（様式４）

学 位 論 文 概 要

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学位論文題目

生体内の酸化ストレスと酸化傷害評価法：百寿者と心肺停止症候群及び筋萎縮性側索硬化症患者への応用

学位論文の要旨

酸化ストレスとは生体内の酸化と抗酸化のバランスが崩れ前者に傾き，生体にとって好ましくない状態と定義されている．我々はこれまで，血漿中酸化ストレスマーカーとしてコエンザイムQ10（CoQ10）の酸化還元バランス，組織の酸化傷害マーカーとして血漿中遊離脂肪酸とその組成に注目し，分析法の確立と応用を行なってきた．還元型CoQ10は強い抗酸化能を持ち，酸化されると定量的に酸化型になる．そのため，酸化ストレスの定義や還元型CoQ10の鋭敏性から，血漿中の全CoQ10中の酸化型の割合（%CoQ10）は生体内の酸化ストレスマーカーとして有用であると思われる．しかし，%CoQ10は全身の酸化ストレスを評価しているが，必ずしも組織の酸化ストレスを評価しているわけではない．生体膜中には非常に酸化されやすい高度不飽和脂肪酸（PUFA）が多い．そのため組織が酸化傷害を受けると，PUFAが減少し細胞膜の流動性の低下が起こる．これを補うために飽和脂肪酸を1価不飽和脂肪酸に変換するステアリルCoA不飽和化酵素によって，オレイン酸やパルミトオレイン酸が増加する．さらに酸化傷害が進むと細胞は死に至り，膜成分は加水分解され遊離脂肪酸として血中に放出されると考えられる．したがって，組織が酸化傷害を受けると血漿中の全遊離脂肪酸量は増加し，そのうちのPUFAの割合（%PUFA）は減少し，16:1や18:1の割合は増加すると考えられる．

この2つの酸化ストレスマーカーを用いて，当研究室では様々な疾病の酸化ストレスを評価してきたが，本研究では心停止後症候群（PCAS）患者，筋萎縮性側索硬化症（ALS）患者，百寿者における酸化ストレスを評価した．PCAS，ALS患者，百寿者において，%CoQ10が健常人に比べ有意に高く%PUFAが有意に低いことから，酸化ストレスが亢進していることが分かった．また，スーパーオキシドとNOの拡散律速によって生成される，ペルオキシナイトライトのスカベンジャーである尿酸がPCASとALSで減少していることが分かった．そのため，これらの疾病ではペルオキシナイトライトの生成も示唆された．さらに全ての例でフリーコレステロール（FC）とコレステロールエステル（CE）の比が有意に高くなっていることもわかった．この比は肝臓からHDLと共に分泌される酵素によって決まる．そのため，肝機能の低下が起きていることが考えられた．これらの結果から，PCAS，ALS患者，百寿者に対する酸化ストレス亢進や肝機能低下に対する対策が必要であることが示唆された．さらに，局所的な酸化ストレスマーカーとして有用になると考えられる脳脊髄液（CSF）中のCoQ10の酸化還元バランスの測定法を開発した．以上の結果と考察は臨床現場での酸化ストレス評価に有用であり，治療方針の策定にも役立

つと考えられる.

備　考

１．要旨は1200字程度にまとめること。

２．本様式により、ワープロで作成することを原則とする。

３．用紙はＡ４版 上質紙を使用すること。

Contents

List of Abbreviations

BR	unconjugated bilirubin
CE	cholesterol esters
CoQ10	oxidized form of coenzyme Q10
$CoQ10H_2$	ubiquinol-10, reduced form of coenzyme Q10
CSF	cerebral spinal fluid
CV	coefficient of variation
ECD	electrochemical detector
FC	free cholesterol
FFA	free fatty acids
HDL	high density lipoprotein
IPA	2-propanol
LCAT	lecithin-cholesterol acyltransferase
MSA	multiple system atrophy
$NaClO_4$	sodium perchlorate
%CoQ10	percentage of oxidized form of coenzyme Q10 in TQ10
%PUFA	percentage of polyunsaturated fatty acids in total FFA
%16:1	percentage of palmitoleic acid in total FFA
%18:1	percentage of oleic acid in total FFA
PCAS	post-cardiac arrest syndrome
Psap	prosaposin
TBHQ	*tert*-butylhydroquinone
TH	therapeutic hypothermia
TC	total cholesterol
TQ10	total coenzyme Q10
UA	uric acid
VC	ascorbic acid
VE	vitamin E

Chapter 1

General Introduction

1-1 Markers of Oxidative Stress

Oxidative Stress

Oxidative stress is defined as a disturbance in the pro-oxidant-antioxidant balance in favor of the former.[1] Increase in oxidative stress has been suggested to cause aging and degenerative diseases such as heart attack, stroke, neurodegenerative diseases, diabetes, and cancer.[2,3]

To evaluate oxidative stress, measurement of free radicals and reactive oxygen species (ROS) is the first choice. However it is difficult to measure them directly because their reactivities are high, and consequently their life-spans are very short and their concentrations are low. Electron spin resonance (ESR) has been used for the measurement of free radicals directly *in vitro*. However, the detection of free radicals *in vivo* has not been successful mainly because of the presence of many antioxidants.

Second choice could be oxidation products of lipids, proteins, and DNA. Kasai *et al.* focused on 8-hydroxydeoxyguanosine (8-OHdG), oxidation product of deoxyguanosine (Fig. 1).[4] Since 8-OHdG is secreted to urine, it is a good advantage that a non-invasive measurement is possible. Sato *et al.* reported that urinary 8-OHdG levels in patients with Parkinson's disease (PD) were significantly higher than healthy controls and increased with the progression of the disease (Fig. 2).[5]
Moreover, Hayakawa *et al.* reported the accumulation of 8-OHdG in mitocondrial DNA of human diaphragm with age (Fig.3).[6]

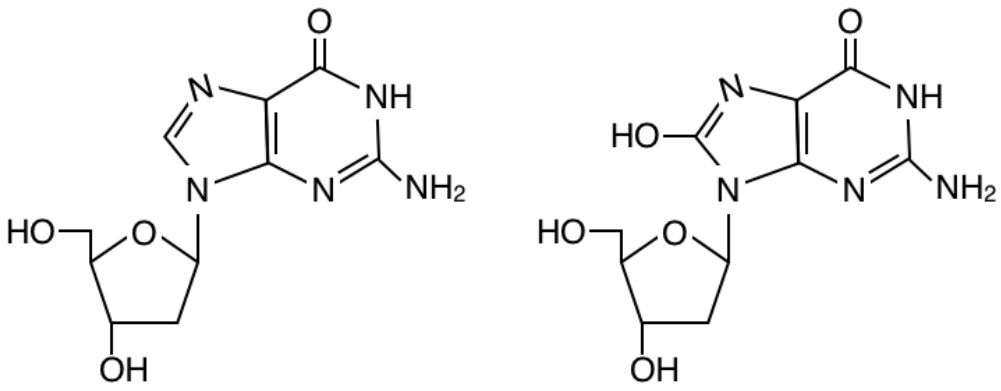
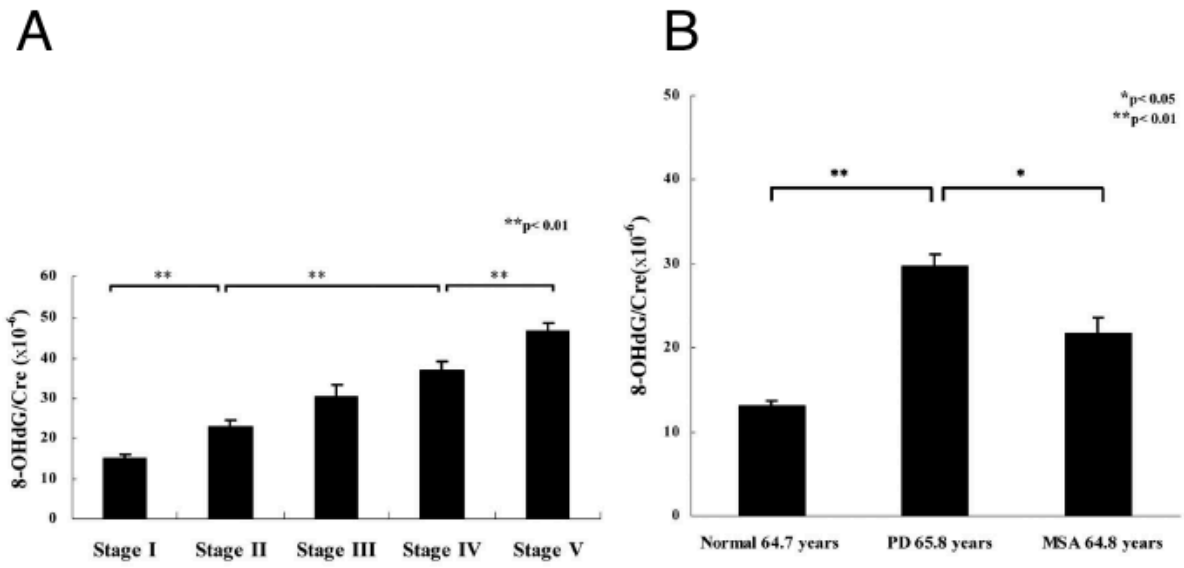
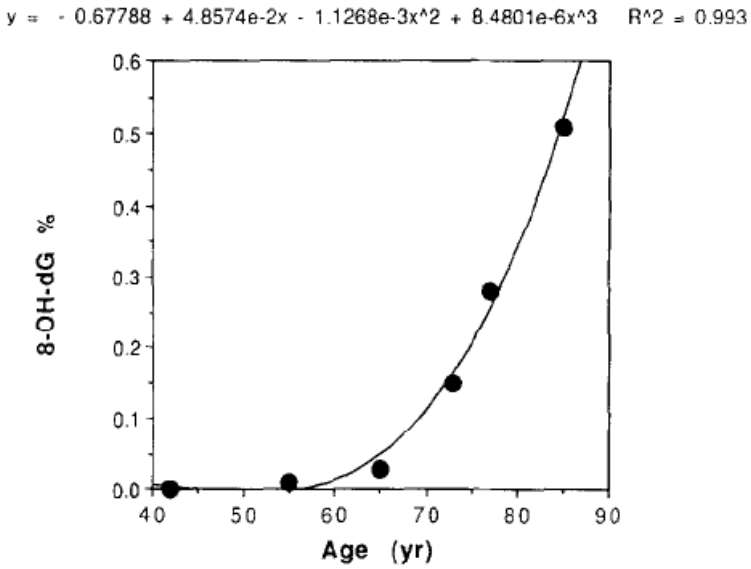


Fig. 1 Structures of deoxyguanosine and 8-OHdG.



from ref.[5], Sato S *et al.* (2015).

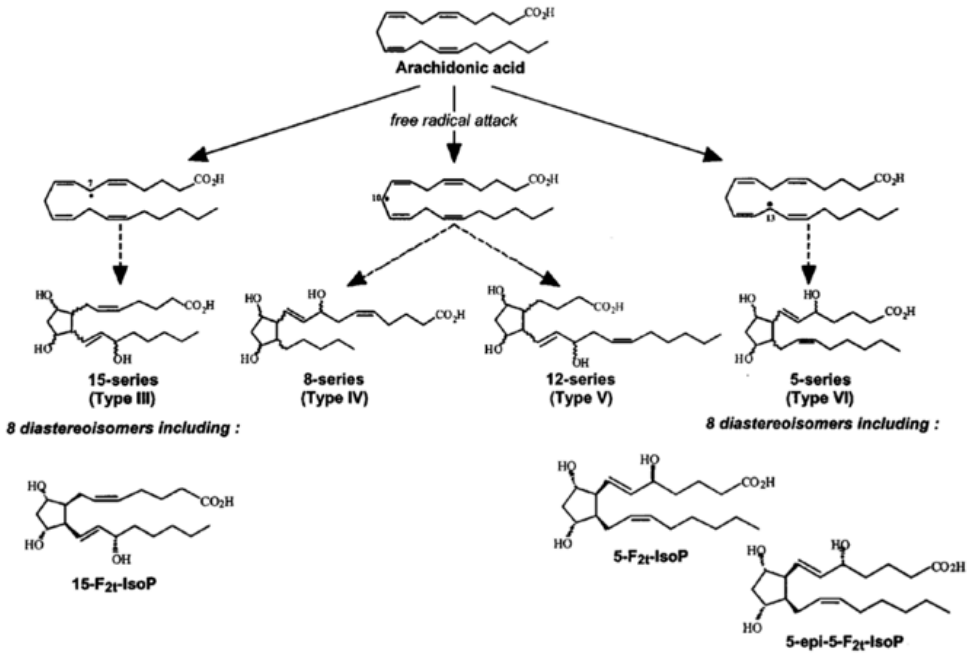
Fig. 2 Comparison of urinary levels of 8-OHdG in PD at different of stages (A) and that with normal and patients with Multiple system atrophy (MSA) (B).



from ref.[6], Hayakawa M *et al.* (1991).

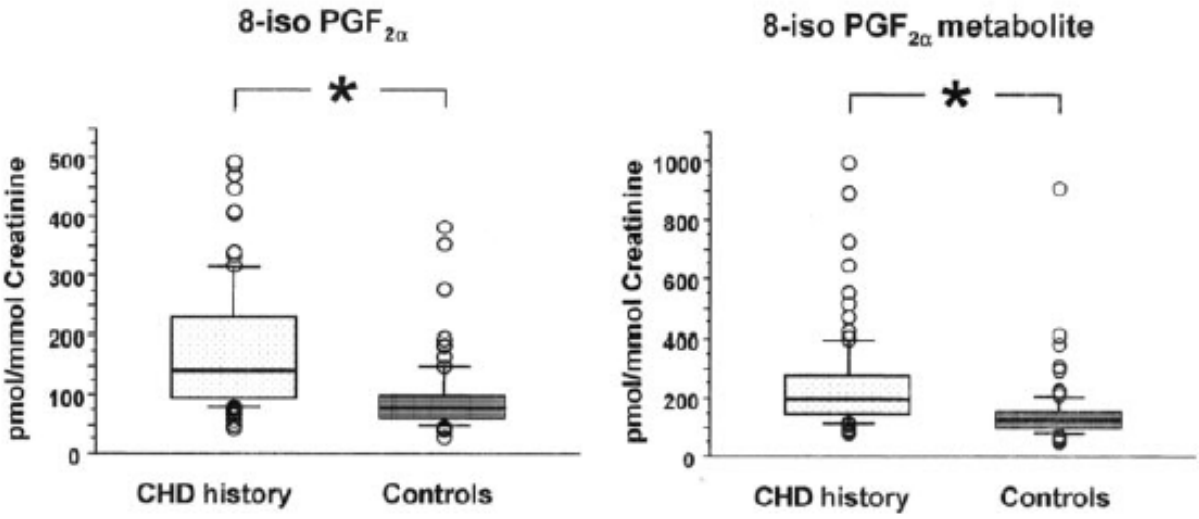
Fig. 3 Changes in 8-ODdG levels in mitochondrial DNA of human diaphragm with age.

8-isoprostane $F_{2\alpha}$ is the prostaglandin-liked compounds produced in the free radical oxidation of arachidonic acid in cell membrane and lipoprotein (Fig. 4).[7] Edzard *et al.* reported 8-isoprostane $F_{2\alpha}$ and that metabolite significantly increased in patients with coronary heart disease that it is known related with diabetes and hypertension (Fig. 5).[8]



from ref.[7], Stéphanie M *et al*. (2002).

Fig. 4 Formation of isoprostanes from autoxidation of arachidonic acid.



from ref.[8], Edzard S *et al*. (2004).

Fig. 5 Levels of urinary 8-iso-Prostaglandin $F_{2\alpha}$ in patients with coronary heart disease as compared with age-matched controls. *$p < 0.001$.

Thiobarbituric acid reactive substances (TBARS) have been used for the evaluation of lipid oxidation for a long time. Although the method is convenient, it lacks specificity and accuracy. To overcome these problems Yamamoto *et al.* developed a postcolumn HPLC detection with chemiluminescence method for the analysis of lipid hydroperoxides (LOOH).[9] LOOH were decomposed by microperoxidase to alkoxyl radicals (LO•) and they oxidized isoluminol to produce light through isoluminolendoperoxide (Fig. 6).[9] Conversion of LOOH to light makes the detection limit of lipid hydroperoxide very low (less than picomole). Yamamoto *et al.* detected cholesterol ester hydroperoxide (CE-OOH) even in healthy human plasma.[10] It is intersting that levels of CE-OOH and the ratio of CE-OOH/CE significantly increased in the order of humans, Sprague-Dawley rats (SDR) and Nagase analbuminemic rat (NAR) (Fig.7).[11] NAR are known to be very sensitive to carcinogen such as *N*-dimethlnitrosamine, suggesting that increased oxidative stress resulted in increasing the risk of carcinogenesis.[11]

$$\text{LOOH} + \text{microperoxidase} \rightarrow \text{LO}^{\bullet} \quad [1]$$

$$\text{LO}^{\bullet} + \text{isoluminol } (\text{QH}^{-}) \rightarrow$$

$$\text{LOH} + \text{semiquinone radical } (\text{Q}^{\bullet-}) \quad [2]$$

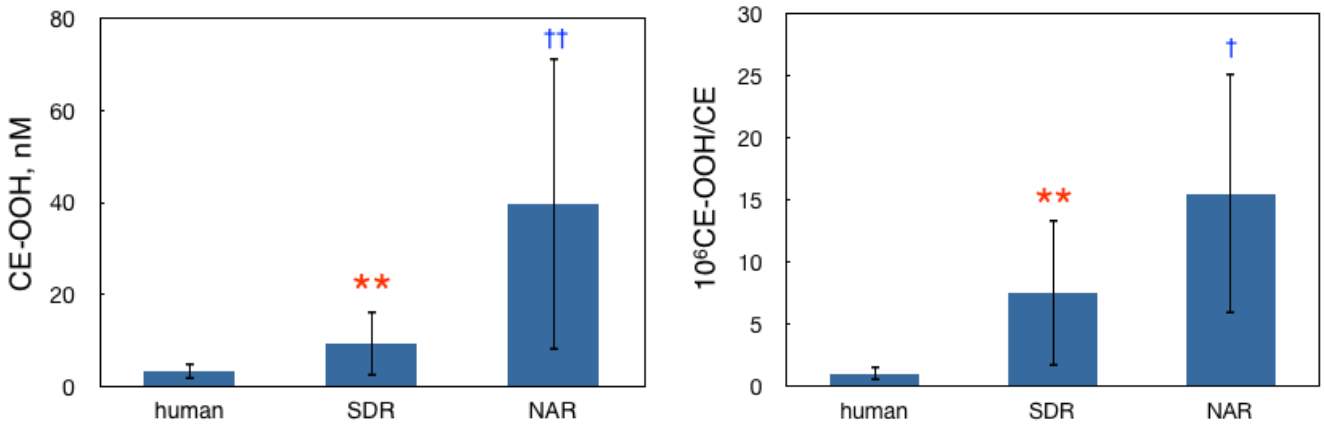
$$\text{Q}^{\bullet-} + \text{O}_2 \rightarrow \text{Q} + \text{O}_2^{\bullet-} \quad [3]$$

$$\text{Q}^{\bullet-} + \text{O}_2^{\bullet-} \rightarrow$$

$$\text{isoluminol endoperoxide} \rightarrow \text{light} \quad [4]$$

from ref.[9], Yamamoto Y *et al.* (1987).

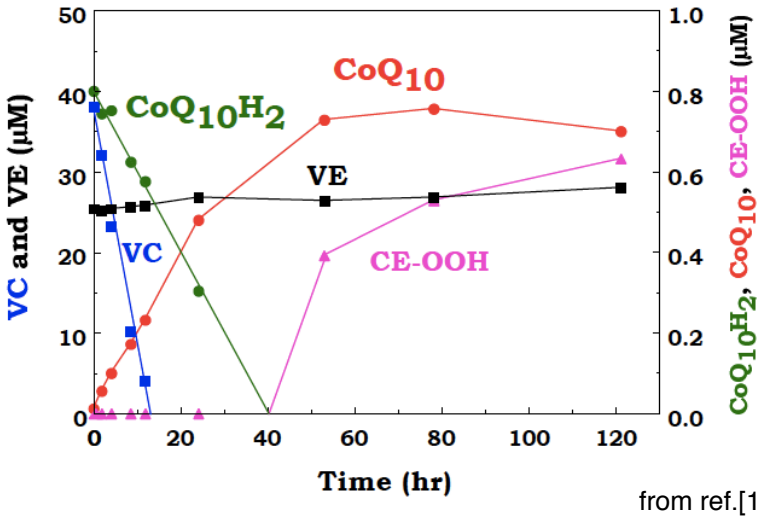
Fig. 6 Conversion of lipid hydroperoxide to light in the presence of microperoxidase and isoluminol.



from ref.[11], Yamamoto Y *et al.* (1992).

Fig. 7 Levels of lipid hydroperoxide in human, SDR, and NAR plasmas. * means human vs SDR and **$p < 0.01$. † means SDR vs NAR and † = 0.011, †† < 0.01.

These biological oxidation products are useful to understand the mechanism of ROS-induced oxidative damage. However, they may not be useful oxidative stress markers at early stage because antioxidants protect the oxidation of lipids, proteins, and DNA. In other words, decrease of antioxidants should be a good marker of early oxidative stress. There is an interesting report by Yamamoto *et al.* showing which is the most sensitive plasma antioxidants against oxidative stress.[12] Figure 8 shows that vitamin C (VC) decreased first and followed by the reduced form of coenzyme Q10 ($CoQ10H_2$). However, no significant decay in vitamin E (VE) was seen. Furthermore a significant increase in CE-OOH was observed after the depletion of VC and $CoQ10H_2$. The above results indicate that VC and $CoQ10H_2$ should be a useful marker of oxidative stress at early stage. However metabolism of VC is rapid and VC is influenced by meals. On the other hand, coenzyme Q10 is present in the reduced form ($CoQ10H_2$) and the oxidized form (CoQ_{10}) (Fig. 9) and $CoQ10H_2$ was converted to CoQ10 quantitatively in plasma oxidation (Fig. 8), the redox balance of coenzyme Q10 would be a sensitive marker of early oxidative stress.



from ref.[12], Yamamoto Y *et al.* (1991).

Fig. 8 Change in plasma antioxidant levels during the aerobic oxidation of human plasma in the presence of 5 μM cupric chloride at 37 °C.

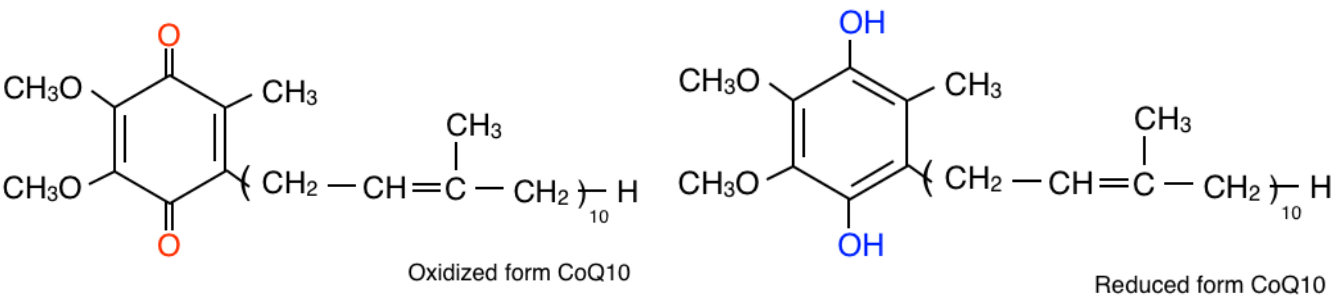
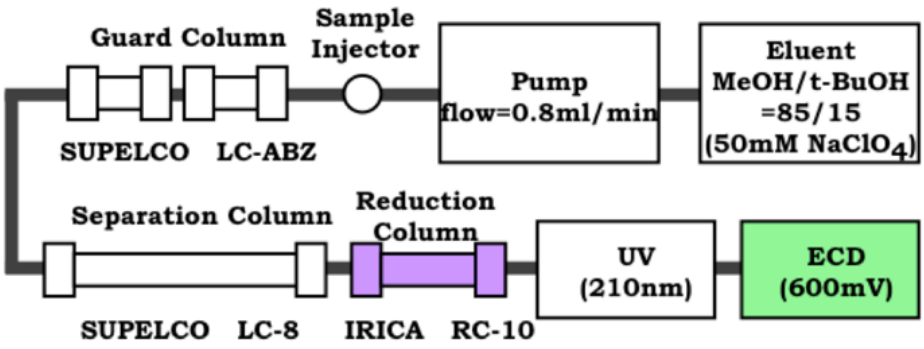


Fig. 9 Chemical structures of reduced and oxidized forms of coenzyme Q10.

