role in
٦٤ preventing local infections by neutralizing invading bacteria, promoting phagocytosis, and
٦٥ stimulating complement activation pathways. The sequential appearance of IgM, IgA, and IgG has
٦٦ been reported in cervico-vaginal secretions following the challenge of the bovine uterus with
٦٧ *Campylobacter fetus* (3). There is evidence to suggest that the concentrations of different classes
٦٨ of immunoglobulins may vary in different regions of the genital tract. In cows with abnormal
٦٩ puerperium, both IgA and IgG concentrations rise rapidly in uterine fluids as endometritis develops
٧٠ (4).

٧١ Administration of Non-steroidal anti-inflammatory drugs (NSAIDs) could balance the immune
٧٢ 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

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

2.2. Preparation of bacterial antigens

116 The used *E. coli* and *T. pyogenes* bacteria were prepared from previously isolated stains at
117 Microbiology Department of the Faculty of Veterinary Medicine, Shahid Chmran University of
118 Ahvaz, Iran. These strains had been characterized through biochemical properties. The isolated
119 strains were cultured individually on blood agar medium enriched with 5% sheep blood for 24 to
120 48 hours in an incubator set at 37°C. Suspensions of *E. coli* and *T. pyogenes* were prepared
121 simultaneously, achieving concentrations of approximately 12×10^8 CFU/mL. Bacterial
122 inactivation was achieved by incubating the bacterial strains in a 1% formalin solution for 24 hours
123 at room temperature. After the elimination of formalin by centrifugation, the bacterial inactivation
124 was confirmed by culture of the formalin-treated bacteria. The antigen solutions were sonicated
125 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*

127 Polyclonal specific IgGs were purified from the rabbit´s hyperimmune serum. Briefly, the
128 concentration of the bacterial antigens was adjusted to a turbidity of 4 McFarland. A suspension
129 of 0.5 mL antigen was mixed with an equal volume of complete Freund's adjuvant and
130 intramuscularly injected to the thigh muscle of two rabbits. In addition, four booster were injected
131 at 10-day intervals, containing 0.5 mL of bacterial suspensions adjusted to a 2 McFarland
132 concentration, along with with incomplete Freund's adjuvant 0.5 mL. An indirect ELISA was used
133 for confirmation of the appropriate antibody titers; after that, blood samples were collected and the
134 hyperimmune sera were collected. The purification of IgG from hyperimmune serum was achieved
135 according to Khosravi et al. (2021) using the ion exchange chromatography method (13). Briefly,
136 the hyperimmune sera were precipitated with a 33% saturated ammonium sulfate. After
137 centrifugation, the sediments were dialyzed against a phosphate buffer (0.02 M, pH 7.2) for 48
138 hours. The ion exchange chromatography was conducted on a DEAE-cellulose column. The resin
139 powder was soaked in a 0.02 M phosphate buffer solution (pH 7.2) for 24 hours and then
140 transferred to the column. The dialyzed solution was added to the column and incubated for 2
141 hours. The IgG fraction was eluted by adding 0.02 M phosphate buffer solution (pH 7.2). The
142 purity and reactivity of the isolated antibodies were evaluated through respectively, dodecyl sulfate
143 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*

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

١٤٨ antibodies from rabbits. Also, in addition to the negative samples, serial dilutions of the specific
١٤٩ rabbit antibodies, without mucus samples, were used as controls for the tests. The prepared
١٥٠ sonicated bacterial antigens concentration were adjusted to 10 μg/mL in carbonate-bicarbonate
١٥١ coating buffer (pH=9.6) and 100 μL were poured into the microplate of ELISA. After 24 hours
١٥٢ incubation at 4°C, three steps of washing were conducted using PBS contain 0.05% Tween 20
١٥٣ solution (PBS-T). The blocking step was conducted by adding 250 μL of skimmed milk 4% to
١٥٤ each well. In a separate plate, 10 μL of the supernatant from the mucus samples were diluted with
١٥٥ 90 μL of PBS in each well and then simultaneously poured into wells. After that, rabbit anti-*E. coli*
١٥٦ IgG or rabbit anti-T. pyogenes IgG 150 μg/mL in volume of 20 μL was added to each well and the
١٥٧ plate was incubated for 1 hour. After washing, 100 μL of horseradish peroxidase-conjugated anti-
١٥٨ rabbit antibody was added to each well and incubated at room temperature for an additional hour.
١٥٩ Subsequent to five washes step, 75 μL of TMB solution was added to each well. After 10 minutes,
١٦٠ the stop solution 2M sulfuric acid 75 μL was poured in each well. The optical densities were then
١٦١ assessed using an spectrophotometer (AccuReader, Taiwan) at a wavelength of 450 nm. The
١٦٢ prepared samples containing rabbit anti-*E. coli* IgG or rabbit anti-*T. pyogenes* IgG 32.5-300 μg/mL
١٦٣ was used as positive controls. Protein concentrations in the analyzed samples were determined
١٦٤ using the Bradford method. The mucosal specimen 20 μL, distilled water 80 μL, and Bradford
١٦٥ buffer 100 μL were mixed in a 96-well microplate wells. Additionally, six dilutions of bovine
١٦٦ serum albumin (BSA) were prepared to serve as standards. Finally, the optical density (OD) values
١٦٧ were measured using a spectrophotometer at a wavelength of 600 nm. There was a inverse
١٦٨ relationship between the optical densities obtained in the competitive ELISA and the antibody
١٦٩ titers; so, the results were normalized by detract from 1 and dividing by their corresponding protein
١٧٠ volume.

