Effects of ferric citrate on intracellular oxidative stress markers after hydrogen peroxide treatment of human U937 monocytes

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Abstract

Phosphate binders, such as iron (III) citrate hydrate (FCH), are essential medications for hemodialysis patients. Some *in vivo* studies have demonstrated that FCH prevented induction of oxidative stress in the presence of transferrin. However, how FCH affects iron-related oxidative stress in the absence of transferrin remains unclear. In the current study, we investigated the effects of ferric citrate (FC) on oxidative stress in the absence of transferrin *in vitro* to address this question. Human U937 monocytes were pretreated with FC, iron (II) chloride tetrahydrate (FeCl$_2$・4H$_2$O), iron (III) chloride hexahydrate (FeCl$_3$・6H$_2$O), or saccharated ferric oxide for 24 h and then treated with 10-mM hydrogen peroxide (H$_2$O$_2$) for 30 min. The final Fe concentrations were adjusted to approximately 200 μg/dl. Iron concentration, intracellular reactive oxygen species (ROS) levels, and intracellular lipid peroxidation of the cell membrane were measured. After treatment with FC, iron concentration and ROS levels increased. Change in lipid peroxidation after treatment with FC was not observed. However, after treatment with H$_2$O$_2$, no change was observed in the intracellular ROS levels in FC-pretreated cells, whereas lipid peroxidation of the cell membrane was decreased. Despite the high iron concentration in FC-pretreated cells, neither intracellular ROS nor cell membrane lipid peroxidation levels were increased with H$_2$O$_2$ treatment. Their results might represent antioxidative effects of FC. The results of this study may contribute to a better understanding of the effects of oxidative stress in hemodialysis patients treated with FCH.

Key words : iron (III) citrate hydrate, human U937 monocytes, hydrogen peroxide, reactive oxygen species, diphenyl-1-pyrenylphosphine

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Introduction

Hemodialysis patients take 3,500 mg of phosphorus per week, and hemodialysis treatment administered three times a week removes approximately 3,000 mg of phosphorus. Therefore, to prevent the absorption of dietary phosphorus from the intestinal tract, phosphate binders are administered to hemodialysis patients, thereby preventing hyperphosphatemia, which could cause various complications such as calcification, fractures, and itching. Oxidative stress, an important atherosclerosis-related factor, is high among hemodialysis patients[1]. Thus, cardiovascular disease prevention could be achieved through treatments that reduce oxidative stress.

Iron is absorbed as a divalent ion through the divalent metal transporter 1 (DMT-1) in intestinal cells ; iron reductase and citric acid reduce trivalent iron to its divalent form and promote iron absorption[2]. Iron transfer from intracellular endosomes requires DMT-1 and acidification of the endoplasmic reticulum, which may be induced by citric acid. Furthermore, by increasing the synthesis of aconitase (an iron response protein), which is one of the enzymes in the citrate cycle and increases transferrin and ferritin synthesis, citric acid may increase intracellular iron concentration.

Iron (III) citrate hydrate (FCH) is a phosphate binder that also alleviates anemia[3-5] by increasing iron absorption in anemic and chronic kidney disease patients. Generally, the superoxide-assisted Fenton reaction (Haber–Weiss reaction) catalyzed by iron *in vivo* produces hydroxyl radicals, which cause tissue damage and increased levels of oxidative stress[6, 7]. In previous studies in which FCH was administered to hemodialysis patients, oxidative stress or inflammation after dosing was not increased[8, 9]. Indeed, the administration of FCH to mice partially alleviates oxidative stress in kidney tissue *in vivo*[10]. Oral FCH prevents oxidative stress induction compared to intravenous sugar iron oxide administration[11, 12]. All of these findings suggest that binding to transferrin determines protection from oxidative stress ; however, the direct effects of FCH in the absence of transferrin were not examined by these studies.

