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Total and reduced/oxidized forms of coenzyme Q_{10} in fibroblasts of patients with mitochondrial disease

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ABSTRACT

Coenzyme Q_{10} (CoQ_{10}) is involved in ATP production through electron transfer in the mitochondrial respiratory chain complex. CoQ_{10} receives electrons from respiratory chain complex I and II to become the reduced form, and then transfers electrons at complex III to become the oxidized form. The redox state of CoQ_{10} has been reported to be a marker of the mitochondrial metabolic state, but to our knowledge, no reports have focused on the individual quantification of reduced and oxidized CoQ_{10} or the ratio of reduced to total CoQ_{10} (reduced/total CoQ_{10}) in patients with mitochondrial diseases.

We measured reduced and oxidized CoQ_{10} in skin fibroblasts from 24 mitochondrial disease patients, including 5 primary CoQ_{10} deficiency patients and 10 respiratory chain complex deficiency patients, and determined the reduced/total CoQ_{10} ratio.

In primary CoQ_{10} deficiency patients, total CoQ_{10} levels were significantly decreased, however, the reduced/total CoQ_{10} ratio was not changed. On the other hand, in mitochondrial disease patients other than primary CoQ_{10} deficiency patients, total CoQ_{10} levels did not decrease. However, the reduced/total CoQ_{10} ratio in patients with respiratory chain complex IV and V deficiency was higher in comparison to those with respiratory chain complex I deficiency.

Measurement of CoQ_{10} in fibroblasts proved useful for the diagnosis of primary CoQ_{10} deficiency. In addition, the reduced/total CoQ_{10} ratio may reflect the metabolic status of mitochondrial disease.

1. Introduction

CoenzymeQ$_{10}$ (CoQ_{10}), also known as ubiquinone, is a lipophilic molecule composed of a redox-active benzoquinone head group and species-specific isoprenoid side chain (10 subunits in humans) [1,2]. CoQ_{10} takes three forms depending on the redox state of the benzoquinone ring; oxidized (CoQ_{10}, Ubiquinone), fully-reduced ($CoQ_{10}H_2$, Ubiquinol), and semi-reduced (CoQ^{+}_{10}, Semiubiquinone) forms [2]. It presents ubiquitously in all cellular membranes and cells [3]. The amount of CoQ_{10} and the proportion of reduced CoQ_{10} differ between organs and cells; CoQ_{10} is distributed in high amounts in the heart, kidneys, liver, and muscles, and the proportion of reduced CoQ_{10} is lower in the brain and lungs [3]. In cells, it is mostly localized in the mitochondria [3].

CoQ_{10} has multiple functions. One of the main roles of CoQ_{10} is as a component of the mitochondrial respiratory chain. As a mobile electron

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carrier, CoQ_{10} accepts electrons from complex I and II and transfers them to complex III [2]. In the mitochondrial inner membrane, CoQ_{10} is proposed to exist as two independent pools: a CoQ_{NADH} pool in the super-complex (CI, CIII, CIV) involved in the oxidation of NADH; and a CoQ_{FADH} pool involved in the oxidation of CII and other enzymes that use CoQ as a cofactor [4]; CoQ_{NADH} receives electrons from NADH, and CoQ_{FADH} receives electrons from $FADH_2$ and other enzymes, such as glycerol phosphate dehydrogenase (GPDH), choline dehydrogenase (CHDH), sulphide:quinone oxidoreductase (SQOR), dihydroorote dehydrogenase (DHODH), and electron transfer flavoprotein dehydrogenase (ETFDH), and is reduced [5]. In respiratory chain complex III, electrons are transferred from reduced CoQ_{10} to cytochrome *c* in a process called the Q cycle. The Q cycle results in the oxidation of two molecules of reduced CoQ_{10}, the reduction of two molecules of cytochrome *c*, and the formation of one additional molecule of reduced CoQ_{10} [2]. This normal forward electron transfer results in the creation of an electrical gradient and a pH gradient (proton gradient) between the mitochondrial matrix and intermembrane space. The energy generated by this proton motive force enables complex V(ATP synthase) to synthesize ATP. On the other hand, reverse electron transfer (RET) of CoQ_{10} is also known to occur; in RET, electrons from reduced CoQ_{10} are returned to complex I, reducing NAD+ to NADH, and generating ROS [6] (Supplemental Fig. 1).

