Oxidative Damage of the Extracts of Condensate, Particulate and Semivolatile Organic Compounds from Gasoline Engine Exhausts on Testicles of Rats

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Abstract Oxidative damage induced by extracts of condensate, particulate matters and semivolatile organic compounds from gasoline engine exhausts were investigated in testicles of adult Sprague-Dawley rats. The results showed that gasoline engine exhaust could increase the contents of malondialdehyde and carbonyl protein, decrease activities of superoxide dismutase and glutathione peroxidase, and induce DNA damage in testicle of rat. Taking together, the gasoline engine exhaust could promote oxidative damage of bio-macromolecular in testicles of rat and oxidative stress might be an alternative mechanism for male reproductive function of male mammals.

Keywords Gasoline engine exhaust · Testicle · Oxidative damage

With rapid development of transportation, the health impacting by air pollution of vehicle emissions has become a more and more serious problem in many cities throughout the world. Epidemiologic studies have proved that long-term exposure to automobile exhausts might be associated with increasing in the risk of pulmonary and extra-pulmonary cancer as well as non-cancer health effects in the general population (De et al. 2003; Guo et al. 2004). Recently study found that vehicle emissions can lead to reproductive toxicity, such as disrupting sex hormones secretion, decreasing daily sperm production and motility, as well as influencing activity of testicular hyaluronidase, inhibiting spermatogenesis, increasing sperm aberration rate and changing Leydig cell ultrastructural in mammal (Watanabe and Oonuki 1999; Takeda et al. 2004; Yoshida et al. 1999; Ono et al. 2007) etc. The investigation results basis on male traffic police or tollgate workers were consistent with the animal experimental ones (Eibensteiner et al. 2005; De et al. 2003; Pei et al. 1993; 1994).

To our knowledge, little is known about the reproductive toxicity of gasoline engine exhausts. Due to the different engine structures and fuel used there might be some difference between the chemical components and physical characteristics in the exhaust. It is possible that gasoline and diesel engine exhaust might exert different biological effects. At present, gasoline engine exhaust had become the major air pollutants in many urban environment atmospheres instead of diesel engine exhaust (Alan and Gertler 2005). More and more studies are focusing on the effects of gasoline engine exhausts on reproductive organs of mammal and humans. Zhong et al. (2003) found that automobile exhaust could induce the contents of reactive oxygen species (ROS) and malondialdehyde (MDA) increase in testicles of mice by inhalation exposure. However, the effects of the extracting of condensate, particulate matters and semivolatile organic compounds from gasoline engine exhausts on protein and DNA in testicles of rats has not been reported. Previously, we learned that induced oxidative damage to biomacromolecules in the brain and lung of
rats exposed to the extracts of condensate, particulate and semivolatile organic compounds from gasoline engine exhausts (Che et al. 2007). The aim of this study was to investigate genotoxic and oxidative damage effects of an intratracheal instillation of the extracts of gasoline engine exhausts in testicles of rats. Biomarkers of oxidative stress included lipid peroxidation, protein oxidation, antioxidant enzyme activity, and DNA single strand breaks were determined in the testicles. We founded the extracts from gasoline engine exhausts could promote lipid peroxidation, oxidative protein damage, DNA single strand break, and inhibit activities of antioxidant enzyme.

Materials and Methods

Chemicals were obtained from the following sources: thiobarbituric acid (TBA), 1, 1, 3, 3-theramethoxypropane (TMP) and 5, 5-dithiobis (2-nitro-benzoic acid) from Sigma-ALDRICH Inc (St. Louis, USA). Normal melting point agarose (NMP) and low melting point agarose (LMP) from Amresco (Solon, USA). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals used including solvents were analytical in grade.

The extracts of condensate, particulate matters and semivolatile organic compounds from gasoline engine exhausts (EGE) were collected and prepared by the method of Che et al. (2007). For the composition of the extracts of gasoline engine exhausts, see Che et al. (2007).

Forty healthy adult male Sprague-Dawley rats (8 weeks of age, weight 190–220 g) were obtained from Laboratory Animal Center of Sichuan University (Chengdu, China). Animals were housed in stainless-steel cages in temperature and humidity regulated air-conditioned room under standard laboratory conditions (12 h light, 12 h dark and (23 ± 3)°C). Body weights were assessed once a week. The protocol of this study was approved by the local Ethics Committee. All experimental procedures were conducted in accordance with the Guide to the Care and Use of Laboratory Animals. The rats were fed with standard commercial rat diet (pellet form, in the sack, laboratory animal center of Sichuan University, Chengdu, China). Feed and tap water were provided ad libitum. Animals were acclimated for 7 days prior to experimentation. The rats were randomly divided into five groups, each group containing eight rats. Various doses for toxicological testing were prepared by diluting 200 L/mL stock solution of extracts with physiological saline. The final volume for instillation was 0.3 mL/rat. The extracts of gasoline engine exhausts which were suspended in 0.3 mL physiological saline were intratracheally instilled to rat lungs at the doses of 5.6, 16.7 and 50 L/kg. The blank and solvent control groups were instilled with 0.3 mL/rat physiological saline and 0.25 mL/kg body weight DMSO with the same pathway respectively. Every group received an intratracheal instillation once a week for 4 weeks. 24 h after the last instillation rats were anesthetized (sodium pentobarbital, 40 mg/kg, ip.) and sacrificed for the experimental procedures.

