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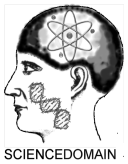
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A Practical Approach for Restoring Homeostasis in Diseases Characterized by a Chronic Oxidative Stress

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Authors' contributions

This work was carried out in collaboration among all authors. Author VB designed the study and wrote the protocol. Author EB did the literature search and also wrote part of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The leading cause of death across the globe is due to cardiovascular diseases complicated by a chronic oxidative stress only partially corrected with orthodox drugs. About 80% of deaths occur in low and middle-income countries of every continent. In order to reduce the burden of these diseases, it appears indispensable to integrate orthodox drugs with novel approaches capable of stimulating the natural defense system, which are able to restore homeostasis. Several approaches have been and ozone therapy has been selected because particularly effective.

Keywords: Ozone therapy; chronic oxidative stress; antioxidants; reactive oxygen substances.

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1. INTRODUCTION

Heart disease is the worst leading cause of death and it is increasing all the time because in low and middle-income countries, where the disease strikes young adults as well as elderly and explains why these deaths shatter families and slow economic growth. In 2011, the World Health Organization reported a statistic showing that ischemic heart disease contributes with 7 millions deaths, stroke with 6.2 million, lower respiratory infections with 3.2 millions, chronic obstructive pulmonary disease (COPD) with 3 millions, diabetes type II with 1.4 millions, neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease and Parkinson's disease with 1,6 millions while diarrheal diseases with HIV/AIDS contribute with 3.5 millions [1]. Reasons for explaining these data are: overeating and obesity, tobacco smoke and alcoholic use, physical inactivity and salt-heavy diets. In spite of significant medical improvements malignant tumors are slowly raising [2].

Orthodox medicine has made available valid drugs such as statins, antihypertensive and platelets antiaggregants, a number of anti-diabetic drugs, and a great number of recommendations for recognizing and reducing chronic diseases, for encouraging healthier eating habits and for improving affordable medicines but this is not enough because poor and low-income countries yield an increasing negative contribution.

Chronic metabolic diseases are characterized by a chronic inflammation interesting either part or the whole circulation, COPD mostly due to smoking effects deeply invalidates the lungs, diabetes type II involves insulin resistance as well as many organs such as the vascular system, the retina and the peripheral nervous system. Age- related macular degeneration (dry form) including about 85% of patients over 60 eventually leads to blindness for a progressive degeneration of the retinal pigment epithelium (RPE) and death of photoreceptors implying a loss of visual acuity, metamorphopsia and paracentral–central scotoma. In COPD the breakage of lung structure causes insufficient oxygenation and inability to walk and work thus making this disease the fourth cause of death. Thus the cardiovascular epidemic including chronic limb ischemia and stroke, the type II diabetes, the COPD and the macular degeneration, multiple sclerosis, Alzheimer and Parkinson's disease [2] are characterized by a progressive chronic oxidative stress due to an excessive production of inflammatory agents (reactive oxygen and nitrogen species) and the progressive loss of defense due to a decreased synthesis of a variety of antioxidants enzymes. This situation causes the invalidity, inability and eventual death of the patient. The question arises: can modern medicine be able to stop or correct the critical loss of homeostasis?

2. A DISCUSSION ON POSSIBLE INTEGRATIVE APPROACHES

In 2011, cardiovascular diseases (CVD) and lower respiratory diseases (COPD), type II diabetes have killed 21 millions people including both rich and poor countries of the world due to genetic predisposition, high cholesterol and blood pressure due to high-fat heavily salted diet, inactivity and tobacco smoking, excess of alcohol, social inequity and poverty. The novelty is that fewer patients are living in opulent western lifestyle while the majority are farmers and herders living in Africa, Asia and South America. The reason is that meals are heavily salted and people eat more fried meat, use too much tobacco and only a few patients can afford medical treatment. The lack of physicians is another problem and in poor countries often there is one for more than 40.000 people.

Which are the possible interventions able to delay the variety of aging-associated diseases? Orthodox medicine must realize the inability to prevent or "cure" these diseases and therefore it is necessary to consider the usefulness of integrating therapeutic schemes such as:

1) The administration of antioxidants
2) The effectiveness of caloric restriction.
3) The value of a daily exercise stress
4) The administration of selected compounds able to reduce the chronic oxidative stress
5) The regular administration of ozone therapy.

