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Oxidative stress and inflammation in mucopolysaccharidosis type IVA patients treated with enzyme replacement therapy

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ABSTRACT

Mucopolysaccharidosis type IVA (MPS IVA) is an inborn error of glycosaminoglycan (GAG) catabolism due to the deficient activity of N-acetylgalactosamine-6-sulfate sulfatase that leads to accumulation of the keratan sulfate and chondroitin 6-sulfate in body fluids and in lysosomes. The pathophysiology of this lysosomal storage disorder is not completely understood. The aim of this study was to investigate oxidative stress parameters, pro-inflammatory cytokine and GAG levels in MPS IVA patients. We analyzed urine and blood samples from patients under ERT ($n = 17$) and healthy age-matched controls ($n = 10$–15). Patients presented a reduction of antioxidant defense levels, assessed by a decrease in glutathione content and by an increase in superoxide dismutase activity in erythrocytes. Concerning lipid and protein damage, it was verified increased urine isoprostanes and di-tyrosine levels and decreased plasma sulfhydryl groups in MPS IVA patients compared to controls. MPS IVA patients showed higher DNA damage than control group and this damage had an oxidative origin in both pyrimidine and purine bases. Interleukin 6 was increased in patients and presented an inverse correlation with GSH levels, showing a possible link between inflammation and oxidative stress in MPS IVA disease. The data presented suggest that pro-inflammatory and pro-oxidant states occur in MPS IVA patients even under ERT. Taking these results into account, supplementation of antioxidants in combination with ERT can be a tentative therapeutic approach with the purpose of improving the patient's quality of life. To the best of our knowledge, this is the first study relating MPS IVA patients with oxidative stress.

1. Introduction

Mucopolysaccharidoses (MPSs) are a heterogeneous group of inherited lysosomal storage disorders (LSDs) caused by deficiency of the enzymes involved in the degradation of glycosaminoglycans (GAGs). MPSs are classified into 11 syndromes according to the specific deficient enzyme [1]. MPS IVA (or Morquio A Syndrome) is a rare disorder with an incidence in the general population estimated in 1:201,000 [2]. However, this incidence ranges among different populations from 1 in 76,000 live births in Northern Ireland to 1 in 640,000 live births in Western Australia [2]. This disorder is caused by a defect on N-acetylgalactosamine-6-sulfate sulfatase enzyme (GALNS; E.C.3.1.6.4),

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CAT, catalase; Cr, creatinine; DI, damage index; Di-Tyr, di-tyrosine; DMB, 1,9-dimethylmethylene blue; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Endo III, endonuclease III; ELISA, enzyme-linked immunoassay; ERT, enzyme replacement therapy; FU, fluorescence units; GAGs, glycosaminoglycans; GALNS, N-acetylgalactosamine-6-sulfate sulfatase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione oxidized; H_2O_2, hydrogen peroxide; IEM, inborn errors of metabolism; IL-6, interleukin 6; LSDs, lysosomal storage diseases; MDA, malondialdehyde; MPS, mucopolysaccharidoses; $OH^{\bullet}$, hydroxyl radical; RNA, ribonucleic acid; SEM, standard error of the mean; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNB, tionitrobenzoic acid

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responsible for chondroitin 6-sulfate and keratan sulfate degradation. The GALNS enzyme deficiency leads to the accumulation of GAGs and subsequent cellular damage, mainly in connective tissues where keratan sulfate is abundant, specially cartilage, cornea and heart valve [3,4]. The main clinical manifestations of MPS IVA patients are: skeletal dysplasia, restricted growth and short stature, joint hypermobility, valvular heart disease, pulmonary disease, corneal clouding, hearing loss, and poorly formed teeth [5]. It was already described about 277 mutations in the *GALNS* gene, and this extensive allelic heterogeneity is consistent with the broad spectrum of phenotypes observed in MPS IVA [6,7]. The characteristic signs and symptoms of this disease appear at around two or three years of age. Phenotypic spectrum may range from a more attenuated phenotype, with patients reaching the age of 70 years, to a more severe phenotype, where patients survive no more than 20–30 years [5,8–10]. MPS IVA patients have no neurological impairment, differently of many other MPSs and inborn errors of metabolism (IEM) [11].

Diagnosis of MPS IVA is typically based on clinical examination, skeletal radiographs, evaluation of the pattern of urinary GAGs and measurement of the enzymatic activity of GALNS in white blood cells or fibroblasts [5,10,12]. Until recently, there was no effective therapy for MPS IVA and treatment was palliative, focusing on management of the multiple clinical symptoms [10,13]. The recent introduction of ERT with recombinant human enzyme (elosulfase alfa), however, has raised the possibility that the burden of GAGs storage can be reduced and that the progressive tissue and organ damage associated with MPS IVA can be slowed or even prevented. In a recent study, MPS IVA patients undergoing ERT showed improvements in 6-minute-walk test and a decrease in keratan sulfate levels [14].

