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Protective effect of n-acetyl-L-cysteine and rosuvastatin against oxidative stress in fibroblasts from asymptomatic patients with X-ALD: a preliminary study

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ABSTRACT

Introduction: Several studies in the literature have evaluated the role of oxidative stress and adjuvant therapies for X-linked adrenoleukodystrophy (X-ALD). Here, we investigated whether n-acetyl-L-cysteine (NAC) and rosuvastatin (RSV) could influence the generation of reactive species, redox status and nitrative stress in fibroblasts from asymptomatic patients with X-ALD.

Methods: Skin biopsy samples were cultured and treated for 2 hours (37 °C) with NAC and RSV.

Results: X-ALD fibroblasts generated high levels of reactive oxygen species. These levels were significantly lower in fibroblasts treated with NAC and RSV relative to untreated samples. The X-ALD fibroblasts from asymptomatic patients also had higher catalase activity, and only NAC was able to increase enzyme activity in the samples.

Conclusions: Our results indicated that NAC and RSV were able to improve oxidative stress parameters in fibroblasts from asymptomatic patients with X-ALD, showing that adjuvant antioxidant therapy may be a promising treatment strategy for asymptomatic patients with this disease.

Keywords: *X-linked adrenoleukodystrophy; fibroblasts; n-acetyl-L-cysteine; rosuvastatin; oxidative stress*

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INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD) is a severe neurodegenerative disorder caused by a defective ABCD1 transporter protein. This gene encodes ALDP, a protein involved in the peroxisomal uptake of very long chain fatty acids (VLCFA) as well as their CoA-esters. This results in a buildup of fatty acids, predominantly hexacosanoic (C26:0) and tetracosanoic acids (C24:0), in body fluids, adrenal glands, tissue and plasma[1,2].

X-ALD is the most common peroxisomal disorder, occurring in all regions of the world with an estimated incidence of 1 per 17,000 births (male and female)[3,4]. Moser et al.[1] described four main phenotypes in patients with X-ALD: childhood cerebral form (CCER), adrenomyeloneuropathy (AMN), asymptomatic and heterozygous (HTZ) females. Asymptomatic individuals have the genetic abnormality and accumulation of VLCFA associated with X-ALD, but no adrenal or neurological involvement[1]. The diagnosis of X-ALD is based on increased concentration of VLCFA in serum, as well as high C24:0/C22:0 (docosanoic acid) and C26:0/C22:0 ratios. However, mutation analysis is considered the best method to establish carrier status in women and currently, over 643 different mutations have been identified in the ABCD1 gene[4,5]. In recent years, LC–MS/MS methods have also been developed to screen dried blood spots for hexacosanoyl-2-lyso-sn-3-glycero-phosphorylcholine,

a biomarker that has been shown to be elevated at birth in the blood of patients with X-ALD and other peroxisomal blood disorders[6].

Oxidative damage caused by reactive oxygen and nitrogen species is an important mediator of neurodegeneration since the brain has relatively low levels of antioxidant defenses, high lipid content and large quantities of catecholamines, which are highly susceptible to free radical attack[7]. The mechanisms responsible for tissue damage in X-ALD are not well elucidated; however, the literature has demonstrated that oxidative stress is involved in the pathophysiology of the disease[8-11].

Many studies have shown the potential effects of antioxidants as adjuvant treatment for symptomatic patients. N-acetyl-L-cysteine (NAC) is a compound with antioxidant and anti-inflammatory activities that have been studied in X-ALD[9,12,13]. It stimulates glutathione synthesis, scavenges free radicals and is hypothesized to play a neuroprotective role[14-16]. Rosuvastatin (RSV) is one of the most potent widely available statins, and is an approved treatment to reduce circulating low-density lipoprotein[17]. The anti-inflammatory and antioxidant effects of RSV on leukocytes from patients with X-ALD and C26:0-enriched glial cells have been established in the literature[9,16].

