

Research Article

Effect of Indoxyl Sulfate on Oxidative Stress, Apoptosis, and Monocyte Chemoattractant Protein-1 in Leukocytes

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This study showed that indoxyl sulfate, an uremic toxin present in the serum of patients with chronic kidney disease, increases oxidative stress and apoptosis in human neutrophils and reduces the production of monocyte chemoattractant protein-1 (MCP-1) by peripheral blood mononuclear cell (PBMC). It is possible that these effects caused by this toxin contribute to vascular injury of the endothelium and decreased response to infectious insults, respectively.

1. Introduction

Uremic toxins are solutes that accumulate in the plasma of patients with loss of renal function [1–4]. More than 100 uremic solutes and toxins have been classified by the “European Uremic Toxin Work Group” (EUTox) [5, 6]. Uremic toxins have been considered one of the main factors that contribute to the state of inflammation [7–9] and have been associated with immune dysfunction in patients with chronic kidney disease (CKD) [10–17]. They have also been associated with cardiovascular disease (CVD), particularly because of their effects on different cells types leading to the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and superoxide anion (O_2^-) [18, 19] that contribute to oxidization of lipids, protein, and DNA damage. In addition, uremic plasma induces synthesis of inflammatory mediators as IL-1 [20], IL-6 [20], IL-12 [21],

TNF- α [22], and IL-10 [23] and chemokine as IL-8 [22] and Monocyte chemoattractant protein-1 (MCP-1) [24, 25].

MCP-1 is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages and it has been demonstrated to be induced and involved in various diseases. Migration of monocytes from the blood stream across the vascular endothelium is required for routine immunological surveillance of tissues, as well as in response to inflammation. Besides, MCP-1 is produced by a variety of cell types, either constitutively or after induction by toxins, oxidative stress, cytokines, or growth factors.

The immunosuppression observed in patients with CKD has also been associated with uremic toxins that contribute to dysregulated apoptosis of leukocytes and other cell types [9, 26–28].

The uremic toxin indoxyl sulfate (IS) has been associated with inflammation and renal interstitial fibrosis and both

processes contribute to progression of CKD [26]. This toxin originates from the intestinal metabolism of tryptophan, which is metabolized into indole by the action of tryptophanase produced by bacteria such as *Escherichia coli* present in the intestinal microbiota. The newly synthesized indole is absorbed by the bowel and metabolized in the liver and then converted into indoxyl sulfate [29]. In CKD, IS accumulates in the uremic plasma due to inadequate renal clearance [29].

The normal concentration of IS from healthy subjects is 0.6 mg/L. However, in CKD patients, the maximum uremic concentration is around 53 mg/L [30]. It has been described that at serum uremic concentration of IS induces an increase of ROS production and deregulations of the antioxidant system in renal tubules, contributing to the renal injury [31–33].

IS has also been associated with increased ROS production and lower proliferation of endothelial cells in uremic patients [34]. It has also been shown to be an independent predictor of vascular calcification in CKD patients [35].

Several studies have reported the association of IS at average serum uremic concentration in CKD patients with inflammatory processes in endothelial and kidney cells [4, 8, 11, 15]. However, there is still no study on the impact of retention of IS in uremia on the neutrophils and peripheral blood mononuclear cell (PBMC). Hence, we sought to investigate *in vitro* whether IS at uremic concentration may have effect on ROS production and apoptosis in neutrophils and MCP-1 synthesis by PBMC.

2. Materials and Methods

Blood samples (30 mL) were collected from 35 healthy donors (36 ± 12 years old) to obtain isolated neutrophils and PBMC. Exclusion criteria were age <18 years, hepatitis B and/or C virus infection, HIV infection, immunosuppressive treatment (chemotherapy, steroids), current use of anti-inflammatory drugs or antibiotics, cancer, diabetes, recent infection (<1 month), active rheumatic disease (e.g., lupus erythematosus, rheumatoid arthritis), and pregnancy.

This study was approved by the Ethics Committee on Research of the Universidade Federal de São Paulo. Healthy volunteers who signed an informed consent were enrolled in the study.

