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ORIGINAL ARTICLE

Detection of extracellular 8-oxo-7,8-dihydro-2′-deoxyguanosine as a biomarker of oxidative damage in X-irradiated fibroblast cultures: optimization of analytical procedure

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Abstract

We have developed a simple methodology, based on single-step solid-phase extraction followed by isocratic high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD), to determine extracellular 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in culture supernatants of normal human dermal fibroblasts. A standard addition method, using externally added 8-oxodG (0.5 and 1 pmol) was employed to eliminate matrix effects arising from the chemically complex, protein-rich medium. Secondly, applying this procedure to X-ray irradiated fibroblasts, we report a significant twofold increase in the levels of 8-oxodG at the radiobiologically relevant dose of 6 Gy. This suggests that extracellular 8-oxodG might be a useful biomarker for oxidative stress following moderate doses of X-irradiation.

Keywords: *8-oxo-7,8-dihydro-2′-deoxyguanosine; oxidative stress; radiobiology; solid-phase extraction; culture supernatant; high-performance liquid chromatography coupled with electrochemical detection*

Introduction

Ionizing radiation is known to induce reactive oxygen species (ROS) which are among the most hazardous human carcinogens (Loft et al. 2008). The hydroxyl radical is the major ROS produced. Among other cellular damages, it causes oxidatively damaged DNA through a nucleophilic attack on DNA bases, giving rise to a variety of oxidation products (Cadet et al. 2003). The most abundant DNA lesion formed is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). If not repaired in time, this lesion leads to G-to-T transversion mutations (Cheng et al. 1992). Hence, after numerous studies, 8-oxodG has been proposed as a key marker of oxidative nucleobase damage and carcinogenesis (Cooke et al. 2008, Valavanidis et al. 2009, Evans et al. 2010).

Radiation-induced increase in the levels of 8-oxodG in DNA have been reported in *in vitro* studies, animal experiments and a few clinical studies (Wilson et al. 1993, Bialkowski et al. 1996, Olinski et al. 1996). Kasai et al. (1984) first identified 8-oxodG in DNA that had been X-ray irradiated in an aqueous solution. 8-OxodG was also found to be generated in cellular DNA of cultured cells exposed to ultraviolet (UV) (A and B) irradiation (Rosen et al. 1996, Wamer & Wei 1997, Budiyanto et al. 2002, Pelle et al. 2003, Svoboda & Harms-Ringdahl 2005). Similarly, Kasai et al. (1986) found elevated levels of 8-oxodG in HeLa cell DNA after exposure to oxygen radical generators, such as γ-radiation. According to Tahara and Kaneko (2004), the content of 8-oxodG increased in DNA immediately after low doses (up to 2 Gy) of X-ray irradiation of mouse splenic cells, while other studies

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reported no production of 8-oxodG at low doses of γ-irradiation (Pouget et al. 1999, 2000).

The 8-oxodG levels in DNA extracted from white blood cells (WBC) often serve as indicators of oxidative stress, especially in population studies. In contrast, examining the products of oxidatively damaged DNA in extracellular matrices such as samples of urine, cerebrospinal fluid or plasma, is a non-invasive method for assessing oxidative stress. This also circumvents the requirement for DNA extraction and its associated risk of inadvertent production of artefacts (Cooke et al. 2009).

Base excision repair (BER), leading to the release of oxidative nucleobase, is by far the most important and the most well-known route for the removal of the majority of oxidatively derived lesions. In contrast, the presence of 2′deoxyribonucleoside modifications in extracellular matrices is less well defined and recent studies have implicated several other DNA repair mechanisms as well, e.g. nucleotide excision repair (NER) and nucleotide incision repair (NIR) pathways, both resulting in the formation of 8-oxodG, although their biological significance is not yet fully elucidated (Haghdoost et al. 2005). The nucleotide pool in cells is an important target for oxidative stress, as the free nucleotide forms of guanine are prone to oxidation leading to the formation of 8-oxodGTP which is a mutagenic substrate during DNA synthesis (Haghdoost et al. 2005, 2006). Thus DNA synthesis may constitute the major source of extracellular 8-oxodG (Haghdoost et al. 2006).

Cell culture medium may be considered as an extracellular matrix that can be used for the assessment of cellular oxidative stress *in vitro*. While there is a lack of consensus between analytical techniques, high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) appears to be the most widely used method for 8-oxodG measurement (for review, see Valavanidis et al. 2009). Cell culture medium is chemically complex, undefined, rich in proteins and therefore a challenging matrix for application in chromatographic techniques. A purification step is necessary to remove interference from high-molecular-weight compounds (e.g. proteins and carbohydrates) present in cell supernatants prior to application. Solid-phase extraction (SPE) for 8-oxodG purification has often been reported, using various extraction mechanisms with different sorbents such as C18 (Teixeira et al. 1995, Bogdanov et al. 1999, Abu-Qare & Abou-Donia 2000, Lengger et al. 2000, Weiss & Lunte 2000, Sangsuwan & Haghdoost 2008) C18/OH (Teixeira et al. 1995, Xu et al. 2004, Yao et al. 2004), C8 (Bogdanov et al. 1999), LiChrolut EN® (Renner et al. 2000), BondElut® Cyclohexyl (Tagesson et al. 1995), ion-exchange (De Martinis & de Lourdes Pires Bianchi 2002), immunoaffinity sorbent (Yin et al. 1995) or 8-oxodG-imprinted sorbent (Ersöz et al. 2008, 2009). Generally, extensive clean-up procedures with multistep SPE and/or semipreparative HPLC are required to obtain clean chromatograms and to extend instrument life (Bogdanov et al. 1999, Lin et al. 2004). These are time-consuming procedures and not amenable to high-throughput analyses. In the present study, 8-oxodG was detected in a protein-rich culture medium using a HPLC-ECD method described previously (Breton et al. 2005) with appropriate modifications. First, a simple clean-up method was developed using a single-step SPE on Oasis® HLB cartridges. Second, a standard addition method was applied to eliminate the matrix effects, which are due to the biological medium, and thus provide an accurate quantification of extracellular 8-oxodG.

