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ORIGINAL ARTICLE – OPEN ACCESS

Potential Genotoxic Effects of Butylparaben (Butyl 4-Hydroxybenzoate) in Lymphocytes and Liver Samples of Pubertal Male Rats

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ABSTRACT

Objective: The aim of this study was to investigate potential genotoxic effects in blood and liver samples of male rats of pubertal age following the administration of butylparaben (BP).

Materials and Methods: A total of 48 6-week-old male rats were divided randomly into 8 equal groups. Three groups received 200, 400, or 800 mg/kg/day BP for 14 days; 3 groups received 100, 200, or 400 mg/kg/day for 28 days; and 2 control groups received corn oil or methyl methanesulfonate. Blood and liver samples were analyzed to determine any genotoxic effect using comet assay parameters of tail moment, tail length, and tail intensity.

Results: Significant differences were observed between the control and treatment groups: liver sample analysis revealed that the tail length of the 28-day dose groups was lower than that of the 14-day dose groups and some tail moment and the tail intensity values were high in the BP treatment groups. The measurements of tail length in the 200 and 400 mg/kg/day BP groups was higher than that of the control groups, while in the 800 mg/kg/day group, it was lower than that of the control group. The lymphocyte values in the 14-day high-dose groups were higher than the 28-day low-dose data.

Conclusion: The results indicated that BP had genotoxic potential in the blood and liver cells of young male rats.

Keywords: Butylparaben, comet assay, genotoxicity, male rats

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INTRODUCTION

Estrogen-like compounds that enter the body through the diet or compounds that interact with estrogen have the potential to disrupt hormonal homeostasis in both males and females. These endocrine-disrupting chemicals can interfere with the important functions of natural estrogen (1–3).

Parabens are man-made chemicals with estrogenic effects that are frequently used as antibacterial and antifungal preservatives in foods, drugs, and cosmetic products (4). A report of the Cosmetic Ingredient Review Expert Panel in the USA recommended a margin of safety for parabens of 1.2 mg/kg daily in adults and 0.3 mg/kg/day in children, assuming a no-observed-adverse-effect level (NOAEL) of 1000 mg/kg/day (5). Long-chain parabens and others with large alkyl groups are more lipophilic and have stronger estrogen-binding capacity. Parabens are rapidly metabolized following either dermal or oral uptake to their primary metabolite, p-hydroxybenzoic acid, via non-specific esterases in the skin and liver. In general, parabens are metabolized in a phase I reaction to p-hydroxybenzoic acid and in a phase II reaction to glucuronide and sulfate conjugates (6, 7). However, they are generally considered safe due to a low bioaccumulation potential.

The exposure to butylparaben (BP) is 1.5 times greater in children than adults since children are more sensitive to chemicals. The guidance of the Scientific Committee on Consumer Safety of the European Commission noted that some metabolic enzymes seem to be less expressed in the skin of children, in particular those aged <1 year. Hence, the very young might have higher internal exposure to certain ingredients after dermal application than adults. (8). The estrogenic properties of BP were assessed using MCF-7 cell lines and BP demonstrated weaker estrogenic activity than 17-beta estradiol (9). Exposure to parabens in the prenatal period may affect thyroid hormone indicators, specifically increased serum TT3 levels, which may be associated with a higher birth weight (10). In another study, liver weight increases observed in rats suggested that the liver was the target organ in BP toxicity (11). Boberg et al. (12) reported that BP exposure led to reduced ovary weight, decreased anogenital distance, and increased mammary gland outgrowth in female rats.

BP has been the subject of some controversy regarding safety; there are some claims that it is carcinogenic. However genotoxic studies remain unsatisfactory. Research and review of the safety of parabens and acceptable daily

intake and NOAEL remains a subject in need of further clarification. The aim of this study was to investigate any effect of BP on DNA chain fractures in a short-term, high-dose application of 14 days and a long-term, low-dose application of 28 days.

MATERIALS and METHODS

Ethics Considerations

Approval for this study was obtained from the Hacettepe University Experimental Animals Ethics Committee date: 05.11.2015 (no: 2015/81-6).

