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Artigo

PLASMID DNA DAMAGE INDUCED BY SINGLET MOLECULAR OXYGEN RELEASED FROM THE NAPHTHALENE ENDOPEROXIDE DHPNO$_2$ AND PHOTOACTIVATED METHYLENE BLUE

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PLASMID DNA DAMAGE INDUCED BY SINGLET MOLECULAR OXYGEN RELEASED FROM THE NAPHTHALENE ENDOPEROXIDE DHPNO$_2$ AND PHOTOACTIVATED METHYLENE BLUE. To investigate oxidative lesions and strand breaks induction by singlet molecular oxygen ($^1O_2$), supercoiled-DNA plasmid was treated with thermo-dissociated DHPNO$_2$ and photoactivated-methylene blue. DNA lesions were detected by Fpg that cleaves DNA at certain oxidized bases, and T4-endoV, which cleaves DNA at cyclobutane pyrimidine dimers and apurinic/apyrimidinic (AP) sites. These cleavages form open relaxed-DNA structures, which are discriminated from supercoiled-DNA. DHPNO$_2$ or photoactivated-MB treatments result in similar plasmid damage profile: low number of single-strand breaks or AP-sites and high frequency of Fpg-sensitive sites; confirming that base oxidation is the main product for both reactions and that $^1O_2$ might be the most likely intermediate that reacts with DNA.

Keywords: oxidative stress; DNA lesions; singlet molecular oxygen.

INTRODUCTION

Living cells are constantly exposed to potentially damaging reactive oxygen species (ROS), whose origin can be intracellular, such as those arising from normal cellular metabolism, and which includes the highly reactive hydroxyl radical ($^{\bullet}OH$) generated by the presence of iron intracellular (Fe^{2+}), the superoxide anion radical ($O_2^{\bullet-}$), and non-radical hydrogen peroxide (H_2O_2).[1] ROS can attack DNA and produce base oxidation besides DNA breaks. Moreover, nitric oxide (NO) and $O_2^{\bullet-}$ react to form peroxynitrite ($ONOO^-$), a potent genotoxic oxidant.[2] Furthermore, oxygen radicals generated during the reduction of O_2 can attack DNA bases or deoxyribose residues to produce damaged bases or strand-breaks. Alternatively, oxygen radicals can oxidize lipid or protein molecules to generate intermediates that react with DNA to form adducts.[3]

Singlet molecular oxygen ($^1O_2$) has also been identified as the ROS involved in numerous biological processes[4] and can be generated in photodynamic therapy, through a photo-sensitization type II mechanism.[5] Among other biological processes, $^1O_2$ is produced by neutrophils in phagocytosis[6] and by many enzymatic processes.[7] It should be added that $^1O_2$ is known to be mutagenic and genotoxic,[8-10] since it is able to react with DNA, thus leading to cell-killing and mutagenesis. In fact, DNA treated with different $^1O_2$ sources was found to contain single- and double-strand breaks,[11] as well as producing base damage which may promote *in vitro* DNA synthesis arrest.[12-14] As a consequence of DNA lesions, $^1O_2$ is proposed to be directly involved in degenerative processes such as cancer and aging.[15]

It was shown that, among the components of nucleic acids, $^1O_2$ oxidizes guanine (G) almost exclusively, thereby generating 8-oxo-7,8-dihydroguanine (8-oxoG) lesions,[16] besides others. Curiously, this ubiquitous oxidation product from guanine is much more susceptible to the oxidizing action of $^1O_2$ than its normal precursor, with the possible formation of other oxidized lesions *in vivo*.[5] However, 8-oxoG cannot be considered as a specific biological marker of $^1O_2$, since this DNA lesion can be formed under various conditions of oxidative stress, including those generated by the one-electron process, hydroxyl radical and Fenton-type reactions.[16] It may be pointed out that the delineation of biochemical features of secondary oxidation products of 8-oxoG, including mutagenicity and DNA-repair substrate specificity, has recently received major attention.[5,17]

