Improved Dialysis Removal of Protein-Bound Uraemic Toxins with a Combined Displacement and Adsorption Technique

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Keywords

End-stage renal disease · Protein-bound uraemic toxins · Haemodialysis · Adsorption technology · Displacer augmented dialysis technique

Abstract

Introduction: Protein-bound uraemic toxins (PBUTs) are poorly removed by conventional dialytic techniques, given their high plasma protein binding, and thus low, free (dialysable) plasma concentration. Here, we evaluated and compared PBUTs removal among conventional haemodialysis (HD), adsorption-based HD, displacement-based HD, and their 2 combinations both in vitro and in vivo. **Methods:** The removal of PBUTs, including 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid (CMPF), p-cresyl sulphate (PCS), indoxyl sulphate (IS), indole-3-acetic acid (3-IAA), and hippuric acid, was first evaluated in an in vitro single-pass HD model. Adsorption consisted of adding 40 g/L bovine serum albumin (Alb) to the dialysate and displacement involved infusing fatty acid (FA) mixtures predialyser. Then, uraemic rats were treated with either conventional HD, Alb-based HD, lipid emulsion infusion-based HD or their combination to calculate the reduction ratio (RR), and the total solute removal (TSR) of solutes after 4 h of therapy. **Results:** In vitro dialy-

sis revealed that FAs infusion prefilter increased the removal of PCS, IS, and 3-IAA 3.23-fold, 3.01-fold, and 2.24-fold, respectively, compared with baseline and increased the fractional removal of CMPF from undetectable at baseline to $14.33 \pm 0.24\%$, with a dialysis efficacy markedly superior to Alb dialysis. In vivo dialysis showed that ω -6 soybean oil-based lipid emulsion administration resulted in higher RRs and more TSRs for PCS, IS, and 3-IAA after 4-h HD than the control, and the corresponding TSR values for PCS and IS were also significantly increased compared to that of Alb dialysis. Finally, the highest dialysis efficacy for highly bound solute removal was always observed with their combination both in vitro and in vivo. **Conclusions:** The concept of combined displacement- and adsorption-based dialysis may open up new avenues and possibilities in the field of dialysis to further enhance PBUTs removal in end-stage renal disease.

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Introduction

Uraemic retention solutes are generally subdivided into 3 major classes based on their removal pattern by dialysis [1, 2]. Class 1 consists of small water-soluble solutes

with a molecular weight (MW) of <0.5 kDa, such as urea, which can be easily removed by any dialysis strategy. Class 2 contains middle and large molecules, with MW between 0.5 and 15 kDa versus 15–60 kDa, respectively, which can only be cleared using large-pore dialyser membranes. Class 3 is composed of protein-bound uraemic toxins (PBUTs). In general, the latter solutes have MWs <500 Da but are difficult to remove through most dialytic procedures, given their high plasma protein binding, especially to human serum albumin (HSA) (MW 65.5 kDa) [3, 4]. Increasing evidence suggests that PBUTs play an important role in various pathologic features of uraemia, and elevated serum concentrations of such toxins have been repeatedly associated with adverse outcomes in chronic kidney disease and end-stage renal disease patients [5–13]. Although many efforts have been made to improve the dialytic removal of PBUTs in recent years, the decrease in concentration was modest and not unanimous [14–23]. Thus, enhancing the removal of such compounds through blood purification-based strategies remains a challenging task.

PBUTs circulate in the bloodstream in 2 forms: a major bound fraction and a minor free fraction. In most cases, this protein binding is reversible, involving non-covalent binding (such as electrostatic or hydrophobic interactions, dipolar and van der Waals forces, and hydrogen bonds) and obeying “the law of mass action” [3, 24–26]. Following the development of theoretical models to describe the kinetics of PBUTs during haemodialysis (HD), it has been found that the elimination of such compounds depends primarily on diffusion of their free fractions, and the overall diffusive removal thus depends on the speed of dynamic equilibration between the bound and free fractions and on the free toxin concentration gradients between blood and dialysate [17, 27, 28]. Based on this principle, various methods for improving extracorporeal removal of PBUTs, such as increasing dialysate flows and dialyser surface area [18, 19], increasing dialysis frequency [21], extending dialysis duration [22, 23], using haemodiafiltration [14, 15], using adsorbent technology [28–31], and changing physical conditions within the dialyser [32–34], have been tested in patient populations or in experimental setups. The ultimate goal of all these strategies is either to keep the free concentrations on the dialysate side consistently low or to increase the free fractions on the blood side and finally to maintain as high a diffusive concentration gradient as possible along the dialyser fibres during the whole course of a dialysis session to permit continuous passage of solutes from the patient’s blood to the dialysate. Nevertheless, the additive effect on

