

Dose ranging and efficacy study of high-dose coenzyme Q₁₀ formulations in Huntington's disease mice

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Abstract

There is substantial evidence that a bioenergetic defect may play a role in the pathogenesis of Huntington's Disease (HD). A potential therapy for remediating defective energy metabolism is the mitochondrial cofactor, coenzyme Q₁₀ (CoQ₁₀). We have reported that CoQ₁₀ is neuroprotective in the R6/2 transgenic mouse model of HD. Based upon the encouraging results of the CARE-HD trial and recent evidence that high-dose CoQ₁₀ slows the progressive functional decline in Parkinson's disease, we performed a dose ranging study administering high levels of CoQ₁₀ from two commercial sources in R6/2 mice to determine enhanced efficacy. High dose CoQ₁₀ significantly extended survival in R6/2 mice, the degree of which was dose- and source-dependent. CoQ₁₀ resulted in a marked improvement in motor performance and grip strength, with a reduction in weight loss, brain atrophy, and huntingtin inclusions in treated R6/2 mice. Brain levels of CoQ₁₀ and CoQ₉ were significantly lower in R6/2 mice, in comparison to wild type littermate control mice. Oral administration of CoQ₁₀ elevated CoQ₁₀ plasma levels and significantly increased brain levels of CoQ₉, CoQ₁₀, and ATP in R6/2 mice, while reducing 8-hydroxy-2-deoxyguanosine concentrations, a marker of oxidative damage. We demonstrate that high-dose administration of CoQ₁₀ exerts a greater therapeutic benefit in a dose dependent manner in R6/2 mice than previously reported and suggest that clinical trials using high dose CoQ₁₀ in HD patients are warranted.

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1. Introduction

Huntington's disease (HD) is a progressive and fatal neurological disorder caused by an expanded CAG repeat in the gene coding for the protein huntingtin. Despite great progress, a direct causative pathway from the HD gene mutation

to neuronal dysfunction and death has not yet been established. It has been postulated, however, that the abnormal aggregation of the mutant huntingtin protein may cause toxic effects in neurons, leading to a cascade of pathogenic mechanisms. There is considerable evidence that a secondary consequence of the gene defect may be impaired energy metabolism via mitochondrial dysfunction [1]. Indeed, N-terminal huntingtin fragments may directly impair mitochondrial function, leading to increased oxidative damage, as mitochondria are a major source of free radicals in the cell [1]. Bioenergetic dysfunction, therefore, may play a role in cell death in HD. No clinically proven treatment for HD currently exists and, as such, therapy that buffers intracellular energy levels and reduces oxidative

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stress may ameliorate the neurodegenerative process. Experimental evidence in animal models of HD suggests that such a strategy may be a fruitful avenue in the treatment of HD patients [2–5].

One such compound is coenzyme Q₁₀ (CoQ₁₀), an important cofactor in the electron transport chain that also acts as an antioxidant. Although experimental evidence shows that CoQ₁₀ is efficacious in animal models of HD and has potential as a therapeutic in HD patients [3,6], it remains unclear whether optimal CoQ₁₀ dosing has been determined. It may well be that higher doses of CoQ₁₀ are necessary to significantly slow the disease process in HD patients. A double-blind, randomized, controlled study trial in Parkinson's disease patients, using CoQ₁₀ at 1200 mg/day, slowed the rate of deterioration in the UPDRS score [7]. Follow up studies in both Parkinson's disease and amyotrophic lateral sclerosis patients have demonstrated safe and tolerable doses up to 3000 mg/day [8,9]. While this evidence suggests that higher doses of CoQ₁₀ may provide greater efficacy in HD patients, there are significant differences amongst commercial preparations, each with its own bioavailability characteristics [10,11].

Recent findings suggest that the R6/2 HD model exhibits a progressive HD-like phenotype that more closely corresponds to human HD than previously believed [12]. There is a clear longitudinal progression of disease along with neuronal death. Recapitulation of the features of HD makes the R6/2 mice highly suitable for pre-clinical therapeutic trials for HD [5,13]. Success using this model has led to a number of ongoing and planned human clinical trials in HD patients [14]. As such, we performed a dose ranging study administering high levels of CoQ₁₀ from two commercial sources in R6/2 mice to determine any enhanced beneficial effects. A preliminary report of our findings has been published [15].

2. Methods and materials

2.1. Animals

Male transgenic HD (R6/2) mice were obtained from an established colony at the Bedford VA Medical Center and were bred with B6CBA females from Jackson Laboratory (Bar Harbor, ME). The offspring were genotyped using a PCR assay on tail DNA. Cohort homogeneity is essential in testing potential therapies in murine models of disease. Minimizing measurement variability increases the power to detect differences. We have standardized criteria to ensure homogeneity of the experimental mice and the cohorts within the testing groups [13]. Mice were randomized from 33 litters all within 4 days of the same age from the same 'f' generation. Any mice that had altered base-paired banding beyond 550 bp and less than 500 bp as identified from PCR analysis were excluded from the study, since increased CAG repeats beyond 148–153 with greater than 550 base pairs on PCR gels result in increased variability in disease severity in the R6/2 mice [12]. Body weights were taken at 20 days and mice were equally distributed according to weight and parentage within each cohort ($n=10$). Mice under 8 g at 20 days were excluded from the experiments. Since others and we have not observed gender differences in survival in the R6/2 transgenic HD mouse model, female mice were used in the experimental paradigms. The mice were housed five in each cage under standard conditions with ad libitum access to food and water. Mice were identified by a randomly assigned code so that the studies were performed blind as to the genetic identity of the mice. The mice were handled under the same conditions by one investigator blinded (by the preclinical technical staff) to the genotype of the mice and the type of diet administered. Each of the clinical/behavioral outcome

assessments was performed on the same day of the week at the same time of day. We do not apply environmental enrichment in our facility, as this is considered a therapeutic treatment that will confound the mouse trials. In our hands, environmental enrichment results in greater heterogeneity of the clinical and neuropathological phenotype, reducing the power to detect differences. All of the experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by both the Veterans Administration and Boston University Animal Care Committees.

2.2. Therapeutic treatment

At 28 days of age, groups of 10 female R6/2 mice and littermate wild-type control mice were placed on an unsupplemented diet and diets supplemented with Chemco CoQ₁₀ formulation at 1000, 5000, 10000, or 20000 mg/kg/day (Chemco Industries Inc, Los Angeles, CA) ($n=60$) or with Tishcon CoQ₁₀ formulation at 400, 1000, and 2000 mg/kg/day (Tishcon Corp., Westbury, NY) ($n=50$). The Tishcon compound is a highly bioavailable formula of CoQ₁₀ and was provided as HydroQsorb[®] powder. The diets were made into pelleted mouse chow (Research Diets, New Brunswick, NJ). Chow was analyzed every 2 weeks to ensure that the levels of CoQ₁₀ in each of the treatment chows remained the same. In order to ensure that CoQ₁₀ was present in the treated mice, CoQ₁₀ doses were confirmed in treated mice by HPLC analysis of serum (see methods below). There was a dose-dependent increase in serum levels of CoQ₁₀ in R6/2 treated mice consistent with the CoQ₁₀ chow doses, confirming that the mice were receiving CoQ₁₀ (1000 mg/kg treated R6/2 mice: 0.133 μ M/ml; 5000 mg/kg treated R6/2 mice: 0.149 μ M/ml; 10000 mg/kg treated R6/2 mice: 0.152 μ M/ml; 20000 mg/kg treated R6/2 mice: 0.191 μ M/ml, Chemco formulation). In addition, a positive therapy control group of R6/2 mice was administered cystamine (225 mg/kg/day) in the drinking water. Cystamine is a useful positive-control therapeutic since it has been consistently verified to be efficacious in multiple different laboratories investigating HD [16–20].

