

Adequacy of phosphodiesterase inhibitor in prevention and treatment of LPS induced organ failure in BALB/c mice

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Received: 03 January 2019

Accepted: 04 February 2019

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ABSTRACT

Background: Even though with immense improvement and extensive understanding of pathophysiology of sepsis induced organ failure and affected population, it continues to put hundreds of people worldwide to eternal sleep due to lack of targeted therapy. Newer treatment modalities is the dire need of time. The present study was aimed to ascertain the adequacy of phosphodiesterases inhibitor - pentoxifylline (75mg/kg i.p) in endotoxin/LPS induced hepatotoxicity in BALB/c mice.

Methods: The number of animals in each group was six. Endotoxin/lipopolysaccharides induced hepatotoxicity was reproduced in mice by giving lipopolysaccharide of serotype *E. coli* intraperitoneally. To ascertain the Preventive role, pentoxifylline was administered forehand LPS injection whereas therapeutic potential adjudged via post LPS delivering. The extent of liver damage was evaluated through serum alanine aminotransferases (ALT) and aspartate aminotransferase (AST) estimation along with histopathological examination of liver tissue.

Results: Results set forth that serum ALT, AST levels and histological alteration abated considerably ($p \leq 0.05$) both in animals subjected to pentoxifylline pre and post-treatment.

Conclusions: Pentoxifylline set up promising results in endotoxin induced hepatotoxicity and can be used therapeutic adjuncts to conventional treatment strategies in sepsis induced liver failure.

Keywords: Endotoxin, Gram negative sepsis, Hepatotoxicity, LPS, Pentoxifylline, Phosphodiesterase inhibitor

INTRODUCTION

Sepsis is major health dilemma, putting hundreds of people to eternal sleep, not only in developing countries like Pakistan but calls a devastating death toll in developed countries like USA. About 50-100 cases per 100,000 population suffered from sepsis induced organ failure in

industrialized countries.¹ The EPISEPSIS study in French ICU's and PROWESS study stated the incidence of hepatic dysfunction in sepsis to be 46.6% and 35.6% respectively.^{2,3} Previously gram negative organisms were the main offender especially in developing countries with *E. coli*, *Klebsiella pneumoniae* and *Enterobacter* mostly involved.^{4,5} But with times the trends have shifted toward

gram positive organisms such that almost equal number of patients are infected by both.

The resident cells of human body utilizing pattern recognition receptors (PRRs) spot the intruding pathogen through signature 'pathogen-associated molecular pattern (PAMP) or microbial associated molecular pattern (MAMP)' molecules and initiate a war against it by producing pro-inflammatory cytokines that results in full blown activation of intrinsic immune response. TLR 4 is gram negative's outer cell membrane molecule called endotoxin or Lipopolysaccharide (LPS) receptors.^{6,7} This LPS activates host intrinsic immune response through mechanisms dependent on lipopolysaccharide binding protein (LBP) and CD14 receptors.⁸

TNF-alpha and IL-1 are antecedent inflammatory cytokines released in 30-60 minutes after LPS administration followed by polymorph nuclear cells and macrophage activation. Following through is induction of prostaglandins, nitric oxide synthase with resultant oxidative stress and induction of cellular adhesion molecules, releases high mobility group box -1 (HMGB1), macrophage migratory inhibitory factor (MIF), platelet activating factor (PAF), IL-6, IL-8. All of this call for hypotension, shock and organ failure. Erroneous and faulty livers activity in sepsis is inferred to trigger other organ failure, considering its significance in endotoxin detoxification, xenobiotic's biotransformation and protein synthesis for immunological and coagulatory function.⁹

The surviving sepsis campaign, survive sepsis, and the global sepsis alliance are in play to augment sepsis recognition, intensify and reinforce guidelines to appreciate the septic patient, to provide emergency treatment and reduce mortality associated with multiple organ failure. But to our dismay, the treatment of sepsis has not thus far expanded and newer diagnostic and treatment modalities are required.

