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Relationship between levels of oxidative DNA damage, lipid peroxidation and mitochondrial membrane potential in young and old F344 rats

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Abstract

The extent of *in vivo* oxidative damage has been known to be cumulative over the period of the life of mammals. Our hypothesis is that there should be a positive correlation between the levels of 8-hydroxy-2′-deoxyguanosine (8OHdG) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in major rat tissues. We also investigated whether increased level of oxidative stress causes a decrease in the mitochondrial membrane potential of peripheral lymphocytes of old rats using the MitoTracker Red fluorochrome. Our results show positive correlations between 8OHdG and 8-iso-$PGF_{2\alpha}$ for liver, brain and kidney measured by HPLC-UV-ECD (electrochemical detector) and EIA methods, respectively. However, heart tissues show a negative correlation. The mitochondrial membrane potential of old rat lymphocytes records significant decrease compared with the young lymphocytes. Based on our results, we conclude that in ageing studies, specific tissues need to be examined in order to measure the localised DNA damage and lipid peroxidation as different tissues display different extent of oxidative damage. We believe this approach of using combined markers is useful to verify the true efficacy of health intervention studies in animals and humans. In addition, the isoprostane assay can be further developed looking at lipid peroxidation as a potential marker in ageing studies.

Keywords: *8OHdG, 8-iso-$PGF_{2\alpha}$, mitochondrial membrane potential, oxidative damage, ageing*

Introduction

Free radicals arising from environmental sources and from metabolism are interacting continuously in biological systems. The balance between oxidants and antioxidants has been known to be essential to minimize molecular, cellular and tissue damage. Oxidative damage to DNA, lipids, proteins and various macromolecules accumulates with age and has been postulated to be major, although not the only endogenous damage leading to ageing [1,2]. 8-hydroxy-2′-deoxyguanosine (8OHdG) is one of the most frequently formed lesions among over 20 known base modifications caused by oxygen radicals [3] and has been found to have a non-linear accumulation in different tissues of rats during ageing [4].

Lipid peroxidation is another well-investigated free radical-induced process. It is also known to be an important factor in the pathophysiological functions of a number of diseases and in the ageing process [5–7]. One of the best biomarkers of lipid peroxidation appears to be the isoprostanes. 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is one major isoprostanes formed *in vivo* [8] and are initially generated *in situ* from esterified arachidonic acid in phospholipids and are released in the free form into the circulation [9]. One of the most useful aspects of measuring 8-iso-$PGF_{2\alpha}$ to assess oxidative stress is the fact that they

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can be accurately and specifically measured and are present in all biological tissues or fluids studied [10,11]. In animals, the levels of 8-iso-$PGF_{2\alpha}$ are increased in oxidative stress models [12] and reduced by dietary antioxidant supplementation [13]. Sensitive gas chromatography-mass spectrometry (GC-MS) assays have been the most widely used method to measure 8-iso-$PGF_{2\alpha}$. Other methods such as enzyme immunoassays (EIAs) and radioimmunoassays (RIAs) have also been reported [14,15].

The continuous generation of reactive oxygen species (ROS) by the mitochondrial respiratory chain throughout cell life produces an age-related "chronic" oxidative stress which plays a role in cellular ageing. Oxidative lesions of mtDNA have been found to accumulate with age in human and rodent tissues [16,17]. With regard to peroxidation of mitochondrial lipids upon ageing, part of it appears to be caused by changes in the membrane lipid composition which enhance its susceptibility to oxidative damage [16]. In some systems, the onset of degenerative conditions seem to coincide with a phenomenon known as permeability transition, which is associated with a collapse of mitochondrial membrane potential (DeltaPsi(M)). Age-related decreases in membrane potential have also been shown to occur in the mitochondria of various tissues [18]. Disruptions in the DeltaPsi(M) can be measured using a number of cationic lipophilic fluorochromes including 3,3′-dihexylocarbocyanine iodide (DiOC6) [19], 5,5′,6,6′-tetracholoro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine (JC-1) [20] and choloromethyl-X-rosamine (MitoTracker Red or CMX-Ros) [21] and Rhodamine 123 [22].

DNA damage caused by peroxidised lipids may be implicated in the ageing process and in tumourigenesis [23]. It has been shown that there is concurrent damage not only to lipids but also DNA during lipid peroxidation [24]. So far, although many reports have measured the 8OHdG levels in various animal tissues but none have correlated this with the levels of 8-iso-$PGF_{2\alpha}$ and mitochondrial membrane potential of young and old animals. This paper addresses the question of whether old rats which record high levels of base oxidation products in DNA also show high levels of lipid peroxidation in the same organ. In this work, we measured the levels of 8OHdG in the liver, kidney, heart and brain of young and old rats using the sensitive and well-established high performance liquid chromatography-electrochemical detector (HPLC-ECD) method which was first developed by Floyd et al. [25]. To investigate the lipid peroxidation status in ageing animals, we employed an optimised EIA method for the measurement of total 8-iso-$PGF_{2\alpha}$ in plasma, liver, kidney, heart and brain of young and old rats. We also examined how the mitochondrial membrane potential of circulating peripheral lymphocytes in the whole body system is affected by ageing. In this experiment, we used the MitoTracker Red fluorochrome which is know to be a structurally novel fluorescent probe with positively charged rosamine derivatives. These derivatives are rapidly taken up into the negatively charged mitochondria suggesting that their loading should be dependent on changes in DeltaPsi(M) and the localisation should be mitochondria specific [26]. The MitoTracker Red or its reduced state has been known to be strongly influenced by the presence of ROS [27]. Thus, the aim is to determine whether the old rats are in the general state of elevated oxidative stress in major physiological organs and if correlations between 8OHdG and 8-iso-$PGF_{2\alpha}$ levels in these organs exist. We believe a study of the relationship between lipid peroxidation and DNA damage is pertinent to understanding ageing and degenerative diseases.

