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

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ABSTRACT

Objectives: To measure plasma nucleosome levels and expression of toll-like receptors (TLRs) in a pilot cohort of patients with severe acute kidney injury (AKI) within a randomised controlled trial of continuous venovenous haemofiltration with high cut-off filters (CVVH-HCO) v standard filters (CVVH-std).

Methods: We measured plasma nucleosome levels using the Cell Death Detection ELISA PLUS (10X) assay kit. We analysed plasma levels for correlation with disease severity and compared the effects of CVVH-HCO and CVVH-std on plasma nucleosome levels over the first 72 hours. We studied cell surface TLR expression on CD14-positive monocytes in a subcohort of CVVH-HCO patients.

Results: We did not detect nucleosomes in normal human plasma, but found elevated nucleosome levels in patients with severe AKI. Nucleosome levels at randomisation correlated weakly with Acute Physiology and Chronic Health Evaluation III scores (Pearson $\rho = 0.475$, $P = 0.016$). Treatment with CVVH-HCO or CVVH-std had no effect on nucleosome levels over 72 hours. The mean fluorescence intensity (MFI) ratios of TLR2 and TLR4 expression were elevated throughout the 72-hour period (range for TLR2, 0.97–3.98; range for TLR4, 0.91–10.18) and did not appear to decrease as a result of treatment with CVVH-HCO.

Conclusions: Nucleosome concentration was elevated in the plasma of patients with severe AKI and mildly correlated with disease severity, but was not affected by treatment with CVVH-HCO or CVVH-std. Similarly, levels of TLR2 and TLR4 expression did not decrease over time during CVVH-HCO treatment.

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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 μL volume of the sample was added to each well of streptavidin-coated 96-well plates, and 80 μL of the immunoreagent added, containing antihistone-biotin and antiDNA-POD monoclonal antibodies. The plates were incubated for 2 hours at 20°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

Table 1. Baseline characteristics and outcomes of study patients

Characteristic	High cut-off haemofiltration ($n = 6$)	Standard haemofiltration ($n = 7$)	*P*
Median age, years (IQR)	57.8 (46.4–64.3)	65 (62.3–72.2)	0.295
Sex (male/female), *n*	5/1	5/2	1.0
Median weight, kg (IQR)	85.5 (72.5–94.75)	80 (75–87.6)	0.731
Median APACHE II score (IQR)	19.5 (12–27)	23 (16.5–23.5)	0.731
Median APACHE 3 score (IQR)	63.5 (54.25–87)	72 (60.5–90.5)	0.731
Median SOFA score (IQR)			
Cardiovascular	4 (4–4)	4 (3.5–4)	0.445
Respiratory	3 (2.25–3)	3 (2–3.5)	1.0
Renal	2.5 (2–3.75)	2 (2–3.5)	0.731
Coagulation	0.5 (0–2.5)	0 (0–0.5)	0.445
Liver	2.5 (1.25–3)	0.5 (0–1)	0.093
Total of 5 SOFA scores	13 (10.5–14.75)	9 (8.5–11.5)	0.051
Median baseline creatinine within 1 year of date of admission, μmol/L (IQR)	86.6 (55.75–106)	80 (66.5–101.5)	0.628
Median RIFLE scores at start of CRRT (R/I/F)	0/2/4	1/2/4	0.629
Shock etiology (sepsis/cardiogenic/other), *n*	3/2/1	2/3/2	0.719
Median baseline mean arterial pressure at enrolment, mmHg (IQR)	70 (66.25–70)	75 (70–77.5)	0.234
Ventilated at enrolment (yes/no), *n*	5/1	5/2	1.0
Median serum urea at enrolment, mmol/L (IQR)	13.8 (10.93–18.33)	22.6 (13.65–26.9)	0.534
Median serum creatinine at enrolment, μmol/L (IQR)	247.5 (220.5–266.3)	217 (179–256)	0.628
Median blood lactate at enrolment, mmol/L (IQR)	2.7 (2.43–4.93)	1.4 (1.1–1.76)	0.002*
Median blood pH at enrolment (IQR)	7.37 (7.31–7.39)	7.34 (7.30–7.42)	0.731
Median international normalised ratio at enrolment (IQR)	1.85 (1.45–2.25)	1.3 (1.2–1.35)	0.022*
Median activated partial thromboplastin time at enrolment, seconds (IQR)	44 (31.25–47)	30 (24–37)	0.295
Median serum albumin at enrolment, g/dL (IQR)	34 (33.25–37.75)	27 (21.5–27.5)	0.014*
Median noradrenaline infusion at enrolment, μg/min (IQR)	17.5 (13.25–23.25)	5 (3–8)	0.051
Intensive care unit mortality, %	33.33%	28.57%	1.00
Hospital mortality, %	50%	28.57%	0.592

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.

