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Low concentrations of citrate reduce complement and granulocyte activation *in vitro* in human blood

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Abstract

Background. The use of acetate in haemodialysis fluids may induce negative effects in patients including nausea and increased inflammation. Therefore, haemodialysis fluids where acetate is substituted with citrate have recently been developed. In this study, we investigated the biocompatibility of citrate employing concentrations used in haemodialysis.

Methods. The effects of citrate and acetate were investigated in human whole blood *in vitro* under conditions promoting biomaterial-induced activation. Complement activation was measured as generation of C3a, C5a and the sC5b-9 complex, and granulocyte activation as up-regulation of CD11b expression. For the experimental set-up, a mathematical model was created to calculate the concentrations of acetate and citrate attained during haemodialysis.

Results. Citrate reduced granulocyte activation and did not induce higher complement activation compared with acetate at concentrations attained during haemodialysis. Investigating different citrate concentrations clearly showed that citrate is a potent complement inhibitor already at low concentrations, i.e. 0.25 mM, which is comparable with concentrations detected in the blood of patients during dialysis with citrate-containing fluids. Increased citrate concentration up to 6 mM further reduced the activation of C3a, C5a and sC5b-9, as well as the expression of CD11b.

Conclusions. Our results suggest that citrate is a promising substitute for acetate for a more biocompatible dialysis, most likely resulting in less adverse effects for the patients.

Keywords: acetate; biocompatibility; citrate; complement; haemodialysis

Introduction

Acetate has been used in haemodialysis fluid since the 1960s, initially as the sole buffer at high concentrations (30–40 mM), but has been found to induce side effects in patients, such as nausea and increased inflammatory response [1, 2]. Although modern haemodialysis fluids contain relatively low amounts of acetate (3–4 mM), the levels are still far from physiological, raising concerns especially for the older and more vulnerable dialysis patients with low muscle mass, and therefore at higher risk for acetate intolerance [3, 4].

In haemodialysis treatment, blood will be in direct contact with foreign surfaces including the dialyser and bloodlines—and indirectly with the dialysis fluid. When in contact with foreign surfaces, the first events in blood are the triggering of the cascade systems, which include the complement, and the contact activation and coagulation systems. As a result numerous proteins are affected and immune cells and platelets are recruited, leading to adverse reactions such as anaphylactic reactions and acute or chronic inflammation [5, 6].

The complement system is highly dependent on the presence of Ca^{2+} and Mg^{2+} for full activity. Citrate binds these divalent cations with high affinity and is also a natural metabolite providing both energy and buffering capacity to the patient [7]. Therefore, citrate is an attractive substitute for acetate, having the potential of reducing the activation of the cascade systems induced by the haemodialysis treatment.

For decades, citrate has been used as an anticoagulant during haemodialysis—in regional citrate anticoagulation (RCA) haemodialysis—to improve dialysis efficiency and reduce intolerance reactions [8]. In RCA dialysis, the dialysis fluid is normally calcium free. Therefore, calcium is infused after the dialyser by a separate pump to maintain calcium balance in the patients. Consequently, RCA is quite laborious with the need for additional equipment and staff training, which might constitute limiting factors for applying RCA on a large scale [9]. Nevertheless, the use of RCA has shown positive effects by reducing oxidative stress and

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abolishing degranulation of polymorphonuclear cells compared with heparin anticoagulation [10]. Furthermore, RCA during haemodialysis has indicated a reduced activation of coagulation when compared with heparin [11]. Haemodialysis fluids where acetate is substituted, in part or completely, with citrate were recently developed. Citrate-containing dialysis fluids have shown promising results in terms of improving efficacy and treatment tolerance albeit the citrate concentrations employed in RCA haemodialysis are substantially higher than those of citrate-containing fluids [12, 13]. Although citrate-containing dialysis fluids have been used in haemodialysis during recent years, there has been no systematic investigation on the effects of different citrate concentrations and optimization thereof.

The purpose of this study was (i) to compare the effects of citrate and acetate (at clinically relevant concentrations combined with a clinically relevant concentration of heparin) on complement and neutrophil activation under conditions which promote surface-induced complement activation; and (ii) to investigate at which concentrations citrate attenuates this activation. A mathematical model was created to determine the concentrations of acetate and citrate in blood during a haemodialysis treatment session—to elucidate whether citrate-containing dialysis fluids may be a biocompatible alternative to conventional acetate-containing fluids regarding complement activation.

