Expression of the Cystine-Glutamate Antiporter with NAC Therapy

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Introduction

- The X-linked condition cerebral-adrenoleukodystrophy (c-ALD) is characterized by the demyelination of neurons and poor adrenal function. The root cause of these symptoms is the accumulation of long chain fatty acids due to a genetic deficiency in the ABCD1 peroxisomal transporter(3). The resulting oxidative stress likely plays a role in the demyelination of neurons, eventually leading to death if left untreated. Recently, the antioxidant, N-acetyl-cysteine (NAC) has been found to increase overall survival of boys with late-stage childhood c-ALD as adjuvant therapy to stem cell transplant(2). While it is thought that NAC acts as a precursor to cysteine which is used for GSH (a potent endogenous antioxidant) synthesis, the mechanisms of action of NAC are not clear (Figure 1).

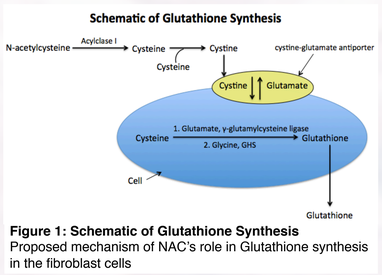

Figure 1: Schematic of Glutathione Synthesis
Proposed mechanism of NAC's role in Glutathione synthesis in the fibroblast cells

- In fibroblast cells, NAC increases GSH under conditions of oxidative stress (Figure 2).

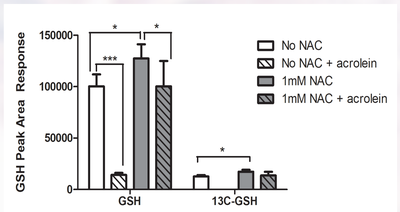

Figure 2: GSH response in ALD-derived fibroblasts after incubation with labeled NAC
ALD-derived fibroblast cells were incubated at 37ºC for 2 hours in media with No NAC, No NAC + 25µM acrolein, 1mM NAC, and 1mM NAC + 25µM acrolein. Cells were lysed and intracellular labeled (^{13}C-GSH) and unlabeled GSH were measured using HPLC-MS. (*) p<0.05; (***) p<0.001.

- The transporter system X_c^- is formed by 2 proteins: a heavy-chain subunit (SLC3A2) and a light-chain subunit, xCT(SLC7A11), and functions as an antiporter transporting cystine into the cell and glutamate out of the cell (Figure 1). This membrane-bound transport system is sodium independent and has been found in several areas of the brain as well as in fibroblast cells. One role of this transporter is to maintain intracellular glutathione levels and the redox balance between cysteine and cystine in extracellular and cerebrospinal fluid. This system may be an important defense mechanism against oxidative stress in the brains of boys with c-ALD (4,5).

- **Hypothesis:** NAC upregulates the human cystine-glutamate antiporter under conditions of oxidative stress, thereby increasing GSH synthesis. The results of this study will help us to better understand the mechanisms of the drug's action in patients with c-ALD.

Literature cited

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Materials and Methods

Preparation of Cell Lysates:

- Induced pluripotent stem (iPS) cells were derived from skin cells collected from boys with c-ALD. The iPS cells were differentiated to fibroblast cells.
- Cells were grown on 24-well plates cultured in DMEM and incubated at 37°C.The following treatments were included in the media and cells were incubated for 24 hours:
  - Control (n=6)
  - 1 mM NAC (n=6)
  - 1 mM NAC + Acrolein (n=6)
  - 500 µM NAC (n=6)
  - 500 µM NAC + Acrolein (n=6)
  - 250 µM NAC (n=4)
  - 250 µM NAC + Acrolein (n=4)

- After 24 hours, the media was removed. Each well was washed twice with cold PBS. 75µL PBS was added to each well and the resulting cell suspensions were transferred to appropriate tubes. The samples underwent 2 freeze-lyse cycles and were stored at -20°C until analysis.

Bradford Assay:

- Total protein content for each sample was determined using a Bradford assay according to directions provided by Bio-Rad Laboratories, Inc.

ELISA:

- The ELISA protocol was followed as outlined in the user manual for the human cystine/glutamate transporter (SLC7A11) ELISA kit from CUSABIO Biotech Co., Ltd. Absorbance was measured at 450nm and 570nm (background) using BioTek Synergy 2 plate reader. Background was subtracted from the 450nm readings.

Data Analysis:

- Descriptive statistics were determined using Excel. The SLC7A11 Calibration Curve was made using linear regression of the log(Absorbance) vs. log(Concentrations). An ANOVA (Tukey) test was performed to evaluate differences between treatment groups using Graph Pad Prism.

