
**Manuscript Type:** Invited Review

**Title:** Potential Genotoxic Effects of Butylparaben (Butyl 4-Hydroxybenzoate) in Lymphocytes and Liver Samples at the Pubertal Stage of Male Rats

**Running Title:** Butylparaben Induced Genotoxic Effects in the Rat Blood and Liver

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ABSTRACT

Objective: We investigated genotoxic effects of butylparaben (BP) in the rat blood and liver samples in male rats at pubertal period.

Materials and Methods: Six weeks old male rats were divided randomly into eight groups as follows; 200, 400 and 800mg/kg/day for 14 days and 100, 200 and 400mg/kg/day for 28 days butylparaben and two control group (corn oil and methyl methanesulfonate). Genotoxic effect was measured in blood and liver samples. Comet parameters including tail moment, tail length and tail intensity were evaluated for possible genotoxic effects.

Results: Significant differences were observed between the control and treatment groups according to the following parameters: In liver parameters, the tail length of the 28-day dose groups was lower than the 14-day dose group and the tail moment and the tail intensity was high in butylparaben treatment groups. However, the lymphocyte values measured higher in 14 days high dose groups than the 28-day low dose data. In the liver measurements of the BP administration groups, the tail length increased in the 200 and 400mg/kg/day groups contrary in the 800mg/kg/day group, it was found to be lower compared to the control group.

Conclusion: These results showed that butylparaben causes a genotoxic potential in blood and liver cells.

Keywords: Butylparaben, genotoxicity, Comet assay, male rats

INTRODUCTION

Estrogen-like compounds that enter the body with diet, or compounds that interact with estrogen, have the potential to disrupt hormonal homeostasis in both male and female because of their important hormonal signaling functions. Therefore, they are called endocrine disruptors with estrogenic effects (1, 2, 3).
People are frequently exposed to these parabens because they are used as antibacterial and antifungal preservatives in foods, drugs and cosmetic products (4). In the Cosmetic Ingredient Review Expert Panel (5) the value Margin of Safety for parabens is 1.2mg/kg daily in adults and 0.3mg/kg/day in children and the NOAEL value is 1000mg/kg/day. Those carrying long chain or very large alkyl groups are more lipophilic and stronger in binding to estrogen receptors. However, they are considered safe because of their low bioaccumulation potential. Parabens, both dermal and via oral uptake, are rapidly metabolized to their primary metabolite, p-hydroxybenzoic acid, via non-specific esterase’s present in the skin and liver. In general, parabens are metabolized in phase I reaction to p-hydroxybenzoic acid and in phase II reaction to glucuronide and sulfate conjugates (6, 7). The exposure to butylparaben is 1.5 times higher in children than adults. Because, children are more sensitive to chemicals compared to at other ages. According to Scientific Committee on Consumer Safety, some metabolic enzymes express less in the skin of children who is under age one with the effect of skin metabolism (8). Thus, the internal exposure to some ingredients in cosmetic products on children is more than adults. The estrogenic properties of butylparaben were determined using MCF-7 cell lines and they demonstrated that butylparaben has weak estrogenic activity compared to 17-beta-estradiol (9). Exposure to parabens in prenatal period may affect thyroid hormone indicators with increased serum TT3 levels and this case caused to higher birth weight (10). Also, liver weights increased due to BP administration and it was stated that the liver was the target organ in BP toxicity (11). Boberg et al. (12) reported that butylparaben caused reduced ovary weights, decreased anogenital distance, and increased mammary gland outgrowth present in exposed female rats. The fact that the values such as methylparaben and ethylparaben are not clearly defined yet and their values such as ADI, NOAEL are not specified in the framework of expert panels are the reasons of the selection of butylparaben. Although it is claimed to be carcinogenic, there is no satisfactory genotoxic studies. In this study, we aimed to investigate whether or not butylparaben has an effect on DNA chain fractures at high doses at 14 and 28 days of application.
MATERIAL and METHODS

Study place

Six weeks-old male rats (*Rattus norvegicus*) (weighed approximately 140–220g) were provided from the Experimental Animal Production Center, (Turkey). All rats were housed in polypropylene cages in an air-conditioned room (12-h light/dark cycle) and the laboratory temperature was 22.4±1.6°C and the relative humidity was 47- 50%. Water and food pellets were given *ad libitum* and consumption were recorded daily. The rats were orally exposed to butylparaben or the vehicle control for 14 and 28 consecutive days.

