A Mouse Model of Familial ALS Has Increased CNS Levels of Endogenous Ubiquinol9/10 and Does Not Benefit from Exogenous Administration of Ubiquinol10

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Abstract

Oxidative stress and mitochondrial impairment are the main pathogenic mechanisms of Amyotrophic Lateral Sclerosis (ALS), a severe neurodegenerative disease still lacking of effective therapy. Recently, the coenzyme-Q (CoQ) complex, a key component of mitochondrial function and redox-state modulator, has raised interest for ALS treatment. However, while the oxidized form ubiquinone10 was ineffective in ALS patients and modestly effective in mouse models of ALS, no evidence was reported on the effect of the reduced form ubiquinol10, which has better bioavailability and antioxidant properties. In this study we compared the effects of ubiquinone10 and a new stabilized formulation of ubiquinol10 on the disease course of SOD1G93A transgenic mice, an experimental model of fALS. Chronic treatments (800 mg/kg/day orally) started from the onset of disease until death, to mimic the clinical trials that only include patients with definite ALS symptoms. Although the plasma levels of CoQ10 were significantly increased by both treatments (from <0.20 to 3.0–3.4 µg/mL), no effect was found on the disease progression and survival of SOD1G93A mice. The levels of CoQ10 in the brain and spinal cord of ubiquinone10- or ubiquinol10-treated mice were only slightly higher (<10%) than the endogenous levels in vehicle-treated mice, indicating poor CNS availability after oral dosing and possibly explaining the lack of pharmacological effects. To further examine this issue, we measured the oxidized and reduced forms of CoQ9/10 in the plasma, brain and spinal cord of symptomatic SOD1G93A mice, in comparison with age-matched SOD1WT. Levels of ubiquinol9/10 but not ubiquinone9/10 were significantly higher in the CNS, but not in plasma, of SOD1G93A mice, suggesting that CoQ redox system might participate in the mechanisms trying to counteract the pathology progression. Therefore, the very low increases of CoQ10 induced by oral treatments in CNS might be not sufficient to provide significant neuroprotection in SOD1G93A mice.

Introduction

Coenzyme Q (CoQ) is an amphipathic molecule structurally composed of a quinone ring synthesized from β-hydroxybenzoate and of a polyisoprenoid chain synthesized from acetil-CoA [1]. There are different forms of CoQ related to the length of polyisoprenoid chain: the predominant form in humans is CoQ9 which contains 10 isoprenoid units, whereas CoQ5 is the predominant form in rodents [2]. CoQ exists in three redox states: a fully oxidized form (ubiquinone), a fully reduced form (ubiquinol) and an intermediate semiquinone radical (CoQ·). The redox state is tissue-specific: for example, in mice ubiquinol is the predominant form in liver and skeletal muscle while ubiquinone is predominant in the brain [3]. Several factors, such as drugs, age, diet and pathologies affect total levels and redox state of CoQ [4,5,6,7] and their alterations may be regarded as markers of oxidative stress and mitochondrial dysfunctions [8].

Amyotrophic Lateral Sclerosis (ALS) is a severe neurodegenerative disorder in which the loss of motor neurons induces a progressive motor paralysis associated with dysphagia, dysarthria, respiratory failure and death within 3–5 years from the diagnosis. Analysis of plasma [9] and cerebrospinal fluid (CSF) [10] of sporadic ALS (sALS) patients have shown an increase of ubiquinone10 with a shift in the redox state of the coenzyme, interpreted as an index of oxidative stress.

Because of the substantial involvement of oxidative stress and mitochondrial impairment in this disease [11] different therapeutic approaches have been aimed towards counteracting these mechanisms. Among them, the use of CoQ10 has raised some interest for the treatment of ALS. In fact, on the basis of a modest but significant increase of survival observed in a mouse model of familial ALS (fALS) after chronic treatment with ubiquinone10 [12], a phase II trial was designed to investigate the effects of a chronic treatment with the same molecule (2700 mg daily) in patients with sALS. Unfortunately, the data collected after 9 months of treatment did not provide sufficient evidence of a benefit to justify a phase III trial [13].
The use of the reduced form ubiquinol, characterized by potent antioxidant properties, was difficult because it is readily oxidized in air. Recently, however, a new stabilized formulation of ubiquinol10 has been produced (Kaneka QH\textsuperscript{TM}) which overcomes this problem [14]. Notably, pharmacokinetic studies showed that ubiquinol10 has a better oral bioavailability than ubiquinone10, in humans [14], dogs and rats [15]. It may be expected that chronic treatment with this formulation results in higher CNS levels of humans [14], dogs and rats [15]. It may be expected that chronic treatment with this formulation results in higher CNS levels of humans [14], dogs and rats [15].

**CoQ10**, with superior neuroprotective properties. Consistently, treatment with this formulation results in higher CNS levels of humans [14], dogs and rats [15]. It may be expected that chronic treatment with this formulation results in higher CNS levels of humans [14], dogs and rats [15].

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model from striatal neurodegeneration induced by acute treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model for Parkinson’s disease [16].

**On this basis, in the present study we evaluated:** 1) the effects of a chronic oral administration with ubiquinol10 or ubiquinone10 on disease progression of transgenic SOD1\textsuperscript{G93A} mice, a model of \( \text{ALS} \) [11], 2) the endogenous levels of total, reduced and oxidized CoQ10 and CoQ10, in plasma, brain and lumbar spinal cord of transgenic SOD1\textsuperscript{G93A} mice at symptomatic stage.

