

## Effects of Ketoprofen on Cellular Immune Responses in Mice

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**Abstract.-** Analgesic and anti-inflammatory properties of ketoprofen are well documented but little is known about its effects on various aspects of immune system. Apart from immune clearance of foreign antigens, cell mediated immunity plays major role in the regulation of immune system. Present study describes the effects of ketoprofen on cell mediated immunity as measured through macrophage engulfment, nitric oxide (NO) detection, cyclophosphamide induced neutropenia and delayed type hypersensitivity assays following injection of 1mg/kg and 5mg/kg of the drug into the mice. Macrophage engulfment activity and ability to produce NO was significantly ( $P < 0.001$ ) reduced in ketoprofen treated groups. There was significant decrease ( $p \leq 0.01$ ) in Total leukocyte count (TLC) after neutropenic dose of cyclophosphamide in control, 1mg/kg and 5mg/kg treated ketoprofen groups. Moreover there was significant decrease ( $p < 0.005$ ) in the phenomena of delayed type hypersensitivity in ketoprofen treated mice. These results provides the basic information of immunosuppression by ketoprofen on cell mediated immunity and supports the potential application of ketoprofen in patients with allo-grafts or autoimmune diseases.

**Key words:** Ketoprofen, immunomodulatory response, cell mediated immunity, macrophages, cyclophosphamide, NSAIDs, prostaglandins, nitric oxide.

### INTRODUCTION

**K**etoprofen belongs to propionic acid class of NSAIDs with analgesic, anti-inflammatory and antipyretic effects (Akural *et al.*, 2009). It is most often prescribed for muscle pain, tissue injury, joints pain and for laminitis (Hiller *et al.*, 2006). There are no intraoral side effects with the solution containing ketoprofen (Liccardi *et al.*, 2003). Ketoprofen developed in the form of nanoemulsion for topical application on the skin is considered very effective (Sakeena *et al.*, 2010).

Ketoprofen acts by inhibiting the body's production of prostaglandins (PGs). PGs are lipid-derived autacoids that modulate many physiological systems including the central nervous system, cardiovascular, gastrointestinal, genitourinary, endocrine, respiratory, and immune systems (Hata and Breyer, 2004). In the immune system, cytokines and PGs are produced by antigen presenting cells

such as macrophages and dendritic cells. Cytokines and PGs regulate immune responses (Kuroda and Yamashita, 2002). Produced by many cell types, PGE<sub>2</sub> has been shown to affect various aspects of the immune and inflammatory responses by acting on all components of the immune system (Hedi, 2013).

Various NSAIDs have been tested for their immunomodulatory effects. TNF- $\alpha$  release from LPS activated RAW 264.7 cells is significantly enhanced at certain concentrations or doses of fenoprofen, indomethacin, piroxicam, aceclofenac, diclofenac and sulindac. Nitric oxide (NO) production was most powerfully intensified by phenylbutazone and aspirin. Phenylbutazone also triggered the phagocytic activity. The results suggesting the role of NSAIDs in immunomodulatory effects on activated macrophages and lymphocytes (Cho, 2007). Similarly the effects of aspirin on cell mediated immunity (CMI) of mice was observed. CMI in mice was determined by delayed type hypersensitivity and phagocytosis assay. Aspirin was shown to cause immunosuppression (Javeed,

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2011). Naproxen has been shown to block the phagocytic activity of circulating blood WBC, reduction of natural killer cell cytotoxicity of splenic mononuclear and delayed type of hypersensitivity responses (Goswami *et al.*, 2012).

Considering the immunomodulating effect of PGs, we evaluated whether ketoprofen affects the immune system optimistically or pessimistically. In the present project ketoprofen effects on the phenomena of cell mediated immunity were studied.