Another important role of CoQ_{10} is as an antioxidant. CoQ_{10} is the sole lipid-soluble antioxidant that is endogenously synthesized. Reduced CoQ_{10} inhibits both the initiation and the propagation of lipid peroxidation [7]. NADH-quinone oxidoreductase 1 and cytochrome *b*5 reductase, have been known to be the major oxidoreductases in the plasma membrane [8]. In addition, ferroptosis suppressor protein 1 (FSP1) was also found to be an important oxidoreductase. FSP1 reduces extra-mitochondrial CoQ_{10} and acts as a lipophilic radical-trapping antioxidant to suppress lipid peroxides, resulting in the inhibition of cell death, called ferroptosis [9,10]. Moreover, reduced CoQ_{10} also regenerates the other antioxidants—α-tocopherol and ascorbate—into an active reduced form [7]. CoQ_{10} is also involved in the β-oxidation of fatty acids [11], de novo pyrimidine biosynthesis [12], sulfide oxidation [13], an essential cofactor for uncoupling proteins (UCPs) [14], and modulation of the mitochondrial permeability transition pore [15]. Thus, the importance of the distinct state of CoQ_{10} is gaining growing attention [5].

Primary CoenzymeQ_{10} (CoQ_{10}) deficiency is an autosomal recessive mitochondrial disease caused by a decrease in CoQ_{10} due to mutations in genes involved in CoQ_{10} biosynthesis (*COQ* genes) [16]. To date, defects in at least 10 *COQ* genes (*COQ2, COQ4, COQ5, COQ6, COQ7, COQ8A, COQ8B, COQ9, PDSS1, PDSS2*) have been found to cause this disease [17]. Primary CoQ_{10} deficiency was first reported in 1989 as familial mitochondrial encephalomyopathy [18]. Currently, over 280 patients from 180 families have been reported [17]. Secondary CoQ_{10} deficiencies occur in a wider variety of pathologies, including mitochondrial disease [19,20]. Measuring the CoQ levels is known to be useful for diagnosing CoQ_{10} deficiency [21]. As described above, the ratio of reduction varies among organs and cells. As a result, in addition to the total amount of CoQ_{10}, evaluating the reduced/oxidized CoQ_{10} ratio may also be useful for further elucidating various pathophysiological states in cells.

Methods to measure CoQ_{10} in fibroblast and the reduced/oxidized state of CoQ_{10} have been reported [22,23]. However, to our knowledge, there are no reports on the measurement of the reduced/oxidized CoQ_{10} ratio in primary CoQ_{10} deficiency or mitochondrial diseases. Therefore, we decided to measure the total CoQ_{10} levels as well as the levels of reduced and oxidized CoQ_{10} in skin fibroblasts from patients affected by various mitochondrial diseases.

2. Material, methods, and patients

2.1. Subjects

We studied fibroblasts from 24 patients with mitochondrial disease, including primary CoQ_{10} deficiency (Table 1). The inclusion criterion for patients with primary CoQ_{10} deficiency was childhood onset with biallelic pathogenic variants in the *COQ* gene encoding proteins for the biosynthesis of CoQ_{10} [16]. Fibroblasts were obtained from patients at Kanagawa Children's Medical Center, Chiba Children's Medical Center, and Jichi Medical University under the approval of the Ethics Committee of Jichi Medical University. Written informed consent was obtained from the parents of each patient.

We obtained fibroblasts from five patients with primary CoQ_{10} deficiency. One patient carries biallelic *COQ2* variants with the c.[349G > C];[912 + 1G > del] (Case 1). Four patients had biallelic *COQ4* mutations: one with compound heterozygous biallelic variants c.[718C > T];[421C > T] (Case 2) [24], one with c.[431C > A];[718C > T] (Case 3) [25], and two with c.[190C > T];[479G > A] (Cases 4, 5).

Ten patients had disorders of mitochondrial respiratory chain subunits. Seven patients had mutations related to complex I: one with a c.[55C > T] mutation in *NDUFA1* (Case 6) [26], one with an m.10158 T>C mutation in *MT-ND3* (Case 7) [26], Three with an m.13513G > A mutation in *MT-ND5* (Case 8) [27], (Case 9), (Case 10) [28], one with a c.[811 T > G];[1766-2A > G] mutation in *ACAD9* (Case 11), and one with a c.[1150G > A];[1817 T > A] mutation in *ACAD9* (Case 12) [28]. NDUFA1, MT-ND3, and MT-ND5 are subunits of complex I, and ACAD9 is the assembly factor of complex I. Two patients had mutations related to complex IV: one with a c.[743C>A] mutation in *SURF1* (Case 13) [29], and one with a c.[367_368delAG]; [572delC] mutation in *SURF1* (Case 14) [25]. SURF1 is the assembly factor of complex IV. One patient had an m.8993 T > G mutation in *MT-ATP6* (Case 15) [25]. MT-ATP6 is a subunit of complex V.

