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Review

Detection and interpretation of 8-oxodG and 8-oxoGua in urine, plasma and cerebrospinal fluid☆,☆☆,★

Henrik E. Poulsen [a,b,*], Laia Llovera Nadal [c], Kasper Broedbaek [a,b], Peter E. Nielsen [c], Allan Weimann [a,b]

[a] Department of Clinical Pharmacology, Bispebjerg Hospital, Copenhagen University Hospital, DK-2200 Copenhagen N, Denmark
[b] Laboratory of Clinical Pharmacology Q7642, Rigshospitalet, Copenhagen University Hospital, DK-2100 Copenhagen, Denmark
[c] Institute of Cellular and Molecular Medicine, The Panum Institute, Health Science Faculty, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark

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ABSTRACT

Background: DNA and RNA oxidations have been linked to diseases such as cancer, arteriosclerosis, neurodegeneration and diabetes. The prototype base modification studied is the 8-hydroxylation of guanine. DNA integrity is maintained by elaborate repair systems and RNA integrity is less studied but relies mainly on degradation.
Scope of review: DNA and RNA oxidations are measured by very similar techniques. The scope of this review is to highlight the preferred methods of measurement of oxidized nucleic acid metabolites, to highlight novel findings particularly in RNA oxidation, and to present the interpretation of the measurements.
Major conclusions: Tissue levels are snap-shots of the level in a specific organ or cell system and reflect the balance between formation rate and elimination rate (repair), and must be interpreted as such. Urinary excretion is a global measure of oxidative stress in an organism and is therefore best suited for situations or diseases where large parts or the entire organism is stressed by oxidation. It represents the body average rate by which either RNA or DNA is oxidized and is interpreted as oxidative stress. Oxidations of RNA and DNA precursors have been demonstrated and the quantitative importance is debated.
General significance: Careful experimental designs and appropriate choice of methodology are paramount for correct testing of hypotheses related to oxidative stress, and pitfalls are plentiful. There is accumulating evidence that DNA oxidation is associated with disease, particularly cancer, and recent evidence points at an association between RNA oxidation and neurodegenerative diseases and diabetes. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

Correct and error-free copying, transcription and translation of the DNA code are essential cellular processes. Previous decade's research has focused on DNA and the processes that ensure a stable genome and correct translation of protein coding genes. The last decade has revealed that the transcription of DNA to RNA is not restricted to the protein coding genes and includes complex regulatory processes where a plethora of non-coding RNAs (ncRNAs) play a central role. The focus on genetic changes in DNA and their relevance for disease development is now expanded to include this ever growing and increasingly complex RNA regulatory network. Perturbations of the RNA regulatory network have already been associated with diseases and many more discoveries in this field are expected. However, RNA modifications such as oxidation are only recently being recognized as disease relevant mechanisms. Nonetheless the few results obtained so far hold promises that further research along these lines could significantly contribute to a deeper understanding of disease processes and unravel new targets for intervention.

The synthesis, maintenance and decay of RNA and DNA differ in many ways. This is summarized in Fig. 1. It is textbook knowledge that phosphorylated ribose (Rib-5P) and ATP initiate the synthesis of the (ribo)nucleoside diphosphates, of the adenine, guanine, thymine and cytosine bases. The bicyclic structure of purines (guanine and adenine) requires more energy for the synthesis than the pyrimidines (thymine, uracil and cytosine), which is probably why pyrimidines are synthesized de novo and purines are salvaged for reutilization during the catabolism of nucleic acids (not shown in Fig. 1). The final catabolic product of purine nucleotides is uric acid (not shown in Fig. 1). De novo synthesis of ribose/2-deoxyribose includes the use of water and this step can be utilized to label the ribose moiety with deuterium for the study of nucleic acid synthesis rates [1].

The (ribo)nucleoside diphosphates are converted to triphosphates and polymerized into RNA by RNA polymerases based on the sequence

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* Corresponding author at: Laboratory of Clinical Pharmacology Q7642, Rigshospitalet, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark. Tel.: +45 3545 7671.
E-mail address: hepo@rh.dk (H.E. Poulsen).

