Correlation between Epididymal Semen Quality Parameters and Malondialdehyde Levels in Adults SD rats Liver after Exposure to Bisphenol A.

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Abstract: It has been shown that reactive oxygen species (ROS) can lead to deleterious effects on a range of sperm parameters. Bisphenol A (BPA) has been shown to cause injury in the liver by forming ROS and increasing levels of lipid peroxidation. The aim of study was to determine the level of lipid peroxidation as indicated by Malondialdehyde (MDA) in the liver tissue of rats treated with BPA. BPA was mixed in corn oil and intra-peritoneal administered every other day for 20 days in dose dependent manner. After 24 h of the last treatment, rats were weighed, sacrificed and liver harvested for analysis. Epididymal semen quality analysis was performed simultaneously using the CASA system (CFT-9200 computer-aided sperm and microorganism test and analysis system). The levels of MDA increased significantly in the treatment group compared to control group (**P < 0.01) and had negative correlation with sperm count, motility and morphology (P<0.001). BPA caused a reduction in the epididymal semen quality and sperm count in a dose dependent manner. Sperm analyses results showed that there was oligozoospermia (<2 x10⁶ spermatozoids/ml) and asthenozoospermia (motility <50%) in the treatment group compared to control groups. These results indicate that exposure of graded doses of BPA may elicit depletion of MDA level and induce oxidative stress in epididymal sperm of rat thereby decreasing sperm count and quality. These findings provide a possible toxicological evidence of an adverse effect of BPA on semen quality.

Keywords: Bisphenol A (BPA, 2, 2-bis (4-hidroxyphenyl) propane); semen quality, sperm count, Liver, Oxidative stress, ROS, MDA.

1. Introduction

Sperm parameters and function evaluation is used to determine if the sperm have the biologic capacity to carry out the tasks necessary to access and fertilize ova and ultimately result in live births. There is growing evidence that damage to spermatozoa by reactive oxygen (ROS) may affect sperm function and antioxidant fight against such reactive oxidant species. 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 [2, 2-bis (4-hydroxyphenyl) propane] is a well-known estrogenic endocrine disruptor used as a 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 [1]. 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 [2,3], and an increase in prostate weight [4].

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) [5-8]. The liver has a range of antioxidant defense system. ROS are scavenged by the endogenous antioxidant defense system. Peroxidation of polyunsaturated fatty acids has been implicated in a wide of pathological conditions including infertility and inflammatory joint disease amongst others. Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS induced sperm damage leading to infertility. Malondialdehyde (MDA), a byproduct of lipid peroxidation represents level of lipid peroxidation [9].

Aitken and Clarkson were demonstrated that the notion that oxidative stress might also be factor in the etiology of defective sperm function in our species [10, 11]. 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 present study was to evaluate the relationship between the presence of oxidative stress indicators in the liver like lipid peroxidation as indicated by MDA during exposure to BPA and its effects on sperm quality.

2. Materials and Methods

2.1 Animals and Treatments

Twenty four healthy male Sprague-Dawley rats (50-days olds) were purchased from the Tongji Medical College Animal Laboratory (Wuhan, China). 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 (list the groups e.g., control group, low dose group, middle dose group and high dose group) was fed different doses of bisphenol A 0, 2, 10, 50 mg/kg body weight respectively in corn oil every forty-eight hours by intra-peritoneal injection for 20 days. After 20-days of treatment, the rats were sacrificed. 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 People’s Republic of China.

2.2 Dose Selection and Preparation

The doses and time used for the present study were derived from published data [12, 13] and the results of our preliminary experiment. BPA was dissolved in corn oil to obtain the desired concentration of BPA dose range, i.e., 0, 2, 10 and 5 mg/kg. An additional control group that had received only corn oil. Dose formulations were mixing well 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.

2.3 Chemicals and Reagents

Bisphenol A (2,2-Di (4-hidroxyphenyl) 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).

2.4 Body weight and organ collection

The weight of each animal was recorded every forty-eight hours and any gross abnormality was noted. The animals were fasted overnight, weighed and killed by cervical dislocation. Liver and other organs were isolated from adhering tissues and weighed independently. The liver was homogenized using lysis buffer (containing 1mM Na2EDTA, 150mM NaCl, 10mM PMSF, 10mM Tris, 1mM aprotin) to evaluate oxidase stress following the protocol of MDA assay kit (Jiancheng Bioengineering Ltd., Nanjing, China). Concentrations of MDA, as proceeding lipid peroxidation (LPO), were measured in the homogenate. MDA level were assessed to determinate the concentration of MDA, measuring thiobarbituric-acid (TBA) reacting substances at 532nm. The level of MDA was expressed as nmol MDA per milligram protein. Protein content was measured according to Bradford method.

