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Abstract *Objective:* To evaluate the effects of hyperbaric oxygen (HBO) therapy on the coagulation cascade using an experimental model of multiple organ failure syndrome (MOFS). *Design:* MOFS was induced by zymosan (500 mg/kg i. p.) in rats. HBO therapy (2 ATA) was administered in a cylindrical steel chamber 4 and 11 h after zymosan administration. In a separate set of experiments animals were monitored for 72 h, and systemic toxicity was scored. *Intervention:* Eighteen hours after zymosan administration, rats were killed and blood samples were used for analysis of hemocoagulative parameters, hemodynamics, and arterial blood gas. *Main results:* Zymosan administration caused MOFS by affecting the coagulation cascade, as shown by a significant increase in plasma levels of fibrinogen, tissue plasminogen activator, inhibitor of tissue plasminogen activator of type 1, and plasma levels of fibrin degradation products vs. control rats. Zymosan-induced MOFS was also characterized by a significant increase in von Willebrand antigen plasma levels vs. controls. Moreover, zymosan administration induced a significant fall in mean arterial blood pressure and alteration in blood gas values. HBO therapy significantly reduced the derangements of coagulation cascade, the fall in mean blood pressure and alteration in blood gas induced by zymosan administration. *Conclusions:* The hypercoagulability induced by zymosan could be responsible for organ failure and death. Our data demonstrate that HBO therapy significantly prevents the alteration in the coagulation cascade and arterial blood gas in an experimental model of MOFS.

Keywords Zymosan · Multiple organ failure syndrome · Hyperbaric oxygen therapy · Hemocoagulation

Introduction

Multiple-organ failure syndrome (MOFS) is one of the most challenging clinical problems in intensive care. This and related conditions such as the systemic inflammatory response syndrome represent the end result of a wide variety of insults, including trauma, sepsis, and poisoning, that can lead to extensive tissue injury and ultimately to organ failure [1]. Studies of hemocoagulation disorders associated with MOFS and related conditions demonstrate that activation of the coagulation cascade and inhibition of fibrinolysis play a critical role in MOFS [2, 3, 4, 5]. The

fact that sepsis can alter the properties of the endothelium and cause it to function as a prothrombotic surface shows that the vascular endothelium can affect the hemostatic functions of vascular beds [6]. For instance, under physiological conditions the vascular endothelium maintains blood fluidity and, through anticoagulant protein C and antithrombin systems, fibrinolysis [7].

The experimental model of MOFS induced by the intraperitoneal administration of zymosan has been validated in several studies [8]. Zymosan, a cell wall component of the yeast *Saccharomyces cerevisiae*, is an inflammatory agent [9]. It is a nonbacterial, nonendotoxic agent that produces acute peritonitis and MOFS characterized by functional and structural changes in lung, liver, kidney, and intestine [10]. In addition, we have reported that zymosan causes signs of both peritonitis and organ injury within 18 h [8]. The onset of the inflammatory response caused by zymosan in the peritoneal cavity was associated with systemic hypotension, high peritoneal, and plasma levels of nitric oxide, maximal cellular infiltration, exudate formation, cyclooxygenase activity, and proinflammatory cytokine production [8]. We have also demonstrated that exposure to hyperbaric oxygen (HBO) blunts vascular derangements and organ failure in the zymosan-induced shock model in rats, thereby resulting in improved survival [11, 12].

The aim of this study was to evaluate the effects of HBO on mortality and morbidity and the role of HBO in the changes in the hemocoagulative cascade that can occur during zymosan-induced MOFS.

Materials and methods

Animals

Sprague-Dawley male rats weighing 250–300 g were used in this study. Animals were housed in compliance with Good Laboratory Practice for the protection of experimentally used animals (Italian Ministry of Health Decree no. 116/92) and with European Economic Community regulations (OJ of ECl 358/1, 18 December 1986). They were kept at a constant temperature of 21 ± 1 °C and relative humidity of 60% under an alternating light-dark cycle (light: 7 am–7 pm) and given free access to water and food.

