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Original Article

No indications for platelet activation in acute clinical myocardial or renal ischemia/reperfusion injury

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Abstract: The pathophysiology of ischemia/reperfusion (I/R) injury is complex and poorly understood. Animal studies imply platelet activation as an initiator of the inflammatory response upon reperfusion. However, it remains unclear whether and how these results translate to clinical I/R. This study evaluates putative platelet activation in the context of two forms of clinical I/R (heart valve surgery with aortic-cross clamping, n = 39 and kidney transplantation, n = 34). The technique of sequential selective arteriovenous (AV) measurements over the reperfused organs was applied to exclude the influence of systemic changes occurring during surgery while simultaneously maximizing sensitivity. Platelet activation and degranulation was evaluated by assessing the expression levels of established markers, i.e. RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), β-thromboglobulin (β-TG), platelet-derived growth factor (PDGF)-BB and CXCL8 (known as interleukin-8), and by employing an in-vitro assay that specifically tests for platelet excitability. Moreover, a histological analysis was performed by means of CD41 staining. Results show stable RANTES, β-TG, PDGF-BB and CXCL8 AV-concentrations within the first half hour over the reperfused organs, suggesting that myocardial and renal I/R are not associated with platelet activation. Results from the platelet excitability assay were in line with these findings and indicated reduced and stable platelet excitability following renal and myocardial reperfusion, respectively. Histological analysis yield evidence of platelet marginalization in the reperfused organs. In conclusion, results from this study do not support a role for platelet activation in early phases of clinical I/R injury.

Keywords: Ischemia, reperfusion, inflammation, platelets

Introduction

Ischemia/reperfusion (I/R) injury is the paradoxical increase of damage upon reperfusion of ischemic tissue [1]. I/R injury is an inherent consequence of various clinical situations, such as therapeutic revascularization procedures (i.e. percutaneous or radiological revascularization after myocardial or cerebral infarction), cardiac surgery with arterial cross-clamping, and organ transplantation. The pathophysiology of I/R injury is complex and only partially understood, and at present no therapy has proven to be clinically effective [2].

It has repeatedly been shown that the earliest phases of I/R are dominated by an acute inflammatory response [3, 4]. Presently, mechanism(s) driving this acute and robust inflammatory response are still unknown. Platelets have been established as key initiators of the innate immune response. Upon activation, platelets release granules containing cytokines, chemokines, leukotrienes, and growth factors into their surroundings through the process of exocytosis. As such, they are implicated as key contributors to the initiation and/or amplification of reperfusion-associated inflammatory responses [5-8].

In spite of a wealth of preclinical evidence [8-11], the studies to date do not clearly define a role for platelets in the context of clinical I/R injury [2]. In this study, we evaluated putative platelet activation in the early phases of clinical I/R in two distinct clinical conditions, namely

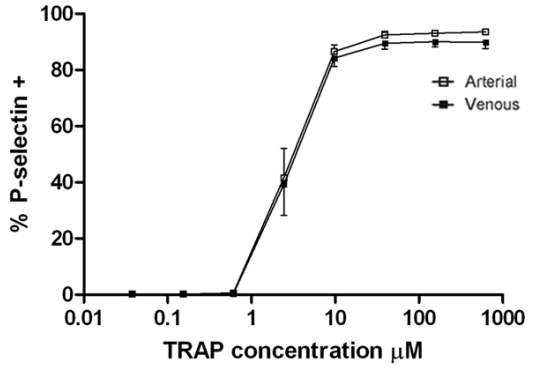

Figure 1. Typical examples of the platelet excitability assay. This assay was performed in order to explore more subtle changes in the activation status of platelets that passed the reperfused organ. Stimulation of platelets with increasing concentrations of thrombin receptor activating peptide (TRAP) results in progressive activation of platelets indicated by increased percentage P-selectin positive platelets. Results of the stimulation series are depicted in sigmoid shaped curves; arterial and venous percentages of P-selectin positive platelets can be compared.

cardiac valve surgery with aortic-cross clamping and kidney transplantation. Sequential arteriovenous measurements over the reperfused organs were applied [12], thereby eliminating systemic influences while also maximizing sensitivity by avoiding sample dilution [3, 4].

Materials and methods

Patient population

The study protocols of this single center study were approved by the Leiden University Medical Center institutional review board (P04.162 and P08.031), and all patients provided written informed consent.

