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ORIGINAL PAPER

Effects of L-Canavanine and ozone on vascular reactivity in septicemic rats

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Abstract Septicemia leads to oxidative stress with overproduction of reactive-oxygen species (ROS) and consumption of endogenous antioxidant enzymes. We tested a twofold hypothesis: (1) does oxidative stress (OxS) induced by sepsis acting alone or in concert with augmented inflammatory processes contributes to sepsis-related vascular dysfunction, and, (2) whether ozone (O_3) and L-canavanine (CAV) mitigate the negative impact of the aforementioned phenomena. We investigated the relative impact of treatment with CAV and/or O_3 on vascular OxS associated vascular functional changes in septicemic rats. For this study, 60 male Sprague–Dawley rats were used and divided into six experimental groups ($n=10$): control group (C), sham-operated (Sham), septicemic rats (S), S rats treated with CAV (100 mg/kg. i.p; S+CAV), S rats treated with O_3 (1.2 mg/kg, i.p.; S+O_3) and S rats treated with both O_3 and CAV (S+O_3+CAV). After 22 h, the mean arterial blood pressure (MAP), the aortic ring vascular reactivity to phenylephrine, abdominal aortic blood flow (AABF), serum tumor necrosis factor-α (TNF-α) and plasma nitrite/nitrate (NOx) concentration were measured. In addition, hepatic antioxidant enzyme activities sodium dismutase (SOD) and glutathione peroxidase (GSH-Px) were estimated. Septicemia caused significant elevation of serum TNF-α ($p<0.001$) and plasma NOx ($p<0.001$) and significant ($p<0.001$) reduction of AABF ($p<0.001$), aortic vascular response to phenylephrine ($p<0.001$), MAP ($p<0.001$) and hepatic SOD and GSH-Px activity ($p<0.001$) compared with the C group, while treatment with O_3 and/or CAV induced significant amelioration of all those increases. Abnormalities were attenuated to a similar extent with treatment with both O_3 and CAV. These results

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suggested that concomitant administration of O_3 and CAV alleviated the compromised vascular reactivity in septicemic conditions and prevent its progression into septic shock compared with each alone.

Keywords Septicemia · Ozone · L-canavanine · Oxidative stress · Hepatic antioxidant enzymes

Introduction

Bacterial sepsis is a systemic inflammatory state characterized by vascular smooth muscle (VSM) dysfunction, leading to hypotension, inadequate tissue perfusion and multiple organ failure [14]. Septicemia not only leads to overproduction of reactive-oxygen species (ROS) but also consumption of endogenous antioxidant enzymes with associated inhibition of the hepatic antioxidant enzyme synthesis such as superoxide dismutase SOD [11] and glutathione peroxidase (GSH-Px) [30].

All pathologic processes in the structure and function of human body during endotoxin shock are a result of the misbalance of a number of mediators with a proinflammatory and anti-inflammatory effects [45]. The proinflammatory cytokines, including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) of the outer membrane of gram-negative bacteria and the anti-inflammatory cytokines including IL-10 and IL-12, and cell adhesion molecules induce vascular inducible nitric oxide (iNOS) expression [31]. iNOS is a key molecule believed to be responsible for several aspects of sepsis [48]. In response to systemic bacterial infection, iNOS is expressed in several diverse cell types resulting in increased nitric oxide (NO) production. NO is thought to have a dual function; i.e., it is protective in terms of its bactericidal actions in host defense [36], but conversely pathogenic in terms of contributing to some of the deleterious effects of endotoxemia, namely: impaired contractile function of VSM with the resultant decreased blood pressure and vascular hyporeactivity [46]. It has been shown that that the components of infectious organisms; such as LPS, directly activate the toll-like receptors in the VSM with subsequent elevation of NF-κB levels which translocates to the nucleus inducing iNOS synthesis as it binds to the promoter regions of various inflammatory genes [32].

L-Canavanine (CAV) is a potent L-arginine antagonist that has a selective inhibitory action on iNOS enzyme activity [18]. Hsieh et al. [24] observed that CAV attenuated the effects of LPS through direct inhibition of iNOS.

The Europe-based Medical Society for Ozone and the National Center for Scientific Research in Cuba currently use O_3 for the treatment of a wide variety of conditions, including wound problems, gastrointestinal disorders, cancer, AIDS, and different types of hepatitis [47]. O_3/oxygen mixture also has a strong microbiocidal activity in vitro and might therefore act as a regulator or modulator of many inflammatory processes in vivo [19]. It also exhibits various effects on the immune system where it modulates the phagocytic activity of the peritoneal and the alveolar macrophages [12, 13]. Therefore, it can be hypothesized that O_3/oxygen mixture may enhance the production/release of proinflammatory cytokines in different organs as liver that could influence the outcome of infection [53]. It was reported that, O_3 increased the effectiveness of the antioxidant enzyme system, which scavenges excess free radicals in the body. Low doses of O_3 could also induce the antioxidant endogenous systems, including GSH-Px, SOD and catalase (CAT) preparing the host to face patho-physiological conditions mediated by ROS [34]. Thus, O_3 by means of an oxidative preconditioning mechanism may produce improvement of antioxidant–prooxidant imbalance with subsequent preservation of cell redox state [6, 8].

