

Review

Measurement and Meaning of Oxidatively Modified DNA Lesions in Urine

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Abstract

Background: Oxidatively generated damage to DNA has been implicated in the pathogenesis of a wide variety of diseases. The noninvasive assessment of such damage, i.e., in urine, and application to large-scale human studies are vital to understanding this role and devising intervention strategies.

Methods: We have reviewed the literature to establish the status quo with regard to the methods and meaning of measuring DNA oxidation products in urine.

Results: Most of the literature focus upon 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), and whereas a large number of these reports concern clinical conditions, there remains (a) lack of consensus between methods, (b) possible contribution from diet and/or cell death, (c) no definitive DNA repair source of urinary 2'-deoxyribonucleoside lesions, and (d) no reference ranges for healthy or diseased individuals.

Conclusions: The origin of 8-oxodG is not identified; however, recent cell culture studies suggest that the

action of Nudix hydrolase(s) on oxidative modification of the nucleotide pool is a likely candidate for the 8-oxodG found in urine and, potentially, of other oxidized 2'-deoxyribonucleoside lesions. Literature reports suggest that diet and cell death have minimal, if any, influence upon urinary levels of 8-oxodG and 8-oxo-7,8-dihydroguanine, although this should be assessed on a lesion-by-lesion basis. Broadly speaking, there is consensus between chromatographic techniques; however, ELISA approaches continue to overestimate 8-oxodG levels and is not sufficiently specific for accurate quantification. With increasing numbers of lesions being studied, it is vital that these fundamental issues are addressed. We report the formation of the European Standards Committee on Urinary (DNA) Lesion Analysis whose primary goal is to achieve consensus between methods and establish reference ranges in health and disease. (Cancer Epidemiol Biomarkers Prev 2008; 17(1):3–14)

Introduction

Reactive oxygen species are produced continually from normal cellular metabolism but may be produced in excess, leading to oxidative stress, after exposure to xenobiotics, radiation, etc. A consequence of reactive oxygen species production is their interaction with, and subsequent modification of, cellular biomolecules, in particular, DNA, lipids, and proteins. Damage to DNA is of

particular importance, in part due to the possibility of inheritable sequence alterations (mutations), although it should not be forgotten that reactive oxygen species-induced damage to DNA may have nonmutational effects, such as the promotion of microsatellite instability and acceleration of telomere shortening (1). Over 30 base modifications, such as 8-oxo-7,8-dihydroguanine (8-oxoGua) and its corresponding deoxyribonucleoside equivalent, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG; Fig. 1), have been described (2) with >70 DNA lesions identified (3). Furthermore, reactive intermediates arising from the interaction of reactive oxygen species with lipids and proteins may also modify DNA, leading to adducts, such as 1,N⁶-etheno-2'-deoxyadenosine (ϵ dA), 3-(2-oxo- β -D-erythro-pentofuranosyl)pyrimido[1,2- α]purin-10(3H)-one, and DNA-protein cross-links (Fig. 1; ref. 4). A great deal of evidence exists, which suggests that this plethora of lesions, arising from oxidative stress, may have an important role in the etiology and/or pathogenesis of many diseases (reviewed in ref. 5), such as cancer and aging.

To fully understand the extent to which such DNA lesions are involved in disease, methods for their analysis are essential. Numerous approaches have been applied to

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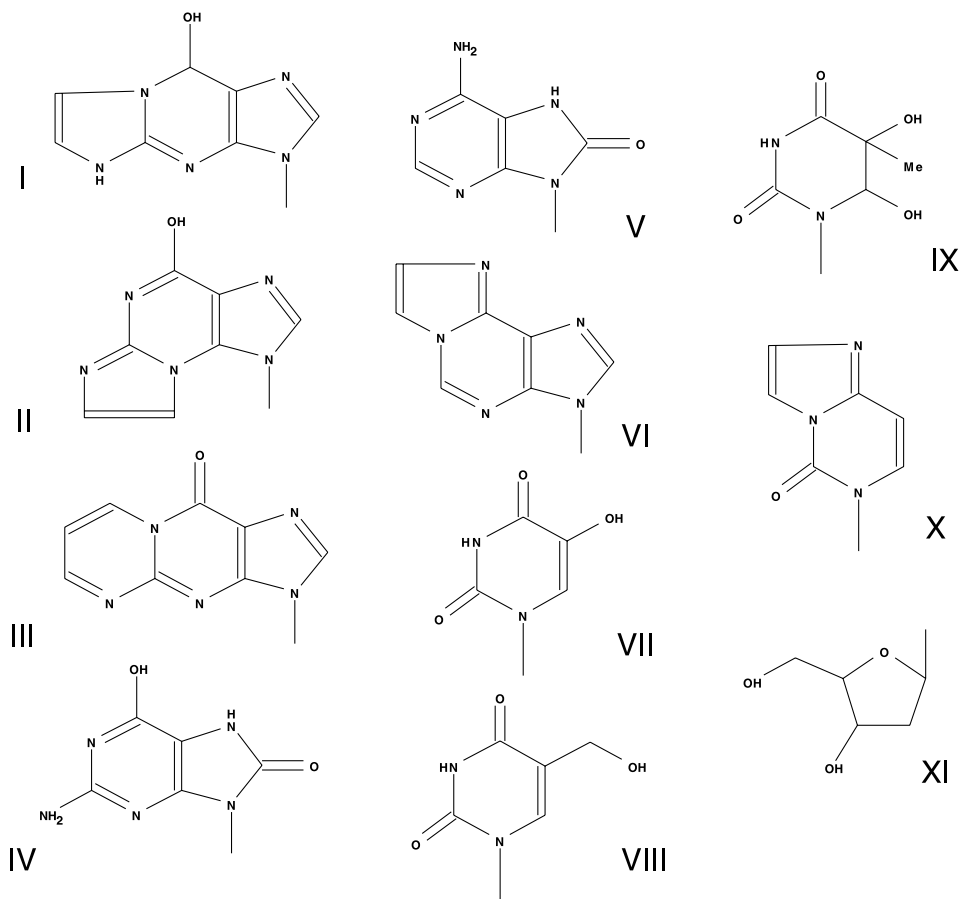