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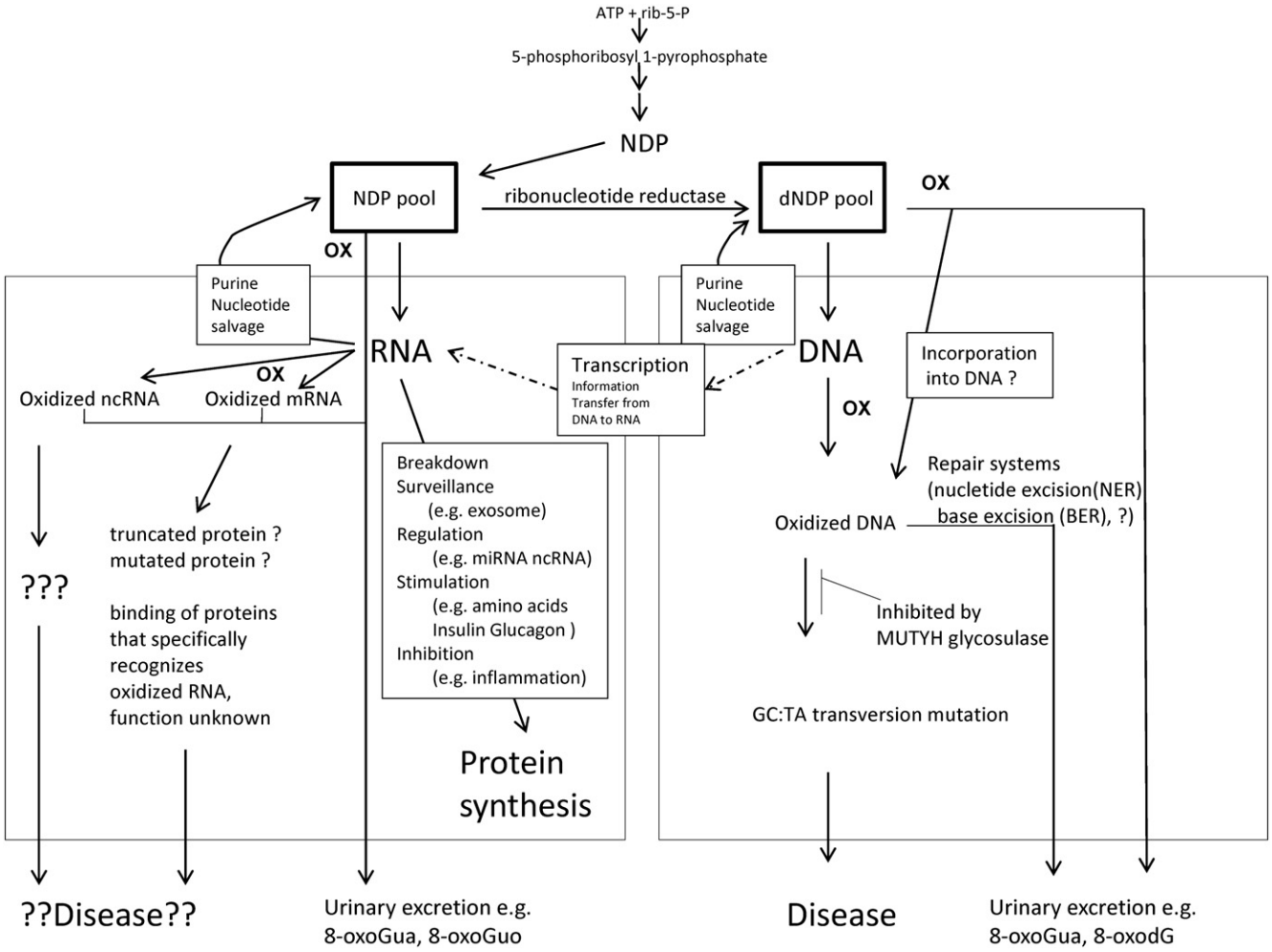

Fig. 1. Depict the common origin of RNA (right box of figure) and DNA (left box of figure), and the locations where oxidation can occur (OX). Note that oxidation can also occur in the precursor pools. The DNA integrity mainly relies on DNA repair mechanisms, whereas the RNA integrity mainly relies on RNA surveillance and breakdown. In DNA integrity failure leads to GC:TA transversion mutations. Failure in RNA integrity can lead to formation of truncated or mutated proteins. The importance and functions of oxidized non-coding RNAs are unknown.

read from DNA. The eukaryotic RNA polymerase II primarily produces mRNA. The steady state concentration of mRNA in the cell is not determined solely by the transcription rate, but to a large extent by RNA turnover, and is controlled by processes including 5′end capping, 3′ polyadenylation and degradation by a dedicated exonuclease in the exosome complex [2–4]. The half-life among the about 20,000 mRNAs varies considerably from minutes to days with a median of about 12 h [5]. The half-life of an mRNA correlates positively with the number of exon junctions per open reading frame, and negatively with the presence of PUF-binding motifs and AU-rich elements in the 3′-untranslated region and CpG dinucleotides in the 5′ UTR region [5]. The functional half-life is increased with the presence of 3′ UTR G-U rich elements. In mitochondria several RNAses are suspected, and some are identified, but the complicated RNAse machinery is still best characterized as a mystery [6]. Even though much is not understood, it is clear that mRNAs are continuously synthesized and degraded to a much larger extent than previously thought. It seems clear that RNA processing and degradation play a critical role in post transcriptional gene regulation and thus protein expression.

