

EFFECT OF COLCHICINE ON CELLULAR IMMUNE RESPONSES IN MICE

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ABSTRACT

Colchicine is microtubule inhibiting agent used for the cure of various skin ailments causing inflammation and reduction of pain in gouty arthritis. The current study was designed to evaluate the effect of colchicine on cellular immune responses in mice. Cellular immune responses were assessed in delayed type hypersensitivity (DTH) assay, macrophage engulfment assay, cyclophosphamide induced neutropenic test and nitric oxide (NO) production assay. Colchicine was administered to mice at doses of 40 µg/kg, 80 µg/kg and 160 µg/kg intraperitoneally. Data were statistically analyzed by ANOVA and chi square test. DTH showed significant ($P < 0.0001$) decrease in skin thickness of colchicine treated groups. In macrophage engulfment assay, significant ($P < 0.0001$) decrease in engulfment of sheep red blood cells (SRBCs) was observed by macrophages in colchicine treated groups. Colchicine treated groups showed significant ($P < 0.0001$) reduction of TLC and neutrophil count. NO production difference was significantly ($P < 0.0001$) reduced in macrophages alone and lipopolysaccharide (LPS) stimulated macrophages in colchicine treated groups. Therefore, it is concluded that colchicine suppresses the cellular immune responses in mice.

Key words: Cellular, Colchicine, Immune, Nitric oxide, Macrophage.

INTRODUCTION

Colchicine is separated from *Colchicum autumnale* plant (Battersby *et al.* 1972). It is an alkaloid approved in 2009 by the Food and drug agency (FDA) for the prophylaxis and the treatment of acute attack of gout and Familial Mediterranean Fever (Chetrit *et al.* 2006; Richette and Bardin 2010). Its mechanism of action includes prostanoid formation and alteration in chemokine function (Molad 2002). Colchicine has been revealed to control manifestation of inflammatory gene (Lee *et al.* 2007). Acquired and innate immunity has played fundamental role against protection of diseases (Von Dossow *et al.* 2008). The immune system of humans and animals is complicated. There is coordination between cell mediated and humoral immunity (Koller 1982). Different cells of immune system are involved in regulation of cell mediated immunity. These cells include neutrophils, macrophages, and T cells. Macrophages along with neutrophil embody second line of host defense after epithelial barrier. Macrophages can act as antigen presenting cells and coordinate with T- lymphocytes to alter the adoptive immune response (Schepetkin *et al.* 2006). Administration of colchicine with antigen has synergistic effect in producing plaque forming response. Induction of T cell mediated delayed type hypersensitivity, and rapid production of T cells in mice was reported previously (Titus 1991). Thymus relied immune mediation was investigated in chickens, bursectomized neonatally by colchicine, showing no considerable effects on T cell functions (Romppanen *et al.* 1983). Considering all these

reports, it was unavoidable to investigate the effect of colchicine on cell mediated immunity in mice model, which in turn will unveil its necessary use in various medical applications.

MATERIALS AND METHODS

Effects of colchicine on cell mediated immunity (CMI): Experimental animals: Albino mice (five-to-seven week-old) were purchased from Department of Theriogenology University of Veterinary and Animal Sciences (UVAS) Lahore. They were kept in animal house of the UVAS, Lahore, by taking into consideration all possible sanitary measures. The animals were kept on standard pelleted diet and water *ad libitum*. All experimental directions were attempted in agreement with the Institutional Guidelines for the Care and Use of Laboratory Animals.

Chemicals: Colchicine (Applichem pvt. Ltd. Germany), Acetone (Merck chemicals Ltd, Cyclophosphamide (Pharmedic pharmaceuticals pvt Ltd), Dinitro-chlorobenzene (DNCB) (Alfa Aesar, A Johnson Mathey Company)

Experimental design: In each test, mice were divided into group I (negative control), groups II, III, IV colchicine-treated i.e. 40 µg/kg, 80 µg/kg and 160 µg/kg respectively and group V (positive control). Groups were administered doses intraperitoneally. Positive control group was administered cyclosporine 100 µg/kg. Negative control group was treated with phosphate buffer saline

(PBS) only. Effect of colchicine was evaluated by performing delayed type hypersensitivity, macrophage engulfment assay, detection of nitric oxide (NO) production and cyclophosphamide induced neutropenia assay.

