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Rational bases for using oxygen-ozonetherapy as a biological response modifier in sickle cell anemia and β-thalassemia: A therapeutic perspective

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ABSTRACT: We are proposing to evaluate whether a complementary approach based on cycles of oxygen-ozone autohemotherapy (O_3-AHT) already performed in millions of patients, can abate the chronic oxidative stress and improve the quality of life of serious hemoglobinopathic patients. Although a preliminary study has yielded encouraging results, it appears appropriate to perform a controlled, randomized and possibly multicentre clinical trial. The long use of this approach in other pathologies has proved to be very useful and it is hoped that scepticism will not prevail over scientific rationale. Ozone, as any other drug, has an intrinsic toxicity that, in the proposed application, is fully tamed by the blood antioxidant system. (J Biol Regul Homeost Agents 2004; 18: 38-44)

KEY WORDS: *Oxygen-ozone therapy, Sickle cell anemia, β-thalassemia, Nitrogen monoxide, Oxidative stress, Ozone tolerance*

INTRODUCTION

Among hemolytic anemias, sickle cell anemia (SCA) and β-thalassemia stand as the most relevant and common hereditary chronic anemias due to either altered or impaired globin chain synthesis. The altered β-globin biosynthesis leads to a series of problems such as ineffective erythropoiesis, accelerated erythrocyte breakdown, iron overload, tissue hypoxia, impaired growth and shortened survival. Besides prevention, blood transfusion, desferrioxamine infusion and whenever possible bone marrow transplantation or gene therapy, orthodox therapy is modestly effective. To date a cytotoxic agent such as hydroxyurea (HU) is used to prevent recurrent vasoocclusive crises in sickle cell anemia (SCA).

Patients with these hemoglobinopathies are at a risk of alloimmunization, viral infections and, most important iron overload, that induce a vicious circle leading to chronic oxidative stress (COS). Indeed oxygen-free radicals and peroxidative tissue injury accompany the anemia and represent an unavoidable complication that accelerates the multi-organ abnormalities (1-3).

Is there any possibility of correcting the chronic oxidative stress that from day to day establishes a negative involution? Improving chelation therapy and a supplement of antioxidants can be useful but they are unable to abate the chronic oxidative stress. During the last decade most of the basic mechanisms of action elicited by the interaction of ozone with blood have been identified: precise and small amounts (μg) of ozone act as a real drug capable of activating in blood cells a number of biochemical pathways leading to enhanced vasodilation, oxygen delivery and induction of an adaptative response with enhanced synthesis of antioxidative enzymes in erythroid precursors.

The ozone therapeutic window has been defined (20-80 μg/ml of ozone per ml of blood) in several pathologies and in the case of hemoglobinopathies a cycle of ozonated autotransfusion (03-AHT) will be performed starting with a very low dose (10 μg/ml) slowly escalated up to 30 μg/ml to allow adaptation to COS. We would like to examine the validity of several mechanisms that represent a rational basis for evaluating the efficacy of ozonetherapy as a real biological response modifier. This paper intends to address this problem and to propose the evaluation of this approach.

The problem of sickle cell anemia

Sickle cell anemia (SCA) or drepanocytosis is a common genetic disease among black population due to an autosomal recessive disorder involving a single amino acid substitution in the beta subunit of a

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peculiar hemoglobin, referred to as sickle hemoglobin (HbS) to distinguish it from the normal adult hemoglobin A (HbA). Vernon Ingram in 1954 made the memorable discovery that HbS contains valine instead of glutamate at position 6 of the β chain and Linus Pauling in 1949 had already shown that HbS has an isoelectric point of 7.09 (oxyHb) and 6.91 (deoxyHb) in comparison to normal Hb (6.87 and 6.68, respectively).

Patients with SCA are homozygous for the abnormal gene and up to 35% of erythrocytes are sickled while heterozygous subjects are normally not symptomatic and 1% only of erythrocytes may become sickled. Homozygous SC patients have usually less than 20% fetal Hb (HbF), 3% HbA_2 and 70-80% HbS. Sickling occurs when the erythrocytes, passing through the capillary circulation (the PO_2 decreases from 98 to about 40), release oxygen to the tissues. The process of deoxygenation causes a brisk change of the tertiary structure of HbS with formation of an intracellular precipitate consisting of fibers 21.5 nm thick. Interestingly HbF inhibits the polymerization of HbS so that erythrocytes with a high content of HbF are somewhat protected from sickling.

