Activities of key glycolytic enzymes in the plasma of Saudi autistic patients

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Objective: Measurement of plasma levels of lactate, lactate oxidase (LOX), pyruvate kinase (PK), and hexokinase (HK) as possible glycolytic parameters to assess brain damage in autistic patients.

Design and methods: Plasmatic levels of lactate, LOX, PK, and HK were determined in 20 autistic children aged 3–15 years and 20 age-matching healthy control subjects.

Results: Plasmatic levels of lactate and LOX were significantly higher in autistic patients compared to healthy subjects and that of PK and HK were significantly lower in these patients as compared to controls. This could reflect the impaired metabolism of astrocytes, the brain cells responsible for the production and provision of lactate, as the primary metabolic fuel for neurons.

Conclusion: Remarkably different levels of plasma glycolytic parameters were recorded in Saudi autistic patients. This could be correlated to the impairment of energy metabolism, glutathione depletion, and lead intoxication previously detected in the same investigated samples. The identification of biochemical markers related to autism would be advantageous for earlier clinical diagnosis and intervention.

Keywords: autism, glycolysis, lactate, lactate oxidase, pyruvate kinase, hexokinase

Introduction

Autism is a disorder of reciprocal social interaction, behavioral repertoire, and language and communication disabilities.¹ Because the phenotype ranges from manifest disability to specific performance elevation, the term autistic spectrum disorder (ASD) is now commonly used to denote the Diagnostic and Statistical Manual of Mental Disorder, 4th Edition (DSM-IV)-defined group of pervasive neurodevelopmental disorders encompassing autistic disorder including Asperger’s disorder, Rett’s disorder, and pervasive developmental disorder not otherwise specified (PDDNOS).¹² A fraction of cases have a defined genetic cause, but the apparent increase in prevalence of ASD as reviewed is suggestive of an environmental contribution.¹³⁻⁵ Changes in awareness and diagnostic criteria may explain some of the rise but a true increase in prevalence has not been excluded.⁶⁻⁷ Elevated ASD rates in urban versus rural areas are consistent with an environmental contribution.⁸⁻⁹ Recently, Weissman and colleagues pointed to several underlying pathophysiological mechanisms in autism, including altered neurite morphology, synaptogenesis and cell migration due to abnormalities in distinct ensembles of proteins and pathways. In a cohort analysis, they reported that defective mitochondrial oxidative phosphorylation is an additional pathogenetic basis for a subset of individuals with autism.¹⁰ Impairment of energy metabolism due to mitochondrial
dysfunction was confirmed by Al-Mosalem and colleagues in a study of 30 Saudi autistic children.11

Glucose had long been thought to fuel oxidative metabolism in active neurons until the recently proposed astrocyte-neuron lactate shuttle hypothesis (ANLSH) challenged this view. According to the ANLSH, activity-induced uptake of glucose takes place predominantly in astrocytes, which metabolize glucose anaerobically. Lactate produced from anaerobic glycolysis in astrocytes is then released from astrocytes and provides the primary metabolic fuel for neurons. The conventional hypothesis asserts that glucose is the primary substrate for both neurons and astrocytes during neural activity and that lactate produced during activity is removed mainly after neural activity.12 The dependence of brain function on blood glucose as a fuel does not exclude the possibility that lactate within the brain might be transferred between different cell types and serve as an energy source. It has been suggested recently that 1) about 85% of glucose consumption during brain activation is initiated by aerobic glycolysis in astrocytes, triggered by demand for glycolytically derived energy for Na+-dependent accumulation of transmitter glutamate and its amidation to glutamine, and 2) the generated lactate is quantitatively transferred to neurons for oxidative degradation. However, astrocytic glutamate uptake can be fueled by either glycolytically or oxidatively-derived energy and the extent to which “metabolic trafficking” of lactate might occur during brain function is unknown.13 The subcellular compartmentalization of pyruvate allows neurons and astrocytes to select between glucose and lactate as alternative substrates, depending on their relative extracellular concentration and the operation of a redox switch. This mechanism is based on the inhibition of glycolysis at the level of glyceraldehyde 3-phosphate dehydrogenase by NAD+ limitation. Following glutamatergic neurotransmission, increased glutamate uptake by the astrocytes is proposed to augment glycolysis and tricarboxylic acid cycle activity, balancing to a reduced cytosolic NAD+/NADH in the glia. Reducing equivalents are then transferred to the neuron resulting in a reduced neuronal NAD+/NADH redox state. This may eventually switch off neuronal glycolysis, favoring the oxidation of extracellular lactate in the lactate dehydrogenase (LDH) equilibrium and in the neuronal tricarboxylic acid cycles. Finally, pyruvate derived from neuronal lactate oxidation, may return to the extracellular space and to the astrocyte, restoring the basal redox state and beginning a new loop of the lactate/pyruvate transcellular coupling cycle.14

