Hemolysis is associated with acute kidney injury during major aortic surgery

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Hemolysis is an inevitable side effect of cardiopulmonary bypass resulting in increased plasma free hemoglobin that may impair tissue perfusion by scavenging nitric oxide. Acute kidney injury after on-pump cardiovascular surgery arises from a number of causes and severely affects patient morbidity and mortality. Here, we studied the effect of acute hemolysis on renal injury in 35 patients undergoing on-pump surgical repair of thoracic and thoracoabdominal aortic aneurysms of whom 19 experienced acute kidney injury. During surgery, plasma free hemoglobin increased, as did urinary excretion of the tubular injury marker *N*-acetyl-β-D-glucosaminidase, in patients with and without acute kidney injury, reaching peak levels at 2 h and 15 min, respectively, after reperfusion. Furthermore, plasma free hemoglobin was independently and significantly correlated with the urine biomarker, which, in turn, was independently and significantly associated with the later postoperative increase in serum creatinine. Importantly, peak plasma free hemoglobin and urine *N*-acetyl-β-D-glucosaminidase concentrations had significant predictive value for postoperative acute kidney injury. Thus, we found an association between increased plasma free hemoglobin and renal injury casting new light on the pathophysiology of acute kidney injury. Therefore, free hemoglobin is a new therapeutic target to improve clinical outcome after on-pump cardiovascular surgery.

Kidney International (2010) **77,** 913–920; doi:10.1038/ki.2010.24; published online 24 February 2010

KEYWORDS: acute kidney injury; cardiovascular; nitric oxide; renal proximal tubule cell; risk factors

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Received 29 July 2009; revised 17 November 2009; accepted 28 December 2009; published online 24 February 2010

Acute kidney injury (AKI) is an important complication of major cardiovascular surgery that affects 3–48% of patients and is independently associated with mortality.[1–3] At present, specific therapies for postoperative AKI are not available, and patients generally receive supportive care until renal function has recovered spontaneously.[4] The development of novel interventions to reduce AKI after major cardiovascular surgery requires a detailed knowledge of its pathophysiology, which is characterized by a complex interplay of renal vascular dysfunction, tubular epithelial injury, and inflammation.[5]

In cardiovascular surgery, the use of cardiopulmonary bypass is associated with AKI.[6,7] Hemolysis is a common consequence of cardiopulmonary bypass that is caused by mechanical stress in the perfusion circuit and results in the release of hemoglobin from lysed erythrocytes into the plasma.[8,9] Cell-free oxyhemoglobin reacts with nitric oxide to form methemoglobin and nitrate. As nitric oxide is an important vasodilator that has a central role in blood flow regulation, reduction of nitric oxide bioavailability by free hemoglobin (fHb) may impair tissue perfusion.[10] Indeed, addition of 6 μmol/l oxyhemoglobin to the perfusion solution of isolated rabbit hearts completely eliminates the activity of endothelium-derived nitric oxide.[11] In line with this finding, patients with chronic hemolysis due to sickle cell disease are characterized by an attenuated increase in forearm blood flow after infusion of the nitric oxide donor sodium nitroprusside as compared with healthy volunteers; forearm blood flow response was reduced by 80% in patients with plasma fHb concentrations >6 μmol/l.[10,12] In addition, after glomerular ultrafiltration, urine fHb may cause AKI by generating reactive oxygen species and by aggregating into casts in the tubular lumen.[13,14]

Until now, human studies have focused on the association between urinary fHb and AKI.[13] The vasoconstrictive effects of fHb in the plasma may provide an additional explanation for hemolysis-induced renal injury. In the current study, we show that increased plasma fHb concentrations are independently associated with injury to the proximal tubular

epithelium during open surgical repair of thoracic and thoracoabdominal aortic aneurysms with cardiopulmonary bypass. Moreover, plasma fHb levels 2 h after surgery predicted AKI in the early postoperative period. This finding sheds new light on the pathophysiology of AKI and offers a novel therapeutic target to improve clinical outcome after major cardiovascular surgery.

