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Title: Coenzyme Q10 distribution in blood is altered in patients with Fibromyalgia

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Abstract: Objective: Coenzyme Q10 (CoQ10) is an essential electron carrier in the mitochondrial respiratory chain and a strong antioxidant. Signs and symptoms associated with muscular alteration and mitochondrial dysfunction, including oxidative stress, have been observed in patients with fibromyalgia (FM). The aim was to study CoQ10 levels in plasma and mononuclear cells, and oxidative stress in FM patients.

Methods: We studied CoQ10 level by HPLC in plasma and peripheral mononuclear cells obtained from patients with FM and healthy control subjects. Oxidative stress markers were analysed in both plasma and mononuclear cells from FM patients.

Results: Higher level of oxidative stress markers in plasma was observed respect to control subjects. CoQ10 level in plasma samples from FM patients was doubled compared to healthy controls and in blood mononuclear cells isolated from 37 FM patients was found to be about 40%

lower. Higher levels of ROS production was observed in mononuclear cells from FM patients compared to control, and a significant decrease was induced by the presence of CoQ10.

Conclusion: The distribution of CoQ10 in blood components was altered in FM patients. Also, our results confirm the oxidative stress background of this disease probably due to a defect on the distribution and metabolism of CoQ10 in cells and tissues. The protection caused in mononuclear cells by CoQ10 would indicate the benefit of its supplementation in FM patients.

Suggested Reviewers:

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Edgard E. Delvin

Editor-in-Chief

Clinical Biochemistry

Dear Editor,

We are submitting the revised work titled Coenzyme Q10 distribution in blood is altered in patients with Fibromyalgia by Cordero and co-workers to be considered for Clinical Biochemistry.

This work tries to introduce the analysis of coenzyme Q10 content in both plasma and mononuclear cells as a marker for the diagnosis of fibromyalgia. We show a significant alteration of the distribution of this antioxidant in these two compartments that are shown to correlate in control conditions, as it has been previously demonstrated in mammals.

We also show results demonstrating the oxidative stress status of the disease.

Your favourable consideration would be appreciated.

Sincerely,

Plácido Navas

Professor of Cell Biology

Cover

Dear Dr Lai,

Thank you very much for your editorial decision. The comments of the reviewers have been very much useful to improve the text. We have modified the text to fit with their comments according to the following responses.

Reviewer 1.

Major comments:

1. We have clarified the hypothesis of this work adding a sentence in page 3 of the text.
2. We have modified the information about the characteristics of muscle in fibromyalgia in page 3 and changed the reference number 3.
3. We have observed a higher level of CoQ in plasma but not in mononuclear cells, which show a deficiency. If mononuclear cells content of CoQ correlates with the content in muscle as it has been demonstrated, then it is expected that muscle should have a deficiency.
4. We have increased the number of controls. These controls were selected as healthy volunteers from the university environment with no muscle pain according to their declaration.
5. There is a significant difference of the concentration of CoQ in plasma between fibromyalgia patients and controls, although some of them are in the same range. We consider a cut-off level at about 200 nM/L.
6. CoQ is a redox compound that can be antioxidant but also pro-oxidant when in excess. We have indicated this idea in page 7.
7. We refer in the text a review by Pieczenik et al. (2007) that indicates the existence of oxidative stress in fibromyalgia.
8. The major finding in this paper is the change of the distribution of CoQ between plasma and mononuclear cells from fibromyalgia patients. It is known that CoQ content is equilibrated between plasma and these cells. Their contents are related and, when mice are supplemented with CoQ, the content of both plasma and mononuclear cells are increased but not in the other blood cells (see works from G. Dallner and I. Hardgreaves). According to our results this relationship is altered in fibromyalgia.

Minor comments:

1. Last 2 lines on Introduction were shifted to the end of Discussion.
2. We have indicated the presence of Gingko in the treatment of reference 10 in page 7 of the text.

Reviewer 2.