Animal experimentation

In the study, there were eight groups, including six rats in each group: BP 200, 400, 800mg/kg/day for the repeated 14-day oral toxicity study and 100, 200 and 400mg/kg/day for the repeated 28-day oral toxicity study. Animals receiving only the vehicle served as two control group (corn oil and MMS=positive control group that received 60mg/kg methylmethanesulfonate intraperitoneally 24 h before dissection). Dose selection and study design as mentioned in Ogiwara et al. (13). Similar to doses used in other studies, we chose three doses and two exposures periods namely, 200, 400, and 800 mg/kg/day for 14 days and 100, 200, and 400mg/kg/day for 28 days of butylparaben. The purpose behind this study is to compare the effects of short-term and high-dose with long-term and low-dose of butylparaben. The applications were started at the same time and terminated on the 14th and 28th days.

Laboratory Analyses

In the current study, possible genotoxic effects of butylparaben were investigated in rats using the Mammalian Alkaline Comet Assay in vivo, the 489 Test Method, which was mainly prepared by the Organisation for Economic Co-operation and Development (OECD), was followed for the Comet Test (14). This technique is a rapid and sensitive fluorescence microscopic method that detects DNA damage. In current study, for lymphocyte isolation,
blood was taken from the heart and diluted with PBS in 1:1 ratio. Histopaque-1077 solution was added to the volume of the blood-PBS solution into the sterile falcon tube and centrifuged for 20 min at 2300 rpm at 25 °C. The lymphocytes that deposited in the middle layer were taken and 5 mL 1M saline phosphate buffer (PBS) was added at pH 7.4 to the Histopaque containing lymphocyte and centrifuged for 10 minutes at 25°C at 2000 rpm. The supernatant was discarded and the lymphocyte pellet was obtained.

For hepatocyte isolation, a small piece of liver was taken into the tube containing 1 mL cold (0-4°C) lysis solution and mechanically cut into small pieces then is kept in the refrigerator for 20 minutes to allow the residues to settle and leave the cell suspension on top. The isolated cell suspensions and trypan blue dye (0.4%) were mixed in a 1:1 ratio. Then, one drop of stained cells was placed on the slide and the coverslip was closed. Uncoated live cells were counted under light microscope. Total number of cells was determined by counting the cells on the lamina to 25 mm².

Total cell count = 10⁴ x mL x Number of cells in slide x Dilution coefficient

For spread of cells, each sample was mixed by pipetting with a 0.5% low melting agarose of 1:10 at a temperature of 37°C, and slides coated with a 1% high-melting agarose overnight. About 5x10³-1x10⁴ cells/µL in one of approximately 75µL cell-agar mixture was placed on the coverslip was covered. To make a protective layer on the cell-agar layer, 80µL of 1% fused low-grade agarose was heated at 45°C and closed again with coverslip and maintained to refrigerator for 5-7 minutes. The slides taken from the filter were lifted and suspended in lysis solution buffer at 4°C in lightproof chambers overnight. Then the samples were kept in the dark to prevent UV-induced DNA damage. The slides removed from the lysis solution then immersed in distilled water, and left for 20 minutes in alkaline treatment in an electrophoresis tank at 4°C.

The slides, which were placed in the electrophoresis tank with DNA migration from the cathode to the anode were subjected to electrophoresis at constant 25 V, 300 mA for 30 minutes. Then slides were washed three times with Tris buffer at 4°C before being stained with ethidium bromide.

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Each slide was stained with 35µL ethidium bromide then covered with coverslip and kept in refrigerator for 15 minutes. Then, 100 randomly selected cells were analyzed at 40 x10 magnification under a fluorescent 2 attachment microscope equipped with an excitation 3 filter of 546 nm and barrier filter of 590 nm and with a 100 W Hg lamp. Three parameters were selected for evaluate DNA damage: tail moment (DNA product contained by the tail), the intensity of the Comet tail (% of migrated DNA) and tail length (µm). These parameters were measured using the Comet Assay IV image analysis system (Perceptive Instruments, UK).

