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Sodium chlorate induces DNA damage and DNA-protein cross-linking in rat intestine: A dose dependent study

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HIGHLIGHTS

- Oral administration of $NaClO_3$ induces DNA damage in rat intestine.
- $NaClO_3$ induces DNA-protein cross-link formation and DNA strand breaks.
- DNA fragmentation was observed from electrophoresis and colorimetric assays.

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ABSTRACT

Sodium chlorate ($NaClO_3$) is widely used in paper and pulp industries and as a non-selective herbicide. It is also a major by-product generated upon disinfection of drinking water by chlorine dioxide. In this study, we have investigated the genotoxicity of $NaClO_3$ on the small intestine of rats. Adult male rats were divided into 5 groups: one control and four $NaClO_3$ treated groups. The $NaClO_3$ treated groups were given a single acute oral dose of $NaClO_3$ (100, 250, 500 and 750 mg/kg body weight) and sacrificed 24 h later. Administration of $NaClO_3$ caused significant DNA damage in a dose dependent manner in the rat intestine. This was evident from the comet assay which showed DNA strand breaks and was further confirmed by agarose gel electrophoresis and release of free nucleotides. Increased DNA protein cross-linking in $NaClO_3$ administered groups showed formation of a critical lesion which hampers activities of proteins/enzymes involved in DNA repair, transcription and replication. Thus, oral administration of $NaClO_3$ induces DNA damage in the rat intestine, probably through chlorate induced production of reactive oxygen species.

1. Introduction

Sodium chlorate ($NaClO_3$) is a white crystalline compound that is commercially used to make chlorine dioxide. About 95% of chlorate use is in the bleaching of paper and pulp (Kaczur and Cawlfield, 1993). $NaClO_3$ is also used as bleaching agent in tanning and leather industry, dying and printing fabrics, uranium ore processing, rocket fuel oxidant, explosives and matches (McCauley et al., 1995). It is primarily used as defoliant and desiccant on rice, corn, soybean, sunflower, grain and wheat. $NaClO_3$ is widely used as non-selective herbicide since it enters the plant body through root absorption and kills it as it is toxic to all green parts of plant (Perry et al., 1994). Almost 56 active registered products contain $NaClO_3$ as the main ingredient (US EPA, 2008). Many incidents of animal poisoning due to accidental ingestion or inclusion in animal feed or improper disposal of herbicides containing $NaClO_3$ have been reported (Blakley et al., 2007; Murphy, 2002).

Chlorate is a major water disinfection by-product formed upon disinfection of drinking water by chlorine dioxide (Bolyard et al., 1993). About 30–50% of chlorine dioxide used as primary water disinfectant is converted to chlorate and chlorite (Tsai et al., 1995). In the presence of free chlorine, chlorite can be further oxidized to chlorate during the disinfection process (Baribeau et al., 2002). Chlorine has been replaced by chlorine dioxide as primary water disinfectant as it does not effectively kill all the microorganisms and produces trihalomethanes and haloacetic acids (Bellar et al., 1974; Michael et al., 1981) which are reported to be both mutagenic and carcinogenic (NTP, 1976). Although, $NaClO_3$ as

Abbreviations: DPC, DNA-protein crosslinks; EDTA, ethylenediaminetetraacetic acid; $NaClO_3$, sodium chlorate; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute 1640; PBS, phosphate buffer saline; SDS, sodium dodecyl sulfate.

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disinfection by-product is present at ppb levels (μg/L) it represents a chemical hazard for public health due to daily consumption which can lead to health problems.

Several studies on oral exposure to $NaClO_3$ in both humans and animals have been reported. The main symptoms/characteristics of chlorate poisoning are gastrointestinal irritation, methemoglobinemia, massive intravascular hemolysis, disseminated intravascular coagulation, cyanosis and renal failure (Abdel-Rahman et al., 1985; Singelmann et al., 1984). $NaClO_3$ also directly affects the proximal tubule of kidney, leading to necrosis (Couri et al., 1982; Thurlow et al., 2013). Animals which survive chlorate poisoning often die later due to chronic renal failure (Couri et al., 1982). Exposure to $NaClO_3$ has detrimental effects on thyroid homeostasis in rats (Hooth et al., 2001; McCauley et al., 1995). Chlorate also induced chromosomal aberration and micronuclei formation in mammalian cells and showed mutagenic activity in *Salmonella* (Richardson et al., 2007). Sodium and potassium chlorate are also found to be promoters of renal tumours (Kurokawa et al., 1985; WHO, 2000).

The widespread use of $NaClO_3$ in different public and agriculture fields has caused severe environmental pollution and potential health hazard. The increased risk of development of malignancies in occupationally exposed populations necessitates further studies in animal models to determine the mechanisms by which $NaClO_3$ induces genotoxicity. The major goal of this work was to evaluate DNA damage induced by $NaClO_3$ in rat intestine since toxicity mechanisms of $NaClO_3$ remain scarce, and there is a lack of information about its adverse effects on intestine.