The excision of some of these lesions in DNA was first demonstrated in *Escherichia coli* (*E. coli*) where a *mutM* gene product, also named Fpg (formamidopyrimidine-DNA glycosylase), removes 8-oxoG paired with cytosine (C), while MutY excises an adenine (A) mispaired with 8-oxoG.[18,19] Another important protein involved in the protection of DNA is MutT which eliminates 8-oxo-7,8-dihydro-2'-dGTP (8-oxodGTP), formed spontaneously by oxidation of dGTP. This process cleans away the nucleoside pool of the oxidized form of guanine nucleoside, thus avoiding incorporation of the damaged base into DNA by replication.[20,21]

More recently, a comprehensive mechanistic study was undertaken using the *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide 1,4-endoperoxide (DHPNO$_2$) or the [^{18}O]-labeled endoperoxide of *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide (DHPN^{18}O$_2$), in order to generate, under mild conditions, either unlabeled or [^{18}O] enriched $^1O_2$.[22] These compounds are clean generators of $^1O_2$ species which are released by thermo-dissociation (Figure 1). These studies made it possible to track the fate of oxygen atoms in several reactions between $^1O_2$ and nucleic acids. The main oxidation products of 8-oxoG detected in nucleosides when using an accurate HPLC-ESI-MS/

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MS method were, in addition to previously identified oxazolone (dZ) and imidazolone (dIz), the two diastereomers of spiroiminodihydantoin (dSp). Moreover, the $^1O_2$ oxidation of an 8-oxoG residue inserted into a single-stranded 15-mer oligonucleotide was found to generate oxaluric acid.[23] It may be added that the formation of secondary $^1O_2$ oxidation products of 8-oxoG, including spiroiminodihydantoin and oxaluric acid that were characterized in nucleosides and oligonucleotide, respectively, have not yet been found in cellular DNA.[5]

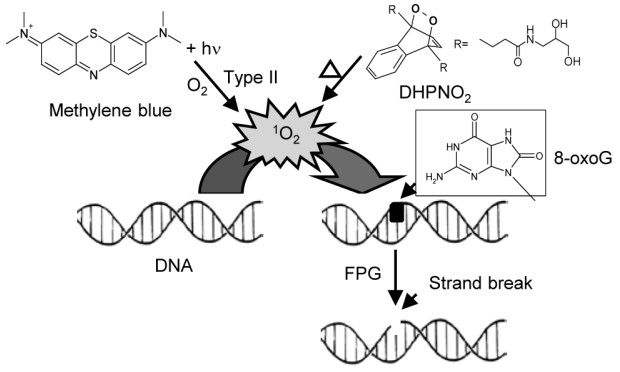

Figure 1. *Schematic representation of singlet molecular oxygen generation ($^1O_2$) by methylene blue photosensitization (type II) or endoperoxide (DHPNO$_2$) thermolysis and DNA oxidation by $^1O_2$, leading to the formation of base oxidation, as for example 8-oxoG, that is recognized by FPG enzyme that cleaves DNA in the site of the lesion*

With the aim of complementing the study of lesions in DNA damaged by $^1O_2$, we treated supercoiled-DNA plasmid with DHPNO$_2$. This is the first time DHPNO$_2$ was analyzed for determining the amount and type of DNA damage produced in plasmidial DNA. Furthermore, the results were compared with the effect of methylene blue (MB) photoactivated on these DNA molecules. MB is a photosensitizer widely used for the photodynamic therapy of cancer[24] and is well known to also generate $^1O_2$ by the photo-sensitization type II mechanism (Figure 1). After thermo-dissociation of either DHPNO$_2$ or MB photoactivation, samples were digested with bacterial and phage endonucleases. The data basically show that most of the lesions probably induced by $^1O_2$ are oxidized and altered bases, including 8-oxoG, in a frequency much higher than breaks or alkali-labile sites. These results also show that this simple and easy methodology can be used to analyze oxidative stress caused in DNA by oxidative agents.

EXPERIMENTAL

DNA treatment with DHPNO$_2$

The water-soluble non-ionic endoperoxide DHPNO$_2$ was prepared by methylene blue-mediated photo-sensitization of DHPN as reported previously.[25] The plasmid DNA used was pBluescript SK (Stratagene, CA, USA) that has 2958 bp. Plasmid DNA was prepared using plasmid mega kits (Plasmid/Midi-Qiagen Inc., USA) according to manufacturer's instructions. Each sample of 50 ng/μL of plasmid in a sodium phosphate buffer (50 mM, pH 7.4) was incubated for 90 min at 37 °C in the presence of various concentrations of DHPNO$_2$. Incubation was performed by soft-shaking every 10 min so as to supply oxygen for the reaction.

