Increase of high mobility group box 1 protein levels (HMGB1) in patients with chronic kidney disease after hemodialysis

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Keywords Chronic kidney disease • Hemodialysis • High mobility group box 1 protein • Thrombomodulin • Innate immunity

1.0 ABSTRACT

1.1 Background: There are few studies approaching the blood level of inflammatory mediators in Chronic Kidney Disease (CKD) patients before and after hemodialysis.

1.2 Objective: With the aim of establishing the inflammatory profile in patients with CKD, the levels of thrombomodulin (TM) (anticoagulant and anti-inflammatory), high mobility group box 1 protein (HMGB1), IL-1β and IL-6 (proinflammatory cytokines), and MDA (malondialdehyde), a biomarker for oxidative stress were determined in a descriptive paired study in CKD patients before and after hemodialysis.

1.3 Methods*:* The study population comprised twenty patients with CKD, each of whom had been undergoing hemodialysis treatment for approximately seven years. Levels of HMGB1, TM, IL-1β, IL-6 and MDA were determined by enzyme-linked immunosorbent assay of blood samples collected from each patient before and after hemodialysis.

1.4 Results: Mean values of HMGB1 increased substantially from 0.48±0.2 to 1.07±0.5ng/mL ($p<0.05$) following hemodialysis, while mean levels of TM showed only a minor increase from 9.01±0.9 to 9.35±0.9 ng/mL ($p>0.05$). The median of IL-6 and IL-1β, expressed as pg/mL, were 15.3 (8.8-40.7) to 16.10 (9.5–51) ($p>0.05$) and 10.3 (5.3–32.3) to 9.7 (5.3–34.2), before and after hemodialysis, respectively. The HMGB1/TM ratio rose by a factor of 2.86 following hemodialysis. No correlations were detected between HMGB-1 and TM, IL-6, IL-1β or MDA levels before or after hemodialysis. Blood urea levels decreased significantly after hemodialysis, as expected.

1.5 Discussion: Hemodialysis decreases toxic components (e.g. urea) in blood but induces a significant increase in HMGB-1 without affecting other proinflammatory biomarkers. The observed increase in HMGB1 concentration could be due to interaction between blood cells and the filter membrane

leading to cellular death without consequent releasing of proinflammatory cytokines and activation of innate immunity, evaluated by IL-1β and IL-6 neither biomarker for oxidative stress (MDA). Hemodialysis did not induce or aggravate inflammatory profile of CKD patients.

Abbreviations: AKI, acute kidney injury, CKD, Chronic Kidney Disease, EN-RAGE, receptor for advanced glycation end-products binding protein, HD, hemodialysis, HDL, high-density lipoprotein, HMGB1, high mobility group box 1 protein, LDL, low-density lipoprotein, MDA malondialdehyde, PAPP-A, plasma protein-A, RAGE, receptor for advanced glycation end products, ROS, Reactive oxygen species, TLR, toll-like receptor, TM, thrombomodulin

2.0 Background

Renal diseases involving nephron dysfunction can be classified into two clinical forms, namely acute kidney injury (AKI) and chronic kidney disease (CKD). AKI, which is reversible and precedes CKD (Malyszko J, et al, 2008), refers to the reduction of glomerular filtration rate associated or not with a decrease in urinary volume. In contrast, CKD relates to the cessation of kidney function with loss of regulatory, excretory and endocrine functions. Both AKI and CKD are caused by inflammatory processes, and these are of pivotal importance in CKD complications involving the participation of proinflammatory cytokines resulting in oxidative stress, endothelial dysfunction and high cardiovascular morbidity (Stenvinkel P, et al, 2005). In CKD patients, interrelationships between chronic inflammation, malnutrition and cardiovascular atherosclerosis have been proposed (Avesani CM, et al, 2006). CKD patients in hemodialysis (HD) therapy suffer from chronic inflammation, thromboembolic events, incidences of fistula thrombosis, ischemic stroke, coronary ischemic and thrombotic episodes.