2.5. Serum antibacterial effects

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

2.6. Data analysis

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

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3. Results

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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
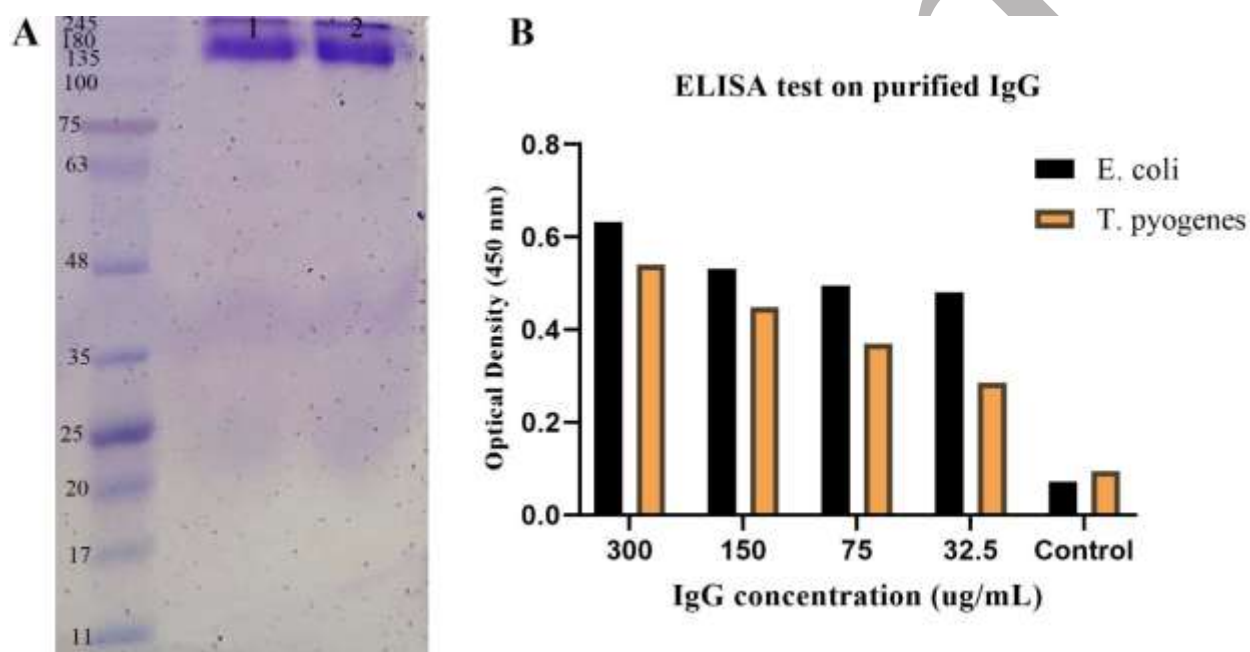the treated rabbits (Figure 1B).

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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*.

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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).

