The use of ozone for the management of primary root caries

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Introduction

Demographic changes have led to a rapid increase in the population of elderly people during the twentieth century. Both the absolute numbers of elderly people and the average life expectancy at birth were recently quoted as 82-86 years for women and 78-84 years for men.[1] This trend towards a rapidly ageing population has drawn attention to the dental requirements of elderly patients. With age, increasing numbers of root surfaces become exposed to the oral environment, which increase with time in surface area, and these are then susceptible to caries. Indeed, root caries is one of the major reasons for tooth loss in adults. The increased number of elderly people and the greater retention of teeth clearly suggest that effective strategies for the prevention and management of root caries are required[2].

Demineralisation starts from the action of acidogenic micro-organisms on the tooth, subsequent to the ingestion of fermentable carbohydrates.[3] The various organic acids diffuse within the plaque to the root surface and produce dissolution of the hydoxyapatite material. Root caries occurs in the same manner as enamel caries, except that demineralisation begins at a higher pH.[4] As the demineralisation proceeds, more mineral is lost exposing collagen in the root surface which is degraded by the plaque-forming micro-organisms.[5] Active root caries may become inactive by formation of a hard outer surface through the mineralisation process. Hence, killing micro-organisms in primary root carious lesions (PRCLs) can prevent acid formation and collagenase production.

To distinguish caries originating in enamel from that commencing in root dentine, the term primary root caries lesion (PRCL) has been introduced by Lynch.[6] The microbiology of PRCLs is well established.[7,8] The primary initiating agent of root caries is generally accepted to be *Streptococcus mutans*;[2,9-11] although animal models and clinical data suggest strongly adjunctive roles for *Lactobacillus* and *Actinomyces* species.[12-15] Based on numerous microbiological studies performed over the past three decades, it is clear that mutans streptococci and lactobacilli are high-risk factors.

Root carious lesions are classified as soft, leathery, or hard based on differences in the degree and pattern of mineralisation. Nyvad and Fejerskov[16] suggested that soft lesions presented extensive demineralisation with no evidence of an intact mineralised surface layer, whilst hard lesions appeared to have a generally uniform distribution of mineral throughout the lesion, and leathery lesions had a broad range of histological appearances. These authors concluded that soft and leathery lesions were active, whilst hard lesions were arrested. Observations suggest that soft lesions can undergo remineralisation and may become hard under favourable conditions.[17-19] When lesions become inactive i.e., hard or arrested, they acquire a smooth and hard surface. It should also be noted that arrested lesions remained unchanged during several years of observation.[20] An anti-microbial method to manage PRCLs could therefore be useful.[21]

Root caries is an emerging challenge to the dental profession in view of the knowledge that the risk factors for developing root caries point to both intra-oral and environmental factors, making management complex and multidisciplinary.[22] Compared to enamel caries, there has been relatively limited research into the pharmaceutical management of root caries, and many of these studies have been conducted *in-vitro,* with limited numbers of clinical trials.

Management of dental caries is expensive and time-consuming. Many restorative materials such as resin-modified glass ionomer, composites and amalgams have been used to restore the root carious lesion but many problems have arisen such as microleakage and poor marginal adaptations which have necessitated the frequent replacement of filling materials. Using antimicrobial agents, fluoride releasing restorative materials, especially applied fluoride and fluoride containing toothpastes to provide effective levels of continuous fluoride release may provide some protection for the high caries risk patients. However, clinical trials have been sparse.

As an alternative management strategy, ozone (O_3) may be considered as a useful therapeutic agent. Treatment may be achieved by increasing the resistance of the tooth against the microbial activity and reducing the extent of microbial activity. In addition to recent materials and techniques, the therapeutic actions of ozone may provide beneficial treatment results by reducing the demineralisation of the tooth.

Treatment of patients may be achieved without placing restorative materials but applying effective level of ozone to root carious lesions with and without placing a sealant. When the disease is active, there is a significant tooth loss to affect the quality of life. This novel non-restorative treatment regime aims to reverse and/or arrest dental caries and will be assessed by investigation of the chemical composition of the caries.

To date, many studies concerning the clinical evaluation of ozone have, unfortunately, been based on assessments of its harmful effects rather than demonstrating any therapeutic benefits that it may offer.[23] In the Earth's biosphere, the role of O_3 is controversial. Indeed, it is one of nature's most powerful oxidants and is reactive towards many biomolecules. Ozone can be produced either by ultra-violet rays of the sun, or artificially with an ozone generator, and in the former case, O_3 is the major constituent of the atmosphere responsible for shielding off ultra-violet light from the sun and oxidising pollutants in the air. O_3 has also been usefully employed by man for purposes of water purification and sewage treatment.

The purpose of this paper is to provide a sound knowledge regarding the use of ozone in dentistry and medicine and to assess the efficacy of ozone as a therapeutic agent on root caries.

History of ozone

Ozone was first discovered and named by C. F. Schonbein in 1840, and the utilisation of O_3 in industrial environments has an impressive history. The American Indian, for whom fishing was a central industry, recognised a correlation between a successful catch of fish and a strong odour released by the action of lightning following an electric storm. Similarly, the ancient Greeks also noticed this odour, which they termed 'ozein'. These subjects preferentially fished subsequent to electric storms, a custom, which prevails today. Since the upper layer of lake water is enriched with dioxygen these phenomena are explicable by an elevated level of ozone generation in this biosphere. It should be further noted that ozone in lake water arises as a consequence of air diffusion in its upper layer rather than as a product arising from chemical reactions therein.

Chemistry of ozone

Ozone is produced by the photodissociation (i.e., bond cleavage induced by light energy) of molecular O_2 into activated oxygen atoms, which then react with further oxygen molecules (equations 1 and 2).

$$O_2 \xrightarrow{\text{(solar energy / uv)}} 2O \qquad (1)$$

$$O + O_2 \longrightarrow O_3 \qquad (2)$$

The two oxygen-oxygen bonds in the O_3 molecule are of equal length and intermediate in nature between those of oxygen-oxygen single and double bonds.[24] Ozone decomposition (liberating O_2) is an exothermic reaction, but in the absence of catalysts and ultraviolet light, the rate of this reaction is very slow, even at temperatures as high as 250°C. Its decomposition is caused by solar ultraviolet radiation in the range 240-300 nm via the reaction depicted in equation 3.

