# An integrated medical treatment for type-2 diabetes

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Perspective

# An integrated medical treatment for type-2 diabetes

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## 1. Introduction

In 2000, with some approximation, it was estimated that there were 170 million people suffering from diabetes. This number has grown to 346 million people in 2012 and it has been supposed that in 2030 there will be 439 million diabetic patients [1]. If indeed about 6% of the world population will suffer from diabetes, by adding the huge number of patients affected by possibly concomitant chronic cardiovascular diseases, it will represent an unsustainable socioeconomic burden. Ironically, in spite of the prolongation of the life-span, an erroneous life style leads to such a high number of patients who will further unbalance the cost of medical expenses.

Diabetes mellitus can mainly manifest into three categories: Type 1-, gestational-, and type 2- diabetes. Type 1 diabetes mostly happens in children (about 10%) and for practical reasons it will be not discussed here. Gestational diabetes needs careful medical supervision throughout the pregnancy, but normally disappears after delivery. On the contrary, type 2 diabetes includes almost 90% of patients and tends to be progressive, unless an effective treatment is adopted. The progression of type 2 diabetes induces a multiform pathology complicated by a chronic oxidative stress. Careful dieting associated with moderate physical exercise and above all antidiabetic drugs have an important role in slowing down the progression of the disease. Medical treatment, though efficacious, does not always normalize the deranged metabolism. Moreover, the biochemical dysfunction is accompanied by a progressively worsening of a chronic oxidative stress, with complex anatomo-pathological lesions at the level of many organs. The concomitant use of oral antioxidants certainly is not harmful, but it is practically ineffective in normalizing the redox system. On the other hand, in the last decade it has been demonstrated that the Nrf2/Keap1 signalling pathway is the master system for cell defence against oxidative stress and, if properly activated, can resurrect the cellular redox balance at physiological levels [2–4].

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During the last few years we have clarified that precisely treating blood with therapeutic ozone concentrations induces the formation of two messengers such as hydrogen peroxide and alkenals [5]. The latter, especially 4-hydroxynonenal (4-HNE), is an electrophile able to inhibit the suppressive action of Keap1 allowing the translocation of Nrf2 into the nucleus and the binding to the Antioxidant Response Elements (ARE) [6,7]. Such a mechanism is optimally activated by ozone therapy, a complementary approach of which the biological and molecular aspects have been thoroughly evaluated during the last two decades [5]. In fact, both preclinical and clinical studies in diabetes as well as in chronic oxidative stress have shown its validity in reactivating the innate antioxidant system [8]. Consequently, the integration of orthodox medical treatment with this approach can normalize the redox system and it is likely to significantly reduce the diabetic dysfunctions.

### 1.1. Anatomo-biochemical alterations of type 2 diabetes

The first problem is usually referred to the hyperglycemia due either to the insulin receptor resistance or to a decreased insulin secretion [9]. As obesity frequently accompanies type 2 diabetes, the release of detrimental adipokines from adipose tissues further complicates the disease [10]. The following biochemical mechanisms are greatly responsible for the glucose-mediated vascular damage:

(a) Increased formation of advanced glycation end products (AGEs). Their development has been recognized as an important pathophysiological mechanism in the development of diabetic complications [11,12]. The Maillard reaction is a non-enzymatic glycosylation occurring when an α-aminogroup of the β-chain of haemoglobin reacts with a reducing sugar such as glucose. The reaction yields Schiff-base intermediates, which undergo the Amadori rearrangements to stable ketoamine derivatives. These compounds degrade into a variety of α-dicarbonyl compounds able to react with proteins forming irreversible AGEs which, taken up by cell receptors (RAGEs), stimulate the synthesis of proinflammatory cytokines such as IL-1 and TNF-α and of matrix proteins able to induce an irreversible damage. AGEs are detrimental for endothelial cells present in the corneal stroma and in the lens, accelerating cataract formation. Moreover, they particularly damage the vascular and neuronal system;
(b) activation of protein kinase C isoforms enhances the amount of diacylglycerol in vascular cells;
(c) the abnormally high glycemia is shunted into the hexosamine pathway leading to increased production of TGF-β1 and plasminogen activator inhibitor-1;
(d) an increased polyol pathway flux. Activation of aldose reductase leads to increased conversion of glucose to sorbitol with concomitant decrease in NADPH useful for regenerating oxidated GSH. The reduced GSH/GSSG ratio decreases the antioxidant defence. Moreover, a decrease of NO synthesis leads to enhanced platelet aggregation and vasoconstriction [13];
(e) type 2 diabetic patients often have infections due to immunological mediated acute phase reactions: experimental data have shown elevated levels of serum amyloid A, C-reactive protein and development of inflammatory responses [14,15]. Long-term type 2 diabetes frequently shows a decreased chemotaxis of neutrophils [16], an impaired monocyte adhesion to vascular endothelium [17] and a reduced phagocytic activity [18]. The increased levels of circulating and pathogenic immune complexes (IC) have been detected [19] and their deposition in the endothelium causes an inflammatory response by the activation of the complement cascade. In comparison to healthy controls, C1, C2, C3 and C4 proteins were significantly higher in type 2 diabetic patients [20] inducing the formation of micro- and macrovascular diseases.

The initial problem of type 2 diabetes is the hyperglycemia frequently due to insulin resistance, possibly combined to reduced insulin secretion. In obesity, there is a different production and release of adipokines in comparison to the release of adiponectin and leptin occurring in normal subjects. The release of resistin and of pro-inflammatory cytokines (IL-1; IL-6 and TNFα) from adipocytes, fibroblasts, macrophages and monocytes present in adipose tissue appears involved in mediating insulin resistance in peripheral tissues [10]. Needless to say that a careful control of glycemia and glycosilated haemoglobin with an appropriate and a fairly restricted diet associated with daily exercise is important. Otherwise, a diffused macrovascular disease can leads to the following complications:

(a) atherosclerosis can become evident with hypertension, myocardial infarction, stroke and limb vascular obstruction complicated with necrotic ulcers. Diabetic foot disease is frequently accompanied by polyneuropathy and infected foot ulcers [21];
(b) neuropathy may involve both the somatic and autonomic nervous system with neuromuscular dysfunction and wasting;
(c) diabetic nephropathy occurs in about 30% of patients and it may lead to end stage renal disease;
(d) diabetic retinopathy may cause blindness in the majority of patients.

### 1.2. Progression of type 2 diabetes is dominated by a chronic oxidative stress

Baynes [22] was one of the first to emphasize the role of a diffused oxidative stress. West [23] suggested a scheme indicating the interaction between hyperglycemia and the increased production of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, $H_2O_2$, $^{\bullet}OH$, $ONOO^-$. An excessive consumption with a decreased synthesis of GSH leads to a lower GSH/GSSG ratio, which is a significant marker of oxidative stress. Another negative aspect is due to the AGE compounds binding to the endothelium and to the erythrocyte membrane favouring a worsening of the oxidative stress because the cellular innate molecular mechanism of restoring antioxidant enzymes (SOD, GSH-reductase and transferase, catalase, etc.), phase-2 enzymes and HO-1 is somewhat inhibited and incapable of neutralizing the excess of oxidants.

Orthodox medicine disposes of excellent drugs as antidiabetics, statin, antihypertensive and anticoagulants but their administration, although slowing down diabetes progression, cannot re-establish a normal redox system because some of these drugs are unable to reactivate the cellular antioxidant system. However, after two decades of intensive work, it is now possible to use a complementary system able to normalize the redox system [5,7]. The integration of this approach with orthodox medicine may indeed interrupt this vicious circle and can be very helpful to the patient.

