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ARTICLE

Free heme and hemopexin in acute kidney injury after cardiopulmonary bypass and transient renal ischemia

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Abstract

Free heme is released from hemoproteins during hemolysis or ischemia reperfusion injury and can be pro-inflammatory. Most studies on nephrotoxicity of hemolysis-derived proteins focus on free hemoglobin (fHb) with heme as a prosthetic group. Measurement of heme in its free, non-protein bound, form is challenging and not commonly used in clinical routine diagnostics. In contrast to fHb, the role of free heme in acute kidney injury (AKI) after cardiopulmonary bypass (CPB) surgery is unknown. Using an apo-horseradish peroxidase-based assay, we identified free heme during CPB surgery as predictor of AKI in patients undergoing cardiac valve replacement ($n = 37$). Free heme levels during CPB surgery correlated with depletion of hemopexin (Hx), a heme scavenger-protein. In mice, the impact of high levels of circulating free heme on the development of AKI following transient renal ischemia and the therapeutic potential of Hx were investigated. C57BL/6 mice were subjected to bilateral renal ischemia/reperfusion injury for 15 min which did not cause AKI. However, additional administration of free heme in this model promoted overt AKI with reduced renal function, increased renal inflammation, and reduced renal perfusion on functional magnetic resonance imaging. Hx treatment attenuated AKI. Free heme administration to sham operated control mice did not cause AKI. In conclusion, free heme is a

Abbreviations: AKI, acute kidney injury; apoHRP, apo-horseradish peroxidase; AUC, area under the curve; CPB, cardiopulmonary bypass; fHB, free hemoglobin; Hx, hemopexin; IL-6, Interleukin 6; IRI, ischemia reperfusion injury; MRI, magnetic resonance imaging; NGAL, neutrophil gelatinase-associated lipocalin; pRBCs, packed red blood cells; qPCR, quantitative polymerase chain reaction.

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predictor of AKI in CPB surgery patients and promotes AKI in transient renal ischemia. Depletion of Hx in CPB surgery patients and attenuation of AKI by Hx in the in vivo model encourage further research on Hx therapy in patients with increased free heme levels during CPB surgery.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Acute kidney injury (AKI) after cardiopulmonary bypass (CPB) surgery is diagnosed by serum creatinine increase after surgery and has multiple contributing factors, such as transient renal ischemia. Free heme can be nephrotoxic and cause AKI in chronic hemolytic disorders. The role of free heme in AKI after transient renal ischemia and CPB surgery is less well understood.

WHAT QUESTION DID THIS STUDY ADDRESS?

Can circulatory levels of free heme during CPB surgery predict subsequent AKI in patients? Does exogenous free heme administration induce AKI in mice with mild transient ischemia? Can hemopexin therapy attenuate AKI in this setting?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

We identified circulatory free heme during CPB surgery as a predictor of AKI in patients and showed that it only promotes AKI in mice with transient renal ischemia, but not in sham mice. The heme scavenger hemopexin was depleted in CPB surgery patients and attenuated AKI in these mice.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

This study encourages further research on targeting the free heme-hemopexin axis in CPB surgery-associated AKI.

INTRODUCTION

Acute kidney injury (AKI) is a major clinical complication of on-pump cardiac surgery[1] and is associated with extended duration of intensive care unit treatment and increased risk of death.[2] AKI has multiple contributing factors which make timely diagnosis and intervention challenging.[3] Major causes of AKI are renal ischemia reperfusion injury (IRI)[4] and hemolysis[5] both of which can occur in cardiopulmonary bypass (CPB) surgery[6,7] and are associated with release of free heme.[8,9]

Free heme is not bound to hemoproteins, such as hemoglobin or cytochromes, and has pro-inflammatory and cytotoxic effects.[10–12] It is known from chronic hemolytic disorders, such as sickle cell disease, that free heme can cause kidney injury.[10–12] Therefore, the investigation of free heme in CPB surgery-associated AKI is of good rationale. So far, a recent study found no correlation between heme and CPB surgery-associated AKI.[13] Importantly, heme was measured after and not during CPB surgery when the hemolysis is ongoing due to contact of the erythrocytes with the artificial surface of the extracorporeal circuit. Thus, it is still unknown whether free heme is increased during CPB surgery and whether this is associated with AKI.

The toxicity of free heme can be contained in the circulation by the serum heme-binding protein hemopexin (Hx)[14–16] and intracellularly via degradation by heme oxygenase 1 (HO-1), the inducible isozyme of heme catabolism.[15] Genetic deficiency of HO-1 has been associated with major heme-mediated endothelial damage[17] and increased renal inflammation.[18,19]

Measurement of heme in its free, non-protein-bound form is challenging and currently no diagnostic tests to determine free heme are available.[16,20] Using an innovative apo-peroxidase based assay,[21] we first tested the hypothesis that free heme elevation occurs during and not after CPB surgery and that free heme during CPB surgery can predict subsequent AKI. Second, a combination of transient renal ischemia and free heme toxicity—both of which are relevant in CPB surgery—was modeled in mice to test the hypothesis that free heme promotes AKI in the presence of transient renal ischemia and to investigate the therapeutic potential of Hx in this specific setting.

MATERIALS AND METHODS

Study design

A roadmap to the study design and the experimental setup is shown in Figure S1. First, free heme was measured in the serum of CPB surgery patients. Second, C57BL/6 mice underwent bilateral renal IRI for 15 min or sham surgery with additional free heme challenge (control groups) to investigate the natural presentation of mild IRI and the effect of increased heme levels separately. In a third step, overt AKI was induced by bilateral renal IRI for 15 min with additional injection of free heme to investigate the combined effects of mild IRI and increased heme levels. Finally, an experimental treatment study with the heme scavenger protein Hx was performed in this model.

Clinical study

CPB surgery patients

On-pump cardiac surgery patients ($n = 37$) were prospectively enrolled into a clinical study at Hannover Medical School, Germany, during 2018 and 2020. Informed consent was obtained from all patients and the study was approved by the local ethics committee (no. 6895). Indications for cardiac surgery were cardiac valve replacement with or without additional coronary bypass grafting. Exclusion criteria were age under 18 years, coronary artery bypass grafting without valve replacement, off pump procedure, or maintenance dialysis prior to surgery. Plasma samples were collected immediately after anesthesia induction (surg.-start), at the end of aortic cross clamping (aort. X-clamp), at the end of CPB (reperfusion), at the end of surgery (surg.-end) and on day 1 after surgery (d1). Patient plasma samples were centrifuged at 200×*g* for 10 min. The supernatant was transferred to Eppendorf tubes and centrifuged at 200×*g* for 10 min to remove residual red blood cells (RBCs). The supernatants obtained were aliquoted and immediately stored at −80°C until further use.

