

EFFECT OF NABUMETONE ON CELLULAR IMMUNE RESPONSES IN MICE

K. Naveed¹, A. Javeed^{1*}, M. Ashraf¹, A. Riaz², S. Khurram¹, A. Sattar¹ and A. Ghafoor³

¹Department of Pharmacology and Toxicology, ²Department of Theriogenology, ³University Diagnostic Lab, University of Veterinary and Animal Sciences, Lahore- Pakistan

*Corresponding author's Email: aqeel.javeed@uvas.edu.pk

ABSTRACT

Nabumetone is used to control pain and inflammation in rheumatoid arthritis. It works through inhibition of cyclooxygenases (COX), especially COX-2. In the current study, Nabumetone immunomodulatory effect was detected on cellular immunity in mice. The control group was administered normal saline orally as placebo. Nabumetone was administered orally in two treatment groups 14mg/kg and 28mg/kg, respectively by gavage needle. Macrophage engulfment, delayed type hypersensitivity (DTH), nitric oxide measurement and cyclophosphamide induced neutropenia assays were used to evaluate the immunomodulatory effects. In DTH, there was more thickness of the skin in control than Nabumetone treatment groups ($p \leq 0.05$). In the macrophage engulfment assay, a significant decrease ($p \leq 0.05$) in engulfment percentage were observed in treatment groups. In cyclophosphamide induced neutropenia, the levels of TLC and DLC were higher in the control group ($p \leq 0.05$). In the nitric oxide measurement assay, there was significant reduction in nitric oxide production at higher doses of Nabumetone ($p \leq 0.05$). Therefore, it is concluded that Nabumetone suppress cellular immune responses in mice.

Key words: Nabumetone, Cellular, Immune, Nitric oxide, Macrophages.

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INTRODUCTION

The food and drug administration gave approval of Nabumetone in 1991. This is a non-acid pro-drug (Walter and Romano 2000). Therefore, it is essentially taken by oral passage. It is useful in conditions like rheumatoid arthritis, osteoarthritis and any other disease associated with pain (Gonzalo *et al.*, 2007). Cyclooxygenase-2 and cyclo-oxygenase enzymes are connected with effectiveness as well as negative effects of anti-inflammatory drugs (Relia *et al.*, 2012). Nabumetone inhibits cyclo-oxygenase enzymes (Trifan and Hla 2003; Schiller *et al.*, 2006; Dierssen 2016). This belongs to 2-6 substituted naphthyl alkaline class of non-steroidal anti-inflammatory drugs. Excessive amounts of its active metabolite: 6-methoxy 2-naphthyl acetic acid reaches fluid of joints (Matsumoto *et al.*, 2011; Pawer *et al.*, 2015). It has been found that Nabumetone has very little negative effects (Kriesten 1996; Donnan *et al.*, 2000; Roy *et al.*, 2001; Ban Warth 2008). Nabumetone is made from 1-naphthyl acetic acid. This can be obtained through a variety of names Relafen, gambaran and relifex.

There are two types of cyclo-oxygenase enzymes cyclo-oxygenase-1 and cyclo-oxygenase-2 (Claria *et al.*, 2005). Prostaglandins, prostacyclines and thromboxanes are included in prostanoids which are inflammatory mediators. Cytokines and prostanoids are made from arachidonic acid. In fact, the immune system raises the levels of cytokines and prostanoids. These

inflammatory mediators completely change the reaction process. PGD₂, PGE₂, 14-PGJ₂, PGI₂ and Thromboxane A₂ are playing an important role in the change of immunogenic reactions, intolerance, non-acceptance of allograft and cancer (Ferreria *et al.*, 2002). The COX-2 effect is enhanced during inflammation and it can play an important role in the regulation of the immune reaction (Bernard *et al.* 2010). Through various assays, the immunomodulatory effects of Nabumetone were studied in the present work to discover its necessary use in various medical applications.

MATERIALS AND METHODS

Experimental animals: Albino mice were procured from Department of Theriogenology, University of Veterinary and Animal Sciences Lahore, Pakistan. In a standard environment, these experimental animals were kept in a separate house meant for research purpose. In present research, mice of five to seven weeks of age and 25±30g of weight were used. All hygienic measures were considered. Clean water and pellet feed was provided. All international procedures for the maintenance and care of laboratory animals were considered.

