Bisphenol A induced reactive oxygen species (ROS) in the liver and affect epididymal semen quality in adults Sprague-Dawley rats

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INTRODUCTION

During the last two decades, it has become evident that environmental contaminants disrupt male reproduction in wildlife and humans and play an important role in the decline of quality and quantity of human semen. Bisphenol A [BPA: 2, 2-bis (4-hydroxyphenyl) propane] is a well known estrogenic endocrine disruptor used as a

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monomer in the manufacture of polycarbonate plastics; it is also released from epoxy resin lining of canned foods, beverages, dental sealants and a multitude of consumer products (Vandenberg et al., 2009). The detection of BPA in biological fluids like maternal plasma, fetal plasma, placental tissue, amniotic fluid and umbilical cord blood have indicated that it can easily transverse the placental barrier (Tsutsumi, 2005; Vandenberg et al., 2007). Numerous toxicological studies have shown that rodents exposed to BPA during the prenatal and perinatal period show a marked negative change in the reproductive system, including decreased epididymal weight and daily sperm production (vom Saal et al., 1998; Salian et al., 2009a,b), and an increase in prostate weight (Nagel et al., 1997). Similarly, BPA was also reported to significantly increase anogenital distance (AGD) and prostate weight, and decrease epididymal weight in postnatal offspring among CD-1 mice fed BPA at 50 µg/kg on day 16 to 18 of pregnancy (Gupta, 2000). Moreover, it interferes with the function of androgen receptors and the production of male sex hormones (Richter et al., 2007; National Toxicology Program, 2008). BPA has been shown to cause injury in the liver, kidney, brain, epididymal sperm in rodents and other organs by forming reactive oxygen species (ROS) (Bindhumol et al., 2003; Chitra et al., 2003; Kabuto, 2003; Kabuto et al., 2004). The liver has a range of antioxidant defense system. ROS are scavenged by the endogenous antioxidant defense system, including superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) in cells. ROS have been shown to play an important role in the defense mechanisms against pathological conditions, but excessive generation of free oxygen radicals may damage tissues (Kitas et al., 1991). The fundamental notion that spermatozoa could generate ROS, specifically hydrogen peroxide, was confirmed by Tasic and Walton (1946). However, Aitken and Clarkson (1987) demonstrated that the notion that oxidative stress might also be factor in the etiology of defective sperm function in our species (Alvarez et al., 1987).

BPA is currently a very controversial subject; any factor that exacerbates the production of oxygen free radicals in the mitochondria is a major source of oxidative stress and apoptosis among sperm cells. Several studies have shown BPA effect on the ARs, on male sex hormone levels, on male reproductive organs including testes, epididymal sperm and seminal vesicles and prostate gland, and on sperm production (Richter et al., 2007; National Toxicology Program, 2008). However, the mechanisms of the adverse effect on semen quality are not yet completely understood. The liver has a range of antioxidant defense systems. The purpose of this study was to evaluate the relationship between the presence of oxidative stress indicators in the liver during exposure to BPA and its effects on sperm quality.

MATERIALS AND METHODS

Animals

Twenty four healthy male Sprague-Dawley rats (50-days olds, weighing 170 to 185 g) were purchased from the Tongji Medical College Animal Laboratory (Wuhan, China) and kept in accordance with the Guide for the Care and Use of Laboratory Animals published by Ministry of Health of People’s Republic of China (Permit Number: 2011-s2456).

Treatments

The animals were housed in plastic cages under a well-regulated light and dark schedule (12 h light:12 h dark) at 24±3°C, humidity (50 ± 5%) environment, and free access to chow and tap water ad libitum. The rats were randomly divided into four groups, each group containing six rats. Each group (e.g. control group, low dose group, middle dose group and high dose group) was fed different doses of BPA: 0, 2, 10, and 50 mg/kg body weight, respectively in corn oil every other day by intraperitoneal injection for 20 days. After 20-days of treatment, the rats were sacrificed; the testes, epididymis, seminal vesicles and ventral prostates were removed, freed of the adhering tissues and weighed. Ethical clearance for the use of animals in the study was obtained from the Institutional Animal Ethics Committee prior to the initiation of the study, and the experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals published by Ministry of Health of People’s Republic of China.