However, nontransferrin-bound iron is rarely found in serum ; nontransferrin-bound iron produces hydroxyl radicals via the Fenton reaction and increases oxidative stress[13]. Therefore, to study more effects through iron-related oxidative stress, which is difficult to assess *in vivo* because of the transferrin in the serum, a nontransferrin condition is necessary. We aimed to investigate the effects of ferric citrate (FC) on oxidative stress using an *in vitro* model in this study as it is difficult to determine the direct antioxidant effects of FCH *in vivo* because of the presence of transferrin in the serum. Moreover, human U937 monocytes of macrophage-based cells are used in this study because macrophages accumulate excess iron in the cells.

Materials and methods

Study materials and cell culture

All chemicals used in this study were of the purest grade available commercially. FC ($C_6H_5FeO_7$) (F3388) was purchased from Sigma-Aldrich Japan

G.K., Tokyo, JAPAN. Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), 99.9% ($FeCl_2$) (091-04412) ; iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), 99.9% ($FeCl_3$) (090-02802) ; citric acid (C6H8O7) (038-06925) ; and hydrogen peroxide (H_2O_2) (080-01186) were purchased from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. Saccharated ferric oxide ($[Fe(OH)_3]_m[C_{12}H_{22}O_{11}]_n$) (product name, FESTIN), which contains 20 mg/ml of Fe, was obtained from Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan. We selected $FeCl_2$, $FeCl_3$, and SFO, representative iron agents, as the controls for FC. Phorbol-12-myristate 13-acetate (PMA) was purchased from Abcam KK, Tokyo, Japan, and human U937 monocytes (JCRB9021) were obtained from the Japanese Collection of Research Bioresources cell bank in the National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan. All the operations were performed under sterile conditions. The cells were cultured at a density of 1×10^6 cells/ml, using routine methods, in Roswell Park Memorial Institute medium from Wako Pure Chemical Industries Ltd., Osaka, Japan containing L-glutamine and phenol red with 10% fetal bovine serum (BIOSERA FRANCE SAS, Nuaille, France), 100-nM PMA, and 1% of antibiotic-antimycotic solution from Life Technologies Japan Co. Ltd., Tokyo, Japan, at 37℃ in a 5% CO_2 atmosphere for 24 h for differentiation into macrophages. These cells were subsequently used in the experiments.

Drug treatment

After cell incubation for 24 h, the medium with FBS was replaced, and the cells were left untreated or treated with each of the Fe-containing compounds such as 10-μg/ml FC, 7 μg/ml $FeCl_2 \cdot 4H_2O$, 10-μg/ml $FeCl_3 \cdot 6H_2O$, and ten-thousand-fold diluted SFO sample (FESIN) containing 20-mg/ml of Fe, in which the final Fe concentrations were adjusted approximately to 200 μg/dl (228.0, 196.7, 206.7, and 200.0 μg/dl, respectively). Cells were also incubated with 7.8-μg/ml citric acid as a control of FC. The medium without FBS was replaced again, and the cells were left untreated or treated with 10-mM H_2O_2 for 30 min. The final concentration of Fe in the medium corresponds to the upper limit of those in the serum of healthy individuals.

Measurement of iron concentration in the cell lysate

Cell lysates were prepared using cell lysis buffer (×10) #9803 (Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's instructions. To obtain 80 μg of the resulting lysate,

1 µl of 5-M HCl was added to dissociate the iron from transferrin and convert it into Fe^{2+} in the presence of a reducing agent to ultimately form a Fe^{2+}-ferrozine complex using Metallo Assay Iron measurement LS from Metallogenics Co., Ltd., Chiba, Japan. The mixture was incubated for 5 min at room temperature (approximately 20–25℃) after adding an R-A buffer. The absorbance was measured at the main wavelength of 560 nm (optical density, OD 1). Then, the chromogenic solution was added, followed by incubation at room temperature (approximately 20–25℃) for 5 min, and the absorbance was measured at the main wavelength of 560 nm (OD 2). Iron standard solution of 200 µg/dl was used for the measurement. The iron concentration was calculated using the following formula: {OD 2(sample) −OD 1 (sample)/OD 2(standard)−OD 1(standard)} × 200.

