PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
SCHOOL OF MEDICINE
GRADUATE PROGRAM IN HEALTH SCIENCES

INVESTIGATION OF ERYPTOSIS MECHANISMS AND REDOX STATE IN UREMIC ERYTHROCYTES IN RESPONSE TO HYPOXIA

Gabriela Ferreira Dias
Advisors: Andréa Novais Moreno-Amaral, Ph.D.
Peter Kotanko, M.D.

CURITIBA
December 2021

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Ph.D. thesis presented to the Graduate Program in Health Sciences at Pontifícia Universidade Católica do Paraná as a partial requirement for obtaining the title of Ph.D. in Health Sciences.

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CURITIBA

December 2021

Dados da Catalogação na Publicação
Pontifícia Universidade Católica do Paraná
Sistema Integrado de Bibliotecas – SIBI/PUCPR
Biblioteca Central
Luci Eduarda Wielganczuk – CRB 9/1118

Dias, Gabriela Ferreira
D541i Investigation of eryptosis mechanisms and redox state in uremic
2021 erythrocytes in responde to hypoxia / Gabriela Ferreira Dias ; advisors: Andréa Novais Moreno-Amaral and Peter Kotanko. – 2021.
96 f. : il. ; 30 cm

Tese (doutorado) – Pontifícia Universidade Católica do Paraná, Curitiba, 2021
Bibliografia: 88-96

1. Insuficiência renal crônica. 2. Anemia. 3. Eriptose. 4. Toxinas. 5. Stress oxidativo. I. Amaral, Andréa Novais Moreno. II. Kotanko, Peter. III. Pontifícia Universidade Católica do Paraná. Programa de Pós-Graduação em Ciências da Saúde. IV. Título.

CDD 20. ed. – 616.414

Pontifícia Universidade Católica do Paraná

Escola de Medicina
Programa de Pós-Graduação em Ciências da Saúde

PUBLIC SESSION OF THESIS EXAMINATION FROM THE GRADUATE PROGRAM IN HEALTH SCIENCES, (DOCTORATE DEGREE), PONTIFICIAL CATHOLIC UNIVERSITY OF PARANA

On december 10, 2021 took place at the Center for Health Sciences the public session of thesis examination entitled, **"ERYPTOSIS MECHANISMS AND REDOX STATE IN UREMIC ERYTHROCYTES IN RESPONSE TO HYPOXIA"** presented by **Gabriela Ferreira Dias**, candidate for a doctor degree in Health Science

The Board of examiners was composed for the following members:

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Profa. Dra. Andrea Novais Moreno Amaral	Evaluation: APPROVED
Prof. Dr. Peter Kotanko	Evaluation: APPROVED
Profa. Dra. Cristina Pellegrino Baena	Evaluation: APPROVED
Profa. Dra. Lia Sumie Nakao	Evaluation: APPROVED
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Final decision: APPROVED

Notes from the Examining Board:

Profa. Dra. Andrea Novais Moreno Amaral
President of the Examining Board

Prof. Dr. Cristina Pellegrino Baena
Coordinator of PPGCS PUCPR

In memory of my grandfather, who was so proud of me and called me doctor from the moment I started my Ph.D. course.

ACKNOWLEDGMENTS

To my alma mater, PUCPR, where I had the opportunity to learn, grow and meet brilliant researchers that guided me to where I am now.

To CAPES, for the scholarship that allowed me to dedicate my time entirely to this research.

To the Renal Research Institute (RRI), for the financial support that was fundamental to the development of this project, and for hosting me for one year of my doctoral course.

To my advisor Dr. Andrea Novais Moreno-Amaral, for the 9 years of supervision, kindness, and friendship. It was an honor to work under your guidance.

To Dr. Peter Kotanko, whose exceptional supervision, enthusiasm, and kindness I will never forget.

To Dr. Nadja Grobe, who is one of the most inspiring people I know. Thank you for pushing me to my full potential and being such a good mentor and friend.

To all my PUCPR lab mates, especially Sara Tozoni, Natalia Bonan and Ana Clara Almeida. Working alongside you was fun and creative. I am grateful for every special moment we shared.

To the RRI lab team, in special Dr. Xia Tao and Dr. Xiaoling Wang, whose enthusiasm and passion for research are contagious.

To my husband Paulo, for truly supporting me in every minute of this journey, helping me to be strong and fight for my goals, and for being by my side celebrating every achievement.

To my family, for their unconditional support. To my father for his hard work in educating me and encouraging me on my decisions. To my mother, for her endless love and for empowering me to be the woman I am today.

BIOGRAPHICAL SKETCH

Gabriela Ferreira Dias received her BS degree in Biology in 2016 from Pontifícia Universidade Católica do Paraná, Brazil. In the second year of college, she joined a research group at the university and started her scientific career. She received a scholarship that supported her during the entire course of her master's (2016-2017) and Ph.D. (2017-present) in Health Sciences, and so she was able to dedicate about six years of full-time commitment to research activities. She joined Renal Research Institute for one year (2019-2020) as a visiting researcher, where she learned how to conduct research in the US and an industry environment. She has experience in different lab techniques (e.g., flow cytometry, LC-MS, ELISA, cell culture, etc.), scientific writing, and presentation of research outcomes. She is motivated to rigorously apply the scientific method and test hypotheses to advance biomedical sciences research.

ABSTRACT

Background: Chronic kidney disease (CKD) is a long-term condition characterized by the gradual loss of kidney function. Its systemic nature negatively impacts multiple organs and leads to anemia, a prevalent comorbidity where red blood cells (RBC) and hemoglobin levels are low. It is caused mainly by erythropoietin deficiency and iron metabolism disorders. In CKD patients, eryptosis – an apoptosis-like RBC death pathway – is increased, particularly for patients treated with hemodialysis (HD). This increase might be partly responsible for the shortened RBC lifespan observed in CKD, and it might contribute to renal anemia. In CKD, the accumulation of uremic toxins, oxidative stress, and hypoxemia might induce eryptosis and worsen renal anemia. **Aim:** We aimed to assess eryptosis, intracellular O_2 levels, oxidative stress, and antioxidants of RBC from HD patients (HD-RBC) and their response to hypoxia, uremic serum (S-HD), and inhibition of uremic toxins uptake. **Methods:** RBC and serum were isolated from the blood of 14 healthy subjects and 22 CKD patients undergoing high-flux HD. RBC were immediately analyzed or incubated for 24h with S-HD in normoxia (21% O_2) and/or hypoxia (5% O_2), with or without ketoprofen (KETO). Phosphatidylserine (PS) and intracellular Ca^{2+} were measured as eryptosis indicators by flow cytometry (FC) using Annexin-V and Fluo-3/AM. Intracellular O_2 levels were estimated by FC with the probe Hypoxia Green, and uremic toxins were quantified in serum and cytosol using high-performance liquid chromatography. Reactive oxygen species (ROS) production, glutathione (GSH) content and heme oxygenase-1 (HO-1) were evaluated by FC using DCFH-DA, Thiol Tracker Violet probe, and HO-1 specific antibody. Enzymatic activity of superoxide dismutase (SOD) and xanthine oxidase (XO) were evaluated by spectrophotometry. **Results:** We found that HD-RBC has low intracellular O_2 levels, especially after HD. Eryptosis was induced by hypoxia and by S-HD; and it was aggravated when these stimuli were combined. Inhibition of uremic toxins uptake with KETO reverted eryptosis and restored O_2 levels. Also, we showed that the increased ROS production in baseline HD-RBC is not mediated by XO. Baseline levels of the antioxidant GSH were low in HD-RBC while SOD and HO-1 were increased. ROS production was enhanced upon stimulation with S-HD and hypoxia and the inhibition of uremic toxins uptake by RBC attenuated this effect. **Conclusions:** Our findings indicate that uremia and hypoxia play an essential role in developing eryptosis, intracellular hypoxemia, and oxidative stress, which might lead to RBC clearance and renal anemia.

Key words: Chronic Kidney Disease, Renal anemia, Eryptosis, Uremic Toxins, Oxidative stress, Intradialytic hypoxemia.

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ABBREVIATIONS AND SYMBOLS LIST

ADMA Asymmetric Dimethylarginine
AGEs Advanced Glycation End Products
Ca^{2+} Calcium ions
CAT Catalase
CKD Chronic Kidney Disease
CXCL16 Chemokine Ligand 16
Epo Erythropoietin
GPx Glutathione Peroxidase
GFR Glomerular Filtration Rate
GSH Reduced Glutathione
GSSG Oxidized Glutathione
H_2O_2 Hydrogen Peroxide
HIF Hypoxia-Inducible Factor
HO-1 Heme Oxygenase-1
IAA Indole-3-Acetic Acid
IL-1 Interleukin-1
IL-6 Interleukin-6
IS Indoxyl Sulfate
K^+ Potassium ions
KRT Kidney Replacement Therapy
MHC Major Histocompatibility Complex
NADPH Nicotinamide Adenine Dinucleotide Phosphate
NO Nitric Oxide
NOS Nitric Oxide Synthases
$O_2^{\bullet-}$ Superoxide
OAT Organic Anion Transporters
$OH^{\bullet}$ Hydroxyl
PAF Platelet-Activating Factor
PBUT Protein-Bound Uremic Toxins
pCS p-Cresyl Sulfate
PHD Prolyl Hydroxylase Proteins
PIH Prolonged Intradialytic Hypoxemia
PS Phosphatidylserine
RBC Red Blood Cells
rHuEpo Recombinant Human Erythropoietin
RNS Reactive Nitrogen Species
ROS Reactive Oxygen Species
SaO_2 Arterial Oxygen Saturation
SOD Superoxide Dismutase
TMAO Trimethylamine-N-oxide
TNF-α Tumor Necrosis Factor
XO Xanthine Oxidase

BACKGROUND

Chronic Kidney Disease (CKD)

Chronic Kidney Disease (CKD) is considered a syndrome that affects the structure and function of the kidneys for at least three months, bringing implications for the patient's health (KDIGO 2013). CKD etiology is multifactorial and can be either inherited or acquired. However, diabetes mellitus and hypertension are known risk factors contributing to CKD development. Additionally, several factors such as obesity, prematurity, and low birth weight contribute to the loss of kidney function and can eventually result in CKD, when the kidney function can no longer be restored. Although CKD pathophysiology is diverse, renal fibrosis is a common manifestation of the disease and leads to glomerulosclerosis, tubular arthropathy, and interstitial fibrosis (Romagnani et al. 2017).

Currently, the overall kidney function is evaluated through two indicators: glomerular filtration rate (GFR) and albuminuria. GFR reflects the total fluid volume that a single nephron can filter per unit of time and is divided into five groups, as depicted in **figure 1**. GFR categories vary from G1, where the filtration is normal or high (>90 mL/min/1.73m^2), to G5, where the filtration is <15 mL/min/1.73m^2 and the kidneys can no longer sustain body homeostasis. The second indicator – albuminuria – shows the degree of kidney barrier disfunction through albumin levels in the urine. The loss of proteins that should be retained in circulation reflects a possible increase in glomerular permeability and is a risk factor for kidney failure. Together, GFR and albuminuria levels predict an individual's risk of progressing to advanced stages of CKD and experiencing adverse outcomes such as hospitalization and death (**Figure 1**) (Webster et al. 2016; KDIGO 2013).

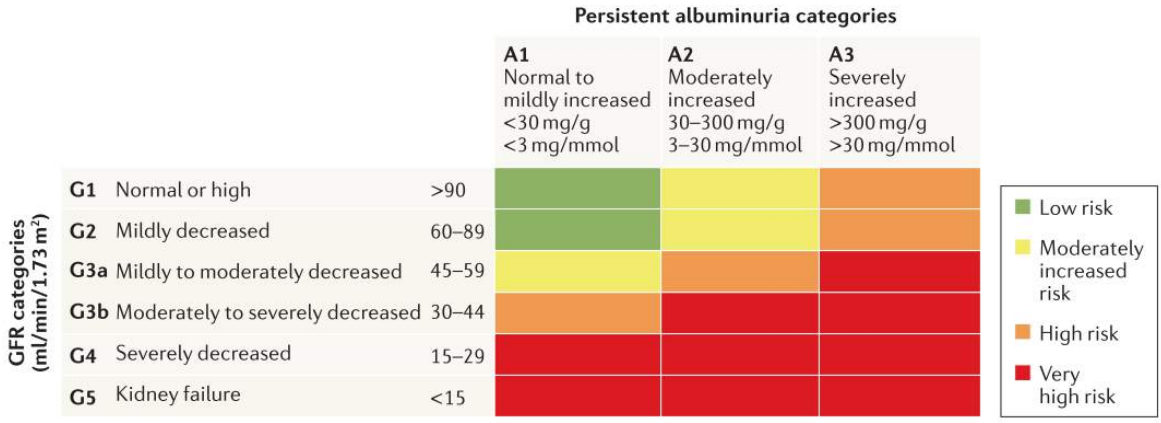

Figure 1. Chronic kidney disease classification according to KDIGO (*Kidney Disease Improving Global Outcomes*). Image from: Romagnani et al. (2017).

CKD stage five is characterized by the invariably need of kidney replacement therapies (KRT) as a life-sustaining treatment that comprises different dialysis modalities and kidney transplant (KDIGO 2013). In 2017, nearly 700 million people were recorded as having CKD among all stages, with a global prevalence of 9.1%. In 2010, there an estimated 2.6 million people received KRT worldwide. However, the number of people requiring KRT varied from 4.9 - 9.7 million. This evidence highlights that a significant portion of the population did not have access to the treatment, especially in low and lower-middle income countries. This situation occurs due to the high costs associated with CKD treatment, leading to a burden in many countries' economies and health care systems. Unfortunately, an estimated 2.3 - 7.1 million people may have died in consequence of the lack of treatment, and the number of people requiring KRT worldwide is expected to more than double by 2030 (Liyanage et al. 2015; Thurlow and Norris 2021; Bikbov et al. 2020).

A key element to reducing CKD and KRT global burden is increasing its awareness as an essential health issue and providing prevention and early detection to slow the disease progression to late stages and KRT (Thurlow and Norris 2021). Commonly, patients are diagnosed when symptoms have become severe, either because they are asymptomatic or due to non-specific symptoms. Therefore, the chance of having a late diagnostic is high for this population (Webster et al. 2016).

For this reason, international guidelines highlight the importance of investigating and treating risk factors to reduce mortality (KDIGO 2013).

Uremic Toxicity

Renal function loss affects several systems and the relationships of the kidney with other organs is an ongoing research subject. Several solutes absorbed from the gut depend on the kidneys for their excretion to not accumulate in the bloodstream (Zoccali et al. 2017). However, with the loss of renal function, these molecules tend to accumulate in the blood, reaching high and harmful concentrations that characterize uremia. When these solutes exert harmful biological activity, they are called uremic toxins. A large body of evidence shows the impact of uremic toxins in several tissues. An instance of this is the cardiovascular system, which is highly affected by these solutes through the dysregulation of endothelial function and enhanced inflammatory activity (Falconi et al. 2021). The adverse outcomes promoted by uremic toxins increase as the GFR declines, hence the importance of early CKD diagnosis and treatment (Ackley et al. 2012).

Uremic toxins are classified into three groups according to their physicochemical properties, and to their behavior during dialysis, they are (1) small water-soluble molecules, (2) middle molecules and (3) protein-bound compounds. The first category covers compounds with molecular weight up to 500 Da, with minimal protein binding. All dialysis strategies effectively remove the toxins in this group. Some examples include urea, creatinine, uric acid, asymmetric dimethylarginine (ADMA), and trimethylamine-N-oxide (TMAO) (Vanholder et al. 2018; Ackley et al. 2012). The toxic effects of these compounds at concentrations seen in CKD are shown in **Table 1**.

Table 1. Characteristics of small water-soluble uremic toxins. Adapted from Ackley et al., 2012.

	MW (Da)	Source	Metabolism	Toxicity
Urea [6]	60.05	Dietary proteins	Hepatic	Vascular disease, insulin resistance (in vivo data)
ADMA [24]	202.25	Protein metabolism	Endogenous enzymes	Vascular disease
TMAO [15]	75.11	Diet	Hepatic	Vascular disease, renal fibrosis
Uric acid [18–20]	168.11	Purine metabolism	Endogenous enzymes	Accelerated CKD, vascular disease, hypertension

URS, uremic retention solutes; MW, molecular weight; ADMA, asymmetric dimethylarginine; TMAO, trimethylamine-N-oxide

The second group – middle molecules – comprises compounds with molecular weight >500 Da that can cross the glomerular filtration barrier in healthy conditions. Their filtration is achieved only with membranes containing large pore sizes during dialysis, the so-called high-flux dialyzers. The prototype and most studied molecule in this group is β2-microglobulin, a component of the major histocompatibility complex (MHC) that was associated with amyloid deposition in bones and joints (**Table 2**) (Vanholder et al. 2018; Ackley et al. 2012).

Table 2. Characteristics of middle molecules uremic toxins. Adapted from Ackley et al., 2012.

	MW (Da)	Source	Toxicity
β_2-Microglobulin [25]	11,729	Major histocompatibility complex	Amyloid bone and joint disease, vascular wall infiltration
Leptin [28]	16,000	Endogenous	Malnutrition

URS, uremic retention solutes; MW, molecular weight

Finally, the protein-bound uremic toxins (**PBUT)** comprise molecules with various molecular weights. Toxins in this group are hardly eliminated through dialysis due to their protein binding affinity, mainly with albumin. PBUT is a broad group composed of phenols, indoles, advanced glycation end products (AGEs), hippurates, and kynurenines (Vanholder et al. 2018). The prototypes and best-studied PBUT are Indoxyl sulfate (IS), indole acetic acid (IAA), and p-Cresyl Sulfate (pCS), all originated from the gut microbiota. Gut bacteria metabolize tryptophan from diet to form indole. Once in circulation, it goes through hydroxylation and sulfation in the liver to form IS. On the other hand, pCS is generated by tyrosine and phenylalanine metabolism, also mediated by the gut microbiota (Laville et al. 2021) (**Figure 2**). The

blood uptake and excretion of these toxins are mediated by the organic anion transporters (OAT) located on proximal tubule cells of the kidney (Deguchi et al. 2004). Similarly, toxins transport in other cell types is also mediated through OATs, such as OAT1 and OAT3 in endothelial cells (Favretto et al. 2017) and OAT2 in erythrocytes (Sager, Smaglyukova, and Fuskevaag 2018). Besides the limited PBUT clearance, it has been shown that CKD patients show alteration in the gut microbiota and dysbiosis. In this condition, levels of beneficial bacteria responsible for the production of essential short-chain fatty acids are reduced. In contrast, the microbial population accountable for producing uremic toxins increases (Lau et al. 2018).

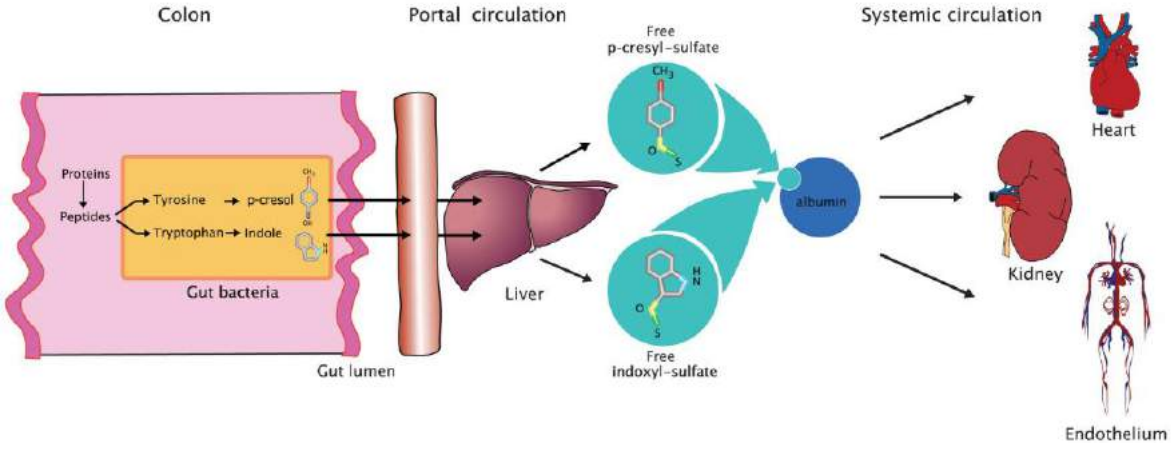

Figure 2. Generation of p-cresyl sulfate and indoxyl sulfate and its impact on different organs and systems. Figure from MEIJERS; EVENEPOEL, 2011.

The PBUT accumulation in the bloodstream results in many complications, including endothelial dysfunction, cardiovascular disease, and the promotion of toxicity in renal, bone, and neurologic organs (Liabeuf, Villain, and Massy 2016). Many of these complications have been attributed to the potential of PBUT in enhancing oxidative stress, thereby modifying macromolecules (Pieniazek, Bernasinska-slomczewska, and Gwozdzinski 2021). Recent studies focus on strategies to increase PBUT clearance and decrease its production to improve patient outcomes. Promising approaches include applying modifications in the traditional hemodialysis setting, such as changes in the number of dialysis sessions per week and the addition of absorption-based techniques to dialysis (Magnani and Atti 2021). Additionally, researchers have been exploring intervention with probiotics,

prebiotics, and symbiotics to re-establish a healthy gut microbiota and reduce PBUT production in CKD patients (Rysz et al. 2021).

CKD co-exists with other diseases: renal anemia in the spotlight

Although efforts have been made to improve health of CKD patients, the last significant advancement in the nephrology field was the introduction of erythropoietin (Epo) therapy for anemia correction back in 1988 (Ng et al. 2003). However, many questions regarding renal anemia are still to be answered.

Anemia is an important comorbidity in patients with impaired renal function and occurs more frequently as the GFR declines. However, an incidence of 10% is already observed in CKD stage 1 and increases to 70% when patients reach stage 5 (Yilmaz et al. 2011). In the population undergoing KRT, the incidence can be as high as 90% (Gluba-Brzózka et al. 2020). Anemia is defined when hemoglobin levels fall below 12g/dL in women and 13g/dL in men. Morphologically, it is characterized as normocytic and normochromic, meaning that red blood cells (RBC) show regular size and color. However, the number of circulating RBC is reduced. The complications associated with anemia include fatigue, shortness of breath, loss of appetite, reduced physical performance etc., besides significant reduction in quality of life (Zadrazil and Horak 2015a). The first symptoms become evident when the GFR decreases to 50mL/min. The average hemoglobin concentration in this group is 2.5 g/dL lower than in individuals with higher GFR (Zoccali 2002).

Renal anemia pathogenesis is multifactorial but is characterized mainly by defective Epo production by renal peritubular cells and iron deficiency, either by deficient iron absorption or functional iron deficiency (KDIGO, 2012; Webster et al., 2016). In addition, iron bioavailability is decreased due to iron sequestration in reticuloendothelial cells, thereby reducing availability to hemoglobin synthesis (Yilmaz et al. 2011). Iron metabolism is regulated by hepcidin, a small peptide that promotes suppression of iron absorption and uptake. Enhanced inflammation induces hepcidin production in liver cells, resulting in dysregulation of iron absorption in the gut and iron mobilization from reticuloendothelial cells (Koury and Haase 2015). In a healthy individual, the hormone erythroferrone controls hepcidin production. Epo-stimulated erythroblasts participate in hepcidin control through the

synthesis of erythroferrone. Therefore, Epo deficiency in CKD contributes to reduced levels of erythroferrone and high levels of hepcidin (Nakanishi et al. 2019; Koury and Haase 2015) (**Figure 3**).

Epo is a glycoprotein containing 165 aminoacids and acts as a hormone stimulating RBC production in the bone marrow. When the body lacks its ability to maintain Epo levels and consequently drop RBC count, the administration of rHuEpo (recombinant human erythropoietin) is required to meet adequate hemoglobin levels. Besides Epo primary function, it also has protective effects on RBC and enhances their lifespan (Macdougall 2007). Also, it was described that rHuEPO treatment in predialysis patients with non-severe anemia slows CKD progression and reduces by 60% the risk of initiation of KRT or death (Gouva et al. 2004).

On the other hand, renal anemia is associated with other factors, such as the accumulation of proinflammatory cytokines during the disease progression that can be harmful to the bone marrow. It is well known that CKD patients have higher levels of inflammatory markers. In advanced stages of the disease, 30%-50% of patients show markers that indicate an active inflammatory state, with high levels of IL-1 (interleukin-1), IL-6, and tumor necrosis factor (TNF-α) (Gluba-Brzózka et al. 2020). Severe inflammation can suppress endogenous Epo production and reduce erythropoiesis and iron levels (Keithi-reddy et al. 2009; Adamson 2009). Other important factors contributing to renal anemia include vitamin deficiency, oxidative stress, accumulation of uremic toxins, and shortened RBC lifespan (Zadrazil and Horak 2015b; Çelic et al. 2011). Taken together, these conditions help explain the Epo hypo-responsiveness observed in 5 to 10% of CKD patients receiving rHuEPO. Studies show an association between Epo hypo-responsiveness with mortality and rapid disease progression (Ogawa and Nitta 2015). A study with a large population of anemic patients receiving rHuEpo found that higher doses correlate with cardiovascular events and toxicity, emphasizing that the administration of this drug in Epo hypo-responsiveness patients must be carefully evaluated (McCullough et al. 2013). Additionally, patients receiving high rHuEpo doses were more likely to show greater levels of inflammatory biomarkers and soluble Epo receptor, which may act as an erythropoiesis inhibitor (Inrig et al. 2011).

