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Sickle-cell anemia

by

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Abbreviations:

- SCA: Sickle-cell anemia
- SCD: Sickle cell disease
- RBC: Red blood cell
- NO: Nitric oxide
- NOS: Nitric oxide synthase
- HbS: sickle cell hemoglobin
- HbA: normal cell hemoglobin
- ATP: Adenosine tri-phosphate

Table of Contents: **Page no.**

1. Abbreviations 1

2. Abstract 2

3. What is sickle cell anemia? 3

4. Origin and history of SCA 4

5. Genetics of SCA 4

6. Treatment of SCA 5

7. Free radicals in SCA 8

8. Antioxidants in SCA 8

9. Nitric oxide and SCA 10

10. Functioning of NO in hydroxyurea therapy 14

11. Proposed mechanism of metabolic pathway to produce NO from hydroxyurea 15

12. Summary 17

13. References 19

Abstract

Sickle cell anemia (SCA) is an inherited blood disorder characterized primarily by chronic anemia and periodic episodes of pain and is caused by the point mutation in the β-chain of the hemoglobin where amino acid glutamine (Glu) is replaced by valine (Val). This results in sickle-shaped erythrocytes after deoxygenation and leads to polymerization, which cause blockages, depriving the organs and tissue of the oxygen-carrying blood. SCA is the most common genetic disease among African Americans, with an 8% incidence the trait among this population. SCA causes higher level of auto-oxidation of hemoglobin, which elevates the level of superoxide species in RBCs and lowers the level of antioxidant enzymes. SCA causes polymerization of sickle cell

hemoglobin (HbS) that leads to the vascular occlusion. Control of vascular tone is mediated by the free radical species nitric oxide (NO). NO has also been found to be the major molecule helping indirectly to cure SCA. This paper primarily focuses on the therapies available for SCA and understanding the functioning of NO in curing SCA.

What is Sickle cell Anemia?

Sickle cell anemia (SCA) or sickle cell disease (SCD) is an inherited blood disorder characterized primarily by chronic anemia and periodic episodes of pain [1]. The underlying problem involves hemoglobin, a component of the red cells in the blood. The hemoglobin molecules in each red blood cell carry oxygen from the lungs to the body organs and tissues and bring back carbon dioxide to the lungs. In SCA, the hemoglobin is defective. After the hemoglobin molecules give up their oxygen, some of them may cluster together and form long, rod-like structures. These structures cause the red blood cells to become stiff and sickle-shaped. Unlike normal red cells, which are usually smooth and donut-shaped, the sickled red cells cannot squeeze through small blood vessels. Instead, they stack up and cause blockages that deprive the organs and tissue of oxygen-carrying blood. This process produces the periodic episodes of pain and ultimately damages the tissues and vital organs and leads to other serious medical problems. Normal red blood cells (RBCs) last about 120 days in the bloodstream, while sickled red cells die after only about 10 to 20 days and because they cannot be replaced fast enough the blood is chronically short of red blood cells.

Origin and history of SCA

Scientists do not know when exactly sickle-cell disease began. As with other genetic diseases it came about because of genetic mutation during man's evolution [6,7]. The first formal description of sickle cell anemia was reported in 1910. After that in various intervals of time the sickling of RBCs with the low oxygen concentrations and change in hemoglobin molecule structures with the absence of oxygen was reported. In 1949, Pauling *et al.* used protein electrophoresis to show that sickle cell hemoglobin differed in structure from normal hemoglobin. This was the first time when cause of a disease was linked to a change in protein structure. Then another major breakthrough came when in 1978, Flavell *et al.* prepared maps of the human beta and delta globin genes. In 1995 Charache *et al.* reported that the anticancer drug hydroxyurea was the first to reduce the frequent, painful complications that characterize sickle cell disease [4]. Recently (2003), Steinberg *et al*. showed that the drug hydroxyurea helps reducing the death rate to a great extent compared to other treatments [5].

Genetics of SCA

SCA is an autosomal recessive disease caused by a point mutation in the *hemoglobin beta gene* (*HBB*) found on chromosome 11p15.4 [1]. Hemoglobin is made up of 4 chains: 2 α and 2 β. In SCA, a point mutation causes the amino acid glutamine (Glu) to be replaced by valine (Val) in the β chains of HbA, resulting in the abnormal HbS (Figure 1a) [8]. Under low oxygen level conditions, RBCs with HbS distort into sickled shapes (Figure 1b). These sickled cells can block small vessels producing microvascular occlusions that may cause necrosis of the tissue (Figure 1c).

