Chapter 5

Organ Perfusion During Cardiopulmonary Bypass: Blood Rheology and Endothelial (Dys)function.

Acute Isovolemic Hemodilution Triggers Pro–Inflammatory and Pro–Coagulatory Endothelial Activation in Vital Organs: Role of Erythrocytes Aggregation

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Abstract

The essential role of erythrocytes as oxygen carriers is historically well established, however their function to aggregate with consequences on homeostasis is under debate. The pathogenic potential of low erythrocyte aggregation might have implications for patients undergoing on–pump cardiopulmonary bypass who are severely hemodiluted due to preoperative isovolemic hemodilution (IHD), circuit priming, and large fluid infusions peri–operatively. Considering the vascular endothelium sensitivity to variations in blood rheology, we hypothesize that low erythrocyte aggregation will be responsible for activation of vascular endothelium during acute IHD. To verify this theory, we induced acute IHD (30 ml/kg exchange–transfusion with colloid–solutions) in an “aggregating species” (pigs, n=15), and investigated the hypoxic oxidative stress (plasma Malondialdehyde, ex–vivo oxygen radicals production in heart, lung, kidney, liver, ileum tissue biopsies), erythrocyte aggregation (LORCA), and endothelial activation (Real Time Quantitative RT–PCR to analyze von Willebrand Factor (vWF), E– and P–Selectins, endothelial nitric oxide synthase gene–expression in tissue biopsies). The production of superoxide and hydroxyl radicals, measured as $\mathrm{H}_2\mathrm{O}_2$ generation, was similar at all times in sham–operated and hemodiluted animals, proving a maintained oxygen delivery to tissues. Acute IHD was followed by a dramatic drop in erythrocyte aggregation and immediate pro–thrombotic (significant vWF mRNA up–regulation in heart, lungs, kidney, liver, ileum) and pro–inflammatory (significant E– and P–Selectins mRNA up–regulation in lungs and ileum) endothelial activation.

Low erythrocyte aggregation was statistically significantly correlated with increased mRNA–expression of vWF (heart, liver, ileum) and P–Selectin (lungs, ileum and heart). These results suggest that low erythrocyte aggregation can actively trigger endothelium–dependent thrombogenic and pro–inflammatory response during acute isovolemic hemodilution.
5.1 Introduction

The essential role of erythrocytes as oxygen carriers is historically well established, however, their function to aggregate with consequences on homeostasis is under debate. The aggregation property of red blood cells (RBC) is mainly considered to be pathophysiologic, since aggregation is elevated in many disease states such as diabetes mellitus\(^1\) and hypertension\(^2\).

Current understandings of blood rheology suggest complex mechanisms related to red blood cell hyper-aggregation. RBC hyper-aggregation is the main cause of increased blood viscosity under low shear conditions\(^3\). Increased aggregation is expected to augment the energy cost for breakdown of aggregates as blood approaches the microcirculation\(^4\). Enhanced RBC aggregation tends to promote axial accumulation of RBC in blood vessels, resulting in a less-viscous, plasma-rich region near vessel walls\(^5\). Decreased local viscosity of the marginal layers in blood vessels might be associated with decreased pressure gradients and hence lower wall-shear stresses for some vessels, thereby affecting vascular control mechanisms that are modulated by shear stress. Studies investigating the response of flow adapted endothelial cells, either in vivo or in vitro, demonstrated that positive or negative variation in shear stress at the vascular wall leads within minutes to membrane depolarization, increased intracellular Ca\(^{2+}\), nitric oxide and reactive oxygen species generation\(^6\). In addition to synthesis and release on demand, several stored compounds are secreted during mechanical endothelial cell stimulation, in a Ca\(^{2+}\) dependent way. Elevation in intracellular Ca\(^{2+}\) triggers release of several vasoactive factors and factors involved in hemostasis and thrombolysis: nitric oxide (NO), prostacyclin, von Willebrand factor, tissue factor, tissue plasminogen activator, adhesion molecules and chemoattractant proteins\(^7\). In this respect, increased RBC aggregation was reported to result in diminished nitric oxide-dependent vascular control and decreased endothelial NO synthase expression\(^8\).