Several biomarkers are reported in both CKD and AKI. Pregnancy associated plasma protein-A (PAPP-A), an extracellular newly identified receptor for advanced glycation end-products binding protein (EN-RAGE), and high mobility group box 1 protein (HMGB1), are elevated in AKI and CKD patients and are related to the inflammatory process (Zakiyanov O, et al, 2013). The role of DAMPs such as HMGB1 in CKD is controversial. HMGB1 is significantly elevated in CKD patients and it is suggested as a possible marker of disease severity and predictor of CKD (Bruchfeld A, et al, 2008). In contrast, Sunden-Cullberg et al. (2005) suggested that CKD patients with high levels of HMGB1 exhibited better outcomes over a 24 month period in comparison with those presenting lower levels of this proinflammatory cytokine. In addition, it has been reported (Sunden-Cullberg J, et al, 2006; Goldstein RS, et al, 2007; Hatada T, et al, 2005) that higher levels of HMGB1 may be correlated with enhanced outcomes in sepsis, a life-threatening condition in which the inflammatory processes induce intravascular coagulation accompanied by a reduction in the production of the thrombin receptor, thrombomodulin (TM) (Yoshihara M, et al, 2005). The incidence of fistula thrombosis in CKD patients is common. The association between TM and HMGB1 is suggested. HMGB1 has been reported to trigger cellular signalling through toll-like receptor (TLR) 2, TLR4, and TLR9 and receptor for advanced glycation end products (RAGE), leading to the recruitment of inflammatory cells and the release of proinflammatory cytokines and chemokines that cause organ damage (Ivanov S, et al, 2007; Schmidt AM, et al, 2001; Yu M et al, 2006). Reactive oxygen species (ROS) production as consequence of TLR or RAGE activation by HMGB1 may induce oxidative stress.

TM is located at the surface of endothelial cells or secreted in a soluble form and is responsible for the regulation of intravascular coagulation (Esmon CT, 2005). Formation of a TM-thrombin complex activates protein C which, in turn, is able to inhibit the proinflammatory and coagulant effect of thrombin. In addition, TM exhibits anti-inflammatory properties by sequestering the proinflammatory HMGB1 via its lectin-like domain (LLD) to form an HMGB1-TM complex, down-regulating proinflammatory cytokine secretions due to thereby inhibiting the activation of RAGE and toll-like receptors (TLR2 and TLR4) by HMGB1 (Abeyama K , et al, 2005; Herzog C, et al, 2014; Lotze MT, et al, 2005). Considering the functional link between the anti-inflammatory role of TM and the proinflammatory role of HMGB1, it is likely that imbalance between these two molecules might give rise to coagulation and/or inflammation. Thus, CKD patients may have problems associated with coagulation and inflammation. On this basis, high levels of TM together with low concentrations HMGB1 could be beneficial to hemodialysis patients by promoting anticoagulation and control of inflammation simultaneously. For this reason, HMGB1/TM ratios should be investigated

during disease, in conjunction with pro inflammatory cytokines to determine the profile pro- or anti-inflammatory after hemodialysis.

2.1 Objective

The aim of the present study was to establish the levels of HMGB1, soluble TM, proinflammatory cytokines and oxidative stress biomarker in a paired evaluation in CKD patients before and after hemodialysis.

3.0 Methods

3.1 Subjects

Details of the study were submitted to and approved by the Ethical Committee of the Hospital Santa Casa de Belo Horizonte (Belo Horizonte, MG, Brazil), and written informed consent was obtained from all individual participants prior to the commencement of the study. All procedures were performed in accordance with the ethical principles contained in the 1964 Declaration of Helsinki. Twenty CKD patients (aged between 18 and 50 years), each of whom had been undergoing hemodialysis treatment for approximately seven years, were selected by Dr. Gustavo Mário Capanema from the Nephrology Service at Hospital Santa Casa de Belo Horizonte. Prior to the study, all volunteers were submitted to physical examination and laboratory assessment, and their medical histories were evaluated in detail.

