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Estimation of hydroxyl radical generation by salicylate hydroxylation method in kidney of mice exposed to ferric nitrilotriacetate and potassium bromate

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Abstract

Hydroxyl radical (·OH) generation in the kidney of mice treated with ferric nitrilotriacetate (Fe-NTA) or potassium bromate ($KBrO_3$) *in vivo* was estimated by the salicylate hydroxylation method, using the optimal experimental conditions we recently reported. Induction of DNA lesions and lipid peroxidation in the kidney by these nephrotoxic compounds was also examined. The salicylate hydroxylation method revealed significant increases in the ·OH generation after injection of Fe-NTA or $KBrO_3$ in the kidneys. A significant increase in 8-hydroxy-2′-deoxyguanosine in nuclei of the kidney was detected only in the $KBrO_3$ treated mice, while the comet assay showed that the Fe-NTA and $KBrO_3$ treatments both resulted in significant increases in DNA breakage in the kidney. With respect to lipid peroxidation, the Fe-NTA treatment enhanced lipid peroxidation and ESR signals of the alkylperoxy radical adduct. These DNA breaks and lipid peroxidation mediated by ·OH were diminished by pre-treatment with salicylate *in vivo*. These results clearly demonstrated the usefulness of the salicylate hydroxylation method as well as the comet assay in estimating the involvement of ·OH generation in cellular injury induced by chemicals *in vivo*.

Keywords: *Hydroxyl radical, oxidative stress, salicylic acid, chemicals, mice*

Abbreviations: *·OH, hydroxyl radical; SA, salicylic acid; ASA, acetylsalicylic acid; 2,3-DHBA, 2,3-dihydroxy benzoic acid; Fe-NTA, ferric nitrilotriacetate; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 2-dG, 2-deoxyguanosine.*

Introduction

The generation of free radicals, such as reactive oxygen species, is well known to produce DNA damage and lipid peroxidation and may be at least partly responsible for the carcinogenicity and/or toxicity of chemical compounds in humans and animals. Among reactive oxygen species, the measurement of hydroxyl radicals (·OH) is generally difficult because of their high reactivity and extremely short lifetimes. In particular, it is almost impossible to detect or measure ·OH directly in *in vivo* experimental systems, therefore determinations of ·OH using several trapping agents are widely accepted.

Salicylic acid (SA) was first used as a trapping agent for detecting ·OH in *in vivo* experimental systems in

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1986 by Floyd et al. [1]. This method has been successfully applied to demonstrate the relation between ·OH generation and pathology in many different organs, such as heart [2], brain [3], lung [4], intestinal tract [5], kidney [6], central nervous system [7] and was also employed to evaluate the possible involvement of ·OH in diabetes [8] as well as in a sepsis model [9]. On the other hand, several studies have reported problems with this method [10–12] and almost all of the research involving *in vivo* experiments adopted similar experimental conditions in which the analyses were made at 1 h after the administration of 100 mg/kg of SA [13–15]. Recently, using the optimum dosage of SA (400 mg/kg) and the appropriate *in vivo* sampling time, we demonstrated the usefulness of salicylate hydroxylation in estimating ·OH generation in multiple organs of mice exposed to X-rays [16].

In the present study, the salicylate hydroxylation method was further applied to estimate ·OH generation in the kidney of mice treated with two representative nephrotoxic compounds, ferric nitrilotriacetate [17] and potassium bromate [18]. Moreover, to investigate the involvement of ·OH in cellular injury, DNA damages and lipid peroxidation caused by these chemicals were also examined under the same experimental condition. Our results demonstrated that the salicylate hydroxylation method is useful for determination of ·OH levels in *in vivo* experimental systems and that the levels of ·OH may be closely related to the induction of DNA breaks, as estimated by the comet assay.

Materials and methods

Chemicals

Salicylic acid (SA), acetylsalicylic acid (ASA), iron (III) nitrate nonahydrate [$Fe(NO_3)_3 \cdot 9H_2O$], nitrilotriacetic acid disodium salt and potassium bromate ($KBrO_3$) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2,3-Dihydroxy benzoic acid (2,3-DHBA) was obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 5,5-Dimethyl-1-pyrrolline-1-oxide (DMPO) was obtained from Dojindo laboratories (Kumamoto, Japan). Ferric nitrilotriacetate (Fe-NTA) was prepared according to the method of Toyokuni et al. [17]. All chemicals used were the highest quality commercially available.

