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EXPERIMENTAL

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Carbamylated erythropoietin-FC fusion protein and recombinant human erythropoietin during porcine kidney ischemia/reperfusion injury

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Abstract *Purpose:* To test the hypothesis that a carbamylated EPO-FC fusion protein (cEPO-FC) or recombinant human erythropoietin (rhEPO) would protect against kidney ischemia/reperfusion (I/R) injury in pigs with atherosclerosis. *Methods:* Anesthetized and mechanically ventilated animals received cEPO-FC (50 μg kg^{-1}), rhEPO (5,000 IU kg^{-1}), or vehicle ($n = 9$ per group) prior to 120 min of aortic occlusion and over 4 h of reperfusion. During aortic occlusion, mean arterial pressure (MAP) was maintained at 80–120 % of baseline values by esmolol, nitroglycerin, and ATP. During reperfusion, noradrenaline was titrated to keep MAP at pre-ischemic levels. Blood creatinine and neutrophil gelatinase-associated lipocalin (NGAL) levels, creatinine clearance, fractional Na^+ excretion, and HE and PAS staining were used to assess kidney function and histological damage. Plasma interleukin-6, tumor necrosis factor-α, nitrate + nitrite and 8-isoprostane levels were measured to assess systemic inflammation, and nitrosative and oxidative stress. *Results:* I/R caused acute kidney injury with reduced creatinine clearance, increased fractional Na^+ excretion and NGAL levels, moderate to severe glomerular and tubular damage and apoptosis, systemic inflammation and oxidative and nitrosative stress, but there were no differences between the treatment groups. Pre-ischemia nitrate + nitrite and 8-isoprostanes levels were lower and higher, respectively, than in healthy animals of a previous study, and immune histochemistry showed higher endothelial nitric oxide synthase and lower EPO receptor

expression in pre-ischemia kidney biopsies than in biopsies from healthy animals. *Conclusions:* In swine with atherosclerosis, rhEPO and cEPO-FC failed to attenuate prolonged ischemia-induced kidney injury within an 8-h reperfusion period, possibly due to reduced EPO receptor expression resulting from pre-existing oxidative stress and/or reduced NO release.

Keywords Glomerular filtration · Tubular reabsorption · Creatinine clearance · Fractional Na^+ sodium excretion · Neutrophil gelatinase-associated lipocalin · HE staining · PAS staining · Apoptosis · Cytokines · 8-isoprostanes · Nitric oxide · Endothelial NO synthase · EPO receptor

Introduction

Aortic crossclamping during aneurysm repair frequently causes kidney ischemia/reperfusion (I/R) injury [1]. In rodent [2–8], large animal [9–12] and primate [13] models, recombinant human erythropoietin (rhEPO) has been shown to protect against kidney I/R injury. Clinical studies, however, have yielded controversial results [14, 15]. While the hematopoietic effects of rhEPO are through the activation of a homodimeric EPO receptor complex (EPO-R/EPO-R), the organ-protective properties are related to activation of a heterodimeric receptor complex consisting of the EPO-R and the common-β-receptor (EPO-R/β_cR) [16]. Stimulation of the latter has no undesired side effects, in contrast to EPO-R/EPO-R homodimer activation [17, 18].

Carbamylated EPO derivatives (cEPO) do not bind to the EPO-R/EPO-R homodimer, but are as cytoprotective as rhEPO [18, 19]. cEPO reduces kidney inflammation in brain dead rats [8], and a newly developed carbamylated EPO-FC fusion protein, consisting of two EPO molecules fused to the Fc part of IgG1 (cEPO-FC) [20], protects against spinal cord I/R injury [21]. All data on cEPO-related organ protection originate from studies involving young healthy animals. Patients undergoing aortic aneurysm repair, however, frequently present with impaired kidney function prior to surgery [22, 23], which in turn is associated with aggravated postoperative acute kidney injury [23, 24]. Therefore, we tested the hypothesis that rhEPO and cEPO-FC would protect against kidney I/R injury in swine with ubiquitous atherosclerosis [25, 26].

Materials and methods

Animals and materials

The University Animal Care Committee and the Federal authorities for animal research approved the experiments, which were performed in adherence to National Institutes of Health Guidelines on the Use of Laboratory Animals. Twenty pigs of either sex (age 13–20 months; body weight, median 68 kg, range 53–85 kg) were used. The pig strain is a cross-bread of Rapacz farm pigs homozygous for the R84C low density lipoprotein (LDL) receptor mutation with the smaller Chinese Meishan and French Bretoncelles strains ("FBM") [25, 26]. Genotypic testing was provided by the breeding institution (Claire Bal dit Sollier, Ludovic Drouet, Institut des Vaisseaux et du Sang, Hôpital Lariboisière, Paris, and Michel Bonneau, Institut National de Recherche Agronomique, Jouy-en-Josas, France). All animals had received an atherogenic diet (1 kg daily, 1.5 % cholesterol, 20 % bacon fat) resulting in hypercholesterolemia (median 11.08 mmol L^{-1}, range 7.39–12.31 mmol L^{-1} vs. 1.41 mmol L^{-1}, 1.35–1.53 mmol L^{-1}, in 15 healthy German Landswine of the same age; $p < 0.001$). cEPO-FC and rhEPO for parenteral injection was produced by Polymun Scientific GmbH, Klosterneuburg, Austria [20, 21].

Anesthesia and surgical preparation

After induction of anesthesia (propofol 2–3 mg kg^{-1}, ketamine 1–2 mg kg^{-1}) and endotracheal intubation, anesthesia was maintained with pentobarbitone (8 mg kg^{-1} h^{-1}) and buprenorphine (30 μg kg^{-1} every 8 h and prior to surgical stimuli) together with muscle relaxation (pancuronium 0.1 mg kg^{-1} h^{-1}). Animals were mechanically ventilated (FiO_2 0.35, tidal volume 8 mL kg^{-1}, PEEP 10 cmH_2O, inspiratory/expiratory time ratio 1:1.5, respiratory rate 13–15 min^{-1} adjusted to maintain an arterial pCO_2 of 35–45 mmHg). Ventilator settings were used because swine are particularly susceptible to atelectasis formation in dependent lung regions due to the lack of alveolar collateral ventilation [27]. Catheters were placed in the arteria carotis dextra for the measurement of blood pressure in the upper body half ($MAP_{proximal}$), transpulmonary single indicator thermodilution-cardiac output (CO) and global end-diastolic volume (GEDV), a marker of cardiac preload, and in the vena jugularis dextra for central venous pressure (CVP) measurement and drug infusion [21, 27]. Catheter sheaths were introduced into both arteriae femorales for distal blood pressure recording (MAP_{distal}) and placement of an inflatable balloon catheter, respectively, and into the vena femoralis dextra. Intra-aortic balloon occlusion was used to avoid mechanical injury from clamp placement [27]. The right kidney was surgically exposed, and a

precalibrated ultrasound flow probe was positioned around the arteria renalis dextra [27]. An aortic balloon catheter was positioned under manual control so that balloon inflation allowed simultaneous occlusion of the orifice of both arteriae renales. Another catheter was advanced into the vena cava inferior and manually guided into a vena renalis dextra under visual control [27]. A catheter was placed in the bladder for urine sampling. At the end of kidney instrumentation, a tissue biopsy was taken for histopathological evaluation and immune histochemistry.

