$B\beta_{15-42}$ (FX06) reduces pulmonary, myocardial, liver, and small intestine damage in a pig model of hemorrhagic shock and reperfusion*

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Objective: **The fibrin-derived peptide $B\beta_{15-42}$ (also called FX06) has been shown to reduce myocardial infarct size following ischemia/reperfusion. Hemorrhagic shock (HS) followed by volume resuscitation represents a similar scenario, whereby a whole organism is vulnerable to reperfusion injury.**

Design: **We subjected male farm-bred landrace pigs (~30 kg) to HS by withdrawing blood to a mean arterial pressure of 40 mm Hg for 60 minutes. Pigs were then resuscitated with shed blood and crystalloids for 60 minutes, and at this time, FX06 (2.4 mg/kg, n = 8) or vehicle control (phosphate buffered saline; 2.4 mg/kg, n = 7) was injected as an intravenous bolus.**

Setting: **University hospital laboratory.**

Subjects: **Anesthetized male farm-bred landrace pigs.**

Measurements and Main Results: **Data are presented as mean ± SD. Five hours after resuscitation, controls presented acute lung injury (Pao_2/Fio_2-ratio <300 mm Hg; extra-vascular lung water index (marker for lung injury): 9.0 ± 1.8 mL/kg) and myocardial dysfunction/damage (cardiac index: 4.3 ± 0.25 L/min/m^2; stroke volume index: 30 ± 6 mL/m^2; cardiac TnT levels: 0.58 ± 0.25 ng/mL). In contrast, FX06-treated animals showed significantly improved pulmonary and circulatory function (Pao_2/Fio_2-ratio >*400 mm Hg; extra-vascular lung water index: *5.2 ± 2.1 mL/kg, cardiac index: *6.3 ± 1.4 L/min/m^2; stroke volume index: *51 ± 11 mL/m^2; cardiac TnT levels: *0.11 ± 0.09 ng/mL; *$p < 0.05$). Also, tissue oxygenation (tpO_2; mm Hg) was significantly improved during reperfusion in FX06-treated pigs when compared with controls (liver 51 ± 4 vs. *65 ± 4; serosa 44 ± 5 vs. *55 ± 7; mucosa 14 ± 4 vs. *26 ± 4). Finally, FX06 reduced accumulation of myeloperoxidase-positive cells (mainly neutrophils) in myocardium, liver, and small intestine and reduced interleukin-6 plasma levels (*$p < 0.05$; compared with controls).**

Conclusion: **We conclude that in a pig model of HS and reperfusion, administration of FX06 during reperfusion protects shock-susceptible organs such as heart, lung, liver, and small intestine. (Crit Care Med 2009; 37:598–605)**

KEY WORDS: **fibrin-derived peptide; pig; hemorrhagic shock; organ injury and reperfusion**

Every year, ~12 million people are injured on European roads. Many victims are polytraumatized and suffer from extensive blood loss. This leads to hemorrhagic shock (HS), followed by multiorgan failure, and results in high morbidity and mortality (1). In other words, HS patients are exposed to two pathogenic hits. First, the reduction in blood volume causes hypoxia, energy depletion of cells, and increased adrenergic activity (2), resulting in vasoconstriction, tachycardia, and increased myocardial contractility. This increases the myocardial oxygen demand to a level that cannot be achieved (3, 4). Concomitantly, precapillary vasoconstriction leads to ischemia, anaerobic metabolism, acidosis, and tissue damage. In this clinical scenario, volume resuscitation is required. Paradoxically, this induces the second hit: the change from hypoxia to normoxia causes the generation of free oxygen radicals, the release of inflammatory mediators, endothelial cell activation, and tissue inflammation (5, 6). Reperfusion by itself causes additional tissue damage and is termed reperfusion injury. The role of reperfusion injury in HS is clearly supported by the clinical observation that aggressive substitution of blood or crystalloid solutions is clearly associated with multiorgan failure (7, 8).

We have recently identified a novel tissue-protective function of the fibrin-derived peptide $B\beta_{15-42}$, which is also called FX06 (9). This peptide is a natural cleavage product of fibrin, following exposure to plasmin. It consists of 28 amino acids corresponding to the N-terminal sequence of the β-chain of fibrin and targets an endothelial adherens junction protein, vascular endothelial-cadherin. FX06 reduces leukocyte transmigration across endothelial junctions and the release of proinflammatory cytokines (9–12). In rodent and pig models of myocardial ischemia/reperfusion (I/R), this peptide—when given concomitantly at the time of restoration of coronary flow—reduces myocardial inflammation and infarct size (9–12). HS followed by volume

*See also p. 771.

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resuscitation represents a similar scenario, whereby a whole organism is vulnerable to reperfusion injury. Therefore, we hypothesized whether administration of FX06 at the time of resuscitation may have circulatory and organ protective effects in a pig model of HS and reperfusion.

MATERIALS AND METHODS

FX06 (amino acid sequence, GHRPLDKKREEAPSLRPAPPPISGGGYR) was produced by solid-phase peptide synthesis and purified with reverse-phase high-performance liquid chromatography using nucleosil 100–10C18 columns (UCB-Bioproducts and PiChem, Graz, Austria). The endotoxin concentration was <0.06 EU/mg, and microbial contamination was <1 colony-forming units/100 mg.

Anesthesia. Animal experiments were approved by the governmental ethical board for animal research in Mecklenburg-Vorpommern and, in accordance with German law on animal protection and the American Association for the Accreditation of Laboratory Animal Care Liaison guidelines and Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, National Institutes of Health, publication no. 86-23).