Study Conditions

Six-week-old male rats (*Rattus norvegicus*), weighing approximately 140–220 g, were obtained from the experimental animal procurement center. The subjects were housed in polypropylene cages in an air-conditioned room (12-hour light/dark cycle) with a laboratory temperature of 22.4±1.6°C and a relative humidity of 47% to 50%. Water and food pellets were available ad libitum and consumption rates were recorded daily. BP or an oil control vehicle was administered orally for 14 or 28 consecutive days.

Animal Experimentation

The study used 8 groups of 6 rats: BP was administered at 200, 400, or 800 mg/kg/day for the 14-day oral toxicity study and 100, 200, or 400 mg/kg/day for the 28-day oral toxicity study. Animals receiving only corn oil or a 60 mg/kg methyl methanesulfonate (MMS) intraperitoneal injection 24 hours before dissection served as control groups. The study design was based on that reported by Ogiwara et al. (13). Three doses and 2 exposure periods were used. The objective was to compare the effects of short-term, high-dose BP with long-term, low-dose BP administration. The BP exposure was initiated at the same time in the treatment groups and terminated on the 14th and 28th day.

Laboratory Analyses

The possible genotoxic effects of BP were investigated using the Organisation for Economic Co-operation and Development (OECD) Test No. 489 guideline: *In Vivo* Mammalian Alkaline Comet Assay guideline (14). This is a rapid and sensitive fluorescence microscopic method to measure and assess DNA damage. In current study, blood was taken from the heart and diluted with phosphate-buffered saline (PBS) in a 1:1 ratio for lymphocyte isolation. Histopaque-1077 solution (Merck KGaA, Darmstadt, Germany) was added to the volume of the blood-PBS solution in a sterile Falcon tube (Corning Inc., Corning, NY, USA) and centrifuged for 20 minutes at 2300 rpm at 25°C. The lymphocytes deposited in the middle layer were collected and 5 mL 1M PBS was added at pH 7.4 to the Histopaque solution containing lymphocytes and centrifuged for 10 minutes at 25oC at 2000 rpm. The supernatant was discarded and the lymphocyte pellet cells were retrieved.

For hepatocyte isolation, a small piece of the liver was added to a tube containing 1 mL cold (0–4°C) lysis solution, mechanically cut into small pieces then refrigerated for 20 minutes to allow the residue to settle and leave the cell suspension on top. Trypan blue dye (0.4%) was added to the isolated cell suspension in a 1:1 ratio. Next, one drop of the stained cells was placed on a slide and a coverslip was mounted in place. The uncoated live cells were counted using a light microscope. The total number of cells was determined by counting the cells on the lamina to 25 mm^2.

Total cell count=10^4×mL×number of cells in slide×dilution coefficient.

Pipetting with 0.5% low-melting-point agarose 1:10 dilution at 37°C was used to mix the samples, and the slides were coated with a 1% high-melting-point agarose overnight to demonstrate the spread of the cells. A cell density of 5x10^3–1x10^4 cells/µL in one of approximately 75 µL cell-agar mixture was used and covered with a coverslip. To make a protective layer on the cell-agar layer, 80µL of 1% fused low-grade agarose at 45°C was applied to the slide, the coverslip was remounted, and the slides were refrigerated for 5–7 minutes. The slides were lifted and suspended in lysis solution buffer at 4°C in lightproof chambers overnight to prevent UV-induced DNA damage. The slides were removed from the lysis solution, immersed in distilled water, and left for 20 minutes in an electrophoresis tank at 4°C.

The slides were placed in the electrophoresis tank with DNA migration from the cathode to the anode and were subjected to electrophoresis at constant 25 V, 300 mA for 30 minutes. The slides were then washed 3 times with Tris buffer at 4°C before being stained with ethidium bromide.

Each slide was stained with 35 µL ethidium bromide then covered with coverslip and kept in the refrigerator for 15 minutes. Next, 100 randomly selected cells were analyzed at 40×10 magnification under a fluorescent microscope equipped with 3 excitation filters of 546 nm, a barrier filter of 590 nm, and a 100 W mercury bulb.

Three parameters were selected to evaluate DNA damage: tail moment (tail length×% of DNA in 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/Instem, Suffolk, UK).