Oxidative stress may play an important role in the pathophysiology of some IEM. Even though this relationship is not well elucidated, the accumulation of toxic metabolites is considered the main responsible for the increased generation of reactive species, which can react with lipids, proteins, DNA and RNA [15–18]. Most studies linking oxidative stress and MPS were performed in animal models. In this context, we can mention the study developed by Villani et al. [19] that showed the presence of oxidative stress even in the early stages of MPS IIIB, and another work demonstrating an oxidative imbalance in an animal model of MPS I [20]. Furthermore, a possible link between inflammation and oxidative stress in MPS IIIA mice was reported by Arfi et al. [21]. In animal models of MPS VI and VII, it was verified an inflammation process caused by intralysosomal accumulation of GAGs, which could trigger the release of cytokines, chemokines, proteases and nitric oxide, culminating in apoptosis and consequent destruction of connective tissue [22]. Ohmi et al. [23] showed microglia activation in mouse models of MPS I and IIIB, and this activation is one possible explanation for the involvement and occurrence of inflammatory processes along with oxidative damage in various diseases. In mice with MPS IIIB, Di Domenico et al. [24] demonstrated a significant reduction in the expression of several genes related to oxidative stress and inflammation, after six months of gene therapy, corroborating with the hypothesis that both processes are involved in the pathogenesis of MPS IIIB.

With regard to research in patients, there are few reports relating MPS with oxidative stress and inflammation. Pereira et al. [25] found high lipid peroxidation levels in MPS I patients. Moreover, they showed that ERT was able to induce an increase of catalase (CAT) and a decrease of superoxide dismutase (SOD) activities at specific times of treatment. However, these enzymatic changes were not sufficient to reduce lipid peroxidation levels, suggesting an important role of oxidative stress in the pathophysiology of MPS I, even during treatment. In a study with MPS II patients, it was observed oxidative damage to proteins and lipids, as well as increased CAT activity and decreased total antioxidant status before treatment when compared to controls. In these patients, until the third month of the ERT, there was a decrease in lipid peroxidation levels and in protein damage, suggesting a possible involvement of oxidative stress in the pathophysiology of MPS II and a protective role of therapy [26]. In addition, ERT was capable to decrease DNA damage [27] and also GAG levels in MPS II patients [28], what suggests that the decrease of metabolites accumulation probably may be related to the improvement of oxidative stress parameters. Nevertheless, patients with Fabry disease, another type of LSD, under ERT, presented high levels of oxidative damage to proteins and lipids, accompanied by low antioxidant defenses and high pro-inflammatory cytokines [29]. This study also showed, in treated Fabry patients, a positive correlation between oxidative stress, inflammation and the metabolites accumulated in this disease.

Considering the involvement of oxidative stress and inflammation in other MPSs and the lack of reports in this issue in MPS IVA, the aim of this study was to evaluate oxidative damage to biomolecules, antioxidant defenses, pro-inflammatory cytokine and GAG levels in patients with Morquio A disease.

2. Materials and methods

2.1. Subjects

For this study it was collected blood and occasional urine samples from 17 MPS IVA patients with ages varying between 6 and 36 years (15.3 ± 8.9 years old, mean ± standard deviation). For the control group we collected blood from 14 healthy individuals with ages ranging between 10 and 28 years (24.0 ± 4.8 years old, mean ± standard deviation) and for comet assay from 10 healthy individuals with ages varying between 19 and 23 years (20.8 ± 1.5 years old, mean ± standard deviation). Urine was collected from 15 healthy individuals with ages between 7 and 32 years (19.7 ± 7.9 years old, mean ± standard deviation). All MPS IV A patients were receiving ERT treatment (elosulfase alfa—Vimizim® 2 mg/kg) every week by intravenous infusion, for about 32 weeks. At the moment of diagnosis, patients presented the classic symptoms, usually including short stature, skeletal deformities (pectus carinatum and genu valgum almost always present), limited ambulation, restrictive airway disease, heart valves problems and corneal clouding. Patients' data were described in Table 1. Diagnosis was confirmed by evaluation of GAGs in urine (increased total content and presence of increased amounts of keratan sulfate) and measurement of GALNS in leucocytes (deficient activity) [10,30].

Informed consent was obtained from all participants. The study was approved (number 13-0246) by The Ethics Committee of the *Hospital de Clínicas de Porto Alegre* (HCPA), RS, Brazil.

2.2. Sample collection and preparation

Occasional urine and heparinized blood samples were obtained from patients immediately before the session of ERT. Urine samples were collected in sterile flask, aliquoted and frozen at −80 °C until analysis. Samples were obtained from controls concomitantly. Whole blood was centrifuged at 1000 ×*g* for 10 min and plasma was removed by aspiration, aliquoted and frozen at −80 °C until biochemical determinations. An aliquot of whole blood was separated for comet assay. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride) and the lysates were prepared by addition of 1 mL of distilled water to 100 μL of washed erythrocytes. The lysates were frozen at −80 °C until determination of GSH and antioxidant enzymes activities. For these determinations, the supernatant (after centrifugation at 13,500 ×*g* for 10 min) was diluted in order to contain approximately 0.5 mg/mL of protein.

2.3. Reduced glutathione (GSH) content in erythrocytes

In order to measure GSH levels, the main intracellular antioxidant, lysates of erythrocytes were processed as described by Browne and Armstrong [31] and the fluorescence measured (λexcitation = 350 nm,

Table 1
Data of studied MPS IVA patients.