2.3. Clinical assessment: motor performance, body weight, grip strength, and survival

Motor performance and body weight was assessed weekly from 28 to 63 days of age and twice weekly from 63 to 90 days of age in the R6/2 and littermate control mice. The mice were given two training sessions to acclimate them to the rotarod apparatus (Columbus Instruments, Columbus, OH). During testing the mice were placed on a rod rotated at a constant speed of 16 rpm. Each mouse had three separate trials at 60 s each. The three results were averaged and recorded. Fore- and hind-limb strength was measured weekly using a grip strength meter (Columbus Instruments, Columbus, OH) [12]. Briefly, mice were held by the scruff of their back and drawn along in a straight line along the sensor to allow the mice to grasp the fore-limb pull bar assembly, recording the force applied by the fore-limbs of the mouse in kilograms force. In a continued sweep, the mouse was drawn along until the hind-limbs grasped and released the hind-limb assembly, again recording the force applied by the hind-limbs of the mouse. Fore-limb and hind-limb strength was measured by the same investigator (KMS) 3 times and averaged during each weekly assessment. It is critically vital that a single investigator perform the grip strength measurements due to interrater differences in tensile strength. R6/2 mice were assessed for morbidity and mortality twice daily, mid morning and late afternoon. Motor performance and ability to feed were closely monitored and used as the basis for determining when to euthanize the mice. The criterion for euthanization was the point in time when the R6/2 mice were unable to right themselves within 30 s after being placed on their back. A limited number of deaths occurred overnight and were recorded the following morning. Two independent observers confirmed the criterion for euthanization (RJF and KMS).

2.4. Neuropathological evaluation

At 28 days of age groups ($n=10$) of R6/2 and littermate wild-type control mice were placed on either an unsupplemented diet or a diet supplemented with the Chemco CoQ₁₀ formulation at 5000 mg/kg. At 90 days of age, all animals from each group were deeply anesthetized and then transcardially perfused with

2% buffered paraformaldehyde (100 ml), with care to avoid the introduction of any perfusion artifact. It has been our experience that poor perfusion or variance in perfusion pressure may result in significant differences in gross and microscopic anatomy. Brains were cryoprotected and serially sectioned (50 μ m). Serial cut mouse tissue sections were stained for Nissl substance using cresyl violet and immunostained for mutant Huntingtin. Sections were rinsed in tris-buffered saline with Tween 20 (TBS-T) containing 10% normal goat serum for 1 h. Sections were then incubated overnight in TBS-T with 0.1% sodium azide and mouse anti-huntingtin (1:500, Chemicon, Temecula, CA). Sections were rinsed three times in TBS-T, followed by a 2–3 h incubation in TBS-T containing a goat anti-mouse peroxidase-conjugated secondary antibody to detect htt. Sections were then rinsed three times in TBS-T. Antibody complexes were visualized using diaminobenzidine. Preabsorption with excess target protein, or omission of either primary or secondary antibody, was used to demonstrate antibody specificity and background generated from the detection assay. Tissue sections were examined using a Nikon Eclipse E800 microscope with a Spot RT digital camera.

2.5. Stereology/quantitation

Serial-cut coronal tissue-sections beginning from the most rostral segment of the neostriatum to the level of the anterior commissure (Interaural 5.34 mm/Bregma 1.54 mm to Interaural 3.7 mm/Bregma -0.10 mm), were used for huntingtin aggregate analysis. Unbiased stereological counts of huntingtin-positive aggregates (≥ 1.0 μ m) were obtained from the neostriatum in 10 mice each from CoQ₁₀-treated (5000 mg/kg Chemco formulation) and untreated R6/2 mice at 90 days using NeuroLucida Stereo Investigator software (MicroBrightfield, Colchester, VT). The total areas of the rostral neostriatum were defined in serial sections in which counting frames were randomly sampled. The optical disector method was employed estimating the number of huntingtin-positive aggregates. Striatal neuron areas were analyzed by microscopic videocapture using a Windows-based image analysis system for area measurement (Optimas, Bioscan Incorporated, Edmonds, WA). The software automatically identifies and measures profiles. All computer identified cell profiles were manually verified as neurons and exported to Microsoft Excel.

2.6. HPLC Analysis of CoQ₉ and CoQ₁₀

At 42 days of age, groups of 8 R6/2 mice were administered either 5000 mg/kg/day CoQ₁₀ (Chemco formulation) or 1000 mg/kg/day (Tishcon formulation) or unsupplemented chow for 30 days, along with age-matched littermate wild-type mice ($n=32$). Maximal serum concentrations are reached by 1 month and plateau with continuous daily dosing [21]. Mice were euthanized at 73 days of age by decapitation. Blood was collected and was allowed to clot at 4 °C and centrifuged. Serum was removed, placed in eppendorf tubes and flash frozen in liquid nitrogen at -80 °C. Brains were immediately removed and divided along the longitudinal sagittal sulcus and further dissected in the coronal plane at the optic chiasm. Each quartered brain area was immediately flash frozen at -80 °C. From decapitation to freezing of brain tissue specimens took no more than 70 s with a team of four investigators. Our experience has been that longer dissection times result in increasing variability in the data that precludes significance. Analysis of the samples began immediately after the dissection procedure. The two rostral areas contained the frontal lobe and neostriatum. The left frontal lobe of the brain was used for CoQ₉, the predominant ubiquinone in mice, and CoQ₁₀ measurements. Extraction took place in cold (4 °C) 2-propanol, sonicated for 3 \times 10 s cycle and centrifuged for 10-min at 12K rpm at 4 °C. CoQ₉ and CoQ₁₀, and analyzed using liquid chromatography with an ESA Coularray (LCEC detection, cell potentials: mV 700, -1000 , -1000 , and 500 (ESA, Chelmsford, MA). Samples were kept at 4 °C on an auto sampler. An ESA MD-150 column was kept at 37 °C with a flow rate of 0.8 ml/min for 30 min, using a mobile phase of 78% methanol, 20% 1-propanol, and 2% ammonium acetate pH 4.4. CoQ₆ was used as an internal standard.

2.7. ATP analysis

The right frontal lobe of the brain from the HPLC cohorts was used for ATP analysis. Four hundred μ l of lysis buffer was added to each tissue sample,

followed by homogenization with the Fisher PowerGen 125 tissue homogenizer. The homogenized samples were centrifuged at 4 °C at 14000 RPM for 10 min. Intracellular ATP levels were determined using a luciferase ATP assay kit (Promega) according to the manufacture's instructions. The amount of ATP-driven light produced was measured using a Luminoskan Ascent luminometer. The amount of total cellular protein was determined with protein assay reagents (Bio-Rad) using bovine serum albumin as a standard.

2.8. Urinalysis and brain levels for 8-hydroxy-2-deoxyguanosine (OH⁸dG)

At 42 days of age, groups of 10 R6/2 mice were administered 5000 mg/kg/day CoQ₁₀ (Chemco formulation) or unsupplemented chow for 35 days, along with age-matched littermate wild-type mice, in order to reach maximal serum concentration levels. It has been our experience that the use of metabolic cages to collect urine results in sample contamination. Alternatively, mice were handled to urinate directly into eppendorf tubes. After 35 days of treatment, approximately 100 μ l of urine was collected, frozen on dry ice at -80 °C, and transferred to a -80 °C freezer. Mice were euthanized at 77 days of age by decapitation. Brains were immediately removed, quartered as above with selective dissection of the striatum, and immediately flash frozen at -80 °C. An HPLC method with electrochemical detection was used for analysis of OH⁸dG levels in both urine and brain samples, as described in detail elsewhere [22,23]. The urinary levels represent nucleotide excision repair for the whole body standardized to creatinine levels within each sample and are measured in ng/mg creatinine. Creatinine levels were measured after OH⁸dG analysis on a 16 channel HPLC-EC Coularray (ESA, Chelmsford, MA) with UV detection at 236 nm, using a mobile phase A buffer (0.02 M NaH₂PO₄, 0.1% Methanol and 120 mg/l of tetrabutyl ammonium phosphate) and mobile phase B buffer (0.1 M NaH₂PO₄, 0.1% Methanol, 20% Acetonitrile and 200 mg/l of tetrabutyl ammonium phosphate) with a Tosohaas 4.6 \times 25 cm TSK-GEL column and autosampler set to 4 °C. The brain levels of OH⁸dG represent unrepaired damage by base or nucleotide excision and are measured in pg/ μ g 2 deoxyguanosine (2dG).

