Medical ozone prevents inflammatory effects from carrageenan-induced knee joint synovitis in rats through A₁ adenosine receptor, as well as lipid and protein oxidative damages

Gabriel Takon Oru¹, Renate Viebhan-Haensler², Yanet Hernández Matos¹, Daylin Díaz Rodríguez³, Mayté Casanova Orta¹ and Olga Sonia León Fernández¹*

¹Pharmacy and Food Institute, University of Havana, Havana 10 400, Cuba.
²Medical Society for the Use of Ozone in Prevention and Therapy, Iffezheim/Baden-Baden D-76473, Germany.
³Center for Control of Drug and Medical Devices (CECMED), Ministry of Public Health, Havana 10 300, Cuba.

*Corresponding author. E-mail: olga@infomed.sld.cu, Tel: (537) 7 272 77 26

Accepted 22 March, 2018

Medical ozone reduced inflammation, IL-1β, TNF-α mRNA levels and oxidative stress in experimental arthritis. The aim of this study was to investigate whether medical ozone could reduce inflammation through A₁ adenosine receptor, as well as by decreasing oxidative stress in carrageenan-induced knee joint synovitis. Wistar rats were used for the study. Synovitis was induced by 50 µl intra-articular injection of 3% carrageenan. The joint inflammation, the histopathological characterization of knee joint and redox markers in supernatants of spleen homogenates were determined. In order to evaluate the role of A₁ adenosine receptor, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was tested. The results show that medical ozone prevented inflammation, reduced cell infiltration, and protected against oxidative protein damage and lipid peroxidation. DPCPX counteracted the protective effects of ozone. In conclusion, medical ozone protected against the inflammatory response in experimental synovitis. The protective effects of ozone involved A₁ adenosine receptor activity and the control of oxidative injury to biomolecules.

Key words: Ozone, inflammation, adenosine, oxidative stress.

INTRODUCTION

Synovitis has been recognized as one of the most important predisposing factors for subsequent structural damage. The synovium is the primary site of the inflammatory process which, if untreated, results into irreversible damage to the adjacent cartilage and bone (Mclnnes and O’Dell, 2010).

An overproduction of reactive oxygen species (ROS) is associated with synovitis. Studies on rheumatoid arthritis (RA) synovium in vivo have suggested that synovial perfusion is directly influenced by high intra-articular pressures, which are further increased by movement. On the basis of these observations, it has been proposed that intermittent joint loading with ambulation due to locomotion, especially in the setting of an effused joint, enhances local joint hypoxia, which in turn is followed by reoxygenation when the joint is unloaded. Repetitive cycles of hypoxia and reoxygenation, along with oxidants produced by phagocytic cells such as macrophages and neutrophils, lead to produce chronic oxidative stress in the RA synovial microenvironment (Hitchon and El-Gabalawy, 2004). Several groups have demonstrated increased oxidative enzyme activity accompanied by decreased antioxidant levels in RA synovium (Taysi et al., 2002). Studies of RA synovial fluid and tissue have demonstrated oxidative damage to hyaluronic acid, lipid peroxidation products and increased carbonyl groups reflective of oxidation damage to proteins (Hitchon and El-Gabalawy, 2004). Besides, adenosine receptors have
been associated with inflammation and autoimmune diseases. All type of adenosine receptors (A₁, A₂A, A₂B and A₃) are expressed in human synoviocytes (Varani et al., 2010). In particular, increased A₁ adenosine receptor expression has reduced neuroinflammation in experimental allergic encephalomyelitis (Shigeki et al., 2004).

Medical ozone is an ozone/oxygen mixture administered at low concentrations and using controlled number of treatments according to the disease involved. Through an ozone oxidative pre-/postconditioning mechanism (León et al., 1998; León, 2014), it has been possible to protect against ischemia/reperfusion injury by means of reestablishing the cellular redox balance and increasing adenosine availability (Peralta et al., 2000), as well as A₁ adenosine receptor activity, which is involved in analgesic, ansiolytic and neuroprotective actions (Detlev et al., 2010). The protective effects of ozone in diabetes and diabetic foot (Martínez-Sánchez et al., 2005) disc hernia and pain (León et al., 2012) and other diseases with oxidative etiology have been demonstrated.

Previous to this study, the protective effects of medical ozone against PG/PS-induced arthritis in rats on inflammation, and the reduction of IL-1β and TNF-α mRNA levels and oxidative stress have been demonstrated (Dranguet et al., 2013) whereby MTX + medical ozone combined therapy increased clinical efficacy and reduced MTX-induced hepatotoxicity risk compared with MTX monotherapy in RA patients. It has been proposed that adenosine and redox status are common therapeutic targets of MTX and medical ozone, meaning that a combination of both therapeutic concepts could increase the beneficial/risk ratio (León et al., 2016; Takon et al., 2017).

