

Effect of Ozone in Freund's Complete Adjuvant-Induced Arthritis

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ABSTRACT

Objectives: This study aims to investigate the effectiveness and reliability of ozone (O₃) in Freund's complete adjuvant (FCA)-induced arthritis, an animal model for rheumatoid arthritis (RA).

Materials and methods: Thirty-six four- to five-month-old male Wistar rats weighing between 274-420 gr were used in this study. Saline was injected into the hind paws of half of these rats, and FCA was injected into the other half. At the end of two weeks, 40 µg of O₃ was administered to nine rats from each group twice a week for seven total doses. The rats were followed-up in terms of clinical findings. At the sixth week, the rats were sacrificed and serum malondialdehyde, glutathione peroxidase, and superoxide dismutase levels were measured. In addition, ankle joints were separated for histopathological examination.

Results: Significant improvement was observed in terms of hind-paw diameter, severity of arthritis, and histopathological findings of inflammation after O₃ treatment in the group with FCA-induced arthritis. Although it was not quite significant, an upward trend was detected in oxidative stress markers with O₃ treatment.

Conclusion: This study, the first to investigate the effects of systemic O₃ on the clinical and histopathological outcomes of RA, indicates that O₃ is a highly effective and reliable treatment method in FCA-induced arthritis in animal models.

Keywords: Freund's complete adjuvant-induced arthritis; ozone; oxidative stress; rheumatoid arthritis.

Rheumatoid arthritis (RA) is a common systemic autoimmune disease that can cause chronic inflammation in the joints and organs such as the lungs, heart, and kidneys. The most significant problem of patients with RA is irreversible joint destruction and functional disability. The aim of RA treatment is to diminish inflammation and decrease the progression of erosion in the joints. Numerous biological and non-biological disease-modifying antirheumatic drugs have been used in the treatment of RA.¹ In some patients, the best therapeutic efforts do not produce the expected results and are often associated with numerous complications and serious side effects.² Therefore,

the search for new treatment options continues today.

In medical applications, ozone (O₃) is a gas administered in an O₃-oxygen mixture (0.05-5% O₃; 95-99.95% oxygen), obtained from pure oxygen with the help of an O₃ generator.¹ Ozone, discovered in 1840, has been used for more than 40 years for therapeutic purposes in the treatment of various disorders, such as ischemic and inflammatory disorders, several metabolic diseases, and cancer.³ It is thought that the pharmacological effect of O₃ occurs through an increase in the production of antioxidant enzymes.⁴ Recent evidence suggests that oxidative

Received: January 24, 2017 **Accepted:** June 13, 2017 **Published online:** September 13, 2017

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stress has an important role in the process of RA.⁵⁻⁷ Therefore, in this study, we aimed to investigate the effectiveness and reliability of O₃ in Freund's complete adjuvant (FCA)-induced arthritis, an animal model for RA.

MATERIALS AND METHODS

This study was conducted between October 2015 and January 2017 and included 36 four- to five-month-old male Wistar rats weighing between 274-420 gr that were obtained from the Experimental Animal Center of Adnan Menderes University. All experiments were performed according to the principles and guidelines of the Animal Ethical Committee's approval (64583101/2014/084). The study was conducted in accordance with the principles of the Declaration of Helsinki.

After being weighed and marked, the animals were divided into four groups, with nine rats in each group. The rats in the control group were injected with saline (0.1 mL) in the palmar surface of the hind paw. The rats in the O₃ group were injected with saline (0.1 mL) in the palmar surface of the hind paw. At the end of two weeks, the rats were given an intraperitoneal injection of O₃ at 40 µg in 2 mL volume twice weekly for a total of seven doses (Ozonosan Photonik, Dr. Hänslér Ozonosan, Germany). In the FCA group, adjuvant arthritis was induced in rats by a single subcutaneous injection of 0.1 mL heat-killed *Mycobacterium tuberculosis* in mineral oil (FCA) (Sigma-Aldrich Chemical Co., Interlab, Istanbul, Turkey) in the palmar surface of the hind paw. The peak of adjuvant arthritis was reached after 14 days from the adjuvant inoculation. FCA-induced arthritis is considered an appropriate animal model of RA for experimental animal studies.⁸⁻¹⁰ In the FCA-O₃ group, adjuvant arthritis was induced as in the FCA group. Then, 40 µg intraperitoneal O₃ (2 mL gas form) was administrated on the 15th day after FCA inoculation. The same dose was given twice a week for a total of seven doses.