2. Methods

2.1. Animal protocol

All animal husbandry and experimental procedures were conducted according to the institutional guidelines and were approved by the Animal Ethics Committee of Aligarh Muslim University that monitors research involving animals (R No: 714/GO/Re/S/02/CPCSEA). Male Wistar rats weighing 150–175 g were acclimatized for one week prior to the experiment. $NaClO_3$ (Sigma-Aldrich, USA), dissolved in drinking water, was administered orally to rats through gavage as single dose of 100, 250, 500 and 750 mg/kg body weight (McCauley et al., 1995). These doses of $NaClO_3$ are toxic but not lethal to the animals since they are well below the LD50 value of 1200 mg/kg body weight (Hayes, 1982); no animal died during the experiment. Control animals were not given $NaClO_3$ but an equivalent volume of water by gavage. Each group contained 6 animals who had free access to water and food during the acclimatization and experimental periods. All animals were sacrificed 24 h after the administration of $NaClO_3$ after anesthetization by ethyl ether. The entire small intestine was carefully removed, flushed with ice-cold physiological saline to remove debris and then completely slit open along its length. A part of intestine (1–2 cm from duodenum) was immediately suspended in RPMI medium for comet assay. The mucosa was scraped gently with a glass slide and used for the preparation of homogenates in suitable buffers for respective assays.

2.2. Isolation of DNA and agarose gel electrophoresis

A part of intestine (1 cm) was homogenized in ice-cold cell lysis buffer containing 100 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 10 mM Tris-Cl, pH 8.0 and 0.5% (w/v) sodium dodecyl sulfate (SDS). The proteins in homogenates were removed by three extractions with phenol-chloroform-isoamyl alcohol (25:24:1). The nucleic acids in clear supernatant were ethanol precipitated, resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8) buffer and then treated for 60 min with DNase-free RNase A (final concentration, 10 μg/ml) at 37 °C and then for 3 h with proteinase K (final concentration, 100 μg/ml) at 56 °C. After extraction with equal volume of tris saturated phenol-chloroform-isoamyl alcohol (25:24:1) the viscous aqueous phase was transferred to a fresh centrifuge tube. The samples were mixed with 1/10th volume of 3 M sodium acetate, pH 5.3, and DNA was precipitated with twice the volume of ethanol. The precipitated DNA was pelleted by centrifugation at 6500 rpm for 10 min at 4 °C. The DNA pellet was washed twice with 70% ethyl alcohol, air dried and dissolved in TE buffer (Evans, 1990). DNA samples (10 μg DNA/lane) were electrophoresed at 80 V on 0.7% agarose gel in Tris-acetate-EDTA buffer, containing 0.5 mg/ml ethidium bromide. DNA bands were visualized under a UV transilluminator and the gel was photographed.

2.3. Comet assay

The intestinal mucosa was removed immediately after sacrifice of animals and transferred into the Roswell Park Memorial Institute (RPMI) medium containing 1 mM EDTA. The solution was sieved through muslin cloth into Petri dishes to collect the cell suspension. The single cell gel electrophoresis (comet) assay was performed as described by Singh et al. (1988) with slight modifications. Briefly, 100 μl suspension of intestinal mucosal cells in RPMI medium was mixed with 75 μl of 0.5% low melting point agarose (LMA; Bio-Rad, Richmond, CA) in phosphate buffer saline (PBS) at 37 °C, layered on a pre-coated slide with 1% normal melting point agarose (NMA; Bio-Rad) in PBS and covered with another layer of LMA. A coverslip was immediately placed and the slides were kept at 4 °C for 10 min. After solidification of agarose, the coverslip was removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 10, with 10% dimethyl sulfoxide and 1% Triton X-100) for 1 h at 4 °C. After cell lysis, slides were placed in a horizontal gel electrophoresis tank with alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.5). The slides were left in the solution at 4 °C for 20 min in the dark to allow DNA unwinding and cleavage of alkali-labile sites. Electrophoresis was conducted at 4 °C for 20 min at 25 V (0.8 V/cm) and 300 mA. The slides were then rinsed gently 3 times with 0.4 M Tris-HCl, pH 7.5, to neutralize the excess alkali. Each slide was stained with 50 μl of ethidium bromide (20 μg/ml) and covered with a cover-slip. Fifty randomly selected cells (25 from each replicate slide) per sample were examined at 100× magnification, on a CX41 fluorescence microscope (Olympus, Japan). The comet tail length was recorded using an automated image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK).

2.4. Quantitative DNA fragmentation assay

DNA fragmentation was quantitated by the colorimetric diphenylamine assay (Burton, 1956). The intestinal homogenates were mixed with equal volume of buffer containing 20 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 7.5, and centrifuged at 15,000 rpm for 15 min at 4 °C to separate intact DNA in the pellet from fragmented/damaged DNA in the supernatant fraction. Perchloric acid (final concentration 0.5 M) was added to the pellet and supernatant samples, which were heated at 90 °C for 15 min and then centrifuged to remove precipitated proteins. The resulting supernatants, whether containing whole or fragmented DNA, were treated with 58.7 mM diphenylamine for 16–20 h at room temperature in dark and the absorbance was recorded at 600 nm. DNA fragmentation was expressed as the percentage of fragmented DNA to total DNA.