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## DNA CLEAVAGE AND DETECTION OF DNA RADICALS FORMED FROM HYDRALAZINE AND COPPER (II) BY ESR AND IMMUNO-SPIN TRAPPING

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### Abstract

Metal ion-catalyzed oxidation of hydrazine and its derivatives leads to the formation of the hydrazyl radical and subsequently to oxy-radicals in the presence of molecular oxygen. Here we have examined the role of  $\text{Cu}^{2+}$ -catalyzed oxidation of hydralazine in the induction of DNA damage. Neither 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO) nor dimethyl sulfoxide (DMSO) were effective in inhibiting hydralazine- $\text{Cu}^{2+}$ -induced DNA damage. Singlet oxygen did not appear to participate in this DNA cleavage. The one-electron oxidation of hydralazine also leads to the formation of DNA radicals as confirmed by immuno-spin trapping with 5, 5-dimethyl-1-pyrroline-*N*-oxide. Electron spin resonance (ESR) and spin trapping studies further confirmed the formation of DNA radicals; predominantly 2'-deoxyadenosine radical adducts were detected, while some radicals were also detected with other nucleosides. Our results suggest that free hydroxyl radicals may not be the main damaging species causing DNA cleavage, and possibly, Cu-peroxide complexes, formed from  $\text{Cu}^+$ - $\text{H}_2\text{O}_2$ , are responsible for this hydralazine- $\text{Cu}^{2+}$ -induced DNA cleavage.

### Keywords

Hydralazine; Free Radicals; Oxy-radicals; ESR; Spin Trapping; DNA Damage; DNA Radicals; Immuno-spin Trapping

## INTRODUCTION

Hydrazine and its derivatives constitute an important class of compounds as they are environmental pollutants, and are used in medicine. Aromatic hydrazines (e.g., agaritine) are found in edible mushrooms (*Agaricus bisporus*). Isoniazid is used for the treatment of tuberculosis.<sup>1</sup> Iproniazid is a potent antidepressant; however, it is not currently in use due to its liver toxicity.<sup>2</sup> Procarbazine is a chemotherapeutic agent used in the treatment of Hodgkin's disease and melanoma.<sup>3</sup> Hydralazine is a potent arterial vasodilator and plays an important role in the management of hypertension and congestive heart failure.<sup>4</sup> Hydrazines in general are toxic, and induce a variety of toxic insults. These include liver toxicity,

carcinogenicity and mutagenicity.<sup>1, 2, 4, 7</sup> Hydralazine, the least toxic of the hydrazine derivatives, induces DNA damage,<sup>8</sup> causes severe forms of systemic lupus erythematosus,<sup>9, 11</sup> and has been shown to increase the incidence of lung tumors in mice.<sup>4, 6</sup> More recently, hydralazine has been shown to inhibit DNA Methyltransferase 1 by preventing transfer of a methyl group to DNA in several cancer silencing genes/tumor suppressor genes.<sup>9, 12</sup> It has, therefore, become an important treatment combination in cancer therapy.<sup>9, 12</sup> Furthermore, hydralazine has also been found to inhibit iron-containing prolyl hydroxylase enzymes, which are important for the induction of hypoxia induced factor (HIF) and vascular endothelial growth factor. HIF1 $\alpha$  is a critical target in cancer chemotherapy as it is believed to be involved in tumor progression.<sup>10</sup>

Because of the significance of hydrazine derivatives as environmental pollutants and food contaminants and their utility in medicine, a large volume of research has been carried out to elucidate the mechanisms of toxicity of these compounds.<sup>1-10</sup> Previous studies from our laboratory and others have clearly shown that hydrazine derivatives generate free radicals upon oxidation, catalyzed by metal ions, cytochrome P-450 and peroxidases, including prostaglandin synthase.<sup>13-22</sup> In addition, certain substituted hydrazines have been proposed to alkylate DNA by nucleophilic attack rather than via free radical generation.<sup>21, 22</sup> Irrespective of mechanisms (free radicals or nucleophilic attack), the consequences of such DNA alkylation may lead to carcinogenic events.

We have previously shown that hydralazine undergoes one-electron oxidation by both metal ions (Cu<sup>2+</sup> and Fe<sup>3+</sup> ions) and enzymatically (horseradish peroxidase and prostaglandin synthase) to form the hydralazyl radical (HY.), which then further decomposes to form various products.<sup>14</sup> Reilly and Aust<sup>23</sup> have reported that hydralazine in the presence of peroxidase/H<sub>2</sub>O<sub>2</sub> induces single-strand DNA damage; however, the presence of a donor such as chlorpromazine is required for damage to occur. Studies of Yamamoto and Kawanishi<sup>24</sup> have shown that the oxidation products of hydralazine and Cu<sup>2+</sup> induce DNA damage, predominantly at guanine residues, while Fe-catalyzed oxidation of hydralazine induces damage at every nucleotide in DNA.

Copper is an essential trace element that is widely distributed throughout the body and forms the essential redox-active reaction center in a variety of metalloenzymes, including ceruloplasmin, superoxide dismutase, cytochrome oxidase, dopamine  $\beta$ -hydroxylase, tyrosinase and lysyl oxidase. Nevertheless, the body's total daily requirement for copper estimated at 1–2 mg<sup>25</sup> for adult humans is much below that for iron, which is estimated at 4–6 gm. Serum levels of copper are significantly elevated in anemia, during pregnancy and during oral contraceptives.<sup>26</sup> It is interesting to note that copper metabolism is significantly altered in tumors and that serum concentrations are correlated with tumor incidence, progression and recurrence in a number of human tumors.<sup>27-29</sup> Serum ceruloplasmin levels increase by a factor of 4–8 during cancer progression.<sup>30</sup> Elevated levels of copper plays an important role in tumor angiogenesis and seems to be necessary for endothelial cell activation. Cu<sup>2+</sup>-dependent DNA fragmentation has been reported to be much more extensive than Fe<sup>3+</sup> than iron,<sup>31</sup> it is important and relevant to examine the toxicity of hydralazine in the presence of copper as this combination may be more potent for the DNA damage and consequently, for the formation of tumors than the single agents alone.

