

## SHORT COMMUNICATION

# X-radiation induces 8-hydroxy-2'-deoxyguanosine formation *in vivo* in rat mammary gland DNA

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**Ionizing radiation is a carcinogen that induces oxidative DNA damage. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is a relatively abundant, mutagenic lesion that is widely regarded as a reliable index of oxidative DNA damage. The purpose of this study was to examine the effects of X-radiation on levels of 8-OHdG in the context of an experimental model for breast cancer in which chronic radiation exposure has been shown to be carcinogenic in Sprague-Dawley rats. A secondary objective of this study was to determine if the use of phenol during DNA isolation affected the concentration of 8-OHdG subsequently measured. Our results indicate that a profoundly carcinogenic dose of radiation induced a small but significant increase in 8-OHdG concentration in mammary gland DNA, and that the use of a phenol-based versus a salt-based method of DNA isolation had no significant impact on the levels of 8-OHdG detected in either control or irradiated tissue.**

Oxidative DNA damage has been implicated in a number of disease syndromes including cancer (1–6) and 8-hydroxy-2'-deoxyguanosine (8-OHdG\*) is generally regarded as a reliable indicator of such damage (4–6). Widely disparate levels of 8-OHdG are reported from different laboratories, however, and a number of important methodological issues that surround 8-OHdG analysis have emerged. Among these, the use of phenol in DNA isolation procedures has been the subject of considerable controversy (5,7–12). Phenol has been reported to induce 8-OHdG formation (7,10) and, conversely, not to artificially elevate 8-OHdG levels (5,8,9). A recent report claims that phenol extraction sensitizes DNA to subsequent oxidative modification, and makes the provocative inference that labile guanine sites are generated under oxidative conditions that can be detected as 8-OHdG after phenol extraction (12).

Ionizing radiation is a well-established carcinogen that induces oxidative DNA damage (13). Treatment with X-radiation has been shown to be a complete carcinogen as well as to greatly enhance MNU-induced carcinogenesis in the rat mammary gland (14). While ionizing radiation has been shown to induce 8-OHdG formation in rat liver (15), the doses employed (30–173 krad) were much larger than those used in the aforementioned rat mammary cancer model (25 doses of 180 rad each over a 5 week interval). We sought to determine if a dose of X-radiation that is profoundly carcinogenic when chronically administered would result in a detectable increase in 8-OHdG concentration in rat mammary gland DNA. The

\*Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; DP, 2,2'-dipyridyl; MNU, 1-methyl-1-nitrosourea; NMWL, nominal molecular weight limit.

acute dose of radiation employed in the study reported herein (390 rad) is approximately equivalent to that administered in two separate treatments in the rat mammary cancer model. In light of the controversy surrounding the use of phenol, we examined both phenol-based and phenol-free DNA isolation procedures.

Nine female Sprague-Dawley rats (Taconic Farms) were fed an AIN-76A diet containing 6% corn oil for 36 days prior to irradiation. At 56 days of age rats were anesthetized with sodium nembutal (7.5 mg/rat; 50 mg/ml) and irradiated along the right mammary gland chain with 390 rads X-radiation. The method for unilateral mammary gland irradiation is as previously described (14). The treatment was delivered at 58.8 rads/min with an orthovoltage instrument (General Electric Maximar 250; 225 kVp, 16 mA DC, 0.25 mm Cu, 1 mm Al). Animals were killed 20 min after irradiation and mammary gland chains excised and frozen in liquid nitrogen.

Frozen mammary gland was pulverized with a ceramic mortar and pestle under liquid nitrogen. Approximately 500 mg of frozen pulverized tissue was added to 12 ml ice-cold nuclei isolation buffer (100 mM NaCl; 5 mM MgCl<sub>2</sub>; 0.5% Nonidet p-40; 50 mM Tris, pH 7.4) and vigorously mixed. The mixture was transferred to an ice-cold Dounce homogenizer and further disrupted with seven strokes of the 'B' pestle prior to removing nuclei from suspension by centrifugation at 5000 g for 10 min. The supernatant was resuspended in 6 ml digestion buffer [100 mM NaCl; 30 mM Tris pH 8.0; 25 mM EDTA, pH 8.0; 10 mM 2-mercaptoethanol; 0.75% sodium dodecyl sulfate; 1 mg/ml proteinase K (E.Merck, Darmstadt, Germany)] and incubated at 50°C for 20–24 h. The extensively digested nuclei preparations were divided into two 3 ml aliquots and DNA was isolated by one of two methods.

In the phenol-based DNA isolation, the 3-ml digested nuclei preparation was extracted sequentially with 1.5 ml phenol (IBI/Kodak, Rochester, NY) and 1.5 ml Sevag (chloroform/isoamyl alcohol, 24:1). The aqueous phase was recovered from the organic extractions and DNA was precipitated by addition of 0.04 and 0.75 vol. of 5 M NaCl and 100% isopropanol respectively.

