Oxidative damage and antioxidant enzymes in blood of patients with Spinocerebellar Ataxia Type 2

Marcadores de daño oxidativo y defensa antioxidante en pacientes con Ataxia Espinocerebelosa tipo 2

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Abstract
Recent evidences suggest that increased oxidative damage as well as deficits in antioxidants defense systems could be related to the pathogenesis of some hereditary ataxias. The aim of this study was to investigate some redox status biomarkers in patient with spinocerebellar ataxia type 2. Samples from 33 patients and 22 control subjects, from Holguín province, were used to determined plasmatic levels of malondialdehyde and enzymatic activities of Cu/Zn superoxide dismutase and catalase by spectrophotometric methods. Besides, DNA damage in peripheral blood cells was evaluated using the Comet assay. Our results evidenced that oxidative damage is higher in spinocerebellar ataxia type 2 Cuban patients in comparison with the control group. In addition, we found an increase of antioxidant activity of evaluated enzymes in affected subjects. These findings suggest that the systemic redox state is altered in patients with spinocerebellar ataxia type 2.

Keywords: ataxia, antioxidant enzyme, comet assay, malondialdehyde, neurodegenerative diseases, oxidative stress.

Resumen
Evidencias recientes sugieren que el aumento del daño oxidativo, así como deficiencias en los sistemas de defensa antioxidantes podrían estar relacionados a la patogénesis de algunas ataxias hereditarias. El objetivo de este estudio fue evaluar algunos marcadores del estado redox en pacientes con ataxia espinocerebelosa tipo 2. En el estudio se incluyeron, muestras de 33 pacientes y 22 sujetos como controles, todos provenientes de la provincia de Holguín. Se determinaron los niveles plasmáticos de malondialdehído y las actividades enzimáticas de las enzimas Cu/Zn superóxido dismutasa y catalasa, por métodos espectrofotométricos. Además, se evaluó el daño del ADN en las células de sangre periférica mediante el ensayo Cometa. Los resultados obtenidos demuestran un aumento del daño oxidativo en los pacientes con ataxia espinocerebelosa tipo 2 en comparación con el grupo control. Además, se observa un incremento en las actividades de las principales enzimas antioxidantes, en los sujetos afectados. Estos hallazgos sugieren que el estado redox a nivel sistémico está alterado en pacientes con ataxia espinocerebelosa tipo 2.

Palabras clave: ataxia, enzimas antioxidantes, ensayo comet, malonildialdehído, enfermedades neurodegenerativas, estrés oxidativo.

Introduction

Reactive oxygen species (ROS) generated within cells or, more generally, in a tissue environment, may easily turn into a source of cell and tissue injury. Aerobic organisms have developed evolutionarily conserved mechanisms and strategies to carefully control the generation of ROS and other oxidative stress-related radical or non-radical reactive intermediates. However, a derangement in redox homeostasis, resulting in sustained levels of oxidative stress and related mediators, can play a significant role in the pathogenesis of major human diseases including cancer, diabetes, cardiovascular diseases and neurodegenerative disorders.1

Oxidative stress, manifested by lipid peroxidation, protein oxidation and DNA oxidation among other indices, is observed in several neurodegenerative disorders including Alzheimer and Parkinson’s diseases, amyotrophic lateral sclerosis (ALS), myotonic dystrophy and hereditary ataxias such as Friedreich’s ataxia (FRDA), ataxia telangiectasia (AT) and ataxia with vitamin E deficiency.2-6 However, in all cases the influence of oxidative stress remains unclear. Recent evidences indicate that loss of neurons in such disorders results from a complex interplay among oxidative injury, excitotoxic stimulation, dysfunction of critical proteins and genetic factors.7

Redox research outside the Central Nervous System (CNS), particularly in blood cells and plasma of patient with neurodegenerative diseases, although controversial, support the presence of peripheral oxidative damage.8 On the other hand, relatively few studies have considered the effect of oxidative stress on the pathogenesis of hereditary ataxias. To our knowledge, no study has so far reported evidenced of an impairment of antioxidant enzymes and DNA damage in blood of Spinocerebellar ataxia type 2 (SCA2) patients. Therefore, the aims of this study were to investigate the levels of antioxidant enzymes like Superoxide Dismutase and Catalasa and also investigate whether lipid peroxidation and DNA damage is related with the pathological events of SCA2.

Methods

Thirty-three patients with SCA2 (16 women and 17 men; mean age 40.1 ± 12.5 years) and twenty-two healthy subjects as controls (10 women and 12 men; mean age 37 ± 10.8 years) were included in the study after obtaining their informed consent. The study was performed in accordance with the ethical guidelines of the World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects.

SCA2's patients were diagnosed in the Clinic for Investigation and Rehabilitation of Hereditary Ataxias (CIRAH) using PCR amplification for detecting CAG repeats described by Imbert et al. in 1996. All patients showed ataxia and related symptoms (slow saccadic eye movements, dysarthria, hyporeflexia and adiadochokinesia) and could be at clinic stages I, II or III. For all patient the age at onset was defined as the onset of motor impairment (mean 27 ± 10.8 years).