For some time, it has been known that in cultured astrocytes, nitric oxide (NO) can upregulate the rate of glucose consumption and lactate production, suggesting glycolysis activation, a phenomenon that is possibly a consequence of the NO-mediated mitochondrial inhibition.15 However, it is surprising that, in contrast to astrocytes, neurons do not display increased glycolytic rate upon mitochondrial inhibition and this leads to neuronal cell death. Only astrocytes respond by activating, very rapidly (ie, within a few minutes), the glycolytic pathway.16 Interestingly, the increased glycolytic rate in astrocytes served to preserve cells from ATP depletion and cell death, possibly because glycolytic ATP served to drive the reverse activity of ATP synthase in order to maintain the mitochondrial membrane potential.16 It is noteworthy that in several neurodegenerative diseases, such as Alzheimers or Huntingtons, decreased neuronal glycolytic activity has been observed in neurons of the degenerating area.17,18 It is therefore of interest to understand the mechanism(s) responsible for the differential glycolytic response of astrocytes and neurons upon mitochondrial inhibition.

ATP is considered to be a feed-back allosteric inhibitor of 6-phosphofructokinase 1 (Pfk1), key rate-limiting step in the

Figure 1 A) Normal distribution of lactate in the control group. B) Normal distribution of lactate in the autistic group.
glycolytic pathway. Accordingly, for many years it has been considered that the decrease in cytosolic ATP concentrations that follows mitochondrial dysfunction would stop Pfk1 inhibition, thus causing a rapid activation of glycolysis. Indeed, inhibition of mitochondrial ATP synthesis which triggers a rapid Pfk1 activation is known to take place in the intact, but not disrupted, astrocytes. The involvement of other glycolytic enzymes, such as hexokinase(s), pyruvate cannot be disregarded as potential targets of NO-mediated glycolysis activation.

Regarding the possibility of using plasma glycolytic enzymes as biomarkers for brain damage, Tadeusz measured the activities of several glycolytic enzymes such as hexokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase, as well as glycerol-1-phosphate dehydrogenase, and (Mg$^{2+}$)ATPase in normal cerebrospinal fluid (CSF) and blood plasma. Samples were drawn from 12 healthy infants and in supernatants from brain tissue slices taken during neurosurgical operations from infants of the same range of age. The values obtained confirm the high activity of the above-mentioned enzymes in human brains and indicate an independence of this activity in blood plasma and CSF. The origin of the activities of the investigated enzymes in CSF seems to be mainly, if not exclusively, from brain tissue. This finding might prove useful for detection of brain tissue damage, as was earlier shown with LDH activity in CSF and plasma.

Selaković et al reported that many substances are released into the CSF and blood during brain damage but the ideal damage marker would have to satisfy certain requirements: to be localized intracellularly, present in high concentration in brain tissue, and to be relatively easy to detect. They reported that neuron-specific enolase (NSE) as a glycolytic pathway isoenzyme, specific for phosphoglycerate and phosphoenolpyruvate (2-phospho-D-glycerate- hydrolase, EC. 4.1.11) has recently been recorded as brain damage marker. The increase in concentration of NSE in CSF and plasma has been detected in patients with brain ischemia and can be significant in the early diagnosis of BI.

