Detection of Genotoxicity of Phenolic Antioxidants, Butylated hydroxyanisole and *tert*-Butylhydroquinone in Multiple Mouse Organs by the Alkaline Comet Assay

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Abstract: In this study we tested the genotoxicity of two widely used phenolic antioxidants, butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ) in multiple mouse organs using the alkaline comet assay. Tissue samples from four organs (stomach, liver, kidney and bone marrow) were collected from male mice at 3 and 24 h post treatment with BHA (800 mg/kg) or TBHQ (400 mg/kg) and examined for genotoxicity. The two compounds induced significant increase in DNA migration in a time dependant manner in specific organs. Extensive DNA damage was observed in stomach cells at 24 h post treatment in treatment groups. In addition to stomach, TBHQ treatment induced significant increase in DNA migration in liver and kidney cells. Although increased DNA damage was found in kidney cells of treatment groups at 3 h time point, at later time point it was persistent only in mice treated with TBHQ and in other treatment group (BHA) it appeared to be recovered with time. Evidently, bone marrow cells did not show genotoxicity in response to treatment with TBHQ and BHA. Considering these findings, although TBHQ and BHA are generally considered non-genotoxic, the DNA damage observed in this experiment may be related to their indirect action on DNA via ROS mechanism. Since toxicity of these compounds are often ascribed to their metabolic products such as quinone thioethers and hence differences in the metabolism of these compounds may play an important role in determining the target organ of toxicity. The present work draws our attention to revising the genotoxicity of the widely used antioxidants and accepted as safe artificial antioxidants.
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Key wards: BHA; TBHQ; comet assay; mice; bone marrow; liver; kidney; stomach; ENU.

1. Introduction

Butylated hydroxyanisole (BHA, 3-*tert*-butyl 4-hydroxyanisole) and its *O*-demethylated metabolite *tert*-butylhydroquinone (TBHQ) are synthetic phenolic food antioxidants widely used to protect oils, fats and shortenings from oxidative deterioration and rancidity (**Finley *et al*., 2011**). Both compounds are generally non-mutagenic and nearly all short-term genotoxicity tests including the classic *Salmonella typhimurium* with or without S9 fraction (**Matsuoka *et al*., 1990**), unscheduled DNA synthesis assay using rat hepatocytes, and except both positive and negative results with and without activation respectively in Chinese hamster cells for chromosomal aberration and sister chromatid exchange are negative (**Matsuoka *et al*., 1990; Williams *et al*., 1990**). On the other hand; chronic dietary administration of BHA resulted in DNA damage in forestomach of rats (**Morimoto et al., 1991**) and papilloma and carcinoma formation in the forestomach of rats, mice and hamsters (**Clayson *et al*., 1990; Chandra *et al*., 2010**). In fact, both BHA and TBHQ have shown to contain both carcinogenic and anticarcinogenic properties (**Kahl, 1997; Gharavi *et al*., 2007**). Anticarcinogenic activity of BHA has been ascribed to its ability to induce Phase II metabolic enzymes and its free radical trapping activity (**Kadoma *et al*., 2008; Cemeli and Anderson, 2011**); its carcinogenicity has been often linked to its quinone-forming metabolites, a property that BHA shares with benzene. Quinones are highly reactive molecules, which can bind to biological macromolecules (**Tu *et al*., 2011**) and induce chromosomal loss or chromosomal breakage (**Jacobus *et al*., 2008**).

The scope of this research is to determine the *in vivo* genotoxic effects of TBHQ and BHA in different organs of mouse. Through conducting the single cell gel electrophoresis assay (Comet assay) on four organs (stomach, liver, kidney and bone marrow) of mice treated with TBHQ (400 mg/kg) or BHA (800 mg/kg). In addition, to identify possible time dependent effect, tissue samples were collected on two occasions, 3 and 24 h post treatment and subjected to the alkaline version of the comet assay. The comet assay is a sensitive cytogenetic assay with advantages of detecting broad spectrum of DNA damages which may lead into DNA strand breaks, the need of small number of cells per sample, and the short time needed to do a complete study (**Tice *et al*.,**

2000).This assay could be applied to any tissue in the given *in vivo* model, and has potential advantages over other *in vivo* genotoxicity tests methods, which are reliably applicable to one or few tissues which is considered a very important matter for investigation of suspected tissue specific-genotoxic activity, which include 'site of contact' genotoxicity.