Delayed Type Hypersensitivity Assay: On day 1, drugs were administered to treated groups. On day 2 of the experiment (i.e. 1 day after treatment with colchicine), a sensitizing dose of 2% DNCB about 0.1 ml dissolved in acetone was applied on the right side of skin of thorax region below arm of each mice in treated groups. At 6th days after sensitization (8th day of the experiment), thickness of left side of skin of thorax region was measured with vernier caliper before treatment. Consequently, 0.2 ml of DNCB was applied as challenging dose on left side of thorax region of the mice. After a time period of 24, 48 and 72 hours, an enhancement in the skin thickness was measured with vernier caliper (Sajid *et al.* 2007; Hamdani *et al.* 2015).

Macrophage Engulfment Assay (MEA): Colchicine treated and control groups were administered doses daily intraperitoneally for 1 week. Mouse peritoneal exudates cells were obtained from the peritoneal exudates of mice from groups I, II, III, IV and V. Macrophages from these peritoneal exudates were separated by method described

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

Phagocytic index = % of macrophage containing RBCs × Mean number of RBCs engulfed/cell

Detection of nitric oxide (NO) production: Colchicine was administered to three groups intraperitoneally for one week. Positive and negative control groups were also administered with respective drugs intraperitoneally daily for one week. Mice peritoneal macrophages were extracted from the peritoneum of mice by the method described (Liu *et al.* 2007; Yang *et al.* 2007; Lahat *et al.* 2008; Javeed *et al.* 2011; Hamdani *et al.* 2015). Macrophages obtained from the peritoneum were then incubated in 24 well plates with the lipopolysaccharide (LPS) 10 µg/ml/well of *Escherichia coli* Serotype 055: B5 (Sigma chemical (company, USA) for 48 hours 5% CO₂ and 37°C for determination of NO production in six well plates. Supernatant from the macrophages were separated for further use. Then, these macrophages were taken in to the 96 flat bottom plates. Nitric oxide production was determined by using Griess reagent kit (Molecular Probes Life technologies).

Supernatants (150 µl) were added in 130 µl of de-ionized water. Then, 20 µl of Griess reagent 1:1 mixture of 1% sulphanilamide dihydrochloride and 0.1% Naphthyl ethylenediamine dihydrochloride in 2.5% H₃PO₄. Well Plates were incubated at 37°C for 30 minutes, and the absorbance at 550 nm was measured by using ELISA reader. A calibration curve for the nitrite

(Liu *et al.* 2007; Lahat *et al.* 2008; Yanget *et al.* 2007; Javeed *et al.* 2011).

Mice peritoneal macrophages were cultured in RPMI1640 medium containing 2.0 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin for two hours so that they were adherent to the sterilized glass slides (Ammon *et al.* 2000; Esashi *et al.* 2003). Trypan blue exclusion dye was used for determination of cell viability (Freshney 2010; Hamdani *et al.* 2015).

$$\% \text{ viable cells} = \frac{\text{Number of viable cells/ml}}{\text{Total number of cells/ml}} \times 100$$

Hemocytometer was used for counting of macrophages and cell number was maintained at 5 × 10⁶ cells/ml. SRBCs were washed three times with PBS. In vitro phagocytosis of SRBCs was performed in colchicine treated groups, negative and positive control groups by macrophages (Javeed *et al.*, 2011; Loike *et al.*, 2004; Seyrantepe *et al.*, 2010). Peritoneal exudates macrophages 1 × 10⁶ were co-incubated with 1 × 10⁷ SRBCs for 3 hours in six well plates. Non-phagocytosed SRBCs were removed with cold PBS. About 200 macrophages were selected for phagocytosis. Macrophage phagocytosis was seen under microscope. Macrophage engulfment percentage and phagocytic index were evaluated by the formulas (Tatarczuch *et al.* 2002).

concentration was made with standard nitrite solution (Monney *et al.* 2002; Hamdani *et al.* 2015).

