## Original Research Article

# Evaluation of immunomodulatory activity of tenoxicam in mice

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## Abstract

*Purpose:* The present study was conducted to evaluate the effect of tenoxicam on cellular and humoral immunity.
*Methods:* Tenoxicam (2.5 - 10mg/kg) was administered at three different doses to three groups of mice and the cellular immune responses were studied using delayed hypersensitivity response (DTH) and cyclophosphamide-induced neutropenia while the humoral immune response was evaluated using hemagglutination test and mice mortality ratio. Normal saline and cyclophosphamide were used as negative and positive controls, respectively.
*Results:* DTH assay resulted in a significant reduction in skin thickness (p < 0.05) for tenoxicam treated groups when compared to the negative control group at 24 h, 48 h and 72 h after administration of challenging dose of dinitrochlorobenzene (DNCB). Cyclophoshamide induced neutropenia showed a significant percentage reduction in total leukocyte count (TLC) and differential leukocyte count (DLC) i.e. lymphocytes and neutrophils (p< 0.05), but an increase in monocytes in all the treatment groups in the following order: 10 mg>5 mg >2.5 mg> negative control group. A dose dependent reduction response was observed (p<0.05) in haemagglutination assay (HA). In mice lethality test mortality ratios of 2.5 mg, 5 mg, 10 mg tenoxicam were 60 %, 80% and 100 %, respectively, compared to 20 % and 100 % for normal saline group and cyclophosphamide, respectively
*Conclusion:* The results suggest that tenoxicam suppresses both cellular and humoral immunity in mice.

*Keywords: Tenoxicam, Cellular immunity, Humoral immunity*

## INTRODUCTION

Infiltration of blood leucocytes to an infected area in the body triggers inflammatory response. This response is initiated when body tissues are damaged by bacteria, trauma, toxins, heat, or any other agent [1]. Inflammatory mediators, which include prostaglandins, leukotrienes, histamines and bradykinins, secreted by the inflamed organs of the body propagate the inflammation [2]. In response to the inflammatory reactions, tissue manifests swelling pain, oedema and hyperthermia. By blocking the synthesis of inflammatory mediators, anti-inflammatory drugs are able alleviate the inflammatory response. These anti-inflammatory

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drugs are mainly steroids and non-steroidal anti-inflammatory drugs (NSAIDs). The NSAIDs has been categorized into different groups on the basis of their variable response and mode of action and exert their actions by blocking cyclooxygenase 1 and 2 (COX 1 and COX 2) enzymes [3]. The enzymatic inhibition of COX causes blocking of prostaglandins that modulate immunity through complicated communication with white blood cells and parenchymal cells in the inflamed organ [4]. Prostaglandins binding receptors involved in inflammation are also expressed throughout the immune system [5].

Oxicams, an important family from the non-steroidal anti-inflammatory drugs, bind to plasma proteins and have weak acid characteristics. Tenoxicam is a new drug from this family which is used to treat many inflammatory disorders. It is often preferred due to less side effects and long half-life (67 h) as compared to other NSAIDs [6] and produces its anti-inflammatory and analgesic effects by inhibiting prostaglandins ($PGE_2$) [7]. Besides its role as an anti-inflammatory drug, an earlier study reported that tenoxicam suppresses the immune system of the subject by inhibiting in vitro functional chemotactic response of neutrophils and monocytes but a detailed work on its role on immune response is yet to be explored [8,9]. In this study, the cellular and humoral immunity response to tenoxicam was evaluated.

# EXPERIMENTAL

The experimental research was performed in the Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan with the collaboration of University Diagnostic Laboratory, UVAS Lahore, Pakistan.

## Animals

A total of 120 healthy albino mice (20 to 30 g) were bought from the Department of Theriogenology, University of Veterinary and Animal Sciences (UVAS) Lahore. They were kept in the laboratory for acclimatization for 48 hrs and then divided randomly into five groups for each test, nourished with standard pellet diet and water during whole experimental period. All the ethical issues were considered as mentioned in the institutional guidelines regarding the experimental use of mice.

## Materials

The following chemicals were used: tenoxicam (Zhejiang Chemicals Imp & Exp. Corp,

Hangzhou, China), normal saline (Medipak Ltd., Lahore, Pakistan), dinitrochlorobenzene (DNCB) (Alfa Aesar, Heysham, Lancaster, England), cyclophosphamide injection in powder form (cyclomide) (Pharmedic laboratory Pvt. Ltd Lahore, Pakistan), acetone and ether solvent (*Sigma-Aldrich Laborchemikalien GmbH Seelze,* Germany) and phosphate buffer saline (PBS) (Bioplus Fine Research Chemicals, Madison, USA).