Patient	Age (years)	Gender	Race and ethnicity	Height (cm)	Weight (kg)
1	34.4	Female	White-Hispanic/Latin	100	21.75
2	8.5	Male	White-Hispanic/Latin	91.1	16.60
3	36.3	Female	White-Hispanic/Latin	97.9	20.45
4	10.6	Female	White-Hispanic/Latin	84.9	14.45
5	13.4	Male	Mulatto-Hispanic/Latin	87.5	15.10
6	12.5	Male	White-Hispanic/Latin	84.3	17.30
7	10.6	Female	White-Hispanic/Latin	110	15.05
8	12.8	Female	Mulatto-Hispanic/Latin	107	27.85
9	9.5	Male	White-Hispanic/Latin	97.2	17.30
10	16.3	Male	White-Hispanic/Latin	107.6	31.10
11	6.7	Female	White-Hispanic/Latin	90.3	14.30
12	14.4	Male	Black-Hispanic/Latin	96.4	21.00
13	9.5	Female	Black-Hispanic/Latin	90.9	13.60
14	13.4	Female	Mulatto-Hispanic/Latin	101.4	25.55
15	17.4	Male	White-Hispanic/Latin	112	29.45
16	6.7	Male	White-Hispanic/Latin	96.2	16.75
17	26.9	Female	White-Hispanic/Latin	89.6	24.3

λemission = 420 nm) was compared to a calibration curve prepared with GSH solutions. Results were expressed as nmol/mg protein.

2.4. Erythrocyte superoxide dismutase (SOD) activity

SOD activity was measured using the RANSOD® kit (Randox Lab, Antrim, United Kingdom). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical, which is produced by the incubation with the xanthine–xanthine oxidase reaction system. The absorbance of the product is measured spectrophotometrically at 505 nm. One unit of SOD corresponds to a 50% inhibition of red formazan formation. The specific activity of SOD was expressed as U/mg protein.

2.5. Erythrocyte glutathione peroxidase (GPx) activity

Erythrocyte GPx activity was measured by using a commercially available kit (RANSEL®; Randox Lab). GPx catalyses the oxidation of glutathione (GSH) to GSSG (oxidized glutathione). In the presence of glutathione reductase (GR) and NADPH, the oxidized GSSG is converted to the reduced form with a concomitant oxidation of NADPH to NADP. The decrease in absorbance after 1 and 2 min at 340 nm was measured and results were expressed in U/mg protein.

2.6. Urine 15-F2t-isoprostane levels

15-F2t-isoprostane, a product of arachidonic acid metabolism and a biomarker of lipid peroxidation, was measured by a competitive enzyme-linked immunoassay (ELISA) (Oxford Biomed, EA 85), according to the kit's instructions. First, the urine samples were mixed with dilution buffer. In this assay, the 15-F2t-isoprostane in the urine samples competes with the 15-F2t-isoprostane conjugated to horseradish peroxidase for the binding to a specific antibody fixed on the microplate. The concentration of 15-F2t-isoprostane was determined spectrophotometrically at 630 nm by the intensity of color developed after the addition of substrate. Results were expressed as picograms of isoprostanes per mg of urinary creatinine (pg/mg Cr).

2.7. Urine di-tyrosine (di-Tyr) levels

In order to determine the levels of protein oxidation in urine, the intensity of di-Tyr fluorescence was measured according to the method described by Kirschbaum [32]. For Di-tyr fluorescence determination, 50 μL of thawed urine was added to 950 μL of 6 mol/L urea in 20 mmol/L sodium phosphate buffer pH 7.4. After 30 min, the concentration was measured using a fluorometer (excitation 315 nm, emission 410 nm). Results were expressed as fluorescence units per mg urine creatinine (FU/mg Cr).

2.8. Total plasmatic level of sulfhydryl groups

The plasmatic concentration of sulfhydryl groups was determined as described by Aksenov and Markesbery [33]. The method is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by sulfhydryl groups into a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as μmol TNB/mg protein.

2.9. Alkaline comet assay in leukocytes

The alkaline comet assay, that measures single and double DNA strand breaks, was performed as described by Singh et al. [34] in accordance with general guidelines for use of the comet assay [35]. Aliquots of 100 μL from whole blood were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Slides were placed in ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10.0–10.5 with 10% DMSO and 1% Triton X-100) to remove cell proteins and leaving DNA as "nucleoids". After the lysis-buffer procedure, the slides were covered with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 15 min to allow DNA unwinding and then, electrophoresis was performed for 15 min (25 V; 300 mA; 0.9 V/cm). Slides were neutralized with 0.4 M Tris (pH 7.5), washed in bi-distilled water and stained using silver nitrate staining protocol [36]. After drying at room temperature overnight, the samples were analyzed using an optical microscope. One hundred 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 (DI) was calculated, ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) [35,37]. The slides were analyzed under blind conditions at least by two different individuals.