Zymosan-induced MOFS

Animals were randomly divided into four groups of ten rats each. One group was injected with saline solution (0.9% NaCl intraperitoneally) and served as control group. The second group received zymosan (500 mg/kg intraperitoneally). The third group received zymosan (500 mg/kg intraperitoneally) and HBO (2 absolute atmospheres, ATA) 4 and 11 h after zymosan-induced shock. The fourth group underwent HBO treatment 4 and 11 h after saline solution administration. Before the study a culture test carried out on a zymosan solution previously sterilized in a waterbath at 100 °C for 80 min demonstrated the absence of contamination. In another set of experiments animals ($n = 10$ in each group) were randomly divided as described above and monitored for systemic toxicity, loss of body weight, and mortality for 72 h after zymosan or saline administration as previously described [10]. The time and the dose of HBO treatment was chosen based on a previous observation in which HBO treatment was shown to prevent MOFS induced by zymosan [11].

Clinical scoring of systemic toxicity

The clinical severity of systemic toxicity was scored for 72 h in rats after zymosan or saline injection on a subjective scale ranging from 0 to 3: 0 = absence, 1 = mild, 2 = moderate, 3 = serious. The scale was used for each of the toxic signs (conjunctivitis, ruffled fur, diarrhea, and lethargy) observed in the animals. Clinical scoring was carried out by an independent investigator who had no knowledge of the treatment regimen received by each animal.

Blood pressure evaluation

The animals were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneally). The femoral artery was cannulated with a 4 cm length of Tygon tubing (0.01 inch inner diameter). The catheter was then sealed with a straight pin, routed subcutaneously to the nape of the neck and through a leather harness fastened around the forequarters of the rat. The animals were allowed to recover for 3 days before experiments were performed. Immediately before the experiments the catheter was connected to a pressure transducer, and the stable baseline of mean arterial blood pressure (MAP) was recorded for 20 min by a polygraph (Hellige, Model 218087, Germany). The MAP of freely moving rats was then recorded 3, 6, 12, and 18 h after treatment.

HBO treatment

HBO was administered in a cylindrical steel chamber (40 cm diameter, 65 cm long; Galeazzi, La Spezia, Italy) with thick glass windows so that animals could be observed during treatment. Before pressurization 100% medical oxygen was flushed through the chamber for 5 min to displace the room air. Oxygen pressure was then increased at a constant rate to reach a pressure of 2 ATA

in 4 min. The animals were treated under compression for 60 min. The chamber was constantly ventilated at a rate of 4 l/min to avoid carbon dioxide accumulation during pressurization. The O_2 concentration was greater than 99% (Taylor Servomex OA272 Oxygen Analyzer, Italy) and carbon dioxide was less than 0.2% (Medical Gas Analyzer LB-2, Model 40 M, Beckman, USA) [11].

Arterial blood gas analysis

Eigtheen hours after the study began blood samples were collected from femoral artery and measuraments of partial arterial pressure of oxygen (PaO_2), partial arterial pressure of carbon dioxide ($PaCO_2$), blood pH and bicarbonates levels were determinated with a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy).

Platelet aggregation ex vivo and platelet count

Blood samples were drawn from the abdominal aorta and collected into a tube containing sodium citrate (3.8%; 1 v for 9 v of blood) 18 h after zymosan administration. Platelet-rich plasma (PRP) was prepared by centrifugation at 2,200 rpm for 3 min at 23 °C. Platelet-poor plasma (PPP) was prepared by centrifugation at 10,000 rpm for 8 min at 23 °C. Platelet aggregation of PRP, induced by 10 μm ADP, was measured by aggregometer (Optical Aggregometer, Model 4902D, Chrono-Log Corp., Havertown, Pa., USA). The tests were performed at 37 °C in 450 μl PRP in a siliconized cuvette with continuous stirring. The platelet count in PRP was adjusted to 350,000/ml by dilution with PPP as required. Ex vivo data of platelet aggregation are presented as the percentage of aggregation relative to PRP. Moreover, an aliquot of blood samples drawn from adbominal aorta was used to count the number of platelets in a Burker chamber as reported elsewhere [13].

Analysis of hemocoagulative parameters

Prothrombin time (PT), activated partial thromboplastin time (apTT), and fibrinogen (FIB) were measured according to the manufacturer's instruction (Roche Diagnostics, Monza, Italy). Plasma levels of tissue plasminogen activator (t-PA), the inhibitor of tissue plasminogen activator of type 1 (PAI-1), and von Willebrand antigen (vWF) were measured by enzyme-linked immunosorbent assay based on specific monoclonal antibodies. Plasma levels of fibrin degradation products were measured with an agglutination test.