2.5 MDA analysis

The liver was homogenized using lysis buffer (containing 1mM Na2EDTA, 150mM NaCl, 10mM PMSF, 10mM Tris, 1mM aprotin) to evaluate oxidase stress following the protocol of MDA assay kit (Jiancheng Bioengineering Ltd., Nanjing, China). Concentrations of MDA, as proceeding lipid peroxidation (LPO), were measured in the homogenate. MDA level were assessed to determinate the concentration of MDA, measuring thiobarbituric-acid (TBA) reacting substances at 532nm. The level of MDA was expressed as nmol MDA per milligram protein. Protein content was measured according to Bradford method.

2.6 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 2ml of 0.9% NaCl solution at 37°C and kept for 15 minutes 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.

3. 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 minutes, it was stained with 2% eosin for 1hour. 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, we considered the sperm head, neck, midpiece and tail must be 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 hook less cells; amorphous shapes and forms; folded, short and double Y tail and other aberrations.

4. Statistical Analysis

Data are presented as the Mean ± S.E.M. 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. A linear regression (Spearman) model was applied to the relationship of MDA levels and sperm count, sperm motility and morphology of spermatozoa. All hypotheses were two-tailed with statistical significance assessed at the p value <0.05 level with 95% confidence intervals.

5. Results

Results were expressed as mean ± SD for each parameter. The results are illustrated in the (Figure 1) the increasing of malondialdehyde (MDA) level was observed in response to BPA treatment when compared with the control group (**P < 0.01).

5.1 Effect of BPA on sperm counts

Figure 2 demonstrates the results obtained after exposure to BPA on epididymal sperm counts of adult male rats. Outcomes according to the percentage strictly Normal Morphology. Total sperm counts were reduced at all doses, but whilst a significant decrease was observed at a dose of 50mg/kg. The semen parameters from a total of twenty four fresh semen samples were examined by CFT-9200 computer-aided sperm analyzer (Table 1). The mean ± SD of total sperm concentration, density, motility, sperm motion

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variables (LIN=VSL/VCL) and (STR=VSL/VAP) P > 0.05, were analyzed by SPSS Student’s t-test.

5.2 Sperm morphology

After observation under the microscope, a significant reduction in the number of normal sperm was observed compared to the control group, (Table 1). Sperm analyses showed oligozoospermia (~20 x10^6 spermatozoids/ml) and asthenozoospermia (progressive motility <50%) in all groups treated by BPA including control groups. Meanwhile, in the 2, 10, 50 mg / kg dose groups percentage of sperm normality decreased gradually 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. Sperm with deformed heads were observed in the groups treated with BPA (10mg/kg and 50mg/kg). As related to tail abnormalities, some had no flagella, and others had proximal and distal cytoplasmic droplets. We also observed a negative correlation between MDA levels with sperm count (P<0.001; Figure 3), sperm motility and morphology (P<0.001; Figure 4 ).

6. Discussion

Oxidative stress as a result of an inappropriate balance between antioxidants and oxidants in the semen can lead to sperm damage, impairs the structure and function of spermatozoa and eventually male infertility [14, 15]. It has been shown that BPA may have effect on liver enzymes and also affect sperm quality. Thus, the tissues (liver) antioxidant evaluation seems to have important role in the etiology of semen quality. Therefore, MDA may be a diagnosis tool for the analysis of infertility patients. In present study we found that MDA levels liver tissue were increased with rats treated with BPA compared with the control groups (Figure 1). There was a negative correlation of sperm count, motility and morphology with MDA levels in rats. One of the byproducts of lipid peroxidation decomposition is MDA. It has been used in biochemical assays at the same time CASA to monitor the degree of peroxidative damage in spermatozoa. The results of such assay exhibit an excellent correlation with the degree to which sperm function and quality. The administration of BPA may induces overproduction of H_2O_2 in the liver; the higher concentrations of hydrogen peroxide induce lipid peroxidation and result in cell death, may due to the activity of another antioxidant enzymes such catalase. Reduction in the activity of catalase may reflect inability of liver to eliminate hydrogen peroxide after exposure to BPA [5, 16]. In rats the main route of elimination of conjugated BPA is by biliary and fecal elimination which enables enterohepatic recirculation [17]. Atkinson and Roy 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 [18].

Cytochrome P-450 once activated, inactivates, and facilitates the excretion of most xenobiotics, thus modulating the intensity and duration of their toxicity [19] such as drugs and environmental chemicals as well as endogenous compounds such as steroids and fatty acids [20]. BPA also induces ROS and disrupt the mitochondria membrane resulting in release of cytochrome-C protein from the mitochondria that activates the caspases, induce apoptosis and increase sperm damage [16, 21, 22].

