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Urinary 8-Hydroxydeoxyguanosine in Infants and Children

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Several diseases of prematurity are thought to be related to oxidative injury and many of the available markers are unsatisfactory. An assay was developed using HPLC with electrochemical detection for the quantitation of urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) as a proposed indicator for oxygen-derived free radical injury to DNA in preterm infants.

A median value of 3.79 μmol/mol creatinine was obtained for normal children (2–15 years old, $n = 14$). Urinary 8-OHdG excretion in neonates ranged from 0–99 μmol/mol creatinine. There were no gestation or birthweight related differences in urinary 8-OHdG, and no correlation with urinary malondialdehyde. Mean 8-OHdG excretion increased with postnatal age ($r = 0.80$, $p < 0.0001$, $n = 15$), mirroring the growth velocity curve. These changes could also be due to changes in the activity of the enzyme responsible for 8-OHdG excision.

Urinary 8-OHdG levels are unlikely to accurately reflect oxygen derived free radical activity given the strength of the relationship with growth.

Keywords: 8-Hydroxy-2′-deoxyguanosine, oxygen derived free radicals, preterm, neonate

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; ODFR, oxygen derived free radical

INTRODUCTION

Preterm infants suffer from a range of complications of intensive care, which have been attributed in part to injury from oxygen derived free radicals (ODFR).[1] Detection of ODFR injury in this population has largely been confined to measurement of products of lipid peroxidation, such as malondialdehyde in plasma[2] and urine,[3] and the alkanes ethane and pentane in exhaled breath.[4] ODFR damage is not confined to lipids and oxidation products from proteins and DNA may also be identified.

One such product is 8-hydroxydeoxyguanosine (8-OHdG) formed by oxidative damage to the nucleoside deoxyguanosine.[5] 8-OHdG is widely used as a sensitive marker of oxidative damage to DNA, particularly as the result of hydroxyl radical activity. The adduct formed has been shown to cause extensive mispairing in *in vitro* DNA replication studies, with G–T and A–C substitutions.[6] Repair of this lesion in DNA

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results in the excretion of free 8-OHdG in urine. It has been implicated in aging and cancer[7] and is also formed in activated neutrophils.[8] Its formation appears to depend to some extent on the metabolic rate[9] and is also increased by smoking,[7,10] a habit known to increase the production of ODFR's.

The aims of this work were (i) to develop a method that could be used to measure 8-OHdG in the urine of children and preterm infants; (ii) to quantify sex and age dependent differences in normal children; (iii) to observe the pattern of 8-OHdG appearance in preterm infants over the first month of life and (iv) to investigate the relationship with the lipid peroxidation marker urinary malondialdehyde.

METHODS

Preparation and Purification of Standard 8-Hydroxy-2′-deoxyguanosine

2.5 mM 2′-deoxyguanosine (Sigma) was hydroxylated as described by Kasai and Nishimura.[11] The resultant reaction mixture was then diluted 1 in 2 with 1 M sodium chloride and applied to an Isolute MF C18 (C18/OH) 10 g/60 ml solid phase extraction column (Jones Chromatography), preconditioned with 100 ml methanol and 100 ml water. The column was washed with 100 ml water and then with 30 ml of 15% methanol.

Purification was carried out by HPLC using a Waters Prep Nova-Pak HR C18 cartridge (100 × 25 mm) with a Prep Nova-Pak HR C18 Guard Pak. Mobile phase was 10% methanol at a flow rate of 5 ml/min. Ultraviolet absorbance of the eluate was read using a Waters Lambda Max 480 spectrophotometer set at 245 nm, and a Waters 740 Data Module. The injector was a Waters U6K, and a Waters multisolvent delivery system was used to pump the mobile phase. The peak following the large deoxyguanosine peak was collected and freeze-dried. A 1 mM stock standard solution was prepared by dissolving a portion of the freeze dried product in methanol. This was diluted in 15% methanol/50 mM KH_2PO_4, pH 5.5 for use as a working standard solution. Towards the end of the study a commercial preparation of 8-OHdG became available (Sigma), which co-eluted under the HPLC conditions used with the substance prepared by the above method.

Solid Phase Extraction of Urine Samples

Urine samples were stored at −20°C whilst awaiting analysis. Solid phase extraction was performed on 2 ml urine samples using a Varian Bond Elut LRC C18/OH solid phase extraction column as described by Shigenaga *et al.*[12] A second sample of urine (2 ml) was spiked with 50 pmol/ml 8-OHdG and extracted in parallel with the first to enable peak identification, and the calculation of the recovery and 8-OHdG concentration.

