The Additive Beneficial Antioxidant Effect of Ozone Therapy to Xanthine Oxidase Inhibitor in Experimentally-Induced Hyperuricemic Rats

SAFAA EL-KOTB, M.D.* and REHAM GALAL, M.D.**
The Departments of Physiology* and Biochemistry**, Faculty of Medicine, Menoufiya University

Abstract
The high serum uric acid level is termed as hyperuricemia, hyperuricemia has been demonstrated to play a pivotal role in the pathogenesis of various cardiovascular disorders and renal diseases. Because ozone therapy can activate the antioxidant system, influencing the level of antioxidant enzymes and some markers of endothelial cell damage, medical ozone treatment may be of help in the treatment of hyperuricemia. The aim of this study was to demonstrate the additive effect of ozone therapy to xanthine inhibitor on hemodynamic parameters, renal dysfunction and oxidative stress markers in hyperuricemic rats. Rats were subdivided into 4 groups: 1- Control rats, 2- Hyperuricemic non-treated rats, 3- Hyperuricemic allopurinol-treated rats and 4- Hyperuricemic allopurinol + ozone-treated rats. At the end of the experiment, all rats were fasted for 12 hours. Systolic blood pressure (SBP) was measured, retro-orbital blood samples were collected for estimation of erythrocytes superoxide dismutase (SOD) activity, plasma malondialdehyde (MAD), serum uric acid (UA), serum urea and creatinine, the animals were then scarified. Renal artery was exposed for direct estimation of renal blood flow velocity (BFV) and renal vascular resistance (RVR). Ozone therapy significantly lowered serum UA, the arterial blood pressure, renal vascular resistance, and the plasma (MAD), also elevated significantly the erythrocytes (SOD) activity by its anti oxidative effect and improved renal blood flow velocity in hyperuricemic rats.

Key Words: Ozone – Hyperuricemia – Allopurinol – Antioxidant and SOD activity.

Introduction
HYPERURICEMIA, characterized by abnormal high levels of uric acid, is a common metabolic disorder with a worldwide distribution [1]. Mild hyperuricemia induced by uricase inhibition causes intrarenal oxidative stress, which contributes to the development of the systemic hypertension and the renal abnormalities induced by increased uric acid. Scavenging of the free radicals in this setting attenuates the adverse effects induced by hyperuricemia. Currently, attention is focused on oxidative stress as one of the major determinants of endothelial dysfunction and cardiovascular senescence [2]. Evidence for microvascular oxidative stress in animals with hypertension has recently accumulated [3]. It is hypothesized that imbalance between production and scavenging of superoxide anion increases oxidative stress and results in hypertension [4]. Elevated uric acid levels must be both a direct and an indirect cause of renal disease and cardiovascular disease. It is not known whether lowering uric acid levels with allopurinol will be effective in people with more severe or longstanding hypertension as compared with those in the preliminary studies cited. Nor do we know whether the beneficial effect of allopurinol observed in completed and preliminary human studies is due to the reduction of uric acid or to a reduction in xanthine oxidase-associated oxidants. Hyperuricemia has been linked to cardiovascular and renal diseases, possibly through the generation of reactive oxygen species (ROS) and subsequent endothelial dysfunction. The enzymatic effect of xanthine oxidase is the production of ROS and uric acid. Studies have shown that inhibiting xanthine oxidase with allopurinol can reverse endothelial dysfunction. Oxygen-ozone therapy is one of the different minimally invasive treatments currently available. Ozone has been used, as a complementary therapeutic agent, in several unrelated pathologies. Parental administration of ozone may represent the key to solve some medical problems when ordinary medicine has failed to do so [5]. Ozone is ten folds more soluble than oxygen in blood, and cannot equilibrate because it reacts with biomolecules (ascorbic & uric acids, free cysteine, reduced glutathione and -SH groups of albumin) present in the plasma [6]. Blood exposed to ozone undergoes a “transitory oxidative stress” necessary to activate biological functions without harmful effects when it is used.
within its therapeutic window. The ozonation process represents an acute oxidative stress. However, it is not deleterious but is actually capable of eliciting a multiple useful biological responses and, possibly, can reverse a chronic oxidative stress due to aging, chronic infections, chronic ischemic conditions, diabetes, atherosclerosis, degenerative processes and hyperuricemia. Indeed, the ozone therapy is interpreted as a toxic but real-therapeutic shock able to restore homeostasis [7].