Materials and method

Animals

Male Fischer 344 rats of two age groups (6- and 24-months old) were obtained from the National Institute of Ageing, USA. Upon arrival, animals were kept in an AAALC-accredited facility at the National University of Singapore on a daily cycle of alternating 12 h periods of light and dark. Rats received food and drinking water *ad libitum*. Animal ages at the time of sacrifice were 8 months (for the young group, $n = 9$) and 26 months (for the old group, $n = 8$), respectively. All procedures were performed in compliance with relevant regulations approved by the Institutional Animal Care and Use Committee of National University of Singapore. After intraperitoneal anaesthesia with a mixture of xylazine (10 mg/kg) and ketamine (40 mg/kg), blood was withdrawn via cardiac puncture into EDTA-vacutainers put on ice. Liver, kidney, heart and brain were subsequently removed from the animals and snapped frozen in liquid nitrogen followed by storage at -80°C until further analysis. All dissection procedures were performed approximately at the same time in the mornings to minimise any day-to-day variations in the levels of oxidative damage products. Blood samples were processed immediately to separate the plasma and lymphocytes using the Ficoll–Paque centrifugation technique. Aliquots of 1 ml of plasma sample were then stored with 20 µl of 5 mM butylated hydroxytoluene and snapped frozen in liquid nitrogen until further isoprostane analysis. The lymphocytes were used immediately for MitoTracker staining.

Materials

8-hydroxy-2′-deoxyguanosine (8OHdG). Tris–HCl and trisodium citrate, HPLC grade were purchased from Fisher Scientific (Pittsburgh, PA, USA). Triton X-100, sucrose, $MgCl_2$, NaCl, diethylenetriamine pentaacetic acid (DTPA), ethylenediamine tetraacetic

acid (EDTA), sodium dodecyl sulphate (SDS) and RNase A each of the highest purity available, were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). HPLC-grade chloroform, isoamyl alcohol, isopropanol and methanol were from purchased from Tedia Co. Inc. (Fairfield, OH, USA). Absolute ethanol and acetic acid glacial were obtained from Merck Chemicals (Darmstadt, Gemany). Protein precipitation solution was purchased from Puregene, Gentra Systems (Minneapolis, USA). DNase-free RNase, DNase I, alkaline phosphatase (AP), phosphodiesterase I (PDE I), and Proteinase-K were purchased from Roche Diagnostics (Mannheim, Germany). Nuclease P1 (NP1) and phosphodiesterase (PDE II) were obtained from US Biological (MA, USA) and Calbiochem (CA, USA), respectively. Standard 8-hydroxy-2′-deoxy guanosine (8OHdG) and nucleosides (2-deoxy cytidine, dC; 2-deoxyguanosine, dG; 2-deoxy thymidine, dT; 2-deoxyadenine, dA) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Deionised water from Milli-Q Synthesis (18 MΩ cm^{-1}) was used for all reagent preparations.

8-iso-$PGF_{2\alpha}$. Indomethacin, KOH, KH_2PO_4, K_2HPO_4, butylated hydroxytoluene (BHT) and EDTA of the highest purity were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). HPLC-grade chloroform and methanol were obtained from Tedia Co. Inc. (Fairfield, OH, USA). 8-Isoprostane EIA Kit, 8-Isoprostane Affinity Column, Eicosanoid Affinity Column Buffer, Eicosanoid Affinity Column Elution Solution and UltraPure Water were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Cayman's UltraPure Water was used for all 8-isoprostane samples and reagent preparations, where applicable.

MitoTracker Red staining and measurements. Ficoll–Paque was purchased from Amersham Bioscience (USA). MitoTracker Red stain (CMXRos) was purchased from Molecular Probe (OR, USA). Dimethyl sulfoxide (DMSO), 99.9% purity and analytical-grade paraformaldehyde was obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). FluorSave reagent was purchased from Calbiochem (CA, USA).