Measurement of 8-OHdG by HPLC with Electrochemical Detection

The residue from the solid phase extraction was dissolved in 200 µl water, filtered using a 0.22 µm Millex GV4 4 mm filter (Millipore) and an aliquot was injected onto the HPLC. The HPLC system was as described for the preparation and purification of 8-OHdG. Two Supelcosil 5 µm LC-18-S 25 cm × 4.6 mm columns (Supelco) were placed in series, with a Supelguard LC-18-S guard column, Waters 460 electrochemical detector with a glassy carbon working electrode set at an oxidation potential of +0.6 V. The flow rate was 1 ml/min and a gradient separation was carried out using 50 mM KH_2PO_4 (pH 5.5) and 70:30 acetonitrile:methanol (v/v) as described by Shigenaga *et al.*[12] The inter-assay coefficient of variation was 4.7%.

Urinary MDA Measurement

Urinary MDA was measured by TBA assay followed by HPLC with detection at 532 nm.[13]

To 600 µl sample/2.5 µM TEP/H_2O was added 60 µl BHT (0.2%) and 3 ml phosphoric acid (1%). Samples were mixed and incubated at room temperature for 10 min. One ml TBA (0.6%) and 340 µl H_2O were added before mixing and incubation in a boiling water bath for 60 min.

All samples were cooled and filtered prior to injection using Millipore Durapore PVDF 0.45 µm pore luer fitting filters. One hundred µl was injected in each case. A Kontron 420 HPLC pump, Waters Lambda Max UV detector set at 532 nm, Hewlett Packard 3392A integrator, Waters µBondapak C18, 10 µm 3.9 × 300 mm HPLC column and Guard column were used. 65%, 50 mM potassium phosphate (pH = 7.0), 35% methanol was the mobile phase at a flow rate of 2 ml/min. The column was washed nightly with 80% methanol. Standard curves were constructed and a correlation coefficient of $r = 0.994$ was obtained. The intra-assay coefficient of variation was 4.6%.

Since 24 h urine collection was not feasible in the preterm infants, 8-OHdG and urinary MDA concentrations were expressed in relation to urinary creatinine concentration. This was determined by spectrophotometric measurement at 520 nm of the red complex formed when creatinine reacts with picric acid under alkaline conditions.[14]

Subjects

Since there are no previously reported data on urinary 8-OHdG in children or preterm infants, we made measurements on several groups of infants.

1. Normal, healthy children age range 2–15 years.
2. One child receiving cytotoxic therapy, and 4 children acutely ill with nephrotic syndrome (expected to have high levels).
3. A group of sick and well preterm and term infants during the first month of life.

RESULTS

Measurements were made in 14 children between the ages of 2 and 15 years; there were 8 boys and 6 girls. A median value of 3.79 (range: 1.09–6.76) µmol/mol creatinine was obtained. No clear relationship with age (see Figure 1) or sex was observed, although boys tended to excrete slightly more 8-OHdG (median 4.6 µmol/mol creatinine for boys, 2.6 µmol/mol creatinine for girls).

Children with nephrotic syndrome or on cytotoxic therapy excreted a median 8-OHdG concentration of 15.5 µmol/mol creatinine (range: 9.5–39.1 µmol/mol creatinine). Urinary creatinine in this population was within the normal range.

Twenty-eight neonates were studied with median gestation of 30.5 weeks (range: 24–40 weeks gestation), and median birthweight of 1469 g (range: 650 – 3680 g). There was a preponderance of boys (20). A total of 53 samples were collected from infants (1–3 samples per baby) on 15 days during the first month of life. Mean 8-OHdG excretion increased linearly with time from birth ($r = 0.80$, $p < 0.0001$, $n = 15$) as shown in Figure 2. There were no notable changes in creatinine excretion over this time period (Figure 3). There were no gestation or birthweight related differences in 8-OHdG excretion,

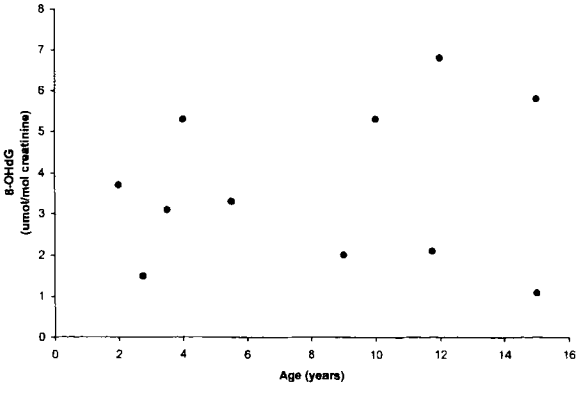

FIGURE 1 Graph to show urinary 8-OHdG excretion in children of different ages. Measurements are from 14 children between the ages of 2 and 15 years.