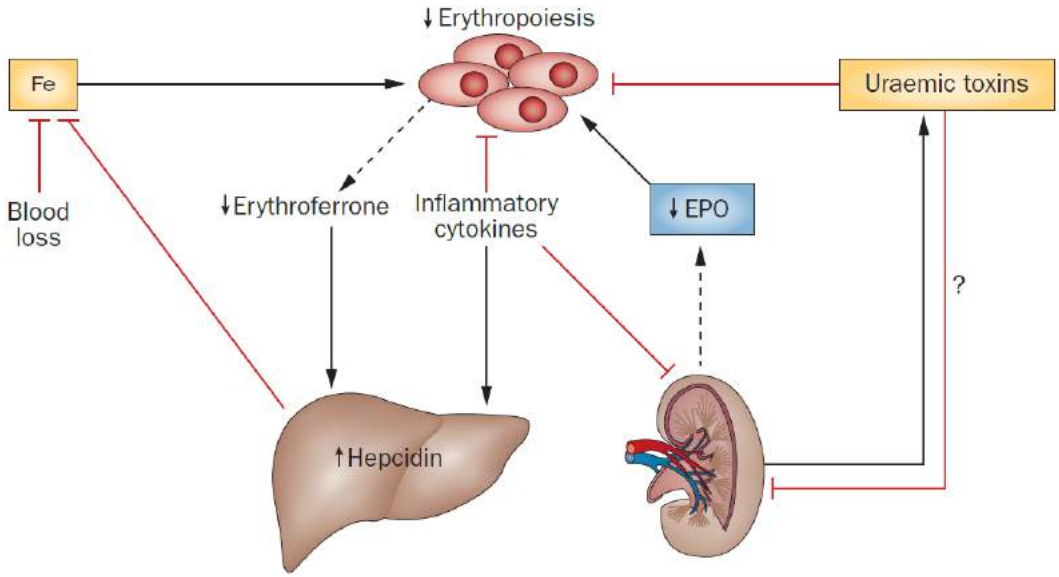

Figure 3. Overview of renal anemia mechanisms. The defective Epo production by kidney cells results in decreased erythropoiesis. In addition, high levels of uremic toxins and inflammatory cytokines present in CKD patients impair erythropoiesis. Iron metabolism is also affected due to reduced levels of erythroferrone, the erythroid regulator of hepcidin. Figure from KOURY; HAASE, 2015.

Interestingly, studies show that acute hypoxia can increase endogenous Epo levels in some CKD patients, indicating that the function of Epo-producing cells is not entirely suppressed (Jelkmann 2007). The hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates several mechanisms, including Epo production and erythropoiesis. It is composed of two subunits: HIF-1β, which is constitutively expressed, and HIF-1α, which is hypoxia sensitive, regulated by oxygen levels. When oxygen levels are normal – in normoxia – HIF-1α goes to a degradation process mediated by HIF prolyl hydroxylases proteins (PHDs), especially PHD2. Conversely, when oxygen levels are insufficient to maintain cellular homeostasis – in a state of hypoxia – PHDs are inhibited, and HIF-1α can escape degradation. Thus, the HIF pathway is activated, allowing the transcription of Epo genes that lead to RBC production by kidney fibroblasts highly sensitive to HIF pathway activation, resulting in increased body oxygen availability. The discovery of this mechanism opened new research strategies to treat CKD-related anemia based on PHDs inhibitors (Schödel and Ratcliffe 2019; W. Li, Zhao, and Fu 2018). This promising new approach has some advantages over rHuEPO, such as decreased hepcidin

production, oral administration, and keeping Epo levels in a physiologic range (Massy and Drueke 2021). Nonetheless, other causes of renal anemia that might occur despite the success of the treatments might as well be investigated.

RBC senescence

RBC are highly specialized cells, consisting of 50% of the cells in the human body. To maximize oxygen delivery, these cells are packed with hemoglobin, comprising 98% of the total protein content in this cell type. High hemoglobin levels are vital since >98% of oxygen in circulation is bound to it. The erythropoietic process is tightly regulated and produces about 2 million RBC per second in healthy humans. (Bogdanova and Kaestner 2020; Kaestner and Bogdanova 2018). Since RBC expel their nucleus and all organelles during their development, all proteins needed to sustain the ~120 days lifespan are synthesized before cell maturation (Moras, Lefevre, and Ostuni 2017). The mechanisms underlying the senescence of RBC are still a subject of ongoing research. It is well known that aging RBC release vesicles, lose surface area and become denser than young RBC, which impairs their deformability capacity and makes them more susceptible to phagocytosis (Kruse et al. 2008). Resident macrophages in the spleen and liver have a significant role in controlling the phagocytosis process by interacting with RBC's surface markers and promoting the clearance of cells that are no longer functional. Indeed, RBC express molecules that act as "eat me" or "don't eat me" signals. For instance, CD47 expression on the RBC membrane has been described as a "self" receptor that interacts in SIRPα present on macrophages surface, inhibiting the phagocytic pathway. Interestingly, older RBC show a decrease in CD47 signaling, facilitating their recognition and engulfment by macrophages. Also, the presence of natural occurring antibodies on the RBC surface has been postulated as a mechanism of RBC clearance from circulation, with the transmembrane protein band 3 being the main target for antibodies binding (de Back et al. 2014; Arias and Arias 2017). Natural occurring antibodies and complement C3 on the RBC surface are recognized by Fcγ and complement receptors on macrophages, facilitating erythrophagocytosis (Qadri et al. 2017).

Eryptosis and shortened RBC lifespan on the development of renal anemia

Despite mechanisms of senescence, RBC can undergo another death pathway termed “eryptosis”. Noteworthy, this pathway may occur early in their life, well before the average of 120 days. Eryptosis is an apoptosis-like death pathway, and the term was created to differentiate from the pathway nucleated cells go through. Although there are similarities between both pathways, such as membrane blebbing and cell shrinkage, the eryptotic process does not include mechanisms involving nuclear fragmentation and chromatin condensation, for instance. Instead, the eryptosis machinery is much more focused on membrane alterations such as calcium influx and phosphatidylserine (PS) exposure on the cell surface (Pyrshev et al. 2018; F. Lang and Qadri 2012).

Interestingly, enhanced eryptosis is seen in several conditions such as inflammation, diabetes, cardiac failure (F. Lang et al. 2017), and CKD patients compared to healthy individuals (ABED et al., 2014; DIAS et al., 2018). This phenomenon was observed in RBC from hemodialysis and peritoneal dialysis (Bissinger et al. 2016) and has been attributed to many insults and stressors experienced by these patients, such as oxidative stress, the dialysis itself, and uremic toxins. A study with peritoneal dialysis patients showed that eryptosis increases progressively as the residual diuresis decrease, possibly by poor uremic toxins removal (Virzì et al. 2019). Among the uremic toxins, IS (Ahmed, Abed, et al. 2013; Tozoni et al. 2019), acrolein (Ahmed, Langer, et al. 2013), and methylglyoxal (Nicolay et al. 2006) could induce eryptosis *in vitro* and therefore might play a role in the process of accelerated RBC death in this population. Efforts have been made to put eryptosis in the spotlight as a potential cause o renal anemia. Since CKD patients commonly cannot keep adequate levels of Epo, an increase in the eryptosis rate could result in low levels of RBC and anemia, particularly for those patients that do not respond well to treatment with rHuEpo (F. Lang et al. 2017; Dias et al. 2020).

Another critical aspect of RBC from CKD patients is their shortened lifespan. Several papers indicate that the period in which uremic RBC remain in circulation is below 120 days. In hemodialysis patients, an average lifespan of 73±18 days (Ma et

al. 2017) and 89±28 days (Sato et al. 2012) and a median of 58 days were reported (Vos et al. 2011). No difference between hemodialysis and peritoneal dialysis was observed (Vos et al. 2011). Another study showed that RBC lifespan decreases progressively with renal function decline and has been observed since the early stages of CKD (J. H. Li et al. 2019). Additionally, a negative correlation between rHuEpo and RBC lifespan was observed (Sato et al. 2012). The causes of this severe lifespan reduction are not entirely understood. However, the premature RBC death by mechanisms of eryptosis might influence the early RBC removal from the bloodstream and young age of these cells in renal failure, contributing to the development of renal anemia (Dias et al. 2020).

Eryptosis mechanisms

Eryptosis is orchestrated by several pathways that consist of morphologic alterations such as cell shrinkage, alterations in ions movement across the membrane, and very notably, the exposure of the phospholipid PS on the cell surface, which is considered the hallmark of eryptosis (Dias et al. 2020).

The influx of calcium ions (Ca^{2+}) from the extracellular space to the cytosol is considered the first step of the eryptotic process that will ultimately lead to a cascade of other modifications. Ca^{2+} influx to the cytosol is mediated by unselective cation channels on the cell membrane. These channels are activated by several stimuli, such as oxidative stress, uremic toxins, shear stress, osmotic shock, prostaglandin E_2 and many others (Dias et al. 2020; Föller and Lang 2020). Once intracellular Ca^{2+} is elevated, it activates scramblase, an energy-independent protein that bidirectionally translocates phospholipids between the membrane monolayers, working like a channel that allows lipids to navigate the lipid bilayer (Segawa and Nagata 2015). Normally, PS is distributed on the inner leaflet of the cell membrane. However, upon Ca^{2+} entry and scramblase activation, PS is exposed on the cell surface. The ATP-dependent enzymes flippase and floppase transport phospholipids - including PS - to the inner and outer membrane leaflet, respectively. When Ca^{2+} levels in the cell increase, flippase is inhibited while scramblase and floppase are activated, resulting in the loss of membrane asymmetry, which is

essential for RBC lipid homeostasis (Pretorius, Du Plooy, and Bester 2016). The “eat me” signal promoted by PS exposure leads to the recognition of RBC by phagocytic cells, culminating in their removal from circulation (de Back et al. 2014; Bonan et al. 2016) through several receptors, including Stabilin-2, BAI1, Tim-1, Tim-4 and CD300 (Qadri et al. 2017).

Ca^{2+} influx impacts cell volume regulation by activating Ca^{2+} sensitive K^+ channels – the Gardos channels – that trigger membrane hyperpolarization and exit K^+ and Cl^- ions to the extracellular space. The efflux of KCl is accompanied by water and results in loss of cellular volume and cell shrinkage (U. E. Lang et al. 2005).

Another major eryptosis trigger is ceramide, a sphingolipid known to participate in apoptosis of nucleated cells and RBC death. Ceramide can be generated through the hydrolysis of sphingomyelin by sphingomyelinases enzymes, or it can be generated *de novo* by the activity of other enzymes, such as serine palmitoyl-transferase and ceramide synthases (E. Lang et al. 2015). Also, ceramide production can be mediated through RBC shrinkage, which induces platelet-activating factor (PAF) formation by phospholipase A2. Next, the interaction of PAF with their receptors on RBC surface stimulates sphingomyelin breakdown leading to ceramide formation (P. a Lang et al. 2005; F. Lang et al. 2010). Although the role of ceramide in eryptosis is not completely understood, its abundance in RBC of CKD patients is enhanced (Abed et al. 2014). Consequences of high ceramide include increased membrane fragility and eventually vesicles release and cell rigidity (Dinkla et al. 2012). Importantly, ceramide induces scramblase activity and thus participates in PS externalization (P. a Lang et al. 2005).

The kinases proteins – enzymes that transfer a phosphate from a high energy molecule to a substrate – p38 mitogen-activated protein kinases (MAPK) and Janus kinase JAK3 were reported to have a link with Ca^{2+} influx on the eryptotic process. The inhibition of these enzymes in RBC resulted in attenuated PS exposure (Qadri et al. 2017).

Besides acting as an “eat me” signal, PS interacts with the transmembrane chemokine ligand 16 (CXCL16), a cytokine present on the surface of endothelial cells. In a condition of accentuated eryptosis, PS-exposing RBC may adhere on CXCL16 of vascular endothelial cells and therefore form aggregates that can interfere with the microcirculation and develop thrombus (Borst et al. 2012; Abed et al. 2013).

Oxidative stress and antioxidant defense

The mechanisms explained above can be triggered in part by oxidative stress. RBC are sensitive cells and very responsive to their environment. Therefore, they respond to oxidative stress leading to a series of alterations in their metabolism and structure (Massaccesi, Galliera, and Corsi Romanelli 2020). Oxidative stress is defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” (Jones 2006; Sies 2019). The primary reaction that generates these oxidants or reactive oxygen species (ROS) consists of molecular oxygen being reduced to a superoxide radical ($O_2^{\bullet-}$) by adding an electron. In the presence of another electron and protons, $O_2^{\bullet-}$ is converted into hydrogen peroxide (H_2O_2), a non-radical ROS that can freely pass through the cell membrane and thus impact the extracellular space, which generally has a weaker antioxidant defense than the cytosol. H_2O_2, in turn, can be converted into hydroxyl radical ($OH^{\bullet}$) in the presence of iron(II) and is far more reactive than $O_2^{\bullet-}$ and H_2O_2, particularly with intracellular components (**Figure 4**). Usually, ROS are formed all the time through different pathways, such as in the respiratory chain, through the exposure to xenobiotics and environmental factors, as well as by the respiratory burst promoted by phagocytic cells (Halliwell and Gutteridge 2015). In RBC, ROS are not generated via mitochondria, which is the primary source in other cells. Instead, it can be formed by the oxidation of hemoglobin, resulting in the non-functional form of this protein, called methemoglobin (Çimen 2008). Besides hemoglobin autoxidation, ROS in RBC can be generated by enzymatic activity, such as through the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidases comprise a family of transmembrane proteins first discovered in phagocytes but found in nearly

all tissues. Their primary function is to transfer electrons to oxygen through cell membranes and generate $O_2^{\bullet-}$ and H_2O_2 using NADPH as a substrate (Lambeth 2004). NADPH oxidase activity has been postulated to regulate intracellular Ca^{2+} levels by interacting with Ca^{2+} plasma proteins channels and pumps. ROS derived from NADPH oxidase activity can activate these channels and enhance Ca^{2+} levels in the cytosol. Concurrently, high Ca^{2+} levels activate NADPH oxidase, producing more ROS (George et al. 2013; Bedard and Krause 2007). Therefore, the alterations in Ca^{2+} homeostasis mediated by NADPH oxidase can either play a role in the development of oxidative stress or promote eryptosis, particularly in cells from individuals with imbalanced Ca^{+2} regulation, such as RBC from CKD patients (Dias et al. 2018). Indeed, the expression of this enzyme is enhanced in CKD while antioxidant enzymes are repressed (Vaziri et al. 2003). Additionally, the accumulation of uremic toxins such as IS has increased NADPH oxidase activity in RBC and nucleated cells (Dias et al. 2018; Dou et al. 2007). Besides NADPH oxidase, $O_2^{\bullet-}$ and H_2O_2 can be produced by xanthine oxidase (XO), another ROS-producing enzyme that is upregulated in inflammatory disorders such as diabetes and atherosclerosis, as well as under hypoxia (Mittal et al. 2014). In hemodialysis and peritoneal dialysis patients, serum levels of XO are elevated and might participate in the oxidative injury in this patient population (Miric et al. 2013). Importantly, researchers indicate that high XO activity can be a predictor of cardiovascular events in CKD (Gondouin et al. 2015).

Despite the harmful effects of exacerbated ROS and reactive nitrogen species (RNS) production, the maintenance of their adequate levels is crucial for the elimination of pathogens and as mediators of cell signaling. For instance, nitric oxide (NO) is a RNS generated by nitric oxide synthases enzymes (NOS) or by the reaction of nitrite with hemoglobin and plays an important role as a second messenger in the process of smooth muscle cells relaxation and control of vascular tone (Valko et al. 2007; Joseph M. Rifkind et al. 2018). To ensure that the production of oxidants remains at physiological levels, all systems in the body have a tightly regulated balance of ROS and antioxidants. The antioxidant defense is often divided into two categories: enzymatic and non-enzymatic antioxidants. Among the antioxidant

enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase, thioredoxin, and peroxiredoxin are determinants for ROS neutralization. SOD function consists of catalyzing the dismutation of $O_2^{\bullet-}$ into H_2O_2 (Ratliff et al. 2016). This enzyme is widely distributed in the body and is a critical element in the antioxidant defense of RBC. SOD knockout mice showed limited erythropoiesis and reduced RBC lifespan, leading to anemia development (Fujii et al. 2021). Other enzymes such as CAT and GPx are essential for the conversion of H_2O_2 into water. GPx is found in several cellular regions and uses glutathione (GSH) as a co-factor, leaving this molecule in its oxidized form (GSSG). The recycling of GSSH back to the reduced form GSH is achieved by the activity of the enzyme glutathione reductase that, in turn, uses NADPH synthesized from the pentose phosphate pathway as a co-factor (Valko et al. 2007; Ratliff et al. 2016) (**Figure 4**).

Some enzymes such as heme oxygenase-1 (HO-1) are induced upon stress conditions, and therefore, serve as indicators of cellular stress. HO-1 cleaves free heme, avoiding the toxic effects of unbound heme. The products of this reaction include a series of beneficial molecules such as bilirubin, which has antioxidant properties (Araujo, Zhang, and Yin 2012). The most abundant non-enzymatic antioxidant is GSH, a tripeptide thiol present at mM levels in RBC, representing the main antioxidant to control the cellular redox state. GSH plays many roles in cellular metabolism, as a co-factor to enzymatic activity and directly preventing the oxidation of molecules such as protein -SH groups.

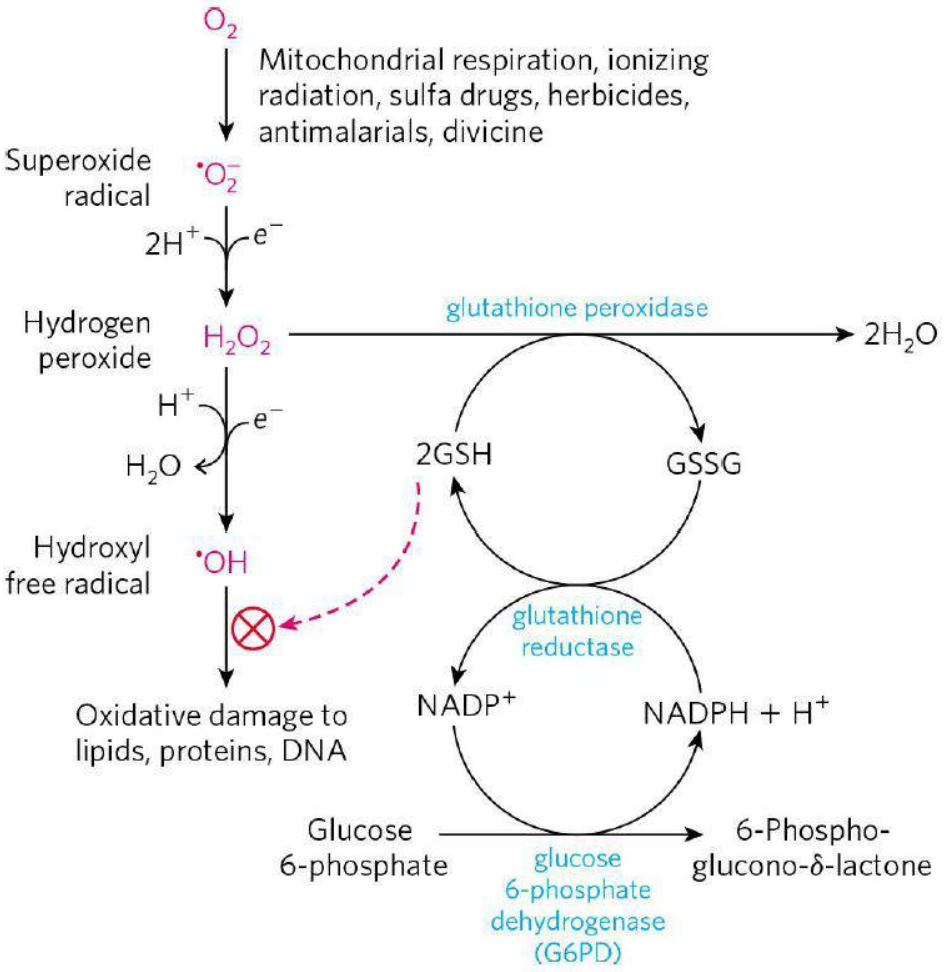

Figure 4. General overview of reactive oxygen species formation and the neutralization by the antioxidant system. Figure from Nelson, D. L., & Cox, M. M. (2017). Lehninger principles of biochemistry (7th ed.). W.H. Freeman.

Although RBC have a great reducing capacity and an extensive antioxidant system to fight exposure to O_2, this defense can be overwhelmed when pathologies such as CKD occur (Verma et al. 2021). It is known that CKD patients have a weaker antioxidant defense since several of these molecules are reduced or non-functional. For instance, SOD and CAT were shown to have reduced activity in RBC of uremic patients, particularly in Epo hypo-responsiveness. Also, a direct correlation of hemoglobin and these antioxidants enzymes was observed (Bissinger et al. 2018; Khalil et al. 2016). Additionally, serum and RBC from CKD patients have consistently reported low levels of GSH, which is described as a marker of oxidative stress in this disease (Ceballos-picot et al. 1996; F. Lang et al. 2014; Dias et al. 2018).

Hypoxemia and CKD

Despite the efficiency of dialysis and its improvement throughout the years, patients still suffer from adverse outcomes experienced only in extreme stress conditions in a healthy person. It includes intradialytic hypotension, thermal stress, and reduction in arterial oxygen saturation (SaO_2) (Kooman et al. 2018). The drop in SaO_2 observed in HD is attributed to factors such as CO_2 diffusion into the dialysate and sleep apnea. Although there is not a consensus on the definition of hypoxemia, it is often defined as SaO_2 ≤90% (O'Driscoll, Howard, and Davison 2008). Additionally, anemic hypoxemia might occur when hemoglobin levels are low, and oxygen delivery to tissues is impaired (Campos et al. 2016). A study with a large population showed that HD patients that experience hypoxemia for at least 1/3 of the time of dialysis session – called prolonged intradialytic hypoxemia (PIH) – have higher hospitalization and mortality rates. PIH patients also required higher doses of Epo and had lower levels of hemoglobin, as well as exhibited an inflammatory phenotype (Meyring-Wosten et al. 2016). Although this finding indicates a certain degree of Epo hypo-responsiveness, hypoxia triggers eryptosis and other alterations in RBC metabolism *in vitro*, as shown by Tozoni et al., which can impact RBC count and hemoglobin levels in hypoxemic patients. (Tozoni et al. 2019). Interestingly, a recent study from Hartzell and co-workers showed that RBC from PIH patients exhibit higher ROS generation than nonhypoxemic HD patients, which may contribute to RBC fragility and clearance from circulation (Hartzell et al. 2021). Nonetheless, it is worth to mention that acute and moderate hypoxia have beneficial effects, such as Epo production. However, severe and chronic hypoxia might trigger pathological effects (Navarrete-Opazo and Mitchell 2014). Additionally, oxygen affinity of hemoglobin depends on several factors altered during dialysis, such as pH variations, partial CO_2 pressure, 2,3-diphosphoglycerate concentration, and blood temperature. Of note, dialysis can increase the pH of the blood, which promotes an increase in the oxygen dissociation curve, reducing oxygen delivery to tissues (Campos et al. 2016).

The relationship of hypoxia, ROS production, and eryptosis is observed when the amount of oxidized hemoglobin increases, leading to the interaction of RBC

cytoskeleton with the membrane, which can then decrease cell deformability and increase Ca^{2+} influx (Kiefer and Snyder 2000). Also, it is well known that under hypoxic conditions, the production of $O_2^{\bullet -}$ is increased in RBC (Balagopalakrishna et al. 1996; J. M. Rifkind et al. 1991). Further, these alterations can promote the externalization of PS and cell death. (Kiefer and Snyder 2000). The uremic toxin IS has been reported to alter oxygen metabolism in tubular cells by increasing oxygen consumption, a phenomenon that was at least in part mediated by oxidative stress (Palm et al. 2010).

Given the evidence presented here, we hypothesize that the association of uremia and hypoxia experienced by CKD patients can trigger the eryptotic process, mediated by mechanisms of increased oxidative stress. Ultimately, this process might lead to RBC loss and worsening of renal anemia.