RESULTS

Open repair of thoracic and thoracoabdominal aortic aneurysms with cardiopulmonary bypass causes AKI

We studied 35 consecutive patients undergoing open repair of a thoracic aortic aneurysm ($N=1$) or thoracoabdominal aortic aneurysm ($N=34$) with cardiopulmonary bypass. Clinical data are presented in Table 1. None of the patients were diagnosed with preoperative hemolytic disease. Overall, the in-hospital mortality was 20% ($N=7$), and there were no intra-operative deaths. A total of 19 patients (54%) developed postoperative AKI (Table 1) as defined by the AKIN (AKI Network) classification. Mortality was numerically higher in AKI patients than in those without AKI (32%, $N=6$; vs 6%, $N=1$), but this difference did not reach statistical significance ($P=0.10$). Renal function fully recovered during hospitalization in 85% ($N=11$ out of 13) of the surviving AKI patients. In the remaining two AKI patients, renal function improved from stage 3 to stage 2. The impact of AKI on overall patient outcome was reflected by a trend toward longer hospitalization times (21 (13–34) days vs 14 (12–16) days, $P=0.05$), and intensive care unit admission times (7 (4–28) days vs 4 (3–7) days, $P=0.11$) in AKI vs non-AKI patients, respectively. Baseline renal function (estimated glomerular filtration rate (eGFR) estimated by the abbreviated Modification of Diet in Renal Disease equation[15]) did not differ between patients with or without postoperative

Table 1 | Characteristics of patients undergoing open surgical repair for thoracic or thoracoabdominal aortic aneurysms with cardiopulmonary bypass

	Non-AKI (*N*=16)	AKI (*N*=19)	*P*-value
Preoperative variables			
Age (years), median (IQR)	58 (54–66)	68 (56–72)	0.14
Male, *n* (%)	9 (56)	14 (74)	0.31
Indication, n (%)	—	—	0.04
TAA	1 (6)	0 (0)	
TAAA type I[a]	2 (13)	1 (5)	
TAAA type II[a]	2 (13)	9 (47)	
TAAA type III[a]	7 (44)	5 (21)	
TAAA type IV[a]	1 (6)	0 (0)	
TAAA type V[a]	3 (19)	5 (26)	
Concomitant aortic disease, n (%)	—	—	0.50
Type B dissection (Stanford classification)	2 (13)	4 (21)	
Marfan disease	2 (13)	1 (5)	
Aortic stenosis (high grade)	2 (13)	0 (0)	
eGFR (ml/min per 1.73 m^2)[b], mean ± s.d.	77 ± 30	95 ± 43	0.28
Chronic kidney disease (eGFR < 60), *n* (%)	8 (28)	9 (31)	0.70
Intra-operative variables			
Selective organ perfusion, *n* (%)	14 (88)	19 (100)	0.20
Total operation time (min), mean ± s.d.	369 ± 92	433 ± 96	0.06
Cardiopulmonary bypass time (min), mean ± s.d.	163 ± 56	195 ± 64	0.14
Aortic cross clamp time (min), mean ± s.d.	114 ± 58	144 ± 71	0.21
Blood loss (L), median (IQR)	8 (5–25)	8 (6–21)	0.86
Packed cell transfusion ($n \times 350$ ml units), median (IQR)	10 (7–16)	10 (7–18)	0.95
Postoperative variables			
In-hospital mortality, *n* (%)	1 (6)	6 (31)	0.10
Total intensive care unit stay (days), median (IQR)	4 (3–7)	7 (4–28)	0.11
Total hospitalization time (days), median (IQR)	14 (12–16)	21 (13–34)	0.06
Creatinine change < 48 h (%), median (IQR)	115 (93–129)	243 (166–300)	< 0.001
Acute kidney injury (stage 1, 2, or 3 of AKIN classification), n (%)	—	19 (100)	—
Stage 1	—	6 (32)	—
Stage 2	—	4 (21)	—
Stage 3	—	9 (47)	—
Dialysis	—	7 (37)	—

Abbreviations: AKI, acute kidney injury; AKIN, Acute Kidney Injury Network; eGRF, estimated glomerular filtration rate; IQR, interquartile range from the 25th to the 75th percentile; TAA, thoracic aortic aneurysm; TAAA, thoracoabdominal aortic aneurysm.
[a]Roman numerals refer to the TAAA classification system by Crawford[37]; see also the 'Materials and Methods' section.
[b]Glomerular filtration rate was estimated by the abbreviated Modification of Diet in Renal Disease (MDRD) as recommended by the National Kidney Foundation.[15]

AKI (95 ± 43 and 77 ± 30 ml/min per $1.73\,m^2$, respectively, $P = 0.28$).

