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SHORT COMMUNICATION

# Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanine assays

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**The DNA base adduct, 8-hydroxyguanine (8-OHGua), has been reported to be a key biomarker relevant to carcinogenesis and cellular oxidative stress important in tumor promotion. Although investigators often report artificially high levels of 8-OHGua in DNA samples that have been exposed to phenol solutions and/or air during processing, few quantitative results are available. We show that routine phenol-based DNA purification procedures can increase 8-hydroxydeoxyguanosine (8-OHdG) levels 20-fold in samples that are exposed to air after the phenol is removed from the solutions. Surprisingly, air exposure alone accounts for a significant portion of this increase (4-fold) when compared to dG or DNA samples that have been solubilized in buffers purged with nitrogen. Most importantly, phenol treatments of DNA are shown to sensitize DNA to 8-OHdG formation by subsequent exposures to air. The sensitization of DNA occurs even though extensive dialysis is used between phenol treatment and enzymatic DNA digestion. Alternate procedures, including chloroform:isoamyl-alcohol extractions, also yield air-sensitive DNA samples. Other artifacts of organic extraction prior to air exposure include alterations in DNA base ratios after nuclease digestions. Overall, these results strongly suggest that studies of 8-OHdG in carcinogenesis should avoid dry conditions, such as lyophilization followed by exposure to air, and that all four of the bases should be monitored before 8-OHdG concentrations are normalized by undamaged deoxynucleoside concentrations. Failure to heed these precautions can lead to 2- to 20-fold overestimates of 8-OHdG in target tissues or *in vitro* models.**

Among a wide variety of 'biomarkers' that indicate target organ damage from reactive oxygen species (ROS*), 8-hydroxyguanine (7,8-dihydro-8-oxoguanine, 8-OHGua) has become increasingly popular as a sensitive, stable and integral marker of oxidative stress in cellular DNA (1,2). Evidence of 8-OHGua in tissues has been reported to be particularly correlated with carcinogenesis (1) and tumor promotion (3), as well as implicated causally in human breast cancer (4).

The widespread interest in using this DNA base product as a biomarker stems in part from the availability of sensitive and specific means of measuring 8-hydroxy-2′-deoxyguanosine (7,8-dihydro-8-oxo-2′-deoxyguanosine, 8-OHdG) in digests of DNA (5–7) or other biological specimens (8). New techniques, such as HPLC with electrochemical detection (LCED; 5) and gas chromatography–mass spectrometry (GC/MS; 6), have enabled quantitation of 8-OHdG in the femtomole range within DNA digests containing nanomoles of undamaged deoxynucleotides. The sensitivity and specificity of LCED for 8-OHdG places this technique among only a few assays that measure oxidative DNA base damage at environmentally relevant levels.

The measurement of trace substances in any experimental system includes the risk of measuring contaminants and/or artifically-induced concentrations of the monitored compound. In particular, if a given biomarker is a sensitive detector of ROS, then it is likely that the biomarker will be formed during any procedure that exposes the precursor compound to transition metals, oxygen and redox-active compounds. In the present communication, we report on a commonly-reported artifact in 8-OHdG studies: phenol purification of DNA under oxygen containing atmospheres.

For our studies on model DNA, we used classical DNA isolation and purification techniques involving phenol-based extraction of proteins (9; Claycamp and Ho, manuscript submitted). Phenol solutions, purified by distillation and preserved using 0.1% (w/v) of 8-hydroxyquinoline and 0.2% (v/v) β-mercaptoethanol, were equilibrated in Tris–hydroxyaminomethane: 10 mM, pH 8; EDTA: 1 mM; or, 'TE' buffer, and kept cold and in the dark until use (9). In some experiments, we replaced the EDTA in TE and TEN (TE buffer to which 100 mM NaCl has been added) buffers with diethylenetriaminepentaacetic acid (DTPA) for reasons discussed below. Prior to enzymatic digestion of DNA samples, the samples were dialyzed to equilibrium in the appropriate buffer (TEN or TDN) for at least two changes of 400 excess volumes. The spectrophotometrically pure, RNA-free DNA samples were enzymatically digested to deoxyribonucleosides using a mixture of DNase I and nuclease P1 at pH 7.0 for 1 h at 55°C. Phosphate residues were removed using both phosphodiesterase and bacterial alkaline phosphatase (BAP). After the enzymatic digestion of DNA to 2′-deoxynucleosides (dNs), ethanol precipitation (5:1 volumes) of viscous salts and proteins was performed followed by drying of the ethanol phase under air or nitrogen streams. Using a multiplace gas manifold and samples in 1.5 ml tubes, drying typically required 1–2 h. The dried dNs were suspended in HPLC mobile phase for injection into the system. Mass balances calculated between the chromatographically measured deoxynucleotides and the original DNA concentration have shown that we routinely recover >95% of the DNA using these procedures (unpublished observations).

