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ORIGINAL RESEARCH

Comparative Effects of Dexamethasone and Meloxicam on Magnitude of the Acute Inflammatory Response Induced by *Escherichia coli* Lipopolysaccharide in Broiler Chickens

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Purpose: Dexamethasone has been widely used to treat acute inflammatory diseases and endotoxic shocks in animal models. Meloxicam is one of the most commonly used anti-inflammatory agents in avian species. However, little is known about the effects of dexamethasone and meloxicam on lipopolysaccharide (LPS)-induced acute inflammatory response in birds. In the present study, LPS-challenged broiler chickens were used to investigate the comparative protective effects of meloxicam and dexamethasone on LPS-induced acute inflammatory responses.

Methods: Lipopolysaccharide (LPS)-induced acute lung injury (ALI) histopathological scores, selected serum acute phase reactants, inflammatory mediators, and gangliosides were evaluated in broiler chickens inoculated with *E. coli* LPS and simultaneously treated with two doses of meloxicam (0.5 and 2 mg/kg BW) and dexamethasone (2 and 4 mg/kg BW).

Results: LPS-induced ALI scores were not significantly different between the meloxicam-treated, dexamethasone-treated, and untreated positive control groups at 4 hours after LPS inoculation. Interleukin-6 concentrations were also statistically the same among the positive control, dexamethasone-treated, and meloxicam-treated groups at 3 and 12 hours after LPS inoculation. However, these anti-inflammatory drugs reduced adenosine deaminase, ceruloplasmin, lipid-bound sialic acid, protein-bound sialic acid, and total sialic acid in LPS-inoculated broiler chickens at 12, 24, and 48 hours after LPS inoculation in a drug- and dose-dependent manner. Ovotransferrin concentrations were not significantly different between positive control and treatment groups at 12 hours after LPS inoculation. However, twenty-four hours after LPS inoculation, all the treated groups, except the one treated with 0.5 mg/kg meloxicam, showed significantly lower concentrations of ovotransferrin as compared with the positive control group.

Conclusion: Our results showed that dexamethasone was more effective than meloxicam in inhibiting the LPS-induced response in broiler chickens by diminishing the serum levels of adenosine deaminase, ceruloplasmin, and gangliosides.

Keywords: lipopolysaccharide, meloxicam, dexamethasone, interleukin-6, acute phase proteins, chickens

Introduction

Lipopolysaccharide (LPS), or bacterial endotoxin, is a major pathogenic part of the outer membrane of Gram-negative bacteria and is considered to have an important role in the onset of Gram-negative sepsis, inflammation, shock, failure of multiple organs, and finally occurrence of high mortality.[1] The inoculation of chickens with

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LPS has led to significant changes in the plasma proteome within 12-hour post infection.[2] Inflammation is a complex homoeostatic process which is associated with many infectious diseases and consequently leads to a prominent systemic critical reaction known as acute phase response (APR).[3,4] The APR, following the inflammatory process triggered by LPS from Gram-negative bacteria involves variations in the serum levels of different acute phase proteins (APPs), cytokines, enzymes, and metabolites with the aim of restoring the physiologic homeostasis of the host organism.[5,6] Therefore, acute endotoxemia could be considered as an appropriate inflammation model for gaining insight into the inflammatory processes.[7] Alterations in the circulating levels of APPs are associated with the onset and durability of LPS inflammation; accordingly, APPs have been widely used as appropriate markers for investigating the health status of humans and other mammals.[8,9]

Upon exposure to various inflammatory conditions, such as acute bacterial endotoxemia resulting from LPS inflammation, circulating leukocytes release pro-inflammatory cytokines, which are necessary for the recruitment of neutrophils to the site of inflammation and the regulation of different metabolic responses.[10–12] These cytokines are involved in the regulation of the synthesis of APPs in the liver and have a mediating role in inducing the febrile response during inflammation and the development of the inflammation process by raising APPs.[13]