Figure 1. Structures of DNA base adducts analyzed in urine. *I*, 1, N^2 - ϵ Gua; *II*, $N^2,3$ - ϵ Gua; *III*, M₁Gua; *IV*, 8-oxoGua; *V*, 8-oxo-7,8-dihydroadenine; *VI*, 1, N^6 -ethenoadenine; *VII*, 5-hydroxyuracil; *VIII*, 5-HMeUra; *IX*, thymine glycol; *X*, 3, N^4 -ethenocytosine; *XI*, 2'-deoxyribose, which forms a N -glycosidic bond with the N^9 in purines and N^1 in pyrimidines in the 2'-deoxyribonucleoside analogues of these base modifications.

the study of oxidatively damaged DNA, including gas chromatography with mass spectrometry (GC/MS; ref. 6), high performance liquid chromatography with electrochemical detection (HPLC ECD; ref. 7), HPLC with single (8) or tandem (9) mass spectrometry, ^{32}P postlabeling (10), immunoassay (11, 12), alkaline elution (13), and Comet assay (14), plus other methods based upon the nicking of DNA at oxidized bases by means of repair enzymes (15, 16). However, after the publication of the findings from the European Standards Committee on Oxidative DNA Damage (17), a number of these techniques have fallen from favor (reviewed in ref. 18).

Assessment of damage to DNA by methods requiring invasive procedures, e.g., blood samples or tissue biopsy, imposes severe limitations in large-scale human studies, requiring staff with specialist training and reducing the likelihood of consent. Furthermore, the possibility of adventitious oxidation during sample storage and DNA extraction has not been entirely ruled out, but methods have been developed to minimize the risk (16). Examining the products of oxidatively generated damage to DNA in extracellular matrices offers a means by which oxidative stress may be assessed noninvasively or minimally invasively and circumvents extraction and the resultant artifacts.

Methods of Analysis

For the most part, methods that have been applied to the analysis of oxidatively damaged DNA lesions in urine

are either chromatographic (principally, HPLC MS/MS; liquid chromatography prepurification before GC/MS, HPLC GC/MS, HPLC ECD, GC/MS) or immunoassay. The majority of assays focus upon 8-oxodG as the analyte of choice, although the following have been reported to be present in urine, in many cases with their corresponding deoxyribonucleoside (Table 1).

Chromatographic Techniques. Urine is a very complex matrix, and therefore, the common challenge for all chromatographic techniques has been to clean-up the urine sufficiently to simplify analysis, which very often also extends instrument life. At its simplest, column switching has meant that, after chromatographic separation of the urine's constituents, only the fraction containing the compound of interest (e.g., 8-oxodG) is applied to the final separation column and detector, either ECD (19, 20) or MS (21); the remainder is diverted to waste.

Using HPLC, thymine glycol, which was a "significant marker" of oxidative stress (22), was one of the first reactive oxygen species-induced DNA lesions to be studied in urine. The methodology required the use of boronate, solid phase extraction columns, and semipreparative HPLC to isolate thymine glycol and thymidine glycol from urine; the lesions were then chemically reduced back to thymine and thymidine before analysis by HPLC with UV detection (23). The widespread preference for studies of 8-oxodG, however, was derived from 1,000-fold greater sensitivity of 8-oxodG detection using HPLC

ECD compared with the UV detection of thymine glycol, along with the predominance of 8-oxodG over thymine glycol, in human urine (24). This predominance may arise for reasons, such as the reported instability of thymine glycol and greater levels of 8-oxodG in DNA, due to the relative ease of formation of the latter (24). Overall, this has led to urinary measurements of thymine glycol being superseded by those of 8-oxodG and the subsequent widespread use of HPLC ECD. A number of methods based on column-switching HPLC with regular reverse phase (usually C18) separative columns and ECD for assay of 8-oxodG have been developed (24-28). The pre-purification, or extraction, columns have differed in their packing material, using generally C18 or ion exchange separation approaches. The switching of the effluent from the first to the second column can be controlled automatically by adding to the sample a detectable marker which elutes close 8-oxodG from the first column and the peak is used as the trigger, and

this has greatly enhanced the analytic capacity by electrochemical detection (29). An approach shown to be successful in a wide variety of biological matrices [including DNA, urine, plasma, RBC extracts, cerebrospinal fluid, saliva, sweat, kidney dialysate, brain and muscle microdialysate, food (rat, mouse, monkey and human chow), *Caenorhabditis elegans*, cell culture medium, and rat feces] uses a column comprising "treated" carbon material, similar to that used for ECD cells, together with CoulArray multichannel electrochemical detection (30).

Most recently, mass spectrometric techniques have been increasingly applied to study urinary lesions. Benefits of mass spectrometry include the use of isotopically labeled internal standards, simplifying quantification and accounting for loss during sample workup (and storage), differences in ionization efficiencies due to matrix effects, and confirmation of analyte identity. The HPLC MS/MS assay described by Weimann et al. (21)

