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Protective effect of pentoxifylline plus thalidomide against septic shock in mice

OSCAR ARRIETA*, ARACELI ORTIZ-REYES*, DANIEL REMBAO†, MINERVA CALVILLO*, ERIKA RIVERA* AND JULIO SOTELO*

**Neuroimmunology Unit and †Experimental Pathology Laboratory, Instituto Nacional de Neurología y Neurocirugía and Universidad Nacional Autónoma de México*

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Summary. Mortality caused by septic shock in experimental animals is reduced by thalidomide, an inhibitor of tumour necrosis factor α. Another drug that could act on the pathopysiological mechanisms of septic shock is pentoxifylline, an inhibitor of platelet aggregation that increases the flexibility of the erythrocyte membrane and has fibrinolytic activity. We studied the effect of pentoxifylline alone and combined with thalidomide in septic shock; 97 NIH mice were injected with lipopolysaccharides of *Salmonella abortus equi* and D galactosamine. Animals were separated in 4 groups; group A ($n = 20$) was used as control, group B ($n = 15$) received thalidomide 50 mg/kg, group C ($n = 20$) received pentoxifylline 40 mg/kg, and group D ($n = 15$) received thalidomide plus pentoxifylline. Mortality was recorded every hour. Additionally, 5 animals from each group were sacrificed 8 h after the induction of septic shock for histological analysis of heart, lung, brain, kidney, small intestine, adrenal glands and liver. Microscopic findings were rated as absent, mild, moderate and severe damage. In control animals histological analysis showed intense haemorrhage and necrosis in all organs studied. When compared with controls, treatment with pentoxifylline plus thalidomide reduced mortality ($P < 0.03$). The tissue damage was less severe in animals from the groups that received pentoxifylline or pentoxifylline plus thalidomide ($P < 0.05$). Pentoxifylline seems to potentiate the beneficial effects of thalidomide, reducing mortality and attenuating the pathological changes produced by septic shock.

Keywords: septic shock, pentoxifylline, thalidomide, histopathology

Septic shock is characterized by hypotension and organic dysfunction (Hardawat 1996), as a consequence of a widespread immune response induced by bacterial lipopolysaccharides. Apparently, the most important mediator is tumour necrosis factor α (TNFα) (Lesslauer *et al.* 1991; Mohler *et al.* 1994), a cytokine that stimulates phospholipase A2 (Danner 1991; Vadas & Pruzanski 1993) and participates in the magnification of the response mediated by the release of other cytokines and active substances such as the proinflammatory

Correspondence: Dr Oscar Arrieta, Instituto Nacional de Neurología y Neurocirugía, Insurgentes Sur 3877, Mexico City 14269, Mexico. Fax: +525 528 00 95;
E-mail: ogar@servidor.unam.mx

Table 1. Survival of mice with septic shock

Treatment	Number	Individual survival (h)	Mean survival/expected* survival (h) (%)	Mortality (%)
None	20	7,7,7,7,7,8,8,8,8,8,9,9,20,28,30,30,30,30,30,30	16/30 (53)	14/20 (70)
Thalidomide	15	7,7,8,8,8,8,9,9,10,30,30,30,30,30,30	17/30 (56)	9/15 (60)
Pentoxifylline	20	7,7,7,7,7,7,8,8,8,10,13,30, 30,30,30,30,30,30,30,30	18/30 (60)	11/20 (50)
Thalidomide/Pentoxifylline	15	7,8,8,8,8,30,30,30,30,30,30,30,30,30,30	23/30 (75)	5/15 (30)†

* Kruskal – Wallis analysis of variance followed by multiple comparations of all treatment groups with the infected control by the Mann – Whitney *U* – test; † $P < 0.03$ with Fisher's exact Test.

interleukines (IL) 1,6 and 8, kalycreine, factor XII, platelet activator factor and nitric oxide.