In this report, we have examined the consequences of  $\text{Cu}^{2+}$ -catalyzed oxidation of hydralazine by assessing the induction of DNA cleavage in supercoiled pHOT1 DNA and the formation of DNA radicals in calf thymus DNA. Furthermore, we have investigated the  $\text{Cu}^{2+}$  ion: hydralazine stoichiometry that induces maximum DNA damage. Our results indicate that reactive species formed from the oxidation of hydralazine by  $\text{Cu}^{2+}$  induce significant DNA cleavage in pHOT1 DNA, and this nicking is dependent upon the ratio of  $\text{Cu}^{2+}$  ions to hydralazine. Using ESR spin-trapping techniques, we found that hydralazine- $\text{Cu}^{2+}$  complexes formed significant amounts of 2'-deoxyadenosine radical spin-adducts while some radical adducts of other bases were also detected. We also found that in calf thymus DNA, formation of oxy-radicals leads to the generation of DNA radicals, which were trapped by DMPO and were easily detected by the immuno-spin trapping technique (IST). Again, the maximum DNA radical formation was dependent upon the ratio of  $\text{Cu}^{2+}$  ions to hydralazine, and this ratio was similar to those found for DNA cleavage.

### Methods and Materials

Hydralazine, calf thymus DNA, 2,2,6,6-tetramethyl-piperidine (TEMP) and Rose Bengal were obtained from Sigma Aldrich (St. Louis, MO). DMPO was purchased from Dojindo (Rockville, MD) and used without any further purification. DNA was dissolved in 100 mM Chelex-treated PBS buffer (pH 7.4), and all incubations were carried out in this buffer. Supercoiled pHOT1 DNA was obtained from Topgen (Port Orange, FL). Copper (II) sulfate (Sigma Aldrich, St. Louis, MO) was dissolved in doubly distilled water prior to use. A fresh solution of hydralazine was prepared before use. 2'-Deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine and thymidine were obtained from MP Biomedicals (Irvine, CA).

### DNA Cleavage Assay with pHOT1

DNA cleavage in the presence of hydralazine and  $\text{Cu}^{2+}$  ion was analyzed using pHOT1 supercoiled DNA according to previously published methods.<sup>32</sup> Briefly, a 20  $\mu\text{l}$  assay mixture contained 0.5  $\mu\text{g}$  of pHOT1 supercoiled DNA and varying concentrations of hydralazine. The reaction was initiated by adding  $\text{Cu}^{2+}$  and incubating the reaction mixture for 30 min at room temperature. The reaction was terminated by adding 2  $\mu\text{l}$  of 0.5 M EDTA. The reaction products were loaded onto 1% agarose gel for the DNA separation in Tris-acetate-EDTA buffer (pH 8.0). The DNA was stained with ethidium bromide and photographed. For the effects of scavengers on DNA damage, scavengers were preincubated for 5–10 min before adding  $\text{Cu}^{2+}$  ions and carrying out gel electrophoresis as before. Quantitation of various forms of DNA cleavage products was carried using an Image J software.

### Oxygen Consumption with Hydralazine

Oxygen uptake assays were carried out with hydralazine and  $\text{Cu}^{2+}$  in the presence or absence of DNA with an YSI Model 52 Oxygen Monitor. Typically, hydralazine, dissolved in 100 mM Chelex-treated PBS buffer (pH 7.4), was equilibrated for 10 min at room temperature before adding  $\text{Cu}^{2+}$  and monitoring  $\text{O}_2$  consumption. When the effects of DNA on oxygen consumption were examined, DNA was first incubated with  $\text{Cu}^{2+}$  (200  $\mu\text{g}/\text{ml}$  calf thymus DNA and 50  $\mu\text{M}$   $\text{Cu}^{2+}$ ), the reaction was initiated by adding hydralazine, and  $\text{O}_2$  consumption was measured. In some experiments, DNA-hydralazine complexes were

performed<sup>15</sup> before the addition of Cu<sup>2+</sup>, and no significant differences in O<sub>2</sub> consumption were observed. The effects of scavengers were examined by preincubating DNA-metal ion mixtures with the scavenger for 10 min before adding hydralazine.

### ESR Studies

ESR studies for the detection of singlet oxygen<sup>33</sup> and DNA radicals were carried out as described previously.<sup>34,35</sup> For the detection of singlet oxygen, TEMP (100 mM) was used in the presence of hydralazine (125 μM) and Cu<sup>2+</sup> (25 μM) in Chelex-treated PBS buffer (pH 7.4). Rose Bengal (20 μM) was used as the positive control in the presence of light (5 min exposure).<sup>36</sup> For the spin trapping studies, reaction mixtures contained in Chelex-treated PBS buffer (pH 7.4) individual 2'-deoxyribonucleosides (5.0 mM), hydralazine (125 μM), and DMPO (100 mM), and the reaction was initiated by adding 25 μM Cu<sup>2+</sup>. The mixture was transferred to ESR flat cells and the spectra were recorded with an ELEXSYS E 500 ESR spectrometer (Bruker Biospin, Billerica, MA) equipped with an ER4122SHQ cavity operating at 9.76 GHz at room temperature. The ESR settings were as follows: scan range 100 G; modulation frequency 100 KHz; modulation amplitude 1.0 G; microwave power 20 mW; receiver gain  $2 \times 10^4$ ; time constant 250 ms and the conversion time 250 ms. ESR spectra were simulated as described previously.<sup>37</sup>

### Formation of DNA-Nitrone Adduct with Hydralazine and Cu<sup>2+</sup>

Calf thymus DNA (0.5 mg-1.0 mg/ml) was mixed with various concentrations of hydralazine and the reaction was initiated by adding Cu<sup>2+</sup> followed by the addition of DMPO; the reaction mixtures were then incubated for 30 min at room temperature. Reactions were terminated by adding EDTA solution (0.5 M), and DNA was precipitated in ethanol. DNA was collected by centrifugation and washed with 75% ethanol, dissolved in the Chelex-treated PBS, and analyzed for DNA-nitrone adducts.

### Immuno-Spin Trapping Analysis of DNA-Nitrone Adducts

For the detection of DNA-nitrone adducts formed in calf thymus DNA by hydralazine, a standard ELISA procedure in a 96-well plate (Greiner-Bio-One) was used as described previously.<sup>38,39</sup> Briefly, DNA samples were diluted to 5 μg/ml in the Chelex-treated PBS buffer and incubated with Reacti-bind DNA coating solution (Thermo Scientific, Rockford, IL) at 37°C for 3 hrs. The plates were washed once with a washing buffer (the Chelax-treated PBS buffer containing 0.05% nonfat dry milk and 0.1% Tween-20) and blocked with a blocking buffer (the Chelax-treated PBS buffer containing 3% nonfat dry milk) for 18 hrs. at 4°C. The plates were then washed once with the washing buffer and incubated with rabbit anti-DMPO serum (diluted 1:10,000) at 37°C for 1 hr. The plates were washed three times with the washing buffer, 100 μl of the secondary antibody (horseradish peroxidase-conjugated, diluted 1:5000 in the washing buffer) was added, and the plates were incubated at 37°C for 1 hr. Following three washes as before, the antigen-antibody complexes were developed with Immobilon Chemiluminescence substrate (Millipore Corp, Billerica, MA), and the light emitted was recorded as arbitrary light units using X-Fluor software (Tecan US, Research Triangle Park, NC).

