Potentiality of oxygen-ozone therapy to improve the health of aging people

Title: Potentiality of oxygen-ozonetherapy to improve the health of aging people

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ABSTRACT

During the last century the lifespan of human beings has increased from about 49 to almost 80 years owing to the great advances of biomedical sciences. This fact has strongly stimulated the idea that it may be possible to prolong productive life for another twenty years but paradoxically the problem of both hyper- and hyponutrition severely jeopardizes this objective all over the world. Many causes of premature aging have been discussed in order to prevent it or find suitable medical treatment. So far only a moderate restriction of caloric intake that does not alter essential nutrients has proved capable of keeping animals and humans healthier for a prolonged time. This modality appears to activate critical longevity genes that can prolong survival: although this is a valuable line of research it will take considerable time to produce valid drugs to selectively activate these relevant genes. Meantime we propose to evaluate an easy and well-accepted treatment based on a weekly quasi-total body exposure to oxygen-ozone inside a thermostatically controlled cabinet. The rational exposure to a minimal amount of ozone acting as a mild stressor induces a striking improvement of crucial metabolic activities capable of preserving a good health for several years.

Keywords: aging; oxidative stress; longevity genes; telomerase; immune system; hormones; oxygen-ozone therapy

INTRODUCTION

The dream of a long and healthy life is probably as old as *Homo sapiens*. In 1546 Lucas Cranach represented a famous painting (Berlin State Museum) where old and crippled people, who after bathing in a magic pool were rejuvenated and ready to begin a new life-cycle. More than 2000 years ago the Latin poet Virgil wrote "Age carries all things away, even the mind" ("Omnia fert aetas, animum quoque" Eclogues IX, line 51). This is so true that during the last decades, especially in well-off people, the dream of overcoming the aging process has almost become an obsession in both sexes to the main advantage of cosmetic surgeons. Although a beautiful appearance seems important, it is really a futile and temporary practice because, in order to buy extra time for our terrestrial life, our fifty trillion cells need far more powerful stimuli than a beauty treatment.

It is well-known that the life expectancy has markedly increased during the last century from an average of 49 to almost 80. Many factors such as the advent of vaccines, antibodies, antibiotics and both medical and surgical progress have been of fundamental relevance and today we can see a dramatic difference between well-off and very poor populations. However, in the former, overfeeding often causes obesity, type-II diabetes and cardiovascular diseases, the first cause of death thus in turn shortening life. This has highlighted the importance of a low fat and low-calorie diet, of moderate physical exercise, and of avoiding smoking, drinking and drug addiction. Interestingly, in isolated villages, in rural areas of the globe, groups of centenarians have been described [1]. While it remains unclear whether these people possess "longevity" genes, they often were shepherds who, by tending their flock, surely had an active but unstressful life probably associated to moderate diets and unpolluted air and water.

In this review the state of our knowledge about some biological features of longevity is briefly described together with the attempts that have been made to prolong life.

PRESENT KNOWLEDGE OF THE AGING PROCESS AND POSSIBILITIES OF LENGTHENING THE LIFE SPAN

During the last fifty years several theories of senescence have been formulated and it appears that aging can be attributed to many causes that are here briefly discussed: 1) The continuous generation of Reactive Oxygen Species (ROS) and of Lipid Oxydation Substances (LOPs); 2) The formation of Advanced Glycosylation End substances (AGEs); 3) The role of genes; 4) The role of the immune system; 5) The relevance of telomerase; 6) The physiologic decline of circulating hormones; 7) The quality of life.

1) The continuous generation of ROS and LOPs

In the fifties, Denham Harman [2] was the first to formulate the free-radical theory of aging. Since radiations cause the production of free radicals, by evaluating short-lived strains of mice subjected to radiation but protected with 2-mercaptoethylamine, he found a 30% increase in average life expectancy. After a few years he also showed that polyunsaturated dietary fats could induce a high cancer rate in mice. Then he turned to study the effect of exogenous antioxidants but he was disappointed when he found that none of them increased the maximum lifespan. Ingeniously, he supposed that mitochondria were the producers of free radicals and that damaging depended on the fact the administered antioxidants could not enter them [3].

Although scientists were slow to accept his theory, Harman was finally acknowledged as the father of the free-radical theory of aging. Indeed, later on other scientists [4-9] showed that, owing to the fact that we breathe oxygen, human mitochondria (complex III) release every day, even at rest, about 5 g anion superoxide ($O^{2.-}$) [5] which, after dismutation to H_2O_2 can impair the mitochondrial activity. Moreover, other subcellular organelles and a variety of NADPH-oxidases also produce ROS, some of which has by now been proved to be either protective or physiologically useful provided the endogenous antioxidant system works well. Indeed as it will be described further, it will become clear in what follows: a well-calibrated, small dose of ozone against the blood

antioxidants can represent a crucial metabolic stimulant. Almost needless to add, a chronic imbalance between the free radicals and the defensive antioxidant system, as manifested in several pathologies (chronic infections diseases, cancer, diabetes, autoimmunity, atherosclerosis), induces a chronic oxidative stress that is synonymous with accelerated aging. Moreover, LOPs represented by toxic aldehydic compounds such as alkenals, pro-inflammatory cytokines and a variety of proteinases, unless properly quenched, can severely alter vital processes [10,11].

