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Effect of α-Phenyl-Tert-Butylnitrone on Endotoxin Toxemia in Horses*

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ABSTRACT. Lipopolysaccharide (LPS), or endotoxin, is a component of the cell wall of gram-negative bacteria and is toxic to humans and animals. The GI tract of horses contains large numbers of endotoxins which may cause disease if gut wall integrity is compromised. The objective of this study was to develop a unique therapeutic approach to the treatment of endotoxemia with a sulfonyl analog of the α-phenyl-N-tert-butyl-nitrone (PBN) spin-trap molecule which may prevent the LPS-induced cytokine cascade. Following challenge with 55 mg/kg LPS, the survivability of ICR Swiss mice was significantly improved after treatment with 100 and 175 mg/kg PBN, although survivability of mice treated with 175 mg/kg PBN was significantly less than those treated with 100 mg/kg PBN. Challenged mice treated with 300 and 1000 mg/kg PBN survived for a significantly shorter period of time (vs control). Horses treated with a sublethal dose (1 µg/kg) of endotoxin experienced 2 periods of distress at 1 and 6 h after challenge. Disulfonyl-PBN significantly reduced the increase in heart and respiratory rates 6 h after challenge. Analogs of PBN appeared to be more beneficial following near-lethal challenge with LPS. Dramatic benefits to horses may only be observed in life-threatening situations.

Endotoxin, also called lipopolysaccharide (LPS), is a component of the outer surface of gram-negative bacterial cell walls (1). Two characteristics of endotoxin make it a critically important toxin in horses and also in humans. First, endotoxin is extremely toxic and can be lethal at concentrations as low as 10^{-9} g/mL. Secondly, endotoxin is quite stable, which means that if antibiotic therapy is used in an affected horse, the endotoxins released from dying bacteria are absorbed and are toxic to the animal (2,3).

Although the GI tract of horses contains large amounts of endotoxins, protective mechanisms protect the animal from systemic distribution of the organisms (4). However, any compromise of gut wall integrity could increase the occurrence of endotoxemia. Furthermore, an invasive organism (eg Salmonella spp) may be the source of endotoxin, thereby increasing the chances of endotoxemia.

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Studies using laboratory animals implicate both reactive oxygen species (free-oxygen radicals) and cytokines in the pathophysiology of endotoxic shock (5,6). Tumor necrosis factor (TNF) is the main cytokine released in association with tissue injury during sepsis (7). Furthermore, reactive oxygen species in combination with LPS as an activating factor amplify TNF release by macrophages thereby increasing tissue injury.

Treatment for endotoxemia in horses is directed toward correcting the underlying condition, counteracting the effects of mediators synthesized in response to endotoxin, and neutralizing endotoxin itself. The spin-trap α-phenyl-N-tert-butly-nitrone (PBN) protects against endotoxic shock, possibly by down-regulation of the LPS-induced cytokine cascade (4). The objective of the present study was to develop a unique therapeutic approach to the treatment of endotoxin toxicity using a sulfonyl analog of the PBN spin-trap molecule.

MATERIAL AND METHODS

All animals used in these experiments were managed according to the rules and regulations of the Institutional Animal Care Use Committee at the University of Kentucky.

Mouse Studies

ICR Swiss mice received endotoxin LPS

(Escherichia coli serotype 055:B5; List Biological Laboratories Inc, Campbell, CA) via ip injection. To establish the toxicity of LPS in ICR Swiss mice, the drug was administered in doses of 2.5, 12.5, 25.0, 35.0, 45.0, 55.0 or 65.0 mg/kg and survivability was determined over the following 7 d.

In a subsequent experiment, mice were administered ip injections of 55.0 mg/kg LPS and then treated with ip doses (50, 100, 175, 300 or 1000 mg/kg) of PBN every 6 h after challenge (qid) to assess the effect of PBN on survivability.

