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Oxidative profile exhibited by Mucopolysaccharidosis type IVA patients at diagnosis: Increased keratan urinary levels

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ABSTRACT

Morquio A disease (Mucopolysaccharidosis type IVA, MPS IVA) is one of the 11 mucopolysaccharidoses (MPSs), a heterogeneous group of inherited lysosomal storage disorders (LSDs) caused by deficiency in enzymes need to degrade glycosaminoglycans (GAGs). Morquio A is characterized by a decrease in *N*-acetylgalactosamine-6-sulfatase activity and subsequent accumulation of keratan sulfate and chondroitin 6-sulfate in cells and body fluids. As the pathophysiology of this LSD is not completely understood and considering the previous results of our group concerning oxidative stress in Morquio A patients receiving enzyme replacement therapy (ERT), the aim of this study was to investigate oxidative stress parameters in Morquio A patients at diagnosis. It was studied 15 untreated Morquio A patients, compared with healthy individuals. The affected individuals presented higher lipid peroxidation, assessed by urinary 15-F2t-isoprostane levels and no protein damage, determined by sulfhydryl groups in plasma and di-tyrosine levels in urine. Furthermore, Morquio A patients showed DNA oxidative damage in both pyrimidines and purines bases, being the DNA damage positively correlated with lipid peroxidation. In relation to antioxidant defenses, affected patients presented higher levels of reduced glutathione (GSH) and increased activity of glutathione peroxidase (GPx), while superoxide dismutase (SOD) and glutathione reductase (GR) activities were similar to controls. Our findings indicate that Morquio A patients present at diagnosis redox imbalance and oxidative damage to lipids and DNA, reinforcing the idea about the importance of antioxidant therapy as adjuvant to ERT, in this disorder.

1. Introduction

Morquio A disease (Mucopolysaccharidosis type IVA, MPS IVA, OMIM #253000) is an autosomal recessive inborn error of glycosaminoglycan (GAG) catabolism due to a defect in *N*-acetylgalactosamine-6-sulfatase enzyme (GALNS; E.C.3.1.6.4) [42]. This enzyme deficiency is responsible for keratan sulfate and chondroitin 6-sulfate accumulation in body fluids and tissues. Keratan sulfate, the main accumulated metabolite, contains a repeating disaccharide unit with alternating D-galactose and *N*-acetyl-D-glucosamine residues. Keratan sulfate accumulates principally in the cartilage, cornea and heart valves of Morquio A patients and excess of tissue accumulation leads to disruption in the cartilage and subsequent increase of keratan sulfate levels in blood and urine [34]. The Morquio A incidence ranges among different populations from 1 per 76,000 live births in Northern Ireland [40] to 1 per 640,000 live births in Western Australia [41]. However, within this

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Cr, creatinine; DI, damage index; 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-sulfatase; GCL, glutamate cysteine ligase; GCLC, catalytic subunit of GCL; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione oxidized; H_2O_2, hydrogen peroxide; IEM, inborn errors of metabolism; LPS, lipopolysaccharide; LSDs, lysosomal storage disorders; MPSs, mucopolysaccharidoses; mRNA, messenger ribonucleic acid; $OH^{\cdot}$, hydroxyl radical; ROS, reactive oxygen species; SEM, standard error of the mean; SOD, superoxide dismutase; TLR4, Toll Like Receptor 4; TNB, tionitrobenzoic acid

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context it is important to mention the study developed by Leadley et al. [28], which shows that definitions for prevalence and incidence are not well understood in rare diseases field. Since overestimation and underestimation of prevalence data can occur, guidelines and quality tools for estimating the prevalence of rare diseases are recommended. Clinical manifestations of Morquio A patients include: skeletal dysplasia, restricted growth and short stature, joint hypermobility, valvular heart disease, pulmonary disease, corneal clouding, hearing loss, and poorly formed teeth [33]. The characteristic signs and symptoms of Morquio A syndrome appear at around two or three years of age [33].

The affected enzyme in Morquio A syndrome is encoded by the *GALNS* gene, which is located on chromosome 16q24.3 and split into 14 exons spanning approximately 50 kb [53]. Until April of 2017, 328 different mutations have been identified in the *GALNS* gene in the Human Gene Mutation Database (www.hgmd.cf.ac.uk [51]).

