Effect of hyperbaric oxygen and ulinastatin on plasma endotoxin, soluble CD14, endotoxin-neutralizing capacity and cytokines in acute necrotizing pancreatitis

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Background: We sought to study the effect of a combination therapy comprised of hyperbaric oxygen (HBO) and ulinastatin on the plasma levels of endotoxin, soluble CD14 (sCD14), endotoxin neutralizing capacity (ENC) and cytokines in acute necrotizing pancreatitis (ANP) in rats.

Methods: We randomly allocated 90 Sprague–Dawley rats into 6 groups: group 1 (ordinary control), group 2 (sham operation), group 3 (ANP), group 4 (ANP with HBO), group 5 (ANP with ulinastatin) and group 6 (ANP with HBO and ulinastatin). We induced ANP by retrograde injection of 3.5% sodium taurocholate (2.5 mL/kg) via the pancreatic duct. Five minutes after induction, animals in groups 5 and 6 were infused with ulinastatin (20 000 U/kg) via the portal vein. Thirty minutes after induction, animals in groups 4 and 6 received HBO therapy. We collected samples 3, 6 and 10 hours after induction of ANP.

Results: We found that the plasma level of endotoxin in group 3 was significantly higher than in group 4 (3, 6 h, both $p < 0.001$), group 5 (3 h, $p < 0.001$; 6 h, $p = 0.014$) and group 6 (both $p < 0.001$). The level of plasma sCD14 in group 3 was significantly higher than in group 4 (3, 6 h, both $p < 0.001$), group 5 (3, 6 h, both $p = 0.001$) and group 6 (3 h, $p < 0.001$; 6 h, $p = 0.001$). The plasma endotoxin and sCD14 levels in group 6 were significantly lower than in groups 4 and 5. The plasma ENC level in group 6 was significantly higher than in groups 3, 4 and 5 ($p < 0.001$). The ENC level in groups 4 and 5 were higher than in group 3, but there was no significant difference. The plasma level of tumour necrosis factor-α (TNF-α) and IL-6 in group 6 were significantly lower than in groups 3, 4 and 5 ($p < 0.001$). The TNF-α and IL-6 levels in groups 4 and 5 were lower than in group 3, but there was no significant difference.

Conclusion: The use of an early combination therapy of HBO and ulinastatin was more effective than either therapy alone in the treatment of ANP.

Contexte : Nous avons voulu étudier l'effet d'un traitement d'association composé d'oxygène hyperbare (OHB) et d'ulinastatine sur les taux plasmatiques d'endotoxine, de CD14 soluble (sCD14), la capacité de neutralisation des endotoxines (CNE) et les cytokines dans la pancréatite aiguë nécrosante (PAN) chez le rat.

Méthodes : Nous avons assigné aléatoirement 90 rats Sprague–Dawley à l'un de 6 groupes : groupe 1 (témoins ordinaires), groupe 2 (intervention factice), groupe 3 (PAN), groupe 4 (PAN et OHB), groupe 5 (PAN et ulinastatine) et groupe 6 (PAN, OBH et ulinastatine). Nous avons induit la PAN au moyen d'une injection rétrograde de taurocholate de sodium à 3,5 % (2,5 mL/kg) par le canal pancréatique. Cinq minutes après l'induction, les animaux des groupes 5 et 6 ont reçu une perfusion d'ulinastatine (20 000 U/kg) par la veine porte. Trente minutes après l'induction, les animaux des groupes 4 et 6 ont reçu le traitement par OHB. Nous avons recueilli des échantillons 3, 6 et 10 heures après induction de la PAN.

Résultats : Nous avons noté que les taux plasmatiques d'endotoxine dans le groupe 3 étaient significativement plus élevés que dans le groupe 4 (3, 6 h, $p < 0.001$), le groupe 5 (3 h, $p < 0.001$; 6 h, $p = 0.014$) et le groupe 6 ($p < 0.001$ pour les deux). Les taux de sCD14 plasmatiques dans le groupe 3 étaient significativement plus élevés que dans le groupe 4 (3, 6 h, $p < 0{,}001$ pour les deux), le groupe 5 (3, 6 h, $p = 0{,}001$ pour les deux) et le groupe 6 (3 h, $p < 0{,}001$; 6 h, $p = 0{,}001$). Les taux d'endotoxine et de sCD14 plasmatiques dans le groupe 6 étaient significativement plus bas que dans les groupes 4 et 5. Les taux de CNE plasmatiques dans le groupe 6 étaient significativement plus élevés que dans les groupes

3, 4 et 5 ($p < 0.001$). Les taux de CNE dans les groupes 4 et 5 étaient plus élevés que dans le groupe 3, sans toutefois atteindre une différence significative. Les taux plasmatiques de TNF-α et d'IL-6 dans le groupe 6 étaient significativement plus bas que dans les groupes 3, 4 et 5 ($p < 0.001$). Les taux de TNF-α et d'IL-6 dans les groupes 4 et 5 étaient plus bas que dans le groupe 3, sans toutefois atteindre une différence significative.