The about 20,000 genes in the human genome only correspond to 1–2% of its entire sequence. The last decade has revealed transcripts of (short) RNAs with regulatory and other functions called non-coding RNAs (ncRNA). The relevance of such ncRNAs in human disease has predominantly been studied for miRNAs, but “we are only beginning to understand the nature and extent of ncRNA in disease” [7]. miRNAs are short 19–24 bp RNAs, and are also implicated in a long list of non-cancer diseases [7] but also >200 bp ncRNA have been described and established as players in epigenetic mechanisms in many cancer types. The miRNAs act as post-transcriptional key regulators of gene expression as part of a complex RNA regulatory network and are particularly prevalent in higher organisms, where at least 1500 miRNA genes have so far been identified. Moreover, it seems that the more complex the organism the more complex its RNA regulatory network has evolved [8]. The complexity of the RNA regulatory network is immense; however modern high through-put sequencing has enabled comparisons based on RNA sequence and identification of motifs involved in diverse cellular processes where one RNA interacts with another RNA (or DNA) in nearly all aspects of regulation in gene expression, including transcription, splicing, localization and decay [9].

The DNA integrity maintenance systems have been known longer than the complex RNA regulatory network. The unique double helix structure of DNA makes it possible to repair single strand lesions using the undamaged strand as template, a process which is not possible for the single stranded RNA. There are multiple DNA repair processes that deal with all possible types of modifications [10,11]. Originally DNA modification by oxidation was linked to the development of cancer [12,13]. More recently deficiencies in the DNA repair systems are also linked to the development of cancer and aging [11,14].

The amount of urinary excreted oxidized bases from DNA, measured as the excretion of the oxidized guanine nucleoside, is up to

about 60 pmol per 24 h [15,16]. This corresponds to at least 500 oxidations of guanine per day per cell, and would result in a doubling of the number of oxidized nucleobases per 3 months, and oxidation of 1:100 bases in DNA after 8 years if not repaired. Evidently, an elaborate repair and maintenance machinery must be effective, including sanitation of oxidized nucleotides in the cellular pool, so that oxidized precursors are not incorporated into DNA during copying or repair (Fig. 2).

The sources of ROS (reactive oxygen species include singlet oxygen, superoxide anion radicals, hydroxyl radicals, hydrogen peroxides, peroxynitrate) capable of oxidizing nucleic acids or their building elements and precursors are multiple. ROS as "by products" of the normal intracellular metabolism [17] is a major endogenous source as are also immune and inflammatory reactions [18]. Exogenous sources include ionizing radiation, UVA and visible radiation (via sensitizers), environmental pollutants including some nano-particles, some carcinogenic compounds, redox-cycling drugs and many more [19].

The number of modifications described in DNA amounts to 100+, and includes modifications of the base, the sugar moiety, and strand breaks [17]. Among the bases, guanine has the lowest redox potential, thereby being the best electron donor and the predominantly oxidized base by $^1O_2$ but not by the hydroxyl radical, where thymine predominantly is the target [20,21]. Furthermore, probably because the first available and sufficiently specific and sensitive analytical method measures guanine modification by oxidation [22,23], it is by far the most extensively studied lesion by oxidation. Novel readers in this field should be aware that the nomenclature used by different authors is not consistent, and that abbreviations for the same chemical entity measured differ over time and between authors. Recently, a unifying terminology, classification and nomenclature have been proposed [24]. For the ease of reading we use 8-oxodG and 8-oxoGuo for the oxidized guanine nucleosides from DNA and RNA, and the oxidized base, 8-oxoGua.

As regards RNA, a much smaller variety of oxidative lesions have so far been characterized, presumably due to little interest in the oxidation of these nucleic acids over the years. Nevertheless, guanine oxidation can also take place in the RNA, being even more prone to oxidation than the DNA because of its cytosolic location closer to the mitochondria, its single stranded structure and its lack of protecting proteins such as histones [25].

2. Recent relevant reviews

A key discovery of thymine hydroxyhydroperoxides was done already in 1959 by Ekert and Monier [26]. The pioneering characterization of 8-oxodG as the main Udenfriend's reagent-mediated oxidation product of 2′-deoxyguanosine in 1984 by Kasai and Nishimura [23,27] has been a major scientific event in the field of base damage to DNA generated by oxidation. The subsequent application of the HPLC-electrochemical detection (ECD) method for monitoring 8-oxodG in cells that was developed by Floyd et al. [22] has provided a very strong impetus to the use of this lesion as an indicator of oxidative stress in cells and animals. And also hydroxyl radical ($HO^{\bullet}$)-mediated precursors of 5,6-dihydroxy-5,6-dihydrothymine have been identified as the main oxidation product to cellular DNA upon exposure to gamma radiation [28]. Many reviews of nucleic acid oxidation have been published within the latest years, indicating that the field has grown to the extent that a review must focus on specific topics, and instead of compiling yet another review we provide a list in Table 1 of selected recent reviews and their focus that the reader with a special interest can consult for detailed information.