The present study was undertaken to determine whether O_3 and/or CAV prevent impairment of vascular reactivity caused by sepsis-induced OxS, and if so, whether TNF-α and iNOS have a prominent role in sepsis-prevented attenuated vascular reactivity. Specifically, we addressed the hypothesis that during sepsis, O_3 and CAV attenuate the ability of induced OxS stress to decrease vascular reactivity by interfering with signaling through the TNF-α and iNOS pathway. To test this hypothesis, we used septimetic Sprague–Dawley rats, rats injected with CAV, O_3 or injected with both O_3 and CAV and compared them with septic (S) and controls (C) in terms of CAV and O_3 regulation of: (1) mean arterial pressure (MAP), (2) vascular reactivity (3) abdominal aortic blood flow (AABF), (4) TNF-α and NOx (5) activity of SOD and GSH-Px.

Materials and methods

Chemicals

CAV was purchased from Sigma, St Constant, QC, Canada. Ketamine hydrochloride was supplied by Pfizer pharmaceutical company, INS, USA. Thiopental sodium was ordered from Biocheme, Austria and recombinant human TNF-α was supplied by BASF/Knoll, Ludwigshafen, Germany.

Ozone (O_3) preparation and administration

O_3 was generated by an ozonator equipment (manufactured by the O_3 Longevity Resources Dwyermade, Canada) and was administered intraperitoneally (i.p.) at a dose of 1.2 mg/kg body weight in a volume of 1 ml as a gas in a gas mixture with O_2. The concentration of O_3 used in our study is identical to that used in other studies [34]. The O_3 concentration in the O_3/O_2 mixture was 50 μg/ml and was measured by using an UV spectrophotometer at 254 nm. O_3 preparation was obtained from medical-grade oxygen and used immediately upon generation [41].

Experimental animals

This study was carried out on 60 male Sprague–Dawley rats (150–200 g). Animals were fed with standard laboratory chow and water ad libitum and housed in the animal house of Menoufiya Faculty of Medicine under artificial light/dark cycle of 12 h. The animals were divided into six equal groups (n=10 each): C-rats had no surgery; Sham–Sham-operated animals were subjected to laparotomy with light manipulation of the cecum without ligation and perforation; S-septicemic-rats were subjected to cecal ligation and perforation (CLP) to induce septicemia; S+CAV rats were treated with a single dose [100 mg/kgi.p.] of CAV [50]; S+O_3-CLP animals were treated with a single dose of O_3 (1.2 mg/kg, i.p.) and, S+CAV+O_3 group-CLP animals received CAV and O_3 in the same doses as groups S+CAV and S+O_3. The concentration of CAV used in our study is identical to that used in other studies [50].

Sepsis was induced by CLP [28]. The rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) [35] and an abdominal incision (3 cm) was made to expose the cecum. The cecum was ligated and punctured once with a 21-gauge needle and a small amount of feces was extruded to ensure patency of the bowel. The bowel was returned to the abdomen, and the abdominal cavity was closed in two layers. The sham operation in group (Sham) consisted of laparotomy and light manipulation without ligation or puncture of the cecum. Without postoperative fluid resuscitation, animals are not able to demonstrate the early, hyperdynamic phase of sepsis studies [25, 50]. Further, it is important that the saline solution is prewarmed (37°C) to avoid iatrogenic hypothermia, which most likely affects the outcome of CLP. Thus, the animals of both procedures were resuscitated with prewarmed (37°C) saline (5 ml/100 g b.w, s.c) postoperatively resembling the first-line of treatment of septic shock in humans [50]. The experiments were conducted in accordance with the ethical guidelines for investigations of laboratory animals and were approved by the ethical committee of the Faculty of Medicine, Menoufiya University.

Parameters measured

At the end of the experimental protocol period (22 h after CLP), the following parameters were measured:

Mean arterial pressure

MAP was measured using tail-cuff plethysmography (Harvard Apparatus Ltd., England) [49] in conscious rats prewarmed for 10 min in a thermostatically controlled restrainer (XBP1000; Kent Scientific). The mean of at least three separate recordings on three occasions was taken.

Abdominal aortic blood flow

After overnight fast, the animals were anesthetized with thiopental sodium (60 mg/kg body wt, i.p.) [26]. The trachea was intubated, the abdominal wall was opened by midline incision and the suprarenal abdominal aorta was gently freed from surrounding tissue. The probe top of Pulsed Doppler Flow-meter (Model T206, Transonic System, and Ithaca, NY) was covered with an ultrasound gel, the volume control was adjusted to maximum and the probe was pressed

gently to the measurement area of the aorta at 45–60°. After hearing the optimal sounds, the freeze key was pressed to freeze the wave form record [22]. From the record, AABF was determined.

Blood sampling

After measuring the abdominal aortic blood flow, blood samples were obtained by direct heart puncture. Samples were divided into two parts; the first part was allowed to clot for 20 min, then, centrifuged at 14,000 rpm for 10 min for serum separation and used for determination of serum TNF-α. The blood of the other part was added to EDTA and centrifuged at 1,000 rpm for 15 min for separation of plasma and stored at −80°C for assay of the total plasma nitrite/nitrate (NOx) level.

Plasma nitrite/nitrate level

The method for estimation of total NOx level was based on the Griess reaction. Plasma nitrite and nitrate level were measured after enzymatic conversion of nitrate to nitrite by nitrate reductase (from *Aspergillus*) in the presence of NADPH. The oxidation of the coenzyme was monitored by the decrease in absorbance at 340 nm. Results are expressed as μmol/L [37].

Estimation of serum TNF-α

TNF-α was measured by cytotoxicity on L929 cells. TNF-α level was determined using recombinant human TNF-α (BASF/Knoll, Ludwigshafen, Germany; specific activity 10^7 U/mg) and expressed in pg/mL [5].

