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Experimental paper

A double blind, single centre, sub-chronic reperfusion trial evaluating FX06 following haemorrhagic shock in pigs☆

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ABSTRACT

Objective: Haemorrhagic shock causes ischaemia and subsequent fluid resuscitation causes reperfusion injury, jointly resulting in high morbidity and mortality. We tested whether the anti-inflammatory fibrin-derived peptide, $B\beta_{15-42}$, also called FX06, is tissue protective in a model of haemorrhagic shock.
Methods: In a pig model, we standardised the severity of haemorrhagic shock by achieving a cumulative oxygen deficit of approximately 100 ml/kg body weight by withdrawing blood over a period of 1 h. This was followed by resuscitation with shed blood and full electrolyte solution, and pigs were monitored for 3 days. At reperfusion, 17 pigs were randomly assigned to FX06 or solvent treatment.
Results: FX06-treated pigs demonstrated improved cardiac function (stroke volume index: 67 ml/m^2 versus 33 ml/m^2), decreased troponin T release in the early reperfusion (0.24 ng/ml versus 0.78 ng/ml), decreased AST levels after 24 h (106 U/l versus 189 U/l) and decreased creatinine levels after 24 h (108 μmol/l versus 159 μmol/l). Furthermore, FX06-treated pigs demonstrated preservation of the gut/blood barrier, while controls demonstrated high endotoxin plasma levels indicating translocation of bacteria and/or its products (0.2 EU/ml versus 24.3 EU/ml) after 24 h. This study also demonstrates a significantly improved neurological performance in the FX06 group as determined by S100β serum levels (0.72 μg/l versus 1.25 μg/l) after 48 h and neurological deficit scores (11 versus 70) after 24 h.
Conclusion: FX06 – when administered as an adjunct to fluid resuscitation therapy – is organ protective in pigs. Further investigations are warranted to reveal the protective mechanism of FX06.

1. Introduction

Haemorrhagic shock is a leading cause of death in trauma patients worldwide.[1–3] The main goals of resuscitation are to stop the haemorrhage and to restore circulating blood volume. Early controlled fluid resuscitation with crystalloids, colloids or blood products can be life saving.

Complicating the management of haemorrhagic shock is the risk of reperfusion injury mediated by fluid resuscitation.[4–9] Although fluid resuscitation is important for survival, it also can cause further damage to various organs such as the lungs, intestine, heart and brain. Currently, there is no known treatment available to prevent or mitigate reperfusion injury.

Recently, we showed that the fibrin-derived peptide $B\beta_{15-42}$ (also called FX06) is tissue protective in a model of acute haemorrhagic shock (blood pressure controlled) followed by a 6 h reperfusion period.[10] FX06, a 28 amino acid peptide corresponding to the N-terminal sequence of the β-chain of fibrin, is a natural cleavage product of fibrin following exposure to plasmin. We have shown that FX06 targets an endothelial adherens junction protein, VE-cadherin, representing a novel tissue-protective function.[11] FX06 reduced leukocyte transmigration across endothelial junctions and reduced the release of pro-inflammatory cytokines.[7,11–13] In the present trial, we have used the method of cumulative oxygen deficit as a measure of haemorrhagic shock.[14] This technique has proven to be an excellent predictor of severity of cellular insult prior to resuscitation. In addition, we have extended the time of reperfusion to 72 h mimicking the early clinical course of a patient suffering

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from haemorrhagic shock and subsequent in-hospital treatment. This study was carried out as a double blinded, single centre, subchronic resuscitation trial. Endpoints of the study were assessment of organ function and damage, inflammatory markers and neurological outcome.

2. Materials and methods

2.1. Animals

This study was performed in accordance with German law on animal protection and approved by the governmental ethical board for animal research in Mecklenburg-Vorpommern. 22 male German landrace pigs (28–32 kg) were anaesthetised and instrumented (intubation and ventilation, arterial and venous lines). Anaesthesia was induced with fentanyl (5 μg/kg i.v.; Janssen-Cilag GmbH, Neuss, Germany), propofol 1% (2 mg/kg i.v.; Braun Melsungen AG, Melsungen, Germany) and pancuronium (0.3 mg/kg i.v.; Delta Select, Pfullingen, Germany). Anaesthesia was maintained with propofol 2% (6–8 mg/kg/h i.v., Braun Melsungen AG).

Analysed parameters were heart rate, mean arterial blood pressure (MAP), cardiac output, blood gases and a variety of biochemical markers. Cardiac output and intrathoracic blood volume were determined by thermodilution using PiCCO monitoring. Animals were connected to a Deltatrac™ II metabolic monitor (Datex-Ohmeda Instrumentation Corp., Helsinki, Finland) to measure oxygen deficit. The full methods have been described by our group previously.