Oxidative stress in the plasma, leukocytes, fibroblasts and post-mortem brain samples of symptomatic patients with X-ALD have been previously reported[8,9,18-21]. Our research team has also found, in previous studies, that NAC and RSV were effective in reducing inflammation, oxidative and nitrative stress in symptomatic patients with X-ALD. As such, in this study, we aimed to analyze the effect of these compounds on oxidative and nitrative stress in fibroblasts from asymptomatic patients with X-ALD.

METHODS

Subjects

Primary fibroblast cell lines were generated from blood and skin samples collected by the Medical Genetics Department of the Hospital de Clínicas de Porto Alegre from asymptomatic patients with X-ALD and healthy participants. Sample characteristics are shown in Table 1. Mutation analysis was performed as previously described by Pereira and colleagues[22] and all diagnoses were confirmed by the analysis of plasma C26:0 and blood C26:0-lysophosphatidylcholine (C26:0-LPC) levels (Table 2). This study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (number 15-0487) and all patients provided written informed consent to participate in the investigation.

Table 1: Asymptomatic X-ALD patients and healthy age-matched controls' data.

Subjects	Ages (years)	Gender	Sample collection
Patient 1	37	female	Blood
Patient 2	39	female	Blood
Patient 3	38	female	Blood
Patient 4	9	male	Blood and fibroblast
Patient 5	10	male	Blood and fibroblast
Patient 6	11	male	Blood and fibroblast
Mean ± SD	24 ± 15.4	-	-
Control 1	35	female	Blood
Control 2	36	female	Blood
Control 3	22	female	Blood
Control 4	21	female	Blood
Control 5	10	male	Blood
Control 6	9	male	Blood and fibroblast
Control 7	8	male	Blood and fibroblast
Control 8	38	female	Blood and fibroblast
Mean ± SD	22 ± 12.7	-	-

Table 2: Concentrations of hexacosanoic acid (C26:0) and lysophosphatidylcholine-C26:0 (LC-C26:0) in controls and X-ALD patients.

Subjects	C26:0 (µmol/L) (in plasma)	LC-C26:0 (µg/mL) (in whole blood)
Healthy controls (n = 8)	0.35 ± 0.10	0.51 ± 0.07
X-ALD males (n = 3)	0.80 ± 0.17	0.80 ± 0.17 *
X-ALD females (n = 3)	1.35 ± 0.45 **	1.12 ± 0.17 *** and #

*p<0.05 and ***p<0.001 compared to controls. #p<0.05 compared to X-ALD females. Results represent mean ± SD (standard deviation). One-way analysis of variance ANOVA followed by Tukey post hoc test.

Sample collection and preparation

Plasma was prepared from whole blood samples collected from fasting individuals (controls and patients with X-ALD) by venipuncture into heparinized vials. Whole blood was centrifuged at 1000 x g for 10 minutes. Plasma was removed by aspiration and frozen at -80°C until use. An aliquot of whole blood was blotted on filter paper to prepare dried blood spots for the measurement of C26:0-LPC levels. Fibroblasts were collected and frozen in liquid nitrogen until analysis.

Cell culture and antioxidant treatment

Skin biopsies were taken from patients under local anesthesia and placed in sterile polyethylene

vials (type Falcon T25). Primary and secondary cells were cultured in DMEM (Dulbecco's modified Eagle medium) (GIBCO, Grand Island, NY, USA) containing 5% fetal bovine serum at 37 °C in a humid atmosphere of 5% CO_2 and harvested by treatment with 0.15 % trypsin–0.08 % EDTA in PBS (phosphate buffered saline). Cells were treated at confluence for 2 hours at 37 °C with NAC (100 µM) and RSV (5 µM)[16]. Cells and supernatants were harvested for the analysis of oxidative stress.

Cell viability assay

Cytotoxicity was evaluated using neutral red uptake assay, as described by Repetto et al.[23].

Nitrite and nitrate assay

The quantification of NO equivalents in cell supernatants was performed using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Protein determination

Protein concentration in the extracts prepared from fibroblasts after treatment was determined according to the method described by Bradford[24], using bovine serum albumin as standard.

