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Oxidative stress in myotonic dystrophy type 1

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Abstract
Myotonic dystrophy type 1 (DM1) is the most common form of muscular dystrophy affecting adults. The genetic basis of DM1 consists of a mutational expansion of a repetitive trinucleotide sequence (CTG). The number of triplets expansion divides patients in four categories related to the molecular changes (E1, E2, E3, E4). The pathogenic mechanisms of multi-systemic involvement of DM1 are still unclear. DM1 has been suspected to be due to premature aging, that is known to be sustained by increased free radicals levels and/or decreased antioxidants activities in neurodegenerative disorders. Recently, the gain-of-function at RNA level hypothesis has gained great attention, but oxidative stress might act in the disease progression. We have investigated 36 DM1 patients belonging to 22 unrelated families, 10 patients with other myotonic disorders (OMD) and 22 age-matched healthy controls from the clinical, biochemical and molecular point of view. Biochemical analysis detected blood levels of superoxide dismutase (SOD), malonilaldehyde (MDA), vitamin E (Vit E), hydroxyl radicals (OH) and total antioxidant system (TAS). Results revealed that DM1 patients showed significantly higher levels of SOD (+40%; $p < 0.003$), MAL (+57%; $p < 0.03$), RAD 2 (+106%; $p < 0.000002$) and TAS (+20%; $p < 0.05$) than normal controls. Our data support the hypothesis of a pathogenic role of oxidative stress in DM1 and therefore confirm the detrimental role played by free radicals in this pathology and suggest the opportunity to undertake clinical trials with antioxidants in this disorder.

Keywords: *Myotonic dystrophy, oxidative stress, superoxide dismutase, free radicals, premature aging*

Introduction

Myotonic dystrophy is an autosomal dominant multi-systemic disease with a severe phenotype characterized by a variable involvement of skeletal muscle, heart, eye, brain and endocrine system. Two different genetic loci have been associated with DM phenotypes. In myotonic dystrophy type 1 (DM1) the gene encoding the myotonin protein kinase (MtPK) is located on chromosome 19q13.3 and has an abnormal CTG repeat expansion in the 3′ untranslated region, whereas the mutation responsible for myotonic dystrophy type 2 (DM2) is a CCTG-repeat expansion in intron 1 of the gene coding for the ZNF9 gene on chromosome 3q21.3 [1]. However, the DM1 pathogenesis and the function of the gene product, MtPK, as well as their relationship with the complex multisystemic features of the disease are still unclear. Different mechanisms have been postulated as haploinsufficiency of the MtPK, altered expression of neighbouring genes and pathogenic effects of the CUG expansion in RNA [2,3,4]. In the last mechanism the enlarged CUG-containing transcripts, that accumulate as foci in the nuclei, exert a trans-dominant effect that disrupts splicing and possibly other cellular functions [5]. In an additive model proposed each of the above mechanisms contributes to DM1 pathogenesis [6]. Previous studies described

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DM1 as a disease due to premature aging [7] and free radicals production, lipid peroxides levels and the antioxidant system have been investigated in DM1, supporting a possible role of oxidative stress in its pathogenesis [8]. Further evidences showed an increased susceptibility to oxidative stress in cells with expanded CTG repeats and that this susceptibility and the signalling pathways evoked by oxidative stress are CTG repeats number-dependent [9,10].

Even if recently the gain-of-function alteration in RNA hypothesis has gained great attention, the downstream effects on the cell are still not clear [3,4]. The aim of our study was to verify if oxidative stress contributes to alter the cellular homeostasis, being a cofactor in DM1 pathogenesis. Therefore we measured several oxidative stress parameters in a large population of DM1 patients, in patients with other different myotonic disorders and in healthy controls.

