Original Article

Efficacy of Melatonin and Pentoxifylline combination therapy in treatment of endotoxin-induced hepatic dysfunction in white albino mice

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Abstract

Introduction: Despite major expansion and elaboration in treatment protocols for septic patients, the mortality rate is still very high due to multiple organ damage including hepatotoxicity. This study evaluated the role of two strong anti-inflammatory agents, melatonin and pentoxifylline, as combined treatment in lipopolysaccharide-induced hepatic dysfunction in white albino mice.

Materials and Methods: Endotoxemia was reproduced in white albino mice through intraperitoneal administration of lipopolysaccharide (LPS) of serotype E.Coli. Therapeutic potential of both melatonin and pentoxifylline alone and as combined therapy was adjudged by administering agents 2 hours after LPS delivering. The extent of liver damage was evaluated via serum alanine aminotransferases (ALT) and aspartate aminotransferase (AST) estimation along with a histopathological examination of liver tissue.

Results: Lipopolysaccharide administration (Group 2) resulted in marked hepatotoxicity as evident by statistically raised serum ALT (($p \leq 0.01$) and AST ($p \leq 0.01$) at the end of experimentation. Also, a liver cross-section examination showed marked distortion of liver parenchyma. Melatonin (Group 3) was prosperous in the aversion of LPS invoked hepatotoxicity as proved by lessening of augmented ALT ($p \leq 0.01$) and AST ($p \leq 0.01$) along with restoration of pathological changes on liver sections ($p \leq 0.05$). Pentoxifylline generated similar results and serum ALT, AST and histological alteration abated considerably ($p \leq 0.05$). Combination therapy in animals of Group 5 also tapered LPS evoked hepatic dysfunction statistically considerably.

Conclusion: Melatonin and pentoxifylline alone and as a combination therapy as effective in countering LPS induced hepatotoxicity. However, the combination therapy did not yield synergistic effects.

Keywords: Lipopolysaccharides, Endotoxin, Hepatotoxicity, Melatonin, Pentoxifylline**.**

Introduction

Sepsis resulting in distant organ failure is one of the leading causes of mortality all around the world, with gram-positive and gram-negative organisms isolated in almost equal amounts from all patients.[1-3] The eventual extinction of sepsis results from multiple organ failures, dawning at a single organ and then spreading to many. Multiple factors are implicated in the development of organ failure including hepatotoxicities such as distorted immune reaction, endothelial dysfunction, microvascular leakage, and cellular metabolic dysfunction.[4]

The Liver where serve as an important site for immune and inflammatory response, there also serve as a front line of defense against circulating endotoxins, antigens, and microorganism. Hepatic sinusoids play an essential role in these immune-mediated reactions with all cells including Kupffer cells, hepatic stellate cells, endothelial cells, and immune cells, playing their part. Lipopolysaccharide Binding Protein is formed by the liver that binds bacterial endotoxin /LPS in hepatic sinusoids and catalyzes its transfer to membrane CD14 receptor with resultant activation of Toll-like receptors (TLR4) on Kupffer cells. This produces inflammatory mediators such as interferons (IFNs), IL-1β, IL-8, and TNF. Additionally, neutrophils release reactive oxygen species to produce oxidative stress.[5]

Despite the immense work done in the laboratory and clinical trials, the treatment of sepsis and sepsis-induced organ failure includes fluid resuscitation, infection control, and cardiac support. Newer treatment strategies are the need time to reduce sepsis-induced mortality.

Melatonin, derived from amino acid tryptophan, performs multiple physiological and pharmacological actions through two G–protein-coupled receptors MT1 and MT2 receptors. Their distribution is related to precise biologic functions within the complexity of central nervous system signaling.[6] Multitude of actions contributes to melatonin efficacy in reducing oxidative stress in immune-mediated reactions. Studies have documented that Melatonin scavenges reactive oxygen species (ROS) and reactive nitrogen species (RNS) very efficiently and even at very low concentrations through a process termed as "free radical scavenging cascade".[7] It has been proved that at higher concentrations Melatonin protects from oxidative stress-induced cellular injury in sepsis by replenishing glutathione and other antioxidant enzymes and protects organs from LPS induced dysfunction.[8,9] Melatonin also holds an anti-inflammatory role through down-regulation of NF-κB, modulation of TLR4 function, and inhibition of apoptosis.[10-13]