Methods

8-hydroxy-2′-deoxyguanosine. DNA was extracted and purified in a method previously described [28] with some modifications. Briefly, 100 mg of frozen tissue (liver, kidney, heart and brain) was pulverised in liquid nitrogen and nuclei were isolated in cold 10 mM Tris–HCl pH 8.0 containing 1% Triton X-00, 0.32 M sucrose solution, 0.1 mM DTPA, 0.2 mM EDTA and 5 mM $MgCl_2$. Pellet was treated with RNase A and DNase-free RNase for 0.5 h at 37°C. Cell lysis solution containing 10 mM Tris–HCl pH 8.0, 1% SDS, 5 mM EDTA and 0.1 mM DTPA was added and samples were digested with 20 μg/μl Proteinase-K for 2 h. Protein precipitation solution was then added to cooled samples to remove proteins. The supernatant containing DNA was extracted twice using equal volumes of chloroform/isoamyl alcohol (24:1 v/v). DNA was precipitated at −20°C overnight in the aqueous phase with equal volumes of ice-cold isopropanol added. After centrifugation, DNA was washed twice with 70% ethanol and dissolved in Milli-Q water. Immediate enzymatic digestion was done after measuring the ratio of the UV absorption at 260/280 nm and determining the concentration of the DNA yield. The enzymatic digestion has also been described elsewhere [43] using 30 μg of DNA and 20 U/μl DNAse I followed by 1 U/μl NP1 in a solution containing 0.1 mM DTPA. Subsequent digestion was completed using 1 U/μl AP, 0.01 U/μl PDE I and 0.0044 U/μl PDE II. Hydrolysates were immediately filtered through the Vivaspin 500 concentrator (MWCO 5000 Sartorius) to remove proteins and interfering molecules. Concentrated samples were placed in the autosampler of the HPLC system at 4°C until injection. Samples were analysed using HPLC coupled with UV-photodiode array (PDA) and electrochemical (EC) detectors. The HPLC system consists of a liquid chromatography separation module (LC) with an autosampler (Model 2695, Waters, USA), a degasser, 2 pumps with 4-way solvent programming, a photodiode array (PDA-UV/VIS) detector (Model 2996, Waters USA) and an amperometric EC detector (Model 2465, Waters, USA) with a glassy-carbon working electrode and an ISAAC reference electrode (Waters, USA). Data from both UV and EC detectors were acquired by Waters Empower Software (Waters Corp., version 1.6). Two HPLC columns were used in series (5 μm, C_{18} 4.6 × 150 mm, Waters, USA) and protected by a similar C_{18} ODS guard column. The mobile phase consisted of 5% methanol and 95% of aqueous buffer containing 50 mM trisodium citrate and 20 mM NaCl, at pH 3.5 adjusted using acetic acid glacial. Mobile phase was filtered through Millipore 0.22 μm GP Express Plus Membrane before using at a flow rate of 0.6 ml/min. Normal nucleosides (dC, dG, dT, dA) were detected by the UV absorption at 254 nm and 8OHdG was monitored with the ECD using the working cell (DC mode at 700 mV). Levels of oxidised nucleosides were quantified using the standard curves of each corresponding compound. Standard samples were injected at the beginning of the whole HPLC run and after every 15 DNA samples. The degree of DNA damage was expressed as 8OHdG/10^6 of dG. Care was taken to process

the samples away from light exposure and at ice cold conditions where possible.

8-iso-PGF$_{2\alpha}$

A amount of 300 mg of tissues (liver, kidney, heart and brain) were used for the 8-isoprostane EIA assay. Tissue samples were homogenised in ice-cold 0.1 M phosphate buffer containing 0.1 M of KH_2PO_4/K_2HPO_4, 1 mM EDTA, 10 μM indomethacin and 0.5% BHT. For tissue samples, Folch extractions were carried out to ensure complete recovery of lipids as described elsewhere [29]. Briefly, 6 ml of chloroform/methanol (2:1 v/v) was added to the samples and vortexed for 5 min and sonicated for another hour. The samples were then centrifuged at 3220*g* for 30 min to recover the lipid phase. The chloroform layer containing the lipids was dried completely under nitrogen gas.

KOH hydrolysis for total and esterified lipids. About 330 μl of 15% (w/v) KOH was added to 0.5 ml of plasma and the extracted tissue lipids. Samples were incubated at 40°C for 1 h. After the alkaline hydrolysis, two volumes of 0.01% BHT in absolute ethanol were added to the samples. Samples were cooled on ice and centrifuged at 3220*g* for 15 min to precipitate proteins. The supernatants were decanted into new tubes and dried off under nitrogen. Samples were neutralised with 2 ml of 1 M of KH_2PO_4 to pH 7.0 followed by 2 ml of Cayman Eicosanoid Affinity Column Buffer. Samples were purified and eluted using the Cayman 8-Isoprostane Affinity Column according to the manufacturer's instructions. The final eluate in Cayman Column Elution Solution (95% ethanol) was evaporated completely under nitrogen gas. The samples were then reconstituted in Cayman EIA Buffer before proceeding with the EIA incubation process. The EIA method was done according to the instructions from the Cayman 8-Isoprostane Kit.

MitoTracker Red staining and measurements

Blood collected in vacutainers containing EDTA were processed immediately in ice cold conditions for the analysis. Blood was diluted with equal volume of 1X phosphate buffer saline (PBS) and separated using centrifugation at 400*g* for 20 min at 18°C using Ficoll-Paque as per the manufacture's instructions. The buffy layer containing lymphocytes was transferred to a new tube containing 1X PBS solution for further MitoTracker staining. The plasma was kept in cryo vials BHT was added to a final concentration of 20 μM and then stored in liquid nitrogen until further 8-iso-PGF$_{2\alpha}$ analysis.