Since we had previously observed a significant artifact induced by air-drying phenol-extracted DNA samples (Claycamp and Ho, manuscript submitted), we wished to determine whether or not air alone, could induce 8-OHdG formation. For these experiments, we dissolved 'off-the-shelf' deoxyguanosine in high purity $H_2O$ before drying aliquots under nitrogen or air. The dried samples were subsequently redissolved in water for analysis by LCED. Figure 1 shows the effect of air drying dG solutions on the estimates of 'background' 8-OHdG. It is apparent that air drying produced up to a 7-fold higher background in dG

*Abbreviations: ROS, reactive oxygen species; 8-OHGua, 8-hydroxyguanine; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; LCED, HPLC with electrochemical detection; GC/MC, gas chromatography–mass spectrometry; DTPA, diethylenetriamine-pentaacetic acid; BAP, bacterial alkaline phosphatase; dN, 2′-deoxynucleoside

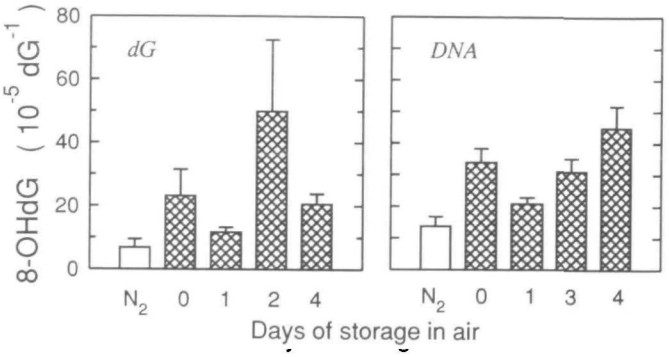


**Fig. 1.** Effects of drying deoxyguanosine or DNA digest samples on the yield of 8-OHdG. Left panel, a solution of 0.1 mM dG (Sigma Chemical Company) was prepared in high purity $H_2O$ and acid-washed glassware. Samples for LCED analysis were dried under $N_2$ in vials and either immediately resolubilized ('$N_2$') or exposed to air at −20°C for up to 4 days after drying ('air, 0−4') prior to resolubilization and analysis by LCED. Right panel, ethanol-extracted deoxynucleosides from DNA digests were dried under $N_2$ and either resolubilized immediately or after up to 4 days exposure to air. Data are means ± SEM ($n$ = 3).

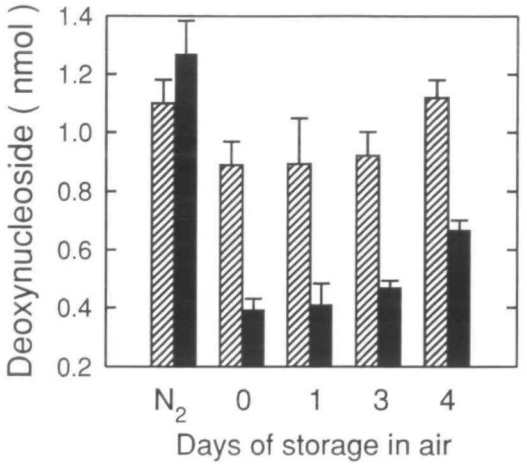


**Fig. 2.** *Differential effects of air exposure on the dG and dT concentrations* in air-exposed DNA digests. The 'undamaged' dG (diagonally-filled bars) and dT (solid bars) quantity in the LCED injectate corresponding to the DNA experiment in Figure 1 are shown. Air exposure significantly lowered the recoverable dT in these dried digests. The putative dG:dT ratio is 0.8.

