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Facts and Artifacts in the Measurement of Oxidative Base Damage to DNA

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This short survey is aimed at critically evaluating the main available methods for measuring oxidative base damage within cellular DNA. Emphasis is placed on separative methods which are currently widely applied. These mostly concern high performance liquid chromatography (HPLC) and gas chromatography (GC) associated with sensitive detection techniques such as electrochemistry (EC) and mass spectrometry (MS). In addition, the comparison is extended to ^{32}P-postlabeling methods, immunoassays and measurement of two main classes of oxidative DNA damage within isolated cells. It may be concluded that the HPLC–electrochemical detection (ECD) method, even if restricted to the measurement of only a few electroactive oxidized bases and nucleosides, is the simplest and safest available method at the moment. In contrast, the more versatile GC–MS method, which requires a HPLC pre-purification step in order to prevent artifactual oxidation of overwhelming normal bases to occur during derivatization, is more tedious and its sensitivity may be questionable. Alternative simpler procedures of background prevention for the GC–MS assay, which, however, remain to be validated, include low-temperature for derivatization and addition of antioxidants to the silylating reagents. Interestingly, similar levels of 8-oxo-7,8-dihydroguanine were found in cellular DNA using HPLC–ECD, HPLC–MS/MS and HPLC/^{32}P-postlabeling methods. However, it should be noted that the level of cellular 8-oxodGuo, thus determined, is on average basis 10-fold higher than that was inferred for more indirect measurement involving the use of DNA repair enzymes with methods on isolated cells. Further efforts should be made to resolve this apparent discrepancy. In addition, the question of the biological validation of the non-invasive measurement of oxidized bases and nucleosides in urine is addressed.

Keywords: DNA base lesions, DNA damage measurement, non invasive assays

Abbreviations: dGuo, 2′-deoxyguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxoAde, 8-oxo-7,8-dihydroadenine; 5-OHCyt, 5-hydroxycytosine; 5-HMUra, 5-(hydroxymethyl)uracil; CE, capillary electrophoresis; GC–MS, gas chromatography associated with mass spectrometry detection; HPLC–ECD, high performance liquid chromatography coupled to electrochemical detection; HPLC–MS/MS, high performance liquid chromatography associated with tandem mass spectrometry detection

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INTRODUCTION

Oxidized bases represent one of the major classes of DNA lesions induced by •OH radicals, hydrogen peroxide, ozone, peroxynitrite, singlet oxygen or one-electron oxidation reactions (for recent comprehensive reviews, see [1,2]). In this respect, more than 60 different modified nucleosides including diastereioisomeric forms have been characterized from detailed model studies. However, there is still a paucity of accurate data on the formation of the latter lesions in cellular DNA upon exposure to a specific physical or chemical oxidizing agent.[2,3] In addition to the still pending validation of the oxidation chemistry of DNA within cells, there is an increasing interest devoted to the assessment of the biological role of oxidative base damage. To achieve such an aim, one requisite is to be able to measure the level of modified bases within cellular DNA under different conditions of chronic or acute oxidative stress. Another important biochemical parameter is the rate of repair of specific base damage which may be inferred from the kinetics of disappearance of the lesions with increasing periods of time following initial DNA oxidative injury.[4] Therefore, major efforts were made during the last decade to promote various chemical and biochemical assays aimed at singling out oxidative base damage to DNA within cells or individuals.[5–8] As two pioneering contributions to the field, we may mention the high performance liquid chromatography–electrochemical detection (HPLC–ECD) for monitoring the formation of 8-oxo-7,8-dihydroguanine (8-oxoGua)[9] and the gas chromatography–mass spectrometry (GC–MS) method aimed at measuring a wide range of modified bases.[10] The two assays have received numerous applications during the last twelve years, some of them are summarized in recent review articles.[3,11–14] However, the validity of most of the earlier GC–MS measurements has been questioned since the levels of 8-oxoGua was found on the average basis to be 10- to 50-fold higher[15,16] than those determined by the accurate HPLC–electrochemical assay. It was shown by several groups that this discrepancy can be rationalized in terms of artifactual oxidation of the overwhelming guanine residues during the silylation step prior to the GC analysis.[17–20] The latter observation was subsequently extended to other DNA oxidized bases including 5-hydroxycytosine (5-OHCyt), 5-(hydroxymethyl)uracil (5-HMUra) and 8-oxo-7,8-dihydroadenine (8-oxoAde).[21] An accurate way to get rid of the artifactual oxidation of normal DNA constituents is to pre-purify the damage of interest prior to the derivatization step. Other possibilities involve lowering of the temperature of derivatization and the addition of antioxidants to the silylating reagent.[22] Another drawback which is further discussed together with major requirements such as the use of [M + 4] isotopically labeled internal standards, deals with the instability of several modified bases under the acid conditions usually applied to hydrolyze DNA prior to the GC–MS analysis.[23,24] Information on recent developments of the highly sensitive ^{32}P-postlabeling method is also provided. It was found that either thin-layer chromatography (TLC) or HPLC pre-purification of the 8-oxo-7,8-dihydro-2′-deoxyguanosine 3′-monophosphate (8-oxo-dGMP-3′) prior to polynucleotide kinase-mediated phosphorylation led to a significant decrease in the level of the oxidized nucleotide.[25–29] Interestingly, the value of 8-oxo-dGMP-3′ measured in control cellular DNA is similar to the level of 8-oxodGuo obtained either by applying the accurate HPLC–ECD method[25] or the promising HPLC–MS/MS assay.[30] However, the latter values of 8-oxodGuo are still, on an average basis, higher by factors of 3 and 10 compared to those inferred from the use of the formamidopyrimidine glycosylase (Fpg) repair enzyme associated with either the comet assay or the alkaline elution technique (*vide infra*). Finally, information is provided on non-invasive methods aimed at measuring oxidized bases and nucleosides in urine.

