Title: Investigating the effects of flunixin meglumine and meloxicam on humoral immune responses against *E. coli* and *T. pyogenes* in postpartum dairy cows

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Abstract

Disruption of the endometrial barrier after gestation leads to devastating effects on the cow's reproductive system. However, the accumulating immune responses help repair and defend the endometrium against microbial infections. Nevertheless, excessive inflammation could lead to chronic inflammation and consequently result in reproductive issues. The administration of NSAIDs can reduce the harmful effects of excessive inflammation, modulate the immune response, and improve reproductive indices in postpartum dairy cows. The aim of this study is to evaluate the side effects of NSAID administration on humoral immune responses, which could potentially inform the

development of more effective treatment strategies for postpartum cows. The study involved a total of 60 apparently healthy cows, which were divided into three groups: flunixin meglumine, meloxicam, and control. The test groups were treated at 3 and 8 days after calving. To assess the humoral immune responses, the IgG and IgM titers against *Escherichia coli* and *Trueperella pyogenes*, were determined on Day 4,9 and 15 after gestations. The results of the study showed that the drugs did not significantly affect the total specific and nonspecific antibody levels against tested pathogens. A time-dependent decrease and increase in the levels of IgM and IgG antibodies against *E. coli* and *T. pyogenes* were observed in all groups. The results showed a reduction in IgM and IgG titers against both pathogens after treatment with meloxicam and flunixin respectively. However, the elevation of IgG and IgM titers indicated that the treatment affected antibody isotype switching rather than suppressing the humoral immune responses.

Keywords: Antibody, Cattle, Flunixin meglumine, Meloxicam, Postpartum

Introduction

The postpartum period is when the reproductive system returns to its normal state before conception. In non-seasonal polyestrous species such as cattle, the normality of the postpartum period is crucial because it enables livestock breeders to re-conceive as soon as possible after delivery. Disruption during this period leads to devastating effects on the efficiency of the cow reproductive system. The anatomical barriers of the bovine reproductive system are the most critical defense mechanisms against ascending microbial infections that open during and after delivery, allowing pathogenic agents to enter the uterus. The immunity against pathogens depends on the resistance of tissues and uterine cells to infection, such as the endometrial mucosal layer, lysozyme, antimicrobial peptides, and mucus glycoproteins. As bacteria overcome the resistance of tissues in the uterus, innate and acquired immunity helps to limit the accruing infection (1).

The most commonly isolated bacteria from animals with uterine disease are *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, *Prevotella*, and *Bacteroides* species. The role

of *E. coli* and *T. pyogenes* in uterine diseases has been proven by injecting these bacteria into the uterus of native cows to produce animal models of endometritis. Uterine-specific strains of these pathogens have virulence factors that enable them to bind to and attack bovine endometrial epithelial and stromal cells (1). The onset of the inflammatory process in the uterus after calving is linked to *E. coli*, and its presence in the uterus during the postpartum period raises the risk of metritis and endometritis. *T. pyogenes* has also been widely isolated from the uterus of cattle with metritis and plays an important role in the etiopathogenesis of endometritis (2).

Many dairy cows develop metabolic or infectious diseases in the first month of lactation, depending on different management systems and milk production. The disease occurrence during the transition period is associated with impaired immune systems. Inadequate immune responses can lead to poor animal health. The risk of retained fetal membranes, metritis, endometritis, and mastitis increases in cows that cannot produce an appropriate immune response when exposed to pathogens (1). The immune system's humoral components, such as natural antibodies and total immunoglobulins, have been used as appropriate safety indicators for dairy cows (3).