2.2. Haemorrhagic shock and fluid resuscitation

Pigs were rapidly bled via an arterial sheath (5 F) in order to achieve a cumulative oxygen deficit of 95–120 ml/kg/h according to the work of Rixen et al.[14] Animals were resuscitated with shed blood and full electrolyte crystalloid solution according to the intrathoracic blood volume index to prevent hypovolemia (using PiCCO monitoring). At reperfusion, animals received in addition to fluid resuscitation an i.v.-bolus injection (20 ml) of vehicle solution (phosphate buffered saline, PBS; $n = 9$) or FX06 ($n = 8$; 3.0 mg/kg) followed by an infusion of either preparation at a rate of 0.3 mg/kg/min for 1 h (50 ml volume). Pigs were allowed to recover and received intramuscular tramadol (1.5 mg/kg Tramal®, Grünenthal, Stolberg, Germany) at 12-h intervals for pain relief. Animals were transferred to the animal unit and were closely observed for 72 h (daily medical assessment, consisting of neurological scoring as well as blood tests). Measurements were performed at baseline, 1 h after inducing shock, as well as 1, 24, 48 and 72 h after the onset of fluid resuscitation. At the end of the experimental period, pigs were anaesthetised as described above and haemodynamic parameters were determined. Hereafter, the animals were killed by increasing anaesthetic depth followed by lethal injection with potassium-chloride (40 ml, 1 mol/l). The protocol is summarised in Fig. 1.

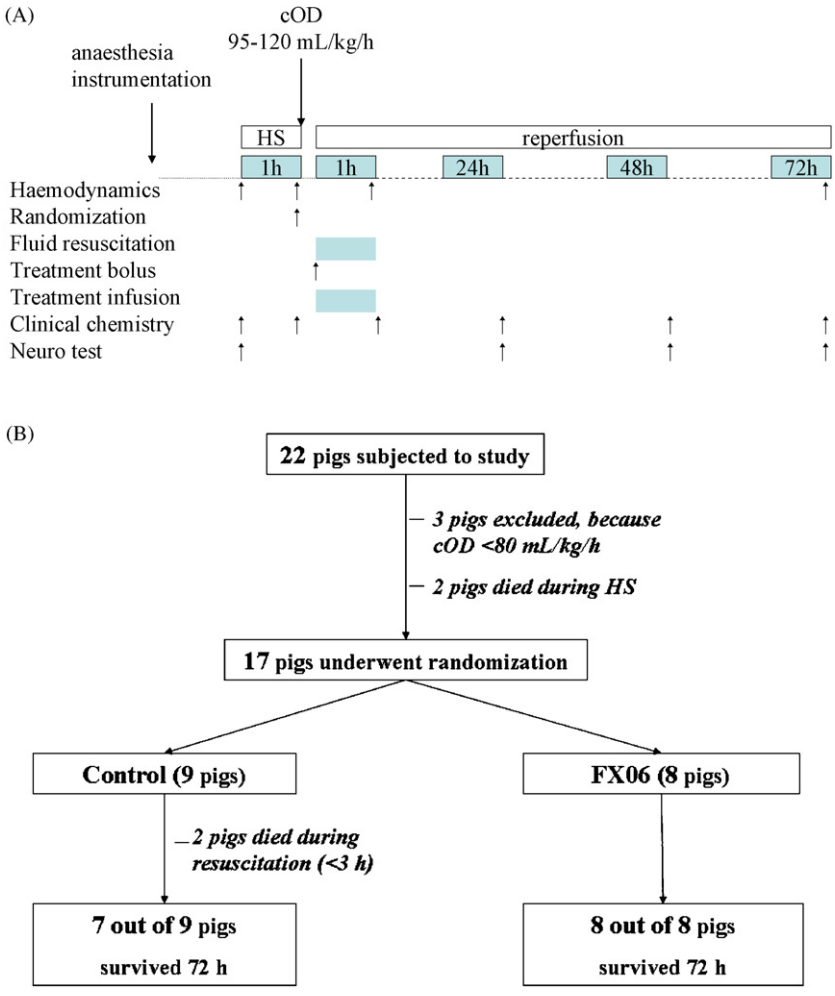

Fig. 1. (A) Time-line depicting experimental protocol and time points of intervention/measurements. (B) Number of study subjects, randomisation and drop-outs.

2.3. Study groups

Treatment (vials numbered from 1 to 22 with a 'white powder') was stored at 4 °C. 5 min prior to reperfusion, vials were filled with phosphate buffered saline (20 ml for bolus injection and 50 ml for infusion). Treatment was allocated in a blinded fashion and the identity of the drugs was uncovered only after all data were obtained and sent to the study controller (independent of authors of this article as well of this study). Pigs were randomly assigned into the following two treatment arms (see Fig. 1B):

(1.) *control* vehicle solution (PBS) bolus injection (20 ml) at the start of fluid resuscitation followed by an infusion of 50 ml for 1 h ($n = 9$).
(2.) *FX06* (3.0 mg/kg) bolus injection at the start of fluid resuscitation followed by an infusion of FX06 (0.3 mg/kg/min; total volume = 50 ml) for 1 h ($n = 8$).

