Nickel(II)-catalysed oxidative guanine and DNA damage beyond 8-oxoguanine

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Abstract

Oxidative DNA damage is one of the most important and most studied mechanisms of disease. It has been associated with a range of terminal diseases such as cancer, heart disease, hepatitis and HIV, as well as with a variety of everyday ailments. There are various mechanisms by which this type of DNA damage can be initiated, through radiation and chemical oxidation, among others, though even still, these mechanisms have yet to be fully elucidated. A HPLC-UV-EC study of the oxidation of DNA mediated by Nickel(II) obtained results that show an erratic, almost oscillatory formation of 8-oxoguanine (8-oxoG) from free guanine and from guanine in DNA. Sporadic 8-oxoG concentrations were also observed when 8-oxoG alone was subjected to these conditions. A HPLC-MS/MS study showed the formation of oxidised-guanidinohydantoin (oxGH) from free guanine at pH 11, and the formation of guanidinohydantoin (GH) from DNA at pH 5.5.

Keywords: Nickel, HPLC-UV-EC, HPLC-MS/MS, oxidative DNA damage, guanine, 8-oxo-7,8-dihydroguanine.

Abbreviations: HPLC-UV-EC, high performance liquid chromatography with ultra-violet and electrochemical detection; HPLC-MS, high performance liquid chromatography with

mass spectrometric detection; HIV, human immunodeficiency virus; DNA, deoxyribonucleic acid; G, guanine; 8-oxoG, 7,8-dihydro-8-oxoguanine; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; SOD, superoxide dismutase; FapyG, formamidopyrimidine; FHIT, fragile histidine triad; LOD, limit of detection; EC, electrochemical; GH, guanidinohydantoin; oxGH, oxidised guanidinohydantoin; Sp, spiroimidodihydantoin; GF-AAS, graphite furnace atomic absorption spectroscopy; ROS, reactive oxygen species.

Introduction

DNA is subjected to thousands of oxidative hits per day.[1] Oxidative DNA damage has been implicated as a factor of cancer, neurodegeneration, and heart disease. It can cause strand breaks, base modifications and base mutations.[2] Diet, lifestyle and other environmental conditions and factors can alter the amount of oxidative stress that a body will undergo.[3-6] There is much research in this area at present, both in trying to determine the causes of oxidative stress and to elucidate the mechanisms of action of these external and internal contributory factors and to determine any roles this oxidative stress may have in various diseases.[7-9]

Most of the research to date has been centred around guanine (G) and 2'-deoxyguanosine (dG), the most easily oxidised of the four DNA bases and the four nucleosides respectively.[10] Their primary oxidation products, 7,8-dihydro-8-oxoguanine (8-oxoG) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) respectively, have been analysed in depth to date, as they are considered to have the potential to lead to the

determination of the degree of oxidative DNA damage. Analysis of oxidative DNA products can also potentially lead to the elucidation of oxidative stress mechanisms.

8-oxoG and 8-OH-dG are also subjected to oxidative attack, and are even more susceptible to oxidative damage, as their oxidation potentials are lower than that of their precursors. This means that there is also a spectrum of potential further oxidation products to investigate, giving an even more in-depth view of the full picture of oxidation of DNA.

The main causes of oxidative DNA damage are irradiation, chemical reactions and oxidation by reactive oxygen species (ROS).[8] One of the most investigated ROS is ·OH.[11,12] One of the methods from which it is produced is the Fenton reaction,[13] where a transition metal is oxidised to a higher oxidised state, by donating an electron to a hydrogen peroxide species, resulting in the formation of a hydroxyl radical and a hydroxyl ion.[13,14] ·OH is one of the most studied reactive biological radicals,[15] and has been implicated in reactions with the nucleic acid bases of DNA [16]. ·OH reacts preferentially with the π-bonds of DNA bases, but can also interact with the sugar units by hydrogen abstraction.[17] ·OH is known to react with each of the four DNA bases, resulting in mutagenic lesions.

·OH attacks the guanine moiety at the C4, C5 or the C8 position. The addition of the radical to the C4 position is in greater yield (60%) than the C8 position (25%). The formation of radicals is seen initially. On C8 oxidation, the resulting 8-hydroxy-7,8-dihydroguanyl radical is redox ambivalent, *i.e.*, it can be oxidised or reduced to form oxidation product, 8-oxoG, or reduction product, formamidopyrimidine (Fapy-G) respectively.[18] The 4- or 5- OH-guanine radical can be dehydrated to form a further

oxyl radical which is then converted to final oxidation products, imidozolone and oxazolone derivatives. These can also decay to reform guanine, in what can be considered as an “auto-repair” mechanism. 8-oxoG is the most abundant oxidation product of ·OH oxidation of guanine, with a 50% yield. Fapy-G had a reported yield of 20%. Cadet *et al.* also implicated the ·OH in tandem DNA base damage.[11]

This damage via the Fenton reaction can be mediated *in vivo* by labile transition metals, such as iron (Fe), copper (Cu) and nickel (Ni).[19] In a study of the effect of carcinogenic nickel compounds, Kawanishi *et al.* looked at the effects of $NiSO_4$ induced oxidative DNA damage. The formation of 8-OH-dG was monitored over time with samples taken at lengthy intervals (2, 4, 16 and 24 hours). Ni was found to induce damage to DNA.[20] Ni is an abundant transition metal in the environment. It is a trace element, present in some chocolate, nuts, oatmeal, beans and pulses, with daily dietary intake varying from about 100 to 900 μg/day.[21-23] Ni has been found in its highest concentrations in the lungs, kidneys and in some hormone-producing tissues.[24-25]

Some Ni compounds are known carcinogens, *e.g.* nickel subsulfide and nickel carbonyl have been reported to cause lung and nasal cancer and have been labelled as Group A and Group B2 carcinogens respectively. Metallic nickel can also cause skin irritations and dermatitis and is a Group C carcinogen.[26-27]

Both soluble and insoluble forms of Ni damage genetic material. Examples of such damage include: DNA strand breaks, mutations, chromosomal damage, cell transformation, and disrupted DNA repair.[11, 28, 29] Ni has also been reported to damage other cellular factors such as the tumour suppressor genes p53 and FHIT (fragile histidine triad) via protein damage.[30]

Due to its ability to vary oxidation states, Ni in certain complexes with natural ligands can also participate in redox reactions at physiological pH and may well be able, therefore, to generate strong oxidising species in its reaction with hydrogen peroxide (H_2O_2).[31] Available nickel *in vivo* should, therefore, be able to cause oxidative DNA damage by the production of noxious hydroxyl radicals and other types of ROS by this reaction. The formation of 8-oxoG from reactions involving DNA and Ni has been reported before, though in relatively low levels. Damage to DNA by different Ni compounds and the enhancement of Ni oxidation by biological ligands resulted in 8-oxoG formation.[19,32-36] It is also noted that there is an association between Ni concentration and the amount of oxidative lesions in urine.[34] The formation of oxidative lesions in DNA bases found in urine over time has not been mapped extensively with min. by min. sampling. Such min. by min. sampling would allow for a more detailed insight into the mechanisms of Ni(II) -mediated damage to DNA bases. Because of its carcinogenic properties and its redox abilities, nickel was chosen for this research. We therefore chose to investigate the mechanisms of oxidative damage to DNA caused by nickel compounds.

This research is focused on the elucidation of the mechanism of *in vitro* oxidation of G; both free in solution and in the DNA backbone, by a Ni(II)-mediated reaction. The methods used in this study were HPLC-UV-EC for the determination of 8-oxoG and G and HPLC-MS/MS for the determination of 8-oxoG and structural determination of its further oxidation products.

Materials and Methods

Materials

All chemicals including the DNA bases guanine (G0381, ≥99%), adenine (A8626, ≥99%), thymine (T0376, ≥99%), cytosine (C3506, ≥99%), and uracil (U0750, ≥99%), 7,8-dihydro-8-oxoguanine (R288608), calf thymus DNA sodium salt (D1501, Type I, fibres) [2,000 av. base pairs, 41.2% G/C] and nickel sulphate hexahydrate (22,767-6, ACS reagent, 99%) were purchased from Sigma-Aldrich (Tallaght, Dublin, Ireland). Ethanol, methanol and HPLC-MS grade methanol were purchased from Labscan Ltd. (Dublin, Ireland). Deionised water was purified using a MilliQ system to a specific resistance of greater than 18 MΩ.cm. All HPLC buffers and mobile phases were filtered through a 47mm, 0.45 μm polyvinylidene fluoride (PVDF) micropore filter prior to use. Fresh solutions of all standards were prepared weekly.