$$O_3 \longrightarrow 3/2\ O_2 \quad \Delta H = -142\ kJ\ mol^{-1} \qquad (3)$$

The reaction of water-soluble electron donors with ozone appears to produce the ozone radical anion, a facile process for reductants with sufficient redox potentials (equation 4).[25] This transient radical anion rapidly becomes protonated, generating $HO_3^{\cdot}$ which, in turn, decomposes to hydroxyl radical (equation 5).

$$X: + O_3 \rightarrow X^+ + O_3^{\cdot -} \qquad (4)$$

$$O_3^{\cdot -} + H^+ \rightarrow HO_3^{\cdot} \rightarrow {}^{\cdot}OH + O_2 \qquad (5)$$

Reactions 4 and 5 convert O_3 to an even more powerful oxidant, the hydoxyl radical ($^{\cdot}$OH). $^{\cdot}$OH radical is an extremely reactive species that can contribute to tissue injury, and O_3 is known to promote free radical generation both *in-vivo* and *ex-vivo*.[26-29] Since the reduction potential of the $O_3/O_3^{\cdot -}$ couple is *ca.* +1.6 volt,[30] electron transfer reactions of ozone with thiols and catechol-like compounds, as well as many other biological electron donors, are thermodynamically feasible.

In view of its powerful oxidising properties, O_3 can attack many biomolecules such as the cysteine, methionine and the histidine residues of proteins. Tyrosine residues in proteins can be cross-linked subsequent to the O_3-mediated oxidation of their phenolic-OH groups, yielding O,O$^{'}$-dityrosine, the oxygen-oxygen bond acting as a cross-linking unit. Oxidation of polyunsaturated fatty acids (PUFAs) by O_3 can lead to lipid peroxidation, a reaction system proceeding via the prior generation of ozonides. There is also evidence that O_3 produces $^{\cdot}$OH radical in aqueous solution, extremely reactive species that contribute to tissue injury.[31] The physiologically relevant oxidising actions of O_3 towards biomolecules can be examined in simple model experiments. Exposure of methyl linoleate to 0.35 ppm O_3 in microimpingers causes lipid peroxidation, a process monitored by peroxide formation (measured iodimetrically), the development of absorption at 223 nm (ascribable to isomeric conjugated hydroperoxydienes) or the production of thiobarbituric acid-reactive substances (TBARS). The rate of development of conjugated hydroperoxydienes during the induction period is directly proportional to the square root of the concentration of gas-phase O_3.[32] As expected, the addition of α-tocopherol at a level similar to that in biomembranes gives rise to a pronounced lengthening of the induction period observed before peroxidation ensues.[33] O_3-induced oxidation of polyunsaturated fatty acids leads to the production of aldehydes,[34] species which have been employed as biomarkers for ozonylation.[35] Ozonylation products derived from unsaturated fatty acids and cholesterol can be isolated from the lungs of rats exposed to O_3, a phenomenon attributable to reaction of the oxidant with pulmonary lipidic components.

The deleterious biological effects of ozone depend upon the dynamic equilibrium between the ozone

concentration, duration of exposure and the nature and level of extra- and intracellular antioxidants. However, during limited periods of exposure, low concentrations of ozone can be restrained against causing damage to the cell membrane where it exerts its non-specific action via induction of the lipid peroxidation cascade. The reaction of ozone with lipids is a classical ozonolysis of carbon-carbon double bonds, and recently stoichiometric analysis of the products that serve as indicators of ozonolysis, and the labelling of products with ^{18}O from ^{18}O-labeled ozone, revealed that hydrogen peroxide and aldehydes were important mediators of the modifications in the lung and extrapulmonary tissues when ozone is inhaled.[36-39] The above controversy has overshadowed reports of the reaction of ozone with amino acids[40-43] and proteins.[44,45] The majority of studies conducted have been employed with pure lipids or pure proteins. Banerjee and Mudd[46] studied the reaction of ozone with glycophorin inserted in a phospholipid vesicle, the asymmetric orientation of this protein allowing a determination of whether ozone oxidatively modified externally exposed amino acids in model membranes or whether this oxidant also reacted with amino acids located in the inside of the vesicle. They found ozone to be capable of preferentially oxidising the first methionine residue located in the vesicle outside of the model membrane and that residues within the vesicle were protected. Therefore, it appears that membrane lipids protect amino acids from oxidation by ozone, i.e., the ozone molecule may not penetrate beyond the unsaturated fatty acids present in the apolar regions of membranes.

The use of ozone

Ozone was first suggested as a disinfectant for drinking water in the 19th Century in view of its powerful ability to inactivate micro-organisms.[47] There is growing evidence that ozone can be employed as a useful therapeutic agent in both dentistry and medicine. The modern development of ozone's application to medicine began in the 1950s in Europe and gradually spread throughout this continent and then to Australia, Israel, Cuba, Brazil and Columbia. In World War I, ozone was used medically to treat wounds and other infections. Indeed, many researchers have emphasised that the utilisation of this oxidant over limited time periods can effectively disinfect water supplies.[48-50] It is used in water purification and sewage treatment and is now being applied to treat patients with inflammatory bowel disorders (specifically, ulcerative colitis, Crohn's disease and chronic bacterial diarrhoea), cancer, stroke

and AIDS in Europe and the United States. [48, 50-52] However, little is known regarding the efficacy of ozone for dental purposes.

Ozone is an agent that disinfects by destroying, neutralising or inhibiting the growth of pathogenic micro-organisms, and has been found to be an effective alternative biocidal agent to chlorine.[53] Ozone acts rapidly and in lower concentrations when compared to chlorine, and has no side effects such as taste and odour which are characteristic of other disinfecting agents.[54]

Clinical applications of ozone

Ozone has the unique feature of decomposing to a harmless, non-toxic and environmentally safe material (oxygen). Interestingly, ozone has been utilised to treat patients with inflammatory bowel disorders, (specifically ulcerative colitis, Crohn's disease and chronic bacterial diarrhoea). The first ozone generator was developed by Werner von Siemens in Germany as early as 1857, and the first report of it being used therapeutically was for the purpose of purifying blood by C. Lender in 1870. In 1885, Dr. Charles J. Kenworthy first published important information regarding the medical applications of O_3.

Historically, ozone was first administered by application to external body surfaces to determine its effects on a variety of lesions. However, currently there are several different types of ozone generators utilised for the purpose of clinical applications: (1) the production of O_3 from O_2 in a narrow frequency bandwidth of ultraviolet light, (2) corona discharge involving a tube with a hot cathode surrounded by a screen anode, and (3) a method described by the term 'cold plasma' which involves a device constructed from two glass rods filled with an inert noble gas excited by a high voltage. In this latter method, the voltage jumps between the rods, forming an electrostatic plasma field, which converts O_2 to O_3. The original cold plasma generator, originally invented by Nikola Tesla in the 1920s, is still in use today.