A digestion step with a bacterial repair enzyme was included in the alkaline comet assay before electrophoresis, in accordance to Dizdaroglu et al. [38]. The enzyme used was endonuclease III (Endo III, also known as Nth) which converts oxidized pyrimidines (including thymine glycol and uracil glycol) to strand breaks. It was obtained from New England Biolabs (NEB, Ipswich, MA). After lysis, each slide was washed for 5 min in an enzyme buffer (40 mM HEPES-KOH, 1 M KCl, 5 mM EDTA, 2.5 mg/mL bovine serum albumin fraction V-BSA, and pH 8.0). The suspension was added to the slide, covered with coverslip, and incubated for 45 at 37 °C. Subsequent steps were the same as in the alkaline version of comet assay. DNA damage with Endo III was calculated as the score obtained with enzyme minus the score without enzyme (basal).

2.10. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels

Urinary 8-OHdG levels, a product of DNA oxidative damage due to the hydroxyl radical attack at the C8 position of deoxyguanosine, were determined by Highly Sensitive 8-OHdG Check ELISA kit (JaICA, Fukuroi, Japan). The 8-OHdG Check ELISA kit is a competitive *in vitro* enzyme-linked immunosorbent assay for quantitative measurement of the oxidative DNA adduct 8-hydroxy-2′-deoxyguanosine. This assay employs a microplate with 96 wells pre-coated with 8-OHdG, a monoclonal antibody specific for 8-OHdG (primary antibody), an HPR-conjugated antibody (secondary antibody) and colorimetric substrate detection whose absorbance was measured in an ELISA microwell reader at 450 nm. The samples 8-OHdG concentrations (ng/mL) were calculated using a polynomial equation from the relative absorbance of standard curve. After creatinine correction, the results were expressed as ng/mg Cr.

2.11. Pro-inflammatory cytokine Interleukin 6 (IL-6)

Plasma IL-6 was measured by commercial kit Human IL-6 ELISA^{PRO} (Mabtech AB, Sweden). The assay utilizes ELISA strip plates pre-coated with a capture monoclonal antibody (mAb), to which samples were added. Captured cytokine was detected by adding a biotinylated mAb followed by streptavidin–horseradish peroxidase. The addition of the enzyme substrate TMB results in a colored substrate product. The color intensity is directly proportional to the concentration of cytokine in the sample, which is determined by comparison with a serial dilution of recombinant cytokine standard analyzed in parallel. The results were expressed as pg/mL.

2.12. Urinary glycosaminoglycans (GAGs)

The measurement of urinary GAGs was performed according to the technique described by de Jong et al. [30]. The technique principle is based on formation of a complex between sulfated GAGs present in urine with stain 1,9-dimethylmethylene blue (DMB). This complex can be detected in a spectrophotometer at 520 nm. The results were expressed in μg GAGs/mg Cr.

2.13. Urinary creatinine (Cr)

Creatinine was determined using *Creatinine K* kit of Labtest® (Labtest Diagnóstica, Lagoa Santa, MG, Brazil), by reaction with picric acid under alkaline conditions, producing an orange color whose absorbance was determined in a spectrophotometer at 492 nm. Results were expressed as mg Cr/dL.

2.14. Protein determination

Plasma and erythrocyte protein concentrations were determined, respectively, by Biuret method — using the commercial kit of Labtest® (Labtest Diagnóstica, Lagoa Santa, MG, Brazil) — and by the method of Lowry et al. [39].

2.15. Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM). Normal distribution was tested by the Shapiro–Wilk test. Logarithmic (log) transformation was done in data not normally distributed in order to transform them in parametric. Unpaired Student's *t* test was used for all comparisons between the two groups. Correlations between parameters were performed by Pearson's correlation test. Differences were considered significant when $p < 0.05$. Analyses were performed by using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA — SPSS version 19.0) software, and graphics were constructed in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA — version 5.0) software.

3. Results

3.1. Antioxidant defenses

The concentration of erythrocyte GSH, the main non-enzymatic antioxidant in cells, was significantly reduced in MPS IVA patients (0.060 ± 0.004 nmol/mg protein) when compared to control group (0.079 ± 0.006 nmol/mg protein) ($p < 0.05$) (Fig. 1A). The SOD activity was significantly increased in patients (5.75 ± 0.31 U/mg protein) when compared to controls (4.45 ± 0.38 U/mg protein) ($p < 0.05$) (Fig. 1B), while there was no significant difference in GPx activity between patients (0.11 ± 0.006 U/mg protein) and controls (0.099 ± 0.006 U/mg protein) ($p > 0.05$) (Fig. 1C). All data were expressed as the mean ± SEM.

3.2. Oxidative damage to biomolecules

Results showed that MPS IVA patients had increased levels of oxidative damage to lipids [urine isoprostanes levels (1233.6 ± 66.6 pg/mg Cr) (Fig. 2A)] ($p < 0.05$), proteins [urine di-Tyr (16,523.6 ± 917.4 FU/mg Cr) (Fig. 2B) and plasma sulfhydryl groups (30.37 ± 0.86 μmol TNB/mg protein) (Fig. 2C)] ($p < 0.01$) when compared to controls (926 ± 93 pg/mg Cr; 11,586.3 ± 1336.4 FU/mg Cr, 41.22 ± 0.88 μmol TNB/mg protein, respectively). In addition, MPS IVA patients showed significant increase in basal DNA damage (98.63 ± 5.07 arbitrary units) when compared to controls (31.5 ± 3.79 arbitrary units) ($p < 0.01$) (Fig. 2D). After Endo III enzyme treatment (that indicates pyrimidines bases damage), MPS IVA patients also showed an increased DNA damage (75.38 ± 5.84 in patients versus 30.67 ± 9.58 arbitrary units in controls) ($p < 0.01$) (Fig. 2E). The urinary excretion of 8-OHdG (biomarker of purine bases oxidative damage) was significantly higher in patients (9.25 ± 0.82 ng/mg Cr) when compared to controls (6.1 ± 0.54 ng/mg Cr) ($p < 0.05$) (Fig. 2F). All data were expressed as the mean ± SEM.

