Ozone inhalation can induce chromosomal abnormalities in bone marrow cells of Wistar rats

Farhang Haddad*, Vajiheh Golami and Maliheh Pirayesh Shirazi Nejad

Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad

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Abstract

Due to the wide range of applications for ozone and its increasing use for medical and industrial purposes, studying its effects has become a very important line of research. The ozone has been suspected to be a carsinogen. Because of the increasing use of ozone, the human could be more and more exposed to this gas. In this study the effects of ozone inhalation on chromosomes and its clastogenic consequences have been investigated using *in vivo* micronucleus assay in bone marrow cells of treated rats.

Animals were treated for 6 hours a day at 3 ppm of ozone during 10 consecutive days. The micronucleus assay was performed immediately and 11 days after the last exposure. The frequency of micronucleated polychromatic erythrocyte of bone marrow (MNPCE) increased in both groups compared to the control. Such increase confirmed the clastogenic effects of ozone. The elevated frequency of MNPCE did not decrease after 11 days of the last ozone exposure.

Results indicate that ozone inhalation could induce persistent chromosomal damages even to bone marrow cells which were not in direct contact to it. Also, once more, the results confirmed the usefulness of the micronucleus assay in toxicological studies.

Key words: ozone inhalation, micronucleus assay**,** chromosomes

Introduction

Studying the substances and factors polluting the environment is getting more and more important in our industrialized life and environment. One of those widely used factors is ozone. Ozone is used for sterilization of operating rooms and surgical tools, as a direct or indirect antiseptic agent of drinking water and preservative of food. Also due to its very strong oxidative capability, it is considered as a very good agent in removal of natural organic substances of swimming pools (Matilainen, 2006; Murphy, 2006).

Because of its special chemical and physical properties, in aqueous solution, ozone is capable of producing free radicals which could cause wide range of damages to cells and tissues. Different studies have presented various results on the effects of ozone on living organism (Victorin, 1992).

Ozone is a very strong oxidant with the ability to interact with biomolecules. Its disintegration in aqueous solutions leads to formation of various free radicals of oxygen such as oxygen super oxide (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^0), and very active single oxygen of superoxide (O^0). These free radicals, in turn, can take part in secondary reactions which produce an oxidative stress (Victorin, 1992). Free oxygen radicals eventually destroy DNA by inducing cleavage in the deoxyribose-phosphate backbone and the chromosome breakages. Also ozone could directly react with DNA and destroy or modify its organic bases (Cataldo, 2006, Ito, 2005). The results of these changes in cells and tissues are oxidative destructions which are effective on aging, cell deformation, mutation, cancer, and eventually cell death and necrosis. In some particular concentrations ozone inhibits DNA replication. The tissue destruction caused by ozone is mostly due to its destructive effects on lipids of cell memebranes

* Corresponding Author: haddad@um.ac.ir

(Steinberg, 1990). It leads to decomposition of fatty acids of cell membrane and inflict serious damages to the airway cells (Leikauft, 1995). Analysis the ozone exposure on airway tissues of several test animal species and humans with regards to the ozone concentration suggest that time and frequency of exposure have a very important role on its induced-destructive effects (Ratto, 2006). The elevated level of ozone in living and working environments may cause serious damages to living cells and chromosomes of the individuals involved in such conditions, and may explain the increasing risk of subsequent lung cancer (Chen, 2006).

In nature ozone is found in concentrations of 0.01 to 0.05 ppm which reaches 10 to 20 ppm in the ozone layer high above the ground. In natural conditions it is produced as a result of solar radiation and thunder storms. The industrial methods of producing ozone are: using UV irradiation on pure oxygen or air and/or passing air or oxygen through electric arch chambers (Sundell, 1996).

Considering the destructive effects of ozone on cells and tissues, there has been extensive research on its mechanisms of inducing damages and effective doses. These studies have mostly performed on target tissues such as cells of pulmonary systems or treated cells in culture media (Leikauf, 1995; Ratto, 2006; Chorvatovicova; 2000). There has been no report concerning the effects of this gas on other tissues which are in no direct contact with it in *in-vivo* conditions.