Aortic ring preparation

After taking blood samples from the heart, rats were killed by lethal dose of thiopental sodium (180 mg/Kg wt, i.p.). The thoracic aorta was removed and placed in cold physiological Kreb's solution. The aorta was cleaned of extraneous tissue, and rings were cut (2–3 mm) and mounted in 10-ml organ baths, under 1 g tension for online isometric tension recording, using specific chart [21]. Aortic rings were bathed in prewarmed physiological Krebs solution and maintained at 37°C and then bubbled with 5% CO_2 in O_2. After 45 min of equilibrium in Krebs solution, control concentration–response curves to phenylephrine (10^{-9}–10^{-5} M) were performed. The dose of phenylephrine that gives 50% of the contraction response is 10^{-7} M [16].

Preparation of liver homogenates for estimation of SOD and GSH-Px enzymatic activity

After removal of the thoracic aorta, the right lobe of the liver was dissected, cleaned from extraneous tissues, cut into small pieces and homogenized by using Omni tissue homogenizer (Omni International, Gainesville, VA). The liver homogenate was centrifuged at 600 g for 60 min at 4°C and the supernatant was stored at −80°C to be used for various measurements [53].

SOD activity in liver

SOD activity in liver homogenates was assayed following the method modified by Kakkar et al. [29]. Each sample (containing 5 μg proteins) was mixed with sodium pyrophosphate buffer, phenazine methosulfate and nitro blue tetrazolium. The reaction was started by addition of NADH, and reaction mixture was incubated for 90 s at 30°C. Then, the reaction was stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in 1 min under the assay condition.

GSH-Px activity in liver

GSH-Px activity was assayed as described by Rotruck et al. [42]. The principle of this method is that the rate of glutathione oxidation by H_2O_2, as catalyzed by the GSH-Px present in the supernatant, is determined; the color that develops is read against a reagent blank at 412 nm on a spectrophotometer. The activity of GSH-Px was expressed in terms of μg of GSH consumed/min/mg protein.

Statistical analysis

Statistical analysis was performed by Kruskal–Wallis one-way ANOVA for multiple comparisons followed

by Fisher's PLSD test. Values are expressed as mean±SD. Statistical analysis was performed for each group to the corresponding control group within that experiment and differences were considered significant if $P<0.05$. Values for untreated control rats were arbitrarily assigned a value of 1, and values for all treatments were normalized to 1 (fold increase versus control), unless otherwise specified.

Results

Light manipulation of cecum in group of Sham controls (Sham) showed no significant change in all parameters measured compared with the control group (C). Induction of septicemia in rats by CLP (S group) produced a significant reduction by 51% of MAP (C=89.9±9.6 and S=45.9±7.6 mmHg, $p<0.001$; Fig. 1a), reduction of vasoconstrictor response to phenylephrine by 44% (C=50.1±5.9 and S=22.1±3.1, $p<0.001$; Fig. 1b) and a reduction of AABF by 39% (C=25.7±3.2 and S=10.1±1.6 ml/min $p<0.001$; Fig. 1c). Septicemic rats were treated with CAV (100 mg/kg. i.p.) or O_3 (1.2 mg/kg, i.p), for 22 h, and values for MAP, vascular reactivity and AABF were obtained. Treatment with CAV and O_3 separately, significantly elevated MAP by 52% and 77%, respectively (C=89.8±9.6, S+CAV=67.2±8.1 and S+O_3=68.5±7.6 mmHg, $p<0.001$) compared with control (Fig. 1a). Also, CAV and O_3 treatments causes decreased in vascular reactivity by 63% and 78%, respectively (C=50.1±5.910^{-7}; S+CAV=31.3±4.310^{-7}; S+O_3=30.2±3.2 10^{-7} M, $p<0.001$; Fig. 1b), and AABF by 55% and 73%, respectively (C=25.7±3.2, S+CAV=14.2±1.7 and S+O_3=18.9±2.1 ml/min, $p<0.001$) compared with control (Fig. 1c). However, combined treatment of septicemic rats with both, CAV and O_3 for 22 h, resulted in a greater significant restoration of MAP (Fig. 1a), vascular reactivity (Fig. 1b), and AABF (Fig. 1c), than either agonist alone. Furthermore, results showed that concomitant administration of both CAV and O_3 caused restoration of MAP (Fig. 1a) and AABF (Fig. 1b) back to control level, while vascular reactivity to phenylephrine was found to increase in comparison to S+CAV, S+O_3 and S groups but did not go back to control level (Fig. 1a,b,c).

Furthermore, induction of septicemia caused significant decrease of hepatic homogenate levels of the antioxidant enzymes SOD by 0.259-fold (C=8.1±1.06 and S=7.8±1.12 U/mgProt, $p<0.001$; Fig. 2a) and GSH-Px, by 0.144-fold (C=36.1±5.93 and S=5.2±0.74 μg/min/mgProt, $p<0.001$; Fig. 2b), while serum level of TNF-α (Fig. 3a) and plasma level of NOx (Fig. 3b) were significantly increased compared with the corresponding values of the control group (TNF-α: C=2.2±0.34; S=21±3.63 pg/ml; NOx: C=19.2±3.3; S=59.3±6.4 μM/L, $p<0.001$).

Treatment of septicemic rats with either CAV (group S+CAV) or O_3 (group S+O_3) resulted in increased hepatic levels of the antioxidant enzymes SOD (Fig. 2a) and GSH-Px (Fig. 2b) compared with the corresponding values in septicemic rats (group S; SOD: S=2.1±0.29.; S+CAV=9.1±1.16; S+O_3=3.9±0.55 U/mgProt, $p<0.001$, GSH-Px: S=5.2±0.74; S+CAV=9.1±1.16; S+O_3=16.3±1.79 μg/min/mgProt, $p<0.001$). Moreover, the serum level of TNF-α (Fig. 2a) and plasma level of NOx (Fig. 2b) were significantly lowered in groups S+CAV and S+O_3 compared with the septicemic group (S; TNF-α: S=21.0±3.63; S+CAV=11.8±2.08 S+O_3=6.8±0.82 pg/ml; NOx: S=59.3±6.4; S+CAV=45.1±4.8; S+O_3=35.2±3.2 μM/L, $p<0.001$).