Methods

Modelling of citrate, calcium and acetate transport across the dialyser membrane

A simulation model of transports of ions and complexes across the dialyser membrane was created in MATLAB (The MathWorks, Inc., Natick, MA, USA). The model equations handle transport of ions through the membrane by calculating a varying Goldman potential along the dialyser fibres to achieve a local net electrical current of zero. It was combined with calculations of evolving local chemical equilibrium on both sides of the membrane. The blood chemistry was modelled by a mixture of substances designated 'pseudo-plasma'. Pseudo-plasma contains calcium, magnesium, potassium, bicarbonate, hydrogen, chloride, sodium, citrate/acetate and albumin dissolved in water including an additional 26 compounds generated when combining these substances. The dialysis fluid in the model contains the same substances except albumin and complexes thereof.

The input plasma values for the patient blood to the dialyser were 2.4 mM total Ca, 0.8 mM total Mg, 26 mM total HCO_3^-, 0.1 mM total citrate, 0.05 mM acetate and 139 mM total Na. The input values for different citrate-containing dialysis fluids in the dialyser were 1.25–1.75 mM total Ca, 0.7 mM total Mg, 37 mM total HCO_3^-, 0–2 mM total citrate and 140 mM total Na. For the acetate-containing dialysis fluid, the input values were identical except that citrate was replaced with 3 mM acetate. Other input parameters included: 300 mL/min blood flow rate, 35% haematocrit, 94% plasma water fraction, 500 mL/min dialysate flow rate, 1000 mL/min k_0A_{urea} (membrane permeability) and 10 mL/min ultrafiltration rate.

Modelling of systemic citrate concentration

In order to simulate the systemic concentration of citrate during a 4-h dialysis session, a single pool model was set up. Input values: distribution volume of citrate in the body: 39 L; citrate generation: 0.88 µmol/min; and blood flow rate: 300 mL/min. Citrate metabolism in the human body varies with average half-life values around 50 min [14]; model input values for citrate half-life were 18, 48 and 131 min.

Blood sampling

Blood was drawn from healthy blood donors who had received no medication for at least 10 days. Vacutainer™ tubes (Becton, Dickinson and Co., Plymouth, UK) containing the specific thrombin inhibitor lepirudin (Refludan, Avenis Pharma, final concentration of 50 µg/mL); which does not inhibit complement activation [15] or heparin (LEO Pharma AB, Malmö, Sweden, final concentration of 1 IU/mL), which at this concentration inhibits complement activation to some extent [15, 16]. Within 5 min, the blood was transferred to polystyrene test tubes. This study was approved by the Ethics Committee at the University of Linköping, Sweden (#03-520).

Blood incubation with citrate or acetate under conditions promoting biomaterial-induced complement and granulocyte activation

Citrate and acetate experiments mimicking dialysis. Stock solutions of citrate and acetate, pH 7.0, were prepared. Veronal buffer (VBS: 5 mM barbiturate, pH7.4; 145 mM NaCl; 0.15 mM Ca^{2+}; 0.5 mM Mg^{2+}) was used as control.

Blood (1 IU/mL heparin; i.e. the concentration in patients during dialysis) with addition of citrate or acetate solutions (final concentrations 0.35 and 1.35 mM, respectively) was incubated in polystyrene test tubes at 37°C with continuous rotation at 20 rpm to enable activation of cascade systems upon contact between blood and the surface for up to 60 min [15, 17]. The reaction was terminated by the addition of 10 mM ethylenediaminetetraacetic acid (EDTA) (final concentration), platelets were counted using a Swelab AC920 EO autocounter, activation of granulocytes was analysed and plasma was obtained by centrifugation (3000 *g*, 20 min, 20°C) and stored at −80°C pending analysis.

Citrate dose-response experiments. In additional experiments, citrate was added to lepirudin blood in polystyrene tubes and incubated for 60 min as described above (final concentrations 0–6 mM).

Complement and coagulation activation

In all ELISAs, PBS with 0.05% Tween 20, 1% bovine serum albumin (BSA) and 10 mM EDTA was used as buffer. PBS containing 0.05% Tween 20 served as washing buffer; and 3,5,3′,5′-tetrametylbenzidin (6 mg/mL dissolved in dimethyl sulphoxide, Sigma-Aldrich) in Na-acetate buffer (0.11 M, pH5.5) containing H_2O_2 (0.5 µL/mL buffer) was used as colour substrate. Results were normalized to the control at 60 min.