Statistical Analysis

IBM SPSS Statistics 13.v0 program (Statistical Package for the Social Sciences, Inc, Chicago, IL, USA). The significance of the differences between the groups was assessed using the Tukey post hoc test and a bidirectional t-test as well as the Bonferroni correction. One-way analysis of variance was applied to the comet test results of tail moment, tail length, and tail percentage. The results were shown as mean±SD. A p value of <0.05 was the criterion for statistical significance.

RESULTS

The food and water consumption of the rats is provided in Table 1. There was no significant difference in the daily water consumption. The 14-day, 400 mg/kg/day BP treatment group showed a decrease in average food consumption. The average food consumption of the 28-day dose groups was significantly higher than that of the 14-day dose groups.

In the 14-day dose groups, the comet test of lymphocytes revealed a dose-dependent increase in tail length (Fig.1, Table 2) compared to that of the oil control, MMS control, and 28-day 100 mg/kg BP groups. The tail moment was increased in the 14-day 400 mg/kg BP group (Fig. 2). Tail intensity was greater in the 14-day BP treat-

Table 1. Effects of orally administered butylparaben on mean daily food intake and mean daily water intake of rats in the control and treatment groups

		14-day treatment			28-day treatment		
	Oil control	200 mg/kg/day	400 mg/kg/day	800 mg/kg/day	100 mg/kg/day	200 mg/kg/day	400 mg/kg/day
Food (g)	14.61±2.82	13.68±2.79	12.51±1.43[a]	13.88±1.66	22.82±2.16[a,b]	22.4±2.32[a,b]	21.58±1.11[a,b]
Water (mL)	30.30±6.82	28.07±5.36	32.24±3.63	38.88±5.97	33.51±4.63	36.33±5.79	36.38±5.77

Data are presented as mean±SD (n=6). [a]: Significantly different from oil control group. [b]: 28-day butylparaben dose groups significantly different from 14-day butylparaben dose groups ($p \leq 0.05$)

ment groups in comparison with the control and 28-day treatment groups. There was no significant difference in the tail intensity seen in 28-day treatment groups (Fig. 3). In the MMS group, the tail length, tail intensity, and tail moment values were significantly different from those of the control and BP groups ($p<0.05$) (Table 2).

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

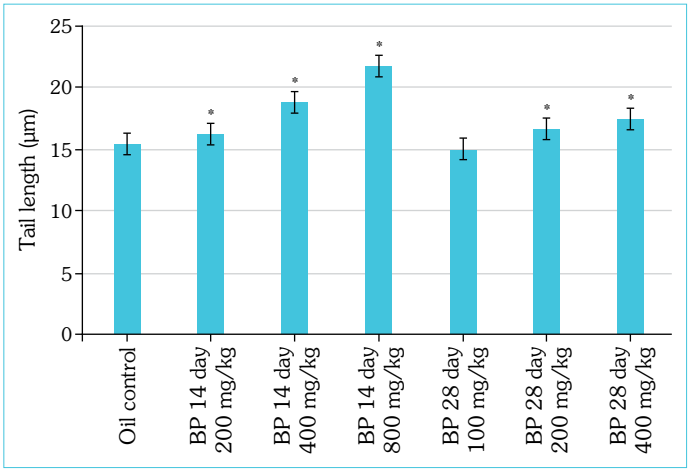

Figure 1. Tail length measurements in the lymphocytes of rats exposed to butylparaben and control group
Data are presented mean±SD; *p≤0.05. BP: Butylparaben

Comparison of the dose groups revealed an increase in tail length in the 14-day exposure groups compared to the 28-day exposure groups. However, there was no difference between the 14-day 200 mg/kg BP group and the 28-day 200 mg/kg BP group. In the 28-day groups, a decrease was observed in tail moment compared with the 14-day groups. Tail intensity in the 14-day dose groups was higher than that of the 28-day dose groups. In particular, the values of the 14-day 200 and 400 mg/kg BP administration groups were significantly higher than the 28-day dose groups (Table 2).