Measurements and calculations

Hemodynamic parameters recorded were: heart rate, $MAP_{proximal}$, MAP_{distal}, CVP, CO, GEDV, and renal blood flow. Arterial and renal venous blood samples were analyzed for blood gases, acid–base status, K^+, hemoglobin content and O_2 saturation. Arterial interleukin-6 and tumor necrosis factor-α concentrations were measured using commercially available species-specific ELISA kits [21, 27]. Arterial blood 8-isoprostane and nitrite/nitrate ($NO_2^- + NO_3^-$) concentrations were measured using a commercially available test kit and a chemiluminescence technique, respectively [21, 27]. Urine was sampled during the 2 h before aortic occlusion and during the 8-h reperfusion period. Urinary and blood creatinine and Na^+ levels were determined to calculate creatinine clearance and fractional Na^+ excretion [27] together with blood neutrophil gelatinase-associated lipocalin (NGAL) [28] using a commercially available ELISA kit (Pig NGAL ELISA kit; BioPorto Diagnostics, Gentofte, Denmark).

At the end of the experiment, immediate post mortem samples of kidney were analyzed for the expression of endothelial constitutive and inducible nitric oxide synthase isoforms (eNOS, iNOS), heme oxygenase-1 (HO-1), Bcl-xL and cleaved caspase-3 and for the activation of the nuclear transcription factor κB (NF-κB) [27]. Tissue samples were homogenized and suspended in lysis buffer, protein concentration was determined, and equal total protein aliquots were separated by SDS-PAGE and transferred by western blotting. After blocking, the membranes were incubated with commercially available primary rabbit anti-cleaved caspase-3, anti-iNOS, anti eNOS, and anti-Bcl-xL and mouse anti-HO-1 antibodies. The primary antibodies were detected using goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies. The membranes were subjected to chemiluminescence using the SuperSignal West Femto chemiluminescent substrate. Exposed films were scanned, and the intensity of immunoreactivity was measured using NIH ImageJ software (http://rsb.info.nih.gov/nih-image). NF-κB activation was determined using an electrophoretic mobility shift assay: kidney lysates (10 μg) were incubated with 0.1 μg/μl poly-dI-dC and 50,000 cpm ^{32}P-labeled double-stranded oligonucleotide containing the NF-κB (HIVκB-site). Complexes were separated in polyacrylamide gels, which were subsequently dried and exposed to X-ray films. A phosphorimager and image analyzer software (AIDA Image Analyzer, Raytest) was used to quantify the labeled NF-κB by autoradiography.

Pyramid-shaped kidney specimens showing kidney cortex, medulla, renal papilla and the corresponding renal calyx were dissected for histopathological examination performed by an experienced pathologist (A.S.) blinded to the sample grouping. Tissues were fixed in paraformaldehyde, paraffin sections were stained with hematoxylin and eosin and periodic acid-Schiff stain, and photomicrographs of three random sampling areas were acquired from each section for determination of signs of tubular and glomerular damage. Histopathological alterations at the glomerular level were analyzed for the degree of "glomerular tubularization", i.e. herniation of proximal tubular epithelial cells into Bowman's capsule along the parietal surface of the capsule [27], dilatation of Bowman's space, and swelling of Bowman's capsule. Data are expressed as the percentage of glomeruli showing the pathological finding in relation to all glomeruli analyzed (30 glomeruli each of the outer cortical and the medullary region) of the three random sections. Tubular histopathological damage was scored from 0 to 4 for cellular edema of the proximal tubule (0 no damage, 1 single cell damage, 2 edema of some adjacent cells, 3 edema of complete single tubules, 4 edema of the whole tubular epithelium and focal separation of cells from the basal membrane), distal tubular dilatation and elongation (0 normal tubuli, 1 single tubular dilatation, 2 up to 25 % of tubules dilated, 3 up to 50 % of tubules dilated, 4 > 50 % of tubules dilated), and tubular necrosis (0 no necrosis/apoptotic event, 1 single-cell apoptosis, 2 single-cell necrosis and desquamation, 3 patchy necrosis of adjacent cells, 4 complete necrosis of the tubular epithelium or complete necrosis of single tubules). Typical examples of glomerular and tubular histopathological alterations are shown in Fig. 1.

The expression of eNOS in vessels and EPO-R were determined on formalin-fixed paraffin-embedded sections of kidney from FBM and young healthy German Landswine using immune histochemistry. Sections were dewaxed in xylene, rehydrated with a graded series of ethanol solutions, incubated in citrate buffer and brought to boiling twice for heat-induced antigen retrieval, and blocked with normal goat serum before incubating with mouse anti-eNOS (Becton Dickinson) and rabbit anti-Epo-R 1:50 (Santa Cruz Biotechnology). Primary antibodies were detected using the APAAP method and visualized with a red chromogen (Dako APAAP REAL^{TM}; Dako Corporation, Carpinteria, CA) followed by counterstaining with hematoxylin. Slides were visualized using a Zeiss Axio Imager A1 microscope (EC

