Recharging Red Blood Cell Surface by Hemodialysis

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Abstract
Background: Similar as in vascular endothelium the negatively charged glycocalyx of erythrocytes selectively buffers sodium. Loss of glycocalyx (i.e. loss of negative charges) leads to increased erythrocyte sodium sensitivity (ESS) quantified by a recently developed salt-blood-test (SBT). The hypothesis was tested whether a regular 4-hour hemodialysis (4h-HD) alters ESS. Methods: In 38 patients with end stage renal disease (ESRD) ESS was measured before and after 4h-HD, together with standard laboratory and clinical parameters (electrolytes, acid-base status, urea, creatinine, hemoglobin, c-reactive protein and blood pressure). Results: Before 4h-HD, 20 patients (out of 38) were classified as “salt sensitive” by SBT. After 4h-HD, this number decreased to 11. Erythrocyte sodium buffering power remained virtually constant in patients with already low ESS before dialysis, whereas in patients with high ESS, 4h-HD improved the initially poor sodium buffering power by about 20%. No significant correlations could be detected between standard blood parameters and the respective ESS values except for plasma sodium concentration which was found increased by 3.1 mM in patients with high salt sensitivity. Conclusions: 4h-HD apparently recharges “run-down” erythrocytes and thus restores erythrocyte sodium buffering capacity. Besides the advantage of efficient sodium buffering in blood, erythrocytes with sufficient amounts of free negative charges at the erythrocyte surface will cause less (mechanical) injury to the negatively charged endothelial surface due to efficient repulsive forces between blood and vessel wall. Hemodialysis improves erythrocyte surface properties and thus may prevent early vascular damage in patients suffering from ESRD.
Introduction

Cardiovascular disease is the major cause of mortality in hemodialysis patients and represents almost 50% of all-cause-death [1, 2]. Beyond others endothelial dysfunction is a mayor pathologic risk factor related to atherogenesis and vascular calcification [3]. Deterioration of the endothelial glycocalyx is one of the earliest alterations in the development of cardiovascular diseases [4].

Anemia is a cardinal symptom in patients with chronic kidney disease (CKD). It is thought to be mainly caused by suppressed erythropoiesis and a reduced life span of circulating erythrocytes [5]. The latter is supposed to due to alterations of the RBC plasma membrane including the negatively charged surface layer, the RBC glycocalyx [6, 7]. Loss of negative surface charges, either from the endothelial and/or the RBC surface, reduce the repulsive forces between erythrocytes and vessel wall. These forces, however, are of eminent functional importance allowing frictionless RBC slipping through narrow blood vessels [8].

There is evidence that RBC surfaces “mirror”, at least to a certain extent, the surface of vascular endothelium. RBC glycocalyx shedding (i.e. loss of negative charges) occurs when “healthy” RBC are exposed to damaged endothelium. On the other hand, the endothelial glycocalyx is also damaged when exposed to poorly charged RBC [9]. In reference to patients with chronic kidney disease, a dual strategy fighting anemia appears most adequate, namely stimulation of erythropoiesis and expanding RBC life span.

Sodium ions are known to selectively bind to the negative charges of the glycocalyx of RBC and of vascular endothelium [10]. A “healthy” glycocalyx has a high sodium buffer capacity, a “poor” glycocalyx has a low one. A recently developed test system, the so-called Salt-Blood-Test (SBT) quantifies this surface property, termed Erythrocyte Sodium Sensitivity (ESS) [11]. ESS is high when sodium buffer capacity of the erythrocytes is low and vice versa. The principle of the SBT is based on the Na⁺ dependent sedimentation velocity of aggregated erythrocytes.

Patients suffering from chronic kidney disease usually undergo periodic hemodialysis three times a week with at least four hours treatment. Therefore, the question has been raised whether RBC surface properties are being altered by the hemodialysis process. Thus, ESS was measured before and after a 4h-hemodialysis. It turned out that RBC glycocalyx function (i.e. electrical surface negativity) improves by hemodialysis (i.e. ESS decreases). This positive effect is pronounced in male end stage renal disease (ESRD) patients.

