Increased Oxidative Damage in RNA in Alzheimer’s Disease Progression

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Abstract

Studies of oxidative damage during the progression of Alzheimer’s Disease (AD) suggest a central role in disease pathogenesis. To determine if RNA oxidation increases in the progression of AD levels of oxidized bases from RNA were measured in the superior and middle temporal gyri (SMTG), inferior parietal lobule (IPL), and cerebellum (CER) throughout the progression of AD including subjects with mild cognitive impairment (MCI), preclinical AD (PCAD), late-stage AD (LAD), diseased control (DC) (Frontotemporal Dementia (FTD) and Dementia with Lewy Bodies (DLB)), and age-matched normal control subjects (NC) were analyzed by gas chromatography mass spectrometry. Median levels of multiple RNA adducts were significantly (p<0.05) elevated in the SMTG, IPL, and CER in multiple stages of AD and in DC subjects. Elevated levels of 4,6-diamino-5-formamidopyrimidine (FapyA) suggest a hypoxic environment early in the progression of AD and in DC subjects. Overall, these data suggest oxidative damage is an early event not only in the pathogenesis of AD, but is present in neurodegenerative diseases in general.

Keywords: RNA; Mild cognitive impairment; Preclinical Alzheimer’s disease; Alzheimer’s disease; Neurodegenerative diseases; RNA oxidation; Gas chromatography-mass spectrometry

Introduction

Alzheimer’s Disease (AD), the most common form of dementia, is estimated to currently affect ~5 million (1 in 9) Americans aged 65 years or older [1]. Antemortem, AD is characterized by a progressive decline in both cognitive and non-cognitive domains following an insidious onset. Despite research endeavors by the scientific community, the etiology of AD remains unclear effectively hindering potential advancements. The study of the underlying disease etiology and pathogenesis of AD is complicated by the multifaceted nature of the disease on a molecular level.

Initial AD diagnostic criteria developed by the Neurological and Communicative Disorders Association (NINCDS) and the Alzheimer’s disease and Related Disorder Association (ADRDA) workgroup in 1984 proposed that the clinical manifestations were synonymous with the underlying pathogenesis of the disease. Subjects with mild cognitive impairment (MCI) experience a perceived and verifiable change in cognition but are not demented and maintain functional independence antemortem [2-4] and postmortem exhibit AD associated pathology but to a lesser extent than demented AD subjects [5]. More recently postmortem analyses have described the presence of senile plaques (SP) and neurofibrillary tangles (NFT) in the brains of cognitively normal subjects suggestive of an extended ‘preclinical AD’ (PCAD) stage of AD in which extensive pathology exists in the absence of obvious clinical manifestations [6-9].

Although multiple hypotheses have been proposed to explain the pathogenesis of AD, no single hypothesis fully encompasses both the clinical and pathological features of AD. There is the distinct possibility that a multifaceted hypothesis can more effectively account for pathogenesis of AD. One particular hypothesis that has received considerable interest is the oxidative damage hypothesis, an extension of the free radical theory of aging (FRTA) initially proposed by Harman [10,11] that attempts to unify themes of AD including the formation of SP and NFT, mitochondrial dysfunction, neuronal excitotoxicity, and neurodegeneration. Although the FRTA proposes that oxidative stress or free radical generation increases throughout the body, the brain is particularly vulnerable due to the increased oxygen consumption/high energy demands and limited antioxidant defense relative to other tissue types. Increased generation or prolonged exposure to reactive oxygen species (ROS) is thought to play a role in a wide range of oxidative damage observed in both early and late stages of AD [12] resulting in compromised cellular function.

