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Effects of drugs which alter blood cell deformability in circulatory shock produced by endotoxin

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University of Hawaii, 1991

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EFFECTS OF DRUGS WHICH ALTER BLOOD CELL DEFORMABILITY
IN CIRCULATORY SHOCK PRODUCED BY ENDOTOXIN

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
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ABSTRACT

Pentoxifylline is a xanthine derivative chemically related to theophylline and caffeine. The drug is said to improve tissue oxygenation in chronic vascular occlusive disease by increasing RBC deformability and has been reported to improve survival in experimental hemorrhagic, septic and endotoxic shock. In the present investigation, the effects of pentoxifylline on endotoxin-induced mortality, disseminated intravascular coagulation (DIC), hypotension, and reduction in blood cell deformability were studied in awake rats and compared with the effects produced by two other xanthine compounds, theophylline and caffeine, and by a non-xanthine compound, buflomedil.

In control rats, endotoxin (25 mg/kg, i.v.) produced a 92% mortality as well as clinical laboratory signs of DIC. The latter included increased serum fibrin(ogen) degradation products (FDP), prothrombin time, and partial thromboplastin time, and decreased plasma fibrinogen and blood platelet count as well as evidence of gross visceral hemorrhage. Pretreatment with pentoxifylline (25-50 mg/kg), caffeine sodium benzoate (25-200 mg/kg) and buflomedil hydrochloride (2.5-50 mg/kg) produced a dose-dependent reduction in the endotoxin-induced mortality and inhibited most of the

manifestations of DIC produced by endotoxin. Pretreatment with theophylline (given in the form of aminophylline, 5-125 mg/kg), did not inhibit the endotoxin-induced DIC and failed to protect against death caused by the lipopolysaccharide.

The effects of drugs on blood pressure and heart rate were studied in awake rats with implanted carotid catheters. All four drugs produced a dose-dependent lowering of arterial pressure when administered as a pretreatment. The administration of endotoxin (25 mg/kg) in control animals caused an immediate fall in arterial pressure, followed by a partial recovery and a later progressive decline until death of the animal. All of the control animals in this series died within 24 hours. Pretreatment with pentoxifylline (50 mg/kg), caffeine sodium benzoate (100-200 mg/kg) and buflomedil hydrochloride (30-50 mg/kg) significantly reduced the endotoxin-induced hypotension as well as the mortality during the 24-hour period of observation. In contrast, pretreatment with aminophylline (50-100 mg/kg) not only failed to antagonize the hypotension caused by endotoxin but actually had a deleterious effect: post-endotoxin blood pressure in the aminophylline pretreated series was significantly lower than in the saline pretreated endotoxin controls throughout the period of observation. All animals in the aminophylline series died. Vasodilator agents are known to have a protective action in circulatory shock. In the present study, the hypotensive effect

produced by aminophylline was comparable to that produced by protective doses of the other agents. The difference in protective efficacy therefore was not related to a difference in vasodilator efficacy.

Heart rate measurements showed that there was no correlation between cardiac stimulation produced by the xanthines and protection against endotoxic shock. All three xanthines caused a dose-dependent increase in the heart rate and antagonized the bradycardia caused by endotoxin. However, only pentoxifylline and caffeine increased survival. Buflomedil actually slowed the heart rate but nevertheless inhibited the endotoxin-induced bradycardia and decreased the mortality.

Endotoxin (25 mg/kg) was found to cause a reduction in RBC deformability. The *in vivo* administration of pentoxifylline, caffeine and buflomedil improved the deformability of both erythrocytes and leukocytes and was found to reverse the endotoxin-induced reduction in RBC deformability. In contrast, aminophylline did not have a significant effect on blood cell deformability and did not affect the rheological effect of endotoxin.

The present investigation thus showed that prevention of endotoxin-induced DIC, circulatory shock and death by pentoxifylline, buflomedil and caffeine was positively correlated with an ability of the drugs to improve blood cell deformability. The hypothesis is

advanced that a causal relationship may exist between the latter action and the protective action of the three drugs in endotoxic shock.

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LIST OF ABBREVIATIONS

AMN	Aminophylline
BFM	Buflomedil
CAF	Caffeine
CCB	Calcium Channel Blocker
DI	Deformability Index
DIC	Disseminated Intravascular Coagulation
ETX	Endotoxin
FDP	Fibrin(ogen) Degradation Products
LPS	Lipopolysaccharide
PAF	Platelet Activating Factor
PT	Prothrombin Time
PTT	Partial Thromboplastin Time
PTX	Pentoxifylline
RBC	Red Blood Cell
WBC	White Blood Cell

I. INTRODUCTION

A. Endotoxic shock and septic shock.

Shock complicating bacteremia presents a dramatic constellation of clinical problems that a clinician must recognize, diagnose and treat. While septic shock has been known and feared by physicians for at least a century, its reported incidence has risen sharply since the classical description of gram-negative sepsis by Weil and his coworkers in the early 1960s (Weil et al., 1964). Circulatory shock is a profound disturbance of the blood circulation over a protracted period, characterized by low blood pressure and insufficient blood flow to tissues and organs, with resultant inadequate delivery of oxygen and nutrients to cells and tissues as well as inadequate elimination of cellular waste products. While the derangement is correctable initially, persistence of the shock state leads to irreversible cell injury and death. The term septic shock refers to circulatory shock associated with infection.

Clinically, gram-negative bacteria are the most common cause of septic shock (McCabe, 1974). Nevertheless, toxic shock syndrome, a disorder associated with gram-positive infection, serves as a reminder of the clinical importance of septic shock unrelated to gram-negative infection. Shock associated with gram-negative bacteremia in

humans is most likely caused by endotoxin (Ziegler et al., 1982). Endotoxin is a lipopolysaccharide released from the cell wall of disintegrating gram-negative bacteria. The key chemical structure of the lipopolysaccharide molecule consists of a lipid A moiety, a core polysaccharide and an O-antigen polysaccharide. The lipid A zone of the lipopolysaccharide molecule is associated with most of the toxicity of endotoxin (Galanos et al., 1971).

Clinical management of life-threatening septic shock includes use of antibiotics in combination with intravenous fluids (Lundberg et al., 1983) and steroids (Schumer, 1976; Weitzman, 1974). The administration of antibiotics is a main stay in the treatment of this disorder. However, Antibiotic therapy of sepsis reportedly resulted in the release of endotoxin, accentuating the condition (Shenep et al., 1985). In recent years, a number of drugs have been reported to exert beneficial effects in experimental endotoxic shock, including naloxone (Gahhos et al., 1982), thromboxane synthetase inhibitors and thromboxane receptor antagonists (Wise et al., 1980) and human endotoxin antibody (Ziegler et al., 1982), anti-tumor necrosis factor monoclonal antibodies (Tracy et al., 1987), interleukin-1 receptor antagonist (Wakabayashi et al., 1991). Previous studies in our laboratory showed that calcium channel blockers (Lee et al., 1986 & 1989) and platelet activating factor antagonists (Tang et al., 1990) were highly effective in endotoxic shock in rats.

Experimentally, the administration of endotoxin in animals produces a wide spectrum of pathophysiological manifestations resembling that seen in septic shock patients. The toxin generates profound alterations in systemic vascular resistance, myocardial contractility (Siegel et al., 1967; Gunnar et al., 1973), pulmonary function (Hinshaw et al., 1957) and cellular oxygen extraction. Circulatory shock occurs as the functions of various organ systems become compromised. While endotoxin is known to trigger a variety of pathophysiological events which purportedly might induce circulatory shock, there is no universal agreement as to the crucial mechanism(s) responsible for the derangement. The review in this introductory chapter will primarily focus on two of the known pathophysiological effects of endotoxin, viz., decreased blood cell deformability and disseminated intravascular coagulation.

B. Erythrocyte deformability.

Erythrocytes, with a mean diameter of about 7 μm , are much larger than the diameter of the capillary lumen (approximately 5 μm for arterial capillaries). Despite the size discrepancy, unimpeded flow of erythrocytes through the capillaries occurs because of the remarkable deformability or flexibility of the RBCs, which allow the latter to alter their normal biconcave shape.

Red cell deformability has been extensively studied since the 1960s by biophysicists, physiologists and hematologists, who

recognized that deformability is a very important determinant of red cell life span and of adequate perfusion of the microcirculation (Burton, 1965; Charache et al., 1967; Chien et al., 1967; Gregersen et al., 1967; Haradin et al., 1969; Jandi et al., 1961; La Celle, 1969; Murphy, 1967 & 1968; Rand, 1964; Rand & Burton, 1964; Schmid-Schonbein et al., 1969; Teitel, 1967; Teitel & Rodulesco, 1952; Weed, 1968; Weed et al., 1966 & 1969).

Drugs and chemicals have been found to alter erythrocyte deformability. Sugiura (1983) first reported that RBC deformability is decreased by endotoxin in vitro as well as in rabbits subjected to endotoxic shock. This observation has since been confirmed by other investigators (Puranapanda et al., 1987; Hurd et al., 1988). A number of drugs have been found to improve RBC deformability. A partial list includes pentoxifylline (Coccia et al., 1989; Puranapanda et al., 1987), buflomedil (Clissold et al., 1987), indobufen (Grasselli et al., 1987), cyclandelate (Timmerman, 1987) and isoxsuprine (Aarts et al., 1986).

Pentoxifylline is a xanthine derivative (Figure 1) which clinically is used for a number of circulatory disorders including the treatment of patients with intermittent claudication due to chronic occlusive arterial disease. The drug is thought to be beneficial because it increases erythrocyte deformability (Rall, 1990). The drug has been reported to improve survival in experimental hemorrhagic and septic

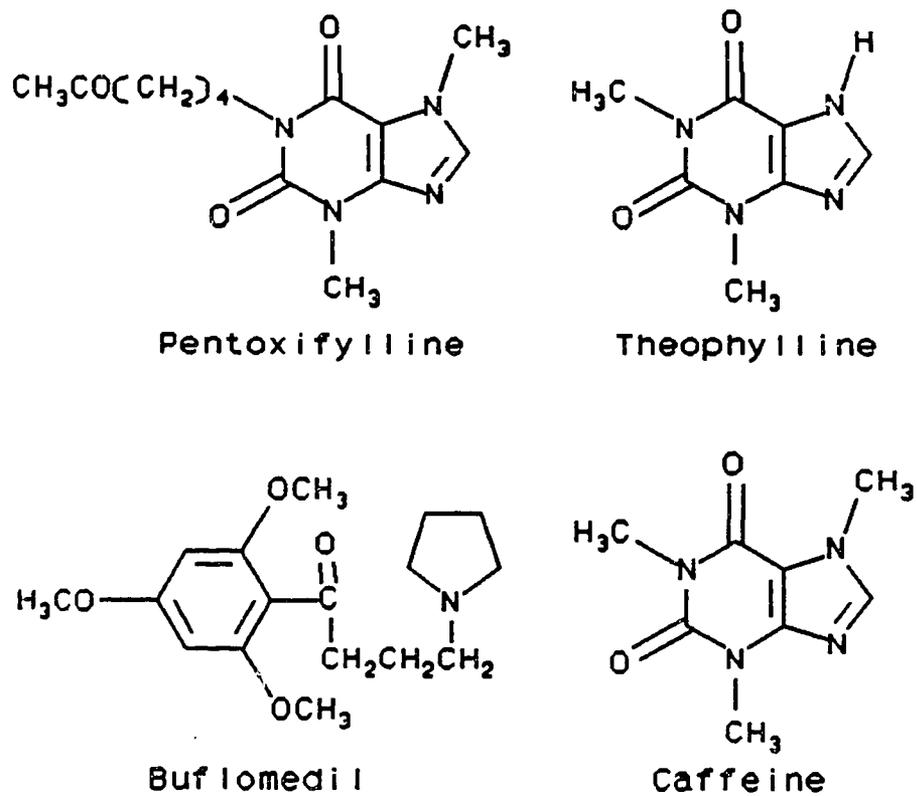


Figure 1. Chemical structures of pentoxifylline, theophylline, caffeine and bufomedil.

shock (Coccia et al., 1989; Puranapanda et al., 1987; Sullivan et al., 1984) and in experimental peritonitis (Chalkiadakis et al., 1985; Bjornson et al., 1985). Studies on the effect of this agent in endotoxic shock have yielded conflicting results, the drug having been reported to protect (Schade et al., 1986; Schonharting et al., 1989) as well as to have no effect (Sugiura, 1983) on endotoxin-induced lethality.

The rate of red cell filtration through a porous membrane has been extensively used to assess erythrocyte deformability (Chien, 1977; Dormandy, 1983). A number of filtration systems, using different pressures, filtered volume, red cell concentrations, and suspending media have been described (Matrai et al., 1984). Most of the methods involve the use of a nuclepore filter, usually with a pore diameter of 5 μm . The methods can be divided basically into two groups: (1) the constant pressure method, where the flow rate is measured and (2) the constant flow method, where pressure is recorded. The latter has the advantage of ease of recording and the disadvantage that at high pressure the shear stress may be unphysiological and unclogging of the pores may occur (Dormandy et al., 1985).

C. Leukocyte deformability.

In comparison to erythrocytes, leukocytes are fewer in number, larger in size and more complex in structure. Chien et al. (1983)

calculated that the resistance of one leukocyte to flow through a 5 μm filter is equivalent to about 700 erythrocytes. The ratio of leukocytes to erythrocytes in the blood is about 1:700; thus, leukocytes can be expected to contribute significantly to the resistance of blood flow through capillaries, which also have a diameter of about 5 μm (Schmalzer & Chien, 1984).

This important influence of leukocytes on blood fluidity has been noted by several investigators (Brooks et al., 1987; Chien et al., 1984). However, relatively scant attention has been paid to the rheological properties of leukocytes because of methodological difficulties. In 1985, Dormandy et al. developed a new blood filtration device, referred to as the St. George's filtrometer, which was found to be suitable for investigations of the mechanical properties of leukocytes. Then in 1986, Mikita et al. described a simple method of isolating and preparing white blood cells for deformability studies.

Leukocyte deformability has been reported to be affected by disease as well as drugs and chemicals. Reduced leukocyte deformability has been observed in patients with acute stroke (Ernst et al., 1987) and chronic cerebrovascular diseases (Vermes & Strik, 1987). A peptide (N-formyl-methionyl-leucyl-phenylalanine) has been reported to increase deformability of polymorphonuclear leukocytes (Kawaoka et al., 1981). The drug, pentoxifylline, has been found to improve leukocyte deformability when administered in vitro (Schmalzer

et al., 1984) as well as in vivo (Matrai & Ernst, 1985).

D. Disseminated intravascular coagulation.

Disseminated intravascular coagulation (DIC) is an acute, subacute, or chronic thrombohemorrhagic disorder occurring as a secondary complication in a variety of diseases (Cotran et al., 1989). However, this type of coagulopathy may be a primary effect of the action of endotoxin and not a secondary terminal phenomenon in endotoxic shock (Garcia-Barreno, 1978). The complex disorder is characterized by microthrombi formation with resultant obstruction of blood flow as well by internal bleeding. The former is caused by activation of the coagulation cascade while the latter relates to activation of fibrinolysis as well as consumption of clotting factors because of activation of the coagulation cascade (Figure 2) (Muller-Berghaus & Hasegawa, 1983).

A variety of clinical disorders can precipitate DIC. Obstetric complications, such as abruptio placenta, septic abortion and retained dead fetus (Schneider, 1951; Bonnar, 1973) can cause DIC because of the entrance of thromboplastin-like substances from the uterus into the maternal systemic circulation. Neoplasms (Gordon, 1975; Sack et al., 1977; Edwards et al., 1978; Weich, 1978; Curatolo, 1979; Edgington, 1980; Colucci et al., 1981), especially acute promyelocytic leukemia (Albarracin et al., 1971; Gralnick, 1975) and carcinomas of the lung, pancreas, colon, and stomach, are frequently associated

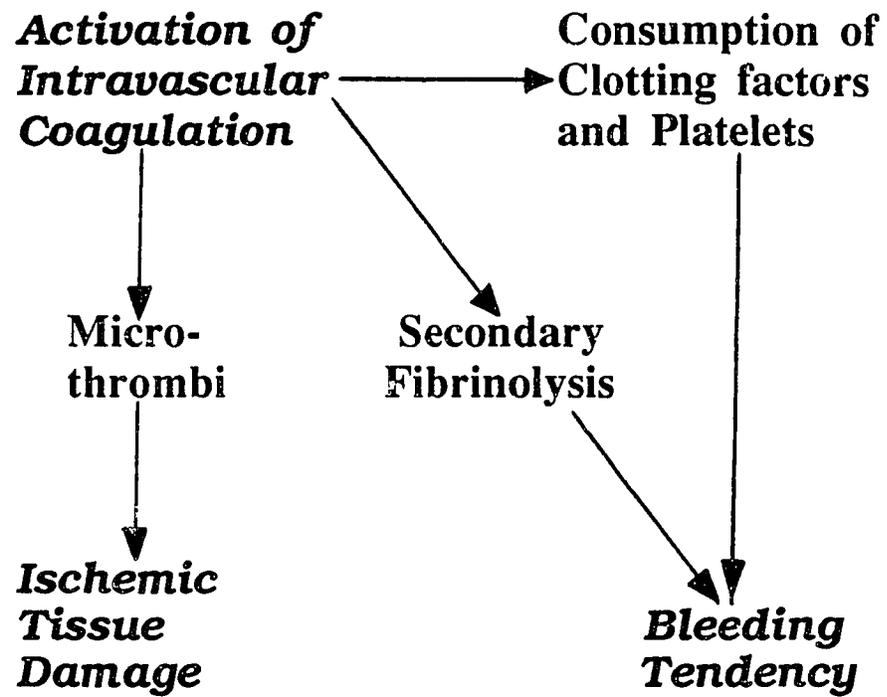


Figure 2. Sequence of events in disseminated intravascular coagulation (DIC).

with DIC. These tumors release a variety of thromboplastic substances including tissue factor, proteolytic enzymes, mucin and other undefined tumor products (Cotran et al., 1989). Massive tissue injury, such as trauma, burns and extensive surgery, can cause DIC probably by autoinfusion of tissue thromboplastin (Cotran et al., 1989). Liver diseases such as hepatic cirrhosis and severe hepatitis, may also bring about DIC (Rake, 1970; Verstraete et al., 1974; Donaldson et al., 1969; Fletcher et al., 1964; Mobarhan et al., 1971; Ogston et al., 1971). The major mechanism is that the damaged liver is compromised in two functions essential to maintenance of normal hemostasis: synthesis of coagulation proteins and clearance of procoagulants and fibrinolytic activators (Minna et al., 1974).

Infection, particularly that caused by gram-negative bacteria is probably the most common clinical cause of DIC (Yoshikawa et al., 1971). Endotoxin released by gram-negative bacteria may activate both intrinsic and extrinsic coagulation pathways by producing endothelial cell injury and by releasing of thromboplastin from inflammatory cells (Cotran et al., 1989).

Mckay and Shapiro (1958) were the first to suggest that DIC can be caused by endotoxemia. Numerous studies have since clearly established that the lipopolysaccharide can induce DIC (Bohn & Muller-Berghaus, 1976; Morrison & Cochrane, 1974; Muller-Berghaus & Lohmann, 1974; Rivers et al., 1975; etc.). Although the precise

mechanism(s) by which endotoxin causes DIC has been a topic of considerable controversy, the lipopolysaccharide is known to activate a number of steps in the coagulation-fibrinolysis cascade, any one of which could potentially trigger the coagulation disorder. These include interaction with platelets and leukocytes, activation of factor XII and the serum complement system, damage to endothelial cells, and inhibition as well as activation of fibrinolysis.

In vitro as well as in vivo studies have clearly shown that the lipopolysaccharide can interact with platelets causing them to aggregate and as well as to release factors which induce coagulation (Davis et al., 1960 & 1961; Des Prez et al., 1961; Horder et al., 1965; Ream et al., 1965; Cohen et al., 1966; Nagayama et al., 1971; Brown et al., 1973).

Endotoxin binds to receptors on WBCs (Springer et al., 1975; Hawiger et al., 1977; Muller-Berghans, 1978) causing aggregation, release of chemical mediators as well as increased procoagulant activity of the leukocytes (Hiller et al., 1977; Lerner et al., 1977; Rickles et al., 1977; Tono-Oka 1978; Edwards et al., 1979). Garner et al. (1974) reported that lipopolysaccharide activated the serum complement system. The latter might play an important role in the process of leukocyte activation and tissue factor release (Prydz et al., 1977; Rothberger et al., 1977; Osterud et al., 1982).

Endotoxin has been reported to cause injury to vascular endothelium (Morrison et al., 1978; MacIntyre et al., 1978). With endothelial damage, exposure of blood to the underlying collagen can activate Factor XII and thus bring about stimulation of coagulation as well as fibrinolysis. In 1983, Colucci et al. reported that human endothelial cells generated procoagulant activity (tissue factor) in response to endotoxin. Morrison (1974) reported that endotoxin activated the fibrinolytic system both by stimulating the release of plasminogen proactivator from endothelial cells and by activating factor XII.

Laboratory diagnosis of DIC is based on various coagulation tests that are capable of reflecting abnormalities in the hemostatic mechanism. Screening tests of DIC include measurements of prothrombin time (PT), plasma fibrinogen and blood platelet count. Abnormalities in all three measurements is considered to be diagnostic of DIC. If one of the three is normal, confirmatory tests, including partial thromboplastin time (PTT) (Colman et al., 1970), serum fibrinogen degradation products (FDP) (Thomas et al., 1970), thrombin time (Thomas et al., 1967), and euglobulin clot lysis time (ELT) (Kowalski et al., 1959), are used. DIC varies markedly in the intensity of clinical manifestations and in the laboratory findings. Clinically, different types of DIC such as acute and chronic (McKay, 1973; Merskey, 1968), and decompensated, compensated or

overcompensated (Cooper et al., 1974; Sharp, 1977) may exist. The only common laboratory finding in these various types of DIC is the elevation of serum FDP (Sharp, 1977; Kobayashi et al., 1983).

