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Application of Ozone Therapy in Patients with Knee Osteoarthritis

José Luis Calunga,[1] Silvia Menéndez,[1] Rodolfo León,[2] Soulien Chang,[3] Dailen Guanche,[1] Alberto Balbín,[4] José Zayas,[4] and Pedro García[4]

[1]Ozone International Clinic, Ozone Research Center, National Center for Scientific Research, Havana City, Cuba
[2]A.A. Aballí Pediatric Teaching Hospital, Havana City, Cuba
[3]Pharmaceutical and Food Institute, Havana University, Havana City, Cuba
[4]"Dr Fructuoso Rodríguez" Orthopedic Hospital, Havana City, Cuba

Osteoarthritis is a common degenerative joint disease. Taking into account the ozone (O_3) effects in cellular redox balance and upon biomarkers of inflammation, the aim of this study was to evaluate the action of ozone therapy in oxidative stress parameters in synovial fluid of patients suffering of knee osteoarthritis and their clinical evolution. In 42 patients, O_3 was administered rectally and by intra-articular applications. Synovial fluid was extracted for the measurement of parameters associated with oxidative stress. Also, evaluation of the joint capacity, pain, and ultrasound imaging were performed. Combined ozone therapy produced an intra-articular redox balance and a significant reduction of pain.

Keywords Ozone Therapy, Osteoarthritis, Synovial Fluids, Pain, Oxidative Stress

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and is defined as a degenerative joint disease that causes pain, stiffness, swelling and loss of motion in the joints which is aggravated by prolonged activity (Martin and Buckwalter 2002; Poole 1999). It is a process of progressive deterioration of articular cartilage (it breaks down and becomes thin) and formation of new bone (osteophyte) at the joint surface.

OA is becoming increasingly prevalent worldwide because of the combination of an aging population and growing levels of obesity. It mainly affects people over the age of 45, but it can develop in younger people. It is estimated that 40 million Americans and 70 to 90% of people older than 75 years are affected by OA. Although symptoms of OA occur earlier in women and appear to be more severe, the prevalence among men and women is equal (Hinton et al. 2002).

Many studies have identified molecular characteristics of ageing in OA cartilage or chondrocytes, which may contribute to the onset of OA, including telomere genomic instability, formation of advanced glycation end products, increased apoptosis and senescence (Carrington 2005). Such changes could be related to the increased levels of oxidative stress that occur in OA, leading to cells unable to respond effectively to normal loading regimens and potentially contributes to disease onset (Henrotin et al. 2005; Ostalowska et al. 2006; Plumb and Aspden 2005). Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and cell damage. The body's defense mechanisms would play an important role in the formation of anti-oxidants and try to minimize the damage, adapting itself to the above stressful situation. Anti-oxidants are able to scavenge and suppress the formation of free radicals or oppose their actions (Cotgreave et al. 1988; Sie 1991).

Oxidative phosphorylation is a major source of reactive oxygen species (ROS); however, chondrocytes also express NADPH oxidase and nitric oxide (NO) synthase family members together with various oxygenases, which principally generate the ROS, NO and the superoxide anion ($O_2^{\bullet -}$) (Henrotin et al. 2005). These ROS generate derivatives including hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and

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Address correspondence to Silvia Menéndez, Ozone International Clinic, Ozone Research Center, National Center for Scientific Research, P.O. Box 6414, Havana City, Cuba. E-mail: silviamenendez@infomed.sld.cu

hydroxyl radicals ($OH^{\bullet}$). Lipid peroxidation products and nitrotyrosine residues have been observed in aged and OA cartilage (Loeser et al. 2002; Tiku et al. 2000). ROS can cause cartilage degradation directly by cleaving collagen and activating matrix metalloproteinases (MMP), a family of enzymes that have a key role in cartilage destruction in OA (Klämfeldt and Marklund 1987; Rowan and Young 2007). To prevent an accumulation of ROS-mediated damage chondrocytes produce a number of anti-oxidant enzymes, including the superoxide dismutase (SOD), catalase, and glutathione peroxidase (Henrotin et al. 2005).

