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Inhibition of Sudan I genotoxicity in human liver-derived HepG2 cells by the antioxidant hydroxytyrosol

XIAOMEI ZHANG[1,2], LIPING JIANG[3], CHENGYAN GENG[3], CUNLI HU[1], HIROYUKI YOSHIMURA [4], & LAIFU ZHONG[1]

[1]*Department of Toxicology, Dalian Medical University, 465 Zhongshan Road, Dalian 116027, Liaoning, China,* [2]*Department of Laboratory Medicine, College of Medicine, Dalian University, Dalian 116622, Liaoning, China,* [3]*China-Japanese Joint Institute for Medical and Phamaceutical Science, Dalian Medical University, 465 Zhongshan Road, Dalian 116027, Liaoning, China, and* [4]*Eisai Food & Chemical Co., Ltd, Nihonbashi, chuo-ku, Tokyo 103-0027, Japan*

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Abstract

The chemoprotective effect of hydroxytyrosol (HT) against Sudan I-induced genotoxicity was investigated in a human hepatoma cell line, HepG2. The comet assay and micronucleus (MN) assay were used to monitor genotoxicity. Intracellular reactive oxygen species (ROS) formation was measured using a fluorescent probe, 2,7-dichlorofluorescein diacetate (DCFH-DA). The levels of oxidative DNA damage and lipid peroxidation were estimated by immunocytochemistry analysis of 8-hydroxydeoxyguanosine (8-OHdG) and by measuring levels of thiobarbituric acid-reactive substances (TBARS), respectively. Intracellular glutathione (GSH) level was estimated by fluorometric methods. The results showed that HT significantly reduced the genotoxicity caused by Sudan I. Furthermore, HT ameliorated lipid pexidation as demonstrated by a reduction in TBARS formation and attenuated GSH depletion in a concentration-dependent manner. It was also found that HT reduced intracellular ROS formation and 8-OHdG level caused by Sudan I. These results strongly suggest that HT has significant protective ability against Sudan I-induced genotoxicity.

Keywords: *Chemoprotective, HepG2 cells, hydroxytyrosol, genotoxicity, Sudan I.*

Abbreviations: *DCFH-DA, dichlorofluorescein diacetate; EtBr, ethidium bromide; MN, micronucleus; 8-OHdG, 8-hydroxydeoxyguanosine; HT, hydroxytyrosol; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis; GSH, glutathione; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; T-BHP, tert-butylhydroperoxide; LMP, low melting point; DMSO, dimethylsulphoxide; NMP, normal melting point; MEM, minimum essential Eagle's medium.*

Introduction

There is general agreement that the Mediterranean diet, characterized by an abundance of fruits and vegetables, contributes to the prevention of various chronic degenerative diseases such as cardiovascular diseases and cancer [1]. Olive oil is the major fat source of this diet and is considered an important component responsible, at least in part, for the low incidence of cardiovascular diseases in the Mediterranean area [2]. The beneficial health effects of olive oil have been mainly attributed to its elevated oleic acid content and the high level of antioxidants in the non-saponifiable fraction, including phenolic compounds absent in seed oils [3,4]. Among them, hydroxytyrosol (HT) is the most representative and

Correspondence: Laifu Zhong, Department of Toxicology, Dalian Medical University, 465 Zhongshan Road, Dalian 116027, China. Fax: +86 411 8472 0661. Email: zxmljw@hotmail.com

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it is present in olive oil in a free form or linked to the dialdehydic form of elenolic acid and as an isomer of oleuropein aglycon [5]. HT has been shown to function as an efficient scavenger of peroxyl radicals [6] and contributes toward determining the shelf-life of the oil, preventing its auto-oxidation [7,8].

HT *in vitro* prevents protein damage in melanoma cells by reactive oxygen species (ROS) induced by long-wave ultraviolet light [9] and DNA damage in Jurkat cells exposed to hydrogen peroxide (H_2O_2) [10]. Pre-incubation of intestinal Caco-2 cells with HT prevents the typical damages of oxidative stress [11]. Similarly, HT exerts a protective effect against the H_2O_2-induced oxidative hemolysis and malondialdehyde (MDA) formation in red blood cells [12]. It also exerts an inhibitory effect on the glutathione (GSH) depletion induced by tert-butylhydroperoxide (t-BHP) in HepG2 cells [13]. In addition, HT *in vitro* prevents LDL oxidation and platelet aggregation and inhibits 5- and 12-lipoxygenases [14]. It also exerts an inhibitory effect on peroxynitrite-dependent DNA base modifications and tyrosine nitration [15].