2.1 The Limited Value of the Administration of Antioxidants

This topic had been considered important during the last four decades but finally mainly negative results have been obtained in several clinical trials [3,4]. Human blood contains a number of antioxidants such as uric acid, ascorbic acid, cysteine, bilirubin, reduced glutathione (GSH)

and plenty of albumin which contains one cysteine and multiple nucleophilic groups able to bind toxic molecules.

Plasma and blood cells contain also a variety of antioxidant enzymes such as superoxide dismutase, catalase, thioredoxin, glutathione peroxidase (GPx), glutathione-S- transferase (GST) and glutathione reductase (GR), that use nicotinamide adenin dinucleotide phosphate (NADPH) as an electron donor to reduce glutathione from its oxidized state of glutathione disulphide (GSSG). Moreover glucose 6 phosphate dehydrogenase continuosly produce reducing equivalents. However when either a vascular, a degenerative, an autoimmune disease or a neoplasm intervenes, the equilibrium between intra and extracellular antioxidants is altered owing to an excessive production of reactive oxygen species (ROS), or a reactive nitrogen species (RNS). A process of chronic inflammation either present in the vascular system or in the brain or in other organs leads to cell death because cells become unable to react against the excessive production of ROS. Unfortunately the administration of antioxidants leads to a surplus of them in the extracellular space that does not protect the cells. Therefore there are several reasons explaining why the antioxidant therapy is not as useful as it was hoped to be. Therefore this approach today has a limited value.

2.2 The Value of Caloric Restriction (CR)

Caloric restriction and insulin / insulin-like growth factor 1 (IGF1) signaling are two independent mechanisms that regulate ageing and lifespan. Insulin and IGF1 promote neuronal growth, survival and differentiation. In a variety of model systems from invertebrates to mammals (yeast, worms, fruit flies, and rodents), data indicate that mutations in genes that share similarities with the human genes involved in the insulin/IGF-I signal response pathway are capable of extended lifespan [5].

Weindruch and Walford [6] in 1988 were the first to show that CR is able to delay aging and diseases in mice. Obviously the diet must be only partially reduced and must remain equilibrated regarding the various components. The value of this approach must be understood if compared to a few million people starving and many millions who over–eat, have a sedentary life and undergo an epidemic of obesity and type II diabetes. Fontana et al. [7] and Holloszy and Fontana [8] have provided important studies performed in humans who accepted CR for about six years and showed a great number of improvements regarding serum total cholesterol, triglycerides, C-reactive protein, both systolic and diastolic blood pressure and carotid intima-media thickness. Moreover Cruzen and Colman [9] have shown that CR delays cardiovascular diseases and mortality of rhesus monkeys.

The mechanism of action of CR are focused on DNA methylation and histone modification. CR has been found to also activate SIRT1 [10], SIRT3 [11] and SIRT6 [12]. These sirtuins, once activated, promote survival and reduce cell damage in age-associated diseases. It is unfortunate that only some patients accept a partial reduction of their diet and therefore long-term effects remains unknown.

2.3 The Value of a Daily Exercise Stress

There is no doubt that a moderate exercise is useful because it induces adaptative responses of muscle fibers and while enhances muscle force production reduces muscle fatigability. The redox signalling in muscle can influence numerous transcriptional activators leading to altered gene expression and changes in muscle phenotype. It has been reported for example, after regular endurance exercise, an increase in both mitochondrial enzymes and antioxidants in the active skeletal muscle [13]. While an exercise of extreme duration causes tissue damage, a number of studies reported, with a regular and moderate aerobic exercise, an increase of resistance to oxidative stress throughout an increased expression of superoxide dismutase (SOD) and Human Mut T Homolog (hMTH), a damaged nuocleotide sanitization enzyme [14]. Additional research is required to determine the exact modality of exercise able to generate the adaptative response.

