Acta Scientific Pharmaceutical Sciences (ISSN: 2581-5423)

Volume 6 Issue 2 February 2022

Research Article

Butyl Cyclohexyl Phthalate (BCHP) Exposure Induces Oxidative Stress on Male Reproductive System

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Received: September 06, 2021

Published: January 12, 2022

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Abstract

Background and Aims: Phthalates member of toxic chemical material have been shown to cause reproductive and developmental toxicity. The study was performed to determine oxidative stress inducing potential in male mice reproductive system of Butyl cyclohexyl phthalate (BCHP).

Methods: Reproductive system toxicity of BCHP was evaluated according to OECD 407 test procedure. Mice were exposed to phthalate at 100, 200 and 400 mg/kg doses. After 28-day subacute toxicity test period, it was evaluated the effects of BCHP on antioxidant enzymes, lipid peroxidation activities, sperm morphologies and histopatological parameters.

Results: BCHP caused dose-dependent histopathological toxicity in the testes tissues. 28-days subacute toxicity study showed that epididymis weights induced significantly in the 200 and 400 mg/kg BCHP treated groups. The sperm count was reduced and the number of abnormal sperm was increased dose-dependenly. Statistically significant decreases in the SOD activities were determined in 200 and 400 mg/kg groups.

Conclusion: The results indicated that the BCHP cause severe damage to testicular tissue and disruption of spermatogenesis and induce oxidative stress. This is the first study in which BCHP has been shown toxicity on male reproductive system.

Keywords: Phthalate; Subacute Toxicity; Oxidative Stress; Sperm Morphology; Histopathological Changes

Introduction

Nowadays the industry generates billions of pounds of synthetic materials every year which are not yet fully known in our environment. They can disrupt endocrine function and induce reproductive and developmental toxicity. As one group of these chemicals, phthalates are widely used as plasticizing agents [1,2].

Phthalates contribute 60% of plastic products by weight according to Committee on the Health Risks of Phthalates. They are used by industry in variable amounts and considered more importantly [3]. With the increasing use of plastic products worldwide, phthalate contamination has become more serious. Due to the lack of chemical bonding with the polymer system, these phthalates easily leach out from such polymer system into the surrounding environments and finally reaches into the human system. In recent years, many studies have documented the toxic effects of phthalates [4-7]. Exposure to some phthalates results in serious and irreversible changes in the development of reproductive tract in males

[8]. *In vivo* studies showed that long-term exposure of phthalates, even at subchorionic levels may lead to endocrine disruption and allied abnormalities, tumorigenesis, and neuro behavioral changes [9].

Increasing risk of hazardous phthalates to the environment and its impact on human health has attracted researchers across the globe to actively engage in the research on protection of phthalate contamination.

According to Material Safety Data Sheet, hazard identification and biodegradation data for BCHP which is an industrial plasticizer, are not available. It is expected to adsorb to suspended solids when released into water. Data indicate that the general population may be exposed to BCHP via dermal contact with products containing BCHP. The main goal of the present research was to evaluate the histopathological-toxic effects and to determine oxidative damage in male reproductive system by subacute exposure of BCHP. This manuscript is helpful to establish the knowledge about reproductive toxicity elicited by BCHP.

Materials and Methods

Material

Butyl cyclohexyl phthalate (BCHP; CAS No: 84-64-0) was purschased from Chemical Service Inc. (West Chester, PA, USA). All of the chemicals were of analytical grade.

Animals and experimental design

The protocol was approved by the Ege University, Local Ethical Committee of Animal Experiment (23.11.2009, 2009-165). Adult male Swiss albino mice (4-5 week-old, 20-25 g) purchased from the KOBAY Laboratory (Izmir, Turkey). Subacute toxicity study was performed according to OECD Guidelines No 407 [10]. After 14 days of acclimation, healthy male mice (n = 20) were divided randomly to either the treatment or the control group. The BCHP treatment groups (100, 200 and 400 mg/kg doses) were administered by oral route for 28 days. It was calculated based on LD_{50} and body weight data.