Liver tissue tail length (Fig. 4), tail moment (Fig. 5), and tail intensity (Fig. 6) parameters are shown in Table 3. The values of the MMS group were significantly different from those of the control and BP groups. The tail length was significantly greater in the 14-day BP-exposure groups. Tail moments and tail intensity were greater in the 200 mg/kg and the 400 mg/kg BP groups, but significantly lower in the 800 mg/kg group.

The 28-day treatment groups demonstrated a significant increase in tail length and tail moment when compared with the control groups. Also, the tail intensity was significantly higher in the 100 mg/kg BP group than in the 200 and 400 mg/kg groups.

Table 2. Comet assay tail length, tail intensity, and tail moment results of lymphocyte samples

Groups	Exposure period	Dose	Tail length (µm)	Tail intensity (%)	Tail moment (µm)
Oil control	14 day	1 mL	15.42±4.32	0.08±0.17	0.005±0.013
Positive control (MMS)	24 hour	60 mg/kg	140.13±8.71	27.07±1.32	20.08±1.3
Butylparaben	14 day	200 mg/kg/day	16.26±4.17[a,b,d,e]	0.24±0.85[a,b]	0.02±0.07[a,b]
Butylparaben	14 day	400 mg/kg/day	18.81±5.3[a,b,c,e]	0.32±0.9[a,b]	0.03±0.11[a,b,c,e]
Butylparaben	14 day	800 mg/kg/day	21.77±5.05[a,b,e]	0.19±0.61[a,b,e]	0.02±0.08[a,b,e]
Butylparaben	28 day	100 mg/kg/day	14.99±4.62[b,g,h]	0.11±0.43[b]	0.01±0.045[b]
Butylparaben	28 day	200 mg/kg/day	16.67±4.94[a,b,f,h]	0.09±0.41[b]	0.009±0.051[b]
Butylparaben	28 day	400 mg/kg/day	17.44±3.96[a,b,f,h]	0.14±0.23[a,b]	0.006±0.012[b]

Data are presented as mean±SD. $P<0.05$; n=6. [a]: Significantly different from oil control group; [b]: Significantly different from positive control group (MMS); [c]: Significantly different from 200 mg/kg/day butylparaben dose group; [d]: Significantly different from 400 mg/kg/day butylparaben dose group; [e]: Significantly different from 800 mg/kg/day butylparaben dose group; [f]: Significantly different from 100 mg/kg/day butylparaben dose group; [g]: Significantly different from 200 mg/kg/day butylparaben dose group; [h]: Significantly different from 400 mg/kg/day butylparaben dose group. MMS: Methyl methanesulfonate

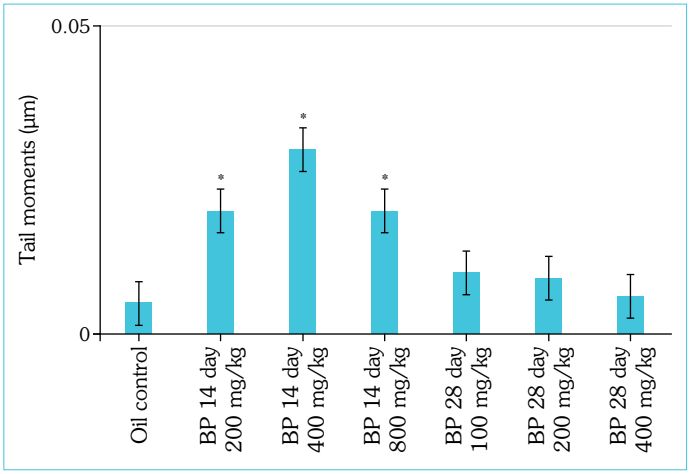

Figure 2. Tail moment measurements in the lymphocytes of rats exposed to butylparaben and control group

