Renal Replacement Therapy

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Hemoglobin-dependent differences in the antioxidant properties of uric acid and albumin: an in vitro analysis

Yoshihiro Tange[1,2*], Hibiki Sakai[1] and Tadashi Tomo[2]

Abstract

Background Oxidative stress is a major contributor to morbidity in patients with end-stage kidney disease. The antioxidant properties of vitamin E-coated hemodialyzers have attracted increasing attention. However, the intrinsic antioxidant capacity of dialysis-related substances themselves remains unclear despite their potential relevance to redox balance during hemodialysis. In this study, we aimed to clarify the antioxidant behavior of several clinically relevant compounds, including uric acid, ascorbic acid, glutathione, glycerin, and recombinant human albumin, by evaluating their ability to suppress chemiluminescence generated by Fenton's reagent ($FeSO_4$ and H_2O_2) in aqueous media.

Methods To assess the influence of hemoglobin, each antioxidant was dissolved in distilled water or a 3 μM human hemoglobin solution. Additionally, recombinant human albumin was tested in the presence of 1, 2, and 30 μM hemoglobin. A 50 μL sample was mixed with 100 μL of Fenton's reagent, and luminescence intensity was quantified using a luminometer. The concentrations of $FeSO_4$ and H_2O_2 were 1 and 10 mM, respectively. The half-maximum inhibitory concentration (IC_{50}) and its negative logarithm (pIC_{50}) were calculated from sigmoidal dose–response curves, and relative activities were compared with those of glycerin, a known hydroxyl radical scavenger.

Results In distilled water, glutathione, ascorbic acid, and uric acid exhibited approximately 100-, 1000-, and 20,000-fold higher antioxidant activities, respectively, than glycerin. Although uric acid substantially suppressed chemiluminescence in distilled water, it showed no antioxidant effect in hemoglobin-containing solution. In contrast, recombinant human albumin increased luminescence intensity when tested alone but markedly suppressed luminescence in the presence of hemoglobin. Based on pIC_{50} values, antioxidant capacity in distilled water decreased in the order uric acid > ascorbic acid > glutathione > glycerin. Albumin showed clear antioxidant activity under hemoglobin-containing conditions, most likely attributable to its heme-binding ability.

Conclusions Uric acid, ascorbic acid, and glutathione exhibited strong antioxidant activity against Fenton's reagent in aqueous systems, with uric acid showing the highest potency. Furthermore, recombinant human albumin showed antioxidant activity, most likely through heme sequestration. These findings provide mechanistic insights into the strongly context-dependent antioxidant behavior of dialysis-related solutes. Further validation in ex vivo and clinical studies is required.

Keywords Uric acid, Ascorbic acid, Glutathione, Glycerin, Albumin, Antioxidant activity, Hemoglobin

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Background

By 2030, approximately 5.4 million patients with renal failure are expected to require dialysis, and this number is projected to continue rising [1]. When kidney function declines, uremic toxins cannot be excreted, leading to uremia. Hemodialysis (HD) and hemodiafiltration (HDF) are used to treat end-stage kidney disease (ESKD). HD and HDF must be performed three times a week for at least 4 h per session for the rest of the patients' lives. The dialysis membrane used in HD and HDF is in direct contact with blood; therefore, it must be highly biocompatible [2]. Poor biocompatibility induces oxidative stress and radical chain reactions, which promote organ injury. Kidney disease is also linked to accelerated aging [3]. The prognosis of patients undergoing dialysis has improved because of the dialysis fluid used in HD and HDF [4] and advances in dialysis membranes. However, no unified method exists for evaluating the antioxidant capacity of biological antioxidants in kidney disease pathology, and no established method exists for stopping radical chain reactions during HD and HDF. Consequently, patients undergoing dialysis experience high levels of oxidative stress.

