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Erstveröffentlichung in / First published in:  

DOI: [https://doi.org/10.1159/000465520](https://doi.org/10.1159/000465520)

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Triple A Syndrome: Preliminary Response to the Antioxidant N-Acetylcysteine Treatment in a Child

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Established Facts

- Fibroblasts of triple A patients exhibit increased oxidative stress in vitro that might be related to progressive neurodegeneration.
- N-acetylcysteine protects renal function in patients with kidney injuries associated with increased oxidative stress and improved viability of AAAS-knockdown adrenal and neuronal cells in vitro.

Novel Insights

- Increased oxidative stress is reported in vivo in a boy with triple A syndrome assessed by increased thiobarbituric acid reactive substances (TBARS) and LDL oxidation.
- Oral administration of N-acetylcysteine was safe and capable of decreasing TBARS, reducing the susceptibility of LDL to oxidation and improving antioxidant protection of HDL. Long-term effects on neurodegenerative manifestations are still unknown.

Keywords
Adrenal insufficiency · Triple A syndrome · Allgrove syndrome · N-acetylcysteine · Thiobarbituric acid

Abstract

Introduction: Triple A syndrome (AAAS) is a rare autosomal recessive disorder characterized by alacrima, achalasia, ACTH-resistant adrenal insufficiency, autonomic dysfunction, and progressive neurodegeneration. Increased oxidative stress, demonstrated in patients' fibroblasts in vitro, may be a central disease mechanism. N-acetylcysteine protects...
renal function in patients with kidney injuries associated with increased oxidative stress and improves viability of AAAS-knockdown adrenal cells in vitro. **Patient and Results:**

A boy diagnosed with AAAS presented with short stature and increased oxidative stress in vivo assessed by increased thiobarbituric acid reactive substances (TBARS), which are markers of lipid peroxidation, and by the susceptibility of LDL to oxidation and the capacity of HDL to prevent it. A homozygous missense germline mutation (c.523G>T, p.Val175Phe) in AAAS was identified. N-acetylcysteine (600 mg orally, twice daily) decreased oxidative stress but did not change the patient’s growth pattern. **Conclusions:** An increase in oxidative stress is reported for the first time in vivo in an AAAS patient. N-acetylcysteine was capable of decreasing TBARS levels, reducing the susceptibility of LDL to oxidation and improving the antioxidant role of HDL. The long-term effect of antioxidant treatment should be evaluated to determine the real benefit for the prevention of the degenerative process in AAAS.

**Case Report**

A boy born to consanguineous parents presented in infancy at a local hospital with jaundice, severe anemia, neurodevelopmental delay, repeated otitis, and elevated hepatic enzymes. By the age of 6–7 months, repeated vomiting and a decline in growth velocity were noted. He was submitted to gastroduodenal serigraphy, and the diagnosis of gastroesophageal reflux was made. Despite several treatments, he had many hospital admissions due to episodes of otitis, diarrhea, and bronchitis.

At 3 years of age, investigation of abdominal pain, hypoglycemia, and seizures led to the diagnosis of isolated primary cortisol deficiency, and replacement with glucocorticoid was started. Despite improvement of the abdominal pain, the patient continued vomiting and underwent a swallowing videogram, which was suggestive of achalasia. The lower esophageal sphincter was endoscopically dilated twice and he underwent Heller’s cardiomyotomy. Since the beginning of the follow-up, the patient presented with severe short stature, with bone age delayed by 3 years, but no signs of malnutrition (serum albumin 5.0 g/dL and hemoglobin 13.6 g/dL before surgery). There was not much improvement in his height after the correction of achalasia (before surgery: 109 cm, SDS −3.7; 1 year after surgery: 117 cm, SDS −3.17; target height: SDS −0.93). The Schirmer test was compatible with alacrima.

Genetic study was approved by the Hospital Ethics Committee. Molecular analysis identified a homozygous missense mutation (c.523G>T) in exon 6 of AAAS gene resulting in a change of valine at amino acid position 175 into phenylalanine (p.Val175Phe). His unaffected mother presented the same mutation in the heterozygous state.

Based on previous studies with NAC in chronic renal failure models and considering the safety of this drug we proposed to the patient’s mother that the patient should be treated with NAC. This was accepted, and a formal term of consent was signed.

**Methods**

**Thiobarbituric Acid Reactive Substances**

Plasma levels of TBARS were determined using the thiobarbituric acid assay. In brief, a 0.2-mL plasma sample was diluted in 0.8 mL of distilled water. Immediately thereafter, 1 mL of 17.5% trichloroacetic acid was added. Following the addition of 1 mL of 0.6% thiobarbituric acid, pH 2, the sample was placed in a boiling water bath for 15 min, after which it was allowed to cool. Subsequently, 1 mL of 70% trichloroacetic acid was added, and the mixture was incubated for 20 min. The sample was then centrifuged for 15 min at 2,000 rpm. The optical density of the supernatant was read at 534 nm against a blank reagent using a spectrophotometer. The concentration of lipid peroxidation products was calculated as malondialdehyde equivalent using a molar extinction coefficient for the malondialdehyde-thiobarbituric acid complex of 1.56 × 10⁵ mol⁻¹ cm⁻¹. Plasma levels of TBARS were expressed in nmol/mL [10]. We have used plasma samples of 43 healthy children (1.60 ± 0.04 nmol/mL) as a control for plasma TBARS levels [11].