Incubation of G, 8-oxoG and DNA with Ni(II) and H_2O_2

For HPLC-UV-EC, a 10 mM solution of G, prepared in 0.1 M NaOH, was incubated at 37 ^{o}C with 1.5 mM $NiSO_4.6H_2O$ and 0.5 M solution of hydrogen peroxide (H_2O_2). A 2.4 mM 8-oxoG standard, also prepared in 0.1 M NaOH, and a 2 mg/ml standard of DNA in 50 mM ammonium acetate buffer pH 5.5 were analysed similarly.

Incubations were carried out from 0-30 min., with duplicate sampling of 100 μl at 1 min. intervals. The reaction was quenched in 1 ml of cold ethanol (cooled to –18 ^{o}C). The solution was then dried under nitrogen and refrigerated until analysis by HPLC. G and 8-oxoG samples were reconstituted in 10% 0.1 M NaOH, 90% 50 mM ammonium acetate, 85 mM acetic acid buffer, pH 5.5 to 1 ml. DNA was hydrolysed with formic acid to release DNA bases and then reconstituted with 50 mM ammonium acetate, 85 mM

acetic acid buffer, pH 5.5 prior to analysis. For mass spectrometric analysis, samples were prepared in 10 mM NaOH and reconstituted in 100 µl 10 mM NaOH and 900 µl 50 mM ammonium acetate. All samples were filtered through a 4.5 µm micropore filter prior to injection.

HPLC-UV-EC analysis of 8-oxoG formation.

Samples were separated by reversed phase HPLC using a Varian ProStar HPLC system with Varian ProStar 230 Solvent Delivery Module and Varian ProStar 310 UV-VIS Detector. The eluent composition was 10% methanol, 90% 50 mM ammonium acetate, 85 mM acetic acid buffer through a Restek reverse phase Ultra C18 5 µm 4.9 x 250 mm column, equipped with Ultra C18 4 x 10 mm guard column. The separation was carried out at 1.0 ml/min. isocratic elution and the run time for the separation was 6 min. G and uracil were detected using UV detection at 254 nm and any 8-oxoG formed was detected by electrochemical (EC) detection, using a CC-4 electrochemical cell comprising of glassy carbon working electrode, stainless steel auxiliary electrode and Ag/AgCl reference electrode at a detection potential 550 mV. EC chromatograms were generated using a Shimadzu integrator. UN-SCAN-IT digitising software was used to digitise integrator chromatograms, which were then imported into SigmaPlot 8.0 or MS Office Excel.

HPLC-MS/MS Analysis of Further Oxidation Products.

Incubated samples were analysed by HPLC-MS-MS using an Agilent 1100 HPLC System with diode array detection coupled to a Bruker Daltonics Esquire 3000 LC-MS. Reconstituted samples were separated by HPLC using gradient elution through a Supelco

Supelcosil LC-18 reversed phase column 5 μm 2.1 mm x 250 mm. Eluent A consisted of 10 mM ammonium acetate buffer pH 5.5. Eluent B was 50/50 methanol/water. A flow rate of 0.2 ml/min. was used with a linear gradient of 0-10% B from 0-22 min., 10-0% B from 22-25 min. DNA bases and oxidation products were also detected by UV detection at 210, 254 and 280 nm. Mass Spectrometric analysis was carried out at an ionisation temperature of 300 ^{o}C and at an ionisation potential of +15 V unless otherwise stated.

Controlled Experiments

Controlled incubations were performed, with both G and 8-oxoG. Each of the oxidation reagents was replaced with deionised water, first singly, to determine whether one of the reagents could generate oxidative damage alone, and then both reagents were replaced to measure how much, if any, artifactual oxidation was caused by the reaction conditions themselves.

A graphite furnace atomic absorption spectroscopy study of nickel sulphate was performed using glassware washed in 20% nitric acid and ultra-pure deionised water. The GF-AAS was calibrated using a "pre-mix" method with prepared standards. A 17 mM sample of the $NiSO_4.6H_2O$ was analysed for iron content.

Results

Determination of 8-oxoG formation over time

Controlled experiments

A number of control experiments were undertaken on G, to ensure that all results were due to Ni/H_2O_2 induced oxidative DNA damage, and not due to artifactual oxidation

from the methodology involved or from residual contaminants such as iron in any of the reactants. EC detection was applied in order to determine the concentration of 8-oxoG production.

A residual concentration of 8-oxoG was generated by the addition of H_2O_2 alone (the same amount of 8-oxoG as was measured in untreated DNA, 0.06 ± 0.02 μM). However, there was no increase in the concentration of 8-oxoG present as the incubation time with H_2O_2 increased and the concentration of 8-oxoG did not fluctuate with increasing incubation time. Neither was there any consumption of free G as the incubation time increased, nor any fluctuation in its concentration.

Significant concentrations of 8-oxoG were not detected in any of the remaining controls, indicating that the oxidative damage caused was by the Ni(II)- H_2O_2 reaction. There was no 8-oxoG detected when G was dried under nitrogen with no incubation performed. G concentration was mapped with UV detection and both G and 8-oxoG concentrations remained constant during all these controlled incubations.

As a further control, graphite furnace atomic absorption spectroscopy (GF-AAS) studies carried out on nickel sulphate samples indicated that there was less than 0.0001% iron in these samples. This means for the 1.5 mM Ni(II) used in the experiments there was less than 0.0000075 mM (7.5 nM) of Fe present. As illustrated in the control experiments, concentration of iron was not responsible for the oxidative damage observed below.

G Incubations

The incubation of G with Ni(II) and H_2O_2 showed an oscillatory concentration of 8-oxoG over the 30 min. incubation period. In the Ni-H_2O_2 mix, the highest oscillations

were found to occur for 8-oxoG at 6, 16 and 21 min., as seen in Fig. 1. Samples were taken in duplicate at one min. intervals; however, they were not taken simultaneously, but rather approximately 10 s apart. Therefore, the reaction would have progressed within these 10 s. The first sample was taken after approximately 15 s and so there was some 8-oxoG formed after the first sample was taken. The resulting plots show large error bars, which gave evidence of this progression of the reaction in between samples, especially in the formation of 8-oxoG where large error bars were seen on the higher peaks. The error bars shown are illustrating standard deviation of duplicate samples injected in triplicate in Fig.'s 1-3. These large differences between runs illustrate the complexity of the reactions involved in this system.

8-oxoG Incubations

The incubation of 8-oxoG showed a similar erratic pattern to that observed from free G. The concentration of 8-oxoG was initially very high, but decreased sharply almost immediately, and illustrated that the reaction proceeded extremely rapidly (Fig. 2). The initial concentration was calculated to be 192 μM (based on initial concentration of 2.4 mM diluted by the addition of the oxidising reagents, and a 1/10 dilution upon reconstitution). However, the 0 min. concentration observed in Fig. 2 (1.4 μM) corresponds to samples taken immediately upon the addition of the reagents, which was about 15 s after the reaction had begun, indicating how rapidly the initial 8-oxoG oxidation proceeded.

A large oscillation was noted at 6 min. 8-oxoG oxidation by the Ni-H_20_2 reaction was monitored to 12 min. This experiment was repeated on a different system using a

CHI Instruments potentiostat and Phenomenex Onyx monolith column of dimensions 250mm, 4.6mm I.D. and the results proved highly reproducible.

DNA Incubations

Ni(II)-mediated oxidative damage to G in DNA was then investigated by incubating calf thymus DNA with $NiSO_4$ and H_2O_2. The incubations were performed over a 30 min. time period and at pH 5.5, with samples taken at 1 min. intervals. Single samples were taken at each min. and analysed in triplicate by HPLC-UV-EC. The concentration of G in DNA and the concentration of 8-oxoG formed were monitored at each min. of the incubation period. The results observed showed chaotic patterns for 8-oxoG as was seen previously in the reaction of free G with the Ni(II) and H_20_2 reagents. The fluctuating pattern of 8-oxoG formation is illustrated in Fig. 3.