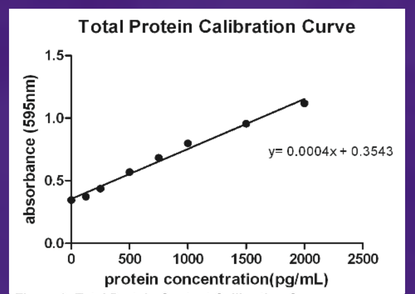

Figure 3: Total Protein Content Calibration Curve
The standard concentration curve used in the Bradford assay to determine total protein content was linear (r^2 = 0.98). The equation for the line of best fit of these standards was used to determine the total protein concentrations of the samples from each treatment group.

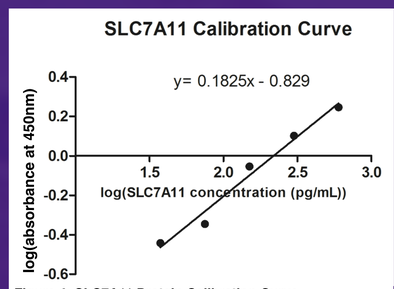

Figure 4: SLC7A11 Protein Calibration Curve
The standard concentration curve used in the ELISA to determine SLC7A11 protein content was linear (r^2 = 0.9784). The equation for the line of best fit of these standards was used to determine the SLC7A11 protein concentrations of the samples from each treatment group.

Table 1: Total Protein Content of each Treatment Group
The mean total protein content values for each treatment group were similar with no significant differences between groups observed.

		Protein Content		
Treatment	**n**	**Mean**	**SD**	**%CV**
Control	6	204.3	56.4	27.6
1mM NAC	6	259.3	71.3	27.5
1mM NAC + Ac	6	273.8	73.9	27.0
500µM NAC	6	302.2	123.9	41.0
500µM NAC + Ac	6	203.0	34.7	17.1
250µM NAC	4	218.6	134.1	61.3
250µM NAC + Ac	4	288.0	138.2	48.0

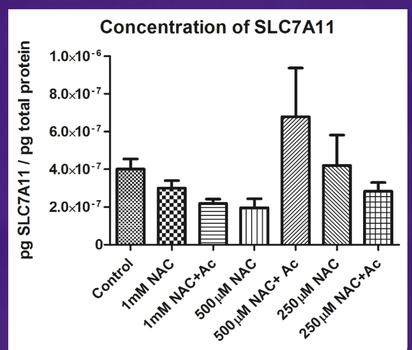

Figure 5: SLC7A11 Protein Expression
There was no detectable difference between SLC7A11 expression and any of the treatment groups. An ANOVA (Tukey) test confirmed that there was no statistically significant difference in SLC7A11 protein expression between any of the treatment groups. *samples below the lower limit of quantitation

Results

Total Protein: A Bradford assay was run to determine the overall protein concentration in each of the samples. The calibration curve determined using the standards is displayed in Figure 3. The data were linear with an r^2 value of 0.98. The equation for the line of best fit was used to determine the concentrations of each of the samples in the treatment groups. The mean total protein concentration, as well as the standard deviation and the coefficient of variation was calculated for each treatment group, as displayed in Table 1. The total protein concentration did not differ significantly between treatment groups. The coefficient of variation was also similar between each treatment group. The treatment groups using 250 µM NAC appeared to have had greater variability in protein concentration than the other treatment groups, as seen in the high standard deviation and coefficient of variation, however, this can be attributed to the lower number of samples (n=4) for this concentration of NAC.

SLC7A11 Protein: The calibration curve and line of best fit for the SLC7A11 protein standards are shown in Figure 4. The SLC7A11 concentrations were normalized by dividing each by the total protein concentrations that were determined from the Bradford assay. The mean + standard deviation SLC7A11 protein concentrations are shown for each treatment group in the bar graph, Figure 5. There were no significant differences in normalized SLC7A11 protein concentration between treatment groups.

Conclusions and Future Applications

There was no statistically significant difference in the SLC7A11 protein expression between any of the treatment groups. Since the SLC7A11 protein is unique to the human cystine-glutamate antiporter (X_c^- system), it can be concluded that NAC treatment, with or without the presence of oxidative stress, has little to no effect on the expression of the X_c^- system. This may mean that NAC is using a different transporter in order to increase the production of glutathione, such as the ASC-1 (alanine-serine-cysteine transporter 1) transporter system, or that NAC increases glutathione levels in another manner and does not increase expression of any transporter at all. Future studies could include evaluating the effect of NAC on other cysteine transporters. In order to fully confirm the findings, this study should be repeated using a higher cell concentration in order to be sure that all absorbance readings are above the lower limit of quantitation.

A better understanding of the mechanisms of NACs actions will allow us to optimize dose, dosage, and route of administration of NAC therapy, with the goal of improving the overall outcome with NAC treatment.

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