The need for gaining knowledge on different aspects of immune reactions of chickens during infections and different environmental or nutritional situations has led to the increased interest for investigations on APPs in chickens.[14] Adenosine deaminase (ADA) is an enzyme that accelerates the conversion of adenosine to inosine and regulates extracellular adenosine and inosine concentrations in mammals.[15] ADA, as an endogenous regulator of the innate immune system, is essential in the proliferation and differentiation of T lymphocytes. In addition, ADA is involved in controlling the magnitude of purinergic response under physiological conditions and, to a larger extent, pathological events, such as inflammation.[16,17] Ovotransferrin, as a positive APP in chickens, increases during the APR triggered by a variety of experimentally induced inflammatory reactions and performs its bacteriostatic role by diffusing through the outer membrane of Gram-negative bacteria.[18,19] Serum ovotransferrin is used as a biomarker of inflammatory diseases in chickens.[20] Ceruloplasmin is a multifunctional antioxidant protein which accumulates and transports the copper within the body. The role of ceruloplasmin as an APP became evident by observing that its level increases in different infectious diseases.[21]

As a family of neuraminic acid derivatives, sialic acids are involved in many biological and pathological phenomena located at the end chain of many APPs. The majority of sialic acids found are either protein-bound or lipid-bound, and only a few of them are in free form.[22] Sialic acids have a principal role in cell-to-cell identification and interaction, which mediate various cell-cell adhesion processes in the inflammation and immune response.[23] Sialic acids concentration increases immediately following various bacterial and viral inflammatory responses associated with various diseases in humans and animals. Hence, the evaluation of sialic acids concentration may be helpful in the diagnosis and prognosis of inflammatory diseases.[24–26]

Non-steroidal anti-inflammatory drugs (NSAIDs) are extensively used as analgesics to alleviate pain and treat inflammatory musculoskeletal diseases and other inflammatory ailments, such as LPS endotoxemia. The NSAIDs mechanism of action is to inhibit prostaglandins and thromboxanes production as the major mediators in the inflammation process. This feature of NSAIDs makes them a more appropriate candidate, compared to their steroidal counterparts, to be applied in a targeted approach for treating inflammation.[27]

Meloxicam is a COX-2-selective NSAID in its therapeutic dose. However, the COX-2 specificity of meloxicam decreases at high doses and the drug could bind to COX-1 with some controversial effects.[28] Pharmacokinetics and toxicological effects of meloxicam have been evaluated in chickens and other avian species.[29] Empirical dose ranges of meloxicam (0.5–2.0 mg/kg BW) has been used for the various inflammatory conditions and postoperative pain managements in different bird species with no adverse effects on either the renal and intestinal organs or the immune and hematopoietic system of birds.[29] These qualifications explain why meloxicam has become the most commonly used anti-inflammatory medication in avian species.[30]

Dexamethasone, as a synthetic derivative of cortisol, targets the phospholipid-arachidonate cascade to control inflammatory reactions.[31] It has been shown that dexamethasone can modulate the production of cytokines and acute phase proteins in the acute phase of inflammation.[32,33] Despite the limitations specified in the pharmacopeia regarding the use of dexamethasone as a steroidal drug, its use has

been approved in few cases such as septic shock and Gram-negative endotoxemia with a dose range of 2–4 mg/kg in different avian species.[30,34,35]

To our knowledge, no report is available about the comparative effects of dexamethasone and meloxicam on magnitude of the acute inflammatory response induced by bacterial LPS in avian species. In the present study, based on the previously described LPS-induced inflammation model in broiler chicken,[36–38] LPS-challenged broiler chickens were used to investigate the comparative protective effects of these anti-inflammatory compounds on LPS-induced acute inflammatory responses.