Sudan I is a synthetic lipid soluble azo pigment and it is extensively used in hydrocarbon solvents, oils, fats, waxes, plastics, printing inks, shoes, floor polishes, cellulose ester varnishes, styrene resins, gasoline and soap [16]. It has also been adopted for colouring various foodstuffs, particularly in those containing chilli powders, because of their intense red-orange colour [17]. Such a wide use of Sudan I can result in a considerable exposure. Nevertheless, it has been recommended as unsafe, because it causes tumours in the liver or urinary bladder in rats, mice and rabbits and is considered a possible carcinogen and mutagen for humans [18]. Sudan I gives positive results in micronucleus test (MNT) in bone marrow cells and peripheral blood reticulocytes of F344 rats exposed to 1000 mg/kg Sudan I [19]. In addition to the tests *in vivo*, positive results were also observed in Salmonella typhimurium mutagenicity tests with S9 activation [20]. Moreover, the comet assay also showed increased DNA-migration in the HepG2 cells exposed to Sudan I [21].

The purpose of the present study was to examine the protective effects of HT on genotoxicity induced by Sudan I and to characterize the mechanisms involved in HepG2 cells. The HepG2 cell line retains many characteristics of hepatocytes such as the activities of phase I and phase II enzymes that play a key role in the activation of genotoxic mutagens or carcinogens [22].

To ascertain the potential chemopreventive properties of HT, the present study investigated HT inhibition of the genotoxicity induced by Sudan I in HepG2 cells, using MNT and comet assay. In short-term genotoxicity assays, the comet assay and MNT were sensitive and easy to perform. In order to clarify the underlying mechanisms, we have studied 8-OHdG, lipid peroxidation, GSH level and ROS level of Sudan I-treated HepG2 cells in the absence or presence of HT. The overall results suggest a protective effect of HT against genotoxicity induced by Sudan I in HepG2 cells.

Materials and methods

Chemicals, materials and mediums

Sudan I (CAS No.842-07-9) was purchased from Sigma-Aldrich (Germany; purity > 97%). Dimethylsulphoxide (DMSO), RNAase A, Cytochalasin B, ethidium bromide (EtBr) and DCFH-DA were bought from Sigma (St. Louis, MO). Low melting point (LMP) agarose and normal melting point (NMP) agarose were obtained from Gibco BRL, Life Technologies (Paisley, UK). Monoclonal 8-OHdG antibody and Ultrasensitive Streptavidin-peroxidase Kit were obtained from JaICA (Fukuroi, Japan) and Maixin-Bio (FuJian, China). The sample of HT was supplied by Eisai Food &Chemical Co, Ltd. (Japan). All tissue culture reagents, i.e. minimum essential Eagle's medium, fetal bovine serum, antibiotics (penicillin, streptomycin) and trypsin-EDTA solution were supplied by Gibco BRL-Life Technologies (Grand Island, NY).

Cell culture and treatment

HepG2 cells (ATCC HB-8065) were obtained from Peking Union Medical College (Peking, China) and cultured in minimum essential Eagle's medium (MEM) (ATCC) containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were sub-cultured every 5–7 days at 1:3 split ratios. Cells with eight passages were used for experiments to ensure cell line stability. Medium was changed every 2 days. Stocks of cells were routinely frozen and stored in liquid N_2. Sudan I was prepared as a 10 mm stock solution in filter-sterilized DMSO and stored at -20°C for the aqueous insolubility. HT was dissolved in ultrapure (Milli-Q) water at a concentration of 100 mm, divided into aliquots and stored at -20°C in the dark. For each experiment, cells were treated with different concentrations of HT for 30 min and then exposed to 100 μM Sudan I. Two vehicle controls consisting of culture medium with or without 1% DMSO were included in all subsequent experiments. In each experiment there were no differences between these controls. Thus, we usually employed culture medium with 1% DMSO as the control for comparison.