Myocardial I/R (mitral valve surgery with aortic-cross clamping, and as a consequence compromised coronary blood flow) was studied in 39 patients undergoing mitral valve surgery for mitral regurgitation in the time period between 2008 and 2010. All patients were scheduled for restrictive mitral annuloplasty ring implantation with (out) additional procedures. As pre-existing conditions of the heart could influence the response to I/R, we studied patients with and without left ventriculair (LV) dysfunction separately. Nineteen patients had normal left ventricular function and underwent reconstructive mitral valve surgery for degenerative, rheumatic, or cured endocarditis mitral valve pathology. The other 20 patients had LV dysfunction, and were scheduled for restrictive mitral annuloplasty ring implantation with concomitant procedures such as implantation of an external cardiac support device, coronary artery bypass surgery and/or ventricular reconstruction surgery. Cardiac specific venous blood sampling was achieved by positioning a 5 French indwelling jugular vein catheter (PICC, Arrow International Inc., REF PS-01651, USA) in the coronary sinus by the surgeon. Since all patients underwent mitral valve surgery using a vertical transseptal incision, the coronary sinus was directly accessible for positioning and securing the catheter. Exclusion criteria and details of the procedure have been described previously [4, 13].

Renal I/R (kidney transplantation) was studied in 24 patients undergoing renal allograft transplantation at our institution between 2004 and 2008. Eight of them received a kidney from a living donor, nine of which were derived from a brain-dead donor, and seven patients a kidney from a cardiac-dead donor. Brain- and cardiac-dead donors are collectively referred to as deceased donors. The platelet excitability assay became available after completion of the arteriovenous sampling for renal I/R. We therefore included an additional 10 patients that received a kidney from a living donor for evaluation of platelet excitability. Details of the arteriovenous sampling procedure over the renal graft have been described elsewhere [3, 14]. During surgery, no heparin or other drugs influencing platelet aggregation were administered. Preservation fluid did not contain anticoagulants either.

Collection of materials

In patients undergoing cardiac surgery or kidney transplantation, paired arteriovenous blood samples (radial artery and coronary sinus; renal artery and vein) were obtained at various timepoints during the first 30 minutes after reperfusion [3, 4]. All samples were collected in pre-cooled EDTA tubes and placed in an ice water bath. A pre-study evaluation excluded an effect of cooling or the use of EDTA as an anti-coagulant on the markers tested. Blood samples were centrifuged and the plasma was re-centrifuged to obtain leukocyte and platelet-free

Table 1. Clinical characteristics of patients with and without left ventricular dysfunction

	LV dysfunction (n = 20)	No LV dysfunction (n = 19)	*p* value
Clinical characteristics			
Age in years (SD)	65.0 (8.9)	63.6 (11.6)	0.78
Male gender (M:F%)	65:35	55:45	0.65
Creatinine in μmol/L (SD)	95.4 (26.7)	89.0 (20.4)	0.42
NYHA functional class (SD)	3.1 (0.3)	2.0 (0.8)	< 0.001*
Comorbidities			
Diabetes, no. (%)	7 (35%)	2 (11%)	0.07
COPD, no. (%)	5 (25%)	2 (11%)	0.24
Medication			
ACE-inhibitors/ARBs, no. (%)	17 (85%)	10 (53%)	0.03*
Beta-blockers, no. (%)	19 (95%)	9 (47%)	0.001*
Statins, no. (%)	16 (80%)	6 (32%)	0.002*
Diuretics, no. (%)	19 (95%)	9 (47%)	0.001*
Antiplatelet therapy			
Acetylsalicylic acid, no. (%)	4 (20%)	4 (21%)	1.00
Clopidogrel, no. (%)	0 (0%)	0 (0%)	1.00
Dual antiplatelet therapy, no. (%)	2 (10%)	0 (0%)	0.49
Anticoagulants			
Anticoagulant alone, no. (%)	6 (30%)	5 (26%)	1.00
Anticoagulant + clopidogrel, no. (%)	2 (10%)	1 (5%)	1.00
Surgical characteristics			
Aortic cross-clamp time in min (SD)	124.2 (50.2)	142.1 (54.2)	0.52
CPB time in min (SD)	189.4 (60.4)	190.4 (63.4)	0.54
Surgery time in min (SD)	339.1 (79.7)	310.7 (84.9)	0.03*

Mean and standard deviations are shown unless stated otherwise. **p* values are given for the comparisons of patients with and without left ventricular (LV) dysfunction. Abbreviations: ACE, Angiotensin Converting Enzyme; ARB, Angiotensin Receptor Blocker; COPD, Chronic Obstructive Pulmonary Disease; CPB, Cardiopulmonary Bypass; NYHA, New York Heart Association.

plasma. Aliquots were stored at -70°C until analysis. Paired renal cortical biopsies were taken before and 45 minutes after reperfusion.