To date and rather remarkable, the scientific approach to unravel this issue has completely ignored the pathogenic potential of low erythrocyte aggregation states. Some authors have suggested that normal levels of aggregation may serve homeostasis, having functional significance for normal physiology, as red cell aggregation is normally present in humans and other “athletic” species\(^9,10\). This hypothesis, however, has never been investigated before, and also, never been placed in a clinical relevant context.

The pathogenicity of low erythrocyte aggregation could have major implications for hemodiluted patients. This situation routinely occurs in cardiac patients undergoing on-pump cardiopulmonary bypass who are severely hemodiluted due to therapeutic preoperative isovolemic hemodilution, priming of the extracorporeal circuit and large fluid infusions peri-operatively. Excessive hemodilution prevails also during sustained fluid resuscitation in traumatic-hemorrhagic shock patients. In addition to the con-
sequences of hypoxic stress, the implications of low erythrocyte aggregation during acute hemodilution might prove to be essential for a full understanding of microcirculation impairment and deteriorated tissue perfusion in these patients. Considering the sensitivity of the vascular endothelium to variations in blood rheology, we hypothesized that low erythrocyte aggregation will be responsible for activating vascular endothelium during acute isovolemic hemodilution.

In this study we address the pathophysiology of acute isovolemic hemodilution in a clinical relevant animal model, studying hypoxic oxidative stress, red blood cell aggregation, and subsequent vascular endothelial activation.

### 5.2 Methods

This study was set up as a comparative, controlled, pseudo–double blind animal study, including a total of 15 adult pigs (60–80 kg). The experiments were in accordance with institutional and legislator regulations and approved by the local Committee for Animal Experiments. Two colloid solutions commonly used in clinical practice as plasma expanders were taken to induce acute isovolemic hemodilution (IHD). This experimental design was also based on our previous studies showing different effects of different molecular weight of hydroxyethyl starches (HES) on human red blood cells, with a pro–aggregatory effect increasing with the molecular weight of the colloid\textsuperscript{11,12}. The animals were randomized in three groups:

- **group 1** (n=6): 30 ml/kg isovolemic exchange transfusion with HAES–sterile 3% (HES 200/0.5, median molecular weight 200 kD, supplemented with Ringer’s lactate to a final concentration of 3%).
- **group 2** (n=6): 30 ml/kg isovolemic exchange transfusion with Voluven 3% (6% HES 130/0.4, median molecular weight 130 kD, supplemented with Ringer’s lactate to a final concentration of 3%).
- **group 3** (n=3): control group sham–operated animals.

Anaesthesia was induced with ketamine (i.m. 10 mg/kg) and diazepam (i.m. 1 mg/kg). Before intubation, the ventilation was performed using a mixture of O\textsubscript{2} and isoflurane 4%. After tracheal intubation, ventilation was performed with isoflurane 1.5–2%.

Isovolemic hemodilution was induced after cannulation of the jugular vein and carotid artery, by infusing HES at the arterial site, and a simultaneous withdrawal of an equal volume of blood. The drops in Hematocrit (Hct) and Hemoglobin (Hb) were monitored throughout the experiment, and adjusted to a constant value of 40% of the initial value (Fig. 5.1). No inotropic support was included in the protocol.

After 3 hours of maintaining the isovolemic hemodilution, tissue biopsies were obtained from the small intestine (ileum, luminal site), a randomly selected kidney
Figure 5.1: Hematocrit (%) variation during three hours of acute IHD, infused with either 3% HES 130/0.4 solution or 3% HES 200/0.5 solution. The controls are represented by sham-operated animals. The values are represented as mean (symbols) and standard error of the mean (bars).

(cortex), liver, lung, and heart. The biopsies were snap frozen in liquid nitrogen and stored at −80°C for real time RT-PCR measurements and histological assessments. Blood samples were collected at three time points: baseline (5 min after placement of the cannulae), post-infusion (5 min after induction of isovolemic hemodilution), and at the end of the experiment (3 hours of isovolemic hemodilution).