3.3. Pro-inflammatory cytokine

The plasmatic IL-6 (Fig. 3) was increased in MPS IVA patients (30.99 ± 4.53 pg/ml) when compared to controls (11.5 ± 0.33 pg/ml) ($p < 0.01$). The data were expressed as the mean ± SEM.

3.4. Correlation between oxidative stress and pro-inflammatory cytokine

Erythrocytes GSH levels were inversely correlated with the plasma IL-6 levels (Fig. 4) in MPS IVA patients ($r = -0.848$ $p < 0.05$).

3.5. GAG levels

Results showed that MPS IVA patients presented significant higher levels of urinary GAGs (2 ± 0.02 μg/mg Cr) when compared to controls (1.57 ± 0.07 μg/mg Cr) ($p < 0.01$), despite they are receiving weekly ERT for about 8 months (Fig. 5). The data were expressed as the mean ± SEM and the values were reported in logarithmic scale.

4. Discussion

The primary biological consequence in LSDs is the increase in size and number of the lysosomes, in an attempt to retain the growing concentration of undegraded macromolecules, due to enzymatic deficiency. However, the biochemical and cellular mechanisms by which this intralysosomal accumulation leads to cell and tissue dysfunction remain not completely clear [40]. Reactive species are formed and degraded during normal aerobic metabolism in cells at physiological concentrations and are required for the proper functioning of the body. However, uncontrolled production of reactive species results in oxidative damage to biomolecules, altering their function and even causing diseases [41].

The involvement of reactive species is described in more than a hundred human diseases, including some IEM [15–18,26–28,42]. Although the relation between oxidative stress and IEM pathophysiology is not well elucidated, the accumulation of toxic metabolites is appointed as the main reason for the increase of free radicals formation [15–18]. Despite the fact that many studies relating oxidative damage to IEM exist, there are few studies on LSDs, especially MPS. It is known that in this diseases group, the imbalance between formation and removal of reactive species might be even more important due to the lysosomal overload [20,25]. Lysosomes are very susceptible to oxidative stress and destabilization of its membranes can result in release of hydrolases and metabolites accumulated in cytosol causing apoptosis or necrosis. Furthermore, the liberation of lysosomal contents can induce mitochondrial damage with secondary enhanced production of superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), suggesting that lysosomal

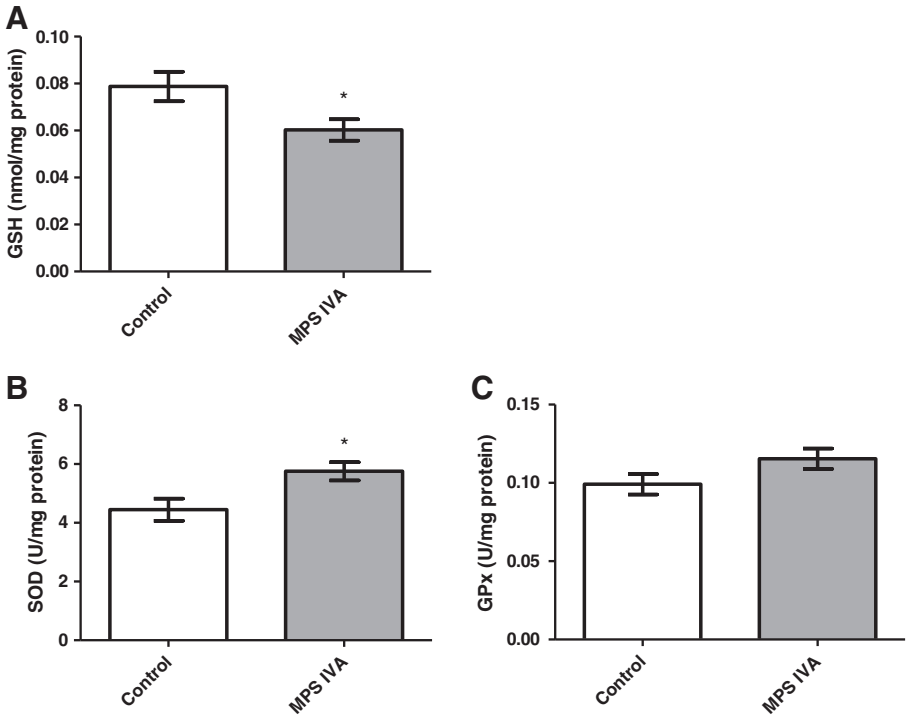

Fig. 1. Antioxidant defenses [reduced glutathione in erythrocytes—GSH (A), superoxide dismutase in erythrocytes—SOD (B), glutathione peroxidase in erythrocytes—GPx (C)] in MPS IVA patients under ERT ($n = 10$–11) and controls ($n = 10$–13). Data represent mean ± SEM. $^*p < 0.05$ (Student's t test for unpaired samples) compared to the control group.

damage seems to cause further oxidative stress amplifying loop process [43–46].