E. Objectives.

The mechanism(s) by which endotoxin produces circulatory shock and death is complex and incompletely understood. The principal objectives of the present investigation were to assess the possible importance of a change of blood cell deformability in (1) the pathogenesis of DIC and circulatory shock induced by the lipopolysaccharide, (2) the mechanism of protection induced by pentoxifylline against endotoxic shock. The following drugs were used as "tools" to accomplish these objectives: (a) pentoxifylline and buflomedil, chemically dissimilar drugs which are known to improve blood cell deformability; and (b) theophylline and caffeine, xanthine compounds which are chemically related to pentoxifylline but have not been studied for effect on blood cell deformability.

II. MATERIALS AND METHODS

A. Studies on endotoxin-induced mortality.

Male Wistar rats, awake, weighing 220 to 270 grams, were fasted overnight and given E. coli endotoxin (Lipopolysaccharide 0127:B8, Difco Laboratories) intravenously via a tail vein at a dose of 25 mg/kg. Pentoxifylline, theophylline (in the form of aminophylline), caffeine and buflomedil (Figure 3) were injected 15 minutes before or 15 minutes after the administration of endotoxin. Control rats were treated with an equivalent volume of normal saline instead of the agents described above. Cumulative mortality was recorded at 6, 24, and 48 hours following the endotoxin administration.

B. Studies on arterial blood pressure and heart rate.

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). An incision was made on the ventral surface of the neck and a heparin-filled (300 units/ml) polyethylene catheter (PE 50), approximately 40 cm in length, was implanted in the right common carotid artery. The other end of the catheter was threaded subcutaneously and exteriorized cervically through a dorsal cutaneous puncture and the incision was closed with wound clips. The rats were allowed to recover from anesthesia and studied the following day. Arterial blood pressure was monitored via the carotid catheter

connected to a Statham P-23 transducer and Grass Model 7 polygraph. Heart rate was measured by counting the pulses on the blood pressure tracing.

C. Hematological measurements.

A polyethylene catheter, approximately 20 cm in length, was implanted into the right common carotid artery one day prior to the experiment, as described above. Blood samples were drawn from the catheter for clinical laboratory tests of DIC and for measurements of erythrocyte and leukocyte deformability. A volume of normal saline, equivalent to the amount of blood removed, was administered via the catheter immediately following blood drawing.

1. Measurements of serum fibrin(ogen) degradation products (FDP), blood platelet count, plasma fibrinogen, prothrombin time (PT), and partial thromboplastin time (PTT). Arterial blood (approximately 1.7 ml) was drawn before and 3 hours after the i.v. administration of endotoxin (25 mg/kg). Drugs being studied were injected intravenously 15 minutes before endotoxin. A 0.2 ml blood sample was placed in an FDP collection tube for measurement of fibrin(ogen) degradation products (FDP); 0.3 ml was anticoagulated with 1.0% EDTA (1:9 V/V) for measurement of hematocrit and platelet count; another 1.2 ml was anticoagulated with 3.8% sodium citrate (1:9 V/V) for determinations of fibrinogen, PT, and PTT.

Serum fibrin(ogen) degradation products (FDP) were measured by means of a commercial kit (American Dade) which utilizes a latex-agglutination test. In the test, FDP present in serum reacts with antibodies on the surface of latex particles and produces visible agglutination. A serial dilution of the serum is used to quantify the serum FDP level.

Plasma platelets were counted with the aid of a platelet determination kit (American Scientific Products) and a Coulter counter (Model ZF). The blood platelet count was calculated from the plasma platelet value and the hematocrit.

Plasma obtained from citrated blood centrifuged at 3800 rpm for 15 minutes was used for measurements of fibrinogen, PT and PTT. Fibrinogen was assayed by means of a thrombin titration method using a commercial kit (American Dade). PT and PTT were measured with commercial kits (Instrumentation Laboratories).

2. Erythrocyte deformability. Filtration rate through a nuclepore membrane (5 μm pore size, Thomas Scientific) was used as a measure of RBC deformability. RBC suspensions were prepared by a modification of the procedure described by Hurd et al. (1988). An arterial blood sample (approximately 2.5 ml), was anticoagulated with disodium EDTA (2 mg/ml). The sample was centrifuged at 1500 g for ten minutes, and the plasma and buffy coat fraction, including some of the uppermost erythrocytes, were aspirated and discarded.

The packed RBCs were washed three times in a Tris-buffered Ringer solution (pH 7.4), consisting of 0.9 gm/dl NaCl, 0.03 gm/dl KCl, 13.33 mg/dl (1.2 mmol/L) Ca_2Cl , 90.83 mg/dl (7.5 mmol/L) Tris and 0.1 gm/dl disodium EDTA and centrifuged at 1500 g for 5 minutes each time. After the final wash, the cells were diluted to a hematocrit of approximately 0.07, using the same buffer and mixed by inversion.

The apparatus (Figure 3) used for this measurement was a modification of that described by Reid et al. (1976). A 1.0 ml sample of the red cell suspension, introduced with a 1 ml syringe, was filtered through the 5 μm nuclepore membrane at room temperature using a filtration pressure of -30 cm H_2O (Figure 3). A stopwatch was used to time the passage of the cell suspension through the membrane.

A deformability index (DI) was calculated using the equation: $\text{DI} = \frac{H(60s)}{t}$, in which H is the hematocrit and t is the time in seconds for the 1.0 ml RBC suspension to pass through the filter. Measurements were made in triplicates and procedures were completed within 3 hours of blood drawing.

3. Leukocyte deformability. White blood cell suspensions were prepared by a procedure modified from that described by Mikita et al., 1986. A 3.0 ml blood sample, drawn from the carotid catheter and anticoagulated with disodium EDTA (2 mg/ml), was centrifuged at

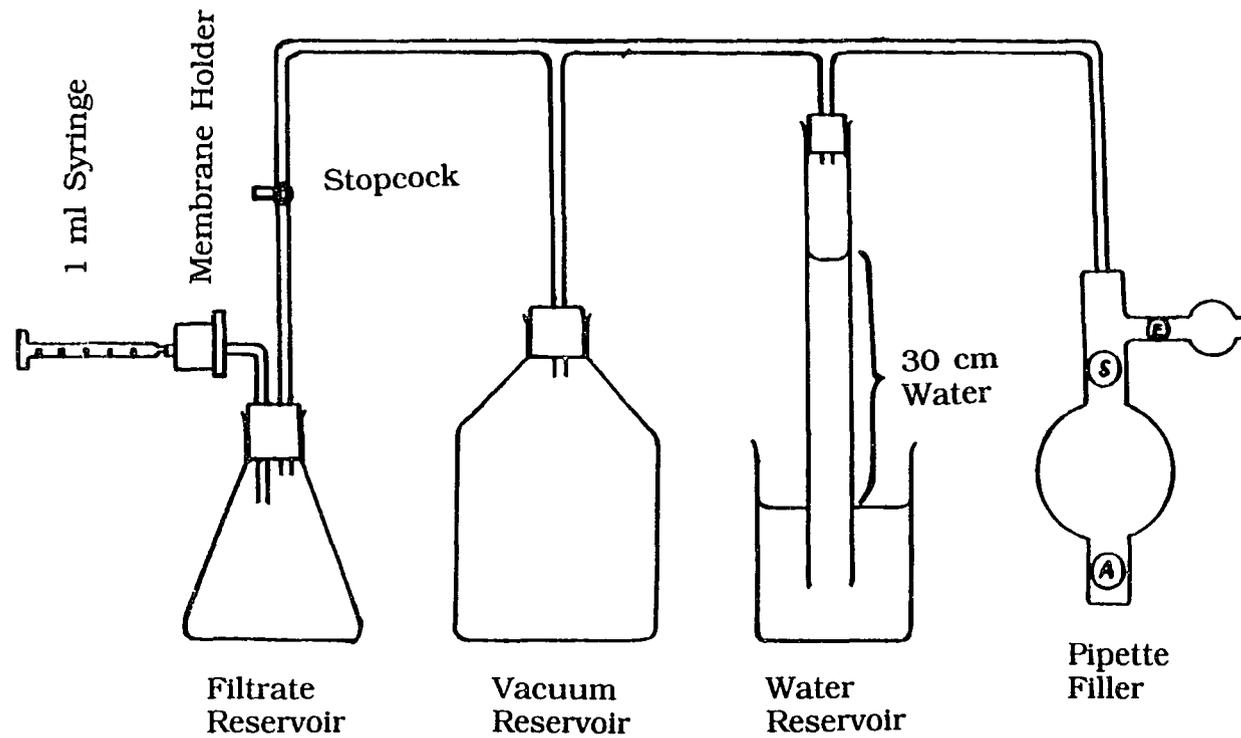


Figure 3. A schematic representation of apparatus for measurement of blood cell deformability.

The pipette filler was used to create a negative pressure of 30 cm H₂O.

1500 g for 10 minutes. The upper two thirds of the buffy (leucocytic) coat was carefully aspirated without disturbing the RBC layer and was resuspended in 2 ml of buffer solution (same as that for the RBC deformability) and centrifuged at 400 g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 2 ml buffer and recentrifuged at 400 g for 5 minutes. The final pellet was suspended in 2 ml of buffer. The WBC count of the suspension was determined with a Coulter counter (Model ZF) and the suspension was diluted with buffer to yield a final count of 200 cells/ μ l. One ml of the WBC suspension was passed through a 5 μ m nuclepore membrane filter at a filtration pressure of -30 cm H₂O using the apparatus described above. The filtration rate (ml/min) was used as a measure for WBC deformability index (DI). The deformability index (DI) was calculated from the equation: $DI = 60s/\underline{t}$, where \underline{t} is the measured time in seconds for 1.0 ml of the WBC suspension to pass through the filter. Measurements were made in triplicates and the DI was determined within 2 hours after drawing of blood.

D. Drugs.

E. coli endotoxin (Lipopolysaccharide 0127:B8, Difco Laboratories), caffeine sodium benzoate (Sigma Chemical Co.), bufloxedil hydrochloride (Abbott Laboratories), and pentoxifylline (Hoechst-Roussel Pharmaceuticals) were dissolved in physiological saline immediately before use. Theophylline was used in the form of

aminophylline, the water soluble complex of theophylline with ethylenediamine (Abbott, 25 mg/ml ampul). Except for pentoxifylline, doses of the drugs utilized in this study are expressed in terms of their salts; their dose equivalences in terms of the free base are shown in Table 1. Drug solutions were injected intravenously over a period of 15 to 20 seconds.

E. Statistical analyses.

Values are given as the mean \pm standard error (S.E.). The Chi-square test with Yates' correction for small numbers was used for the mortality data. Student's t test for grouped comparisons, the t test for paired data and ANOVA with the Newman-Keuls Studentized range test for multi-group comparisons were used as appropriate. P values of less than 0.05 were considered significant.

Table 1. Dose equivalencies

Drug	Dose as Salt (mg)	Dose as Active Base (mg)
Aminophylline (theophylline ethylene- diamine)	5	3.9
	10	7.9
	25	19.7
	50	39.4
	100	78.8
	125	98.5
Buflomedil hydrochloride	2.5	2.2
	5	4.5
	7.5	6.7
	10	8.9
	15	13.4
	30	26.8
Caffeine sodium benzoate	25	12
	50	24
	100	48
	200	96
Pentoxifylline	---	25
	---	50

III. RESULTS

A. Studies on endotoxin-induced mortality.

1. Dose-mortality relationship in control rats. The intravenous administration of endotoxin produced a dose-dependent mortality in control animals, awake, pretreated with physiological saline (Figure 4). The 48-hour mortality was 50%, 60%, 64%, and 92% for the doses of 10, 15, 20, and 25 mg/kg of endotoxin, respectively. The LD50 (lethal dose-50) of endotoxin at 48 hours was approximately 10 mg/kg. The 25 mg/kg dose, which produced a 92% mortality, was selected for use in subsequent studies on the protective effects of drugs.

2. Effect of pretreatment with pentoxifylline. Pentoxifylline was given intravenously 15 minutes before endotoxin (25 mg/kg). The drug produced a dose-dependent protection against endotoxin-induced death. No deaths occurred in the series of rats pretreated with 50 mg/kg of pentoxifylline, in contrast to the 92% mortality observed in control rats at 48 hours post-endotoxin (Figure 5). Pretreatment with a smaller dose (25 mg/kg) significantly reduced the mortality from 50% to 0% at 6 hours post-endotoxin; however, no significant reduction in mortality was observed at 24 and 48 hours.

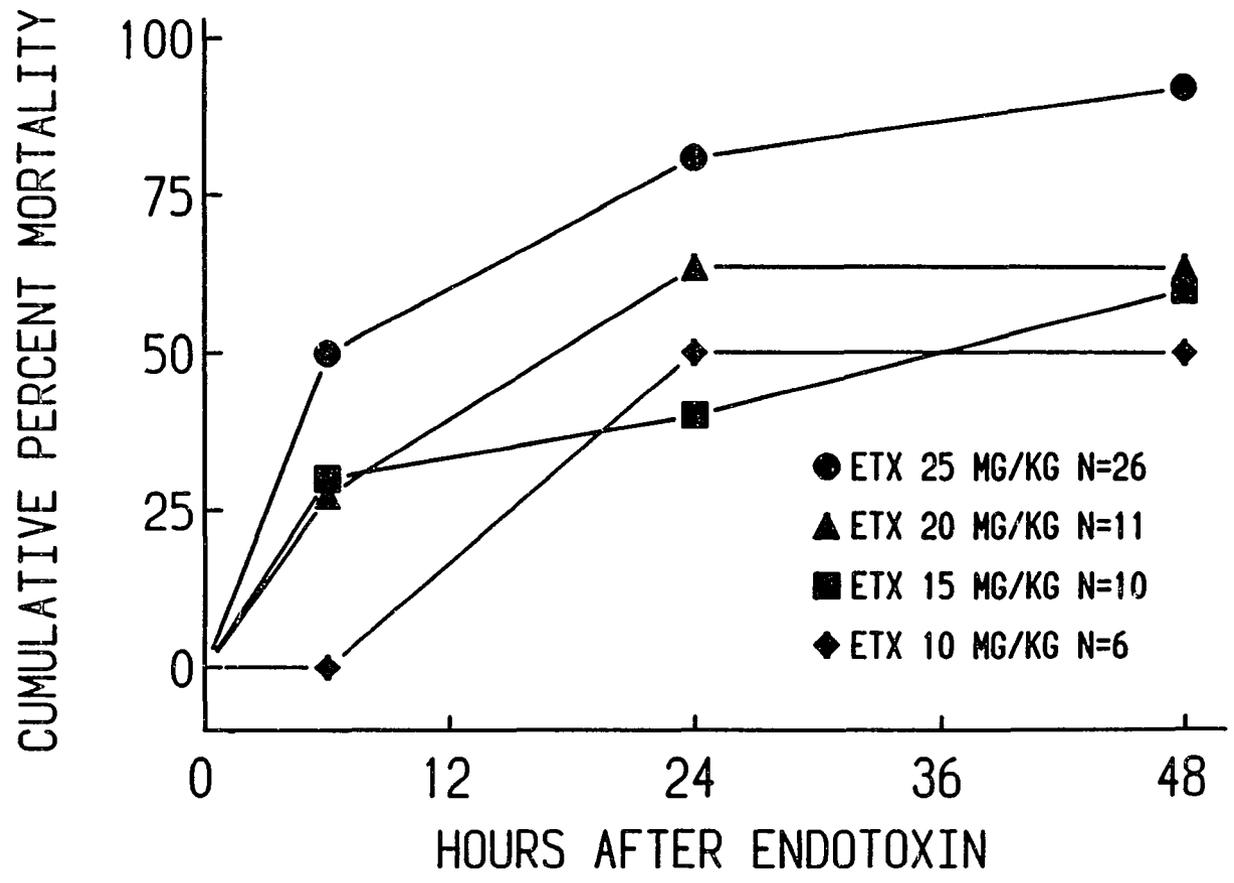


Figure 4. Dose-mortality relationship of intravenous administration of endotoxin in rats.

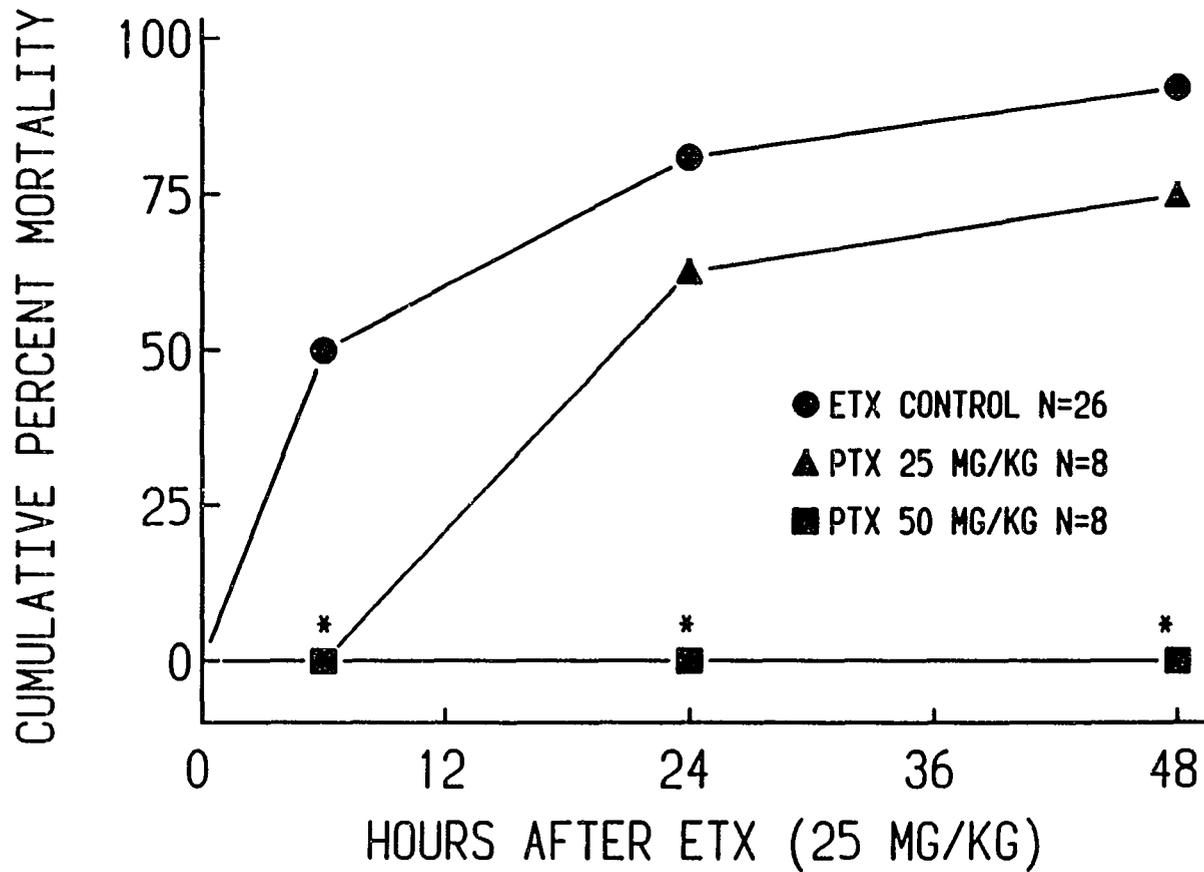


Figure 5. Effect of pretreatment with pentoxifylline (PTX) on mortality induced by endotoxin.

PTX given 15 minutes before ETX.

* Significantly different from the control.

3. Effect of pretreatment with buflomedil. Treatment with intravenous buflomedil hydrochloride (2.5-50 mg/kg), 15 minutes prior to endotoxin injection, produced a dose-dependent protection. A dose of 50 mg/kg of the drug significantly reduced the mortality from a control of 92% to 12.5% at 48 hours post-endotoxin (Figure 6). Smaller doses of the drug had little or no effect.

4. Effect of pretreatment with caffeine. Caffeine sodium benzoate (25-200 mg/kg), injected intravenously 15 minutes before endotoxin (25 mg/kg), produced a dose-dependent protection in the endotoxin-induced mortality. The 200 mg/kg dose of the drug reduced the mortality to 14% at 6, 24, and 48 hours post-endotoxin (Figure 7). At 100 mg/kg dose, it produced a significant decrease in the mortality at 6 hours but not at 24 and 48 hours. Other doses of the drug had no effect.

5. Effect of pretreatment with aminophylline. Pretreatment with aminophylline (5-125 mg/kg), 15 minutes before endotoxin, did not significantly affect endotoxin-induced mortality (Figure 8). Some of the animals exhibited transient grand mal convulsions with the 125 mg/kg dose of the drug (4 out of 6 in the series); for this reason, higher doses were not studied.