Data are presented mean±SD; *p≤0.05. BP: Butylparaben

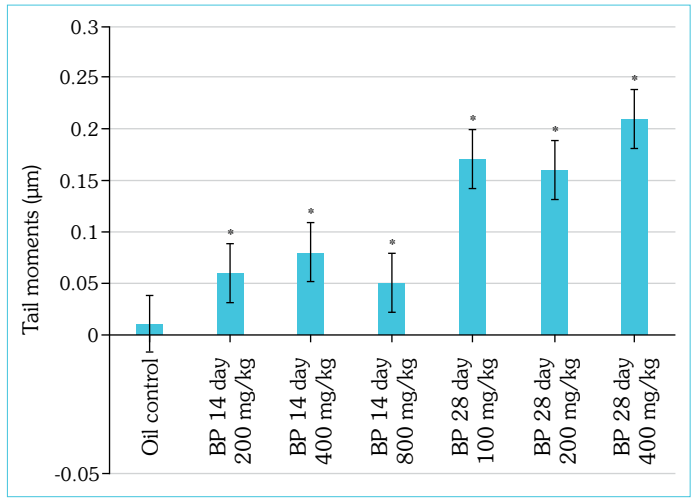

Figure 5. Tail moment measurements in the liver of rats exposed to butylparaben and control group

Data are presented mean±SD; *p≤0.05. BP: Butylparaben

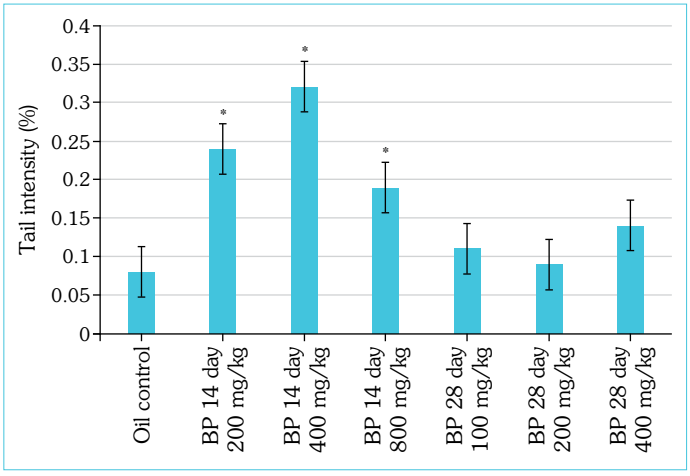

Figure 3. Tail intensity measurements in the lymphocytes of rats exposed to butylparaben and control group

Data are presented mean±SD; *p≤0.05. BP: Butylparaben

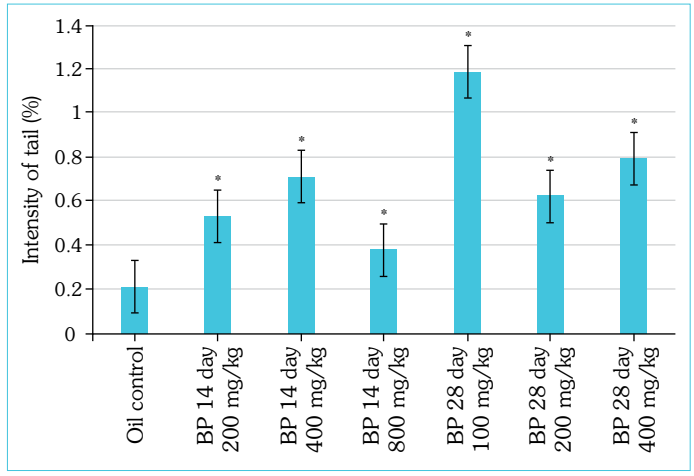

Figure 6. Tail intensity measurements in the liver of rats exposed to butylparaben and control group

Data are presented mean±SD; *p≤0.05. BP: Butylparaben

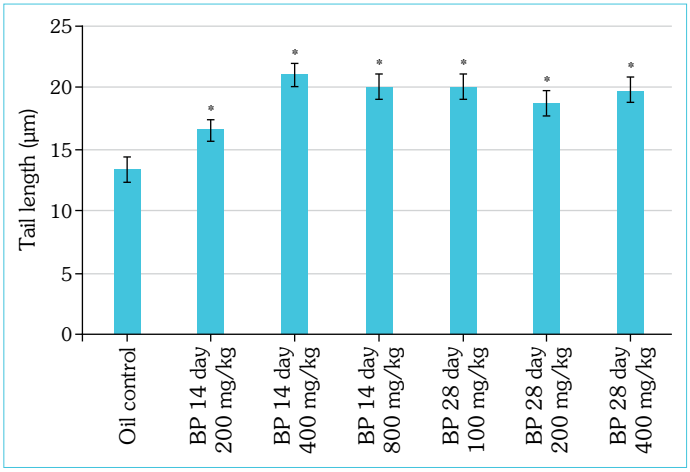

Figure 4. Tail length measurements in the liver of rats exposed to butylparaben and control group