Recent studies of our group showed evidence that oxidative stress may be involved in the pathophysiology of MPS II, and that ERT protected against lipid, protein and DNA damage in these patients [26,27]. Furthermore, another study carried out by our group verified that patients with Fabry disease presented oxidative damage to proteins and lipids, low antioxidant defenses and high pro-inflammatory cytokine levels, even under ERT [29].

Considering this scenario, the aim of this study was to evaluate oxidative stress parameters and inflammation biomarkers in MPS IVA patients under ERT in order to better understand the mechanisms related to the pathophysiology of this complex syndrome.

It is widely established that several reactive oxygen species react with membrane lipids (altering cell membrane fluidity), proteins (giving rise to carbonyl group formation into side-chains, reducing sulfhydryl groups of susceptible amino acids) and DNA (for example causing mutations) [41,47]. In our study we found decreased levels of antioxidant defenses (erythrocyte GSH content) in MPS IVA patients when compared to controls. The low GSH content implies a higher susceptibility to oxidative damage because GSH is the most important non-enzymatic antioxidant, being preferentially oxidized by reactive species and then preserving more important biomolecules [48]. On the other hand, we observed high SOD activity in MPS IVA patients, showing, probably, a response of the body to the increase of reactive species. However, the high SOD activity is not accompanied by an increase of GPx activity, which would be required for the degradation of H_2O_2 formed by the reaction of SOD. Thus, the H_2O_2 remains free and, by Fenton reaction in the presence of Fe^{2+}, yields the highly toxic hydroxyl radical ($OH^{\bullet}$). This radical attacks all types of biomolecules around it, being extremely dangerous to the cell [48]. Then, increased activity of SOD could be deleterious and the balance between SOD and the peroxide-removing enzymes like GPx may imply on redox status [49]. Hence, the low GSH content and the high SOD activity presented by MPS IVA patients probably contribute to the oxidative stress process. Confirming our results, an increase of SOD activity in different organs was already verified in animal models of MPS I and, probably, contributed to oxidative imbalance found in this model [20]. Regarding GSH, Arfi et al. [21] verified a strongly lower ratio of GSH over the oxidized derivative (GSSG) in MPS IIIA mouse model when compared to normal controls. Furthermore, aspirin treatment completely normalizes GSH values, indicating a possible link between inflammation and oxidative stress in this disease [21].

With respect to oxidative damage in biomolecules, we found increased urine isoprostane levels in MPS IVA patients when compared to controls. Isoprostanes are products derived from the free radical-catalyzed peroxidation of arachdonic acid, independent of the cyclooxygenase enzyme. The continued oxidation and fragmentation of fatty acid side chains can produce aldehydes like isoprostanes, malondialdehyde (MDA) and 4-hydroxynonenal (formed from peroxidation of linolenic, arachidonic or docosahexaenoic acids). These aldehydes may cause rupture of lysosomal (and other organelles) membrane and can bind to membrane proteins, inactivating enzymes and receptors and even attack DNA, forming mutagenic lesions [48,50]. Thus, the increased isoprostane levels verified in MPS IVA patients could be a result of the lysosome destabilization and also may be acting in the maintenance of chain reactions with other molecules. Corroborating with these findings, MPS I patients under ERT also showed damage to lipids, measured by increased TBARS (thiobarbituric acid reactive substances) levels [25]. However, MPS II patients under ERT presented decrease of MDA when compared to patients without treatment [26].

In this study, we also observed increased levels of di-Tyr in urine and decreased levels of sulfhydryl groups in plasma of MPS IVA patients when compared to controls. Sulfhydryl groups can be reversibly oxidized by reactive species, suggesting that they represent a potential antioxidant, acting in cellular defense against oxidative stress [49]. Since two-thirds of sulfhydryl groups are bonded to proteins, a reduction of these groups also characterizes a protein oxidation [51–53]. The increase of urine di-Tyr levels reinforces protein oxidation found in MPS IVA patients, since di-Tyr is formed by the oxidation of adjacent protein tyrosine residues leading to the formation of a highly stable inter-phenolic bond that does not undergo further metabolism [54].