Animals

After approval by the Committee of Animal Experimentation, Kitasato University School of Veterinary Medicine, experiments were performed in accordance with Japanese legislation on protection of animals and the 'Guide for the Care and Use of Laboratory Animals' (NIH publication no. 86–23, revised 1985). ICR male mice 8 weeks of age were purchased from SLC Inc. (Japan). The mice were fed a standard cubed diet (MF, Oriental Yeast Co., Japan) maintained on distilled water *ad libitum* and exposed to a 12 h light (7:00 am to 7:00 pm) and 12 h dark (7:00 pm to 7:00 am) cycle. Ambient temperature during the study was maintained at 21°C.

For salicylate hydroxylation analysis *in vivo*, mice were first intraperitoneally injected with ASA (400 mg/kg) and this was followed 10 min later by intraperitoneal injections of either Fe-NTA (5 mg Fe/kg) [17] or $KBrO_3$ (400 mg/kg). Kidneys were collected under anaesthesia at 30 min after the ASA treatment in the salicylic hydroxylation method. Measurements of other parameters of oxidative stress were performed in kidneys collected at 20 min after the chemical treatments with or without ASA pre-treatment.

Determination of SA and 2,3-DHBA and estimation of ·OH generation in the kidney

SA and 2,3-DHBA were measured according to the methods of Tsai et al. [19] and Yamamoto et al. [20] with some modifications. For determination of SA and 2,3-DHBA in the organs, tissue (around 0.5 g) from each mouse was homogenized with ice-cold saline (0.5 ml) and deproteinized by addition of 10% (v/v) perchloric acid containing 1 mM EDTA-2Na and 100 μM sodium pyrosulphite (1.5 ml). HCl (1 M, 0.4 ml) was added to the supernatant fraction obtained by centrifugation. The resulting solution was extracted with 10 ml of diethyl ether and dried under a N_2 evaporator.

According to the method of Yamamoto et al. [20], the samples were analysed using a Gilson high performance liquid chromatography (HPLC) system equipped with a Model 307 pump (Gilson, Inc., Middleton, WI) and an electrochemical detector (ECD; Nanospace SI-2, SHISEIDO Co. Ltd., Tokyo, Japan). The dried samples were dissolved in the mobile-phase for HPLC (30 mM Sodium citrate/27.7 mM Sodium acetate, pH4.75) and reverse-phase HPLC analysis was carried out using a YMC-Pack ODS-AA-312 column (150 × 6.0 mm, YMC Co., Ltd., Kyoto, Japan). The glassy carbon working electrode was set at a potential of + 0.85 V and the flow rate was 1.0 ml/min. The concentrations of SA or 2,3-DHBA in the samples were measured by the absolute standard curve method.

To evaluate ·OH generation, the ratios of 2,3-DHBA to SA were compared between the treated mice and the control mice according to our previous study [16]. Further, to estimate the amount of increase in ·OH corresponding to the 2,3-DHBA increase in the samples, we derived two linear-regression equations from our *in vitro* experiments and the G-value of ·OH (2.7/100eV) by gamma-radiolysis of water at neutral pH [21]. Using these linear-regression

equations, the equivalent amounts of ·OH in the samples were calculated as follows assuming a specific gravity of 1 [16]:

1. The expected values of ·OH generated by 24 Gy of irradiation were calculated from the concentration of SA in the samples.

$$c = 0.1224x + 1.9391$$

where c is the predictive amounts of increase in 2,3-DHBA in the buffer after 24 Gy of X-irradiation (ng/ml) and x is the concentration of SA in the buffer before the X-irradiation (μg/ml).

2. The equivalent amounts of ·OH corresponding to the 2,3-DHBA increases in the samples (nmol/g) were calculated by subtracting the background (un-irradiated samples) from the concentrations of 2,3-DHBA in the samples.

$$y = 6.7190 \div c \times z$$

where c is the predictive amounts of increase in 2,3-DHBA in the buffer after 24 Gy of X-irradiation (ng/ml) and z is amounts of increase of 2,3-DHBA in the tissue (ng/g or ng/ml).

Measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine

Nuclear DNA was isolated from the kidney by the NaI method using DNA Extractor WB Kit (Wako Pure Chemical Industries, Ltd. Osaka, Japan). After the enzymatic treatment procedures with Nuclease P1 (Sigma-Aldrich Corp. St. Louis, MO) and Alkaline phosphatase (Sigma-Aldrich Corp. St. Louis, MO) according to Kasai et al. [22], 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 2-deoxyguanosine (2-dG) were resolved by HPLC and quantified by electrochemical detection as described by Floyd et al. [23]. The samples were analysed using a Gilson HPLC system equipped with a Model 307 pump (Gilson, Inc., Middleton, WI) and an electrochemical detector (ECD; Nanospace SI-2, SHISEIDO Co. Ltd., Tokyo, Japan). HPLC analysis was carried out using a CAPCELL PAK C18 UG120 (5 μm, 250 × 4.6 mm, SHISEIDO Co. Ltd., Tokyo, Japan). The nucleotides were eluted from the column with an isocratic mobile phase consisting of 50 mM sodium acetate pH 5.2, 5.0% methanol at a flow rate of 1.0 ml/min. The glassy carbon working electrode was set at a potential of + 0.70 V for measurement of 8-oxodG and the concentration of 2-dG was monitored by UV detection at 290 nm. The identities of 8-oxodG and 2dG on the chromatograms were determined by co-injection of standards. The data were expressed as the ratio of nmoles of 8-oxodG to 10^5 nmoles of 2-dG.

Comet assay

This experiment followed the method of Ueno et al. [24] with multiple mouse organs. The kidneys were minced, suspended in 4 ml chilled homogenizing solution (pH 7.5) containing 0.075 M NaCl and 0.024 M EDTA-2Na and then homogenized gently using a Potter–Elvehjem type homogenizer at 500 rpm in ice. To obtain nuclei, the homogenate was centrifuged at 700 × g for 10 min at 0°C and the precipitate was re-suspended in chilled homogenizing buffer at approximately 5×10^5 nuclei/ml. Glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan) were pre-coated with agarose GP-42 (Nacalai Tesque, Inc., Kyoto, Japan) and dried. Seventy-five microlitres of agarose GP-42 was quickly layered onto a pre-coated slide and covered with another slide. The slide sandwiches were placed horizontally to allow the agarose to solidify. The nucleus suspension was mixed 1:1 (v/v) with 2%, 45°C, agarose LGT (Nacalai Tesque, Inc., Kyoto, Japan) and 75 μl of the nucleus mixture was quickly layered in the same manner after removal of the covering slide. Finally, 75 μl of agarose GP-42 was quickly layered on again. Slides prepared from nuclei isolated by homogenization were placed in a chilled lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Trizma, 1% Sarkosyl, 10% DMSO and 1% Triton X-100, pH 10) and kept at 0°C in the dark for 1 h, then in chilled alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 30 min in the dark at 0°C to unwind. Electrophoresis was conducted at 0°C in the dark for 30 min at 25 V (0.97 V/cm) and approximately 300 mA in an electrophoretic chamber (FA-8420, System Instruments Co., Ltd., Tokyo, Japan). The slides were neutralized by 0.4 M Tris-HCl buffer, pH7.4 and left to dry overnight. The slides were stained with 50 μl of 20 μg/ml ethidium bromide (Nacalai Tesque, Inc., Kyoto, Japan) and covered with a coverslip for 20 min. They were then destained for 10 min in deionized water. Fluorescence images were observed using a fluorescence microscope (Nikon Co., Tokyo, Japan) and more than 100 nuclei per slide were collected as digital images (DC-200, Laica Microsystems GmbH, Wetzlar, Germany). Images were saved as electronic files and the comets were measured for the tail length (total length – nuclear diameter) and the percentage DNA in the tail (sum of tail intensity ÷ sum of cell intensity × 100) using image processing software (Comet analyser v1.5, Youworks Co., Tokyo, Japan). Fifty isolated comets were randomly selected and the mean values of the parameters were calculated from the results of two slides per individual animal. The experiments were repeated more than four times and the data were expressed as the mean ± standard deviation of the animals in the same treatment group.