3. The use of the oxidized nucleotides from DNA and RNA as biomarkers

Although many different modifications in DNA [29] and a few in RNA [25,30] have been described, the vast majority of investigations have focused on guanine oxidation. This is most probably because of the particular susceptibility of guanine to oxidation, and the earlier developed HPLC-ECD methodology, which requires less expertise and cheaper equipment, and therefore is easier to implement than the cumbersome mass spectrometric techniques.

In the following decades most investigations were done by analysis of the nucleoside 8-hydroxydeoxyguanosine (nomenclature for the 8-oxo-tautomer is 8-oxo-7,8-dihydro-2′-deoxyguanosine, abbreviated 8-oxodG) or the corresponding base 8-hydroxyguanine in tissue or cells. It was, however, realized that measurements in many cases resulted in overestimated and poorly reproducible values because of artificial oxidation during the procedures before the actual measurements, and the European Standards Committee on Oxidative DNA Damage (ESCODD) network of more than 25 laboratories worked out procedures that reduce or eliminate these artifacts [31–34].

In the early 1990es we published the first practical assay for measuring 8-oxodG in urine, where we investigated the factors influencing its excretion from 83 randomly selected healthy subjects [16]. The rationale for choosing the nucleoside was that the diet and gut bacteria contained oxidized DNA. The nucleoside bond between base and sugar is broken in the gut and therefore the oxidized base, 8-oxo-7,8-dihydroguanine (8-oxoGua) is absorbed from the gut into the organism and thereafter excreted into urine, whereas the corresponding nucleoside, 8-oxodG, is not absorbed and therefore can only originate from the body's cells, i.e. the excretion is independent of the content in the food and gut bacteria. This was originally advocated for [35], but later it has been suggested that this is not the case

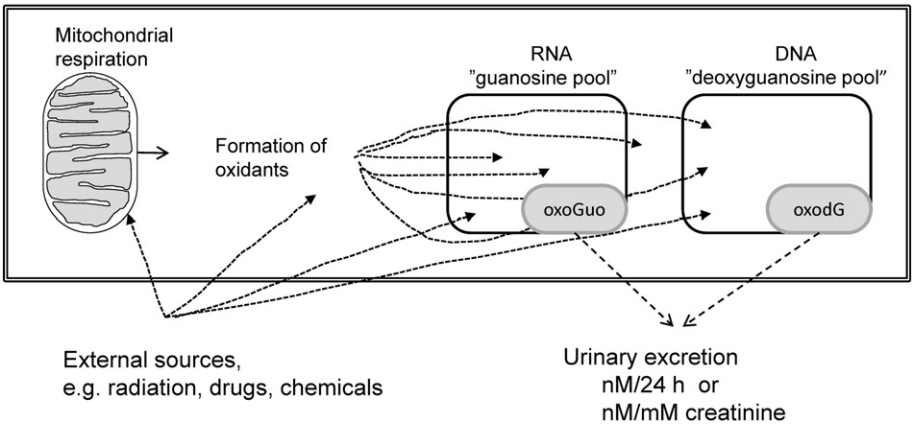

Fig. 2. Mitochondrial dysfunction leads to increase production of oxidants that can oxidize RNA and DNA. Note that in the text we state that oxidized bases in the DNA or RNA also can originate from the oxidation of precursor pools (not shown in the figure). The quantitative contribution from the oxidation of precursor pools is not known.

Table 1
Focus of recent selected reviews on nucleic acid (NA) oxidation.

Review focus	Reference
Special section on oxidation–reduction of nucleic acids and biophysics of nucleic acids	[75]
Analysis of urinary nucleic acid oxidation biomarkers, both DNA and RNA biomarkers, and chromatographic methods	[15,76]
Discussion of artifacts in the measurement	[37]
Relation between DNA repair and cancer	[77,78]
Measurement in cellular DNA	[36]
Comprehensive book covering all aspects of oxidative stress, particularly suited for researchers new in the field	[19]
RNA oxidation and disease mechanisms	[25,30]
Latest standardization schemes for analysis in tissue and urine	[79]
Latest suggestion for nomenclature	[24]
Urinary excretion of the oxidized guanine base	[43]
Problems with the ELISA analysis	[51,80]
Sensitivity and specificity issues	[36,62]
Alzheimer's disease	[81,82]
Diabetes	[83]
Comprehensive lists of findings in various diseases	[84,85]
Cardiovascular diseases	[86,87]
Aging	[88]
Comprehensive lists of lesions, methodologies and much more	[29]

in humans. This issue is still controversial and not subjected to rigorous investigation. Many factors and diseases are associated with high or low excretion of 8-oxodG (see the reviews in Table 1). A large number of intervention studies with 8-oxodG as a biomarker have been published, but a review of these is beyond the present scope.