Open repair of thoracic and thoracoabdominal aortic aneurysms results in hemolysis

To study the development of hemolysis during surgery, plasma fHb levels were analyzed at eight preset perioperative time points from preoperative until the second postoperative day. Preoperative fHb levels were comparable with values measured in healthy subjects ($<4\,\mu mol/l$). At the end of cardiopulmonary bypass, plasma fHb levels had increased significantly in AKI patients ($P<0.05$) compared with baseline values (Figure 1a). Plasma fHb values further increased in both groups in the first few hours after cessation of cardiopulmonary bypass ($P<0.001$) and remained elevated above the preoperative level during the first two postoperative days. Comparison of plasma fHb levels between patients with and without postoperative AKI (Figure 1b) showed that at 2 h after reperfusion, plasma fHb levels were significantly higher in AKI patients (16.6 ± 12.4 vs $7.9 \pm 4.2\,\mu mol/l$, $P<0.05$). Overall, plasma fHb concentrations of $\geqslant 6\,\mu mol/l$, levels associated with a reduction in microcirculatory blood flow, were measured in 83% of patients at any time point during the study period. Urine fHb was undetectable ($<2\,\mu mol/l$) in all patients, suggesting that glomerular filtration of fHb is unlikely to be a major contributor to AKI after cardiovascular surgery.

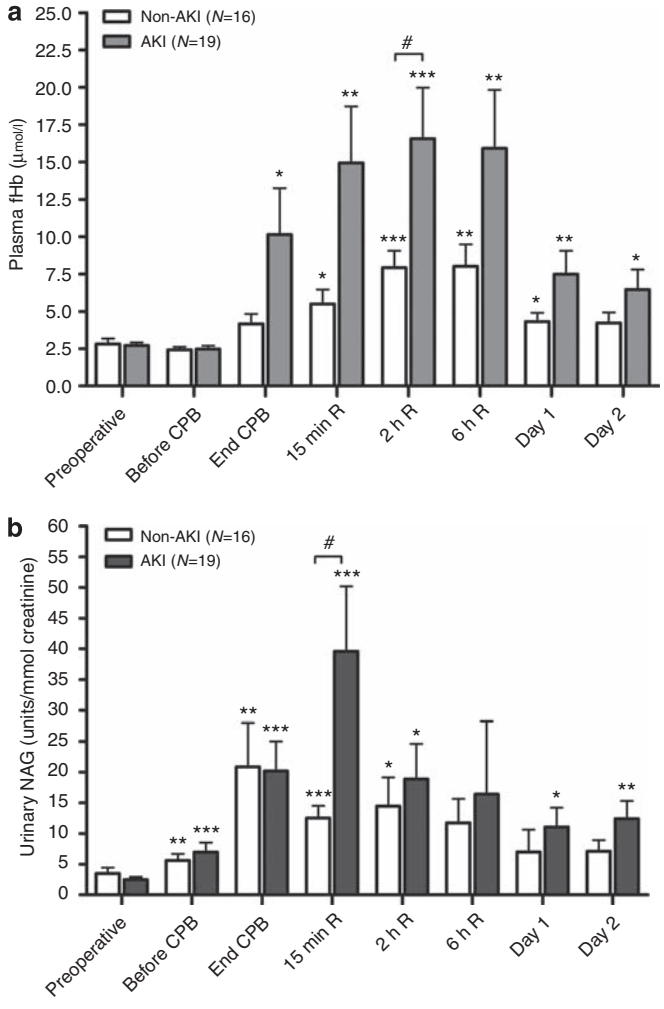

Figure 1 | Hemolysis and renal damage during open thoracic or thoracoabdominal aortic repair. (**a**) Hemolysis and (**b**) renal proximal tubular epithelial damage during open thoracic or thoracoabdominal aortic repair in patients with ($N = 19$) and without ($N = 16$) acute kidney injury. Urinary NAG concentrations were corrected for urine creatinine values. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with preoperative levels. $^{\#}P<0.05$ between AKI and non-AKI patients. AKI, acute kidney injury; CPB, cardiopulmonary bypass; fHb, free hemoglobin, NAG, *N*-acetyl-β-D-glucosaminidase; R, reperfusion.