The "therapeutic window" for O₃ has been defined as 10-80 µg/mL.⁴ The response of organisms to O₃ is dose-dependent. O₃ acts as an immunostimulant at low doses (10-20 µg/mL) and as an immunosuppressant at high doses

(40-80 µg/mL). In a previous study, the most effective and reliable dose in acute arthritis was reported to be 40 µg/mL.¹¹ Because Bocci¹² recommended that O₃ should be applied at least twice a week, O₃ was administrated twice a week at 40 µg/mL for this study.

Arthritis severity was assessed by paw swelling and an arthritis index. The assessments were made every Monday during the experimental period. Paw swelling was measured by a micrometric Vernier caliper. Measurements were taken three times at each evaluation, and the average of the measurements was recorded. Arthritis severity was evaluated by an experienced observer using an arthritis index according to the standardized method.⁷ The arthritis index was scored from 0 to 4 points as follows:

0: no evidence of hyperemia and/or inflammation; 1: hyperemia with little or no paw swelling; 2: swelling confined predominantly to the ankle region, with modest hyperemia; 3: increased paw swelling and hyperemia of the ankle and metatarsal regions; 4: maximal paw swelling and hyperemia involving the ankle, metatarsal, and tarsal regions.

Extra-articular findings were evaluated during each experimental week. Rats were sacrificed under ketamine and xylazine anesthesia (50 mg/kg and 5 mg/kg, respectively) at the end of the sixth week of the experiment. Blood samples were collected by cardiac puncture on the day of sacrifice and centrifuged at 1000 g for 10 minutes. The supernatant was collected and stored at -80 °C. The contents of malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD) in serum were measured by an enzyme-linked immunosorbent assay (ELISA) using commercial kits purchased from Sunred Biological Technology (Baoshan District, Shanghai, Chinese). Procedures were performed according to the manufacturer's instructions. The absorbance from each sample was measured using a spectrophotometric microplate reader at a wavelength of 450 nm (ELX800, BioTek Instruments Inc., Winooski, Vermont, USA). The calculated overall intra-assay coefficient of variation (CV) was 10%, and the inter-assay CV was 12% for all three ELISA kits. The assay ranges were between 0.75-100 nmoL/mL, 0.8-200 ng/mL, and 0.5-150 ng/mL for MDA, GPx, and SOD, respectively.

Table 1. Effect of ozone on paw diameter (cm)

	1 st week	2 nd week	3 rd week	4 th week	5 th week	6 th week
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Control	0.4±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0
Ozone	0.4±0.0	0.4±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0
FCA-induced arthritis	0.5±0.1	0.8±0.1*	0.8±0.1*	0.7±0.1*	0.8±0.1*	0.7±0.1*
FCA-induced arthritis-O ₃	0.5±0.0	0.8±0.1*	0.8±0.1*	0.6±0.1*‡	0.6±0.1*‡	0.5±0.1*‡

SD: Standard deviation; FCA: Freund's complete adjuvant-induced arthritis; * p<0.05 compared with control group; O₃: Ozone; ‡ p<0.05 compared with Freund's complete adjuvant-induced arthritis group.

The ankle joints were separated and kept in 10% neutral buffered formalin for 24 hours prior to placement in a Surgipath Decalcifier (Surgipath Medical Industries, Inc., Richmond IL, USA) for approximately one week. The paws were decalcified with a solution containing hydrochloric acid and 0.1 ethylenediaminetetraacetic acid. After the completion of the decalcification process, the ankle joints were transected into two equal halves along the longitudinal plane, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Synovial inflammation, synovial hyperplasia, and cartilage damage were evaluated separately (0: absent; 1: mild; 2: moderate; 3: severe).¹³

Statistical analysis

Data were analyzed using PASW software package version 18.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as the mean ± standard deviation. Differences between the groups were examined with a Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

RESULTS

The measurements of paw diameters were summarized in Table 1. The paw swelling caused by the FCA application regressed with O₃ treatment. Arthritis was not detected in the control and O₃

groups. The arthritis index in the second week after FCA administration was 3.1±0.6 in the FCA group and 3.0±0.6 in the FCA-O₃ group, with no statistically significant difference between groups (*p*>0.05). The arthritis index in the sixth week of the study was 3.1±0.6 in the FCA group and 1.4±0.8 in the FCA-O₃ group, with statistically significant improvement achieved in the FCA-O₃ group (*p*<0.001). One rat from the FCA-O₃ group was observed to have a full clinical recovery. No adverse effects were detected in any of the study groups (Table 2).

The levels of oxidative stress markers, including MDA, SOD, and GPx, were determined to show an upward trend in both the O₃ and FCA-O₃ groups (Table 3). However, the differences among the groups were not found to be statistically significant (*p*>0.05).