Dose selection and preparation

The doses and time used for the present study were derived from published data (You et al., 1998; Yamasaki et al., 2009) and the results of our preliminary experiment. BPA was dissolved in a in corn oil to obtain the desired concentration of BPA dose range, that is, 0, 2, 10, and 5 mg/kg. An additional control group that received only corn oil. Dose formulations were well mixing and stored in crystal bottles at 37°C overnight and were subsequently kept at room temperature throughout the study. Solutions were mixed thoroughly before use.
Chemicals and reagents

BPA (2,2-Di (4-hydroxyphenyl) propane) was purchased from DR Co., Augsburg, Germany, purity: 98.5%. Corn oil was obtained from Sigma-Aldrich, St. Louis, MO, USA. Sigma Chemical Co. (St. Louis, MO) USA, Collagenase, Trypsin-EDTA were obtained from GIBCO (Grand Island, NY, USA), sodium lauryl sulphate from SRL, Eosin stain, Hematoxylin stain, Orange G stain from HiMedia (Mumbai). GSH-Px, MDA and SOD assay kit (Jiancheng Bioengineering Ltd., Nanjing, China).

Body weight and organ collection

The weight of each animal was recorded every two days and any gross abnormality was noted. The animals were fasted overnight, weighed and killed by cervical dislocation. Testes, epididymis, liver and other organs, were isolated from adhering tissues and weighed independently. The liver and testes were quickly frozen at -70°C for later use for biochemical assays, while epididymal sperm was used immediately for sperm analysis (CASA).

Parameters of oxidative stress

Glutathione peroxidase (GSH-Px) activity, SOD activity and malondialdehyde (MDA) level were measured. The liver was homogenized using lysis buffer (containing 1 mM Na2EDTA, 150 mM NaCl, 10 mM PMSF, 10 mM Tris, 1 mM aprotin). The homogenates was centrifuged at 10,000 rpm at 10 min at 4°C and the supernatant was recovered for use to evaluate oxidative stress following the protocol of GSH-PX, SOD and MDA assay kit (Jiancheng Bioengineering Ltd., Nanjing, China).

GSH-Px

GSH-Px activities were assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H2O2. One unit of GSH-Px was defined as the amount that reduced the level of GSH by 1 µM in 1 min/mg protein at 412 nm absorbance.

MDA

MDA level were assessed to determined the concentration of MDA, measuring thiobarbituric-acid (TBA) reacting substances at 532 nm. The level of MDA was expressed as nmol MDA per milligram protein. Protein content was measured according to Bradford method.

SOD

SOD activity in supernatant was determined by determining the reduction of nitro blue tetrazolium (NBT) by O2- produced from the xanthine-xanthineoxidase system. One unit of SOD was defined as the amount of protein inhibited in the rate of NBT reduction by 50%. Results were defined as U/mg protein.

Analysis of semen quality

Semen quality analysis was performed simultaneously using the CASA system (CFT-9200 computer-aided sperm and microorganism test and analysis system). After the animals were sacrificed, the epididymis was immediately removed and the tissues were minced with surgical scissors to extract the sperm cells into 2 ml of 0.9% NaCl solution at 37°C and kept for 15 min to allow the sperm to disperse. The sperms were counted with CASA to evaluate the specific parameters of sperm quality, sperm motility, density and motion including beat cross frequency VCL, straight line velocity (VSL), average path velocity (VAP), linearity (LIN=VSL/VCL), and straightness (STR=VSL/VAP). The CASA settings were followed according to the manufacturer’s instructions.

Morphology and sperm normality criterion

A small amount of sperm suspension was smeared on to a slide using a pipette and fixed with methanol; after drying for 10 min, it was stained with 2% Eosin for 1 h. Each of the stained slides was analyzed. The images were captured by a color by light microscopy (Olympus IX-71, Tokyo, Japan) for high quality image production. Morphological evaluation was accomplished on a monitor screen and the total calculated magnification was (x400). For a spermatozoon to be considered normal, the sperm head, neck, mid piece and tail must be considered normal. The head should be oval in shape. The percentage of normal sperm cells was calculated. It showed normal looking hook-shaped heads and the shape and thickness of the tail was thin uniform. Abnormal sperm cells included headless and hookless cells; amorphous shapes and forms; folded, short and double Y tail and other aberrations.

