Free Radical Research, May 2005; 39(5): 499–505

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Differential DNA strand breaking abilities of $^{\cdot}OH$ and ROS generating radiomimetic chemicals and γ-rays: Study of plasmid DNA, pMTa4, *in vitro*

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Accepted by Professor N. Taniguchi

(Received 14 November 2004)

Abstract
Reactive oxygen species (ROS) interact with components of a living cell. Among them $^{\cdot}OH$ is known to cause major oxidative damage to living cells and is proposed to be involved in pathogenesis including carcinogenesis. Proper understanding of consequences of such damage is, therefore, medically relevant. In this report, aqueous solution of plasmid DNA, pMTa4, has been exposed to Fenton oxidant and Haber–Weiss oxidant mediated free radical generating chemical systems, and ^{60}Co γ-rays *in vitro* either alone or in combination to study their strand breaking abilities. The exposed pMTa4 was analyzed by agarose gel electrophoresis. The results show qualitative differences in the induction of strand breaks on the plasmid pMTa4 molecule by the iron (Fe^{2+}), copper (Cu^{2+}) or γ-rays mediated $^{\cdot}OH$ and other ROS.

Keywords: *pMTa4 DNA, Fenton oxidant, Haber–Weiss oxidant, γ-Rays, In vitro*

Introduction

Reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) and superoxide anion radical ($O_2^{\cdot -}$), interact with components of a living cell. Among them $^{\cdot}OH$ is known to cause major oxidative damage to living cells. Due to this, it has been a subject of intense investigations [1]. For obvious reasons oxidative damage to DNA, the genetic material, has always been core of such investigations to understand the process (reviewed in Ref. [2]). Interestingly, the major source of generation of such reactive oxidative species is internal—the metabolic process itself [2,3], besides other exogenous triggers like radiation. The cellular concentration of $^{\cdot}OH$ correlates rather well with pathological conditions, ageing and other degenerative processes including mutagenesis and carcinogenesis [4–6]. It is known that cellular H_2O_2 is involved in reducing transition metals to generate $^{\cdot}OH$ via classical Fenton reactions (reviewed in Ref. [7]. Fenton reported, towards the end of nineteenth century, oxidation of tartaric acid or other hydroxy acids in the presence of low amounts of ferrous salts and H_2O_2. Haber and Weiss, in 1930s, identified the oxidizing intermediate in Fenton or Fenton-type reactions as $^{\cdot}OH$. Later it was found that in lower oxidation states, several metal ions participated in Fenton-type reactions (see details in Ref. [7]). Due to the fundamental contributions of Fenton as well as Haber and Weiss, such reactants are referred to as Fenton, Fenton-type or Haber–Weiss reactants/reaction. These reactants are reported to inflict strand breaks in DNA [8]. The transition metals of biological relevance are iron (Fe^{2+}) and copper (Cu^{2+}), which normally exist as adducts to membrane, proteins, nucleic acids and low-molecular weight chelators [9].

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760500072305

The free metal ions of both of these readily participate in the degenerative activity mediated by co-metabolic product, H_2O_2. The highly reactive $^{\cdot}OH$ produces a plethora of damages on the DNA, notably oxidation of bases and sugars that also result in single strand breaks (SSB) and double strand breaks (DSB) [1,10]. The induced strand breaks, especially DSB, are of immense biological consequence because of its potential to cause large mutations and major metabolic upheavals [11]. Exogenous factors may also cause oxidative damages of similar kind. Among them ionizing radiation is of particular interest as it damages DNA by direct ionization of the molecule (direct effect) as well as through reactive species generated in the overwhelming aqueous medium (indirect effect) in which DNA functions [12]. The damage inflicted upon DNA via Fenton reactions and that by ionizing radiation are similar, thereby, qualifying to be called radiomimetic, but not identical [8]. It has been shown that the major component of damage by ionizing radiation comes from the $^{\cdot}OH$ since estimated 80% of DNA damage is oxidative in nature [13]. On the other hand the living systems have developed appropriate counter-metabolic strategies, like repair pathways, to reduce the oxidative stress for the well being of the cell [8]. Depending on the prevalence of the two opposing metabolic processes, the fates of living system in terms of degeneration, ageing, pathogenesis, mutagenesis or carcinogenesis are decided. Undoubtedly, therefore, proper understanding of oxidative damage to DNA and its consequences have applied medical potentials in human health and welfare.