Comet assay

The comet assay was carried out according to the protocol of Singh and Stephens [23]. HepG2 cells (1×10^6 cells) were suspended in 2 ml MEM and

incubated with HT (0, 25, 50 and 100 μM) at 37°C for 30 min. Then Sudan I (100 μM) was added for another 1 h. After washing twice with PBS, the cells were suspended in 1 ml PBS. To avoid artifacts resulting from necrotic and apoptotic cells, the cell suspensions (50 μl) were mixed with Hoechst 33342 (8 μg/ml) and trypan blue (50 μg/ml). After 15-min treatment the cells were observed under a fluorescent microscope (U-MWU2 filters). Only cell suspensions with viabilities > 90% and no apoptotic cells were used to determine DNA migration. Then the cell suspension in 1% LMP agarose dissolved in PBS was spread onto microscope slides pre-coated with 1.5% NMP agarose. After solidification the slides were immersed in cold fresh lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100 and pH 10) for 1 h. Afterwards, the slides were placed for 20 min in a horizontal gel electrophoresis tank filled with cold electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) to allow DNA unwinding and then they were electrophoresed for 30 min at 200 mA. Cells were neutralized using 0.4 M Tris (pH 7.5) and stained with 50 μl of EtBr (20 μg/ml). Slides were viewed at ×400 magnification using fluorescent microscopy with excitation filter of 549 nm and barrier filter of 590 nm. Then 150 randomly selected cells (50 cells from each of the three replicate slides) were quantitatively analysed using CASP Comet Assay Software casp-1.2.2 (University of Wroclaw, Poland).

MNT

The method of Natarajan and Darroudi [24] for measuring MN assay in HepG2 cells was employed with slight modification. In the present experiment, HepG2 cells were seeded at a density of 5.0×10^5 onto 25 cm^2 tissue flasks and incubated at 37°C for 24 h. The cells were pre-treated with different concentrations of HT (0, 25, 50 and 100 μM) for 30 min. After washing twice with PBS, the cells were added to 100 μM Sudan I and incubated for 24 h. After washing twice with PBS, the cytokinesis blocking agent cytochalasin B (final concentration: 4.5 μg/ml) was added in fresh medium for another 24 h. Then the cells were trypsinized and exposed to cold hypotonic solution (KCl 5.6 g/l) for 20 min. Afterwards, the cells were fixed in a methanol-glacial acetic acid (3:1) solution twice. After air-drying on conventional slides, the cells were stained with 2% Giemsa solution. All slides were coded and the number of cells with MN were recorded based on the observation of 1000 binucleated cells (BNC) for each treatment of concentration. Three independent experiments were performed ($n = 3$).

Measurement of ROS

The intracellular ROS production was measured using the 2′-7′-dichlorofluorescein diacetate (DCFH-DA) by the method of Bass et al. [25], with some modification. DCFH-DA penetrates the cells and is hydrolysed by intracellular esterases to the non-fluorescent DCFH, which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. HepG2 cells (5×10^5 cells/ml) were suspended in 2 ml mediun and incubated with HT (0, 25, 50 and 100 μM) at 37°C for 30 min before adding Sudan I (100 μM) for 1 h. Cells were washed twice with cold PBS and then suspended in PBS and incubated with DCFH-DA at a final concentration of 5 μM for an additional 40 min at 37°C in the dark. The fluorescent intensity of the cell suspensions was then monitored with a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan, excitation wavelength of 485 nm, emission wavelength of 530 nm).

Immunocytochemistry detection of 8-OHdG

A modification of the method of Halliwell and Whiteman [26], for detecting 8-OHdG level as a marker of oxidative damage in single cells was employed. Briefly, exponentially growing HepG2 cells were incubated with HT (0, 25, 50 and 100 μM) for 30 min before adding Sudan I (100 μM) for 3 h on a coverslip and washed with PBS twice. Then the coverslips were fixed with cold acetone and treated with RNAse A (100 μg/ml) for 1 h at 37°C. DNA was denatured for 5 min at 4°C and treated with 0.1% TritonX-100 for 5 min at 4°C. After blocking non-specific antibody binding sites using 10% normal horse serum, the slides were incubated with the primary antibody at 4°C overnight and subsequently added to biotin-conjugated second antibody for 10 min and streptavidin-peroxidase for another 10 min. Diaminobenzidene was applied as colour presentation (3–10 min). The images were recorded by a camera fitted to a microscope (Olympus BX-51, Omachi, Japan) and the relative intensity of the nuclear staining of 50 randomly chosen cells per group was subsequently quantified using a multiparameter analysis program, Image-Pro Plus 4.5.1. The staining data represented the average absorbance multiplied by 1000.

