Original Article
Intrinsic Susceptibility of Uremia RBC for Hemolysis

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A R T I C L E   I N F O

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A B S T R A C T

Using in *vitro* system; the compounds sodium dodecyl sulphate , nitrosocysteine, nitrosoarginine, cysteine and salicylic acid caused more than 60% hemolysis of RBC from uremia patients. This hemolysis was concentration dependent and showed more severity on patients with chronic kidney disease than on dialysis group. The IC50 values of hemolytic in RBC of uremia patients were significantly lower (p < 0.05) than their corresponding values in normal controls. Dialysis managed to decrease the potency of RBC hemolysis when compared with patients having chronic kidney disease. All these hemolytic changes were associated with a defect in total antioxidant capacities of uremia groups. However, some radical scavenger agents like trolox, ascorbic acid and uric acid were partially able to protect the uremia RBC from hemolysis. The current in *vitro* system has the potential to assess the susceptibility of uremia RBC for hemolysis, and may help to evaluate the degree of RBC exposure to harmful endotoxins or oxidative agents.

1. Introduction

Anemia is a common complication of patients with chronic kidney disease (CKD) [1] and its severity is well correlated with the extent of renal insufficiency [2 , 3]. In these patients, the incidence of anemia is frequently associated with an increase in blood urea nitrogen concentration [1] or a fall in creatinine clearance level below 20 mL/minute [4].The impairment in renal excretion of uremia patients can retain several toxins [5] that may inhibit erythropoiesis and reduce the RBC life span [2] or rendering cellular membranes more vulnerable for oxidation [6]. A further defect in total antioxidant capacity [7] can aggravate the condition of oxidative stress and provokes vicious cycle of chronic free radicals formation, facilitated by certain renal inflammatory mediators [8]. Insupport of this view, a diminution in plasma of some elements belong to total antioxidantsystem such as reduced glutathione, glutathione peroxidase and superoxide dismutase were significantly detected in CKD patients [9 ,10 , 11] . Also, these patients are characterized by a short lifespan of their red blood cells compared with healthy subjects [12].Dialysis may partially adjust the correction of anemia state, apparently through removal of certain toxic substances including some hemolytic agents [1] while; themanagement of anemia itself could ultimately improve the disease outcomes and delays the progression of uremia patients toward renal end stage [13].

In current work; we examined the vulnerability of uremia RBC for hemolysis by investigating their in vitro exposure to several hemolytic agents. This investigation may help to gain better understanding regarding the factors that trigger the incidence of anemia in uremia patients.

Methods:

Patients:

84 patients attended Al-Karak general hospital in Jordan and they were clinically diagnosed with abnormal renal damage according to routine kidney function tests. They were divided into two groups,55 patients (65%) were under pre-dialysis condition and categorized as a group of chronic kidney disease (CKD). Another 29 patients (35%) had renal failure and they were maintained on hemodialysis treatment(dialysis group). The CKD group included patients who had kidney damage with structural or functional abnormalities that produced prolonged glomerular filtration rate (GFR) of <60 mL/min.

The average age of these patients was 54 years old, with a gender distribution of 56% females and 44% males. Matched individuals (107) with no previous history of kidney diseases were included in this study as normal control.

All participants gave fully informed consent to join in the study, taking in consideration the compliance to ethical criteria issued by Al-karak hospital regulations in Jordan.

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Preparation of human erythrocytes:[14]

Fresh heparinized blood sample from both healthy controls and renal patients were centrifuged under cooled temperature for 5 min at 2 000 r/m. After removing plasma and buffy coat, the collected pellet was washed three times with phosphate buffered saline

(PBS) at pH 7.4 and then re-suspended in PBS buffer to give 10 % erythrocytes suspension.

