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Bronco T (Shirisadi kasaya), a polyherbal formulation prevents LPS induced septicemia in rats

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Abstract. – OBJECTIVE: Here, Bronco T (BT), a polyherbal formulation developed in 1984 for treating asthma, has been repurposed against septicemia-induced ALI.

MATERIALS AND METHODS: Lipopolysaccharides (3 mg/kg BW) were injected intraperitoneally before 24 hours of surgery to assess the cardiorespiratory parameters, blood PaO_2/FiO_2 and MPO, pulmonary water content and histological changes in the lungs. The pentoxifylline (PTX) (25 mg/kg BW) was used as the positive control and given one hour before LPS. BT was given 3 hours (orally at different doses of 3, 1.5 and 0.75 g/kg BW) before LPS.

RESULTS: The LPS treated group showed significant bradypnea, hypotension and bradycardia, through elongated peaks (RR) and (MAP) respectively and finally death after 95 minutes of LPS injection. The PTX and BT (3 g/kg BW) pretreatment significantly prevented these changes (dose-dependent in the BT group). The survival in these groups was maintained up to 190 min after LPS. The Pentoxifylline showed a better response (75%) than Bronco T (72%). In both the treatments, a significant decrease in pulmonary water content and minimal neutrophil infiltration and intact alveoli-capillary membrane was seen in the transverse section (T.S) of the lungs.

CONCLUSIONS: Significant improvement was noted in survival time with lesser tissue damage and improved pulmonary function was observed by pre-treating with Bronco T in LPS induced septicemia.

Key Words:
Septicemia, ARDS, LPS, Pentoxifylline, Bronco T, Cardiorespiratory, Herbal formulation.

Abbreviations

ALI: Acute lung injury; ARDS: Acute respiratory distress syndrome; BT: Bronco T; BW: Body weight; HR: Heart rate; LPS: Lipopolysaccharides, MAP: Mean atyerial pressure; MPO: Myeloperoxidase; PaO_2/FiO_2: Arterial oxygen partial pressure/Fractional inspired oxygen partial pressure; RR: Respiration rate.

Introduction

Uncontrolled infection results in septicemia which induces ARDS involving cardiorespiratory failure and hypoxemia[1-3]. The increased inflammation and oxidative stress provokes physiological and anatomical changes[4]. The mortality rate due to ARDS is high despite the current therapeutic protocol. This needs to be addressed by target-based newer therapies. The pentoxifylline, a standard drug for septicemia has been used as a positive control. It is a competitive nonselective phosphodiesterase inhibitor[5]. Reduced surfactant production by pneumocytes, formation of hyaline membrane, mixed infiltration of inflammatory cells in the interstitium, alveoli, and perivascular areas are key histological observations in these patients[6].

Here we have used lipopolysaccharide (LPS) induced septicemia which acts through TLR4 receptors and induces pro-inflammatory cytokines like IL6 and TNF alpha[7].

The Bronco T is an ayurvedic polyherbal decoction in clinical use since 1984 to treat asthma. It consists of five plants including *Albizia lebbeck, Solanum xanthocarpum, Justicia adhatoda, Glycyrrhiza glabra,* and *Cinnamomum tamala*[8,9].

For the first time, we are repurposing it for sepsis mediated respiratory distress and ARDS which has not been done earlier. In this work, the LPS model of septicemia was used and changes in terms of cardio-respiratory, biochemical and histological parameters were evaluated.

Materials and Methods

Materials

Urethane and LPS (*Escherichia coli* 0111:B4,) were obtained from Sigma-Aldrich, St. Lou-

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is, MO, USA. Trentrol 400 tablet (Pentoxifylline-400 mg, Sanofi) and Bronco-T (Surya Pharmaceuticals, Varanasi) were purchased from the local market.

Methodology

Experimental Protocol was approved by the Ethical Committee of IMS-BHU (Dean/2021/IAEC/2550). The inbreed Charles-Foster rats weighing (175-225 g) were purchased from a central facility, acclimatized for 7 days in our laboratory condition. They were divided into the groups mentioned in Table I. LPS (3 mg/kg body weight) was administered intraperitoneally for 24 hours. Pentoxifylline was pre-administered at the dose of 25 mg/kg body weight (BW) through intraperitoneal route in the rats one hour before LPS administration. The BT decoction was orally given three hours before LPS and after six hours to maintain a steady Cmax of BT. The selected doses were 3, 1.5 and 0.75 g/kg of rat body weight for experimental study[9]. This dose was calculated, based on a human dose i.e., one teaspoon thrice a day for an adult (60 Kg), which corresponds to approximately 3.196 g, which is equivalent to 150 mg/kg of human body weight. The rat dose was 10 times higher than the human dose because rats have a metabolic rate, about ten times higher than humans[10,11].

The dose and duration of treatment were made based on the biological half-life of the active constituents.

After 24 hours, urethane (1.5 g/kg BW i.p.) was given to anaesthetize the animals. The animals were stabilized and dissected out. The trachea, jugular vein and carotid artery were cannulated in the same order to connect the animal to the computerized chart recorder (Lab Chart 7, AD Instrument, Australia). The cardio-respiratory parameter was recorded for the observation period of 190 min. At the end of each experiment, the lungs were excised, and one part of the lung was kept for the estimation of pulmonary water content, MPO and the other part was used for histological examination. Blood from the right internal carotid artery was collected for blood gas analysis.