Measurement of lipid peroxidation

Lipid peroxidation was determined by measurement of thiobarbituric acid-reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides [27]. Briefly, the cells were lysed with TritonX-100. The lysed cells (250 μl) were placed into glass tubes and incubated in a water bath at 37°C for 1 h and followed by the addition of 400 μl 35%

perchloric acid. Mixtures were centrifuged at 5000 × g for 10 min at room temperature and the supernatants (600 μl) were added to 200 μl of 1.2% thiobarbituric acid and then placed in a boiling water bath for 30 min. After cooling, the absorbance was measured at 535 nm with BIO-RAD microplate reader Model 3550.

Determination of the intracellular GSH content

Intracellular GSH content was measured as described by previous studies with some modification [28]. Briefly, the cells were incubated with HT for 30 min before exposure to Sudan I (100 μM) for 1 h. After washing twice with PBS (pH 7.4), the cells was added to 400 μl 5% trichloroacetic acid (TCA) at 4°C for 30 min. The final assay mixture contained 50 μl of the diluted supernatant, 0.8 ml of phosphate EDTA buffer (pH 8.3) and 50 μl (final 50 μg/ml) of the o-phthalaldehyde solution. After thorough mixing and incubation at 37°C for 15 min in the dark, the fluorescence was read at an emission wavelength of 420 nm and an excitation wavelength of 350 nm.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed with Student's *t*-test using SPSS v8.0 software (SPSS Inc., Chicago, IL). Differences were considered statistically significant when $p < 0.05$.

Results

The inhibition of HT on the DNA breakage induced by Sudan I

The effect of HT on Sudan I-induced DNA damage is presented in Table I. It was found that HepG2 cells treated with 100 μM Sudan I resulted in serious DNA damage. In contrast, DNA damage was significantly reduced in cells pre-treated with HT. Pre-treatment with 25–100 μM HT for 30 min inhibited Sudan I-induced DNA damage in a concentration-dependent manner (Table I). Pre-treatment of cells with HT at 100 μM for 30 min significantly inhibited comet tail to a maximum value of 77.2% ($p < 0.01$) (Table I). The results also indicated that treatment with HT up to 100 μM did not cause obvious DNA damage in HepG2 cell (data not shown).

The effect of HT on the MN frequencies induced by Sudan I

To evaluate the effect of the HT on the chromosome breakage induced by Sudan I, MNT was measured. Results show that HT can reduce the increase of the MN frequencies induced by Sudan I in a concentration-dependent manner (Table II).

Reductive effect of HT on intracellular ROS level in Sudan I-treated cells

The concentration of intracellular ROS was evaluated by the changes in DCF fluorescence intensity. Table II shows concentration-dependent reduction of the intracellular ROS level in Sudan I-treated cells by HT. DCF fluorescence intensity dropped significantly from 9.03 ± 1.05 in cells treated with Sudan I only to values between 6.08 ± 0.78 ($p < 0.05$) and 2.68 ± 0.24 ($p < 0.01$) in cells to which 25 and 100 μM HT was added, respectively.

The effect of HT on 8-OHdG formation induced by Sudan I

8-OHdG is widely used as a biomarker for oxidative gene damage including cancer. In the present study, blocking of 8-OHdG by prior supplementation of cells with 25, 50 and 100 μM HT resulted in different extent protection, by 27.7% ($p < 0.05$), 37.7% ($p < 0.05$) and 77.7% ($p < 0.01$), respectively (Table II).

The effect of HT on lipid peroxidation in Sudan I-treated HepG2 cells

Lipid peroxidation is the result of interactions between free radicals of diverse origins and unsaturated fatty acids or lipids. In this study, lipid peroxidation was determined by measurement of TBARS. As shown in Table II, pre-treatment of cells with 25–100 μM HT significantly reduced the TBARS formation induced by Sudan I. Meanwhile, compared to cells without HT (control), obvious changes in TBARS formation treated with HT up to 100 μM only were not found.

Table I. Protective effect of HT on Sudan I-induced DNA breakage in HepG2 cells.