REVIEW ARTICLE:

"The role of eryptosis in the pathogenesis of renal anemia: insights from basic research and mathematical modeling"

frontiers in Cell and Developmental Biology

REVIEW
published: 09 December 2020
doi: 10.3389/fcell.2020.598148

The Role of Eryptosis in the Pathogenesis of Renal Anemia: Insights From Basic Research and Mathematical Modeling

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Red blood cells (RBC) are the most abundant cells in the blood. Despite powerful defense systems against chemical and mechanical stressors, their life span is limited to about 120 days in healthy humans and further shortened in patients with kidney failure. Changes in the cell membrane potential and cation permeability trigger a cascade of events that lead to exposure of phosphatidylserine on the outer leaflet of the RBC membrane. The translocation of phosphatidylserine is an important step in a process that eventually results in eryptosis, the programmed death of an RBC. The regulation of eryptosis is complex and involves several cellular pathways, such as the regulation of non-selective cation channels. Increased cytosolic calcium concentration results in scramblase and floppase activation, exposing phosphatidylserine on the cell surface, leading to early clearance of RBCs from the circulation by phagocytic cells. While eryptosis is physiologically meaningful to recycle iron and other RBC constituents in healthy subjects, it is augmented under pathological conditions, such as kidney failure. In chronic kidney disease (CKD) patients, the number of eryptotic RBC is significantly increased, resulting in a shortened RBC life span that further compounds renal anemia. In CKD patients, uremic toxins, oxidative stress, hypoxemia, and inflammation contribute to the increased eryptosis rate. Eryptosis may have an impact on renal anemia, and depending on the degree of shortened RBC life span, the administration of erythropoiesis-stimulating agents is often insufficient to attain desired hemoglobin target levels. The goal of this review is to indicate the importance of eryptosis as a process closely related to life span reduction, aggravating renal anemia.

Keywords: kidney failure, anemia, eryptosis, erythropoietin, phosphatidylserine, calcium, hypoxia, oxidative stress

OPEN ACCESS

Edited by:
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Reviewed by:
Etheresia Pretorius,
Stellenbosch University, South Africa
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Specialty section:
This article was submitted to Cell Death and Survival, a section of the journal Frontiers in Cell and Developmental Biology

Received: *23 August 2020*
Accepted: *16 October 2020*
Published: *09 December 2020*

Citation:
Dias GF, Grobe N, Rogg S, Jörg DJ, Pecoits-Filho R, Moreno-Amaral AN and Kotanko P (2020) The Role of Eryptosis in the Pathogenesis of Renal Anemia: Insights From Basic Research and Mathematical Modeling. Front. Cell Dev. Biol. 8:598148. doi: 10.3389/fcell.2020.598148

INTRODUCTION

Red blood cells (RBCs) are vital to life, and their oxygen carrying role is indispensable to the function of tissues and organs. In healthy humans, RBCs undergo senescence and cell death after around 120 days. RBCs can also undergo a distinct mechanism of death, a process of programmed RBC death similar to apoptosis, called eryptosis (Qadri et al., 2017). This may occur throughout the

RBC lifetime, even before senescence under various stress conditions, and it is increased in kidney failure patients for reasons only partially understood (Abed et al., 2014; Bissinger et al., 2016; Dias et al., 2018). The RBC plasma membrane acts as a selective barrier that ensures a constant internal composition, by controlling the active and passive transfer of ions and molecules. Composed of more than 50 transmembrane proteins, the membrane regulates RBC shape, as well as mobility, deformability, and ion and macromolecules transport (Mohandas and Gallagher, 2008). Membrane proteins play an important role in regulating RBC volume by controlling the movement of ions and thus assure cell deformability while traversing capillaries and spleen sinusoids (Gallagher, 2013; Glogowska and Gallagher, 2015). Fluidity of the cytoplasm and cell volume regulation are necessary for capillary transit and transport of O_2 and CO_2 (Narla and Mohandas, 2017). Beyond their primary O_2-carrying function, RBCs are essential for systemic metabolic processes, such as pH regulation, nitric oxide production, and immune responses (Nemkov et al., 2018; Rifkind et al., 2018).

The capability to perform these functions decreases as RBCs approach senescence. In healthy subjects, a delicate balance between RBC death and production rates is maintained, resulting in stable RBC counts in the peripheral blood.

In kidney failure, the erythropoiesis rate is reduced, leading to fewer circulating RBCs. It is well established that the main cause of renal anemia is an inadequately low renal erythropoietin (Epo) synthesis combined with functional iron deficiency. Additionally, kidney patients show a dysregulation of oxygen sensing via the hypoxia-inducible factor pathway (Guedes et al., 2020). Erythropoiesis-stimulating agents (ESAs) are routinely used to compensate for the shortfall in endogenous Epo production. However, hypo-responsiveness to ESA is seen in 5–10% of anemic CKD patients. These patients do not achieve prescribed hemoglobin targets despite high ESA doses. This can be partially explained by accentuated inflammation and iron deficiency (Ogawa and Nitta, 2015). A less recognized potential cause of ESA hypo-responsiveness is decreased RBC life span. Eryptosis may reduce RBC survival and contribute to renal anemia since in some patients the erythropoiesis rate cannot compensate for this increased loss (Lang et al., 2017). In this review, we describe the role of eryptosis in the pathogenesis of renal anemia, an aspect frequently neglected in the clinical practice.

THE PATHOGENESIS OF RENAL ANEMIA: THE EPO-CENTRIC VIEW

Most chronic kidney disease (CKD) patients suffer from anemia at some point in the course of their illness. Epo levels in CKD patients are well below those seen in anemic non-kidney failure patients at the same level of hematocrit. The first cases of patients with renal anemia treated with ESA showed a dramatic effect: A few days after initiation of ESA therapy, their hematocrit approached normal levels, necessitating a reduction in ESA dose. The marked increase in RBC mass following treatment with ESA was accompanied by enhanced utilization of iron stores, as reflected in a decline in serum iron and serum ferritin (Eschbach et al., 1987; Bunn, 2013). To many of those who witnessed these first results, the challenges of treating renal anemia may have become a matter of the past. However, decades later, many open questions have remained.

ERYPTOSIS PATHWAYS: OVERVIEW

Anemia is considered as a non-conventional risk factor in patients with CKD, especially in those on dialysis (Iseki and Kohagura, 2007). As discussed above, renal anemia is mainly attributed to decreased production of Epo by diseased kidneys, compromising erythropoiesis. In addition, several studies indicate that eryptosis is increased in CKD patients leading to early and accelerated elimination of circulating RBCs (Abed et al., 2014; Bissinger et al., 2016; Bonan et al., 2016; Dias et al., 2018).

Eryptosis is characterized by RBCs undergoing morphologic changes such as cell shrinkage, membrane scrambling, and the exposure of phosphatidylserine (PS) (Lang and Qadri, 2012). These changes are stimulated by Ca^{2+} influx into the RBCs through non-selective Ca^{2+} channels, which in turn can be activated by prostaglandin E2 (PGE2) formation, oxidative and osmotic stress, as well as Cl^- efflux (Lang et al., 2007). Activation of Ca^{2+} channels results in an increase of cytosolic Ca^{2+}, which can further induce floppase to expose PS on the cell surface and subsequent recognition, engulfment, and degradation of RBCs by macrophages (Lang et al., 2012) and pro-inflammatory monocytes (Bonan et al., 2016). Ca^{2+} may also stimulate sphingomyelinase to form ceramide, which in turn activates scramblase and culminates in loss of asymmetry of the RBC cell membrane and PS exposure (Lang et al., 2010).

Although eryptosis and senescence result in RBC death and clearance from circulation, the mechanisms driven by each pathway differ considerably. While eryptosis is orchestrated by the mechanisms mentioned above, the removal of aged RBCs relies mainly on the reduced deformability of the cells and macrophage recognition of immunoglobulin G and complement factor 3 on the surface of the senescent RBC. Externalization of PS was shown to be negligible in old RBCs (Franco et al., 2013). However, this RBC population is more susceptible to eryptosis induced by energy depletion (Ghashghaeinia et al., 2012). Thus, PS exposure seems to be more relevant for eryptosis rather than RBC senescence (Qadri et al., 2017).

Eryptosis is considered a useful mechanism to avoid a potentially fatal complication of hemolysis, by starting a cell death program with controlled removal before any damage can cause uncontrolled hemolysis (Föller et al., 2008). Since most of the iron content in the body is bound to hemoglobin, phagocytosis and the degradation of RBCs represent an important source of iron. The amount of recycled iron is sufficient to maintain the daily iron requirement for erythropoiesis (Ginzburg and Li, 2010). However, excessive eryptosis can compromise microcirculation through the adhesion of RBCs exposing PS to endothelial receptors of the vascular wall (Borst et al., 2012) and lead to anemia due to the exacerbated RBC

clearance by the immune system (Bonomini et al., 2001; Bonan et al., 2016). Enhanced eryptosis has been observed in some clinical conditions, such as diabetes, uremic hemolytic syndrome, sepsis, sickle cell anemia, and CKD, among others (Lang and Lang, 2015). PS exposure was observed to be significantly increased in RBCs from patients undergoing hemodialysis (HD) compared to RBCs from healthy individuals (Abed et al., 2014; Bissinger et al., 2016; Dias et al., 2018). Also, PS exposure was significantly higher in patients on peritoneal dialysis (PD) compared with HD patients (Bissinger et al., 2016). In PD patients, the residual glomerular filtration rate was inversely correlated with percentage of eryptosis. This correlation might be explained by a better clearance of retention solutes in patients with residual kidney function (Virzì et al., 2019). The question whether the HD therapy ameliorates or triggers eryptosis remains controversial. Results suggesting both an increase (Abed et al., 2014) and a reduction (Meyring-Wösten et al., 2017) of PS exposure post HD session were reported.

Eryptosis can be triggered by a range of both endogenous and exogenous insults, including toxins, drugs, and acute and chronic diseases (Lang and Lang, 2015). Among the uremic solutes that accumulate in CKD, acrolein (Ahmed et al., 2013b), methylglyoxal (Nicolay et al., 2006), and indoxyl sulfate (IS) (Ahmed et al., 2013a; Dias et al., 2018; Tozoni et al., 2019) were shown to increase eryptosis. Moreover, stressors including osmotic shifts, oxidative stress, and energy depletion may also contribute to a shortened RBC survival (Lang et al., 2006).

A reversion of PS exposure was shown by the addition of the antioxidant *N*-acetyl-L-cysteine to senescent RBCs (Ghashghaeinia et al., 2012) and incubation of uremic RBCs in healthy plasma (Bonomini et al., 1999). Also, the PS exposure induced by IS in healthy RBCs was attenuated by diphenyleneiodonium chloride (an NADPH oxidase inhibitor) and by ketoprofen (an organic anion transporter 2 inhibitor) (Dias et al., 2018).

RBC MICROVESICLES RELEASE

Microvesicle (MV) release is part of the physiological RBC aging process *in vivo*, which indicates a disruption of the network between the lipid bilayer and the cytoskeleton. Moreover, the presence of PS on the surface of MV allow for their recognition by the immune system (Burnier et al., 2009; Leal et al., 2018). The addition of Ca^{2+} to RBC media promotes MV release (Nguyen et al., 2016). This finding suggests the participation of MV in eryptosis when RBC Ca^{2+} is increased. However, the vesiculation process in eryptosis is still poorly understood.

The involvement of PS translocation in MV generation remains controversial. Some studies claim that MV release occurs independent of PS (Williamson et al., 1992; Bucki et al., 1998). Conversely, other authors reported that scramblase inhibition reduced MV release from Ca^{2+}-stimulated RBCs, suggesting the participation of PS translocation (Gonzalez et al., 2009). In contrast to cells undergoing apoptosis, RBC form MV from the plasma membrane with minute loss of the lipid order, possibly due to the absence of intracellular organelle membranes (Pyrshev et al., 2018).

MV release was shown to be increased in RBCs from HD patients and attributed to an impacted membrane–cytoskeleton interaction, such as the proteolytic breakdown of band 3 (Antonelou et al., 2011). The uremic solutes IS and indol acetic acid (IAA) induced PS exposure and MV release from healthy RBCs (Gao et al., 2015). PS inhibition with lactadherin reduced MV release, reinforcing the participation of PS and micro-vesiculation in eryptosis. The authors also implicated MV release from RBC in thrombus formation, which may aggravate cardiovascular events in CKD (Gao et al., 2015).

THE ROLE OF IRON IN ANEMIA AND ERYPTOSIS

Another relevant aspect of renal anemia is the functional iron deficiency due to increased iron storage in the reticuloendothelial system. In addition, increased hepcidin levels are frequently observed in CKD patients, resulting in poor intestinal iron absorption. CKD patients are also prone to iron loss from (micro)bleeds and iatrogenic causes, such as frequent blood draws and blood loss in the extracorporeal system of dialysis machines. Poor iron availability contributes to impaired eryptosis in concert with the elevated levels of pro-inflammatory cytokines and hepcidin. Hence, ESA therapy is commonly accompanied by iron supplementation (Wish et al., 2018). RBCs from mice fed with an iron-deficient diet showed an increased Ca^{2+} uptake, RBC PS exposure, and eryptosis. Eryptotic RBCs were rapidly cleared from the circulation and thus may have amplified iron deficiency (Kempe et al., 2006). However, excessive iron administration may result in iron store pathologies driven by intracellular iron accumulation. As a consequence of inflammation and reticuloendothelial blockade of iron release, patients might still experience low erythropoiesis rate despite increased iron content (Wish, 2006). RBCs from patients with hemochromatosis showed increased PS exposure, mostly as a result of oxidative stress (Du Plooy et al., 2018).

THE ROLE OF REACTIVE OXYGEN SPECIES IN ERYPTOSIS

Patients with CKD, especially when on dialysis, are exposed to a variety of stimuli that change RBC number and phenotype. A critical contributor to eryptosis in CKD is the enhanced oxidative stress. The overproduction of pro-oxidant molecules in CKD is multifactorial and HD itself can activate inflammatory responses; in addition, essential antioxidants, such as vitamins, may be cleared by HD (Bissinger et al., 2018). Oxidative stress is classically defined as the imbalance between pro-oxidants and antioxidants in favor of the former. Oxidative stress exerts its detrimental effects through oxidation of macromolecules. However, it is now clear that oxidative stress is a compartmentalized event that occurs at different levels,

from cellular compartments to cells to the whole organism (Santolini et al., 2019). A more recent definition of oxidative stress indicates the importance of a disruption of redox signaling and control and/or molecular damage (Jones, 2006). This broader definition recognizes that damage to macromolecules is not the only pathway by which oxidative stress promotes disease. In fact, changes in cell signaling mediated by ROS can develop in a series of alterations and affect the body in a pathway- and organ-specific manner (Jones, 2006; Halliwell and Gutteridge, 2015).

Both enzymatic and non-enzymatic antioxidant systems are altered in CKD patients (Ling and Kuo, 2018). The thiol glutathione (GSH) is important for the maintenance of RBC redox homeostasis, where it is present at higher concentrations in the cytosol (Valko et al., 2007). This powerful redox buffer system provides an overall picture of the organism's redox state (Jones, 2006). In one study, the GSH concentration in RBCs from HD patients and healthy subjects was similar while the ratio of GSH and its oxidized form, glutathione disulfide (GSSG), was 40% lower (Khazim et al., 2013). We recently found that the GSH content in RBCs from HD patients is halved compared to RBCs from healthy subjects (Dias et al., 2018); however, the fact that the control subjects were significantly younger may have impacted the results since RBCs from elderly individuals tend to have lower GSH levels (Lupescu et al., 2015). Consistent with this finding, previous studies in uremic and HD patients reported low activity of γ-glutamylcysteine synthetase, a key enzyme in GSH biosynthesis (Alhamdani, 2005). Antioxidant enzymes, such as glutathione peroxidase, which detoxifies hydrogen peroxide (H_2O_2), also show a reduced activity in RBCs and plasma of uremic patients (Zachara et al., 2004). Although HD partially increased plasma antioxidant enzyme activities immediately after treatment, their function is not completely restored compared to healthy controls (El-Far et al., 2005).

Different from their non-uremic counterparts, uremic RBCs show activated non-selective Ca^{2+} transporters and subsequent increased Ca^{2+} influx, which triggers eryptosis (Abed et al., 2014). Oxidative stress participates in this process by maintaining Ca^{2+} levels high through the inhibition of the enzyme Ca-ATPase (Mohanty et al., 2014). Autoxidation of hemoglobin is the main pathway of free radical production in RBCs, leading to anion superoxide formation (Çimen, 2008). It was observed that RBCs can release H_2O_2 (Huertas et al., 2013; Rifkind et al., 2018). Under hypoxic conditions, RBCs increased superoxide formation and further dismutation to H_2O_2. The latter was then diffused from the RBCs and promoted inflammation in the lung microvascular endothelium (Kiefmann et al., 2008). Given the high concentration of hemoglobin in the blood, it is conceivable that even a minor increase of hemoglobin autoxidation could trigger an imbalanced redox state, especially in a population with an already defective antioxidant system, such as CKD.

Moreover, iron facilitates redox reactions, and its accumulation leads to the generation of hydroxyl radical, a powerful ROS, via Fenton's reaction (Nakanishi et al., 2019). Ferritin levels not only reflect body iron stores but also serve as a biomarker of inflammation. Ferritin and oxidative stress markers are correlated, possibly due to an increased dissociation of iron from ferritin. An increase in unbound iron catalyzes oxidative reactions and promotes cell damage (Kell, 2009; Kell and Pretorius, 2014). The addition of physiological iron levels results in slight RBC shape changes (Pretorius, 2013). Scanning electron microscopy showed that generation of hydroxyl radicals induced by iron overload triggers the aggregation of RBC with fibrin-like fibers, resulting in a pro-thrombotic state (Lipinski et al., 2012).

Despite substantial evidence regarding oxidative biomarkers in CKD, some findings remain controversial and their significance is unclear (Tucker et al., 2013). The high levels of pro-oxidants in tandem with the defective antioxidant machinery found in CKD may contribute to oxidative stress, an enhanced susceptibility of RBCs for eryptosis and worsening of renal anemia.

OXIDATIVE STRESS, INFLAMMATION, AND AGING

The triad of aging, inflammation, and oxidative stress reduces the quality of life of CKD patients. The emblematic term *inflammaging* has been introduced to highlight the role of these factors in the often rapid deterioration of the patient's health, longevity, and well-being (Ebert et al., 2020). Interestingly, inflammatory biomarkers such as interleukin 6 and C-reactive protein are not correlated with RBC life span in HD patients (Ma et al., 2017). Aging and oxidative stress are processes that can take place at the cellular level or the whole-body level and aging is associated with an imbalanced redox environment and rise in inflammatory biomarkers (Maurya et al., 2015).

The oxidative injury during the RBC life span may lead to dysfunction and cell death. Although oxidative stress is fundamental to RBC senescence, it is not the sole reason for its progression. CD47, a constitutive membrane receptor, acts as a protective ("do not eat me") signal against phagocytosis by interacting with the macrophage receptor SIRPα. Conversely, the expression of PS on RBC surface has the opposite effect and acts as an "eat me" signal to phagocytic cells (Lutz and Bogdanova, 2013; Arias and Arias, 2017; **Figure 1**). Moreover, Burger et al. found that aging promoted by oxidative stress induces a conformational change of CD47, which enables this molecule to bind to thrombospondin-1, converting CD47 into an "eat me" signal (Burger et al., 2012). The role of O_2 is highlighted by the observation that RBCs from male subjects performing hypoxic exercise training (15% O_2) showed less CD47 expression as well as a reduction of the cytoskeleton proteins actin and spectrin. RBC deformability, which is essential for their physiological function, was compromised in hypoxic conditions by the activation of Gardos channel (Mao et al., 2011). Moreover, in RBCs from CKD patients, a reduced expression of CD47 was observed (Antonelou et al., 2011). These different mechanisms underlying RBC senescence are still subject of ongoing research.

From an inflammation perspective, there are several molecules that play a role in renal anemia. The lipid peroxidation

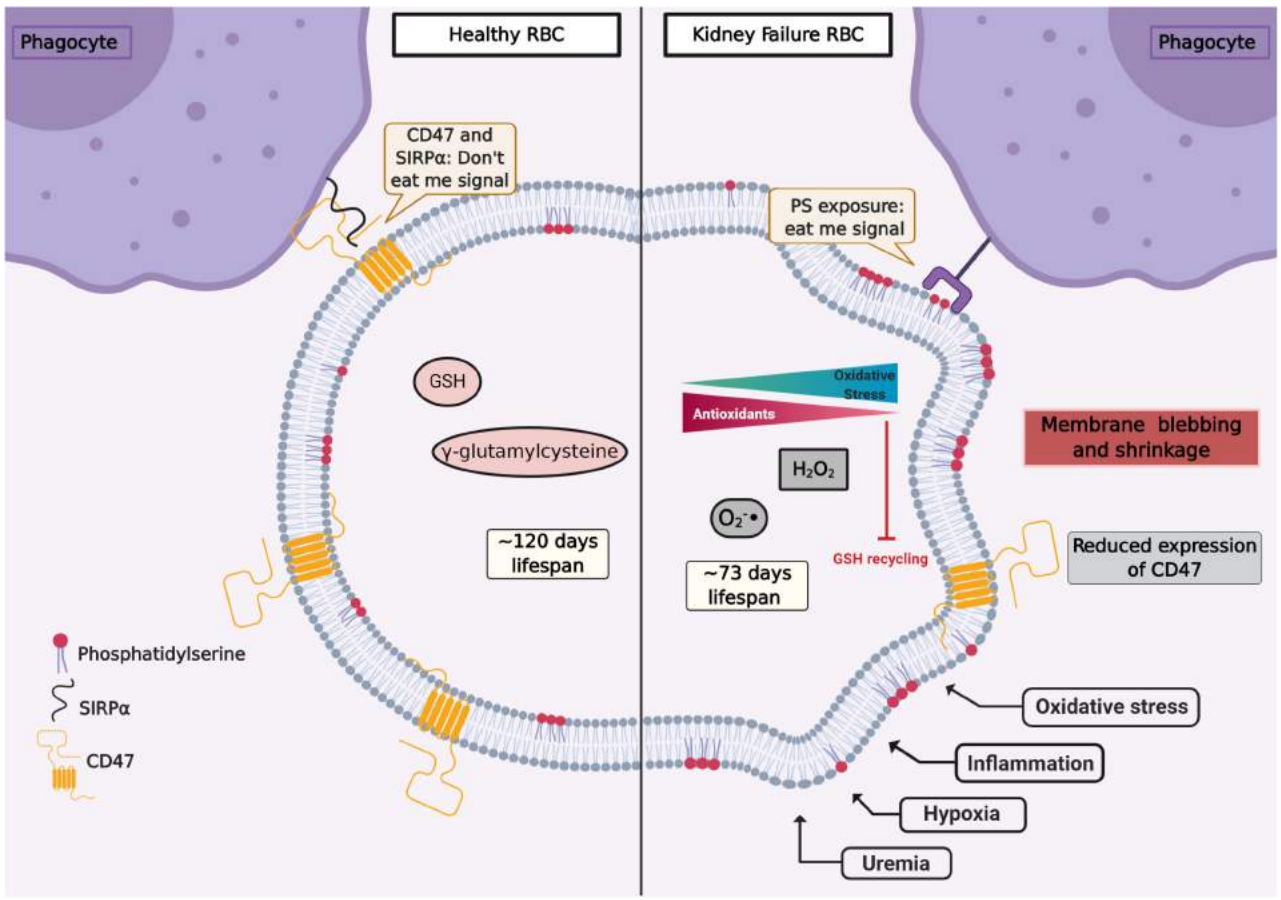

FIGURE 1 | Representation of RBC signaling, life span, and redox environment in health (left) and kidney failure (right). Several factors such as uremia, hypoxia, inflammation, and oxidative stress can induce signals of cellular damage and instigate eryptosis. CD47 is a constitutive transmembrane protein that interacts with receptors on the surface of phagocytes, preventing erythrophagocytosis ("don't eat me signal"). In health, after about 120 days, CD47 expression is reduced and RBCs are removed by the monocytic–macrophage system, especially in the spleen. The RBC life span is reduced in chronic kidney disease (CKD). RBCs from CKD patients show an overproduction of oxidants and an impaired antioxidant recycling. In CKD, circulating RBCs increase the translocation of phosphatidylserine (PS) from the inner membrane leaflet to the cell surface. Pro-inflammatory monocytes and resident macrophages recognize PS, and RBCs are cleared from circulation. Figure created with BioRender.com.

product 4-hydroxy-trans-2-nonenal (HNE) contributes to several inflammatory and degenerative processes. It is overexpressed in kidneys from aged rats and leads to NF-κB activation (Jang et al., 2016). In RBCs, HNE exerts a pro-eryptotic effect, initiating the classical eryptotic markers as well as agglutination elements and adhesion molecules, resulting in binding of RBCs to endothelial cells, possibly promoting thrombosis (Allegra et al., 2020).