Conclusion : L'utilisation d'un traitement d'association hâtif composé d'OHB et d'ulinastatine s'est révélée plus efficace que l'un ou l'autre des traitements utilisés seuls pour la PAN.

System inflammatory reaction syndrome (SIRS) is the leading cause of morbidity and mortality in acute necrotizing pancreatitis (ANP).[1] There is a large amount of experimental data which suggests that endotoxin, endotoxin-specific high-affinity receptor (soluble CD14 [sCD14]) and plasma endotoxin-neutralizing capacity (ENC) play critical role in SIRS.[2–7] Therefore, endotoxin–sCD14 complexes must be blocked, plasma ENC must be improved and SIRS must be controlled in the management of ANP.

Hyperbaric oxygen therapy (HBO) may improve pancreatic microcirculation and lung edema in experimental ANP. This type of therapy has been successfully used to treat refractory pancreatic abscess.[8,9] Ulinastatin is a multivalent knitz-type serine protease inhibitor, which has been shown to inhibit the activity of inflammatory proteases and limit the enhanced production of inflammatory cytokines.[10,11] However, the effect of single therapy has not been satisfactory. Therefore, we hypothesized that a combination of therapies with different mechanisms might be an effective strategy for treatment of ANP. Thus, we designed an experimental combination therapy comprised of HBO and ulinastatin to treat plasma endotoxin, sCD14 and plasma ENC in sodium taurocholate–induced experimental ANP.

Methods

Animals

We acquired 90 male Sprague–Dawley rats (mean weight 286 g, standard deviation [SD] 30 g) from the Experiment Center of the Second Xiangya Hospital, Central South University, China. Before the experiment, the animals were fed standard rat chow and water. They were housed in metal cages with a controlled temperature and a 12-hour light/dark cycle for at least 1 week. We performed all studies in accordance with the national guideline for the use and care of laboratory animals, and our study was approved by the University Animal Care Committee.

Induction of ANP

Starting 12 hours before the experiment, we deprived the animals of food but allowed them free access to water. We induced anesthesia by intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg; Sigma). We performed laparotomy via a midline incision, and we placed a microaneurysm clip around a biliopancreatic duct at its entry into the duodenum to avoid reflux of enteric contents into the duct. We cannulated the common biliopancreatic duct with a 28-gauge, 1/2-inch microfine catheter. A solution of 3.5% sodium taurocholate (2.5 mL/kg; Sigma) was slowly infused into the common biliopancreatic duct via a microinjection pump at a speed of 0.2 mL/min. After the injection was complete, the microclips were removed, and the abdomen was closed in 2 layers. All procedures were performed using sterile techniques.[12]

Study protocol

After a stabilization period, the rats were randomly divided into 6 groups: group 1 (ordinary control, $n = 15$) received neither operation nor anesthesia, group 2 (sham operation, $n = 15$) underwent laparotomy with manipulation of the pancreas, group 3 (ANP without therapy, $n = 15$), group 4 (ANP with HBO therapy, $n = 15$), group 5 (ANP with ulinastatin therapy, $n = 15$) and group 6 (ANP with HBO and ulinastatin therapy, $n = 15$). After observation periods of 3, 6 and 10 hours after the induction of pancreatitis, the rats were exsanguinated under anesthesia by aortal puncture. We collected the blood, which was centrifuged, and the serum was stored at –20°C.

Hyperbaric oxygen therapy and ulinastatin therapy

We performed HBO 30 minutes after the induction of ANP using an animal hyperbaric chamber at more than 97% oxygen at 253 kPa (2.5 ATA) for 120 minutes.

Five minutes after the induction of ANP, we infused ulinastatin (20 000 U/kg; Tianpusheng Pharmaceutical Corporation) into the portal vein.