Sudan I (μM)/HT (μM)	Tail length (μm)	Tail DNA/head DNA (%)	Tail moment (μm)
100/0	46.49 ± 2.06	82.96 ± 4.67	19.99 ± 1.44
100/25	29.23 ± 5.34*	66.19 ± 2.77*	11.66 ± 0.90*
100/50	14.12 ± 3.20**	24.70 ± 5.34**	5.03 ± 1.04**
100/100	10.23 ± 1.45**	11.23 ± 1.56**	1.12 ± 0.78**
0/0	4.64 ± 0.46	9.01 ± 0.36	0.47 ± 0.05

DNA breakages were estimated by Comet assay. Cells were exposed to 0, 25, 50 and 100 μM HT 30 min before treatment with μM Sudan I. Results are the mean ± SD ($n = 3$). * $p < 0.05$; ** $p < 0.01$ significantly different from cells treated with Sudan I only.

Table II. Effects of HT on the increases of MN frequencies, intracellular ROS level, formation of 8-OHdG, lipid peroxidation and GSH depletion induced by Sudan I in HepG2 cells.

	Concentration of HT (μM)			
	0	25	50	100
MN frequency (%)				
HT	23±1.03	21±1.08	22±1.14	20±0.86
HT+Sudan I	50±3.04	36±2.02*	29±3.05*	29±2.25*
DCF fluorescence intensity				
HT	2.94±0.56	2.97±0.32	2.33±0.25	205±0.18
HT+Sudan I	9.03±1.05	6.08±0.78*	5.28±0.64**	2.68±0.24**
Relative staining intensity of 8-OHdG				
HT	9.36±1.42	10.24±1.35	9.78±1.65	9.91±1.29
HT+Sudan I	90.40±1.40	65.02±1.6*	56.20±0.64**	20.51±0.09**
Intracellular GSH (% of control)				
HT	100±5.42	95±3.35	98±2.65	96±5.29
HT+Sudan I	47.6±3.40	62.15±5.64*	74.32±4.64**	100±5.09
TBARS absorbance 535 nm				
HT	0.18±0.13	0.17±0.09	0.18±0.08	0.18±0.11
HT+Sudan I	0.41±0.15	0.30±0.11*	0.23±0.09**	0.18±0.08**

Cells were exposed to 0, 25, 50 and 100 μM HT for 30 min before treatment with 100 μM Sudan I for 1 h. Results are the mean±SD. * $p < 0.05$; ** $p < 0.01$ significantly different from respective control.

The effect of HT on the GSH depletion induced by Sudan I

GSH is an important cellular antioxidant. To determine the effect of HT on the redox status of cells induced by Sudan I, GSH level was evaluated in the cells treated with Sudan I. GSH decreased dramatically when the cells were treated with 100 μM Sudan I. When pre-treated with 25–100 μM HT, GSH depletion was greatly prevented in the cells treated with 100 μM Sudan I (Table II).

Discussion

Previous studies have clearly demonstrated a strong protective effect of HT against the DNA oxidative damage induced by hydrogen peroxide in Jurkat cells in a concentration-dependent manner [10]. Similar results were obtained in blood cells [29] and in human prostate cells (PC3) [30]. However, to date, no researches on the protective effect on the DNA damage in cultured cells from liver origin have been reported. However, the liver is not only the main target for phenolic antioxidants once absorbed from the gastrointestinal tract but also is the major place for phenolic metabolism [31]. Moreover, the liver is the target site of Sudan I toxicity and the CYP1A1 is assumed to play a role in the oxidative metabolism of Sudan I in this organ [32]. Experiments from the laboratory demonstrated that Sudan I induced genotoxic effects depending on the ROS-induced oxidative DNA damages in HepG2 cells [21]. The HepG2 cell line was originally derived from a human hepatoblastoma, retaining many of the functions of normal liver cells [33] and expressing the activities of several phase I and II xenobiotic metabolizing enzymes. HepG2 cells have been shown to be a suitable system for genotoxicity testing. Based on the above observations, we decided to ascertain whether HT can equally inhibit Sudan I-induced genotoxicity in a human liver cell line and the probable molecular mechanisms of action and thus to determine its effect on modulating Sudan I carcinogenicity.