Despite the wealth of information on oxidative damage to DNA and its biological consequences, the overall picture is not clear yet and there is urgent need to assess oxidative damage to cellular DNA [1]. The situation is further complicated because it has been shown that nucleotides, oligonucleotides and DNA sustained qualitatively and quantitatively different damage by Fenton oxidants [10]. Furthermore, in an *in vivo* system, wherein repair systems are simultaneously operational, it is difficult to decipher exact extent of damage by oxidants. In that sense an *in vitro* model is useful, as one can control possible biological repair of the inflicted damage. This sort of freezes the damage, which can be analyzed to get clearer picture of the events. Using a plasmid construct, pMTa4, *in vitro* we have shown earlier that low and high LET radiation caused both oxidative and strand break kind of damages to DNA [14,15]. The plasmid, by acquiring different topological forms due to SSB or DSB, is a convenient tool to monitor strand break kind of damage. In this study, we elucidate strand break type of DNA damage induced by oxidative species using pMTa4 in aqueous solution (*in vitro*). To create oxidative damage onto plasmid DNA molecule, two transition metals of biological relevance, Fe^{2+} and Cu^{2+}, were used in Fenton-type reactions. For convenience, the Fe^{2+}- and Cu^{2+} mediated reactions have been referred to as Fenton and Haber–Weiss reactions, respectively, in this report. Parallel to this, the plasmid DNA *in vitro* was also exposed to ^{60}Co γ-radiation either alone or in combination with the radiomimetic chemical systems. The damaged plasmid was analyzed to study the strand breaking ability.

Materials and methods

Chemicals

Analytical grade $CuSO_4$ (S. D. Fine Chem. Ltd), $FeCl_2$ (Fluka) and H_2O_2 (Qualigen) were used in the investigation without further purification. Other chemicals used in the investigation were of highest purity grades. All solutions were made in sterile ultrapure (Milli-Q) water.

Plasmid DNA isolation

The plasmid, pMTa4 (6173 bp), used in this investigation has been described earlier [14,15]. The pMTa4 was propagated in and routinely purified from *Escherichia coli* K12 strain AB1157 (wild type) growing on LB medium supplemented with 100 μg ml^{-1} Ampicillin under standard conditions. It was isolated by a modified alkaline lysis method [16] in which EDTA, a metal chelator, was not used at any stage. The isolated pMTa4 was dissolved in sterile water and refrigerated.

Exposure of pMTa4 to Fe^{2+} mediated Fenton oxidant

The plasmid aqueous solution (5 μg) was exposed to Fenton reactants (88 mM H_2O_2 and 20 μM $FeCl_2$ in 10 μM Tris–Cl buffer, pH 7) and incubated at 37°C for 10, 20, 30, 40, 50 and 60 min. The reaction was stopped by addition of 10 mM EDTA to the reaction mixture and pMTa4 was analyzed.

Exposure of pMTa4 to Cu^{2+} mediated Haber–Weiss oxidant

The plasmid aqueous solution (5 μg) was exposed to Haber–Weiss reactants (2.8 mM H_2O_2 and 25 μM $CuSO_4$ in 10 μM Tris–Cl buffer, pH 7) and incubated at 37°C for 10, 20, 30, 40, 50 and 60 min. The reaction was stopped by addition of 10 mM EDTA to the reaction mixture and pMTa4 was analyzed by electrophoresis.

Exposure of pMTa4 to ^{60}Co γ-radiation

The plasmid aqueous solution (5 μg) was irradiated with ^{60}CO γ-rays in a Gamma chamber (dose rate: 3 Gy min^{-1}) accumulating doses of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 Gy. The plasmid was analyzed.