2) The formation of AGEs

Another problem is represented by the formation of advanced glycosylation end substances. The glycation reaction is a non-enzymatic process occurring when a protein, such as hemoglobin, is in solution with a key reducing sugar such as glucose. Free amino groups of proteins react with the carbonyl group of reducing sugars and form Schiff-base intermediates which undergo Amadori rearrangement to stable ketoamine derivatives. These degrade into α-dicarbonyl compounds such as methylglyoxal and glyoxal which finally form inter- and intra-molecular cross-links with long-lived proteins and form AGEs. These compounds, deposited along the arterial wall, collagens and nerve proteins can switch on the oxidant stress accelerating the aging of noble structures. Their progressive accumulation represents the key event in the establishment of a vicious and self-propelled inflammatory loop [12].

3) The role of genes

There is no doubt that some genes associated to a good quality of life are important. Numerous and excellent studies [13-21] have contributed to assess the relevance of some genes in senescence. Sardinian centenarians as well as other groups have been studied: while gene polymorphism of cytokines plays an important role in inflammation it does not affect life expectancy. On the other hand, HLA polymorphism, paraoxonase-1 (PON-1), an enzyme protecting lipids from peroxidative damage, the usual tumor suppressor p53 as well as several other genes (HRAS1, SIRT1, SIRT3, TH, INS, and IGF2) are likely to play a role in favoring longevity. The human SIRT1 (SIR2

homolog 1) gene encodes a protein capable of activating several transcription factors involved in improving glucose metabolism and cell defenses [22,23]. Sinclair's group [24,25] was the first to report that resveratrol extends the lifespan of the yeast *Saccharomyces cerevisiae*, of the worm *Caenorhabditis elegans*, and of the fruit fly *Drosophila melanogaster*. However, other researches have called into question the theory connecting resveratrol to the activation of the SIRT1 [26-28]. One aspect of this controversy may be due to the poor resveratrol bioavailability in small mammals. Indeed, a recent study [29] has clarified that, although resveratrol has some beneficial effects in elderly mice, it does not increase the longevity of *ad libitum*-fed animals.

Furthermore, a process of redox regulation of Forkhead proteins through a p66shc-dependent signaling pathway has been extensively analyzed [30-34].

4) The role of the immune system

To respond more positively or more negatively throughout life the immune system ought to be ready in such a way to neutralize noxious stimuli and restore homeostasis. It is a constantly dynamic and self-renewing system where the T-cell compartment and the thymus undergo changes with age [35-39] and may lead to autoimmunity. It is not surprising that the rate of aging of immune system has been considered a prognostic factor for human longevity [40]. Immunosupportive therapies such as the administration of thymic hormones [35], cytokines and even the use of vaccine have been proposed and partly evaluated [35,41,42] but no definitive conclusion has been reached about the validity of a harmonic and effective intervention.

5) The relevance of telomerase

The contribution of telomerase is another important point and it provides a clear reason why normal cells ultimately lose their capacity to divide [43]. The 2009 Nobel Prize in Physiology or Medicine has been awarded to Elisabeth Blackburn, Carol Greider and Jack Szostak "for the discovery of how chromosomes are protected by telomers and telomerase" [44,45]. This enzyme is a ribonucleoprotein that adds telomeric repeats to the 3'end of chromosome to stop DNA degradation,

hence to the progressive disappearance of telomeres because the shortest telomere is critical for chromosome stability [46]. Telomerase is active in immortal cells but it is also found in some 85% of cancer cells [47]. It seemed logical to assume that a prolonged persistence of the enzyme may favor longevity because the introduction of telomerase reverse transcriptase (TERT) extended the cell lifespan [48]. It remains to be seen how we could therapeutically improve TERT activity for achieving DNA repair.

6) The physiologic decline of circulating hormones.

On the whole both hormone synthesis and release progressively decline after 30-50 years of life with the significant exception of the sexual hormones which in men start to decline testosterone at the age of 60-70 years old [49]. Of the 5-7 mg of daily testosterone [50] only about 2% as 5α-dihydrotestosterone (DHT) is free and available for biologic activity. DHT is responsible for the induction and maintenance of the sexual male organs, secondary sexual characters and frequently aggressive behavior [51]. Interestingly, about 40 µg of testosterone are reduced to the typical female hormone, estradiol, in adipose tissue, in bone and in brain. This process performed by cytochrome P450 aromatase [52] does not imply feminization. In aging men a reduced aromatization, hence a reduced lack of estrogen, causes osteoporosis, vascular diseases, insulin resistance, dyslipidemia and deterioration of cognitive functions which are typical markers of the metabolic syndrome. In women, the relative deficiency of estradiol and progesterone occurs earlier with the menopause. Dehydroepiandrosterone (DHEA) synthesized from cholesterol in the cortex (reticular zone) of the adrenal gland, reaches high levels in the foetus, decreases until puberty, reaches high levels in young adulthood but then starts to decrease again reaching negligible levels at about 70 [53-55]. Growth hormone (hGH) is a polypeptide hormone produced in the anterior pituitary gland, with crucial anabolic activities. It is synthesized in a pulsatile manner throughout the day and after the onset of sleep. During adolescence hGH is secreted at an average of 700 µg/day and decreases (about 400 µg/day) in healthy adults.

Insulin is also a proteic hormone produced by the β-cells of the endocrine pancreas and its secretion is regulated by the plasmatic glucose level. With the exception of both typeI- and typeII-diabetes its variable levels are maintained throughout life. This necessarily brief endocrinologic digression serves to emphasize that hormonal activities vary during the day according to the circadian rhythm and tend to decrease with aging. The reasons of their use as anti-aging agents will be discussed later.