After overnight fasting and receiving water *ad libitum*, 16 male German Landrace pigs with body weights ranging from 27 to 32 kg were premedicated intramuscularly with 0.3 mg/kg of midazolam (Dormicum; Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany) and 10 mg/kg of ketamine (Ketanest; Parke-Davis, Freiburg, Germany). Anesthesia was induced intravenously via an ear vein with 3 μg/kg of fentanyl (Fentanyl-Janssen; Janssen-Cilag, Neuss, Germany), 2 mg/kg of ketamine, and 0.25 mg/kg flunitrazepam (Rohypnol, Hoffmann-La Roche AG). The trachea was intubated after an injection of pancuronium (0.3 mg/kg; DeltaSelect, Dreieich Germany). One pig could not be intubated, and emergency tracheotomy was performed. This animal was excluded from the study to avoid additional inflammatory damage. Anesthesia was maintained by a continuous intravenous infusion of flunitrazepam (0.1 mg/kg/hr), ketamine (8.0 mg/kg/hr), and pancuronium (0.15 mg/kg/hr). Pressure-controlled mechanical ventilation was provided by a Servo900 ventilator (Siemens, Erlangen, Germany). Respiratory rate and pressure were adjusted at the beginning of the experiment to maintain an arterial partial pressure of carbon dioxide ($Paco_2$) at 4.8–5.6 kPa. A positive end-expiratory airway pressure (PEEP) of up to 7 cm H_2O was applied to maintain an arterial partial pressure of oxygen (Pao_2) at 12–15 kPa. In case the increase in PEEP was not sufficient to maintain Pao_2, inspired oxygen fraction (Fio_2) was adjusted. One pig was ventilated with a Fio_2 of 0.3 and PEEP of 5 cm H_2O from the beginning of the experiment onwards; however, $Paco_2$ and Pao_2 were within study limits.

Instrumentation. After the induction of anesthesia, animals were placed in supine position on a heating pad to keep body temperature constant at 37°C. A double-lumen catheter (7F Two-Lumen Central Catheterization Set; Arrow, Reading, PA) and an 8.5F introducer (Percutaneous Sheath Introducer Set; Arrow, Reading, PA) were inserted into the right internal jugular vein. Both catheters were advanced 11–13 cm to guarantee correct positioning of the tip in the superior vena cava. A Swan-Ganz thermodilution catheter (7F Swan-Ganz Thermodilutions Catheter; Arrow, Reading, PA) was introduced into the pulmonary artery. Body temperature was monitored continuously with a thermistor in this flow-directed catheter. The right femoral artery was cannulated with a 4.5F introducer set (Percutaneous Sheath Introducer Set; Arrow, Reading, PA) and COLD-catheter (4F Oxymetrie-Thermo-Dye-Dilutionssonde; Pulsion Medical Systems, Munich, Germany). The left femoral artery was cannulated with an 18 G arterial catheter (Vyggon, Aachen, Germany) for continuous monitoring of the arterial blood pressure.

After a median laparotomy, the left hepatic vein and portal vein were cannulated as previously described (13). Ultrasonic perivascular flow-probes (Transonic Systems, Ithaca, USA) of appropriate size were placed around the hepatic artery and the portal vein. The vessel had to fill 75% to 100% of the probe's acoustic window. An improved signal quality was provided with perivascular ultrasonic gel. Care was taken to preserve the perivascular nerve plexus and to place the flow probe of the hepatic artery behind the departure of the superior gastro duodenal artery (13). The bladder was drained with a urinary catheter (Curity 14 CH; TycoHealthcare, Neustadt, Germany) and the abdominal cavity closed after ~90 minutes. All intravascular catheters were connected to pressure transducers and signals recorded via PO-NE-MAH (Digital Acquisition Analysis and Archive Systems, Simsbury, CT). Analyzed parameters were heart rate, mean arterial pressure, central venous pressure, mean pulmonary artery pressure, and pulmonary capillary occlusion pressure, cardiac output and hepatic arterial blood flow, portal venous blood flow, and blood gases. Equations for derived variables are listed in the appendix. For maintenance of normovolemia, all animals received an intravenous full-electrolyte solution (Jonosteril; Fresenius, Bad Homburg, Germany) at 12 mL/kg/hr before the laparotomy and 15–20 mL/kg/hr during the laparotomy to maintain mean arterial pressure, central venous pressure, hemoglobin, and hematocrit values (measured after insertion of the artery catheter). In addition, serum electrolytes, glucose (data not shown), and lactate concentrations were also measured using an ABL715 Autoanalyzer (Radiometer Copenhagen, Denmark). Cardiac output was determined by thermodilution (mean of three injections of 10-mL ice-cold saline using Baxter Explorer CO-computer; Unterschleissheim, Germany). Intrathoracic blood volume was measured to obtain information about the volume status of animals to prevent hypovolemia following reperfusion by means of the COLD-System (Oxymetrie-Thermo-Dye-Dilutionssonde; Pulsion Medical Systems, Munich, Germany) and indocyanin-green dilution technique. Furthermore, extravascular lung water index (marker for lung injury) and pulmonary vascular resistance index (PVRI) were determined using the COLD-System.

Experimental Protocol and Study Groups. The animal model of HS was adapted to pigs from previous experiments by our group (14). Pigs were rapidly bled via the arterial sheath to a mean arterial pressure of 40 mm Hg within 10 minutes and maintained at 40 ± 3 mm Hg for 60 minutes. Additional blood was withdrawn at a mean arterial pressure >44 mm Hg, or crystalloid solution was infused at a mean arterial pressure <36 mm Hg. Shed blood was stored in sterile bags (4R3615NM, Single Blood-Pack Unit 500-mL citrate phosphate dextrose adenine-1, Baxter, Round Lake, IL) containing citrate-phosphate dextrose solution (0.14 mL/mL shed blood). After 60 minutes, animals were resuscitated with twice the volume of shed blood as crystalloid solution and 60% of shed blood during the first hour of reperfusion. Ionized calcium was kept within physiologic range by adding calcium (calcium gluconate 10%; B. Braun Melsungen AG, Melsungen, Germany). In the second hour of reperfusion, the rate of crystalloid infusion was lowered to a volume equaling that of shed blood. Afterward, animals received a basal crystalloid infusion of 10 mL/kg/hr.