Staining and flow cytometry measurements. CMX-Ros was stored at − 20°C and dissolved in DMSO to give a stock solution of 1 mM. A subsequent dilution was made from the stock solution with 1X PBS to obtain a working solution of 10 μM. (final DMSO content in the MitoTracker working solution is less than 5%). 1×10^6 lymphocytes were incubated with 10 μM MitoTracker Red to a final concentration of 200 nM for 30 min at 37°C. Control samples containing only the lymphocytes in 1X PBS (without MitoTracker Red staining) were also incubated using the same method. Excess MitoTracker Red was washed off using 1X PBS after incubation. Cells were immediately resuspended in 500 μl of 2% paraformaldehyde in 1X PBS and analysed immediately using Dako CyAN LX high performance flow cytometry system, equipped with an air-cooled argon laser (488 nm, 20 mW). Forward scatter (FS) and side scatter (SC) were collected and analysed using Summit Software for Acquisition and Sort Control (version 3.1, licensed to Cytomation Inc.) Acquisition was performed on 10,000 cell counts. The remaining cells not used in the flow cytometer were pelleted by centrifugation and mounted on microscope slides and cover slips with a drop of FluorSave and viewed under a Carl Zeiss LSM 510Meta confocal-Axiovert 200 M microscope using an Ar air-cooled laser (488 nm, 25 mW). Care was taken to ensure all the steps in MitoTracker staining and preparations were done with minimal exposure to light.

Statistics

All data were analysed using SPSS for Windows version 13.0. All data are presented as means ± SD. Variables were continuous and normality was ascertained using Kolmogorov–Smirnov test. All data has been screened and any statistical outliers have been discounted before running the correlation tests. All data are found to be normally distributed but since not all groups meet the criteria of equal variances, nonparametric Mann–Whitney *U* test and Spearman's correlations were used. Correlations statistics were performed on the results from the 8OHdG and 8-iso-PGF$_{2\alpha}$ measurements from liver, kidney, heart and brain tissues of the young and old rats.

Results

Prior to euthanasia, the physical conditions of the rats were seen to be normal but with the old rats being less responsive and less agile compared to the young rats. No abnormal lesions were observed in general for all the tissues analysed.

8-hydroxy-2′-deoxyguanosine

The method employed in this experiment for the DNA extraction and digestion has been reported to give minimal artifactual formation of 8OHdG and

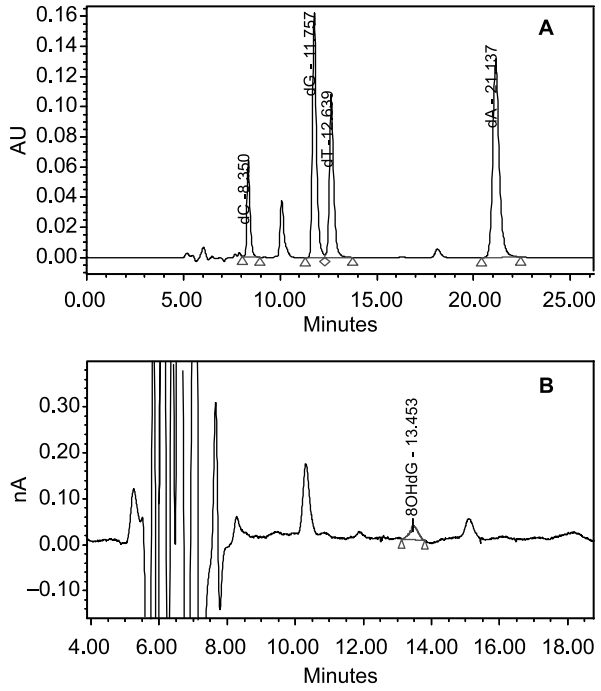

Figure 1. HPLC chromatogram from the PDA detector for normal dC, dG, dT and dC nucleosides from young rat liver monitored at $\lambda = 254$ nm in (A). HPLC chromatogram from the ECD for 8OHdG detection from the same young rat liver in (B).

a complete DNA hydrolysis to release all the nucleosides for a more accurate and reproducible detection of 8OHdG in tissues [28]. Final detection of the 8OHdG was done using the sensitive HPLC-ECD method where baseline noise from the ECD was found to be minimal after performing a passivation process on all the metal components of the LC system. The detection limit of our ECD system was down to 0.5 fmol for 8OHdG. The normal nucleosides (dA, dC, dG and dT) and oxidised base (8OHdG) were detected by the PDA and ECD, respectively (Figure 1).

The levels of 8OHdG obtained for the old rats were significantly higher (approximately two times more) compared to the young rats for the liver, heart, kidney and brain tissues. The mean levels of 8OHdG in the 8-month old rats were 16.51 ± 2.24, 29.18 ± 4.77, 19.34 ± 4.80 and 15.12 ± 1.91, whereas the 26-month old rats recorded values of 31.94 ± 7.24, 59.21 ± 7.87, 42.21 ± 10.03 and 28.99 ± 4.59 in terms of 8OHdG/ × 10^6 dG in the liver, heart, kidney and brain, respectively (Figure 2). The 8OHdG content was found to be highest in the heart tissues (approximately 1.5–2 times more) for both young and old rats compared to the contents in liver, kidney and brain. The second highest 8OHdG level was found in kidney followed by liver and brain having similar values in the young and old rats. The kidney tissue was found to have the highest percentage of increase (118% higher) in the old rats compared to the young rats followed by the increase in heart (103%), liver

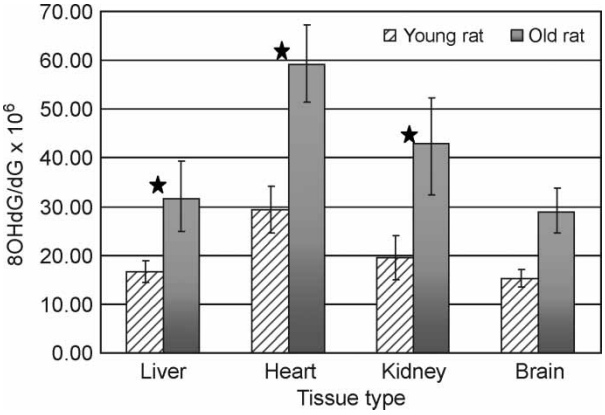

Figure 2. 8OHdG/10^6 dG levels in young and old rat liver, heart, kidney and brain. Data represent mean ± SD, error bars indicate SD, ⋆ indicates significant difference between young and old rats using independent-sample Mann–Whitney U test ($p < 0.05$).