Contradictory clinical data have been published on the use of 8-oxodG as a biomarker for exploring possible associations between oxidative DNA damage and response to treatment after radiotherapy. *In vitro* studies are warranted to better understand and assess DNA oxidation during irradiation. Measurement of oxidative DNA damage in irradiated cells by a relatively simple and sensitive technique is of great interest in radiobiology and radiotherapy. It may be used to optimize conditions of irradiation and reduce the risk of secondary cancers which may be unavoidably associated with radiotherapy. The purpose of the present study was to develop an easy and reliable method to determine 8-oxodG in an extracellular matrix such as a culture supernatant. Using this method, we were able to detect changes in 8-oxodG levels in supernatants of fibroblast cultures that had been exposed to X irradiation at radiobiologically relevant doses (6, 10 Gy).

Materials and methods

Cell culture

Normal human dermal fibroblast cell line (NHDF) was obtained from Lonza (Verviers, Belgium). The fibroblasts were cultured in Dulbecco's modified eagle medium (DMEM 41965-039; Gibco BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS), antibiotics (2%, penicillin and streptomycin mix), 1% L-glutamine and 1% HEPES and were maintained at 37°C in humidified atmosphere containing 5% CO_2 in air. Cells were allowed to reach confluence, typically, 10^6 per 9.6 cm^2, in six-well plates and fresh culture medium was provided immediately before irradiation.

X-ray irradiation of cells

Cell culture irradiations were performed using a megavoltage X-ray clinical apparatus (Orion-CGR MEV; energy: 5 MV, Orion, Riverside, California, USA) at a dose rate of 1.4 Gy min^{-1}. Selected doses were 6 and 10 Gy that correspond to 10% and close to 0% of cell survival, respectively, in clonogenicity assays. Clonogenicity of

the cells was estimated by a modification of the method described by Puck et al. (1956). Briefly, 24 h after irradiation, a known number of confluent fibroblasts, ranging from 400 to 5000 corresponding to 0–6 Gy doses, respectively, were seeded in 25 cm^2 tissue culture flasks. After 10 days of incubation, colonies that appeared were stained with crystal violet (2.5 g l^{-1} in 45:5 methanol/paraformaldehyde 30%). Those containing 50 or more cells were scored and the surviving fraction was calculated. The experiment was repeated three times, each with triplicate radiation doses. Experiments were performed at passages 4–6. Cell monolayers were maintained at room temperature during irradiation. Non-irradiated cells were held in the same room and processed similarly to serve as control. Each dose was given in triplicate. Cultures were incubated under standard culture conditions for 24 hours and then centrifuged at 1000 *g*, 10 min, to collect the supernatants. The latter were stored at -80°C until further use.

Sample preparation

Prior to application in HPLC-ECD analysis, the cell culture supernatant was purified by SPE using an Oasis® HLB cartridge as reported previously for preprocessing samples of saliva and urine destined for 8-oxodG measurement by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Cooke et al. 2006). The SPE procedure was performed with modifications aimed at optimizing 8-oxodG extraction in culture supernatants. Various procedures for preconditioning of the columns, sample loading and recovery were evaluated. The optimised conditions employed are detailed below.

Samples were adjusted for the standard addition method (see below) before being subjected to SPE. Each fortified sample (500 µl) was first acidified with 1400 µl of 20 mM formic acid and then loaded onto a 3 ml (60 mg of packing material) SPE cartridge (Waters Oasis® HLB; Waters Ltd, Elstree, UK) preconditioned with 5 ml of methanol followed by 5 ml of sterilized water. After sample loading, 2 ml of 20 mM formic acid was added to flush the cartridges. The fraction containing 8-oxodG was eluted with 4 × 500 µl of 17.5% (v/v) methanol in 20 mM formic acid and the fractions collected were reduced to dryness at 60°C in a vacuum centrifuge concentrator overnight. The dry residues were dissolved in 500 µl of sterilized water and subjected to HPLC-ECD analysis. Experiments on 8-oxodG recovery after SPE were performed.

8-oxodG HPLC-ECD analysis

An optimised method for the quantification of 8-oxodG in WBC DNA hydrolysate has been described previously (Breton et al. 2005). Briefly, the SPE eluate was analysed by HPLC with an electrochemical detector (Coulochem III, ESA Inc., Chelmsford, MA, USA) using an Uptispher ODB C18 HPLC column (150 × 2.1 mm, 3 µm; Interchim, San Pedro, CA, USA) equipped with a C18 guard column. The eluant was 10 mM potassium dihydrogen phosphate, pH 4.6, containing 11.2% methanol, at a flow rate of 0.2 ml min^{-1}. The potentials applied to the analytical cell (ESA 5011) were + 50 mV and + 350 mV for E1 and E2, respectively. Acquisition and quantitative analyses of chromatograms were carried out using Azur v 4.6 software (Datalys, St Martin d'Heres, France). 8-OxodG standards in an aqueous solution were used to estimate 8-oxodG in biological samples based on its retention time of 11.75 min.

