



Review

Diabetes and chronic oxidative stress. A perspective based on the possible usefulness of ozone therapy

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ABSTRACT

It is now well established that hyperglycemia, present in both type 1 and type 2 diabetes, causes a variety of biochemical derangements leading to a diffused vascular damage responsible for several pathologic manifestations. Although preclinical and clinical studies have been performed by an unreliable administration route, the correct approach of oxygen-ozonotherapy may break a vicious circle. Messengers, released by a precise interaction *ex vivo* of the patient's blood with an equivalent calculated dose of ozone (0.42–0.84 mM), react with a variety of cells after blood infusion and restore a number of functions went astray. This paper aims to open a debate on this new therapy for improving the prognosis of diabetes.

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1. Introduction

Type 2 diabetes is one of the most rapidly increasing chronic diseases in the world, a fact that has led to consider it a real epidemic [1]. Associated to type 1 or insulin-dependent diabetes, makes the disease one of the worst by considering the human suffering and the socio-economic burden. In developed countries the number of diabetic patients is increasing all the time and both inability and mortality values are staggering. There is a fervour of studies aiming first to block or slow down the onset of type 1 diabetes, secondly to identify the numerous environmental and genetic factors causing type 2 diabetes and thirdly to suggest possible ways for the prevention or the postponement of crippling complications [2–4].

The initial problem of diabetes is the hyperglycemia due to the inability of several control systems to maintain a normal glycemic plasma level. A first question is: can diabetic complications be prevented or delayed by normalizing hyperglycemia? This can be achieved at least in part if a meticulous control of glycemia is kept with an appropriate diet, oral antidiabetic drugs, or insulin administration [4–7] associated with daily exercise and a correct lifestyle. However, owing to genetic factors and in spite of a serious control [4], complications are found even in patients with a transitory and slight hyperglycemia. Circulatory abnormalities are the common denominator [8] and they are present under the form of micro- and macrovascular disease. Throughout the years the following complications may develop with different intensity and localization:

- (1) Diabetic retinopathy is a leading cause of blindness in about 85% of patients aged 20–75 years [4].

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- (2) Diabetic nephropathy occurs in 20–40% of patients and when the GFR is <15 ml/min, the end stage renal disease (ESRD) is a leading cause of disability and premature death [4,9].
- (3) Diabetic foot disease normally caused by several factors such as peripheral vascular disease (PAD), altered biomechanics, possibly polyneuropathy and infected foot ulcers [10].
- (4) Neuropathy involving both the somatic and autonomic nervous system with neuromuscular dysfunction and muscular wasting is another major cause of morbidity [4,11].
- (5) Accelerated atherosclerosis frequently manifests itself with myocardial infarction, stroke and limb vascular occlusion complicated with necrotic ulcers [4].
- (6) Lipodystrophy, seemingly due to ineffective leptin activity or/and fatty acids dysmetabolism [12–14], represents another facet of the metabolic syndrome.

During the last decade the biochemical mechanisms implicated in glucose-mediated vascular damage have been clarified as follows:

- Increased advanced glycation end products (AGEs) formation. Intracellular hyperglycemia is the initiating event in the formation of intra- and extracellular AGEs. The nonenzymatic glycosylation (Maillard reaction) occurs when an alpha-amino group of the beta chain of hemoglobin, reacts with a reducing sugar such as glucose. The reaction yields Schiff-base intermediates, which undergo Amadori rearrangements to stable ketoamine derivatives. These compounds degrade into a variety of alpha-dicarbonyl compounds able to react with proteins forming irreversible AGEs, that after being taken up by cell receptors (RAGE), stimulate the synthesis of proinflammatory cytokines and matrix proteins able to induce a biological damage.
- Increased polyol pathway flux. Activation of aldose reductase leads to increased conversion of glucose to sorbitol with concomitant decrease in NADPH useful for regenerating GSH. Moreover, a decrease of NO synthesis leads to enhanced platelet aggregation and vasoconstriction [15].
- Activation of protein kinase C isoforms. Intracellular hyperglycemia increases the amount of diacylglycerol in vascular cells of diabetics.
- Increased hexosamine pathway flux. Excess of intracellular glucose is shunted into the hexosamine pathway leading to increased production of transforming growth factor β 1 and plasminogen activator inhibitor-1.