HYPOXIA IN DIALYSIS PATIENTS AND ITS ASSOCIATION WITH ERYPTOSIS

Hypoxia *per se* is well known to provoke oxidative stress. In the early 1990s, Rifkind et al. (1991) showed that the hypoxemic condition facilitates hemoglobin autoxidation and, as a result, the free radical anion superoxide is exacerbated despite the high levels of antioxidants normally present in RBC. Importantly, about 10% of patients undergoing HD experience prolonged intradialytic hypoxemia (PIH), a clinical phenotype characterized by an arterial oxygen saturation below 90% for at least one third of the dialysis treatment time (Meyring-Wosten et al., 2016). Previously, we explored the effect of low oxygen partial pressure and the uremic toxin IS on eryptosis (Tozoni et al., 2019). Interestingly, we found that hypoxemia and IS independently increase eryptosis and ROS generation and decrease GSH levels, possibly contributing to the reduced RBC life span observed in CKD (**Figures 1**, **2**). Of note, high altitude, another hypoxia model, had a different effect on RBC homeostasis. Epo produced in response to hypoxia inhibits Ca^{2+} channels and thus attenuated eryptosis (Myssina et al., 2003). In rats kept at high altitude (5,000 m) for 30 days, chronic hypoxia inhibited eryptosis, possibly by increasing CD47 expression and decreasing intracellular Ca^{2+} levels (Tang et al., 2018). This observation is possibly caused by the protective effect of Epo and production of new RBC with high CD47 expression.

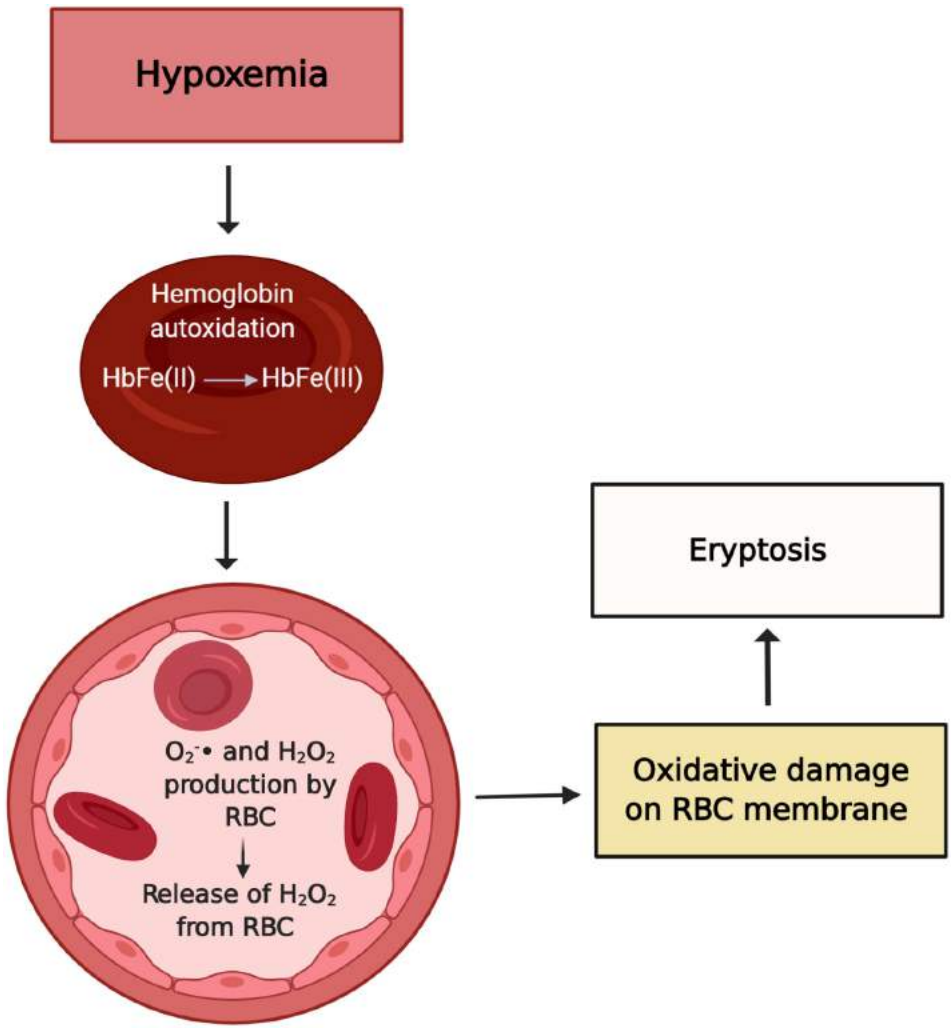

FIGURE 2 | Possible effects of hypoxemia on RBC. Hypoxia leads to hemoglobin autoxidation and formation of O_2^- and H_2O_2. In CKD, a weakened antioxidant system fails to prevent injury by oxidants. The exacerbated oxidative stress is a stimulus to eryptosis. Figure created with BioRender.com.

Any harmful insult to RBC will generate membrane modifications and possibly signal an eryptotic event, reversible or not (Pretorius et al., 2016a). Raman spectroscopy reveals several changes in hemoglobin morphology and function of RBCs under hypoxia. According to Revin et al. (2017), not only the lipid composition of RBC is profoundly altered in hypoxia, but also the ability of hemoglobin to bind and select ligands is reduced, including its affinity for oxygen (Chowdhury and Dasgupta, 2017). Morphological modifications of RBCs in tandem with low hemoglobin concentration can result in poor O_2-carrying capacity and compound tissue hypoxia.

ION CHANNEL MODIFICATIONS IN ERYPTOSIS

The disturbance of membrane asymmetry that favors PS exposure and subsequent eryptosis is caused by the increase of cytosolic Ca^{2+} (Segawa and Nagata, 2015; Pretorius et al., 2016a). Ca^{2+} enters RBC through several ion channels, pumps, and exchangers that transport Ca^{2+} through the plasma membrane (Polak-Jonkisz et al., 2010b; Brini and Carafoli, 2011; Polak-Jonkisz and Purzyc, 2012). Several factors such as oxidative stress, energy depletion, or uremic toxins activate Ca^{2+} influx into the RBC (Lang and Lang, 2015). Ca^{2+} efflux occurs mainly by a high-affinity, low-capacity Ca^{2+}-ATPase, the plasma membrane Ca^{2+} pump (PMCA) (Brini and Carafoli, 2011).

The distribution of PS on the inner and outer membrane leaflet is determined by the activity of translocase proteins in the RBC membrane: flippase, floppase, and scramblase. Flippase is an ATP-dependent transporter that transfers phospholipids from the extracellular leaflet to the cytoplasm, while floppase is an ATP-binding cassette transporter that catalyzes the movement of phospholipids in the opposite direction (Hankins et al., 2015). To maintain an optimal distribution of lipid bilayer phospholipids, scramblases

regulate PS movement in both directions, independent of ATP. These enzymes are regulated by intracellular Ca^{2+} levels (Bitbol et al., 1987; Pretorius et al., 2016a; Föller and Lang, 2020). A high concentration of cytosolic Ca^{2+} inhibits flippase, which results in the activation of the scramblase, followed by the translocation of PS from the internal leaflet to the surface of the eryptotic RBC (Williamson et al., 1992; **Figures 3**, **4**).

Intracellular Ca^{2+} regulation is mediated mainly by its low passive permeability and its active removal by the calcium ATPase pump dependent on Ca^{2+}-Mg^{2+} and by the Na^{+}/Ca^{2+} exchanger (Carafoli, 1987; Blaustein, 1988; Reeves et al., 1994). The Ca^{2+} PMCA1 and PMCA4 regulate and maintain the internal concentration of Ca^{2+}. About 10% of the plasma RBC membrane proteins are PMCAs (Rothstein et al., 1976). These enzymes are important regulators of Ca^{2+} homeostasis, being activated by a series of mechanisms, some of them though still unknown.

Ca^{2+} efflux from the cytosol is against a steep chemical gradient and hence PMCA requires ATP. RBCs from CKD patients have a decreased PMCA activity as well as reduced calmodulin concentration as renal failure progresses (Polak-Jonkisz et al., 2010a). Thus, elucidating the relationship between the systems that control both Ca^{2+} influx and efflux in eryptosis would be an important step in determining potential inhibitory targets for the accelerated RBC death in CKD.

Although RBC's PMCA has been well characterized, the knowledge of ion transport systems that mediate Ca^{2+} uptake in RBC is quite limited. The incubation of RBC with Ca^{2+} ionophore ionomycin causes exposure of PS in the outer membrane leaflet (Föller et al., 2009b). In addition to PS exposure, elevated levels of intracellular Ca^{2+} promote oxidative stress by directly activating NADPH oxidase and nitric oxide synthase in uncoupled mode (Özüyaman et al., 2008; George et al., 2013). Among the transport systems that contribute to the uptake of Ca^{2+} in human RBCs, there are several classes of cation channels (Kaestner, 2011). Some ionotropic receptors have been described in RBCs, like the GluA1, the AMP glutamate ionotropic receptor subunit (Makhro et al., 2016; Kaestner et al., 2020), as well as N-methyl-D-aspartate (NMDA) (Makhro et al., 2017), contributing to Ca^{2+} homeostasis in these cells. Moreover, after removal of Cl^{-} or extracellular glucose, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) antagonist receptors drive the increase in cytosolic Ca^{2+} and induce eryptosis by stimulating Ca^{2+} influx (Föller et al., 2009a).

Circulating RBCs are exposed to significant mechanical forces that influence their physiology and function in several ways, including deformability, the release of products such as ATP (Sprague et al., 2001), and the Ca^{2+} influx. Ca^{2+} influx also influences cell volume. Changes in RBC volume affect their ability

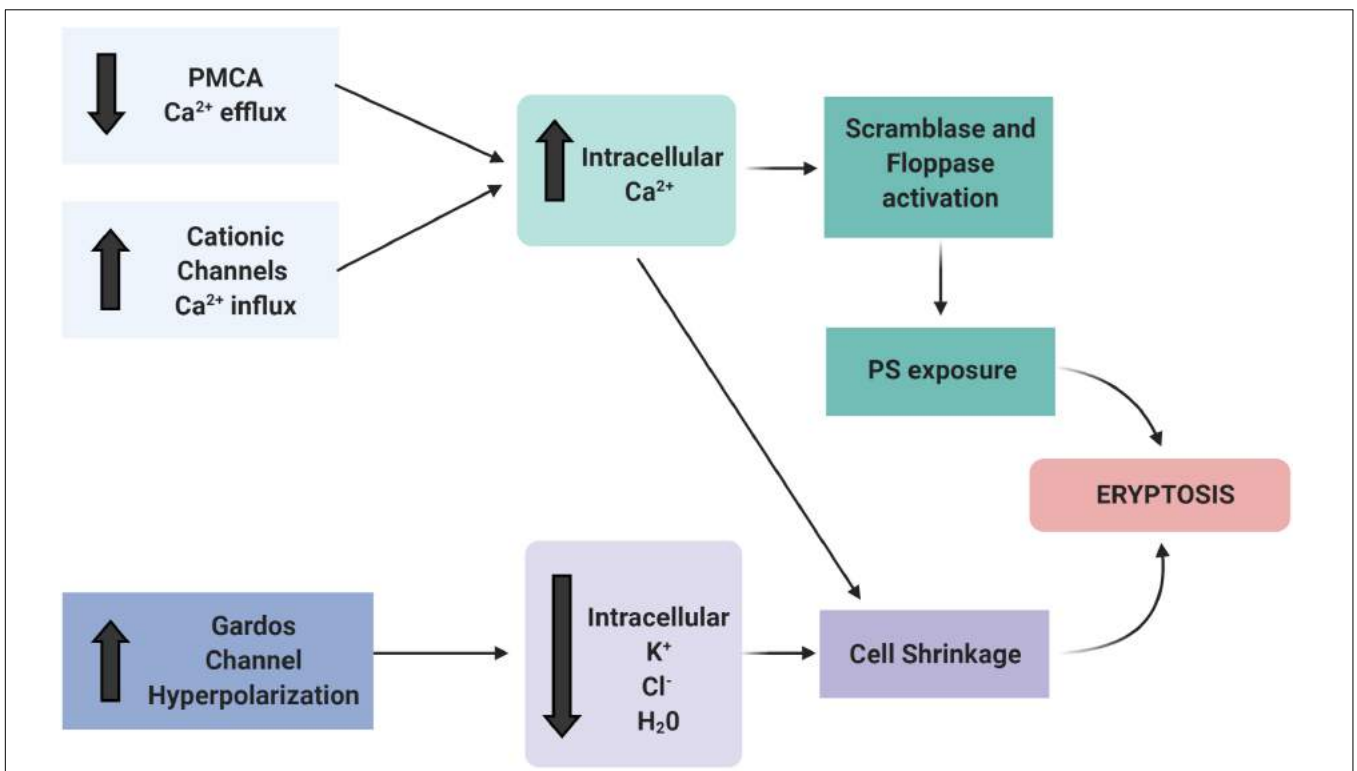

FIGURE 3 | Ion channels involved in eryptosis. The activation of Ca^{2+} channels (e.g., by uremic toxins, oxidative stress, hypoxia) is the main trigger to initiate the events that culminate in eryptosis. The increased intracellular Ca^{2+} activates scramblase and floppase proteins that promote PS exposure on the cell surface. Patients with CKD have PMCA inactivation and subsequent reduced Ca^{2+} efflux. In addition, other ion channels, such as the Gardos channel, are altered. The resulting efflux of K^{+}, Cl^{-}, and water result in RBC shrinkage and induce eryptosis. Figure created with BioRender.com.

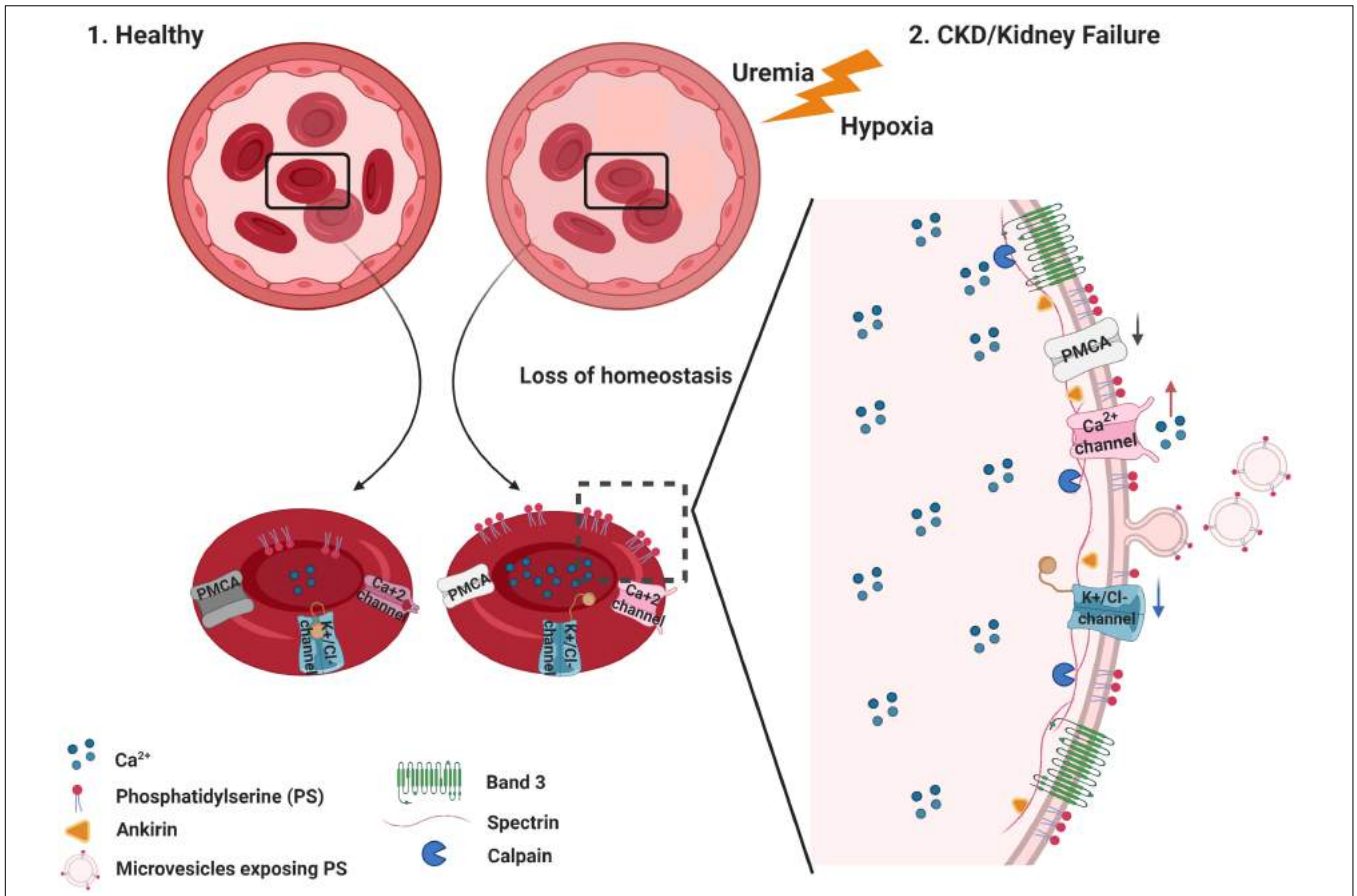

FIGURE 4 | Summary of membrane and cytoskeleton modifications in eryptosis.(1) Healthy RBC maintain normal intracellular levels of Ca^{2+} by active mechanisms controlling its efflux and influx. Under these circumstances, phosphatidylserine (PS) is located in the inner leaflet of the RBC membrane. (2) In CKD, uremic toxins accumulate and low hemoglobin oxygen saturation (hypoxemia) is observed in some patients. This loss of homeostasis favors significant changes in the stability of the RBC, with an increase in the Ca^{2+} influx and, consequently, an increase in its intracellular concentration. Increased intracellular Ca^{2+} trigger PS translocation to the outer leaflet of the RBC. Several processes follow the Ca^{2+} increase, such as the activation of calpains (calcium-activated proteases) that target cytoskeletal proteins such as spectrin, ankyrin, as well as band 3/AE1. These processes result in the degradation of cytoskeletal proteins, microvesicle release, and changes in cell shape and deformability. Figure created with BioRender.com.

to traverse capillaries. The molecular mechanisms involved in sensing mechanical forces and their effects on RBC volume are not fully understood. The mechanosensitive Ca^{2+} channel PIEZO1 is located on the RBC membrane. PIEZO1 is an important regulator of cell volume in response to mechanical stress (Cahalan et al., 2015). The connection between mechanical forces and RBC volume via Ca^{2+} influx through PIEZO1 is closely linked to the cells' ability to change shape and reduce cell volume and enable passage through small-diameter capillaries. Of note, the intensity of the mechanical force can induce the initial steps of eryptosis, since an increase of PIEZO1-dependent Ca^{2+} influx stimulates Gardo's channels and subsequent RBC shrinkage (Cahalan et al., 2015).

Increased Ca^{2+} influx due to Ca^{2+} channel activation combined with reduced Ca^{2+} efflux from the cytosol may affect the homeostasis of other ions, most prominently K^+. Activation of the Gardos channel, a K^+ efflux channel activated by intracellular Ca^{2+} increase, results in membrane hyperpolarization and increased Cl^- efflux. Finally, the loss of water leads to cell shrinkage (Thomas et al., 2011; Boulet et al., 2018; **Figures 3**, **4**). Even a local membrane deformation can trigger this process and induce cell dehydration, which may explain the higher density of senescent RBCs (Dyrda et al., 2010).

CYTOSKELETON MODIFICATIONS

The RBC cytoskeleton plays an important role in cell homeostasis. The spectrin-actin network interacts with ankyrin and controls RBC deformability (Pretorius et al., 2016b). This interaction of the cytoplasmatic domain of membrane proteins with cytoskeleton proteins prevents membrane vesiculation and breakup (Mohandas and Gallagher, 2008). Besides the role of Ca^{2+} on the eryptotic process, the prolonged Ca^{2+} permeability activates μ calpain, which can degrade cytoskeleton components, such as the ankyrin R complex that forms bridges to connect membrane proteins to the spectrin-based skeleton, assuring membrane stability and assembly of signaling and

structural components on the inner membrane surface (Berg et al., 2001; **Figure 4**).

The band 3 protein, also called anion exchanger 1 (AE1), is one of the transport proteins that mediate the exchange of Cl^- and HCO_3^-. Through interaction with lipids and proteins, the multifunctional band 3 unites the multiprotein complex of the cytoskeleton and confers mechanical and elastic properties to RBC and thus blood viscosity (Burton and Bruce, 2011). Moreover, studies of RBC membrane proteins in CKD stage 5 patients showed lower levels of ankyrin and spectrin, as well as altered ankyrin/band 3 ratio. In addition to these alterations, the same study showed that patients in CKD stage 5 who do not respond to ESA have a lower spectrin/ankyrin ratio (Costa et al., 2008).

RBC LIFE SPAN IN HEALTH AND CKD

Aging RBCs lose the flexibility needed to traverse the network of tight capillaries. Senescent RBCs are removed from circulation by splenic red pulp macrophages. While the key role of Epo deficiency in renal anemia is undisputed, the impact of RBC life span has received only little attention (Dou et al., 2012). One reason is that RBC life span measurements are impractical in the clinical environment. Studies show that the life span of RBC from HD patients is dramatically reduced. Several groups have measured RBC life span in HD patients and arrived, for example, at values of 73 ± 18 days (Ma et al., 2017) and 89 ± 28 days (Sato et al., 2012). While there are rare obvious reasons for hemolysis in dialysis patients (e.g., contaminations with chloramine or nitrate; overheated dialysate) (Saha and Allon, 2017), the pathogenesis of the reduced RBC life span is ill-defined. The shortened RBC life span in CKD has been attributed to the uremic environment rather than mechanical stress induced by HD (Vos et al., 2011). As the RBC life span declines, higher doses of ESAs are needed to attain hemoglobin target levels in HD patients (Sato et al., 2012). Interestingly, ESA administration was positively correlated with PS exposure on RBC from HD and PD patients (Bissinger et al., 2016). On the other hand, eryptosis in healthy RBCs induced by osmotic shock showed the opposite effect, where PS exposure was ameliorated in the presence of Epo (Myssina et al., 2003). These contradictory findings demonstrate that the response of the RBCs to Epo may be dependent on the nature of the eryptotic trigger and the prevailing milieu interieur. Although the administration of ESAs is crucial for the correction of renal anemia, it may promote the clearance of young RBCs, a process called neocytolysis (Alfrey and Fishbane, 2007).

Of note, serum Epo levels do not differ substantially across CKD stages. Interestingly, reticulocyte count was reduced only in CKD stage 5 when compared with stage 1 (Li et al., 2019). Such findings suggest that other factors besides Epo deficiency, such as RBC life span, compound renal anemia. Indeed, the decline of kidney function is correlated with a progressive shortening of RBC life span (122 ± 50, 112 ± 26, 90 ± 32, 88 ± 28, and 60 ± 24 days, from CKD stages 1–5, respectively) (Li et al., 2019), and the prevalence of anemia also increases with the progression of the disease (KDIGO, 2012).

RELATION BETWEEN RBC LIFE SPAN AND EPO REQUIREMENT: INSIGHTS FROM BIOMATHEMATICAL MODELING

The higher ESA requirement in patients with a lower RBC life span can also be elucidated using computational models of erythropoiesis. Such models encapsulate key features of human erythropoietic physiology and, through simulations, enable one to study how physiological factors such as RBC life span determine a patient's response to ESA administrations. Furthermore, they provide a tool to augment the design and interpretation of clinical and laboratory studies and aid the development of treatment algorithms. Phenomenological approaches have focused on an abstract description of the hematocrit as the only model variable, in which ESA administrations lead to an effective increase in hematocrit, without resolving the underlying physiological mechanisms (Uehlinger et al., 1992; Kalicki and Uehlinger, 2008). In this scheme, the duration and progression of the ESA-induced hematocrit increase is determined by RBC life span and its variability, whereas the speed of the increase is determined by effective parameters accounting for ESA efficacy and the concentration threshold for ESA response. Using model simulations, it has been illustrated that a lower RBC life span results in lower hematocrit levels for the same ESA dose (Kalicki and Uehlinger, 2008), consistent with higher ESA doses being required to achieve a desired hematocrit target for lower RBC life span.