Protocol for Parameter Estimation

Physiological parameters

Determination of survival time: It was estimated for the observation period of 190 min as the LPS treated group died around 95 min after 24 hours, therefore the rationale for twice the survival time of the LPS group was chosen. Mortality was characterized by flat line response in cardio-respiratory responses (RR, MAP, and HR) as observed from the Lab Chart 7, AD Instrument.

Determination of Respiratory Rate: It was recorded by securing the skin over the xiphisternum with help of a thread and connecting it to the chart recorder (Lab Chart 7, AD Instrument) through a force-displacement transducer.

Determination of Mean Arterial Pressure (MAP): It was estimated from blood pressure, firstly the pressure transducer was calibrated and the carotid artery was connected to it through a three-way stop clock. Blood pressure was recorded by connecting the pressure transducer to a chart recorder (Lab Chart 7, AD Instrument) through a bridge amplifier. MAP was determined by computing the data obtained.

MAP= Diastolic Pressure + 1/3
(Pulse Pressure), where PP is obtained from,
PP= Systolic Pressure-Diastolic Pressure

Determination of Heart Rate (HR): It was manually calculated from R-R interval by recording the electrocardiograph by connecting the needle electrode to the chart recorder (Lab Chart 7, AD Instrument) through bio amplifier using standard limb lead II configuration in the rats.

Determination of PaO_2/FiO_2 ratio: PaO_2/FiO_2 ratio was estimated by ABG analyzer (Roche OMNI

Table I. Division of animals in different groups based on the intervention used.

S.No	Groups	Vehicle	Route
1	Group I (Control)	Normal Saline (75 µl)	Peroral
2	Group II (Lethal)	LPS (3 mg/kg) 24 hours	Intraperitoneal
3	Group III (Positive control)	Pentoxifylline (25 mg/kg BW) i.p. 1 hour before LPS injection.	Intraperitoneal
4	Group IVa (BT-Treatment)	(3 g/kg BW) orally 3 hours before LPS injection and after 6 hours.	Peroral
5	Group IVb (BT-Treatment)	(1.5 g/kg BW) orally 3 hours before LPS injection and after 6 hours.	Peroral
6	Group IVc (BT-Treatment)	(0.75 g/kg BW) orally 3 hours before LPS injection and after 6 hours.	Peroral

Table II. Effect of Bronco T on oxygenation index (PaO_2:FiO_2) of rat in LPS induce septicemia.

S.No	Group	Interventions	PaO_2:FiO_2
1	I	Normal Saline (75 µl) 24 hours	458 +/- 0.2
2	II	LPS (3 mg/kg) 24 hours	274^{****} +/- 0.2
3	III	Pentoxifylline (25 mg/kg BW) + LPS 24hours	$358.6^{\#\#\#\#}$+/- 0.3
4	IV a	BT (3 g/kg BW) + LPS 24hours	$348.7^{\#\#\#\#}$+/- 0.2
5	IV b	BT (1.5 g/kg BW) + LPS 24hours	$312.4^{\#\#\#}$+/- 0.2
6	IV c	BT (0.75 g/kg BW) + LPS 24hours	$298.8^{\#\#}$+/- 0.3

Here data is represented as mean ± SD where***, p<0.001 comparison to Normal saline treated group and $^{\#\#\#\#}$, p< 0.0001, $^{\#\#\#}$, p<0.001 and $^{\#\#}$, p<0.01 in comparison to LPS 3 mg/kg treated group following one-way ANOVA Turkey test.

gas analyzer) in the blood sample from the right internal carotid artery using a heparinized syringe.

Pulmonary parameters

Determination of pulmonary water content: It was determined by the physical method as described earlier[12]. At the end of each experiment, the lungs were excised. One lung was preserved in formalin for histological examination and the other was weighed and dried in an electric oven (at 90ºC for 48 h) to a constant weight. The difference between wet weight and dry weight was calculated to determine the water content.

Pulmonary Water content =
[(Wet lung weight- Dry lung weight)/Wet lung weight]*100

Surface Morphology and Histology of Lungs

Surface morphology was observed and photographed, at the end of the experiment. The lung tissue was washed with ice-cold phosphate buffer saline (PBS) and preserved in a 10% formalin solution. Further, it was subjected to standard histological protocol and 5-micron lung tissue sections were stained with hematoxylin (H) and eosin (E) for microscopic examination (10X, 40 X magnifications)

Myeloperoxidase Level

The MPO level serves as a marker for neutrophilic sneaking at the site of inflammation. The level of myeloperoxidase (MPO) was estimated in lung homogenate in freshly prepared lysis buffer, which was centrifuged for 5 min at 5000 RPM. The supernatant was collected and immediately estimated through the procedure stated by the Real gene rat MPO ELISA kit (Cat No 3100574, Lot No.17517601575) at 435 nm.

Results

A) Effect of Bronco T and PTX in LPS Treated Rats on Physiological Parameters

Bronco T (3 g/kg BW) significantly improved the physiological parameters in LPS treated rats. On observation, rats in Group I moved freely after 24 hours and did not show any sign of respiratory distress. However, LPS treated rats were comparatively less active and showed shortness of breath. BT pretreated rats were significantly in better condition with 100 per cent survival.

Survival time

All the animals in Group-I survived throughout the experimental period (190 min). In LPS treated group-II, the survival time was reduced to 92 min +/- 0.3. Further, in Pentoxifylline (25 mg/kg) treated group-III, survival time was significantly higher than the LPS treated group, but lower than group I. In the BT treated group (3 g/kg BW *vs.* 1.5 g/kg BW *vs.* 0.75 g/kg BW), dose-dependent enhancement in survival time was recorded, however, in group IVc (0.75 g/kg) the survival time was 110 min +/- 0.3 (Figure 1A-F).

