

# An Investigation of Micronucleus Induction by Butylated Hydroxytoluene in Wistar Rat Bone Marrow Cells <sup>[1]</sup>

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## Summary

This study aims to investigate whether Butylated Hydroxytoluene (BHT), the synthetic antioxidant and food additive, increases the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in rat bone marrow. BHT, dissolved in corn oil, was administered intraperitoneally to 8-10 week old male and female Wistar rats (n=36) in three different doses (125, 250 and 500 mg/kg b.w.) for two different time periods. 12- and 24-h after BHT treatment, the bone marrow samples were analyzed for the frequency of MNPCEs. Additionally, by evaluating the ratio of polychromatic erythrocyte to normochromatic erythrocyte (PCE/NCE), the cytotoxic effect of BHT on bone marrow was tested. It was found that all BHT doses at two time periods significantly increased the MNPCEs frequency about 1.9-2.84 fold. On the other hand, BHT caused significant decreased in the PCE/NCE ratio, which is indicative of bone marrow cytotoxicity when compared to the control groups. This study showed that BHT increased the formation of MNPCEs in rat bone marrow and we think that this increase might be related to the applied doses and the administration way BHT.

**Keywords:** Butylated hydroxytoluene, Cytotoxicity, Genotoxicity, Micronucleus

## Bütıl Hidroksitoluen Tarafından Mikronükleus İndüksiyonunun Wistar Sıçan Kemik İliği Hücrelerinde Araştırılması

### Özet

Bu çalışma ile sentetik antioksidan ve yiyeceklerde katkı maddesi olarak kullanılan Bütıl Hidroksitoluenin (BHT) rat kemik iliğinde mikronükleuslu polikromatik eritrositlerin sayılarında herhangi bir artışa neden olup olmadığı araştırıldı. Mısır yağında çözölen BHT, iki farklı zaman periyodu ve üç farklı dozda (125, 250, 500 mg/kg v.a.), 8-10 haftalık erkek ve dişi Wistar albino sıçanlara (n=36) intraperitoneal olarak verildi. BHT muamelesinden 12 ve 24 saat sonra kemik iliği örnekleri MNPCE sayısı için analiz edildi. Ayrıca, polikromatik eritrositlerin normokromatik eritrositlere oranı (PCE/NCE) değerlendirilerek BHT'nin kemik iliğindeki sitotoksik etkisine bakıldı. Bütün BHT dozları ve iki farklı zaman periyodunda MNPCE'lerin sayısında 1.9-2.84 kat önemli artışlar bulundu. Diğer taraftan BHT, kemik iliği sitotoksiste belirteci olan PCE/NCE oranını kontrol grubuna kıyasla düşürdü. Sonuç olarak bu çalışmada BHT'nin rat kemik iliğinde MNPCE oluşumunu artırdığını ve bu artışın, uygulanan dozlar ve verilme şekliyle ilişkili olabileceğini düşünmekteyiz.

**Anahtar sözcükler:** Bütıl Hidroksitoluen, Sitotoksiste, Genotoksiste, Mikronükleus

## INTRODUCTION

Butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-*p*-cresol), a synthetic antioxidant that is widely used in the food industry around the world, is used to maintain and protect foods' color, taste, nutritive value, and freshness. It is also used to preserve drugs, fat-soluble vitamins and cosmetics for long periods of time. BHT helps to increase the life extension of rubber, elastomers and plastics <sup>[1,2]</sup>.

BHT's genotoxicity was investigated in a large number of *in vitro* and *in vivo* test systems. The negative results were obtained from the mutagenicity studies performed by adding BHT to plates with different strains of *Salmonella typhimurium* <sup>[3-6]</sup>. The micronucleus test conducted by the Stanford Research Institute (S.R.I.) also presented negative results for BHT administered with doses of 30, 90, and



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1400 mg/kg (acute and subacute) in rat bone marrow cells [7]. Jung and Ryu did not find any increase in micronucleus frequency of mouse peripheral reticulocytes in BHT applications [8]. According to the World Health Organization, International Agency for Research on Cancer (WHO/IARC), BHT does not stimulate the DNA damage in *Bacillus subtilis*, mutations in *Salmonella typhimurium*, chromosomal aberrations in plants and *Drosophila melanogaster*, dominant lethal mutations in mice and micronucleus formation in bone [9]. The present study aims to investigate whether BHT is genotoxic in three different doses and two different time periods in rat bone marrow cells by applying micronucleus assay.