2.4 The Administration of Selected Compounds Able to Reduce the Chronic Oxidative Stress

At this stage only the value of curcumin in turmeric [15], resveratrol in grapes [16,17], L-sulphoraphane in cruciferous vegetables [18,19], tea polyphenols in green tea [20] and genistein in soybean [21] will be briefly commented. All of these compounds can be daily taken orally but have some cost. All of these compounds, expecially curcumin, have been used for a long

time but the bioavailability remains variable and ought to be improved. Curcumin that is a component of turmeric (curcuma longa), has been used for centuries in Asia and Middle East and, if used continuosly in a dose of 500 mg/day, is well tolerated and displays anti inflammatory, antiproliferative and antiparasitic properties [15]. Resveratrol may modulate lifespan and regulate metabolic disordes [16,17]. L-sulphoraphane is an isothiocyanate compound found in broccoli, which acts as a selective inducer of phase II detoxification enzymes with anticarcinogenic properties [18,19]. Moreover it protects against the toxicity of electrophiles and ROS [20,21]. Resveratrol, if used regularly, may regulate metabolic disorders and modulate lifespan.

Genistein and lunasin [22] are isoflavones present in soybeans and in plants such as clover. Genistein binds to estrogen receptors and may block the effect of natural estrogen. They may lower the rate of breast cancer in women and prostate cancer in men.

Daily drinking several cups of green tea benefits patients with rheumatoid arthritis because green tea contains epigallocatechin-3-gallate which exerts antinflammatory activity and restore normal levels of NO [23].

2.5 The Regular Administration of Ozone Therapy

Christian Friedrich Schonbein discovered ozone in 1840 but he could not use this gas medically. Werner von Siemens invented the “super induction tube” which is fundamental for producing ozone. Joachim Hansler (1908-1981) was the first who invented a reliable medical ozonizer, but only a physician, Hans Wolff deserves the credit for having developed the ozonated autohaemotherapy (AHT) by applying small volumes of ozone to human blood collected in a sterile glass bottle in 1974. However modern ozone generators have been amply perfected to make sure that ozone is precisely measured when added to human blood. Thus ozone therapy consists in the infusion of a small volume of ozonated blood (50-150 ml) in the donor patient. Only an autotransfusion is permitted for avoiding any problem. The present generators are able to precisely measure both the volume and ozone concentration for avoiding any damage. While ozone therapy was used empirically until 2000, after our studies on the biochemical, physiological and pharmacological aspects of ozone reacting with human blood, ozone therapy has become scientifically precise, effective and completely atoxic [24-32]. In consideration of different body weights, blood is withdrawn from 50 up to 150 ml in a sterile glass bottle containing Na citrate as anticoagulant (9 ml of blood and 1 ml of Na citrate) and a corresponding gas volume (O_2: 95% + O_3: 5%). While oxygen is scarcely soluble, ozone dissolves immediately in the plasma (solubility of ozone in 100 ml water is equivalent to 49 ml pure ozone, while oxygen is 10 fold less soluble).

Ozone concentrations range from the initial 10 microgram/ml for ml of blood during the first two AHTs. Below this dosage, ozone remains a placebo. AHTs are normally performed twice weekly and therefore every successive weeks, the ozone dosage may be increased in small steps: 15 µg of O_3 /ml of blood in the 2nd week, 20 µg/ml in the 3rd week, 25 µg/ml in the 4th week, 30 µg/ml in the 5th week, 35 µg/ml in the 6th week and possibly 40 µg/ml in the 7th week. The low ozone levels used are consonant with the hormetic mechanism observed during ozone therapy [33,34]. Consequently 14 AHTs have been performed almost at the end of the 2nd months and, by this time, patients show an objective and subjective improvement. After the 16 sessions, the frequency may be reduced to one session per week for the next two months. At this stage most of the patients show both a marked improvement of their pathology and therefore the frequency may be reduced to two sessions per month for an undetermined period.

This is a general scheme that can be modified depending upon the pathology and the patient. The selected diseases to be treated with great benefit are all cardiovascular diseases (CD), the chronic obstructive pulmonary disease (COPD), the age-related macular degeneration (dry form only), the multiple sclerosis (MS), possibly the Parkinson’s and Alzheimer’s diseases and the type II diabetes [32]. Almost needless to say is that all of these diseases are characterized by a chronic oxidative stress that is hardly modified by the simultaneous use of specific orthodox drugs. The integration of specific drugs with ozone therapy is the key for correcting the chronic inflammatory pathologies and able to normalize the redox system especially if the pathology has not created irreversible damages. Ozone should NEVER be inhaled. Unfortunately ground-level concentrations of ozone and fine particulate matter have increased since preindustrial times in urban and rural regions and are associated with cardiovascular and respiratory mortality

[35,36]. However normally neither the patient nor the physician should inhale a trace of ozone.

