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Original Research Article

Evaluation of the immunomodulatory activity of meloxicam *in vitro* and *in vivo*

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Abstract

Purpose: *To demonstrate the immunomodulatory activity of meloxicam based on cellular and humoral immune responses and in mice.*
Methods: *Cyclophosphamide-induced neutropenia assay and delayed-type hypersensitivity assay (DTH) were carried out to assess cellular immunity. In addition, mouse lethality and haemagglutination assays were carried out to investigate humoral immunity. Meloxicam was administered intraperitoneally in two doses, i.e., 5 mg/kg and 10 mg/kg to mice.*
Results: *Cyclophosphamide-induced neutropenia assay data showed a significant decline in differential leukocyte count (DLC) and total leukocytes count (TLC) in the meloxicam administered groups when compared with control group ($p < 0.05$). In DTH test, meloxicam showed a significant reduction in skin thickness against dinitrochlorobenzene than the control group, respectively ($p < 0.05$). A significant dose-dependent decline in antibody titre in the meloxicam-treated groups was observed ($p < 0.05$), while a gradual decrease in antibody titre occurred with increasing dose. However, there was significant rise in mortality ratio with increasing dose of meloxicam ($p < 0.05$).*
Conclusion: *The results indicate that meloxicam has immunosuppressive activity in mice, and therefore, can potentially be developed for use in countering organ transplant rejection.*

Keywords: *Meloxicam, Cellular, Humoral, Neutropenia, Haeamagglutination, Immunity*

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INTRODUCTION

All over the world, inflammatory and painful situations are often cured with non-steroidal anti-inflammatory drugs (NSAIDs) [1]. Meloxicam belongs to the enolic acid (oxicam) class of NSAIDs and is a potent inhibitor of cyclooxygenase-2 (COX-2) enzyme [2]. Cyclooxygenase-2 COX-2 acts on arachidonic acid to produce prostaglandins (PGs). Prostaglandins regulate a large number of physiological systems, i.e., the immune system, gastrointestinal system, respiratory system, central nervous system, cardiovascular system and endocrine system. Prostaglandin also plays a major role in many pathological conditions including cancer, hypertension and inflammation. The inhibition of PGs synthesis by meloxicam

suggests its immunomodulatory activity. The immune system is the basic host defensive system. A competent immune system protects against the harmful effects of the transformed cells as well as invading pathogens [3].

Prostaglandin E2 regulates the immune responses, including the production of cytokines and chemokines [2]. The inhibition of PGs synthesis by meloxicam suggests its immunomodulatory activity. Immune-mediated reactions are of two main types. Humoral immunity is controlled by antibodies while cellular immunity is determined through T-cells [6]. The humoral immune system is very diverse in its ability to produce antibodies against antigens which are recognized as foreign to the body [7]. The goal was to demonstrate the role of meloxicam on immune system.

EXPERIMENTAL

Immunomodulatory activity of meloxicam was evaluated on cellular and humoral immunity in mice. The animals were kept in the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan animal house. All the animals were nourished on a pelleted diet and water.

Chemicals

Meloxicam (Hilton Pharmaceuticals, Pakistan), dimethylsulphoxide (DMSO, Sigma-Aldrich, USA). Phosphate buffer saline (PBS) (Thomas Fischer Scientific, USA) and ether solvent (Merck Sharp & Dohme, Germany), dinitrochlorbenzene (DNCB, Sigma-Aldrich, USA), Cyclophosphamide powder for injection (Cytoxan Bristol Mayer squib, USA), Acetone (Riedel-de Haen AG, USA), water for injection (Otsuka Pakistan) were used in the study.

Animals

Albino mice aged 5 to 6 weeks were purchased from the Department of Theriogenology, UVAS, Lahore, and were kept under hygienic conditions at the animal room of UVAS Lahore in cages made of stainless-steel rods. The mice were given regular pellet diet and water. All trials were in accordance with the Institutional Rules for the Care and Use of Animals (approval no. DR/595/2018), and carried out following the National Institutes of Health Laboratory Animal Care and Use Guidelines. [6]. Mice in every protocol were divided into three groups, with five mice in each individual group. Group I was established as a control group. Then, 1 % dimethylsulphoxide (DMSO) was administered to the control group intraperitoneally. Non-toxic doses of DMSO and meloxicam were selected after the pilot study. Meloxicam (5 and 10 mg/kg) was injected intraperitoneally to groups II and III, respectively. All trial procedures were completed according to the established rules concerning the right use of animals.

