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7,8-hydroxy-2′-deoxyguanosine/2′-deoxiguanosine ratio determined in hydrolysates of brain DNA by ultrachromatrography coupled to tandem mass spectrometry

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ABSTRACT

7,8-hydroxy-2′-deoxyguanosine (8-OHdG) is an abundant DNA lesion formed by oxidation of the nucleoside 2'-deoxyguanosine (2-dG) and one of the most studied and accepted oxidative stress biomarkers. 8-OHdG has a strong carcinogenic potential, and prolonged oxidative stress heightens pathological conditions and especially cancer risk. Our aim was to develop, validate and apply a reliable method to assess DNA oxidation in genomic cellular DNA of sensible target organs such as brain. A procedure to isolate and digest the DNA of brain tissue properly for further detection of 8-OHdG and 2-dG by Ultra Performance Liquid Chromatography–tandem Mass Spectrometry (UPLC-MS/MS) was optimized. The UPLC-MS/MS was validated following the American Food and Drug Administration (FDA) Guidelines using mice pups' brain samples. To demonstrate the applicability of the UPLC-MS/MS method, 8-OHdG/2-dG ratio was determined in brain tissue of 1 day old newborn mice pups (P1) in a model of hypoxia pre-conditioning during fetal-to-neonatal transition. We found that hypoxia at birth (FiO_2 0.14) and for 8 h thereafter induced lower levels of DNA oxidation in mice pups and rendered even protective against a postnatal asphyxia/reoxygenation insult compared with fetal to neonatal transition in room air.

We conclude that the UPLC-MS/MS method developed has proven suitable for the analysis of DNA oxidation biomarker 8-OHdG/2-dG ratio in tissue samples from newborn mice pups. We aim to apply this method in future studies aiming to provide a deeper insight into the mechanisms of oxidation DNA caused during neonatal asphyxia and resuscitation.

1. Introduction

Reactive Oxygen Species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$) or peroxynitrite ($ONOO^-$) are toxic molecules highly reactive that are formed in living organisms during normal metabolic processes. ROS can react with lipids, proteins, carbohydrates and nucleic acids altering their structure and function, transient- or permanently. However, in normal healthy cells ROS production and antioxidant defences represented by the Redox Defence System (RDS) are extremely well balanced [1]. DNA is one of the preferred substrates for ROS including HO. or $ONOO^-$. HO. may attack deoxyribose phosphate backbones as well as the nucleobases of DNA nucleotides, generating a broad variety of base and sugar modification products [2]. Under physiological conditions, oxidative DNA damage is being produced continuously and RDS but simultaneously enzymatic DNA repair mechanisms are capable of maintaining a low steady state of oxidative DNA damage [3].

An abundant DNA lesion formed *in vivo*, by oxidation of the nucleoside 2′-deoxyguanosine (2-dG), is 8-oxo-2′-deoxyguanosine (8-oxodG), also known as 7,8-hydroxy-2′-deoxyguanosine (8-OHdG). On the one hand, 8-OHdG plays essential biological functions regulating gene expression which including epigenetic mechanisms interfering [4,5] and DNA relaxation for binding of transcription factors [6,7]. On the other hand, 8-OHdG has a strong carcinogenic potential. If not

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repaired, 8-OHdG increases the DNA base mutagenic frequency by promoting G > T/C > A transversion, which may affect oncogenes and tumour suppressor genes. Therefore, prolonged impairment of redox homeostasis, known as oxidative stress as well as defects on DNA repair heightens cancer risk [4]. 8-OHdG has been detected in numerous biological matrices including cells, tissue, plasma, urine or cerebrospinal fluid [8,9]. It is broadly accepted that 8-OHdG within the DNA is repaired and released from cells to the blood stream and rapidly excreted into the urine by the kidneys (few hours), and thus, urinary excretion is interpreted as a measure of DNA oxidation. Notably, oxidation of the nucleotide precursors' pool and of RNA also occurs; however, their contribution to the urinary 8-oxodG excretion is still not fully elucidated [10]. In addition, special attention must be taken to artefactual formation of 8-OHdG during the DNA isolation from cells or tissues [11]. In this regard, especial efforts were made by the European Standards Committee on Oxidative DNA Damage (ESCODD) to reduce inter-laboratory variability and to establish suitable methods for DNA samples preparation for 8-oxodG measurement [12–14].

Since the first description of 8-OHdG formation in DNA in 1984 [15], several analytical methods have arisen for its detection. Among them, the most widely employed are enzyme-linked-immune-absorbent-assay (ELISA), gas chromatography coupled to mass spectrometry (GC-MS), and high performance liquid chromatographic coupled to electrochemical detection (HPLC-ECD) and to tandem mass spectrometry (HPLC-MS/MS). There are many commercial ELISAs for measuring 8-OHdG that have the advantages of being faster and cheaper than chromatographic methods. By contrast, it has been demonstrated that none of the anti-8-OHdG are as specific as desired resulting in an overestimation of the 8-OHdG levels [16,17]. GC-MS requires a laborious and time-consuming sample treatment. In addition, artefacts can be also formed during the derivatization procedure for measuring 8-OHdG by GC-MS. This may be overcome by adding a pre-purification step by HPLC but at the expense of increasing the technique tediosity [18,19]. Of note, for sensitivity, reproducibility and accuracy, the use of liquid chromatographic methods such as HPLC-ECD or HPLC-MS/MS are considered the gold-standard for the assessment of 8-OHdG [16]. Furthermore, both ECD and MS approaches have shown a close agreement in the values during inter-laboratory assays [12,14–17]. HPLC-MS/MS also has the advantage of giving unambiguous information about analyte identification. Furthermore, additional strengths brought to MS by stable isotope internal standardization makes it the method of choice [3,17].