Cyclophosphamide induced neutropenia assay: Colchicine treated and control groups were administered daily intraperitoneally for 10 days. Cyclophosphamide (200 mg/kg s.c) as neutropenic dose was given on 10th day to colchicine treated groups, positive control group and negative control. This day was defined as day 0. Blood was collected from the heart of mice. Differential leukocyte count (DLC) and total leukocyte count (TLC) were performed before and on day 3 after administration of cyclophosphamide. The neutrophil count and TLC percentage in colchicine treated groups and positive control were compared with the values in negative control group (Licto Thomas *et al.* 2007; Hamdani *et al.* 2015).

Statistical analysis: The data was analyzed by one way analysis of variance (ANOVA) followed by multiple comparison test i.e. LSD (Least significant difference) and Chi square test with the use of statistical package of social sciences (SPSS). The values were considered significant at P < 0.05. The values were expressed as mean ± S.E.

RESULTS

Delayed type hypersensitivity assay: There was change in skin thickness observed in all groups at 24, 48 and 72 hours following application of DNCB (Dinitrochloro benzene). Groups II, III and IV received colchicine 40µg/kg, 80µg/kg and 160µg/kg body weight respectively. Skin thickness of colchicine treated groups II, III and IV were increased after 24 hours. There was decrease in skin thickness of group II, III and IV at 48 and 72 hours. In negative control group, there was continuous increase in thickness of skin after 48 and 72 hours as compared to colchicine treated groups. Significant ($P<0.0001$) reduction of skin thickness was observed to all colchicine treated groups to negative control. Positive control showed significant ($P<0.0001$) reduction in skin thickness to negative control after 24 hours.

After 48 hours, skin thickness started to decrease in the colchicine treated groups II, III, IV but there was increase in thickness of negative control group. There was significant ($P<0.0001$) reduction in difference of skin thickness in all colchicine administered groups as compared to negative control. Positive control showed significant ($P<0.0001$) reduction in skin thickness after 48 and 72 hours as compared to negative control. After 72 hours, there was further reduction in skin thickness of the colchicine treated groups II, III and IV as compared to negative control. Highest skin thickness was observed after 72 hours in the negative control. There was significant skin reduction after 72 hours in all colchicine treated group but most significant skin reduction was found in group IV as shown in Figure 1.