Laboratory measurements

Plasma measurements: Plasma levels of RANTES were determined using a semi-automated enzyme-linked immunosorbent assay (ELISA; anti-hCCL5/RANTES, MAB278 and AB-278-NA, R&D systems) on a TECAN Freedom Evo robot. Plasma levels of β-thromboglobulin (β-TG) were measured manually by ELISA (human β-TG, MAB393; R&D systems). Platelet derived growth factor-BB (PDGF-BB) and CXCL8 were measured in a multiplex assay (X-plex, BioRad, Veenendaal, the Netherlands). Platelet counts in the arterial and venous samples were assessed with a certified routine laboratory test (Sysmex XE-2100, Sysmex Corporation, Kobe, Japan).

Platelet excitability assay: Changes in platelet excitability were explored by a state-of-the-art assay for platelet excitability [15]. Arterial and venous samples from ten patients undergoing living donor kidney transplantation and from five patients undergoing cardiac surgery were collected in citrate-coated tubes at 0 and 30 minutes after reperfusion. Whole blood was then added to tubes containing either the platelet agonist adenosine diphosphate (ADP), collagen-related peptide (C-RP) or increasing concentrations of a thrombin receptor activating peptide (TRAP). Incubations were performed in the presence of fluorescent anti-P-selectin and anti-GP1b antibodies for subsequent flow cytometry analysis. Activation status and excitability of platelets were evaluated by assessing GP1b positivity and P-selectin positivity in a FACS-Calibur flow cytometer adjusted in a standard configuration (Cytomics FC 500 flow cytometer, Beckman and Coulter, Krefeld, Germany). Analysis included assessment of the maximum percentage of activated platelets (% P-selectin positive), and intensity of the signal of activated platelets (mean fluorescence index; MFI). The percentage of activated platelets increased with concentration of the agonist in a sigmoid shaped curve (**Figure 1**).

Platelet staining: Pre- and post-reperfusion cortical kidney biopsies (45 min. post reperfusion) were collected from living donor kidneys. Biopsies were snap frozen, after which 5 μm sections were fixed in acetone for 10 min. Slides were incubated with an anti-CD41 anti-

Table 2. Transplantation and outcome characteristics in living donor (LD), brain-dead donor (BDD) and cardiac-dead donor (CDD) kidney transplantation

	LD PEA, IHC (n = 10)	LD (n = 8)	BDD (n = 9)	CDD (n = 7)
Donor age: mean (SD)	53.1 (10.0)	43.9 (10.6)	54.1 (17.1)	52.7 (15.3)
Donor gender (M:F%)	30:70%	75:25%	44:56%	43:57%
Preservation fluid	HTK (n = 10)	HTK (n = 8)	UW (n = 9)	UW (n = 2) HTK (n = 5)
WIT1 in min. (SD)	N/A	N/A	N/A	23.1 (7.7)
CIT in h. (SD)	2.8 (0.4)*,▽	3.0 (0.3)*,▽	19.7 (6.2)	17.3 (2.6)
WIT2 in min. (SD)	30.9 (8.1)	34.0 (6.3)	33.0 (6.1)	34.1 (6.4)
Recipient age: mean (SD)	47.8 (16.1)	41.1 (10.5)	55.1 (13.5)	54.0 (11.2)
Recipient gender (M:F%)	70:30%	38:62%	44:56%	71:29%
Creatinine clearance day 30 (ml/min)	49.8 (6.1)▽	73.3 (20.5)*,▽	49.3 (15.3)▽	27.1 (10.3)
DGF (%)	0%	0%	56%	86%
DGF: dialysis after transplantation (days) (SD)	0 (0)*,▽	0 (0)*,▽	7.0 (5.3)▽	17.2 (7.2)

Mean and standard deviations are shown. LD procedures that were exclusively sampled for platelet excitability assay (PEA) and biopsy collection (IHC) are presented in a separate column. *P < 0.05 compared to BDD, ▽P < 0.05 compared to CDD. Abbreviations: BD, the total duration of brain dead of the donor; WIT1, first warm ischemia time; CIT, cold ischemia time; WIT2, second warm ischemia time (anastomosis time); DGF, delayed graft function (the need for dialysis within one week after transplantation.

body (ab30434, Abcam, Cambridge, United Kingdom), and CD41 positivity visualized using Envision and DAB (DAKO, Glostrup, Denmark).

Statistical analysis

All statistical analyses were performed with SPSS 20.0 (SPSS Inc., Chicago, USA). Clinical characteristics were compared using a Chi-Square-, Fisher's exact- or Mann-Whitney *U*-test. Areas under the curve (AUCs) were calculated for arterial and venous blood levels of all platelet activation markers. Arteriovenous AUCs and platelet counts were analysed by a paired *t*-test, and values expressed as mean ± SEM. Platelet excitability max. % P-selectin positive, and max. MFI were analysed by Wilcoxon-signed rank test. *P* values < 0.05 were considered significant.