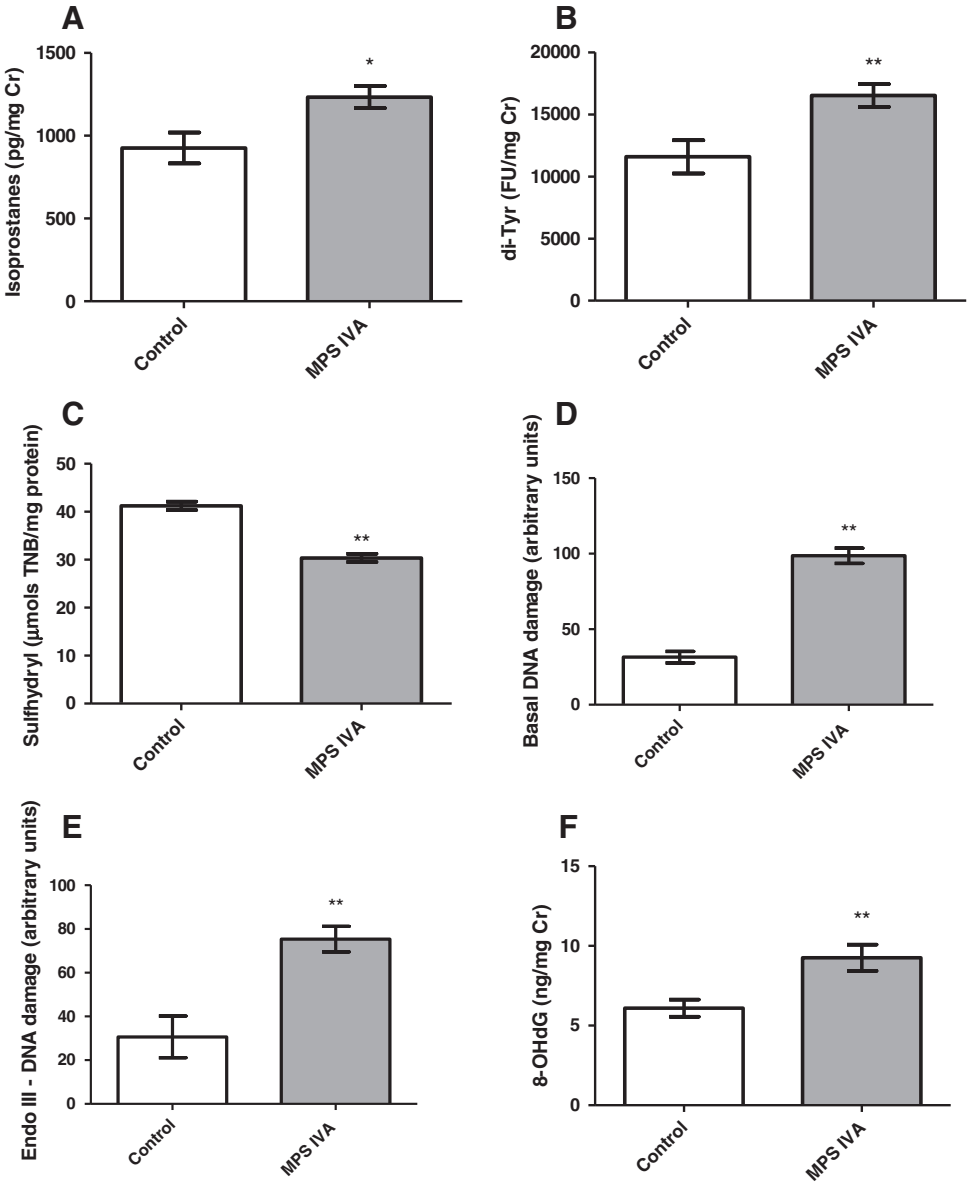

Fig. 2. Damage to lipids [isoprostanes in urine (A)], proteins [di-tyrosine in urine—di-Tyr (B), sulfhydryl groups in plasma—SH (C)] and DNA [basal DNA damage in leukocytes (D); DNA damage with Endo III in leukocytes (E); 8-hydroxy-2′-deoxyguanosine in urine—8-OHdG (G)] in MPS IVA patients under ERT ($n = 7–13$) and controls ($n = 6–13$). Data represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's t test for unpaired samples) compared to the control group.

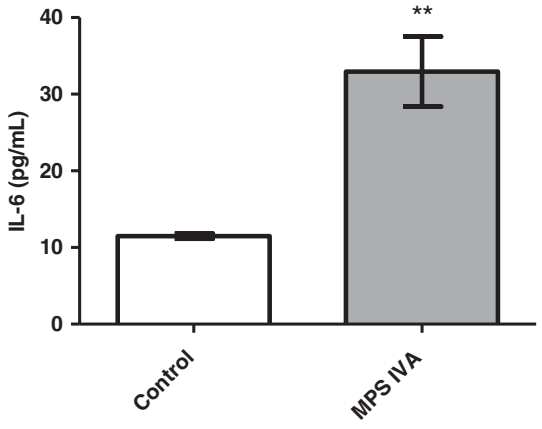

Fig. 3. Pro-inflammatory cytokine interleukin 6 (IL-6) in plasma of MPS IVA patients under ERT ($n = 10$) and controls ($n = 12$). Data represent mean ± SEM. $^{**}p < 0.01$ (Student's t test for unpaired samples) compared to the control group.

Oxidative damage to proteins was also observed in animal models of MPS I [20] and in MPS II patients [26]. During the first three months of treatment, the ERT therapy was able to increase sulfhydryl levels. However, after three months, the sulfhydryl groups returned to the values presented before treatment [26].