Taking into account the fact that medical ozone protective effects involve a regulation of cellular redox balance, adenosine accumulation and A₁ adenosine receptors (León, 2014), the aim of this study has been to investigate whether anti-inflammatory effects of ozone are mediated by A₁ adenosine receptors and the reduction of biomolecules oxidative damage in carrageenan-induced knee joint synovitis in rats.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (180–200 g) were used for this study. The rats were kept in an air-filtered and temperature-conditioned (20–22°C) room at a relative humidity of 50–52%. The animals were fed with standard commercial pellets and water ad libitum. All procedures were performed as approved (March 9, 2016, Code, CIEB/046) by the Institutional Review Board (Scientific and Ethics Committees from Pharmacy and Food Institute, University of Havana, Cuba) and in accordance with the European Union Guidelines for animal experimentation.

Chemicals

Medical Ozone (O₃) was generated using an OZOMED unit as manufactured by CNIC, Cuba and was administered via rectal insufflation. Ozone was obtained from medical grade oxygen, and was used immediately as it was generated and represented only about 3% of the gas (O₃+O₂) mixture. The ozone concentration was measured using a built-in UV spectrophotometer set at 254 nm (with an accuracy of 0.002 A at 1A, a repeatability of 0.001 A, and calibrated with internal standard). The ozone dose was the product of the ozone concentration [expressed as mg by the gas (O₂+O₃) volume (l)]. As assessed from the body weight of the rat, the ozone dose was calculated. DPCPX (8-cyclopentyl-1,3-dipropylxanthine) had been obtained from the Sigma-Aldrich, Germany.

Induction of carrageenan-induced knee joint synovitis in rats

The standard protocol for carrageenan-induced knee joint synovitis was followed as described (Ekundi-Valentim et al., 2010). Briefly, the left knee received an intra-articular (i.art.) injection of carrageenan (50 µL of a 3% solution in a sterile 0.9% saline solution). Control animals were injected with an equal volume of saline. Joint swelling and pain score were evaluated after 1, 2, 3 and 4 h, and tactile allodynia after 2 and 4 h. The change in knee diameter was assessed by applying a digital calliper (Starret, São Paulo, Brazil) and histopathological criteria.

Treatment schedule

The protocol consisted of four experimental groups (n=20). Group 1 (n=5): Control (-) receiving saline only; Group 2 (n=5): Carrageenan, in which the rats received 50 µL of a 3% carrageenan solution i.art; Group 3 (n=5): Medical Ozone+Carrageenan, prior to carrageenan treatment as in group 2 in which the animals were treated with ozone via rectal insufflations at 1 mg/kg. The rats received 15 ozone treatments, one per day, of 5–5.5 ml at an ozone concentration of 50 µg/ml; Group 4 (n=5): Oxygen+Carrageenan, as in Group 3, but the ozone treatment was here substituted by oxygen (the ozone carrier) instead.

Influence of ozone treatment on A₁ adenosine receptors

In this investigation, injection of the A₁ receptor
antagonist DPCPX was not proconvulsant at the doses used (León et al., 2008; Li et al., 2008).

The protocol comprised of four groups (n=20, Wistar rats): Group 1 (n=5): Control (-) received saline only; Group 2 (n=5): Carrageenan, rats received 50 µL of a 3% carrageenan solution in a sterile 0.9% saline solution i.art.; Group 3 (n=5): Medical Ozone+Carrageenan, prior to carrageenan treatment as in group 2 in which the animals were treated with ozone via rectal insufflation at 1 mg/ml; Group 4, the same as in group 3 in which rats here received 1 mg/kg (subcutaneous) DPCPX after the final ozone treatment and 30 min prior to carrageenan administration (Li et al., 2008). Inflammatory response was assessed at the end of the experiment (4 h after carrageenan treatment).

**Evaluation of carrageenan-induced knee joint synovitis**

Measurements of the mediolateral diameter (at the level of the patellar ligament) of each rat knee joint were carried out in the rats prior to carrageenan injection and once per hour for a period of 4 h (i.e. up to the end of the experiment). Knee swelling was expressed as the change in knee diameter (in mm).