Due to the extended uses of ozone and its known effects on tissues we decided to study the effects of ozone inhalation in long-term treatment on the chromosomes of rat bone marrow cells which are not in direct contact to inhaled ozone. In this study the micronucleus assay has been performed.

The *in vivo* micronucleus assay was introduced by Schmid in 1975. The *in vivo* micronucleus is able to reveal the structural and numerical chromosomal damages induced by physical or chemical stimuli. The *in vivo* micronucleus has significant advantages over analysis of metaphase chromosome. In terms of preparation and scoring the probable damages, this method is easier and faster than metaphase chromosomal analysis while keeping the accuracy intact (Heddle 1973). It is widely used in toxological study for analysis the effect of physical and chemical agents in our environment. In this method, any damages to chromosomes which may lead to chromosome breakage or loss, could be detected by scoring the small nucleous (micronucleus) in cytoplasm of the damaged cell (Heddle, 1991; Gocke, 1996; Hayashi, 1994; Mutsuki, 1993). The frequency of micronucleus reflects the rate of chromosomal damages. In *in vivo* systems this method is applicable to sample from different tissues such as skin, spleen, bone marrow, and blood (Abramsson-Zetterberg, 1999).

Materials and Methods

Male Wistar rats with the age of 7 to 8 weeks and weight of 250-300 grams were kept in animal house with the standard condition of 12h dark/12h light with the temperature of 20 ± 2 °C for a week to adapt before treatment. They were divided into three groups of control, treatment1 and treatment2. Total of 12 rats were divided into these three groups. All the experimental procedure were performed according to the Guide for the Care and Use of Laboratory Animals by National Academy Press Washington, D.C. in 1996.

Ozone treatment: To provide the required dose of ozone, the ozone producing chamber (Teb-e-Razi Mashhad) was used which could produce ozone by electrical discharge of the air. The dose of 3 ppm of ozone was used in this study. The calibration of 3 ppm ozone was performed by chemical titration of iodine released from KI solution (recommended by the manufacturer). Briefly, passing the ozone gas through KI solution resulted in releasing of iodine according to the following formula:

$$O_3 + 2KI + H_2O \rightarrow I_2 + 2KOH + O_2$$

The sodium thiosulfate is capable to scavenge the iodine from the above solution. Decrease of the free iodine would change the solution color from yellow to blue. The dose of ozone used to free the iodine from KI could be calculated by measuring the amount of sodium thiosulfate solution consumed to scavenge the iodine.

The treatment chamber with dimensions of 70×110×50 cm was used for ozone exposure. The samples were treated separately inside the chamber. The rats from each treatment groups 1 and 2 were exposed to 3ppm of ozone to inhale for 10 consecutive days and 6 hours daily from 12:00 to 18:00.

Sampling: Micronucleus assay were performed on control as well as treatment groups 1 and 2. The rats from treatment group 1 were sacrificed immediately after last ozone inhalation. The rats of the group 2 were sacrificed 11 days after the last ozone inhalation.

The micronucleus assay was performed according to Hayashi et al (1994), and Schmid (1975); briefly, rats were euthanized by chloroform inhalation. The femoral bone marrow cells were gently flushed out by a 5 ml syringe containing 3 ml fetal bovine serum (gibco) and smeared on clean slides. The smeared cells were left 24 h to air dry and fixed with absolute methanol for 5 min and stained according to May-Grünwald – Gimsa technique. Observations were made within 24 h. The coverslipped slides were blindly scored on coded slides at ×1000 magnification. At least 2000 polychromatic erythrocytes (PCEs) with or without micronuclei and normochromatic erythrocytes (NCE) were scored per slide. At least two slides per animal were scored. The ratio of micronucleated polychromatic erythrocytes (MNPCEs) to PCEs was calculated after simultaneously recording both PCE and MNPCE on each slide.

Statistical analysis: The statistical analysis was performed using software MINITAB. The differences between treated groups and control and also between treated groups themselves were analyzed by the one way analysis of variance (ANOVA).