OA is becoming increasingly prevalent worldwide because of the combination of an aging population and growing levels of obesity. Despite the increasing number of OA patients, treatments to manage this disease are limited in controlling pain and improving function and quality of life while limiting adverse events (Hinton et al. 2002). Effective therapies to regenerate damaged cartilage or to slow its degeneration have not been developed. Current treatments such as: rehabilitation, exercise, modification of activities of daily living, pharmacotherapy, alternative medicine or surgery are focused on symptomatic relief but they lack efficacy to control the progression of this disease, which is a leading cause of disability (Alcaraz et al. 2010). Therefore, the development of effective disease-modifying drugs is urgently needed.

Taking into account different biological effects of ozone, such as: the stimulation of the anti-oxidant defense system counteracting the intracellular pro-oxidant status, improvement in the oxygen deliver to tissues, the immunological modulation, among others (Ajamieh et al. 2002, 2003, 2004, 2005; Al-Dalain et al. 2001; Bocci 2002, 2006; Borrego et al. 2004; Candelario-Jalil et al. 2001; Hernández et al. 2005; León et al. 1988, 2008; Martínez et al. 2005; Menéndez et al. 2008; Valacchi and Bocci 2000; Zamora et al. 2005), the aim of this study was to evaluate the action of ozone therapy in oxidative stress parameters in synovial fluid of patients suffering of knee osteoarthritis and their clinical evolution.

PATIENTS AND METHODS

This controlled clinical trial was approved by an institutional review board (Scientific and Ethics Committees of the Institutions) in accordance with the principle of the Declaration of Helsinki (1997). All patients provided informed consent after receiving appropriate information about the study (characteristics, benefits, and possible side effects). Before enrolling, all participants attended a training program designed to familiarize them with the study objectives and treatment plans. A complete clinical and personal history of all the subjects involved in the study was recorded.

Inclusion criteria were: Adult patients (45–65 years old), of both sexes and different ethnic origins that were clinically and radiologically diagnosed as patients with knee osteoarthritis. Exclusion criteria were: patients that present severe hypertension, septic conditions, diabetic complications, liver/hematological/ cardiovascular diseases, hypersensibility to the medication that will be used, inability to cooperate with the requirements of the study or recent history of alcohol or drug abuse. Subjects with supplementing anti-oxidant vitamins or receiving anti-inflammatory drugs during the last 3 months were excluded. Forty-two patients were involved in this study. There were two study groups: (1) Control: 10 healthy age- and sex-matched subjects, and (2) Ozone: 42 patients with clinically diagnosed osteoarthritis.

Treatment

Ozone was administered by rectal way (20 sessions, daily, Monday to Friday) at scaling doses, using ozone concentrations between 25 and 40 mg/L and volumes of 100 to 200 mL and by intra-articular (15 sessions, twice per week) applications with an ozone concentration of 20 mg/L and volumes between 5 and 10 mL.

Evaluation Criteria

The evaluation criteria were based in the oxidant-antioxidant status and in the clinical evolution of patients with knee osteoarthritis. For the evaluation of the oxidant-antioxidant status, 21 patients treated with ozone and a sample of 10 healthy volunteers (control group), 1–2 mL of synovial fluid was extracted, at the beginning and at the end of the study, for the measurement of different parameters related to oxidative stress, as: superoxide dismutase (SOD), catalase (CAT), CAT/SOD ratio, reduced glutathione (GSH), malondialdehyde (MDA), advanced oxidation protein products (AOPP), total hydroperoxides (ROOH) and peroxidation potential (PP).

For the clinical evolution, the pain, the evaluation of the joint capacity and the imaging studies performed in all patients were considered, at the beginning and at the end of the study.

- For the pain measurement, the visual analog scale (VAS) was used (10-maximum of pain and 0-no pain).
- For the evaluation of joint capacity, four aspects were taken into account: (1) Articular movements (movements of flexion and complete extension), classifying the patients into 3 categories, according to the movement limitation: null (between 0 and 10%), partial (between 11 and 65 %) and total (between 65 and 100%); (2) Presence of clover sign: In the knee appear 3 bulky zones in form of a clover due to the presence of adiposis; (3) Grade of tumefaction (swelling): severe, moderate or slight; and, (4) Presence of palpable crepitations.
- In respect to the imaging studies, they were made by means of the ultrasound diagnose, at

the beginning and one month after the end of the treatment. The radiologist classified the patient synovitis into three levels: slight (scarce quantity of synovial fluid localized in the suprapatellar region), moderate (when the synovial fluid covers the articular capsule in its external and internal parts) and severe (when the synovial fluid covers all the articular spaces with swelling of the articular capsule that extends to the popliteal fossa and prepatellar cavity).