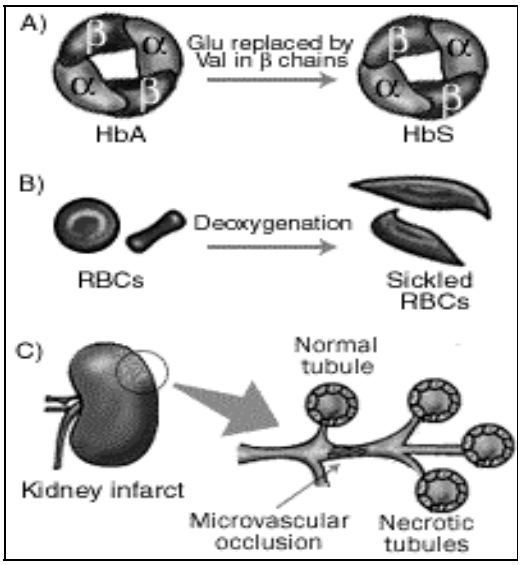


Figure 1: Point mutation in hemoglobin results in SCA forming sickled-RBCs blocking small vessels causing necrosis [8]

Treatment of SCA

Few treatments are available for Sickle cell disease with a low survival rate. The major treatments used clinically are stem cells transplantation and hydroxyurea therapy.

- **Marrow Stem cells Transplantation:** Marrow transplantation is used to replace genetically abnormal blood-forming stem cells with normally functioning cells [2]. A sibling with a matching tissue type is the donor of the stem cells. The dissimilarity of certain characteristics between two siblings is an advantage in this situation. Marrow transplantation is even possible when the donor may be a carrier of the gene and have sickle cell trait or have received the mutant gene from mother or father.
- **Hydroxyurea Therapy:** Hydroxyurea drug resulted in an almost 50 percent reduction in the number of painful crises and episodes of chest syndrome and patients required about 50 percent fewer transfusions and hospitalizations [2,3]. Thus

hydroxyurea became the only drug approved by FDA, in 1995, for the treatment of SCA for adult patients, but due to the concerns of potential toxicity and effect on growth and development it has not yet been used on children. Very recently, Steinberg *et al*. showed even better results with hydroxyurea [4]. They designed a long-term observational experiment and found out that SCA patients who took the hydroxyurea over a 9-year period experienced a 40 percent reduction in deaths (out of 299 adult patients across United States and Canada), which is better then any other therapy available.

- **Gene therapy:** Gene therapy, though slow progressing, is promising to treat SCA. The problems arise due to inadequate gene transfer and efficacy and low gene-expression [9]. However, the identification of a locus control region (LCR), essential for transcription activity of the globin cluster, has accelerated the field. In addition, advances in vector research have resulted in recombinant lentiviruses, with large fragments of the human β-globin gene and needed regulatory elements are now available. Recently, Pawliuk *et al.* demonstrated the ability of gene therapy to correct the disease's pathophysiology [10]. The gene responsible for the antisickling effect of γ-globin was inserted to prevent sickling. Several steps are still needed to make the gene therapy useful at the clinical level, including improvement in the quantity and safety of lentiviral production, and the development of safer myeloablation regimens.
- **Other therapies:** Several therapeutic options are available which interrupt the sickling process at various key pathways. Short-chain fatty acids, 5-azacytidine, decibatine, clotrimazole, magnesium pidolate etc. are the drugs that are being into

consideration to use clinically [9]. These emerging therapeutic agents and their mechanisms are summaized below in Table 1. The drugs are catagorized according to their mode of action.

Table 1: Emerging therapeutics agents in Sickle cell disease [9]

