Mutation Research 743 (2012) 83–90

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Clastogenic and mutagenic effects of bisphenol A: An endocrine disruptor

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ARTICLE INFO

Article history:
Received 24 August 2011
Received in revised form 3 December 2011
Accepted 29 December 2011
Available online 9 January 2012

Keywords:
Bisphenol A
Endocrine disruptor
Clastogenicity
Mutagenicity
Oxidative stress

ABSTRACT

Bisphenol A (BPA) is a well-known endocrine disruptor (ED) which represents a major toxicological and public health concern due to its widespread exposure to humans. BPA has been reported to induce DNA adduct and aneuploidy in rodents. Recent studies in humans depicted its association with recurrent miscarriages and male infertility due to sperm DNA damage indicating that BPA might have genotoxic activity. Hence, the present study was designed to determine genotoxic and mutagenic effects of BPA using in-vivo and in-vitro *assays*. The adult male and female rats were orally administered with various doses of BPA (2.4 μg, 10 μg, 5 mg and 50 mg/kg bw) once a day for six consecutive days. Animals were sacrificed, bone marrow and blood samples were collected and subjected to series of genotoxicity assay such as micronucleus, chromosome aberration and single cell gel electrophoresis (SCGE) assay respectively. Mutagenicity was determined using tester strains of *Salmonella typhimurium* (TA 98, TA 100 and TA 102) in the presence and absence of metabolically active microsomal fractions (S9). Further, we estimated the levels of 8-hydroxydeoxyguanosine, lipid per-oxidation and glutathione activity to decipher the potential genotoxic mechanism of BPA. We observed that BPA exposure caused a significant increase in the frequency of micronucleus (MN) in polychromatic erythrocytes (PCEs), structural chromosome aberrations in bone marrow cells and DNA damage in blood lymphocytes. These effects were observed at various doses tested except 2.4 μg compared to vehicle control. We did not observe the mutagenic response in any of the tester strains tested at different concentrations of BPA. We found an increase in the level of 8-hydroxydeoxyguanosine in the plasma and increase in lipid per-oxidation and decrease in glutathione activity in liver of rats respectively which were exposed to BPA. In conclusion, the data obtained clearly documents that BPA is not mutagenic but exhibit genotoxic activity and oxidative stress could be one of the mechanisms leading to genetic toxicity.

1. Introduction

There is an increasing concern about the exposure to environmental estrogenic chemicals in a number of human health disorders viz. testicular cancer, precocious puberty, low sperm count, hypospadias, and cryptorchidism [1]. One such estrogenic chemical is bisphenol A (BPA), a synthetic monomer used in production of polycarbonate plastics, epoxy resins, food packaging, dental sealant and lacquers for food cans [2]. Human beings are exposed to BPA, as it leaches from the inner lining of tin cans and microwave containers during heating into the food materials [3], from dental sealant into saliva [4] and into beverages from the polycarbonate bottles due to the repeated usage or contact with any acidic/alkaline contents [5]. BPA concentration has been detected in human serum and in 95% of the urine samples obtained from a reference population in the USA [6]. The presence of BPA has been reported in maternal and fetal plasma [7], placental tissue [8] and in the milk of lactating mothers [9]. Higher levels of urinary BPA has been correlated with cardiovascular disease and diabetes and may be associated with increased risk of miscarriages with abnormal embryonic karyotype [6,10,11]. All these reports confirm that indeed human beings are getting exposed to BPA. This has raised a great concern regarding human health and environmental exposure to BPA.