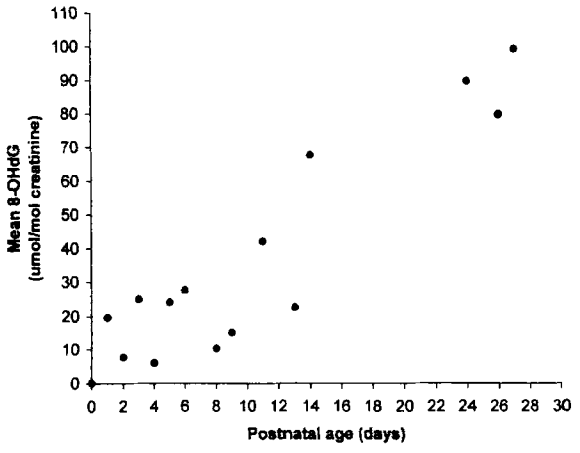

FIGURE 2 Graph showing relationship between mean urinary 8-OHdG excretion and postnatal age. 53 samples were collected from 28 infants on 15 different days during the first month of life ($r = 0.80$, $p < 0.0001$, $n = 15$). The mean value obtained on each postnatal day was used.

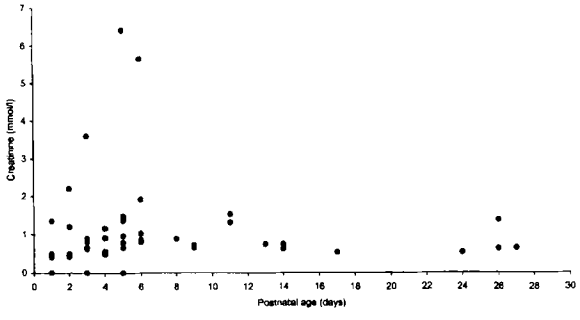

FIGURE 3 Graph showing changes in urinary creatinine excretion during the first month of life for preterm infants ($n = 51$).

and there was no correlation between urinary 8-OHdG excretion and malondialdehyde ($r = -0.19$, $p = 0.40$, $n = 23$). There was a weak inverse correlation between mean plasma creatinine recorded and mean 8-OHdG over the first week of life ($r = -0.56$, $p = 0.02$, $n = 16$).

DISCUSSION

Levels of urinary 8-OHdG in adult humans are generally low at between 1.0 and 7.0 μmol/mol creatinine,[15,16] but with raised values in some occupations involving exposure to azo dyes[17] and benzene.[18] Higher levels have also been described in cancer patients both before and after treatment with chemotherapy or irradiation.[15] The median value seen in healthy children in this study is within the range of published values for adults. It was expected that the excreted 8-OHdG concentration would increase with increasing age, but no evidence of such a relationship was observed. In a study investigating the role of oxidative damage in Alzheimer's disease, investigators were also unable to find a relationship between brain cell 8-OHdG levels and age or sex.[19] Slightly higher (although statistically insignificant) values were obtained from male than female children, a finding in agreement with results from Loft *et al.*[7] The values seen in children undergoing cytotoxic therapy or acutely ill with nephrotic syndrome were, as expected, about 4 times higher, demonstrating DNA damage. In the neonates, there was a gradual increase in urinary 8-OHdG over the first month of life *ex utero* which seems to mirror the growth velocity curve. This increase does not reflect changes in rates of creatinine excretion, since this was essentially constant over the studied time period. The growth velocity curve for neonates increases steadily until a peak is reached at between 4 and 6 weeks of age, with a gradual decrease thenceforth until about 6 months when the velocity is comparatively stable.[20] Neonates who are growing rapidly are likely to have a larger pool of free nucleotides that are much more prone to oxidative damage than nucleotides incorporated into DNA. Additionally, because of the high rate of cell division, resulting in the removal of the nuclear membrane and histones, DNA may be exposed to a much higher concentration of oxygen derived free radicals and thus be more vulnerable to oxidative damage. Unlike other markers of oxidative free radical damage, there was no correlation with degree of prematurity, and no relationship between urinary malondialdehyde and urinary 8-OHdG. We have previously shown that urinary MDA as

determined by this method in preterm infants, correlates with plasma MDA concentrations 24 h earlier.[13] It is likely that the concentration of excreted 8-OHdG in neonates is fairly low and constant during the first week of life in preterm infants, since they have very low velocity growth in the first week of postnatal life. We would expect excreted 8-OHdG in neonates to plateau at between 4 and 6 weeks of life, and then decline steadily to adult values. There may be a further increase in urinary 8-OHdG excretion during the adolescent growth spurt. It is also possible that the activity of enzymes that repair 8-OHdG from DNA or its precursor might be rising over that time, thus giving more excretion. Studies in cystic fibrosis patients, who have inflammatory lung disease, found elevated urinary 8-OHdG compared to control children, but this did not correlate with clinical status.[21]

In conclusion, we have reported here the first measurements of urinary 8-OHdG in a neonatal population. In normal, healthy children we were unable to demonstrate a significant age or sex difference in urinary 8-OHdG excretion. There was a gradual increase in excreted urinary 8-OHdG over the first month of life, mirroring the velocity growth curve, but no correlation was found between urinary 8-OHdG and urinary malondialdehyde. It seems from these results, that urinary 8-OHdG levels are unlikely to be a useful marker of oxygen derived free radical activity in neonates.

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