To date, O_3 therapy has been a recognised modality in 16 nations, but properly controlled double-blind trials are urgently required in order ascertian the efficacy of ozone to treat these conditions.

The four primary methods of administering medical ozone are:

Autohaemotherapy, of which there are two classes. Major therapy involves the removal of approximately 200 ml of blood from a patient, adding O_3 and O_2 to it and infusing the mixture back into the individual. Heparin is required as an anticoagulant to prevent the blood from clotting. Minor autohaemotherapy involves the process of withdrawing only 5-10 ml of blood from patients for the treatment of ozone and treating with the application of ozone. Subsequently, infusing the ozone-treated blood back into the indivual.

Rectal insufflation, in which ozone and oxygen are administered as a rectal enema. The O_2/O_3 mixture is then absorbed through the larger intestine.

O_3 "bagging" which involves having an airtight bag placed around the area to be treated. A mixture of O_3 and O_2 is pumped into the bag and absorbed through the skin.

O_3 is also utilised externally in the form of **ozonylated olive** or **sunflower oils**. In this respect, medical treatment with ozone appears to be safe, therapeutically beneficial and cost-effective.

- Autohaemotherapy

Autohaemotherapy involves the treatment of up to 200 ml of pre-isolated human blood with a gaseous mixture of oxygen and O_3 and has been used since 1950 in Central Europe.[55] Many unrelated diseases such as acute and chronic viral diseases, neoplasia,[56] vascular disorders such as obstructive arteriopathies, venous insufficiency and vascular degenerative diseases, ulcers and cutaneous infections have been treated with ozone. There are several mechanisms of autohaemotherapy by which ozonised blood can improve the circulation and oxygenation of hypoxic tissues.

Major autohaemotherapy was employed in the treatment of acute chronic viral diseases (herpes, hepatitis) and neoplasia by activating the induction mechanism of the phagocytic and bactericidal

functions of leukocytes, with a concomitant enhancement of immunoglobulin production. In this context, autohaemotherapy is a promising treatment based on the use of inducers to elicit the endogenous production of cytokines. The advantages are lack of toxicity and a resulting equilibrated stimulation of cytokines, the latter being a phenomenon accompanied by improved oxygenation and metabolism. Paulesu *et al*.,[57] studied the action of O_3 as a potential inducer of tumour necrosis factor (TNF-alpha) in human blood and Ficoll-purified blood mononuclear cells (PBMC). All samples were exposed to O_3 concentrations ranging from 2.2 to 108 mg/ml and tested for TNF activity, whilst some PBMC cultures were analysed for their capacity to synthesise DNA. The applied O_3 concentration was found to be critical in terms of TNF production and cell mitogenesis. Furthermore, in blood a high ozone concentration was found to be more effective than it was in PBMC. Subsequently, in one of their series of experiments regarding the biological effects of O_3 in human blood, these researchers have also found that there was a significant release of transforming growth factor beta (TGF-beta 1) in volunteers' blood following exposure to O_3 concentrations ranging from 22 to 126 mg/ml (obtained via the utilisation of autohaemotherapy). In comparison to TGF-beta 1, TGF-beta 2 production was not influenced by O_3 exposure. It appeared that blood, in the presence of heparin and 5.0 mM Ca^{2+}, allowed a consistent production of tumour necrosis factor a (TNF alpha) and the release of a low and non-hazardous level of haemolysis. These data supported the notion that autohaemotherapy may promise a valuable therapeutic approach to achieve immunoregulatory effects. Indeed, Bocci *et al*.,[58] found that autohaemotherapy had a therapeutic value in viral diseases and neoplasms since O_3 acted as a mild inducer of cytokines. They studied the influence of increasing levels of O_3 exposure on human blood and attempted to correlate the production of cytokines with the depletion of reduced erythrocyte glutathione (GSH) and haemolysis. Erythrocytes were found to be a useful marker of ozone's oxidative activity since they constitute the bulk of blood cells and represent the main target of O_3-mediated oxidative damage. Transient exposure (30 sec.) of human blood to 78 mg/ml O_3 showed no depression in the production of cytokines even though there was a slight increase in haemolysis and a small decrease in intracellular GSH. It should be noted, however, that either a constant (up to 30 sec.) O_3 exposure, or a high O_3 concentration (108 mg/ml) markedly reduced GSH levels and depressed cytokine production.

The effects of autohaemotherapy on the human hair cycle in 42 subjects suffering from adrogenetic alopecia were studied by Riva Sanseverino *et al*.[59] The microscopic observation of hairs (trichogram) was carried out before and after autohaemotherapy according to a European Scientific Protocol (O_3 dosage 2500-3000 micrograms for each treatment, one cycle consisting of 16 treatments), and the investigators concluded that there was a marked improvement in the hair cycle.

Recently, Cooke *et al.,*[60] revealed that a therapy involving the combination of heating, ozonylation and exposure to ultraviolet light (H-O-U) may exert a therapeutic effect in the treatment of Raynaud's syndrome. Four patients with severe Raynaud's syndrome for the duration of more than five years and presenting with more than five daily attacks were selected for this study. Patients were treated daily, or on alternate days, for a 2-3 week period by the re-injection of citrated, autologous blood pre-treated with heat, ozone and ultraviolet light. A reduction or abolition of Raynaud's attacks for at least three months was observed following this treatment.

- Rectal insufflation

Özmen *et al.,*[61] studied the peritoneal cavities of 240 rats following faecal-capsule implantation. Ozonylated saline proved to be an effective irrigating solution for reducing abscess formation in survivors when compared to normal saline solution, and saline-cephalothin irrigation in the treatment of faecal peritonitis. Subsequently, Romero Valdes *et al.,*[62] reported that the least uncomfortable, more harmless and more economically feasible manner of O_3 administration was rectal insufflation in the treatment of 72 non-diabetic patients with obliterant atherosclerosis, rather than the endovenous and intramuscular methods of employing O_3 and conventional medical treatment (control group). Daily doses of O_3 ranging from 2.7 to 30 mg were employed in the treatment of intractable diarrhoea associated with AIDS. Carpendale *et al.,*[51] claimed that rectal insufflation was simple, safe and effective. Since O_3 administration by rectal insufflation can infiltrate the parenchyma of the liver to inactivate the Hepatitis B virus, it should be noted that the ability of this oxidant to inactivate HIV or any enveloped virus in the wall of the colon is feasible.