## MATERIALS AND METHODS

### *Experimental animals*

Albino mice, 5 weeks old, were purchased from Veterinary Research Institute, Lahore and kept in animal house of the University of Veterinary and Animal Sciences, Lahore, by taking into consideration all possible hygienic measures. Mice colony was developed and adult mice were housed in comfortable cages (5 to 7 mice per cage) and maintained on standard pellet diet and water ad libitum during the entire trial period. All manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

### *Experimental design*

Ketoprofen was purchased from Sigma Aldrich (catalogue No. k1751-5G). Mice were acclimatized for one week and afterwards three groups were made, control group and two ketoprofen-treated groups each with five to seven mice. Ketoprofen was administered intraperitoneally at the dose of 1mg/kg/day and 5 mg/kg/day. Control mice were injected with phosphate buffer (PBS) saline only.

### *Macrophage engulfment assay (MEA)*

Mouse peritoneal exudates cells were obtained from the peritoneum of mice inoculated with both doses of the ketoprofen as well as from the control mice. Macrophages were separated as described by Yang *et al.* (2007).

### *Morphology observation*

Mice peritoneal macrophages (PEMs) were cultured in RPMI1640 (Catalogue # 21870-076 Life

technologies) medium for two hours so that they adhered to the sterilized glass slides. For microscopic studies, the adherent cells were stained with Giemsa and Wright staining solution after fixing in methanol (Esashi *et al.*, 2003).

### *Engulfment by sheep RBCs*

Sheep red blood cells (SRBCs) suspension containing  $1 \times 10^7$  cells/ml were prepared by diluting the pelleted RBCs in PBS. The engulfment of SRBCs by macrophage *in vitro* was performed in both control and treated groups (Javeed, 2011). An average of 200 macrophages were counted adherent and engulfment percentage was counted in both control and ketoprofen treated groups. Following formula was used to calculate the engulfment percentage

$$\text{Engulfment percentage} = \frac{\text{Number of macrophages engulfing SRBCs}}{\text{number of adherent macrophages}} \times 100$$

### *Detection of Nitric oxide (NO) production*

NO detection kit (Cat#A013-1, Nanjing Jiancheng) was used for assay. PEMs were separated. The macrophages were then incubated for 3-4 h at 37°C and 5% CO<sub>2</sub>. Supernatant from the macrophages was added to 96 well flat bottom plates. Nitrite was determined using the Greiss reagent. Supernatant (100 µl) each from control and treated group was added to 100 µl mixture of Greiss reagent containing equal volume of 0.1% naphthyl ethylene diamine, 1% sulphanilamide in 3% phosphoric acid. Plates were incubated at 37°C for 10 min. Absorbance at 570nm was measured with ELISA reader. Nitrite concentration was determined with reference to a standard curve generated by using standard NO solution from the kit (5-100µM) (Bryan and Ghisam, 2007).

### *Cyclophosphamide induced neutropenia assay*

Mice were weighed and were given ketoprofen 1mg/kg/day and 5mg/kg/day intraperitoneally for 10 days. Control mice was given phosphate buffer saline only. On the 10th day neutropenic dose of cyclophosphamide (200 mg/kg s.c) was injected both in ketoprofen treated group and in control group. This day was labeled as day 0. Blood was collected and the total leucocyte count (TLC) and differential leucocyte count (DLC) were

performed prior to and on day 3 after injection of cyclophosphamide. The TLC and neutrophil count percentages in treated group were compared with the values in the control group (Licto Thomas *et al.*, 2007).

#### Delayed type hypersensitivity assay

Prior to sensitization; mice were weighed. On day 1 ketoprofen 1mg/kg/day and 5mg/kg/day were given intraperitoneally to ketoprofen treated groups. On 2nd day of the experiment (*i.e.* 1 day after treatment with ketoprofen), a sensitizing dose of 2% DNCB in acetone was applied to mice in both control and ketoprofen treated groups. On day 6 post sensitization (8th day of the experiment), the pre-treatment thickness of ears was measured. Subsequently, a challenging dose of 2% DNCB was applied to the mice. After a period of 24, 48 and 72 h, an increase in the thickness of ear was measured using a vernier caliper (Sajid *et al.*, 2007).

#### Statistical analysis

Data collected was analyzed using the SPSS for Windows version 13. One-way ANOVA and LSD post hoc tests were applied to see statistical differences between groups. Differences were considered significant at  $P < 0.05$ .

