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Original Contribution

8-HYDROXYDEOXYGUANOSINE IN VITRO: EFFECTS OF GLUTATHIONE, ASCORBATE, AND 5-AMINOSALICYLIC ACID

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Abstract—Oxidative DNA damage, as expressed by 8-hydroxydeoxyguanosine (8-OHdG), was investigated in calf thymus DNA exposed to either ultraviolet radiation or to $FeCl_2/H_2O_2$ in a Fenton-like reaction. The influence of iron (absent in the UV system and present in the $FeCl_2/H_2O_2$ system) and pH (7.4 and 4.0) on the effect of glutathione (GSH), ascorbate, and 5-aminosalicylic acid (5-ASA, a drug used in the treatment of chronic inflammatory bowel diseases) was examined in these systems. Without iron, all three compounds considerably reduced 8-OHdG formation (i.e., acted as scavengers), while in the presence of iron salts, 8-OHdG formation was accelerated (except for GSH at pH 7.4), i.e., the compounds acted as prooxidants. This effect was augmented at low pH. The prooxidant property of 5-ASA may have implications for its clinical use. Maximum scavenging effect for all the compounds investigated was obtained at much lower doses than the maximum enhancing effect. This demonstrates that to the end of oxy-radical scavenging, the concentration of the GSH, ascorbate, and 5-ASA, respectively, should be chosen to obtain maximum antioxidant effect and minimum prooxidant effects. The significance of this finding for the selection of antioxidant dose is important but remains to be investigated further.

Keywords—Oxygen free radicals, 8-Hydroxydeoxyguanosine, Scavengers, Prooxidants, Glutathione, Ascorbate, 5-Aminosalicylic acid, Free radicals

INTRODUCTION

Reactive oxygen species (ROS) have been proposed to contribute to aging as well as to cancer, ischemic heart disease, cataract, and certain chronic inflammatory disorders.[1–7] ROS are formed continuously from cellular metabolism, radiation, cooxidation of xenobiotics, metabolism of arachidonic acid, the respiratory burst in phagocytic cells, and hypoxanthine metabolism by the xanthine oxidoreductase enzyme. The latter is suggested to play a key role in the postischemic reperfusion injury because of conversion to the oxidase form.[5–8]

Along with this possible role of ROS as a pathogenetic factor in a number of otherwise disparate disease processes, increasing attention is focused on antioxidant acting substances as potential therapeutic agents.[7,9,10]

The overall effects of such compounds therefore become important, and indeed some may have dual functions acting as prooxidants just as well as antioxidants, depending on the environmental conditions. Particularly, the presence of iron (or other transition metals), participating in a Fenton-like reaction, may favor the prooxidant effect, whereby oxidized metal ions are reduced by the anti-/prooxidant, leading to a recycling with increased formation of ROS.[11] This influence of iron may have relevance in conditions that involve hemorrhagia, hemolysis, cell death, and iron overload. Also, pH influences scavenger function,[12] and especially acidic pH is pertinent for ROS generation during ischemia/reperfusion.

ROS are known to induce damage to DNA, yielding strand breaks as well as specific modified bases.[13] One of the targets of such oxidative DNA damage is C8-hydroxylation of deoxyguanosine (dG). The resulting 8-hydroxydeoxyguanosine (8-OHdG) may be used both as an in vitro and an in vivo biological marker of oxidative stress, measured either in DNA isolated from tissues or as a repair product excreted in the urine.[14–19]

The purpose of the present study was to examine the formation of 8-OHdG in calf thymus DNA in the presence of glutathione, ascorbate, and 5-amino salicylic acid. Particularly, the influence of iron and pH on the effect of these compounds was investigated. GSH was chosen because of its importance as an intracellular scavenger; ascorbate was chosen as an important intra- and extracellular scavenger with well-known prooxidant properties and therefore as a positive assay control; and 5-ASA was chosen as a drug widely used in the treatment of chronic inflammatory

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bowel diseases (CIBD),[20] presumably in part acting by scavenging oxy-radicals.[14,21,22]

METHODS

Materials

Calf thymus DNA and Nuclease P_1 was purchased from Sigma Chemical Co. (St. Louis, MO) and alkaline phosphatase from Boehringer Mannheim, Germany. Glutathione came from Serva, Germany; ascorbate from Struers Laboratory, Denmark; $FeCl_2$ from Merck, Germany; and hydrogen peroxide from the dispensary of Rigshospitalet, Copenhagen. 5-ASA was a kind gift from Ferring A/S, Copenhagen.