Rodriqueuz *et al.,*[63] recently performed rectal insufflation on a daily basis to patients suffering from

senile dementia (vascular, degenerative or mixed aetiology). An improvement was obtained according to psychometric and Hamilton tests, and parameters such as the Hachinsky scale and Katz index. Since no side effects were observed, this class of O_3 therapy was recommended for the treatment of senile dementia.

- Ionozone Therapy

Ionozone therapy was first utilised in Germany. It has been found to have a bactericidal effect, particularly on staphylococcal, streptococcal and protean infections, and has been employed in the treatment of open wounds, together with ulcers such as varicose, diabetic and pressure sores. An ionozone apparatus was designed to generate steam which is then ionised via passage over a mercury vapour arc (producing a mixture of ionised water, ozone and oxygen). The physiological effects observed can be considered as a sedatory action on sensory nerve endings, and/or a stimulation of superficial blood flow. However, a bactericidal effect was observed in conjunction with the use of antibiotics. Following three years of treatment, Dolphin and Walker[64] indicated that over 75% of lesions were completely healed and the condition of the skin was found to have no transparent appearance that can often be seen within the short period of healing. A preliminary investigation was designed to determine the effect of Ionozone therapy for treating skin lesions in elderly people in a geriatric unit.[65] Pressure sores were defined as an area of tissue necrosis with underlying ulceration of the skin, a consequence of ischaemia related to the sustained pressure and ulceration of the lower leg regarding venous pressure. 17 out of 23 patients showed a rapid improvement and were discharged mobile, although 6 were referred to other units. Of the 11 surviving patients with ulcers, ten healed rapidly and were discharged (one was referred to another unit). It should also be noted that no member of staff or patient complained of any adverse symptoms when the Ionozone apparatus was employed.

A Bioozon U type apparatus (produced by B. Prochazka GmbH, Reutlingen, Germany) was employed to test the effects of an O_3/O_2 mixture in rats. Rats poisoned with cadmium acetate during 12 weeks, at a dose of 50 mg/dm^3 (administered in drinking water), were treated with an O_3/O_2 mixture as an intraperitoneal injection during the last 10 days of the experiment at a daily dose of 1 ml of an O_3 concentration of 40 mg/ml. Two control groups included animals treated with this mixture except

cadmium ion, and rats poisoned with this metal ion without the subsequent O_3/O_2 treatment. Liver and cardiac muscle were examined using transmission electron microscopy. Morphological traits of a protective effect of the administered mixture against cadmium-poisoning (expressed as less destructive changes within the endoplasmic reticulum, basal cytoplasm and lysosome of the hepatocytes) were observed in both organs.[66]

Furthermore, O_3 therapy has been carried out on the forehead of 16 male test persons suffering from acne vulgaris on 7 consecutive days with the employment of Vapozone 9 which is an instrument in normal commercial usage. Surface lipids on the unchanged skin, and 2.0 hr. after defatting the skin, were directly extracted and analysed by means of thin layer chromatography. As expected, a decrease in the content of free fatty acids was induced by this therapy.[67]

Medical ozone is a mixture of O_2 and O_3, which can be employed for several therapeutic applications. In a recent study, 50 patients were treated by the employment of intradiscal O_3 inflitration as an alternative to surgery in the treatment of lumbar sciatic pain supported by an intradiscal hernia.[68] Following local anaesthesia, 12 ml of a mixture of O_2 and O_3, the former at a concentration of 20-30 mg/ml, was injected into the disk with 18-20 G needles under computer tomography (CT) or fluoroscopic guidance. This treatment was repeated two or three more times at intervals of 3, 15 or 30 days. Following each treatment, CT examinations were conducted and these revealed 82% positive results (36% excellent, 46% good), whilst there were no major changes between pre- and post-treatment CT in the remaining (18% of) cases. However, clinical examinations gave 68% positive results (40% excellent, 28% good) and 32% negative results (10% of patients underwent surgery and 22 were taken under medical treatment without surgery). In view of its ease of execution and non-invasiveness, O_3 therapy was found to permit the successful out-patient treatment of lumbar sciatic pain. Moreover, the lack of major complications and the promising results obtained compared favourably with those obtained from other methods such as chemonucleolysis, percutaneous automated discectomy, microsurgery and conventional surgery. Hence, O_3 therapy can be considered a useful treatment for lumbar sciatic pain, and can, in principle, offer a valid alternative to surgery in many cases.

Potential applications of ozone treatment in dentistry

Ozone is approximately 10 times more soluble in water than oxygen. Mixed into pyrogen-free water, the half-life of O_3 is nine to ten hours (at pH 7 and 20°C), and at 0°C this value is doubled. Ozonated water was found to be effective in dental surgery where it is reported to promote hemostasis, enhance local oxygen supply and inhibit bacterial proliferation. Therefore, ozone can be applied during dental surgery, or following tooth extraction processes.[65,69]

A denture cleaner using O_3 bubbles (O_3 concentration approximately 10 ppm) was considered as clinically appropriate in view of its strong disinfecting and deodorising power, and high biological safety.[70] The effectiveness of this cleaner against *Candida albicans* was investigated and levels of this microbe were found to decrease to about 1/10 of their initial value after 30 min., and to $1/10^3$ after a 60 min. period of exposure.

Subsequently, Suzuki *et al.,*[71] examined the influence of ozone on the surface of removable partial denture (RPD) alloys to determine its usefulness as a cleaning method for RPDs. The researchers reported that ozone treatment caused a slight change in the Au-Cu-Ag-Pd alloy in terms of reflectance. However, the changes were significantly less than those caused by acid-electrolyzed water and one of the commercial denture cleaners.

Safety of ozone

Ozone is often found in ambient air at levels exceeding the National Air Quality Standard of 0.12 ppm averaged over a period of 1 hr. Many detailed reviews of studies regarding ozone health effects on humans and animals have been published.[72]

The main purposes of these studies were to identify the biological molecules that react with ozone as it crosses the air/pulmonary tissues barrier and the products formed in these reactions. Ozone is so reactive that it cannot penetrate far into the air-tissue boundary before it reacts.[73] Therefore, the principal targets for the reaction of ozone probably lie in the fluid layer covering the internal surfaces of

the lung. The lung lining fluid layer is a patchy and highly dynamic material consisting lipids, proteins, and antioxidants such as ascorbate, glutathione and uric acid.