Cardiorespiratory parameters

In group I, after 24 hours and surgical stabilization, no significant change was observed in the RR, MAP and HR. However, in group II there was a significant reduction in RR, followed by death after 90 minutes. Interestingly, in group III, the RR was reduced in the beginning but later on maintained throughout the observation period. In the BT (3 g/kg) pretreatment group, RR was maintained to data of group I (normal saline-treated rats) (Figure 2A). A similar observation was noted on HR (Figure 2B) and MAP (Figure 2C).

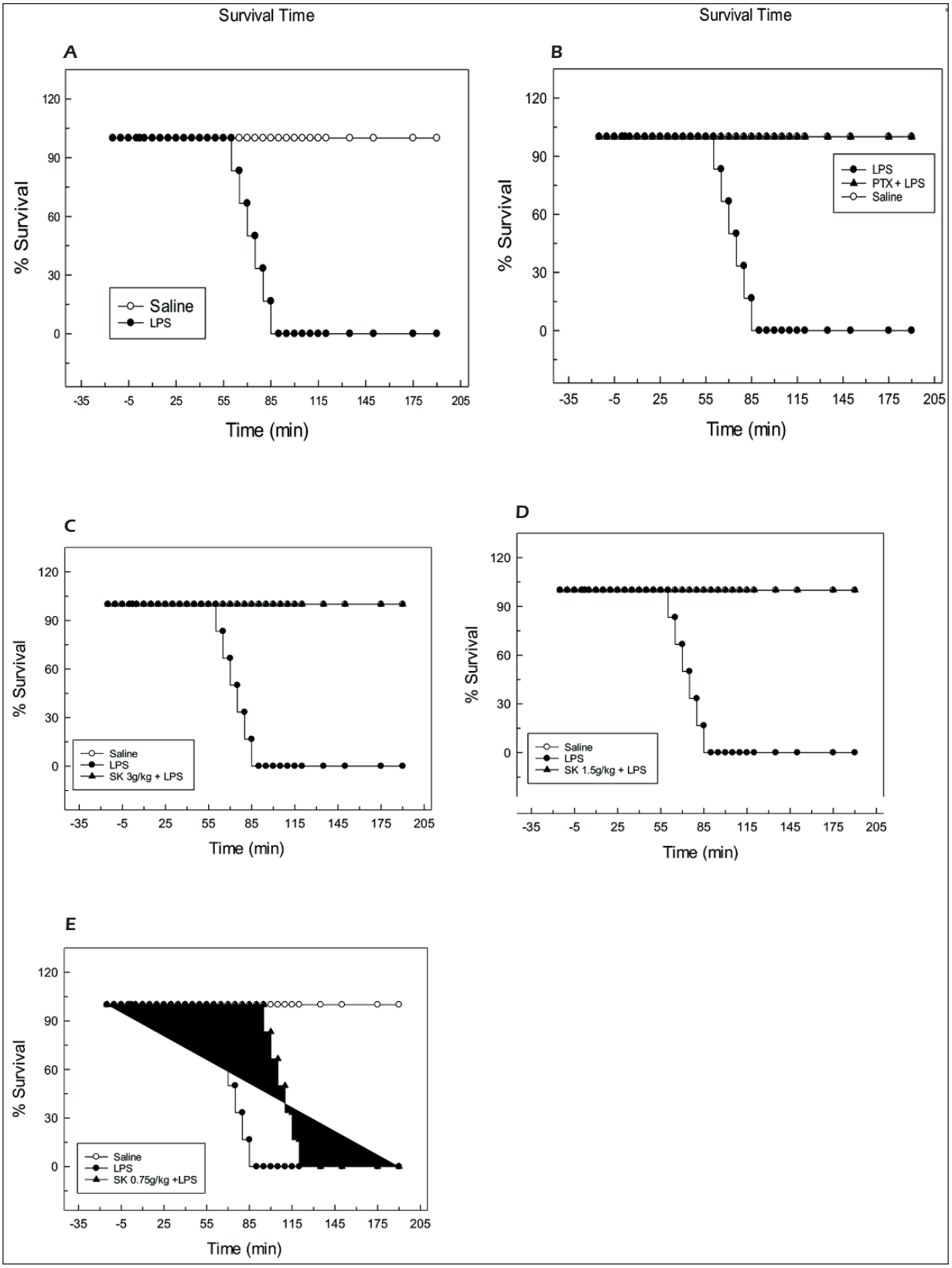

Figure 1. A-E, Survival time comparison in each group as obtained from Kaplan-Meier plot. 1A comparison between normal saline and LPS. 1B, comparison between LPS and PTX.1C-E, denotes comparison between LPS and BT (3, 1.5 and 0.75 g/kg BW).