Chemicals: Nabumetone (Alfa Aesar, A Johnson Matthey Company), Cyclophosphamide (Pharmedic Pharmaceuticals Pvt Ltd), Acetone (Merk chemicals Ltd), Dinitrochlorobenzene (DNCB) (Alfa Aesar, A Johnson

Methey Company), Griess reagent (Life Technologies Ltd USA)

Experimental design: A completely randomized design (CRD) was employed to this study. The mice were divided into three clusters, each group comprising of five mice. There were two treatment groups and one control group. A treatment group (n=5) was given Nabumetone @14mg/kg. The second treatment group was given 28mg Nabumetone/kg. (Robert P. 2003; Prieto A *et al.*, 2007). Normal saline was given in control group as placebo using gavage needle. In treatment groups, Nabumetone was given through oral route by gavage needle.

Delayed type hypersensitivity assay: Dinitrochlorobenzene was used for sensitization of skin of mice. On day one of the experiment, treatment groups were administered with Nabumetone. Then 2% of dinitrochlorobenzene was prepared. On right side of skin of mice, 2% solution of dinitrochlorobenzene was applied. The size of this mediator used was solitary single tenth portion of a milliliter. It caused skin sensitization in mice. On 8th day, inflamed site of mice was measured. Then 0.2 ml of 2% dinitrochlorobenzene solution was pragmated on leftward of skin of mice. Then measurement of this left lateral of skin was carried out after 24, 48 and 72 hrs. The percent increase in skin swelling was measured as described earlier (Umair *et al.*, 2016; Khurram *et al.*, 2017).

Macrophage engulfment assay: Nabumetone was given orally in treatment assemblies for one week. Control mice were given standard normal saline for one week (mice without Nabumetone). Mouse peritoneal exudate cells were taken out from peritoneal exudate of mice of all groups. Macrophages were extracted as mentioned earlier (Javeed *et al.*, 2011; Khurram *et al.*, 2017). RPMI 1640 medium was used for macrophage dissolution. These cells were colored with wright and giemsa stains. The macrophages were kept placed by the use of methanol, as described earlier (Khurram *et al.*, 2017). After staining, blue color was the parameter for differentiation of dead and live cells. Live cells did not accept that stain so they were not blue (Freshney 2010). By the aid of hemocytometer, counting of macrophages was carried out. The cell numbers were maintained at 5×10^6 cells per milliliter. Sheep red blood cells (SRBCs) suspension having 1×10^7 million cells/ml was shaped by collaborating buffer saline in pelleted SRBCs. Phagocytic cells were co-incubated with SRBCs for three hours in six well plates. Later, macrophage engulfment percentage and phagocytic index was calculated as described earlier (Khurram *et al.*, 2017).

Cyclophosphamide induced neutropenia assay: All three groups of mice were weighed individually to calculate dose. Nabumetone was administered to treatment groups of mice for ten days by gavage needle.

In the control group mice were with placebo. On 10th day of trial, 200mg/kg of cyclophosphamide neutropenic dose was injected subcutaneously in all three groups of mice at day zero. Blood was collected from all mice earlier to cyclophosphamide injection and three days later so that the total number and differential number of leukocytes were counted. As a final step these counting were compared in Nabumetone treated and non- treated group (Thomas *et al.*, 2007).

Assay of nitric oxide determination: The mice were weighed and divided in to two treatment sets and one control set. Treatment sets were given Nabumetone @14mg/kg and @28mg/kg body weight of mice for seven days. In the control group, the mice were given normal saline for seven days. The above mentioned method was adopted for taking macrophages from mouse peritoneum. The macrophages were incubated in 24 well plates which contained ten microliters lipopolysaccharide in each well of E-coli serotype 055:B5 from Sigma chemical company USA for 48 hours, at 5% carbon dioxide and at 37° C for the production of nitric oxide in six well plates. The supernatant was separated from the macrophages for the further work. The macrophages were taken in 96 well plates with lower surface flat. Ultimately, liberation of the macrophages was determined by the measurement of nitric oxide concentration with griess reagent. Supernatants 150 microliters were added in 130 microliters of deionized water. Then 20microliters of griess reagent 1:1 mixture of 1% sulfanilamide dihydrochloride in 2.5% hypochloricacid Plates of 96 well, were incubated at 37°C temperature for thirty minutes and the absorbance was measured at 550nanometer with the help of enzyme linked immunosorbant assay reader. A calibration for the nitrite concentration was made using standard nitric oxide solution. A standard curve was made by using the absorbance. Different concentrations of standard solution were made from 0 to 60 microliters. One well of each row of 96 well plate was used as a control with deionized water. Then curve was drawn between different concentrations of standard solution and absorbance (Bryan and Ghisma 2007; Javeed *et al.*, 2011; Khurram *et al.*, 2017).