How does RBC life span affect Epo requirements within an entire patient population, taking into account inter-patient variability in other physiological parameters related to RBC fate as well? Modeling approaches based on "virtual patient populations" can provide insights into this question, as can be illustrated considering an established physiological model of erythropoiesis (Fuertinger et al., 2013, 2018a). This model explicitly represents the proliferative hierarchy of erythroid progenitor populations in the bone marrow including the dynamics of cell birth, maturation, differentiation, and apoptosis; in this modeling scheme, Epo acts as a regulator of apoptosis of the colony-forming unit-erythroid (CFU-E) and erythrocyte populations. Previously, this model has been adapted on a patient-individual level to a large population of HD patients treated with methoxy polyethylene glycol-epoetin beta (6,659 patients randomly sampled from a U.S. HD population comprising over 37,000 patients) (Fuertinger et al., 2018b). These model adaptations were carried out such that previously recorded hemoglobin responses to ESA therapy for the specific patient were described by the model within a predefined accuracy. Individual patients were represented by a patient-specific parameter set (RBC life span, ESA half-life, endogenous Epo levels, and effective parameters accounting for the ESA's effect on erythroid progenitor apoptosis and maturation velocity) capturing their erythropoiesis-related physiology (Fuertinger

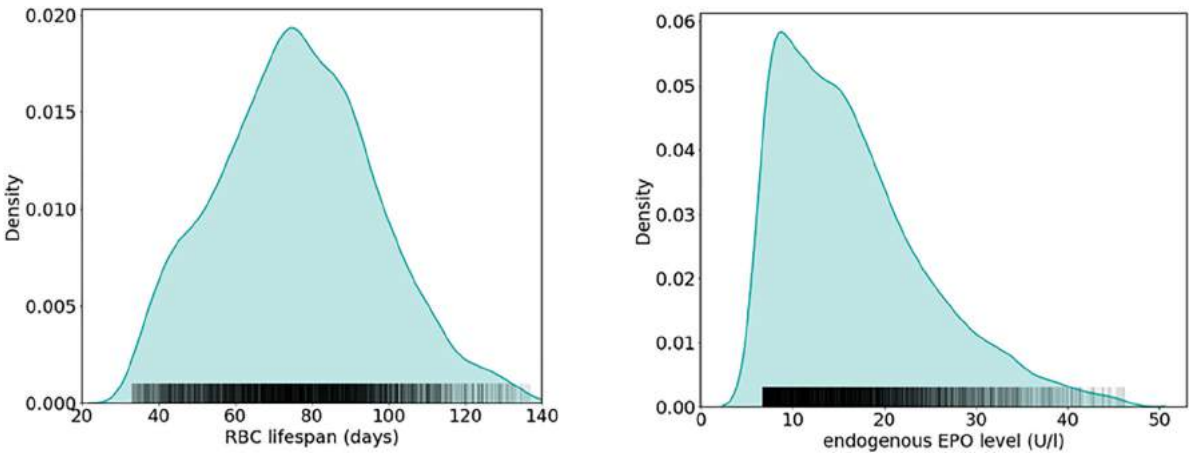

FIGURE 5 | Estimation of physiological parameters in a HD patient population using a physiology-based model of erythropoiesis (Fuertinger et al., 2013). Shown are relative frequencies of estimated RBC life span (left) and endogenous Epo levels (right) obtained from patient-individual model adaptations to hemoglobin and ESA administration data of 6,659 HD patients, resulting in a virtual patient population (Fuertinger et al., 2018b) (see the main text for details).

et al., 2018b). Although iron availability is not an explicit part of the model, its effects are implicitly present in the effective bone marrow-related parameters. These patient representations through patient-specific parameter sets within the model paradigm are termed "anemia avatars" or "virtual patients." Estimated mean RBC life span across all virtual patients was 76 ± 21 days (mean ± SD; range: 33–137 days), close to reported values in urban HD centers (see, e.g., Ma et al., 2017; mean ± SD: 73 ± 18 days; range: 38–116 days); the median estimated endogenous Epo level is 15 U/L (25th, 75th percentiles: 10.2 U/L, 21.1 U/L). The relative frequencies of estimated RBC life spans and endogenous Epo levels among the population of virtual patients reported in Fuertinger et al. (2018b) are shown in **Figure 5**.

Making use of this established set of virtual patients, it is straightforward to illustrate how RBC life span affects the amount of Epo required for simulated patients to meet a specific hemoglobin level (10.49–10.51 g/dl). To this end, for each virtual patient, required total Epo levels[1] are determined. **Figure 6** shows

[1]Total Epo levels instead of ESA utilization are chosen to eliminate the effects of ESA half-life, which has a high inter-patient variability that would confound the relation between RBC life span and Epo requirement.

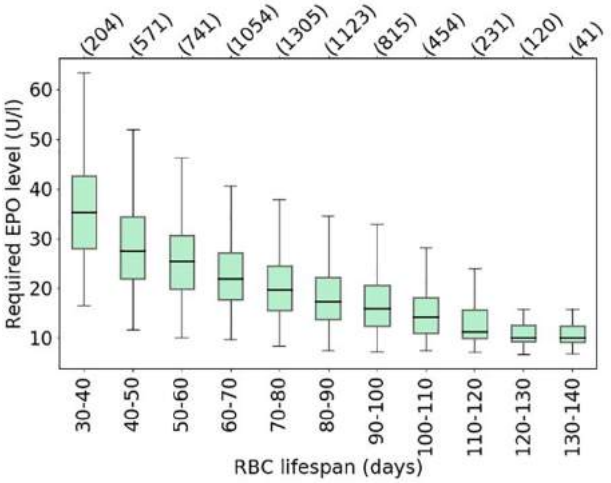

FIGURE 6 | Model-based estimates of the average required Epo levels to achieve a hemoglobin target of 10.49–10.51 g/dl across the virtual patient population shown in **Figure 5**, binned by patient-specific RBC life span. Boxes show the interquartile range (IQR); whiskers show the full range of values for all virtual patients in the respective bin (bin population indicated in parentheses) excluding outliers defined as being more than ± 1.5 IQR outside the box.

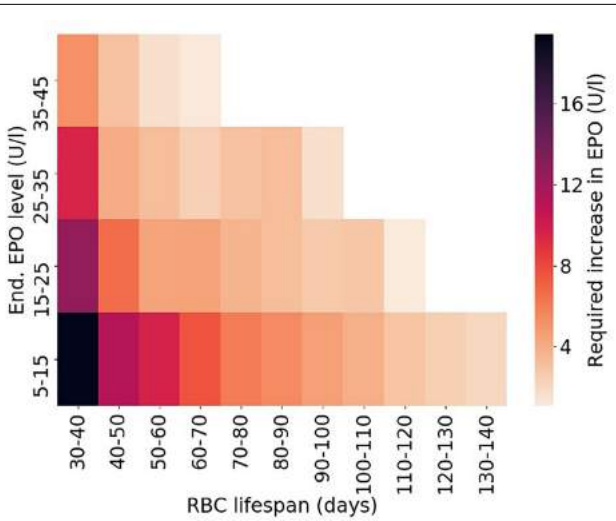

FIGURE 7 | Required average increase in endogenous Epo levels depending on RBC life span and endogenous Epo levels for the virtual patient population shown in **Figures 5**, **6**. Only bins populated with at least 25 virtual patients are included in the density plot.

the distribution of required Epo serum concentrations to achieve the hemoglobin target, with virtual patients being grouped into RBC life span bins of 10 days. The model analysis suggests a systematic increase in Epo requirement with decreasing RBC life span, a marked trend despite the variability of the virtual patient population in other physiological parameters affecting RBC generation and fate. This inter-patient variability is responsible for the partially large spread of required Epo levels within each binned group, an effect that becomes more prominent for small RBC life spans. Dissecting the virtual patient population by RBC life span and endogenous Epo levels, the mean required increase in Epo concentration to achieve the hemoglobin target is shown in **Figure 7**: Within the same Epo range, the shorter the RBC life span, the higher the required increase in Epo level, as is most clearly visible for the smallest Epo level range (5–15 U/L). Notwithstanding the effects of ESA half-life, these insights obtained from modeling approaches involving "virtual patient populations" illustrate that (i) a reduced RBC life span may necessitate frequent and high-dose ESA administrations and may present a cause of Epo hypo-responsiveness; (ii) within some (virtual) patients, the effects of a reduced RBC life span on Epo requirements are partially compensated by other physiological factors affecting RBC generation and fate such as endogenous Epo levels.

CONCLUSION

Among the many causes that contribute to anemia in kidney failure, eryptosis is a key process whose significance has not been fully acknowledged so far. Although improving hemoglobin levels, ESA and iron administration alone are only one strategy to correct renal anemia that can be limited by a decreased RBC life span. Oxidative stress, inflammation, hypoxemia, and accumulation of uremic solutes promote an imbalance of RBC homeostasis and need to be considered. Even in a scenario where ESA administration increases erythropoiesis rate, the newly formed RBCs can undergo eryptosis within a few days in circulation, resulting in ESA hypo-responsiveness and thus preventing attainment of desired hemoglobin targets. These mechanisms center around (i) increased Ca^{2+} influx and reduced activity of enzymes mediating Ca^{2+} efflux (PMCA), (ii) Gardos channels activation and RBC volume loss, (iii) PS exposure on cell surface and subsequent RBC clearance from circulation. More research in this field is needed to further elucidate these processes and develop potential therapeutic interventions. Extending RBC life span in uremia may evolve as a novel therapeutic strategy for renal anemia.

AUTHOR CONTRIBUTIONS

GD and AM-A wrote, reviewed and edited, contributed to the discussion, and created figures. NG wrote, reviewed and edited, and contributed to the discussion. SR wrote, reviewed and edited, contributed to the discussion and carried out the mathematical analysis. DJ and RP-F reviewed, edited, and contributed to the discussion. PK conceptualized, wrote, reviewed and edited, and contributed to the discussion. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Renal Research Institute, New York, United States. GD was a recipient of research fellowship from the Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior—Brasil (CAPES).

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Conflict of Interest: PK holds stock in Fresenius Medical Care. The Renal Research Institute is a wholly owned subsidiary of Fresenius Medical Care.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

JUSTIFICATION

The treatment of renal anemia with rHuEpo and iron administration has tremendously improved CKD patients' survival and quality of life and is effective for most of them. However, many patients (5-10%) do not respond well to the treatment and cannot reach adequate hemoglobin levels. Renal anemia is a multifactorial comorbidity, and the contribution of eryptosis to it is still not completely understood. It is well known that an increase in eryptosis might shorten the RBC lifespan. Once the production of new RBC by the Epo-stimulated bone marrow cannot surpass RBC loss, the establishment of renal anemia might occur. In this circumstance, it is crucial to understand the factors that trigger eryptosis. Oxidative stress, hypoxia, and uremia play an essential role in the pathophysiology of CKD. However, their impact on RBC and their relationship with renal anemia are poorly understood and therefore were considered for investigation in the present study.

GENERAL AIM

To evaluate the effects of hypoxia and uremia in the development of eryptosis and RBC resistance to oxidative stress.

Specific aims

1. To evaluate phosphatidylserine exposure and intracellular Ca^{2+} levels in healthy and uremic RBC submitted to *in vitro* hypoxia and/or uremia.
2. To analyze reactive oxygen species production and antioxidant defense mechanisms in healthy or uremic RBC submitted to *in vitro* hypoxia and/or uremia.
3. To quantify intracellular oxygen levels in healthy or uremic RBC submitted to *in vitro* hypoxia and/or uremia.
4. To analyze the concentration of protein-bound uremic toxins in RBC and serum of healthy subjects and hemodialysis patients.
5. To measure phosphatidylserine exposure, intracellular Ca^{2+} and intracellular oxygen levels after the inhibition of protein-bound uremic toxins entry in RBC.
6. To measure reactive oxygen species production and glutathione levels after the inhibition of protein-bound uremic toxins entry in RBC.

RESULTS

Article 1: “Uremia and inadequate oxygen supply induce eryptosis and intracellular hypoxia in red blood cells”

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2021;55:449-459
DOI: 10.33594/000000396
Published online: 14 July 2021
Accepted: 8 May 2021

Original Paper

Uremia and Inadequate Oxygen Supply Induce Eryptosis and Intracellular Hypoxia in Red Blood Cells

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Key Words
Eryptosis • Uremic toxins • Chronic kidney disease • Anemia • Hypoxia

Abstract
Background/Aims: Chronic kidney disease is frequently accompanied by anemia, hypoxemia, and hypoxia. It has become clear that the impaired erythropoietin production and altered iron homeostasis are not the sole causes of renal anemia. Eryptosis is a process of red blood cells (RBC) death, like apoptosis of nucleated cells, characterized by Ca^{2+} influx and phosphatidylserine (PS) exposure to the outer RBC membrane leaflet. Eryptosis can be induced by uremic toxins and occurs before senescence, thus shortening RBC lifespan and aggravating renal anemia. We aimed to assess eryptosis and intracellular oxygen levels of RBC from hemodialysis patients (HD-RBC) and their response to hypoxia, uremia, and uremic toxins uptake inhibition. ***Methods:*** Using flow cytometry, RBC from healthy individuals (CON-RBC) and HD-RBC were subjected to PS (Annexin-V), intracellular Ca^{2+} (Fluo-3/AM) and intracellular oxygen (Hypoxia Green) measurements, at baseline and after incubation with uremic serum and/or hypoxia (5% O_2), with or without ketoprofen. Baseline levels of uremic toxins were quantified in serum and cytosol by high performance liquid chromatography. ***Results:*** Here, we show that HD-RBC have less intracellular oxygen and that it is further decreased post-HD. Also, incubation in 5% O_2 and uremia triggered eryptosis *in vitro* by exposing PS. Hypoxia itself increased the PS exposure in HD-RBC and CON-RBC, and the addition of uremic serum aggravated it. Furthermore, inhibition of the organic anion transporter 2 with ketoprofen reverted eryptosis and restored the levels of intracellular oxygen. Cytosolic levels of the uremic toxins pCS and IAA were decreased after dialysis. ***Conclusion:*** These findings suggest the participation of uremic toxins and hypoxia in the process of eryptosis and intracellular oxygenation.

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Introduction

The primary function of human red blood cells (RBC), which comprise approximately 40% of the blood volume, is the hemoglobin-mediated oxygen transport [1] from the lungs to organs and tissues [2]. The RBC production by the bone marrow is regulated by erythropoietin (Epo), a hormone that maintains an adequate erythropoiesis rate. Anemia is observed in 70% of the patients undergoing dialysis and is mainly attributed to the lack of Epo production by the diseased kidneys and impaired iron homeostasis [3]. Despite the administration of high doses of erythropoietin stimulating agents (ESA), factors such as inflammation, accumulation of uremic toxins (UT), and reduced RBC lifespan [4] contribute to ESA hypo-responsiveness in 5-10% of the kidney failure population [5]. The reduced RBC lifespan might occur due to an apoptosis-like RBC death, called eryptosis. This premature RBC death can be triggered by increased cytosolic Ca^{2+} content and is initiated through phosphatidylserine (PS) exposure on the outer RBC membrane leaflet [6]. Phagocytic cells recognize PS on the RBC surface and upon receptor-mediated binding remove these cells from circulation. This process may contribute to worsening renal anemia [7, 8]. It is known that hemodialysis (HD) patients have accentuated eryptosis compared to healthy individuals [9, 10]. This is attributed, in part, to the action of protein-bound uremic toxins (PBUT), such as indoxyl sulfate (IS) [10, 11].

About 10% of the patients undergoing HD show prolonged intradialytic hypoxemia (PIH), defined as arterial oxygen saturation below 90%, during at least a third of the dialysis session. PIH is associated with higher all-cause mortality and hospitalization as well as higher doses of ESA administration, indicating some degree of ESA hypo-responsiveness [12]. This clinical observation gave rise to studies of the combined effects of hypoxia and uremic toxins. We found evidence that hypoxia and uremia may increase eryptosis *in vitro* through an imbalance of the intracellular redox environment [13]. This study aimed to explore the role of hypoxia and uremia in the genesis of eryptosis and to evaluate RBC intracellular oxygen levels in HD patients. We hypothesized that *(i)* hypoxia and uremia increase eryptosis in RBCs from healthy subjects (CON-RBC) and HD patients (HD-RBC) *in vitro*; *(ii)* low oxygen levels are observed in HD-RBC, particularly after dialysis; (*iii*) increased eryptosis and decreased oxygen levels are partially attributed to the extra- and intracellular accumulation of PBUT.

Materials and Methods

Subjects and blood sampling

The study was approved by the ethics committee of Pontifícia Universidade Católica do Paraná (registration number 1.752.213). Participants gave informant consent before blood collection. Demographic and biochemical data were collected from patients' medical records or certified laboratory results for healthy subjects. Healthy subjects were required to have no history of renal or inflammatory disease and to have not received anti-inflammatory medication or blood transfusion one month prior to enrollment. All patients were undergoing HD with high-flux dialyzers for at least 3 months prior to enrollment. In hemodialysis patients, blood was drawn pre- and post-HD. In healthy subjects, blood was drawn by venipuncture. Samples were collected in tubes containing 3.2% sodium citrate and in SST tubes for serum collection. Blood samples were centrifuged (3000 rpm, 15 min, 4°C), buffy coat and plasma were discarded. RBCs were washed twice with cold phosphate-buffered saline (PBS) (1500 rpm, 10 min, 4°C) and immediately analyzed.

RBC treatments

RBCs from HD patients and healthy subjects were incubated for 24 hours under hypoxic conditions in an atmosphere comprising 5% O_2 and 5% CO_2 using the controllers ProOx 110 and ProCO2 120 (Biospherix, Redfield, NY, USA), respectively. Normoxic cells were incubated under physiological conditions, with 21% O_2 and 5% CO_2. The effect of uremia was tested by the addition of 10% healthy (S-CON) or HD (S-HD) serum to the incubation medium. To each patient, a healthy subject was randomly assigned, forming a patient-donor pair. To increase randomness in the study, before every experiment new patient-donor pairs were selected.

RBC from healthy donors (CON-RBC) were incubated with autologous serum (S-CON) or S-HD serum from a randomly assigned HD patient. RBC from HD patients (HD-RBC) were incubated with autologous serum (S-HD) or healthy serum (S-CON) from a randomly assigned healthy subject (Fig. 1). Serum was diluted with Tris-Glucose-BSA buffer (composition (in mM): 21.0 tris [hydroxymethyl] aminomethane; 4.7 KCl; 2.0 $CaCl_2$, 140.5 NaCl; 1.2 $MgSO_4$, 5.5 glucose and 4% of bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.4. Additionally, we incubated RBC for 24 hours with or without the addition of 30 µM ketoprofen (KETO), an organic anion transport 2 (OAT2) inhibitor [14] to block the entry of UT.

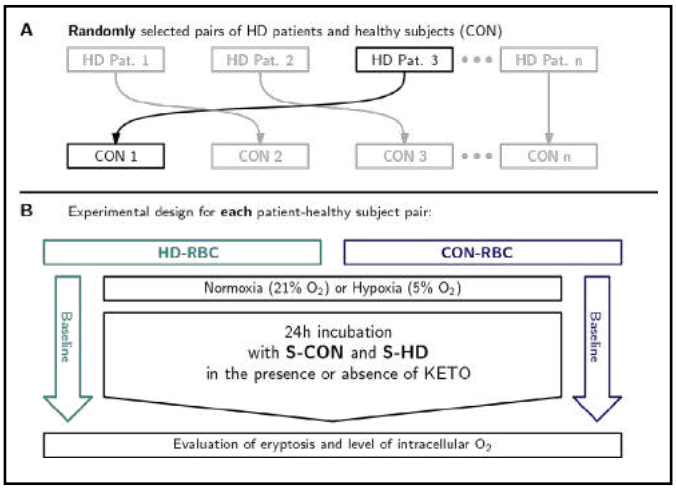


Fig. 1. Schematic overview of RBC incubations. A: each HD patient (HD Pat.) was randomly assigned to a healthy subject (CON), forming a patient-healthy subject pair. B: The HD-RBC and CON-RBC were separately incubated with HD serum (S-HD) or healthy serum (S-CON), from within the respective patient-healthy subject pair. In this setting, the incubation occurred for 24 hours with or without ketoprofen (KETO), in 5% O_2 or 21% O_2. Eryptosis and levels of intracellular O_2 were evaluated immediately after blood collection (baseline) and after incubation.

Measurement of PS exposure

The incubation with Annexin-V PE (BD Bioscience, Sparks, MD, USA) was performed for 15 minutes in the dark and washed once with PBS. Cells were fixed with 200 µl of PBS 4% formaldehyde (Fix-FACS) and analyzed by flow cytometry (FACSCalibur BD Bioscience, Sparks, MD, USA).

Measurement of intracellular Ca^{2+}

According to the manufacturer's instructions, erythrocytes were stained with 2 µM Fluo-3/AM (Thermo Fisher Scientific, Waltham, MA, USA) in Tris-glucose buffer without BSA. After incubation for 40 min at 37°C, cells were washed thrice with PBS and resuspended in 200 µl Tris-glucose buffer without BSA. Ca^{2+} dependent fluorescence intensity was measured by flow cytometry (FACSCalibur BD Bioscience, Sparks, MD, USA).

Determination of O_2 levels

RBCs were loaded with Hypoxia Green probe (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. With a reduced intracellular oxygen content, the hypoxia green probe releases rhodamine, emitting fluorescence. Cells were incubated with 1 µM of the probe diluted in Tris-glucose buffer without BSA for 2 hours (at 37°C in the dark). Then, cells were washed once with PBS, fixed with 200 µl Fix-FACS, and analyzed by flow cytometry (FACSCalibur BD Bioscience, Sparks, MD, USA).

Serum and RBCs concentration of protein-bound uremic toxins

The PBUT indoxyl sulfate (IS), indole 3-acetic acid (IAA), and p-cresyl sulfate (pCS) were quantified in serum and cells by high-performance liquid chromatography (HPLC) and fluorescence detection, as described by Stockler-Pinto et al. [15] and Rodrigues et al. [16], respectively. The analytical method was developed on a Shimadzu Prominence system and detected by a fluorescence detector (Shimadzu RF-20A). Analytes were separated using a C8 Luna column 150 × 4.6 mm, 5 µm (Phenomenex, Torrance, CA, USA). The gradient mobile phase consisted of 50 mM ammonium formate pH 3.0 and methanol, with a linear gradient proportion increasing from 25 to 70% (v/v) along to the run, at a flow rate of 0.7 mL/min. The fluorescence wavelengths for IS and IAA were λ excitation = 280 nm/λ emission = 383 nm. For pCS it was λ excitation = 265 nm/λ emission = 290 nm. The concentration of PBUT in RBCs was normalized by protein content, determined by Bradford assay.

Data analysis

The results are presented as mean ± SD or median (interquartile range). The statistical software was SPSS Statistics version 20 (Chicago, Ill, USA). The graphs were created using GraphPad Prism 5 (La Jolla, CA, USA). We evaluated the data distribution using the Shapiro-Wilk test. Multiple comparisons between groups were done by one-way ANOVA and post hoc least significant difference test. Paired data were analyzed using paired samples t-test. A $p< 0.05$ was considered statistically significant.

Results

Baseline clinical characteristics of study subjects

The clinical characteristics of the healthy subjects (n=14) and HD-patients (n=22) are shown in Table 1. Additional biochemical and treatment parameters from HD-patients are shown in Table 2. HD-patients were older compared to healthy subjects (58.1±18.1 vs. 34.8±17.3 years). Also, we found a significant predominance of male sex in HD patients. The CKD etiology showed a high prevalence of hypertension (40%) and diabetes type II (30%). HD patients were anemic (Table 1). Dialysis vintage was 41±35.6 months (Table 2).

Table 1. Baseline characteristics of participants. Data expressed as mean ± SD, or binary variables (frequency). BMI = Body mass index; Hb = hemoglobin; HD = hemodialysis; NA= not applicable. * p<0.05, ** p<0.01, *** p<0.001 compared to healthy subjects; [a] p<0.001 compared to pre-HD. [b] Pre-dialysis in HD patients

Parameters	Healthy Subjects (n=14)	HD Patients (n=22)
Demographics		
Age (years)	34.8±17.3	58.1±18.1*
Males (%)	20	73*
Caucasians (%)	100	88
BMI (kg/m²)	23.17±2.6	25.7±4.5
CKD Etiology		
Hypertension %	NA	40
Diabetes type II %	NA	30
Obstructive uropathy %	NA	5
Chronic glomerulonephritis %	NA	5
Polycystic kidney %	NA	5
Pyelonephritis %	NA	5
Uncertain %	NA	5
Biochemical Parameters		
Urea [b] (mg/dl)	22.6±1.1	109.2±25.6***
Urea post HD (mg/dl)	NA	31.4±11.8[a]
Creatinine (mg/dl)	0.8±0.05	8.1±2.5***
Hb (g/dl)	13.7±0.7	11±2.2**

Baseline characteristics of eryptosis

Our results showed that both markers of eryptosis – PS exposure and intracellular Ca^{2+} levels - were increased in HD-RBC compared to CON-RBC. The mean of the fluorescence intensity for PS was 12.8 higher on the RBC surface from post-HD compared to pre-HD, while Ca^{2+} influx demonstrated no difference between pre- and post-HD (Fig. 2).