7) The quality of life.

Although the life span of humans has progressively increased during the last century, there are many factors that could erode the so far observed steady improvements in health. Either denutrition or malnutrition or hypernutrition leading to obesity are deleterious. Type-II diabetes is very often linked to obesity and almost 21 million Americans suffer from it. This is a minimal number because the number of obese people in Europe, Asia and South America increases every year. On the other hand, almost a billion people suffer from quantitative and qualitative limitations of nutritional compounds and at least one million are starving and die each year. Diabetes is not simply due to resistance to insulin but it is related to a chronic inflammatory state due to an excessive presence of macrophages which, by releasing ROS and pro-inflammatory cytokines (TNFα, IL-1β), reinforces insulin resistance [8,56,57]. Vascular diseases culminate with heart infarction, stroke, chronic limb ischemia, macular degeneration and blindness, uremia, polyneuropathy and dementia.

Chronic viral, bacterial, and parasitic diseases such as hepatitis B and C, HIV-AIDS, HPV, tuberculosis, gastro-intestinal infections, malaria, toxoplasmosis, listeriosis, amebiasis, leishmaniasis, Chagas disease and the grisly seven tropical diseases (ascariasis, trichuriasis; hookworm, schistosomiasis, lymphatic filariasis, onchocerciasis, trachoma) affect almost two billion people and strikingly reduce the life span [58,59].

Moreover, smoking, drug and alcohol addiction, air and water pollution have marked negative effects and reduce survival [60-64]. Finally, cancer is a great killer with about 7 million deaths

(excluding emerging Countries) each years and with the grim forecast of 11.5 million deaths in 2030. On the other hand, the whole life of Sardinian centenarians, mostly shepherds and peasants, is spent in a peaceful country side with pure air and no stressful situations; their diet is modest but the healthy Mediterranean one and they practice a moderate but constant physical exercise. This particular diet contains a variety of antioxidants present in virgin olive oil, red wine, garlic and fresh vegetables. Resveratrol, with many other compounds, is present in red wine and partly justifies the well-known "French paradox" [65]. On the whole, the average life span all over the world population must be well below the one registered in Italy of 78-81 (male-female).

IS IT POSSIBLE TO PREVENT OR DELAY AGING BY: i) A NATURAL METHOD; ii) PHARMACOLOGICAL MEANS; iii) HORMONES?

i) A natural method

Recent evidence suggests that the reduced (-35%) food intake is important because it acts as a biological stressor capable of inducing a series of positive metabolic responses [66,67]. Indeed calorie restriction (CR) is the only experimental tool known to extend the lifespan from yeast to rodents and humans. It has been long demonstrated that in rats, mice, dogs, rhesus monkeys and humans a limited, but balanced diet, with daily physical exercise, significantly prolongs the life span [68-71]. Moreover, recent studies have shown that either calorie restriction or, at the other extreme, overfeeding not only have a positive or negative impact, respectively, on aging but surprisingly act as critical epigenetic factors on future generations [72]. Unfortunately modern life does not allow to avoid several risk factors.

ii) Pharmacological means

iia) Oral administration of antioxidants and minerals.

Especially in aged people a daily intake of a Recommended Daily Allowance (RDA) of antioxidants including essential vitamins and minerals (as Se, Fe, Zn) can improve a

possibly deficient diet. Megadoses are unnecessary because they often can be toxic. A large number of patents describing the beneficial effects of antioxidants have been published [73] but in general the results are rather disappointing because the endogenous upregulation of antioxidant enzymes is not stimulated by the administration of supplementary antioxidants.

iib) During the last decade, almost every month a new supplemental nutrient with a different biochemical activity such as L-carnitine orotate, or omega-3 fatty acids, or coenzyme Q, or resveratrol is publicized as a wonderful prevention for cancer, cardiovascular diseases, diabetes, or immune disorders. If taken correctly, they can be useful, but they do not represent the "Fountain of youth".

iic) Some twenty years ago melatonin (N-acetyl-5-methoxytriptamine) [74-76], a natural antioxidant regulating the circadian rhythms of several biological functions was celebrated as a "wonder drug", but recently its fame is on the wane.

iii) Hormones

During the last three decades, the theory that hormonal decline may be an important cause of aging has gathered importance, and hormonal replacement has been assumed to restore youth in frail or ailing elderly people [77]. Thus, numerous hormones have been proposed and variably tested. In the first study, hGH was used to treat 12 men over 60 [78]. Although hGH did not reverse the aging process, a misinterpretation of the clinical results suggested that this hormone could be an effective anti-aging compound. Indeed, a recent study [79] performed on 220 participants treated with hGH led to the conclusion that the hormone, while causing small changes in body composition, increased adverse effects and it could not be recommended as an anti-aging therapy. In a recent editorial, Zs-Nagy [80] has tried to compose such a controversy between the American Academy of Anti-Aging Medicine and the American Medical Associations.

Dehydroepiandrosterone (DHEA-sulphate), a sort of mother steroid, and testosterone have been also touted as anti-aging hormones [81,82]. However, a controlled, randomized study involving

both elderly men and women receiving either DHEA or low-dose testosterone did not show beneficial effects on body composition, physical performance or quality of life [83,84]. In either post-menopausal or ovariectomized women, small doses of estrogens may produce some benefits (improvement of memory and of the HDL/LDL ratio) but if taken for a long time may also have serious drawbacks as breast cancer and vascular disorders [85,86].