Ozone is very reactive towards unsaturated lipids, certain amino acid residues in proteins and many antioxidants.[74] Studies concerning the reactions of ozone with lipids have identified the lipid ozonation products that can relay the toxic effects of ozone to deeper tissue strata where ozone cannot reach.[73] Similarly, studies regarding the reactions of ozone with proteins and amino acids and their compounds indicate the types of oxidative damage that can be expected when proteins undergo ozonation.[42]

Ozone is a major component of environmental photochemical smog and can exert toxic effects on erythrocytes, the lungs and other organs after prolonged exposure.[75] However, there is evidence that Nature produces and uses this reactive oxygen species, together with nitric oxide[76] for bactericidal and virucidal purposes, and possibly for killing infected and neoplastic cells.

Bocci[77] reported that in autohaemotherapy, O_3 dissolves in water more abundantly than oxygen and decomposes rapidly. Therefore, when the gas mixture composed of 95% oxygen and of no more than 5% ozone, is mixed with blood *ex-vivo* for a short time before reinfusion, there was not any genetic damage in lymphocytes.

Bocci[78] suggested that treating human blood with low ozone concentrations for the management of vascular disorders, chronic viral and autoimmune diseases can actually activate cells of the immune system and exert beneficial effects. However, it should be noted that ozone concentrations and the time of exposure to this agent should be considered. In principle, the potential toxicity of O_3 should not prevent its use as a therapeutic agent. At the correct dose, ozone can be useful as a therapeutic agent.

Indeed, humans are continually exposed to ozone during their daily lives, a phenomenon that raises doubts about the deleterious health effects of ozone. Occupational exposure to O_3 includes electric arc welding, mercury vapour lamps, laser printers, some office photocopying equipment, X-ray generators

and other high voltage electrical equipment. Recently, Brown[79] found that mall emissions of nitrogen dioxide, ozone and formaldehyde were generated by a dry-process photocopier in a controlled room dynamic environmental chamber. Subsequently, the emission of ozone was measured from one photocopier and four laser printers to assess the impact of office equipment on the quality of indoor air. The laser printers equipped with traditional technology emitted significant amounts of ozone and formaldehyde.[80]

In maintenance, construction, and cleaning departments, high exposures to asbestos, chromium compounds, copper, nitrogen dioxide, ozone, styrene, sulfur dioxide, trichloroethylene, and welding fumes have been observed.[81] Interestingly, artists are also susceptible to the occupational exposure to ozone, cadmium, and molybdenum in their daily lives.[82]

Ozone has been used in the US food processing industry. Indeed, Moore *et al.,*[83] indicated that low levels of ozone has a significant bactericidal effect on the micro-organisms that are responsible for causing common foodborne illnesses and infections. They concluded that the efficacy of O_3 coupled with its relatively low running costs, known deodorising properties, lack of environmentally sensitive residues, and its ability to kill resistant bacteria should lead to an increase in the use of ozone as a disinfectant.

It has been estimated that over ten million people (primarily in Germany, Russia and Cuba) have been given bio-oxidative therapies over the past seventy years to treat over fifty different diseases. They include heart and blood vessel diseases, lung diseases, infectious diseases and immune-related disorders. For many years ozone had a very limited application in buildings for the elimination of micro-organisms. It is now accepted as a good odour eliminator in bars, restaurants, kitchens, homes, etc. The Occupational Safety and Health Administration (OSHA) has established levels of 0.10 ppm of ozone for indoor air and 0.12 ppm for ambient air.

Potential applications of ozone to the treatment of primary root carious lesions (PRCLs)

Recent investigations conducted by the authors have demonstrated that exposure of carious dentine specimens to O_3 exposure produced by a novel ozone delivery system (HealOzone CurOzone, USA) for periods of either 10 or 20 s substantially reduced the levels of pathogenic micro-organisms in these samples. The following will be part of these studies.[84]

Use of ozone on the management of primary root carious lesions (PRCLs) *in-vitro*

The ozone delivery system

The ozone delivery system is a portable apparatus (Figure 4). It includes a source of oxidizing gas and a dental handpiece for delivering the gas/water to the target tooth. A cup attached to the handpiece is provided for receiving the gas and exposing a selected area of the tooth to the gas. The tightly fitting cup includes a resilient edge for sealing the edge of the cup against the selected area on the tooth to prevent escape of gas (Figure 5).

Figure 5. Ozone delivery system

Figure 6. Handpiece with a cup

Study design 1.

40 freshly extracted teeth with soft PRCLs requiring restorations were collected from the Department of Oral and Maxillofacial Surgery at St. Bartholomew's and the Royal London School of Medicine and Dentistry. Immediately after extraction, these teeth were randomly divided into two groups to test the anti-microbial effect on PRCLs from exposure to ozonated water for either 10 or 20 s. Plaque was removed using a hand held standard fine nylon fibre sterile toothbrush with water as a lubricant. Each tooth was dried using dry sterile cotton wool rolls and a dental 3 in 1 air syringe. The excavator blade was used to traverse the lesion in line with long axis of the tooth across the maximum gingival/occlusal dimension. Half of each lesion was removed using a sterile excavator. Following samplings, water without ozone from the ozone generator was applied to the samples in the control group. Subsequently, the remaining lesion was exposed to the ozonised water for a period of either 10 or 20 s at room temperature (23°C) and a further sample was taken. Each sample was immediately put into a preweighed sterile vial and weighed. 1 ml of fastidious anaerobe broth (FAB, Lab M Ltd., Bury, Lancs, UK) with sterile glass beads (3.5 - 4.5 mm in diameter, BDH, Poole, Dorset, UK) was added to these vials and vortexed for 30 s to facilitate the extraction of any micro-organisms from carious dentine and disperse any aggregates. Dilutions were performed by transferring 1 ml of the resulting suspensions into 9 ml of FAB and this process was repeated in 10 fold dilution to 10^4. After decimal dilution with FAB, 100 μl aliquots (for both test and control groups) were spread on fastidious anaerobe agar (FAA, Lab M, Bury, Lancs, UK) supplemented with 5% (v/v) horse blood and placed in an anaerobic chamber at 37°C for four days. The total number of colony forming units (cfu) was calculated.

Study design 2.

Test micro-organisms

Streptococcus mutans (NCTC 10449) and *Streptococcus sobrinus* (TH 21) were maintained by subculturing on 5% blood agar (Oxoid, Basingstoke, UK) every 7 days. Cultures were grown anaerobically for 16 h at 37°C in 5 ml Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md., USA).