Biochemical Determinations

All biochemical parameters were determined by spectrophotometric methods using an Ultrospect Plus Spectrophotometer from Pharmacia LKB, Sweden. CAT activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 sec intervals for 1 min (Boehringer 1987). SOD was measured using kits supplied by Randox Laboratories Ltd., Ireland. Concentrations of MDA were analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA). In the assay, the production of a stable chromophore, after 40 min of incubation at 45 °C, was measured at 586 nm. For standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma, St. Louis, MO, USA) were employed and assayed under identical conditions (Esterbauer and Cheeseman, 1990).

Quantification of ROOH was measured by Bioxytech H2O2–560 kit (Oxis International Inc., Portland, OR, USA) using xylenol orange to form a stable colored complex, which can be measured at 560 nm. Total protein concentration was determined by the method of Bradford with bovine serum albumin as standard (Bradford 1976). PP was measured by inducing lipid peroxidation by adding Cu^{+} (2 mM) to serum (incubated for 24 h at 37 °C). The difference between malondialdehyde levels, measured at 0 and 24 h after induction, for each sample, was calculated (Özdermirler et al. 1995). After precipitation of thiol proteins using trichloroacetic acid 10%, GSH was measured according to the method of Sedlak and Lindsay (1968), with Ellman's reagent [5′5 dithiobis (2-nitrobenzoic acid) 10^{-2}M (Sigma, St. Louis, MO, USA). AOPP was measured as the oxidation of iodide anion to diatomic iodine by advanced oxidation protein products (Witko-Sarsat et al. 1998).

Statistical Analysis

The OUTLIERS preliminary test for detection of error values was initially applied. Afterwards, data were analyzed by one-way analysis of variance (ANOVA) followed by a homogeneity variance test (Bartlett-Box). In addition, the Wilcoxon rank-sum test (Dalle-Donne 2006) and Student's *t*-tests were performed. The data were expressed as mean ± SD (standard deviation). The level of statistical significance used was $p < 0.05$.

RESULTS AND DISCUSSION

Reactive oxygen species (ROS) are involved in both bone and cartilage physiology and play an important role in the pathogenesis of osteoarthritis (Baur et al. 2011; Jokić et al. 2010). ROS produced by abnormal chondrocyte metabolism exceeds the physiological buffering capacity and results in oxidative stress, causing cartilage degradation directly by cleaving collagen and activating matrix metalloproteinases (Klämfeldt and Marklund 1987). The excessive production of ROS can damage proteins, lipids, nucleic acids, and matrix components. They also serve as important intracellular signaling molecules that amplify the inflammatory response (Sutipornpalangkul et al. 2009a). Similar results of elevated ROS levels have been reported in patients with rheumatic disease (Mezes and Bartosiewicz 1983).

In this study, patients with knee osteoarthritis presented a remarkable oxidative stress in synovial fluid (high values of AOPP and ROOH, as well as consumption of GSH) with negative effects upon joint recovery. The mean ± SD of the redox balance parameters, measured in synovial fluid in both groups, is shown in Table 1.

AOPP is an indicator of protein oxidative damage and precursor of advanced glycation end products (AGEs). Rise in AOPP and ROOH could be due to the increased generation of ROS and consequent excessive oxidative damage generated in these patients, affecting the functions of bonding, transportation and protein structure (García Villanova 1994). These ROS, in turn, can oxidize many other important biomolecules, including membrane lipids. It was demonstrated that at the end of the ozone combined treatment, AOPP and ROOH levels decreased significantly in respect to the initial values, obtaining for ROOH, similar figures to that of the control group, which suggest the preservation of membrane integrity.

GSH presented significant differences at the end of the treatment, with values more similar to those of the control group. The level of erythrocyte GSH was significantly decreased in patients with osteoarthritis compared to controls, at the beginning of the treatment. At the end of the study, even maintaining a significant low level in respect to the control group, it had increased significantly in respect to its initial one, suggesting an increased defense against oxidant damage in osteoarthritis. The decrease in the level of this nonenzymatic anti-oxidant parameter may be due to the increased turnover for preventing oxidative damage in these patients (Surapaneni and Venkataramana 2007).