Aerobic oxidative phosphorylation by mitochondria is essential to meet energetic demands of neurons. Unfortunately, mitochondrial by-products of cellular respiration are considered to be the primary endogenous source of ROS, including hydrogen peroxide (H2O2), superoxide (O2•−), and hydroxyl (HO•) radicals [13]. It is estimated that ~2% of total oxygen consumed by the cell is converted to superoxide radical during respiration [14]. Although superoxide can be detoxified to water by sequential activities of superoxide dismutase and glutathione peroxidase, in the presence of reduced transition metals (copper and iron) hydrogen peroxide can generate hydroxyl radicals via Fenton [15] and/or Haber-Weiss reactions [16]. Increased oxidative damage to mitochondrial DNA (mtDNA), (−10X levels observed in nuclear DNA (nDNA)) is attributed to the close proximity to ROS generation and a lack of protective histones [14,17] and may contribute to the decreased glucose consumption and depletion of cellular energy noted in the earliest stages of AD [18-20]. Although multiple ROS are produced in the brain, the highly reactive hydroxyl radical is the primary initiator of damage to heterocyclic nucleic acids and associated sugar moieties at or near diffusion controlled rates [21,22]. Because markers of oxidative damage are an indirect measurement of oxidative damage, it remains unclear if the oxidative damage observed in AD is the direct result of increased generation or reduced efficiency in elimination or some combination thereof.

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Multiple by-products of ROS-mediated damage to lipids, proteins, and DNA have been studied during the pathogenesis of AD. Oxidation of nucleic acids, initiated by highly reactive hydroxyl radical is a result in over 20 potential adducts [23]. However, modification of guanine is considered the preeminent marker of nucleic acid oxidation because of its low reduction potential. Levels of oxidized guanine have been reported to be significantly increased in both nDNA [24-27] and mtDNA [26] in the vulnerable brain regions in AD.

In contrast, quantification of nucleic acid adducts in RNA has not been reported in either the early or late stages of AD. To fully characterize the extent of nucleic acid oxidation in RNA during the progression of AD, levels of multiple nucleic acid adducts were quantified from two vulnerable brain regions, the superior and middle temporal gyri (SMTG) and inferior parietal lobule (IPL), and a non-vulnerable brain region, the cerebellum (CER) of subjects with mild cognitive impairment (MCI), preclinical AD (PCAD), late-stage AD (LAD), diseased control (DC) subjects that included frontotemporal dementia (FTD) and dementia with Lewy bodies (DLB), and age-matched normal control subjects (NC) by gas chromatography mass spectrometry (GC/MS) operated in selective ion monitoring mode (SIM).

Methods

Brain specimen sampling

Tissue specimens were obtained through the Neuropathology Core of the University of Kentucky Alzheimer’s Disease Center (UK-ADC) under University of Kentucky IRB approval protocols. Tissue specimens of short postmortem interval (PMI) autopsies from seven subjects with MCI (2 men [M]: 5 women [W]), fourteen PCAD subjects (3 M: 11 W), fifteen LAD subjects (7 M: 8 W), six DLB subjects (4 M: 2 W), six MCI (2 men [M]: 5 women [W]), fourteen PCAD subjects (3 M: 11 W), and fifteen age-matched NC subjects (4 M: 11 W) were flash frozen in liquid nitrogen and maintained at -80°C until processed for analysis.

All subjects were characterized as previously described [28] based on existing criteria for MCI [4], PCAD [6,29], and LAD [30,31]. Postmortem DLB pathology was characterized by the presence of ubiquitin and α-synuclein positive Lewy bodies (LB) and by ubiquitin positive Lewy neurites (LN) in a semi-quantitative grading density in paralimbic and neocortical structures. DLB diagnosis was according to the current guidelines proposed by the International Workshop for the Consensus Guidelines for the Clinical and Pathological Diagnosis of DLB [32,33]. Postmortem DLB was characterized by symmetric focal atrophy of the frontal and/or temporal lobes, atrophy of the basal ganglia, and loss of pigmentation in the substantia nigra. Hematoxylin and eosin (H&E) staining of the cerebral cortex highlight microvacuolation and neuron loss, whereas immunostaining for α-β-crystallin reveal swollen cortical neurons. Pathological diagnosis of FTD was based on current standards [34,35]. Neuritic plaque (NP) and Braak staging scores for all subjects were provided by the Neuropathology Core of the UK-ADC.