Materials and methods

We selected 3 groups of subjects to be analysed: (1) 36 patients (mean age: 27 years; SD: 11), belonging to 22 unrelated families, that met the diagnostic criteria for definite DM1 [11]; (2) 10 disease controls with other myotonic disorders (mean age: 34.5 years, SD:17), as Thomsen and Becker diseases (other myotonic disorders); 3) 22 age and sex-matched healthy controls (mean age:32.5 years, SD: 11,9). All the subjects were not on medications or supplements at the time of the study.

DM1 patients were subdivided in four groups according to CTG repeat expansions criteria [12]: E1 (from 50 to 500 triplets), E2 (from 501 to 1000), E3 (from 1001 to 1500) and E4 (above 1500).

Biochemical analysis

We performed an extensive biochemical analysis to determine the levels of superoxide dismutase (SOD), malondialdehyde (MDA), vitamin E (Vit E), hydroxyl radicals (OH) production before (RAD 1) and after (RAD 2) treatment with salicylic acid and total antioxidant status (TAS) in blood samples. Technical description of specific assays are herein reported as follows.

SOD analysis. SOD activity was evaluated in order to estimate endogenous defences against superoxide anions. Blood sample (1.0 ml) was collected in polyethylene tubes with previous addition of 10 μl of heparin solution (1,000 IU). The plasma samples, obtained after centrifugation at 3,000*g* for 10 min at 4°C, were discarded and red cells were cleaned, about five times, with isotonic solution (NaCl 0.9%). SOD activity was assayed spectrophotometrically at 505 nm by using a commercial kit (Ransod assay kit, cat. No. Sd 125, Randox Laboratories, Crumlin, UK). Briefly, 50 μl of diluted samples (1:200, *w*/*v* with 0.01 M phosphate buffer, pH 7.0) were mixed with 1.7 ml of solution containing 0.05 mM xanthine and 0.025 mM iodonitrotetrazolium chloride. After mixing for 5 s, 250 μl of xantine oxidase (80 U/l) were added. Initial absorbance was immediately read and the final absorbance was read after 3 min. A standard curve of SOD solution (from 25 to 400 U/ml) was also run for quantitation. All standard and sample rates were converted into percentage of sample diluent rate and subtracted from 100% to give a percentage inhibition. Sample SOD activities were obtained from a plotted curve of the percentage inhibition for each standard.

MDA measurement. Assessment of the MDA levels was carried out in plasma sample as index of lipid peroxidation in cell membranes. Blood sample (1.0 ml) was collected in polyethylene tubes with previous addition of 10 μl of heparin solution (1,000 IU). The plasma samples, obtained after centrifugation at 3,000*g* for 10 min at 4°C, were frozen at −70°C until the analysis. The assay was performed using a colorimetric commercial kit (Lipid peroxidation assay kit, cat. No. 437634, Calbiochem, La Jolla, CA, USA). Briefly, 0.65 ml of 10.3 mM N-methyl-2-phenyl-indole in acetonitrile were added to 0.2 ml of homogenate supernatant. After vortexing for 3–4 s and adding 0.15 ml HCl 37%, samples were mixed well and closed with a tight stopper and incubated at 45°C for 60 min. The samples were then cooled on ice and the absorbance was measured spectrophotometrically at λ: 586. A calibration curve of an accurately-prepared standard MDA solution (from 0.5 to 35 μM) was also run for quantification.

Vitamin E evaluation. Plasma Vit E levels were measured in order to determine the cell oxidative state. Blood sample (2.5 ml) was collected in polyethylene tubes with previous addition of 10 μl of heparin solution (1,000 IU). The plasma samples, obtained after centrifugation at 3,000*g* for 10 min at 4°C, were frozen at −70°C until the assay. The analysis was carried out by using a high performance liquid chromatography (HPLC) method with some modifications [13]. Briefly, 1.0 ml of plasma samples, contained in polycarbonate tubes lined with tin foil, was treated with 200 μl of tocopherol acetate (25 μg/ml in ethanol), which has been used as an internal standard, and with 2.0 ml of butanol/ethyl acetate (1:1, *v*/*v*). After vortexing for 20 s, 100 mg of sodium sulfate were added and the sample was shaken on vortex mixer for additional 60 s. After centrifugation at 15,000*g* for 5 min at 4°C, the organic layer was recovered and 50 μl aliquot was injected into the HPLC apparatus. The column used was an ultratechsphere C18, 250 × 4.6 mm, 5 μm

particle size. The mobile phase was methanol/water (97:5, *v*/*v*) at a flow rate of 1 ml/m at room temperature. The UV detector was set at wavelength of 280 nm.