A histopathological examination of the paws in the O₃ group revealed no pathology different from the control group. In the FCA and FCA-O₃ groups, prominent synovial inflammation, synovial proliferation, and cartilage damage were observed. However, all of these findings were decreased in the FCA-O₃ group compared to the FCA group (Figure 1, Table 4). In addition, granulomatous changes involving Langhans cells were detected in the FCA groups (Figure 1). It was observed that these granulomas also decreased in the FCA-O₃ group.

Table 2. Effect of ozone on arthritis index

	FCA group	FCA-O ₃ group	<i>p</i>
	Mean±SD	Mean±SD	
Before O ₃ treatment	3.1±0.6	3.0±0.6	>0.05
After O ₃ treatment	3.1±0.6	1.4±0.8	<0.001
<i>p</i>	>0.05	<0.001	

O₃: Ozone; FCA: Freund's complete adjuvant-induced arthritis; SD: Standard deviation.

DISCUSSION

Freud's complete adjuvant-induced arthritis was considered as an animal model of RA, because it shows some common characteristics with human RA (e.g., proliferative synovial tissue and cartilage destruction).^{9,10,14} In this study, the arthritis and histopathological findings that are expected in

RA were observed in FCA-induced arthritis. These findings show a successfully established rat model of RA. In addition, diffuse granulomatous changes were also observed. These granulomatous changes may be rarely observed in autoimmune disorders such as RA, but after a local injection of FCA, granulomas may also occur due to foreign-body reactions. These granulomas rarely

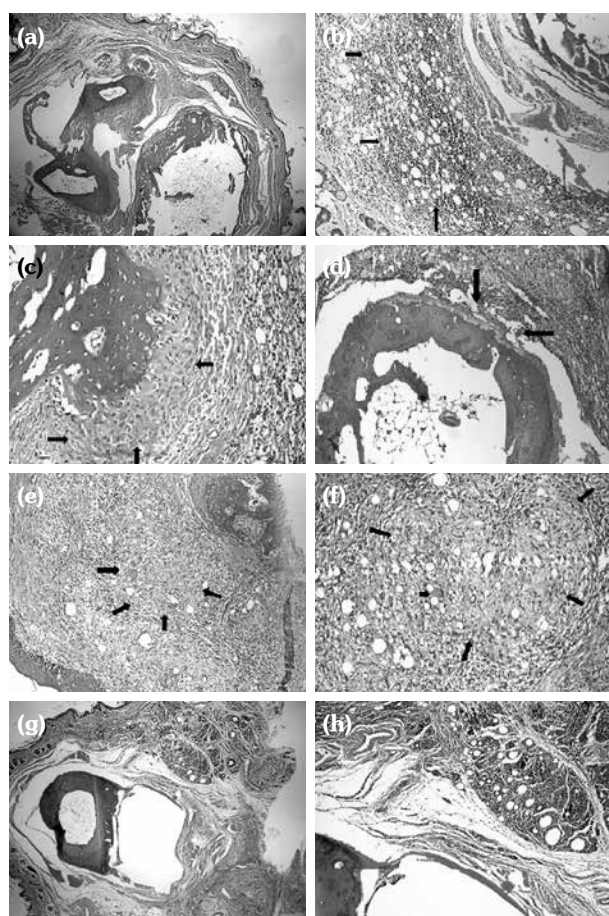


Figure 1. Photomicrographs for sections stained with hematoxylin & eosin (H-E). **(a)** Normal skin, subcutaneous tissue and normal metatarsal joint in intact rat (H-E×40). **(b)** Severe inflammation (arrows) comprised of lymphocytes and polymorphonuclear leukocytes, after Freund's complete adjuvant injection (H-E×100). **(c)** Synovial cell proliferation (arrows). Picture shows increased cellular stratification in synovial cells (H-E×200). **(d)** Cartilage damage after Freund's complete adjuvant injection (H-E×40). **(e)** Granuloma formation in inflammation (H-E×40). **(f)** Granulomas composed of groups of epithelioid histiocytes (big arrows) and Langhans type giant cells (small arrow) (H-E×200). **(g, h)** Minimal inflammation composed of lymphocytes and polymorphonuclear leukocytes. The granulomas disappeared after ozone treatment (H-E×40 and H-E×100, respectively).

have Langhans cells, as found in this study.¹⁵ For this reason, it is possible that the granulomatous changes that occurred in this arthritis model may be due to FCA-related reactions.

This study determined that O₃ therapy significantly reduced arthritis severity (paw diameter and arthritis indexes) in FCA-induced arthritis. Histopathological findings also showed regression. Any complications or side effects in terms of O₃ treatment were not detected.