Our data show that exogenous administration of ubiquinol10 or ubiquinone10 to SOD1\textsuperscript{G93A} mice, starting at the onset of symptoms, had no effect on the disease course. Since the basal levels of reduced CoQ10/10 are increased in the CNS of symptomatic SOD1\textsuperscript{G93A} mice, suggesting an attempt of equilibrium toward a protective antioxidant state, we think that the small increase of ubiquinol after the oral treatment is insufficient to exacerbate this effect.

**Materials and Methods**

**Animals and Treatments**

Transgenic SOD1\textsuperscript{G93A} and SOD1\textsuperscript{WT} mice on 129S2/SvHsd genetic background, and non-transgenic littermates were used in this study. The SOD1\textsuperscript{G93A} mouse line derives from the line originally obtained from Jackson Laboratories (B6SJL-TgNSOD1-SOD1G93A-1Gur) expressing approximately 20 copies of mutant human SOD1 with a Gly93Ala substitution (SOD1\textsuperscript{G93A}) or wild-type human SOD1 (SOD1\textsuperscript{WT}) and were maintained on 129S2/SvHsd background for more than 15 generations at Harlan Italy S.R.L., Bresso (MI), Italy [17]. We observed no differences between the males and females as regards the disease progression and survival, as reported in supporting figure 1 (Fig. S1). Therefore, to reduce at minimum the number of transgenic mice used, chronic treatments were carried out in female whereas the biochemical profile was examined in males.

All mice were maintained at a temperature of 21±1°C with a relative humidity 55±10% and 12 h of light. Food (standard pellets) and water were supplied ad libitum.

Ubiquinone10 (Ubidecarenone from Kaneka) and ubiquinol10 (Kaneka QH\textsuperscript{TM} from Kaneka) were administered by gavage after solubilization in sunflower seed oil (vehicle); preparation procedures were carried out at room temperature protecting from light. All the solutions were administered within four hours from preparation.

**Ethics statement.** Procedures involving animals and their care were conducted in conformity with the institutional guidelines of “Mario Negri” institute (IRFMN), that are in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circular No.3, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). All the experiments and the protocol proposed in the projects were reviewed and approved by the IRFMN Animal Care and Use Committee (IACUC), that includes members “ad hoc” for ethical issues. The mice were bred and maintained in a SPF environment. Animals with substantial motor impairment had food on the cage bottom and water bottles with long drinking spouts. The survival time was defined as the time when the animals were unable to right themselves within 10 s after being placed on either side. At this point, the animals were deeply anesthetized with Equithesin and then sacrificed by decapitation before proceeding to the dissection of tissues for biochemical analyses.

**Preliminary acute treatment in 129Sv mice.** Two preliminary pharmacokinetic studies were carried out in non-transgenic 129Sv female mice aging 9-11 weeks. For the time-course study, mice were treated orally with 200 mg/kg of ubiquinol10 or ubiquinone10 and killed at various times thereafter (30, 60, 180, 360 min and 24 hours after dosing, four mice/group). In a separate single-dose study, ubiquinone10 and ubiquinol10 were administered orally at various doses (200, 400, 800 and 1600 mg/kg) and the animals (4 mice/dose) were killed 3 hours after dosing. Four mice were treated with vehicle alone.

**Chronic treatment in 129Sv SOD1G93A mice and analysis of their motor dysfunction and survival.** Female 129Sv SOD1\textsuperscript{G93A} mice were treated by oral gavage with ubiquinone10 or ubiquinol10 (800mg/kg), or vehicle (n = 15 each group). The treatment started when the mice were 13 weeks old, which is the mean age that the growth of their body weight starts to differentiate from that of non-transgenic littermates and they show first alterations of hindlimbs abduction when raised by tail. This occurs just before the appearance of motor impairments (onset of motor deficit). Mice were treated daily until the end-stage of the disease, when they were sacrificed (see below).

Disease progression was assessed two times a week from the start of the experiment by an operator blinded to the treatment. The parameters included body weight, latency on rotating bar (rotarod) and paw grip endurance (PaGE) and were evaluated as previously described [17]. The motor symptoms onset was determined by the first impairment in grip strength or on rotarod performance for two consecutive time points. The mice were sacrificed when they were unable to right themselves within 10 seconds after being placed on both sides. This time point was considered as the end-stage of the disease and was used to estimate the survival time.

Statistical analysis of the data of body weight, grip strength and rotarod performance was carried out by Two-way ANOVA for repeated measures (time) and different groups (treatments), followed by post-hoc Bonferroni’s test to compare the effect of treatments in respect to vehicle at each time point. The analysis was applied at the time points when all animals for each group were still alive in order to maintain the number per group balanced. The age at onset and the survival length were statistically evaluated by the Log-rank test to compare probabilities.

**HPLC Analysis of CoQ10 and CoQ9 Levels**

Animals were sacrificed by decapitation. Blood was collected from the heart in heparinized tubes. After centrifugation at 5000 g for 10 min, plasma was collected and stored at -80°C. CNS tissues were removed immediately, blotted with paper to remove surface blood, quickly frozen in dry ice and stored at -80°C.