Results showed that administration of both CAV and O_3, together, caused increased liver SOD (Fig. 2a) and GSH-Px (Fig. 2b) and decreased serum TNF-α (Fig. 3a) and plasma NOx (Fig. 3b) level in comparison to S+CAV, S+O_3 and S group but did not go back to control level.

These results suggest that concomitant administration of O_3 and CAV alleviated the compromised vascular reactivity in septicemic conditions and prevent its progression into septic shock in comparison to each alone.

Discussion

The principal new findings of this study are that O_3 and CAV prevent impairment of vascular reactivity caused by induced OxS in septicemic rats. Specifically, we demonstrated that during sepsis, O_3 and CAV attenuate the ability of OxS stress to decrease vascular reactivity by interfering with signaling through the TNF-α and iNOS pathway, in experimental model of sepsis, S rats.

CLP is a model of septicemia that is acceptable for studying the systemic response to local infection

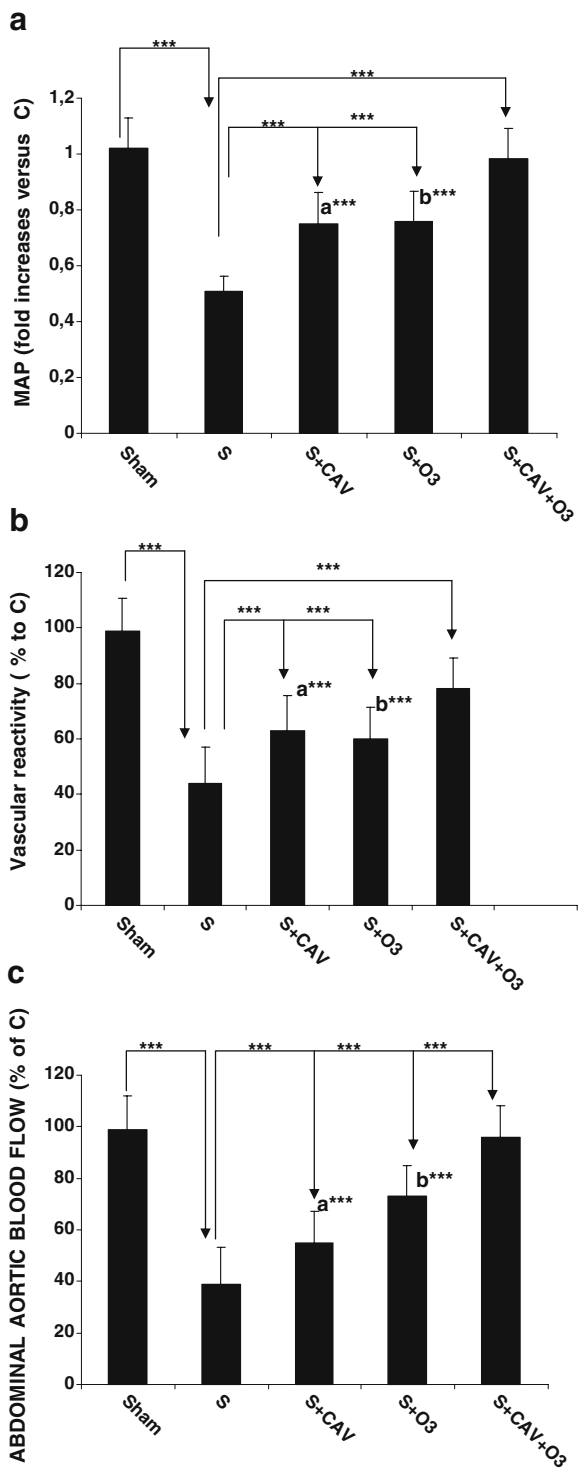

Fig. 1 Effects of CAV and/or O_3 on mean arterial blood pressure (*MAP*), vascular reactivity and AABF. MAP (**a**), Vascular reactivity (**b**) and AABF (**c**) in Sham control (Sham), Septic (S) and S treated with CAV (S+CAV), S treated with O_3 (S+O_3) and S treated with both CAV and O_3 (S+CAV+O_3) rats (were assessed as described in the "Materials and methods" section. The *y* axis represents MAP (**a**), vascular reactivity (**b**) and AABF (**c**) and is expressed as a -fold vs. control (**a**) or % of control (**b**, **c**).Control value for each experiment has been set at 1 (**a**) or 100 (**b**, **c**), and all treatments are normalized to that value. Data are expressed as means±SD of at least ten animals/group. **a** Significantly different between S+CAV and S+CAV+O_3. **b** Significantly different between S+O_3 and S+CAV+O_3. All treated groups except Sham were significantly different compared with (**c**). ***$p<0.001$. Survival rates (%) for each experimental group was as follows: C=100%; Sham=95%; S=45%; S+CAV=60%; S+O_3=67% and S+CAV+O_3=85%.