Pentoxifylline (PTX) is an all-around and wide range phosphodiesterase inhibitor and adenosine receptor agonist/antagonist that has been utilized clinically for hemorrhheological diseases and proved beneficial through heterogeneous pathways for treatment of sepsis. Studies have demonstrated that PTX down regulates NF-κB activity by diminishing phosphorylation and ensuing degradation of inhibitory complex I-κBα, nuclear translocation and DNA binding of NF-κB after LPS administration.¹⁰⁻¹² Also optimal activity of NF-κB requires interaction and acetylation of p65 subunit with CBP/p300. However, the binding site of NF-κB and phosphorylated CREB is same on co-activator, CBP. Thus PTX may inhibit NF-κB activity by increasing concentration and DNA binding capacity of CREB that compete with it for co-activators.^{13,14} Moreover several researcher have showed that activator protein-1 (AP-1) and CREB share similar association site on promoter region of TNF-alpha gene and PTX may produce anti-inflammatory action through facilitation of CREB binding

to DNA and diminishing TNF-alpha production.¹⁵⁻¹⁷ Also pentoxifylline is shown to decrease superoxide anion production in PMN and A2A receptor invigoration that causes anti-inflammatory effects and constrains overzealous immune response in sepsis.¹⁷⁻¹⁹ This drug has reported to decreases anorexia, muscle wasting and weight loss in septic rats, has influential nephroprotective role in septic mouse, ameliorated pulmonary dysfunction following *E.coli* intraperitoneal inoculum and curtails LPS-mediated heightening of ally-alcohol hepatotoxicity.²⁰⁻²³

This study has been designed on these convincing grounds that pentoxifylline might prove beneficial in treatment and prevention of endotoxin/ LPS induced hepatotoxicity .

METHODS

This study was a randomized controlled laboratory trial carried out in Department of Pharmacology and Therapeutics, after study protocols approval from the Ethical committee of "Centre of Research in Experimental and applied Medicine (CREAM)" Army Medical College. Mice (BALB/c) of both genders, segregated, were elected through non-probability convenience method and then were sectioned randomly into 4 groups, with at least 6 in each group. Contamination free fresh water and nutritionally adequate rodent pellet diet (containing 4-7% fat and 11-15% protein) was provided ad libitum during the entire time period

Agents used in the study

Lipopolysaccharides (LPS) From *Escherichia Coli* O111:B4- provided by Sigma chemicals, USA used at dose of 10mg/kg intraperitoneally.^{24,25}

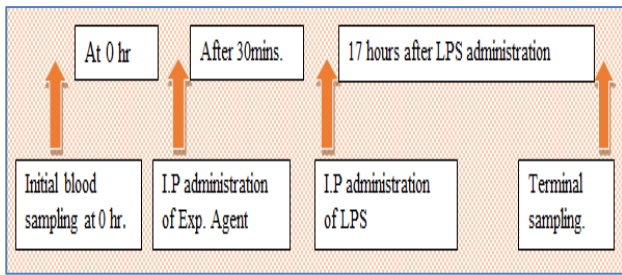
Pentoxifylline was purchased from Sigma Aldrich chemicals (USA). The dose of 75 mg/kg of body weight was well handled by mice and was taken as our research dose.

Phosphate Buffered Normal saline was utilized for preparation of lipopolysaccharide solution whereas 10% formaldehyde for preserving tissues.

Experimental approach

Generation of LPS Induced hepatotoxic model

A pilot project was conducted to estimate the hepatotoxic dose of LPS in our white albino mice and a dose of 10mg/kg of body weight with time interval of 17 hours between LPS administration and terminal sampling was the effective strategy to produced marked hepatotoxicity. For preventive role appreciation, Pentoxifylline was administered prior to LPS (Figure 1) and for therapeutic role appraisal pentoxifylline was administered after LPS administration (Figure 2).



IP: intraperitoneal, LPS: lipopolysaccharide, Exp. Agent: experimental agent- (pentoxifylline). Terminal Sampling-cardiac puncture, laparotomy, organ removal, termination.

Figure 1: Schematic diagram of pentoxifylline pre-treatment group study design.

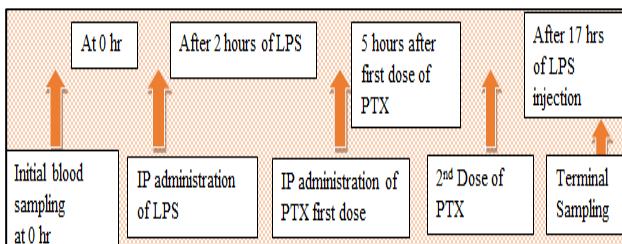


Figure 2: Schematic diagram of pentoxifylline post-treatment group study design.

Grouping outline

Group 1 (control group)

Animals (n=6) served as vehicle control group and received single intraperitoneal injection of normal saline.