Hemolysis is correlated with renal tubular injury during open repair of thoracic and thoracoabdominal aortic aneurysms

To study renal tubular injury during surgery, urinary *N*-acetyl-β-(D)-glucosaminidase (NAG) concentration was measured and corrected for urinary creatinine. NAG is a lysosomal brush border enzyme present in proximal tubular epithelial cells that are particularly vulnerable to ischemic injury.[16] Parallel to the development of hemolysis, a significant increase in urine NAG was observed during cardiopulmonary bypass (Figure 1b). NAG levels remained elevated above preoperative levels until the second postoperative day. Moreover, when comparing patients with and without AKI, significantly higher urine NAG levels were detected in patients with AKI at 15 min after reperfusion (39.6 ± 45.0 vs 12.5 ± 7.7 Units/mmol creatinine, $P<0.05$).

Next, we analyzed the association between hemolysis and renal tubular injury. To this end, the area under the curve of fHb (AUC_{fHb}) and NAG (AUC_{NAG}) was calculated, reflecting total fHb release and NAG excretion during the study period. Both AUC_{fHb} and AUC_{NAG} showed a strong positive correlation (Figure 2, Pearson's $r = 0.75$, $P<0.0001$). This

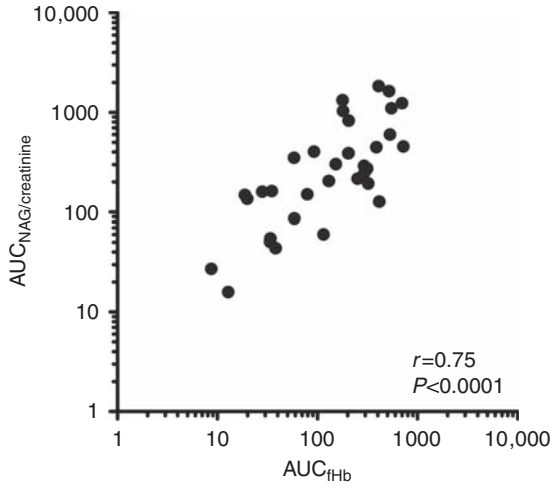

Figure 2 | Correlation of total plasma fHb release and urinary NAG excretion during the entire study period. Data were log transformed before entering into the Pearson's correlation test. AUC, area under the curve; fHb, free hemoglobin; NAG; *N*-acetyl-β-D-glucosaminidase.

Table 2 | Multivariable linear regression analysis for AUC_{NAG}

Variable	Beta	95% CI	*P*-value
Cardiopulmonary bypass time (min)	0.11	−2.30–2.51	0.93
AUC_{fHb}	1.31	0.57–2.05	0.001

Abbreviations: AUC, area under the curve; CI, confidence interval; fHb, plasma free hemoglobin; NAG, *N*-acetyl-β-D-glucosaminidase.

Table 3 | Multivariable linear regression analysis for perioperative creatinine change[a]

Variable	Beta	95% CI	*P*-value
Age (years)	3.34	0.97–5.71	0.01
Preoperative eGFR (ml/min per 1.73 m^2)	0.91	0.10–1.73	0.03
Cardiopulmonary bypass time (min)	−0.01	−0.54–0.52	0.97
$NAG_{15\,min\ R}$ (Units/mmol creatinine)	1.53	0.61–2.46	0.002

Abbreviations: CI, confidence interval; eGFR, estimated glomerular filtration rate; NAG, *N*-acetyl-β-D-glucosaminidase; 15 min R, 15 min after reperfusion.
[a]Creatinine change expressed as change from baseline to highest value reached during the first two postoperative days in percentage (%).

correlation remained when the subgroups of AKI and non-AKI patients were analyzed separately (Pearson's $r = 0.79$, $P < 0.001$ and $r = 0.64$, $P < 0.01$, respectively). Finally, AUC_{fHb} remained significantly associated with AUC_{NAG} ($P = 0.001$, Table 2) after adjustment for cardiopulmonary bypass time—a potential confounder—in multivariable linear regression.