In a third experiment, the effect of sulfonyl-phenyl-N-tert-butyl-nitrone (sulfonyl-PBN) on survivability following LPS challenge was assessed. Two ip treatments of sulfonyl-PBN (300, 500, 1000 or 3000 mg/kg) were administered at 1 and 6 h post-LPS challenge. In a final experiment, mice were given 4 treatments of sulfonyl-PBN (1000 mg/kg) at 1, 3, 5 and 7 h post-LPS challenge.

Equine Studies

Six mature Thoroughbred mares weighing 413-602 kg were used in another series of experiments. To assess the safety of disulfonyl-PBN, the 6 horses were administered the drug in increasing doses (5, 10, 40 and 100 mg/kg). All concentrations appeared safe.

In a cross-over pattern, the 6 horses were challenged with an iv sublethal dose of LPS (1 µg/kg) and treated with disulfonyl-PBN or saline to assess the effects of those treatments on clinical signs of the disease. The horses received 50 mg disulfonyl-PBN/kg or saline 15 min before LPS challenge and 10 mg disulfonyl-PBN/kg or saline at hourly intervals for 5 h after challenge. Blood samples were collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h after injection. Blood samples were used to perform a complete blood count (CBC) which measured red blood cell count, white blood cell count and a differential, and general chemistry panel (CHEM) which measured alkaline phosphatase, GGTP, BUN, LDH, SGOT, CPK, blood glucose, total protein, albumin, calcium, creatinine, sodium, potassium, chloride, total carbon dioxide, and total bilirubin. Additionally, a physical exam assessing attitude, mucous membrane refill, bowel sounds, temperature and respiratory and heart rates was performed at 15 min and immediately before administration and at 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 h after administration.

Statistical Analysis

Data are presented as means ± SEM. The LIFEREG procedure was used to compare survivability of mice between control (LPS only) and treatment (LPS and PBN) values. The LIFEREG procedure fits parametric models to failure time for interval censored data. Fisher's Exact Test was used to compare survivability of mice between control (LPS only) and treatment (LPS and sulfonyl-PBN) values. Analysis of variance with repeated measures (8) was used to compare control (LPS only) and treatment (LPS and disulfonyl-PBN) values for horses for CBC, CHEM and physical exam at each measuring time. Significance was set at $P < 0.05$.

RESULTS

Figure 1 is the dose-related survivability of the control mice following ip injection of LPS. All mice survived following administration of 2.5 or 12.5 mg/kg. Following doses of 25.0, 35.0, 45.0, 55.0 or 65.0 mg LPS/kg, the mice survival rates were 71, 69, 43, 25 and 10%, respectively.

Figure 2 is the effect of PBN on survivability following challenge with 55 mg LPS/kg. Doses of 1000 mg PBN/kg were acutely toxic to the mice, and mice died within 10 min of dosing. Doses of 300 mg PBN/kg qid also significantly reduced the duration of survivability following LPS challenge. Treatment with 50 mg PBN/kg qid did not significantly affect survivability. There was a significant increase in survivability following treatment with 100 or 175 mg PBN/kg qid. However, all mice treated with 175 mg PBN/kg qid were dead by 96 h post-LPS challenge, whereas 50% of the mice treated with 100 mg PBN/kg qid survived the entire 7-d period. There was a significant improvement in survivability for mice treated with 100 mg PBN/kg qid when compared with mice treated with 175 mg PBN/kg qid.

Figure 3 is the effect following 2 treatments of sulfonyl-PBN after LPS challenge. Following challenge with 20-25 mg/kg (A), there was no difference in survivability between control mice and mice treated with sulfonyl-PBN. Although there appeared to be