2.9. Statistics

The data are expressed as the mean \pm standard error of the mean. Statistical comparisons of rotarod, weight data, grip strength, and neuropathological data were compared by ANOVA or repeated measures of ANOVA and by non-paired Student's *t*-test (Statview, CA). Survival data were analyzed by the Kaplan–Meier survival curves.

3. Results

Diets supplemented with Chemco CoQ₁₀ formulation at 1000, 5000, 10000, or 20000 mg/kg/day and with Tishcon CoQ₁₀ formulation HydroQ-sorb at 400, 1000, and 2000 mg/kg/day significantly improved the clinical phenotype of R6/2 mice in a dose dependent manner (Fig. 1A and B). Significant prolongation of survival was observed in all doses using each formulation. Using the Chemco formulation, the greatest prolongation in survival (21.3%) was observed at the 5000 mg/kg/day dose, with less efficacy at higher doses (unsupplemented R6/2 mice: 101.2 ± 2.9 days; 1000 mg/kg/day CoQ₁₀-treated R6/2 mice: 117.0 ± 6.0 days, $P < 0.001$; 5000 mg/kg/day CoQ₁₀-treated R6/2 mice: 122.7 ± 7.4 days, $P < 0.001$; 10000 mg/kg/day CoQ₁₀-treated R6/2 mice: 112.8 ± 6.1 days, $P < 0.01$; 20000 mg/kg/day CoQ₁₀-treated R6/2 mice: 107.5 ± 7.4 days, $P < 0.01$). The Tishcon formulation also significantly extended survival in the R6/2 mice, however, to a greater degree (25.3%) and at a lower dose concentration of 1000 mg/kg/day (unsupplemented R6/2 mice: 101.4 ± 2.6 days; 400 mg/kg/day

CoQ₁₀-treated R6/2 mice: 114.4 ± 5.5 days, $P < 0.01$; 1000 mg/kg/day CoQ₁₀-treated R6/2 mice: 127.0 ± 7.4 days, $P < 0.001$; 2000 mg/kg/day CoQ₁₀-treated R6/2 mice: 123.6 ± 7.2 days, $P < 0.001$). Each of these CoQ₁₀ formulations resulted in greater survival extension than a previously reported lower dose of CoQ₁₀ using a different formulation (Vitaline, Ashland, OR) [3]. The positive control using oral cystamine treatment in R6/2 mice resulted in an 18.0% extension in survival and was consistent with previous work, providing context to the current study [4] (unsupplemented R6/2 mice: 101.4 ± 2.6 days; 225 mg/kg/day cystamine-treated R6/2 mice: 119.7 ± 5.5 days).

There was a marked overall loss of body weight in the unsupplemented R6/2 mice in comparison to wild type littermate control mice (Fig. 1C). The effect of daily dietary supplementation of high dose CoQ₁₀ (Chemco formulation) from each dosing paradigm on body weight was significantly improved from 7 weeks of age through 13 weeks when compared to the unsupplemented R6/2 mice (1000 mg/kg/day CoQ₁₀-treated R6/2 mice: $F_{(89,826)} 17.34$, $P < 0.01$; 5000 mg/kg/day CoQ₁₀-treated R6/2 mice: $F_{(89,826)} 19.22$, $P < 0.01$; 10000 mg/kg/day CoQ₁₀-treated R6/2 mice: $F_{(89,826)} 21.16$, $P < 0.01$; 20000 mg/kg/day CoQ₁₀-treated R6/2 mice: 17.6 ± 0.8 days, $F_{(89,826)} 24.08$, $P < 0.01$) (Fig. 1C). There were no significant differences, however, in the amelioration of body weight loss between CoQ₁₀ doses. The weight curves of CoQ₁₀-treated mice closely paralleled one another. The effect on body weight loss using the Tishcon CoQ₁₀ formulation was not different than the Chemco formulation. Administration of the Tishcon CoQ₁₀ resulted in reduced body weight loss in the R6/2 mice (1000 mg/kg dose $F_{(49,634)} 18.29$, $P < 0.01$). There was no dose response to treatment. Interestingly, improvement of weight loss profiles using both CoQ₁₀ formulations in this study was the same as that previously reported at a lower dose using a different CoQ₁₀ formulation (400 mg/kg/day; Vitaline, Ashland, OR) [3].

An analysis of motor performance on the rotarod revealed significant differences between R6/2 mice and wild-type littermates (Fig. 1D). Using the Chemco formulation, CoQ₁₀ treated R6/2 mice significantly improved in rotarod performance starting at 5 weeks through 13 weeks of age, in comparison to untreated R6/2 mice (1000 mg/kg/day CoQ₁₀-treated R6/2 mice: $F_{(59,1023)} 25.18$, $P < 0.01$; 5000 mg/kg/day CoQ₁₀-treated R6/2 mice: $F_{(59,1023)} 20.67$, $P < 0.01$; 10000 mg/kg/day CoQ₁₀-treated R6/2 mice: $F_{(59,1023)} 27.32$, $P < 0.01$; 20000 mg/kg/day CoQ₁₀-treated R6/2 mice: $F_{(59,1023)} 26.54$, $P < 0.01$) (Fig. 1D). The greatest improvement in rotarod occurred at the 5000 mg/kg/day dose. The performance in rotarod at 80 days for the 5000 mg/kg dose was over three-fold greater than the unsupplemented R6/2 mice. The data for the Tishcon formulation was no greater than that observed with the Chemco formulation, however, with the greatest improvement occurring at the 1000 mg/kg dose ($F_{(49, 876)} 29.84$, $P < 0.01$).

A grip strength meter was used to assess fore- and hind-limb grip strength. Analysis is presented in Fig. 1E and F. There was no significant difference between CoQ₁₀-treated (Chemco 5000 mg/kg/day) and unsupplemented R6/2 mice in hind-limb

grip strength over the entire course of treatment. There was, however, a significant difference in fore-limb strength after 11 weeks of age in the CoQ₁₀-treated R6/2 mice ($F_{(29,347)} 18.36$, $P < 0.01$). Grip strength was not performed using the Tishcon formulation.

At 90 days, marked neuroprotection was observed. Serial cut coronal tissue sections revealed gross brain atrophy, bilateral ventricular hypertrophy, and flattening of the medial aspect of the striatum in the unsupplemented R6/2 brains in serial coronal-cut step sections, in comparison to wild type littermate mice (Fig. 2). CoQ₁₀ treatment (Chemco, 5000 g/kg/day) ameliorated the gross brain atrophy in R6/2 mice, in comparison to untreated mice at this time point ($F_{(2,27)} 17.49$, $P < 0.01$). Consistent with the attenuation of gross atrophy, the neuroprotective effects of CoQ₁₀ treatment significantly reduced striatal neuron atrophy observed in the R6/2 mice (Wt littermate control: $138.9 \pm 12.6 \mu\text{m}^2$; CoQ₁₀-treated R6/2 mice: $116.8 \pm 17.1 \mu\text{m}^2$; unsupplemented R6/2 mice: $54.9 \pm 26.3 \mu\text{m}^2$; $F_{(2,30)} 21.61$, $P < 0.001$) (Fig. 3). Since high dose administration of therapeutic agents may result in liver toxicity and subsequent damage, we performed a histopathological analysis of liver in treated mice. No evidence of necrosis or inflammation was observed in CoQ₁₀-treated mice (data not shown).