Materials and Methods

Animals

Fifty-four one-day-old Ross 308 male chicks were provided by a local commercial poultry farm (Fars province, Iran) and raised in an environmentally controlled poultry house in the Animal Research Unit of Shiraz University Veterinary School for five weeks under the standard environmental conditions and in compliance with the production parameters recommended by the broiler producer company.[39] All the birds used in this experiment were handled in accordance with the technical regulations and the guidelines set out by the committee of animal ethics of Shiraz University, Iran. The protocols of the study were approved by the Ethics Committee of Shiraz University (IACUC no: 4687/63).

Experimental Group

The chickens, aged five weeks, were divided into six groups of 9 birds each. The mean body weight of all chickens was 1.65 ± 0.2 kg. The positive LPS group was intravenously (IV) injected with LPS of *E. coli* O55 B5 (Sigma-Aldrich, USA) at a dose of 0.5 mg/kg. The birds in the negative control group were injected with the same volume of pyrogen-free water.

The other four groups were treated with meloxicam 5% (Razak, Iran) and dexamethasone 0.2% (Razak, Iran), as anti-inflammatory drugs, in combination with LPS. Immediately after the IV inoculation of LPS, meloxicam and dexamethasone were intramuscularly (IM) injected in the pectoral muscles at two different doses, ie 0.5 mg/kg, 2 mg/kg and 2 mg/kg, 4 mg/kg, respectively.[40]

Histological Examination

Four hours after LPS administration, three chickens were randomly selected from each group and euthanized. Their upper right lung lobes were removed and stored in 10% buffered formalin for one week. The samples were dehydrated, embedded in paraffin, cut into 5-µm sections, and stained with hematoxylin and eosin (H&E) according to standard procedures. Four microscopic fields (100×), which contained at least a tertiary bronchus per field, per tissue sample for each bird were randomly chosen and observed by a light microscope. Five high-power fields (HPF), containing an interatrial septum, were randomly assigned to each field. All sections were scored according to the previously described criteria for LPS-induced acute lung injury,[41] with some modifications for chicken lungs. In brief, the thickness of the interatrial septum, infiltration of inflammatory cells, and hemorrhage were scored in each HPF using a blinded approach. Acute lung injury scores (ALI scores) for each item was categorized according to the following scale: 0: minimum damage; 1: mild damage; 2: moderate damage; 3: severe damage; 4: maximum damage. Accordingly, the final ALI score for each field varied within a range from 0 to 12.

Serum Sampling

Blood was collected from the jugular vein at 3 h and 12 h after LPS injection to determine IL-6 concentration and at 12, 24, and 48 h after LPS injection to measure the adenosine deaminase, ceruloplasmin, ovotransferrin and, gangliosides (TSA, LBSA, PBSA). The sera were separated by centrifugation at 750g for 15 min and stored at −20 C until further use.

IL-6 and Ovotransferrin Assays

The serum levels of IL-6 and ovotransferrin were measured using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) and commercial chicken-specific kits (Shanghai Crystal Day Biotech, Shanghai, China). The sensitivity of IL-6 and ovotransferrin kits was 0.53 ng/L and 0.024 mg/mL, respectively. The intra-assay and inter-assay precision of these kits were CV < 8% and CV < 10%, respectively.

Gangliosides (TSA, LBSA, PBSA) Assay

Serum total sialic acid (TSA) concentration was determined by the thiobarbituric acid method. Lipid-bound sialic acid (LBSA) concentration was determined using the method described by Katopodis et al.[42] Protein-bound sialic acid (PBSA) concentration was measured by subtracting LBSA from the serum TSA.

Adenosine Deaminase (ADA) Assay

ADA concentration was assessed by an enzymatic-calorimetric assay kit (Diazyme Laboratories, Gregg Court, California, USA).