Table 1. DNA markers of oxidative stress reported in the urine of healthy humans

Nucleobase	Modification	Abbreviation	Representative method(s) of analysis	Reported levels
Gua	8-oxo-7,8-dihydroguanine	8-oxoGua	HPLC GC/MS (110)	9.4 nmol/mmol Cr
			LC-MS/MS (21) HPLC GC/MS (87) HPLC ECD (75)	136 nmol/24 h 130 nmol/24 h ~ 308 pmol/kg/24 h
	8-oxo-7,8-dihydro-2'-deoxyguanosine	8-oxodG	HPLC GC/MS (110)	1.5 nmol/mmol Cr
			LC-MS/MS (35)	0.4 nmol/mmol Cr
			ELISA (111) GC/MS (34) CE-EC (112) HPLC GC/MS (113) HPLC MS/MS (9)	19.4 nmol/mmol Cr 1-3 nmol/mmol Cr 13.5 nmol/L 35 nmol/24 h 20 nmol/L
	8-oxo-7,8-dihydroguanosine	8-oxoG	HPLC EC (114)	405 pmol/kg/24 h
	Pyrimido[1,2-a]purin-10(3H)-one	M ₁ Gua	LC-MS/MS (21) LC-MS (115)	48 nmol/24 h <LOD (500 pmol/L)
3-(2-deoxy-β-D-erythro-pentofuranosyl)-pyrimido[1,2-α]purin-10(3H)-one	M ₁ dG	LC-MS/MS (116)	Not reported	
Ade	1, N ² -ethenoguanine	1, N ² -εGua	LC/APCI-MS/MS (117) LC/electrospray ionization/MS/MS (60)	12 fmol/kg/24 h <0.3 (LOD)-10 nmol/L
	N ² -3-ethenoguanine	N ² -3-εGua	LC/electrospray ionization/MS/MS (60)	<0.3 (LOD) - 10 nmol/L
Ade	8-oxo-7,8-dihydroadenine	8-oxoAde	HPLC GC/MS (33)	7 nmol/L
		8-oxodA	LC-MS/MS (36)	<LOD
	1,N ⁶ -etheno-2'-deoxyadenosine	εdA	LC/APCI-MS/MS (56)	38.6 pmol/24 h
Thy	Thymine glycol Thymidine glycol 5-(hydroxymethyl)uracil 5-hydroxymethyl-2'-deoxyuridine 5-hydroxyuracil	Tg	Immuno-HPLC fluorescence detection (51)	6-113 pmol/24 h (1.2-17 pmol/mmol Cr)
		dTg	Immuno-HPLC fluorescence detection (52)	6.4 pmol/mmol Cr
		5-HMUra	HPLC UV (23)	1.9 nmol/mmol Cr
		5-HMdUrd	HPLC UV (23)	1.2 nmol/mmol Cr
			HPLC GC/MS (79, 118) HPLC GC/MS (118)	8.5 nmol/mmol Cr At LOD (5 nmol/L)
Cyt	3,N ⁴ -ethenocytosine 3,N ⁴ -etheno-2'-deoxycytidine	5-OHUra	HPLC GC/MS (33)	58 nmol/L
		εCyt	GC/NICI/MS (119)	84.6 nmol/L/mmol Cr
		εdC	³² P-postlabeling (120)	2.5 pmol/mmol Cr

Abbreviation: LOD, limit of detection.

was the first report for the simultaneous analysis of the oxidized (8-oxoGua, 8-oxoGuo, and 8-oxodG) and native (Gua, Guo, and dG) moieties. Inclusion of the latter provided useful data for subsequent hypotheses (see Cell Death/Turnover). Similarly, the HPLC GC/MS assay of Olinski's laboratory entails HPLC prepurification of the compounds of interest, which again includes native compounds namely 8-oxoGua, 8-oxodG, Gua, dG, and 5-(hydroxymethyl)uracil (31, 32), before derivatization and GC-MS. This approach was first applied to the simultaneous analysis of five urinary, oxidatively modified DNA base products, including 8-oxoGua, 5-(hydroxymethyl)uracil, 5-hydroxyuracil, 8-oxodG, and 8-oxo-7,8-dihydroadenine (33). Analysis time for HPLC GC/MS is said to be comparable with that of HPLC MS/MS and, at present, is capable of the simultaneous analysis of the greatest number of lesions.

Whereas solid-phase extraction (SPE) had been used to isolate 8-oxodG, a major drawback of many SPE columns is the need to keep them wet during use. The Waters Oasis HLB SPE column, first used by Lin et al. (34), before GC-MS analysis of urinary 8-oxodG, benefits from allowing to become dry during use. These columns have also been used before HPLC MS/MS, which has minimized the incidence of interfering peaks reported by Lin et al. (34) in 10% to 20% of their urine samples (35). The analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyadenosine has been attempted previously (36), but levels were reported to be at or below the limit of detection for the HPLC MS/MS assay (0.3 nmol/L, 7.5 fmol injected, S/N = 3). A similar finding was noted with the SPE-based HPLC MS/MS assay.⁵ We predict that SPE will receive increasing application in the future, particularly when on-line SPE is introduced.

Immunoassay. Competitive ELISA is invariably the format of choice for the analysis of lesions in extracellular matrices, when antibody-based methods are used. Predominantly, 8-oxodG has been the lesion of choice for commercially available [e.g., from the Japanese Institute for the Control of Aging (JICA), Stressgen, and Oxis; the latter simply being a distributor of the JICA kit] and custom-made (37, 38) ELISAs. It seems that there are very few commercially available kits presently on the market. Two are available from JICA, named the "new" and "highly sensitive" formats (calibration ranges are 0.5-200 ng/mL and 0.125-10 ng/mL, respectively). These kits are remarkably similar in description (including references cited) to those marketed by Northwest Life Science Specialties, and indeed both utilize the monoclonal antibody denoted N45.1 and have the same concentration range of standards. The other kit available is from Stressgen Bioreagents, now known as Nventa (DNA damage StressXpress ELISA kit), which is remarkably similar to that from Trevigen, Inc. (HT 8-oxodG kit). Both have a range of standards in their calibration curve of 1.9 to 60 ng/mL and essentially the same Microsoft Excel calculation spread-

sheet, although the source of the primary antibody used is less clear.

Simply, the assay format of these ELISAs comprise 8-oxodG-protein conjugate bound to the bottom of a 96-well plate, a calibration curve of buffered aqueous 8-oxodG standards containing 0.9% NaCl, and a monoclonal antibody. Both 8-oxodG in the samples and on the plate compete for antibody binding. After a wash step, antibody that remains bound to the plate can be detected using an enzyme-labeled secondary antibody. Color intensity (absorbance) of the enzyme-catalyzed product is proportional to the amount of primary antibody bound, which, in turn, is inversely proportional to the concentration of antigen in the test sample.