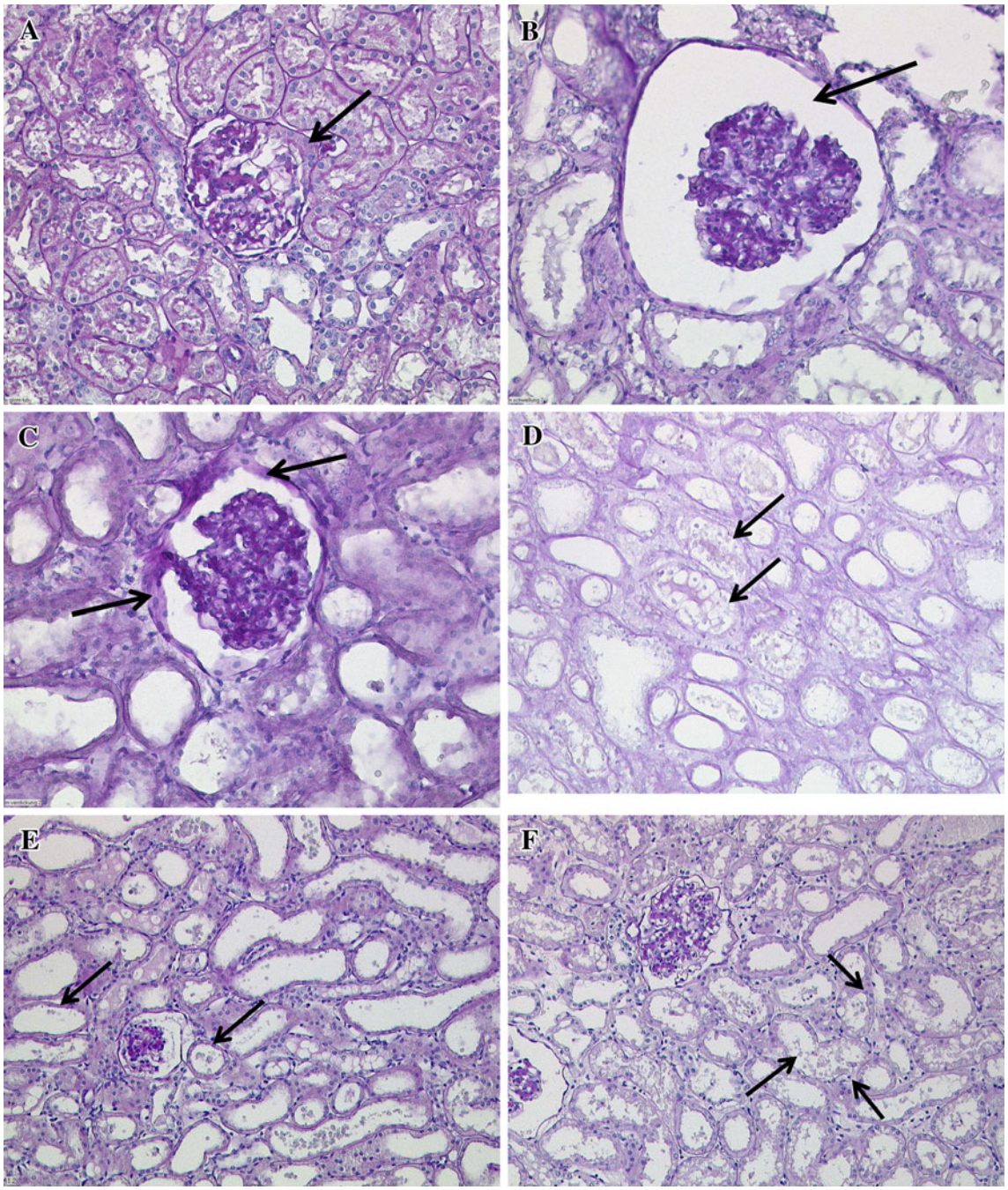


Fig. 1 Tissue sections showing "glomerular tubularization" (PAS staining, ×40): **a** herniation of proximal tubular epithelial cells into Bowman's capsule along the luminal surface of the capsule, **b** dilatation of Bowman's space, **c** swelling of Bowman's capsule, **d** tubular cytoplasmatic edema, **e** tubular dilatation and elongation, **f** tubular necrosis. *Arrows* specified histopathological feature

Plan-NEOFLUAR). The results are presented as mean densitometric sum red.

Experimental protocol

Room temperature was kept at 24–26 °C. Animals randomly received either rhEPO (body weight. median 74 kg, range 56–80 kg; six males, no females), cEPO-FC (76 kg, 56–85 kg; six males, one female) or vehicle (57 kg, 53–72 kg; six males, one female). Two doses of rhEPO (5,000 IU kg^{-1} to 50 μg kg^{-1}) or cEPO-FC (50 μg kg^{-1}) were infused over 30 min immediately before aortic occlusion, and during the first 4 h of reperfusion. The identical protocol previously allowed

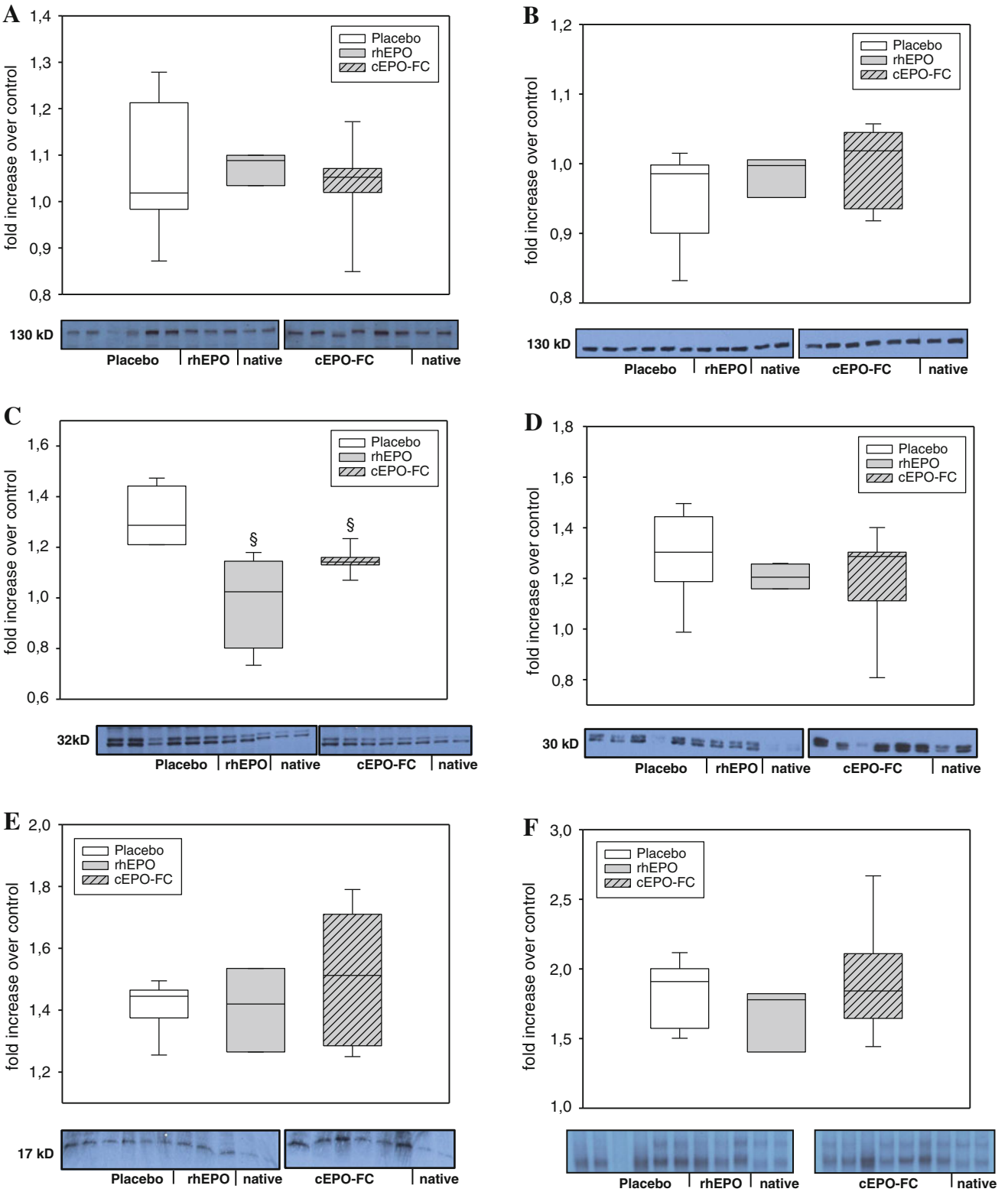


Fig. 2 Representative immune blots and gel shifts and as well as quantitative analysis of the tissue expression of the eNOS (**a**), iNOS (**b**), HO-1 (**c**), markers of apoptosis Bcl-xL (**d**) and cleaved caspase-3 (**e**), and activation of NF-κB (**f**) in immediate post mortem (after 8 h of reperfusion) samples of kidney in the control (*open boxes*, $n = 7$), rhEPO (*gray boxes*, $n = 6$), and cEPO-FC (*hatched gray boxes*, $n = 7$) groups. The data presented are medians, quartiles and ranges, and are the fold increases in relation to values from animals which had only undergone surgical instrumentation (*native*)