Calibration curves and standard addition method

Standard calibration curves were established using solutions of 8-oxodG, 0–5 nM, either in an aqueous solution (without SPE purification) or in culture supernatants of non-irradiated fibroblast (with SPE purification). Experiments were performed in triplicate to obtain 8-oxodG values for mean and standard deviation. Calibration curves of the area of peak corresponding to 8-oxodG were generated. The objective of this preliminary step was to evaluate the matrix effect in culture supernatants.

Next, in order to eliminate the matrix effects in measuring 8-oxodG from culture supernatants, the standard addition method was used. The standard addition method may be employed instead of a calibration curve in instrumental analysis, to determine the concentration of an analyte in a given sample, wherein the matrix effects are significantly high (Harris 2003). The standard solutions (here, solutions of known concentrations of 8-oxodG) are added to the test sample before taking measurements and a calibration curve is established, which accounts for background contribution from the matrix. By extrapolation of the calibration curve, the absolute value of the *x*-intercept gives the concentration of the analyte (here, 8-oxodG). In order to validate a standard addition method for the measurement of extracellular 8-oxodG, for each supernatant, two aliquots of 500 µl each were taken and 0.5 or 1 pmol of 8-oxodG was added as an external standard. These additions were done before SPE purification of the samples. HPLC-ECD signals were recorded in the culture supernatants spiked with the external standard and values for 8-oxodG peak areas plotted against 8-oxodG concentration (in nM). The linear plot thus obtained was extrapolated to determine the value of the *x*-intercept, which represents the absolute concentration of 8-oxodG in the test samples.

Statistical analysis

Standard curves were generated by linear regression analysis. In irradiated samples, statistical significance versus control and between each dose was determined

using an unpaired Student's *t*-test and significance was set at $p < 0.05$.

Results

SPE of fibroblast culture supernatants

The extraction protocol using Oasis® HLB cartridges described by Cooke et al. (2006) for clean-up of saliva or urine samples was adapted for application to cell culture supernatants. Different conditions for column preconditioning, sample loading and elution, were tested in order to obtain the best recovery of 8-oxodG. Among other parameters, we tested the possibility of avoiding the sample acidification because it appeared to require drying of the samples overnight before HPLC analysis. However, sample acidification with 20 mM formic acid was found to be essential to remove proteins efficiently from the cell culture supernatants. Next, the sample loading volume was optimised. Culture supernatant volumes of 250 µl, 500 µl and 1 ml were tested. In terms of quantification of 8-oxodG, 500 µl was judged to be optimum under the experimental conditions used here. Different sample elution conditions were also tested, notably the elution volume (1.5–3 ml). Elution in successive fractions of small volume appeared to be more efficient than a single elution in a large volume. Altogether, four fractions of 500 µl eluted in 17.5% (v/v) methanol in 20 mM formic acid allowed recovery of about 74% of the 8-oxodG from culture supernatants. The eluted fractions were dried under vacuum and resuspended in deionised water. A volume of 500 µl of deionised water was found to be necessary to maintain an adequate signal-to-noise ratio in the subsequent chromatographic analysis. The SPE procedure performed as described above, was found to be a highly effective sample preparation method, producing a clean chromatogram showing a good separation and resolution of 8-oxodG in this complex matrix (Figure 1).

Matrix effect on the quantification of 8-oxodG in fibroblast culture supernatants

Standard calibration curves were generated for HPLC-ECD signals plotted against 8-oxodG concentration (0–5 nM) either as an aqueous solution or in culture supernatants of non-irradiated fibroblasts after SPE purification. As shown in Figure 2, the calibration curves showed a good linearity for both, an aqueous solution of 8-oxodG ($y = 404\times - 3.6$, $R^2 = 0.998$) and for 8-oxodG in a control supernatant ($y = 288\times + 358$, $R^2 = 0.999$).

However, the slope of the standard curve for 8-oxodG in the non-irradiated culture supernatant (288 ± 3.6) was lower than that in water (404 ± 8.5, $p < 0.05$), reflecting a matrix effect on the measurement. Second, the background level of 8-oxodG formed in non-irradiated control culture supernatant, which was 1.24 nM in this experiment, showed a variation of about 15-fold in different trials. (The origin of this background level of 8-oxodG is unknown, but is probably due to the presence of FBS in the culture medium. It may vary according to the experimental conditions, e.g. culture medium, incubation time, etc.) Also, the chromatographic detection limit, determined as the lowest concentration that can be detected at a signal-to-noise ratio of at least 3, was about 0.4 nM. Based on these observations the standard addition method was employed to correct for the interferences introduced by the components of the culture supernatant in the measurement of 8-oxodG.