## RESULTS

We used supercoiled pHOT1 DNA (which contained 80–85% supercoiled DNA, according to the manufacturer and confirmed in our experiments) to examine the induction of DNA cleavage by hydralazine (25  $\mu\text{M}$ –1000  $\mu\text{M}$ ) in the presence of 25  $\mu\text{M}$   $\text{Cu}^{2+}$  ions. Copper ion alone induced some DNA cleavage (Figure 1A, lane 1), which was significantly enhanced in the presence of hydralazine (Figure 1A). While higher concentrations of hydralazine also induced some DNA cleavage (e.g.,  $35 \pm 5\%$  at 1000  $\mu\text{M}$ ), complete DNA cleavage was observed at 125–250  $\mu\text{M}$  hydralazine. At these concentrations of hydralazine, formation of both open circular and linear DNA was observed. Furthermore, as little as 10  $\mu\text{M}$   $\text{Cu}^{2+}$  was sufficient to induce this damage (data not shown).

We next examined the effects of various scavengers of oxy-radicals on the hydralazine- $\text{Cu}^{2+}$ -complex-induced DNA cleavage. As shown in Figure 1B, 1 mM GSH (an OH radical scavenger, lane 3), SOD (a scavenger of superoxide anion radical, 50 U/incubation; lane 4), catalase (a scavenger of hydrogen peroxide, 2500 U/ incubation; lane 5) or a combination of SOD and catalase (lane 6) significantly ( $75 \pm 10\%$ ) inhibited DNA cleavage. These observations indicate the participation of both superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in the DNA damage induced by the  $\text{Cu}^{2+}$ -hydralazine complex (lane 2). It is interesting that DMPO (lane 7), and DMSO (lane 8) both scavengers of free  $\cdot\text{OH}$ , were not effective in inhibiting this DNA cleavage. This would suggest that the damaging radical (free  $\cdot\text{OH}$ ) if formed is in close proximity to the DNA binding site (s) of hydralazine or the hydralazine-Cu-DNA complex that DMSO/DMPO is unable to inhibit it. Alternatively, it is also possible that the species that induce DNA cleavage are not free  $\cdot\text{OH}$  and are derived from copper-peroxide complexes.

Scavengers of free  $\cdot\text{OH}$  were not very effective in preventing DNA cleavage from hydralazine in the presence of  $\text{Cu}^{2+}$ . Since singlet  $\text{O}_2$  has been implicated in  $\text{Cu}$ - $\text{H}_2\text{O}_2$ -mediated DNA damage,<sup>33</sup> experiments were carried out to determine whether  $^1\text{O}_2$  was formed from hydralazine- $\text{Cu}^{2+}$  under our experimental conditions. In order to confirm this, we used TEMP as a probe. TEMP has been successfully used for the detection of singlet  $\text{O}_2$ <sup>33,36</sup> from the  $\text{Cu}^{2+}$ - $\text{H}_2\text{O}_2$  system. As a positive control we used Rose Bengal, a dye that has been shown to generate  $^1\text{O}_2$  in the presence of light.<sup>36</sup> Figure 2 shows that Rose Bengal generated large amounts of the TEMP nitroxide (note the difference in receiver gain), the oxidation product of TEMP by  $^1\text{O}_2$ . In contrast, the hydralazine- $\text{Cu}^{2+}$  system generated very little  $^1\text{O}_2$  under the conditions where significant DNA cleavage was observed. These observations would suggest that  $^1\text{O}_2$  did not participate significantly in inducing DNA cleavage from the hydralazine- $\text{Cu}^{2+}$  system under our experimental conditions.

### Oxygen Consumption of Hydralazine in the Presence of $\text{Cu}^{2+}$

The ratio dependence for the maximum DNA cleavage would suggest that at these ratios the  $\text{Cu}^{2+}$  ion may undergo recycling for the maximum formation of oxy-radicals. In order to confirm this, we examined  $\text{O}_2$  consumption at various metal ion-to-hydralazine ratios. The maximum  $\text{O}_2$  consumption was obtained at a ratio of 1: 2.5–5 of  $\text{Cu}^{2+}$ : hydralazine (Figure 3A, Tracing 1 and 2), while at higher ratios, significantly decreased  $\text{O}_2$  was consumed (Figure 3A, Tracings 3–5). These observations are similar to our cleavage data where

complete DNA degradation was observed (e.g., 125–250  $\mu\text{M}$  hydralazine). This consumption of  $\text{O}_2$  was inhibited by the presence of SOD (250U/ml), while catalase (6,000 U/ml) was only partially effective (Figure 3B). GSH (1000 $\mu\text{M}$ ) was also very effective in inhibiting  $\text{O}_2$  consumption, while DMPO had no effect (Figure 3B). These observations implicate  $\text{O}_2^{\cdot-}$  as the intermediate in the formation of  $\text{H}_2\text{O}_2$  and other reactive intermediates from hydralazine and  $\text{Cu}^{2+}$ .

The effect of DNA on  $\text{O}_2$  consumption was also examined, and the data is presented in Figure 2C. Addition of DNA increased  $\text{O}_2$  consumption from hydralazine and  $\text{Cu}^{2+}$ , and the maximum  $\text{O}_2$  was consumed at a  $\text{Cu}^{2+}$ :hydralazine ratio of 1:5. Again, these observations are consistent with our cleavage reactions suggesting that  $\text{Cu}^{2+}$  is accessible for cycling to generate the maximum reactive intermediates.

### ESR Spin Trapping Studies

In order to detect free radicals formed in DNA following oxidation of hydralazine, spin trapping studies with DMPO were carried out with nucleosides and calf thymus DNA (native and sonicated DNA). Typically, the reaction mixtures contained 5 mM individual nucleosides, dissolved in the Chelex-treated PBS buffer (pH 7.4), 125 $\mu\text{M}$  hydralazine and 100 mM DMPO. The reactions were initiated by adding 25  $\mu\text{M}$   $\text{Cu}^{2+}$  and recording the spectra.