3.2 inclusion criteria: male or female patients with chronic renal insufficiency who were receiving hemodialysis treatment and had agreed to sign the informed consent form.

3.3 Exclusion criteria: Smokers, pregnant women, patients with cancer or clinically diagnosable active inflammatory processes, subjects who were unable to exercise autonomy and individuals who did not agree to sign the informed consent form were excluded from the study.

3.4 Biochemical analyses: Prior to submission of patients to their first procedures using a hemodialyser supplied by Fresenius Medical Care (Bad Homburg, Germany), blood samples (10 mL) were collected via the arteriovenous fistula (hemodialysis vascular access) and transferred to Vacutainer® tubes without anticoagulant in order to obtain blood serum. Following hemodialysis, further blood samples (10 mL) were collected from the heparinized patients in order to obtain plasma. Serum and plasma samples were separated and stored at -80 °C until required for analysis. Determinations of urea, creatinine, triglycerides and albumin, along with low-density lipoprotein (LDL), high-density lipoprotein (HDL) and total-cholesterol, were performed using standard automated methods followed by the Laboratory of Clinical Analyses of Hospital Santa Casa de Belo Horizonte. Enzyme linked immunosorbent assay (ELISA) kits were used to quantify TM (R&D Systems, Minneapolis, MN, USA), HMGB1 (MyBioSource, San Diego, CA, USA), IL-1β and IL-6 (Enzo Life Sciences, Inc., New York, USA). MDA concentration was measured using the TBARS Assay Kit (ZeptoMetrix Corp., New York, USA). A decreased level of urea after hemodialysis was used as reference of treatment success.

3.5 Statistical analyse: Values were presented as the means ± standard deviation (SD) or as median and limits. The nonparametric Kolmogorov-Smirnov test was used to assess the normal distribution of the continuous variables. Comparisons between data from parameters before and after HD were performed using paired Student t-test or Mann-Withney test. Within-group correlations were performed using Spearman's correlation coefficient (r). All analyses were considered significant at P-values <0.05 using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

4.0 Results

The demographic and biochemical characteristics of the study population are shown in Table 1, while the effects of hemodialysis on the levels of urea, TM and HMGB1 are presented in Table 2. The expected decrease in blood urea concentration following hemodialysis was 72% and the difference in urea levels before and after dialysis was statistically significant ($P < 0.05$). The concentration of HMGB1 showed a significant increase ($P < 0.05$) of two-fold

(123%) during hemodialysis such that the after/before ratio for HMGB1 was 2.23. In contrast, hemodialysis exerted no significant effect ($P > 0.05$) on the level of TM and, as a consequence, the after/before ratio of HMGB1/TM remained high at 2.14. No significant correlations ($P > 0.05$) could be established between the levels of HMGB1 and TM, IL-6, IL-1β or MDA before or after hemodialysis (Table 3).

The results with proinflammatory cytokines are shown in the Figure 2 panels A and B. The levels of IL-6 and IL-1β were not changed before and after HD procedure ($p>0.05$). Similar result was observed with biomarker of oxidative stress, MDA. The comparison of MDA concentration before and after HD were not significant ($p>0.05$) (Figure 3). No correlation was observed when HMGB-1 and TM, IL-1β, IL-6 and MDA were studied (Table 3).

5.0 Discussion

The balance between cytokines could define the pro- or anti-inflammatory profile of a disease. In hemodialysis, the interaction between blood cells and the dialysis membrane may generate changes in blood profile, possibly leading to the activation or inhibition of soluble mediators secretion, oxidative stress and/or cell death. Clearly, the potential consequences of alterations in blood profile need to be considered. The levels of the inflammatory mediators MDA, IL-6, IL-1β, TM and HMGB1 were compared in CKD patients before and after hemodialysis. The results obtained demonstrated that HMGB1 levels increased substantially after hemodialysis while those of MDA IL-6, IL-1β and TM were essentially unaltered.

