L-ARGININE SUPPLEMENTATION ENHANCES ANTIOXIDANT ACTIVITY AND ERYTHROCYTE INTEGRITY IN SICKLE CELL ANEMIA SUBJECTS

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INTRODUCTION

Sickle cell hemoglobinopathy – common among black populations of the world

• Arises from a replacement on the beta chain of hemoglobin of gluthamic acid by valine at the 6th position (Ingram 1956).

• Sickle cell anemia (SCA) - characterized by chronic hemolytic anemia and periodic, painful vaso-occlusive events (Raghupathy and Billet 2009).
Incidence of sickle cell disease (SCD) in Africa

- between 5% and 40% (Diallo and Tchemia 2001).

Estimated new births:

- In Africa - 120,000 to 200,000 babies each year with SCD (WHO 1994).

- In Nigeria - 45,000 to 90,000 babies each year (Shenoy 2007).
There is no known cure for sickle cell disease (SCD). **Hydroxyurea** currently used in the management of SCD

- Considered safe.

- Decreases the polymerization rate of Hb S by increasing Hb F concentration (Kato 2008; Raghupathy and Billet 2009).

- May possess myelosuppressive effects on leucocytes and platelets.

- Does not prevent stroke even with elevation of Hb F levels. (Atweh and Schechter 2001).
Inhaled nitric oxide.

In transgenic sickle mice, nitric oxide inhalation:

- Reduced red cell density

- Increased perfusion and

However, clinical studies on nitric oxide inhalation had provided divergent results.

- Inhaled NO significantly reduced pain scores in adult patients (Head et al, 2010) and children with acute VOC pain (Weiner et al, 2003).

- Gladwin et al, (2011) showed that inhaled NO did not reduce VOC severity in SCD.

In spite of these controversies its application may limited by the following reasons:
• It is cumbersome

• May have side-effects that require close monitoring in an acute care setting (Morris et al, 2000).

• In Africa where the SCD burden is high, manpower, technological and infrastructural support may be lacking.

• The cost may also be prohibitive.
**Sildenafil** amplifies the effect of endogenous NO by inhibiting breakdown of cyclic GMP (Machado et al, 2005).

- Sildenafil was discontinued due to safety concerns (Machado et al, 2011).

- **Stem cell transplantation** (Shenoy 2007) and **gene therapy** (Townes 2008)
  - Novel methods
  - In their experimental stages
  - Very expensive and sophisticated for the African population that has a high SCD sub-population.
Arginine therapy:
In sickle cell transgenic mice:

• Improved microvascular function (Kaul et al, 2008)

• Decreased inflammatory cytokines and c-reactive protein (Archer et al, 2008),

• Inhibited red cell Gardos channels and red cell density (Romero et al, 2002)

• Protected against oxidative stress (Dasgupta et al, 2006).
In humans with SCD, Arginine:

- Increased erythrocyte glutathione levels (Little et al, 2009).

- Increased nitric oxide metabolites ($\text{NO}_x$) (Morris et al, 2000).

- Significantly reduced total opioid use and pain scores in children that had acute pain crises (Morris et al, 2013).
However, the doses of L-Arginine used in these studies were high and for variable periods.

- Low-dose arginine therapy may be sub-therapeutic in SCD (Morris et al, 2013)
  
  - Earlier studies had shown that low-dose arginine did not affect NO synthesis (Maxwell and Cooke, 1998; Styles et al, 2007).
Higher plasma concentrations of arginine may be needed to produce beneficial effects in SCD subjects (Archer et al, 2008)

- Arginine metabolism had been shown to be different between sickle cell disease subjects in the steady state and those in vaso occlusive crisis or normal non sickle cell disease subjects (Morris et al, 2000; Lopez et al, 2008).
Since earlier authors had employed high dose and short term administration of arginine

We rationalized that:
  • low dose
  • long term administration of arginine

May enhance plasma concentrations of arginine which may be beneficial to the disease sufferers.
AIM OF STUDY
This study therefore sought to investigate in sickle cell anemia subjects the effect of a low-dose, oral supplementation with L-Arginine (1g/day for 6 weeks) on:

- Plasma arginine concentration ([R]).
- Total anti-oxidant enzymes (TAE) level.
- Malondialdehyde concentration ([MDA]),
- Osmotic fragility,
- Some hematological parameters,
- Red blood cell indices
- Percent irreversibly sickled cells (%ISC).
MATERIALS AND METHODS

• Twenty eight (28) sickle cell anemia subjects were recruited for the study after due:

• Institutional Ethical approval had been obtained from the Research Grant and Experimentation Committee of the College of Medicine of the University of Lagos.

