Role of Plasma Protein and Low-Molecular Weight Substances in the Change of Hydroxyl Radical Scavenging Activity in Hemodialysis Patients

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Summary While it is well known that hemodialysis (HD) patients with end stage renal failure are exposed to high oxidative stress, there is not a general opinion regarding whether antioxidant activity is high or low in these patients. We evaluated the variation of plasma hydroxyl radical scavenging activity (p-HRSA) by a single-session of HD in 69 patients by using a new system, reactive flow-injection electron spin resonance. And then comparing p-HRSA with their biochemical parameters, we tried to elucidate what components affected p-HRSA in the HD patients. The average of p-HRSA significantly increased after HD and the variation of p-HRSA by HD was correlated with that of plasma total protein (TP). In 5 patients however, their p-HRSA decreased after HD, in spite of increasing TP. In pre-HD, the p-HRSA values and hydroxyl radical scavenging activity of low-molecular weight fraction of plasma were significantly higher in these 5 patients than in patients whose p-HRSA increased after HD. These 5 patients were in an inflammatory state. These findings suggest that p-HRSA is mainly affected by TP, but caution should be exercised in patients who have high p-HRSA before HD and whose p-HRSA does not increase after HD.

Key Words: hemodialysis, plasma hydroxyl radical scavenging activity, flow-injection electron spin resonance, total protein, low-molecular weight substance

Introduction

The high incidence of early cardiovascular disease in patients with chronic renal failure, either on dialysis or not, is well documented [1–3]. This phenomenon is regarded as being caused by increasing oxidative damage due to reactive oxygen species (ROS). The over production of ROS in these patients is evidenced by several oxidative stress markers [3–7]. In addition, hemodialysis (HD) patients are exposed to more oxidative damage because neutrophils and the complement pathways may be activated during HD with a subsequent increase in the neutrophil free radical production [2,6–8]. On the other hand, Soejima et al. reported that HD procedure reduced oxidative albumin [9]. Given that HD removes uremic toxins, a hemodialysis session does not always induce malignancies.

To date, there are several reports about antioxidants and antioxidant capacity in HD patients. Some reports have said that antioxidant capacity was higher in HD patients
compared with healthy subjects [1, 2, 10, 11], but others have stated that antioxidant status was not different [12, 13]. It is difficult to attribute whether antioxidant activity is stronger or not because their methods had different approaches against ROS. And it was unclear what kinds of ROS were scavenged in each report. Thus, in HD patients there is an increase of oxidative stress and the impairment of antioxidant defense. The imbalance between overproduction of oxidants and antioxidant defense mechanism is well established in HD patients [6–8].

Electron spin resonance (ESR) is the only direct method for both detection and identification of free radicals. In previous reports, we have already demonstrated by ESR measurement that hydroxyl radical scavenging activity (HRSA) of plasma in HD patients significantly decreased compared with controls and recovered after HD [14]. Shirai et al. also reported that, by using ESR in HD patients, HRSA was higher than that in healthy subjects but it never changed by HD session [15]. These differences may contribute to defects in the conventional ESR method. In addition, the existing X-band ESR method has such difficulties as low-sensitivity, a longer time requirement, and variable of sensitivity. Therefore, we developed a reactive flow-injection ESR system (FI-ESR), using three high-pressure liquid chromatography (HPLC) pumps and ultraviolet (UV) lamp. The problems were solved by holding flow-cell in the cavity, and measuring of fixing magnetic field. When hydrogen peroxide is exposed to UV in the cell, hydroxyl radical is generated and is transiently trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO). This system makes it possible to measure HRSA for various antioxidants. In this study, we investigated the variation of plasma HRSA (p-HRSA) by a single-session of HD by using this new system, FI-ESR. Moreover, by comparing it with their biochemical parameters, we tried to elucidate what components affected p-HRSA in HD patients.