RNA isolation

RNA samples were prepared as described by Chomczynski and Sacchi [36]. Briefly, tissue specimens (~100 mg) were homogenized in 1 ml of TRI Reagent® in a polypropylene microcentrifuge tube and incubated at room temperature for 5 min. Chloroform (200 μL) was added, mixed by hand, incubated for 5 min at room temperature, and centrifuged at 9000×g for 15 min at 4°C. Isopropl alcohol (500 μL) was added to the resulting clear aqueous phase containing RNA, incubated at room temperature for 10 min and centrifuged at 9000×g for 10 min at 4°C. RNA pellets were washed in 75% ethanol, air dried, and stored at -80°C until analysis. Samples were resuspended in autoclaved distilled/deionized water and the concentration and purity determined using a NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA).

RNA integrity

Representative RNA isolates were submitted to the UK MicroArray Core Facility for electrophoretic analysis. Sharp peaks representing the eukaryotic 18S and 28S ribosomal subunits along with the absence of smaller well-defined peaks between the two ribosomes peaks are characteristics associated with high quality isolates. In addition, the RNA integrity number (RIN), an algorithm based product, designed to communicate the level of RNA degradation, on a scale of 1 (degraded) to 10 (intact) was also determined.

RNA derivatization

For analysis, RNA (100 μg) samples were prepared for GC/MS analysis as previously described by Wang et al. [26,37]. Ten nmol of each stable-labeled internal standard: 8-[8-13C, 7, 9-15N2] hydroxyguanine; 8-[8-13C, 6, 9-diamo-15N2] hydroxyadenine; 5-[2,13C, 1, 3-15N2] hydroxythymidine; [formyl-13C, diamino-15N2] fapyadenine; [formyl-13C, diamino-15N2] fapyguanine were added for quantification of oxidized bases. Briefly, samples were subjected to acid hydrolysis with formic acid (250 μL, 90%) at 145°C for 30 min in evacuated tubes. Following hydrolization individual bases were derivatized with a pyridine:N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)/1% trimethylchlorosilane (TMCS) solution (200 μL, 1:1) in evacuated sealed tubes for 2 hr at room temperature. The derivatized samples were dried under a stream of nitrogen in a water bath at 37°C, and 20 μL BSTFA/1% TMCS added immediately before GC/MS injection.

Gas chromatography/mass spectrometry (GC/MS) with selective ion monitoring (SIM)

Derivatized samples were analyzed using an Agilent 7890A gas chromatograph on a HP-5ms capillary column (0.25 mm internal diameter, 0.25 μm film thickness, and 30 m length; Hewlett Packard, Palo Alto, CA, USA) as previously described by Wang et al. [26,37]. Derivatized nitrogenous base spectra were acquired in SIM mode at the following m/z ratios: 443 (8-[8-13C,7,9-15N2] hydroxyguanine) and 440 (8-hydroxyguanine (8OHG)); 445 ([formyl-13C, diamino-15N2] fapyguanine) and 442 (fapyguanine (FapyG)); 355 (8-[8-13C,6,9-diamo-15N2] hydroxyadenine) and 352 (8-hydroxyadenine (8OHA)); 357 ([formyl-13C, diamino-15N2] fapyadenine) and m/z 354 (fapyadenine (FapyA)); and 331 (5-[2,13C,1,3-15N2] hydroxythymidine) and m/z 328 (5-hydroxythymidine (5OH)). Instrument response plots of integrated peak intensities for stable-labeled analyte signals were determined over a range of 0.5 nmol to 100 nmol per stable labeled isotope standard. The integrated area of each analyte was normalized with respect to the integrated area of the corresponding internal standard for all samples and corrected based on instrument response plots for a given internal standard.

Measurement of Aβ1-40 and Aβ1-42 levels

Total levels of Aβ1-40 and Aβ1-42 in phosphate buffered saline (PBS)-, sodium dodecyl sulfate (SDS)-, and formic acid (FA)-soluble fractions were provided by the Sanders-Brown Center on Aging Amyloid Core as previously described [38] for the SMTG, IPL, and CER. Briefly, tissue samples (200 mg) were homogenized in PBS with a complete protease inhibitor cocktail (PIC) via apolystron and centrifuged at 20,800×g for 20 min.
30 min at 4°C. The supernatant (PBS-soluble pool) was collected and the remaining pellet sonicated (10×0.5 sec pulses at 100 W) in 2% (w/v) SDS with PIC and centrifuged at 20,800×g for 30 min at 14°C. The supernatant (SDS-soluble pool) was collected and the remaining pellet was sonicated as previously described in 70% (v/v) formic acid, and centrifuged at 20,800×g for 60 min at 4°C. The supernatant (FA-soluble pool) was collected. All samples were stored at -80°C until analysis.