Statistical analysis: The data recorded on DTH assay skin thickness were measured. In MEA assay, number of macrophages were counted. In CIN assay, number of neutrophils were counted. In nitric oxide assay, nitric oxide production was measured. All these data were analyzed through one-way analysis of variance (ANOVA) followed by multiple comparison test i.e. LSD (Least significant difference) to understand statistical difference between groups with the help of Statistical Package of Social Sciences (SPSS) for windows version 13. The differences were considered significant ($P \leq 0.05$).

RESULTS AND DISCUSSION

Delayed type hypersensitivity assay (DTH): First of all, a known allergen dinitrochlorobenzene (DNCB) was applied on the skin of mice. Therefore, the thickness of mouse skin was measured after 24, 48 and 72 hours. In all mice, the thickness of skin had been increased up to 24 hours and in the treatment group continued to decline post 48 and 72 hours. However there was more reduction in the thickness of skin in Nabumetone treatment clusters, compared to non-treatment group (without Nabumetone). The probability was less than $P \leq 0.05$. This means that Nabumetone bottle-up cellular immune response in mice. Noticeably, the maximum thickness of skin in the control group was noticed after 72 hours. Lowering in skin thickness in treatment clusters compared to control group was very important. The highest reduction in skin thickness was seen in group of mice to which dose rate of Nabumetone was 28mg/kg, which was observed 72 hours later.

Typically, delayed type response means a response that comes later as compared to earlier. The response of inflammation that comes after 48 to 72 hours of first antigenic exposure is delayed type response. Within 12 minutes of antigenic exposure, the immediate hypersensitivity is produced. T-lymphocytes identify antigen and this act is compulsory for delayed type hypersensitivity. After recognition of antigen by T-lymphocytes, secretion of cytokines takes place (Singhal *et al.*, 2013) as a consequence. In the organ rejection process, the role of delayed type hypersensitivity is undeniable. This type of hypersensitivity is very important in the development of tumors and tuberculosis which is caused by intracellular organism (Actor *et al.*, 2007; Kumar 2008). After transfusion with the skin dinitrochlorobenzene produce dinitrophenyl protein. This protein encourages T-cells and after sensitivity of T-cells there is liberation of cytokines. Owing to these lymphokines, the scavenger cells are attracted to the place where dinitrochlorobenzene was applied (Trifen and Hla 2003; Gieger *et al.*, 2012; Dierssen *et al.*, 2016).

Macrophage engulfment assay (MEA): The trypan blue dye was used to eliminate dead macrophages from counting. In the present experiment, the viability of macrophages was 99%. Only 200 macrophage cells were selected for observation of engulfment of red blood cells of sheep (SRBCs). The macrophage engulfment percentage varied significantly in control and Nabumetone treated groups. After statistical analysis, probability was less than $P \leq 0.05$, indicating effect of Nabumetone on immunomodulation in mice. Percentage of phagocytosis was 59.8 ± 2.08 in control group, 35.2 ± 2.26 in 14mg/kg Nabumetone treated group and 15.2 ± 1.67 in 28mg/kg Nabumetone treated group perceived after 45 minutes. Percentage of engulfment was

63.7 ± 1.53 in non Nabumetone group, 38.2 ± 2.51 in 14mg/kg Nabumetone treated group and 18.7 ± 1.87 in 28mg/kg Nabumetone treated group observed 90 minutes after initiation of engulfment. It was noticeable that at higher dose of Nabumetone, percentage of phagocytosis of macrophages reduced more as compared to low dose of Nabumetone. So Nabumetone decreased cellular immunity in dose dependent manner.