(septicemia) as it is similar to the clinical situation of bowel perforation, inducing peritonitis [17]. It has been shown that endotoxemia, sepsis, and septic shock are associated with the generation of ROS that leads to a very high level of OxS as indicated by lipid peroxidation, high blood levels of malondialdehyde and conjugated dienes, as well as the consumption of the endogenous antioxidant vitamins C and E [20]. Pretreatment with antibodies against TNF-α prevented death both in mice and in non-human primates that received endotoxin; however, in humans, monoclonal antibodies directed against TNF-α failed to produce substantial benefit [1]. This controversy suggested that there are other mediators that may play more important role in human septicemia and septic shock outcome [39]. Senoglu et al. [44], showed that TNF-α and IL-1 can induce the production of free radicals, NO and eicosanoids from various cells with consumption of the endogenous antioxidant enzyme system produces most of the pathophysiologic changes seen during sepsis and septic shock. Since it is not always possible to access the site of inflammation, circulatory cytokine levels could be used to predict prognosis in septic patients. Increasing concentration of the proinflammatory cytokines as TNF-α have been shown to correlate with the severity of sepsis and its clinical complications [4]. This observation was also confirmed in the present work by significant elevation of serum TNF-α. Calandra et al. [10] suggested that, exposure to bacterial toxins, such as LPS, toxic shock syndrome toxin-1 and cytokines TNF-α and IFN-γ, incites macrophages to produce macrophage migration inhibitory factor which promotes inflammation by stimulating the

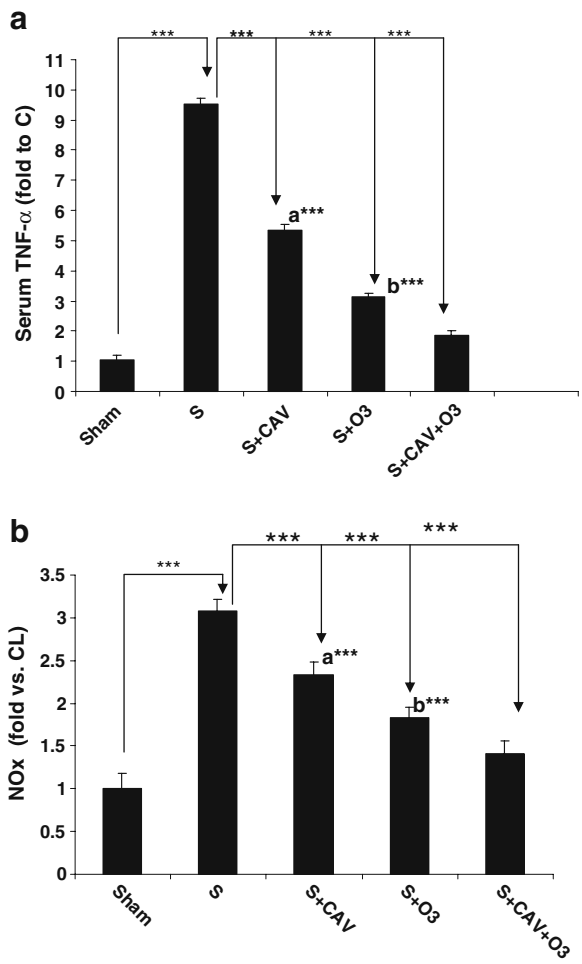

Fig. 2 Effects of CAV and/or O_3 on serum tumor necrosis factor-α (TNF-α and plasma nitrite/nitrate (NOx) level. Serum TNF-α (**a**) and plasma NOx (**b**) level of Sham control (Sham), Septic (S) and S treated with CAV (S+CAV), S treated with O_3 (S+O_3) and S treated with both CAV and O_3 (S+CAV+O_3) rats were assessed within as described in the "Materials and methods" section. The *y* axis represents TNF-α (**a**) and NOx level (**b**) and is expressed as a -fold vs. control. Control value for each experiment has been set at 1 and all treatments are normalized to that value. Data are expressed as means±SD of at least 10 animals/group. **a** Significantly different between S+CAV and S+CAV+O_3. **B** Significantly different between S+O_3 and S+CAV+O_3. All treated groups except Sham were significantly different compared with (**c**). ***$p<0.001$