Category	Therapeutic Agent	Mechanism
Red-cell rehydration	Clotrimazole,	Gardos channel inhibition
	Mg pidolate	K:CL co-transport inhibition
Antiadhesion	RGD peptide, Antiadhesion antibodies	Red-cell endothelial adhesion
	Anti-von-Willebrand factor	PAF-induced adhesion
	Anti-integrin receptors	Anti-white-cell adhesion
	Sulphasalazine (inhibitor of NF-kb)	Endothelial activation
HbF augmentation	Hydroxyurea	Ribonucleotide reductase
	Short-chain fatty acids	Histone deacetylase
	2-deoxy-5-azacytidine	DNA hypomethylation
	Erythropoietin	Stress erythropoiesis
Antioxidative therapy	Deferiprone	Chelate membrane iron
	Glutamine	Glutathione metabolism, N-acetyl-cysteine
Antithrombotic therapy	Acenocoumarol, heparin	Decrease thrombin
	N3 fatty acids	Inhibition of platelet activation
Antisickling through multiple pathways	Nitric oxide	Vasodilation
	Arginine	Decreased adhesion,
	Flocor (non-ionic surfactant)	Polymerisation Antiadhesion, anti-inflammation, increased flow
Transplantation	Allogeneic, nonmyeloablative	Haemopoietic stem cell
Gene therapy	Viral delivery of β-globin Gene	Direct gene replacement
	Erythropoietin delivery Cord blood	Indirect gene therapy DNA, RNA repair
Transfusion therapy	Pheresis, Simple transfusion	Decrease HbS cells

Free radicals in SCA

Superoxide is produced by the auto-oxidation of hemoglobin present in RBCs if there is an alteration in the normal electron transfer reaction between the heme iron and oxygen in oxygenated hemoglobin [11]. Along with superoxide, auto-oxidation of hemoglobin produces methemoglobin, a crystalline blood pigment that differs from hemoglobin in containing ferric iron and in being unable to combine reversibly with molecular oxygen [3], which provides a continual source of superoxide production that in turn generates hydrogen peroxide (H_2O_2) as product of dismutation reaction. The auto-oxidation of hemoglobin is found to be 1.7 times faster in sickle cell patients (HbS) than in normal patients (HbA). Also, HbS generate 2-fold greater extent of superoxide ($O_2^{\bullet -}$), H_2O_2, hydroxyl radical and lipid oxidation products compared with HbA-containing cells [12].

- **Mechanism of membrane damage**

Iron associated with red cell membrane stays in free Fe(III) state. Fe(III) can initiate peroxidation reaction in the presence of reducing agent. Excess H_2O_2 decomposes heme, induces iron release which reacts with H_2O_2 to form hydroxyl radicals or perferryl species [13]. Sickle red cells shows increased endogenous extent of oxidized lipid and a greater tendency for further lipid peroxidation, when compared to normal RBCs [11].

Antioxidants in SCA

RBCs have an array of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), that protects them from free radical damage [13]. SOD dismutates superoxide to yield H_2O_2 and H_2O. GPX catalyzes the reduction of H_2O_2 by reduced glutathione (GSH). NADPH, derived in large part from the pentose-

phosphate pathway reduces the resulting glutathione disulfide (GSSG). GPX has a lower K_m for H_2O_2 than CAT and therefore it can scavenge at lower H_2O_2 concentrations (Figure 2). Sickle erythrocytes have increased levels of SOD and decreased levels of GPX and CAT (heme containing enzymes) suggesting that endogenously produced H_2O_2 cannot be removed by sickle erythrocytes as efficiently as for normal red blood cells [11]. In addition to this, there is also a decrease in the low molecular weight antioxidant like α-tocopherol (vitamin E) and β-carotene (vitamin A). There is 40 % reduction in plasma carotene levels and 30 % reduction in plasma vitamin E levels in sickle cell disease. RBC membrane vitamin E levels are also depleted [14]. In addition, the level of RBC GSH is also lowered by 50% in HbS cells as compared to HbA cells.