Animals were randomly assigned into the following: Group 1: Control animals (HS; n = 7) were instrumented and subjected to HS and received vehicle solution intravenously (phosphate buffered saline [PBS], 2.4 mg/kg; 20 mL total volume) at reperfusion, followed by a second injection of vehicle solution (PBS, 2.4 mg/kg; 20 mL total volume) 2 hours after the onset of reperfusion. Group 2: FX06-treated animals were instrumented and subjected to HS and received the peptide (FX06) at a dose of 2.4 mg/kg dissolved in 20 mL PBS at initiation of reperfusion and 2 hours thereafter. The dose of 2.4 mg/kg was chosen based on previous experiments in models of myocardial I/R, where a dose of 0.9 mg/kg gave insignificant effects, and a dose of 7.2 mg/kg was not superior to 2.4 mg/kg (9, 12).

Measurements were performed at baseline, 60 minutes after inducing shock (40 mm Hg), as well as 1, 3, and 5 hours after the onset of reperfusion. At the end of the experimental period, animals were killed by lethal injection with potassium chloride (40 mL, 1 mol/L). Specimen samples of lung, myocardium, liver, and small intestine were obtained and stored in formaldehyde (Merck KG, Darmstadt, Ger-

many). Another set of specimen samples were frozen in liquid nitrogen and stored at −80°C.

Myeloperoxidase (MPO) Measurements in Lung Lysates. Lung tissue was homogenized, and supernatants were analyzed in 96-well plates. MPO activity (U/g, wet weight) was calculated as the change in absorbance (ΔA450) between 1 and 3 minutes (after the initial reaction) multiplied by the coefficient 13.5, where 1 unit of MPO activity is the amount of enzyme that will reduce 1 μmol peroxide/min (15). Results are expressed as x-fold of control to account for any variation between plate measurements.

MPO-Positive Cell Accumulation in the Myocardium, Liver, and Small Intestine. After 5 hours of reperfusion, myocardial, liver, and small intestine tissue were incubated in paraformaldehyde and embedded in paraffin. Slices (5 μm) were stained with hematoxylin and eosin. For neutrophil count, immunohistochemistry was performed. Briefly, tissues were deparaffinized and incubated with an anti-MPO antibody, followed by an appropriate secondary antibody, according to standard protocols. MPO is mainly expressed in neutrophils but also at a lower degree in monocytes and macrophages. A total of five microscopic fields (in each organ of the seven control and eight FX06-treated pigs) covering 1 mm^2 were photographed, and MPO-positive cells were counted by a blinded investigator. Data are expressed as numbers of MPO-positive cells per area.

Measurements of Tissue Po_2. Intermittently, a multi-wire surface electrode was placed onto the liver as well as serosa and mucosa of the small intestine to measure tissue surface Po_2. For the latter, a 1-cm transmural anti-mesenteric incision was made, and a spacer was introduced to get uncompromised access to the mucosa. After each measurement, the incision in the intestine was sutured. A microprocessor-supported system (Ingenieurbüro Meß- und Datentechnik Mußler, Aachen, Germany) with eight-channel multiwire platinum surface electrodes (Eschweiler, Kiel, Germany) was used for measuring liver surface oxygen partial pressure. During each measurement, ~200 individual Po_2-values were registered at ten different electrode locations to receive representative data of Po_2 distribution. The mean values of these data reflect tissue oxygenation, which is the net result of nutritive blood flow and tissue oxygen consumption (VO_2). Methods were described in detail previously (13).

Determination of Biochemical Plasma Markers. Venous blood samples (3 mL) were collected and plasma obtained (centrifuged at 3000 × *g* for 5 minutes at 4°C) and stored at −20°C until assayed. Cardiac troponin T levels were measured using the Troponin T STAT System (Roche Diagnostics, Mannheim, Germany) (10). Immunoassay for interleukin (IL)-6 (Catalog Number P6000) was purchased from R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany, and performed in duplicates (50-μL samples) (10). Aspartate aminotransferase was determined at the Zentrallabor of the University Hospital Rostock according to standard protocols (http://www.ilab.med.uni-rostock.de/_sgg/m1_1.htm).

Statistics. Data are presented as mean ± SD of n observations. Comparisons between groups were made using Prism 4.03 (GraphPad Software, USA) after datasets passed normality testing—using the Wilcoxon matched pair test (leukocytes and MPO). In both cases, we were not sure whether the assumption of a Gaussian distribution was met. By doing so, we accepted the fact that we used a nonparametric test with possible data from a Gaussian population and that *p* values tend to be too high. In all other cases, we used analysis of variance, followed by Bonferroni's *post hoc* test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Weight, Hemodynamics, Shed Blood Volume, and Hemoglobin. The mean values, SD, and statistical analysis are given in Table 1. Body weight and intrathoracic blood volume (indicator for cardiac preload) were similar in both groups. Mean arterial pressure did not differ between experimental groups at baseline, during shock, or following reperfusion. Mean pulmonary artery pressure and pulmonary capillary occlusion pressure did not differ between experimental groups at baseline, during shock, or following reperfusion. Shed blood volume was similar in both groups, indicating a severe blood loss of ~60% of a pig's total blood volume. Hemoglobin levels were also similar in both groups during the course of the experiments.

