Nucleosome levels and toll-like receptor expression during high cut-off haemofiltration: a pilot assessment

Rafidah Atan, Clive May, Simon R Bailey, Marcel Tanudji, Kumar Visvanathan, Narelle Skinner, Rinaldo Bellomo, Hermann Goehl and Markus Storr

Apoptosis\(^1,2\) likely contributes to organ injury in the setting of sepsis and systemic inflammatory response syndrome (SIRS).\(^3\) Direct assessment of apoptosis is difficult\(^4-6\) and one important marker is DNA fragmentation into nucleosomal units,\(^7,8\) which may predict outcomes.\(^9\) Recent research also established the crucial role of toll-like receptors (TLRs) in sepsis and SIRS.\(^10-17\)

High cut-off (HCO) haemofilters\(^18\) have improved performance in removing middle molecules (0.5 to 60kDa).\(^19-21\) We designed a trial involving HCO filters (continuous venovenous haemofiltration [CVVH]-HCO) in critically ill patients with acute kidney injury (AKI) requiring vasopressor support. In a subset of patients, we performed a pilot assessment on plasma nucleosome levels and changes in TLR4 and TLR2 expression.

Methods

Our pilot investigation was nested within a randomised, double-blind, controlled trial (ClinicalTrials.gov/NCT00912184) approved by the Austin Hospital Human Research Ethics Committee (H2008/03400). Written informed consent was obtained from the patient or person responsible.

Patients with AKI and underlying shock requiring vasopressor infusion were recruited within 12 hours of commencing haemofiltration. Patients were randomised to either CVVH-HCO, using polyethersulfone filters with a nominal cut-off point of 100 kDa (Polyflux P2SH filters, 1.12 m\(^2\), Gambro), or to standard CVVH (CVVH-std), using custom-manufactured control polyethersulfone filters with a nominal cut-off point of 30 kDa. The two study filters were identical in appearance and surface area.

The settings for CVVH included a blood flow of 200 mL/min, an ultrafiltration dose of 25 mL/kg/h and use of predilution bicarbonate-buffered replacement fluids. We excluded patients on maintenance dialysis and those who received CVVH during the same hospitalisation. Arterial blood samples were taken at baseline (T0), at 24 hours after initiation of CVVH (T24), and 72–96 hours after initiation of CVVH (T72). Blood was also sampled from the postfilter port at T24.

Measurement of plasma nucleosomes

We used the Cell Death Detection ELISA PLUS (10X) kit (Roche Diagnostics). A 20\(\mu\)L volume of the sample was added to each well of streptavidin-coated 96-well plates, and 80\(\mu\)L of the immunoreagent added, containing antihistone-biotin and antiDNA-POD monoclonal antibodies. The plates were incubated for 2 hours at 20\(\degree\)C on a shaker at 300 rpm, washed, and the reaction was developed with 2,2’-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt solution (ABTS) (a water-soluble horseradish peroxidase substrate). Stop solution was added after 10–20
minutes (once adequate colour development was present) and plates were then read on a colorimetric plate reader (Synergy H1, BioTek) at 405 nm, with a reference wavelength of 490 nm. Nucleosome levels from one healthy human volunteer were also measured.

**Validation**

We conducted assay validation with purified nucleosomes provided in the kit as positive control. Values were normalised to the absorbance of the positive control sample and expressed as relative units.

To establish the intra-assay coefficient of variation, the positive control sample was analysed five times on three separate plates. To determine the interassay coefficient of variation, five different dilutions of the control nucleosomes were assayed in triplicate on three separate plates. The coefficients were defined by the standard deviation as a percentage of the mean. Each sample was assayed in triplicate on each plate (taking the mean value), and the mean and standard error from three separate assay runs were calculated.

**Measurement of nucleosomes from patients with sepsis**

We added a 20 μL volume of the sample to each well in triplicate, and added 80 μL of immunoreagent. The assay then proceeded as described above. None of the samples gave values outside the working range of the assay.

**Toll-like receptor analysis**

To measure peripheral blood mononuclear cell (PBMC) TLR expression, we performed cell surface staining on frozen PBMCs using human CD14-allophycocyanin/cyanine7