Measurement of OH formation. In order to quantify OH production in the muscle of patients, we used the "aromatic trap" technique [14]. Sodium salicylate or acetyl salicylic acid serves as a specific trap for hydroxyl radicals because it can react chemically with the OH. Radicals produced, yielding 2,5-dihydroxybenzoic acid (2,5-DHBA), 2,3-dihydroxybenzoic acid (2,3-DHBA) and catechol as its hydroxylation derivatives in an approximate proportion of 40, 49 and 11%, respectively [15,16]. In the present study we measured only 2,5-DHBA, since it better reflects the salicylate-hydroxyl radical reaction. To allow the chemical reaction, each subject received a single dose of acetyl salicylic acid load (800 mg). Blood samples (1.5 ml) were drawn at time 0 (basal control - RAD1) and two hours after (RAD2) acetyl salicylic acid administration, respectively. The blood was collected in polyethylene tubes with previous addition of 10 μl of heparin solution (1,000 IU). The plasma samples, obtained after centrifugation at 3,000*g* for 10 min at 4°C, were frozen at −70°C until the assay. To measure 2,5-DHBA formation, an HPLC technique was used [14,15]. Briefly, 0.5 ml of plasma was treated with 20 μl of 100 μM 2,4-dihydroxybenzoic acid (2,4-DHBA), which has been used as an internal standard and 20 μl of 40% $HclO_4$. Then plasma was extracted with 5.0 ml HPLC grade diethylether and mixed on a vortex for 2 min. After centrifugation for 15 min at 15,000*g* at 4°C, the diethylether layer was then evaporated in a vacuum concentrator system. The residue obtained was dissolved in 60 μl of 0.1 N HCl and 65 μl of mobile phase, then 50 μl of the solution was injected into the HPLC apparatus. The column used was a Lichrosorb- 10-RP18, 10 μm 250 × 4.6 mm, attached to a precolumn. The mobile phase was 80% 0.03 M citric acid, 0.03 M acetic acid buffer (pH 3.6) and 25% methanol at a flow rate of 1.3 ml/min. The UV detector was set at a wavelength of 315 nm.

TAS evaluation. The total antioxidant status was assayed in order to evaluate the global antioxidant defences of the subject's blood. Blood sample (1.0 ml) was collected in polyethylene tubes with previous addition of 10 μl of heparin solution (1,000 IU). The plasma samples, obtained after centrifugation at 3,000*g* for 10 min at 4°C, were frozen at −70°C until the analysis. The assay was performed by using a colorimetric commercial kit (Total antioxidant status assay kit, cat. No. NX2332, Randox Laboratories, Crumlin, UK). Briefly, 0.25 ml of 610 μM ABTS and 6.1 mM peroxidase were added to 0.2 ml of plasma. After vortexing for 3–4 s, initial absorbance (600 nm) was read (A1). Then, 50 μl H_2O_2 250 μM were added and after 3 min the final absorbance was read again (A2). Values of unknown samples were obtained by a multiplication of the differences in absorbance (A2–A1) for a factor obtained by the division of standard concentration and the differences between standard absorbance and blank absorbance.

Statistical analysis

Multiple comparisons were evaluated statistically by the analysis of variance. Two-group comparisons were analysed by the two-tailed Student's *t* test for independent samples. For all procedures, probability values of less than 0.05 were considered statistically significant.