Results

Patient population

Myocardial I/R (mitral valve surgery): Age, gender and ischemia times in the two patient groups undergoing mitral valve surgery were similar (**Table 1**). As expected, patients with LV dysfunction were on a more intense medical regimen. By definition, LV ejection fraction in patients with LV dysfunction was lower (32%) as compared to patients without LV dysfunction, (54%; P < 0.001). Follow-up of patients without LV dysfunction was uneventful; in the LV dysfunction group two patients died within a year due to ventricular fibrillation and therapy-resistant heart failure.

Renal I/R (kidney transplantation): Recipient and donor age and gender were similar in all donor groups (**Table 2**). As expected, the warm and cold ischemia duration differed between the groups, with shorter cold ischemia times in living donor kidney transplantation. A significantly higher incidence of delayed graft function (DGF) was observed in the deceased donor groups as compared to living donor kidney transplantation. There were no direct transplantation-related complications. With the exception of a kidney derived from a cardiac-dead donor where the recipient was not compliant with immunosuppressive medication, all grafts were still functional one year after transplantation.

No release of α-granule content following myocardial or renal I/R

Platelet activation in the first 30 minutes following reperfusion was assessed by comparing arteriovenous plasma concentration differences of the platelet α-granule contents. Surprisingly, the absence of net coronary RANTES, β-TG, PDGF-BB or CXCL8 release in patients with (P = 0.51, P = 0.67, P = 0.56, P = 0.25, respectively; **Figure 2**) and without LV dysfunc-