Dichlorofluorescein Diacetate Oxidation (H2DCF-DA) measurement

The generation of reactive oxygen species (ROS) can be estimated as described by LeBel et al.[25] using H_2DCF-DA. ROS exposure induces the oxidation of H_2DCF-DA to dichlorofluorescein (DCF), a fluorescent product that can be quantified by fluorimetry. A calibration curve was determined with standard DCF (10 µM) and the levels of ROS expressed as µmol DCF / mg protein.

Superoxide Dismutase (SOD) activity

SOD activity was measured as described by Misra and Fridovich[26]. A unit (U) of SOD is defined as the amount of enzyme required to inhibit 50% of adrenaline oxidation, so that activity is expressed as U SOD / mg protein.

Catalase (CAT) activity

CAT activity was determined as described by Aebi[27]. One unit (U) of CAT is defined as the amount required to catalyze the decomposition of 1 µmol H_2O_2 per minute, with activity expressed as U CAT / mg protein.

Fatty acid analysis

Blood levels of C26:0-LPC

Dried blood spot samples from control subjects and patients with X-ALD were analyzed using liquid chromatography coupled to mass spectrometry (LC/MS/MS). The samples were processed using the method described by Turgeon and collaborators[6], with some adaptations. The results were expressed as µg/mL.

Plasma C26:0 levels

Plasma levels of C26:0 were determined according to the technique described by Moser and Moser[5]. A total lipid extract was prepared and treated with methanolic HCl (3N) for the formation of fatty acid methyl esters, which were then purified by thin-layer chromatography. The purified fatty acid methyl esters were extracted with hexane and analyzed by gas chromatography. This was carried out using a Varian gas chromatograph equipped with an HP-5 column (5% methylphenyl silicone, 0.33 mm film thickness, 0.2 mm inner diameter and 25 m length), a flame ionization detector, a split/splitless injector, and helium as the mobile phase. C26:0 concentrations were expressed in µmol/L. Heptacosanoic acid was used as an internal standard.

Statistical analysis

Results were expressed as mean ± SEM (standard error of the mean). Comparisons between mean values were made using one-way analysis of variance ANOVA followed by Tukey post-hoc tests. Results with $p < 0.05$ were considered significant. Data analysis and plotting were performed using GraphPad Prism, version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cell viability assay

Fibroblast viability was determined by neutral red assay. Figure 1 demonstrates the absence of significant differences between the control group, untreated X-ALD fibroblasts, and X-ALD fibroblasts treated with 100µM of NAC and 5µM of RSV, respectively ($F[3,8] = 1.36$, $p > 0.05$). These results indicate that the antioxidants were not cytotoxic and did not impair cell growth.

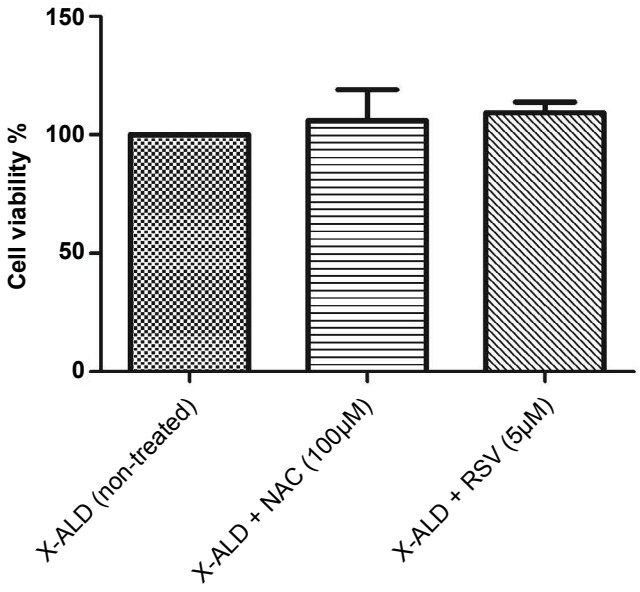

Figure 1: Neutral red viability assay in untreated X-ALD fibroblasts, X-ALD fibroblasts treated with 100 μM n-acetyl-L-cysteine (NAC) and X-ALD fibroblasts treated with 5μM rosuvastatin (RSV) (n = 3 per group). Results represent mean ± SEM (standard error of the mean), compared by one-way analysis of variance (ANOVA) followed by Tukey post hoc tests.