## RESULTS

#### Macrophage engulfment

The viability of macrophages was 99 % when checked by trypan blue exclusion dye method. There was significant difference ( $p < 0.001$ ) in engulfment percentages between control, 1mg/kg and 5mg/kg ketoprofen treated groups. Macrophage engulfment was significantly decreased ( $P < 0.001$ ) in 5mg/kg ketoprofen treated group (Fig. 1). At 45min macrophage engulfment percentage in control 1 mg/kg and 5 mg/kg treatments group was  $58.35 \pm 2.74$ ,  $47.85 \pm 3.71$ ,  $32.21 \pm 5.31$ , respectively indicating with the increase in dose of ketoprofen macrophage ability to engulf sheep red blood cells is effectively decreased. At 90 min mean macrophage engulfment percentage in control 1 mg/kg and 5 mg/kg treated group was  $65.25 \pm 2.17$ ,  $49.25 \pm 1.79$  and  $32.95 \pm 2.12$ , respectively indicating the difference in engulfment in control and ketoprofen

treated groups.

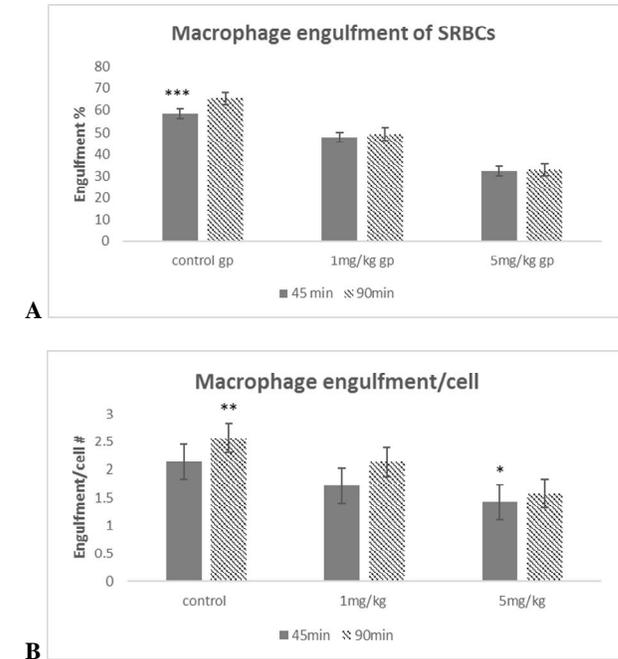


Fig. 1. Macrophage engulfment (% + SUE) and engulfment/cell ( $\# \pm$  S.E) observed at 45 and 90min in control, 1mg/kg and 5mg/kg ketoprofen treated group  $**p \leq 0.005$ ;  $*p < 0.05$  compared with corresponding groups.

Macrophage engulfment/cell was significantly decreased ( $p < 0.05$ ) in 5mg/kg ketoprofen treated group as compared to control but there was no significant difference in macrophage engulfment/cell of 1mg/kg ketoprofen treated group with control and 5mg/kg ketoprofen treated group. Overall mean engulfment/cell was significantly ( $p \leq 0.005$ ) more after 90 min of co-incubation with SRBCs (Fig. 2).

#### Detection of nitric oxide (NO) production

NO production by macrophages was significantly ( $p < 0.001$ ) decreased in ketoprofen treated group as compared to control group. There was significant difference ( $p < 0.001$ ) in NO production by macrophages between control, 1mg/kg and 5mg/kg treated groups (Fig. 2). Stimulation of macrophages by SRBCs showed significant ( $p < 0.01$ ) high value of NO production by macrophages as compared to non-stimulated macrophages. However, macrophage when

stimulated with SRBCs also showed similar trend as observed in macrophages that were not stimulated. There was decrease in NO production in ketoprofen treated group in comparison with the control group. The result suggested that ketoprofen caused decrease in NO production by macrophages in dose dependent manner.

