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Impaired microcirculatory perfusion in a rat model of cardiopulmonary bypass: the role of hemodilution

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Koning NJ, de Lange F, Vonk AB, Ahmed Y, van den Brom CE, Bogaards S, van Meurs M, Jongman RM, Schalkwijk CG, Begieneman MP, Niessen HW, Baufreton C, Boer C. Impaired microcirculatory perfusion in a rat model of cardiopulmonary bypass: the role of hemodilution. Am J Physiol Heart Circ Physiol 310: H550–H558, 2016. First published January 8, 2016; doi:10.1152/ajpheart.00913.2015.—Although hemodilution is attributed as the main cause of microcirculatory impairment during cardiopulmonary bypass (CPB), this relationship has never been investigated. We investigated the distinct effects of hemodilution with or without CPB on microvascular perfusion and subsequent renal tissue injury in a rat model. Male Wistar rats (375–425 g) were anesthetized, prepared for cremaster muscle intravital microscopy, and subjected to CPB (n = 9), hemodilution alone (n = 9), or a sham procedure (n = 6). Microcirculatory recordings were performed at multiple time points and analyzed for perfusion characteristics. Kidney and lung tissue were investigated for mRNA expression for genes regulating inflammation and endothelial adhesion molecule expression. Renal injury was assessed with immunohistochemistry. Hematocrit levels dropped to 0.24 ± 0.03 l/l and 0.22 ± 0.02 l/l after onset of hemodilution with or without CPB. Microcirculatory perfusion remained unaltered in sham rats. Hemodilution alone induced a 13% decrease in perfused capillaries, after which recovery was observed. Onset of CPB reduced the perfused capillaries by 40% (9.2 ± 0.9 to 5.5 ± 1.5 perfused capillaries per microscope field; P < 0.001), and this reduction persisted throughout the experiment. Endothelial and inflammatory activation and renal histological injury were increased after CPB compared with hemodilution or sham procedure. Hemodilution leads to minor and transient disturbances in microcirculatory perfusion, which cannot fully explain impaired microcirculation following cardiopulmonary bypass. CPB led to increased renal injury and endothelial adhesion molecule expression in the kidney and lung compared with hemodilution. Our findings suggest that microcirculatory impairment during CPB may play a role in the development of kidney injury.

acute kidney injury; cardiopulmonary bypass; endothelium; microcirculation; hemodilution

NEW & NOTEWORTHY

The current study shows that impaired microcirculatory disturbances during cardiopulmonary bypass can only partially be explained by hemodilution. Endothelial and inflammatory activation are likely important contributors to microvascular perfusion deterioration. We further show that impairment of microvascular perfusion during cardiopulmonary bypass is associated with increased markers for renal injury.

MICROCIRCULATORY PERFUSION is impaired during and after cardiac surgery with cardiopulmonary bypass (CPB). We have previously shown that onset of CPB results in reduction of microcirculatory perfusion (31), and increased heterogeneity in the distribution of microcirculatory perfusion (30), which may lead to disturbances in tissue oxygenation and subsequent tissue injury (20, 24, 44).

Since impairment of microcirculatory perfusion is a negative predictor of outcome in critically ill patients (42) and is thought to play an important role in the development of organ dysfunction (21, 25, 37), insight in the mechanisms underlying perioperative microvascular dysfunction is warranted. Many factors related to cardiopulmonary bypass play a role in mi-
crocirculatory disturbances at the initiation of CPB, but hemodilution is attributed a central role (7, 11, 30). We previously showed preserved microvascular perfusion throughout the perioperative period in patients undergoing off-pump cardiac surgery without hemodilution (30). Findings from experimental studies showed that acute normovolemic hemodilution resulted in a reduction of perfused vessel density (9), increased endothelial activation (39), and impaired renal oxygenation (32). In addition, an increase in hematocrit by red blood cell transfusions during CPB improved microcirculatory perfusion in cardio surgical patients (2, 46). These findings suggest that hemodilution itself is associated with disturbances in microcirculatory perfusion. It is however unknown whether hemodilution is explanatory for all of the detrimental effects of CPB on the microcirculation, as CPB induces an additional inflammatory and endothelial activation that may contribute to microcirculatory impairment (1). This study therefore investigated the role of hemodilution in the impairment of microcirculatory perfusion observed during cardiopulmonary bypass in a rat model using intravital microscopy. We hypothesized that the deteriorating effects of CPB on microcirculatory perfusion are not completely explained by hemodilution, but additionally involve systemic inflammation and endothelial activation.

