



# Evaluation of Cellular and Humoral Immune Responses of Piroxicam in Murine Model

Bushra Zahoor<sup>1</sup>, Aqeel Javeed<sup>1\*</sup>, Naeem ur Rehman<sup>2</sup>, Ahmed Tokhi<sup>2</sup>, Zainab Ahmed<sup>2</sup>, Khadijeh Esmaeilpour<sup>3\*</sup>, Muhammad Ashraf<sup>1</sup>, Jawad Nazir<sup>1</sup> and Ahmad Hamdani D<sup>1</sup>

<sup>1</sup>Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences, Pakistan

<sup>2</sup>Department of Pharmacy, COMSATS University Islamabad, Pakistan

<sup>3</sup>Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Iran

## Abstract

**Introduction:** Piroxicam has analgesic, anti-inflammatory and antipyretic activity. It inhibits the formation of prostaglandins, important regulator of the immune system. The current study was conducted to assess immunomodulatory effects of piroxicam on cell mediated as well as humoral immunity in mice.

**Material and Method:** Type 4 Delayed Hypersensitivity reaction (TDH) assay along with cyclophosphamide induced neutropenia were performed for the study of cell mediated immunity. The effect of the drug on humoral immunity was studied by the Hemagglutination Assay (HA) and mice lethality test. Mice were treated with piroxicam at a dose 5 mg/kg and 10 mg/kg intraperitoneally.

**Result:** The data was evaluated and analyzed via ANOVA (\*P<0.05). There was significant reduction in mean skin thickness and neutrophil count in DTH assay and cyclophosphamide induced neutropenia respectively when compared to the control group. The dose dependent reduction was observed in HA assay. In mice lethality assay, the mortality ratio was higher in 10 mg/kg piroxicam treated group.

**Conclusion:** It is concluded from present study that piroxicam may have immunosuppressant effects addition to its analgesic action and it may be clinically effective in autoimmune diseases and organ transplantation.

**Keywords:** Piroxicam; Cell mediated immunity; Humoral immunity

## Introduction

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are the most commonly used drugs world widely and they are as effective as opioids and acetaminophen in treating acute musculoskeletal pain [1,2]. Piroxicam is an oxicam derivative, a NSAID having analgesic, anti-inflammatory and antipyretic property that is generally used to mitigate many inflammatory diseases like rheumatoid arthritis, osteoarthritis, musculoskeletal disorders and postoperative pain [3,4]. *In vitro*, the potency of piroxicam as an inhibitor of Prostaglandin (PG) biosynthesis is similar to indomethacin. Piroxicam is a preferential Cyclooxygenase-1 (COX-1) inhibitor [2]. The drug inhibits the formation of prostaglandins which plays a vital role in immune system [5-7]. Piroxicam stimulates or modulates the immune functions requiring lymphoproliferation by suppressing the formation of PG-E2 that is a natural inhibitor of both LC (Lympho Proliferative Cytokines) and LC-induced lymphoproliferation production [8,9].

Immune system plays an important role for fighting against various diseases. Regulation of immune response can help in the treatment and prevention of a number of diseases [9]. In the immune system, antigen presenting cells are dendritic; macrophages produced cytokines and Prostaglandins (PG). Cytokines and PGs regulate immune responses [10]. Immune system has two main components that are adaptive and innate immunity. Acquired immunity has been intensively studied. The roles and mechanisms of innate immunity responses might be different in all organisms that have both innate and adaptive immunity and also those that possess only innate immunity. On the other hand, invading pathogenic microorganisms use several strategies to inhibit the host immune system for their survival [11].

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### \*Correspondence:

Aqeel Javeed, Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, Pakistan,  
E-mail: aqeel.javeed@uvas.edu.pk  
Khadijeh Esmaeilpour, Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran,  
E-mail: Esmaeilpour@kmu.ac.ir

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DTH is a protecting localized cell-mediated immune response mainly against intracellular pathogens. It is commonly used for research to find immune reactions and it is an important diagnostic test in commercial settings [12]. Hemagglutination is a process of agglutination in which antibodies are bind with Red Blood Cells (RBCs). RBCs act as an antigen and are very suitable targets due to their availability. It is commonly used assay for the determination of the antibodies titer in a serum, viral quantification and blood grouping [13]. To measure serological responses in experimental models that are vaccinated with different vaccines, commonly employed method is mice lethality test [14].