Table 1 Systemic hemodynamic, gas exchange, and acid–base status

Parameter	Group	Before aortic occlusion	4 h reperfusion	8 h reperfusion
Core temperature (°C)	Control	33.8 (32.3–36.0)	32.8 (31.1–35.3)	33.1 (30.9–36.4)
	rhEPO	33.5 (33.0–34.8)	32.6 (31.1–34.6)	32.1 (30.8–34.6)
	cEPO-FC	33.7 (33.1–36.9)	32.4 (31.7–35.0)	32.3 (31.2–34.8)
Hemoglobin (g L^{-1})	Control	83 (68–91)	105 (76–162)*	95 (21–185)
	rhEPO	85 (74–105)	105 (74–119)	92 (80–120)
	cEPO-FC	81 (71–89)	118 (89–148)*	105 (87–135)
Heart rate (beats min^{-1})	Control	92 (79–113)	138 (121–168)*	145 (78–178)*
	rhEPO	100 (85–115)	109 (74–147)**	97 (69–142)
	cEPO-FC	103 (83–128)	112 (82–134)**	123 (72–140)
Mean arterial pressure (mmHg)	Control	90 (78–126)	82 (69–99)	72 (63–105)
	rhEPO	98 (88–105)	102 (67–122)	94 (63–112)
	cEPO-FC	93 (68–105)	84 (75–97)	85 (71–104)
Central venous pressure (mmHg)	Control	11 (7–13)	11 (6–15)	12 (8–18)
	rhEPO	12 (8–15)	13 (9–16)	13 (8–16)
	cEPO-FC	12 (10–14)	13 (11–16)	14 (12–16)
Cardiac index (mL kg^{-1} min^{-1})	Control	67 (57–121)	85 (51–132)	56 (39–117)
	rhEPO	70 (62–115)	80 (35–104)	71 (33–93)
	cEPO-FC	75 (68–106)	89 (59–106)	90 (62–112)
Stroke volume (mL kg^{-1})	Control	50 (36–72)	37 (20–41)	33 (15–35)*
	rhEPO	53 (39–74)	56 (19–72)	58 (19–66)
	cEPO-FC	53 (47–67)	59 (34–67)**	59 (34–68)**
Intrathoracic blood volume (mL)	Control	603 (508–888)	645 (425–789)	542 (351–891)
	rhEPO	783 (496–950)	792 (557–1,048)	752 (480–1,106)
	cEPO-FC	797 (687–1,098)	772 (530–1,015)	802 (517–1,166)
Arterial PO_2 (mmHg)	Control	159 (142–206)	193 (124–248)	177 (127–243)
	rhEPO	172 (157–217)	158 (113–236)	152 (108–236)
	cEPO-FC	171 (135–231)	64 (121–246)	169 (114–245)
Arterial PCO_2 (mmHg)	Control	37 (35–38)	36 (31–42)	36 (26–44)
	rhEPO	37 (32–40)	37 (35–39)	35 (33–41)
	cEPO-FC	36 (33–41)	36 (35–43)	36 (34–38)
Arterial pH	Control	7.44 (7.39–7.50)	7.04 (6.92–7.38)*	6.95 (6.83–7.35)*
	rhEPO	7.43 (7.31–7.47)	7.31 (6.92–7.40)*	7.30 (6.78–7.38)*
	cEPO-FC	7.43 (7.39–7.45)	7.20 (6.95–7.33)*	7.23 (7.30–6.92)*
Arterial base excess (mmol L^{-1})	Control	−0.1 (−10.1–2.3)	−18.0 (−23.8 to −2.8)*	−22.0 (−26.2 to −5.6)*
	rhEPO	0.6 (−5.2–1.6)	−7.7 (−22.2 to −1.4)*	−9.4 (−25.5 to −3.5)*
	cEPO-FC	−0.6 (−2.6–1.8)	−12.8 (−20.6 to −6.6)*	−10.7 (−22.6–7.8)*
Na (mmol L^{-1})	Control	144 (141–146)	145 (131–151)	146 (134–152)
	rhEPO	144 (143–145)	143 (139–146)	144 (143–149)
	cEPO-FC	143 (141–147)	140 (136–144)	144 (138–148)
K (mmol L^{-1})	Control	3.3 (3.0–3.5)	4.1 (3.3–5.5)*	5.2 (3.8–6.2)*
	rhEPO	3.4 (2.9–3.6)	4.4 (4.1–5.3)*	4.3 (3.8–5.7)*
	cEPO-FC	3.3 (3.0–3.5)	4.2 (3.8–6.1)*	4.3 (3.9–6.1)*

All data are median (range); control $n = 7$, cEPO-FC $n = 7$ and rhEPO $n = 6$
* $p < 0.05$ versus before aortic occlusion, ** $p < 0.05$ versus control animals

attenuation of ischemic spinal cord damage [21]. The cEPO-FC molecule used is a fusion protein comprising two rhEPO molecules connected to the Fc domain of a human antibody IgG1 [20]. The whole complex is carbamylated until no erythropoietic potency remains. The same amount of protein was administered to the cEPO-FC and rhEPO groups. Taking into account the steric molecular structure and the molecular weight of cEPO-FC, the number of EPO subunits administered to the cEPO-FC group was approximately 44 % of that administered to the rhEPO group. Baseline data were collected, and immediately after the first rhEPO, cEPO-FC or vehicle administration, animals underwent 120 min of aortic occlusion by inflation of the balloon catheter until cessation of renal blood flow and disappearance of the MAP_{distal} trace. We chose to study this ischemia period because in two pilot experiments, 90 min of kidney ischemia only moderately increased creatinine blood levels (from 87 and 106 to 101 and 113 μmol L^{-1}, respectively), and was associated with only minor histological damage (glomerular tubularization 5 and 6 %, respectively, some minor widening of Bowman's capsule, but no tubular cell death at all). Animals received 10 and 20 mL kg^{-1} h^{-1} of lactated Ringer's solution prior to and during reperfusion, respectively, to ensure constant fluid administration. Cardiac preload was maintained at comparable central venous pressures prior to the reperfusion by infusing 30 mL kg^{-1} h^{-1} of hydroxyethyl starch