## Experimental design

Tenoxicam was administered at three different doses to mice and the cellular immune response was studied using delayed hypersensitivity test (DTH) and neutropenic effect of cyclophosphamide while the humoral immune response was evaluated using hemagglutination test and mice mortality test. Animals treated with normal saline and cyclophoshamide served as negative and positive control groups, respectively.

## Delayed type hypersensitivity (DTH) assay

A total of 25 mice organized into five groups (A, B, C, D and E; five mice in each group) were used for the delayed type hypersensitivity (DTH) assay as previously reported [10]. On the 1st day of experiment tenoxicam at the doses of 2.5 mg, 5mg and 10mg in group C, D and E respectively were administered intraperitoneally (i.p.) while to the negative control group (group A) only normal saline was administered. Cyclophoshamide, at dose of 150 mg / kg, was given i.p. to the positive control group (group B). On the 2nd day of experiment (i.e. one day after the treatment with tenoxicam), hair were removed (shaved) from left side of all the mice with the help of a scissor. Measurement of skin thickness was taken with the help of digital vernier calliper (mm). Dinitrochlorobenze (DNCB, 2 %) solution in acetone at measured amount of 0.1 ml was applied to the shaved area of the skin (4 $cm^2$) marked with permanent marker. After the 12th day post sensitization (14th day of experiment) skin thickness of mice was measured from the shaved area and a challenging dose (0.2ml) of 2 % DNCB in acetone was applied. Skin thickness was measured again using a digital vernier callipers (mm) after 24, 48 and 72 h.

## Cyclophosphamide induced neutropenia assay

All the experimental animals (20 albino mice) were weighed and sorted out into four groups (A, B, C and D) with each group having five mice [11]. Treatment groups B, C and D were

administered with tenoxicam (2.5 mg, 5mg and 10mg respectively) intraperitoneally daily for total of 13 days while the mice for the control group (group A) were given saline solution for the same duration. On 10th day of experiment 200 mg/kg cyclophosphamide was administered subcutaneously once to both tenoxicam treated groups and to the control group. Blood sample was collected and total leukocyte count (TLC) as well as differential leukocyte count (DLC) for lymphocytes, monocytes and neutrophils were performed by using automatic haematological analyzer (Dawn Analytical Supplies & Calibrators) prior to the administration of cyclophosphamide. On day 3 after injection of cyclophoshamide i.e. 13th day (last day) again blood samples were analyzed for TLC and DLC. Percentage values of total and differential leucocytes in tenoxicam treated groups and the control group were compared prior to and after the cyclophosphamide administration.

### Haemagglutination assay

Five groups of mice (A, B, C, D, and E; five mice in each group) were selected for assay [12]. Each animal in the tenoxicam treated group (C, D and E) was administered tenoxicam (2.5 mg, 5mg and 10mg) respectively through intraperitoneal route daily for 28 days. Each of the mouse in groups A and B was administered normal saline and cyclophoshamide (150 mg/kg) i.p. for the same duration (28 days) while all the mice in treatment groups and control groups were injected intraperitoneally with $0.5 \times 10^9$ sheep's red blood cells/ mouse i.p in phosphate buffer saline on 14th and 21st day of the test. On last day of treatment (28th day) blood serum was separated from blood and HA titre was performed. The maximum serum dilution (minimum volume of serum) in which haemagglutination appeared was known as haemagglutination (HA) titre.

### Mice lethality test

This test was done with a total of 25 mice, which were arranged into five groups (A, B, C, D and E) each having five mice [13]. All the mice in the tenoxicam treated groups (C, D and E) were received daily doses of 2.5 mg, 5mg and 10mg of tenoxicam intraperitoneally for 21 days while those in groups A and B were administered with normal saline and cyclophoshamide (150 mg/kg) i.p. respectively. All the experimental mice were immunized with 0.2 ml haemorrhagic septicemia vaccine (HS vaccine) subcutaneously on the 7th and 17th day of the test. Challenging dose of

0.2ml of *Pasteurella multocida* culture ($10^7$ cells per ml) was given to all of the mice subcutaneously on the 21st day of experiment. The mice were then observed for three days and the mortality rate calculated as reported previously [13].

### Statistical analysis

All the results were assessed by one way analysis of variance (ANOVA) followed by Duncan's post-test. Results were compared with negative control group at significance level of *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Statistical Package for Social Sciences (SPSS for Windows version 16, SPSS Inc, and Chicago, IL, USA) was used.

# RESULTS

Delayed type hypersensitivity showed a significant difference between the mean values of skin thickness of all the treatment and control groups at one, two and three days intervals ($P<0.05$). The percentage reduction in the mean skin thickness was dose dependent. Maximum percentage reduction was observed in group E and the lowest was observed in group C when compared with the negative control group (Table 1). Mice of group B showed the maximum change in skin thickness in comparison with the mice treated with tenoxicam.

