Plasma marker of tissue oxidative damage and edaravone as a scavenger drug against peroxyl radicals and peroxynitrite

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The percentage of the plasma oxidized form of coenzyme Q10 in the total amount of coenzyme Q10 (%CoQ$_{10}$) is a useful marker of oxidative stress in the circulation. Plasma free fatty acids and their composition can be used as markers of tissue oxidative damage, as demonstrated in patients suffering from a wide variety of diseases and in humans and rats under oxidative stress. Edaravone was approved for the treatment of stroke in Japan in 2001 and its mechanism of action is based on scavenging lipid peroxyl radicals. In 2015, edaravone was also approved for the treatment of ALS patients. Edaravone functions therapeutically as a scavenger of peroxynitrite, as demonstrated by the finding that its administration raises plasma uric acid levels and decreases 3-nitrotyrosine in cerebrospinal fluid.

Key Words: coenzyme Q10 redox balance, plasma levels of free fatty acids and their composition, edaravone, ALS, peroxynitrite

Plasma Markers of Oxidative Stress

Reactive oxygen and nitrogen species have been suggested to increase oxidative stress and to cause aging and degenerative diseases such as heart attack, stroke, neurodegenerative diseases, diabetes, and cancer. Oxidative stress is defined as a disturbance in the pro-oxidant-antioxidant balance in favor of pro-oxidants.$^{(1)}$ The redox balance of coenzyme Q10 (CoQ$_{10}$) could be a good marker of oxidative stress because its reduced form (ubiquinol-10, CoQ$_{10}$H$_2$) is highly reactive with oxygen radicals and is converted to the oxidized form (ubiquinone-10, CoQ$_{10}$). The incubation of human plasma at 37°C under aerobic conditions in the presence of 5 μM cupric ion resulted first in a decrease in vitamin C (VC; ascorbic acid),$^{(2)}$ followed by a rapid decrease in CoQ$_{10}$H$_2$ and concomitant production of an equal amount of CoQ$_{10}$ (Fig. 1). Interestingly, no significant decrease in vitamin E (VE) was observed despite VE being a self-sacrificing strong antioxidant against lipid peroxidation (eqs. 1 and 2). However, when the lipid peroxyl radical (LOO$^\cdot$) concentration is much smaller than the VE concentration, equation 2 does not hold and the VE radical (VE$^\cdot$) abstracts a proton from lipid to give lipid radical (L$^\cdot$) and VE (eq. 3).$^{(3)}$ Although this reaction is very slow. The coupling of eqs. 1, 3, and 4 creates a new cycle (tocopherol-mediated peroxidation) in which lipid hydroperoxide (LOOH) accumulates without any loss of VE, as observed in the second half (after 40 min) of plasma oxidation (Fig. 1).$^{(4)}$ The large amount of cholesterol ester in plasma supports the formation of its hydroperoxide (CE-OOH) (Fig. 1).

\[ \text{LOO}^\cdot + \text{VE} \rightarrow \text{LOOH} + \text{VE}^\cdot \].................................(1)

\[ \text{VE}^\cdot + \text{LOO}^\cdot \rightarrow \text{VE} + \text{OOL} \]..............................................(2)

\[ \text{VE}^\cdot + \text{LH} \rightarrow \text{VE} + \text{L}^\cdot \]..............................................(3)

\[ \text{L}^\cdot + \text{O}_2 \rightarrow \text{LOO}^\cdot \]..............................................(4)

The above results indicate that the percentage of CoQ$_{10}$ in total plasma (%CoQ$_{10}$) could be a good marker of early stage oxidative stress. We therefore developed a simple and reliable method for the simultaneous detection of plasma CoQ$_{10}$H$_2$ and CoQ$_{10}$ and applied the method to patients with various diseases,$^{(5)}$ together with human and rats under oxidative stress conditions (Table 1). Significant increases in %CoQ$_{10}$ were observed in patients with hepatitis,$^{(6)}$ cirrhosis,$^{(7)}$ hepatoma,$^{(8)}$ juvenile fibromyalgia,$^{(9)}$ amyotrophic lateral sclerosis (ALS),$^{(10)}$ post cardiac arrest syndrome (manuscript in preparation), and heart attack (manuscript in preparation) as compared to age-matched healthy controls. It is interesting that newborn babies have significantly higher plasma %CoQ$_{10}$ than adults.$^{(9)}$ Plasma %CoQ$_{10}$ increases in triathlon athletes the day after a race (manuscript in preparation).

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Fig. 1. Changes in plasma antioxidant levels during the aerobic oxidation of human plasma in the presence of 5 μM cupric chloride at 37°C. VC, ascorbic acid; CoQ$_{10}$H$_2$, reduced form of coenzyme Q10; CoQ$_{10}$, oxidized form of coenzyme Q10; VE, vitamin E; CE-OOH, cholesterol ester hydroperoxide.