In the salt (ammonium acetate)-based DNA isolation, an aliquot of 1 ml of 7.5 M ammonium acetate was added to 3 ml of digested nuclei preparation and thoroughly mixed. The resulting precipitate was removed by centrifugation at 10 000 g for 10 min. The supernatant was decanted and extracted with 2 ml Sevag, and DNA was precipitated from the aqueous phase by addition of 0.75 vol. 100% isopropanol. Nucleic acid clumps from both isolation methods, treated identically henceforth, were transferred to 1.5 ml microcentrifuge tubes (Fisher) and rinsed sequentially with 70 and 100% ethanol. After drying under vacuum, the samples were reconstituted with 100 µl 20 mM sodium acetate buffer containing 5 mM 2,2'-dipyridyl (DP). DP is an iron-stabilizing reagent that has been shown to inhibit free radical producing reactions initiated by both FeII and FeIII (16).

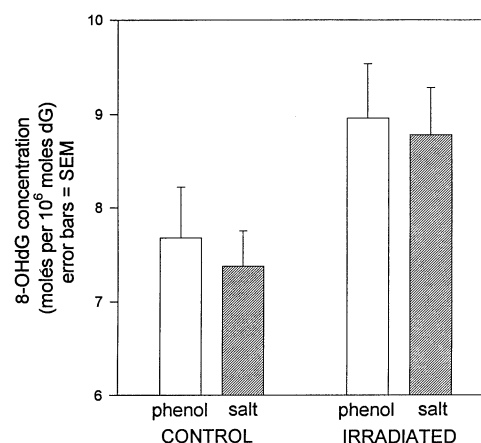
Dissolution was allowed to proceed overnight at room temperature in the dark, followed by sequential digestion with nuclease P1 (Sigma #N-8630; 4 units delivered in 10  $\mu$ l 20 mM sodium acetate, pH 4.8) for 12 min at 65°C and alkaline phosphatase (Sigma #P-0280; 5 units delivered in 10  $\mu$ l 1 M Tris, pH 8.7) for 30 min at 37°C. An aliquot of 125  $\mu$ l of 100 mM sodium acetate, pH 4.8, was then added and the solution filtered through a 10 000 NMWL filter (Millipore #UFC3LGCNB). The samples were kept on ice and protected from light prior to HPLC analysis, which was completed within 8 h of nucleic acid digestion.

8-OHdG and dG were quantitated by use of reverse phase HPLC that utilized electrochemical and spectrophotometric detection. The method employed is based on that of Floyd *et al.* (17), with some modifications. The separation was performed isocratically on a 4.6 $\times$ 250 mm Rainin Microsorb C18 column (5  $\mu$ m, 100 Å) with a mobile phase of 8.2% methanol in 50 mM potassium phosphate buffer, pH 5.5, delivered at 1 ml/min. Detection of 8-OHdG was achieved on an ESA Coulochem Model 5100A electrochemical detector equipped with a model 5011 analytical cell. Detector potentials were set as follows: guard cell +0.43 V, detector one +0.12 V, detector two +0.38 V. 8-OHdG was measured as current at detector two. dG was monitored by absorbance at 290 nm with a Shimadzu SPD-10AV spectrophotometric detector installed downstream from the electrochemical detector. Results were reported as moles 8-OHdG per 10<sup>6</sup> moles dG. 8-OHdG was generously provided by R.A.Floyd; dG was purchased from Boeringer Mannheim (Mannheim, Germany).

A repeated measures multivariate analysis of variance was employed to examine the effects of irradiation and DNA isolation method on 8-OHdG concentration. In this model, the response variable is 8-OHdG concentration (moles per 10<sup>6</sup> moles dG) and the main effects are the categorical variables irradiation (yes/no) and DNA extraction method (phenol/salt). A repeated measures model was deemed appropriate because all measurements are not independent: the four values reported for each animal, i.e. the two measurements (phenol and salt based) performed on both control and treated mammary gland chains, are correlated. Because the interaction between irradiation and DNA isolation was not statistically significant, the interaction term was not included in the final model.

Concentrations of 8-OHdG in DNA isolated from irradiated mammary glands and from controls (lead-shielded mammary glands) from nine animals are shown in Figure 1 and Table I. The increase in 8-OHdG concentration associated with X-ray treatment was highly significant ( $P = 0.0002$ ) whereas the method of DNA isolation was found not to have a significant effect ( $P = 0.45$ ). The interaction between irradiation and DNA isolation method on the concentration of 8-OHdG detected was not statistically significant, which indicates that phenol-based DNA isolation is not required in order to observe an effect of irradiation on 8-OHdG concentration.

The level of X-radiation imposed on the irradiated mammary gland is in a range that has previously been reported by our laboratory to be a potent carcinogen when chronically administered (14). In this previous account, treatment with X-radiation significantly enhanced MNU-induced mammary carcinogenesis as well as causing a dramatic increase in mammary cancer when administered in the absence of MNU. It is noteworthy that the treatment reported herein resulted in only a modest (~18%) increase in 8-OHdG concentration, given that the carcinogenic effects in tissue exposed to ionizing



**Fig. 1.** Comparison of 8-OHdG concentration in DNA isolated from control and irradiated mammary glands by phenol- and salt-based procedures ( $n = 9$  per group). 8-OHdG concentration in DNA from irradiated mammary glands was significantly higher ( $P = 0.0002$ ) than that from control glands by repeated measures multivariate analysis of variance. DNA isolation method was without effect ( $P = 0.45$ ). There was no significant interaction between irradiation and DNA isolation method.

