IMMUNOMODULATORY ACTIVITY OF FLURBIPROFEN IN MICE

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ABSTRACT

Flurbiprofen is a propanoic acid derivative which is widely used and has well-known anti-inflammatory and analgesic effects. Many new studies have emerged which display the immuno-pharmacological behavior of NSAIDs. The immunomodulatory activity of flurbiprofen was evaluated in this work by using immunological experimental models like cyclophosphamide induced neutropenia assay, delayed type hypersensitivity assay, haemagglutination assay and mice lethality test. Cyclophosphamide induced neutropenia and delayed type hypersensitivity assays were performed to evaluate cell mediated immunity while the other two models were used to evaluate the effect of flurbiprofen on humoral immunity. Flurbiprofen was administered intraperitoneally into swiss albino mice at the doses of 5 mg/kg and 10 mg/kg body weight of mice. The drug in both doses decreased the circulating antibodies, and did not prevent the mortality induced by a deadly bovine *Pasteurella multocida*, evaluated by hemagglutination antibody titer and mice lethality test respectively. Similarly, flurbiprofen did not show any significant effect on T cells. The decrease in skin thickness in delayed type hypersensitivity assay indicated the inability of flurbiprofen to stimulate the T cells. WBCs and neutrophil count reduction showed lack of flurbiprofen effectiveness in preventing cyclophosphamide induced neutropenia. In conclusion, flurbiprofen decreased both humoral and cell mediated immunity in mice.

Key words: Humoral immunity, Flurbiprofen, Cellular Immunity, Cyclophosphamide, *Pasturella multocida*.

INTRODUCTION

Host survival depends upon immune system that prevents harmful substances from penetrating into the body. Protection against invading organisms and infections is provided by collaboration between first, second and third lines of defense (Talaro *et al*., 2002). The defensive system of humans and animals is a complex system that works by cooperation between humoral and cell mediated immune systems. Humoral immunity provides protection by different antibodies production, whereas, the cell mediated defends by bringing in action different types of cells like T-cells, macrophages, eosinophils, basophils, and neutrophils (Koller, 1982).

The immune system under normal circumstances does not induce any rejection against the body and its organs by the process called self-immune tolerance (Van Parijs and Abbas, 1998; Goodnow *et al*., 2005). A competitiveness between the immune system and invading substance, whether, an allograft may result in activation of inflammatory cascades involving release of different mediators that may ensue in a good effect or destructive complication to the host. Any defect in immune tolerance triggers a response against the body, whether organs are transplanted or not may lead to autoimmunity (Ring and Lakkis, 1999). A disruption in immune tolerance, results in excitement that may involve multiple pathways such as production of cytotoxic antibodies, activation of T-cells (Feghali and Wright,

1997; Barton, 1999) cytokines and prostanoids (prostaglandins, prostacyclin, and thromboxane) production from arachidonic acid during chronic inflammation. Such complex relationship of immune cells, antibodies and inflammatory mediators are of significant importance in modulation of not only usual immune reactions, but also in self-immune tolerance, autoimmunity, allograft rejection and cancers (Tilley *et al*., 2001; Rocca and FitzGerald, 2002).

Flurbiprofen (2-[3-Fluoro-4-biphenyl] propanoic acid) belongs to propanoic acid derivatives (Chunhua *et al*., 2012) which represent the largest class of NSAIDs (Elliott *et al*., 1988). It is a non-selective cyclooxygenase (COX-1 and COX-2) inhibitor (Hailong *et al*., 2012) having analgesic, anti-pyretic and anti-inflammatory effects (Adams *et al*., 1975). It was first presented as an anti-rheumatic agent, but later in a double blind controlled trial it was found efficacious in rheumatoid arthritis (Chalmers *et al*., 1972). It was assumed that flurbiprofen may prevent the formation of prostanoids and may provide immuno-protective effects (Tegeder *et al*., 2001). Nevertheless, the effects of flurbiprofen on the immune system have not been fixed. The primary purpose of the study was to access the immunomodulatory activity of flurbiprofen, which is an extension of its pharmacological actions, by investigating its effects on humoral and cell mediated immunity in experimental animals after treating them with a range of low to high doses.

