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Redox Abnormalities as a Vulnerability Phenotype for Autism and Related Alterations in CNS Development

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### Title and Subtitle
Redox Abnormalities as a Vulnerability Phenotype for Autism and Related Alterations in CNS Development

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### Abstract
We hypothesize that low systemic redox potential (GSH/GSSG; cysteine/cystine) reflects a vulnerability phenotype that is associated with regressive autism and is predictive of the risk of developing autism. The redox vulnerability phenotype is associated with epigenetic alterations in primary immune cells that may be reversible with restoration of intracellular redox potential. The hypothesis predicts that children with regressive autism and high risk (developmentally-delayed) children who are subsequently diagnosed with autism will exhibit lower redox potential compared to age-matched unaffected control children. It also predicts that low redox potential from these children will be associated with epigenetic modifications in DNA methylation and histone acetylation/methylation that are reversible with treatment to restore redox potential. In Aim 1 we will determine whether redox potential in immune cells can be used as a biomarker for regressive autism and whether it is predictive of the subsequent diagnosis of autism. We will also evaluate immune redox potential from high risk developmentally delayed children to determine whether redox status is predictive of subsequent development of autism. In Aim 2, we will determine whether immune cells from autistic children are associated with altered cytokine patterns, macrophage/T cell DNA methylation, and chromatin histone methylation compared to control children.

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### Security Classification
None provided.
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INTRODUCTION:
Based on our preliminary studies, we hypothesized that children with autism spectrum disorders (ASD) have a more oxidized metabolic status than normal children. The goal of Aim 1 of this project is to better define the functional implications of redox abnormalities associated with autism and to study the predictive potential of the GSH/GSSG redox ratio as a biomarker for autism. The goal of Aim 2 is to determine whether targeted treatment to increase redox potential will alter cytokine balance and epigenetic alterations in primary immune cells from children with ASD. A summary of our progress to date is summarized below in the body of the report following the format of Project 1 SOW and is accompanied by data generated this year with interpretation of these results.

BODY:
*Aim 1: Determine whether redox potential in plasma can be used as a biomarker for regressive autism and whether it is predictive of the subsequent diagnosis of autism.*

a) DoD regulatory review and approval of our UAMS IRB-approved protocol and consents for our ongoing NIH grant (1RO1HD051873) (months 1-4) **Done**

b) Biomarkers for regressive autism: Selection of children with 50 regressive autism, 50 infantile autism and 50 age-matched control boys; Selection of 30 children with developmental delay (DD) with diagnosis of autism; 30 children with DD without autism; 30 age-matched control children (on-going years 1-3)

**Progress:** We have increased our number of autistic children recruited this year and have identified 24 children with sudden onset regression, 35 children with infantile autism and 73 control children. Figure 1 presents our current data comparing plasma concentrations of total glutathione (GSH) between children with regressive onset and age matched unrelated control children. In Figure 2, we have added, a new biomarker of oxidative protein damage. Nitrotyrosine is an oxidative by-product of protein tyrosine oxidative damage.

**Interpretation:** Compared to unaffected control children, the autistic children who underwent regression had lower levels of plasma glutathione which would be consistent with a more oxidized internal environment. Lower mean levels of glutathione were accompanied by an increase in nitrotyrosine, a biomarker of oxidative protein damage. Together these data suggest that the decrease in antioxidant “capacity” is accompanied by an increase in oxidative damage in children with autism. The functional consequence, if any, cannot be determined.
from this data but it indicates that the more oxidizing conditions are associated with altered macromolecular structure.

In Figures 3-5, we divided the regression cohort into “sudden” regression and “gradual” regression and find that both subgroups have decreased plasma levels of glutathione and increased nitrotyrosine protein oxidative damage relative to unaffected controls (figures 3 and 4). Interestingly, low levels of SAM/SAH, an indication of reduced methylation capacity was associated with a significant decrease in global DNA methylation (Figure 5).

**Figure 3**

**Figure 4**

**Figure 5**

c) **Predictive biomarkers in toddlers** (age 18-30 months) with developmental delay who fail a standardized autism screening test (MCHAT) and are at high risk of developing autism. We will use extracts from plasma and primary cells to identify redox-related predictive biomarkers of regressive autism in newly recruited cases and controls (ongoing; years 1-3)

**Progress:** Because extracts from frozen RBC pellets proved to be unstable and not reproducible, we have been collecting data on fresh primary leukocytes from newly recruited patients. Preliminary results are presented below comparing plasma GSH/GSSG and nitrotyrosine levels in developmentally delayed children who failed the MCHAT (high risk) with those that passed the M-CHAT (low risk). Currently we have analyzed data on 59 children who have undergone regression of which 24 experienced sudden regression and 35 experienced gradual onset regression. To date we have data on age-matched unrelated unaffected control children. The data in Figure 6 below indicate that the glutathione redox status (GSH/GSSG) is significantly decreased in children who failed the MCHAT and are at higher risk of developing autism. In Figure 7 below, nitrotyrosine levels are shown to be significantly increased among the high risk children who failed the MCHAT compared to those who passed.
**Interpretation:** These preliminary results are consistent with a lower GSH/GSSG redox potential and elevated nitrotyrosine levels in children who are at high risk of developing autism. These results may serve as predictive biomarkers if they are maintained as we increase our sample number in the next year.

e) HLPC-ESI-MS analysis of redox couples GSH/GSSG and cysteine/cystine in blood samples and mouse tissue from Dr. Noble (ongoing; years 1-3).