Macrophages are stimulated by antigens and allergy causing agents and then they provide resistance by the stimulation of B- and T-lymphocytes. This is due to involvement of lymphocytes that the strong ability to kill microorganisms. They are talented to destroy bacteria in this way. In the current study Nabumetone impacted the capability of macrophages to engulf SRBCs.

Macrophage engulfment per cell: In addition, macrophage engulfment per cell observed after 45 minutes was higher in control group as compared to the groups of mice with Nabumetone treatment. Per cell macrophage engulfment was 3.1 ± 0.025 in the control group. In 14mg/kg and 28mg/kg Nabumetone groups, it was 2.3 ± 0.017 and 1.2 ± 0.012 , respectively. The significant immunomodulatory effect of Nabumetone was obvious by results. Nabumetone caused reduction in engulfment per macrophage dependent on dose. The macrophage engulfment observed after 90 minutes was 3.4 ± 0.034 in control group, 2.6 ± 0.019 in 14mg/kg and 1.4 ± 0.015 in 28mg/kg Nabumetone treated group.

Cyclophosphamide induced neutropenia assay(CIN): Cyclophosphamide reduced the number of leukocytes in all groups of mice. When compared with control group, the total leukocyte count had been reduced in both Nabumetone treated groups. In this situation, probability was less than $P \leq 0.05$ indicating Nabumetone important immunosuppressant effect. This decrease in total leukocytic count in control group was 68.41%, while 79.48% and 82.82% in 14 mg/kg and 28mg/kg groups, respectively. The neutrophils numbers were decreased in the groups of Nabumetone as compared to control group. The reduction in the number of neutrophils was 60.42% in the control group, 68.17% in the group of 14mg/kg and 89.77% in 28mg/kg of Nabumetone treatment group. So Nabumetone gave a discount to neutrophils.

Cyclophosphamide effects bone marrow in the mice is experimentally approved. This causes DNA alkalinity effecting DNA function badly. Therefore, cyclophosphamide effect (Abhigna *et al.*, 2012) is the cause of immunosuppression. So neutropenia which is caused by cyclophosphamide can be failed with the inspiration of macrophages. Macrophage produces several materials such as the colony motivational factor and interleukin-1 (Lee *et al.*, 2011).

After the introduction of infection causing agent, antibodies were given to understand neutropenia due to these drugs (Elio *et al.*, 2007). In a study, neutropenia which was caused by cyclophosphamide was blocked by methanolic extract of *Moringaolifera* due to inhibition of cyclophosphamide action on haemopoietic system (Bharani 2010). Myelosuppression was caused by cyclophosphamide (Hui W *et al.*, 2011). Nabumetone's current study has discovered that this reduced the total number of leukocytes and the number of differential leukocytes. This reduction was increased with increasing dose of Nabumetone due to the weak work of immune system. Nabumetone also reduced the number of cells

when cyclophosphamide was not given to mice and therefore, it is proposed that Nabumetone is immunosuppressant in cellular immunity. After using cyclophosphamide, the cell number was further discounted. After the introduction of cyclophosphamide, the effect of immunosuppression was increased. Cyclophosphamide might be replaced with other clinical immunosuppressive drugs which are widely used in clinical practices. However, all these parameters suggested the qualitative analysis, determination of cytokines that affect the proliferation of immune cells including lymphocytes, neutrophils and monocytes should be observed to support these results.

Table1. Effect of Nabumetone on cyclophosphamide induced neutropenia (Please add superscripts for significant differences between groups Effect of Nabumetoneon Cyclophosphamide Induced neutropenia.

Type of Group	Total Leukocyte count (Cells/mm ³)				Neutrophil count			
	Prior Count	Later Count	Reduction in Numbers	Percent reduction	Prior count	Later Count	Reduction in numbers	Percent reduction
Control Group	4597 (±98)	1442 (±95)	3145 (±81.3)	68.41	2792 (±53)	1105 (±68)	1687 (±46)	60.42
14mg/kg Group	3386 (±76)	695 (±47)	2691 (±49)	79.47	1899 (±47)	604 (±38)	1294 (±27)	68.17
28mg/kg Group	2731 (±24)	569 (±80)	***2262 (±52)	82.82	806 (±74)	82 (±51)	***724 (±31)	89.77

Nitric oxide determination assay (NOD): Production of nitric oxide by nonstimulated macrophages decreased to 5.7 μ M from 10.4 μ M in 28 mg/kg Nabumetone treated group, whereas it decreased to 5.2 μ M from 10.4 μ M in LPS stimulated macrophages in the same group.