In the past, experiments conducted in rodents, frequently using pharmacological doses of hormones, led to trust the usefulness of hormones. It is now understood that it is incorrect to extrapolate apparently positive results obtained in these non-primate models to humans also because rodents have a hormonal pattern different from that of man. Transitory improvements of mental or of sexual activities are not consistent findings and long-term treatments are often associated with adverse effects such as irsutism, diabetes, and prostatic hypertrophy. Another problem is that, in order to achieve striking results, enthusiastic clinicians tend to administer pharmacological doses of a single hormone, thus possibly disrupting the physiological equilibrium with unforeseeable consequences. Indeed, exogenous and excessive doses exert a negative feedback with the consequent inhibition of the natural secretion. Creams or slow-release patches have been tried in order to solve this problem and these may be better than oral administration or injection. Apart from the research about chronopharmacologically relevant drug delivery systems [87], all these attempts retain the disadvantage of modifying the natural endogenous secretion pattern.

The search for a safe and non-invasive intervention to slow the aging process continues because it is obviously important to improve the quality of life and reduce health expenditures for old people. Every year, the American National Institute on Aging's Intervention Testing Program evaluates in mice up to five compounds with the aim to discover an agent capable of increasing lifespan and delay the appearance of age-related diseases. Surprisingly rapamycin, an inhibitor of the mTOR pathway, already approved for clinical use, has been found to extend lifespan in both male and female mice when administered beginning either at 270 or 600 days of age. Rapamycin led to a

survival increase of 14% for females and 9% for males [88]. Rapamycin was firstly identified as a natural product of the bacterium *Streptomyces hygroscopicus* in soil samples and it is a well-known inhibitor of a kinase, target of rapamycin. Therefore this result indicates that TOR is a protein that can modulate lifespan in invertebrates and now in mice. Although this remains uncertain, rapamycin may work as a true dietary-restriction mimetic without either changing food consumption or reducing body weight. It is necessary to add that rapamycin is an immunosuppressive agent and cannot be used in normal people, but it may allow to discover an ideal compound capable of inhibiting relevant downstream targets of TOR [89].

After this prologue, the presumable corrections can be summarized in Table 1. The question is if there is any possibility of inducing an active and harmonious process that may physiologically stimulate all biological functions in such a way as to rejuvenate the organism or at least to keep it in a healthy state.

THE EVALUATION OF OXYGEN-OZONETHERAPY

As ozone has strong oxidizing properties, it seems paradoxical that this gas may display beneficial effects. However, on the basis of this synthesis:

$3\ O_2 + 68{,}400\ \text{Cal} \rightleftharpoons 2O_3$

and the reversibility of the reaction, it has been shown that ozone readily dissolves into the blood water, releases its energy into the hematic components, hence into the body during the reinfusion procedure in the blood donor. Ozone is about ten-fold more soluble in water than oxygen [90,91] and all the basic chemical reactions concerning it have been clarified during the last decade [92-98]. The therapeutic range has been precisely defined to be within ozone concentrations of 20 (0.42 μM) - 80 (1.68 μM) μg/mL of gas (pure O_2: 95% and O_3: 5%) per mL of human blood. Owing to the potent antioxidant power of blood due to its hydrophilic, lipophilic and cellular enzymes, a small part of the ozone dose dissolved into the water of plasma is instantly quenched by free antioxidants

(mainly uric acid, ascorbic acid and albumin) while the bulk of ozone reacts with polyunsaturated fatty acids (PUFA) mostly present in the three hydrophobic tasks of albumin [99].

$-R-CH=CH-R + H_2O + O_3 \rightarrow 2\ RCHO + H_2O_2$

Thus the potential energy of ozone is finally transferred into two fundamental messengers such as hydrogen peroxide as a ROS and aldehydic molecules of which 4-hydroxynonenal (4-HNE) is the quantitatively relevant lipid oxidation product:

$O_3 + plasma \rightarrow H_2O_2 + 4\text{-}HNE$

Thanks to the high ozone reactivity these biochemical reactions occur in few seconds, in fact within the canonical five minutes during which an average 200 mL of human blood *ex vivo* in a sterile glass bottle with the 200 mL corresponding volume of the gas mixture (O_2+O_3), ozone is totally exhausted while oxygen is solubilized in plasma and fully oxygenates hemoglobin:

$Hb_4O_{4\text{-}6} + O_2 \rightarrow Hb_4O_8$

Blood oxygenation is useful, but it has little practical relevance because oxygenated-ozonated blood is reinfused *via* venous route into the donor during the next 20 minutes and it is abundantly diluted with venous blood.