Progressive accumulation of huntingtin-immunoreactive aggregates has been shown to begin at postnatal day one in R6/2 mice [12]. A number of compounds that have been efficacious in the R6/2 transgenic mice, inclusive of lower dose CoQ₁₀, significantly reduce huntingtin-positive striatal aggregates or ubiquitin-positive inclusions [5]. High dose CoQ₁₀ was no exception. In comparison to age-matched unsupplemented R6/2 mice, CoQ₁₀-treated R6/2 mice (Chemco, 5000 mg/kg/day) demonstrated a marked reduction in huntingtin aggregate (huntingtin aggregates in unsupplemented R6/2 mice: $5.78 \times 10^6 \pm 1.39$, 5000 mg/kg/day CoQ₁₀-treated R6/2 mice: $4.89 \times 10^6 \pm 1.07$; $F_{(2,32)} 9.43$, $P < 0.01$) (Fig. 4). These findings, however, were not significantly different than those reported using a lower dose concentration of CoQ₁₀ [3].

Two major functions of CoQ₁₀ are as an electron carrier in the mitochondrial respiratory chain and as a lipid-soluble antioxidant. While in humans CoQ₁₀ is the dominant species, CoQ₉ is the predominant ubiquinone in rodents, with as much as 70% CoQ₉ making up the total [24]. It was unclear at the onset of these preclinical trials whether increased levels of CoQ₁₀ would have an effect upon CoQ₉ levels in the R6/2 mice. We show a comparison of both CoQ₉ and CoQ₁₀ in both serum and brain of unsupplemented wild type littermate and R6/2 mice and in R6/2 mice at their optimal survival dosing concentrations of 5000 mg/kg/day and 1000 mg/kg/day using the CoQ₁₀ Chemco and Tishcon formulations, respectively, on day 73 after 30 days treatment (Fig. 5). As measured by HPLC, baseline serum levels of CoQ₁₀ in wild type littermate and R6/2 mice were $0.004 \mu\text{M/ml}$ and $0.002 \mu\text{M/ml}$, respectively (Fig. 5A). The Chemco and Tishcon CoQ₁₀ formulations increased CoQ₁₀ serum levels by 70 and 300 fold, respectively, in R6/2 mice (Fig. 5A) (Littermate wild type mice: $0.004 \pm 0.0004 \mu\text{M/ml}$; untreated R6/2 mice: $0.002 \pm 0.0005 \mu\text{M/ml}$; Chemco CoQ₁₀-treated R6/

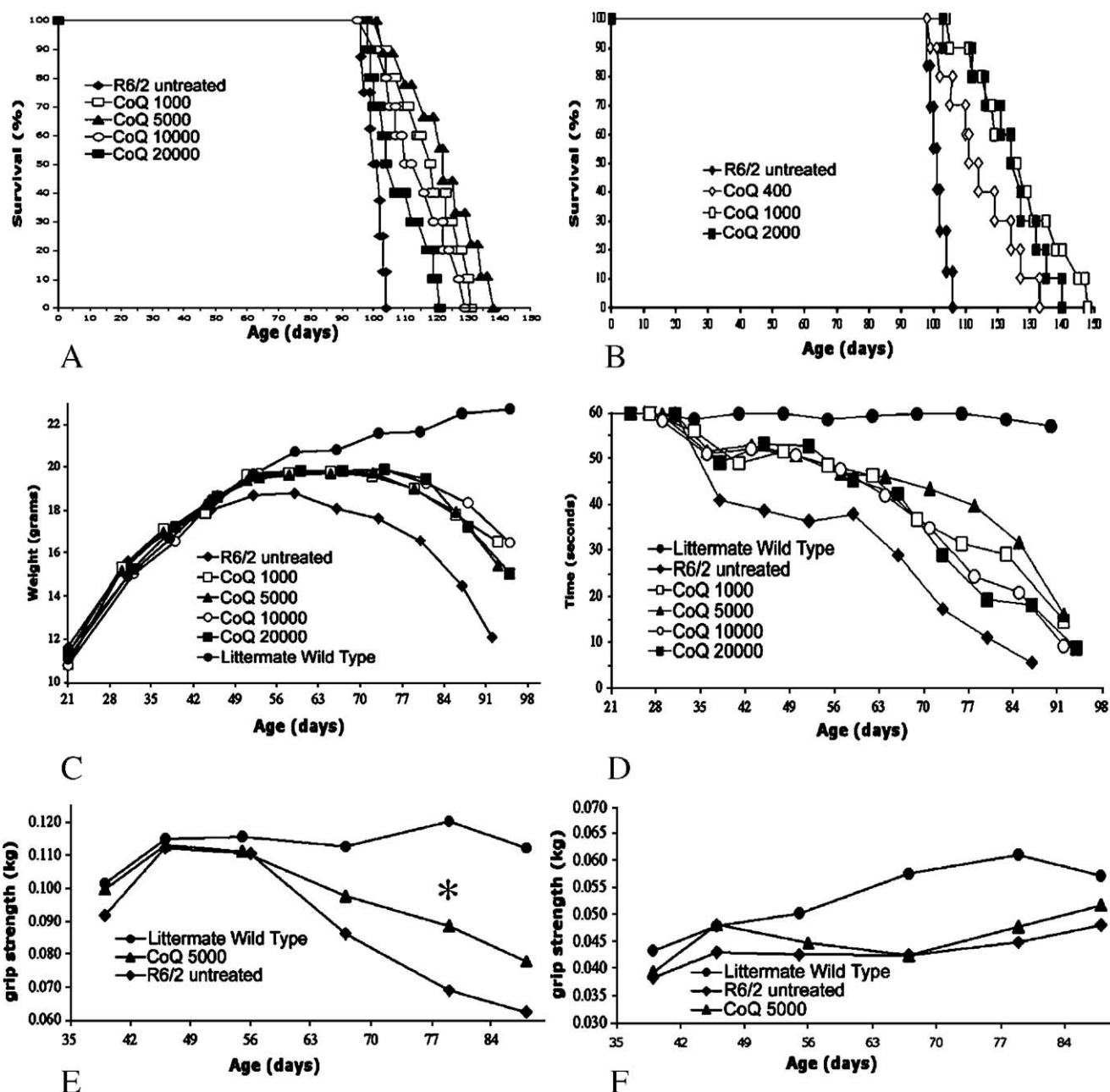


Fig. 1. Survival, body weight, rotarod, and grip strength in untreated and coenzyme Q₁₀ (CoQ₁₀) treated R6/2 transgenic mice. Cohorts of R6/2 mice ($n=10$) were treated from day 28 with oral administration of CoQ₁₀ (1000, 5000, 10,000, and 20,000 mg/kg/day, Chemco). Kaplan–Meier probability of survival analyses of CoQ₁₀ treatment and untreated R6/2 mice are presented in A. Survival was significantly extended using all CoQ₁₀ doses in R6/2 mice in comparison to untreated R6/2 mice, with greatest efficacy in the 5 gm/kg/day dose by 21.3%, $P<0.001$ (untreated R6/2 mice: 101.2 ± 2.9 days; CoQ₁₀ 1000 mg/kg R6/2 mice: 117.0 ± 6.0 days; CoQ₁₀ 5000 mg/kg R6/2 mice: 121.3 ± 7.4 days; CoQ₁₀ 10000 mg/kg R6/2 mice: 112.8 ± 6.1 days; CoQ₁₀ 20000 mg/kg R6/2 mice: 107.5 ± 7.4 days). The Tishcon formulation provided greater survival extension (25.3%, $P<0.001$) at lower dose (untreated R6/2 mice: 101.4 ± 2.6 days; 400 mg/kg/day CoQ₁₀-treated R6/2 mice: 114.4 ± 5.5 days; 1000 mg/kg/day CoQ₁₀-treated R6/2 mice: 125.8 ± 7.4 days; 2000 mg/kg/day CoQ₁₀-treated R6/2 mice: 123.6 ± 7.2 days) (B). The body weight, rotarod, and grip strength outcome measures are shown using the Chemco formulation only. Effects of CoQ₁₀ treatment on the body weight in R6/2 mice are shown in C. Significant body weight differences between untreated and CoQ₁₀ treated R6/2 mice occurred in all doses from 7 weeks through end stage disease. Motor performance using rotarod was improved using all doses starting at 5 weeks through 13 weeks as shown in D. Grip strength was significant in fore-limb analysis (E) after 79 days (asterisk), with no significant differences in hind-limb analysis (F). Asterisk (*) denotes significance as compared untreated R6/2 mice.