Test of red blood cell aggregation

The RBC aggregation measurements were performed on fresh arterial blood samples, using a Laser-assisted Optical Rotation Cell Analyzer (LORCA R&R Mechatronics, Hoorn, The Netherlands), and quantified as Aggregation Index (AI). This method closely mimics the in vivo blood flow conditions by applying a large range (0 to 500 s$^{-1}$) variations in shear rate and measuring the response in erythrocyte aggregation as indicated by the variation in the backscattered intensity from the blood layer$^{13}$. In short, for the determination of red cell aggregation, the blood was brought under a shear rate of 500 s$^{-1}$, after which the shear was stopped. The backscattered intensity from the blood layer was measured during 120 s after shear stop. The intensity drops because of red blood cell aggregation$^{14}$.

Viscosity measurements of plasma samples were performed with an automated dy-
namic shear rheometer with cone–plate geometry (AR1000 Rheometer, TA Instruments). During measurements the temperature was set at $37^\circ$C and the shear rate of operation at $100\text{s}^{-1}$.

**Test of hypoxic oxidative stress**

*Plasma Malondialdehyde (MDA)* – enzymatic detection, according to the method described by Esterbauer and Cheeseman$^{15}$.

$H_2O_2$ production in bioptic tissues: a fluorophore–nitroxide (Molecular Probes, Eugene, OR, USA) was used to image ex–vivo superoxide and hydroxyl radicals generated by cells$^{16}$. The reaction of fluorophore–nitroxide with superoxide results in a loss of electron spin resonance signal intensity concurrent with an increase in fluorescence emission. The fluorophore–nitroxide also reacts with methyl radicals generated by the reaction of hydroxyl radicals with DMSO$^{17}$. Biopsies from tissue of approximately 2 mm$^3$ and dry weight of 2–5 mg were incubated for 10 minutes in a microtiterplate in 50 µl of 0.1 M Tris–HCl buffer (pH 8.0) containing 2.5 mM pyruvate and 5 mM succinate to stimulate mitochondrial activity$^{18}$. Then 50 µl Tris–buffer containing 2 µM fluorescamine and DMSO (final concentration 2.5%) was added. The reaction was started after the addition of 5 µl FeII-EDTA (final Fe concentration 2 µM) in Tris buffer. In this way, both superoxide and hydroxyl radicals were converted and measured as $H_2O_2$.$^{19}$ The biopsies were incubated in this mixture for 10 min at room temperature on a plate shaker. After removal of the biopsies the fluorescence was measured in a multilabel counter (Victor2, EG&G Wallac, Turku, Finland) by using 390 nm excitation and 510 nm emission filters. Standard curves were obtained by adding known amounts of $H_2O_2$ to the assay medium. During incubation hemoglobin was released from the biopsies, resulting in quenching of the fluorescence signal. Thus, a separate standard curve was prepared including stepwise diluted hemoglobin ranging from 0.1 to 1.2 g/L. The linear relationship between hemoglobin concentrations and fluorescence signal was used to correct for the hemoglobin signal quenching. Hemoglobin concentration in the supernatant of the incubated biopsies was measured by the method of Harboe$^{20}$. Finally, measured $H_2O_2$ concentration was corrected for the dry weight of the biopsy.

*Diaminobenzidine (DAB) staining* – the production of $H_2O_2$ by cells in paraformaldehyde–fixed sections of ileum mucosa was histochemically demonstrated by incubating them for 30 min with 25 mg DAB/50 ml Tris/HCL pH 7.6, at 60$^\circ$C. Catalase (150 µg/ml, 1400 U/ml) inhibited the reaction, indicating that $H_2O_2$ was required to produce the chromogenic DAB staining.

**Endothelial activation:** Real–Time Quantitative Taqman RT–PCR on von Willebrand factor, E–Selectin, P–Selectin, and endothelial nitric oxide synthase (eNOS)
gene expression in heart, lung, kidney, liver and intestinal tissue biopsies. Total RNA was extracted using RNeasy Mini Kits (Qiagen, Venlo, The Netherlands), as recommended by the supplier. Total RNA was treated with 2 U of DNase I (RNase–Free DNase, Qiagen, Venlo, The Netherlands) in a volume of 15 µl to remove contaminating DNA (15 min at 37°C). First–strand cDNA synthesis: the mix of RNA (1 µg), 0.25 µg random hexamer primers and 2 ng of dNTPs (Promega, Leiden, The Netherlands) was heated for 5 min at 65°C and incubated on ice for at least 1 min, subsequently. The master mix [200 U SuperScript III (Invitrogen, Breda, The Netherlands) with 4 µl of 5× first–strnad buffer, 1 µl 0.1 M dithiothreitol, and 40 U RNase–OUT ribonuclease inhibitor (Invitrogen)] was added to the samples in a total volume of 20 µl; finally a reverse transcriptase program was performed (5 min at 25°C, 60 min at 50°C, 15 min at 70°C, ∼ at 4°C).