Plasma Mareker of Oxidative Stress

Percentage of CoQ10 in total coenzyme Q10 (%CoQ10) would be a good oxidative stress marker. Yamashita and Yamamoto developed the HPLC method for the simultaneous detection of plasma $CoQ10H_2$ and CoQ_{10} by an electrochemical detector.[13] CoQ10 was reduced to $CoQ10H_2$ after the HPLC separation by a reduction column as shown in Fig. 10.[13] Introduction of column switching system makes the method more practical and reliable (unpublished data). The method was applied to various subjects and an increase of oxidative stress was observed in patients with hepatitis, cirrhosis, and hepatoma,[14] juvenile fibromyalgia (JFM),[15] and PD.[16] Table 1 summarizes the representative results.



from ref.[13], Yamashita S *et al.* (1997).

Fig. 10 Schematic diagrams of HPLC system for simultaneous detection of $CoQ10H_2$ and CoQ10.

Table 1 Change 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. Ref means reference, in prep means manuscript in preparation.

Diseases or conditions	%CoQ10(9)	Ref	FFA	%PUFA	%16:1	%18:1	Ref
Hepatitis, cirrhosis, and hepatoma	↑	(14)	↑	↓	↑	↑	in prep
Juvenile fibromyalgia	↑	(15)	↑	↓	↑	↑	(15)
Newborn babies at day 1	↑	(24)	↑	↓	↑	NS	(24)
Rat, brain attack model	NS	(23)	↑	NS	↑	↑	(23)
Above rat with edarabone treatment	NS	(23)	NS	NS	↓	↓	(23)

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[3] and the resulting FFAs may enter the bloodstream through leakage or lysis of oxidatively damaged tissues. 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[17] since they are highly susceptible to oxidation. 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.[18] Such changes in the plasma were observed in rats with carbon tetrachloride poisoning,[19] in LEC rats which are spontaneous liver injury model,[20] and patients with hepatitis, cirrhosis, and hepatoma.[14]

Stearoyl-CoA desaturase (SCD) is a membrane-boud enzyme that catalyzes the biosynthesis of monounsaturated fatty acids from saturated acids.[18] Thus, 18:1 and 16:1 are produced frome stearic acid (18:0) and palmitic acid (16:0), respectively. SCD expression is regulated by various factores. Oxidative stress is a general stimulant of SCD activity,[19] since decreased levels of PUFA due to its high susceptibility to oxidation need to be compensated for by an increase in monoenoic acids to maintain membrane fluidity. [18] High SCD activity has been implicated in oxidative stress-related diseases such as diabetes, atherosclerosis, and cancer.[21]

These results suggest that plasma FFA and their composition are good markers of tissue oxidative damage (Fig. 11). Lee *et al.* developed a fluorometric derivatization method of carboxylic acid with monodansyl cadaverine for HPLC analysis(Fig. 12).[22] Their method was modified and applied to the plasmas obtained from the rats with a middle cerebral artery occlusion,[23] patients with JFM,[15] and newborn babies.[24] Table 1 summarizes the representative results.

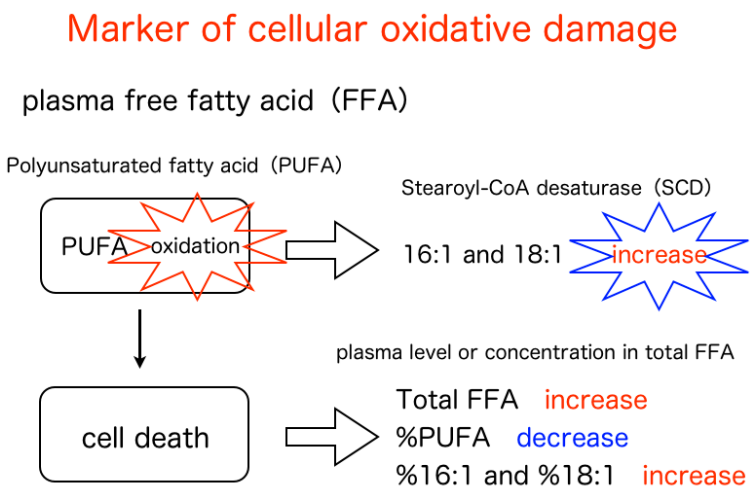
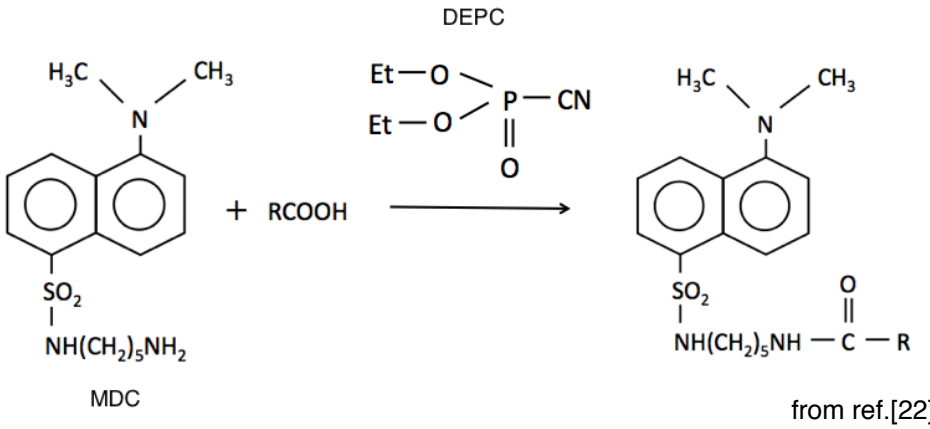


Fig 11 Plasma FFA and their compositions as a marker of tissue oxidative damage.



from ref.[22], Lee Y *et al*. (1989).

Fig 12 Conversion of FFA to a fluorometric derivatives.

Oxidative Stress Marker in Cerebrospinal Fluid

Edaravone is a low-molecular-weight antioxidant drug targeting peroxyl radicals among many types of reactive oxygen species. In 2001, Edaravone was approved in Japan as a drug to treat acute-phase cerebral infarction, and then in 2015 it was approved for amyotrophic lateral sclerosis (ALS). In 2017, the U.S. Food and Drug Administration also approved edaravone for treatment of patients with ALS. Therefore, oxidative stress in brain has received much attention.

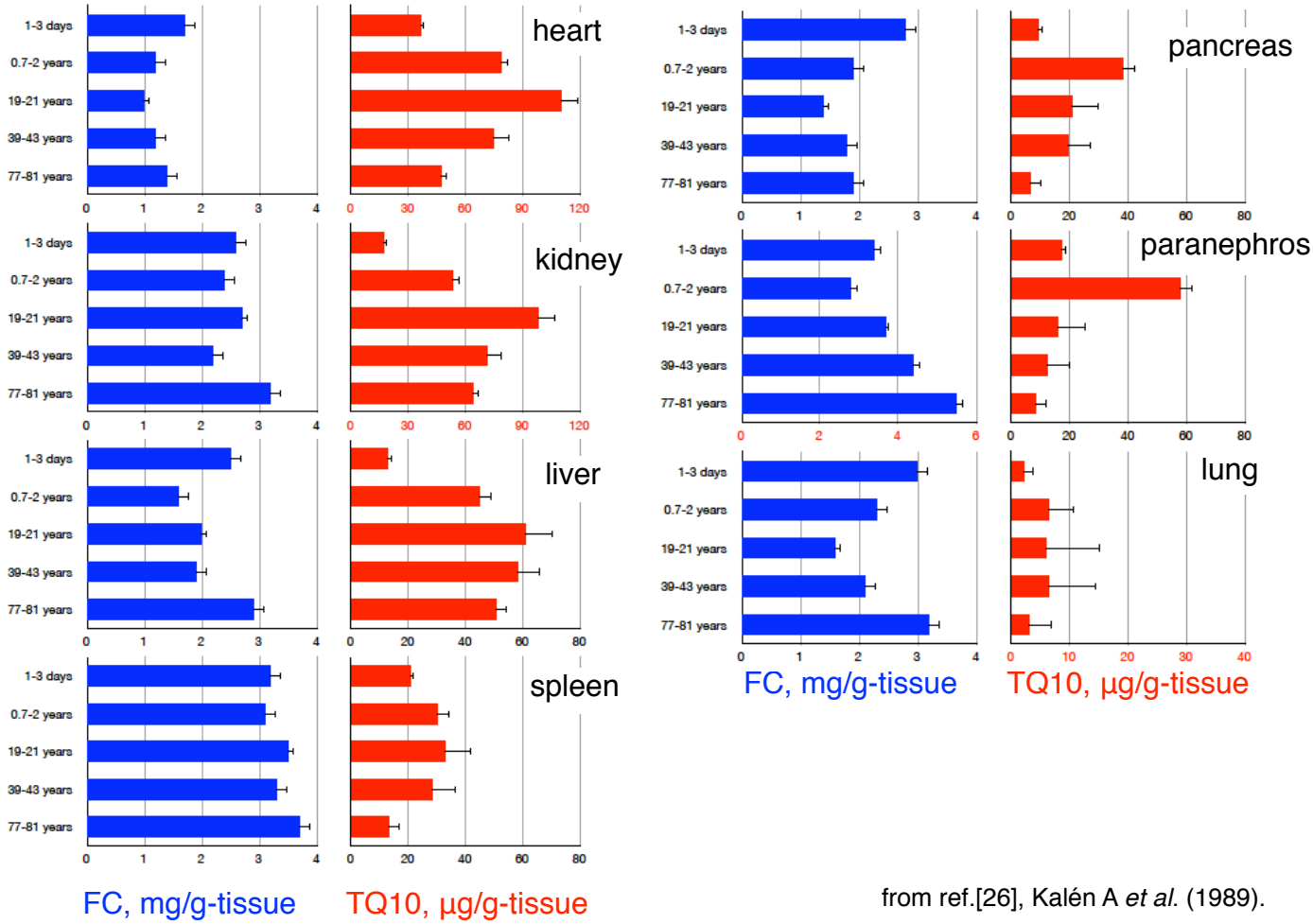
Mutation in coenzyme Q10 biosynthesis enzyme gene was found in patients with multiple system atrophy (MSA) and brain level of coenzyme Q10 in homozygous patient was very low as compared with those of control subject.[25] Now a phase 2 clinical trial employing a high dose $CoQ10H_2$ for patients with MSA is ongoing.

In order to discuss oxidative stress in brain, cerebrospinal fluid (CSF) should be a good target. However, the levels of total coenzyme Q10 (TQ10) in human CSF are less than 1/500 of those in human plasma. In this study, we developed a method for the simultaneous electrochemical detection of $CoQ10H_2$ and CoQ10 in CSF.

1-2 Coenzyme Q10 as a Antioxidant

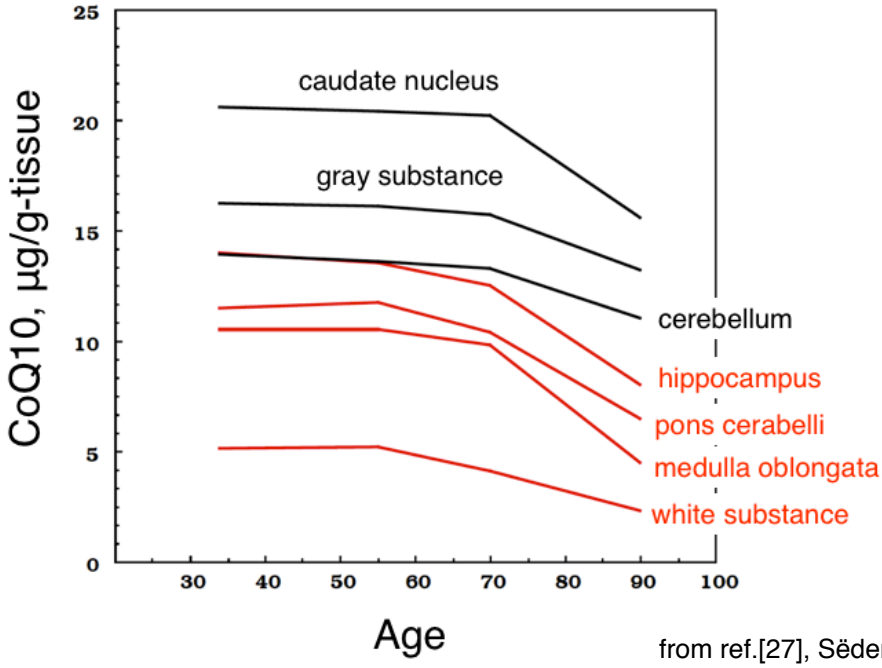
Tissue Coenzyme Q10 Levels

Coenzyme Q10 is an essential component of the mitochondrial electron transport system. $CoQ10H_2$ is one of the most important lipid-soluble antioxidant. Changes in tissue levels of free cholesterol (FC) and coenzyme Q10 was shown in Fig. 13.[26] Tissue levels decrease in the order of heart, kidney > liver > spleen, pancreas > adrenal > lung. Tissue levels are the highest at the age of 20's and decrease with age (Fig. 13). Contrary, FC levels increase with age. Since biosynthetic pathways of coenzyme Q10 and FC are common, increase in tissue FC level retards the biosynthesis of coenzyme Q10. Figure 14 shows the changes in the levels of coenzyme Q10 in various regions of the huan brain.[27] They decrease with age and their drops are apparent after age 70. More than 60% decline in hippocampus should be very serious since it controls our memory.



from ref.[26], Kalén A *et al.* (1989).

Fig. 13 Changes in human tissue levels of FC and coenzyme Q10 with age.



from ref.[27], Sëderberg M *et al.* (1990).

Fig. 14 Changes in coenzyme Q10 levels at various regions in human brain with age.

Coenzyme Q10 binding/transfer Protein

Since coenzyme Q10 is not soluble in water, it is located in lipoproteins in blood. Moreover, coenzyme Q10 binding/transfer proteins were identified as saposin B and its precursor prosaposin (Psap).[28] Psap is the precursor protein for saposins A, B, C, and D. These glycoproteins were isolated as activators of sphingolipids hydrolytic enzymes in lysosome. Psap also exists as a secreted protein, which has been found in various bodily fluids such as milk, serum, seminal plasma, and cerebrospinal fluid. Despite a lot of previous works, many physiological functions of Psap have not been elucidated to date. In this study, we measured Psap in human plasma and discussed the role of Psap under oxidative stress.

1-3 The Objective of This Study

Since useful methods for the evaluation of oxidative stress in circulation (plasma %CoQ10) and tissue oxidative damage (plasma FFA and their composition), I applied these methods to human subjects under oxidative stress such as patients with post-cardiac arrest syndrome (PCAS) and ALS, and centenarians. Water-soluble antioxidants such as VC and uric acid, Psap, FC, and CE were also measured.
Taken all data, we discussed the characteristic of the diseases and aging.
For the future study, we also developed a method for simultaneous electrochemical detection of $CoQ10H_2$ and CoQ10 in CSF.

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Chapter 2

Oxidative Stress and Abnorma Cholesterol Metabolism in Patients with Post-cardiac Arrest Syndrome

Introduction

Ischemia/reperfusion is an important trigger of reactive oxygen species (ROS) formation.[1-3] As direct evidence, Maekawa *et al.* observed the continuous formation of superoxides in the rat jugular vein after forebrain ischemia and subsequent reperfusion promoted its production.[4,5] Newborn babies suffer from whole body ischemia/reperfusion injury since their oxygen tensions change from 2-8% to 21% at birth.[6] We previously demonstrated a significant increase in oxidative stress by measuring plasma antioxidants at 0, 1, 3, and 5 days after birth.[7] Plasma levels of the most sensitive antioxidant, ascorbic acid, decreased daily to equilibrium values at 3 and 5 days.[7] Percentages of the oxidized form of coenzyme Q10 (%CoQ10) in total coenzyme Q10, another blood marker of oxidative stress, in infants (25-31%) were significantly higher than those in healthy young adults (4.5%).[7] We also measured plasma free fatty acids (FFA) and their composition as markers of tissue oxidative damage.[7] FFA levels were highest at day 1 and decreased rapidly thereafter, whereas the content of oxidatively vulnerable polyunsaturated fatty acids (%PUFA) in total FFA was lowest at day 1 and then increased.[7]

Patients with post-cardiac arrest syndrome (PCAS) also suffer from whole body ischemia/reperfusion. Therefore, their oxidative stress is likely severe. However, few papers describe a decrease of antioxidants and an increase of oxidation products from lipids and proteins. In this paper, we evaluated oxidative stress in patients with PCAS by measuring plasma markers of oxidative stress in the circulation (antioxidants) and tissues (FFA and their composition). We also measured plasma levels of free cholesterol (FC) and cholesterol esters (CE). Their time course changes (0, 6, and 24 hrs, and 2 and 7 days after hospitalization) were compared among 6 groups of patients divided according to outcome severity as follows: died within a day; died within a week; died within a month; hospitalized for more than 2 weeks; hospitalized for less than 2 weeks; and discharged in a week. We will discuss important factors determining the survival of patients.

The survival rate to hospital discharge from PCAS in Japan was only 7.9% in 2014.[3] To increase the survival rate, we treated about 70% of patients with therapeutic hypothermia (TH). Surprisingly, TH treatment enhanced the increase in plasma ratio of total coenzyme Q10 (TQ10) to total cholesterol (TC) at the end of rewarming. This implies that coenzyme Q10 was introduced to blood circulation by a lipoprotein-independent pathway not previously discussed.

Subjects and Methods

Study design

The present study was carried out in the Division of Emergency and Critical Care Medicine, Department of Acute Medicine, Nihon University School of Medicine during the period from 29 November 2005 to 4 August 2015. The study protocols were approved by the Ethical Committee of Nihon University School of Medicine, and patient samples were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2001. Forty subjects (27 males aged 66.0 ± 15.8 years (mean ± SD) and 13 females aged 63.4 ± 20.8 years) were enrolled. The causes of PCAS were acute myocardial infarction (number of patients =10), ventricular fibrillation (5), suffocation (5), congestive heart failure (2), hyperkalemia (2), complete atrioventricular block (1), sick sinus syndrome (1), coronary spastic angina (1), chronic obstructive pulmonary disease (1), diffuse interstitial fibrosing pneumonia (1), pulmonary embolism (1), necrotizing fasciitis (1), sepsis (1), acute pancreatitis (1), diabetic ketoacidosis (1), gastric ulcer bleeding (1), subarachnoid hemorrhage (1), double outlet right ventricle (1), and unknown (3).

Patients were treated with conventional resuscitation methods and, if possible, TH treatment (34°C for 24 hr and gradual rewarming to 36°C for 24 hr) was introduced. In 32 of 40 cases, TH treatment was applied; however, in 5 cases treatment was not completed because of unstable blood pressure (4 cases) and low temperature caused by infection (1 case) (Table 1). TH treatment was not applied to 8 cases.

Heparinized plasma was collected when patients were hospitalized and at 6 and 24 hrs, and 2 and 7 days, and stored at -80°C until analysis.

Table 1 Outcomes of the 40 PCAS patients and therapeutic hypothermia treatment.

Outcome	Died/ Hospitalized	therapeutic hypothermia treatment			Total
		performed	ceased	untreated	
Died	< 1 day	2	1	3	6
Died	< 1 week	3	2	1	6
Died	< 1 month	2	0	2	4
Survived	> 2 week	9	2	0	11
Survived	1-2 week	10	0	0	10
Survived	1 week	1	0	2	3
Total		27	5	8	40

Analytical procedure

Plasma levels of vitamin E (VE), ubiquinol-10, ubiquinone-10, FC, and CE were determined as previously described[8] with some modifications. In brief, plasma was extracted with 19 volumes of 2-propanol and the extract was analyzed by HPLC using an analytical column (Supelcosil LC-8, 5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan, Tokyo, Japan), a reduction column (RC-10-1; Irica, Kyoto, Japan) and an amperometric electrochemical detector (Model Σ985; Irica) with an oxidation potential of +600 mV (vs. Ag/AgCl) on a glass carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/2-propanol (9/1, v/v), delivered at a flow rate of 0.8 ml/min.

Plasma levels of ascorbic acid (VC), uric acid (UA) and unconjugated bilirubin (BR) were determined by HPLC on a bonded-phase aminopropylsilyl column (Supelcosil LC-NH_2, 5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan) with UV/VIS detection (265 nm for 0-15 min and 460 nm for 15-22 min) as described previously.[9]

Plasma FFA were derivatized with monodansylcadaverine for analysis by HPLC.[10] Briefly, plasma samples (50 μl) were mixed with 200 μl of methanol and then centrifuged at 13,000 x g for 5 min. Aliquots (50 μl) of supernatants were mixed with 20 μl of methanol containing 25 μM tridecanoic acid (internal standard) and dried under a stream of nitrogen gas, and the residue was admixed with diethyl phosphorocyanidate (1 μl) and N,N-dimethylformamide (50 μl) containing monodansylcadaverine (2 mg/ml) and kept at room temperature in the dark for 20 min. A 5-μl sample was injected onto an octadecylsilyl column (3 μm, 3.3 cm x 4.6 mm i.d.; Supelco Japan) and a pKb-100 column (5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan) connected in tandem. The FFA components were measured by fluorescence detection (Model 821-FP; Japan Spectroscopic, Tokyo, Japan) with excitation at 320 nm and emission at 520 nm. The mobile phase consisted of acetonitrile/methanol/water (17.5/65.0/17.5, v/v/v) delivered at a flow rate of 1.5 ml/min. The analytical columns were heated to 40°C.

Plasma levels of prosaposin (Psap), a coenzyme Q10 binding and transfer protein, were measured by a sandwich ELISA using monoclonal and polyclonal antibodies against human saposin B.[11] Plasma was diluted 100 times with a phosphate-buffer saline containing 0.1% Triton X-100, 1 g/L NaN_3, 10 g/L BSA, and 1 mM EDTA. Purified saposin B was used as a standard.[11]

Statistical analysis

Data presented are mean values and standard deviations. Statistical analysis was performed with a paired Student's t-test for two comparisons and one-way repeated measures ANOVA followed by the Tukey–Kramer multiple comparisons test. $p < 0.05$ was considered statistically significant.

Results and Discussion

Oxidative stress in PCAS patients

Table 2 shows plasma levels of antioxidants and lipids in PCAS patients at the time of hospitalization and those in age-matched healthy controls. A significant increase in %CoQ10 was observed in PCAS patients compared to healthy controls, indicating that the redox balance of coenzyme Q10 shifted to the oxidized form, confirming increased oxidative stress in the blood of PCAS patients. A significant increase in plasma FFA levels suggests that considerable tissue damage occurred in PCAS patients. This damage is likely oxidative because a significant decrease in %PUFA was observed. Tissue damage results in the decomposition of DNA and the conversion of purines to UA. This is consistent with the observed significant increase in plasma UA. In contrast, plasma levels of VC, VE, and TQ10 in PCAS patients were similar to healthy controls. The significant decrease in BR is notable, since decreased BR is recognized as a risk factor for coronary artery disease.[12-13]

It is noteworthy that infants also exhibited high %CoQ10 (25-31%) at birth.[7] Plasma FFA level were the highest at day 1, decreasing thereafter.[7] Whereas, plasma %PUFA were the lowest at day 1 and then increased,[7] and plasma levels of UA were the highest at day 1.[7] The similarity in data between patients with PCAS and newborns is reasonable, since both are exposed to ischemia/reperfusioninduced oxidative stress.