Circulation. The mean values, SD, and statistical analysis are given in Table 2. Heart rate did not differ at baseline and doubled after the induction of HS. However, after 5 hours of reperfusion, heart rate remained high in controls and returned almost back to normal in FX06-treated animals. Cardiac index did not differ at baseline or during shock. After 5 hours of reperfusion, cardiac index decreased significantly by ~25% in control pigs but returned to almost baseline values in FX06-treated animals. Stroke volume index remained low in controls and recovered in FX06-treated animals. All experimental animals had normal TnT levels at baseline. In control pigs, we saw a significant increase in plasma TnT 5 hours after reperfusion, and this increase

Table 1. Weight, intrathoracic blood volume, mean arterial pressure, mean pulmonary artery pressure, pulmonary capillary wedge pressure, shed blood volume, and hemoglobin concentration in control (n = 7) and FX06-treated (n = 8) pigs at different time points

		Shock	Reperfusion		
	Baseline	1 hr	1 hr	3 hrs	5 hrs
Weight (kg)					
Control	29.9 ± 2.1				
FX06	29.4 ± 1.9				
Intrathoracic blood volume (mL)					
Control	795 ± 73	440 ± 65[a]	728 ± 125	705 ± 100	716 ± 75
FX06	860 ± 97	432 ± 78[a]	784 ± 98	772 ± 113	767 ± 86
Mean arterial pressure (mm Hg)					
Control	96 ± 10	40 ± 1[a]	80 ± 5	88 ± 12	85 ± 9
FX06	93 ± 7	41 ± 2[a]	88 ± 8	88 ± 15	90 ± 17
Mean pulmonary artery pressure (mm Hg)					
Control	12.6 ± 2.3	8.2 ± 1.9[a]	18.3 ± 2.5	14.9 ± 2.3	16.4 ± 3.5
FX06	13.0 ± 3.2	8.4 ± 2.1[a]	21.8 ± 3.2	15.6 ± 2.1	16.8 ± 3.2
Pulmonary capillary wedge pressure (mm Hg)					
Control	3.2 ± 1.0	1.2 ± 0.9[a]	3.4 ± 1.2	3.7 ± 1.3	3.3 ± 1.0
FX06	4.2 ± 1.3	1.7 ± 0.9[a]	3.2 ± 1.2	3.9 ± 1.1	3.9 ± 1.4
Shed blood (mL/kg)					
Control		39 ± 2.1			
FX06		41 ± 2.7			
Hemoglobin (mmol/L)					
Control	5.8 ± 0.6	5.5 ± 0.7	5.7 ± 0.8	5.6 ± 0.9	5.5 ± 0.7
FX06	5.9 ± 0.6	5.8 ± 0.9	5.9 ± 1.0	5.6 ± 0.7	5.6 ± 0.7

Data are presented as mean ± SD. Baseline, postlaparotomy, and instrumentation.
$p < 0.05$ ([a]vs. baseline within the group).

was significantly lower in FX06-treated animals ($p < 0.05$). Also, myocardial inflammation was high in controls and significantly lower in FX06-treated pigs ($p < 0.05$). Reduced tissue inflammation in FX06-treated pigs correlated with significantly lower plasma levels of IL-6 compared with controls ($p < 0.05$). We have also determined tumor necrosis factor (TNF)-α and IL-1β plasma levels. TNFα levels were high at baseline (postlaparotomy and instrumentation) and did not change during the course of the experiment and were not different in both groups (the TNFα release following surgery being the dominant source of TNFα and this is pre-FX06 treatment). IL-1β levels were lower (trend) in FX06-treated pigs at the end of reperfusion; however, this was not of statistical significance.

Lung. The mean values, SD, and statistical analysis are given in Table 2. At baseline and during HS, all animals maintained a Pao_2 of 12–15 kPa, with a PEEP of 4 cm H_2O and an FIO_2 of 0.21. Then, to maintain the target Pao_2, the PEEP had to be increased in control animals to a maximum of 7 cm H_2O but not in FX06-treated pigs ($p < 0.05$). When maximum PEEP was achieved, FIO_2 was adjusted to meet the target Pao_2. Control pigs received a FIO_2 of 0.5 after 5 hours of reperfusion, whereas FX06-treated animals were sufficiently oxygenated with a FIO_2 of 0.21. The adjustment of the aforementioned parameters resulted in a decrease of the Pao_2/FIO_2 ratio in controls but not in FX06-treated animals (Table 2). To assess pulmonary inflammation, MPO activity was measured in lung lysates. Its activity was reduced by ~40% in FX06-treated animals, but this effect was not statistically significant when compared with controls (Table 2). Lungs (n = 3) from untreated and not instrumented pigs ("healthy control") demonstrate an MPO activity (mean ± SD) of 7 ± 2 U/g. In control pigs, the value almost doubles after HS/R and is back to normal in FX06-treated pigs (Table 2).

Extravascular lung water index did not differ at baseline or during shock (Table 2). After 5 hours of reperfusion, extravascular lung water index significantly increased in controls and was comparable with baseline values in FX06-treated animals (Table 2). PVRI increased significantly during shock in both groups (Table 2). After 5 hours of reperfusion, PVRI was significantly increased in controls and comparable with baseline values in FX06-treated (Table 2).