Definition of AKI

AKI was graded according to the Kidney Disease Improving Global Outcomes (KDIGO) criteria[22] into stage I-III based on serum creatinine (sCr) elevation during 48 h post-surgery as follows: stage I: sCr increase by 1.5 to 1.9-fold from baseline or absolute sCr increase by greater than or equal to 0.3 mg/dL (≥26.5 µmol/L), stage II: sCr increase by 2.0 to 2.9-fold from baseline and stage III: sCr increase by 3.0-fold from baseline or absolute sCr increase by greater than or equal to 4.0 mg/dL (≥353.6 µmol/L), or initiation of renal replacement therapy.

Measurement of free heme and Hx

The measurement of free heme and Hx is detailed in the Supplementary Methods.

Experimental mouse study

Reagents

Human Hx was purchased from Athens Research and Technology. Hemin was obtained from Frontiers Scientific. All other materials and reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Animals

Adult male C57BL/6 mice (11–13 weeks of age, body weight 23–28 g) housed and bred at the Institute of Laboratory Animal Sciences, Hannover Medical School, and purchased from Charles River were used for the experiments. The day/night cycle was 14/10 h and all mice had free access to drinking water and food. Mice were monitored daily for the physical condition after surgery. Reasons for study termination were visible behavioral changes, such as scrubby appearance, reduced motility, reduced food uptake, reduced activity, or body weight reduction of greater than 20%. All experiments were approved by the local animal protection committee of the Lower Saxony State department for animal welfare and food protection (33.19-42502-04-14/1657).

Renal ischemia reperfusion injury

Isoflurane was used for anesthesia (3% induction, 1%–2% maintenance) and butorphanol (1 mg/kg) for analgesia. IRI was induced by bilateral renal pedicle clamping with a micro-aneurysm clip for 15 min. Reperfusion was controlled visually. Sham surgery with opening of the abdominal cavity, but without manipulation of the renal vessels served as control. Free heme (20 mg/kg) was diluted in phosphate buffered saline (PBS) and injected via the retro-orbital venous plexus immediately after reperfusion. The vehicle group received PBS injection. In the treatment study, Hx (2 mg/mouse) was injected via tail vein 1 h prior to IRI. Then, IRI was induced in 32 mice. Of those, eight

mice in each group received vehicle, free heme, Hx, or free heme + Hx. Sham surgery was performed in 18 mice. Of those, six mice in each group received vehicle, Hx, or free heme. In vivo imaging studies by functional magnetic resonance imaging (MRI) prior to and 24 h after the respective intervention were performed in separate experiments in five to 10 mice in each group. MRI perfusion data were correlated with sCr, Gr-1 neutrophil score, and HO-1 positive tubuli in a subgroup of 10–20 animals.

Organ preservation

Mice were euthanized in deep general anesthesia (5% isoflurane) at 24 h after IRI and organ retrieval was performed. After midline laparotomy, whole body perfusion with ice-cold 0.9% PBS via the left cannulated ventricle resulted in a circulatory arrest. Organs were dissected and fixed in RNA later, 4% paraformaldehyde, or shock frozen in liquid nitrogen.

Renal morphology and immunofluorescence

After paraffin embedding, 2 µm sections were cut and stained as detailed in the Supplementary Methods.

Cell staining and flow cytometry

Cell staining and flow cytometry was performed as detailed in the Supplementary Methods.

Cytokine expression

Cytokine expression levels were determined by quantitative polymerase chain reaction (qPCR) using a protocol detailed in the Supplementary Methods.

Renal function

Serum creatinine was measured using the Olympus AU400 Chemistry Analyzer.

Perfusion MRI

In a separate experimental series, MRI was performed 1 day after IRI using a 7 Tesla small-animal MRI scanner (Bruker; Pharmascan, PHS701) with a circular polarized volume coil as detailed in Supplementary Methods.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software) and IBM SPSS Statistics 26 (SPSS, IBM Corporation). Multiple comparisons of normally distributed data were analyzed by one-way analysis of variance (ANOVA) and group means were compared using the Tukey's post hoc test. Nonparametric analyses were performed using the Kruskal-Wallis test and the Friedman's ANOVA for related samples. Single comparisons were analyzed by Mann–Whitney *U* test where appropriate. Continuous data with normal distribution are reported as mean value ± standard error of the mean. Data with non-normal distribution are reported with median and interquartile range (IQR). Categorical data were analyzed by the chi-square test and Fisher's exact test. The Spearman rank test was used for correlation analyses. The *p* values less than 0.05 were accepted as significant.

RESULTS

Plasma levels of free heme and Hx in CPB surgery patients with and without AKI

In the clinical part of this work, an apo-horseradish peroxidase (apoHRP) assay was applied to measure free—non-protein-bound—heme in CPB surgery patients with and without AKI (Table 1). Patients with AKI had similar baseline renal function parameters relative to no-AKI patients. All patients with AKI had KDIGO stage I AKI. Duration of surgery, CPB, and aortic cross clamping were similar in AKI as compared to patients without AKI (data not shown).

Levels of free heme were determined in plasma at various times during and after CPB (Figure 1a). The time schedule for sample collection did not differ between AKI and no-AKI patients (Table 2). Baseline levels of free heme at the onset of surgery were similar in patients with and without postoperative AKI. During the CPB procedure, plasma levels of free heme markedly increased, with a maximum at reperfusion and return to baseline values 24 h post-surgery (Figure 1a). Patients with postoperative AKI had higher levels of free heme compared to non-AKI patients at the end of aortic cross clamping (2.02 ± 1.22 vs. 1.23 ± 1.00; $p = 0.033$) and at reperfusion (2.35 ± 1.30 vs. 1.46 ± 1.12; $p = 0.049$; Figure 1a). A moderate correlation between levels of free heme and free hemoglobin (fHb) at the respective timepoints was observed (Figure 1b,c).

Receiver operating characteristic curve analysis was performed to determine whether free heme levels during ongoing surgery are predictive of the postoperative development of AKI. Increased levels of free heme

TABLE 1 Pre-, peri- and postoperative data of the study cohort (mean ± standard deviation).