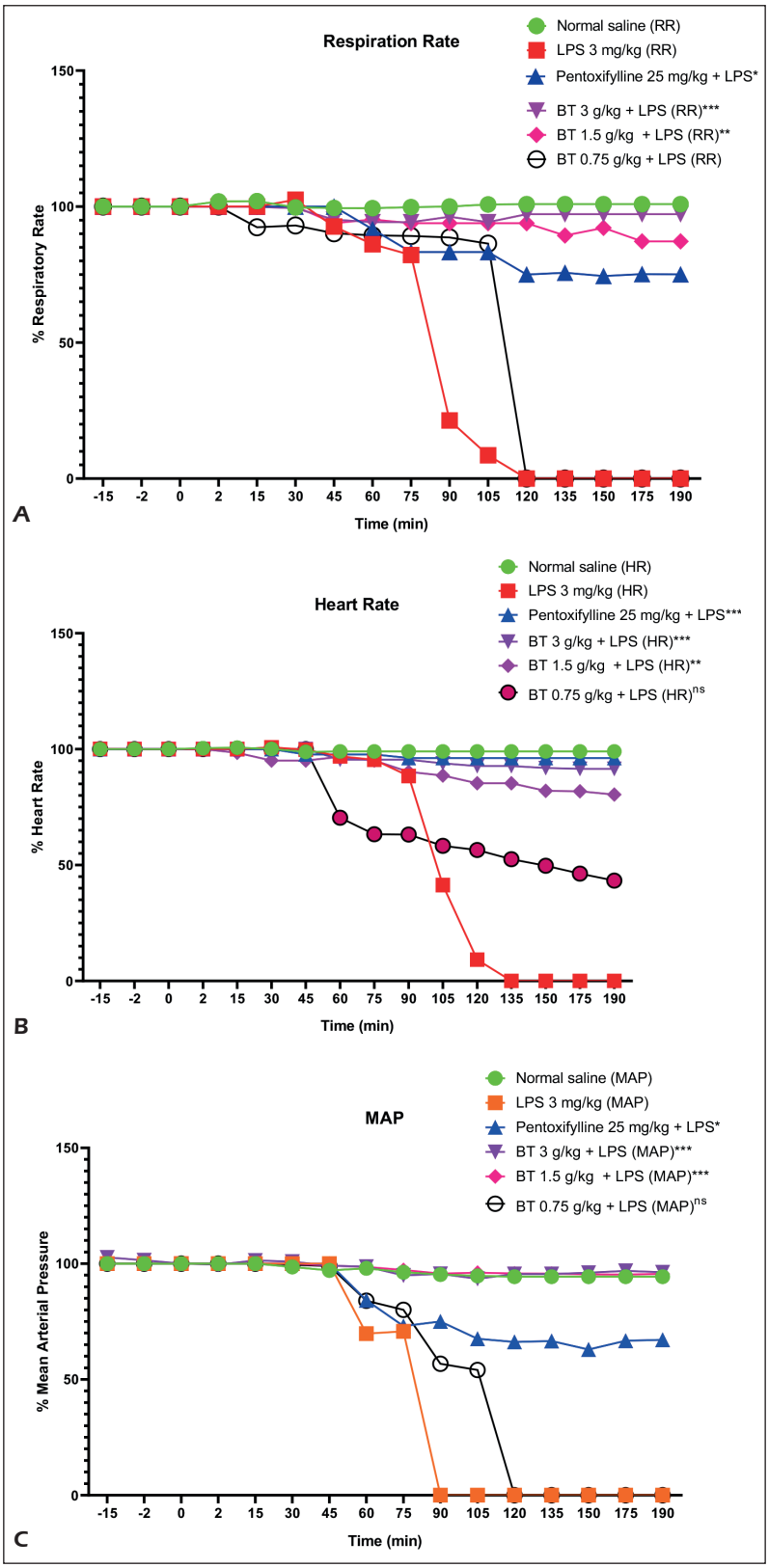

Figure 2. A,. Respiration Rate in each group. LPS treated group suffered bradypnea from starting and represented flat line response after 95 minutes (Red blocks). BT treated group (3 g/kg) showed significant improvement in comparison to PTX treated group. Data represented as percentage initial mean ± SD where $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ in comparison to LPS 3 mg/kg treated group. **B,** Heart Rate in each group. LPS treated group suffered bradycardia from starting and represented flat line response after 120 minutes (Red blocks). BT treated group (3 g/kg) showed significant improvement in comparison to PTX treated group. Data represented as percentage initial mean ± SD where $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ in comparison to LPS 3 mg/kg treated group. **C.** Mean arterial pressure in each group. LPS treated group represented flat line response after 75 minutes (Orange blocks). BT treated group (3 g/kg) showed significant improvement in comparison to PTX treated group. Data re presented as percentage initial mean ± SD where $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ in comparison to LPS 3 mg/kg treated group.