DNA treatment with MB

Each sample of pBluescript SK plasmid DNA (225 ng/μL) was incubated with MB (Sigma-Aldrich Chemical Company, USA) in a final concentration of 10 μM. MB was purified through recrystallization in ethanol. White light from two 15 W Phillips lamps (with emission between 400-700 nm), situated 10 cm above the samples was used to photoactivate-MB. The fluence used was 0.31; 0.63; 1.27; 1.9 and 2.54 J/cm^2. The light dose was measured with a Power Max 500A laser meter (Molecton Detector, Inc, USA). The temperature of the system was monitored during the irradiation but no heating effect was observed. The reaction was stopped by placing the mix on ice in the dark.

DNA damage detection

Immediately after treatment, samples were incubated with two different enzymes. One was T4 PDG (pyrimidine dimer glycosylase) endonuclease V (T4-endoV), prepared in our laboratory from an *E. coli* strain carrying a plasmid encoding the phage *T4 DEN V* gene,[26] and the other formamidopyrimidine [fapy]-DNA glycosylase (Fpg) (from New England BioLabs, Inc).[27]

For the digestion assay, 100-200 ng of plasmid DNA was diluted in the corresponding enzyme buffer. For T4-endoV, this was 10 mM Tris-HCl, 10 mM EDTA and 100 mM NaCl, pH 8.0, and for Fpg, 40 mM Tris-HCl, 2 mM EDTA and 100 mM KCl, pH 8.0. The enzymes were added (0.8 U/reaction of Fpg and 70 ng/reaction of T4-endoV) and the samples incubated for 30 min at 37 °C for digestion. The enzymes were tested up to saturation, so that all lesions were cleaved, but in amounts where no non-specific cleavage was observed.[28] Damaged plasmid DNA samples were subject to electrophoresis in 0.8% agarose gels already stained with ethidium bromide (0.5 μg/mL) in a Tris-borate buffer (pH 7.5). After migration, the DNA was visualized under UV light using an Image Quant 300 (GE Healthcare, USA), to obtain digital images of the stained gels. Fluorescent band intensities were determined using Image Quant-RT ECL Capture software (GE Healthcare, USA).

DNA cleavage was determined by measuring conversion from the supercoiled form (form I) to the relaxed form (form II). On assuming Poisson damage distribution, the average number of enzyme sensitive sites per plasmid is given by the expression X = -ln (1.4 x SC/(1.4 x SC + OC)), where SC and OC are the fluorescence of the supercoiled and relaxed forms, respectively, the factor 1.4 correcting for the difference in ethidium bromide binding to supercoiled compared to relaxed-DNA.[11,29] As a control group for linear-DNA (form III), plasmid was digested with *Bam*HI enzyme (Promega, Southampton, UK) for 1 h at 37 °C. Note that pBluescript SK contains a unique *Bam*HI restriction site.

RESULTS AND DISCUSSION

The detection of DNA damage induced by plasmid treatment with DHPNO$_2$ is illustrated in Figure 2A with one representative experiment, and summarized after quantification in Figure 2B. The endonucleases employed in this work permitted detecting base damage. In fact, upon lesion recognition T4-endoV and Fpg cleave one strand of the DNA molecule, this resulting in the generation of form II (open relaxed circle) from form I (supercoiled-DNA) plasmid DNA.

T4-endoV normally recognizes CPDs caused by ultraviolet (UV) irradiation and cleaves DNA by two-step activities, DNA glycosylase and AP-lyase activities. The enzyme cleaves the glycosyl bond of the 5' end of the CPD generating an AP site, whereas the endonucleolytic activity cleaves the phosphodiester bond of the DNA backbone at the AP site itself.[30] Thus, this protein also cleaves DNA that contains AP sites.