Hemolysis and renal tubular injury are associated with postoperative renal dysfunction

To assess whether intra-operative renal tubular injury was associated with postoperative AKI, we analyzed the relationship between NAG levels (at 15 min reperfusion) and perioperative serum creatinine changes (the highest value reached within the first two postoperative days as a percentage from baseline). Using multivariable linear regression, we studied the association of NAG with creatinine increase independently of three established risk factors for AKI, namely preoperative age, preoperative eGFR, and intra-operative cardiopulmonary bypass time. NAG levels ($P = 0.002$), preoperative eGFR ($P = 0.03$), and age ($P = 0.01$) were significantly associated with serum creatinine increases (Table 3).

To study the prognostic value of plasma fHb and urine NAG for postoperative AKI, we analyzed the sensitivity and specificity of peak plasma fHb and urine NAG using receiver-operating characteristic curves (Figure 3). Peak fHb and NAG values were observed at 2 h and 15 min of reperfusion, respectively. Peak plasma fHb and urine NAG both held significant diagnostic power for the prediction of AKI (AUC 0.73, $P = 0.04$ and AUC 0.76, $P = 0.01$, respectively). Optimal cutoff values to discriminate between patients with and without AKI were 10 μmol/l for plasma fHb (sensitivity: 79%, specificity: 69%) and 14 Units/mmol creatinine for urinary NAG (sensitivity: 73%, specificity: 72%). This indicates that it is possible to differentiate between patients with and without postoperative AKI on the basis of plasma fHb levels shortly after surgery.

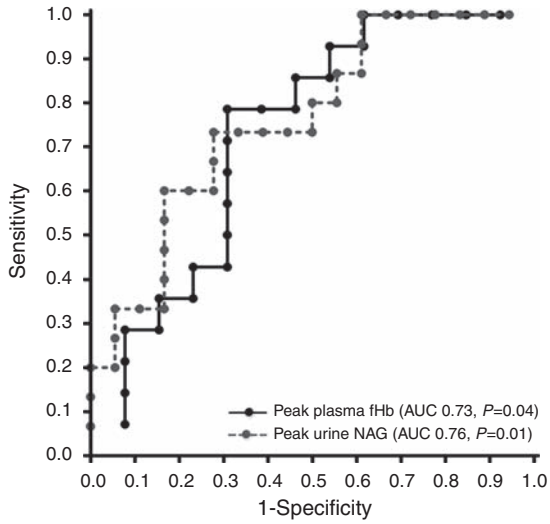

Figure 3 | Receiver-operating characteristics (ROC) curve presenting sensitivity and 1-specificity for peak plasma fHb levels at 2 h reperfusion (black line) and peak urine NAG levels at 15 min reperfusion (dotted line). AUC, area under the receiver-operating characteristics curve; fHb, free hemoglobin; NAG, *N*-acetyl-β-D-glucosaminidase.

DISCUSSION

AKI is a common complication after surgery using cardiopulmonary bypass with a profound impact on postoperative morbidity and mortality.[17] Despite major improvements in biocompatibility and flow performance of the bypass circuits, the incidence of AKI after cardiovascular surgery has not decreased significantly over the past decade.[18] Therefore, development of novel interventions to reduce AKI after major cardiovascular surgery requires further study of its pathophysiology.

In the current study, we show that the concentrations of plasma fHb and urine NAG increased during on-pump repair of thoracic and thoracoabdominal aortic aneurysms, which indicates that hemolysis and tubular epithelial injury occurred. Hemolysis during cardiac surgery has previously been reported and is considered to be caused by cardiopulmonary bypass circuits, transfusion of stored red blood cells, and cell salvage devices.[8,19–21] Interestingly, we found that the total release of plasma fHb was independently correlated with urine NAG, which in turn was independently associated with the postoperative increase in serum creatinine. This clinical observation is supported by rodent experiments in which intravenous infusion of fHb increased urine NAG concentrations 10-fold, while decreasing GFR by 30%.[22] Furthermore, scavenging of plasma fHb by infusion of haptoglobin in patients undergoing cardiac surgery was associated with a four-fold reduction in urine NAG as compared with a historical cohort without haptoglobin infusion.[23] In addition, cardiac surgery without cardiopulmonary bypass, which is expected to attenuate intravascular hemolysis, causes a significant reduction in postoperative AKI as compared with on-pump surgery.[24] In our study, peak plasma fHb levels $\geqslant 10$ μmol/l held a significant diagnostic value for the

prediction of AKI. The sensitivity and specificity of this marker to predict AKI in clinical practice should be validated in future studies. Taken together, these findings indicate that plasma fHb is a risk factor for tubular epithelial damage and AKI after cardiovascular surgery.