**METHODS**

**Animals.** All experiments were approved by the Institutional Animal Care and Use Committee of the VU University, and were conducted following the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the Dutch Animal Experimentation Act. Male Wistar rats of 375–425 g (Charles River Laboratories, Brussels, Belgium) were housed four per cage with unrestricted access to food and water in a temperature-controlled room (20–23°C; 40–60% humidity) with a 12:12-h light-dark cycle. Animal care was performed according to the national guidelines for care of laboratory animals. Twenty-four rats were randomly allocated to undergo a cardiopulmonary bypass (n = 9), hemodilution (n = 9), or sham protocol (n = 6). Additionally, six rats were euthanized immediately after induction of anesthesia for baseline assessment of RT-PCR analyses.

**Anesthesia and surgery.** Anesthesia was induced with 5% isoflurane in 100% oxygen followed by endotracheal intubation with a 16G catheter (Venflon Pro, Becton Dickinson, Helsingborg, Sweden). Volume controlled mechanical ventilation (UMV-03, UNO Roestvaststaal BV, Zevenaar, The Netherlands; tidal volume 10 ml/kg, ventilator frequency 60 min−1, positive end-expiratory pressure 2–4 cmH2O) was initiated with 2–3% isoflurane in an oxygen/air mixture with an FiO2 of 0.35. Rats were placed on a heating pad, the rectal temperature probe was inserted to maintain body temperature at 36.5°C, and ECG electrodes were connected.

The tail artery was cannulated with a 22G catheter (Venflon Pro, Becton Dickinson) for continuous arterial blood pressure monitoring. Fentanyl boluses (12 μg/kg, Janssen-Cilag, Tilburg, the Netherlands) were then administered intermittently at set time points in all groups, followed by a reduction of inhaled isoflurane concentration (1.5–2.0%). The left femoral muscle was isolated under warm saline superfusion according to the technique previously described by Baez (4), and with covered gas-impermeable plastic film (Saran Wrap, presoaked for 24 h in distilled water). Heparin (500 IU/kg, LEO Pharma, Amsterdam, The Netherlands) was administered. Subsequently, the right femoral artery was cannulated with a 20G catheter (Arterial Cannula, Becton Dickinson) for arterial inflow of the CPB circuit. Arterial blood gas analysis (Radiometer ABL-50, Radiometer, Brønshøj, Denmark) and hematocrit measurement were performed at baseline and repeated at 10, 30, and 60 min of extracorporeal circulation and 10 and 60 min after discontinuation of CPB, or at corresponding time points for hemodilution or sham experiments. Before initiation of a study protocol, a repeat-dose heparin (500 IU/kg) was given, in combination with pancuronium bromide (0.5 mg/kg, Organon, Oss, The Netherlands). Rats were euthanized, and renal and pulmonary tissue was harvested directly after the last microcirculatory recordings.

**Hemodilution protocol.** The right jugular vein was cannulated with an 18G catheter (Venflon Pro, Becton Dickinson). Six milliliters venous blood was withdrawn and exchanged simultaneously with 6 ml 6% hydroxyethyl starch (HES; Voluven, Fresenius Krabi, Halden, Norway). Hematocrit was controlled 10 min after hemodilution; additional blood was exchanged if hematocrit levels were higher than 0.25, to aim for a hematocrit of 0.22–0.24. Ventilation and temperature settings remained unaltered compared with baseline. In concordance with the CPB-group, protamine hydrochloride (2 mg/kg, Meda Pharma BV, Amstelveen, The Netherlands) was infused 90 min after initial hemodilution.

