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Association between Machado–Joseph disease and oxidative stress biomarkers

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ABSTRACT

Spinocerebellar ataxia type 3, also called Machado–Joseph disease (MJD), is an hereditary autosomal dominant neurodegenerative disease that affects the cerebellum and its afferent and efferent connections. Since the mechanism by which mutant ataxin-3 eventually leads to neuronal death is poorly understood, additional investigations to clarify the biological alterations related to Machado–Joseph disease are necessary. Recent investigations suggest that oxidative stress may contribute significantly to Machado–Joseph disease. We compared markers of oxidative stress between Machado–Joseph disease and healthy control subjects. The results showed that Machado–Joseph patients have higher catalase levels and lower thiol protein levels compared to control subjects. The peripheral blood lymphocyes of MJD patients also showed higher levels of DNA damage by the comet assay than control subjects. Our results corroborate the hypothesis that the oxidative stress is associated with MJD patients. However, whether strategies to increase cellular antioxidative capacity may be effective therapies for the treatment of Machado–Joseph disease is an open question.

1. Introduction

Machado–Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is an hereditary autosomal dominant neurodegenerative disease that affects the cerebellum and its afferent and efferent connections. The ataxin-3 protein that is altered by the mutation in MJD has been linked to protein homeostasis maintenance, transcription, cytoskeleton regulation, and myogenesis. However, its biologic function remains obscure, limiting understanding of the mechanisms by which the altered protein leads to the selective neuronal death profile observed in MJD patients [1].

This disorder occurs later in life, with clinical manifestations characterized by a progressive and selective loss of neural cell bodies, axons, dendrites and/or synapses. Most frequently, in affected subjects, slowly progressive ataxia symptoms appear between the ages of 20 and 50 years, including cerebellar ataxia, progressive external ophthalmoplegia, dysarthria, dysphagia, pyramidal signs, dystonia, rigidity and distal muscle atrophies [2,3].

Although MJD is a relatively rare disease, it is the most frequent spinocerebellar ataxia with a worldwide distribution [4]. Its prevalence, however, varies according to the population studied [4]. MJD belongs to a special class of inherited neurodegenerative diseases caused by CAG trinucleotide repeat expansion in the coding region of different genes. An unstable CAG trinucleotide repeat expansion in the 10th exon of the MJD gene (*MJD1* or *ATXN3*, *locus* 14q32.1) has been identified as the pathological mutation in affected persons, so far [4,5]. The *MJD1* gene encodes for ataxin-3, with normal alleles from 12 to 47 CAG repeats, and pathogenic alleles larger than approximately 51 repeats [4]. In rare instances, the (CAG) repeat count falls into the 47 and 51 interval (intermediate or premutation alleles) [4]. These alleles are probably associated with an increased risk of developing clinical disease, but penetrance is incomplete [4]. When mutated, the *MJD1* gene produces a protein that shows an expanded polyglutamine tract (polyQ) leading to neurodegeneration. Aggregation of expanded polyQ-containing proteins into the nucleus is also a pathological hallmark of these diseases [4,5].

Since the mechanism by which mutant ataxin-3 leads to neuronal death is poorly understood, additional investigations to

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clarify the biological alterations related to MJD are necessary. Recent studies suggest that oxidative stress may contribute to MJD, as noted in other neurodegenerative disorders, including Alzheimer's disease and Huntington's disease [6–8]. These diseases present a chronic oxidative stress state induced by reactive oxygen species (ROS) or free radicals that play an important role in the pathogenesis of these conditions. Araujo et al. suggest that an impaired antioxidative capacity and increased susceptibility toward oxidative stress promotes neuronal cell death in SCA3 [9]. Since the brain has the highest oxygen consumption metabolic rate of any organ in the body, deleterious effects of oxidative stress stimuli can be expected to be implicated in the neurodegenerative process in SCA3 [9].

Furthermore, investigations have showed that activation of mitochondrial apoptotic pathways, reduction in antioxidant enzyme activity, and increased mitochondrial DNA damage are associated with MJD [10–13]. Among these studies, Yu et al. [12] have shown the potential association of some oxidative metabolism biomarkers in cell culture investigations of the mutant MJD cell line, SK-N-SH-MJD78, compared to wild-type cells. These findings strongly support the idea that MJD cells exhibit decreased activity of antioxidant enzymes, e.g., glutathione reductase (GSSG-R), catalase, and superoxide dismutase (SOD), when compared to wild-type cells. Therefore, these results suggest that the MJD mutation(s) significantly impairs the ability of the cells to respond to stress by altering antioxidant enzyme activities and damaging mitochondrial DNA, which, in turn, may lead to increased stress-induced cell death during the progression of MJD.

However, the potential association between oxidative stress status and MJD clinical symptoms, including balance and gait disturbance, is yet to be examined. In the present study, we performed an exploratory case-control study to determine whether MJD patients present alterations of oxidative stress biomarkers, compared to healthy controls with similar age and sex.

2. Materials and methods

2.1. Subjects and clinical data

A case-control study was performed involving 14 subjects, including seven patients with Machado–Joseph disease (MJD), with age of onset between 33 and 59 years, and seven healthy controls (HC) with similar sex, age, and smoking habit profiles. The normal alleles of the MJD patients showed between 23 and 28 CAG repeats, while mutant alleles ranged from 65 to 67 CAGs.

The study compared the balance (static/dynamic) condition as well as blood oxidative stress biomarkers between the two groups. The study was conducted in the Fonoaudiology and Biogenomic Laboratories of the Federal University of Santa Maria, Brazil, and based on a protocol that was approved by the Ethical Board Committee (No. 23081.013318/2010-83). Written informed consent to enroll in the study was obtained from all participants. MJD subjects with advanced disease that did not present the conditions to perform the balance tests were not included in the study.