These four mechanisms have been extensively reviewed by Brownlee [16,17]. In other words, they induce overproduction of anion superoxide ($O_2^{\bullet-}$) and it must be said that since 1991, Baynes postulated that the alteration in diabetic patients may depend on an increased oxidative stress [18]. West [19] has proposed a scheme clearly indicating the interaction between hyperglycemia and the enhanced production of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, H_2O_2 , and $OH^\bullet$ in opposition to a depletion of antioxidant compounds and enzymes.

An increased level of xanthine oxidase at the endothelial site [20,21] increases the local release of $O_2^{\bullet-}$ that, by rapidly reacting with nitric oxide (NO), decreases vascular relaxation, enhances platelet aggregation and allows the formation of peroxynitrite anion ($ONOO^-$). This compound is highly toxic and inactivates several enzymes crucial for correct cell signalling [22,23].

The unbalanced equilibrium between ROS and antioxidants, particularly a lower GSH/GSSG ratio, usually precedes hypertension in diabetes. Another vicious circle may start when AGEs bound to the erythrocytes membrane stimulate production of liperoxides and adhesion to the endothelium. This process in turn favours transendothelial migration of monocytes with a consequent exacerbation of the oxidative stress [24,25]. In order to

interrupt this involution there is an urgent need for devising more effective therapeutic interventions.

2. Which are the current therapeutic approaches?

As it is clear that hyperglycemia represents a continuous risk factor, modalities for improving glycemic control are necessary and they differ for type 1 and type 2 diabetes. A low fat and a balanced low diet intake (1200–1500 kcal) are effective in reducing weight and may reverse insulin resistance in patients with type 2 diabetes [4]. A correct lifestyle including smoking cessation is useful but long-term compliance is usually poor and consequently the prevalent trend is to adopt a pharmacological approach that may reduce the burden of morbidity and mortality due to hyperglycemia. Thus the use of either insulin or/and oral antidiabetic drugs appears to have a beneficial impact on disease progression. A non-removable plaster cast, modelled to the form of the foot, appears useful to reduce the risk of diabetic ulcers [26].

How to re-equilibrate the redox potential remains an open problem. An obvious approach is the life-long oral administration of an equilibrated multi-antioxidant diet that has provided mixed results. However, while it may be useful in several oxidative stress-related conditions, there is little evidence that for several reasons it can be a definitive remedy [27–33].

Thus an appropriate oral supplementation [4], while not harmful, is not sufficient to block the complications of diabetes. This becomes understandable bearing in mind that most of the cell damage is due to an intracellular excessive production of oxidants and peroxidation compounds remaining unquenched by both an abnormally low GSH content and an impaired enzymatic function carried out by several enzymes (superoxide dismutase; GSH peroxidase; GSH reductase; GSH-S transferase, catalase; glucose-6-phosphate dehydrogenase), acting in a cooperative fashion. Moreover, at least two of the main enzymes degrading 4-hydroxynonenal, namely GSH-S-transferase and aldehyde dehydrogenase have been found reduced in liver microsomes and mitochondria of diabetic rats [34]. Moreover iron overload in diabetic patients with haemolytic diseases, although usefully treated with chelation therapy [35], may make worsen oxidative stress because residual Fe^{2+} catalyzes the formation of $OH^\bullet$ from H_2O_2 .

It must be emphasized that even a normal plasma level of antioxidants is unable to abate the intracellular oxidative stress that is a continuous process leading to a diffused damage and eventually cell death. On the basis that AGEs have been implicated in vascular pathologies, several clinical studies have been published [36–40] showing the potential usefulness of compounds such as aminoguanidine, benfotiamine and carnosine somewhat able to cleave covalent AGE-derived protein crosslinks. However the compliance to these drugs is not satisfactory.