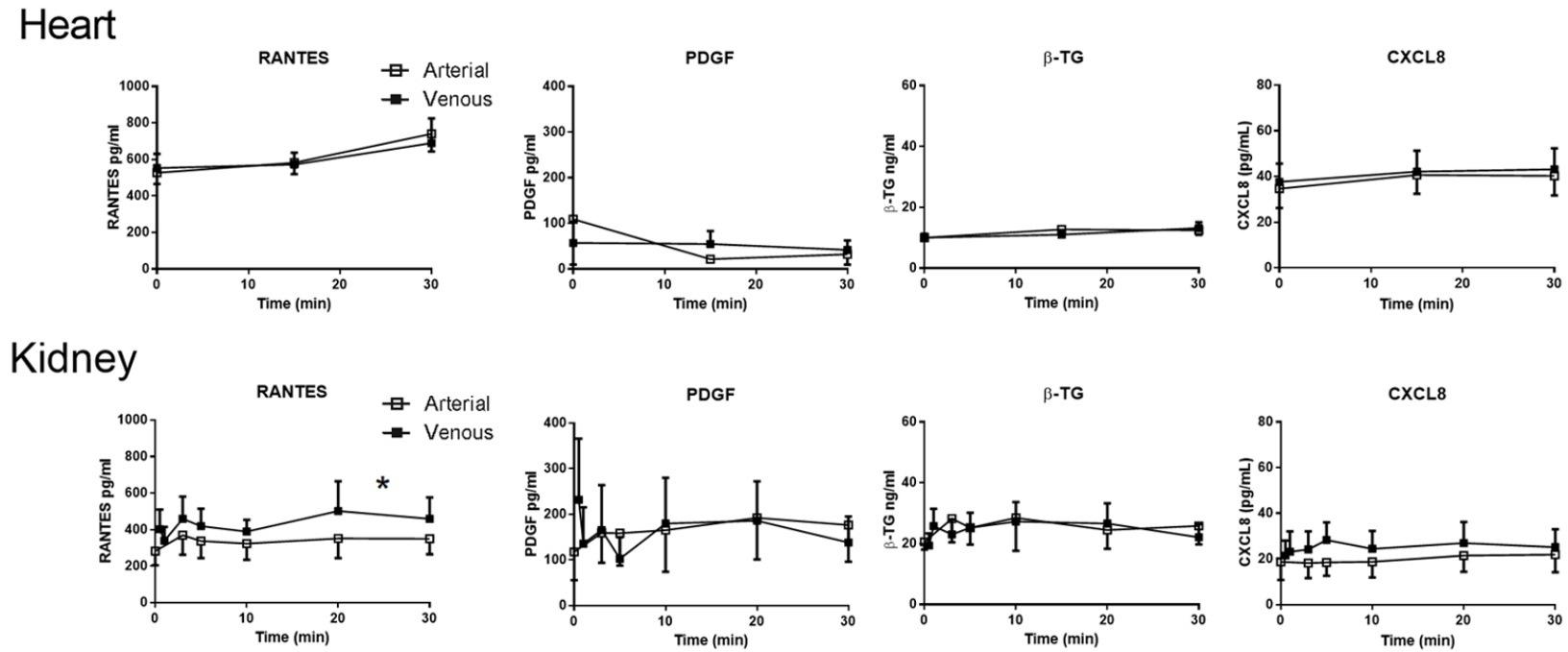

Figure 2. No release of α-granule content following myocardial or renal I/R. Arterial and venous concentrations of RANTES, platelet derived growth factor (PDGF)-BB, β-thromboglobulin (β-TG) and CXCL8 for the first 30 minutes of reperfusion are shown for patients with LV dysfunction (myocardial I/R) and patients receiving a brain-dead donor kidney graft (renal I/R). Data from other patient groups are not shown. Reperfusion was not associated with a myocardial release of RANTES, β-TG, PDGF-BB or CXCL8 in patients with left ventricular dysfunction (P = 0.51, P = 0.67, P = 0.56, P = 0.25, resp., n = 20). RANTES was released significantly from the reperfused kidney (P = 0.03), however other platelet degranulation markers β-TG, PDGF-BB or CXCL8 were not (P = 0.72, P = 0.51, P = 0.06, resp. (n = 9)). Bars represent SEM.

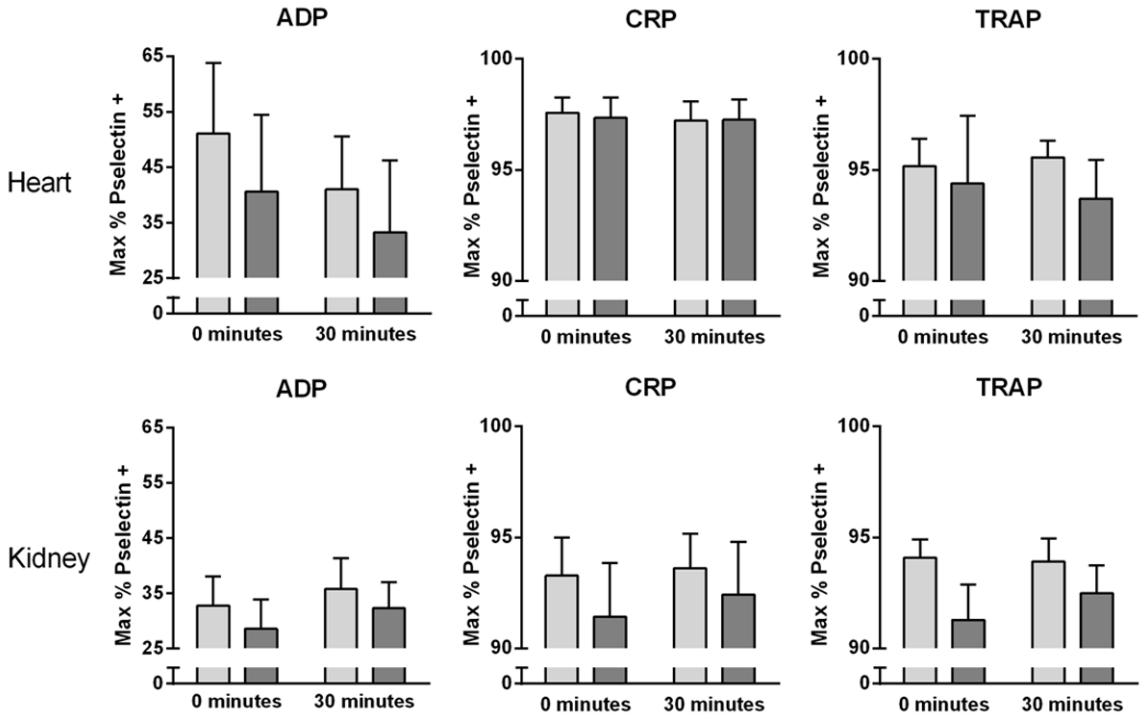

Figure 3. Maximum percentage activated platelets after stimulation with agonists adenosine diphosphate (ADP), collagen-related peptide (C-RP) or thrombin receptor activating peptide (TRAP) remained constant. In other words, transition through the reperfused myocardium did not influence the maximum percentage of activated platelets at 0 and 30 minutes after reperfusion for all three agonists; ADP (P = 0.14 and P = 0.29, resp.), TRAP (P = 0.69 and P = 0.11, resp.) and CRP (P = 0.14 and P = 0.71, resp.). Immediately after reperfusion (0 min.), a significant decrease in the maximum percentage of activated platelets over the kidney was observed for both C-RP (P = 0.013) and TRAP (P = 0.005). After 30 minutes of reperfusion changes were not statistically different (n = 10). Bars represent SEM.

tion (P = 0.73, P = 0.29, P = 0.32, P = 0.90, respectively, data not shown) refutes significant platelet activation upon myocardial reperfusion.