4. Interpretation of urinary excretion of oxidized nucleic acid metabolites versus tissue levels

Nucleic acid oxidation levels can be measured in two principally different ways: levels in specific tissues or cells, and urinary excretion. Some authors also use the plasma levels of oxidized nucleic acid metabolites, and a few authors report on concentrations in CSF and plasma.

Tissue measurements, as performed in the ESCODD program and by others [31–34,36,37], are often presented as number of oxidized guanines per total number of guanines, usually about 1–5 per 10^6 in DNA. As such it is a concentration measurement at the time of the biopsy of tissue or harvesting the cells. These levels represent the actual balance between formation and repair rates, but can fluctuate quite dramatically e.g. after the administration of agents that induce guanine oxidation [38], or changes in the repair mechanisms. Conceptually, one would interpret the rate of oxidation as oxidative stress, but according to the original definition by Helmut Sies oxidative stress is the balance between oxidative and anti-oxidative processes [39]. In knock-out animals the lack of OGG1 repair system leads to increased levels of oxidized guanine in DNA [40,41] and in yeast OGG1 knock-out to increased accumulation of GC:TA transversion mutations [40]. Surprisingly, OGG1 knock-out animals exhibit normal life-span [40], but show increased metabolic syndrome and diabetes development [42]. This means that the interpretation of the levels of oxidized guanine in DNA or RNA is difficult and cannot alone be used to draw conclusions about long term effects. Furthermore, there is no evidence, i.e. no data, that concentrations in target organs are predictive for development of e.g. cancer in that particular organ. In cellular systems and model organisms like yeast, investigations of the level of oxidative modification in nucleic acids can provide important information about basic mechanisms, but cannot per se be taken as evidence for predicting disease development in vivo.

Measurement of urinary excretion of nucleic acid metabolites, e.g. 8-oxodG and 8-oxoGua, has gained increasing interest, probably because it is a non-invasive method that can be used in vivo, in humans as well as animals, and because of the advancement and improved sensitivity and user-friendly interface of liquid-chromatography with electrospray-tandem-mass spectrometry detection. Fig. 1 depicts the basic interpretation of the urinary excretion of 8-oxodG and 8-oxoGuo. A detailed review of the use of the oxidized base as a biomarker has recently been published [43] (Table 1). The traditional view has been that the oxidation of guanine in DNA/RNA will eventually result in the release of such nucleosides from the cell to the blood stream and their excretion into urine by the kidneys, with a half-life in plasma of a few hours [44], and therefore their urinary excretions are interpreted as a measure of DNA/RNA oxidation. Detailed knowledge of this breakdown/repair mechanism is still deficient and poorly understood, as are the transport systems. Furthermore, it has recently been demonstrated that oxidation of nucleotide precursors can also take place in the nucleotide pool leading to incorporation into nucleic acids, although enzymes are present that sanitize the pool in order to minimize the process [45–48]. The quantitative contributions between oxidation of guanine in the DNA/RNA and the precursor pools are uncertain, and in the case of RNA it is limited to few studies. The biochemical pathways that produce 8-oxodG and 8-oxoGuo are thought to be the nucleotide excision repair and RNA breakdown enzymes. This is not fully elucidated. Very recently, it has been demonstrated in *Escherichia coli* that the rate of incorporation of 8-oxo-GPT is about 4% [49]. This however is at variance with the findings by Tim Hofer et al. [50] who reported about 3 oxidized guanines per 10^6 guanines in RNA, which corresponds to unpublished results from our lab. On the other hand, unpublished preliminary results from our lab indicate that in rat liver the ratio of 8-oxoGuo/Guo in the pool is about 1:20,000, which indicates that the mononucleotide pool is oxidized about 15 times more than RNA. However, the total content of guanine in the nucleotide pools is much smaller than in the DNA and RNA in a cell. Therefore, in order to contribute significantly to the total amount of oxidized bases in the cell, both a very high oxidation rate in the pool and a very high incorporation rate into RNA and/or DNA would be required, probably higher than the 4% reported.

Presently it is the accepted interpretation that the excretion of the oxidized nucleosides, 8-oxodG and 8-oxoGuo into urine represents the rate of oxidation of guanine in the respective nucleic acid and procure pools. This interpretation has the assumption that an individual, man or animal, is at a steady state with no change in the rate of oxidation for a reasonable time period of days or weeks. In that situation, the number of guanine moieties oxidized in the nucleic acid and its precursor pool is constant and the number of oxidations per time unit must equal to the number removed/excreted from the cell. Therefore the urinary excretion equals the guanine oxidation rate, usually expressed in nanomoles per 24 h. This requires constant nucleic acid repair (for DNA) and breakdown (for RNA) rates. However, if the oxidative stress is unchanged and the repair/breakdown is changed to another level, after some time a new steady state will occur where the number of oxidized moieties again will equal the number excreted. Therefore the urinary excretion is a measure of oxidative stress that is independent of changes in repair/breakdown. If fluctuations occur in oxidative stress and/or repair/breakdown, the interpretation is difficult. It is therefore important to assure a steady state when designing the experiment so that the data can be interpreted correctly.

