EFFECT OF OZONE OXIDATIVE PRECONDITIONING ON OXIDANT STATUS OF ADJUVANT ARTHRITIC RATS

By

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Abstract

Controlled ozone administration has been shown to promote an oxidative preconditioning or adaptation to oxidative stress by increasing endogenous antioxidant systems. In the present study, the effects of ozone administration either prophylactically or therapeutically on the alterations of oxidant status in adjuvant arthritic 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 ozone/oxygen mixture at 0.5, 0.7 and one mg/kg b.wt. 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 as vehicle for ozone in a manner similar to group 3; (5) control arthritic group (24 days); (6) therapeutic-ozone group: arthritic rats received ten intra-rectal applications of ozone/oxygen mixture at 0.5, 0.7 and one mg/kg b. wt. in a 5-6 ml volume daily for ten days starting fourteen days after adjuvant inoculation; (7) oxygen-treated group: received oxygen as vehicle for ozone in a manner similar to group 6. The effect of ozone administration was assessed by measuring: blood glutathione (GSH), erythrocyte glutathione peroxidase (GPx) and catalase (CAT) activities, serum levels of protein thiols (PrSHs), malondialdehyde (MDA) and nitrite/nitrate (NOx), as well as serum ceruloplasmin activity (CP). Results of the present study showed that adjuvant induced arthritis in rats caused a significant reduction in blood GSH, serum PrSHs levels and erythrocyte antioxidant enzyme activities accompanied by a marked increase in serum levels of MDA, NOx and CPactivity. Ozone administration either prophylactically or therapeutically succeeded to normalize blood GSH, serum PrSHs and MDA levels and restored erythrocyte antioxidant enzyme activities. However ozone did not significantly modify elevated serum NOx level but augmented the increased CP activity in arthritic rat serum. So it could be concluded that ozone oxidative preconditioning effectively improved the antioxidant/oxidant imbalance associated with adjuvant arthritis in rats.

Key words

Ozone
Oxidative preconditioning
Adjuvant arthritis

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 (Bocci, 1999). With the advent of precise medical ozone generator, the recent years of ozone therapy have been marked with growing recognition of the use of appropriate and judicious doses; making that therapy useful with valuable biological effects (Bocci, 2004). 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 (Bocci, 2006).

Several experimental studies have demonstrated that controlled ozone administration could bring about a state of ozone oxidative preconditioning (O_3OP) 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 (Candelario-Jalil et al., 2001), hepatic ischemia-reperfusion injury (Ajamieh et al., 2004), cisplatin-induced acute renal failure (Borrego et al., 2004), chronic renal failure induced by subtotal nephrectomy (Calunga et al., 2005) and streptozotocin-induced diabetes in rats (Al-Dalain et al., 2001).

Adjuvant arthritis 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 (Billingham and Davies, 1979). 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 (Rosenberg, 1999).

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 (Wade et al., 1987) and experimental arthritis (Yoshikawa et al., 1985; Symous et al., 1988). 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.

Aim of the Work

The aim of the current work was directed towards investigating the role of ozone as prophylactic or therapeutic application, in correcting the redox imbalance and certain biochemical changes associated with adjuvant arthritis in rats.

Materials and Methods

a) Animals: Adult male albino rats of Wistar strain, 200-250 g weight were obtained from laboratory animals farm of Egyptian Organization for Biological Products and Vaccines – Cairo – Egypt. Rats were fed standard rat chow diet and water ad-libitum and were kept under controlled temperature and humidity throughout the experimental work.

b) 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).

c) Ozone generation: Ozone was generated by ozone generator system [EXT120-T]. It is an oxygen fed ozone generator for medical applications (Longivity-resorce3s Inc., Canada – ETL Approved for proven quality and safety). It produces (one µg/ml – 120 µg/ml) ozone in oxygen concentrations.

d) Experimental design:

I. Induction of adjuvant arthritis: It was induced in rats by a single subcutaneous injection of 0.25 mg complete Freund`s adjuvant into the palmer surface of the right hind foot pad. The peak of adjuvant polyarthritis was reached after 14 days from adjuvant inoculation (Piliero et al., 1966).