**Cardiopulmonary bypass protocol.** The protocol for cardiopulmonary bypass was based on a previous report (35). The CPB circuit, as previously described, consisted of a Plexiglas open venous reservoir, a roller pump (Pericor SF70, Verder, Haan, Germany), and a 4 ml Plexiglas oxygenator-heat exchanger (Ing. M. Humbs, Valley, Germany) with a three-layer hollow fiber membrane (Oxyphan, Membrana, Wuppertal, Germany) for gas exchange (12). A 1.0-mm-diameter arterial line (LectroCath, Vygon, Écouen, France) was connected to the femoral inflow catheter. The circuit was primed with 10 ml of 6% HES.

After cannulation of the right jugular vein with a modified multiorifice 4.5 French catheter (Desilets-Hoffman, Cook, Bloomington, IN) that was advanced into the right atrium, CPB was initiated. Flow rates of 150–200 ml·kg⁻¹·min⁻¹ were maintained during extracorporeal circulation, corresponding with 100% of the normal rat cardiac output (34). The venous cannula was positioned to minimize residual blood flow through the heart, which was associated with minimal residual arterial pulsations. Ventilation was discontinued; a mixture of oxygen and carbon dioxide was led through the oxygenator membrane to maintain P O2 values between 150 and 250 mmHg and P CO2 levels between 32 and 45 mmHg. Isoflurane (1.0–1.5%) was added to the gas mixture. Temperature was maintained between 35.0 and 35.5°C. At 65 min of CPB, ventilation was restarted at a frequency of 30 min⁻¹ and rats were rewarmed to 36.5°C. Weaning from CPB occurred after 75 min of extracorporeal circulation. The venous cannula was removed and the jugular vein was clamped. Protamine hydrochloride (2 mg/kg) was administered to neutralize heparin 15 min after weaning from CPB.

**Sham protocol.** Instrumentation of rats undergoing a sham procedure was identical to the hemodilution group, including heparin and protamine administration. No other interventions were made throughout the procedure, and all analyses occurred identical as in the other protocols.

**Intravital microscopy.** The microvasculature of the cremaster muscle was observed with a 10× objective (WN-Achromat, Zeiss, Oberkochen, Germany; numerical aperture 0.30) on an intravital microscope (AxioTechVario 100HD, Zeiss) connected to digital camera (scA640, Basler, Ahrensburg, Germany). Final magnification was 640×. Three regions of adequate tissue quality and perfusion were selected for baseline and all of the subsequent measurements. In each region, four videos of 10–15 s were recorded, so that 12 videos per time point were obtained. The camera was aligned to observe capillaries in a horizontal fashion on the screen. Baseline measurements were performed after a 30-min stabilization period of the cremaster muscle. Subsequent measurements were made at 10, 30, and 60 min of CPB and 10 and 60 min after discontinuation of CPB, or corresponding time points in other groups.
Microcirculation measurements. Microcirculatory analyses were performed off-line, and the investigator was blinded for the allocated treatment protocol. Similar to previously described methods, two vertical test lines were drawn on the screen, on which the capillary crossings were counted and averaged to obtain number of vessels per video screen (43). Vessels were subdivided in continuously perfused, intermittently perfused, and nonperfused capillaries (16). Spatial heterogeneity of microcirculatory perfusion was assessed by calculation of the coefficient of variation (SD/mean) of the number of perfused vessels over the 12 recordings per time point.

Gene expression analysis by real-time RT-PCR. Total RNA was isolated from cryosections from kidney and lung and isolated using the RNeasy Mini Plus Kit (Qiagen; Westburg, Leusden, The Netherlands) according to the manufacturer’s instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield and purity were measured with NanoDrop ND-1000 UVvis spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA was reverse-transcribed in cDNA using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The following Assays-on-Demand primers (Applied Biosystems Systems, Foster City, CA) were used for quantitative PCR: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (assay ID Rn01775763_g1), E-selectin (assay ID Rn00594072_m1), P-selectin (assay ID Rn00565416_m1), ICAM-1 (assay ID Rn00564227_m1), VCAM-1 (assay ID Rn00563627_m1), tumor necrosis factor-α (TNF-α) (assay ID Rn00562055_m1), interleukin-6 (IL-6) (assay ID Rn00561420_m1), and interleukin-10 (IL-10) (assay ID Rn00563409_m1). Samples were run in duplicate and the obtained threshold cycle values (CT) were averaged. Gene expression was normalized to the expression of the housekeeping gene GAPDH (∆CT). mRNA levels relative to GAPDH were calculated by 2^−∆CT values and averaged per group.