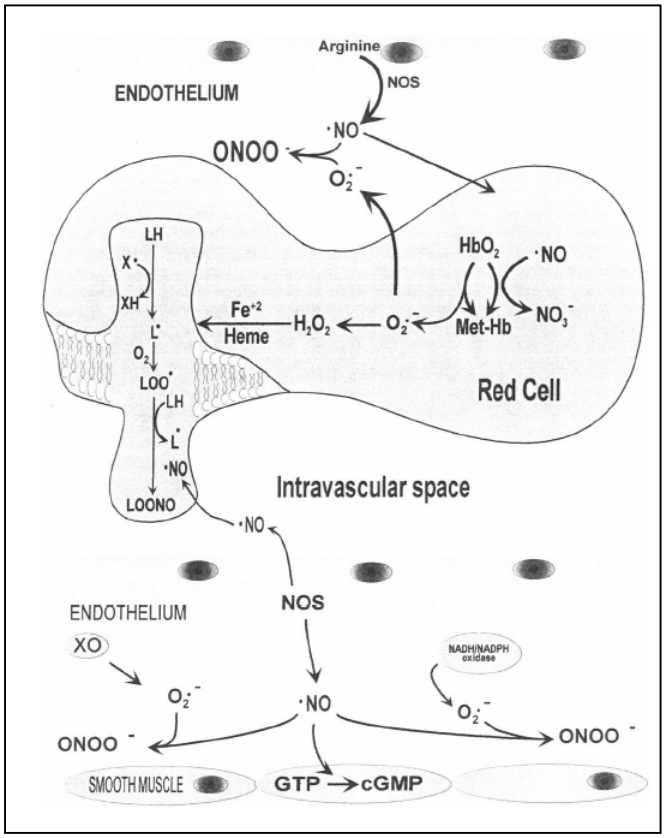


Figure 2: Antioxidant enzymes in erythrocytes [11]

Nitric oxide (NO) and SCA

SCD causes polymerization of HbS that leads to the vascular occlusion [15]. There are a variety of abnormalities including hemoglobin polymerization, increased endothelial cell adherence, increased blood viscosity, coagulopathy, endothelial dysfunction and altered vascular tone that are caused by SCD [11]. Control of vascular tone is mediated by the free radical species nitric oxide (NO), that is released by endothelial cell nitric oxide synthase (NOS). NO is a freely diffusible intercellular messenger produced by a variety of cells, including vascular endothelium, neurons, smooth muscle cells, macrophages, neutrophils, platelets and pulmonary epithelium. NO regulates blood vessel tone, endothelial adhesion, severity of ischaemia-reperfusion injury and anaemia in SCD [9].

- **NO generation and vascular relaxation**

The generation of NO occurs by the oxidative de-amination of L-arginine to L-citrulline in the presence of the cofactor NADPH by nitric oxide synthases (Figure 3) [16].

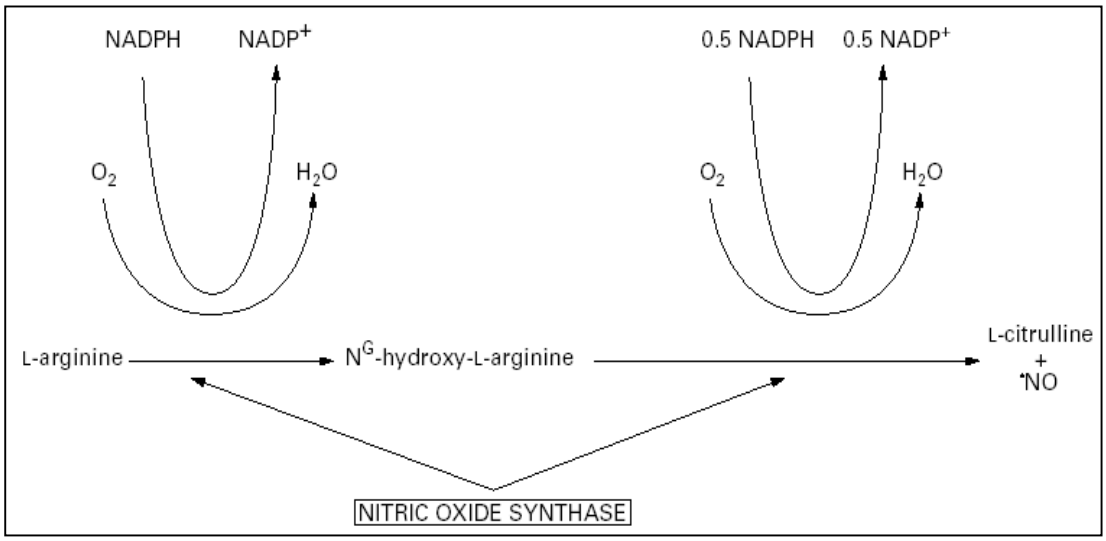


Figure 3: Synthesis of NO. First step is a 2-electron and second is 3-electron oxdiation [16]

The NO generated then diffuses to the smooth muscle cells in the vicinity, in which it reacts with the ferrous iron in the heme group of guanylate cyclase, resulting in enhanced synthesis of cyclic-GMP from guanosine triphosphate [17]. cyclic-GMP then interacts with cGMP-dependent protein kinases, cGMP-regulated cyclic nucleotide phosphodiesterases and c-GMP-regulated ion channels. Cyclic GMP-dependent kinase stimulates Ca^{2+}-ATPase and lowers intracellular calcium levels, thus mediating smooth muscle cell relaxation (Figure 4). Stimulation of guanylate cyclase also inhibits platelet aggregation and adhesion to endothelium [11,17]