II. Ozone treatment: Ozone was given by intra-rectal application. This route of administration is considered as most useful and easy procedure to perform in rats (Gonzalez et al., 2004).

For studying the prophylactic and therapeutic effects of ozone on adjuvant arthritis, the arthritic rats were divided equally into six groups of eight rats each. The first (prophylactic ozone group) received 15 intra-rectal applications of ozone/oxygen mixture over three weeks starting one day before adjuvant inoculation. Ozone/oxygen mixture was given as five applications per week. It was started with a relatively low dose of ozone as 0.5mg/kg b. wt. /day in the first week, increased to 0.7mg/kg b. wt/day in the second week and ended with one mg/kg b. wt/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 manner similar to the first group. The third group of arthritic rats was kept without

treatment throughout the 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 O_3/O_2 mixture starting fourteen days after adjuvant inoculation. The treatment was started by a daily dose of 0.5 mg/kg b. wt for 3 days, followed by 0.7 mg/kg b. wt. for another 3 days and ended with one mg/kg b. wt. for 4 days. The volume of O_3/O_2 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 manner similar 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.

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 for the estimation of glutathione (GSH) (Beutler et al, 1963). 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) (Aebi, 1974). The remaining heparinized blood was centrifuged for 10 minutes at 3000 rpm for the separation of red cells used for the estimation of glutathione peroxidase activity (GPx; EC 1.11.1.9) (Paglia and Valentine, 1967) On the other hand the non-heparinized blood was allowed to clot and the separated sera were used for the estimation of malondialdehyde (MDA) (Uchiyama and Mihara, 1978; Yoshioka et al; 1979); protein thiols (PrSHs) (Koster et al., 1986) and nitrite/nitrate (NO_x) levels (Miranda et.al., 2001) as well as ceruloplasmin (CP) activity (Schosinsky et al., 1974).

Statistical Analysis

Values are given as means ± SDM. 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.

Results

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

Intra-rectal application of O_3/O_2 mixture as prophylactic therapy (table1), caused a significant elevation in blood GSH, serum PrSHs, erythrocyte GPx and CAT activities as compared with the arthritic values.

On the same direction, therapeutic intra-rectal application of O_3/O_2 mixture (table 2) successfully restored these blood antioxidants to levels approaching or exceeding the normal values.

Serum levels of MDA and NO_x as well as CP activity in arthritic rats subjected to prophylactic and therapeutic intra-rectal application of ozone: As indicated in table 3 & 4, adjuvant arthritis caused a significant increase in serum levels of MDA, NO_x and CP activity after both 21 and 24 days of adjuvant inoculation. Data also, demonstrated that prophylactic intra-rectal application of O_3/O_2 mixture (table 3) succeeded to normalize serum MDA level of arthritic rats, but failed to exert any change in serum NO_x level of these rats. Meanwhile, O_3 pretreatment provided a further elevation of serum CP activity to a level exceeding the arthritic values. Therapeutic intra-rectal application of O_3/O_2 mixture (table 4) caused a significant reduction in serum MDA level to approach the normal value, together with further elevation of CP activity than the arthritic levels. Meanwhile serum NO_x of arthritic rats was not significantly changed in response to that therapy.

The results also clearly showed that intra-rectal application of O_2 (as a vehicle for O_3) in prophylactic and therapeutic treatment did not affect any of the measured parameters compared with the values of arthritic rats.