Various animal models of BPA exposure have revealed multiple effects on the male and female reproductive system. Inhibition of the development of seminiferous tubule and spermatogenesis was observed after BPA exposure in male chick [12] and impaired semen quality in brown trout [13]. Exposure of adult male rats

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doi:10.1016/j.mrgentox.2011.12.023

with BPA resulted in decrease in sperm count and motility and also affected sperm morphology [14,15]. The oral exposure of pregnant mice to BPA at doses of 2–20 μg/kg/day caused enlargement of prostate gland, decrease in size of seminal vesicle and epididymis, and daily sperm production in male offsprings [16]. BPA has been associated with declined semen quality and increased sperm DNA damage among men from an infertility clinic [17]. The review of the literature and our own studies in rats confirmed that BPA is an endocrine disruptor and at low dose it causes long lasting organizational effects in response to developmental exposure which occurs on brain, male and female reproductive system and metabolic process [18,19]. The above mentioned reports are also supporting the fact of germ cell toxicity potential of BPA.

BPA was shown to induce aneuploidy and DNA adducts formation in Syrian hamster embryo cells [20]. It was found to disrupt the organization of the cytoplasmic microtubular complex, mitotic and meiotic spindle formation [21,22]. BPA was also reported to induce aneuploidy and chromosome congression failure in oocytes of mice accidentally exposed to low concentration of BPA [23]. BPA has been observed to induce micronuclei formation in organisms like mussel gills [24] and fish erythrocytes [25]. BPA has been evaluated in standard screens for mutagenicity including Ames test, mouse lymphoma, sister chromatid exchange and mammalian gene mutation assay and most of the results indicated that BPA is not mutagenic [26,27]. However, some reports have indicated that BPA has the potential to induce point mutation, double stranded DNA breaks and aneuploidy [28–30]. National Toxicology Programme has evaluated the carcinogenic activity of BPA and concluded that it was not a robust carcinogen in the context of adult exposure [31]. However, careful analysis of the same data documented several shortcoming of the NTP study with respect to effects observed on hematology of female mice and testicular tumor, age of animal as well as use of strain of rats and mice and their susceptibility to carcinogenic agent [32]. Recent studies have shown that prenatal exposure to BPA causes breast cancer in adult female rats [33] and hyperplasia of prostate in male rats resulting in greater risk of prostate cancer [34]. Moreover, BPA has the potential to form DNA adduct in both liver and mammary cells of female CD-1 mice [35]. Therefore, in order to label BPA as carcinogen one requires more indepth assessment of its genotoxic activity and mode of action.

As far as genotoxic and mutagenic effects of BPA are concerned, most of the studies are carried out in in-vitro systems, which do not mimic the in-vivo environment. Therefore, it is important to study the genotoxic activity of BPA in an in-vivo mammalian system. Currently there are few in-vivo genotoxicity studies carried out in bone marrow cells of mice upon BPA exposure at different time interval (one to five days), which document that BPA failed to induce chromosomal aberrations and micronuclei formation [36,37]. Based on a few in-vivo studies it is not possible to draw a definite conclusion about genotoxic activity of BPA as it is estrogenic in nature. Further, the susceptibility of the species and the sensitivity of the strains of animals used play a pivotal role in determining activity of such chemicals. These differences warrant the need for the further studies evaluating genetic toxicity in another rodent strain which is susceptible to estrogen. Such studies will be helpful in determining the human risk assessment to environmental estrogens.

Aim of the present study was to assess the possible genotoxic effects of BPA exposure by measuring the frequency of micronucleus (MN) in polychromatic erythrocytes (PCEs), structural chromosome aberrations in bone marrow cells and DNA damage in blood lymphocytes using single cell gel electrophoresis (SCGE or "Comet") assay while the mutagenicity was assessed by Ames assay. Furthermore, the levels of 8-hydroxydeoxyguanosine, lipid per-oxidation and glutathione activity were also estimated to decipher the genotoxic mechanism of BPA.

2. Materials and methods

2.1. Chemicals

Bisphenol A (~99% purity) was purchased from Sigma Chemical Company (St Louis, MO, USA). Dimethyl sulphoxide, nitro-o-phenylenediamine, methyl methanoate sulphate, 2-anthramine, ethidium bromide and low melting point agarose (LMP) from Sigma Aldrich (Germany). Giemsa stain, May–Gruenwald stain, colchicine powder from HiMedia (Mumbai). RPMI-1640 media and fetal bovine serum (FBS) from GIBCO (Grand Island, NY, USA), 8-OHdG (Bioxytech, USA). The *Salmonella typhimurium* strains used for Ames assay were procured by Dr. Maru (ACTREC), Kharghar, Mumbai. 1,1,3,3-Tetraethoxy propane, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) and 5,5-dithiobis (2-nitrobenzoic acid (DTNB) from Sigma Aldrich. Phosphoric acid, meta-phosphoric acid and N-butanol were purchased from Merck.