**Sample preparations and biochemical determinations**

At the end of the experiment, the rats received terminal diethyl ether anesthesia. The spleen was promptly removed for biochemical studies. Spleen homogenates were obtained as previously described (Dranget et al., 2013). Redox parameters were determined spectrophotometrically using a plate reader (SUMA, Cuba) and a Model S 220 Spectrophotometer, BOECO, Germany. After precipitating the thiol proteins, the reduced glutathione (GSH) was measured using Ellman's reagent; absorbance was measured at 412 nm (Sedlak and Lindsay, 1968). The Griess reaction (Granger et al., 1995) was used to determine nitrite/nitrate levels as a measure of nitric oxide (NO).

The advanced oxidation protein products (AOPP) were measured as the oxidation of iodine anion to diatomic iodine by advanced oxidation protein products (Witko-Sarsat et al., 1998). Nitrotetrazolium Blue (NBT) redox indicator was used to measure the relative fructosyllamine content (Amadori’s product of glycated serum protein) at 530 nm (Thorne et al., 1996). To quantify the total hydroperoxides (TH), a Bioxytech H₂O₂-560 kit (Oxis International Inc., Portland, OR, USA) was used. The concentrations of malondialdehyde (MDA) were analysed using an LPO-586 kit as obtained from Calbiochem (La Jolla, CA). The ferric reducing ability of the supernatant of spleen homogenates was determined as described by Benzie and Strain (1996).

**Histological study**

Rats’ hind paws were fixed in formaldehyde at 4% for a period of 24 h. The tissue was decalcified with formic acid and sodium citrate. After dehydration and xylol treatment, samples were included in paraffin wax using a tissue processor unit (Sakura, Model RH-12EP-2). Haematoxylin-eosin (H&E) staining was also used. Visualization and image capture were obtained using a Digital Chamber DP72 (Olympus; Center Valley, PA) matched on an Olympus Microscope BX51.

**Statistical analysis**

The OUTLIERS preliminary test for detecting error values was first applied for statistical analysis. Following this, the ANOVA method (single way) was applied, followed by the homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test (Duncan test) was included. Data were expressed as the mean ± standard deviation of 5 animals. The level of statistical significance employed was at least P < 0.05 for all experiments.

**RESULTS**

Figure 1 shows the ankle’s thickness in each experimental group. Ozone treatment was able to prevent the joint swelling induced by carrageenan and there was no difference to the control group at the end of the experiment. By contrast, in the carrageenan and carrageenan+oxygen groups, the ankle’s thickness was increased and there were no differences after 4 h of carrageenan treatment.

The histological results are shown in Figure 2. In Figure 2A, the control group shows a normal morphology that is very similar to Figure 2B, in which O₃+carrageenan group also showed no alterations. Cell infiltrations (T lymphocytes and macrophages) in the synovial tissue were observed in Figure 2C as carrageenan, and in Figure 2D as carrageenan+oxygen groups.

Redox marker levels in the supernatant of spleen homogenate are given in Table 1. The injury markers AOPP, fructolysine and MDA showed no difference (P >0.05) in the O₃+carrageenan and control groups. The opposite was observed in O₂+carrageenan group where these redox markers (AOPP, fructolysine and MDA) increased with regards to ozone-treated rats and control groups and showed no difference to the carrageenan group. These results agree with those for ankle’s thickness. TH increased while NO decreased compared with the remaining experimental groups. There was no change in FRAP levels and GSH had decreased in O₂+carrageenan group.

DPCPX completely suppressed the protective effect of ozone against inflammatory reaction in carrageenan-
Figure 1. Medical ozone effects on ankle’s thickness in carrageenan-induced knee joint synovitis. Data represent the mean ± SD of each group (n = 5). Means having different letters indicate significant differences (P < 0.05) between groups. Statistical tests: ANOVA method (single way) followed by Bartlett-Box, as well as a multiple comparison test (Duncan test) were used.

Figure 2. Histological results of the experimental groups in carrageenan-induced synovitis treated with ozone/oxygen. (A) Control and (B) Ozone+Carrageenan groups. Normal morphology was observed. In (C) Carrageenan and (D) O2+Carrageenan groups; black arrows show cell infiltrations in synovial tissue (HE 20X)
Table 1. Redox marker levels in rat’s spleen homogenates in carrageenan-induced synovitis treated with ozone/oxygen.