All solvents and reagents used were HPLC grade (Carlo Erba, Milan, Italy). Ubiquinone10, ubiquinone10, FeCl3, 1,4-benzoquinone and sodium perchlorate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Plasma levels of total CoQ10 and CoQ9 in treated SOD1G93A mice.** The method described by Mosca et al. [18] was used. Fifty µL of plasma were pre-incubated for 10 min at
Figure 1. Ubiquinol\textsubscript{10} chronic treatment has no effect on disease course in SOD\textsubscript{1}\textsuperscript{G93A} mice. Effect of oral treatment with 800 mg/kg/day ubiquinone\textsubscript{10}, ubiquinol\textsubscript{10} or vehicle, on motor dysfunction and disease progression of 129Sv SOD\textsubscript{1}\textsuperscript{G93A} mice (n = 15 mice per group). Treatment started at the age of 91 days (arrows) until the sacrifice (at the end-stage of the disease, when mice were unable to right themselves within 10 seconds after being placed on both sides). Treatment with ubiquinone\textsubscript{10} or ubiquinol\textsubscript{10} had no significant effects on body weight (A), on the latency of the rotarod test (B), on the latency of the PaGE test (C), and on the onset of motor deficit (D). Treatment with ubiquinol\textsubscript{10} also had no significant effects on survival (E). The table shows the mean ± SD of the onset of symptoms and life-span for each group.

| Treatment     | Onset of symptoms (days) | Life-span (days) |
|---------------|--------------------------|------------------|
| Vehicle      | 101 ± 7                  | 126 ± 10         |
| Ubiquinone\textsubscript{10} | 105 ± 5                  | 126 ± 7          |
| Ubiquinol\textsubscript{10}   | 102 ± 4                  | 123 ± 7          |
room temperature with 15 μL of 1,4-benzoquinone (2 mg/mL in ethanol), in order to allow a complete oxidation of ubiquinol9/10. The samples were then added to 235 μL of 1-propanol, vortexed and then centrifuged at 10000 g for 2 minutes. Two hundred μL of supernatant were then injected into the HPLC system with UV detection at 275 nm (Waters 1525 Binary Pump; Waters 2487 Dual λ Absorbance Detector; Waters mod. 717-plus autosampler). Separation was carried out using a Supelcosil LC 18-DB column (15×0.46 cm, i.d. 5 μm, Supelco Inc., Bellefonte, PA, USA), with a Supelcosil LC 18-DB Supelguard Cartridge (2×0.4 cm, i.d. 5 μm, Supelco Inc., Bellefonte, PA, USA) by isocratic elution with ethanol/methanol (65:35 v/v) at flow rate of 1 mL/min. The total run time was 19 min and the retention times for CoQ9 and CoQ10 were 7.1 and 8.9 min, respectively.

Total levels of CoQ9 and CoQ10 were quantified by reference to calibration curves with ubiquinol9 or ubiquinone9 run in parallel with each analytical session. Calibration curves were linear in the range 0.2–3.2 μg/mL for both coenzymes, with \( r^2 \) values >0.99.

CNS levels of total CoQ10 and CoQ9 in treated SOD1G93A mice. CoQ9 and CoQ10 were extracted from CNS tissues and measured by HPLC with UV detection at 275 nm, based on the method described by Kitano et al. [14], with slight modifications.

CNS tissues were homogenized in ultrapure water (1 g in 20 mL), aliquots of 0.15 mL were added to 1 mL of acetonitrile and the samples were centrifuged at 2500 rpm for 5 min; the supernatants were collected and 1 mL aliquots were added to 0.1 mL of 2% FeCl3 to allow the oxidation of ubiquinol to ubiquinones. After 20 minutes incubation at room temperature the extraction procedure started by adding 2 mL methanol, 0.5 mL purified water and 5 mL hexane, followed by 15 minutes shaking, centrifugation (1200 g, 5 min) and collection of the organic phase. This procedure was repeated twice and the combined extracts were evaporated to dryness, the residue was dissolved in 150 μL of water/methanol (20:80 v/v), transferred in Eppendorf tubes, centrifuged (2500 rpm, 30 sec) and 100 μL were injected into the chromatographic system (Waters 1525 Binary Pump; Waters 2487 Dual λ absorbance detector; Waters mod. 717-plus autosampler). Separation was carried out on a Kinetex 2.6 μm PFP 100 Å column (100×4.6 mm, Phenomenex Inc., Torrance, CA, USA), using gradient elution with water/methanol (20:80 v/v; solvent A) and acetonitrile/methanol (4:96 v/v; solvent B). During HPLC analysis the solvent gradient was programmed to operate at a flow rate of 1 mL/min for 25 minutes at the following gradient conditions: step 1 - from the initial condition of 100% solvent A to 100% solvent B over 16 min; step 2 - from 100% solvent B to 100% solvent A over 1 min. Retention times of CoQ9 and CoQ10 were 17.8 and 18.3 minutes, respectively.

Total levels of CoQ10 and CoQ9 were quantified by reference to calibration curves with ubiquinol9 or ubiquinone9 run in parallel with each analytical session. Calibration curves were linear in the range 4.0–16.0 μg/mL (CoQ9) and 10.0–60.0 μg/mL (CoQ10), with \( r^2 \) values >0.99.