Exposure of pMTa4 to a priming dose of Fenton or Haber–Weiss oxidants followed by ^{60}Co γ-ray

The plasmid aqueous solutions (5 μg) were exposed to a priming dose of either Fe^{2+} or Cu^{2+} mediated Fenton oxidant/Haber–Weiss oxidant for 20 min followed by exposure to increasing doses of ^{60}CO γ-rays. The plasmid was analyzed after that.

Exposure of pMTa4 to a priming dose of ^{60}Co γ-rays followed by Fenton or Haber–Weiss oxidant

The plasmid aqueous solution (5 μg) was exposed to a priming dose of 30 Gy of γ-ray followed by exposure to either Fe^{2+} or Cu^{2+} mediated Fenton oxidant. The plasmid was analyzed after the treatment.

Agarose gel electrophoresis

The pMTa4 samples were analyzed by 1% agarose gel electrophoresis using tris–acetate–EDTA buffer system as described earlier [13,14]. The gels were stained with 0.3 μg ml^{-1} ethidium bromide and visualized over a UV-transilluminator.

Analysis

The gel images were digitized using a Kodak digital camera and the net pixel density of bands of topological forms of the plasmid was calculated using KDS-1D Image Analysis (Kodak) software. Each experiment had at least three independent replicates. The mean and SD were calculated from each of the three independent experimental sets and plots were made in the form of bar diagrams.

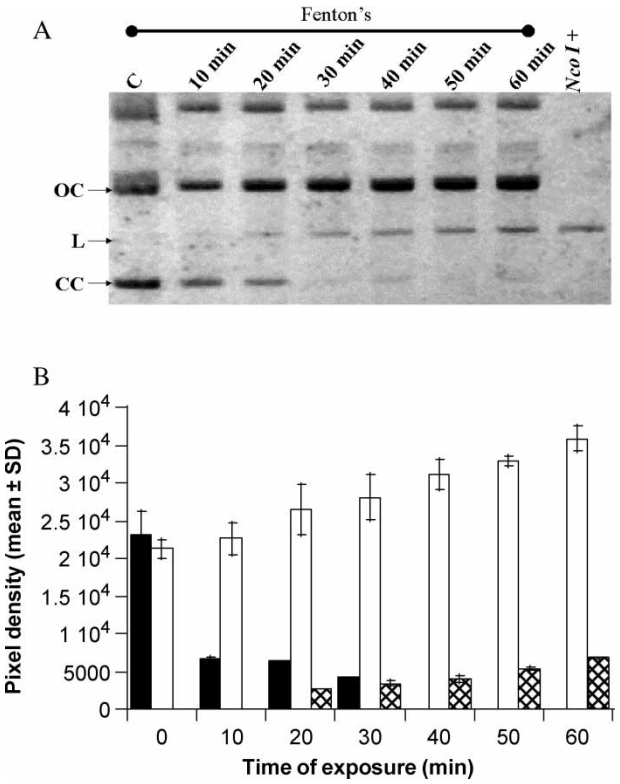

Figure 1. DNA strand breaks induced by Fenton reactants. Panel A shows the electrophoreogram of plasmid pMTa4 unexposed (C) or exposed to Fenton oxidant from 10 to 60 min. The bands marked CC, OC and L represent the undamaged native, SSB induced and DSB induced forms of the plasmid, respectively. The last lane marked *Nco I* + was loaded with pMTa4 linearized with restriction enzyme *Nco I*. Panel B shows the net intensities of the bands (mean ± SD) in form of a bar diagram. The closed, open and crossed bars represent the CC (native), OC (SSB), L (DSB) forms, respectively.

Results

Effect of Fenton reactants on pMTa4 DNA

Gel electrophoreogram of plasmid DNA treated with the Fenton reactants (Figure 1A) showed a dose dependent increase in OC band with appearance of L band after about 20 min of exposure and progressive decline in the native CC band of the plasmid. In about 40 min following exposure to Fenton's reactants, all native plasmid DNA molecules, the CC band, was fully converted into either OC or L forms of the plasmid. The plot of pixel densities of the bands versus time of exposure has been shown in Figure 1B.