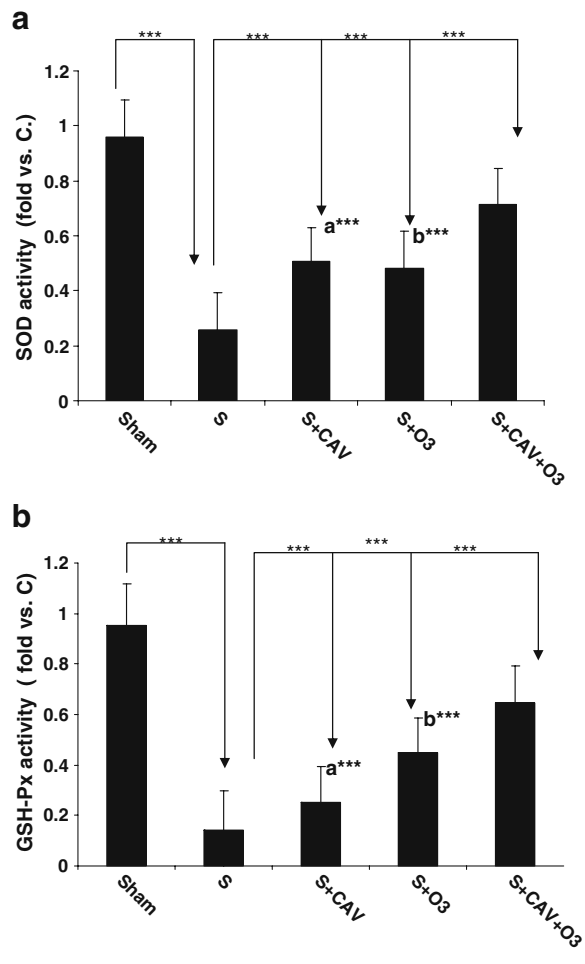

Fig. 3 Effects of CAV and/or O_3 on serum tumor sodium dismutase (SOD) and glutathione peroxidase (GSH-Px) activity. SOD (**a**) and GSH-Px (**b**) activity of Sham control (Sham), septic (S) and S treated with CAV (S+CAV), S treated with O_3 (S+O_3) and S treated with both CAV and O_3 (S+CAV+O_3) rats were assessed within as described in the "Materials and methods" section. The *y* axis represents SOD (**a**) and GSH-px (**b**) and is expressed as a -fold vs. control. Control value for each experiment has been set at 1 and all treatments are normalized to that value. Data are expressed as means±SD of at least 10 animals/group. **a** Significantly different between S+CAV and S+CAV+O_3. **b** Significantly different between S+O_3 and S+CAV+O_3. All treated groups except Sham were significantly different compared with (**c**). *** $p<0.001$

production of eicosanoids that in turn stimulate the production of more TNF-α. In addition, it is possible that if nitrite/nitrate and TNF-α measurements are performed earlier, their levels may be more altered in tissues than in plasma.

Meanwhile, it was documented that sepsis is associated with increased iNOS activity [52]. Scott et al. [43] has demonstrated that 6 h following induction of sepsis, iNOS activity and expression is elevated and remains up to 48 h. Our results are in agreement with those of others, which showed elevation of plasma NOx level; a mediator of OxS. It has been shown by Liu et al. [33] that these inflammatory mediators that are released during

septicemia could affect microvasculature reactivity with subsequent microvascular failure. During septicemia, the intracellular Ca^{2+} sensitivity of VSM cell (VSMC) contraction were lowered and the mechanisms responsible for this event have not yet been elucidated [9]. One of the possible mechanisms could be that sepsis may induce production of NO that decreases intracellular Ca^{2+} sensitivity of VSMC [3]. Another explanation could be that NO-induced membrane hyperpolarization opening of Ca^{2+}-activated K^+ ATP-sensitive channels [15]. This explanation might account for the reduction of the aortic ring response to phenylephrine documented in our study.

In accordance with our results, it was suggested that the higher level of OxS with the reduction of hepatic antioxidant enzyme activity (SOD and GSH-Px) may be responsible for the significant reduction of abdominal aortic blood flow in septicemia [23]. Administration of O_3 in our study (group S+O_3) induced significant reduction of the plasma level of the inflammatory mediator TNF-α. The O_3/oxygen mixture has been documented to have a strong microbiocidal activity in vitro [19]. It might indirectly act as a regulator or modulator of many inflammatory processes in vivo [7]. Zamora et al. [53] suggested that the inhibitory effects of the O_3/oxygen mixture on serum TNF-α levels in septicemic mice might relate to the inactivation of the NF-κB, which is activated by ROS with the subsequent reduction in generation and expression of various cytokines and enzymes such as TNF-α and GSH-Px, respectively. In addition, it has been reported that O_3 could stimulate and or preserve endogenous hepatic antioxidant enzymes activity after hepatic reperfusion injury [40] and work done by Alvarez et al. [2] documented that this may occur through different mechanisms such as oxidative preconditioning mechanism and/or by blocking the xanthine/xanthine oxidase pathway for ROS generation with subsequent adenosine accumulation and decrease ROS generation. Candelario-Jalil et al. [11] suggested that low doses of O_3 may increase the antioxidant endogenous systems including GSH-Px, SOD and CAT, preparing the host to face physiopathological conditions mediated by ROS. This is in accordance with our work, where we showed significant enhancement of hepatic antioxidant enzyme activity SOD and GSH-Px. O_3 not only has antioxidant activity but also might produce a direct effect on peritoneal macrophages modulating their phagocytic activity [12]. CAV, the inhibitor of iNOS, has been demonstrated to restore vascular reactivity in the experimental models of sepsis [38] as well as in human septic shock [51].

Our results showed that administration of CAV alone produced significant reduction of the plasma NOx with significant improvement of the hepatic antioxidant enzyme activity and vascular reactivity. However, it was found that improvement of oxidative status and antioxidant condition in O_3 treated rats is rather more effective than administration of CAV. Administration of both CAV and O_3 showed more improvement than administration of each alone with subsequent restoration of the MAP, aortic vascular reactivity to phenylephrine and AABF back to control levels. This may prove that O_3 could act synergistically with CAV, as O_3 has an effective antioxidant activity and resist the generation of ROS in septicemia [11], while CAV inhibits the activity of iNOS [27]. Another explanation for restoration of the septicemic hypovascular reactivity in aortic rings is that O_3 might lead to inhibition of the NO-induced membrane hyperpolarization of the vascular system through its inhibitory effect on plasma NOx level [35]. O_3 may also act through improving oxygen transport and/or restoring adequate perfusion pressure to tissue that improves the OxS and antioxidant status with the resultant improvement in the hypovascular reactivity and AABF [27].

The present study has several limitations. These include limited timing of sampling and absence of measuring routine liver function tests. However, the present study now provides a basis for estimating the timing and statistical power of future studies.

Understanding the mechanism(s) by which sepsis disrupts the TNF-α and iNOS regulatory pathway will be important in identifying novel anti-inflammatory agents that are both specific and effective in inhibiting the initiation and progression of inflammation. To this, we are in favor of the concept that CAV and O_3 exert vasculoprotective effect, possibly via mechanisms involving the down regulation of the TNF-α and iNOS signaling pathway. Result from our study show that combined administration of O_3 and CAV alleviated the compromised vascular reactivity in septicemic rats suggesting their role in the treatment of septicemic complications. However, extrapolating from rats to humans has its limitations. More research in this field is required.