The neutrophils and PBMC (monocytes plus lymphocytes) were harvested by Ficoll-Hypaque separation [36] (Sigma Chemical Co., St. Louis, MO, USA) and hypotonic lysis of erythrocytes was performed. In brief, each 10 mL sample of heparinized (10 UI/mL) blood was diluted in 20 mL of normal saline, underlayered with 10 mL of Ficoll-Hypaque, and centrifuged at 500 ×g for 45 minutes at room temperature. The pellet of PBMC was separated in another tube and it was washed with phosphate buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO, USA) and we counted by using a standard hemocytometer. The purity of PBMC was greater than 93% and viability greater than 97% as judged by trypan blue exclusion method. PBMC was suspended at

1×10^6 cells/mL in sterile culture medium containing RPMI 1640 pH 7.4 (Sigma Chemical Co., St. Louis, MO, USA), 10 mM/L L-glutamine, 24 mM/L NaHCO₃ (Mallinckrodt, Paris, KY), 10 mM/L Hepes (Sigma Chemical Co., St. Louis, MO, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Irvine Scientific, Santa Ana, CA, USA) in the absence or presence at 53 mg/L of indoxyl sulfate (molecular weight: 251.3 g/mol, Sigma-Aldrich Co. St. Louis, MO, NY, USA), at 5% CO₂ at 37°C for 18 hours. The IS was diluted from stock solution immediately before each assay. In the remaining neutrophils and erythrocytes, 25 mL of PBS and 25 mL of 3% dextran (Sigma Chemical Co., St. Louis, MO, USA) were added to the buffy coat, to separate neutrophils from erythrocytes. After 15 minutes, the leukocyte-rich supernatant was harvested and centrifuged at 500 ×g for 10 minutes at 4°C. Residual erythrocytes were subjected to sequential hypotonic lysis procedure. After centrifugation, the cell pellet was resuspended in 25 mL ice-cold 0.2% NaCl and gently agitated for 30 seconds and osmolality was restored to normal by immediate addition of 1.6% NaCl. When the cell pellet (neutrophils) was free of erythrocytes, cells were suspended in ice-cold PBS, and neutrophils were counted by using a standard hemocytometer. The purity of neutrophils was greater than 95%, and viability was greater than 99% as judged by trypan blue exclusion method. Neutrophils were suspended at 1×10^6 cells/mL in sterile culture medium containing RPMI 1640, 10 mM/L L-glutamine, 24 mM/L NaHCO₃, 10 mM/L Hepes, 100 U/mL penicillin, and 100 mg/mL [37, 38].

Isolated neutrophils and PBMC were incubated in the presence and absence of IS at uremic concentrations average (53 mg/L) referred to the list of uremic toxins provided by EuTox group [30, 39] at 5% CO₂ at 37°C for 18 hours. All cells were incubated with IS plus 50% of RPMI medium and 50% of autologous serum (of each donor).

Apoptosis of neutrophils was measured by annexin V-FITC (Kit I Annexin V-FITC Apoptosis Detection, Pharmingen, Becton Dickinson Company, Franklin Lakes, NJ, USA). One of the cell-membrane changes during the early and intermediate stages of cell apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer layer of the cell membrane. Annexin V binds to the PS residue. To evaluate apoptosis, neutrophils were washed in PBS and density was adjusted to 1×10^6 /mL. One hundred microliters of the solution (1×10^5 cells) was transferred for a tube and 5 μL annexin V-FITC (Pharmingen, BD, USA) and 2 μL of propidium iodide (PI) were added. The neutrophils were incubated for 15 min at room temperature in the dark; 400 μL of binding buffer was added to each tube. The following controls were used: unstained neutrophils, neutrophils stained with annexin V-FITC (no PI), and neutrophils stained with PI (no annexin V-FITC). The degree of apoptosis was assessed by flow cytometry (FACS Canto Analyzer; Becton Dickinson Immunocytometry Systems, CA, USA) within 1 h. Neutrophils staining positive for PI were considered as dead cells (necrosis), neutrophils staining positive only for annexin V were considered as apoptotic, and neutrophils staining for PI plus Annexin V were considered as late apoptosis. The results of viability and apoptosis were expressed as

percentage. In addition, CD95 assay was performed to identify the degree of activation of the death pathway by staining neutrophils with CD95/Fas monoclonal antibody-FITC (CD95-FITC, PharMingen, Becton Dickinson, San Diego, CA, USA) according to the manufacturer. The expression of CD95 was assessed by mean fluorescence intensity (MFI) by flow cytometry. High MFI means more expression of respective monoclonal antibody.

ROS production was analysed by assays that measure the production of hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and nitric oxide (NO) by neutrophils, stimulated or unstimulated by IS. Hydrogen peroxide and superoxide anions were quantified by measuring the oxidation by 2,7 dichlorofluorescein diacetate (DCFH-DA) (Sigma, St Louis, MO, USA) and nitric oxide was quantified by measuring the oxidation by 4,5 diaminofluorescein diacetate (DAF) (DAF, Sigma, St. Louis, MO, USA) in neutrophils. Both DCFH-DA and DAF are stable, nonfluorescent, nonpolar compounds that can diffuse through the neutrophils membranes.

