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 myelosuppresive 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

- Decreased lung injury, microvascular vaso-occlusion and mortality (Martinez-Ruiz, 2001; de Franceschi *et al*, 2003).

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.

Inspite 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 (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.

Anthropometic 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.

Age (yrs)	24.5 ± 1.2
Height (m)	1.7 ± 0.02
Weight (kg)	61.7 ± 1.1
Body mass index (kg/m2)	21.8 ± 0.6

Data are presented as means ± SEM.

Table 2. Effect of L-Arginine supplementation on measured parameter.

Parameter	Before Suppl.	After Suppl.	p-value
[R] (mg/dL)	8.3 ± 0.7	15.2 ± 0.4	< 0.001
TAE (mmol/L)	1.0 ± 0.1	1.6 ± 0.2	< 0.05
MDA (mM/mg/prot)	41.4 ± 0.4	14.9 ± 1.1	< 0.001
RBC (10^6/mm^3)	3.0 ± 0.2	3.1 ± 0.2	NS
[Hb] (g/dL)	8.9 ± 0.3	8.7 ± 0.4	NS
PCV (%)	25.1 ± 1.1	26.7 ± 1.1	NS
MCHC (g/dL)	35.4 ± 0.01	32.7 ± 0.01	< 0.001
MCV (fL)	85.1 ± 0.4	86.2 ± 0.3	< 0.05
MCH (pg)	30.1 ± 0.01	28.2 ± 0.1	< 0.001
%ISC	8.1 ± 0.5	2.0 ± 0.1	< 0.001

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 Δ[R] or ΔTAE with other measured variables.

Parameters	Correlation Coefficient (r)
Δ[R] Vs ΔTAE	+ 0.8
Δ[R] Vs ΔMDA	- 0.5
Δ[R] Vs ΔRBC count	- 0.01
Δ[R] Vs ΔISC	- 0.5
ΔTAE Vs ΔMDA	- 0.7
ΔTAE Vs ΔRBC	- 0.3
ΔTAE Vs Δ%ISC	- 0.6