Effect of Haber–Weiss reactants on pMTa4 DNA

Gel electrophoresis of the plasmid DNA exposed to Haber–Weiss reactants exhibited essentially similar albeit milder expression of effects (Figure 2A). The plot of pixel densities of the bands versus time of exposure has been shown in Figure 2B. The dose dependent increase in the OC and L band intensities of the plasmid was accompanied with progressive reduction in its native, CC band with some noticeable differences as compared to the patterns exhibited by Fenton's oxidant (Figure 1). Even though the L band made its appearance faster, in about 10 min after exposure to Haber–Weiss reactants, the native, CC band of the plasmid did not disappear completely even after 60 min of exposure. This suggests that not all plasmid DNA molecules sustained damage by Haber–Weiss oxidant.

Effect of γ-rays on pMTa4 DNA

The gel electrophoresis of the plasmid DNA exposed to increasing doses of γ-rays (Figure 3A) showed progressive increase in OC band intensity up to 45 Gy followed by a slight decrease. The L band of the plasmid made its appearance upon exposure to 20 Gy and exhibited a dose dependent increase. This was accompanied by progressive decrease in native CC band of the plasmid. However, the CC band did not

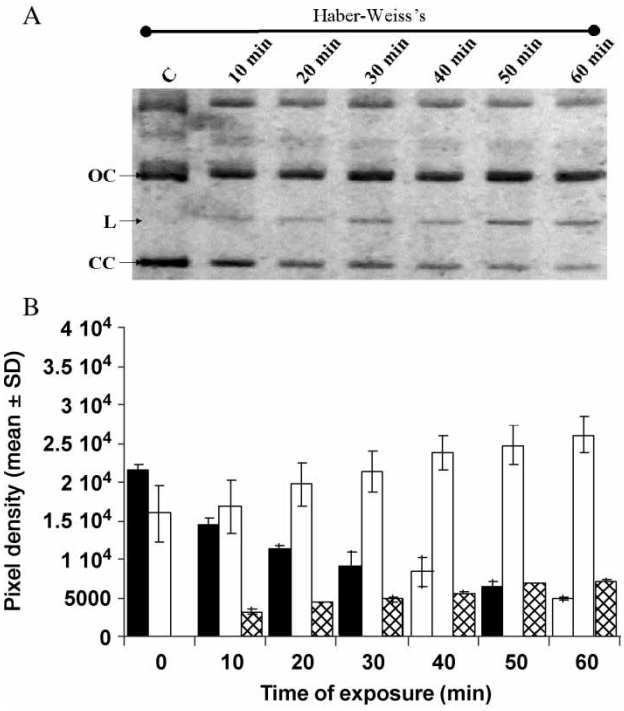

Figure 2. DNA strand breaks induced by Haber–Weiss reactants. Panel A shows the electrophoreogram of plasmid pMTa4 unexposed (C) or exposed to Haber–Weiss oxidant from 10 to 60 min. The bands marked CC, OC and L represent the undamaged native, SSB induced and DSB induced forms of the plasmid, respectively. Panel B shows the net intensities of the bands (mean ± SD) in form of a bar diagram. The closed, open and crossed bars represent the CC (native), OC (SSB), L (DSB) forms, respectively.

completely disappear even after exposure to 60 Gy. The plot of pixel density of the plasmid bands versus dose is shown in Figure 3B.

Effect of combined exposure to Fenton reactants and γ-radiation on pMTa4 DNA

In combined exposure regime, plasmid DNA was first exposed to a priming dose of Fenton's reactants for 20 min followed by exposure to increasing doses of γ-rays. The samples run on agarose gel were quantified and plotted in Figure 4. Fenton oxidant primed plasmid DNA showed increase in OC band intensity even for 5 Gy dose and remained essentially invariant at higher doses. The density of L band of plasmid increased in a dose dependent manner. However, the native CC band of the plasmid remained visible even after 60 Gy exposures to γ-rays. In contrast, the plasmid DNA primed to 30 Gy of γ-rays and then exposed to Fenton reactants for up to 60 min showed a different pattern of damage (Figure 5). The densities of OC and L bands of plasmid DNA were statistically invariant for increasing time of exposure to Fenton's oxidant even though the CC band disappeared completely after 30 min of exposure. The results suggest degradation of plasmid DNA.