• Informed consent was also obtained from each subject.
Subjects were patients regularly attending Out-Patients’ Sickle Cell Clinic of the Lagos University Teaching Hospital (LUTH), Lagos.

• Clinical History including family history
• Blood counts and
• Blood film were done for the red cell morphology (which showed sickle cell and some target cells).
• Sickle solubility test using a freshly prepared buffer mixture and packed red cell from EDTA anticoagulation blood.
• Hard red band on top and colorless solution was obtained
• The hemoglobin electrophoretic pattern was determined for confirmation. (Dacie and Lewis, 1991).

• Subjects had not undergone any blood transfusion in the last six months.

• No vaso-occlusive crisis in the past six months.

• Persons with the sickle cell trait (HbAS) were excluded from the study.
Anthropometric measurements:

- Age (years)
- Height (meters)
- Weight (kilograms) of each subject was recorded.

Five (5) milliliter of blood was withdrawn from an ante-cubital vein of each subject
**Determination of Plasma L-Arginine level ([R])**

Plasma L-Arginine concentration ([R]) (µM/L) was determined using a modification of the Sakaguchi reaction (Li *et al*, 2008).

Absorbance was read at 500nm with a UV-1700 Spectrophotometer (SpectrumLab S23A, Medical Globe, England).

L-Arginine concentration was determined based on a prepared standard curve.
**Determination of total anti-oxidant enzymes (TAE) level**

Total anti-oxidant enzymes level was assessed using the method of Koracevic (Koracevic *et al.*, 2001).

Absorbance was read at 532 nm with a UV-1700 Spectrometer (SpectrumLab S23A, Medical Globe, England) using deionized water as control.
Determination of osmotic fragility of red cells.

Osmotic fragility of red cells was determined by the method of Dacie and Lewis as modified by Jaja et al, (2002).

Hematological and Red blood cell Indices were estimated using an automated counter (Mindray BC 2800 Haematology Automated System, China).

Identification of irreversibly sickled cells was carried out using a modified method of Jensen et al, (1960)
Estimation of malondialdehyde
Malondialdehyde, (MDA), was determined using the thiobarbituric acid method. (Titus et al, 2004).

Data Analyses
Statistical comparisons using the paired t’test and results expressed as Mean ± SEM.

Significance was accepted when p < 0.05.

Correlation coefficients, (r), calculated between change (Δ) in [R] or Δ in TAE level with other measured variables.
RESULTS

Table 1. Physical Characteristics of the subjects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>24.5 ± 1.2</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7 ± 0.02</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>61.7 ± 1.1</td>
</tr>
<tr>
<td>Body mass index (kg/m2)</td>
<td>21.8 ± 0.6</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.
Table 2. Effect of L-Arginine supplementation on measured parameter.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before Suppl.</th>
<th>After Suppl.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[R] (mg/dL)</td>
<td>8.3 ± 0.7</td>
<td>15.2 ± 0.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TAE (mmol/L)</td>
<td>1.0 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>MDA (mM/mg/prot)</td>
<td>41.4 ± 0.4</td>
<td>14.9 ± 1.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>RBC (10^6/mm³)</td>
<td>3.0 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>[Hb] (g/dL)</td>
<td>8.9 ± 0.3</td>
<td>8.7 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>25.1 ± 1.1</td>
<td>26.7 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>35.4 ± 0.01</td>
<td>32.7 ± 0.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>85.1 ± 0.4</td>
<td>86.2 ± 0.3</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.1 ± 0.01</td>
<td>28.2 ± 0.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>%ISC</td>
<td>8.1 ± 0.5</td>
<td>2.0 ± 0.1</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Note: Data are presented as means ± SEM. [R] = arginine concentration; TAE = total antioxidant enzymes; NS = not significant; ISC = irreversibly sickled cells.
Table 3. Correlation coefficients between $\Delta[R]$ or $\Delta$TAE with other measured variables.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta[R]$ Vs $\Delta$TAE</td>
<td>+ 0.8</td>
</tr>
<tr>
<td>$\Delta[R]$ Vs $\Delta$MDA</td>
<td>- 0.5</td>
</tr>
<tr>
<td>$\Delta[R]$ Vs $\Delta$RBC count</td>
<td>- 0.01</td>
</tr>
<tr>
<td>$\Delta[R]$ Vs $\Delta$ISC</td>
<td>- 0.5</td>
</tr>
<tr>
<td>$\Delta$TAE Vs $\Delta$MDA</td>
<td>- 0.7</td>
</tr>
<tr>
<td>$\Delta$TAE Vs $\Delta$RBC</td>
<td>- 0.3</td>
</tr>
<tr>
<td>$\Delta$TAE Vs $\Delta$%ISC</td>
<td>- 0.6</td>
</tr>
</tbody>
</table>