The benefits of ELISA are (a) ease of use, (b) no specialist (or indeed expensive) equipment is required, (c) potential application to numerous extracellular matrices (serum, ref. 39; plasma, ref. 40; saliva, refs. 41, 42; urine, ref. 43; cerebrospinal fluid, refs. 44, 45; cell culture medium, refs. 46-48; and sputum, ref. 49), (d) other than centrifugation of cloudy specimens, no pretreatment of urine is required, and (e) high throughput. The kit from JICA has, by far, received the most published use and represents the basis of the bulk of observations to follow. It should be noted that only the JICA kit is recommended for use in plasma; such an application has only recently been reported in the literature, unlike serum.

In the case of plasma, the manufacturer recommends ultracentrifugation of the specimen through a 10-kDa filter (JICA kit insert) to remove "interfering substances," the identity of which is unknown to us. This has been discussed recently by Cooke et al. (35), who speculated that the competitive format of the ELISA is prone to interference from high molecular weight compounds (e.g., proteins and carbohydrates) present in some body fluids that resulted in high 8-oxodG readings (e.g., serum) or readings in the absence of significant amounts of 8-oxodG (e.g., saliva). Presently, no ELISA kit has been proved to possess sufficient specificity and sufficiently high correlation to the established chemical methods, e.g., HPLC ECD with column switching or HPLC MS/MS.

Analysis of Secondary DNA Products of Oxidation Reactions in Urine. Although not as prevalent as methods for analyzing primary DNA products of oxidation in urine, this is a rapidly growing area of interest. Several methods have been developed for the measurement of lipid peroxidation-induced etheno-DNA adducts in human urine. An HPLC fluorescence detection method has been developed for ϵ dA (50) and applied in several studies. For example, in Japanese women, urinary ϵ dA levels were positively associated with increased ω -6 polyunsaturated fatty acid intake and NaCl excretion, providing evidence of salt-induced inflammation and lipid peroxidation (51). It has also been reported that short-term fasting does not affect urinary ϵ dA adduct or, indeed, 8-oxodG levels in healthy Korean women despite a reduction in primary lipid peroxidation products (e.g., urinary MDA and 8-isoprostaglandin $F_{2\alpha}$; ref. 52). Isotope dilution GC-negative ion chemical ionization/MS methodology have been developed for $3,N^4$ -ethenocytosine and $3,N^4$ -ethenodeoxycytidine, with higher levels detected in the urine of smokers compared with

⁵ M.D. Evans, R. Singh, V. Mistry, K. Sandu, P.B. Farmer, M.S. Cooke. Analysis of urinary 8-oxopurine-2'-deoxyribonucleosides by liquid chromatography tandem mass spectrometry and enzyme-linked immunosorbent assay, submitted for publication.

nonsmokers (53, 54). Furthermore, there was good agreement between urinary levels of 1,*N*⁶-ethenoadenine in healthy individuals, measured by HPLC–electrospray ionization–MS/MS method and isotope dilution GC–negative ion chemical ionization/MS methods (55). An HPLC MS/MS method has been developed to analyze urinary ϵ dA (56) and 3,*N*⁴-ethenodeoxycytidine (57) and have been applied to show a significant association between etheno-DNA adduct excretion rate and the dietary intake of linoleic acid in healthy men (58). A ³²P postlabeling method has also been reported, which measures 3,*N*⁴-ethenodeoxycytidine in human urine (59).

Background levels of 1,*N*²-ethenoguanine and *N*²,3-ethenoguanine have been detected in the urine from healthy subjects who have not been exposed to the recognized sources of these adducts, e.g., vinyl chloride and ethyl carbamate (60). An HPLC–electrospray ionization–MS/MS method was developed and used for the detection of low levels of 1,*N*²-ethenoguanine, which were found to be higher in cigarette smokers (61).

Method Comparison. A principal source of criticism of ELISA derives from the discrepancy between chromatography and immunoassay in the determination of “background” or baseline levels of urinary 8-oxodG in healthy individuals. This difference can be anywhere between 4-fold and 10-fold, although recent improvements in the JICA kit (narrowing calibration curve range; recommendation for strict temperature control), have reduced this margin (Fig. 2). For the most part, however, 8-oxodG measurements by the two approaches have shown significant correlation: $r = 0.46$, $P < 0.001$ using HPLC ECD (62) and $r = 0.73$, $P = 0.002$ using HPLC GC/MS (63); although, this is not always the case (64).⁶ Nevertheless, this does suggest that both techniques share a common analyte, with the ELISA perhaps recognizing additional compounds. In accounting for the discrepancy, some reports in the literature fail to distinguish between antibodies and their respective specificities, applying a blanket statement to the unsuitability of all ELISAs. Whereas some of this criticism is well-founded, clearly some antibodies are highly specific and their application to urine seems entirely appropriate.

For example, according to characterization data reported by Toyokuni et al. (65), N45.1 is highly specific for 8-oxodG. In addition to being specific for the hydroxylated C8 position of Gua, it would seem to discriminate the C6 carbonyl group of 8-oxoGua (and C2 NH₂) from the C6 amino group of 8-oxo-7,8-dihydroadenine. Furthermore, unlike many antibodies, N45.1 displays preference for the 2-deoxyribose moiety of 8-oxodG until recently, a prerequisite to the assessment of urinary DNA oxidation products. The closest competitor for the antibody is 8-oxo-7,8-dihydroguanosine (8-oxoG), which needs to be present in concentrations two orders of magnitude higher than 8-oxodG to compete to the same extent. Weimann et al. (20) later showed that 8-oxoG was present at a concentration not dissimilar to 8-oxodG itself (8-oxoG, 48 nmol/24 h versus 8-oxodG, 28 nmol/24 h), and hence, it was concluded that 8-oxoG was unlikely to be a competitor (66).

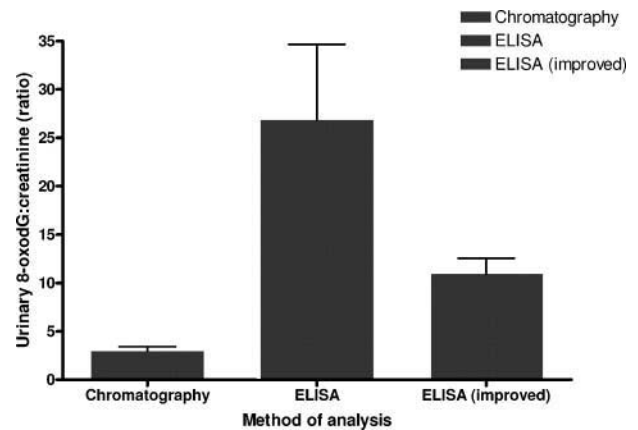


Figure 2. Comparison of urinary 8-oxodG levels by chromatographic techniques (HPLC ECD, LC-MS/MS) and JICA ELISA (original 8-OHdG check and new 8-OHdG check).