CNS levels of ubiquinol9/10 and ubiquinone9/10. The method described by Takada et al. [19] was used with slight modifications. Tissues were homogenized (1 g in 10 mL of ultrapure water) and divided in two aliquots, one to measure total CoQ9/10 levels (aliquot A) and the other to measure ubiquinone9/10 only (aliquot B). 100 μL of aliquot A were added to 10 μL of ultrapure water and 60 μL of 1,4-benzoquinone (2 mg/mL in ethanol) to allow a complete oxidation of ubiquinol. In parallel, 100 μL of aliquot B were added to 10 μL of 10% Na2EDTA (to protect ubiquinol from oxidation), and 60 μL of ethanol. After 10 min, both aliquots were added to 750 μL of ethanol/hexane (2:5, v/v), shaken, and centrifuged at 4000g for 3 min (4°C). The supernatants were dried under nitrogen flow, reconstituted with 100 μL of ethanol and 50 μL were injected into a HPLC system (Waters 1525 Binary pump; Waters mod. 717-plus autosampler) coupled to a coulometric detector (ESA Coulombic 5100A with ESA analytical cell mod. 5011). The electrochemical analytical cell consisted of a series of two coulometric electrodes: the first one (−650mV) was for ubiquinone9/10 reduction and the second one (+400mV) was for detection of CoQ9/10 in the reduced form. The chromatographic column was a reverse phase Supelcosil LC-18 DB (15×0.46 cm, i.d. 3 μm; Supelco Inc., Bellefonte, PA, USA). The separation was carried out in isocratic operational mode with ethanol/methanol/HClO4 (700:300:1, v/v/v) +7% sodium perchlorate at 0.8 mL/min for 13 minutes. Retention times were 5.3 min (ubiquinol9), 6.3 min (ubiquinone9), 7.1 min (ubiquinol10) and 8.8 min (ubiquinone10).

Total levels of CoQ9/10 (aliquot A), and ubiquinone9/10 (aliquot B) were quantified by reference to calibration curves with ubiquinol9/10, run in parallel with each analytical session. Calibration curves were linear in the range 5–50 μg/g tissue (ubiquinol9) and 20–100 μg/g tissue (ubiquinone9), with \( r^2 \) values >0.99. Endogenous levels of ubiquinol9/10 were obtained as difference between total CoQ9/10 levels (determined in fully oxidized aliquot A) and ubiquinone9/10 levels (determined in aliquot B).

All the procedures were carried out rapidly, on ice, with protection from light, and in the presence of Na2EDTA, to limit the possibility that endogenous ubiquinol oxidizes to ubiquinone during the sample preparation. In these conditions, we verified that the storage of CNS homogenate at 4°C, for up to one hour, did not affect ubiquinol/total CoQ ratios (data not shown). We also note that the ubiquinol/total CoQ ratios detected in our study are very similar to published data [3,19,20]. Most importantly, we operated to ensure reliable comparisons between the different groups (SOD1G93A, SOD1WT and non-transgenic), always including matched samples in the same analytical session, with random processing.

Plasma levels of ubiquinol9 and ubiquinone9. The method described by Takada et al. [21] was used with slight modifications. Plasma samples were divided in two aliquots, one to measure total CoQ9 levels (aliquot A) and the other to measure ubiquinone9 only (aliquot B). 50 μL of aliquot A were added to 50 μL of ultrapure water and 25 μL of 1,4-benzoquinone (2 mg/mL ethanol), to allow a complete oxidation of ubiquinols. In parallel, 50 μL of aliquot B were added to 50 μL of 10% Na2EDTA (to protect ubiquinol from oxidation), and 25 μL of ethanol. After 10 min, both aliquots were added to 750 μL of ethanol/hexane (2:5, v/v), shaken, and centrifuged at 4000 g for 3 min (4°C). The supernatant was dried under nitrogen flow, reconstituted with 200 μL of H2O/2-propanol (1:5, v/v) and 150 μL were injected in a HPLC system as reported for CNS samples. The chromatographic separation was carried out in...
isocratic operational mode with ethanol/methanol/H₂O/HClO₄ (700:300:10:1, v/v/v/v) +7% sodium perchlorate at 1 mL/min for 15 minutes. Retention times for ubiquinol₉ and ubiquinone₉ were 6.2 and 8.6 minutes, respectively.

Total levels of CoQ₉ (aliquot A), and ubiquinone₉ (aliquot B) were quantified by reference to calibration curves with ubiquinone₉, run in parallel with each analytical session. Calibration curve was linear in the range 0.05–1.20 μg/mL, with r² values >0.99. Endogenous levels of ubiquinone₉ were obtained as difference between total CoQ₉ levels (determined in fully oxidized aliquot A) and ubiquinone₉ levels (determined in aliquot B).

All the procedures were carried out rapidly, on ice, with protection from light, and in the presence of Na₂EDTA. The ubiquinon/total CoQ₉ ratios detected in our study are very similar to published data [3,19,20]. Most importantly, we operated to ensure reliable comparisons between the different groups (SOD1G93A, SOD1 WT and NTg), always including matched samples in the same analytical session, with random processing.

Results

Mice Treatment with Ubiquinone₁₀ and Ubiquinol₁₀

Since available data indicated that the stabilized formulation of ubiquinol₁₀ has a better oral bioavailability than ubiquinone₁₀ in different animal species [14,15], preliminary studies were carried out to verify if this was true also in 129Sv mice. With this aim, plasmatic CoQ₁₀ levels were measured in wild-type mice treated acutely with both ubiquinol₁₀ and ubiquinone₁₀. Fig. S2A shows that, after oral treatment with 200 mg/kg, the area under the plasma concentration-time curve over the dosing interval (AUC₀–24h), was 1.5-fold higher for ubiquinol₁₀ (14.2 μg·h/mL) than ubiquinone₁₀ (9.8 μg·h/mL). Fig. S2B shows the plasma CoQ₁₀ levels in mice treated with different doses of the two compounds, three hours before sacrifice, confirming higher CoQ₁₀ levels in the plasma of ubiquinol₁₀-treated mice than in the plasma of ubiquinone₁₀-treated mice, except at the highest dose tested (1600 mg/kg). In particular, treatment with 800 mg/kg ubiquinol₁₀ resulted in CoQ₁₀ levels double than those found after treatment with the same dose of ubiquinone₁₀ (1.5±0.2 and 0.8±0.4 μg/mL, respectively, p<0.05 Student’s t test).