Table 2 Renal hemodynamic, oxygen exchange, gas exchange, and function

Parameter	Group	Before aortic occlusion	4 h reperfusion	8 h reperfusion
Right kidney blood flow (mL min^{-1})	Control	265 (38–303)	83 (70–306)	67 (37–263)
	rhEPO	169 (55–293)	138 (57–260)	115 (10–255)
	cEPO-FC	190 (153–429)	151 (55–325)	146 (95–325)
Renal venous pO_2 (mmHg)	Control	55 (35–57)	59 (47–80)	52 (31–68)
	rhEPO	49 (44–73)	73 (47–77)*	60 (46–75)*
	cEPO-FC	47 (41–51)	66 (56–77)*	70 (57–82)*
Renal venous pH	Control	7.43 (7.38–7.50)	7.06 (6.91–7.37)*	7.00 (6.75–7.35)*
	rhEPO	7.43 (7.27–7.46)	7.30 (6.90–7.40)*	7.27 (6.49–7.37)*
	cEPO-FC	7.42 (7.40–7.45)	7.21 (6.95–7.33)*	7.25 (6.92–7.30)*
Renal venous base excess (mmol L^{-1})	Control	2.3 (−1.1–4.5)	−15.8 (−22.1 to −1.7)*	−17.7 (−26.1 to −3.7)*
	rhEPO	1.7 (−5.9–3.2)	−7.2 (−20.2 to −0.8)*	−9.2 (−28.0 to −4.0)*
	cEPO-FC	1.3 (0.1–2.9)	−10.4 (−18.6 to −5.3)*	−10.3 (−20.3 to −7.1)*
Plasma creatinine (μmol L^{-1})	Control	101 (91–115)	152 (131–214)*	169 (135–230)*
	rhEPO	104 (100–128)	158 (145–182)*	169 (146–217)*
	cEPO-FC	112 (98–116)	152 (122–224)*	150 (119–244)*
Urine flow (mL min^{-1})	Control	3.2 (1.3–7.5)	2.5 (1.0–12.7)	
	rhEPO	3.5 (1.2–9.0)	7.9 (2.9–13.3)	
	cEPO-FC	2.9 (1.4–4.1)	7.9 (1.1–17.4)	
Creatinine clearance (mL min^{-1})	Control	77 (14–124)	7 (2–28)*	
	rhEPO	81 (40 –109)	13 (4–22)*	
	cEPO-FC	72 (61–123)	25 (2–34)*	
Fractional Na excretion (%)	Control	5 (1–6)	37 (17–51)*	
	rhEPO	4 (1–9)	53 (45–67)*	
	cEPO-FC	2 (1–6)	39 (17–66)*	
NGAL (ng mL^{-1})	Control	10 (4–31)	n.d.	551 (284–1,503)*
	rhEPO	11 (3–250)	n.d.	463 (161–679)*
	cEPO-FC	15 (9–19)	n.d.	551 (88–1,189)*

The data for urine flow, creatinine clearance and fractional Na^+ excretion refer to the 2 h before aortic occlusion and the 8 h of reperfusion, respectively. All data are median (range); control $n = 7$, cEPO-FC $n = 7$ and rhEPO $n = 6$

NGAL Neutrophil gelatinase associated lipocalin, *n.d.* not determined

* $p < 0.05$ versus before aortic occlusion

Table 3 Inflammation, oxidative and nitrosative stress in the blood

Parameter	Group	Before aortic occlusion	4 h reperfusion	8 h reperfusion
Tumor necrosis factor-α (pg mL^{-1})	Control	15 (11–36)	72 (60–116)*	59 (24–101)*
	rhEPO	23 (10–37)	89 (50–158)*	68 (48–181)*
	cEPO-FC	16 (10–30)	130 (50–167)*	118 (29–200)*
Interleukin-6 (pg mL^{-1})	Control	0 (0–80)	1,200 (115–5,174)*	1,431 (117–3,912)*
	rhEPO	0 (0–391)	343 (100–1,926)*	306 (61–7,424)*
	cEPO-FC	0 (0–44)	1,073 (96–3,818)*	1,961 (56–9,920)*
Nitrate + nitrite (μmol L^{-1})	Control	5 (1–9)	25 (12–76)*	19 (9–51)*
	rhEPO	7 (3–12)	15 (7–64)*	13 (4–51)*
	cEPO-FC	4 (2–11)	14 (8–43)*	11 (6–32)*
8-Isoprostane (pg mL^{-1})	Control	98 (77–233)	410 (110–1,379)*	280 (83–924)*
	rhEPO	130 (66–169)	119 (79–550)*	138 (62–599)*
	cEPO-FC	102 (58–156)	189 (91–866)*	161 (97–435)*

All data are median (range); control $n = 7$, cEPO-FC $n = 7$ and rhEPO $n = 6$

* $p < 0.05$ versus before aortic occlusion

during the aortic occlusion. In order to control blood pressure and based on our previous studies [10, 21, 27], nitroglycerin (1.7 mg min^{-1}), esmolol (16.5 mg min^{-1}) and ATP (2–10 mg min^{-1}) were infused to maintain $MAP_{proximal}$ at 80–120 % of the baseline value during aortic balloon occlusion. During reperfusion, noradrenaline was titrated to keep MAP_{distal} at the value before aortic occlusion [10, 21, 27] or until a maximum heart rate of 160 beats min^{-1} had been reached, the latter in order to avoid tachycardia-induced heart ischemia. Additional data were collected at 4 and 8 h of reperfusion. The animals were then killed under deep anesthesia by administration of 20–30 mg kg^{-1} Na-pentobarbitone and 30 mL KCl.

Table 4 Histopathological analysis of immediate post mortem samples of kidney

Parameter	Group	Before ischemia	8 h reperfusion
Glomerular tubularization	Control	3 (0–13)	2 (0–20)
	rhEPO	3 (0–17)	3 (0–15)
	cEPO-FC	0 (0–10)	3 (0–7)
Dilatation of Bowman's space	Control	0 (0–10)	42 (13–93)*
	rhEPO	0 (0–13)	45 (15–77)*
	cEPO-FC	0 (0–7)	40 (15–72)*
Swelling of Bowman's capsular cells	Control	3 (0–17)	12 (7–42)*
	rhEPO	3 (0–10)	15 (3–33)*
	cEPO-FC	2 (3–17)	17 (5–25)*
Tubular cell edema	Control	1.2 (1.0–2.2)	1.2 (1.0–1.6)
	rhEPO	1.0 (1.0–1.1)	1.0 (1.0–1.6)
	cEPO-FC	1.2 (1.0–1.6)	1.1 (1.0–1.3)
Tubular dilatation/elongation	Control	1.0 (1.0–1.0)	1.5 (1.0–3.4)*
	rhEPO	1.0 (1.0–1.0)	2.3 (1.4–3.2)*
	cEPO-FC	1.0 (1.0–1.0)	1.9 (1.4–3.0)*
Tubular cell death	Control	1.1 (1.0–1.3)	1.3 (1.1–2.7)*
	rhEPO	1.0 (1.0–1.3)	1.2 (1.0–1.3)*
	cEPO-FC	1.0 (1.0–1.3)	1.5 (1.1–1.7)*

Glomerular pathology parameters (glomerular tubularization, dilatation of Bowman's space, swelling of Bowman's capsule) are expressed as percentages of all glomerulae analyzed; tubular pathology parameters (cell edema, dilatation/elongation, cell death) are expressed as a score ranging from 0 (no alteration) to 4 (near complete tubular damage; for details see text). All data are median (range); control $n = 7$, cEPO-FC $n = 7$ and rhEPO $n = 6$

* $p < 0.05$ versus before aortic occlusion

Statistical analysis

All data are presented as median (range). After exclusion of a normal distribution using the Kolmogorov–Smirnov test, within-group data were analyzed by Friedman repeated measures analysis of variance on ranks and a subsequent post hoc multiple comparison procedure (Dunn's method). Differences between groups at identical time points were analyzed with a one-way Kruskal–Wallis analysis of variance on ranks followed by a post hoc Dunn test. Because of the multiple statistical testing resulting from the numerous variables measured, all results have to be interpreted in an exploratory rather than a confirmatory manner.