samples compared with samples that were dried in $N_2$ prior to resolubilization and analysis. Results in Figure 1 show that air exposure not only increased the absolute magnitude of 8-OHdG in the samples, but they also show that the magnitude of the sample variation within replicate data increased. The analogous experiment was performed for 2′-deoxynucleoside digests of DNA from salmon testes (ST-DNA) that had not been exposed to either phenol or chloroform in our laboratory. (It is possible that the manufacturer of the salmon testes DNA had used the phenol−chloroform purification. This DNA was typically contaminated with RNA which we removed with RNase treatment followed by dialysis.) In this experiment, the ethanol-extracted DNA digests were dried under either $N_2$ or air prior to resuspending the samples for LCED analysis (Figure 1).

Most investigators have chosen to normalize 8-OHdG concentrations by either the original mass of DNA or the amount of deoxynucleoside (dG or dT) measured during the chromatographic analysis. In our view, the latter method is preferable, since deoxynucleosides serve as pseudo internal standards controlling for the amount of digested DNA injected into the chromatograph. However, this is a suitable quality control procedure only if all four major bases are monitored in each sample. For example, we observed previously that dG was selectively lost in a DNase I digest of calf thymus DNA, yet *Escherichia coli* DNA was efficiently digested by the same procedure (unpublished observations); and, Floyd (10) and Frenkel *et al.* (11) have also reported similar enzymatic digestion artifacts.

In the present study, we found that dT was disproportionately lost in air-dried DNA digests from salmon testes (Figure 2). Normalizing the 8-OHdG concentration by the dT concentration (in place of dG) would have tripled the apparent yield in Figure 1. Thus we strongly agree with Frenkel *et al.* (11) that a mass balance among all four bases be used to determine the extent of enzymatic digestion prior to using any particular normalization scheme. Fortunately, the use of tandem UV and electrochemical detectors enables relatively facile quantitation of all four (major) deoxynucleosides in each sample.

Perhaps most commonly cited among potential sources of artificially-induced 8-OHGua is phenol−chloroform based purification of DNA during sample processing (1). Some investigators have even attempted to limit a 'phenol artifact' by

**Table I.** 8-Hydroxydeoxyguanosine in phenol exposed DNA[a]

| Buffer and drying gas | 8-OHdG ($10^{-4}$$dG^{-1}$) (mean ± SD) | $n$ |
|---|---|---|
| Phosphate buffer, pH 7; $N_2$ | 1.37 ± 0.74 | 6 |
| Phosphate buffer, pH 7; air | 3.27 ± 1.13 | 12 |
| TEN[b]; phenol; $N_2$ | 3.06 ± 0.79 | 6 |
| TEN; phenol; $N_2$; air | 23.5 ± 10.6 | 6 |
| TDN[b]; phenol; $N_2$ | 3.18 ± 1.26 | 6 |
| TDN; phenol; $N_2$; air | 28.8 ± 5.59 | 6 |

[a]Phenol solutions were removed by dialysis prior to enzymatic digestion of the DNA samples and exposure of the dN (digests) to $N_2$ or air.
[b]TEN = Tris−hydroxyaminomethane (10 mM); EDTA (1 mM) and NaCl (100 mM). In TDN, DTPA was used in place of EDTA.

purging phenol solutions with an inert gas prior to and during use (12). In order to quantitate the effects, we treated DNA (100 μg/ml) in aqueous media with air- and buffer-equilibrated phenol solution and monitored 8-OHdG formation as a function of time at 37°C. Parallel samples were utilized for the time points studied so that dilution volumes between the organic and aqueous layers remained constant. DNA samples exposed to phenol solutions in this manner were enzymatically digested to dNs, extracted with 5 volumes of 100% ethanol according to our standard protocol. The ethanol extracts ('digests') were divided into two portions and dried under nitogen stream. One-half of the dried digests were immediately resolubilized in mobile phase buffer for direct injection into the HPLC. The remaining replicate samples were exposed to air at −20°C and in the dark for 3 days prior to solubilization for 8-OHdG determination by LCED.