	All patients $n = 37$	No AKI $n = 28$	AKI $n = 9$	p value
Patients				
Age (years)	71.0 ± 9	70.5 ± 9.2	72.1 ± 8.3	0.958
Gender, n (%), male/female	24/13 (64.9/35.1)	19/5 (67.9/32.1)	5/4 (55.6/44.4)	0.386
Weight (kg)	81.2 ± 17.3	77.9 ± 11.8	91.7 ± 26.8	0.302
Height (cm)	170.9 ± 9.2	170.4 ± 9.3	172.3 ± 9.4	0.589
BMI (kg/m^2)	27.8 ± 5.1	26.9 ± 3.8	30.7 ± 7.5	0.240
Indication for surgery, n (%)				
Valvular heart disease	20 (54.1)	15 (53.6)	5 (55.6)	
Valvular heart disease and coronary heart disease	17 (45.9)	13 (46.4)	4 (44.4)	0.612
Comorbidities, n (%)				
Arterial hypertension	30 (81.1)	25 (89.3)	5 (55.6)	0.045
Peripheral artery disease	3 (8.1)	3 (10.7)	0 (0.0)	0.422
Diabetes mellitus	10 (27.0)	7 (25.0)	3 (33.3)	0.679
Cardiac ejection fraction <30% prior to surgery	4 (10.8)	4 (14.3)	0 (0.0)	0.310
History of smoking	10 (27.0)	8 (28.6)	2 (22.2)	0.586
Hyperlipidemia	19 (51.4)	13 (46.4)	6 (66.7)	0.447
Pre-operative data				
Baseline serum creatinine (μmol/L)	113.7 ± 25	114.3 ± 25	111.6 ± 31	0.651
Baseline eGFR (mL/min)	53.5 ± 21	53.3 ± 12.6	54.3 ± 14.6	0.724
Serum C-reactive protein (mg/L)	4.3 ± 5.7	4.6 ± 6.2	3.1 ± 2.8	0.875
Intra-operative data				
Duration of surgery (min)	231 ± 78.9	235.0 ± 75.7	218.9 ± 91.9	0.392
Number of transfused packed red blood cells (units)	3 ± 2	3 ± 2	2 ± 3	0.286
Postoperative data				
Serum creatinine 48 h (μmol/L)	115.5 ± 30.1	107.8 ± 27.2	139.6 ± 27.2	<0.002
eGFR 48 h (ml/min)	53.4 ± 15.3	57.8 ± 14.4	39.6 ± 8.6	<0.000
Re-operation, n (%)	11 (29.7)	9 (32.1)	2 (22.2)	0.454
Intensive care unit treatment (days)	2.1 ± 2.5	2.3 ± 2.8	1.4 ± 1.0	0.362

Note: AKI is defined according to the Kidney Disease Improving Global Outcomes -criteria.Abbreviations: AKI, acute kidney injury; BMI, body mass index; eGFR, estimated glomerular filtration rate.

at the end of aortic cross clamping (area under the curve [AUC] = 0.74, $p = 0.044$; Figure 1d) and at reperfusion (AUC = 0.73, $p = 0.048$; Figure 1e) were indeed predictive of the development of AKI within 48 h after surgery. In summary, the data indicate that increased plasma levels of free heme during CPB surgery could be a marker for early identification of patients at risk of AKI following CPB surgery.

In addition to CPB surgery-induced hemolysis and IRI-mediated tissue release of free heme, circulatory free heme levels may also be affected by the transfused units of packed RBCs (pRBCs). However, the number of transfused pRBCs during surgery did not correlate with free heme levels in patient plasma at aortic cross-clamping (Figure 2a) and reperfusion (Figure 2b). Moreover, free heme levels did not correlate with aortic cross clamp time, CPB surgery time or duration of surgery, but a strong correlation with lactate dehydrogenase levels at reperfusion—a common marker of hemolysis and cell damage—was observed ($r = 0.8$, $p < 0.001$, data not shown). Levels of the heme-scavenger protein Hx were comparable in both patients with AKI and no AKI patients under basal conditions. In both groups, levels of Hx decreased significantly after aortic cross-clamping and remained low until the

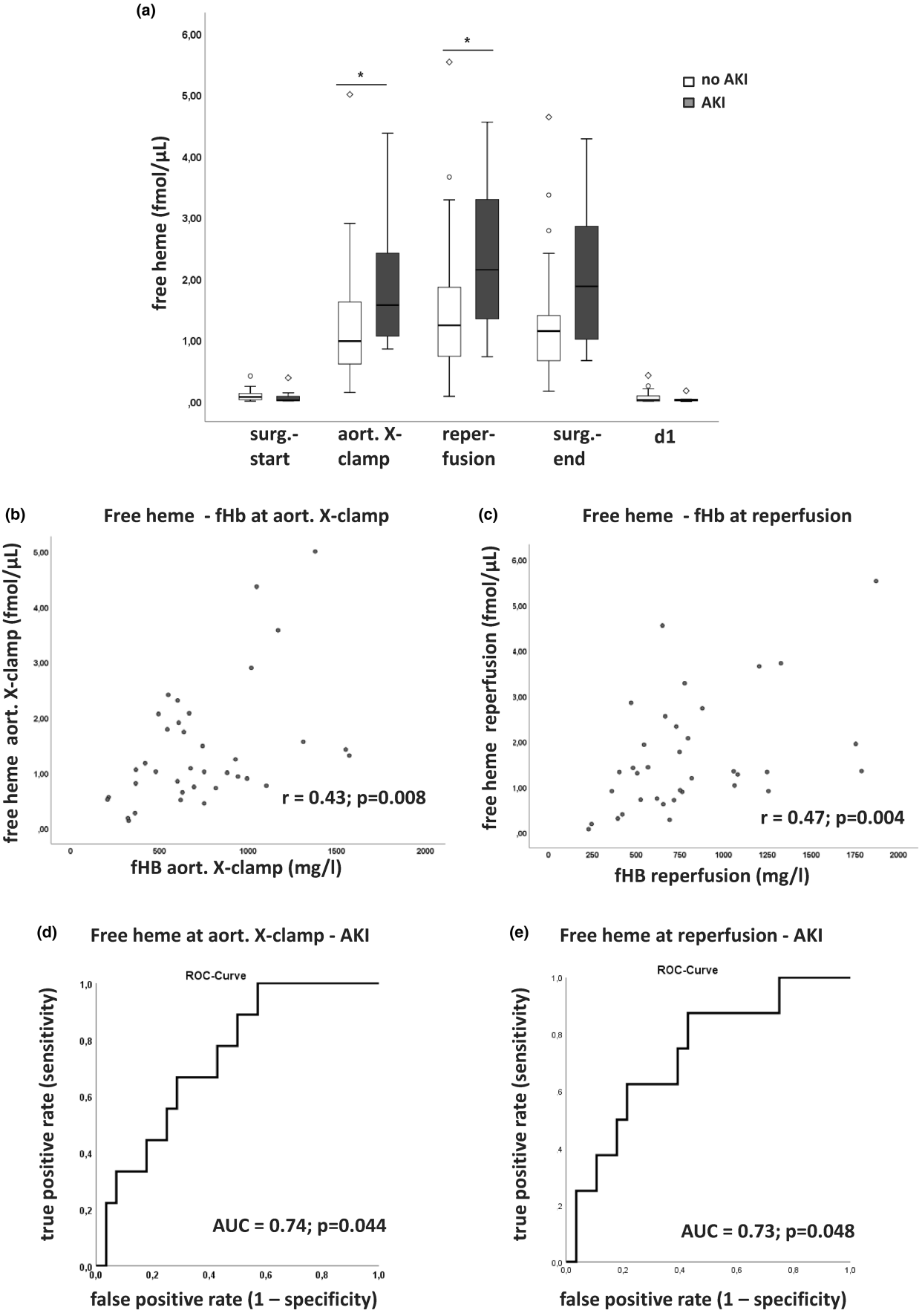
(a)
free heme (fmol/µL)
6,00
5,00
4,00
3,00
2,00
1,00
,00
*
*
no AKI
AKI
surg.-start
aort. X-clamp
reper-fusion
surg.-end
d1
(b)
Free heme - fHb at aort. X-clamp
free heme aort. X-clamp (fmol/µL)
r = 0.43; p=0.008
fHB aort. X-clamp (mg/l)
(c)
Free heme - fHb at reperfusion
free heme reperfusion (fmol/µL)
r = 0.47; p=0.004
fHB reperfusion (mg/l)
(d)
Free heme at aort. X-clamp - AKI
ROC-Curve
true positive rate (sensitivity)
AUC = 0.74; p=0.044
false positive rate (1 – specificity)
(e)
Free heme at reperfusion - AKI
ROC-Curve
true positive rate (sensitivity)
AUC = 0.73; p=0.048
false positive rate (1 – specificity)