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Evans cinnamon (LEC) rats accumulate copper, especially in the liver, and develop hepatitis by 24 weeks of age. Treatment with trientine, a copper chelator, prevents the onset of hepatitis and an increase in plasma percent coenzyme Q9 (%CoQ9). However, an elevation of plasma %CoQ10 does not reflect oxidative damage inside a specific tissue because plasma CoQ10 is located in circulating lipoproteins. For example, no significant elevation of plasma %CoQ9 was observed in CCl4-poisoned rats because CCl4 is metabolized to trichloromethylperoxyl radical (Cl3COO•) in the liver. Moreover, rats with a middle cerebral artery occlusion did not show any increase in plasma %CoQ9.

**Table 1.** Changes in plasma markers of oxidative stress and cellular oxidative damage in patients with various diseases as compared to healthy controls and humans or rats under oxidative stress conditions

| Diseases or conditions                          | %CoQ9 (9) | Ref | FFA | %PUFA | %16:1 | %18:1 | Ref |
|------------------------------------------------|-----------|-----|-----|-------|-------|-------|-----|
| Hepatitis, cirrhosis, and hepatoma             | ↑         | (5) | ↑   | ↓     | ↑     | ↑     | in prep |
| Juvenile fibromyalgia                          | ↑         | (6) | ↑   | ↓     | ↑     | ↑     | (6) |
| ALS                                            | ↑         | (7) | NS  | ↓     | NS    | NS    | (7) |
| Post-cardiac arrest syndrome                    | ↑         | in prep | ↑   | ↓     | NS    | NS    | in prep |
| Heart attack                                    | ↑         | in prep | ↑   | NS    | ↑     | NS    | in prep |
| Newborn babies at day 1                         | ↑         | (8) | ↑   | ↓     | ↑     | NS    | (8) |
| Right after a triathlon race                    | NS        | in prep | ↑   | ↓     | ↑     | ↑     | in prep |
| 1 day after a triathlon race                    | ↑         | in prep | ↑   | ↓     | ↑     | ↑     | in prep |
| LEC rat at 24 weeks of age with trientine treatment| ↓         | (9) | NS  | ↑     | ↓     | ↓     | (9) |
| Rat, chronic CCl4 poisoning                     | NS        | in prep | ↑   | ↓     | ↑     | ↑     | in prep |
| Rat, single CCl4 poisoning                      | NS        | in prep | ↑   | ↓     | ↑     | ↑     | in prep |
| Rat, brain attack model                         | NS        | (11) | ↑   | NS    | ↑     | ↑     | (11) |
| Above rat with edaravone treatment              | NS        | (11) | NS  | NS    | ↓     | ↓     | (11) |

Ref, reference; in prep, manuscript in preparation.

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![Diagram](https://via.placeholder.com/150)

**Fig. 2.** Pathways for the reduction of lipid peroxyl radicals (LOO•) by the edaravone anion and formation of its oxidation products, 4-oxoedaravone and OPB.

Evans cinnamon (LEC) rats accumulate copper, especially in the liver, and develop hepatitis by 24 weeks of age. Treatment with trientine, a copper chelator, prevents the onset of hepatitis and an increase in plasma percent coenzyme Q9 (%CoQ9).

However, an elevation of plasma %CoQ10 does not reflect oxidative damage inside a specific tissue because plasma CoQ10 is located in circulating lipoproteins. For example, no significant elevation of plasma %CoQ9 was observed in CCl4-poisoned rats because CCl4 is metabolized to trichloromethylperoxyl radical (Cl3COO•) in the liver. Moreover, rats with a middle cerebral artery occlusion did not show any increase in plasma %CoQ9.

**Plasma Marker of Tissue Oxidative Damage**

As discussed above, %CoQ10 is useful for evaluating the formation of oxygen radicals in blood plasma, but it would be more...
practical to have a plasma marker of tissue oxidative damage. We focused on plasma total free fatty acids (FFAs) because the activities of phospholipase A\(_2\) and A\(_1\) increase under oxidative stress and the resulting FFAs may enter the bloodstream through leakage or lysis of oxidatively damaged tissues.\(^{12-14}\) If this were indeed the case, we would expect an increase in plasma FFA concentration and a decrease in polyunsaturated fatty acids (PUFAs) such as linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4) and docosahexaenoic acid (22:6) in the blood plasma since they are highly susceptible to oxidation.\(^{15}\) The oxidative loss of PUFAs should be compensated by an increase in monoenoic acids such as palmitoleic acid (16:1) and oleic acid (18:1) due to the action of stearoyl-CoA desaturase.\(^{16}\) Table 1 shows that plasma FFA levels and the percentages of 16:1 and 18:1 in total FFA (%16:1 and %18:1, respectively) were elevated and that the percentages of PUFAs in total FFA (%PUFA) decreased in all diseases and oxidative stress conditions studied (Table 1).