Cyclophosphamide-induced neutropenia assay

Groups II and III were administered 5 and 10 mg/kg meloxicam, while group I which was selected as control, was administered 1 % DMSO intra-peritoneally for 13 days. On day 10 of the test, blood samples were drawn directly from the retro-orbital plexus of every mouse in order to perform DLC and TLC. Cyclophosphamide was injected subcutaneously in a dose of 200 mg/kg. On the 13th day of the test, Blood was withdrawn to carry out DLC and TLC [7]. Reduction in TLC and DLC was determined using Eq 1 [7].

$$PR (\%) = \{(N1 - N2)/N1\}100 \quad \ldots\ldots\ldots\ldots (1)$$

where PR is reduction, N1 and N2 are the number of cells before and after cyclophosphamide treatment.

Delayed type hypersensitivity (DTH) assay

The protocol was designed for the evaluation of delayed-type hypersensitivity with dintrochlorobenzene (DNCB) as an antigen. On the 1st day of the experiment, meloxicam5 and 10 mg/kg of body weight was injected intraperitoneally, while dimethylsuphoxide (DMSO) was administered to the control group. For DTH assay, two areas on the skin of each mouse were selected. The left side of each mouse in all the groups were selected for the sensitizing dose, and the right side for the challenging dose. On the second day of the trial, 0.1 mL of 2 % DNCB mixed with acetone was used as an immunizing dose on the skin of each mouse in all three groups on the left side.

Before applying DNCB, skin thickness was determined using vernier calipers. Six days after sensitization (8th day of experiment), 0.2 mL of 2 % DNCB was used as a challenging dose on the mice on the right side, and skin thickness (mm) was noted again after a period of 24, 48 and 72 h [9].

Haemagglutination assay (HA)

There were three groups i.e., control and meloxicam treated groups. DMSO was administered to the control group, and other groups received meloxicam for a period of 28

days. Every animal in the meloxicam-administered groups and the control group was immunized using 0.5 × 10^9 sheep's red blood cells (SRBCs) and with normal saline on 14th and 21st day of animal trial. On the 28th day of the experiment, blood was drawn from the retro-orbital plexus of all mice.

Round-bottomed 96-well plates (microtitre plates) were used for the HA titre. Different dilutions of serum were admixed with 0.025 x 10^9 red blood cells of sheep. Clumping in microtiter plates was evaluated after 2 h incubation period at 37 °C temperature. The results of HA titre of meloxicam-treated groups were compared with those of the control group [9].

Mice lethality test

Two doses of meloxicam, i.e., 5 and 10 mg/kg were administered to groups II and III. Control group was given 1 % DMSO via intraperitoneal route for a period of 21 days. All groups were given 0.2 mL of hemorrhagic septicaemic (HS) vaccine subcutaneously on the 7th and 17th day of the test. These groups were administered 10^7 cells/mL of *Pasteurella multocida* culture (0.2 mL). Mortality of mice was determined after 24, 48 and 72 h. Mortality ratio (R), assessed as ratio of number of dead mice to the total number of mice, expressed as a percentage [10].

Data analysis

Data were evaluated by one-way analysis of variance (ANOVA) with multiple comparison test, i.e., least significance difference (LSD), except for mice lethality assay data. The results were presented as mean ± standard deviation (SD). Differences were compared with control at $p < 0.05$.

RESULTS

Cyclophosphamide-induced neutropenia

The results showed a decline in TLC in the control group and meloxicam-administered group II (5 mg/kg) and group III (10 mg/kg) during the cyclophosphamide-induced neutropenia assay. There was also a reduction in TLC in the control group, group II and group III after cyclophosphamide administration (64.85, 66.82, and 67.2 %, respectively). There was also significant decrease ($p < 0.05$) in TLC among control and treatment groups (5 and 10 mg/kg), as well as in neutrophil count in groups II and III in comparison with control group. Decrease in neutrophil count was 19.29, 22.6, and 28 % in control, group II and group III, respectively after cyclophosphamide administration. (Table 1).

Delayed-type hypersensitivity

The results of skin thickness test revealed significant change in all groups at 24, 48 and 72 h after the application of dinitrochlorobenzene ($p < 0.05$). Groups II and III received meloxicam at a dose of 5 mg/kg and 10 mg/kg. There was an increase in the skin thickness in the control and meloxicam treated groups (5 and 10 mg/kg) after 24 h. The highest skin thickness level was noted in the control group compared to meloxicam treated groups.

There was a decline in skin thickness of mice in the control and meloxicam treated groups after 48 and 72 h. There was also a significant decrease in skin thickness in the meloxicam-treated groups II and III in comparison with negative control after 72 h ($p < 0.05$), as shown in Figure 1.