Measurement of plasma endotoxin and ENC

We measured plasma endotoxin concentration by use of the Tachypleus Amebocyte Lysate Kit (Shanghai Medical Science and Technology Corporation).[13] Plasma samples treated with heparin were diluted 1:10 with pyrogen-free water and heated for 10 minutes at 75°C. We added 50 μL of the inactivated sample to 50 μL of solution A, and the mixture was incubated for 33 minutes at 37°C. Next, we added 100 μL of solution B diluted 1:2 with solution C and

incubated the mixture for 3 minutes at 37°C. The extinction of endotoxin was determined by reading the absorbance using a spectrophotometer at 405 nm. We determined the endotoxin concentration of a known sample by use of a simultaneously established standard curve developed from a pool of plasma from 20 healthy volunteers; this plasma was free of endotoxins. The endotoxin concentrations were expressed as endotoxin units (EU) per mL.

We quantified plasma ENC by measuring the endotoxin concentration using the Limulus Amebocyte Lysate test after adding 4 EU of the lipopolysaccharide to 100 μL of each plasma sample. We subtracted the endotoxin concentration of the nonspiked plasma sample as previously described.[6,14]

Measurement of plasma tumour necrosis factor-α, interleukin-6 and sCD14

We measured the concentrations of plasma tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) (Jingmei Bioengineer Corporation) and sCD14 (IBL Company) by use of enzyme-linked immunosorbent assay kits, in accordance with the manufacturers' instructions.

Statistical analysis

We used analysis of variance to compare means. We considered p values less than 0.05 to be significant. We used SPCC version 10.0 (SPSS Inc.) for all statistical measurements.

Results

By use of the standard curve we created, we determined that about 94% (SD 10%) of a known quantity of endotoxin could be recovered. The variation was 7.6% (SD 2.5%).

The plasma endotoxin level in group 3 was significantly higher than in group 4 (3, 6 h, both $p < 0.001$), group 5 (3 h, $p < 0.001$; 6 h, $p = 0.014$) and group 6 (both $p < 0.001$). The levels in group 6 were significantly lower than in group 4 (3 h, $p = 0.048$; 6 h, $p = 0.070$) and group 5 (both $p < 0.001$). There were no significant difference between groups 4 and 5 (3 h, $p = 0.42$; 6 h, $p = 0.07$; Table 1) .

The level of plasma sCD14 in group 3 was significantly higher than in group 4 (3, 6 h, both $p < 0.001$), group 5 (3, 6 h, both $p = 0.001$) and group 6 (3 h, $p < 0.001$; 6 h, $p = 0.001$). The level in group 6 was significantly lower than in group 4 (3 h, $p = 0.039$; 6 h, $p = 0.035$) and group 5 (3 h, $p = 0.016$; 6 h, $p = 0.022$). There were no significant differences between groups 4 and 5 (3 h, $p = 0.24$; 6 h, $p = 0.79$; Table 2).

The plasma ENC level in group 6 was significantly higher than in groups 3, 4 and 5 ($p < 0.001$). The levels in groups 4 and 5 were higher than in group 3, but there was no significant difference (3 h: group 3 v. group 4, $p = 0.09$; group 3 v. group 5, $p = 0.74$; 6 h: group 3 v. group 4, $p = 0.97$; group 3 v. group 5, $p = 0.06$). There were no significant differences between groups 4 and 5 (3 h, $p = 0.08$; 6 h, $p = 0.07$; Table 3).

The levels of plasma TNF-α and IL-6 in group 6 were significantly lower than in groups 3, 4 and 5 ($p < 0.001$). The plasma TNF-α and IL-6 levels in groups 4 and 5 were lower than in group 3, but there was no significant difference. There were no significant differences between groups 4 and 5 (Table 4).

Discussion

About 20%–30% of patients with acute pancreatitis have severe ANP, and SIRS triggered by bacterial infection is a serious complication responsible for up to 80% of deaths among patients with ANP.[15] There is consensus that endotoxin–sCD14 complexes, plasma ENC and cytokines secreted by activated cells initiate the cascade of the SIRS and multiple organ failure.[15,16]

Endotoxin, namely lipopolysaccharide, is a constituent of the outer membrane of gram-negative bacteria. The