Table 2. Circulation (TnT; MPO-positive cell accumulation; myo. MPO+ accum.), lung (Pao_2/FIO_2 ratio, extravascular lung water index, pulmonary vascular resistance index, positive end-expiratory pressure; MPO), liver (hepatic arterial blood flow index, portal venous blood flow index; total hepatic blood flow index), and small intestine (superior mesenteric artery blood flow index; superior mesenteric artery) parameters in control (n = 7) and $B\beta_{15-42}$-treated (n = 8) pigs at different time points

		Shock	Reperfusion		
	Baseline	1 hr	1 hr	3 hrs	5 hrs
Circulation					
Heart rate (beat/min)					
Control	110 ± 10	220 ± 16[a]	174 ± 16	161 ± 32[a]	155 ± 33[a]
FX06	105 ± 9	211 ± 16[a]	148 ± 26[a]	139 ± 30[a]	120 ± 29[b]
Cardiac index (mL/kg/min)					
Control	5.8 ± 0.5	2.0 ± 0.6[a]	6.2 ± 1.0	4.9 ± 0.5	4.3 ± 0.3[a]
FX06	6.0 ± 07	2.1 ± 0.6[a]	6.5 ± 1.4	6.1 ± 1.2[b]	6.3 ± 1.4[b]
Stroke volume index (mL/m^2)					
Control	52 ± 5	9 ± 3[a]	36 ± 7[a]	32 ± 9[a]	30 ± 6[a]
FX06	57 ± 6	10 ± 3[a]	44 ± 12[a]	46 ± 13[a]	51 ± 11[b]
TnT (ng/mL)					
Control	0.01 ± 0.01				0.58 ± 0.25[a]
FX06	0.04 ± 0.02				0.11 ± 0.09[a,b]
Myo. MPO+ accum (/1 mm^2)					
Control					97 ± 26
FX06					60 ± 19[b]
Tumor necrosis factor α (pg/mL)					
Control	73 ± 15				69 ± 28
FX06	92 ± 57				84 ± 57
Interleukin-1 (pg/mL)					
Control	12 ± 9				48 ± 45
FX06	11 ± 14				32 ± 33
Interleukin-6 (pg/mL)					
Control	26 ± 7				131 ± 53[a]
FX06	13 ± 11				24 ± 13[b]
Lung					
Pao_2/FIO_2 ratio (mm Hg)					
Control	462 ± 60	395 ± 156	424 ± 79	363 ± 142	233 ± 82[a]
FX06	474 ± 72	448 ± 64	420 ± 40	407 ± 58	415 ± 86[b]
Extravascular lung water index (mL/kg)					
Control	5.5 ± 3.1	3.8 ± 1.7	5.3 ± 0.9	5.7 ± 1.2	9.0 ± 1.3[a]
FX06	5.7 ± 1.1	6.1 ± 1.5	5.5 ± 1.2	5.8 ± 1.1	5.2 ± 1.6[b]
Pulmonary vascular resistance index (dyn^a $s/cm^5/m^2$)					
Control	128 ± 22	286 ± 119[a]	176 ± 59	199 ± 66	250 ± 68
FX06	120 ± 32	253 ± 96[a]	225 ± 74	170 ± 68	162 ± 37[b]
Positive end-expiratory pressure (cm H_2O)					
Control	4.0 ± 0.6	4.3 ± 0.6	5.0 ± 1.0	5.4 ± 1.4	6.7 ± 1.3[a]
FX06	3.9 ± 0.4	3.9 ± 0.4	3.9 ± 0.5[b]	3.9 ± 0.6[b]	3.9 ± 0.5[b]
FIO_2					
Control	0.21 ± 0.0	0.32 ± 0.2	0.24 ± 0.1	0.28 ± 0.1	0.46 ± 0.1[a]
FX06	0.21 ± 0.0	0.21 ± 0.0	0.21 ± 0.0[b]	0.21 ± 0.0[b]	0.21 ± 0.0[b]
MPO activity (U/g)					
Control					13 ± 2
FX06					8 ± 1
Liver					
Hepatic arterial blood flow index (mL/kg)					
Control	6 ± 2	3 ± 1	6 ± 2	5 ± 2	7 ± 1
FX06	6 ± 2	4 ± 2	9 ± 2	9 ± 1	10 ± 3
Portal venous blood flow index (mL/kg)					
Control	31 ± 4	14 ± 8[a]	48 ± 9[a]	38 ± 9	35 ± 9
FX06	28 ± 4	12 ± 2[a]	35 ± 5	32 ± 7	34 ± 10
Total hepatic blood flow index (mL/kg)					
Control	37 ± 5	14 ± 8[a]	50 ± 9	43 ± 7	42 ± 8
FX06	34 ± 7	17 ± 4[a]	42 ± 7	40 ± 7	41 ± 8

Table 2. —*Continued*

Circulation	Baseline	Shock 1 hr	Reperfusion 1 hr	Reperfusion 3 hrs	Reperfusion 5 hrs
DO_2 (mL/kg)					
Control	50 ± 4	9 ± 5[a]	64 ± 11	50 ± 14	52 ± 8
FX06	47 ± 11	14 ± 4[a]	48 ± 15	47 ± 9	54 ± 15
VO_2 (mL/kg)					
Control	10 ± 2	6 ± 2	13 ± 3	12 ± 4	13 ± 6
FX06	11 ± 4	7 ± 2	14 ± 2	11 ± 2	12 ± 4
tpO_2 (mm Hg)					
Control	74 ± 8	52 ± 12[a]	63 ± 11	61 ± 7	51 ± 4[a]
FX06	71 ± 5	46 ± 14[a]	70 ± 9	66 ± 9	65 ± 4
AST (U/L)					
Control	37 ± 7				129 ± 9[a]
FX06	43 ± 11				77 ± 7[a,b]
Lactate (mmol/L)					
Control	2.8 ± 1.0	10.2 ± 1.5[a]	10.7 ± 2.6[a]	5.1 ± 2.8	2.9 ± 2.3
FX06	2.6 ± 1.0	9.3 ± 1.7[a]	10.9 ± 3.3[a]	6.8 ± 3.9	3.6 ± 2.6
Small intestine					
Superior mesenteric artery blood flow index (mL/kg)					
Control	18 ± 2	8 ± 4[a]	29 ± 7[a]	24 ± 7	22 ± 8
FX06	17 ± 4	8 ± 2[a]	23 ± 4	20 ± 4	21 ± 7
DO_2 (mL/kg)					
Control	31 ± 2	15 ± 8	43 ± 8	35 ± 11	35 ± 10
FX06	30 ± 7	18 ± 9	38 ± 7	30 ± 7	34 ± 12
VO_2 (mL/kg)					
Control	9 ± 2	9 ± 3	14 ± 4	9 ± 3	10 ± 4
FX06	11 ± 4	11 ± 2	13 ± 4	11 ± 3	10 ± 3
Serosa tpO_2 (mm Hg)					
Control	64 ± 7	43 ± 12[a]	43 ± 8[a]	42 ± 7[a]	44 ± 5[a]
FX06	61 ± 4	45 ± 10[a]	64 ± 5	55 ± 6	55 ± 7
Mucosa tpO_2 (mm Hg)					
Control	33 ± 4	7 ± 5[a]	21 ± 8	20 ± 4[a]	14 ± 4[a]
FX06	30 ± 5	8 ± 5[a]	24 ± 4	24 ± 7	26 ± 4