Markers of oxidative and nitrative stress

As can be seen in Figure 2a, higher levels of ROS were generated by X-ALD fibroblasts from asymptomatic patients than control samples. The comparison of untreated X-ALD fibroblasts to the two antioxidant groups showed that both treatments were able to reduce ROS levels, though this effect was more pronounced for NAC than RSV ($F[3,9] = 40.73$, $p < 0.0001$).

Figures 2b and 2c show that only CAT activity was altered in X-ALD fibroblasts compared to controls. Additionally, only NAC was able to increase CAT and SOD activity ($F[3,8] = 21.18$, $p < 0.001$) and ($F[3,8] = 13.79$, $p < 0.01$), respectively, relative to untreated X-ALD fibroblasts and fibroblasts treated with RSV.

Nitrative stress was determined by measuring NO equivalents. Figure 2d shows that there were no differences between groups, suggesting that NAC and RSV were unable to suppress nitrative stress in X-ALD fibroblasts from asymptomatic patients ($F[3,8] = 0.82$, $p > 0.05$).

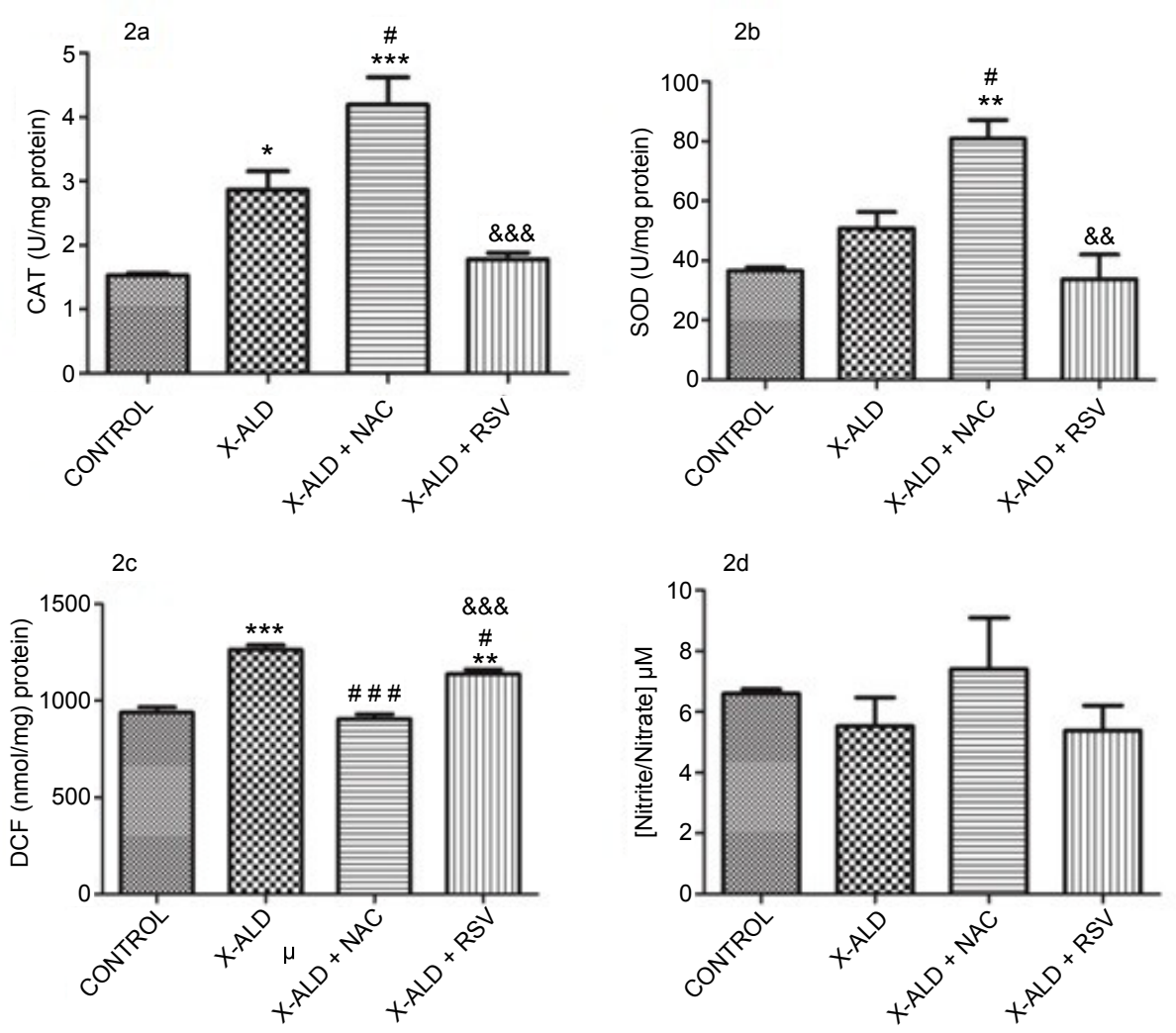

Figure 2: Oxidative and nitrative stress in control fibroblasts, untreated X-ALD fibroblasts, X-ALD fibroblasts treated with 100 µM n-acetyl-L-cysteine (NAC) and X-ALD fibroblasts treated with 5µM rosuvastatin (RSV) (n = 3 per group). A: catalase (CAT) activity; B: superoxide dismutase (SOD) activity; C: reactive oxygen species (ROS) production; D: nitrite/nitrate levels. Results represent mean ± SEM (standard error of the mean). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to controls. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to X-ALD group. $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$ compared to X-ALD + NAC groups. Data analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc tests.

DISCUSSION

Many studies have reported that oxidative stress may be involved in the pathophysiology of neurodegenerative disorders. The chemical structure of free radicals includes a single unpaired electron in the outermost shell, resulting in a highly reactive molecule that can combine nonspecifically with proteins, lipids and DNA[7]. To avoid the cell