The healthy control subjects were interviewed about their medical history and physically examined. They were excluded both if considered to be malnourished and with evidence of significant medical problems or if they were taking antioxidant supplements.

Blood samples were drawn from the cubital median vein of the subjects and were collected into K2 EDTA tubes. For Comet Assay ten microliters of whole blood were used, the rest of blood samples were centrifuged at 1000 x g for 10 min at 4 °C and stored at -20 °C until their processing (before 10 days). The plasma was used for the determination of the antioxidant enzymes activity, as well as to determine the concentration of malondialdehyde (MDA), product of oxidative damage to lipids.

Biochemical measurements

Determination of Lipid peroxidation (LPO)

Malondialdehyde (MDA), a product of LPO, was determined by the spectrophotometric method described by Beuge JA and Aust SD.9 This assay is based on the reaction of a thiobarbituric acid with MDA yielding a stable chromosphore with a maximum absorbance at 532 nm. Concentration of MDA was calculated by a calibration curve using 1,1,3,3 tetramethoxypropane (TMP) as a standard in terms of nmol/mL.

Measurement of Superoxide Dismutase Activity

Extracellular Superoxide Dismutase (ec-SOD) activity was assayed according to the method of Marklund and Marklund.10 It was based in the ability of SOD to inhibit auto-oxidation of pyrogallol, considering that one unit of SOD activity is the amount of the enzyme that inhibits the 50 % auto-oxidation rate of pyrogallol under standard condition at 420 nm, and was expressed as Units/mL.

Measurement of Catalase Activity

Catalase (CAT) activity was determined according to Aebi’s procedure.11 The method is based on the capacity of the enzyme to transform H2O2 into H2O and 1/2 O2, in 1 min under standard conditions, decreasing absorbance at 240 nm. One unit of enzymatic activity
was considered as the quantity of enzyme necessary to transform 1 µmol of H₂O₂ in 1 minute at 37 °C. A molar extinction coefficient of 43.6 M⁻¹ cm⁻¹ was used.

All biochemical measurements were carried out at room temperature using a Spectronic™ GENESYS™ 8 UV/VIS spectrophotometer.

**Measurement of DNA damage in peripheral blood by Comet Assay**

The Comet assay, which is also called the single-cell gel electrophoresis technique, is a simple, sensitive and rapid method that can be used to estimate DNA damage at individual cell level through strand breaks, open repair sites, cross-links and labile sites. This assay was performed as described previously by Collins et al. with slight modifications. Prepared slides are examined using the optical microscope. Stained nuclei with DNA damage exhibit migration of single stranded DNA toward the anode forming a comet tail (% of DNA in the tail is related to DNA break frequency). One hundred nucleoids were scored per slide and it was classified according to the extent of tail DNA and given a value 0-4. DNA damage was expressed in Arbitrary Units (UA).

**Statistical analysis**

Data are expressed as mean values ± SD. The comparison between values obtained in patients and controls was performed by Student’s *t* test for unpaired data. Significance was taken as *p* < 0.05. The statistical program SPSS v10.0 for Windows was used in all the analyses.

**Results**

SCA2 patient showed significantly elevated levels of LPO in plasma compared to healthy subjects (1.05 ± 0.09 vs 0.75 ± 0.09 nmol/L, *p*=0.012), as illustrated in figure 1.

We found that the ec-SOD activity in plasma of SCA2 patients was higher than in the control group (19.85 ± 1.50 vs. 9.73 ± 1.64 U/mL, *p*<0.001) and also found differences in CAT activity between groups (601.59 ± 71.3 vs. 388.22 ± 59.7 U/mL, *p*=0.038) (Figure 2).

The DNA damage determined in our study is shown in figure 3. There was a significant difference in DNA damage between SCA2 patients and controls (246 ± 11, 4 vs. 197 ± 11,3 UA, *p*=0.005). Interestingly, in the group of patients, the levels of the DNA damage increased as the disease progressed (Table 1).
Discussion

Increased production of ROS can induce oxidative damage to lipids and as a result of this are formed lipid peroxidation products such as MDA. Our results showed that MDA levels were significantly increased in all SCA2 patients as compared with the control group. This could be due to an extensive generation of ROS, thus leading to the uncontrolled progression of peroxidative damage to lipids and cellular membranes. Oxidative stress increases MDA and the cytotoxic effects of this peroxidation product can induce a reduction of membrane fluidity, cause DNA damage and directly inhibit several proteins.\(^\text{15}\)

In this study, increased of MDA levels in the plasma of SCA2 patient was consistent with the finding of Delgado \textit{et al.} (2002) in a complementary study with SCA2 patients.\(^\text{16}\) According with our results, increased MDA concentration has been reported in other hereditary ataxias. Bradley \textit{et al.} (2004) reported elevated MDA levels were in the plasma from FRDA patients.\(^\text{17}\) Reichenbach \textit{et al.} (2002), who report higher levels of MDA in patients with AT.\(^\text{6}\) Also elevated levels of MDA have been observed in other neurodegenerative pathologies, such as PD and ALS, confirming a pathophysiological role of oxidative stress in these diseases.\(^\text{2,18,19}\)