2- Material and Methods

Chemicals

All chemicals used in this study were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and treatments.

Twenty male ddY mice were obtained from Japan SLC (Shizuoka, Japan) at 7 weeks of age and used after one week of acclimatization. They were maintained at 20 -24ºC and 55-65 % humidity with a 12-hr light-dark cycle and fed commercial pellets (Orintal Yeast Industries Co., Tokyo, Japan) and tap water *ad libitum.* Aqueous solutions of TBHQ (400 mg/kg) and BHA (800 mg/kg) were administered intragastrically to two groups of three mice at a dose level of 10 ml/kg. A positive control (ENU, 20 mg/kg) group of two mice were treated at the same time in the same manner. The animals were sacrificed by cervical dislocation at 3 h and 24 h after treatment and four organs - bone marrow, liver, kidney and stomach - were dissected out. Tissue samples were collected from four untreated mice at the latter sampling point (24 h) and treated as the negative control.

The tissue samples except bone marrow were washed in saline, minced, suspended at a concentration of 1 g/ml in ice cold homogenizing buffer (HBSS with 20 mM EDTA, 10% DMSO, pH 7.5) and homogenized gently using a Dounces' homogenizer. The marrow was collected from the femur bones and suspended in 1 ml of chilled homogenizing buffer. The cell suspensions were diluted in chilled homogenizing buffer appropriately and subjected to the alkaline comet assay immediately (**Tice *et al*., 2000**).

Comet assay

The alkaline comet assay was preformed basically as described by **Miyamae *et al*. (1998**) with few modifications. The cell suspensions prepared as above were mixed 1: 1 (v/v) with 1% low melting point (37°C) agarose (LMA) prepared in PBS, pH 7.4 and 75 µl of the mixture was quickly layered on a 1% normal melting point agarose (NMA) (prepared in distilled water) precoated and overnight dried slides and covered with a coverslip. Then the slides were placed on a chilled plate to allow complete polymerization of agarose. Finally 75 µl of 0.5% NMA in PBS was quickly layered in the same manner after removing the coverslip and allowed to solidify on chilled plate. The slides were immersed in freshly made chilled lysis solution (2.5M NaCl, 100mM Na_2EDTA, 10mM Trizma base, 10% DMSO and 1% Triton X-100; pH 10.0) in dark at 4°C for 60 min. The slides were then placed in a horizontal electrophoresis tank containing electrophoresis buffer (300mM NaOH and 1mM Na_2EDTA; pH 13.0) for 10 min, allowing salt equilibration and further DNA unwinding before electrophoresis at 25 V (0.8V/cm), 300 mA for 20 min. The slides were washed three times (5 min each) with chilled neutralizing buffer, 0.4M Tris-HCl (pH 7.5). After the third wash, the slides were stained with 50 µl EtBr (20 µl/ml) and covered with a coverslip. To prevent drying, the slides were stored in a humidified container until microscopic examination.

The slides were examined at 200x magnification using Olympus fluorescent microscope. All slides were coded and examined blindly. A total of 1000 randomly selected cells from two replicate slides (500 cells per slide) were examined per sample. The comets were classified into five categories (Type1 – Type 5) depending on the fraction of DNA migrated out into the tail, and thus increasing degrees of damage (**Miyamae *et al*., 1998**). The comets were assigned a value of 0, 1, 2, 3 or 4 (from undamaged, 0, to maximally damaged, 4). Thus, the total score for 1000 comets ranged from 0 (all undamaged) to 4000 (all damaged) in arbitrary units (**Visvardis *et al*., 1997; Piperakis *et al*., 1998**). In this method a large number of cells can be examined in a short time.

Statistical analysis

Results of the different treatment groups were compared to data obtained from four untreated mice using Students' one-tailed *t*-test (**Fowler *et al.*, 1998**). Significance was indicated by P values <0.05

3. Results

As shown in table 1; the comet data measured by visual scoring from four organs (bone marrow, liver, kidney and stomach) from mice intragastrically treated with TBHQ (400mg/kg b.w.) or BHA (800 mg/kg b.w.) for 3 and 24 hr. In this experiment comet data obtained from four untreated mice were treated as the negative control and used for comparison. Among TBHQ treated animals, 24 hr after exposure mean DNA migration score was significantly ($P<0.05$) high in 3 organs (stomach, liver and kidney), although at earlier time point relative to control value it was different significantly only in kidney. In bone marrow the induced DNA strand breaks were not significant at both time points.