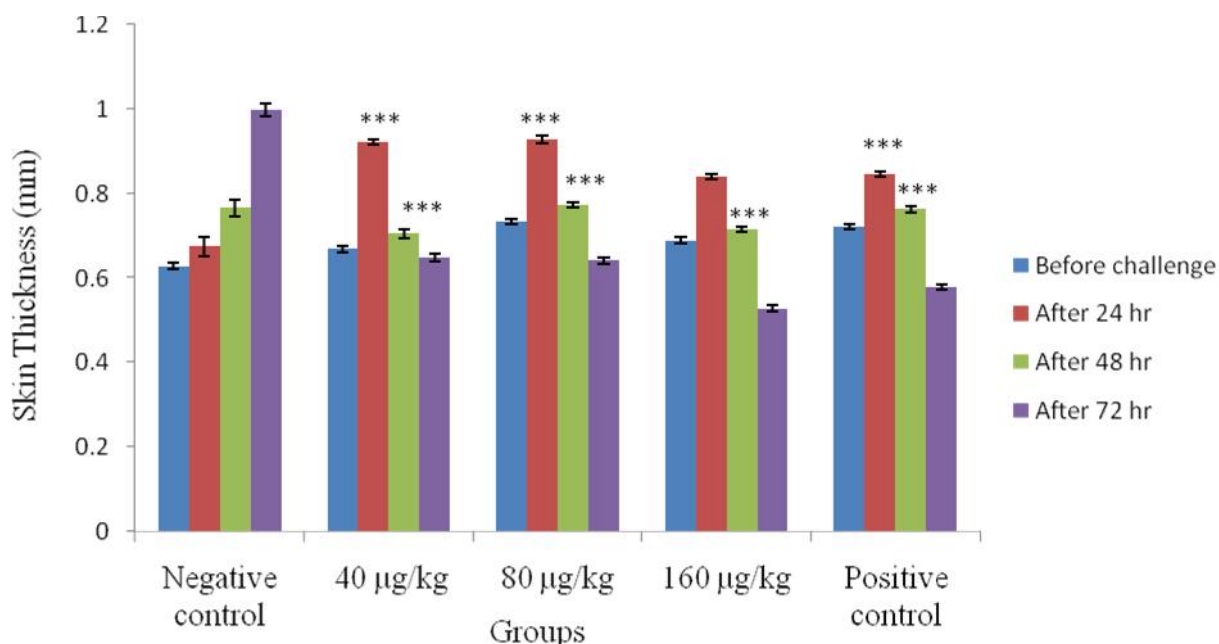


Figure 1. Overall mean thickness $mm \pm S.E.$ ($n=7$) after challenge with DNCB
 $p<0.0001$ compared to control group at 24 & 48 hrs of treatments by using repeated measure design

Macrophage engulfment assay: Macrophages were studied for their viability by using trypan blue exclusion dye. The viability of macrophages was 99%. About 200 macrophages were selected for engulfment with sheep red blood cells. There was significant ($P<0.0001$) difference of engulfment in colchicine group II (40µg/kg), group III(80µg/kg) and group IV(160µg/kg) as compared to negative control. There was significant ($P<0.0001$) reduction in engulfment of colchicine treated groups to negative control. Macrophage engulfment was more significantly reduced in group IV (160 µg/kg) as compared to other colchicine treated groups. After 45 minutes, the macrophage engulfment percentage and

standard error mean was 61.5 ± 0.85 for negative control, 52.75 ± 0.94 for group II (40µg/kg), 39.58 ± 0.71 for group III (80µg/kg) group and 24.25 ± 0.44 for group IV (160 µg/kg) colchicine. There was reduction in phagocytosis percentage of macrophages with increase in dose. There was significant ($P<0.0001$) decrease in macrophage percentage of positive control to 18.75 ± 0.62 as compared to negative control at 45 minutes.

At 90 minutes, engulfment percentage in negative control was 71.08 ± 0.58 to 45.83 ± 1.01 for 40µg/kg, 30.5 ± 0.76 for the 80 µg/kg and 17.66 ± 0.44 for 160 µg/kg dose of colchicine. There was significant reduction in difference of the engulfment percentage at

different doses indicating that increase in dose resulted in reduction in the engulfment percentage of macrophages. There was also significant ($P < 0.0001$) decrease in macrophage engulfment of positive control compared to negative control at 90 minutes. When engulfment was compared at 45 minutes and at 90 minutes, it was observed that engulfment percentage of macrophages was

decreased with passage of time. There was significant difference between 45 minutes and 90 minutes of engulfment as shown in Figure 2. Phagocytic index was significantly reduced ($P < 0.0001$) observed at 45 and 90 min in group II (40 $\mu\text{g}/\text{kg}$), group III (80 $\mu\text{g}/\text{kg}$) and group IV (160 $\mu\text{g}/\text{kg}$) of colchicine treated to group I (negative control) as shown in Figure 3