Comet assay has been shown to be a very sensitive technique for measuring DNA damage [34]. And the MN assay is very sensible for detecting the chromosome breakage induced by various mutagens and carcinogens [35]. In the present study, HT was found to concentration-dependently inhibit the DNA strand breaks and MN formation in HepG2 cells. Our findings showed that HT at 100 μM could not exert 100% protection against the genotoxicity of Sudan I and the same result was obtained even at 200 μM (data not shown). In addition, the same result of protection was observed in MNT.

In recent years, ROS-induced oxidative DNA damage has been implicated in mutagenesis and carcinogenesis and has attracted extensive attention. It is well known that ROS induce a number of molecular alterations on cellular components, leading to changes in cell morphology and viability [36]. Over-production of these ROS induces cell oxidative injury, such as DNA damage, oxidation of proteins and lipid peroxidation [37]. Lipid peroxidation is initiated by ROS attacking unsaturated fatty acids and is propagated by a chain reaction cycle involving lipids, peroxy radicals and lipid hydroperoxides [38]. Peroxidation of the cell membrane phospholipids and

accumulation of lipid peroxides are expected to alter the membrane fluidity and permeability, consequently leading to disruption of membrane structure and function. Sudan I is metabolized by several routes, such as oxidation by cytochrome P-450 monooxygenases and peroxidase [39]. Upon redox cycling of Sudan I, ROS are produced, i.e. superoxide radical anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) [40]. In addition, Sudan I can be metabolited by prostaglandin H synthase (PHS) in the presence of arachidonic acid or H_2O_2 *in vitro* and the oxidation gives rise to free radicals, inhibited by radical scavengers, such as GSH and NADH [37]. In another aspect, considerable experimental evidence has been adduced in support of the protection of HT against cytotoxicity induced by ROS in different cell systems [12]. In addition, results from two studies demonstrated that HT was likely to prevent H_2O_2-induced cytotoxicity by acting as a chain-breaking inhibitor of lipid peroxidation and chelating iron ions [12,41]. With regard to all the above aspects, to further explore the mechanisms of the protective effect of HT on Sudan I-induced oxidative damage, intracelluar ROS level and lipid peroxidation were determined, respectively. The present study showed that HT strongly reduced the generation of ROS induced by Sudan I in HepG2 cells, achieving a maximum value of 100% reduction in DCF fluorescence intensity. A similar inhibitory effect of HT on Sudan I-induced lipid peroxidation was also observed.

It is well established that intracellular GSH, the most important biomolecule protecting against chemically induced oxidative stress, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction [42]. It is usually assumed that GSH depletion reflects intracelluar oxidation whereas an increase in GSH concentration could be expected to prepare the cell against a potential oxidative insult [43]. Moreover, pre-treatment with HT significantly restore the GSH level reduced by t-BHP treatment in HepG2 cells [13]. In our experimental conditions, treatment of HepG2 cells with 100 μM Sudan I induced a dramatic decrease in the concentration of GSH, which was prevented by pre-treatment with all three doses of HT.

Immnocytochemistry staining for 8-OHdG is a reliable marker for oxidative DNA damage *in vivo* [44] and *in vitro* [45]. 8-OHdG production is induced by the oxidation of deoxyguanosine (dG), which is one component of DNA. $OH^{\bullet}$ directly act on dG to form 8-OHdG. Up to the present, kinds of physical and chemical factors produce $OH^{\bullet}$ directly and indirectly, which damages DNA, with this being the ordinal mechanism of formation of 8-OHdG. Moreover, GSH depletion has previously been found to be associated with elevated levels of 8-OHdG and DNA single strand breaks (SSB) in cell culture and chromatid breaks and sister chromatid exchanges *in vivo* [46]. In the present study we conclude that HT could inhibit the genotoxicity of Sudan I through preventing 8-OHdG formation induced by Sudan I, which was related to the decrease of ROS and increase of GSH.

In summary, the present study suggested the protective effect of HT on genotoxicity induced by Sudan I in human hepatoma cells. The Sudan I genotoxicity was associated with elevated ROS level, lipid peroxidation and 8-OHdG formation and GSH depletion. Pre-treatment of HT protected against all these alterations induced by Sudan I, probably acting mainly to quench radical species. In view of the results obtained in the present study, HT may therefore merit further consideration as an edible phytochemical with the potential for a beneficial application in the chemoprevention of Sudan I genotoxicity and, possibly, human carcinogenesis.