Group 2 (lipopolysaccharide group)

Chemical shock model was reproduced in this study by single intraperitoneal injection of lipopolysaccharide (LPS)-10mg/kg of body weight.²⁵

Group 3 (pentoxifylline pre-treatment group)

Animals (n=6) in Group 5 were administered pentoxifylline 75mg/kg of body weight. This was followed 30 minutes after by a single intraperitoneal injection of LPS.

Group 4 (pentoxifylline post-treatment group)

Six mice in this group were intervened by twice giving intraperitoneal injection of pentoxifylline (75mg/kg of body weight). First dose was injected two hours after LPS, and subsequent dose was given 5 hours later.

Blood sample and tissue collection

Primary blood sampling in all animals was done from lateral tail vein of mouse. Blood was collected in microcapillary inserted into pipette bulb followed by

sample dispensing into eppendorf tube. Closed ventral approach was adopted for terminal sampling by cardiac puncture followed latter by midline laparotomy. Liver was identified, removed from abdominal cavity and immediately fixed in 10% neutral buffered formalin.

Determination of hepatic dysfunction

Estimation of serum alanine amino transferase (ALT) and AST

Serum separation was performed by centrifuging blood at 4000 revolution per min at 8 degree centigrade for 10 minutes. International Federation of Clinical Chemistry (IFCC) endorsed methods were employed for ALT and AST measurement in automatic analyzer, via commercially available MECK kits.

Histological examination and grading

Liver tissue blocks were placed in tissue cassettes to be processed through Leica TP1020 Tissue Processor ensued by hemotoxylin and eosin staining through LEICA autostainer XL. Stained sections were covered with Canada balsam and covered with cover slip. As demonstrated by previous studies sepsis resulted in varied histopathological changes in liver, we utilized Ishak Modified Histological Activity Index to grade histopathological changes. Fibrosis was excluded due to short study duration.²⁶⁻²⁸

Statistical analysis

Data was entered in statistical package for social sciences (SPSS) version 20. The results of the serum analysis were expressed as Means±Standard Error of Means. ANOVA followed by post hoc Tukey test was put to use for between group serum markers levels where as unpaired t test analyzed statistically significant difference in serum marker levels at 17 hours of LPS group with treatment group. Histopathological results were compared using chi square test. Results significance was set at p value of ≤ 0.05.

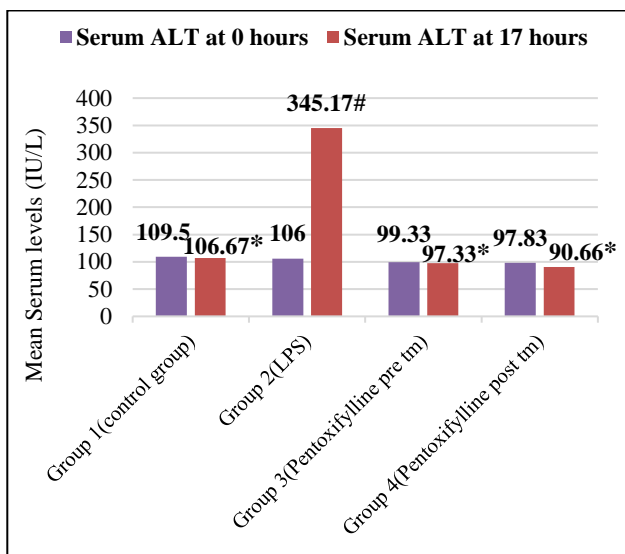
RESULTS

Chemical parameters analysis

Six mice of both gender that served as controls in group 1 received intraperitoneal normal saline at 0 hour and were dissected at 17 hours. Mean Serum ALT and AST levels at 17 hours have not significantly risen from those at 0 hours yielding a p value of 0.276 and 0.137 respectively.