Consequently the sickled erythrocyte becomes rigid and deformed and by obstructing the circulation provokes ischemia and infarction. The vessel occlusive crises due to physical trapping or increased adhesion of the sickled erythrocytes to the vascular endothelium occur in various organs and can be painful, particularly those consequent on bone marrow necrosis. The enhanced hemolysis is accompanied by hemochromatosis, anemia and a chronic inflammatory disease. Indeed there is an activation of macrophages, an increase of leukocytes with release of cytokines and consequently an alteration of cell adhesion regarding monocytes and neutrophils (4, 5). Although any organ may be involved, impairment of cardiopulmonary, renal hepatic, skeletal, ocular and neurologic functions are most common (6).

Thus SCA is a serious disease and only 2% of about 120,000 affected babies born in Africa survive to the age of five. Conventional medicine does practically nothing to help patients in poor countries. In theory, African Americans could undergo bone marrow transplantation but this is rarely performed and is accompanied by significant mortality (7). In the future, gene therapy may become useful (8). Administration of an oral drug could be practical but to date, among potentially ameliorating agents such as urea, (9) cyanate, (10) methylprednisolone (11) and Polaxamer 188, (12) only one drug is widely used: hydroxyurea (HU) increases the percentage of HbF, reduces HbS and the rate of painful crisis (13) but the drug is somewhat toxic, mutagenic, (14, 15) and possibly immunosuppressive (16). Clotrimazole, a specific Ca^{2+}-activated K^+ channel inhibitor, may reduce the deleterious dehydration of sickled erythrocytes but it remains to be validated (17). Similarly the use of antibodies against adhesive integrins, although it may work as an anti-occlusive strategy remains to be tested (18). Painful crises can be treated with an analgesic, hydration and oxygen administration. A daily oral supplement of folic acid is somewhat helpful and blood transfusions must be used sparingly to avoid isoimmunization, hepatitis and iron overload.

The problem of β-thalassemia

This disease is one of the thalassemias that ranges from small erythrocytes abnormalities to a life-threatening disease due to wide differences in the synthesis of the globin chains. In contrast to the previously discussed SCA, the β chains of patients with B thalassemia have a normal structure but are often almost undetectable. The gene frequency for B thalassemia is about 0.1 in Sicily and other Mediterranean islands but the disease is also present in Asia and Africa. Two heterozygote parents (B-thalassemia trait) statistically will generate one in four children in the homozygous state with B thalassemia major (TM) or Cooley's anemia. Erythrocytes contain an excess of α-chains and practically little or no β-chains. Owing to decrease solubility, free α-chains form insoluble aggregates within the erythrocyte precursors in the bone marrow (19). The result is an extensive intramedullary erythroid destruction and in any case a short life span of the circulating erythrocytes. These defects cause severe anemia, peripheral hemolysis, release of free iron, hemosiderosis, impaired growth, abnormal development and short life expectancy. Hepatic and splenic extramedullary hematopoiesis is to no avail. Patients with TM, who are able to upregulate γ-chain production have a less severe clinical course because γ-chains combine with the free α-chains to form the stable HbF, which is however unsuitable to perform the oxygen delivery as requested in normal life.

For preventing β thalassemia, genetic counselling and antenatal diagnosis are essential but not always sufficient. Patients can be supported with daily supplement of folic acid and in order to maintain at least a level of 9 g Hb/dL, transfusion therapy from normal donors is necessary but this, in the long run implies alloimmunization, risk of viral infections and unavoidably fatal iron overload. Constant infusion of desferrioxamine as well as phlebotomy are effective (20-22) and oral administration of deferiprone appears useful in removing myocardial iron (23). Thus, in some patients, an excess of Fe^{2+} enhances the formation of radical species not sufficiently neutralized by the antioxidant system (24, 25). Bone marrow transplantation, even though with some risk, is able to modify the prognosis but it cannot be applied on a large scale. Interestingly Kihm et al (26) have now discovered that alpha hemoglobin stabilizing protein (AHSP) acts as a chaperone and blocks the deleterious effects of free αHb precipitation. If the lack