Experimental procedure

Calf thymus DNA was solubilized in phosphate buffer (10 mM, pH 7.4) to a concentration of approximately 400 μg/mL as estimated by UV absorbance at 260 nm (20 OD units/mg DNA). For experiments performed in acid environment, the DNA solution was adjusted with phosphoric acid to pH 4.0 just before use. Dilution of the DNA to 300 μg/mL was used in all experiments.

$FeCl_2/H_2O_2$ exposure. Reaction mixtures (2 mL) containing 600 μg DNA, 25 μM $FeCl_2$, and 0.03% H_2O_2 were shaken at 37°C for 30 min in the dark. Where indicated, reaction mixtures were supplemented with GSH (0.1 to 10 mM), ascorbate (0.02 to 1 mM), or 5-ASA (0.005 to 5 mM).

UV exposure. Six hundred micrograms DNA in 2 mL phosphate buffer without or with scavengers as described above was placed in 12.5-mL standard plastic containers (2.6-cm diameter) and exposed to light from a sunlamp 35 cm from the solution surface for 2 min (Höhensonne 1000 Impuls, without infrared bulbs. Light emitted from 240 to 580 nm, major peaks from 240 to 320 nm, at 365 nm, 405 nm, 435 nm, 550 nm, and 580 nm. The effect was 14 mW/cm^2 35 cm from the lamp. No temperature increase of the samples was measured during the exposure). The lamp was switched on for 10–15 min before use for stabilization. A maximum of nine samples were exposed simultaneously, and for every exposure period controls (i.e., without scavengers) were coexposed. Reactions were stopped by cessation of UV exposure followed by the procedure described next. All scavenger solutions were adjusted to the relevant pH just before use.

Exposure of DNA in either system was terminated by adding 5 M NaCl to a final concentration of 1 M (500 μL) and then 2 vol (5 mL) ice-cold 96% ethanol. The DNA was allowed to precipitate at −20°C overnight followed by centrifugation at 3000 rpm for 5 min. In experiments performed at pH 7.4, 25 μL of 0.25 N HCl was added just before DNA precipitation to ensure the following enzymatic digestion. The DNA precipitate was washed with 70% ethanol, dried with a stream of nitrogen gas, and solubilized in 1–8 mL 20 mM sodium acetate buffer (pH 4.8). Two hundred microliters of this solution was digested to nucleoside level at 37°C with 5 U Nuclease P_1 (in 20 μL 20 mM sodium acetate, 10 mM $ZnCl_2$, 15% glycerol, pH 4.8) for 30 min and 1 U alkaline phosphatase (in 20 μL 1 M Tris-HCl, pH 8.0) for 1 h.

Determination of 8-hydroxydeoxyguanosine (8-OHdG) and deoxyguanosine (dG). The amount of 8-OHdG and dG in the DNA was measured using a high-performance liquid chromatography (HPLC) system with electrochemical detection originally described by Floyd et al.[23] For analysis, 10 μL of the nucleoside mixture was injected into a reverse-phase C18 Nucleosil column (15 cm, 5 μm) eluted with 1 mM ethylenediaminetetraacetic acid (EDTA), 2% (v/v) methanol, and 3% (v/v) acetonitrile in phosphate buffer (pH 2.5). 8-OHdG was determined by an ESA Coulochem II electrochemical detector (ESA, Inc., Bedford, MA) with a 5010 analytical cell (porous graphite) run in the oxidative mode (E_1 = 120 mV, E_2 = 280 mV) and dG by ultraviolet absorbance at 254 nm. Quantitation of 8-OHdG was performed by injection of known amounts of pure 8-OHdG kindly supplied by Dr. David W. Potter, U.S.A., and Dr. Peter Leanderson, Sweden.

Statistical analysis. When necessary for homogeneity of variance, logarithmic transformation was performed. Data were analyzed with one-way analysis of variance, and individual groups were compared by the method of least significant difference (LSD). *P* values less than .05 were considered statistically significant.