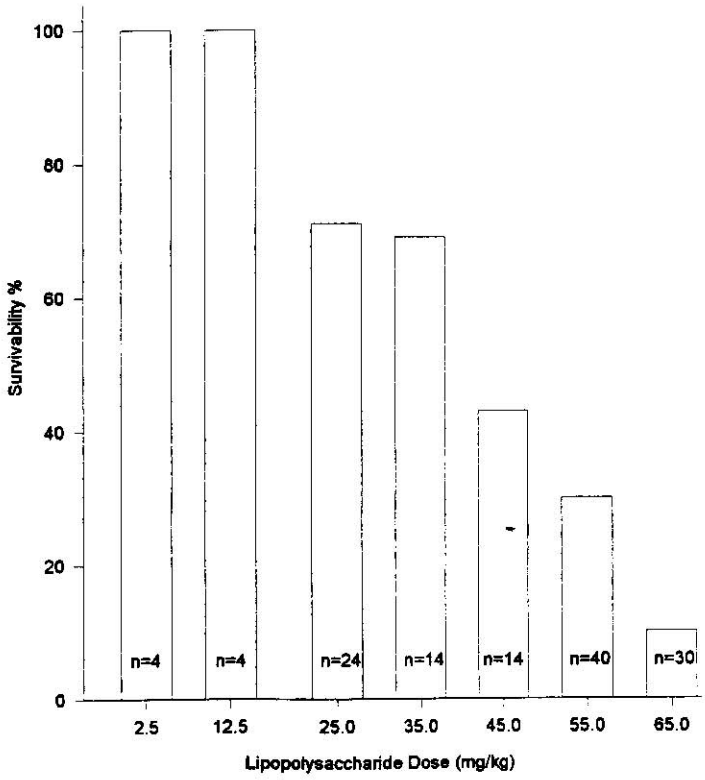

Figure 1. Survivability of control mice following LPS challenge.

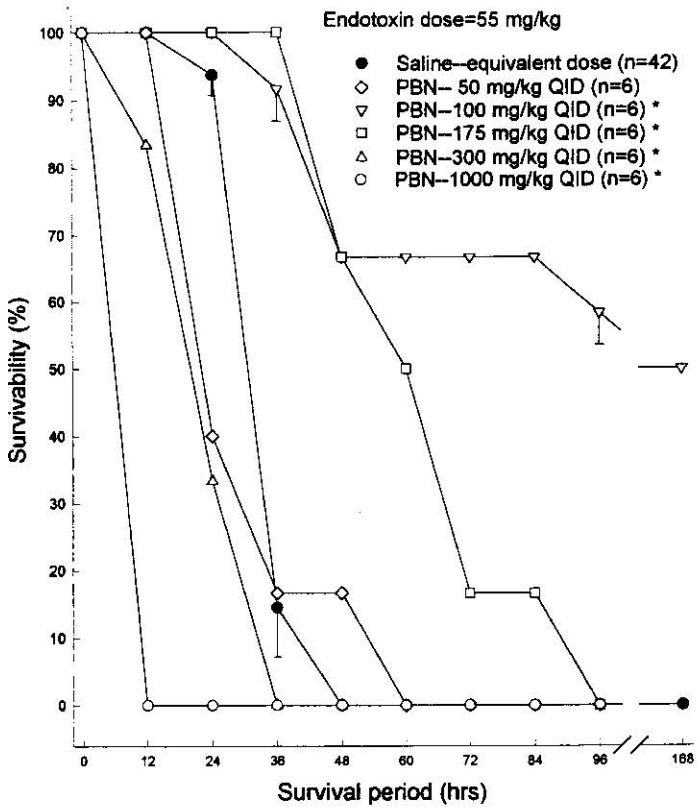

Figure 2. Survivability of PBN-treated mice following LPS challenge. *=Significantly different from control values.

improvement in survivability between control mice receiving 3000 mg sulfonyl-PBN/kg, the improvement was not significant. Following challenge with 30-35 mg/kg (B), there was no difference in survivability between control mice and mice treated with 300, 500, 1000 or 3000 mg sulfonyl-PBN/kg.

Following challenge with 30-35 mg LPS/kg, there was no significant difference in survivability after 4 treatments of 1000 mg sulfonyl-PBN/kg. Likewise, when mice were challenged with 50-55 mg LPS/kg, there was no significant difference in survivability between mice treated with saline or with sulfonyl-PBN qid.