MATERIALS AND METHODS

Chemicals: Flurbiprofen was kindly gifted by 3S pharmaceuticals Pvt. Ltd (Raiwind Manga Road, Lahore). Other materials like dimethylsulfoxide (DMSO), dinitrochlorobenzene, cyclophosphamide injection, ether solvent, and phosphate buffer saline were obtained from the Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences, Lahore (Syed Abdul Qadir Jillani (Out Fall) Road, Lahore – Pakistan).

Organism: *Pasteurella multocida* of bovine origin was obtained from the Department of Microbiology, University of Veterinary and Animal Sciences (Syed Abdul Qadir Jillani (Out Fall) Road, Lahore – Pakistan).

Experimental Animals: Swiss albino mice (weight ranges from 20-25g) were purchased from the animal house of University of Agriculture, Faisalabad-Pakistan. The animals were given standard diet and water *ad libitum* and kept under standard conditions in animal house approved by the ethical committee of the University of Veterinary and Animal Sciences, Lahore (Syed Abdul QadirJillani (Out Fall) Road, Lahore – Pakistan).

Antigen: Dinitrochlorobenzene and Sheep red blood cells were used as antigens. Dinitrochlorobenzene was used at 0.1 mL in delayed type hypersensitivity test, in order to sensitize and challenge the skin.

Sheep red blood cells (SRBCs) were collected in vacutainers and washed three times in phosphate buffer saline. The anti-body titers were determined against SRBCs in flurbiprofen treated and control mice by haemagglutination assay. *Pasteurella multocida* bacterial strain was used to challenge the mice in mice lethality test after immunizing them with hemorrhagic septicemia vaccine (HS vaccine).

Animal grouping, doses and exposure schedule: Mice were randomly divided into three groups (five mice per group). Two groups were treated with the doses (5 mg/kg and 10 mg/kg body weigt) of flurbiprofen intraperitoneally and the control group with DMSO intraperitoneally alone. It was found in preliminary experiments that 0.05 CC of DMSO was not lethal and did not affect the parameters evaluated during an immunomodulatory study of flurbiprofen. So, flurbiprofen was dissolved in undiluted dimethylsulfoxide (DMSO). Antigens, SRBCs and *Pasteurella multocida* were injected 6 hours after the daily injection of flubiprofen in correspondent mice groups.

Evaluation of immunomodulatory activity

Cyclophosphamide induced neutropenia assay: Swiss albino mice were treated with flurbiprofen (dose calculated according to the weight of each mouse @ 5 mg/kg/day and 10 mg/kg/day) or solvent (DMSO) intraperitoneally for 12 days. On 10th and 13th day of the test, blood samples from each mouse were collected. However, on the 10th day after blood collection, a neutropenic dose of cyclophosphamide (200 mg/kg body weight) was injected subcutaneously. Total leukocyte and neutrophil count were performed prior to i-e on 10th day before administration of cyclophosphamide and after injection of cyclophosphamide i-e on 13th day (Thatte *et al.*,1987).

Delayed type hypersensitivity assay: Mice were divided into groups and treated accordingly for eight days. Rings on right and left side of shaved abdomen having a diameter of one inch each were drawn, with permanent marker, of each mouse. Sensitizing and challenging dose of Dinitrochlorobenzene (DNCB) was applied at the marked places at right and left sides on 2nd and 8th days respectively with the help of tuberculin syringe. Skin thickness was measured immediately prior to and after applying the challenging dose of DNCB on 8th day of the experiment. The thickness was measured after 24, 48 and 72 hours using digital vernier caliper (Sajid *et al.*, 2007; Omer *et al.*,2012).