Brain CoQ₁₀ levels (13 μg/g tissue on average) were not affected by acute treatment with either ubiquinol₁₀ or ubiquinone₁₀, at any time-point or any concentration (data not shown).

Chronic Treatment with Ubiquinone₁₀ and Ubiquinol₁₀ in SOD1G93A Mice

Effect on motor dysfunction, body weight and survival of SOD1G93A mice. Fig. 1 shows that chronic oral treatment of SOD1G93A mice with 800 mg/kg/day ubiquinone₁₀ or ubiquinol₁₀, started at the onset of disease symptoms, did not modify the motor impairment progression assessed by rotarod performance (fig. 1B) and grip strength (fig. 1C), in respect to the vehicle-treated mice. The progressive loss of body weight (fig. 1A) was also unaffected by both treatments. The lack of effect was clearly evidenced by the overlapping Kaplan-Meyer curves and the similar mean for motor symptom onset (fig. 1D) and survival length (fig. 1E).

Total CoQ₁₀ and CoQ₉ plasma levels. Table 1 reports the plasma concentrations of total CoQ₁₀ and CoQ₉ after chronic administration of ubiquinone₁₀ and ubiquinol₁₀ in the same SOD1G93A mice used for behavioral tests. The last dose of both formulations was administered two hours before sacrifice.

As expected, no detectable levels of CoQ₉, were found in mice chronically treated with vehicle (<0.20 μg/mL), whereas levels of

| Table 1. Levels of CoQ₁₀/CoQ₉ in the plasma of SOD1G93A mice treated chronically with ubiquinone₁₀ or ubiquinol₁₀.
| Chronic treatment | Total CoQ₁₀ (μg/mL) | Total CoQ₉ (μg/mL) |
|-------------------|---------------------|---------------------|
| Vehicle           | <0.20 (5)           | 1.63±0.40 (5)       |
| Ubiquinol₁₀       | 3.40±0.22 (13)      | 1.25±0.12 (13)      |
| Ubiquinone₁₀      | 3.03±0.26 (9)       | 1.19±0.20 (9)       |

Female 129Sv SOD1G93A mice were treated orally with 800 mg/kg of ubiquinol₁₀ or ubiquinone₁₀, or vehicle (sunflower seed oil), once a day, starting from age of 91 days until the last stage of the disease (age of 125 days, on average) when they were sacrificed, two hours after the last treatment. Values are means±SEM of (n) mice.

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Levels of Reduced and Oxidized Forms of CoQ₁₀ and CoQ₉ in SOD1G93A Mice

Basal levels of reduced and oxidized forms of both coenzymes were measured in male 129Sv SOD1G93A mice at the symptomatic stage of the disease (16 weeks), and in age-matched 129Sv SOD1 WT and non-transgenic 129Sv mice. In figures 3, 4, and 5, the levels in transgenic mice are shown as a percentage of levels in non-transgenic mice (absolute values in table S1), in order to highlight the effect of the over-expression of the human SOD1 transgene, and any difference related to the G93A mutation.

Effect on endogenous CoQ₁₀/CoQ₉. Basal levels of reduced and oxidized forms of both coenzymes were measured in male 129Sv SOD1G93A mice at the symptomatic stage of the disease (16 weeks), and in age-matched 129Sv SOD1 WT and non-transgenic 129Sv mice. In figures 3, 4, and 5, the levels in transgenic mice are shown as a percentage of levels in non-transgenic mice (absolute values in table S1), in order to highlight the effect of the over-expression of the human SOD1 transgene, and any difference related to the G93A mutation.

Brain. Figure 3 shows that over-expression of human SOD1WT enzyme does not alter the endogenous levels of oxidized and reduced CoQ₁₀/CoQ₉ in comparison with non-transgenic mice. Over-expression of the mutated protein (SOD1G93A) was instead associated with a significant increase (10–25%) of endogenous total levels of both CoQ₁₀ and CoQ₉, in comparison with age-matched SOD1 WT mice (Fig. 3A,D). This effect was associated with a significantly increased (30–40%) of the reduced forms only (Fig. 3B,E), whereas no significant changes were observed for the oxidized forms (Fig. 3C,F).

Lumbar spinal cord. Endogenous levels of CoQ₁₀ and CoQ₉ in lumbar spinal cord of 16 weeks-old SOD1 WT mice...
were significantly lower (−20%), than in age-matched non-transgenic mice, a difference due to a decrease of the oxidized forms. In line with the results found in the brain, basal levels of CoQ_{9} and CoQ_{10} in SOD1^{G93A} were significantly higher (16–20%) than corresponding levels in age-matched SOD1^{WT} mice (Fig. 4A,D) and this increase was associated with a significant increase (36–60%) of the reduced forms only (Fig. 4B,E), whereas no or much lower changes where observed for the oxidized forms (Fig. 4C,F).

**Plasma.** Figure 5 shows that over-expression of human SOD1^{WT} enzyme does not alter the endogenous plasma levels of total CoQ_{9}, ubiquinol_{9} and ubiquinone_{9}, in comparison with non-transgenic mice. Moreover, no significant differences were observed between SOD1^{WT} and SOD1^{G93A} mice. CoQ_{10} levels were under the limit of detection.