**Subjects and Methods**

**Patients**

All studies were performed after 48 hours passed since the last HD. Sixty-nine hemodialysis patients (39 male, 30 female) were studied. Sixty-eight patients underwent hemodialysis three times a week and one patient did so twice. The age was 63 ± 10 (mean ± SD, range 36–81) years and the duration of HD treatment was 96 ± 79 (5–330) months. Origins of the end stage renal disease (ESRD) in the group were: chronic glomerulonephritis (n = 24), diabetic nephropathy (n = 19), polycystic kidney (n = 3), autoimmune disease (n = 2), chronic pyelonephritis (n = 1), ureter tumor (n = 1), reflux nephropathy (n = 1), pregnancy-induced hypertension syndrome (n = 1), and unknown (n = 17). Clinical characteristics are shown in Table 1.

**Measurement of hydroxyl radical scavenging activity (HRSA)**

HRSA was observed by reactive flow-injection electron spin resonance (FI-ESR) system. It consisted of three inert HPLC pumps (PU-2080i; JASCO, Tokyo, Japan), automatic injector (AS-2057i; JASCO), ultraviolet lamp (RUVF-203S; Radical Research Inc., Tokyo, Japan), and ESR spectrometer (RRX-1X; Radical Research Inc.) (Fig. 1). Ultrapure water, 10 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap agent, and 20 mM hydrogen peroxide, were flown into a capillary quartz cell at each flow rate of 0.4 ml/min. The cell was exposed to ultraviolet light and generated hydroxyl radical reacted DMPO. The formed DMPO-OH adduct was detected by ESR and we obtained its spectra. Then, we fixed a magnetic field at the second peak. Samples were then injected into an ultrapure water line. When antioxidant was injected, reduced signal peak was observed (Fig. 2). The HRSA was the negative peak of the FI-ESR signal, and the HRSA value was calculated as relative intensity against 0.5 mmol dimethyl sulfoxide.

**Samples**

Before measurement all plasma was stored at −24°C. In plasma HRSA (p-HRSA) determination, plasma was undiluted and used directly. Low-molecular weight fraction.

### Table 1. Clinical characteristics of hemodialysis patients before (pre-HD) and after (post-HD)

| Measurement                              | pre-HD     | post-HD    |
|------------------------------------------|------------|------------|
| Total Protein (g/dl)                     | 6.58 ± 0.41| 7.71 ± 0.66|
| Albumin (g/dl)                           | 3.84 ± 0.31| 4.46 ± 0.45|
| Glucose (mg/dl)                          | 124.4 ± 45.1| 97.5 ± 43.9|
| Uric Acid (mg/dl)                        | 8.2 ± 1.4  | 2.5 ± 0.6  |
| Creatinine (mg/dl)                       | 11.9 ± 2.5 | 4.9 ± 1.3  |
| Plasma Urea Nitrogen (mg/dl)             | 70.3 ± 14.11| 24.5 ± 6.7 |
| CRP (mg/dl)                              | 0.29 ± 0.5 | —          |
| Hemoglobin (g/dl)                        | 10.3 ± 1.0 | —          |
| Hematocrit (%)                           | 32.6 ± 3.0 | —          |

**Fig. 1.** Schematic flow diagrams of reactive flow-injection ESR system. P1, P2, and P3 pumps sent ultrapure water, 10 mM DMPO, and 20 mM H2O2, respectively. Each pump of flow rate was 0.4 ml/min.
UV lamp on ——— Before HD
0.5 mmol DMSO
UV lamp off ——— After HD

Fig. 2. FI-ESR chart of plasma and 0.5 mmol DMSO. When UV lamp was turned on, hydroxyl radical generated and upper baseline was shown, and when it was turned off, hydroxyl radical stopped generating and the detected point went down. Hydroxyl scavenging activity represented these downward peaks. These peak areas were calculated and p-HRSA values were elucidated by plasma/DMSO ratio.

of plasma was collected by ultrafiltration (Ultrafree-MC filter, Millipore Corporation, Billerica, MA; cut off M.W. 10,000).