PBUTs removal may be limited by only lowering free concentrations on the dialysate side because restoration of equilibrium may not be immediate, which results in an increase in the percentage of protein binding during HD sessions [35], and merely increasing the free fractions on the blood side may also have its own limitations in exerting a clinically significant effect. Herein, the aim of the study was to evaluate and compare PBUTs removal between conventional HD, adsorption-based HD (dialysis with dialysate rich in Alb), free fatty acid (FFA) displacement-based HD, and their 2 combinations in both in vitro and in vivo experiments.

Materials and Methods

Materials

All reagents, unless otherwise indicated, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). p-cresyl sulphate (PCS) and 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid (CMPF) were purchased from ApexBio (ApexBio Technology, Houston, TX, USA). Intralipid™ (refined soybean oil 20%) was obtained from Fresenius Kabi SSPC (Jiangsu, China). HSA (≥96%, low fatty acid [FA]) was obtained from Absin (Absin, Shanghai, China), predialysed to remove impurities in the commercial Alb preparation that may compete for Alb-binding sites and confirmed to possess high binding activity for CMPF, PCS, and indoxyl sulphate (IS). High-performance liquid chromatography-grade methanol was purchased from Sinopharm Chemical Reagent Company (Shanghai, China). All other chemicals were of analytical grade, and all solutions were prepared in deionized and distilled water.

The experimental setup for the in vitro and in vivo circulation systems consisted of miniature peristaltic pumps (VWR International, Irving, TX, USA) and a miniaturized polysulfone dialyser specially developed for small animals. All parts of the setup were connected via sterilized silicone tubing. The specifications of the minidialyser and the HD setup have been previously described [31]. In vitro, 2 long-chain FA mixtures, prepared by mixing oleic acid and linoleic acid ([linoleic acid]/[oleic acid] = 2), were selected as binding competitors to displace PBUTs from Alb according to preliminary experiments [36, 37]. In vivo, we compared the removal capacity of displacement and adsorption techniques using Intralipid™ infusion in the blood side and Alb-rich dialysate in the dialysate side, respectively.

In vitro Dialysis of Alb-Uraemic Toxin Mixtures

In vitro dialysis experiments were conducted with a volume of 200 mL of HSA solution spiked with uraemic toxins in the blood compartment and standard bicarbonate dialysate with or without bovine serum albumin (BSA, 40 g/L) in the dialysate compartment. We chose concentrations of CMPF, PCS, IS, indole-3-acetic acid (3-IAA), hippuric acid (HA), and urea to be comparable to the mean levels in dialysis patients (CMPF: 255 µmol/L; PCS: 200 µmol/L; IS 150 µmol/L; 3-IAA: 15 µmol/L; HA 400 µmol/L; and urea: 30 µmol/L) [1, 2]. Experiments were conducted at ambient temperature with an Alb flow rate of 6.0 mL/min and a counter-

Fig. 1. Schematic representations of the experimental setups for in vitro and in vivo dialysis. **a** A reservoir fluid composed of HSA-uraemic toxin mixtures represented a uraemic patient's plasma on the blood side. The dialysate side consisted of standard bicarbonate dialysate with or without BSA (40 g/L) in the dialysate compartment. In vitro dialysis was conducted at ambient temperature with a volumetrically measured Alb flow of 6.0 mL/min and counter-current dialysate flow of 10.0 mL/min for 30 min. **b** Setup of the in

vivo HD model in uraemic rats. A single-lumen PE50 vascular catheter for removing and returning blood was inserted into the right carotid artery and left jugular vein, respectively. The blood rate and counter-current dialysate flow were set at 1.0 and 5.0 mL/min, respectively. Blood purification therapy was performed for 240 min with single-pass counter-current dialysate flow. HSA, human serum albumin; BSA, bovine serum albumin; HD, haemodialysis; Alb, albumin.