Plasma levels of FC, CE, and TC were significantly lower than those in agematched healthy controls. Moreover, the FC/CE ratio was significantly greater than that in controls, indicating decreased activity of lecithin-cholesterol acyltransferase (LCAT), which catalyzes the conversion of FC to CE.[14,15] Since LCAT is secreted from the liver, an increased FC/CE ratio suggests impairment of liver function.[14,15]

Table 2 Levels of plasma antioxidants and lipids in patients with PCAS at the time of hospitalization as compared to age-matched healthy controls (average ± SD). *p* values were determined using a Student's *t*-test. VC, ascorbic acid; UA, uric acid; BR, unconjugated bilirubin; VE, vitamin E; TQ10, total coenzyme Q10; %CoQ10, ratio of oxidized form of coenzyme Q10 to TQ10; FFA, free fatty acids; %PUFA, ratio of polyunsaturated fatty acids to total free fatty acids; %16:1, ratio of palmitoleic acid to total FFA; %18:1, ratio of oleic acid to total FFA; FC, free cholesterol; CE, cholesterol esters; TC, total cholesterol.

	PCAS	normal control	*p*
n	40	55	
male/female	27/13	38/17	
age	65.2 ± 17.4	60.1 ± 9.3	
VC, μM	36.5 ± 28.9	31.1 ± 21.0	
UA, μM	406 ± 191	317 ± 86	< 0.05
BR, μM	4.1 ± 3.7	6.9 ± 3.6	< 0.01
VE, μM	26.0 ± 8.8	28.5 ± 7.3	
TQ10, nM	667 ± 393	710 ± 206	
%CoQ10	20.1 ± 21.0	3.9 ± 1.3	< 0.05
FFA, μM	709 ± 608	457 ± 288	< 0.001
%PUFA	19.1 ± 3.5	23.6 ± 4.6	< 0.001
%16:1	3.9 ± 1.7	3.9 ± 1.4	
%18:1	33.8 ± 6.6	34.4 ± 5.1	
FC, mM	1.17 ± 0.35	1.37 ± 0.25	< 0.001
CE, mM	2.54 ± 0.95	3.36 ± 0.72	< 0.001
TC, mM	3.71 ± 1.20	4.72 ± 0.94	< 0.001
FC/CE	0.53 ± 0.33	0.41 ± 0.05	< 0.05

Time course changes in plasma antioxidants and cholesterol

Next, we examined time course (0-7 days after hospitalization) changes in plasma antioxidants and lipids as shown in Figs. 1-9. Patients with PCAS were divided into 6 groups according to outcome severity as follows: died within a day (6 cases); died within a week (6); died within a month (4); hospitalized for more than 2 weeks (11); hospitalized for less than 2 weeks (10); and discharged in a week (3). We expect that comparisons among these 6 groups could reveal important factors in determining the survival of patients.

Figure 1 shows the time course changes of the oxidative stress marker %CoQ10 in the circulation. %CoQ10 values increased with time in patients who went on to die. In contrast, %CoQ10 values decreased in patients who were discharged within one or two weeks. These results clearly show that control of oxidative stress in blood circulation is important for patient survival.

Figures 2 and 3 show the time course changes in lipid-soluble antioxidants, TQ10 and VE, respectively. Surprisingly, more than 50% of TQ10 and VE levels were lost over 6 hr in patients who died within a day. However, these declines may not be associated with oxidative stress, since ~50% of TC also disappeared in 6 hr (Fig. 4). Lipoprotein secretion from the liver and other organs was obviously impaired in patients who died within a day.

In contrast, plasma levels of TC, TQ10, and VE in patients who were discharged in a week remained constant and within normal ranges (Figs. 2-4).

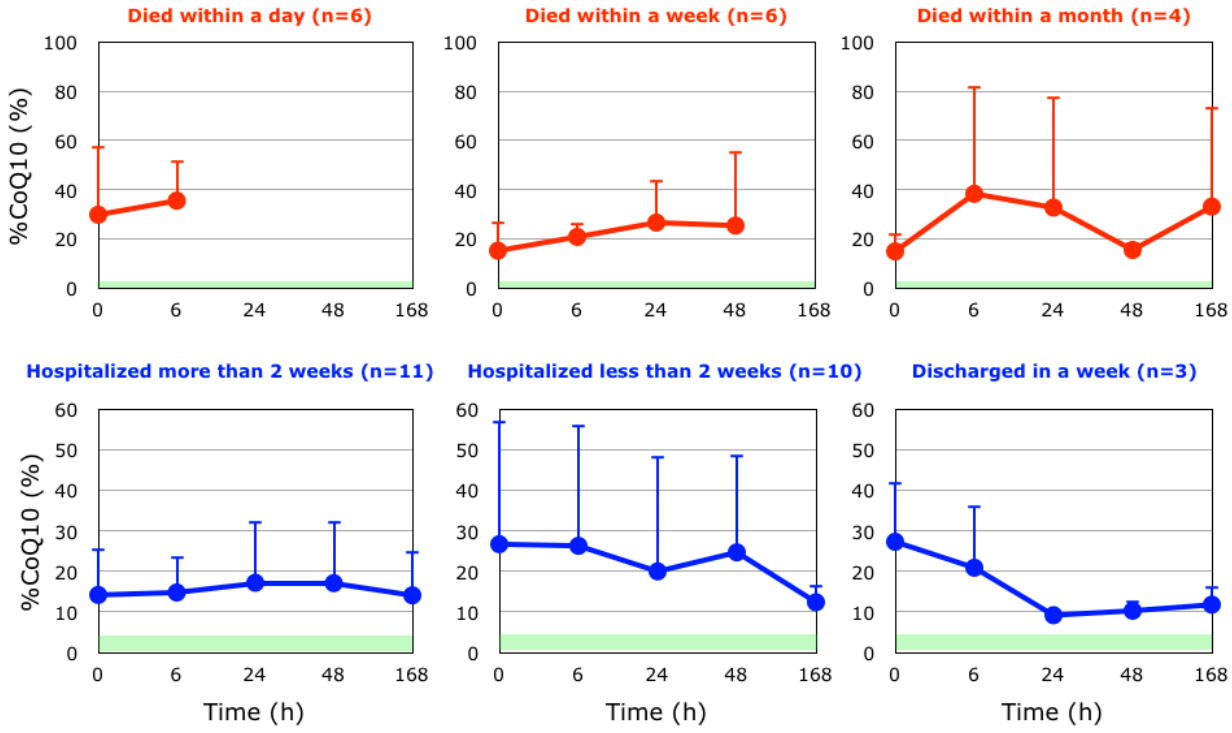

Fig. 1 Time course of changes in the percentage of the oxidized form of coenzyme Q10 in TQ10 (%CoQ10) after hospitalization. Patients were divided into six groups according to outcome. Average %CoQ10 in age-matched healthy controls was 3.9 ± 1.3 (± SD, n=55); this range is shaded in green.

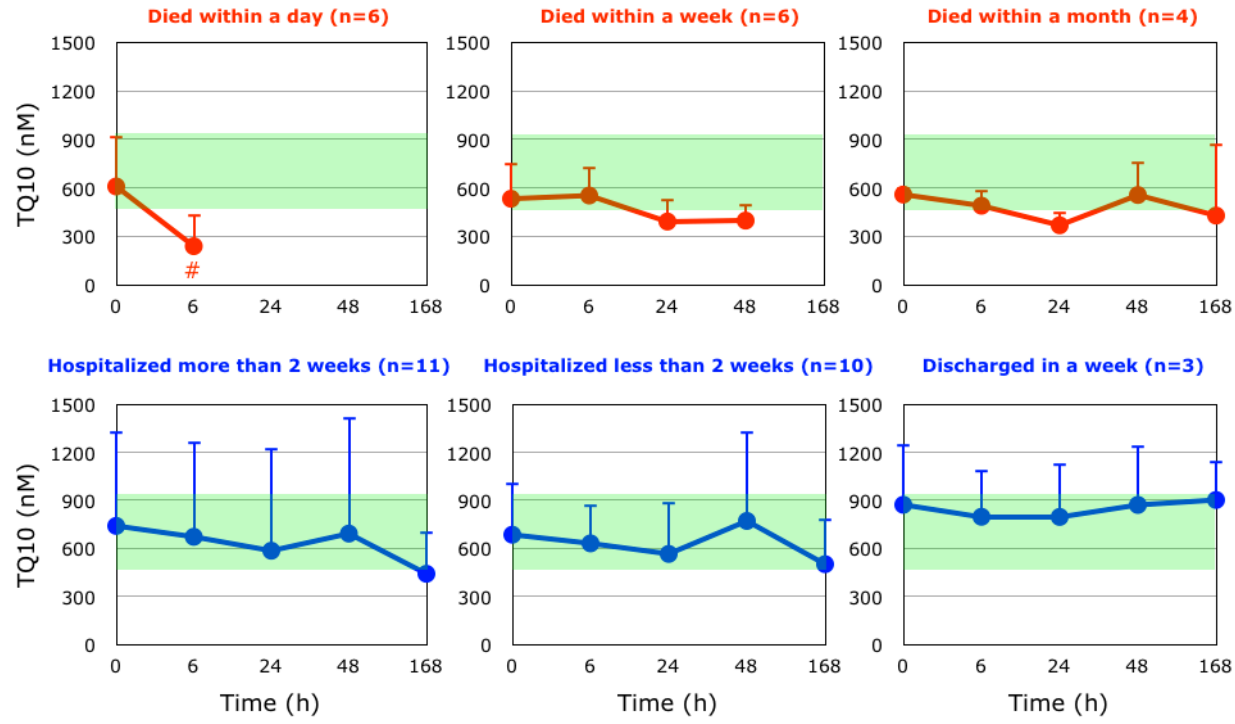

Fig. 2 Time course of changes in plasma total coenzyme Q10 (TQ10) after hospitalization. Patients were divided into six groups according to outcome. The average TQ10 level in age-matched healthy controls was 710 ± 206 μM (± SD, n=55); this range is shaded in green. # $p < 0.05$, significant differences compared to values at 0 hr as determined by a paired Student's *t*-test.

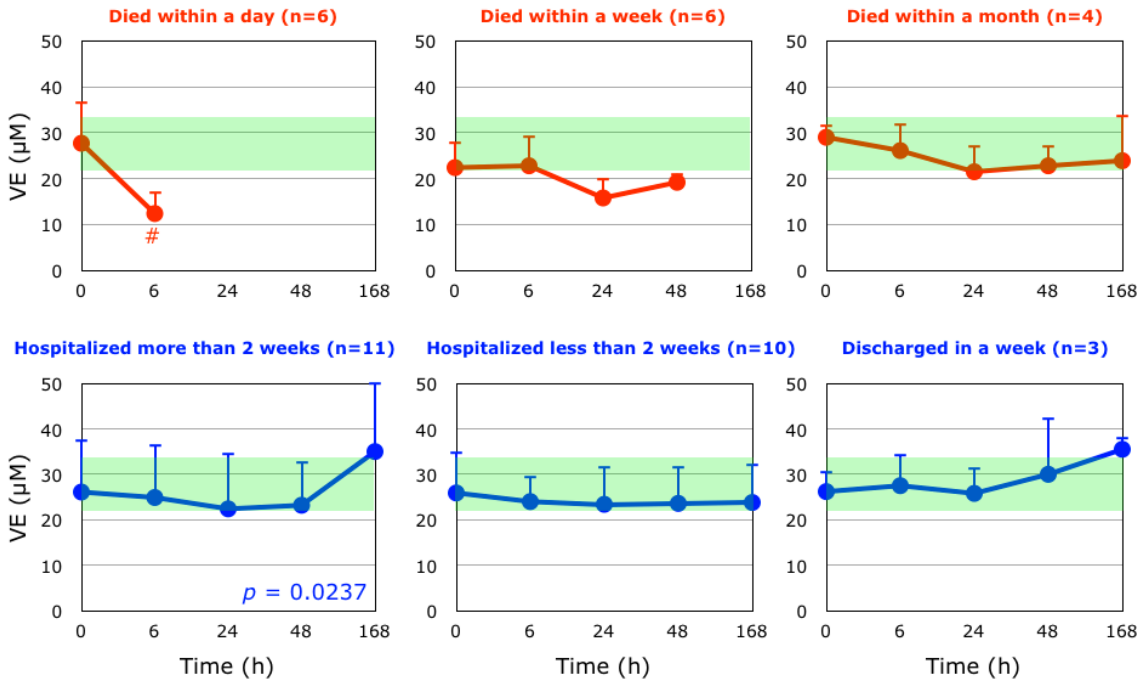

Fig. 3 Time course of changes in plasma vitamin E (VE) after hospitalization. Patients were divided into six groups according to outcome. The average VE level in age-matched healthy controls was 28.5 ± 7.3 μM (± SD, n=55); this range is shaded in green. # $p < 0.05$, significant differences compared to values at 0 hr as determined by a paired Student's *t*-test. p values are indicated when one-way repeated ANOVA analysis was significant.

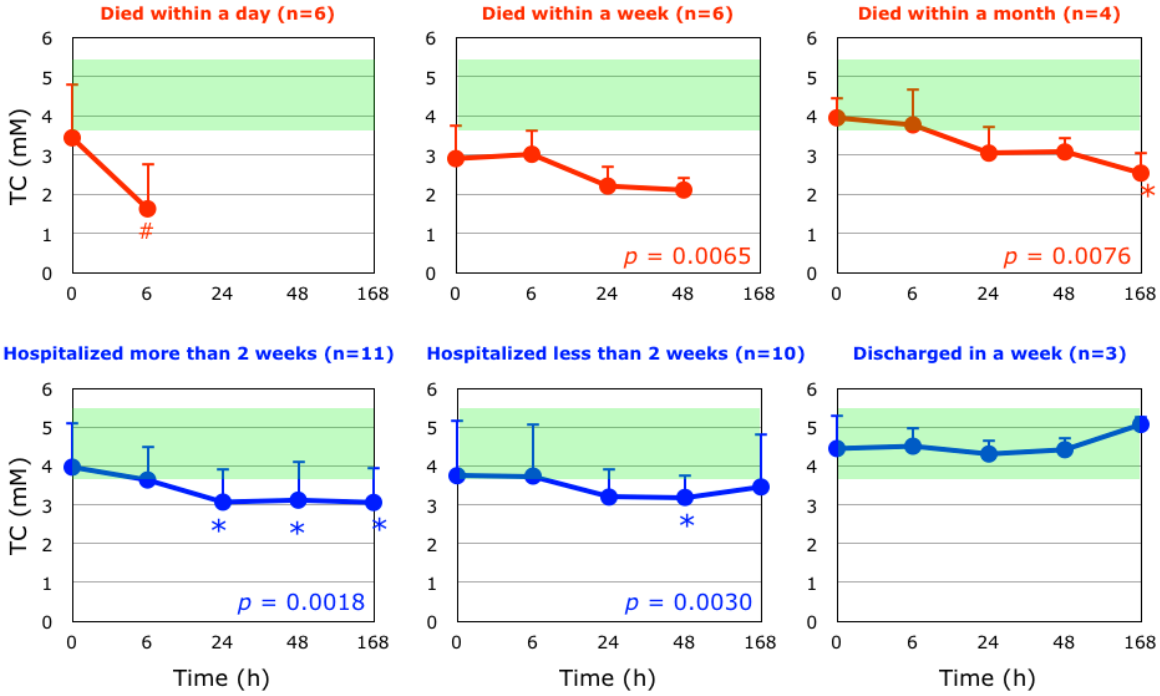

Fig. 4 Time course of changes in plasma total cholesterol (TC) after hospitalization. Patients were divided into six groups according to outcome. The average TC level in age-matched healthy controls was 4.72 ± 0.94 mM(± SD, n=55); this range is shaded in green. # $p < 0.05$, significant differences compared to values at 0 hr as determined by a paired a Student's *t*-test. *p* values are indicated when one-way repeated ANOVA analysis was significant.
* $p < 0.05$, significant differences compared to values at 0 hr as determined by the Tukey–Kramer multiple comparisons test.

Figures 5 and 6 show the time course changes in plasma CE and FC, respectively. It is apparent that the declines of CE were more profound than those of FC. This can be seen more easily in the FC/CE ratios shown in Fig. 7. The FC/CE ratios remained constant and within a normal range in PCAS patients who were discharged in a week. On the other hand, the FC/CE ratios were extremely high in PCAS patients who died within a day. This ratio increased with time in PCAS patients who died and those hospitalized for more than 2 weeks. Since the FC/CE ratio is determined by LCAT activity, which converts FC to CE, and LCAT is secreted with HDL from the liver, a high FC/CE ratio indicates some impairment of liver function. Supplementation with coenzyme Q10 could be one approach to preserve liver function, since improved FC/CE ratios were observed in patients with fibromyalgia upon supplementation.[16]

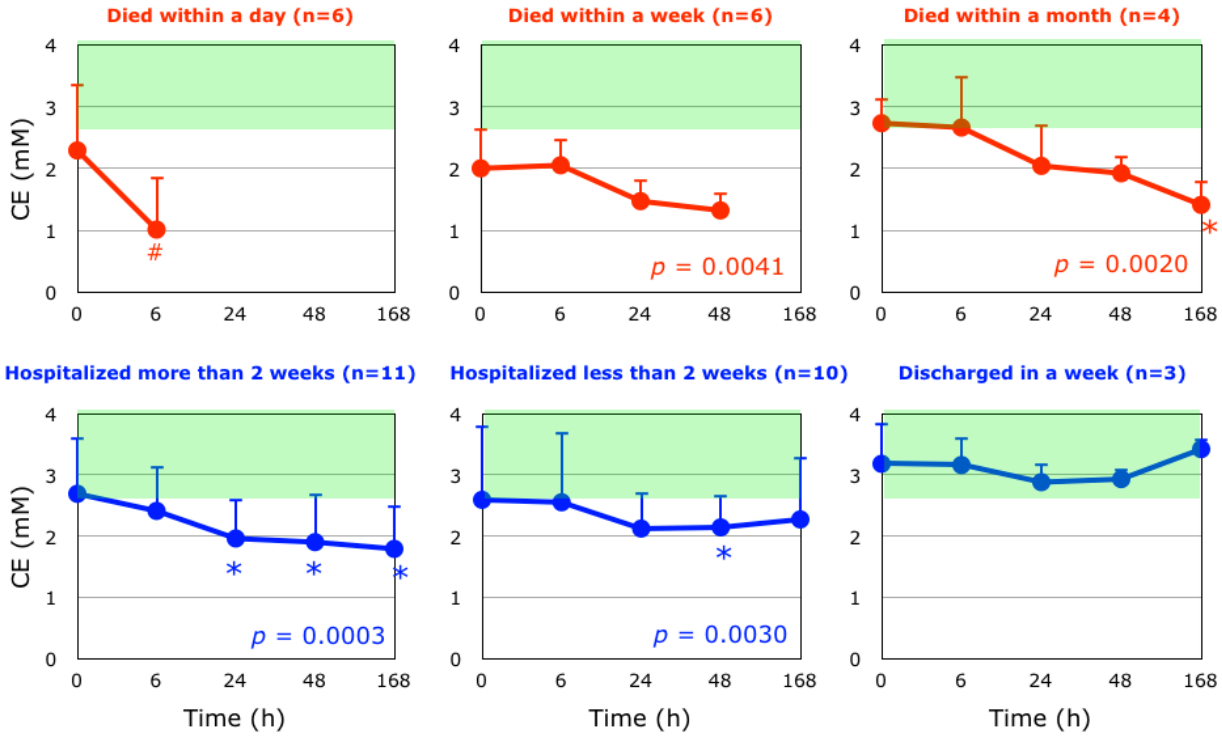

Fig. 5 Time course of changes in plasma cholesterol esters (CE) after hospitalization. Patients were divided into six groups according to outcome. The average CE level in age-matched healthy controls was 3.36 ± 0.72 mM (± SD, n=55); this range is shaded in green. # $p < 0.05$, significant differences compared to values at 0 hr as determined by a paired Student's *t*-test. *p* values are indicated when one-way repeated ANOVA analysis was significant.
* $p < 0.05$, significant differences compared to values at 0 hr as determined by the Tukey–Kramer multiple comparisons test.

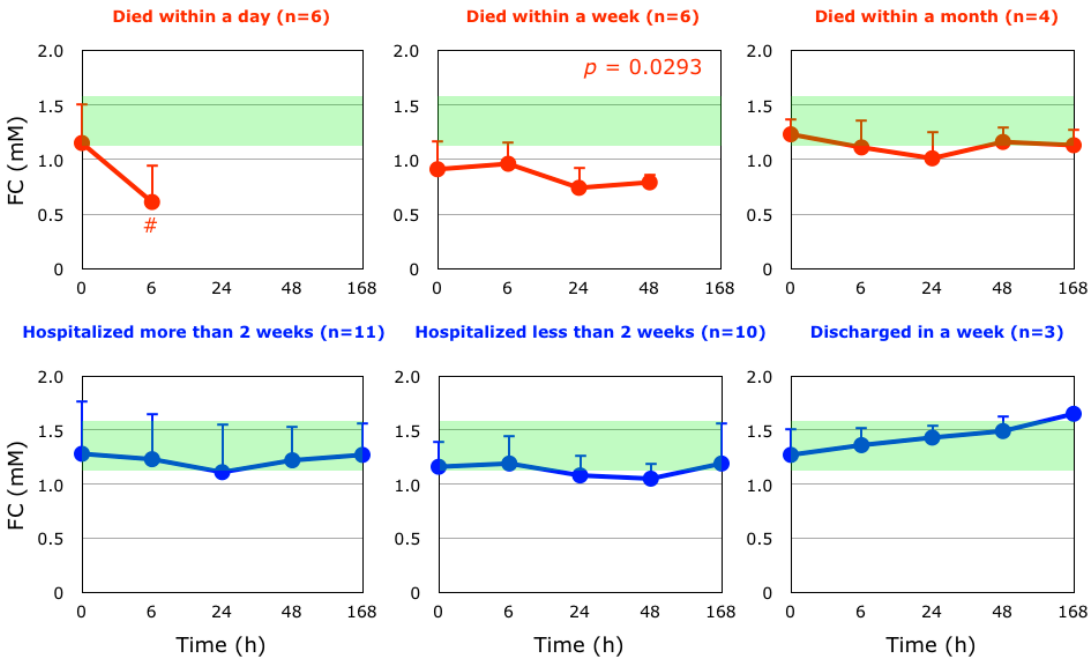

Fig. 6 Time course of changes in plasma free cholesterol (FC) after hospitalization. Patients were divided into six groups according to outcome. The average FC level in age-matched healthy controls was 1.37 ± 0.25 mM (± SD, n=55); this range is shaded in green. # $p < 0.05$, significant differences compared to values at 0 hr as determined by a paired Student's *t*-test. p values are indicated when one-way repeated ANOVA analysis was significant. * $p < 0.05$, significant differences compared to values at 0 hr as determined by the Tukey–Kramer multiple comparisons test.