Materials and Methods

Hemodialysis protocol

Blood samples were obtained from 38 patients (15 females, 23 males) who underwent in-patient hemodialysis between May and August 2014. All patients signed consent forms approved by the hospital ethics committee and all procedures were performed in accordance with the declaration of Helsinki. Patients were included independent of the underlying renal disease and the reason for current hospital admission. Standard dialysis method was postdilutional hemodiafiltration for 4 hours using the Fresenius 5008 machine and a FX600 filter (both Fresenius Medical Care, Bad Homburg, Germany). Blood flow was at least 250 ml/min. Ultrafiltration was individually adjusted as appropriate (0 to 3 l / dialysis session). Anticoagulation was performed with heparin or, alternatively with argatroban in those patients diagnosed with heparin-induced thrombocytopenia. Intradialytic medication (mainly erythropoietin and iron substitution) was administered after taking the second blood sample for the study. Blood parameters (acid-base status, electrolytes, creatinine, urea, c-reactive protein, hemoglobin concentration, hematocrit) and blood pressure measurements were evaluated before 4-HD using standard methods.

Salt-Blood-Test protocol

The SBT has been published in detail recently [11]. In short, 2ml of blood was drawn from CKD patients before and after 4-HD using heparinized monovettes (Sarstedt Company, Sarstedt, Germany). Blood was
stored overnight at 4°C. The following day, blood was transferred into plastic vials and centrifuged. Plasma was removed and erythrocytes washed in buffered saline including 1% bovine serum albumin. For a single measurement 80 µl of washed RBC were suspended in 120 µl of two separate NaCl solutions, (125 and 150 mM NaCl; fixed hematocrit: 0.4) containing 3% dextran (Sigma 4486, MW: 70,000 D). Dextran is adsorbed to the RBC plasma membrane surface, a crucial prerequisite necessary for triggering RBC aggregation. Constant osmolality was maintained by addition of appropriate amounts of sucrose. Hematocrit capillary tubes (Safecap P75-2000; length: 75 mm; Scholz Company, Neubiberg, Germany) were filled by capillary forces with the respective RBC suspensions as appropriate. Hematocrit capillary tubes, closed at the lower end, were put on stands in an upright position. RBC sedimentation rates were measured after 60 minutes. In principle, as smaller the number of free negative charges as more aggregates are being formed and as larger sedimentation velocity comes about. Performing this sedimentation procedure in two solutions containing different amounts of Na⁺ ions, erythrocyte sodium sensitivity can be derived. The dimensionless ESS values of the individual blood samples were calculated (triple measurements) as the ratio of the respective RBC sedimentation velocities in 150 and 125 mM Na⁺ solutions (ESS = L₁50/L₁25) as described previously [11].

**Results**

Figure 1 displays the relationship between the ESS values measured before and after 4-HD in 38 patients suffering from ESRD. ESS values located on or close to the line of identity indicate "no change" in erythrocyte salt sensitivity induced by hemodialysis. ESS values below the red-dotted line indicate a decrease of salt sensitivity by 4h-HD, values above the dotted line indicate the opposite. The data show that 4h-HD improves (i.e. lowers) the ESS values in a majority of patients, particular in those who start with initially high ESS.

**Fig. 1.** Relationship between erythrocyte sodium sensitivity (ESS) measured before and after 4h-hemodialysis. 38 chronic kidney disease patients were studied. ESS values below the line of identity indicate an improvement of the erythrocyte sodium buffering power, ESS values above this line indicate the opposite.

**Fig. 2.** Histograms based on 38 chronic kidney disease patients showing erythrocyte sodium sensitivity (ESS) before and after 4h-hemodialysis.
Two observations can be made. (i) There are two peaks, a major one at low ESS and a minor one at high ESS. This confirms a previous finding obtained from a healthy population indicating that about two thirds of the people are rather salt sensitive (large peak). (ii) 4h-HD leads to a left-shift of the large peak and a concomitant decrease of the amplitude of the small peak indicating that dialysis lowers ESS, particularly of those patients with an initially high salt sensitivity.

Figure 3 shows the individual ESS values obtained before 4-HD. The black-dotted line indicates the so-called reference ESS value (= 4.3) obtained in healthy volunteers published previously. For the sake of clarity, patients with ESS values below this line were termed "salt resistant", patients with ESS values above this line were termed "salt sensitive". As evident, 53 % of dialysis patients were found salt sensitive before 4h-HD. However, after 4h-HD this picture has changed. As derived from Figure 4 the percentage of salt sensitive patients decreased to 34 %.