Pentoxifylline (PTX) is a phosphodiesterase inhibitor and adenosine receptor agonist/antagonist. It functions in diverse cellular functions by increasing the intracellular concentration of cAMP and cGMP. Worth mentioning are inhibition of PDEs 4 type expressed on inflammatory cells line and PDE7 that causes T-cell activation. 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.[14] It possesses strong anti-inflammatory effects as it downregulates TLR4 signaling in monocytes. This results in diminishing LPS-induced interleukin (IL)-1β, IL-6, and TNF-α levels.[15,16]

In this study, we aim to comprehend if the combined treatment of Melatonin and Pentoxifylline is more beneficial in LPS induce hepatic dysfunction than the individual drug therapy in white albino mice.

Aims and Objectives:

In the present study, we aim to evaluate the effectiveness of Melatonin and Pentoxifylline combination therapy in the treatment of lipopolysaccharide (LPS) induced hepatic dysfunction.

Our objectives are as follows:

1. To reproduce LPS induced hepatic dysfunction model in white albino mice.
2. To evaluate the individual role of melatonin and pentoxifylline in LPS induced hepatoxicity in our study animals
3. To evaluate combined melatonin and pentoxifylline effectiveness in LPS induced hepatoxicity
4. To compare individual drug roles with combination therapy in LPS induced hepatoxicity

Materials and Methods

This was an experimental study carried out in the Department of Pharmacology and Therapeutics, Army Medical College, NUST, Rawalpindi after ethical approval from the Ethical committee of "Centre of Research in Experimental and Applied Medicine (CREAM)" Army Medical College.

White albino mice of both genders were divided randomly into five groups. Each group, having at least six animals, was housed in the traditional wire-topped cages with the temperature kept at 20-22°C and humidity maintained at 40-70%. Contamination-free

fresh water and a nutritionally adequate rodent pellet diet (containing 4-7% fat and 11-15% protein) were provided during the entire time period.

Lipopolysaccharide, Pentoxifylline, and Melatonin were all purchased from Sigma Aldrich chemicals, USA, and stored according to the given protocols. A pilot project was run to devise an appropriate septic model for our animals. The dose and time duration after LPS administration were explored and after three weeks, the appropriate and satisfactory hepatotoxic model was reproduced with 10 mg/kg of body weight as dose and 17 hours time interval between LPS administration and terminal sacrifice. Furthermore, the 2 hour time interval between LPS and drug administration was chosen on basis of previous studies which indicate that inflammatory cytokine levels peak after 1 hour of LPS administration.[17] Melatonin and pentoxifylline were both dosed twice after LPS administration due to rapid elimination kinetics.

Experimental outline:

Sr. no.	*Group name*	*Treatment received*
1.	Group 1(control group)	Single intraperitoneal injection of normal saline.
2.	Group 2 (LPS group)	Single intraperitoneal injection of lipopolysaccharide (LPS)-10mg/kg of body weight.
3.	Group 3 (Melatonin group)	In a separate set of 6 animals, melatonin was given intraperitoneally in a double dose of 10 mg/kg of body weight, initially two hours after injecting LPS and a second dose after an additional one hour.
4.	Group 4 (Pentoxifylline group)	The first dose of 75 mg/kg of body weight was injected two hours after LPS and the subsequent dose was given 5 hours later the first dose intraperitoneally.
5.	Group 5 (Melatonin +Pentoxifylline group)	Both agents at the same time, again 2 hours after LPS injection, followed by melatonin's second dose (after 1 hour of 1st dose) and pentoxifylline next dose (additional 5 hours after 1st dose).