Generation of C3a. C3a in plasma was measured by sandwich ELISA using the monoclonal antibody mAb 4SD17.3 directed against a neo-epitope in human C3a for capture and polyclonal anti-Hu-C3a followed by horseradish peroxidase (HRP)-conjugated streptavidin (GE Healthcare, Uppsala, Sweden) for detection. Zymosan-activated

serum calibrated against purified C3a was used as standard and results are presented as ng/mL [18].

Generation of C5a. C5a in plasma was quantified using a commercial ELISA kit (HK349 human C5a, Hycult Biotech, Uden, the Netherlands) according to the product manual and results are presented as ng/mL.

Generation of sC5b-9. sC5b-9 was detected by sandwich ELISA using the mAb anti-neo-Hu-C9 mAb aEII (Diatec Monoclonals AS, Oslo, Norway) for capture and biotinylated polyclonal anti-Hu-C5 (The Binding Site, Birmingham, UK), for detection. Zymosan-activated serum served as standard and results are presented as arbitrary units/mL [19].

Generation of thrombin-AT. Thrombin-AT (TAT) was captured by anti-human thrombin mAb and detected by HRP-coupled anti-human AT mAb (Enzyme Research Laboratories, South Bend, IN, USA) [17].

Granulocyte activation

After incubation, 50-µL aliquots of blood were incubated with 5 µL anti-CD11b-RPE (Dako A/S, Glostrup, Denmark) for 1 h at room temperature, and then lysed by 2 mL AKC (0.16 M NH_4Cl with 10 mM $NaHCO_3$, 0.12 mM EDTA and 0.04% paraformaldehyde) for 10 min in the dark. After centrifugation at 400 *g* for 5 min, the sediment cells were washed with PBS containing 1% BSA. After fixing with 1 mL 0.5% paraformaldehyde in PBS, a minimum of 5000 cells was collected and mean fluorescent intensity (MFI) of CD11b was measured by flow cytometer CUBE8 (Partec, Münster, Germany).

Statistics

Each experiment was performed separately using blood from different donors and data are presented as mean values ± SEM. Statistical calculations (one-way ANOVA with Dunnetts's *post hoc* test) were made using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA USA), and $P < 0.05$ was considered significant.

Results

Modelling of calcium and citrate transport across the dialyser membrane

Simulation of dialysis fluids containing different concentrations of citrate and Ca^{2+} was performed to predict the concentrations of total and ionized calcium, and citrate in the extracorporeal blood plasma returning to the patient. As expected, the ionized calcium concentration was decreasing with increasing citrate concentration when the total calcium level was kept constant through the dialyser.

A dialysis fluid containing 1 mM citrate and 1.5 mM Ca^{2+} was predicted to return a concentration of 0.58 mM citrate and a total and ionized calcium level of 2.25 and 0.98 mM, respectively, in the blood plasma at the dialyser outlet. The citrate concentration of blood plasma in the dialyser outlet was similar (i.e. 0.58 mM) for a fluid containing 1 mM citrate with a Ca^{2+} concentration of 1.25–1.75 mM. As a result, the concentration of ionized calcium extracorporeally is expected to decrease within the range of 0.2–0.3 mM.

Modelling of acetate transport across the dialyser membrane

Simulation of a dialysis fluid containing 3 mM acetate and 1.25–1.75 mM Ca^{2+} was performed to predict the concentration of acetate in the extracorporeal blood plasma returning to the patient. The mathematical model predicted an acetate concentration of 2.25 mM in the extracorporeal blood plasma returning to the patient.

Modelling of systemic citrate concentration

A single-pool model was created to predict the concentration of citrate systemically in a patient during dialysis with a 1 mM citrate-containing dialysis fluid. The model revealed a systemic citrate concentration of between 0.10 and 0.42 mM at the end of a 4 h session, depending on the rate of citrate metabolism (i.e. half-life of 18–131 min).