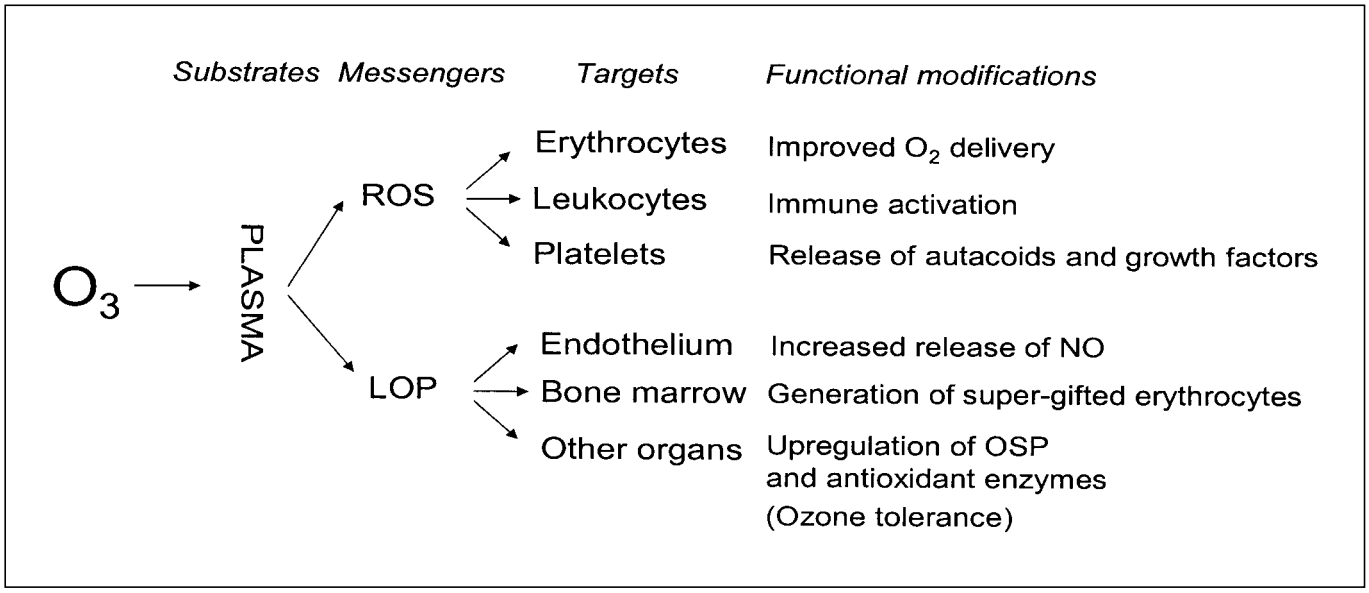

Fig. 1 - A summary of the biological effects elicited during exposure of human blood to O_2-O_3 *ex vivo* and during its reinfusion in the donor.

or a mutation of AHSP in TM proves to be really detrimental, gene therapy may help these patients for the future.

Can oxygen-ozone therapy represent a supporting therapy and why?

We already have the availability of some clinical data provided by the National Center for Scientific Research at Havana. Cuban physicians performed a randomized clinical trial in 55 SCA patients (30 experimental and 25 control). A gas mixture composed of about 97% oxygen and 3% ozone was administered daily (5 days per week) for 3 weeks in 30 patients through the rectal route by insufflation. The control group received only analgesics, vasodilators and IV saline infusion. The ozone treated group displayed a rise in arterial pO_2 and a significantly reduced (by about 50%) frequency and severity of painful crises. No side effects were recorded (27). Recent basic advancements and clinical results achieved in vasculopathies using ozonated autohemotherapy (O_3-AHT)appear very encouraging (28, 29) and they entice to test it in hemoglobinopathies. Thus, on the basis of this encouraging experience, it appears reasonable to carry out a controlled, randomized clinical trial in several hematological institutions.

Let us examine pros and cons of a treatment cycle based on the well standardized procedure of the ozonated autohemotherapy (O_3-AHT) by using 225 ml blood (+25 ml Na citrate at 3.8%) and 225 ml of O_2-O_3 at low O_3 concentration (starting with 10 µg/ml per ml of blood and slowly escalating up to 30 µg/ml). The autologous transfusion is quite safe (30) and the use of low ozone concentrations does not cause any damage to either normal or pathologic erythrocytes and infact the increase of hemolysis remains negligible (±0.2%). This is because the oxidizing activity is exhausted when ozone solubilized in the plasmatic water, instantaneously reacts with a variety of biomolecules, namely polyunsaturated fatty acids (PUFA), hydrosoluble antioxidants (uric acid, ascorbic acid, cysteine, reduced glutathione, GSH, albumin, etc) and generates reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and a variety of lipid oxidation products (LOP).

On the basis of our working hypothesis, ROS and LOP are the ozone messengers able to activate multiple biochemical and immunological pathways in blood cells (Fig. 1). Moreover upon blood reinfusion in the donor, the endothelium at first and then parenchymal cells interact with LOP that have a far longer half-life than H_2O_2. The scheme (Fig. 2) shows that a prolonged course of O_3-AHT is able to reactivate a number of biological processes that, either simultaneously or successively, combine to improve the physiology of circulation and to reduce the chronic oxidative stress. Needless to say, ozonetherapy cannot modify the genetic irregularities. However we have shown that owing to the upregulation of antioxidant enzymes (superoxide dismutase, SOD; GSH peroxidase, GSHPx, GSH reductase, GSHR; GSH transferase, GSH Tr) coadiuvated by glucose 6-phosphate dehydrogenase (G6PD), newly formed erythrocytes dubbed as “super-gifted erythrocytes” are more resistant to oxidative stress and more or less rapidly depending upon the therapeutic schedule, become a large proportion of circulating cells (29).