Statistical Analyses
Statistical analysis of the data was done using Microsoft Office Excel 2013 program. The significance of the differences between the groups was done by using the Tukey post hoc test and the bi-directional t test was analyzed at the level of significance with the help of Bonferoni correction. In the Comet test, the results were recorded as tail moment, tail length and tail percentage and ANOVA test was applied. The results are shown as mean ± standard error. A p-value of less than 0.05 is set as the criterion for statistical significance.

RESULTS
The food and water consumption of rats are given in Table 1. There was no significant difference in daily water consumption of animals. In 14 day, 400mg/kg/day of BP treatment group showed a decrease in average food consumption. The average food consumption of 28-day dose groups showed a significant increase compared to 14-day dose groups.

[Insert Table 1.]
In 14-day dose group; the Comet test applied to lymphocytes resulted in a dose-dependent increase in tail length (Fig.1, Table 2) compared to the oil control, MMS control and 28-day 100mg/kg BP group. Tail moments were increased especially in 400mg/kg BP group of 14-day treatment (Fig. 2). Also, tail intensity was increased in 14-day BP treatment groups according to control and 28-day treatment groups. There was no significant difference between tail...
intensity in 28-day treatment groups (Fig. 3). In MMS-receiving group, tail length, tail intensity and tail moment values were also significantly different from control and butylparaben groups ($P < 0.05$) (Table 2).

[Insert Fig. 1.2, 3] and [Insert Table 2.]

In 28-day dose group, in terms of tail length, no significant difference was found between the 100mg/kg BP group according to oil control group (Fig. 1). In contrast, a significant increase was observed in 200 and 400mg/kg BP treatment group according to the oil control group. No significant difference was found between the application groups in tail moment and tail intensity parameters (Fig. 2, 3).

Dose groups compared to each other; there was an increase in tail length for 14-day exposure groups compared to 28-day exposure groups. However, there was no difference between the 200mg/kg BP group for 14 days and the 200mg/kg BP group for 28 days. In the 28-day groups, a decrease was observed in tail moments compared to the 14-day groups. Tail intensity; in the 14-day dose groups, there was an increase compared to the 28-day dose groups. In particular, 14-day 200 and 400mg/kg butylparaben administration groups were significantly higher than the 28-day dose groups (Table 2).

In liver tissue, tail length (Fig. 4), tail moment (Fig. 5) and tail intensity (Fig. 6) parameters were shown in Table 3. Values of MMS-receiving group were significantly different from control and butylparaben groups. In 14-day treatment group, tail length significantly increased in BP exposure groups. Tail moments and tail intensity were increased in 200mg/kg and 400mg/kg BP administration groups but in 800mg/kg group significantly decreased.

Although 28-day treatment groups showed a significant increase in tail length and tail moments compared to the control groups. Also, tail intensity was significantly higher in the 100mg/kg BP exposed group than in the 200 and 400mg/kg groups.

When dose groups compared to each other; the tail length was increased in 14 days 400 and 800mg/kg dose groups, according to 28 days 200 and 400mg/kg groups. In contrary, it was decreased in 14 days 200mg/kg group compared to 28-day dose groups. The tail moment
parameter observed a significant increase in the application groups of 28 days compared to 14-day exposure groups. Although there was no statistically significant difference between groups in terms of tail intensity, a significant increase was observed in the 100mg/kg group in 28 days compared to the other groups (Fig. 6). Also, Figure 7 (14 days) and 8 (28 days) show images of alkaline comet analysis in lymphocyte and liver samples.

[Insert Fig. 4, 5, 6, 7, 8] [Insert Table 3.]

DISCUSSION

For more than 50 years, parabens have been widely used in cosmetics and pharmaceuticals, and in many foods, as an antimicrobial preservative (15). These substances, which are taken into the body together with food and beverages, medicines or cosmetic use, have serious issues that need to be investigated for human and environmental health. Parabens do not accumulate in the body in general and have been disintegrated in a short time.