Enzyme analyses and lipid peroxidation of testes

Enzymatic parameters of testes tissues analyse according o Lowry method [11]. Moreover, SOD Assay Kit (Fluka, 19160-1KT-F) for SOD activiy and CAT activity was measured. TBARS was used to calculate the lipid peroxidation with trichloroacetic acid (TCA) [12]. The results were monitored by absorbance at 532 nm after incubation period.

Sperm morphology test

According to Wyrobek and Bruce method, sperm morphology test was evaluated [13]. The abnormalities of sperms such as neck, heads and tails were evaluated.

Histological examinations

To measure the sperm reserves, cauda epididymis and vasa deference were minced gently, and homogenized in physiological saline solution [14,15]. Testes tissues were stained with haematoxylin and eosin [16].

Statistical analyses

Data are presented as the mean ± standard deviation (SD) from three independent experiments. Comparisons were analyzed using Student's t-test or one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Values of $p \leq 0.05$ were regarded as statistically significant.

Results

Body, testes and epididymidis weights

After 28-days BCHP treatment period, in comparison with control animal, there was no significant changes in body weight gains absolute and relative weights of testes of BCHP treated mice. However, epididymidis weights increased significantly ($p < 0.05$) in the 200 and 400 mg/kg groups (Table 1).

Groups	Body Weight (g)	Testes weights (g)	Relative testes weights (g/g bw)	Epididymidis weights (g)	Relative epididymidis weights (g/g bw)
Control	29.2±2.58	0.176 ± 0.035	0.0060 ± 0.0011	0.072 ± 0.017	0.0025 ± 0.0008
100 mg/kg	27.4±2.19	0.172 ± 0.033	0.0063 ± 0.0012	0.076 ± 0.016	0.0028 ± 0.0007
200 mg/kg	26.8±4.54	0.168 ± 0.030	0.0062 ± 0.0008	0.108 ± 0.038*	0.0041 ± 0.0016*
400 mg/kg	27.6±3.28	0.160 ± 0.031	0.0058 ± 0.0009	0.104 ± 0.032*	0.0038 ± 0.0015*

Table 1: Testes and epididymis weights of control and BCHP treated groups.

Values are given as mean ± SEM. bw: body weight.

* Statistically significant difference from the control group ($p < 0.05$).

SOD, CAT, lipid peroxidation

The activities of SOD of 200 and 400 mg/kg BCP exposure group were significantly lower than those of the control ($p < 0.05$). The activities of CAT of all exposure groups were higher than those of the controls, but not significant ($p > 0.05$). TBARS contents were not chaned in testes of BCHP exposure groups compared with the control group (Table 2). In our study, a dose-dependent reduction in the number of sperm count in cauda epididymidis was recorded in all BCHP treatment groups compared to control animals ($p < 0.05$). Figure 1 reveals the effect of BCHP on sperm count. Sperm morphology data was shown table 3 and figure 2. A dose-dependent increase in the number of abnormal sperm in cauda epididymidis was recorded in all BCHP treatment groups compared to control animals ($p < 0.05$). Also, it was shown intense coagulation in 400 mg/kg BCHP treated group.

Groups	SOD (U/ mg protein)	CAT [µmol/(mg min)]	TBARS (nmol/mg protein)
Control	129.59 ± 1.62	21.64 ± 3.78	16.77 ± 0.25
100 mg/kg	125.68 ± 2.03	23.15 ± 3.14	14.45 ± 0.17
200 mg/kg	119.13 ± 2.27*	25.38 ± 4.68	17.29 ± 0.19
400 mg/kg	117.32 ± 2.71*	28.59 ± 5.11	16.77 ± 0.26

Table 2: Effects of BCHP exposure on SOD, CAT and Lipid peroxidation level in testes of mice.
Values are given as mean ± SEM. SOD: superoxide dismutase, CAT: catalase, TBARS: thiobarbituric acid reactive substances.* Statistically significant difference from the control group ($p < 0.05$).