The lymphoid follicles of the uterus subepithelial stroma are likely the inductive site where immune cells are activated and where the antibody response occurs during uterine infections. Systemic immunity against pathogenic factors and inactive cells, such as *E. coli*, *T. pyogenes*, and *F. necrophorum*, increases serum IgG levels against these antigens, consequently reducing the risk of metritis (2). Previous studies have suggested that the infiltration of polymorphonuclear leukocytes (PMNL) into the endometrial surface and the uterine wall is essential for the immune defense of the uterus (1). Although immunoglobulins are not necessary for the phagocytosis of bacteria by PMNL, they significantly enhance PMNL phagocytic activity. Previous studies have suggested that natural antibodies can neutralize pathogens directly and activate the complement system, thereby reducing the presence of bacteria in the uterus after parturition (4). Therefore, it helps to limit uterine infections and consequently reduce the occurrence of metritis and clinical endometritis. The notion that antibodies are crucial in resisting uterine diseases has been supported by another study that

reported systemic immunization against intrauterine pathogens led to an increase in serum levels of specific antibodies and a decrease in the occurrence of metritis (5).

Excessive systemic inflammation around the time of calving can impact milk production, animal health, and the efficiency of the reproductive system in dairy cows. There are some methods to modulate the immune response and minimize the adverse effects of postpartum systemic inflammation. One approach that has garnered significant attention in recent years is the use of non-steroidal anti-inflammatory drugs (NSAIDs) after delivery. Eighty percent of the veterinarians in the United States who participated in a survey prescribed NSAIDs in over 50% of dystocia cases (6). However, the treatment of dairy cows with NSAIDs after parturition shows promising results. However, comparing studies with each other is complex due to the use of different drugs, variations in the timing of administration, and differences in dosage. Therefore, further studies are needed to determine the most appropriate NSAIDs and the optimal time of administration. Treatment with NSAIDs can affect transcription factors such as NF-κB, which regulate the expression of many genes involved in inflammation, such as TNF-α and interleukin-1β (7). Meloxicam selectively inhibits cyclooxygenase-2, exhibits 100% average bioactivity, and has a half-life of 27 hours. Flunixin meglumine inhibits cyclooxygenase 1 and 2 non-selectively and has an average bioactivity of 76%, and also its half-life ranges from 3.1 to 26 hours (8).

Therefore, NSAID administration can reduce the harmful effects of continuous excessive inflammation, modulate the immune response, and improve reproductive indices in postpartum dairy cows. Also, the side effects of this administration on humoral immunity need to be studied. This study aimed to investigate the impact of different NSAIDs on humoral immune responses against *E. coli* and *T. pyogenes* in postpartum dairy cows.

Materials and methods

This study was conducted at the Animal Husbandry and Agriculture of FKA, where about 14,000 Friesian Holstein cows are kept in Isfahan province, Iran. Enrollment occurred from August 2023

to September 2023. In this study, 60 healthy cows with their second, third, or fourth parturition were divided into three groups with 20 cows in each group. Cows with dystocia, twinning, mastitis, lameness, retained placenta, or any other clinical diseases were excluded from this study.

- Flunixin meglumine was administered intravenously (IV) at a dose of 2.2 mg/kg on days 3 and 8 postpartum (Fig. 1).

- The meloxicam group (0.5 mg/kg) received IV injections on days 3 and 8 postpartum.

- Control Group: This group did not receive any drug injections.

Figure 1.

Sampling

Blood samples were collected from each cow on day 4 (24 hours after the first administration), day 9 (24 hours after the second administration), and day 15 after calving (Fig. 1). The samples were collected using a clot activator vacuum blood collection tube (gel-free) and a 21-gauge needle from the coccygeal vein. After collecting the samples, blood tubes were promptly transferred to the farm's laboratory. To obtain serum, the tubes containing blood were centrifuged at 1200g for 10 minutes. The resulting serum was then transferred to 2 mL microtubes and stored in a freezer at -20°C until the sampling was completed. After collecting all the samples, the serum samples were placed in a cooler box with dry ice and transferred to the immunology laboratory of Veterinary Medicine at Shahid Chamran University of Ahvaz. The samples were then stored in a -20°C freezer until the start of the experiments.