Blood sample and tissue collection: Primary blood sampling in all animals was done from the lateral tail vein of a mouse according to protocols approved by Institutional Animal Care and Use Committee (IACUC).[19] Terminal sampling was done by cardiac puncture. All blood samples were dispensed into an Eppendorf tube followed by centrifugation and chemical analysis for serum ALT and AST levels. This was followed by terminal anesthesia using the Drop jar method utilizing 20% v/v isoflurane in propylene glycol solution soaked cotton gauze. After midline laparotomy, the liver was removed, washed off excess blood, and immediately fixed in 10% neutral buffered formalin in a plastic container labeled with group name and treatment received. Tissue blocks, placed in labeled tissue cassettes were processed in LEICA TP 1020 Automatic Tissue Processor. We decided to use Ishak Modified Histological Activity Index to grade histopathological changes in the liver as recommended by several studies.[19]

Statistical analysis

Data were entered in the statistical package for social sciences (SPSS) version 20. The results of the serum analysis were expressed as Means + Standard Error of Means. The statistical significance of differences between means was analyzed with a one-way analysis of variance (ANOVA) followed by a Post-hoc Tukey test. Statistical differences between serum markers at initial and final hours were found using Paired T-test. An Independent/unpaired T-test was utilized to access the difference in serum markers at 17 hours in the LPS group with another. The result of histopathology was analyzed using the "Chi-square test". All values were considered statistically significant if the "p-value" was equal to or less than 0.05.

Results

Chemical Parameters Analysis:

Chemical parameter analysis in animals of Group 1(control) resulted in a statistically nonsignificant p-value of 0.276 for serum ALT (Figure 1) and 0.137 (Figure 2). for serum AST. Histopathological analysis showed normal lobular organization of mouse liver. Hence these animals served our control group (Figure 3).

LPS administration in mice of Group 2 produced marked hepatotoxicity as evident by escalated serum ALT and AST levels and clear marked inflammatory changes in liver cross-section examination. Serum ALT and AST levels at 17 hours were statistically significant when compared to levels at 0 hours yielding a significant p-value of ≤0.01 each (Figures 1 & 2). Histological Examination revealed Moderate inflammatory cells infiltrated almost all portal areas, apoptotic changes in cells, and mild periportal inflammation in a few portal areas were predominant inflammatory changes (Figure 3). Four slides were classified as moderate inflammation, one slide showed

severe necrotic changes and the remaining one had minimal inflammatory changes (Figure 3).

Melatonin administration after LPS in animals of Group 3 protected them from the development of hepatotoxicity. Serum ALT levels didn't rise significantly at 17 hours yielding a two-tailed significance p-value of 0.262 (Figure 1).

Similar results were seen with serum AST levels with a p-value of 0.117 (Figure 2). Light microscopy of liver tissue also revealed minimal to moderate inflammation showing melatonin's protective role in LPS induced hepatotoxicity (Figure 3).

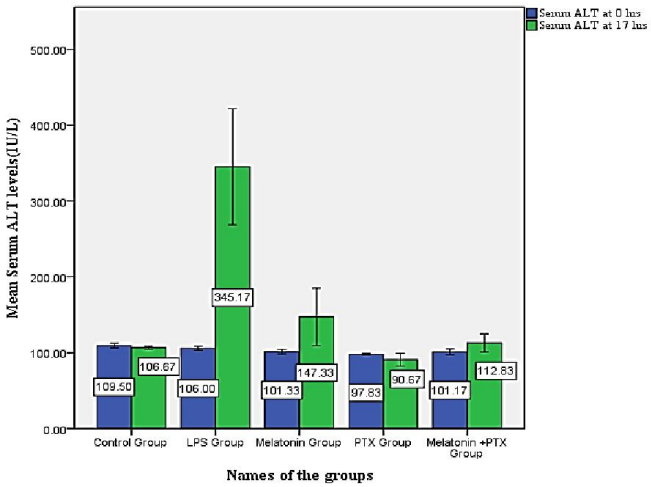

Figure 1: Comparison of serum ALT mean (IU/L) of each group at the start (0 hour) and end of the experiment (17 hour). There is no statistically significant disparity in reading except for group 2 (LPS group), p value is ≤ 0.001.