The strong antioxidant properties of vitamin E-coated hemodialyzers have attracted considerable attention, and the number of substances targeted for removal in HD/HDF therapy is increasing. In our recent study, we demonstrated that coating a dialysis membrane with vitamin E enhanced its antioxidant capacity, as shown using an experimental system with Fenton's reagent [5]. Many patients undergoing dialysis suffer from anemia and, therefore, receive frequent iron (Fe) supplementation [6]. Excess iron can lead to oxidative stress and accelerate aging [7]. In kidney disease models, the Fenton reaction, in which iron reacts with hydrogen peroxide (H_2O_2) to generate highly reactive hydroxyl radicals (·OH), is likely to occur ($H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$). However, to our knowledge, no study has systematically evaluated the effects of antioxidants on kidney disease using a standardized experimental system. Meyer et al. [8] reported that cell-free hemoglobin (Hb) levels were 196 ± 43 mg/L (approximately 3 μM) predialysis and 285 ± 109 mg/L postdialysis. In addition, Cho et al. [9] reported that cell-free Hb levels in patients undergoing HD were 54.6–116.7 mg/L. Therefore, in this study, we aimed to simulate a kidney disease model and quantitatively compare the antioxidant activities of various compounds with Fenton's reagent in aqueous media. Furthermore, we aimed to examine Hb concentrations of 1, 2, and 3 μM—reflecting the levels reported in patients undergoing dialysis, and of 30 μM, which simulates severe hemolysis. At 1, 2, and 30 μM Hb, only albumin was evaluated, because these conditions were used specifically to characterize the albumin response across the Hb range.

Methods

Materials

Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), H_2O_2, luminol, uric acid, ascorbic acid, glutathione, glycerin, and recombinant human albumin were purchased from Fujifilm Wako Pure Chemical Co. Ltd. (Tokyo, Japan). Diethylenetriamine-N,N,N,N,N-pentaacetic acid (DTPA) was purchased from Dojindo Laboratories (Kumamoto, Japan). A 70% solution of *tert*-butyl hydroperoxide (*tert*-BuOOH) and lyophilized Hb powder were purchased from Sigma-Aldrich, LLC (Steinheim, Germany). Distilled water was purchased from Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan).

Preparation of Fenton's reagent

Fenton's reagent was prepared by dissolving $FeSO_4 \cdot 7H_2O$ and DTPA in phosphate buffer (PB, pH 7.4), each to a concentration of 2 mM. An equal volumes of 20 mM H_2O_2 was then added, giving final concentrations of 1 and 10 mM $FeSO_4$ and H_2O_2.

Sample preparation

Distilled water and Hb solutions of 1, 2, 3, and 30 μM were used as solvents. To prepare stock solutions, uric acid, ascorbic acid, glutathione, glycerin, and recombinant human albumin were adjusted to 0.45, 100, 100, 6.85, and 0.6 mM, respectively. The resulting stock solutions were subsequently diluted with the solvent to obtain the final samples. The Hb concentrations used in this study (1–30 μM) are several orders of magnitude lower than the intracellular concentration in erythrocytes (approximately 5 mM); however, the mean plasma free Hb concentration in patients undergoing dialysis has been reported to be approximately 3 μM [8]. Therefore, the experimental conditions in this study mimic the low levels of extracellular Hb resulting from microhemolysis or extravasation.

Measurement of luminescence intensity

Antioxidants, including uric acid, ascorbic acid, glutathione, and glycerin, were dissolved in distilled water and a 3 μM Hb solution. Additionally, human Hb was added at 1, 2, and 30 μM to the samples containing recombinant human albumin. A 50 μL sample was injected into each microplate well, and 100 μL of Fenton's reagent was then added. Immediately before measurement, 25 μL of 0.1% luminol plus 25 μL of 0.14% *tert*-BuOOH were added to each well. After mixing samples with the Fenton's reagent, the pH of the final reaction mixture was estimated to be 7.4. Reactions were

conducted at 25 °C, and luminescence was recorded 30 s after albumin was mixed with Fenton's reagent. The Fe^{2+}/H_2O_2 Fenton's reagent was freshly prepared immediately before each experiment. Background luminescence was determined using a blank well and subtracted from all measurements. The luminescence intensity was measured at 1-min intervals using a luminometer (GloMax® Navigator, Promega Corporation, Madison, WI, USA), and the area under the curve (AUC) over 60 min was used to quantify the amount of oxidation generated [5].