Haemagglutination assay: Mice were pre-treated with doses of flurbiprofen (5 mg/kg and 10 mg/kg body weight) for 27 days. Mice in all groups (treated and control) were immunized with 0.5×10^9 SRBCs (Sheep red blood cells) intraperitoneally on 14th and 21st days of the experiment. On 28th day, blood was collected; serum was separated and collected to determine haemagglutinating (HA) antibody titre against 1% suspension of SRBCs in multiwell micro titre plates. The plates were incubated for 2 hours and HA titre was noted (Fulzele *et al.*, 2003).

Mice lethality test: Swiss albino mice were injected with flurbiprofen for 20 days. Mice in the control group were treated with DMSO accordingly. Each mouse was immunized with hemorrhagic septicemia vaccine (HS vaccine) on the 7th and 17th days of the experiment. On 21st day, all experimental animals were challenged subcutaneously with 0.2 mL of *Pasteurella multocida* culture containing 10^7 cells per mL. The animals were observed for 72 hours (time intervals of 24, 48, and 72 hours after injecting *Pasteurella multocida*) to detect mortality (Ramanatha *et al.*, 1995). The mortality ratio was determined by using the following formula:

$$\text{Mortality ratio} = \frac{\text{No. of animals died}}{\text{Total no. of animals}} \times 100$$

An identification test for *Pasturella mutocida* was performed. Organs of mice which were most commonly affected by *Pasturella mutocida* included heart, lungs and liver. Blood samples from these organs were taken within a few hours of death. Biochemical characterization and identification (catalase, oxidase, and indole production test) of the isolated strain of *Pasteurella multocida* from dead mice was performed (Rashida *et al*., 2006).

RESULTS

Cyclophosphamide induced neutropenia assay: Results explicated marked reduction in WBCs and neutrophil count before and after injecting cyclophosphamide in the groups treated with flurbiprofen (5 mg/kg and 10 mg/kg body weight) when compared with the values of the control group (Table I and Table II). At dose of 5 mg/kg and 10 mg/kg body weight, flurbiprofen caused a statistically significant decrease in WBCs and neutrophil count.

The mean values and percentage reduction of WBCs and Neutrophil count before and after administration of cyclophosphamide in all groups is shown in table I and II.

Delayed type hypersensitivity: Delayed type hypersensitivity (DTH) was observed by measuring and comparing the skin thickness of left and right side of the abdomen of albino mice. Decrease in skin thickness was observed in the groups administered with 5 mg/kg and 10 mg/kg body weight of flurbiprofen immediately before and after the application of the challenging dose of Dinitrochlorobenzene (DNCB) on 8th day.

It was anticipated that only the group of mice administered with 10 mg/kg showed a significant reduction in the mean values of skin thickness when compared with the mean values of other groups after applying a sensitizing dose of DNCB (Table III). The mean skin thickness measured at time intervals of 24, 48 and 72 hours after applying a sensitizing dose of DNCB in the groups is shown in table III.

Haemagglutination test: Flurbiprofen (5 mg/kg and 10 mg/kg body weight) treatment exhibited a significant reduction in serum anti-SRBCs antibody titer when compared with the control group. HA titer was comparatively more reduced in 10 mg/kg flurbiprofen treated group than in 5 mg/kg flurbiprofen treated group. There was also a significant difference in HA titer between 5 mg/kg and 10 mg/kg flurbiprofen treated group (Table IV).

Mice lethality test: Mortality ratio, in the group of mice injected with flurbiprofen @ 10 mg/kg body weight was 100 % within 24 hours of *pasturella multocida* injection while the group treated with 5 mg/kg of flurbiprofen resulted in 80% deaths within 48 hours of administration of the microbe (*Pasturella multocida).* In the control group the mortality percentage was 60% (Table V).

