Research Article

Iron Inhibits Respiratory Burst of Peritoneal Phagocytes In Vitro

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Objective. This study examines the effects of iron ions Fe3+ on the respiratory burst of phagocytes isolated from peritoneal effluents of continuous ambulatory peritoneal dialysis (CAPD) patients, as an in vitro model of iron overload in end-stage renal disease (ESRD).

Material and Methods. Respiratory burst of peritoneal phagocytes was measured by chemiluminescence method.

Results. At the highest used concentration of iron ions Fe3+ (100 µM), free radicals production by peritoneal phagocytes was reduced by 90% compared to control.

Conclusions. Iron overload may increase the risk of infectious complications in ESRD patients.

1. Introduction

Anemia is a common complication of end-stage renal disease (ESRD) and results from decreased erythropoietin (EPO) production, reduced bone marrow response to EPO, shortened red blood cell survival, and chronic iron loss. Anemia can be treated by administration of erythropoiesis-stimulating agents, that is, EPO [1].

The efficacy of EPO treatment in dialysis patients is determined mainly by the availability of iron [2]. Hence, intravenous iron supplementation is recommended in ESRD patients to support erythropoiesis [3]. However, iron supplementation can cause iron overload, occurring typically when the plasma iron content exceeds the iron-binding capacity of transferrin. Iron overload can also be a consequence of increased erythrocyte destruction [4].

Iron and its binding proteins have immunoregulatory properties, and altering the balances in immune system by iron overload or deficiency may produce detrimental physiological effects [5]. As infection contributes greatly to mortality and morbidity in ESRD patients [6], the potential for iron therapy to impair immune system and thus to increase the risk of infection deserves special consideration.

Reactive oxygen species produced by phagocytic leukocytes (macrophages, monocytes, and neutrophils) play a primary role in antimicrobial defense of the host. Phagocytosis of bacteria, viruses, and so forth is accompanied by a sharp increase in oxygen uptake, a process known as “respiratory burst,” resulting in the production of the toxic oxygen compounds, that is, H2O2, hydroxyl radical, and superoxide anion, which kill phagocytosed microorganisms [7].

The aim of the study was to investigate in vitro the effects of iron ions Fe3+ on the respiratory burst of phagocytes isolated from peritoneal effluents of continuous ambulatory peritoneal dialysis (CAPD) patients.

2. Methods

2.1. Isolation of Peritoneal Phagocytes. Peritoneal phagocytes were obtained from CAPD patients not receiving iron and/or EPO cotreatment and not suffering from any infection during the study. Cells were isolated from peritoneal overnight dwell time effluents by centrifugation (1200 × g, 10 min, 4 °C), followed by removal of the contaminating erythrocytes by hypotonic lysis. To harvest sufficient number of cells, effluents of five different patients were used for obtainment of each cell pool. Following isolation, cells were resuspended in Krebs-Ringer buffer, counted, and analysed using May-Grünwald Giemsa staining performed concomitantly with the trypan blue exclusion viability test.
The study was approved by the ethics committee of Jagiellonian University in Cracow, and informed consent was obtained from each of the patients.

2.2. Measurements of Respiratory Burst. Respiratory burst of peritoneal phagocytes was measured using chemiluminescence (CL) method. Briefly, cells were plated in 96-well white plates (1.5 × 10^5/well) and incubated (30 min, 37°C) in the presence or absence of urea (5–50 mM), creatinine (50–1000 µM), or iron ions Fe^{3+} (10–100 µM; as FeCl₃ solution), before addition of Krebs-Ringer buffer containing luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; 2.3 mM) and glucose (5 mM). In the separate series of experiments, cells were exposed for 20 min to lipopolysaccharide (LPS; E. coli 0111:B4; 100 ng/mL) prior to the treatment with the above-mentioned agents. Respiratory burst was initiated by addition of latex beads suspension (polystyrene, 0.8 µm, 3% aqueous suspension; 10 µL/well). CL was measured for 60 min at 37°C using Microlumat LB96P luminometer (Berthold, Austria).

2.3. Statistics. All measurements were performed in triplicates. Respiratory burst results are expressed as area under curve (AUC), being the difference between time-response curves of CL intensities recorded in the presence and absence of latex. Results are given as mean ± SEM, calculated on the basis of five independent experiments, each experiment being performed on cells pooled from dialysates of five patients. Unless indicated otherwise, statistical analyses were performed on nonnormalized data using one-way ANOVA followed by Tukey-Kramer multiple comparison test. IC_{25} and IC_{50} values were estimated based on interpolations of data from concentration-response curves. *, **, and *** indicate \( P < 0.05, 0.01 \) and \( 0.001 \), respectively.

3. Results

In the present study, the effects of iron ions Fe^{3+} on latex-induced respiratory burst of peritoneal phagocytes were investigated. As bacterial infection is considered as a major complication of CAPD [8], respiratory burst was also measured in the presence of LPS, one of the most powerful bacterial virulence factors with proinflammatory properties [9]. The effects of iron were compared with phagocyte response to two classical uremic toxins, that is, urea and creatinine.