Nitric oxide plays its part in all organs of the body and is vigorous biological agent. It has a necessary function in innate immunity, autoimmune diseases of skin, any sort of inflammation and skin cancer (David *et al.*, 2014). Numerous sicknesses are identified on the base of manufacture of nitric oxide. This mechanism of macrophages may be cGMP dependent or independent. The enzyme nitric oxide synthase is stimulated in contact hypersensitivity. Reduction of contact hypersensitivity is produced by nitric oxide (Feng *et al.*, 2011). When motivated macrophages were allowed to act in soya bean diffusate, their ability to produce nitric oxide was reserved. For the period of infection nitric oxide played very vital part. The level of nitric oxide upsurges in the body throughout malaria, tuberculosis, rheumatoid arthritis and any inflammation (Yette *et al.*, 2010). When the dose of Nabumetone was enlarged progressively, there was steady reduction of nitric oxide production. In LPS stimulated macrophages, the consequences were similar; with increasing dose of Nabumetone production of nitric oxide was reduced.

Cyclooxygenase enzymes are essential to produce thromboxane and prostaglandins. Some drugs suppress or block the above said enzymes to show their treatment response against inflammatory syndrome. This is the reason, these medicines can reduce the pain, fever and swelling (Blobaum and Marnett 2007). Nabumetone is found among non-steroids list. It is used to treat inflammation and is very effective in painful symptoms in rheumatoid arthritis. Clinical efficacy and toxic effects of inflammation inhibiting drug depend upon proportional discrimination against formation of certain enzymes (Relia *et al.*, 2012). Nabumetone is not acidic. It is soon absorbed and changed in the liver due to metabolism, in its smart structure. Its active metabolite shows anti-inflammatory, antipyretic and analgesic effects. However, Nabumetone itself has a weak restriction in the way of formation on of cyclo-oxygenase byproducts, especially prostaglandins. Nabumetone is relatively safe for kidneys, when matched with indomethacine (Hedner *et al.*, 2004). Considering therapeutic effects of Nabumetone, it was expected before starting this effort that Nabumetone may modulate the cellular immune system. These results were also in agreement with therapeutic effects of Nabumetone.