Intracellular oxygen level

Intracellular oxygen levels were decreased in RBCs from HD patients pre- and post-HD when compared to CON-RBC. It is shown as an increase in hypoxia level (Fig. 3). Additionally, HD treatment was associated with a significant rise in hypoxia level (mean difference = 7.5) (Fig. 3).

Serum and RBC PBUT concentration

Serum PBUT was significantly decreased after dialysis but remained high compared to healthy serum (Table 3). Intracellularly, only pCS was detected in CON-RBC. Pre-HD RBCs had a higher concentration of all PBUT compared to control. Additionally, pCS and IAA decreased significantly compared to pre-HD (Table 3).

Table 2. Additional biochemical and treatment parameters in HD patients. PTH = parathyroid hormone; 25(OH)D = 25-hydroxyvitamin D; EPO = epoetin alfa

Parameters	HD-Patients (n=22)
Biochemical Parameters	
Serum albumin (g/dl)	3.7±0.3
Serum iron (µg/dl)	74±17.7
Ferritin (µg/ml)	327.4±240.5
Transferrin saturation (%)	31.5±12.6
Platelets (x10³/µl)	235.3±111.8
Leucocytes (x10⁹/L)	7±2.1
PTH (pg/dl)	215.9±136.1
Serum potassium (mmol/L)	4.9±0.6
Serum phosphorus (mg/dl)	4.5±1.1
Serum calcium (mg/dl)	9.1±0.8
25(OH)D (ng/mL)	22.8±11
Glucose (mg/dL)	116.6±29.7
Treatment Parameters	
Time in hemodialysis (months)	41±35.6
Treatment time (minutes)	216.3±19.9
Kt/V	1.68±0.26
Heparin (ml)	1.24±0.48
EPO (units per week)	7000±3861
Intravenous iron (mg per treatment)	10.4±3.7

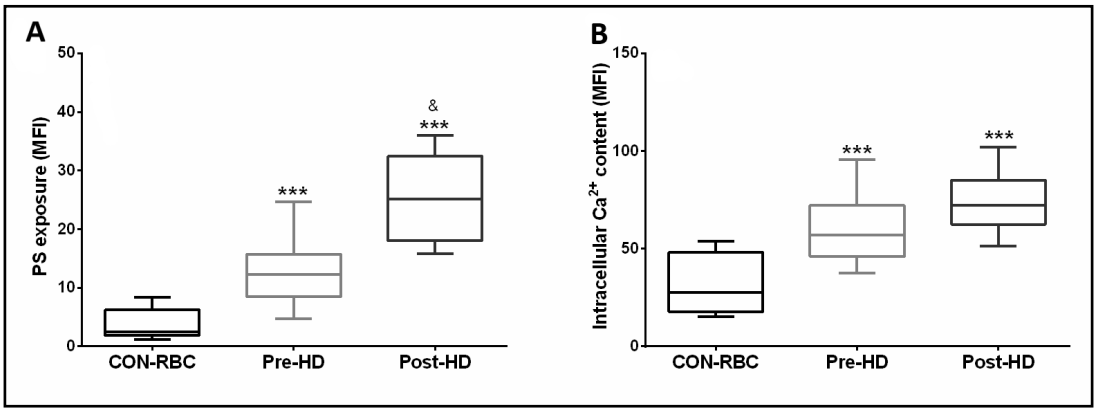
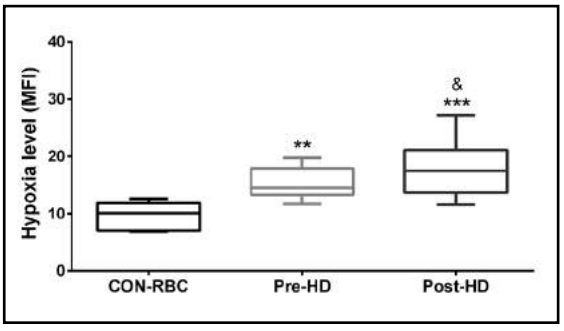


Fig. 2. Levels of eryptosis as indicated by PS exposure (A) and RBC intracellular Ca^{2+} content (B). Fresh isolated RBCs from healthy individuals (CON-RBC, n=12) and HD patients - before (Pre-HD, n=18) and after (Post-HD, n=12) HD - were loaded with Annexin-V and Fluo-3AM. Data are expressed as mean fluorescence intensity (MFI). ***p<0.001 compared to CON-RBC. & means p<0.05 comparing pre vs post HD.

Fig. 3. Hypoxia level in response to HD. Fresh isolated RBCs from healthy individuals (CON-RBC, n=6) and ESRD patients – before (Pre-HD, n=9) and after (Post-HD, n=8) hemodialysis session – were loaded with Hypoxia Green. Data are expressed as mean fluorescence intensity (MFI). ** p<0.01; *** p<0.001 compared to CON-RBC. &p<0.05 comparing post- vs pre-HD.



Effect of hypoxia on PS exposure

CON-RBC were exposed to 5% O_2 and HD serum by 24h incubation. We observed the 5% O_2 condition itself promoted an increase of PS exposure (11.3%) compared to CON-RBC in 21% O_2 (4±1.7%). In addition, CON-RBC in 21% O_2 incubated with S-HD increased the eryptosis from 4±1.7 to 17.6±6.5%. When both factors – 5% O_2 and S-HD – were combined, eryptosis increased to 25% (Fig. 4A). In HD-RBC, the incubation with 5% O_2 promoted a 6.3% increase of PS exposure compared to HD-RBC in 21% O_2 and healthy serum. S-CON decreased eryptosis of HD-RBC in 21% O_2 (from 27.4±9.2% to 16.2±6%) and 5% O_2 (from 37.1±10.6% to 22.6±6.8%). The UT inhibitor ketoprofen (KETO) decreased eryptosis in both cell types and treatments, except CON-RBC in 21% O_2 (Fig. 4A and B).

Table 3. PBUT levels in serum and RBC cytoplasm of healthy controls and HD patients before and after hemodialysis

PBUT concentration	Control	Pre-HD	Post-HD
In serum			
IS (µM)	2.6±1.8	100.9±66.9 ***	60.0±28.8 &***
pCS (µM)	15.7±10.2	243.4±180.8 ***	143.1±94.6 & ***
IAA (µM)	1.3±0.9	12.1±5.7***	7±4.3 & ***
In RBC			
IS (pmol/µg of protein)	ND	0.3±0.4 **	0.16±0.17 **
pCS (pmol/µg of protein)	0.16±0.12	2.44±1.89 *	0.42±0.28 &
IAA (pmol/µg of protein)	ND	0.16±0.18 **	ND &

Effect of hypoxia on Ca^{2+} content

S-HD promoted an increase in Ca^{2+} concentration in CON-RBC, and this effect was amplified in 5% O_2. Surprisingly, KETO increased Ca^{2+} in CON-RBC incubated in 21% O_2 and had no impact on CON-RBC in 5% O_2. When CON-RBC were incubated with S-HD, KETO reduced Ca^{2+} concentration in both 21% O_2 and 5% O_2 conditions. The same experimental design was performed using HD-RBC. S-CON diminished intracellular Ca^{2+} concentrations in normoxic HD-RBC. In HD-RBC, 5% O_2 incubation did not change Ca^{2+} levels when compared

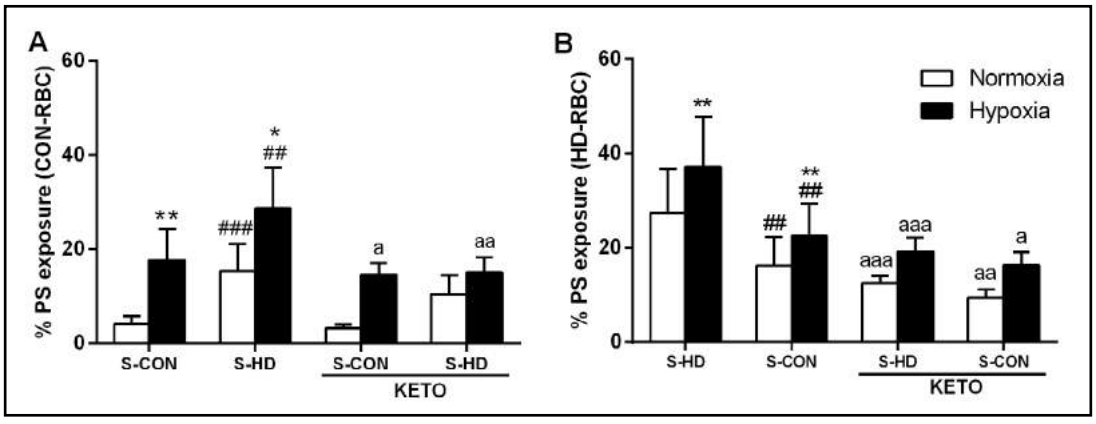


Fig. 4. Phosphatidylserine exposure in response to hypoxia, uremia, and UT entry inhibitor. Isolated RBCs from healthy individuals (CON-RBC, n=6) and patients pre-HD (Pre/HD-RBC, n=6) were incubated for 24h under normoxic (21% O_2) or hypoxic (5% O_2) conditions, in the presence or absence of the UT entry inhibitor ketoprofen (KETO). All treatments contain 10% serum from the patient-healthy subject pair, which can be pre hemodialysis serum (S-HD) or healthy individuals' serum (S-CON). Data are expressed as mean fluorescence intensity (MFI). ** means p<0.01 and *** p<0.001 comparing normoxia vs hypoxia; "a" means p<0.05, "aa" p<0.01 and "aaa" p<0.001 comparing KETO group vs no KETO group; ## means p<0.01 and ### p<0.001 comparing autologous serum vs S-HD or S-CON.

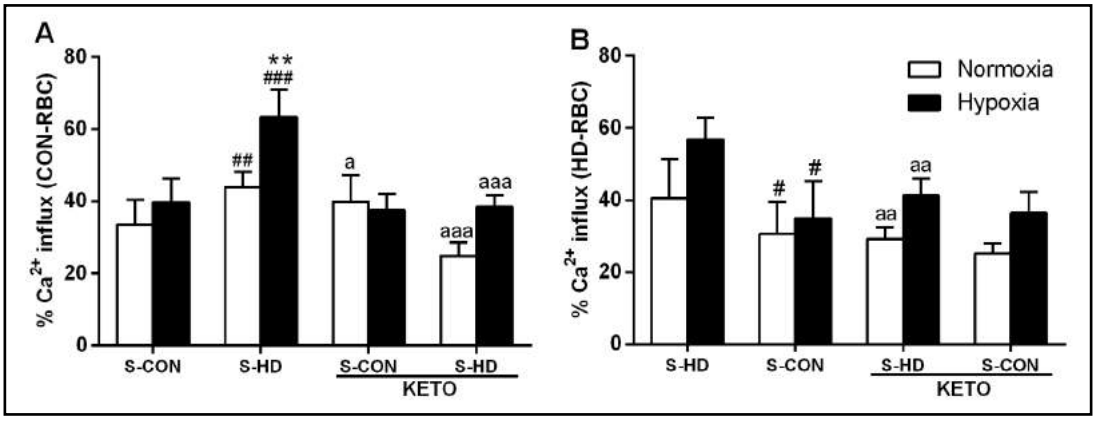


Fig. 5. Ca^{2+} influx into RBCs in response to uremia, hypoxia, and UT entry inhibitor. Isolated RBCs from healthy individuals (CON-RBC, n=6) and patients pre-HD (Pre/HD-RBC n=6) were incubated for 24h under normoxic (21% O_2) or hypoxic (5% O_2) conditions, in the presence or absence of the UT entry inhibitor ketoprofen (KETO). All treatments contain 10% serum from the patient-healthy subject pair, which can be pre hemodialysis serum (S-HD) or healthy individuals' serum (S-CON). Data are expressed as mean fluorescence intensity (MFI). ** means p<0.01 comparing normoxia vs hypoxia; "a" means p<0.05, "aa" p<0.01 and "aaa" p<0.001 comparing keto group vs no keto group; # means p<0.05, ## means p<0.01 and ### p<0.001 comparing autologous serum vs S-HD or S-CON.

to the same treatment in 21% O_2. In the presence of KETO, HD-RBC treated with S-CON presented no difference in Ca^{2+} levels. However, in HD-RBC incubated with autologous serum, KETO reduced Ca^{2+} content (Fig. 5).

Effect of uremia on RBC oxygen level

S-HD *per se* decreased intracellular oxygen levels in CON-RBC as indicated in Fig. 6A, represented by increased intracellular hypoxia (from 15.28±1.7 to 23.49±2.2). As expected, when incubated in 5% O_2, the increase in CON-RBC intracellular hypoxia was more evident (25±6.7), particularly in the presence of S-HD (41.68±8) (Fig. 6A). The intracellular oxygen

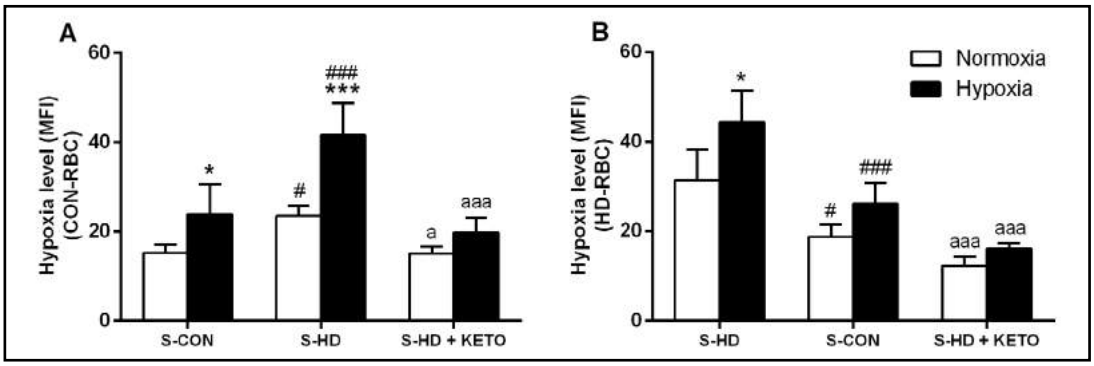


Fig. 6. Intracellular hypoxia level in response to uremia, hypoxia, and UT entry inhibitor. Isolated RBC from healthy individuals (CON-RBC, n=6) and patients pre hemodialysis (HD-RBC n=6) were incubated for 24h under normoxic (21% O_2) or hypoxic (5% O_2) conditions, in the presence or absence of the UT entry inhibitor ketoprofen (KETO). All treatments contain 10% serum from the patient-healthy subject pair, which can be pre hemodialysis serum (S-HD) or healthy individuals' serum (S-CON). Data are expressed as mean fluorescence intensity (MFI). * means p<0.05, ** p<0.01 and *** p<0.001 comparing normoxia vs hypoxia; "a" means p<0.05 comparing keto group vs no keto group; ## means p<0.01 and ### p<0.001 comparing autologous serum vs S-HD or S-CON.

levels in these cells were restored by the incubations with KETO, both in 21% O_2 (15±1.5) and 5% O_2 (19.8±3.8). Additionally, a significant decrease in oxygen levels, and therefore, increased intracellular hypoxia was observed in HD-RBC incubated with S-HD in 21% O_2 (31.33±6.8) and 5% O_2 (44.2±7.0). In the presence of S-CON, the intracellular oxygen levels increased almost 40%, both in 21% O_2 and 5% O_2 conditions (Fig. 6B). It was even more evident in the presence of KETO (12.3±1.9; 16.1±1.1, respectively).

Discussion

In this study, we demonstrated for the first time that HD-RBC had reduced intracellular oxygen levels compared to CON-RBC, particularly after dialysis. Oxygen levels were restored with the inhibition of OAT2 or absence of UT. Moreover, we found that incubation in 5% O_2 and uremia contribute to the increased eryptosis in RBCs from healthy subjects and HD patients *in vitro*.

The participation of eryptosis in the context and progression of different pathologies has been studied in the past decades. Eryptosis was observed in clinical conditions such as diabetes type II [17], liver disease [18], hypertension, and dyslipidemia [19]. In accordance with previous findings, our data shows that eryptosis is higher in RBCs from pre-HD patients compared to healthy subjects [10]. PS expression is accentuated after HD, while the increase in Ca^{2+} levels is observed in pre-HD only [20]. Although it might lead to the interpretation that HD partially regulates Ca^{2+} concentration and eryptosis would be consequently decreased, PS remains highly exposed.

We showed the incubation in 5% O_2 itself enhanced eryptosis in CON-RBC and HD-RBC by exposing PS. In previous studies, S-HD [9] and hypoxia incubation [13] increased PS exposure on healthy RBCs. Here, the highest percentage of PS and Ca^{2+} levels were observed when both stimuli - S-HD and 5% O_2 - were combined. Interestingly, the incubation in 5% O_2 did not augment Ca^{2+} concentration either in CON-RBC or HD-RBC, showing that uremia might play a more critical role in this process. However, previous findings indicated that hypoxia increases intracellular Ca^{2+} content in healthy RBC incubated in media instead of autologous serum [13]. We interpret that the sustained PS exposure caused by hypoxia might have other triggers, such as the accumulation of UT, or Ca^{2+} might occur as an early event that is already regulated after 24 hours of incubation. Clinical evidence suggest that acute and moderate hypoxia might have beneficial results, such as the promotion of erythropoiesis.

On the other hand, chronic and prolonged hypoxemia tend to be pathological [21, 22]. Therefore, studies assessing the effects of hypoxia in different regions of the body are of significant importance. Apart from the main cause of anemia in CKD, which is decreased Epo synthesis and functional iron deficiency [23], we suggest that the increased eryptosis rate and the shortened RBC lifespan [24] reduce the number of circulating RBCs in CKD patients. However, the mechanisms underlying eryptosis in renal anemia and how to correct it are still poorly understood.

In addition to the increased eryptosis by S-HD, isolated UT have been described to trigger eryptosis. IS [10, 11, 13] and acrolein [25] promoted cell shrinkage and PS exposure. Additionally, IS and indole-3 acetic acid (IAA) induced a procoagulant phenotype through microparticles release by RBC [26]. Here, we showed an accumulation of IS, pCS, and IAA in HD-RBC pre-dialysis compared to control. Deltombe et al. measured PBUT in RBC, and they found that the rate of transport of the PBUT in pre-HD RBC increases as follows: HA < IS < pCS < IAA. This corroborates our findings, where we observed that only IAA and pCS, not IS, are reduced after HD, indicating different behaviors among these toxins and high transport capacity for pCS and IAA, respectively [27]. As expected, PBUT serum levels were elevated in pre-HD, and although a visible decrease was found after HD, that still significantly high compared to healthy RBC. This is attributed to the PBUT properties, where the binding to albumin disable their clearance by dialysis membranes [28].

We found that the inhibition of the organic anion transporter 2 (OAT2) – a transmembrane RBC protein [29] – by KETO and the incubation with S-CON have the potential to attenuate eryptosis. HD-RBC incubated with S-CON showed a significant decrease in PS exposure. However, it was not comparable to healthy cells, and hypoxia still increased eryptosis in this condition. This evidence suggests the participation of serum components in RBC death. The reduced PS exposure caused by the inhibition of OAT2 indicates the involvement of UT in the process of eryptosis. Our group's previous findings showed an anti-eryptotic effect of KETO in healthy RBCs incubated with IS [10]. Interestingly, KETO reduced PS in non-uremic RBCs in 5% O_2, showing a protective effect against eryptosis promoted by hypoxia. S-CON and KETO acted independently in reducing Ca^{2+} levels in HD-RBC. Although KETO reduced Ca^{2+} in CON-RBC incubated with S-HD, it showed an opposite effect by increasing Ca^{2+} when these cells were in a healthy environment. Decreasing PBUT entry to levels lower than its physiological concentration might be unfavorable since molecules such as IS are known to be beneficial at normal-physiological levels [30].

Herein, we found that RBC from HD patients have low intracellular oxygen levels than healthy RBCs and that this is particularly accentuated post-dialysis. This important finding leads to the assumption that HD-RBC might have disorders in oxygen metabolism either by intrinsic factors, such as the accumulation of UT, or extrinsic factors, such as the extracorporeal circuit. Indeed, our data from 24 hours incubation supports that S-HD promotes a drop in intracellular oxygen levels in CON-RBC in 21% O_2. The increased intracellular oxygen levels in HD-RBC promoted by S-CON and KETO indicate the crucial role of uremic solutes in developing intracellular hypoxemia. In fact, IS induces abnormal oxygen consumption in renal tubular cells and correlates with renal function deterioration [31]. Also, a connection between IS and impaired Epo production through the desensitization of the oxygen-sensing system in erythropoietin-producing cells was indicated [32]. Experimental analysis revealed that RBC in hypoxia condition show less oxygen binding affinity, probably due to a Hemoglobin conformation modification [33]. Hypoxia was also shown to promote iron release from RBCs and methemoglobin formation, which render RBCs unable to deliver oxygen to the tissues [34].

We recognize that this work has limitations. First, a study evaluating eryptosis in an elderly population found that elderly subjects have higher PS externalization than younger individuals [35]. In the present work, the HD population is significantly older than the healthy population, and therefore some of the findings presented here might have age as a contributor. A second limitation of this study is attributed to the fact that our hypoxia

model (5% O_2) is much more aggressive than the oxygen levels experienced by HD patients. Therefore, further research is needed to better translate the effects of hypoxemia to CKD patients.

Conclusion

In conclusion, we found that HD-RBC have impaired RBC survival by mechanisms of eryptosis and show reduced intracellular levels of oxygen, particularly after the HD session. Also, the incubation in 5% O_2 *per se* promoted an increase in eryptosis in both CON-RBC and HD-RBC. In addition, S-HD decreased intracellular oxygen levels. These effects were partially inhibited by healthy serum and OAT2 inhibition. Together, these findings indicate the importance of uremic toxins in driving eryptosis and aggravating intracellular hypoxemia, possibly contributing to RBC clearance and renal anemia.

Acknowledgements

We would like to thank all patients and healthy subjects that were willing to donate blood samples to the study. We also thank the staff from the dialysis center at the Hospital Santa Casa de Curitiba, in special Dr. Ana Claudia Dambiski. Finally, the authors thank Prof. Márcia Olandoski from PUCPR, who was essential in advising the statistical analysis.

Author Contributions

Dias, G.F. performed the experiments and prepared the manuscript. Tozoni, S. S., Bohnen, G., Rodrigues, S.D., Meireles, T. and Nakao, L.S. performed the experiments and reviewed/edited the manuscript. Pecoits-Filho, R. and Grobe. N. reviewed/edited the manuscript. Kotanko, P. and Moreno-Amaral, A.N. conceived the idea and reviewed/edited the manuscript. All authors participated in discussions during the progress of the study.

Funding Sources

This study was funded by Renal Research Institute, NY, USA. Dias, G.F. was a recipient of research scholarship from the Fundação Araucária/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES). Bohnen, G. and Tozoni, S. S. were recipients of research scholarship from CAPES.

Statement of Ethics

The study was approved by the ethics committee of Pontifícia Universidade Católica do Paraná (registration number 1.752.213). Participants gave written informed consent before blood collection.

Disclosure Statement

Dias, G.F. has been a consultant for Renal Research Institute, NY, USA. Kotanko, P. is an employee of the Renal Research Institute, a wholly owned subsidiary of Fresenius Medical Care; he holds stock in Fresenius Medical Care. The other authors have no conflicts of interest to declare.