damage caused by free radical formation, biological systems have developed antioxidant defenses capable of converting these reactive species into inactive derivatives. Antioxidants may be enzymatic or non-enzymatic. Enzymatic antioxidants include catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase[7]. There are also non-enzymatic antioxidants, such as metal binding proteins and vitamins (such as E, A, C)[7]. The literature has shown that many compounds, such as NAC and RSV, have antioxidant properties and can be used in the treatment of several diseases, since they have the ability to chelate ROS and convert them into inactive derivatives. Oxidative stress occurs when the balance between antioxidant capacity and reactive species formation shifts in favor of the latter, due to a decrease in antioxidant defenses and/or an increase in the intracellular concentration of ROS[7].

In this study, we investigated the effects of NAC and RSV on oxidative and nitrative stress in X-ALD fibroblasts from asymptomatic patients. We verified

that these fibroblasts generated higher levels of ROS than those of control participants, and that NAC and RSV were able to reduce ROS formation relative to untreated fibroblasts. Moreover, NAC was more effective than RSV in this regard. We also showed that X-ALD fibroblasts had higher CAT activity compared to control samples, and when treated with NAC, the fibroblasts showed an increase in SOD and CAT activity relative to untreated X-ALD fibroblasts. Nitrative stress markers did not significantly differ between X-ALD fibroblasts and the other study groups, and antioxidants were not able to alter NO production. Overall, our results suggest that NAC seems to be more potent than RSV as an inhibitor of oxidative stress in X-ALD fibroblasts from asymptomatic patients. Previous studies have demonstrated that NAC provides cysteine for glutathione synthesis and thereby contributes to the treatment of disease-associated oxidative stress[28,29]. Moreover, NAC has been shown to reduce disulfide bonds in proteins, scavenge free radicals and bind metals to form complexes[28].