Preparing bacterial antigens

The *E. coli* and *T. pyogenes* strains were obtained from the archives of the Microbiology department at the Veterinary Medicine Faculty. These strains were characterized using biochemical and bacteriological tests. The isolates were removed from the -80°C deep freezer and cultured separately on a blood agar medium containing 5% sheep blood for 24 - 48 hours in a 37°C incubator. After ensuring purity using sterile PBS, suspensions of *E. coli* and *T. pyogenes* with concentrations of

approximately 4 McFarland units (12×10^8 CFU/mL) were prepared simultaneously. To inactivate these bacteria, the suspensions were stored in PBS containing 1% formalin at room temperature for 24 hours. The formalin was removed through three washes with a PBS solution. The absence of bacterial growth after 24 hours of culture under the previous conditions indicates bacterial inactivation (9). Then, the antigen solutions were irradiated ten times for 15 seconds using a sonicator (product number 4B200, Sigma-Aldrich). The prepared antigens were transferred to a -20°C freezer (10).

Preparation of specific antibodies

The *E. coli* suspension (0.5 mL) was mixed with 0.5 mL of complete Freund's adjuvant and injected intramuscularly (IM) into the thigh muscle of 2 rabbits. Subsequently, four booster injections were administered at 10-day intervals, each consisting of 0.5 mL of *E. coli* suspension with 0.5 mL of incomplete Freund's adjuvant to enhance antibody titer. After confirming the appropriate antibody levels using the indirect ELISA method, blood samples were collected from the rabbit's ear vein, and the hyperimmune serum was stored in a freezer at -20°C. To produce hyperimmune serum against *T. pyogenes*, the above procedure was performed simultaneously by injecting inactivated *T. pyogenes* suspension into two other rabbits (9).

Isolation of IgG antibodies from hyperimmune rabbit serum against *E. coli* and *T. pyogenes* was performed using the ammonium sulfate precipitation method. Four milliliters of saturated ammonium sulfate solution were added to a 50 mL beaker containing 8 mL of hyperimmune serum on ice. The mixture was placed on a shaker set at 90 rpm and was thoroughly mixed with the serum for 30 minutes. The solution was centrifuged for 15 minutes at 1200g. The supernatant was discarded, and the remaining sediment was combined with a 40% ammonium sulfate solution. It was centrifuged again as before, and the supernatant was discarded. The remaining sediment was dissolved in 0.02 M phosphate buffer (PB) with a pH of 7.2. Finally, for the purification of the salt, the sediment of ammonium sulfate in PB was dialyzed for 48 hours. Chromatography of ion exchange was performed using DEAE-cellulose powder. After soaking DEAE-Cellulose powder in a 0.02 M PB for 24 hours, the soaked powder was placed in a column and washed with a phosphate

buffer containing 1 M KCl, followed by washing with a 0.02M phosphate buffer (pH 7.2). The solution from the dialysis bag was transferred to the pillar and left closed for 2 hours. The column was washed with 0.02 M phosphate buffer (pH 7.2), and the IgG fraction obtained was collected in 2 ml microtubes. Finally, after checking the purity and yield percentage, the microtubes were transferred to a -20°C freezer (10). The purity of the isolated antibodies was determined through electrophoresis on a polyamide gel (9).