## MATERIAL and METHODS

### Animals

Healthy adult male and female *Wistar albino* rats (n=42), 8-10 weeks of age, with an average body weight of 180-200 g, were used in this study. Rats were obtained from the Experimental Medical Research Unit at Kocaeli University, Turkey. Rats were randomly selected and housed in polycarbonate cages with free access to tap water and rat chow with a 12 h dark/light cycle. The temperature value of the animal laboratory was 22±2°C and the relative humidity was 50-70%. For each dose group, six animals were used and were allowed one week to adjust to their new environment. The Ethics Committee of Kocaeli University School of Medicine gave ethical approval for this research (Ethical Approval No: 2011-24) and all procedures on animals were performed in accordance with the guidelines of this ethics unit.

### Experimental Design and Doses

In this study, the food preservative BHT (Sigma - B1378) (E321) was used as the test substance. BHT was dissolved in corn oil. A total of 42 animals were randomly divided into seven groups, each including three female and three male rats (n=6 per group). Six groups (n=36) served as BHT-treated group while one group served as control group (n=6). Rats in BHT-treated groups were administered a single dose of BHT intraperitoneally at concentrations of 125, 250, and 500 mg/kg b.w. for 12 and 24 h before sacrifice. Rats in control group were given only corn oil. The control group was also referred to the solvent control. Rats were sacrificed by cervical dislocation at 12 and 24 h after BHT treatment. Femurs of each rat were bilaterally harvested and cleaned of any adhering muscle. Bone marrow cells were bilaterally collected from the rats' femurs.

### Micronucleus Test

The frequency of micronucleated erythrocytes in femoral bone marrow was evaluated according to the procedure of Schmid with slight modifications of Agarwal

and Chauhan [10,11]. The bone marrow was flushed out from both femora using 1 ml of fetal calf serum and centrifuged at 336 g for 10 min and the supernatant was discarded. Evenly spread bone marrow smears were stained using the May-Grünwald and Giemsa protocol [12]. Slides were scored at a magnification of 1000x using a light microscope.

### Scoring

For the analysis of MN, 2000 polychromatic erythrocytes (PCEs) per animal were scored to calculate the MN frequencies. The PCE/NCE (Normochromatic erythrocyte) ratio was also determined based on a total of 1000 erythrocytes counted. PCEs appear as blue and NCEs appear as orange/pink in bone marrow.

### Statistical Analysis

The Kruskal Wallis Method was used because each group consisted of four animals and the difference between the groups was identified with the method. Then, statistical analysis was performed using the SPSS 18 package program. To determine the statistical significance between dosage and effect, depending on the time, the Mann-Whitney U Test was used. P<0.05 was considered as the level of significance.

## RESULTS

The investigation of the genotoxic effects of BHT in rats was evaluated by the detection of micronucleus frequency in PCEs in bone marrow. No sign of sickness, decreased activity, or mortality was observed with the rats used during the study.

BHT induced a significant increase of MNPCEs for all treatment groups when compared with control groups (Table 1, Fig. 1) (P<0.05). In Table 1, in contrast to an increase in doses, a decrease was observed in the number of MNPCEs in 12- and 24-h experimental groups. Within the examination of the 12-h groups, it was found that the decrease in the number of MNPCEs at 500 mg/kg was statistically different from doses of 125 and 250 mg/kg. On the other hand, when 24-h experimental groups were examined, in contrast to an increase in doses, a decrease was observed in the numbers of MNPCEs. This, however, was not statistically significant. Comparing the experimental groups of the same BHT doses at 12 and 24 h, statistically no difference was found in terms of the numbers of MNPCEs.