Two pigs died during the shock phase and an additional two pigs from the control group died during the early resuscitation period.

2.4. Reagents

All reagents were obtained from Sigma–Aldrich, Munich, Germany, unless otherwise stated. FX06 (peptide $B\beta_{15-42}$ with the sequence GHRPLDKKREEAPSLRPAPPPISGGGYR) was obtained from UCB, NL.

2.5. Statistics

Statistical analysis was performed using a one way-ANOVA followed by a Bonferroni's post-test if appropriate using SPSS 14.01 for Windows (SPSS Inc. Headquarters, Chicago, IL, USA) and Prism 4.03 for Windows (GraphPad Software Inc., San Diego, CA, USA). Data are derived from controls ($n = 7$) or FX06-treated ($n = 8$) animals. Experimental time points measured were baseline, 1 h after haemorrhagic shock, as well as 1, 24, 48 and 72 h of reperfusion (see Fig. 1A). Data are expressed as mean ± SD. $^{*}P < 0.05$ versus baseline within the group; $^{+}P < 0.05$ versus control at the same time point.

3. Results

3.1. Weight, ITBVI, haemodynamics, EVLWI, shed blood volume, haematocrit, haemoglobin, and cardiac troponin T

For mean values, SD and statistical analysis see Table 1.

Heart rate and cardiac index did not differ at baseline and doubled after the induction of haemorrhagic shock in both groups. However, after 72 h of reperfusion, heart rate remained high in the control group and returned to baseline values in FX06-treated animals. The cardiac index was comparable between groups at baseline and decreased to 25% during the shock phase in both groups. After resuscitation, the cardiac index in the control group almost doubled compared to baseline value indicating a hyperdynamic state, whereas the cardiac index in FX06-treated animals returned to baseline levels after fluid resuscitation. Stroke volume index did not differ at baseline but decreased significantly in both groups during haemorrhagic shock. After 72 h stroke volume index in the control group was 50% of the baseline value whereas in the FX06 group baseline levels were observed. Extra-vascular lung water index (EVLWI) did not differ at baseline or during shock. 72 h after fluid resuscitation, EVLWI was increased in control animals and was comparable to baseline values in FX06-treated animals.

Shed blood volume was similar in both groups indicating a severe blood loss of approximately 60% of a pig's total blood volume. Haemoglobin and haematocrit levels were similar in both groups

Table 1
Weight, intrathoracic blood volume index (ITBVI), mean arterial pressure (MAP), extra vascular lung water index (EVLWI), shed blood volume, haematocrit and haemoglobin concentration, heart rate, cardiac index, stroke volume index and troponin T (TnT) parameters in control ($n = 7$) and FX06-treated ($n = 8$) pigs at different time points. $P < 0.05$ (*versus baseline within the group; $^{+}$versus control at the same time point). Baseline = post-surgical instrumentation. Body surface area (control group: 0.76 ± 0.01; FX06 group: 0.75 ± 0.02).

		Baseline	Shock	Reperfusion			
			1 h	1 h	24 h	48 h	72 h
Weight (kg)	Control	32.4 ± 0.9					
	FX06	31.4 ± 1.3					
ITBVI (ml/m^2)	Control	939 ± 93	408 ± 120*	838 ± 52			820 ± 51
	FX06	918 ± 52	414 ± 97*	780 ± 54			819 ± 83
MAP (mmHg)	Control	83 ± 8	21 ± 5*	88 ± 15			65 ± 15
	FX06	82 ± 12	23 ± 8*	77 ± 12			80 ± 8
EVLWI (ml/kg/m^2)	Control	8.3 ± 0.8	10.7 ± 2.4	10.9 ± 1.6			11.9 ± 2.1*
	FX06	8.7 ± 0.9	11.3 ± 3.4	9.4 ± 0.9			8.4 ± 0.7^{+}
Shed blood (ml/kg)	Control		46 ± 6				
	FX06		47 ± 6				
Haematocrit (%)	Control	26 ± 2	26 ± 4	28 ± 3	31 ± 4	30 ± 2	25 ± 3
	FX06	27 ± 2	28 ± 4	29 ± 3	32 ± 3	33 ± 2	24 ± 2
Haemoglobin (g/dl)	Control	8.1 ± 0.6	7.7 ± 1.3	8.3 ± 1.0	9.5 ± 1.1	9.1 ± 0.8	7.6 ± 1.0
	FX06	8.2 ± 0.3	8.3 ± 1.3	8.7 ± 1.1	9.7 ± 0.9	9.9 ± 0.5	7.5 ± 0.4
Heart rate (beats/min)	Control	83 ± 7	147 ± 45*	170 ± 23*			134 ± 14*
	FX06	82 ± 7	145 ± 29*	145 ± 21*			91 ± 13^{+}
Cardiac index (l/min/m^2)	Control	5.2 ± 0.5	1.4 ± 0.4*	8.1 ± 1.5*			4.4 ± 0.5
	FX06	4.8 ± 0.4	1.1 ± 0.4*	5.8 ± 1.2^{+}			5.3 ± 1.2
Stroke volume index (ml/m^2)	Control	64 ± 11	11 ± 6*	49 ± 11			33 ± 6*
	FX06	58 ± 6	11 ± 4*	40 ± 8*			67 ± 15^{+}
TnT (ng/ml)	Control	0.01 ± 0.00	0.06 ± 0.08	0.78 ± 0.38*	0.32 ± 0.13	0.36 ± 0.21*	0.21 ± 0.22
	FX06	0.01 ± 0.00	0.08 ± 0.13	0.24 ± 0.18^{+}	0.17 ± 0.14	0.13 ± 0.12	0.08 ± 0.11