Standard addition method for the quantification of 8-oxodG in fibroblast culture supernatants

For validating the standard addition method, measurements were taken on supernatants of non-irradiated cultures in triplicate. Before SPE and application to

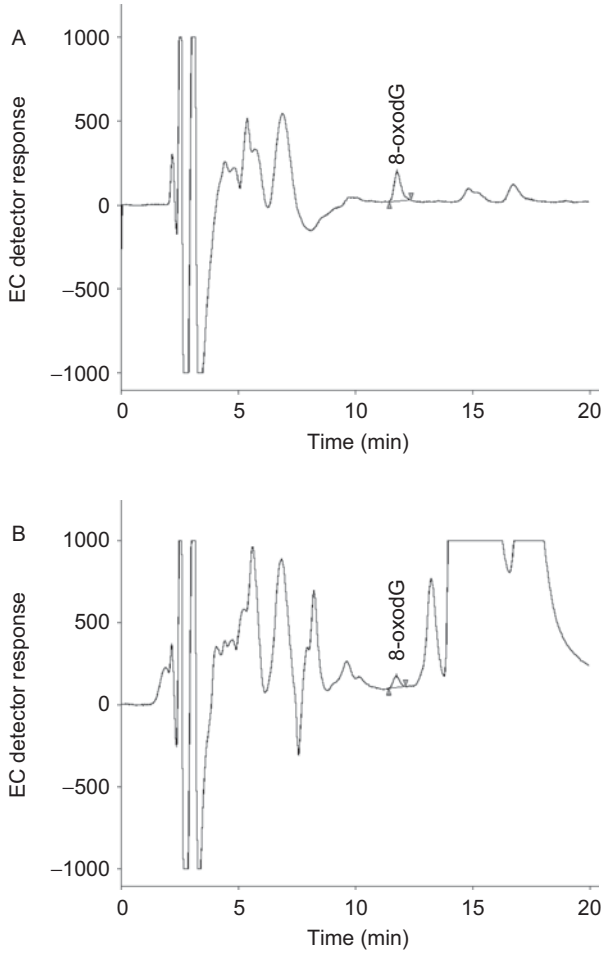

Figure 1. Representative chromatogram of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (5 pmol) in water (A), or in fibroblast culture supernatant after solid-phase extraction purification (B). 8-oxodG was detected at a retention time of 11.75 min, as indicated.

HPLC-ECD, 0.5 or 1 pmol of 8-oxodG, was added to two independent aliquots (500 µl) of the samples to achieve final concentrations of 1 or 2 nM, respectively. These concentrations were chosen for two reasons. First, the 8-oxodG levels in our samples were expected to be below the detection limit and, second, the calibration curve was linear until 5 nM. The plot of measurements taken on the spiked samples ($y = 308\times + 333$, $R^2 = 1.00$), was extrapolated to obtain the value for the x-intercept, which represented the concentration 8-oxodG concentration in the sample (1.1 ± 0.2 nM). It was thus confirmed that extracellular 8-oxodG from culture supernatants can be estimated accurately by this method.

Levels of 8-oxodG in irradiated cells

As described above, for supernatants of irradiated cultures also, two aliquots of 500 µl were independently spiked with 0.5 or 1 pmol of 8-oxodG before SPE and application to HPLC-ECD. This allowed quantification of 8-oxodG in clean chromatographic conditions. The 8-oxodG levels in supernatants of cultures exposed to X-ray irradiation (triplicate cultures for each dose) were determined as described above. As shown in Figure 3, the 8-oxodG concentration was twofold higher in supernatants of cultures exposed to 6 Gy dose of X-rays (1.75 ± 0.01 nM; $p < 0.05$), compared with the non-irradiated control (0.82 ± 0.13 nM). However, there was no statistically significant difference between the levels corresponding to 6 or 10 Gy doses ($p = 0.258$), indicating a lack of dose-dependence at higher doses.

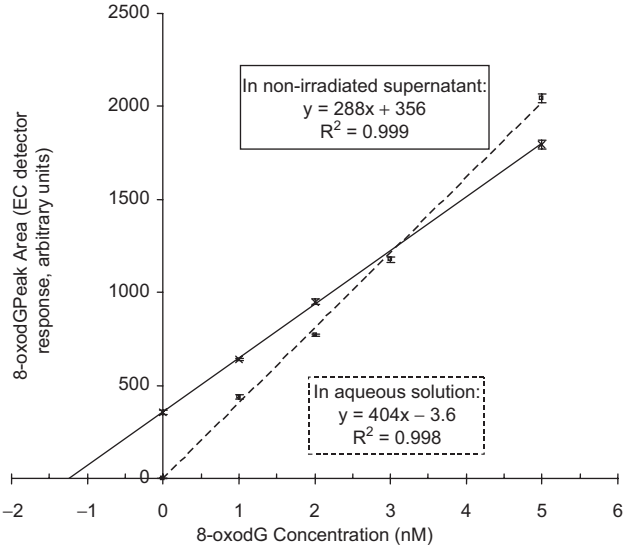

Figure 2. Validation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) analysis in culture supernatant. 8-OxodG (0–5 nM) calibration curves for an aqueous solution (---) or in non-irradiated control fibroblast culture supernatant (—). Values for mean ± standard deviation of the peak areas obtained for triplicate determination are plotted against the 8-oxodG concentration (nM).

Discussion

During the past two decades, 8-oxodG has served extensively as an indicator of oxidative stress, but its choice and reliability are still debated (Cooke et al. 2008, Sajous et al. 2008, Valavanidis et al. 2009, Evans et al. 2010). A prerequisite for such a biomarker is that it should be well validated in order to improve our understanding of its biological significance. The assessment of oxidative damage requires reliable analytical methods. Analysis of 8-oxodG using HPLC-ECD is a highly sensitive technique and is the most frequently used (Hwang & Bowen 2007). The first objective of our work was to develop a simple methodology based on single-step SPE and isocratic HPLC-ECD to determine 8-oxodG in an extracellular matrix such as cell culture supernatants.