The significant decreases in the ratio of PCE to NCE were recorded between experimental and control groups while there are significant increases in the MNPCEs frequency (Table 1). In the 12-h experimental group, decreases were observed in the PCE/NCE ratio in contrast to an increase in BHT doses with 125, 250 and 500 mg/kg. These decreases were statistically significant at the 0.05 level. In the

**Table 1.** The induction of micronucleus in rat bone marrow erythrocytes after butylated hydroxytoluene treatment**Tablo 1.** Bütil hidroksitoluen muamelesinden sonra sıçan kemik iliği eritrositlerinde mikronükleus indüksiyonu

Treatments	Dose (mg/kg)	Time (h)	Total PCE/n	MNPCE/2000 PCE	PCE/NCE
Control <sup>a</sup>	-	-	12000/6	6.25±1.25	1.59±0.140
BHT	125	12	12000/6	17.75±1.5 <sup>††</sup>	1.14±0.044 <sup>#</sup>
	250	12	12000/6	15.7±1.25 <sup>††</sup>	0.75±0.016 <sup>††#</sup>
	500	12	12000/6	12.25±0.96 <sup>*</sup>	0.68±0.031 <sup>*°</sup>
	125	24	12000/6	14.75±0.95 <sup>*</sup>	0.92±0.042 <sup>*</sup>
	250	24	12000/6	14.30±0.96 <sup>*</sup>	0.93±0.018 <sup>*</sup>
	500	24	12000/6	13.75±0.96 <sup>*</sup>	0.59±0.047 <sup>†</sup>

*n*: number of animals per group; MNPCE, micronucleated polychromatic erythrocyte; PCE: polychromatic erythrocyte; NCE: normochromatic erythrocyte. All data are presented as mean ± standard deviation, <sup>a</sup> Corn oil; \* *P*<0.05: significantly different from control groups; <sup>†</sup> *P*<0.05: significantly different from groups with BHT doses of same time periods; <sup>#</sup> *P*<0.05: significantly different from groups with time periods of same BHT doses



**Fig 1.** White arrow refers to MNPCE; black arrow refers to NCE; gray arrow refers to PCE

**Şekil 1.** Beyaz ok MNPCE; siyah ok NCE; gri ok PCE'yi işaret etmektedir

case of the 24-h experimental group, the statistically significant, approximately 1.5 fold decrease, was observed in the 500 mg/kg BHT dose compared with 125 and 250 mg/kg BHT doses. Comparing within the experimental groups of matching BHT doses at 12 and 24 h, the PCE/NCE ratio in animals given 125 and 250 mg/kg BHT doses was statistically significant at the 0.05 level, though no difference was found in the application of 500 mg/kg.

The PCE/NCE ratio in *Table 1* shows whether BHT had a cytotoxic effect on bone marrow. Accordingly, the PCE/NCE ratio was decreased in all applied BHT doses. This is an indication of the cytotoxicity of the bone marrow. The highest reduction was observed in rats treated with the highest dose of BHT.

## DISCUSSION

A micronucleus test is a very useful biomonitoring test which is used to predict a cancer risk, screen for cancer and for researching the genotoxic and carcinogenic potentials and reabilities of all kinds of chemical substances such as physical factors, drugs, environmental pollutants and food additives that we are often exposed in our daily lives. Because of its advantages such as simplicity, reliability, validity and applicability in different cell types, the micro-

nucleus test has been used for many years [12-17].

As reported in Bomhard et al.<sup>[6]</sup>, with regard to point mutation assays, negative results were obtained in most of the genotoxicity tests with BHT. These tests include *in vitro* studies performed with various bacterial species, strains and various mammalian cell lines as well as *in vivo* studies with *Drosophila melanogaster*, silk worms and mouse specific locus tests<sup>[6,18]</sup>. According to the European Food Safety Authority Panel (EFSA Panel), effects of BHT on tumor formation reported by Olsen et al. are subject to a threshold because the genotoxicity studies generally show a lack of potential for BHT to induce point mutations, chromosomal aberrations, or to interact with or damage DNA<sup>[18,19]</sup>. The Benchmark dose (BMD) analysis from the Panel shows that the Benchmark dose, lower 95% confidence limit at 10% extra risk (BMDL<sub>10</sub>) value was 247 mg/kg b.w./day for BHT derived from the data reported by Olsen et al.<sup>[18]</sup>.