AST, aspartate aminotransferase; TnT, troponin T; MPO, myocardial myeloperoxidase; DO_2, oxygen delivery; VO_2, oxygen uptake; tpO_2, tissue oxygen tension.

$p < 0.05$ ([a]vs. baseline within the group; [b]vs. control at the same time point). Data are presented as mean ± SD. Baseline, post laparotomy, and instrumentation.

Liver. For mean values, SD, and statistical analysis, see Table 2 and Figure 1*A* and *B*. The hepatic artery blood flow index decreased during the shock phase by ~40% but recovered during reperfusion in both study groups. The portal vein blood flow index was reduced by 50% during HS but recovered to baseline values with a phase of hyperperfusion in the control group during early reperfusion. Total hepatic blood flow index (hepatic artery blood flow index + portal vein blood flow index) did not differ when compared with hepatic artery blood flow index or portal vein blood flow index alone. No differences were detectable between groups. Liver oxygen delivery (DO_2) was decreased to 20% during the shock phase, almost reaching the baseline level of liver VO_2, which decreased to 60% during the shock phase. During reperfusion, liver-DO_2 and -VO_2 returned to baseline levels without showing differences between groups. The liver surface oxygenation (tpO_2) decreased during HS from 70 to 50 mm Hg. In control animals, the liver-tpO_2 remained at a decreased level at 50 mm Hg at the end of 5-hours reperfusion period. In the FX06 group, liver-tpO_2 was restored to baseline level. At 5-hour reperfusion, AST levels were significantly higher in controls when compared with FX06-treated pigs, indicating more pronounced liver damage in controls. The number of MPO-positive cells infiltrating the liver was significantly higher in control pigs compared with FX06-treated animals (Fig. 1*A* and *B*). In healthy animals without laparotomy or shock, number of MPO-positive cells within the liver was 16 ± 5/mm^2.

Small Intestine. For mean values, SD, and statistical analysis, see Table 2 and Figure 2*A* and *B*. Superior mesenteric blood flow index decreased during HS to 50% and recovered during the phase of reperfusion, showing hyperperfusion in control animals in the early reperfusion period. The DO_2 to small intestine via the superior mesenteric artery (DO_2) decreased during HS by 50% but recovered during reperfusion without showing differences between groups. The small intestine VO_2 was unchanged during HS and reperfusion. The small intestine serosa surface oxygenation (serosa-tpO_2) decreased during HS from 60 to 45 mm Hg. In the FX06 group, serosa-tpO_2 was restored to baseline level. The mucosa-tpO_2 decreased from 32 to 8 mm Hg during HS and recovered to only 14 mm Hg in controls, whereas in the FX06 group, mucosal-tpO_2 recovered to 26 mm Hg after 5-hour reperfusion. In the small intestine, numbers of MPO-positive cells were lower in the mucosa and submucosa of FX06-treated pigs compared with controls (Fig. 2*A* and *B*). The muscularis contained only minimal numbers of MPO-positive cells, and this did not differ between study groups (1 ± 1/mm^2). In untreated, healthy pigs (no surgical intervention), there were some MPO-positive cells detectable in the mucosa (4 ± 5/mm^2), submucosa (4 ± 5/mm^2), and muscularis (1 ± 1/mm^2).

DISCUSSION

We show in an acute pig model of HS and reperfusion that administration of FX06 during reperfusion protects shock-susceptible organs such as heart, lung, liver, and small intestine.

Effects on FX06 on HS and Reperfusion. HS and reperfusion caused cardiac damage in control pigs as confirmed by elevated TnT levels. In contrast, FX06-treated animals had significantly less damage to the heart. Treatment was given at the time of resuscitation, which favors the concept that peptide FX06 reduces myocardial damage during the reperfusion period. Left ventricular dysfunction in settings of I/R has been correlated with both the increased number of leukocytes infiltrating the myocardium (9) and the elevated levels of plasma IL-6 (16). Both surrogate parameters were decreased in the FX06-treated animals. With regard to the lung, HS/reperfusion resulted in an elevated extravascular lung water index, which has been previously shown to correlate with severity of acute respiratory distress, length of ventilation days, and mortality (17, 18). This parameter was significantly improved by peptide FX06. Although PVRI did not differ between controls and peptide-treated animals during HS and during the early phase of reperfusion, PVRI values were significantly lower in