Zakiyanov O, et al, (2013) reported that levels of HMGB1 and EN-RAGE were increased in the serum of AKI patients while the amounts of soluble RAGE (sRAGE) remained unaltered. Augmented HMGB1 is correlated with inflammation and malnutrition and, for this reason, this DAMP has been used as a marker of such conditions in patients undergoing peritoneal dialysis (Zhu N, et al, 2011). High levels of HMGB1 were observed in CKD blood in paired study with hemodialysis patients (Table 2 and Figure 1). In CKD patients, inflammation appears to be associated with morbidity and mortality (Chawla LS, et al, 2009; den Elzen WP, et al, 2006; Racki S, et al, 2006), but in such cases EN-RAGE and sRAGE can neutralize the effect of AGEs and HMGB1, thereby decreasing proinflammatory activity (Kalousova M, et al, 2007).

The results presented herein (Figure 1) demonstrated that the concentrations of TM were unchanged after hemodialysis, a finding similar to that reported by Keven K, et al, (2010) following a unpaired design experiment involving 15 hemodialysis patients. In contrast, Sioulis A, et al (2009) found that the levels of TM decreased in chronic patients after hemodialysis in unpaired analysis. According to these authors, high plasmatic levels of von Willebrand factor (vWF) and TM at the end stage of CKD in patients under hemodialysis treatment suggest endothelial dysfunction and coagulation disorders, while restoration of the balance of these factors could prevent clot formation (Sioulis A, et al, 2009). The discrepancies between results relating to the change in TM levels following hemodialysis could be due to experimental design rather than to the characteristics of the studied populations. In contrast to the other studies, a paired design was employed in which the same CKD patients were assessed before and after hemodialysis. Thus, our present results cannot be directly compared to other authors (Chawla LS, et al, 2009; den Elzen WP, et al, 2006).

There were no significant correlations between the levels of HMGB1 and TM, IL-6, IL-1β or MDA (Table 3). Physical contact between blood cells and dialysis membrane could have been influenced the levels of HMGB1 since it can be released from necrotic cells. Thus, the dialysis membrane could play an active role in hemodialysis by adsorbing and/or activating protein and/or leukocytes. For example, Omichi M, et al, 2010) has shown that the physical adsorption of human TM onto the dialysis membrane prevents blood coagulation, and this could constitute a better method of coagulation control than the administration of heparin. However, our

results with IL-6, IL-1β and MDA suggest that filter membrane was not able to activate cytokine secretions or MDA formation (Figure 2 and Figure 3). Similar results in a paired study were described by Tarakçıoğlu M, et al, (2003) that described a decreased of IL-8 levels without any change in IL-6, IL-1β and TNF-α when these cytokines levels were compared before and after HD. The correlation results (Table 3) showed that levels of HMGB1 were not correlated with any other biomarker. It suggests that HMGB1 did not activate cytokines secretion. Several authors have reported that cytokines such as IL-2, IL-6, IL-10, IL-12, TNF-α, and IFN-γ are increased in uremic CKD patients compared to healthy controls (Sester U, et al, 2000; Nagy E, et al, 1994; Girndt M, et al, 1995). These results are not directly comparable to our present data due to methodological approach. The authors concluded based on cytokine production between healthy controls and HD patients, and our results were from the same CKD patients before and after hemodialysis (paired test).

Based on the present results CKD patients did not show a pro-inflammatory profile after hemodialysis even with increased HMGB1. Furthemore, it is important to evaluate TM and HMGB1 together since the TM can modulates the proinflammatory effects of HMGB1 (Abeyama K, et al, 2005; Herzog C, et al, 2014; Lotze MT, et al, 2005). In spite a greater level of HMGB1 no significant difference between proinflammatory cytokines levels was observed before and after HD. Thus, the ratio between HMGB1 and TM could be used as a biomarker of the final blood profile after HD than individual levels of modulators. As shown in Table 2 the concentration of HMGB1 after HD was some two-fold higher than that recorded before the procedure, and this increase was likely related with the process of dialysis. Furthermore, the HMGB1/TM ratio increased by a factor of 2.14 after HD, indicating that the amount of HMGB1 per unit volume of blood was much greater than that of TM resulting in a predominance of the proinflammatory modulator. However, it appears that TM was enough to neutralize HMGB1 and to block RAGE or TLR activation. Although it has been reported that high levels of HMGB1 correlate with better outcomes in renal disease and sepsis (Zakiyanov O, et al, 2013; Sunden-Cullberg J, et al, 2005; Goldstein RS, et al, 2007; Hatada T, et al, 2005).