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References

[1] Trichopoulou A, Lagiou P. Healthy traditional Mediterranean diet: an expression of culture, history, and lifestyle. Nutr Rev 1997;55:383–389.
[2] Katan MB, Zock PL, Mensink RP. Dietary oils, serum lipoproteins, and coronary heart disease. Am J Clin Nutr 1995;61:1368s–1373s.
[3] Bravo L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutr Rev 1998;56:317–333.
[4] Visioli F, Galli C. The effect of minor constituents of olive oil on cardiovascular disease: new findings. Nutr Rev 1998;56:142–147.
[5] Montedoro GF, Servili M, Baldioli M. Simple and hydrolyzable compounds in virgin olive oil. Spectroscopic characterization of the secoiridoids derivatives. J Agric Food Chem 1993;41:2228–2234.
[6] Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. Biochem Biophys Res Commun 1998;247:60–64.
[7] Papadopoulos G, Boskou D. Antioxidative effect of natural phenols on olive oil. J Am Oil Chem Soc 1991;68:669–671.
[8] Baldioli M, Servili M, Perretti G, Montedoro GF. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. J Am Oil Chem Soc 1996;73:1589–1593.
[9] Stefania DA, Diego I, Valentina M, Alvara S, Giovanna D, Adone B, Lucia M, Maria AT, Marcello Z, Patrizia G. Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells. Free radical Bio Med 2005;38:908–919.
[10] Lambros N, Paschalis TD, Nektarios A, Dimitrios B, Apostolos A, Dimitrios G. DNA protecting and genotoxic effects of olive oil related components in cells exposed to hydrogen peroxide. Free Radic Res 2005;39:787–795.

[11] Manna C, Galletti P, Cucciolla V, Moltedo O, Leone A, Zappia V. The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl) ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. J Nutr 1997;127:286–292.

[12] Manna C, Galletti P, Cucciolla V, Montedoro G, Zappia V. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. J Nutr Biochem 1996;10:159–165.

[13] Goya L, Mateos R, Bravo L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells, protection against oxidative stress induced by tert-butylhydroperoxide. Eur J Nutr 2007;46:70–78.

[14] De la Puerta R, Ruiz Gutierrez V, Hoult RS. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. Biochem Pharmacol 1999;57:445–449.

[15] Deiana M, Aruoma OI, Bianchi MP, Spencer JPE, Kaur H, Halliwell B, Banni S, Dessi MA, Corongiu FP. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. Free Radic Biol Med 1999;26:762–769.

[16] Moller P, Wallin H. Genotoxic hazards of azo pigments and other colorants related to 1-phenylazo-2-hydroxynaphthalene. Mutat Res 2000;462:13–30.

[17] Capitan F, Capitan-Vallvey LF, Fernadez MD, Orbe De, Avidad R. Determination of colorant matters in foods by solid-phase spectrophotometry. Anal Chim Acta 1996;331: 141–148.

[18] Westmoreland C, Gatehouse DG. The differential clastogenicity of Solvent Yellow 14 and FD & C Yellow No. 6 *in vivo* in the rodent micronucleus test (observation of species and tissues specificity). Carcinogenesis (Lond) 1991;12: 1403–1407.

[19] Tsuda S, Matsusaka N, Madarame H, Ueno S, Susa N, Ishida K, Kawamura N, Sekihashi K, Sasaki YF. The comet assay in eight mouse organs: results with 24 azo compounds. Mutat Res 2000;465:11–26.

[20] Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K. Mortelmans, salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. Environ Mol Mutagen 1998;12(Suppl.11):1–157.

[21] An Y, Liping J, Jun C, ChengYan G, Laifu Zh. Sudan I induces genotoxic effects and oxidative DNA damage in HepG2 cells. Mutat Res 2007;627:164–170.

[22] Mersch-Sundermanna, V, Knasmüller S, W XJ, Darraoudi F, Fekadu K, Kassie F. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. Toxicology 2004;198:329–340.

[23] Singh NP, Stephens RE. Microgel electrophoresis: sensitivity, mechanisms, and DNA electrostretching. Mutat Res 1997;383:167–175.

[24] Natarajan AT, Darroudi F. Use of human hepatoma cells for *in vitro* metabolic activation of chemical mutagens/carcinogens. Mutagenesis 1991;5:399–403.