(93%) and brain (92%). In general, the old rats also showed greater levels of variation in 8OHdG content compared to the young rats regardless of tissue type.

8-iso-PGF$_{2\alpha}$

From our results, we see a wide variability of 8-iso-PGF$_{2\alpha}$ levels in tissues and plasma. The level in tissues was found to be an order of magnitude higher than levels found in plasma, especially for the old rats. There was significant difference between the young and old rats in terms of their 8-iso-PGF$_{2\alpha}$ contents for liver, kidney, heart and plasma (Figure 3). For liver, kidney, brain and plasma the levels were 4.7, 2.2, 1.2 and 1.6 times higher for the old rats compared to the young rats, respectively. The brain, however, showed a lack of significant difference between the young and old groups although, 8-iso-PGF$_{2\alpha}$ was higher for the latter. The 8-iso-PGF$_{2\alpha}$ level in the young heart recorded the

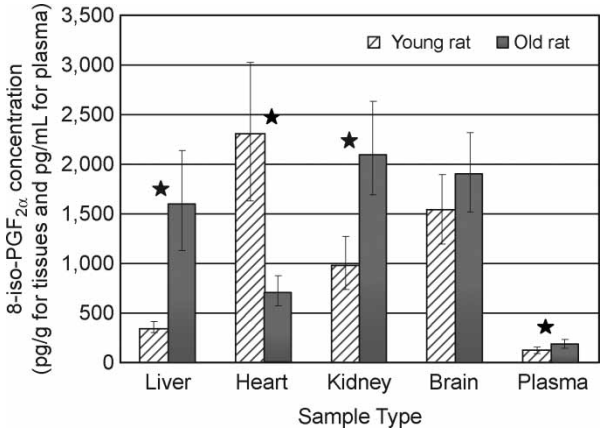

Figure 3. Levels of 8-iso-PGF$_{2\alpha}$ in old and young rat liver, heart, kidney, brain and plasma. An increased level was found in the brain of the old rat although not significantly different ($p = 0.068$). Data represent mean values ± SD, ⋆ indicates significant difference between young and old rats using independent-sample Mann–Whitney U test ($p < 0.05$).

highest value in contrast to the general trend of the data, where the rest of young tissues have lower amounts of 8-iso-$PGF_{2\alpha}$ compared to the old tissues. In addition, the young heart was found to have three times higher 8-iso-$PGF_{2\alpha}$ compared to the old heart. The recovery results (recovery ± CV)% for this assay using spiked standard 8-iso-$PGF_{2\alpha}$ after Folch extraction were found to be 79.02 ± 3.5, 68.11 ± 6.25, 72.85 ± 4.62, 62.26 ± 4.81 and 81.14 ± 3.17 for liver, heart, kidney, brain and plasma, respectively.

Mitochondrial membrane potential

By using the MitoTracker Red (CMX-Ros) probe as a fluorochrome in the assessment of the mitochondrial membrane potential, median intensity counts acquired from the flow cytometer for the MitoTracker Red staining were found to be 24.11 ± 6.19 and 13.45 ± 6.27 (mean ± SD) for the young and old rat lymphocytes, respectively. The data from the MitoTracker intensity histograms, showed a significant intensity difference between the young and old lymphocytes (Figure 4). This decrease in intensity of the old lymphocytes represents a 44% decline in fluorescence from that of the young rat lymphocytes. However, the coefficients of variations for these values are large (46.6%) for the young lymphocytes and the old lymphocytes (25.7%). To examine the isolated lymphocytes morphology and the cellular uptake distribution of the MitoTracker Red fluorochrome, we viewed the cells under a laser confocal microscope (Figure 5).

Relationship between 8OHdG, 8-iso-$PGF_{2\alpha}$ and mitochondrial membrane potential

Overall, significant correlations between 8OHdG and 8-iso-$PGF_{2\alpha}$ levels in young and old rats were observed for liver, heart and kidney tissues regardless of their chronological age. Strong positive correlations were obtained for the liver and kidney ($r = 0.654$, $p < 0.01$ and $r = 0.733$, $p < 0.01$). Levels in heart recorded a strong negative correlation ($r = -0.846$, $p < 0.01$). Levels in brain did not show any significant correlations, with $r = 0.292$, $p = 0.256$ but with the $r > 0.20$, this still indicates a moderate association between these two markers (Figure 6). It is noteworthy to mention that weaker correlations between 8OHdG and 8-iso-$PGF_{2\alpha}$ were found within some of the age groups. The lack of correlation in these individual young and old groups implies that the relationship between 8OHdG and 8-iso-$PGF_{2\alpha}$ may not be that of a perfect linear relationship. With the plasma 8-iso-$PGF_{2\alpha}$ found at a higher level in the old rat compare to the young rat, this increased lipid peroxidation may be associated with the lymphocytes reduced mitochondrial membrane potential found in the old rats which recorded an overall decreased MitoTracker Red intensity.