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.

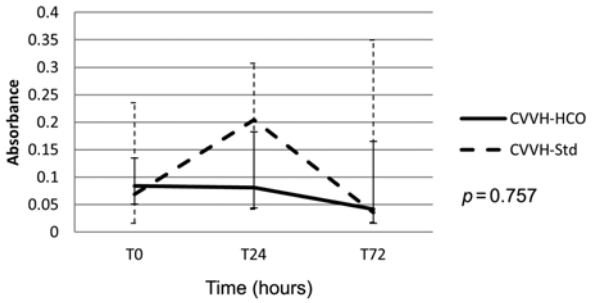
Measurement of nucleosomes from patients with sepsis

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

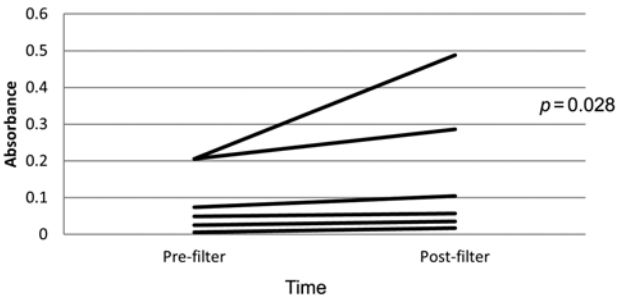
To measure peripheral blood mononuclear cell (PBMC) TLR expression, we performed cell surface staining on frozen PBMCs using human CD14-allophycocyanin/cyanine7

Figure 1. Median nucleosome levels in plasma at T0, T24 and T72, patients on CVVH-HCO v patients on CVVH-std*



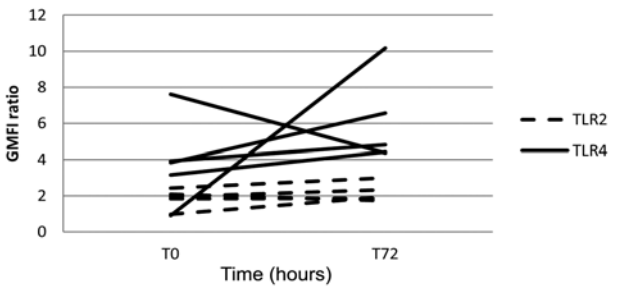
T0 = prefilter plasma at 0–12 hours after randomisation. T24 = prefilter plasma at 24 hours after randomisation. T72 = prefilter plasma at 72–96 hours after randomisation. CVVH-HCO = high cut-off continuous venovenous haemofiltration. CVVH-std = control/standard continuous venovenous haemofiltration. Absorbance = $[A_{405\,nm} - A_{490\,nm}]$.
* Nucleosome levels not detected in normal human plasma.

Figure 2. Median nucleosome levels in plasma sampled at 24 hours from the CVVH-HCO patients, prefilter v postfilter



CVVH-HCO = high cut-off continuous venovenous haemofiltration. Absorbance = $[A_{405\,nm} - A_{490\,nm}]$. Pre-filter = prefilter plasma at 24 hours after randomisation. Post-filter = postfilter plasma at 24 hours after randomisation.

Figure 3. TLR2 and TLR4 expression, T0 v T72 (CVVH-HCO)



CVVH-HCO = high cut-off continuous venovenous haemofiltration. GMFI = geometric mean channel fluorescence intensity (in normal control = 2.03). T0 = prefilter plasma at 0–12 hours after randomisation. T72 = prefilter plasma at 72–96 hours after randomisation.

(CD14-APCcy7), TLR2-fluorescein isothiocyanate (TLR2-FITC), and TLR4-R-phycoerythrin (TLR4-PE) conjugated monoclonal antibodies, as previously described.[22] 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 χ^2 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 ($\rho = 0.475$; $P = 0.016$).