Macrophage engulfment of SRBCs

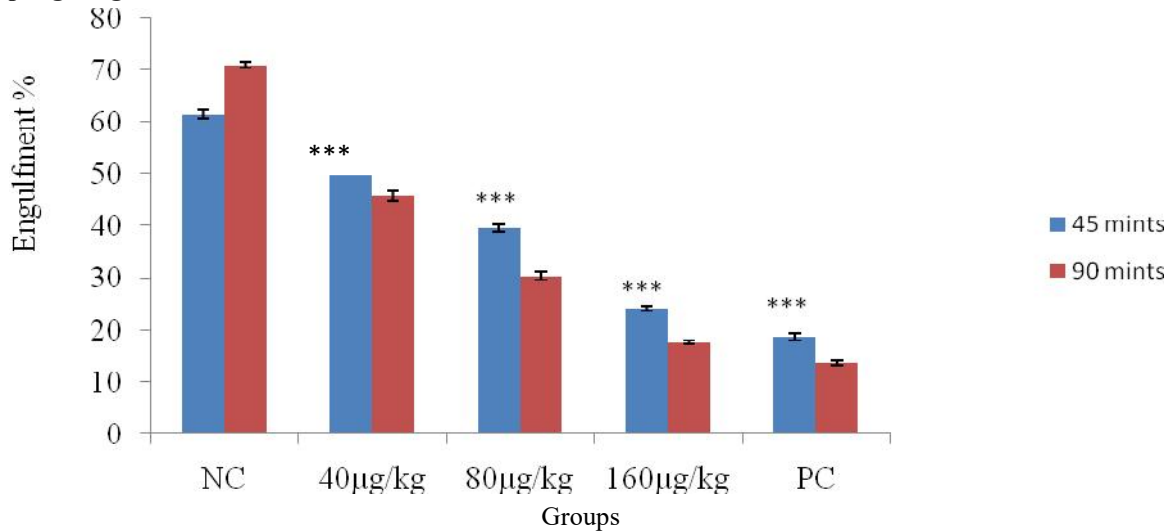


Figure 2. Macrophage engulfment \pm SEM (n=6) observed at 45 and 90min in Negative control, 40 $\mu\text{g}/\text{kg}$, 80 $\mu\text{g}/\text{kg}$ and 160 $\mu\text{g}/\text{kg}$ colchicine treated group $p < 0.0001$, by using Chi Square compared with corresponding groups. NC = Negative control, PC = Positive control