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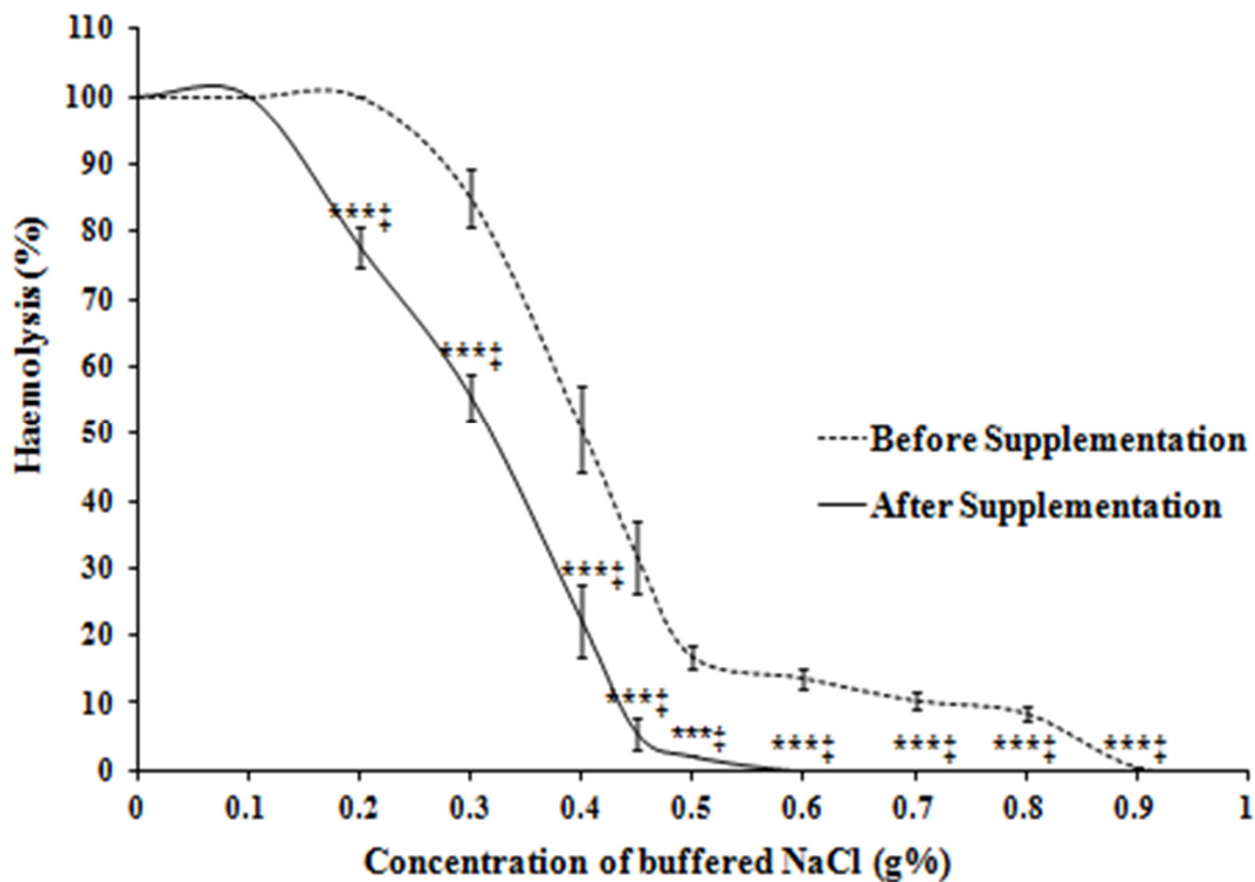
Fig. 1. Effect of L-Arginine supplementation on osmotic fragility of erythrocytes of sickle cell anemia subjects.

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).

Morris *et al,* (2000)

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

Archer, D.R., Stiles, J.K., Newman, G.W., Quarshie, A., Hsu, L.L., Sayavongsa, P., Perry, J., Jackson, E.M. & Hibbert J.M.(2008) C-Reactive Protein and Interleukin-6 Are Decreased in Transgenic Sickle Cell Mice Fed a High Protein Diet. *J. Nutrition,* **138,** 1148–1152.

Atweh, G.F., and Schechter, A.N. 2001. Pharmacological induction of fetal hemoglobin: raising the therapeutic bar in sickle cell disease. Curr. Opin. Hematol. **8**: 123 – 130.

Dacie J.V. & Lewis S.M. (1991). Practical Hematology, 7th Ed.., Livingstone, Edinburgh, Churchill, pp. 755–756.

Dasgupta, T., Hebbel, R.P. and Kaul, D.K. 2006. Protective effect of arginine on oxidative stress in transgenic sickle mouse models. Free. Radic. Biol. Med. **41**(12):1771 – 80.

De Franceschi, L., Bachir, D., Galacteros, F., Tchernia, G., Cynober, T., Neuberg, D., Beuzard, Y. and Brugnara, C. 2000. Oral magnesium pidolate: effects of long-term administration in patients with sickle cell disease. Brit. J. Haematol. **108**: 284 – 289.

Diallo, D., and Tchernia, T. 2002. Sickle cell disease in Africa. Curr. Opin. Hematol. **9**: 111 – 116.

Gladwin, M.T., Kato, G.J., Weiner, D., Onyekwere, O.C., Dampier, C., Hsu, L., Hagar, R.W., Howard, T., Nuss, R., Okam, M.M., Tremonti, C.K., Brian Berman, B., Anthony Villella, A., Krishnamurti, L., Lanzkron, S., Castro, O., Gordeuk, V.R. & Wynona A. (2011) Nitric oxide for inhalation in the acute treatment of sickle cell pain crisis: A randomized clinical trial. *J. Am. Medical Association,* **305,** 893–902.