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.[9,23] 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.[23-25] We previously found that HCO haemofiltration is superior in removing middle molecules.[19-21] 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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References

1 Kellum JA, Bellomo R, Mehta R, Ronco C. Blood purification in non-renal critical illness. *Blood Purif* 2003; 21: 6-13.

2 Rimmelé T, Kellum JA. Clinical review: blood purification for sepsis. *Crit Care* 2011; 15: 205.

3 Hotchkiss RS, Swanson PE, Freeman BD, et al. Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. *Crit Care Med* 1999; 27: 1230-51.

4 Lerolle N, Nochy D, Guérot E, et al. Histopathology of septic shock induced acute kidney injury: apoptosis and leukocytic infiltration. *Intensive Care Med* 2010; 36: 471-8.

5 Niu G, Chen X. Apoptosis imaging: beyond annexin V. *J Nucl Med* 2010; 51: 1659-62.

6 Atan R, Virzi GM, Peck L, et al. High cut-off hemofiltration versus standard hemofiltration: a pilot assessment of effects on indices of apoptosis. *Blood Purif* 2014; 37: 296-303.

7 Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. *Trends Cell Biol* 1995; 5: 21-6.

8 Zeerleder S, Zwart B, Wuillemin WA, et al. Elevated nucleosome levels in systemic inflammation and sepsis. *Crit Care Med* 2003; 31: 1947-51.

9 Chen Q, Ye L, Jin Y, et al. Circulating nucleosomes as a predictor of sepsis and organ dysfunction in critically ill patients. *Int J Infect Dis* 2012; 16: e558-64.

10 Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol* 2001; 1: 135-45.

11 Lorne E, Dupont H, Abraham E. Toll-like receptors 2 and 4: initiators of non-septic inflammation in critical care medicine? *Intensive Care Med* 2010; 36: 1826-35.

12 Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; 388: 394-7.

13 O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors — redefining innate immunity. *Nat Rev Immunol* 2013; 13: 453-60.

14 Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* 2009; 21: 317-37.

15 Fenhammar J, Rundgren M, Hultenby K, et al. Renal effects of treatment with a TLR4-inhibitor in conscious septic sheep. *Crit Care* 2014; 18: 488.

16 Fenhammar J, Rundgren M, Forestier J, et al. Toll-like receptor 4 inhibitor TAK-242 attenuates acute kidney injury in endotoxemic sheep. *Anesthesiology* 2011; 114: 1130-7.

17 Lin Q, Li M, Fang D, et al. The essential roles of Toll-like receptor signaling pathways in sterile inflammatory diseases. *Int Immunopharmacol* 2011; 11: 1422-32.

18 Boschetti-de-Fierro A, Voigt M, Storr M, Krause B. Extended characterization of a new class of membranes for blood purification: the HCO membranes. *Int J Artif Organs* 2013; 36: 455-63.

19 Atan R, Crosbie D, Bellomo R. Techniques of extracorporeal cytokine removal: a systematic review of the literature. *Blood Purif* 2012; 33: 88-100.

20 Atan R, Crosbie D, Bellomo R. Techniques of extracorporeal cytokine removal: a systematic review of the literature on animal experimental studies. *Int J Artif Organs* 2013; 36: 149-58.

21 Atan R, Crosbie DC, Bellomo R. Techniques of extracorporeal cytokine removal: a systematic review of human studies. *Ren Fail* 2013; 35: 1061-70.

22 Visvanathan K, Skinner NA, Thompson AJ, et al. Regulation of Toll-like receptor-2 expression in chronic hepatitis B by the precore protein. *Hepatology* 2007; 45: 102-10.

23 Zeerleder S, Stephan F, Emonts M, et al. Circulating nucleosomes and severity of illness in children suffering from meningococcal sepsis treated with protein C. *Crit Care Med* 2012; 40: 3224-9.

24 D'Auria F, Rovere-Querini P, Giazzon M, et al. Accumulation of plasma nucleosomes upon treatment with anti-tumour necrosis factor-alpha antibodies. *J Intern Med* 2004; 255: 409-18.

25 Stoetzer OJ, Fersching DM, Salat C, et al. Prediction of response to neoadjuvant chemotherapy in breast cancer patients by circulating apoptotic biomarkers nucleosomes, DNAse, cytokeratin-18 fragments and survivin. *Cancer Lett* 2013; 336: 140-8. ❑