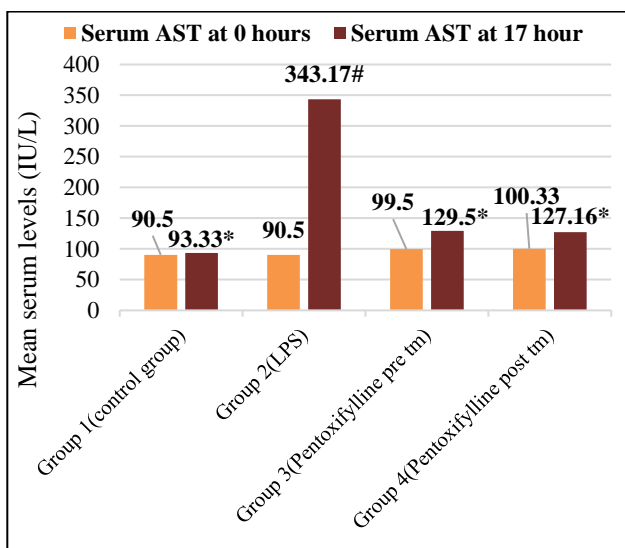
LPS administration in animals of group 2 resulted in highly deranged serum ALT and AST levels at terminal blood sampling. Mean serum ALT levels risen from to 345.17IU/L±76.51 at 17 hours from 106.00 IU/L±2.81 at 0 hours. Analogous observation was with serum AST levels that yielded a highly statistically considerable p value of

≤0.001. likewise serum marker comparison of GROUP 2 (LPS group) at 17 hours with that of group 1 (control) generated a statistically significant p value of ≤0.05 for both serum AST and ALT levels (Figure 3 and 4).



*p ≤0.05 is significant when compared to LPS group at 17 hours. #p ≤0.05 between start at 0 hour and end of experiment at 17 hours

Figure 3: Comparison of serum ALT level (IU/L) of each group at 0 hour and 17 hours.



#p value is ≤0.001. *p ≤0.05 is significant when compared to LPS group at 17 hours.

Figure 4: Comparison of serum AST mean (IU/L) of each group at 0 hour and 17 hours.

Chemical parameters, strictly speaking serum ALT and AST, levels analysis in mice of group 3, where pentoxifylline was given prior to LPS, undoubtedly showed statistically insignificant raise of both at 17 hours. Our study also showed that animals receiving Pentoxifylline after LPS administration in group 4 results in statistically inconsequential rise of serum ALT and AST

at 17 hours. Serum ALT levels altered from 97.83IU/L±1.14 at 0 hour to 90.67IU/L±8.43 at 17 hours, an inconsiderable (p value=0.369) change. Serum AST also did not escalate significantly at 17 hours, as paired student t-test yielded insignificant p value (Figure 3 and 4). 3.2

Histopathology analysis

Hepatic triad consisting of portal vein, a branch of hepatic artery along with bile duct and also hepatocytes cord, separated by sinusoids radiating from central venule were exhibited during light microscopy of group 1 liver sections slides (Figure 5). All slides were graded as normal according to Ishak’s criteria. Mice of group 2 became sluggish, inert, prostrated and weakened with shivering and piloerection visible soon after LPS administration. LPS resulted in severe hepatotoxicity as marked inflammatory changes were revealed in almost all liver section slides that were accordingly graded to have moderate to marked inflammation ; again, a statistically compelling difference (p ≤0.01) from group 1 (Figure 6).

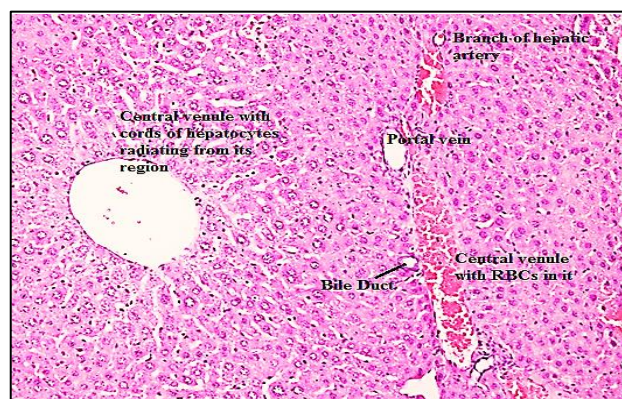
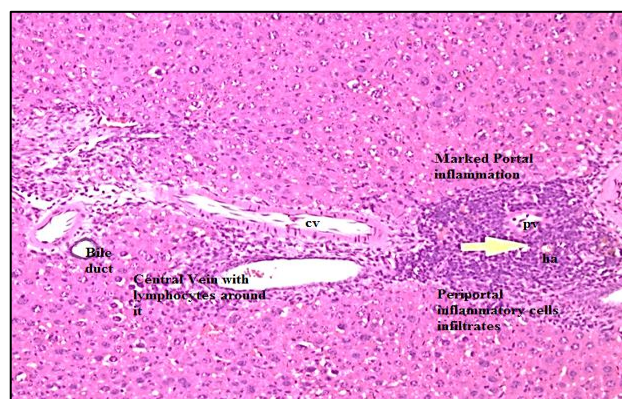


Figure 5: Group 1- normal histology of mouse liver at 20x magnification.