The levels of TM and HMGB1 were not quantified simultaneously in these studies and the results could be explained on the basis of high levels or activity of TM. Thus, it has been suggested that, in sepsis, a TM value that is significantly higher than HMGB1 indicates the absence of intravascular coagulation and control of inflammation, whereas low TM together with high HMGB1 enhances inflammation and coagulation leading to death. On this basis, the HMGB1/TM ratio could be used as a biomarker to evaluate inflammatory and infectious diseases. In spite of a high ratio between HMGB1/TM, the levels of biomarker for oxidative stress and pro-inflammatory cytokines were not changed before and after hemodialysis (Table 2, Figure 1-3).

The results of the present study show that hemodialysis induces an increase in HMGB1 in CKD patients without affecting the level of TM, IL-1β, IL-6 and MDA and that elevation of the HMGB1/TM ratio is not associated with the inflammatory profile. In conclusion, our present results suggest that hemodialysis does not aggravates neither down-regulates inflammatory response in CKD patients.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors wish to thank Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Rede Mineira de Toxinas com Ação Terapêutica for financial support.

Previous Presentations

None.

6.0 References

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Figures

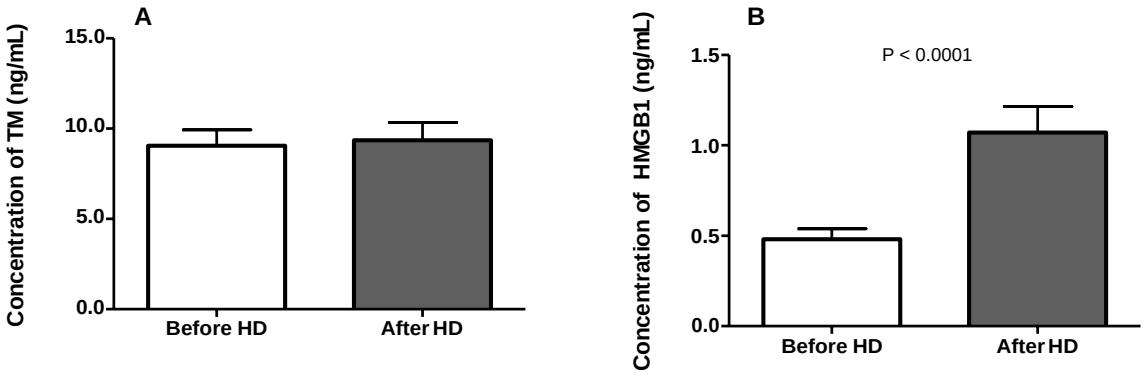

Figure 1
Blood levels of thrombomodulin and HMGB-1 before and after haemodyalisis
HMGB-1 = High mobility group box 1; TM = thrombomodulin. Levels of thrombomodulin (TM) (panel A) and high mobility group box 1 protein (HMGB1) (panel B) in blood of patients with chronic kidney disease (*n* = 20) sampled before and after hemodialysis (HD). Bars show mean ± standard deviation.