## Materials and Methods

### Chemicals

Piroxicam was provided by Pfizer pharmaceuticals and polyethylene glycol 600 (PEG used as vehicle) purchased from Applichem industry, acetone from Riedel-de-Haen, Dinitrochlorobenzene (DNCB) from Lancaster, cyclophosphamide powder for injection (cyclomide) from Pharmedic laboratory Pvt. Ltd Lahore, water for injection from Elixir Laboratories Pvt. Ltd. Lahore, ether solvent from Den Norsken Eterfabrikk oslo-Norway, Phosphate Buffer Saline (PBS) from Oxoid limited Basingstoke Hampshire (England).

### Organism and vaccine

*Pasteurella multocida* strain of bovine origin was obtained from Veterinary Research Institute (VRI) Lahore, Pakistan. Hemorrhagic Septicemia (HS) vaccine against *Pasteurella multocida*. Sheep Red Blood Cells (SRBCs) used as antigen.

### Experimental animal

Swiss Albino mice having weight range 25 gm to 35 gm and aged 6 to 8 weeks were purchased from VRI. All the experimental protocols were approved from Animal Ethical Committee of UVAS. Mice were kept in transparent plastic cages with 12 h of dark-light cycle starting 8 am to 8 pm in the animal house of UVAS, Lahore. Animals were provided a saw dust bedding which was cleaned on every alternative day taking into consideration all possible hygienic parameters. Animals were provided with pathogen free standardized mice food along with water ad libitum.

### Piroxicam treatment/selection of dose

In every experiment 15 mice were used and divided into three groups (A, B and C) consisting 5 mice in each group. The first group (group A) was used as a control group (received PEG intraperitoneally), the second group and the third group (B and C) received 5 mg/kg and 10 mg/kg IP of Piroxicam respectively.

### Assessment of cell mediated immunity (CMI)

**Delayed type hypersensitivity (TDH):** Delayed type4 hypersensitivity assay was performed as prescribed by with some modifications [15]. After treatment with Piroxicam on day 1, day 2 was followed by 0.1 mL of sensitizing dose of DNCB 2% in acetone with application at 4 cm<sup>2</sup> shaved area of the skin on left side of every mice in all groups (A, B and C). Immediately before applying DNCB, digital vernier scale was used to measure the thickness of mice skin (mm). On 8<sup>th</sup> day of experiment i.e. Day 6 after sensitization, a boosted dose (0.2 ml) of DNCB 2% was applied on right side of the skin. Skin thickness was measured (mm) after 24, 48 and 72 h, using digital vernier scale. In control group, acetone 0.1 mL excluding DNCB was incorporated on skin, serving as placebo during the test.

**Cyclophosphamide induced neutropenia:** Swiss albino mice were pre-treated with the piroxicam and solvent for ten days. On day 10<sup>th</sup> of experimental protocol, cyclophosphamide at a dose (200 mg/kg subcutaneously) produced neutropenia and was considered day zero. Each mouse heart was punctured to collect the blood and Total Leukocyte Count (TLC) along with Differential Leukocyte Count (DLC) was performed prior to administration of cyclophosphamide. On 13<sup>th</sup> day of the test (on 3<sup>rd</sup> day) after injection of cyclophosphamide, same procedure was repeated as performed on 10<sup>th</sup> day. The TLC and DLC of piroxicam treated groups were compared with the values of the control [16].

### Assessment of humoral immune immunity

**Hemagglutination assay (HA):** Albino mice were weighed and divided into three groups (A to C), each group has 5 mice. Groups C and D were pretreated with piroxicam for 14 days and group A received vehicle. On 14<sup>th</sup> day, each mouse was immunized with  $0.5 \times 10^9$  Sheep Red Blood Cells (SRBCs) intraperitoneally. The day of immunization was referred to as day 0. On 28<sup>th</sup> day, blood samples were collected from each mouse, centrifuged the blood sample at 5000 rpm, 4°C for 10 min and serum was separated properly. Round-bottom 96-well plates (microtiter plates) was preferred for this assay. In each well, 50 (µl) of Phosphate Buffer Saline (PBS) was added. In the first column of the plate, added 50 µl of sample (serum of mice). Mixed each well and transfer 50 µl to the next well on its right. Added 50 µl of 0.5% sheep red blood cell working solution to each well and mixed properly. Hemagglutination was determined by titrating serum dilutions with sheep's red blood cells ( $0.025 \times 10^9$  cells). Incubated the microtitre plates at room temperature for two hours without any disturbance. The highest dilution of serum show hemagglutination was expressed as HA titre [17].