Results

Table 1 presents data on systemic hemodynamics, gas exchange, acid–base status, and electrolytes. While heart rates were higher in the control group at 4 h of reperfusion, most likely due to the higher noradrenaline infusion rates required to achieve the hemodynamic targets (vehicle group 1.3 μg kg^{-1} min^{-1}, 0.4–17.8 μg kg^{-1} min^{-1}; cEPO-FC group 0.4 μg kg^{-1} min^{-1}, 0.1–2.2 μg kg^{-1} min^{-1}; rhEPO group 0.9 μg kg^{-1} min^{-1}, 0.3–2.6 μg kg^{-1} min^{-1}; $p = 0.063$), stroke volumes were higher in the cEPO-FC group. None of the other parameters showed any significant differences between groups.

Table 2 presents data on kidney blood flow, O_2 exchange, metabolism, and organ function. All animals developed acute kidney injury stage II or III (acute rise in plasma creatinine >0.3 and >0.5 mg dL^{-1}) and organ injury or failure (fall in creatinine clearance >50 % and >75 %) according to the AKIN and RIFLE criteria [29], respectively. Accordingly, both fractional Na^+ excretion and blood NGAL levels showed a several-fold increase. However, there were no significant differences between groups.

The data presented in Table 3 and Fig. 2 demonstrate that kidney I/R caused systemic inflammation, oxidative and nitrosative stress, and tissue apoptosis. Except for lower HO-1 expression in the two treatment groups, there were no significant differences between groups.

The histopathological analyses (Tables 3 and 4) confirmed the findings on kidney function: I/R injury caused moderate (maximum score 3) glomerular and tubular damage, again with no significant differences among the groups. Of note, some degree of histological damage was already present in the biopsies taken prior to organ ischemia.

Figures 3 and 4 show examples of the immune histochemical detection of renal vascular eNOS (Fig. 3a) and EPO-R (Fig. 4a) in pre-ischemia biopsies in comparison to biopsies taken at the same time point during our previous study in young healthy German Landswine [27]. Quantitative image analysis showed that eNOS expression (Fig. 3b) was tenfold higher and EPO-R expression (Fig. 4b) 50-fold lower in the FBM swine.

Discussion

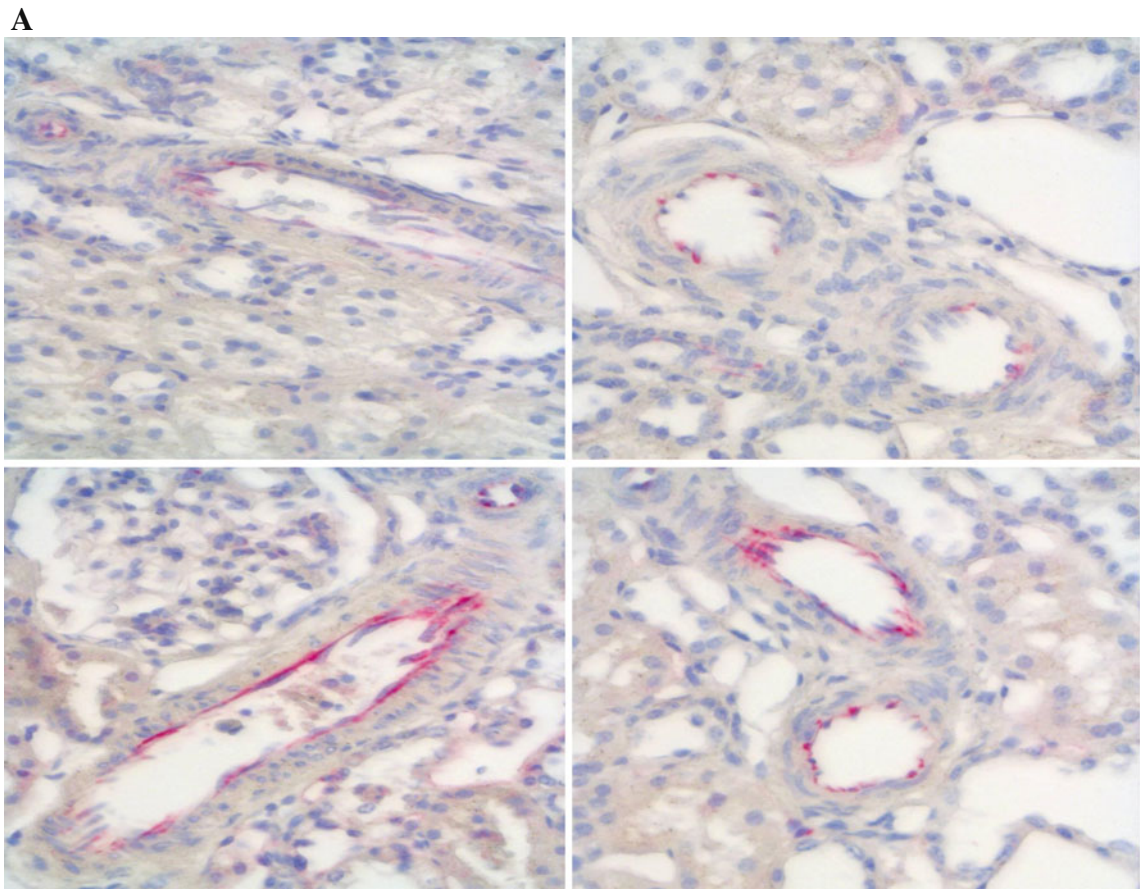
This purpose of this study was to test the hypothesis that the newly developed cEPO-FC and rhEPO, which protect against spinal cord I/R injury to comparable extents, would also attenuate kidney I/R injury. To mimic the clinical scenario of patients with atherosclerosis, who frequently present with impaired kidney function prior to surgery [22, 23], we studied swine with ubiquitous atherosclerosis [25, 26]. The major finding was that (1) both cEPO-FC and rhEPO failed to attenuate I/R injury-induced organ dysfunction and histological damage, which (2) coincided with unchanged parameters of inflammation, oxidative and nitrosative stress.