Measurement of lipid peroxidation of cell membrane using diphenyl-1-pyrenylphosphine (DPPP)

U937 cells were incubated with 50 µM of DPPP for 10 min at 37℃ (Molecular Probes, Eugene, OR, USA) and subsequently exposed to H_2O_2 for 30 min to detect phospholipid peroxidation in cell membranes. Lipid peroxidation was measured using a microplate reader (Spectra MAXi3; Molecular Devices, San Jose, CA, USA; excitation wavelength: 351 nm; emission wavelength: 380 nm). Lipid peroxidation of the cell membrane was expressed as the fluorescence intensity (FI) per 1 µg of intracellular protein, and changes in lipid peroxidation were evaluated as the difference between FIs before and after treatment with H_2O_2.

Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were evaluated using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA; Molecular Probes, Eugene, OR, USA), as previously described[14]. FI was measured using a plate reader (excitation wavelength: 488 nm; emission wavelength: 525 nm). The intracellular ROS level was expressed as the FI per 1 µg of intracellular protein, and changes in these levels were evaluated as the difference between FIs before and after treatment with H_2O_2.

Statistical analysis

Statistical analysis was performed using JMP (version 14) statistical software (SAS Institute, Cary, NC, USA). All data are reported as means ± standard error of the means. Results for various treatments were compared with those of other groups using one-way analysis of variance and Tukey-Kramer tests. $p < 0.05$ was considered statistically significant.

Results

Intracellular iron concentration

Treatment with FC significantly increased the iron concentration (97.0 ± 25.5 µg/dl) compared with that of the control (5.6 ± 1.2 µg/dl), $FeCl_2$ (41.3 ± 9.5 µg/dl), SFO (6.5 ± 0.5 µg/dl), and citric acid-treated cells (4.8 ± 0.7 µg/dl). Treatment with FC increased the iron concentration compared with that of $FeCl_3$ (55.2 ± 7.1 µg/dl) although not significantly (Figure 1).

Effects of iron-containing preparations on intracellular lipid peroxidation

No change was observed in the intracellular lipid peroxidation after treatment with FC (3,535 OD/µg protein), $FeCl_2$ (3,170 OD/µg protein), $FeCl_3$ (2846 OD/µg protein), SFO (2,992 OD/µg protein), and citric acid (2,853 OD/µg protein) compared to the control (3,177 OD/µg protein) (Figure 2).

Effects of iron-containing preparations on intracellular ROS levels

Intracellular ROS levels were significantly increased after treatment with FC (148 FI × 10^5/µg protein), $FeCl_2$ (148 FI×10^5/µg protein), and SFO (142 FI × 10^5/µg protein) compared with those in the control (104 FI × 10^5/µg protein) and citric acid treatment (107 FI × 10^5/µg protein) (Figure 3).

Change in lipid peroxidation after treatment with H_2O_2

Lipid peroxidation changes after treatment with H_2O_2 in FC-pretreated cells (−98 OD/µg protein) were lower compared to those in the control (898 OD/µg protein), $FeCl_2$ (806 OD/µg protein), and citric acid-treated cells (1,467 OD/µg protein). Lipid peroxidation level changes after treatment with citric acid were significantly higher than those after treatment with $FeCl_3$ (591 OD/µg protein) and SFO (565 OD/µg protein) (Figure 4).