As reported in Bomhard et al.<sup>[6]</sup> study, there were a large number of genotoxicity studies done with BHT in *Salmonella typhimurium* in the 1980's and 1990's. The study conducted by Shelef and Chin, indicated that the addition of BHT (5-20 µg/plate) caused a 2-fold increase on mutagenic potential of aflatoxin B1 using *Salmonella typhimurium* TA98 and TA100 strains<sup>[20]</sup>. However, in a

similar study performed by Redy et al.<sup>[21]</sup> it was reported that the mutagenicity induced by 3,2'-dimethyl-4-aminobiphenyl in the presence of rat liver S-9 fraction on *Salmonella typhimurium* TA98 and TA100 strains was inhibited by BHT. Similarly, Ames test results were found negative in point mutation studies performed by adding different amounts of BHT to plates with the same bacterial species and strains<sup>[3-5]</sup>.

A study performed by using Djungarian hamster fibroblast cultures transformed SV-40, it was found that a concentration as low as 10 µg/ml BHT exerted a strong inhibitory effect on cell to cell dye transfer (Lucifer yellow transfer). However, it was suggested that this effect was reversible and BHT shared this effect with a series of well-known tumor promoters<sup>[22]</sup>.

In the studies conducted by S.R.I. in 1972, the result of chromosomal aberration assay using 2.5-250 µg/ml BHT with human WI-38 (embryonic lung cell) was positive, and MN assay using rat bone marrow applied BHT at concentrations of 30, 90 and 1400 mg/kg (acute and sub-acute) gave negative results<sup>[7]</sup>.

Jung and Ryu applied the micronucleus test in mouse peripheral reticulocytes *in vivo*. They injected single dose BHT at doses of 17.3, 34.5 and 69.0 mg/kg intraperitoneally. In the evaluation of the frequency of MNRETs at 48 h after BHT, they found that BHT administration did not cause a dose-dependent increase in the frequency of MNRETs<sup>[8]</sup>.

In this study, male *Wistar albino* rats were injected intraperitoneally with BHT at doses of 125, 250 and 500 mg/kg b.w., and after 12 and 24 h. of BHT treatment the sampling of bone marrow was taken for evaluation. At the end of the study, it was found that all administered BHT doses and time periods caused an increase in the frequency of MNPCEs (Fig. 1). The highest frequency of MNPCEs was in 125 mg/kg b.w dose at the 12 h time period; this increase was approximately 3 fold when compared to control groups (Table 1). We might say that the dose of 500 mg/kg showed a similar effect on both MNPCEs numbers and PCE/NCE ratio at 12 h and 24 h time periods. In this study, we also determined that BHT caused a decrease in the ratio of PCE/NCE, demonstrating the cytotoxic effect of BHT in rat bone marrow cells. The ratio of PCE to NCE is an important index in showing the toxicity of chemical substances affecting bone marrow cells. The significant decrease, which was observed in the PCE/NCE ratio in the chemically treated group when compared to control group, is an indication that the administered chemical reached to bone marrow, caused a decrease in the erythrocyte formation by inhibiting the maturation and division of the nucleated erythrocyte precursor cells, and caused a toxic effect<sup>[23-26]</sup>.

As presented in Table 1, the statistically significant increases in the number of MNPCEs were observed in all

doses and two time periods when compared to control groups. However, these increases interestingly reduced with an increased dosage. The statistically significant decreases seen in PCN/NCE ratios when compare to control groups, are parallel to the decrease in the number of MNPCEs in experimental groups.

In comparison to this study in which we clearly found positive results with the studies having negative results for micronucleus frequency. Those differences depend on several factors that are range of dose, way of application, contact time, cell types, and *in vivo* or *in vitro* study. Paschin et al. injected intraperitoneally single dose of 75 mg/kg b.w. BHT into male and female mice<sup>[27]</sup>. Jung and Ryu injected single dose BHT at doses of 17.3, 34.5 and 69.0 mg/kg BHT into mice intraperitoneally and examined the MNRETs frequency for 48 h after BHT<sup>[8]</sup>. Contrary to all these studies, in our study, BHT was administered to *Wistar albino* rats intraperitoneally and bone marrow samples were taken 12 and 24 h after BHT treatment. The applied doses were 125, 250 and 500 mg/kg b.w. All these applications might have caused positive results identified in micronucleus test in our study.

In conclusion, this study which is performed using an *in vivo* micronucleus test demonstrated that the treated dose of BHT at experimental time periods caused an increase in the formation of MNPCEs. Given that BHT shows a promoter effect in carcinogenesis studies, solitary or combined effects of BHT should be considered as the exposure to many different chemicals in cosmetic products and food additives in our daily lives is highly probable.

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