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MEASUREMENT OF OXIDATIVE BASE DAMAGE USING CHROMATOGRAPHIC METHODS

The bulk of the available methods aimed at measuring individual DNA base lesion requires either chemical hydrolysis or enzymatic digestion of oxidized DNA subsequent to its extraction from cells or tissues.[6] As it will be discussed later more specifically, several drawbacks may occur during the latter initial steps. These involve artifactual oxidation of the overwhelming normal purine and pyrimidine DNA components due to the occurrence of Fenton type reactions. In addition, enzymatic digestion of modified nucleosides or nucleotides may be not quantitative, whereas conditions of acid hydrolysis may promote the decomposition of unstable compounds. Then, the complex mixture of released DNA components is usually resolved by efficient separative methods. These include HPLC, GC and capillary electrophoresis (CE) which have to be associated with a sensitive and specific method of detection. The limit of sensitivity which is required at the ouput of the column should be, at least, 1 lesion per 10^6 normal bases in a sample size of less than 50 μg of DNA. Other difficulties to be overcome, deal with the multiplicity and, sometimes, the lability of the lesions.

HPLC–ECD Methods

As already mentioned, the measurement of 8-oxodGuo which requires the association of an amperometric detection with an efficient HPLC separation on octadecylsilyl silica gel columns has been introduced by Floyd *et al.*[9] This represents a simple and accurate method to measure 8-oxodGuo. This has been extended to a few other electroactive oxidized lesions including 8-oxoAde, 5-OHCyt, 5-hydroxyuracil and the corresponding 2′-deoxyribonucleosides.[31,32] The sensitivity of the assay, which is within the subpicomole range for a DNA sample size of, at least, 20 μg, has been improved with the advent of the coulometric detection technique. Usually, oxidized nucleosides which are more retained on the ODS columns that the related bases are measured by the HPLC–ECD assay. This allows a better separation of the targeted lesion from electroactive contaminants which are eluted near the void volume of the HPLC column. The quantitative aspect of the enzymatic release of 8-oxodGuo from DNA has been questioned as a possible source of underestimation of the oxidized nucleoside. In fact, it was shown from various studies including time course experiments with increased quantities of exonuclease and comparison with acidic hydrolysis that the nuclease P1-mediated release of 8-oxodGuo was complete.[24,33]