Incubation of hydralazine (0.5 mM or more) with  $\text{Cu}^{2+}$  (25  $\mu\text{M}$ ) rapidly formed DMPO-adducts as previously reported;<sup>14</sup> however, when the hydralazine concentration was decreased to 125  $\mu\text{M}$  (ratio of 5:1 with  $\text{Cu}^{2+}$ ), the DMPO-adduct (94%) formed had the following coupling constants  $a^{\text{N}} = 14.14$  G;  $a^{\text{H}}_{\beta} = 11.3$  G and  $a^{\text{H}}_{\gamma} = 1.24$  G (Figure 3A). These coupling constants are similar to those described for DMPO adducts of superoxide anion radicals.<sup>40</sup> When SOD (500 U/ml) was included in the reaction mixtures, very little DMPO-adduct was detected, indicating that the DMPO-adducts trapped were derived from the trapping of superoxide anion radicals (Figure 3B). Formation of DMPO adducts required the presence of both hydralazine and  $\text{Cu}^{2+}$ .

Additionally, when the incubations were carried out for more than 20 min, a small amount (<10%) of another DMPO-adduct with coupling constants of  $a^{\text{N}} = 15.4$  G and  $a^{\text{H}} = 22.86$  G was also trapped. The identity of this radical is not known but it appears to be derived from trapping of a carbon-centered radical with DMPO. Finkelstein et al.<sup>40</sup> have described the appearance over time of a DMPO-adduct with  $a^{\text{N}} = 15.31$  and  $a^{\text{H}} = 22.0$  during trapping of superoxide anion radical and have indicated that it was formed from the decomposition of DMPO. These coupling constants are similar to those of the DMPO-adduct trapped in our system and thus may represent this decomposition product of DMPO.

Inclusion of 2'-deoxyadenosine in the reaction mixtures containing hydralazine and  $\text{Cu}^{2+}$  also resulted in the formation of several different DMPO-adducts, including the 2'-deoxyadenosine radical adduct (Figure 4Ac). This radical adduct (40%) had the following coupling constants:  $a^{\text{N}1} = 15.42$  G;  $a^{\text{N}2} = 2.58$  G and  $a^{\text{H}} = 19.95$  G. These coupling constants are similar to those described for the N-centered exocyclic  $\text{NH}_2$  of the 2'-deoxyadenosine moiety.<sup>34,41</sup> DMPO-OH (53%), DMPO-decomposition products and some nitroxide radical

were also present. Simulated spectra of these radical adducts with their coupling constants are shown in Figure 4.

When incubations were carried out with other nucleosides, various DMPO adducts were detected, these coupling constants are in agreement with those previously published,<sup>34,41</sup> and are summarized in Table-1. It should be noted that these radical adducts were minor products (less than 10% of the total adducts formed) as DMPO-OH was the major adduct detected.

It is interesting to note that when the spin-trapping experiments were carried out under conditions such as those described by Yamamoto and Kawanishi,<sup>24</sup> no radicals originating from nucleosides were trapped with DMPO. More importantly, when incubations were carried out at 37°C, only exocyclic NH<sub>2</sub> from 2'-deoxyadenosine was detected with DMPO, suggesting that this radical (or the DMPO-adduct) was significantly more stable than those formed from other nucleosides. When calf thymus DNA or sonicated calf thymus DNA was used, no radical was detected with DMPO.

### Formation of DNA-Nitron Adducts

Because oxy-radicals are formed from hydralazine-Cu<sup>2+</sup>-DNA complexes, it is possible that this oxy-radical formation would also lead to the formation of DNA radical (s) in spite of our negative ESR spin-trapping results. This possibility was investigated by examining the formation of stable DNA-nitron adducts by a highly sensitive immuno-spin trapping method.<sup>38,39</sup> Data presented in Figure 5 clearly show the formation of stable nitron adducts in calf thymus DNA. Interestingly, at high hydralazine concentrations, very few DNA-nitron adducts were formed, and the highest amounts of adducts were detected at a Cu<sup>2+</sup>:hydralazine ratio of 1:5, a ratio at which the maximum O<sub>2</sub> consumption was also observed in the presence of DNA (Figure 3C, Tracing 1). The time course indicated that this DNA-nitron adduct formation was rapid and reached a maximum within 5–10 minutes (data not shown). Furthermore, when we examined the dependence of this adduct formation on DMPO concentrations, the highest amounts of adducts were formed at 50–100 mM, and no significant adduct formation was observed at 1–10 mM of DMPO.

## DISCUSSION

Hydrazine and its derivatives react with metal ions (Cu<sup>2+</sup> and Fe<sup>3+</sup>) to generate hydrazyl radical, which then reacts with molecular O<sub>2</sub> to generate superoxide anion radicals, H<sub>2</sub>O<sub>2</sub> and, finally, ·OH.<sup>13-15</sup> Our previous studies<sup>13-15</sup> as well as this study shows that hydrazyl radical, superoxide anion radicals, and ·OH are formed from hydralazine under aerobic conditions at pH 7.4 during one-electron oxidation of hydralazine. In this study, hydralazine in the presence of Cu<sup>2+</sup> ions induced significant DNA cleavage in the supercoiled pHOT1 DNA. Furthermore, we found that the DNA damage was O<sub>2</sub><sup>·-</sup> and H<sub>2</sub>O<sub>2</sub> dependent as both SOD and catalase were effective in preventing DNA damage from hydralazine and Cu<sup>2+</sup> ions. While it has been suggested that hydroxyl radicals, once formed, will react with any species present in high concentrations in a diffusion-control manner, two typical hydroxyl radical scavengers (DMSO and DMPO) exhibited no significant inhibition of the DNA damage induced by hydralazine-Cu<sup>2+</sup> complexes in our hands. There are a number of reports

in which the addition of ·OH scavengers (ethanol, mannitol, sodium formate and DMSO) conferred only partial protection or had no effect on DNA damage.<sup>33, 42-47</sup> Because of the small or insignificant protection against DNA damage by hydroxyl radical scavengers, it has been proposed that the active species causing DNA damage are more likely copper-peroxide complexes (Scheme 1) with reactivity similar to hydroxyl radical and/or singlet oxygen rather than a free hydroxyl radical.<sup>44,47</sup> In this study, we found no inhibition of Cu<sup>2+</sup>-hydralazine-induced DNA damage by DMSO and DMPO as observed by others.<sup>44,47</sup> These observations lead us to suggest that free hydroxyl radicals are not the main DNA damaging species, and participation of Cu<sup>+</sup>-peroxo species (Scheme 1) in inducing DNA cleavage cannot be ruled out this time.