Quantitative PCR amplifications were performed on an ABI Prism 7900HT Sequence Detection System (Applera Nederland, Nieuwekerk a/d IJssel, The Netherlands). Primers and probes for von Willebrand factor, E– and P–Selectin, eNOS, CD31 (endothelial marker) and GAPDH (house keeping gene) were developed commercially (Custom TaqMan® Assays, Applied Biosystems–Applera Nederland BV, Nieuwekerk a/d IJssel, The Netherlands). The mRNA coordinates for the exon–exon boundaries were determined by aligning the human genomic sequences with pig mRNA sequences (Spidey alignment program, http://www.ncbi.nlm.nih.gov). As a precaution to prevent amplification of genomic DNA, primer/probe sequences were chosen such that they span exon junctions or lie in distant exons separated by long introns. The PCR step contained 1 µl of the appropriate RT reaction, 10 µl of TaqMan universal PCR master mix (Applied Biosystems), 200 nM primers, and 100 nM TaqMan probe in a final volume of 20 µl. The PCR cycling conditions were 2 min at 50°C, 10 min at 95°C, and 40 two–step cycles of 15 s at 95°C and 60 s at 60°C. All samples were assayed in triplicate.

Relative quantification of the mRNA levels was done by subtracting the GAPDH C_T (threshold cycle) from the investigated gene C_T value (ΔC_T = C_T gene – C_T GAPDH). Results were normalized with the average value of the respective gene in control sham–operated animals, arbitrarily set to 1. Results were finally expressed as \(2^{-\Delta C_T \text{ gene}} / 2^{-\Delta C_T \text{ CD31}}\) which represents an index of the relative amount of mRNA expressed in each tissue, corrected for the number of endothelial cells presented in each biopsy.

Plasma concentrations of endothelial vWF were investigated by means of ELISA (Coamatic von Willebrand Factor kit, Nodia BV, Amsterdam, The Netherlands).

**Statistical Analysis**

The statistical analysis was performed using SPSS (Statistical Package for the Social Sciences). Before analysis, the data was tested for distribution according to
Kolmogorov–Smirnov goodness of fit test. The variations over the study period were investigated using repeated measures ANOVA. To investigate differences between groups, continuous variables where compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). Correlation between non–parametric variables was performed with Sperman’s correlation test. Results are presented as mean±SEM (unless stated otherwise). Statistical significance was accepted at p<0.05.

5.3 Results

Immediately post–infusion the hematocrit (Hct, Fig. 5.1) reached 39.9±1.9% of baseline values in HES 130/0.4 hemodiluted animals and 41.3±2.2% of baseline values in HES 200/0.5 hemodiluted animals, but recovered by the end of the 3 experimental hours to 65.5±5.9% and 57.9±4.3% of baseline values, respectively. The extraordinary compensating capacity of the circulating number of erythrocytes was probably achieved by way of mobilizing spleen–trapped erythrocytes. This observation was supported by a smaller size and pale color of spleens in hemodiluted animals, as compared with those of sham–operated animals. To exclude the possibility of hemoconcentration due to loss of infused fluid through urine or extravascular extravasations, we performed plasma viscosity measurements. The baseline plasma viscosity levels (1.7±0.05 mPa.s) dropped in the hemodiluted animals immediately after infusion (1.39±0.06 mPa.s HES 130/0.4; 1.4±0.07 mPa.s HES 200/0.5) and remained low until the end of the experiment (1.35±0.13 mPa.s and 1.44±0.06 mPa.s, respectively) proving a comparable level of plasma dilution during the entire experiment.