[25] Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometry studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 1983;130:1910–1917.

[26] Halliwell B, Whiteman M. Measuring reactive species and oxidative damage *in vivo* and cell culture. How should you do it and what does it mean? Br J Pharmacol 2004;142:231–255.

[27] Leal LKAM, Junior HVN, Cunhaa GMA, Moraes MO, Pessoa C, Oliveira RA, Silveira ER, Canuto KM, Viana GSB. Amburoside A, a glucoside from Amburana cearensis, protects mesencephalic cells against 6-hydroxydopamine-induced neurotoxicity. Neurosci Lett 2005;388:86–90.

[28] Hissin PJ, Hilf RA. Fluorimetric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976;74:214–226.

[29] Grasso S, Siracusa L, Spatafora C, Renis M. Hydroxytyrosol lipophilic analogues: enzymatic synthesis radical scavenging activity and DNA oxidative damage protection. Bioorg Chem 2007;35:137–152.

[30] Quiles JL, Farquharson AJ, Simpson DK, Grant I, Wahle KW. Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. Br J Nutr 2002;88:225–234.

[31] Stupans I, Kirlich AL, Tuck KJ, Hayball P. Comparison of radical scavenging effect, inhibition of microsomal oxygen free radical generation, and serum lipoprotein oxidation of several natural antioxidants. J Agric Food Chem 2002;50: 2462–2469.

[32] Stiborova M, Martinek V, Rydlova H, Hodek P, Frei E. Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. Cancer Res 2002;62:5678–5684.

[33] Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980;209:497–499.

[34] Fairbairn DW, Olive PL, O'Meill KL. The comt assay: a comprehensive review. Mutat Res 1995;339:37–59.

[35] Kirsch VM, Elhajouji A, Cundari E, Van HP. The *in vitro* micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction. Mutat Res 1997; 392:19–30.

[36] Sies H (ed). Oxidative stress, oxidants, and antioxidants. London and New York: Academic Press; 1991.

[37] Mates JM. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 2000;153:83–104.

[38] Haraguchi H, Ishikawa H, Sanchez Y, Oqura T, Kubo Y, Kubo I. Antioxidative constituents in Heterotheca inuloides. Bioorg Med Chem 1998;6:339–347.

[39] Stiborava M, Frei E, Schmeiser HH, Wiessler M, Hradec J. Mechanism of formation and 32P-postlabeling of DNA adducts derived from peroxidative activation of carcinogenic non-aminoazo dye 1-phenylazo-2-hydroxynaphthalene (Sudan I). Carcinogenisis 1990;11:1843–1848.

[40] Mϕller P, Wallin H. Genotoxic hazards of azo pigments and other colorants related to 1-phenylazo-2-hydroxynaphthalene. Mutat Res 2000;462:13–30.

[41] CaterinaM, Fulvio DR, Valeria C, Adriana B, Stefania DA, Patrizia G, Vincenzo Z. Biological effects of hydroxytyrosol, a polyphenol from olive oil endowed with antioxidant activity. In: Zappoa V, editor. Advances in nutrition and cancer. New York: Kluwer Academic/Plenum Publishers; 1999.

[42] Alia M, Ramos S, Mateos R, Bravo L, Goya L. Response of the antioxidant defense system to t-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). J Biochem Mol Toxicol 2005;19:119–128.

[43] Scharf G, Prustomersky S, Knasmuller S, Schulte HR, Huber WW. Enhancement of glutathione and g-glutamylcysteine synthetase, the rate limiting enzyme of glutathione synthesis, by chemoprotective plant-derived food and beverage components in the human hepatoma cell line HepG2. Nutr Cancer 2003;45:74–83.

[44] Sai K, Umemura T, Takagi A, Hasegawa R, Kurokawa Y. The protective role of glutathione, cysteine and Vitamin C against oxidative DNA damage induced in rat kidney by potaassium bromate. Jpn J Cancer Res 1992;1:45–51.

[45] Kasai H, Nishimura S. Hydroxylation of deoxyguanosine at the C8 position by ascorbic acid and other reducing agents. Nucleic Acids Res 1984;12:2137–2145.

[46] Reliene R, Schiestl RH. Glutathione depletion by buthionine sulfoximine induces DNA deletions in mice. Carcinogenesis 2006;27:240–244.