Reduced glutathione was extremely effective in preventing DNA cleavage. This would suggest that GSH rapidly traps any Cu<sup>+</sup> formed, resulting in the formation of a stable Cu<sup>+</sup>-GSH complex and preventing the formation of reactive species. It is of interest to note that studies reported from our laboratory have shown that Cu<sup>+</sup>-GSH complexes are stable and do not undergo oxidation to generate Cu<sup>2+</sup>. Using the spin-trapping technique, we have shown that these Cu<sup>+</sup>-GSH complexes do not form DMPO-OH spin adducts in the presence of H<sub>2</sub>O<sub>2</sub>.<sup>48</sup> Concentrations of GSH in cells and *in vivo* are extremely high, and it would appear that reactive species generated from hydralazine-Cu complexes may be ineffective in causing any DNA damage. However, it should be noted that many reactive species formed from various toxic drugs (e.g., cis-platin, Adriamycin) induce significant damage at submicromolar concentrations *in vivo*. Furthermore, it is now well established that oxidative stress causes a significant decrease in reduced GSH in cells and *in vivo*.<sup>49,50</sup> Repeated use of hydralazine in the presence of elevated levels of copper ions *in vivo* would result in depletion of GSH, causing increased toxicity of hydralazine.

It is noteworthy that DNA cleavage studies, oxygen consumption studies, ESR spin trapping studies and immunological detection of DNA-DMPO adducts all show a very similar stoichiometry for Cu<sup>2+</sup> ion with hydralazine for the maximum oxy-radicals formation, DNA cleavage, oxygen uptake and nitron formation at a ratio of 5–10. The reason for this is not clear at the present; however, it would suggest that hydralazine at these concentrations (e.g. 125–250µM) is extremely effective in cycling Cu<sup>2+</sup> ions for Fenton-type chemistry and facilitates the maximum formation of oxy-radicals. We found that this ratio of hydralazine to Cu<sup>2+</sup> ions of between 5–10 is extremely effective in inducing DNA damage when this ratio is maintained throughout the range of concentrations e.g., 50 µM hydralazine and 10 µM Cu<sup>2+</sup> or 1mM hydralazine and 100 µM Cu<sup>2+</sup> (data not shown). It is possible, then, that hydralazine, at higher concentrations, acts as an antioxidant inhibiting cycling of copper ions and formation of oxy-radicals, and consequently, decreasing O<sub>2</sub> consumption, DNA cleavage and DNA radical formation, as observed in this study. This would be similar to ascorbic acid, which can act both as an antioxidant and as a pro-oxidant, causing significant DNA damage in the presence of Fe or Cu ions.<sup>51</sup> It should be noted that hydralazine concentrations greater than 100 µM are extremely difficult to achieve in patients undergoing treatment with hydralazine even at the highest doses of hydralazine used.<sup>52</sup> Furthermore, due to acetylation and rapid metabolism, the concentration of hydralazine is significantly lower<sup>52</sup> than 100 µM and thus, at these lower concentrations, hydralazine is expected to act more as



a pro-oxidant, inducing significant oxidatively generated damage in vivo in the presence of  $\text{Cu}^{2+}$ .

The most important finding is that hydralazine in the presence of  $\text{Cu}^{2+}$  induces significant DNA radical formation, detected as DNA-DMPO nitron adducts as shown in Scheme 1. The implications of this finding are that DNA radicals, once formed, may induce formation of 8-oxo-7,8-dihydroguanine (8-oxoGua), and DNA-aldehydes.<sup>53,54</sup> Formation of DNA aldehydes (or 4,6-diamino-5-formidopyrimidine and/or 2,6-diamino-4-hydroxy-5-formamidopyrimidine) is common to many oxidizing systems such as ionizing radiation and some chemotherapeutic agents, e.g., bleomycin.<sup>55,56</sup> We have previously shown that hydralazine binds to DNA and induces DNA aldehyde formation in the presence of metal ions.<sup>15</sup> As copper is distributed throughout the body, DNA damage involving copper would be relatively favored in tissues/cells where copper concentration is elevated. Furthermore, in tissues where  $\text{O}_2$  concentration is low, the oxidation of Cu (I) by  $\text{H}_2\text{O}_2$  would be favored, resulting in DNA damage as studied in this paper. Repeated use of drugs such as hydralazine is expected to further enhance DNA damage, resulting in more serious adverse effects, e.g., tumor formation and possibly have implications in autoimmune disease.

Using spin trapping studies, we show here direct trapping of DMPO-nucleoside adducts from hydralazine- $\text{Cu}^{2+}$  complexes. Furthermore, we clearly show that the  $\text{Cu}^{2+}$ -hydralazine system induces adduct formation predominantly at the 2'-deoxyadenosine moiety of the DNA. Thomas et al.<sup>55</sup> have shown that hydralazine binds to the poly dA-poly dT sequences of DNA, and it is possible that hydralazine- $\text{Cu}^{2+}$  also binds to these sequences in DNA and induces radical formation in 2'-deoxyadenosine similar to that induced by the  $\text{Cu}^{2+}$ - $\text{H}_2\text{O}_2$  system.<sup>35</sup> Yamamoto and Kawanishi<sup>24</sup> have presented evidence for the formation of nucleoside radicals from hydralazine-peroxidase/ $\text{H}_2\text{O}_2$  and hydralazine- $\text{Cu}^{2+}$  complexes. However, no nucleoside radical adducts of DMPO were trapped in their studies. Furthermore, both 2'-deoxyguanosine and 2'-deoxyadenosine were equally effective in producing radicals.<sup>24</sup> In contrast, our studies suggest that the exocyclic  $-\text{NH}_2$  adenosine radical was predominantly formed and trapped (40%) by DMPO, and only a small amount of radicals (5%) was detected from 2'-deoxyguanosine. However, it should be noted that other nucleoside radicals may have been formed in calf thymus DNA but not trapped/detected at 37°C, as these radical adducts of DMPO were not stable at 37°C as observed with our spin-trapping studies.

Work is in progress to understand how DNA radical formation may be linked to inhibition of DNA Methyltransferase 1. In this regard, formation of 8-oxoGua has been implicated in decreasing the ability of DNA Methyltransferase 1 to methylate neighboring cytosines.<sup>58,59</sup> Furthermore, the formation of 8-oxoGua in the CpG island significantly alters binding of the enzyme to DNA, resulting in the inhibition of methylation.<sup>60,61</sup> It is not known at this time whether hydralazyl radical or hydralazine diazonium radical<sup>23</sup>, or the phthalazinyl radical derived from the diazonium radical (Scheme 1), can directly alkylate DNA. Furthermore, it is also not known if the diazonium radical can generate oxy-radicals that could induce DNA damage as found in this study.