Results

DM1 patients showed significantly higher levels of SOD (+40%; $p < 0.003$), MAL (+57%; $p < 0.03$), RAD 2 (+106%; $p < 0.001$) and TAS (+20%; $p < 0.05$) than normal controls. Interestingly, RAD 2 level, after an oral salicylate load, was also significantly increased in DM1 versus OMD (+87%; $p < 0.001$) (Figure 1).

In DM1 group there was a significant inverse relationship between CTG-repeats and age of onset (r: −0.35; $p < 0.05$), but biochemical parameters of oxidative stress were not correlated to any clinical or laboratory variable. When DM1 patients were divided in four groups according to CTG repeat size, E1 patients had a significantly higher age of onset than E2, E3, and E4 groups (respectively $p < 0.005$, $p < 0.02$, $p < 0.02$). Among the different markers of oxidative stress measured, only SOD value was significantly higher in E4 versus E3 group ($p < 0.02$) (Table I).

Discussion

The oxidative damage is an irreversible phenomenon, that exponentially accumulates with age, resulting in the senescence-associated attenuation of various vital mechanisms. Oxidative stress has been postulated to play an important role in several neurodegenerative conditions [17–19] and in some neuromuscular disorders such as mitochondrial disorders and dystrophinopathies [20,21]. DM1 has been suspected to be a multisystemic disorder due to premature aging and that oxidative stress could be involved in the pathogenesis of the disease [7]. Ihara et al. demonstrated increased levels of free radicals and lipid peroxides as well as a reduced cellular antioxidant activity [8]. Moreover, further evidences came from Usuki et al. in two consecutive papers suggesting

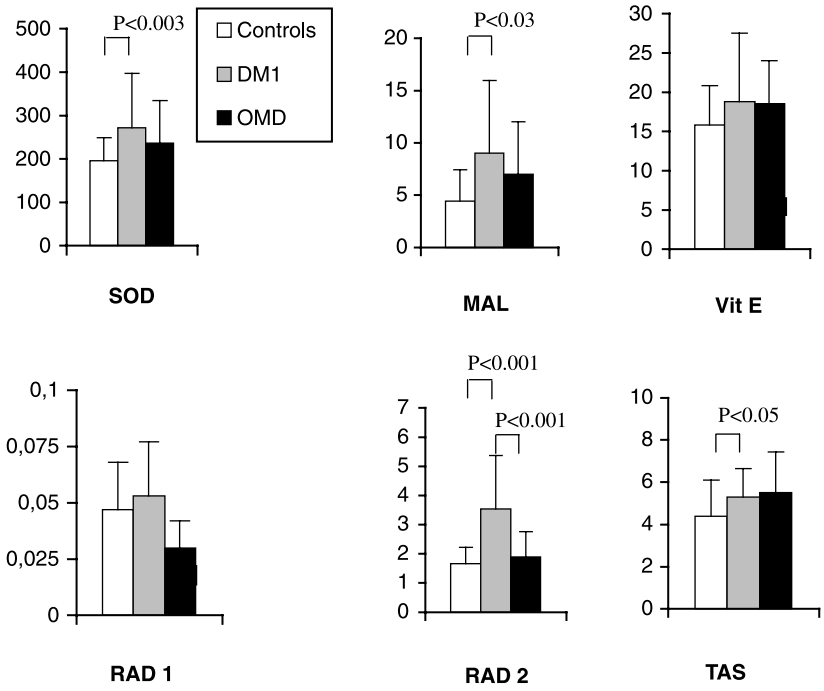

Figure 1. Values (mean ± SD) of SOD, MAL, Vit E, RAD1, RAD 2, TAS in normal controls, DM1 and OMD patients.