RBC engulfment/cell

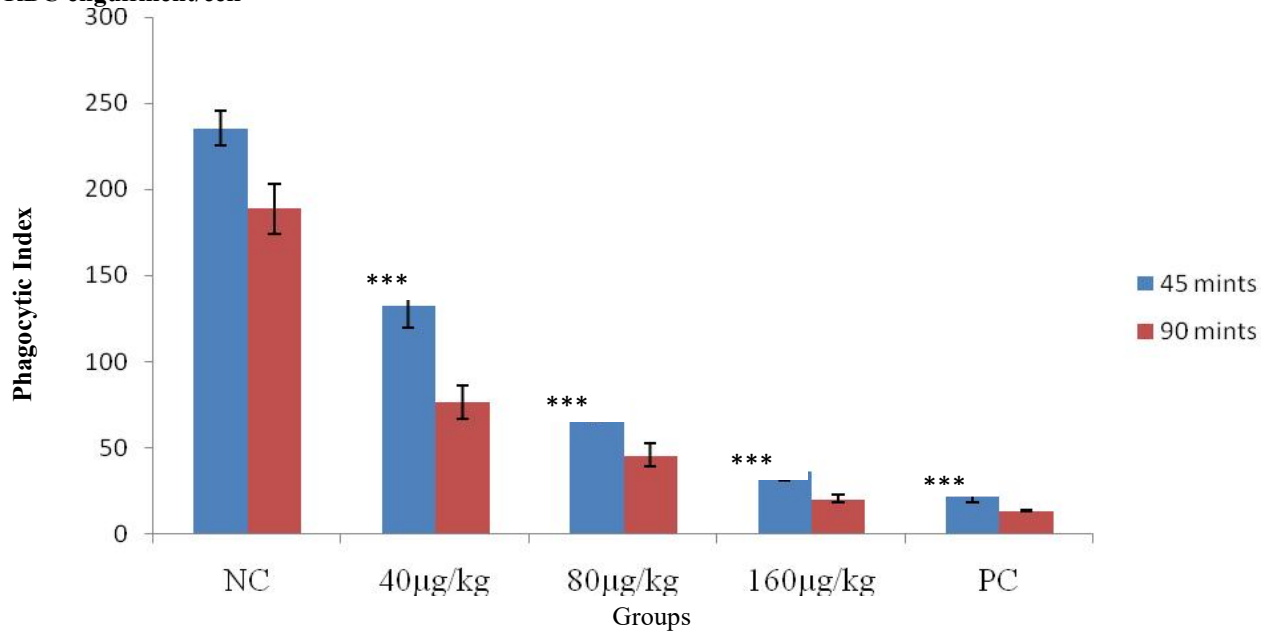


Figure 3. Macrophage engulfment \pm SEM (n=6) observed at 45 and 90min in Negative control 40 $\mu\text{g}/\text{kg}$, 80 $\mu\text{g}/\text{kg}$, 160 $\mu\text{g}/\text{kg}$ colchicine treated group $***p < 0.0001$, compared with corresponding groups using One way ANOVA. NC: Negative control, PC: Positive control

Detection of nitric oxide production: Nitric oxide produced by macrophages obtained from mice that were treated with colchicinewas evaluated by griess reagent shown in figure 4. NO production by alone macrophages was significantly ($P<0.0001$) decreased in colchicine administered groups as compared to negative control. There was significant ($P<0.0001$) reduction in NO production by macrophagesof colchicine treated group II (40 $\mu\text{g}/\text{kg}$), group III (80 $\mu\text{g}/\text{kg}$), group IV (160 $\mu\text{g}/\text{kg}$) to negative control. With the increase in dose of colchicine, NO production by macrophages was decreased. NO produced by macrophages activated by LPS in negative control and colchicine treated groups were also

determined. Activation of macrophages by LPS showed significant ($P<0.0001$) higher production of NO from macrophages as compared to non-activated macrophages. However macrophages when activated with LPS also showed similar trend as seen in macrophages that were not activated. There was decrease in NO production in colchicine treated groups compared to negative control. There was significant ($P<0.0001$) reduction in NO production in positive control both with LPS stimulation and without LPS stimulation to negative control. The results suggested that colchicine caused reduction in NO production by macrophages in dose dependant manner as shown in Figure 4