Statistical analysis
Data were expressed as mean ± SD. Correlation coefficient was obtained using simple regression analysis (Excel software). Statistical significance was determined using paired or un-paired t-test. The differences were considered statistically significant when the calculated p value was less than 5%.

Results

p-HRSA value of pre- and post-HD
Using this new system, FI-ESR, the significant increase in the average of p-HRSA values was observed after HD (from 0.44 ± 0.06 to 0.49 ± 0.06, n = 69). The p-HRSA value increased over 5% after HD in 46 patients (increased group), and in 18 patients, their p-HRSA values showed little variation (invariant group; within 5% variation). Interestingly enough, in the remaining 5 patients, this value significantly decreased over 5% (reduced group) (Table 2). In a comparison of the p-HRSA values between each group, p-HRSA of pre-HD in the invariant group and the reduced group were stronger than in the increased group (p = 0.002, and p<0.01, respectively). Furthermore, in post-HD, the value of the reduced group was lower than that of the increased group (p<0.05).

Relationship between p-HRSA and plasma total protein
Protein is a major component in plasma and an easy target of hydroxyl radicals. In addition, plasma total protein becomes thicker by HD procedure because of hemo-concentration. We investigated the relationship between p-HRSA and the concentration of plasma total protein (TP). Fig. 3 shows the correlation of the before-after HD ratio between the p-HRSA and the concentration of TP. Variation of p-HRSA was correlated with that of TP (r = 0.69, p<0.0001). Both pre- and pro-HD p-HRSA values were also correlated to plasma TP levels significantly (r = 0.41, p = 0.0005; r = 0.69, p<0.0001, respectively) (Fig. 4).

The ratio of p-HRSA/TP of the increased, invariant, and decreased group
To eliminate the influence of HRSA of plasma protein, we calculated p-HRSA value/TP for each group (Fig. 5). In the

| Group               | p-HRSA Value |
|---------------------|--------------|
|                     | pre-HD       | post-HD      |
| All patient (n = 69)| 0.44 ± 0.06  | 0.49 ± 0.06***|
| Increased group (n = 46) | 0.42 ± 0.01  | 0.51 ± 0.01***|
| Invariant group (n = 18) | 0.47 ± 0.01† | 0.48 ± 0.01   |
| Reduced group (n = 5)  | 0.50 ± 0.02** | 0.44 ± 0.02***|

Table 2. p-HRSA value of hemodialysis patients

Statistical significance: *p = 0.001, **p<0.001 vs pre-HD, †p = 0.002, ‡p<0.01, §§p<0.05 vs increased group.
The grouping was done in over 5% increase, within 5% invariant, and over 5% decrease after HD.
increased group, means of the p-HRSA value/TP were the same as pre- and post-HD (0.064 ± 0.008 and 0.064 ± 0.006, respectively). In the invariant and reduced group, these values were higher in pre-HD than in post-HD (0.071 ± 0.006 vs 0.065 ± 0.006, p<0.001; 0.079 ± 0.01 vs 0.063 ± 0.007, p = 0.001). Calculation of p-HRSA value/TP in pre-HD revealed a significant difference in each group (invariant group; p = 0.002, reduced group; p<0.001 vs increased group, respectively).

Correlation between the p-HRSA value and the plasma concentration of albumin

Plasma protein includes many kinds of proteins but albumin is known to be the most abundant protein. We also investigated the possibility that there is correlation between p-HRSA value and plasma albumin. Before HD they were not correlated (r = 0.02, p = 0.88), whereas after HD, p-HRSA values were correlated with plasma albumin levels (r = 0.45, p=0.0001) (Fig. 6).