Head, C.A., Swerdlow, P., McDade, W.A., Joshi, R.M., Ikuta, T., Cooper, M.L. & Eckman, J.R. (2010) Beneficial effects of nitric oxide breathing in adult patients with sickle cell crisis. *Am. J. Hematol.* **85,** 800-802.

Ingram, V.M. 1956. A specific chemical difference between the globins of normal human and sickle-cell anemia haemoglobin. Nature **178**: 792 - 794.

Jaja. S.I., Ikotun, A.R., Gbenebitse, S. and Temiye, E.O. 2002. Blood pressure, hematologic and erythrocyte fragility changes in children suffering from sickle cell anemia following ascorbic acid supplementation. J. Trop. Paed. **48**: 366 – 370.

Jaja, S.I., Kehinde, M.O., Gbenebitse, S.O., Mojiminiyi, F.B.O. and Ogungbmi, S.O. 2000. Effect of Vitamin C on arterial blood pressure, irreversibly sickled cells and osmotic fragility in sickle cell anemia subjects. Niger. J. Physiol. Sci. **16 (1-2)**: 14 - 18.

Jensen, W.N., Rucknagel, D.L. and Taylor, W.J. 1960. In vivo study of the sickle cell phenomenon. J. Lab. Clin. Med. **56**: 854 – 865.

Kato, G.J. 2008. Novel small molecule therapeutics for sickle cell disease: Nitric oxide, carbon monoxide, nitrate and Apolipoprotein A-1. Hematology Am. Soc. Hematol. Educ. Program 186 – 192.

Kaul, D.K., Zhang, X., Dasgupta, T., and Fabry, M.E. 2008. Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress. Am. J. Physiol. Heart. Circ. Physiol. **295**: H39 – H47.

Kaul, D.L., Nagel, R.L., Cen, D., and Tsai, H.M. (1993). Sickle eythrocyte-endothelial interaction in microcirculation: The role of Von Willebrand factor and implications for vaso-occlusion. Blood. **81**; 2429 – 2438.

Koracevic, D.C., Koracevic, A., Djordjevic V., Andrejevic S and Cosic V. 2001. Method for the measurement of antioxidant activity in human fluids. J. Clin. Pathol. **54**: 356 – 361.

Li, H., Liang, X., Feng, L., Liu, Y. and Wang, H. 2008. A simple and fast method for arginine determination in grape juice. J. Food and Drug Analysis. **16(3)**: 53 - 58.

Little, J.A., Hauser, K.P., Martyr, S.E., Harris, A., Maric, I., Morris, C.R., Suh, J.H., Taylor, J., Castro, O., Machado, R., Kato, G., and Gladwin, M.T. 2009. Hematologic, biochemical, and cardiopulmonary effects of L-arginine supplementation or phosphodiesterase 5 inhibition in patients with sickle cell disease who are on hydroxyurea therapy. Eur. J. Haematol. **82(4)**: 315 – 321.

Lopez B.L., Kreshak A.A., Morris C.R., Davis-Moon L., Samir K. Balla S.K. and Ma X (2003). L-arginine levels are diminished in adult acute vaso-occlusive sickle cell crisis in the emergency department. Brit. J. Haemat. 120, 532–534

Machado R.F., Barst R.J., Yovetich N.A., Hassell K.L., Kato G.J., Gordeuk V.R., Gibbs J.S., Little J.A., Schraufnagel D.E., Krishnamurti L., Girgis R.E., Morris C.R., Rosenzweig E.B., Badesch D.B., Lanzkron S., Onyekwere O., Castro O.L., Sachdev V., Waclawiw M.A., Woolson R., Goldsmith J.C., and Gladwin M.T., on behalf of the walk-PHaSST Investigators and Patients (2011). Hospitalization for pain in patients with sickle cell disease treated with sildenafil for elevated TRV and low exercise capacity. Blood. 118 (4), 855

Machado RF, Martyr S, Kato GJ, et al. (2005). Sildenafil therapy in patients with sickle cell disease and pulmonary hypertension. *Br J Haematol.* 130(3):445-453.