The aim of the present investigation is to evaluate the possible beneficial effect of ozone therapy on renal dysfunction in experimentally-induced hyperuricemic rats.

**Material and Methods**

**Animals:** This study was carried out on 32 adult male albino rats (150-200g). Animals were fed with standard laboratory chow and water ad libitum and housed in animal house of Menoufiya faculty of Medicine at (2012) under artificial light/dark cycle of 12h. The animals were divided into 4 groups (n=8 each): 1- C-rats were intraperitoneal (i.p) injected with saline in a dose of (250mg/kg B.W). 2- Hyperuricemic rats which were made hyperuricemic by injection of oxonic acid potassium salt dissolved in 0.9% saline solution in a dose of (250mg/kg B.W).i.p, [8] daily for 4 weeks. 3- Hyperuricemic- allopurinol- treated rats where hyperuricemic rats were treated with allopurinol (5mg/kg orally) [8] daily for 4 weeks. 4- Hyperuricemic-allopurinol-ozone-treated rats, where hyperuricemic rats were submitted to Allopurinol in the same dose used before combined with Ozone/ oxygen mixture (i.p) at doses of 1.2mg/Kg) [9]. The volume of gaseous mixture administered to each animal was approximately 2-2.5mL/daily/ three applications weakly for 4 weeks.

**Chemicals:** Chemicals used for preparation of Krebs- Hanseleit solution were purchased from Sigma (St Louis, Mo, U.S.A. oxonic acid potassium salt (Sigma-Aldrich Chemical Co. Steinheim, Germany), Allopurinol in form of zyloric tablet 100mg/ tab (GlaxoSmithline S.A.E. El Salam City, Cairo, A.R.E.). kits for estimation of serum UA (El-Gomhoria Company, Egypt). Kits for estimation of serum urea (El-Gomhoria Company, Egypt). Kits for estimation of serum creatinine (El-Gomhoria Company, Egypt). Kits for estimation of serum MAD (Biodiagnostic Company, Egypt) and Kits for estimation of erythrocytes SOD activity (Biodiagnostic Company, Egypt).

**Ozone injection:** 2ml of O2-O3 mixture at concentration of 100ug/ml; obtained from longevity ozone generator (EXT120, Canada) from ozone unit. Intraperitoneal ozone is applied by injecting 2cc of O2-O3 mixture one dose. The concentration of ozone used will be 100µg/ml. The output concentration was calculated from a special table depending on the flow rate of O2 and ozone concentration in the generator. A disposable syringe must be used in order to properly administer the ozone mixture. Each rat will receive 2mL/daily/ three applications weekly.

**Blood pressure measurement:** At the end of experimental protocol period (4 weeks), blood pressure was measured using rat tail sphygmomanometer (Harvard apparatus Ltd, Aden Berge, England) and a pneumatic pulse transducer (Harvard U.K.) [10], in conscious rats pre-warmed for 10 min in thermostatically controlled restrainer (XBP1000; Kent Scientific). Mean systolic blood pressure of at least 3 separate recordings on 3 occasions was estimated from the recorded graph [11].

**Blood sampling and biochemical analysis:** After measuring MSBP retro-orbital blood samples (each 2ml) were obtained through heparinized capillary tubes, samples were allowed to clot at room temperature in water bath for 15 minutes. The supernatant serum was collected in a dry tube [12]. Serum samples were used for estimation of uric acid, urea, creatinine, erythrocytic (SOD) activity and malondialdehyde (MAD) using Novaspectra Spectrophotometer (Shimadzu/Double beam Spectro-photometer U.V. 15 0, Germany).