RESULTS

Hydroxylation of DNA occurred in two oxy-radical-generating systems, ultraviolet radiation (UV) and $FeCl_2/H_2O_2$. Table 1 depicts the hydroxylation of dG in DNA caused by either UV or $FeCl_2/H_2O_2$ exposure at pH 7.4 and 4.0. The two exposure systems were adjusted at pH 7.4 to generate approximately equal amounts of 8-OHdG. To eliminate the differences in absolute values, the following results are expressed relative to controls (i.e., without added scavengers). All data in the figures are corrected for the background level of 8-OHdG found in the nonexposed calf thymus DNA corresponding to each group (Table 1).

Table 1. Formation of 8-OHdG in Calf Thymus DNA Exposed to $FeCl_2/H_2O_2$ or Ultraviolet Radiation

	8-OHdG/10^5 dG	
	Controls	$FeCl_2/H_2O_2$[a]
pH 7.4	13.6 ± 0.99	769.0 ± 11.28
pH 4.0	15.1 ± 3.39	1534.9 ± 61.48
		UV radiation
pH 7.4	11.3 ± 1.76	684.3 ± 57.46
pH 4.0	12.1 ± 0.65	239.1 ± 12.83

The values are given as mean ± SD of three experiments. The dG and 8-OHdG content was determined by HPLC separation followed by UV absorbance detection and electrochemical detection, respectively.

[a] Reaction mixtures were exposed to $FeCl_2$ and H_2O_2, as described in Methods. Controls were not exposed to $FeCl_2/H_2O_2$ but were otherwise treated as described.

The effects of GSH, ascorbate, and 5-ASA were investigated in the two oxy-radical-generating systems, which mainly differed by the presence and absence of iron. The presence of iron salts markedly changed the formation of 8-OHdG. Without iron, a considerable reduction of 8-OHdG formation occurred, while the presence of iron with one exception (GSH at pH 7.4) accelerated 8-OHdG formation.

Figure 1 depicts the effects of GSH, ascorbate, and 5-ASA in equimolar concentrations (1 mM). During UV exposure, all three compounds served as excellent scavengers. The 8-OHdG formation in the presence of GSH, ascorbate, or 5-ASA at pH 7.4 was 0.12, 0.03, and 0.02 times control values, respectively ($p < .01$). At acid pH, too, the UV-induced formation of 8-OHdG was reduced; however, GSH had a less scavenging effect compared with neutral pH (0.49 times versus 0.12 times unscavenged value).

In the presence of iron, GSH prevented 8-OHdG formation at pH 7.4 (0.68 times controls, $p < .01$). In contrast, 5-ASA basically exhibited the same characteristics as ascorbate and accelerated the generation of 8-OHdG to 1.3 times controls ($p < .05$); the corresponding value for ascorbate was 3.7 ($p < .01$). An augmented formation of 8-OHdG for these latter two compounds was seen at pH 4.0, where the reaction was further accelerated and 8-OHdG formation increased to 3.5 times controls for 5-ASA and 13 times for ascorbate ($p < .01$). For GSH, the change in pH from 7.4 to 4.0 converted the overall function from scavenging to enhancing hydroxylation of dG to 1.6 times controls ($p < .01$).

The effect of the concentration of the three compounds was investigated in the two experimental systems at pH 7.4. In general, the effects of added GSH, ascorbate, and 5-ASA were augmented with increasing concentrations. However, in the absence of iron, maximum scavenging effect of ascorbate and 5-ASA was achieved even at very low concentrations.

Figure 2 shows the formation of 8-OHdG at pH 7.4 by UV or $FeCl_2/H_2O_2$ exposure and in the presence of varying concentrations of GSH, ascorbate, and 5-ASA.

