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Effect of ozone therapy on redox status in experimentally induced arthritis

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Keywords

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Abstract

Controlled ozone administration has been shown to promote an adaptation to oxidative stress by increasing endogenous antioxidant systems. In the present study, the effects of O2/O3 administration either prophylactically or therapeutically on the alterations of oxidant status in adjuvant-induced arthritis in rats have been studied. Seven groups of rats were used: 1) normal control group; 2) control arthritic group (21 days); 3) prophylactic ozone group: arthritic rats received fifteen intra-rectal applications of O2/O3 at 0.5, 0.7 and 1 mg/kg b.w. in a 5-6 mL volume starting one day before adjuvant inoculation and continued as five applications/week over 21 days; 4) oxygen group: received oxygen (vehicle of ozone) in a similar schedule to group 3; 5) control arthritic group (24 days); 6) therapeutic-ozone group: arthritic rats received 10 intra-rectal applications of O2/O3 at 0.5, 0.7 and 1 mg/kg b. w. in a 5-6 mL volume daily for 10 days starting fourteen days after adjuvant inoculation; 7) oxygen-treated group: received oxygen in a similar schedule of group 6. The effect of O2/O3 administration was assessed by measuring: blood glutathione (GSH), erythrocyte glutathione peroxidase and catalase activities, serum levels of protein thiols (PrSH), malondialdehyde (MDA) and nitrite/nitrate (NO2/NO3), as well as serum ceruloplasmin activity (CP). The present study showed that adjuvant-induced arthritis in rats caused a significant ($p<0.05$) reduction in blood GSH, serum PrSH levels and erythrocyte antioxidant enzyme activities accompanied by a significant ($p<0.05$) increase in serum levels of MDA, NO2/NO3 and CP activity. Ozone administration either prophylactically or therapeutically normalize blood GSH, serum PrSH and MDA levels and restored erythrocyte antioxidant enzyme activities. However ozone did not significantly ($p>0.05$) modify serum NO2/NO3 level in induced rat but significant ($p<0.05$) increase CP activity. So it could be concluded that O2/O3 oxidative preconditioning / postconditioning effectively modulate the antioxidant/oxidant balance associated with adjuvant arthritis model in rats.

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Introduction

Ozone therapy as a complementary medical approach has been known for more than four decades. The main areas where that kind of treatment could be useful include resistant infectious diseases, autoimmune diseases, neurodegenerative diseases, orthopedic pathologies and vascular disorders[1] . With the advent of precise medical ozone generator, currently ozone therapy have been marked with growing recognition of the use of appropriate and judicious doses making this therapy useful with valuable biological effects[2]. The use of calculated, standardized ozone doses has been found to induce a transient acute oxidative stress condition which is not deleterious but is capable of eliciting a multiple useful biological responses. The effect could be seen in activation of antioxidant defense system, improvement of circulation, oxygen delivery, and trophic processes in tissues as well as enhancement of autocoids, growth factors and cytokine release[3].

Several experimental studies have demonstrated that controlled ozone administration could bring about a state of ozone oxidative preconditioning (O3OP) or adaptation to oxidative stress, preventing the damage caused by reactive oxygen species (ROS) generated in various experimental models. These include; carbon tetrachloride-induced hepatotoxicity[4], hepatic ischemia-reperfusion injury[5], cisplatin-induced acute renal failure[6] , chronic renal failure induced by subtotal nephrectomy[7] and streptozotocin-induced diabetes in rats[8]. More recently is also demonstrated that the oxidative postconditioning can be cytoprotective in different experimental model of diseases[9], [10]. Experimental arthritis induced by adjuvant is an experimental model of systemic inflammatory autoimmune disease that shares many features with human rheumatoid arthritis. It involves most of the joints and associated tissues[11]. Although the etiology of rheumatoid arthritis is not fully elucidated, autoimmune destruction of the affected tissues plays a pivotal role in the incidence and progression of the diseases[12].

In addition excessive generation of free radicals and formation of lipid peroxide in target tissues of inflammation are, also, considered as the most common factors implicated in tissue damage in rheumatoid arthritis[13] and experimental arthritis[14]. Thus, a state of oxidative preconditioning such that achieved with controlled ozone therapy may potentially be able to readjust the redox imbalance in adjuvant arthritis and attenuate the progression of the disease.

The aim of the current work was to investigate the role of ozone, as prophylactic or therapeutic agent, in correcting the redox imbalance and the biochemical changes associated with adjuvant-induced arthritis in rats.