Similarly, whereas 8-oxoGua is present in urine at significant concentrations (136 ± 12 nmol/24 h), characterization data suggests reactivity of N45.1 toward the modified base to be, at worst, negligible. Some of the comments in the literature, which state that N45.1 recognizes 8-oxoGua, are likely derived from misinterpretation of a statement in the abstract of the paper by Toyokuni et al. (65), “N45.1 recognized both the modified base and 2-deoxyribose structure of 8-oxodG.” Clearly, some authors have taken this to refer to the modified base and 2'-deoxyribonucleoside as separate structures, not in combination, as intended by Toyokuni et al. (65). It is important to note that whereas the JICA assay is reported to be specific for 8-oxodG, the specificity of the Trevigen/Stressgen kit(s) includes recognition of 8-oxoGua and 8-oxoG, likely to account for the higher levels of urinary 8-oxodG reported by these kits. The appropriateness of a calibration curve consisting solely of 8-oxodG, as used by these latter two suppliers' kits, is also questionable.

Identifying the basis of the discrepancy has proved to be problematic. We have speculated that, as chromatographic assays can only detect “free” 8-oxodG, other compounds structurally related to 8-oxodG, recognizable by N45.1 specifically, may exist and be present in urine (67). For example, 8-oxodGMP, a product of the activity of enzymes, such as the human Mut T homologue (hMTH1; ref. 68), which may have the potential to leave the cell (69); along with 8-oxodG-containing oligomers, putative products of nucleotide excision repair (67). The issue 8-oxodG containing oligomers in urine does not seem to have been resolved, as there have been two reports both indicating (70) and disputing their presence (71), although both reports agree that oligomers are present. Patel et al. (70) ascribe the failure of Weimann et al. (71) to detect 8-oxodG-containing oligomers to a sensitivity issue, arising from the absence of sample concentration steps despite LC-MS/MS having greater than thrice the sensitivity of the ELISA. At best, this would indicate that, if present, 8-oxodG-containing oligomers are not at a concentration detectable in urine by the ELISA and hence not the source of the discrepancy between this and chromatographic techniques. Furthermore, use of an independent technique, i.e., not one

⁶ Cooke et al., unpublished data.

relying upon antibody specificity, would provide a more definitive answer.

Reardon et al. (72) showed that 8-oxodG is a substrate for nucleotide excision repair, which excises lesions as a single-stranded, lesion-containing oligomer (see DNA Repair). Galloway et al. (73) showed that, postexcision, cyclobutadithymine (T<>T)-containing oligomers, at least, are subsequently degraded to lesion-containing 7-mers. What then happens to the oligomers is unknown, although from the findings of Le Curieux and Hemminki (74), who detected the presence of urinary T<>T as a monophosphate nucleotide dimer, it may be implied that further processing does occur, the nature of which remains to be described. Despite all this circumstantial evidence, the precise reason for the higher ELISA values remains unknown and represents a severe problem that needs to be clarified before its use can be recommended without comparison with established chromatographic methods.

The HPLC ECD method is not without analytic difficulties, Bogdanov et al. (30) reported the presence of peaks which coelute with 8-oxodG identified by multichannel colometric electrochemical detection. Furthermore, if DNA oxidation products are indeed present in urine in forms other than bases or 2'-deoxyribonucleosides, such as oligomers, monophosphates, or diphosphates, then the vast majority of chromatographic methods will not recognize these products. Despite these issues, within technique agreement of levels in control subjects between laboratories is strong and supports the continued use of both chromatographic and ELISA approaches. Furthermore, in longitudinal or comparative studies, the significance of absolute levels is of less importance compared with the ability to detect differences in 8-oxodG levels.

Urinary Lesions: Issues of Artifact and Stability. The measurement of oxidatively generated damage to DNA has been plagued by the issue of artifact-generated, for example, during DNA extraction, or sample workup. This issue has been critically examined in detail, European Standards Committee on Oxidative DNA Damage, as part of its attempts to achieve consensus between different techniques and different laboratories (the objectives of European Standards Committee on Oxidative DNA Damage are outlined in Table 2). Similarly, urinary lesions may arise from the artifactual oxidation of DNA or its constituent nucleobases or 2'-deoxyribonucleosides after exposure to metabolic enzymes or other oxidizing species after release into the systemic circulation or in the urine. However, Shigenaga et al. (75) showed that dG is not artifactually oxidized in the systemic circulation or after incubation with microsomal enzymes. Equally, 8-oxodG, thymine glycol, and thymidine glycol are not subject to degradation upon release into the systemic circulation (23, 75), the stability of 8-oxoGua not having been examined. Experimental studies with 8-oxodG injected i.v. have shown that recovery is almost complete in urine in pigs, indicating that further metabolism is an unlikely event (76).

Furthermore, as significant concentrations of hydrogen peroxide have been reported to be present in urine (77), Lin et al. (34) examined the potential for 8-oxodG to be generated from dG present in the urine. Combinations of dG (1 nmol) and hydrogen peroxide (50 or 100 $\mu\text{mol/L}$) were incubated in a number of urine

samples for 24 h at room temperature (34). The authors concluded that hydrogen peroxide has a negligible effect upon 8-oxodG production, under these circumstances, agreeing with previous results from Ames' laboratory.

As part of a study, representing a great deal of foresight, Loft et al. (78) reported that storage of urine samples at -20°C and repeat measurements did not lead to a decrease in the concentration of 8-oxodG over a 15-year period, confirming the stability of this lesion in urine. This also applies to 5-(hydroxymethyl)uracil that has been stable upon storage at -80°C for periods up to 4 months (79).