Immunohistochemical analysis. Renal immunohistochemical analysis was performed to assess glomerular neutrophil infiltration with myeloperoxidase (MPO) staining and for detection of an inflammatory status of the glomerular endothelium with N(epsilon)-carboxymethyllysine (CML) staining (28, 45). Following termination of the experiments, the left kidney was harvested and preserved in 4% formalin, embedded in paraffin, and cut into 4-μm-thick sections. The sections were dewaxed, rehydrated, and incubated in methanol/H2O2 (0.2%) for 30 min to block endogenous peroxidases. Next, antigen retrieval was performed either by heat inactivation in citrate buffer (pH 6.0) [myeloperoxidase (MPO), or enzymatic (CML)] at 37°C for 30 min using pepsin-HCl 0.1% solution. Slides to be stained with (pH 6.0) were incubated with Envision (MPO slides, undiluted, anti-mouse/rabbit, 0.02% H2O2) and counterstained with hematoxylin, dehydrated, and Cambridge, UK) or CML (1:2,000) for 1 h. After weaning from CPB, a 22% decrease in perfused capillaries compared with baseline was still observed (7.2 1.1 perfused capillaries per microscope field; P = 0.01 vs. baseline). The impairment of microcirculatory perfusion persisted at all measurements during and after extracorporeal circulation. At 1 h after weaning from CPB, a 22% decrease in perfused capillaries compared with baseline was still observed (7.2 1.1 perfused capillaries per microscope field; P = 0.01 vs. baseline).

No differences between groups in intermittently perfused vessels (Fig. 2B) could be detected. Hemodilution alone or the sham procedure did not increase the number of nonperfused microvessels at any time point (Fig. 2). However, immediately after initiation of cardiopulmonary bypass a fourfold increase in nonperfused microvessels was observed [from 0.2 (0.1–0.4) to 0.9 (0.5–2.0) nonperfused capillaries per microscope field; P = 0.014]. The number of stagnant capillaries remained increased, even after disconnection from cardiopulmonary bypass.

Spatial heterogeneity of microcirculatory perfusion did not alter compared with baseline in the hemodilution group or in the sham group. In the CPB group, spatial heterogeneity increased after onset of extracorporeal circulation (coefficient of variation 0.36 ± 0.05) compared with sham (0.34 ± 0.04).

RESULTS

The rats weighed 393 g (372–412), 415 g (386–419), and 410 g (392–424) for sham, hemodilution, and CPB groups, respectively (P = 0.286). All animals completed the experimental protocol and were analyzed on an intention to treat basis.

Systemic variables. In the hemodilution and CPB group, we aimed for a similar drop in hematocrit levels. Baseline hematocrit (0.43 ± 0.04 vs. 0.43 ± 0.02 l/l; P = 0.870) and hematocrit values at 10 min following initiation of CPB (0.24 ± 0.03 vs. 0.22 ± 0.02 l/l; P < 0.001 vs. baseline for both groups; P = 0.122 between groups) were comparable between the hemodilution and CPB groups, respectively (Fig. 1A). In the sham group, hematocrit levels remained unaltered throughout the experiment (ANOVA RM within-group effect P = 0.335). There was a transient decrease in mean arterial pressure (MAP) during hemodilution and CPB, but this returned back to baseline values toward the end of the experiment (Fig. 1B).