The diagnosis of AKI using serum creatinine is difficult, as serum creatinine is a poor marker of early renal dysfunction in the nonsteady state of the surgical patient. As the impact of AKI on the outcome of the patient is profound, the quest for sensitive and specific biomarkers has been designated as priority. During the past decade, several candidate biomarkers (such as neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, and interleukin-18) were reported; however, none of them have been generally accepted for clinical use. The diagnostic accuracy of urine NAG and plasma fHb in the current study might provide a valuable tool for early detection of postoperative AKI, enabling timely therapeutic interventions.[25] Nevertheless, sensitivity and specificity may be overestimated when calculated with the same data set that has been used to determine the optimal cutoff point for a diagnostic test. Therefore, the diagnostic accuracy of our suggested cutoff point for plasma fHb should be validated in future studies.

The observed relationship between plasma fHb and renal damage in this human setting is important as earlier studies on hemolysis-induced AKI focussed traditionally on the role of filtered fHb in urine.[13,26,27] Indeed, urine fHb and its catabolic products iron and heme catalyze the generation of reactive oxygen species that damage the tubular epithelium when present in the glomerular ultrafiltrate.[14] In support of this, administration of the iron scavenger deferoxamine attenuated the glomerular and tubular dysfunction induced by intravenous administration of fHb in rats.[28] Similarly, reduced plasma ferritin concentrations—which may be associated with reduced iron scavenging—were associated with increased AKI after human cardiovascular surgery.[21] Second, fHb may precipitate in the acidic ultrafiltrate, forming casts that obstruct the tubular lumen and reduce glomerular filtration.[14,29] Prevention of cast formation by alkalinization of the urine reduced tubular injury and glomerular dysfunction after intravenous fHb infusion in rats.[14] As fHb was not detected in urine samples of our patients, we propose that the glomerular filtration of fHb is unlikely to be a major contributor to AKI.

An additional and complementary explanation for the induction of renal injury by hemolysis is provided by the recent discovery that plasma oxy-fHb converts the vasodilator nitric oxide into biologically inactive nitrate.[10] As nitric oxide has a central role in blood flow regulation, reduction of nitric oxide bioavailability by fHb may impair tissue perfusion and induce AKI. In line with this observation, induction of hemolysis by water infusion or direct administration of fHb in dogs increased plasma nitric oxide consumption and resulted in elevated systemic vascular resistance and reduced creatinine clearance. These hemodynamic effects of acute hemolysis were attenuated by nitric oxide inhalation, which converts oxy-fHb into biologically inactive methemoglobin.[12] Furthermore, in patients with chronic hemolytic disorders such as sickle cell disease and malaria, the extent of hemolysis was correlated with endothelial dysfunction, pulmonary hypertension, and coagulation.[30–32]

In the light of these findings, we propose that the association between acute hemolysis and renal injury in patients undergoing on-pump cardiovascular surgery may be caused by scavenging of nitric oxide through fHb. First, plasma fHb levels in our study are comparable with concentrations reported to reduce blood flow, nitric oxide bioavailability, and impair renal function in animal experiments and men.[10,12] Second, in patients after on-pump surgical repair of thoracic and thoracoabdominal aortic aneurysms, we found that the increase in forearm blood flow after infusion of the nitric oxide donor sodium nitroprusside was significantly lower at the time of peak plasma fHb concentration as compared with the increase at 1 day after surgery. As the vascular response to acetylcholine—with vasodilatory effects that are largely independent of nitric oxide—was similar at these time points, the impaired reactivity to sodium nitroprusside is most likely not caused by vascular dysfunction but rather by reduced bioavailability of the administered nitric oxide donor (Hanssen *et al.*, submitted). These data, although providing indirect proof, support our findings and suggest that scavenging nitric oxide by plasma fHb may contribute to the association between acute hemolysis and renal injury.