current dialysate flow rate of 10.0 mL/min. HSA solution and dialysate were perfused through each compartment in a single-pass procedure. The system was primed with HSA solution in the blood circuit and with dialysate in the dialysate circuit prior to each experiment, during which we manually adjusted the transmembrane pressure between 2 circuits so that there was zero net ultrafiltration at the beginning of each experiment. A schematic diagram of the in vitro dialysis system is shown in Figure 1a.

HSA solution containing uraemic toxins was first circulated for 10 min without FAs infusion to measure the baseline removal. At 10 min, FAs or phosphate-buffered saline were continuously infused into the blood line between the reservoir and dialyser blood inlet to allow a 15- to 20-s transit time before arriving at the dialyser and to maintain a constant concentration of 1.0 mmol/L for the infused FAs. Samples were collected from the dialyser blood inlet, blood outlet and dialysate outlet ports at 2-min intervals throughout the experiment. The removal of PBUTs was expressed as fractional removal (%), calculated according to Tao et al. [33]. PBUT fractional removal (%) = $100 \times (C_{do} \times Q_d) / (C_{alb-in} \times Q_{alb})$, where C_{alb-in} and C_{do} are the total uraemic toxin concentrations in the dialyser blood inlet and dialysate outlet ports, respectively. Q_d and

Q_{alb} are the dialysate and Alb flow rates, respectively. Urea removal was specified as the single-pass extraction ratio, calculated as extraction ratio (%) = $100 \times (C_{alb-in} - C_{alb-out}) / C_{alb-in}$, where C_{alb-in} and $C_{alb-out}$ are the concentrations in the dialyser blood inlet and blood outlet ports, respectively.

In vivo Dialysis in Uraemic Rats

All animal experiments were performed with approval from the Medical Animal Care and Welfare Committee of Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University, School of Medicine (HKDL [2018] 38). The animals were reared at $\pm 20^\circ\text{C}$ with 12-h light/dark cycles and free access to food and water. Five-sixths of the normal kidney mass was removed from 6-week-old male Sprague-Dawley rats ($n = 40$, Chinese Academy of Science, Shanghai, China) to establish end-stage renal failure. Sixteen weeks after 5/6 nephrectomy, the uraemic rats were randomly assigned to the following 4 groups and were administered blood purification therapy: (1) control group ($n = 10$): dialysis with standard bicarbonate dialysate; (2) Alb dialysis group ($n = 10$): dialysis with BSA (40 g/L) added to bicarbonate dialysate; (3) intravenous lipid emulsion (ILE) group ($n = 10$); and (4) ILE + Alb

dialysis group ($n = 10$). Blood purification therapy was performed for the former 2 groups 30 min after the injection of saline (3.0 mL/kg) and for the latter 2 groups 30 min after the injection of Intralipid™ (3.0 mL/kg) via the femoral vein. A schematic diagram of the in vivo dialysis system is presented in Figure 1b. To circulate blood, a single-lumen cannula was inserted approximately 1 cm into the right carotid artery and left jugular vein and secured. We initially washed the low-capacitance circuit, including the miniaturized haemodialyser, with heparinized saline in the blood circuit and with bicarbonate dialysate in the dialysate circuit prior to each experiment. During the preparation, we also manually adjusted the transmembrane pressure between the 2 circuits so that there was zero net ultrafiltration at the beginning of each experiment. The blood circuit was finally replaced with whole blood from uraemic rats at the beginning of each experiment. The blood flow rate was set at 1.0 mL/min, and the counter-current dialysate flow was 5.0 mL/min. Blood purification therapy was conducted for 240 min with single-pass counter-current dialysate flow. Blood was collected before and after blood purification therapy via the cannula inserted into the right carotid artery, and the dialysate used was collected after dialysis. Serum was obtained by centrifuging for 10 min at 1,503 g. Samples were stored at -80°C until analysis.