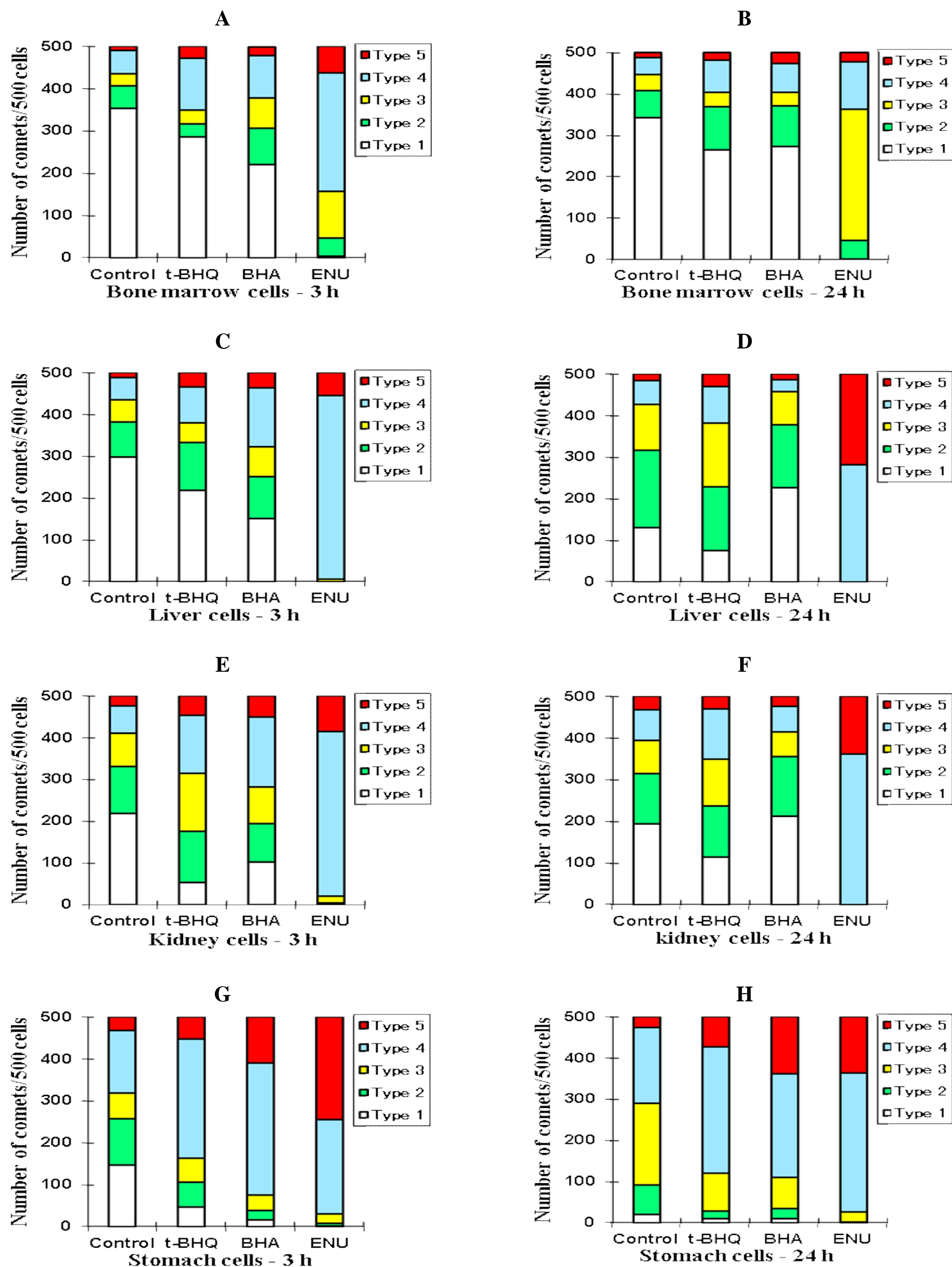

Fig. 1. Incidences of comet cells in mice treated with TBHQ (400 mg/kg b.w.), BHA (800 mg/kg b.w.) and the positive control ENU (20 mg/kg b.w.) for 3 and 24 h. The comet cells derived from bone marrow (Figs. 1A and 1B), liver (Figs. 1C and 1D), kidney (Figs. 1E and 1F) and stomach (Figs. 1G and 1H).