Effects of citrate and acetate on complement and granulocyte activation at concentrations relevant in haemodialysis

The mathematical modelling of dialysis with fluids containing 1 mM citrate and 3 mM acetate predicted citrate and acetate concentrations of 0.58 and 2.25 mM, respectively, in the blood plasma leaving the dialyser. Consequently, these concentrations were selected to investigate the effect on complement and granulocyte activation. With a haematocrit value of 40%, the final concentrations of citrate and acetate added to blood were 0.35 and 1.35 mM, respectively. Concentrated solutions of citrate and acetate were added to the blood to minimize dilution since complement activation is highly concentration dependent [20]. Notably, dialysis concentrates could not be used as the concentrations of ions such as Ca^{2+} and Mg^{2+} would not reflect those obtained during dialysis.

Platelet consumption. Similar platelet loss was observed in blood incubated with either citrate or acetate and a platelet loss up to 28% was observed after 30 min. The corresponding value for control samples were 40% (data not shown). TAT was analysed to evaluate the activation of the coagulation cascade without any difference between acetate/citrate and the control (data not shown).

Complement activation. Similar levels of activated complement factors were found in blood after 1 h incubation with citrate, or acetate; C3a: 76 versus 86% of uninhibited control; C5a: 90 versus 88% of control; sC5b-9: 77 versus 106% of control (Figure 1A–C). After 60 min of incubation the concentrations of C3a and C5a were ≈85 and ≈0.05 nM respectively, corresponding to ≈1 and ≈0.2% of the total amount of C3 and C5 in blood.

Granulocyte activation. There was a significant difference in the expression of CD11b between citrate-containing and acetate-containing blood (87 versus 119% of the uninhibited control) $P = 0.0079$ after 1 h of incubation (Figure 1D).

Effect of citrate on complement and granulocyte activation in whole blood

Platelet loss. Citrate concentrations up to 6 mM did not affect platelet consumption (data not shown).