Fpg, also known as 8-oxoguanine DNA glycosylase, acts both as an N-glycosylase and an AP-lyase. N-glycosylase activity releases damaged purines from DNA, thus generating an AP site. The AP-lyase activity of Fpg cleaves both 3' and 5' to the AP site, thereby

removing this and leaving a one base gap. However, this enzyme has low specificity, some of the damaged bases recognized and removed by Fpg including 8-oxoG, 8-oxoadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapy-guanine), methy-fapy-guanine, 4,6-diamino-5-formamidopyrimidine (fapy-adenine), aflatoxin B1-fapy-guanine, 5-hydroxy-cytosine and 5-hydroxy-uracil.[31]

As shown in Figure 2, there is a small increase in the number of single-strand breaks (SSBs) by increasing concentration of $DHPNO_2$, even in the absence of enzymes. These results are in agreement with previous data indicating that $^1O_2$ generates low levels of breaks in the DNA chain.[11,32] Addition of T4-endoV leads to a further, but still small, increase in the number of SSBs. Since $^1O_2$ should not yield CPDs in DNA, this increase is most likely due to the presence of AP sites in the treated DNA, in agreement with the observation that $^1O_2$ induces strand breaks as well as alkali-labile sites (AP sites lead to breaks in alkali).[32] However, the most prominent effect is observed with Fpg endonuclease. Clearly, there is a strong endoperoxide dose-dependent increase in the number of Fpg-sensitive sites per plasmid molecule. The detection of these lesions increases at least up to 20 mM, where a *plateau* is observed. This *plateau* may simply indicate a limit in methodology, as at this dose or more, most of these molecules (close to 100%) are already in form II. These data strongly suggest that thermo-dissociation of $DHPNO_2$ can release mainly $^1O_2$, as previously described,[22] and thus generate base damages in double-stranded plasmid DNA. The great majority of these lesions are recognized by Fpg confirming the formation of potential oxidative base-damage.

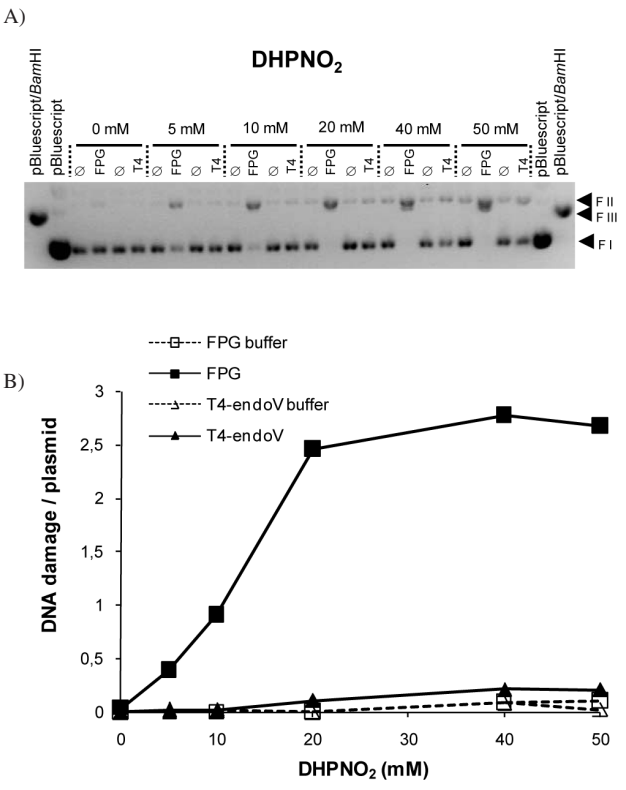

Figure 2. *DNA lesions induced in plasmid DNA treated with $DHPNO_2$. (A) Plasmid was incubated at 37 °C for 90 min with the indicated concentration of $DHPNO_2$, then digested with Fpg or T4-endoV at 37 °C for 30 min, and after analyzed by agarose gel electrophoresis. ∅: incubation with the corresponding enzyme buffer. (FI) supercoiled form; (FII) relaxed form; (FIII) linearised form. (B) DNA damage induced by $DHPNO_2$. Band intensity was determined for the quantification of damage, as described in Experimental. Values correspond to an average from two experiments*

Plasmid DNA damage detection, as a result of treatment with photoactivated-MB, is illustrated in Figure 3A and 3B with a representative experiment, and summarized after quantification in Figure 3C. As a control group, samples that were not photoactivated were kept in the dark during the same period of time used for photoactivation. Visibly, in the presence of Fpg, there is an increase in the amount of form II plasmid DNA in relation to form I, when the samples were incubated with MB plus light this increase depending on the intensity of photoactivation. In these experiments, the frequency of lesions generated was lower than what was observed for $DHPNO_2$, and did not reach a *plateau*. This indicates that in the tested conditions the relation between effect/damage provoked by MB photoactivation is lower than $DHPNO_2$ thermo-dissociation. However, it is important to emphasize that fluence rate of photoactivation may be the limiting factor, and higher fluence rate could be enough to generate the same effect/damage relation observed after $DHPNO_2$ thermo-dissociation.