**Progress:** The preliminary results for the GSH/GSSG and Cysteine/Cystine redox ratio in 55 blood samples from autistic children and 51 age-matched control children are presented in Figures 8 and 9 below.

**Interpretation:** Both GSH/GSSG (reflection of intracellular redox status) and Cysteine/Cystine (reflection of extracellular redox status) were significantly lower in autistic compared to control children.

**Deliverables:** We anticipate 2 publications in major peer-reviewed journals once data collection is complete. The discovery of a more oxidized phenotype among children with regressive autism and/or as a biomarker for the risk for developing autism would provide new insights into the etiology of autism as well as earlier detection and new treatment strategies

**Aim 2:** Determine whether targeted treatment to increase glutathione redox potential in autistic children will restore cytokine balance and reverse epigenetic alterations in primary immune cells.

a) DoD regulatory review and approval of our UAMS IRB-approved protocol and consents for our ongoing Arkansas Children’s Hospital Foundation intervention study (months 1-4) **Done**
b) Sample collection before and after nutritional intervention to increase GSH/GSSG redox in 30 autistic (total 60 samples) and 30 controls in our IRB-approved clinical trial (Ongoing; yrs 1-3)

**Progress:** We have screened 15 and recruited 10 autistic children into our double-blind placebo controlled study to date. We are unable to break the code until we have 15 children who have completed the trial so we are unable to analyze effect of intervention at this time.

c) Purification of monocyte/macrophages and T cells from fresh blood samples and determine intracellular GSH/GSSG status (years 1-3)

**Progress:** Methodology for macrophage and T cell isolation has been successfully accomplished using monoclonal antibodies and flow cytometry. We are able to obtain 75% pure monocytes and 90% pure T cells. We have measured intracellular glutathione redox ratio in total PBMCs and in purified primary monocytes and T cells from control children and children with autism. The results shown in Figures 9 and 10 indicate that the intracellular GSH/GSSG ratio is lower and the percent oxidized glutathione (GSSG) is increased in PBMCs, purified monocytes and purified T cells from children with autism compared to unaffected control children.

![Figure 9](image1)

**Interpretation:**
1. PBMC from children with autism have a significantly increased concentration of GSSG as well as more oxidized glutathione equivalents and significantly decreased GSH/GSSG compared to unaffected control children.
2. Following PMA/ionomycin stimulation, CD4\(^+\) T cells from children with autism have significantly decreased GSH and GSH/GSSG, as well as significantly more oxidized glutathione equivalents compared to unaffected control children.
3. Following LPS stimulation, monocytes from children with autism have a significantly increased concentration of GSSG, significantly decreased GSH/GSSG, and significantly more oxidized glutathione equivalents compared to unaffected control children.

d) **Leukocyte global DNA methylation** determination using HLPC-ESI-MS technology before and after targeted intervention to increase GSH. (ongoing; years 1-3)

**Progress:** We have completed DNA extraction and DNA methylation on ~100 cases and controls – recruitment is on-going. Preliminary results from 50 case and 50 control children are presented below.
**Figure 11:** SAM/SAH methylation capacity and global DNA methylation levels in case and control children

**Figure 12:** GSH/GSSG redox ratio, and protein oxidative damage (nitrotyrosine) and DNA oxidative damage (8-oxo-dG)

**Interpretation:** The results presented in Figure 11 suggest that global DNA methylation is significantly decreased in autistic compared to control children and is associated with lower methylation potential (SAM/SAH). Similarly, a decreased in glutathione redox ratio was accompanied by an increase in protein oxidative damage (nitrotyrosine) and DNA oxidative damage (8-oxo-dG). These results suggest that a decrease in methylation and redox potential may have a functional consequence in terms of oxidative damage.

e) **Determine intracellular cytokine patterns** with flow cytometry in stimulated leukocytes from 30 controls and 30 autistic children before and after intervention to increase GSH (yrs 1-3)

**Progress:** We have measured intracellular TNFα, IL-1 and IL-6 cytokine production in LPS stimulated monocytes from autistic children, their siblings and unaffected control children.