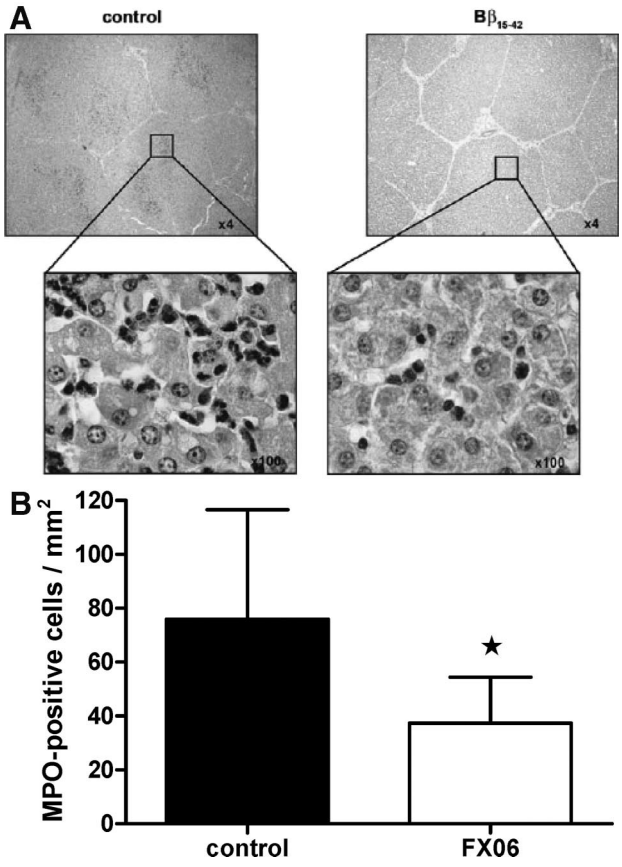

Figure 1. (*A*) Depicted are representative liver slices (×4 or ×100) stained with an anti-myeloperoxidase antibody (*MPO*) after 5 hours of hemorrhagic shock /reperfusion. Livers were obtained from pigs treated either with phosphate buffered saline (control) or FX06. MPO-positive cells appear brown and can be seen best in cartoons with magnification, ×100. (*B*) Liver MPO-positive cell count in control (n = 7) or FX06-treated (n = 8) pigs after 5 hours of reperfusion. Data are expressed as mean ± SD ⋆$p < 0.05$ vs. control.

FX06-treated pigs when compared with controls 5 hours after reperfusion. High PVRI values can be associated with pulmonary hypertension or increased left atrial pressure, leading, for example, to cardiac and pulmonary vascular damage.

With regard to the splanchnic region, HS dramatically decreases the blood flow, and blood is redistributed to vital organs such as heart and brain (19, 20). Hypoperfusion of the splanchnic region impairs function of small intestine and liver, which has been correlated with increased mortality (1, 21, 22). Here, HS decreased the DO_2/consumption to/of the liver and gut, equal in both treatment groups. In our study protocol, the Pao_2 has been kept within physiologic ranges. Therefore, the decrease of liver surface oxygenation must have been due to a decrease in liver tissue oxygenation. Liver damage was demonstrated by elevated serum levels of aspartate amino transferase, which was significantly lower in FX06-treated animals. The DO_2 to the small intestine via the superior mesenteric artery was severely compromised in both treatment groups during HS. However, at the end of the reperfusion period, FX06-treated pigs had significant higher tpO_2 levels, therefore reducing intestinal hypoxia and damage. These results confirm previous findings (23) that tissue oxygenation at the microcirculatory level cannot be predicted from macrocirculatory hemodynamics and suggest that FX06-treated animals suffered less microcirculatory impairment.

With regard to a role of neutrophils, it is well described that they rapidly enter the pulmonary parenchyma following hypovolemic shock (24, 25), where they directly and indirectly participate in cytokine release (26). The role of neutrophils in the mechanism of lung injury has been demonstrated in neutropenic animals, where lung damage was reduced when compared with control animals. IL-6 appears to play a major role in neutrophil recruitment to lungs, and even intratracheal instillation of IL-6 alone into healthy rats results in neutrophil infiltration into lung interstitium and alveoli (27, 28). Similar observations have been published for the liver and small intestine (14, 29–33). In our model, we found reduced inflammation and reduced IL-6 associated with an improved outcome in FX06-treated animals. We found no differences in TNF or IL-1 levels, which we attributed to the fact that they were already high at baseline (after surgery/instrumentation and before FX06 was given) and not further increased during reperfusion. We have shown previously that FX06 protects the heart in acute and chronic myocardial I/R models in mice, rats, and pigs (9, 10, 12). Also in this model, we found reduced inflammation and reduced IL-6 levels associated with an improved outcome in FX06-treated animals. HS shares a number of similarities with myocardial I/R. In contrast to myocardial I/R, hemorrhage acts globally by decreasing circulating blood volume, cardiac output, and tissue DO_2. This causes ischemic injury to numerous vital organs. The subsequent volume resuscitation causes "iatrogen" injury via reperfusion (5, 34–38). Several approaches have been suggested to improve the outcome following HS. For example, volume resuscitation with crystalloids, blood, plasma, colloids, and artificial hemoglobin solutions have been used (39). However, although DO_2 and overall cardiovascular performance have been improved, reperfusion injury could not be ameliorated. Approaches such as cooling (40) or treatment with recombinant IL-11 or with diaspirin cross-linked hemoglobin have reduced reperfusion injury in experimental settings, but so far, these interventions have not been licensed to treat injury following HS/reperfusion in man (41–44).