Solute removal was calculated by the reduction ratio (RR) on the blood side and total solute removal (TSR) in the dialysate. RR was defined as a function of pre-dialysis (C_{pre}) and post-dialysis (C_{post}) concentrations ($\text{RR} = [1 - (C_{\text{post}}/C_{\text{pre}})] \times 100$). For protein-bound solutes, the concentration of C_{post} was corrected based on the total protein concentration at the start versus at the end of the dialysis session. TSR was calculated by multiplying the dialysate solute concentration by the volume of dialysate used.

Sample Analysis

To establish the total (free + bound) concentrations of PBUTs, samples of HSA-uraemic toxin mixtures, dialysate, and rat serum were deproteinized by the addition of 3 parts of cold methanol to 1 part of samples followed by centrifugation at 10,000 g for 30 min, and then were analysed by reversed-phase high-performance liquid chromatography as described previously [31, 37]. Briefly, a 2- μL aliquot of specimen was injected into an Agilent 1100 high-performance liquid chromatography system (Agilent Technologies, Dover, DE, USA), which was equipped with a Model G1311A quaternary pump, a Model G1322A degasser, a Model G1313A autosampler, and a Model G1314A variable wavelength detector, and the separation for all targets of interests was performed on a reversed-phase C18 column (Kromasil 100–3.5, 150 mm $\times$ 2.1 mm, 3 μm , AkzoNobel, Sweden). For PCS, IS and 3-IAA, the mobile phase used for isocratic elution consisted of 45% solvent A (containing 20 mmol/L of NaH_2PO_4 and 7.0 mmol/L tetrabutyl ammonium iodide as ion-pairing agent in water) and 55% methanol as solvent B at a flow rate of 0.16 mL/min at 30°C . Quantitative detections for IS, 3-IAA, and PCS were monitored using a fluorescence detector (model RF-20, Shimadzu Corp., Kyoto, Japan). The excitation and emission wavelengths were set at 278 and 348 nm, respectively. The retention time was about 4.80 min, 5.83 min, and 7.68 min for IS, 3-IAA, and PCS under these chromatographic circumstances, respectively. For CMPF and HA, the chromatographic conditions were the same as those for IS, 3-IAA, and PCS, except for the ratio of the solvent A changed to 36% and accordingly B to 64% for CMPF, and the ratio of solvent A changed to 72% and accordingly B to 28% for HA. CMPF was monitored at 16.36 min and

HA was monitored at 6.85 min under these chromatographic circumstances, respectively, by UV absorbance at 254 nm. Measurements were calibrated with standard curves for all protein-bound solutes and were linear (all r^2 values >0.999) over the concentration range of the experiments. Samples with concentrations below the minimum levels of the calibration curves were evaporated to dryness in a gentle stream of nitrogen gas and then dissolved in water for reversed-phase high-performance liquid chromatography analysis. Quantitative results were obtained and calculated as concentrations in milligrams per litre. Urea nitrogen and creatinine were measured using standard laboratory methods.

Statistical Analysis

The Kolmogorov-Smirnov test was used to assess the normal distribution of the data. Data are expressed as the means $\pm$ standard deviations and were analysed using one-way analysis of variance followed by Bonferroni's comparison test. Results with $p < 0.05$ were considered statistically significant. The IBM SPSS Statistics 21 program was used for statistical analyses.

Results

PBUTs Removal in the in vitro Single-Pass HD Model

To adjust to the main ingredients of ω -6 soybean oil-based lipid emulsion (Intralipid™), FAs were prepared by mixing oleic acid and linoleic acid ($[\text{linoleic acid}]/[\text{oleic acid}] = 2$) at a final concentration of 1 mmol/L in the inlet blood line of the in vitro HD model based on the established preliminary data [36, 37]. As conformed previously, CMPF, which possesses the highest binding affinity to HSA among the PBUTs tested in the study, was undetectable in the standard bicarbonate dialysate of the control group. Adding BSA (40 g/L) to the dialysate-simulating Alb dialysis significantly increased the removal of CMPF to $2.91 \pm 0.15\%$, whereas 1 mmol/L FAs infusion into the blood line significantly increased the removal of CMPF to $14.33 \pm 0.24\%$ ($p < 0.001$ vs. Alb dialysis). Moreover, the fractional removal of CMPF further increased to $33.31 \pm 0.94\%$ when FAs were infused into the blood line and BSA was added to the dialysate side simultaneously (Fig. 2a).