**Discussion**

Oxidative stress and mitochondrial impairment are the most prominent pathogenic mechanisms of ALS. The CoQ complex is a key component of the mitochondrial function, and its reduced form ubiquinol is fundamental for the antioxidant response, by direct interaction with radical species or by regenerating antioxidant agents [22].

It had been previously reported that chronic treatment with ubiquinone_{10} had a significant, although slight, effect on the lifespan of a mouse model of familial ALS [12]. Regrettably, this finding was not confirmed in a phase II trial in humans [13]. One of the potential reasons underlying these results regarded the poor pharmacokinetic profile of ubiquinone_{10}. In fact, a stabilized formulation of ubiquinol_{10}, with a better bioavailability in respect to the oxidized form [14,15] showed a better neuroprotective activity on MPTP-induced degeneration in mice [16].

On these basis we chronically treated a mouse model of familial ALS (SOD1^{G93A}) with ubiquinol_{10} and ubiquinone_{10} in parallel, to increase CNS CoQ_{10} levels with the aim to obtain beneficial effect on the disease course.

Chronic treatment with both formulation resulted in a significant increase of plasmatic CoQ_{10} levels (from <0.2 to ≥3 μg/mL) that was associated to a very mild increase (10% on average) of the CoQ_{10} levels in CNS tissues (brain and spinal cord). Despite the better pharmacokinetic profile of the new formulation of ubiquinol_{10}, we found no differences in the CoQ_{10} levels after chronic treatment with both formulations.

No effect of the chronic treatments with either ubiquinone_{10} or ubiquinol_{10} (both at 800 mg/kg/day) was observed on the disease progression or the life-span of SOD1^{G93A} mice. This finding is at variance with previous data showing a 8% increase of survival after chronic treatment with a lower dose (200 mg/kg) of ubiquinone_{10} [12]. Unfortunately, that study did not report the levels of CoQ_{10} reached in the brain of SOD1^{G93A} mice after the chronic treatment. Two possible reasons may account for the discrepancy: 1) the strain of SOD1^{G93A} mice is different (C57BL6/SJL in [12] and 129Sv in our study), although the disease onset and progression is very similar in the two strains; and 2) in our study, the treatment started at the appearance of the first symptoms of disease (13 weeks of age), while Matthews et al. [12] started the treatment when mice were 7-weeks old, well before the onset of symptoms. According to the recent guidelines for preclinical animal research in ALS [23], the poor impact of preclinical studies on clinical practice is in part due to the fact that most of the pharmacological interventions are intended to be disease-preventive rather than being disease-modifying once the...
symptoms are evident. In this respect, since we start treatment when the mice exhibited the first disease symptoms, our data are in line with the lack of effect found in phase II clinical trials with ubiquinone10 [13].

The observation that the chronic treatments with ubiquinone10 and ubiquinol10 resulted in a very small increase of CoQ10 levels in the CNS of SOD1G93A mice (≤10%) may provide an explanation for the lack of effect on disease progression and life span. In fact, it was previously shown that the neuroprotection observed after 1-week pretreatment of non-transgenic rats with 200 mg/kg ubiquinone10, i.e. prevention of the neurotoxicity induced by 3-nitropropionic acid, was associated with a ~30% increase of brain CoQ10 [12]. All these data may suggest that an high enough increase of CNS concentrations of CoQ10 over basal levels is required to support neuroprotection and that the increase associated with our treatments in 129Sv SOD1G93A mice, started at the time of the onset of symptoms, was too low to provide an efficient neuroprotection.

To better understand this point, in the second part of the study we measured the endogenous levels of CoQ10 and CoQ10 in CNS tissues and plasma of symptomatic SOD1G93A mice, in comparison with age- and sex-matched SOD1WT mice, in 16 week-old male 129Sv SOD1WT and SOD1G93A mice are shown as percentage of the levels measured in age-matched 129Sv non-transgenic mice (absolute values in table S1). Panels A, D show the total CoQs levels, whereas panels B, E and panels C, F show the reduced and oxidized forms, respectively. Each value is the mean±SEM of 4–7 mice. * P<0.05, ** P<0.01 compared to SOD1WT mice (Student’s T-test). doi:10.1371/journal.pone.0069540.g003

Coenzyme-Q in a Familial ALS Mouse Model

Figure 3. Increased levels of ubiquinol9/10 in the brain of symptomatic SOD1G93A mice. Endogenous brain levels of CoQ9 (A–C) and CoQ10 (D–F) in 16 week-old male 129Sv SOD1WT and SOD1G93A mice are shown as percentage of the levels measured in age-matched 129Sv non-transgenic mouse (absolute values in table S1). Panels A, D show the total CoQs levels, whereas panels B, E and panels C, F show the reduced and oxidized forms, respectively. Each value is the mean±SEM of 4–7 mice. * P<0.05, ** P<0.01 compared to SOD1WT mice (Student’s T-test).