6. Effect of post-treatment with pentoxifylline. This group of animals received a dose of 50 mg/kg of pentoxifylline, 15 minutes after the administration of endotoxin. No significant protective

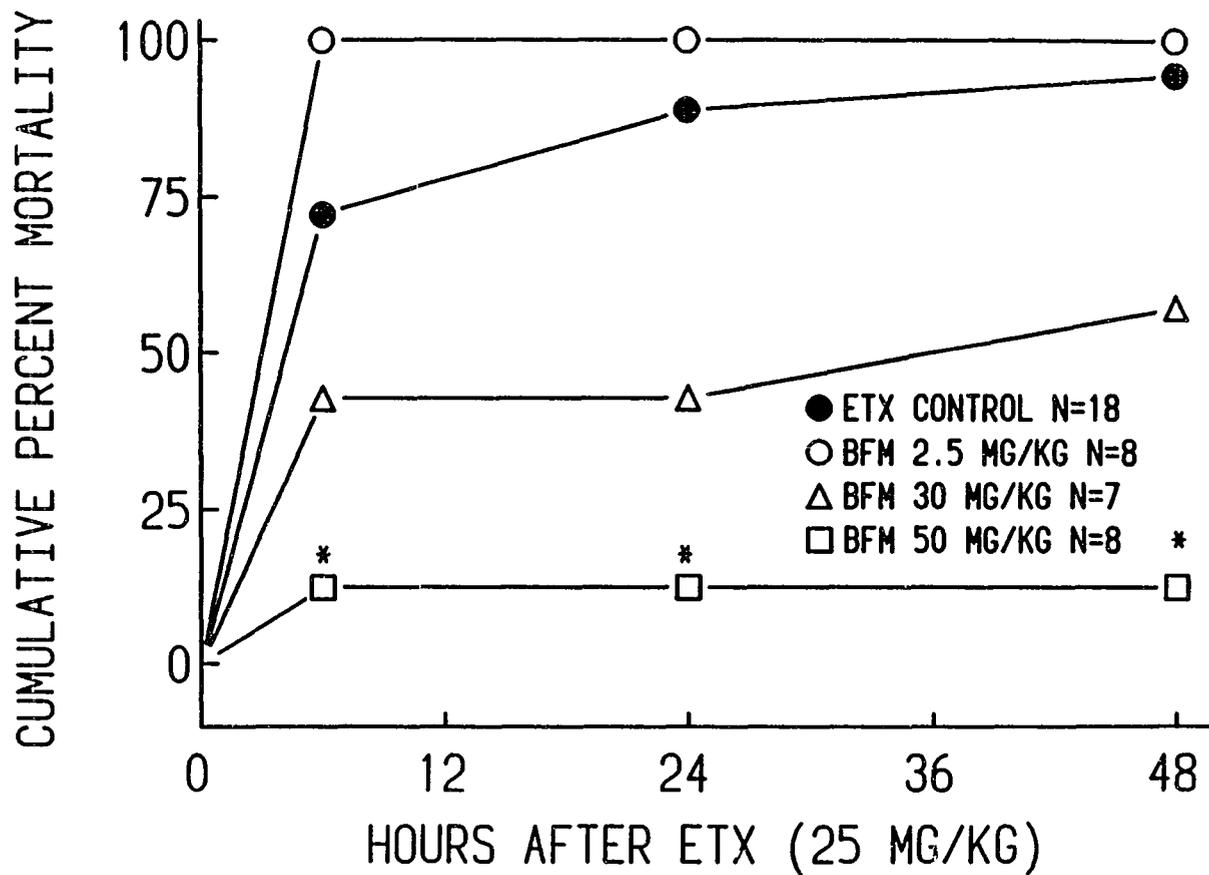


Figure 6. Effect of pretreatment with buflomedil (BFM) on mortality induced by endotoxin.

BFM given 15 minutes before ETX. *Significantly different from control. Series pretreated with the doses of 5, 7.5, 10 and 15 mg/kg not shown. No significant protection observed with the unshown doses.

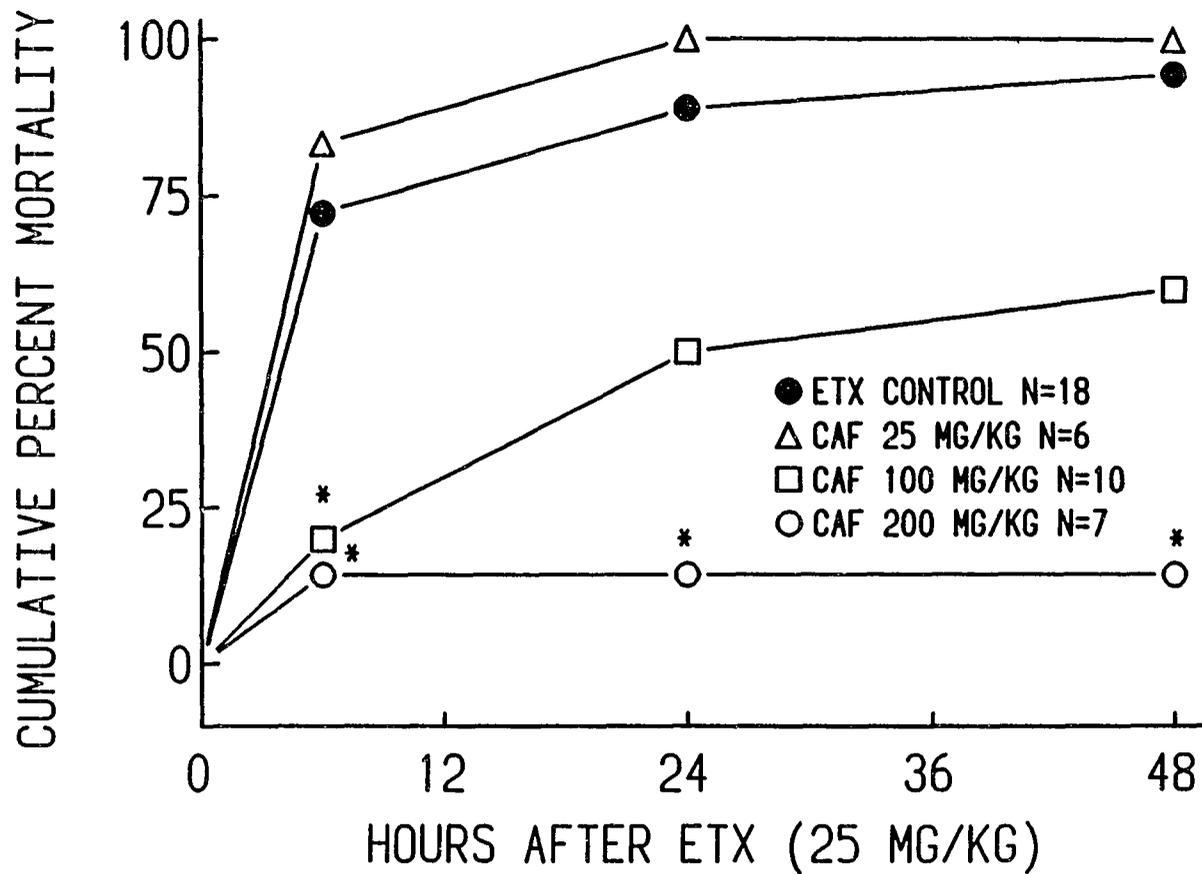


Figure 7. Effect of pretreatment with caffeine (CAF) on mortality induced by endotoxin.

CAF given 15 minutes before ETX. *Significantly different from control. Series pretreated with the dose of 50 mg/kg not shown. No significant protection observed with the unshown dose.

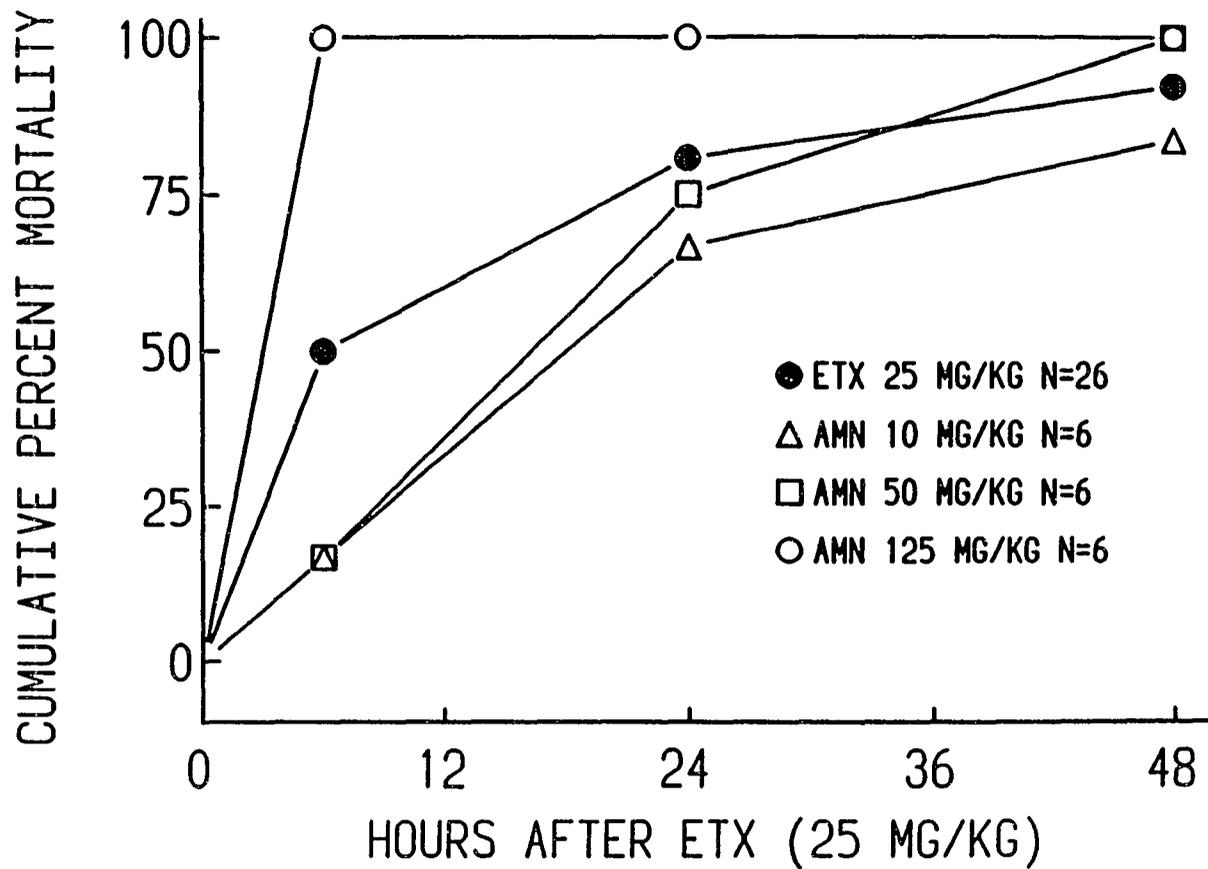


Figure 8. Effect of pretreatment with aminophylline (AMN) on mortality induced by endotoxin.

AMN given 15 minutes before ETX.

Series pretreated with the doses of 5, 25 and 100 mg/kg not shown.

No protection was produced by pretreatment with any of the doses of AMN.

action was observed (Figure 9).

B. Studies on arterial blood pressure.

Mean arterial pressure and heart rate were recorded periodically over a 24-hour period in animals, awake, with an implanted carotid catheter. In saline-pretreated controls, endotoxin (25 mg/kg, i.v.) caused an immediate fall in arterial pressure, from a baseline of 142 ± 4 mm Hg to a value of 57 ± 3 mm Hg (Figure 10). A partial recovery of blood pressure quickly ensued and remained fairly stable at approximately 85 mmHg for about 4.5 hours; the blood pressure then declined progressively until death of the animal. All of the endotoxin controls in this series of animals with surgically implanted catheters died within 24 hours.

The xanthine compounds and buflomedil, administered intravenously 15 minutes before endotoxin, produced a dose-dependent fall in blood pressure (Table 2). Pentoxifylline (50 mg/kg), aminophylline (50 mg/kg), caffeine sodium benzoate (100 mg/kg) and buflomedil hydrochloride (30 mg/kg) had approximately equal hypotensive effects, producing mean decreases in pressure of 46 to 53 mm Hg, within a minute after drug administration. Blood pressure returned to approximately baseline levels within 15 minutes after the administration of caffeine and buflomedil. The hypotensive effect of pentoxifylline and aminophylline persisted longer, with the mean pressures being 21 and 15 mm Hg below baseline, 15 minutes after

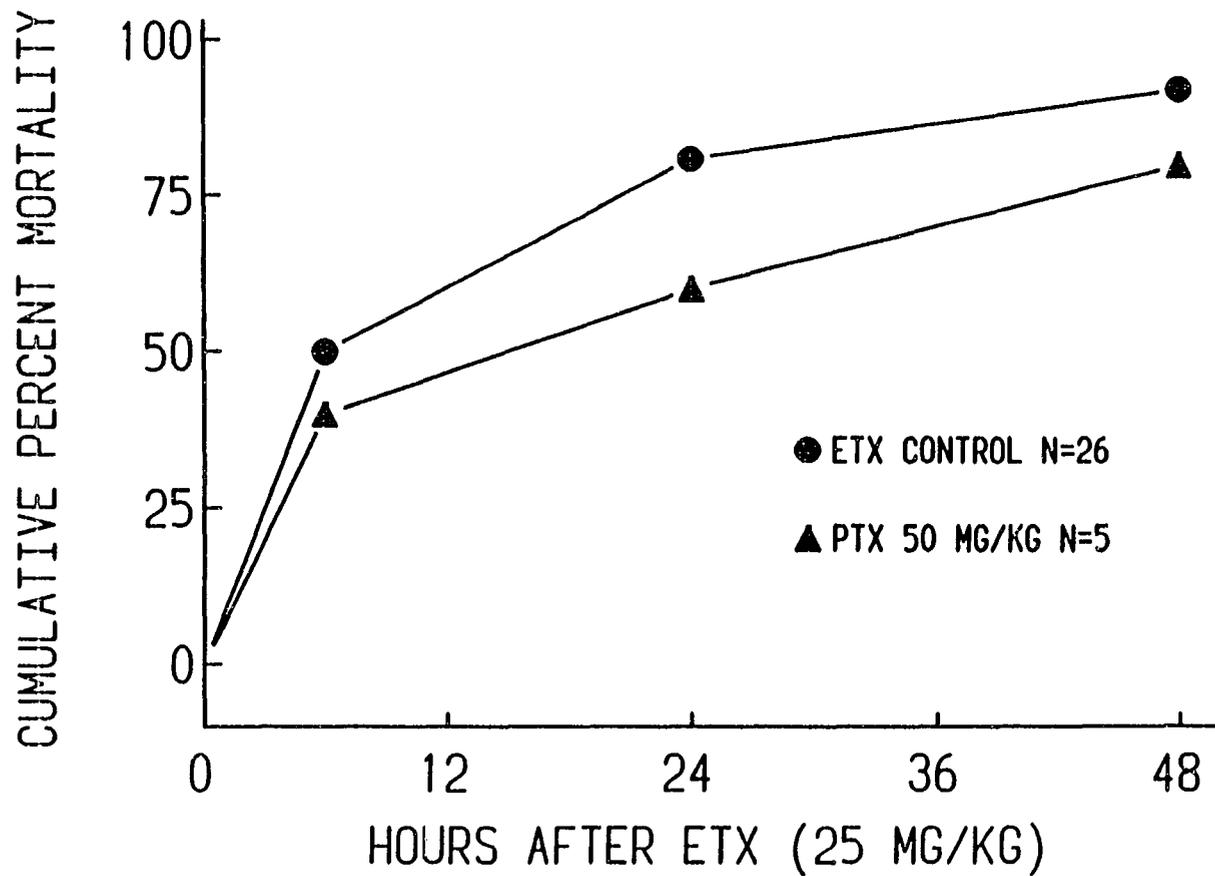


Figure 9. Effect of post-treatment with pentoxifylline (PTX) on mortality induced by endotoxin.

PTX given 15 minutes after ETX. No significant protection.

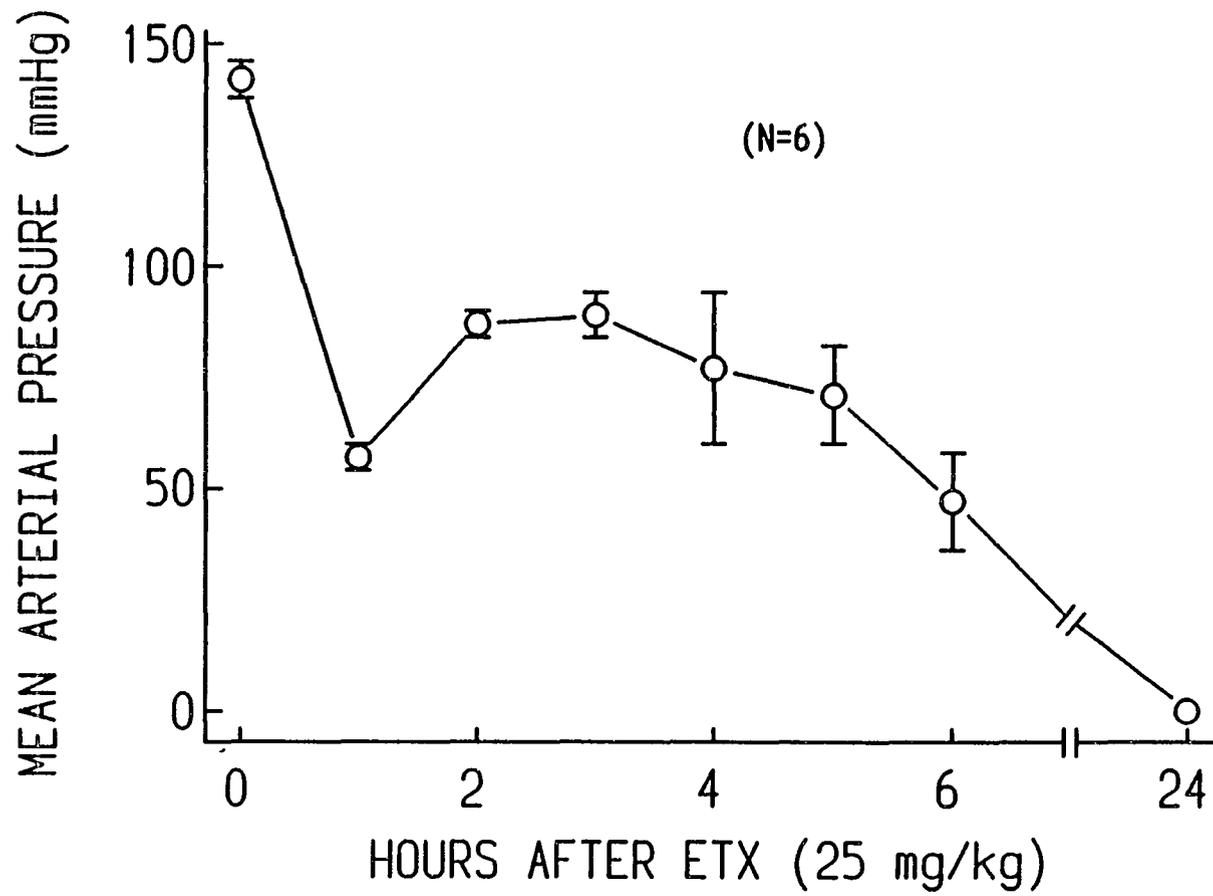


Figure 10. Effect of intravenous administration of endotoxin on mean arterial pressure.

Table 2. Effects of drugs on mean arterial Pressure

Drugs	Mean Arterial Pressure (Mean \pm S.E. mmHg)			
	Minutes After Drug Administration			
	0	1	5	15
Pentoxifylline				
50 mg/kg (N=8)	129 \pm 4	83 \pm 4*	99 \pm 5*	114 \pm 4*
Buflomedil				
30 mg/kg (N=8)	133 \pm 2	80 \pm 4*	121 \pm 3*	127 \pm 2*
50 mg/kg (N=8)	135 \pm 3	65 \pm 4*	106 \pm 2*	119 \pm 2*
Caffeine				
100 mg/kg (N=8)	135 \pm 3	83 \pm 3*	132 \pm 3	130 \pm 3
200 mg/kg (N=8)	133 \pm 3	67 \pm 3*	124 \pm 3*	126 \pm 3*
Aminophylline				
50 mg/kg (N=8)	132 \pm 4	85 \pm 4*	102 \pm 4*	111 \pm 4*
100 mg/kg (N=8)	138 \pm 1	70 \pm 6*	107 \pm 4*	102 \pm 4*

* Significantly different from base line (Zero time).

administration of the two drugs, respectively (Table 2).

Pretreatment with pentoxifylline (50 mg/kg) and buflomedil hydrochloride (30-50 mg/kg) significantly diminished the fall in blood pressure caused by endotoxin during most of the period of observation (Figures 11-12). Two of eight animals in the pentoxifylline pretreated series died within 24 hours post-endotoxin. No deaths were observed in the buflomedil hydrochloride groups 24 hours after endotoxin.

Caffeine sodium benzoate (100-200 mg/kg) was less effective than the other two agents in antagonizing the hypotensive effect of endotoxin during the early periods of observation; however, blood pressure values in the caffeine-pretreated series were substantially better than in the saline-pretreated controls beyond 4 hours post-endotoxin (Figure 13). In the caffeine pretreated series, no animals died within 24 hours after the endotoxin administration. Post-endotoxin blood pressure readings were significantly lower in the aminophylline (50-100 mg/kg) pretreated series than in the controls throughout most of the period of observation (Figure 14); as in the controls, all of the animals in the aminophylline-pretreated series died within 24 hours.

C. Studies on heart rate.

Heart rate was measured in the blood pressure experiments (above) by counting the pulses in arterial pressure tracing.

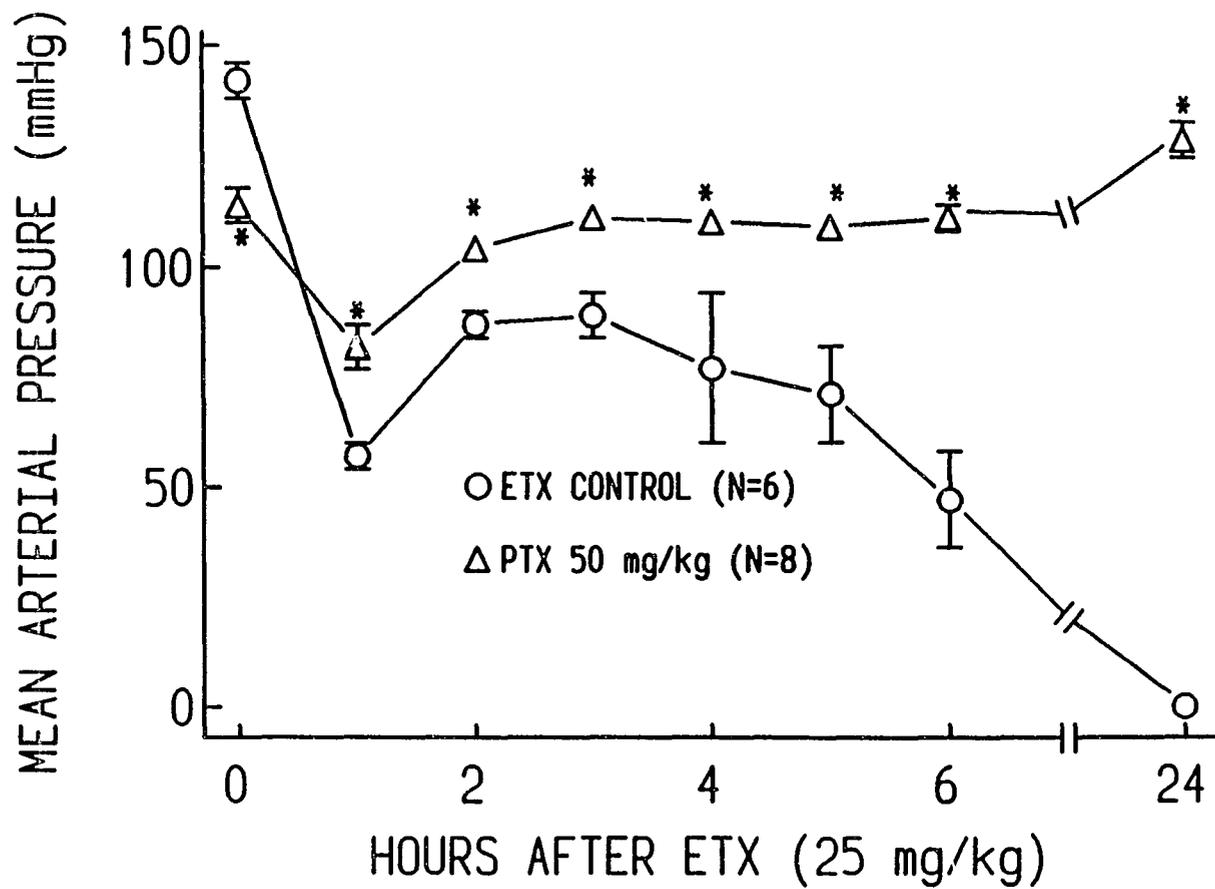


Figure 11. Effect of pentoxifylline on mean arterial pressure in endotoxic shock.