### 1.3. The integration of orthodox drugs with ozone therapy

Nowadays, ozone therapy is used every day in most public hospitals in India, China, Russia and Cuba and in private clinics by many physicians, particularly in Germany. However, orthodox medicine disregards ozone therapy either owing to the lack of knowledge or simply by preconception based on the well-known



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ozone toxicity. This view is completely outdated because firstly ozone, like other gases such as NO, CO, $H_2S$ and $H_2$ are only used in therapeutic doses [24] and it is known that the toxicity of any chemical compound depends upon the dosage [25]. The second important remark is that ozone toxicity for the pulmonary system [26] depends upon critical parameters such as the ample alveolar surface, the minimal volume of the alveolar surface lining (about 30 mL), its minimal antioxidant capacity, many fold less than plasma, and the cumulative effect of inhaling ozone per months. Thus, the dogma that ozone is always toxic is untenable [27] and indeed the performance of ozone therapy during the last three decades has shown to be effective and without side effects. This is due to the potent antioxidant capacity of human plasma [28] and the use of ozone concentrations within the therapeutic range (10–80 μg/mL of gas per mL of blood, or 0.21–1.68 mM per mL of blood). Moreover, thanks to the modern ozone generators, the ozone concentration is precisely measured in real time within the Hartley band at 253.4 nm. The volume of the gas mixture composed of medical oxygen (≥95%)-ozone (≤5%) is controlled by a calibrated ozone-resistant syringe at normal atmospheric pressure.

### 1.3.1. Mechanisms of action of ozone in blood

These aspects have been already extensively discussed elsewhere [5,8,29,30] and they will be summarized. Two phases need to be described: the first phase happens ex vivo in a 250–500 mL sterile glass bottle where a volume of blood (100–150 mL) comes in close contact with an equal volume of the oxygen–ozone mixture. As blood has a potent antioxidant capacity, equivalent to 1.28–1.83 mmol/l plasma [28], some of the ozone dose dissolved in the water of plasma is immediately quenched by uric acid, ascorbic acid, GSH, cysteine and albumin, while the remaining ozone reacts with polyunsaturated fatty acids (PUFA), giving a mixture of heterogenous lipid oxidation products including alkenals, schematically as follows:

$$R{-}CH{=}CH{-}R' + H_2O + O_3 \rightarrow H_2O_2 + RCHO + R'CHO$$

Owing to the high ozone solubility and reactivity, this reaction is completed in 30–50 s and ozone is totally exhausted while oxygen, dissolved in the plasma water, fully saturated haemoglobin. Blood oxygenation is however irrelevant because the ozonated blood is slowly infused into the donor patient within a few minutes and mixes with venous blood. At this stage, the two important messengers generated by the ozone reactions are hydrogen peroxide and aldehydes as the end products of the PUFA peroxidation. While half of the uric acid is oxidized to allantoin and excreted, dehydroascorbate and a trace of GSH disulfide are reduced back to their normal value within a few minutes by a well-coordinated sequence of electron donations by thioredoxin, vitamin E and NADPH [31].

The unionized hydrogen peroxide enters into all blood cells (at most about 4 μM) because the chemical gradient between plasma and cells is only about 10% of its extracellular concentration [32,33]. The sudden and very brief inflow of hydrogen peroxide inside blood cells is the indispensable stimulus to activate a number of biochemical reactions as follows:

(i) in the erythrocytes it causes the activation of glycolysis with a significant increase of ATP and 2,3-diphosphoglycerate. Consequently the oxyhemoglobin sigmoid curve shifts to the right and increases the release of oxygen especially at the level of ischaemic tissues. Due to the great number of erythrocytes, hydrogen peroxide is promptly reduced to water by GSH. The altered GSH/GSSG ratio is quickly corrected by either extruding some glutathione disulphide, or by reducing it via GSH reductase at the expense of either ascorbic acid or thioredoxin. Moreover, the activation of the G6PDH provides reducing power and activate glycolysis [7,30];

(ii) In the leukocytes: neutrophil phagocytic activity is enhanced. Hydrogen peroxide entering into lymphocytes and monocytes activate a tyrosin-kinase with consequent phosphorylation of IkB, one of the trimeric components at rest of the transcription factor NF-kB. The phosphorylated IkB becomes free and is lysed in the proteasome. The remaining eterodimer p50–p65 translates into the nucleus and activates a variety of genes leading to a small release of γ-interferon and IL-8 [34–36]. The slight immune enhancing effect remains limited to the ozonated leukocytes.