Table 1. Incidences of comet cells without type 1 in bone marrow, liver, kidney and stomach of mice treated with t-BHQ and BHA for 3 and 24 h.

Treatment	Pooled calculations of incidences of comets without type 0 (Average/500 cells ± S.D.)							
	Bone marrow		Liver		Kidney		Stomach	
	3 h	24 h	3 h	24 h	3 h	24 h	2h	24 h
Control (Dis. H2O)	146.3 ± 125.3	156.5 ± 16.3	202.0 ± 116.0	369.5 ± 99.3	283.3 ± 110.3	305.3 ± 94.6	350.0 ± 109.4	479.5 ± 14.5
t-BHQ (400 mg/kg)	213.7± 136	234.3 ± 37.7	274.5 ± 61.3	425.2 ± 59.6*	447.5 ± 66.1	385.2 ± 66.1*	453.5 ± 29.1	490.3 ± 8.1*
BHA (800 mg/kg)	279.3 ± 171.5	226.0 ± 45.1	345.0 ± 40.5	237.2 ± 72.2	397.2 ± 79.1*	287.3 ± 97.7	483.3 ± 13.6*	490.1 ± 11.6*
ENU (20 mg/kg)	495.0 ± 6.0**	498.5 ± 3.0**	500.0 ± 0.0**	500.0 ± 0.0***	500.0 ± 0.0**	500.0 ± 0.0***	498.25 ± 2.1**	500.0 ± 0.0***

n.b.
ns = non significant; * = (P< 0.05) ; * = (P< 0.01) ; * = (P< 0.001)

Exposure to BHA resulted in significant (P<0.05) increase in DNA migration in a time dependent manner in specific organs which was observed among stomach tissues at both sampling points, in kidney it occurred only at 3 hr time point and then decreased with time. Following BHA treatment liver and bone marrow cells did not yield any significant increase in mean DNA migration. ENU (20 mg/kg) treatment produced highly significant (P<0.001) DNA damage in all four tested organs at both time points.

Figure 1 shows the distribution pattern of nuclear DNA expressed as the percent cells in the five comet classes from Type 1 (undamaged) to Type 5 (maximally damaged) in various organs of mice belonged to different treatment groups. Except in ENU (positive control) treated group, in other samples some cells/nuclei remained undamaged (category Type 1). At both sampling time points in all treatment groups' greater numbers of comets showed high levels of DNA damage (category Type 4) in stomach sells. The frequency distribution of DNA damage among individual hepatocytes showed that the number of damaged cells and the extent of damage were greatest among mice treated with TBHQ (Figures 1-C and 1-D). Similarly in renal cells the extent of DNA damage decreased with time in BHA treated animals (Figures 1-E and 1-F). Higher levels of damage were observed in renal cells from TBHQ treated mice. Among bone marrow cells in treatment groups (TBHQ and BHA) over 50% of the cells showed no damage (Figures 1-A and 1-B)