The endogenous oxidative stress is life-time long and cannot be compared to an extremely transitory and calculated oxidative stress occurring during exposure *ex vivo* of the patient's blood to a gas mixture composed of 98% oxygen and 2% ozone [41]. This problem will be discussed in the next section.

3. Oxygen-ozone therapy seems able to correct the oxidative stress in diabetes

We would like to propose the evaluation of a scientifically proved application of a complementary medical approach based on performing cycles of oxygen-ozone therapy. We shall discuss how this therapy can abate the chronic oxidative stress and possibly attenuate diabetic complications. At first sight the use of a gas mixture composed of oxygen-ozone (98% O_2 and 2% O_3) is surprising because ozone is a powerful oxidant. However owing

to recent studies on its biological activity reviewed elsewhere [42–44], it is becoming clear that a small and precisely calibrated ozone dose paradoxically induces an adaptative response capable of reducing the endogenous oxidative stress.

The treatment is based on briefly (about 10 min) exposing a volume of the patient's heparinized blood (at most 225 ml) to an equal volume of the gas mixture (98% O₂ and 2% O₃) with an ozone concentration ranging from 20 to 40 µg/ml of gas per ml of blood (0.42–0.84 mM). The equivalence of the system, the safety of the autologous transfusion [45] and the use of low ozone concentrations do not cause any damage to blood cells and plasma components [44,46], and in fact only a negligible increase of haemolysis is detectable. This is because the oxidizing activity is exhausted when ozone, solubilized in the plasmatic water, reacts with a variety of biomolecules such as hydrosoluble antioxidants (uric acid, ascorbic acid, cysteine, GSH, albumin) and polyunsaturated fatty acids bound to albumin [44] and generates reactive oxygen species, mainly hydrogen peroxide as well as a variety of lipid oxidation products (LOPs).

That traces of reactive oxygen species, particularly hydrogen peroxide can act as physiological messengers is no longer surprising [47] because it is now clear that they are able to activate multiple biochemical and immunological pathways in blood cells [44]. Moreover when the ozonated blood is reinfused in the donor, the endothelium at first and then several organs interact with submicromolar concentrations of LOPs that have a longer half-life than H₂O₂ but nonetheless are metabolized or excreted [44]. The interaction leads to a reactivation of a number of biological processes that combine to ameliorate the chronic oxidative stress, to improve the physiology of circulation and, possibly, to improve the insulin secretion, decrease the resistance to insulin action and enhance hormonal secretion. In other words the major O₃-autohemotherapy (AHT) treatment appears to turn a "vicious" into a "virtuous" circle.

It has been already demonstrated that ozonotherapy induces an up-regulation of antioxidant enzymes, among which superoxide dismutase; GSH peroxidase; GSH reductase; GSH transferase, as well as glucose-6-phosphate dehydrogenase in erythrocytes [43,44,48]. This important modification is due to the stimulatory activity of submicromolar levels of LOPs in the bone-marrow during erythroblast differentiation leading to a progressively increasing proportion of circulating erythrocytes more resistant to oxidative stress. Moreover the phenomenon of adaptation to chronic oxidative stress [44] evaluated also as "oxidative preconditioning" [49], or as "hormesis" [50] implies that the repeated and calculated therapeutic events induces the synthesis of oxidative stress proteins of which heme-oxygenase-I is a prototypic example [51,52]. This protective enzyme will enhance heme breakdown and it will yield a higher level of bilirubin (an equally potent lipophylic antioxidant as α -tocopherol) and carbon monoxide. The enzyme indirectly reduces vascular constriction because it suppresses the gene expression of endothelin-I and inhibits the proliferation of smooth muscle cells. It is known that NO is the most important physiological vasodilator and inhibitor of platelet and leukocyte aggregation and adhesion to the endothelium and certainly traces of CO cooperates with NO[•] in enhancing vascular relaxation [53–55]. Interestingly, we have shown that human endothelial cells in culture briefly exposed to ozonized plasma increase the release of NO [56]. Although some of the released NO is immediately scavenged by the Fe²⁺ heme of hemoglobin, some is converted into more stable compounds as S-nitrosohemoglobin and a variety of S-nitrosothiols [54,55], which can relax and increase the flow of blood and release of oxygen in ischemic vessels distant from the site of origin. In diabetes, the endothelium generates more anion superoxide (O₂^{•-}) which counteracts the functional activities of NO[•] and causes vessel