(iii) as platelets are sensitive to even a slight oxidation, they tend to release their growth factors, which is relevant in enhancing the healing of ulcers in peripheral obstructive arterial disease [37].

The chemical reactions between blood and ozone are happening in a few minutes and then the ozonated blood is quickly infused into the donor patient. During this important second phase, some of the lipoperoxides formed during the ozonation phase are either reduced to hydroperoxide, further broken down by GSH transferase, or leading to the formation of 4-HNE. This alkenal is an electrophilic, amphipathic molecule which forms an adduct with either GSH, or cysteine, or preferentially with Cys34 present in the domain-1 of albumin. Owing to the amount of both intra and extravascular albumin (about 280 g), alkenals undergo a great dilution in body fluids. Alkenals are also broken down by GSH-S-transferases, aldehyde dehydrogenase and other enzymes [38] and some are also eliminated via renal and bile excretion [39]. The remaining submicromolar concentration of alkenals bound to albumin become the most critical ozone messengers because they are being released at many sites and inform a variety of cells of a transitory, acute oxidative stress. Albumin can transport alkenal adducts in all body tissues, from liver to endocrine glands and the hypothalamus. Once they are internalized, this electrophilic molecule reacts with –SH and $NH_2$ groups of the inert complex Nrf2-Keap1in the cytosol where Keap1 is bound to the actin cytoskeleton. Keap 1 (Kelch-like ECH-associated protein 1) is a protein molecule with many –SH groups but Cys272 and Cys288 are important for the repression of Nrf2 activity [4]. Normally the complex Nrf2-Keap1 has a half life of about 20 min because Keap1 is readily ubiquitinated and digested in the proteasome. However the alkenal interaction with Cys 151 of Keap-1 allows the release of Nrf2, which escapes proteasomal degradation and translocates into the nucleus, heterodimerizes with a small Maf protein and binds to the Antioxidant Response Element (ARE or EHRE) on DNA [2–4].

On this basis, it is clear that the Nrf2-Keap1 system is now correctly recognized as the master cellular defence system against oxidative and xenobiotic stresses. Moreover it is reassuring that this defence system can be activated in hepatic, renal, pulmonary, cardiac, ovarian and neuronal cells.

The infusion of the ozonated blood into the donor patient expresses also other beneficial effects: one is the stimulation of endothelial NO synthase. Consequently the release of NO, S-nitrosothiols and a trace of CO released with bilirubin, via the upregulation of HO-1 activity, improves vasodilation and oxygenation of ischaemic tissues. Another advantage is the enhanced release of prostacyclin (PGI2) due to a stimulation of cyclo-oxygenase 1 [40]. In conclusion the mild oxidative stress induced by the infusion of ozonated blood can activate several biological responses of which the most important are characterized by the synthesis of several protective pathways as follows:

(a) the up-regulation of the synthesis of antioxidant enzymes such as catalase, SOD, GSH-peroxidases, GSH-reductase, NADPH-quinone oxidoreductase able to neutralize the excess of ROS;
(b) the enhancement of synthesis and levels of phase-II enzymes;
(c) upregulation of HO-1 so that a trace of CO in synergy with NO allows vasodilation of ischaemic tissues;
(d) an increased synthesis of GSH as well as an increased reduction of GSSG via ascorbic acid, and thioredoxin reductase;
(e) an inhibition of the synthesis of pro-inflammatory cytokines via the induction of leukotriene B4 reductase;
(f) a reduction of $Fe^{2+}$ overload via an increased synthesis of ferritin;
(g) a generation of a feeling of well-being throughout the ozone therapy sessions.