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

For this purpose, an in-house indirect competitive ELISA was used. The above-mentioned *E. coli* suspension (10 µg/mL) was diluted with carbonate-bicarbonate buffer (pH = 9.5). Subsequently, 100 µL of this diluted antigen solution was added to each well of an ELISA plate and stored at 4°C for 24 hours for coating. After that, the samples were washed three times with a PBS-Tween 20 solution. Subsequently, 250 µL of 4% skimmed milk was added to each well for blocking. The samples were stored overnight at 4°C and washed three times as previously described. In a separate plate, 2 µL of bovine serum was diluted with 78 µL of PBS in each well and then immediately added to the ELISA plate by multichannel sampler. Subsequently, 20 µL of rabbit anti-*E. Coli* IgG with a 20 µg/mL concentration was added to each well. The plate was then stored at room temperature for 1 hour, resulting in a final concentration of 1/50 for rabbit IgG and bovine serum. After rinsing the plate, 100 µL of anti-rabbit antibody conjugated with horseradish peroxidase was added to each well and incubated at room temperature for 1 hour. After five washes, 75 µL of TMB solution was added to each well. Ten minutes later, 75 µL of the stop solution (2 M sulfuric acid with pH = 3) was added to each well to halt the reaction. The optical absorption values were then promptly measured using a spectrophotometric method in an ELISA reader (AccuReader®) at the wavelength of 450 nm. A sample containing rabbit anti-*E. Coli* IgG with a concentration of 20 µg/mL was used as a positive control. For the detection of antibody titer against *T. pyogenes*, the stages were similar to the *E. coli;* instead of using an *E. coli* suspension, a *T. pyogenes* suspension and *T. pyogenes* IgG were utilized (9).

Detection of IgG titer against *E. coli* and *T. pyogenes*

For this purpose, an ELISA test was performed to detect a specific antibody against both bacteria, following the method described above. Serum samples were diluted with PBS containing 0.1 M β-mercaptoethanol at 1/40. The diluted serum samples were then incubated for 1 hour on a separate plate at room temperature to degrade IgMs. We measured the specific IgM levels by reducing the specific IgG levels, from total specific antibodies levels (11).

Total serum immunoglobulin levels

Total serum immunoglobulin level was evaluated using a modified zinc sulfate precipitation method. Zinc sulfate buffer 0.7 mM with pH 5.8 was prepared. Ten microliters of each serum sample were poured into a 96-chamber plate, and then 190 µL of 0.7 mM zinc sulfate buffer with pH 5.8 was added in each well and mixed well with the serum sample. For the positive sample, samples were taken from the control groups, mixed, added to the plate simultaneously, and read according to the mentioned method (12).

Statistical analysis

The immunoglobulin levels were analyzed at various time points post-parturition using the repeated measures method. Also, group comparisons at specific time points were conducted with the one-way ANOVA method with Tukey's post hoc test between the groups using SPSS software version 27. The P-value < 0.05 was considered significant. Diagrams were drawn using Excel software version 19 (Microsoft).

Results

The mean immunoglobulin titers of total specific antibody, IgG, and IgM are separately presented against *E. coli* and *T. pyogenes* in Figure 2. The total specific antibody level against *E. coli* in the flunixin, meloxicam, and control groups at different times revealed no significant differences (P = 0.798, 0.745, and 0.885, respectively). Group comparisons at specific times on days 4, 9, and 15 also indicated no significant differences between groups (P = 0.838, 0.717, and 0.219, respectively). Comparison of IgG concentration against E. *coli* in groups at different times revealed a significant increase in the meloxicam group (P =0.032) and control group (P = 0.034), while this increase wasn't

significant in the flunixin group (P = 0.122). Group comparisons on days 4 (P = 0.579) and 9 (P = 0.564) showed no significant difference, but we observed a significant difference on day 15 between all groups (P = 0.022), as well as a significant difference between flunixin and control group (P = 0.031). A time-dependent decrease in IgM against *E. coli* was observed in all three groups; This decrease was significant in the meloxicam group (P = 0.004), while no significant difference was noted in the flunixin (P = 0.352) and control groups (P = 0.053). On day 4, we observed no significant difference (P = 0.853), while one was revealed on day 9 between all groups (P = 0.036) and also between the flunixin and meloxicam group (P = 0.031). On day 15, we observed significant differences between 3 groups (P = 0.002), as well as the flunixin group against the meloxicam group (P = 0.002) and control group (P = 0.039).