Materials and Methods

Animals: Adult male albino rats of Wistar strain, 200-250 g weight were obtained from Egyptian Organization for Biological Products and Vaccines (Cairo, Egypt). Rats were housed in plexiglass cages, maintained in an air-filtered and temperature-conditioned (20 oC - 22 oC) room with a relative humidity of 50 % - 52 % and under an artificial light/dark cycle of 12 h. Animals were fed with standard laboratory chow and water ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international (EEC Council Directive 86/ 609, OJL 358, 1, 12 December 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996) laws and policies.

Chemicals: Complete Freund`s adjuvant (Difco laboratories, Detroit, USA) was used for induction of arthritis in rats. It consists of 0.05% heat killed Mycobacterium butyricum suspended in mineral oil. All other chemicals were of analytical pure grade supplied from Sigma-Aldrich St. Louis (USA).

Ozone generation: Ozone was generated just before applied by ozone generator system [EXT120-T] (Longevity-resovces Inc., Canada – ETL approved for proven quality and safety). Ozone obtained from

medical grade oxygen represented about 0.4-0.5 % (1 µg/mL – 120 µg/mL) of the gas mixture. The ozone concentration was measured by using a build-in UV spectrophotometer at 254 nm.

Experimental design

Induction of adjuvant arthritis: It was induced in rats by a single subcutaneous injection of 0.25 mL of complete Freund`s adjuvant into the palmer surface of the right hind foot pad[15]. The peak of adjuvant polyarthritis was reached after 14 days from adjuvant inoculation.

Ozone treatment

Ozone was given by intra-rectal application using 20 mL silicone-coated disposable syringe and rectal catheter. Fixed volumes of the O3/O2 mixture were administered according to the animal weight so as to reach a final O3 dose. This route of administration was considered as most useful and easy procedure in rats[16].

For studying the prophylactic or therapeutic effects of ozone on the adjuvant arthritis model, the arthritic rats were divided equally into six groups of eight rats each. The first (prophylactic O3/O2 group) received 15 intra-rectal applications of O3/O2 over three weeks starting one day before adjuvant inoculation. O3/O2 mixture was given as five applications per week. It was started with a relatively low dose of ozone as 0.5 mg/kg b.w. /day in the first week, increased to 0.7 mg/kg b.w./day in the second week and ended with 1 mg/kg b. w./day in the third week. The volume of O3/O2 mixture administered was 5-6 mL/rat according to the animal weight. The second arthritic group received oxygen only (as a vehicle for ozone) in a similar schedule to the first group. The third group of arthritic rats was kept without treatment throughout 21 days and served as a control (arthritic 21 days) for the above two groups. The fourth arthritic group (therapeutic ozone group) received 10 intra-rectal applications of O3/O2 mixture starting fourteen days after adjuvant inoculation. The treatment was started by a daily dose of 0.5 mg/kg b.w. for 3 days, followed by 0.7 mg/kg b.w. for another 3 days and ended with one mg/kg b.w. for 4 days. The volume of O3/O2 mixture administered was 5-6 mL/rat according to the animal weight. The fifth arthritic group received oxygen only 14 days after adjuvant inoculation in a similar way to the fourth group. The sixth arthritic group was kept without treatment throughout 24 days and served as a control (arthritic 24 days) for the fourth and fifth groups. A group of normal rats left without any treatment and served as a control (non arthritic) group for all the above groups. All the groups were kept under the same conditions during the whole experiment.

At the end of the experimental periods, the animals were sacrificed and the blood was collected in heparinized and non-heparinized tubes, an aliquot of heparinized blood was used to assay glutathione (GSH)[17]. Another aliquot of heparinized blood was lysed directly in ice cold distilled water (5% v/v) and used for the determination of catalase activity (CAT; EC 1.11.1.6)[18]. The remaining heparinized blood was centrifuged for 10 min at 3000 g for the separation of red cells used to measure the glutathione peroxidase activity (GPx; EC 1.11.1.9)[19]. On the other hand the non-heparinized blood was allowed to clot and the separated serum were used for the estimation of malondialdehyde (MDA)[20, 21]; protein thiols (PrSH)[22] and nitrite/nitrate (NO2/NO3) levels[23] as well as ceruloplasmin (CP) activity[24].

Statistical Analysis

Initially the OUTLIERS preliminary test for detection of error values was applied as a first step in the statistical analysis. After this, the homogeneity of variance test (Bartlett-Box) was used. Values are given as means ± SD. The level of statistical significance was taken at $p<0.05$, using one way ANOVA followed by Tukey-Kramer`s multiple comparisons test to judge the difference between various groups. The SPSS software package version 10, 2000 was used for all statistical analyses.