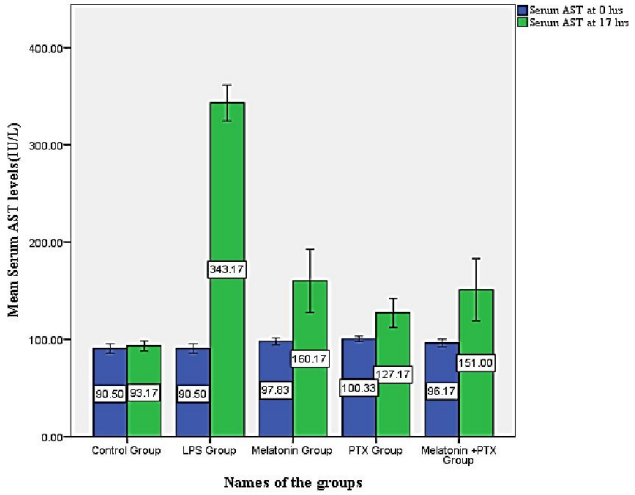

Figure 2: Comparison of serum AST mean (IU/L) of each group at the start (0 hour) and end of the experiment (17 hour). There is no statistically significant disparity in reading except for group 2 (LPS group), the p-value is ≤ 0.001.

Likewise, Pentoxifylline administration after LPS in Group 5 resulted in an insignificant p-value of 0.369 for serum ALT and a p-value of 0.147 for serum AST levels through paired sample t-test. A liver cross-section examination indicated similar results where all six slides were labelled to have minimal inflammation according to Ishak criteria (Figure 3).

Eventually coming to our last Group 5 (Pentoxifylline + Melatonin combination), the difference between ALT (0.280) and AST (0.112) levels at the start and end of the experimental study was, like other groups, insignificant (Figure 1,2). Slight inflammatory changes were seen in all slides under light microscopy. Lymphocytic infiltrates were visible around portal areas and in hepatic sinusoids. Every slide was ranked as minimal inflammation (Figure 3).