The results in Table I show that phenol treatment elevated the background of 8-OHdG from 3.06 to 23.5 × $10^{-4}$ $dG^{-1}$ in TEN buffer. More importantly, the 8-OHdG background increased ~20-fold from $N_2$-dried (control) samples to 'phenol-exposed, $N_2$-dried' samples that were subsequently exposed to air (Table I). Both the use of DTPA in place of EDTA in TEN (Table I) and prolonged time in contact (24 h) with phenol (not shown) had no significant effect on the final 8-OHdG yield. Thus the major portion of the increase in 8-OHdG yield occurred after

the removal of the phenol solution. Also supporting the observation that oxidative damage of dG occurred apart from the period of direct contact with phenol and chloroform solutions, were the observations that DTPA did not lower the 8-OHdG background: DTPA–metal complexes have been shown to catalyze reactions of ROS at ~10-fold lower rates than EDTA–metal complexes (13).

It is apparent that air-induced 8-OHdG occurred even though phenol–chloroform solutions were removed by dialysis before enzymatic digestion and air exposure. Previously we had observed that phenol 'sensitized' calf thymus DNA to a 30-fold increase in 8-OHdG yield from ionizing radiation (Claycamp and Ho, manuscript submitted), and a similar increase from $H_2O_2$ treatment. Similarly, results here (Table I) suggest that pre-treatment with phenol–chloroform solutions sensitizes DNA to subsequent $O_2$ induced damage. (Sensitization refers to modification of the dose–yield relationship by a constant factor.)

Although many investigators have implicated phenol solutions in particular as the source of artifactual 8-OHdG, little documentation of phenol solutions compared directly with other DNA purification solutions has been available. In order to compare phenol-based DNA purification with alternate techniques removing proteins, we treated DNA samples in TEN buffer using several popular combinations of organic phases. One source of calf thymus DNA was used for the entire procedure in which aliquots were extracted using routine, buffer-equilibrated phenol mixtures (9, 'P'), phenol followed by extraction with chloroform:isoamyl 24:1 (PC), chloroform:isoamylalcohol extraction alone (C), phenol followed by chloroform:isoamyl-alcohol and subsequent ether extraction (PCE), or DNA precipitation using isopropanol (IPA). The latter (IPA) samples required the addition of molar NaCl to facilitate precipitation. Triplicate control and treated samples were digested to dNs and then dialyzed using spin-dialysis cartridges (Amicon, Inc., 10 kDa filters) in place of the ethanol precipitation step. The digests were dried under air or $N_2$ streams at room temperature after whih they were immediately redissolved in high purity $H_2O$ for analysis by HPLC.

Results of the comparative DNA isolation experiment are shown in Figure 3. These results show clearly that air exposure to the digested DNA samples is at least as important as organic phase exposure in causing increases in 8-OHdG. Most important are the observations confirming those in Figure 1 that air leads to a much greater relative variance among replicate samples than variances among $N_2$ exposed samples. In fact, the means of the phenol- and air-exposed groups (PHE, PC and PC) were not statistically different from the CHL, control and IPA means, due primarily to the large variances among the air-exposed samples.

Finally, it is reasonable to question whether or not a component of the organic extraction medium could survive our extensive dialysis and ethanol precipitation procedure in order to react with oxygen during drying (or lyophilization) procedures. Since phenol is readily observed using electrochemical and/or UV detectors, contamination of samples is easily monitored and results in rejection of a given sample as 'contaminated'. Yet, when we have looked at chromatograms of contaminated samples, we have not detected any consistent or reliable relationship for yield of 8-OHdG with increasing contaminant concentration. Figure 4 illustrates this observation showing that phenol contamination can even (and often) be inversely related to the quantity of 8-OHdG in the samples.