Oligomeric Aβ was quantified as previously described using a single-site sandwich ELISA (4G8/4G8). Immunol 4HBX plates were coated with 0.5 μg/well antibody 4G8 (Covance, Princeton, NJ, USA) and incubated overnight at 4°C. Wells were blocked with Synblock® (AbD Serotec; Oxford, UK) according to manufacturer's instructions. Standard curves were prepared using synthetic Aβ peptide (rPeptide; Bogart, GA, USA). PBS samples were diluted 1:4 in Antigen Capture (AC) buffer (20 mM Na2PO4, 0.4% Block Ace (AbD Serotec), 0.05% NaN3, 2 mM EDTA, 0.4 M NaCl, 0.2% BSA, 0.05% CHAPS, pH 7). SDS samples were diluted between 1:20 and 1:100 in AC buffer and formic acid samples were first neutralized 1:20 in TP buffer (1.0 M Tris base, 0.5 M Na2HPO4) then further diluted between 1:5 and 1:20 in AC buffer. Standards and samples were analyzed in duplicate and were incubated at 4°C overnight. Biotinylated detection antibodies were Ab13.1.1 (Aβ1-40-end specific), 12F4 (Aβ 1-42-end specific), or 4G8 (Aβ17-24-end specific) followed by the addition of NeurtAvidin-HRP (Pierce Biotechnologies, Rockford, IL, USA). Colorimetric detection used 3,3′,5,5′-tetramethylbenzidine reagent (TMB; Kirkegaard and Perry Laboratories; Gaithersburg, MD, USA). The reaction was stopped via acidification (6% o-phosphoric acid) and read using a BioTech Powerwave XS (Winooski, VT, USA) plate reader at 450 nm.

Statistics

All data were tested for normality using the Wilkes-Shapiro test. Levels of oxidized RNA ribonucleic acid adducts, PBS-, SDS-, and FA-soluble Aβ1-40 and Aβ1-42, and Braak staging scores demonstrated non-normal distributions and were analyzed using the Mann-Whitney U-test and are reported as median values with range. All other data including, age and PMI, demonstrated a normal distribution and are reported as mean ± standard error of mean (SEM). No significant differences were observed for any measure between FTD and DLB subjects, therefore the two neurodegenerative diseases were pooled to create a diseased control (DC) subject pool. All statistical comparisons were carried out using Sigma Plot. Statistical significance was set at p ≤ 0.05 and trending toward significance was set at p ≤ 0.1.

The magnitude of Pearson’s r value was determined for levels of nucleic acid adducts and neuropathological markers including, NFT counts, SP counts, and levels of PBS-, SDS-, and FA- soluble Aβ1-40 and Aβ1-42. Type one errors arising from multiple comparisons per brain region studied were corrected through the use of the Bonferroni method. Recalculated P-values for significance for each brain region studied were 0.0045 (SMTG), 0.00625 (IPL), and 0.0045 (CER).

Results

Subject demographic data are shown in Table 1. Neither PMI nor age of subjects were significantly different except for a significant (P<0.05) increase in the age of PCAD (85.8 ± 1.7 y), MCI subjects (91.0 ± 1.9 y), LAD (80.8 ± 1.4 y) and NC subjects (86.3 ± 1.4 y) compared to DC subjects (68.9 ± 16.6 y) and a significant increase in the age of MCI subjects compared to LAD subject. Median Braak staging scores were significantly higher for MCI (IV), PCAD (V), LAD (VI), and DC subjects (II) compared to age-matched NC subjects (I).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SEM age (years)</th>
<th>Sex</th>
<th>Mean ± SEM PMI (hr)</th>
<th>Median Braak Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>86.3 ± 1.4</td>
<td>N=15; 4M, 11W</td>
<td>2.8 ± 0.2</td>
<td>I</td>
</tr>
<tr>
<td>PCAD</td>
<td>85.8 ± 1.7</td>
<td>N=14; 3M, 11W</td>
<td>2.8 ± 0.2</td>
<td>I*</td>
</tr>
<tr>
<td>MCI</td>
<td>91.0 ± 1.9</td>
<td>N=7; 2M, 5W</td>
<td>2.6 ± 0.2</td>
<td>IV</td>
</tr>
<tr>
<td>LAD</td>
<td>80.8 ± 1.4</td>
<td>N=15; 7M, 8W</td>
<td>3.6 ± 0.4</td>
<td>V/II</td>
</tr>
<tr>
<td>DC</td>
<td>68.9 ± 4.8</td>
<td>N=12; 7M, 5W</td>
<td>3.5 ± 0.4</td>
<td>II</td>
</tr>
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</table>