On the other hand, the fractional removal of PCS, IS, 3-IAA, and HA was $9.51 \pm 0.12\%$, $11.71 \pm 0.22\%$, $15.53 \pm 0.16\%$, and $24.15 \pm 0.34\%$, respectively, in the control group of the in vitro HD model (compared with each other, all $p < 0.001$). These results indicated stronger protein binding is correlated with worse PBUT removal. Figure 2b–d shows that a 2.31-fold increase in PCS removal, a 1.78-fold increase in IS removal and a 1.46-fold increase in 3-IAA removal were achieved by Alb dialysis observed during minutes 12–30 compared with the baseline interval (minutes 0–10). Infusion of 1 mmol/L FAs prefilter increased the removal of PCS, IS, and 3-IAA approxi-

Fig. 2. Time courses of the fractional removal of PBUTs during the in vitro single-pass HD experiments. CMPF (**a**), PCS (**b**), IS (**c**), 3-IAA (**d**), HA (**e**). Control group (red): dialysis with standard bicarbonate dialysate; FAs group (blue): dialysis with FAs infusion into the prefilter blood line during minutes 12–30; Alb group (light green): dialysis with BSA added to the dialysate side during minutes 12–30; FAs + Alb group (dark green): FAs were infused into

the prefilter blood line and BSA was added to the dialysate side simultaneously during minutes 12–30; Each point represents the mean $\pm$ SEM ($n = 4$). PBUTs, protein-bound uraemic toxins; CMPF, 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid; PCS, p-cresyl sulphate; IS, indoxyl sulphate; 3-IAA, indole-3-acetic acid; HA, hippuric acid; FAs, fatty acids; Alb, albumin; BSA, bovine serum albumin; HD, haemodialysis.

mately 3.23-fold, 3.01-fold, and 2.24-fold, respectively, compared with the respective baseline, which were also markedly superior to Alb dialysis (all $p < 0.001$). On the other hand, HA removal was also significantly increased by both infusion of FAs in the blood side (an approximately 1.27-fold increase) and addition of BSA in the dialysate side (an approximately 1.25-fold increase). However, no significant differences in HA removal were noted between the Alb group and FAs group (Fig. 2e). Similar to CMPF, the fractional removal of PCS, IS, 3-IAA, and HA increased more efficiently when FAs were infused into the blood line and BSA was added to the dialysate side simultaneously, suggesting additive or synergistic effects of these 2 approaches (Fig. 2b–e). Infusion of phosphate-buffered saline as control did not lead to an increase in removal of any of the PBUTs among all groups. In addition, no significant differences in the urea extraction ratio were noted among the 4 groups.

PBUTs Removal in Uraemic Rats by HD

In uraemic rats, the RRs of urea and creatinine were $44.33 \pm 3.56\%$ and $40.42 \pm 2.87\%$, respectively, in the control group when HD was performed at a Q_b of 1.0 mL/min and a Q_d of 5.0 mL/min for 240 min using the standard bicarbonate dialysate (Fig. 3a, b). Compared with the 2 small water-soluble molecules, the RRs of PBUTs were significantly reduced in the control group (all $p < 0.05$), except for HA, which had an RR similar to that of non-protein-bound compounds (Fig. 3). Neither Alb dialysis nor lipid emulsion infusion led to a significant change in the RRs of BUN and Scr, whereas both approaches resulted in significantly higher RRs for PCS, IS and 3-IAA compared with the control group (all $p < 0.001$, Fig. 3c–e). Similarly, the highest RRs of PCS IS, and 3-IAA were achieved by infusing lipid emulsion into the blood line and adding BSA to the dialysate side simultaneously.