Once inside the neutrophils, the acetyl groups are cleaved by cytosolic enzymes to form the polar nonfluorescent dichlorofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2,7 dichlorofluorescein (DCF) in the presence of hydrogen peroxide or superoxide anion thus providing a quantitative assessment of the ROS in individual neutrophils through measure of mean fluorescence intensity (MFI) using flow cytometry [40, 41].

For the H_2O_2 and O_2^- production assays, a mixture of 100 μ L of neutrophils (1×10^6 cell/mL), 200 μ L of 0.3 mM of DCFH-DA, 700 μ L of PBS, and 100 μ L of IS (at final concentration of 53 mg/L) was prepared in separated tubes. To evaluate unstimulated ROS production, one of the tubes was free of IS. The tubes were incubated with agitation for 30 min at 37°C in a shaking water bath, and then 2 mL of ethylenediaminetetraacetic acid (EDTA) (Sigma, St Louis, MO, USA) was added. The same procedure was done to detect the production of nitric oxide by DAF at a concentration of 1 mM. For the NO production assay, a similar procedure was performed with DAF. Histograms of the MFI for each tube were constructed for both methods (DCFH and DAF) to detect ROS.

The levels of monocyte chemoattractant protein-1 (MCP-1) were measured in the supernatant of PBMC by enzyme immunoassay technique according to the manufacturer's recommendations CCL2/MCP-1 Human Quantikine kit (R&D Systems, Inc., Minneapolis, USA). The minimum detectable concentration by this kit is 5.0 pg/mL.

3. Statistical Analysis

Statistical analysis was performed by SPSS for Windows, version 16.0. The results were expressed as mean \pm standard deviation and percentage. To test differences between groups, we used the Mann-Whitney test.

The Spearman correlation test was used to verify the correlation between nonparametric variables. The results were considered statistically significant when $P < 0.05$.

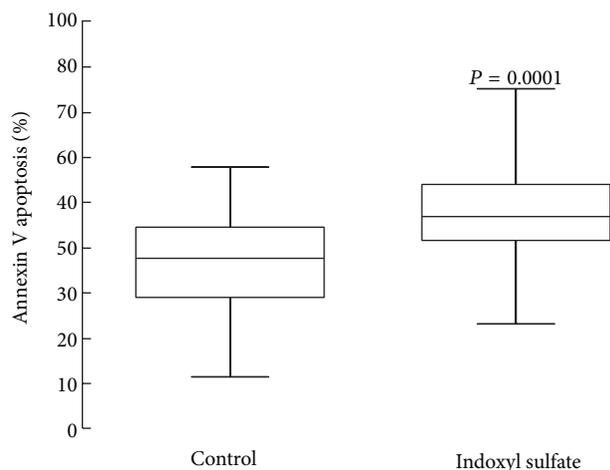


FIGURE 1: Evaluation of apoptosis (%) of PMN labeled by annexin V-FITC in the absence and presence of indoxyl sulfate.

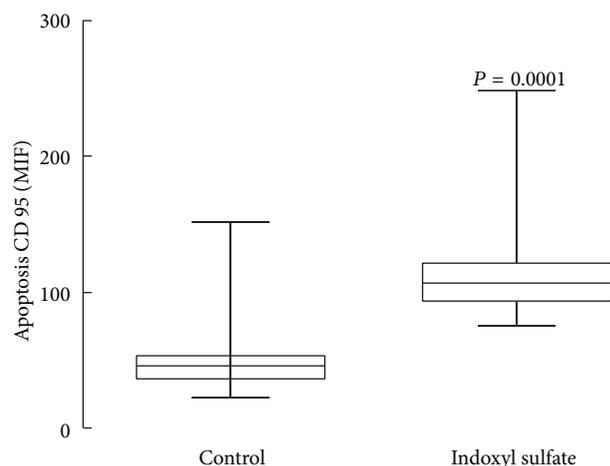


FIGURE 2: Evaluation of apoptosis (MIF) of PMN through the expression of Fas (CD 95-FITC) in the absence and presence of indoxyl sulfate.

4. Results

Apoptosis detected by annexin V ($47\% \pm 11\%$ versus $36\% \pm 11\%$; $P = 0.0001$) and CD95 expression (110 ± 29 versus 48 ± 21 MFI; $P = 0.0001$) were higher in neutrophils incubated in the presence of IS (Figures 1 and 2, resp.). In addition, IS induced higher ROS production (detected by DCFH-DA method) in neutrophils compared to control ($43,200 \pm 31,488$ versus 8633 ± 5138 MFI; $P = 0.0001$) (Figure 3). On the other hand, there was no difference in NO generation detected by DAF between the groups ($1,595 \pm 694$ versus $1,413 \pm 609$ MFI; $P = 0.33$) (Figure 4).