An increase of ubiquinone10 with shift in the redox state of the coenzyme has been reported in the plasma [9] and CSF [10] of sALS patients and was suggested as a marker of the underlying oxidative stress. Our data, showing that the increase of ubiquinol9/10 in the CNS of SOD1G93A mice was not paralleled by changes in plasma levels, indicate that the peripheral and central compartments react differently to the oxidative stress induced by mutant SOD1, and suggest that plasmatic CoQ levels do not represent
sensitive markers for the changes associated to oxidative stress in the CNS. This is in line with the observations of Kontush et al. [28] who could not detect changes of ubiquinol_{10} in the plasma of patients with Alzheimer’s disease (AD), another neurodegenerative condition associated with increased free radical production in the

Figure 4. Increased levels of ubiquinol_{9/10} in the lumbar spinal cord of symptomatic SOD1^{G93A} mice. Endogenous levels of CoQ_{9} (A–C) and CoQ_{10} (D–F) in the lumbar spinal cord of male 16 week-old 129Sv SOD1^{WT} and SOD1^{G93A} mice are shown as percentage of the levels measured in age-matched 129Sv non-transgenic mice (absolute values in table S1). Panels A, D show the total CoQs levels, whereas panels B, E and panels C, F show the reduced and oxidized forms, respectively. Each value is mean±SEM of 4–7 mice. * P<0.05, ** P<0.01 compared to SOD1^{WT} mice; † P<0.05 compared to 129Sv NTg mice (Student’s T-test).

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Figure 5. Unchanged plasma levels of ubiquinol_{9} in symptomatic SOD1^{G93A} mice. Levels of CoQ_{9} (A), ubiquinol_{9} (B) and ubiquinone_{9} (C) in plasma of male 16 week-old 129Sv SOD1^{WT} and SOD1^{G93A} mice are shown as percentage of the levels measured in age-matched 129Sv non-transgenic mice (table S1). Each value is mean±SEM of 4–7 mice.

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CNS, even if higher levels of total CoQ were reported in post mortem brain tissues from AD patients [29].

In summary, our data show that chronic treatment of SOD1G93A mice with ubiquinone10 or ubiquinol10 had no effect on the disease progression. This lack of efficacy may be due to the negligible changes of CoQ9/10 levels in the brain and spinal cord of treated mice, which in turn may be the consequence of a poor CNS availability of the compounds and/or the fact that endogenous CoQ9/10 levels are already significantly increased in SOD1G93A mice.

Supporting Information

Figure S1 No gender effect on disease progression in SOD1G93A mice. Disease progression and survival length in males (gray) and females (black) SOD1G93A mice on 129Sv background. Each point represent the mean of n = 10 male and n = 13 female. Table reports the mean and standard deviations of symptoms onset, life-span and disease duration for each group.

(TIF)

Figure S2 Ubiquinol10 shows a better oral pharmacokinetic profile than ubiquinone10. Plasma CoQ10 levels in female 129Sv non-transgenic mice treated orally with ubiquinone10 (grey) or ubiquinol10 (black). (A) Time-course after acute treatment with 200 mg/kg. (B) Dose-dependency after acute treatment with 200, 400, 800 and 1600 mg/kg and sacrifice 3 hours later. Each value is the mean±SEM of 4 mice. Plasma basal levels of CoQ10 were under the limit of quantification (<0.2 µg/mL).

(TIF)

Table S1 Endogenous levels of CoQ9/10 in 129Sv NTg mice. Endogenous levels (µg/g tissue or ng/mL) of total CoQ9/10, ubiquinol9/10 and ubiquinone9/10 in brain, lumbar spinal cord (L-SpC) and plasma of 16 week-old male 129Sv non-transgenic mice. Values are expressed as means±SEM of (n) mice.

(DOC)

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Author Contributions

Conceived and designed the experiments: JL SC EB CB MG. Performed the experiments: JM MM S. Papa MT GG S. Pozzi. Analyzed the data: JL MM MT VB CB MG. Wrote the paper: JL CB MG.

References

1. Tran UC, Clarke CF (2007) Endogenous synthesis of coenzyme Q in eukaryotes. Mitochondrion 7: 587–602.

2. Lass A, Agarwal S, Sohal RS (1997) Mitochondrial ubiquinone homologues, superoxide radical generation, and longevity in different mammalian species. J Biol Chem 272: 19199–19204.

3. Tang PH, Miles MV, Miles I, Quinlan J, Wong B, et al. (2004) Measurement of reduced and oxidized coenzyme Q and coenzyme Q10 levels in mouse tissues by HPLC with coulometric detection. Clin Chim Acta 341: 173–184.

4. Littarru GP, Langgjøen P (2007) Coenzyme Q10 and statins: biochemical and clinical implications. Mitochondrion 7 Suppl: S160–174.

5. Pravst I, Zmitek N, Zmitek J (2010) Coenzyme Q10 contents in foods and fortification strategies. Crit Rev Food Sci Nutr 50: 269–280.

6. Huetras JR, Battino M, Lenzaj G, Matias FJ (1991) Changes in mitochondrial and microsomal rat liver coenzyme Q9 and Q10 content induced by dietary fat and endogenous lipid peroxidation. FEBS Lett 287: 89–92.

7. Eilhuf C, Soderberg M, Kristensen K (1994) Isoprenoids in aging and neurodegeneration. Neurochem Int 25: 35–38.

8. Lagunidj J, Ubbink JR, van der Velden WJ (1996) Measurement of the ratio between the reduced and oxidized forms of coenzyme Q10 in human plasma as a possible marker of oxidative stress. J Lipid Res 37: 67–75.

9. Suhana N, Tanaka M, Suzuki Y, Tanino Y, Okamoto K, et al. (2005) An increase of oxidized coenzyme Q10 occurs in the plasma of sporadic ALS patients. J Neurol Sci 228: 49–53.