Change in intracellular ROS levels after treatment with H_2O_2

The lowest change in intracellular ROS levels was observed after treatment with citric acid (23 FI × 10^5/µg protein), followed by FC (40 FI × 10^5/µg protein), which is both significant, compared with $FeCl_3$ (98 FI × 10^5/µg protein). Change in intracellular ROS levels after treatment with H_2O_2 in FC- and citric acid-pretreated cells were lower compared to those in the control (62 FI × 10^5/µg protein), $FeCl_2$ (66 FI × 10^5/µg protein), and SFO (64 FI × 10^5/µg protein) although not significantly (Figure 5).
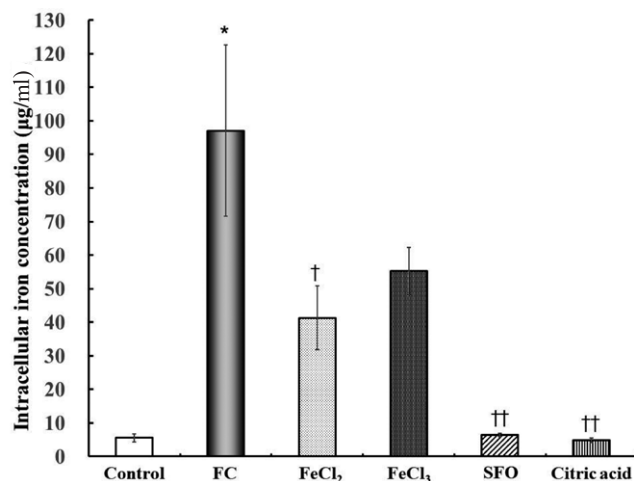
Fig. 1. Intracellular iron concentration. Human U937 monocytes were treated with each compound, and intracellular iron concentration levels were evaluated. $^*p < 0.0005$ versus control; $^†p < 0.05$ versus ferric citrate; $^{††}p < 0.0005$ versus ferric citrate. FC, ferric citrate; FeCl$_2$, iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O) ; FeCl$_3$, iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O) ; SFO, saccharated ferric oxide.
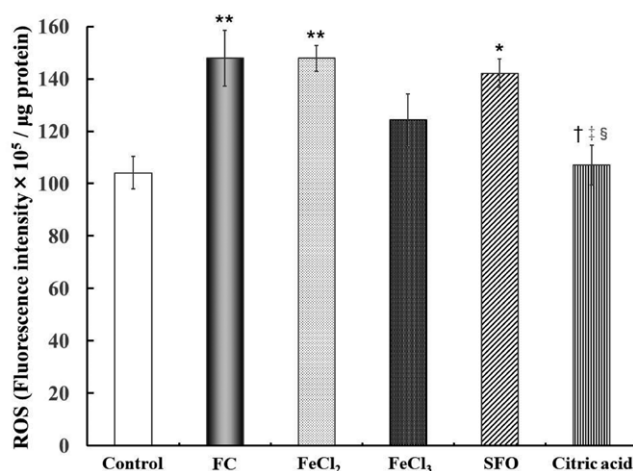

Fig. 3. Intracellular reactive oxygen species (ROS) levels. Human U937 monocytes were treated with each compound, and intracellular ROS levels were evaluated. $^*p < 0.05$ versus the control; $^{**}p < 0.01$ versus control; $^†p < 0.05$ versus ferric citrate; $^‡p < 0.05$ versus iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O) ; $^§p < 0.05$ versus saccharated ferric oxide. FC, ferric citrate; FeCl$_2$, iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O) ; FeCl$_3$, iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O) ; SFO, saccharated ferric oxide.
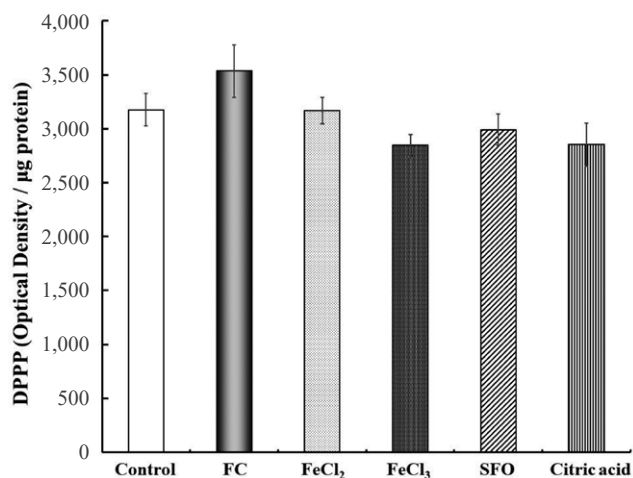