FIGURE 1 Free heme in CPB surgery patients could serve as a predictive marker for AKI.Plasma free heme levels before, during, and 1 day after CPB are shown in (a) for patients with AKI ($n = 9$, black bar) or without AKI ($n = 28$, white bar) within 48 h after surgery according to KDIGO criteria. Data are shown as median ± IQR. Free heme levels at aort. X-clamp and reperfusion were significantly higher in patients with AKI (a). Correlation of free heme levels with fHb at the respective timepoints are shown in (b) and (c). Plasma free heme levels correlated significantly with fHb levels albeit this correlation was only moderate. Spearman correlation coefficient (r) and p value is shown in (b) and (c). receiver operating characteristic curve analysis was used to test the predictive ability of plasma free heme for AKI development at aortic X-clamp (d) and reperfusion (e). *$p < 0.05$. AKI, acute kidney injury; AUC, area under the curve; CPB, cardiopulmonary bypass; fHb, free hemoglobin; IQR, interquartile range; KDIGO, Kidney Disease Improving Global Outcomes.

TABLE 2 Time periods between sampling (mean ± SD).

Time period	All patients	No AKI	AKI	*p* value
Start-surgery—aortic X-clamp (min)	148 ± 42.7	144 ± 38.3	158 ± 51.5	0.86
Aortic X-clamp—reperfusion (min)	46 ± 33.2	48 ± 36.9	41 ± 15.3	0.94
Reperfusion—end-surgery (min)	34 ± 13.6	34 ± 14.8	34 ± 8.9	0.97
End-surgery—d1 (min)	1041 ± 155.3	1045 ± 158.3	1026 ± 144.2	0.94

Note: Samples were taken at different timepoints during and after surgery. The time periods between sampling were comparable in no-AKI and patients with AKI.

Abbreviation: AKI, acute kidney injury.

end of surgery (Figure 2c). Levels of Hx were not restored within 24 h after CPB surgery.

Free heme reduces kidney perfusion and function in transient renal ischemia

To investigate whether increased circulating free heme aggravates ischemic kidney damage, we used a mouse model of short-term transient bilateral renal ischemia and administration of exogenous free heme. In B6 mice with bilateral renal ischemia or sham surgery free heme (or vehicle) was administered intravenously immediately after reperfusion or at the end of sham surgery, respectively (Figure S1). Renal perfusion was evaluated prior to and 24 h after surgery using in vivo imaging by functional MRI. When exogenous heme was administered in mice with experimental IRI a marked reduction of renal perfusion was observed (Figure 3a–e) in comparison to IRI and treatment with vehicle alone (Figure 3c,e). Sham surgery with or without (Figure 3a,b,e) administration of heme did not affect renal perfusion (Figure 3e). Bilateral IRI for 15 min without exposure to heme did not result in AKI as demonstrated by normal sCr concentration (Figure 3f). Free heme-induced AKI with significantly elevated levels of sCr (Figure 3f). Renal perfusion correlated with renal function (Figure 3g), neutrophil infiltration (Figure 3h), and tubular HO-1 expression (Figure 3i). The data indicate that increasing circulatory levels of free heme by exogenous free heme administration causes severe AKI in mild transient renal ischemia.

Free heme induces blood neutrophilia and kidney neutrophil infiltration in transient renal ischemia

Quantification of neutrophil infiltration in whole kidney lysates was performed by flow cytometry after free heme or vehicle administration in sham conditions or transient renal ischemia (Figure S2). The gating strategy is shown in Figure S2A–C. Neutrophil counts were significantly decreased in bone marrow (Figure S2D) and increased in blood (Figure S2E) and kidney (Figure S2F) following free heme administration in renal IRI. Transient renal ischemia for 15 min without free heme administration and free heme administration without transient renal ischemia did not alter neutrophils in bone marrow, blood, or kidneys (Figure S2d–f). Blood neutrophilia correlated with increased neutrophil infiltration of the kidneys (Figure S2G).

Heme-mediated inflammation and HO-1 expression in transient renal ischemia is alleviated by Hx

To determine whether free heme toxicity in transient renal ischemia can be modulated via scavenging therapy, Hx, a serum protein with exceptionally high binding affinity for heme,[13] was applied 1 h prior to IRI with and without administration of exogenous heme, respectively. To investigate renal inflammation, IL-6, a pro-inflammatory cytokine, and infiltrating neutrophils (Gr-1 positive cells) were analyzed in the kidney tissue (Figure 4a,c,e,g,i). Free