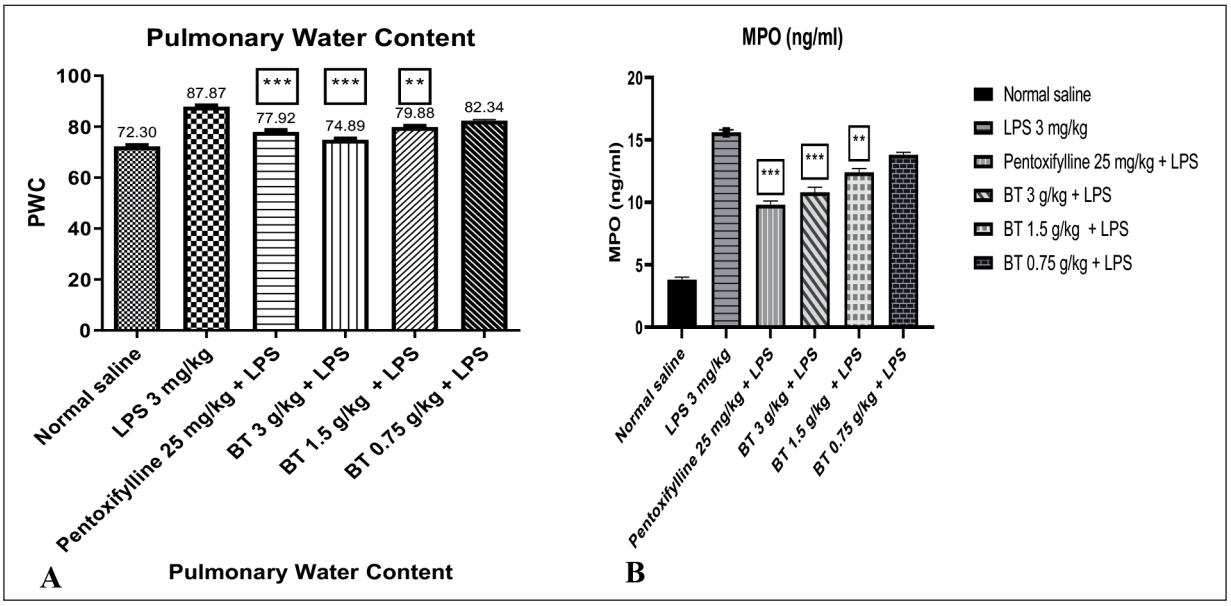

Figure 3. Effect on lung wet to dry ratio **(A)** and MPO **(B)** in each group. Here BT (3 g/kg) showed significant improvement in comparison to PTX treated groups. Data represented as mean ± SD where $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ in comparison to LPS 3 mg/kg treated group following one-way ANOVA Turkey test.

PaO_2:FiO_2 (Oxygenation Index)

The PaO_2:FiO_2 represents the oxygenation capacity of the lungs, which indirectly determines the mortality and directly determine the ARDS incidence. The hypoxemic condition will lead to respiratory distress and multi-organ failure if not treated. As shown in Table II, LPS treated group have PaO_2:FiO_2 less than 300 signifying the acute lung injury condition. This was increased in both PTX and BT (3 g/kg) treated groups suggesting their preventive role against acute lung injury.

Here data is represented as mean ± SD where ***, $p<0.001$ comparison to Normal saline treated group and $^{####}$, $p< 0.0001$, $^{###}$, $p<0.001$ and $^{##}$, $p<0.01$ in comparison to LPS 3 mg/kg treated group following one-way ANOVA Turkey test.

Effect of Bronco T and PTX in LPS Treated Rats on Pulmonary Parameters

Lung Wet to Dry ratio (Pulmonary water content) and Myeloperoxidase level (MPO)

PWC indicates the level of oedema in the pulmonary region, which increases lung stiffness in ALI. The wet to dry ratio for all the treated groups was determined after 24 h following the LPS challenge. A significant increase in lung W/D weight ratio was observed for the LPS group as compared to normal saline. However, BT pre-treatment (3 g/kg) significantly reduced lung W/D weight ratios (n=6, $p<0.001$) as compared to the LPS group. Similarly, the PTX treated group also markedly reduced the wet to dry weight ratio compared to the LPS as shown in the Figure 3A. The MPO level was elevated in the LPS group; however, BT and PTX pretreated group markedly reduce the level of MPO as observed in the Figure 3B.

Surface Morphology

The surface morphology analysis revealed (Figure 4) in group I (A), the tissue was dry and without hyperemia; however, in group II (B), the surface appeared to be dark red to black with a hardened and congested appearance a typical characteristic of ALI. The morphological presentation was better and there was significantly less bleeding in the PTX (group III) (C) and BT (group IV) (D- 3g/kg, E- 1.5g/kg & F- 0.75g/kg BW) groups as observed in Figure 4.

Histological Examination

H&E staining was used to reveal the pathological changes. As shown in Figure 5 (10x), LPS administration was associated with significantly more fluid accumulation and inflammatory cells in the alveolar region. There is thickened alveoli-capillary membrane and hemorrhagic manifestation in this group. In contrast, BT and PTX treated groups showed varying degrees of protec-