**Measurement of renal blood flow velocity and renal vascular resistance parameter:** The rat was anaesthetized with urethane 25% in a dose of (0.6ml/100gm) by intraperitoneal injection. Then the animal was laid on its back and mid line laparotomy was made to expose the renal artery. After setting the mode of pulsed blood flow meter (Doppler) (Bi-directional blood flow meter with FFT-analysis (HADECO, Japan), ultrasonic gel was used on the probe top and turn the volume control to the maximum. The probe pressed softly to the measured area at an angle of 45-50°. After hearing the optimal sounds, wait for 5seconds without moving the probe then press the freeze key to freeze the waveform and recorded [13]. Data were printed on the graph for blood flow velocity and vascular resistance.

**Statistical analysis:** It was performed by Kruskal Wallis one -way ANOVA for multiple comparisons followed by fisher’s PLSD test. Values are expressed as mean±SD. Post-hoc Scheffe test was applied to identify the source of statistical
significance, $p$-values $<0.05$ were considered statistically significant.

**Results**

In the present work, induction of hyperuricemia produced significant changes in all measured parameters compared to the control group. Changes in serum UA, SBP, BFV, RVR, plasma MAD and erythrocytes SOD activity. The experimental animals were monitored for changes in different parameters that indicate the hyperuricemia is not associated with renal dysfunction. Serum urea and creatinine were insignificantly differ ($p>0.05$) compared to the control, allopurinol-treated and to allopurinol+ozone- treated group. Fig. (2-A,B).

Serum UA $3.60±0.1$, SBP $11.8±0.29$ and plasma MAD $19.96±1.4$ were significantly higher ($p<0.001$) and RVR $2.01±0.07$ was significantly higher ($p<0.01$) compared with the control group. Figs. (1,3,5-A,4-B respectively) while BFV $4.32±0.067$ and erythrocytes SOD activity $0.27±0.2$ were significantly lower ($p<0.01$) and ($p<0.001$) respectively compared with the control group. Fig. (2-A,B).

A significant decrease ($p<0.001$) in serum UA $1.49±0.9$ and $1.44±0.04$, SBP $102±2.91$ and $86.25±1.44$ and plasma MAD $10.6±1.1$ and $8.14±0.7$ and a significant decrease ($p<0.01$) in RVR $1.71±0.07$ and $0.93±0.02$ while a significant increase ($p<0.01$) in BFV $5.003±0.08$ and $6.02±0.11$ were observed in hyperuricemic-allopurinol treated and hyperuricemic allopurinol+ozone-treated group, respectively when compared with the corresponding values in the hyperuricemic group. Figs. (1,3,5-A,4-A,B respectively).

The hyperuricemic allopurinol treated group showed insignificant change ($p>0.05$) in erythrocytes SOD activity $0.55±0.06$ compared to the hyperuricemic group. The hyperuricemic allopurinol+ozone treated group showed a significant reduction ($p<0.001$) in erythrocytes SOD activity $1.29±0.20$ compared to the hyperuricemic group. Fig. (5-B).

![Fig. (1): Effect of Allopurinol and ozone treatment in experimentally induced hyperuricemic rats for 4 weeks on serum UA level on control group, hyperuricemic group, hyperuricemic+allopurinol group, hyperuricemic-allopurinol+ozone-treated group.](image1)

* : Significant when compared to the corresponding value in control group.

* : Significant when compared to the corresponding value in hyperuricemic group.

![Fig. (2-A): Effect of Allopurinol and ozone treatment in experimentally induced hyperuricemic rats for 4 weeks on serum urea level on control group, hyperuricemic group, hyperuricemic+allopurinol group, hyperuricemic-allopurinol+ozone-treated group.](image2)

![Fig. (2-B): Effect of Allopurinol and ozone treatment in experimentally induced hyperuricemic rats for 4 weeks on serum creatinine level on control group, hyperuricemic group, hyperuricemic-allopurinol group, hyperuricemic-allopurinol+ozone-treated group.](image3)
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Fig. (4-A): Effect of Allopurinol and ozone treatment in experimentally induced hyperuricemic rats for 4 weeks on SBP on control group, hyperuricemic group, hyperuricemic+allopurinol group, hyperuricemic+allopurinol+ozone-treated group.