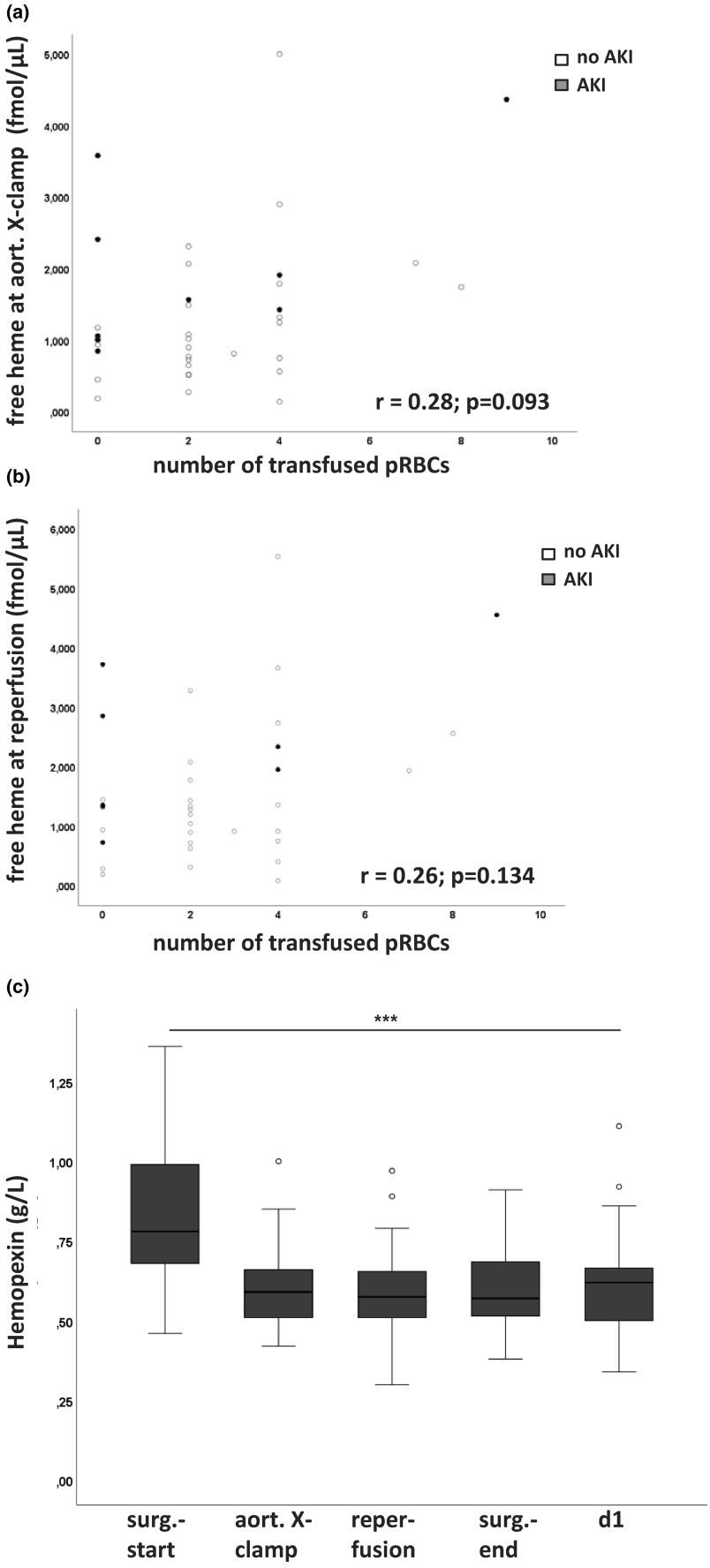

FIGURE 2 Association of free heme levels with blood transfusion and levels of the heme binding protein Hx in CPB surgery patients. The amount of pRBCs administered during CPB was correlated with free heme levels at aort. X-clamp (a) and at reperfusion (b). White dots represent no AKI-patients and dark gray dots patients with AKI. Zero to nine pRBCs were transfused during surgery. Spearman's correlation and p value is reported for the respective timepoints. There was no significant correlation between the amount of pRBCs during CPB and levels of free heme in the circulation. Plasma levels of the heme binding protein Hx is shown in patients before, during and 24 after CPB. Hx levels are depleted during CPB and not restored 24 h after CPB. Data is expressed as median ± IQR. ***$p < 0.001$. AKI, acute kidney injury; CPB, cardiopulmonary bypass; IQR, interquartile range; pRBCs, packed red blood cells.

heme administration in transient renal ischemia caused upregulation of kidney tissue IL-6 levels which were attenuated by Hx treatment (Figure 4a). Moreover, administration of heme in IRI led to a marked increase of neutrophil infiltration in the outer medulla of the kidneys (Figure 4e,i) which was reduced by Hx (Figure 4g,i). Sham surgery or treatment with IRI and vehicle did not cause significant renal infiltration by neutrophils (Figure 4c,i).

HO-1 was markedly induced in tubular cells in the presence of increased free heme levels, as determined by qPCR (Figure 4b) and immunofluorescence (Figure 4d,f,h) in the kidneys of sham and IRI mice and was reduced by Hx treatment (Figure 4b,d,f,h,j).

Renal expression of COX-2, a pro-inflammatory enzyme not directly induced by free heme,[23] was markedly higher in renal tubules after transient renal ischemia plus free heme (Figure 5c) and was attenuated by treatment with Hx (Figure 5e,g).

NGAL, a common AKI marker indicating tubular damage, was determined by immunofluorescence 24 h after surgery. In contrast to IRI alone (Figure 5b,h), administration of free heme in transient kidney ischemia led to a marked increase of NGAL staining in tubuli of the renal cortex (Figure 5d,h). NGAL positivity in tubular cells was markedly reduced by pretreatment with Hx in animals with IRI plus heme (Figure 5f,h). Sham surgery with vehicle, heme, or Hx treatment did not affect NGAL expression (Figure 5h). Taken together, the findings indicate an aggravating role of free heme in IRI-mediated renal injury that is attenuated by the heme-binding serum protein Hx.

DISCUSSION

In the current work, we identified free heme as a predictor of AKI in patients undergoing CPB surgery. Depletion of the specific free heme scavenger Hx in this patient cohort suggests a mechanistic role of free heme/Hx in this setting. Using a mouse model combining free heme and transient renal ischemia—a relevant injury factor associated with CPB surgery—we showed that free heme mediates AKI solely in the setting of transient renal ischemia. The causal role of free heme in promoting AKI in this setting was demonstrated by the alleviation of AKI via supplementary administration of Hx. Free heme toxicity has been demonstrated to cause AKI in chronic hemolytic disorders, such as sickle cell disease,[24–26] but this is—to our knowledge—the first study to show that free heme during CPB is a predictor of AKI after CPB surgery.