Table 1. Plasma endotoxin levels in rats after the induction of pancreatitis

Group, $n = 15$	Time; endotoxin level, μg/mL		
	3 h	6 h	10 h
Group 1: Control	0.16 (0.04)	0.17 (0.03)	0.16 (0.07)
Group 2: Sham surgery	0.21 (0.06)	0.23 (0.11)	0.17 (0.04)
Group 3: ANP with no treatment	0.46 (0.13)	0.62 (0.26)	0.83 (0.17)
Group 4: ANP with HBO	0.27 (0.09)*	0.32 (0.16)*	0.71 (0.24)
Group 5: ANP with ulinastatin	0.29 (0.03)*	0.43 (0.11)*	0.67 (0.15)
Group 6: ANP with HBO and ulinastatin	0.19 (0.12)*†	0.21 (0.16)*†	0.37 (0.22)*†

ANP = acute necrotizing pancreatitis; HBO = hyperbaric oxygen.
*Significantly different from group 3 ($p < 0.05$).
†Significantly different from groups 4 and 5 ($p < 0.05$).

Table 2. Levels of plasma-soluble CD14 in rats after the induction of pancreatitis

Group, $n = 15$	Time; CD14 level, μg/mL		
	3 h	6 h	10 h
Group 1: Control	2.4 (1.3)	2.6 (1.5)	2.7 (1.7)
Group 2: Sham surgery	2.6 (1.5)	3.4 (1.1)	3.2 (1.8)
Group 3: ANP with no treatment	5.4 (1.3)	6.4 (1.2)	8.1 (1.9)
Group 4: ANP with HBO	2.9 (1.1)*	4.1 (1.8)*	6.9 (1.7)
Group 5: ANP with ulinastatin	3.5 (1.6)*	3.9 (2.3)*	6.4 (2.5)
Group 6: ANP with HBO and ulinastatin	2.6 (1.8)*†	3.1 (0.9)*†	4.6 (2.1)*†

ANP = acute necrotizing pancreatitis; HBO = hyperbaric oxygen.
*Significantly different from group 3 ($p < 0.05$).
‡Significantly different from groups 4 and 5 ($p < 0.05$).

endotoxin–sCD14 complex mediates host responses to gram-negative infections by stimulating the release of inflammatory mediators, including cytokines (e.g., IL-1, IL-6, IL-8 and TNF-α); it also downregulates HLA-DR expression on monocytes and contributes to immune paralysis.[15–18]

Soluble CD14 is an endotoxin-related signalling molecule. The exquisite sensitivity of macrophage activation to endotoxin stimulation requires sCD14.[19] Moore and colleagues[19] reported that sCD14 sensitized macrophages to purified endotoxin by more than 2 orders of magnitude. Endotoxin–sCD14 complexes combine with the coreceptor TLR4, leading to endotoxin-induced recruitment of IL-1–associated kinase, mediating nuclear factor (NF-κB) activation and triggering the cytokine cascade in ANP;[20,21] this is a central mechanism in the pathogenesis of multiple organ failure.[22]

Human serum contains endogenous factors that may neutralize endotoxins and limit endotoxin-mediated inflammatory response, including endogenous antiendotoxin antibodies, high-density lipoprotein and transferrin.[23,24] Previous studies have shown an association between the neutralization of endotoxin with serum and protection from lethal challenge with endotoxin.[25] Endotoxin-neutralizing capacity is a novel marker of immune function and is inversely proportional to endotoxin recovery.

In the present study, we focused on molecules in the endotoxin-related signal transduction pathway, especially endotoxin–sCD14 complexes, ENC and cytokines. We investigated the early use of a combination therapy of HBO and protease-modulating therapy as a potentially effective strategy in the treatment of ANP.

Hyperbaric oxygen therapy comprises the intermittent inhalation of 100% oxygen at a pressure of more than 101 kPa (1 ATA), which increases the concentration of plasma and tissue oxygen to more than 10 times the normal level. A single HBO treatment 6 hours after the induction of acute pancreatitis in rats reduced lung edema and histological severity and improved in vivo pancreatic perfusion.[26] Twice-daily HBO therapy initiated 6 hours after induction of pancreatitis significantly improved 7-day mortality in a rat model.[27] The inhibition of bacterial translocation, neutrophil chemotaxis and oxidative stress may be the key to the effect of HBO therapy in ANP.[28–30] This therapy should be initiated as early as possible. Compared with other studies, we applied HBO therapy much earlier, and the effects were obvious.