Statistical analysis

Data were presented as the mean ± standard error of mean (SEM) and were analyzed using the GraphPad PrismTM software version 5.0 (San Diego, USA) and SPSS statistical package 17.0 (SPSS Inc, Chicago, IL, USA). Comparison of means for treatment and control groups were done by independent-sample T-test. semen quality analysis was performed simultaneously using the CASA system (CFT-9200 computer-aided sperm and microorganism test and analysis system). Levels of significance were set at P ≤ 0.05.

RESULTS

The results are illustrated as shown in Figure 1. The body weights of BPA-treated rats did not show significant changes as compared to the corresponding control groups except for a slight difference with the low dose group. The same behavior was observed in testicular weight; in this case, a significant difference was observed (P < 0.05) (Figure 2). However, the weights of the liver, decreased significantly when the concentration of BPA was gradually increased to 50 mg/kg, P < 0.05 (Figure 3). Among the BPA treated rats, the activities of superoxide dismutase (SOD), malondialdehyde (MDA) increased significantly (**P < 0.01, *P < 0.05), respectively (Table 1). A dose dependent decrease in the levels of glutathione peroxidase (GSH-Px) was observed in response to BPA treatment when compared with the control group (**P < 0.01) (Table 1).

Effect of BPA on sperm counts

Figure 4 demonstrates the results obtained after exposure to BPA on epididymal sperm counts of adult male rats. Outcomes according to the percentage were strictly
strictly normal morphology. Total sperm counts were reduced at all doses, but whilst a significant decrease was observed at a dose of 50 mg/kg. The semen characteristics from a total of twenty four fresh semen samples were examined by CFT-9200 computer-aided sperm analyzer (Table 2 and Figure 5). The mean ± standard deviation (SD) of total sperm concentration, density, motility, and sperm motion variables (LIN=VSL/VCL and STR=VSL/VAP; P > 0.05) were analyzed by SPSS Student’s t-test.

**Table 1.** The effect of BPA on antioxidant enzymes, GSH-Px, MDA and SOD on rat SD liver tissues.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>GSH-Px (U/mg prot)</th>
<th>MDA (nmol/mg prot)</th>
<th>SOD (U/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>47.83 ± 4.08</td>
<td>2.47 ± 0.39</td>
<td>49.94 ± 2.82</td>
</tr>
<tr>
<td>Low 2 mg/kg</td>
<td>6</td>
<td>42.93 ± 2.83*</td>
<td>2.85 ± 0.18</td>
<td>54.81 ± 6.61*</td>
</tr>
<tr>
<td>Middle 10 mg/kg</td>
<td>6</td>
<td>37.13 ± 2.17*</td>
<td>3.30 ± 0.82</td>
<td>65.71 ± 8.74**</td>
</tr>
<tr>
<td>High 50 mg/kg</td>
<td>6</td>
<td>29.16 ± 2.35**</td>
<td>3.32 ± 0.72*</td>
<td>73.38 ± 6.97**</td>
</tr>
</tbody>
</table>

Effect of BPA on the activity of the antioxidant enzymes, glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and superoxide dismutase (SOD) in liver tissues. Data are presented as the mean ± standard deviation (SD). *Indicate significant change compared with control group, by means of Independent-Samples T test. *P < 0.05 versus control; **P < 0.01 versus control.

**Sperm morphology**

After observation under the microscope, a significant reduction in the number of normal sperm was observed compared to the control group (Table 2). Sperm analyses showed oligozoospermia (<20 × 10^6 spermatozoids/ml) and asthenozoospermia (progressive motility <50%) in all groups treated by BPA including control groups. Meanwhile, in the 2, 10, and 50 mg/kg dose groups, percentage of sperm normality decreased gradually to 15.00, 6.50 and 2.33%, respectively; compared with the control group, the differences were statistically significant (P < 0.05 and P < 0.01). Finding on sperm abnormalities showed that, headless sperm cells were the most common abnormality followed by amorphous cells, bent tail, coiled tail, pyriform head abnormal midpiece detached head and highly unusual double tail (Figures 4 and 5). Sperm with deformed heads were observed in all four groups,
Table 2. This table shows caudal epididymal semen characteristics in the experimental adult male rats SD using CASA CFT-9200.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>Low 2 mg/kg (n=6)</th>
<th>Middle 10 mg/kg (n=6)</th>
<th>High 50 mg/kg (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (M/ml)</td>
<td>21.23 ± 2.44</td>
<td>20.02 ± 1.51</td>
<td>12.35 ± 2.62*</td>
<td>9.33 ± 2.77**</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>55.21 ± 6.57</td>
<td>44.82 ± 9.86</td>
<td>34.72 ± 3.04**</td>
<td>32.51 ± 5.88**</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>40.30 ± 6.07</td>
<td>30.53 ± 7.67*</td>
<td>34.42 ± 3.66</td>
<td>34.89 ± 2.04</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>19.82 ± 6.15</td>
<td>15.65 ± 4.02</td>
<td>19.09 ± 6.17</td>
<td>18.41 ± 4.50</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>20.85 ± 6.08</td>
<td>17.96 ± 5.18</td>
<td>22.93 ± 6.35</td>
<td>21.56 ± 3.03</td>
</tr>
<tr>
<td>LIN</td>
<td>2.18 ± 0.75</td>
<td>2.05 ± 0.66</td>
<td>1.93 ± 0.48</td>
<td>1.98 ± 0.41</td>
</tr>
<tr>
<td>STR</td>
<td>2.05 ± 0.53</td>
<td>1.72 ± 0.17</td>
<td>1.57 ± 0.31</td>
<td>1.64 ± 0.17</td>
</tr>
</tbody>
</table>