Luminescence was normalized to relative ratios using the following formula:

$$\text{Luminescence ratio} = (\text{AUC of the sample})/(\text{AUC of the solvent}) \times 100$$

For each Hb condition (0, 1, 2, 3, and 30 μM), the luminescence intensity of the antioxidant-free reaction mixture was defined as 100%, and antioxidant-containing samples were expressed as relative percentages of this control. For all Hb concentrations (0, 1, 2, 3, and 30 μM), the recorded relative luminescence unit (RLU) values remained well below the saturation threshold of the detector, confirming that luminescence varied linearly with logarithm Hb concentration tested.

The half-maximum inhibitory concentration (IC_{50}), the negative logarithm of the IC_{50} (pIC_{50}), the 95% confidence interval (95% CI), and maximal effect (E_{max}, defined as the percentage inhibition of luminescence relative to that of the solvent) were calculated from the luminescence ratios for each sample using a sigmoidal dose–response curve. All experiments were performed as $N=6$ independent biological replicates, using freshly prepared reagents. Each replicate consisted of a complete reaction setup and luminescence measurement performed independently. Each parameter obtained from the dose–response curve was calculated using the method described by Allijn et al. [10]. Dose–response curves were fitted using a three-parameter logistic model with the bottom based on the observed minimal response. IC_{50} values represent the sample concentration at which the response is halfway between 100% and the bottom. The IC_{50} values and pIC_{50} were calculated using GraphPad Prism (version 10; GraphPad Software Inc., La Jolla, CA, USA). Because luminescence was enhanced by albumin alone, an IC_{50} value could not be defined for that condition; IC_{50} values were therefore calculated solely from the inhibitory phase observed in the presence of Hb.

Results

Based on the IC_{50} and pIC_{50} values, uric acid had the highest antioxidant capacity, followed by ascorbic acid, glutathione, and glycerin in distilled water. The calculated IC_{50} values for uric acid, ascorbic acid, glutathione, and glycerin in distilled water were approximately 26.6, 647, 6067, and 571,000 μM, respectively (Table 1).

Table 1 Antioxidant capacity of each solute in distilled water

Compound	IC_{50} (μM)	95% CI (μM)	E_{max} (%)	R^2	$pIC_{50} \pm SE$
Uric acid	26.6	19.5–35.8	90	0.94	4.58 ± 0.07
Ascorbic acid	647.1	526–794	99	0.98	3.19 ± 0.04
Glutathione	6067	4323–8638	100	0.89	2.22 ± 0.08
Glycerin	571,000	487,200–666,500	84	0.99	0.24 ± 0.03

IC_{50}, half-maximum inhibitory concentration; 95% CI, 95% confidence interval; E_{max}, maximal effect; SE, standard error. Compounds with an $E_{max} < 30\%$ and/or $R^2 < 0.70$ were considered not to be inhibitory even if an IC_{50} could be calculated

The calculated IC_{50} values for ascorbic acid and glutathione in the 3 μM Hb solution were approximately 2635 and 592 μM, respectively. For uric acid and glycerin, the E_{max} was < 30%, and no obvious luminescence suppression was observed. Based on pIC_{50} values, glutathione showed higher antioxidant activity than ascorbic acid in the 3 μM Hb solution (Table 2).