vasoconstriction, as well as platelet activation and therefore is at least in part responsible for the microvascular damage. The excessive production of O₂^{•-} and the consequent lack of balance of the physiological equilibrium between NO[•] and O₂^{•-} is not only due to the dysmetabolic consequences of hyperglycemia [16,18] but also to an increased amount of xantine oxidase bound to endothelial cells [20]: this phenomenon does not occur only in diabetes but in several pathologies such as chronic hepatitis, ischemia-reperfusion and hemolytic anaemia. Thus, one of the scope for the proposed therapy is to interrupt this involutive cycle of events by renormalizing the balance NO[•]/O₂^{•-} ratio at the endothelial level, that eventually should restore a normal blood flow and slowdown the subtle inflammatory state that, via the release of proinflammatory cytokines, perpetuates the process.

4. Is there experimental and clinical evidence that ozonotherapy is useful in diabetes?

Two papers dealing with ozonotherapy have shown that streptozotocin-diabetic rats treated 10 times in two weeks with oxygen-ozone (1.1 mg Kg⁻¹ with an ozone concentration of 50 µg ml⁻¹) via rectal insufflation showed, in comparison to controls, a reduced level of hyperglycemia and of biomarkers (aldose reductase, fructolysine and AGEs) related to diabetes [57,58]. Concurrently total hydroperoxides, malondialdehyde and the levels of NO₂/NO₃ did not differ from the control group. By considering that rectal insufflation of O₂-O₃, in comparison to the O₃-AHT method is an empirical approach [59] as well as the short time of therapy, these results need to be kept in mind. Another experimental study in diabetic rats has examined the marked cardioprotective effect of a long-term ozone therapy [60]. Moreover, in a very recent experimental work also performed in streptozotocin-induced diabetes rats and currently submitted for publication, researchers have verified that treatment daily for six weeks of both insulin and ozone via intraperitoneal route for 42 days significantly improves the diabetic outcome and alleviates the oxidative stress.

On the other hand, clinical evidence that ozonotherapy is useful has been anecdotically reported several times by ozonotherapists but a controlled clinical trial has yet to be performed. Chronic limb ischemia is often accompanied by type 2 diabetes and these patients have been advantageously treated with O₃-AHT [43,44]. The need of reducing the insulin dose, suggesting either an improved insulin secretion or/and an increased receptor sensitivity, has become a common but useless observation.

As ozone is used in all Cuban hospitals, Cuban scientists have performed a randomized controlled clinical trial in diabetic patients with peripheral arterial diseases and diabetic foot [61]. One group (n = 51) was treated with ozone administered via rectal insufflation of 200 ml of gas with an ozone dose of 10 mg together with topical ozone both as gas and ozonated oil. The control group (n = 49) was treated with systemic and topical antibiotics. The efficacy of the treatments was evaluated by comparing the glucose level, the area of the ulcers and several biochemical markers after only 20 days of treatment. A drawback was the lack of a follow-up after the 20 days therapy. The experimental group, after receiving a total dose of 200 mg ozone showed an improved glycemic control, a reduced oxidative stress, an increase of SOD, a significant improvement of the lesions without any side effects and with a fewer amputations than the control group. Human diabetic foot is a chronic disease and these results are even more surprising not only because they were achieved only after 20 days therapy, but also because among the five routes of ozone administration, i.e.: (1) the major and minor ozonated autohemotherapy; (2) the extracorporeal circulation of blood against oxygen-ozone; (3) the quasi-total body exposure to oxygen-ozone; (4) the intraca-