2 mice: 0.146 ± 0.058 $\mu\text{M}/\text{ml}$, $F_{(2,32)}16.27$, $P<0.01$; Tishcon CoQ₁₀-treated R6/2 mice: 0.679 ± 0.054 $\mu\text{M}/\text{ml}$, $F_{(2,32)}7.16$, $P<0.0001$), while not significantly increasing serum CoQ₉ levels (Fig. 5B) (Littermate wild type mice: 0.075 ± 0.006 $\mu\text{M}/\text{ml}$ untreated R6/2 mice: 0.066 ± 0.009 $\mu\text{M}/\text{ml}$; Chemco CoQ₁₀-treated R6/2 mice: 0.082 ± 0.008 $\mu\text{M}/\text{ml}$, $P<0.10$;

Tishcon CoQ₁₀-treated R6/2 mice: 0.084 ± 0.011 $\mu\text{M}/\text{ml}$, $P<0.08$).

Brain levels of CoQ₁₀ were significantly lower in R6/2 mice by 20%, in comparison to wild type littermate mice (Fig. 5C). Administration of the Chemco and Tishcon CoQ₁₀ formulations significantly increased brain CoQ₁₀ levels in R6/2

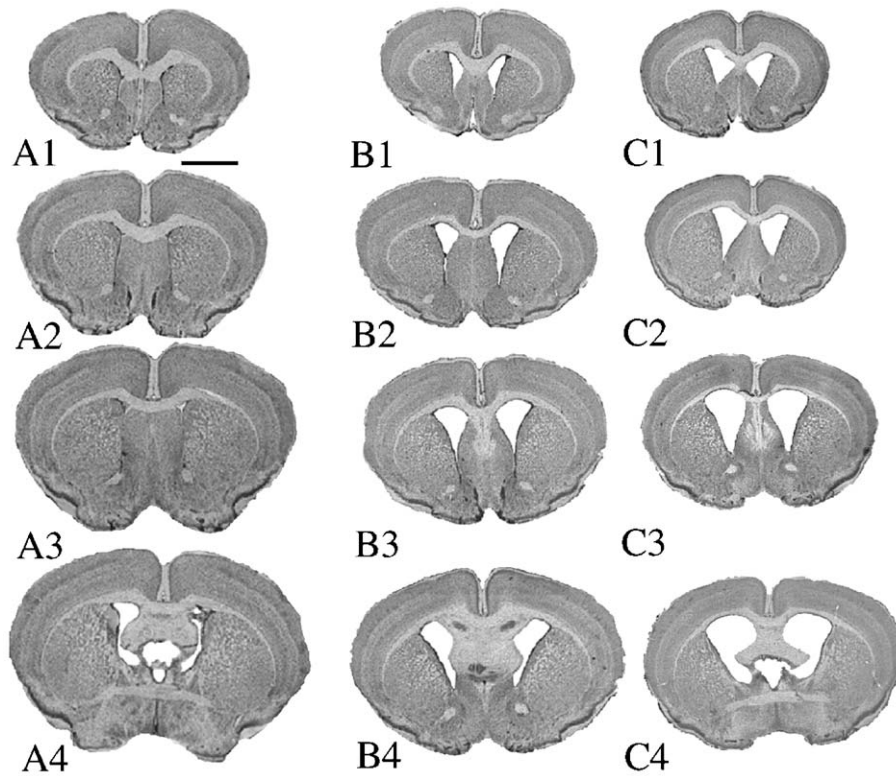


Fig. 2. Gross brain neuroprotection with coenzyme Q₁₀ (CoQ₁₀) treatment. Photomicrographs of coronal serial step sections from the rostral neostriatum through the level of the anterior commissure in a wild-type littermate mouse (A1–A4), CoQ₁₀ treated (Chemco, 5 mg/kg/day) R6/2 HD transgenic mouse (B1–B4), and untreated R6/2 HD transgenic mouse (C1–C4) at 90 days. There is gross atrophy of the brain and ventricular enlargement in the untreated R6/2 mouse (C1–C4) compared with the wild type littermate control (A1–A4). In contrast, the CoQ₁₀ treated R6/2 (B1–B4) shows reduced gross brain atrophy and reduced ventricular enlargement compared to the untreated R6/2 mouse (C1–C4). Scale bar in A1 equals 2 mm.

2 mice by 22.4% and 52.1%, respectively, in comparison to untreated R6/2 mice (Fig. 5C) (Littermate wild type mice: 5.25 ± 0.21 ng/mg; untreated R6/2 mice: 4.20 ± 0.39 ng/mg; Chemco CoQ₁₀-treated R6/2 mice: 5.14 ± 0.26 ng/mg, $F_{(3,32)} 19.29$, $P < 0.01$; Tishcon CoQ₁₀-treated R6/2 mice: 6.39 ± 0.21 ng/mg, $F_{(3,32)} 15.10$, $P < 0.001$). Brain levels of CoQ₉ were also significantly lower in R6/2 by 19.7%, in comparison to wild type littermate mice (Fig. 5D). Both the Chemco and Tishcon formulations significantly increased CoQ₉ brain levels by 38.3% and 40.1%, respectively (Littermate wild type mice: 10.13 ± 0.62 ng/mg; untreated R6/2 mice: 8.14 ± 0.74 ng/mg; Chemco CoQ₁₀-treated R6/2 mice: 11.26 ± 0.55 ng/mg, $F_{(3,32)} 14.53$, $P < 0.01$; Tishcon CoQ₁₀-treated R6/2 mice: 11.40 ± 0.61 ng/mg, $F_{(3,32)} 12.78$, $P < 0.01$). These findings are consistent with the neuroprotective effects of CoQ₁₀ in R6/2 mice.

Using HPLC analysis, we previously showed a significant decrease in both creatine and ATP levels in the R6/2 mice, in comparison to littermate control mice, suggesting that mitochondrial dysfunction was present in the R6/2 HD model [4]. We now confirm and extend the ATP results using a luciferase method. There was a 35.6% reduction in ATP levels in R6/2 mice, in comparison to littermate wild-type mice (Fig. 6). Both Chemco (5000 mg/kg/day) and Tishcon (1000 mg/kg) CoQ₁₀ supplementation significantly increased ATP levels in brain tissue samples from R6/2 mice, in comparison to unsupple-

mented R6/2 mice, to those levels detected in normal littermate control mice (littermate control: 9.11 ± 0.22 pmol/mg; untreated R6/2 mice: 5.87 ± 0.41 pmol/mg; Chemco CoQ₁₀-treated R6/2 mice: 9.61 ± 0.32 pmol/mg, $F_{(3,32)} 15.72$, $P < 0.01$; Tishcon CoQ₁₀-treated R6/2 mice: 9.68 ± 0.39 pmol/mg, $F_{(3,32)} 19.01$, $P < 0.01$).