### Free Radical Scavenger Drug

Table 1 shows that trientine and edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) ameliorated oxidative damage in rats induced by copper and middle cerebral artery occlusion,\(^{6,11}\) respectively, suggesting that an appropriate drug should be effective for treating human diseases. Many companies have attempted to develop a reactive oxygen scavenging drug.

In late 1980s, Mitsubishi Chemical Corporation was developing an antioxidant as a drug for the treatment of stroke, and animal experiments indicated that edaravone was the most promising lead.\(^{17,18}\) In 1990, I was asked to elucidate the mechanism by which edaravone prevents lipid peroxidation.\(^{19}\) The pKa value of edaravone is 7.0, and the rate of edaravone oxidation initiated by an azo compound increases with increasing pH, suggesting that the anionic form of edaravone is more reactive than the non-dissociated form. Donation of an electron to a lipid peroxyl radical converts edaravone anion to the edaravone radical, which is oxidized to produce 3-methyl-1-phenyl-2-pyrazolin-4,5-dione (4-oxoedaravone) and its hydrolysate, 2-oxo-3-(phenylhydrazono) butanoic acid (OPB), as major products of peroxyl radical-induced oxidation (Fig. 2).\(^{19}\) Edaravone inhibits lipid peroxidation as efficiently as do well-known antioxidants such as VE and VC. Furthermore, a combination of edaravone with VC or VE almost completely inhibits the oxidation of phosphatidylcholine liposomal membrane by lipid-soluble and water-soluble azo initiators.\(^{19}\)

Intravenous injection of edaravone at a dose of 30 mg twice a day for 14 days in patients with acute ischemic stroke, commencing...
within 24 h after onset, significantly improved the functional outcome in a multicenter, randomized, placebo-controlled, double-blind study. Accordingly, in 2001 the Japanese Ministry of Health, Labor, and Welfare approved intravenous injection of 30 mg edaravone twice a day for a maximum of 14 days for patients with acute brain infarction within 24 h after onset, and edaravone is now widely used in Japan for the treatment of acute stroke.

To obtain direct evidence that edaravone serves as an antioxidant in vivo, four groups of rats were prepared: an ischemia/reperfusion (I/R) group receiving 2 h occlusion-reperfusion of the middle cerebral artery, a single administration group treated by intravenous injection of edaravone (3 mg/kg) immediately after I/R, a repeated treatment group receiving twice daily edaravone administrations for 14 days, and a sham operation group without occlusion. Repeated treatment with edaravone significantly improved the neurological symptoms and decreased the impairment of motor function as compared to the I/R group, while single administration demonstrated limited efficacy. No significant differences in plasma antioxidants such as VC, urate and VE, or in the redox status of CoQ_{10}, were observed among the four groups. In contrast, the plasma %18:1 was significantly increased in the I/R group for the first 7 days as compared to the sham operation group (Fig. 3). The above results suggest that cellular oxidative damage in the rat brain is evident for at least 7 days after I/R (Fig. 3). Repeated treatment suppressed the increase in %18:1, whereas a single administration did not, which is consistent with the limited efficacy of single administration (Fig. 3).

**Edaravone as a Scavenger of Peroxynitrite**

The success of edaravone in the treatment of stroke prompted Dr. Hiide Yoshino at Yoshino Neurology Clinic in Chiba to use it as an ALS treatment since oxidative stress is believed to play an important role in this disease. After positive preliminary results, we and Dr. Yoshino began a collaboration in 2011 to determine the mechanism underlying the drug’s efficacy. I believed our plasma oxidative markers would be useful in this investigation.

We recruited 26 ALS patients: 17 received edaravone (30 mg/day, 1–4 times a week) for at least 3 months, and 13 underwent this treatment regime for 6 months. Changes in the revised ALS functional rating scale (ALSFRS-R) were significantly smaller in the patients treated with edaravone than in the edaravone-untreated ALS patients (n = 19) (Fig. 4). Based on the ΔALSFRS-R results at 6 months (Δ), the patients were divided into 3 groups: a satisfactory progress group (Δ≥0), an ingravescent group (Δ<–5), and a middle group (Δ = –1––4). Fig. 4 shows that there were 6 patients in the satisfactory progress group of the 13 patients treated with edaravone for 6 months (or 7 of the 17 patients), but none out of the total of 19 untreated patients. Further, there were 3 patients in the ingravescent group among the total 13 edaravone-treated patients (or 4 out of 17 treated patients), but there were 12 out of a total of 19 untreated patients. These data indicate that edaravone treatment may have a significant beneficial outcome for ALS patients.