<table>
<thead>
<tr>
<th>Redox Marker</th>
<th>Control Group</th>
<th>Carrageenan Group</th>
<th>O$_3$+Carrageenan Group</th>
<th>O$_2$+Carrageenan Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH (µM/mg)</td>
<td>43.3 ± 4.4</td>
<td>52 ± 2.8</td>
<td>39.6 ± 0.07</td>
<td>45.4 ± 0.32</td>
</tr>
<tr>
<td>MDA (µM/mg)</td>
<td>1.6 ± 0.01</td>
<td>1.99 ± 0.01</td>
<td>1.4 ± 0</td>
<td>1.81 ± 0.06</td>
</tr>
<tr>
<td>Fructosylamine(*)</td>
<td>5.3 ± 0.002</td>
<td>10 ± 0.03</td>
<td>6.1 ± 0</td>
<td>8.3 ± 0</td>
</tr>
<tr>
<td>AOPP (µM/mg)</td>
<td>14.6 ± 1.2</td>
<td>31.1 ± 2.5</td>
<td>14 ± 8.2</td>
<td>26.5 ± 2.5</td>
</tr>
<tr>
<td>NO (µM/mg)</td>
<td>55 ± 15</td>
<td>41 ± 3.6</td>
<td>57 ± 6</td>
<td>72 ± 22</td>
</tr>
<tr>
<td>FRAP (µM/mg)</td>
<td>1761 ± 247</td>
<td>1732 ± 63</td>
<td>1991 ± 92</td>
<td>2000 ± 134</td>
</tr>
<tr>
<td>GSH (µM/mg)</td>
<td>2201 ± 21</td>
<td>2354 ± 128</td>
<td>2393 ± 89</td>
<td>1954 ± 98</td>
</tr>
</tbody>
</table>

TH, Total Hydroperoxides; GSH, reduced Glutathione; MDA, Malondialdehyde; AOPP, Advanced Oxidation Protein Products; NO, Nitric Oxide; FRAP, Ferric Reducing Ability of Plasma; (*), Relative fructosylene content. mg protein$^{-1}$ x 10$^{-4}$. Data represent the mean ± SD of each group (n = 5). The mean values with different letters indicate significant differences (P < 0.05) between groups. As statistical tests, the ANOVA method (single way) followed by Bartlett-Box, as well as a multiple comparison test (Duncan test) were used.

Figure 3. Effects of A$_1$ adenosine receptor blockage by DPCPX (8-cyclopentyl-1,3-dipropylxanthine) on medical ozone protective effects 4 h after carrageenan-induced knee joint synovitis. Data represent the mean ± SD of each group (n = 5). Means having different letters indicate significant differences (P < 0.05) between groups. Statistical tests: ANOVA method (single way) followed by Bartlett-Box, as well as a multiple comparison test (Duncan test) were used.

induced knee joint synovitis (Figure 3). No differences in ankle thickness between carrageenan and O$_3$+DPCPX+carrageenan groups were observed (P>0.05). Figure 4A and B shows the cellular redox balance in O$_3$+DPCPX+carrageenan compared with O$_3$+carrageenan group. DPCPX increased the level of oxidized proteins (AOPP) and lipid peroxidation (MDA). TH levels decreased in O$_3$+DPCPX+carrageenan compared with the remaining experimental groups, whereas NO only decreased in the carrageenan group whereby there was no change in fructosylamine. A dramatic decrease in the reduction ability (FRAP, measure of antioxidant power) in spleen homogenates was found when the A$_1$ adenosine receptor was blocked.

**DISCUSSION**

Synovitis is a critical pathological event preceding the clinical onset of RA. In this study, medical ozone was able to counteract the carrageenan-induced inflammatory response. Ozone anti-inflammatory effects were mediated by A$_1$ adenosine receptor activity, a reduction in oxidative protein damage and lipid peroxidation. Under our experimental conditions, the main impact of ROS in carrageenan-induced synovitis was to promote protein
and lipid oxidative damage (increase in AOPP, fructosylamine and MDA concentrations).

A reduction in inflammatory is probably the most important target in synovitis. Under normal physiological conditions, the levels of adenosine in the tissue microenvironment are relatively low and certainly below the sensitivity threshold of immune cells. However, during hypoxia, ischemia, inflammation and other pathological conditions, adenosine levels increase rapidly (Cronstein, 1994). When present in the extracellular environment, the nucleoside adenosine protects cells and tissues from excessive inflammation and immune-mediated damage while promoting healing processes (Serena Longhi, 2013).

A<sub>1</sub> adenosine receptor displays antinflammatory effects. Its activity has been linked to the reduction of ROS and IL-8 levels in T vaginalis-stimulated neutrophils (Frasson et al., 2017), while systemic inflammatory response syndrome-related lymphopaenia associated with adenosine A<sub>1</sub> receptor dysfunction has been demonstrated (Riff et al., 2017).

mRNA and the protein for A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> adenosine receptors are expressed in human synoviocytes. Our data now show that the A<sub>1</sub> adenosine receptor is involved in the antinflammatory effects of medical ozone in carrageenan-induced knee joint synovitis in Wistar rats (Figures 3 and 4). These results agree with the disappearance of antinflammatory effects and the protection provided by ozone against oxidative damage when DPCPX was tested as demonstrated by ankle thickness, protein and lipid oxidative injury, as well as a dramatic reduction of the plasma antioxidant capacity (FRAP).