Biochemical characterization and identification of the isolated strain of bacteria (*Pasturella multocida)* showed a positive oxidase, catalase and indole test which confirmed that the death of mice was due to *Pasturella multocida.*

Table 1. The effect of Flurbiprofen on WBCs count in Cyclophosphamide induced neutropenia assay.

Treatment	White Blood Cells		
	WBCs Count Before Cyclophosphamide Administration (cells/mm^3)	WBCs Count After Cyclophosphamide Administration (Cells/mm^3)	Percentage Reduction Of WBCs
Control group (Vehicle 0.05 mL, Intraperitoneally)	9225 ± 1196.5	5262.5 ± 58.0	42.95 %
Flurbiprofen Dose (5 mg/kg, Intraperitoneally)	4150 ± 130.38 [***]	2307.5 ± 61.42[***]	44.39 %
Flurbiprofen Dose (10 mg/kg, Intraperitoneally)	1640 ± 112.24 [***++]	427.5 ± 67[***+++]	73.93 %

All values are mean $\pm$ SD, [***]P<0.001 when compared to control group. [++]P<0.01 and [+++]P<0.001 when values of groups administered flurbiprofen (5 or 10 mg/kg) were compared.

Table 2. The effect of Flurbiprofen on neutrophil count in Cyclophosphamide induced neutropenia assay.

Treatment	Neutrophils		
	Neutrophil Count Before Cyclophosphamide Administration	Neutrophil Count After Cyclophosphamide Administration	Percentage Reduction Of Neutrophils
Control group (Vehicle 0.05 mL, Intraperitoneally)	2868.24 ± 68.20	1064.20 ± 42.18	62.89 %
Flurbiprofen Dose (5 mg/kg, Intraperitoneally)	1957.97 ± 75.71 [*]	623.55 ± 22.75 [*]	68.15 %
Flurbiprofen Dose (10 mg/kg, Intraperitoneally)	714.85 ± 75.01 [***+]	77.83 ± 17.32 [***+]	89.11 %

All values are mean $\pm$ SD, [*]P<0.05, [***]P<0.001 when compared to control group. [+]P<0.01 when values of groups administered flurbiprofen (5 or 10 mg/kg) were compared.

Table 3. The effect of Flurbiprofen on skin thickness measured in different time intervals.

Treatment	Skin Thickness (mm)			
	Before Second Exposure Of DNCB	24 hours After Second Exposure Of DNCB	48 hours After Second Exposure Of DNCB	72 hours After Second Exposure Of DNCB
Control group (Vehicle 0.05 mL, Intraperitoneally)	0.36 ± 0.02	1.31 ± 0.028	1.63 ± 0.02	1.45 ± 0.04
Flurbiprofen Dose (5 mg/kg, Intraperitoneally)	0.3 ± 0	1.25 ± 0.05	1.3 ± 0.14	1.11 ± 0.23
Flurbiprofen Dose (10 mg/kg, Intraperitoneally)	0.25 ± 0.02	0.45 ± 0.04 [*+]	0.86 ± 0.08 [**+]	0.66 ± 0.04 [**+]

All values are mean $\pm$ SD, [*]P<0.05, [**]P<0.01 when compared to control group. [+]P<0.05, test results of the studied groups were compared to each other.

Table 4. The effect of Flurbiprofen on haemagglutination.

	Hemagglutination Titer	Log$_2$ Values Of HA Titer
Control group (Vehicle 0.05 mL, Intraperitoneally)	224 ± 64	7.75 ± 0.5
Flurbiprofen Dose (5 mg/kg, Intraperitoneally)	96 ± 36.95 [*]	6.5 ± 0.5
Flurbiprofen Dose (10 mg/kg, Intraperitoneally)	6 ± 2.3 [***+]	2.5 ± 0.5

All values are mean $\pm$ SD, [*]P<0.05, [***]P<0.001 when compared to control group. [+]P<0.05 Test results of the studied groups were compared to each other.

Table 5. The effect of Flurbiprofen on mice mortality in lethality test.