Data are presented as mean±SD; *p≤0.05. BP: Butylparaben

When dose groups were compared, the tail length was greater in the 14-day 400 and 800 mg/kg dose groups than in the 28-day 200 and 400 mg/kg groups. Tail length was lower in the 14-day 200 mg/kg group compared with the 28-day dose group. The tail moment parameter was significantly greater in the 28-day treatment when compared with the 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 28-day 100 mg/kg group compared with the other groups (Fig. 6). Figure 7 (14 days) and Figure 8 (28 days) show the alkaline comet analysis of lymphocyte and liver samples.

DISCUSSION

For more than 50 years, parabens have been widely used in cosmetics, pharmaceuticals, and in many food products as an antimicrobial preservative (15). The use of these substances that have known endocrine-disrupting properties deserves careful and thorough investigation. While parabens have a short half-life and do

Table 3. Comet assay results of tail length, tail intensity, and tail moment

Groups	Exposure period	Dose	Tail length (μm)	Intensity of tail (%)	Tail moment (μm)
Oil control	14 day	1 ml	13.35±7.02	0.21±0.17	0.01±0.05
Positive control (MMS)	24 hour	60 mg/kg	147.08±6.34	32.19±1.71	25.42±0.3
Butylparaben	14 day	200 mg/kg/day	16.65±5.76[a,b,d,e]	0.53±1.54[a,b]	0.06±0.22[a,b]
Butylparaben	14 day	400 mg/kg/day	21.0±5.99[a,b,c,e]	0.71±0.6[a,b,e]	0.08±0.21[a,b,e]
Butylparaben	14 day	800 mg/kg/day	20.06±6.04[a,b,c,d]	0.38±0.97[b,d]	0.05±0.15[a,b,d]
Butylparaben	28 day	100 mg/kg/day	20.06±5.9[a,b,g]	1.18±2.49[a,b,g,h]	0.17±0.52[a,b]
Butylparaben	28 day	200 mg/kg/day	18.7±6.82[a,b,f,h]	0.62±1.87[a,b,f]	0.16±0.68[a,b]
Butylparaben	28 day	400 mg/kg/day	19.78±6.85[a,b,g]	0.79±2.12[a,b,f]	0.21±0.94[a,b]

Data are presented as mean±SD; $p<0.05$; n=6. [a]: Significantly different from oil control group; [b]: Significantly different from positive control group (MMS); [c]: Significantly different from 200 mg/kg/day butylparaben dose group; [d]: Significantly different from 400 mg/kg/day butylparaben dose group; [e]: Significantly different from 800 mg/kg/day butylparaben dose group; [f]: Significantly different from 100 mg/kg/day butylparaben dose group; [g]: Significantly different from 200 mg/kg/day butylparaben dose group; [h]: Significantly different from 400 mg/kg/day butylparaben dose group. MMS: Methyl methanesulfonate