The concentration of 8-oxoG was seen to fluctuate over the incubation period. The concentration at the initial sampling was below the LOD of the EC detector. The concentration then proceeded to increase slightly, 1 min. into the incubation, but decreased again for the next two min. The concentration then increased dramatically and the erratic, almost oscillatory pattern continued but the concentration of 8-oxoG did not fall below 0.2 μM, which was the workable LOD of the EC system used. The highest concentrations of 8-oxoG observed were at 5 min., 18 min. and 29 min., where the concentration of 8-oxoG exceeded approximately 0.8 μM.

Mass Spectrometric Analysis of Further Oxidation Products

G Analysis

The presence of suspected oxidative DNA damage products guanidinohydantoin (GH), oxidised guanidinohydantoin (oxGH), spiroimidodihydantoin (Sp) and other similar products were investigated by analysing *m/z* values 156, 157, 158 and 159.[37-40] The retention time of the peak at *m/z* 156 was just over 6 min. A peak of *m/z* 157 was also detected. This peak was not observed at a higher skim voltage of +30 V. There was no *m/z* 158 peak found in the sample. The peak at *m/z* 159 eluted with the solvent front, suggesting that it was not retained on the column, or was a component of the mobile phase. There was no peak at *m/z* 168, which would have corresponded to 8-oxoG observed in any of the G incubation samples.

The peak observed at *m/z* 152 corresponded to the [G + H^+] adduct. A negative ionisation potential skim voltage was then applied, in order to confirm results obtained from the mass spectrometric analysis obtained using the previous positive ionisation potentials. The extracted ion chromatograms obtained, post HPLC separation, showed the occurrence of a peak retained at less than 6 min., which corresponded to *m/z* 154, the corresponding negative ion of the *m/z* 156 product. The negative ionisation potential scan produced a deprotonated internal standard uracil at *m/z* 111, and a deprotonated G at *m/z* 150.

8-oxoG Analysis

8-oxoG was analysed in positive scan mode at an ionisation skim voltage of +15V. A peak at *m/z* 168 was observed, corresponding to the [8-oxoG + H^+] adduct, as

would be expected in the sample. There were no quantifiable peaks at *m/z* 158 or *m/z* 194, which, if present, would imply that 8-oxoG was fully oxidised to GH and Sp respectively. The peak that emerged at *m/z* 156 was detected, this would suggest the formation of oxGH.

MS/MS Analysis of Product of m/z 156, Detected in both G and 8-oxoG

A tandem mass spectrometric scan (MS/MS) was performed on Product 1 (*m/z* 156), at retention time 6.2 min. in order to identify this product. The MS/MS spectrum of the *m/z* 156 peak, illustrating fragmentation is shown in Fig. 4. The base peak of the spectrum was *m/z* 113, and other peaks observed were *m/z* 114 and 139.6. The peak at *m/z* 86.1 seen below in Fig. 4 was due to background or matrix effects, and was not a fragment ion of the product compound.

G oxidation product, *m/z* 156, was observed over the 120 min. incubation period with the Ni(II) mediated reagents. The product concentration was seen to increase on increasing incubation time up to 60 min., after which the concentration appeared to level off. (Fig. 5)

Controlled experiments showed no formation of a peak at *m/z* 156, indicating that it was indeed a product of oxidative DNA damage via the Ni (II)-mediated reaction.

DNA Analysis

A similar experiment was undertaken for the analysis of G oxidation products formed from the DNA backbone by mass spectrometry. There was no peak at *m/z* 156 observed in this experiment, which indicated that the product at *m/z* 156 was not formed from G in the DNA backbone. Similarly this experiment was carried out over an

incubation period of 120 min. The G peak was observed at *m/z* 152, as was expected. A peak at *m/z* 158 was detected. Other peaks observed in the DNA sample were at *m/z* 168 corresponding to 8-oxoG. A negative ion mode analysis was also carried out on the DNA samples. This scan showed peaks of *m/z* 150 and *m/z* 166 corresponding to G and 8-oxoG, respectively.

As the peak at *m/z* 168, corresponding to 8-oxoG, was formed from DNA incubations with Ni(II) and H_2O_2 reagents, the formation of this compound was monitored. It can be seen in Fig. 6 that there was a slight oscillatory-type pattern of 8-oxoG formation observed. The concentration of 8-oxoG decreased to a minimum at 15 min., followed by a sharp increase to its peak concentration at 20 min.

The formation of the product at *m/z* 158 was then monitored, over the 120 min. incubation period, and is illustrated in Fig. 7. The formation of this product was detected in positive ion mode (*m/z* 158) 20 min. into the incubation, was not detected in negative ion mode.

Discussion

Nickel-Mediated Oxidative DNA Damage

The results described in this study support the idea that 8-oxoG is not a suitable biomarker for the accurate detection of these diseases due to its instability and almost oscillatory formation. An erratic pattern of 8-oxoG formation, shows a complex mechanism which involves the degradation of G resulting in the formation of 8-oxoG, and subsequent further oxidation of 8-oxoG.

In a study of iron Fenton chemistry, previously reported by this laboratory,[41] a major erratic change in concentration was observed at just under 5 min., 14 min. and 20 min., which compared well with the sharp increases noted at 6 min., 16 min. and 20 min. in this study, suggesting a similar reaction process.

8-oxoG is subject to further oxidation by the same ROS, due to its lower oxidation potential in comparison to G. The 8-oxoG moiety is an intermediate in the G oxidation scheme, and this is illustrated by the vagarious 8-oxoG concentration over the incubation periods monitored.

This result, as was seen with the iron mediated Fenton reaction, illustrates a complicated mechanism which may involve numerous processes and reactions resulting in oscillatory reaction switching similar to that seen in such reactions as the Belusov Zhabotinsky reaction.[42,43] This laboratory is currently working to elucidate this fluctuant mechanism in order to determine the exact mechanism behind this complicated nature of 8-oxoG concentration.

The concentration of 8-oxoG formed from the nickel experiment; however, was significantly lower than the concentrations noted for the previous iron experiments, probably as nickel is a much weaker oxidant. There is a possibility that nickel binds to the G molecule to enhance its oxidative abilities, thus causing G to enhance its own oxidation. Nickel's oxidation powers are known to be enhanced when it binds to certain biomolecules, such as peptides.[19, 31-36]

When this formation of 8oxoG was noted as a result of G oxidation at pH 11, the experiment was then carried forward to DNA at pH 5.5. The experiments on G alone were performed at pH 11 due to solubility issues. This limited the biological significance

of the study, but was deemed necessary to conduct a preliminary study to ensure that the method was applicable to the detection of both G and 8-oxoG, and to ensure that 8-oxoG was actually being formed from the Ni(II)-catalysed oxidation of G.

On incubation of DNA with the reagents at physiological pH, 8-oxoG formation was monitored over a 30 min. incubation period, and again a chaotic pattern was observed. The 8-oxoG concentrations from G in DNA are approximately 2/3 of that from free G, as the concentration of G was less than that studied in free G solutions. There may also have been some protection given by the DNA backbone, and the other DNA bases could have reacted with the oxidising agents in solution, though in this study there was no investigation into oxidation products of the other DNA bases. This may be an interesting area of future study. The highest concentration of 8-oxoG concentration from Ni(II) mediated oxidative DNA damage were found to occur at 5, 18, 22 and 29 min. The maximum 8-oxoG concentration recorded was 0.91 μM.

A ratio of [8-oxoG]/{[G]+[8-oxoG]} against reaction time is shown in Fig. 8. The fact that the ratio does not result in a linear increase in 8-oxoG concentration, and instead oscillations are seen even here, further confirms the suggestion that the many reactions involved here are occurring simultaneously and 8-oxoG is being formed and consumed and regenerated. It also implies that 8-oxoG is not final product of the reaction, and is just an intermediate. This evidence prompts investigation of further oxidation products.

Mass Spectrometric Analysis of Further Oxidation Products

The mass spectrometric analysis of the reaction of G, 8-oxoG and DNA with the reagents $NiSO_4$ and H_2O_2 gave a valuable insight into the mechanism of oxidation to G and 8-oxoG when free in solution and also when in the DNA backbone. It was evident

that 8-oxoG was further reacting as an intermediate in the overall process, and therefore further investigation was necessary to determine any further oxidation products of 8-oxoG. Previously identified 8-oxoG oxidation products, as well as a short summary of G oxidation from various mechanisms are illustrated in Fig. 9.