Fig. 4. Erythrocyte sodium sensitivity (ESS) measured after 4h-hemodialysis. The dotted horizontal line indicates the ESS reference value (=4.3) obtained in a previous study. ESS values below the dotted line (green columns) indicate patients with low salt sensitivity, ESS values above the dotted line (red columns) indicate the opposite.
Table 1. Parameters measured in the blood/plasma of 38 patients before a 4h-hemodialysis

| Parameter                | Patients with low salt sensitivity (n = 18; ± sem) | Patients with high salt sensitivity (n = 20; ± sem) | p-value          |
|--------------------------|---------------------------------------------------|---------------------------------------------------|------------------|
| ESS (150 Na⁺/125 Na⁺)    | 3.3 ± 0.12                                        | 5.9 ± 0.24                                        | 0.001            |
| Na⁺ [mM]                 | 136.8 ± 0.93                                      | 139.9 ± 0.77                                      | 0.034            |
| K⁺ [mM]                  | 4.4 ± 0.22                                        | 4.6 ± 0.15                                        | 0.620            |
| pH                       | 7.39 ± 0.011                                      | 7.40 ± 0.013                                      | 0.370            |
| pCO₂ (mm Hg)             | 41.4 ± 1.05                                       | 37.5 ± 1.25                                       | 0.023            |
| HCO₃⁻ [mM]               | 24.9 ± 0.69                                       | 23.2 ± 0.54                                       | 0.068            |
| Base excess [mM]         | 0.0 ± 0.88                                        | -1.5 ± 0.63                                       | 0.178            |
| Creatinine [mg/dl]       | 5.2 ± 0.42                                        | 5.7 ± 0.45                                        | 0.444            |
| Urea [mg/dl]             | 50 ± 4.9                                          | 48 ± 4.4                                          | 0.747            |
| C-reactive protein [mg/dl]| 3.2 ± 0.82                                        | 2.0 ± 0.42                                        | 0.189            |
| Hb [g/dl]                | 10.3 ± 0.49                                       | 10.2 ± 0.40                                       | 0.888            |
| Hct (%)                  | 32 ± 1.5                                          | 31 ± 1.2                                          | 0.884            |
| Systolic RR (mm Hg)      | 127 ± 6.6                                         | 130 ± 4.8                                         | 0.643            |
| Diastolic RR (mm Hg)     | 62 ± 4.9                                          | 62 ± 3.4                                          | 0.952            |
| BMI                      | 24.9 ± 1.00                                       | 25.4 ± 1.28                                       | 0.771            |
| Age (years)              | 68 ± 3.7                                          | 68 ± 2.8                                          | 0.905            |
| Sex                      | females:males = 50:50                             | females:males = 30:70                             | -                |

Fig. 5. Erythrocyte sodium sensitivity (ESS) measured before and after 4h-hemodialysis. 38 patients have been studied, 15 females and 23 males. ESS values (before dialysis) in male patients are significantly larger as compared to ESS values of females.

In parallel to ESS measurements several standard parameters were measured. On the basis of the before mentioned data the results were split into "patients with (initially) low ESS" and "patients with (initially) high ESS". Table 1 allows a comparison of these data. Clearly, ESS values were significantly different in the two groups. Moreover, plasma sodium concentration was significantly increased by about 3 mM in patients with high
Fig. 6. Plasma sodium concentration measured before and after 4h-hemodialysis. 38 patients have been studied, 15 females and 23 males. Plasma sodium concentration in male patients is significantly larger before dialysis compared with that after dialysis. Female patients do not show any differences of sodium concentration before and after hemodialysis.

salt sensitivity. Although this change in plasma sodium is quite small it still may have considerable impact on vascular function. It has been shown that such a small change in plasma sodium stiffens vascular endothelial cells leading to endothelial dysfunction [12-14]. The only other significant change was found in the acid-base status \( pCO_2 \) of patients with high salt sensitivity indicating a mild metabolic acidosis, compensated by hyperventilation.

Finally, the data were split in a male and a female cohort. As indicated in Figure 5, male patients tend to have a higher ESS compared to female patients. Possibly due to the small cohort, this comparison misses statistical significance. However, male patients significantly responded to 4h-HD by a significant ESS decrease probably due to high initial ESS values. Female patients had lower initial ESS values and thus did obviously not respond in a similar way. Figure 6 shows plasma sodium concentrations in male and female patients before and after 4h-HD. Probably due to the scatter of the data and the rather low number of patients we found no significant difference in plasma sodium concentration between male and female patients. However, in male patients plasma sodium concentration significantly decreased after 4h-HD.

