

IN VITRO EFFECT OF N-ACETYL-L-CYSTEINE ON GLUTATHIONE AND SULFHYDRYL LEVELS IN X-LINKED ADRENOLEUKODYSTROPHY PATIENTS

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ABSTRACT

Introduction: Recent evidence shows that oxidative stress seems to be related with the pathophysiology of X-linked adrenoleukodystrophy (X-ALD), a neurodegenerative disorder.

Methods: In the present study, the *in vitro* effect of N-acetyl-L-cysteine (NAC) on glutathione (GSH) and sulfhydryl levels in X-ALD patients was evaluated.

Results: A significant reduction of GSH and sulfhydryl content was observed in X-ALD patients compared to the control group. Furthermore, 5 mM of NAC, *in vitro*, led to an increase in GSH content and sulfhydryl groups in these patients.

Conclusion: These data probably indicate that an adjuvant therapy with the antioxidant NAC could improve the oxidative imbalance in X-ALD patients.

Keywords: X-linked adrenoleukodystrophy; N-acetyl-L-cysteine; glutathione; sulfhydryl

X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal disorder that affects at least 1 in 17,000 males¹. Phenotypic variability is remarkable, ranging from severe and rapidly progressive forms (i.e., childhood cerebral form, CCER) to slowly progressive paraparesis compatible with life¹.

Hexacosanoic acid (C26:0) and tetracosanoic acid (C24:0) are saturated fatty acids that accumulate in tissues and body fluids¹. The exact mechanisms underlying brain damage in X-ALD are poorly elucidated. Some researchers have proposed that oxidative stress represents a hallmark in the pathogenesis of X-ALD²⁻⁴.

Glutathione (GSH) deficiency is associated with several pathological conditions, since the glutathione system provides the main cellular protection against oxidative damage. Cellular redox balance is assured by equilibrated ratios among glutathione forms; therefore, a decrease in GSH or an increase in its oxidized form (GSSG) reflects an oxidative perturbation in cell metabolism⁵.

The antioxidant capacity of NAC has been proposed based on data from *in vitro* studies, in which this compound has been shown to reduce oxidant-induced cell damage, acting as a cysteine prodrug and a GSH precursor⁶. NAC can also reduce disulphide bonds in proteins, scavenge free radicals and bind metals to form complexes⁷. There are several *in vitro* and *in vivo* studies involving NAC action on X-ALD treatment⁸⁻¹¹. Considering that NAC has been proposed as a GSH precursor, the purpose of this study was to analyze the *in vitro* effect of NAC on GSH and sulfhydryl levels in X-ALD patients.

METHODS

Subjects

A total of eight X-ALD patients [four heterozygotes (HTZ), one Addison-only and three CCER] were included in this study. These patients had their diagnosis confirmed by determination and mutation analysis of very long chain fatty

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acids. The control group consisted of eight healthy subjects. This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil (protocol number 13-0247), and all subjects or parents gave written informed consent.

Mutation Analysis

Using 10 polymerase chain reactions (PCRs), it was possible to screen the entire coding sequence of the ABCD1 gene and intron-exon boundaries. All samples were screened by single strand conformational polymorphism analysis followed by automated DNA sequencing to establish the specific mutation. Amplicons with mobility shift were purified with Exo-SAP (GE Healthcare) and submitted to automated sequencing on ABI 3100 Genetic Analyzer using BigDye, version 3.0 (Life Technologies). Mutations were confirmed by reverse strand PCR sequencing.

Sample Collection and in Vitro Assay

Venous blood was collected under sterile conditions in heparinized vials. Immediately, aliquots of whole blood cells were incubated with NAC (2.5 and 5 mM) for 6 hours at 37 °C^{9,12}. Then, the blood was centrifuged at 1000 ×g for 10 minutes and plasma was removed by aspiration, aliquoted and frozen at -80 °C until sulfhydryl determination. Erythrocytes were washed three times with cold saline solution, lysated with water and frozen at -80 °C until GSH analysis. NAC was diluted with phosphate-buffered saline (PBS buffer) on the day of use. The proportion of blood and antioxidant solutions diluted in PBS buffer was 1:3.

Reduced GSH Content in Erythrocytes

To measure GSH levels, lysates of erythrocytes were processed as described by Browne and Armstrong¹³. Results were expressed as nmol/mg protein.

Total Plasmatic Level of Sulfhydryl Groups

The plasmatic concentration of sulfhydryl groups was determined as described by Aksenov and Markesbery¹⁴. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as μmol TNB/mg protein.