Fig. 2. Intracellular lipid peroxidation of cell membrane by measurement of fluorescence of diphenyl-1-pyrenylphosphine (DPPP). Human U937 monocytes were treated with each compound, and intracellular DPPP levels were evaluated. There was no change in the intracellular DPPP levels after treatment with FC, FeCl$_2$, FeCl$_3$, SFO, and citric acid compared to those in the control. FC, ferric citrate; FeCl$_2$, iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O) ; FeCl$_3$, iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O) ; SFO, saccharated ferric oxide.
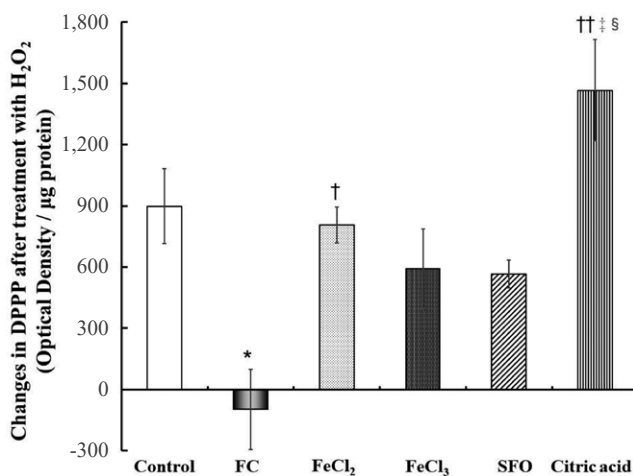

Fig. 4. Changes in intracellular lipid peroxidation after treatment with hydrogen peroxide (H$_2$O$_2$). Human U937 monocytes were treated with each compound, and intracellular DPPP levels were evaluated. Changes in lipid peroxidation of the cell membrane were evaluated after treatment with H$_2$O$_2$. $^*p < 0.0005$ versus control; $^†p < 0.05$ versus ferric citrate; $^†p < 0.0001$ versus ferric citrate; $^‡p < 0.05$ versus iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O) ; $^§p < 0.0001$ versus saccharated ferric oxide. DPPP, diphenyl-1-pyrenylphosphine; FC, ferric citrate; FeCl$_2$, iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O) ; FeCl$_3$, iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O) ; SFO, saccharated ferric oxide.
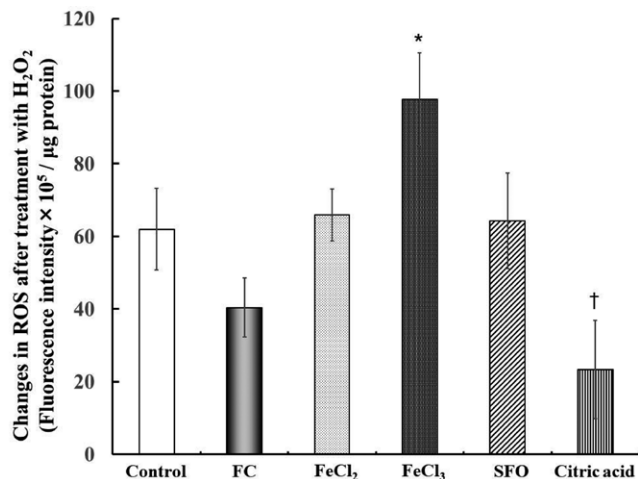
Fig. 5. Changes in intracellular ROS levels after treatment with H_2O_2. Human U937 monocytes were treated with each compound, and intracellular ROS levels were evaluated. After treatment with H_2O_2, changes in intracellular ROS levels were evaluated. *$p < 0.05$ versus ferric citrate; †$p < 0.05$ versus iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$). FC, ferric citrate; $FeCl_2$, iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$); $FeCl_3$, iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$); SFO, saccharated ferric oxide.

Discussion

In this study, intracellular ROS and lipid peroxidation levels were not increased by H_2O_2 in comparison to those of the control despite an increase in intracellular ROS levels after treatment with FC compared to the control and the high FC iron concentration. In fact, treatment with FC significantly decreased lipid peroxidation levels. To the best of our knowledge, this is the first study evaluating the direct effects of FC on oxidative stress using an *in vitro* model.