Drugs

Zymosan A was obtained from Sigma (Milan, Italy). All reagents for the determination of hemocoagulative parameters were purchased from Bouty (Sesto San Giovanni, Italy) or bioMérieux (Florence, Italy).

Statistical analysis

All standard error of the values in the figures and text are expressed as mean ± standard error of the mean (SEM) of n observations. For the in vivo studies n represents the number of animals studied. The results were analyzed by one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparisons. Differences at the level of $p < 0.05$ were considered statistically significant. Statistical significance for survival data was calculated by Fisher's exact test; for such analyses $p < 0.05$ was considered significant. The Mann–Whitney U–test (two-tailed, independent) was used to compare medians of the clinical score; when this test was used, $p < 0.05$ was considered significant.

Results

Effects of HBO therapy on systemic toxicity, body weight loss, and mortality rate induced by zymosan

The administration of zymosan caused severe illness in the rats, namely ruffled fur, lethargy, conjunctivitis, diarrhea, and a significant loss of body weight ($p < 0.01$; Table 1, Fig. 1a). Systemic toxicity appeared 6 h after the intraperitoneal injection of zymosan and gradually became more severe. As shown in Fig. 1b, animals began to die about 18 h after treatment, and more than 50% of the zymosan-treated animals had died at the end of the observation period. Treatment with HBO exerted a protective effect. Symptoms were less severe in zymosan-treated rats exposed to HBO than in zymosan-treated rats not exposed to HBO (Table 1). Moreover, there was no significant loss of body weight (Fig. 1a) and no mortality (Fig. 1b) in HBO-treated animals. The protective effect of HBO treatment on these parameters is in agreement with our previous observation [11].

Effect of HBO therapy on zymosan-induced hypotension

MAP decreased within 6–7 h of zymosan injection and reached its lowest values (45 ± 2.6 mmHg) at 18 h (Fig. 1c). HBO treatment of rats blunted the severe