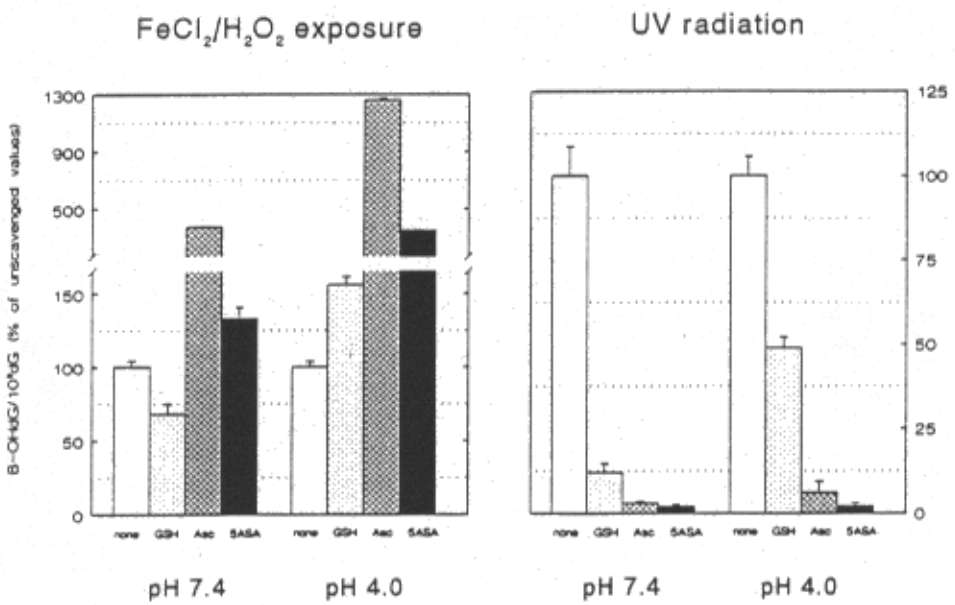

Fig. 1. Formation of 8-OHdG in calf thymus DNA (300 μg/mL) exposed to UV radiation for 2 min or $FeCl_2/H_2O_2$ (25 μM $FeCl_2$, 0.03% H_2O_2) for 30 min at pH 7.4 or pH 4.0. Reaction mixtures of 2 mL contained either no scavenger (none), 1 mM reduced glutathione (GSH), 1 mM ascorbate (Asc), or 1 mM 5-amino salicylic acid (5-ASA). All values are significantly different from corresponding unscavenged values ($p < .05$).

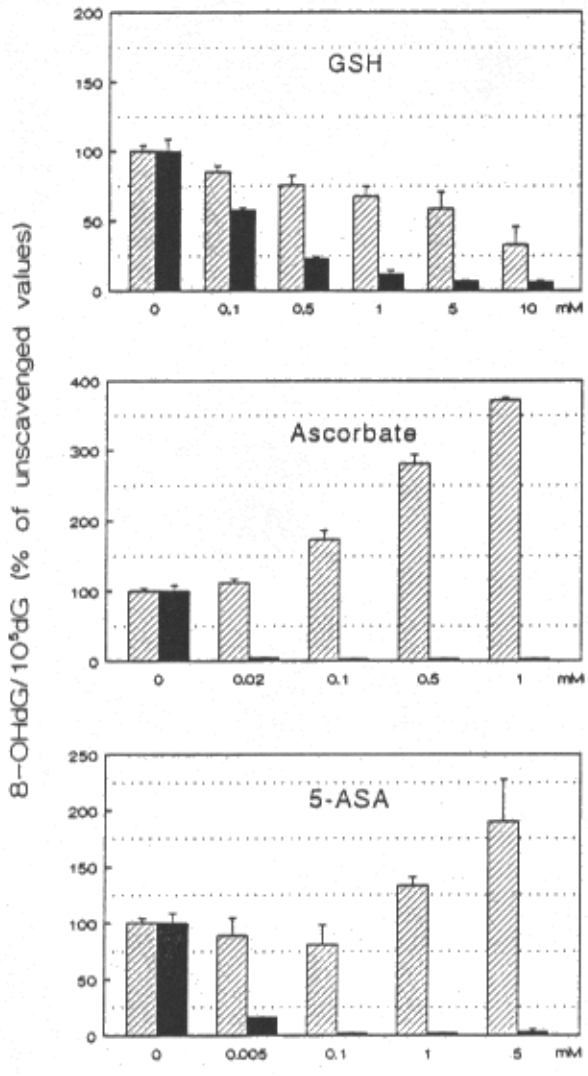

Fig. 2. Formation of 8-OHdG in DNA exposed, as in Figure 1, to $FeCl_2/H_2O_2$ (□) or UV radiation (▨) at pH 7.4. Reaction mixtures contained indicated concentrations of reduced glutathione (GSH), ascorbate, or 5-amino salicylic acid (5-ASA). All values are significantly different from corresponding unscavenged values ($p < .05$) with the following exceptions in the $FeCl_2/H_2O_2$ system: GSH 0.1 mM, ascorbate 0.02 mM, 5-ASA 0.005, and 0.1 mM.