Table 2
Oxygenation ratio (paO_2/FIO_2) and biochemical parameters (v = venous) in control ($n = 7$) and FX06-treated ($n = 8$) pigs at different time points. $P < 0.05$ (*versus baseline within the group; $^+$versus control at the same time point). Baseline = post-surgical instrumentation.

		Baseline	Shock	Reperfusion			
			1 h	1 h	24 h	48 h	72 h
PaO_2/FiO_2 (mmHg)	Control	499 ± 16	444 ± 88	486 ± 86			340 ± 70*
	FX06	461 ± 32	397 ± 39	535 ± 94			429 ± 51
CK (U/l)	Control	1296 ± 540	1281 ± 432	1677 ± 700	6115 ± 2306*	1456 ± 701	1309 ± 476
	FX06	1424 ± 480	1482 ± 324	1990 ± 514	7573 ± 2949*	2489 ± 1124	1055 ± 342
LDH (U/l)	Control	920 ± 190	817 ± 83	1031 ± 55	2672 ± 691*	2339 ± 885*	1310 ± 615
	FX06	1099 ± 324	1274 ± 364	1331 ± 398	2506 ± 732*	2153 ± 525*	1109 ± 236
AST (U/l)	Control	37 ± 7	39 ± 9	79 ± 29	189 ± 63*	84 ± 18	52 ± 12
	FX06	43 ± 11	43 ± 8	81 ± 25	106 ± 31^{*+}	61 ± 19	41 ± 7
ALT (U/l)	Control	41 ± 6	34 ± 7	41 ± 7	82 ± 17*	67 ± 13*	55 ± 12
	FX06	41 ± 10	35 ± 10	43 ± 12	55 ± 5$^+$	55 ± 5	45 ± 5
GLDH (U/l)	Control	1.3 ± 0.7	2.0 ± 1.1	3.6 ± 2.1	8.2 ± 3.2	11.5 ± 6.5*	7.3 ± 3.3
	FX06	1.7 ± 0.4	2.2 ± 0.5	2.6 ± 0.7	3.1 ± 1.3	3.5 ± 1.4	2.1 ± 0.5
TNFα (pg/ml)	Control	64 ± 53	119 ± 55	125 ± 66	4 ± 3	5 ± 3	11 ± 11
	FX06	68 ± 45	131 ± 51	115 ± 45	5 ± 3	5 ± 3	4 ± 2
IL-4 (pg/ml)	Control	12 ± 13	10 ± 7	12 ± 11	27 ± 24	26 ± 18	65 ± 13
	FX06	9 ± 6	8 ± 6	9 ± 6	15 ± 11	15 ± 15	10 ± 7
IL-10 (pg/ml)	Control	31 ± 36	28 ± 36	47 ± 63	35 ± 39	37 ± 37	34 ± 28
	FX06	18 ± 28	28 ± 28	32 ± 34	26 ± 29	26 ± 29	17 ± 22
BUN (mmol/l)	Control	4.5 ± 1.1	5.2 ± 1.1	5.6 ± 0.9	9.1 ± 2.4*	7.5 ± 1.2	5.5 ± 1.4
	FX06	4.5 ± 1.7	5.5 ± 1.5	5.9 ± 1.6	6.1 ± 1.4	5.5 ± 1.4	4.2 ± 1.1
Creatinine (μmol/l)	Control	94 ± 19	129 ± 25	128 ± 23	159 ± 46*	146 ± 38*	140 ± 26
	FX06	92 ± 18	132 ± 16	117 ± 15	109 ± 12$^+$	102 ± 11	87 ± 17$^+$
Lipase (U/l)	Control	2.6 ± 1.5	1.9 ± 1.3	5.3 ± 2.9	49.4 ± 17.2*	11.6 ± 4.0	4.9 ± 1.6
	FX06	3.0 ± 0.8	3.0 ± 0.8	3.8 ± 1.0	12.6 ± 6.9*	9.8 ± 3.9	3.8 ± 1.3
v BE (mmol/l)	control	4.7 ± 2.8	−14.2 ± 4.8*	−7.4 ± 4.6*	5.3 ± 3.0	5.4 ± 3.3	4.8 ± 1.9
	FX06	4.9 ± 2.0	−10.8 ± 2.0*	−4.6 ± 3.9*	8.2 ± 2.0	6.1 ± 1.0	7.6 ± 2.7
v Lactate (mmol/l)	Control	1.4 ± 0.4	14.2 ± 2.8*	10.0 ± 2.9*	2.5 ± 0.6	2.1 ± 0.6	2.2 ± 0.6
	FX06	1.5 ± 0.3	11.4 ± 1.0*	6.6 ± 2.7^{*+}	1.5 ± 0.4	1.5 ± 0.3	1.3 ± 0.5
v Glucose (mmol/l)	Control	4.9 ± 0.9	15.7 ± 6.1*	11.9 ± 5.1	5.4 ± 0.5	5.6 ± 0.7	4.5 ± 0.8
	FX06	5.1 ± 1.0	13.3 ± 7.1*	11.3 ± 4.7	6.4 ± 0.7	6.4 ± 1.0	5.1 ± 0.9