In many cases it is difficult to collect urine for 24 h, and the use of a spot urine sample has been advocated in order to correct variability in fluid intake/excretion by the kidneys. The usefulness of this methodology, relying on a creatinine correction approach, has recently been confirmed by a large study [51]. Nevertheless, it should be recognized that the creatinine correction method where the spot urine 8-oxo concentration is divided by the creatinine concentration in the same urine sample, has some pitfalls. Considering the ratio 8-oxo(dG or Guo)/creatinine it is clear that the ratio can increase because of a higher excretion of 8-oxo(dG or Guo), or a lower creatinine, and vice versa for a decreased ratio. The use of the ratio therefore assumes that creatinine excretion is constant. The total creatinine excretion is mainly determined by the muscle mass and the concentration in urine

modified by the water intake/excretion, e.g. a person excretes the same amount of creatinine per 24 h, but the concentration is doubled if urine output is 1 l/24 h compared with 2 l per 24 h. Likewise the creatinine concentration is very low shortly after water intake when very "thin" urine is produced. The spot urine with creatinine correction method is therefore particularly useful e.g. when making a paired experiment (persons or animals serving as their own control), or in randomized studies where the determinants for creatinine can be assumed to be equally distributed in the groups that are compared. The use of creatinine correction will introduce bias when it cannot be assumed that the creatinine is constant or even distributed between the groups to be compared. Examples of bias include comparison of children with adults, controls with patients with cachexia, and gross weight loss. Considerable care must be taken to ensure an experimental design avoiding bias from differences in creatinine excretion. Similar considerations must be given when comparing spot urine samples corrected by fluid intake by specific gravity [51].

As indicated previously the interpretation of 8-oxodG level is global DNA oxidation and of 8-oxoGuo level is global RNA oxidation. The method represents an average of all tissues in the organism under investigation, which at the same time represents a limitation of its use. Assume that prostate cancer is associated with a 20 fold increase in oxidative stress in the prostate gland only. Since the prostate contributes about 25 g to the 70–90 kg body weight of a man, the increase will be only $(25 * 20 / 75000) * 100\% = 0.7\%$, clearly a difference that will be impossible to detect. The method is therefore most useful in situations where large organs or the entire organism is responsible for the increase. Still, it should be borne in mind that the contribution from oxidation in the nucleic acid and the precursor nucleotide pools is uncertain.

Empirically, urinary excretion of 8-oxodG predicts lung cancer in non-smoker [52] and breast cancer I women [53], and urinary 8-oxoGuo excretion predicts death and death from complications in type 2 diabetes [54,55].

5. Interpretation of plasma or cerebro-spinal fluid (CSF) levels of oxidized guanine nucleosides

Some researchers have used plasma levels of 8-oxodG as a measure of oxidative stress. We do not think that this is a valid measure for the following reasons. 8-oxodG is produced by the cells, transported in the blood and excreted by the kidneys. This is a process that is well described in many physiological textbooks. Such substances, either endogenously produced or infused, are used to determine kidney function and have gained widespread clinical use. The level in plasma is mainly determined by the kidney function and when comparing different individuals the measure will not provide information about oxidative stress, but of excretory kidney function. Severe kidney function deterioration, i.e. kidney failure or chronic kidney disease is associated with severe diseases such as increased atherosclerosis, so it would be easy to establish an association between blood levels of 8-oxodG and mortality. However, this cannot be interpreted as increased oxidative stress. Only in certain cases, e.g. paired experiments where one can assume that the kidney function is identical in two situations; it might be possible to interpret changes in plasma levels as oxidative stress. Detailed considerations or empirical evidence for such use does not exist, and the variability is unknown and might be large, and thereby prohibits its use. Sadly, plasma levels measured by ELISA have been commercially advocated as a measure of oxidative stress — we do not find this sufficiently documented, and for the reasons given above it cannot be recommended. The conventional chromatographic methods do not have the sensitivity to measure plasma levels, but require either specialized set-up or may be achieved with the latest generation UHPLC with tandem mass spectrometry.

A few publications report on the concentrations in CSF [56–61]. As indicated below there is no agreement on the levels and the analysis is a challenge, but increased levels probably represent oxidative stress in the central nervous system. Both increased 8-oxodG and 8-oxoGua concentrations have been reported in CSF from patients with neurodegenerative diseases, see reviews [25,30] in Table 1.