This information initiated our interest in measuring three glycolytic enzymes (hexokinase, pyruvate kinase and lactate dehydrogenase) in Saudi autistic children compared to healthy age-matching control subjects in a trial. Our aim was to investigate a potential correlation between the activity of these enzymes with the previously measured parameters related to energy metabolism and oxidative stress in the same investigated samples.

**Material and methods**

**Patients and subjects**
The subjects enrolled in this study were 20 children with autism (16 males and 4 females) ranging in age from 3–15 years, and another 20 age-matching children (15 males and 5 females) as a control group. The diagnosis of autism was made by child neuropsychiatrists based on the criteria of autistic disorder as defined in the DSM-IV. Complete diagnostic work-ups including medical, neurological, psychiatric, and psychological evaluations were done for all of the studied children with autism. All were of good physical health and were not taking any medications or nutrient supplements. Written consent was obtained from the parents of each subject, according to the guidelines of the ethical committee of King Khalid Hospital, King Saud University, Riyadh, Saudi Arabia.

**Blood samples**

After an overnight fast, 10 mL blood samples were collected from both groups in test tubes containing heparin as an anticoagulant. Centrifugation was done at 3000 rpm. Plasma was obtained and deep frozen (–80°C) until analysis time.
Chemicals
All chemicals used in this study were of analytical grade and were products of Sigma (St. Louis, MO, USA), or Merck (Darmstadt, Germany). Lactate oxidase and lactate kits were products of the United Diagnostics Industry (UDI), Kingdom of Saudi Arabia.

Biochemical analyses
Measurement of lactate
Lactate present in the samples was determined according to the method of Brandt and colleagues using a diagnostic kit.

Measurement of LOX
Quantitative determination of LOX in plasma was performed according to the method of Henry using a lactate to pyruvate kinetic method.

Measurement of HK
Hexokinase was assayed in plasma according to the method of Abraham-Neto and colleagues in which a reduction of NADPH through a coupled reaction with glucose-6-phosphate dehydrogenase (G-6-PDH), is determined spectrophotometrically by measuring the increase in absorbance at 340 nm.

Measurement of PK
Pyruvate kinase was determined in plasma according to the method of Malcovati and Valentini by which the rate of decrease in absorption at 340 nm, due to oxidation of NADH by coupling the system with an excess of LDH, was followed.

Statistical analysis
SPSS software (SPSS Inc., Chicago, IL) was used to analyze the data. Results were expressed as mean ± standard deviation (SD). The data from the patient group was compared with data from the control group using Student’s t-test. A P value of <0.05 was considered statistically significant. Pearson correlations between the measured parameters are presented.

Results
Levels of lactate, LOX, PK and HK for both the control and autistic groups are presented in Tables 1 and 2. Results are given as Mean ± S.D. Normal distribution of the measured parameters in control and autistic Saudi patients together with the percentage change of the measured parameters in autistic compared to control subjects are presented in Figures 1–7.

Discussion
The concept of brain injury is heterogeneous in terms of etiology as well as type and severity of motor and associated disabilities. At this point, because of the survival of extremely premature infants and severely hypoxic neonates, the risk of brain damage has not been eliminated. Lifelong disabilities such as autism, cerebral palsy, epilepsy, behavioral and learning disorders are still some of the consequences of brain injury acquired in fetal life or the perinatal and neonatal periods. Efforts to understand and prevent neonatal cerebral injury are therefore worthwhile.

Finding a single biochemical marker which is both sensitive and specific for brain injury is unlikely because the brain contains many different types of cells, each with a different threshold for injury and different sensitivities to various types of injury. Because of the complexity of the brain, it may be important to develop a panel of markers rather than a single marker to be used as a screening tool. This panel would need to include indicators of neuronal and glial cell injury, as well as markers that are sensitive to direct trauma, hypoxia, and oxidative stress.