DNA damage has been associated with various pathologies, including some IEM [17,27,55]. In this work, we investigated DNA damage in MPS IVA patients under ERT by comet assay (single-cell electrophoresis), which is a widely used method, extremely sensitive for DNA damage, inexpensive and quick [56]. In order to investigate the mechanisms underlying DNA damage, we assessed DNA repair using Endo III enzyme which recognizes oxidized pyrimidines bases and converts them into breaks reflected in comet tail [57]. Our results showed higher DNA damage levels in MPS IVA patients when compared to control group and this damage has an oxidative origin in pyrimidines bases. DNA damage was also observed in a study with MPS II patients and in this study ERT was able to decrease, but not reverse the levels of DNA damage [27]. For a more accurate comparison with the data reported by Filippon et al. [27] an investigation of this parameter in MPS IVA patients at diagnosis

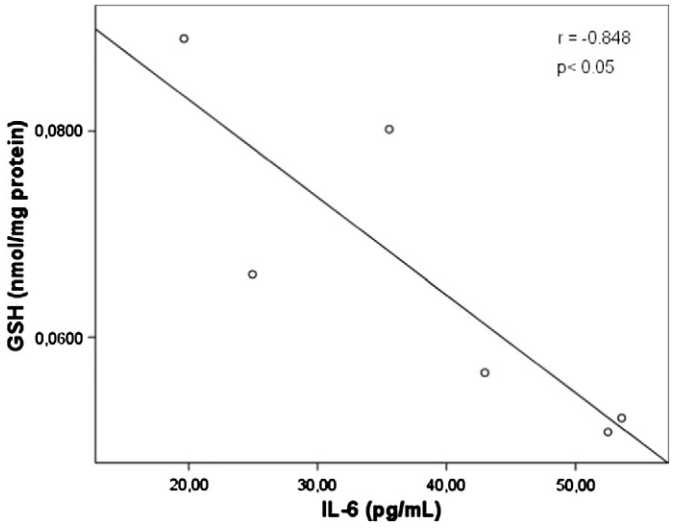

Fig. 4. Correlation between GSH in erythrocytes and IL-6 in plasma of MPS IVA patients under ERT (Pearson's correlation).

is necessary. The product of guanosine oxidation (purine base), 8-OHdG, is formed by attack of reactive oxygen species in nuclear and mitochondrial DNA [41]. The oxidized DNA is continuously repaired and the injured nucleoside is excreted into the bloodstream and urine [58]. Phenylketonuric patients with uncontrolled diet, presented high 8-OHdG levels in serum and this oxidative damage to DNA may be induced by the high phenylalanine levels and the low total antioxidant status verified in the plasma of these patients [59]. Our results are in agreement with the above findings, since MPS IVA patients had higher urinary excretion of 8-OHdG than control group. Combining this result to those obtained by enzymatic comet assay, we can presume that the MPS IVA patients exhibit oxidative damage to purine and pyrimidine bases.

In order to investigate the mechanisms underlying inflammation, we assessed IL-6 inflammatory cytokine. MPS IVA patients showed increased plasma IL-6 levels compared to controls. This result provides evidence that the pro-inflammatory state occurs in these patients. There are few studies about inflammatory mediators in MPSs and most of them were performed in animal models. These studies indicated a high expression of inflammatory molecules and cytokines secondary to the accumulation of GAGs in MPS IIIB, VI and VII [19,22,60]. In our work, we also observed an inverse correlation between GSH and IL-6. These data may suggest that the inflammatory process is associated to

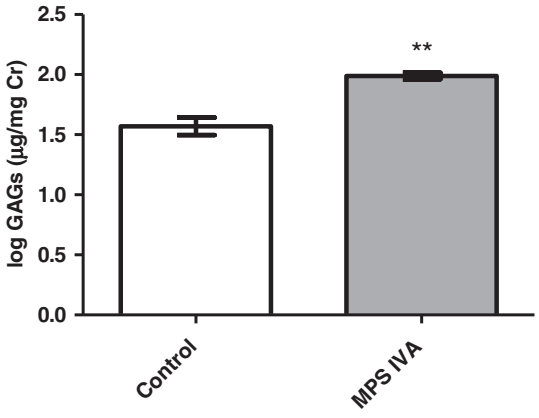

Fig. 5. Glycosaminoglycan (GAG) urinary levels in MPS IVA patients under ERT ($n = 12$) and controls ($n = 13$). Data represent mean ± SEM. $^{**}p < 0.01$ (Student's t test for unpaired samples) compared to the control group.

oxidative state in MPS IVA patients and is consistent with studies involving animal model of MPS I, IIIA and IIIB [21,23,24].

Considering that GAG urinary levels in our study were still high in patients compared to the control group, we propose that GAGs are related, at least in part, with the oxidative damage found in MPS IVA patients. The present results for MPS IVA patients are in agreement with those found in treated Fabry patients, once they also showed high lipid and protein oxidative damage, decreased antioxidant defenses and increased inflammatory biomarkers [29].

To the best of our knowledge, this is the first study that describes the increase of oxidative damage to biomolecules in MPS IVA patients. Possibly, these data may provide new targets to future therapeutic strategies in order to improve the life quality of MPS IVA patients. Further researches and clinical trials are needed to reveal how safe and effective would be the supplementation of antioxidants in combination with ERT in MPS IVA disease.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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