In cyclophosphamide induced neutropenia assay, a dose dependent reduction response was observed in tenoxicam treated groups. Group D showed a maximum reduction in TLC and DLC when compared to the control group (Figure 1). Group B showed minimum reduction of cell count (lymphocytes, monocytes and neutrophils) in comparison to the control group (figure 2). Percentage reduction for TLC in mice (A, B, C and D) was 62.67 %, 64.47 %, 70.11 % and 73.2 % respectively while decrease in differential leucocytes count (DLC i.e. lymphocytes, neutrophils and monocytes ) in the group A was 12.72%, 31.2% and 30.28%, in group B was 14.05%, 35.5% and 37.1%, in group C was 21.1%, 37.6% and 55.4%, and in group D was 23.4%, 62.6% and 54.7% after cyclophoshamide administration.

TLC decreases after administration of cyclophoshamide in all the control and tenoxicam treated groups .Data is presented as mean and standard deviation.

**Table 1:** Effect of tenoxicam on skin thickness

| Time for skin thickness measurement | Negative Control group (Normal Saline) | Positive control group (cyclophosphamide) | Tenoxicam groups | | |
|---|---|---|---|---|---|
| | | | 2.5 mg/kg | 5mg/kg | 10mg/kg |
| Before challenge with DNCB | 0.59±0.055 | 0.53±0.067 | 0.64±0.042 | 0.64±0.042 | 0.56±0.072 |
| After 24h | 0.77±0.038* | 0.62±0.051+ | 0.80±0.023* | 0.75±0.045 | 0.64±0.065 |
| After 48h | 0.77±0.037* | 0.58±0.053+ | 0.78±0.035* | 0.73±0.039 | 0.63±0.069 |
| After 72h | 0.76±0.030* | 0.56±0.043+ | 0.77±0.030* | 0.71±0.038 | 0.59±0.055 |

*Values were shown as mean value ± standard deviation (SD) n= 5 in skin thickness, the change in skin thickness of low, standard and high dose tenoxicam treated group (2.5 mg, 5 mg and 10 mg), negative and positive control group was significant at \*p < 0.05 when compared to the normal saline group. +p < 0.05when compared to the positive control group. \*p < 0.05 when compared to the low dose of tenoxicam (2.5 mg)*
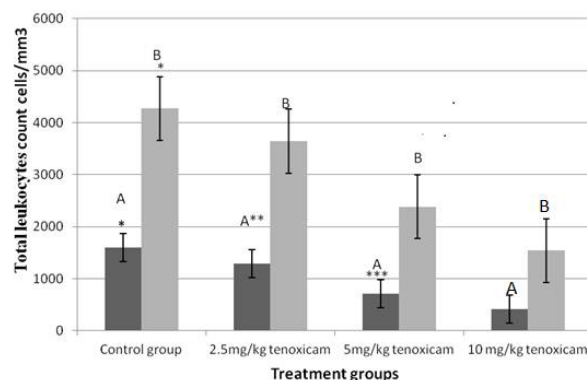


**Figure 1:** Total leukocyte count (TLC) before and after cyclophoshamide administration. A = After cyclophoshamide administration, B = Before cyclophoshamide administration

High dose tenoxicam group showed the maximum decrease as compared to low (2.5 mg) and standard (5 mg) tenoxicam treated group. Control group showed minimum change in TLC. Control group showed significance at p < 0.05, low dose group at p < 0.01 and high dose group at p < 0.001.
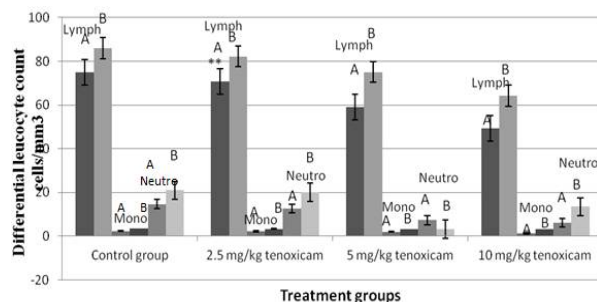


**Figure 2:** Differential leukocyte count (DLC) decreases after administration of cyclophoshamide in all the control and tenoxicam treated groups. **A** = After cyclophoshamide administration, **B** = Before cyclophoshamide administration

DLC decreases after administration of cyclophoshamide in all the control and tenoxicam treated groups. Data is presented as mean and standard deviation. High dose tenoxicam group showed the maximum decrease as compared to low (2.5 mg) and standard (5 mg) tenoxicam treated group. Control group showed minimum change in DLC. Tenoxicam at low dose was significant at p < 0.01. All the other results were significant at p < 0.05 except the monocytes level after cyclophoshamide administration

The value of HA titre revealed that the high dose group E in the experiment showed the minimum HA titre. Low dose group C showed maximum titre among the treated tenoxicam groups when compared to the negative saline group. Cyclophosphamide group and group E showed equal mean values of HA titre (Table 2).