Expression levels of β-TG, PDGF-BB and CXCL8 in kidney I/R followed those of the myocardium (P = 0.72, P = 0.51, P = 0.06 respectively) with the sole exception of net RANTES release from reperfused grafts derived from brain dead donors (P = 0.03, **Figure 2**). We did not detect evidence of enhanced N RANTES, β-TG, PDGF-BB or CXCL8 release in living- (P = 0.11, P = 0.68, P = 0.28, P = 0.06, respectively, data not shown) or cardiac-dead donor kidneys (P = 0.34, P = 0.17, P = 0.14, P = 0.44, respectively, data not shown). Moreover, incident delayed graft function, the clinical manifestation of I/R injury in the context of kidney transplantation, was not preceded by platelet α-granule release (data not shown).

Platelet excitability assay

A platelet excitability assay was performed in order to explore more subtle changes in the activation status of platelets that passed the reperfused organ. Baseline (i.e. unstimulated) percentages of spontaneously P-selectin positive platelets were all < 1% and were not influenced by crossing the reperfused heart (arterial 0.87%, venous 0.96%; P = 0.50, at t = 0 minutes, and arterial 0.87%, venous 0.94%; P = 0.75 at t = 30 minutes) or kidney (arterial 0.24%, venous 0.22%; P = 0.36 at t = 0 minutes, and arterial 0.25%, venous 0.23%; P = 0.21 for 30 minutes after reperfusion). Maximum platelet excitability was subsequently assessed by stimulating the blood samples with established platelet activation agonists, namely ADP, C-RP and TRAP. The percentage of activated platelets and the mean fluorescence index (MFI) of activated platelets were used as a read out.

The transition through the reperfused myocardium did not influence the maximum percentage of activated platelets. In fact, at baseline as well as at 30 minutes post-reperfusion, platelet activation for the myocardial venous samples was similar to that observed in the

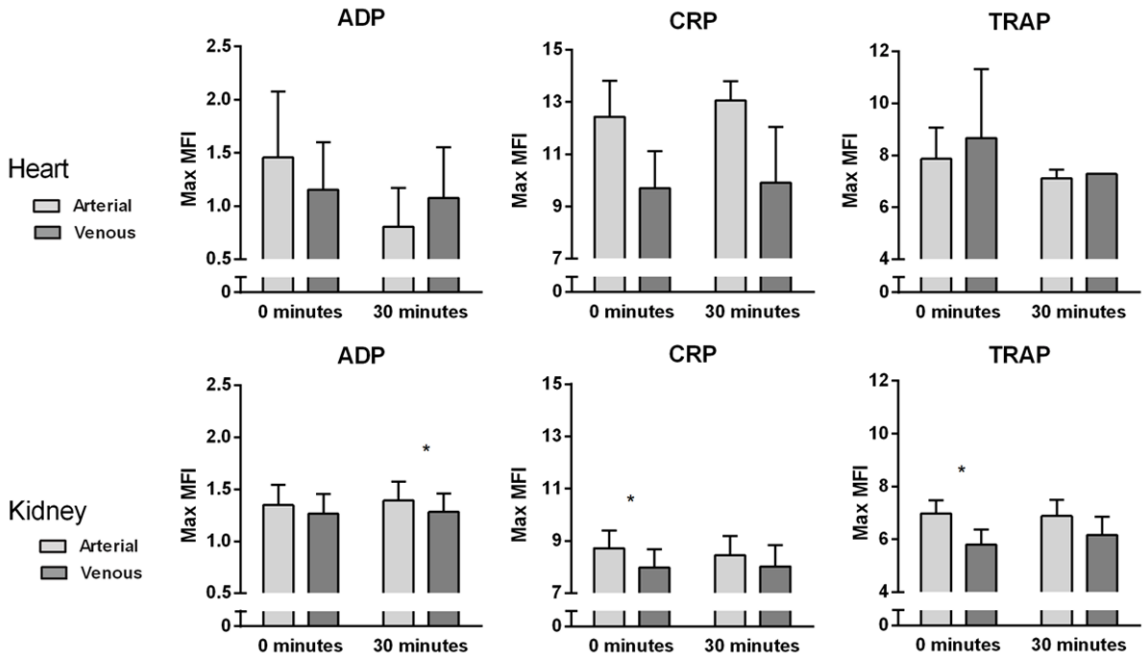

Figure 4. Maximum intensity of activated platelets was equal in myocardial venous blood compared to arterial blood after stimulation with agonists adenosine diphosphate (ADP, P = 0.29; P = 0.18 resp.), collagen-related peptide (C-RP, P = 0.11; P = 0.66 resp.) or thrombin receptor activating peptide (TRAP, P = 1.0; P = 0.32 resp.) immediately after reperfusion (0 min.) and after 30 minutes, respectively (n = 5). Directly after reperfusion (0 min.) there was a significant decrease in maximum intensity over the kidney for both C-RP (P = 0.047) and TRAP (P = 0.009). After 30 minutes of reperfusion only ADP activated platelets showed a significant decrease in intensity (P = 0.013, n = 10). Bars represent SEM.