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## Abbreviations

<b>DMPO</b>	Dimethyl-1-pyrrolidine <i>N</i> -oxide
<b>GSH</b>	reduced glutathione
<b>DMSO</b>	dimethylsulfoxide
<b>SOD</b>	Superoxide dismutase
<b>OH</b>	hydroxyl radical
<b>IST</b>	Immuno-spin trapping
<b>TEMP</b>	2,2,6,6-tetramethyl-piperidine

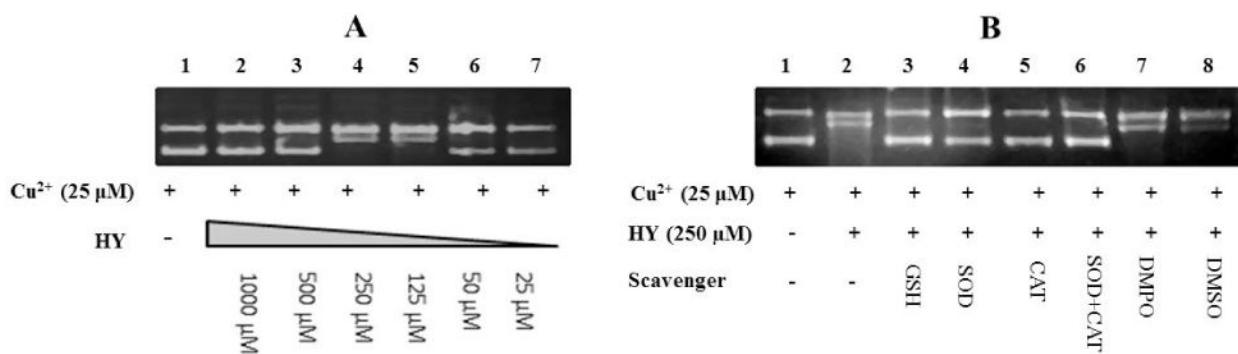
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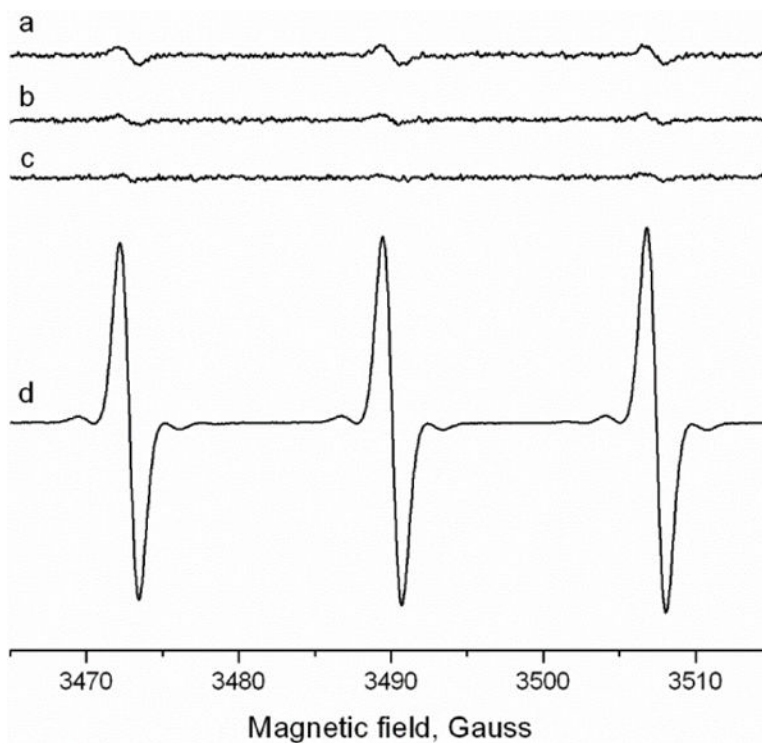
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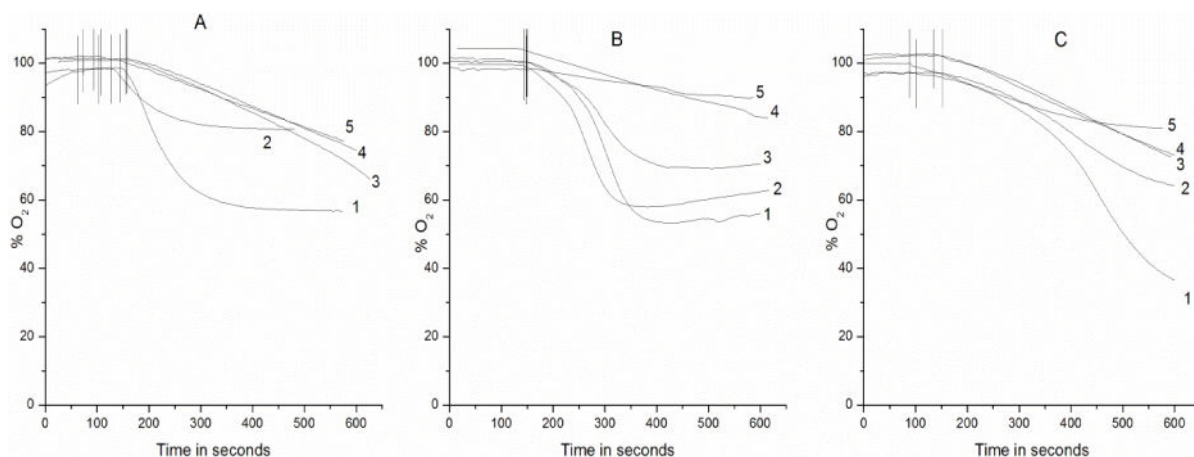
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**Figure 1.** Hydralazine-mediated cleavage (**A**) in pHOT1 DNA following oxidation with Cu<sup>2+</sup> (25 μM). The DNA cleavage was examined as described in the Methods section. Lane 1: DNA in the presence of Cu<sup>2+</sup> alone; Lane 2: Hydralazine (1000 μM) and Cu<sup>2+</sup>; Lane 3: Hydralazine (500 μM) and Cu<sup>2+</sup>; Lane 4: Hydralazine (250 μM) and Cu<sup>2+</sup>; Lane 5: Hydralazine (125 μM) and Cu<sup>2+</sup>; Lane 6: Hydralazine (50 μM) and Cu<sup>2+</sup>; and Lane 7: Hydralazine (25 μM) and Cu<sup>2+</sup>. (**B**) Effects of scavengers on the DNA cleavage induced by hydralazine (250 μM) and Cu<sup>2+</sup> (25 μM). Scavengers were incubated with hydralazine-DNA complexes for 5–10 min before adding Cu<sup>2+</sup>. Lane 1: control DNA; Lane 2: hydralazine in the presence of Cu<sup>2+</sup>; Lane 3: reduced GSH (1000 μM); lane 4: SOD (50 U/incubation); Lane 5: catalase (2500 U/incubation); Lane 6: SOD and catalase; Lane 7: DMPO (100 mM) and Lane 8: DMSO (1 mM).