While any O_3-AHT represents a little oxidative stress, this is quite transitory, calculated and promptly

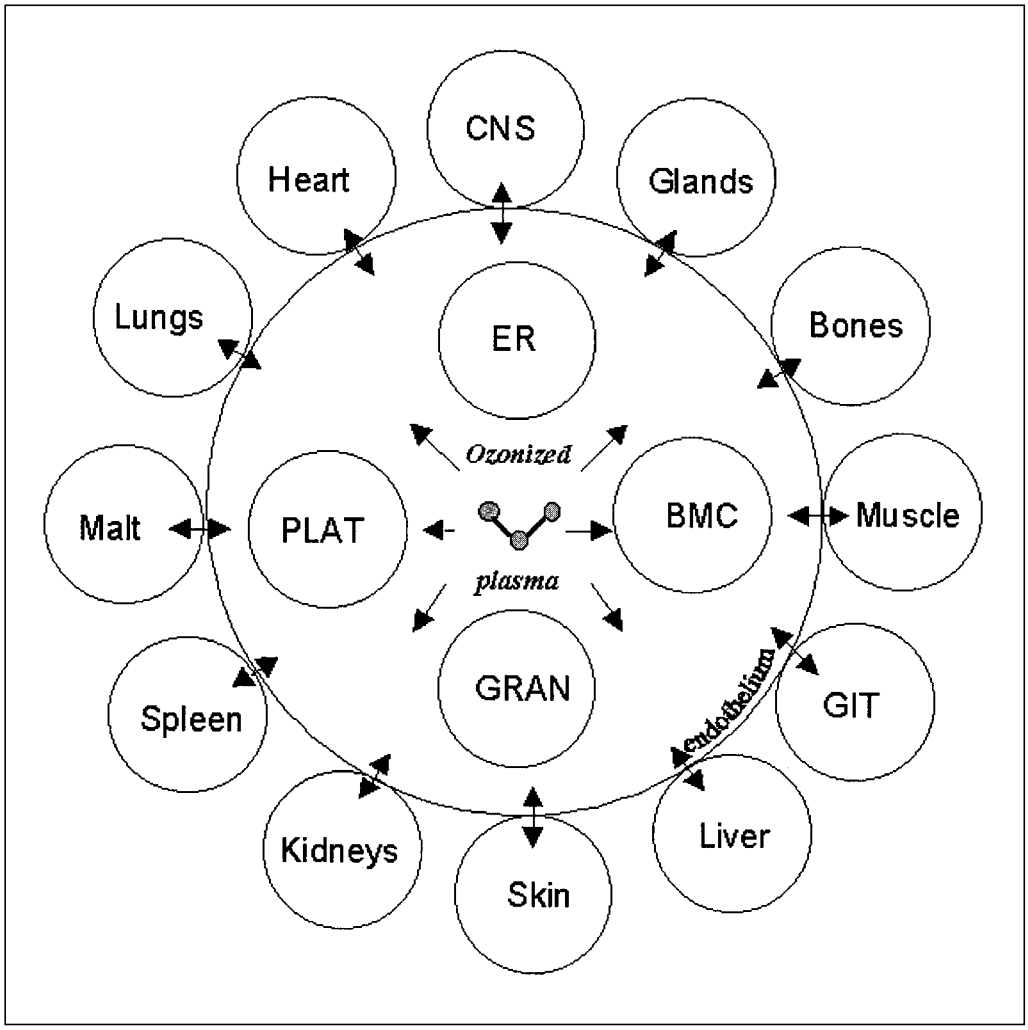

Fig. 2 - The multivaried biological response of the organism to ozonized blood can be envisaged by considering that ozonized blood cells and compounds interact with a number of organs. Some of these represent real targets (liver in chronic hepatitis, vascular system for vasculopathies), while other organs are probably involved in restoring normal homeostasis. ER: erythrocytes, PLAT: platelets, BMC: blood mononuclear cells, GRAN: granulocytes, CNS: central nervous system, GIT: gastrointestinal tract, MALT: mucosal associated lymphoid tissue.

corrected by the antioxidant system. The treatment is interpreted as a "therapeutic shock" occurring *ex vivo* during the exposure of blood to O_2-O_3 and transmitted into the donor during blood reinfusion. It must be clear that without stress, no biological effect will ensue. The synthesis of oxidative stress proteine (OSP), particularly of heme-oxygenase I (HO-I) or HSP-32, is a clear example. HO-I will enhance heme breakdown, hence will yield a higher level of bilirubin (a powerful lipophylic antioxidant) and carbon monoxide (CO), which, in this case, acts as a local hormone. It has been shown that HO-I expression reduces vascular constriction probably because it suppresses the gene expression of endothelin-1 and inhibits the proliferation of smooth muscle cells (31, 32).