**Mice lethality test:** All albino mice were divided into three groups (A to C), each having 5 mice. Group C and D were treated with 5 mg/kg and 10 mg/kg of piroxicam intraperitoneally respectively and group A received solvent. On the 7<sup>th</sup> and 17<sup>th</sup> day of the experiment, each mouse of all groups (A to C) was immunized with Hemorrhagic Septicemia vaccine (HS vaccine). On 21<sup>st</sup> day the mice were challenged subcutaneously with 0.2 ml of *Pasteurella multocida* culture containing  $10^7$  cells per ml. The animal was observed for a period of 72 h (24, 48 and 72 h after injecting of *Pasteurella multocida*) to detect any mortality in the inoculated mice. The mortality ratio was determined by using the following formula [18].

$$\text{Mortality ratio} = \frac{\text{No. of animals dead}}{\text{Total no. of animals}}$$

**Statistical analysis:** One-way Analysis of Variance (ANOVA) followed by multiple comparison test i.e. LSD (Least Significant Difference) was used for statistical evaluation. The values are expressed as mean  $\pm$  SD and \*P<0.05 value was considered significant.

## Results

### Delayed type hypersensitivity (DTH) assay

Cellular immune response was measured by DTH. The mean difference of skin thickness was highly reduced at dose of 10 mg/kg than 5 mg/kg of piroxicam, administered intraperitoneally in group B and group C when compared against control group A. There was significant difference between the values of all the groups on 24, 48 and 72 h at the significant level of \*P<0.05 (Table 1).

### Cyclophosphamide induced neutropenia assay

The required dose of cyclophosphamide to produce neutropenia

**Table 1:** Effect of piroxicam on delayed type hypersensitivity assay.

Parameters	Changes in skin thickness of control group	Changes in skin thickness of 5 mg group	Changes in skin thickness of 10 mg group
Before challenging with DNCB	0.648 ± 0.025884	0.592 ± 0.0311	0.556 ± 0.0089
After 24 hrs	0.618 ± 0.021679	0.568 ± 0.03033**	0.522 ± 0.0130***+
After 48 hrs	0.6 ± 0.014142	0.544 ± 0.0378**	0.502 ± 0.01643***+
After 72 hrs	0.586 ± 0.016733	0.528 ± 0.0376**	0.482 ± 0.0204***+

Effect of piroxicam on mean difference in skin thickness of mice was assessed by delayed type of hypersensitivity. Results are expressed as mean ± SD (n=5). Data were analyzed by one-way Analysis of Variance (ANOVA) followed by multiple comparison test that is LSD \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 when compared against control group. \*P<0.05 when 10 mg/kg of piroxicam treated group compared with 5 mg/kg piroxicam treated group

**Table 2:** Effect of piroxicam cyclophosphamide induced neutropenia.

Treatment	Total leukocytes		Percentage reduction	Lymphocytes		Percentage reduction	Monocytes		Percentage reduction	Neutrophils		Percentage reduction
	Before treatment	After treatment		Before treatment	After treatment		Before treatment	After treatment		Before treatment	After treatment	
Control group	448 ± 533.66	247 ± 552.96	44.86%	61.78 ± 2.54	47.8 ± 5.61	22.66%	8.64 ± 2.828	6.4 ± 2.125	25.46%	22.9 ± 1.635	16.1 ± 2.723	29.69%
	3520 ± 589.06	934 ± 92.08		58.4 ± 2.72	38.16 ± 3.722		8.04 ± 2.688	5.64 ± 0.79		18.56 ± 1.066771	12.5 ± 1.593738	
5 mg/kg	2792 ± 280.12	570 ± 114.23	73.46%	47.86 ± 3.647	28.52 ± 3.248	34.65%	7.42 ± 0.967	5.04 ± 0.24	29.85%	16.56 ± 1.244	9.68 ± 1.241	32.65%
10 mg/kg			79.58%			40.41%			32.07%			41.54%

Results are expressed as mean ± SD (n=5). Data were analyzed by one-way Analysis of Variance (ANOVA) followed by multiple comparison test i.e. LSD \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 when compared against control group \*P<0.05 when 10 mg/kg of piroxicam treated group compared with 5 mg/kg piroxicam treated group