A recent improvement in the quantitative accuracy of the overall HPLC–ECD assay is the use of 2,6-diamino-8-oxopurine as an internal standard.[34] It should be pointed out that no major drawback is associated with the application of HPLC–ECD unless the chromatographic system including the loop injector and the column have been previously contaminated with relatively large amounts of the compounds to be measured. The main pending problem to be resolved is that of the extraction of DNA and its subsequent work-up, which is also shared by other chromatographic methods including GC–MS and HPLC–^{32}P-postlabeling. Further efforts are required to prevent more efficiently Fenton type reactions from occurring during the steps preceding the HPLC or GC analysis. Interestingly, a significant lowering in the level of 8-oxodGuo in control cellular DNA, as inferred from HPLC–ECD measurement, was recently reported.[35] This was achieved using desferoxamine in order to chelate transition metals together with the chaotropic NaI extraction method. It was also shown that a critical parameter to lower artifactual oxidation is the amount of DNA to be extracted which has to be, at least, 100 μg.[35]

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Artifacts of GC–MS Method and Improved Assays

The coupling of a mass spectrometer operating in the electron impact ionization mode to the GC technique constitutes a powerful and versatile method for monitoring the formation of oxidized bases within cellular DNA. Interestingly, the sensitivity of the assay is significantly enhanced by applying the selective ion monitoring detection mode. Usually, the bases rather than the nucleosides are analyzed by this method, mostly for reasons of higher sensitivity.[12] Less fragmentation is observed during the GC–MS analysis for the former compounds with respect to the corresponding 2′-deoxyribonucleosides. It should be noted that derivatization, usually silylation, of the modified bases is required to make the compounds volatile for the GC–MS analysis. Unfortunately, the method suffers from several drawbacks as already discussed in several recent review articles. The major flaw in the application of the earliest version of the GC–MS assay deals with the overestimation of the values of the oxidized bases. Thus, on average, the level of 8-oxoGua in control samples of cellular DNA was found to be higher, by a factor of at least 10–50 when compared to that measured by HPLC–ECD.[16] It was shown and there is now a large consensus on this observation that the higher values of the GC–MS analysis are mostly explained by the occurrence of an artifactual oxidation of the overwhelming unmodified guanine bases during the silylation reaction of the DNA acidic hydrolysate.[17,18] In fact this oxidation reaction which was already observed during the derivatization of 5,6-dihydropyrimidine bases, almost 20 years ago, also occurs to other DNA bases in nitrogen purged solutions. Therefore, 8-oxoAde, 5-OHCyt, 5-formyluracil and 5-HMUra were found to be generated during the silylation of adenine, cytosine and thymine respectively.[21] A suitable way to prevent the formation of artifactual background is to prepurify by HPLC the targeted lesion prior to the derivatization reaction.[18,20,21] Interestingly, comparison of HPLC/GC–MS and HPLC–ECD measurements of acidic hydrolysates of calf thymus DNA gives rise to similar values of 8-oxoGua, 8-oxoAde and 5-OHCyt respectively.[20,21] Other means of preventing artifactual oxidation of normal bases during the derivatization step have been proposed. These include application of room temperature for the silylation of bases in the whole acidic hydrolysate[17] and/or addition of antioxidants such as ethanethiol[22] or *N*-phenyl-1-napthylamine[37] in the derivatization mixture. The use of guanase[38] is another possibility to remove guanine which however has been questioned. It should be pointed out that the validation of the latter analytical and biochemical approaches is still pending. This could be achieved by comparing the results of the latter methods with those involving the HPLC pre-purification step. Other sources of possible drawbacks have been identified. One deals with the lack of utilization of suitable internal standards until 1993[12] or still in some current analyses. It was found that 8-azaadenine which is used as an internal standard is unstable under conditions of formic acid hydrolysis of DNA,[23] thus preventing accurate quantitative determinations. It should be added that formamidopyrimidine derivatives of guanine[23,24] and to a lesser extent of adenine[23] were found to undergo partial cyclization under conventional HCOOH treatment. This is likely to induce flaws in the GC–MS measurement particularly when the [M+4] isotopically labeled Fapy derivatives are not used as internal standards.[20,24] Interestingly, HF-pyridine is an appropriate substitute to HCOOH hydrolysis for inducing the quantitative release of Fapy derivatives under mild conditions.[24] Therefore, in light of the above observations, three major requirements have to be fulfilled to achieve accurate GC–MS measurements. These include: optimized conditions of acidic hydrolysis of DNA which have to be checked specifically for each modified base, HPLC pre-purification of

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the target lesion(s) and the use of [M + 4] authentic standards.