Routine measurement of 8-oxodG in sources other than DNA digests, such as urine or plasma samples has been problematic (Bogdanov et al. 1999) due to the inherent low levels of the analyte and the complexity of the biological matrices which contain high and variable levels of potential interferences. Usually SPE is needed before the assay of 8-oxodG. The extraction columns differ in their packing material, using generally C18 or ion exchange separation approaches. Time-consuming extraction using multiple-step SPE is usually carried out to achieve selectivity by reducing the complexity of the chromatograms (Tagesson et al. 1995, Germadnik et al. 1997, Bogdanov et al. 1999). Oasis® HLB columns with hydrophilic–lipophilic balance (HLB) copolymer were previously used to purify urinary 8-oxodG prior to GC-MS (Lin et al. 2004) or LC-MS/MS (Cooke et al. 2006, Wu & Ho 2009) analyses. Lin et al. (2004) reported that the recovery of 8-oxodG after SPE extraction using

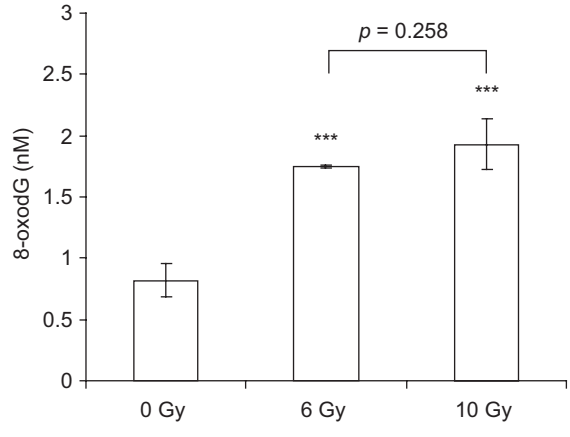

Figure 3. Analysis of extracellular 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in fibroblast culture supernatants after X-ray irradiation at 6 and 10 Gy as indicated compared with non-irradiated cultures (0 Gy). Values are mean ± standard deviation from triplicate cultures for each dose. p-Values were determined by an unpaired Student's t-test. ***$p < 0.05$, compared with corresponding non-irradiated control.

HLB cartridges ranged from 70% to 80% in urine samples as shown by using heavy isotope-labelled 8-oxodG and that even 200 pmol of 8-oxodG did not overload the SPE cartridge. They found that single extraction with HLB cartridges was more efficient than with classical C18 SPE cartridges, even for double extraction. Furthermore, compared with traditional C18 cartridges, the HLB contains water-wettable material and can be dried during extraction. These characteristics of HLB made the extraction procedure easier and faster than with classical C18. In this report, for the first time, Oasis® HLB SPE columns were used to extract 8-oxodG from fibroblasts culture supernatant and allowed its detection by HPLC-ECD thereafter. We adapted and optimised the procedure described previously by Lin et al. (2004) and Cooke et al. (2006) so that it can be applied to the purification of cell culture supernatant, a protein-rich, complex matrix with a large number of potentially interfering compounds and low levels of 8-oxodG. While mass spectromic detection methods can use deuterated 8-oxodG as an internal standard, other methods such as the HPLC-ECD used here, need to rely on an externally added standard solution to assess recovery. Adding 8-oxodG (1–2 nM, final concentration) to the culture supernatant was necessary and sufficient to correct for the matrix effects contributed by the culture medium constituents and/or other experimental confounding parameters. Combined with the fast and simple preprocessing of samples, this method allowed a recovery of around 74% of the extracellular 8-oxodG, a value similar to that noted by Lin et al. (2004) and Cooke et al. (2006).

Few studies have reported extracellular 8-oxodG analysis in cellular model systems (Cooke et al. 2001, Haghdoost et al. 2005, 2006, Sangsuwan & Hagdoost, 2008). This approach confers several advantages: (1) it circumvents issues of DNA extraction and the frequently cited resultant artefactual overestimation of 8-oxodG (reviewed by Collins et al. 2004, Gedik & Collins 2005); (2) it represents a non-invasive measurement of whole cell oxidative stress; (3) extracellular 8-oxodG is present at a relatively high abundance (compared with nuclear 8-oxodG), resulting from oxidation of both the nucleotide pool and DNA. Extracellular 8-oxodG sources could derive from DNA repair, diet, cell death/turnover, mitochondrial turnover and, as shown recently, via cellular uptake and salvage of products of DNA damage (reviewed by Cooke et al. 2009). It has also been proposed that extracellular 8-oxodG originates from dying cells but no convincing experimental results are available to prove these hypotheses (Lindahl 1993, Cooke et al. 2002). Haghdoost et al. (2005) suggested that the eventual contribution of necrotic and/or apoptotic cells to radiation-induced levels of 8-oxodG should be minimal. Recent data suggest that the nucleotide pool could constitute the major source of radiation-induced, extracellular 8-oxodG with a minimal contribution from DNA repair (Cooke et al. 2001, Haghdoost et al. 2006). The nucleotide pool is indeed mainly located in the cytoplasm and lacks structural protection unlike the nucleus, resulting in its higher vulnerability to attack by free radicals (Sangsuvan & Haghdoost 2008).