The major strength of the current study is its translation of experimental findings on the effects of hemolysis on renal function into the clinical setting of on-pump cardiovascular surgery. Patients undergoing open repair of thoracic and thoracoabdominal aortic aneurysms are particularly suitable for clinical investigation of the pathophysiology of AKI, as these individuals are at exceptionally high risk of postoperative renal dysfunction. The strong and significant correlations between hemolysis and renal injury in our patients should not be interpreted as causal relationships. The results from the current study require further analysis in larger patient groups, and interventional studies using haptoglobin infusion or nitric oxide inhalation to inactivate fHb or using nitrite administration to increase nitric oxide bioavailability may provide further support for a causal relationship between nitric oxide scavenging by plasma fHb and AKI after on-pump cardiovascular surgery.[12,23,33,34] The pathophysiology of AKI is highly complex and involves multiple factors other than intravascular hemolysis, such as ischemia and reperfusion, hemodilution, inflammation, and baseline renal function.[5] The current study provides additional insights into the pathophysiology of this condition relevant for the design of multivariable risk scores and future therapeutic studies.

In conclusion, we show that increased plasma fHb concentrations are independently associated with injury to the proximal tubular epithelium during surgery with

cardiopulmonary bypass. Peak plasma fHb concentrations 2 h after surgery were predictive of AKI within the first postoperative days. This finding sheds new light on the pathophysiology of AKI during on-pump cardiovascular surgery that may also have a role in other conditions associated with acute hemolysis, such as hemodialysis and major trauma.[35,36]

MATERIALS AND METHODS

Patients

Patients admitted to the Maastricht University Medical Center or University Hospital Aachen between May 2006 and November 2008 to undergo elective open repair of a thoracic aortic aneurysm or thoracoabdominal aortic aneurysm with cardiopulmonary bypass were included. The study was approved by the Institutional Review Board of both institutes, and written informed consent was obtained from every patient before surgery.

Patients requiring preoperative dialysis due to preexistent renal failure were excluded.

Preoperative clinical data were prospectively collected from the patient's medical record.

eGFR was estimated by the abbreviated Modification of Diet in Renal Disease as recommended by the National Kidney Foundation.[15] Thoracoabdominal aortic aneurysm type was classified according to Crawford's classification. Type I thoracoabdominal aortic aneurysms extend distally from the left subclavian artery, in any case above the sixth intercostal space, down to encompass the aorta at the origin of the celiac trunk and superior mesenteric artery. The renal arteries may also be involved, but the aneurysm does not extend into the infrarenal segment. Type II thoracoabdominal aortic aneurysms extend distally from the left subclavian artery till the aortic bifurcation. Type III thoracoabdominal aortic aneurysms begin in the distal half of the descending thoracic aorta, below the sixth intercostal space, and extend to the aortic bifurcation. Type IV aneurysms involve the entire abdominal aorta from the diaphragm to the bifurcation. Type V thoracoabdominal aortic aneurysms originate from the lower thoracic aorta to the aortic section just below the visceral arteries.[37]

Surgical procedure

An indwelling radial artery catheter was placed as part of standard anesthetic care. After induction, patients were placed in a left helical position. Thoracolaparotomy in types I, II, and III thoracoabdominal aortic aneurysms was performed through the sixth intercostal space. In type IV thoracoabdominal aortic aneurysms, a small thoracolaparotomy through the eighth intercostal space sufficed. In patients with an isolated thoracic aneurysm, a small laparotomy was performed. Surgical repair was typically performed from proximal to distal. After heparinization (0.5 mg/kg), cardiopulmonary bypass was established using a centrifugal pump with membrane oxygenation (Jostra Rotaflow with Jostra Quadrox, Maquet Cardiopulmonary AG, Hirrlingen, Germany). Depending on the extent of the aneurysm, cardiopulmonary bypass was expanded with a four-branched tubing system enabling pressure-controlled perfusion with oxygenated blood of the celiac trunk, superior mesenteric artery, renal arteries, and if necessary intercostal arteries, during aortic cross clamping. During aortic cross clamping, renal blood flow and perfusion pressure was closely monitored and kept at 200–280 ml/min, and 60–70 mm Hg, respectively. A cell-salvage device (CATS, Fresenius SE, Bad Homburg, Germany) was used in every patient. The exact surgical protocol was identical in both institutions and has been described in detail elsewhere.[38,39] Postoperatively, all patients were transported to the Cardiothoracic Intensive Care Unit for postoperative monitoring, circulatory, and ventilatory support.