and did not change during the course of the experiments. All experimental animals had normal TnT levels at baseline. In control pigs, haemorrhagic shock followed by resuscitation caused a significant increase in plasma TnT after 1 h, while FX06-treated animals had significantly lower levels. After 72 h, both groups showed normal values which were similar to baseline levels.

3.2. Lung, liver, kidney and other plasma parameters

For mean values, SD and statistical analysis see Table 2. At baseline, haemorrhagic shock and early fluid resuscitation period, all animals maintained a PaO_2/FiO_2 ratio >400 mmHg. However, at 72 h control pigs showed a decreased PaO_2/FiO_2 ratio, while FX06-treated animals demonstrated normal values. Plasma creatinine kinase (CK) and lactate dehydrogenase (LDH) levels were significantly elevated in both groups at 24 h (CK and LDH) and 48 h (LDH). After 72 h of resuscitation, both parameters were back to baseline levels in both study groups. Various plasma markers for liver and kidney dysfunction/damage (e.g. aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), glutamatedehydrogenase (GLDH), blood–urea–nitrogen (BUN), creatinine) were significantly increased at 24 and 48 h of reperfusion. However, there were significant differences between study groups indicating less dysfunction/damage in FX06-treated animals. Base excess (BE), lactate and glucose levels were significantly elevated during haemorrhagic shock and/or 1 h following reperfusion. All three parameters went back to baseline after 24 h and remained normal until 72 h.

3.3. Biochemical parameters

The following parameters did not change during the course of the experiment (baseline versus haemorrhagic shock versus 1, 24, 48 or 72 h following fluid resuscitation): amylase, total bilirubin, GGT, quick, aPTT, fibrinogen, IL-10, TNFα, total cholesterol, HDL cholesterol, LDL cholesterol, LDL/HDL ratio.

3.4. Study numbers and mortality

22 pigs were enrolled in the study (see Fig. 1B). Three pigs were killed prior to randomisation because they did not fulfill the inclusion criteria of a cumulative oxygen deficit of approximately 95–120 ml/kg. Two pigs died during the haemorrhagic shock phase subjecting 17 pigs to the randomisation process. Nine pigs were treated as control animals and eight pigs with FX06 as indicated in the method section. Two pigs of the control group died during the early phase of resuscitation (<3 h) giving a 22% mortality rate. All FX06-treated pigs survived the follow-up of 72 h.

3.5. Systemic inflammatory response syndrome (SIRS)

At baseline and during haemorrhagic shock, plasma endotoxin levels (Fig. 2A), plasma leukocyte levels (Fig. 2B), IL-1β levels (Fig. 2C), IL-2 levels (Fig. 2D) and IL-6 levels (Fig. 2E) were comparable in both groups.