2.2. Animal handling and care

Healthy Holtzman adult male and female rats (8 weeks of age, body weight ≈ 250 g) that were randomly bred at the institutional animal house were used for the study. The animals were kept in cages with autoclaved paddy husk for bedding and maintained under standard laboratory conditions (14 h: 10 h dark/light cycle, a temperature of (22 ± 2 °C), and 50–70% humidity). The animals were fed a diet of soy-free, in-house-prepared rat pellets (consisting of crude protein, fiber and nitrogen free extract) and water (purified by UV and reverse osmosis) ad libitum throughout the study. The quality of food and water provided was routinely monitored by qualitative and quantitative analysis. The ethical clearance for the use of animals in the study was obtained from the institutional animal ethics committee. The experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India.

2.3. Dose selection

The doses of BPA in the present study were selected based on NTP 1982 report of acute, sub-acute and chronic toxicity study wherein these doses were considered to be safe. This document the overall oral no observed adverse effect level (NOAEL) for the BPA was considered to be 5 mg/kg bw/day, low observed adverse effect level (LOAEL) 50 mg/kg bw/day at which pivotal effects were significant reductions in adult body weight and pup body and organ weights [31]. BPA exerts its biological effects at very low doses similar to amounts typically found in environment, which give rise to much concern on the risk of human exposure to BPA [38]. The dose 2.4 μg/kg bw was decided as the environmental exposure dose, which was considered as a safe for human being on daily exposure [31]. Earlier studies by our group have demonstrated that 2.4 μg/kg and 10 μg/kg dose of BPA is capable to act as a reproductive toxicant [39,40]. Based on the above information following doses viz. 2.4 μg, 10 μg, 5 mg and 50 mg/kg bw respectively were selected for current study. The toxicity study was not performed as the doses were reported to be nontoxic in the animal study [31].

2.4. Dose preparation

BPA was dissolved in distilled ethyl alcohol (99.9% purity) and diluted with sesame oil to obtain a final concentration (2.4 μg–50 mg/kg bw) of BPA. The dose formulations were stored in an amber colored bottle and kept at 37 °C overnight. They were subsequently kept at room temperature throughout the study. Cyclophosphamide was dissolved in sterile distilled water (40 mg/kg bw) and used as positive control in all genotoxicity assays. Sesame oil was used as a vehicle control.

2.5. Treatment schedule

The animals were randomly selected and divided into six groups (groups I–VI) each consisting five male and five female rats respectively. The group I was administered with sesame oil and served as vehicle control. Groups II, III, IV and V were administered with different concentrations of BPA (2.4 μg, 10 μg, 5.0 mg, and 50 mg/kg bw) orally once a day for the period of 6 days. Group VI was administered with cyclophosphamide (40 mg/kg bw) which served as positive control. Micronucleus test and comet assay were conducted in same set of animals, whereas chromosome aberration test was performed in another set of animals having identical groups.

2.6. Micronucleus test (MN test)

The MN test was carried out in rat femoral bone marrow cells and frequencies of micronucleated-PCE were evaluated with slight modifications [41]. Animals were sacrificed by cervical dislocation, 24 h after the last dose administered. The femoral bone marrow cells were aspirated using syringe and needle (21 G) with 3.0 ml of FBS and centrifuged at 800 × *g* for 10 min. The supernatant was discarded, pellet was mixed, smeared on clean glass slides and fixed in methanol for 5 min. The fixed smear was stained with undiluted May–Gruenwald stain for 5 min followed by diluted