The luminescence intensity of recombinant human albumin increased upon exposure to Fenton's reagent, with no detectable antioxidant activity (Fig. 1). Because albumin enhanced luminescence in the absence of Hb, an IC_{50} value based on a monotonic decrease could not be defined. IC_{50} values were therefore calculated only for the conditions in which the Hb concentration was 1 μM or higher, where albumin showed a luminescence-inhibiting (antioxidant) phase. In the presence of Hb, antioxidant activity was observed (Fig. 2), and the IC_{50} values were 60.2, 79.6, 75.5, and 195.1 μM at 1, 2, 3, and 30 μM of Hb, respectively (Table 3). Based on pIC_{50} values, the antioxidant activity of albumin was higher at 1 μM Hb than at 2, 3, and 30 μM. In the absence of Hb (0 μM Hb), albumin enhanced chemiluminescence, acting as a prooxidant. Conversely, at Hb concentrations of 1 μM or higher, albumin suppressed luminescence, functioning as

Table 2 Antioxidant capacity of each solute in the 3 μM Hb solution

Compound	IC_{50} (μM)	95% CI (μM)	E_{max} (%)	R^2	$pIC_{50} \pm SE$
Uric acid	–	–	< 30	–	–
Ascorbic acid	2635	1972–3523	100	0.94	2.58 ± 0.08
Glutathione	592.4	519.2–671.8	100	0.99	3.23 ± 0.03
Glycerin	–	–	< 30	–	–

IC_{50}, half-maximum inhibitory concentration; 95% CI, 95% confidence interval; E_{max}, maximal effect; SE, standard error. Compounds with an $E_{max} < 30\%$ and/or $R^2 < 0.70$ were considered not to be inhibitory even if an IC_{50} could be calculated

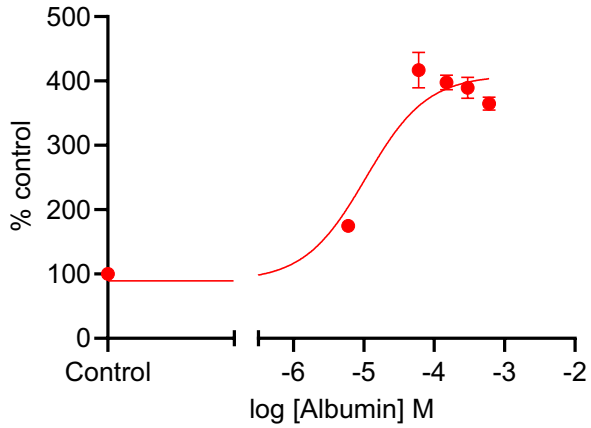


Fig. 1 Chemiluminescence response of recombinant human albumin in distilled water. The chemiluminescence values obtained at various concentrations were normalized to those of the blank (solvent) sample ($N = 6$, mean ± SD). When albumin was used alone, the luminescence intensity increased with increasing concentration, and no antioxidant activity was observed. SD, standard deviation

an antioxidant. Therefore, the action of albumin in this assay system is interpreted as a biphasic dose–response, characterized by a functional switch from prooxidant to antioxidant activity in the presence of Hb.

Discussion

Hydroxyl radicals (·OH) are highly reactive oxidizing agents that are continuously generated in vivo during metabolic processes [11] and can be produced via the Fenton reaction, which involves the reaction of Fe^{2+} with H_2O_2. Patients with ESKD often experience anemia, and oxidative stress caused by iron supplementation is a major concern [6, 12]. Iron supplements given to patients undergoing dialysis can induce the Fenton reaction in vivo. To date, no standardized method exists for evaluating antioxidant activity, as radical-scavenging capacity varies depending on the antioxidant used. In this study, we evaluated the activity of antioxidants in a simulated kidney disease model using Fenton's reagent.

Some substances, including uric acid, exhibit antioxidant properties in patients with kidney disease. In aqueous experiments, the biological antioxidant potential increases with increasing concentrations of uric acid and ascorbic acid [13]. In the current study, the relative activity ratio of uric acid, calculated on a molar basis, was approximately 20,000 times that of glycerin, a hydroxyl