We have demonstrated (33) that human endothelial cells coming in contact with ozonized plasma, hence LOP, enhance the release of nitric oxide (NO). This compound, after binding to the receptor on smooth muscle cells activates guanylate cyclase, so that an increased level of cyclic guanosine monophosphate (cGMP) causes relaxation and thus vasodilation. It is well known that NO inhibits platelet and leukocyte aggregation and adhesion and certainly cooperates with CO in enhancing vascular relaxation. Although the intravascular half-life of NO is about 2 msec, (34) important biochemical pathways describing the formation of 5-nitrosohemoglobin and S-nitrosothiols have been described (35-39) for relaxing and increasing blood flow in vessels of ischemic tissues distant from the site of origin. The possibility of an increased vasodilation cannot be underestimated because in SCA, vaso-occlusion is not only caused by sickle erythrocytes but is facilitated by vasoconstriction and obstruction due to adhesion of platelets and leukocytes to the endothelium (5). A subtle inflammatory state with release of pro-inflammatory cytokines and platelet activation does further aggravate the process.

An initial report showed that low concentrations of NO would augment HbS oxygen affinity when SCA patients inhaled NO at 80 p.p.m. in air (40). This would have been a useful therapeutic approach but recent data (41, 42) have clarified that the induced left shift in P50 correlates with an unacceptable increase of methaemoglobin formation. Another mechanism that has been pursued is the possibility that a high plasma level of arginine may increase NO production

(43-45). Interestingly HU metabolism in rat (46) and in SCA patients enhances the release of NO, and detectable amounts of nitrosyl hemoglobin (47). Thus HU efficacy may be due not only to the ability of stimulating the production of HbF but also to induce vasodilation and decrease platelet activation.

However the important role of NO may be jeopardized by an excessive release of O_2^-: in physiological conditions, the endothelium produces minute amounts of 1-10 µM NO and 1 nM O_2^- (48) but NO is rapidly scavenged by erythrocytes (actually the iron II heme of Hb) that explains its short half-life (34). Although O_2^- displays functional activities (vasoconstriction, platelet activation etc) just the opposite of NO, in normal conditions there is a sort of equilibrium. However in pathological circumstances such as chronic hepatitis, ischemia-reperfusion and severe hemoglobinopathies, the liver, which is the main repository of xantine dehydrogenase (XDH) allows its conversion to xantine oxidase (XO) and to its release in the circulation (49-51). When an excess of XO binds to endothelial cells (52) the consequent increase of O_2^- and H_2O_2 generation impairs vascular function and establishes a chronic oxidative stress. Moreover O_2^- enhances NO consumption and formation of peroxynitrite ($ONOO^-$), a deadly compound inducing protein and lipid oxidation, thus extending tissue injury (53). Not to be forgotten that in SCA, sickle erythrocytes are already generating great amounts of O_2^-, H_2O_2, OH and LOP (54). Clearly the unbalanced NO/O_2^- production contributes greatly to the diffused vascular damage and to a progressive involution of SCA.

In spite of chelation therapy and phlebotomy, TM patients present a progressive oxidative stress generated by the imbalance between the α and β chains and worsened by hepatic and cardiac iron overload (1-3).

Conclusions

The answer to the question posed is yes for several reasons: life-long ozonetherapy is feasible as we have shown in age-related macular degeneration (ARMD), in chronic limb ischemia and in angina abdominis (29, 55). After an initial cycle including 24 treatments in three months (twice weekly), the therapeutic effect can be maintained with three treatments per month. Upregulation af antioxidant enzymes and 2,3-diphosphoglycerate (2,3-DPG) is likely to occur during the first two months while rheological improvement (decrease of arterial pressure is the norm) due to NO/O_2^- rebalance may take two-to three months.

Ozonization of patient's blood must be carefully performed, first evaluating the antioxidant capacity in order to employ the optimal ozone concentration. The usual strategy "start low, go slow" is the most idoneous for inducing ozone tolerance and the rebalance of the redox system. This approach will likely diminish the frequency of allotransfusion, the severity of painful vaso-occlusive crises in SCA and will lead to a metabolic improvement. Chelation therapy with desferrioxamine must be continued regularly and for potentiating the plasma antioxidant capacity we prescribe the following oral daily supplementation one week before starting the therapy:

- 0.5 g of vitamin C (morning). This dose saturates the body (56);
- 0.6 g of NAC (either morning or evening) (57, 58);
- an approved multivitamin complex (RD dose) including vitamin E, selenium and alpha lipoic acid;
- a rich dietary intake of fresh fruit and vegetables.