Preparation of saliva-coated glass beads

5 ml of unstimulated human saliva from one donor was collected into a sterile container for each experiment. 1 ml of saliva was clarified by centrifugation for 2 min. The saliva supernatant was pipetted into a sterile universal bottle and filtered using 0.45 μm and 0.2 μm filters. Three glass beads (3.5 - 4.5 mm in diameter, BDH, Poole, Dorset, UK) were put into a sterile bijou bottle. Immediately, 0.5 ml-filtered saliva was added and left for 5 min.

Test procedure

40 sterile saliva-coated glass beads were randomly divided into two groups (test and control) for *S. mutans* and *S. sobrinus*. Each glass bead was put into a sterile bijou bottle with 3 ml of Todd-Hewitt broth for control and test groups and agitated for 2 s. Each bijou was inoculated with either *S. mutans* or *S. sobrinus* and inoculated anaerobically overnight. The glass beads were then washed with 2 ml of PBS and transferred to sterile bijoux. Immediately, ozone gas was applied for 10 s to each glass bead in the test groups for either *S. mutans* or *S. sobrinus* at room temperature (23°C) while shaking the bijoux to deliver the ozone gas to all surfaces of the test glass beads, whilst control glass beads for each micro-organism were left in the sterile bijoux and shaken for 10 s. Subsequently, these glass beads (control and test groups) were placed in 3 ml of Todd-Hewitt broth with six more sterile glass beads and vortexed for 30 s. Samples were serially diluted with FAB up to 10^8, and 100 μl aliquots were spread on blood agar plates supplemented with 5% (v/v) horse blood and placed in an anaerobic chamber at 37°C for two days. The number of each colony type was counted and calculated.

Results

Indeed, the number of colony forming units (cfu + 1) were reduced to < 1% of their control (untreated) values at both dose levels applied (Table 1) and these data indicate that O_3 successfully penetrates into the lesion and kills the great majority of micro-organisms therein, presumably via a mechanism involving the rupture of their membranes. The means ± SE of test and control groups for both micro-organisms are shown in Table 2. There was a significant (p<0.0001) reduction in ozone treated samples compared with the control samples for both micro-organisms.

Table 1. Mean ± SE $\log_{10}$ (cfu + 1) and $\log_{10}$ (cfu + 1)/mg before and after ozone application for either 10 or 20 s

	10 s	**20 s**
Groups	**$\log_{10}$ (cfu + 1)/mg**	**$\log_{10}$ (cfu + 1)/mg**
Control Samples	8.99 ± 0.39	9.19 ± 0.36
Test Samples	6.79 ± 0.39	6.31 ± 0.12

Table 2. Mean ± SE $\log_{10}$ (cfu + 1) before and after ozone application for *S. mutans* and *S. sobrinus*

Groups	***S. mutans***	***S. sobrinus***
Control	3.92 ± 0.07	4.61 ± 0.13
Test	1.01 ± 0.27	1.09 ± 0.36

Ozone exposure to PRCLs consistently achieved microbial reduction in the test groups regardless of the application time. The rapid inactivation of micro-organisms is one of ozone's outstanding characteristics. Presumably, ozone dissipates quickly in water[85] and kills micro-organisms via a mechanism involving the rupture of their membranes in the lesions. In this study, the mechanism of killing of ozone on PRCLs was not investigated. However, Yamayoshi and Tatsumi[86] demonstrated that ozone was a strong oxidiser to cell walls and cytoplasmic membranes of bacteria. Such an ozone solution could be used to disinfect medical instruments and similar equipment.

^{1}H NMR analyses of products arising from the interaction of ozone with PRCLs components

Nuclear magnetic resonance (NMR) spectroscopy uses energy from the radiofrequency region of the electromagnetic spectrum to detect changes in the alignment of nuclear magnets during exposure to a powerful external magnetic field. The absorption frequency is dependent on the magnetic (and,

therefore, chemical) environment of nuclei. Moreover, the appearance (multiplicity) of a resonance in the ^{1}H (proton) NMR spectrum of a particular chemical component is influenced by neighbouring hydrogen nuclei in a well-characterised way. Hence, much useful information about the molecules present in a biological sample can be obtained from NMR spectroscopic techniques.[87]

The recent development of high field NMR spectrometers with increased resolution, dynamic range and sensitivity has permitted the rapid and simultaneous determination of a wide range of components present in biological samples or alternative complex multicomponent systems. The technique is non-invasive since it involves only minimal sample preparation prior to analysis. [88]

The authors have also recently performed a multicomponent evaluation of O_3-mediated oxidative consumption of salivary and PRCL biomolecules using high resolution proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy.[89,90] The results acquired from these investigations revealed that treatment with O_3 gave rise to 1) the oxidative decarboxylation of the electron-donor pyruvate to acetate and CO_2, 2) oxidation of the volatile sulphur compound precursor methionine to its corresponding sulphoxide, and 3) the oxidative consumption of polyunsaturated fatty acids, a reaction presumably giving rise to the generation of fatty acid ozonides which, in turn, degrade to a variety of secondary oxidation products (Figure 1-3). Moreover, evidence for the O_3-induced oxidation of 3-D-hydroxybutyrate was also obtained. Clearly, the technique provides much useful analytical data regarding the fate of O_3 in human PRCLs and saliva, information which may be of relevance to its potential therapeutic actions *in-vivo*.

Use of ozone on the management of PRCLs *in-vivo*

The data with which this whole work is concerned has been obtained from a total of 26 PRCLs in 26 patients. Each PRCL was classified subjectively in terms of colour, texture, cavitation, size, hardness and severity. These clinical criteria were as follows:

- **Colour.** A shade guide was employed with typical examples of ‘yellow’, ‘light brown’ ‘dark brown’ and ‘black’ lesions. This shade guide was used as a reference in classifying the lesions.

- **Texture.** Each lesion was classified as either rough or smooth by passing a dental explorer over its surface.
- **Cavitation.** An estimate of the depth of the PRCL was made by recording the greatest distance between the existing surface of the lesion and what was judged to have been the original root surface.
- **Size.** A standard periodontal probe marked at 1-mm intervals was used to determine the dimensions of each lesion. The maximum occluso-gingival and mesio-distal or labio/buccal-lingual/palatal dimensions were assessed as well as the minimum distance from the gingival margin of the lesion and the crest of the gingivae itself. The product of the first two figures was used as an indicator of its overall size.
- **Hardness.** Soft PRCLs permitted a sharp probe to penetrate the surface at 100 g pressure with ease and there is definite resistance to its withdrawal, whilst leathery root carious lesions permitted a sharp probe to penetrate the surface at 100 g pressure and there is a slight resistance to its withdrawal. Hard PRCLs were comparable to the surrounding sound root dentine.
- **Severity index.** All PRCLs were classified according to the severity index as follows:

  0 All 'Hard' lesions

  1 'Leathery' lesions, which were considered to be small, easily cleansable and approaching a 'Hard' texture

  2 'Leathery' lesions, which were judged to be shallow and where the surface of the exposed sound dentine could be easily maintained plaque free

  3 'Leathery' lesions judged to be in surfaces, which were difficult to maintain plaque free and large, cavitated 'leathery' lesions where pulpal integrity was judged to be at risk.