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Table 1. Baseline characteristics and outcomes of study patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>High cut-off haemofiltration (n = 6)</th>
<th>Standard haemofiltration (n = 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (IQR)</td>
<td>57.8 (46.4–64.3)</td>
<td>65 (62.3–72.2)</td>
<td>0.295</td>
</tr>
<tr>
<td>Sex (male/female), n</td>
<td>5/1</td>
<td>5/2</td>
<td>1.0</td>
</tr>
<tr>
<td>Median weight, kg (IQR)</td>
<td>85.5 (72.5–94.75)</td>
<td>80 (75–87.6)</td>
<td>0.731</td>
</tr>
<tr>
<td>Median APACHE II score (IQR)</td>
<td>19.5 (12–27)</td>
<td>23 (16.5–23.5)</td>
<td>0.731</td>
</tr>
<tr>
<td>Median APACHE 3 score (IQR)</td>
<td>63.5 (54.25–87)</td>
<td>72 (60.5–90.5)</td>
<td>0.731</td>
</tr>
<tr>
<td>Median SOFA score (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>4 (4–4)</td>
<td>4 (3.5–4)</td>
<td>0.445</td>
</tr>
<tr>
<td>Respiratory</td>
<td>3 (2.25–3)</td>
<td>3 (2–3.5)</td>
<td>1.0</td>
</tr>
<tr>
<td>Renal</td>
<td>2.5 (2–3.75)</td>
<td>2 (2–3.5)</td>
<td>0.731</td>
</tr>
<tr>
<td>Coagulation</td>
<td>0.5 (0–2.5)</td>
<td>0 (0–0.5)</td>
<td>0.445</td>
</tr>
<tr>
<td>Liver</td>
<td>2.5 (1.25–3)</td>
<td>0.5 (0–1)</td>
<td>0.093</td>
</tr>
<tr>
<td>Total of 5 SOFA scores</td>
<td>13 (10.5–14.75)</td>
<td>9 (8.5–11.5)</td>
<td>0.051</td>
</tr>
<tr>
<td>Median baseline creatinine within 1 year of date of admission, μmol/L (IQR)</td>
<td>86.6 (55.75–1062)</td>
<td>80 (66.5–101.5)</td>
<td>0.628</td>
</tr>
<tr>
<td>Median RIFLE scores at start of CRRT (R/W/F)</td>
<td>0/2/4</td>
<td>1/2/4</td>
<td>0.629</td>
</tr>
<tr>
<td>Shock etiology (sepsis/cardiogenic/other), n</td>
<td>3/2/1</td>
<td>2/3/2</td>
<td>0.719</td>
</tr>
<tr>
<td>Median baseline mean arterial pressure at enrolment, mmHg (IQR)</td>
<td>70 (66.25–70)</td>
<td>75 (70–77.5)</td>
<td>0.234</td>
</tr>
<tr>
<td>Ventilated at enrolment (yes/no), n</td>
<td>5/1</td>
<td>5/2</td>
<td>1.0</td>
</tr>
<tr>
<td>Median serum urea at enrolment, mmol/L (IQR)</td>
<td>13.8 (10.93–18.33)</td>
<td>22.6 (13.65–26.9)</td>
<td>0.534</td>
</tr>
<tr>
<td>Median serum creatinine at enrolment, μmol/L (IQR)</td>
<td>247.5 (220.5–266.3)</td>
<td>217 (179–256)</td>
<td>0.628</td>
</tr>
<tr>
<td>Median blood lactate at enrolment, mmol/L (IQR)</td>
<td>2.7 (2.43–4.93)</td>
<td>1.4 (1.1–1.76)</td>
<td>0.002*</td>
</tr>
<tr>
<td>Median blood pH at enrolment (IQR)</td>
<td>7.37 (7.31–7.39)</td>
<td>7.34 (7.30–7.42)</td>
<td>0.731</td>
</tr>
<tr>
<td>Median international normalised ratio at enrolment (IQR)</td>
<td>1.85 (1.45–2.25)</td>
<td>1.3 (1.2–1.35)</td>
<td>0.022*</td>
</tr>
<tr>
<td>Median activated partial thromboplastin time at enrolment, seconds (IQR)</td>
<td>44 (31.25–47)</td>
<td>30 (24–37)</td>
<td>0.295</td>
</tr>
<tr>
<td>Median serum albumin at enrolment, g/dL (IQR)</td>
<td>34 (33.25–37.75)</td>
<td>27 (21.5–27.5)</td>
<td>0.014*</td>
</tr>
<tr>
<td>Median noradrenaline infusion at enrolment, μg/min (IQR)</td>
<td>17.5 (13.25–23.25)</td>
<td>5 (3–8)</td>
<td>0.051</td>
</tr>
<tr>
<td>Intensive care unit mortality, %</td>
<td>33.33%</td>
<td>28.57%</td>
<td>1.0</td>
</tr>
<tr>
<td>Hospital mortality, %</td>
<td>50%</td>
<td>28.57%</td>
<td>0.592</td>
</tr>
</tbody>
</table>

IQR = interquartile range. APACHE = Acute Physiology and Chronic Health Evaluation. SOFA = sequential organ failure assessment. RIFLE = risk, injury, failure, loss of kidney function, end-stage kidney disease. CRRT = continuous renal replacement therapy. * Statistically significant.
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(CD14-APCcy7), TLR2-fluorescein isothiocyanate (TLR2-FITC), and TLR4-R-phycocerythrin (TLR4-PE) conjugated monoclonal antibodies, as previously described. Expression was assessed on the CD14+ monocyte. Relative fluorescence intensity was expressed as a ratio of the geometric mean fluorescence intensity (MFI) of the test sample to that of an isotype control stained sample.