Whereas the above provide evidence of stability in extracellular matrices, it is worth noting that 8-oxodG is more prone to oxidation than dG due to its lower redox potential (80, 81) and is preferentially oxidized even in the presence of an excess of Gua (82, 83). By the same token, oxidation of Thy to thymine glycol is less likely to occur than Gua to 8-oxoGua due to the lower oxidation potential of Gua compared with Thy. However, it would seem that most of these studies have examined the oxidation of 8-oxoGua *in situ* in DNA (84), rather than as a postexcision product of DNA repair, which may alter the likelihood of oxidation and its "oxidizability." In contrast, M₁G and 3-(2-deoxy- β -D-erythro-pentofuranosyl)pyrimido[1,2- α]purin-10(3H)-one both seem to undergo further oxidative metabolism in rat liver cytosol, with the base adduct being a better substrate for such enzymic oxidation than the deoxyribonucleoside adduct (85, 86). There is also some evidence to suggest that M₁G is further oxidized when given i.v., although 3-(2-deoxy- β -D-erythro-pentofuranosyl)pyrimido[1,2- α]purin-10(3H)-one was not examined (85).

Overall, it would seem that 8-oxodG, thymine glycol, and thymidine glycol, and probably 8-oxoGua, are not formed artifactually *in vivo* in biological matrices of mammals, including humans, and there is no published evidence for their degradation upon release. In contrast, there is strong evidence that M₁G and 3-(2-deoxy- β -D-erythro-pentofuranosyl)pyrimido[1,2- α]purin-10(3H)-one may undergo oxidation, either enzymically or in the systemic circulation.

Sources of Extracellular, Oxidatively Modified DNA Lesions

Broadly speaking, the possible sources of extracellular, oxidatively modified DNA lesions are (a) diet, (b) cell death/turnover (mitochondrial turnover), and (c) DNA repair.

Diet. Urine is the extracellular matrix upon which dietary influence has been most studied. The majority of reports measuring oxidatively modified DNA lesions in urine have focused upon 2'-deoxyribonucleoside lesions and 8-oxodG specifically. This is largely a consequence of early work from Bruce Ames' laboratory in rats, showing that diet could affect levels of urinary 8-oxoGua, but not 8-oxodG, and they concluded thymine glycol to be unaffected by diet (discussed in detail in ref. 67). There is little human data on the subject of dietary contribution and some disagreement with the animal findings. Gackowski et al. (87) have presented evidence to suggest that, in humans, neither 8-oxoGua nor 8-oxodG is

Table 2. Overview of the remit of ESCOLA and comparison with the European Standards Committee on Oxidative DNA Damage

	ESCODD	ESCOLA
Assay (s)	HPLC ECD, GC-MS, LC-MS/MS and repair endonuclease-based methods (e.g., Comet assay)	HPLC ECD, LC-GC-MS, LC-MS/MS, ELISA
Matrix of study	DNA*	Urine
Primary lesion investigated	8-oxoGua	8-oxodG [†]
Objectives		
1	Validate above methods for 8-oxoGua analysis	To achieve consensus between above methods
2	Increase sensitivity and reliability of above methods, addressing issues of artifact.	Investigate any discrepancy between methods
3	Standardize protocols for preparation of samples, DNA	Provide validated methods for wider dissemination
4	To compare methods on identical samples to achieve consensus	Establish a reference range for urinary 8-oxodG, accounting for factors known to affect levels
5	To reach consensus on average levels of 8-oxoGua in normal human DNA	Investigate the use of other oxidized nucleobases and nucleosides as possible biomarkers
6		Elucidate extent of contribution from possible confounders of urinary lesion measurement (e.g., diet and cell turnover)

Abbreviation: ESCODD, European Standards Committee on Oxidative DNA Damage.

*Chromatographic methods that require extraction of DNA will report on levels of damage from both mitochondrial and nuclear DNA. Repair endonuclease-based methods will predominantly address nuclear DNA levels of damage.

[†]ESCOLA's remit is proposed to extend beyond this lesion.

affected by diet. Pivotal to this study was the assumption that, like rats (88), humans reach a minimum level of 8-oxoGua excretion 2 to 3 days after switching to a nucleic acid-free diet. Similarly, it was assumed that a maximum level of 8-oxoGua excretion was reached 3 to 5 days after reverting to a normal diet. Additional sampling points throughout this study would make these conclusions more definitive.

Studies using radiolabeled lesions are preferred over those involving nucleic acid-free diets as the use of radiolabels provides a degree of stringency not afforded by nucleic acid-free diets. However, for ethical reasons, radiolabeled lesions are to be avoided in human studies. To circumvent this issue, Cooke et al. (32) recently completed a feeding study using heavily oxidized, stable isotopically labeled [¹⁵N]-DNA (98% incorporation). Healthy male volunteers were fed oxidized [¹⁵N]-DNA, and first void, midstream urine samples were collected for up to 14 days later. The presence of [¹⁵N₅]-8-oxodG and [¹⁵N₅]-8-oxoGua in urine was examined using HPLC GC/MS. Neither lesion was detected in any of the urine samples. To confirm that the absence of lesion was not a sensitivity issue, urinary [¹⁵N₅]-dG and [¹⁵N₅]-Gua, which should be present at significant levels, was also examined. Again, neither of these labeled compounds were detected in any of the urine samples. The authors also investigated whether any of the ingested [¹⁵N₅]-Gua/[¹⁵N₅]-dG had been degraded to [¹⁵N₅]-uric acid, the final product of purine metabolism, before excretion.

Again, no labeled material was detected. As with previous studies (75), the authors could only presume that the isotopically labeled DNA components/adducts pass through the gastrointestinal tract to appear in feces. Nevertheless, these results, coupled with the findings of Gackowski et al. (87), provide the most compelling

argument that diet is not a significant contributor to both urinary 8-oxoGua and 8-oxodG levels in human urine. This is consistent with the animal data relating to 2'-deoxyribonucleoside lesions (75), which includes thymidine glycol (23), but disagrees with early data for 8-oxoGua and thymine glycol (23, 89). Species differences may be proposed to account for this discrepancy, although the focus of our attention must be upon the more relevant human data. Earlier animal studies should be repeated using the highly specific and sensitive mass spectrometric techniques.

