Ultrasound Monitoring of RBC Aggregation as a Real-Time Marker of the Inflammatory Response in a Cardiopulmonary Bypass Swine Model*

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Objectives: In many pathological conditions, including high-risk surgery, the severity of the inflammatory response is related to the patient outcome. However, determining the patient inflammatory state presents difficulties, as markers are obtained intermittently through blood testing with long delay. RBC aggregation is a surrogate marker of inflammation that can be quantified with the ultrasound Structure Factor Size and Attenuation Estimator. The latter is proposed as a real-time inflammation monitoring technique for patient care.

Design: Ten swine underwent a 90-minute cardiopulmonary bypass, and surveillance was maintained during 120 minutes in the postbypass period. To promote the inflammatory reaction, lipopolysaccharide was administered two times prior to surgery in six of those swine (lipopolysaccharide group). During the whole procedure, the Structure Factor Size and Attenuation Estimator cellular imaging method displayed a RBC aggregation index ($W$) computed from images acquired within the pump circuit and the femoral vein. Interleukin-6, interleukin-10, C-reactive protein, haptoglobin, immunoglobulin G, and fibrinogen concentrations were measured at specific periods.

Main Results: Compared with controls, the lipopolysaccharide group exhibited higher $W$ within the pump circuit ($p < 0.05$). In the femoral vein, $W$ was gradually amplified in the lipopolysaccharide group during cardiopulmonary bypass and the postbypass period ($p < 0.05$), whereas interleukin levels were higher in the lipopolysaccharide group but only at the end of cardiopulmonary bypass and beginning of postbypass ($p < 0.05$).

Conclusions: Continuous RBC aggregation monitoring can characterize the evolving inflammatory response during and after cardiopulmonary bypass. The Structure Factor Size and Attenuation Estimator is proposed as a real-time noninvasive monitoring technique to anticipate inflammation-related complications during high-risk surgery or critical care situations. Because RBC aggregation promotes vascular resistance and thrombosis, $W$ could also provide early information on vascular disorders in those clinical situations. (Crit Care Med 2013; 41:e171–e178)

Key Words: cardiopulmonary bypass; critical care; erythrocyte aggregation; perioperative care; systemic inflammatory response syndrome; ultrasonography

*Crit Care Med 2013; 41:e171–e178

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This work was performed at the Montreal Heart Institute and University of Montreal Hospital Research Center.

Supported, in part, by the Natural Sciences and Engineering Research Council of Canada (R2PJ-phase 1 grant #372485-08) and the Canadian Institutes of Health Research (grant # MOP-84358).

The authors have disclosed that they do not have any potential conflicts of interest.

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DOI: 10.1097/CCM.0b013e31828a2354

Cardiopulmonary bypass (CPB) surgery can lead to perioperative endotoxemia and triggers a cascade of events that includes a systemic inflammation sometimes leading to poor patient outcome (1–3). The early diagnosis and treatment when a disproportionate inflammatory reaction is noticed is therefore critically important. However, because of laboratory processing time, cytokine measurements correspond to an inflammatory process that has taken place before the time when the results are obtained. Thus, a noninvasive tool able to monitor real-time inflammation and to recognize it at the earliest state could result in improvement of patient outcome in case of sepsis or high-risk surgery by allowing the most appropriate intervention as early as possible.

Many studies have proposed to use instruments evaluating the microcirculation to detect inflammatory-related ischemic indices in both critically ill and high-risk surgical patients.
(4–6). Most of them monitor real-time microvascular perfusion of skin (e.g., with laser Doppler flowmetry and nailfold videocapillaroscopy), perfusion of other superficial tissues (e.g., with orthogonal polarization spectral imaging), or tissue oxygenation (with near-infrared spectroscopy, oxygen/carbon dioxide pressure, cerebral oxygen saturation, etc.), but they do present several theoretical or practical limitations (7–11). They all rely on measures sampled within localized ischemic regions presumably affected by the inflammatory process, and for some of these techniques, microcirculatory abnormalities are not associated with other ischemic indices (12, 13). Therefore, there is a need for other inflammation monitoring methods.