Results

Treatment with ozone had a significant effect on increased frequency of micronucleus in bone marrow cells compared to the control group (Figures 1, 2). Micronucleus frequency in the control group was 1.12%. Long term treatment with ozone could significantly elevate the micronucleus frequency to 4.88% (Table 1). The rats treated with similar dose and time of ozone, the elevated frequency of micronucleus did not decreased even after 11 days of the last exposure. The frequency of micronucleus in treatment 1 and 2 did not show any significant differences.

In both treatment groups 1 and 2 the ratio of polychromatic erythrocytes to the total number of normochromatic and polychromatic cells was decreased significantly, representing the toxicity of the ozone inhalation in bone marrow cells (Table1). Comparing the values from the two treatment groups showed a significant increase for the treatment group 2, representing recovery from ozone treatment and returning to natural cellular conditions in bone marrow after 11 days of last exposure.

Table1: Frequency of MNPCE** in Rat Bone Marrow Cells

	MnPCE/100 PCE	PCE/NCE***+PCE
Control	1.12±0.29	54.45±0.092
Treatment 1	4.88*±0.88	46.34*±1.02
Treatment 2	5.62*±1.34	51.77*±0.88

*Significant difference with control (0.05)

** Micronucleated polychromatic erythrocyte

*** Normochromatic erythrocyte

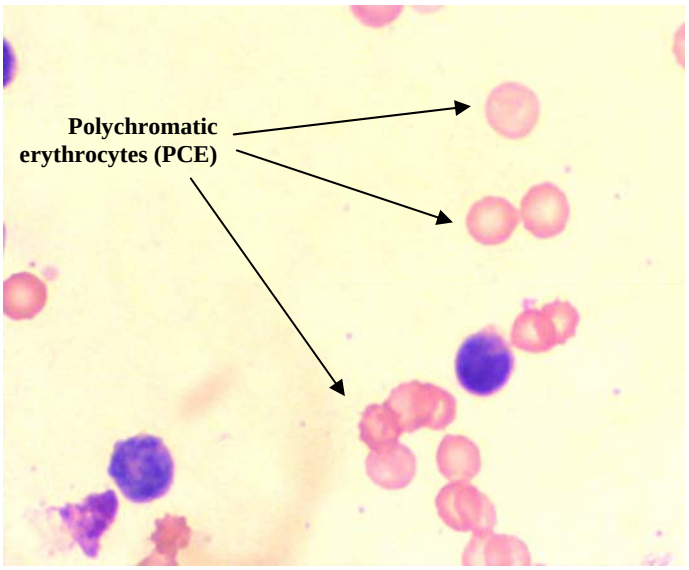

Figure 1: Bone marrow smear from control rat. PCE are stained light purple.

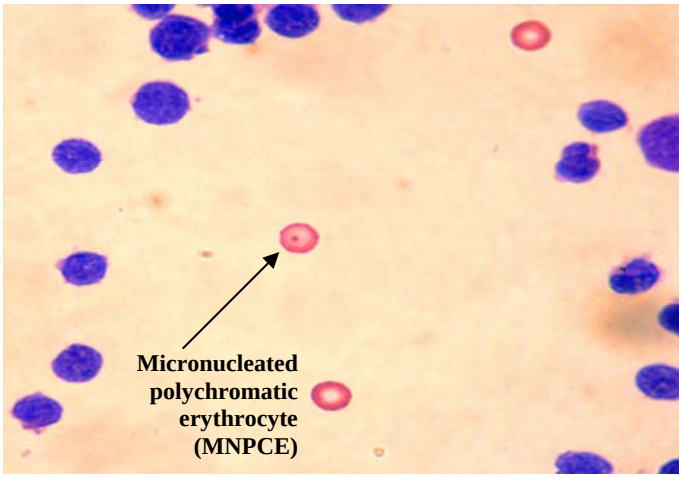

Figure 2: Bone marrow smear of treated rat. MNPCE is at the center.