<table>
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<th>Baseline</th>
<th>Post–infusion</th>
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<tbody>
<tr>
<td><strong>MAP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td>71.8±13.1</td>
<td>64.3±6.3</td>
<td>53.5±2.7</td>
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<td>HES 130/0.4</td>
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<td>48.3±5.5</td>
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<td>49.5±5.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>controls</td>
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<td>99±1</td>
<td>106±9</td>
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<tr>
<td>HES 130/0.4</td>
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<td>108±13</td>
<td>138±11**</td>
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<tr>
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<td>106±10</td>
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<td><strong>Arterial PO₂ (mmHg)</strong></td>
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<tr>
<td>controls</td>
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<td>70.7±3.1</td>
<td>69.1±1.6</td>
</tr>
<tr>
<td>HES 130/0.4</td>
<td>63.9±6.5</td>
<td>70.1±6.8</td>
<td>70.4±5.3</td>
</tr>
<tr>
<td>HES 200/0.5</td>
<td>62.4±7.1</td>
<td>68.4±7.8</td>
<td>69.6±7.1</td>
</tr>
</tbody>
</table>

Table 5.1: Mean arterial pressure (MAP), heart rate (HR) and arterial partial oxygen pressure (PO₂) during three experimental hours of isovolemic hemodilution
**Chapter 5**

**Hemodynamics**

Mean arterial pressure (MAP) and heart rate (HR) were monitored throughout the experiment (Table 5.1). MAP decreased gradually and significantly (Wilks Sig.<0.001) in all animals during the experiment. Immediately post-infusion, MAP was significantly lower in the HES 130/0.4 group (Mann-Whitney p=0.024) than the control group; after 3 hours of hemodilution no significant differences were seen anymore. The heart rate increased gradually, with a stronger rise registered in hemodiluted animals (Wilks Sig. p=0.009). At the end of experiment, the HES 130/0.4 group had significantly higher heart rates than the control group (Mann-Whitney p=0.024).

**RBC Aggregation**

RBC Aggregation (Fig. 5.2) decreased significantly after induction of hemodilution (Wilks Sig.=0.002), with an overall lower aggregation index (AI) in the experimental animals as compared with sham-operated animals (between subjects effect sig.=0.001). In HES 130/0.4 group, AI dropped to 39.2±4.8% of baseline values and maintained low during the experiment with values of 37.05±3.3% of baseline values at the end of experiment. In HES 200/0.5, AI declined post-infusion to 49.3±5.9% of baseline values and maintained low with 47.7±6.5% of baseline values at the end of experiment. Although the AI tended to be higher in the HES 200/0.5 group than in the HES 130/0.4 group, the differences were not significant at any time point.

![Figure 5.2: RBC aggregation during three hours of acute IHD, infused with either 3% HES 130/0.4 solution or 3% HES 200/0.5 solution. The controls are represented by sham-operated animals. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median of 6 measurements, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Symbol ◦ represents the outliers.](image)
**Hypoxic oxidative stress**

Arterial \( PO_2 \) increased moderately but not significantly after hemodilution (Table 5.1), expressing either an improvement in pulmonary gas exchange or a decreased diffusional oxygen exit.

**Plasma Malondialdehyde (MDA),** (Fig. 5.3a) dropped significantly right after infusion, due to the dilution effect. The relative increase in plasma MDA during the 3 experimental hours was comparable in the sham–operated animals (0.34±0.09 µmol), HES 130/0.4 infused animals (0.30±0.16 µmol) and HES 200/0.5 infused animals (0.40±0.18 µmol).

**Oxygen radicals production** (Fig. 5.3b). All animals showed a significantly higher \( H_2O_2 \) production in abdominal organs (ileum, kidney, liver) than in heart and lung tissues. Oxygen radicals production was comparable in all animals with no significant difference at any time point between hemodiluted and sham–operated animals.

A **DAB staining** of \( H_2O_2 \) producing cells in the ileum was performed, as the ileum seemed to be one of the organs exposed to oxidative stress. Fig. 5.3(c,d) shows a similar villi morphology, comparable staining intensity and distribution of \( H_2O_2 \) producing cells in both hemodiluted (Fig. 5.3c) and sham–operated animals (Fig. 5.3d).