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Article 2: (submitted to the Journal Biochimica et Biophysica Acta): “Effect of hypoxia and uremia on oxidative stress on erythrocytes from hemodialysis patients”

Effect of hypoxia and uremia on oxidative stress on erythrocytes from hemodialysis patients

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HIGHLIGHTS

- HD-RBC (red blood cells from hemodialysis patients) overexpress heme oxygenase-1 (HO-1)
- High superoxide dismutase activity and HO-1 levels do not prevent the elevated ROS seen in HD-RBC
- HD-RBC show higher ROS and lower GSH levels than healthy RBC; HD amplifies ROS production
- HD serum and hypoxia induce ROS generation in healthy and HD-RBC
- Blocking RBC uremic toxins uptake in hypoxemic conditions reduces intracellular ROS levels

ABSTRACT

Background: Oxidative stress (OS) plays an important role in uremia-associated comorbidities, such as renal anemia. Complications experienced by hemodialysis (HD) patients, such as hypoxemia and uremic toxins accumulation induce OS and premature death of red blood cells (RBC). We aimed to characterize reactive oxygen species (ROS) production and antioxidant pathways in HD-RBC and healthy RBC (CON-RBC) and evaluate the role of uremia and hypoxia in these pathways. **Methods:** ROS production, xanthine oxidase (XO) and superoxide dismutase (SOD) activity, glutathione (GSH), and heme oxygenase-1 (HO-1) levels were measured using flow cytometry or spectrophotometry in CON-RBC and HD-RBC (pre and post-HD), at baseline or after 24h incubation with uremic serum (S-HD) and/or hypoxia. Ketoprofen was used to inhibit RBC uremic toxins uptake. **Results:** HD-RBC showed higher ROS levels and lower XO activity than CON-RBC, particularly post-HD. GSH levels were lower, while SOD activity and HO-1 levels of HD-RBC were higher than control. Hypoxia itself triggered ROS production in CON and HD-RBC. Adding S-HD to this condition intensified ROS levels. Inhibition of uremic toxins uptake attenuated ROS of CON and HD-RBC under hypoxia and uremia. CON-RBC in uremia and hypoxia showed lower GSH levels than cells in normoxia and non-uremic conditions. **Conclusions:** Redox mechanisms of HD-RBC are altered and prone to oxidation. Uremic toxins and hypoxia play a role in unbalancing these systems. **General significance:** Hypoxia and uremia participate in the pathogenesis of OS in HD-RBC and might induce RBC death and renal anemia.

Keywords: Uremic toxins, hypoxemia, oxidative stress, anemia, eryptosis

OS: oxidative stress; HD: hemodialysis; RBC: red blood cells; ROS: reactive oxygen species; CON-RBC: red blood cells from healthy subjects; HD-RBC: red blood cells from hemodialysis patients; XO: xanthine oxidase; SOD: superoxide dismutase; GSH: reduced glutathione; HO-1: heme oxygenase-1; S-HD: serum from hemodialysis patients.

1. BACKGROUND

In a balanced redox system, reactive oxygen species (ROS) are tightly regulated to defend the body against infections and participate in cellular signaling pathways [1]. In chronic kidney disease (CKD), the exacerbated ROS production surpasses the buffering capacity of the antioxidant system, resulting in a pro-oxidative state. Oxidative stress is a non-traditional risk factor contributing to CKD progression [2]. Solutes that otherwise would be cleared by the kidneys accumulate in CKD. These uremic retention solutes may cause harmful effects throughout the body, being called uremic toxins. Previous studies found that uremic toxins such as advanced glycation end products are associated with oxidative stress [3]. The primary function of red blood cells (RBC), to deliver oxygen to tissues, is itself a source of ROS production. Therefore, robust antioxidant systems are required to protect RBC from internal and external ROS and avoid premature RBC death or eryptosis [4]. Nevertheless, in hemodialysis (HD) patients, the RBC normal lifespan of ~120 days is reduced to 73.2 ± 17.8 days [5]. Our group has previously shown that RBC from HD patients produce more ROS compared to healthy RBC and that the uremic toxin indoxyl sulfate (IS) induces ROS production in RBC from healthy subjects [6].

Additionally, hypoxemia, a condition frequently observed in HD patients [7] can impact RBC physiology, promote oxidative stress and possibly shorten RBC lifespan [7,8]. We previously showed that hypoxia induces ROS production and eryptosis in healthy RBC [9]. In the present study, we explore additional redox pathways that might be altered by hypoxia and uremic toxicity. The activity of xanthine oxidase (XO), a redox enzyme capable of producing significant amounts of superoxide or hydrogen peroxide, is increased in the plasma of CKD patients [10]. Important defense mechanisms include the enzymes superoxide dismutase (SOD) and heme oxygenase-1 (HO-1). SOD facilitates ROS neutralization through the dismutation of superoxide to hydrogen peroxide, which will be converted to water by other enzymes such as catalase and glutathione peroxidase [11]. HO-1 is a stress-induced enzyme whose primary function is to catalyze free heme degradation, preventing heme toxicity. During the heme cleavage steps, HO-1 releases iron, CO, and bilirubin, products with antioxidant, anti-inflammatory, and anti-apoptotic effects [12]. Besides the enzymatic defense, plasma and cells contain high levels of reduced glutathione (GSH) to protect cellular components from damage caused by ROS. GSH is the major non-enzymatic antioxidant, and it functions as a co-factor or directly donates electrons, thus turning oxidants into less harmful molecules [11].

Given the importance of a balanced redox system to maintain RBC homeostasis, this study aimed to explore levels of ROS, HO-1, GSH and activity of XO and SOD in RBC from healthy subjects and HD patients pre- and post-dialysis. We hypothesized that uremia and hypoxia are key players in the development of oxidative stress, that can lead to RBC death and reduced lifespan.

2. METHODS

2.1. Subjects and blood samples

The study was approved by the ethics committee of Pontifícia Universidade Catótica do Paraná (registration number 1.752.213) and all participants gave written informed consent before blood collection. Demographics and laboratory data were obtained from the medical record closest to the blood draw date. Healthy subjects were without a history of renal or inflammatory diseases and did not receive anti-inflammatory medication or blood transfusion one month prior to blood draw. All patients were undergoing high-flux HD for at least 3 months prior to enrollment. Venous blood from HD patients pre (n=17) and post HD session (n=12) and from healthy subjects (n=14) was drawn into tubes containing 3.2% sodium citrate for RBC isolation and SST tubes for serum isolation. Serum was isolated from whole blood by centrifugation (3000 rpm, 15 min, 4°C) and stored at -80°C or immediately used in the experiments. For RBC isolation, whole blood was centrifuged (3000 rpm, 15 min, 4°C), and the plasma and buffy coat was discarded. The isolated RBC were washed twice with cold phosphate-buffered saline (PBS) (1500 rpm, 10 min, 4°C) and submitted to the incubations or immediately analyzed.

2.2. RBC incubation

The incubations were performed as described by Dias et al., 2021. Briefly, RBC from HD patients and healthy subjects were incubated for 24 hours under hypoxic conditions with 5% O_2 and 5% CO_2 using the controllers ProOx 110 and ProCO_2 120 (Biospherix, Redfield, NY, USA). Normoxic cells were incubated with 21% O_2 and 5% CO_2. The effect of uremia was tested by adding 10% healthy (S-CON) or HD (S-HD) serum. A randomly assigned healthy subject formed a patient-donor pair. Before every experiment, new patient-donor pairs were selected. Healthy RBC (CON-RBC) were incubated with autologous (S-CON) or S-HD serum from a randomly assigned patient. RBC from HD patients (HD-RBC) were incubated with autologous serum (S-HD) or healthy serum (S-CON) from a randomly assigned healthy subject **(Figure 1)**.

Serum was diluted with Tris-Glucose-BSA buffer (composition [in mM]: 21.0 tris [hydroxymethyl] aminomethane; 4.7 KCl; 2.0 CaCl2, 140.5 NaCl; 1.2 MgSO4, 5.5 glucose, and 4 g/dL of bovine serum albumin [Sigma-Aldrich, St. Louis, MO, USA] at pH 7.4). Additionally, we incubated RBC for 24 hours with or without 30 µM ketoprofen (KETO), an organic anion transporter 2 (OAT2) inhibitor [13], blocking the entry of uremic toxins.

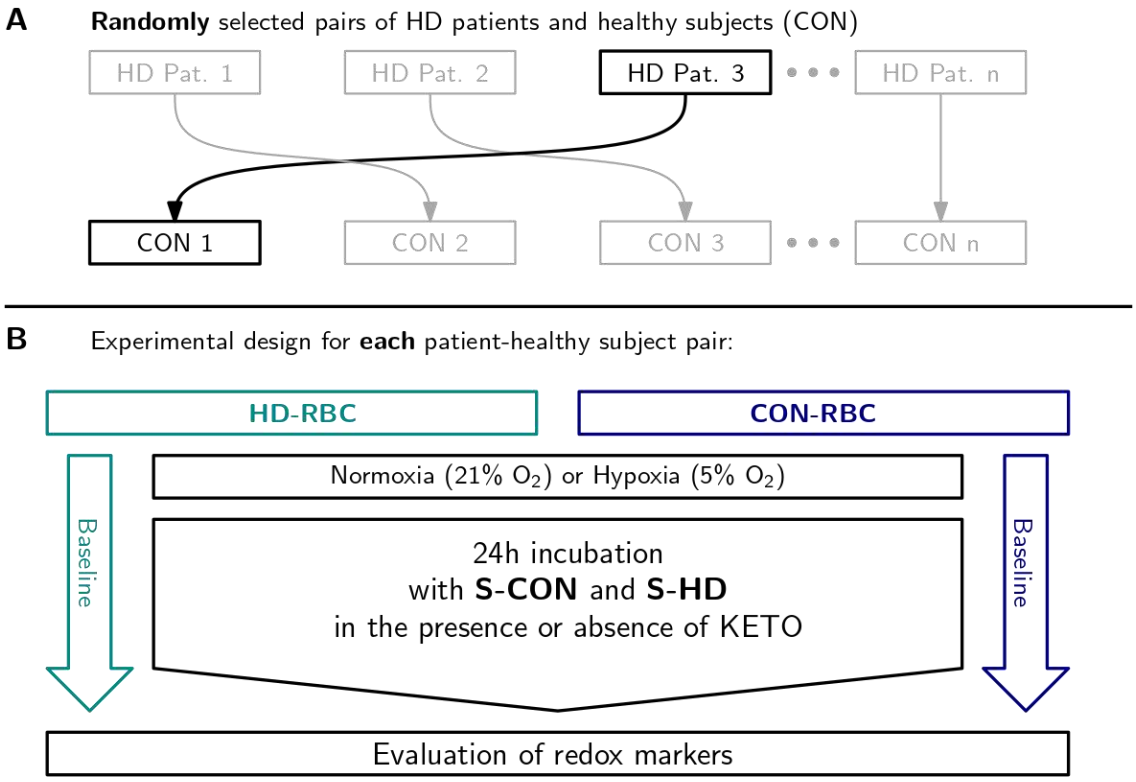

Figure 1. Schematic overview of RBC incubations was adapted from Dias et al. 2021. Panel **A**: A HD patient (HD Pat.) randomly assigned to a healthy subject (CON) forming patient-CON pair. Panel **B**: Incubation with HD serum (S-HD) or healthy serum (S-CON) were used in HD-RBC and CON-RBC separately in the respective patient-CON pairs. After 24h incubation with or without ketoprofen (KETO) using 5% O_2 or 21% O_2, redox markers were evaluated. Baseline concentrations were evaluated immediately after blood collection.

2.3. Quantification of intracellular ROS levels

To estimate intracellular ROS levels, we stained RBC with 2 mM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Abcam, Cambridge, MA, USA) for 30 minutes in the dark at 37°C. RBC were then washed with PBS and fixed with 200 µl of PBS 4% formaldehyde (FixFACS). The analysis occurred using flow cytometry (FACS Calibur BD Bioscience, Sparks, MD, USA).

2.4. Measurement of intracellular XO activity

XO activity was measured using the Amplex Red Xanthine/Xanthine Oxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Briefly, 50 µl of RBC (100% hematocrit) were lysed using 500 µl of 4 mM diethylene triamine pentaacetic acid (DTPA) diluted in dH_2O. From this step on, all procedures were conducted on ice and protected from light. We performed a XO standard curve from 0 to 10 mU/mL for further calculations. 4 µl of the cell lysate were added in 100 µM of Amplex Red reagent containing 0.4 U/mL HRP and xanthine. 1X reaction buffer was used as a negative control. The reaction occurred for 30 minutes at 37°C, and the absorbance was read at 560 nm on the VersaMax microplate reader with SoftMax Pro Software (Molecular Devices, San Jose, CA, USA). All samples were performed in duplicates and normalized by the Bradford protein assay (Bio-Rad, Milan, Italy) [15], using bovine serum albumin as standard. Absorbance was read at 595 nm using the microplate reader mentioned above.

2.5. Evaluation of reduced glutathione (GSH)

GSH quantification was carried out using 10 µM of Thiol Tracker Violet (Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes in the dark, at 37°C. After washing with PBS, RBC were resuspended in 200 µl Fix-FACS. The mean fluorescence intensity (MFI) was analyzed by flow cytometry (FACS Calibur BD Bioscience, Sparks, MD, USA).

2.6. Assessment of intracellular superoxide dismutase activity

SOD activity was tested with the Assay Kit-WST 19160 (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. The principle of this assay consists of the reduction of WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt) with a superoxide anion, producing a formazan dye. RBC were lysed as described for XO. The absorbance was read at 450 nm on the VersaMax microplate reader with SoftMax Pro Software (Molecular Devices, San Jose, CA, USA). All samples were duplicates. The SOD activity (inhibition rate) was calculated through the equation:

$$\text{inhibition rate } \% = \frac{(\text{blank}_1 - \text{blank}_3) - (\text{sample} - \text{blank}_2)}{\text{blank}_1 - \text{blank}_3} \times 100$$

Blank 1 contains the enzyme working solution and water; blank 2 contains the RBC sample and dilution buffer; blank 3 contains water and dilution buffer; sample consists of RBC sample and enzyme working solution. WST was added to all plate wells.

2.7. HO-1 measurement

To verify the presence of HO-1 on the cell membrane, freshly isolated RBC were incubated with 5 µg/mL of anti-HO-1 antibody (Abcam, Cambridge, MA, USA) for 30 minutes, washed once, and incubated for 30 minutes with 1 µg/mL secondary antibody m-IgGκ BP-CFL 488 (Santa Cruz Biotechnology, Dallas, TX, USA). After incubation, RBC were rewashed and fixed with 200 µL Fix-FACS. Samples were analyzed by flow cytometry.

2.8. Data analysis

The results are presented as mean ± SD or median (interquartile range). SPSS Statistics version 20 (Chicago, Ill, USA) and R version 4.0.4 were used for statistical analysis. The graphs were created using GraphPad Prism 5 (La Jolla, CA, USA) and Python's library Matplotlib [16]. We evaluated the data distribution using the Shapiro-Wilk test. Multiple comparisons between groups were done by one-way ANOVA and post hoc least significant difference test. Paired data were analyzed using paired samples t-test. A $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Clinical characteristics of study subjects

The clinical characteristics of the healthy subjects (n=14) and HD-patients (n=17) are shown in Table 1. HD-patients were older and predominantly male compared to healthy subjects (59.1±18.8 vs. 34.2±16 years). The CKD etiology constituted mostly hypertension (35.2%) and diabetes type II (35.2%). Hemoglobin levels from HD patients were low compared to healthy subjects (10.7±2.1 vs. 13.9±0.9) **(Table 1)**.

Table 1. Baseline Characteristics of Participants.

Parameters	Healthy Subjects (n=14)	HD Patients (n=17)
Demographics		
Age (years)	34.2±16	59.1±18.8*
Female (%)	71	35.2*
Caucasians (%)	86	76.4
BMI (kg/m^2)	22.9±2.6	24.4±3.4
CKD Etiology		
Hypertension, N (%)		6 (35.2)
Type 2 Diabetes %		6 (35.2)
Obstructive uropathy %		1 (5.8)
Chronic glomerulonephritis %		1 (5.8)
Polycystic kidney %		1 (5.8)
Uncertain %		2 (11.7)
Biochemical Parameters		
Urea [b] (mg/dl)	22.6±1.1	103.2±19.6***
Post HD urea (mg/dl)		30.6±11.4[a]
Creatinine (mg/dl)	0.8±0.05	8.2±2.7***
Hb (g/dl)	13.9±0.9	10.7±2.1**
Treatment Parameters		
Dialysis vintage (months)		41.1±36.5
Treatment time (minutes)		214.9±20
Kt/V		1.73±0.25
EPO (units per week)		6909±4036
Intravenous iron (mg per treatment)		10.1±3.6

Data expressed as mean ± SD, or binary variables (frequency). BMI = Body mass index; Hb = hemoglobin; HD = hemodialysis; EPO = epoetin alfa. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to healthy subjects; [a] $p<0.001$ compared to pre-HD. [b] Pre-dialysis in HD patients.

3.2. Baseline ROS production and XO activity

We observed a 4-fold increase in ROS levels from immediately analyzed RBC pre-HD compared to CON-RBC (**Figure 2**). The highest level of ROS was found in RBC post-HD, 6.6-fold higher than CON- RBC and 1.5 higher than pre-HD-RBC. XO activity was comparable between CON-RBC and RBC pre-HD, having its activity reduced only in RBC post-HD compared to CON-RBC.

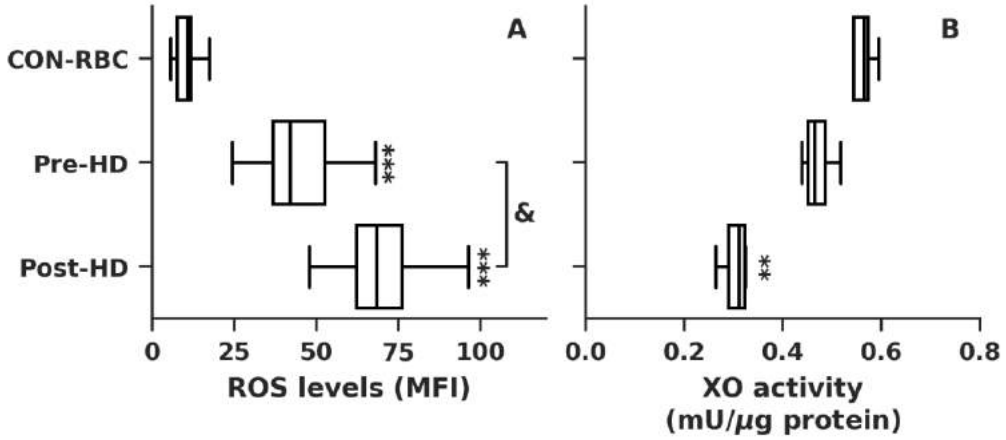

Figure 2. **(A)** Levels of reactive oxygen species (ROS) in RBC from healthy subjects (CON-RBC, n = 14) and CKD patients pre hemodialysis (Pre-HD, n = 17) and post hemodialysis (Post-HD, n = 12). ROS levels were obtained using the probe DCFH-DA and analyzed by flow cytometry. **(B)** Xanthine oxidase (XO) activity from CON-RBC (n = 4), pre-HD RBC (n = 4) and post-HD RBC (n = 4) was measured using the Amplex Red Xanthine/Xanthine Oxidase Assay Kit, analyzed with a microplate reader. Data are expressed as mean fluorescence intensity (MFI) and enzyme activity normalized by protein content. ***p<0.001 and **p<0.01 compared to CON-RBC. & means p<0.05 comparing pre *vs.* post HD.

3.3. Baseline levels of antioxidant markers

The MFI of GSH baseline in pre-HD (11± 0.8) and post-HD (12.3±3) was reduced compared to CON-RBC (37.2±7.3). Intracellular GSH did not differ between the start and end of HD **(Figure 3-A)**.

SOD activity was measured as the ability to inhibit the formation of formazan, which depends on superoxide production. As illustrated in **Figure 3-B**, there was an increased % of inhibition (*i.e.*, increased SOD activity) in pre-HD RBC comparing to CON-RBC. There was no difference in post-HD RBC compared with pre-HD RBC and CON-RBC.

HO-1 was determined by the binding of the HO-1 antibody on the RBC surface. We observed elevated levels of HO-1 on RBC from HD patients, either before or after dialysis, when compared to CON-RBC. No significant change was found in pre-HD *vs.* post-HD (**Figure 3-C**).

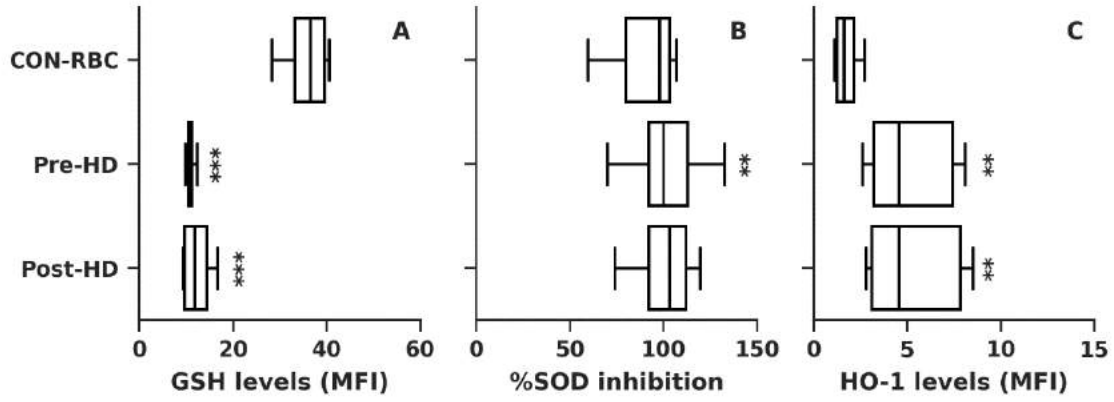

Figure 3. **(A)** Levels of intracellular reduced glutathione (GSH) in RBC from healthy subjects (CON-RBC, n=6) and CKD patients pre hemodialysis (Pre-HD, n=14) and post hemodialysis (Post-HD, n=8). GSH levels were measured using the probe Thiol Tracker Violet analyzed by flow cytometry. **(B)** Superoxide dismutase inhibition rate in CON-RBC (n = 10), pre-HD RBC (n = 8) and post-HD RBC (n = 8). The SOD inhibition rate was obtained using SOD Assay Kit-WST 19160 analyzed with a microplate reader. **(C)** Heme Oxygenase-1 (HO-1) levels on the membrane of CON-RBC (n = 6), pre-HD RBC (n = 6) and post-HD RBC (n = 6). Cells were stained with HO-1 antibody and analyzed by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) for GSH and HO-1 and as % of SOD inhibition. ** means p<0.01, and *** means p<0.001 compared to CON-RBC.

3.4. ROS levels in response to hypoxia and uremia

We measured ROS levels in CON-RBC and HD-RBC after 24h incubation in uremic and hypoxic conditions. CON-RBC incubated with serum from HD patients (S-HD) showed higher levels of ROS in normoxia (17.7±3.8%) than cells incubated with healthy serum (S-CON). In a non-uremic hypoxic environment, CON-RBC produced elevated levels of ROS, from 5.5±2.3% in normoxia to 10.8±3.1% in hypoxia (**Figure 4-A)**. The combination of S-HD and hypoxia resulted in 27.3±6.3% of ROS production in CON-RBC, the most pro-oxidative condition tested in CON-RBC. In addition, we found that the uremic toxin uptake inhibitor KETO reduced ROS levels in CON-RBC under the uremic hypoxic condition to 19.4±8.9%. Conversely, KETO increased ROS levels of hypoxic CON-RBC under non-uremic conditions (**Figure 4-A**). HD-RBC incubated for 24h with S-HD in normoxia showed 35.6±10.7% of

ROS production, while the same cells in hypoxia had 56.1±2.6% (**Figure 4-B**). Interestingly, S-CON and KETO reduced ROS levels of HD-RBC when in hypoxia (**Figure 4-B**).

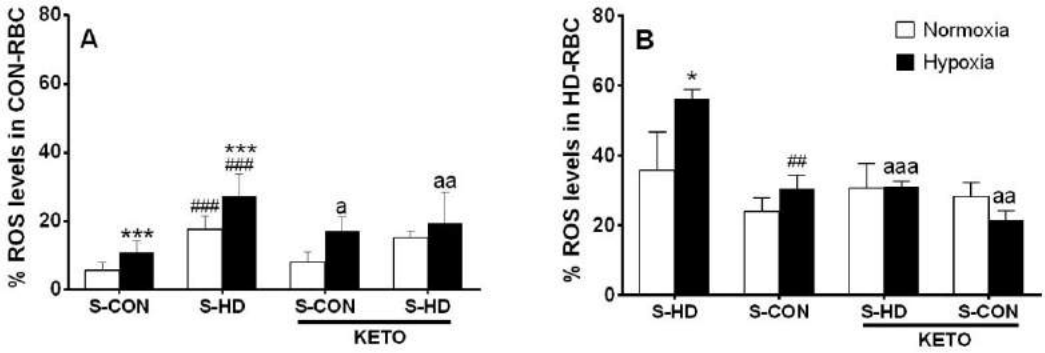

Figure 4. Levels of reactive oxygen species (ROS) in RBC in response to uremia, hypoxia, and uremic toxins entry inhibition with KETO. Isolated RBC from healthy individuals (**A**: CON-RBC, n=10) and patients pre hemodialysis (**B**: HD-RBC, n=4) were incubated for 24h under normoxic (21% O_2) or hypoxic (5% O_2) conditions, in the presence or absence of KETO (n=6). All treatments contained 10% serum from the patient-healthy subject pair, which can be pre hemodialysis serum (S-HD) or healthy individuals' serum (S-CON). Data are expressed as a percentage of positivity to the DCFH-DA probe, analyzed by flow cytometry. * means $p<0.05$, and *** means $p<0.001$ comparing normoxia *vs.* hypoxia; "a" means $p<0.05$, and "aa" $p<0.01$ comparing the presence *vs.* absence of KETO; ## means $p<0.01$, and ### means $p<0.001$ comparing autologous serum *vs.* S-HD or S-CON.