The findings have significant clinical implications since currently no causal treatment for CPB-associated AKI exists. Measurement of free heme during CPB by the apoHRP-based assay applied in this study would enable prediction of AKI and identification of patients which would have potential benefit from Hx treatment. The proof of concept that Hx treatment attenuated AKI mediated by exogenous free heme administration in mice with transient renal ischemia may stimulate further studies on the therapeutic administration of Hx in patients with high levels of free heme during CPB surgery. However, Hx treatment in the current study was performed 1 h prior to IRI induction and free heme administration. Therefore, the efficacy of Hx as a rescue treatment during or after surgery is yet to be explored. Moreover, it is noteworthy that multiple factors can contribute to the development of AKI after CPB surgery and that this is a relatively small patient cohort. Patient-related risk factors, such as impaired renal function prior to CPB surgery,[27] high age,[27] and presence of diabetes mellitus,[23] are well known risk factors for AKI in cardiac surgery.[28] In addition, procedure-specific risk factors for AKI, such as duration of CPB surgery and aortic cross clamping time, have been described.[29] These risk factors were comparable in patients with and without AKI in our cohort and did not correlate with levels of free heme. Factors associated with higher free heme release remain elusive in this study, but notably other factors, such as pump speed and the mode of surgical suction which were shown to affect hemolysis,[30] are not recorded in the current work. All patients had impaired renal function prior to surgery and 22% developed AKI following CPB surgery. These findings are in line with previous data from independent studies.[31] A limitation of the current study is that all patients with AKI had KDIGO stage I AKI and it is therefore not possible to draw conclusions on a potential link of free heme with severe, dialysis-requiring, AKI. To this end, further studies are required with larger numbers of patients and various stages of AKI.

In contrast to our study, a recent study found no correlation between heme and renal injury after CPB surgery.[13] However, there are apparent differences in study design and conduct which might explain these differences. First, we chose to measure free heme during CPB surgery when the hemolysis is ongoing and not after CPB surgery,[13] because free heme has a reported half-life of 2 h[32] and measurement of free heme at the end of surgery might miss the free heme peak. Second, the study by Pat et al.[13] did not address patients with AKI according to the KDIGO definition because the sCr pre- and post-surgery was unchanged in their study. Third, heme was measured by a commercially available enzyme-linked immunosorbent assay in the study by Pat and colleagues.[13] Because commercially available heme assays do not differentiate free from protein-bound heme in serum or urine,[16,20,33] an apoHRP-based assay for determining free, non-protein-bound, heme in patient plasma was applied in our study. This assay was adapted

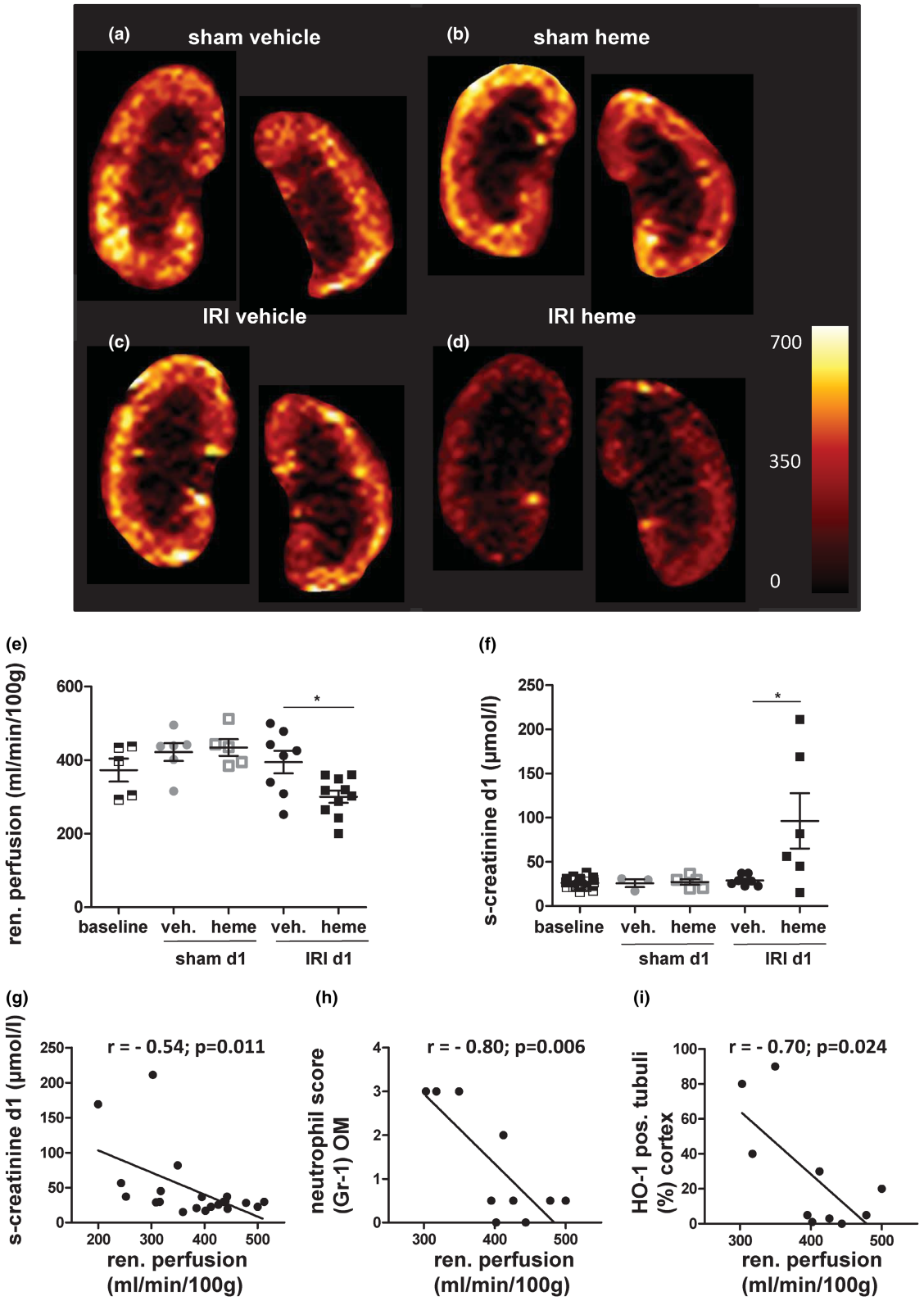

FIGURE 3 Free heme reduced kidney perfusion and function after IRI. Renal perfusion was assessed by arterial spin labeling on functional magnetic resonance imaging prior to (baseline) and on day 1 in mice treated with or without free heme after sham surgery (a, b) or IRI (c, d). Representative color-coded maps of renal perfusion (a–d, bright color indicates strong perfusion and dark red poor perfusion, renal perfusion is normalized to kidney weight as mL/min/100 g and the mean perfusion of the right and left kidneys is shown in (e) because a bilateral clipping model was applied). Data is expressed as mean ± SEM. Serum creatinine is shown in (f). Reduced renal perfusion correlated with serum creatinine elevation (g). In a subgroup of animals, correlation of renal perfusion with neutrophil infiltration (h) and renal HO-1 expression (i) is shown. *$p < 0.05$. IRI, ischemia/reperfusion injury.

