Is Inflammation a Mitochondrial Dysfunction-Dependent Event in Fibromyalgia?

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Abstract

Fibromyalgia (FM) is a complex disorder that affects up to 5% of the general population worldwide. Both mitochondrial dysfunction and inflammation have been implicated in the pathophysiology of FM. We have investigated the possible relationship between mitochondrial dysfunction, oxidative stress, and inflammation in FM. We studied 30 women diagnosed with FM and 20 healthy women. Blood mononuclear cells (BMCs) from FM patients showed reduced level of coenzyme Q10 (CoQ10) and mtDNA contents and high level of mitochondrial reactive oxygen species (ROS) and serum tumor necrosis factor (TNF)-alpha and transcript levels. A significant negative correlation between CoQ10 and TNF-alpha levels ($r = -0.588; p < 0.01$), and a positive correlation between ROS and TNF-alpha levels ($r = 0.791; p < 0.001$) were observed accompanied by a significant correlation of visual analogical scale with serum TNF-alpha and transcript levels ($r = 0.4507; p < 0.05$ and $r = 0.7089; p < 0.001$, respectively). TNF-alpha release was observed in an in vitro (BMCs) and in vivo (mice) CoQ10 deficiency model. Oral CoQ10 supplementation restored biochemical parameters and induced a significant improvement in clinical symptoms ($p < 0.001$). These results lead to the hypothesis that inflammation could be a mitochondrial dysfunction-dependent event implicated in the pathophysiology of FM in several patients indicating at mitochondria as a possible new therapeutic target. Antioxid. Redox Signal. 18, 800–807.

Introduction

Fibromyalgia (FM) is a common chronic pain syndrome accompanied by other symptoms such as fatigue, headache, sleep disturbances, and depression, the pathophysiological mechanisms of which have not yet been identified. Although FM is diagnosed according to the classification criteria established by the American College of Rheumatology (ACR) (9), the diagnosis is not easy and may be frequently overlooked; this is why new diagnostic markers in FM are needed. Therefore, despite being a common disorder that affects at least 5 million individuals only in the United States (9), its pathogenic mechanism remains elusive. There are some hypotheses and pilot studies suggesting that cytokines may play an important role in FM (8), and correlation has been observed between cytokines and several symptoms common in FM (8). However, there are discrepant findings related to whether pro-inflammatory and anti-inflammatory cytokines are elevated or reduced in people with FM and whether their levels correlate with the core symptoms of this disorder (8).

Reactive oxygen species (ROS) have an established role in inflammation and host defense, and are involved in the pathophysiology of several diseases including rheumatoid arthritis, multiple sclerosis, and diabetes (1). Moreover, in addition to the presence of oxidative stress, these diseases are also characterized by deregulated inflammatory responses including, but not limited to, pro-inflammatory cytokine production.

It is believed, that in cells undergoing aerobic metabolism, the majority of ROS are produced as a byproduct of the

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Innovation

Inflammation has been indicated as being in the pathophysiology of fibromyalgia (FM) in some patients, and it has been suggested that oxidative stress and mitochondria could be among the factors responsible for development of inflammation. This is an interesting issue that warrants consideration to design new experiments to gain further insight into its potential therapeutic applications, given that systemic antioxidant status is an exogenously modifiable factor. The results described in this article may serve as a new way for designing experiments to better understand the influence of oxidative stress on the development of FM and generate new therapeutic strategies.

Results and Discussion

Mean age in the FM group was 43.8 ± 13.1 years and in the control group it was 44.9 ± 10. Patient routine laboratory tests yielded normal results (data not shown). The mean duration of symptoms in the FM group was 8.2 ± 4.4 years. The mean tender point score in the FM group was 15.1 ± 2.2 points (Table 1).

| Table 1. Characteristic Findings of Fibromyalgia Patients and Control Group |
|-----------------------------|----------------|
|                             | Patients | Controls |
| Age (years)                 | 43.8 ± 13.1 | 44.9 ± 10 |
| Tender points               | 15.1 ± 2.2  | 0         |
| Duration of disease (years) | 8.2 ± 4.4   | 0         |
| BMI (kg/m^2)                | 25.8 ± 3.3  | 24.6 ± 2.1 |
| VAS total score             | 6.9 ± 0.8^a | 0.4 ± 0.1 |
| FIQ total score, range 0–80 | 56.1 ± 11.1^a | 2.1 ± 1.1 |

^p < 0.001.
BMI, Body Mass Index; FIQ, Fibromyalgia Impact Questionnaire; VAS, Visual Analogue Scale.