*P<0.05 compared to DC
+P<0.05 compared to LAD
**P<0.05 compared to NC

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<td>3.5 ± 0.4</td>
<td>II</td>
</tr>
</tbody>
</table>

*P<0.05 compared to DC
+P<0.05 compared to LAD
**P<0.05 compared to NC

NC=Normal Control; MCI=Mild Cognitive Impairment; PCAD=Preclinical Alzheimer's Disease; LAD=Late-stage Alzheimer’s Disease; DC=Diseased Control; PMI=Postmortem Interval.

Table 1: Subject demographic.

Representative RNA isolates exhibited signature characteristics, including 18S and 28S ribosomal RNA units and high RINs, indicating a highly pure and intact RNA (Figure 1). Oxidized RNA adducts quantified were based on previous studies that indicated significant changes in oxidized nucleic acids of nDNA and mtDNA between age-matched NC subjects and LAD subjects. The five stable isotope labeled internal standards were well separated chromatographically at the following retention times: 8-[8-13C, 7, 9-15N2] hydroxyguanine (47.2 min), [formyl-13C, diamino-15N2] fapyguanine (42.2 min), 8-[8-13C, 6, 9-diamino-15N2] hydroxyadenine (34.6 min), [formyl-13C, diamino-15N2] fapyadenine (29.3 min), and 5-[2-13C, 1,3-15N2] hydroxycytosine (23.5 min) (Figure 2A).

Three replicate analyses were performed to determine the dynamic range of each stable isotope labeled internal standard. Positive significant correlations were observed between the instrument response over a range of 500 pmol to 100 nmol for: 8-[8-13C, 7, 9-15N2] hydroxyguanine (r=0.99), [formyl-13C, diamino-15N2] fapyguanine (r=0.99), 8-[8-13C, 6, 9-diamino-15N2] hydroxyadenine (r=0.97), [formyl-13C, diamino-15N2] fapyadenine (r=0.99), 5-[2-13C, 1,3-15N2] hydroxycytosine (r=0.99). Representative mass spectra for each stable labeled adduct
of interest are shown in Figures 2B-2F. For quantitative purposes the dominant daughter ion for each oxidized ribonucleic acid adducts were monitored. To illustrate the percentage of change, levels of ribonucleic acid oxidation are expressed levels as median [range] % of NC subjects. Median levels of oxidized bases measured in each identifiable stage of AD and in DC subjects are shown in Table 2.

Significant (p ≤ 0.05) elevations in median levels of 8-OHG were observed in the SMTG of MCI and LAD subjects compared to NC subjects. Although median levels of 8-OHG in DC subjects were not significantly different, levels trended toward significance (p ≤ 0.1) compared to NC subjects. In contrast, no significant changes were detected in PCAD in the SMTG. No significant changes in
Significant \( p \leq 0.05 \) elevations of median levels of 8-OHA were observed in only PCAD subjects, although they trended toward significance \( p \leq 0.1 \) in MCI, LAD, and DC subjects compared to NC subjects. Similarly to 8-OHG, no significant changes in the median level of 8-OHA were noted during the progression of AD or in DC subjects compared to NC subjects in the IPL or CER. Median levels of FapyA were not significantly different in the SMTG of any disease stage studied but were significantly elevated in the two early stages (PCAD and MCI) as well as LAD and DC subjects compared to NC subjects in the IPL. No significant changes were noted in the CER during AD pathogenesis or in DC subjects compared to NC subjects.