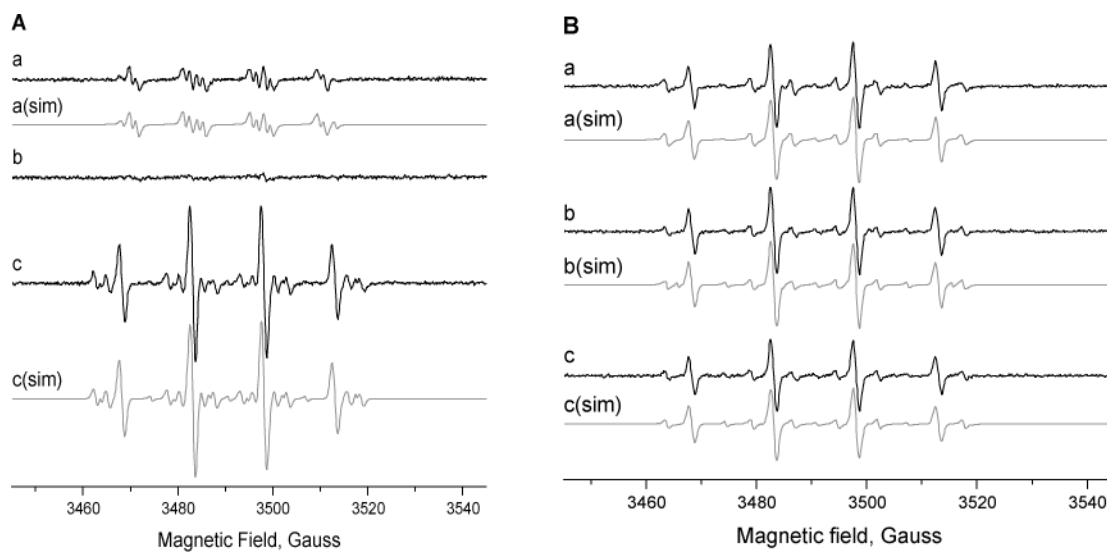
**Figure 2.** Detection of  $^1\text{O}_2$  from hydralazine (125  $\mu\text{M}$ ) in the presence of  $\text{Cu}^{2+}$  (25 $\mu\text{M}$ ) and TEMP (100 mM). **a:** complete System; **b:** Hydralazine alone; **c:**  $\text{Cu}^{2+}$  alone and **d:** in the presence of Rose Bengal (20  $\mu\text{M}$ ) and light for 5 min. No signal for the nitroxide was detected in the absence of light with Rose Bengal. The ESR studies were carried out as described in the Methods section; a–c: conversion time = time constant = 163 ms. d: 81 ms.



**Figure 3.**

Oxygen consumption (**A**) with different concentrations of hydralazine in the presence of Cu<sup>2+</sup> (50 μM). The oxygen consumption was carried out as described in the Methods section. Tracing 1: 125 μM hydralazine; Tracing 2: 50 μM hydralazine; Tracing 3: 250 μM hydralazine; Tracing 4: 500 μM hydralazine; and Tracing 5: 1000 μM hydralazine. (**B**) Effects of oxy-radical scavengers on oxygen consumption from 125 μM hydralazine and 50 μM Cu<sup>2+</sup>. Tracing 1: control; Tracing 2: 100 mM DMPO; Tracing 3: catalase (6000 U/ml); Tracing 4: SOD (500 U/ml); and Tracing 5: 1000 μM GSH. (**C**) Effect of DNA (200 μg/ml) on the oxygen consumption by various concentrations of hydralazine in the presence of Cu<sup>2+</sup> (50 μM). Tracing 1: 250 μM hydralazine; Tracing 2: 125 μM hydralazine; Tracing 3: 500 μM hydralazine; Tracing 4: 1000 μM hydralazine and Tracing 5: 50 μM hydralazine. DNA was added to the hydralazine solutions and incubated for 10 min before adding Cu<sup>2+</sup> and measuring the O<sub>2</sub> consumption.