10. Murata T, Otsubo C, Terayama Y (2008) Increased mitochondrial oxidative damage in patients with sporadic amyotrophic lateral sclerosis. J Neurol Sci 267: 60–65.

11. Bendotti C, Carri MT (2004) Lessons from models of SOD1-linked familial ALS. Trends Mol Med 10: 393–400.

12. Matthews RT, Yang L, Browne S, Baik M, Beal MF (1998) Coenzyme Q10 administration increases brain mitochondrial concentrations and exerts neuroprotective effects. Proc Natl Acad Sci U S A 95: 8892–8897.

13. Kaufmann P, Thompson JL, Levy G, Buchsbaum R, Shefner J, et al. (2009) Phase II trial of CoQ10 for ALS finds insufficient evidence to justify phase III. Ann Neurol 66: 235–244.

14. Mosca F, Fattoni D, Bompard S, Littarru GP (2002) Assay of coenzyme Q10 in plasma by a single dilution step. Anal Biochem 305: 49–54.

15. Takada M, Ikemoto S, Yuzuriha T, Katayama K (1984) Simultaneous determination of reduced and oxidized ubiquinones. Methods Enzymol 105: 147–155.

16. Polda M, Weber C, Traber MG, Packer L (1996) Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. J Lipid Res 37: 893–901.

17. Takada M, Ikemoto S, Yuzuriha T, Katayama K (1982) Studies on reduced and oxidized coenzyme Q (ubiquinone): II. The determination of oxidation-reduction levels of coenzyme Q in mitochondria, microsomes and plasma by high-performance liquid chromatography. Biochim Biophys Acta 769: 308–314.

18. Benstetter M, Brismar K, Dallner G (2007) The antioxidant role of coenzyme Q. Mitochondrion 7 Suppl: S41–50.

19. Laidoloph AC, Bendotti C, Blangrand E, Chio A, Greensmith L, et al. (2010) Guidelines for preclinical animal research in ALS/MND: A consensus meeting. Amyotroph Lateral Scler 11: 38–45.

20. Poderoso JI (2009) The formation of peroxynitrite in the applied physiology of mitochondrial nitric oxide. Arch Biochim Biophys 404: 214–220.

21. Schopfer F, Raabo N, Carreras MC, Alvarez R, Kadi R, et al. (2000) Oxidation of ubiquinol by peroxynitrite: implications for protection of mitochondria against nitrosative damage. Biochem Biophys 349: 33–42.

22. Caso F, Basso M, Massipian T, Gianazza E, Cherini C, et al. (2005) Protein nitration in a mouse model of familial amyotrophic lateral sclerosis: possible multifunctional role in the pathogenesis. J Biol Chem 280: 16295–16304.

23. Basso M, Samengo G, Nardo G, Massipian T, D’Alessandro G, et al. (2009) Protein nitration increases brain mitochondrial concentration in SOD1 mutant mice. Amyotroph Lateral Scler 10: 221–229.

24. Batterman S, Soderberg M, Kristensen K (2000) Oxidation of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. J Lipid Res 37: 893–901.

25. Pizzasegola C, Caron I, Daleno C, Ronchi A, Minoia C, et al. (2009) Treatment with lithium carbonate does not improve disease progression in two different strains of SOD1 mutant mice. Amyotroph Lateral Scler 10: 221–229.

26. Mosca F, Fattoni D, Bompard S, Littarru GP (2002) Assay of coenzyme Q10 in plasma by a single dilution step. Anal Biochem 305: 49–54.

27. Bendotti C, Brismar K, Dallner G (2007) The antioxidant role of coenzyme Q. Mitochondrion 7 Suppl: S41–50.

28. Laidoloph AC, Bendotti C, Blangrand E, Chio A, Greensmith L, et al. (2010) Guidelines for preclinical animal research in ALS/MND: A consensus meeting. Amyotroph Lateral Scler 11: 38–45.

29. Poderoso JI (2009) The formation of peroxynitrite in the applied physiology of mitochondrial nitric oxide. Arch Biochim Biophys 404: 214–220.

30. Schopfer F, Raabo N, Carreras MC, Alvarez R, Kadi R, et al. (2000) Oxidation of ubiquinol by peroxynitrite: implications for protection of mitochondria against nitrosative damage. Biochem Biophys 349: 33–42.

31. Caso F, Basso M, Massipian T, Gianazza E, Cherini C, et al. (2005) Protein nitration in a mouse model of familial amyotrophic lateral sclerosis: possible multifunctional role in the pathogenesis. J Biol Chem 280: 16295–16304.

32. Basso M, Samengo G, Nardo G, Massipian T, D’Alessandro G, et al. (2009) Protein nitration increases brain mitochondrial concentration in SOD1 mutant mice. Amyotroph Lateral Scler 10: 221–229.

33. Batterman S, Soderberg M, Kristensen K (2000) Oxidation of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. J Lipid Res 37: 893–901.

34. Pizzasegola C, Caron I, Daleno C, Ronchi A, Minoia C, et al. (2009) Treatment with lithium carbonate does not improve disease progression in two different strains of SOD1 mutant mice. Amyotroph Lateral Scler 10: 221–229.

35. Mosca F, Fattoni D, Bompard S, Littarru GP (2002) Assay of coenzyme Q10 in plasma by a single dilution step. Anal Biochem 305: 49–54.

36. Bendotti C, Brismar K, Dallner G (2007) The antioxidant role of coenzyme Q. Mitochondrion 7 Suppl: S41–50.