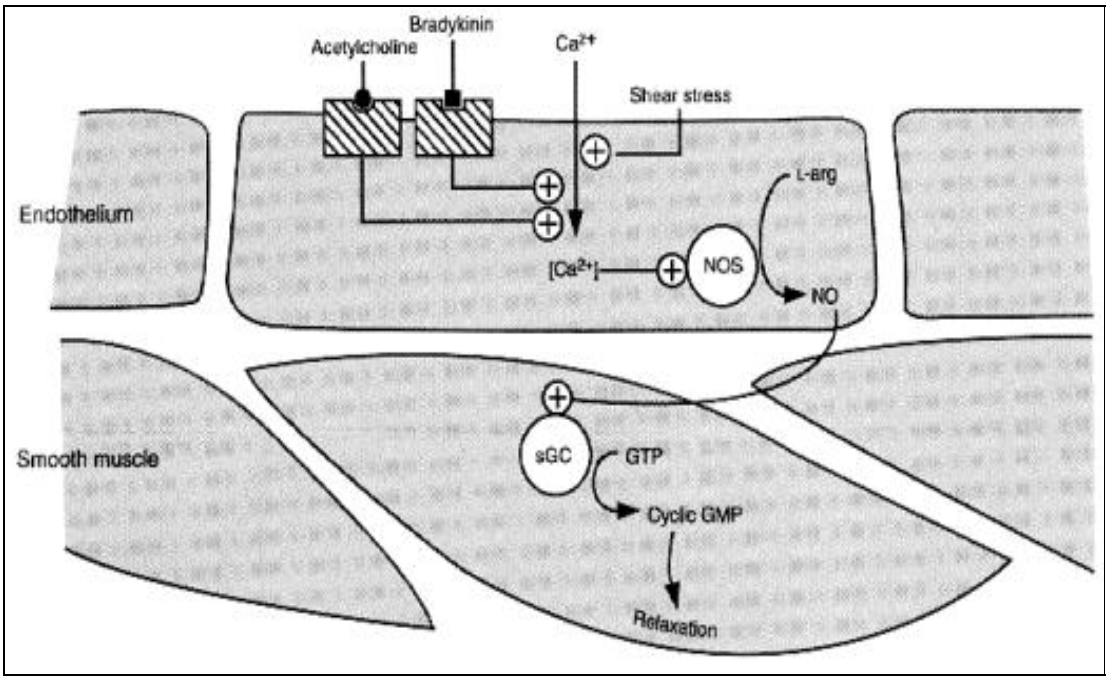


Figure 4: Vascular relaxation mediated by NO [17]

- **Oxidative stress regulation by NO**

The reaction of NO with metalloproteins, protein sulfhydryls and oxygen-derived free radicals enables it to modulate inflammation and oxidative stress [11]. However, NO acts as a prooxidant when the concentration of superoxide is greater than or equal to that

of NO. $O_2^{\bullet-}$ inhibits NO-dependent vascular relaxation by reacting with NO to give peroxynitrite and its conjugate acid peroxynitrous acid (ONOOH, pK_a=6.8). The rate constant for this reaction is $2\times10^{10}M^{-1}sec^{-1}$, which is even faster than the SOD-catalyzed enzymatic dismutation of superoxide ($2\times10^{9}M^{-1}sec^{-1}$). Peroxynitrous acid reacts by two pathways, first pathway yields nitrate (NO^{3-}) while the second pathway forms hydroxyl radical and nitrogen dioxide (NO_2^-), initiating fatty acid oxidation and nitration of aromatic amino acids. Peroxynitrite reactivity is also influenced by CO_2, with the formation of a reactive nitrosoperoxocarbonate ($ONOOCO^{2-}$) intermediate. CO_2 stimulates both peroxynitrite decay and enhances peroxynitrite-mediated nitration of molecules by nearly 2-fold. The prooxidant versus antioxidant effects of NO depends on the relative concentrations of reactive oxygen species (ROS) and NO synthesis. As an antioxidant, NO serves as a potent terminator of radical chain propagation reactions and yields novel nitrogen compounds containing lipid oxidation products. It has been shown that the reaction between NO and chain propagating peroxyl radical species is three times faster than that with vitamin E. NO has also been shown to prevent oxidative injury associated with oxoferryl-hemoglobin formation via the reduction of ferryl hemoglobin to methemoglobin [18]. It binds rapidly to deoxyhemoglobin forming a stable hemoglobin (Fe^{+2})-NO complex, and also reacts with and converts oxygenated hemoglobin to methemoglobin and nitrate [11].