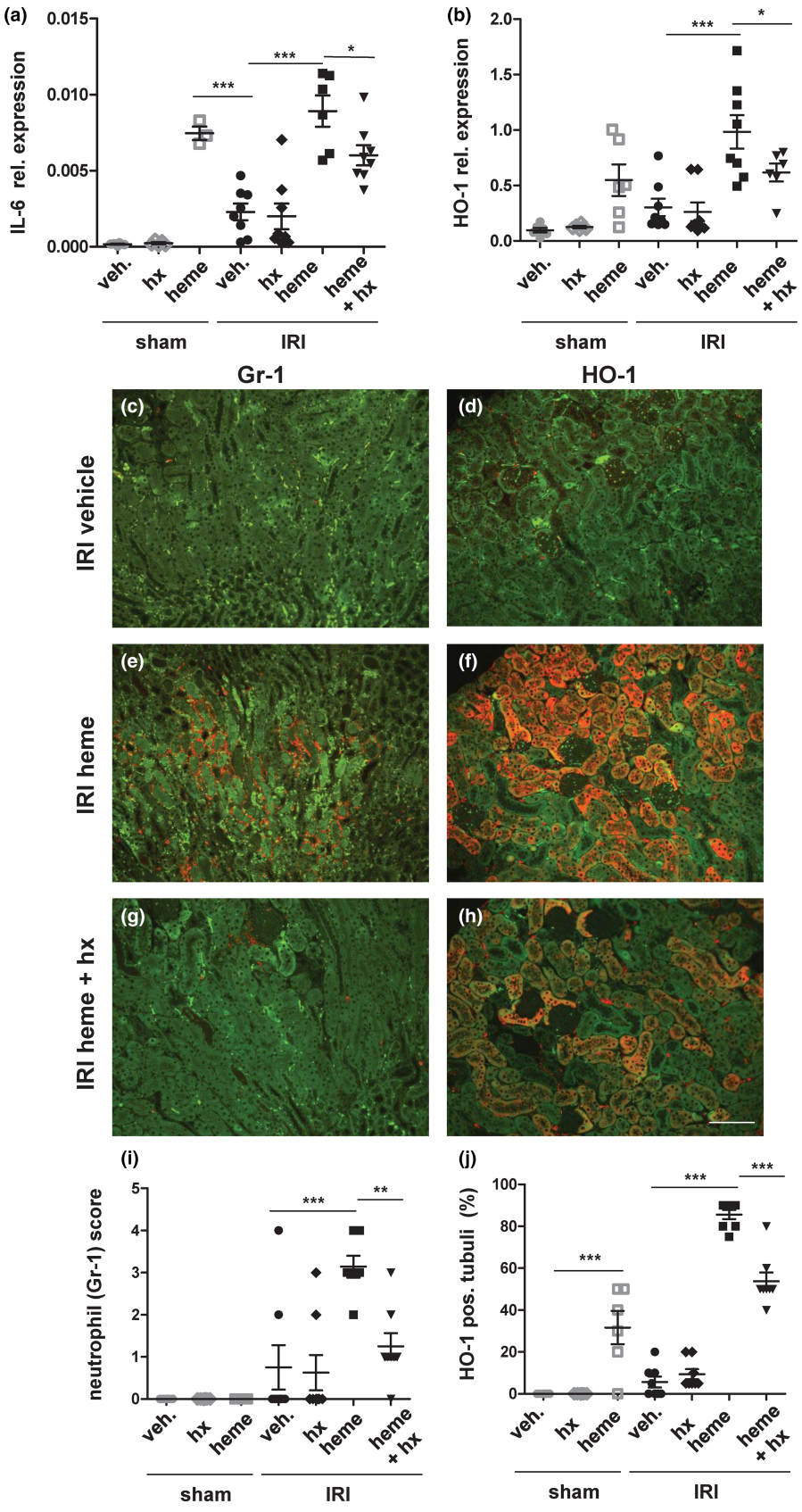

FIGURE 4 Free heme-mediated neutrophil infiltration and HO-1 expression after IRI is mitigated by Hx treatment. Renal IL-6 (a) and HO-1 (b) levels were measured by quantitative polymerase chain reaction. Immunofluorescence for Gr-1 (c, e, g) and HO-1 (d, f, h) are shown 24 h after IRI and vehicle (c, d), heme (e, f), and heme + Hx (g, h) treatments (specific signal is in red, auto fluorescence in green). Gr-1 scoring is shown in (i) including sham groups which did not have relevant Gr-1 positive cells (pictures not shown). Free heme administration after IRI resulted in severe neutrophil infiltration in the outer medulla (e, i) and HO-1 expression (f, j). This was reduced by Hx treatment (g, h, i, j). Heme administration in sham animals also resulted in significant renal HO-1 expression (b, j, picture not shown) but lower compared to IRI + heme. Data is expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $N = 3$–8 mice in each group were analyzed. Scale bar 100 μm. IRI, ischemia/reperfusion injury.

from a method previously established for measuring free heme in various experimental settings and is based on the relatively high heme-binding affinity of apoHRP to free heme (K_D—10^{-7})[21] and has been successfully used for measuring free heme in various previous in vitro and in vivo settings.[9,34,35]