Lipid peroxidation

Lipid peroxidation in the kidney was measured according to the method of Kikugawa et al. [25]. A 30% homogenate of kidney was prepared with 1.15% KCl solution. The homogenate (0.1 ml) was mixed with 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of acetate buffer, 0.05 ml of 0.8% butylated hydroxytoluene in acetate, 1.5 ml of 0.8% tiobarbituric acid (TBA) solution and 0.7 ml of distilled water and heated at 100°C for 60 min after incubation at 5°C for 60 min. After cooling, 5 ml of butanol-pyridine (15:1) was added to the sample and TBA reactive substances (TBA-RS) were extracted to the organic phase. The concentration of TBA-RS was determined by the absorbance at 532 nm using the molecular extinction coefficient (1.56×10^5 M^{-1} cm^{-1}) and expressed as malondialdehyde (MDA) values per wet tissue weight.

Detection of radical adducts in the kidney by ESR

The relative intensity of ESR spectra was measured according to the method of Toyokuni et al. [17]. The kidney samples were sliced to 150 μm thickness by Microslicer (DTK-2000, D.S.K., Kyoto, Japan). The tissue was placed in the tissue-type quartz cell for ESR (LTC-10, Labotec, Tokyo, Japan) and 10 μl of DMPO was added. At 10 min after the addition of DMPO, X-band ESR spectra were recorded using a JES-FA 100 spectrometer (JEOL Ltd., Tokyo, Japan). ESR measurements were performed under the following conditions: modulation frequency, 9.4 GHz; field modulation, 100 kHz; modulation amplitude, 0.08 mT; microwave power, 4 mW; centre field, 334.5 mT; sweep width, 5 mT; sweep time, 4 min; time constant, 0.1 s. All spectra were recorded in the tissue-type cells at room temperature using manganese oxide as an internal standard and normalized by the sample weight.

Statistical analysis

The differences between the averages of the treated animals and the untreated control animals were compared with the Tukey's test after one-way ANOVA and a p-value less than 0.05 was considered statistically significant. Data from studies with only two groups were analysed by the Student t-test for equal variance or Welch t-test for unequal variance after Bartlett's test.

Results and discussion

According to our previous findings on suitable experimental conditions for the salicylate hydroxylation method in *in vivo* experimental systems [16], mice were injected with the optimum dose of 400 mg/kg ASA and then treated with Fe-NTA or $KBrO_3$ at 10 min after ASA, because at least 10 min is required for full distribution of intraperitoneally injected ASA to the organs of mice [16]. Following the intraperitoneal treatment with the chemicals for 20 min, we evaluated the ·OH generation, the formation of DNA damages such as 8-oxodG in nuclei and DNA breaks estimated by the comet assay and the amounts of TBA-RS as well as the appearance of ESR spectra by alkylperoxyl radical adduct in the kidney of mice.

SA can undergo ·OH attack to produce 2,3-DHBA, a compound that has not been reported as a product of enzymatic metabolism [26]. As shown in Table I, the concentrations of 2,3-DHBA in the kidney were significantly increased by the Fe-NTA or $KBrO_3$-treatment, as compared to the levels of the untreated control. With respect to SA levels, previous studies showed that differences in 2,3-DHBA levels among animals are closely associated with the concentration of SA [27] and that the increases in the amount of 2,3-DHBA are dependent on the concentration of SA even *in vitro* [16], indicating that the ratios of 2,3-DHBA to SA might be useful as an indicator of ·OH generation *in vivo*. However, there was no significant difference in SA concentration in the kidney among these treated and untreated mice and the values of 2,3-DHBA/SA were significantly increased in the Fe-NTA or $KBrO_3$ treated mice (Table I). Collectively, these results strongly indicated that the salicylate hydroxylation method is useful for detection of chemically-induced ·OH generation in the kidney *in vivo*.

When the equivalent amounts of ·OH in the kidneys were calculated according to our previous study [16], the amounts of ·OH generated in the kidney after the chemical treatment were 81.22 and 41.03 nmol/g in the Fe-NTA treated and the $KBrO_3$-treated mice, respectively. In our previous study, the equivalent amount of ·OH formed by 24 Gy of X-irradiation

Table I. Concentration of SA and 2,3-DHBA in the kidney at 30 min after ASA administration with the chemical treatments and the equivalent amounts of hydroxyl radicals for 2,3-DHBA increase in the kidney.