Three patients had mitochondrial DNA (mtDNA) depletion syndrome: one with a c.[143-307_170del335];[143-307_170del335] mutation in *DGUOK* (Case 16) [30], one with a c.[451dupC];[308_310del] mutation (Case 17) [30] and one with a c.[148C > T];[149G > A] mutation in *MPV17* (Case 18) [30]. Both DGUOK and MPV17 are involved in the maintenance of mtDNA. One patient had Kearns-Sayre syndrome with a single mtDNA deletion (5513 bp del; m.8290–13,802) (Case 19). Two patients had MELAS: one with an m.3243 A > G mutation of tRNA-Leu (Case 20) [26] and one with an m.5541C > T, mutation of tRNA-Trp (Case 21) [26]. One patient had short-chain enoyl-CoA hydratase (ECHS1) deficiency with heterozygous mutations in maternal c.[832G > A] in *ECHS1* (Case 22) [31]. ECHS1 plays a role in valine and fatty acid catabolism in mitochondria. Two patients had c.[287A > G]; [287A > G] mutation in *BOLA3* (Cases 23) [28],(Case 24). BOLA3 is related to iron-sulfur cluster production and is involved in the assembly of the mitochondrial respiratory chain complex.

Five fibroblasts from healthy individuals were purchased: two fibroblasts from the PromoCell Company (#C-12300, GmbH, Heidelberg, Germany), two fibroblasts from Japanese Collection of Research Bioresources Cell Bank (#TIG-120, #HT-2020, Japan), and fibroblasts from Lonza Japan (#CC-2509, Tokyo, Japan). Another five fibroblasts from patients without mitochondrial disease were used as controls. Cells from passages 4–29 were used for assays.

2.2. Cell culture and growth conditions

The fibroblasts were maintained in 1.0 g/L low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 °C under 5% CO_2.

Table 1
Fibroblast cell lines from patients with mitochondrial disease, including primary CoQ_{10} deficiency.

Case [ref]	diagnosis	DNA mutation	variants, heteroplasmy rate[a]	function
1	primary CoQ_{10} deficiency	*COQ2*	c.[349G > C];[912 + 1G > del]	CoQ_{10} biosynthesis
2 [24]	primary CoQ10 deficiency	*COQ4*	c.[718C > T];[421C > T]	CoQ_{10} biosynthesis
3 [25]	primary CoQ_{10} deficiency	*COQ4*	c.[431C > A];[718C > T]	CoQ_{10} biosynthesis
4	primary CoQ_{10} deficiency	*COQ4*	c.[190C > T];[479G > A]	CoQ_{10} biosynthesis
5	primary CoQ_{10} deficiency	*COQ4*	c.[190C > T];[479G > A]	CoQ_{10} biosynthesis
6 [26]	Leigh syndrome	*NDUFA1*	c.[55C > T], 100% (X-linked)	Respiratory chain subunits, complex I
7 [26]	Leigh syndrome	*MT-ND3*	m.10158 T>C, heteroplasmic (F; 90%)	Respiratory chain subunits, complex I
8 [27]	neonatal cardiomyopathy	*MT-ND5*	m.13513G > A, heteroplasmic (F; 78.87%)	Respiratory chain subunits, complex I
9	infantile mitochondrial disease	*MT-ND5*	m.13513G > A, heteroplasmic (B; 77%)	Respiratory chain subunits, complex I
10 [28]	Leigh syndrome	*MT-ND5*	m.13513G > A, heteroplasmic (F; 26%)	Respiratory chain subunits, complex I
11	mitochondrial cardiomyopathy	*ACAD9*	c.[811 T > G];[1766-2A > G]	Respiratory chain assembly factor, complex I
12 [28]	non-lethal infantile mitochondrial disease	*ACAD9*	c.[1150G > A];[1817 T > A]	Respiratory chain assembly factor, complex I
13 [29]	Leigh syndrome	*SURF1*	c.[743C>A], homoplasmy	Respiratory chain assembly factor, complex IV
14 [25]	Leigh syndrome	*SURF1*	c.[367_368delAG];[572delC]	Respiratory chain assembly factor, complex IV
15 [25]	Leigh syndrome	*MT-ATP6*	m.8993 T > G, homoplasmy	Respiratory chain subunits, complex V
16 [30]	mtDNA depletion syndrome	*DGUOK*	c.[143-307_170del335];[143-307_170del335]	Deoxynucleotide triphosphate synthesis
17 [30]	mtDNA depletion syndrome	*MPV17*	c.[451dupC];[308_310del]	mitochondrial protein synthesis
18 [30]	mtDNA depletion syndrome	*MPV17*	c.[148C > T];[149G > A]	mitochondrial protein synthesis
19	Kearns-Sayre syndrome		Single mtDNA deletion (5513 bp del; m.8290–13,802)	
20 [26]	MELAS	(tRNA-Leu)	m.3243 A > G, heteroplasmic (F; 21%)	Mitochondrial tRNA
21 [26]	MELAS	(tRNA-Trp)	m.5541C > T, heteroplasmic (F; 49%)	Mitochondrial tRNA
22 [31]	ECHS1 deficiency	*ECHS1*	c.[832G > A]	Metabolism of toxic compounds
23 [28]	cardiomyopathy	*BOLA3*	c.[287A > G];[287A > G]	Iron-sulfur protein assembly
24	cardiomyopathy	*BOLA3*	c.[287A > G];[287A > G]	Iron-sulfur protein assembly