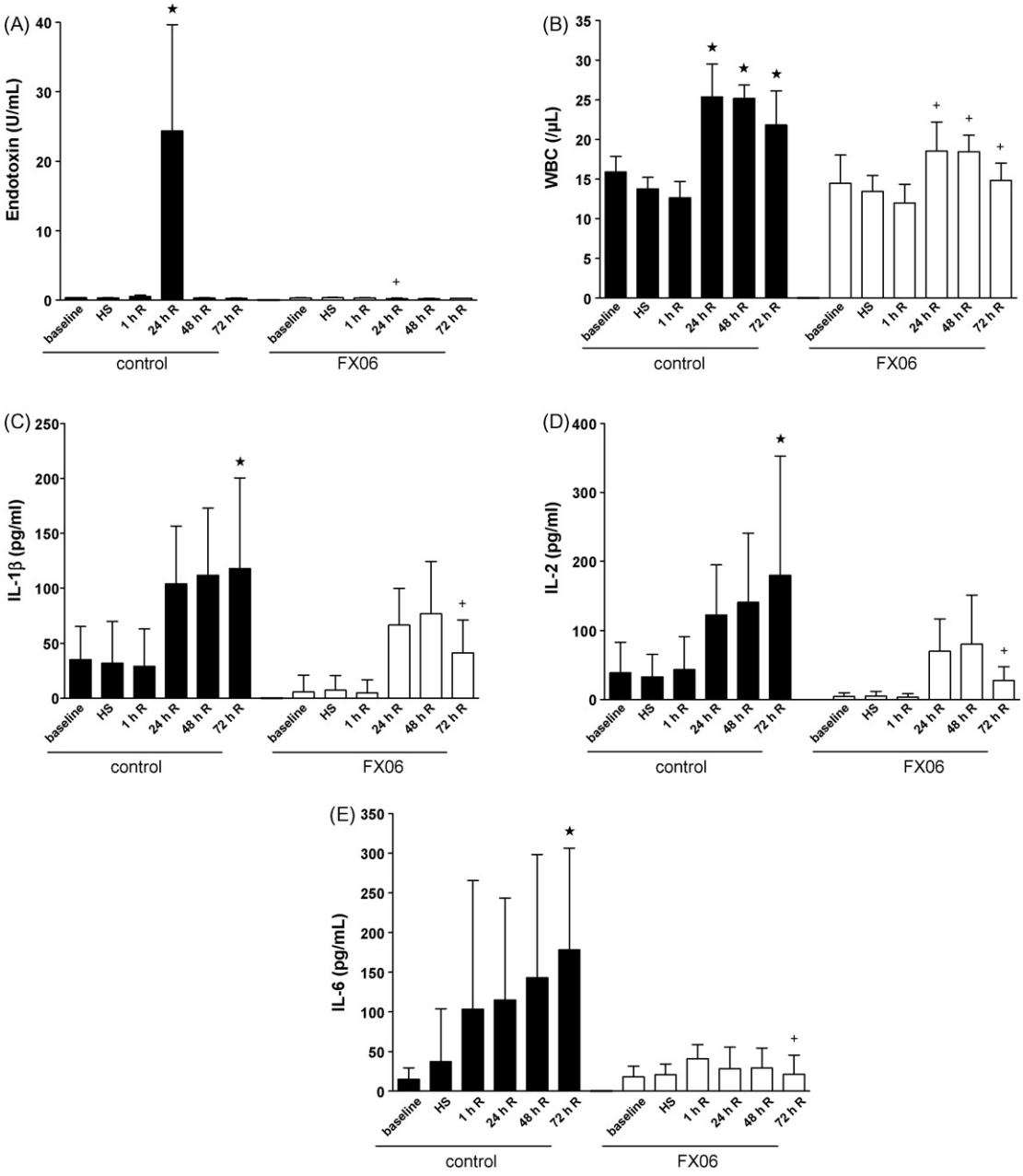

Fig. 2. Depicted are markers of inflammation in control ($n = 7$) or FX06-treated ($n = 8$) animals. (A) Plasma endotoxin levels indicating bacterial translocation via the intestine. (B) Leukocyte count. Plasma levels of IL-1β (C), IL-2 (D) or IL-6 (E). Experimental time points measured were baseline (=post-surgical instrumentation), 1 h after haemorrhagic shock as well as 1, 24, 48 and 72 h of reperfusion. Data are expressed as mean ± SD. $^{*}P < 0.05$ versus baseline within the group; $^{+}P < 0.05$ versus control at the same time point.

Plasma endotoxin levels increased significantly in control pigs 24 h following fluid resuscitation. This indicates bacterial translocation into the blood stream most likely due to splanchnic hypoperfusion during haemorrhagic shock and subsequent reperfusion injury. There were no detectable endotoxin levels in FX06-treated pigs at any time point throughout the experiment.

White cell count in control pigs almost doubled at 24 and 72 h following fluid resuscitation. This did not occur in FX06-treated pigs at any time point studied.

IL-1β, IL-2 and IL-6 plasma levels rose in control pigs and remained high 72 h following fluid resuscitation indicating systemic inflammation. In contrast, in FX06-treated pigs, IL-1β, IL-2 and IL-6 plasma levels did not change in a statistically significant fashion during the course of the experiment. 72 h after fluid resuscitation FX06-treated pigs demonstrated significantly lower levels of the latter cytokines compared to control pigs. TNFα levels (Table 2) were high at baseline (post-surgical instrumentation) and did not change during haemorrhagic shock and fluid resuscitation. TNFα levels were almost undetectable at 24–72 h. IL-4 and IL-10 levels did not change and were similar in both groups during the course of the experiment.