In vitro RBC hemolysis assay:

The erythrocytes suspension was incubated with different concentrations of the hemolytic agents at 37 °C for 60 min. Aliquots of the hemolyzed mixture were taken at appropriate time intervals, diluted with 0.15 mol/L NaCl, and centrifuged at 2 000 r/m for 10 min. The supernatant was collected and its absorbance was measured spectrophotometrically at 415 nm .The extent of RBC hemolysis by the chemical agent was expressed as a percentage value relative to complete hemolysis of similar erythrocyte sample (blank) carried out in deionized water alone. Solutions of nitroso-amino acids were prepared freshly, as described previously [14].

Antioxidants protection of RBC hemolysis:

For determining the protection effect of antioxidants, 10% of the RBC suspension was pre-incubated at 37 °C with 500μM antioxidant (uric acid or ascorbic acid or trolox) for 30 minutes. After centrifugation, the RBC pellet was re-suspended in 15mM cysteine and the suspension was incubated for one hour before assaying the hemolysis extent as described above. Similar hemolytic test with cysteine but lacking the anti-oxidant was used as a control.

Ferric Reducing/Antioxidant Power (FRAP) assay:

The FRAP assay [15], depends upon the reduction of ferric [Fe (III)-TPTZ] to [Fe(II)- TPTZ] complex that gives intense blue color absorbed at 593 nm. Due to possible interference of the endogenous antioxidants uric acid, it was necessary to consider the level of this interfering substance in plasma during the calculation of total reducing power by the FRAP method. In uremia patients, such discrepancy is particularly aggravated by the increase in tissue destruction which tends to elevate the concentration of uric acid in plasma (data not shown). Hence, we used the ratio Fe II/ uric acid concentration to express total antioxidant capacity by FRAP method instead of using the FeII concentration alone.

All experiments in this investigation were repeated in triplicate and values were expressed as the mean ± SD.

Statistical analysis

The values were expressed as means ± SD using SPSS software for data analysis. One way analysis of variance was used to assess group means and P <0.05 was considered statistically significant. Linear regression analysis was used to calculate the concentration of compound that gives 50% hemolysis of RBC (IC 50).

Results:

Blood chemistry of uremia groups and control subjects

Blood obtained from uremia patients with CKD or under dialysis managements displayed high concentrations of urea; creatinine and uric acid, when compared with similar samples collected from healthy control (Table1). Also, these patients showed abnormal levels of electrolytes, in particular the CKD patients had significantly high concentration of plasma potassium (p < 0.05). The blood hemoglobin (Hb) scored another abnormal deviation, emphasizing the dominant association of anemia state with currently investigated uremia patients.

Susceptibility of uremia RBC for hemolysis:

Screening of hemolytic potency:

The hemolytic potential of various chemical compounds were screened in vitro against fresh preparations of erythrocytes from renal patients (Fig 1). During one hour exposure, the compounds SDS, nitrosocysteine, nitrosoarginine, cysteine, and salicylic acid produced strong hemolysis of more than 60% , but other compounds like spermidine, SNP, arginine , urea and methionine showed weak hemolytic power of less than 10% and their action is not pursued any further.

Comparison of strong hemolytic agents:

Despite their versatile chemical structure, all strong hemolytic agents exhibited distinctive action on uremia RBC when compared with the same hemolysis action on RBC of normal controls (Figs 2-6). This hemolysis was concentration dependent and its severity was more pronounced on RBC of CKD patients than on RBC of dialysis group. Further quantitative assessment of the hemolysis potency was susceptibility achieved by estimating the concentration of hemolytic agent that produces 50 % hemolysis of RBC (IC50 values). Data in table 2 show that IC50 values of all chemical agents determined from the hemolysis of uremia RBC is significantly different (p < 0.05) from similar values of RBC hemolysis in normal controls. This pattern of differential potency by the hemolytic agents was also observed between RBC of CKD patients and those under dialysis. In particular, the cytotoxic agent SDS scored the lowest IC50 values while salicylate expressed the highest IC50 on corresponding RBC of uremia patients.