Discussion

One of the molecular theories of ageing is that DNA is progressively damaged and these DNA errors that accumulate can shorten life-span [30]. Evidence supporting this theory of free radical-mediated damage

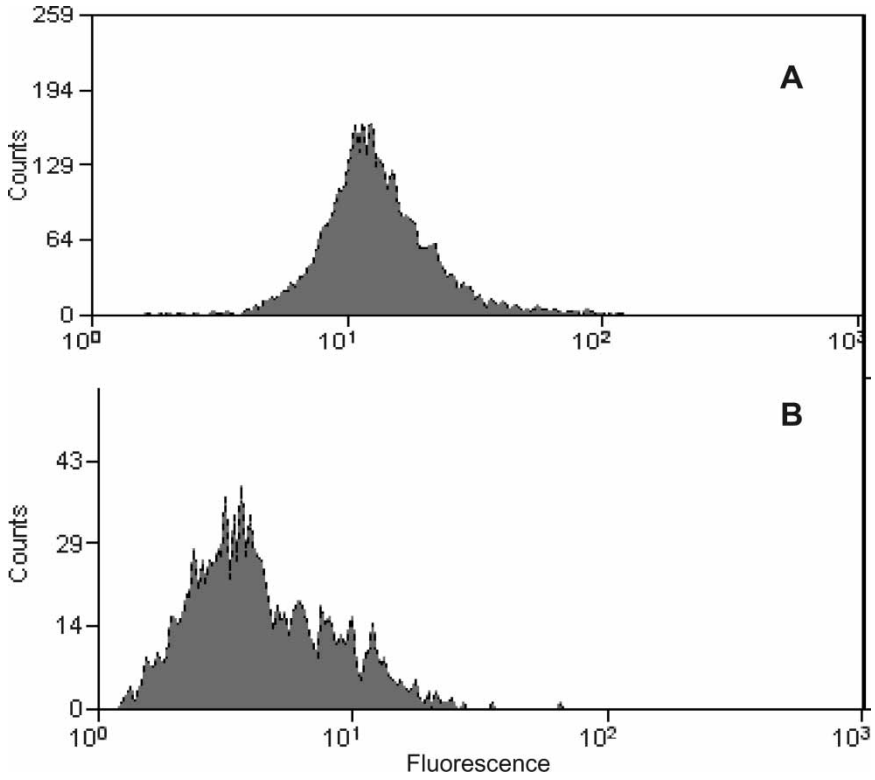

Figure 4. Flow cytometry histogram of young (A) and old (B) rat lymphocytes stained with 200 nM MitoTracker Red fluorochrome.

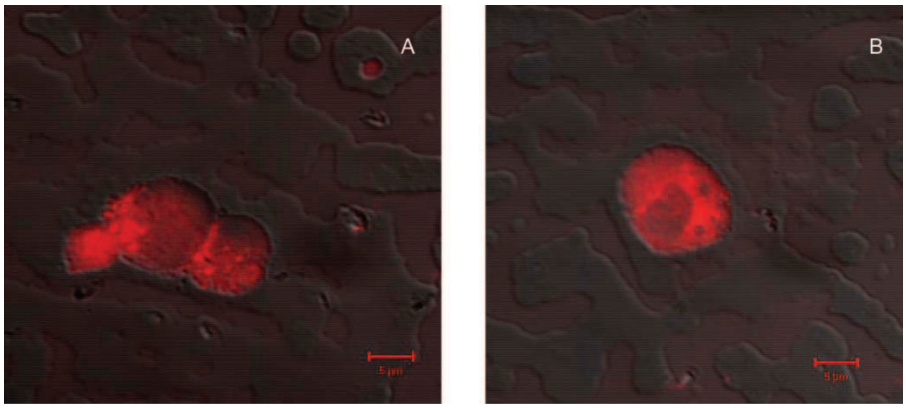

Figure 5. (A) Young and (B) old lymphocytes stained with 200 nM MitoTracker Red fluorochrome viewed under Zeiss laser confocal microscope with 63 × magnification and 3 × zoom. Bar represents 5 μm. The MitoTracker stained only the mitochondria in the cytoplasm and not the nuclei.

in ageing includes an increase in 8OHdG levels in aged specimens [2,4], increase lipid peroxidation in liver [7] and gluthathione oxidation increase in mitochondria [17]. Thus, it is not intrinsically reliable to measure a single reaction product as an index of oxidative DNA damage, but this is what is usually performed due to the technical challenges of some of these methods of measurement. Quantitative relationships between biomarkers of ageing are still controversial, involving complex and long-lasting pathophysiological