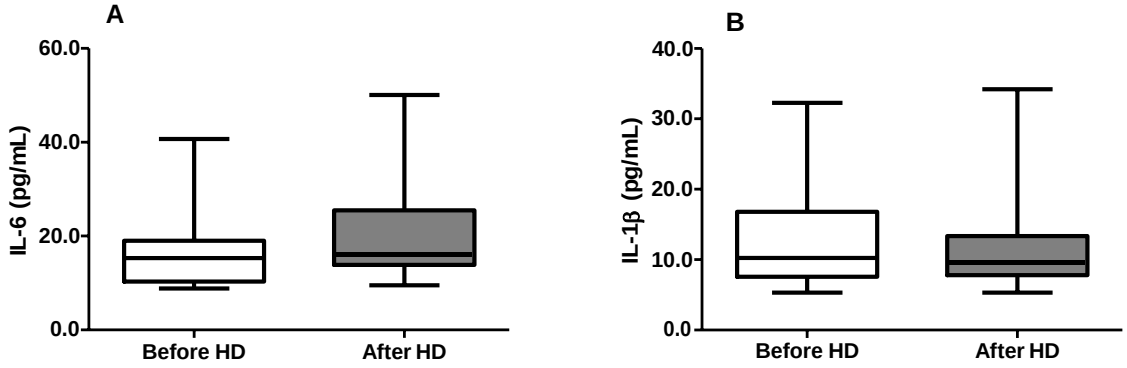

Figure 2
Levels of IL-6 (panel A) and IL1-β (panel B) levels in blood of patients with chronic kidney disease (*n* = 20) paired samples before and after haemodialysis (HD). Box show median with interquartile range.

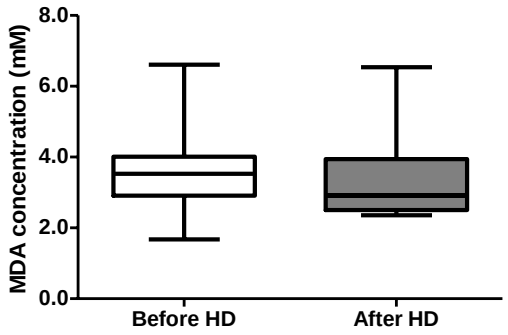

Figure 3
Levels of MDA levels in blood of patients with chronic kidney disease (n = 20) paired samples before and after hemodialysis (HD). Box show median with interquartile range.

Table 1

Characteristics of the population of patients with chronic kidney disease (*n* = 20)

Age	43.8 ± 3.5 years
Duration of hemodialysis	7.9 ± 5.0 years
Body mass index	24.5 ± 6.1 kg/m^2
Total cholesterol	156 ± 36.2 mg/dL
Low-density lipoprotein cholesterol	81.0 ± 27.1 mg/dL
High-density lipoprotein cholesterol	42.0 ± 13.2 mg/dL
Triglycerides	192.7 ± 33.0 mg/dL
Albumin	3.7 ± 0.3 g/dL

Values shown are mean ± standard deviation. This population as used before and after hemodialysis in an unpaired test.

Table 2

Levels of thrombomodulin (TM) and high mobility group box 1 protein (HMGB1) and HMGB1/TM ratios in patients with chronic kidney disease (n = 20) before and after hemodialysis

	Urea g/dL	TM ng/mL	HMGB1 ng/mL	HMGB1/TM ratio
Blood before hemodialysis	145.7 ± 29.0	9.48 ± 0.8	0.48 ± 0.2	0.051
Blood after hemodialysis	41.4 ± 12.0	9.92 ± 0.9	1.07 ± 0.5	0.109
Ratio of parameters after/before hemodialysis	0.28	1.05	2.23	2.14

Values shown are mean ± standard deviation

Table 3

Spearman correlations coefficients (*r*) for linear associations between levels of high mobility group box 1 (HMGB1) protein and the markers of kidney function, blood urea and thrombomodulin (TM)

	HMGB1			
	Before hemodialysis		After hemodialysis	
	r	*P*	*r*	*P*
TM	-0.31	0.19	0.16	0.49
IL-6	-0.06	0.82	-0.29	0.29
IL-1β	-0.12	0.66	-0.21	0.42
MDA	-0.34	0.21	-0.15	0.58

Correlations are significant at $P \leq 0.05$