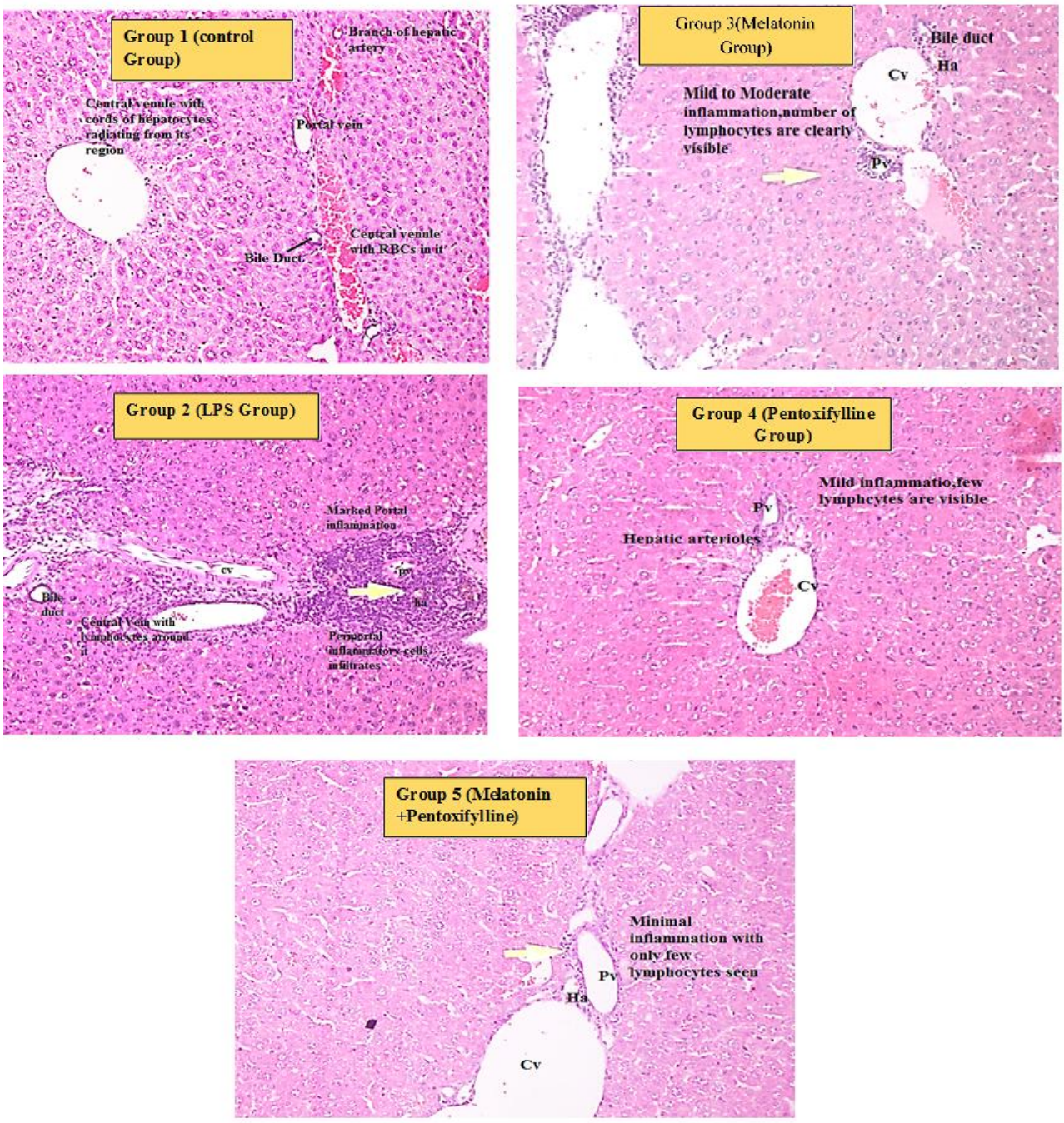

Figure 3: Mouse Liver Histology at 20X of all Groups (labelled). Pv-Portal Vein, Cv Centarl Vein And Ha-Hepatic Arteriole

The one-way analysis of variance (ANOVA) was carried out to determine whether there are any statistically significant differences between the means serum ALT and AST levels of our study groups. There was a statistically significant difference between groups as indicated by results for serum ALT levels at 17 hours (F (4,25) =7.388, p = .000) and for Serum AST levels at 17 hours (F(4,25) =17.807, p = .000). This was followed by a post hoc Tukey test to determine whether significant differences occur between which groups. The results demonstrated that a significant difference occurred in serum ALT levels at 17 hours between Group 2(LPS) and Group 1(control), Group 3(Melatonin), Group 4(Pentoxifylline), and Group

5(Melatonin and Pentoxifylline) yielding a statistically significant p-value (Table 1 & 2). However our combination treatment Group 5 (Melatonin +Pentoxifylline) demonstrated no added benefit with an insignificant p-value when compared to individual treatment groups 3 and 4.

Table 1: Post hoc Tukey Comparisons of mean serum ALT levels (IU/L) at 17 hours of each group

Groups	*Mean Serum ALT(IU/L) at 17 hrs*	*SEM*	*Tukey's HSD Comparisons(Sig.)*				
			Group1 (control)	Group2 (LPS)	Group3 (MELA)	Group4 (PTX)	Group5 (MELA+PTX)
Group 1 (Control)	106.67	±2.23		0.002	0.944	0.998	1.000
Group 2 (LPS)	345.17	±76.5	0.002		0.011	0.001	0.002
Group 3 (MELA)	147.33	±37.81	0.944	0.011		0.837	0.969
Group 4 (PTX)	90.67	±8.43	0.998	0.001	0.837		0.994
Group 5 (MELA+PTX)	112.83	±28.92	1.000	0.002	0.969	0.994	

Table 1: Post hoc Tukey Comparisons of mean serum AST levels (IU/L) at 17 hours of each group

Groups	*Mean Serum ALT(IU/L) at 17 hrs*	*SEM*	*Tukey's HSD Comparisons(Sig.)*				
			Group 1 (Control)	Group 2 (LPS)	Group 3 (MELA)	Group 4 (PTX)	Group 5 (MELA+PTX)
Group 1 (Control)	93.16	±5.0		<0.001	.273	.834	.413
Group 2 (LPS)	343.2	±18.5	<0.001		<0.001	<0.001	<0.001
Group 3 (MELA)	160.17	±32.54	0.273	<0.001		0.849	0.999
Group 4 (PTX)	127.2	±14.9	0.834	<0.001	0.849		0.948
Group 5 (MELA+PTX)	151.0	±31.98	.413	<0.001	.999	.948	

Discussion

Our study clearly demonstrates that in LPS induced hepatotoxicity, Melatonin and Pentoxifylline administration proved beneficial as evidenced by significantly reduced serum ALT and AST levels and minimal inflammation on liver cross-sections histology. However, the combined treatment with Melatonin and Pentoxifylline did not result in added benefits.