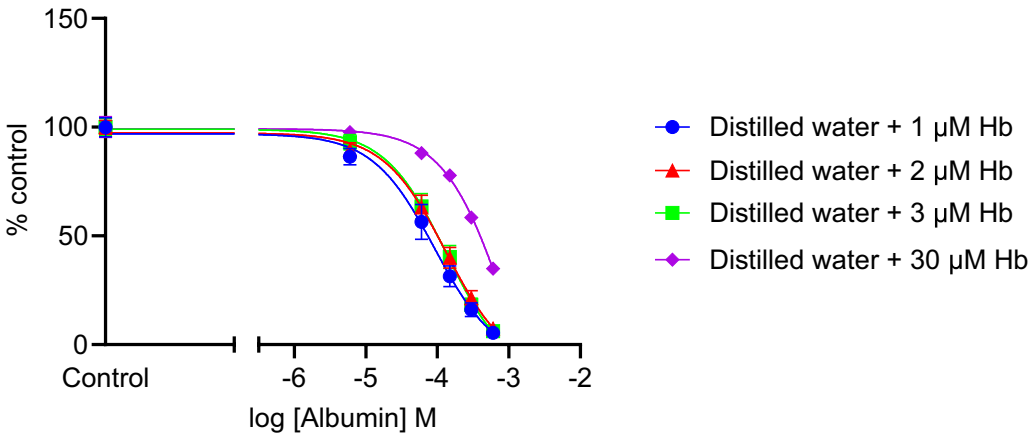


Fig. 2 Chemiluminescence of recombinant human albumin in distilled water containing hemoglobin. The chemiluminescence values obtained at various concentrations were normalized to those of the blank (solvent) sample ($N = 6$, mean ± SD). Hemoglobin was present at 1, 2, 3, and 30 μM. The luminescence intensity decreased with increasing albumin concentration, confirming its antioxidant activity. SD, standard deviation

Table 3 Antioxidant capacity of recombinant albumin in Hb solution

Compound	Solvent	IC_{50} (μM)	95% CI (μM)	E_{max} (%)	R^2	pIC_{50} ± SE
Albumin	1 μM Hb	60.2	50.8–71.0	96	0.97	4.22 ± 0.04
	2 μM Hb	79.6	68.5–95.5	95	0.97	4.10 ± 0.03
	3 μM Hb	75.5	63.9–89.0	96	0.97	4.12 ± 0.04
	30 μM Hb	195.1	158.7–241.3	67	0.92	3.71 ± 0.05

IC_{50}, half-maximum inhibitory concentration; 95% CI, 95% confidence interval; E_{max}, maximal effect; SE, standard error; Hb, hemoglobin

radical scavenger [14–16]. Confirming that uric acid has strong antioxidant properties. As uric acid is a small molecule (molecular weight of 168), it can be easily removed by dialysis (> 70%). However, the solubility of uric acid in plasma is limited to approximately 8.0 mg/dL (480 μM). In addition, it is a causative agent of gout; therefore, its removal by dialysis is necessary in patients with ESKD. In the solution containing Hb, the E_{max} of uric acid was < 30%, indicating that its antioxidant activity was lost in the presence of Hb.

Ascorbic acid exhibits strong antioxidant activity [17]. In the current study, the antioxidant activity was approximately 1000 times higher than that of glycerin in distilled water. The clinical benefits of ascorbic acid treatment in patients with kidney disease are thought to be due to its antioxidant activity [18–21]. Although the decrease in antioxidant capacity after dialysis [13] is generally not attributed primarily to the loss of small molecules, such as uric acid and ascorbic acid, our experiments using Fenton's reagent suggest that their removal may contribute to this effect. The decrease in pIC_{50} in the solution containing Hb may be because ascorbic acid reduces heme, thereby promoting the Fenton reaction and increasing luminescence intensity.

Glutathione is a strong antioxidant [22]. Although the antioxidant capacity is lower than that of uric acid and ascorbic acid, it is approximately 100 times that of glycerin. The high pIC_{50} of glutathione in the Hb solution may be due to the function of glutathionylated Hb [23]. Both ascorbic acid and glutathione are readily soluble in water at 20 °C; therefore, these compounds could be used to develop infusion fluids with antioxidant properties.