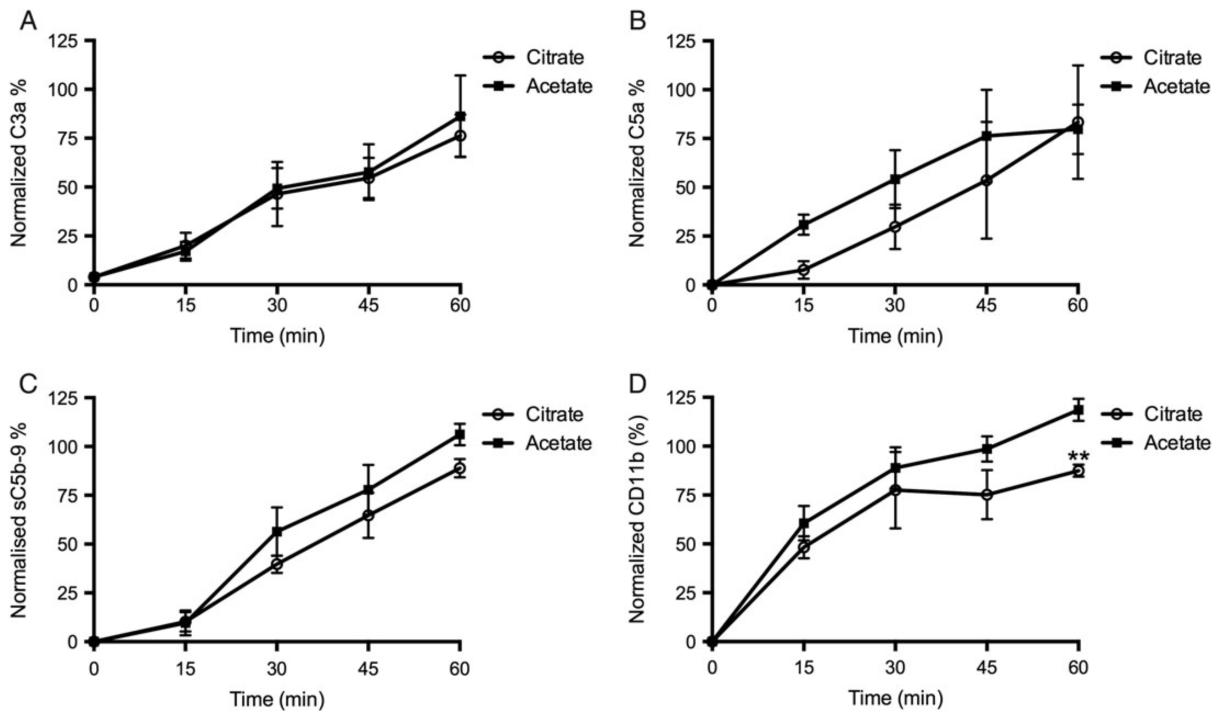

Fig. 1. Effects of citrate and acetate on complement and granulocyte activation at concentrations relevant in haemodialysis. Activation of complement was measured as the generation of (**A**) C3a, (**B**) C5a and (**C**) sC5b-9 in blood with 1 IU/mL heparin after incubation with 0.35 mM citrate or 1.35 mM acetate. Data are presented as mean ± SEM (n = 5). Activation of control samples (with the addition of VBS) at 60 min was set to 100%. (**D**) Activation of granulocytes was quantified as the expression of CD11b after incubation in blood as described for A–C. Mean fluorescence intensity (MFI) of each sample was analysed immediately after incubation at indicated time points. Data are presented as mean ± SEM (n = 5). Activation of control samples (with the addition of VBS) at 60 min was set to 100%. Significant difference between citrate- and acetate-containing blood is indicated as **P < 0.01.

Complement activation. *Already in the presence of 0.25 mM citrate, the generation of* C5a and sC5b-9 was significantly reduced, to 52 and 73%, respectively, compared with the control without citrate. The generation of C5a and sC5b-9 was further inhibited at higher citrate concentrations up to 2 and 19%, respectively, in the presence of 6 mM citrate compared with control. Generation of C3a decreased significantly only at the higher concentrations of citrate. A reduction to 59–41% compared with the control was observed in the presence of 4–6 mM citrate (Figure 2A–C).

Granulocyte activation. Expression of CD11b was significantly inhibited at a citrate concentration from 2 mM. The activation was inhibited in a dose-dependent manner with increasing citrate concentration from 92% expression in 0.25 mM citrate to 11% in 6 mM citrate as compared with the control without citrate (Figure 2D).

Discussion

In addition to acetate intolerance, common side effects in patients on dialysis include amyloidosis, arteriosclerosis [21] and higher risk of myocardial infarction compared with healthy subjects [22]. Many of these effects are related to complement activation during the haemodialysis treatment when the blood is in direct or indirect contact with artificial surfaces such as those of the dialyser, bloodlines and dialysis fluid. Upon activation, complement activation products such as C3b/iC3b and C4b/iC4b may bind to the dialysis membrane; soluble anaphylactic toxins C3a and C5a (as well as the less potent C4a) will be generated; and leukocytes will become activated and trigger an inflammatory response [5]. Therefore, a dialysis fluid that induces a minimal level of activation, or even reducing the complement activation in the blood, would be of great benefit for haemodialysis patients.

Low doses of citrate, which is a natural metabolite as well as an anticoagulant, have been used in dialysis fluids for several years, providing high efficiency of dialysis and less intolerance reactions in the patients [23, 24]. In the present study, investigation whether citrate may be a more biocompatible substance compared with acetate regarding complement and granulocyte activation was performed *in vitro* at concentrations similar to those attained in the blood during a dialysis session. Therefore, based on mathematical modelling, citrate and acetate was added to heparinized whole blood to yield final concentrations of 0.35 and 1.35 mM, respectively. No significant difference was observed for platelet loss or complement activation between citrate and acetate. However, citrate showed a significantly lower activation of granulocyte compared with acetate, suggesting that citrate is more biocompatible for haemodialysis. This is in agreement with earlier studies which have shown that citrate has a direct inhibitory effect on granulocyte function by reversing the leukotirene B4-induced adherence and interfering with their activation, e.g. by zymosan [25, 26].

To date, citrate-containing haemodialysis fluids contain up to 1 mM citrate while the citrate concentrations when using RCA haemodialysis are significantly higher. Specifically,