**Table 3:** Effect of piroxicam on haemagglutination titre.

Treatment	Log 2 value of HA
Control	41.6 ± 21.46625
5 mg/kg piroxicam treated group	22.4 ± 8.763561*
10 mg/kg piroxicam treated group	2.4 ± 2.19089**+

Comparison of Log 2 values of HA titre of piroxicam treated groups against control group. Results are expressed as mean ± SD (n=5). Data were analyzed by one-way Analysis of Variance (ANOVA) followed by multiple comparison test that is LSD \*p<0.05, \*\*p<0.05 and \*\*\*p<0.01 when compared against control group. \*P<0.05 when 10 mg/kg of piroxicam treated group compared with 5 mg/kg piroxicam treated group.

resulted in reduction of Total Leukocyte Count (TLC) in control group, 5 mg/kg piroxicam treated group and 10 mg/kg piroxicam treated group by 44.86%, 73.46% and 79.58% respectively and lymphocytes was reduced in control group, 5 mg/kg and 10 mg/kg piroxicam treated group by 22.66%, 34.65% and 40.41% respectively while neutrophils were reduced by 29.69%, 32.65% and 41.54% respectively. Statistical analysis showed that there was significant difference in the TLC and DLC i.e. neutrophils and lymphocytes counts before and after cyclophosphamide administration in drug treated groups (at dose 10 mg/kg showed significant decreased in TLC as well as in DLC than at dose 5 mg/kg). There was no prominent effect on monocytes when compared against control group. All values were significantly different from control group \*P<0.05 (Table 2).

### Hemagglutination assay

All values of piroxicam at 5 mg/kg and 10 mg/kg are expressed as HA titre ± standard deviation when compared with control. Table 3 represents log2 value. At the high dose in the experiment (10 mg/kg) showed the minimum HA titre as compared to other dose (5 mg/kg) and the control group. The change in HA titre was significant i.e.

**Table 4:** Effect of piroxicam on mice in mice lethality assay.

Sr. No.	Groups	Mice No.	No. of mice died in 24 h	No. of mice died in 48 h	No. of mice died in 72 h	Percentage mortality ratio
1	Control group (PEG + vaccine)	5	1	1	2	40%
2	5 mg/kg piroxicam treated group + vaccine)	5	3	4	4	80%
3	10 mg/kg piroxicam treated group + vaccine)	5	5	0	0	100%

Mortality ratio of piroxicam treated groups against control group. All values were significantly different from control \*P<0.05, (n=5). Piroxicam showed significant increase in mortality rate (100%) at dose 10 mg/kg and lower in 5 mg/kg (80%) treated group when compared against control group

\*P<0.05.

### Mice lethality test

Table 4 represented the percentage mortality ratio of piroxicam treated groups against control group. There was significant increase in mortality rate in piroxicam group treated at dose 10 mg/kg (100%). Mortality rate was seen less in 5 mg/kg treated group (80%) when compared against control group (40%).

## Discussion

Non-Steroid Anti-Inflammatory Drugs (NSAIDs) show analgesic, anti-inflammatory and anti-pyretic effect by inhibiting Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) [6]. They also modulate a number of immunological responses and play a key role as antitumor in the host immunity [19]. NSAIDs also inhibit NF-κ B activation and thus suppress cancer cell proliferation due to stopping the expression of survival related gene products [20-22].

This study was conducted on Albino mice, divided into 3 groups to explore the effects of piroxicam on cellular and humoral immunity. Results of this study showed that in addition to effective reduced the inflammation, fever and pain etc., piroxicam also has immunosuppressive effect. Different experimental models including Delayed Type Hypersensitivity reaction (DTH), cyclophosphamide induced neutropenia, Hemagglutination Assay (HA) and mice lethality test were performed to evaluate the effect of piroxicam on cellular and humoral immune system by comparing treated groups with the control group.

DTH is an important for the indication of cell-mediated immunity *in vivo*. We determined the DTH responses against DNCB by using previous method with some modifications [14]. DNCB