Improved ^{32}P-postlabeling Assays for Measuring 8-oxo-7,8-dihydro-2′-deoxyguanosine 3′-monophosphate

Major efforts have been made in recent years to develop ^{32}P-postlabeling assays for monitoring the formation of 8-oxodGuo. The method, which is one of the most sensitive analytical approaches for measuring a single base damage in cellular DNA, is based on the enzymatic radioactive postlabeling of nucleoside 3′-monophosphates.[39] On average, the sensitivity of the method is, at least, of one modification per 10^7 normal bases in a sample size of a few μg of DNA. However, application of the assay to the measurement of oxidized nucleotides may also be faced with artifactual oxidation of the overwhelming normal DNA components. In this respect, polynucleotide kinase-mediated phosphorylation of the bulk of micrococcal nuclease hydrolysate of control DNA was found to give rise to levels of 8-oxodGMP in the range of, at least, 3 modifications per 10^4 normal nucleotides.[40,41] These high values were diagnostic of the occurrence of artifacts as the result of both self-radiolysis and autoxidation of dGMP. Pre-purification of 8-oxodGMP by either TLC, HPLC or capillary electrophoresis (CE)[25–29] allows the measurement of levels of 8-oxodGMP in cellular DNA which are similar to those determined by HPLC–ECD. The measurement of adenine N-1-oxide in cellular DNA exposed to hydrogen peroxide constitutes another example of the application of the HPLC–^{32}P-postlabeling method.[42] Efforts are currently made to use an internal standard in the assay in order to make it more quantitative.

ASSAYS INVOLVING THE USE OF DNA REPAIR GLYCOSYLASES

A different strategy than those requiring DNA extraction involves single cells and the use of DNA repair enzymes. For this purpose, either the comet assay (single cell electrophoretic method)[43] or sedimentation techniques including the alkaline elution assay[44] and the alkaline unwinding method[45] are available. As common features of the assays, the cells are lysed *in situ* and the modified bases are converted in a relatively specific way into strand breaks upon incubation of DNA with repair enzymes.

Alkaline Elution Technique Associated with DNA Repair Endonucleases

An optimized version of the alkaline elution technique including the use of the Fpg protein, endonuclease III (Endo III) and exonuclease III as DNA repair enzymes is now available.[7] This allows the measurement of modified purine bases, oxidized pyrimidine bases and abasic sites respectively. One of the major observations, which also applies to the comet assay (*vide infra*) and the alkaline unwinding method, is that the steady-state level of the two main classes of DNA base damage is very low. Particularly striking is the number of Fpg sensitive sites which was found to be at a level of between 0.08 and −0.25 lesion per 10^6 bp depending on the cells.[13] Interestingly, the lesions recognized by the Fpg protein are mostly 8-oxodGuo and to a lesser extent Fapy derivatives and abasic sites.

Alkaline Comet Assay Involving the Inclusion of DNA Repair Endonucleases

The alkaline comet assay has received numerous applications, at least, for monitoring the formation of chemically generated strand breaks and alkali-labile sites, together with enzymatic cleavages of DNA mediated by DNA repair proteins. The method is highly sensitive but, however, suffers from a lack of specificity. In this respect, the assay was significantly improved by the introduction of DNA repair enzymes aimed at targeting, at least, two classes of modified bases. Typically, this involves the incubation of DNA