What is the fate of H_2O_2 and 4-HNE? H_2O_2, being unionized, rapidly enters all blood cells and the chemical gradient between plasma-cells is about 10% of the extracellular concentration [100-102]. In other words, when the highest ozone concentration is mixed with blood, depending upon the modest interindividual variability of antioxidant potency (1.28-1.83 mmol/L plasma) [103] the highest H_2O_2 concentration measured in plasma is 40-50 μM [104] and therefore it is at most 4-5 μM inside the cells. This sudden inflow of H_2O_2 inside blood cells is the stimulus necessary to activate a series of biochemical reactions as follows:

a) In **erythrocytes**: activation of glycolysis with increase of ATP and 2,3-diphosphoglycerate. Functionally, the oxyhemoglobin sigmoid curve shifts to the right and increases the release of oxygen at the tissue level. H_2O_2 is promptly reduced to water by GSH, thioredoxin, catalase and

GSH-peroxidase. GSH-disulphide is reduced by GSH-reductase or by reducing equivalents (NADPH) generated by dehydrogenation of glucose-6-phosphate at C-1, a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PD).

b) In **leukocytes**: neutrophil phagocytic activity is enhanced. Inside monocytes and lymphocytes, H_2O_2 activates a tyrosin-kinase with subsequent phosphorylation of IkB, one of the trimeric components at rest of the NF-kB [105,106]. The phosphorylated IkB detaches from the trimer and it is broken down in the proteasome. The remaining eterodimer p50 - p65 is transferred into the nucleus where it can activate about 100 genes. Of great significance is the final release of some cytokines (IFNγ, TNFα and IL-8) and of some acute-phase proteins [97].

c) In **platelets**. In relation to the ozone concentration, we have measured the release of PDGF-AB, TGFβ-1 and IL-8 [107].

It must be said that the H_2O_2 concentration in the cells (4-5 μM) is the minimal necessary to switch on cellular responses and it probably lasts few seconds since GSH-Px and catalase promptly reduce it to H_2O. In plasma, the H_2O_2 half-life is less than 1 minute and it is absent during blood reinfusion. On the other hand, among a variety of LOPs, 4-HNE remains fairly stable. A small part is broken down at once by enzymes such as GSH-S-transferases and aldehyde dehydrogenase [108] but the bulk is bound to the -SH group of Cys34 present in domain-I of albumin. Furthermore, eleven nucleophilic residues (Lys199 and His146) can also bind up eleven 4-HNE molecules. Thus, owing to the high albumin amount (about 125 g intravascular and 160 g extravascular) the bound alkenals undergo a great dilution in the body fluids causing a most important loss of toxicity.

An interesting aspect is that albumin can transport 4-HNE in ALL body tissues, from liver to endocrine glands and the CNS. Thus 4-HNE-Cys adducts can be released at many sites and inform a variety of cells of a transient, acute oxidative stress. At submicromolar or picomolar levels, 4-HNE can act as a signaling molecule capable of activating the synthesis of γ-glutamate cysteine ligase, γ-glutamyl transferase, γ-glutamyl transpeptidase, HSP-70, heme-oxygenase-I (HO-1), and

antioxidant enzymes such as SOD, GSH-peroxidase, catalase and last but not least, G6PDH, a critical electron-donor enzyme during erythropoiesis in the bone marrow. There is a wide consensus on the relevance of the induction of protective molecules during small but repeated oxidative stress [109-115]. In other words, the concept that a precisely controlled oxidative stress can strengthen the antioxidant defenses is well accepted today. At the time of ozonated blood infusion, 4-HNE-Cys adduct can also act on the vast expanse of endothelial cells and enhance the production of NO. This crucial mediator on its own or as a nitrosothiol, with a trace of CO released with bilirubin *via* HO-1 activity allows vasodilation, thus improving tissue oxygenation in ischemic tissues. H_2S is another potentially toxic molecule that, when released in trace amounts, it becomes an important physiological vasodilator like NO and CO [116,117]. Moreover, as it happens for the just mentioned physiological traces of gases, the minimal amount of ozone necessary to trigger useful biological effects fits is wholly consistent with the concept of the xenohormesis theory [118,119].

Another very interesting aspect observed in 67-78 years old subjects affected by the dry form of age-related macular degeneration (ARMD) is that the majority of them report a feeling of euphoria and a sense of wellness and physical energy throughout the ozonetherapy cycle of 14-16 treatments lasting about two months [93]. Whether these feelings are simply due to faith in the medical treatment (the power of the mind!), i.e. the power of the placebo effect [120] or is caused by the generated ozone messengers that can modify or improve the hormonal secretion is not yet known. Unfortunately, lack of funds has always prevented researchers from performing a study in normal volunteers where, before and after ozonetherapy, the complete hormonal pattern and cycling in the plasma throughout the 24 hours could have been determined. This study would be very informative and helpful to understand why the patients experiment a feeling of well-being. This may be due to improved oxygenation or/and enhanced secretion of GH, cortisol, and DHEA. If this proves to be true, the treatment would be preferable to the pharmacological ones. Patients with pain caused by arthrosis have also noticed a marked improvement and less pain which may be due to release of

ACTH-cortisol or to a limited stimulation of COX-2 with enhanced release of prostacyclin (PGI2). It is also possible for LOPs, by reaching the hypothalamic area, to improve the release of neurotransmitters such as serotonin. dopamine, and endorphins as has been observed after intense physical exercise [121-123].

It is important to note that neither acute nor chronic toxicity has ever been observed during or after ozone therapy. Several studies [96,99,124] performed to evaluate possible hematochemical, biochemical or enzymatic modifications have clearly demonstrated that ozone concentrations of 20-80 (even up 160) μg/mL of gas per mL of blood do not damage blood cells or other components.