3.6. Neurological outcome

Haemorrhagic shock and fluid resuscitation were associated in our model with neurological damage/dysfunction. Control pigs demonstrated significantly elevated levels of S100β (marker of brain damage) after 24 and 48 h (Fig. 3A). After 48 h, FX06-treated pigs had significantly lower levels of S100β than control animals. We also used a neurological deficit score[15] for pigs to further

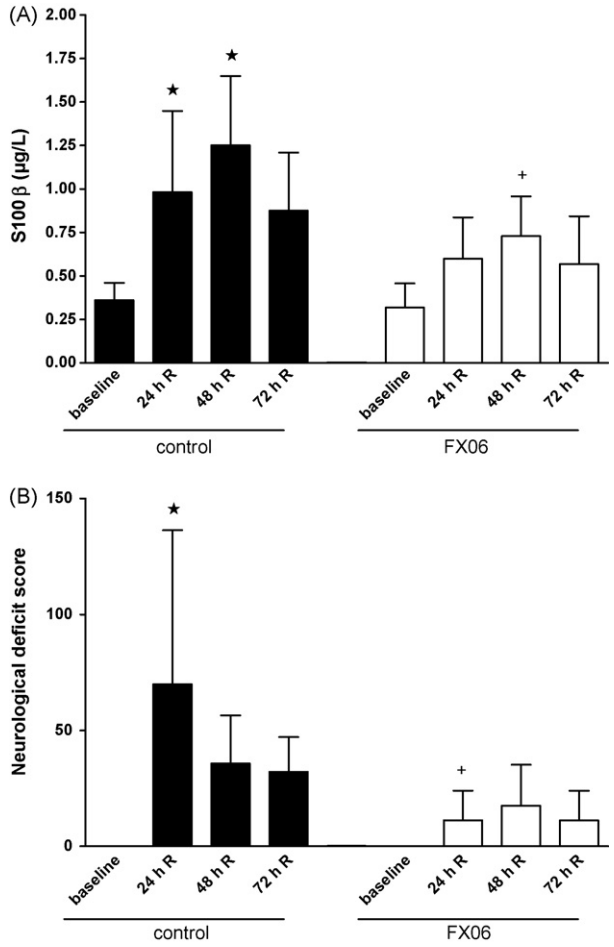

Fig. 3. Depicted are the neurological deficit score for pigs and markers of neurological damage in control ($n = 7$) or FX06-treated ($n = 8$) animals. (A) Plasma levels of S100β. (B) Neuro scores for pigs during reperfusion. Experimental time points measured were baseline, 1 h after haemorrhagic shock, as well as 1, 24, 48 and 72 h after reperfusion. Data are expressed as mean ± SD. $^{*}P < 0.05$ versus baseline within the group; $^{+}P < 0.05$ versus control at the same time point.

evaluate neurological outcome. After 24 h, control pigs showed a neurological deficit score of 70, which was statistically significant when compared to baseline (score = 0). After 24 h, FX06-treated pigs had a significantly lower neurological deficit score than control animals.

4. Discussion

The peptide FX06 mediates organ protection during ischaemia and reperfusion injury such as myocardial infarction[7,11,13] or acute pressure controlled haemorrhagic shock[10]. Here we studied whether FX06 mediates tissue protection in a sub-chronic porcine haemorrhagic shock model. All animals were subjected to the same traumatic insult of haemorrhagic shock evidenced by cumulative oxygen deficit. Hereafter, pigs were randomised to either control or FX06 treatment. Participating scientists were blinded to this process excluding any potential bias.

FX06 application at the time of fluid resuscitation improved cardiac, lung and neurological function and preserved gut–blood barrier function during the following 72 h. Beneficial effects of FX06 were further supported by improvement in surrogate parameters such as plasma levels of troponin T, cytokines, creatinine and liver enzymes.

Our study adds further information to the understanding of injury due to haemorrhagic shock and fluid resuscitation. In addition to death, lactate and base excess we have documented several other endpoints for the assessment of reperfusion injury. Plasma cytokine levels demonstrate the magnitude of the inflammatory response to haemorrhagic shock and the time-course over which it occurs. The role of cytokines in haemorrhagic shock remains controversial, with some studies showing an elevation of cytokines, whereas others do not. We provide the time-course of six cytokines for this model (TNFα, IL-1β, IL-2, IL-4, IL-6 and IL-10). TNFα levels were high at baseline which can be explained by surgical instrumentation. During haemorrhagic shock and following fluid resuscitation TNFα remained elevated initially. After 24–72 h, TNFα levels decreased again indicating that TNFα release following surgery is the dominant source of TNFα. It is of interest that markers such as IL-1β, IL-2 and IL-6, and white cell count were markedly raised at 24 h and in the case of the latter cytokines were raised even further after 72 h in the control group. The markers of neurological injury were also striking with levels of the protein S100β and neurological scores remaining elevated at 72 h in both groups. This suggests a clear relationship between cumulative oxygen deficit and neurological damage for at least 72 h following haemorrhagic shock, although how this might recover in subsequent days is unknown.

All of our findings confirm that haemorrhagic shock and subsequent fluid resuscitation lead to a significant inflammatory response with associated myocardial, neurological and gastrointestinal impairment. The fact that so many of these inflammatory markers were attenuated by FX06 is of genuine interest and represents the basis for further research on the molecule.