Evidence has accumulated over the last two decades indicating that L-lactate (L-LAC) is an important cerebral oxidative-energy substrate. The brain can take up L-LAC from blood, particularly during intense exercise, as well as in the initial minutes of recovery. Moreover, an “astrocyte-neuron L-LAC shuttle” has been proposed, in which astrocytes take up glucose from blood, convert it into L-LAC via glycolysis and then export L-LAC into the extracellular phase through the isof orm 1 of monocarboxylate transporter (MCT1). In turn, neurons take up extracellular L-LAC via the isof orm 2 of monocarboxylate transporter (MCT2) and use it as a fuel for mitochondrial respiration. Recently, it was hypothesized that, in the brain, L-LAC is the principal product of glycolysis, whether or not oxygen is present. The significant increase of plasma lactate found in Saudi autistic children involved in the present study compared to control subjects could possibly reflect the impairment of neuron cell integrity in these patients. This theory is supported by considering the work of Al-Mosalim and colleagues which proposes there is energy metabolism impairment in Saudi autistic children.

Table 1 Lactate levels in serum of control and autistic children

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>0.872 ± 0.335</td>
<td>0.495</td>
<td>1.626</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Autistic</td>
<td>15</td>
<td>1.398 ± 0.819</td>
<td>0.424</td>
<td>3.312</td>
<td></td>
</tr>
</tbody>
</table>

Note: This table describes the independent t-Test between the control and autistic groups in lactate determination levels.
Table 2 Lactate oxidase (LOX), pyruvate kinase (PK), and hexokinase (HK) levels in plasma of control and autistic children

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>Min</th>
<th>Max</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate oxidase (LOX) (µ moles NAD reduced/min/L)</td>
<td>Control</td>
<td>15</td>
<td>133.82 ± 60.56</td>
<td>82.40</td>
<td>266.00</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Autistic</td>
<td>24</td>
<td>237.71 ± 89.11</td>
<td>112.06</td>
<td>448.20</td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase (µ moles NADH oxidized/min/L)</td>
<td>Control</td>
<td>11</td>
<td>73.84 ± 6.80</td>
<td>64.84</td>
<td>88.56</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Autistic</td>
<td>17</td>
<td>28.17 ± 5.55</td>
<td>20.30</td>
<td>38.54</td>
<td></td>
</tr>
<tr>
<td>Hexokinase (µ moles NAD reduced/min/L)</td>
<td>Control</td>
<td>9</td>
<td>106.60 ± 16.28</td>
<td>88.56</td>
<td>138.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autistic</td>
<td>17</td>
<td>25.22 ± 5.07</td>
<td>18.04</td>
<td>34.60</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: This table describes the independent t-Test between the control and autistic groups in LOX, PK, and HK activity levels.

These authors encountered significantly higher activity levels of Na⁺/K⁺ ATPase, creatine kinase, and NTPdase, together with lactate in the plasma of autistic patients. Increased glycolysis confers adaptive advantages if it allows the availability of excess pyruvate for lipid synthesis or by providing essential anabolic substrates. Glucose consumption through the pentose pathway may also provide essential reduction equivalents (ie, NADPH) to decrease the toxicity of reactive oxygen species conferring resistance to senescence.

Table 2 demonstrates the activity levels of lactate oxidase, hexokinase, and pyruvate kinase are three critically important glycolytic enzymes to monitor in Saudi autistic children as compared to the control group. While lactate oxidase was significantly elevated, both hexokinase and pyruvate kinase was unexpectedly lower in the plasma of the autistic children. Figure 3b shows that 15 autistic patients had LOX activity higher than 200 µmoles/minute/L compared to the control subjects represented in Figure 3a. This figure indicates that 11 out of the 15 control subjects had LOX activities less than 175 µmoles/minute/L. Figure 4b illustrates that all the investigated samples from autistic children had PK activity levels less than 40 µmoles/minute/L while all the control subjects (Figure 4a) demonstrated significantly higher activities (ie, more than 65 µmoles/minute/L). A significantly lower activity of HK in autistic patients can be seen in Figure 5b in which 17 autistic exhibited HK activity levels less than 34 µmoles/minute/L compared to significantly higher HK levels in the control subjects (ie, more than 90 µmoles/minute/L).

