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Original Paper

Uremia and Hypoxia Independently Induce Eryptosis and Erythrocyte Redox Imbalance

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Key Words
Indoxyl sulfate • Eryptosis • Hypoxia • ROS • Glutathione • Uremia

Abstract
Background/Aims: Red blood cell (RBC) death could contribute to anemia in chronic kidney disease (CKD) patients. Recent observational research has suggested a relationship between RBC death (eryptosis) and hypoxemia in hemodialysis patients. Thus, we studied the isolated and joint effects of a uremic toxin (indoxyl sulfate; IS) and hypoxia on RBC biology. ***Methods:*** We incubated RBC from healthy donors with IS at concentrations of 0.01mM, 0.09mM and 0.17mM under both normoxic (21% O_2) and hypoxic (5% O_2) conditions for 24 hours. Eryptosis was evaluated by RBC phosphatidylserine (PS) exposure, cell volume, and cytosolic calcium which were quantified by Annexin-V+, forward scatter, and Fluo-3AM+ binding, respectively. RBC redox balance was reported by reactive oxygen species (ROS) production and intracellular reduced glutathione (GSH). Analyses were performed by flow cytometry. ***Results:*** Hypoxia induced a 2-fold ROS production compared to normoxia. PS exposure and cytosolic calcium increased, while cell volume decreased by hypoxia and likewise by IS. IS increased ROS production in a dose-dependent manner under conditions of both normoxia and hypoxia. The same conditions promoted a GSH decrease with IS intensifying the hypoxia-induced effects. ***Conclusion:*** In summary, our results indicate that the concurrent presence of hypoxia and uremia augments RBC death and may therefore, contribute to the genesis of anemia in CKD.

Introduction

After maturation in the bone marrow, red blood cells (RBC) are released into the blood where they live for approximately 100 to 120 days [1]. In patients with chronic kidney disease (CKD) anemia is frequently observed. Anemia in CKD is multifactorial in origin and thought to be predominantly caused by insufficient production of erythropoietin (EPO) in the kidneys and a shortened RBC life span (RBCLS). We [2] and others [3-5] have shown that

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RBCLS in hemodialysis (HD) patients is reduced to about 73 ± 18 days (range 38–116) [3]. Both mathematical models [6] and clinical observation [3] show that a shortened RBCLS requires more erythropoiesis-stimulating agents (ESA) to achieve hemoglobin targets. In uremia, RBCLS may be reduced not only by uremic solutes such as acrolein [7], methylglyoxal [8], and indoxyl sulfate (IS) [9], but also by stressors, including osmotic shifts, oxidative stress, and energy depletion [10].

RBC death (eryptosis), can be triggered by increased cytosolic Ca^{2+} content and is indicated by the exposure of phosphatidylserine (PS) and ceramide formation on the RBC membrane [11, 12, 13]. We have shown that in RBC from healthy subjects (CON-RBC) the uremic toxin IS increases the generation of reactive oxygen species (ROS) and eryptosis by a pathway dependent on the influx of IS through organic anion transporter 2 (OAT2) as well as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity-dependent and glutathione (GSH)-independent mechanisms [14]. These findings lend support to a putative role of IS in the pathogenesis of renal anemia.

We have reported recently that approximately 10% of 983 chronic hemodialysis patients experienced prolonged intradialytic hypoxemia (PIH). These hypoxemic patients presented with a pro-inflammatory phenotype, characterized by increased inflammatory markers, lower albumin levels, anemia despite higher ESA utilization (indicative of ESA hyporesponsiveness), and higher morbidity and mortality [15]. Hypoxemia decreases oxygen delivery to the periphery and tissue hypoxia is further compounded by the presence of fluid overload, reduced cardiac output, capillary rarefication, and anemia. A reduction in the oxygen-carrying capacity may be related to a lack of hemoglobin caused by anemia. Patients with PIH had higher EPO use and lower hemoglobin and serum iron levels [15]. These clinical observations led us to hypothesize that the concurrent presence of hypoxemia, i.e., a reduced hemoglobin oxygen saturation, and uremic toxins may increase eryptosis and thus promote anemia and ESA hyporesponsiveness.

Although both eryptosis and intradialytic hypoxemia are not recent discoveries, basic research on these topics and their relationship to uremic toxicity has been scarce over the years. The goal of our study was to evaluate the effects of the uremic toxin IS in the presence or absence of hypoxic conditions on eryptosis and RBC redox status.

Materials and Methods

Subjects

This study was approved by the Ethics Committee of Pontifícia Universidade Católica do Paraná (PUCPR) (registration number 1.752.213). We obtained informed consent from healthy subjects that fulfilled the following inclusion criteria: men or women aged ≥18 years with normal creatinine and urea levels, normal hemoglobin (Hb) levels and absence of acute or chronic inflammation.

RBC preparation and incubation

Blood samples were drawn in 3.2% sodium citrate tubes (BD, Biosciences) and centrifuged at 3000 rpm for 15 min at room temperature. The buffy coat was carefully removed and discarded. The remaining RBC were washed twice with cold phosphate buffered saline (PBS). RBC were incubated for 24h at 37°C and 5% CO_2 in normoxic (21% O_2) or hypoxic (5% O_2) conditions, using the Culture Chamber ProOx 110 and ProCO2 120 (Biospherix, Redfield, NY, USA). RBC were incubated with IS at concentrations within the range observed in stage 5 CKD patients (0.01mM, 0.09mM, and 0.17mM) [16]. IS was diluted in Tris-Glucose-BSA buffer (all concentrations in mM; 21.0 tris [hydroxymethyl] aminomethane; 4.7 KCl; 2.0 CaCl2, 140.5 NaCl; 1.2 MgSO4, and 5.5 glucose, containing 4% of bovine serum albumin (BSA) [Sigma-Aldrich, St. Louis, MO, USA]—pH 7.4). Negative control RBC were incubated with Tris-Glc-BSA only. After incubations, RBC were prepared for assays. Fig. 1 shows a schematic of the study design.