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with either Fpg protein or Endo III subsequent to the lysis of isolated cells on agarose gel.[8,46] As already mentioned, the level of both Fpg and Endo III sensitive sites within cellular DNA is about 10-fold lower than that inferred from HPLC–ECD. Thus, in a recent study on the effects of ionizing radiation on lymphocytes, the level of 8-oxodGuo in control DNA was estimated to be as low as $0.14/10^6$ bp. This is in agreement with similar values in DNA of monocytes reported by others using the alkaline elution technique ($0.24/10^6$ bp)[13] and the comet assay ($0.14/10^6$ bp)[19] in association with the Fpg protein. An interesting application of the DNA repair enzyme–comet assay allowed the detection of both Endo III and Fpg sensitive sites in the DNA of monocytes exposed to γ-rays at a dose as low as 2 Gy.[47] In contrast, at least, 80 Gy are required to observe a significant increase in the level of 8-oxodGuo using the HPLC–ECD method of measurement. This apparent discrepancy is likely to be explained in terms of higher level of artifactual background in the HPLC–ECD method with respect to that of the comet assay. It may be suggested that autooxidation processes are expected to be minimized in both the comet and alkaline elution assays. Another possibility provided by the comet assay is the use of specific immunofluorescent detection against a targeted base lesion. This was recently illustrated by the immunofluorescent detection of cyclobutadithymine in UV-irradiated cells through a double labeling staining experiment.[48] Further work is aimed at extending the application of the assay to the measurement of oxidative DNA damage as soon as highly specific antibodies are available.

NON-INVASIVE ASSAYS

Significant efforts were made during the last decade to develop non-invasive assays for assessing the effects of oxidative stress on DNA in humans and animals (for a comprehensive review, see Ref. [49]). Emphasis was placed on the measurement of excreted oxidized bases and 2′-deoxyribonucleosides in urine as bioindicators of DNA injuries using mostly accurate HPLC–ECD and GC–MS assays. In this respect, several oxidized DNA compounds including 8-oxoGua,[50,51] 5-HMUra and their corresponding nucleosides[52–54] were measured. One major difficulty encountered with the application of the currently available assays is that, in most cases, a tedious pre-purification step of the targeted lesions from urine is required prior to either the HPLC–ECD or GC–MS analysis. This makes difficult the analysis of the large numbers of samples usually associated with epidemiological studies. It should be added that the biological validation of these non-invasive assays remains to be established. One way to approach the problem would be the simultaneous measurement of several oxidized bases and nucleosides in biological fluids[55] together with the determination of the level of related modifications in cellular DNA. The use of the promising HPLC–MS/MS method with the electrospray ionization source[30] should be very useful in this respect.

CONCLUSION

The measurement of oxidative base damage in cellular DNA remains, at least, partly an analytical challenge. This is mostly due to the possible occurrence of artifactual oxidation of the normal DNA constituents at different steps of the experimental protocols of the chromatographic assays. A major source of errors has been recently identified in the GC–MS and ^{32}P-postlabeling assays respectively. In both cases, the lack of prepurification of the targeted lesion to be measured prior to either the GC–MS analysis or the polynucleotide kinase-mediated phosphorylation leads to an overestimation of the levels of oxidized bases. The extent of the overestimation, which is between 10- and 50-fold with respect to the average values provided by HPLC–ECD, is likely to be further increased by, at least, a factor

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of 5 if the background associated with DNA extraction and subsequent work-up is considered. Interestingly, there is a general agreement on the fact that pre-purification is necessary for preventing artifactual oxidation to occur in the ^{32}P-postlabeling methodology. Unfortunately, this does not apply to the GC–MS measurements since data obtained using this questionable assay are still reported in the literature. This is illustrated in particular by the claim that excess of supplementation with vitamin C may have pro-oxidant effects as inferred from the overestimation of the level of 8-oxoAde in the DNA of human lymphocytes.[56] Another questionable result deals with the huge value of FapyGua, close to 1 lesion per 10^2 bp, in lymphoma L5178Y (LY-S) cells exposed to 400 Gy of γ-radiation.[57] In addition, 1.7 8-oxoGua residue per 10^4 bp was measured in the same irradiated cells, which is ten times the value that was determined in human monocytes using the HPLC–ECD assay. Other inconsistency concerns the 50-fold decrease in the level of FapyGua in the LY-R sublines which was explained by differences in their cellular redox status with respect to that of LY-S cells! Other matters of concern are the high levels of $^{\bullet}$OH-mediated decomposition products of purine bases in the DNA of human blood cells which are the following: 0.7 8-oxoGua, 1 8-oxoAde, 1 FapyGua and 1.6 FapyAde residues per 10^4 bp[58] (Figure 1). This was achieved by derivatization of the DNA acidic hydrolysate at low temperature in the presence of ethanethiol prior to GC–MS analysis. Intriguing huge level values of modified bases were reported in the DNA of primary hepatocytes supplemented with 100 μM ferric nitrilotriacetate for 24 h.[59] For example, 3.2 8-oxoGua, 10 FapyAde, 4.1 5-HMUra and 4.0 5-OHCyt per 10^3 bp were measured by GC–MS after derivatization at low temperature. This clearly illustrates the strong need to further evaluate the new derivatization procedures aimed at preventing artifactual oxidation of normal bases during silylation.