Mortality caused by septic shock is high, treatments are frequently ineffective (American College of Chest Physicans/Society of Critical Care Medicine Consensus Conference Committee 1992). Thalidomide, an inhibitor of the synthesis of TNFα has been used in the treatment of *leprosy* lupus, AIDS, billiary cirrhosis, experimental brain tumours, and bone marrow transplant (Randall 1990; Koch 1994; Palencia *et al.* 1998). In experimental models of septic shock, previous administration of thalidomide reduced mortality (Schmidt *et al.* 1996). Pentoxifylline, a dimethylxanthine with fibrinolytic activity an inhibit of platelet aggregation increases the flexibility of erythrocytes thus improving the tissue perfusion in patients with arterial stenosis (Frampton & Brogden 1995); it also decreases the inflammation associated with experimental bacterial meningitis (Saez-Llorens *et al.* 1994; Ostrosky *et al.* 1996). Our aim was to asses the effect of pentoxifylline alone and combined with thalidomide in a murine model of septic shock.

Materials and methods

Pharmacological compounds

Thalidomide was synthesized in the Orphan Drug laboratory at the Universidad Autónoma Metropolitana (Mexico City), the isomer used was the teratogenically active one. Pentoxifylline was the commercially available compound (Hoeschst-Roussel, Mexico City).

Induction of septic shock

97 male NIH mice aged 8–10 weeks, were injected intraperitoneally with 10 μg of lipopolysaccharides (LPS)

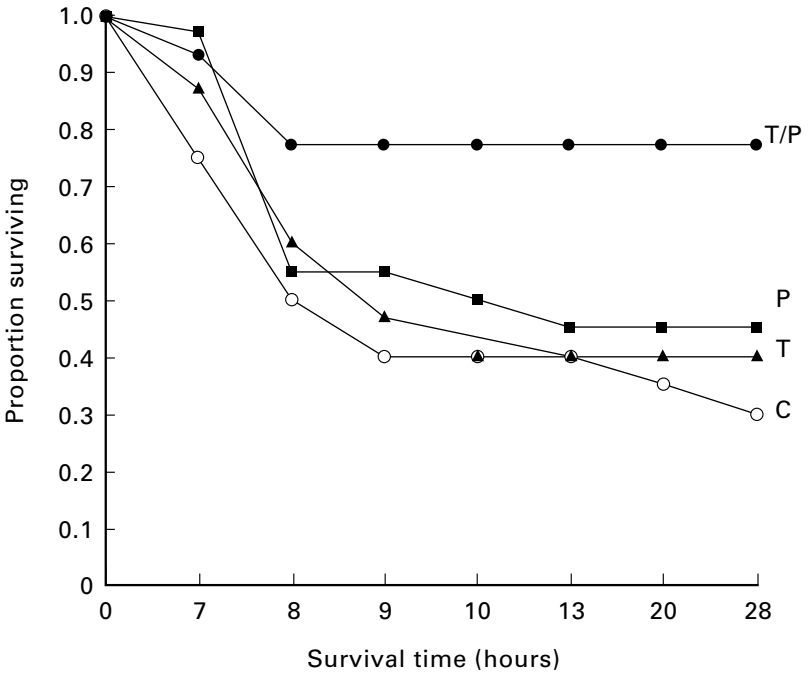


Figure 1. Survival data after induction of septic shock are shown as a Kaplan Meier plot, cummulative survival in animals treated with thalidomide/pentoxifylline was significantly greater than the other groups ($P < 0.03$). ○ controls; ▲ thalidomide; ■ pentoxifylline; ● thalidomide + pentoxifylline