Eryptosis

a) Measurement of annexin-V binding to quantify phosphatidylserine (PS) exposure: Cells were incubated with annexin-V conjugated with phycoerythrin (PE) (BD, Biosciences, Sparks, MD, USA) for 15 min in the dark, according to the manufacturer's instructions, then washed once with PBS and resuspended in FixFACS. PE fluorescence was analyzed by flow cytometry (FACS, Calibur BD Bioscience, Sparks, MD, USA); PS expression was expressed as Mean Fluorescence Intensity (MFI).

b) Cell volume analysis: Data were collected by flow cytometry (FACS, Calibur BD Bioscience, Sparks, MD, USA), and the forward light scatter channels (FSC) were set to linear gain. Cell size is the principal component of the FSC signal and the principles of the FACScan flow cytometer to measure cell volume have been described previously [9, 17, 18]. Briefly, approximately 100,000 RBC were analyzed per experiment and a histogram was generated and analyzed using FACScan analyzing software.

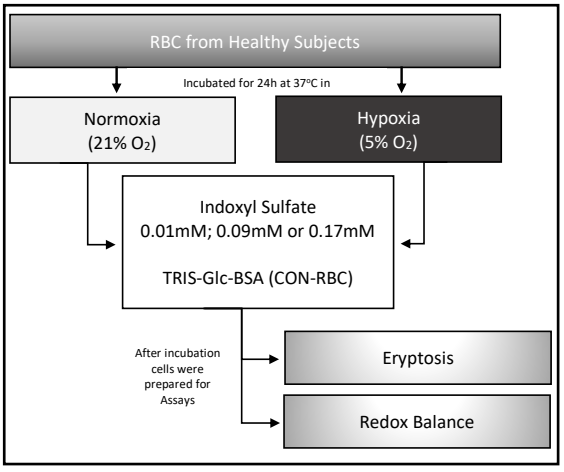

Fig. 1. Schematic view of the RBC incubations and Assays evaluated.

c) Measurement of intracellular calcium (Ca^{2+}): RBC were labeled with Fluo-3AM+ (Thermo Fischer Scientific, Waltham, MA, USA) for 40 min in 37°C in the dark, as per manufacturer's instructions, then washed 3x with PBS and resuspended in FixFACS. Fluo-3AM+ fluorescence was analyzed by flow cytometry (FACS, Calibur BD Bioscience, Sparks, MD, USA) and expressed as fold-change from CON-RBC in normoxia.

Redox Balance

a) Measurement of ROS production: RBC were resuspended in 2mM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Abcam, Cambridge, MA, USA) for 30 min in the dark. Then, RBC were resuspended in PBS + 0.4% formaldehyde (FixFACS). DCFH-DA fluorescence levels were quantified by flow cytometry (FACS Calibur BD Bioscience, MD, USA); results were expressed as MFI.

b) Measurement of GSH: RBC were incubated with ThiolTracker Violet (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min in the dark, according to the manufacturer's instructions, then washed once with PBS and resuspended in FixFACS. Fluorescence was determined by flow cytometry (FACS, Calibur BD Bioscience, MD, USA) and expressed as Relative % of ThiolTracker Violet after the samples were normalized against CON-RBC in normoxia.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Groups were compared by repeated measurement one-way analysis of variance (ANOVA) and post-hoc Tukey multiple comparison test. Paired t-test was performed to assess significant differences between normoxia and hypoxia. Furthermore, in order to explore if hypoxia intensified the IS effect, a t-test was used to determine the significance between the difference of normoxia vs hypoxia at different IS concentrations, denoted delta (Δ). Statistical analyses were performed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA). A p-value of $p<0.05$ was considered statistically significant.

Statement of Ethics

The Ethics Committee of Pontifícia Universidade Católica do Paraná (PUCPR) approved this study (Approved on August 29, 2016, under registration number 1.752.213).

Informed consent was obtained from all individuals enrolled in the present study.

Results

Description of the study population

RBC were obtained from healthy men (n=3) and women (n=7). Their mean age was 23.3±4.4 years. Table 1 presents the baseline characteristics of the study population.

Table 1. Baseline characteristics of participants. BMI: Body mass index. Hb: Hemoglobin. Data expressed as mean±SD or binary variables (frequency)

Laboratory characteristics of the study individuals	Healthy Subjects [n=10]
Age (years)	23.3± 4.44
Gender (Male %)	30
Caucasians (%)	100
BMI (Kg/m^2)	22± 2.53
Hb (g/dL)	14.1± 1.05
Creatinine (mg/dL)	0.7± 0.01
Urea (mg/dL)	24± 2.82

Eryptosis

Phosphatidylserine (PS) exposure. Increased PS exposure was found in RBC incubated with the two highest IS concentrations (IS 0.09 mM: 11.2±4.4 MFI; IS 0.17 mM: 14.2±5.8 MFI). PS exposure increased in hypoxic RBC, particularly with the two highest IS concentrations (IS 0.09 mM: 17.3±2.0; IS 0.17 mM: 19.5±5.1 MFI). Following previous results, the hypoxic environment per se, without IS addition, was able to induce more PS exposure than normoxia in CON-RBC (hypoxia: 11.5±2.6 MFI; normoxia 5.9±1.7 MFI). Similarly, increased PS exposure in hypoxia was significantly different in all IS concentrations compared to normoxia (Fig. 2). To find if hypoxia intensified the IS effect seen in normoxia, we calculated the delta, which is the variation of the increase in ROS production between CON-RBC and each IS concentration. When the deltas of the elevated PS exposure induced by hypoxia between CON-RBC and IS concentrations were compared, we found no significant difference between them. This result suggests that uremia and hypoxia act in an independent manner, although, when both are present, they confer additional RBC damage, resulting ultimately in premature cell death.