Median levels of 5-OHC were not significantly different in any brain region studied, although median levels were significantly \( p \leq 0.05 \) elevated in the CER only of DC subjects compared to NC subjects. Median levels of PBS-soluble Aβ_{1-40} and Aβ_{1-42} in each identifiable stage of AD and in DC subjects are shown in Tables 3-5. In the SMTG, levels of PBS-soluble Aβ_{1-40} were significantly \( p \leq 0.05 \) elevated in both MCI and LAD subjects compared to NC subjects. Median levels of PBS-soluble Aβ_{1-42} were significantly \( p \leq 0.05 \) elevated in only LAD subjects compared to NC subjects.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>LESION</th>
<th>Group</th>
<th>SMTG</th>
<th>IPL</th>
<th>CER</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOHG</td>
<td>NC 100.0 ([33.6-205.3])</td>
<td>100.0 ([12.3-293.5])</td>
<td>100.0 ([38.0-109.3])</td>
<td></td>
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<tr>
<td>PCAD</td>
<td>113.0 ([38.3-388.8])</td>
<td>96.2 ([23.0-254.9])</td>
<td>133.6 ([87.5-165.0])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCI</td>
<td>250.5 ([33.6-573.1])</td>
<td>124.5 ([26.6-276.8])</td>
<td>117.3 ([89.6-261.4])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>599.3 ([106.0-2741.3])</td>
<td>117.5 ([27.6-297.0])</td>
<td>89.3 ([66.1-198.8])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FapyG</td>
<td>NC 100.0 ([24.9-202.8])</td>
<td>100.0 ([45.0-199.4])</td>
<td>100.0 ([57.6-263.5])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCAD</td>
<td>113.5 ([81.1-196.2])</td>
<td>118.4 ([12.4-240.0])</td>
<td>150.1 ([62.2-546.7])</td>
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<tr>
<td>MCI</td>
<td>60.2 ([24.2-105.5])</td>
<td>121.7 ([85.0-148.9])</td>
<td>119.2 ([57.2-611.4])</td>
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<tr>
<td>LAD</td>
<td>102.5 ([52.2-216.2])</td>
<td>141.8 ([105.7-517.3])</td>
<td>171.4 ([83.3-876.2])</td>
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<tr>
<td>DC</td>
<td>344.8 ([32.9-641.3])</td>
<td>100.5 ([30.2-266.8])</td>
<td>330.4 ([113.3-1067.1])</td>
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**Table 2:** Levels of oxidized nucleic acid base adducts in RNA expressed as % of NC (Median [Range]).

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Aβ</th>
<th>Group</th>
<th>SMTG</th>
<th>IPL</th>
<th>CER</th>
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<tr>
<td>PBS Aβ_{1-40}</td>
<td>NC</td>
<td>0.0 ([0.0-179.0])</td>
<td>52.7 ([0.0-103.5])</td>
<td>3.4 ([0.0-3.6])</td>
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<tr>
<td>PCAD</td>
<td>12.8 ([0.0-122.6])</td>
<td>0.0 ([0.0-257.4])</td>
<td>14.75 ([0.0-66.5])</td>
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<tr>
<td>MCI</td>
<td>50.6 ([0.0-66.4])</td>
<td>0.0 ([0.0-117.1])</td>
<td>0.0 ([0.0-0.8])</td>
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<td></td>
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<tr>
<td>LAD</td>
<td>80.6 ([0.8-281.1])</td>
<td>163.4 ([125.2-1412.3])</td>
<td>58.2 ([0.0-60.16])</td>
<td></td>
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<tr>
<td>DC</td>
<td>22.7 ([6.8-34.6])</td>
<td>0.0 ([0.0-173.2])</td>
<td>0.0 ([0.0-0.8])</td>
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</table>