4. Discussion

Considering the strong correlation between organ specific genotoxicity and organ specific carcinogenicity, the assessment of genotoxicity in multiple organs *in vivo* may indicate that it would be the target organ(s) in humans and provide useful information for the evaluation of chemical safety. While other *in vivo* genotoxicity tests are limited to one or few tissues, the comet assay can be applied to any tissue provided that a single cell/nucleus suspension can be obtained. Therefore, in the present research, to test the tissue specific genotoxic effect of TBHQ and, BHA mice were administered intragastrically with the test compounds and various organs were examined for genotoxicity using the comet assay. Results of this research suggest that the test compounds induced significant DNA strand breaks in stomach cells and the effect was more pronounced among BHA treated animals at both sampling occasions. With TBHQ, a significantly higher degree of DNA damage was observed at 24 h time point, although at earlier time point (3 h) the induced DNA damage in these treatment groups were not significantly different from control data. These observations again acknowledge the earlier findings that stomach in particular forestomach in rodent is the target organ for BHA induced genotoxicity. It is also important to remember that there are several bioassay reports describing neoplasm-promoting effects of BHA and TBHQ in the fore stomach when given after initiating carcinogens such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (**Kuroiwa *et al*., 2007; Liu and Russell, 2008**), N-methyl-N-nitrosourea (NMU) and N,N-dibutylnitrosamine (DBN) (**Fukushima *et al*., 1987**). In this respect, there are studies, which demonstrated that BHA inhibits cell-to-cell communication (**Williams *et al*., 1990**), which is often considered a marker for tumor promoters (**Leithe *et al*., 2006; Vinken *et al*., 2009**). In the bone marrow cells, although at earlier time point a non-significant increase in DNA migration was observed in BHA and t-BHQ treated animals, it was not persistent at latter time point and appeared to be recovered with time. *In vivo* BHA is metabolized to t-BHQ in the liver (**Peters *et al*., 1996; Moridani *et al*., 2002**). TBHQ and HQ are further oxidized to their respective quinone species, 1,4-benzoquinone and tertiary-butylquinone, which in turn may enter

into a redox cycling between quinone and hydroquinone via semiquinone radicals and can generate reactive oxygen species (**Peters *et al*., 1996; Li *et al*., 2002**). These ROS may then react with cellular protein and DNA and thus cause large-scale chromosomal alterations (**Cerutti, 1985**). The test compounds used in this study have been reported to be nephrotoxic and can induce tumor in rodent kidney (**Shibata *et al*., 1991; Kari *et al*., 1992**). Nonetheless, the evidence for genotoxicity as increased level of DNA migration in the liver and kidney was observed only in mice treated with t-BHQ. The nephrotoxicity of these compounds are often linked to their quinone thioether metabolites (**Monks *et al*., 1992**) and their ability to react with DNA either directly (**Jacobus *et al*., 2008**) or indirectly via reactive oxygen generation (**Li *et al*., 2002; Li *et al*., 2007**).Hence, differences in metabolism of these compounds and ability of their metabolites to generate ROS could be a possible reason for the observed differences in toxicity (**Kadoma *et al*., 2008**).

DNA strand breaks are associated both to the removal of DNA adducts by endonuclease and tothe direct action of the chemicals and/or the free radicals produced during their metabolism, which then creates both single- and double-strand breaks in DNA (**Bohr, 1991**). Investigations of BHA and its metabolites have not demonstrated DNA adducts formation, as measured by the sensitive [^{32}P] postlabelling assay (**Saito *et al*., 1989**). Previous research has showed that enzymatic oxidation of TBTQ in rodent forestomach may occur via prostaglandin H synthase PHS (**Schildermanet *al*., 1993**) or possibly a gastric peroxidase (**Banerjee, 1988**). It is widely believed that TBHQ; an *O*-demethylated metabolite of BHA probably produces many of the toxic effects ascribed to BHA. It is also postulated that BHA and TBHQ act through ROS induced oxidative damage including DNA damage (**Inverson, 1995**). Supporting this suggestion studies have demonstrated the formation of 8-hydroxydeoxyguanosine, a marker for reactive oxygen formation and for DNA strand breaks (**Schilderman *et al*., 1995; Okubo *et al*., 1997**), in calf thymus DNA (**Nagai *et al*., 1996**) and in isolated DNA and cultured rat hepatocytes (**Li *et al*., 2002**) treated with TBHQ. **Peters *et al.* (1996)** have demonstrated that TBHQ and its glutathione conjugates are capable of catalyzing 8-hydroxydeoxyguanosin formation and thus induce oxidative DNA damage. Further, **Dobo and Eastmond (1994)** have demonstrated the role of reactive oxygen species in the chromosomal breakage induced by TBHQ in a prostaglandin H synthase containing Chinese hamster V79 cell line. Considering these findings, although TBHQ and BHA are generally considered non-genotoxic, the DNA damage observed in this experiment may be related to their indirect action on DNA via ROS mechanism. Since toxicity of these compounds are often ascribed to their metabolic products such as quinone thioethers and hence differences in the metabolism of these compounds may play an important role in determining the target organ of toxicity.

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