4.1. Reperfusion therapy

The peptide FX06 was given concomitantly with the onset of fluid resuscitation to evaluate its effect on reperfusion-dependent injury. Tissue damage during and following haemorrhagic shock arises from hypovolaemia and hypoxia (shock phase) as well as from fluid resuscitation. Reperfusion injury is a complex phenomenon seen in various acute conditions such as coronary artery occlusion followed by reperfusion, organ transplantation and haemorrhagic shock followed by fluid resuscitation. The precise patho-physiological mechanism is still unclear. Reperfusion injury appears to be initiated by the generation of reactive oxygen species and is followed by tissue inflammation, vascular damage and a 'no-reflow' phenomenon.[5,16–20]

We have previously shown that FX06 protects against reperfusion injury in myocardial ischaemia and reperfusion.[7,11,13] In the current study, haemorrhagic shock followed by fluid resuscitation induced cardiac damage in pigs, as determined by TnT levels and compromised stroke volume index. Such damage could be due to myocardial ischaemia during the low flow phase and subsequent reperfusion. We observed less cardiac damage as demonstrated by surrogate parameters such as TnT, heart rate and stroke volume index in FX06-treated animals. FX06 was given at the time of fluid resuscitation, which strongly favours the concept that the peptide FX06 protected the myocardium by reducing reperfusion injury. Left ventricular dysfunction has been correlated with both an increased number of leukocytes infiltrating the myocardium[11] and elevated levels of plasma IL-6.[21] Both surrogate parameters were decreased in our previous experiments and correlated with improved outcome in FX06-treated animals compared to controls. It should be noted that the role of IL-6 in myocardial reperfusion injury is controversial. Administration of IL-6 did not increase myocardial infarct size[22], while IL-6/sIL-6R complex reduced myocardial damage.[22] However, there is evidence that IL-6

promotes leukocyte recruitment into tissue sites via induction of IL-8[23], thereby promoting tissue damage. Plasma IL-6 is an important surrogate marker for reduced tissue damage in other organs.[7,11–13] Higher IL-6 levels after haemorrhagic shock and trauma are associated with an increased gut barrier dysfunction and consequently translocation of endotoxin[24] which is consistent with our findings of high endotoxin blood levels 24 h after fluid resuscitation. IL-6 is also associated with a higher rate of multi-organ failure and mortality in male trauma patients.[25] We observed improvement in several additional pro-inflammatory cytokines (IL-1β, IL-2 and IL-4) and markers for organ function in FX06-treated animals compared to controls. In the early post-injury phase, higher IL-1β levels are associated with an increased mortality rate and risk for subsequent ARDS and MOF.[26]

Although we detect clear beneficial effects of FX06 on the pro-inflammatory cytokine pattern, we still do not know the underlying mechanism of action. However, this report provides a time-course of numerous markers in the pig helping to understand the condition of haemorrhagic shock followed by fluid resuscitation in more detail. The next step would be to analyse how FX06 reduces cytokine levels.

Haemorrhage acts globally by decreasing circulating blood volume, reducing cardiac output and tissue oxygen delivery, causing ischaemic injury in numerous organs. The subsequent fluid resuscitation causes 'iatrogenic' injury via reperfusion.[5,16–20] Several approaches have been attempted to improve the outcome following haemorrhagic shock and fluid resuscitation. Methods for volume resuscitation with crystalloids, blood, plasma, colloids and artificial haemoglobin solutions have been compared[27,28]. While all increase oxygen delivery and overall cardiovascular performance, they have little or no impact on the reduction of reperfusion injury. Approaches such as cooling[29] and drug treatment (e.g. recombinant IL-11, diaspirin cross-linked haemoglobin) have been shown in animal models to reduce reperfusion injury following haemorrhagic shock and fluid resuscitation[30–34], but so far none of these approaches have gained marketing authorisation to treat or prevent reperfusion injury following haemorrhagic shock and fluid resuscitation in man.

Of particular interest is the finding that we have observed a clear neurological deficit during the course of reperfusion underlining that haemorrhagic shock followed by fluid resuscitation reflects a "whole body" condition including the brain. This is further supported by the detection of high levels of endotoxin in the blood indicating a breakdown of gut-barrier function.

5. Conclusions

We have demonstrated that therapy with FX06 during reperfusion improves pulmonary, circulatory, gut–blood barrier and neurological functions in a pig model of haemorrhagic shock followed by fluid resuscitation. Besides the reduction of leukocyte infiltration in affected tissues, one of the key mechanisms of action seems to be the reduction of inflammatory cytokines after resuscitation. Further investigations are warranted to reveal the protective mechanism of FX06.

Conflict of interest

This study has been partially funded by a research grant from Fibrex Medical Research & Development GmbH. Peter Petzelbauer receives consultancy fees and owns shares in Fibrex Medical Inc. Kai Zacharowski receives consultancy fees and owns stock options in Fibrex Medical Inc.

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