1. We have indicated in the text that the excess of CoQ contributes to pro-oxidant action and increases the oxidative stress markers. We have also incorporated in page 7 a sentence related to the cooperation of CoQ and tocopherol in the antioxidant properties of plasma. We have included in the Results the ratio of CoQ to cholesterol in plasma. We have not measured the production of oxygen radicals in plasma but we have done it in mononuclear cells.
2. We have included in Results the final contents of CoQ in mononuclear cells after their incubation with external CoQ.

Minor point:

The references were included.

Coenzyme Q₁₀ distribution in blood is altered in patients with Fibromyalgia

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Disclosure: The authors report no conflicts of interest.

ABSTRACT

Objective: Coenzyme Q₁₀ (CoQ₁₀) is an essential electron carrier in the mitochondrial respiratory chain and a strong antioxidant. Signs and symptoms associated with muscular alteration and mitochondrial dysfunction, including oxidative stress, have been observed in patients with fibromyalgia (FM). The aim was to study CoQ₁₀ levels in plasma and mononuclear cells, and oxidative stress in FM patients.

Methods: We studied CoQ₁₀ level by HPLC in plasma and peripheral mononuclear cells obtained from patients with FM and healthy control subjects. Oxidative stress markers were analysed in both plasma and mononuclear cells from FM patients.

Results: Higher level of oxidative stress markers in plasma was observed respect to control subjects. CoQ₁₀ level in plasma samples from FM patients was doubled compared to healthy controls and in blood mononuclear cells isolated from 37 FM patients was found to be about 40% lower. Higher levels of ROS production was observed in mononuclear cells from FM patients compared to control, and a significant decrease was induced by the presence of CoQ₁₀.

Conclusion: The distribution of CoQ₁₀ in blood components was altered in FM patients. Also, our results confirm the oxidative stress background of this disease probably due to a defect on the distribution and metabolism of CoQ₁₀ in cells and tissues. The protection caused in mononuclear cells by CoQ₁₀ would indicate the benefit of its supplementation in FM patients.

Key words: Coenzyme Q₁₀, fibromyalgia, mononuclear cells, oxidative stress

Fibromyalgia (FM) is a common chronic pain syndrome accompanied by other symptoms such as fatigue, headache, sleep disturbances, and depression. It affects predominantly females, but its pathogenesis is still unknown. FM is diagnosed according to the classification criteria established by the American College of Rheumatology (ACR) [1] and routine laboratory investigations usually yield normal results [2], therefore new diagnostic markers for FM are needed. **Characteristics of muscle in fm patients are muscle pain, fatigue and weakness, which could correspond to the abnormalities observed such as disorganization of Z bands and an altered number and shape of mitochondria. These muscles also show abnormalities of ATP and phosphocreatine levels [3].** Coenzyme Q₁₀ (CoQ₁₀) is often reduced in patients with myopathy, either as a primary or secondary event [4], and also there is a positive correlation of the content of CoQ₁₀ in plasma and mononuclear cells in rats supplemented with this compound [5], and there is a positive correlation between mononuclear cells and skeletal muscle [6]. **We hypothesize that an altered homeostasis of CoQ₁₀ would lead to an increased oxidative stress in plasma and a dysfunction in muscle and mononuclear cells.** We have found that plasma CoQ₁₀ levels were higher in FM patients than control but blood mononuclear cells showed a decrease of CoQ₁₀ indicating a dysfunction of CoQ₁₀ distribution in the blood of these patients.

PATIENTS AND METHODS

Patients and controls. The study was performed with the informed consent of all participants and the approval of the local ethical committee. We have studied 40 patients (males/4, females/36) recruited from the database of the Sevillian Fibromyalgia Association (AFIBROSE) and **30 healthy controls with no pain declared (males/7, females/23)**. The diagnosis of FM was established by an experienced rheumatologist according to ACR

criteria [1]. All patients had not taken any drug during a 15 days period before the collection of the blood samples. All patients reported following a standard balanced diet.