Microcirculatory perfusion during and after CPB. In the sham group, microcirculatory perfusion did not change during the experimental protocol (Fig. 2A). Hemodilution per se led to an initial reduction in perfused capillaries of 13% (from 8.8 ± 0.6 to 7.7 ± 1.1 perfused capillaries per microscope field; P = 0.028). This transient reduction in microcirculatory perfusion was only observed in the first two measurements after hemodilution, followed by restoration of the microcirculation to baseline values. Onset of cardiopulmonary bypass induced a 40% decrease in the number of perfused microvessels (from 9.2 ± 0.9 to 5.5 ± 1.5 perfused capillaries per microscope field; P < 0.001). The impairment of microcirculatory perfusion persisted at all measurements during and after extracorporeal circulation. At 1 h after weaning from CPB, a 22% decrease in perfused capillaries compared with baseline was still observed (7.2 ± 1.4 perfused capillaries per microscope field; P = 0.01 vs. baseline).
Renal and pulmonary inflammatory cytokine and endothelial adhesion molecule expression during and after CPB. Levels of renal and pulmonary pro- and anti-inflammatory cytokines were assessed using real-time RT-PCR and are presented in Fig. 3. CPB increased TNF-α (Fig. 3, A and B) and IL-6 (Fig. 3, C and D) mRNA expression in both kidney and lung tissue compared with the other groups (ANOVA \( P < 0.001 \) for both renal and pulmonary TNF-α, IL-6, and IL-10 mRNA expression). Hemodilution alone was associated with increased pulmonary TNF-α mRNA expression compared with baseline only. Additionally, the anti-inflammatory cytokine IL-10 mRNA expression (Fig. 3, E and F) was increased in the kidneys and lungs in the CPB group only.

The early endothelial adhesion molecules P-selectin mRNA (Fig. 4A) and E-selectin mRNA (Fig. 4C) showed increased expression after CPB in the kidney compared with all other groups (ANOVA \( P < 0.001 \) for renal P-selectin and E-selectin mRNA expression). In lung tissue, E-selectin mRNA was increased after CPB compared with baseline and sham group (Fig. 4D), whereas P-selectin mRNA expression was higher in both CPB and hemodilution groups than in sham and baseline groups (Fig. 4B; ANOVA \( P < 0.001 \) for pulmonary P-selectin and E-selectin mRNA expression). ICAM-1 mRNA (Fig. 4E) and VCAM-1 mRNA (Fig. 4G) were increased in the CPB group compared with all other groups in renal tissue (ANOVA
In this study we questioned whether hemodilution is explanatory for impaired microcirculatory perfusion following cardiopulmonary bypass. In a rat model for extracorporeal circulation we showed that cardiopulmonary bypass led to more microcirculatory disturbances, which were paralleled by increased endothelial activation and increased markers for renal damage, than hemodilution alone. Our findings suggest that the damage of the microcirculation as observed during and after cardiopulmonary bypass can only partially be explained by a dilutional component, but involves a cumulative effect of hemodilution with activation of inflammatory pathways.

The mechanistic cause of impaired microcirculatory perfusion during cardiopulmonary bypass has not yet been elucidated. First, it was assumed that microcirculatory perfusion disturbances originate from systemic hemodynamic alterations, but several strategies with vasoactive medications have failed to preserve perfusion of the microvasculature (8, 15, 36). In the current study, minor decreases in mean arterial pressure were observed after both hemodilution and onset of CPB, whereas particularly in the CPB group, the reduction in microcirculatory perfusion was substantial and did not recover after normalization of mean arterial pressure. Moreover, several studies have demonstrated that raising the mean arterial pressure target to 80 mmHg does not improve clinical outcome or splanchnic function (3, 38). Another cause of microcirculatory impairment may be nonpulsatile flow during CPB. Our group previously showed that pulsatile flow during cardiopulmonary bypass,
which is associated with improved endothelial function (33), improved the recovery of microcirculatory perfusion after cardiac surgery (31). A reduction of intraoperative hemodilution showed similar benefits (13), but neither intervention could prevent the initial decrease in microcirculatory patency.

Increased organ inflammatory cytokine expression and endothelial adhesion molecule expression activation induced by CPB (23), as we observed in the CPB group, are known to trigger processes that interfere with microcirculatory perfusion (16). These processes include increased adhesion of erythro-

Fig. 4. Renal and pulmonary endothelial adhesion molecule expression. mRNA expression of the endothelial activation markers P-selectin (A and B), E-selectin (C and D), ICAM-1 (E and F), and VCAM-1 (G and H) assessed in kidney and lung tissue. CPB was associated with increased endothelial activation compared with the other groups, whereas the hemodilution group showed increased activation of P-selectin and VCAM-1 relative to the sham and baseline groups. *P < 0.05, **P < 0.01, ***P < 0.001 between groups, as tested with Bonferroni post hoc tests.
cytes (14), and leukocytes (19), activation of the coagulation system, and endothelial swelling (6). Our findings demonstrate that the negative impact of cardiopulmonary bypass on microcirculatory perfusion and renal injury is more extensive than that of hemodilution alone. Therefore, other strategies to reduce inflammatory activation, and cytokine and endothelial adhesion molecule expression during cardiopulmonary bypass should be investigated to preserve the microcirculation and reduce organ dysfunction.