Despite the efforts of Surviving Sepsis of setting treatment protocols, sepsis with organ dysfunction as hepatotoxicity is a primary health hazard in developing countries like Pakistan inflicting especially among the elderly population. Due to the liver's considerable role in the immune system; it's malfunctioning in sepsis commences and exacerbates other organ failures.

Gram negative's outer cell membrane molecule called endotoxin or Lipopolysaccharide (LPS) receptors in combination with acute phase reactant protein LPS-binding protein, interact with the CD4 receptor and Toll-like receptor to stimulate host innate immune response through activation of kinases cascade with phosphorylation of series of adaptor proteins. Nuclear factor Kappa B (Nf-κB) translocates to the nucleus causing inflammatory gene transcription like cytokines (TNF-alpha, ILs), adhesion molecules, acute phase reactant proteins, and enzymes which produce mitochondrial dysfunction through reactive oxygen and nitrogen species. This leads to hypotension, fever, sepsis-like syndrome, and shock.[20]

Melatonin, an indole hormone of the pineal gland, has well-established anti-oxidant properties accounting for its beneficial role in sepsis-induced multiple organ failure. Melatonin has radical scavenging capacity. Melatonin also enhances the organism's capacity to protect itself from oxidative damage by up-regulating the activity and synthesis of important antioxidant enzymes like Glutathione peroxidase and glutathione reductase. Superoxide dismutase (SOD) and catalase (CAT) activity is also potentiated by melatonin by virtue of membrane receptors. Similar to our study, other researchers have documented the beneficial role of melatonin in sepsis-induced hepatotoxicity via reduction of inflammatory mediators TNF-α, IL-12, IL-6, IL-8, and anti-apoptotic activity via escalation of IL-10.[8,13,21,22] In line with our data, studies have documented that melatonin has improved survival, reduced recovery time and protected animals from LPS induced inflammatory changes.[23] Melatonin administration in septic rats improved insulin resistance, reduced LPS induced elevated serum ALT and AST levels, and increased SIRT1 and STAT3 with reduced mortality.[24]

Many clinical trials and laboratory animal studies have substantiated the remedial role of pentoxifylline in inflammatory diseases and sepsis. Supporting our study results, it is demonstrated that Pentoxifylline's beneficial effects are attributed to the reduction of LPS induced production of TNF-α followed by a decline in IL-1β, IL-12, IL-8, and inducible nitric oxide synthase production with up-gradation of an anti-inflammatory circle through IL-10. Supporting our results, another study demonstrated that PTX induced anti-inflammatory cytokine IL-10 and protected from sepsis-induced organ failure.[25] It has also proved effective in reducing inflammatory cytokines through Toll-like receptors and holds a promising role as a neonatal anti-inflammatory agent.[26] Pentoxifylline abated thioacetamide-induced liver damage in rats as evident by declined serum transaminase levels and improved histopathological changes on liver cross-sections. These findings support our study results. This could be achieved through inhibition of the oxidative stress activation and reduction in NF-κB with pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6)[27].

Despite their distinctive mechanism of action, Pentoxifylline and melatonin both failed to produce additive effects in animals of Group 5. However, this combination was put to test by Zaitone et al with pioglitazone for a non-alcoholic fatty liver disease where they proved that these agents in combination can be used therapeutically for the above-mentioned liver disorder .[28] Also Noyan deduced from his study results that melatonin and pentoxifylline simultaneous dosing resisted carbon tetrachloride caused the liver malfunction.[29]

We appreciate our study constraints. Although the endotoxemia model used in our study is popular and sterile but it is associated with rapid progression of sepsis in rodents, as opposed to a slow disease process in humans. Also, therapies proven beneficial in LPS models not always turn out in the same manner when tested in humans. But still, animal models are only tools to access the role of new therapies that can own power and adequacy for medical world application.

Conclusion

Melatonin and pentoxifylline alone and in combination therapy as effective in countering LPS induced hepatotoxicity. However, the combination therapy did not yield synergistic effects.

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