Of the several procedures currently used for ozone administration on the basis of previous data for anti-aging purposes, it is suggested that either the classical major ozonated autohemotherapy [93] or the quasi-total body exposure to oxygen-ozone (QTBE) be used [125]. This second approach is very appealing because with the ozonetherapist's guidance it can be done at home at any time. With the exception of the neck and head (to absolutely prevent ozone inhalation) all the remaining body surface (about 1.4-1.6 m^2) comes in contact with a final concentration of 0.5-0.9 μg/mL within a tightly closed ozone resistant cabinet where the temperature, via a thermostat, can be regulated between 30-42 °C and 100% relative humidity. This ozone concentration is about 30-fold lower than the one used in the topical treatment of skin ulcers [126]. The subject is comfortably seated inside almost naked and with her/his skin surface wet. This is important because ozone immediately reacts with sweat and lipids normally secreted by sebaceous glands (Figure 1). The skin, while notoriously sensitive to high level of ozone [127-130], tolerates environmentally realistic level (0.8 ppm for 2 hour) [131]. As opposed to oxygen, ozone is never absorbed, while a variety of LOPs are, as shown by our experimental data [125]. In 1999 several physicians volunteered to evaluate the modification of some metabolic parameters. In normal subjects, after a period of 20-minutes in the cabinet, the following variations have been observed: a transient increase (about 1.5 °C) of the body temperature, a slight decrease of the body weight (about 0.5 Kg), a marked increase of the venous

pO_2 (up to 68 ± 9 mmHg) to indicate a greatly improved oxygenation (Figure 2a), and an increased IL-8 level with moderate leucocytosis (Figure 2b). It is very important to consider: i- the total antioxidant status (TAS) that decreased slightly at the end of the session (1.52 ± 0.12 mM) but returned to normal values after 1 hour (Figure 3a); ii- the protein thiol group levels (GTP) that decreased for 1 hour but were back to normal values after 24 hours (Figure 3b); iii- the progressively steady increase of thiobarbituric acid reactive substances (TBARS) (LOPs as markers of peroxidation) up to 1 hour after the end of the session (Figure 3c). The variation of these markers is relevant because measurements were performed in venous blood clearly indicating the multiform modifications of crucial parameters during the 20 minute exposure. O_2 practically did not change LOPs' level. An extensive hematochemical analysis did not show any acute or late toxic effect [125]. As a consequence, QTBE appears to have a significant systemic effect with an excellent patient's compliance and appreciation of the procedure because there is no need of venipuncture. After purchasing the cabinet and a simple ozone generator it is possible to perform a free and easy treatment weekly throughout the year. On the other hand, it is believed that this practice may easily be performed in a spa when the subject may receive a cycle of at least 12-15 treatments (six weekly) during Spring and Fall. The session may last 20-30 minutes depending upon the desired temperature. No preclinical studies on laboratory mammals have been done, but at the spa, where we did the first experimental study, the chief doctor has continued to use the QTBE for a decade with great patients' satisfaction. Similarly, the QTBE approach is widely and successfully used in Canada and England by naturopathics. It is regretful that they do not publish their results.

Thus, after almost five centuries from the famous rejuvenating pool painted by Cranach we are back to a bit more sophisticated Turkish bath strengthened by ozone!

CONCLUSIONS AND PERSPECTIVES

Several aging factors have been briefly reviewed and it appears that it is a multi-factorial process. On the market there is a great number of dietary supplements and exists the suggestion of using hormones to slow aging but their validity remains uncertain, if not deleterious in the long run. During the last decade, the affluent society has tried to preserve good health for a longer time. Ironically, hypernutrition causes several problems and shortens the lifespan whereas in villages, almost secluded in rural areas of the globe, several people have lived up to 100 years by leading a humble life. It is believed that firstly, this people can thank their genes and then surely an unstressful life associated to a moderate, if not limited, dieting. Indeed, it has been well demonstrated that several mammals, including Rhesus monkeys, kept to a low caloric intake for life, live longer than controls fed *ad libitum*. The evaluation of the metabolic profile of 18 men and women who had been on self-imposed caloric restriction for 3-15 years is truly remarkable: it has shown significant beneficial effects with respect to the major atherosclerosis risk factors and a decrease of inflammation. There is no doubt that dietary restrictions reduce oxidative stress. Besides genes, which at the moment cannot be safely modified or substituted, today we can try to prolong our life-time with a moderate, well-balanced diet, daily physical exercise, a correct life style and, if necessary, good drugs to preserve the efficiency of the cardiovascular system. Prevention is the key to success. Exogenous administration of hormones can certainly yield an illusory period of youth but, in the long run, may have the opposite effect. For people closely observing the rules of prevention, a mild form of ozone therapy may be helpful because ozone detains several fundamental requirements for keeping active or for revitalizing critical physiological functions.

Ozone therapy may work better than any other exogenous therapy because a few milligrams of ozone are capable of simultaneously reactivating several functions, such as antioxidant defenses, immune functions, the network of enzyme repair, a sustained and balanced hormonal and neuro-transmitter release, with the inherent benefits of an increase in energy, an improved mood and memory, prevention of cancer and atherosclerosis, and a minimal retention of sexual activity. We

hope to be able to perform a pilot study in humans to evaluate if ozonetherapy can activate the telomerase reverse transcriptase.

More than prolonging the lifespan it would be relevant to prolong life in a state of good health with reasonably preserved mental and physical independence. After all, we have a most serious problem regarding overpopulation and it is illogical to keep alive almost mummified ultra-centenarians while young talented people cannot express their abilities. Thus the idea of prolonging the lifespan at any cost must be tempered by common sense. Furthermore, to state that ozone will represent the eternal "fountain of youth" (as it was hyped for melatonin) would be a mistake unless we could provide a significant clinical evidence in at least 10,000 people. The performance of a such complex study, if successful, would have a theoretical and an economic value, but it is beyond our possibility. We would be very glad to collaborate with clinical scientists if a multicenter trial lasting several years could be performed.