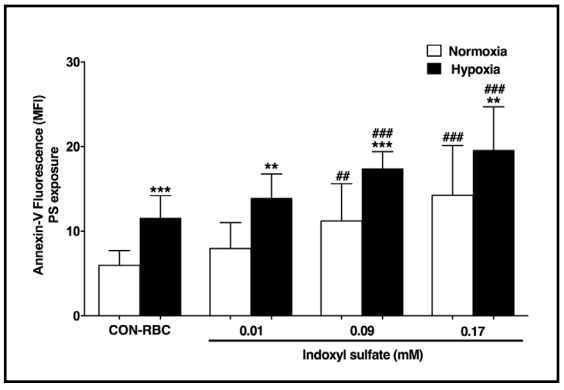

Fig. 2. IS promotes phosphatidylserine (PS) exposure in a time-dose response. RBC obtained from healthy subjects (n=10) were treated with different concentrations of IS (0.01, 0.09, 0.17mM) for 24h at 37°C in an incubator with controlled oxygen in under normoxic (21% O_2) (white bars) or hypoxic (5% O_2) (black bars) conditions. Control RBC (CON-RBC) were incubated with TRIS-Glc-BSA in normoxia and hypoxia. RBC were analyzed by flow cytometry, and the results were expressed as Mean Fluorescence Intensity (MFI). **p<0.01; ***p<0.001 between normoxia versus hypoxia in the same group. ##p<0.01; ###p<0.001 between CON-RBC versus IS concentration in the same group (normoxia vs normoxia; hypoxia vs hypoxia).

Cell volume. RBC volume was decreased with increasing IS concentration. A significant difference was found with the two highest IS concentrations (0.09 and 0.17mM), both in the normoxic (283±17.6 and 270.4±22.6, respectively) and in hypoxic conditions (232.5±19.5 and 214.7±14.7, respectively). Consistent with the PS exposure results, in both normoxia or hypoxia condition, cell volume decreased in a uremic toxin dose-dependent manner (Fig. 3). Following previous results, hypoxia per se induced a decrease of cell volume in the absence of IS (hypoxia: 272.3±26.1 versus normoxia: 317.8±27.2). Additionally, no significant difference between the deltas was observed, demonstrating that the decrease in the RBC volume seen in the CON-RBC in hypoxia is not different from the decrease observed after treatment with IS in hypoxia. These results reinforce the idea that these stimuli act independently.

Calcium influx. Under normoxic conditions cytosolic calcium levels increased in RBC incubated with IS when compared to control cells (IS 0.01mM 1.2±0.25-fold; IS 0.09 mM 1.5±0.2-fold; 0.17 mM 1.8±0.3-fold). In hypoxia, only the two highest IS concentrations

caused a significant increase in calcium influx compared to the control without IS (IS 0.09 mM 1.7±0.25-fold; 0.17 mM 2±0.25-fold). Additionally, calcium influx was significantly increased in hypoxia compared to normoxia when RBC were incubated with Tris-Glc-BSA (1.3±0.17-fold) or with the two lower IS concentrations (1.5±0.15-fold; 1.7±0.25-fold, respectively) (Fig. 4). Although hypoxic condition and IS increased cytosolic calcium in RBC, the deltas between control and IS concentrations in hypoxia compared with normoxia were not different. This suggests that hypoxia and uremia act independently, but when both stimuli are present calcium influx is increased, inducing PS exposure and ultimately eryptosis.

Redox Balance

ROS Generation. RBC incubated with TRIS-Glc-BSA (CON-RBC) under hypoxic conditions showed an increased ROS production compared to normoxia (17± 6.5 vs. 8.8±3.5 MFI) (Fig. 5). The addition of IS further augmented that ROS increase (IS 0.01 mM: 23.6±8.5 (hypoxia) vs 11.4±3.5 (normoxia); IS 0.09 mM: 31.1±6.9 vs 20.8±5.5; IS 0.17 mM: 32.8±4.7 vs 22.5±5.9).