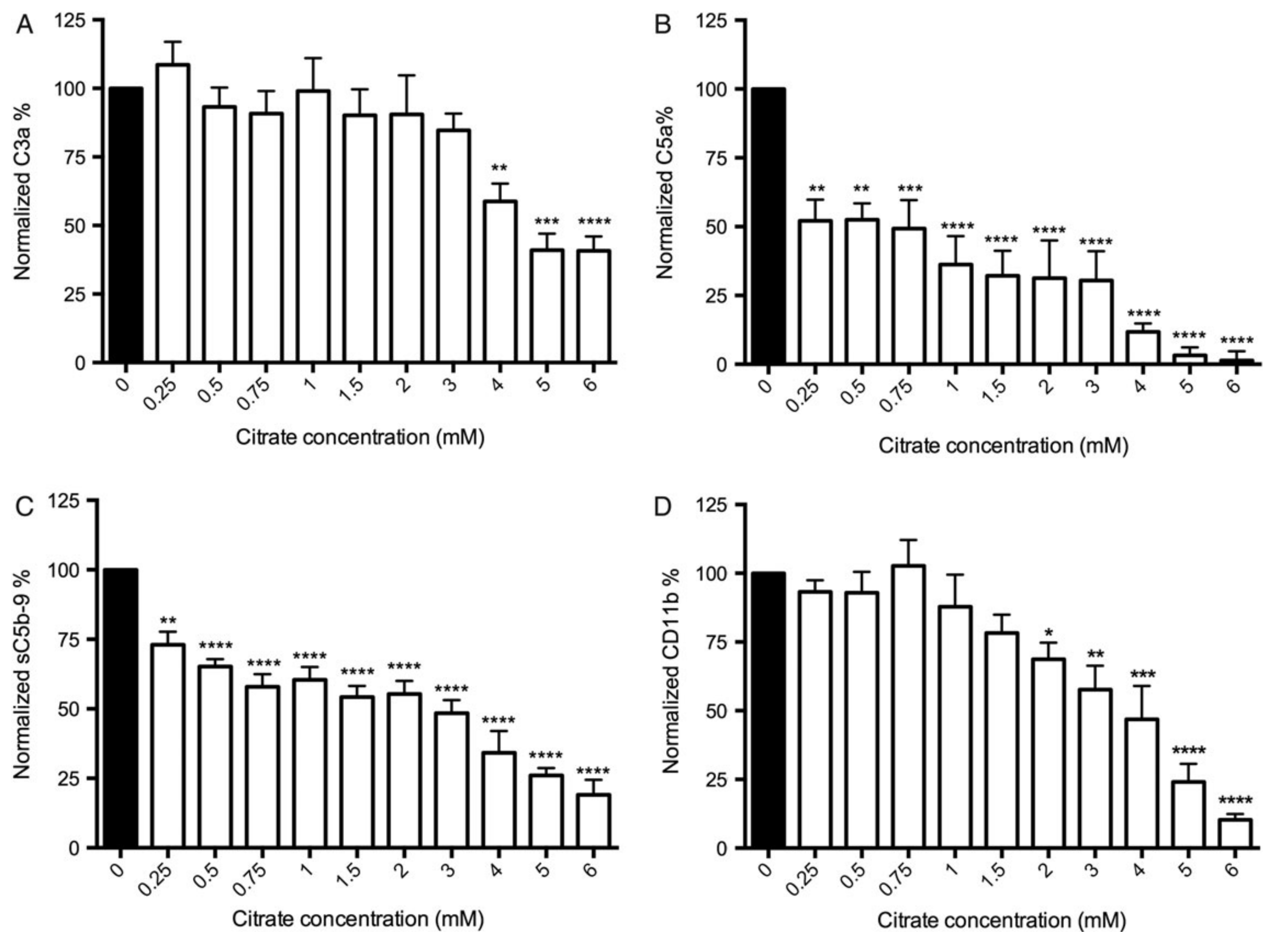

Fig. 2. Effect of different citrate concentrations on complement and granulocyte activation in whole blood. Generation of (**A**) C3a, (**B**) C5a and (**C**) sC5b-9 in lepirudin blood (50 µg/mL) after incubation with increasing concentrations citrate for 60 min at 37°C. Data are presented as mean + SEM ($n = 5$). (**D**) Expression of CD11b on granulocytes in lepirudin blood (50 µg/mL) after incubation with citrate. Data are presented as mean + SEM ($n = 5$). Results significantly different compared with the control without citrate (100%) are indicated as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