Lactate oxidase activation can be explained on the basis of substrate availability, since lactate, as a substrate, was found to be significantly higher in the plasma of the Saudi autistic children compared to the healthy age-matched control group. It is known that brain energy supply requires the oxidative metabolism of glucose in mitochondria, and when neural energy demands transiently exceed the rate of oxidative metabolism, l-Lac is produced to supply energy as a result of glycolytic processes. Increased lactate level is related to the reduced use of pyruvate in the citric acid cycle and the increase of anaerobic glycolysis. It is well known that oxidative stress increases the concentrations of lactate dehydrogenase and thus induces the increment of the lactate level. Therefore, significantly increased lactate and LOX observed in the present investigated samples (Figures 1 and 3) might indicate the deficiency of mitochondria function or over-expression of lactate oxidase in autistic children. This explanation is supported by considering the work of...
Al-Gadani and colleagues, and Al-Mosalim and colleagues in which they recorded oxidative stress and the disturbance of energy metabolism in Saudi autistic children compared to age-matching control subjects. Lower activities of HK and PK observed in this study could reflect the less-adaptive capacity of autistic children to cope with energy metabolism impairment, as was previously documented by Al-Mosalim and colleagues. In addition, glycolysis enhancement has recently been reported to cooperate with autophagic mechanisms in preventing caspase-independent cell death, further supporting the notion that glycolysis activation is an important neuronal survival pathway. The relationship between lower plasma HK and PK reported in the present study and the lipoxidative stress data.

Figure 4 A) Normal distribution for control group in pyruvate kinase levels. B) Normal distribution for autistic group in pyruvate kinase levels.

Figure 5 A) Normal distribution for control group in hexokinase levels. B) Normal distribution for autistic group in hexokinase levels.

Figure 6 Percentage change of lactate oxidase, pyruvate kinase, and hexokinase in autistic group compared to control.
Figure 7 A) Correlation between lactate and lactate oxidase (LOX) with best fit line curve (positive correlation). B) Correlation between pyruvate kinase and hexokinase with best fit line curve (positive correlation). C) Correlation between lactate oxidase and pyruvate kinase with best fit line curve (negative correlation). D) Correlation between lactate oxidase and hexokinase with best fit line curve (negative correlation).
previously found in Saudi autistic patients could be supported by considering the recent work of Gomez and Ferrer in which they note lipoxidative damage of three enzymes linked with glycolysis and energy metabolism in the adult human brain. Gomez and Ferrer further observe that increased oxidation of aldolase A, enolase 1, and glycolaldehydes dehydrogenase may result in decreased activity and may partly account for impaired metabolism and function of the frontal lobe in Parkinson’s disease and dementia with Lewy bodies (DLB).

The lower PK activity found in the plasma of Saudi autistic children compared to normal healthy control could be correlated to the gastrointestinal disturbances that often coexist with autism. Czub and colleagues demonstrated that the dimeric isofrom of pyruvate kinase (PK) detected in the stool of children suffering from inflammatory bowel disease (IBD) might serve as a potential non-invasive screening tool for inflamed pouch mucosa. Enzyme immunoreactivity was found to be significantly higher in all IBD patients than in healthy subjects. Lower activity of plasma PK of autistic children could therefore be inversely related to the higher fecal level.

Figures 7a–d demonstrate the correlations between the measured parameters. The positive correlations recorded between lactate and LOX confirmed the possibility of relating these two plasmatic parameters to the etiopathology of autism since higher lactate could explain the induced activity of LOX. Moreover, the positive correlation between PK and HK and the negative correlation between each of these two enzymes and LOX could confirm the importance of lactate and LOX as metabolic markers related to the disease.

In addition, the recorded lower activity of plasma HK and PK could be attributed to the significantly high concentrations of lead previously detected by the authors when using the same investigated samples as the present study. Lead is known to be a potent inhibitor of two sulfhydryl enzymes: hexokinase and pyruvate kinase. Moreover, Hunaiti and Soud reported that lead may bind to and deplete glutathione and generate reactive oxygen species. This finding correlates to the recorded differences in glycolytic enzymes found between autistic and control subjects, as well as the significant depletion of reduced glutathione, and the H$_2$O$_2$ stress previously detected in Saudi autistic children.

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**Disclosures**

The authors report no conflicts of interest in this work.

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