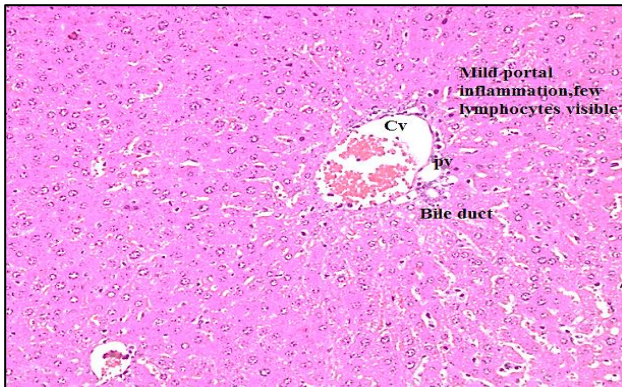


Cv-central vein, Pv-portal vein and Ha- hepatic artery.

Figure 6: Group 2-LPS treated mouse liver histology at 20x .

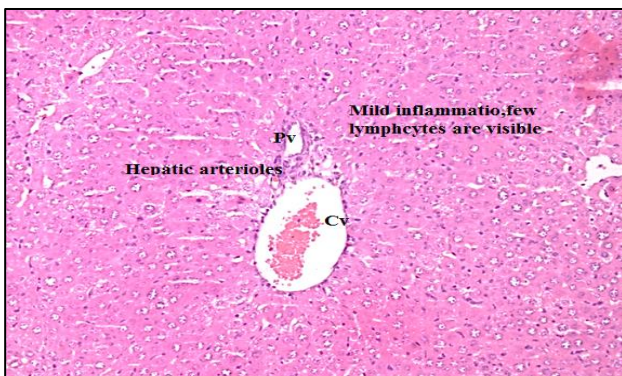
Pentoxifylline administration, before LPS is given, contracted histological distortion. Portal and sinusoidal

inflammatory cell infiltration, cellular ballooning and necrosis was alleviated by pentoxifylline pre-treatment in animals of group 3 (Figure 7). Pearson chi square test between group 2 and 3 yielded a statistically significant p value of ≤ 0.05 . Pentoxifylline after treatment also abolished LPS induced liver dystrophy and all H&E stained slides had minimal inflammation as observed under light microscopy (Figure 8). All six slides were assorted to have minimal inflammation according to Ishak criteria and generated a statistically considerable p value of ≤ 0.05 with Pearson chi square analysis amid group 2 and group 4.



Pv-portal vein, Cv central vein and Ha-hepatic arteriole.

Figure 7: Group 5 (pentoxifylline pretreatment) mouse liver histology at 20x (above) and 40x (below).



Pv-portal vein, Cv central vein and Ha-hepatic arteriole.

Figure 8: Group 6 (pentoxifylline post-treatment) mouse liver histology at 20X.

DISCUSSION

Acting as a major exterminator, especially in elderly population in our part of world, sepsis is a dysfunctional organ system associated with metabolic abnormalities. This bizarre host immune response is mostly seen with Gram negative bacteria liable for 30-50% of cases due to high predominance of genitourinary infection in our area.²⁹ To due promising and efficient liver role in endotoxin detoxification, and protein synthesis for immunological and coagulatory function; its malfunctioning in sepsis is thought to commence and aggravate other organ failure.⁹

Despite the enormous mortality load, treatment guidelines to date include early resuscitation, source control, intravenous fluids and antibiotics only. Expansion and evolution of current therapies in the need of time.

White albino mice were favored in present study as they have manageable housing and maneuvering, inherent defiance to endotoxin and being a non-companion species, their use in survival studies more acceptable and ethical.³⁰ Serum ALT and AST estimation to date is a gold mark for hepatic function evaluation and numerous studies categorized ISHAK /KNODEL'S to be finest hepatic histomorphological alteration scoring system, not only in man but also in mouse.^{31,32} With this support we decided to go for serum ALT and AST along with histopathological analysis of liver section for determination liver dysfunction induced by LPS/endotoxin.

Chemical shock mode was reproduced in our laboratory with intraperitoneal administration of LPS, the functional part of endotoxin as documented by other study, animal of this group became sluggish, prostrated and had considerable hepatic dysfunction evident from statistically considerably elevated serum ALT and AST levels and marked inflammatory changes on liver sections.³³⁻³⁵

Pentoxifylline is one of the most potent xanthine derivatives in suppression of TNF- α production and PDE inhibition; dose required is much lower than that of theophylline.³⁶ Authors decided to use this non-specific phosphodiesterase inhibitors as it has been investigated in many inflammatory disorders including sepsis and maintains promising results.