- **Effect of SCA on functioning of nitric oxide**

SCD leads to the increased levels of xanthine-oxidase (XO) into the circulation (Table 2), which results in the elevated levels of $O_2^{\bullet-}$ and H_2O_2 [19]. These reactive species then hinders with the normal functioning of NO-dependent vascular functions and

activate tissue inflammatory responses. Increased rates of production of reactive oxygen- and nitrogen- derived species in tissues mediate the oxidation and nitration of lipids, nucleotides, and susceptible protein amino acid residues. Circulating XO binds to vessel luminal cells and impair vascular function by creating an oxidative environment and catalytically consuming NO via diffusion-limited reaction with $O_2^{\bullet-}$. In addition, liver and kidney inducible-NOS (iNOS or NOS2) expression and tissue 3-nitrotyrosine (NO_2Tyr, a protein nitration product) immunoreactivity increases significantly in SCD mice and humans, but not in non-diseased tissues (Table 2) [20]. NOS2 is one of the three isoforms of enzyme NOS. Other isoforms are neuronal-NOS (nNOS or NOS1) and endothelial-NOS (eNOS or NOS3) [16]. NOS2 is absent in resting cells, but the gene is rapidly expressed in response to stimuli such as pro-inflammatory cytokines. Once present, NOS2 synthesizes 100–1000 times more NO than the constitutive enzymes and does so for prolonged periods. This high concentration of NO inhibits a large variety of microbes, but also potentially damage the host, thereby contributing to pathology.

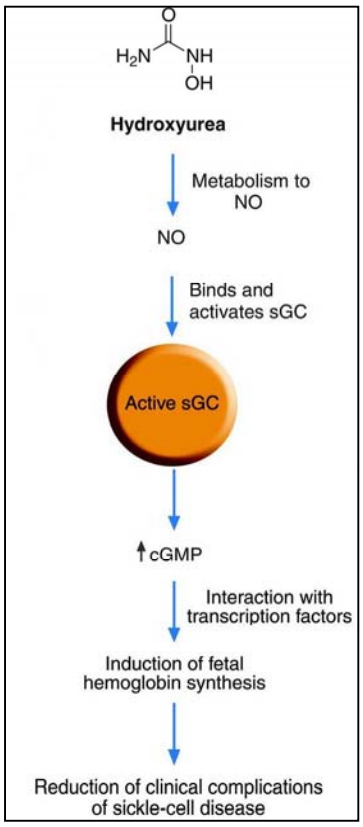
Table 2: XO activity and 3-nitrotyrosine content in sickle cell disease [19,20]

Xanthine oxidase			**3-Nitrotyrosine**		
Measurement	**Control**	**SCD**	**Measurement**	**Control**	**SCD**
Human plasma XO (μU/ml)	0.89±03	3.3±0.9	Human Plasma (ng/mg protein)	10.1±3.2	24.7±1.7
Mouse plasma XO (mU/ml)	2.2±0.26	5.6±1.5	Mouse plasma (ng/mg)	13.1±2.2	37.7±1.7
Mouse Liver XO	53.9±7.8	18.6±4.4	Mouse liver (ng/mg)	8.2±2.2	21.4±2.6
Mouse Aortic XO	0.29±0.01	0.46±003	Mouse kidney (ng/mg)	10.0±1.2	37.5±7.8
			Mouse actin-enriched fraction		
			Liver (μg/mg)	0.13±0.03	0.34±0.06
			Kidney (μg/mg)	0.17±0.03	0.92±0.08

Aslan *et al.* observed that the nitration led to morphologically distinct disorganization of filamentous actin [20]. Actin is a cytoskeletal protein and constitutes 5% or more of cell protein and serves as a critical target for oxidation and nitration-induced functional impairment. Actin contains a high percentage of tyrosine residues, many of which are crucial participants in protein-protein recognition motifs. The introduction of an electronegative NO_2 group onto a tyrosine ring reduces the pK_a of the phenolic hydroxyl to values in the range of 6.8–7.0. Electrospray ionization and fragment analysis by tandem mass spectrometry revealed that 3 of 15 actin tyrosine residues were nitrated (Tyr91, Tyr198, and Tyr240) at positions that significantly modify actin assembly.