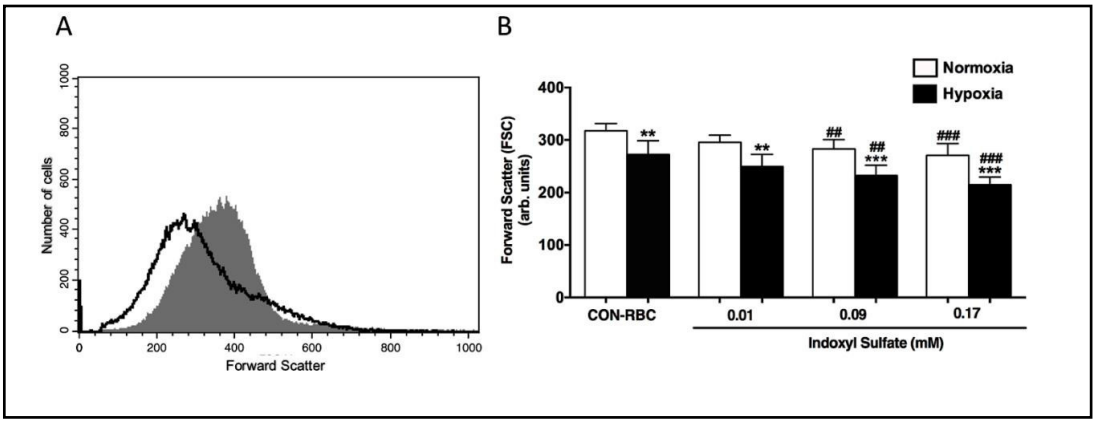

Fig. 3. Hypoxia and Uremia effects on RBC volume. A. Representative histogram of forward scatter of erythrocytes following 24-h exposure of RBC to normoxia (gray area) or hypoxia (black line). B. RBC obtained from healthy subjects (n=10) were treated with different concentrations of IS (0.01, 0.09, 0.17mM) for 24h at 37°C in an incubator with controlled oxygen under normoxic (21% O_2) (white bars) or hypoxic (5% O_2) (black bars) conditions. Control RBC (CON-RBC) were incubated with TRIS-Glc-BSA in normoxia and hypoxia. RBC were analyzed by flow cytometry. **p<0.01; ***p<0.001 between normoxia versus hypoxia in the same group. ##p<0.01; ###p<0.001 between CON-RBC versus IS concentration in the same group (normoxia vs normoxia; hypoxia vs hypoxia).

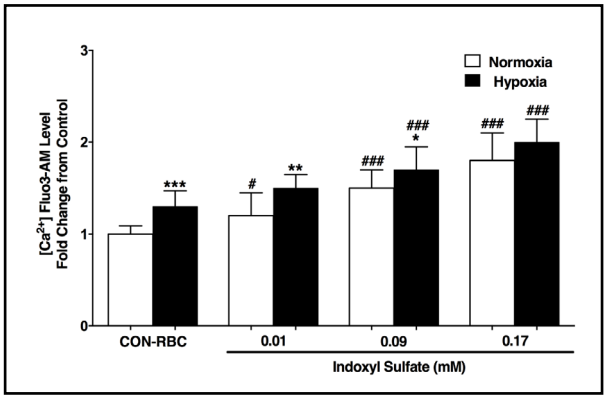

Fig. 4. RBC cytosolic calcium in response to the uremic and hypoxic condition. RBC obtained from healthy subjects (n=10) were treated with different concentrations of IS (0.01, 0.09, 0.17mM) for 24h at 37°C in an incubator with controlled oxygen under normoxic (21% O_2) (white bars) or hypoxic (5% O_2) (black bars) conditions. Control RBC (CON-RBC) were incubated with TRIS-Glc-BSA in normoxia and hypoxia. The analysis was realized by flow cytometry through Fluo-3AM fluorescence and the results were expressed as fold changes in fluorescent levels against CON-RBC in normoxia. *<0.05; **p<0.01; ***p<0.001 between normoxia versus hypoxia in the same group. #p<0.05; ###p<0.001 between CON-RBC versus IS concentration in the same group (normoxia vs normoxia; hypoxia vs hypoxia).

The results showed no significant difference between the deltas, demonstrating that the increased ROS production seen in the CON-RBC in hypoxia is not different from the increase observed with the three different concentrations of IS in hypoxia. Therefore, elevated ROS production is derived only by the rising concentrations of IS. This finding indicates that hypoxia and uremia act independently. However, in the presence of both, RBC increase ROS production in response to both stimuli. In normoxic conditions, IS at 0.09mM or 0.17mM increased ROS production by about 2.4-fold compared with the initial value measured for CON-RBC. Similarly, ROS production was about 1.7 times higher at 0.09 mM, or 0.17 mM IS in hypoxia compared to the initial ROS production in CON-RBC.

Intracellular reduced glutathione levels. In hypoxia, CON-RBC showed a slight increase in ThiolTracker-labeled cells when compared with the normoxic condition (118%±0.8 vs 100%±1.5, respectively). When IS was added to the incubations at different concentrations, the level of GSH decreased even further in a dose-dependent manner in both normoxia and hypoxia. While in normoxia only the highest IS concentration significantly decreased GSH levels (IS 0.17 mM: to 85%±0.7), in hypoxia GSH levels decreased at all IS concentrations (IS 0.01 mM 82%±1.2; 0.09 mM 68%±1.2; 0.17mM 60%±1.0). Again, we investigated the deltas of the decreased GSH levels between CON-RBC and IS concentrations in hypoxia. We found that there were significant differences in delta GSH levels between CON-RBC and IS concentrations demonstrating that hypoxia intensified the effect of IS seen in normoxia (Fig. 6).

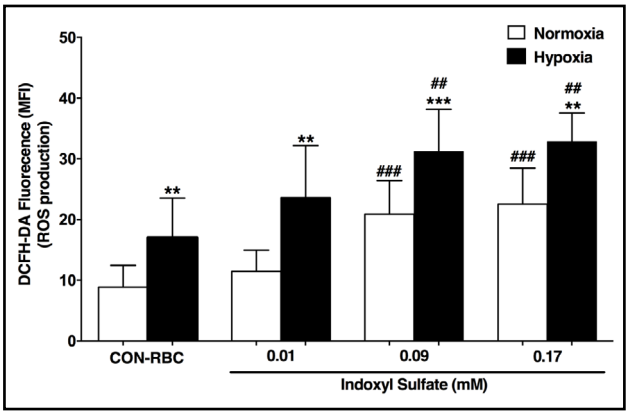

Fig. 5. ROS production in response to IS and hypoxia. RBC obtained from healthy subjects (n=10) were treated with different concentrations of IS (0.01, 0.09, 0.17 mM) for 24h at 37°C in an incubator with controlled oxygen partial pressure under normoxic (21% O_2) (white bars) or hypoxic (5% O_2) (black bars) conditions. Control RBC (CON-RBC) were incubated with TRIS-Glc-BSA in normoxia and hypoxia. ROS production was analyzed by flow cytometry through DCFH-DA probe fluorescence and results were represented as Mean Fluorescence Intensity (MFI). **p<0.01; ***p<0.001 between normoxia versus hypoxia in the same group. ##p<0.01; ###p<0.001 between CON-RBC vs. IS concentration in the same group (normoxia vs. normoxia; hypoxia vs. hypoxia).