In the current study, possible genotoxic effects of butylparaben were investigated in hepatocyte cells and lymphocytes of rats. Considering the regeneration ability of the liver, as mentioned in Ogiwara et al. (13) it is stated that it would be more appropriate to divide the application into two different time periods. Thus, in our study, it was decided to administer BP 14 days at 200, 400 and 800mg/kg doses and 28 days at 100, 200 and 400mg/kg doses. Aydemir et al. reported that administration of BP causes to defects in antioxidant enzyme activities and damage to liver, kidney, brain and testis tissue in rats (16) and oxidative stress metabolism is impaired in spleen, kidney and liver tissue (17). Bayülken and Tüylü (18) showed that butyl-, propyl-, isobutyl- and isopropyl paraben esters have genotoxic and cytotoxic effects on human lymphocytes cells in vitro. We observed a significant increase in liver samples, 28-day groups, in tail moment and tail intensity. The highest value was measured in the 100mg/kg/day group, especially when the tail intensity values were considered. Considering that the liver plays a primary role in the degradation of parabens like other chemicals, we can predict that DNA damage may be more in this organ. Findings from existing in vivo carcinogenicity studies with methyl- and propylparaben have led to the conclusion that these compounds cannot be considered carcinogenic (19). Parabens may induce breast cancer risk, particularly if exposure
occurs during childhood periods (20). In the current study, we evaluated genotoxic activity in liver and lymphocyte we can say that high doses of butylparaben can cause DNA damage. Todorovac et al (21) demonstrated that 0.25 and 0.50 mg/L butylparaben caused genotoxic effects in lymphocyte by increasing the tail intensity. The metabolic defense of mammals has up to a point the enzymes and mechanisms to eliminate these effects. Therefore, it is very unlikely that parabens alone will cause cancer. Nevertheless, there are many different endocrine disrupters in the body, and long-term exposure to the cocktail must also be considered. It is known that parabens interfere with the normal functioning of endocrine hormones in living things. The fact that there are a lot of studies the negative effects of parabens on the functioning of the endocrine system brings along the search for a safer preservative that may be an alternative to parabens.

CONCLUSION
In butylparaben dose groups in blood and liver samples, DNA damage was observed. In this study, we chose three doses and two-exposure periods. The objective behind this study is to compare the effects of short-term and high-dose with long-term and low-dose of butylparaben. In recent years, there have been many studies on the toxicity of parabens, but the number of studies on the genotoxic effects is very few. Also, none have investigated the genotoxic effects of butylparaben from the pre-pubertal period into puberty. In this context, our study supports many studies on paraben toxicity. Possible potential genotoxic effects on blood and liver cells were discussed as a result of exposure to butylparaben which are commonly used in cosmetics, food and pharmaceutical products. In the light of this study, we can say that butylparaben has the potential to cause DNA damage.

Ethics Committee Approval:
Permission required for the studies was obtained from Hacettepe University Experimental Animals Ethics Committee with the number of 2015/81-6.
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Figure Legends

Figure 1. Tail length (µm) measurements in the lymphocytes of rats exposed to butylparaben and control (Data are presented ±SD) (*p≤ 0.05).

Figure 2. Tail moments (µm) measurements in the lymphocytes of rats exposed to butylparaben and control (Data are presented ±SD) (*p≤ 0.05).

Figure 3. Tail intensity (%) measurements in the lymphocytes of rats exposed to butylparaben and control (Data are presented ±SD) (*p≤ 0.05).

Figure 4. Tail length (µm) measurements in liver of rats exposed to butylparaben and control (Data are presented as tail length ±SD) (*p≤ 0.05).

Figure 5. Tail moments (µm) measurements in liver of rats exposed to butylparaben and control (Data are presented ±SD) (*p≤ 0.05).

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Figure 6. Tail intensity (%) measurements in liver of rats exposed to butylparaben and control (Data are presented ±SD) (*p≤ 0.05).

Figure 7. Representative Comet images in lymphocytes and liver samples from oil control, 200, 400 and 800mg/kg butylparaben treatment group for 14-days (DNA stained with ethidium bromide; x40)

Figure 8. Representative Comet images in lymphocytes and liver samples from oil control, 200, 400 and 800mg/kg butylparaben treatment group for 28-days (DNA stained with ethidium bromide; x40)
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Table 1. Effects of orally administered butylparaben on mean of daily food intakes and mean of daily water intakes of rats in the control and treatment groups (±S:D).

<table>
<thead>
<tr>
<th>Oil Control</th>
<th>14-day treatment</th>
<th>28-day treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>200 mg/kg/day</td>
<td>400 mg/kg/day</td>
</tr>
<tr>
<td></td>
<td>200 mg/kg/day</td>
<td>200 mg/kg/day</td>
</tr>
<tr>
<td></td>
<td>400 mg/kg/day</td>
<td>400 mg/kg/day</td>
</tr>
<tr>
<td></td>
<td>800 mg/kg/day</td>
<td>800 mg/kg/day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.4±2.32a</td>
</tr>
</tbody>
</table>

Data are presented as mean and standard deviation (n=6)

\( ^a \) Different from oil control group.