Via the Fenton reaction, high levels of divalent iron in the body generate hydroxyl radicals from H_2O_2, which induce lipid peroxidation[6, 7]. As mentioned earlier, a decrease in lipid peroxidation represented by the lower OD of DPPP, an indicator of lipid peroxidation, after FC treatment in the presence of H_2O_2 suggests that FC prevented hydroxyl radical production. Citric acid and ascorbic acid jointly prevent the peroxidation of linoleic acid[15]. However, in our study, treatment with citric acid alone increased hydroxyl radical production due to its activity as a reducing agent. Therefore, from the study findings, whether treatment of citric acid only is involved in reducing oxidative stress *in vivo* remains unclear. The suppression of hydroxyl radical production by FC might be attributed to the chelation of iron

and citric acid in FC. In fact, there are no reports of antioxidant effects of sucroferric oxyhydroxide, another iron-based phosphate binder, suggesting that the antioxidant activity is affected by iron-binding molecules, such as citric acid or sucrose.

ROS levels, an indicator of total oxidative stress in the cell, increased in the presence of all iron-containing preparations before treatment with H_2O_2. Because the levels of hydroxyl radicals did not increase, the increase in ROS levels was attributed to the increase in oxidative stress caused by factors other than hydroxyl radicals despite the high levels of iron. However, the decrease in ROS levels after the FC-pretreated cell treatment with H_2O_2 might be attributed to a decrease in the levels of hydroxyl radicals generated from H_2O_2 by the superoxide-assisted Fenton reaction (Haber-Weiss reaction). In this study, four different treated iron agents, including bivalent iron and trivalent iron, were used and the amount of iron uptake into the cells also differs between the agents. A uniform evaluation of the H_2O_2 reaction to the iron is difficult. However, in this study, the most interesting is the decrease in hydroxyl radical levels after treatment with H_2O_2 in FC-pretreated cells whose intracellular iron concentration is the highest.

Nontransferrin-bound iron produces hydroxyl radicals via the Fenton reaction, thereby increasing oxidative stress[13]. Hence, intravenous iron administration induces oxidative stress in patients with chronic kidney disease, which is also related to liver damage, hyperglycemia, endocrine disorders, and carcinogenesis via DNA damage[16, 17]. A study has shown that malondialdehyde levels increased transiently after intravenous administration of SFO, but not after oral administration of FCH. In that study, the antioxidant effects of FCH were due to iron binding to transferrin[12]. However, it is necessary to study these effects in the absence of transferrin, which is difficult to assess *in vivo* because of the transferrin in the serum, to evaluate the antioxidant effects of FCH. Therefore, in this study, we investigated the effects of FC on oxidative stress using an *in vitro* model and found no increase in intracellular ROS levels and a significant decrease in lipid peroxidation after H_2O_2 treatment and FC pretreatment. This is the first *in vitro* study evaluating the direct effects of FC on oxidative stress.

One limitation of this study is that we used FC (SIGMA F3388-250G; molecular weight 244.94; Fe 55.75) and not FCH. FC contains 59.44% of the iron in FCH, which is used in clinical

practice. However, FCH (the hydrated form of FC) will not act differently from FC; the only difference is the molecular weight and the amount of iron taken in the study. Another limitation of the current study is that the dose-dependency of the iron-containing compounds, which is critically important for cell biological experiments, were not shown in the experiment. Although many of the previous studies on the effects of FC on oxidative stress were conducted by *in vivo* experiments, *in vitro* studies were limited in number. As the first step to such *in vitro* experiments, we adjusted the final Fe concentrations approximately to $200\,\mu g/dl$, which corresponds to the upper limit of serum Fe concentration in healthy individuals.

In conclusion, the study findings reveal that neither intracellular ROS nor lipid peroxidation levels were increased after H_2O_2 treatment and pretreatment with FC compared to the control, despite the high iron concentration. The suppression of hydroxyl radical production by FC might be due to the iron chelation and citric acid in FC. FCH is an iron-based drug used as a phosphate binder and is essential for hemodialysis patients. The results of this study may contribute to a better understanding of the effects of oxidative stress in hemodialysis patients treated with FCH.

Acknowledgement

Conflicts of interest disclosure

None of the authors have any conflicts of interest to disclose.

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