In line with previous studies, hemodilution to a hematocrit of 24% led to decreased microcirculatory perfusion (9) and increased expression of inflammation and endothelial activation (39). Hemodilution may lead to a reduction in the number of perfused capillaries through the Fahraeus and phase separation effects in the microcirculation (40). Alternatively, reduced blood viscosity, independently from the hematocrit level, may impair capillary perfusion during hemodilution, possibly because of reduced endothelial shear stress levels and reduced intracapillary pressure (10). Finally, reduced erythrocyte deformability during hemodilution may interfere with perfusion of capillaries with low diameters (27). The current study shows that hemodilution per se is already associated with renal endothelial inflammation compared with the sham group. From clinical studies, it is known that hemodilution during cardiopulmonary bypass might be a risk factor for acute kidney injury (22), probably through reduced tissue oxygenation following the drop in hematocrit (41). Although the use of hydroxyethyl starches is controversial nowadays with respect to kidney injury, recent experimental evidence shows that hemodilution with colloids leads to less renal injury than crystalloid hemodilution (32). As hematocrit correction with perioperative blood transfusion is by itself an independent risk factor for renal failure after cardiac surgery, it is of importance that the associated microcirculatory disturbances due to hemodilution are minimized to reduce additional defects in tissue oxygenation (25).

Albeit no causal relationship, microcirculatory impairment in the rats subjected to CPB was paralleled by inflammatory and endothelial activation and an increased expression of markers for renal injury, while these observations were completely absent in the sham group. It has been established that cardiac surgery-associated acute kidney injury is more prevalent following on-pump than off-pump coronary artery bypass grafting (18). It is likely that the observed microcirculatory disturbances are an important contributor to acute kidney injury observed following cardiac surgery (21,25). Our findings warrant further exploration of the association between
endothelial injury and microcirculatory dysfunction and the consequences for renal function following CPB.

Our study has several limitations. First, although the current rat cardiopulmonary bypass model is a modification of a previously described protocol (35), and enables direct in vivo microcirculatory observations, cardioplegic arrest was not induced in the current study. However, residual flow through the heart was only minimal as there was abolished arterial pulse pressure during CPB. Second, the current study is limited by the use of a closed-chest CPB with the accompanying reduction of surgical trauma compared with the clinical situation; our results show that microcirculatory perfusion (11,31), endothelial activation, and inflammation (26) are well in concordance with results from clinical investigations. Additionally, confounding influence of heparin and protamine on microcirculatory perfusion and endothelial and inflammatory activation were eliminated by equal administration of both drugs in all groups (23). Since intravital microscopy during CPB in a small animal model has not been described previously, the current setup may help to elucidate the mechanisms behind microcirculatory disturbances during CPB and its consequences for organ dysfunction.

In conclusion, the decrease in microcirculatory perfusion observed during cardiopulmonary bypass can only partly be attributed to hemodilution. Instead, increased cytokine expression and endothelial adhesion molecule expression during CPB are likely more important contributors to the deterioration of the microcirculation. We further showed that impairment of microvascular perfusion is associated with increased markers for renal damage, which is supportive for the hypothesis that microcirculatory perfusion impairment might contribute to the development of postoperative acute kidney injury (21, 25). However, as these hypotheses are based on associative data, further studies are warranted to prove the direct relationship between microcirculatory perfusion disturbances and postoperative renal dysfunction. Our findings furthermore suggest that interventions aimed at the preservation of capillary patency during CPB to improve postoperative organ failure should target cytokine expression and endothelial adhesion molecule expression rather than prevention of hemodilution.

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