Regarding this last point it is very likely that alkenals bound to GSH stimulate the neurons of the supra-aortic nucleus in the hypothalamus with a release of CRF-ACTH and, finally, cortisol [7]. This may explain the feeling of wellness reported by the majority of patients during ozone therapy.

The very low level of therapeutic ozone is consistent with the pharmacological concept of hormesis which clarifies how a therapeutic effect results from the response of an organism to a low-intensity biological stressor [41,42]. In line with this concept, the initial ozone dosage must be at the lowest levels of 10–15 μg/mL per mL of blood. Indeed the axiom: "start low, go slow" has been adopted and only during the following sessions the ozone dosages can be slowly upgraded up to a maximum of 35–40 μg/mL per mL of blood. Previous clinical experience has indicated that the very moderate oxidative stress induced by ozone therapy must be repeated for a prolonged time, especially for diabetes, before noticing an improvement and possibly a normalization of the redox state [5]. The protocol foresees the application of ozonated autohemotherapy either three times, or twice weekly, depending upon the stage of the disease for at least five months. Almost needless to say is that the sooner the ozonetherapy begin, the better. Then, on the basis of the clinical and hematochemical results, the frequency of the treatment may be reduced to once weekly for the following six months. In order to maintain an efficient antioxidant level, autohemotherapy at a slower pace can be continued for life. There other several modalities of ozone administration [6,43] but the autohemotherapy approach is the most valid and the compliance of the patient is excellent. It is worth mentioning that infections and ulcers of the diabetic foot can be effectively treated [44,45] and topical application of ozonated oils [46–48]. It is also necessary to mention that experimental studies performed in laboratory animals have already shown that a minimal oxidative preconditioning with low ozone dosages yields a significant induction of antioxidant enzymes [49–51]. There are also a number of anecdotic studies performed by physicians, who privately have evaluated the value of ozonated autohemotherapy in diabetic patients. The results have shown that there is a marked reduction of insulin-resistance and often the need to suspend insulin therapy [8]. In spite of the dictum "*in patients veritas*", anecdotic results have a minimal value and it appears therefore necessary and urgent to perform randomized and well-controlled clinical trials. The results of these studies would be very informative especially because in leptin deficient (ob/ob)-Keap1-knockdown mice a constant activation of Nrf2 activity is generated and appears counterproductive [52,53]. Needless to say that ozone therapy can be easily modulated on the basis of the clinical and hematologic parameters.

## 2. Concluding remarks

In this paper we have briefly reviewed the progressive stages of type 2 diabetes. Hyperglycemia, AGE compounds and other metabolic disturbances are able to induce a micro- and macro-vascular dysfunction leading to a great number of complications. One considerable problem is the progressive development of a chronic oxidative stress and antidiabetic drugs, though effective, are unable to readjust the redox system. It appears useful to integrate the orthodox therapy with an appropriate complementary approach of which today we exactly know the biochemical pathway, the molecular mechanisms of action, and the lack of toxicity. Administration of oral antioxidants is certainly useful, but it is unable to correct the chronic oxidative stress because it does neither allow the neutralization of intracellular oxidative species, nor it is able to actively stimulate the innate antioxidant system. To the best of our knowledge, ozone therapy is the unique procedure able to reactivate the depressed antioxidant system and to improve hemorheological parameters. Therefore by considering the diabetic epidemiology and the urgency to fully correct the complex dysfunction, it appears necessary to integrate orthodox medications with ozone therapy.

## Author contributions

VB conceived the paper, collated and analyzed the data, wrote manuscript, gathered references. IZ helped to critical revision of the drafts, gathered references. MH assisted with the critical editing of the paper. VT planned/drafted the paper, refined the search for information, analyzed the data, wrote manuscript, gathered references. All the Authors approved the final manuscript.

## Conflict of interest

The authors have not received any fund for this work and declare that they have no conflict of interest.

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