Total antioxidant capacity (TAC):

Total antioxidant capacity (TAC) in plasma was determined by FRAP method, and expressed as FeII/ uric acid ratio. The highest value of this ratio was markedly detected in plasma of normal subjects followed by dialysis group and then CKD renal patients, respectively (Fig 7).

In vitro Protection of RBC hemolysis:

The in vitro protection of uremia RBC against cysteine hemolysis was attempted using the antioxidants ascorbic acid, uric acid and trolox chemical agents. Each antioxidant agent was pre-incubated with uremia RBC before the initiation of hemolysis by cysteine.A control of cysteine treated RBC sample from uremia patients but lacking the corresponding antioxidant was run in parallel. All tested antioxidants produced a decrease in cysteine hemolysis of renal RBC when compared with the control(Fig 8).The uric acid exhibited the highest potential of hemolysis protection (76 %), followed by ascorbic acid (68%) and then trolox (55%), respectively.

Table 1: Blood chemistry of uremia groups and control subjects

Blood test	Normal control	CKD	Dialysis
Urea	30± 5 mg/dl	193±25 *	144±31 *
Creatinine	1 ± 0.1 mg/dl	9.2±5.3 *	7.9±6.2 *
Na+	140 ± 2 mmol/L	136±2	132±2
K+	4.5 ± 0.5mmol/L	6.5± 1 *	5.9±0.3
Hemoglobin	13 ± 2 g/dl	11±3.5	9.2±2.5*

(*) Indicates a significant difference between patient groups and normal controls

(p < 0.05)

Table 2: IC50 of hemolytic agents

| Chemical compound	Control [a]	Dialysis [a]	CKD [a]
SDS	0.14 ± 0.001	0.053 ± 0.004*	0.048 ± 0.002*
Nitrosocysteine	0.73 ± 0.2	0.64 ± 0.13*	0.59 ± 0.15*
Nitrosoarginine	1.26 ± 0.18	1.020 ± 0.20 *	1 ± 0.10*
Cysteine	13.6 ± 1.3	12 ± 0.5*	11.4 ± 0.7*
Salicylic acid	32.7 ± 1.9	29 ± 1.5*	25 ± 1.7*

(a) IC50 value = mM the concentration of compound that produces 50% RBC hemolysis.

The values expressed as a mean of 10 individuals ± SD

(*) A significant difference between patient groups and normal controls.