vitary, subcutaneous, intramuscular administration, and (5) the ozone insufflation via rectum is the most erratic administration route. These routes with their relative mechanisms of action have been already extensively analyzed [62,63]. Moreover, a few caveats need to be discussed: (i) in Europe at least 40% of patients refuse the administration of ozone via rectum; (ii) part of the ozone dose may be unwillingly eliminated immediately after the gas introduction; (iii) the fecal content and the abundant mucoproteins certainly neutralize part of the ozone dose, and (iv) in all cases ozone, unlike oxygen, is not absorbed by the rectal mucosa. In previous rabbit experiments [59], it was clarified that only peroxidation products could be detected in portal blood. This is because ozone immediately reacts with a variety of biomolecules present in the luminal content and only some of the generated compounds are absorbed and may exert therapeutic activity. The concentration of ozone (50 µg/ml) used in the Cuban patients is too high and unwise because it can cause painful intestinal cramps and moreover it must keep in mind that Eliakim et al. after repeated enema in rats with ozonated water (20 µg/ml) have reported the appearance of a microscopic colitis [64]. As a consequence, even though results are spectacular, they need to be confirmed not only by repeating the trial but preferably by using the reliable strategy of ozonated autohemotherapy.

On this basis it appears urgent to organize an appropriate clinical trial in order to evaluate whether an initial cycle including 32 treatments during four months (twice weekly) can modify critical parameters including glycemic and C-reactive peptide levels, nonenzymatic glycosilation, aldose reductase activity, AGEs and the antioxidant-prooxidant balance. Although a little more expensive, the precise stoichiometry of the O₃-AHT method can give far more precise information than the rectal insufflation. The adopted strategy “start low, go slow” appears the most idoneous for inducing ozone tolerance and the rebalance of the redox system [42–44]. Bearing in mind that O₂-O₃ therapy may modify glycemic levels, a strict control of it is imperative.

During the last four decades million of O₃-AHT have been performed in Europe without any problem and the recorded four deaths [65] were due to direct intravenous administration of O₂-O₃, a practice prohibited since 1984. 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 [42]. 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 of eleven euros and a trained nurse can easily perform the whole procedure in about 35 min. The only inconvenience is the venipuncture to which patients are nonetheless compliant. It must be emphasized that the treatment is not only perfectly tolerated but most age-related macular degeneration (ARMD) and vasculopathic patients have reported a feeling of wellness and euphoria throughout the cycle. This fact explains why the compliance of the patients remains excellent throughout the years. Almost needless to say that if ozonotherapy improves the diabetic condition, it must be continued by adopting the maintenance therapy (two–three monthly sessions) for an undefined period. In ARMD and chronic limb ischaemia we have observed that after an initial cycle (14–17 treatments), two O₃-AHT treatments per month are sufficient to maintain the clinical improvement.

5. Conclusions

We are proposing to open a debate on a scientifically proved application of a complementary approach based on the knowledge that O₃-AHT can abate the chronic oxidative stress, delay serious complications and improve the quality of life of diabetic patients.

The long use of this approach in other pathologies has proved to be very useful [43,44] and it is hoped that scepticism will not prevail over a scientific rationale. Chronic ozone inhalation is very toxic for the lungs but, as it has been explained before [42], the single and calibrated ozone dose used in blood is fully tamed by the antioxidant system. Neither acute nor chronic adverse effects have ever been noted. Unfortunately orthodox medicine has overlooked this complementary approach because, only recently, the mechanisms of action have been clarified [44] and are in the realm of established biochemistry. It has now been proved that O₃-AHT: (a) improves blood circulation and oxygen delivery to ischemic tissues; (b) corrects the chronic oxidative stress by upregulating the antioxidant system; (c) induces, without side effects, a states of wellness and euphoria and (d) may improve insulin secretion or its effectiveness.

Regarding the foot ulcers, both ozonated water and different gradation of standardized ozonated vegetable oils [66] will be used twice daily until complete healing. Both ozonated water and oils have been already proved to be excellent disinfectants and healing stimulators [44,66], more effective than topical antibiotics [67], growth factors [68], maggot [69] and negative pressure wound [70] therapies. In conclusion, this paper aims to prospect the possibility of a new approach for improving the gloomy prognosis of diabetes. All of us are very much interested in collaborating with diabetologists for verifying how ozone therapy performed with a reliable method can really help diabetic patients.

Conflict of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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