Heparinised and coagulated bloods were collected from each patient, centrifuged at 3800g for 5 min, and plasma and serum were stored at -80°C until testing. Serum biochemical parameters were assayed by routine analytical methods.

Blood mononuclear cells preparation and cultures. Peripheral blood mononuclear cells were purified from heparinised blood by isopycnic centrifugation using Histopaque-1119 and Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA).

Measurement of CoQ₁₀ levels. Both plasma and cellular CoQ₁₀ contents were analysed by HPLC (Beckmann 166-126 HPLC) with ultraviolet detection (275 nm) according to the method of Montero et al. [7].

Malondialdehyde levels. TBARS levels were determined by a method based on the reaction with thiobarbituric acid (TBA) at 90–100°C [8]. Shortly, the reaction was performed at 90°C for 15 min pH 2-3. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. This precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. Results were expressed as nmol/ml.

Protein carbonyl content. Plasma protein carbonyl content was quantified by spectrophotometric measurement of 2, 4-dinitrophenylhydrazine derivatives of protein carbonyls. Samples were precipitated with trichloroacetic acid at a final concentration of 20%, centrifuged at 16400 g for 10 min and protein pellets allowed to react with 2,4-dinitrophenylhydrazine. Pellets were then dissolved in sodium hydroxide and the

concentration of protein carbonyls measured spectrophotometrically at 360 nm, according to the method of Harma et al. [9]. Results are expressed as nmol/ml.

Intracellular ROS production. Reactive oxygen species (ROS) production were measured according to Quinzii *et al.* [4].

Statistical analysis. All results are expressed as means \pm SD unless stated otherwise. The unpaired Student's *t* test was used to evaluate the significance of differences between groups, accepting $P < 0.05$ as level of significance.

RESULTS

Mean age was 47.5 ± 11 for the FM group and 44.2 ± 13 years for the control group.

Routine laboratory test yield normal results for glucose $93,15 \pm 18,06$ mg/dL (normal values 76-110), urea $35,97 \pm 10,39$ mg/dL (n.v. 10-45), uric acid $4,65 \pm 1,51$ mg/dL (n.v. 2.5-7.5), total protein $7,64 \pm 0,40$ g/dL (n.v. 6.6-8.7), creatinine $0,77 \pm 0,11$ mg/dL (n.v. 0.5-1.1), aspartate aminotransferase $22,94 \pm 7,94$ mU/mL (n.v. 10-40), alanine aminotransferase $21,21 \pm 12,33$ mU/mL (n.v. 10-40), cholesterol $227 \pm 46,07$ mg/dL (n.v. >220), and triglycerides $109,92 \pm 65,5$ mg/dL (n.v. 150-200).

On average FM patients showed higher level of oxidative stress markers in plasma respect to control subjects (malondialdehyde: FM 14.2 ± 3.7 nmol/mL; controls: 8 ± 1.2 nmol/mL, $P < 0,005$), protein carbonyls: FM 40.6 ± 7.6 nmol/mL and control 18.3 ± 2.2 , $P < 0,001$). However, antioxidant CoQ₁₀ level average in plasma samples from FM patients was doubled compared to healthy controls (figure 1). **The ratio of CoQ₁₀ to cholesterol in plasma were of 1.56 in FM patients and 0.76 in control.**

Interestingly, CoQ₁₀ concentration determined in blood mononuclear cells isolated

from 37 FM patients was found to be about 40% lower than in control cells ranging from 138.4 ± 48.5 pmol CoQ₁₀/mg protein in FM samples to 225.1 ± 12.1 pmol CoQ₁₀/mg protein in controls ($P < 0,01$).