Results

Blood antioxidant levels in arthritic rats subjected to prophylactic and therapeutic intra-rectal application of ozone: The results for these parameters are shown in table 1 and 2. Data demonstrated that 21 or 24 days after adjuvant inoculation, arthritic rats exhibited a significant ($p<0.05$) reduction in blood GSH and serum PrSH levels. The reduction was extended, also, to include GPx and CAT activities as compared to the normal values.

Intra-rectal application of O3/O2 as prophylactic therapy (table1), caused a significant ($p<0.05$) elevation in blood GSH, serum PrSH, erythrocyte GPx and CAT activities as compared to the values present in arthritic animals. In the same way, therapeutic intra-rectal application of O3/O2 mixture (table 2) successfully restored these blood antioxidants to levels approaching or exceeding the normal values.

Serum levels of MDA and NO2/NO3 as well as CP activity in arthritic rats subjected to prophylactic or therapeutic intra-rectal application of ozone: As indicated in table 3 and 4, adjuvant-induce arthritis caused a significant ($p<0.05$) increase in serum levels of MDA, NO2/NO3 and CP activity after both 21 or 24 days of adjuvant inoculation. These data, demonstrated that prophylactic intra-rectal application of O3/O2 mixture (table 3) normalize serum MDA level of arthritic induced rats, but failed to exert any change in serum NO2/NO3 level of these rats. Meanwhile, O3/O2 pretreatment provided a further elevation of serum CP activity to a level exceeding the arthritic values. Therapeutic intra-rectal application of O3/O2 mixture (table 4) caused a significant ($p<0.05$) reduction in serum MDA level to approach the normal value, together with further elevation of CP activity than the arthritic levels. Meanwhile serum NO2/NO3 of arthritic rats was not significantly changed in response ozone treatment.

The results clearly showed that intra-rectal application of O2 (as a vehicle of O3) in prophylactic and therapeutic treatment did not affect any of the measured parameters compared to the values of arthritic rats.

Discussion

The involvement of ROS in chronic inflammatory conditions such as rheumatoid arthritis and adjuvant induced-arthritis is well documented. ROS once generated provoke deleterious effects on various cellular components, among which are membrane lipids that are extensively subjected to peroxidation. Aggravation of arthritis was reported to be associated with enhancement of lipid peroxidation[25].

In the present study, overproduction of ROS in adjuvant arthritis model leads to a considerable oxidant stress as indicated by a high serum level of MDA, a marker of lipid peroxidation, as well as consumption of blood antioxidants such as GSH and PrSH. The marked increase in serum MDA was observed in arthritic rats in line with our results; in arthritic rats model[26-28], and in rheumatoid arthritis patients[29]. Increased lipid peroxide in arthritic rats is exacerbated by the decline in blood antioxidants. Similar results about GSH were reported in arthritic rats model and rheumatoid arthritis patients respectively[30], [31]. In the same line, a marked decrease in GSH concentration was observed in the joint articular cartilage of arthritic rats[32]. The reduction of GSH might be attributed to the increased consumption for counteracting oxidative stress during inflammation. Increased oxidative stress was reported to enhance the formation and efflux of glutathione disulfides[33]. Moreover, the observed reduction in serum PrSH is in line with previous studies[30], [34]. Such reduction could be attributed to the excessive consumption by peroxide[35] and/or to a low serum albumin reported in other studies, since the greatest majority of serum SH (85-90%) are found in albumin[36].

In the current investigation, the decline in blood antioxidants was, also, extended to include erythrocyte GPx and CAT activities. The observation is consistent with previous manuscript[26], [27]. A defective antioxidant enzyme machinery had been observed in erythrocytes of rheumatoid arthritis patients[37] and in liver, kidney

and heart of adjuvant arthritic rats[38]. The increased production of superoxide anion, H2O2 and hydroxyl radicals demonstrated by Ramprasath et al. (2005)[39] might be responsible for inhibition of GPx and CAT activities.

The role of NO and other reactive nitrogen species in inflammation has not been conclusively established. However, evidences for the implication of NO in the process of inflammation and that NOS inhibitors possess potential-anti inflammatory effects have been presented[40, 41].

The present results revealed a significant ($p<0.05$) increase in serum NO level in arthritic rats (measured as total NO2/NO3). Such result is in line with previous reports[27, 41] in arthritic rats and in rheumatoid arthritis patients[42]. The over expression of iNOS in arthritis might result from increased production of IL-1 and other pro-inflammatory cytokines characteristic of that disease[40].