  4 All 'Soft' lesions

Overlying plaque was then removed using a hand held standard fine nylon fibre sterile toothbrush with water as a lubricant. Each tooth was dried using dry sterile cotton wool rolls and a dental 3 in 1-air syringe. The excavator blade was used to traverse the lesion in line with long axis of the tooth across the maximum gingival/occlusal dimension. Half of each lesion was removed using a sterile excavator.

Subsequently, the remaining lesion was exposed to the ozone gas for a period of either 10 or 20 s at room temperature (23°C) and a further sample was taken.

Microbiological analysis

Each sample was put into a preweighed sterile vial and weighed. 1 ml of fastidious anaerobe broth (FAB, Lab M Ltd., Bury, Lancs, UK) with sterile glass beads (3.5 - 4.5 mm in diameter, BDH, Poole, Dorset, UK) was added to these vials and vortexed for 30 s to facilitate the extraction of any micro-organisms from carious dentine and disperse any aggregates. Dilutions were performed by transferring 1 ml of the resulting suspensions into 9 ml of FAB. This process was repeated in 10-fold dilution to 10^4. After decimal dilution with FAB, 100 μl aliquots (for both test and control groups) were spread on fastidious anaerobe agar (FAA, Lab M, Bury, Lancs, UK) supplemented with 5% (v/v) horse blood and placed in an anaerobic chamber at 37°C for four days. The total number of colony forming units (cfu) was calculated.

Statistical analyses

Microbiological counts from test and control groups for each study were transformed as $\log_{10}$ (colony forming units + 1) prior to statistical analyses in order to normalise their distributions. Statistical analyses of the data were obtained by paired Student t-tests to determine differences between test and control groups, with the threshold of significance chosen at 0.05. Means and standard errors were also recorded.

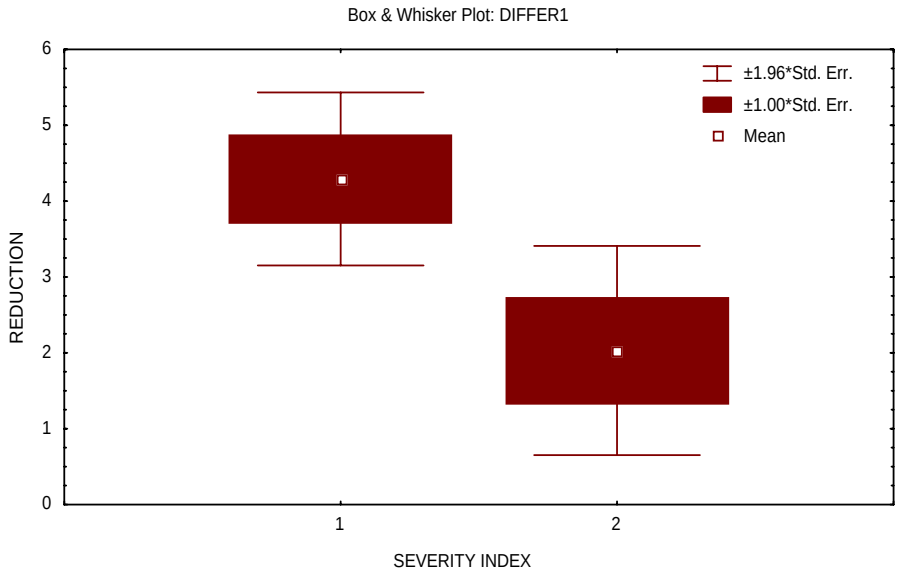
Results

Results revealed that there was a significant (p<0.00001) difference between the control and test samples in $\log_{10}$ (cfu + 1). Means and SE for the $\log_{10}$ (cfu + 1) data are shown in Table 3 (p<0.0001). There were also significant correlation for reduction and severity, size, distance from gingival margin and cavitation (Figure 6).

Table 3. Mean ± SE log_{10} (cfu + 1) and log_{10} (cfu + 1)/mg before and after ozone application for a period of 10 s (p < 00001)

	10 s	
Groups	**log_{10} (cfu + 1)**	**log_{10} (cfu + 1)/mg**
Control Samples	6.84 ± 0.27	7.69 ± 0.32
Ozonated Samples	3.54 ± 0.51	3.64 ± 0.69

Figure 6. Reduction of log_{10} (cfu + 1) according to the severity of PRCLs



Conclusion

In this study, ozone exposure for a period of 10 s substantially reduced micro-organisms in the PRCLs *in-vivo*. Only 10 seconds of ozone application is an effective, rapid, simple and conservative method to kill micro-organisms in PRCLs. This proposed novel treatment regime may therefore be considered as an effective alternative to conventional "drilling and filling" for the management of root caries.

Ozone detection (ppm) around the cup using a ozone analyser after 10 of ozone application *in-vivo*

The quantification of ozone during the *in-vivo* study was measured using a ozone analyser (Enviro Technology, UK).

Study design

8 patients for whom microbiological tests were carried out on their carious samples were randomly selected. Plaque was removed using a hand held standard fine nylon fibre sterile toothbrush with water as a lubricant. Each tooth was dried using dry sterile cotton wool rolls and a dental 3 in 1 air syringe. During the cross-sectional study, ozone gas was delivered into a cup closely adapted to each PRCL in the extracted teeth for a period of 10 s after the lesion was dried for 3 s with a standard 3 in 1 dental syringe. Next a reductant filled the cup and a suction system removed any possible remaining ozone whilst the cup was still adapted to the PRCL. The suction system passed the gas from the delivery system through manganese. The tip of the ozone analyser's sensor was held within 2 mm of the edge of the cup throughout and after the procedure, during the application of ozone for 10 s at room temperature (23°C) and maximum detectable ozone level was measured using this ozone analyser. The model 450 UV Photometric Ozone Analyser detects low level-ppm ozone for safety applications. The lowest detectable level is 0.003 ppm with a flow rate 1 to 2.5 l/min. The repeatability of the readings is 0.1% of full scale.