Methodologic Considerations. In this study, we used pigs as a large animal model because of their physiologic similarity to humans regarding cardiovascular, pulmonary, and splanchnic circulation (45). In contrast to a clinical scenario in humans, animals were, in general, given anesthesia throughout the procedure. We have used ketamine and flunitrazepam, which are known to have only marginal effects on investigated parameters, such as splanchnic circulation (46). We have withdrawn ~40 mL/kg blood, which corresponds to a reduction of blood volume by 60% (47). This was followed by a mean arterial blood pressure of 40 mm Hg, a compromised macro-circulation and a severe hypovolemic shock. State-of-the-art treatment in such a condition requires substitution of fluids and red blood cells (48). We have, there-

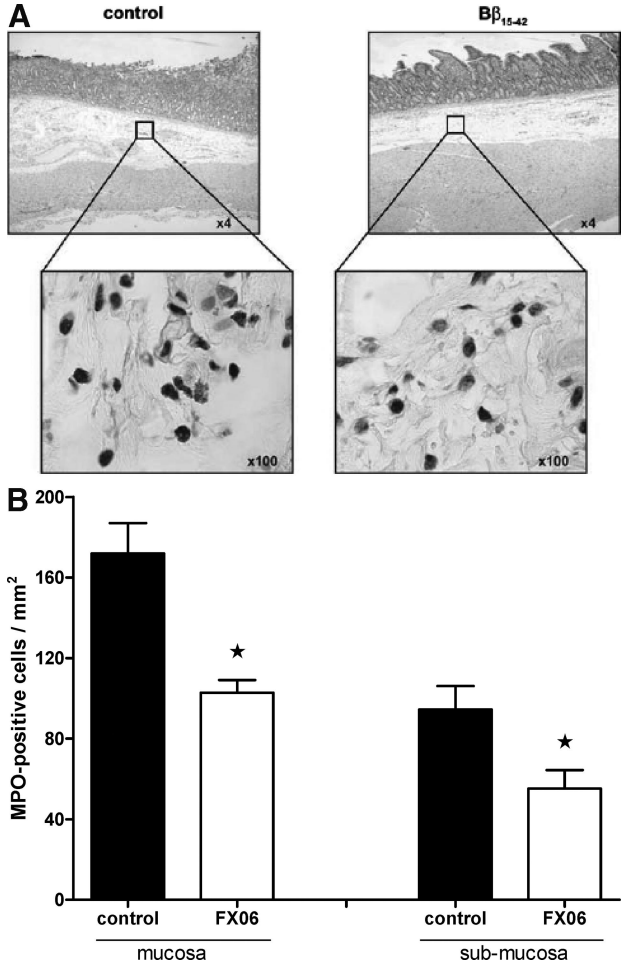

Figure 2. (*A*) Depicted are representative small intestine slices (×4 or ×100) stained with an anti-myeloperoxidase antibody (*MPO*) after 5 hours of hemorrhagic shock/reperfusion. Small intestine was obtained from pigs treated either with phosphate buffered saline (control) or FX06. MPO-positive cells appear brown and can be seen best in cartoons with magnification, ×100. (*B*) Small intestine MPO-positive cell count (mucosa and submucosa) in control (n = 7) or FX06-treated (n = 8) pigs after 5 hours of reperfusion. Data are expressed as mean ± SD ★$p < 0.05$ vs. control.

fore, initiated reperfusion by transfusing parts of shed blood and crystalloid solution. Importantly, hemoglobin concentration did not differ between treatment groups and time points (see Table 1). Also, intrathoracic blood volume, mean pulmonary artery pressure, and pulmonary capillary occlusion pressure did not differ between groups, underlining the physiologic relevance of this model. Animals were observed for a reperfusion period of 5 hours. However, our setup depicts only acute hemorrhage without severe tissue injury and not long-term outcome. This is a clear limitation and difference to clinical traumatic shock and clinical relevant animal models of complex polytrauma (49). However, our model represents an accepted model of HS (50), and we have provided some additional degree of trauma (median laparotomy and manipulation of abdominal vessels and intestine). Despite the latter limitations of this model, our results are very encouraging that peptide FX06-treated animals presented almost "back to baseline" respiratory, splanchnic, and circulatory functions at the end of the 5-hour reperfusion period. This suggests that FX06-treated pigs may have passed the critical time frame of tissue damage. However, to demonstrate long-lasting tissue protective effects, reperfusion periods of several days are required.

In conclusion, we present a novel option using the fibrin fragment FX06 for the treatment of reperfusion injury following HS. Our previous data in myocardial I/R models have resulted in the initiation of the F.I.R.E. trial in human infarct patients (www.clinicaltrial.gov). The effect of FX06 in HS as presented in this work encourages further preclinical development of this peptide for a potential use in human reperfusion injury.

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APPENDIX

Equations used for calculation of oxygen supply/uptake

$$\text{O}_2\text{ content (C-O}_2\text{) [mL O}_2\text{/dL]} = \text{Hb [g/dl]} \times \text{SO}_2\,[\%] \times 1.34\text{ mL O}_2\text{/g Hb} + 0.0031\text{ mL O}_2\text{/dl/mmHg PO}_2 \times \text{PO}_2\,[\text{mmHg}]$$

$$\text{O}_2\text{ delivery (DO2)} = \text{C-O}_2 \times \text{flow [mL O}_2\text{/min]}$$

$$\text{DO}_2\text{SMA} = \text{C-O}_2\text{A} \times \text{SMABF} \times 10^{-2}\,[\text{mL O}_2\text{/min}]$$

$$\text{DO}_2\text{TH} = \text{DO}_2\text{HA} + \text{DO}_2\text{PV [mL O}_2\text{/min]}$$

$$\text{VO}_2\text{SI} = (\text{C-O}_2\text{A} - \text{C-O}_2\text{SMV}) \times \text{SMAwBF} \times 10^{-2}\,[\text{mL O}_2\text{/min}]$$

$$\text{VO}_2\text{TH} = (\text{C-O}_2\text{PV} - \text{C-O}_2\text{HV}) \times \text{PVBF} \times 10^{-2} + (\text{C-O}_2\text{A - CO}_2\text{HV}) \times \text{HABF} \times 10^{-2}\,[\text{mL O}_2\text{/min}]$$

DO_2SMA = superior mesenteric arterial oxygen delivery

DO_2TH = total hepatic oxygen delivery

VO_2SI = total small intestinal oxygen uptake

VO_2TH = total hepatic oxygen uptake