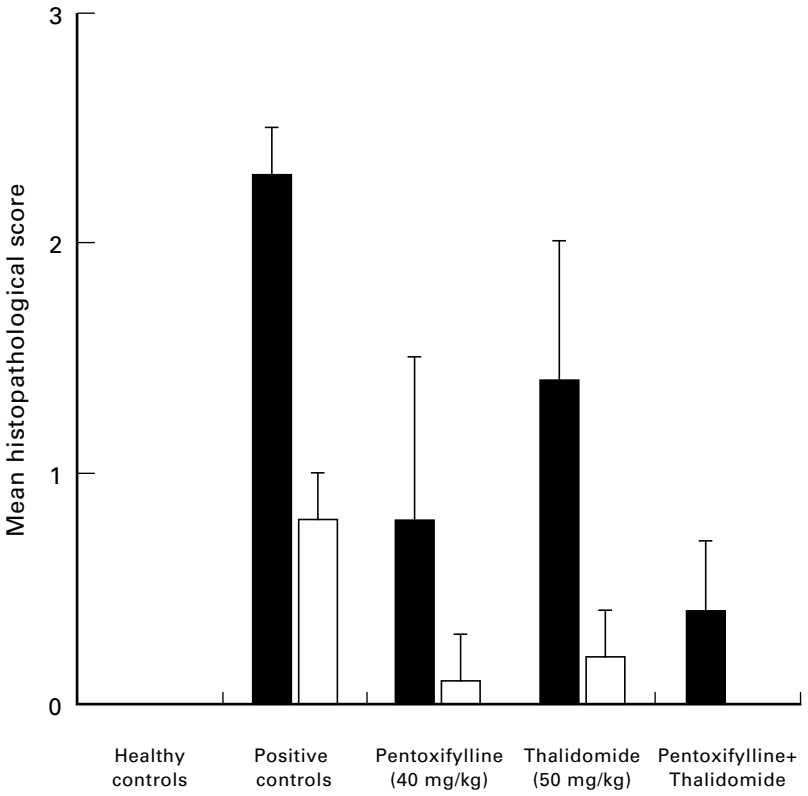


Figure 2. Mean results of histopathological findings in the liver (■) and kidney (□) of healthy animals and in animals with septic shock treated with thalidomide and/or pentoxifylline. * $P < 0.05$ when compared with positive controls.

from S*almonella abortus equi* (Sigma-Aldrich Chemical Co., Mexico City) diluted in 0.1 ml of PBS simultaneously, 18 mg of D-galactosamine (D-GalN, from Sigma, St Louis, MO) diluted in 0.1 ml of PBS were injected in the another side of the peritoneal cavity (Lehmann *et al*. 1987). Mortality was monitored every hour over a period of 30 h. No further mortality was observed after this time.

Drug treatments

The treatment schedule was initiated immediately after induction of septic shock. 70 animals were randomly assigned to four groups. Animals from group A ($n = 20$) were orally treated with nonpyrogenic saline solution (positive controls); group B ($n = 20$) received 40 mg/kg body weight of pentoxifylline by intraperitoneal injection; group C ($n = 15$) were orally treated, through a catheter, with thalidomide at 50 mg/kg; group D ($n = 15$) received thalidomide plus pentoxifylline in the same doses as animals from groups B and C.