Recent studies have suggested that proteases contribute to endotoxin-induced SIRS.[31] Ulinastatin (urinary trypsin inhibitor), an acidic glycoprotein with 2 Kunitz-type domains, protects against systemic inflammatory response and subsequent organ injury induced by bacterial endotoxin by inhibiting the enhanced expression of proinflammatory cytokines.[32,33] Clinical trials have confirmed that ulinastatin reduces the incidence of pancreatitis following endoscopic retrograde cholangiopancreatography.[34]

The results of our study showed that, compared with those in the single-therapy groups, the rats in the combination therapy group had decreased plasma levels of endotoxin, sCD14 and inflammatory cytokines (TNF-α, IL-6) and increased levels of plasma ENC at all times points. There was no significant difference between the 2 single-therapy

Table 3. Levels of plasma endotoxin-neutralizing capacity in rats after induction of pancreatitis

	Time; ENC level, EU/mL		
Group, *n* = 15	3 h	6 h	10 h
Group 1: Control	0.149 (0.010)	0.155 (0.030)	0.146 (0.050)
Group 2: Sham surgery	0.168 (0.006)	0.175 (0.01)	0.157 (0.008)
Group 3: ANP with no treatment	0.193 (0.009)	0.163 (0.006)	0.116 (0.010)
Group 4: ANP with HBO	0.226 (0.006)	0.189 (0.007)	0.146 (0.009)
Group 5: ANP with ulinastatin	0.241 (0.011)	0.201 (0.017)	0.167 (0.015)
Group 6: ANP with HBO and ulinastatin	0.337 (0.019)*†	0.412 (0.021)*†	0.364 (0.023)*†

ANP = acute necrotizing pancreatitis; ENC = endotoxin-neutralizing capacity; EU = endotoxin units; HBO = hyperbaric oxygen.
*Significantly different from group 3 ($p < 0.05$).
†Significantly different from groups 4 and 5 ($p < 0.05$).

Table 4. Levels of plasma tumour necrosis factor-α and interleukin-6 in rats after the induction of pancreatitis

	Time; cytokine level, pg/mL		
Group; factor	3 h	6 h	10 h
TNF-α, *n* = 15			
Group 1: Control	10.28 (2.06)	13.33 (2.13)	10.46 (2.05)
Group 2: Sham surgery	22.68 (3.56)	25.75 (5.31)	27.57 (6.58)
Group 3: ANP with no treatment	92.93 (21.90)	269.67 (35.64)	329.16 (23.11)
Group 4: ANP with HBO	75.96 (5.36)	219.29 (9.37)	262.76 (12.39)
Group 5: ANP with ulinastatin	65.61 (7.71)	189.29 (8.17)	278.19 (23.15)
Group 6: ANP with HBO and ulinastatin	34.33 (5.59)*†	55.42 (8.21)*†	64.34 (9.63)*†
IL-6, *n* = 15			
Group 1: Control	67.2 (8.8)	58.8 (6.9)	64.8 (7.6)
Group 2: Sham surgery	158 (12.5)	179.8 (13.6)	212.8 (19.4)
Group 3: ANP with no treatment	793.9 (98.9)	935.93 (82.9)	1136.93 (96.6)
Group 4: ANP with HBO	592.68 (53.2)	735.6 (75.3)	875.2 (68.8)
Group 5: ANP with ulinastatin	628.6 (73.7)	825.6 (95.3)	877.6 (86.7)
Group 6: ANP with HBO and ulinastatin	347.8 (13.9)*†	357.5 (15.5)*†	428.7 (25.4)*†

ANP = acute necrotizing pancreatitis; ENC = endotoxin-neutralizing capacity; HBO = hyperbaric oxygen; IL= interleukin; TNF = tumour necrosis factor.
*Significantly different from group 3 ($p < 0.05$).
†Significantly different from groups 4 and 5 ($p < 0.05$).

groups. This suggests that early combination therapy has a more efficient action in the progression of ANP than either of therapy alone. This is the first demonstration of the protective effect of early combination HBO and protease-modulating therapy on ANP.

Conclusion

The results from our study indicate that a combination of oxygen therapy and protease-modulating therapy can effectively decrease the plasma level of endotoxin–sCD14 complexes, inhibit the enhanced expression of proinflammatory cytokines and improve immune function; this finding may be of clinical use. To date, similar research has not been performed, and the relevant mechanism for the additive effect of combination therapy is not clear. The efficacy and safety should be determined in clinical trials, and further studies of pathophysiologic efficacy are required.

Competing interests: None declared.

Contributors: Drs. Li and Tang designed the study. Drs. Hou, He and Zhu acquired data. Drs. Wei and Li analyzed the data. Drs. Tang and Hou wrote the article. All authors reviewed the article and approved its publication.

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