**Vascular endothelial activation**

**Von Willebrand Factor (vWF) mRNA** (Fig. 5.4a) was significantly up–regulated in HES 130/0.4 hemodiluted animals when compared with sham–operated animals in all organs studied (Mann–Whitney: ileum, kidney, lung and heart \( p=0.024 \), liver \( p=0.048 \)). The same outcome was found in HES 200/0.5, with the exception of the lungs, where differences did not reach significance (Mann–Whitney: ileum, kidney, and heart \( p=0.024 \), liver \( p=0.048 \), lung \( p=0.095 \)). vWF mRNA responses did not differ between HES 130/0.4 and HES 200/0.5 treated animals.

**Plasma vWF systemic release** (Fig. 5.4b) translates the information found at mRNA level. Indeed, the relative increase in vWF plasma concentrations during three experimental hours in HES 130/0.4 group (30.32±4.6%) and in HES 200/0.5 group (27.9±1.3%) were significantly higher than the control values (0.1±0.01%) in sham–operated animals (Mann–Whitney \( p=0.024 \) for both comparisons).

**E–Selectin mRNA** (Fig. 5.5a) was significantly up–regulated in the ileum and lungs of HES 130/0.4 hemodiluted animals, as compared with the sham–operated ones (Mann–Whitney \( p=0.048 \), and \( p=0.024 \), respectively). HES 200/0.5 hemodilution resulted in significantly up–regulated E–Selectin mRNA in the ileum (Mann–Whitney \( p=0.024 \)).
when compared with control levels.

<table>
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<th>Sperman’s correlation</th>
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<tr>
<td><strong>P Selectin–ileum</strong></td>
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<tr>
<td>Correlation Coefficient</td>
<td>–0.614</td>
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<td>Sig. (2–tailed)</td>
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<td>Sig. (2–tailed)</td>
<td>0.011</td>
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</table>

Table 5.2: Statistical significant correlations found between RBC aggregation and markers of endothelial activation in different organs

**P–Selectin mRNA** (Fig. 5.5b) was up–regulated significantly in the ileum and lungs of both groups of hemodiluted animals (HES 130/0.4: ileum, lung Mann–Whitney p=0.024; HES 200/0.5: Mann–Whitney ileum p=0.024, lung p=0.048). Additionally for HES 200/0.5, the levels measured in the kidney reached significance when compared with controls (Mann–Whitney p=0.048).

**Endothelial nitric oxide synthase (eNOS) mRNA** (Fig. 5.6) was up–regulated significantly in the ileum and lungs in the HES 130/0.4 group (Mann–Whitney p=0.024 for both organs). In the HES 200/0.5 group, eNOS was up–regulated significantly only in the lungs (Mann–Whitney p=0.024).

**Correlations**

A significant negative correlation was found between the RBC aggregation index and levels of different markers of endothelial activation (Table 5.2).
Figure 5.3: Hypoxic oxidative stress during 3 hours of acute IHD (a) Plasma Malondialdehyde (MDA): The values are represented as mean (symbols) and standard error of the mean (bars); (b) Hydrogen peroxide ($H_2O_2$) production in heart, lung, kidney, liver, ileum tissue biopsies. Box plots graph data represent statistical values (see legend Fig. 5.2). (c) Diaminobenzidine (DAB) staining of $H_2O_2$-producing cells (brown coloration) in paraformaldehyde–fixed sections of ileum mucosa of hemodiluted and (d) sham–operated animals.
5.4 Discussion

Using our experimental model of acute isovolemic hemodilution we documented an immediate pro-thrombotic and pro-inflammatory endothelial activation in heart, lung, kidney, liver, and ileum, accompanied by a dramatic drop in erythrocyte aggregation. Erythrocyte aggregability correlated significantly with markers of endothelial activation suggesting a causality effect.