[a] F; fibroblasts, B; blood

2.3. CoQ_{10} measurement in fibroblasts

2.3.1. CoQ extraction

The methods for the extraction CoQ_{10} were based on a previously reported method with slight modifications [32]. To extract CoQ_{10} from fibroblast in 60 mm dishes, cells were washed twice with PBS, and pellets were re-suspended in 500 μL of lysis buffer (0.25 mM Sucrose, 2 mM EDTA, 10 mM Tris, and 100 UI/mL heparin, pH 7.4.), and sonicated twice for 5 s. These homogenates were also used to citrate synthase and protein quantification. To measure CoQ_{10}, nine hundred microliters of ethanol containing internal standard CoQ_{10}-d9 (IsoSciences, Ambler, PA) and 20 μM *tert*-butyl hydroquinone (TBHQ) (FUJIFILM Wako, Osaka, Japan) was added to 100 μL of homogenates. TBHQ was added to prevent oxidation of reduced CoQ_{10}. The cell suspensions were vortexed and centrifuged at 15,700 ×*g* for 10 min (4 °C).

2.3.2. Reduction of ubiquinone

Reduced CoQ_{10} was required for use in the calibration curve measurement. However, since reduced CoQ_{10} is easily oxidized, reduced CoQ_{10} was prepared just before the analysis by reducing oxidized CoQ_{10} following a previously reported method with slight modification [33]. Briefly, 50 μL of CoQ_{10} was diluted in 1.95 mL hexane in a glass tube. Twenty milligrams of $NaBH_4$ was added and followed by the addition of 100 μL methanol, vortexed for 3 min, then placed in the dark for 5 min at room temperature. After reduction, 1 mL of water containing 100 μM EDTA was added to stop the reaction, vortexed for 1 min, and centrifuged 1500 ×*g* for 5 min at 4 °C. The upper layer containing reduced CoQ_{10} was transferred to a glass tube.

2.3.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The method for measuring reduced and oxidized CoQ_{10} was based on the previously reported method with slight modifications [23]. An LC-MS/MS analysis was performed on an LC-electrospray ionization-MS (LC-ESI-MS) with triple quadrupole (Nexera X2 and LCMS-8060, Shimadzu, Kyoto, Japan). A Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm, Phenomenex) and a guard column filled with the same packing material were used. The column temperature was kept at 40 °C. The mobile phase was isocratic with 2 mM ammonium formate in methanol. The flow rate was 0.8 mL/min, the injection volume was 2 μL, and the run time was 6 min. The interface temperature was 300 °C, the desolvation line temperature was 250 °C, and the heat block temperature was 400 °C. The nebulizing gas flow was 3 L/min, the heating gas flow was 10 L/min, and the drying gas flow was 10 L/min. The samples were kept at 4 °C before injection by the autosampler. The MS/MS conditions for each target were optimized using the automated multiple reaction monitoring (MRM) optimization procedures in LabSolutions (Shimadzu). The MRM used for quantification was m/z 880.5 > 197.1 for oxidized CoQ_{10}, 882.4 > 197.0 for reduced CoQ_{10}, and 890.4 > 206.2 for CoQ_{10}-d9 (internal standard). Standards and samples were quantified using the LabSolutions software program to determine the peak area for oxidized CoQ_{10}, reduced CoQ_{10}, CoQ_{10}-d9, and the standard curves were used to determine the total amount of CoQ present in the samples.