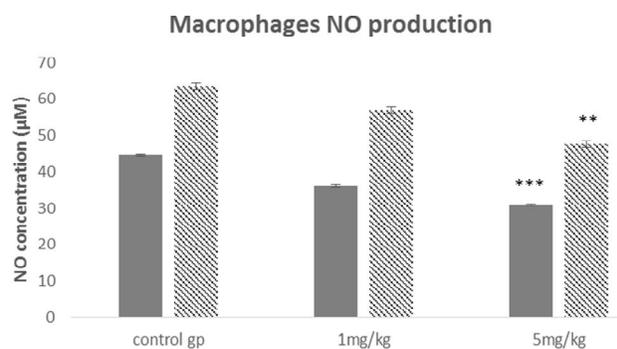


Fig. 2. Nitric Oxide ( $\mu\text{M} \pm \text{S.E}$ ) production by macrophages alone and with SRBCs stimulation in control, 1mg/kg and 5mg/kg ketoprofen treated groups (gp) \*\*\* $p < 0.001$ ; \*\* $p < 0.01$  compared with corresponding macrophages group producing NO.

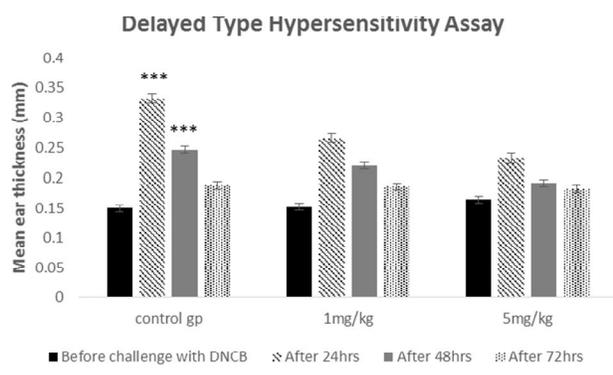


Fig. 3. Overall mean ear thickness ( $\text{mm} \pm \text{S.E}$ ) after challenge with DNCB. \*\*\* $p < 0.005$  compared to control group (gp) at 24 and 48 h of treatments

#### Cyclophosphamide induced neutropenic assay

There was a significant decrease ( $p \leq 0.01$ ) in TLC after neutropenic dose of cyclophosphamide. The TLC was reduced in control mice to 67.77 %, 79.65% in 1mg/kg and 82.03% in 5mg/kg treated groups compared to initial values it. There was

significant difference ( $p < 0.005$ ) in TLC reduction between 5mg/kg ketoprofen treated group and control group but there was no statistical difference in TLC values between 5mg/kg and 1mg/kg treated groups. Moreover, there was no statistical difference in TLC values between 1mg/kg ketoprofen treated group and control group. The neutrophil % was reduced to 61.88% in control group 69.75% in 1mg/kg ketoprofen treated group and 88.13 % in 5mg/kg ketoprofen treated group when compared to initial values, which indicates that after administration of ketoprofen there is significant % reduction in neutrophils (Table I).

#### Delayed type hypersensitivity assay

Ketoprofen at 1mg/kg, and 5mg/kg suppressed the DTH response 24hrs after the application of DNCB challenge. Ear thickness was significantly increased after administration of challenge dose of DNCB in all three groups (control, 1mg/kg, and 5mg/kg) but the highest ear thickness was observed in control group ( $p < 0.005$ ) compared to 1mg/kg and 5mg/kg ketoprofen treated groups. Overall ear thickness was significantly higher after 24 h of DNCB application (challenge dose) that further decreased after 48hrs and 72 h in all three groups. There was significant ( $p < 0.005$ ) difference in ear thickness between control, 1mg/kg and 5mg/kg after 24 h of challenge dose with the highest thickness was observed in control. After 48hrs ear thickness started to reduce but there was significant difference ( $p < 0.005$ ) in ear thickness between control, 1mg/kg and 5mg/kg ketoprofen treated groups. After 72 hrs ear thickness reduced further compared to 48hrs but this reduction in ear thickness was not statistical significant (Fig. 3).