RBC hyperaggregation is a well-known hemorheologic disorder that leads to microvascular dysfunctions (14) and venous thrombosis (15). The two current explicative theories for RBC hyperaggregation emphasize the role of inflammatory plasmatic macromolecules, such as fibrinogen, immunoglobulin G (IgG), haptoglobin, and C-reactive protein (CRP) (16–20). RBC hyperaggregation is a global marker of inflammation during illness or surgery (21–24), and Reggiori et al (12) described a strong association between early alterations of RBC aggregation and sepsis occurrence in critically ill patients. Because abnormal RBC aggregation is a precursor of microvascular disorders and thrombosis, it leads to ischemic events and tissue damages that are further reinforcing the existing inflammatory condition (14).

In the context of postoperative sepsis, monitoring RBC aggregation could be a relevant marker of inflammation and tissue perfusion. To continuously evaluate RBC aggregation, we propose to use the Structure Factor Size and Attenuation Estimator (SFSAE). This new cellular ultrasonic in vivo imaging modality was recently developed and validated by our group (15, 25–27). We propose to use it to assess real-time variations of RBC aggregation as a surrogate marker of inflammation in a CPB swine model.

**MATERIAL AND METHODS**

The experimental procedures were approved by the institutional animal care committees of the Montreal Heart Institute and of the Research Center of the University of Montreal Hospital in accordance with the recommendations and guidelines on the care and use of laboratory animals issued by the Canadian Council on Animals and the U.S. National Institutes of Health (assurance number A5377-01).

**CPB and Perioperative Procedure**

Ten swine from a slaughterhouse (weight 70.3 ± 1.2 kg) experienced a 90-minute CPB procedure, as previously described (28). Twenty-four hours before and at the beginning of the surgical procedure, six swine received lipopolysaccharide (LPS) injection (0.8 μg/kg) to increase the likelihood of a significant inflammatory response, as described elsewhere (29–32). These six swine constituted the LPS group, whereas the four remaining ones formed the control group. Swine were anesthetized with a perfusion of propofol (0.1–0.2 mg/kg/min). A median sternotomy was performed immediately after anesthesia, ventilator connection, heparin injection (400 UI/kg), and the pericardium opening. Both the aorta and right atrium were cannulated and connected to a hollow fiber membrane oxygenator (Monolynth, Sorin, Irvine, CA) and a roller pump (Sarns 7000, Ann Harbor, MI) in a close circuit (Fig. 1). The heart was left beating during the whole CPB (90 min). Then, animals were reperfused for 120 minutes. At that time, swine were still ventilated, and anesthesia was maintained with a 2% isoflurane air mix. We tried to maintain the mean systemic arterial pressure between 50 and 70 mm Hg. Animals were euthanized at the end of the postbypass period.

Venous blood samples were drawn from the right leg before the surgery \(T_0\) and after 30- and 120-minute postbypass (respectively, \(T_30\) and \(T_90\), where subscripts are in min from \(T_0\)). During CPB, venous blood was drawn directly from the pump circuit after 30 and 90 minutes (respectively, \(T_30\) and \(T_90\)). The mean arterial pressure (MAP), HR (heart rate), and lingual pulse oximetry (Spo2) were recorded every 15 minutes.

**Ultrasound Acquisitions**

Ultrasound imaging was performed with a high-frequency scanner (Vevo 770; Visualsonics, Toronto, ON, Canada) equipped with two mono element oscillating probes. During the CPB, the first probe (RMV770; central frequency of 25 MHz) was placed over a tube deviation (length 50 cm, diameter 6 mm) connected to the inlet circuit of the pump, whereas the second probe (RMV707B; central frequency of 30 MHz) was placed over the exposed femoral vein during both CPB and postbypass periods (Fig. 1). RBC aggregation is a flow shear rate-dependent phenomenon. We therefore momentarily normalized the blood flow for 120 seconds before each measurement (33). In the pump deviation, an electromagnetic flowmeter (Cliniflow II; Carolina Medical Electronics, East Bend, NC) was used to monitor flow that was set at 3 mL/min before ultrasound measurements. In the femoral vein, a vascular occluder (Harvard apparatus, Saint-Laurent, Québec, Canada) was placed downstream of the ultrasound probe to adjust the flow. A Doppler velocity measurement provided feedback for adjusting the degree of occlusion (target velocity 1.5 mm/s for a typical vein diameter of 6 mm). Mean shear rates (γ) were postprocessed, as done by Yu et al (27), in both the pump and
femoral vein to control that every aggregation measurement was made under similar shear conditions. Venous instead of arterial monitoring was preferred because low shear rates offer favorable conditions for the formation and maintenance of aggregates.