Moreover, MB can aggregate according to some environmental characteristics as for example, salt concentration. The photochemical reactions are rather different between these two forms. While dimeric form can predominantly generate free radical species by primary reaction mechanism, monomeric form can mainly generate $^1O_2$ by secondary mechanisms in the presence of molecular oxygen (Figure 1).[33] In the MB concentration used in this work (10 μM), MB is most likely in the monomeric form,[33] thus, generating predominantly $^1O_2$ after photoactivation. Nevertheless, photo reduction of oxygen may be responsible for the production of some ROS, therefore in considering possible reactive species in photosensitized reactions, the participation of species such as H_2O_2, $O_2^{\bullet-}$ and $^\bullet OH$ must be considered along with $^1O_2$, and direct dye/substrate processes.[34] However, some authors have already described that the nicking rate observed in supercoiled plasmid DNA treated with extensive exposure to light in the presence of MB is not prevented by inhibitors of the iron-catalyzed Fenton reaction or by scavengers of hydroxyl free radicals.[35] These results suggest that $^\bullet OH$ are an unlikely cause of the DNA nicking observed under their tested conditions. In addition, the formation of 8-oxoG is not dependent upon the binding of MB to DNA, since this is formed in polydeoxyguanylic acid.[35] As expected, there is no Fpg-sensitive site detection in the dark. These data are in agreement with previous reports expressing that exposure to MB plus light mediates the formation of high levels of oxidative bases including 8-oxoG in DNA, thus indicating light dependence.[27,35-37]

As observed for treatment with $DHPNO_2$, the increase of MB light-exposure generated almost no increase in the number of SSBs, even in the absence of enzymes. Moreover, addition of T4-endoV leads to a further, although still small, increase in the number of recognition sites which may represent AP sites (Figures 3B and 3C). Nevertheless, there is no doubt that these damages are not the main products of the oxidative effects of $DHPNO_2$ thermo-dissociation and also for MB plus light treatment, as previously described.[35]

Interestingly, the reduction pathway leading to the formation of 8-oxodG is predominant in $^1O_2$ oxidation of double-stranded DNA, even in the absence of any added reducing agent.[16,36,38,39] It was also found that fapy-guanine, a degradation product that may be formed by hydration of radical guanine cation followed by the opening of the imidazole ring according to a reductive pathway, is not generated within isolated DNA,[5] at least in any detectable amount. These observations suggest that in these assays, lesion recognition by Fpg enzymes may include 8-oxoG. This is also in complete agreement with previous work disclosing that DNA treated with MB plus light yields more 8-oxoG than SSBs.[35] In contrast OhUigin *et al.*,[40] described SSBs formation after MB photoactivation, although these cleavages are only formed in guanine residues. They suggest direct electron transfer between MB and DNA as responsible for strand breakages especially in anoxic solutions, which is not the case here as oxygen is present in our reactions. As is

shown in this work, results with $DHPNO_2$ were similar to those of MB plus light, the number of Fpg sensitive sites exceeding the number of SSBs or AP-sites at least 20-fold.