Dietary supplementation of antioxidants is certainly useful but likely unable to counteract the intracellular oxidative stress due to increased iron concentrations (59).

During the last three decades, O_3-AHT has been amply performed in Europe (seven million treatments in Germany each year) without any problem and the recorded four deaths (60) where due to direct intravenous administration of O_2-O_3, a practice prohibited since 1984. At our University polyclinic, since 1995 ARMD patients have been treated with about 6,000 treatments without any adverse effect. Ozone, generated *ex tempore* from medical oxygen, must be used immediately and represents about 2% of the gas mixture. Erythrocytes, after ozonization, maintain their usual life-span in the circulation (29, 61). The method used since 1999 using neutral, sterile glass bottles under vacuum is absolutely toxic-free, the cost of the disposable set for each treatment is eleven euros and a trained nurse can easily perform the whole procedure in about 40 min. The only inconvenience is the venipuncture (performed with a 19 G needle) to which patients are nonetheless compliant. It must be emphasized that the treatment is not only perfectly tolerated but most ARMD and vasculopathic patients report a feeling of wellness and euphoria throughout the cycle. This fact explains why the compliance of the patients remains excellent throughout the years. Hemoglobinopathies are often complicated by chronic hepatitis C infection and although the combination of pegylated interferon alpha and ribavirin is effective, (62) the O_3-AHT treatment displays antiviral activity.

The treatment proposed by Cuban physicians of O_2-O_3 insufflation via the rectal route has been evaluated in the rabbit (63) but in comparison to the stoichiometry of O_3-AHT, is too empirical. However it is even cheaper and amenable to self-administration. If ozonetherapy now proves to be useful in hemoglobinopathies, a re-evaluation may be warranted also because the patient, once properly instructed, can do it at home. One drawback of ozonetherapy is that lack of electricity and medical oxygen may impede ozone production for SCA therapy in remote parts of Africa.