	Control	Treated	
	(n=5)	5 mg/kg (n=5)	10 mg/kg (n=5)
Mortality Within 24 hours	0	1	5
Mortality Within 48 hours	1	3	0
Mortality Within 72 hours	2	0	0
Mortality (%)	60	80	100

Percentage mortality of control group and flurbiprofen treated groups

DISCUSSION

There is a growing concern that along with anti-pyretic and analgesic effects of NSAIDs, they also possess immunomodulatory activity. They produce effects by inhibiting COX enzyme responsible for the production of inflammatory mediators. In this study the immunomodulatory response of flurbiprofen on two main components of immune system i.e. humoral and cellular mediated immunity was investigated. It is indicated that along with the anti-inflammatory and anti-nociceptive effects of flurbiprofen, it also possesses the suppressive effect on cellular and humoral immunity. Effect of flurbiprofen on cellular immunity was investigated by using delayed type hypersensitivity and cyclophosphamide induced neutropenia assay whereas HA titer and mice lethality test was performed to investigate the effect of flurbiprofen on humoral immunity.

Cell mediated immunity plays a defensive role not only against infectious organisms but tissue grafting, tumor immunity, and delayed type hypersensitivity with the aid of T lymphocytes and lymphokines. Some cytokines regulate maturation, development and responsiveness of T-cells. Prostaglandin E$_2$, prostaglandin D$_2$, and prostaglandin I$_2$ are among those that regulate T-cell functions (Issa *et al.*, 2010). T-cells exhibit the ability to regulate the immune responses against different protein antigens. In our study, DNCB was used because it activates T-cells by forming di-nitro phenyl protein complexes with various skin proteins and thus develops delayed type hypersensitivity responses. These protein complexes act as protein antigens. Collateral evidences from previous researches indicate that NSAIDs possesses both types of actions, i.e. functional activation and inactivation (Muzammal *et al.*, 2012). Fate of T cells (functional incitement or suppression) mainly depends on the type and timings of signals generated by the receptors that recognize antigen patterns (Padovan *et al.*, 2007). Our study has shown a decrease in skin thickness in mice administered with flurbiprofen which indicates that it suppresses the functional activity of T cells, which are usually involved in establishing a delayed type response. This might be due to non-selective inhibition of cyclooxygenases that provides support in the synthesis of prostanoids necessary for generating signals by APCs

(Antigen presenting cells) and transmitting these signals to T cells. Proliferation and proper functioning of T-cells depend upon cyclooxygenases. Flurbiprofen showed a dose dependent decrease in hypersensitivity responses. Paccani *et al.* (2002) suggested that NSAIDs are responsible for the inhibition of cyclooxygenase dependent pathways responsible for the transcription of proteins that trigger T cells. This might be the cause of suppression of T cell activity and their proliferation. There is also a growing concept that NSAIDs at some doses inhibits the transcription factor NF-kB that is responsible for the development, maturation and antigen specific proliferation of T and B cells by expressing cytokines and chemokines (Yan Liang *et al.*, 2004).