PTX given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

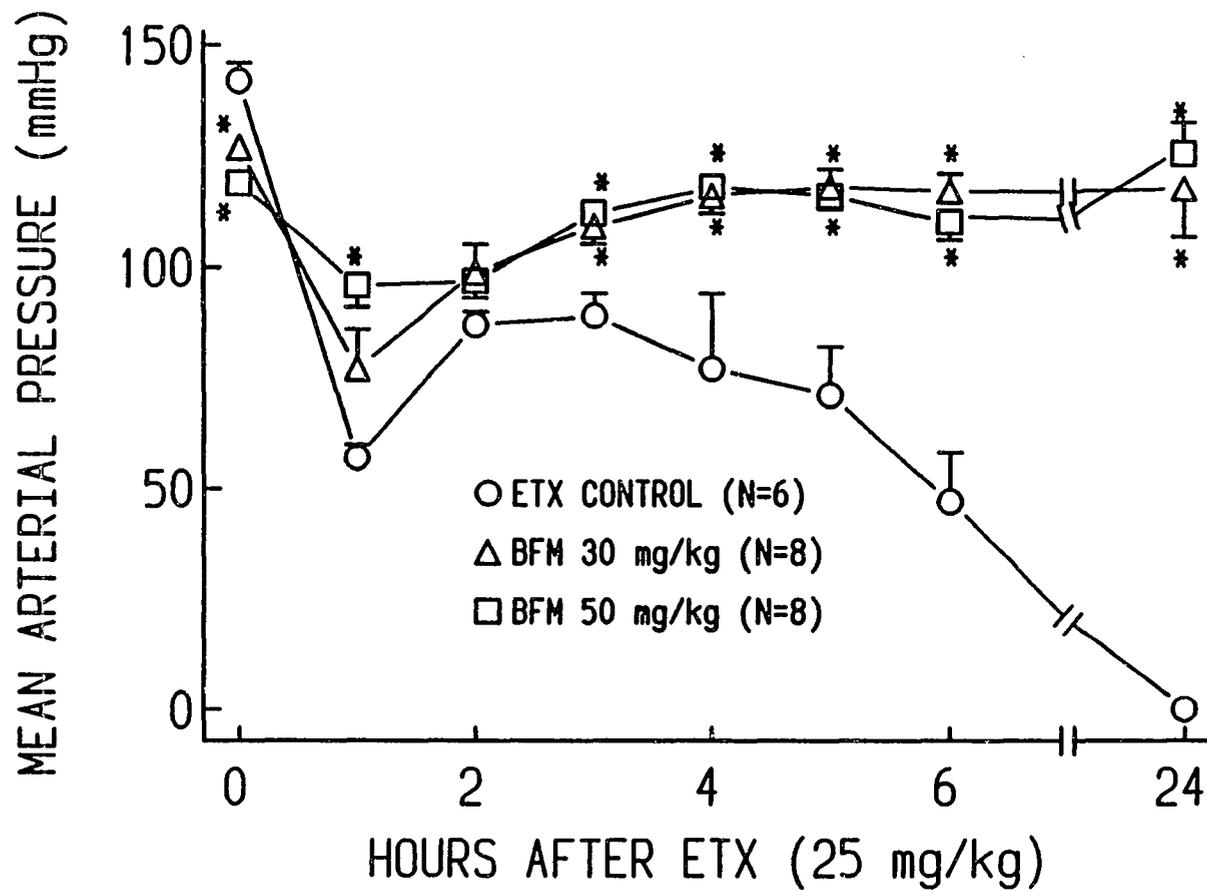


Figure 12. Effect of buflomedil on mean arterial pressure in endotoxic shock.

BFM given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

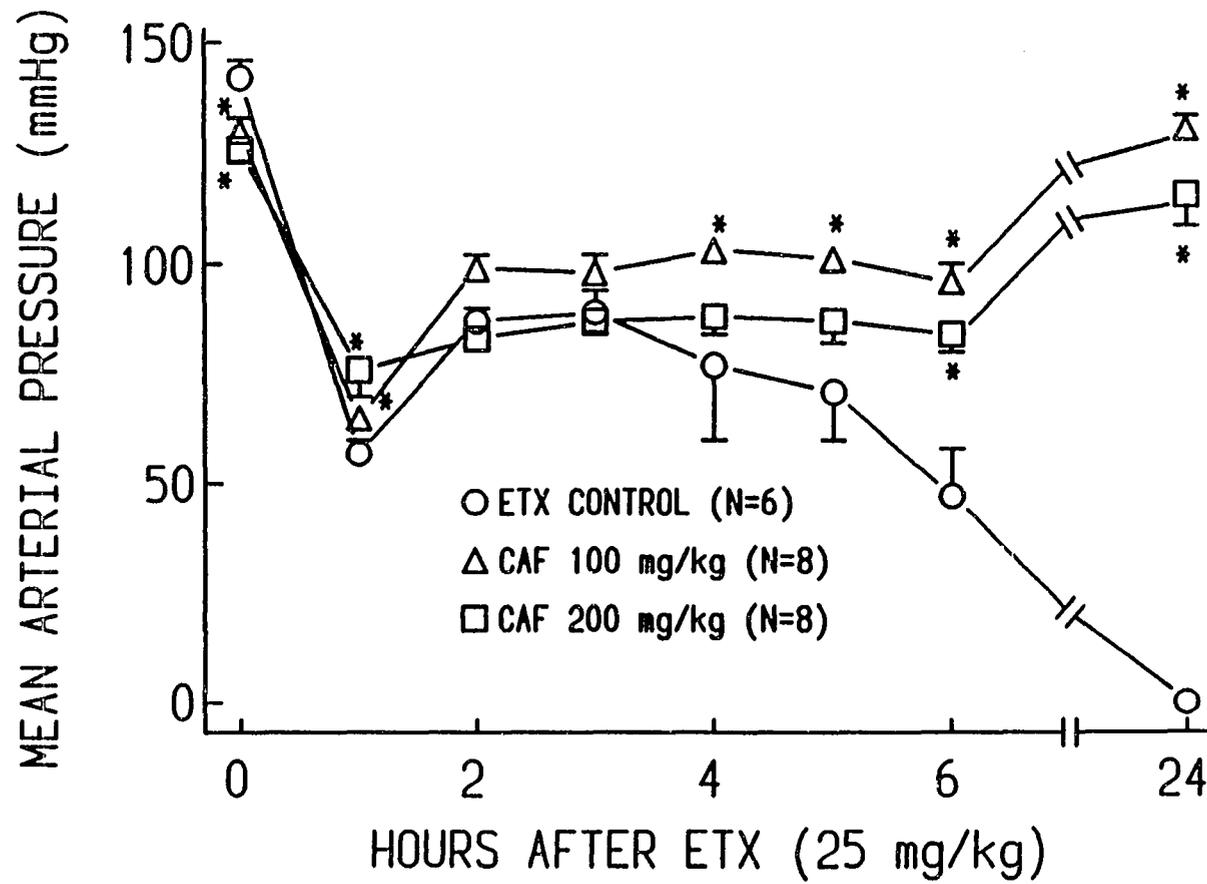


Figure 13. Effect of caffeine on mean arterial pressure in endotoxic shock.

CAF given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

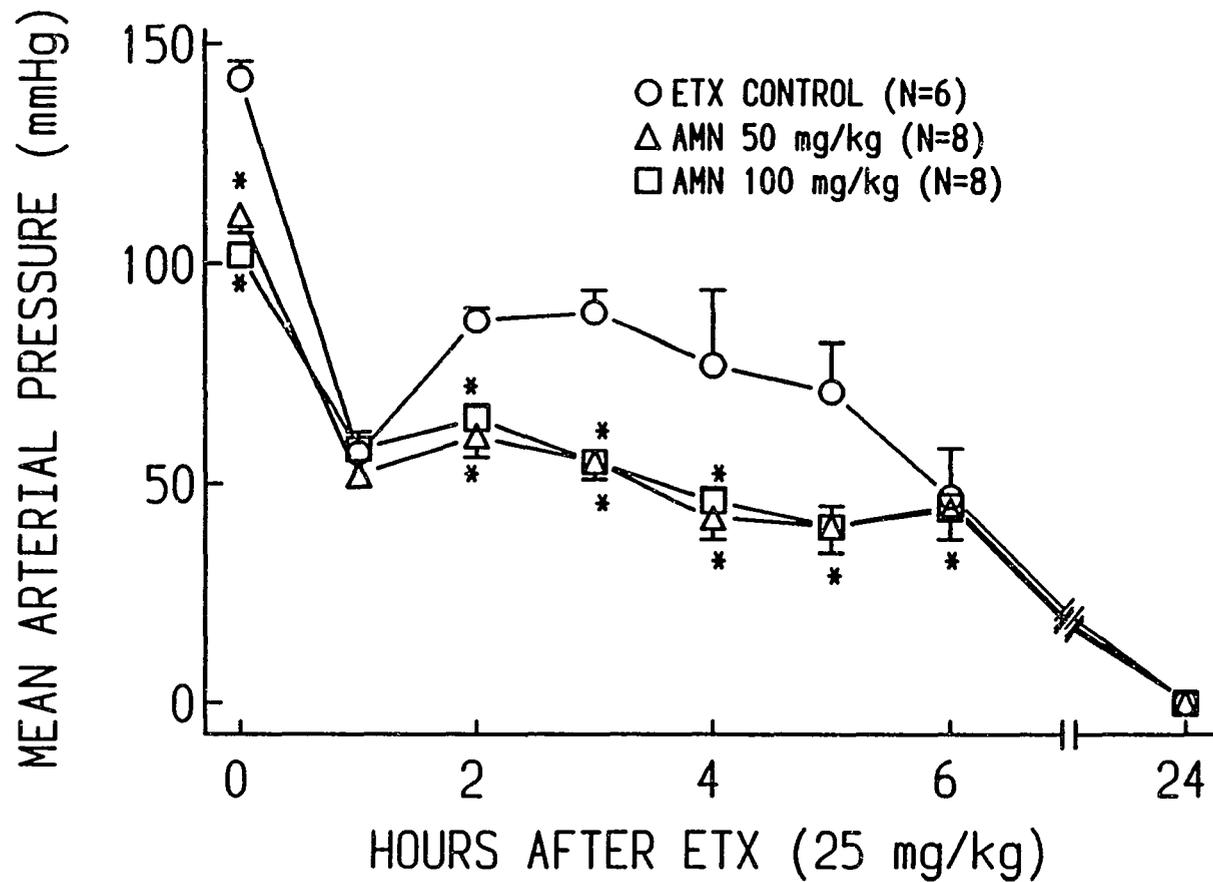


Figure 14. Effect of aminophylline on mean arterial pressure in endotoxic shock.

AMN given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

Endotoxin produced a progressive decline in the heart rate in control animals pretreated with saline (Figure 15).

The three xanthine compounds, given as pretreatment, produced an immediate increase in the heart rate (Table 3). All three drugs also dose-dependently antagonized the bradycardia caused by endotoxin (Figures 16-18).

The lower dose of buflomedil (30 mg/kg) produced a mild increase in the heart rate; the higher dose (50 mg/kg) produced a decrease in the rate (Table 4); nevertheless, the endotoxin-induced bradycardia was diminished in animals pretreated with both doses of the drug, particularly during the later periods of observation (Figure 19).

D. Studies on clinical laboratory tests for DIC.

Blood samples were drawn prior to and at three hours after the intravenous administration of endotoxin for measurements of serum fibrin(ogen) degradation products (FDP), blood platelet count, plasma fibrinogen, prothrombin time (PT) and partial thromboplastin time (PTT). In saline-pretreated controls, endotoxin (25 mg/kg) produced changes in clinical laboratory tests which were pathognomonic of DIC, including an elevation in serum FDP, increases in PT and PTT and reductions in plasma fibrinogen and blood platelet count (Figures 20-31).

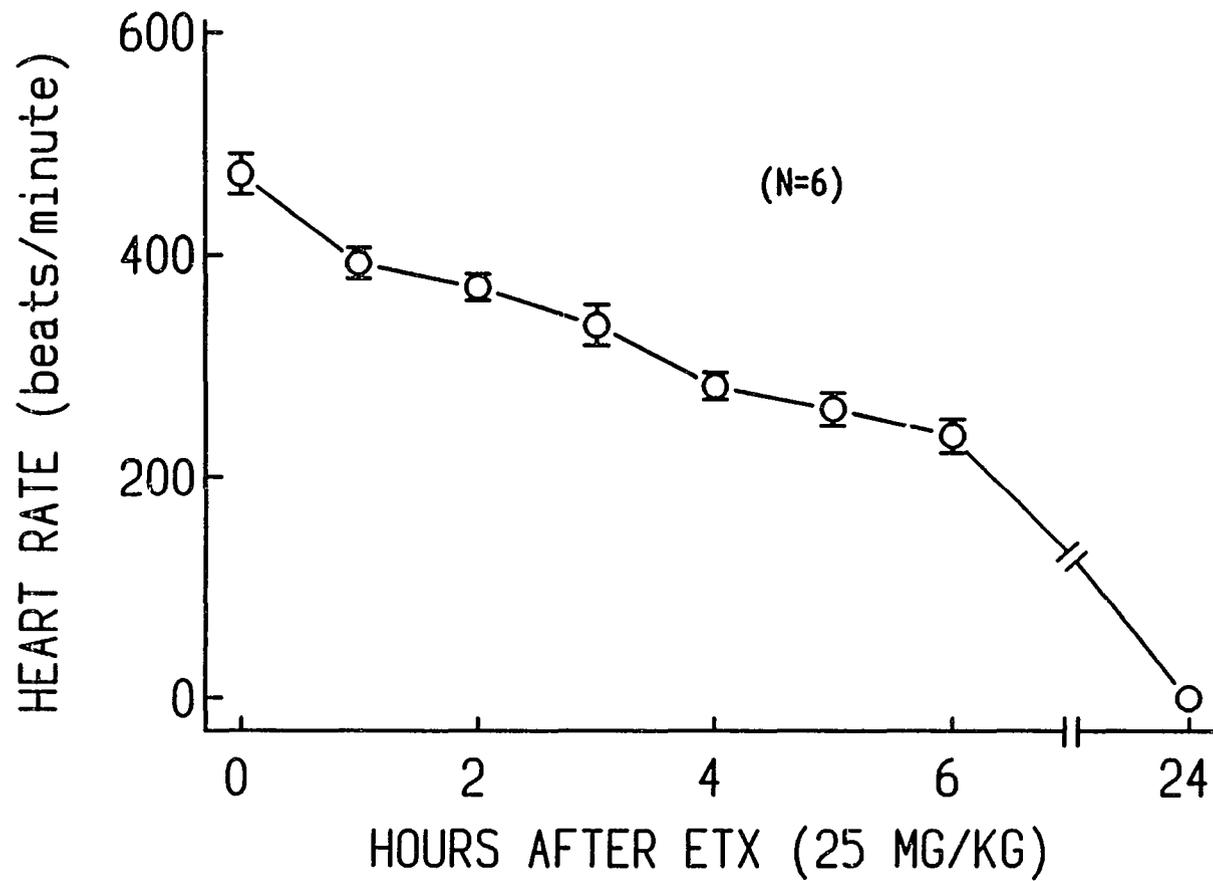


Figure 15. Effect of intravenous administration of ETX on heart rate.

Table 3. Effects of drugs on heart rate

Drugs	Heart Rate (Mean \pm S.E. Beats/Min)			
	Minutes After Drug Administration			
	0	1	5	15
Pentoxifylline				
50 mg/kg (N=8)	479 \pm 8	565 \pm 7*	---	516 \pm 9*
Buflomedil				
30 mg/kg (N=8)	446 \pm 14	463 \pm 16	470 \pm 8	477 \pm 11*
50 mg/kg (N=8)	480 \pm 9	372 \pm 23*	435 \pm 7*	444 \pm 8*
Caffeine				
100 mg/kg (N=8)	453 \pm 19	530 \pm 10*	497 \pm 12	491 \pm 17*
200 mg/kg (N=8)	458 \pm 16	512 \pm 15*	512 \pm 6*	510 \pm 7*
Aminophylline				
50 mg/kg (N=8)	446 \pm 16	549 \pm 9*	---	545 \pm 10*
100 mg/kg (N=8)	443 \pm 14	594 \pm 18*	563 \pm 5*	554 \pm 6*

* Significantly different from base line (Zero time).

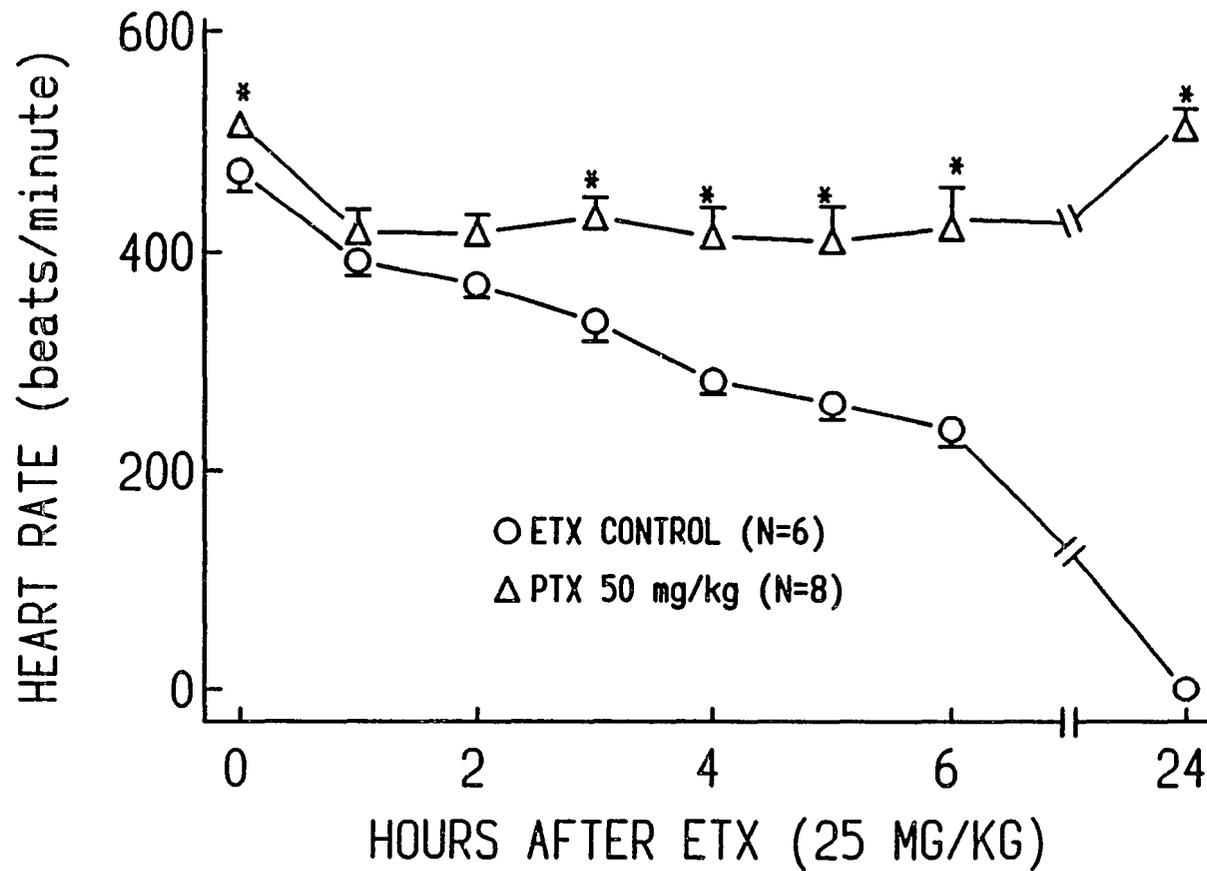


Figure 16. Effect of pentoxifylline on heart rate in endotoxic shock.