Functioning of NO in hydroxyurea therapy

As stated earlier, hydroxyurea drug resulted in an almost 50 percent reduction in the number of painful crises and episodes of chest syndrome and patients required about 50 percent fewer transfusions and hospitalizations [2,3]. Hydroxyurea increases the levels of fetal hemoglobin, which has a large solubilizing effect on sickle-cell hemoglobin and reduces polymerization [21]. Fetal hemoglobin is different from the adult hemoglobin in the chain structure. Adult hemoglobin has α and β chains, while fetal hemoglobin has α and γ chains and has more affinity for oxygen then adult hemoglobin [22].



The mechanism of how hydroxyurea increases fetal hemoglobin levels, however, remains unclear. Cokic *et al*. presented results that provide a partial explanation of this increase. Fetal hemoglobin increases in response to the activation of soluble guanylyl cyclase (sGC) by hydroxyurea-derived NO [23]. King SB derived a mechanism of fetal hemoglobin induction by hydroxyurea on the basis of the work done by Cokic *et al.* (Figure 5) [24], which however, does not explain how NO is produced from hydroxyurea metabolism. Chemically, conversion of hydroxyurea to NO requires a three-electron oxidation and a number of heme- and copper- containing proteins react with hydroxyurea to produce NO in vitro. There is little information available regarding hydroxyurea metabolism in-vivo.

Figure 5: Mechanism of fetal hemoglobin induction by hydroxyurea [24]

Proposed mechanism of metabolic pathway to produce NO from hydroxyurea

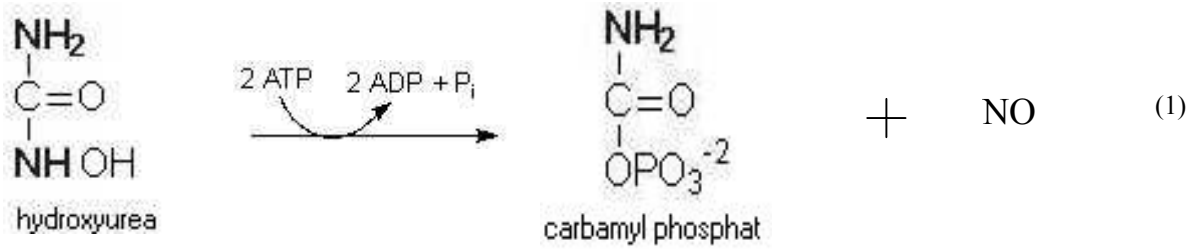
The mechanism of the metabolism of hydroxyurea to produce NO will provide a new insight into the development of NO-based therapies for treatment of sickle-cell disease. Early work shows the ability of urease to catalyze the hydrolysis of hydroxyurea to hydroxylamine, which reacts rapidly with heme proteins to release NO, but this would not happen anywhere other than the areas confined to bacterial colonization (such as gut) as urease is not a mammalian enzyme [24]. Moreover, Yarbro JW suggested that hydroxyurea inhibits ribonuclotide reductase to produce NO, but it could not be confirmed [25]. However, there is no record of hydroxyurea reacting with ornithine or metabolizing to produce carbamyl phosphate with the help of ATP.

Two mechanisms of hydroxyurea metabolism are being proposed in this report to produce nitric oxide. Both the mechanisms are based on the hindrance of hydroxyurea in

urea cycle. Urea cycle is the mechanism by which body gets rid of the excess nitrogen produced in the form of urea [26]. Ornithine arising in the cytosol is transported to the mitochondrial matrix and condenses with carbamyl phosphate producing citrulline. The synthesis of citrulline requires a prior activation of carbon and nitrogen as carbamyl phosphate. The activation step requires 2 equivalents of adenosine tri-phosphate (ATP). The product, citrulline, is then transported to the cytosol. In a 2-step reaction, citrulline and aspartate are condensed to form argininosuccinate that in turn forms arginine and fumarate. Arginine is cleaved to generate urea and ornithine. In the final step of the cycle cytosolic ornithine regenerates, which can be transported to the mitochondrial matrix for another round of urea synthesis [26].