Previous studies have reported that spermatozoa from oligozoospermic or asthenozoospermic men showed a higher production of oxidative stress [23-25]. In our study, we found high levels of MDA in rats treated with BPA compared against control groups, and it was negatively correlated with sperm count (Figure 3; r = -0.769, P < 0.0001) and sperm motility and morphology (Figure 4; r = - 0.689, P=0.0001), so they suggested damaging effect of free radicals on sperm membrane integrity. Our results of MDA are in accordance with previous study in rats [6, 26] in human [27-29]. However, study shown on the absence of adverse effects of BPA in two and three generation studies, which have considered BPA as safe for human use, have stirred up a lot of controversy [30, 31] and human [32]. 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 [29] which may corroborate our findings of the low percent active sperm motility in rats. The consequences of such oxidative stress include a loss of motility and fertilizing potential, and the induction of DNA damage in the sperm nucleus. The loss of sperm function is due to the peroxidation of unsaturated fatty acids in the sperm plasma membrane, as consequences of which the latter loses its fluidity and the cell lose their function [33]. Unfortunately the spermatozoa are unable to repair the damage induced by oxidative stress because they lack the cytoplasmic enzymes systems that required accomplishing this repair [34].

In our study high levels of MDA suggests that lipid peroxidation of the membrane lipid may disturb the function accomplish by the sperm membrane and negative correlation with sperm count, motility and morphology indicates free radicals may have role by altering semen quality parameters. MDA can be used as a marker of oxidative stress a potential marker for predicting assisted techniques (ART) outcomes [35, 36] like CASA. The common sperm parameters of CASA have shown significant correlation of sperm concentration in all groups treated with BPA against control group (Table 1). Observed values are below the values of references of semen analysis [37]. Sperm density < 20 x10^6/ml sperm motility <50%, VCL<70 µm/s in most cases except VSL> 25 µm/s. This confirms is similar to findings of a previous in vivo study on murine, but they are different with respect to the dose and time of exposure to BPA [6, 38] and in humans [39, 40]. The majority of epididymal sperm from adult rat had normal morphology (77.44%). In this study, we strictly considered only the percentage normal sperm to determine the etiology of semen quality. Therefore, MDA may be a diagnosis tool for the analysis of infertility patients.
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 interpreting such results.

7. Conclusion

In summary, the present study provides evidence that exposure of adult male rats to low dose of BPA induces oxidative stress in the liver, and may have significant role in the etiology of sperm abnormality. Negative correlation of sperm parameters with MDA levels indicates, oxidative stress adversely affects rat’s semen quality. 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, on male reproductive organs including semen quality may help to explain the observed abnormalities and exploration of future treatments.

8. Author’s Contributions

All authors contributed to the experimental process and in data collection, analysis and interpretation. Yang Kedi designed the research; A. Kourouma performed the statistical analyses and wrote the manuscript; Yaima M.L.T revised the manuscript. All authors read and approved the final manuscript.

9. Acknowledgments

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References

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Table 2: This table shows caudal epididymal semen characteristics in the experimental adult male rats SD using CASA CFT-9200

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=6)</th>
<th>Low 2mg/kg (n=6)</th>
<th>Middle 10mg/kg (n=6)</th>
<th>High 50mg/kg (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (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 ± S.E.M. (n= 6 rats per group). *P < 0.05 and **P < 0.01 denotes significant difference compared with controls.

Figure 1: The Effect of BPA on MDA activity in the adult SD rat liver. BPA (0, 2, 10 and 50 mg/kg/ day) was administrated ip. every forty-eight hours for 20 days. After the last administration, the rats were sacrificed decapitation and liver tissue was carefully dissected and stored at -70 °C until analyzed. Data represent as means ± S.E.M. (n= 6 rats per group). *P < 0.05 denotes significant difference compared with controls.
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**Figure 2:** 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 ± S.E.M. (n = 6 rats per group). *P < 0.05 and **P < 0.01 denotes significant difference compared with controls.

**Figure 3:** Correlation between MDA level in the liver tissue and epididymal sperm count. A negative correlation (r = -0.769; P < 0.0001) was observed between sperm count and MDA level. Regression analysis is for total treated with BPA against control groups.

**Figure 4:** Correlation between MDA level in the liver tissue and epididymal sperm motility. A negative correlation (r = -0.689; P < 0.0001) was observed between sperm motility and MDA level. Regression analysis is for total treated with BPA against control groups.

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- Investigation of a possible effect of Bisphenol A (BPA) to cause injury in the liver by forming ROS.
- Male reproductive function can be also disrupted by exposure to Bisphenol A (BPA).
- Investigation of a possible effect of Nonyphenol to cause injury in the liver by forming ROS and its effect on sperm quality after exposure.

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