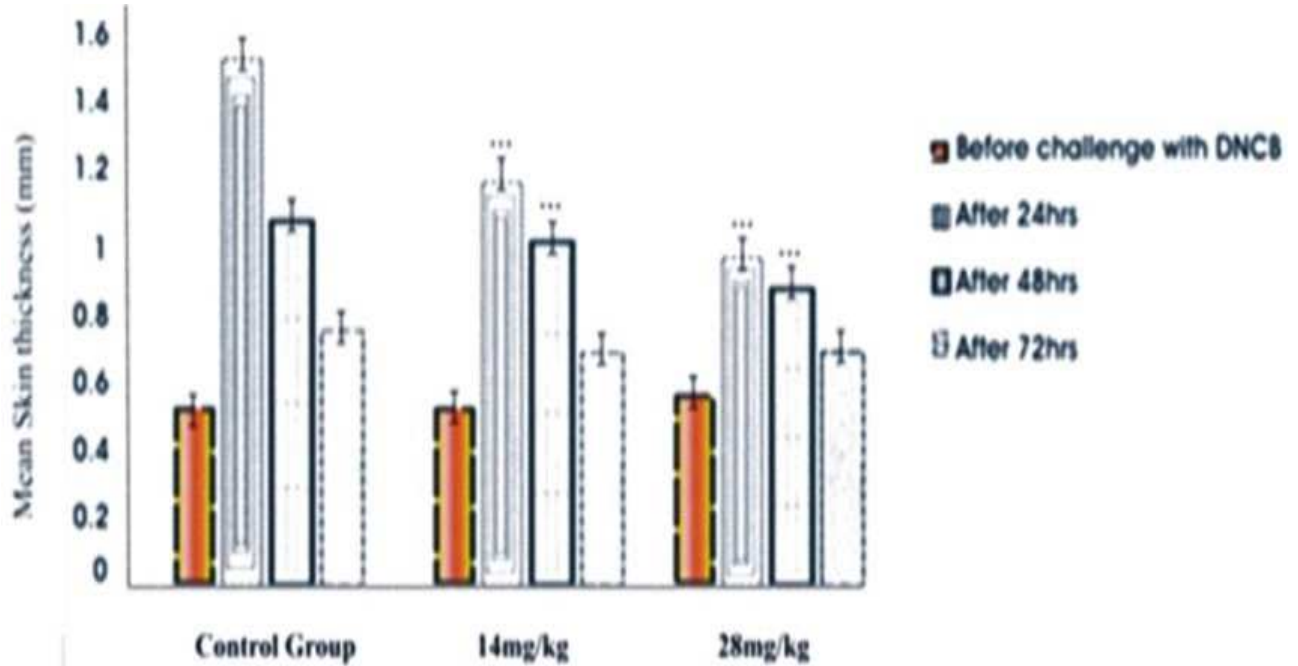


Figure 1. (Mean \pm S.E, n=5) Overall mean thickness of skin of mice in Nabumetone treated groups and control group, after challenge dinitrochlorobenzene. *** $P \leq 0.05$ at 24 and 48 hours of treatment, compared with corresponding groups.

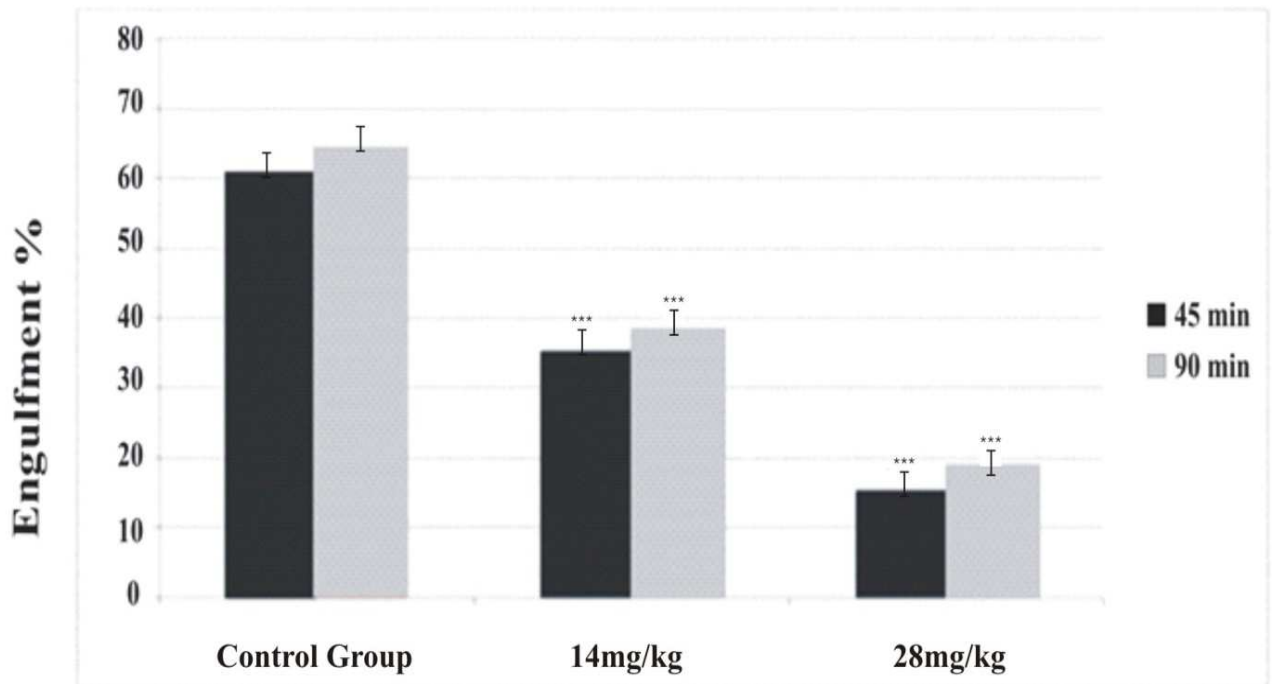


Figure 2. Engulfment percentage by macrophages (\pm S.E, n=5) observed at 45 and 90 minutes in control and in treated (14mg and 28mg of Nabumetone/kg body weight of mice) groups*** $P \leq 0.05$ matched with corresponding groups.