Several potential mechanisms exist for the 'phenol sensitization' of DNA including (i) the phenol mixture might increase

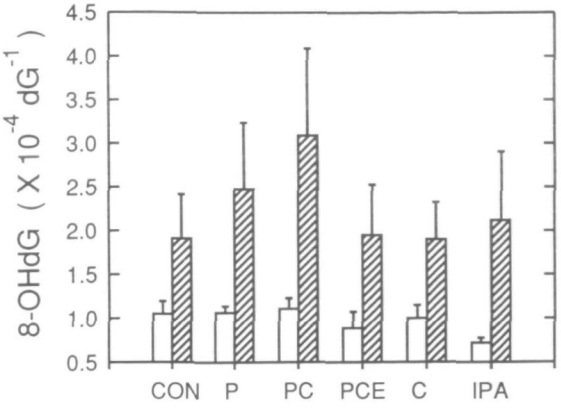


**Fig. 3.** Effects of DNA purification techniques on 8-OHdG in $N_2$- or air-dried DNA samples. Organic phase treatment of calf thymus DNA in TEN buffer in included standard 'phenol' mixtures (P); phenol followed by chloroform:isoamyl-alcohol 24:1 (PC); the preceding followed by (excess) ether extraction (PCE); chloroform:isoamyl-alcohol extraction alone (C); or precipitation of DNA using isopropanol (IPA). Dialyzed dN digests were dried using $N_2$ (open bars) or air (diagonal bars) streams prior to HPLC analyses. Control (CON) DNA samples that were not exposed to organic extraction are also shown. Means ± SEM for three independent samples per point are shown.

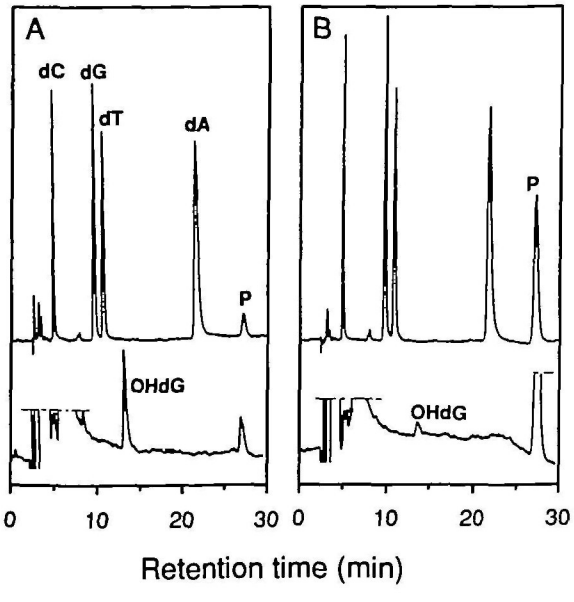


**Fig. 4.** HPLC chromatograms of phenol-contaminated DNA digests. Upper traces in both panels are UV absorption at 260 nm whereas the lower traces are electrochemical detector current (nA) recorded in series with the UV scan. **Panel A** shows a large 8-OHdG concentration in the presence of a relatively small phenol (P) concentration. The inverse situation is shown in **panel B**. These observations typified chromatograms for 'contaminated' samples which generally did not reveal a consistent correlation between contaminant and 8-OHdG concentrations.

the amount of site-coordinated Fe(II) or Fe(III); (ii) a component in the phenol mixture might chemically add to dG residues and subsequently be eliminated by reactions with active oxygen species; or (iii) a component of the phenol mixture might intercalate near dG residues and sensitize dG to oxidant damage much like methylene blue intercalates and sensitizes dG to photo-induced 8-OHdG formation (14). Thus far we have made confirming observations pertaining only to the first alternative: phenol sensitizes DNA to thymine glycol formation which is generally not considered to have a site-coordinated metal

requirement (data not shown). We have not been able to observe direct evidence for the remaining alternatives even though analyses are already performed at trace (p.p.m.) levels. Furthermore, the DNA in these samples is otherwise considered 'pure' by molecular biology standards (i.e. UV absorbance ratios). For example, 100 residues of 8-OHdG in $10^5$ dG corresponds to 99.9% of the dG remaining 'undamaged'.

In conclusion, we have provided quantitative estimated of the often-cited 'phenol artifact' in studies on 8-OHdG from DNA. More importantly, we have shown that dG, either within or apart from DNA is exquisitely sensitive to oxygen- (air)-induced 8-OHdG formation after phenol exposures: phenol (in air) exposures only doubled 8-OHdG backgrounds. While these results suggest that some reported 8-OHdG levels might be of questionable value—if the samples were exposed to air while in a lyophilized state—they also confirm that dG is a sensitive detector of generic oxidative stress. Thus the compound should be monitored in any *in vivo* or *in vitro* experiment in which oxidative damage is anticipated to influence the outcome.

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