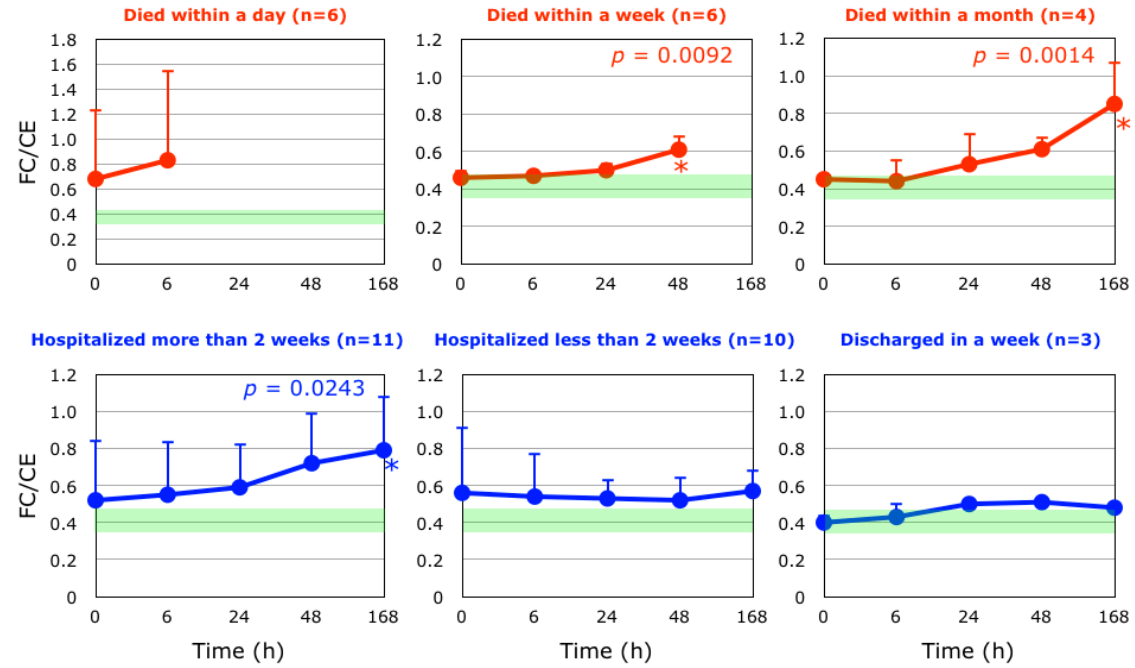

Fig. 7 Time course of changes in plasma ratio of free cholesterol to cholesterol esters (FC/CE) after hospitalization. Patients were divided into six groups according to outcome. The average FC/CE ratio in age-matched healthy controls was 0.41 ± 0.05 (± SD, n=55); this range is shaded in green. # $p < 0.05$, significant differences compared to values at 0 hr as determined by a paired Student's *t*-test. p values are shown when one-way repeated ANOVA analysis was significant. * $p < 0.05$, significant differences compared to values at 0 hr as determined by the Tukey–Kramer multiple comparisons test.

Figures 8 and 9 show the time course changes in water-soluble antioxidants, VC and UA, respectively. Plasma levels of VC can be increased when many tissue cells are disrupted, because tissue cells contain mM levels of VC while plasma contains levels of ~30 μM. Such increases in VC levels were observed at 48 hr in PCAS patients who died within a week (Fig. 8). It is reasonable that UA levels also increased at this point, because tissue disruption results in the conversion of purines to UA (Fig. 9). However, a significant decrease in UA was observed in PCAS patients who were hospitalized for more than a week (Fig. 9). This may due to the formation of peroxynitrite since UA is a specific inhibitor of peroxynitrite.[17,18]

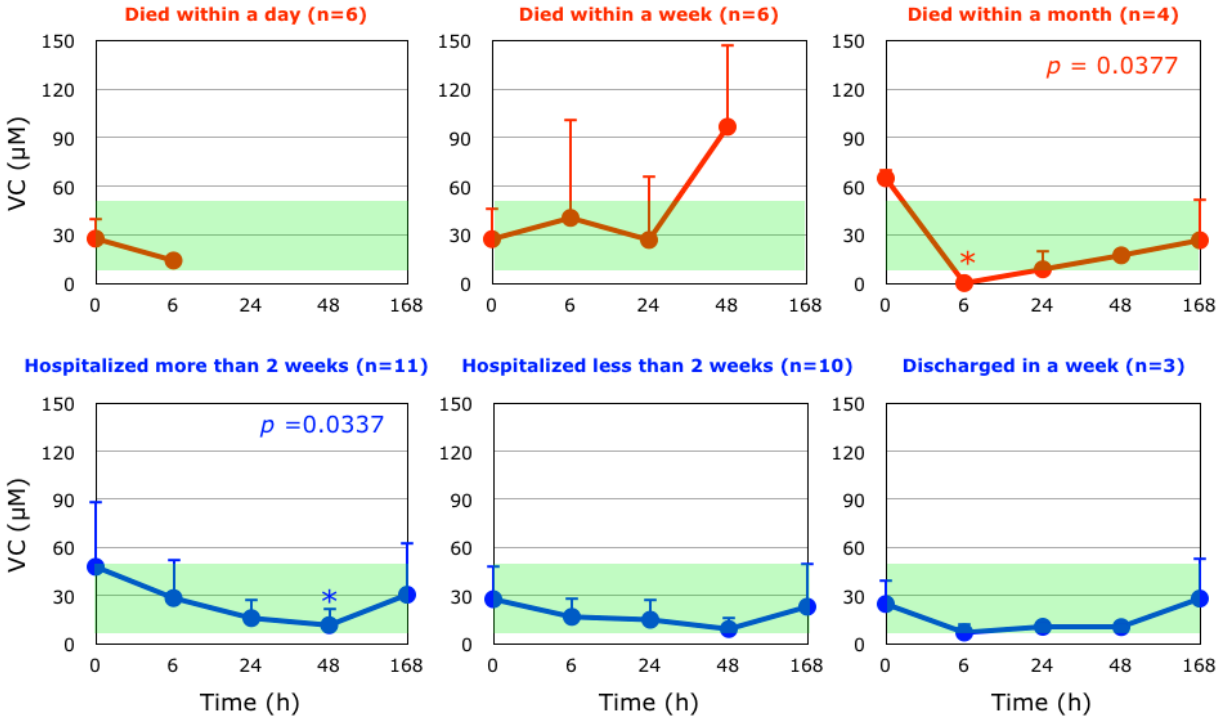

Fig. 8 Time course of changes in plasma ascorbic acid (VC) after hospitalization. Patients were divided into six groups according to outcome. The average VC level in age-matched healthy controls was 31.1 ± 21.0 μM (± SD, n=55); this range is shaded in green. p values are shown when one-way repeated ANOVA analysis was significant. * $p < 0.05$, significant differences compared to values at 0 hr as determined by the Tukey–Kramer multiple comparisons test.

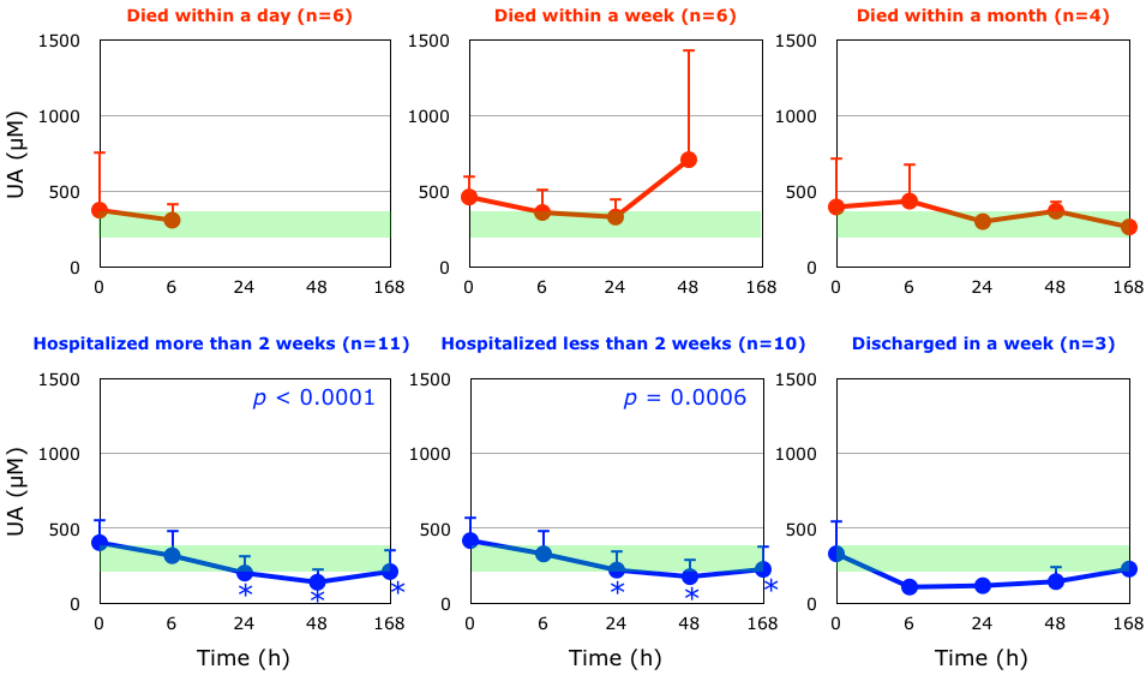

Fig. 9 Time course of changes in plasma uric acid (UA) after hospitalization. Patients were divided into six groups according to outcome. The average UA level in age-matched healthy controls was 317 ± 86 μM (± SD, n=55); this range is shaded in green. # $p < 0.05$, significant differences compared to values at 0 hr as determined by a paired Student's *t*-test. p values are shown when one-way repeated ANOVA analysis was significant. * $p < 0.05$, significant differences compared to values at 0 hr as determined by the Tukey–Kramer multiple comparisons test.

We have employed plasma FFA and the content of oxidatively vulnerable PUFA in total FFA as markers of tissue oxidative damage.[15] It is common that stearoyl-CoA desaturase is activated to compensate for the loss of PUFA; therefore, the percentages of palmitoleic acid and oleic acid in total FFA (%16:1 and %18:1, respectively) are also appropriate markers of tissue oxidative damage.[15] Figure 10 shows the time course changes in FFA, %PUFA, %16:1 and %18:1. Here, we divided patients into 3 groups: those who died within a day (6 cases), died within a month (10), and survived (26). No significant changes were observed in patients who survived. In contrast, %PUFA decreased, and %16:1 and %18:1 increased with time in patients who died within a month, indicating ongoing tissue oxidative damage in these patients. However, all parameters significantly decreased at 6 hr in patients who died within a day, suggesting that the above lipid preserving reactions had ceased in those patients.

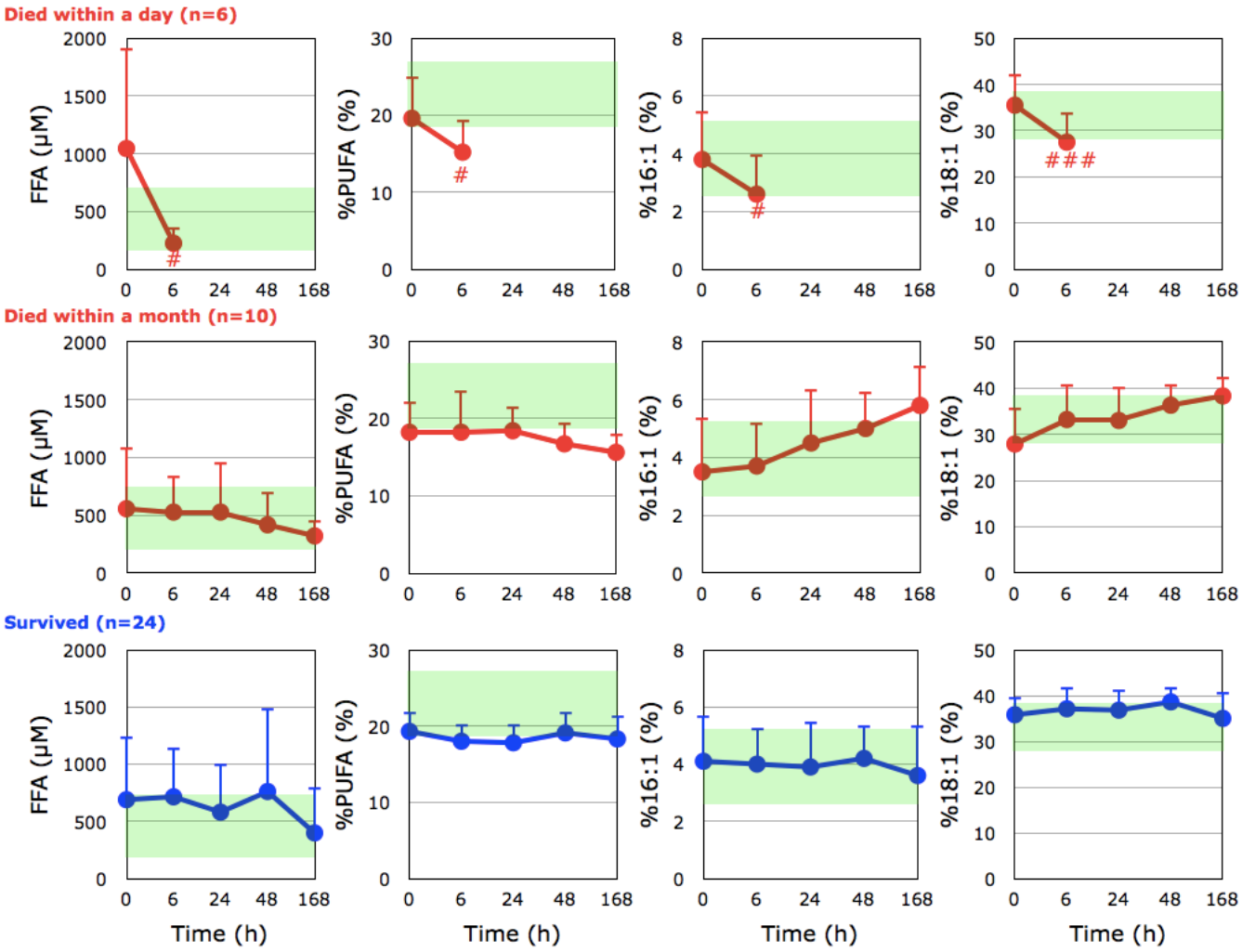

Fig. 10 Time course of changes in plasma free fatty acids (FFA), the percentage of polyunsaturated fatty acids in total FFA (%PUFA), the percentage of palmitoleic acid in total FFA (%16:1), and the percentage of oleic acid in total FFA (%18:1) after hospitalization. Patients were divided into three groups according to outcome. The average FFA level, %PFA, %16:1, and %18:1 in age-matched healthy controls were 457 ± 288 μM, 23.6 ± 4.6, 3.9 ± 1.4, and 34.4 ± 5.1, respectively (± SD, n=55); these ranges are shaded in green. # $p < 0.05$ and ### $p < 0.001$, significant differences compared to values at 0 hr as determined by a paired Student's *t*-test.

Effect of therapeutic hypothermia (TH) treatment

The efficacy of TH treatment against PCAS is generally acknowledged in Japan.[20,21] TH treatment is employed to reduce the formation of oxygen radicals. Infact, TH treatment decreased superoxide formation in an animal model of ischemia/reperfusion injury.[22] Moreover, we observed that TH treatment induced a significant increase in plasma TQ10/TC at the end of rewarming (48 hr) compared to the 24 hr values and this ratio was significantly decreased at day 7 (Fig. 11). This was not the case in the absence of TH treatment (Fig. 11). Since the ratios of TQ10/TC were increased, this indicates that the increment is not lipoprotein-dependent. Thus, we focused on the levels of Psap as a coenzyme Q10 binding and transfer protein[11,23,24] in plasma.

Psap is a multifunctional glycoprotein present in all organs as the lysosomal precursor of four small sphingolipid activator proteins, known as saposin A, B, C, and D, and also exists as a secreted protein, which has been found in various bodily fluids such as serum, milk, and seminal fluid.[25,26] Notably, plasma levels of Psap in PCAS patients at the time of hospitalization were 47.3 ± 15.1 (± SD, n=36), significantly higher than in age-matched healthy controls (27.2 ± 5.8, n=80) ($p < 0.001$). Since plasma Psap levels decreased with time (Fig. 11), the elevation of Psap levels preceded the alterations in TQ10/TC ratio. Although the detailed mechanism requires further study, it is of interest that the human body appears to require coenzyme Q10 under critical conditions such as PCAS.

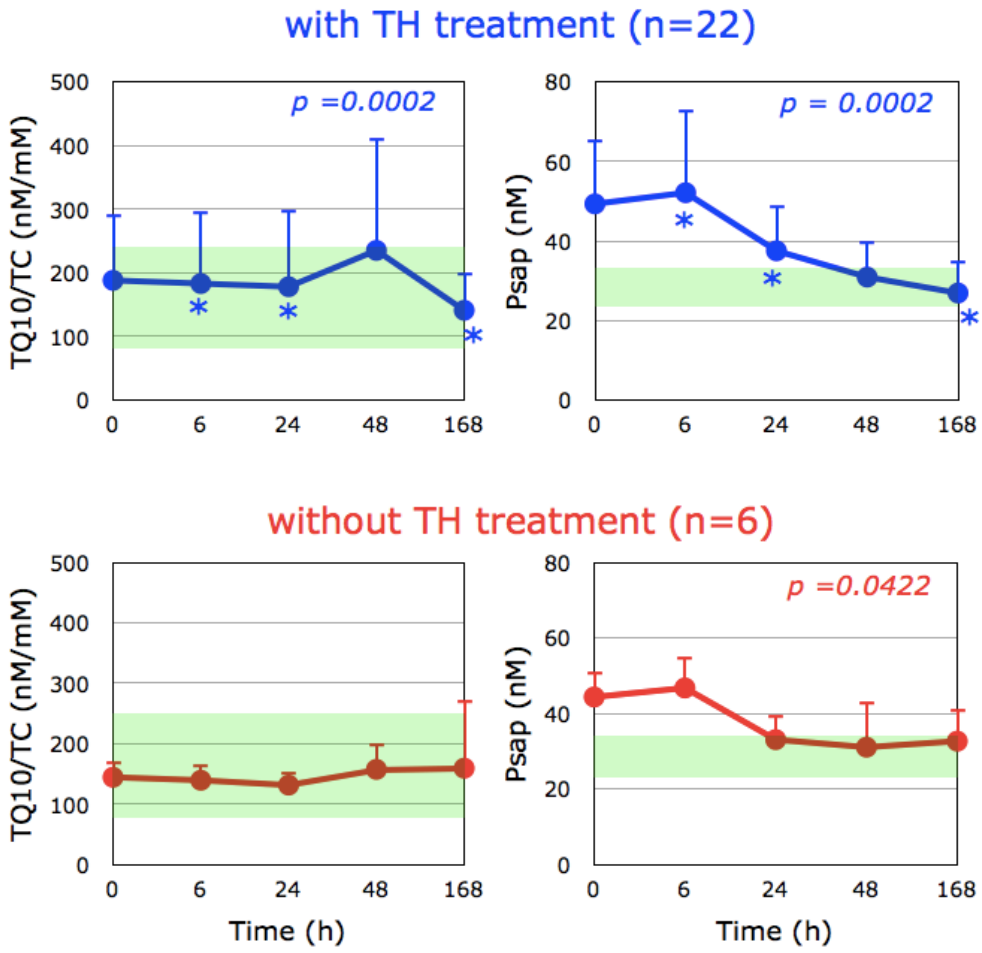

Fig. 11 Time course of changes in ratio of plasma total coenzyme Q10 to total cholesterol (TQ10/TC) and plasma prosaposin (Psap) after hospitalization. Patients were divided into groups with (n=22) and without (n=6) therapeutic hypothermia (TH) treatment. The average Psap level and TQ10/TC ratio in age-matched healthy controls were 27.2 ± 5.8 nM (± SD, n=80) and 180 ± 86 nM/mM (± SD, n=55), respectively; these ranges are shaded in green. *p* values are shown when one-way repeated ANOVA analysis was significant. * $p < 0.05$, significant differences compared to values at 48 hr as determined by the Tukey–Kramer multiple comparisons test.

Conclusion

In summary, increased oxidative stress was confirmed in PCAS patients at the time of hospitalization by the significant increases in plasma %CoQ10 and FFA, and the significant decrease in %PUFA. Impairment of liver function was suggested by an increase in FC/CE ratio. A time course study revealed that this ratio is one of the key factors in determining the survival of patients. TH treatment enhanced increases in the plasma ratio of TQ10 to TC at the end of rewarming via a lipoprotein-independent pathway.

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Chapter 3

Increased oxidative stress in patients with amyotrophic lateral sclerosis and the effect of edaravone administration

Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by muscular atrophy and reduced muscle strength, and it is an adult-onset neurodegenerative disease that causes selective degeneration of upper and lower motor neurons.[1] Disease progression is usually rapid and respiratory disturbance results in death within 20 to 48 months.[2] Despite extensive research, the cause of the disease is unknown and no effective treatment is available.[3] Although familial ALS is known, sporadic ALS accounts for the majority of ALS cases. Beal et al. reported an increase in 3-nitrotyrosine[4] and 8-hydroxyguanine[5] in
the spinal cord of both sporadic and familial ALS patients. 3-Nitrotyrosine is formed by peroxynitrite-mediated nitration of tyrosine[6] and 8-hydroxyguanine is generated from guanine by oxidants such as hydroxyl radical. An increase of 3-nitrotyrosine was confirmed in the cerebrospinal fluid (CSF) of patients with sporadic ALS.[7] We have also reported an increase of oxidative stress in patients with sporadic ALS, based on measurements of the redox balance of plasma coenzyme Q10.[8]

These observations prompted us to evaluate the efficacy of edaravone, a free radical scavenger,[9] in patients with ALS. Edaravone has been shown to inhibit lipid peroxidation as efficiently as well-known antioxidants such as vitamin E (VE) and ascorbic acid (VC).[10] In April 2001, the Japanese Ministry of Health, Labor, and Welfare approved intravenous infusion of 30 mg edaravone twice a day for a maximum of 14 days in patients with acute brain infarction within 24 hr after onset, and edaravone is now widely used in Japan. Therefore, it is considered to be safe.

We have demonstrated that edaravone (60 mg) administration 5-6 times a week for 6 months significantly reduced both the decline of the revised ALS functional rating scale (ALSFRS-R) and 3-nitrotyrosine levels in CSF of treated patients.[11]
In this study we evaluated oxidative stress in ALS patients by measuring the redox balance of plasma coenzyme Q10, plasma uric acid (UA being the predominant endogenous scavenger of peroxynitrite[6]), and tissue oxidative damage markers such as the percentage of polyunsaturated fatty acids in the total plasma free fatty acids (FFA). We also evaluated the effect of edaravone administration on these markers.

Subjects and Methods

Study design

The present study was carried out at the Yoshino Neurology Clinic during the period from 26 October 2011 to 31 May 2014. The study protocols were approved by the Ethical Committee of Tokyo University of Technology, and samples from patients were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2001. Twenty-six subjects (14 males aged 61.5 ± 12.4 years (mean ± SD) and 12 females aged 60.2 ± 16.5 years) were enrolled after they had given informed consent to participate in the study. The inclusion criterion was a diagnosis of sporadic or familial ALS. Exclusion criteria were compromised respiratory function that include tracheotomy, artificial respiration, or dyspnea. Additional exclusion criteria were complications such as advanced cancer, severe cardiac insufficiency, and age of less than 20 years. Edaravone (30 mg) dissolved in 100 ml of saline was administered via an intravenous drip 1-4 times a week; among the 26 patients, 17 received edaravone administration for at least 3 months and 13 continued treatment for 6 months. Heparinized blood was collected before and after edaravone administration at week 0, week 1, month 1, month 3, and month 6; the collected plasma samples were stored at -30 °C until analysis. ALSFRS-R scores were also measured before edaravone administration at week 0, week 1, month 1, month 3, and month 6.

Analytical procedures

Plasma levels of VE, ubiquinol-10, ubiquinone-10, free cholesterol (FC), and cholesterol esters (CE) were determined as previously described[12] with some modifications. In brief, plasma was extracted with 19 volumes of 2-propanol and the extract was analyzed by HPLC using an analytical column (Supelcosil LC-8, 5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan, Tokyo), a reduction column (RC-10-1; Irica, Kyoto, Japan) and an amperometric electrochemical detector (Model Σ985; Irica) with an oxidation potential of +600 mV (vs. Ag/AgCl) on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/2-propanol (9/1, v/v), delivered at a flow rate of 0.8 ml/min.