When evaluating the safety of disulfonyl-PBN in horses, we found no significant differences in CBC, CHEM and physical exam values between control horses and horses treated with 5-100 mg disulfonyl-PBN/kg. The clinical signs associated with sublethal challenge to LPS in horses included colic-like symptoms such as sweating, pawing, flank checking and rolling. The heart and respiratory rates showed biphasic increases at 1 and 6 h post-challenge. Intestinal motility was greatly decreased from 30 min until about 6 h after challenge. Body temperatures increased steadily until reaching a peak 5 h after challenge, and then gradually decreased until reaching control levels 24 h after challenge.

Figure 4 illustrates the changes in heart rates, respiratory rates, temperatures and bowel sounds associated with LPS challenge and the effects of 100 mg disulfonyl-PBN/kg on those changes. Both heart and respiratory rates were significantly less for horses treated with LPS and disulfonyl-PBN at 6 h after challenge when compared to control (only LPS-treated) horses. Although the temperature of horses treated with LPS and disulfonyl-PBN appeared less than in control horses, there was no significant difference between the 2 groups. Treatment with disulfonyl-PBN did not significantly affect bowel sounds.

When evaluating the effects of endotoxin challenge on the measured chemistry variables, there were no significant differences between horses treated with LPS and disulfonyl-PBN and the control horses at the dosages of LPS and disulfonyl-PBN tested.

DISCUSSION

Since there was only 25% survival following 55 mg LPS/kg in mice (Fig 1), we chose that dose as our challenge dose. Earlier studies showed that PBN treatment before (9) and after (10) LPS challenge improved survivability in mice. However, the data in Fig 2 suggest there is a narrow window of improved survivability following PBN treatment. A qid dose of 50 mg PBN/kg provided no protection over control values, and qid doses of 300-1000 mg/kg were toxic resulting in decreased

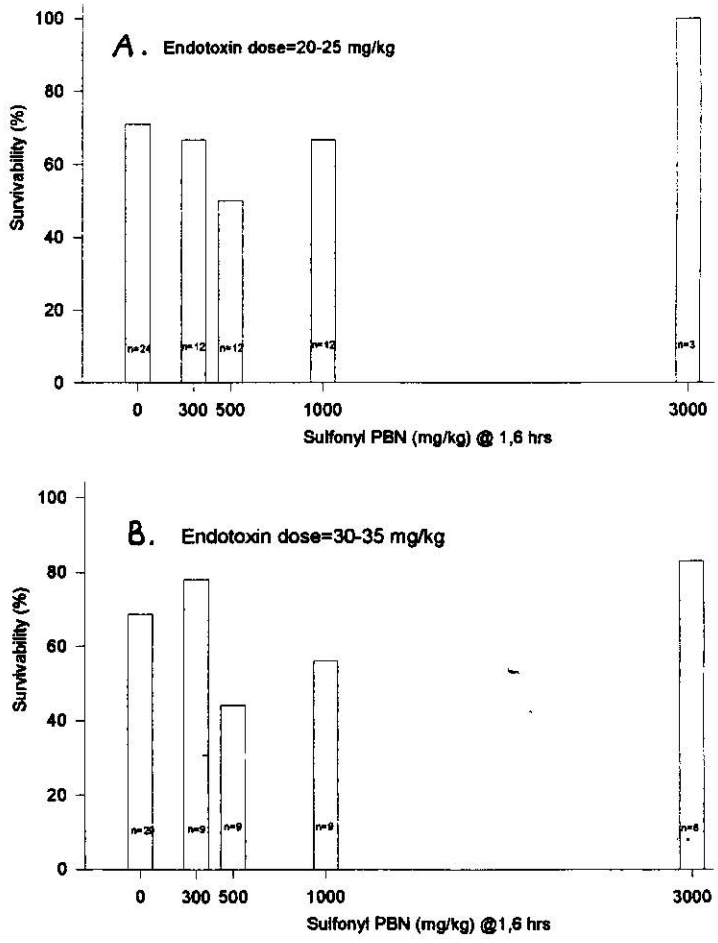

Figure 3. Survivability of LPS-challenged mice following 4 treatments of sulfonyl-PBN.