Results

The maximum ozone detectable level (ppm) around the cup from lesions for a period of 10 s (Table 4) ozone application during the treatment of root carious lesions were as follows:

Table 4. Maximum ozone detectable level (ppm) after a 10 s of ozone application

Teeth types	Sites	Ozone detection (10 s)
Upper left incisor	Mesial	0.066
Upper right 1. premolar	Buccal	0.001

Upper right canine	Distal	0.002
Upper right 1. molar	Buccal	0.006
Upper left 2. premolar	Buccal	0.076
Lower right 2. premolar	Mesial	0.058
Upper right lateral	Distal	0.001

In conclusion, the use of a cup is a safe way of delivering ozone when ozone was applied for a period of 10 s on the root carious lesions.

CONCLUSION

In principle, the potential toxicological actions of ozone should not prevent its use as a therapeutic agent. Indeed, at the correct dose, O_3 can be a useful therapeutic agent. The modern development of ozone's application to Medicine began in the 1950s in Europe and gradually spread throughout this continent and then to Australia, Israel, Cuba, Brazil and Columbia. In World War I, O_3 was used medically to treat wounds and other infections. Over 5,000 physicians world wide routinely use O_3 in their medical practice. Research concerning the anti-microbial efficacy of ozone has continued over the last twenty years and has conclusively shown the ability of both gaseous and dissolved ozone to extinguish a wide range of bacteria, bacterial spores and viruses. However, although the oxidative modification of essential biomolecules are primarily responsible for the biocidal actions of ozone, cellular DNA may also be damaged when concentrations of this oxidant at levels greater than those permitted by the European Union are employed.

Ozonation is recognized as an environmentally safe and effective process for the treatment of industrial effluents, drinking water and sewage. Ozonation has the potential to reduce odours in stored livestock waste by chemically oxidising malodorous metabolites produced during anaerobic storage. Ozone is a very strong oxidant that attacks the cell wall and the cytoplasmic membrane of the cell, thereby inactivating the organism. Therefore, ozone has the potential to reduce the number of micro-organisms present in waste, and thereby control the rate of production of these malodorous metabolites. Previous

studies indicated that ozone preferentially oxidises odour-producing metabolites, reduces odour intensity and inactivates micro-organisms.[91]

Ozone exposure for either 10 or 20 s substantially reduced micro-organisms in the PRCLs *in-vitro* and *in-vivo*. Ozone application for a period of 10 s was also capable of reducing the numbers of *S. mutans* and *S. sobrinus* on saliva-coated glass beads *in-vitro*. High field ^{1}H NMR spectroscopy provided much useful analytical data regarding the fate of ozone in human primary root carious lesions, information which may be of relevance to its potential therapeutic actions *in-vivo*. This proposed treatment regime with the employment of an ozone delivery system to kill micro-organisms associated with PRCLs may therefore be considered to be an effective alternative to conventional "drilling and filling" for the management of root caries.

We believe that O_3 deserves a place in the management of health and disease, and an increased awareness of the molecular mechanisms underlying the therapeutic and toxicological properties should establish suitable applications and dosage limitations regarding its future clinical use. Indeed, various therapeutic regimens have successfully been tested *ex-vivo* and *in-vivo*. However, further clinical investigations are warranted to determine O_3's efficacy and tolerance in human beings.

To date, there is no universally accepted management strategy with a pharmaceutical approach to treat root caries. Application of ozone is very promising, since there is growing evidence that ozone can be employed as a therapeutic agent in both dentistry and medicine.

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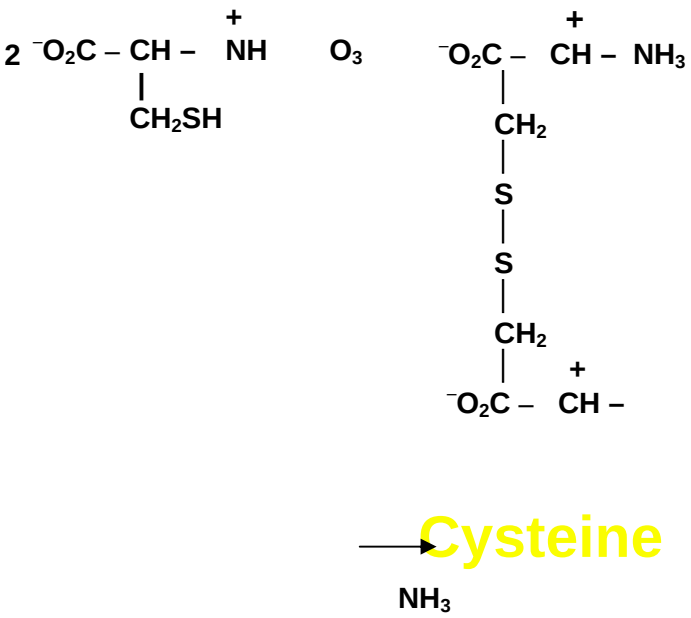
Figure 1.



Cysteine + O_3 → Cystine

Figure 2.

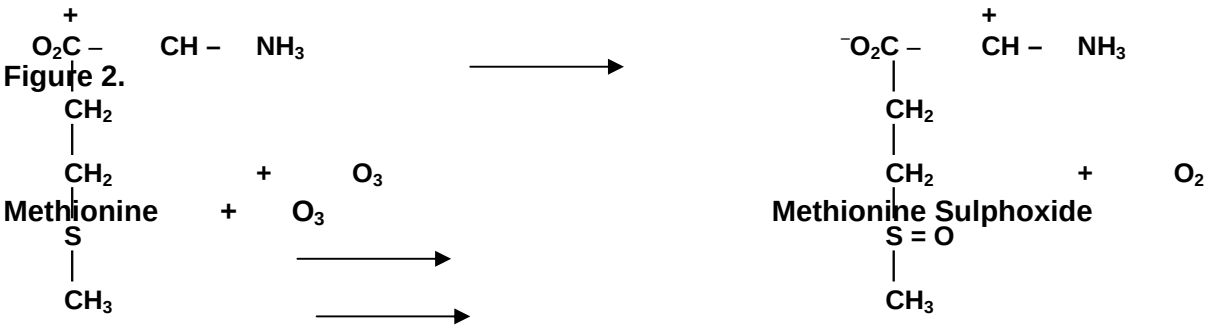


Figure 3.

$CH_3CO.CO_2^-$ + O_3 → $CH_3CO_2^-$ + CO_2 + O_2

Pyruvate + O_3 → Acetate

Ozone effects the oxidative decarboxylation of pyruvate, generating acetate and CO_2 as products