In a previous study, Chen found that intra-articular O₃ injection was clinically ameliorated in FCA-induced arthritis as animal model of RA.¹⁶ Furthermore, Vaillan also observed similar findings in peptidoglycan-polysaccharide-induced arthritis with the same treatment.⁹ Chang demonstrated that the administration of O₃ to the RA synovial fibroblastic cell culture showed significant improvement in the histopathological findings of inflammation.¹⁷ However, RA is a systemic and polyarticular disease, especially involving small joints. For this reason, intra-articular O₃ injection alone may not be sufficient in the treatment of RA. Therefore, this study aimed to investigate the effectiveness of systemic O₃ treatment in an animal model for RA. Previous studies have shown that systemic O₃ administration could be used safely in some clinical conditions, such as diabetes mellitus and renal disorders,^{18,19} but a literature review indicates that this study is the first to evaluate the effect of systemic O₃ on both clinical and histopathological findings in an animal model of RA.

It is believed that O₃ used within therapeutic dose limits leads to transient oxidative stress, which may trigger antioxidant enzymes.^{20,21} Several experimental studies have demonstrated that controlled O₃ administration could prevent the damage of reactive oxygen species.⁵⁻⁷ Although the etiopathogenesis of RA has not been fully identified yet, oxidative stress plays an important role in its underlying pathogenesis.^{11,22-24} MDA, the oxidation product, is considered an index of lipid oxidation.²⁵ In this study, MDA levels increased from 8% to 11% after O₃ administration; however, with no statistical significance. As Bocci³ stated, even small increases in lipid oxidation products such as MDA could trigger the enzymatic and non-enzymatic antioxidant system. GPx and

Table 3. Effect of ozone on serum malondialdehyde, superoxide dismutase and glutathione peroxidase in Freund's complete adjuvant-induced arthritis

	Control group	O ₃ group	FCA group	FCA-O ₃ group
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Malondialdehyde	14.2±2.7	15.4±4.3	14.4±3.3	16.8±3.2
Superoxide dismutase	4.4±0.5	4.5±0.9	4.5±0.7	4.6±0.4
Glutathione peroxidase	4.5±2.3	7.8±8.8	5.7±3.8	7.8±4.1

O₃: Ozone; FCA: Freund's complete adjuvant-induced arthritis; SD: Standard deviation.

Table 4. Effect of ozone on histopathological findings in Freund's complete adjuvant-induced arthritis in rats

	Synovial inflammation	Synovial proliferation	Cartilage damage
	Mean±SD	Mean±SD	Mean±SD
Control group	0†	0†	0†
O ₃ group	0.2±0.4†	0†	0†
FCA group	2.9±0.3*	1.9±0.6*	1.7±0.5*
FCA-O ₃ group	1.6±0.5*†	1.2±0.4*†	1.0±0.0*†

SD: Standard deviation; O₃: Ozone; FCA: Freund's complete adjuvant-induced arthritis; * p<0.005 compared with control group; † p<0.05 compared with Freund's complete adjuvant-induced arthritis group.

SOD are powerful antioxidant enzymes that are affected by the activity of the disease in RA.²³ In this study, upward trends of serum SOD and GPx levels were observed in both the O₃ and FCA-O₃ groups; however, with no statistical significance. Mawsouf et al.²² detected a significant increase in antioxidant levels with O₃ in FCA-induced arthritis, but unlike the current study, Mawsouf applied O₃ rectally for 10 sessions. The difference between the results may be related to these differences.

The significant improvement of clinical and histopathological findings in the current study, despite minor changes in oxidative stress markers, is suggestive that this effect is not exclusively processed through the antioxidant system. Several studies have demonstrated that O₃ treatment decreases tumor necrosis factor-alpha in adjuvant arthritis.^{9,26} It has also been observed that O₃ increases the release of various growth factors (platelet-derived growth factor, transforming growth factor-beta), autacoids, and cytokines; regulates tissue circulation and oxygen utilization; and improves the tropic process.^{1,3,26} Ozone enables the individual to feel better throughout the treatment period by stimulating the neuroendocrine axis.²⁷ For this reason, the effect of O₃ in FCA-induced arthritis may also depend on other factors besides antioxidants.

The limitation of this study is that the effect of O₃ observed in the animal model of RA may not be similar to those observed clinically. Therefore, human studies should be done in the future.

In conclusion, this study determined that systemic O₃ treatment suppressed the inflammatory process both clinically and histopathologically in FCA-induced arthritis. In addition to its effectiveness, it was observed that O₃ could be used reliably in the treatment of arthritis. However, there is need for additional studies on O₃ treatment in RA.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

This study was supported by ADU Research Funding (TPF-15047).

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