Consistent with previous reports, both brain and urinary OH⁸dG levels in R6/2 mice were significantly increased in comparison to wild type littermate mice, suggesting that oxidative stress plays a role in the pathogenesis of neuronal degeneration in the R6/2 transgenic mouse model of HD [23]. After clinical disease onset (42d), R6/2 mice were treated with CoQ₁₀ (Chemco 5000 mg/kg/day) for 35 days, until 11 weeks. OH⁸dG levels in CoQ₁₀-treated R6/2 mice were significantly reduced, as compared to untreated R6/2 mice in brain (wild type littermate mice: 12.5 ± 3.7 pg/μg 2dG; untreated R6/2 mice: 48.6 ± 9.6 pg/μg 2dG; CoQ₁₀-treated R6/2 mice: 25.4 ± 5.8 pg/μg 2dG, $F_{(2,58)} 16.29$, $P < 0.01$) and urine (wild type littermate mice: 6.6 ± 2.3 ng/mg creatinine; untreated R6/2 mice: 23.6 ± 5.4 ng/mg creatinine; CoQ₁₀-treated R6/2 mice: 7.6 ± 3.1 ng/mg creatinine, $F_{(2,58)} 19.34$, $P < 0.01$) (Fig. 7). The urinary levels measure the aggregate effect of damage and nucleotide excision repair for the whole body, while the brain levels measure the amount of damage that has not been repaired by either base or nucleotide excision. The OH⁸dG levels in wild type and R6/2 mice are comparable to those previously reported

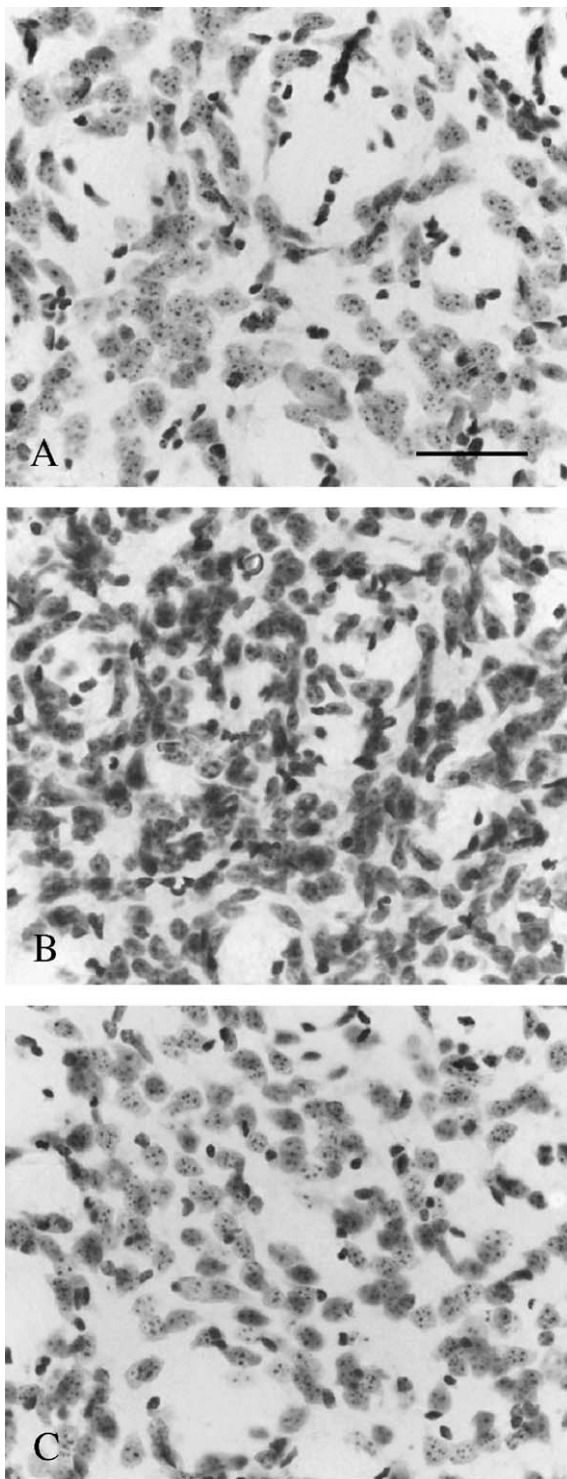


Fig. 3. Neuronal protection after coenzyme Q₁₀ (CoQ₁₀) administration. Nissl-stained tissue sections from the dorsomedial aspect of the neostriatum at 90 days in wild-type littermate control (A), untreated R6/2 (B), and a CoQ₁₀ treated R6/2 mice (C) (Chemco, 5000 mg/kg/day), show marked neuronal atrophy in the untreated R6/2 mouse (B) with significantly less neuronal atrophy in the CoQ₁₀ treated mouse (C), as compared with the control WT mice (Wt littermate control: $138.9 \pm 12.6 \mu\text{m}^2$; CoQ₁₀-treated R6/2 mice: $116.8 \pm 17.1 \mu\text{m}^2$; unsupplemented R6/2 mice: $54.9 \pm 26.3 \mu\text{m}^2$; $F_{(2,30)} 21.61$, $P < 0.001$). Greater cell packing density occurs in the untreated R6/2 mice as a consequence of tissue loss and neuronal atrophy (B). Scale bar in A equals 100 μm .

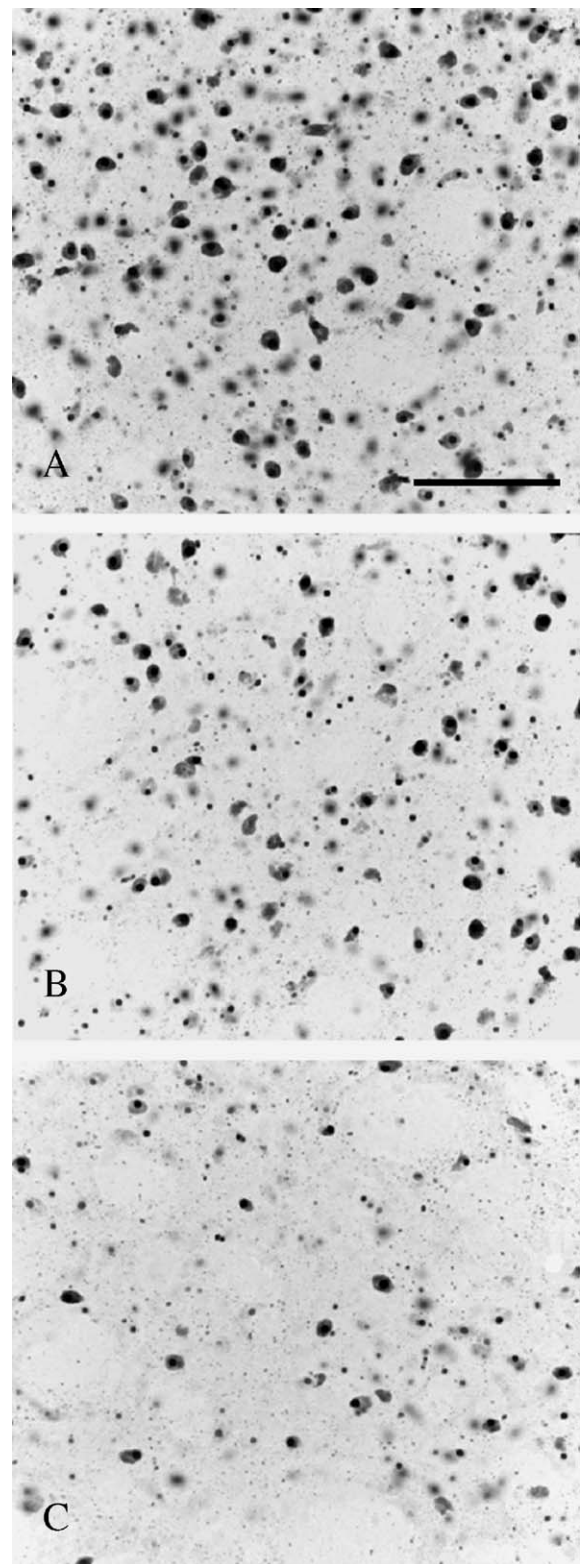


Fig. 4. Huntingtin (Htt) immunoreactivity in coenzyme Q₁₀ (CoQ₁₀) treated R6/2 mice. Htt immunostained tissue sections from the striatum at 90 days in untreated (A) and 1 gm/kg CoQ₁₀ (B) and 5000 mg/kg treated (C) (Chemco formulation) R6/2 transgenic mice. The number and size of htt aggregates, as well as intensity of immunostaining, is significantly reduced in both CoQ₁₀ doses with a dose response reduction in htt aggregates greatest in the 5000 mg/kg dose. Scale bar in A equals 100 μm .