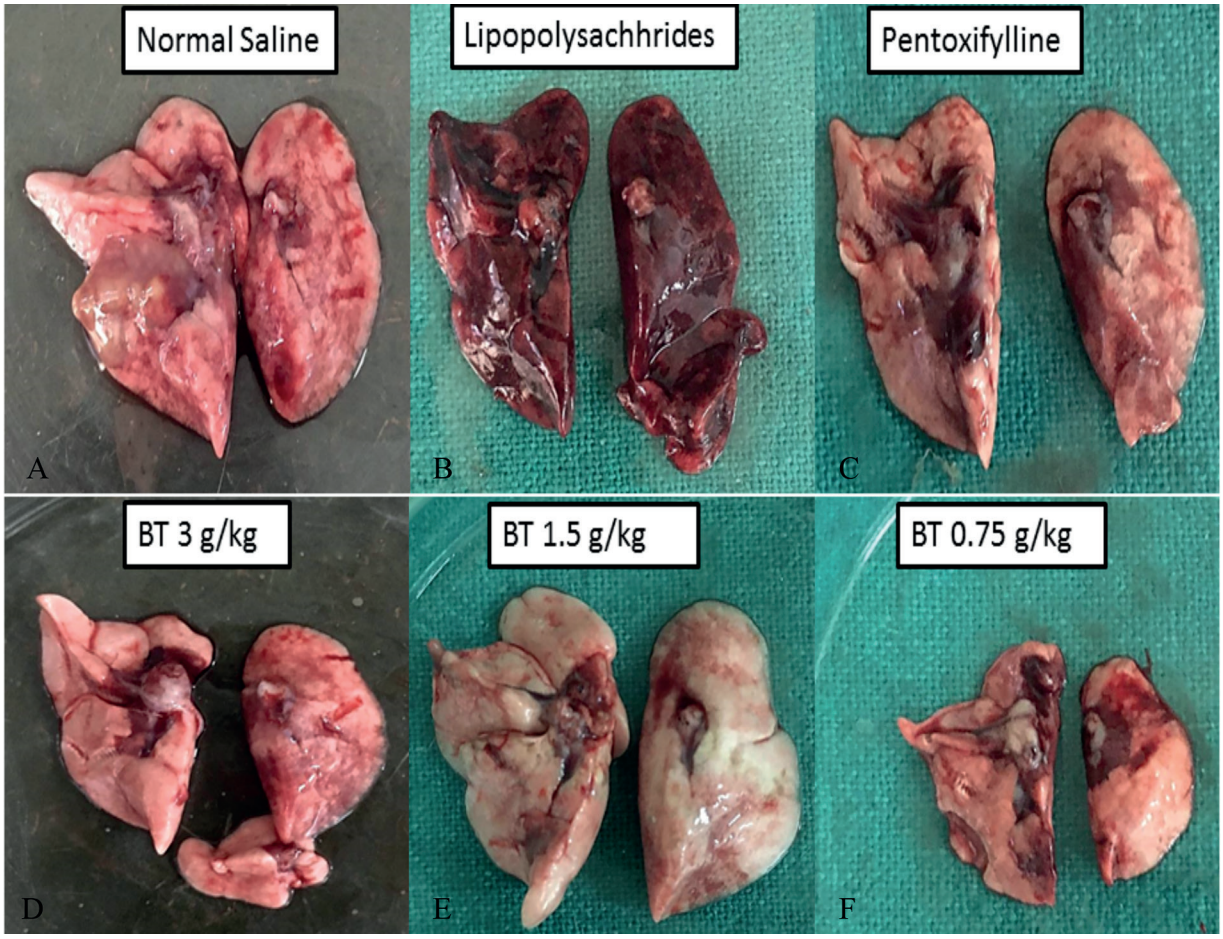

Figure 4. Surface morphology of lungs of each group with clear hemorrhagic manifestations in LPS treated group. **A,** group I (NS), **B,** denotes group II (LPS), C, denotes group III (PTX 25 mg/kg BW), **D,** denotes group IV (BT 3 g/kg BW), **E,** denotes group IV (BT 1.5 g/kg BW) and **F,** denotes group IV (BT 0.75 g/kg BW) respectively.

tion against LPS. BT (3 g/kg BW) treated group have fewer cell infiltration due to intact alveoli-capillary membrane. Their histological appearance is closer to group I as observed in Figure 6.

At 40 (X) magnification as observed in Figure 6, infiltration of neutrophil along with fluid can be seen in air space of lungs of LPS treated rat,-suggesting breach of the alveolo-capillary membrane. The honeycomb presentation of parenchymal is lost with alveolar flooding in this group. However in PTX treated group,the normal parenchymal structure is visible with no fluid accumulation. Similar presentation is visble in BT treated group suggesting its lung protective role.

Discussion

Septicemia is the leading cause of mortality due to ARDS related respiratory distress and pentoxifylline is a common dependable pharmacological intervention[13]. Here, we have used it as a positive control. The heart-lung interaction is difficult to evaluate during mechanical ventilation in sepsis-induced ARDS patients[14], so here we have estimated them in an experimental model using Charles foster rats. In the present work, we have demonstrated the protective role of Bronco T (3 g/kg BW) on the physiological, pulmonary and histological parameters in LPS induced sepsis in rats.

Respiration rate tends to rise initially followed by progressive fall in septicemia. This leads to lowering of oxygen in the pulmonary region and cause severe hypoxemia. In this study, similar observations were reported after 24 hours of LPS administration. Pentoxifylline treatment significantly reverses this effect by inhibiting pro-inflammatory cytokines including TNF alpha