**Reduced GSH**

Reduced GSH was determined in total blood by the method of Sedlak and Lindsay [12]. Whole blood was processed by the addition of 4 volumes of ice-cold 5% (W/V) metaphosphoric acid (Sig-
ma-Aldrich, Germany) and centrifuged at 14,000 g for 3 min. This assay consists of the supernatants of the total blood reacting with Ellman’s reagent to produce a yellow pigment measured spectrophotometrically at 412 nm. The GSH was quantified with a standard curve and reported as µmol/mL [13].

**LDL Oxidation**

To test the ability of the patient’s HDL in inhibiting LDL oxidation, pooled LDL (40 mg protein) from healthy donors (C-LDL) was incubated with CuSO₄ (10 mmol/L final concentration) at 37 °C in the presence of HDL (80 mg protein) isolated from the patient prior and after NAC treatment. In another set of incubations, the susceptibility of the patient’s LDL to oxidation was analyzed by incubating pooled HDL from healthy donors (C-HDL) with the patient’s LDL (isolated prior and after NAC treatment) in the presence of CuSO₄.

The kinetics of conjugated diene formation was monitored at 234 nm for 4 h at 3-min intervals in order to calculate the lag phase time (the time – in minutes – elapsed between the beginning of the reaction and the propagation phase) and the maximal rate of conjugated diene formation (Δabsorbance/time elapsed in minutes between the initiation phase and the maximal absorbance phase) according to Esterbauer et al. [14].

**Results**

TBARS and GSH levels demonstrated an increased oxidative stress in the patient’s plasma (Fig. 1). TBARS levels decreased during NAC treatment (600 mg orally, twice daily) despite the suboptimal compliance of the patient during follow-up. No side effects or adverse events of NAC were reported during treatment.

Before and after NAC treatment, LDL and HDL were obtained from the patient’s plasma after discontinuous density ultracentrifugation, in order to assess the susceptibility of LDL oxidation as another marker of oxidative stress. As shown in Figure 2a, the lag time (minutes; mean ± SEM) for C-LDL oxidation in the presence of HDL isolated after NAC treatment was increased (20.6 ± 0.4) compared to that when incubated with HDL isolated prior to treatment (19.4 ± 0.7). In addition, the susceptibility of the patient’s LDL to oxidation after NAC treatment was diminished as shown by the enhanced lag time (min-
utes; mean ± SEM) for conjugated diene formation (Fig. 2b: 8.8 ± 2.1 vs. 14.9 ± 1.9).

The short stature of the patient persisted even with the decrease in oxidative stress and the spontaneous onset of puberty. Treatment with growth hormone 0.15 U/kg/day was started, with little improvement of height SDS (height before rhGH 124.0 cm, SDS –2.89; height 1 year after rhGH 129.6 cm, SDS –2.55). During follow-up, the progression of puberty was slow.

**Discussion**

Reactive oxygen species (ROS) are produced primarily by mitochondria as a byproduct of normal metabolism during the conversion of molecular oxygen to water. Cells have several antioxidant defense mechanisms, such as superoxide dismutase and GSH. Oxidative stress is generated when an imbalance exists between production of oxidants and the antioxidative defense. ROS excess may damage cellular macromolecules with DNA oxidation [15]. The adrenal cortex is a tissue with high metabolic demand and high turnover of lipids by the mitochondria during steroidogenesis and is particularly susceptible to oxidative stress caused by the excessive ROS production. Triple A syndrome is a rare, autosomal recessive cause of adrenal failure that affects steroidogenesis because of the imbalance in redox homeostasis, impairing the function of several enzymes such as CYP17A1, CYP21A2, and POR [3]. In about 70% of cases, the defect is due to mutations in the AAAS gene [1]. Its product, the ALADIN protein, has a role in preventing DNA damage of the cell and consecutive apoptosis under oxidative stress [3]. Dermal fibroblasts of triple A patients have higher basal intracellular ROS and are more sensitive to oxidative stress than wild-type fibroblasts [2]. NAC, which is a precursor of L-cysteine and reduced GSH, has been extensively studied as an antioxidant. NAC is a source of sulphydryl groups in cells and a scavenger of free radicals, such as hydroxyl radicals and hydrogen peroxide. In addition, NAC can triple endothelial nitric oxide synthase expression as well as increase nitric oxide bioavailability [16, 17]. Several studies using animal and human models with increased oxidative stress showed benefits of NAC when compared to placebo [18, 19].

Our patient was treated with NAC administration by oral route, because it is noninvasive and has been proved to be as effective as the intravenous route [16]. No side effects were detected during follow-up. We employed a NAC dose of 600 mg twice daily based on a previous study by Tepel et al. [4] who demonstrated a reduction in cardiovascular events in patients with end-stage renal failure on this dose of NAC. ROS are unstable compounds with a half-live of seconds; thus, indirect methods have been developed to measure lipoperoxidation products, such as TBARS, or antioxidant products, such as GSH. In the presented case, NAC was safe and managed to reduce TBARS levels as expected and enhanced the HDL protection against LDL oxidation. This finding shows that NAC increased the antioxidant property of HDL minimizing LDL oxidation. In addition, NAC decreased the susceptibility of LDL to oxidation [20]. Oxidized LDL reflects oxidative stress, and its concentration in plasma is related to chronic inflammation and development of several chronic diseases [20, 21]. In conclusion, we present the first in vivo study of ROS reduction by NAC treatment in a boy with triple A syndrome. The long-term effect of this treatment should be evaluated to determine its real benefit for the prevention of the degenerative process in triple A syndrome.

**Disclosure Statement**

The authors declare no conflict of interest regarding this paper.

**References**