Mechanism 1: Hydroxyurea metabolism to produce carbamyl phosphate

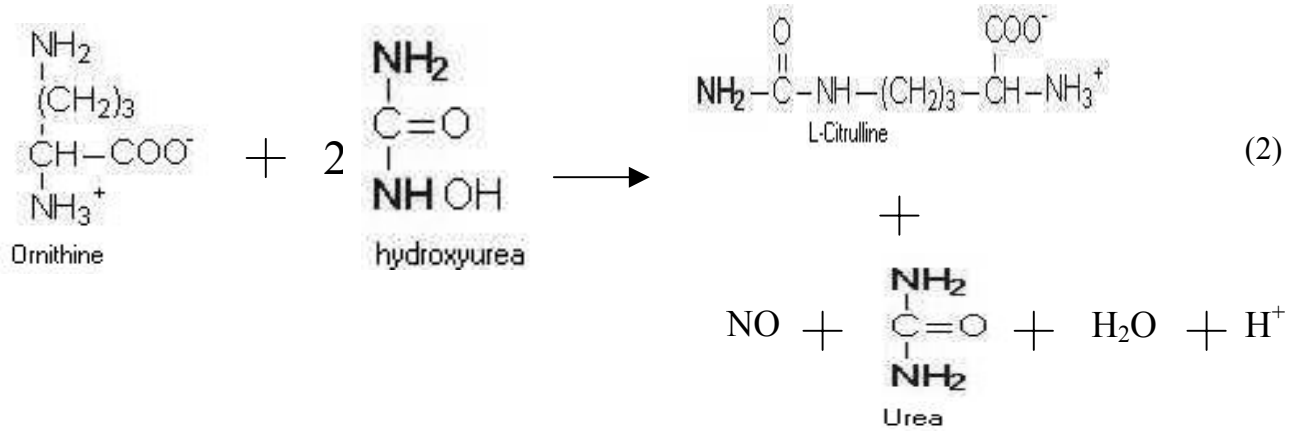
First mechanism that has been proposed is that hydroxyurea is metabolized in the presence of ATP to produce carbamyl phosphate and nitric oxide which is shown below.



(1)

Mechanism 2: Reaction of hydroxyurea with ornithine

Second mechanism that has been proposed here is that hydroxyurea reacts with ornithine to give citrulline with NO and urea as a byproduct. It can be shown as:



(2)

These mechanisms could be the key source of the metabolism of hydroxyurea to NO. Mechanism 1 can be analyzed by the measuring the concentration of carbamyl phosphate produced. Increase in the concentration would support the mechanism. Mechanism 2 can be analyzed by measuring the concentration of citrulline or ornithine. Increase in citrulline concentration or decrease in ornithine concentration would support the mechanism.

Summary

Sickle cell anemia (SCA) is an inherited blood disorder characterized primarily by chronic anemia and periodic episodes of pain and is caused by the point mutation in the β-chain of the hemoglobin where amino acid glutamine (Glu) is replaced by valine (Val). This results in sickle-shaped erythrocytes after deoxygenation and leads to

polymerization, which cause blockages, depriving the organs and tissue of the oxygen-carrying blood. Few treatments are available for Sickle cell disease with a low survival rate. The major treatments that are used clinically are stem cells transplantation, gene therapy and hydroxyurea therapy. Hydroxyurea therapy has emerged as a promising drug towards the cure of SCA. Hydroxyurea increases the levels of fetal hemoglobin, which has a large solubilizing effect on sickle-cell hemoglobin and reduces polymerization. Fetal hemoglobin increases in response to the activation of soluble guanylyl cyclase (sGC) by hydroxyurea-derived nitric oxide (NO). Control of vascular tone is mediated by the free radical species nitric oxide (NO), that is released by endothelial cell nitric oxide synthase (NOS). NO regulates blood vessel tone, endothelial adhesion, and the severity of ischaemia-reperfusion injury and anaemia in SCD. King SB derived a mechanism of fetal hemoglobin induction by hydroxyurea on the basis of the work done by Cokic *et al.* (Figure 5) [king], which still does not explain how NO is produced from hydroxyurea metabolism. Two mechanisms of hydroxyurea metabolism are being proposed in this report to produce nitric oxide. Both the mechanisms are based on the hindrance of hydroxyurea in urea cycle. In first mechanism, carbamyl phosphate and NO are the products of the reaction, while in the second mechanism hydroxyurea is metabolized by ornithine to give citrulline and NO. These will provide a new insight into the development of NO-based therapies for treatment of sickle-cell disease.

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