Mitochondrial dysfunction has recently been proposed as a relevant event in the pathogenesis of this disorder (2). Mitochondria generate energy primarily in the form of electrochemical proton gradient, which fuels ATP production, ion transport, and metabolism. At the same time, mitochondria are also the major source of ROS. Both complexes I and III, along with CoQ10 leak electrons to oxygen (7). CoQ10 deficiency has been associated with a variety of human disorders, some of them caused by a direct defect of CoQ10 biosynthesis genes or as a secondary consequence of other diseases (7). Recent findings have shown that CoQ10 deficiency alters mitochondrial function and mitochondrial respiratory complexes organization, leading to increased ROS generation and activation of mitochondrial transition pore, increasing autophagy of dysfunctional mitochondria by mitophagy (2,7).

Since it has been suggested that CoQ10 levels could be a useful biological marker of mitochondrial function (2,7), we measured CoQ10 levels (Fig. 1A) in BMCs from healthy controls and FM patients as a feature of mitochondrial dysfunction. BMCs from FM patients showed a decrease of CoQ10 levels compared with controls (51% reduction of the average controls value, Fig. 1A). Further, significant decrement in mtDNA content in BMCs from FM patients was also observed (46.7% of the value found in the control group, Fig. 1B).

On one hand, it is known that mitochondrial dysfunction is often associated with an induction of ROS production in mitochondria and on the other hand, oxidative stress has been proposed as a relevant event in the pathogenesis of FM (2,4). To assess the oxidative stress level, we determined mitochondrial ROS in BMCs from FM patients and controls. On average, FM patients showed higher levels of mitochondrial ROS production in BMCs compared with control subjects (Fig. 1C).

Inflammation in FM patients is modulated by mitochondrial dysfunction

Several hypotheses have been made around the etiological origin of FM and its symptoms. Clinical studies have produced evidence that FM may be associated with immune dysregulation of circulatory levels of pro-inflammatory cytokines, affecting the neural dysfunction of pain-related neurotransmitters (8). Cytokines, depending on their concentration, induce symptoms, such as fatigue, fever, sleep, pain, and myalgia (8), all of which usually developed in FM patients. Alterations in pro-inflammatory cytokine levels have been observed in serum and biopsies of FM patients (8). In addition, increased levels of IL-1Ra and IL-6 have been found in the supernatants of cells from FM patients in vitro (8).

To assess inflammation in FM patients, we determined TNF-alpha levels in serum from controls and FM patients. FM patients showed higher level of TNF-alpha in serum compared with control subjects (Fig. 1D). Further, to corroborate inflammation in FM patients we analyzed the expression of tnf-alpha mRNA. Transcripts of tnf-alpha increased in BMCs from FM patients (Fig. 1E). Recent evidence has shown the possible involvement of ROS in inflammation suggesting that mitochondrial ROS may be a novel therapeutic target for inflammatory diseases (1). In this respect, inflammation as a consequence of oxidative stress and mitochondrial dysfunction has been indicated as the cause of many human diseases.
Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) levels, mitochondrial reactive oxygen species (ROS) production, and tumor necrosis factor (TNF)-alpha levels in blood mononuclear cells (BMCs) from fibromyalgia (FM) patients and healthy control individuals. (A) CoQ<sub>10</sub> levels were measured by high-performance liquid chromatography, as described in Materials and Methods. (B) Reduced mtDNA contents in BMCs from FM patients compared with healthy controls. (C) Mitochondrial ROS production was analyzed in BMCs from controls and FM patients by flow cytometry as described in Material and Methods. (D) Serum TNF-alpha levels in FM and control patients were measured as described in Material and Methods. (E) tnf-alpha transcripts were analyzed by SYBR Green quantitative polymerase chain reaction as described in Materials and Methods. Data represent the mean±SD of three separate experiments. *p<0.001; **p<0.05 between controls and FM patients.