ACKNOWLEDGMENTS

We are grateful to prof. Stefania D'Agata D'Ottavi for her careful linguistic revision of the manuscript.

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Table 1. Cause of aging and presumed interventions.

Cause	Presumable therapies
Generation of ROS and LOPs	Antioxidants and upregulation of antioxidant defenses
Formation of AGEs	Strict control of glycemia. Administration of benfotiamine, aminoguanidine and carnosine
The role of genes	Stimulation of longevity genes. Silencing deleterious genes
The immune system	Stringent control of cytotoxic CD24+ T-cells and enhancement of Tregs
The telomerase activity	Pharmacological stimulation of telomerase
The hormonal decline	Difficult of achieve pharmacologically
The quality of life	Decrease the calorie intake. Daily physical activity. Avoid stress, smoking alcohol and drugs

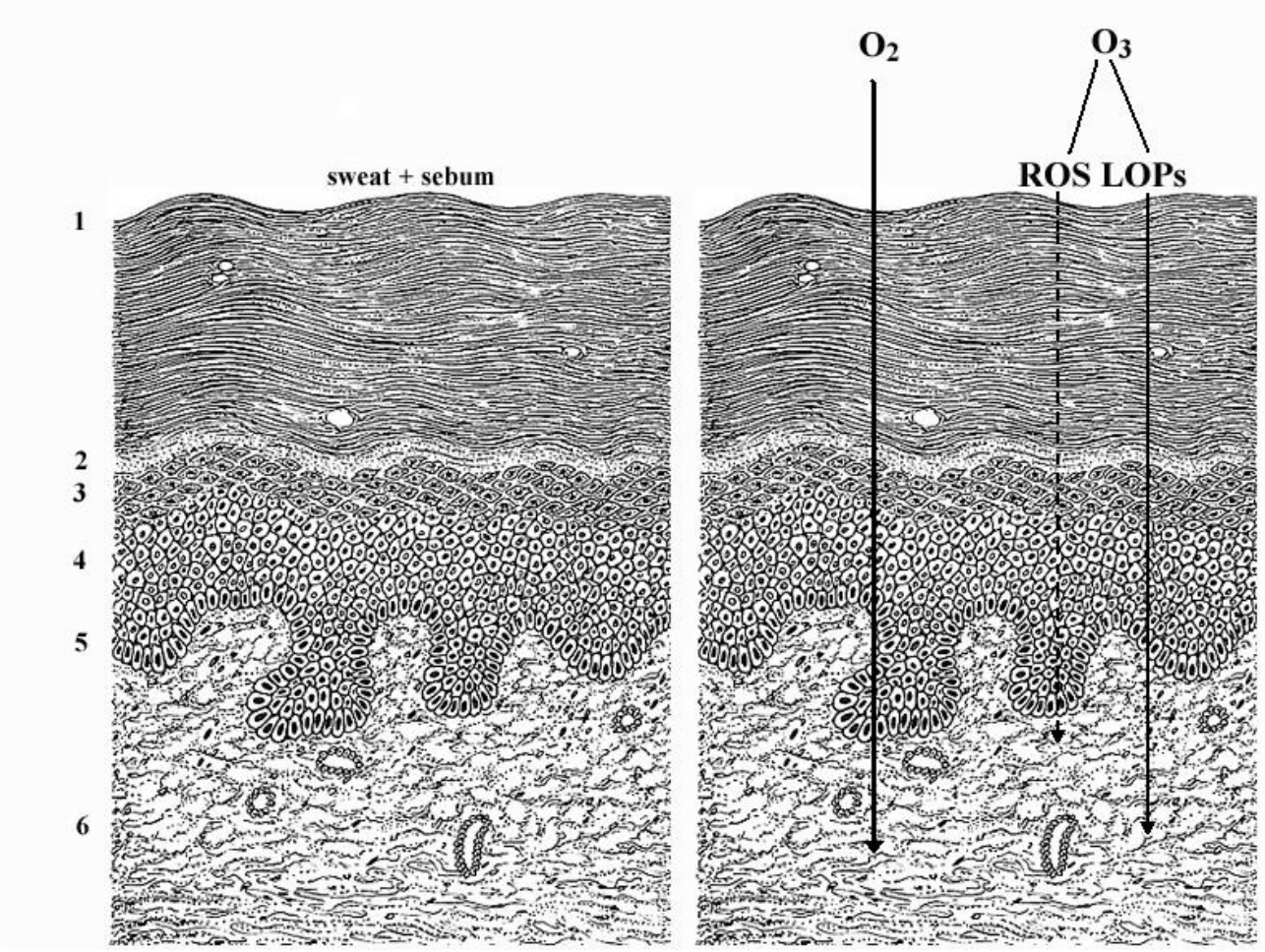

Figure 1. A schematic view of the fate of ozone in the skin during QTBE. 1– Stratum corneum; 2- Stratum lucidum; 3- Stratum granulosus; 4- Stratum spinosus; 5- Stratum basale; 6- Dermis.

When ozone comes in contact with the skin, it reacts totally and immediately with sweat and fatty acids present on its surface. While gaseous oxygen is absorbed, only ROS and LOPs, i.e. the ozone messengers, are absorbed through the skin, although ROS are rapidly quenched and do not reach the circulation.