Lipid peroxide was measured by thiobarbituric acid test for MDA according to a modified method of Ohkawa et al. (1979). Procedure was as follows: 0.1 mL 10% tissue homogenate of testicle was added 0.2 mL 8.1% SDS, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5, and 1.5 mL of 0.8% aqueous solution of TBA. The mixture was made up to 4.0 mL with distilled water, and then heated in water bath at 95°C for 60 min. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured, TMP was used as an external standard, and the level of lipid peroxides was expressed as nmol MDA/mg protein.

The levels of protein carbonyls (CP) were analyzed by 2, 4- dinitrophenylhydrazine (DNPH) method as described by Levine et al. (1990). Briefly, 300 μL of tissue supernatant was pipetted into the tubes, to which 300 μL of 10 mM DNPH in 2N HCL was added. Blank was made by adding only 2 N HCL. Samples were then incubated at room temperature for 1 h, stirred every 10 min, then the proteins were precipitated with 10% trichloroacetic acid (final concentration) and subsequently washed three times with 1 mL ethanol/ethyl acetate (1:1, v/v). The pellet was dissolved in 1.0 mL of 6 M guanidine HCL in 10 mM sodium phosphate buffer (pH 2.3). Insoluble debris was removed by centrifugation. The difference in absorbance at 366 nm between the DNPH-treated sample and the HCL treated control was determined. Results were expressed as nmol carbonyl protein/mg protein based on an extinction coefficient of 22.0 mM⁻¹ cm⁻¹ for aliphatic hydrazones.

The activity of SOD and GPx was determined spectrophotometrically according to the method of the Nanjing Jiancheng Bioengineering Institute with a spectrometer. SOD activity was assayed spectrophotometrically at 550 nm by use of a xanthine and xanthine oxidase system. One unit of SOD activity was defined as the amount of SOD required for 50% inhibition of the xanthine and xanthine oxidase system reaction in 1 mL enzyme extraction of per milligram of protein. GPx was assayed spectrophotometrically by use of glutathione as substrate by measurement of the decrease of enzymatic reaction of glutathione (except the effect of nonenzymatic reaction) at 412 nm. One unit of GPx activity was defined as the decrease amount of 1 nmol/L glutathione (except the effect
of non-enzymatic reaction) in system of enzymatic reaction of 1 mg protein per minute.

The alkaline version of the comet assay was carried out according to the procedure of Singh et al. (1988) with some modifications. Briefly, the single-cell suspensions were mixed with 0.6% molten LMP at 37°C and spread on the ground microscope slides precoated with 0.8% NMP. The slides were covered with a coverslip and allowed to solidify for 10 min at 4°C; then the coverslips were removed and the slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA, pH 10, 1% Triton X-100, 10% DMSO; the last two components were freshly added) for 1 h at 4°C. After lysis, the slides were subjected to freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 30 min to allow DNA unwinding before electrophoresis. To electrophorese the DNA, an electric field of 25 V and 300 mA was applied for 30 min. All of these steps were conducted in the dimmed light to prevent additional DNA damage. After electrophoresis, the microgels were neutralized in distilled water for three times. The DNA was stained with 30 μL of ethidium bromide (20 μg/mL) just prior to analysis. The comets were analyzed at 200× magnification in a fluorescence microscope (Leica, Germany) attached to digital camera (Nikon, Japan) and connected to a personal computer. Two hundreds randomly selected cells were scored from each slide (two slides per dose) and rate of caudate cells was calculated. Quantification of the DNA damage from each slide (two slides per dose) and rate of caudate cells were analyzed at 200× magnification in a fluorescence microscope. The DNA migration length was estimated by measuring DNA migration length (μm) according to the equation: DNA migration length or tail length (μm) = (total length of the comet) − (head length). Tail length of 30 randomly selected caudate cells was measured by calibrated reticule to evaluate the length of DNA migration.

The protein contents in homogenates of testicle were examined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Data were expressed as the mean ± standard deviation. One-way analysis of variance was used for statistical difference between exposure groups and the control group. p < 0.05 was considered significant difference.