G Analysis

The Mass spectrometric analysis of oxidised G illustrated the formation of two products of *m/z* 156 and 157.The molecular weight of oxGH is 155 g mol^{-1}, and so the protonated adduct [oxGH + H^+] is expected at an *m/z* value of 156. The structure of oxGH is shown in Fig. 10. The peak at *m/z* 157 was not observed at the higher skim voltage of +30V, indicating that the soft ionisation technique possibly allowed for double protonation of the oxGH moiety. It was therefore suspected that this product corresponds to the formation of oxGH.

The formation of oxGH, as well as GH (157 g mol^{-1}) and Sp (193 g mol^{-1}) involves the oxidation of 8-oxoG to 5-hydroxy-8-oxoG and then subsequent formation of these oxidation products. 5-hydroxy-8-oxoG is a highly reactive intermediate and depending on the pH of the solution will form GH, oxGH, or Sp.[40] The *m/z* 156 product G oxidative damage product adduct, [oxGH + H^+], formed at alkaline pH 11 indicates that oxGH was the product formed. In order to confirm that this was indeed the final product of nickel mediated oxidative G damage, a tandem mass spectrometric analysis was performed. MS/MS was used to determine if the structure of the molecule of *m/z* 156 compared with that of oxGH. The resulting mass spectrum illustrates the fragmentation pattern observed (Fig. 4). There are two fragment peaks observed, at *m/z* 113.1 and 139.6.

Proposed fragmentation ions of ox-GH are illustrated in Fig. 11. These fragments of the product are consistent with the fragmentation observed in the mass spectrum obtained for oxGH strongly corroborating the proposal that this product is in fact oxGH.

The formation of the *m/z* 156 product, formed from free G, was then monitored over the course of the 120 min. incubation period, at intervals of 0, 10, 30, 30, 60, and 120 min. The concentration was seen to increase, linearly, to 60 min., after which it reached a plateau, indicating the reaction may be complete at this point, or had slowed down considerably. As the previous incubations were only up to 12 min. for 8-oxoG and 30 min. for G and DNA, the reaction after this point cannot be compared to the results obtained in the HPLC-EC assay. Further investigation of the formation of oxGH was carried out by incubation of G for 96 hr. Samples were analysed using both mass spectrometry and HPLC-EC up to this extended time. The formation of the *m/z* 156 product was noted after 96 hr. incubation with the reagents. It should also be noted that a small concentration of 8-oxoG was also detected (*m/z* 168), indicating that even after 96 hr., the reaction was still continuing at this point. Further work to elucidate this mechanism is currently underway in order to gain a better understanding of the extent of this reaction.

8-oxoG Analysis

The incubation of 8-oxoG with the Ni(II) and H_20_2 reagents yielded a peak at *m/z* 156, indicating that oxGH was formed. This is indicative that this product does, in fact, come from the further oxidation of 8-oxoG, and further illustrates the intermediate role of 8-oxoG in G oxidation.

DNA Analysis

The incubations of DNA with the reagents resulted in the formation of two major oxidation products, at *m/z* 168 and *m/z* 158. The peak at *m/z* 168 was due to the formation of 8-oxoG and the peak at *m/z* 158 was believed to be due to the formation of GH. There was no oxGH formation from DNA at this lower pH. This would indicate that, therefore, the main final product of DNA oxidation in physiological conditions mediated by Ni(II) was most likely GH (Fig. 12).

The concentration of 8-oxoG fluctuated over the incubation period, as before. The concentration of the *m/z* 158 peak, proportional to the intensity of the peak, increased with increasing incubation time. This final oxidation product did not form in a variable pattern similar to 8-oxoG and this indicated that it was not an intermediate, and therefore a potential biomarker of oxidative DNA damage.

A simplified summary of preliminary of a potential mechanism of G and 8-oxoG oxidation is illustrated in Fig 13. In Scheme 1, the guanine is oxidised at the C8 position and the species formed is further oxidised to 8-oxoG. The 8-oxoG is then quickly further oxidised to form another intermediate compound, which has been named 8-oxoG+ (Scheme 2). It is the reaction of two of these highly reactive intermediates which can go on to form the further oxidation products GH, oxGH or Sp, depending on pH, as well as reforming 8-oxoG, as seen in Scheme 3. 5-hydroxy8-oxoguanine may be an intermediate in the formation of the final oxidation products. The reformation of 8-oxoG will feed back into Scheme 2, causing the fluctuation of 8-oxoG concentration over the course of the reaction. It is for this reason we see a very complicated pattern of 8-oxoG formation.

Conclusion

The results that have been obtained in this study give some enlightenment into the mechanism of oxidative DNA damage by Ni(II) and H_2O_2, that to our knowledge, have previously gone unreported. This work has shown that the Ni(II)-mediated oxidation of G in DNA resulted in oscillatory pattern of 8-oxoG concentration, indicative of a complex reaction mechanism, which is currently under further investigation. It was also found, by HPLC-MS/MS analysis, that the major product of G oxidation was ox-GH at pH 11 and GH at pH 5.5. 8-oxoG is merely an intermediate in this complicated mechanism of Ni(II)-mediated DNA oxidation. These results are an important stepping stone in fully elucidating the mechanisms of oxidative DNA damage by Ni(II). There appears to be a complex, multifaceted reaction mechanism, incorporating numerous reactants, intermediates and a variety of possible products. Further work is currently being performed in this laboratory to investigate possible mechanisms behind the oscillatory pattern of 8-oxoG formation, as well as performing the incubations of DNA with $NiSO_4$ and H_2O_2 over extended incubation periods.

Figures

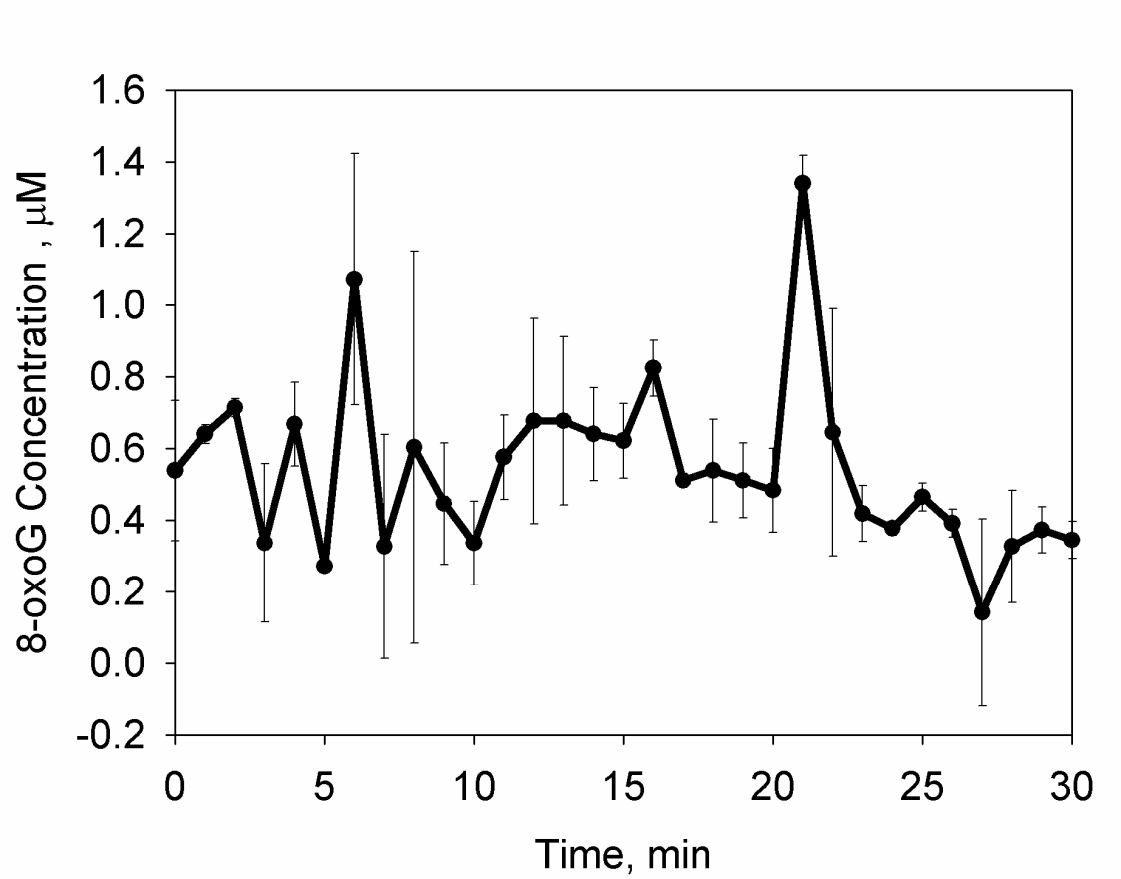

Fig. 1: 8-oxoG concentration as a function of time, after incubation of free G with reagents Ni(II) and H_2O_2 at 37 ^oC. The mean of each of the duplicate samples injected in triplicate is taken. (N=6)