Martinez-Ruiz R, Montero-Huerta P, Hromi J, Head CA. (2001). Inhaled nitric oxide improves survival rates during hypoxia in a sickle cell (SAD) mouse model. Anesthesiology. 94(6):1113– 1118.

Maxwell A. J and Cooke J.P. (1998). Cardiovascular effects of L-arginine. Curr Opin Nephrol Hypertens. 7(1):63-70.

Morris, C.R., Kuypers, K.A., Larkin, S., Sweeters, N., Simon, J., Vichinsky, E.P. and Styles, L.A. 2000. Arginine therapy: a novel strategy to induce nitric oxide production in sickle cell disease. Br. J. Haem. **111**: 498 -500.

Morris, C.R., Kuypers, F.A., Lavrisha, L., Ansari, M., Sweeters, N., Stewart, M., Gildengorin, G., Lynne Neumayr, L. & Vichinsky, E.P. (2013) A randomized, placebo-controlled trial of arginine therapy for the treatment of children with sickle cell disease hospitalized with vaso-occlusive pain episodes. *Haematologica* **98,** 1375-1382.

Morris, C.R., Morris, S.M., Hagar, W., van Warmerdam, J., Claster, S., Kepka-Lenhart, D., Morris, S. M. & Kuypers, F.A. (2003) Arginine therapy: a new treatment for pulmonary hypertension in sickle cell disease? *Am. J. Respir. Crit Care Med.* **168,** 63-69.

Raghupathy, R., and Billett, H. 2009. Promising therapies in sickle cell disease. Cardiovasc. Haemtol. Disorders-Drug Targets **9**: 1-8.

Reiter, C.D,,Wang, X., Tanus-Santos, J.E., Hogg, N., Cannon, R.O., Schecheter, A. N., (2002) Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nature Medicine,* **8,** 1383-1389.

Romero, J.R., Suzuka, S.M., Nagel, R.L., and Fabry, M.E. 2002. Arginine supplementation of sickle transgenic mice reduces red cell density and Gardos channel activity. Blood. **99(4)**:1103–8.

Scavella, A., Leiva, L., Monjure, H., Zea, A.H. & Gardner, R.V. (2010) Effect of L-Arginine supplementation on immune responsiveness in patients with sickle cell disease. *Pediatr. Blood Cancer,* **55,** 318-323.

Shenoy, S. 2007. Has stem cell transplantation come of age in the treatment of sickle cell disease? Bone Marrow Transplantation. **40**: 813 – 821.

Styles, L.A., Kuypers, F., Kesler, K., Reiss, U., Lebeau, P. & Nagel, R. (2007) Arginine therapy does not benefit children with sickle cell anemia: Results of the comprehensive sickle cell multi-center study. 35th convention of National Sickle Cell Disease Program and the Sickle Cell Disease Association of America. Washinton, DC: p 114.

Titus, J., Chari, S., Gupta, M., and Parekh, N. 2004. Pro-oxidant and anti-oxidant status in patients of sickle cell anemia. Ind. J. Clin. Biochem. **19**: 168-172.

Townes, T.M. 2008. Gene replacement therapy for sickle cell disease and other blood disorders. Hematology Am Soc Hematol Educ Program 193 -196

Weiner DL, Hibberd PL, Betit P, Cooper AB, Botelho CA, Brugnara C. (2003). Preliminary assessment of inhaled nitric oxide for acute vaso-occlusive crisis in pediatric patients with sickle cell disease. JAMA. 289(9):1136–1142.

World Health Organization. 1994. Updated estimates of the frequency of the hemoglobin disorders in each country. In Guidelines for the Control of Hemoglobin Disorders. Edited by Model B Geneva: WHO Publications; WHO/HDP/HB/GL/94.1.

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