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References

1. Abraham E (1999) Why immunomodulatory therapies have not worked in sepsis. Intensive Care Med 25:556–566
2. Alvarez RG, Zamora ZB, Borrego A, Delgado R, Schulz S, Alonso Y (2009) Ozone oxidative preconditioning reduces nitrite levels in blood serum in LPS: induced endotoxic shock in mice. Inflamm Res 58(8):441–443
3. Arid WC (2003) The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood 101:3765–3777
4. Baigrie RJ, Lamont PM, Kwiatkowski D, Dallman MJ, Morris PJ (1992) Systemic cytokine response after major surgery. Br J Surg 79(8):757–760
5. Barber E, Menendez S, León OS, Barber MO, Merino N, Calunga JL, Cruz E, Bocci V (1999) Prevention of renal injury after induction of ozone tolerance in rats submitted to warm ischaemia. Mediat Inflamm 8(1):37–41
6. Bette M, Nüsing RM, Mutters R, Zamora ZB, Menendez S, Schulz S (2006) Efficiency of tazobactam/piperacillin in lethal peritonitis is enhanced after preconditioning of rats with O_3/O_2-pneumoperitoneum. Shock 25(1):23–29
7. Billiar TR (1995) Nitric oxide: novel biology with clinical relevance. Ann Surg 221(4):339–349
8. Bocci V (1996) Does ozone therapy normalize the cellular redox balance? Implications for therapy of human immunodeficiency virus infection and several other diseases. Med Hypotheses 46(2):150–154
9. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ (1992) Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest 101:1644–1655
10. Calandra T, Spiegel LA, Metz CN, Bucala R (1998) Macrophage migration inhibitory factor (MIF) is a critical mediator of the activation of immune cells by exotoxins of Gram-positive bacteria. Proc Natl Acad Sci USA 95 (19):11383–11388
11. Candelario-Jalil E, Mohammed-Al-Dalain S, Fernández OS, Menéndez S, Pérez-Davison G, Merino N, Sam S, Ajamieh HH (2001) Oxidative preconditioning affords protection against carbon tetrachloride induced glycogen depletion and oxidative stress in rats". J Appl Toxicol 21(4):297–301
12. Canning BJ, Hmieleski RR, Spannhake EW, Jakab GJ (1991) Ozone reduces murine alveolar and peritoneal macrophage phagocytosis: the role of prostanoids. Am J Physiol 261(4 pt 1):L277–L282
13. Chatterjee D, Mukherjee SK (1993) Destruction of phagocytosis-suppressing activity of aflatoxin B1 by ozone. Lett Appl Microbiol 17(2):52–54
14. Chen D, Wang L (2009) Mechanisms of therapeutic effects of rhubarb on gut origin sepsis. Chin J Traumatol 12 (6):365–369
15. Damas P, Ledoux D, Nys M, Vrindts Y, De Groote D, Franchimont P, Lamy M (1992) Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. Ann Surg 215(4):356–362
16. Damiani CE, Rossoni LV, Vassallo DV (2003) Vasorelaxant effects of eugenol on rat thoracic aorta. Vasc Pharmacol 40 (1):59–66
17. Ebong SJ, Call DR, Bolgos GL, Newcomb DE, Granger JI, O'Reilly M, Remick DG (1999) Immunopathologic responses to non-lethal sepsis. Shock 12(2):118–126
18. Furuichi M, Yokozuka M, Takemori K, Yamanashi Y, Sakamoto A (2009) The reciprocal relationship between heme oxygenase and nitric oxide synthase in the organs of lipopolysaccharide-treated rodents. Biomed Res 30(4):235–243
19. Gazin IK: Criteria for intoxication in the evaluation of severity of endotoxicosis, the efficiency of ozone therapy and traditional treatment in patients with diabetes mellitus complicated by pyonecrotic lesions of the lower extremities. Klin Lab Diagn 2008;(6): 21–23.
20. Goraca A, Asłanowicz-Antkowiak K (2009) Prophylaxis with alpha-lipoic acid against lipopolysaccharide-induced brain injury in rats. Arch Immunol Ther Exp (Warsz) 57 (2):141–146
21. Gros R, Borkowski KR, Feldman RD (1994) Human insulin-mediated enhancement of vascular beta-adrenergic responsiveness. Hypertension 23(5):551–555
22. Haywood JR, Shaffer RA, Fastenow C (1981) Regional blood flow measurements with pulsed Doppler flowmeter in rats. J Physiol 241(2):273–278
23. Ho CH, Hsu SP, Yang CC, Lee YH, Chien CT (2009) Sialic acid reduces acute endotoxemia-induced liver dysfunction in the rat. Shock 32(2):228–235
24. Hsieh YH, Su IJ, Lei HY, Lai MD, Chang WW, Huang W (2007) Differential endoplasmic reticulum stress signaling pathways mediated by iNOS. Biochem Biophys Res Commun 359(3):643–648
25. Hubbard, William J; Choudhry, Mashkoor; Schwacha, Martin G; Kerby, Jeffrey D; Rue, Loring W III; Bland, Kirby I; Chaudry, Irshad H. Cecal ligation and puncture. Shock 2005; 24: 52–57.
26. James WL, Martha LB, Mary A, James M, Margaret A, Gali R (1991) Effects of vanadate on renal hypertrophy and sorbitol accumulation in streptozotocin induced diabetes in rats. Res Comm Path Pharmacol 72(2):180–189
27. Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216
28. Joy RM, Albertson TE (1993) NMDA receptors have a dominant role in population spike-paired pulse facilitation in the dentate gyrus of urethane-anesthetized rats. Brain Res 604:273–282
29. Kakkar P, Das B, Viswanathan PN (1984) A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys 21(2):130–132
30. Kim DH, Jung YJ, Lee AS, Lee S, Kang KP, Lee TH, Lee SY, Jang KY, Moon WS, Choi KS, Yoon KH, Sung MJ,