Intra-assay coefficients of validation (CVs) and relative errors (REs), as measurements of precision and accuracy, respectively, were determined in five parallel analyses of the same cell. To evaluate inter-assay precision and accuracy, one cell line was independently evaluated on three different days. Precision was calculated as (standard deviation/mean concentration) × 100 (%), and accuracy was calculated as (quantitative value/theoretical value) × 100 (%). The intra-assay precision (CV) of reduced CoQ_{10} and oxidized CoQ_{10} was 0.58% and 1.39%, respectively. The intra-assay accuracy (RE) of reduced CoQ_{10} and oxidized CoQ_{10} was 4.20% and 2.50%, respectively. The inter-assay precision (CV) of reduced CoQ_{10} and oxidized CoQ_{10} was 1.27% and 1.84%, respectively. The intra-assay accuracy (RE) of reduced CoQ_{10} and oxidized CoQ_{10} was 10.54% and 1.18%, respectively (Supplemental Table 1, QC1).

2.3.4. Citrate synthase and protein quantification

Fibroblast CoQ_{10} levels were expressed as citrate synthase (CS) activity (measured CoQ_{10} values/CS units, nmol/CS units). CS activity was measured spectrophotometrically referring to the method described by Srere (1969), with 0.1 mM DTNB, 0.3 mM Acetyl-CoA, 0.5 mM Oxaloacetate, and 12–20 μg protein in 200 μL total incubation volume. CS units are determined as follows: CS Units (μmol/min/mL) = (ΔA_{412}/min x V (mL) × dil)/ 13.6 × L (cm) × V_{enz} (mL), V (mL); the reaction volume, dil; the dilution factor of the original sample, 13.6(mM^{-1} cm^{-1}); the extinction coefficient of TNB at 412 nm, L (cm); pathlength for

absorbance measurement (0.552 cm), V_{enz}(mL); the volume of the enzyme sample. Protein concentrations were quantified using Qubit™ Protein Assay Kits and a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

2.4. Statistical analyses

Statistical analyses were performed using the GraphPad Prism software program (version 9.01, GraphPad Software Inc., La Jolla, CA). Comparisons between samples were performed using a one-way ANOVA. The results are expressed as the mean (standard deviation). *P* values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. Total CoQ_{10} levels were observed to decrease in all patients with primary CoQ_{10} deficiency

We showed the reduced, oxidized, and total (sum of reduced and oxidized) CoQ_{10} values corrected for the CS unit (nmol/CS unit) (Table 2). In addition, we also measured the CoQ_{10} values corrected for protein levels (nmol/g protein) (Supplemental Table 2). Six patients showed decreased the total CoQ_{10} values (<70% of the control): five with primary CoQ_{10} deficiency (Cases 1–5), and one with Kearns-Sayre syndrome (Case 19) (Table 2). The total CoQ_{10} levels were significantly decreased in primary CoQ_{10} deficiency than in controls (primary CoQ_{10} deficiency($n = 5$)1.00 ± 0.19 nmol / CS unit (mean ± SD), controls ($n = 10$)2.30 ± 0.24 nmol / CS unit, $p < 0.0001$) (Fig. 1). However, total CoQ_{10} levels were the same in mitochondrial disease other than primary CoQ_{10} deficiency as in controls (mitochondrial disease($n = 19$)2.23 ± 0.26 nmol / CS unit, $p = 0.93$).

Table 2
Reduced and oxidized CoQ_{10} values and total CoQ deficiency in fibroblasts.