It is already described that the large number of mutations in *GALNS* are consistent with the broad spectrum of phenotypes observed in Morquio A patients, ranging from mild to severe based on the residual *GALNS* activity [14,34,35]. In almost all cases, Morquio A patients have no neurological impairment, differently from most MPS types [42].

Since 2014, enzyme replacement therapy (ERT) for Morquio A is approved in many countries, including Brazil. This treatment is based on the intravenous administration of a recombinant enzyme, similar to the native GALNS, aiming to reduce the high levels of GAGs accumulated inside the lysosomes [22]. Morquio A patients submitted to ERT showed improvements in 6-minute-walk test and presented a decrease in keratan sulfate excretion levels [22]. However, it is important emphasize that recent works are showing that ERT do not reverse most of the features present before the treatment and also not shown good clinical efficiency in Morquio A patients [43,56].

Reactive species, that are formed and degraded in cells during normal aerobic metabolism, are required for the correct functioning of the organism. However, an imbalance in the rate of synthesis and detoxification of these reactive species cause a disruption of redox metabolism with oxidative damage to biomolecules like proteins, lipids and DNA [20]. The involvement of reactive species is described in more than a hundred human diseases, including some IEM, and in the majority of published works about IEM, the accumulated metabolites are indicated as main responsible for the increase of reactive species [3,10,17,44,48]. An abnormal accumulation of GAGs not degraded within the lysosomes can lead to an increase of reactive oxygen species (ROS), which has a great impact on lysosomal function since lysosomes lack hydrogen peroxide degrading enzymes and have high content of iron, making them organelles extremely susceptible to oxidative damage [49,50]. A destabilization of lysosomal membranes can cause an overflow of lysosome contents into the cytoplasm which may trigger peroxidation cascades causing, at the end, cell apoptosis or necrosis [49,50].

We have been focusing our research on the mechanisms underlying the pathophysiology of Morquio A and, in a previous study we demonstrated that oxidative and inflammatory imbalance occurs in Morquio A patients even after eight months of ERT [13]. Therefore, the aim of the present study was to evaluate antioxidant defenses and oxidative damage to lipids, proteins and DNA in Morquio A patients without treatment.

2. Materials and methods

2.1. Subjects

The study was performed with 15 Morquio A patients with ages varying between 5 and 43 years (18.1 ± 11.25 years old, mean ± standard deviation) and with 39 healthy individuals with ages ranging between 7 and 32 years (21.31 ± 5.97 years old, mean ± standard deviation). 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. Diagnosis was confirmed by evaluation of GAGs in urine (increased both total content and keratan sulfate) and measurement of GALNS in leukocytes (deficient activity) [32,55].

The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and informed consent was obtained from all participants. The study was approved (project number 13-0246) by The Ethics Committee of the *Hospital de Clínicas de Porto Alegre* (HCPA), RS, Brazil.

2.2. Samples preparation

Urine and heparinized blood samples were obtained from patients and healthy individuals at the same time. The urine samples were collected in sterile flask, aliquoted and frozen at − 80 °C until analysis. Whole heparinized 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. Biochemical determinations

2.3.1. 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 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 to the microplate. The concentration of 15-F2t-isoprostane was determined spectrophotometrically at 630 nm by the intensity of color developed after the substrate had been added. Results were expressed as picograms of isoprostanes per mg of urinary creatinine (pg/mg Cr).

2.3.2. Total plasmatic level of sulfhydryl groups

The plasmatic concentration of sulfhydryl groups was performed as previously described. [1]. 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.

2.3.3. Urinary di-tyrosine levels

In order to determine the levels of protein oxidation in urine, the intensity of di-tyrosine fluorescence was measured according to method previously described [26]. In brief, 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 of urinary creatinine (FU/mg Cr).

2.3.4. Alkaline comet assay in leukocytes

The alkaline comet assay, that measures single and double DNA strand breaks, was performed as previously described and in accordance with general guidelines for the comet assay [47,52]. 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