**Table 3:** Levels of PBS Aβ_{1-40} and Aβ_{1-42} expressed as Median [Range] in pM.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Aβ</th>
<th>Group</th>
<th>SMTG</th>
<th>IPL</th>
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<tr>
<td>PBS Aβ_{1-42}</td>
<td>NC</td>
<td>0.0 ([0.0-179.0])</td>
<td>52.7 ([0.0-103.5])</td>
<td>3.4 ([0.0-3.6])</td>
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<tr>
<td>DC</td>
<td>22.7 ([6.8-34.6])</td>
<td>0.0 ([0.0-173.2])</td>
<td>0.0 ([0.0-0.8])</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4:** Levels of SDS-soluble Aβ_{1-40} and Aβ_{1-42} expressed as Median [Range] in pM.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Aβ</th>
<th>Group</th>
<th>SMTG</th>
<th>IPL</th>
<th>CER</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS Aβ_{1-40}</td>
<td>NC</td>
<td>0.0 ([0.0-179.0])</td>
<td>52.7 ([0.0-103.5])</td>
<td>3.4 ([0.0-3.6])</td>
<td></td>
</tr>
<tr>
<td>PCAD</td>
<td>12.8 ([0.0-122.6])</td>
<td>0.0 ([0.0-257.4])</td>
<td>14.75 ([0.0-66.5])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCI</td>
<td>50.6 ([0.0-66.4])</td>
<td>0.0 ([0.0-117.1])</td>
<td>0.0 ([0.0-0.8])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>80.6 ([0.8-281.1])</td>
<td>163.4 ([125.2-1412.3])</td>
<td>58.2 ([0.0-60.16])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>22.7 ([6.8-34.6])</td>
<td>0.0 ([0.0-173.2])</td>
<td>0.0 ([0.0-0.8])</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5:** Levels of FA soluble Aβ_{1-40} and Aβ_{1-42} expressed as Median [Range] in pM.
Table 6: Summary of Significant Correlations between levels of PBS-, SDS-, and FA-soluble Aβ1-40 and Aβ1-42 and Individual RNA Adducts.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Aβ1-40</th>
<th>Aβ1-42</th>
<th>Aβ1-42</th>
<th>Aβ1-42</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMTG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IPL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CER</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PBS Aβ1-40</td>
<td>8-OHA</td>
<td>8-OHA</td>
<td>8-OHA</td>
<td>8-OHA</td>
</tr>
<tr>
<td>PBS Aβ1-42</td>
<td>r=0.526 p&lt;0.002; FapyG r=0.508 p=0.004</td>
<td>r=0.55 p&lt;0.002; FapyG r=0.087 p=0.00002</td>
<td>r=0.55 p&lt;0.002; FapyG r=0.087 p=0.00002</td>
<td>r=0.55 p&lt;0.002; FapyG r=0.087 p=0.00002</td>
</tr>
<tr>
<td>SDS Aβ1-40</td>
<td>FapyG r=0.653 p&lt;0.002</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SDS Aβ1-42</td>
<td>FapyG r=0.773 p&lt;0.00008</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FA Aβ1-40</td>
<td>8-OHG</td>
<td>r=0.55 p&lt;0.005; FapyG r=0.877 p&lt;0.00002</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FA Aβ1-42</td>
<td>8-OHG</td>
<td>r=0.60 p&lt;0.006; FapyG r=0.730 p&lt;0.00008</td>
<td>FapyG r=0.645 p=0.00005</td>
<td>-</td>
</tr>
</tbody>
</table>

Discussion

Despite extensive study, the etiology of AD remains unclear. The indirect relationship between clinical manifestations and pathological features and an extended prodromal phase challenges researchers. In the current study, two distinct early stages of AD progression were studied including subjects who demonstrate substantial AD pathology but remain cognitively normal (PCAD) and the earliest detectable clinical stage (MCI) concurrent with LAD subjects in both vulnerable and non-vulnerable brains regions. Furthermore, inclusion of FTD and DLB subjects as a pooled diseased control group allowed us to assess RNA oxidation in additional neurodegenerative diseases to determine if RNA oxidation is unique to AD.