Ceruloplasmin Determination

The measurement of the serum ceruloplasmin level was performed with the method suggested by Bestujeva and Kolb.[43]

Statistical Analysis

Results are expressed as mean ± SD. For the analysis of serum data, one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used. In cases of a failed normality test, Kruskal–Wallis ANOVA was performed followed by Dunn's post hoc test. For histopathological scoring, non-parametric Mann–Whitney *U*-test was performed. Statistical analyses were conducted with SPSS software (version 16.0, SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered significant.

Results

Histopathology

The histopathological examination of H&E-stained lungs showed that LPS treatment, compared to the control condition (negative control: Figure 1A), clearly stimulated a diffuse inflammatory response, severe hemorrhage, and thicker interatrial septum as observed in the positive control (Figure 1B), meloxicam-treated, and dexamethasone-treated groups (Figure 1C–F).

Lipopolysaccharide-induced ALI scores were not significantly different between the meloxicam-treated, dexamethasone-treated, and untreated positive control groups ($P > 0.05$) (Figure 2).

Interleukin-6 and Acute Phase Proteins

The concentration of ADA in the positive control at 12, 24, and 48 hours after LPS inoculation was significantly higher than that of the negative control ($P \leq 0.05$). Meloxicam treatment (0.5 and 2 mg/kg) had no significant effects on ADA concentration in comparison with the positive control group ($P > 0.05$). In contrast, dexamethasone treatment (2 and 4 mg/kg) significantly lowered the ADA concentration in comparison with the positive control and meloxicam-treated groups at 12, 24, and 48 hours after LPS inoculation ($P \leq 0.05$) (Figure 3A). Ceruloplasmin concentrations were not significantly different between the positive control group and 0.5 mg/kg meloxicam-treated group at 12, 24, and 48 hours after LPS inoculation ($P > 0.05$). However, 2 mg/kg meloxicam significantly decreased ceruloplasmin concentrations in comparison with the positive control group at the same time points mentioned above ($P \leq 0.05$). On the other hand, ceruloplasmin concentrations were significantly reduced in 2 and 4 mg/kg dexamethasone-treated groups compared with those of the positive control group ($P \leq 0.05$) (Figure 3B). Ovotransferrin concentrations were not significantly different between the positive control and treatment groups at 12 hours after LPS inoculation ($P > 0.05$). Twenty-four hours after LPS inoculation, all the treated groups, except the one treated with 0.5 mg/kg of meloxicam, had significantly lower concentrations of ovotransferrin compared with the positive control group ($P \leq 0.05$). Unfortunately, it was not possible to measure ovotransferrin level at 48 hours after inoculation because of inadequate serum storage (Figure 3C).

The injection of 0.5 and 2 mg/kg meloxicam did not significantly alter the lipid-bound sialic acid concentration compared with that of the positive control group ($P > 0.05$). On the other hand, the injection of 2 and 4 mg/kg dexamethasone significantly changed the lipid-bound sialic acid concentration in comparison with that of the positive control group at 12, 24, and 48 hours after LPS inoculation ($P \leq 0.05$) (Figure 3D). Different doses of meloxicam and dexamethasone significantly decreased the concentration of protein-bound sialic acid in comparison with that of the positive control group ($P \leq 0.05$). Dexamethasone was significantly more effective than meloxicam in decreasing the concentration of protein-bound sialic acid at 12, 24, and 48 hours after LPS inoculation ($P \leq 0.05$) (Figure 3E). The total sialic acid level was not significantly different between the positive control and meloxicam-treated groups at 12 hours after LPS inoculation ($P > 0.05$). However, dexamethasone significantly decreased the total sialic acid level in comparison with that of the positive control and meloxicam-treated groups at 12 hours after LPS inoculation ($P \leq 0.05$). Twenty-four hours after LPS inoculation, dexamethasone and meloxicam significantly reduced the total sialic acid level in a dose-dependent manner compared with that of the positive control group. Forty-eight hours after LPS inoculation, the effect of 0.5 mg/kg meloxicam treatment on the total sialic acid level was not significantly different from that of the positive control ($P > 0.05$). On the other hand, 2 mg/kg meloxicam and dexamethasone treatments significantly decreased the total sialic acid level in comparison with

that in the positive control and 0.5 mg/kg meloxicam-treated groups (P≤0.05) (Figure 3F).