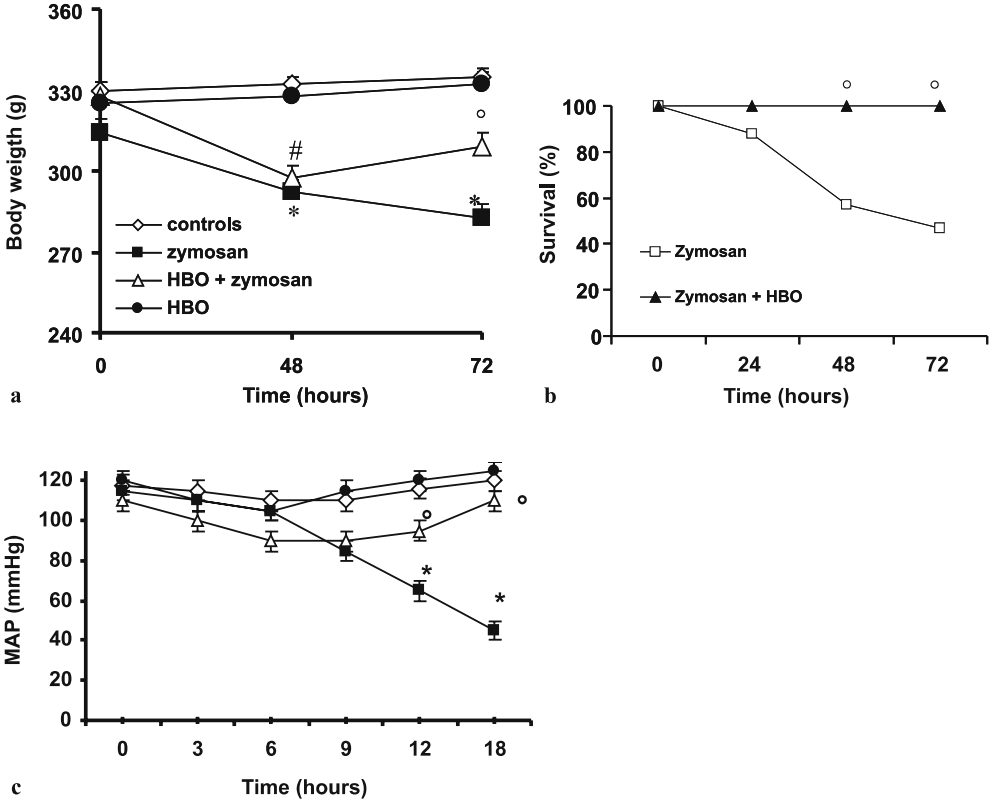

Fig. 1 **a** Changes in body weight (g) during zymosan-induced multiple organ failure syndrome in rats treated or not with hyperbaric oxygen therapy (*HBO*). Each value is the mean ± SEM of $n = 10$. * $p < 0.01$ vs. controls, # $p < 0.05$ vs. zymosan, ° $p < 0.01$ vs. zymosan. **b** Survival of zymosan-induced multiple organ failure syndrome in rats treated or not with HBO. ° $p < 0.01$ vs. zymosan. For clarity, the values of control and HBO saline groups are not shown. Survival was 100% in both groups. Each group consisted of ten animals. **c** Modification in mean arterial pressure (*MAP*) during zymosan induced multiple organ failure syndrome in rats treated or not with HBO. Each value is the mean ± SEM for $n = 10$. * $p < 0.01$ vs. controls, ° $p < 0.05$ vs. zymosan

Table 1 Systemic toxicity of zymosan-induced multiple organ failure syndrome in rats treated or not with hyperbaric oxygen therapy; score: 0 = absence of symptomatology, 1 = mild, 2 = moderate, 3 = severe

	Hours after zymosan injection					
Symptom	3	6	18[a]	24[a]	48[a]	72[a]
Control group						
Ruffled fur	0.5 ± 0.05	0.4 ± 0.03	0.5 ± 0.04	0.3 ± 0.02	0.5 ± 0.05	0.5 ± 0.05
Lethargy	0	0	0	0	0	0
Conjunctivitis	0	0	0	0	0	0
Diarrhea	0	0	0	0	0	0
Zymosan group						
Ruffled fur	0.8 ± 0.1	1.48 ± 0.1	2.3 ± 0.3	2.6 ± 0.2	2.6 ± 0.2	1.2 ± 0.1
Lethargy	0.2 ± 0.08	0.8 ± 0.1	1.6 ± 0.1	2.3 ± 0.2	2.3 ± 0.2	1.3 ± 0.2
Conjunctivitis	0.27 ± 0.06	0.38 ± 0.08	1.8 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	0.8 ± 0.1
Diarrhea	0.2 ± 0.08	0.2 ± 0.09	0.8 ± 0.1	1.9 ± 0.1	0.7 ± 0.1	0.4 ± 0.03
Zymosan+HBO						
Ruffled fur	0.7 ± 0.1	0.8 ± 0.1	1.5 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	0.7 ± 0.1
Lethargy	0.2 ± 0.05	0.5 ± 0.03	1.2 ± 0.1	1.4 ± 0.1	0.8 ± 0.03	0.3 ± 0.03
Conjunctivitis	0.2 ± 0.06	0.5 ± 0.03	0.8 ± 0.1	1.2 ± 0.2	0.6 ± 0.02	0.2 ± 0.02
Diarrhea	0.2 ± 0.06	0.2 ± 0.06	0.7 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.4 ± 0.05

[a] Data obtained from survivor rats

zymosan-induced hypotension, and MAP was maintained at control levels (110 ± 5.7 mmHg). HBO treatment did not cause significant changes in MAP in control rats (Fig. 1c). The protective effect of HBO treatment on this parameters is in agree with our previously observation [12].

Effects of HBO therapy on zymosan-induced arterial blood gas analysis alterations

As shown in Table 2, $PaCO_2$, and HCO_3^- were significantly ($p < 0.01$) reduced while PaO_2 was significantly ($p < 0.01$) increased 18 h after zymosan administration