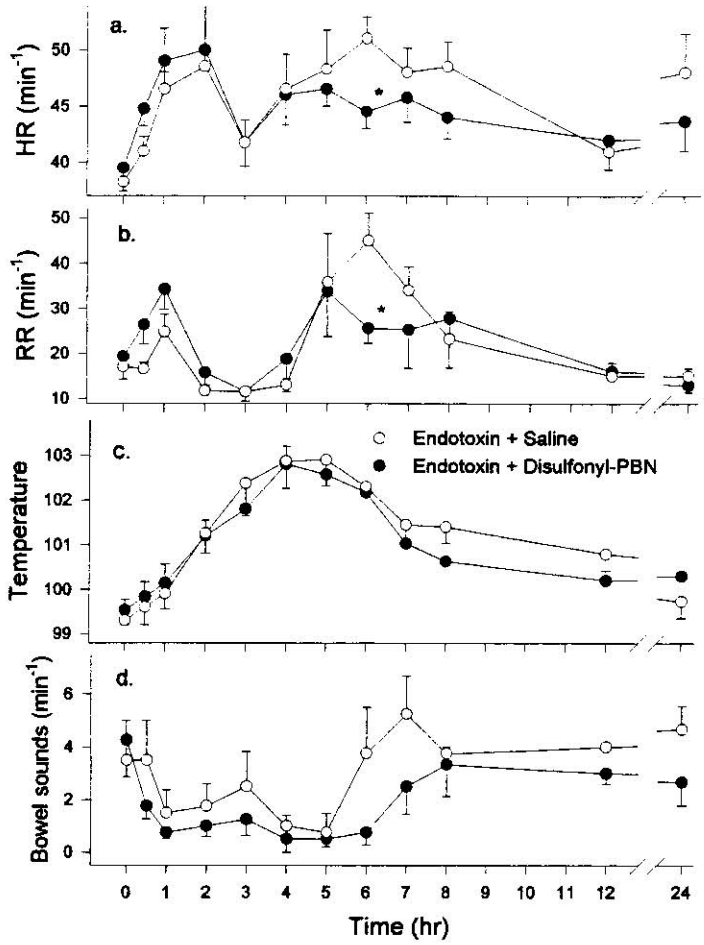

Figure 4. Changes in clinical findings in horses following LPS challenge and LPS challenge with disulfonyl-PBN treatment. *=Significantly different from endotoxin + saline control treatment.

survivability vs control. Although qid doses of 100 or 175 mg/kg improved survivability, doses of 175 mg/kg had a cumulative toxic effect, since all mice treated with that dose were dead within 96 h, and mice treated with 100 mg/kg had 50% survival. These results were sufficiently promising for us to test the disulfonyl-PBN analog in a model of clinical endotoxemia in the horse.

Previous studies challenged ponies and horses with a wide range of doses (0.03-300 µg/kg 11,12), with the higher doses usually resulting in death or euthansia of the challenged animal. The equine endotoxin shock model described in this paper was designed to be comparable with a sublethal endotoxemia infection in horses. Therefore, a low dose of endotoxin (1.0 µg/kg) was administered. Horses experienced 2 periods of distress, as exhibited by increases in heart and respiratory rates 1 and 6 h after LPS challenge. The rapid decrease in bowel sounds was consistent with previous findings (13) and was attributed to intestinal vasoconstriction and reduction of intestinal smooth muscle activity. These events are mediated primarily by prostaglandin (PG) E_2, with lesser contributions from $PGF_{2\alpha}$, thromboxane and prostacyclin (13). Although treatment with disulfonyl-PBN did not affect the increase in heart and respiratory rates at 1 h, it did significantly reduce those variables 6 h post-challenge. Furthermore, disulfonyl-PBN caused a nonsignificant decrease in temperature. These findings suggest that disulfonyl-PBN reduces the stress/discomfort associated with endotoxin toxemia.

We observed more clear-cut and clinically suggestive results during the mouse study than during the equine study. Since the PBN analogs appeared more beneficial following near-lethal challenges of LPS, dramatic benefits to horses from this treatment may only be observed in life-threatening situations (14,15).

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