To confirm this deficiency detected, blood mononuclear cells from six representative FM patients were incubated with 10 μ mol/L CoQ₁₀ and the analysis of ROS production was determined in both treated and non treated cells. Mononuclear cells isolated from FM patients showed higher levels of ROS production compared to control cells (FM: $16,8 \pm 3$ a.u.; control: $10,4 \pm 0,1$ a.u., $P = 0,025$), and a significant decrease was induced by the presence of CoQ₁₀ (FM plus CoQ₁₀: 11.9 ± 0.9 a.u., $P \leq 0.03$) . **CoQ₁₀ concentration in mononuclear cells from FM patients was increased by 35% (189.2 ± 36 pmol CoQ₁₀/mg protein) and by 10% in control (246 ± 14 pmol CoQ₁₀/mg protein).**

DISCUSSION

CoQ₁₀ plays a crucial role in cellular metabolism acting as the electron carrier between complexes I and II and the complex III of the mitochondrial respiratory chain; and regulates uncoupling proteins, the transition pore, β -oxidation of fatty acids, and nucleotide pathway [5].

CoQ₁₀ deficiency has been associated to a variety of human disorders, some of them caused by a direct defect of CoQ₁₀ biosynthesis genes or as a secondary event [4]. Interestingly, patients with CoQ₁₀ deficiency display improvement of symptoms, sometimes dramatic, after oral CoQ₁₀ supplementation [10].

Despite being a common disorder, FM affects at least 5 million individuals in the United States [1], its pathogenic mechanism remains elusive. Recently oxidative stress markers have been proposed as a relevant event in the pathogenesis of this disorder [11].

Major antioxidant factors in plasma are α -tocopherol and CoQ₁₀, and cooperate to prevent oxidative damage. In the present study we have confirmed the significant increase of serum markers associated with oxidative stress in FM patients, and provide for the first time direct evidence of increased ROS production at the cellular level. In fact we have found a lower content of CoQ₁₀ in blood mononuclear cells of these patients correlating with increased ROS production in these cells, which could be corrected by CoQ₁₀ supplementation in the culture medium. This situation is not contradictory because CoQ₁₀ shows both pro-oxidant and antioxidant activities depending on its concentration and membrane environment [5]. Furthermore, fibroblasts of some patients with CoQ₁₀ deficiency syndrome show a higher production of ROS in mitochondria [4].

It should be noted that in general there is a poor correlation between plasma and tissues levels of CoQ₁₀, and even patients with genetically proven CoQ₁₀ deficiency may have plasma CoQ₁₀ levels in the normal range [7]. It is also important to consider that both endogenous biosynthesis and diet contribute to the final content of CoQ₁₀ in blood. Both patients and control did not show significant differences in their diets in this work. However, there is a positive correlation between the content of CoQ₁₀ in skeletal muscle and mononuclear cells [6], and it has been demonstrated that human supplementation with CoQ₁₀ induced an increase in both plasma and mononuclear cells, but not in polinuclear cells [5]. Furthermore, mononuclear cells were successfully used to determine CoQ₁₀ deficiency [6]. The protective effect of CoQ₁₀ supplementation on mononuclear cells could explain the previous pilot study that has reported beneficial effects of CoQ₁₀ administration to FM patients, although this could be also due to the presence of Gingko [12]. In these patients the increased plasma levels does not imply the accumulation in mononuclear cells indicating that cell uptake and/or metabolism of CoQ₁₀ is altered showing a new marker for

this disorder. If this defect is affecting to other tissues would contribute to understand the pleiotropic aspect of the disease.

Because FM is diagnosed only on the basis of clinical criteria it is necessary to find novel diagnostic markers. According to our results, the dysfunction of the CoQ₁₀ distribution between plasma and mononuclear cells, and the correlation between these cells and muscle [6], would represent a good marker for the diagnosis of FM. In conclusion, our results confirm the oxidative stress background of this disease probably due to a defect on the distribution and metabolism of CoQ₁₀ in cells and tissues. **We propose the analysis of CoQ₁₀ levels as a biochemical marker for FM, which could represent a defect of antioxidant homeostasis in this disease.**

Acknowledgements:

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Figure 1. Coenzyme Q₁₀ levels in plasma from fibromyalgia patients and healthy control subjects. The analysis of CoQ₁₀ content in FM patients shows a significant increase of the concentration relative to control samples.

Figure 1
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