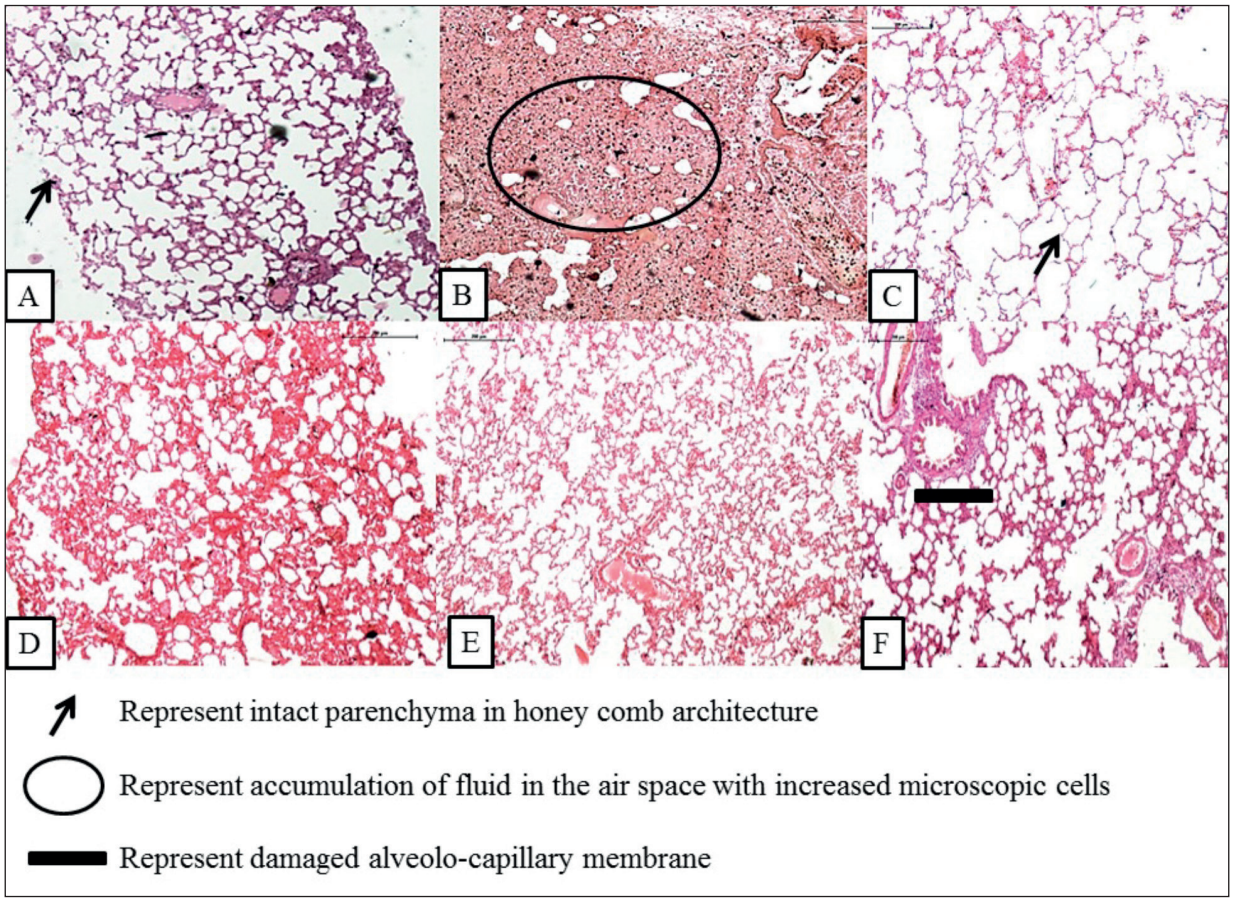

Figure 5. Histological section (H&E staining) of lungs in each group at 10X magnification reveals the following information. 1) In Group I **(A)** normal honeycomb presentation of parenchymal cells could be observed which in Group II **(B)** is flooded with protein-rich fluid and there is clear sloughing of the alveolar region. 2) In Group III **(C)** PTX treatment prevented the sloughing of the alveolar region but honeycomb presentation is altered. 3) In Group IV (**D,** 3 g/kg & **E,** 1.5 g/kg) airspace are visible in comparison to group II (B) which prevents the stiffening of lungs. However, in BT 0.75 g/kg pretreated group **(F)** damaged alveoli-capillary membrane is visible.

and leukotriene synthesis[15]. Similarly, BT (3 g/kg BW) treatment reversed this effect with near to normal peak presentation suggesting that the herbal decoction was able to save the lungs from the deleterious effect of LPS.

Mean Arterial Pressure (MAP) is the main hemodynamic variable, indicating the driving pressure for organ perfusion. In the patient with septicemia, all blood vessels dilate due to the release of nitric oxide, causing blood pressure to drop. Infection coupled with lack of blood flow to vital organs leads to septic shock and organ failure[16]. PTX was able to maintain MAP >65 mmHg due to inhibition of nitric oxide[17] and improving hemodynamic capacity[18]. Similar observations were found with BT (3 g/kg BW) treatment indicating the same mechanism of action which needs to be further explored.

In severe sepsis, initially, the heart beats rapidly, followed by bradycardia where the heart is unable to pump blood to vital organs irrespective of any interventions[19]. In our study, a similar presentation was found in LPS treated group where there was persistent hypotension and bradycardia during recording after 24 hours. PTX and BT (3 g/kg BW) had maintained the heart rate to the normal range of 96.7% and 91.5%, respectively. These suggest that BT (3 g/kg BW) had a cardioprotective role against LPS in a similar manner to PTX.

The ratio of the partial pressure of arterial oxygen to fraction of inspired oxygen (P/F) determines the severity of sepsis[20]. Hypoxemia presents various complications including an elevated level of inflammatory cytokines, reactive oxygen species, and neutrophils and reduced lung com-

pliance. LPS treatment for 24 hours lowered the P/F ratio to severe grade. This was rectified in BT (3 g/kg BW) and PTX treated group implicating the role of BT against hypoxemia and its detrimental effect.

Septicemia produces symptoms of pneumonia due to alveolar flooding. It leads to stiffening of lung and indirectly plays a key role in the failure of the respiratory system. This happens due to altered membrane permeability[21]. The lung wet to dry ratio determines this condition objectively. LPS treatment increased pulmonary water content as obtained from lungs wet to dry ratio, therefore, stiffening the lung and leading to poor exchange of gases of the lungs. BT (3 g/kg BW) treated groups significantly lowered the lung wet to dry ratio suggesting it maintain the integrity of the alveoli-capillary membrane in LPS treated rats. However, further studies are required to pinpoint the exact mechanism behind this.