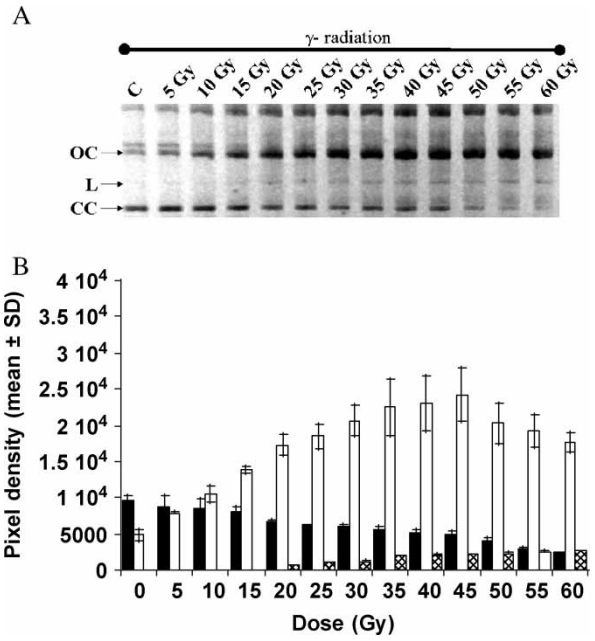

Figure 3. DNA strand breaks induced by γ-rays.. Panel A shows the electrophoreogram of plasmid pMTa4 unexposed (C) or exposed to different doses of γ-rays (5–60 Gy). The bands marked CC, OC and L represent the undamaged native, SSB induced and DSB induced forms of the plasmid, respectively. Panel B shows the net intensities of the bands (mean ± SD) in form of a bar diagram. The closed, open and crossed bars represent the CC (native), OC (SSB), L (DSB) forms, respectively.

Effect of combined exposure to Haber–Weiss reactants and γ-radiation on pMTa4 DNA

In this set of experiments, plasmid DNA was first exposed to a priming dose of Haber–Weiss reactants

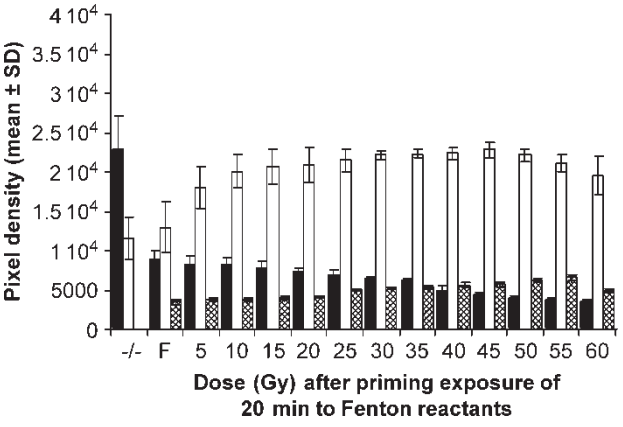

Figure 4. Bar diagrams showing the net intensity of the OC, L and CC bands of pMTa4 resolved on agarose gel (not shown) after exposure to a priming dose of Fenton reactants for 20 min followed by exposure to γ-rays from 5 to 60 Gy. The closed, open and crossed bars represent the CC (native), OC (SSB) and L (DSB) forms, respectively. The first set of bars marked −/− is for sham-exposed control, the second set of bars marked F is for pMTa4 exposed only to Fenton oxidants from 20 min while the remaining sets of bars are for samples exposed to Fenton oxidants followed by increasing doses of γ-rays.