arterial samples following treatment with all three agonists (ADP (P = 0.14 and P = 0.29, respectively), TRAP (P = 0.69 and P = 0.11, resp.) and C-RP (P = 0.14 and P = 0.71, resp.; **Figure 3**)). Similar results were observed for the MFI of activated platelets (myocardial venous blood compared to arterial blood at baseline and 30 minutes post reperfusion) for ADP (P = 0.29 and P = 0.18, resp.), C-RP (P = 0.11 and P = 0.66, resp.) and TRAP (P = 1.00 and P = 0.32, resp.) (**Figure 4**). Altogether, this platelet excitability assay strongly suggest that platelet excitability remains stable upon passing the reperfused heart.

In contrast, our kidney reperfusion data revealed significant reductions of in C-RP (P = 0.01) and TRAP-induced platelet excitability (P = 0.005) immediately following reperfusion. Of note, this effect was lost at 30 minutes following reperfusion (**Figure 3**). Similarly, the MFI of activated platelets was significantly lower in renal venous blood compared to arterial blood at 0 minutes after reperfusion for both C-RP (P = 0.047) and TRAP (P = 0.009). After 30 minutes, only ADP activated platelets were characterized by a significant decrease in MFI (P = 0.013, **Figure 4**). Overall, these results indicate that the passing of platelets through the reperfused kidney reduces their excitability.

Absent thrombocyte retention in the reperfused organs

Possible thrombocyte retention in the reperfused organs was assessed by quantifying arterial and venous thrombocyte counts upon reperfusion, as well as 30 minutes after reperfusion. No arteriovenous differences in the number of thrombocytes were detected following myocardial and renal reperfusion (data not shown). In parallel, we tested for the possibility that platelets could potentially be sequestered-ed in reperfused organs performing immunohistochemical analysis for thrombocytes in pre- and post-reperfused renal biopsies (45 minutes after reperfusion). These studies tested for expression of the cell surface marker CD41, and provided evidence of occasional platelet clusterings, mostly in glomeruli (**Figure 5**). Pre-

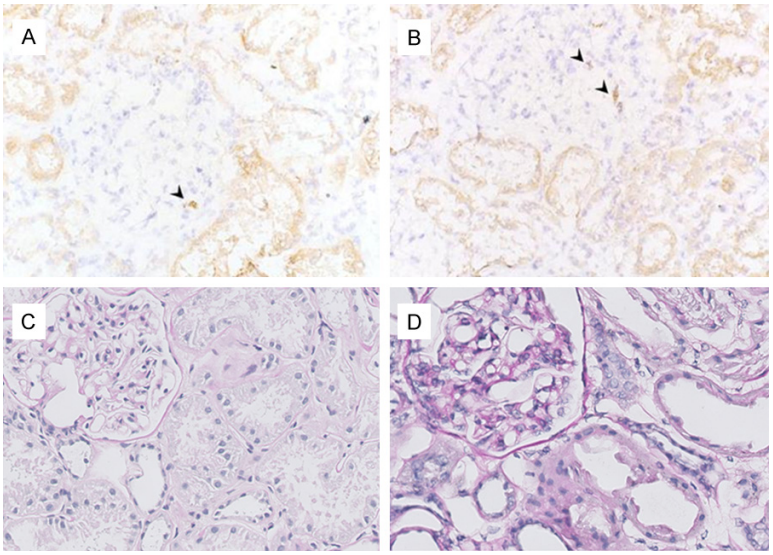

Figure 5. CD41 staining in renal biopsy tissue. Representative sections showing CD41 staining in living donor renal biopsy tissue collected (A) pre, and (B) post-reperfusion. Occasional CD41 positive cells were detected, mostly in glomeruli (arrows). In H&E stained pre- and post-reperfusion biopsies no platelet aggregates were detected (C: pre- and D: post-reperfusion), further indicating that the sporadic CD41 positive cells reflect adhering or rolling platelets as a physiological phenomenon instead of platelet sequestration in the kidney. Original magnification 200×.

and post-reperfusion staining patterns were similar, supporting the quantitative data for the arteriovenous thrombocyte counts.

Discussion

Preclinical studies identify activated platelets as a contributing factor to I/R injury [1, 9-11, 16]. However, a potential role for platelets in human I/R, and hence a place for anti-platelet agents in the prevention of clinical I/R injury remains unclear [2]. In this study, we evaluated the possible involvement of platelets in initiating the early phases of clinical I/R injury. Our evaluation of I/R injury in two different organs refutes a role for platelet activation in early I/R injury.