PTX given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

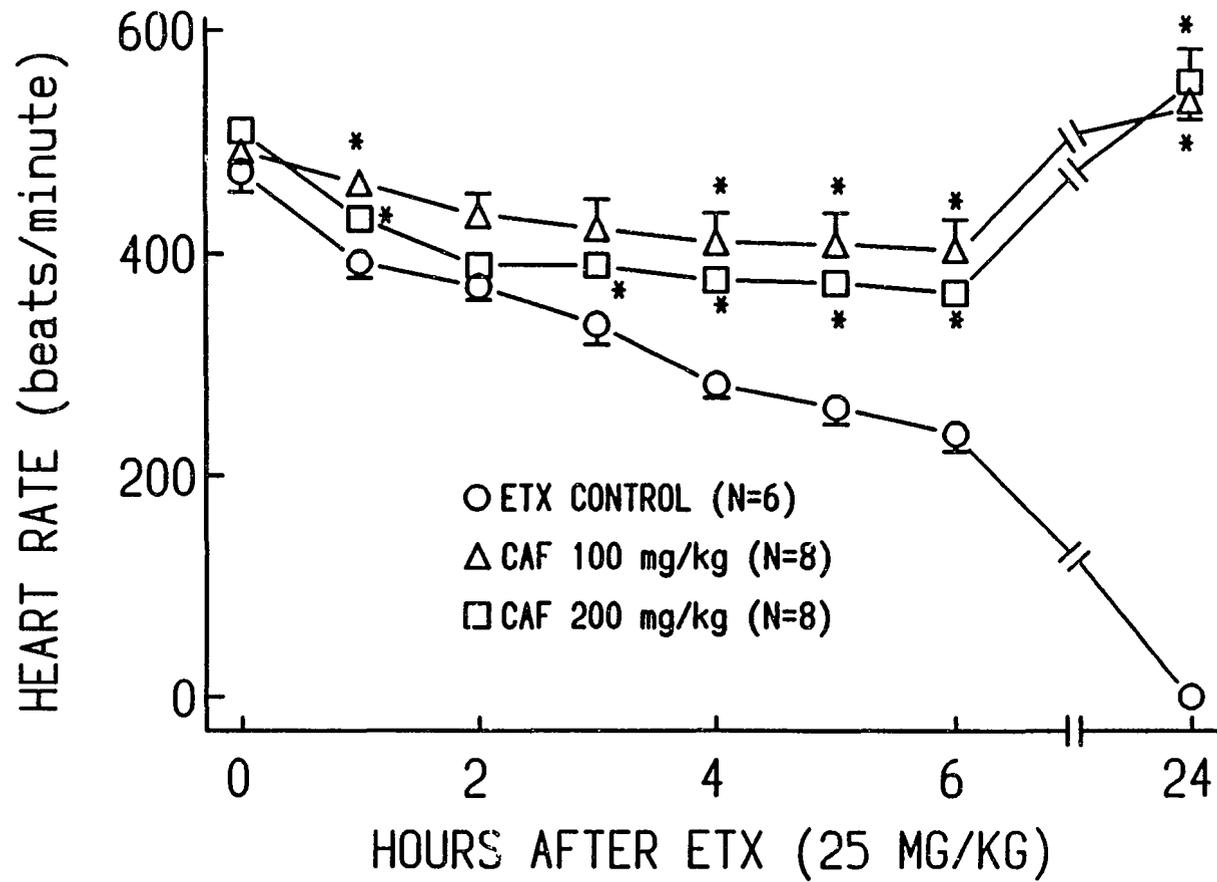


Figure 17. Effect of caffeine on heart rate in endotoxic shock.

CAF given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

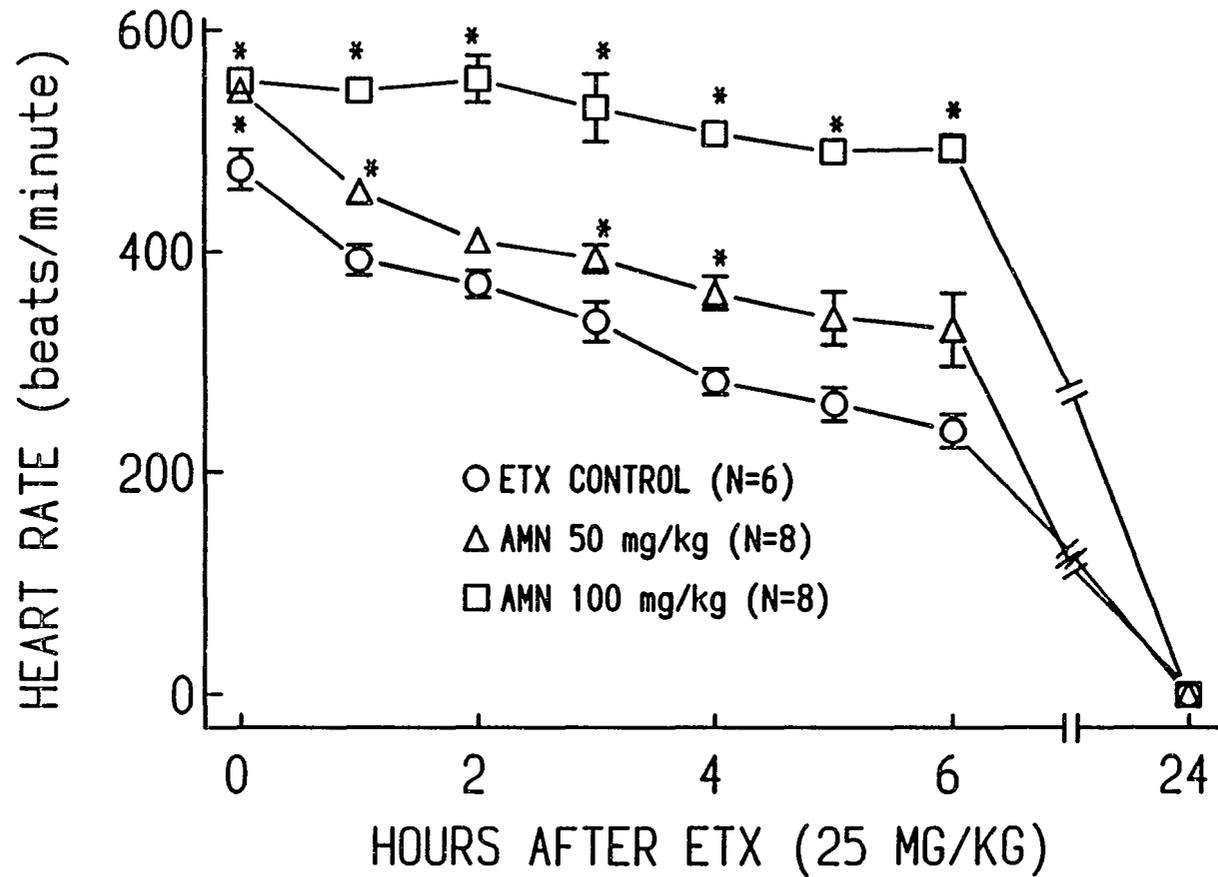


Figure 18. Effect of aminophylline on heart rate in endotoxic shock.

AMN given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

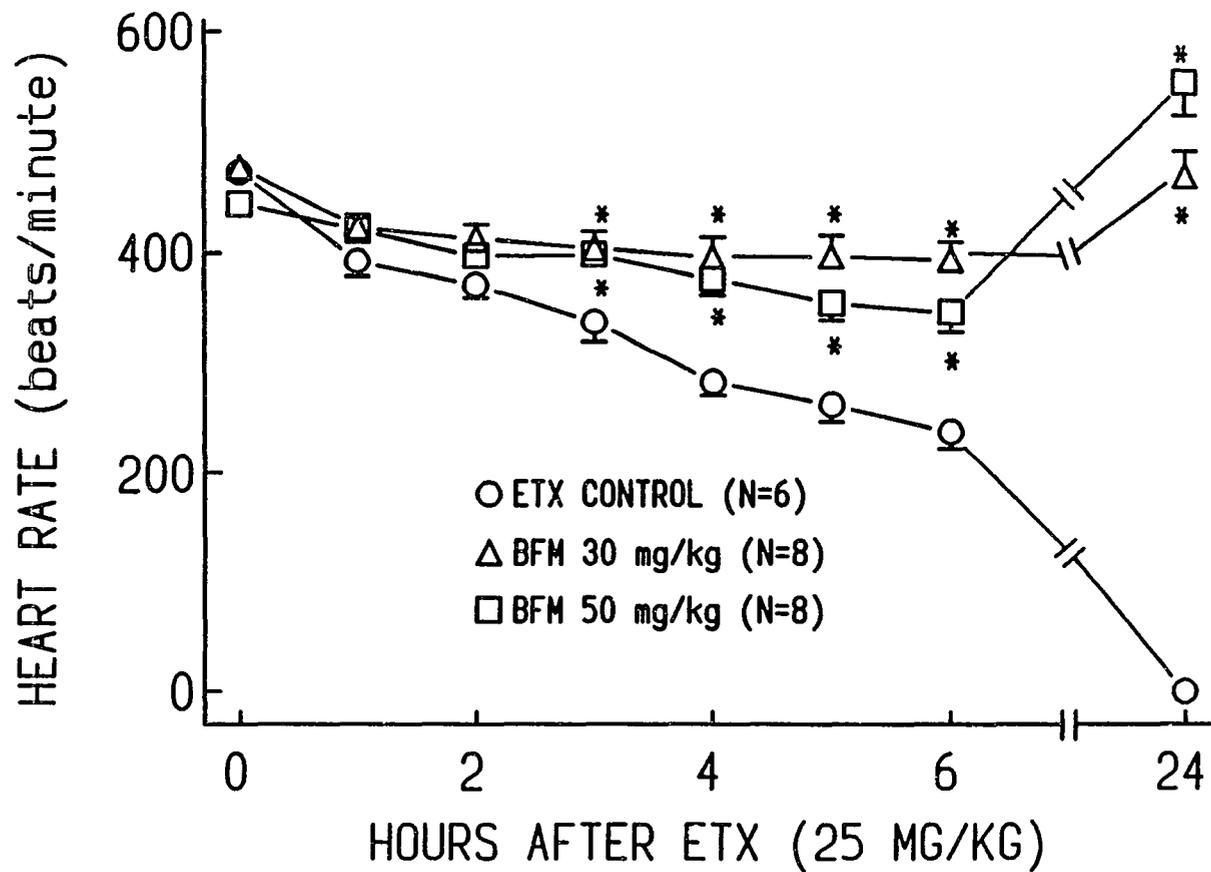


Figure 19. Effect of buflomedil on heart rate in endotoxic shock.

BFM given i.v. 15 minutes before ETX (i.v.).

* Significantly different from the ETX control series.

Pretreatment with pentoxifylline (50 mg/kg), caffeine sodium benzoate (200 mg/kg) and buflomedil hydrochloride (50 mg/kg) significantly reduced the endotoxin-induced increases in serum FDP, PT and PTT (Figures 20, 21, 23, 24, 26, 27). The endotoxin-induced decreases in plasma fibrinogen and blood platelet tended to be smaller in the drug pretreated groups than in the controls; however, the differences were not statistically significant (Figures 22, 25, 28).

In contrast to the other three agents, aminophylline (50 mg/kg) failed to affect significantly any of the endotoxin-induced changes in the clinical laboratory tests for DIC (Figures 29-31).

E. Gross pathology of visceral organs.

1. Endotoxin control. Six endotoxin control animals, sacrificed with an overdose of sodium pentobarbital (i.p.) three hours following the lipopolysaccharide injection, exhibited diffuse hemorrhagic congestion of the stomach, small intestine, colon, cecum, and mesentery and multiple small hemorrhagic spots visible on the surface of the heart, lungs, liver, spleen, pancreas, and kidneys (Figure 32).

2. Effects of drug pretreatment. Rats pretreated with pentoxifylline (50 mg/kg, N=6), buflomedil (50 mg/kg, N=4) and caffeine (200 mg/kg, N=4) exhibited little or none of the visceral hemorrhagic changes seen in the controls (Figure 32). On the other hand, the gross pathologic changes in the aminophylline (125 mg/kg,

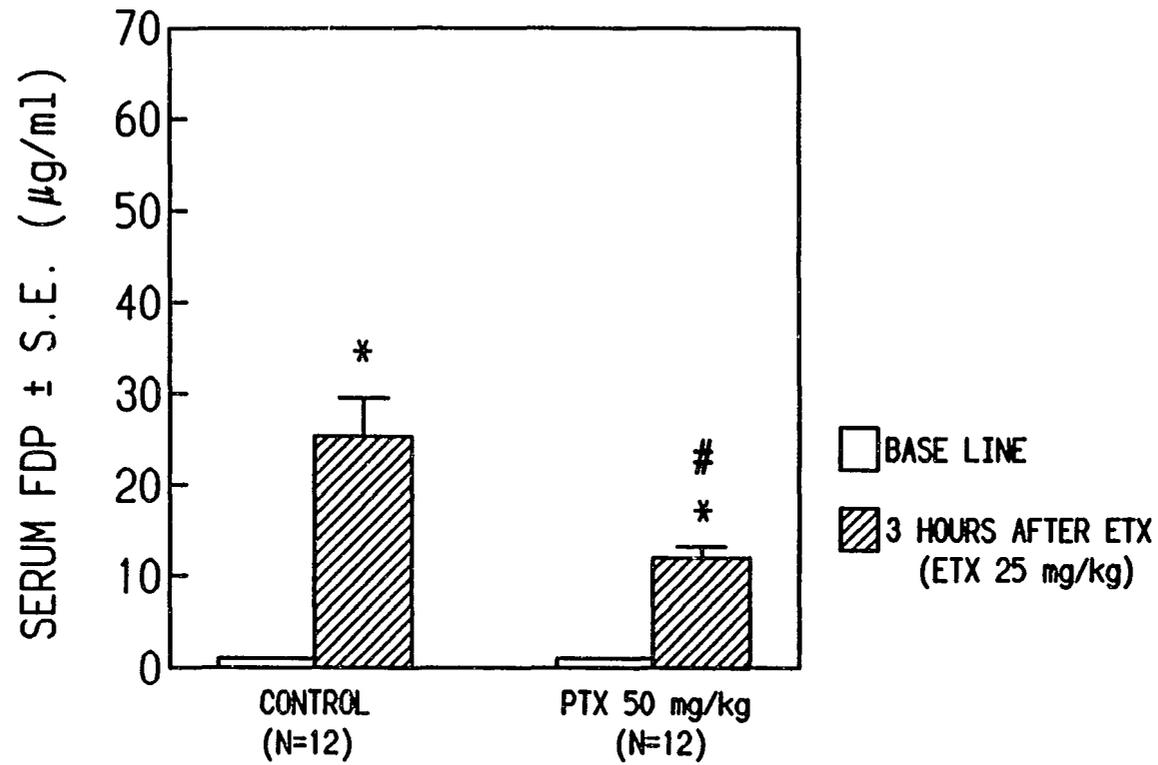


Figure 20. Effect of pretreatment with pentoxifylline (PTX) on the elevation of fibrin(ogen) degradation products (FDP) caused by endotoxin.

PTX given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

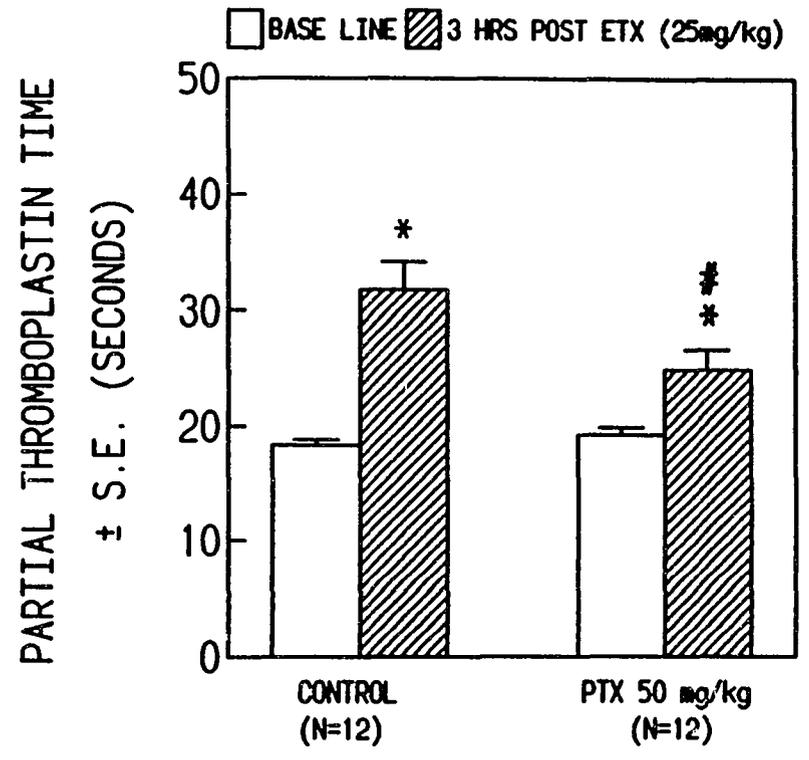
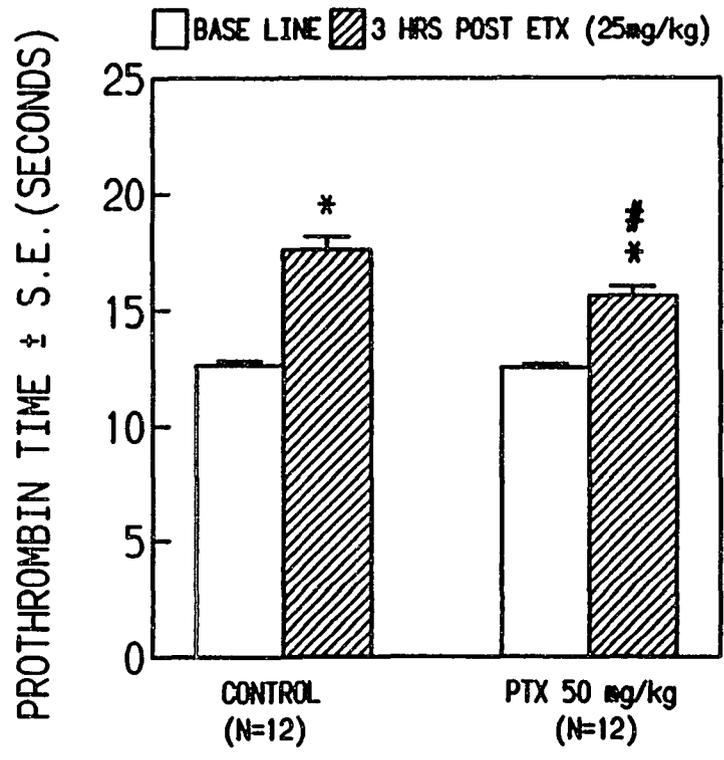


Figure 21. Effect of pretreatment with pentoxifylline (PTX) on the prolongation of prothrombin time (PT) and partial thromboplastin time (PTT) caused by endotoxin.

PTX given 15 minutes before ETX.
 * Significantly different from base line.
 # Significantly different from the control series.

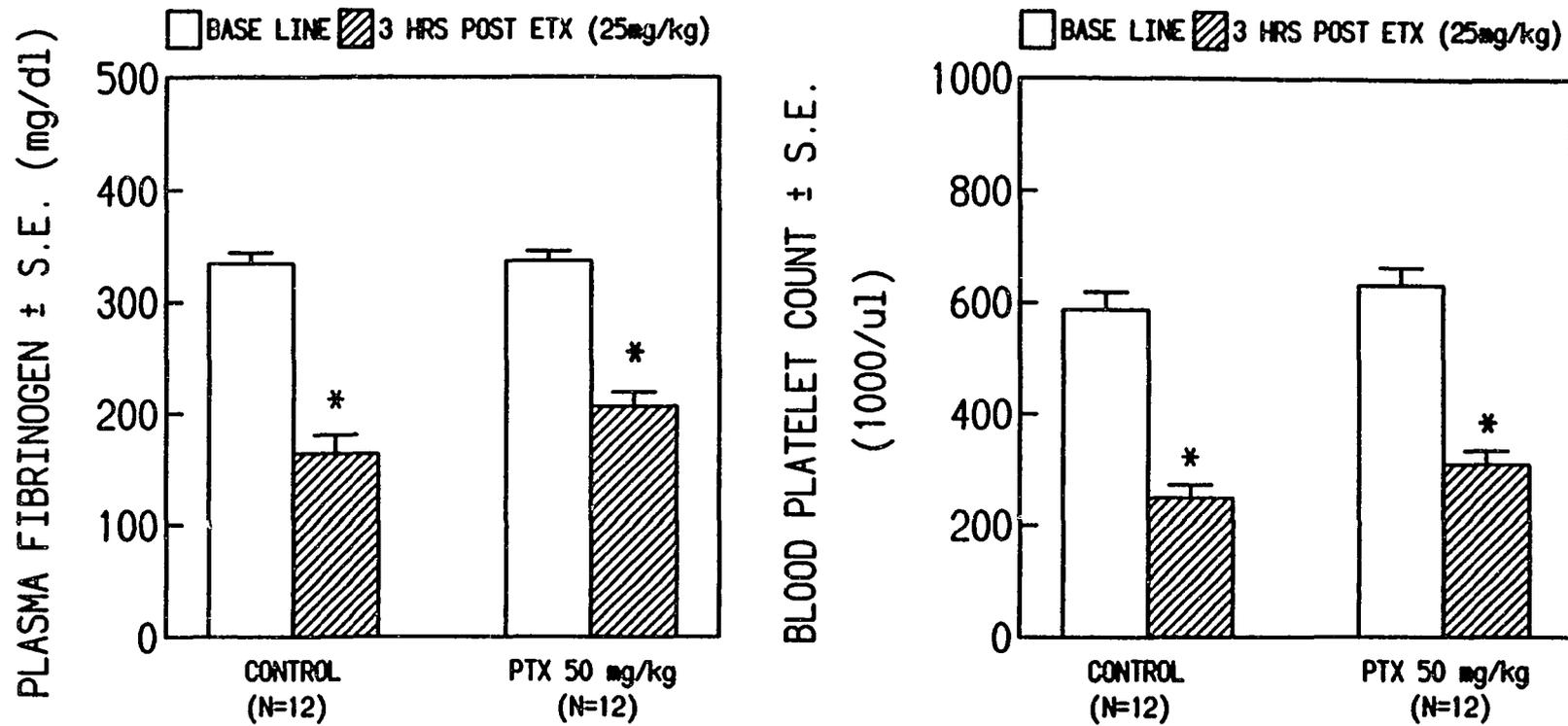


Figure 22. Effect of pretreatment with pentoxifylline (PTX) on the decrease of plasma fibrinogen and blood platelets caused by endotoxin.