Fig. 1 Hemolysis of RBC by various agents: 10% RBC suspension was incubated for 60 min with different concentration of the hemolytic agent. At the end of incubation period the sample was centrifuged and the supernatant absorbance at 415 nm was measured.The extent of hemolysis was expressed as a percentage value of absorbance at 415 nm relative to complete hemolysis of similar sample in deionized water.
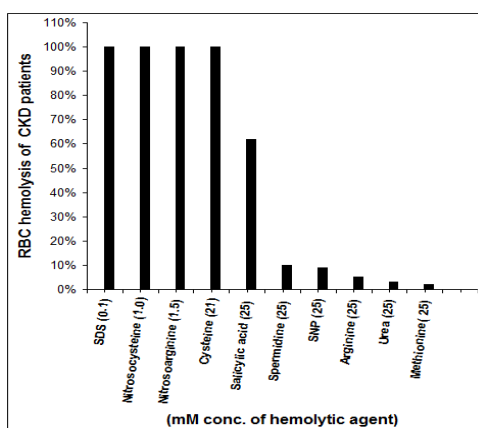
Fig2: RBC hemolysis by salicylic acid
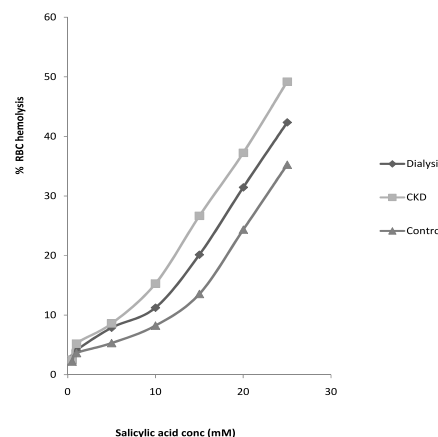
Fig 3: RBC hemolysis by cysteine
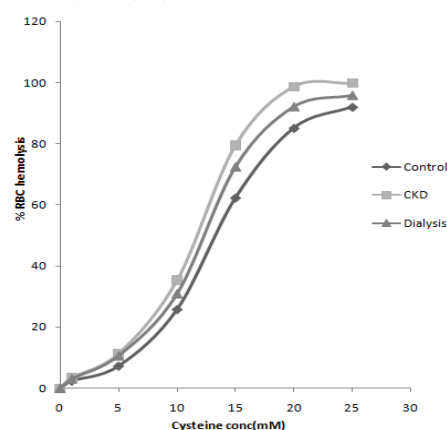
Fig 4: RBC hemolysis by nitrosoarginine
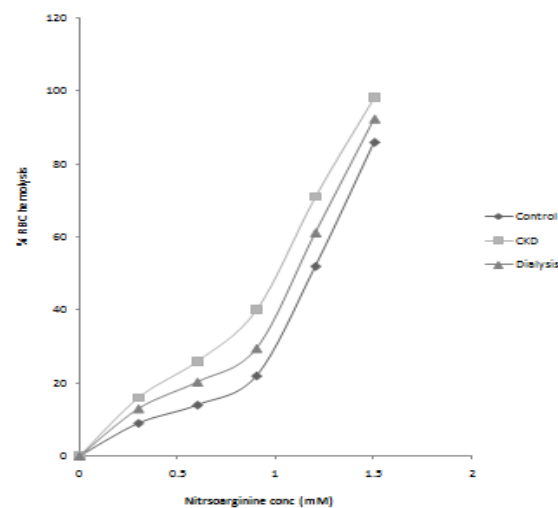
Fig 5: RBC hemolysis by nitrosocysteine
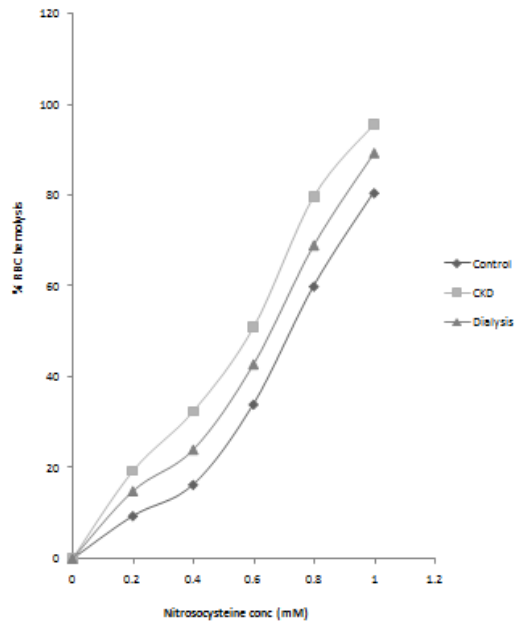
Fig 6:RBC hemolysis by SDS