Plasma levels of VC, UA and unconjugated bilirubin were determined by HPLC on a bonded-phase aminopropylsilyl column (Supelcosil LC-NH_2, 5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan) with UV/VIS detection (265 nm for 0 - 15 min and 460 nm for 15-22 min) as described previously.[13]

Plasma FFA were derivatized with monodansylcadaverine for analysis by HPLC.[14] Briefly, plasma samples (50 μl) were mixed with 200 μl of methanol containing 25 μM margaric acid (internal standard) and then centrifuged at 13,000 x g for 5 min. Aliquots (50 μl) of supernatants were dried under a stream of nitrogen gas, and the residue was admixed with diethyl phosphorocyanidate (1 μl) and N,N-dimethylformamide (50 μl) containing monodansylcadaverine (2 mg/ml) and kept at room temperature in the dark for 20 min. A 5-μl sample was injected onto an octadecylsilyl column (3 μm, 3.3 cm x 4.6 mm i.d.; Supelco Japan) and a pKb-100 column (5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan) connected in tandem. The FFA components were measured by fluorescence detection (Model 821-FP; Japan Spectroscopic, Tokyo, Japan) with excitation at 320 nm and emission at 520 nm. The mobile phase consisted of acetonitrile/methanol/water (17.5/65.0/17.5, v/v/v) delivered at a flow rate of 1.5 ml/min. The analytical columns were heated to 40°C.

Statistical analysis

Data presented are mean values and standard deviations unless otherwise specified. The statistical significance of differences was evaluated with Student's *t*-test. To assess the time course of efficacy of edaravone administration, post-treatment data were assessed using repeated-measures analysis of variance (ANOVA). Group differences were analyzed by means of Fisher's exact probability test. $P < 0.05$ was considered statistically significant.

Results and Discussion

Oxidative stress in ALS patients

Oxidative stress markers such as 3-nitrotyrosine,[4,7,11] 8-hydroxyguanine,[5] and protein carbonyl[5] are typically increased in ALS patients. Here, we examined a range of markers of circulatory oxidative stress and tissue oxidative damage in 26 patients with ALS as compared to those of 55 age-matched healthy controls,[8,15,16] as shown in Table 1. There were no significant differences in the plasma antioxidants, VC, VE, and total coenzyme Q10 between the two groups. However, a significant decrease was observed in UA, a peroxynitrite scavenger, and this is consistent with increased CSF levels of 3-nitrotyrosine observed in patients with ALS.[4,7,11] A significant decrease in plasma UA was also previously reported in ALS patients.[17] There was also a significant decrease in plasma unconjugated bilirubin, which is an important physiological antioxidant.[18] Strikingly, we found that the ratio of the oxidized form of coenzyme Q10 (%CoQ10) to total coenzyme Q10 was significantly increased, corroborating an increased level of oxidative stress in patients with ALS.

Table 1 Levels of plasma antioxidants and lipids in patients with ALS as compared to age-matched healthy controls (average ± SD). *P* values were determined using the Student's *t*-test. VC, ascorbic acid; UA, uric acid; BR, unconjugated bilirubin; VE, vitamin E; TQ10, total coenzyme Q10; %CoQ10, ratio of oxidized form of coenzyme Q10 to TQ10; FFA, free fatty acids; %PUFA, ratio of polyunsaturated fatty acids to total free fatty acids; %16:1, ratio of palmitoleic acid to total FFA; %18:1, ratio of oleic acid to total FFA; FC, free cholesterol; CE, cholesterol esters; TC, total cholesterol.

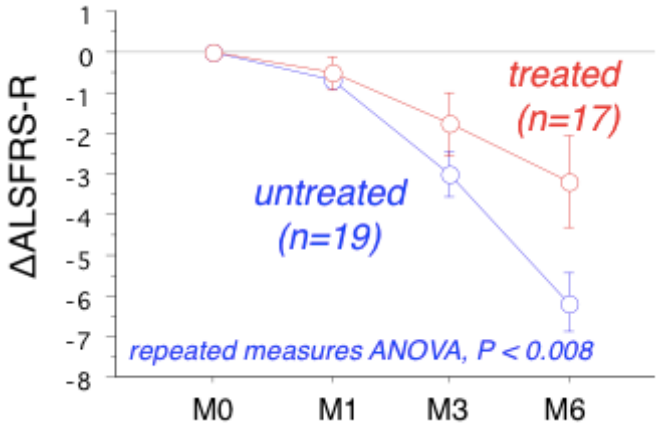
	ALS	normal control	*P*
n	26	55	
male/female	14/12	38/17	
age	60.9 ± 11.2	60.1 ± 9.3	
VC, μM	24.9 ± 15.0	31.1 ± 21.0	
UA, μM	215 ± 94	314 ± 93	< 0.001
BR, μM	3.7 ± 3.3	6.9 ± 3.6	< 0.001
VE, μM	33.5 ± 10.2	28.5 ± 7.3	
TQ10, nM	829 ± 332	710 ± 206	
%CoQ10	20.9 ± 10.3	3.9 ± 1.3	< 0.001
FFA, μM	486 ± 377	457 ± 288	
%PUFA	19.0 ± 4.1	23.6 ± 4.6	< 0.001
%16:1	2.9 ± 1.5	3.9 ± 1.4	
%18:1	33.8 ± 8.1	34.4 ± 5.1	
FC, mM	1.55 ± 0.39	1.37 ± 0.25	< 0.05
CE, mM	3.52 ± 0.95	3.36 ± 0.72	
TC, mM	5.07 ± 1.33	4.72 ± 0.94	
FC/CE	0.44 ± 0.04	0.41 ± 0.05	< 0.01

Plasma level and composition of FFA are good markers of tissue oxidative damage, since polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), and docosahexaenoic acid (22:6) are highly susceptible to cellular oxidation. The oxidative loss of PUFA reduces membrane fluidity, but this can be compensated by the conversion of stearic acid (18:0) to oleic acid (18:1) or of palmitic acid (16:0) to palmitoleic acid (16:1) by stearoyl-CoA desaturase.[19] Acceleration of oxidative stress causes hydrolysis of tissue and membrane lipids to FFA, which enter the blood stream. Under condition of oxidative stress, a lower percentage of PUFA to total FFA (%PUFA) and/or higher percentages of 18:1 and 16:1 to total FFA (%18:1 and %16:1, respectively) would be expected. Indeed, such modifications have been observed in patients with adult respiratory distress syndrome,[20] multiple sclerosis,[21] Papillon-Lefevre syndrome,[22] and juvenile fibromyalgia,[23] and in newborn babies.[24] We recently observed elevation of plasma FFA, %16:1, and %18:1 in rats after a 2-hour occlusion-reperfusion of the middle cerebral artery, and we showed that these changes were attenuated by administration of edaravone.[25]

Although plasma levels of FFA, %16:1, and %18:1 were the same in ALS patients as in healthy controls, a significant decrease in %PUFA was observed in the patients, suggesting an increased level of tissue oxidative damage (Table 1). ALS patients also showed small but significant increases in FC and the FC/CE ratio, suggesting some impairment of lecithin-cholesterol acyltransferase and liver function does occur.[23]

Efficacy of edaravone administration

Since oxidative damages are indicated in the pathogenesis of ALS, administration of a free radical scavenger drug seems a rational theraputic strategy for diesease management. Indeed, we obtained a positive response to edaravone treatment in our phase II trial (60 mg/day 5-6 times a week for 6 months).[11]
In the present study, edaravone was administered only 1-4 times a week for up to 6 months, since patients had joined this study independently. Among the 26 ALS patients, 17 received edaravone administration (30 mg/day, 1-4 times a week) for at least 3 months and 13 continued treatment for 6 months. Missing values of ΔALSFRS-R at 6 months for the 4 patients having an administration time between 3 and 6 months were estimated as twice the value of ΔALSFRS-R at 3 insetmonths. We observed a significant improvement in ΔALSFRS-R at 6 months in these patients as compared to that of edaravone-untreated patients,[11] as shown in Fig. 1 (the *P* value of repeated measures ANOVA was lower than 0.008). There was no significant difference in ALSFRS-R at month 0 between the two groups (the values are shown in the inset table in Fig. 1).



	untreated	treated
M0	41.9 ± 1.4	40.1 ± 1.2
M1	41.2 ± 1.5	39.6 ± 1.4
M3	38.9 ± 1.7	38.4 ± 1.6
M6	35.8 ± 1.6	36.9 ± 2.0

Fig. 1 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 the raw ALSFRS-R values.

Based on the ΔALSFRS-R results at 6 months (Δ), patients were divided into 3 groups, a satisfactory progress group (Δ ≥ 0), an ingravescent group (Δ < -5), and a middle group (Δ = -1 ~ -4). Table 2 shows there were 6 patients in the satisfactory progress group among the total 13 edaravone-treated patients (or 7 out of 17 treated patients), but none out of total 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 Classification of 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 4 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.

group	Δ	untreated	treated
satisfactory progress group	≧ 0	0	6 (7) ↑
middle group	-1 ~ -4	7	4 (6)→
ingravescent group	≦ -5	12	3 (4) ↓
	total	19	13 (17)

Scavenging of peroxynitrite by edaravone

Keizman et al. reported low levels of serum UA in patients with ALS.[17] Of significance, UA levels in 36 patients out of 46 patients had decreased 6 months later, while those in 9 out of 46 patients were increased (one remained unchanged). Peroxynitrite is likely responsible for the predominant decline of UA, since VC and VE levels are identical in control subjects (Table 1) and 3-nitrotyrosine was detected in CSF of patients with ALS. Edaravone administration increased the plasma levels of UA in 10 out of 12 ALS patients (Fig. 2), and notably this occurred in 5 out of 5 subjects in the satisfactory progress group. We have previously reported that edaravone administration decreased 3-nitrotyrosine levels in the CSF of ALS patients. [11] We have also found that edaravone scavenges peroxynitrite approximately 30 times greater than does UA (A. Fujisawa and Y. Yamamoto, submitted).[26] These results are consistent with our contention that scavenging of peroxynitrite by edaravone may contribute significantly toward inhibiting the progression of ALS.

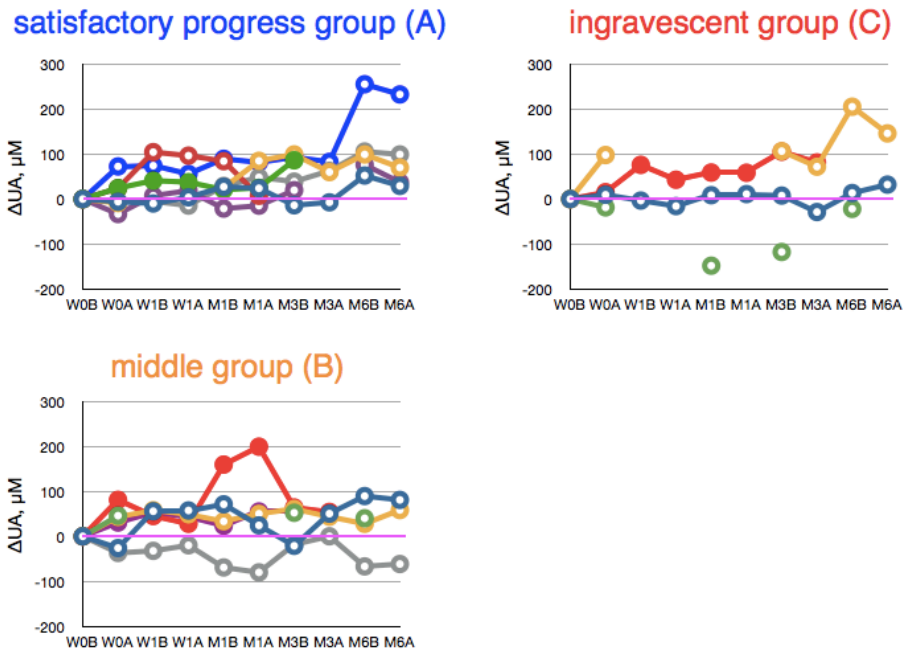


Fig. 2 The increase or decrease of plasma urate levels, calculated from values at week 0 (before edaravone administration), during the course of edaravone treatment in ALS patients. Patients were divided into a satisfactory progress group, an ingravescent group, and a middle group according to their values of ΔALSFRS-R determined at 6 months of treatment. W0B/A and W1B/A are data for week 0 and week 1 before (B) and after (A40) edaravone treatment, respectively; M1-6B/A are data for months 1-6 before (B) and after (A) edaravone treatment. Data for patients for whom the month 6 observation was missing are indicated by solid symbols.

Effects of edaravone treatment on plasma markers of oxidative stress and tissue damage

Edaravone had no significant effect on plasma VC levels (Fig. 3) or plasma %CoQ10 (Fig. 4), suggesting that the dosage of edaravone was insufficient to reduce circulatory oxidative stress. Therefore, additional treatment, such as CoQ10 administration, could be considered. It has been reported that CoQ10 supplementation reduced %CoQ10 and ameliorated the impairment of cholesterol metabolism in patients with juvenile fibromyalgia.[23]

Figures 5-8 shows the time course of changes in plasma markers of tissue oxidative damage, FFA, %PUFA, %16:1, and %18:1, respectively. Red background in Figs. 5, 7, and 8 indicates values above the mean + 1 SD, while the red background in Fig. 6 indicates values below the mean - 1 SD; such can thus be considered abnormal ranges. There were no significant difference in %PUFA among the satisfactory progress, middle and ingravescent patient groups (Fig. 6). However, there were significantly fewer excursions of FFA, %16:1, and %18:1 outside the range of the mean plus one standard deviation in the satisfactory progress group and middle group, compared to that of the ingravescent group (Figs. 5, 7, and 8). These data support the idea that edaravone reduces oxidative tissue damage in the satisfactory progress group, but is not effective in the ingravescent group. Therefore, we contend additional treatment with other antioxidants such as coenzyme Q10 may be required to ameliorate oxidative damage in non-responders to edaravone treatment.

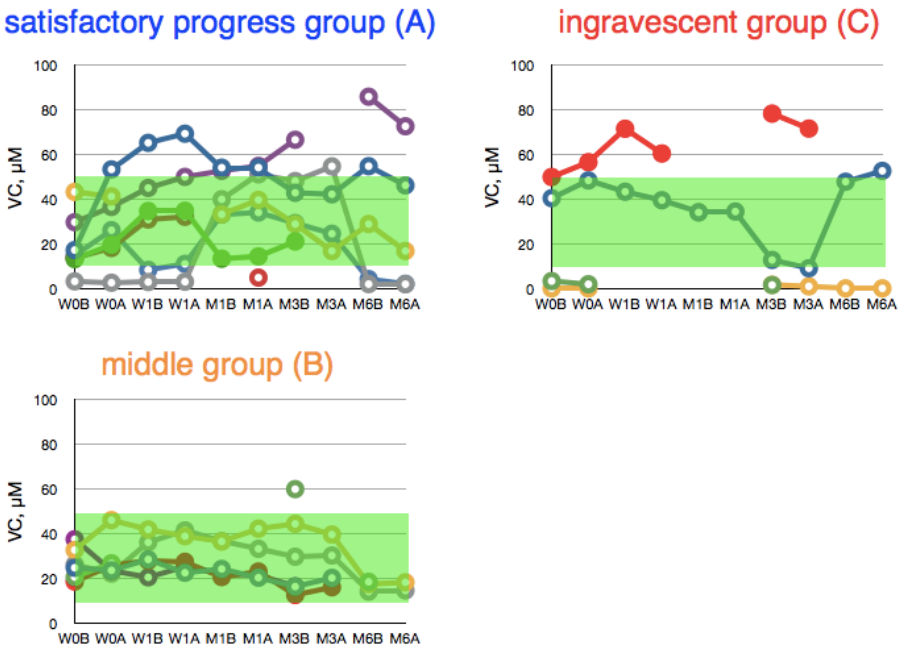


Fig. 3 Time course of changes in plasma ascorbate (VC) during edaravone treatment in ALS patients. Patients were divided into three groups according to the value of ΔALSFRS-R at 6 months of treatment. The average VC level in age-matched healthy controls was 31.1 ± 21.0 μM (± SD, n=55); this range is shaded in green. W0B/A and W1B/A are data for week 0 and week 1 before (B) and after (A) edaravone treatment, respectively; M1-6B/A are data for months 1-6 before (B) and after (A) edaravone treatment. Data for patients for whom the month 6 observation was missing are indicated by solid symbols.

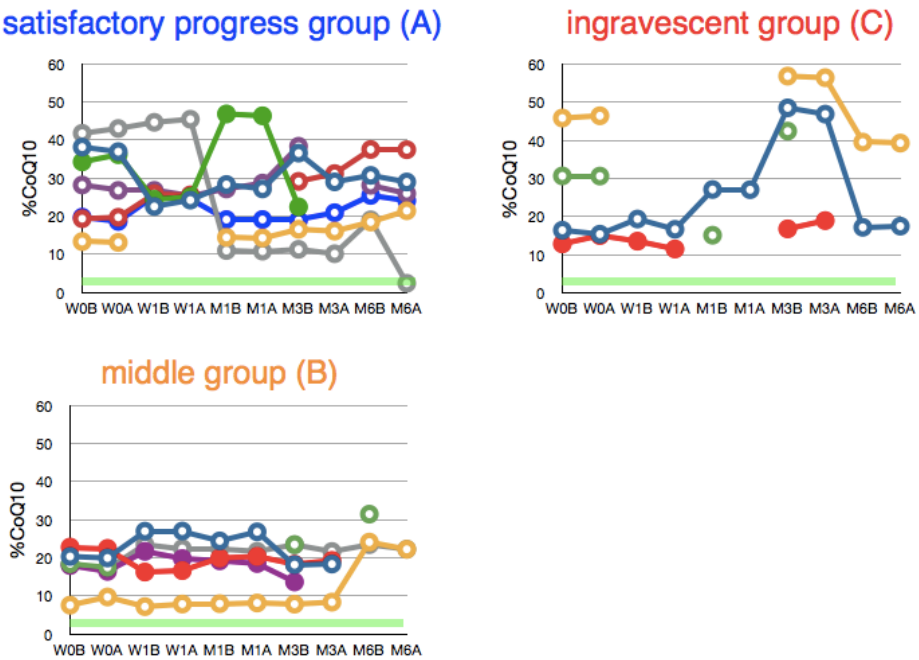
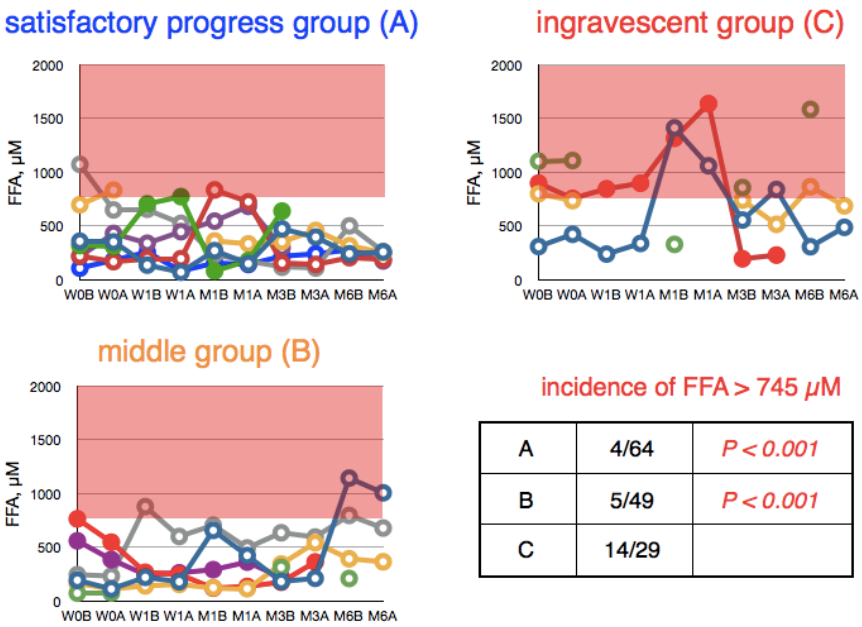


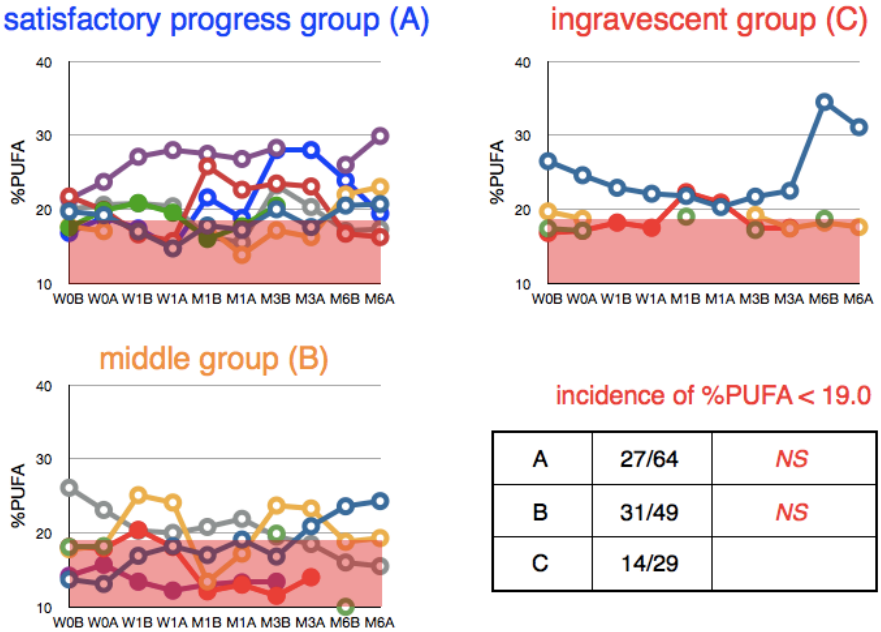
Fig. 4 Time course of changes in the ratio of ubiquinone-10 to TQ10 (%CoQ10) during edaravone treatment in ALS patients. Patients were divided into three groups according to their values of ΔALSFRS-R determined at 6 months of treatment. Average %CoQ10 in age-matched healthy controls was 3.9 ± 1.3 (± SD, n=55); this range is shaded in green. W0B/A and W1B/A are data for week 0 and week 1 before (B) and after (A) edaravone treatment, respectively; M1-6B/A are data for months 1-6 before (B) and after (A) edaravone treatment. Data for patients for whom the month 6 observation was missing are indicated by solid symbols.



incidence of FFA > 745 μM

A	4/64	*P* < 0.001
B	5/49	*P* < 0.001
C	14/29	

Fig. 5 Time course of changes in plasma FFA during edaravone treatment in ALS patients. Patients were divided into three groups according to their values of ΔALSFRS-R determined at 6 months of treatment. FFA level in age-matched healthy controls was 457 ± 288 μM (average ± SD, n=55); the region above this range is shaded in red. W0B/A and W1B/A are data for week 0 and week 1 before (B) and after (A) edaravone treatment, respectively; M1-6B/A are data for months 1-6 before (B) and after (A) edaravone treatment. Data for patients for whom the month 6 observation was missing are indicated by solid symbols. Inset table shows the incidence of FFA levels greater than 745 μM (= average + SD) and the *P* value (Fisher's exact probability test) versus data for the ingravescent group.



incidence of %PUFA < 19.0

A	27/64	*NS*
B	31/49	*NS*
C	14/29	

Fig. 6 Time course of changes in plasma ratio of polyunsaturated fatty acids to total FFA (%PUFA) in ALS patients. Patients were divided into three groups according to their values of ΔALSFRS-R determined at 6 months of treatment. %PUFA in age-matched healthy controls was 23.6 ± 4.6 % (average ± SD, n=55); the region below this range is shaded in red. W0B/A and W1B/A are data for week 0 and week 1 before (B) and after (A) edaravone treatment, respectively; M1-6B/A are data for months 1-6 before (B) and after (A) edaravone treatment. Data for patients for whom the month 6 observation was missing are indicated by solid symbols. Inset table shows the incidence of %PUFA smaller than 19.0 % (= average - SD) and the *P* value (Fisher's exact probability test) versus data for the ingravescent group.