**Note:** Data are presented as means ± SEM. $\Delta$ = change; [R] = Arginine concentration; TAE = total antioxidant enzymes; ISC = irreversibly sickled cells.
Fig. 1. Effect of L-Arginine supplementation on osmotic fragility of erythrocytes of sickle cell anemia subjects.
DISCUSSION

Results of this study show that a 6-week oral supplementation with L-arginine:

• Increased plasma arginine concentration in the subjects.

• Result agrees with earlier studies in humans (Morris et al, 2003; Scavelle et al, 2010) and mouse model of sickle cell (Romero et al, 2002).
Results also show that arginine supplementation:

- Increased total antioxidant enzymes level but reduced MDA level in the subjects i.e. reduced oxidative stress burden in SCD subjects.

- These agree with earlier results in:
  - Transgenic sickle cell mice (Dasgupta et al, 2006; Kaul et al, 2008)
  - Humans (Little et al, 2009).

- Results are further reinforced by correlation coefficients on Table 3.
Supplementation significantly:
• Increased mean corpuscular volume (MCV)
• Decreased mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)
  • These suggest a reduction in red cell density.

L-Arginine had been shown to reduce MCHC and red cell density by inhibiting Ca^{2+} activated K^{+} channels in sickle cell transgenic mice (Romero et al, 2002).

Reduction in red cell density would reduce polymerization of the Hb S and thus reduce the tendency of the cell to sickle (Jaja et al, 2000; Romero et al, 2002).
Further, reduction in percent irreversibly sickled cell count as seen in this study would:

• Reduce stasis,
• Improve blood flow
• Reduce the frequency of painful crises since irreversibly sickled cells are involved in the initiation of painful crises (Kaul et al, 1993).

Shift in osmotic fragility curve to the left indicates:

• An increase in resistance to lyses
• Attributed to increased antioxidant activity and reduced plasma MDA concentration (reduced oxidative stress burden) elicited by L-Arginine supplementation.
DOSAGE

(Dasgupta et al, 2006; Kaul et al, 2008).
- Fed transgenic sickle mice for 15 days (short term) with mouse chow supplemented with 5% (high dose) arginine.

- Arginine increased the catalytic antioxidants (Dasgupta et al, 2006).
- Normalized several markers of oxidative stress (Kaul et al, 2008).
In children:
• Oral L-arginine at a dose of 0.5 g/kg, 0.1 g/kg or 0.25 g/kg as a one-time dose:
  • Increased NO$_X$ formation in normal controls
  • Dose dependently in patients with VOC
  • Decreased it in steady-state SCD patients.

Little et al, (2009)
In adults:
• High dose of L-Arginine (0.1 – 0.2 g/kg, thrice a day for 3 months) on adults with SCD who were on hydroxyurea.
  • Restoration of total erythrocyte glutathione.
Morris *et al*, 2013 - Clinical trial.

In children:

- Oral, acute supplementation (100mg/kg thrice daily for 5 days; i.e. high dose in a short period) to SCD children who had acute pain crises
  - Caused a significant reduction in total opioid use and pain scores
In this study:
• Very low dose chronic supplementation (1g/day, for 6 weeks) led to:
  • Recovery of arginine stores.
  • Reduced oxidative stress burden.
  • Increased resistance to lyses.
  • Reduction in red cell density and percent irreversibly sickle cell count.

Thus low-dose, long-term supplementation may be beneficial to sickle cell disease subjects in the steady state.
LIMITATIONS
(1) Study planned as an open label study and not a clinical trial. Results have no links to clinical outcomes.

(2) L-Arginine, (Mason Vitamins, Inc. Miami Lakes, Florida, USA.) - over the counter preparation.

Two capsules contain L-Arginine HCl (1000 mg) and calcium (as dicalcium phosphate, 120 mg).

Gelatin (bovine), magnesium stearate, stearic acid, and silica - concentrations not stated by manufacturer and contributions to the results are unknown.
(3) We used the method as reported by Li et al, (2008) for the estimation of L-arginine.

HPLC amino acid analyser or capillary electrophoresis are more commonly reported.
REFERENCES


THANKS FOR THE ATTENTION