	SA (μg/g)	2,3-DHBA (ng/g)	2,3-DHBA/SA ($\times 10^3$)	·OH (nmol/g)
Control	73.7±27.3	38.4±9.2	0.567±0.172	
Fe-NTA	80.4±11.1	180.8±45.3**	2.231±0.606**	81.22
$KBrO_3$	65.9±10.3	99.5±30.1**	1.497±0.310**	41.03

** Significant increase compared with the control ($p < 0.01$, $n < 4$).

Table II. Effects of Fe-NTA and $KBrO_3$ on the formation of 8-oxodG, the parameters of the comet assay, lipid peroxidation and the relative intensity of ESR signal in the kidney of mice.

		Comet assay			
	8-oxodG/2-dG ($\times 10^5$)	Tail length (μm)	Ratio (%)	Lipid peroxidation (nmol MDA equivalents/g)	Relative intensity of ESR signal
Control	0.85±0.36	4.99±1.74	2.6±0.9	150.75±13.49	14.27±4.76
Fe-NTA	1.44±0.98	14.41±3.69**	12.6±4.3**	529.27±18.07**	141.53±45.25**
$KBrO_3$	2.67±0.76**	12.35±3.52**	8.1±2.5**	145.43±23.58	47.95±16.48

** Significant increase compared with the control ($p<0.01$, $n=4$).

was 7.39 nmol/g in the kidney [16], so the estimated amounts of ·OH generation by Fe-NTA or $KBrO_3$ in the kidney were 5–10-fold higher than those by the 24 Gy of X-irradiation. The higher levels of ·OH generation by chemicals might be one of the reasons why the salicylate hydroxylation method had been so successful for detection of ·OH generation by chemicals in the many previous *in vivo* experiments [2–9].

In this study, we also examined the induction of oxidative damages such as DNA lesions and lipid peroxidation by these chemicals *in vivo*. As shown in Table II, $KBrO_3$ treatment resulted in a significant increase of 8-oxodG in the nuclei of mouse kidney, however, Fe-NTA treatment (5 mg/kg) failed to increase the levels of 8-oxodG in the kidney. Since a previous study reported that treatment of rats with Fe-NTA (40 mg/kg) for 13 days increased the levels of 8-oxodG in the kidney [28], it is possible that the short treatment time (20 min) and the smaller dosage in the present study may be responsible for the failure of this chemical to increase the levels of 8-oxodG. However, in the Fe-NTA treated mice, the levels of ·OH in the kidney were about 2-fold greater than those in mice after the $KBrO_3$ treatment (Table I). Another study showed that the mechanism of $KBrO_3$-induced oxidative DNA damage is different from general types of oxidative stress such as ·OH [29]. These results suggested that the levels of ·OH are not directly related to the induction of 8-oxodG in the kidney *in vivo*.

Previous studies showed that the comet assay is highly sensitive to DNA damages expressed as DNA strand breaks and alkali-labile sites induced by chemical compounds and X-irradiation [30]. In contrast to 8-oxodG formation, the comet assay revealed that both Fe-NTA and $KBrO_3$ treatment caused significant increases of DNA breaks in the kidney, as estimated by tail length and ratio (Table II). These results were consistent with those of previous studies which reported the induction of DNA damage in rat kidney by Fe-NTA [31] or $KBrO_3$ [32]. Since the levels of both DNA breaks and ·OH after Fe-NTA treatment were higher than those after $KBrO_3$ treatment (Tables I and II), the levels of ·OH may be closely associated with the induction of DNA breaks, as evaluated by the comet assay.