Several studies have reported oxidative changes in the plasma, fibroblasts and leukocytes of symptomatic patients with X-ALD[8-10,20,21], and many investigations have demonstrated the protective effect of antioxidants such as NAC and RSV on X-ALD, both in vivo and in vitro[8,9,13,16]. Marchetti et al.[9] demonstrated that the antioxidants NAC, Trolox (water soluble analog of vitamin E-TRO) and RSV were able to reduce DNA damage in leukocytes from symptomatic patients with X-ALD. Likewise, Marchetti et al.[16] verified that glial cells enriched with C26:0 induced oxidative DNA damage, lipid oxidative damage, antioxidant enzyme imbalance, NO release and increased levels of IL-1β. Furthermore, these authors found that NAC, TRO and RSV were able to reduce some of the damage caused by C26:0 in glial cells[16].

In line with the present findings, Vargas and colleagues[18] observed a significant increase in CAT activity in fibroblasts from patients with symptomatic X-ALD compared to controls. Additionally, Powers et al.[19] examined Mn-SOD expression in skin fibroblasts derived from control individuals and symptomatic patients with X-ALD and found that X-ALD was associated with increased Mn-SOD expression in X-ALD consistent with a response to oxidative stress. On a similar note, Fourcade et al.[8] reported that fibroblasts from patients with X-ALD (CCER and AMN phenotypes) showed markedly increased oxidative, glicoxidative and lipoxidative damage, as evidenced by N1-malondialdehyde-lysine (MDAL), N1-carboxyethyl-lysine (CEL), N1-carboxymethyl-lysine (CML), aminoadipic semialdehyde (AASA) and glutamic semialdehyde (GSA) levels. The authors also observed an antioxidant response after incubating X-ALD and control fibroblasts with C26:0, since the presence of C26:0 induced the expression of SOD2. After incubation, lipoxidative (MDAL), glicoxidative/lipoxidative (CEL, CML) and protein oxidative (GSA, AASA) marker levels nearly doubled in the fibroblast samples from symptomatic patients with X-ALD. Additionally, TRO prevented SOD2 induction in human fibroblasts, and corrected levels of oxidative damage markers[8]. In 2010, Fourcade and colleagues[30] also reported that valproic acid induces ABCD2 gene expression in the fibroblasts of symptomatic patients with X-ALD and reduces oxidative damage to proteins.

López-Erauskin et al.[31] suggested that an early and carefully tailored intervention using an antioxidant mixture could be a possible therapeutic option for AMN patients, who do not suffer from severe neuroinflammation and demyelination, given the ability of NAC, TRO and lipoic acid (LA) to scavenge the ROS caused by excess C26:0 in the fibroblasts of symptomatic patients with X-ALD. All three antioxidants were able to individually normalize ROS levels after the addition of C26:0. Combining the antioxidants at low doses led to a synergistic effect, resulting in the full prevention of ROS accumulation in X-ALD fibroblasts. In 2012, the authors verified that X-ALD fibroblasts from symptomatic patients could not survive when forced to rely on mitochondrial energy production. Furthermore, treatment with antioxidants (NAC and LA) rescued mitochondrial damage markers in X-ALD, including oxidative modifications of cyclophilin D[32]. Other studies in the literature have also shown that the excess C26:0 in X-ALD fibroblasts led to mtDNA oxidation and specifically impaired oxidative phosphorylation triggering mitochondrial ROS production from electron transport chain complexes[33].

In light of our findings, we may conclude that NAC and RSV have the potential to improve oxidative imbalance in fibroblasts from asymptomatic patients with X-ALD. Future studies with a larger number of X-ALD fibroblasts should be conducted to better elucidate our preliminary results. Nevertheless, these results allow us to infer that antioxidants may be considered as an adjuvant treatment for this severe neurogenetic disorder, especially in asymptomatic patients that can be treated early, before the onset of neurologic symptoms.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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