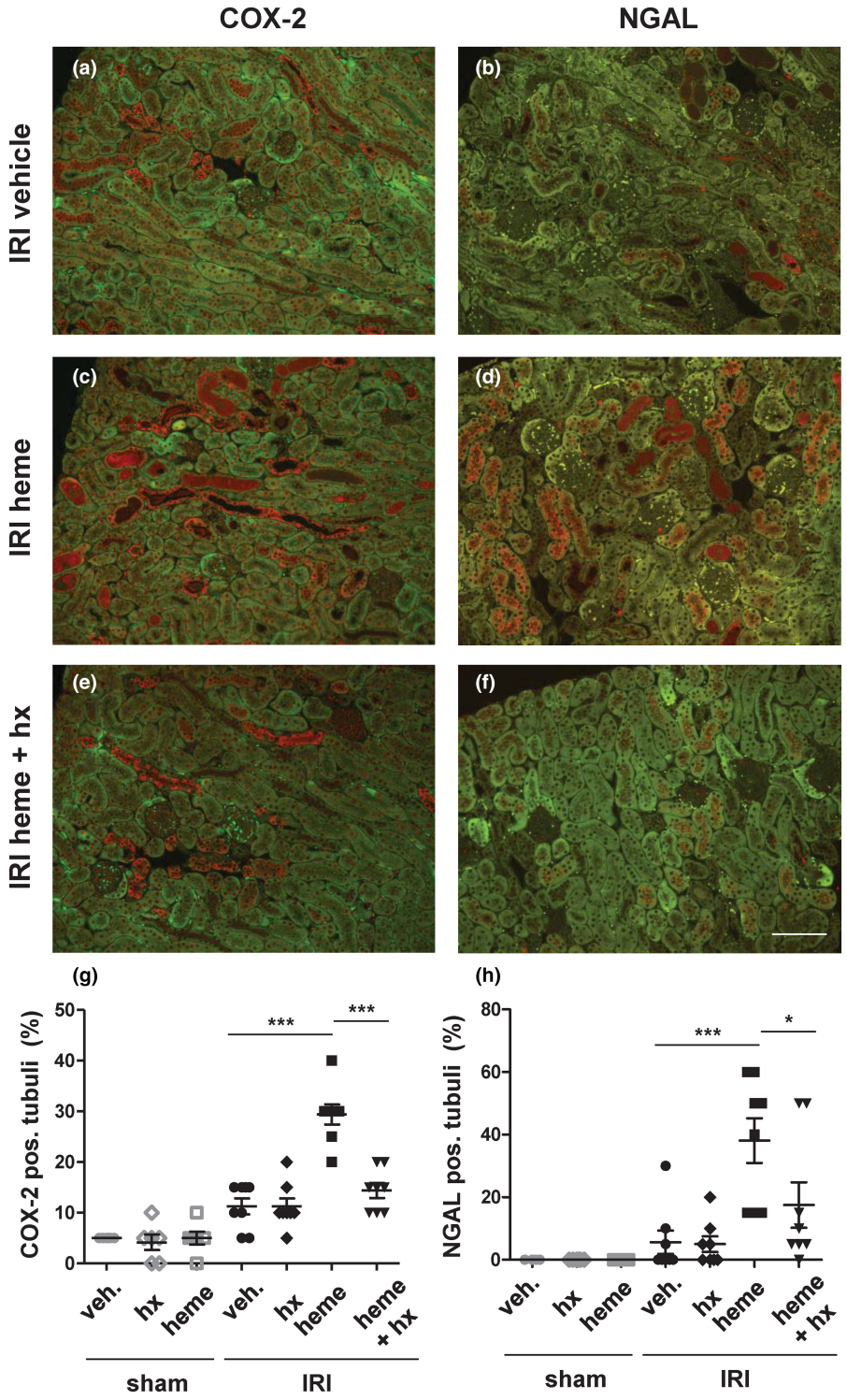

FIGURE 5 Free heme-induced renal upregulation of COX-2 and NGAL after IRI is counter-acted by Hx treatment. Renal COX-2 expression as another inflammatory marker was strongly induced after IRI + heme (c, e), but abrogated by additional Hx treatment (e, g). Sham treated animals (pictures not shown) and mice treated with IRI and vehicle (a, g) did not show major COX-2 immunofluorescence (specific signal in red, autofluorescence in green). Local renal NGAL expression as a marker for tubular damage was upregulated after IRI and heme administration (d, h). Hx treatment reduced renal NGAL expression (f, h). Sham surgery and IRI along with vehicle treatment did not cause NGAL expression (b, h) $^{*}p < 0.05$, $^{***}p < 0.001$. Five and eight mice in each group were analyzed on day 1 after surgery. Scale bar 100 μm. IRI, ischemia/reperfusion injury.

Previously, an inverse correlation of increased serum levels of free heme with decreased levels of Hx was reported in hemolytic conditions such as sickle cell disease.[15,20,36] In sickle cell disease, depleted Hx levels in patients or genetic Hx deficiency in mice were associated with AKI.[24] In contrast to these and our data, a recent study showed in mouse models of ischemic kidney injury and cisplatin toxicity that genetic deletion of Hx protected from AKI.[37] A possible explanation for these apparent differences could be the different injury models. In the models used by Fan et al.,[37] high circulatory levels of free heme do not play a role. The sickle cell disease data and our results suggest that in presence of high plasma free heme levels the beneficial effects of Hx by scavenging free heme outweigh any potential adverse effects. This underlines the importance of free heme measurement to identify patients which might benefit from Hx treatment.

As a limitation of this study, it is important to note that we did not use a mouse model of CPB surgery. However, CPB surgery in a small animal is extremely challenging and additionally the amount of free heme release cannot be controlled. We therefore chose to model transient renal ischemia which is relevant in patients during initiation of/weaning from CPB surgery in combination with a standardized dose of free heme. Whether transient renal ischemia per se causes AKI depends on its duration.[38] In C57BL/6 mice, bilateral IRI for 25 min causes severe AKI with sCr elevation.[39] However, our mouse model of 15 min bilateral renal ischemia only caused minor morphological kidney damage without functional relevance (Figure 3f). We acknowledge that 15 min of complete ischemia with subsequent reperfusion does not exactly match the clinical situation of CPB where renal perfusion is rather impaired for a prolonged time. However, it is virtually impossible to model this in an experimental setting with sufficient precision. We therefore believe that a well standardized complete IRI of 15 min is best suited to mimic mild, subclinical renal injury by reduced perfusion and to examine the effects of a second hit: free heme. The mechanisms of AKI induction via free heme as a second hit in transient ischemia included reduction of renal perfusion as measured in vivo by functional MRI (Figure 3). This sensitive MRI technique has been shown to detect severity of AKI in previous studies.[40–42] Moreover, free heme led to a marked upregulation of renal HO-1 expression after IRI that was attenuated by Hx. Numerous studies have shown a protective role of HO-1 in AKI indicating that HO-1 upregulation in the kidneys is a defense mechanism against renal toxicity of free heme.[43–46] In contrast to HO-1, COX-2 is not inducible by free heme per se, but is expressed in the kidneys in response to a variety of inflammatory stimuli. In line with the known pro-inflammatory properties of free heme, COX-2 was upregulated in the post-ischemic kidneys after free heme challenge and COX-2 expression was attenuated by Hx.

In summary, this is the first study to identify free heme during CPB as a predictor of AKI in patients undergoing CPB surgery. Depletion of the specific heme-scavenger protein Hx in this patient cohort suggests a mechanistic role of free heme/Hx which was demonstrated in an experimental proof-of-concept study. Alleviation of AKI in this model of transient renal ischemia and exogenous free heme administration via treatment with Hx encourages further research on Hx therapy for AKI in patients with high free heme levels during CPB surgery.

AUTHOR CONTRIBUTIONS

R.G., W.G., and S.I. wrote the manuscript. R.G., W.G., and S.I. designed the research. R.G., S.S., L.W., L.G., K.K., K.D., M.G., M.S., A.L., I.T., J.S., F.I., R.N., C.F., R.L., A.M.H., S.v.V., R.S., N.S., S.R., M.G., V.V., I.S., W.G., and S.I. performed the research. R.G., S.S., L.W., L.G., K.K., K.D., M.G., M.S., A.L., I.T., J.S., F.I., R.N., C.F., A.H., R.L., A.M.H., S.v.V., R.S., N.S., S.R., H.H., K.S.O., M.G., V.V., I.S., W.G., and S.I. analyzed the data.

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CONFLICT OF INTEREST STATEMENT

The authors declared no competing interests for this work.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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