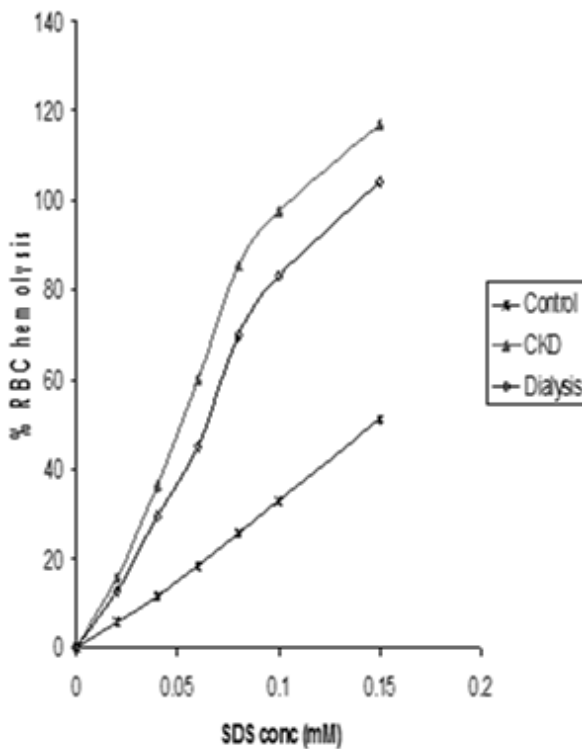
Fig 7: Total antioxidant capacity (TAC): Plasma TAC was measured among normal, CKD and dialysis patients.The antioxidant a capacity was expressed as the mean ± SD values for FeII/Uric acid ratio.
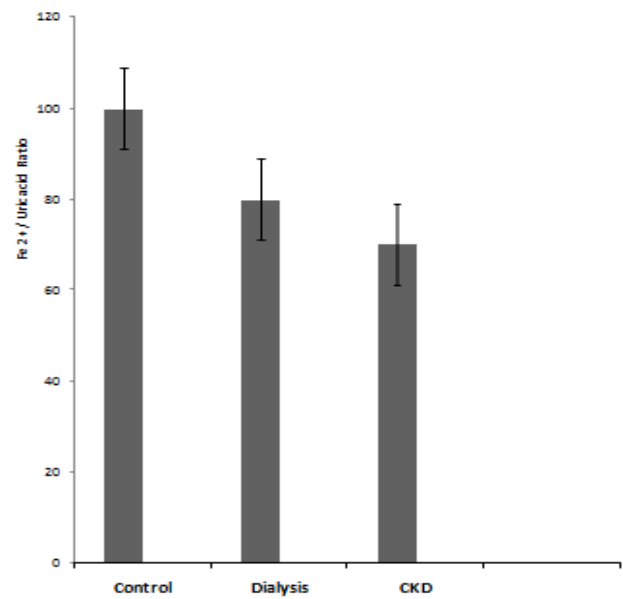
Fig 8: Protection of RBC hemolysis.10% of RBC suspension was pre-incubated for 30 minutes with 500 µM antioxidant (uric acid or ascorbic acid or trolox). After centrifugation, the RBC pellet was re-suspended in 15mM cysteine and the suspension was further incubated for one hour before assaying the hemolysis percentage as described in fig 1. Similar hemolytic test with cysteine but lacking the anti-oxidant was used as a control..Asc aci= Ascorbic acid
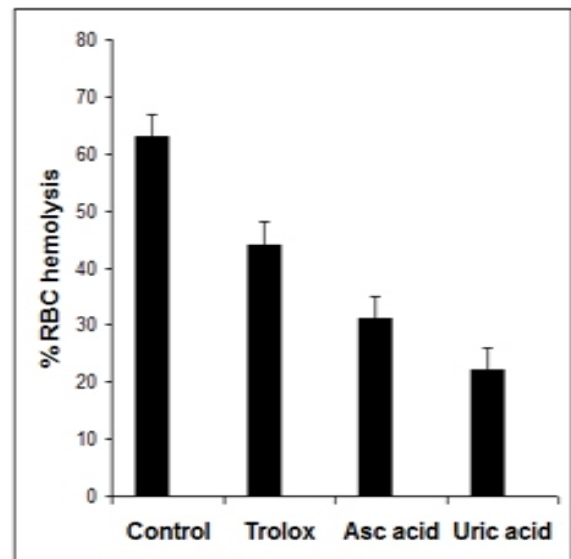
Discussion:

A main objective addressed in present work was to explore possible intrinsic biochemical changes of RBC during their exposure to the stress conditions of uremia.

Our data revealed a distinctive susceptibility of uremia RBC for in vitro hemolysis which was persistent regardless of the variations in type of hemolytic agents or their mode of actions. This hemolytic susceptibility suggests an inherent fragility of uremia RBC probably as an inevitable outcome of their continuous exposure to endotoxins [5]. It is inclined to augment previous reports implying the association of pre-altered rheological properties of RBC with chronic renal failure.[16 , 17].A similar rheological distortion of RBC with increased risks of hemolytic susceptibility have been conveyed with various pathologic conditions such as thalassemia, sickle cell disease, diabetes mellitus and G6PD deficiency [18 , 19, 20].

Another persistent finding observed here, involving the action of dialysis to reduce the uremia RBC susceptibility for hemolysis. This finding emphasizes the correlation of RBC fragility with non-clarified toxins under uremia conditions, since dialysis is considered an important filtering management for clarifying some endotoxins in patients with end stage uremia. [21 , 22].

Sodium dodecyl sulphate showed the most extensive hemolytic effects on uremia RBC investigated in present work. This anionic surfactant can intercalate into the lipid bilayer of RBC membrane and causing hemolysis by altering the erythrocyte cellular shape from biconcave to the echinocyte form [23]. Such hemolysis needs the contribution of surfactant hydrophobic moiety [24, 25] as indicated here by the weak activity of salicylate, which is a similar non polar compound .However, hydrophobicity alone might not be an adequate contributor to the high potency of SDS hemolysis. Iglic et al [23] pointed to an essential role can be played by the polar moiety of anionic amphiphile in the electric repulsion between surfactant negative charge and RBC phospholipids polar group, that can lead to cell shape transformation and initiation of hemolysis. Besides, such cellular transformation can be facilitated by a direct interaction between the SDS and RBC membrane proteins [26].

In renal diseases, oxidative stress is frequently an accomplice to uremia toxicity and both can cause pronounced morphological transformation [6] , [27] which is a pre-request requirement to trigger the hemolysis of RBC [28]. These cells can be shifted towards a state of instability [29] by membrane lipid peroxidation [30] or proteins oxidation or alterations in the cation permeability of uremia RBC [31] .Our data demonstrated a strong hemolytic activity against uremia RBC by the oxidative agents nitrosocysteine and nitrosoarginine,respectively .These NO generating compounds are able to react with plasma membrane thiols through a transnitrosation reaction or cross the RBC membranes and exchange their NO groups [32 , 33]. Under uremia stress, they have the potential to produce peroxidation of protein thiols membrane [34] or induce the formation of methemoglobin [35] and thus rendering RBC more fragile. Unlike these nitroso-compounds; the sodium nitroprusside has failed to produce

significant hemolytic effect on uremia RBC, probably due to its poor ability for generating the appropriate cellular membrane transformations [36].

Numerous disturbances in the metabolism of plasma sulfur amino acids including cysteine have been implicated in patients with chronic kidney disease, making these aminothiol compounds potential candidates for in vivo uremia toxicity [37].

The cytotoxic effect of cysteine is often mediated through reactive oxygen species [38]before targeting the correspondng protein disulphide [37]. Our data indirectly emphasized this oxidative cytotoxic effects of the aminothiol compound, by demonstrating a partial protection of several antioxidant agents such as trolox (vit E);ascorbic acid(vit C) and uric acid against the cysteine hemolysis of uremia RBC. Furthermore, present data augment previous reports regarding the association between low levels of total antioxidant capacity and renal failure [1 , 6] by revealing a clear differences in total antioxidant capacity between uremia and normal groups as well as between CKD and dialysis uremia subgroups, respectively.

Conclusion:

1. RBC of uremia patients is highly prone for hemolysis susceptibility than erythrocytesof normal subjects.

2.Dialysis treatment of uremia patients managed to reduce the RBC susceptibility for hemolysis.

3. The RBC susceptibility for hemolysis in uremia groups is concomitant with a defect in total antioxidant capacity.

4. Some antioxidant can partially protect the hemolysis of uremia RBC.

5. Further work is still needed to correlate the in vitro hemolysis with in vivo hemolysis using animal experimental model.

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