Comparison of the HRSA of low-molecular weight fraction (lmf-HRSA) of plasma for the increased group and reduced group

In an attempt to compare the lmf-HRSA between the increased and reduced group, we randomly selected eight patients in the increased group. The lmf-HRSA of the reduced group was stronger than the increased group in pre-HD (0.18 ± 0.03, n = 5 vs 0.13 ± 0.03, n = 8, p = 0.02) (Fig. 7). However, levels of the conceivable low molecular weight substances that expressed hydroxyl radical scavenging activity (i.e., glucose, uric acid, and creatinine) were not different (data not shown).

Relationship between p-HRSA and C-reactive protein

In the grouping described above, quite a few patients of
the invariant and reduced groups had high C-reactive protein (CRP) level (≥0.3 mg/dL), leukocyte count, or inflammatory diseases (invariant group: 7 of 18 subjects, reduced group: 5 of 5 subjects). We then divided patients into normal (<0.3 mg/dL) and high (≥0.3 mg/dL) CRP group, and investigated pre- and pro-HD p-HRSA values of each group. In normal CRP patients, both pre- and pro-HD p-HRSA values were correlated to plasma TP levels significantly (r = 0.55, p<0.0001; r = 0.69, p<0.0001, respectively). But in high CRP patients there was not a significant correlation for pre-HD (r = 0.25, p = 0.33) (Fig. 8). In pre-HD, the ratio of p-HRSA/TP in high CRP patients was significantly larger than that in the normal CRP patients (p = 0.01) (Table 3).

**Discussion**

In conventional ESR measurement, radical sources, a spin-trapping reagent, and samples are first mixed and they reacted for a set time in a test tube. Then, the mixture is transferred into a cell and inserted into the ESR spectrometer. These operations were not only troublesome but also caused measurement error because observed ·OH was easily affected by the measurement time. Our new method, the reactive FI-ESR system, gives hydroxyl radical generation first in the cell and is able to measure scavenging activity of samples in real time. In addition, holding the cell avoids measurement error by moving over in the cavity and mixing reaction solutions before the cell insures shorter and more accurate reaction time. Measurement of fixing magnetic field at the point of DMPO-OH adduct peak specifically does not need sweeping time, therefore, the measuring time can be shortened. This reactive FI-ESR solves several problems and is a not only practical but also more powerful system for measuring hydroxyl radical.

In the present study, we showed that the average value of p-HRSA of post-HD was significantly higher than that of pre-HD. This result corresponded with previous data [14]. However, comparing patients individually, in 33% of the patients the p-HRSA did not increase after dialysis. This result indicates that a single-session of HD did not always make HRSA stronger.

The variation of p-HRSA by a single-session of HD had a positive correlation to that of plasma TP level. It suggests that the variation of p-HRSA is affected by TP concentration. But in albumin, which accounts for 60% of TP, p-HRSA of pre-HD was not correlated with the albumin level. Human serum albumin is composed of mercaptalbumin (reduced form) and nonmercaptalbumin (oxidized form), that is, a protein redox couple. Soejima et al. have recently shown that the percentage of the reduced form of human albumin

| Table 3. Comparison between normal and high CRP patient |
|---------------------------------------------------------|
|                                                        |
|                                                                                       |
| normal CRP group (n = 51)               | high CRP group (n = 18) |
| pre-HD       | post-HD     | pre-HD       | post-HD     |
| CRP (mg/dL)  | 0.093 ± 0.066 | —           | 0.85 ± 0.79 | —           |
| Total Protein (g/dL) | 6.57 ± 0.37  | 7.75 ± 0.63 | 6.62 ± 0.51 | 7.59 ± 0.73 |
| albumin (g/dL) | 3.91 ± 0.27  | 4.57 ± 0.37 | 3.66 ± 0.36** | 4.14 ± 0.52 |
| p-HRSA value | 0.43 ± 0.06  | 0.49 ± 0.06 | 0.47 ± 0.05* | 0.50 ± 0.07 |
| p-HRSA/TP    | 0.065 ± 0.009 | 0.063 ± 0.006 | 0.071 ± 0.008** | 0.066 ± 0.006 |