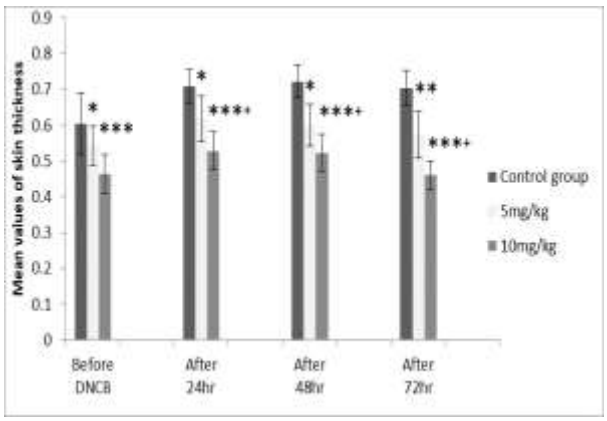

Figure 1: Skin thickness after application of DNCB

Table 1: Effect of meloxicam (0, 5 and 10mg/kg doses) on cyclophosphamide-induced neutropenia in mice

Group	Reduction in leukocyte count (%)	Reduction in lymphocyte count (%)	Reduction in monocyte count (%)	Reduction in neutrophil count (%)
I (Control)	64.85	12.91	20.25	19.29
II (5mg/kg dose)	66.81	18.20	21.70	22.60
III (10mg/kg dose)	67.20	19.52	34.88	28.00

Haemagglutination assay

Antibodies were obtained from serum of the mice and titrated against sheep red blood cells. Formation of agglutination was an indication of the presence of antibodies in the serum. There was a dose-dependent decrease in HA titre of meloxicam. With increasing dose, there was decline in the HA titre of meloxicam. The meloxicam-treated groups (5 and 10 mg/kg) showed a reduction in HA titre compared to the control group. There was also a significant ($P<0.05$) decrease in HA titre in meloxicam 5 mg/kg dose compared to the control group. Figure 2 shows that there was a significant reduction in t HA titre ed compared to control group ($p < 0.05$).

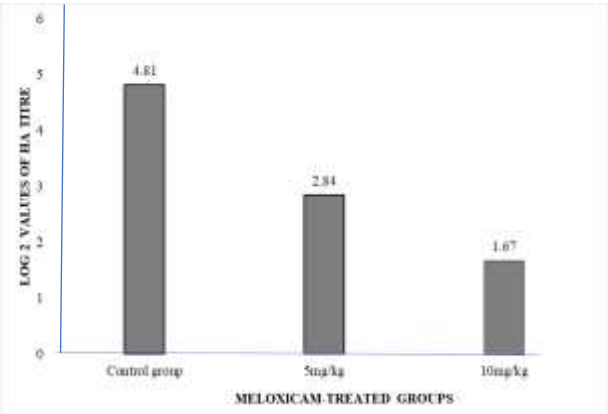

Figure 2: Antibody titre of meloxicam administered groups. There was significant decline in antibody titre with increasing dose compared to control ($p < 0.05$)

Mouse lethality

The mortality rate of mice was due to a decreased production of antibodies. There was a dose-dependent increase in the mortality ratio of mice with an increasing dose of meloxicam. Its mean highest mortality ratio was found between control and 10 mg/kg dose of meloxicam. In mice, lethality test mice exhibited 100 % death rate at a dose of 10 mg/kg, 80 % at a dose of 5 mg/kg, and 40 % mortality ratio was detected in the control group after a duration of 72 h (Figure 3).

DISCUSSION

Meloxicam belongs to the enolic acid (oxicam) class of NSAIDs, and is a potent inhibitor of cyclooxygenase-2 (COX-2) [2]. Cyclooxygenase-2 acts on arachidonic acid (a polyunsaturated fatty acid) to produce prostaglandins, which regulates a large number of physiological and pathological systems [12]. Prostaglandin E2 inhibits the production of cytokines and chemokines thereby regulating the immune system. [3]. Currently, the immunomodulatory activity of meloxicam was evaluated. The immune system is divided into two main components: cellular immunity and humoral immunity. T cells regulate cellular immunity while B cells regulate humoral immunity [6].