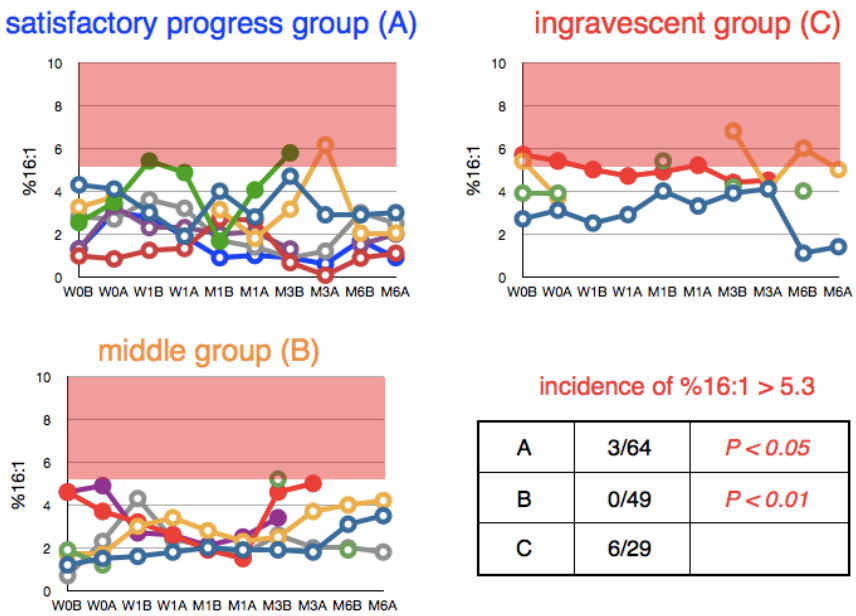


Fig. 7 Time course of changes in plasma ratio of palmitoleic acid to total FFA (%16:1) during edaravone treatment in ALS patients. Patients were divided into three groups according to their values of ΔALSFRS-R determined at 6 months of treatment. %16:1 in age-matched healthy control was 3.9 ± 1.4 % (average ± SD, n=55); the region above this range is shaded in red. W0B/A and W1B/A are data for week 0 and week 1 before (B) and after (A) edaravone treatment, respectively; M1-6B/A are data for months 1-6 before (B) and after (A) edaravone treatment. Data for patients for whom the month 6 observation was missing are indicated by solid symbols. Inset table shows the incidence of %16:1 greater than 5.3 % (= average + SD) and the *P* value (Fisher's exact probability test) versus data for the ingravescent group.

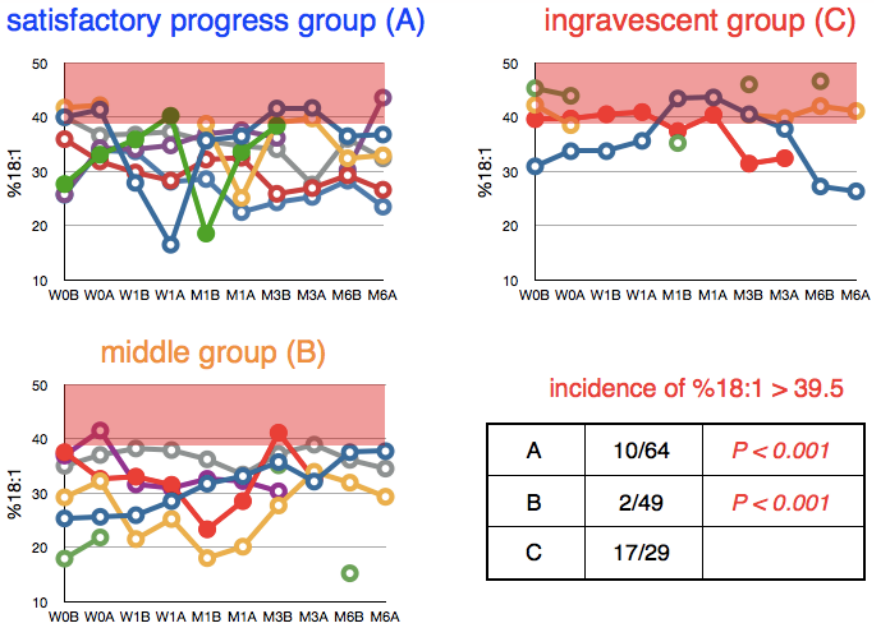


Fig. 8 Time course of changes in plasma ratio of oleic acid to total FFA (%18:1) during edaravone treatment in ALS patients. Patients were divided into three groups according to their values of ΔALSFRS-R determined at 6 months of treatment. %18:1 in age-matched healthy controls was 34.4 ± 5.1 % (average ± SD, n=55); the region above this range is shaded in red. W0B/A and W1B/A are data for week 0 and week 1 before (B) and after (A) edaravone treatment, respectively; M1-6B/A are data for months 1-6 before (B) and after (A) edaravone treatment. Data for patients for whom the month 6 observation was missing are indicated by solid symbols. Inset table shows the incidence of %16:1 greater than 39.5 % (= average + SD) and the *P* value (Fisher's exact probability test) versus data for the ingravescent group.

Conclusion

We observed an increase in %CoQ10, a decrease in UA levels, and a reduction of %PUFA occur in the pathophysiology of ALS patients, consistent with increased oxidative stress. Administration of edaravone, a potent radical and a peroxynitrite scavenger, significantly reduced disease progression, as compared to that of untreated patients. It also increased plasma UA by scavenging peroxynitrite in ALS patients. However, edaravone treatment did not reduce the plasma %CoQ10. Co-treatment of edaravone with other antioxidants such as coenzyme Q10 might be beneficial for non-responders to edaravone treatment.

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Chapter 4

Increased Oxidative Stress and Coenzyme Q10 Deficiency in Centenarians

Introduction

Aging populations are expanding worldwide, and the increasing requirement for nursing care has become a serious problem. Successful aging without cognition loss and physical deficiencies is one of the highest priorities for individuals and societies. Arai *et al*. found that only 20% of centenarians enjoyed physical and cognitive independence at the age of 100 years, although most remained independent in daily living into their 90s.[1] Those who maintained physical independence at 100 years of age were highly likely to become semi-supercentenarians (over 105 years) or supercentenarians (beyond 110 years).[1]

To identify key factors in successful aging, Arai *et al*. focused on the characteristics of centenarians, semi-supercentenarians, and supercentenarians.[2] They found that inflammation predicted cognition and physical capabilities in (semi-) supercentenarians better than chronologic age or gender. Interestingly, the inflammation score was lower in centenarian offspring compared to age-matched controls.[2] They concluded that inflammation is an important malleable driver of aging up to extreme old age in humans.[2] Other reviews also emphasize that the suppression of chronic inflammation is an important driver of successful aging at extreme old age.[1,3,4]

In the above study, an inflammation score was estimated using cytomegalovirus immunoglobulin G antibody titers and plasma levels of interleukin- 6, tumor necrosis factor-α, and C-reactive protein.[2] Acute inflammation, such as sepsis, is characterized by the formation of reactive oxygen and nitrogen species such as superoxide and nitric oxide.[5-7] Therefore, peroxynitrite is also an important reactive molecule since it is produced from the combination of superoxide and nitric oxide.[5-7] In fact, we observed a decline in plasma antioxidants, namely vitamin E (VE), ubiquinol-10 ($CoQ10H_2$), vitamin C (VC), and uric acid (UA), in patients with sepsis (manuscript in preparation). However, no comprehensive study has been reported for centenarians. In this study, we compared serum levels of antioxidants in centenarians and 76-year-old controls. We found a significant decrease in VC and unconjugated bilirubin (BR) and a significant increase in the percentage (%CoQ10) of the oxidized form of coenzyme Q10 (CoQ10) to total coenzyme Q10 (TQ10) in centenarians, suggesting an increase of oxidative stress.

The plasma levels of high density lipoprotein (HDL) were reported to be decreased in centenarians.[2] We, therefore, measured serum free cholesterol (FC) and cholesterol esters (CE) because their ratio (FC/CE) is determined by the activity of lecithin-cholesterol acyltransferase (LCAT) secreted with HDL from liver.[8,9] We confirmed a significant decrease in CE and total cholesterol (TC) and a significant increase in FC/CE ratio in centenarians.

We also measured serum free fatty acids (FFA) and the content of oxidatively vulnerable polyunsaturated fatty acids in total FFA (%PUFA) as markers of tissue

oxidative damage.[10] It is common that stearoyl-CoA desaturase is activated to compensate for the loss of PUFA; therefore, the percentages of palmitoleic acid and oleic acid in total FFA (%16:1 and %18:1, respectively) are also appropriate markers of tissue oxidative damage.[10]

It is well known that human tissue levels of TQ10 decrease with age after the age of 20.[11] For example, decreases in TQ10 of > 30% and 50% in human heart were observed at ages 40 and 80, respectively.[11] However, such a decline of TQ10 was not observed in human plasma within the range of 20 to 60.[12] In this study, we found a significant decrease in serum TQ10 levels in centenarians as compared with 76-year-old controls. On the other hand, a significant increase of the coenzyme Q10 binding and transfer protein prosaposin (Psap)[13-15] was observed. The possible role of Psap will be discussed.

Subjects and Methods

Study design

This study comprised 99 Japanese centenarians (25 males aged 100.8 ± 1.3 years and 74 females aged 100.9 ± 1.5 years; mean ± S.D.) and 62 Japanese controls (25 males aged 74.6 ± 8.5 years and 37 females aged 76.2 ± 8.0 years). The above 4 groups are abbreviated as 101M, 101F, 75M, and 76F, respectively. Written informed consent to participate was obtained either from the participants or a proxy when individuals lacked the capacity to consent. The protocol of this study was approved by the Ethical Committee of the Keio University School of Medicine. Non-fasting venous blood was sampled. Serum was collected and stored at -80°C until analysis. We also compared with previously reported 60-year-old controls[16] (consisting of 38 males and 17 females, 60.1 ± 9.3 years).

Analytical procedures

Serum levels of VE, $CoQ10H_2$, CoQ10, FC, and CE were determined as previously described with some modifications.[17] In brief, serum was extracted with 19 volumes of 2-propanol and the extract was analyzed by HPLC using two analytical columns (Supelcosil ABZ+, 3 μm, 3.3 cm x 4.6 mm i.d. and Ascentis LC-8, 5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan, Tokyo, Japan) connected in tandem, a reduction column (RC-10-1; Irica, Kyoto, Japan) and an amperometric electrochemical detector (Model Σ985; Irica) with an oxidation potential of +600 mV (vs. Ag/AgCl) on a glass carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/2-propanol (78/22, v/v), delivered at a flow rate of 0.8 ml/min. The analytical columns were cooled to 25°C.

Serum levels of VC, UA and BR were determined by HPLC on a bonded-phase aminopropylsilyl column (Supelcosil LC-NH_2, 5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan) with UV/VIS detection (265 nm for 0-15 min and 460 nm for 15-22 min), as described previously.[18]

Serum FFA were derivatized with monodansylcadaverine for analysis by HPLC.[19] Briefly, serum samples (50 μl) were mixed with 200 μl of methanol and then centrifuged at 13,000 x g for 5 min. Aliquots (50 μl) of supernatants were mixed with 20 μl of methanol containing 25 μM tridecanoic acid (internal standard) and dried under a stream of nitrogen gas, and the residue was admixed with diethyl phosphorocyanidate (1 μl) and N,N-dimethylformamide (50 μl) containing monodansylcadaverine (2 mg/ml) and kept at room temperature in the dark for 20 min. A 5-μl sample was injected onto an octadecylsilyl column (3 μm, 3.3 cm x 4.6 mm i.d.; Supelco Japan) and a pKb-100 column (5 μm, 25 cm x 4.6 mm i.d.; Supelco Japan) connected in tandem. The FFA components were measured by fluorescence detection (Model 821-FP; Japan Spectroscopic, Tokyo, Japan) with excitation at 320 nm and emission at 520 nm. The mobile phase consisted of acetonitrile/methanol/water (17.5/65.0/17.5, v/v/v) delivered at a flow rate of 1.5 ml/min. The analytical columns were heated to 40°C.

Serum levels of Psap were measured by a sandwich ELISA using monoclonal and polyclonal antibodies against human saposin B.[13] Plasma was diluted 100 times with a phosphate-buffer saline containing 0.1% Triton X-100, 1 g/L NaN_3, 10 g/L BSA, and 1 mM EDTA. Purified saposin B was used as a standard.[13]

Statistical analysis

Data are presented as mean values with standard deviations. Statistical analysis was performed using one-way ANOVA followed by the Scheffe's multiple comparisons test. $p < 0.05$ was considered statistically significant.

Results and Discussion

Serum antioxidants and oxidative stress

Figure 1 shows serum %CoQ10 and serum levels of VC, BR, and UA among male and female centenarians (101M and 101F, respectively) and 75-year-old male and 76-year-old female controls (75M and 76F, respectively). There were no significant differences between the male and female groups in each age category.
However, a significant increase in %CoQ10 was observed in centenarians compared with 76-year-old controls, indicating that the redox balance of coenzyme Q10 shifted to the oxidized form. This confirms an increase in oxidative stress in centenarians and agrees with a significant decrease in serum antioxidants such VC[10] and BR[20] in centenarians. These results are also consistent with the observation that chronic inflammation is present in centenarians.[2]

Under acute inflammatory conditions like sepsis, the substantial formation of superoxide and nitric oxide, and their product peroxynitrite, is expected. In fact plasma UA levels declined significantly in patients with sepsis during a stay at an intensive care unit (manuscript under preparation) because UA is a good inhibitor of peroxynitrite.[21-23] However, serum UA levels remained constant in centenarians and 76-year-old controls, suggesting that inflammation in centenarians is moderate and chronic.

Since there were no significant differences in %CoQ10, VC, BR, and UA between the male and female groups of centenarians and 76-year-old controls, the data were combined into centenarians and 76-year-old controls (abbreviated as 101 and 76) and compared with 60-year-old controls[16] as shown in Fig. 2. %CoQ10 consistently increased with age, while levels of VC and BR in centenarians were significantly lower than 60- or 76-year-old controls. These data confirm that centenarians are under oxidative stress. However, levels of UA remained unchanged, suggesting that the formation of peroxynitrite is not very significant in centenarians and they are under moderate, chronic inflammatory conditions.

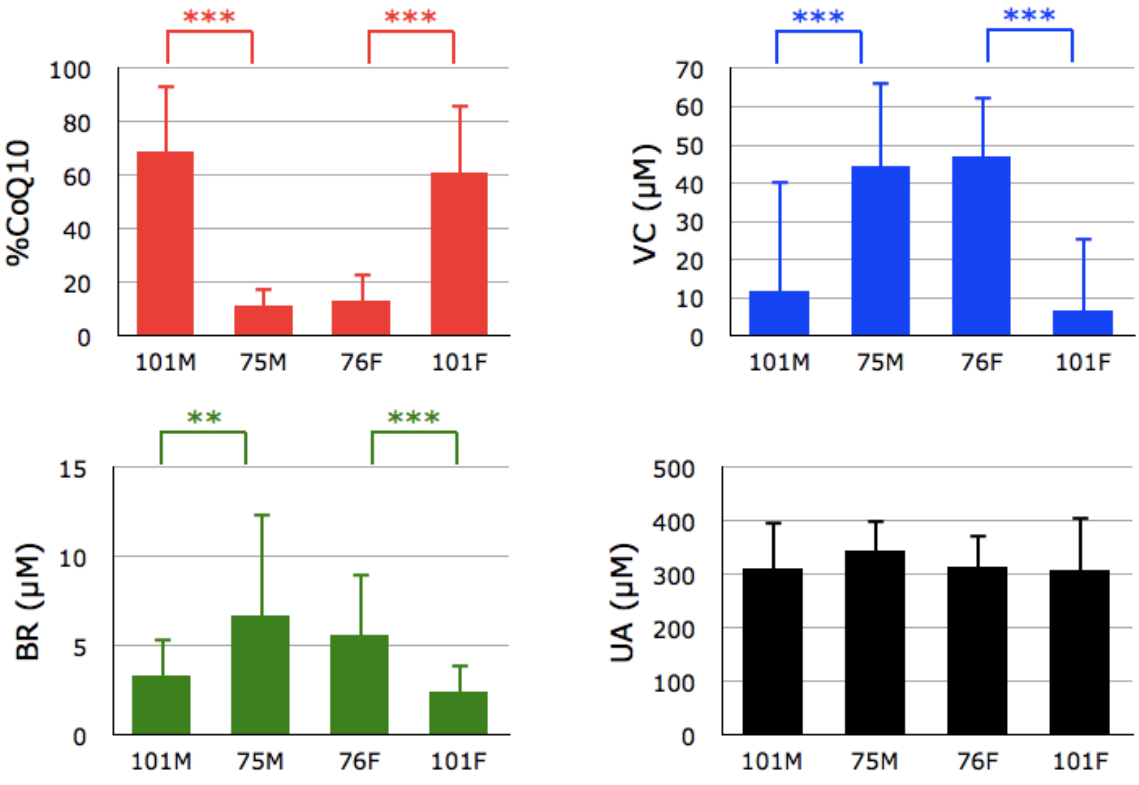

Fig. 1 Comparison of the percentage of oxidized coenzyme Q10 to total coenzyme Q10 (%CoQ10) in serum, and serum levels of ascorbic acid (VC), unconjugated bilirubin (BR), and uric acid (UA) among male centenarians (101M), male 75-year-old controls (75M), female 76-year-old controls (76M), and female centenarians (101F). Data are presented as mean + S.D. ** $p < 0.01$ and *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test.

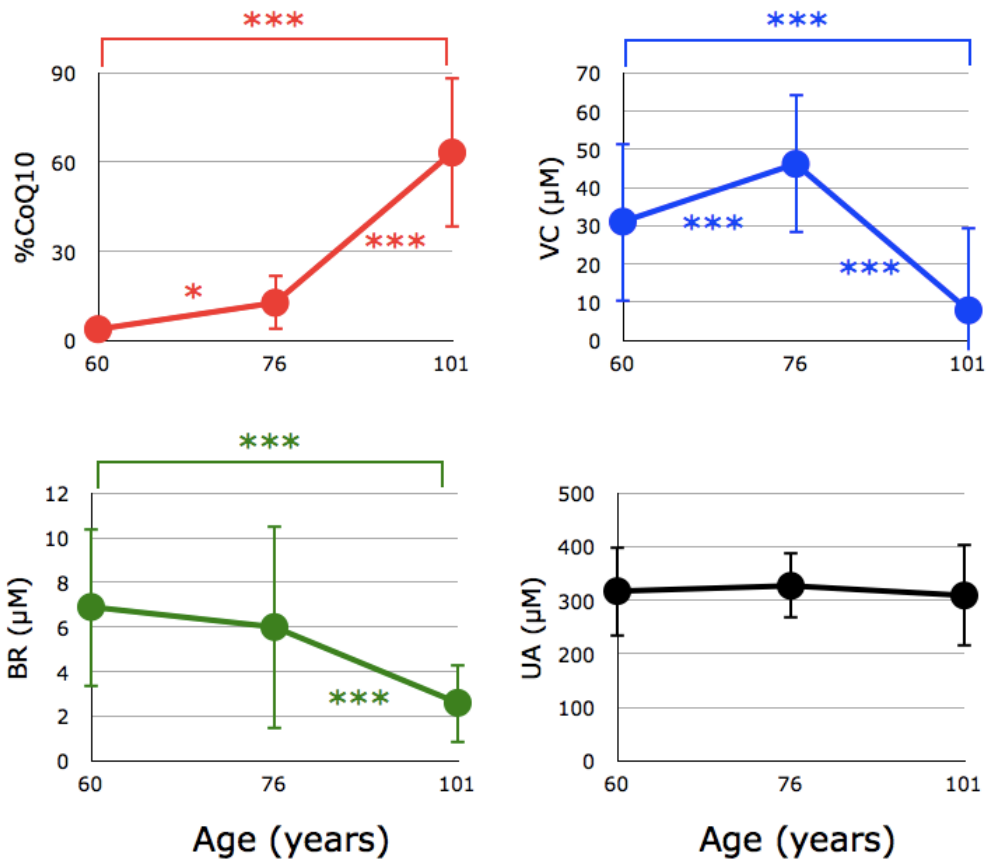

Fig. 2 Changes in the percentage of oxidized coenzyme Q10 to total coenzyme Q10 (%CoQ10) in serum, and serum levels of ascorbic acid (VC), unconjugated bilirubin (BR), and uric acid (UA) with age. Data are presented as mean ± S.D. * $p < 0.05$ and *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test.

Serum levels of cholesterols

Figure 3 shows serum levels of FC, CE, and TC, as well as the FC/CE ratio. A slight decrease in FC was observed in centenarians, however the difference was not significant. A significant decrease in CE and TC was observed, resulting in an increase in the FC/CE ratio. There were no significant differences in the levels of FC, CE, and TC, and the FC/CE ratio between male and female groups in each age category. Thus, we plotted the pooled data against age (Fig. 4). FC, CE, and TC were all observed to decrease with age. Since the decline of CE was more profound than FC, the FC/CE ratio increased with age. The FC/CE ratio is determined by the activity of LCAT secreted with HDL from liver.[8,9] Therefore, these data indicate a degree of impairment in the secretion of LCAT with HDL and liver function. This is consistent with the previous observation that serum levels of HDL are low in centenarians.[2]

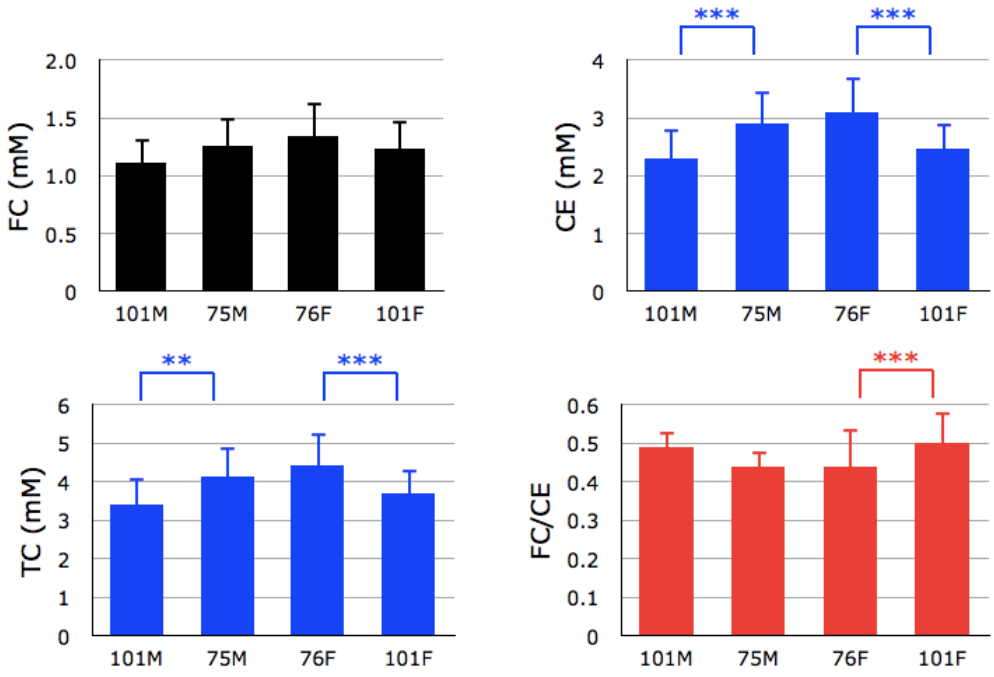

Fig. 3 Comparison of serum free cholesterol (FC), cholesterol esters (CE), total cholesterol (TC), and the FC/CE ratio among male centenarians (101M), male 75-year-old controls (75M), female 76-year-old controls (76M), and female centenarians (101F). Data are presented as mean + S.D. ** $p < 0.01$ and *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test.