Induced CoQ deficiency leads to mitochondrial ROS production and TNF-alpha synthesis

To verify the role of CoQ<sub>10</sub> in the increase to TNF-alpha levels observed in FM patients, we induced CoQ<sub>10</sub> deficiency by inhibiting endogenous biosynthesis in BMCs from healthy controls with P-aminobenzoate (PABA) treatment, a competitive inhibitor of polypropyl-4-hydroxybenzoate transferase (Coq<sub>2</sub>), in vitro assay (Fig. 3A). PABA treatment affected oxidative stress, inducing an increment of mitochondrial ROS levels (p<0.001) (Fig. 3B). CoQ deficiency provoked an important increment of TNF-alpha in the supernatant of cell cultures (Fig. 3C). Since CoQ<sub>10</sub> is also an important antioxidant, we evaluated the effect of treatment with CoQ<sub>10</sub> and other antioxidants in the in vitro model of CoQ<sub>10</sub> deficiency. BMCs were incubated with PABA in the presence or absence of three antioxidants, CoQ<sub>10</sub>, alpha-tocopherol (z-toc), and N-acetylcysteine (N-Acet). Mitochondrial ROS and TNF-alpha production were then measured. CoQ<sub>10</sub> attenuated ROS production (p<0.001) more significantly than z-toc and N-acet (p<0.05).

CoQ<sub>10</sub> has been shown to have a connection with inflammation: a significant negative correlation has been observed between CoQ<sub>10</sub> and pro-inflammatory markers in septic shock patients (1). Moreover, expression profiling revealed that CoQ<sub>10</sub> influences the expression of inflammatory genes suggesting that CoQ<sub>10</sub> exerts anti-inflammatory properties (6). This influence has been associated via NF-kappaB-dependent gene expression. Immunohistochemical studies in FM tissues revealed a stronger expression of NF-kappaB in muscle (5), and TNF-alpha is an NF-kappaB-dependent pro-inflammatory cytokine (1).

To study the role of CoQ<sub>10</sub> in the pathogenesis of FM and inflammatory process, and according to in vitro results, we induced a partial depletion of CoQ<sub>10</sub> in mice by sub-chronic PABA administration. CoQ depletion might provoke an increment of serum TNF-alpha in mice with respect to controls (Fig. 4A). When compared with vehicle-treated controls, PABA-injected mice showed an increase in serum TNF-alpha levels (Fig. 4B); and, interestingly, also showed high levels of hyperalgesia and reproduced a correlation between TNF-alpha levels and pain. Finally, TNF-alpha levels showed an important correlation with pain assayed in mice, (negative correlation between TNF-alpha and time latency in hot place test response, r = -0.6187; p<0.01).

Oral CoQ<sub>10</sub> supplementation in FM patients reduced TNF-alpha levels and improved clinical symptoms

According to our results, oxidative stress, mitochondrial dysfunction, and also CoQ<sub>10</sub> could play a role in the inflammatory response found in FM. CoQ<sub>10</sub> may have two roles in this pathological process: first of all, CoQ<sub>10</sub> is a mitochondrial cofactor with the potential to boost mitochondrial function and, second, CoQ<sub>10</sub> is a powerful free radical scavenger that can mitigate lipid peroxidation and DNA damage caused by oxidative stress (3). To study the possible effect of oral CoQ<sub>10</sub> supplementation in inflammatory process and mitochondrial...
dysfunction of FM and evaluate the improvement in clinical symptoms, eight volunteer FM patients were supplemented with CoQ\textsubscript{10}. CoQ\textsubscript{10} supplementation counteracted lipid peroxidation ($p < 0.001$), and it restored CoQ\textsubscript{10} levels and mtDNA content in BMCs from FM patients ($p < 0.001$). Interestingly, TNF-alpha levels were significantly reduced together with a marked improvement in FM clinical symptoms (Table 2).

Our patients, after oral CoQ\textsubscript{10} supplementation showed an important improvement in clinical FM symptoms, reduced levels of TNF-alpha and lipid peroxidation, indicating once again the possible role of CoQ\textsubscript{10} in the pathogenesis of FM. In other conditions of inflammation and oxidative stress, such as in the case of strenuous exercise, CoQ\textsubscript{10} supplementation before exercise has been shown to decrease oxidative stress and modulate inflammatory signaling, reducing the subsequent muscle damage (1). CoQ\textsubscript{10} analogues, such as mitoquinone, block transcription of inflammatory cytokines involved in the mitochondrial ROS-dependent inflammatory process (1).

**Concluding Remarks and Future Directions**

Most studies that have up to now examined the role of inflammation in FM have been incomplete and contradictory showing several discrepancies (8). However, according to these results, the correlation between TNF-alpha, CoQ\textsubscript{10}, and mitochondrial ROS levels may explain that inflammation in several FM patients could depend on mitochondrial dysfunction, thus identifying a new subgroup of patients in FM. Increased mitochondrial ROS in FM would result from enhanced oxidative phosphorylation. Recently, we demonstrated that oxidative stress could be implicated in the severity of clinical symptoms in FM (2), therefore, antioxidant therapy should be examined as a possible treatment in FM. Blockade of ROS production by mitochondria would provide a new therapeutic strategy to decrease symptoms in FM and other inflammatory states. Moreover, CoQ\textsubscript{10} treatment could be used as an alternative therapy in FM and this should be the aim of further studies. Further analysis involving double-blind placebo-controlled clinical trials will be required to confirm this observation. Indeed, our research group is currently working in this direction, on the basis of the conclusions of the exploratory work discussed in this article.