Histological results revealed cell infiltrations (T lymphocytes and macrophages) in the synovial tissue of rats treated with carrageenan and carrageenan+oxygen, whereas normal morphology was observed in the control and ozone+carrageenan groups. In the very early stage of the disease, the sublining infiltrates may be minimal or modest (Singh et al., 2004). At this stage, macrophages (Kraan et al., 1998) and an infiltration by natural killer cells have been described (Hitchon and El-Gabalawy, 2011).

The A<sub>1</sub> adenosine receptor is closely associated with ozone protective effects. In liver ischaemic reperfusion injury, the activation of A<sub>1</sub> receptors with specific agonist CCPA (2-chloro-N6-cyclpentyladenosine) were in line with transaminases reduction; the blockage of these

Figure 4. Influence of A<sub>1</sub> adenosine receptor blockage on (A) injury and (B) protective redox markers in rats spleen homogenates in carrageenan-induced synovitis model. AOPP (Advanced Oxidation Protein Products, µM), MDA (Malodialdehyde, µM), TH (Total Hydroperoxides, µM), NO (Nitric Oxide, µM), GSH (reduced Glutathione, µM), FRAP (Ferric Reducing Ability of Plasma, µM), Fructolysine (relative fructolysine content). Data represent the mean ± SD of each group (n = 5). Means having different letters indicate significant differences (P < 0.05) between groups. Statistical tests: ANOVA method (single way) followed by Bartlett-Box, as well as a multiple comparison test (Duncan test) were used.
receptors by DPCPX increased liver damage (León et al., 2008). In another study, ozone increased the latency of the first seizure and also reestablished cellular redox balance in PTZ-induced convulsions in mice. DPCPX completely abolished the protection afforded by ozone, which has evidenced the role of A₁ adenosine receptor against brain damage (Mallok et al., 2015).

The anti-inflammatory effects of ozone, as mediated by the A₁ adenosine receptor, may be the consequence of transient oxidative stress. This stress when induced by certain specific antineoplastic agents and H₂O₂, up-regulated the A₁ adenosine receptor in hamster ductus deferens smooth muscle cells. A role of ROS generation in mediating the increase in A₁ adenosine receptor expression was supported by the finding that incubating cells with H₂O₂ and catalase, a scavenger of hydrogen peroxide, attenuated the response to H₂O₂ (Ramkumar et al., 2001). By promoting a slight and transient ROS generation, ozone is able to regulate the cellular redox balance (León et al., 1998) and A₁ adenosine receptor levels.

AOPPs are markers of oxidative protein damage and their level increases in RA patients. In addition, fibroblast-like synoviocytes (FLSs) are related to oxidative stress. An exposure of FLSs to AOPPs upregulated the mRNA and protein expression of proinflammatory cytokines, matrix metalloproteinases and vascular endothelial growth factor in a concentration-dependent manner. IkB degradation and nuclear translocation of NF-κB p65 induced by AOPPs were significantly blocked by the activity of antioxidants such as superoxide dismutase, N-acetyl-L-cysteine and NADPH oxidase inhibitors (Zheng et al., 2013). In effect, medical ozone is capable of stopping an increase in AOPP levels; thanks to oxidative preconditioning mechanism whereby the antioxidant endogenous systems are stimulated.

Fructosylamine (Amadori’s compound) is a precursor of Advanced Glycation End Products (AGE), which alter unique three dimensional structures of various plasma proteins such as IgG (Ahmad et al., 2012). The results suggest that a glycation of IgG results in the generation of neo-epitopes, making it a potential immunogen. AGE-IgG has been proposed as being one of the factors inducing the circulation of RA autoantibodies (Ahmad et al., 2013).

Lipid peroxidation leading to the formation of protein adducts promotes proinflammatory responses that characterize a variety of chronic conditions (Thiele et al., 2008). Malondialdehyde (MDA) is one of such ubiquitous product implicated in disease pathogenesis, and its level increases in synovial fluid.

Conclusion

In conclusion, under our experimental conditions, medical ozone was able to protect against the inflammatory response in carrageenan-induced knee joint synovitis in rats. The protective effects of ozone were mediated by A₁ adenosine receptor activity, in addition to the control of oxidative protein and lipid peroxidation damage. These results have been the basis of another study which investigates the anti-inflammatory effects of medical ozone in the synovitis involved in knee osteoarthritis patients.

REFERENCES