Macrophage NO production

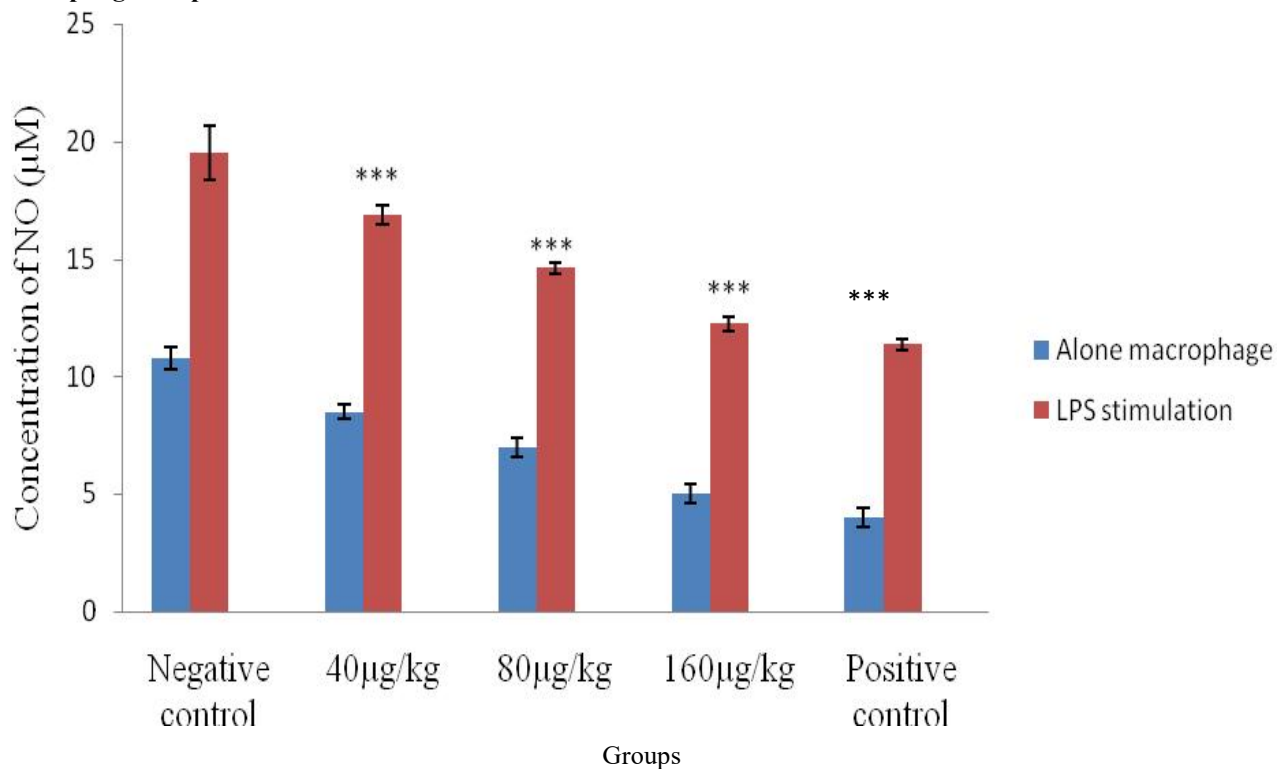


Figure 4: Nitric oxide $\mu\text{M}\pm\text{SEM}$ (n=6) production by unstimulated, LPS stimulated macrophages in negative control, colchicine treated groups $p<0.0001$

Cyclophosphamide induced neutropenia: There was reduction in neutrophil count in five groups after administration of cyclophosphamide. There was significant ($P<0.0001$) decrease in the total leukocyte count (TLC) in the colchicine treated groups II (40 $\mu\text{g}/\text{kg}$) and III (80 $\mu\text{g}/\text{kg}$) but highly significant difference was found in group IV (160 $\mu\text{g}/\text{kg}$) as compared to negative control There was significant ($P<0.001$) decrease in TLC count of positive control from 616 \pm 142 to 199 \pm 46 as compared to negative control. There was reduction in TLC to about 45.3% in control to 48.3%, 54.68% and 65.42% in group II, III and IV respectively when these

were compared with primary values of TLC. There was also statistically significant ($P<0.0001$) reduction in difference of TLC count between groups II, III and IV. There was significant difference of reduction in the neutrophil count of negative control 1057 (\pm 120) to 902 (\pm 67) in group II, 734(\pm 69) in group III and 609^{***} (\pm 71) in group IV of colchicine. There was significant ($P<0.0001$) reduction in neutrophil count of positive control from 369(\pm 85) to 119(\pm 27) as compared to negative control. This result showed that administration of the colchicine resulted in reduction of neutrophil count as shown in Table 1.

Table 1. Effect of Colchicine on Cyclophosphamide induced neutropenia.

Effect of Colchicine on Cyclophosphamide Induced neutropenia								
Groups	Total Leukocyte count (Cells/mm ³)				Neutrophil count			
	Count Before	Count After	Reduction in cell number	% age Reduction	Count Before	Count After	Reduction in Neutrophil number	% reduction
Negative control	4460 (±97.185)	2438 (±98.189)	2022 (±82.276)	45.3	2676 (±58.307)	1462 (±59.018)	1057 (±49.349)	39.51
40µg	3594 (±77.127)	1858 (±45.554)	1736 (±46.329)	48.3	2156 (±46.229)	1114 (±27.258)	902 (±27.737)	41.87
80µg	2525 (±124.379)	1144 (±80.558)	1380 (±48.336)	54.68	1531 (±70.762)	686 (±48.335)	734 (±28.557)	47.94
160µg	1782 (±75.0552)	616 (±55.588)	1166*** (±48.784)	65.42	1069 (±45.046)	369 (±33.358)	609*** (±29.303)	57.0
Positive control	616± 58.223	199± 19.051	416± 45.991	67.63	369 (±34.982)	119 (±11.342)	224 (±27.646)	60.61

TLC count and neutrophil count are represented as mean ± SE (n=6) of colchicine 40µg/kg, 80µg/kg, 160µg/kg, groups are p<0.0001, to negative control by using one way ANOVA