Cell Death/Turnover. In 1993, it was asserted that urinary 8-oxodG did not reflect DNA repair, as it was not a product of base excision repair (90) rather it was a product of nonspecific nucleases acting upon DNA released from during cell death, liberating dG, which is subsequently oxidized. This discounted the potential existence of other, as yet undiscovered, repair pathways which may yield 8-oxodG. Whereas repair processes, whose reaction product may be 8-oxodG, have been described (see below), the possible contribution from cell turnover remains to be addressed, although some data are beginning to emerge. For example, it has been reported recently that rat liver homogenates can release 8-oxodG from oxidatively modified DNA (91). The authors suggest that, during DNA degradation, most of the 8-oxodG, present in DNA, is released by enzymes and subsequently appears in the urine, implying this to be a major source. However, they do acknowledge that their system does not accurately represent the *in vivo* situation, not in the least as it is unrealistic — multiple un compartmentalized nucleases acting upon circulating DNA, particularly as the majority of cell death in healthy humans will be via apoptosis.

The evidence against a contribution from cell turnover has, for the most part, been anecdotal. There exists a number of reports in which urinary 8-oxodG has been measured in patients undergoing chemotherapy in the absence of concomitant increase in urinary 8-oxodG (reviewed in ref. 66). As yet, the most decisive argument against the contribution of cell death to urinary levels of 8-oxodG and 8-oxoGua comes from a recently published report from the Olinski group (92). The findings from this study are supported by the results of Weimann et al. in a study examining oligonucleotides in urine, which concluded that the limited excretion of oligonucleotides into urine argues against oligonucleotides or, indeed, nucleosides originating from cell death (71). On balance, these results suggest that the contribution of cell death to urinary 8-oxodG levels is minimal, but clearly further work needs to be done.

DNA Repair. With respect to DNA repair, the source of oxidatively modified DNA bases in extracellular matrices seems clear. The DNA N-glycosylases responsible for removing modified bases are increasingly well defined (93) and have been discussed thoroughly elsewhere (2). 8-Oxoguanine DNA glycosylase 1 (OGG1) is the major enzyme involved in the removal of 8-oxoGua from cellular DNA. Recently, levels of 8-oxoGua were determined in *OGG1*^{-/-} mice and compared with a wild-type strain. There was ~26% reduction in levels of urinary 8-oxoGua in the deficient strain compared with the wild type, and no significant changes in 8-oxodG were observed (94). This indicates that mouse OGG1 glycosylase is a significant, but by no means unique, source of urinary 8-oxoGua.

The results clearly suggest the existence of back-up DNA glycosylase(s) that cannot entirely compensate for OGG1 deficiency. The reversion to a less efficient back-up system should result not only in reduction of 8-oxoGua in urine but is also expected to lead to the simultaneous, gradual increase in the background level of 8-oxoGua in cellular DNA (discussed fully in ref. 95). In female *OGG1*^{-/-} mice of 8 to 10 weeks of age, no significant increase in 8-oxoGua, assessed as formamidopyrimidine DNA glycosylase-sensitive sites, was found in the liver compared with wild-type mice, whereas the level in lung tissue was 3-fold greater than wild type (96). The faster accumulation in lung could be due to high level of oxygen exposure compared with liver.

The *OGG1*^{-/-} mice also accumulate much more 8-oxodG in target organs after exposure to oxidative stress-inducing agents, such as diesel emission particles or potassium bromate. In the mitochondrial genome from liver tissue, a 20-fold increase in the 8-oxoGua level was observed in the *OGG1* defective mice compared with wild type (97). Taken together, these data suggest the existence of another repair pathway, the product of which is not 8-oxoGua or indeed 8-oxodG that maintains lowered 8-oxoGua levels in most tissues even in the absence of OGG1. It is worth considering that the rate of 8-oxoGua accumulation in DNA may be determined by the proliferation rate of the tissue so that rapid proliferation may dilute the effect of OGG1 deficiency, whereas 8-oxodG accumulates in liver, lung, and muscles with a low proliferation rate.

The presence of 2'-deoxyribonucleoside lesions in extracellular matrices is very much less well defined, as

there are no reports of a single-DNA repair enzyme whose activity yields 8-oxodG. Based upon existing evidence, we have proposed the following three DNA repair routes (Fig. 3) as the most likely contributors to the presence of oxidatively modified 2'-deoxyribonucleosides in urine.

Nudix Hydrolases. There is an imperative for preventing modified DNA precursors from being incorporated into the genome. The best characterized enzyme which performs such a role is the 8-hydroxy-2'-deoxyguanosine triphosphatase (68) activity of NUDT1 (MutT homologue, MTH1), hydrolyzing 8-hydroxy-2'-deoxyguanosine triphosphatase to 8-oxodGMP (Fig. 3). It has been suggested that further processing, perhaps by 5'(3')-nucleotidases, may give rise to 8-oxodG, which is not charged and can be removed from the cell, ultimately appearing in the urine (68). Recent data from the group of Harms-Ringdahl strongly imply that NUDT1 activity and, thus, the nucleotide pool represent a major source of extracellular 8-oxodG (98). These studies in cell culture used small interfering RNA gene expression knockdown methodology directed at human *NUDT1*. However, the roles of other Nudix hydrolases, such as NUDT15 (MTH2) and NUDT5, which include 8-hydroxy-2'-deoxyguanosine triphosphatase and 8-oxodGDP among their substrate repertoire, respectively, remain to be defined (99, 100). These new observations on the contribution of NUDT1 to extracellular 8-oxodG, coupled with any potential negation of the role of diet and cell turnover, could mean that we are very close to determining the biological significance of urinary 8-oxodG levels as markers of oxidation in and sanitization of the nucleotide pool.