Moreover, a major systemic event that happens in the rat following the induction of inflammation is the marked change in the level of serum CP, an acute phase protein[43]. In the present study, a marked elevation in serum CP activity was observed. Such elevation is consistent with previous observations[30, 34] in arthritic rats. The increase in serum CP activity might be due to increase in its hepatic synthesis triggered by increased secretion of IL-1, epinephrine and glucocorticoids[44]. Furthermore, such an increase in CP activity has been reported to have a role in down regulating the inflammatory mediators and inhibiting the lipid peroxidation[43].

In the present study, prophylactic intra-rectal application of O2/O3 to arthritic rats over three weeks exerted protective effects on some important blood antioxidants (GSH, PrSH, GPx and CAT) and preserved them to pre-arthritic values (table 1). The present data, demonstrated that therapeutic intra-rectal application of O2/O3 for 10 days after development of adjuvant arthritis attenuated the reduction in blood antioxidants and restored the levels of these defense constituents to values close to or above the normal ones (table 2). Moreover, these stimulant effects of O2/O3 therapy on blood antioxidants were accompanied by a decrease in serum MDA level to reach the normal levels (tables 3 and 4). These positive experimental observations could be explained at the light of the ozone abilities to up-regulate the antioxidant system, a state reached under controlled use of O2/O3[45]. Ozone post or preconditioning is analogous to other phenomena such as ischemic preconditioning[46], thermal preconditioning[47], chemical preconditioning[48], ischemic protconditioning[10, 49]. All of these processes have in common that a repeated and controlled stress is able to provide protection against a prolonged and severe stress.

A point that should not be overlooked is that O3 adaptation caused by judicious use of O3 is due to the fact that O3, instantaneously reacts with biomolecules generating ROS, among which are H2O2 and lipid peroxidation products (LOP). These molecules can elicit the up-regulation of antioxidant enzymes such as SOD, GPx, GSH-reductase and CAT. In bone marrow cells, particularly during erythrogenesis, submicromolar concentrations of LOP can up-regulate the synthesis of antioxidant enzymes[2]. Interestingly, Iles and Liu50 have demonstrated that some LOP by inducing the expression of glutamate cysteine ligase cause an intracellular increase of GSH. These aforementioned findings might account for the generation of biochemically improved erythrocytes during prolonged O3 therapy. Erythrocytes have been shown to respond to O3 therapy with activation of glycolysis and pentose phosphate pathway[51].

In the current study, up-regulation of erythrocytes GPx and CAT by O2/O3 might be responsible for the preservation of blood GSH and serum PrSH from oxidation by ROS in arthritic rats. Furthermore, the reported activation of pentose phosphate pathway might play a role in restoring GSH level from its oxidized form.

On the other hand, prophylactic and therapeutic rectal applications of O2/O3 therapy provided further elevation of serum CP activity than the arthritic levels (tables 3 and 4). That effect could be explained on

the basis that O3 acts as a mild enhancer of immune system through activation of gene/regulatory nuclear factor kappa B (NF-κB) by H2O2, one of the major decomposition products of O3. Activation of that transcription factor switches on some genes that are responsible for the synthesis of several proteins, among which are the acute phase reactants and numerous interleukins[52]. The increased CP activity might reflect improved antioxidant status of animals subjected to O3 therapy. Moreover, O3-induced increase in CP activity could be beneficial to prevent against oxidative stress observed in adjuvant-induced arthritic rats.

In the present study, the remarkable enhancement of antioxidant status of arthritic rats has provided a protection against ROS and suppressed the process of lipid peroxidation leading to normalization of serum MDA level. Another point which should be considered in is the inability of O3 therapy to change the serum level of NO than was raised the arthritic induced rats; such effect might be related to the stimulatory effect of O3 on blood GSH. It has been stated that NO readily reacts with GSH and other cysteine containing compounds forming S-nitrosothiols with half lives of 5-50 min, in contrast to the very short half-life of NO[53]. Thus, formation of S-nitrosothiols in response to O3 therapy may allow more pharmacological effects at distant sites.

Conclusions

It can be concluded that O2/O3 pre or postconditioning effectively improved the antioxidant/oxidant imbalance associated with adjuvant arthritis in rats. These results potentially support the use of ozone therapy as a complementary medical approach in rheumatoid arthritis. However, further studies are needed to verify the benefit of O3 therapy in rheumatoid arthritis at biochemical and clinical levels.