PTX given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

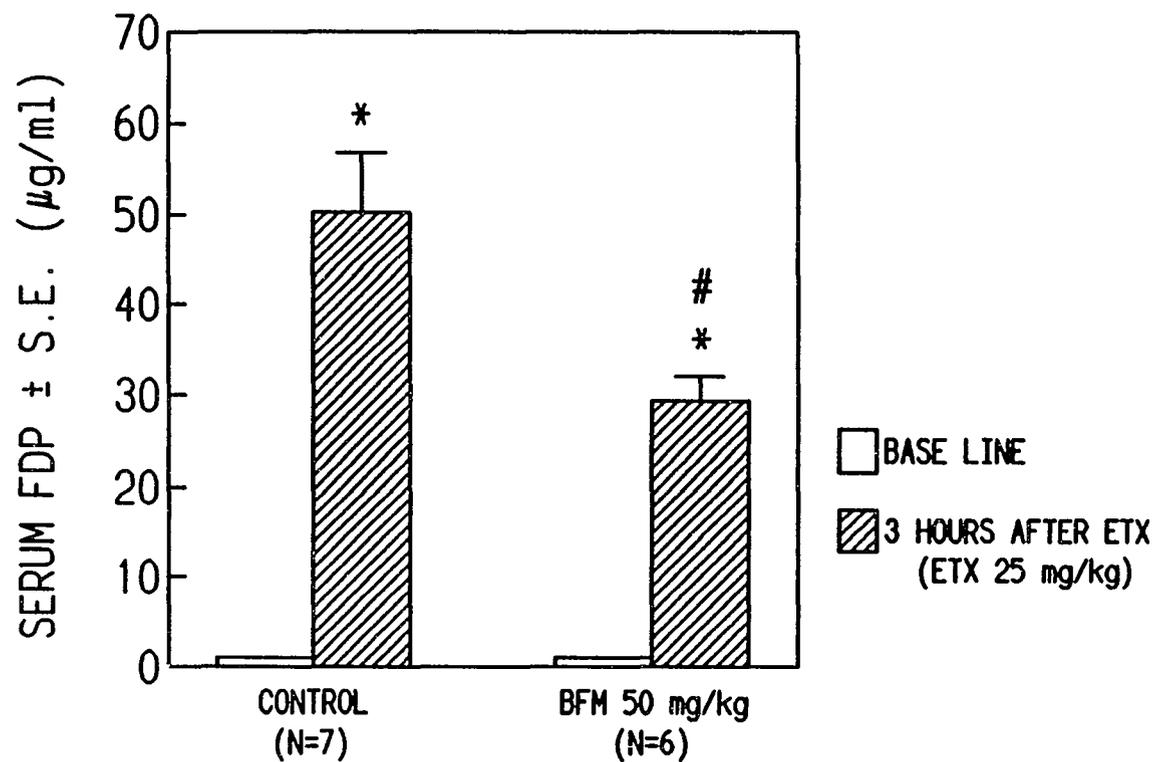


Figure 23. Effect of pretreatment with buflomedil (BFM) on the elevation of fibrin(ogen) degradation products (FDP) caused by endotoxin.

BFM given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

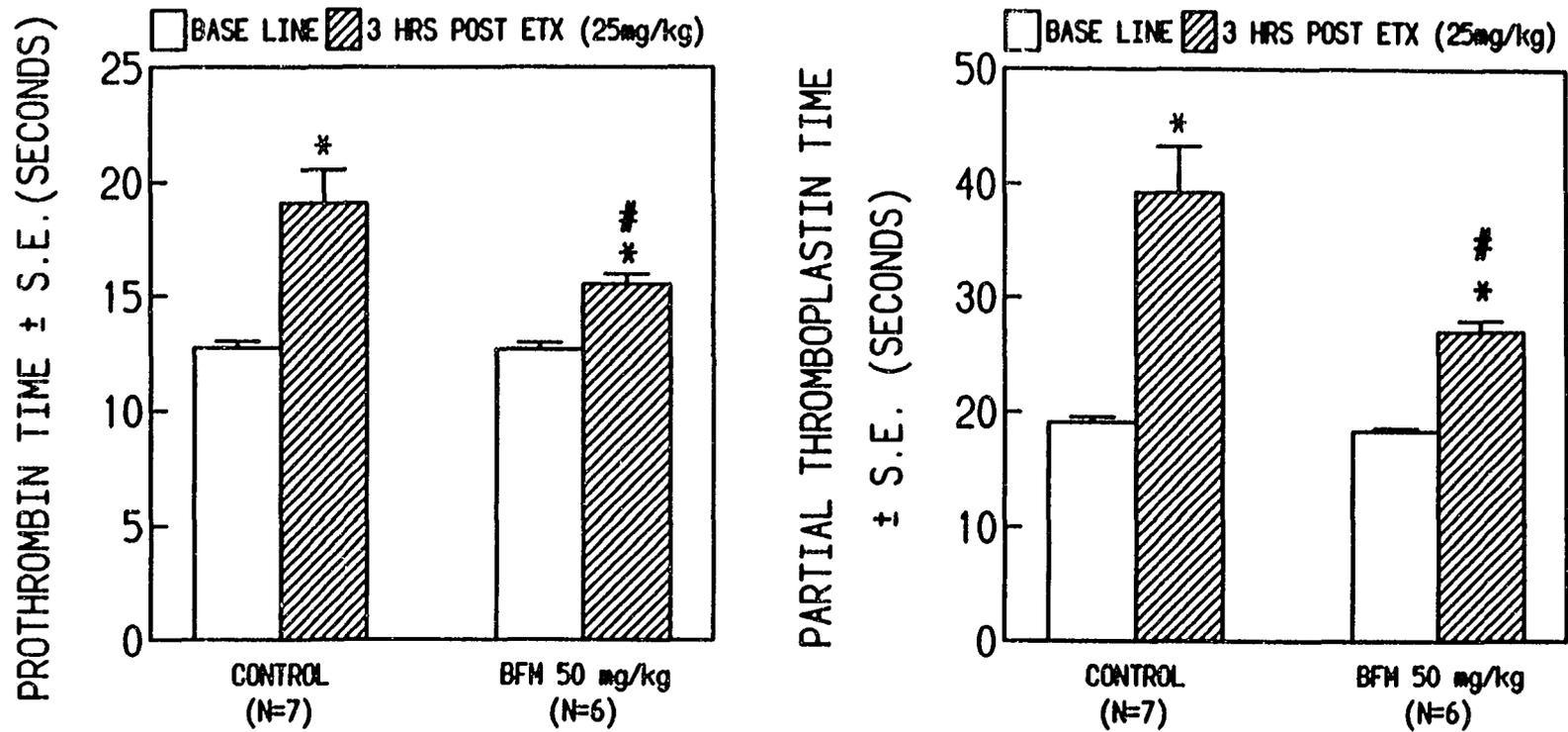


Figure 24. Effect of pretreatment with buflomedil (BFM) on the prolongation of prothrombin time (PT) and partial thromboplastin time (PTT) caused by endotoxin.

BFM given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

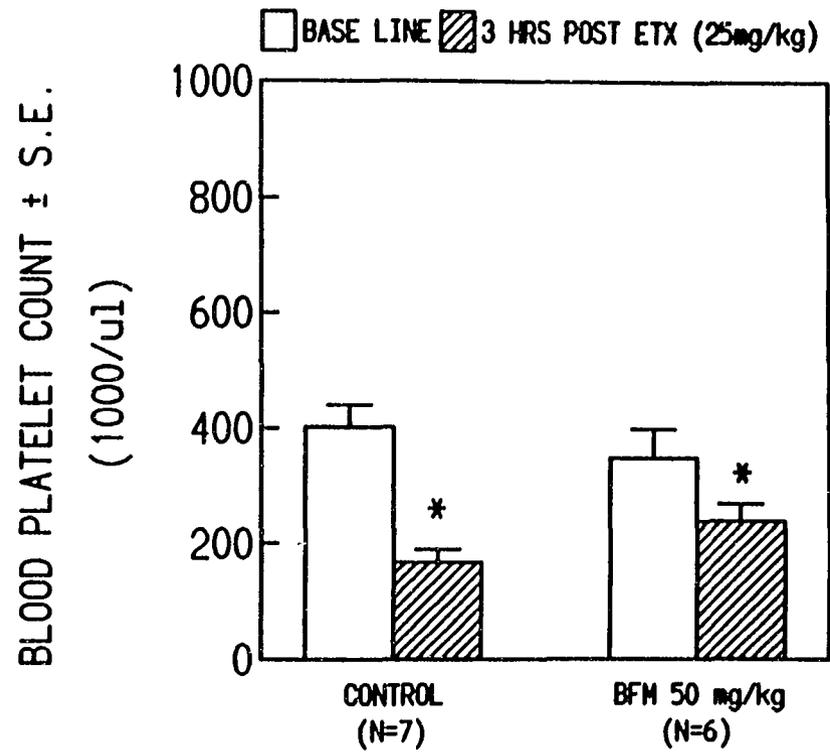
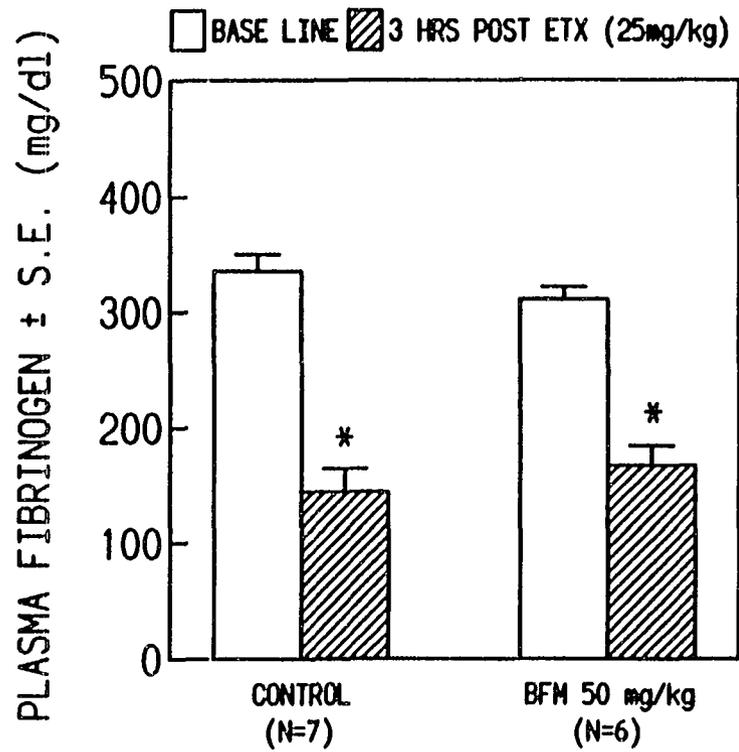


Figure 25. Effect of pretreatment with buflomedil (BFM) on the decrease of plasma fibrinogen and blood platelets caused by endotoxin.

BFM given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

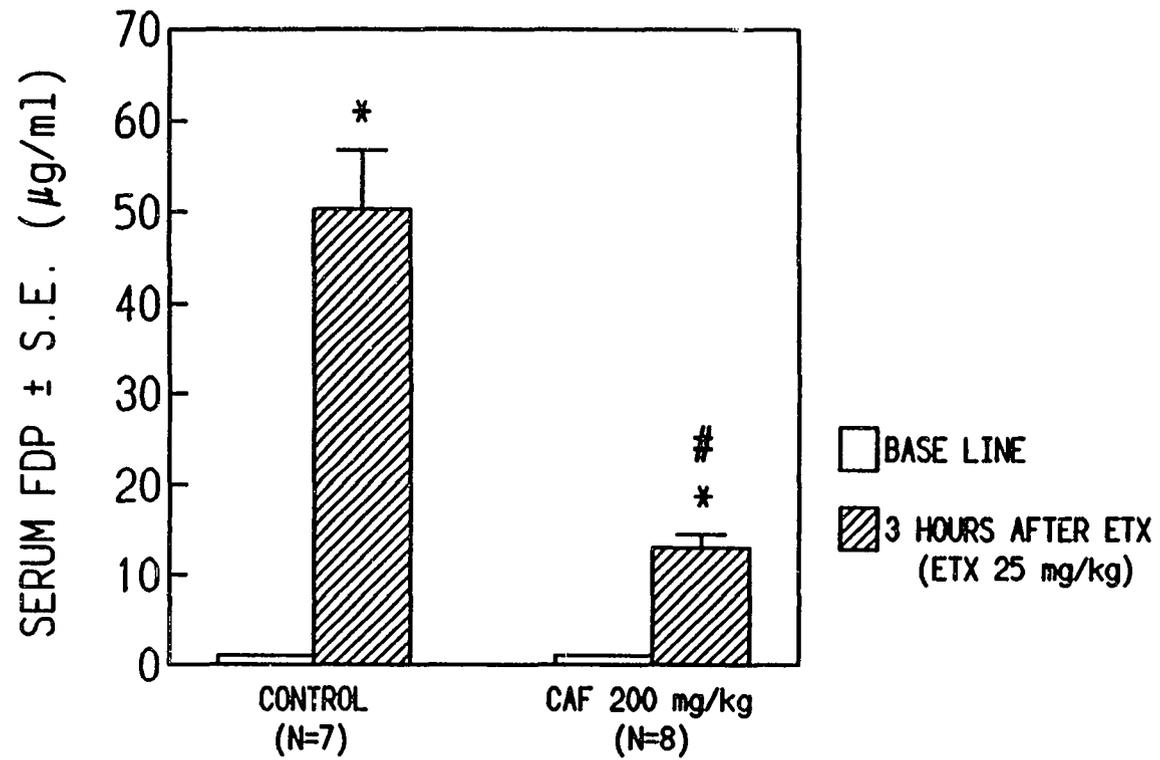


Figure 26. Effect of pretreatment with caffeine (CAF) on the elevation of fibrin(ogen) degradation products (FDP) caused by endotoxin.

CAF given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

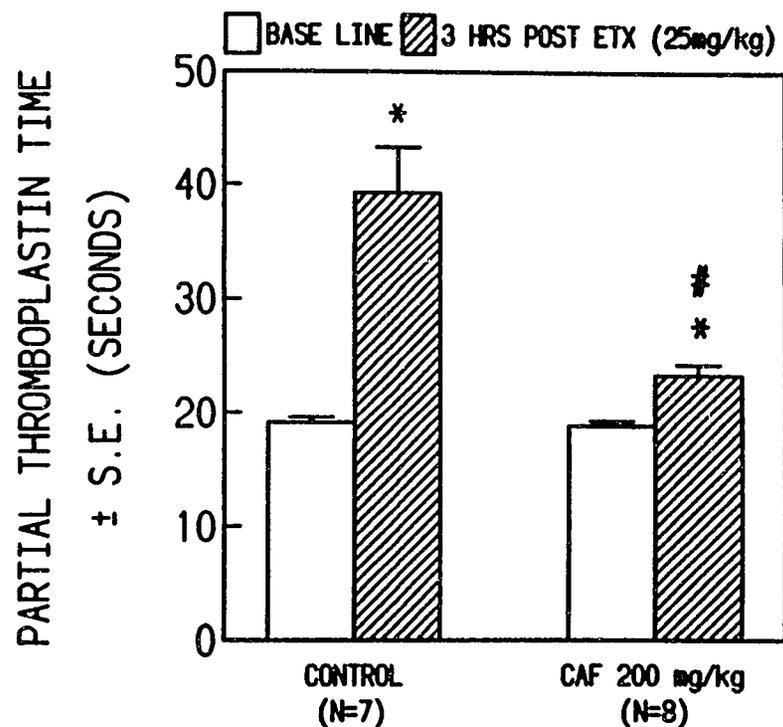
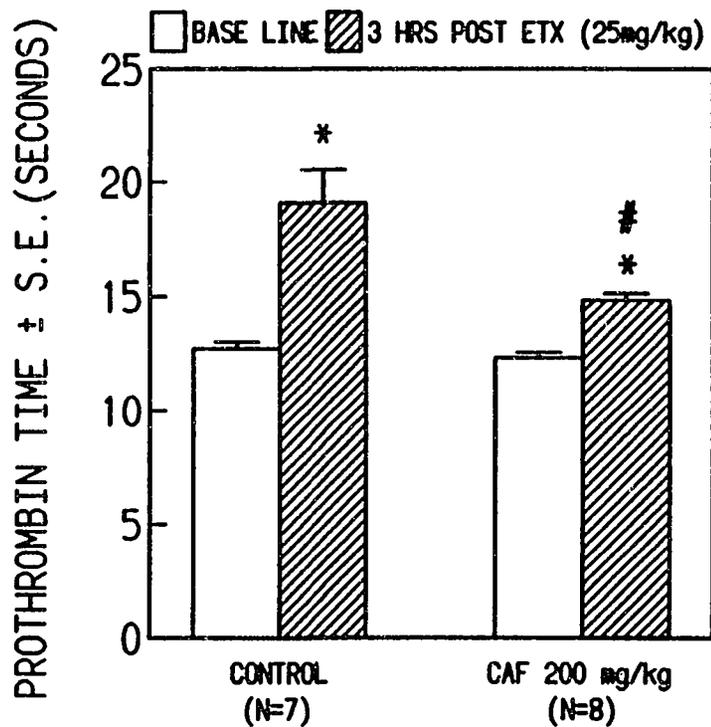


Figure 27. Effect of pretreatment with caffeine (CAF) on the prolongation of prothrombin time (PT) and partial thromboplastin time (PTT) caused by endotoxin.

CAF given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

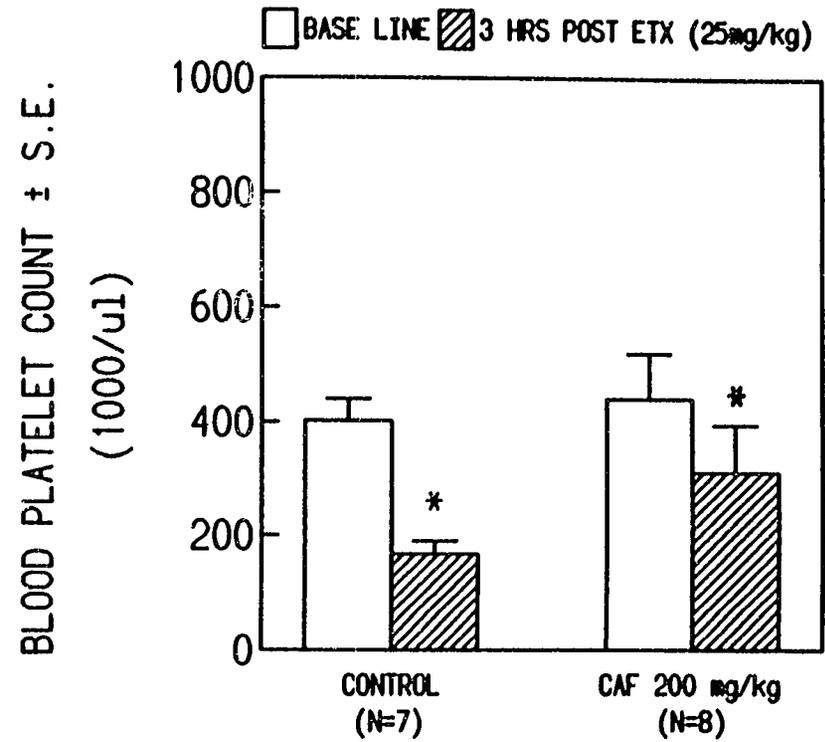
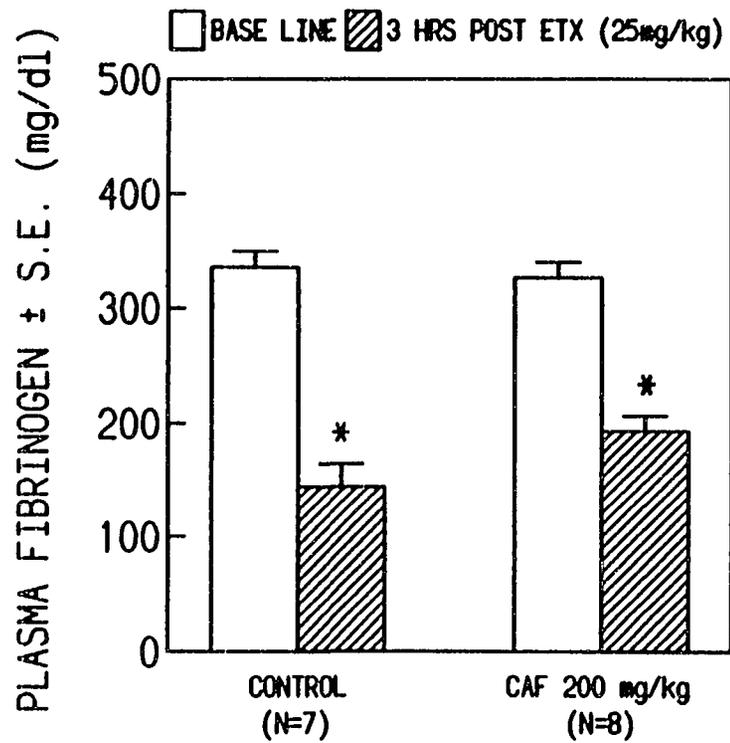


Figure 28. Effect of pretreatment with caffeine (CAF) on the decrease of plasma fibrinogen and blood platelets caused by endotoxin.