GSH prevented 8-OHdG formation in a dose-dependent manner (0.1 to 10 mM) in the presence as well as in the absence of iron. Formation of 8-OHdG in the $FeCl_2/H_2O_2$ system ranged from 0.85 times controls (n.s.) to 0.33 times ($p < .01$) with increasing concentration of GSH, and in the UV system from 0.58 times ($p < .01$) to 0.06 times controls ($p < .01$). Thus there was a higher degree of inhibition in the UV-exposed DNA, and maximum scavenging was achieved at lower concentrations. An increase in the GSH concentration from 5 to 10 mM diminished the response in the $FeCl_2/H_2O_2$-exposed DNA from 0.59 to 0.33 times controls, whereas corresponding values in the UV system were about equal (0.07 and 0.06, respectively).

Ascorbate increased the yield of 8-OHdG in the $FeCl_2/H_2O_2$-exposed DNA in concentrations from 0.02 to 1 mM, values ranging from 1.1 (n.s.) to 3.7 times controls ($p < .01$). At all concentrations, ascorbate almost completely inhibited 8-OHdG formation from UV radiation; relative values ranged from 0.05 to 0.03 ($p < .01$).

For 5-ASA, the increase in 8-OHdG generation in the presence of iron was not seen for the lowest concentrations (0.005 and 0.1 mM), where a slight scavenging effect could not be excluded, though it was not statistically significant. A rise in the concentration to 1 and 5 mM, however, resulted in an increased 8-OHdG formation of 1.3 ($p < .05$) and 1.9 times controls ($p < .01$), respectively. On the other hand, in the absence of iron even 0.005 mM 5-ASA exhibited a remarkable inhibitory effect, 8-OHdG formation being 0.16 times controls ($p < .01$). At 0.1 mM maximal scavenging effect was achieved, the yield of 8-OHdG relative to controls being only 0.02 ($p < .01$).

DISCUSSION

The major finding in the present study is that the effect of three major antioxidant acting compounds on hydroxylation of dG residues in DNA is markedly altered and even reversed in the presence of iron salts. This prooxidant effect was augmented at low pH.

All three compounds, GSH, ascorbate, and 5-ASA, accelerated 8-OHdG formation in the presence of iron and inhibited 8-OHdG formation in the absence of iron, with one exception: GSH at pH 7.4, where 8-OHdG generation was inhibited even in the presence of iron.

The scavenging properties of 5-ASA have previously been reported in several studies,[21,22,24,25] but to our knowledge this is the first demonstration of a prooxidant effect. Maximum scavenging effect of 5-ASA was obtained at a concentration of 100 μM, while in the presence of iron salts the prooxidant effect began at about 1 mM and increased further at 5 mM.

The concentration of 5-ASA in fecal dialysate ranges from about 5 to 15 mM in CIBD patients given an oral 2 g daily dose of sulfasalazine.[26] Plasma concentrations up to 5 μM have been reported in patients treated with slow-release 5-ASA tablets (500 mg).[27] Our results thus indicate that at maximum therapeutic plasma concentrations of 5-ASA, a remarkable scavenging effect is obtained with no concomitant risk of prooxidation. In the intestinal lumen of patients treated with sulfasalazine, however, minimum concentrations (i.e., 5 mM) imply a considerable risk of enhancing oxy-radical formation in the presence of iron salts and hydrogen peroxide. In the present

study, no such effect was seen at a concentration of 100 μM, where on the other hand maximum scavenging effect was achieved. Therefore, concerning the scavenger effect, intraluminal concentrations of 100 μM would benefit just as much and in the presence of iron salts there would be no risk of harmful effects. However, other functions of 5-ASA, such as inhibition of arachidonic acid lipoxygenation and restricting the migration of macrophage cells[20] which probably contribute to the therapeutic effect, may necessitate higher concentrations.

The question then arises to what extent the iron-dependent prooxidant effect of 5-ASA could take place in vivo. Under physiological circumstances, the body is well protected from iron-catalyzed Fenton-like reactions.[28] However, oxidant stress itself can provide Fenton-reactive iron—for example, from ferritin (O_2-) or hemoglobin (H_2O_2).[28] Also, low pH values can release iron from transferrin (pH < 5.6) and lactoferrin (pH < 4.0),[28] and during cell injury iron may be released from its binding proteins.[29]

The major content of the intestinal lumen consists of nonabsorbed and excreted material, including ingested nonabsorbed iron. Therefore, even in normal physiological conditions, iron salts might be available intraluminally. The hemorrhagia and necrosis of the mucosa seen in CIBD patients possibly lead to higher iron concentrations, which in the presence of neutrophil-granulocyte-derived H_2O_2 could accelerate ROS formation at high 5-ASA concentrations.