Table 1. Blood antioxidant levels in arthritic rats subjected to prophylactic intra-rectal application of O3/O2 mixture

Groups	GSH mg/dL	PrSH µmol/L	GPx (nmoles NADPH /min/gHb)	CAT (µmoles H_2O_2/ min/gHb)
Normal Control	23.9 ± 1.16 [a]	346.6 ± 15.3[a]	274.4± 17.4[a]	124.5 ± 16.7[a]
Arthritic (21 days)	19.3± 2.39[b]	276.5± 26.4[b]	175.7± 27.3[b]	87.6± 15.34[b]
Arthritic pretreated with: O_2	19.9± 1.56[b]	280.7± 24.2[b]	191.6± 25.2[b]	94.6 ± 9.07[b]
Arthritic pretreated with: O_3/O_2 mixture	23.4± 1.27[a]	333.3± 65.04[a]	244± 16.3[a]	130.7± 18.3[a]

Legend: Data are expressed as mean of (7) observations ± SD; Values with non-identical superscripts are significantly different p < 0.05 / within the same set. Reduced glutathione, GSH; protein thiols, PrSH; glutathione peroxidase, GPx; catalase, CAT.

Table 2. Blood antioxidant levels in arthritic rats subjected to therapeutic intra-rectal application of O3/O2 mixture.

Groups	GSH mg/dL	PrSH µmol/L	GPx (nmoles NADPH /min/gHb)	CAT (µmoles H_2O_2/ min/gHb)
Normal Control	23.9 ± 1.16^a	346.6 ± 15.3^a	274.4 ± 17.4^a	124.5 ± 16.7^a
Arthritic (24 days)	18.3 ± 3.19^b	290.1 ± 13.9^b	178.7 ± 39.9^b	100.8 ± 8.1^b
Arthritic treated with: O_2	18.1 ± 2.2^b	295.7 ± 9.44^b	193.7 ± 43.7^b	104.1 ± 20.1^b
Arthritic treated with: O_3/O_2 mixture	24.6 ± 3.9^a	338.9 ± 17.9^a	249.6 ± 15.4^a	128.7 ± 8.8^a

Legend: Data are expressed as mean of (7) observations ± SD; Values with non-identical superscripts are significantly different p < 0.05 / within the same set. Reduced glutathione, GSH; protein thiols, PrSH; glutathione peroxidase, GPx; catalase, CAT.

Table 3. Serum levels of MDA, NO2/NO3 and CP activity in arthritic rats subjected to prophylactic intra-rectal application of O3/O2 mixture

Groups	MDA nmol/mL	NO_2/NO_3 nmol/mL	CP U/L
Normal Control	3.62 ± 0.36^a	23.2 ± 1.94^a	127.4 ± 17.04^a
Arthritic (21 days)	4.83 ± 0.8^b	33.4 ± 4.4^b	210.6 ± 31.05^b
Arthritic pretreated with: O_2	4.26 ± 0.49^b	31.6 ± 5.28^b	217.9 ± 27.5^b
Arthritic pretreated with: O_3/O_2 mixture	3.3 ± 0.51^a	35.7 ± 4.36^b	$282.1 \pm 42.4^{b,c}$

Legend: Data are expressed as mean of (7) observations ± SD; Values with non-identical superscripts are significantly different p < 0.05 / within the same set. Malondialdehyde, MDA; nitrite/nitrate NO2/NO3; ceruloplasmin activity CP.

Table 4. Serum levels of MDA and NO2/NO3 as well as CP activity in arthritic rats subjected to therapeutic intra-rectal application of O3/O2 mixture

Groups	MDA nmol/mL	NO_2/NO_3 nmol/mL	CP U/L
Normal Control	3.62 ± 0.36^a	23.2 ± 1.94^a	127.4 ± 17.04^a
Arthritic (24 days)	4.7 ± 0.66^b	33.4 ± 6.29^b	199.1 ± 36.4^b
Arthritic treated with: O_2	4.12 ± 0.37^b	29.7 ± 2.88^b	187.6 ± 22.6^b
Arthritic treated with: O_3/O_2 mixture	3.43 ± 0.21^a	30.1 ± 3.95^b	252 ± 51.9^c

Legend: Data are expressed as mean of (7) observations ± SD; Values with non-identical superscripts are significantly different p < 0.05 / within the same set. Malondialdehyde, MDA; nitrite/nitrate NO2/NO3; ceruloplasmin activity CP.

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