Noradrenaline requirements needed to achieve the hemodynamic targets during reperfusion were lower in the cEPO-FC group, which coincided with a higher stroke volume despite unchanged central venous and mean arterial pressure. This finding of improved heart function agrees with the findings of previous studies of myocardial I/R injury in rodents, which demonstrated that cEPO reduces infarct area and increases left heart contractility [30–32], underscoring the kidney–heart crosstalk during renal I/R injury [33]. Interestingly, rhEPO did not affect the catecholamine response, which is in contrast to the findings of other studies showing a vasopressor effect of rhEPO [17]. The underlying comorbidity may have assumed importance in this context: in young healthy swine, rhEPO enhances the response to catecholamine infusion [10, 34].

Neither cEPO-FC nor rhEPO prevented kidney dysfunction or organ damage, which is in contrast to the findings of previous studies in swine [9–12] and primates [13]. All these studies used young healthy animals, whereas our FBM swine already showed reduced creatinine clearance before aortic occlusion (baseline value of all groups pooled 72 ± 23 vs. 97 ± 26 mL min^{-1} in the German Landswine investigated previously [27], $p = 0.004$). The lower creatinine clearance values coincided to some degree with histological damage seen in the biopsies taken prior to ischemia. In addition, EPO-R expression was markedly attenuated in renal biopsies taken during surgical instrumentation when compared to similar biopsies in our previous study [27]. We can only speculate as to whether the moderate depression of glomerular filtration and the histological damage that were already present prior to aortic occlusion had any effect on EPO-R expression since EPO-R downregulation has been proposed as a putative mechanism of EPO resistance in patients with heart and kidney failure [35, 36]. EPO resistance in patients with end-stage renal disease is related to inflammation and increased oxidative stress [37]. In our FBM swine, baseline isoprostane levels were higher than in the young healthy German Landswine studied previously [27] (111 ± 47 vs. 74 ± 16 pg mL^{-1}, $p = 0.005$), indicating pre-existing oxidative stress. A reduced rate of endogenous NO production in the FBM swine as indicated by the lower nitrate + nitrite baseline levels (14 ± 36 vs. 77 ± 80 μmol L^{-1}, $p < 0.001$) may also have contributed to the low efficacy of rhEPO and cEPO-FC treatment because it has been shown in rats with heminephrectomy-induced polycythemia that EPO combined with nonselective NOS inhibition with L-NAME causes more severe arterial hypertension and only partially attenuates impairment of glomerular filtration rate [38]. In mice lacking constitutive endothelial NOS, EPO even worsens remodeling after vascular injury [39].

Interestingly, in contrast to other studies showing decreased eNOS expression in kidney arteries of hypercholesterolemic swine [40], renal vascular eNOS expression was higher in our FBM swine than in the German Landswine studied previously. The previous authors studied a short period (5–8 weeks) of high-cholesterol feeding and found a moderate rise in blood cholesterol levels (from 1.6 to 5.0 mmol L^{-1}). Longer high-cholesterol feeding (over 12 weeks) resulting in more pronounced hypercholesterolemia (8.4–9.7 mmol L^{-1}) was associated with unchanged renal artery eNOS expression [41, 42]. Nevertheless, the lower nitrate + nitrite and the higher isoprostane levels agree well with the "two faces" [43] attributed to eNOS during hypercholesterolemia, in which activation of eNOS leads to "uncoupling of NOS" with reduced NO release and aggravated oxidative stress resulting from superoxide radical formation [43].

It could be argued that 120 min of kidney ischemia rendered organ injury irreversible [44], since other authors have reported that ischemia beyond 90 min causes permanent organ dysfunction [45–48]. However, 180 min of ischemia is required for major histological damage [49], and all the above-mentioned studies used complete hilar, i.e. arterial and venous, clamping. Arterial clamping alone during open abdominal surgery, i.e. without increasing intra-abdominal pressure and thus similar to the aortic balloon occlusion in our experiments, causes a 50 % smaller increase in creatinine levels and is ultimately associated with complete recovery of organ function [50]. Moreover, all these studies were performed during normothermia. Hypothermia attenuates organ dysfunction and histological damage after 120–180 min of ischemia [51–55]. During the surgical instrumentation, all animals developed hypothermia unresponsive to external heating (baseline core temperature 33.8 ± 1.1 °C), which most likely improved ischemia tolerance since in normothermic (baseline core temperature 37.2 ± 1.0 °C [27]) German Landswine studied previously, 90 min of kidney ischemia only, which was responsive to Na_2S treatment, was associated with a virtually identical creatinine clearance (12 ± 12 vs. 14 ± 10 mL min^{-1}) and even higher creatinine blood



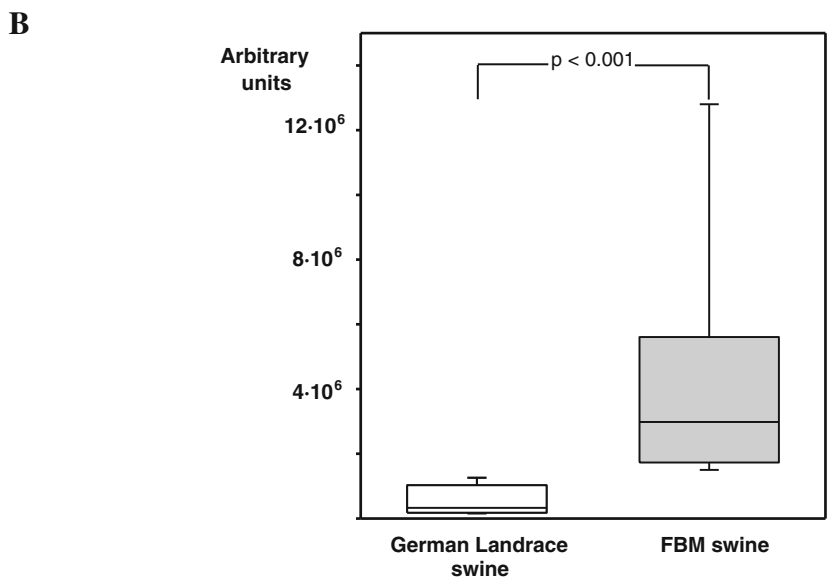
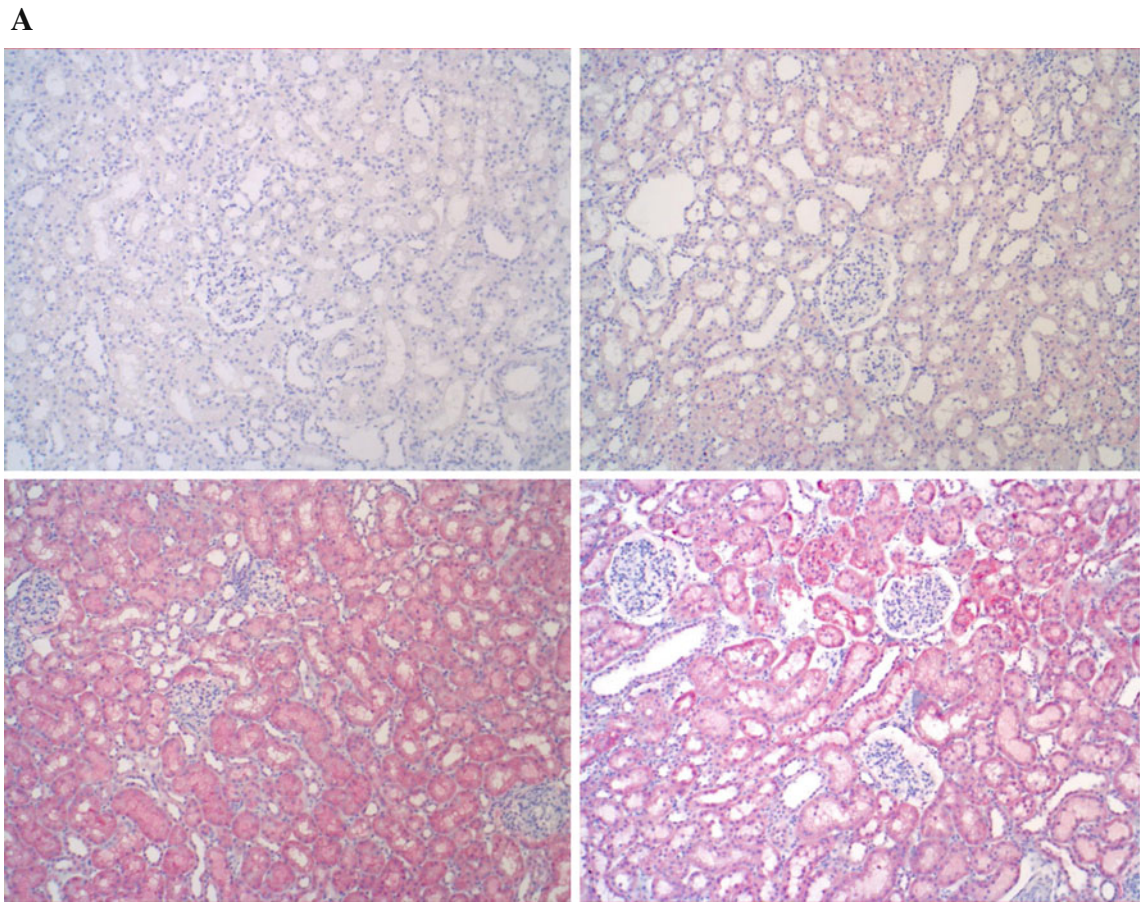