Fig. 3. RRs of small water-soluble solutes and total PBUTs after 4-h HD therapy. BUN (a); Serum creatinine (b); PCS (c); IS (d); 3-IAA (e); HA (f). *** $p < 0.001$ versus the control group; ## $p < 0.01$ versus the Alb group; §§ $p < 0.01$ versus ILE group; § $p < 0.05$ versus ILE group. PBUTs, protein-bound uraemic toxins; CMPF, 3-carboxy-

4-methyl-5-propyl-2-furan-propanoic acid; PCS, p-cresyl sulphate; IS, indoxyl sulphate; 3-IAA, indole-3-acetic acid; HA, hippuric acid; FAs, fatty acids; Alb, albumin; BSA, bovine serum albumin; HD, haemodialysis; BUN, blood urea nitrogen; ILE, intravenous lipid emulsion; RRs, reduction ratios.

The TSRs of small water-soluble solutes and protein-bound compounds are presented in Figure 4. No significant differences in the TSRs were noted for urea and creatinine among the 4 groups. However, both Alb dialysis and lipid emulsion infusion exhibited significantly increased TSRs for PBUTs, except for HA, compared with the control (all $p < 0.001$). Of note, the corresponding TSR values of PCS and IS were superior in the ILE group compared to the Alb dialysis group (both $p < 0.05$). Finally, we found that the highest TSRs of PCS, IS, and 3-IAA were also achieved by their combination simultaneously.

Discussion

PBUTs are primarily bound to the most abundant plasma protein, namely, albumin, at different binding domains with different binding affinities. Sudlow's sites I and II on HSA are the primary binding sites for most pro-

tein-bound compounds with protein-bound fractions ranging from 10% (e.g., p-cresyl glucuronide) to approximately 100% (e.g., CMPF) [4]. Within the large family of PBUTs, we chose CMPF (240 Da, PB ~100%), PCS (188 Da, PB ≥90%), and IS (213 Da, PB ≥90%) as examples of tightly Alb-bound toxins, 3-IAA (175 Da, PB ~70–75%) as an example with medium binding affinity, and HA (179 Da, PB ~40–50%) as an example with weak binding affinity to HSA. In terms of HSA binding sites, CMPF is described to bind to Sudlow's site I [38], whereas PCS, IS and 3-IAA are reported to bind to Sudlow's site II [39]. HA binds to both site I and site II but relatively more strongly to the latter [40]. Our results suggested that both adding a binder to the conventional dialysate to decrease the free concentrations of PBUTs on the dialysate side and infusing a competitive displacer to increase the free fractions of PBUTs on the blood side could efficiently improve the dialytic removal of PBUTs. Meanwhile, the latter might be superior to the former, especially for those with higher protein binding. In particular, the highest di-

Fig. 4. TSR in the collected dialysate for small water-soluble solutes and total PBUTs after 4-h HD therapy. Urea (**a**); creatinine (**b**); PCS (**c**); IS (**d**); 3-IAA (**e**); HA (**f**). *** $p < 0.001$ versus the control group; * $p < 0.05$ versus the control group; ## $p < 0.01$ versus the Alb group; # $p < 0.01$ versus the Alb group; §§ $p < 0.01$ versus ILE group;

§ $p < 0.05$ versus ILE group. PBUTs, protein-bound uraemic toxins; PCS, p-cresyl sulphate; IS, indoxyl sulphate; 3-IAA, indole-3-acetic acid; HA, hippuric acid; FAs, fatty acids; Alb, albumin; BSA, bovine serum albumin; HD, haemodialysis; ILE, intravenous lipid emulsion; TSR, total solute removal.

alysis efficacy for PBUTs removal was always observed when combining the 2 strategies.

As mentioned above, PBUTs exist in the bloodstream in 2 forms: a bound (non-diffusible) fraction and a free (diffusible) fraction. In most cases, this protein binding involves a dynamic and reversible equilibrium [24–26]. As only the free fraction can cross the HD membrane, the major limiting factors during dialysis for the overall dialytic removal of PBUTs will be a combination of the fact that (i) the speed of equilibration between the bound and free fractions and (ii) the free toxin concentration gradients between blood and dialysate [17, 27, 28, 35]. To circumvent this problem, many attempts have been made, including increasing dialysate flows and dialyser surface area [18, 19], increasing the dialysis duration and/or dialysis frequency [21–23], and adding convection by haemodiafiltration [14, 15]. However, the decrease in serum concentrations remains modest and not sufficient [14–23]. Therefore, to further enhance PBUTs removal, it may