3. THE MAIN PHASES OF MAJOR OZONATED AUTOHAEMOTHERAPY

1) 50-100 ml of venous blood is collected from a peripheral vein of the patient in a sterile vacuum glass bottle containing Na citrate (9 ml of blood: 1 ml Na citrate)
2) A corresponding volume (50-100 ml) of oxygen ozone mixture with an ozone concentration from 10 to 40 micrograms/ml of gas mixture is added in the bottle
3) After 5 minutes the blood is reinfused in the patient with a transfusion tube

3.1 Which are the Mechanisms of Action of Ozone Therapy and Why This Integrative Treatment should be Used?

The electrochemical potential value of ozone is: E° = +2.076 V indicating that it is a highly reactive gas. Breathing even a very low dose of ozone mixed with air for months procures pulmonary toxicity and this happens in large metropolis. This situation has created the dogma that ozone is always toxic but, in 2006, it was shown that this dogma is untenable for blood [25]. The 70 m^2 surface of the human lungs, even if exposed to a very low ozone concentration undergo a severe toxicity with the cardiovascular system. This is so because the alveolar lining layer (ALL) is only 0.14 micron thick amounting to a total volume of about 30 ml. Moreover the ALL contains only a trace amount of antioxidants and therefore ozone dissolved in the ALL, generates a toxic amounts of ROS and lipid oxidation products (LOPs). These and not ozone are adsorbed via the endothelial cells and, by stimulating an inflammatory response, cause serious damage to the whole body. On the other hand the plasma has a potent antioxidant capacity (1.28-1.83 mmol/l plasma) including uric acid (1.10^9 molecules), ascorbic acid ($1.7x10^{10}$ molecules), GSH (0.11×10^{18} molecules) cysteine, plus Cys 34 of albumin able to immediately neutralize the oxidant effect of ozone. Consequently the minimal amounts of ozone used for AHT is inactivated partly by the antioxidants present in plasma and partly because ozone reacts with polyunsaturated fatty acids (PUFA) of plasma:

$$-R\text{-}CH{=}CH\text{-}R + H_2O + 0_3 \text{ -}\rightarrow\text{- } H_2O_2 + 2RCHO.$$

This is the basic reaction explaining that, within a few minutes, the peroxidation reaction does completely exhaust the ozone present in the glass bottle and generates hydrogen peroxide and alkenals of which the most important is 4-hydroxy-nonenal (4-HNE). Moreover about 10-40% of uric acid is oxidized to allantoin and excreted while GSH disulfide and dehydroascorbate are reduced back to their normal value in few minutes by a coordinated sequence of electron donations by NADPH, vitamin E and thioredoxin [37,38].

This reaction explains why, after 5 min, the oxygenated-ozonated blood, being reinfused into the donor contains only H_2O_2 and peroxidated PUFA. Ozone has acted as pro drug and is no longer present to clarify that ozonated blood cannot exert any bactericidal or antiviral activity either in blood or in organs which may host hepatitis or HIV viruses. Paradoxically bacteria and intracellular viruses are well protected by either plasma or cellular antioxidants. This explains the possibility that AHT may exert a direct therapeutic activity in both bacterial and viral diseases where antibiotics and antivirals should be used.

3.2 What is the Fate of H_2O_2 and 4-HNE?

No more than 3-5 μM of H_2O_2 easily enter into all blood cells [39,40]. In erythrocytes H_2O_2 activates glycolysis with a transitory increase of ATP and 2,3-diphosphoglycerate [26-30]. The increase of 2-3DPG causes a shift to the right of oxyemoglobin and this process facilitates an increased release of oxygen into ischemic tissues [26,27]. H_2O_2 is therefore rapidly exhausted and if reduced to H_2O by GSH, the GSSG is reduced back to GSH by ascorbic acid or thioredoxin.

Water entering into leukocytes may activate phagocytosis and platelets may be activated and release some of their growth factors. Unsaturated lipoperoxides formed during the ozonation process leads to the final formation of 4-hydroxynonenal (from n-6 PUFA). 4-hydroxynonenal (4HNE) is an electrophilic, amphipatic molecule which binds to cysteine 34 of albumin or to GSH. It is partially broken down by GSH transferases or eliminated via the kidney or liver. The remaining submicromolar quantity of 4 HNE, either bound to Cys 34 of albumin or to GSH, is able to reach a great number of cells all

over the body and in doing so it becomes the critical messenger of ozone.