The increased luminescence intensity of recombinant human albumin indicated a low antioxidant capacity against Fenton's reagent. Ivanova et al. [24] conducted a similar study and reported that the addition of low concentrations of albumin to Fenton's reagent did not affect chemiluminescence, whereas higher albumin levels enhanced luminescence through reactions with peroxyl radicals. In their system, luminescence increased up to ten-fold at the highest albumin concentrations tested. These findings are similar to those observed in the present study. Dissolving albumin in distilled water versus preparing it in PB creates different solution environments, which may affect the redox state of iron and albumin–metal interactions. As an additional experiment to evaluate these effects, the same chemiluminescence assay was performed under conditions with albumin dissolved directly in PB (Additional File 1). In that experiment, albumin showed luminescence-enhancing (prooxidant) behavior even under PB dissolution conditions. Therefore, we confirmed that the luminescence-enhancing response of albumin alone is reproducible and independent of the solvent (distilled water versus PB). In contrast, in the presence of Hb, a marked inhibition of luminescence by albumin was observed. The pIC_{50} decreased as the Hb concentration increased. When the Hb concentration reached 30 μM, the inhibitory effect of albumin on luminescence diminished, and the E_{max} was 67%. These data indicate that the antioxidant effect of albumin depends on the presence of Hb but is subject to an upper limit. To aid interpretation, a schematic illustration has been included to summarize the hypothetical model of the interaction among Hb, reactive oxygen species (ROS), and albumin. This model is intended as a conceptual framework and should be viewed as a hypothesis rather than a mechanism directly demonstrated in the present experiments (Fig. 3). Therefore, the interpretation that albumin attenuates luminescence through heme sequestration should be considered a proposed mechanism rather than an experimentally verified conclusion. Additional studies using purified heme, spectroscopic heme-binding assays, or competitive heme transfer experiments will be required to confirm this mechanism.

Meyer et al. [8] reported that cell-free Hb levels were approximately 3 μM predialysis, consistent with the present study (3 μM Hb). These values are much higher than the 0.2 μM in healthy individuals [25], suggesting that patients with ESKD have higher free-heme concentrations. Okada et al. [26] reported that the extent of albumin leakage during dialysis may be associated with mortality. Therefore, controlled increases in albumin loss, along with promotion of albumin regeneration, could enhance antioxidant capacity and potentially improve clinical outcomes.

This study provides a unified method for comparing the antioxidant capacities of substances associated with kidney disease. As Nakayama et al. [13] noted, these solutes are primarily low-molecular-weight substances, except for albumin, and are therefore removed in large amounts during blood purification therapy. This removal may contribute to the decrease in the antioxidant capacity observed during dialysis.

The first limitation of this study is its in vitro, single-component design. Nevertheless, this design allowed antioxidant activity to be evaluated in isolation and showed that albumin exhibits antioxidant properties in the presence of Hb. In the body, the presence of many other substances may give rise to more complex reactions. The biphasic redox response of albumin observed in this study reflects the response to the low concentrations of Hb (approximately 3 μM) that may be present in plasma and does not directly mimic the high Hb concentrations found within erythrocytes. Therefore, while the findings of this study are useful for understanding the redox effects of cell-free Hb in patients undergoing