Discussion

Due to the way of exposure of living organisms to ozone, a ubiquitous air pollutant, the study of clastogeneic effect of this gas in *in vivo* are mostly devoted to the cells and tissues directly exposed to it (Haney et al., 1999; Chorvatovicova, 2000). In this study the cytogenetic effects of long term ozone inhalation were analysied on bone marrow cells of rat. The cells studied here are not directly exposed to ozone.

The frequency of micronucleus in PCE of control rats was 1.12. In toxicological studies on rats the reported frequency for control group covers a wide range. Such studies report frequencies of 0.01 to 1 percent (Suzuki, 2006; Zhong, 2000). Therefore the observed base line micronucleus frequency in the present study is in the range reported by others.

In this study ozone inhalation clearly increased the frequency of micronucleus in polychromatic erythrocyte of rat bone marrow. In *in vivo* micronucleus assay increase in the frequency of MN represents the structural and numerical chromosomal damages in cells affected by the stimuli. Lost or broken parts of chromosome in cytoplasm of the cell form a small nuclei which is visible as micronucleus. Here the small size of MN suggests the structural damages to the chromosomes (Wakata and Sasaki, 1987). Due to its chemical and physical properties, ozone is capable of producing free radicals when it comes in contact with biologic systems (Victorin, 1992).

Free oxygen radicals may destroy DNA, cleave the deoxyribos-phosphate bonds of DNA, and break the chromosomes. Clastogenic properties of ozone through its ability to break DNA chain have already been established in different studies (Haney, 1999; Diaz-Liera, 2002; Bornholdt 2002). Increased frequency of micronucleus in rats of this experiment represents the ozone-caused structural damages to chromosomes by direct or indirect exposure to free radicals induced by long term ozone inhalation which could reach the bone marrow cells.

In-vitro studies of effects of ozone on leucocytes of peripheral blood have demonstrated temporary effects of ozone in inducing damages to the DNA. The clastogenic effect of ozone treatment was reversed when treatment stopped (Diaz-Liera, 2002). What is significant here and has not been reported before is the persistent harmful effect of long time ozone treatment. The stable frequency of induced micronucleus even after 11 days from last ozone exposure is a proof that the damages induced to chromosomes or bone marrow cells are some how permanent. It is expected that frequency of micronucleus will decrease when the induction of chromosome damages is stopped. It is reported in other studies that the effects of chromosomal damaging factors on micronucleus frequency are reversible and reduces as factors creating chromosome disorders are eliminated (Haddad et al., 2004; Malvandi et al., 2006). Reduction of micronucleus frequency after stopping the induced-damages to chromosome is due to the following reasons:

- Random integration of micronucleus to one of the main nuclei (Gustavino, 1994)
- Disintegration of micronucleus by cytoplasm nucleases (Granetto, 1996)
- Replacement and repairing of the tubulin reservoir and remaking of the dividing spindle needed for correct chromosome separation in the following cell division (Nichol, 1988)
- Activities of monitoring mechanism for preventing the division of damaged cells and induction of apoptosis in cells unable of repair (Sablina, 1998)

Due to the persistence elevated level of micronucleus frequency after 11 days of the last ozone exposure, it can be concluded that despite stopping the ozone exposure there are still factors inducing chromosome damages inside the bone marrow of the treated rats. Induction of chromosome damages, which is due to the attack of the free radicals to DNA, might be because of the deposit of free radicals in fat tissues of rats. The ability to induce clastogenic damages to bone marrow cells and the long lasting effects of ozone inhalation both refer to the existence of a mechanism that transfers ozone and/or ozone-produced free radicals to internal tissues of the body and continuously induces the damages to the chromosome structure.

Although more investigation regarding the analysis of the induced abnormalities to the tissues which are not in direct contact to inhaled ozone are required. In general the proposed model demonstrates that people who are exposed to ozone inhalation because of their jobs requirement may suffer chromosomal damages which may remain for long period of time. Thus care must be taken to not be exposed to ozone inhalation in particular cases such as planning for pregnancy.

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