3.3 What is the Function and Fate of 4-HNE?

3.3.1 The phase II response

Nrf2 is a member of the Cap'n'Collar transcription factor family with a basic region-leucine zipper domain able to bind in the nucleus to the antioxidant response element (ARE) of gene promoters capable of inducing expression of over 230 genes, while about 30 genes are inhibited in an Nrf2-dependent manner. The activation of Nrf2 induces the most potent cellular antioxidant and detoxification systems [41-45]. Chronic inflammation of organs such as the vascular system, the lungs, the retina, the brain are responsible of an excess production of ROS and RNS causing cell apoptosis and dysfunctions. The damage is progressive and leads to inability and cell death. Excellent orthodox drug such as statins, antiaggregants, antidiabetics, antihypertensive are being used but unable to normalize the chronic oxidative stress. Action of Nrf2 by either ozonetherapy, or probably by other described approaches, is able to activate the phase II antioxidant and detoxification response including GSH synthesis genes, GR, GST, GPx, NADPH quinone reductase 1 (NQO1), thioredoxin and thioredoxin reductase, multidrug resistance protein transports and heme oxygenase 1 (HO1) [46]. There are also other inducible genes such as carbonyl reductase and peroxiredoxin genes which are useful, while insulin, growth hormone and insulin-like growth factor (IGF) are inhibited, a fact that may contribute to longevity and stress resistance.

It must be clear that normally Nrf2 is inhibited by the binding to Keap-1 bound to actin cytoskeleton. Normally the complex Nrf2-Keap1 is readily ubiquinated and digested in the proteasome every about 20 minutes. Keap-1 has many SH groups and an electrophilic molecule like 4HNE that easily bind to Cys272 and Cys288. This allows the release of Nrf2 which translocates into the nucleus, heterodimerizes with a small Maf protein and binds to the antioxidant response element (ARE) on the DNA [41-45]. It has been shown that endothelial cells incubated with either H_2O_2, or 4HNE or ozonated human serum induces a rapid Nrf2 activation [47-49]. It is interesting noting that Muthusamy et al. [50] have shown that acute exercise stress is able to promote Nrf2 activation. The molecular weight of Nrf2 ranges from 95 to 110 KDa [51]. The Nrf2 is now recognized as the master cellular defense system present in practically all organs against xenobiotic and oxidative stresses. In conclusion the ability to activate Nrf2 is very important for detoxification and prevention of aging associated diseases while a constitutive activation has serious consequences. Thus the relevance of ozone therapy is that is able to activate Nrf2 for the enzyme activation and protection for about 24-36 hours. An on and off mechanism appears to be very useful for restoring a normal redox system of the body. On the other hand it seems better not to activate it in cancer.

Clinical trials have already evaluated the activation of Nrf2 by ozone therapy in the following diseases: chronic limb ischemia [52], macular degeneration (dry form) [30,53,54], and COPD [55]. In all cases patients have shown a perfect compliance mostly because after 6-9 AHTs, they noted a marked improvement and observed a state of well-being. Particularly the COPD patients became able to work and walk normally.

4. CONCLUSION

How important will be to use either ozone therapy or related approaches is clear when worldwide more than 1.4 billion adults are overweight and 500 million are obese. In 2011 causes of death in the world were 13.2 millions for ischemic heart disease and stroke, 6.2 million for COPD and lower respiratory infections and 1.4 million for type II diabetes. In comparison 7.5 million deaths for HIV/AIDS, cancer, road injury, diarrheal diseases, maturity indicate the absolute need to integrate ozone therapy with orthodox drugs for degenerative diseases. In people between 35-40 years of age, the application of a moderate caloric restriction combined with a moderate physical exercise every day and associated with a daily administration of either curcumin or resveratrol or sulphoraphane may be preventive and reduce the risk of chronic degenerative diseases. It is moreover important to abolish smoking, alcohol and drug addiction. The cost of ozone therapy is either free of charge or comparatively acceptable. It is important to explain and educate people to accept the integration of ozone therapy that able to activate useful biological response for prolonging lifetime in healthy conditions. However these are big problems difficult to solve because of prejudice,

indifference and the idea that medical drugs alone will solve any problem.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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