Interleukin-6 concentrations were not significantly different between the positive control and treatment groups at 3 and 12 hours after LPS inoculation (P> 0.05) (Figure 3G).

Discussion

The present study was designed to explore the extent that dexamethasone or meloxicam could modulate LPS-induced lung inflammation, serum APPs concentration, and IL-6 level in broiler chickens. Some special features of avian lungs, such as a single basal lamina and a thin squamous epithelium layer at the blood-gas interface, predispose their respiratory systems to bacterial pathogens and possibly LPS-induced acute lung injury.[44] In some previous studies, *E. coli*-derived LPS were used to induce the model of acute lung injury in chickens.[37,44,45] In the current experiment, LPS treatment in the positive control group clearly stimulated a diffuse inflammatory response, severe hemorrhage, and thicker interatrial septum, which resulted in higher ALI scores in the positive control group as compared with those in the negative control group. Ansari et al[44] found indistinguishable margins between different parts of the pulmonary lobules, along with a narrowed lumen of the pulmonary atria and obvious congestion and heavy leukocytes infiltration of the pulmonary parenchyma 12 hour after LPS inoculation to broiler chickens. Intravenous LPS administration to the broiler chickens also caused a decrease in circulating white blood cells and appreciable sequestration of the leukocytes to the lungs.[37] In our study, LPS-induced ALI scores were not significantly different among the dexamethasone-treated, meloxicam-treated, and untreated positive control groups. The effects of dexamethasone and meloxicam administrations on the LPS-induced acute lung injuries has not been studied previously in avian species. However, some previous evidence show that pre- and simultaneous treatment with dexamethasone can attenuate LPS-induced acute lung injury in mouse models.[46,47] In addition, Dexamethasone inhibited LPS-induced hydrogen sulphide biosynthesis in a mouse model which can contribute to the anti-inflammatory effect of dexamethasone in endotoxic shock.[48] Moreover, it has