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.¹⁵.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Comparisons between means were analyzed by one-way analysis of variance (ANOVA)

followed by Duncan's *post hoc* test. Differences were considered statistically significant when *P* value was lower than 0.05. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA — SPSS, version 19.0) software, and graphics were produced using the GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA — version 5.0) software.

RESULTS

Patient and Control Information

Table 1 shows patient and control information (average and standard deviation of age, gender and phenotype).

In vitro effect of NAC on GSH content

Figure 1 shows GSH erythrocytes levels and the *in vitro* effect of NAC (2.5 and 5 mM) on GSH content in X-ALD patients (HTZ, CCER and Addison-only). These three phenotypes were included in the same group because there was no significant difference in GSH levels between them [mean and SEM of female HTZ: 0.87 ± 0.82; mean and SEM of male patients (including the Addison-only patient): 0.66 ± 0.90, *P* = 0.68, Duncan's *post hoc* test]. GSH concentration significantly decreased in these patients and 5 mM of NAC increased these levels, reaching control values [*F*(3, 19) = 10.41, *P* < 0.001].

Table 1: Patients and controls' information.

Subjects	Ages (years-old)	Phenotypes/ genders
Patient 1	29	Heterozygote woman
Patient 2	38	Heterozygote woman
Patient 3	49	Heterozygote woman
Patient 4	50	Heterozygote woman
Patient 5	9	Cerebral form male
Patient 6	9	Cerebral form male
Patient 7	14	Cerebral form male
Patient 8	14	Addison-only male
Mean ± SD	27.12 ± 16.77	-
Control 1	23	Male
Control 2	23	Male
Control 3	23	Male
Control 4	24	Male
Control 5	26	Woman
Control 6	26	Woman
Control 7	27	Woman
Control 8	30	Woman
Mean ± SD	25.25 ± 2.49	-

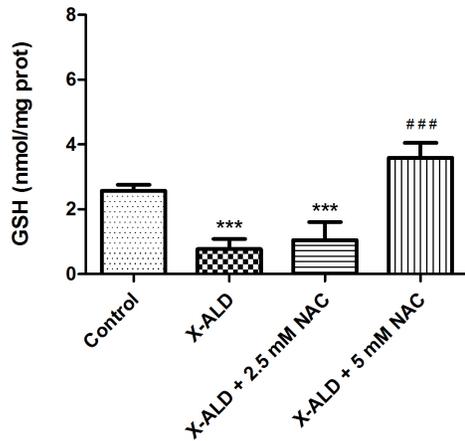


Figure 1: The *in vitro* effect of N-acetyl-L-cysteine (NAC) (2.5 and 5 mM) on GSH levels in erythrocytes from X-ALD patients. Data represent mean \pm standard error of the mean. *** $P < 0.001$ compared to the control group and ### $P < 0.001$ compared to X-ALD patients with NAC 2.5 mM and X-ALD patients [one-way analysis of variance (ANOVA) followed by Duncan's multiple range test].

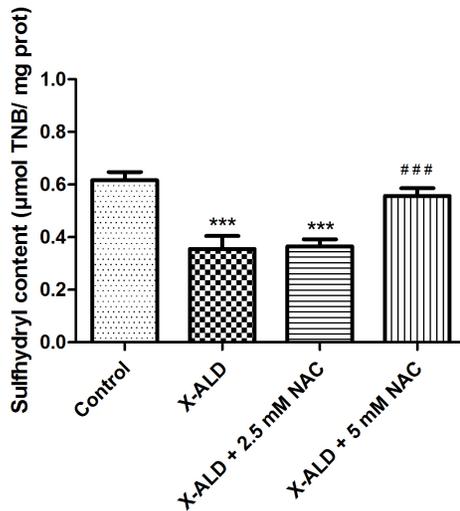


Figure 2: The *in vitro* effect of N-acetyl-L-cysteine (NAC) (2.5 and 5 mM) on sulfhydryl content in plasma from X-ALD patients. Data represent mean \pm standard error of the mean. *** $P < 0.001$ compared to the control group and ### $P < 0.001$ compared to X-ALD patients with NAC 2.5 mM and X-ALD patients [one-way analysis of variance (ANOVA) followed by Duncan's multiple range test].