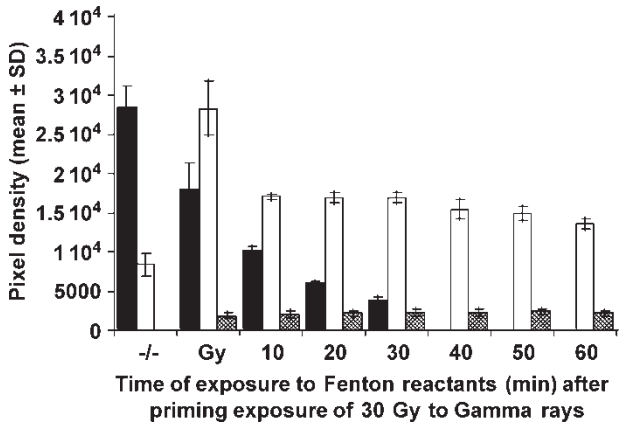

Figure 5. Bar diagrams showing the net intensity of the OC, L and CC bands of pMTa4 resolved on agarose gel (not shown) after exposure to a priming dose of 30 Gy of γ-rays followed by Fenton oxidants from 10 to 60 min. The closed, open and crossed bars represent the CC (native), OC (SSB) and L (DSB) forms, respectively. The first set of bars marked −/− is for sham-exposed control, the second set of bars marked Gy is for pMTa4 exposed only to γ-rays (30 Gy) while the remaining sets of bars are for samples exposed to γ-rays followed by increasing periods of Fenton oxidants.

for 20 min followed by exposure to increasing doses of γ-rays. Figure 6 shows that upon exposure to γ-rays the Haber–Weiss oxidant primed plasmid showed a dose dependent increase in L band and decrease in CC band densities while the OC band remained essentially invariant. Upon reversal, the 30 Gy γ-ray primed plasmid upon exposure to Haber–Weiss reactants showed nearly invariant OC and L bands while the density of CC band decreased rapidly up to 60 min (Figure 7). This also suggests degradation of the plasmid DNA.

Discussion

The CC topological form of a plasmid represents its natural, covalently closed and native status. Upon sustaining a SSB, the form undergoes relaxation creating the OC topological form while a DSB or two proximal SSB creates its linearized L topological form. All three forms of the plasmid have very distinct migration on agarose gel (Figures 1A–3A; CC, OC and L bands, respectively). The quantities of these bands can be measured using the pixel densities of the bands (Figures 1B–3B). Therefore, it becomes immensely convenient tool to follow SSB and DSB types of damage to plasmid DNA molecule [12,14,15]. This investigation has used these characteristics of plasmid, pMTa4, DNA molecules to study effects of oxidative damage in an *in vitro* system. By choosing *in vitro* system, influences of inherent repair pathways of an *in vivo* situation have been completely removed. Two biologically relevant metal ions, iron (Fe^{2+}) and copper (Cu^{2+}), are known to be the major inherent source of generation of ROS and free radicals via classical Fenton reaction [9] while radiation is one such exogenous source [12]. Further oxidative damage is reported to be qualitatively different for mono, oligo or poly NT [10]. Therefore, in this study, these sources of ROS and free radical generation and their combinations have been used to induce oxidative damages on plasmid pMTa4, biologically active DNA molecules, to gain deeper insight into damage process. For convenience, the iron (Fe^{2+}) and copper (Cu^{2+}) mediated chemical reactions have been addressed as Fenton and Haber–Weiss reactions/oxidants, respectively. Since metal

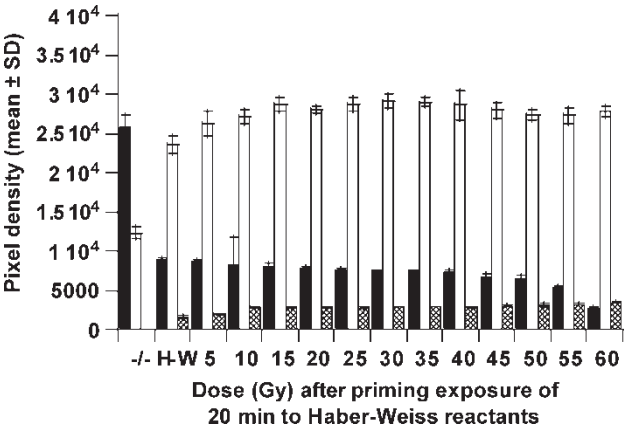

Figure 6. Bar diagrams showing the net intensity of the OC, L and CC bands of pMTa4 resolved on agarose gel (not shown) after exposure to a priming dose of Haber–Weiss reactants for 20 min followed by exposure to γ-rays from 5 to 60 Gy. The closed, open and crossed bars represent the CC (native), OC (SSB) and L (DSB) forms, respectively. The first set of bars marked −/− is for sham-exposed control, the second set of bars marked H–W is for pMTa4 exposed only to Haber–Weiss oxidants from 20 min while the remaining sets of bars are for samples exposed to Haber–Weiss oxidants followed by increasing doses of γ-rays.