Statistical significant: *p = 0.01. **p<0.01 vs normal CRP group
serum albumin increased transiently after hemodialysis [9]. In our study, similarly, the correlation between p-HRSA and albumin level recovered after the HD session. An increase of the rate of reduced form albumin may partly contribute to our results. In addition, Roxborough et al. reported that plasma gultathione peroxidase (GPX) activity increased following HD [16]. These results supported the correlation between p-HRSA values and TP levels, which became strong after HD. On the other hand, lmf-HRSA decreased after HD. It is conceivable that low molecular weight antioxidants, such as vitamin C, uric acid, creatinine, and some other uremic toxins are removed by HD sessions. Overall, although the value of p-HRSA before HD depends on low molecular weight antioxidants and protein, post-HD is affected mainly by concentration and recovery of reducing capacity of plasma total protein.

When p-HRSA value was divided by TP level, this corrected value of pre-HD was the same as post-HD in the increased group. But in the invariant and reduced group it was bigger than post-HD. This result suggests that some substances influence p-HRSA of pre-HD, besides TP, in these groups. Additionally, in the reduced group, lmf-HRSA was higher, especially pre-HD, compared with the increased group. However, the levels of uric acid, creatinine, and glucose did not make any difference at all between these groups. Presumably, these low molecules did not contribute to the varying of p-HRSA changes. Further studies are awaited to find out which molecules affect hydroxyl radical scavenging activity.

Each patient of the reduced group had a high CRP level (≥0.3 mg/L) and severe or inflammatory complication, rheumatoid arthritis, renal tumor, etc. There is a high prevalence of inflammation and oxidative stress in the maintenance hemodialysis population. Haghdooost et al. reported that serum 8-OHdG was significantly elevated in persistent inflammation in comparison to noninflamed patients and a significant correlation was found between serum 8-oxo-dG and high sensitivity CRP [17]. High CRP increases immune activation and such patients are seemingly apt to be exposed to more oxidative stress. Alternatively, in HD patients if the CRP level is high, the serum albumin level is often lower. Our results also proved that the plasma albumin concentration of high CRP patients was low. Therefore, it was considered that their antioxidant activity declined because of hypoalbuminemia, despite strong oxidative state. However, in the present study, their p-HRSA was higher than that of normal CRP patients. Moreover, in high CRP patients correlation between p-HRSA value and TP level of pre-HD was weak and the corrected value by TP of pre-HD was high. It suggests that another factor, besides TP or albumin, may contribute to the high value of p-HRSA before HD in high CRP patients. Fujii et al. showed that CRP altered in vitro antioxidant enzymes in endothelial progenitor cells, i.e., superoxide dismutase increased and GPX decreased [18]. Rufail et al. found that CRP inhibited in vitro oxidation of low-density lipoprotein [19]. These reports indicated that CRP changed antioxidant defense. Nevertheless, there are few papers referring to the relationship between CRP and antioxidant activity in vivo. Our present study developed a new point of view that plasma hydroxyl radical scavenging activity increased before HD in high CRP patient. An increase of oxidative stress by inflammation may stimulate antioxidant pathways.

In conclusion, p-HRSA in hemodialysis patients increased by a single-session of HD, with an increasing concentration of total protein. The patients whose p-HRSA did not increase by HD, however, had some inflammatory complications. It is important to know the variation of p-HRSA in the study of pathological condition in HD patients. These findings lead to a new question, i.e., why p-HRSA, especially pre-HD, is higher in inflamed patients. Further studies are required to resolve this problem.

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Abbreviations

CRP, C-reactive protein; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; ESR, electron spin resonance; GPX, gultathione peroxidase; HPLC, high-pressure liquid chromatography; ROS, reactive oxygen species; UV, ultraviolet.

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