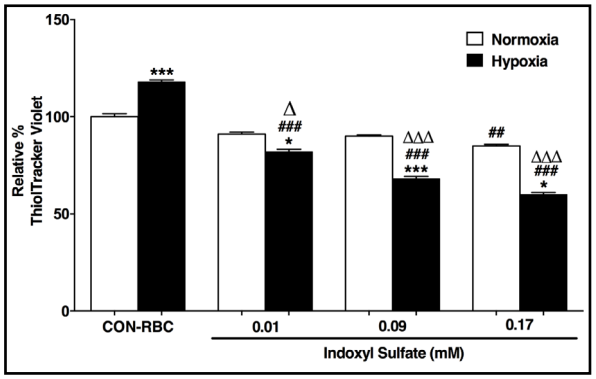

Fig. 6. Effect of IS on RBC GSH levels. RBC obtained from healthy subjects (n=10) were treated with different concentrations of IS (0.01, 0.09, 0.17 mM) for 24h at 37°C in an incubator with controlled oxygen under normoxic (21% O_2) (white bars) or hypoxic (5% O_2) (black bars) conditions. Control RBC (CON-RBC) were incubated with TRIS-Glc-BSA in normoxia and hypoxia. Thiol Tracker-labeled cells were analyzed by flow cytometry and results were represented as Relative % against CON-RBC in normoxia. *p<0.05; ***p<0.001 between normoxia versus hypoxia in the same group. ##p<0.01; ###p<0.001 between CON-RBC versus IS concentration in the same group (normoxia vs normoxia; hypoxia vs hypoxia). $^{\Delta}$p<0.05; $^{\Delta\Delta\Delta}$p<0.001 variation on GSH levels between IS concentrations and controls in normoxia and hypoxia.

Discussion

The main finding of our study is that hypoxia and the uremic toxin IS increase eryptosis and thus shorten RBCLS. Our research was motivated by the clinical observation that about 10% of chronic HD patients show a prolonged intradialytic hypoxemia (PIH), a clinical phenotype that is associated with inflammation, ESA hyporesponsiveness and increased morbidity and mortality [15]. We were particularly intrigued by the ESA hiporesponsiveness seen in PIH patients, because in healthy subject hypoxemia is the primary physiological stimulus of endogenous EPO production. This observation led us to hypothesize that in HD patients hypoxemia and uremic retentions solutes my act synergistically and reduce RBCLS.

In the present study we describe for the first time that hypoxia and uremia promoted alterations in healthy RBC characterized by an increased eryptosis and disturbance in redox balance compared to normoxia and non-uremic conditions. Uremic toxins have already been described as inducers of eryptosis characterized by increased PS exposure, reduced cell volume [7, 8, 9] and increased cytosolic calcium [9]. Our results indicate that PS exposure was elevated and cell volume was decreased in the presence of IS in a dose-dependent manner, confirming that IS is an essential promoter of eryptosis. Hypoxia also contributed to enhanced PS exposure and cellular shrinking compared to normoxia. Besides that, IS was able to increase cytosolic Ca^{2+} in a dose-dependent manner in both normoxia and hypoxia. Although hypoxia acts independently of the toxin concentration, both stimuli together increase PS exposure, promote cellular shrinkage and augment calcium influx into the RBC –concurrent events in the eryptosis process.

A previous study has demonstrated that IS can induce eryptosis and described that an increase in ceramide could play a role in the red cell death process [9]. Another study showed that ceramide synthesis in erythrocyte was associated with PS exposure [19]. Finally, ceramide has been reported to initiate the apoptosis in the PC12 cell line under hypoxic conditions in a p53-independent manner via caspase-3 activation [20]. It is possible that in a hypoxic and uremic condition, ceramide can participate in the eryptosis, which deserves further investigation. To the best of our knowledge, our study is the first report of erythrocyte cell death induced by hypoxia is associated with uremia. In addition, both hypoxia and the exposure to IS independently increased PS on the cell surface and decreased cell volume, both characteristics of eryptosis. Both stimuli increased calcium influx, an endogenous inducer of eryptosis.

Elevated PS exposure and cellular shrinkage associated with increased calcium influx have been shown in erythrocytes before [7, 9, 21]. Calcium efflux also contributes to RBC damage [17, 18, 22, 23], and the balance between calcium influx and efflux can be increased by hypoxia. Uremia is an essential contributor to apoptosis in various cell types [24, 25, 26] and hypoxia is a stimulus that could aggravate this scenario. RBC exhibit diverse oxygen-sensitive responses that autonomously regulate their properties and functions [27]. On the other hand, prolonged hypoxia leads to accumulation of deoxyhemoglobin and its interaction with the cytoplasmic domain of Band3 protein. This interaction triggers RBC deformability, membrane vesiculation, and ATP release via hemolysis reducing RBCLS [28].