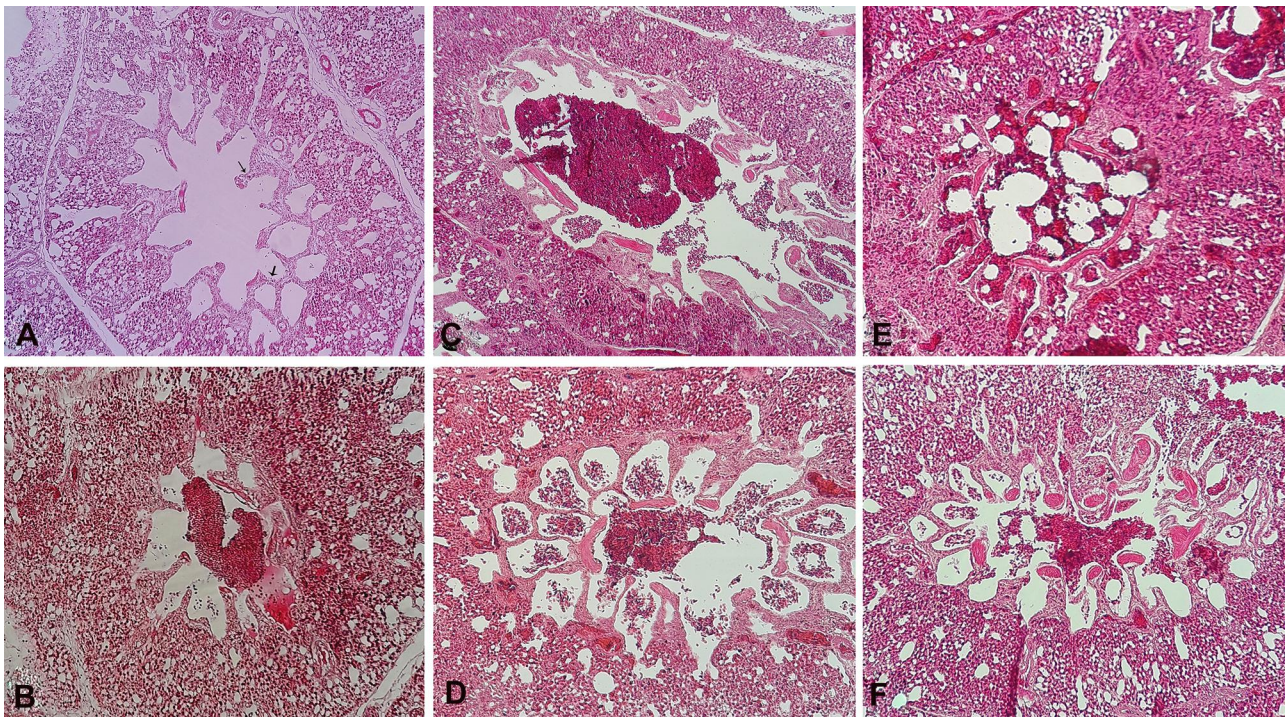

Figure 1 The histopathological features of IV injection of LPS *E. coli* O55 B5 with or without meloxicam and dexamethasone treatment on lung injury (H&E, ×100). (**A**) Negative control: four hours after IV injection of pyrogen-free water, arrow: interatrial septum; (**B**) Positive control: four hours after IV injection of LPS *E. coli* O55 B5; (**C**) LPS+ meloxicam 0.5: four hours after IV injection of LPS *E. coli* O55 B5 and IM injection of 0.5 mg/kg meloxicam; (**D**) LPS+ meloxicam 2: four hours after IV injection of LPS *E. coli* O55 B5 and IM injection of 2 mg/kg meloxicam; (**E**) LPS+ dexamethasone 2: four hours after IV injection of LPS *E. coli* O55 B5 and IM injection of 2 mg/kg meloxicam; (**F**) LPS+ dexamethasone 4: four hours after IV injection of LPS *E. coli* O55 B5 and IM injection of 4 mg/kg meloxicam.
Abbreviations: *E. coli, Escherichia coli*; H&E, hematoxylin and eosin; IM, intramuscular; IV, intravenous; LPS, lipopolysaccharide.

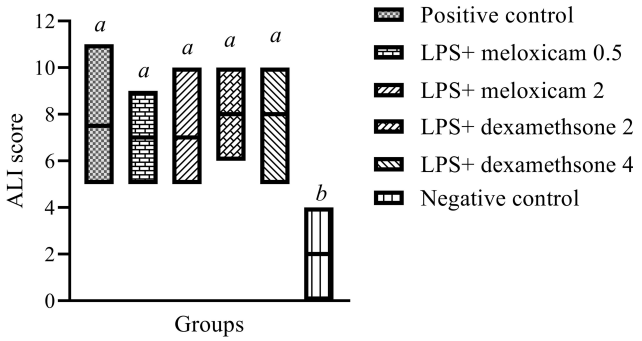

Figure 2 Histopathological LPS-induced ALI scores in lung 4 hours after IV injection of LPS *E. coli* O55 B5 with or without meloxicam and dexamethasone treatment. For histopathological scoring, the results were expressed as box plots with median (minimum to maximum) and comparison was made using the non-parametric Mann–Whitney *U*-test.
Note: [a,b]Columns with different superscripts are statistically different at P<0.05.
Abbreviations: ALI, acute lung injury; *E. coli, Escherichia coli*; IV, intravenous; LPS, lipopolysaccharide.

been shown that meloxicam could decrease endotoxin-induced acute lung injury in rabbits.[49]