cause contact hypersensitivity is generally regarded as a cell mediated rather than antibody mediated immune response. Previously it has been known that when DNCB and other chemicals, of having same structure, were applied topically, they might bind with various skin proteins to form macromolecules (dinitrophenol complex). These macromolecules are affected by skin Langerhans cells, macrophages and dermal dendritic cells. These macromolecules are subsequently processed [23]. The sensitized T-lymphocytes are converted to lymphoblasts and secrete lymphokines, these lymphokines cause the infiltration of inflammatory cells to show the defensive response and attract more scavenger cells at the site of reaction. Thus sensitive cells are immobilized to promote defensive (inflammatory) reaction [24]. This immunosuppressive effect may be started from the raise in CD8+ T-Cell percentage which could be lead to the suppression in cellular immune response [25]. The results of DTH suggested that piroxicam may suppress the cell mediated immunity as shown by decrease in skin thickness of mice. Decrease in skin thickness of mice showed that piroxicam has a suppressive effect on lymphocytes and accessory cell types required for the expression of reaction. The mean skin thickness values in mice treated with 10 mg/kg was significantly ( $*P<0.05$ ) lower than 5 mg/kg piroxicam treated when compared with control group. There was significant decreased in skin sensitivity at 24, 48 and 72 h. Results of DTH response resembles with results of a study conducted by [26].

In cyclophosphamide induced neutropenia, cyclophosphamide injection induced myelosuppression in mice. It belongs to nitrogen mustard subclass of alkylating agents and acts as an immunosuppressive agent and interferes in DNA synthesis by alkylation of DNA and prevents growth of tumor cells. At the same time it can inhibit the rapid cell division of the normal tissues, such as the hematopoietic system and neutropenia occurred [27]. Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC) were affected before and after administration of cyclophosphamide injection in piroxicam treated groups as well as in control group. However percentage reduction in TLC and DLC were significantly caused more suppression at dose 10 mg/kg of piroxicam ( $*P<0.05$ ) than 5 mg/kg piroxicam when compared with the control group. Piroxicam has no prominent effect on monocytes in drug treated groups before and after of TLC and DLC. The most important component of first line of cellular defense against invading microbes is neutrophils or Polymorphonuclear leukocytes (PMNs). Circulating neutrophils are triggered towards the site of infection by chemicals released by host or invading pathogen. Neutrophils ingest the microbes by phagocytosis process [28]. The neutrophil is a critical effector cell in cellular as well as in humoral immunity and has important function in bacterial killing and phagocytosis [29]. Results of a previous study were in-line with the results of cyclophosphamide induced neutropenia assay performed in this research [30].

To evaluate the effect of piroxicam on humoral immunity, hemagglutination assay and mice lethality test were performed. In the hemagglutination test, interaction of B cells with the antigen is responsible for the secretion of antibodies and also causes differentiation and propagation into these cells. These antibodies bind with their specific antigens, neutralize them and facilitated them for their elimination by forming latex [31]. In mice, antibodies produced against sheep red blood cells after sensitization was determined by Hemagglutination Assay (HA). B lymphocytes are responsible for their production. Antibodies are the integral part of humoral immune system [32]. Piroxicam produced Hemagglutination titre

(HA titre) but it was lower in mice treated at a dose 10 mg/kg than 5 mg/kg piroxicam treated group when compared with the control group. Agglutination occurs if antibodies are formed against antigen. Significant decreased in HA titre indicate that there was decreased production of IgG and IgM antibodies in the serum of mice against sheep Red Blood Cells (RBCs). In some plates latex was appeared as a button indicates that there was no any agglutination of sheep RBCs. Shariffar et al. [33] reported in their study that HA titre was markedly decreased at 3 doses of *Achillea wilhelmsii* that supports the results of HA of my study.

Mice lethality test is one of the important tests in world that are conducted to evaluate serological responses in animals immunized with vaccines. In mice lethality test, mice were immunized by Hemorrhagic Septicaemia (HS) against bacterial culture *Pasteurella multocida* to all experimental animals. *Pasteurella multocida* is an organism that is very pathogenic for the mice and act on the mice by causing infection in the airway of the mice [31]. Piroxicam caused significant reduction in mortality ratio ( $*P<0.05$ ), showed 100% mortality at dose 10 mg/kg and 80% at dose 5 mg/kg while only 40% mortality was observed in control group. The results of present research showed that production of antibodies by B-lymphocytes of test animal against *Pasteurella multocida* was reduced at highest dose of piroxicam (10 mg/kg) than other dose of 5 mg/kg when compared against control group. Results of a previous study on methanolic extract of *Moringa oleifera* were in-line with the results of this research to assess humoral immune response [34].

## Conclusion

The results of the present study showed that piroxicam has significant effects on both the cell mediated as well as on the humoral immunity and suppress these arms of immune system, which may add to its potential role as a immunosuppressant agent and thus may play important role in the treatment of autoimmune diseases and also help in organ transplantation.

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