RBC could be considered “reporter cells” for the oxidative state [29] since they may be particularly vulnerable to oxidative stress due to constant exposure to oxygen radicals generated by autoxidation of hemoglobin [30], an event amplified under hypoxic conditions [31]. Although ROS production is required to modulate metabolism or cellular signalling, excess levels of ROS can harm cellular structures and biomolecules [32]. The present study showed that IS increased ROS production in RBC in a dose-dependent manner under both normoxic and hypoxic conditions. Similarly, Dou et al. described that IS in endothelial cells enhanced ROS production, increased NAD(P)H oxidase activity, and decreased GSH levels. The authors concluded that IS modifies the balance between pro- and antioxidant mechanisms in these cells by promoting oxidative stress and decreasing levels of GSH [33].

The same pattern was observed in RBC when incubated in the presence of IS, since the levels of GSH were diminished in a dose-dependent manner and this reduction was intensified

by hypoxia. Interestingly, ROS production was increased in RBC by hypoxia while the levels of GSH only decreased when IS was present in the RBC incubation. These results suggest that RBC are less protected from the toxic effects caused by the oxidative stress promoted by IS in normoxia or hypoxia, as compared to the impact caused by increased intracellular ROS generation generated only by IS in normoxic condition. It has been suggested that hypoxia influences the antioxidant defense system in RBC through O_2-responsive cycling in the glucose metabolism [34, 35], which is linked to reduced recycling of antioxidant molecules. Our results indicate that the increased ROS production in hypoxia is independent of the toxin concentration. Therefore, patients who have PIH may demonstrate higher ROS production in RBC compared to the ones who do not suffer from PIH during dialysis treatment.

In RBC, glucose is metabolized by the Embden-Meyerhof and hexose monophosphate (HMP) pathways, and only through the latter NADPH is generated. The generated NADPH acts as a cofactor since it donates electrons to glutathione reductase. This enzyme reduces oxidized glutathione (GSSG) to the active and antioxidant form of glutathione (reduced glutathione; GSH). GSH generation produces NADP, which in turn stimulates glucose metabolism in the HMP. This mechanism equips RBC with an adequate reduction capacity during an oxidative challenge [30]. Our results demonstrate that the decreased GSH levels observed during periods of oxidative stress, such as in hypoxia, are dependent on the uremic environment. This effect of IS can be explained by a limited HMP activity under hypoxic conditions and, thus, a decreased supply of NADPH in RBC. This inability to generate NADPH results in a limited capacity to make GSH through glutathione reductase [35]. Since hypoxic erythrocytes lose their antioxidant defense, which is linked to the reduction of equivalent recycling [30], it is suggested that RBC do not recover from the oxidative damage generated by uremic toxicity. This inability to recycle may be associated with the constant oxidative environment IS promotes inside these cells related to the stimulus of hypoxia in constraining antioxidant defense generation.

Furthermore, our results show that CON-RBC in hypoxia had a slight increase in GSH levels, which can be explained by the fact that we used healthy RBC as a model. Thus, these cells have their antioxidant defense mechanism intact and can compensate for the hypoxic challenge through an adaptive response. It is known that HD patients have diminished GSH/GSSG redox potential compared to healthy individuals [36], which may render these patients more susceptible to oxidative damage caused by uremia. Here we show that uremic toxins like IS contribute to the inability of RBC to maintain a proper redox balance during the progression of the disease. However, the mechanism in which IS interferes in the GSH recycling should be further investigated. In summary, our results suggest that RBC incubated with different concentrations of IS show decreased GSH content and at the same time increased and sustained oxidative stress, probably due to altered recycling of antioxidant molecules in a hypoxic environment.

Admittedly, our study has some limitations. First, our blood donors were much younger than most HD patients [37]. Second, we studied only Caucasians. Third, our study population of 10 subjects is comparably small. On the other hand, this is the first study to identify hypoxia and uremia as synergistic triggers of eryptosis and oxidative stress, resulting in shortened RBCLS and possibly aggravating anemia in CKD and hemodialysis patients.

Conclusion

In summary, our investigation into the triad eryptosis, uremia, and hypoxia suggest: 1) an alteration in the recycling of antioxidant defense molecules during the increased oxidative stress inside the RBC; 2) the ability of IS to promote ROS production and increased PS exposure, both of which were raised under hypoxic conditions; and 3) the possibility that hypoxic stress may synergistically contribute to RBC changes induced by uremia (Fig. 7). Thus, our results support the idea that uremic toxins possibly play an important role in the