that: (1) mild expanded CTG repeats in MtPK may amplify cell susceptibility to oxidative stress [9]; (2) cells transfected with MtPK cDNAs containing a different number of CTG repeats may evoke different signalling pathways after oxidative stress [10]. Only in one study, by Rodriguez and Tarnopolsky, it has been demonstrated an increase in oxidative stress in patients with Duchenne muscular dystrophy, but not in patient with DM1 [21]. However, these data come as a result of a different experimental approach used to study the oxidative stress. In fact the utilization of 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is a specific indicator of DNA damage, cannot evidence the total antioxidant status neither the cell membrane integrity. Recently, the use of transgenic models in which CTG repeats, expressed at RNA level, cause myotonia and muscular dystrophy [22,23] and the fact that altered CUG- and CCUG-induced splicing changes are directly related to phenotypic features together with other evidences, have provided a convincing model of DM1 and DM2 pathogenesis. A lot of discussion still revolves around the role of RNA foci in disease onset and progression [3,4]. Similarly in other neurodegenerative diseases, such as Alzheimer disease and those caused by polyglutamine expansions, protein aggregates or plaques accumulate in affected tissues and have led investigators to debate their role in disease pathogenesis [24,25]. Interestingly, in these and other degenerative disorders, i.e. Parkinson's disease, Huntington's disease,

Table I. Clinical and biochemical data in the four groups of DM1 patients according to CTG repeat size.

	Age (years)	Disease onset (years)	Disease duration (years)	SOD (μM)	
E1	44.2± 10.5	34.8 ± 10.9	9.4 ± 4.7	260.4 ± 113.1	
E2	32.2 ± 11.3	19.5 ± 6.9	12.7 ± 8.3	301.7 ± 150.8	
E3	33.45 ± 14.2	19.1 ± 10.7	14.4 ± 7.7	207.1 ± 70,43	
E4	32.1 ± 13	19.5 ± 7.76	12.6 ± 9.2	327.5 ± 129,5	
Significane	NS	E1 > E2:p < 0.005 E1 > E3:p < 0.02 E1 > E4:p < 0.02	NS	E3 > E4:p < 0.02	
	MAL (μM)	VIT E (mg/l)	RAD1 (μM)	RAD2 (μM)	TAS (mM)
E1	8.41± 7.51	14.2 ± 2.4	0.046 ± 0.015	2.6 ± 1.1	5.1 ± 0.58
E2	5.5 ± 2.7	18.9 ± 6.79	0.06 ± 0.02	4.2 ± 2.3	5.6 ± 1.4
E3	5.1 ± 1.8	17.8 ± 10.3	0.04 ± 0.02	3.17 ± 1.9	4.7 ± 1.2
E4	10.3 ± 8	23.1 ± 10.5	0.05 ± 0.02	3.64 ± 0.9	5.7 ± 1.6
Significance	NS	NS	NS	NS	NS

Creutzfeldt-Jacob disease and ALS (Amyotrophic Lateral Sclerosis)[17,18], it has been also demonstrated an increase in free radicals levels and/or a decrease in antioxidant activities, supporting the role of oxidative stress in their pathogenesis. Moreover, in many of them, therapeutic approaches with antioxidants have been attempted [26–28].

Our study on a larger cohort of DM1 patients showed a significant increase of several markers of oxidative stress such as SOD, MAL, RAD 2 and of the whole antioxidant system. These markers were strongly increased in DM1 patients when compared to normal controls and RAD 2 was also significantly higher than in the group of OMD. These data well support the hypothesis that oxidative damage is present in the cells of DM1 patients and might contribute to the disease progression.

Given the fact that RNA misregulation might play a central role in DM1 pathogenic mechanism, our study identifies an other concomitant factor, the oxidative stress, that can be involved in disease onset and progression and that is potentially treatable. Recently, gene therapy attempts have been tried to overcome the RNA defects. Two studies demonstrated that viral vectors producing antisense RNA and ribozyme-mediated mutant DMPK mRNAs cleavage can improve human DM1 myoblast functions [29,30].

On the basis of our results and of previous observations and waiting for a possible gene therapy, the possibility to undertake therapeutic trials with combined treatments with dietary management, supplemental vitamins, cofactors (i.e. coenzyme Q10) and antioxidants should be considered.

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