Table 2 shows a comparison of plasma markers of circulatory oxidative stress and tissue oxidative damage between ALS patients and age-matched healthy controls. ALS patients had significantly increased plasma %CoQ_{10} and %PUFA as compared to the controls, corroborating an increased level of oxidative stress and tissue oxidative damage in patients with ALS. However, administration of edaravone did not change %CoQ_{10} and %PUFA (data not shown), indicating that the mechanism of action of edaravone does not depend on scavenging free radicals.

It is interesting that the plasma level of uric acid (UA) in ALS patients was significantly lower than that in the controls (Table 2). UA is a peroxynitrite scavenger, and thus this observation is consistent with increased cerebrospinal fluid levels of 3-nitrotyrosine observed in patients with ALS. Keizman et al. also reported low levels of serum UA in patients with ALS. Significantly, the UA levels in 36 out of 46 patients had decreased 6 months later, while the UA levels increased in 9 out of 46 patients and remained unchanged in one patient. Peroxynitrite is likely responsible for the prominent decrease in UA, since the VC and VE levels were similar in the control subjects (Table 2). Edaravone administration increased the plasma levels of UA in 10 out of 12 ALS patients (Fig. 5), and, notably, in 5 out of 5 subjects in

#### Table 2. Levels of plasma antioxidants and lipids in patients with ALS as compared to age-matched healthy controls (average ± SD)

| Group                      | Δ   | Untreated | Treated |
|----------------------------|-----|-----------|---------|
| Satisfactory progress group| ≥0  | 0         | 6 (7)  |
| Middle group               | –1––4| 7         | 4 (6)–4|
| Ingravescent group         | <–5 | 12        | 3 (4)–1|
| Total                      | 19  | 13        | (17)   |

* Values were determined using the Student's t test. VC, ascorbic acid; UA, uric acid; VE, vitamin E; TQ10, total coenzyme Q10; %CoQ_{10}, ratio of oxidized form of coenzyme Q10 to TQ10; FFA, free fatty acid; %PUFA, ratio of polyunsaturated fatty acids to total FFA; %16:1, ratio of palmitoleic acid to total FFA; %18:1, ratio of oleic acid to total FFA.

Fig. 4. Time course of changes of ALSFRS-R at 6 months with and without edaravone treatment (mean ± SE). Repeated-measures ANOVA shows a significant difference in ΔALSFRS-R between the two groups (p<0.008). Inset table provides a classification of the patients according to the value of ΔALSFRS-R (Δ) at 6 months in edaravone-treated and untreated groups of ALS patients. ΔALSFRS-R at 6 months was missing for four patients in the treated group, and classifications were estimated on the assumption that ΔALSFRS-R at 6 months was twice the ΔALSFRS-R determination at 3 months, as indicated in parentheses.
We have also found that edaravone scavenges peroxynitrite approximately 30 times more efficiently than does UA. The little formation of 4-oxoedaravone and OPB was observed, suggesting that free radicals are not involved in the reaction of edaravone and peroxynitrite. Instead, the major product was 4-NO-edaravone, probably formed by the electrophilic addition of peroxynitrite and the removal of hydroperoxy anion (HOO\(^-\)). Moreover, Yoshino and Kimura previously reported that edaravone administration decreased 3-nitrotyrosine levels in the cerebrospinal fluid of ALS patients. These results are consistent with our contention that scavenging of peroxynitrite by edaravone may contribute significantly toward inhibiting the progression of ALS.

The Japanese government approved edaravone for the treatment of ALS patients on June 26, 2015. This will significantly benefit ALS patients because no other efficacious treatment is available. This development may be of interest to doctors who study other neurodegenerative diseases such as multiple sclerosis, multiple system atrophy, Parkinson’s disease and Alzheimer’s disease because the plasma urate levels of patients with these diseases are significantly lower than those of age-matched healthy controls.

**Abbreviations**

- %16:1: percentage of palmitoleic acid in total free fatty acids
- %18:1: percentage of oleic acid in total free fatty acids
- ALS: amyotrophic lateral sclerosis
- ALSFRS-R: revised ALS functional rating scale
- CoQ\(_{10}\): oxidized form of coenzyme Q10
- CoQ\(_{10}\)H\(_2\): reduced form of coenzyme Q10
- %CoQ\(_{10}\): percentage of CoQ\(_{10}\) in total coenzyme Q10
- FFA: free fatty acid
- %PUFA: percentage of polyunsaturated fatty acid in total free fatty acids
- UA: uric acid
- VC: vitamin C
- VE: vitamin E

**Conflict of Interest**

No potential conflicts of interest were disclosed.

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