Cyclophosphamide induced neutropenia assay focuses on the effect of drugs on the homeopathic system (Diwanay *et al.*, 2004). Cyclophosphamide was used because it is an alkylating agent. It works by forming cross-links between and within a DNA strand at the guanine N-7 position. These cross links are irreversible and lead to cell death. It suppresses bone marrow and possesses dose dependent effects on different types of immune cells (Takimoto and Calvo, 2008). In the present protocol Cyclophosphamide was administered in the mice to evaluate the production and proliferation of WBCs and neutrophils in flurbiprofen administered mice after their complete eradication from blood stream. A significant decrease in WBCs and neutrophil count in the groups of mice received flurbiprofen (5 mg/kg and 10 mg/kg) before administration of cyclophosphamide, would be due to flurbiprofen induced apoptosis. Negrotto *et al.* (2006) observed a decrease in neutrophils after treated with acetyl salicylic acid. Further investigation lead to a conclusion that decrease in their number is due to acetyl salicylic acid induced COX-1 inhibition that acts as a cytoprotective. Similar results were observed in the groups of mice that were administered with flurbiprofen after administration of cyclophosphamide. Reduction in WBCs and neutrophils in the groups administered with flurbiprofen (5 mg/kg and 10 mg/kg) after administration of cyclophosphamide revealed that flurbiprofen affects the homeopathic system which inhibits the proliferation and production of WBCs and neutrophils. Anti-proliferating effect of NSAIDs revealed that some of them decreased the secretion of granulocyte colony stimulating factor that is responsible for the synthesis and maturation of new neutrophils in the bone marrow (Calatayud *et al.*, 2001). We didn't investigate the flurbiprofen induced apoptosis and its effect on haemopoitic system. Whereas some previous studies concluded that an inhibition in COX-1 and Cox-2 enzymes disturbed the factors responsible for proper functioning of the immune system. However, this area needs further investigation to justify its effect on apoptosis and homeopathic system and it is clearly observed that flurbiprofen therapy in mice resulted

in immunosuppression and didn't improve cell mediated immunity parameters.

B cells and plasma cells produce antibodies. They play an important role in humoral immunity (Mauri *et al.*, 2012). The mice lethality test is a tool used to evaluate the serological responses in already vaccinated animals. In our study, animals were vaccinated against *Pasteurella multocida* and then injected with the pure culture of bacteria, which was lethal to mice and hence mortality was determined. Substances that hinder the production of antibodies possess humoral immunosuppressive effects and the animals injected with such substances would die earlier than the control group when a pure culture of pathogenic antigen (lethal bacterial culture in our case *Pasteurella multocida*) is administered in them. In the present investigation, flurbiprofen at 5 mg/kg and 10 mg/kg showed 80% and 100% mortality respectively. This reflects a decreased production of IgG and IgM antibodies in the serum (of mice against lethal micro-organism Pasteurella multocida) after flurbiprofen administration.

Agglutination is the result of antigen-antibody interaction. HA titer is assayed to quantify the changes in the concentration of antibodies against an antigen. In this specific study, sheep red blood cells (SRBCs) were used as an antigen for the production of specific antibodies in mice. A decrease in HA titer refers to decrease in antibody production. Inhibition of humoral response to SRBCs indicated decrease in the response of macrophage, T and B lymphocytes which are key players in antibody synthesis (Benacerraf, 1978). B cells express high amount of COX-2. NSAIDS like indomethacin and COX-2 inhibitors diminishes the ability of B cells to produce anti-bodies. Genetically modified mice with COX-2 knockout have 64% less IgM and 35% less IgG antibodies (Ryan *et al.*, 2005). Decrease in frequency of B cells and T helper cells was also observed by Bernard *et al.* (2010) and concluded that COX-2 inhibition resulted in decrease production of anti-bodies against the virus. Flurbiprofen (5 mg/kg and 10 mg/kg) decreased the HA titer significantly, which depicted the decreased production of antibodies against SRBCs.

Based on the results of all investigations of present study, it is concluded that flurbiprofen at dose of 5 mg/kg and 10 mg/kg showed a dose dependent suppression of immune system by suppressing the components of cellular and as well as of humoral immunity in mice. In the course of this project, we also found out that flurbiprofen also exhibited nonspecific immunosuppressive activity in various models including cyclophosphamide induce neutropenia assay, delayed type hypersensitivity assay, mice lethality test and haemagglutination test. The conclusions dealing with our experimental data can be extrapolated to study in further details and to human situation reconfirming the immunosuppressive activity of flurbiprofen which is not

directly mentioned in clinical data. There is still a room to find the level up to which flurbiprofen would interfere with the components of cell mediated and humoral immunity. Our findings may provide further experimental evidences for further researches on immunological applications of flurbiprofen.

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