The dynamic rheological properties of blood are defined mainly by the coordinated self-organization of RBCs advancing in the arterio-venular direction. RBC hyper-aggregation is nowadays a generally recognized pathogenic factor, mainly due to clinical observation of increased RBC aggregation during disorders associated with macro and/or microvascular impairment, e.g. hypertension, diabetes mellitus, and chronic venous insufficiency. Hypo-aggregation of RBCs has been never described in a pathologic context. Given the strong conditioning effect of RBC aggregation on blood rheology, and thus on mechanistic endothelial activation, we hypothesized low RBC ag-

Figure 5.4: von Willebrand factor (vWF) (a) vWF relative gene expression of in the heart, lung, kidney, liver, ileum tissue biopsies after 3 h IHD. HES 130/0.4 Mann-Whitney: ileum, kidney, lung and heart $p=0.024$, liver $p=0.048$. HES 200/0.5 Mann–Whitney: ileum, kidney, and heart $p=0.024$, liver $p=0.048$, lung $p=0.095$. (b) vWF plasma concentrations.
Figure 5.5: E–Selectin (a) and P–Selectin (b) relative gene expression of in the heart, lung, kidney, liver, ileum tissue biopsies after 3 h IHD. E–Selectin: Mann–Whitney HES 130/0.4: ileum p=0.048, lungs p=0.024; HES 200/0.5: ileum p=0.024. P–Selectin: Mann–Whitney HES 130/0.4: ileum, lung p=0.024; HES 200/0.5: ileum p=0.024, lung p=0.048, kidney p=0.048.

Figure 5.6: Endothelial nitric oxide synthase (eNOS) relative gene expression in the heart, lung, kidney, liver, ileum tissue biopsies after 3 h IHD. HES 130/0.4 Mann–Whitney: ileum, lungs p=0.024. HES 200/0.5 Mann–Whitney lungs p=0.024.
Aggregation to be a pathogenic co-factor in endothelial activation during acute isovolemic hemodilution. To verify this hypothesis, we induced acute isovolemic hemodilution in an “aggregating species”, the pig\textsuperscript{23}, and investigated simultaneously the hypoxic oxidative stress, red blood cell aggregation, and gene regulation of von Willebrand factor, E– and P–Selectin, and eNOS, as markers of endothelial activation.

Hemodilution, by reducing the number of circulating RBCs is expected to decrease the oxygen–carrying capacity of blood and oxygen delivery to the tissue. However, during moderate levels of hemodilution, reduction of the systemic hematocrit up to 50% is compensated with an increased blood flow velocity and decreased diffusional oxygen exit from arterioles, resulting in augmented or maintained oxygen delivery to tissue\textsuperscript{24}. In addition, reduction of systemic Hct during intentional hemodilution is not mirrored at the microcirculatory level, with capillary Hct sustained near control levels\textsuperscript{25}, thus maintaining tissue oxygenation.

In an experimental animal study, Deem en al.\textsuperscript{26} showed that acute normovolemic hemodilution in healthy rabbits resulted in improved gas–exchange efficiency, as shown by higher arterial PO\textsubscript{2}, lower alveolar–arterial PO\textsubscript{2} difference, and increased expired NO. They postulated that the improvement in oxygenation appeared to be related to increased uniformity of pulmonary blood flow, and/or an increase in concentration of the vaso– and bronchodilator substance NO. Our data support this assumption and consistently show an up–regulation of eNOS in the lung tissue during acute hemodilution.

In our approach to detect changes in tissue oxygenation, we tested ex–vivo the mitochondrial (dys)function in the vital organs (heart, lung, kidney, liver, ileum) of hemodiluted animals, reflected by the production of reactive oxygen species when oxidizing pyruvate and succinate. Thus, we aimed at detecting mitochondria that were pre–exposed to hypoxia during hemodilution. The production of superoxide and hydroxyl radicals, measured as H\textsubscript{2}O\textsubscript{2} generation, was similar at all time points in sham–operated and hemodiluted animals, which indicates that a similar hypoxic oxidative stress was present, and oxygen delivery to the tissue during hemodilution was maintained. However, different organs seemed to have different exposure to hypoxia, with a more profound mitochondrial dysfunction in abdominal organs (ileum, kidney, liver) versus a preserved function of mitochondria in the myocardium and lung tissue.

The results found in tissue biopsies were mirrored by the plasma MDA determinations, that showed similar relative increase in systemic lipid peroxidation products during three experimental hours when hemodiluted animals and sham–operated animals were compared. These results suggest that, at least in this animal model, the perioperative stress and the anesthetic management are more important triggers of oxidative stress in abdominal organs, than hemodilution per se.