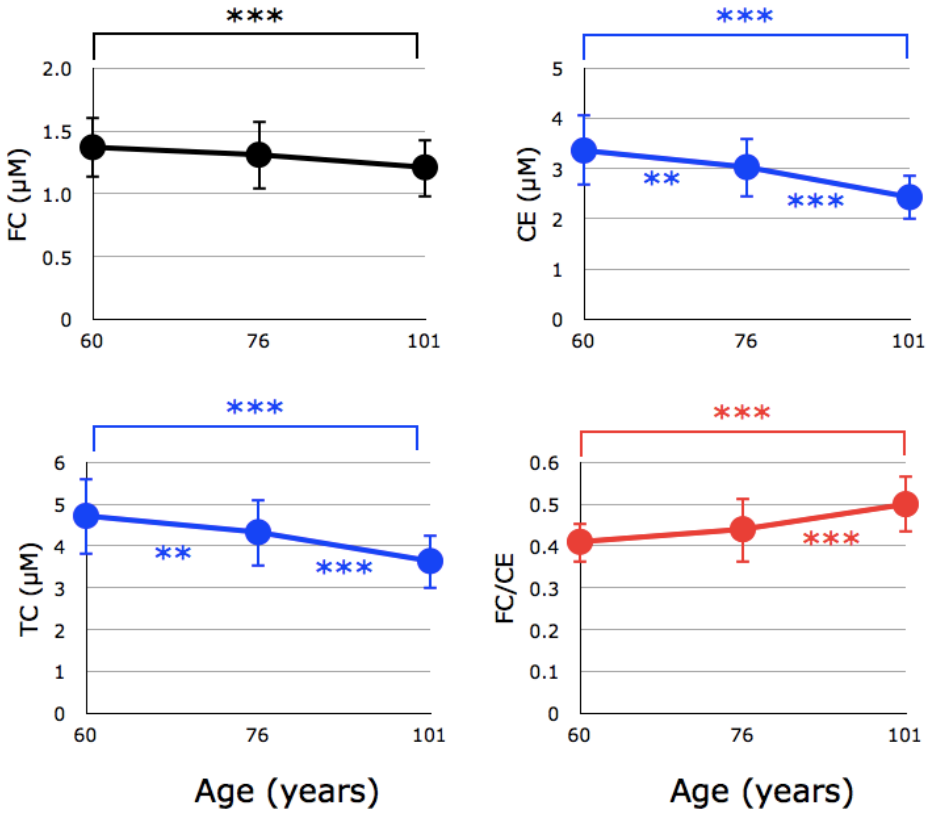

Fig. 4 Changes in serum free cholesterol (FC), cholesterol esters (CE), total cholesterol (TC), and the FC/CE ratio with age. Data are presented as mean ± S.D. ** $p < 0.01$ and *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test.

Serum FFA composition and tissue oxidative damage

Figure 5 shows serum levels of FFA, %PUFA, %18:1, and %16:1. A significant decrease in FFA was observed in centenarians. Under oxidative stress, plasma levels of FFA have been observed to increase in many cases, such as in newborn babies[24] and patients with hepatitis,[25] cirrhosis, [25] hepatoma, [25] juvenile fibromyalgia,[26] and post-cardiac arrest syndrome.[27] Elevated plasma FFA was observed in the rat middle cerebral artery occlusion model of stroke.[28] It is of interest that repeated administration of the antioxidant edaravone significantly improved the neurological symptoms and impairment of motor function induced by a middle cerebral artery occlusion, and reduced the levels of FFA to those of a sham operation.[28] Formation of FFA under oxidative stress is assumed to be a result of phospholipase activity,[29-32] therefore, a significant decrease in serum FFA in centenarians must be ascribed to impairment of the repair system that counteracts increases in oxidative stress.

Under conditions of elevated oxidative stress, oxidatively vulnerable PUFA is selectively damaged which results in decreased membrane fluidity.[10] To compensate for the loss of PUFA, stearoyl-CoA desaturase is activated and converts stearic and palmitic acids to 18:1 and 16:1, respectively.[33] Accordingly, a decrease in %PUFA and an increase in %18:1 and %16:1 have been observed under oxidative stress.[24-28]

Since there were no significant differences in FFA levels, %PUFA, %18:1, and %16:1 between the male and female groups in each age category, we plotted the pooled data against age (Fig. 6). No significant changes in %PUFA were observed in centenarians. In contrast, significant decreases in %18:1 and %16:1 were observed in centenarians, indicating impairment in the oxidative repair system. However, this hypothesis should be investigated further.

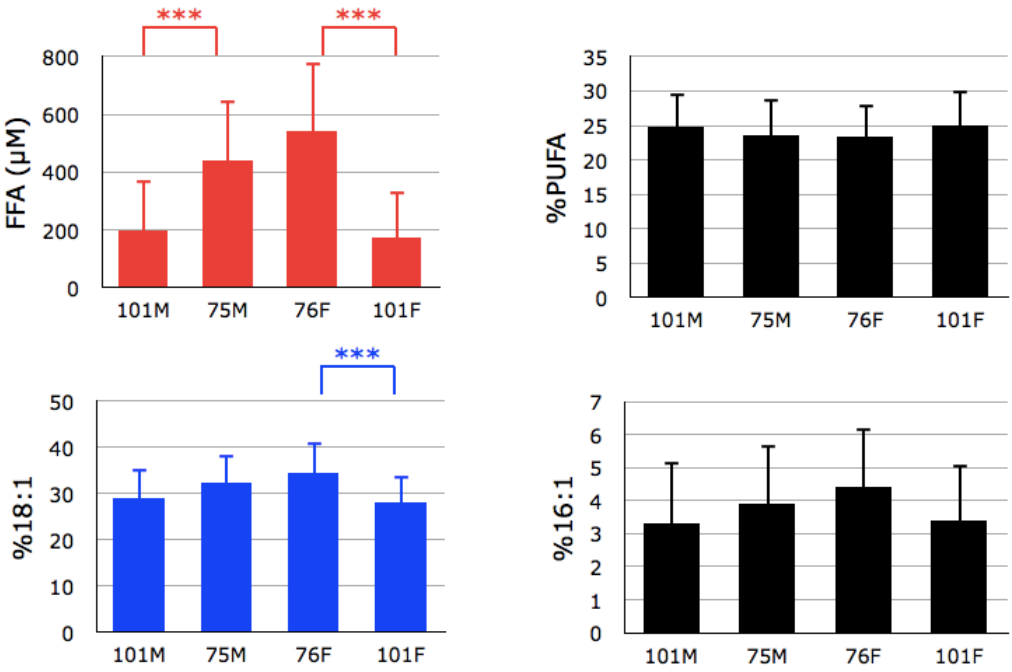

Fig. 5 Comparison of serum free fatty acids (FFA), the percentage of polyunsaturated fatty acids in total FFA (%PUFA), the percentage of palmitoleic acid in total FFA (%16:1), and the percentage of oleic acid in total FFA (%18:1) among male centenarians (101M), male 75-year-old controls (75M), female 76-year-old controls (76M), and female centenarians (101F). Data are presented as mean + S.D. *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test.

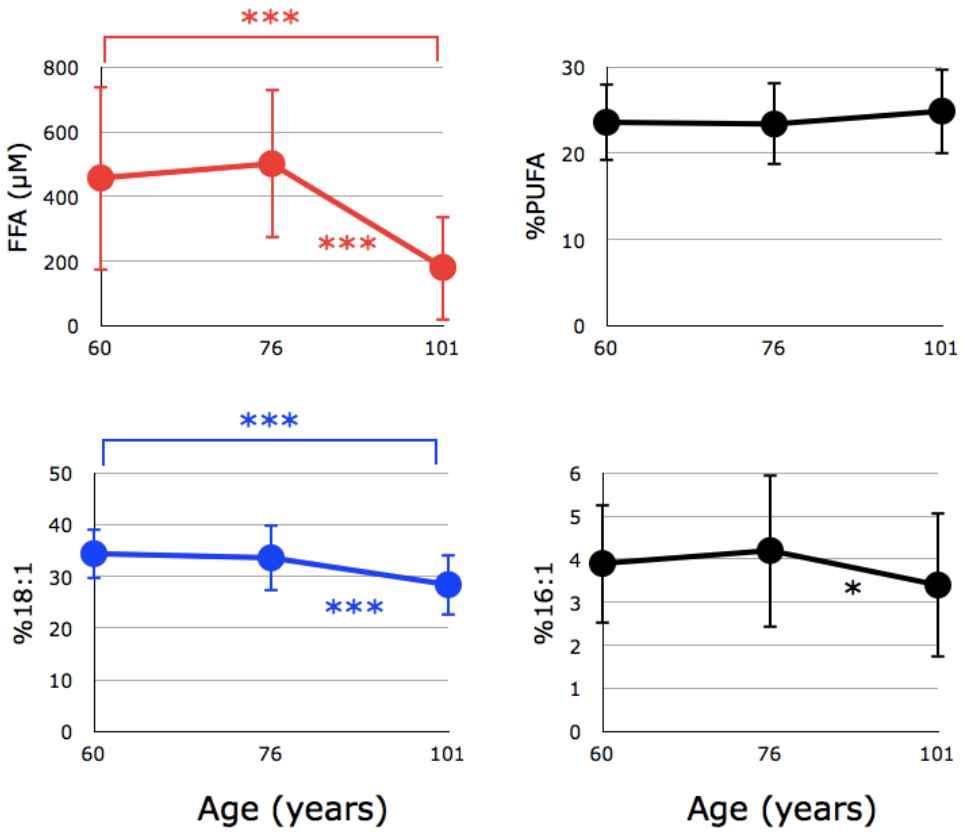

Fig. 6 Changes in serum free fatty acids (FFA), the percentage of polyunsaturated fatty acids in total FFA (%PUFA), the percentage of palmitoleic acid in total FFA (%16:1), and the percentage of oleic acid in total FFA (%18:1) with age. Data are presented as mean ± S.D. * $p < 0.05$ and *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test

Serum TQ10 and Psap

Figure 7 shows serum levels of TQ10 and Psap, as well as the ratio of VE/TC and TQ10/TC in centenarians and 76-year-old controls. A significant decrease in TQ10 was observed in centenarians compared with 76-year-old controls, suggesting a coenzyme Q10 deficiency in centenarians. This is also the case in male centenarians even if TQ10 was normalized to TC, however, female centenarians were not significantly different. A similar trend was observed in VE/TC values.

On the other hand, a significant increase in coenzyme Q10 binding and transfer protein (Psap) [13-15] was observed in female centenarians compared with 76-year-old female controls, and male centenarians showed a similar tendency. Figure 8 shows the combined male and female data of 60- and 76-year-old controls, and centenarians. Psap levels increased progressively and significantly with age while TQ10 levels and the TQ10/TC ratio reached a maximum at 76 years and subsequently decreased. It is well known that tissue TQ10 levels decrease with age; for example > 30% and 50% decreases in TQ10 were observed at the ages of 40 and 80, respectively, in human heart.[11] Furthermore, the rate of coenzyme Q biosynthesis in rat heart is much less than that in rat kidney.[34] This should be also the case in human. These observations suggest that the human heart in octogenarians has a serious requirement for exogenous TQ10. Therefore, coenzyme Q10 should be transferred from its pool (most likely to be kidney) to heart using Psap. This is a likely explanation for the observed increase in serum Psap levels in 76-year-olds, and the consequent increase in serum TQ10 levels, compared with 60-year-old controls.

In centenarians, it is reasonable for Psap levels to increase in order to compensate for the loss of coenzyme Q10. Despite further elevation of Psap in centenarians, their TQ10 levels were decreased, indicating their tissue TQ10 levels were likely to be critically low.

Coenzyme Q10 is essential for ATP production in the mitochondria and is an important antioxidant in every cellular membrane and lipoprotein.[35] Its importance is also suggested by the observation that serum coenzyme Q10 levels were inversely associated with the risk of disabling dementia.[36] Furthermore, a mutation in the coenzyme Q10 synthesis enzyme was identified in patients with familial multiple system atrophy (MSA).[37] Plasma levels of coenzyme Q10 in patients with MSA were significantly lower than controls.[38,39] Now the phase 2 clinical study of coenzyme Q10 supplementation to patients with MSA is on going. Therefore, coenzyme Q10 supplementation would be beneficial for centenarians, although this hypothesis requires further serious investigation.

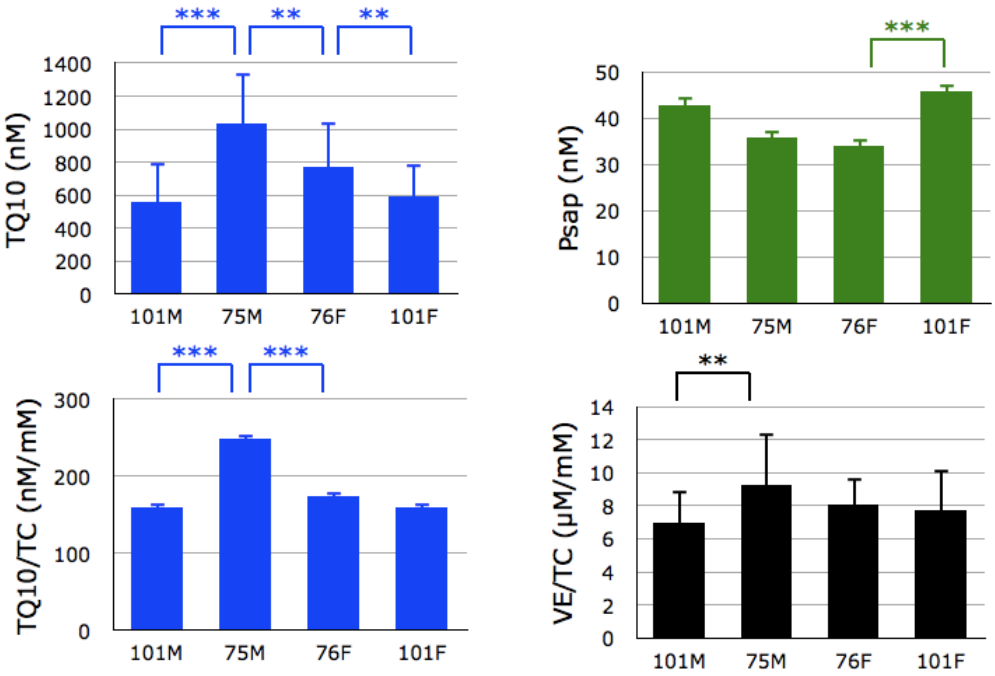

Fig. 7 Comparison of serum total coenzyme Q10 (TQ10), the ratio of vitamin E to total cholesterol (VE/TC), the ratio of TQ10/TC, and serum prosaposin (Psap) among male centenarians (101M), male 75-year-old controls (75M), female 76-year-old controls (76M), and female centenarians (101F). Data are presented as mean + S.D. ** $p < 0.01$ and *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test.

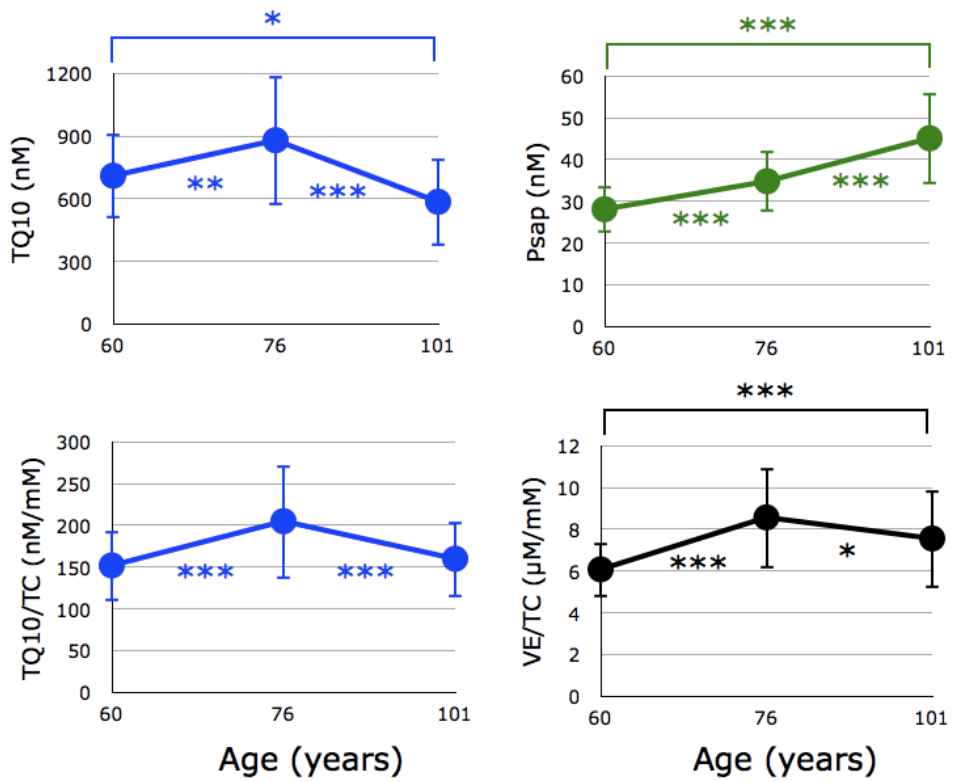

Fig. 8 Changes in serum total coenzyme Q10 (TQ10), the ratio of vitamin E to total cholesterol (VE/TC), the ratio of TQ10/TC, and serum prosaposin (Psap) with age. Data are presented as mean ± S.D. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, significant differences as determined by Scheffe's multiple comparison test.

Conclusion

Oxidative stress in centenarians was demonstrated as an increase in serum %CoQ10 and a decrease in VC compared with 76-year-old controls. Centenarians are suggested to exist in a moderate, chronic inflammatory condition because serum levels of UA were similar to those in 76-year-old controls. Centenarians exhibit a hypocholesterolemic condition and the observed increase in the FC/CE ratio suggests some impairment of liver function. A significant decrease in serum FFA, %18:1, and %16:1 also indicates impairment of the tissue repair system in centenarians. Despite an elevation of the coenzyme Q10 binding protein Psap, serum TQ10 levels decreased in centenarians, suggesting a serious TQ10 deficiency in tissues. Therefore, coenzyme Q10 supplementation is likely to be beneficial for centenarians.

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Chapter 5

Simultaneous detection of reduced and oxidized forms of coenzyme Q10 in human cerebral spinal fluid as a potential marker of oxidative stress

Introduction

Increases in oxidative stress have been suggested to contribute to aging and degenerative diseases such as heart attack, stroke, neurodegenerative diseases, diabetes, and cancer.[1] Oxidative stress is defined as a disturbance in the pro-oxidant-antioxidant balance in favor of pro-oxidants.[2] The redox balance of coenzyme Q10 (CoQ10) is a good marker of oxidative stress because its reduced form ($CoQ10H_2$) is highly reactive with oxygen radicals and is converted to the oxidized form (CoQ10).[1] In fact, the incubation of human plasma at 37°C under aerobic conditions in the presence of 5 μM cupric ion initially produces a decrease in vitamin C (VC), followed by a decrease in $CoQ10H_2$ and the concomitant production of an equal amount of CoQ10.[3] This observation indicates that the percentage of CoQ10 in total coenzyme Q10 (%CoQ10) represents a good marker of early stage oxidative stress. We therefore developed a simple and reliable method for the simultaneous electrochemical detection of plasma $CoQ10H_2$ and CoQ10[4] and applied the method to patients with various diseases. Significant increases in %CoQ10 were observed in patients with hepatitis,[5] cirrhosis,[5] hepatoma,[5] juvenile fibromyalgia,[6] amyotrophic lateral sclerosis (ALS),[7] Parkinson disease,[8] post cardiac arrest syndrome,[9] and sepsis[10] as compared to age-matched healthy controls. It is interesting that newborn babies have significantly higher plasma %CoQ10 than adults[11] and %CoQ10 increases with age.[12,13]

Recently, the measurement of local oxidative stress is sought in order to gain a more precise understanding of the role of reactive oxygen species. For this purpose human cerebral spinal fluid (CSF) represents a good target compartment. However, the levels of total coenzyme Q10 (TQ10) in human CSF are less than 1/500 of those in human plasma. Therefore, CSF extracted with 2-propanol will require concentration prior to electrochemical detection. We prepared pseudo-CSF by preparing a 500 fold dilution of human plasma, which was used to optimize the analytical procedure.

Plasma VC was useful to prevent the oxidation of the reduced form of coenzyme Q10 in 2-propanol extracts (unpublished data). Although, CSF contains ~300 μM ascorbic acid, we found that the addition of *tert*-butylhydroquinone (TBHQ) was effective in preventing the oxidation of the reduced coenzyme Q10 during the extraction and concentration steps. We found the optimal TBHQ concentration to be 20 μM. A column switching system was useful in avoiding interference by TBHQ in the detection of vitamin E (VE), $CoQ10H_2$, and CoQ10. Good within-day reproducibility was observed and day-to-day variance of the analytical method was excellent. Finally, we demonstrated that our method is applicable to human CSF.

Materials and methods

Reagents, pseudo-CSF and human CSF

$CoQ10H_2$ and CoQ10 were generous gifts from Kaneka (Osaka, Japan). TBHQ, 2-propanol (IPA), sodium perchlorate ($NaClO_4$) and other chemicals were of the highest grade commercially available. We prepared pseudo-CSF by diluting human plasma with 499 volumes of 0.9 % physiological saline. Human CSF was obtained from the Department of Neurology, Graduate School of Medicine, The University of Tokyo. Human CSF samples were obtained after written informed consent was obtained from participants and the research project was approved by the institutional review board of the University of Tokyo, School of Medicine.

Optimized Analytical Procedure

Three hundred µl of Pseudo-CSF (or CSF) and 1.2 ml of 20 μM TBHQ in IPA were mixed in a 2.0 ml-polypropylene tube. After centrifugation at 3,000 rpm for 10 min at 4°C, 1.2 ml of supernatant was transferred into a 1.5 ml-LoBind tube (Eppendorf, Hamburg, Germany). The supernatant was dried under a stream of nitrogen gas, and the residue was admixed with 120 µl of IPA. Aliquots (60 μl) were injected into the HPLC system described below.

HPLC system for simultaneous detection of $CoQ10H_2$ and CoQ10

In order to avoid interference by water-soluble antioxidants such as VC and TBHQ in the analysis of $CoQ10H_2$ and CoQ10 using an octylsilyl column, a column switching method was employed (Fig. 1). Nanospace SI-2 systems (Shiseido, Tokyo, Japan) consisting of a 3033 autosampler, two 3011 pumps, a 3004 column oven, a 3011 high pressure valve, and a 3005 electrochemical detector (ECD) (600 mV vs. Ag/AgCl) were configured. A UV detector (210 nm; Model UV-970, Japan Spectroscopic, Tokyo, Japan) and an integrator (Model S-MC 21, Shiseido) were also used. A cleanup column (Supelcosil LC-18, 3 µm, 33 mm × 4.6 mm i.d.; Supelco Japan, Tokyo, Japan), two separation columns (Ascentis LC-8, 5 µm, 250 mm × 4.6 mm i.d. and Supelcosil ABZ+Plus, 3 µm, 33 mm × 4.6 mm i.d.; Supelco Japan), and a reduction column (RC-10, 15 mm × 4 mm i.d.; IRICA, Kyoto, Japan) were employed.

The mobile phase for the cleanup column was 50 mM $NaClO_4$ in methanol/H_2O (95/5, v/v) and was delivered at a flow rate of 0.4 ml/min. The mobile phase for the separation columns was 50 mM sodium $NaClO_4$ in methanol/IPA (78/22, v/v) and was delivered at a flow rate of 0.8 ml/min. The columns were maintained at 20°C.