be necessary to consider alternative approaches, such as the use of adsorbent technology. Adsorptive devices can be coupled to the bloodstream or can be directly brought into the dialysate to generate a virtual sink, and thus to maintain a diffusive concentration gradient across the dialyser fibre during the HD session [28–31, 41–45]. So far, 2 major types of adsorbents for the removal of PBUTs have been developed. One is mainly based on the hydrophobic interactions between PBUTs and hydrophobic adsorbents, such as activated carbon, cellulose beads, and liposome, and the other is based on the size selectivity of porous materials, such as zeolite materials, metal-organic frameworks, and poly-cyclodextrin adsorbents. In vitro HD experiments have revealed that the addition of activated charcoal to the dialysate could significantly increase the clearance of PCS compared to conventional HD, and mathematical modelling predicted that the maximal effect of the addition of a sorbent to the dialysate is equivalent to that of an unlimited increase in Q_d [28]. Given that

carbon has poor biocompatible properties, and thus hampers clinically safe applications, we selected Alb as an adsorbent-simulating sorbent technology in this study. Results showed that adding Alb to the dialysate significantly increased the removal of protein-bound solutes without greatly altering that of unbound solutes both in vitro and in vivo.

On the other hand, although adsorptive-based HD can be efficient in the removal of the available predialysis-free fraction, its ability to restore the bound and free fraction equilibrium during dialysis to achieve continuous free toxin adsorption may remain a challenge given that a significant increase in the percentage protein binding occurred during HD sessions [35]. Another potential strategy that has been developed with the aim of improving the efficiency of PBUTs removal from plasma is to change physical conditions within the dialyser to enhance diffusive removal of PBUTs by triggering the release of toxins from Alb and increasing their free fractions [32–34]. For example, Böhringer et al. [32] demonstrated that infusion of a hypertonic saline solution directly into the prefilter replacement fluid to enhance the ionic strength of plasma facilitated the release of solutes from Alb, and thus significantly increased the removal of some PBUTs in vitro. However, only clearance of free IS was significantly increased by this technique in a pilot clinical study, where the mass of all protein-bound toxins collected in spent dialysate was not significantly increased compared with conventional HD [46]. Later, Tao et al. [33] tested the displacement of IS and PCS by infusion of 3 Alb-binding competitors, ibuprofen, tryptophan, and furosemide, each at a concentration of 1 $\mu\text{mol/L}$. Results showed that ibuprofen, which possesses the highest binding affinity among the displacers tested, increased the free fractions of both IS and PCS in uraemic plasma by approximately 3-fold followed by tryptophan and furosemide. In the same study, they found that the simultaneous use of furosemide and ibuprofen significantly improved the removal of IS and 3-IAA by respective 2.9- and 2.1-fold ratios in an in vitro HD model. Madero et al. [34] further reported that infusion of ibuprofen into the arterial blood line during HD significantly increased the dialytic removal of IS and PCS, and thereby led to a greater reduction in their serum levels in 18 stable uraemic HD patients. Recently, Li et al. [47] reported on binding competitors obtained from natural products of phytomedicine, namely, Danhong injection. In vivo studies performed in chronic kidney disease rats showed that infusion of Danhong significantly increased the dialytic removal of IS and PCS. Despite these positive results reported on the increased

clearance of certain PBUTs by Alb displacers, it is noticeable that the mechanisms and locations of protein binding are not the same for all compounds belonging to the PBUTs family; therefore, not all toxins are equally affected by a certain Alb displacer. For example, ibuprofen, which binds to Sudlow site II, will not displace CMPF, which binds to Sudlow site I, but can compete with IS and PCS [13]. As the understanding of protein binding improves, it is apparent that to achieve removal of a broad spectrum of PBUTs through this approach, concomitant use of multiple displacers is necessary, taking into account clinically acceptable plasma concentrations. However, the simultaneous infusion of multiple displacers may increase the risk of toxicity with a more unpredictable global effect [26, 48].