Preoperative kidney dysfunction and postoperative AKI

All individuals with a GFR <60 ml/min per 1.73 m^2, irrespective of the presence or absence of kidney damage, were classified as having chronic kidney disease in accordance with the National Kidney Foundation Guidelines.[40] Postoperative AKI was defined according to the AKIN classification (Table 1).[41] The AKIN classification provides three grades of postoperative AKI of increasing severity (stage 1, stage 2, and stage 3) and is based on relative changes in either serum creatinine or urine output (whichever is the worst) compared with baseline (preoperative) levels. All patients requiring (transient) postoperative dialysis were classified as stage 3 AKI, irrespective of creatinine change. We stratified patients into the AKIN stages based on creatinine changes because of the fact that urine output data was not sufficiently recorded in all patients.

Blood sampling, urine sampling, and sample processing

Arterial blood was collected preoperatively and at eight preset perioperative time points; T1, preoperative; T2, before start of cardiopulmonary bypass; T3, end of cardiopulmonary bypass; T4, 15 min reperfusion (15 min after cessation of cardiopulmonary bypass); T5, 2 h reperfusion; T6, 6 h reperfusion; T7, day 1 postoperatively; and T8, day 2 postoperatively. Whole blood was collected in EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA). Simultaneously, a fresh spot urine sample was collected from the bladder catheter. Whole blood and urine samples were immediately put on ice and centrifuged within 15 min after collection (1500 g at 4°C for 15 min), aliquoted, and stored at −80°C until further analysis.

Laboratory analysis of hemolysis and renal tubular damage

fHb concentrations, indicating hemolysis, were measured in all patients ($N=35$) by derivative spectrometry as described elsewhere.[42] The detection limit of the assay was 2 μmol/l.

To assess renal tubular damage, urinary NAG concentrations were determined by an enzyme colorimetric assay according to the manufacturer's instructions (HaemoScan, Groningen, The Netherlands). NAG is excreted predominantly by the proximal renal tubular cells. Owing to its relatively large molecular weight (>130 kDa), high levels of urinary NAG preclude glomerular filtration. Therefore, increased levels of NAG imply renal tubular damage. Furthermore, NAG is not reabsorbed by the tubules.[43] Results were normalized to urinary creatinine values and expressed as Units/mmol creatinine.

Statistics

Continuous data were presented as median (interquartile range from the 25th to the 75th percentile) or mean ± s.d, depending on Gaussian distribution (checked using histograms and normal Q–Q plots), and as N (%) for dichotomous data. Differences in patient characteristics between study groups were compared using Pearson's χ^2 test with Fisher's correction, when appropriate, for dichotomous variables. Continuous data were analyzed using independent sample t-test or Mann–Whitney U-test depending on Gaussian distribution. Subsequently, changes in fHb and NAG levels compared with

preoperative values were tested using the paired *t*-test. To characterize the total amount of hemolysis and tubular injury during the study period, the AUC for log-normalized fHb (AUC_{fHb}) and NAG (AUC_{NAG}) was calculated for each patient using trapezoidal analysis with time as a baseline. Subsequently, the correlation between hemolysis and tubular injury was analyzed using Pearson's correlation in the total patient population ($N = 35$) and subgroups of AKI and non-AKI. To correct for other risk factors, the relationship between AUC_{NAG} and AUC_{fHb}, and the relationship between NAG values and changes in perioperative serum creatinine levels were assessed by multivariable linear regression analysis (raw data were used). On the basis of the number of included patients, we could adjust for three other covariates in the latter multivariable linear regression analysis. To analyze the predictive value of plasma fHb and urinary NAG for AKI, receiver-operating characteristics curves were drawn by plotting sensitivity values against 1-specificity for all possible thresholds. The overall accuracy of the markers in detecting AKI was represented by AUC. Best cutoff points were defined as the maximum sum of sensitivity and specificity. Statistical calculations were made using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA), and Prism 4.03 for Windows (GraphPad Software, San Diego, CA, USA). A *P*-value <0.05 was considered to indicate statistical significance.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank Dr S Rensen for critical review of the paper.

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