**Table 2:** Mean values of HA titre of control and tenoxicam treated groups

| Group | Mean HA titre value |
|---|---|
| Negative control group (Normal Saline) | 57.6±14.31* |
| Positive control group (cyclophosphamide) | 2.4±1.67+ |
| 2.5mg/kg tenoxicam | 44.8±17.52* |
| 5mg/kg tenoxicam | 16.8±9.9 |
| 10mg/kg tenoxicam | 2.4±0.89 |

*HA mean ± standard deviation (SD), n=5, the change in HA titre (2.5 mg, 5mg and 10 mg) tenoxicam groups was significant at \*p < 0.05 compared to the normal saline group. +p < 0.05 when compared to the cyclophosphamide group. p < \*0.05 when compared to the low dose tenoxicam group (2.5 mg)*

In the mice lethality test, the mice treated with high dose of tenoxicam had comparable mortality ratio when compared to the positive control group (Table 3).

## DISCUSSION

In this study tenoxicam suppressed both the cellular and humoral immunity in mice in response to DTH and cyclophoshamide induced neutropenia. The maximum inflammatory response was observed in DTH against DNCB

**Table 3:** Effect of tenoxicam and control groups on mice lethality

| Group | Number of mice that died | | | Total number of mice died after 72 hours | Percentage mortality ratio |
|---|---|---|---|---|---|
| | *Between 0-24 hours* | *Between 24- 48 hours* | *Between 48-72 hours* | | |
| Normal saline | - | 1 | - | 1/5 | 20% |
| Cyclophosphamide | 3 | 1 | 1 | 5/5 | 100% |
| 2.5mg/kg tenoxicam | - | 3 | - | 3/5 | 60% |
| 5mg/kg tenoxicam | - | 4 | - | 4/5 | 80% |
| 10mg/kg tenoxicam | 4 | 1 | - | 5/5 | 100% |

in negative control group while the minimum response was observed in the mice treated with tenoxicam high dose (group E).

Tenoxicam has demonstrated inhibition of the production of prostaglandins from inflammatory tissues [14,15]. Less production of inflammatory mediators could be the reason of decreased response to DNCB. Previously research described the effect of aspirin on immune cells of mice; treatment with aspirin in vivo has the ability to reduce the number of macrophages and nonopsonic phagocytosis [16,17].

Cyclophoshamide used in this experiment is a pro-drug which produces active alkylating species after metabolic transformation. These species attach to DNA, causing strand breakage and cross linking, and resulting in death of fast replicating cells [18]. The observed dose dependent percentage reduction in total leucocytes count (TLC) in cyclophosphamide treated animals observed in groups (A, B, C and D) was dose dependent. This suppression of leucocytes was prominent for group D. Reduction in neutrophil count suggests neutropenia which consequently results in immunosuppression. Humoral response works by the continuous specialized conversion of memory B cells to antibody-secreting plasma cells. Antibodies defend the body against antigen [19]. Production of antibodies against the antigen presented to immune system is a manifestation of active humoral immunity. Antibodies against sheep RBCs were produced and measured by haemagglutination assay. An effect of tenoxicam on humoral arm of immune system was observed by haemagglutination assay. The titre was high in negative control group as compared to treated groups. Results showed that tenoxicam at high dose (group E) had a decreased HA titre which could be due to decreased production of immunoglobulins [20]. Humoral antibodies detected in response to killed *Pasteurella multocida* vaccine showed that the post-challenge death ratio was high in tenoxicam treated groups as compared to the control group. Possibly this could be due to less production of antibodies against *Pasteurella multocida* by B lymphocytes of mice at high dose (10 mg/kg body weight).

## CONCLUSION

Tenoxicam is known to possess analgesic, anti-inflammatory and anti-pyretic activity. This study has shown that it also possesses immunosuppressive activity (both cellular and humoral arms of immunity). Thus the compound may be of useful in organ transplant rejection and autoimmune diseases.

## DECLARATIONS

### Conflict of interest

No conflict of interest associated with this work.

### Contribution of authors

We declare that this work was done by the authors mentioned in this article and all liabilities regarding claims relating to the content of this article will be borne by the authors. Aqeel Javeed conceived and designed the study. Fatima Nasim collected, analysed, and wrote the manuscript with the collaboration of Aamir Ghafoor and Muhammad Ashraf. All authors read and approved the manuscript for publication.

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