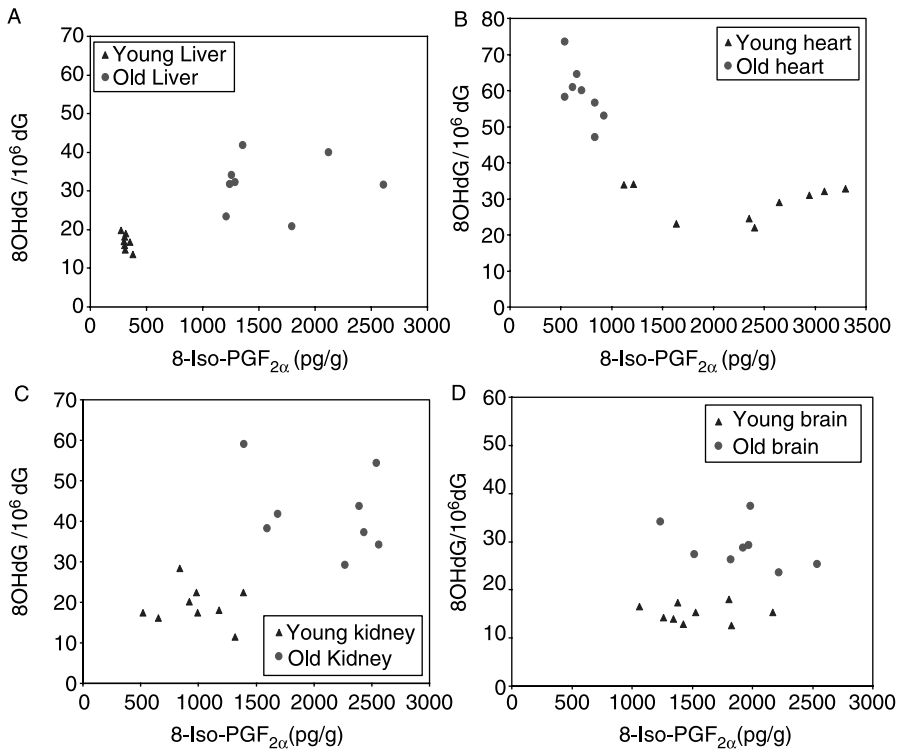

Figure 6. Correlations between 8OHdG and 8-iso-PGF$_{2\alpha}$ in rat liver, heart, kidney and brain from young and old rats. Spearman correlation coefficients: $r = 0.654$, $p < 0.01$ for liver in (A), $r = -0.846$, $p < 0.01$ for heart in (B), $r = 0.733$, $p < 0.01$ for kidney in (C) and $r = 0.292$, $p = 0.256$ for brain in (D).

conditions. Prompted by these considerations, we designed a study aimed at evaluating two most widely established quantitative biomarkers of ageing, i.e. 8OHdG and 8-iso-$PGF_{2\alpha}$ with a semi-quantitative, but relatively simple method of determining mitochondrial membrane potential using the MitoTracker Red fluorochrome in a single experimental model in young and old rats. By quantification of 8OHdG and total amounts of 8-iso-$PGF_{2\alpha}$ (free and esterified) in tissues, it may be possible to investigate the location of oxidative injury in different ageing organs and to ascertain if some tissues are more prone to oxidation than others.

The range of 8OHdG levels obtained in our experiments are consistent with those reported elsewhere when compared with rat tissues of similar age [2,4,31]. This shows that our method is reliable and comparable to others who have used similar HPLC detection although different DNA extraction and digestion methods were used. From our results, it is not surprising that the liver, heart and kidney tissues showed marked increase of 8OHdG levels in the old rat as it is also well-reported that this oxidative DNA damage product accumulates with age in these tissues [2,4,32]. Substantially higher levels of 8-iso-$PGF_{2\alpha}$ were also found in the old rat liver and kidney and this was strongly correlated with the 8OHdG levels. The higher 8-iso-$PGF_{2\alpha}$ in old rat tissues could mean that the rate of hydrolysis of 8-iso-$PGF_{2\alpha}$ from tissue lipids is slower than the rate of its formation. This could also lead to the accumulation of esterified lipids, which can cause loss of structural integrity of cell membranes in old tissues. The age-dependent decline in enzymatic antioxidant defences such as superoxide dismutase, catalase and glutathione peroxidase observed in kidney and liver of rats have also led to an increased rate of oxidative DNA damage [33].

Steady-state cell populations in the heart are well-characterised and are not influenced by mitosis and *de novo* DNA synthesis. The heart also has a particular high oxygen consumption and energy requirement. This could possibly explain the high levels of 8OHdG and 8-iso-$PGF_{2\alpha}$ found in the rat heart. Higher 8-iso-$PGF_{2\alpha}$ level was found in the young heart compared to the old heart. The aged rat hearts have the capability to alleviate oxidative stress by maintaining GSH homeostasis where GSH peroxidase, GSH reductase and GSH S-transferase activities have been found to be augmented, with no decrease in SOD activity in ageing F344 rats [34–36]. These antioxidant enzyme adaptations, despite a general decline of myocardial oxidative capacity and energy production [37] may have prevented lipid peroxidation accumulation in the old heart. Basal release of nitric oxide (NO) by coronary endothelium has also been reported to deteriorate with ageing rats [38]. NO has been known to oxidise low-density lipoprotein and activate cyclooxygenase and lipoxygenase causing the production of prostaglandins and leukotrienes [39]. With a reduced NO production with ageing, it is likely that lipid peroxidation is also attenuated in the ageing heart. In contrast to the age-related increase in the levels of 8OHdG, the levels of 8-iso-$PGF_{2\alpha}$ did not increase with age in the heart. This is because these lipid peroxidation damage moieties are located primarily in lipophilic environments and our data suggests that the effects of endogenous oxidants may be more confined to the aqueous milieu of the heart. This is consistent with another study, where myocardial levels of F_2-isoprostanes did not increase with age although there is a rise in 8OHdG levels between young and old F344 rats [40]. Therefore, we obtained a negative correlation between the 8OHdG and the 8-iso-$PGF_{2\alpha}$ levels in the rat heart.