Data represent as means ± SEM (n= 6 rats per group). *P < 0.05 and **P < 0.01 denotes significant difference compared with controls.

Figure 2. Effect of BPA on weight of the testis of adult SD rats. Data represent as means ± standard error of mean (SEM) (n= 6 rats per group).

*P < 0.05 denotes significant difference when compared with controls.

groups, but most notable in the groups treated with BPA (10 and 50 mg/kg). As related to tail abnormalities, some had no flagella, and others had proximal and distal cytoplasmic droplets.

DISCUSSION

It has been shown that BPA may have effect on liver enzymes and also affect sperm quality. Less is known about effects of BPA on the liver, and there are only a few animal studies done to show for instance formation of DNA adducts and impaired mitochondrial functioning (Ronn et al., 2013) exposure route. These studies are not compared with our study. Our study suggests that ROS may be associated with biochemical markers of liver damage. In this study, a weight gain was observed during the administration of BPA as well as testicular volume of rats which was statistically significant between low dose and control groups (P < 0.05). BPA has been reported to interfere with the function of Leydig cells resulting in a reduction of testosterone biosynthesis (Akingbemi et al., 2004).

In this study, it was observed that BPA treatment did not affect the body weight of rats except the low dose, and testis. In the liver, a decrease in weight of rats treated with BPA compared with those of control group was observed (*P < 0.05) (Figure 3). Thus, the tissues (liver)
antioxidant evaluation seems to have important role in the etiology of semen quality. The levels of enzymatic antioxidants GSH-Px, SOD and MDA activity were determined to evaluate the stability of ROS production in liver. In this study, the BPA (10 to 50 mg/kg) groups significantly increased in MDA and SOD in liver tissues. Whilst GSH-P decreased GSH-Px levels in liver tissues. This reduction in activities of antioxidant enzymes shows the failure of primary antioxidant system to act against free radicals. Decrease in the activity of GSH-Px indicates either reduced synthesis, may be elevated degradation or inactivation of the enzyme and excessive ROS production. So, the GSH-Px deficiency can result in the emergence of morphologic abnormalities in sperm cell mitochondria (Imai et al., 2001; Stradaioli et al., 2009). The increase in the activity of SOD, may be due to higher enzyme activity, but do not mean better antioxidative protection of spermatozoa. The over expression of SOD may reflect a defect in the development or maturation of spermatozoa, as well as sperm cellular damage, resulting in decreased sperm fertilization potential (Sinha et al., 1991; Gavella et al., 1996). The beneficial effect of SOD activity may concern only sperm movement, but has no influence on sperm count. It has been reported that there is relationship between the rate of lipid peroxidation and some morphological characteristics of spermatozoa, such as motility loss (Alvarez et al., 1987) or occurrence of midpiece defects (Rao et al., 1989; Aitken et al., 1993), which could explain the distortion level of sperm flagella in our study (Figure 5).