Case	reduced CoQ_{10}		oxidized CoQ_{10}		total CoQ_{10} (nmol/CS unit)		% CoQ deficiency (%)
	mean	SD	mean	SD	mean	SD	
1	0.37	0.15	0.37	0.02	0.74	0.13	32
2	0.12	0.00	0.16	0.00	0.28	0.00	12
3	0.57	0.45	0.59	0.18	1.16	0.63	50
4	0.64	0.03	0.74	0.20	1.38	0.17	60
5	0.78	0.01	0.64	0.02	1.42	0.01	62
6	0.48	0.01	1.65	0.00	2.13	0.01	
7	1.03	0.04	1.00	0.32	2.03	0.29	
8	1.22	0.04	0.82	0.18	2.04	0.22	
9	1.40	0.54	0.96	0.03	2.36	0.51	
10	0.86	0.16	1.34	0.30	2.20	0.46	
11	1.02	0.08	2.17	0.53	3.19	0.44	
12	1.18	0.56	1.31	0.17	2.49	0.38	
13	1.33	0.03	0.57	0.02	1.90	0.00	
14	1.77	0.31	0.58	0.10	2.35	0.21	
15	1.54	0.09	0.71	0.00	2.25	0.09	
16	0.86	0.02	1.46	0.01	2.32	0.01	
17	1.37	0.40	1.28	0.14	2.65	0.25	
18	1.01	0.20	1.52	0.08	2.53	0.29	
19	0.57	0.01	0.72	0.02	1.29	0.02	56
20	1.41	0.40	0.51	0.11	1.92	0.51	
21	1.24	0.28	0.65	0.28	1.89	0.56	
22	1.19	0.11	0.87	0.23	2.06	0.34	
23	0.81	0.19	1.84	0.46	2.65	0.27	
24	0.92	0.01	1.23	0.00	2.15	0.01	
Reference (n = 10)	1.19	0.15	1.11	0.10	2.30	0.24	

% CoQ deficiency: <70% of control CoQ_{10} value.

3.2. The reduced/total CoQ_{10} ratio was unchanged in primary CoQ_{10} deficiency, but was higher in complex IV or V deficiency

We showed the ratio of reduced CoQ_{10} to total CoQ_{10} (reduced/total CoQ_{10}) of fibroblasts (Table 3). The ratio of reduced and oxidized CoQ_{10} to total CoQ_{10} in control fibroblasts was 52% and 48%, respectively (Table 3). In primary CoQ_{10} deficiency, the reduced/total CoQ_{10} ratio did not change compared to the control (reduced/total CoQ_{10} ratio of primary CoQ_{10} deficiency ($n = 5$) 49 ± 7% (mean ± SD), controls ($n = 10$) 52 ± 1%, $p = 0.92$) (Fig. 2). Regarding the cases with respiratory chain complex deficiency, there was no difference between control and complex I deficiency (reduced/total CoQ_{10} ratio of complex I deficiency ($n = 7$) 44 ± 7%, $p = 0.37$). However, the reduced/total CoQ_{10} ratio in complex IV or V deficiency was increased in comparison to the control (complex IV or V deficiency ($n = 3$) 71 ± 3%, $p = 0.022$). In addition, the reduced/total CoQ_{10} ratio in complex IV or V deficiency was higher in comparison to complex I deficiency and primary CoQ_{10} deficiency (complex IV or V deficiency vs. complex I deficiency: $p = 0.0021$, complex IV or V deficiency vs. primary CoQ_{10} deficiency: $p = 0.015$).

In individual cases, the reduced/total CoQ_{10} ratio decreased (<80% of the reduced/total CoQ_{10} ratio in the control) in six cases; three cases with complex I deficiency (Cases 6, 10, 11), two cases with mtDNA depletion syndrome (Cases 16, 18), one case with *BOLA3* mutation (Case 23)(Table 3). On the other hand, the reduced/total CoQ_{10} ratio increased (>120% of the reduced/total CoQ_{10} ratio in the control) in five cases; two cases with complex IV deficiency (Cases 13, 14), two cases with MELAS(Cases 20, 21), one case with complex V deficiency (Case 15) (Table 3).

4. Discussion

We showed here that the total CoQ_{10} values of fibroblasts were significantly lower in patients with primary CoQ_{10} deficiency. In this syndrome, early recognition and therapy can stop progression and improve the prognosis; however, established severe symptoms cannot be reversed [16,34]. Biochemical measurements of low CoQ_{10} levels in muscle biopsy have been utilized for the diagnosis of this syndrome

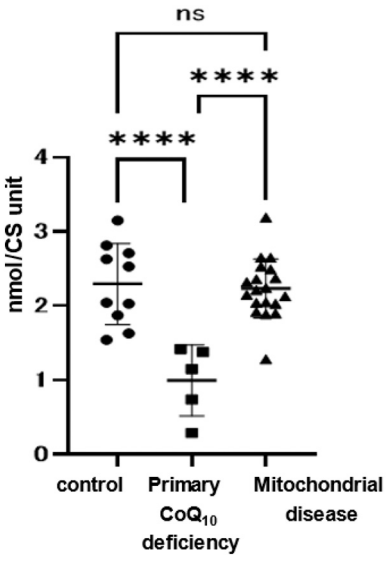

Fig. 1. Total CoQ_{10} values.
Total (sum of reduced and oxidized) CoQ_{10} values of mitochondrial patient fibroblasts. CoQ_{10} deficiency (CoQ_{10} levels <70% of control CoQ_{10}) was found in six cases. All cases of primary CoQ_{10} deficiency showed decreased CoQ_{10} levels. In mitochondrial disease, CoQ_{10} values were not decreased, with the exception of Case 19 (Kearns-Sayre syndrome). Data are expressed as **** $P <$ 0.0001, and n.s. indicates no significance.