Table (1): Blood antioxidant levels in arthritic rats subjected to prophylactic intra-rectal application of O_3/O_2 mixture

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

Data are expressed as mean of (7) observations ± SDM
(a) Significant difference from normal group at P≤ 0.05
(b) Significant difference from arthritic (21 days) group at P≤ 0.05

Table (2): Blood antioxidant levels in arthritic rats subjected to therapeutic intra-rectal application of O_3/O_2 mixture

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

Data are expressed as mean of (7) observations ± SDM
(a) Significant difference from normal group at P≤ 0.05
(c) Significant difference from arthritic (24 days) group at P≤ 0.05

Table (3): Serum levels of MDA and NOx as well as CP activity in arthritic rats subjected to prophylactic intra-rectal application of O_3/O_2 mixture

Parameter / Groups	MDA nmol/ml	NOx nmol/ml	CP U/l
Normal Control	3.62 ± 0.36	23.2± 1.94	127.4 ± 17.04
Arthritic (21 days)	4.83± 0.8 [a]	33.4± 4.4 [a]	210.6± 31.05 [a]
Arthritic pretreated with: O_2 (vehicle for O_3)	4.26± 0.49	31.6± 5.28 [a]	217.9 ± 27.5 [a]
Arthritic pretreated with: O_3/O_2 mixture	3.3± 0.51 [b]	35.7± 4.36 [a]	282.1± 42.4 [a], [b]

Data are expressed as mean of (7) observations ± SDM
(a) Significant difference from normal group at P≤ 0.05
(b) Significant difference from arthritic (21 days) group at P≤ 0.05

Table (4): Serum levels of MDA and NOx as well as CP activity in arthritic rats subjected to therapeutic intra-rectal application of O_3/O_2 mixture

Parameter / Groups	MDA nmol/ml	NOx nmol/ml	CP U/l
Normal Control	3.62 ± 0.36	23.2± 1.94	127.4 ± 17.04
Arthritic (24 days)	4.7± 0.66 [a]	33.4±6.29 [a]	199.1± 36.4 [a]
Arthritic treated with: O_2 (vehicle for O_3)	4.12± 0.37	29.7± 2.88	187.6 ± 22.6 [a]
Arthritic treated with: O_3/O_2 mixture	3.43± 0.21 [c]	30.1± 3.95	252± 51.9 [a]

Data are expressed as mean of (7) observations ± SDM
(a) Significant difference from normal group at P≤ 0.05
(c) Significant difference from arthritic (24 days) group at P≤ 0.05

Discussion

The involvement of reactive oxygen species (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 (Yoshikawa et *at.,* 1985; Symons et al., 1988).

In the present study, overproduction of ROS in adjuvant arthritis 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 PrSHs. The marked increase in serum MDA observed in arthritic rats is in agreement with the results of Agha et al. (1999); Chamundeeswari et al. (2003) and Ramprasath et al. (2005) in arthritic rats, and Gambhir et at. (1997) in rheumatoid arthritis patients. Increased lipid peroxide formation in arthritic rats is exacerbated by the significant decline in blood antioxidants. Similar results about GSH were reported by Fahim et al. (1995) and Chilies et al. (1990) in arthritic rats and rheumatoid arthritis patients respectively. On the same line, a marked decrease in GSH concentration was observed in the joint articular cartilage of arthritic rats (Campo et al., 2003). 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 (Eklow et al., 1984). Moreover, the observed reduction in serum PrSHs is in harmony with the study of Kheir El-Din et al. (1992) and Fahim et al. (1995). Such reduction could be attributed to the excessive consumption by peroxide (Hall et at., 1984) and/or to a low serum albumin reported in other studies, since the greatest majority of serum SH (85-90%) are found in albumin (Thomas and Evans, 1975).

In the current investigation, the decline in blood antioxidants was, also, extended to include erythrocyte GPx and CAT activities. That observation is consistent with those of Chamundeeswari et al. (2003) and Ramprasath et al. (2005). A defective antioxidant enzyme machinery had been observed in erythrocytes of rheumatoid arthritis patients (Imadaya et at., 1988) and in liver, kidney and heart of adjuvant arthritic rats (Vijayalakshmi et al., 1997). The increased production of superoxide anion, H_20_2 and hydroxyl radicals demonstrated by Ramprasath et al. (2005) 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 (Jarvinen et at., 1995; Gad and Khattab, 2000).