At doses 10 and 50 mg/kg of BPA, there was decreased epididymal sperm count which may have been due to increased MDA (Thiele et al., 1995; Bindhumol et al., 2003). It is well known that sperm cell membranes are rich in polyunsaturated fatty acids and are very susceptible to free radical attack. Lipid peroxidation affects the sperm concentration, motility, morphology and related with poor sperm quality. Increased MDA level might represent the pathologic lipid peroxidation of spermatozoa membrane and inhibition of sperm motility (Hsieh et al., 2006) which may corroborate our findings of the low percent active sperm motility in rats. MDA is one of the major end products of lipid peroxidation, especially the polyunsaturated fatty acid peroxidation. Lipid peroxidation is used to monitor the oxidative stress in cells and tissues and it is a well developed way of describing cellular injury which causes endothelial damage, vascular inflammation and cell membrane injury (Subermaniam et al., 2014). Other studies have shown that BPA in combination with carbohydrates can affect fat mass or liver fat content during prenatal and perinatal periods (Marmugi et al., 2012; Ronn et al., 2013). In rats, the main route of elimination of conjugated BPA is by biliary and fecal elimination which enables enterohepatic recirculation.
Figure 4. Effect of BPA on the epididymal sperm count of adult rats SD. (A) Outcomes according to the percentage strictly normal morphology. (B) Mean and standard error of normal sperm cells (%) of the semen of adult rats SD after 20 days treatment with BPA. Data represent as means ± SEM (n= 6 rats per group). *P < 0.05 and **P < 0.01 denotes significant difference compared with controls.

(Volkel et al., 2002). Atkinson and Roy (1995) have reported that BPA accumulates in fatty tissues and is metabolized to 5-hydroxybisphenol by Cytochrome P-450 dependent enzymes and further converted to 4,5-bisphenol-O-quinone. Cytochrome P-450 has been shown to induce ROS that permanently impairs sperm function thereby resulting in decline of sperm counts in men and laboratory animals. Cytochrome P-450 once activated, inactivates, and facilitates the excretion of most xenobiotics, thus modulating the intensity and duration of their toxicity (Aitken et al., 1989) such as drugs and environmental chemicals as well as endogenous compounds such as steroids and fatty acids (Hanioka et al., 2000).

It has been shown that CASA is likely to be of greater value in predicting male fertility than the routine semen examination (Suzuki et al., 2002). The common sperm parameters of CASA have shown significant correlation of sperm concentration in all groups treated with BPA against control group (Table 2). Observed values are below the values of references of semen analysis (Cooper et al., 2010). Sperm density <20 × 10⁶/ml, sperm motility <50%, VCL <70 µm/s in most cases except VSL >25 µm/s; this result is similar to the findings of a previous in vivo study on murine, but they are different with respect to the dose and time of exposure to BPA (Ashby et al., 2003; Bindhumol et al., 2003) and in humans (Meeker et al., 2010; Li et al., 2011). Majority of the epididymal sperm from adult rat had normal morphology (77.44%). This study strictly considered only the percentage normal morphology to be the outcomes. However, there was a decrease in the epididymal sperm count among the animals treated with BPA dose dependent manner (Figure 4). The morphological study showed abnormalities related to spermatozoon. The most abundant abnormalities were bent tail, coiled tail, detached head and a highly unusual double tail (Figure 5; M). The coiled tail was seen in control group and low dose group (Figure 5; C, L). An increasing number of kinked sperm were seen in these rats treated with BPA when compared with the control group (Figure 5, C). Additionally, an increase in detached head sperm was seen in rats treated with BPA of 50 mg/kg (Figure 5, H). These abnormalities may be attributed to damage of DNA by BPA during the process of spermatogenesis. This study provides toxicological evidence that exposure to BPA has an adverse effect on semen male rat. Also, the interesting remark in our study was the observation of significant difference in the sperm morphology between the groups treated with BPA against the control groups. The high prevalence of
oxidative stress in the spermatozoa may have effect on male infertility and implications in reproductive health. High ROS in the liver due to high dose of BPA could cause damage to sperm production and fertility and need to be taken into consideration when handling the interpretation of such results.

Conclusion

Conclusively, this study provides evidence that exposure of adult male rats to low dose of BPA induces oxidative stress in the liver, and impairs spermatogenesis through decreasing epididymal sperm count. However, the differences between humans and animals in terms of kinetics may make it difficult to transpose the effects observed in animals to humans directly. The analyzed semen parameters using CASA might be useful in planning the strategy of screening for semen quality. A clear understanding of the potential mechanisms of observed adverse effects of BPA exposure in the liver and on male reproductive organs including semen quality may help to explain the observed abnormalities and exploration of future treatments.

Competing interests

The authors declare that they have no competing interests.

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