The total specific antibody levels against *T. pyogenes*, increased over time in the meloxicam (P = 0.516) and control groups (P = 0.072) at the time of calving, while in the flunixin group, it remained almost constant (P = 0.923). The total specific antibody titers against *T. pyogenes* at a specific time on days 4 (P = 0.435), 9 (P = 0.538), and 15 (P = 0.425) demonstrated no significant differences between groups. IgG titers on days 4, 9, and 15 did not differ significantly among the three groups (P = 0.985, 0.261, and 0.703, respectively). However, within each group, the IgG titer showed a significant increase over time (P = 0.012 in the flunixin group, P = 0.003 in the meloxicam group, and P = 0.001 in the control group). The IgM levels of all three groups on day 15 were lower than day 9, and day 9 was lower than day 4 postpartum. These decreases were significant in all three groups (P = 0.001 in the flunixin and meloxicam groups, and P = 0.002 in the control group). the IgM levels on days 4 (P = 0.544) and 9 (P = 0.179) were not significant between groups, while there was a significant difference between 3 groups on day 15 (P = 0.012) and also between meloxicam and control groups (0.009).

Figure 2.

The results of the total immunoglobulin levels, which were measured using the zinc sulfate turbidity test (ZST), are shown in Figure 3. The total serum immunoglobulin level in the flunixin group increased but was insignificant (P = 0.364). In contrast, there was a significant decrease in the

immunoglobulin levels in the control group (P = 0.031), especially between days 4 and 9. However, the immunoglobulin titer in the meloxicam group decreased (P = 0.272). The immunoglobulin titers at specific times on day 4 (P = 0.069), day 9 (P = 0.559), and day 15 (P = 0.205) did not show significant differences among all three groups.

Figure 3.

Discussion

The treatment of dairy cows with NSAIDs after calving is promising, but due to the use of different drugs, administration time and the dosage used makes comparing studies with each other complicated. Therefore, the selection of the most appropriate NSAID, dosage and administration time, require more studies (13). The aim of this study was to investigate the effects of administration of two different NSAIDs on the humoral immunity of postpartum dairy cows after parturition in different days, dosage and method of injection compared to previous studies.

Anatomical barriers of the bovine reproductive system are the most critical defense factors for preventing ascending microbial infections that can occur during and after calving, allowing pathogenic agents to enter the uterus. Furthermore, an important concept is that all normal, healthy animals have a substantial load of bacteria in the uterus after normal parturition. Hence, contamination of the uterus after calving is inevitable. It has been suggested that *E. coli* initiates the infectious process in the postpartum uterus. Its presence in the uterus a few days after parturition is associated with an increased risk of metritis and endometritis. However, *T. pyogenes* has been consistently isolated from the uterus of cows affected with metritis (2).

One study reported that total serum IgG levels increased from parturition to the fourth week, while total serum IgM levels remained almost constant during these four weeks, and did not change much. Based on the results of our study, the level of specific IgG against *T. pyogenes* and *E. coli* was in agreement with the mentioned study; The related titers were significantly higher on the day 15 postpartum than on day 4 postpartum. However, the levels of specific IgM against the mentioned

bacteria decreased in all groups, which were significant in all groups against *T. pyogenes* and also in the meloxicam and control groups against *E. coli* (3).

NSAIDs are used in veterinary medicine for various reasons, such as relieving transient pain or treating serious inflammatory diseases. A wide range of NSAIDs reduced the synthesis of antibodies in humans, with the most significant effect being Ibuprofen, which tends to inhibit Cox-2 (14). In our study, there was no significant difference in the level of total specific and non-specific antibodies between flunixin, meloxicam, and control groups at the same time, which is contrary to this study's finding that NSAID administration is associated with decreased antibody levels.

In one study meloxicam administration before calving resulted in more milk production with no effects on health; the treated animals were less active than the control group, so they suggested treatment of the cattle before parturition (15). According to our aims, the first inflammatory responses in the uterus are required for the development of the immune responses against pathogens and the repairment phase after parturition. However, Mainau et al. (16) reported more postpartum cattle activity after the administration of meloxicam in the first 2 days post-parturition. Another study showed that meloxicam treatment resulted in more feeding and milk production (17).