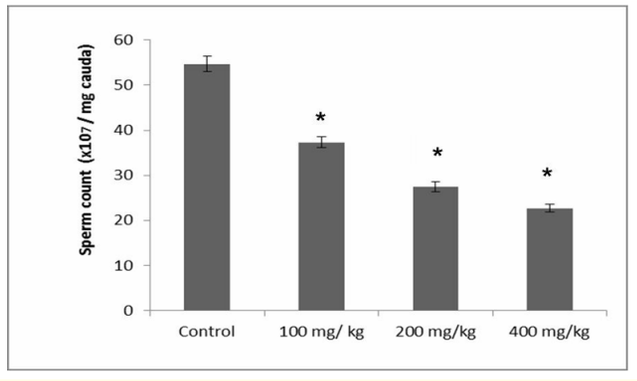

Figure 1: Sperm count in cauda epididymis of control and BCP treated groups. *Statistically significant difference from control ($p < 0.05$).

Figure 2: Sperm morphological abnormalities. a. Normal sperm cell; b. Bend and kinked Mid-piece; c. Tailless normal heads; d. Bend and kinked mid-piece, terminally coiled tails e. Sitoplasmic droplet, intensive sperm coagulation in semen; f. Bend and kinked mid-piece, terminally coiled tails, intensive sperm coagulation in semen. (Diffquick Staining, 100 X).

Groups	Number of sperm abnormalities								
	Head			Neck and mid-piece			Tail		Total
	Large	Small	Double	Bend mid-piece	Kinked mid-piece	Cytoplasmic droplet	Tailless	Coiled	
Control	12	2	4	20	26	6	6	2	%39
100 mg/kg	27	4	4	25	36	7	13	4	%60*
200 mg/kg	23	4	5	23	32	7	32	12	%69*
400 mg/kg	22	4	4	26	40	11	29	32	%84*

Table 3: Number of sperm morphological abnormalities.
* Statistically significant difference from the control group ($p < 0.05$).

Histological examinations

In light microscopic examinations, the testes of the control animals showed a normal histological pattern (Figure 3a). Histopathological observation of the treated mice revealed that BCHP caused dose-related testicular damage such as intercellular disassociation between spermatogenic cell lines in seminiferous tubules, germ cells eruption in lumen of seminiferous tubules, common edema in the interstitial spaces, deformations and degenerations of seminiferous tubules, venous distension and hyperemia (Figure 3b, c and d). All histopathological changes above were potent in the 400 mg/kg group than in the 100 and 200 mg/kg groups.

Figure 3: The light microscopic images of the testes. a. Testicular sections of control mice which show normal spermatogenesis. Note the normal cell arrangement in the seminiferous tubules. The interstitial spaces also appear normal. b. Testicular sections of mice treated with 100 mg/kg BCP. The interstitial spaces appear normal, intercellular disassociation between spermatogenic cell lines in seminiferous tubules and germ cells eruption in lumen of seminiferous tubules. c. Testicular sections of mice treated with 200 mg/kg BCP. Note edema in the interstitial spaces, intercellular disassociation of between spermatogenic cell lines in seminiferous tubules and germ cells eruption in lumen of seminiferous tubules. d. esticular sections of mice treated with 400 mg/kg BCP. Common edema in the interstitial spaces, intercellular disassociation of between spermatogenic cell lines in seminiferous tubules, deformations and degenerations of seminiferous tubules, venous distension and hyperemia, germ cells eruption in lumen of seminiferous tubules. t, seminiferous tubules; i, interstitium; x, spermatogenic cell lines; L, lumen of seminiferous tubules; →, leydig cell; o, intersititial edema (40X, H&E).

Discussion

The reproductive toxicity caused by phthalates is an issue with increasing importance such as decline in the number of sperm, cryptorchidism, hypospadias, and testicular cancer, symptoms together described as the testicular dysgenesis syndrome [4].

Researchers indicated that male reproductive system is considerably sensitive to phthalates exposure. Several phthalates have been detected with the levels from μg/kg (μg/L) to mg/kg (mg/L) in many products. Studies to simulating the actual situations of people exposure to phthalates have shown that the subchronic exposure (90 days) to low-doses (160 mg/kg bw) phthaltes were impaired reproductive function in male rats [2,17]. Dibutyl phthalate (DBP) and diisobutyl phthalate (DiBP) which are frequently detected in the environment were disrupted spermatogenesis and elicit male reproductive toxicity in zebrafish with very low concentrations. Also, it was shown that juvenility DEHP exposure may alter sex steroid hormones over time which may pose potential reproductive health risks.