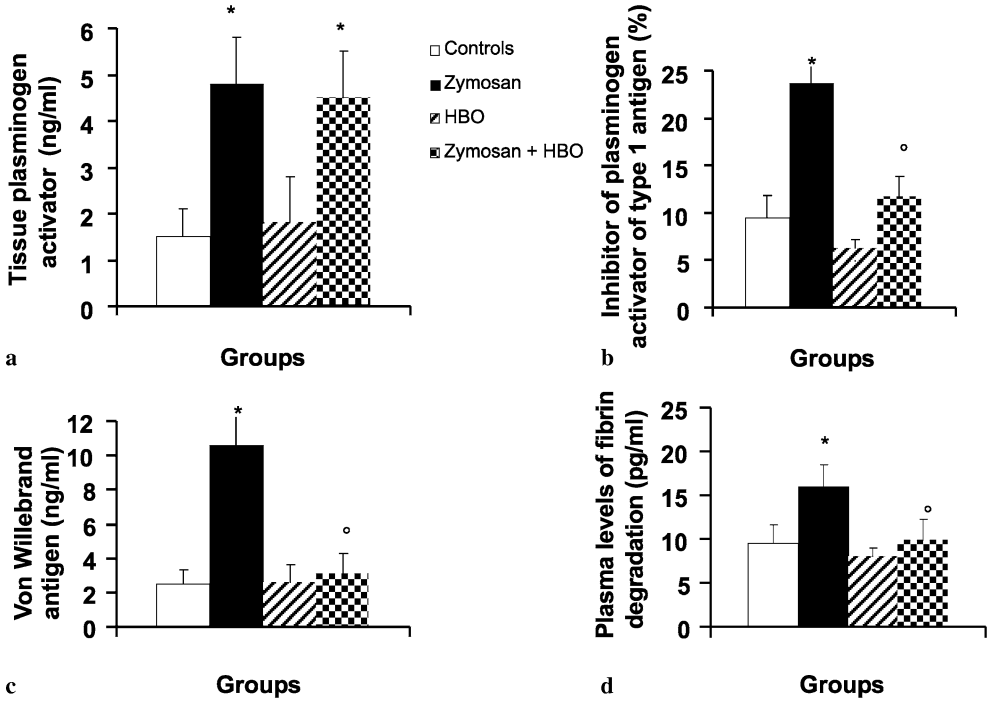

Fig. 2 **a** Evaluation of tissue plasminogen activator during zymosan-induced multiple organ failure syndrome in rats treated or not with hyperbaric oxygen therapy (*HBO*). * $p < 0.01$ vs. controls. **b** Evaluation of the inhibitor of plasminogen activator of type 1 antigen during zymosan-induced multiple organ failure syndrome in rats treated or not with HBO. * $p < 0.01$ vs. controls; ° $p < 0.01$ vs. zymosan. **c** Evaluation of von Willebrand antigen during zymosan-induced multiple organ failure syndrome in rats treated or not with HBO. * $p < 0.01$ vs. controls; ° $p < 0.01$ vs. zymosan. **d** Plasma levels of fibrin degradation products during zymosan-induced multiple organ failure syndrome in rats treated or not with HBO. * $p < 0.01$ vs. controls; ° $p < 0.01$ vs. zymosan. Each value is the mean ± SEM for $n = 10$

Table 2 Measurements of pH, PaO_2, $PaCO_2$, and HCO_3^- in rats with in zymosan-induced multiple organ failure syndrome, treated or not with hyperbaric oxygen therapy (*HBO*)

Group	pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	HCO_3^- (mmol/l)
Control	7.32 ± 0.14	140.3 ± 7.4	56.2 ± 4.1	27.7 ± 5.2
HBO	7.31 ± 0.13	142.4 ± 7.3	54.2 ± 4	26.5 ± 4.8
Zymosan	7.46 ± 0.15	203.7 ± 16.4[a]	15.5 ± 5.5[a]	9.75 ± 3.1[a]
Zymosan+HBO	7.33 ± 0.1	144.3 ± 9.5[b]	49.3 ± 4.3[b]	23.75 ± 3.2[b]

[a] $p < 0.01$ vs. controls, [b] $p < 0.01$ vs. zymosan

compared with control rats. Treatment with HBO prevented this effect (Table 2). Blood pH was not altered in any of the experimental groups (Table 2). HBO therapy did not modify the arterial blood gas parameters in sham-treated rats.