6. Quantification of oxidized nucleosides in biological matrices

Deoxyribonucleosides and ribonucleosides have been quantified in several different biological matrices, of which tissue (or cells), urine and cerebrospinal fluid (CSF) have received the main attention. These are analytically challenging in different ways.

Tissue: its main obstacles are sensitivity and artificial oxidation. There is often more than 1,000,000 fold excess of unmodified nucleosides relative to the nucleosides damaged by oxidation, typically a few modifications within the range 10^6 to 10^8 normal nucleosides can be detected in DNA [62].

In order to measure the ratio between damaged and non-damaged nucleosides, the first step is extracting the nucleic acids from the tissue. Following this, nucleic acids have to be hydrolyzed either enzymatically to free nucleosides or by acid hydrolysis to free nucleobases. As a last step, before analysis by GC–MS, free nucleosides or nucleobases have to be derivatized. All of these steps can induce artificial oxidation and since the ratio between oxidized and non-oxidized analytes is high, the risk that even a small amount of artificial oxidation may affect the result is significant. The problem has been very well described by Cadet et al. [63,64] and has been one of the main objectives of the ESCODD project [65–68].

Urine: the major problem encountered in urine analysis is to obtain sufficient specificity, especially for ELISA. No pure extract of nucleic acids can be made, meaning that free nucleosides or nucleobases have to be separated and/or distinguished from an overwhelming amount of possible interfering substances in the urine.

Moreover, in contrast to the situation in tissue, nucleobases or nucleosides are already present as free analytes and thus there is no need for nucleic acid extraction and hydrolysis. Moreover, the ratio of oxidized to non-oxidized nucleosides is in the range of 1 to 20 [69], which means that the risk for a small degree of artificial oxidation to affect the final result is low.

Cerebrospinal fluid: CSF measurements have shown high variability among different reports. The situation resembles a bit that of the urine, where nucleosides are present as free analytes and there is no problem with overwhelming amounts of non-oxidized analytes. Neither is specificity expected to be an issue. Nonetheless, widely different concentrations of 8-oxodG have been detected in CSF. Bogdanov et al. [58] report a value for healthy adults of approximately 3.5 pM. In contrast to this Gackowski et al. [59] report a value of 0.26 nM, Gmitterová et al. [60] report a value of 2.5 nM and Lovell and Markesbery [61] report 683.7 nM as means for control subjects, and Abe reports about 0.1 nM 8-oxoGuo [56,57]. Preliminary and unpublished results from our own laboratory indicate about 1 nM dG and similar 8-oxodG levels to those of Bogdanov. The high variability with a range of 2×10^5 fold most probably indicates an analytic specificity problem rather than biological variation.

7. Quantification of oxidized nucleobases and nucleosides by different analytical methods (strengths and weaknesses)

The quantification of oxidized nucleobases and nucleosides can be divided into biological and chemical methods. The biological methods can be subdivided into ELISA, Comet assay, alkaline elution and alkaline unwinding methods, and the chemical methods can roughly be subdivided into HPLC with electrochemical detection (LC-EC), HPLC with amperometric detection, GC–MS and electrospray LC–MS/MS methods. We will only comment on the chromatographic methods in this review. Standardization schemes for the Comet assay are being worked out presently in ECNIS collaboration [70]. Still we would like

to point out that the immunological detection methods lack specificity and sensitivity, and there is a growing consensus that these methods should be questioned and not used. In our recent standardization the lacking capabilities of the ELISA assay are evident and support this conclusion [51].

LC-EC: This technique is quite sensitive, provided that the analyte in question is electrochemically active. In addition to 8-oxoGua, 5-OHCyt and the related nucleosides, 2,6-diamino-4-hydroxy-5-ormamidopyrimidine, 4,6-diamino-5-formamidopyrimidine, 8-oxo-7, 8-dihydroadenine and 8-oxo-7,8-dihydro-2′-deoxyadenosine (8-oxodA) can be measured by HPLC-ECD in the oxidative mode of detection. The technique has mainly found use for the detection of the guanine modification by oxidation, whether it is as 8-oxoGua, 8-oxodG or 8-oxoGuo. 5-hydroxy-2′-deoxycytidine (5-OHdC) is also electrochemically active, but since the levels of this damage found in vivo are very low and lower than the corresponding 8-oxodG damage, the level of 5-OHdC will often be below the limit of detection by LC-EC. Another limitation of this method is the lack of specificity of the detector, which may not cause major problems for analysis of the hydrolysate of extracted nucleic acids, but can be a significant drawback for complex matrices like urine, where extensive multi-dimensional chromatography or sample preparation is required in order to reduce the risk of overlapping peaks. An advantage of the LC-EC method is that the electrochemical detector is fairly cheap and that the skills required to operate the LC-EC system are limited to ordinary chromatography skills.