In *in vitro* experiments it has been shown that ionizing radiations generate ROS thereby inducing the formation of 8-oxodG (Fischer-Nielsen et al. 1994, Schuler et al. 1997). Radiation-induced increase in 8-oxodG levels in the extracellular medium exceeded by several fold that expected to be induced in cellular DNA (Haghdoost et al. 2005), suggesting that extracellular 8-oxodG could be a more sensitive marker for oxidative stress than 8-oxodG from cellular DNA. Similarly, γ-irradiation of whole blood and isolated lymphocytes at therapeutically relevant doses induced a dose-dependent excretion of extracellular 8-oxodG (Haghdoost et al. 2005) and in irradiated fibroblasts it was shown to mainly originate from the nucleotide pool rather than from cellular DNA (Haghdoost et al. 2006). Moreover, in terms of practicality, it has been reported that UV-irradiated DNA is more resistant to digestion, requiring additional enzymatic hydrolysis (Liuzzi & Paterson 1992). Enzymatic digestion of fibroblast cellular DNA appears to be difficult and irreproducible, generating nucleosides at levels below the detection limit (Liuzzi & Paterson 1992). Hence extracellular 8-oxodG is an attractive candidate as a biomarker of oxidative damage from ionising radiations.

Here we report on the detection of extracellular 8-oxodG after X-irradiation of cultured human dermal fibroblasts. It is known that the radiation resistance of cells in culture increases with the monolayer confluency. Hence radiobiologically relevant doses of 6 and 10 Gy, corresponding to 10 and ~0% cell survival, respectively, were chosen for these experiments. The radiation doses were chosen to mimic tissue exposure and be appropriate to confluent fibroblast cultures. As the latter are more radiation resistant than exponential cells, radiation doses > D_0 (radiation dose corresponding to 37% survival) seemed more suitable for our study. We demonstrate a significant (p <0.05) two-fold increase in the levels of 8-oxodG in culture supernatants of irradiated fibroblasts compared to the non-irradiated controls. At a dose of 6 Gy, oxidative damage was indeed induced, and 8-oxodG levels appeared to reach a plateau. It would be informative to measure 8-oxodG at very low doses (<6 Gy) because doses of up to 30 Gy resulted in the same level of extracellular 8-oxodG as at 6 Gy (data not shown). Sangsuwan & Haghdoost (2008) and Haghdoost et al. (2005) also found a non-linear dose–response relationship between 8-oxodG in supernatants of γ-irradiated isolated human lymphocytes at doses below 2 Gy. It is likely that this reflects the saturation of mitochondrial response induced by radiation (Leach et al. 2001). Other explanations for this

effect may be linked to the nature of the material, e.g. limitations imposed by the amount of substrate and/or turnover rate of accessible repair enzymes. In view of these uncertainties, determination of the absolute level of 8-oxodG may be of less importance than the ability to discriminate between its levels in irradiated and control samples. In this respect, our results indicate that extracellular 8-oxodG represents a sensitive marker for radiation-induced oxidative stress.

To better understand the biological significance of extracellular 8-oxodG measurement and to allow its application in clinical studies, cellular response to low-dose radiation *in vitro* needs to be fully explored. The kinetics of 8-oxodG formation and excretion in the supernatant following cell exposure to ionizing radiation is not known. The optimal time for sampling which is likely to be critical for 8-oxodG measurements, remains to be determined. In the present study, the 24-h recovery period between the termination of radiation exposure and the harvesting of the cultures may be a critical factor in terms of 8-oxodG determination. Work is in progress to optimize conditions, both in the time frame for sampling and in radiation protocols. In the case of treatment for radio-resistant tumours, a prerequisite for a promising radiotherapy such as hadrontherapy will be to compare the biological effects of carbon ions versus X-rays. It will be interesting to evaluate the potential of 8-oxodG as a radiosensitive biomarker of cellular response to both types of radiation. Moreover, we wonder if extracellular 8-oxodG could constitute a promising clinical tool for evaluating individual response to radiotherapy and help in designing personalised radiation modalities.

In conclusion, we have developed a simple methodology based on a modified single-step SPE for sample preparation and isocratic HPLC-ECD protocol to detect low levels of 8-oxodG from cell culture supernatants. Following this protocol, a significant twofold increase in the levels of 8-oxodG was detectable in culture supernatants of fibroblasts that had been exposed to X-irradiation at the radiobiologically relevant dose of 6 Gy. Our work demonstrates that determination of extracellular 8-oxodG can be used as a sensitive parameter for assessing radiation-induced oxidative stress *in vitro*.

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Declaration of interest

The authors report no declarations of interest.

References

Abu-Qare A, Abou-Donia M. (2000). Increased 8-hydroxy-2′-deoxyguanosine, a biomarker of oxidative DNA damage in rat urine following a single dermal dose of DEET (N, N-diethyl-m-toluamide), and permethrin, alone and in combination.*Toxicol Lett* 117:151–60.

Bialkowski K, Kowara R, Windorbska W, Olinski R. (1996). 8-Oxo-2′-deoxyguanosine level in lymphocytes DNA of cancer patients undergoing radiotherapy. *Cancer Lett* 99:93–7.

Bogdanov MB, Beal MF, McCabe DR, Griffin RM, Matson WR. (1999). A carbon column-based liquid chromatography electrochemical approach to routine 8-hydroxy-2′-deoxyguanosine measurements in urine and other biologic matrices: a one-year evaluation of methods. *Free Radic Biol Med* 27:647–66.

Breton J, Sichel F, Pottier D, Prevost V. (2005). Measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine in peripheral blood mononuclear cells: optimisation and application to samples from a case–control study on cancers of the oesophagus and cardia. *Free Radic Res* 39:21–30.

Budiyanto A, Ueda M, Ueda T, Ichihashi M. (2002). Formation of cyclobutane pyrimidine dimers and 8-oxo-7,8-dihydro-2′-deoxyguanosine in mouse and organ-cultured human skin by irradiation with broadband or with narrowband UVB. *Photochem Photobiol* 76:397–400.