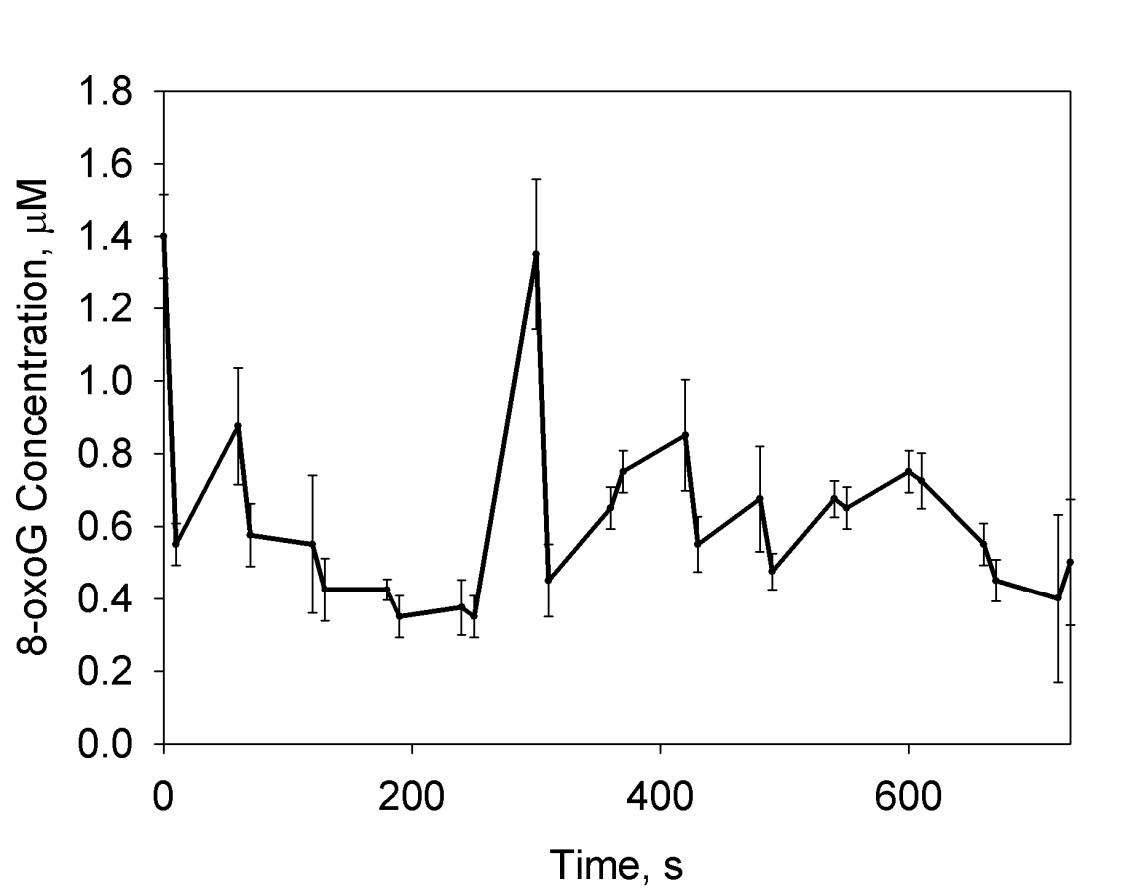

Fig. 2: Concentration of 8-oxoG monitored over 12 min. incubation of 8-oxoG with Ni(II) and H_2O_2 at 37 ^{o}C. (N=3)

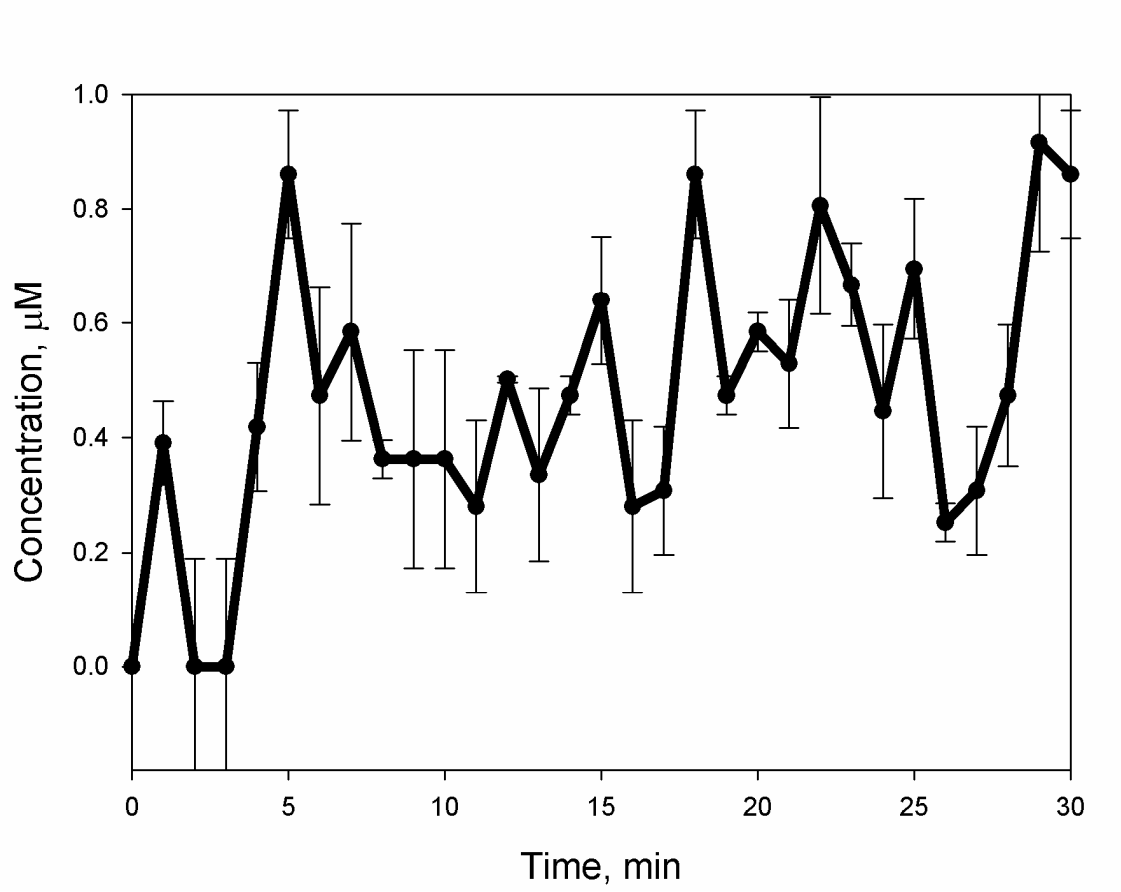

Fig. 3: 8-oxoG concentration as a function of time, after incubation of DNA with Fenton reagents Ni(II) and H_2O_2 at 37 ^{o}C. (N=3)