*: Significant when compared to the corresponding value in control group.
**: Significant when compared to the corresponding value in hyperuricemic group.
***: Significant when compared to the corresponding value in hyperuricemic-allopurinol group.

Fig. (4-B): Effect of Allopurinol and ozone treatment in experimentally induced hyperuricemic rats for 4 weeks on RVR on control group, hyperuricemic group, hyperuricemic+allopurinol group, hyperuricemic-allopurinol+ozone-treated group.

*: Significant when compared to the corresponding value in control group.
**: Significant when compared to the corresponding value in hyperuricemic group.
***: Significant when compared to the corresponding value in hyperuricemic-allopurinol group.

Fig. (5-A): Effect of Allopurinol and ozone treatment in experimentally induced hyperuricemic rats for 4 weeks on serum MAD on control group, hyperuricemic group, hyperuricemic+allopurinol group, hyperuricemic-allopurinol+ozone-treated group.

*: Significant when compared to the corresponding value in control group.
**: Significant when compared to the corresponding value in hyperuricemic group.
***: Significant when compared to the corresponding value in hyperuricemic-allopurinol group.

Fig. (5-B): Effect of Allopurinol and ozone treatment in experimentally induced hyperuricemic rats for 4 weeks on erythrocytes SOD activity on control group, hyperuricemic group, hyperuricemic+allopurinol group, hyperuricemic-allopurinol+ozone-treated group.

*: Significant when compared to the corresponding value in control group.
**: Significant when compared to the corresponding value in hyperuricemic group.
***: Significant when compared to the corresponding value in hyperuricemic-allopurinol group.
Discussion