Interleukin-6 (IL-6) is a multifunctional cytokine that plays a major role in regulating immune responses, acute phase reactions, and hematopoiesis. In accordance to our study, De Boever et al[37] showed maximum levels of secreted IL-6 at 3 h after intravenous LPS administration in the broiler chickens. In the current study, dexamethasone and meloxicam administrations did not significantly affect the concentration of LPS-induced IL-6 in the serum of chickens at 3 and 12 hours after LPS injection. The effects of dexamethasone and meloxicam administrations on the LPS-induced IL-6 secretion had not been studied previously. However, De Boever et al[38] showed that the administration of other anti-inflammatory drugs, including tepoxalin, sodium-salicylate, and ketoprofen did not influence the concentration of IL-6 in plasma 3 hours after LPS administration.

In a previous study, Adanin et al[50] showed that the prevention of adenosine degradation could attenuate proinflammatory cytokine responses after LPS inoculation in rats. They suggested that the inhibition of ADA could be a novel therapeutic approach to control the systemic inflammatory response. In this study, serum ADA activity was significantly higher in the *E. coli* LPS-treated group than in the negative control group. Meloxicam administration was not associated with a significant effect on ADA concentration. However, the ADA concentration in the dexamethasone-treated groups was lower than that of the positive control and meloxicam-treated groups. Yazar et al[51] indicated that