Because hypoxic stress seems to be negligible in this model of acute isovolemic hemodilution, we suggest that the effects observed in endothelial activation were mainly due to the drop in RBC aggregation.
Blood Rheology and Endothelial (Dys)function

Erythrocyte aggregation and endothelium–dependent pro–thrombotic activation

First reliable observations on the involvement of red blood cell in the process of clot formation were made by Turitto et al. who showed that under flow conditions platelet adhesion and thrombus formation increase as hematocrit values increase from 10% to 70%. They hypothesized that red cells may have a significant influence on hemostasis and thrombosis and the nature of this effect is apparently related to the flow conditions. More recently, it was demonstrated that erythrocytes markedly increase platelet eicosanoid formation, promote release of intracellular platelet granule components, and induce recruitment of additional platelets from the microenvironment into the forming thrombus.

The data presented in this study suggest a new pathway for erythrocyte involvement in clot formation: due to their function to aggregate, erythrocyte could modulate endothelial activation with von Willebrand factor release, with a subsequent pro–thrombotic effect. von Willebrand factor, which is stored in the endothelial Weibel–Palade storage granules, has unique biomechanical properties and a critical biological role as an adhesive protein. It mediates the adhesion of platelets to an injured vascular wall by binding on platelet surface and to collagen in the subendothelium. vWF is one of the most potent activators of platelets. Activation of platelets causes them to release additional vWF from their α–storage granules. Increased levels of plasma von Willebrand factor contribute directly to thrombosis, impeding the normal flow of circulating blood. In our experiment, acute isovolemic hemodilution was followed by a dramatic drop in red blood cell aggregation, which resulted in immediate pro–thrombotic endothelial activation as shown by a systemic increase in plasma vWF levels. Analysis of vWF mRNA expression levels in different vital organs showed a concomitant up–regulation in heart, lungs, kidney, liver, and small intestine. In addition, low red blood cell aggregation states were significantly associated with high vWF mRNA expression in heart, liver and ileum suggesting maybe a causality effect. An understanding of how disturbed blood flow might lead to disease is now emerging. Transferring this knowledge to a clinical relevant situation, as the one of the patients undergoing on–pump cardiopulmonary bypass, we hypothesize that lower incidence of thrombotic events could be achieved by avoiding excessive peri–operative hemodilution.

Low erythrocyte aggregation and endothelium–dependent pro–inflammatory response

The presence of a high RBC aggregation proved already its relevance in diagnosing the patients’ inflammatory status, using clinical observations of positive correlations between enhanced RBC aggregation and high plasma levels of C–reactive protein and fibrinogen. There is also evidence that RBC hyper-aggregation enhances the ten-
dencies of leukocytes to adhere to the postcapillary endothelium, a process recognized as essential in inflammation. Pearson et al.\textsuperscript{32} reported that increased RBC aggregation was associated with increased adhesion of white blood cells to the endothelium, possibly because of an enhanced probability of contact between leukocytes and the postcapillary venular wall.

In this study we discovered that RBC \textit{hypo}-aggregation, documented in our model of acute isovolemic hemodilution, was statistically significant correlated with up-regulation of endothelial adhesion molecules, E– and P–Selectins, especially in lungs and small intestine. E– and P–Selectins belong to the Selectin family of adhesion molecules and both have been reported to increase in circulation or at lesion sites of several diseases reflecting endothelial activation. Selectins play important roles in the inflammatory responses by facilitating leukocyte rolling and leukocytes activation\textsuperscript{33,34}.

Translation of these data in clinical terms suggests that acute hemodilution may lead to inflammatory stress of pulmonary capillaries. Subsequent diffusion limitation may be expected. Similar, an increased inflammatory response in the small intestine associated with acute hemodilution, might contribute to a loss in barrier function of the intestinal mucosa with subsequent translocation of endotoxins and/or bacteria.

\section*{Conclusions}

The data presented in this study show that acute isovolemic hemodilution definitely triggers endothelial activation. Since the effects of hypoxic oxidative stress seem to be negligible in this model, red blood cell \textit{hypo}-aggregation could be considered as a new pathophysiologic mechanism which could be held responsible for pro-inflammatory and pro-coagulatory endothelial activation. We hypothesize that a reduced inflammatory response and a lower incidence of thrombotic events will be achieved by avoiding excessive peri-operative hemodilution during on-pump cardiopulmonary bypass.

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