Fig. 3 **a** Renal vascular eNOS expression in pre-ischemia biopsies taken during surgical instrumentation (*lower panel*) in comparison to biopsies taken in young healthy German Landswine (*upper panel*) undergoing a similar surgical instrumentation for subsequent thoracic aortic occlusion-induced kidney I/R injury (×40). **b** Results of quantitative image analysis of eNOS in pre-ischemia biopsies taken during surgical instrumentation in seven FBM swine and seven German Landswine. All data are medians (quartiles, range)



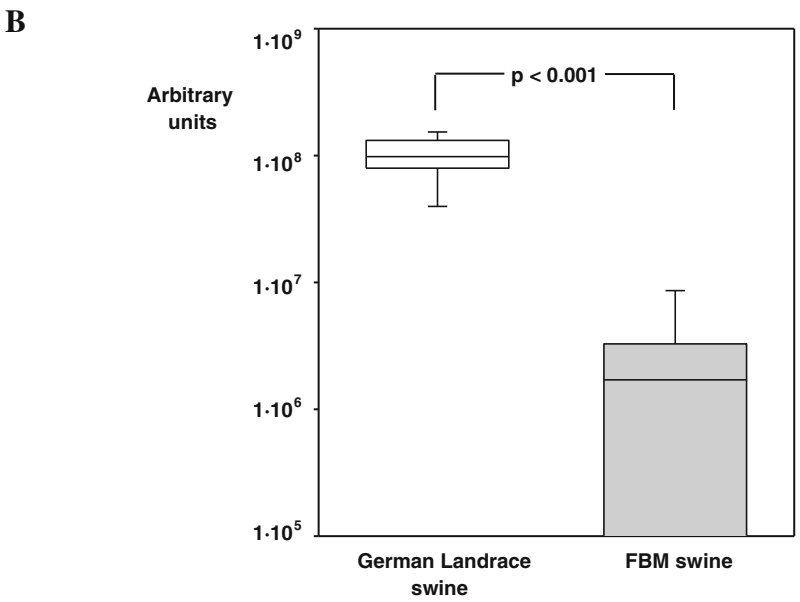


Fig. 4 **a** EPO-R expression in pre-ischemia biopsies taken during surgical instrumentation (*upper panel*) in comparison to biopsies taken in young healthy German Landswine (*lower panel*) undergoing a similar surgical instrumentation for subsequent thoracic aortic occlusion-induced kidney I/R injury (×20). **b** Results of quantitative image analysis of EPO-R in pre-ischemia biopsies taken during surgical instrumentation in ten FBM swine and eight German Landswine. All data are medians (quartiles, range)

levels (230 ± 48 vs. 170 ± 33 μmol L^{-1}, $p < 0.001$) [27]. In a further study in eight German Landswine, 90 min of normothermic ischemia caused the same histological damage as in the present experiments (glomerular tubularization 4 %, range 0–13 %, tubular cell death score 1.3, range 0.5–2.5, vs. 3, range 0–20 %, and 1.4, range 1.0–2.4, respectively). Reducing core temperature by 2–4 °C attenuates kidney damage induced by complete ischemia [56–59] or hemorrhagic shock [60]. Nonetheless, we must be cautious in drawing any definite conclusion as to the reversibility of kidney injury in our model: (1) up to now, there are no data available on the effects of EPO after comparably long ischemia periods, or with shorter ischemia in animals with pre-existing kidney disease, and (2) we may have missed beneficial effects on kidney function due to the short reperfusion period (in previous studies serum creatinine and creatinine clearance reached their peak and nadir values, respectively, at 12–72 h [9, 45–48, 50]). The impact of the duration of reperfusion is supported by the only moderate histological damage. Other authors have reported more pronounced overall damage (e.g. multifocal interstitial nephritis [49], severe tubular necrosis [49, 61]), but histological evaluation was performed at 18 h [61] to 2 weeks [49] after kidney ischemia.

In summary, neither rhEPO nor cEPO-FC protected against kidney I/R injury in FBM swine with ubiquitous atherosclerosis due to familial hypercholesterolemia and an atherogenic diet. This finding is in contrast to those of other preclinical studies and most likely results from the decreased tissue EPO-R expression when compared to young healthy animals. Furthermore, FBM swine already showed reduced creatinine clearance and some degree of histological damage prior to aortic occlusion, which mimics the clinical scenario of patients with atherosclerosis, who also frequently present with impaired kidney function prior to the surgical intervention. Further investigation as to whether pre-existing impairment of kidney function and/or decreased tissue EPO-R expression explains the controversial effects of rhEPO in clinical trials is warranted.

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Conflicts of interest Brigitta Vcelar is responsible for preclinical research at and is an employee of Polymun Scientific GmbH (Klosterneuburg, Austria), a company involved in the commercial development of cEPO-FC, but holds no equity in that company nor related to the molecules investigated. The other authors declare that they have no competing interests at all.

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