Results and Discussion

The mean body weight gain and organ coefficient of testicles were not different in rats exposed to extracts of gasoline engine exhausts compared with blank or solvent control groups during the experimental period (data not shown). No deaths, morbidity, or distinctive clinical signs were observed after any treatment with EGE.

Table 1 showed the results of the contents of MDA and carbonyl protein in testicles of the rats exposed to EGE and the controls. EGE exposure at all tested doses caused an increase in contents of MDA. Compared with solvent control, the proportions increased 4.7%, 30.5% and 54.2%, respectively. There has significant difference between 16.7 and 50.0 L/kg of MDA dose (p < 0.05). In addition, there was a dose-response relationship between contents of MDA in testicles and exposure doses.

For CP contents, the carbonyl protein contents in testicles of rats exposed to 16.7 and 50.0 L/kg of EGE have increased significantly by 172.8% and 574.8% relative to the solvent controls. In addition, compared with solvent control, there was no statistical significance in testicles at 5.6 L/kg treatment groups.

Results of SOD and GPx activities in testicles were presented in Table 2. In contrast to MDA and carbonyl protein, SOD activities progressively decreased with the increase of EGE doses. In addition, there were significant differences in testicle SOD activities between all three treatment groups and solvent group (p < 0.05).

GPx activities showed a different profile. All groups were treated by EGE, the SOD activities of testicles were significantly modified with the solvent control. But for
Data are expressed as mean ± standard deviation (n = 8). The rates of tailed cells and length of DNA migration are expressed as percentage of testicle cells and micron respectively. Significantly different from solvent control without gasoline engine exhausts exposure were analyzed by ANOVA at *p < 0.05.

Table 3 Effects of gasoline engine exhausts on DNA damage in cells of testicle in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Rates of tailed cells (%)</th>
<th>Lengths of DNA migration (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>12.6 ± 3.4</td>
<td>20.6 ± 8.3</td>
</tr>
<tr>
<td>Solvent</td>
<td>7.4 ± 3.1</td>
<td>35.8 ± 6.3</td>
</tr>
<tr>
<td>5.6 L/kg</td>
<td>10.7 ± 2.4</td>
<td>25.4 ± 9.2</td>
</tr>
<tr>
<td>16.7 L/kg</td>
<td>50.1 ± 7.6*</td>
<td>43.3 ± 11.5</td>
</tr>
<tr>
<td>50.0 L/kg</td>
<td>83.2 ± 9.0*</td>
<td>86.8 ± 16.7*</td>
</tr>
</tbody>
</table>

DNA damage in testicles was summarized in Table 3. After exposure to EGE, the dose-dependently relationship being in rates of tailed cells. EGE at the dose of 16.7 and 50.0 L/kg caused a significant increase in rates of tailed cells in testicles of rats compared with the solvent group. But at low dose (5.6 L/kg) the increase of rates of tailed cells was not statistically significant.

Moreover, for the length of DNA migration in tailed cells from testicles, at the dose of 50.0 L/kg group has statistical significance in comparison to the solvent control.

Gasoline engine exhausts is one of the most important pollutant mixtures which contains many known or suspected carcinogens. Owing to automobiles fueled with gasoline are widely used in China, the gasoline engine exhausts have been the most mainly atmospheric pollutant in many cities. Its adverse effects on health have been understood gradually. In this study, oxidative damage induced by EGE in testicles of rats was evaluated to explore the roles of EGE on reproductive toxicity of mammals. The results suggested that gasoline engine exhausts exposure could cause oxidative damage. The DNA damage exists in single strand breaks by testing of testicles of rats, which may be the mechanisms of reproductive toxicity with gasoline engine exhausts.

There was still no consensus about the mechanism of toxicity of gasoline engine exhausts. An alternative mechanism was correlated with the generation of reactive oxygen species (ROS). ROS possess high chemical reactivity with biological macromolecules, which may result in oxidative damage. Most of all, ROS was also known to play an important role in tumor promotional stage of carcinogenesis. ROS could attack DNA bases or deoxyribose residues to produce base oxidation, DNA adducts formation and single strand DNA break. It was possible that an accumulation of oxidative macromolecules damage would increase probability of mutagenesis and carcinogenesis.