Discussion

Generally, senescent cells are supposed to gradually lose their electrical surface charges. This has been shown for vascular endothelium [15, 16] and also for erythrocytes [17]. In CKD patients these deterioration processes are supposed to be accelerated. Most likely this is caused by a mechanical interaction between RBC and vessel wall due to insufficient repulsive forces between the erythrocytes and the endothelial cell surface [8]. In the early days of hemodialysis mechanical destruction of erythrocytes during frequent hemodialysis was thought to be a major cause for anemia observed in these patients [7-18]. Along with the technical improvement of the dialysis equipment the adverse (mechanical) influence of the dialysis process by itself on erythrocyte survival became less [19, 20]. Today, anemia in CKD patients is mainly explained by reduced erythropoiesis and the presence of uremic toxins which trigger early eryptosis [21].

Accumulation of sodium in the human body, often caused by excessive high salt (NaCl) intake creates serious cardiovascular problems [22] particularly in CKD patients. Sodium ions deposited in skin [23], lymphatics [24] and the vasculature [25] neutralize the natural electrically negative surface charges of the glycocalyx that covers most cells. Usually, the blood vessel system is one of the “first stations” where several grams of sodium arrive after a salty meal. The large surfaces of the endothelium and the RBC, estimated to be several hundreds of square meters, can transiently buffer this ‘incoming’ sodium [25]. Thus,
sodium remains “in circulation” until it is finally excreted by the kidneys. Based on this view it can be concluded that particularly ESRD patients face a serious problem. Moreover, excessive sodium damages the glycocalyx [26], with the consequence that sodium buffering power gradually decreases. Deterioration of the vascular glycocalyx, however, has serious consequences. Plasma proteins, normally retained in the glycocalyx, are getting detached from the endothelial cell surfaces. This leads in the capillaries to a loss of “local” oncotic pressure (i.e. loss of the Starling forces) followed by edema formation [27-30]. In larger blood vessels the deterioration of the glycocalyx promotes atherogenesis [4].

Hemodialysis compensates for renal insufficiency and removes excess water, electrolytes and uremic toxins from the patient’s blood. Nevertheless, RBC show early senescence in CKD patients [6, 19, 31] and the question was raised whether frequent hemodialysis contributes (positively or negatively) to this process.

Data of the present study show that a 4h-HD improves sodium buffering capacity of erythrocytes. Patients with a ‘good’ sodium buffer capacity prior to dialysis benefit less than those with a ‘bad’ one. High salt sensitivity (i.e. poor sodium buffer capacity) turned out to be more pronounced in male patients. At first sight this seems to contradict former studies on 61 healthy volunteers where sodium sensitivity was indistinguishable between sexes [11]. However, male volunteers in the former study were significantly younger (on average 23 years of age) than the male ESRD patients analyzed in the present study (on average 68 years). Therefore, this apparent discrepancy could find an explanation in that salt sensitivity may have changed over lifetime [11]. Aged male ESRD patients may have maintained a less favorable life style (e.g. excess dietary salt intake) that differs from that of female ESRD patients. Then, gradually (over time) differences in ESS could be expected. Evidence supporting this view is derived from the present data that male ESRD patients tend to have a slightly higher plasma sodium concentration which can be significantly lowered by 4h-HD whereas female ESRD patients have a low plasma sodium concentration already before 4h-HD which remains constant (i.e. low) despite dialysis. Taken together, these arguments emphasize the view that salt sensitivity cannot be exclusively taken as an invariable property of an individual but can be indeed influenced by exogenous factors. This assumption is in accordance with findings of a previous study showing that ESS can be indeed reduced by long-term application of polyphenols [10].

We found no significant difference in arterial blood pressure between salt sensitive and salt insensitive ESRD patients as determined by SBT. This is not surprising because repetitive hemodialysis and antihypertensive treatment is supposed to mask any such changes. We also could not find any significant correlations between ESS and urea/creatinine/hemoglobin/hematocrit/c-reactive protein (Table 1). Again, this was not unexpected because periodic dialysis and therapeutic measures will not allow strong deviations of these parameters among patients.

**Conclusions and Perspectives**

Erythrocytes of ESRD patients, particularly those of male patients, gain better function by dialysis. The erythrocyte glycocalyx is refurbished indicating that sodium buffering power has improved. Recharged erythrocytes are expected to show better performance in the vascular system since adequate repulsive forces maintain a “security distance” (several hundreds of nanometers) between RBC and vessel wall. This behavior prevents vascular endothelial damage and possibly blood clotting processes. Application of the SBT in ESRD patients could give valuable information on erythrocyte surface properties. Since RBC “mirror” properties of the endothelial glycocalyx, the SBT reports any (positive or negative) changes in the status of the inner vessel wall and thus may be helpful in deciding what therapeutic measures fits best for a specific patient.
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