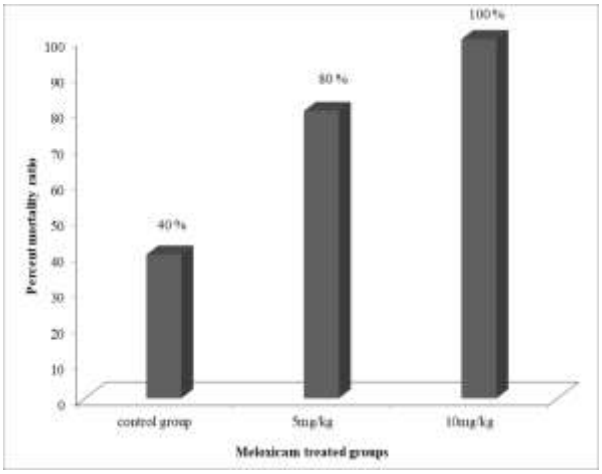

Figure 3: Mortality in meloxicam-treated and control groups

In the current study, the administration of cyclophosphamide reduced differential Leukocyte count and total leukocyte count in all treated groups. Cyclophosphamide is an alkylating agent which causes the myelosupression by interference with DNA synthesis and function [13] and also causes neutropenia [14]. Neutropenia is defined as a reduction in neutrophil count in circulating blood. Severe and persistent neutropenia increased exposure to bacterial and fungal infections [15]. Neutrophils are a component of the immune system and are responsible for killing microorganisms [16].

The percentage decrease in differential leukocytes count and total leukocytes count (except monocytes) was higher in meloxicam-treated groups compared to the control group. This suppression was noticeable at a higher dose, which suggests the influence of meloxicam on hematopoietic cells. The decrease in TLC was due to a decline in the count of neutrophils and lymphocytes. Neutrophils are the most prominent components of the first line of cellular defense against invading microbes which ingest the microbes by phagocytosis [17]. Decreased neutrophil count suggests immunosuppression.

Delayed type hypersensitivity assay effect of DNCB was evaluated. It formed a dinitrophenyl complex with proteins of the skin [9]. Dinitrochlorobenzene was used first as a

sensitizing dose, and when it was applied to the skin of mice, it initiated the activation of T cells. These stimulated T cells changed into lymphoblasts and resulted in the release of cytokines. Cytokines were responsible for attracting more immune cells at the site antigen application to show a protective response [18].

Inflammation in the DTH test was highest in the control group which was injected with DMSO. On the other hand, there was less inflammation in groups II and III. The thickness of the skin was increased within 24 h after administration of challenge dose, and progressively reduced after 48 and 72 h. There was a significant reduction in the inflammation at a higher dose in comparison with the lower dose. In the current research work, a gradual decrease in DTH response was found in dose dependent manner of meloxicam [20].

Haemagglutination test was carried out to determine the influence of meloxicam on humoral immunity. It is responsible for the contact of antigen with plasma B-lymphocytes that could later change into antibody secreting cells. Antibodies protect in two ways: by deactivating the antigen or by assisting its phagocytosis [17]. Antibody production against sheep's red blood cells in mice sensitization was determined using haemagglutination assay [21]. Haemagglutination test was carried out to determine the influence of meloxicam on humoral immunity. Antibodies bind with antigens to neutralize them [22]. HA titre observed at lower dose of meloxicam, was less than the HA titre in the control group, but was greater than the values of HA titre at a high dose in the meloxicam treated group. Results revealed that meloxicam at a high dose significantly decreased HA titre. It was decreased gradually by increasing the dose of the meloxicam, which recommended the lessening of the formation of immunoglobulin G and immunoglobulin M antibodies in the serum obtained from mice due to antigen sheep's RBCs.

Mice lethality assay is employed to assess the immunological response in mice that are previously vaccinated. *Pasteurella multocida* culture is toxic to mice. The mice were immunized with the vaccine before the injection of bacteria (*Pasteurella multocida*) culture, and then the death ratio was determined [23]. Mice lethality test determines the ability of mice to produce antibodies against antigen [24]. A vaccine is responsible for the formation of antibodies against the culture of bacteria. If enough antibodies are produced to counteract the action of *Pasteurella multocida* culture, then there will be no death of mice deaths. However, if insufficient antibodies are produced, then there will be more mice deaths. The mortality of mice was at a higher rate than in meloxicam injected group. This result suggests the reduced formation of antibodies with *Pasteurella multocida* through the B-lymphocytes of mice protected with hemorrhagic septicemia vaccine at a high and low dose of meloxicam. The findings of this study indicate that immunosuppression may be the result of COX-2 inhibition by meloxicam [25].

CONCLUSION

The findings of this study show that meloxicam suppresses both arms of the immune system, i.e., the cellular and the humoral. Therefore, it may be beneficial as an adjunct in the transplantation of organs and in autoimmune disease besides being an active analgesic, antipyretic and anti-inflammatory agent. Further studies on its immune suppressive properties are, however, required.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The content was entirely written by the author(s). No work has been completed by any persons other than

the author(s). The contributing authors are responsible for the claims mentioned within this article. These authors contributed equally.

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