**Figure 13:** Intracellular TNF-α cytokine production in LPS-stimulated monocytes

**Interpretation:** Decreases in IL-6 and IL-1 were observed suggesting a dysregulated immune response to immune stimulation.

f) **Determine gene-specific histone acetylation/methylation** patterns in T cells using chromatin immunoprecipitation (ChIP) in lymphoblastoid cells from autism and control cell lines (ongoing years 1-3)

**Progress:** We have worked up methodology of native ChIP and quantification of histone H3K9 and H3K27, and H3K4 methylation and acetylation in the promoter region of the TNF-α gene in lymphoblastoid cells derived from autistic and unaffected control individuals.
**Interpretation:** Representative results in Figure 14 and 15 are consistent with epigenetic alterations that increase expression of the TNF-α gene in autism cells compared to control cells. We anticipate additional comparisons form at least 6 pairs of case and control cell lines.

**g) Statistical Analysis and manuscript writing: (Year 3)**

**Deliverables:** We anticipate 2-3 publications in major peer-reviewed journals. We expect that targeted nutritional intervention previously shown to increase plasma GSH/GSSG will increase leukocyte GSH/GSSG and will also restore cytokine balance/epigenetic dysfunction to improve immune function in autistic children. We anticipate that this project will be the first to provide definitive evidence for epigenetic dysregulation of immune function in autism.

**Problems encountered and solutions:**
1. We were unable to get reproducible results using our stored frozen cell pellets as originally described in the protocol. Instead, we will only be able to measure intracellular GSH/GSSG on fresh primary cells from newly recruited children. Recruitment is ongoing and we anticipate we will have sufficient number of subjects for statistically meaningful results. Because we cannot use previously frozen cells and must rely on newly recruited children, our total samples numbers will be lower than originally projected.
2. We have found that the volume of blood obtainable varies between children. Some children are more difficult to stick and we may only get 3 ml instead of 20ml needed to do full analysis. We have established a priority of assays depending on final volume obtained: PBMCs > monocyte > T cells.
3. We have found that histone purification from primary cells is also dependent on cell volume obtained and has not been reproducible. We have successfully purified histones from lymphoblastoid cell lines obtained from children with autism and unaffected controls. We will do assays on cell lines rather than primary cells to assure reproducibility and meaningful results.

**KEY RESEARCH ACCOMPLISHMENTS**
- Autistic children who underwent regression had lower levels of plasma glutathione and increased levels of nitrotyrosine, a biomarker of protein oxidation. These results are consistent with a more oxidized internal environment in children with autism.
- The DNA of children who experienced regression was globally hypomethylated compared to unaffected control children. These results are consistent with epigenetic dysregulation.
- Developmentally delayed children who failed a standardized screening test for autism (high risk for autism) had lower plasma glutathione levels and increased nitrotyrosine levels compared to low risk children who passed the screening test.
The two major redox couples in plasma, GSH/GSSG (reflection of intracellular redox) and cysteine/cystine (extracellular redox) were decreased in children with autism compared to controls. Intracellular GSH/GSSG redox was measured in isolated primary PBMCs and purified monocytes and T cells and found to be significantly decreased relative to control children. Intracellular GSSG equivalents were significantly increased in these primary immune cells. Biomarkers of protein and DNA oxidative damage (nitrotyrosine and 8-oxo-deoxygaunosine) were increased in children with autism suggesting that the decrease in methylation and redox “capacity” may have a functional consequence in terms of oxidative “damage”.

The production of cytokines IL-6 and IL-1 were decreased in children with autism. Methylation of histones in the promoter region of the TNFα cytokine gene were hypomethylated which is consistent with increased expression of this inflammatory cytokine.

REPORTABLE OUTCOMES
1. Poster Presentations:
   IMFAR:
   SOCIETY FOR NEUROSCIENCE:
   “Glutathione Redox Imbalance and Altered TNFα Production in Peripheral Blood Mononuclear Cells from Children with Autism”  Shannon Rose, Stepan Melnyk, Oleksandra Pavliv, Timothy A. Trusty, and S. Jill James
   ARKANSAS BIOSCIENCES INSTITUTE (2):
   “Glutathione Redox Imbalance and Cytokine Production in Peripheral Blood Mononuclear Cells from Children with Autism”  Shannon Rose, Stepan Melnyk, Timothy A. Trusty, Oleksandra Pavliv, and S. Jill James


3. Plasma samples have been sent to Co-I Dr. Hepel at SUNY New York.

4. We have received 13 autism and control matched pairs of frozen brain tissues from the Autism Tissue Program.

5. NIH R01 has been submitted entitled “Oxidative stress and epigenetic dysregulation in the autism brain”  Our data showing histone methylation in the TNF-a promoter was used as preliminary evidence to study similar modifications in frozen brain tissue from autistic and control individuals.

CONCLUSION: Definitive conclusions not possible until data collection is complete.

REFERENCES: None

APPENDICES: None