**In Vivo Ultrasonic Radiofrequency Measurements**

Ultrasound acquisitions were performed every 15 minutes from $T_{15}$ to $T_{16}$ during the CPB and from $T_{105}$ to $T_{216}$ during the postbypass period. Baseline measurements were therefore performed soon (~15 min) after the beginning of surgery. Radiofrequency (RF) ultrasound echoes from longitudinal views of each vessel were acquired as described elsewhere (15). RF data correspond to the raw ultrasound signal that has not been modified by any signal processing normally used to obtain classical B-mode images. We used the frequency content of RF echoes to compute the proposed tissue characterization variable $W$. The ultrasound RF data were processed with the SFSAE (34) to compute 1) the mean packing factor of RBC aggregates ($W$), which is a dimensionless measure increasing proportionally with RBC aggregation, and 2) parametric images (filtered with the method [35]), as in Figure 2. A sequence of 25 vascular images of $W$ was spatially averaged to monitor the RBC aggregation process. $W$ time-varying curves were smoothed using a moving average filter with a span of three data points.

**Blood Sampling Analyses**

The hematocrit was measured immediately after every sampling time by microcentrifugation. The plasma was separated from the collected blood by centrifugation and stored at −80°C. Plasma concentrations of interleukin (IL)-6, IL-10, IgG, haptoglobin, and CRP were measured by enzyme-linked immunosorbent assay (Biovet, Sainte-Hyacinthe, Québec, Canada). Fibrinogen plasma concentrations were tested using the heat precipitation method (Biovet). Data were corrected for changes in plasma volume during CPB and postbypass periods using the method of van Beaumont (36).

![Figure 2. Examples of Structure Factor Size and Attenuation Estimator parametric images of red blood cell aggregation ($W$, longitudinal views) in case of low (left) and high inflammation (right). Data were acquired within the pump circuit deviation (top) and the femoral vein (bottom). The color maps vary from 0 (blue) to 40 (red). Variable $W$ has no unit. LPS = lipopolysaccharide; CPB = cardiopulmonary bypass.](image-url)
Statistical Analyses
Results are presented as mean ± SEM. The effects of LPS and CPB on RBC aggregation, IL-6, IL-10, fibrinogen, IgG, haptoglobin, CRP, and hemodynamic and oximetric changes were investigated separately using two-way analyses of variance (ANOVA) for repeated measures, using logarithmic transformations whenever the dependent variable was not normally distributed. The factors included in the ANOVA were the group, the time (considered a categorical variable), and their interaction. The interaction terms provided time-specific group differences. The Holm-Sidak method was used to correct the p values of these tests for multiple comparisons. Further investigations of the effect of CPB on RBC aggregation were conducted using linear mixed models with a group and time interaction to test for a group-specific time trend. Analyses were conducted using R (v.1.4.0, R Foundation for Statistical Computing, Vienna, Austria) and Sigma Stat (v. 3.1, San Jose, CA) packages. In figures and tables, statistical significance was defined as a p value of less than 0.05.