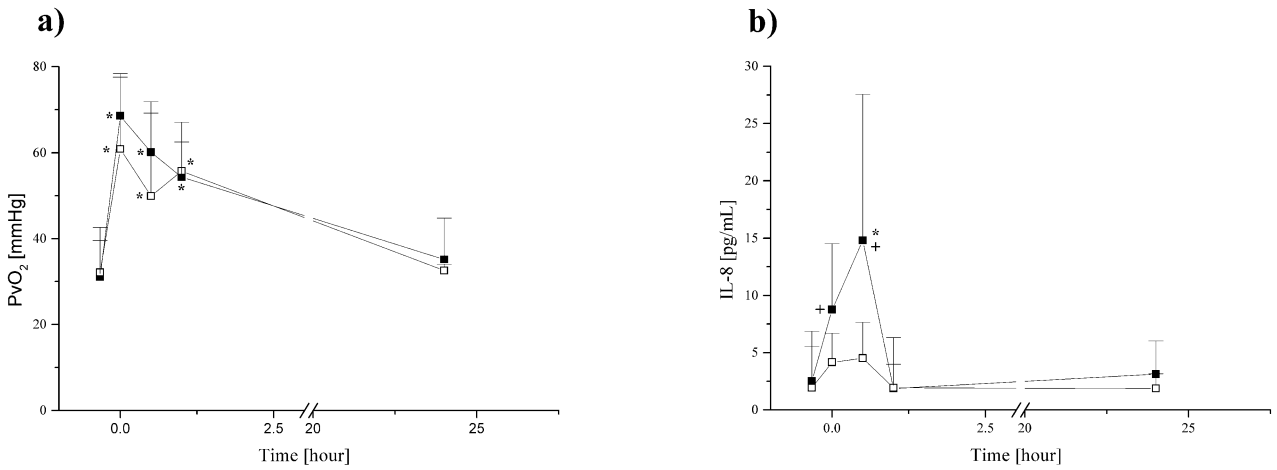

Figure 2. a) Modification of partial pressure of O_2 in the venous blood (PvO_2) and b) modification of interleukin 8 (IL-8) plasma levels of six subjects at the start of the session, at the end (time 0) and 0.5 1.0 and 24 hours after a period in the cabinet in the presence of either O_2-O_3 (solid square) or O_2 only (control, open square). Values represent the mean + SD. Asterisks indicate statistical difference ($p<0.05$) for the intragroup comparison; Crosses indicate statistical difference ($p<0.05$) for the intergroup comparison.

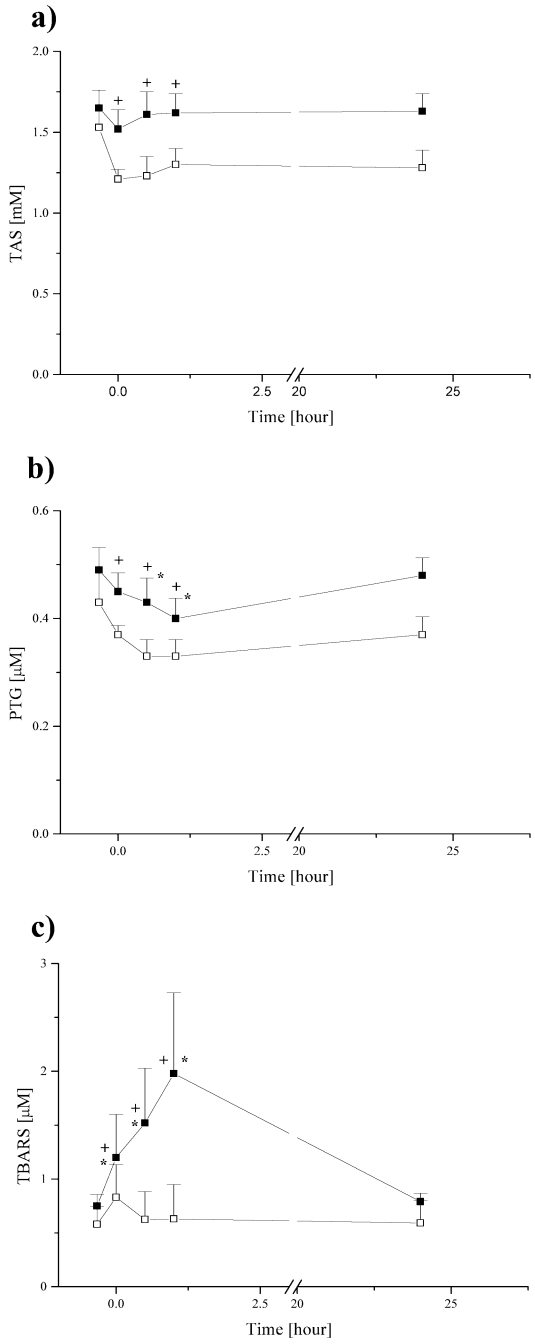

Figure 3. a) Modification of total antioxidant status (TAS), b) protein thiol groups (PTG) and c) thiobarbituric acid reactive substances (TBARS) in the plasma of six subjects at the start of the session, at the end (time 0) and 0.5 1.0 and 24 hr after a period in the cabinet in the presence of either O_2-O_3 (solid square) or O_2 only (control, open square). Values represent the mean + SD. Asterisks indicate statistical difference ($p<0.05$) for the intragroup comparison; Crosses indicate statistical difference ($p<0.05$) for the intergroup comparison.