Cadet J, Douki T, Gasparutto D, Ravanat JL. (2003). Oxidative damage to DNA: formation, measurement and biochemical features. *Mutat Res* 531:5–23.

Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. (1992). 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions. *J Biol Chem* 267:166–72.

Collins AR, Cadet J, Möller L, Poulsen HE, Viña J. (2004). Are we sure we know how to measure 8-oxo-7,8-dihydroguanine in DNA from human cells? *Arch Biochem Biophys* 423:57–65.

Cooke MS, Patel K, Ahmad J, Holloway K, Evans MD, Lunec J. (2001). Monoclonal antibody to single-stranded DNA: a potential tool for DNA repair studies. *Biochem Biophys Res Commun* 284:232–8.

Cooke MS, Lunec J, Evans MD. (2002). Progress in the analysis of urinary oxidative DNA damage. *Free Radic Biol Med* 33:1601–14.

Cooke MS, Singh R, Hall GK, Mistry V, Duarte TL, Farmer PB, Evans MD. (2006). Evaluation of enzyme-linked immunosorbent assay and liquid chromatography–tandem mass spectrometry methodology for the analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine in saliva and urine. *Free Radic Biol Med* 41:1829–36.

Cooke MS, Olinski R, Loft S, European Standards Committee on Urinary (DNA) Lesion Analysis. (2008). Measurement and meaning of oxidatively modified DNA lesions in urine. *Cancer Epidemiol Biomarkers Prev* 17:3–14.

Cooke MS, Henderson PT, Evans MD. (2009). Sources of extracellular, oxidatively modified DNA lesions: implications for their measurement in urine. *J Clin Biochem Nutr* 45:255–70.

De Martinis BS, de Lourdes Pires Bianchi M. (2002). Methodology for urinary 8-hydroxy-2′-deoxyguanosine analysis by HPLC with electrochemical detection. *Pharmacol Res* 46:129–31.

Ersöz A, Diltemiz SE, Ozcan AA, Denizli A, Say R. (2008). Synergie between molecular imprinted polymer based on solid-phase extraction and quartz crystal microbalance technique for 8-OHdG sensing. *Biosens Bioelectron* 24:742–7.

Ersöz A, Diltemiz SE, Özcan AA, Denizli A, Say R. (2009). 8-OHdG sensing with MIP based solid phase extraction and QCM technique. *Sensors Actuors B* 137:7–11.

Evans MD, Singh R, Mistry V, Farmer PB, Cooke MS. (2010). Analysis of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine by liquid chromatography–tandem mass spectrometry. *Methods Mol Biol* 610:341–51.

Fischer-Nielsen A, Jeding IB, Loft S. (1994). Radiation-induced formation of 8-hydroxy-2′-deoxyguanosine and its prevention by scavengers. *Carcinogenesis* 15:1609–12.

Gedik CM, Collins A; ESCODD (European Standards Committee on Oxidative DNA Damage). (2005). Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study. *FASEB J* 19:82–4.

Germadnik D, Pilger A, Rüdiger HW. (1997). Assay for the determination of urinary 8-hydroxy-2′-deoxyguanosine by high-performance liquid chromatography with electrochemical detection. *J Chromatogr B Biomed Sci Appl* 689:399–403.

Haghdoost S, Czene S, Näslund I, Skog S, Harms-Ringdahl M. (2005). Extracellular 8-oxo-dG as a sensitive parameter for oxidative stress *in vivo* and *in vitro*. *Free Radic Res* 39:153–62.

Haghdoost S, Sjölander L, Czene S, Harms-Ringdahl M. (2006). The nucleotide pool is a significant target for oxidative stress. *Free Radic Biol Med* 41:620–6.

Harris DC. (2003). *Quantitative Chemical Analysis, 6th edn*. New York: W.H. Freeman.

Hwang ES, Bowen PE. (2007). DNA damage, a biomarker of carcinogenesis: its measurement and modulation by diet and environment. *Crit Rev Food Sci Nutr* 47:27–50.

Kasai H, Tanooka H, Nishimura S. (1984). Formation of 8-hydroxyguanine residues in DNA by X-irradiation. *Gann* 75:1037–9.

Kasai H, Crain PF, Kuchino Y, Nishimura S, Ootsuyama A, Tanooka H. (1986). Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis* 7:1849–51.

Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R, Mikkelsen RB. (2001). Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. *Cancer Res* 61:3894–901.

Lengger C, Schöch G, Topp H. (2000). A high-performance liquid chromatographic method for the determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine in urine from man and rat. *Anal Biochem* 287:65–72.

Lin HS, Jenner AM, Ong CN, Huang SH, Whiteman M, Halliwell B. (2004). A high-throughput and sensitive methodology for the quantification of urinary 8-hydroxy-2′-deoxyguanosine: measurement with gas chromatography-mass spectrometry after single solid-phase extraction. *Biochem J* 380:541–8.

Lindahl T. (1993). Instability and decay of the primary structure of DNA. *Nature* 362:709–15.

Liuzzi M, Paterson MC. (1992). Enzymatic analysis of oligonucleotides containing cyclobutane pyrimidine photodimers with a cleaved intradimer phosphodiester linkage. *J Biol Chem* 267:22421–7.

Loft S, Møller P, Cooke MS, Rozalski R, Olinski R. (2008). Antioxidant vitamins and cancer risk: is oxidative damage to DNA a relevant biomarker? *Eur J Nutr* 47 (Suppl. 2):19–28.