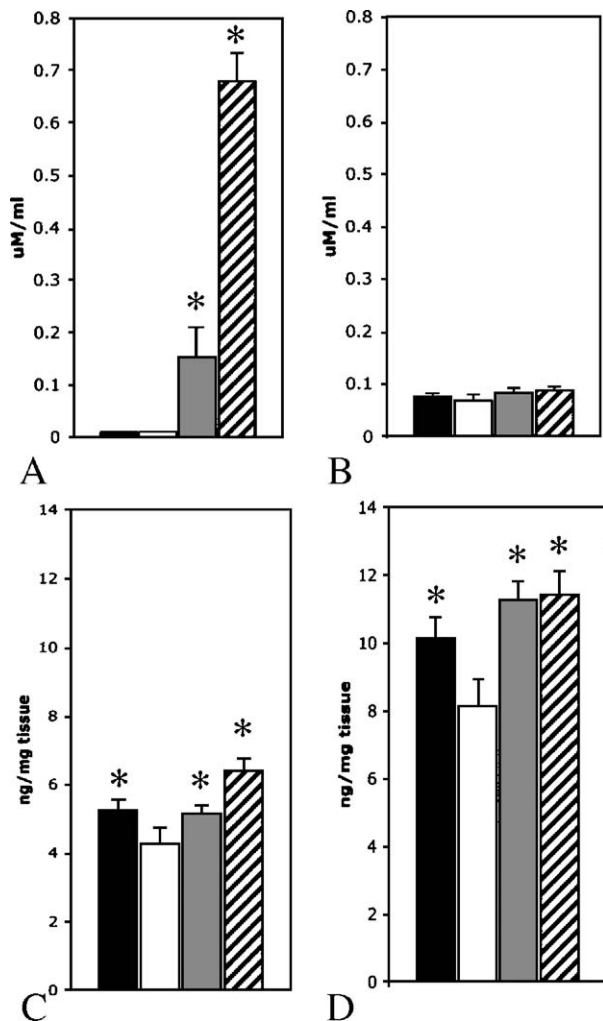


Fig. 5. Serum and brain levels of coenzyme Q₁₀ (CoQ₁₀) and coenzyme Q₉ (CoQ₉) in R6/2 mice. As measured by HPLC, serum levels of CoQ₁₀ in both WT and R6/2 mice were 0.004 and 0.002 μ M/ml, respectively (A). Doses of the Chemco (5000 mg/kg) and the Tishcon (1000 mg/kg) formulations increased CoQ₁₀ serum levels by 70 and 300 fold, respectively (A). CoQ₉ serum levels were not significantly increased in comparison to untreated R6/2 mice (B). Brain levels of CoQ₁₀ were significantly reduced in R6/2 mice, in comparison to wild type (WT) littermate control mice (C). Administration of the Chemco and Tishcon CoQ₁₀ compounds significantly increased CoQ₁₀ brain levels in R6/2 mice by 22.4% and 52.1% ($P < 0.01$), respectively, in comparison to untreated R6/2 mice (C). Brain levels of CoQ₉ were significantly reduced in R6/2 mice (19.7%, $P < 0.01$), in comparison to WT mice (D). Both the Chemco and Tishcon compounds significantly increased CoQ₉ brain levels by 38.3% and 40.1%, respectively, consistent with the neuroprotective effects of CoQ₁₀ supplementation in the R6/2 mice. Wild Type littermate ■, untreated R6/2 □, Chemco CoQ₁₀-treated ▒, Tishcon CoQ₁₀-treated ▨. Asterisk (*) denotes significance as compared untreated R6/2 mice.

[23]. Urinary OH⁸dG levels in R6/2 mice using the Tishcon formulation were not performed.

4. Discussion

We show that that high-dose administration of CoQ₁₀ exerts a greater therapeutic benefit in a dose-dependent manner in R6/2 mice than previously reported at lower CoQ₁₀ doses and that

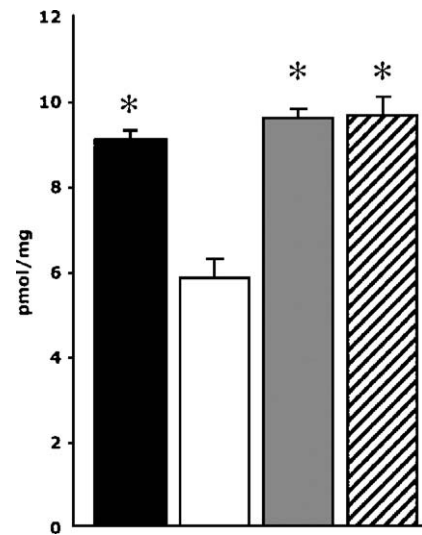


Fig. 6. ATP levels after coenzyme Q₁₀ (CoQ₁₀) administration. Using a luciferase technique, there was a significant decrease (35.6%) in ATP levels in the R6/2 mice, in comparison to littermate control mice, suggesting mitochondrial dysfunction in the R6/2 HD model. In contrast, there was a significant increase in ATP levels in brain tissue samples from both the Chemco and Tishcon CoQ₁₀-treated R6/2 mice, in comparison to unsupplemented R6/2 mice ($P < 0.01$). The ATP levels found in CoQ₁₀ supplemented R6/2 mice were not significantly different from littermate control mice (littermate control: 9.11 ± 0.22 pmol/mg; unsupplemented R6/2 mice: 5.87 ± 0.41 pmol/mg; CoQ₁₀ supplemented R6/2 mice: 9.61 ± 0.32 pmol/mg; Tishcon CoQ₁₀-treated R6/2 mice: 9.68 ± 0.39 pmol/mg). Wild Type littermate ■, untreated R6/2 □, Chemco CoQ₁₀-treated ▒, Tishcon CoQ₁₀-treated ▨. Asterisk (*) denotes significance as compared untreated R6/2 mice.

significant, yet variable, dose-dependent efficacy may result from different commercial sources. High-dose CoQ₁₀ significantly improved both the behavioral and neuropathological phenotype of R6/2 mice, delaying the development of weight loss, motor deficits, grip strength, gross brain atrophy, striatal neuron atrophy, and huntingtin aggregates in R6/2 mice. The

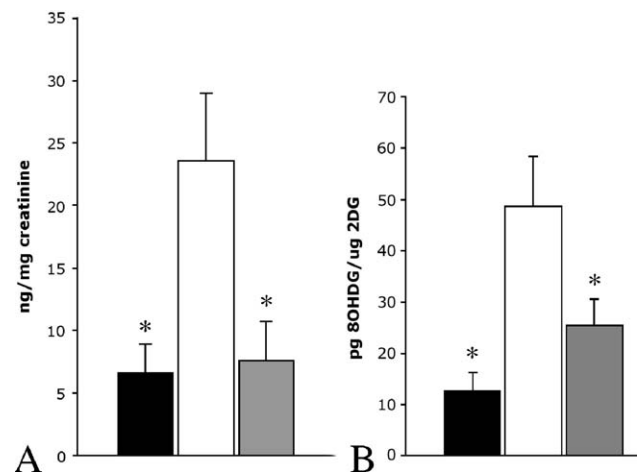


Fig. 7. OH⁸dG urine and brain levels after coenzyme Q₁₀ (CoQ₁₀) administration. There were marked increases in OH⁸dG urine (A) and brain (B) levels in R6/2 mice, in comparison to littermate wild-type control mice, with a significant reduction to normal levels in the CoQ₁₀ treated R6/2 mice. Wild Type littermate ■, untreated R6/2 □, Chemco CoQ₁₀-treated ▒. Asterisk (*) denotes significance as compared untreated R6/2 mice.