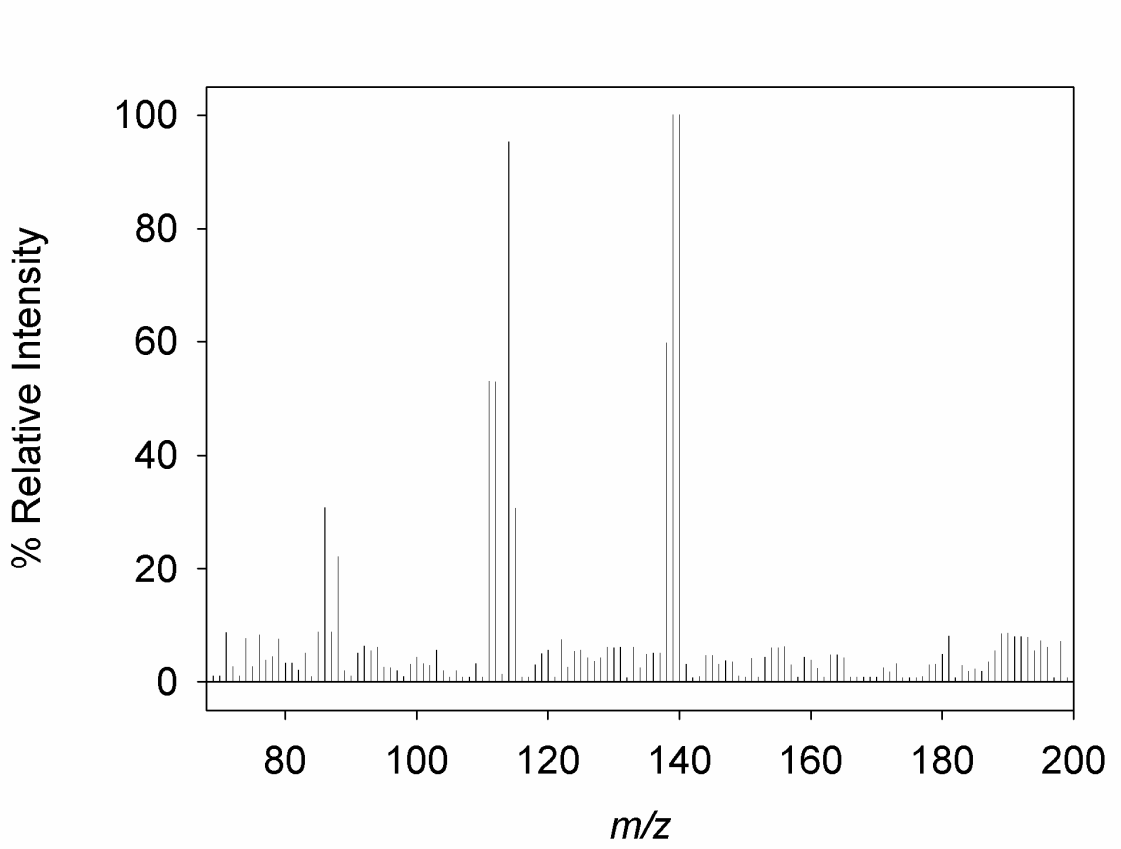

Fig. 4: MS/MS spectrum of peak found at *m/z* 156, illustrating fragment ions at *m/z* 113.3 and *m/z* 139.6. The MS/MS spectrum was measured after incubation of G with reagents Ni(II) and H_2O_2 at 37 ^{o}C.

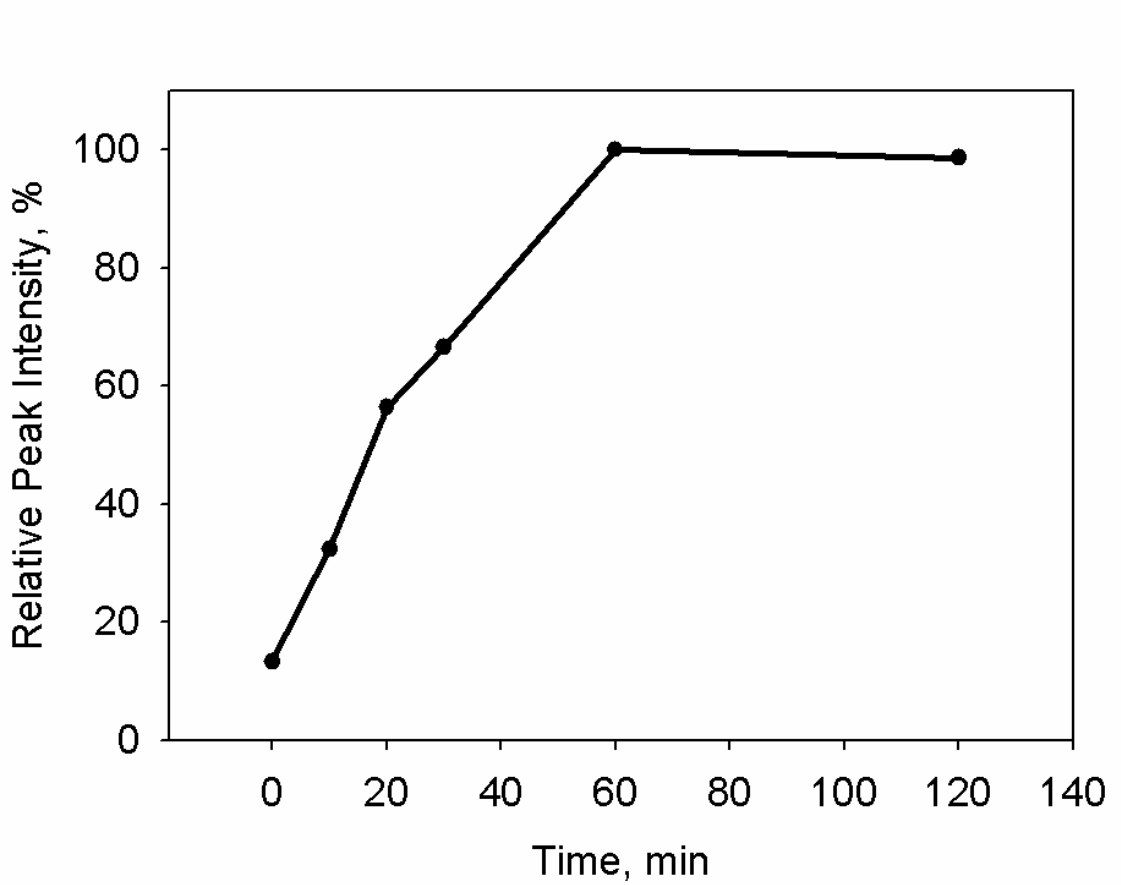

Fig. 5: Formation of product at *m/z* 156 from G over 120 min. with Ni(II) and H_2O_2 at 37 ^{o}C.

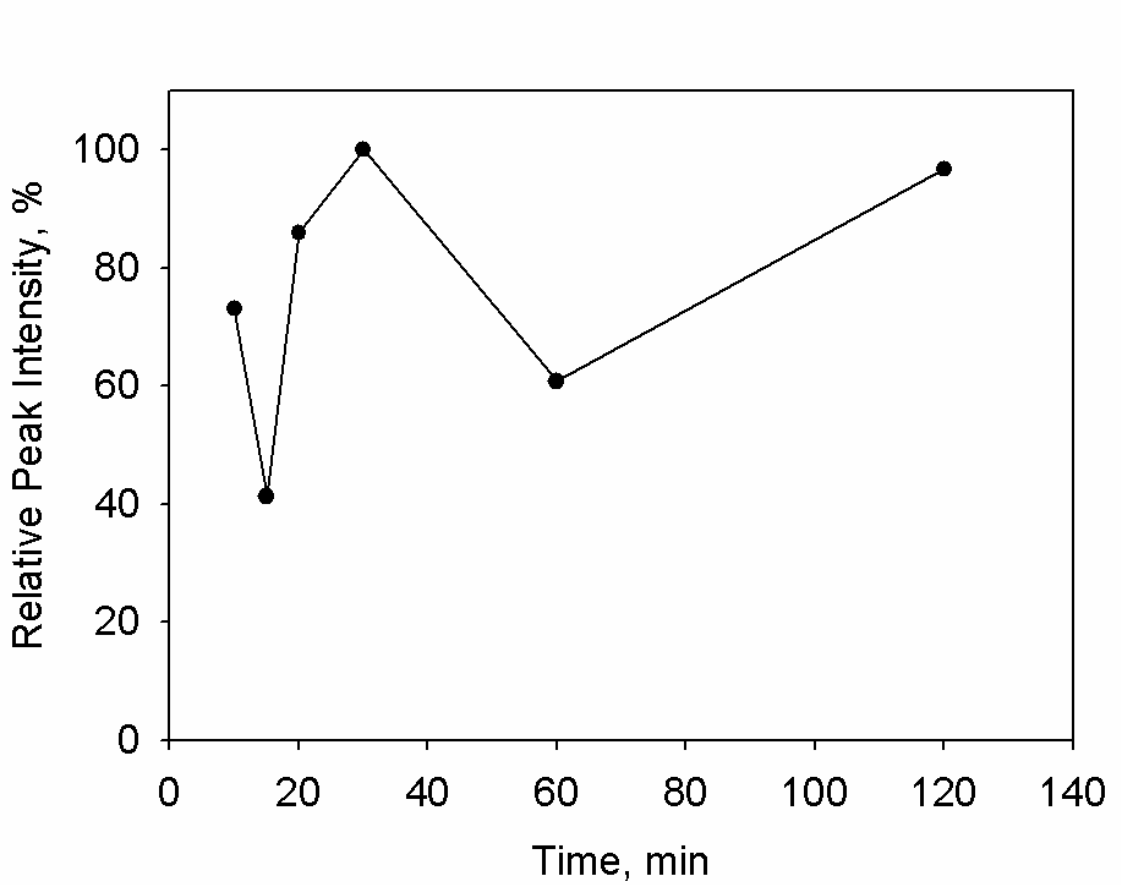

Fig. 6: Formation of 8-oxoG (*m/z* 168) from G in DNA on incubation over 120 min. with Ni(II) and H_2O_2 at 37 ^{o}C. The skim voltage used for the analysis was +15V.