Accordant to previous studies, our data strongly suggests that pentoxifylline (PTX) holds preventive role in LPS mediated hepatic damage. Serum ALT and AST levels of animals of group 5 (pentoxifylline pre-treatment group) raised insignificantly ($p=0.788$ and $p=0.113$ respectively) during 17 hours of experimental period. Alternatively, statistically considerable difference ($p \leq 0.01$) resulted when serum ALT and AST levels at 17 hours of group 5 were compared with those of group 2 (LPS group). Pentoxifylline administration, before LPS is given, also contracted histological distortion. Portal and sinusoidal inflammatory cell infiltration, cellular ballooning and necrosis was alleviated by pentoxifylline pre-treatment in animals of group 5.

The outcome of study carried out by Coimbra et al, was in harmony with present study. They demonstrated that Pentoxifylline pre-treatment declined ALT, AST, TNF- α , IL-6 concentrations and compressed histological liver injury score.³⁷ Same group of researchers also concluded that pentoxifylline through non-specific phosphodiesterases inhibition declined transcription factor NF- κ B activity with resultant reduction of endotoxin induced lung injury.³⁸

Wang et al, demonstrated pentoxifylline nephroprotective role in acute endotoxemia via down-regulation of TNF- α , IL-1 β , iNOS and endothelial adhesion molecules.²³ Pentoxifylline also affords protection against LPS potentiated allyl alcohol induced hepatotoxicity. Sneed et al, testified that pre-treatment of pentoxifylline prevented liver injury caused by co-treatment of rats by LPS and allyl alcohol, although this is due to effect apart from pentoxifylline mediated decline of serum TNF- α .²¹

Similar deduction was drawn from orient study data when the same agent that is pentoxifylline, was given twice after sepsis induction in group 6 (pentoxifylline post-treatment group).

Markers of liver injury, ALT and AST, were not risen sharply (p=0.369 and p=0.147 respectively) at 17 hours when compared to readings at 0 hours. Nevertheless, serum aminotransferases values at 17 hours of group 6 and group 2 (LPS group) clearly indicated pentoxifylline therapeutic capability in sepsis induced liver dysfunction. Pentoxifylline restored liver function and reduced the degree of inflammation in the liver when it was given after LPS injection (p \leq 0.01) as demonstrated by group 6 animals' liver section histological examination.

Backing up present study results were the study's conclusion of Oliveira-Junior et al, who showed pentoxifylline's protective effect in acute lung injury after intraperitoneal administration of LPS. Pentoxifylline successfully reversed bronchoalveolar lavage neutrophil count and total MDA concentration along serum TNF- α levels.²² Pentoxifylline administration after sepsis has been proved to restore distorted hepatic blood flow, oxygen delivery and consumption, serum ALT and lactate levels and hepatocyte necrosis. Another beneficial effect of pentoxifylline is augmented *E. coli* clearance from blood followed by reduced bacterial colonization of liver in endotoxemia. This effect was observed with pentoxifylline concomitant pre and post-treatment of septic animals.³⁹ However, Azevedo et al, negated our study results by exhibiting lack of preventive and therapeutic ability of pentoxifylline in sepsis induced liver injury validated by insignificant reduction serum ALT and AST. This discrepancy may be the result of lower dose (40mg/kg i.p) utilization by Azevedo as compared to ours (75mg/kg i.p).⁴⁰

CONCLUSION

Intraperitoneal administration of 10 mg/kg Lipopolysaccharide of serotype *E. coli* in mice produced a good model to study sepsis induced liver failure. Pentoxifylline assumedly through cAMP inhibition with resultant TNF- α decline has potential part in LPS/Endotoxin induced hepatotoxicity.

This study has proved that Pentoxifylline, given before or after LPS, protected white type albino mice from LPS induced hepatic damage. However future studies on

pentoxifylline role in surgical and polymicrobial model of sepsis should be conducted along with molecular mechanism of action of pentoxifylline.

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Khan A, Afzal A, Butt HA, Waheed A. Adequacy of phosphodiesterase inhibitor in prevention and treatment of LPS induced organ failure in BALB/c mice. *Int J Basic Clin Pharmacol* 2019;8:549-56.