A special LC-EC method was published several years ago by Bogdanov et al. [58] where a particular porous carbon column was used in a LC column switching system. The used carbon columns are reported to have a unique purine selectivity, and thus it is possible to up-concentrate 8-oxodG on the columns. By using this technique 8-oxodG concentrations below 1 pM can be measured. The carbon columns are, however, not commercially available, so the technique never gained widespread use.

Mass spectrometry based methods: Electrospray mass spectrometry based methods offer the possibility of using stable isotope labeled internal standards, in contrast to the other detectors in chromatographic methods that use analog compounds as internal standards. As a mass spectrometer differentiates between compounds based on mass differences (mass/charge), it is capable of distinguishing between two different isotope compositions of the same compound. A stable isotope labeled internal standard can be considered as an ideal standard since it behaves identically to the analyte in question, in contrast to the analog compounds that do not behave exactly like the analytes (a small isotope effect may, however, be observed especially if deuterium is used for the labeling). The best way to compensate losses during sample preparation and changes in detector sensitivity etc., is adding the stable isotope labeled internal sample prior to sample preparation.

GC–MS: GC–MS is suitable for the measurement of a wide variety of oxidative damages to nucleobases. The technique, however, requires that the analytes are volatile or can be made volatile by derivatization, which puts limits to the versatility of the technique. This means that nucleosides are not readily measured. In order to measure nucleosides in urine or nucleobase adducts from nucleic acids the nucleobases first have to be cleaved from the riboses or 2-deoxyriboses by acid hydrolysis. Following this a derivatization step is required before the actual analysis by GC–MS. As mentioned previously this is a potential source of error, especially for tissue extracts, since there is a high risk of artificial oxidation of the overwhelming excess of unmodified nucleobases that can result in an overestimation of the amount of oxidized nucleobases. In order to prevent this some researchers have pre-purified the liberated nucleobases by HPLC and thereby separated the unmodified from the oxidized nucleobases before derivatization. In this way similar results to the ones obtained by LC-EC can be achieved [64]. Although initial attempts to introduce this methodology were successful [71–73], the method is replaced by electrospray LC–MS/MS techniques.

Difficulties are also encountered in urine. If urine is hydrolyzed directly, it is no longer possible to distinguish the analytes that were previously present as nucleobases, 2-deoxy-nucleosides or ribonucleosides from one another, since they will now all be present as nucleobases. In order to avoid this problem the urine must first be fractionated by HPLC into fractions containing the nucleobase, the deoxynucleoside and the ribonucleoside.

LC–MS/MS: Electrospray LC–MS/MS is a very versatile and suitable technique for all biological matrices and for a wide variety of damage to DNA and RNA by oxidation. The technique may be very specific, especially if some basic conditions are met. In order to meet the European Communities requirement for the identification of an analyte by LC–MS/MS, one precursor ion and at least two characteristic product ions are required (one quantifier MRM (multiple reaction monitoring) pair and one qualifier MRM pair). Moreover, the ratio between the product ions of the analyte should be consistent with the ratio in a pure standard of the analyte [74]. This criterion can only be met if the analyte elutes as a narrow peak where no other chemical compounds with any of the same MRM pairs are present. It is easier to avoid interfering peaks by using UHPLC for the separation. An added benefit by using UHPLC is that the peak is narrower (and higher since the area remains constant), and thus improved detection limits can be obtained. In LC–MS, ion suppression from co-eluting peaks is often encountered, thus by using high peak resolution in the chromatography, the risk of having ion suppression is reduced which is an extra bonus. The use of UHPLC and qualifier ions is especially useful in a complex matrix as for instance urine. In a simple matrix such as the hydrolysis product of extracted nucleic acids the benefit is smaller and some researchers use only one MRM pair (only quantifier ions) since using a qualifier ion pair often results in a loss of sensitivity.

We conclude that the methodologies for investigating oxidative stress to DNA and RNA are no longer in their infancy and can be used to investigate the relationship to mechanisms of disease and their development. There are well described procedures and quality control schemes and a fair agreement on the values in normal tissues and in urine. In CSF the levels still show considerable variation.

8. Overall conclusion

RNA and DNA oxidations can be measured in specific tissues and in urine. The urinary excretion reflects global oxidative stress, to DNA or RNA, and can be interpreted as general oxidative stress. The measurement of the concentration of the modifications from oxidation is a snapshot at the time of sampling and represents the balance between formation and repair rates. Protocols exist for tissue DNA extraction and measurement to avoid artificial oxidation during these processes; they are less developed for RNA measurements. HPLC coupled with electrochemical detection and electrospray mass spectrometry is the preferred method. For urine measurements 24 hour collection is the preferred design, however, under certain circumstances spot urine samples with correction for urinary creatinine correction to compensate for differences in fluid intake can be used.

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