The aim of the present study was to develop a reliable and accurate analytical method to determine the 8-OHdG/2-dG ratio in hydrolysates of DNA isolated from mice brain samples.

2. Material and methods

2.1. Reagents and material

Standards of 2-dG and 8-OHdG (96% w/w purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 8-oxo-2′-deoxyguanosine-^{13}C–$^{15}N_2$ (8OHdG-$^{13}C^{15}N_2$) (Chemical purity: 98%; Isotopic purity: 99%) was used as internal standard (IS) and was obtained from Toronto Research Chemicals (Toronto, Canada). Acetonitrile (ACN) (LC-MS grade), methanol (MeOH) (LC-MS grade), formic acid (HCOOH) (analytical grade), Phenol solution, Phenol: choloroform:isoamyl alcohol, isopropanol, ethanol (EtOH), *Proteinase K* from tritirachium album, Deoxyribonucleic acid, single stranded from calf thymus, *Nuclease P1* from Penicillium citrinum lyophilized powder (≥200 units/mg protein), *Phosphodiesterase I* from Crotalus adamanteus venom (≥0,01 units/mg solid) and Normal Melting Temperature agarose were purchased from Sigma Aldrich Química SA (Madrid, Spain). *Alkaline phosphatase* from calf intestine (2000 units/mg protein), Orthophosphoric-monoester phosphohydrolase was purchased from Roche (Mannheim, Germany). TAE 50X (Norgen Biotek, Thorold, Canada), GelRed™ Nucleic Acid Gel stain (Biotium, Fremont, USA), GeneRuler 1 Kb DNA ladder (Fermentas, Vilnius, Lithuania) and water was Milli-Q grade (18.2 MV) from a Millipore purification system were also used.

2.2. Solutions

Lysis buffer (Tris-HCl 10 mmol L^{-1}, EDTA 50 mmol L^{-1}, NaCl 50 mmol L^{-1}, SDS 0.2% (w/v), pH 7.6) and Digestion buffer (Tris-HCl 40 mmol L^{-1}, $MgCl_2$ 10 mmol L^{-1}, pH 7.5) were prepared for tissue homogenization and DNA digestion respectively. The DNA electrophoresis buffer was TAE 1X (Tris 40 mmol L^{-1}, Acetic acid 20 mmol L^{-1}, EDTA 1 mmol L^{-1}). Individual stock solutions of 2dG (2 mmol L^{-1}), 8-OHdG (1 mmol L^{-1}) and 8-OHdG-$^{13}C^{15}N_2$ (1 mmol L^{-1}) were prepared by accurately weighting the standards and dissolving them in H_2O (0.1% HCOOH,v/v). They were stored at −20 °C. Working solutions obtained by dilution of the stock solutions in H2O (0.1% v/v HCOOH) were used to prepare the standards and spiked samples. Calibration curves were prepared daily by serial dilution of working solutions. Concentration ranges were selected from concentrations found in brain during a pre-validation study (data not shown).

2.3. Animal model

Biological samples (Brain tissue) were obtained from Wild-type C57BL/6 mice pups at one day after birth (P1). Wild type C57BL/6 timed-pregnant mice were fed food and water *ad-libitum* and exposed to regular dark/light cycles of 12/12 h. A total of 28 neonatal mice pups were used for this study, fourteen in the control and fourteen in the experimental group. The Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural (Community of Valencia) and the University of Valencia accepted the research protocol. Briefly, 8–12 h prior to delivery at G18 pregnant mice randomly assigned to an oxygen chamber cycler (O_2 range from 0% to 99'9%; *ad hoc* design) kept either at FiO_2=0.21 (normoxic group) or FiO_2=0.14 (hypoxic group). Thus, newborn mice pups perform the fetal-to-neonatal transition under normal or mild hypoxic conditions and were kept for 8 h in those conditions. Thereafter, all the pups were put in room air (FiO_2 =0.21). Afterwards, pups were subjected or not, to an hyperoxic insult, FiO_2=1.0 (reoxygenation) for one hour and then reset to 0.21. At the end of the experiment the mice pups of the four groups (N=7) were sacrificed at one day of life (P1) by decapitation, and the brain was taken out and snap-frozen quickly and kept at −80 °C until analyzed.

2.4. Sample treatment

Brain samples were homogenized in lysis buffer adding 1 ml per 100 mg of tissue. Thereafter, they were incubated with *proteinase K* at 50 °C overnight. This was followed by a phenolization step, adding to the sample an equivalent volume (1:1) of 100% pure phenol at pH 8.0 and mixing gently, by inversion 40 times, until a homogeneous emulsion was obtained. After that, samples were centrifuged at 250 rcf for 3 min, collecting the upper phase with a sterile cut off tip and transferring it to a sterile tube.

Then, the process was repeated but adding an equal volume of phenol: chloroform:isoamyl alcohol (1:1) instead of phenol, collecting the upper phase after centrifugation (25,000 rcf for 3 min). Next, DNA within the samples was precipitated by adding 1 ml of isopropanol and centrifuged at 25,000 rcf for 15 min. If the procedure is well carried out, a white mesh (precipitated DNA) should be observed clearly. Subsequently, isopropanol was removed from samples with caution avoiding any contact with the precipitated DNA. Thereafter, DNA samples were washed by adding 1 ml of ethanol 70% to the DNA samples, centrifuging at 25,000 rcf for 10 min, removing as much ethanol as possible carefully, and finally allowing the rest of ethanol to