Lipid emulsions have been used as parenteral nutrition with FDA indications for caloric supplementation and essential FA deficiency [49]. FFAs are released from the emulsion by lipoprotein lipases in blood, and serum Alb is able to bind up to 9 equivalents of long-chain FAs at multiple binding sites from FA1 to FA9, including both Sudlow sites I and II [50–53]. In a recent study, we chose CMPF as a representative with high binding affinity at site I of HSA as well as PCS and IS as prototypes tightly binding at site II of HSA. Then, we evaluated the effects of higher ionic strength, pH change, and different Alb displacers on the dissociation of these PBUTs from Alb simultaneously in vitro [36]. Results showed that the inhibitory effects were more remarkable with the addition of binding competitors than that of ionic strength and pH changes. In addition, the protein binding of CMPF, PCS, and IS decreased most remarkably in the presence of FFAs, especially oleic acid and linoleic acid, compared to all studied drugs. In the present study, we found that FFA displacement-based HD markedly increased the removal of all tested PBUTs in both in vitro and in vivo dialytic experiments, with a dialysis efficacy even superior to Alb dialysis. These data suggested that both adding an adsorbent to the dialysate side and infusing a competitive displacer in the blood line could efficiently improve the removal of PBUTs with a better dialysis efficacy in the latter compared with the former, especially for toxins with higher protein binding. These findings are consistent with the model prediction of Maheshwari et al. [54], who provided an in silico comparative assessment of the efficacy of different extracorporeal euration modalities for PCS and IS removal, and concluded that competitive binding substantially improved PCS and IS removal outstrip conventional HD, pre-HDF, post-HDF, and ideal membrane adsorption. The

latter scenario was considered equivalent to hypothetical infinite dialysate flow, which will result in zero toxin concentration in the dialysate. Finally, we further found that the highest dialysis efficacy for highly bound solute removal was always observed when combining displacement and adsorption in both in vitro and in vivo dialyses. Although more complex than the standard procedure due to some technical reasons, this approach offered proof of concept that the removal of PBUTs in the conventional dialysis setting has not yet reached its maximum capacity.

Our study has some limitations. First, a desirable displacer should at least possess the following properties: (1) it can significantly inhibit the binding of a broad spectrum of PBUTs to HSA, involving toxins bound to both Sudlow's site II and Sudlow's site I; (2) it should be quite safe and suitable for repeated administration in dialysis patients. Second, as an initial proof of concept, our in vivo experiments in uraemic rats were performed only in 1 session. Long-term effects of the combined displacement- and adsorption-based dialysis technique on these PBUTs levels could not be assessed. Third, it remains unclear what the potential side effects of long-term treatment with this technique would be in HD patients. Future efforts will have to focus on the efficacy and safety of this strategy for long-term clinical practice.

Conclusion

In conclusion, both adsorbent technology to decrease the free concentrations of PBUTs on the dialysate side and a competitive displacement approach to increase the free fractions of PBUTs on the blood side could efficiently improve the dialytic removal of PBUTs. Meanwhile, the dialysis efficacy of the latter might be superior to that of the former, especially for toxins with higher protein binding. As a host of compounds is retained in uraemia, it seems best to focus on removing a broad spectrum of

PBUTs by a certain Alb displacer, which is similar to the FFA used in our study. However, future studies are required to identify more desirable binding competitors that are both efficacious and safe for long-term routine clinical use. In addition, because the toxicity of many PBUTs follows a dose-response gradient, further steps to decrease the levels of uraemic toxins appear to be necessary, and the concept of combined displacement- and adsorption-based dialysis may open up new avenues and possibilities in the field of dialysis to further enhance PBUT removal in end-stage renal disease.

Statement of Ethics

All animal experiments were performed with approval from the Institutional Animal Care and Use Committee of Shanghai JiaoTong University School of Medicine (HKDL [2018] 38).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

F.D. conceived and designed the research. Y.Y.S. and J.Y.X. performed the research. Q.Y.Z. and H.J.T. performed the HPLC measurements. Y.F.W. and Y.S. analysed the data. Y.Y.S. wrote the manuscript. All authors read and approved the final manuscript.

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