Table 3
Ratio of reduced / total CoQ_{10}.

Case	reduced/total CoQ_{10} (%)		Reduced/total CoQ_{10} in cases versus reduced/total CoQ_{10} in controls (%)
	mean	SD	
1	50	11	96
2	43	1	83
3	49	14	95
4	47	8	90
5	55	1	106
6	23	0	44
7	51	9	97
8	60	4	115
9	59	10	114
10	39	1	75
11	32	7	61
12	47	15	91
13	70	1	135
14	75	7	145
15	68	1	131
16	37	1	71
17	52	10	100
18	40	3	77
19	44	0	85
20	74	1	142
21	66	5	127
22	58	4	111
23	30	10	59
24	43	0	83
Reference (n = 10)	52	1	

The reduced/total CoQ_{10} ratio decreased (< 80% of control value); cases 6, 10, 11, 16, 18, 23.
The reduced/total CoQ_{10} ratio increased (120% < of control value); cases 13, 14, 15, 20, 21.

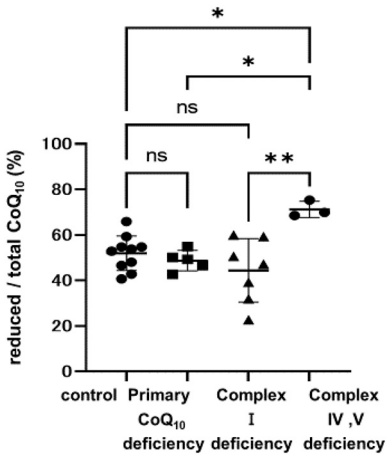

Fig. 2. Comparison of the reduced/total CoQ_{10} ratio.
Comparison of the reduced/total CoQ_{10} ratio in cases with primary CoQ_{10} deficiency, complex I deficiency, and complex IV or V deficiency. In primary CoQ_{10} deficiency, the reduced/total CoQ_{10} ratio was the same as that of the controls. In complex I deficiency, 3/7 cases showed a decreased (< 80% of control value) reduced/total CoQ_{10} ratio. In complex IV or V deficiency, 3/3 cases showed an increased (120% < of control value) reduced/total CoQ_{10} ratio. Data are expressed as $^{*}P < 0.05$, $^{**}P < 0.01$, and n.s. indicates no significance.

[16]. Moreover, the identification of biallelic pathogenic variants in the *COQ* genes, which encode proteins involved in coenzyme Q biosynthesis, enables a definitive diagnosis [16]. However, the invasiveness of muscle biopsy hampers this procedure and can delay the diagnosis. CoQ_{10} levels in fibroblasts were examined and implicated in their usefulness [21]. Our data with LC-MS/MS for the measurement of CoQ_{10} from fibroblasts also supported the usefulness for detecting CoQ_{10} deficiency [32,35]. The fibroblasts from Case 2 showed the lowest CoQ_{10} concentration and showed a very severe phenotype. A correlation between CoQ_{10} levels and phenotype has been suggested [24,36,37]. Therefore, the CoQ_{10} value from skin fibroblast may reflect the clinical severity.

In addition to primary CoQ_{10} deficiency, various mitochondrial diseases have also been reported to decrease CoQ_{10} in fibroblasts and muscle, particularly in mtDNA depletion syndrome [20,38]. However, in our analysis, three patients with mtDNA depletion, including *DGUOK* and *MPV17* mutations, showed no decrease in CoQ_{10} (Cases 16–18). Only one patient with large deletions of mtDNA showed markedly decreased CoQ_{10} (Case 19). Therefore, decreased CoQ_{10} was only a constant feature in primary CoQ_{10} deficiency syndrome in our analysis. Our results support the widely accepted idea that early CoQ_{10} therapy should is therefore indicated if decreased levels of CoQ_{10} are found in the fibroblasts of patients with suspected mitochondrial disease.