A problem common to all chromatographic assays, which is, however, rather minor with respect to the background induced by derivatization (*vide supra*), concerns the prevention of autooxidation processes during DNA extraction and subsequent work-up. Interestingly, several groups are attempting to define conditions

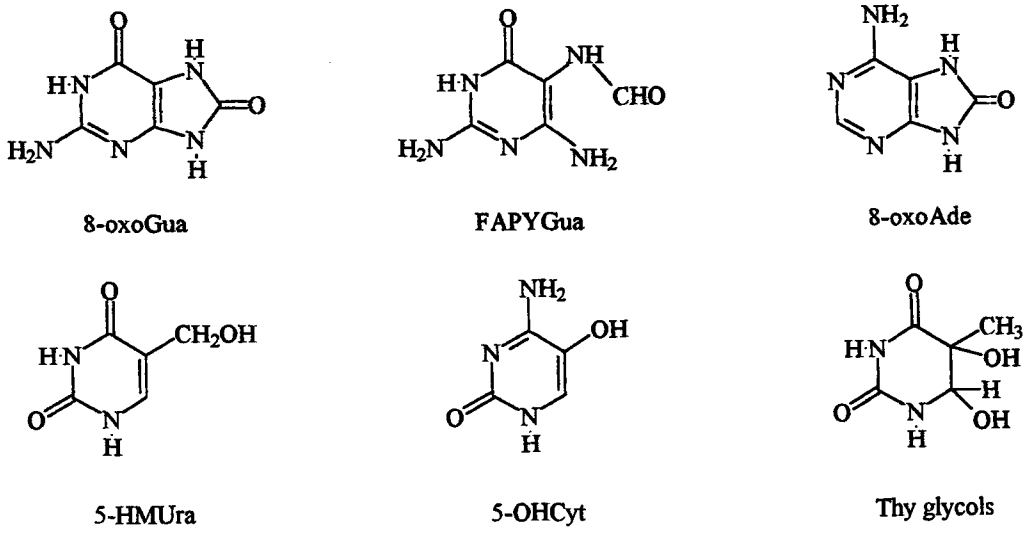

FIGURE 1 Chemical structure of some of the main $^{\bullet}$OH radical-mediated decomposition products of purine and pyrimidine DNA bases.

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where artifactual oxidation of the overwhelming DNA bases is minimized. In this respect, the use of an improved chaotropic NaI method was found to provide a very low value of steady-state level of 8-oxodGuo which is close to that inferred from other experiments including Fpg associated to the comet assay and the alkaline elution technique respectively.[35]

Further optimization and validation of the various chromatographic and enzymatic assays will require extensive and specific inter-laboratory trials. This could be achieved through the ESCODD group and/or direct cooperation between two or several groups. In addition to the already mentioned methods, we may note the emergence of two new promising assays. One concerns the HPLC–MS/MS technique which has shown its high potentiality to measure 8-oxodGuo in the DNA of pig liver and human urine.[30] The other deals with the advent of an ultrasensitive assay which couple the highly resolutive capillary electrophoresis technique to the immunofluorescence detection of 5,6-dihydroxy-5,6-dihydrothymine or thymine glycol.[60] The limit of detection of the assay for this major $^{\bullet}OH$-mediated oxidation product of thymine is about 2 lesions per 10^9 bp, which is by far less the most sensitive method currently available for monitoring oxidative base damage to DNA. Interestingly, exposure of A549 human lung carcinoma cells to 1 Gy of γ-rays resulted in the formation of 1.8 thymine glycol per 10^7 bp. This is similar to the value of radiation-induced 8-oxodGuo in the DNA of monocytes.[47]

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