This physiological change has been further supported by histological studies in which we found a peculiar presentation of hyaline membrane formation, with an increased number of inflammatory cells in the interstitium and alveolar area[6]. This is a typical illustration of ARDS due to injured Type I and II pneumocytes which leads to insufficient surfactant production. This was visible in LPS treated rats where the lungs were hardened with hemorrhagic manifestation. There was an increased fluid accumulation and number of infiltrating cells in the lungs. This was rectified in BT (3 g/kg BW) and PTX treated rats significantly. On histological analysis, there was intact endothelial and epithelial barrier in BT (3 g/kg BW) treated group along with visible air space and less

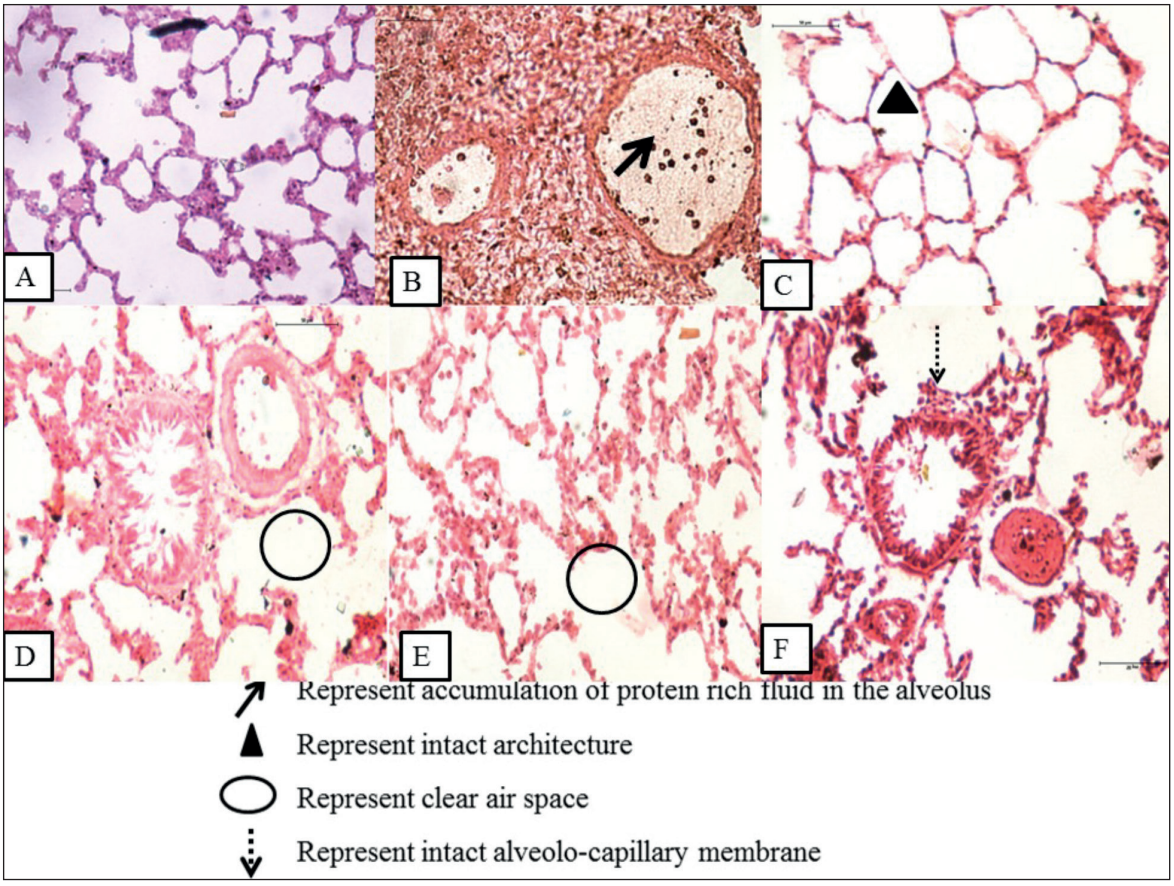

Figure 6. Histological section of lungs in each group at 40X magnification reveals the following information. 1) In Group II **(B)** the alveoli-capillary membrane is breached with the intrusion of neutrophils and fluid accumulation in this region. 2) In Group III **(C)** the intact structure of parenchymal tissue could be observed. 3) In Group IV (**D**, 3 g/kg & **E**, 1.5 g/kg) visible airspace and intact endothelial layer could be observed. There is no hemorrhagic manifestation with less number of intruding cells in the alveolar region. However, in group IV (**F**, 0.75 g/kg) injurious conditions are close to the LPS treated group with exception of fluid accumulation.

number of infiltrating cells. The level of neutrophil infiltration is correlated by myeloperoxidase level in this study which suggests that BT (3 g/kg BW) treatment significantly lower the sneaking of neutrophil in the alveolar region. This indicates the lung-protective role of BT in LPS treated rats in comparison to PTX treatment.

Conclusions

From analyzing the above-stated parameters, it can be safely suggested that Bronco T (BT) (3 g/kg BW) significantly improved survival time, cardio-respiratory, physiological, pulmonary and histological parameters in the LPS induced animal model of septicemia. Therefore BT (3 g/kg BW) can be used as add on therapy with conventional strategies. This need to be done under clinical supervision, for patients, admitted to ICU on mechanical support. Further study is required to elucidate the molecular mechanism of this preparation.

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Conflicts of Interest

The authors declare no conflicts of interest.

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