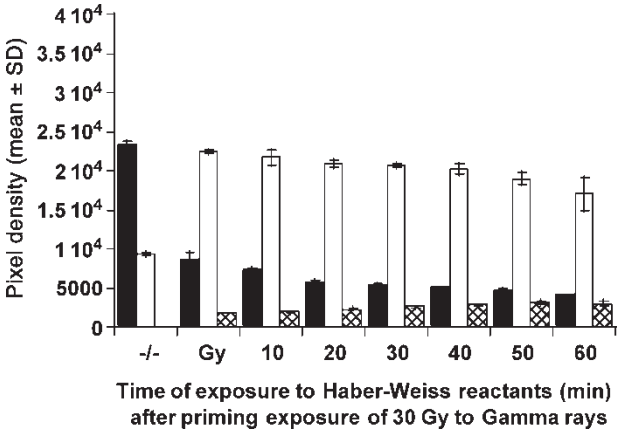

Figure 7. Bar diagrams showing the net intensity of the OC, L and CC bands of pMTa4 resolved on agarose gel (not shown) after exposure to a priming dose of 30 Gy of γ-rays followed by Haber–Weiss oxidants from 10 to 60 min. The closed, open and crossed bars represent the CC (native), OC (SSB) and L (DSB) forms, respectively. The first set of bars marked −/− is for sham-exposed control, the second set of bars marked Gy is for pMTa4 exposed only to γ-rays (30 Gy) while the remaining sets of bars are for samples exposed to γ-rays followed by increasing periods of Haber–Weiss oxidants.

chelators seriously affect Fenton/Haber–Weiss chemical reactions, no metal chelator has been used during isolation of pMTa4 from *E. coli*. It is to be noted that during isolation of pMTa4 from *E. coli*, the procedural stress induces some SSB in the native plasmids. As a result, it is fairly routine to find OC band along with CC band in controls. This is evident from the band profiles in C (control) lanes of electrophoreograms (Figures 1A, 2A, and 3A). Any further strand break type of damage to plasmid DNA should increase band intensities of OC (representing SSB) and introduce L band (representing DSB). However, as it happens, the band intensity of DNA molecule in CC band should correspondingly decrease. It should be kept in mind that two closely placed SSB on a plasmid DNA can potentially cause linearization of plasmid DNA, thereby appearing as L band.

Under the specified conditions of the investigation the results show qualitative differences in the induction of strand breaks on pMTa4 DNA molecules by the Fenton and Haber–Weiss mediated $^{\cdot}OH$ and other ROS (Figures 1 and 2). While both chemical systems induced almost similar kinetics of SSB (Figures 1B and 2B—the open bars) and DSB (crossed bars), the native CC form (closed bars) of the plasmid was rapidly and totally consumed only by Fenton oxidant in about 30 min. The CC form of pMTa4 should normally get converted to OC or L forms upon sustaining SSB or DSB, respectively. This does not seem to be happening as quantities of neither OC nor L bands increased proportionately (Figure 1). Therefore, the result suggests that Fenton reactants might have also induced partial degradation of pMTa4 DNA molecules. The Haber–Weiss reactant induced damage to pMTa4 DNA molecules seem unlike this (Figure 2). The difference in induction of damage to circular pMTa4 DNA molecule by these Fe^{2+} and Cu^{2+} mediated ROS can be explained by assuming that the distribution of damage sites was more uniform in case of Cu^{2+} mediated Haber–Weiss reactants (Figure 2) while Fe^{2+} mediated Fenton reactants induced damage clustered in certain locations within the plasmid DNA molecule.