RESULTS
No Differences in Most Hemodynamic and Oximetric Variables Between Groups
As summarized in Table 1, MAP remained stable throughout CPB and the postbypass period up to T210, where lower values were noticed in both groups (69 ± 7 mm Hg at T0 vs 36 ± 3 mm Hg at T210; p < 0.001). There was no difference between groups except at T90 where the control group presented higher MAPs (p = 0.013). HR remained constant during all the procedure (even at T210) and did not exhibit any difference between groups. SpO2 did not present any time or group effect. Values typically remained close to 100% with some nonsignificant variations at the end of the CPB. Shear rates did not show any group or time effect in the femoral vein or in the pump circuit deviation, indicating no confounding influence of hemodynamic variables on RBC aggregation measurements. Following the initial perfusion, the hematocrit increased slightly in both groups, exhibiting higher values compared with baseline at T150 and T180 (minimum value of 26% ± 1% after the initial perfusion and maximum value of 29% ± 2% at T180; p < 0.001), but there were no differences between groups (data not shown). The native hematocrit of pigs before CPB was 30% ± 1%.

A Higher Blood-Marker-Sensitive Inflammatory Response in the LPS Group
As illustrated in Figure 3A, the plasma concentration of IL-6 increased during the postbypass period (T120 and T210) in both groups (p < 0.0001). This increase occurred earlier (at T90) in the LPS group (p < 0.001). Differences in IL-6 values between groups were only noted early in the postbypass period (T150; p = 0.007). IL-10 displayed in Figure 3B increased for both groups at T90 and T120 (p < 0.001) before returning to baseline at T210. This increase was more important in the LPS group compared with controls (p = 0.019 at T90 and p < 0.001 at T120). To summarize, the cytokine-sensitive inflammatory state was found higher in the LPS group but only at the end of CPB and early in the postbypass period.

As summarized in Table 2, fibrinogen also increased compared with baseline in the LPS group, early in the postbypass period (T120; p = 0.03), and differences between groups were only noticed at this time point (p < 0.001). IgG and haptoglobin did not show any time or group effect. CRP did not vary over time, but the LPS group presented with higher values throughout all time points during CPB and postbypass periods (p = 0.014). It can thus be noticed that fibrinogen and CRP

| TABLE 1. Hemodynamic and Oximetric Changes |
|----------|---------|---------|---------|---------|---------|---------|
| Variable           | Group    | T0 (or T10) | T30 | T90 | T120 | T210 |
| Mean arterial pressure (mm Hg) | Control | 73 ± 13 | 56 ± 4 | 68 ± 7 | 47 ± 3 | 33 ± 3 |
|                   | LPS      | 61 ± 5 | 55 ± 4 | 44 ± 3a | 44 ± 3 | 38 ± 4a |
| Heart rate (beats/min) | Control | 82 ± 11 | 64 ± 3 | 126 ± 14 | 95 ± 11 | 124 ± 18 |
|                   | LPS      | 84 ± 6 | 90 ± 6 | 99 ± 8 | 88 ± 4 | 99 ± 12 |
| SpO2 (%) | Control | 100 ± 0 | 99 ± 1 | 95 ± 4 | 100 ± 0 | 100 ± 0 (n = 1) |
|                   | LPS      | 100 ± 0 | 94 ± 2 | 92 ± 4 | 100 ± 0 | 100 ± 0 |
| γ in the vein (s⁻¹) | Control | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.3 ± 0.1 |
|                   | LPS      | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.2 | 0.4 ± 0.1 |
| γ in the circuit (s⁻¹) | Control | 0.9 ± 0.2 | 1.4 ± 0.4 | 0.9 ± 0.2 | – | – |
|                   | LPS      | 1.1 ± 0.0 | 1.0 ± 0.1 | 1.3 ± 0.1 | – | – |

LPS = lipopolysaccharide, SpO2 = lingual pulse oxygen saturation, γ = mean shear rates within the vessel; – = no measurement performed in the cardiopulmonary bypass circuit during the postbypass period.

aDifferent from T0 (p < 0.05).

bDifference between the two groups (p < 0.05).

Values are means ± SEM.
acute-phase plasma proteins could detect the highest inflammatory condition in the LPS group, but only the fibrinogen changes could identify a temporal evolution early in the post-bypass period.