With respect to the generation of ROS, we observed a positive correlation between ROS production and apoptosis (both for expression of CD95 and annexin V) and generation of NO (Table 1).

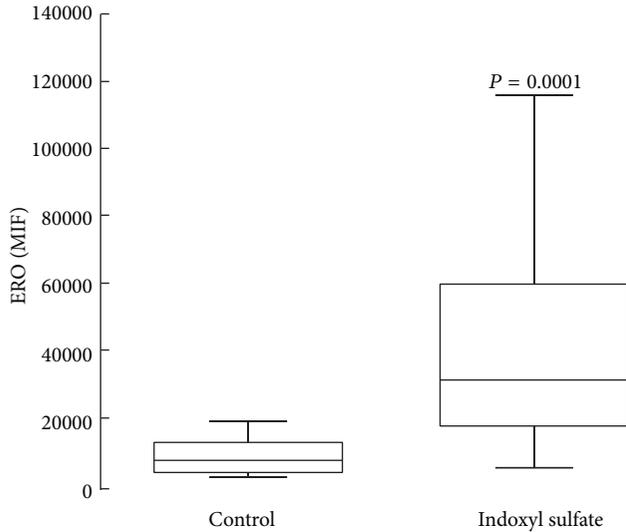


FIGURE 3: Production of ROS (staining by DCFH) from PMN incubated in the absence and presence of indoxyl sulfate (MIF).

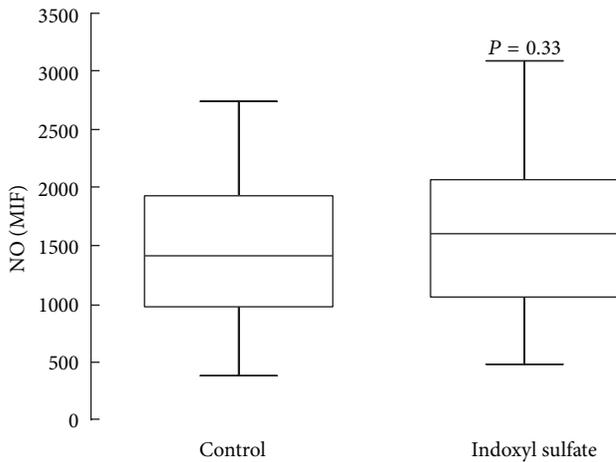


FIGURE 4: Production of nitric oxide (NO) PMN incubated in the absence and presence of indoxyl sulfate.

As for the effect of indoxyl sulfate on monocytes, we observed decreased expression of MCP-1 in the presence of this toxin compared to the controls (979 ± 526 versus $1,673 \pm 1,232$; $P = 0.01$) (Figure 5).

5. Discussion

This is the first report that evaluated the *in vitro* effect of indoxyl sulfate on apoptosis, ROS production in neutrophils, and the expression of MCP-1 in PBMC.

We observed increased apoptosis in neutrophils incubated with IS compared to control. Uremia has been linked to immune deficiency, and dysregulation of apoptosis in leukocytes has been suggested as one of the mechanisms involved. Thus, the *in vitro* effect of IS on neutrophils, causing apoptosis

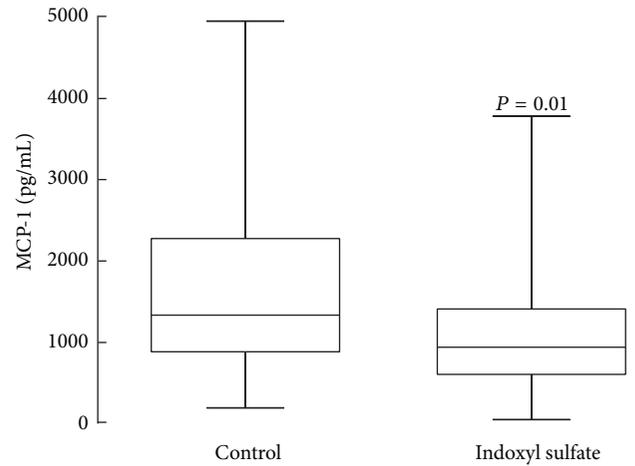


FIGURE 5: Expression of MCP-1 in PBMC incubated in the absence and presence of indoxyl sulfate.