Olinski R, Zastawny TH, Foksinski M, Windorbska W, Jaruga P, Dizdaroglu M. (1996). DNA base damage in lymphocytes of cancer patients undergoing radiation therapy. *Cancer Lett* 106:207–15.

Pelle E, Huang X, Mammone T, Marenus K, Maes D, Frenkel K. (2003). Ultraviolet-B-induced oxidative DNA base damage in primary normal human epidermal keratinocytes and inhibition by a hydroxyl radical scavenger. *J Invest Dermatol* 121:177–83.

Pouget JP, Ravanat JL, Douki T, Richard MJ, Cadet J. (1999). Measurement of DNA base damage in cells exposed to low doses of gamma-radiation: comparison between the HPLC-EC and comet assays. *Int J Radiat Biol* 75:51–8.

Pouget JP, Douki T, Richard MJ, Cadet J. (2000). DNA damage induced in cells by gamma and UVA radiation as measured by HPLC/GC-MS and HPLC-EC and Comet assay. *Chem Res Toxicol* 13:541–9.

Puck TT, Marcus PI, Cieciura SJ. (1956). Clonal growth of mammalian cells *in vitro*; growth characteristics of colonies from single HeLa cells with and without a feeder layer. *J Exp Med* 103:273–83.

Renner T, Fechner T, Scherer G. (2000). Fast quantification of the urinary marker of oxidative stress 8-hydroxy-2′-deoxyguanosine using solid-phase extraction and high-performance liquid chromatography with triple-stage quadrupole mass detection. *J Chromatogr B Biomed Sci Appl* 738:311–17.

Rosen JE, Prahalad AK, Williams GM. (1996) 8-Oxodeoxyguanosine formation in the DNA of cultured cells after exposure to H2O2 alone or with UVB or UVA irradiation. *Photochem Photobiol* 64:117–22.

Sajous L, Botta A, Sari-Minodier I. (2008). [Urinary 8-hydroxy-2′-deoxyguanosine: a biomarker of environmental oxidative stress?] *Ann Biol Clin (Paris)* 66:19–29.

Sangsuwan T, Haghdoost S. (2008). The nucleotide pool, a target for low-dose gamma-ray-induced oxidative stress. *Radiat Res* 170:776–83.

Schuler D, Otteneder M, Sagelsdorff P, Eder E, Gupta RC, Lutz WK. (1997). Comparative analysis of 8-oxo-2′-deoxyguanosine in DNA by 32P- and 33P-postlabeling and electrochemical detection. *Carcinogenesis* 18:2367–71.

Svoboda P, Harms-Ringdahl M. (2005). Influence of chromatin structure and radical scavengers on yields of radiation-induced 8-oxo-dG and DNA strand breaks in cellular model systems. *Radiat Res* 164:303–11.

Tagesson C, Källberg M, Klintenberg C, Starkhammar H. (1995). Determination of urinary 8-hydroxydeoxyguanosine by automated coupled-column high performance liquid chromatography: a powerful technique for assaying *in vivo* oxidative DNA damage in cancer patients. *Eur J Cancer* 31A:934–40.

Tahara S, Kaneko T. (2004). Susceptibility of mouse splenic cells to oxidative DNA damage by x-ray irradiation. *Biol Pharm Bull* 27:105–8.

Teixeira AJ, Ferreira MR, van Dijk WJ, van de Werken G, de Jong AP. (1995). Analysis of 8-hydroxy-2′-deoxyguanosine in rat urine and liver DNA by stable isotope dilution gas chromatography/mass spectrometry. *Anal Biochem* 226:307–19.

Valavanidis A, Vlachogianni T, Fiotakis C. (2009). 8-Hydroxy-2′-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 27:120–39.

Wamer WG, Wei RR. (1997). *In vitro* photooxidation of nucleic acids by ultraviolet A radiation. *Photochem Photobiol* 65:560–3.

Weiss DJ, Lunte CE. (2000). Detection of a urinary biomaker for oxidative DNA damage 8-hydroxydeoxyguanosine by capillary electrophoresis with electrochemical detection. *Electrophoresis* 21:2080–5.

Wilson VL, Taffe BG, Shields PG, Povey AC, Harris CC. (1993). Detection and quantification of 8-hydroxydeoxyguanosine adducts in peripheral blood of people exposed to ionizing radiation. *Environ Health Perspect* 99:261–3.

Wu JZ, Ho PC. (2009). Comparing the relative oxidative DNA damage caused by various arsenic species by quantifying urinary levels of 8-hydroxy-2′-deoxyguanosine with isotope-dilution liquid chromatography/mass spectrometry. *Pharm Res* 26:1525–33.

Xu GW, Yao QH, Weng QF, Su BL, Zhang X, Xiong JH. (2004). Study of urinary 8-hydroxydeoxyguanosine as a biomarker of oxidative DNA damage in diabetic nephropathy patients. *J Pharm Biomed Anal* 36:101–4.

Yao QH, Mei SR, Weng QF, Zhang PD, Yang Q, Wu CY, Xu GW. (2004). Determination of urinary oxidative DNA damage marker 8-hydroxy-2′-deoxyguanosine and the association with cigarette smoking. *Talanta* 63:617–23.

Yin B, Whyatt RM, Perera FP, Randall MC, Cooper TB, Santella RM. (1995). Determination of 8-hydroxydeoxyguanosine by an immunoaffinity chromatography-monoclonal antibody-based ELISA. *Free Radic Biol Med* 18:1023–32.