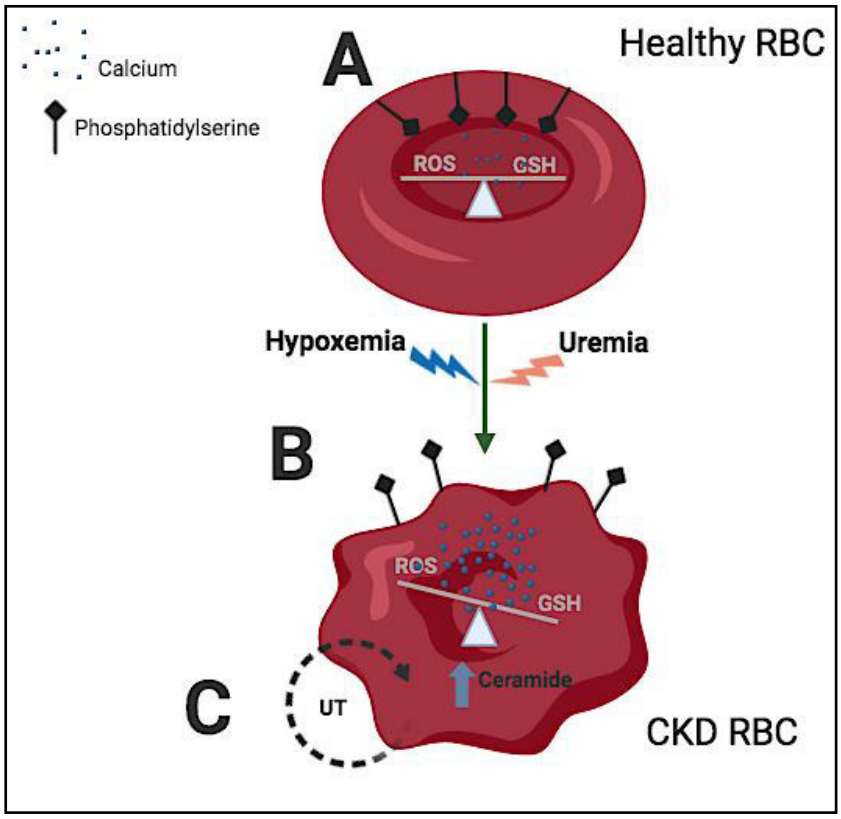

Fig. 7. Disturbance of red blood cell (RBC) homeostasis by uremia and hypoxemia. (A) Under normal conditions, healthy RBC maintain a normal calcium influx and a physiological balance between reduced glutathione (GSH) and reactive oxygen species (ROS). Under these circumstances, phosphatidylserine (PS) is located at the inner leaf of the RBC cell membrane and not exposed to the extracellular milieu. (B) In CKD, uremic toxins (UT), such as indoxyl sulfate (IS) accumulate; in addition, a low hemoglobin oxygen saturation (hypoxemia) is observed in a number of patients. These conditions in CKD RBC, favor an increased calcium influx and consequently a rise in intracellular calcium concentration. With the cellular redox state-maintained, the amount of ROS generation increases and GSH recycling is impaired. When the redox balance is disturbed, high ROS levels can disrupt the normal cellular machinery, eventually triggering the translocation of PS to the RBC surface, a signal that initiates RBC death (eryptosis). (C) Undertaking further study are necessary to understand 1. levels of ceramide formation under uremic and hypoxemic conditions, and 2. the impact of blocking the transport of the uremic toxins into RBC via organic anion transporter 2 (OAT2) on cell homeostasis. Fig. created using BioRender (https://app.biorender.com).

redox imbalance and reduction of RBCLS, predominantly in patients that suffer from chronic hypoxemia during dialysis.

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Author Contributions

Sara Soares Tozoni and Gabriela Ferreira Dias performed the experiments and participated in the preparation of the manuscript. Gabriela Bohnen performed the experiments. Nadja Grobe, Peter Kotanko and Roberto Pecoits-Filho prepared the manuscript for publication, specifically in a critical review by commentary and revision. Andréa Novais Moreno-Amaral was responsible for supervising the study, coordinating the research activities, and preparing the manuscript for publication.

Disclosure Statement

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References

1 Kiefer CR, Snyder LM: Oxidation and erythrocyte senescence. Curr Opin Hematol 2000;7:113–116.
2 Ma J, Dou Y, Zhang H, Thijssen S, Williams S, Kuntsevich V, Ouellet G, Wong MM, Persic V, Kruse A, Rosales L, Wang Y, Levin NW, Kotanko P: Correlation between Inflammatory Biomarkers and Red Blood Cell Life Span in Chronic Hemodialysis Patients. Blood Purif 2017;43:200-205.
3 Sato Y, Mizuguchi T, Shigenaga S, Yoshikawa E, Chujo K, Minakuchi J, Kawashima S: Shortened red blood cell lifespan is related to the dose of erythropoiesis-stimulating agents requirement in patients on hemodialysis. Ther Apher Dial 2012;16:522-528.
4 Strocchi A, Schwartz S, Ellefson M, Engel RR, Medina A, Levitt MD: A simple carbon monoxide breath test to estimate erythrocyte turnover. J Lab Clin Med 1992;120:392–399.
5 Takemoto Y, Okumiya T, Tsuchida K, Nakatani T, Yamamoto K: Erythrocyte creatine as an index of the erythrocyte life span and erythropoiesis. Nephron 2000;86:513–514.
6 Fuertinger DH, Kappel F, Thijssen S, Levin NW, Kotanko P: A model of erythropoiesis in adults with sufficient iron availability. J Math Biol 2013;66:1209-1240.
7 Ahmed M, Langer H, Abed M, Voelkl J, Lang F: The uremic toxin acrolein promotes suicidal erythrocyte death. Kidney Blood Press Res 2013;37:158–167.
8 Loss K, Schneider J, Nicolay JP, Föller M, Wieder T, Lang :. Stimulation of Suicidal Erythrocyte Death by Methylglyoxal. Cell Physiol Biochem 2006;18:223–232.
9 Ahmed MS, Abed M, Voelkl J, Lang F: Triggering of suicidal erythrocyte death by uremic toxin indoxyl sulfate. BMC Nephrol 2013;14:244.
10 Lang F, Lang KS, Lang PA, Huber SM, Wieder T: Osmotic shock-induced suicidal death of erythrocytes. Acta Physiol (Oxf) 2006;187:191–8.
11 Briglia M, Antonia Rossi M, Faggio C: Eryptosis: Ally or Enemy. Curr Med Chem 2017;24:937-942.
12 Mischitelli M, Jemaà M, Almasry M, Faggio C, Lang F: Triggering of Erythrocyte Cell Membrane Scrambling by Emodin. Cell Physiol Biochem 2016;40:91-103.
13 Fazio A, Briglia M, Faggio C, Alzoubi K, Lang F: Oxaliplatin induced suicidal death of human erythrocytes. Cell Physiol Biochem 2015;37:2393-2404.
14 Dias GF, Bonan NB, Steiner TM, Tozoni SS, Rodrigues S, Nakao LS, Kuntsevich V, Pecoits-Filho R, Kotanko P, Moreno-Amaral AN: Indoxyl Sulfate, a Uremic Toxin, Stimulates Reactive Oxygen Species Production and Erythrocyte Cell Death Supposedly by an Organic Anion Transporter 2 (OAT2) and NADPH Oxidase Activity-Dependent Pathways. Toxins (Basel) 2018;10:280.
15 Meyring-Wosten A, Zhang H, Ye X, Fuertinger DH, Chan L, Kappel F, Artemyev M, Ginsberg N, Wang Y, Thijssen S, Kotanko P: Intradialytic Hypoxemia and Clinical outcomes in Patients on Hemodialysis. Clin J Am Soc Nephrol 2016;11:616-625.
16 Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, Argiles A: Normal and Pathologic Concentrations of Uremic Toxins. J Am Soc Nephrol 2012;23:1258–1270.