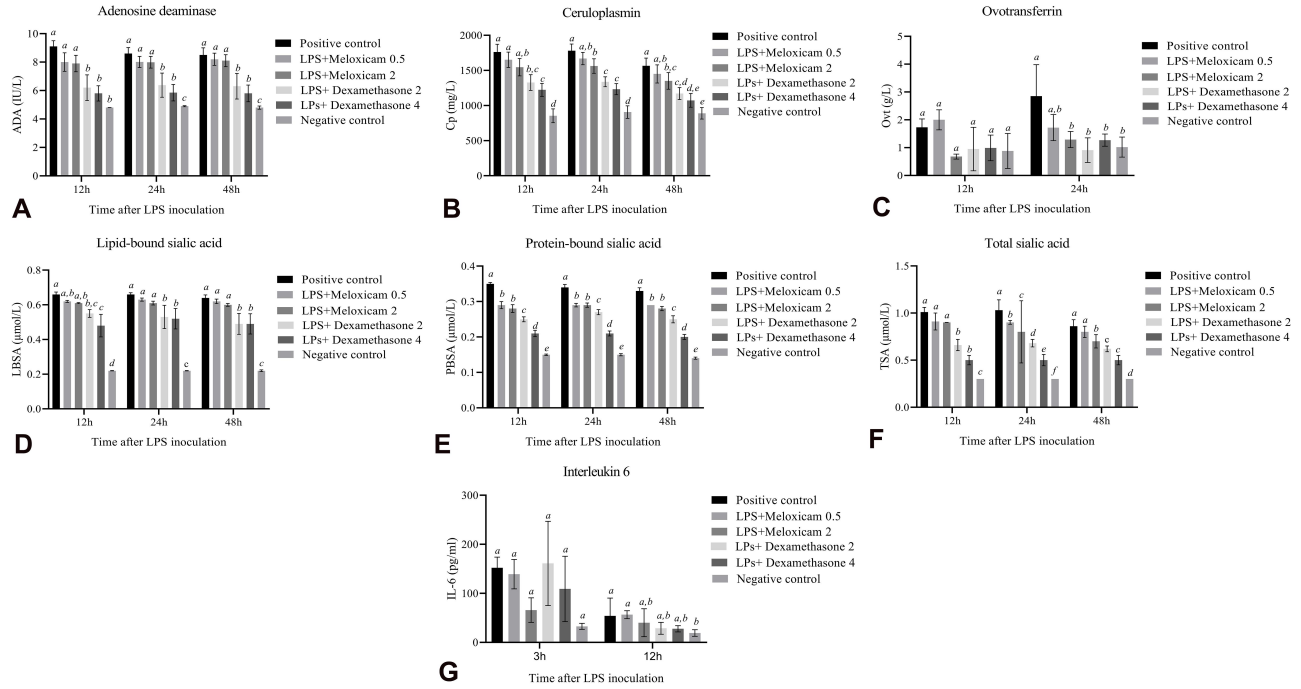

Figure 3 The effects of meloxicam and dexamethasone treatments on the serum concentration of IL-6 (3 and 6 hours after IV injection of LPS *E. coli* O55 B5) and acute phase proteins (12, 24, and 48 hours after IV injection of LPS *E. coli* O55 B5). (**A**) ADA, (**B**) Cp, (**C**) Ovt, (**D**) LBSA, (**E**) PBSA, (**F**) TSA, and (**G**) IL-6. Data are expressed as means ± SD (n=9).
Note: [a,b,c,d,e,f]Columns with different superscripts are statistically different at P<0.05.
Abbreviations: ADA, adenosine deaminase; Cp, ceruloplasmin; *E. coli, Escherichia coli*; IL-6, interleukin-6; IV, intravenous; LBSA, lipid-bound sialic acid; LPS, lipopolysaccharide; Ovt, ovotransferrin; PBSA, protein-bound sialic acid; TSA, total sialic acid.

dexamethasone could significantly decrease ADA levels in *E. coli* LPS endotoxemia in rats.

It is well documented that the concentration of sialic acid immediately rises following different inflammatory stimuli.[52–54] In the present study, the concentrations of total sialic acid, lipid-bound sialic acid, and protein-bound sialic acid were significantly higher in the LPS-challenged groups than in the negative control group. The administration of meloxicam did not significantly affect LBSA level but reduced PBSA in comparison with the positive control group. Dexamethasone was more effective than meloxicam in reducing LBSA, PBSA, and TSA.

In this study, the ceruloplasmin concentration was significantly higher in LPS-inoculated chickens in comparison with that of non-inoculated birds. The increase in the ceruloplasmin concentration in the serum of chickens after LPS injection was consistent with the findings of Butler et al,[21] Curtis and Butler,[55] and Baert et al.[36] Moreover, dexamethasone, as a corticosteroid, showed greater effects on ceruloplasmin concentrations than did meloxicam. Baert et al[36] showed that different doses of sodium salicylate, as an NSAID, did not have significant effects on *E. coli* LPS endotoxemia in broiler chickens. Our findings are in accordance with that of a previous study, which showed that anti-inflammatory drugs, including tepoxalin, sodium salicylate, and ketoprofen, did not have significant effects on LPS-induced ceruloplasmin concentrations.[38]

Rath et al[20] reported that the concentration of ovotransferrin in chickens increases during inflammatory processes and microbial infections. Furthermore, they suggested that ovotransferrin concentration could be considered a diagnostic marker of infection and inflammation. In our study, 24 h after LPS injection, ovotransferrin concentration in *E. coli* LPS-inoculated positive control group was significantly higher than that of the negative control group. Horvatić et al[2] recorded the maximum concentration of ovotransferrin at 24 h after *E. coli* LPS inoculation in chickens. In our study, all the treated groups, except the one treated with 0.5 mg/kg meloxicam, showed significantly lower ovotransferrin concentrations compared with those of untreated positive control group.

Conclusion

Different doses of dexamethasone and meloxicam did not have significant effects on LPS-induced ALI scores and serum IL-6 levels. However, these anti-inflammatory drugs reduced adenosine deaminase, ceruloplasmin, ovotransferrin, lipid-bound sialic acid, protein-bound sialic acid, and total sialic acid in LPS-inoculated broiler chickens. Dexamethasone was more effective than meloxicam in the reducing of these LPS-induced markers.

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Disclosure

The authors declare no conflicts of interest.

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