**Notes**

**Ethical statements**

Written informed consent and the approval of the ethical committee of the University of Seville were obtained, according to the principles of the Declaration of Helsinki.
Pain studies in mice were performed in accordance with the European Union guidelines (86/609/EU) and Spanish regulations for the use of laboratory animals in chronic experiments (BOE 67/8509-12, 1988). All experiments were approved by the local institutional animal care committee.

Patients

The study consisted of 30 women diagnosed with FM and 20 healthy women. Inclusion criteria were FM that had been diagnosed in the previous 2 to 3 years, based on the current ACR diagnostic criteria (9). Exclusion criteria were acute FIG. 3. Induced CoQ deficiency in vitro leads to mitochondrial ROS production and TNF-alpha release. 1 mM of P-aminobenzoate (PABA) induced partial CoQ10 deficiency (A), and high levels of mitochondrial ROS production, being restored by antioxidants (B). CoQ deficiency provoked an important increment of TNF-alpha in the supernatant of cell cultures, counteracted by antioxidants (C). CoQ10 produced a more significant protection than other antioxidants. Data represent the mean ± SD of three separate experiments. *p < 0.001 between control and PABA; **p < 0.01 between PABA with absence or presence of CoQ10; ***p < 0.05 between presence of CoQ10 or presence of BHA or N-acetylcysteine.

FIG. 4. TNF-alpha release in a partial CoQ10 depletion mice model. PABA was administered at a dose of 20 mg/kg/day for 15 days. (A) Levels of CoQ9 and CoQ10 measured in BMCs from mice treated with vehicle or PABA (mammalian species such as the mouse and the rat that have a relatively short lifespan primarily contain CoQ9 and low levels of CoQ10). Data represent the mean ± SD of three separate experiments. *p < 0.001 of CoQ9 and CoQ10 between vehicle or PABA. (B) TNF-alpha levels were measured in serum from mice treated with the vehicle or PABA. Data represent the mean ± SD of three separate experiments. *p < 0.001 between vehicle or PABA. (C) Correlation of TNF-alpha serum levels and pain in animal model. (n = 5 per group).
infectious diseases in the previous 3 weeks; past or present neurological, psychiatric, metabolic, autoimmune, allergy-related, dermal, or chronic inflammatory diseases; undesired habits (e.g., smoking, alcohol, etc.); oral diseases (e.g., periodontitis); medical conditions that required glucocorticoid treatment, use of analgesics, statins, or antidepressants; past or current substance abuse or dependence; and pregnancy or current breastfeeding. Healthy controls had no signs or symptoms of FM and were free of any medication for at least 3 weeks before the study began. All patients and controls had taken no drugs or vitamin/nutritional supplements during the 3 weeks prior to the collection of blood samples. However, before the study, patients reported they used paracetamol on demand. Clinical data were obtained from physical examination and evaluated using the Fibromyalgia Impact Questionnaire (FIQ) including visual analogue scales regarding general and diffuse pain typical of FM (visual analogical scale [VAS]).

**Animals and drug administration**

Two groups (control and treated) of 5 six-week-old male Swiss mice weighing 25–30 g were maintained on a 12 h light/dark cycle. PABA, a competitive inhibitor of polypropenyl-4-hydroxybenzoate transferase (7), was dissolved in saline (vehicle) and intra-peritoneally administered at a dose of 20 mg/Kg/day for 15 days. Behavioral tests were performed 5 days after the first drug administration. After testing, mice were anesthetized with CO₂ and sacrificed by decapitation. The lipid extract was suspended in 1 ml of ethanol, dried in a speed vac, and kept at -20°C. Samples were suspended in a suitable volume of ethanol before high-performance liquid chromatography (HPLC) injection. Lipid components were separated by a Prominence Shimadzu HPLC system (Shimadzu Scientific Instruments) equipped with a Shimadzu Shim-pack XR-ODS column. CoQ levels were analyzed using an ultraviolet SPD-20A detector.