SOD activity had increased significantly in synovial fluid of patients with osteoarthritis. Similar results of raised SOD activity had been reported in patients with osteoarthritis and rheumatic diseases (Mezes and Bartosiewicz 1983; Surapaneni and Venkataramana 2007). SOD is an important anti-oxidant enzyme having an antitoxic effect against superoxide anion and to counter the effect of increased oxidative stress. The over-expression of SOD might be an adaptive response, and it results in increased dismutation of superoxide to hydrogen peroxide. However, at the end of the treatment, its

TABLE 1. Results of the Redox Balance Parameters Measured in Synovial Fluid after the Combined Ozone Treatment (Rectal and Intra-articular Applications)

Redox Parameters	Healthy Control (n = 10)	Ozone (n = 21)	
		Before the Treatment	After the Treatment
MDA (μM)	3.35 ± 0.17	2.6 ± 0.9	3.07 ± 0.89
PP (μM)	7.72 ± 1.17	5.4 ± 1.5	6.02 ± 1.70
CAT (U/L/min)	3810 ± 156	3069 ± 289	3668 ± 307
SOD (U/L/min)	4.86 ± 1.35[a]	22. 9 ± 7.5[b]	12.5 ± 3.8[c]
CAT/SOD	3.20[a]	0.22[b]	1.89[c]
AOPP (μM)	2.50 ± 0.70[a]	21.8 ± 6.3[b]	11.3 ± 2.4[c]
ROOH (μM)	33.9 ± 11.5[a]	64.5 ± 15.5[b]	40.7 ± 10.7[a]
GSH (mg/L)	570.2 ± 37.2[a]	48.9 ± 17.0[b]	268.3 ± 44.0[c]

Note: Superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), malondialdehyde (MDA), advanced oxidation protein products (AOPP), total hydroperoxides (ROOH), and peroxidation potential (PP). Different letters indicate significant differences, $p < 0.05$. Different letters indicate significant differences.

level decreased significantly, obtaining values more similar to those of the control group, indicative of a better anti-oxidant balance and therefore, a modulation of redox-controlled signalling pathways. Perhaps this can be an attempt to reduce the expression of collagenases, enzymes that contribute to cartilage degradation. Ostalowska et al. (2006) have also reported increased activities of SOD, glutathione peroxidase and glutathione reductase in synovial fluid of patients with primary and secondary osteoarthritis of the knee joint.

No significant differences were observed in MDA, PP and CAT in respect to the different groups, concentrations were maintained to control levels. In synovial fluid from primary knee osteoarthritis patients with severe cartilage damage, undergoing total knee replacement, in comparison with those in the synovial fluid from injured knee joint patients with intact cartilage undergoing knee arthroscopy, no significant differences in MDA levels were also achieved (Sutipornpalangkul et al. 2009a). Similar results, regarding MDA and CAT levels, were reported by Ostałowska in his knee posttraumatic arthritis patient study (Ostałowska et al. 2007).

The CAT/SOD ratio, which presented significant decreased value at the beginning of the treatment in comparison with the control group, increased significantly its level at the end of the study, with values more similar to those of the control, but with still significant differences. This ratio can be considered as a risk factor in the development of complications. All these suggest a regulation cellular redox balance.

With respect to pain evaluation, the results in the visual analog scale (VAS) after 20 sessions of ozone by rectal insufflation (administered daily) and the 15 sessions of intra-articular ozone applications (twice per week) are shown in Figure 1. At the beginning of the study, the mean value was in 9 according to the VAS; however, at the end of the treatment, it diminished up to 1, demonstrating a significant decrease of pain with the application of the combined ozone treatment.

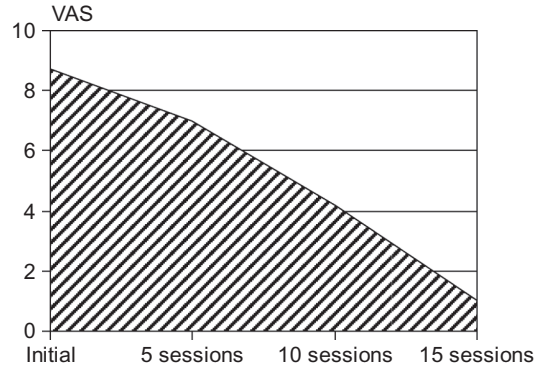

FIGURE 1. Pain evolution according to the Visual Analogous Scale (VAS), after 15 sessions (twice per week) of ozone by intra-articular knee application. Those patients have also received 20 sessions of rectal ozone administration (one daily).