CAF given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

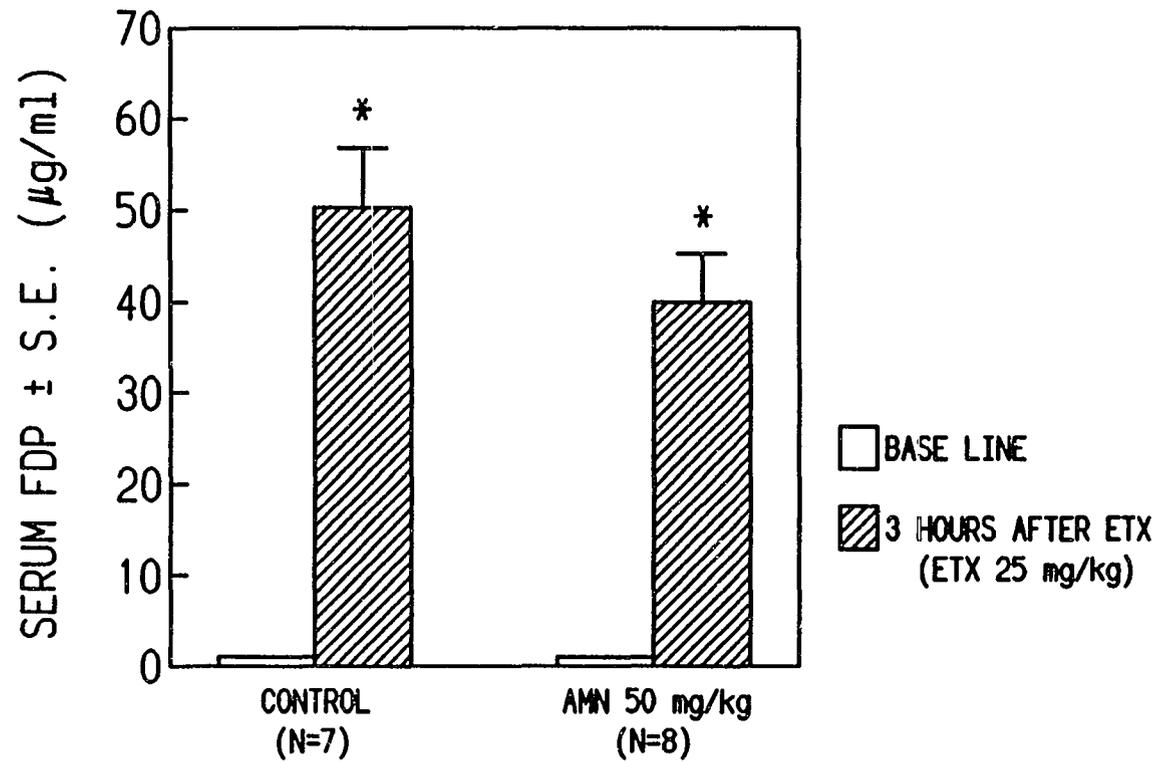


Figure 29. Effect of pretreatment with aminophylline (AMN) on the elevation of fibrin(ogen) degradation products (FDP) caused by endotoxin.

AMN given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

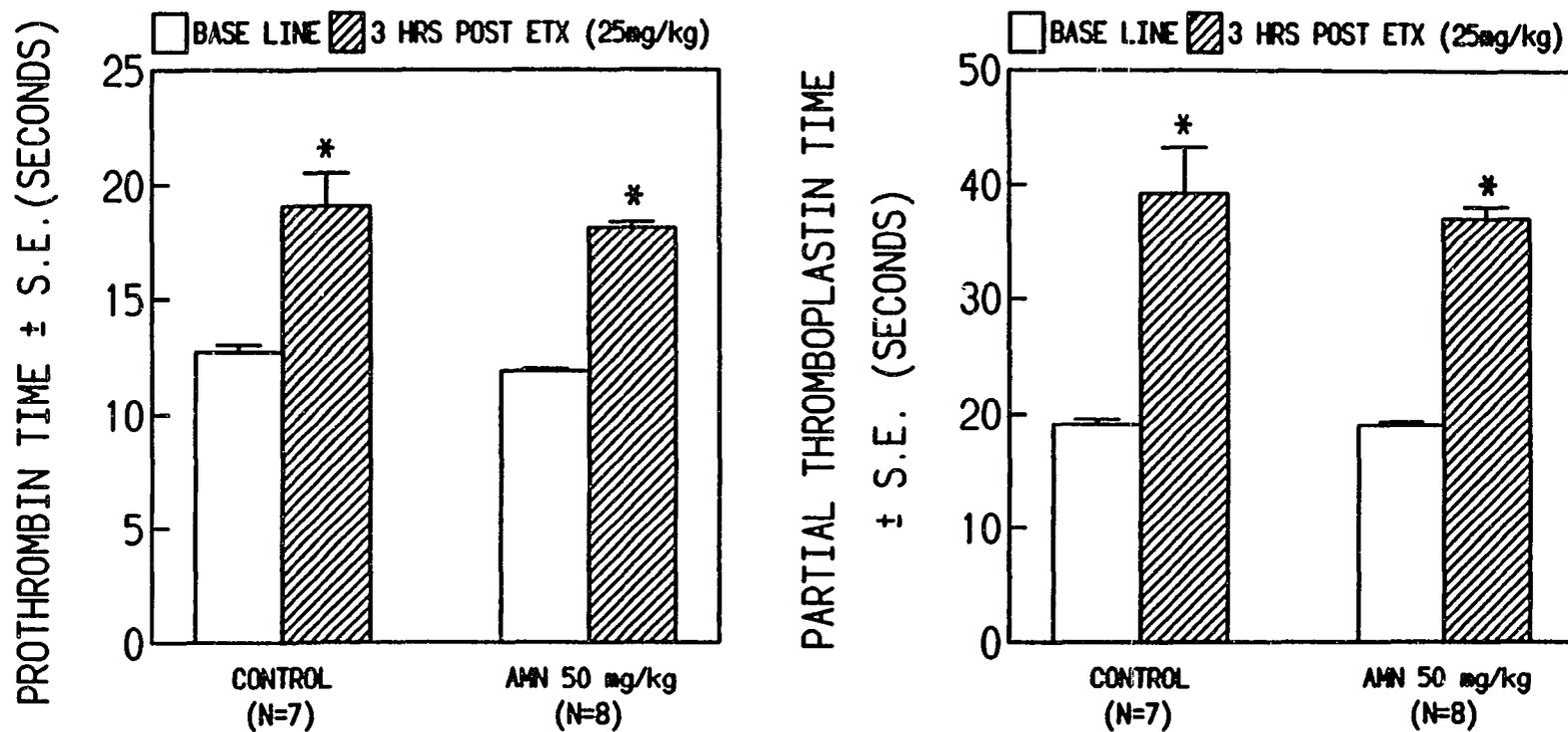


Figure 30. Effect of pretreatment with aminophylline (AMN) on the prolongation of prothrombin time (PT) and partial thromboplastin time (PTT) caused by endotoxin.

AMN given 15 minutes before ETX.

* Significantly different from base line.

Significantly different from the control series.

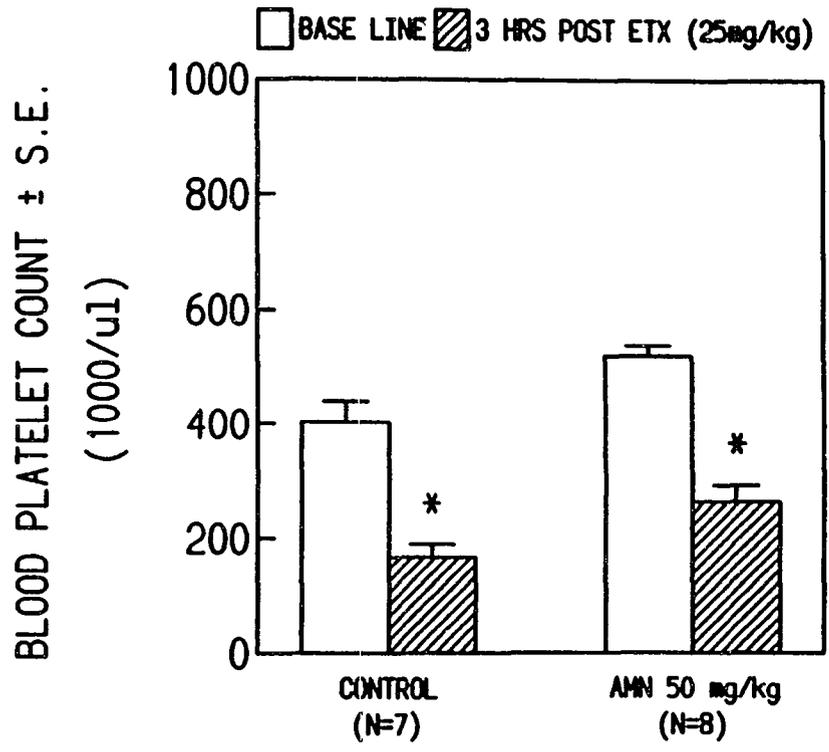
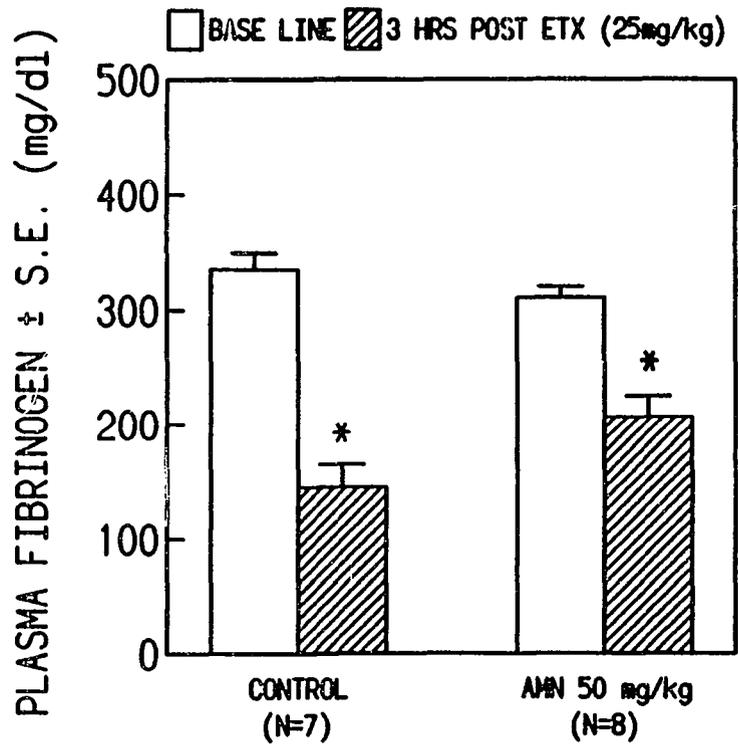


Figure 31. Effect of pretreatment with aminophylline (AMN) on the decrease of plasma fibrinogen and blood platelets caused by endotoxin.

AMN given 15 minutes before ETX.
 * Significantly different from base line.
 # Significantly different from the control series.

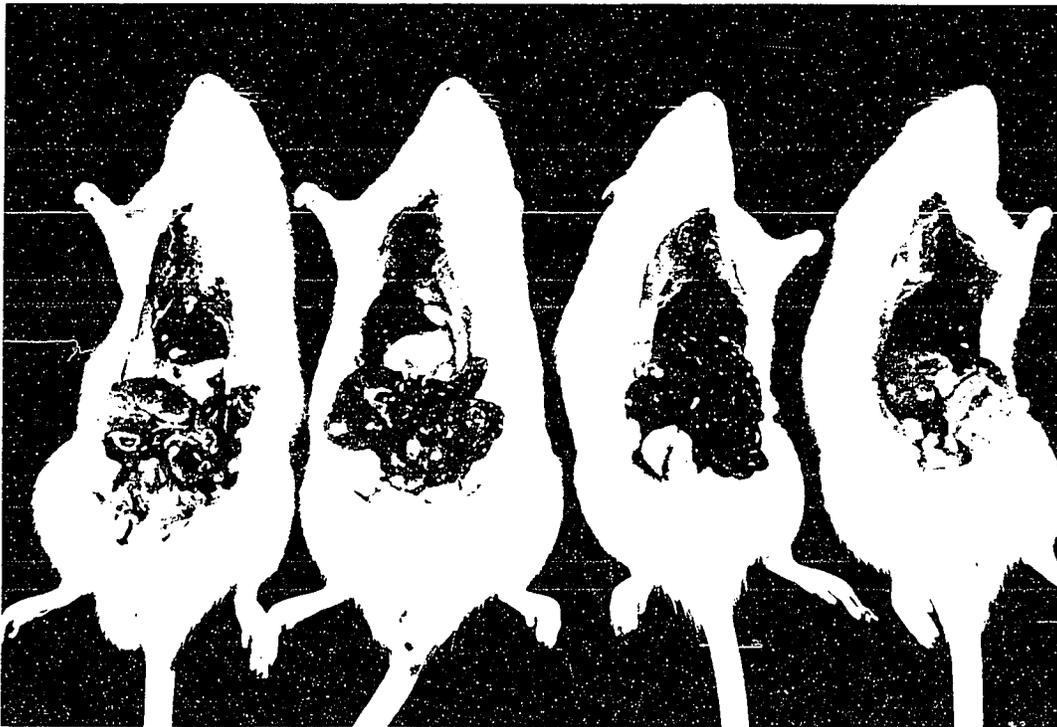


Figure 32. Effect of pretreatment with caffeine and pentoxifylline on gross pathological changes produced by ETX.

From left to right:
CAF pretreated, PTX pretreated, ETX control, Untreated control.

Animals were sacrificed and examined 3 hours after endotoxin administration (25 mg/kg).

Prominent hemorrhagic congestion of the gastrointestinal tract in the ETX control animal can be seen in the photograph.

The pathological change is markedly less in the animals pretreated with caffeine and pentoxifylline.

N=4) pretreated series were similar to the endotoxin controls.

F. Studies on erythrocyte deformability.

1. Effects of drugs on RBC deformability. Drug induced changes in the deformability index (DI) were quantified by measuring the erythrocyte DI of blood samples obtained before and 30 minutes after drug administration and were treated as paired data to normalize individual differences in baseline DI values.

Pentoxifylline (50 mg/kg), caffeine sodium benzoate (200 mg/kg) and buflomedil hydrochloride (50 mg/kg) produced a significant increase in the erythrocyte DI (Figure 33). On the other hand, the changes induced by aminophylline (100 and 125 mg/kg) were not statistically significant (Figure 33).

2. Effect of drugs on endotoxin-induced decrease in RBC deformability. Blood samples were obtained immediately before and 3 hours after the administration of endotoxin (25 mg/kg) in this set of experiments. Endotoxin produced a significant reduction in RBC deformability in saline-pretreated control animals (Figure 34). The decrease in deformability was not only antagonized but was actually reversed by pretreatment with pentoxifylline (50 mg/kg). Caffeine sodium benzoate and buflomedil hydrochloride similarly reversed the effect of endotoxin dose-dependently. On the other hand, aminophylline (50-100 mg/kg) failed to affect significantly the endotoxin-induced reduction in erythrocyte DI (Figure 34).

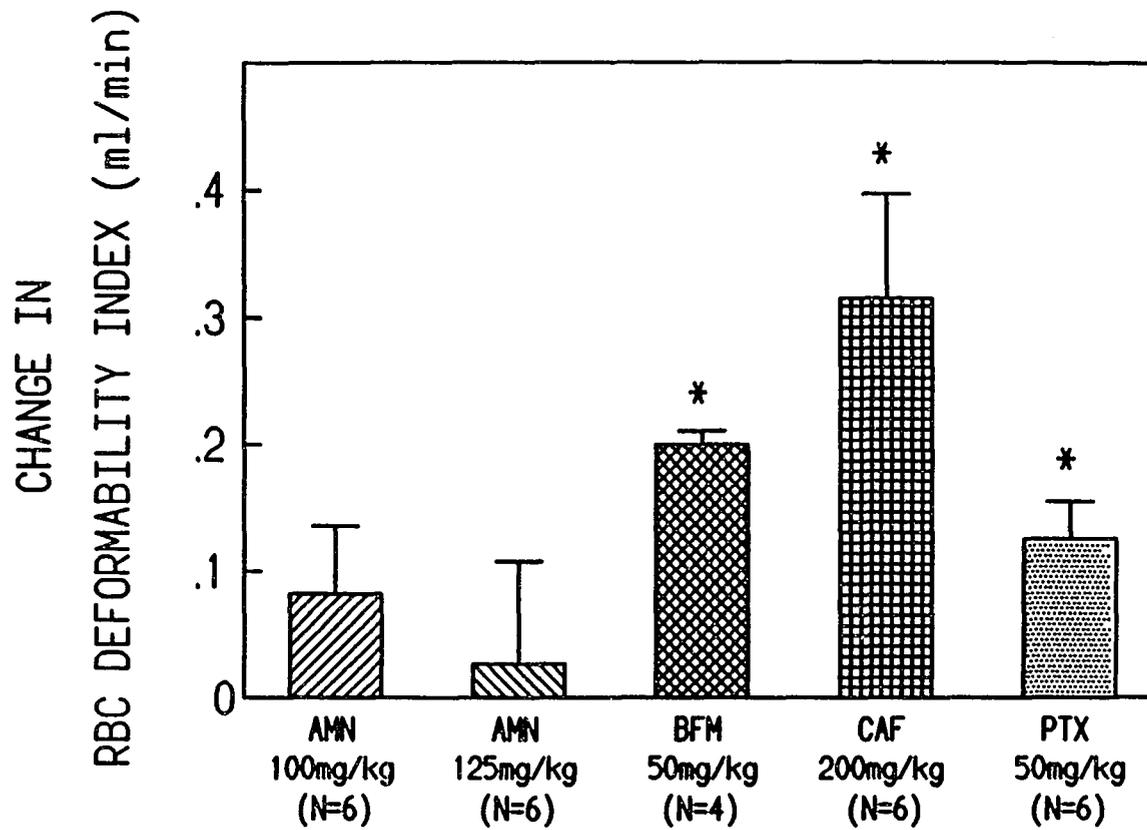


Figure 33. Effects of aminophylline, buflomedil, caffeine and pentoxifylline on erythrocyte deformability in normal rats.

Blood samples drawn before and at 30 minutes after drug.

* Significant change from base line (before drug) value.

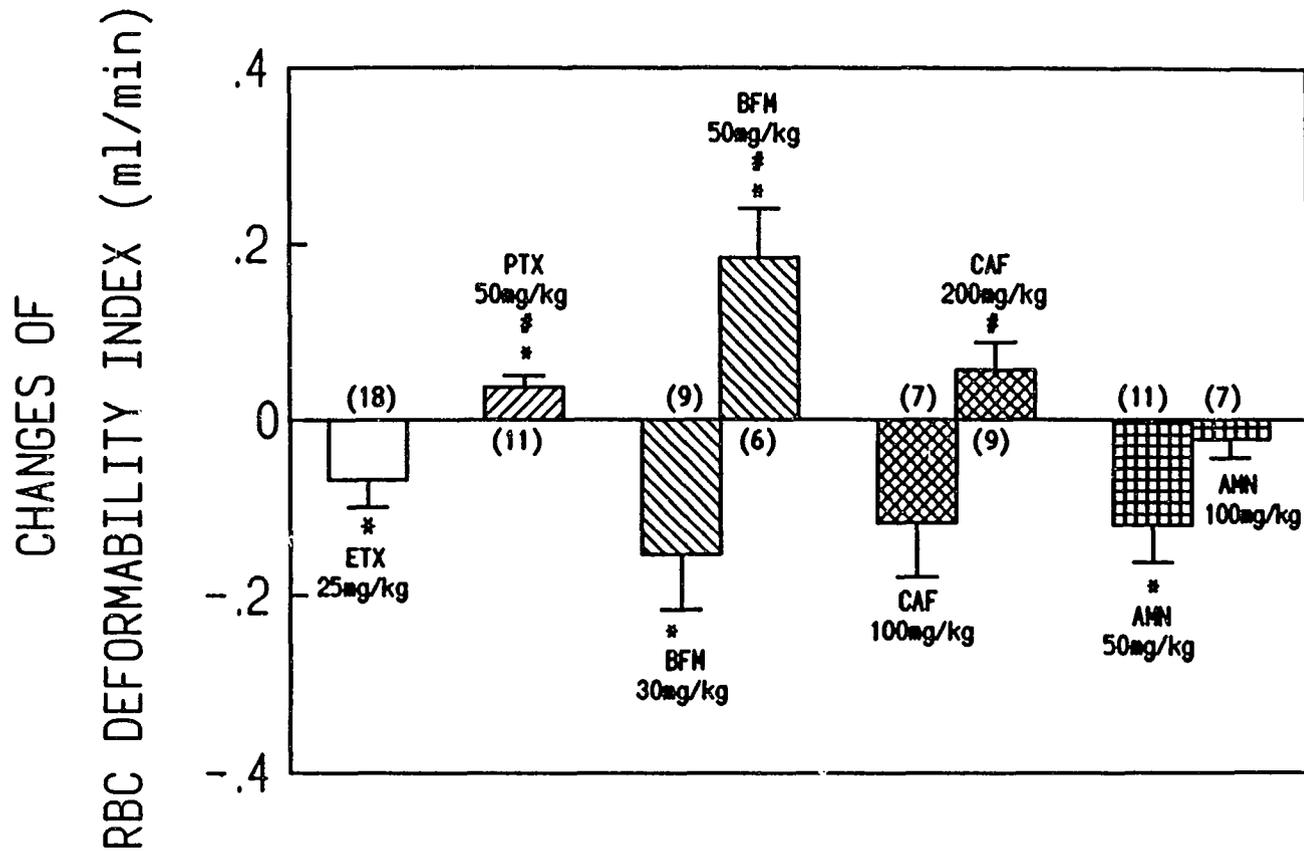


Figure 34. Effect of pretreatment with pentoxifylline, buslomedil, caffeine and aminophylline on the reduction of RBC deformability produced by endotoxin (25 mg/kg).

Blood samples drawn before and at 3 hours after ETX.

* Significant change from base line (before drug) value.

Significantly different from ETX control.

G. Studies on leukocyte deformability.

Leukocytes were isolated from blood samples obtained before and 30 minutes after i.v. drug administration for measurements of drug-induced changes in WBC deformability. Pentoxifylline (50 mg/kg), caffeine sodium benzoate (200 mg/kg) and buflomedil (50 mg/kg) produced marked increases in the leukocyte DI. On the other hand, aminophylline failed to exert a significant effect (Figure 35).