DISCUSSION

Colchicine is microtubule inhibiting agent that has been used several times for the cure of number of skin ailments causing inflammation like Psoriasis, Behcet disease, leukocytoclasticvasculitis, palm plantacpustulosis and Sweet's syndrome (Sullivan *et al.*, 1998). It was investigated that colchicine reduced pain of gouty arthritis (Lidar and Livneh, 2007).The mode of action of colchicine includes modulation of chemokine and prostanoid production and inhibition of neutrophil and endothelial cell adhesion molecules by which it interferes with the initiation and amplification of the joint inflammation (Molad, 2002).Acquired and innate immunity has played fundamental role against protection of diseases (Von Dossow *et al.*,2008).The current study was performed to observe the effect of colchicine on cellular immunity.

Dinitrochloro benzene (DNCB) was used for induction of delayed type hypersensitivity. DNCB forms dinitrophenyl protein by interacting with the skin (Sajid *et al.*, 2007).This protein is responsible for stimulating T-cells. After T-cells sensitization, lymphokines are produced. These lymphokines attract more scavenger cells toward site of application (Kuby, 1997).Locally produced chemokine caused activation of T cells. T cells are responsible for regulation of graft survival (Tan *et al.*, 2013).In currentstudy,the effect of colchicine was investigated on the delayed type hypersensitivity by application of DNCB on the skin. There was reduction in skin thickness with increasing dose of colchicine.The reduction of inflammation caused by the colchicine was due to inhibition of activity of macrophages. Colchicine is involved in inhibition of delayed type hypersensitivity

(Mekori *et al.*, 1989;Sosroseno, 2008). The results of present study are correlated with the previous findings.

Macrophages are activated by the cytokines produced from T helper cells (Noel *et al.*, 2004). Macrophages have played important role in host defense against microbe (Zhao *et al.*, 2010). In present study, macrophage engulfment percentage and phagocytic index were studied.The effect of colchicine on macrophages was observed. There was reduction in macrophage engulfment percentage. The macrophages were allowed to engulf SRBCs in vitro.Similarly, phagocytic index was decreased with increasing dose of colchicine. The reduction in engulfment of macrophages was due to decrease in activity of macrophages to engulf SRBCs. This effect decreased activity of immune system resulting in immunosuppression.

Various diseases can be diagnosed on the basis of nitric oxide (NO) production. NO is biological molecule for regulation of function of immune, cardiovascular and nervous systems(Blaise *et al.*,2005).Inducible NO synthase protein appearance activated by LPS in the macrophages was depressed by colchicine (Kirikae *et al.*,1996). In current study, colchicine caused reduction of NO in alone and LPS stimulated macrophages with increasing dose of colchicine. The result of current study was in accordance to findings (Kirikae *et al.*, 1996) who reported that colchicine partially suppressed NO production by macrophages stimulated by LPS.The suppression of LPS-induced NO production by colchicinewas due to post transcriptional processes.

Cyclophosphamide is responsible for alkylation of DNA causing inhibition of DNA synthesis and function resulted in immunosuppression (Thatte *et al.*, 1987). Cyclophosphamide was used to induce

neutropenia(Zuluaga,2006). The present study has shown that administration of colchicine resulted in reduction of differential leukocyte count (DLC) and total leukocyte count (TLC). This may be due to inhibition of macrophages. Colchicine caused reduction in the cell count before administration of cyclophosphamide. This reduction in cell number may be considered as immunosuppressive effect of colchicine. After administration of cyclophosphamide, there was more reduction in number of cells. The effect of colchicine was enhanced by administration of cyclophosphamide. Neutropenia was caused due to inhibition of macrophages which produced a large number of substances including interleukin 1 and colony stimulating factor (Heppner *et al.*,1996).Cyclophosphamide induced neutropenia assay focused on defensive effect against myelosuppression(Diwanay *et al.*,2004).The results of current studydraw the conclusion that colchicine is involved in dose dependent suppression of cell mediated immune responses.

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