Our previous study showed the oxidative stress and histological damage in liver by subacute doses of BCHP [18]. During 28-day subacute toxicity test period, animals were weighed weekly. Results indicated that there was no significant change of body weight bw among groups. However, it was reported that testes and epididymis weights can vary according to phthalates applied and their treatment times. It is reported that while some phthalates were not changed in body and testes weights, some of them were significantly reduced in weights [19].

Supporting our results, Xue., *et al*. 2019 [17] reported that phthalates such as di-isononyl phthalate (DINP), and di (2-ethylhexyl) phthalate (DEHP) has been determined that the bw of the groups treated with different doses of DINP and the group treated with 500 mg/kg DEHP were not significantly different from each other.

One of the important mechanisms of toxicity is oxidative stress which results form an imbalance between the excessive formation of reactive oxygen species (ROS) and limited antioxidant defenses. It is known that developing mammalian germ cells produce SOD but not CAT. Also, because of the poor vascularization of the testes, oxygen tensions in this tissue are low and competition for vital elements within the testes is extremely intense [20,21]. This implies that immature germ cells may vulnerable to H_2O_2 since CAT is required to reduce toxic H_2O_2. Normal epididymis with antioxidant

defense system protects sperms during their maturation process. Oxidative stress in epididymis might be lead to the amount of antioxidant defense system insufficient to protect sperm and the epididymal epithelial cells themselves also in the risk of increased levels of ROS [22]. ROS production in the testis is associated with several pathological conditions such as a decrease sperm production and fertility. These results are in accordance with previous reports, which found that BCHP induce oxidative stress in the testes in concert with the suppression of antioxidant mechanisms especially SOD during the 28 days exposure period.

Although testicles are known to be sensitive to phthalates, the main effects of them are suppression of testosterone production, abnormal Leydig cell aggregation, and the presence of intratubular Leydig cells. Degeneration in Sertoli cells can directly affect sperm counts. In our study, a dose dependent reduction in the number of sperm count, increase in the number of abnormal sperm in cauda epididymidis was recorded in all BCHP treatment groups compared to control animals. Also, it was shown intense coagulation in 400 mg/kg BCHP treated group. These results suggest that phthalates on sperm parameters in male rats have adverse effects. In the light of the results of this study, we ascribe those severe degenerations of seminiferous tubules in all BCHP treated groups might cause the reduction in sperm counts. Phthalates are known as Sertoli cells toxicants and a reduction in the Sertoli cells number and proliferation in some studies have been reported [22].

(Hutchison., *et al.* 2008). Connately our findings, germ cells eruption in lumen of seminiferous tubules with influence of toxic substances are reported by Creasy, 2001 [23]. Testes are sensitive to toxicants, such as phthalates. Our findings showed that BCHP-induced tissue damage of mice testis may associated with blood-testis barrier (BTB) damage.

Conclusion

The present results indicate that BCHP has toxic effects on testes, sperm counts and morphology of mice and BCHP impairs reproductive function, elicits a depletion of antioxidant defence system, especially in high doses. According to our searches, this is the first manuscript to show reproductive system toxicity for BCHP.

Acknowledgment

This study was funded by Ege University Scientific Research Project with the grant number 2010/FEN/019.

Ethics Committee Approval

The *in vivo* protocol was approved by the Ege University, Local Ethical Committee of Animal Experiment (23.11.2009, 2009-165).

Author Contributions

Conception/Design of Study- N.U.K.Y., C.K.K.; Data Acquisition- N.U.K.Y., M.D., H.A., A.Y.; Data Analysis/Interpretation- N.U.K.Y., E.E.B.D. C.K.K.; Drafting Manuscript- N.U.K.Y., C.K.K.; M.D., H.A., A.Y.; Critical Revision of Manuscript- N.U.K.Y., E.E.B.D. C.K.K.; Final Approval and Accountability- N.U.K.Y., C.K.K.; Technical or Material Support- N.U.K.Y., E.E.B.D. C.K.K.; M.D., H.A., A.Y. Supervision- N.U.K.Y.

Conflict of Interest

The authors report no conflicts of interest.

Financial Disclosure

This study was supported by Ege University Scientific Research Project (Grant number: 2010/FEN/019).

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