**A Higher Time-Evolving Ultrasound-Sensitive Inflammatory Response in the LPS Group**

As seen in Figure 4A, W showed higher values in the LPS group at every time point in the pump circuit deviation (p < 0.05); however, no time-varying W behavior could be noticed for both groups. In the femoral vein, W values were higher in the LPS group compared with the control group from T_{45} to T_{210} (Fig. 4B; p < 0.05 from T_{45} to T_{90} and p < 0.001 from T_{105} to the end). Linear mixed models revealed a significant time increase of W in the LPS group only (p < 0.0001). The ultrasound SFSAE method confirmed the highest inflammatory state in the LPS group and was more sensitive to temporal evolution than classical blood markers.

**DISCUSSION**

This study is the first to demonstrate the feasibility of in vivo and real-time ultrasound monitoring of RBC aggregation (cf. W) during surgery involving experimental CPB. To do so, we used a specific model that has been described as typical of postpump syndrome in humans (30–32). Enhanced RBC aggregation coincided with both LPS- and CPB-related inflammation, emphasizing the usefulness of SFSAE images to follow the inflammatory response. Ultrasound results revealed to be consistent with cytokine and plasma protein responses but advantageously allowed staging changes in inflammation. RBC aggregation is a reversible process modulated by the membrane surface change density, membrane strain, and plasma proteins (37). The sensitivity of the SFSAE method to the time-varying inflammatory state likely results from the cumulative impact of those mechanisms. This new cellular imaging modality might be applicable to various critical care situations, as discussed below.

**RBC Aggregation, Inflammation, and Risk for Thrombosis and Tissue Ischemia**

This is the first study to monitor together RBC rheology with a noninvasive method and inflammatory markers during and after surgery. Our results are in accordance with the literature describing an increase of RBC aggregation in response to surgery including or not including CPB (22, 24, 38–40). Baskurt et al (22) proposed two complementary pathways to explain the increase of RBC aggregation concomitantly to inflammation in postoperative sepsis. First, the inflammatory response induces RBC aggregation via the release of plasmatic macromolecules, as observed in our study for CRP. One may also notice a slight time correspondence

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**Figure 3.** Time course of cytokines during and after the cardiopulmonary bypass surgery. Interleukin (IL)-6 (A) and IL-10 (B).

* Differences between lipopolysaccharide (LPS) and control (CONT) groups (p < 0.05).
† Different from baseline (p < 0.05).
‡ Different from baseline in the LPS group only (p < 0.05).
between the increase in fibrinogen, usually described as the main plasmatic factor responsible for RBC aggregation and the increase of \( W \) in the LPS group. Second, oxidative stress, which in this study could have been induced by both LPS and CPB, promotes cellular alterations further enhancing aggregation (41). Both mechanisms were likely involved in the LPS- and CPB-related inflammation, and in the concomitant RBC aggregation response measured with the SFSAE imaging method. Finally, Piagnerelli et al (42) described a reduction of sialic acid content of the RBC membrane in septic patients. This alteration is known to conduct to enhanced RBC aggregation (43) and may have been responsible for the higher \( W \) observed in the LPS group.

Blood rheology alterations have known impact on venous thrombosis and microcirculatory blood flow. Abnormal RBC aggregation increases blood viscosity and peripheral vascular resistance and has been described to play a significant role in tissue-perfusion disturbances encountered in sepsis (12, 22, 44, 45). A “hemorheologic vicious circle” has been described, emphasizing the fact that enhanced RBC aggregation can further promote the physiopathological process (22, 38). In addition to its relevance for inflammation monitoring, the continuous