Nucleotide Excision Repair. Despite seeming to be directed principally toward bulky lesions, such as cyclobutane thymine dimers (T<>T), there is some evidence that nucleotide excision repair (Fig. 3) may act upon nonbulky lesions, such as 8-oxoGua (72). Indeed, the rate of 8-oxoGua removal seems comparable with that for T<>T (72), seemingly generating a lesion-containing oligomer, ~24 to 32 nucleotides long (101). A recent report failed to show 8-oxoGua-containing oligomers in urine (71), implying that further processing occurs, perhaps ultimately yielding 8-oxodG, or that they do not exist. However, under normal circumstances, the role of nucleotide excision repair in the removal of 8-oxoGua and perhaps other small oxidatively generated DNA lesions would seem to be negligible (102-106), although results from xeroderma pigmentosum cell lines have not entirely excluded the possibility (72, 103, 107, 108).

Endonuclease(s). A poorly characterized endonuclease has been reported by Bessho et al. (109), which, lacking a glycosylase activity, is predicted to give rise to 3',5'-8-oxodGDP as the putative product. We have previously proposed that this may be subsequently hydrolyzed to 8-oxodG by nucleotidase(s). The recently described nucleotide incision repair process generates lesion-containing X'-dNMP products; again, these could be subsequently metabolized to a 2'-deoxyribonucleoside. However the activity of nucleotide incision repair toward 8-oxodG-containing substrates is reported to be negligible. Whereas this may be the case, it is entirely feasible that this process could be a potential source of other extracellular lesion-containing 2'-deoxyribonucleosides.

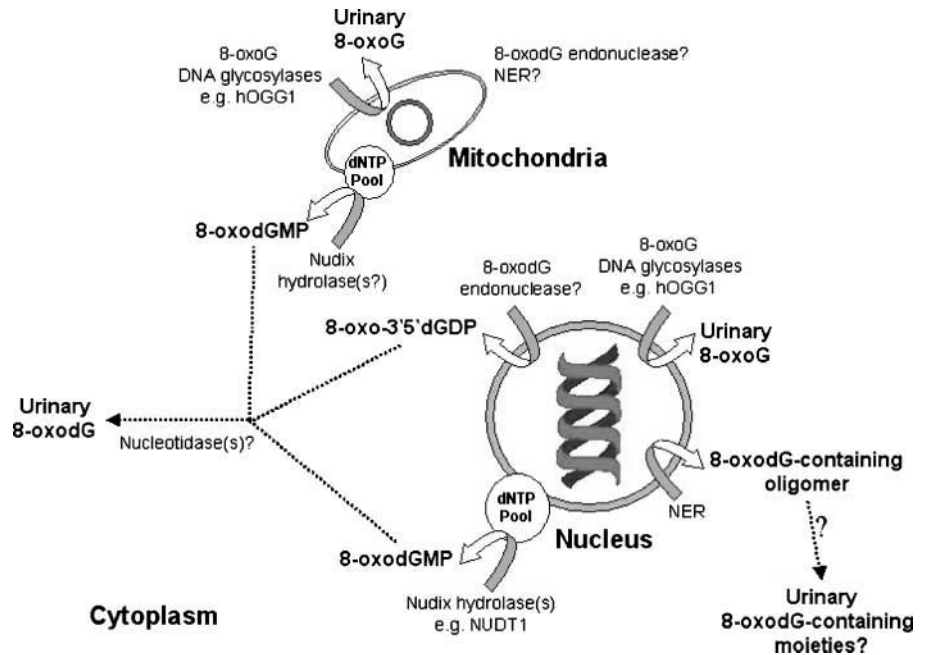


Figure 3. DNA repair sources of extracellular 8-oxodG and 8-oxoGua. *NER*, nucleotide excision repair; *hOGG1*, human 8-oxoguanine glycosylase.

Although the types of lesion that have been examined as substrates have yet to be detected in urine.

European Standards Committee on Urinary (DNA) Lesion Analysis

There is a pressing need for (noninvasive) biomarkers of oxidative stress that are sufficiently validated to be applied to large-scale, molecular epidemiology studies. Validation should include a detailed understanding of the biomarker's provenance plus robust and reproducible methods for analysis. The noninvasive nature of such biomarkers minimizes ethical issues and allows studies to incorporate vulnerable subjects, such as the very young and the very old. Furthermore, the biomarkers should have long-term stability under routinely used conditions, reducing the need for new, prospective studies by allowing retrospective analysis of existing cohorts. Widespread agreement between methods for biomarker analysis will allow multiple accredited centers to perform analyses, the results of which may hence be pooled and reduce the burden on any one laboratory.

Urinary 8-oxodG is an ideal candidate for fulfilling these criteria. European Standards Committee on Urinary (DNA) Lesion Analysis (ESCUA) has been formed to complete the validation of this biomarker of oxidative stress. As this review indicates, the origin of 8-oxodG in urine is perhaps not as simple as first thought. ESCULA is currently addressing this issue through [$^{15}\text{N}_5$]-labeled DNA feeding studies, experiments incorporating knockout mice and markers of cell turnover. As noted above, there is a discrepancy in basal urinary 8-oxodG levels when comparing chromatographic techniques with ELISA, although all techniques have been shown to discriminate between diseased and healthy subjects. ELISA has received widespread use and is clearly amenable to the greatest number of laboratories; however, this discrepancy continues to raise questions

regarding its utility. To continue the validation of urinary 8-oxodG, ESCULA is presently conducting a multicenter study, incorporating numerous platforms and methodologies, including ELISA (see Table 2). We propose that understanding the basis of this discrepancy will aid our understanding of the significance of urinary lesions. An additional outcome of this interlaboratory study will be the provision of validated assays for urinary 8-oxodG measurement for widespread dissemination and application. Hitherto, this has been done in a limited fashion, and the discrepancies remain unaddressed. ESCULA will also provide advice concerning best practice, for example, serious consideration needs to be given to how results for urinary lesion measurements are expressed and the applicability of correcting for creatinine. Finally, there is growing clinical interest in the measurement of urinary 8-oxodG as a means to determine the role of oxidative stress in disease and evaluate intervention strategies. With assays validated, numerous laboratories may work together to establish reference ranges for normal and pathologic conditions and conduct the new molecular epidemiologic studies.

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