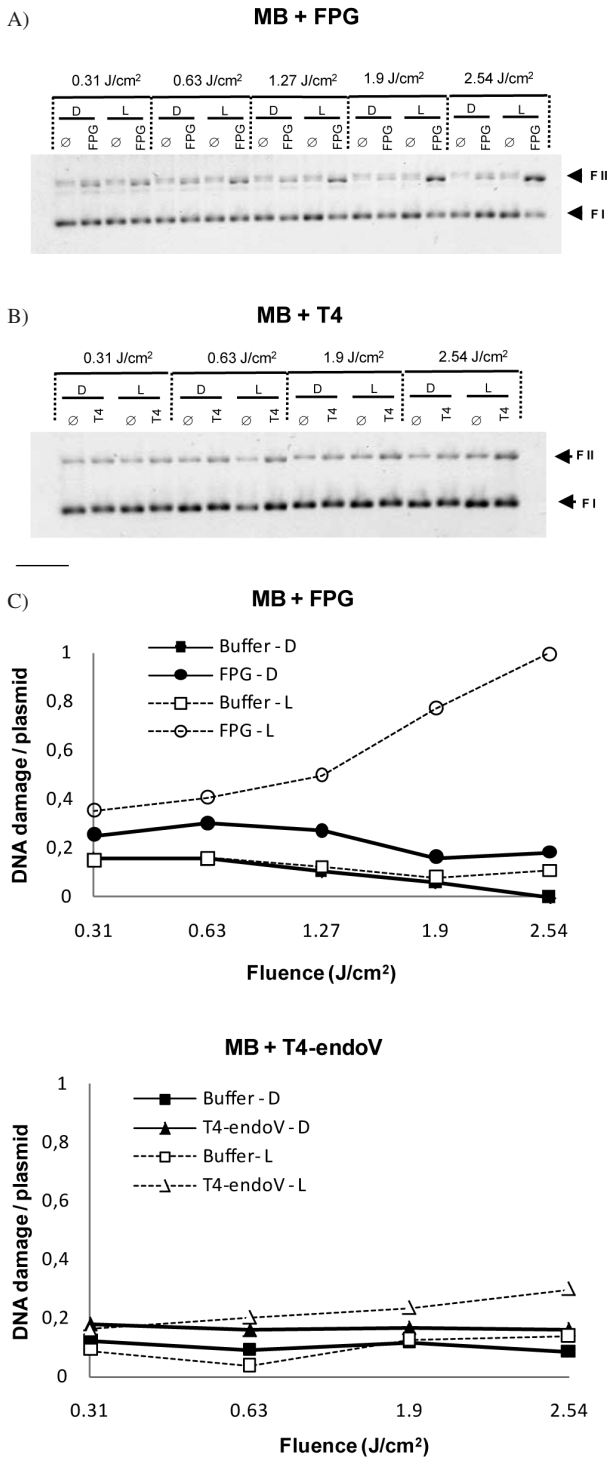

Figure 3. *DNA lesions induced in plasmid DNA treated with photoactivated-MB. Plasmid was incubated with 10 μM MB, then photoactivated (L) or not (D). After digestion with Fpg (A) or T4-endoV (B) at 37 °C for 30 min, samples were analyzed by agarose gel electrophoresis. ∅: incubation with the corresponding enzyme buffer. (FI) supercoiled form; (FII) relaxed form. (C) DNA damage induced by photoactivated-MB. Band intensity was determined for the quantification of damage, as described in Experimental. Values correspond to an average from two experiments*

On the other hand, when using Fpg and gas chromatography/mass spectrometry, Boiteux *et al.*,[18] demonstrated that DNA treated with MB plus light generated fapy-guanine and 8-oxodG, both well-known substrates of Fpg. But the amount of induced fapy-guanine was approximately 20-fold less than that of 8-oxoG. An interesting possibility to be further explored is that the less frequent lesions detected in this work (SSBs and T4-endoV sensitive sites - AP sites) may have been generated due to a secondary oxidation reaction between $^1O_2$ and 8-oxoG. However, the biological relevance of these lesions remains to be established.

CONCLUSION

The data presented here confirm that oxidative base damages are the predominant product of the reaction of DNA with both photoactivated-MB and the novel endoperoxide $DHPNO_2$. It was the first time that thermo-dissociation of $DHPNO_2$ was analyzed for determining the amount and type of DNA damage produced in DNA plasmid digested with specific enzymes which detect specific DNA lesions. The type of damage detected is similar (Fpg-sensitive sites) after both $DHPNO_2$ thermo-dissociation and MB photoactivation treatments. This suggests that $^1O_2$ is possibly the main intermediary product responsible for DNA damages induced by both $DHPNO_2$ and photoactivated-MB. Moreover, this methodology is an appropriate, easy and fast methodology that can be used in analysis of oxidative effect of some oxidative DNA damage agents.

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