Treatment of Holstein Friesian dairy cows with flunixin meglumine in the early postpartum period (day 2) reduced inflammatory markers and improved the metabolic profile. The treated cows showed a lower risk of metritis starting from day 15 postpartum (13). However, treating cows within the first 5 hours postpartum and administering daily injections on days 2 and 3, led to febrile induction and Disruption of the fetal membrane expulsion (18). However, treatment of cows immediately before calving with Flunixin increased the incidence of stillbirths. Animals treated immediately after calving had increased odds of having a retained placenta and, in turn, increased risk of a high temperature, decreased milk production, and an increased risk of developing metritis. The administration of flunixin meglumine within 24 h of parturition is not recommended in dairy cattle (19). In the current study, the selection of the first treatment on day 3

was based on the mentioned facts and the occurrence of the required inflammatory phase for the induction of uterine repair.

According to the current results, the drugs did not significantly affect the total specific antibody levels against both tested pathogens. However, on day 15, a significant reduction was observed in the levels of specific IgM against *E. coli* and *T. pyogenes* in the meloxicam group and IgG levels against *E. coli* in the flunixin group. In an *in vitro* study, the dose-dependent immunosuppressive effects of flunixin and meloxicam on calf leukocytes were demonstrated (20). Bednarek et al. (21) also reported the immunosuppressive benefits of both drugs on calves suffering from enzootic bronchopneumonia. In another study, Compiani et al., (22) suggested using meloxicam administration to prevent bovine respiratory disease in imported male cattle. In contrast to the current study, the treated animals exhibited higher antibody titers following *bovine herpes virus-1* vaccination and increased serum bactericidal activity. Additionally, the reduction in haptoglobin levels indicated successful management of transportation stress. The differences in animal sex, health, and physiological conditions could be involved in the contrasting results.

Oral administration of meloxicam to the treated beef cattle by LPS had no effects on the induced neutralizing antibody titer after vaccination against respiratory pathogens, acute-phase protein concentration, and TNF-α level (23). The current study found no statistical difference in total antibody titer against *E. coli* and *T. pyogenes*. However, the study revealed significant effects on IgM and IgG titers. Transdermal injection of flunixin meglumine did not significantly affect antibody titers against experimentally treated beef heifers against *M. haemolytica* (24). Pascottini et al. (7) reported that administration of meloxicam for four days, days 10 to 13, reduced systemic inflammatory responses but did not affect the endometrial inflammatory status. The current study focuses on the early stages of induced inflammatory responses following calving. Bednarek et al. (21) suggested a combination treatment of antibiotics and meloxicam for calves suffering from enzootic bronchopneumonia. The treated group showed an increased level of interferon-gamma, but no effects on gamma-globulin concentration were observed four days after treatment. Another study demonstrated the adverse effects of meloxicam on weaned calves. Additionally, it revealed that the

treatment did not affect the antibody titers against *bovine herpesvirus type 1*, *bovine respiratory syncytial virus*, *parainfluenza virus type 3*, or *coronavirus* (25).

The results showed respective reductions in IgM and IgG titer against both pathogens after treatment by meloxicam and flunixin. however, according to elevation of the IgG and IgM titer, the results indicated the treatment affected antibody isotype switching of the antibodies and not suppression of the humoral immune responses. The possible different treatment effects on cytokine profiles of the immune cells resulted in dissimilar effects on antibodies and isotype switching.

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Conflict of Interest The authors declare that they have no conflict of interest.

Author Contribution SG, SME: Data curation; Software; Formal analysis; Investigation; Visualization and Writing -original draft. MK, MN: Project administration and supervision, methodology, review & editing. BM: Methodology, investigation.

Ethics This research was conducted on animals by the Helsinki Declaration of 1975, as revised in 2013.

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Data Availability The data that support the findings of this study are available on request from the corresponding author.

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