3.5. GSH levels in response to hypoxia and uremia

Levels of GSH were measured in CON-RBC after 24h incubation in hypoxia and uremia. Hypoxia played a role in elevating GSH under a non-uremic hypoxic condition, from 23.5±2% in normoxia to 28.6±3.3% in hypoxia (**Figure 5-A**). On the other hand, the presence of S-HD reduced GSH levels in either normoxia (15.8±0.5%) or hypoxia (18.3±1.5%). The addition of KETO reduced antioxidant levels when under uremia and normoxia (**Figure 5-A**). HD-RBC incubated for 24h in hypoxia had lower GSH levels than the same cells in normoxia (**Figure 5-B**). The incubation with S-CON did not change GSH levels in normoxia but showed a slight increase in hypoxia. KETO increased GSH levels of HD-RBC incubated with S-HD in hypoxia but had the opposite effect in normoxia in the incubation with both S-CON and S-HD (**Figure 5-B**).

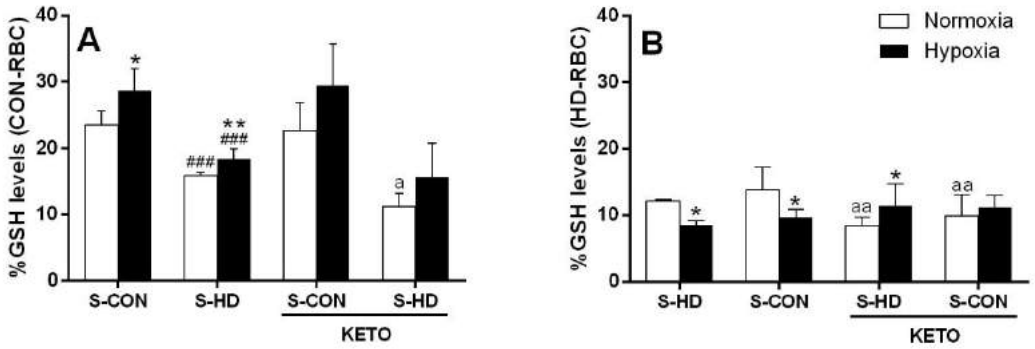

Figure 5. Levels of intracellular reduced glutathione (GSH) in RBC in response to uremia, hypoxia, and uremic toxins entry inhibition with KETO. Isolated RBC from healthy individuals (**A**: CON-RBC, n=6) and patients pre hemodialysis (**B**: HD-RBC, n=4) were incubated for 24h under normoxic (21% O_2) or hypoxic (5% O_2) conditions, in the presence or absence of KETO (n=6). All treatments contained 10% serum from the patient-healthy subject pair, which can be pre hemodialysis serum (S-HD) or healthy individuals' serum (S-CON). Data are expressed as a percentage of positivity to the Thiol Tracker probe. * means $p<0.05$, and ** means $p<0.01$ comparing normoxia *vs.* hypoxia; "a"means $p<0.05$, and "aa" $p<0.01$ comparing the presence *vs.* absence of KETO; ### means $p<0.001$ comparing autologous serum *vs.* S-HD or S-CON.

4. DISCUSSION

In this study, we aimed to characterize some components of the RBC redox system from HD patients and their response to hypoxia and uremia. Although several studies analyzed redox enzymes and glutathione in RBC in this population, it is still poorly defined [6,17–19].

First, we characterized the overall ROS production in RBC from a healthy and HD patient population. We found that (i) HD-RBC contain higher levels of ROS compared to CON-RBC, and (ii) ROS production is amplified after HD. The impaired redox balance in HD patients has been investigated extensively for many years and has been associated with morbidity and mortality in this population [2,20]. The contribution of oxidative stress to CKD was shown by its correlation with the level of renal function [21]. In previous work, our group demonstrated the increased ROS generation in pre-HD RBC and CON-RBC incubated with the uremic toxin IS [6]. The chronic inflammatory state of HD patients - generated in part by dialysate constituents, central venous catheters and bioincompatible components of the extracorporeal circuit - triggers oxidative stress pathways, such as activating NADPH

oxidase [22]. This may help explain the RBC ROS increase post-HD shown in the present study. Contrary to our findings, Abed et al. (2014) did not find a significant difference in RBC ROS levels between pre and post-HD [23]. High levels of RBC ROS in CKD may affect RBC rheology, as shown by increased membrane osmotic fragility and decrease of cytoskeleton protein-protein interaction [17]. Other oxidative stress markers such as malondialdehyde – a lipid peroxidation product – are increased in kidney patients and increase with dialysis vintage [24]. The imbalanced redox state of HD-RBC correlates with phosphatidylserine exposure and may lead to eryptosis, possibly aggravating renal anemia [25,26].

We hypothesized that XO could be a potential source of superoxide and hydrogen peroxide in HD-RBC, playing a role in the elevated ROS levels. However, our data suggest a reduced XO activity pre-HD. In addition, a statistically significant decrease was found post-HD when compared to CON-RBC. It was shown that eryptosis induced by the cholesterol metabolism products 7-keto- cholesterol and cholestane-3beta, 5alpha, 6beta-triol (TRIOL) is mediated mainly by NADPH oxidase and nitric oxide synthase, with a complementary action of XO [27]. Miric et al. showed an increase in XO activity in serum of HD patients. They suggested that XO could mediate oxidative damage and inflammation in HD patients [28]. However, liver and kidneys from 5/6 nephrectomized rats showed a lower XO activity compared to controls, while the oxidant-producing enzyme NADPH oxidase was highly expressed [29]. We speculate that other enzymes such as NADPH oxidase may play a more critical role in RBC ROS genesis [6].

Our second goal was to understand the impact of hypoxia and uremic toxins on the overall RBC redox state. It is well known that hypoxia is involved in the ROS generation of RBC [30]. We have shown recently that hypoxia and IS promote eryptosis and alter the RBC redox system by increasing ROS formation and decreasing GSH content [9]. Here, we highlight the role of hypoxia in the establishment of oxidative stress and show for the first time that uremic serum potentiates these effects. This finding is particularly interesting for HD patients, since arterial O_2 saturation can drop below 90% for more than 1/3 of treatment time in about 10% of patients [7], promoting increased RBC ROS production [31]. Interestingly, OAT2 inhibition - and consequently uremic toxin uptake by the cell [32] - ameliorated oxidative stress promoted by hypoxia in both CON-RBC and HD-RBC incubated with S-HD, showing that uremic toxins in a hypoxemic scenario might mediate ROS generation. Incubation of hypoxic HD-RBC with S-CON had a similar effect, suggesting that components in the uremic serum may play a role in oxidative stress development.

RBC are responsive to their external environment, thus, are susceptible to the oxidants and toxins circulating in the plasma of CKD patients. To counteract this toxicity, their cytoplasmatic redox balance is maintained by a number of enzymatic and non-enzymatic antioxidant defense mechanisms [33]. We previously described that GSH levels in pre-HD RBC are reduced by ~50% [6]. The present study confirms this finding and shows that the GSH decline is sustained post-HD. In the study of Khazim et al., no GSH alteration was found in HD-RBC compared to CON-RBC, but the ratio of GSH and its oxidized form GSSG was 40% lower in patients, reflecting a loss of the reducing capacity of these RBC [18]. Koca et al. found that total thiol levels are reduced in HD patients [24]. A possible explanation for low GSH in CKD RBC could be an impaired *de novo* biosynthetic pathway [34]. It is known that RBC from elderly subjects have lower GSH levels [35]. This represents a limitation of the results obtained here, since the healthy subjects from the control group were significantly younger than the patient group (34.2±16 and 59.1±18.8 years, respectively) (**Table 1**).

Our data suggest that in a non-uremic environment, hypoxia increases GSH levels in CON-RBC (**Figure 5-A**), probably as a protective response to the hypoxic insult. The same does not occur in HD-RBC, where hypoxia decreases GSH levels, demonstrating the inefficiency of the uremic RBC to fight the hypoxic stress (**Figure 5-B**). Opposite to our findings, Rogers et al. demonstrated impairment in the antioxidant status of healthy RBC in hypoxia conditions, but incubated for a short period of time (25 minutes) [36]. In the presence of S-HD, however, CON-RBC in both normoxia and hypoxia showed reduced GSH levels. Tozoni et al. also found an increase in RBC GSH promoted by hypoxia. However, the presence of IS reduced GSH in a dose-dependent fashion [9].

We also assessed the activity of important antioxidant enzymes in HD-RBC. SOD plays a critical role in redox homeostasis by converting superoxide into hydrogen peroxide and preventing oxidation of hemoglobin to methemoglobin, which cannot carry oxygen [33]. We found higher SOD activity in pre-HD RBC compared to CON-RBC. Similarly, another study showed that SOD activity in blood was increased in HD compared to healthy individuals, possibly to compensate the oxidative stress associated with CKD [37]. However, there is evidence that RBC SOD activity is reduced in HD patients, irrespective of HD vintage [24]. Also, SOD was down-regulated in renal and liver tissue of 5/6 nephrectomized rats [29]. Interestingly, Rusu et al. found a positive correlation of SOD and hemoglobin in HD patients and a negative correlation with the rHuEPO responsiveness index [38]. The work

of Aziz et al. showed reduced plasma levels of not only SOD but also glutathione peroxidase and vitamin C and E in RBC from patients with impaired kidney function [39]. In most studies, SOD activity was down-regulated. However, there is still no consensus on the behavior of this enzyme in CKD [40].

HO-1 is a cytoprotective enzyme that prevents the toxic effects of free heme. It is induced upon increased free heme and cellular stress, with relatively low expression in physiological conditions. HO-1 has beneficial characteristics due to the generation of bilirubin and carbon monoxide (CO) - both products with antioxidant properties – through the degradation of free heme [41]. Our data shows that the membrane HO-1 in HD-RBC is increased pre and post-HD compared to CON-RBC. Elevated levels of HO-1 could reflect a defense mechanism against ROS. Despite the protective role of HO-1, its overexpression could also trigger an excessive iron release with consequent iron sequestration within the reticuloendothelial system, aggravating renal anemia. Interesting work from Fraser et al. showed that RBC lifespan is prolonged in HO-1 deficient mice [42]. The high HO-1 levels in HD-RBC may contribute to the short RBC lifespan found in CKD patients [5]. Moreover, a previous report showed that peroxynitrite could react with IS and form 2-nitro-IS *in vitro*, generating higher ROS levels than IS and activating HO-1 in human kidney proximal tubule cells [43]. Also, studies suggest that HO-1 is upregulated in erythroid differentiation and that its deletion could cause a premature release of erythroblasts to the circulation [44,45]. On the other hand, HO-1 overexpression in erythroid cells caused impairment in hemoglobin synthesis [44,45] Taken together, evidence suggests both a protective and harmful role of HO-1. Whether the high content of HO-1 in mature HD-RBC can catabolize heme-associated hemoglobin and contribute to renal anemia remains unclear.

5. CONCLUSION

Understanding the redox state of HD-RBC and its response to stressors such as hypoxia and uremia is of great interest, mainly because RBC are health indicators that could help identify and monitor oxidative stress-related pathologies [46]. In this study, we concluded that the impaired redox state in HD-RBC is partially mediated by the elevated ROS production - which was not induced by XO – and reduced levels of GSH. The high levels of HO-1 in HD-RBC found in this study need further investigation to understand the role of this enzyme in RBC homeostasis and renal anemia. Importantly, we show that uremia and hypoxia are pivotal in developing oxidative stress, which in turn might lead to RBC vulnerability and worsening renal anemia.

Funding

This research was supported by grants from the Renal Research Institute, New York, NY. Dias, G.F. was a recipient of a research scholarship from the Fundação Araucária/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES). Bohnen, G. and Tozoni, S. S. were recipients of research scholarship from CAPES.

Declaration of competing interest

Dias, G.F. has been a consultant for Renal Research Institute, NY, USA. Kotanko, P. is an employee of the Renal Research Institute, a wholly owned subsidiary of Fresenius Medical Care; he holds stock in Fresenius Medical Care. Pecoits-Filho, R. received research grants from Fresenius Medical Care, National Council for Scientific and Technological Development, grants (paid to employer) from Astra Zeneca, Boehringer-Lilly, Novo Nordisk, Akebia, Bayer for participation in advisory Boards and educational activities. Pecoits-Filho, R. is employed by Arbor Research Collaborative for health, who runs the DOPPS studies. Global support for the ongoing DOPPS Programs is provided without restriction on publications by a variety of funders. Funding is provided to Arbor Research Collaborative for Health and not to Dr. Pecoits-Filho directly. For details see https://www.dopps.org/AboutUs/Support.aspx

Acknowledgements

We thank all patients and healthy subjects that were willing to participate in the study donating blood samples. We also thank the staff from the dialysis center at the Hospital Santa Casa de Curitiba, in special Dr. Ana Claudia Dambiski.

CRediT author statement

Gabriela Ferreira Dias: Methodology, Formal Analysis, Validation, Investigation, Writing - Original Draft, Visualization. **Sara Soares Tozoni**: Methodology, Investigation. **Gabriela Bohnen**: Methodology, Investigation. **Nadja Grobe**: Writing - Review & Editing, Supervision. **Lia S. Nakao**: Writing - Review & Editing, Supervision. **Roberto Pecoits-Filho**: Writing - Review & Editing, Supervision. **Peter Kotanko**: Conceptualization, Writing - Review & Editing, Supervision. **Andréa Novais Moreno-Amaral**: Conceptualization, Writing - Review & Editing, Supervision

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CONCLUSION

The present study shows the ability of hypoxia and uremia to promote eryptosis, impair intracellular O_2 metabolism and leave RBC prone to oxidative damage. Also, we show that high concentrations of uremic toxins play an essential role in the translocation of PS, promotion of oxidative stress, and decreasing intracellular O_2 levels. The imbalance between oxidants and antioxidants seen in RBC from hemodialysis patients may induce eryptosis, leading to the accelerated clearance of RBC from circulation and worsening of renal anemia.

Although we found that hypoxia and uremia are triggers of eryptosis, oxidative stress, and low O_2 levels, it is essential to investigate how these stressors can be prevented or controlled in CKD. Nonetheless, eryptosis should be considered a key element in the pathogenesis of renal anemia since it may clear RBC from circulation early in their lifespan, and the formation of new RBC is deficient in many CKD patients. New strategies to control intradialytic hypoxemia and promote an adequate clearance/lower the production of uremic toxins might be fundamental to regulate RBC survival and extend their lifespan. Also, it could improve intracellular O_2, leading to a better O_2 delivery to tissues and amelioration of anemia symptoms.

COMPLIMENTARY ACTIVITIES AND SUPPLEMENTARY MATERIAL

Gabriela Ferreira Dias spent one year (2019-2020) of her doctoral course at Renal Research Institute (RRI), New York, under the supervision of Nadja Grobe, Ph.D., Xia Tao, Ph.D. and Peter Kotanko, M.D. At RRI, she was part of a highly diverse team and worked on several projects that aimed to provide new insights into the nephrology field and improve the quality of life of patients with CKD. During this time, she attended trainings, conferences and learned new laboratory techniques. Some results of this collaboration are shown on the following pages.

Paper published in the journal Kidney 360

Original Investigation

Kidney360

SARS-CoV-2 in Spent Dialysate from Chronic Peritoneal Dialysis Patients with COVID-19

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Abstract

Background To date, it is unclear whether SARS-CoV-2 is present in spent dialysate from patients with COVID-19 on peritoneal dialysis (PD). Our aim was to assess the presence or absence of SARS-CoV-2 in spent dialysate from patients on chronic PD who had a confirmed diagnosis of COVID-19.

Methods Spent PD dialysate samples from patients on PD who were positive for COVID-19 were collected between March and August 2020. The multiplexed, real-time RT-PCR assay contained primer/probe sets specific to different SARS-CoV-2 genomic regions and to bacteriophage MS2 as an internal process control for nucleic acid extraction. Demographic and clinical data were obtained from patients' electronic health records.

Results A total of 26 spent PD dialysate samples were collected from 11 patients from ten dialysis centers. Spent PD dialysate samples were collected, on average, 25±13 days (median, 20; range, 10–45) after the onset of symptoms. The temporal distance of PD effluent collection relative to the closest positive nasal-swab RT-PCR result was 15±11 days (median, 14; range, 1–41). All 26 PD effluent samples tested negative at three SARS-CoV-2 genomic regions.

Conclusions Our findings indicate the absence of SARS-CoV-2 in spent PD dialysate collected at ≥10 days after the onset of COVID-19 symptoms. We cannot rule out the presence of SARS-CoV-2 in spent PD dialysate in the early stage of COVID-19.

KIDNEY360 2: 86–89, 2021. doi: https://doi.org/10.34067/KID.0006102020

Introduction

Currently, individuals suspected of coronavirus disease 2019 (COVID-19) are tested for the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleic acid, mainly in nasopharyngeal-swab, oropharyngeal-swab, nasopharyngeal-aspirate, and bronchoalveolar-lavage specimens. SARS-CoV-2 was also detected in the blood of patients positive for COVID-19 (1,2). Viremia of SARS-CoV-2 has been proposed as a predictor of a severe clinical course (3,4).

Patients with kidney disease who are treated with peritoneal dialysis (PD) are a high-risk, vulnerable population. Previously, several viruses have been detected in spent PD dialysate, including hepatitis C virus and HIV (5,6). Data on the presence or absence of SARS-CoV-2 in spent PD dialysate are scarce; to date, results from only five patients on chronic PD have been reported. SARS-CoV-2 was found to be absent in four patients (7,8) and present in one (9). Therefore, it is currently unclear whether SARS-CoV-2 is present in spent PD dialysate, causing uncertainty and concerns for patients and their caregivers. To address these concerns, we collected and tested serial PD dialysate samples from patients on PD with confirmed COVID-19 for the presence of SARS-CoV-2.

Materials and Methods

Patient Selection

Collection of spent peritoneal dialysate from patients on PD who were positive for SARS-CoV-2 was done as a part of a quality-improvement project. The quality-improvement project was approved by the respective clinic governing bodies after legal and compliance review. Patients on PD who were positive for SARS-CoV-2 were contacted by US Fresenius Medical Care North America (FMCNA) healthcare professionals, who assisted with collecting the spent dialysate. Spent peritoneal dialysate was collected from patients on PD with a confirmed, positive, nasopharyngeal-swab test for SARS-CoV-2 from March until August 2020. Up to five spent PD dialysate samples per patient were collected. Patients received collection kits with

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Conference paper 1 (American society of nephology – kidney week 2020)

PO1171

Tryptophan Removal in ESRD Patients Treated with High-Flux and Medium Cut-Off Dialyzers During Hemodialysis and Hemodiafiltration

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Background: Tryptophan (Trp) loss in kidney failure patients is likely to be associated with poor nutritional status and depletion due to dialysis. However, Trp removal with medium cutoff (MCO) membranes has not been investigated. Here, we compared Trp reduction ratios (RR) between an MCO dialyzer and a high-flux polysulfone (HFPS) dialyzer

Methods: Clinically stable, anuric hemodialysis patients on thrice-weekly HD were enrolled. Over the course of 4 weeks, each subject traversed through the following combinations (with 2 study treatments per week, 4 hours per HD session): post-dilution hemodiafiltration (HDF) with FX120 (Fresenius Medical Care), HD with FX120, HDF with Theranova 400 (Baxter), HD with Theranova 400 (Fig. 1). All subjects exercised using stationary bicycles during HD. Blood samples were collected before dialysis (B0) and at 230 min (B230) upstream of the dialyzer. Trp in plasma was analyzed by liquid chromatography–mass spectrometry. RR was calculated using signal intensities for Trp according to RR=(B0-B230)/B0, with correction for hemoconcentration using hemoglobin levels (Schneditz, ASAIO 2012)

Results: Twelve subjects completed the study (50% female, 43.8±18.5 years old). With HD, RR was comparable between the MCO dialyzer and the larger HFPS dialyzer (median RR 0.29 for MCO, 0.33 for HFPS; surface areas 1.7 m^2 vs. 2.5 m^2, respectively). In HDF, our data suggest somewhat greater Trp loss with the MCO dialyzer despite its smaller surface area compared to the HFPS dialyzer

Conclusions: Use of an MCO dialyzer may result in similar or greater Trp loss as use of an HFPS dialyzer with a much larger surface area. When considering the use of MCO dialyzers, clinicians should consider the potential impact on removal of salutary substances (incl. protein-bound substances), an area that deserves further research

Funding: Private Foundation Support

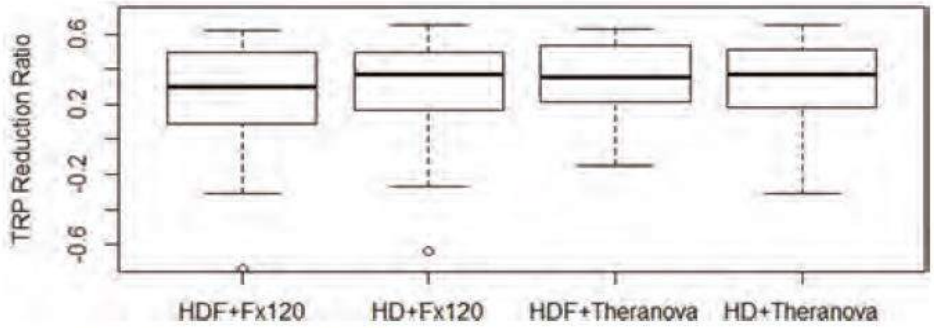

Figure 2. Box-Whisker plot of Trp RR.

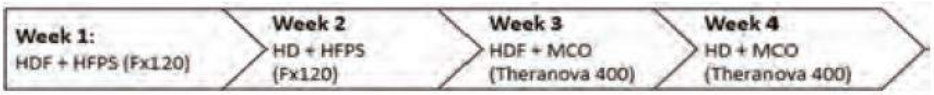

Fig 1. Design of clinical study.

Conference paper 2 (American society of nephology – kidney week 2020)

PO1172

Effect of Hemodiafiltration with Medium Cut-Off Dialyzer on Uremic Toxins Removal

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Background: To our knowledge no study has ever evaluated the use of middle cut-off membranes (MCO) with online hemodiafiltration (OL-HDF). This study aims to show if the combination of OL-HDF with MCO can achieve a higher reduction ratio of some uremic toxins in comparison to regular OL-HDF

Methods: Patients from our hemodialysis unit were treated twice with four different modalities, namely combinations of post-dilutional OL-HDF or hemodialysis (HD) with a high-flux dialyzer (CordiaxFX120, area 2.5 m^2) or the MCO (Theranova 400; area 1.7 m^2), respectively. We analyzed the reduction ratios (RR) of erythropoietin, beta2-microglobulin (B2M), phosphate, and urea.

Results: Twelve anuric patients were studied (6 females; mean age 43.818.5 years; HD vintage 35.2 ± 27.8 months.) Mean blood flow (Qb) was 367 23 ml / min, dialysate flow (Qd) was 493 57 ml / min, ultrafiltration volume was 2382 5683 ml. B2M RR of HDF+HiFlux was higher than HD+MCO **(p=0.003)**, and HDF+MCO vs. HD+MCO **(p=0.029)**. There was no difference in EPO, phosphate, and urea RR between any of the four groups.

Conclusions: Adding a medium cut-off (MCO) dialyzer to HDF does not add benefit. The B2M RR with HiFlux exceeds the one of a MCO dialyzer. HDF provides benefit over HD regarding the B2M RR regardless of the dialyzer used.

Funding: Commercial Support - Fresenius

Reduction (%)	HDF + HiFlux	HD + HiFlux	p*	HDF + MCO	HD + MCO	p*	p value +
EPO[a]	15 (-44-60)	36 (-10 to 48)	0.95	40 (-16 to 53)	10 (1 to 35)	0.52	0.91
B2M[a]	92 (89-93)	88 (86 to 90)	0.018	90 (88 to 92)	85 (77 to 89)	0.005	**0.002‡**
B2M[b]	89 (86-92)	82 (82 to 86)	0.015	88 (83 to 90)	81 (79 to 89)	0.042	**0.023•**
Phos	57 (43-65)	55 (44 to 62)	0.6	53 (36 to 58)	58 (33 to 66)	0.57	0.7
Urea	81 (76-83)	81 (78 to 84)	0.68	83 (78 to 84)	81 (75 to 85)	0.71	0.9

Medians (interquartile ranges) [a]Adjusted to plasma volume, [b]Adjusted to body weight *= Kruskal Wallis test ‡ Dunns comparison test HDF+HiFlux vs. HD + MCO (p=0.003), HDF+ MCO vs. HD+ MCO (p=0.029) • Dunns comparison test HDF+HiFlux vs. HD + MCO (p=0.048)

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