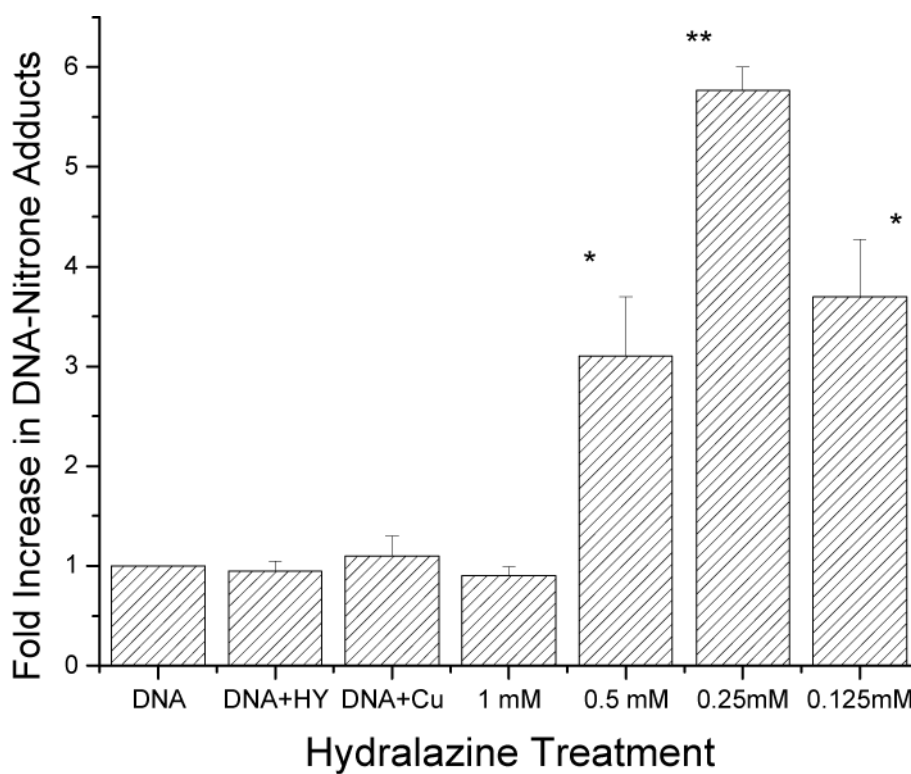




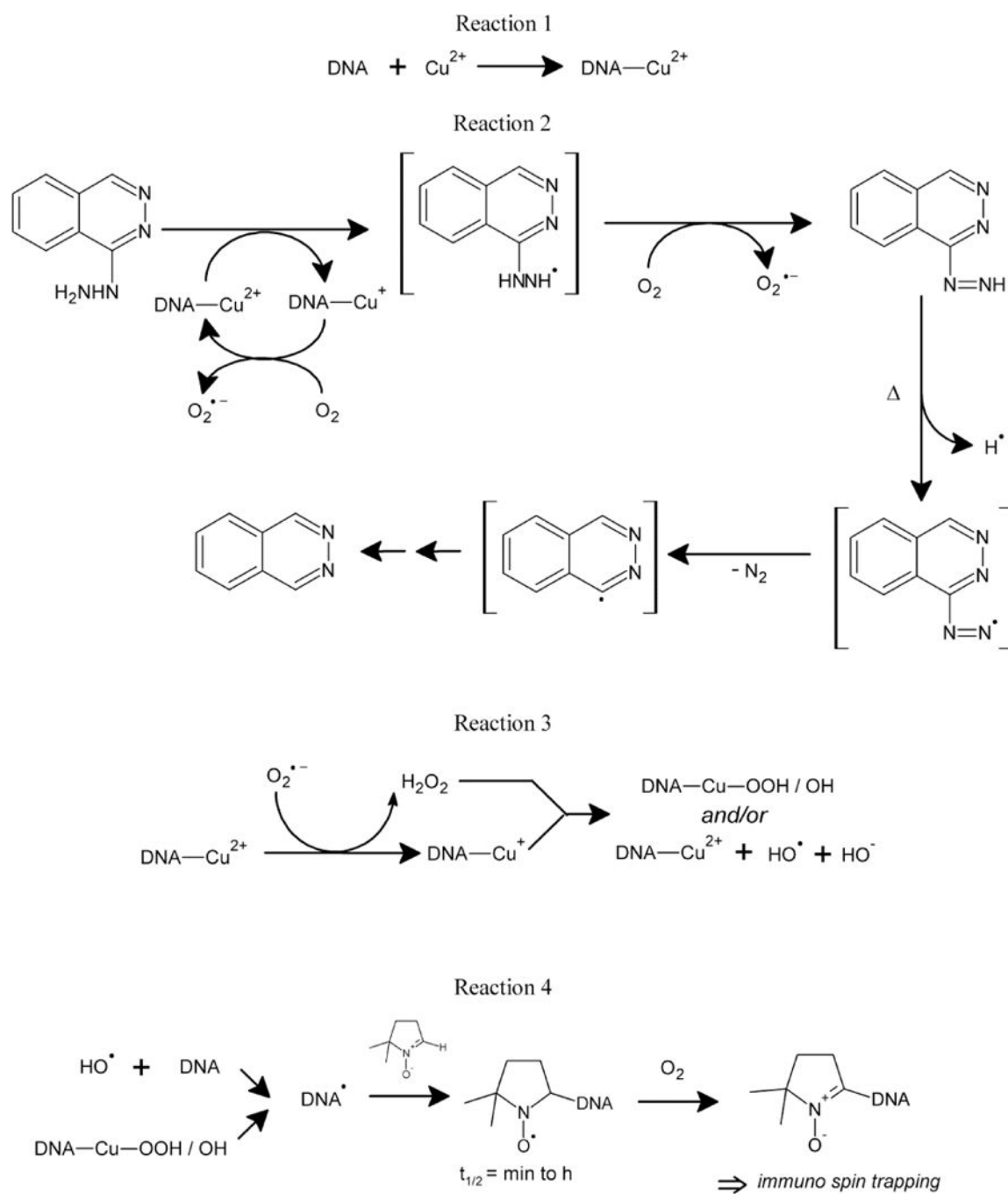
**Figure 4.**

Detection of free radicals in the presence of DMPO (100 mM). **Panel A**, (a) hydralazine (125 μM) and Cu<sup>2+</sup> (25 μM); (b) + SOD (500 U/ml); and (c) + 2'-deoxyadenosine (5 mM).

**Panel B**, Radical adducts obtained from (a) 2'-deoxyguanosine (5 mM), (b) 2'-deoxycytidine (5 mM) and (c) thymidine (5 mM) from hydralazine and Cu<sup>2+</sup>. These studies were carried out at pH 7.4 as described in the Methods section.



**Figure 5.** Immunological detection of the DNA radicals formed in calf thymus DNA (0.5 mg/ml) by various concentrations of hydralazine in the presence of  $\text{Cu}^{2+}$  (50  $\mu\text{M}$ ) and DMPO (100 mM). The detection of these DNA radicals was carried out as detailed in the Methods section. Starred values were significantly different (\* $<0.05$ ; \*\* $<.005$ ,  $n = 3$ ) from DNA alone or DNA in the presence of hydralazine and DNA in the presence of  $\text{Cu}^{2+}$ . The significance was determined by Student's t-test.

**Scheme 1.**

Structure of hydralazine, and DNA nitrene, and proposed formation of various free radical species from hydralazine that may induce DNA cleavage following oxidation by  $\text{Cu}^{2+}$  ion.

**Table 1**

Parameters of DMPO-adducts formed from nucleosides following treatment with hydralazine (125  $\mu\text{M}$ ) and  $\text{Cu}^{2+}$  (25  $\mu\text{M}$ ) at room temperature.

Substrate	Hyperfine Coupling Constants, G			Assignment
	$a^{\text{N}}$	$a^{\text{H}}$	$a^{\text{N}}$	
2'-deoxyadenosine	15.42	19.95	2.58	N-centered, exocyclic $\text{NH}_2$
2'-deoxycytidine	15.38	18.95	2.02	N-centered
Thymidine	15.88	19.5	2.71	N-centered
2'-deoxyguanosine	15.6	20.27	2.56	N-centered, $\text{N}_3$ position

ESR-Spin trapping studies were carried out as described in the Methods Section.