The present results revealed a significant increase in serum NO_X level in arthritic rats. Such result is on line with the reported data of Gad and Khattab. (2000) and Ramprasath et at. (2005) in arthritic rats and Ueki et at. (1996) in rheumatoid arthritis patients. The over expression of iNOS in

arthritis might result from increased production of IL-1 and other pro-inflammatory cytokines characteristic of that disease (Jarvinen et at., 1995).

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 (Moak and Greenwald, 1984). In the present study, a marked elevation in serum CP activity was observed. Such elevation is consistent with the observation of Kheir El-Din et at. (1992) and Fahim et at. (1995) 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 (Denko, 1979). 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 (Moak and Greenwald, 1984).

In the present study, prophylactic intra-rectal application of 0_3 to arthritic rats over three weeks exerted protective effects on some important blood antioxidants (GSH, PrSHs, GPx and CAT) and preserved them to pre-arthritic values (table 1). The present data, also, demonstrated that therapeutic intra-rectal application of 0_3 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 0_3 therapy on blood antioxidants were accompanied by a decrease in serum MDA level to reach the normal levels (tables 3 & 4). These positive experimental observations could be explained in the light of ozone oxidative preconditioning (0_30P), a state obtained on judicious and controlled use of 0_3 (Leon et al., 1998). 0_30P is analogous to other phenomena such as ischemic preconditioning (Murry et at., 1990), thermal preconditioning (Neschis et at., 1998) and chemical preconditioning (Riepe and Ludolph, 1997). All of these processes have in common that a repeated and controlled stress is able to provide protection against a prolonged and severe stress.

Previous studies have demonstrated that 0_30P restored the levels of antioxidant defense constituents (GSH, SOD, CAT and GPx) and depressed lipid peroxidation in rat hepatic tissues after ischemia-reperfusion injury (Ajamieh et at., 2004), in renal tissues of rats subjected to acute and chronic renal failure (Borrego et at., 2004; Calunga et at., 2005) and in pancreatic tissues of diabetic rats (Al-Dalain et at., 2001).

A point that should not be overlooked is that 0_30P caused by judicious use of 0_3 is due to the fact that 0_3, once dissolved in the plasmatic water, instantaneously reacts with biomolecules generating ROS, among which are H_20_2 and lipid peroxidation products (LOPs). 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 LOPs can up-regulate the synthesis of antioxidant enzymes (Bocci et al., 2004). Interestingly, Iles and Liu (2005) have demonstrated that some LOPs 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 0_3 therapy. Erythrocytes have been shown to respond to 0_3 therapy with activation of glycolysis and pentose phosphate pathway (Bocci, 1996).

In the current study, up-regulation of erythrocytes GPx and CAT by 0_30P might be responsible for the preservation of blood GSH and serum PrSHs 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 side, prophylactic and therapeutic rectal applications of 0_3 therapy provided further elevation of serum CP activity than the arthritic levels (tables 3 & 4). That effect could be explained on the basis that 0_3 acts as a mild enhancer of immune system through activation of gene/regulatory nuclear factor kappa B (NF-κB) by H_20_2, one of the major decomposition products of 0_3. 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 (Bocci, 2006). The increased CP activity might reflect improved antioxidant status of animals subjected to 0_3 therapy. Moreover, 0_3-induced increase in CP activity could be beneficial to guard against oxidative stress observed in adjuvant 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 the present study is the inability of 0_3 therapy to change the serum NO_x than the arthritic level; such effect might be related to the stimulatory effect of 0_3 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 (Pawloski et al., 2001). Thus, formation of S-nitrosothiols in response to 0_3 therapy may allow more pharmacological effects at distant sites.

Conclusions

It can be concluded that 0_3OP 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 0_3 therapy in rheumatoid arthritis at biochemical and clinical levels.

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