Effects of HBO therapy on zymosan-induced hemocoagulative parameter alterations

Eighteen hours after zymosan administration there were significant ($p < 0.01$) reductions in PT (50.6 ± 7.3% vs. 98 ± 12.8%), apTT (60.6 ± 7.5 vs. 19.3 ± 2.5 s), and fibrinogen (150 ± 15.8 vs. 45.7 ± 5.4 mg/dl) compared with control rats. Treatment with HBO prevented the changes in PT (77.2 ± 4.5% vs. 50.6 ± 7.3%), apTT (20.5 ± 2.7 vs. 60.6 ± 7.5 s), and fibrinogen (50 ± 7.2 vs. 150 ± 15.8 mg/dl) observed in zymosan-treated rats ($p < 0.01$). Moreover, plasma levels of t-PA (Fig. 2a), PAI-1 (Fig. 2b), vWF (Fig. 2c), and fibrin degradation products (Fig. 2d) were significantly higher ($p < 0.01$) in zymosan-treated rats than in controls. HBO significantly restored plasma levels of PAI-1 (Fig. 2b), vWF (Fig. 2c), and fibrinogen degradation products (Fig. 2d) in treated animals. On the other hand, HBO therapy did not reduce the increase in t-PA plasma level induced by zymosan administration (Fig. 2a). HBO therapy did not affect the hemocoagulative parameters in sham-treated rats.

Effects of HBO therapy on zymosan-induced thrombocytopenia and platelet aggregation

As shown in Fig. 3a, there was significant thrombocytopenia 18 h after zymosan administration. HBO treatment significantly reduced the zymosan-induced thrombocytopenia (Fig. 3a). HBO therapy did not affect the number of platelets in sham-treated animals (Fig. 3a). In addition, the ex vivo platelet aggregation in response to ADP (10 μm) was significantly increased in blood

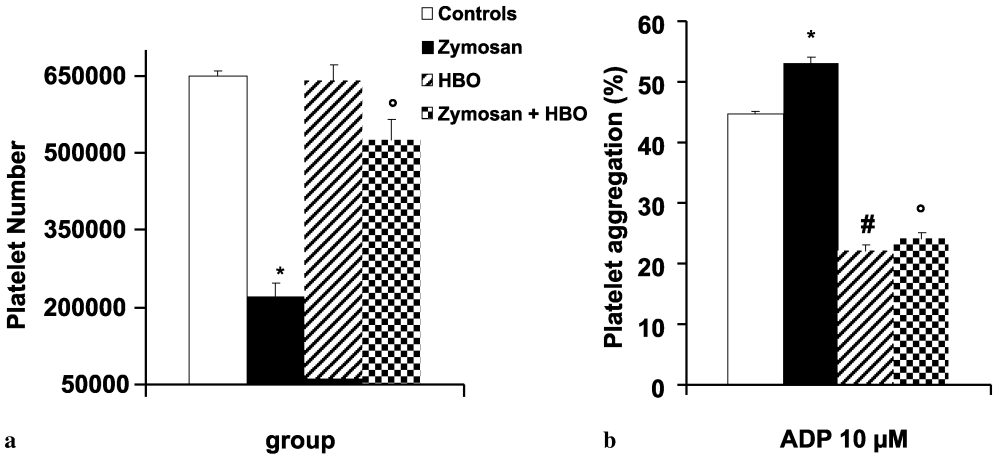

Fig. 3 **a** Platelet number during zymosan-induced multiple organ failure syndrome in rats treated or not with hyperbaric oxygen therapy (*HBO*). * $p < 0.01$ vs. controls, ° $p < 0.01$ vs. zymosan. **b** Platelet aggregation during zymosan-induced multiple organ failure syndrome in rats treated or not with HBO. * $p < 0.01$ vs. controls, ° $p < 0.01$ vs. zymosan, # $p < 0.01$ vs. controls. Each value is the mean ± SEM for $n = 10$

from zymosan-treated rats (Fig. 3b). However, in vivo HBO significantly reduced platelet hyperresponsiveness in zymosan-treated rats (Fig. 3b). It also significantly reduced the aggregation ability of platelets from sham-treated rats (Fig. 3b). The effect of HBO in vivo treatment on platelet aggregation coincides with previously reported results [14].

Discussion

We evaluated the effect of HBO therapy on zymosan-induced nonseptic shock, which is a well-characterized model for the assessment of mediators involved in this condition. We provide the first evidence that HBO treatment attenuates: (a) the fall in MAP, (b) systemic toxicity, (c) mortality, (d) the mixed acid base disorder, (e) inhibition of fibrinolysis (as evaluated by PA-I), (f) activation of the coagulation system (PT, aPTT, and fibrinogen), and (g) thrombocytopenia and platelet aggregation caused by zymosan. Together these findings support the view that HBO attenuates the coagulation disorder induced by zymosan in rats.