Park SK, Kim W (2009) COMP-angiopoietin-1 decreases lipopolysaccharide-induced acute kidney injury. Kidney Int 76(11):1180–1191
31. Kleinert H, Schwarz PM, Forstermann U (2003) Regulation of the expression of inducible nitric oxide synthase. Biol Chem 384(10–11):1343–1364
32. Li X, Stark GR (2002) NFkappa B-dependent signaling pathways. Exp Hematol 30(4):285–296
33. Liu YC, Chang AY, Tsai YC, Chan JY (2009) Differential protection against oxidative stress and nitric oxide overproduction in cardiovascular and pulmonary systems by propofol during endotoxemia. J Biomed Sci 15:16–18
34. Madej P, Plewka A, Madej JA, Nowak M, Plewka D, Franik G, Golka D (2007) Ozonotherapy in an induced septic shock. Effect of ozonotherapy on rat organs in evaluation of free radical reactions and selected enzymatic systems. Inflammation 30(1-2):52–58
35. Mansart A, Bollaert P, Giummelly P, Capdeville-Atkinson C, Atkinson J (2006) Effects of dexamethasone and L-canavanine on the intracellular calcium-contraction relation of the rat tail artery during septic shock. Am J Physiol Heart Circ Physiol 291(3):H1177–H1182
36. Moncada S, Palmer RM, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43(2):109–142
37. Moshage H, Kok B, Huizenga JR, Jansen PL (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. Clin Chem 41(6 Pt 1):892–896
38. Oberholzer A, Oberholzer C, Moldawer LL (2001) Sepsis syndromes: understanding the role of innate and acquired immunity. Shock 16(2):83–96
39. Oz HS, Chen TS, Neuman M (2009) Nutrition intervention: a strategy against systemic inflammatory syndrome. JPEN J Parenter Enteral Nutr 33(4):380–389
40. Peralta C, León OS, Xaus C, Prats N, Jalil EC, Planell ES, Puig-Parellada P, Gelpí E, Roselló-Catafau J (1999) Protective effect of ozone treatment on the injury associated with hepatic ischemia-reperfusion: antioxidant prooxidant balance. Free Radic Res 31(3):191–196
41. Peralta C, Xaus C, Bartrons R, Leon OS, Gelpi E, Rosello-Catafau J (2000) Effect of ozone treatment on reactive oxygen species and adenosine production during hepatic ischemia–reperfusion. Free Radic Res 33(5):595–605
42. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG (1973) Selenium: biochemical role as a component of glutathione peroxidase. Science 179 (73):588–590
43. Scott JA, Mehta S, Duggan M, Bihari A, McCormack DG (2002) Functional inhibition of constitutive nitric oxide synthase in a rat model of sepsis. Am J Respir Crit Care Med 165:1426–1432
44. Senoglu N, Yuzbasioglu MF, Aral M, Ezberci M, Kurutas EB, Bulbuloglu E (2008) Protective effects of *N*-acetylcysteine and beta-glucan pretreatment on oxidative stress in cecal ligation and puncture model of sepsis. J Invest Surg 21(5):237–243
45. Sherman MP, Aeberhard EE, Wong VZ, Griscavage JM, Ignarro LJ (1993) Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. Biochem Biophys Res Commun 191 (3):1301–1308
46. Stoclet JC, Fleming I, Gray GA, Julou-Schaeffer G, Schneider F, Scott CA, Schott C, Parratt JR (1993) Nitric oxide and endotoxemia. Circulation 87:77–88
47. Sunnen GV (1988) Ozone in medicine: overview and future directions. J Adv Med 1(3):159–174
48. Vo PA, Lad B, Tomlinson JA, Francis S, Ahluwalia A (2005) Autoregulatory role of endothelium-derived nitric oxide (NO) on lipopolysaccharide-induced vascular inducible NO synthase expression and function. J Biol Chem 280 (8):7236–7243
49. Wang C, Chao L, Chao J (1995) Direct gene delivery of human tissue kallikrein reduces blood pressure in spontaneously hypertensive rats. J Clin Invest 95(4):1710–1716
50. Wichterman KA, Baue AE, Chaudry IH (1980) Sepsis and septic shock: a review of laboratory models and a proposal. J Surg Res 29(2):189–201
51. Xie XQ, Shinozawa Y, Sasaki J, Takuma K, Akaishi S, Yamanouchi S, Endo T, Nomura R, Kobayashi M, Kudo D, Hojo N (2008) The effects of arginine and selective inducible nitric oxide synthase inhibitor on pathophysiology of sepsis in a CLP model. J Surg Res 146(2):298–303
52. Yamashita T, Kawashima S, Ohashi Y, Ozaki M, Ueyama T, Ishida T, Inoue N, Hirata K, Akita H, Yokoyama M (2000) Resistance to endotoxin shock in transgenic mice overexpressing endothelial nitric oxide synthase. Circulation 101(8):931–937
53. Zamora Z, Borrego A, López O, Delgado R, González R, Menéndez S, Hernández F, Schulz S (2005) Effects of ozone oxidative preconditioning on TNF-α release and antioxidant-prooxidant intracellular balance in mice during endotoxic shock. Mediat Inflamm 1:16–22