TABLE 1: Correlation between ROS production and viability, apoptosis by annexin V, CD 95 expression, and nitric oxide in neutrophils incubated in the presence of indoxyl sulfate.

	ROS production	
	<i>r</i>	<i>P</i>
Viability (%)	0.12	0.35
Annexin V apoptosis (%)	0.38	0.02
CD 95 apoptosis (MFI)	0.60	<0.001
Nitric oxide (MFI)	0.35	0.005

Spearman correlation.

and consequently decreasing their function, suggests that this toxin may be associated with decreased immune response and contribute to increased risk for infections observed in CKD and end stage renal disease (ESRD) patients.

We also observed a higher ROS production in neutrophils incubated with IS. There was a positive correlation between ROS production and neutrophil apoptosis, suggesting that IS may cause mitochondrial injury by increasing ROS production, which might in turn activate the caspase cascade, thereby leading to the apoptosis of these cells. However, the effect of IS on apoptosis and ROS production may not be specific for neutrophils. In fact, this occurs in other cells type, as ROS production by IS has also been shown on proximal tubular cells and was associated with kidney fibrosis [42, 43].

Additionally, based on our *in vitro* results, it is possible to speculate that these effects of IS on neutrophils may also have implications and contribute to the pathophysiology of endothelial vascular cells injury observed in uremia. Chronic kidney disease (CKD) is recognized as a common condition that elevates the risk of atherosclerotic cardiovascular disease (CVD). Evidence suggests that increased oxidative stress is an emerging key mechanism of atherosclerosis in CKD. Moreover, we observed that the expression of ROS by neutrophils was higher than the expression of nitric oxide. Some

authors have reported that nitric oxide binds to superoxide anion, resulting in the formation of peroxynitrite (ONOO⁻), suggesting that the large generation of superoxide anion can be an important mechanism in reducing concentrations and activity nitric oxide [44]. In fact, in our study we found that indoxyl sulfate induced an increase of O₂⁻ and H₂O₂ detected by DCFH, which could result in lower concentration of NO. So, this could be one of the IS pathways that also contribute to endothelial injury, inducing increased ROS production and indirectly reducing concentrations of NO, due to the increased expression of superoxide anion. It is worthwhile to note that we performed an *in vitro* study, so it will be necessary an *in vivo* investigation to prove the association between the effects of IS causing a high ROS production and consequently an oxidative damage to endothelial cells. However, our *in vitro* findings raise the possibility that IS plays an important pathophysiological role in the development of CVD in individuals with CKD.

With respect to the *in vitro* effect of IS on the production of MCP-1 by PBMC, surprisingly, we observed that the production of MCP-1 was significantly lower in cells that were incubated with IS, in contrast with what we expected. Zager et al. observed a downregulation of MCP-1 mRNA in mice HK2 cell previously incubated with peritoneal dialysate [45]. These authors concluded that this effect was influenced by the presence of uremic toxins. It is important to note that high serum levels of MCP-1 observed in CKD patients might be from injury of other tissues in uremic environment and may not reflect a specific cell response, as investigated in our study. Additionally, the *in vitro* results about MCP-1 levels probably depend on the cell types, stimulus, and dose. It is also possible that toxicity of IS on monocytes has resulted in the diminishing MCP-1 production.

As MCP-1 is a chemokine, the effect of IS may contribute to a lower recruitment of leukocytes and consequently to reduced response to infectious challenges, a state that is frequently observed in patients with CKD.

There were some limitations in our study: (1) we investigated apoptosis only on neutrophils, because we used annexin-V and CD95 to evaluate apoptosis, and both methods have not been described as good markers of apoptosis on monocytes; (2) IS is a uremic toxin binding protein, and despite the fact that we used autologous serum from healthy individuals with albumin concentration at 4.0–4.5 mg/dL to incubate cells, we did not perform an isolated assay with albumin at physiologic concentrations (4.0 g/dL) and we did not include a group of cells incubated with a sulfate radical in the same conditions to indoxyl sulfate to eliminate the presence of a toxic effect of this compound.

In conclusion, our original *in vitro* study showed that IS increased neutrophils ROS production and this effect may be one of the mechanisms by which this toxin may contribute to vascular endothelium injury. Additionally, the IS induced apoptosis of neutrophils and decreased production of MCP-1 by PBMC, suggesting that this toxin may also contribute to a reduced response to infectious challenges, thereby increasing the risk of infections in CKD patients. However, our findings merit further *in vivo* investigations.

Conflict of Interests

The authors declare that there is conflict of interests regarding the publication of this paper.

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