2.2. Health conditions evaluation

A self-reported structured interview was applied to all participants who were asked questions related to their health and lifestyle, including smoking habit, regular physical activity, fruits and vegetables consumption and the existence of other chronic illnesses such as hypertension, type 2 diabetes mellitus, dyslipidemia, angina pectoris, depression, allergies, daily medicine ingestion. Additionally, the participants were asked about their self-perception related to their balance when walking, difficulty to climb and descend stairs and to cross the street.

2.3. Balance evaluation

The Berg balance score (BBS) and posturography were performed on the MJD patients and normal volunteers. The BBS is a simple test of functional balance composed of 14 items based on activities of daily living. Each item is rated using an ordinal scale, ranging from 0 to 4, and a maximum score of 56 [14]. The posturography was evaluated by the Sensory Organization Test (SOT) previously validated in Brazil by Castagno et al. [15]. The SOT involves six test conditions of increasing difficulty. In condition 1 (SOT 1) the patients are in the starting position with open eyes and in condition 2 (SOT 2), they are with the eyes closed. For SOT 1 and 2 both the platform and the surround remain immobilized. In condition 3 (SOT 3) the patients are in starting position; however, the surround moves. In condition 4 (SOT 4) the platform moves, but the surround remains fixed. In condition 5 (SOT 5) the platform moves while the subjects keep their eyes closed, and in condition 6 (SOT 6) both the surround and the platform move. Adaptation scores of the six conditions as well as the composite equilibrium were evaluated. The composite equilibrium score is a mathematical or analytic indicator of balance. It is calculated by independently averaging the scores achieved under conditions 1 and 2, adding these two values to the sum of all scores from sensory conditions 3, 4, 5, and 6, and then dividing this sum by the total number of trials. The highest possible score is 100 and this test is the best method of assessing how an individual processes and organizes sensory information.

2.4. Biochemical biomarkers evaluation

After a 12 h overnight fasting period, blood samples were collected from study participants by venous puncture in Vacutainers® tubes (BD Diagnostics, Plymouth, UK). Specimens were routinely centrifuged within 1 h of collection for 15 min at $2500 \times g$. The precipitate was discarded and the serum was used to determine levels of glucose, cholesterol, triglycerides, HDL-cholesterol, uric acid and creatinine using standard enzymatic methods involving Ortho-Clinical Diagnostics® reagents and a spectrophotometer. High-density lipoprotein cholesterol was measured in the plasma supernatant after precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride, as previously described [16]. Low-density lipoprotein cholesterol was estimated using the Friedewald equation [17].

Serum thiobarbituric acid reactive substances (TBARS) were measured according to the modified method of Jentzsch et al. [18] using whole blood collected in citrated saline solution with a 1:10 final dilution. The carbonylation of serum proteins was determined by modifications of the Levine method [19]. Whole blood catalase activity was determined using the method of Aebi [20] by measuring the decomposition rate of H_2O_2 at 240 nm. Whole blood superoxide dismutase activity was measured as described previously by McCord and Fridovich [21] and thiols were assayed in plasma by the method of Ellman [22].

Total polyphenols were spectrophotometrically determined in plasma by reading the absorbance at 750 nm (Folin–Ciocalteau method) and using gallic acid as a standard, following a method described by Chandra and de Mejia [23]. Total phenol concentrations of plasma samples were determined after a procedure of acid extraction/hydrolysis and protein precipitation with 0.75 M metaphosphoric acid (MPA). For hydrolyzing the conjugated forms of polyphenols, HCl was added to the sample, followed by NaOH in methanol. This step breaks the links of polyphenols with lipids and provides a first extraction of polyphenols. MPA was used in this procedure to remove plasma proteins. The final extraction of polyphenols was performed by adding a 1:1 (v/v) solution of acetone:water. The results were expressed as the gallic acid equivalent (GAE) in mg/L as described previously by Ellman [22].

The reactive oxidative species (ROS) levels were measured in the plasma of MJD and healthy subjects using the non-fluorescent cell permeating compound 2′,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolyzed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cellular oxidants. The plasma samples were treated with DCFH-DA (10 μM) for 60 min at 37 °C. The fluorescence was measured at an excitation of 485 nm and an emission of 520 nm. The calibration curve was performed with standard DCF (0–1 mM), and the level of ROS production was calculated as nmol DCF formed/mg protein [24].

2.5. Single cell gel electrophoresis (comet assay)

The comet assay was used to evaluate whether the leukocytes obtained from MJD patients exhibit higher levels of DNA damage when compared to healthy controls. The assay was performed as described by Singh et al. [25] in accordance with general guidelines. 100 cells (50 cells from each of the two replicate slides) were selected and analyzed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (cells with no migration) to 400 (cells with maximal migration). The slides were analyzed under blind conditions (as to the status of the subjects) by at least two different researchers.

2.6. Statistical analysis

Data are presented as means and standard deviations (±SD). Statistical differences between groups were evaluated by Student *t*-test. The potential association between oxidative stress and balance of MJD subjects was evaluated using Pearson correlation test. Multivariate analysis was performed to determine whether certain factors (i.e., sex, age and other biochemical biomarkers such as glucose and lipid profile) may affect the oxidative stress biomarkers that showed differences between the two groups examined here using a logistic regression analysis (*Backward Wald*). All statistical analyses were performed where all p values were two-tailed, and $p < 0.05$ was considered statistically significant. All analyses were carried out using the statistical package for social studies SPSS version 12.0 (SPSS Inc., Chicago, IL).