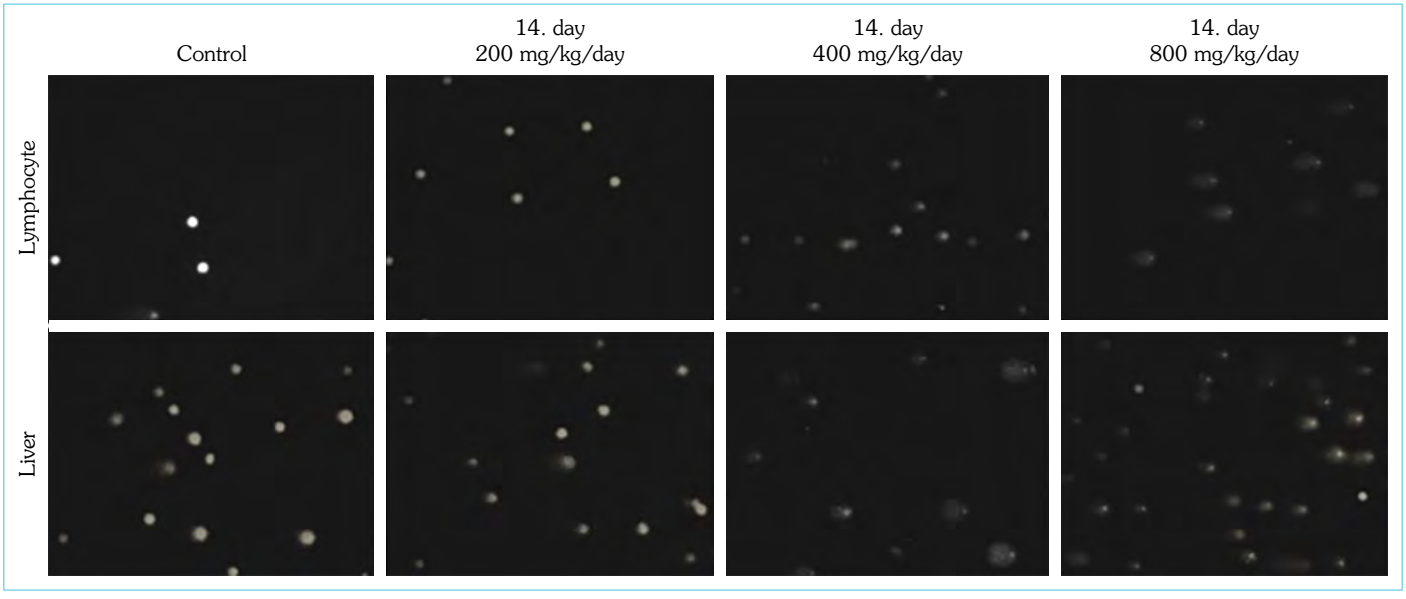

Figure 7. Comet images of lymphocyte and liver samples from oil control group and 200, 400, and 800 mg/kg 14-day butylparaben treatment groups. (DNA stained with ethidium bromide; ×40)

not generally accumulate in the body, it remains a complex subject of some dispute for a variety of reasons.

The current study was designed to investigate the possible genotoxic effects of BP in the hepatocyte cells and lymphocytes of rats. Considering the regenerative ability of the liver, as noted by Ogiwara et al. (13), the study used 2 treatment periods. BP was administered for 14 days at 200, 400, and 800 mg/kg doses and for 28 days at 100, 200, and 400 mg/kg doses. Bayülken and Tüylü (16) found that butyl-, propyl-, isobutyl- and isopropyl paraben esters had genotoxic and cytotoxic effects on human lymphocytes cells *in vitro*. Aydemir et al. (17) reported that BP affected antioxidant enzyme activity and damaged the liver, kidney, brain, and testis tissue in rats, and Aydemir et al. (18) noted that oxidative stress metabolism was impaired in spleen, kidney, and liver tissue. We observed a significant increase in tail moment and tail intensity in liver samples in the 28-day groups. The highest value was measured in the 100 mg/kg/day group, particularly the tail intensity values. Considering that the liver plays a primary role in the degradation of parabens, the DNA damage may be greater in this organ. Findings from *in vivo* carcinogenicity studies with methyl- and propylparaben led to the conclusion that these compounds are not considered carcinogenic (19). Parabens may, however, be linked to breast cancer, particularly if exposure occurs during childhood (20). The current study evaluated genotoxic activity in the liver and lymphocytes and the results suggest that high doses of BP can cause DNA damage. Todorovac et al. (21) demonstrated that 0.25 and 0.50 mg/L administration of BP resulted in increased tail intensity and genotoxic effects in lymphocytes. The metabolic defense of mammals includes enzymes and mechanisms to eliminate these effects. Therefore, it is very unlikely that parabens alone will cause cancer. Nevertheless, many different endocrine disrupters may be introduced into the body in various forms and long-term exposure to such a cocktail must also be considered. It has been