During nonseptic shock endotoxemia, cardiac dysfunction, progressive hypotension, coagulopathies, and organ perfusion defects are frequent sequences that lead to multiple organ failure. In animal studies coagulopathy has been reported to be the most critical manifestation during MOFS, in which platelets and fibrin are deposited in blood vessels, thereby leading to disseminated intravascular coagulation (DIC). In turn DIC leads to thrombotic and hemorrhagic events, which are the main causes of death in MOFS [15, 16, 17, 18]. DIC is a systemic syndrome characterized by enhanced activation of coagulation with some intravascular fibrin formation and deposition, depending on the degree of activity [19]. Intravascular fibrin has been often found in patients who died from an illness associated with evidence of DIC [20]. In addition, in cohort studies mortality was higher in patients with DIC than in those with the same underlying disease but no evidence of DIC [21]. Similarly, experimental studies of DIC associated with sepsis or low-grade activation of coagulation have demonstrated that inhibition of DIC reduces mortality [22, 23]. The microvasculature is the critical interface for oxygen and energy delivery to tissues. Thus any damage to or obstruction of the microvasculature may have harmful consequences.

DIC, diffuse microvascular injury and obstruction, increased vascular permeability, perfusion failure, and organ dysfunction in sepsis and associated syndromes may be related to widespread endothelial apoptosis. The generation of proinflammatory cytokines has several consequences for the microvasculature with relationship to blood coagulation and DIC. Vascular endothelial cells may be perturbed by the action of cytokines such as interleukins 1, 6, and 8, as well as by tumor necrosis factor α [24]. These cytokines change the general anticoagulant phenotype of the endothelium into a procoagulant phenotype, at least under in vitro conditions. This results in reduced expression of thrombomodulin [25], heparan sulfates [26], and potentially upregulating tissue factor [27]. We recently demonstrated that HBO therapy significantly reduced the release of tumor necrosis factor α at 18 h after zymosan administration [11].

DIC occurs when there is an inappropriate widespread activation of the coagulation cascade [28, 29]. In MOFS activation of the extrinsic pathway combined with depression of the inhibitory mechanisms of coagulation and of the fibrinolytic system result in a procoagulant state that may lead to microvascular thrombosis and organ dysfunction. It has recently been demonstrated that PT, aPTT, and fibrinogen are deranged in over 90% of patients with decompensated DIC [30, 31]. The present study demonstrates that zymosan administration alters the coagulation pathway. Treatment with HBO significantly restored the coagulation function.

A number of prospective and case-control studies have shown that fibrinolysis is impaired in subjects with

myocardial infarction, ischemic stroke [32, 33, 34, 35, 36] and, although controversially, peripheral artery disease [37, 38]. This occurs because of increased activity of plasmatic PAI-1, a glycoprotein produced by the endothelium and present in the platelets. Here we demonstrate that PAI-1 is significantly increased during zymosan-induced nonseptic shock and that HBO counteracts this effect. Differently, we found that the zymosan-induced increase in t-PA levels was not reduced by HBO therapy. Recently Yamami and colleagues [39] reported that HBO therapy significantly increased t-PA activity in healthy volunteers. This observation may partially explain the fact that HBO therapy did not reduced t-PA level during zymosan-induced MOFS. However, further studies are required to clarify this point. Generally MOFS is characterized by activation of the humoral cascade system, which may subsequently influence platelet aggregation ability. Activation of the coagulation cascade during systemic inflammation is well known to be the major trigger of DIC [24]. In agreement with this general clinical observation we demonstrate that zymosan-induced MOFS increased platelet aggregation ex vivo as well as vWF. Moreover, HBO treatment significantly reduced the increase in vWF and the platelet aggregation in sham-treated animals as well as in shocked-rats. The ability of HBO to reduced platelet aggregation is in agreement with a previous study [14]. Consequently HBO therapy significantly reduced thrombocytopenia induced by zymosan.

In conclusion, we provide the first evidence that HBO therapy attenuates: (a) inhibition of fibrinolysis, (b) activation of the coagulation system, and (c) thrombocytopenia and platelet hyperaggregation caused by zymosan. Undoubtedly there is activation/dysfunction of both coagulation and fibrinolysis in this model. However, based on the parameters now available it is not easy to clarify the balance between coagulation and fibrinolysis. Future studies are needed to clarify this point, but our results demonstrate an additional antishock property of HBO therapy and supports the use of HBO in DIC conditions associated with nonseptic shock.

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