Histopathological evaluation

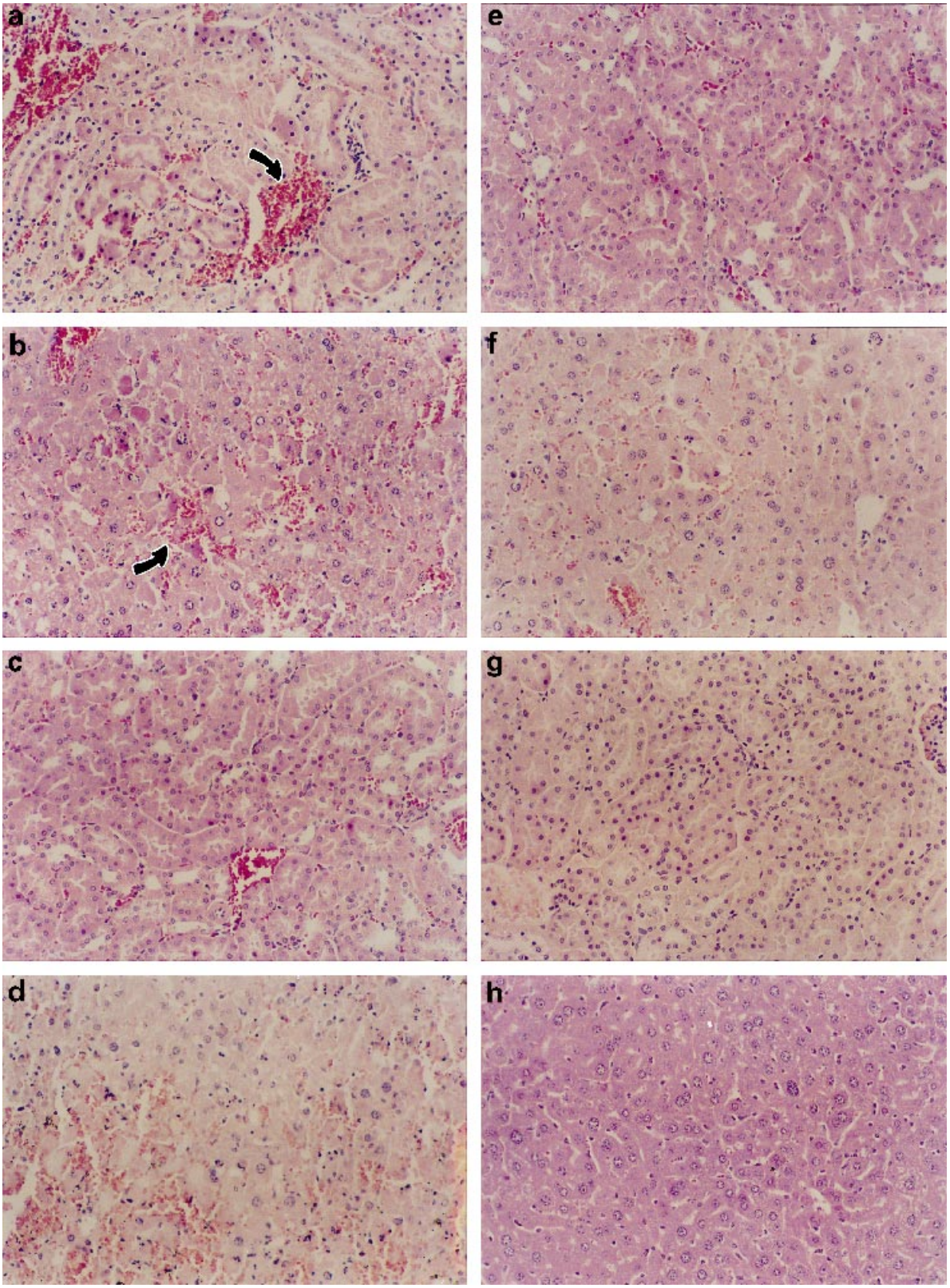
Additional to the experiments of survival, five healthy animals (negative controls), 10 mice from the positive control group, and five animals from each treatment group were sacrificed by cervical dislocation 8 h after induction of septic shock and immediately perfused with a solution of 10% formaline in saline solution. The brain, lungs, kidneys, adrenals glands, heart, and liver were dissected and fixed in the same solution for 2 weeks. Afterwards, the specimens were dehydrated, embedded in paraffin, sectioned and stained with haematoxilin and eosin by the conventional histological procedures. All tissue samples were assessed blindly by the same pathologist (D.R.), who graded the main alterations, necrosis and haemorrhage, observed on every organ according to the following scale: absent (0), mild (1), moderate (2) and severe (3).

Statistical analysis

Survival time and histophatological damage were compared between groups by the Kruskal–Wallis analysis of variance and the Mann-Witney *U*-test; mortality was compared by the Fisher's exact test. α level was set at $P < 0.05$.

Results

The model of septic shock used in these experiments induced a high mortality of 77% in untreated mice;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mortality in animals treated with thalidomide or with pentoxifylline alone was similar. However, the group of animals treated with thalidomide plus pentoxifylline had a significant survival increase ($P < 0.03$) compared with controls or with animals treated with each drug alone (Table 1). Survival according to the Kaplan Meier plot showed significant differences of cummulative survival in animals treated with thalidomide/pentoxifylline when compared with controls or with animals treated either with thalidomide or with pentoxifylline alone (Figure 1). Histopathological analysis in controls with septic shock showed intense of haemorrhage and necrosis in all organs studied, particularly liver and kidney (Figure 2). Degree of damage was significantly minor in animals treated either with pentoxifylline or with pentoxifylline plus thalidomide ($P < 0.05$); no difference was found in animals treated with thalidomide alone (Figure 3).