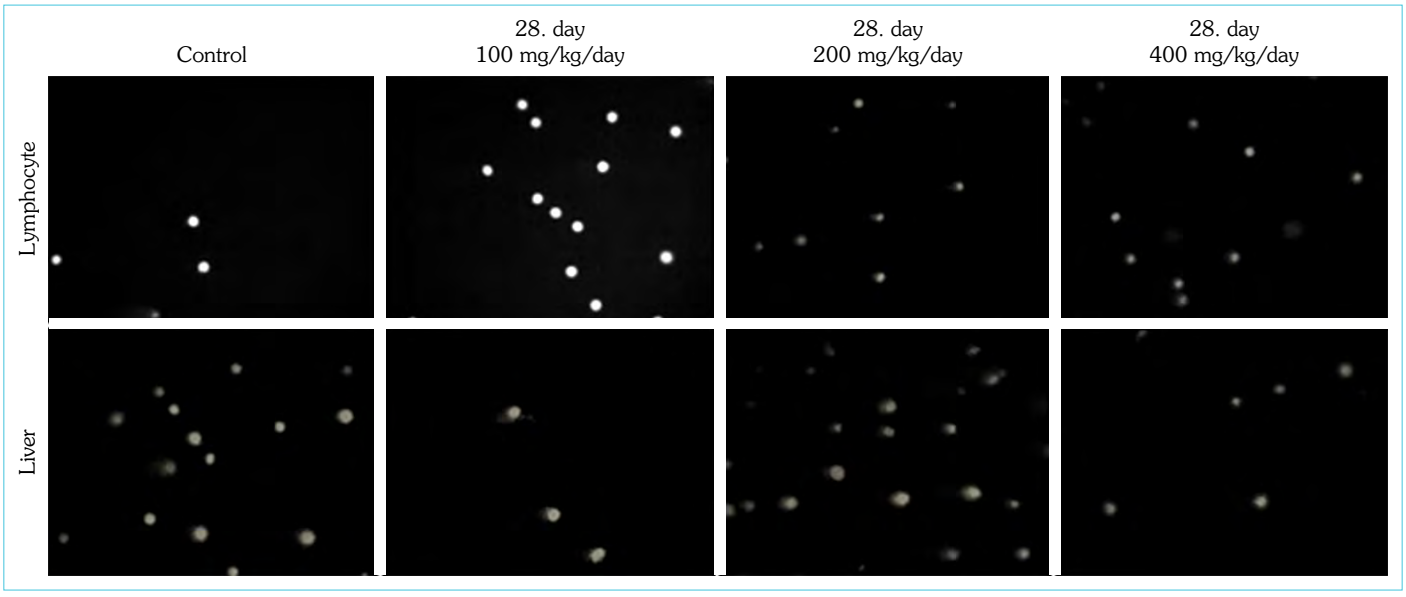

Figure 8. Comet images of lymphocyte and liver samples from oil control group and 200, 400, and 800 mg/kg 28-day butylparaben treatment groups. (DNA stained with ethidium bromide; ×40)

established that parabens interfere with the normal functioning of endocrine hormones in living things, and numerous studies have suggested negative effects as a result of accumulation. Additional research seeking a safer preservative that could be an alternative to parabens is warranted.

CONCLUSION

This study used 3 dose levels and 2 exposure periods to evaluate the effects of BP exposure. DNA damage was observed in the blood and liver samples of groups treated with BP.

In recent years, there have been many studies of the toxicity of parabens, but few studies of the genotoxic effects. Also, to the best of our knowledge, none have investigated the genotoxic effects of BP from the pre-pubertal period to puberty. Our study findings support previous research findings of paraben toxicity. There may be genotoxic effects on blood and liver cells as a result of exposure to BP, which is commonly used in cosmetics, food products, and pharmaceutical products. Therefore, BP may have the potential to cause DNA damage.

Ethics Committee Approval: The Hacettepe University Experimental Animals Ethics Committee granted approval for this study (date: 05.11.2015, number: 2015/81-6).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – NB; Design – NB, BÇ; Supervision – NB; Resource – BÇ; Materials – BÇ; Data Collection and/or Processing – NB, BÇ; Analysis and/or Interpretation – BÇ; Literature Search – NB, BÇ; Writing – NB; Critical Reviews – NB.

Conflict of Interest: The authors have no conflict of interest to declare.

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