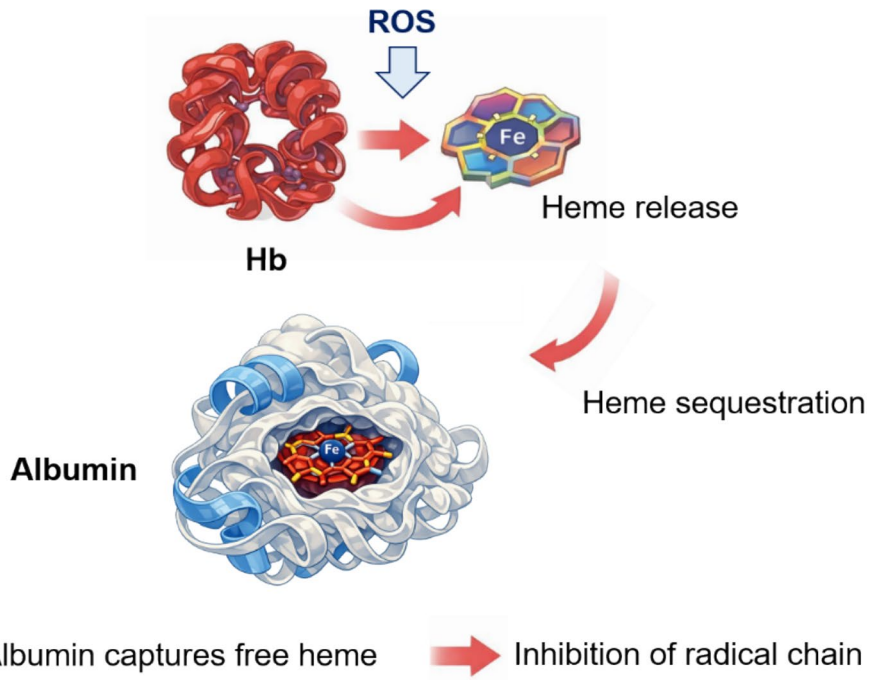


Fig. 3 A hypothetical model of the interaction among Hb, ROS, and albumin. The diagram shows hypothesized redox pathways based on prior literature and the in vitro findings of this study. Hb, hemoglobin; ROS, reactive oxygen species; Fe, iron

dialysis and in individuals with hemolytic diseases, caution is required when extrapolating these results to the behavior of intracellular Hb.

Second, this study used lyophilized hemoglobin as a standardized reagent. However, such preparations may differ from freshly isolated, red-blood-cell-derived Hb in oxidation state, in particular in methemoglobin (MetHb) content, and in heme lability, which could influence both oxidative reactions and heme scavenging by albumin.

As this study was conducted entirely in vitro, the implications for oxidative stress during renal replacement therapy should be interpreted with caution. The experimental system did not include dialysis membranes, circulating antioxidants, or patient-derived samples, and therefore does not directly model the complexity of in vivo dialysis conditions. The present findings should be viewed as mechanistic insights into redox interactions among dialysis-related solutes under controlled aqueous conditions, rather than evidence of clinical oxidative stress. Further validation in ex vivo dialysis models and clinical studies will be necessary to determine whether similar interactions occur in patients undergoing renal replacement therapy.

Conclusions

Uric acid, ascorbic acid, and glutathione exhibited markedly higher antioxidant activity than glycerin, with approximately 20,000-, 1000-, and 100-fold greater potency, respectively. Recombinant human albumin showed minimal antioxidant activity in isolation but substantial suppression of Fenton-derived luminescence in the presence of Hb. This phenomenon may be attributable to the sequestration of heme by albumin.

These findings highlight that the antioxidant behavior of dialysis-related solutes is strongly context-dependent and governed by distinct mechanisms, including direct radical scavenging and heme sequestration. The results provide mechanistic insight into oxidative stress during hemodialysis, particularly under conditions of microhemolysis and free-heme accumulation, and may contribute to the development of strategies aimed at improving redox balance in patients undergoing renal replacement therapy.

Abbreviations

AUC	Area under the curve
CI	Confidence interval
DTPA	Diethylenetriamine-N,N,N,N,N-pentaacetic acid
E_{max}	Maximal effect
ESKD	End-stage kidney disease
IC_{50}	Half-maximum inhibitory concentration
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HD	Hemodialysis
HDF	Hemodiafiltration
MetHb	Methemoglobin
PB	Phosphate buffer
pIC_{50}	Negative logarithm of the half-maximum inhibitory concentration value
RLU	Relative luminescence unit

ROS	Reactive oxygen species
SD	Standard deviation
SE	Standard error
tert-BuOOH	*tert*-Butyl hydroperoxide

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41100-026-00765-5.

Additional file 1.

Additional file 2.

Additional file 3.

Additional file 4.

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Author contributions
Y.T.: Conception, analysis, data collection, assembly, manuscript drafting, and supervision. H.S.: Data collection and assembly. T.T.: Supervision. All the authors have read and approved the final version of the manuscript.

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Availability of data and materials
The data supporting the findings of this study are available in the Supporting Information of this article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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