**Mitochondrial ROS production**

Mitochondrial ROS generation in BMCs was assessed by MitoSOX™ (Invitrogen/Molecular Probes), a red mitochondrial superoxide indicator. BMCs were incubated with 1 μM MitoSox for 30 min at 37°C and washed twice with PBS. Cells were analyzed by flow cytometry. To assay ROS production with antioxidants, BMCs were incubated for 24 h with 10 μM CoQ₁₀ (Pharma Nord), 30 μM vitamin E, and 10 mM N-Acet (Sigma Chemical Co.).

**Quantification of mtDNA**

Nucleic acids were extracted from BMCs by standard cellular lysis. The primers used were as follows: for mitochondrial DNA, mtF3212 (5'-CACCCAAGACAGGGTTTTG-3') and mtR3319 (5'-TGGGGCATGGGTATGTGTTA-3') and those for nuclear DNA for loading normalization, 18S rRNA gene 18S1546F (5'-CGCTGACCCAGTGTTGAA-3') and 18S1650R (5'-CGCTGACCCAGTGTTGAA-3'). Arbitrary units were computed as the ratio between the optical density band...
corresponding to the mitochondrial DNA studied in the 20–30th cycle and that of nuclear DNA in the 15th amplification cycle. One unit was considered to be the ratio corresponding to the control patient.

**Lipid peroxidation**

Lipid peroxidation in cells was determined by analyzing the accumulation of lipoperoxides using a commercial kit from Cayman Chemical. TBARS are expressed in terms of malondialdehyde levels.

**Real-time quantitative polymerase chain reaction**

The expression of tnf-alpha gene was analyzed by SYBR Green quantitative polymerase chain reaction using mRNA extracts of BMCs from patients and controls. The thermal cycling conditions used were as follows: denaturation at 95°C for 20 s, alignment at 54°C for 20 s, and elongation at 72°C for 20 s, for 40 cycles. Tnf-alpha primers were as follows: 5′-GCT GCA CTT TGG AGT GAT CG-3′ (forward) and 5′-TTT ACC TAC AAC ATG GGC TAC AG-3′ (reverse). As an internal control, we used beta-actin primers; forward: 5′-CCA GAT CAT GTT TGA GAC C-3′ and reverse: 5′-ATG TCA CGC ACG ATT TCC C-3′. All reactions were performed in duplicate. Reaction mixtures without RNA were used as negative controls in each run.

**Behavior studies of pain sensibility in mice**

For the hot-plate test, a glass cylinder (16 cm high and 16 cm in diameter) was used to retain the mice on the heated surface of the plate, which was kept at the temperature of 45°C ± 0.5°C. The time latency for paw licking was measured. The cut-off for licking responses was 50°C for 20 s, for 40 cycles. Tnf-alpha primers were as follows: 5′-GCT GCA CTT TGG AGT GAT CG-3′ (forward) and 5′-TTT ACC TAC AAC ATG GGC TAC AG-3′ (reverse). As an internal control, we used beta-actin primers; forward: 5′-CCA GAT CAT GTT TGA GAC C-3′ and reverse: 5′-ATG TCA CGC ACG ATT TCC C-3′. All reactions were performed in duplicate. Reaction mixtures without RNA were used as negative controls in each run.

**Oral CoQ₁₀ supplementation**

Eight volunteer patients were supplemented with CoQ₁₀ (purchased from Pharma Nord), in soft gel capsules for 3 months (300 mg/day CoQ₁₀ divided into three doses). After 3 months of treatment, heparinized blood samples were collected after 12-h fasting and 24 h after the last dose, and clinical symptoms were evaluated. The CoQ₁₀ formulation consisted of soft gelatin capsules containing 100 mg of ubiquinone emulsified with diglyceryl monooleate, beeswax, soy lecithin, and canola oil.

**Statistical analysis**

Data in figures are given as mean ± SD. Data between different groups were statistically analyzed by using analysis of variance (ANOVA) on Ranks with Sigma Plot and Sigma Stat statistical software (SPSS for Windows, 19, 2010, SPSS Inc.). For cell-culture studies, Student’s t test was used for data analyses. A value of p < 0.05 was considered significant. To compare the behavioral results from animals treated with vehicle alone or with PABA a two-way variance (ANOVA) analysis was used. Statistical analyses included Pearson’s correlations between CoQ₁₀ levels and mitochondrial ROS levels in BMCs compared with TNF-alpha, and between VAS pain compared with serum TNF-alpha peptide and mRNA.

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**References**


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### Abbreviations Used

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<tr>
<td>a-toc</td>
<td>alpha-tocopherol</td>
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<td>ACR</td>
<td>American College of Rheumatology</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BMCs</td>
<td>blood mononuclear cells</td>
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