Antioxidant enzymes such as SOD and CAT play an important role in protecting cells from oxidative damage; SOD converting superoxide radicals into hydrogen peroxide, which is then further metabolized by CAT. The rate of dismutation of the superoxide anion results in an increase of \(\text{H}_2\text{O}_2\) and protection from this reactive species would be conferred only by an increased CAT and Glutathione Peroxidase activities.\(^\text{20}\) Theoretically at least, the balance between the first and second step antioxidant enzymes may be critical.\(^\text{21}\) Our data showed a concomitant increase in SOD and CAT activities in SCA2 patients compared with healthy control subjects. Considering these results it might be suggested that the ratios of activi-
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On the first and second steps antioxidant enzymes were increased in order to protect the organism from oxidative damage and could be a compensatory mechanism by the body to prevent tissue damage. Our results are in agreement with previous reports in other hereditary ataxias. Tozzi et al. reported evidence of an impairment of enzymatic antioxidants in FRDA, supporting a relevant role of free radical damage in the pathophysiology of neurodegeneration.1 On the other hand, Aksoy et al., found significantly higher SOD and Catalase activities of erythrocytes in AT patients, compared to healthy control subjects and they suggest that increased antioxidant activities of erythrocytes in these patients may be an indication of the presence of constant oxidative stress.2

Myotonic Dystrophy type 1 (DM1) is a disease with genetic basis similar to SCA2, that consists of a mutational expansion of a repetitive trinucleotide sequence. Previous study has revealed that DM1’s patients showed significantly higher levels of SOD than normal controls.3

Cellular DNA is continuously exposed to insults from exposure to endogenous and exogenous electrophilic agents and oxidative stress.4 ROS also induce oxidative damage of DNA, including strand breaks and base and nucleotide modifications, particularly in sequences with high guanosine content.5 Our study is the first attempt to investigate peripheral cell blood DNA damage in patients with SCA2 using the Comet assay. We found that peripheral blood cell from patients with SCA2 exhibited higher levels of DNA damage than those from controls.

The consequences of DNA damage are manifold, in addition to mutagenic lesions, blocks of transcription or replication leading to altered gene regulation and expression are probable.6 A major source of damage could be the genotoxic action of lipid peroxidation compounds. These are, however, very complex and heterogeneous.7 Our studies presented here have shown that the DNA endogenous damage could be due to actions of lipid peroxidation products.

On the other hand, the degree of DNA damage is based on the genotoxic or oxidative stress prevailing in a given tissue, the levels of endogenous detoxifying systems, and the DNA repair capacity.6 Therefore, we suggested that DNA damage observed in these patients could be a consequence of an imbalance between lesion inductions from radical processes and repair. It has been reported that reduced repair will result in elevated lesions and an increased risk of disease.8

In a number of genetically determined disorders, collectively known as the chromosome breakage syndromes or DNA-repair disorders, such as Ataxia Telangiectasia, Xeroderma Pigmentosum, Trichohydystrophy, and the Cockayne syndrome, neurological symptoms as primary feature of their phenotypes have been described. They are characterized by spontaneous or induced susceptibility to chromosomal breakage and represent a well-known connection between DNA damage and neurodegeneration.9 One possibility to account for this neurodegeneration is the potential genotoxicity of endogenous ROS, generated as byproducts of cellular metabolism; in repair-compromised individuals, these agents could exert a genotoxic role in the nervous system.29-31

The relationship between DNA damage and increasing disease duration suggested that DNA damage increased as the disease progressed. It was suggested that oxidative damage may play a more important role as the disease progresses and antioxidant therapy may help to alleviate this facet of disease progression. The absence of any correlation with CAG size or age at onset suggested the severity of the genetic abnormality was less influential in determining the extent of DNA damage (data not shown). Similar findings have been obtained previously by means of different methods on peripheral DNA damage, among neurodegenerative diseases such as AD, PD, ALS, FRDA and AT.2, 7, 27, 32, 33

In conclusion, this study provides evidence of oxidative damage to different molecules and its impact on antioxidant system in SCA2 patients. Our findings show evidence of an increased sensitivity to oxidative stress in these patients as compared with healthy people, suggesting that oxidative stress could be participating in the SCA2 pathogenesis, at least partially.

Furthermore, the methods used are relatively inexpensive, easily performed and require non-time-consuming procedures. The biochemical tests evaluated could contribute to an integral overview in SCA2 patients and could be used as indices of treatment efficacy. Nowadays, there isn’t an effective treatment of SCA2 and we found that oxidative stress is implicated in the progression of the disease; perhaps antioxidant consumption could ameliorate the symptoms. Further studies, particularly, clinical trials will be needed to probe this hypothesis.

References


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