\( ^b \) 28-day butylparaben dose group different from 14-day butylparaben dose groups. (p≤0.05).
Table 2. Comet assay results of lymphocyte samples regarding tail length, tail intensity and tail moment of the male rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Exposure period</th>
<th>Dose</th>
<th>Tail length (µm)</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil control</td>
<td>14 day</td>
<td>1 ml</td>
<td>15.42±4.32</td>
<td>0.00</td>
</tr>
<tr>
<td>Positive control (MMS)</td>
<td>24 hour</td>
<td>60 mg/kg</td>
<td>140.13±8.71</td>
<td>27.07</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>14 day</td>
<td>200 mg/kg/day</td>
<td>16.26±4.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>14 day</td>
<td>400 mg/kg/day</td>
<td>18.81±5.3</td>
<td>0.31</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>14 day</td>
<td>800 mg/kg/day</td>
<td>21.77±5.05</td>
<td>0.19</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>28 day</td>
<td>100 mg/kg/day</td>
<td>14.99±4.62</td>
<td>0.19</td>
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<tr>
<td>Butylparaben</td>
<td>28 day</td>
<td>200 mg/kg/day</td>
<td>16.67±4.94</td>
<td>0.90</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>28 day</td>
<td>400 mg/kg/day</td>
<td>17.44±3.96</td>
<td>0.19</td>
</tr>
</tbody>
</table>

(n=6)  

a Different from oil control group.  
b different from positive control group (MMS).  
c different from 200 mg/kg/day butylparaben dose group.  
d different from 400 mg/kg/day butylparaben dose group.  
e different from 800 mg/kg/day butylparaben dose group.  
f different from 100 mg/kg/day butylparaben dose group.  
g different from 200 mg/kg/day butylparaben dose group.

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different from 400 mg/kg/day butylparaben dose group (p<0.05). Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Exposure period</th>
<th>Dose</th>
<th>Tail length (µm)</th>
<th>Intensity of tail (%)</th>
<th>Tail moment (µm)</th>
<th>Intensity of tail moment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil control</td>
<td>14 day</td>
<td>1 ml</td>
<td>13.35±7.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control (MMS)</td>
<td>24 hour</td>
<td>60 mg/kg</td>
<td>147.08±6.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butylparaben</td>
<td>14 day</td>
<td>200 mg/kg/day</td>
<td>16.65±5.76</td>
<td>a.b.d.e</td>
<td>0.53±1.54</td>
<td>a.b</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>14 day</td>
<td>400 mg/kg/day</td>
<td>21.0±5.99</td>
<td>a,b,c,e</td>
<td>0.71±0.6</td>
<td>a,b,e</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>14 day</td>
<td>800 mg/kg/day</td>
<td>20.06±6.04</td>
<td>a,b,c,d</td>
<td>0.06±0.22</td>
<td>a,b</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>28 day</td>
<td>100 mg/kg/day</td>
<td>20.06±5.9</td>
<td>a,b,g</td>
<td>1.18±2.49</td>
<td>a,b,g,h</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>28 day</td>
<td>200 mg/kg/day</td>
<td>18.7±6.82</td>
<td>a,b,f,h</td>
<td>0.62±1.87</td>
<td>a,b,f</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>28 day</td>
<td>400 mg/kg/day</td>
<td>19.78±6.85</td>
<td>a,b,g</td>
<td>0.79±2.12</td>
<td>a,b,f</td>
</tr>
</tbody>
</table>

**Table 3.** Comet assay results of liver samples regarding tail length, tail intensity and tail moments of the male rats

(n=6) a Different from oil control group.

b different from positive control group (MMS).

c 200 mg/kg/day butylparaben dose group.
d different from 400 mg/kg/day butylparaben dose group.
e different from 800 mg/kg/day butylparaben dose group.
f different from 100 mg/kg/day butylparaben dose group.
g different from 200 mg/kg/day butylparaben dose group.

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different from 400 mg/kg/day butylparaben dose group. (p<0.05). Data are presented as mean ± SD.