the citrate concentration post-filter has been measured in a study of 41 patients on continuous renal replacement therapies (CRRT) to 5.1–5.3 mM [27]. Unexpectedly, a study comparing citrate- and heparin-anticoagulation strategies during CRRT revealed better survival when citrate rather than heparin was used [28]. Therefore, the impact of higher citrate concentrations on complement and granulocyte activation in human lepirudin-anticoagulated whole blood was investigated. The results showed that low concentrations of citrate provided a significant reduction of complement activation, as well as reduction of granulocyte activation. For the complement activation markers C5a and sC5b-9, the reduction was significant already at the lowest concentration applied, i.e. 0.25 mM citrate. The seemingly more pronounced inhibitory effect of citrate on the generation of C5a compared with C3a might be explained by differences in binding of the two anaphylatoxins for their respective receptors. They have similar affinities for their receptors with $K_d \approx 1$ nM [29], but the expression of these receptors on activated granulocytes differ considerably; with a 20-fold higher expression of C5aR [30]. Consequently, it is likely that a higher proportion of the generated C5a compared with C3a is bound to granulocytes, resulting in misleadingly low levels of C5a in the plasma. An observation which speaks in favour of this hypothesis is that the granulocytes are fully activated at citrate concentrations up to 1.5 mM (Figure 2D), where the levels of generated C5a has declined by 2/3 (Figure 2B). The decline in activation at higher concentrations may be due either to decreased generation of C5a or citrate induced inhibition of granulocyte function [25, 26] or a combination of both.

Mathematical modelling, presented here, shows that during a typical dialysis treatment with a fluid containing 1 mM citrate the extracorporeal blood plasma returning to the patient will contain 0.58 mM citrate. When blood returns to the patient this concentration will instantly decrease since the normal blood concentration of citrate is about 0.1 mM. The exact concentration of citrate systemically will be dependent on the patient's ability to metabolize citrate, and modelling suggested a systemic citrate concentration of 0.1–0.4 mM after a 4-h dialysis session (citrate half-life 18–131 min). This is well in agreement with values measured during dialysis with 1 mM citrate; the systemic concentration after 4 h was 0.25–0.30 mM (values 0–0.5 mM) [31]. Taken together, the levels of citrate modelled and measured in human blood are within the range where positive effects of citrate could be seen as a reduction of the complement activation *in vitro* in human blood (Figure 2). The model created here is based on a transport algorithm of total mass transport across the dialyser that includes all major ions and complexes

present in the dialysis fluid. Several models for prediction of ionized Ca and citrate levels have been published [32, 33]. Notably, these models were created for RCA—a method that includes infusion of both calcium and citrate—hence, the concentration of these two compounds differ significantly from those obtained during haemodialysis without RCA. The model developed by Brandl *et al.* merely calculates the distribution of ionized Ca and citrate in the blood [33], while the model of Thijssen *et al.* includes differences across the dialyser—similar to our model [32, 34]. However, three major differences between our model and that of Thjissen can be noted: firstly, while the previous model includes ionized Ca, citrate and complexes thereof, our model includes all ions relevant to haemodialysis such as K^+, Na^+, Mg^{2+} and bicarbonate ions; secondly, while the previous model includes the dialyser clearance, our model includes calculation of the mass balance in every point along the dialyser and calculation of the local membrane potential along the dialyser fibres to keep electro-neutrality in every point across the dialyser; thirdly, while the previous model calculates chemical equilibrium with the dialyser as one single unit, our model calculates chemical equilibrium locally along the dialyser fibres. As previously mentioned, the citrate values predicted by our model for systemic citrate concentration correlated well with *in vivo* measurements from citrate-based haemodialysis treatments [31]. However, our dialyser model will have to be more thoroughly validated against calcium and citrate values obtained from future clinical studies.

Citrate has been proven to reduce complement activation in human blood and several studies have reported beneficial effects when replacing acetate- with citrate-containing dialysis fluid [13, 24, 35–39]. However, whether the positive effects observed with citrate-based haemodialysis fluids are due to reduction/elimination of acetate or due to citrate *per se*—or a mixture of both—remains to be elucidated. In this study, positive effects of citrate were observed using physiological buffer as control indicating that citrate alone accounts for at least part of the reported beneficial effects. Also, the higher the citrate concentration the more pronounced the effect on reduction of the complement and granulocyte activation.

In conclusion, the present work demonstrates that low concentrations of citrate can reduce complement and granulocyte activation in human whole blood *in vitro*. Also, the effects were further enhanced with increasing citrate concentration. However, future studies will have to elucidate whether these beneficial effects of citrate on complement also can be ascertained *in vivo*. Dialysis fluids containing citrate are promising alternatives for acetate dialysis fluids showing improved biocompatibility dialysis, hopefully with less adverse effects for the patients.

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