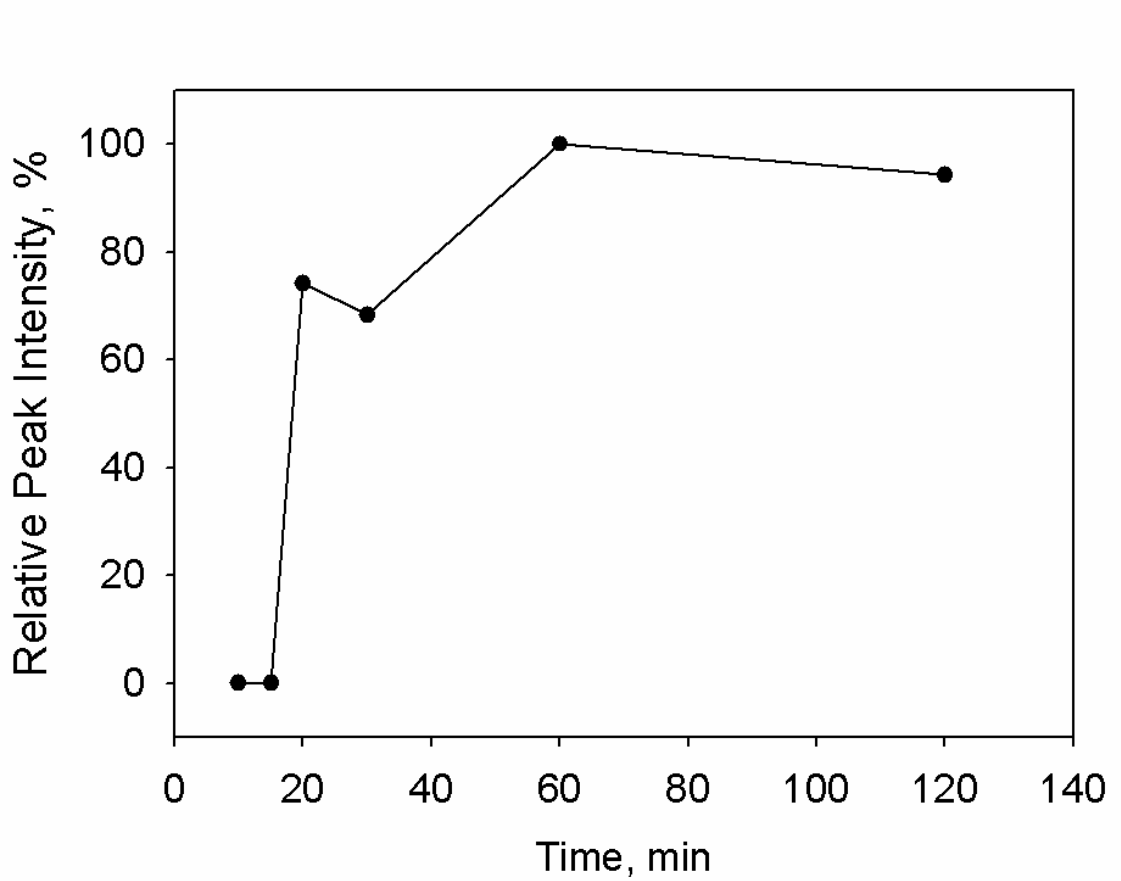

Fig. 7: Formation of product at *m/z* 158 from G in DNA, over 120 min. incubation period with Ni(II) and H_2O_2 at 37 ^{o}C.

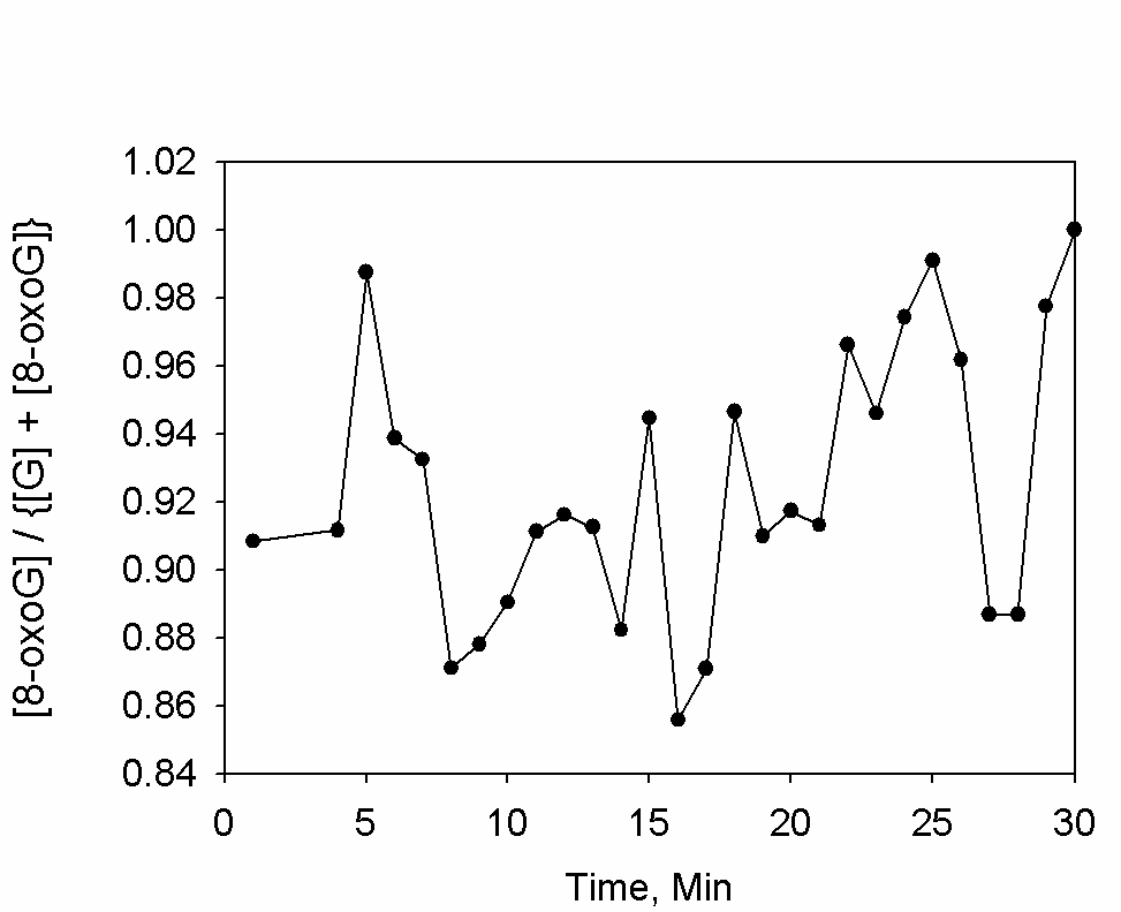

Fig. 8: Monitoring the concentration of 8-oxoG as a ratio [8-oxoG]/{[G]+[8-oxoG]} over a 120 min. incubation period on incubation of DNA with 1.5 mM Ni(II) and 500 mM H_2O_2 at 37 ^{o}C. The concentrations displayed are in mM for both 8-oxoG and for G.