GSH plays a major role in protecting cellular macromolecules from free radicals and other reactive intermediates.[30] The present data show that the pure scavenging function of GSH (i.e., in the absence of iron) was increased slightly when the concentration was raised from 1 to 5 mM, but not further at 10 mM. Therefore, beneficial effects of increasing intracellular GSH concentration (which physiologically ranges from about 1 to 5 mM), as developed by various means,[31] most likely is due to elevated substrate supply for glutathione peroxidase (GPx), which detoxifies H_2O_2, or to conjugation hindering depletion of GSH in circumstances where synthesis cannot resupply. On the other hand, lowering the GSH concentration to levels easily obtained in GSH-depleted cells[32,33] not only removes the substrate for GPx but also attenuates the direct radical scavenging effect.

In the presence of iron, GSH still inhibited 8-OHdG formation at pH 7.4, though to a lesser degree. This possibly reflects a net outcome of two opposite directed effects (i.e., scavenging and enhancing) which at acid pH is pushed further toward the latter. Also, differences in oxidants formed in the two experimental systems could account for the variation in scavenging effect.

In contrast to our findings at pH 7.4, Rowley and Halliwell[34] found a stimulative effect of GSH (100 μM) in the presence of $FeCl_2$ (100 μM) and H_2O_2 (100 μM). This discrepancy might be explained by the GSH:$FeCl_2$ ratio, which in their study was 1, whereas in our study ranged from 4 to 400, possibly leading to an overall quenching effect of GSH (at pH 7.4).

As expected, ascorbate acted as a prooxidant in the presence of iron salts and showed excellent scavenging properties in the absence of iron. The prooxidant action increased from 0.02 to 1 mM, whereas the scavenging function exerted almost inclusively inhibition of 8-OHdG formation at concentrations as low as 0.02 mM. Ascorbate concentration in blood plasma is normally about 0.03 to 0.085 mM[35] and ranges from about 0.25 mM in heart and kidney to 0.85 mM in liver, pancreas, and brain; 1.4 mM in the eye lens; and 2.3 mM in the adrenal glands.[36] To the end of scavenging oxy-radicals, the present study indicates that no further benefit could be achieved by increasing the intake of ascorbate, while the prooxidant action will increase, the net effect shifted to the deleterious side. The latter, however, is unlikely to be relevant to the in vivo situation in healthy organisms, where most transition metal ions are not free.

As mentioned earlier, ROS have been implicated in a number of disease processes, including carcinogenesis. Several studies have shown a correlation between the latter and 8-OHdG formation in DNA.[37] The consequence of oxidative changes of guanine bases in DNA, and presumably of the three other DNA bases as well,[38] could explain the high incidence of colon cancer associated with ulcerative colitis, where ROS are suggested as a pathogenetic factor.

Two experimental systems were used to generate oxy-radicals: UV radiation and exposure to $FeCl_2$/H_2O_2. The exact nature of the oxy-radicals generated was not investigated, but most likely, the hydroxyl radical, which is considered to be the most deleterious one, was produced in both systems. In any case, a well-defined oxidative DNA damage (i.e., 8-OHdG) was generated in either system at either pH. This fulfills demands on a system designed to investigate the effect of anti-/prooxidants in the presence or absence of iron salts.

The DNA solution was not purified for iron ions before use, and a possible contamination could, in the UV system, favor a prooxidant effect of the compounds investigated. However, ascorbate, the most potent prooxidant in this study, almost completely inhibited the formation of 8-OHdG in this system, indicating that a possible iron contamination was of no significance for the results.

We conclude from the present study that GSH, ascorbate, and 5-ASA, in the presence of iron, are able

to accelerate hydroxylation of dG residues in DNA (GSH only at pH 4.0). The maximum scavenging effect was obtained at much lower doses than the enhancing effect. In addition, low pH accelerated 8-OHdG formation in the presence of iron and reduced the scavenging effect of GSH.

This finding may have implications for the incorporation of antioxidants as therapeutic means.

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