## DISCUSSION

Ketoprofen inhibits COX enzyme thereby inhibits prostaglandin synthesis (Sommerauer *et al.*, 2001). In the present study the effect of ketoprofen on the phenomenon of cell mediated immunity were studied.

Macrophages are important key part of immune responses as they are being involved in antigen presentation and in phagocytosis (Noel *et al.*, 2004). Circulating monocytes turn in to resident

**Table I.- Effect of ketoprofen on cyclophosphamide induced neutropenia.**

Treatment	Total leukocytes count (cells/mm <sup>3</sup> )		Reduction in cell number	%age reduction	Neutrophils count		Reduction in neutrophil count	% age reduction
	Before	After			Before	After		
Control	4612 (±203.8)	1486 (±323.92)	3126 (±447.41)	67.77	2878.24 (±668.2)	1064.20 (±122.18)	1814.04 (±351)	61.88
1mg/kg	3440 (±466.9)	700 (±68.92)	2740± (402.80)	79.65	1927.97 (±705.71)	621.55 (±437.75)	1364.42 (±123)*	69.75
5mg/kg	2728 (±249.5)	490 (±79.05)	2238± (279.4)**	82.03	794.85 (±175.01)	97.83 (±127.3)	677 (±234)***	88.13

All values are means ± S.E, \*\*\*p≤0.001, \*\*p≤0.05 compared to control group, \*p≤0.01 when values of groups administered with doses were compared

macrophages (Kaufmann and Schaible, 2005; Belardelli and Ferrantini, 2002). In the present study ketoprofen effects on murine macrophages ability to engulf sheep red blood cells and number of red blood cell engulfment were studied. Engulfment by macrophages is an essential step and a part of innate immunity for protection against foreign pathogens, microorganisms, or dead cells (Gordon and Taylor, 2005). Ketoprofen was previously shown to decrease intracellular cAMP and engulfment by leucocytes due to its anti-inflammatory properties (Ruyu, 1985). This significant decrease in engulfment capability of host macrophages exerted by ketoprofen may be due to its immunosuppressive effects protecting allograft function and survival as well as reducing complication in transplant reception.

NO plays important part in regulating different physiological processes including neural responses, blood pressure control and immune responses (Bryan and Grisham, 2007). Macrophages produce nitric oxide and reactive oxygen species for killing of microorganisms (DiSalvo, 1971). The decrease in NO production by macrophages is believed to be due to reduction in nitric oxide synthases (iNOS) and NO secretion (MacMicking *et al.*, 1997). Our results are in accordance with the findings of (Cirino *et al.*, 1996) which tested different NSAIDs on the mice macrophage cell line J774 and reported the reduction in NO production by the macrophages when treated with the different NSAIDs such as aspirin, naproxen, flurbiprofen, ketoprofen, ketorolac and diclofenac (Cirino *et al.*, 1996).

Cyclophosphamide by interfering in DNA synthesis and function causes alkylation of DNA which results in immunosuppression effect (Thatte *et al.*, 1987). The prevention of neutropenia induced by cyclophosphamide may be through activation of macrophages, which secrete a large number of substances including colony stimulating factor and interleukin 1 (Heppner, 1976). Our study shows that there is significant percentage reduction in total leukocyte count and differential leukocytes count after ketoprofen administration which might be due to suppression of macrophage activity and resulted in increased suppressive effect of cyclophosphamide. Topical application of DNCB results in activation of T-cells. Monocytes and monocytes derived macrophages accumulate at the DTH reaction place (Dannenbergh, 1994). T cells because of their cytotoxicity properties play an important role in transplantation rejection (Tan *et al.*, 2013). The ability of an individual to develop contact sensitivity is a measure of cellular immunity to a new antigen (Gordienko, 1986). In the present study the decrease in DTH response indicates that ketoprofen has suppression effects on macrophage activity. Ketoprofen has been shown to causes delayed type hypersensitivity reactions on skin (Foti *et al.*, 2008).

Results of the present study suggest that ketoprofen has significantly suppressive effect on the cellular immunity.

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#### Conflict of interest

The authors declare that there is no conflict of interest.

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