Discussion

In humans, mortality from sepsis remains close to 50%, this unacceptable rate has stimulated interest in the search for pharmacological agents that might reduce morbility and mortality (Glauser *et al*. 1994). One option is the combination of drugs that interferes with the physiopathological mechanisms of immune response triggered by systemic sepsis. Our results show that the combination of pentoxifylline plus thalidomide has a protective effect on mortality and on tissue damage produced by septic shock in mice. This protective effect was particularly evident in the damage induced in liver and kidney, the most severely affected organs; protection seemed to be more conspicuous in the kidney than the liver. We found histophatological changes characteristic of severe hypoperfusion (Krausz & Cohen 1992), the microscopical examination reveals tubular necrosis, sinusoidal dilatation, congestion with necrosis that affects acinar zone 3 and 2. In contrast, administration of thalidomide alone had minor efficacy compared with administration of pentoxifylline or pentoxifylline plus thalidomide. Other reports have shown that thalidomide administered as pretreatment for septic shock increases survival from acute endotoxaemia, this contrasting finding may be due to the blockage of TNF when thalidomide is given before the induction of toxaemia (Schmidt *et al*. 1996). In this study, however, thalidomide was administered after the induction of septic shock, where it had only a moderate but nonsignificant protective effect. We chose to administer thalidomide not as a preventive drug but as treatment for septic shock after its mechanisms had already been incited. The compound used by us is in a crystal/powder form, virtually insoluble in aqueous, alcohol and oil medium; therefore, it only achieves adequate serum concentrations by oral administration. Serum levels are detectable by liquid chromatography within 1 h of oral administration and the peak levels are seen after 2–4 h. The serum half life is between 12 and 18 h (Eriksson *et al*. 1992a, b). These pharmacodynamic characteristics may diminish the interaction timing of thalidomide with peak levels of TNFα production during the course of septic shock, thus explaining its lack of effectiveness in our experiments. Other studies have found a favourable effect of pentoxifylline in the course of septic shock, particularly when combined with methylxantine (Jilg *et al*. 1996).

Pentoxifylline is a dimethylxantine known for fibrinolytic activity and its ability to inhibit platelet aggregation (Ambrus *et al*. 1995), it is widely used in humans for treatment of vascular diseases such as intermittent claudication (Hood *et al*. 1996). Other studies have found a favourable effect of pentoxifylline in the course of septic shock, particularly when combined with methylxantine (Jilg *et al*. 1996). Pentoxifylline regulates the release of IL-1β, IL-6, IL-8 and TNFα in blood by mononuclear cells (Neuner *et al*. 1994), it also suppresses Interleukin-2 by inhibition of endogenous TNFα (Jewett & Bonavida 1994), additionally pentoxifylline reduces adhesion molecule expression (Mc Bride *et al*. 1996). These effects on cytokines could interfere with complex immune mechanisms incited by toxaemia, thus explaining the decrease in mortality and reduction of histopatological changes obtained when the drug was administrated jointly with thalidomide perhaps as a consequence of mutual pharmacological potentiation.

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Figure 3. Microscopic appearance of kidney and liver of animals after septic shock. Positive controls show (a) in kidney severe tubular necrosis, desquamated cells in the lumen, haemorrhage and necrosis (arrow) and (b) in liver haemorrhage and disorganization into the parenchyma with degenerating hepatocytes (arrow). In animals treated with thalidomide (c) the kidney still shows tubular necrosis with hyperchromatic nuclei and haemorrhage and (d) the liver had eosinophilic cytoplasm, shrunken hepatocytes and haemorrhage. In animals treated with pentoxifylline the above mentioned abnormalities are minor (e) in kidney and (f) in liver. In animals treated with a combination of pentoxifylline/thalidomide the signs of damage caused by septic shock are notably attenuated (g and h). (HE, 200 x)

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