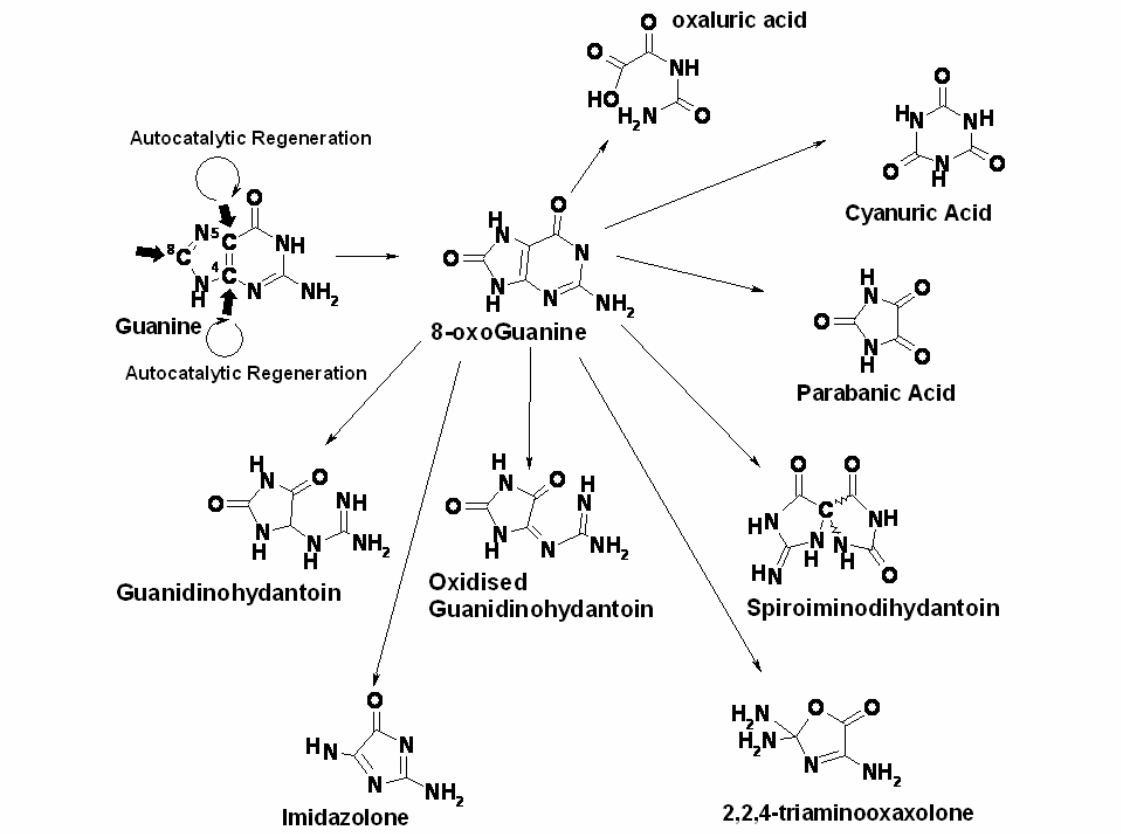

Fig. 9: Summary of G oxidation and further oxidation products of 8-oxoG. [36-39, 43]

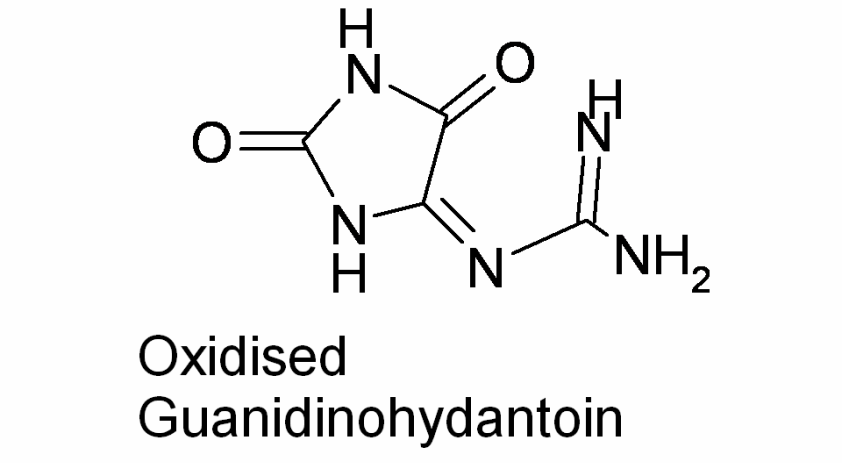

Fig. 10: Oxidised Guanidinohydantoin, (oxGH) [36]

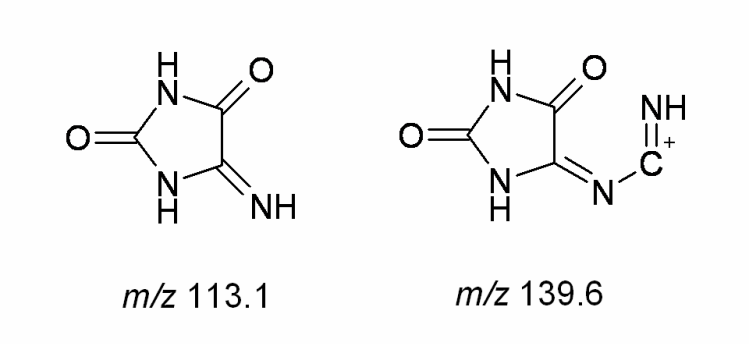

Fig. 11: Proposed structure of fragment ions of oxGH seen in mass spectrum obtained from MS/MS scan of peak at *m/z* 156.

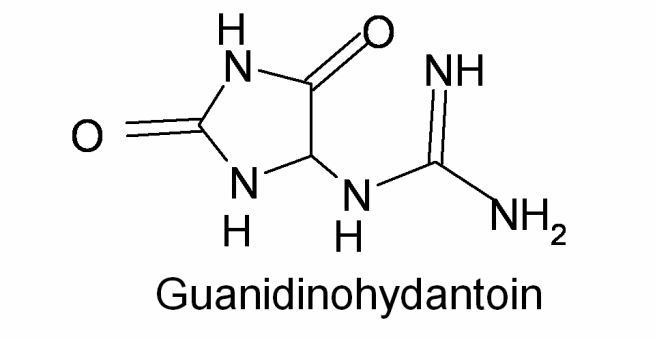

Fig. 12: Guanidinohydantoin (GH) [39]

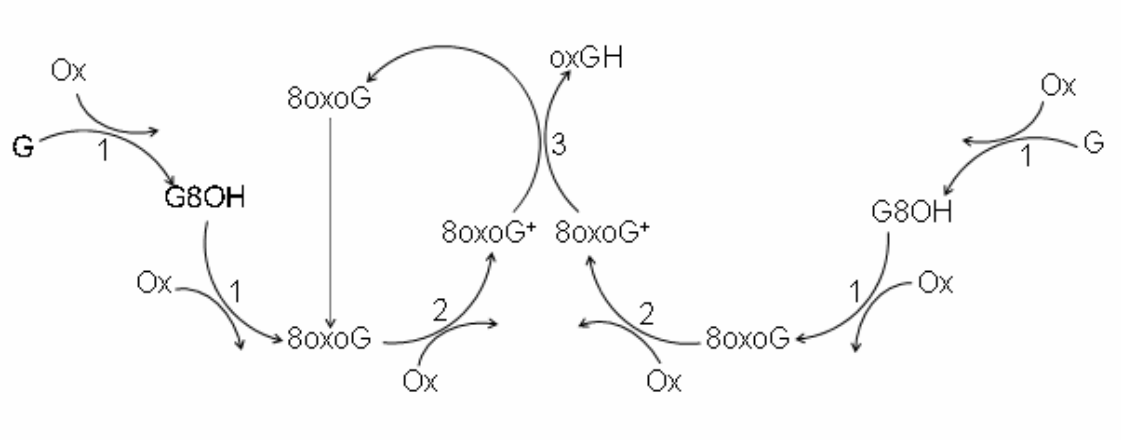

Fig. 13: Summary diagram illustrating a potential simplified mechanism of oxidation of G. Scheme 1 shows oxidation of G to 8-oxoG in a two step oxidation process. Scheme 2 shows the further oxidation of the 8-oxoG molecule to form an oxidised, highly reactive 8-oxoG+ moiety. Scheme 3 shows the reaction of two 8-oxoG+ to form a further oxidation product, oxGH, as well as reforming an 8-oxoG molecule, which can feed back into the reaction at the start of Scheme 2.

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