Oxonic acid induced hyperuricemia with preservation of renal function agreed with Mazzali et al., [14] who reported that administration of low doses of oxonic acid induced mild hyperuricemia (an increase of 1.5 to 2 fold in serum UA levels) without intrarenal urate crystal deposition that lead to acute renal failure. This was approved by Roncal et al., [15] and Sanchez-Lozada et al., [16] who stated that mild hyperuricemia did not affect renal function (assessed by serum blood urea nitrogen and creatinine levels) or cause proteinuria. Increase of systolic blood pressure in oxonic acid treated rats at 4 weeks showed a direct correlation with UA levels. Moreover, these results coincide with Feig et al., [17] who reported that UA causes hypertension in a rat model through the activation of the renin-angiotensin system, down regulation of no, and induction of endothelial dysfunction and vascular smooth muscle proliferation. Pathophysiological mechanisms by which high levels of UA can lead to hypertension have been explained in experimental animal studies. Blood pressure elevation was shown to be due to UA mediated systemic and renal vasoconstriction. Renal arteries are functionally constricted resulting in a decline in renal plasma flow, but are structurally normal [18].Hyperuricemia induces arteriopathy of preglomerular vessels, which impairs the autoregulatory response of afferent arterioles, resulting in glomerular hypertension [18]. With persistent and chronic hyperuricemia, hypertension is associated with the development of preglomerular arteriopathy and tubulointerstitial disease, the classic lesions of essential hypertension. Coupled with reported direct actions of UA on endothelial and vascular smooth muscle cells, these observations suggest that UA may induce microvascular disease independently of hypertension, decrease in the renal blood flow and increase in renal vascular resistance. Moreover, hyperuricemia promotes free radical formation, which stimulates the lipid peroxidation that increases the thickness of intima [19,20]. Furthermore, oxidized LDL has been implicated in the inhibition of endothelial no synthase transcription and expression [21]. Lipid peroxidation alters the structure and the fluidity of biological membranes, which ultimately affect vascular function [22]. In this study, regarding markers of oxidative stress, increase of plasma MDA as a marker of oxidant and decrease of erythrocyte SOD activity as a marker of antioxidant agreed with Haidari et al., [8] & Haidari et al., [23] who reported that in hyperuricemic rats the level of serum MDA as a biomarker of lipid peroxidation was statistically higher than that of normal rats and serum total antioxidant capacity (TAC) was statistically lower in hyperuricemic rats compared to normal rats. Also, they reported that UA was positively correlated with MDA level. This may be explained by that, the enzymes involved in UA production are also responsible for oxidative stress [24]. Increased UA level has been associated with increased production of oxygen free radicals; due to the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) that plays a pivotal role in progression of oxidative stress condition [25]. XO is a source of ROS and may explain the link between hyperuricemia and oxidative stress-induced diseases [25,26]. Concurrent administration of allopurinol (5mg/kg orally) with oxonic acid potassium salt daily for 4 weeks, caused significant lower mean values of serum UA, plasma MDA and systolic blood pressure but, insignificant difference in the mean values of serum urea & creatinine, SOD activity. The improvement in the mean values of serum UA and systolic blood pressure were consistent with Mazzali et al., [14] and Sanchez-Lozada et al., [27]. In this study, the significant decrease in the mean value of plasma MDA by concurrent administration of allopurinol disagreed with Haidari et al., [8,23] who reported that, allopurinol could not significantly change serum MDA level in oxonate induced hyperuricemic rats (250mg/Kg for 2 weeks). This may be explained by longer duration of allopurinol treatment in this investigation for four weeks instead of 2 weeks. On the other hand, insignificant increase in the mean value of SOD activity by concurrent administration of allopurinol concises with Haidari et al., [8,23] who reported that, allopurinol treatment could not significantly increase serum total antioxidant capacity. Parental administration of ozone may represent the key to solve some medical problems when ordinary medicine has failed to do so [8]. A reasonable interpretation of this state is that the presence of erythrocytes in the blood, in spite of ozonation, is able to regenerate antioxidants and quickly normalize TAC levels because erythrocytes can rapidly reconstitute the antioxidant reservoir [28]. Addition of ozone (1.2mg/kg B.W.) for 4 weeks revealed significant decrease plasma (MAD) and significant rise in erythrocytic SOD, the capacity of ozone to enhance antioxidant endogenous systems, in front of oxidative stress by oxidative preconditioning or adaptive mechanisms, has been demonstrated [29]. Therefore, these results suggest that ozone protective effects on antioxidant endogenous defenses improve glucose metabolism. These results were also supported by Al-Dalain et al., [30] who showed that with Ozone treatment there was a reduction of total peroxides and the concentrations of MDA.
and increase in antioxidant systems. Ozone can exert protective effects by oxidative preconditioning stimulating and/or preserving the endogenous antioxidant systems and by blocking the xanthine/xanthine oxidase pathway for reactive oxygen species generation [31]. Moreover, ozone oxidative preconditioning has been shown to preserve glycogen content and to reduce lactate and uric acid formation, controlling oxidative stress [32]. In line with the present results, Martinez et al., [33], conclude that peroxidation potential (PP) which represents a balance between antioxidant and pro-oxidant influences on serum lipids was increased with ozone treatment indicating that ozone treatment promotes a prevalence of antioxidant effects over those pro-oxidants improving the redox cellular status. According to Clavo et al., [34] the capacity of ozone to increase blood flow velocity is compatible with a decrease in vascular resistance. On the basis of the mechanism of action; ozone therapy can improves blood circulation and oxygen delivery to tissue owing to the concerted effect of no and an increased vascular resistance [35]; that could produce a decrease in the friction between the blood and the glomerular vascular walls, decreasing the flow resistance, increasing the RPF and GFR; The flow rise contributes to diminishing the endothelial injuries [36]. Adenosine, prostaglandins and, especially, nitric oxide released from endothelial could collaborate in affecting the micro-circulation and lead to a decrease in vascular resistance [37]. Combined therapy with allopurinol and antioxidants normalized all measured antioxidant enzyme protein expression and activities. Thus hyperuricemia associated reductions in antioxidant state can be ameliorated by xanthine oxidase inhibitor combined with antioxidant therapy. This can be explained on the bases of combination of medical actions of allopurinol and the ability of ozone to enhance antioxidant endogenous systems, in front of oxidative stress by oxidative preconditioning mechanisms.

References