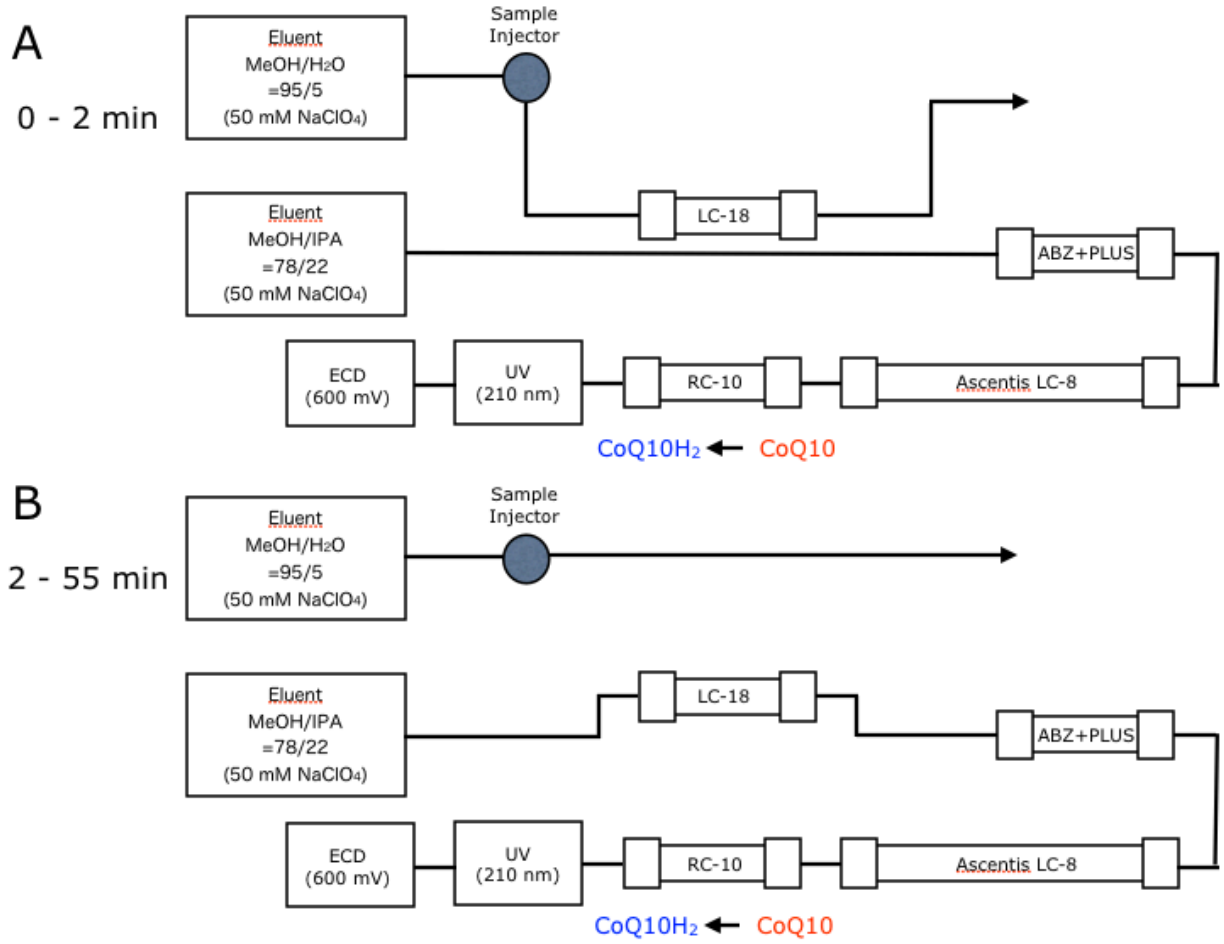

Fig. 1 Column switching system for the analysis of $CoQ10H_2$ and CoQ10 in human CSF.

Results and discussion

Calibration curves

Calibration curve for CoQ10 is shown in Fig. 2A. CoQ10 shows a good linear relationship over a range of 0.015 - 3 pmol, indicating that CoQ10 can be detected over a wide range. CoQ10 is not detectable with an ECD in oxidation mode. However, CoQ10 was converted to $CoQ10H_2$ by the in line reduction column, thereby making the resulting $CoQ10H_2$ detectable with the ECD. Almost identical slopes for CoQ10 and $CoQ10H_2$ were observed (Fig. 2B), indicating that the reduction of CoQ10 to $CoQ10H_2$ was quantitative.

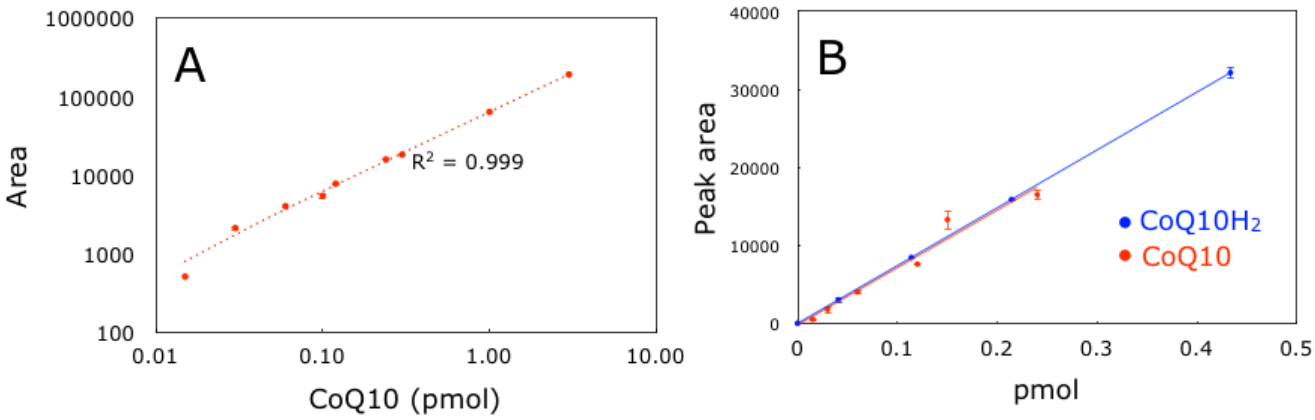

Fig. 2 Calibration curves for $CoQ10H_2$ and CoQ10.

Separation of analytes from TBHQ and VC

We set the column-switching time to 2 min (Fig. 1). For the first 2 min after the sample injection, analytes were introduced to the cleanup LC-18 column, which retains non-polar compounds such as VE, FC, $CoQ10H_2$, and CoQ10, but not polar compounds such as VC and TBHQ. After 2 min, the cleanup column was connected to analytical columns and more non-polar mobile phase (IPA containing methanol) was delivered. In this condition, analytes eluted in the following order: VE, $CoQ10H_2$, and CoQ10 as shown in the typical HPLC chromatograms of extract from pseudo-CSF (Fig. 3).

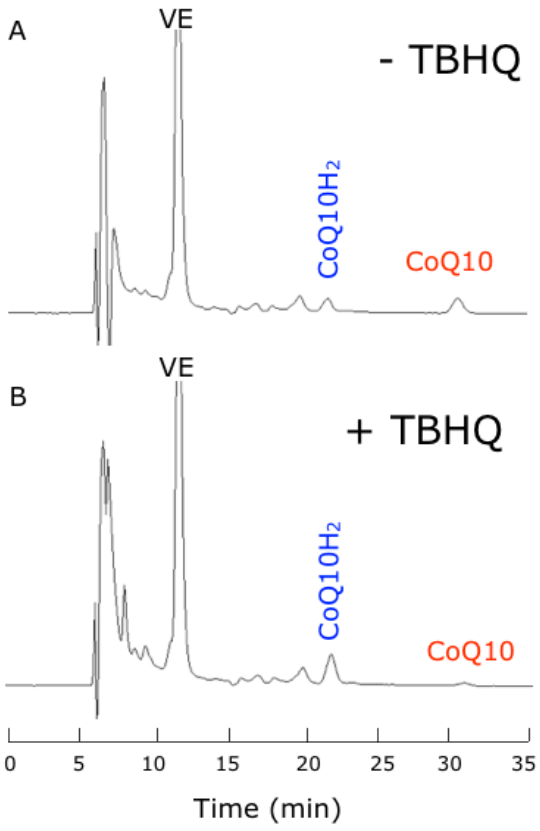

Fig. 3 Typical HPLC chromatograms of IPA extracts of pseudo-CSF. Extraction was carried out without (A) and with (B) 20 μM TBHQ.

Prevention of the oxidation of $CoQ10H_2$ by reductants

Figure 3 also shows that the presence of 20 μM TBHQ in the extraction solvent (IPA) clearly inhibited the oxidation of $CoQ10H_2$ to CoQ10. This is consistent with expectations as pseudo-CSF contained very low VC concentrations. The VC level in human plasma is ~30 μM, thus the VC concentration in pseudo-CSF is calculated to be 30/500 = 0.06 μM. Figure 4 shows the effect of the TBHQ concentration in the extraction solvent on the measurement of TQ10, $CoQ10H_2$, and CoQ10 in pseudo-CSF. Levels of TQ10 were the same, suggesting that TQ10 was well recovered. However, the amount of detected $CoQ10H_2$ increased with increasing TBHQ concentrations in the extraction solvent (up to 20 μM). Therefore, %CoQ10 was lowest at 20 μM TBHQ in the extraction solvent. Under this condition, the molar ratio of TBHQ to $CoQ10H_2$ is calculated as 20 μM x 1200 μl/(6 nM x 300 μl) = 13000, indicating that sufficient amounts of TBHQ were present to inhibit the oxidation of $CoQ10H_2$. However, more than 50 μM TBHQ in the extraction solvent appeared to induce autoxidation of TBHQ and consequently resulted in an increase of %CoQ10. Therefore, the optimal TBHQ concentration in the extraction solvent was deemed to be 20 μM.

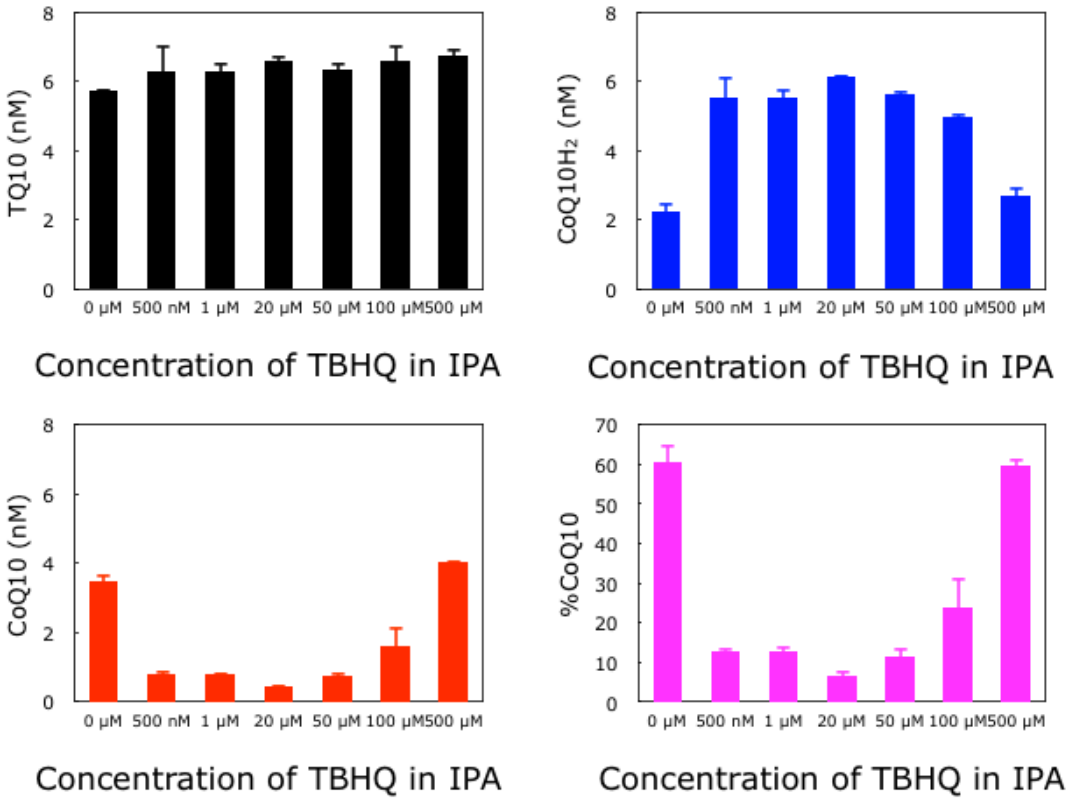

Fig. 4 Effect of TBHQ concentrations in the extracting IPA on the detected levels of TQ10, $CoQ10H_2$, and CoQ10, and %CoQ10 in pseudo-CSF.

Next we compared TBHQ, VC, and TBHQ + VC as reductants (20 μM each) in the extraction solvent, and the results are shown in Fig. 5. The coefficients of variation (CV) for each condition are presented in the figure. There were no significant difference in the levels of TQ10, $CoQ10H_2$, CoQ10 and %CoQ10 values in the presence of either the individual reductants or both reductants. However, poor reproducibility in CoQ10 detection was observed when VC was used as a reductant in the extraction solvent. When TBHQ was employed as the reductant, the reproducibility in CoQ10 detection was excellent. More importantly, the results in the presence of both VC and TBHQ were identical to those with TBHQ alone, indicating no interference with the detection of $CoQ10H_2$ and CoQ10. Levels of VC in human CSF are reported to be about 200 μM,[14] and we confirmed this observation. 200 μM VC in CSF corresponds to 50 μM VC in the extraction solvent. This may be sufficient to prevent $CoQ10H_2$ oxidation during the extraction and concentration procedure. However, we chose to add 20 μM TBHQ to the extraction solvent to prepare for the case of minute VC levels being present in patients' CSF. We would like to also emphasize that the addition of 20 μM TBHQ gave excellent reproducibility.

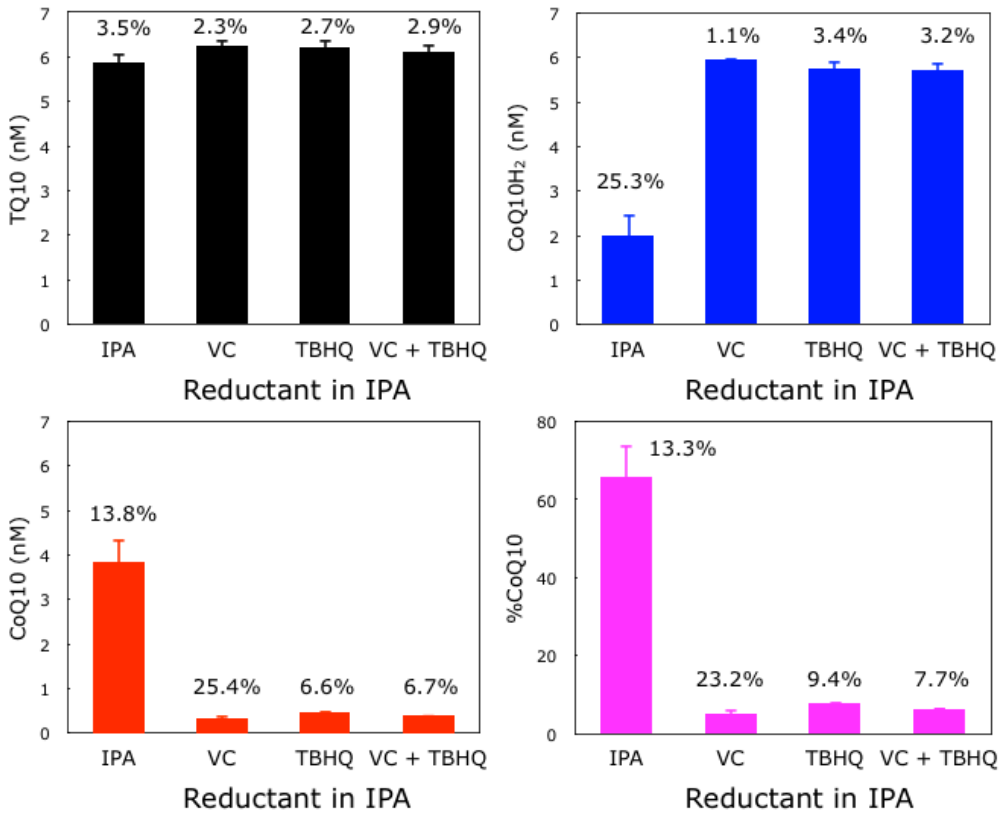

Fig. 5 Effect of VC, TBHQ, and VC + TBHQ (20 μM each) in the extracting IPA on the detected levels of TQ10, $CoQ10H_2$, and CoQ10, and %CoQ10 in pseudo-CSF. Numbers show the coefficients of variation (CV).

Spiking and recovery

Table 1 shows the results of spiked 3.43 nM $CoQ10H_2$ and 1.24 nM CoQ10 added to pseudo-CSF. The recovery of spiked $CoQ10H_2$ and CoQ10 to pseudo-CSF were excellent (n=3), as was TQ10 by extension.

Table 1 Recovery of spiked $CoQ10H_2$, CoQ10, and TQ10 added to pseudo-CSF (n=3).

	$CoQ10H_2$ (nM)	CoQ10 (nM)	TQ10 (nM)
Pseudo-CSF	5.74 ± 0.62	1.08 ± 0.45	6.82 ± 0.17
Additive	3.43	1.24	4.67
Spiked sample	9.64 ± 0.22	2.23 ± 0.27	11.87 ± 0.24
Calculated	9.17	2.32	11.49
Recovery (%)	105.1	96.2	103.3

Reproducibility of the analysis

Table 2 shows the reproducibility of the detection of VE, $CoQ10H_2$ and CoQ10 in pseudo-CSF (n=3). The CV for VE, $CoQ10H_2$, CoQ10, TQ10 and %CoQ10 were less than 10%, showing excellent within-day reproducibility. Table 3 shows the day-to-day variance of the analysis. The CVs for VE, $CoQ10H_2$, CoQ10, TQ10 and %CoQ10 were less than 15%, indicating that the method is reliable.

Table 2 Reproducibilities of the analysis of VE, $CoQ10H_2$, CoQ10, TQ10 and %CoQ10 in pseudo-CSF (n=3).

	VE (nM)	$CoQ10H_2$ (nM)	CoQ10 (nM)	TQ10 (nM)	%CoQ
Average ± SD	59.2 ± 1.1	5.90 ± 0.06	0.44 ± 0.03	6.3 ± 0.04	7.0 ± 0.56
CV (%)	1.9	1.1	7.6	0.6	8.0

Table 3 Day-to-day variance of the analysis of VE, $CoQ10H_2$, CoQ10, TQ10 and %CoQ10 in pseudo-CSF.

	VE (nM)	$CoQ10H_2$ (nM)	CoQ10 (nM)	TQ10 (nM)	%CoQ
Average ± SD	59.7 ± 0.68	5.73 ± 0.21	0.49 ± 0.05	6.2 ± 0.1	7.9 ± 0.9
CV (%)	1.1	3.6	9.3	1.6	11.5

Application of the method to human CSF

Figure 6 shows a typical HPLC chromatogram of the IPA extract of human CSF. The obtained chromatogram was identical to that of pseudo-CSF (Fig. 3), suggesting that interference by the components in human CSF was not observed. Good reproducibility was also observed (Table 4). Thus, we concluded that the present method is useful for the evaluation of oxidative stress in human CSF.

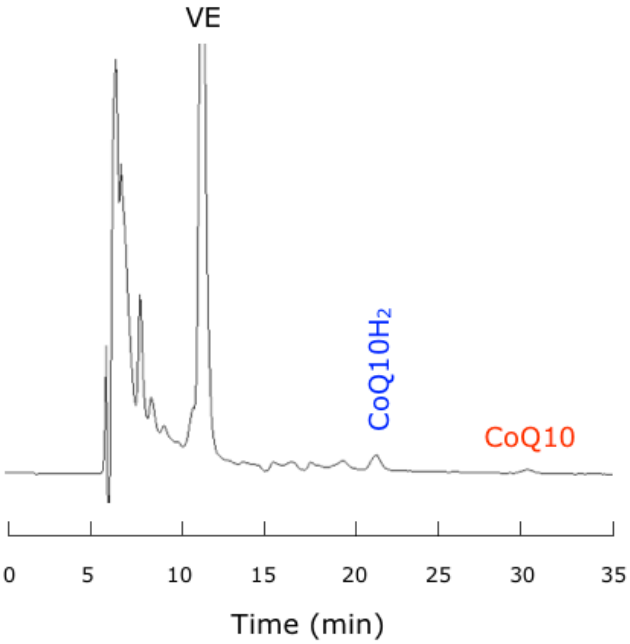

Fig. 6 A typical HPLC chromatogram of an IPA extract of human CSF. Extraction was carried out with IPA containing 20 nM TBHQ.

Table 4 Reproducibilities of the analysis of VE, $CoQ10H_2$, CoQ10, TQ10 and %CoQ10 in human CSF (n=3).

	VE (nM)	$CoQ10H_2$ (nM)	CoQ10 (nM)	TQ10 (nM)	%CoQ
Average ± SD	48.7 ± 5.2	1.67 ± 0.22	0.49 ± 0.02	2.16 ± 0.22	22.8 ± 2.2
CV (%)	10.8	13.1	3.4	10.3	9.5

Conclusion

A method for the simultaneous electrochemical detection of $CoQ10H_2$ and CoQ10 was described. Since the levels of TQ10 in human CSF is ~2 nM, the IPA extract required concentrations prior to analysis. To prevent the oxidation of $CoQ10H_2$ during extraction and concentration, 20 μM TBHQ was added to the extraction solvent (IPA). Good within-day and day-to-day reproducibility was observed and the analytical variance was excellent. We demonstrated our method is applicable to human CSF.

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Chapter 6

Conclusion

We applied plasma FFA and their composition, and plasma %CoQ10 as a marker of tissue oxidative damage and oxidative stress in circulation, respectively, to human subjects under oxidative stress such as patients with PCAS and ALS, and centenarians. We observed significantly higher %CoQ10 levels and significantly lower %PUFA levels as compared with suitable controls as shown in Table below. Furthermore, the ratio of FC to CE were significantly higher than suitable controls. This indicates some impairments of liver function since this ratio is determined by the activity of LCAT secreted from the liver. Especially, this ratio was found to be one of the key factors determining the survival of PCAS patients. In addition, edaravone administration was effective for the treatment of ALS patients and we concluded that it functioned therapeutically as a scavenger of peroxynitrite since edaravone administration raised plasma levels of uric acid, an endogenous scavenger of peroxynitrite. We also concluded CoQ10 deficiency in centenarians. Finally, we developed the method for simultaneous detection of reduced and oxidized forme CoQ10 in cerebrospinal fluid to meet a recent demand for measuring local oxidative stress. We believe that our findings are novel and will provide useful suggestions for future treatments.

Table1 Plasma oxidative markers in patients with PCAS and ALS, and centenarians.

	PCAS	**ALS**	**Centenarians**
VC			↓
UA	↑ but decreased thereafter	↓	
%CoQ10	↑	↑	↑
TQ10/TC			↓
FFA	↑		↓
%PUFA	↓	↓	
%16:1	(↑ thereafter)		↓
%18:1	(↑ thereafter)		↓
FC/CE	↑	↑	↑
Psap	↑	(↑)	↑

↑ and ↓ show a significant increase or decrease as compared with suitable controls.

List of Publications

1. Midori Nagase, Yorihiro Yamamoto, Yusuke Miyazaki, Hiide Yoshino
   Increased oxidative stress in patients with amyotrophic lateral sclerosis and the effect of edaravone administration. Redox Report 2016; 21: 104-112.

2. Midori Nagase, Atsushi Sakurai, Atsunori Sugita, Nozomi Matsumoto, Airi Kubo, Yusuke Miyazaki, Kosaku Kinoshita and Yorihiro Yamamoto
   Oxidative Stress and Abnormal Cholesterol Metabolism in Patients with Post-cardiac Arrest Syndrome. J. Clin. Biochem. Nuin. 2017; 61: 108-117.

3. Midori Nagase, Yorihiro Yamamoto, Nozomi Matsumoto, Yasumichi Arai and Nobuyoshi Hirose
   Increased Oxidative Stress and Coenzyme Q10 Deficiency in Centenarians.
   J. Clin. Biochem. Nutr in press.

4. Midori Nagase, Yorihiro Yamamoto, Jun Mitsui, and Shoji Tsuji
   Simultaneous detection of reduced and oxidized forms of coenzyme Q10 in human cerebral spinal fluid as a potential marker of oxidative stress.
   J. Clin. Biochem. Nutr in press.