**Table I.** Mean ( $\pm$ SE) 8-OHdG concentrations (moles per 10<sup>6</sup> moles dG) in DNA isolated from control and irradiated mammary glands by phenol- and salt-based procedures

Treatment		8-OHdG
Control	Phenol <sup>a</sup>	7.68 $\pm$ 0.54
	Salt <sup>a</sup>	7.38 $\pm$ 0.38
Irradiated	Phenol <sup>b</sup>	8.96 $\pm$ 0.58
	Salt <sup>b</sup>	8.78 $\pm$ 0.50

$n = 9$  per group. Irradiated glands<sup>b</sup> contained significantly more 8-OHdG than controls<sup>a</sup> ( $P = 0.0002$ ).

radiation are thought to be primarily the result of oxidative DNA damage (13), and that chronic treatment with radiation in this dose range is strongly carcinogenic. This observation suggests that increased levels of oxidative stress that cause no overt, acute symptoms, but are nevertheless associated with the induction of cancer may result in oxidative DNA damage increases that are small and difficult to detect. These data also provide a basis for assessing work in which elevated levels of oxidative DNA base damage (including 8-OHdG) were reported in malignancies and surrounding uninvolved tissue in humans (18). There has been concern that artifacts of the analysis procedure may contribute substantially to those values (10,19,20). Although the authors emphasize differences in the spectrum of DNA damage between normal and malignant or surrounding tissue rather than the absolute levels of such damage, and indeed those differences are intriguing, the values reported are much higher (20- to 200-fold for 8-OHdG) than we have observed, even in response to a focally administered dose of ionizing radiation. Our data suggest that the high levels reported in the aforementioned account primarily reflect artifact and that such high levels are not obligatory to influence the carcinogenic process.

The issue of methodology used in the analysis for 8-OHdG is critical and a subject of considerable discussion and controversy (5,7–12,15,17–19). It has been reported that the use of phenol in the isolation of DNA artificially induces the formation of 8-OHdG (7,10), and it has also been argued that

phenol is necessary for obtaining DNA of suitable quality for 8-OHdG analysis (11). Evidence has also been reported of phenol labile sites, i.e. oxidative DNA modifications that are detectable as 8-OHdG only if phenol is used to isolate DNA (12). The data reported herein are in agreement with a number of accounts in which no induction of 8-OHdG attributable to exposure of DNA to phenol was observed (5,8,9). Ames *et al.* report that even phenol that has been intentionally aged under undesirable conditions, failed to induce 8-OHdG formation in DNA to which it was exposed (4). They also note that phenol itself is an antioxidant with an oxidation potential well below that of dG. We were nonetheless fastidious in our use of properly handled redistilled phenol (21). Our use of two different DNA isolation procedures was intended to maximize our ability to observe X-ray-mediated effects on 8-OHdG abundance, and to facilitate direct comparison of our phenol-based and phenol-free DNA isolation procedures. It should be noted that our tissue samples were treated identically (tissue disruption and digestion) prior to DNA isolation, in contrast to some literature comparisons of phenol-based and phenol-free methods (8,9,12). Moreover, the tissue digestion employed in our laboratory is extensive compared with most accounts in the literature. Consequently, methodological differences exist that preclude direct comparison of our results with those obtained by other methods, and it is possible that our exhaustive digestion makes subsequent DNA purification less critical. Oxidative conditions are known to give rise to DNA-protein complexes. Given that methods specifically designed for precipitating such complexes utilize buffers of high salt and detergent concentrations (22,23), procedures employing 'salting out' for protein removal risk loss of complexed DNA in samples that have not been completely digested. In DNA where oxidative damage is distributed heterogeneously, this could result in selective loss of more highly damaged regions, which would presumably contain higher levels of both 8-OHdG and DNA-protein complexes. The low solubility of DNA in phenol at basic pH may facilitate more complete DNA recovery with phenol based methods, thereby offering an advantage over salt-based procedures. Our data do in fact show a slight trend toward higher and less variable DNA recovery for the phenol- versus salt-based methods ( $62.8 \pm 18.3$  versus  $58.25 \pm 19.0$   $\mu\text{g}$  DNA recovered respectively), but the difference is not statistically significant.

In summary, we observed a significant increase in 8-OHdG concentration in DNA from rat mammary gland associated with *in vivo* X-ray treatment. The increase appears modest with respect to a dose of radiation that has been shown to be highly carcinogenic. The phenol- and ammonium acetate-based DNA isolation procedures employed are statistically equivalent. We observed scant evidence for the existence of phenol labile sites or artificial induction of 8-OHdG by use of phenol.

## Acknowledgement

This work was supported by PHS grant number CA 69241 from the National Cancer Institute.

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Received on January 8, 1998; revised on March 27, 1998; accepted on April 1, 1998