It is generally accepted that cellular lipid is one of the main targets of free radicals generated in cells. Thus, we examined the formation of TBA-RS as well as alkylperoxyl radicals, as measured by ESR spin trapping technique [17], in the kidney of mice treated with these chemicals. The results showed that only Fe-NTA treatment induced a significant increase in TBA-RS in the kidney, while the $KBrO_3$ treatment could not (Table II). Furthermore, ESR analysis clearly showed the presence of alkylperoxyl radicals ($a_N = 1.361$ mT, $a_H = 1.118$ mT) in the kidney of Fe-NTA treated animals, while only traces of the similar ESR spectra were observed in the $KBrO_3$ treated mice kidney (Figure 1 and Table II). Since only Fe-NTA treatment resulted in an increase in lipid peroxidation and the formation of alkylperoxyl radicals, larger quantities of ·OH might be needed for the

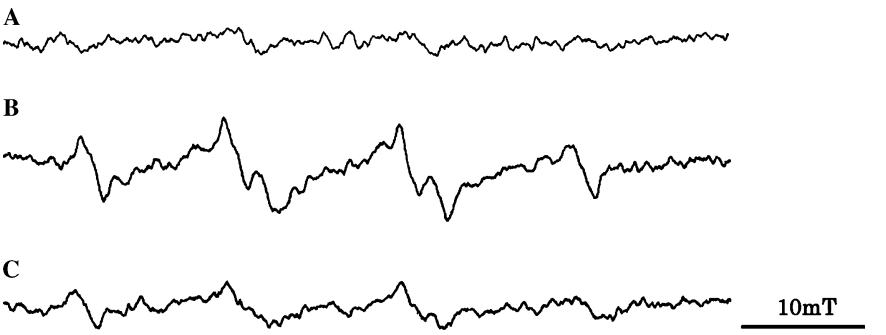

Figure 1. ESR spectra of the kidney tissues obtained from the Fe-NTA or $KBrO_3$-treated mice. The kidneys were collected from the mice at 20 min after the start of treatment and DMPO (10 μl) was added to the tissue slice (150 μm thick) in the tissue-type quartz cell for ESR (LTC-10, Labotec, Tokyo, Japan). (A) Control, (B) Fe-NTA, (C) $KBrO_3$.

Table III. Inhibitory effects of the ASA pre-treatment on the increase in the two parameters for oxidative stress induced by Fe-NTA and $KBrO_3$ in mice kidney.

	Comet assay tail length (μm)	Lipid peroxidation (nmol MDA/g)
Control	4.99±1.74	122.77±40.94
ASA	5.63±0.58	102.90±32.07
Fe-NTA	14.41±3.69**	447.19±80.20**
+ ASA[a]	9.22±1.07[#]	115.75±73.49[##]
$KBrO_3$	12.35±3.52**	
+ ASA[a]	8.18±1.30	

[a] Mice were injected intraperitoneally with ASA (400 mg/kg), and then received the injection of chemicals at 10 min after ASA. The kidney samples were collected at 30 min after the ASA injection.
** Significant increase compared with the control ($p<0.01$, $n=4$).
[#, ##] Significant decrease compared with the treated animals without ASA pre-treatment ($p<0.05$ or 0.01, $n=4$).

induction of lipid peroxidation than for induction of DNA breaks *in vivo*.

Concerning the superiority of salicylate as a powerful probe for ·OH inside the cell, we examined the effect of ASA on ·OH-mediated DNA breaks and lipid peroxidation in the kidney of mice treated with these chemicals *in vivo*. As expected, as shown in Table III, the pre-treatment of mice with ASA at 10 min before the chemical treatments effectively prevented the increases in DNA breaks caused by Fe-NTA and $KBrO_3$ and in the lipid peroxidation induced by Fe-NTA. These results demonstrated that salicylate treatment might be useful of preventing cellular damages caused by ·OH generation *in vivo*.

Taken together, the salicylate hydroxylation method appears to be a relatively sensitive parameter for determination of ·OH in the kidney by Fe-NTA and $KBrO_3$ *in vivo*. The results also suggested that the *in vivo* formation of ·OH in the kidney may be more closely associated with the induction of DNA breaks and lipid peroxidation than with induction of 8-oxodG and implied that DNA breaks, as evaluated by the comet assay, are the most sensitive parameter tested for the investigation of cellular damages caused by ·OH *in vivo*. The detection of ·OH by the salicylate hydroxylation method together with the estimation of DNA damage by the comet assay may be useful in examining the involvement of ·OH in the cellular damages induced by various carcinogenic and/or toxic compounds *in vivo*.

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