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References

1. Livrea MA, Tesoriere L, Pintaudi AM, et al. Oxidative stress and antioxidant status in beta-thalassemia major: Iron overload and depletion of lipid-soluble antioxidants. Blood 1996; 88: 3608-14.
2. Tavazzi D, Duca L, Graziadei G, Comino A, Fiorelli G, Cappellini MD. Membrane-bound iron contributes to oxidative damage of β-thalassemia intermedia erythrocytes. Br J Haematol 2000; 111: 1-4.
3. Cighetti G, Duca L, Bortone L, et al. Oxidative status and malondialdehyde in beta-thalassaemia patients. Eur J Clin Invest 2002; 32 (Suppl. 1): S55-60.
4. Muller WA. Leukocyte-endothelial cell interactions in the inflammatory response. Lab Invest 2002; 82: 521-33.
5. Turhan A, Weiss LA, Mohandas N, Coller BS, Frenette PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: A new paradigm. Proc Natl Acad Sci USA 2002; 99: 3047-51.
6. Prengler M, Pavlakis SG, Prohovnik I, Adams RJ. Sickle cell disease: The neurological complications. Ann Neurol 2002; 51: 543-52.
7. Hoppe CC, Walters MC. Bone marrow transplantation in sickle cell anemia. Curr Opin Oncol 2001; 13: 85-90.
8. Pawliuk R, Westerman KA, Fabry ME, et al. Correction of sickle cell disease in transgenic mouse models by gene therapy. Science 2001; 294: 2368-71.
9. Cooperative Urea Trials Group. Treatment of sickle cell crisis with urea in invert sugar: A controlled trial. JAMA 1974; 228: 1125-8.
10. Charache S, Duffy TP, Jander N, Scott JC, Bedine M, Morrell R. Toxic-therapeutic ratio of sodium cyanate. Arch Intern Med 1975; 135: 1043-7.
11. Griffin TC, McIntire D, Buchanan GR. High-dose intravenous methylprednisolone therapy for pain in children and adolescents with sickle cell disease. N Engl J Med 1994; 330: 733-7.
12. Orringer EP, Casella JF, Ataga KI, et al. Purified poloxamer 188 for treatment of acute vaso-occlusive crisis of sickle cell disease: A randomized controlled trial. JAMA 2001; 286: 2099-106.
13. Moore RD, Charache S, Terrin ML, Barton FB, Ballas SK. Cost-effectiveness of hydroxyurea in sickle cell anemia. Investigators of the Multicenter Study of Hydroxynrea in Sickle Cell Anemia. Am J Hematol 2000; 64: 26-31.
14. de Montalembert M, Begue P, Bernaudin F, Thuret I, Bachir D, Micheau M. Preliminary report of a toxicity study of hydroxyurea in sickle cell disease. French Study Group on Sickle Cell Disease. Arch Dis Child 1999; 81: 437-9.
15. Steinberg MH. Management of sickle cell disease. N Engl J Med 1999; 340: 1021-30.
16. Venigalla P, Motwani B, Allen S, Agarwal M, Alva M, Westerman M, Feldman L. A patient on hydroxyurea for sickle cell disease who developed an opportunistic infection. Blood 2002; 100: 363.
17. Brugnara C, Gee B, Armsby CC, et al. Therapy with oral clotrimazole induces inhibition of the Gardos channel and reduction of erythrocyte dehydration in patients with sickle cell disease. J Clin Invest 1996; 97: 1227-34.
18. Kaul DK, Tsai HM, Liu XD, Nakada MT, Nagel RL, Coller BS. Monoclonal antibodies to alphaVbeta3 (7E3 and LM609) inhibit sickle red blood cell-endothelium interactions induced by platelet-activating factor. Blood 2000; 95: 368-74.
19. Scott MD, van den Berg JJ, Repka T, et al. Effect of excess alpha-hemoglobin chains on cellular and membrane oxidation in model beta-thalassemic erythrocytes. J Clin Invest 1993; 91: 1706-12.
20. Olivieri NF, Brittenham GM. Iron-chelating therapy and the treatment of thalassemia. Blood 1997; 89: 739-61.
21. Angelucci E, Brittenham GM, McLaren CE, et al. Hepatic iron concentration and total body iron stores in thalassemia major. N Engl J Med 2000; 343: 327-31.
22. Angelucci E, Muretto P, Lucarelli G, et al. Phlebotomy to reduce iron overload in patients cured of thalassemia by bone marrow transplantation. Italian Cooperative Group for Phlebotomy Treatment of Transplanted Thalassemia Patients. Blood 1997; 90: 994-8.
23. Anderson LI, Wonke B, Prescott E, Holden S, Walker IM, Pennell DI. Comparison of effects of oral deferiprone and subcutaneous desferrioxamine on myocardial iron concentrations and ventricular function in beta-thalassemia. Lancet 2002; 360: 516-20.
24. Comporti M, Signorini C, Buonocore G, Ciccoli L. Iron release, oxidative stress and erythrocyte ageing. Free Radic Biol Med 2002; 32: 568-76.
25. Eaton JW, Qian M. Molecular bases of cellular iron toxicity. Free Radic Biol Med 2002; 32: 833-40.
26. Kihm AJ, Kong Y, Hong W, et al. An abundant erythroid protein that stabilizes free alpha-haemoglobin. Nature 2002; 417: 758-63.
27. Gomez M, Espinosa E, Caplan IA. Application of medicinal ozone/oxygen in patients with sickle cell anemia. Townsend Letter for Doctors 1995; 48-52.
28. Bocci V. Biological and clinical effects of ozone. Has ozonetherapy a future in medicine? Br J Biomed Sci 1999; 56: 270-9.
29. Bocci V. Oxygen-ozone therapy. A critical evaluation. Dordrecht: Kluwer Academic Publishers, 2002.
30. Vanderlinde ES, Heal JM, Blumberg N. Autologous transfusion. Br Med J 2002; 324: 772-5.
31. Morita T, Kourembanas S. Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. J Clin Invest 1995; 96: 2676-82.
32. Duckers HJ, Boehm M, True AL, et al. Heme