**Statistical analysis**

Baseline characteristics were analysed using the Mann–Whitney U test for continuous data and χ² analysis for categorical data. Data on nucleosome levels were analysed using repeated-measures analysis of variance for between-group analysis. The Friedman test was used for within-group analysis. Prefilter v postfilter samples at T24 were compared using the paired t test. Correlations were analysed using the Pearson correlation test and statistical significance was defined as P<0.05.

**Results**

We obtained samples from 13 patients for nucleosome analysis: six in the CVVH-HCO group and seven in the CVVH-std group. There were significant baseline differences between the groups in blood lactate levels (P=0.002), serum albumin levels (P=0.014) and international normalised ratio for prothrombin time (P=0.022) (Table 1).

**Validation experiments**

Serially diluting the nucleosomes in assay buffer produced a linear decrease in absorbance down to a 1:50 dilution, and the nucleosomes in the positive control sample could be detected at a 1:100 dilution. Dilution in 10% or 25% human plasma did not affect the linearity of the assay. The assay showed excellent reproducibility, with an intra-assay coefficient of variation of 2.07±0.22 %, and an interassay coefficient of variation of 6.96±0.53 %.

**Measurement of plasma nucleosomes**

Nucleosomes were not detected in the plasma of the normal volunteer. There were no significant changes in median plasma levels over 72 hours within either group (CVVH-HCO, P=0.607; CVVH-std, P=1.00). There was also no significant difference in plasma levels over 72 hours between the two groups (P=0.757) (Figure 1). There was a statistically significant but weak correlation between nucleosome levels at baseline and Acute Physiology and Chronic Health Evaluation (APACHE) III scores (ρ=0.475; P=0.016).

At T24, we analysed 15 samples for prefilter v postfilter nucleosome levels (six patients from the CVVH-HCO group [Figure 2] and nine patients from the CVVH-std group) and found that nucleosome levels increased significantly across
the filter for the CVVH-HCO group ($P = 0.016$), but did not in the CVVH-std group ($P = 0.294$).

**TLR2 and TLR4 expression in the CVVH-HCO group**

We analysed five patients in the CVVH-HCO group for TLR2 and TLR4 expression at T0 and T72 (Figure 3). We also analysed five patients for changes in TLR2 and TLR4 expression across the filter at T24 (prefilter v postfilter). TLR2 expression was increased in 40%–60% of samples (MFI ratio range, 0.97–3.98) compared with a reference MFI ratio of 2.0 (SD, 0.66). TLR4 expression showed a greater increase, involving 95% of analysed samples. The MFI ratio for TLR4 ranged from 0.91 to 10.18, compared with a normal reference MFI ratio of 1.55 (SD, 1.81).

There was no reduction in TLR2 and TLR4 expression over 72 hours of treatment. There was no reduction in median TLR2 and TLR4 expression due to passage of blood through the filter. There was no significant correlation between plasma nucleosome levels, TLR2 ($P = 0.304$) or TLR4 ($P = 0.235$) expression, or between TLR2 and TLR4 levels at baseline ($P = 0.068$).

**Discussion**

**Key findings**

Nucleosome levels were elevated and correlated weakly with APACHE III scores at randomisation. Treatment over 72 hours did not reduce plasma nucleosome levels, which increased following passage across HCO filters. TLR2 and TLR4 expression was also increased and did not decrease over 72 hours of treatment with CVVH-HCO.

**Implications**

Previous studies found correlation between nucleosome levels and patient outcome as well as disease severity. We also found that nucleosome levels mildly correlated with illness severity. Our study, which was conducted under conditions of double-blind randomisation, found no effects of CVVH-HCO on circulating nucleosomes, which was similar to other interventions. We previously found that HCO haemofiltration is superior in removing middle molecules. Our findings suggest a lack of effect on nucleosome levels when using HCO filters, despite their increased pore size. We also found no effect on both TLR2 and TLR4 levels.

In summary, we could not find any benefit of HCO haemofiltration in terms of reducing a key aspect of the underlying apoptotic process.

**Strengths and limitations**

Our measurements over 72 hours of treatment provided an extended view of the effects of the interventions, but our sample size was small and subject to a high risk of errors.

**Conclusions**

Our pilot assessment of the effect of CVVH-HCO on plasma nucleosome levels and TLR expression found increased levels of proapoptotic mediators in patients with severe AKI and multiorgan failure, and weak correlation with illness severity. We found that CVVH-HCO had no effect on overall plasma nucleosome levels. Our findings also suggest that previous assessments of blood purification techniques focused on cytokines failed to capture changes in important components of the innate immune response to injury.

**Competing interests**

None declared.

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