The level of 8OHdG in the brain depends critically on the sampling age of the animals measured [41] as the change may only be evident at a later stage in life. As shown by our results, the 8OHdG level in brain was significantly elevated in the old rats. This is consistent with the observation in a study which reported various DNA brain lesions accumulate during ageing [42]. Nevertheless, the content of 8OHdG in brain was lowest among all the tissues measured. This suggests the possible efficient repair system of the brain and that the blood barrier may limit the exposure to endogenously produced mutagens or xenobiotics. The correlation between the 8OHdG and 8-iso-$PGF_{2\alpha}$ in the brain was found to be moderately associated but not statistically significant. This could be due to the reason that the high lipidic contents in the brain may have different susceptibility to the endogenous oxidative attack that occurs in the DNA. Alternatively, the correlation may prove to be stronger if neuroprostanes instead of 8-iso-$PGF_{2\alpha}$ were used as these may possibly better reflect the level of oxidative stress in neuronal membranes. Thus, the insignificant increase of brain 8-iso-$PGF_{2\alpha}$ in the old rat brain could also be attributed to the fact that this isoprostane which is derived from arachidonic acid is an index of whole tissue oxidation and may have less evident age-related changes compared to neuroprostanes which, are generated from docosahexaenoic acid. Another possible reason for this observation could be because marked difference is only detectable in cases where brain lesions and inflammatory diseases such as in Alzheimer disease and amyotrophic lateral sclerosis are present or when the neurodetoxification mechanisms decline drastically.

8-iso-$PGF_{2\alpha}$ measured in plasma is an index of systematic lipid peroxidation level which may not be specific to any organ of origin. The majority of isoprostanes are esterified to plasma lipids while free isoprostanes are formed by oxidation of tissue or plasma lipids and are released as the free acid by the action of phospholipase [10]. The level of total plasma 8-iso-$PGF_{2\alpha}$ was found to be significantly increased

in the old rat compared to the young rat in our study. An increased level could be due to several possible factors related to the interaction of plasma esterified isoprostane and blood at the endothelial interface and the free isoprostane equilibrium between the formation, release, metabolism and excretion of isoprostanes. Enhanced lipid peroxidation in endothelial cells may have occurred in older endothelial cells due to accumulated oxidative damage.

The degree of cellular dysfunction due to mitochondrial damage or how such mitochondrial changes affect organ function in ageing is still not well-understood. Decreased DeltaPsi(M) has been found in a variety of ageing cell types from several mammalian species [43]. To determine the importance of mitochondrial reactive oxygen species toxicity in ageing, we analysed changes in mitochondrial function with age in rat lymphocytes. In this study, MitoTracker Red was used as a probe for mitochondrial membrane potential changes because the dye causes no loss of mitochondrial coupling, is not toxic at low concentrations and has dye accumulation and fluorescence intensity that are stable [21,44]. Thus, these attributes allow easy measurement of fluorescence characteristics. Nevertheless, we are also aware that there are controversial reports which comment otherwise about MitoTracker Red staining and the pitfalls in using fluorochromes in assessing ageing cells [45]. To date, studies have generally used isolated mitochondria to determine age-related changes. However, due to their fragility and loss upon isolation, the purified mitochondrial fractions may not accurately represent the mitochondrial diversity found *in vivo*, leading to misinterpretation of results. In the current study, we examined mitochondrial function in intact rat lymphocytes. Overall, isolated lymphocytes from the old rats exhibited a lower average fluorescence intensity in the MitoTracker Red stain compared to that from the young rats. Precautions in staining the cells were also taken ensuring that quenching in the matrix did not occur because of excess probe loading and aggregation which may lead to the insensitive detection of DeltaPsi(M). The mitochondrial depolarization accompanied by decrease in mitochondrial function has been reported to be more 8OHdG, prominent in older subjects [46]. It has also been reported that in lymphocytes from old mice, electron transport is inhibited, mitochondrial flavin adenine dinucleotide (FAD) is more oxidized and the mitochondrial permeability transition pore (PTP) is more activated [47]. The activation of PTP collapses the mitochondrial membrane potential, inhibits oxidative phosphorylation and Ca^{2+} sequestration by mitochondria, and may induce apoptosis or necrosis. Overall, our results support the view that the bioenergetic functions of mitochondria are more susceptible to oxidative injury in aged individuals. Work is now underway in our group to address possible links between respiratory chain complex activity, ROS generation, glutathione redox status, membrane potential, proton leak and ATP demand in an integrated network for mitochondrial ageing studies.

Conclusions

The well-established method of 8OHdG measurements has enabled it to be used as a marker of ageing in many gerontology models. On the other hand, lipid peroxidation has been used mainly as a marker of oxidant injury and in many of pathophysiological diseases. In this experiment, we have demonstrated strong correlations between the 8OHdG and 8-iso-$PGF_{2\alpha}$ levels in different young and old rat tissues, which paves the way to look at developing lipid peroxidation assay as another possible biomarker of ageing. The measurement and correlations between the extent of oxidative DNA damage and lipid peroxidation in different tissues are important indicators of the individual oxidative stress levels in different physiological systems.

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