This result was maintained in 80% of cases in 1-year follow-up. It was also demonstrated that ozone administered by rectal application favored the effect of intra-articular ozone injection, improving the clinical symptoms, although this procedure needed more ozone sessions, but is the least invasive and cheapest method.

The results of the evaluation of the joint capacity, according to the limitation of articular movements and grade of tumefaction, at the beginning and at the end of the study, are shown in Table 2.

In respect to limitation of articular movements, all the patients that presented a total limitation at the beginning of the study improved significantly their condition after the ozone treatment, achieving 57% of patients without limitations in their articular movements. Similar results were obtained in respect to grade of tumefaction. At the end of the study, no severe grade of tumefaction was observed, 50% of patients

TABLE 2. Evaluation of the Joint Capacity, According to the Limitation of Articular Movements and Grade of Tumefaction, at the Beginning and at the End of the Study

Evaluation of the Joint Capacity	Beginning of the Study			End of the Study		
Limitation of articular movements (%)	Total	Partial	Null	Total	Partial	Null
	20	75	5	0	43	57
Grade of tumefaction (%)	Severe	Moderate	Slight	Severe	Moderate	Slight
	35	55	10	0	5	45

TABLE 3. Results of the Radiological Study, at the Beginning and One Month after Finishing the Ozone Therapy Treatment

	Beginning of the Study			End of the Study		
Radiological Evaluation	Severe	Moderate	Slight	Severe	Moderate	Slight
Presence of synovitis (%)	40	55	5	0	15	85

presented moderate or slight tumefaction, and in the other 50% of patients, no signs of tumefaction were observed in the articular clinical exam.

The presence of clover sign and crepitations were also improved at the end of ozone therapy. At the beginning of the study, clover sign and crepitations were observed in 77% and 85% of patients, respectively; however, at the end of the treatment, they were seen only in 5%.

The results of the radiological studies performed one month after finishing ozone therapy are shown in Table 3. For all the patients that presented a severe synovitis, it disappeared after the ozone therapy treatment, achieving 85% of patients with only a slight synovitis remaining. A remarkable improvement has been demonstrated in the clinical evolution of patients with knee osteoarthritis. Patients presented less disability, increasing their quality of life.

An understanding of oxidative stress involved in this disease might allow the use of anti-oxidant therapies in the prevention and/or treatment of knee osteoarthritis (Afonso et al. 2007). Ex vivo SOD3 gene transfer or SOD mimetics (e.g., M40403) can reduce the severity of collagen-induced arthritis in models (Cuzzocrea et al. 2005a, 2005b) and recombinant SOD1 can inhibit cartilage damage in hens. A trial of intra-articular injections of bovine SOD1 (Orgotein) has also been carried out in patients with OA with some success; however, the drug was withdrawn, due to adverse side effects (Afonso et al. 2007). As dietary supplements, vitamins have been shown to decrease OA development and increase the expression of anti-oxidant enzymes in an OA model (Afonso et al. 2007; Sutipornpalangkul et al. 2009b).

However, epidemiological studies examining the benefits of anti-oxidants in human OA, especially vitamin E (α-tocopherol), are contradictory (Henrotin et al. 2005). Also, the failure of conventional treatments (analgesics or nonsteroidal anti-inflammatory drugs) to satisfactorily control OA progression, combined with their frequent adverse side effects, justify to recommend the use of ozone therapy in the treatment of patients suffering of knee osteoarthritis. Ozone therapy was able to control the oxidative stress achieving an intra-articular redox balance and clinically, it reduced pain, decreased patient disability and increased their quality of life, without the presence of side effects.

CONCLUSIONS

The results of our study demonstrated higher oxygen-free radical production in patients with knee osteoarthritis, as it has been seen in the increased values of total hydroperoxides and advanced oxidation protein products, as well as a decreased reduced glutathione level, obtained at the beginning of the study, supporting the higher oxidative stress hypothesis in osteoarthritis. This situation can contribute to the complications and progression of the disease. Combined ozone therapy diminished the oxidative stress, achieving an intra-articular redox balance, as well as a significant reduction of pain, with a maintained satisfactory response in 80% of patients in 1-year follow-up. An increase in the quality of life of patients with knee osteoarthritis was observed, without the presence of side effects.

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