***In vitro* effect of NAC on Sulfhydryl content**

Figure 2 shows sulfhydryl plasma levels and the *in vitro* effect of NAC (2.5 and 5 mM) on sulfhydryl content in X-ALD patients (HTZ, CCER and Addison-only). These three phenotypes were included in the same group because there was no significant difference in sulfhydryl content between them [mean and SEM of female HTZ: 0.29 ± 0.11 ; mean and SEM of male patients

(including the Addison-only patient): 0.38 ± 0.10 , $P = 0.25$, Duncan's *post hoc* test]. Sulfhydryl content was significantly reduced in these patients and 5 mM of NAC increased these levels until they reached control values [$F(3,20) = 16.47$, $P < 0.001$].

DISCUSSION

Several studies have shown that oxidative stress is induced in X-ALD patients, including oxidative damage to protein, lipids, and reduction of antioxidant capacity^{4,5,16}. Previous research has demonstrated a reduction in GSH and sulfhydryl content in X-ALD patients^{5,17}. Interestingly, low levels of GSH have also been reported in an animal model for X-ALD⁹. The present study showed that X-ALD patients had lower GSH and sulphhydryl levels compared to the control group, which is consistent with the literature. Besides, 5 mM of NAC, *in vitro*, increased GSH content and sulfhydryl groups in these patients.

GSH plays a wide variety of physiological roles and its antioxidant effects depend on the presence of the free sulfhydryl group as a ready source of reducing equivalents to quench radical species. GSH also acts as a "scavenger" of free radicals¹⁸. The limiting step of the GSH synthesis involves conjugation of cysteine with L-glutamate, while L-glycine is added in a subsequent synthetic step by GSH synthase. GSH depletion is a feature of a wide range of neuropsychiatric disorders, including Alzheimer and Parkinson diseases, and in some inborn errors of metabolism^{5,19}.

Adjuvant therapies with antioxidant have been studied in disease-associated oxidative stress. NAC is a cysteine prodrug with an antioxidant activity attributed to its sulfhydryl group, which provides protection against oxidative and metabolic processes. NAC supplies the cysteine necessary for GSH synthesis and has proven to be effective in treating disease-associated oxidative stress⁷.

It has been shown that NAC crosses the blood-brain barrier, accumulated in the brain, and reverses memory impairment and brain oxidative stress in aged mice²⁰. Additionally, in some murine models of Alzheimer disease, the administration of NAC buffered oxidative damage²¹. In an animal model of Duchenne muscular dystrophy, the treatment with NAC significantly decreased the oxidation of GSH and protein thiols, and enhanced muscle protein thiol content²².

In a clinical trial with Gaucher and Parkinson patients, NAC increased whole blood GSH/GSSG ratio and GSH concentration in the brain²³. Rushworth et al.²⁴ suggested that NAC might be useful not only for promoting GSH depletion, but also because NAC-derived cysteine can potentially lead to an increase in the release of glutamate from astrocytes resulting in activation of neuronal glutamate receptors.

Regarding studies involving the effect of NAC on X-ALD, Tolar et al.¹¹ reported that peri-transplant administration of NAC was protective from fulminant demyelination in advanced symptomatic X-ALD patients. It was also seen that a pre-treatment with arginine and NAC inhibited nitrite and superoxide production in glial cells enriched with C26:0⁸. Additionally, NAC in combination with other antioxidants reversed oxidative damage and energetic failure in an animal model for X-ALD¹⁰.

As to the underlying mechanisms by which NAC increases sulfhydryl and GSH levels, it is possible to assume that this action is probably due to stimulation of enzymes related to the GSH synthesis or to non-enzymatic mechanisms related to scavenger properties of free radicals.

Finally, the present results confirm former reports showing the role of oxidative stress on X-ALD and demonstrate, for the first time in the literature, the *in vitro* protective effect of NAC on GSH and sulfhydryl content in this disease. Therefore, this study underscores the pertinence of using antioxidants as an adjuvant therapy for X-ALD to improve oxidative imbalance in X-ALD patients. However, the present *in vitro* study should be interpreted with caution and more *in vivo* experiments are necessary to elucidate this issue.

INFORMED CONSENT

All procedures followed in this study were in accordance with the ethical standards of the responsible (institutional and national) committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for inclusion in the study.

ABBREVIATIONS

C24:0: Tetracosanoic acid; C26:0: Hexacosanoic acid; CCER: Childhood cerebral form; GSH: Glutathione; GSSG: Oxidized glutathione; NAC: N-acetyl-L-cysteine; PBS: Phosphate-buffered saline; X-ALD: X-linked adrenoleukodystrophy.

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