Platelets contribute to the inflammatory response through their release of cytokines, chemokines, and growth factors from their granules [17]. Numerous animal studies support a role for platelets in the post-reperfusion inflammatory response in the context of experimental I/R injury; a first study using intravital microscopy during mouse gut I/R injury showed that platelets roll and adhere to post-reperfusion endothelium through a P-selectin dependent mechanism [18]. On this basis, it was suggested that platelet recruitment and subsequent activation plays an important role in the pathogenesis of I/R injury [18]. Later studies showed that platelet adherence contributes to hepatic allograft damage in rats [19]. Moreover, a clinical association was reported between preoperative von Willebrand Factor-dependent platelet aggregation and functional von Willebrand Factor plasma levels, and hepatocellular damage in the context of liver transplantation [20]. A possible role for platelets in myocardial infarction has been studied by several groups such as Xu et al. [16], Liu et al. [21] and Seligmann et al. [22]. Using P-selecting knock-out mice, Xu et al. showed that interference with platelet adherence reduced infarct size in a murine model of myocardial reperfusion injury whereas Seligmann et al. showed that platelet-derived but not heart-derived reactive oxygen species contribute to myocardial I/R injury in a guinea pig model. Liu et al. showed that platelets mediate inflammatory responses and ventricular rupture or remodeling following myocardial infarction. In the context of experimental kidney I/R, vascular platelet sequestration has been shown to occur during early renal reperfusion in the mouse [23].

Despite this wealth of preclinical findings, a role of platelet activation in the context of clinical I/R is unclear with the current knowledge. Findings from a study evaluating the presence of P-selectin-positive platelets in post-reperfusion kidneys are inconsistent [24]. Similarly, while intervention studies using anti-platelet agents in myocardial infarction have generated beneficial effects [25], it is difficult to discern the possible direct effects on platelet activation from anti-inflammatory effects and effects on the prevention and formation of secondary microthrombi [26, 27]. In earlier studies we confirmed the non-specific inflammatory reaction after reperfusion [3, 4]. However, the inflammatory process was not instigated by

complement activation or oxidative damage [13, 14, 28]. In this study, we specifically addressed a possible role for platelets in the initiation of the post-reperfusion inflammatory response in two different organs. For myocardial I/R injury, we studied patients with and without LV dysfunction separately, since patients with LV dysfunction may be more susceptible to myocardial I/R injury [29]. For kidney transplantation, we expected platelet activation to be more pronounced in procedures involving kidney grafts of deceased donors. These kidneys are thought to experience a more intense I/R injury due to less favourable donor characteristics, and the longer periods of warm and cold ischemia.

This study uses sequential arteriovenous blood sampling in order to organ-specifically and sensitively assess the release of established platelet degranulation products from the reperfused organ. Findings for the myocardium were highly consistent and provided us with no evidence of release of all four markers tested. Findings for the kidney followed those for the myocardium, with the notable exception of decreased RANTES release from kidney grafts derived from brain-dead donors. In the absence of release of other platelet degranulation markers, it is plausible that this release reflects activation of T-lymphocytes and/or macrophages rather than platelets. In fact, T-lymphocyte and macrophage content is increased in kidneys from brain-dead donors [3].

High renal and myocardial blood flow may obscure low levels of platelet activation. In order to test for more delicate changes in platelet activation we used a novel and sensitive test to determine platelet excitability [15]. The results showed stable platelet excitability upon passage of the reperfused heart, and even decreased excitability upon passing the reperfused kidney graft. This inhibitory effect may well reflect endothelial release of short-acting inhibitors of platelet activation, such as nitric oxide and prostacyclin [30-32]. Due to their extreme short half-life such a mechanism is difficult to confirm in our arteriovenous samples.

Sequestration of activated platelets as an alternative cause of decreased excitability was excluded by analysis of thrombocyte counts and immunohistochemistry. Lack of sequestration of CD41-positive cells excludes platelet deposition as an indirect cause of reduced platelet excitability.

Limitations of our study include the sampling times, which were restricted to thirty minutes following reperfusion. As such, we cannot exclude the possibility that secondary platelet activation may occur in later stages of I/R. Nevertheless, such a scenario is not plausible, since all preclinical findings indicate instantaneous platelet activation after the first contact between blood and reperfused tissue. This study focuses on reperfusion-associated platelet activation; therefore, differences in activity of circulating platelets before start of sampling are not a parameter in this study. Although sample sizes for each condition are limited, results from all different conditions are highly consistent. As such, we consider it unlikely that a larger study size would result in different conclusions. Finally, results might be influenced by the antiplatelet/anticoagulation regimens in cardiac surgery patients. However, these regimens are not used in the context of kidney transplant patients in which we obtained similar results.

In summary, results from this study do not support a role for platelet activation in the initiation of clinical I/R injury or in the acute inflammatory responses that occurs after reperfusion.

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Disclosure of conflict of interest

None.

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