To verify it, identical pMTa4 preparations were exposed to increasing doses of γ-rays (Figure 3). γ-Rays are known to randomly damage DNA molecule by both direct ionization of the DNA molecule as well as by indirect mode involving ionization of water. The result shows a dose dependent increase in OC band intensity signifying induction of SSB up to 45 Gy followed by slight decline up to 60 Gy (Figure 3A and B). Concomitantly, the CC form declined proportionately (Figure 3B). The L band made its appearance only at 20 Gy dose and increased in a dose dependent manner up to 60 Gy. The decline in OC band intensity beyond 45 Gy dose suggests that some proximally placed SSBs might have produced L form showing apparent DSB. The electrophoreogram and its quantification data do not suggest degradation of pMTa4 DNA molecule in line with the thinking that random effect of γ-ray produced uniform damage sites on pMTa4 DNA molecule.

Upon combining the chemical and radiation induced ROS and free radical generating systems to create damage on pMTa4, interesting results are obtained (Figures 4–7). The Fenton oxidant primed pMTa4 shows high level of SSB even after 5 Gy γ-ray irradiation and continued to exhibit the same level up to 60 Gy with progressively increasing DSB and decreasing CC form (Figure 4). The reverse is not true for γ-ray primed pMTa4 exposed to Fenton oxidant (Figure 5). In this case, all native CC form of pMTa4 was consumed in about 30 min of exposure to Fenton oxidant but, there was no significant change in SSB or DSB. This suggests degradation of pMTa4 under the experimental conditions. Haber–Weiss oxidant primed pMTa4 upon exposure to 5 Gy or more of γ-ray did not show significant changes in induction of either SSB or DSB with marginal reduction in CC form of pMTa4 (Figure 6). Reversal of treatment, that is, γ-radiation primed pMTa4 exposed to Haber–Weiss oxidant produced essentially similar results (Figure 7).

The exact nature and extent of $^{\cdot}OH$ and other ROS induced base damage in DNA is still not fully elucidated [1]. Despite use of several methods, including spin trapping, appropriate scavengers or product analysis, the nature and extent of intermediates in Fenton or Haber–Weiss reactions are not clear [7]. Nonetheless, pMTa4 DNA molecule clearly show that qualitatively different extent of strand breaks were induced by Fenton (Figure 1), Haber–Weiss (Figure 2) and γ-ray (Figure 3) mediated oxidants in the experimental system used in our investigation. Damage to bases of DNA can potentially cause strand breaks [1,10,12]. We have chosen the minimum concentration of reactants in Fenton and Haber–Weiss reactions to get measurable strand breaks on pMTa4 DNA molecule in this investigation. Even then, we see that while Fenton oxidant completely converted the CC form of pMTa4 to either OC or L forms or degraded it in about 40 min (Figure 1). Haber–Weiss oxidant did not do so (Figure 2). γ-rays, which randomly damage DNA by direct and indirect actions, also did not completely convert the CC form (Figure 3). Further, strand breaks induced by γ-rays following Fenton (Figure 4) or Haber–Weiss oxidant (Figure 6) primed pMTa4 essentially exhibited the trend of γ-ray induced damage. Even reversal of this protocol, that is, γ-ray primed pMTa4 exposed to either Fenton (Figure 5) or Haber–Weiss oxidant (Figure 7) exhibited the trends of damage resembling that of only Fenton or Haber–Weiss oxidant, respectively. These results suggest that at least in an *in vitro* system devoid of any repair pathway, the

damage to DNA is dictated by the secondary or final damaging entity and not the primary ones. Use of metal chelator, EDTA, or radical scavenger, EtOH, in our experimental system significantly, but not completely, abolished induction of strand breaks, especially DSB (M.M. Odyuo and R.N. Sharan—unpublished results). More work is under progress to further understand the response of genetic materials to damaging effects of ˙OH and other ROS species generated by different chemical or physical systems.

Acknowledgements

The authors gratefully acknowledge receipt of partial financial support for this work from CSIR, India in form of a doctoral fellowship to MMO.

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