To our knowledge, this is the first report to describe the reduced/total CoQ_{10} ratios in patients with mitochondrial diseases, including primary CoQ_{10} deficiency. In primary CoQ_{10} deficiency, the reduced/total CoQ_{10} ratio did not change. On the other hand, the reduced/total CoQ_{10} ratio was decreased in 3/7 of cases of complex I deficiency and was increased in 3/3 of cases of complex IV or V deficiency. Primary CoQ_{10} deficiency is caused by impaired CoQ_{10} biosynthesis, which results in a decrease in the absolute value of CoQ_{10}; however, as expected, our data suggest that it does not affect the redox reaction in mitochondria. Since CoQ_{10} changes from the oxidized form to the reduced form by accepting electrons from complex I, complex II and other dehydrogenases, the reduced/total CoQ_{10} ratio is expected to decrease in complex I deficiency. However, only 3 out of 7 complex I patients in our cohort showed disturbed Q reduced/total ratios. In contrast, CoQ_{10} changes from the reduced form to the oxidized form in complex III, and the reduced/total CoQ_{10} ratio is expected to increase in complex III and later complex deficiencies (Supplemental Fig. 1, left panel). In fact, the reduced CoQ_{10} ratio was significantly decreased with complex I inhibitor, whereas the reduced CoQ_{10} ratio was increased with complex IV inhibitor [23]. Some (but not all) of our results support these observations. Among complex I deficiency patients, patients with isolated complex I deficiency tended to have a decreased reduced/total CoQ_{10} ratio (3/4 cases; Cases 6, 10, 11) (Supplemental Table 3). On the other hand, the reduced/total CoQ_{10} ratio was unchanged in a patient who also had a decreased complex III and IV enzyme activity (Case 8), in a patient without a decreased CI enzyme activity in fibroblasts (Case 12), and in a patient with a decreased CI enzyme activity in muscle (Case 7).

In recent years, it has been reported that the reduced/oxidized CoQ_{10} ratio may be a marker of mitochondrial metabolic status [39]. In situations where CoQ_{FADH} can be excessively reduced, RET is induced, and the production of reactive oxygen species (ROS) from complex I is stimulated, which causes complex I destruction [4,6]. On the other hand, oxidizing the CoQ pool by alternative oxidase (AOX) of *Ciona intestinalis* xenotopically expressed in mouse mitochondria induces forward electron transport (FET) from RET [40]. Our system of measuring the reduced/total CoQ_{10} ratio of skin fibroblasts provide the amount and redox states of CoQ_{10}.

As a limitation of our study, CoQ_{10} is mostly localized in the mitochondria in subcellular fractions but it has also been shown to localize in Golgi, lysosomes, and other organelles [3]. In this study, we measured the whole cell CoQ_{10} level without separating the mitochondrial and non-mitochondrial fractions. Moreover, we examined an only limited number of patients affected by only some mitochondrial diseases, which can be caused by >400 gene mutations [41].

In conclusion, we measured the reduced/total CoQ_{10} ratio in fibroblasts from a cohort of patients with mitochondrial disease for the first time. The reduced/total CoQ_{10} ratio tended to show no change in many of the cells that we measured. However, the reduced/total CoQ_{10} ratio was increased in complex IV or V deficiency, while the reduced/total CoQ_{10} ratio tended to decrease in some cases of complex I deficiency.

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Author contributions

CW, HO contributed to the conceptualization and performance of the statistical analysis of the data and wrote the manuscript; MW, AM, EFJ, TT, HU, OT, ET, and KA performed sampling and data acquisition; YK, YO performed genetic testing; KM, AO recruited patients, provided clinical information, collected samples; TY conducted supervision of the project. All authors read and approved the final manuscript.

Ethics

All procedures followed were by the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of the World Medical Organization. This study was approved by the Ethics Committee of Jichi Medical University and written informed consent was obtained from all participants.

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CRediT authorship contribution statement

Chika Watanabe: Conceptualization, Writing – original draft. **Hitoshi Osaka:** Conceptualization, Writing – review & editing, Funding acquisition. **Miyuki Watanabe:** Investigation. **Akihiko Miyauchi:** Investigation. **Eriko F. Jimbo:** Investigation. **Takeshi Tokuyama:** Resources. **Hideki Uosaki:** Resources. **Yoshihito Kishita:** Resources. **Yasushi Okazaki:** Resources. **Takanori Onuki:** Resources. **Tomohiro Ebihara:** Resources. **Kenichi Aizawa:** Resources. **Kei Murayama:** Resources. **Akira Ohtake:** Resources. **Takanori Yamagata:** Supervision.

Declaration of Competing Interest

None.

Data availability

No data was used for the research described in the article.

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