oxygenase-1 protects against vascular constriction and proliferation. Nat Med 2001; 7: 693-8.
33. Valacchi G, Bocci V. Studies on the biological effects of ozone: 11. Release of factors from human endothelial cells. Mediators Inflamm 2000; 9: 271-6.
34. Thomas DD, Liu X, Kantrow SP, Lancaster IJ, Jr. The biological lifetime of nitric oxide: Implications for the perivascular dynamics of NO and O_2. Proc Natl Acad Sci USA 2001; 98: 355-60.
35. Jha L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. Nature 1996; 380: 221-6.
36. Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N, Jr. Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. J Lipid Res 2001; 42: 1257-65.
37. Rafikova O, Rafikov R, Nudler E. Catalysis of S-nitrosothiols formation by serum albumin: The mechanism and implication in vascular control. Proc Natl Acad Sci USA 2002; 99: 5913-8.
38. Rassaf T, Preik M, Kleinbongard P, et al. Evidence for *in vivo* transport of bioactive nitric oxide in human plasma. J Clin Invest 2002; 109: 1241-8.
39. Han TH, Hyduke DR, Vaughn MW, Fukuto JM, Liao JC. Nitric oxide reaction with red blood cells and hemoglobin under heterogeneous conditions. Proc Natl Acad Sci USA 2002; 99: 7763-8.
40. Head CA, Brugnara C, Martinez-Ruiz R, et al. Low concentrations of nitric oxide increase oxygen affinity of sickle erythrocytes *in vitro* and *in vivo*. J Clin Invest 1997; 100: 1193-8.
41. Gladwin MT, Schechter AN, Shelhamer JH, et al. Inhaled nitric oxide augments nitric oxide transport on sickle cell hemoglobin without affecting oxygen affinity. J Clin Invest 1999; 104: 937-45.
42. Hrinczenko BW, Alayash AI, Wink DA, Gladwin MT, Rodgers GP, Schechter AN. Effect of nitric oxide and nitric oxide donors on red blood cell oxygen transport. Br J Haematol 2000; 110: 412-9.
43. Enwonwu CO. Increased metabolic demand for arginine in sickle cell anaemia. Med Sci Res 1989; 17: 997-8.
44. Rees DC, Cervi P, Grimwade D, et al. The metabolites of nitric oxide in sickle-cell disease. Br J Haematol 1995; 91: 834-7.
45. Morris CR, Kuypers FA, Larkin S, et al. Arginine therapy: A novel strategy to induce nitric oxide production in sickle cell disease. Br J Haematol 2000; 111: 498-500.
46. Jhang J, Jordan SJ, Barr DP, Gunther MR, Maeda H, Mason RP. *In vivo* production of nitric oxide in rats after administration of hydroxynrea. Mol Pharmacol 1997; 52: 1081-6.
47. Glover RE, Jvy ED, Orringer EP, Maeda H, Mason RP. Detection of nitrosyl hemoglobin in venous blood in the treatment of sickle cell anemia with hydroxyurea. Mol Pharmacol 1999; 55: 1006-10.
48. Wink DA, Grisham MB, Mitchell JB, Ford PC. Direct and indirect effects of nitric oxide in chemical reactions relevant to biology. Methods Enzymol 1996; 268: 12-31.
49. Parks DA, Granger DN. Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. Am J Physiol 1983; 245: G285-9.
50. Sarnesto A, Linder N, Raivio KO. Organ distribution and molecular forms of human xanthine dehydrogenaseIxanthine oxidase protein. Lab Invest 1996; 74: 48-56.
51. Tan S, Yokoyama Y, Dickens E, Cash TG, Freeman BA, Parks DA. Xanthine oxidase activity in the circulation of rats following hemorrhagic shock. Free Radic Biol Med 1993; 15: 407-14.
52. Houston M, Estevez A, Chumley P, et al. Binding of xanthine oxidase to vascular endothelium. Kinetic characterization and oxidative impairment of nitric oxide-dependent signaling. J Biol Chem 1999; 274: 4985-94.
53. Aslan M, Ryan TM, Adler B, et al. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. Proc Natl Acad Sci USA 2001; 98: 15215-20.
54. Kuross SA, Hebbel RP. Nonheme iron in sickle erythrocyte membranes: Association with phospholipids and potential role in lipid peroxidation. Blood 1988; 72: 1278-85.
55. Amato G. Uso dell'ozonoterapia mediante grande autoemotrasfusione nella terapia delltangina abdominis. Proceedings: I Congresso IMOS, Italia, Siena, 2-4 novembre 2000.2000: 10.
56. Levine M, Conry-Cantilena C, Wang Y, et al. Vitamin C pharmacokinetics in health volunteers: Evidence for a recommended dietary allowance. Proc Nat Acad Sci USA 1996; 93: 3704-9.
57. Bridgeman MM, Marsden M, MacNee W, Flenley DC, Ryle AP. Cysteine and glutathione concentrations in plasma and bronchoalveolar lavage fluid after treatment with N-acetylcysteine. Thorax 1991; 46: 39-42.
58. Hack V, Breitkreutz R, Kinscherf R, Rohrer H, Bartsch P, Taut F, Benner A, Droge W. The redox state as a correlate of senescence and wasting and as a target for therapeutic intervention. Blood 1998; 92: 59-67.
59. Asplund K. Antioxidant vitamins in the prevention of cardiovascular disease: A systematic review. J Intern Med 2002; 251:372-92.
60. Jacobs M-T. Untersuchung uber Zwischenfalle und typische Komplikationen in der Ozon-Sauerstoff-Therapie. OzoNachrichten 1982; 1: 5.
61. Bocci V. Determinants of erythrocyte ageing: A reappraisal. Br J Haematol 1981; 48: 515-22.
62. Li CK, Chan PK, Ling SC, Ha SY. Interferon and ribavirin as frontline treatment for chronic hepatitis C infection in thalassaemia major. Br J Haematol 2002; 117: 755-8.
63. Bocci V, Borrelli E, Corradeschi F, Valacchi G. Systemic effects after colorectal insufflation of oxygen-ozone in rabbits. Int J Med Biol Environ 2000; 28: 109-13.