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٢٠٧ The specific mucosal antibody titer against *T. pyogenes* showed a non-significant elevation over
٢٠٨ time in both the control and flunixin groups at the time of calving. In contrast, the meloxicam
٢٠٩ group exhibited nearly constant titers on day 9, with a non-significant decrease in antibody titer
٢١٠ observed on day 15. The specific mucosal antibody titers against *T. pyogenes* on days 4 and 9
٢١١ demonstrated significant differences between the meloxicam and flunixin groups (P = 0.199 and P
٢١٢ = 0.013, respectively) (Figure 2B). The antibacterial activity against *E. coli* revealed no statistically
٢١٣ significant differences among the analyzed groups. However, the meloxicam group exhibited
٢١٤ significantly higher antibacterial activity against *T. pyogenes* (P = 0.008) on day 9, with non-
٢١٥ significantly higher activity on other days compared to the flunixin and control groups (Figures
٢١٦ 2C and 2D).
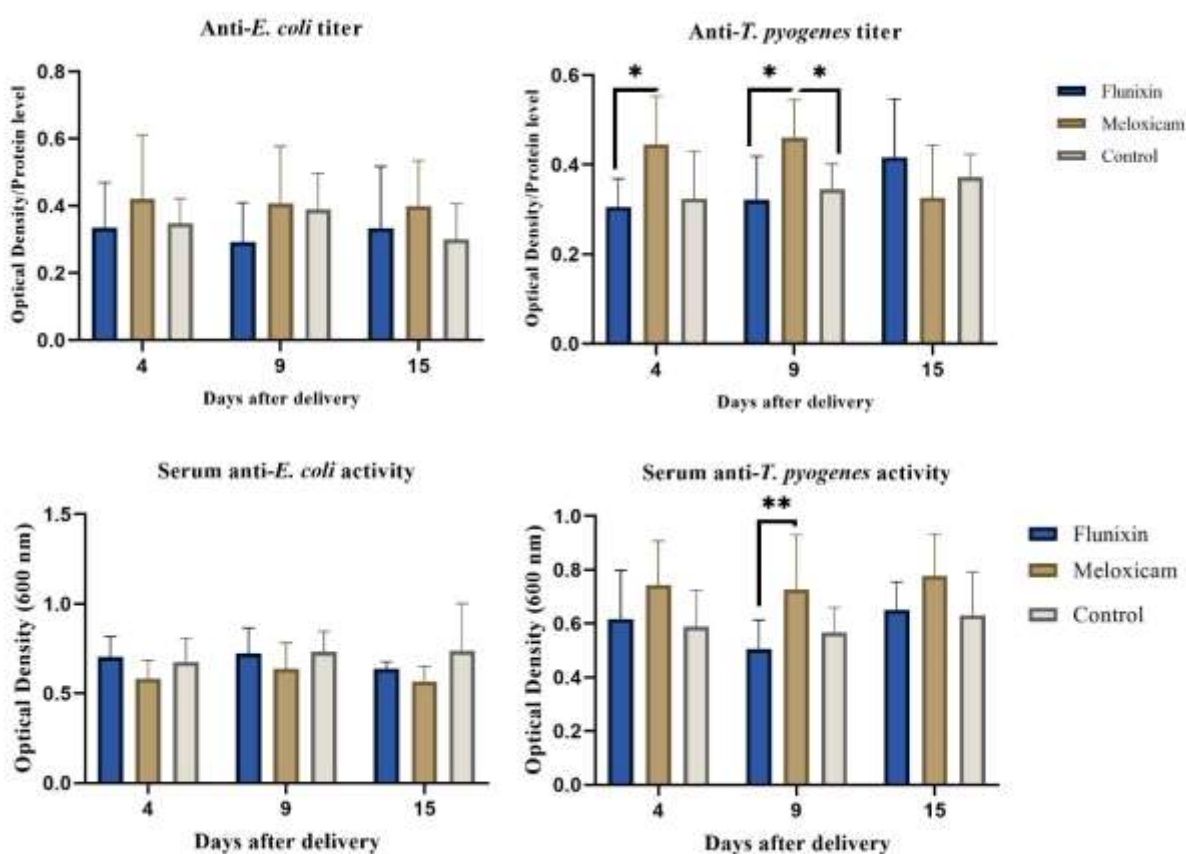
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٢٢٠ **Figure 2.** The cattle serum immunoglobulin (mean ± standard deviation) titer (A and B) and
٢٢١ antibacterial activity (C and D) against *E. coli* and *T. pyogenes* in cows treated by flunixin,
٢٢٢ meloxicam and control groups. The single asterisk (*) denotes a significance level of p<0.033, two
٢٢٣ asterisks (**) indicate a significance level of p<0.002, and three asterisks (***) represent a
٢٢٤ significance level of p<0.001.

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4. Discussion

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

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

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

٢٦٣ were more likely to retain their fetal membranes and had an increased risk of developing metritis
٢٦٤ (20).

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

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

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

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

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Author Contribution

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

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Conflict of Interest

٣٢٤ The authors declare that they have no conflict of interest.

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Data availability

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

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Ethics approval

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

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