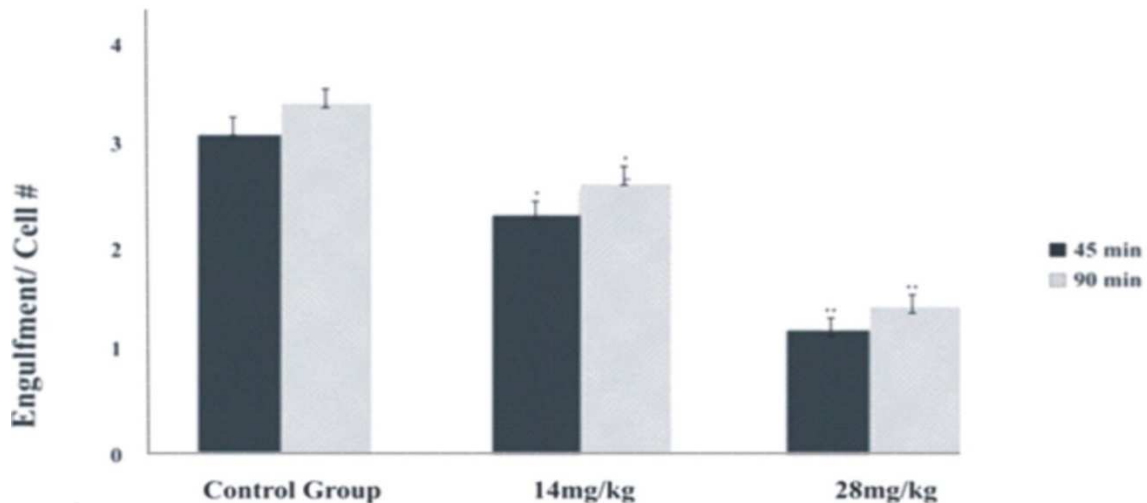


Figure 3. Engulfment per macrophage (Mean \pm S.E n=5) noted at 45 and 90 minutes in control and treated 14mg/kg and 28mg/kg Nabumetone treated groups * $P \leq 0.05$, compared with corresponding groups.

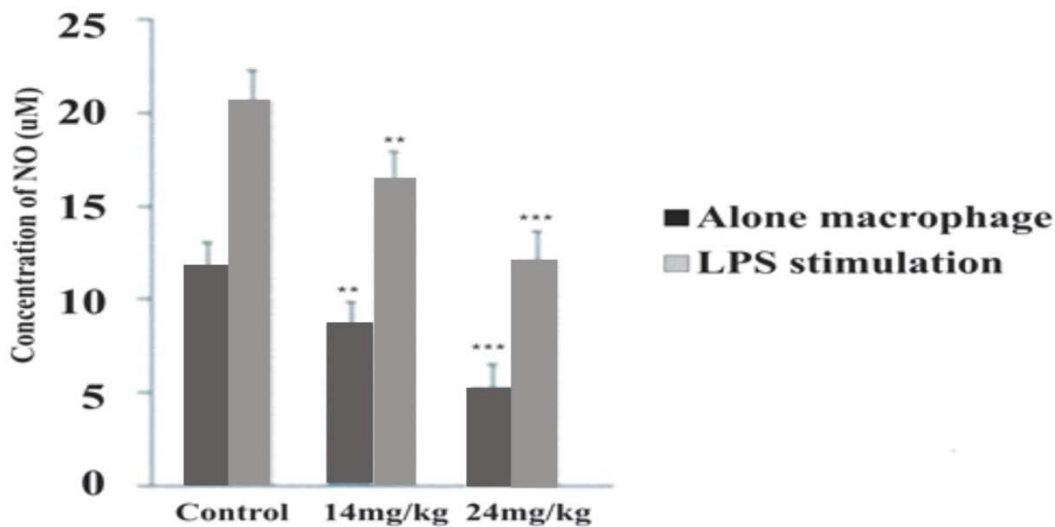


Figure 4. Production of nitric oxide (μ M \pm S.E, n=5) by un-stimulated and LPS stimulated macrophages determined in control, 14mg/kg and 28mg/kg Nabumetone treated groups ** $P \leq 0.05$, compared with corresponding macrophage groups, producing nitric oxide.

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Conflict of interest: Author has no conflict of interest.

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