The dose range from 5.56 to 50.0 L/kg was selected for the present study to examine the effects of rat testicles. According to vehicle emissions inhalation exposure guideline (EPA 2001), the maximum concentration should lie in the range of a ratio between 1:5 and 1:50 emissions to clean air, the minimum should be in the range of a ratio between 1:100 and 1:150. The mean values of rat tidal volume and respiration frequency respectively are 1.5 mL and 85 times per min. So 50.0 L/kg corresponded to exposure 8 h/day a ratio about 1:40 raw exhaust to clean air. However, gas inhaled would be largely trapped in respiratory tract and then cleaned in inhalation exposure. Based on the conclusion of Leong et al. (1998), the dose of 50 L/kg approximately corresponded to exposure to less than 14% dilution by nose-only inhalation. The concentration of benzo [a] pyrene was 5.9 µg/m³ in test gasoline engine exhausts. Based on these, the concentrations of benzo[a]pyrene in 50.0, 16.7 and 5.56 L/kg group were 826.0, 275.3 and 91.8 ng/m³, respectively. These were all far more than that of China or WHO’s limit values of atmospheric air of residential area (5 and 1 ng/m³). But in some cities in China, concentration of atmospheric benzo[a]pyrene was even up to 330 ng/m³. So the maximum dose probably coincides with some high occupational exposure level known to lead to acute poisoning, middle dose was close to reproductive toxicity in some occupational population (Pei et al. 1994).

MDA was a major aldehyde product of lipid peroxidation which was known to have deleterious effects on structure and function of cell membrane and also a marker of oxidative damage in vitro and in vivo, as well as in human studies. Our results showed exposure to gasoline engine exhausts at the doses of 16.7 and 50.0 L/kg significantly increased the levels of MDA in rat testicles. Consistent with our findings, Zhong et al. (2003) also showed that gasoline engine exhausts exposure by inhalation could induce ROS and MDA contents increase in testicles of mice. The gasoline engine exhausts was characterized by mixture with many organic chemical compounds. After respiratory exposure, some chemical compounds could be absorbed into the blood or other body fluids and induced oxidative damage by intracellular ROS generation either via auto-oxidation or by enzyme-catalyzed oxidation.

The increase in protein carbonyl levels was further evidenced that enhanced intracellular oxidative stress occurs in testicles of rats treated with gasoline engine exhausts. Protein carbonyls resulted from oxidative damage to proteins and were widely used as the biomarkers of oxidative stress. The possible sources of the carbonyl protein generation were as follows: ROS react with the side...
chains of lysine, arginine, proline, threonine and glutamic acid residues of proteins leads to the formation of carbonyl derivatives. Furthermore, aldehydes, such as 4-hydroxy-2-nonenal or malondialdehyde produced during lipid peroxidation could be incorporated into proteins by reaction with either the -amino moiety of lysine or the sulphydryl group of cysteine residues to form carbonyl derivatives. Carbonyl groups could also be introduced into proteins by glycation and glycoxidation reactions. Our results showed that carbonyl protein significantly increased at dose of 16.7 and 50.0 L/kg. Since carbonyl levels were relatively stable under nonpathological conditions, the increase of 172.8% and 574.8% indicated a significant intensification of intracellular oxidative stress in testicles. Therefore, these results suggested that the accumulation of peroxidized was over antioxidants and destroyed the balance of oxidative stress and protein oxidation, with the increase of concentration of gasoline engine exhausts.

SOD and GPx were principally protective enzyme against oxygen free radical-induced damage. The change in activities of the two antioxidative enzymes might predispose the testicles to increased free radical damage, because SOD could catalyze decomposition of superoxide radicals to produce hydrogen peroxide, and GPx, catalyzes the reduction of hydrogen peroxide to water, which should result in decrease of the formation of hydrogen radical. Under normal conditions, cells possess enzymatic and nonenzymatic defenses to cope with ROS, such as SOD, GPx and glutathione. Oxidative damage, might occur when antioxidative potential was changed and oxidative stress was increased. In this study, the treatment of gasoline engine exhausts lowered SOD at all test concentrations in testicles of rats. But for GPx, so did it only at 50.0 L/kg. The causes might be that chemicals from gasoline engine exhausts promoted ROS production and accumulation to deplete more antioxidants. Moreover, oxidative stress could modify enzyme proteins to make enzyme structural alteration and functional inactivation. These would result in decreasing of activities of the enzymes in tissues.

DNA breaks in testicle cells of rats treated with gasoline engine exhausts could be detected by using comet assay. This research indicate that rate of tailed cells and length of DNA migration were dose-dependently increased in cells of testicles of rats’ exposure to gasoline engine exhausts with intratracheal instillation, which was in agreement with that gasoline engine exhausts exposure increased the rate of sperm deformity in mice (He et al. 2003). The cause for the DNA damage resulting from gasoline engine exhaust was still unknown. ROS could react with DNA to produce damage. If the damage was not correctly repaired, it could lead to the formation of DNA single strand breaks and/or induction of mutations.

In summary, the present study had demonstrated that extracts of condensate+particulate matters+semivolatile organic compounds from gasoline engine exhausts could induce lipid peroxidation and oxidative protein damage, promoted DNA single strand break, and reduced the activities of SOD and GPx in the rat testicle tissue. These results implied that oxidative damage may be one of the mechanisms of action of reproductive toxicity of gasoline engine exhausts.

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References