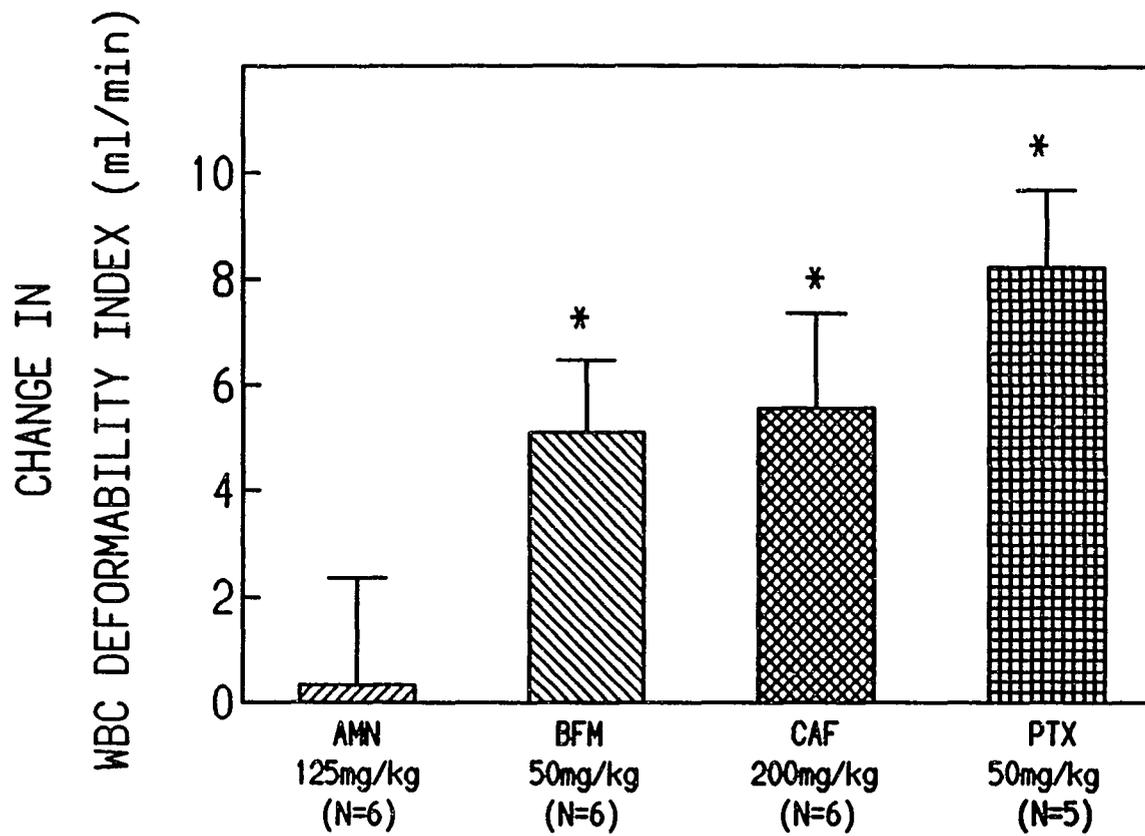


Figure 35. Effects of aminophylline, buflomedil, caffeine and pentoxifylline on leukocyte deformability in normal rats.

Blood samples drawn before and at 30 minutes after drug.

* Significant change from base line (before drug) value.

IV. DISCUSSION

A. Endotoxic shock and mortality.

Endotoxemia/sepsis, especially complicated with circulatory shock, carries a very high mortality despite a number of therapeutic methods used in handling this disorder (Kiani, 1979). Therefore, there is a continued need to search for more effective therapeutic agents.

In the present investigation, three xanthine compounds (pentoxifylline, aminophylline and caffeine) and one non-xanthine compound (buflomedil) were studied. Dose-dependent protection against death caused by endotoxin was observed in animals pretreated with pentoxifylline, caffeine and buflomedil. Aminophylline in doses up to 125 mg/kg (equivalent to 99 mg/kg of theophylline base) failed to reduce the endotoxin-induced mortality. While not statistically significant, the mortality in animals pretreated with the highest doses of aminophylline (100 and 125 mg/kg), was greater than in the controls. The results with aminophylline were in distinct contrast to those seen in animals pretreated with pentoxifylline (50 mg/kg as the base), caffeine sodium benzoate (200 mg/kg, equivalent to 96 mg/kg caffeine base) and buflomedil hydrochloride (50 mg/kg, equivalent to 45 mg/kg buflomedil base), with 48-hour post-endotoxin

mortalities being 0%, 14%, and 12.5% for the three series, respectively.

While highly effective when given as pretreatment, pentoxifylline was found to be ineffective when given 15 minutes after endotoxin. The latter finding indicates that the pathophysiological changes of endotoxic shock quickly become irreversible and/or that pentoxifylline exerts its salutary effect at a very early phase of the pathophysiological sequelae of endotoxic shock. Previous investigations in our laboratory showed that calcium channel blockers and PAF-antagonists behaved similarly in that post-treatment was much less effective than pretreatment (Lee & Lum, 1986; Tang et al., 1990). The ineffectiveness of post-treatment with the various agents does not necessarily suggest that the drugs would be of no value in the clinical management of sepsis and septic shock. In our endotoxic model, the toxin was injected intravenously in the form of a single lethal bolus. On the other hand, the endotoxemia associated with clinical gram-negative infection ordinarily would rise to a lethal level at a comparatively slow rate. Under the latter circumstance, pentoxifylline, particular in combination with antibiotics, may well be life-saving.

B. Arterial pressure and heart rate studies.

The xanthine compounds are known to produce qualitatively similar pharmacological effects including vasodilatation and cardiac

stimulation. Vasodilator agents have been reported to protect against death caused by circulatory shock. The possibility was thus considered that the difference in the protective efficacy of the xanthines may have been related to a difference in the magnitude of their vasodilator effect. Pentoxifylline, aminophylline, caffeine and buflomedil were all found to produce a dose-dependent lowering of the arterial pressure. Pentoxifylline (50 mg/kg), buflomedil hydrochloride (30 mg/kg), caffeine-sodium benzoate (100 mg/kg) and aminophylline (50 mg/kg) produced approximately equivalent lowering of the arterial pressure. With these doses, only pentoxifylline protected against endotoxin-induced death. In studies with higher doses, buflomedil hydrochloride (50 mg/kg), caffeine-sodium benzoate (200 mg/kg) and aminophylline (100 mg/kg), were found to be equi-effective in lowering the blood pressure; however, only buflomedil hydrochloride (50 mg/kg) and caffeine-sodium benzoate (200 mg/kg) were effective in reducing endotoxin-induced mortality; aminophylline (100 mg/kg) failed to reduce the mortality. The hypotensive action of these agents are related to their vasodilator action. Thus, the difference in the protective efficacy of the drugs is not due to a difference in the magnitude of the vasodilatation produced by the drugs.

The administration of endotoxin produced a rapid and dramatic fall in mean arterial pressure in control animals. Blood pressure partially recovered and remained stable for a number of hours before

declining progressively until death of the animal. Pretreatment with protective doses of pentoxifylline, buflomedil and caffeine significantly reduced the endotoxin-induced hypotension, including the initial rapid fall in blood pressure. The latter again suggests that the drugs exert their salutary effect at a very early phase of the pathophysiological sequelae of endotoxic shock. In contrast to the other drugs, pretreatment with aminophylline (50-100 mg/kg) not only failed to antagonize the hypotension caused by endotoxin but actually had a deleterious effect: post-endotoxin blood pressure in the aminophylline pretreated series was significantly lower than in the saline pretreated endotoxin controls throughout the 6-hour period of observation. The reason for the apparent toxic effect of aminophylline is not clear since the hypotension produced by these doses of aminophylline was comparable to that produced by the other agents.

Heart rate measurements showed that there was no correlation between cardiac stimulation produced by the xanthines and protection against endotoxic shock. All three xanthines caused a dose-dependent increase in heart rate and antagonized the bradycardia caused by endotoxin. However, only pentoxifylline and caffeine increased survival. Buflomedil hydrochloride (50 mg/kg) actually slowed the heart rate but nevertheless inhibited the endotoxin-induced bradycardia and decreased the mortality.

C. Disseminated intravascular coagulation (DIC).

DIC frequently complicates clinical cases of endotoxemia and septic shock. In our experiments, injection of endotoxin into rats yielded prominent signs of DIC including thrombocytopenia, prolongation of PT and PTT, hypofibrinogenemia, elevated level of serum FDP and gross evidence of visceral hemorrhage. These findings are indicative of massive activation of both the blood coagulation and fibrinolytic systems. Most of the signs of the endotoxin-induced DIC, including the elevation of FDP, prolongation of PT and PTT and the gross visceral hemorrhage, were reduced or abolished by pretreatment with pentoxifylline, buflomedil and caffeine but not by pretreatment with aminophylline. Pentoxifylline, caffeine and buflomedil did not significantly alter the endotoxin-induced hypofibrinogenemia and thrombocytopenia. However, the most significant sign of DIC is the elevation in serum FDP; antagonism of the latter together with the other findings thus provide ample evidence of inhibition of endotoxin-induced DIC by pentoxifylline, buflomedil and caffeine.

Previous studies done in this laboratory showed that CCBs and PAF antagonists similarly prevented endotoxin-induced DIC without antagonizing the thrombocytopenia (Lee et al., 1989; Tang et al., 1990). The latter, together with the present findings suggest that

platelet aggregation is not importantly related to DIC produced by endotoxin.

DIC is considered to be an important cause of circulatory shock and death caused by the lipopolysaccharide (Hardaway et al., 1959; McGovern, 1971 & 1972). The coagulopathy is thought to be a primary effect of the endotoxin and not a secondary terminal phenomenon in endotoxic shock (Garcia-Barreno, 1978). Our results thus suggest that inhibition of the DIC caused by endotoxin is one mechanism by which pentoxifylline, caffeine and buflomedil protect against endotoxic shock.

The mechanism(s) by which endotoxin produces DIC has been a topic of considerable debate. Some proposed sites of endotoxin action in developing DIC are depicted in Figure 36. The lipopolysaccharide can interact with platelets causing them to aggregate, bind to receptors on WBCs causing them to aggregate and release tissue factor (thromboplastin) and chemical mediators, and injure vascular endothelium resulting in release of tissue factor and exposure of the underlying collagen to blood. All these actions of endotoxin can activate the coagulation system and thus cause DIC.

The possibility that endotoxin-induced DIC might be related to an effect of the toxin on blood cell deformability has received little or no attention in the literature. This possibility will be discussed in the sections to follow.

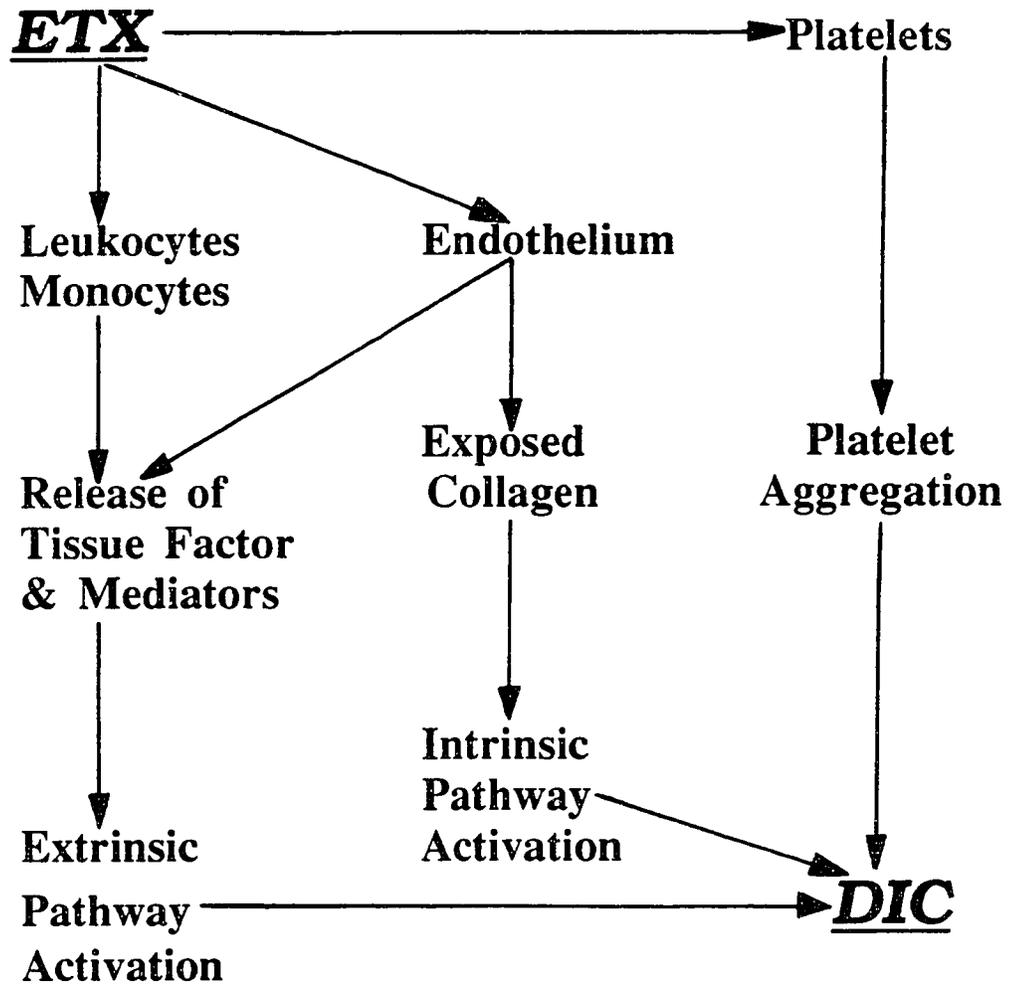


Figure 36. Mechanism for activation of DIC by endotoxin.

D. Erythrocyte deformability.

An increase in RBC deformability can be produced by a number of drugs. Of these, the one that has been studied most extensively is pentoxifylline. The drug has been reported to improve RBC deformability both in vitro (Nishio et al., 1982; Dormandy et al., 1981; Isogai et al., 1981) and in vivo (Schonharting & Schade, 1989; Puranapanda et al., 1987; Sugiura, 1983; Schubotz & Muhlfellner, 1977).

Endotoxin has been reported to cause a reduction in RBC deformability in vitro (Dzhenev et al., 1989) as well as in vivo (Hurd et al., 1988; Puranapanda et al., 1987) and pentoxifylline has been found to antagonize this effect (Sugiura, 1983; Puranapanda et al., 1987). In the present experiments, pentoxifylline, buflomedil and caffeine by themselves produced an increase in RBC deformability. Given as pretreatment, the three agents not only inhibited but actually reversed the reduction in RBC deformability caused by endotoxin. This rheological effect of the three agents was found to be dose-dependently correlated with the protective effect of the drugs against DIC and death caused by endotoxin. Thus, doses which prevented the latter were also effective in the reversing the endotoxin-induced reduction in deformability and doses which were ineffective in preventing death failed to inhibit the rheological effect of the toxin. In contrast to the other three agents, aminophylline did not

significantly affect the rheology of RBCs and failed to inhibit the endotoxin-induced reduction in deformability index.

The mechanism by which pentoxifylline, caffeine and buflomedil affect RBC deformability is not presently clear. Weed et al. (1969) reported that prolonged incubation of RBCs in vitro resulted in a progressive reduction in deformability which paralleled a progressive reduction in cellular ATP and an increase in cellular calcium. In a later publication, Weed (1970) concluded that the deformability of erythrocytes depends on at least three factors: (1) maintenance of the biconcave shape, which in turn is dependent on a high ratio of surface area to volume; (2) normal internal fluidity of the cell, which is primarily dependant on the normal properties of hemoglobin; and (3) an intrinsic deformability of the membrane which can be affected by changes in intracellular ATP, calcium and magnesium, as well as by changes in pH and oxygen tension in local regions of the microcirculation. Endotoxin can bind rapidly and reversibly to the red cell membrane (Godin et al., 1982), cause erythrocyte aggregation and change their normal biconcave shape (Baker et al., 1986). Previous studies in our laboratory showed that calcium channel blockers were highly effective in preventing DIC and death caused by endotoxin (Lee & Lum, 1986; Lee et al., 1989) and recently we found that the calcium channel blocker, nilvadipine, improved RBC deformability and reversed the rheological effect of endotoxin in rats

(Yao and Lum, unpublished results). The reports that pentoxifylline inhibits shear-induced calcium entry and calcium-dependent cross linking in erythrocytes (Swislocki & Tierney, 1989), and that the drug increases the ATP content of RBCs (Nishio et al., 1982; Buchanan & Moodley, 1977; Stefanovich, 1975) and inhibits RBC shape changes (discocyte-echinocyte transformation) caused by hyperosmolarity (Nishio et al., 1982) thus provide a basis for explaining the rheological effect of the drug. Reports that buflomedil has calcium antagonistic activity (Clissold et al., 1987) may similarly explain the effect of this agent on deformability.

E. Leukocyte deformability.

Schmalzer and Chien (1984) have reported that pentoxifylline, added to WBC preparations in vitro, produced an increase in the filterability of leukocytes through a polycarbonate filter. Their results suggested that the drug affected the filterability of monocytes and neutrophils but not lymphocytes. In the present study, WBC deformability was found to be increased by the in vivo administration of pentoxifylline, buflomedil and caffeine but not by aminophylline. The effects of the various agents on WBC deformability were thus positively correlated with their effects on DIC and death caused by endotoxin.

F. Hypothesis: role of reduced blood cell deformability in the pathophysiology of endotoxic shock.

The results of this investigation showed that prevention of endotoxin-induced DIC, circulatory shock and death by pentoxifylline, buflomedil and caffeine was positively correlated with an ability of the drugs to improve blood cell deformability. Thus, a possible causal relationship may exist between the latter action and the protective action of the three drugs in endotoxic shock.

The following sequence of events in the pathophysiology of endotoxic shock is hypothesized (Figure 37):

- (1) The reduced blood cell deformability caused by endotoxin is postulated to be a crucial event in the pathogenesis of endotoxic shock.
- (2) The "stiffening" of the erythrocytes and leukocytes impedes the passage of the blood cells through capillaries and thus impairs the delivery of oxygen and nutrients to tissues and organs, causing multi-organ dysfunction.
- (3) A reduction in blood cell deformability by endotoxin is postulated to induce DIC by a number of possible mechanisms. These include:
 - (a) Damage of the microcirculatory endothelium caused by the traumatic passage of "stiff" red and white blood cells, thereby releasing procoagulant tissue factor from the

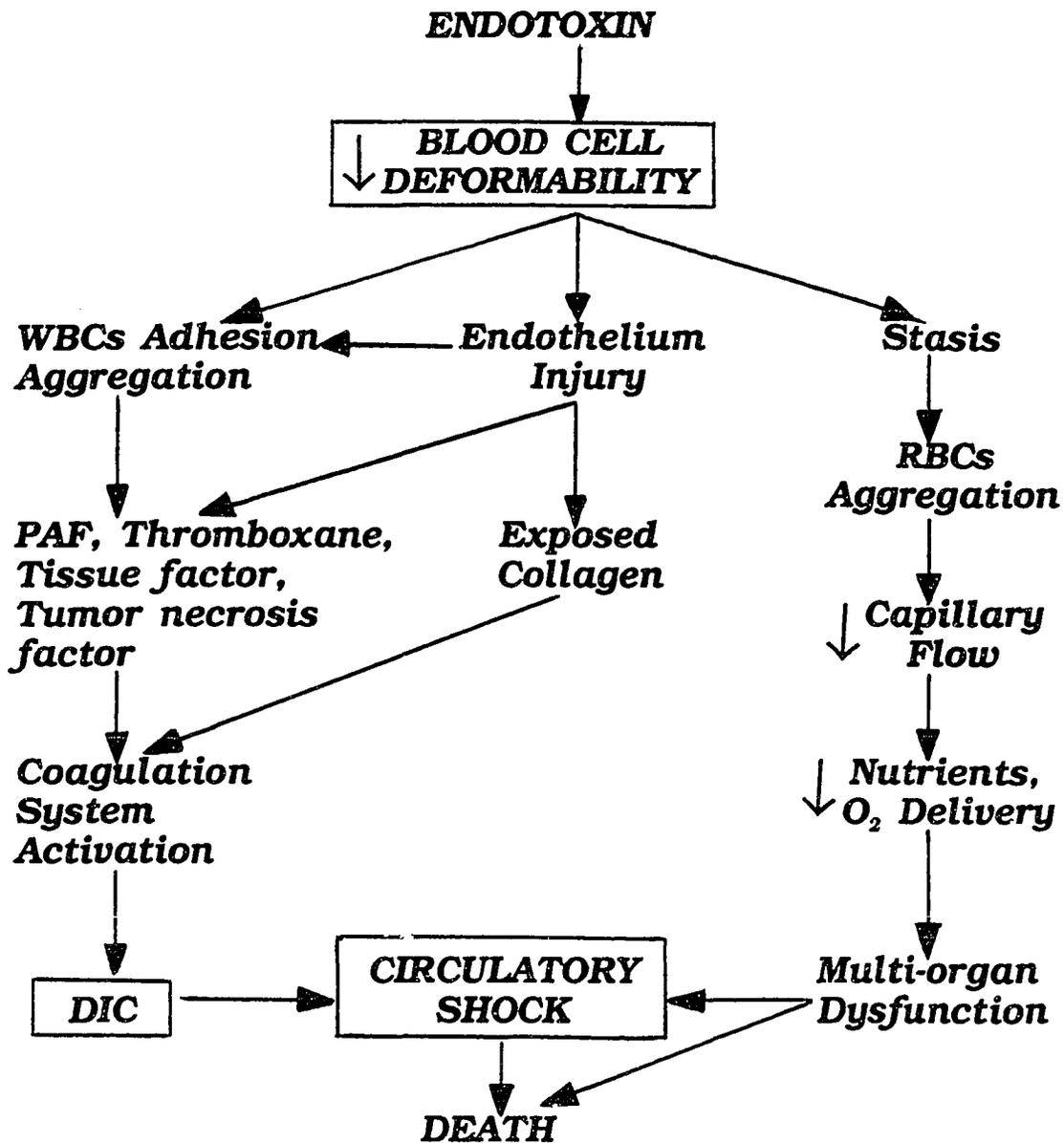


Figure 37. Hypothesis: role of reduced blood cell deformability in the pathophysiology of endotoxic shock.

endothelium as well as causing exposure of the underlying collagen to blood. Collagen is known to activate Factor XII and thus could trigger the coagulation cascade.

(b) Stasis of blood flow in the microcirculation causing RBC aggregation and clotting.

(c) Impeded passage of leukocytes causing aggregation of leukocytes, adhesion to damaged endothelium, activation of leukocytic release reaction, including release and/or increased formation of procoagulant tissue factor, thromboxane, platelet-activating factor, tumor necrosis factor, etc., thereby inducing DIC.

(4) Pentoxifylline, buflomedil and caffeine inhibit or reverse the rheological effects of endotoxin, thereby preventing the sequelae leading to DIC, circulatory shock and death.

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