17 Bissinger R, Modicano P, Alzoubi K, Honisch S, Faggio C, Abed M, Lang F: Effect of saponin on erythrocytes. Int J Hematol 2014;100:51-59.
18 Signoretto E, Honisch S, Briglia M, Faggio C, Castagna M, Lang F: Nocodazole induced suicidal death of human erythrocytes. Cell Physiol Biochem 2016;38:379-392.
19 Jilani K, Enkel S, Bissinger R, Almilaji A, Abed M, Lang F: Fluoxetine induced suicidal erythrocyte death. Toxins (Basel) 2013;5:1230-1243.
20 Yoshimura S, Banno Y, Nakashima S, Takenaka K, Sakai H, Nishimura Y, Sakai N, Shimizu S, Eguchi Y, Tsujimoto Y, Nozawa Y: Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. Inhibitory effects of Bcl-2 on ceramide formation and caspase-3 activation. J Biol Chem 1998;273:6921-6927.
21 Briglia M, Fazio A, Faggio C, Lang F: Triggering of Suicidal Erythrocyte Death by Zosuquidar. Cell Physiol Biochem 2015;37:2355-2365.
22 Polak-Jonkisz D, Purzyc L: Ca influx versus efflux during eryptosis in uremic erythrocytes. Blood Purif 2012;34:209–210.
23 Briglia M, Calabró S, Signoretto E, Alzoubi K, Laufer S, Faggio C, Lang F: Fucoxanthin induced suicidal death of human erythrocytes. Cell Physiol Biochem 2015;37:2464-2475.
24 Cohen G, Michael R, Hörl WH: Uremic toxins modulate the spontaneous apoptotic cell death and essential functions of neutrophils. Kidney Int 2001;59:48-52.
25 Fernández-Fresnedo G, Ramos MA, González-Pardo MC, de Francisco AL, López-Hoyos M, Arias M: B lymphopenia in uraemia is related to an accelerated *in vitro* apoptosis and dysregulation of Bcl-2. Nephrology Dial Transplant 2000;15:502–510.
26 Tanaka T, Hanafusa N, Ingelfinger JR, Ohse T, Fujita T, Nangaku M: Hypoxia induces apoptosis in SV40-immortalized rat proximal tubular cells through the mitochondrial pathways, devoid of HIF1-mediated upregulation of Bax. Biochem Biophys Res Commun 2003;309:222–231.
27 Mohanty JG, Nagababu E, Rifkind JM: Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. Front Physiol 2014;5:84.
28 Grygorczyk R, Orlov SN: Effects of Hypoxia on Erythrocyte Membrane Properties—Implications for Intravascular Hemolysis and Purinergic Control of Blood Flow. Front Physiol 2017;8:1110.
29 Minetti M, Agati L, Malorni W: The microenvironment can shift erythrocytes from a friendly to a harmful behavior: pathogenetic implications for vascular diseases. Cardiovasc Res 2007;75:21-28.
30 Cimen MY: Free radical metabolism in human erythrocytes. Clin Chim Acta 2008;390:1-11.
31 Balagopalakrishna C, Manoharan PT, Abugo OO, Rifkind JM: Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions. Biochemistry 1996;35:6393-6398.
32 Brigelius-Flohe R., Flohé L: Basic principles and emerging concepts in the redox control of transcription factors. Antioxid Redox Signal 2011;15:2335–2381.
33 Dou L, Jourde-Chiche N, Faure V, Cerini C, Berland Y, Dignat-George F, Brunet P: The uremic solute indoxyl sulfate induces oxidative stress in endothelial cells. J Thromb Haemost 2007;5:1302–1308.
34 Messana I, Orlando M, Cassiano L, Pennacchietti L, Zuppi C, Castagnola M, Giardina B: Human erythrocyte metabolism is modulated by the O2-linked transition of hemoglobin. FEBS Lett 1996;390:25-28.
35 Rogers SC, Said A, Corcuera D, McLaughlin D, Kell P, Doctor A: Hypoxia limits antioxidant capacity in red blood cells by altering glycolytic pathway dominance. FASEB J 2009;23:3159–3170.
36 Khazim K, Giustarini D, Rossi R, Verkaik D, Cornell JE, Cunningham SE, Mohammad M, Trochta K, Lorenzo C, Folli F, Bansal S, Fanti P: Glutathione redox potential is low and glutathionylated and cysteinylated hemoglobin levels are elevated in maintenance hemodialysis patients. Transl Res 2013;162:16–25.
37 Thomé FS, Sesso RC, Lopes AA, Lugon JR, Martins CT: Brazilian chronic dialysis survey 2017. J Bras Nefrol 2019;41:208-214.