Peritoneal cell populations used in the study contained 42–52% macrophages, 36–42% lymphocytes, and 5–7% neutrophils, and their overall viability was 93–97%. The highest respiratory burst intensities were observed for 1.5 × 10^5 cells/well and 10 µL latex suspension/well (data not shown), and these conditions were used in subsequent experiments.

LPS was found to be a strong primer of latex-induced respiratory burst. Figures 1(a) and 1(b) show time-response curves of CL for nonprimed and LPS-primed (100 ng/mL, 20 min) cells, respectively, with the AUC value calculated for the primed cells being \( \sim 2.5 \times \) higher than in the absence of LPS.

Iron ions Fe^{3+}, urea, and creatinine were used at the concentrations ranging from physiological to maximal (but
Figure 2: Effects of urea ((a) and (b)), creatinine ((c) and (d)), and iron ions Fe\(^{3+}\) ((e) and (f)) on respiratory burst of peritoneal phagocytes. Dose-response curves showing the intensities of respiratory burst calculated from AUC values for nonprimed ((a), (c), and (e)) and LPS-primed (100 ng/mL, 20 min; (b), (d), and (f)) cells. Cells were exposed to tested agents for 30 min prior to CL measurements. Results are given as mean ± SEM relative to controls on the basis of five independent experiments.
Achieving and maintaining iron sufficiency is crucial for the efficacy of EPO treatment in dialysis patients [2]. Available reports on the effects of iron supplementation on immunity of ESRD patients are inconclusive. Several large-scale prospective studies showed no relationship between risk of bacteremia and iron administration [10, 11]. In contrast, retrospective trial in hemodialysis patients reported a possible increase in infection rate associated with iron therapy [12]. As the biologically active iron plays an important role in the immunity [5], the controversy surrounding the effects of iron supplementation on risk of infections in ESRD patients requires special attention.

In this study, we investigated in vitro effects of iron on respiratory burst of phagocytes obtained from CAPD patients. Investigations were performed on both nonprimed and LPS-primed phagocytes and compared to the effects of urea and creatinine. The lowest concentrations of urea and creatinine used in our study reflected the physiological plasma levels found in healthy subjects while middle and the highest concentrations were within the range observed in ESRD patients [13]. Iron was used at the concentrations comparable with the plasma levels of non-transferrin-bound Fe3+ in CAPD patients (10 mM) or in iron overload patients (50–100 mM) [14].

Both urea and creatinine inhibited the respiratory burst of phagocytes in a concentration-dependent manner, even at concentrations comparable to physiological. This observation is in agreement with the results obtained by Daniels et al. showing the inhibitory effects of low-molecular-weight constituents of peritoneal dialysis effluent on respiratory burst of PMNL in vitro [15]. The effects of non-transferrin-bound Fe3+ on respiratory burst were much more pronounced as compared to urea and creatinine. At the highest used concentration of iron, free radicals production by peritoneal phagocytes was reduced by 90%.

The mechanism by which iron inhibits respiratory burst of peritoneal phagocytes remains unclear. A possible explanation could be peroxidation of membrane lipids mediated by iron ions Fe3+ as some products of lipid peroxidation have been reported to suppress respiratory burst of macrophages [16]. Further investigations are needed to verify this hypothesis. Future studies should also address the possibility of additive or synergistic interactions between uremic toxins and iron ions Fe3+.

The results obtained in this study suggest that iron supplementation can suppress respiratory burst of phagocytes. As the iron deficiency can also impair immune system, careful assessing of iron status in ESRD patients can be of importance to reduce the risk of infections. However, data obtained in vitro must be considered with appropriate limitations when we try to extrapolate them directly to in vivo situation.

### Authors’ Contribution

K. Gotfryd and A. Jurek contributed equally to this work.

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**Table 1: IC25 and IC50 values for the effects of urea, creatinine, and iron on respiratory burst of nonprimed and LPS-primed peritoneal phagocytes.**

| Tested agent | Used phagocytes | IC25 | IC50 |
|--------------|-----------------|------|------|
| Urea         | Nonprimed       | 18.3 mM (17.3–19.5) | >50 mM |
|              | LPS-Primed      | 6.1 mM (5.3–6.9)    | >50 mM |
| Creatinine   | Nonprimed       | 973.3 µM (940.1–1007.6) | >1000 µM |
|              | LPS-Primed      | 455.3 µM (444.9–465.8) | >1000 µM |
| Iron ions Fe3+ | Nonprimed      | 9.5 mM (5.3–10.0)   | 34.5 mM (32.8–36.2) |
|              | LPS-Primed      | 7.3 mM (4.7–8.3)    | 26.8 mM (26.2–27.5) |

Numbers in parentheses indicate interpolated values for IC25 or IC50 ± SEM (based on AUC values from five independent experiments).
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