HydroQSorb formulation (Tishcon Corp) was as effective at a lower dose. CoQ₁₀ also ameliorated biomarkers of bioenergetic dysfunction and oxidative stress found in R6/2 mice. This ergogen dramatically improved serum CoQ₁₀ and brain CoQ₁₀ and CoQ₉ levels, while increasing brain ATP levels in treated R6/2 mice to normal wild type littermate control concentrations, cohering to the observed neuroprotective effects. In addition, CoQ₁₀ decreased elevated OH⁸dG levels in R6/2 mice, a finding consistent with reduced oxidative injury.

High doses of orally ingested therapeutic agents, both drug and natural compounds, may irritate the gut and alter gut flora, resulting in gastritis, increased elimination of food products, and reduced absorption. Gastrointestinal upset is one of the most common adverse events in both human and animal therapeutic trials and may occur subclinically without obvious evidence. This may have been the cause of reduced efficacy at the highest CoQ₁₀ doses in this mouse trial. Dose escalation, starting at lower more tolerable doses while advancing to the higher doses, may have resulted in greater improvement in outcome measures in the R6/2 HD mice than that reported herein.

CoQ₁₀ is a lipid-soluble benzoquinone derivative that resides in the inner mitochondrial membrane and is an essential coactivator in shuttling electrons from complexes I and II to complex III of the electron transport chain during oxidative phosphorylation. CoQ₁₀ plays a vital role in ATP production and serves as an anti-oxidant in both mitochondrial and lipid membranes [21,25,26], directly scavenging free radicals in the inner mitochondrial membrane by mediating uncoupling through superoxide production [27,28]. CoQ₁₀ has been used to treat a number of human medical conditions, the most prominent of which are mitochondrial disorders, and is most efficacious where there is an inherited deficiency of CoQ₁₀ [29–34]. CoQ₁₀ is effective in slowing the progression of neurological disease [34,35]. There is also substantial evidence that CoQ₁₀ is efficacious in mouse models of HD and may have benefit in HD patients [3,6,36–38]. Oral administration of CoQ₁₀ significantly improved both the clinical and neuropathological phenotype in R6/2 mice using a different formulation at a lower dose [3]. As such, a recent multicenter clinical trial (CARE-HD) of CoQ₁₀ (600 mg/day) in HD patients showed a trend towards slowing in total functional capacity decline over 30 months, significance in slowing decline on the independence scale, and a significant beneficial effect on measures of cognitive function, including Stroop color naming and word reading tasks [6]. Since the single target dose, however, did not provide significance in the specified primary outcome of the trial, it remains unclear whether a higher CoQ₁₀ dose would provide greater efficacy in HD patients.

An important advance in developing drug compounds for clinical trials in HD patients has been the introduction of genetic mouse models of HD. Of the available HD murine models, the R6/2 transgenic mouse has been the most widely used in preclinical trials testing potential therapies [5]. The efficiency and clear experimental endpoints of the R6/2 mice remain a major advantage over most HD mouse models in preclinical testing, with recent evidence suggesting that the behavioral and neuropathological changes more accurately replicate those

observed in human HD [12]. Phenotype homogeneity is essential in testing potential therapies in these mice, minimizing measurement variability and increasing the power to detect differences between the test groups [13]. For example, variability in phenotype severity in R6/2 mice may be dependent on CAG repeat size. There is evidence that increased CAG repeats beyond that originally reported in the R6/2 mice result in marked heterogeneity of the R6/2 phenotype, with the potential of reducing their utility in therapeutic trials [12]. In addition, the practice of using environmental enrichment in some laboratories can be considered as a therapeutic treatment that may confound mouse trials. Enrichment results in greater heterogeneity of the clinical and neuropathological phenotype, lowering the power to detect differences.

Methodological variances in testing paradigms can also result in differing expression of outcome responses that may produce differences in data interpretation, with the potential to obscure therapeutic efficacy. In N171-82Q transgenic HD mice, significant survival extension was not found combining CoQ₁₀ and remacemide treatment, with only transient improvement of motor performance [37]. Others, however, have showed significant efficacy in survival, in addition to other behavioral analyses in N171-82Q mice [3]. Similar divergent results in the efficacy of minocycline in R6/2 mice have been reported and are most likely the result of different methods of treatment and measuring outcomes [39,40]. In contrast, there are classes of agents, such as transglutaminase inhibitors (cystamine) and histone deacetylase inhibitors (butyrates) where the results of efficacy in outcome measures are agreed upon across laboratories [16–20,41–43]. The latter may be due to the proximity of these agents to the pathophysiological mechanisms associated with HD. Consistent with previous studies, and that mitochondrial dysfunction may play a proximal role in the pathogenesis of HD, we show a robust effect of high-dose CoQ₁₀ in R6/2 mice with greater than 25% extension in survival.

While survival is an important surrogate indicator for neuroprotection, it may not be possible to use this outcome measure in some laboratories. Longitudinal biomarkers of disease that measure pathological phenotype can validate therapeutic efficacy. We have identified two such biomarkers in the R6/2 mice, ATP levels characterizing the bioenergetic defect and OH⁸dG levels as a marker of oxidative stress. We have previously showed that creatine and ATP levels are reduced in R6/2 mice [4]. We confirm this finding and additionally demonstrate that ATP levels are recovered by CoQ₁₀ treatment. A useful biomarker of oxidative damage to DNA is OH⁸dG. Significant increases occur in OH⁸dG levels in nuclear DNA in the caudate nucleus in post-mortem tissue from HD patients [44], as well as in mitochondrial DNA from parietal cortex [45]. OH⁸dG levels are elevated in urine, serum, and brain samples from R6/2 mice [23]. We show that CoQ₁₀ administration reduces OH⁸dG to normal levels, consistent with a neuroprotective effect. Of great interest is a recent study demonstrating that serum OH⁸dG levels are elevated in HD patients and that creatine treatment, a similar acting ergogen, ameliorated OH⁸dG increases [46]. This is the first such instance of parallel efficacy, using a common

peripheral biomarker, in the administration of a therapeutic agent in HD mice and patients.

Successful preclinical trials demonstrating improved phenotype in HD transgenic mice have yet to be validated in HD patients. This may be the consequence of insufficient fully-powered clinical trials in humans to permit a comparison of therapeutic efficacy between mouse and man. Alternatively, optimal therapeutic dosing may be underestimated, as perhaps was the case for CoQ₁₀ supplementation in HD patients in the recent CARE-HD trial [6]. Human Equivalent Dose extrapolation measurements derived from body surface area criteria in animals [47] may not accurately predict the maximum-recommended safe dose in neurological disorders. This is evident in human trials where human equivalent dosing of bioenergetic agents comparable to that given to mice, while considered safe and tolerable, have not demonstrated significant efficacy in patients [48–50]. There is, however, a growing body of evidence suggesting that the phenotypes from mouse models of neurological diseases closely correlate with human diseases and may validate known CNS drug targets in a therapeutically relevant manner [5,14,51]. The strengths of the HD mouse models are in their utility to provide parallel pathophysiological targets that are present in HD patients, in their potential as sensitive predictors for therapeutic intervention, and their promise in the development of novel drug agents. While drug trials in mice confirm therapeutic direction, the challenge is in determining what dose might be of value in patients since the pharmacokinetics of mice and man is dissimilar.

The present results suggest that higher dose CoQ₁₀, consistent with that shown in safety and tolerability trials of other age-related neurodegenerative disorders [8,9], may provide greater efficacy in HD patients. Administration of CoQ₁₀ at high doses, however, should be closely monitored in test patients, largely because the safety of high-dose administration over a long period of time has yet to be established. It is likely that a bioenergetic agent, such as CoQ₁₀, will represent one of the cornerstone defenses in ameliorating the progression of HD. The present study provides a rationale for high-dose CoQ₁₀ as an immediate candidate for clinical trials in HD patients.

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