**TABLE 2. Time Course of Acute-Phase Proteins Corrected for Dilution During and After the Cardiopulmonary Bypass**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>( T_0 )</th>
<th>( T_{30} )</th>
<th>( T_{90} )</th>
<th>( T_{120} )</th>
<th>( T_{210} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (g/L)</td>
<td>Control</td>
<td>2.0±0.0</td>
<td>2.4±0.1</td>
<td>2.4±0.7</td>
<td>1.9±0.2</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>2.4±0.4</td>
<td>3.7±0.6</td>
<td>3.2±0.5</td>
<td>4.4±0.3 ( ^{ab} )</td>
<td>2.5±0.6</td>
</tr>
<tr>
<td>Immunoglobulin G (g/L)</td>
<td>Control</td>
<td>9.8±1.0</td>
<td>11.3±1.2</td>
<td>9.7±1.1</td>
<td>9.2±0.5</td>
<td>9.1±0.1</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>12.4±1.6</td>
<td>14.6±1.0</td>
<td>11.6±1.1</td>
<td>11.8±0.8</td>
<td>12.5±1.2</td>
</tr>
<tr>
<td>Haptoglobin (mg/dL)</td>
<td>Control</td>
<td>23.7±8.1</td>
<td>17.7±6.4</td>
<td>18.4±7.3</td>
<td>16.3±5.4</td>
<td>22.6±14.1</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>32.0±5.7</td>
<td>34.9±5.7</td>
<td>32.1±5.5</td>
<td>27.5±3.6</td>
<td>27.7±4.4</td>
</tr>
<tr>
<td>C-reactive protein (µg/mL)</td>
<td>Control</td>
<td>47.8±13.0</td>
<td>53.6±26.8</td>
<td>48.8±17.0</td>
<td>49.0±20.3</td>
<td>56.2±20.5</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>675.7±98.2</td>
<td>621.0±176.4</td>
<td>562.8±168.2</td>
<td>566.4±128.2</td>
<td>670.7±102.0</td>
</tr>
</tbody>
</table>

LPS = lipopolysaccharide.

\(^{ab}\)Different from \( T_0 \) (\( p < 0.05 \)).

\(^{abc}\)Difference between the two groups (\( p < 0.05 \)).

Values are plasma concentrations expressed as mean ± sem.
assessment of RBC aggregation could thus be of interest to predict thrombotic side effects and tissue hypoperfusion in critical care situations.

**Clinical Perspectives**

This study proposed a preclinical evaluation of the SFSAE imaging method in a swine CPB model. In humans, CPB can trigger a cascade of events that includes systemic inflammatory response syndrome, which is generally well tolerated. In 10% to 15% of cases, multiple organ dysfunctions (MODS) lead to poor patient outcomes and increased mortality (1–3). However, the contribution of the inflammatory response to adverse patient outcomes is potentially remediable. The extent through which the continuous monitoring of the inflammatory response may be useful to recognize early shocks and guide therapy therefore requires further investigation. Therapeutic strategies involving anti-inflammatory (i.e., steroidal or nonsteroidal) and vasoactive drugs could be used more efficiently by considering a proper timing for therapy initiation and a selection of patients who might optimally respond. This would allow a better risk balance between alterations leading to organ failure versus drug-related side effects (46–48). The SFSAE W monitoring within the systemic venous circulation may anticipate high-risk surgery-related MODS and concomitant circulatory-related adverse consequences, such as venous thrombosis and tissue-perfusion defects. It thus constitutes a promising avenue complementary to existing methods.

One limitation of the current protocol is the exposure of the femoral vein to allow imaging. However, the human skin presents less attenuation to ultrasound than in swine, which gives the opportunity to use the SFSAE in clinical practice and benefits from its real-time potential. The noninvasive monitoring of inflammation with a high-frequency probe (e.g., 25 MHz) indeed is feasible on human superficial vessels. It has already been used to monitor RBC aggregation in the cephalic and great saphenous veins of patients with diabetes (49). To control the flow shear rate, the vascular occluder used in this study could be replaced by a cuff inflated intermittently at the time of the measurement.

**CONCLUSION**

This study used a swine CPB model to demonstrate the feasibility of using the SFSAE ultrasonic cellular imaging method to continuously monitor RBC aggregation as a marker of the inflammatory response and of the risk of tissue hypoperfusion and venous thrombosis. The technique can be viewed as a very promising complementary real-time noninvasive approach to anticipate shock occurrence in surgery with postoperative sepsis risk. Further evidences are nevertheless needed to know if the W index could be useful in the surveillance of critically ill patients.

**ACKNOWLEDGMENTS**

We thank Stéphanie Blanchet, Marie-Pierre Mathieu, Chlo- tile Perrault-Hébert, Thierry Lamarre-Renaud, and Zhao Qin for their technical assistance and Drs François Yu and Hélène Héon for their precious advices.

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