To our knowledge, this study is the first to quantify levels of multiple oxidized nucleic acids in RNA throughout the pathogenesis of AD as well as in DC subjects (DLB and FTD). In an effort to minimize potential analytical biases, subjects from each group and multiple brain regions were analyzed in parallel. We analyzed nucleotides isolated from bulk tissue composed of a mixture of glia, neurons and vasculature, however previous immunohistochemical studies by Nunomura et al. [39] demonstrated that 8-hydroxyguanine immunoreactivity was restricted to neuronal cells. Yet, the detection of multiple mRNA species sequestered in SPs of AD [40] suggest total RNA isolated during that bulk tissue processing includes remnant RNA incorporated in SP following neuron degeneration. Furthermore, the current study did not distinguish between different types of RNA isolated. Therefore median levels reported here represent total RNA including messenger (mRNA), ribosomal (rRNA), and transfer (tRNA). Previous studies by Shan and Lin [41] reported mRNA accounts for less than 5% of total RNA isolates and reported significant oxidation of polyribosomal RNA fractions 40S, 60S, and 80S in LAD [42] suggesting changes observed in their study likely reflect rRNA and tRNA oxidation.

Although pure intact RNA as indicated by a RIN of 9.8 was analyzed, nucleic acid adduct quantification by GC/MS as described in the current study, has the potential to introduce artifactual oxidation during isolation of as well as derivitization [43-45]. Although the use of phenol during nucleic acid isolation has been suggested to contribute to artifactual oxidation, Wang et al. [26] demonstrated that detectable DNA lesions were not significantly different when extracted with a phenol:chloroform extraction compared to the popular NaI based 'salting out' method consistent with the findings of Rehann et al. [46]. Furthermore, atmospheric oxygen was excluded during nucleic acid derivatization and carried out at room temperature to minimize artifactual oxidation [46-48].

Similar to previous studies that demonstrated increased nucleic acid in both nuclear and mitochondrial DNA [25,37,49], we found that median levels of multiple oxidized RNA base adducts were significantly elevated in vulnerable brain regions in LAD consistent with Weidner et al. [38] who observed increased levels of 8-hydroxyadenine in RNA from LAD subjects. Additionally, median levels of 8-OHA and 8-OHA were significantly increased or trended toward significance in the SMTG. These findings are consistent with those of Nunomura et al. [50] who demonstrated increased RNA oxidation in vulnerable neurons of MCI subjects in temporal cortex pyramidal neurons suggesting the earliest clinically identifiable stage of AD is characterized by nucleic acid oxidation. Additionally, median levels of 8-OHA were significantly elevated in PCAD subjects suggesting RNA oxidation may be a feature of the prodromal phase of AD (PCAD) consistent with Nunomura et al. [50] who reported RNA oxidation is an event preceding a transition from normal aging during the extended prodromal phase of AD.

While 8-hydroxyguanine is considered to be the most common marker of oxidative damage, our study suggests levels of 8-hydroxyguanine are not significantly different than levels of 8-hydroxyadenine. Although elevated levels of 8OHG and FapyA were detected in both early stages of AD (PCAD and MCI), levels were not significantly different from those in LAD subjects. These findings suggest RNA oxidation reaches a peak early in AD progression without further significant elevation later in disease. Furthermore, significantly elevated levels of 8-hydroxyguanine and 8-hydroxyadenine observed in DC subjects were comparable to levels observed in both early and late stages of AD suggesting oxidation is a common feature of neurodegeneration, rather than an AD specific event. These findings are consistent with a previous study of nuclear and mitochondrial DNA nucleic acid oxidation in the same subject pool [51].

Interestingly, levels of FapyA were only significantly increased in the IPL. Formation of FapyA following the initial insult is dependent on environmental conditions and elevation suggests damage occurred in
a hypoxic environment. Again median levels of nucleic acid oxidation observed in PCAD and MCI subjects were not significantly different from levels observed in LAD subjects. Additionally, levels of FapyA in the IPL were also significantly increased in DC subjects. Collectively these data suggest a hypoxic environment may be an early event in AD pathogenesis but is not an exclusive feature of AD but rather a common feature of neurodegenerative disease consistent with reports of RNA oxidation in Down syndrome [52, 53], familial AD [54], DLB [55], and Parkinson’s disease [56].

The cerebellum is widely regarded as an internal control with minimal changes associated with AD. However, in the current study levels of FapyG were significantly elevated in the CER of PCAD, LAD, and DC brain. While it remains unclear why significant oxidation was detected in the CER, studies of nuclear DNA and mitochondrial DNA isolated from the same subject pool also exhibited elevated levels of multiple markers of oxidative damage in the CER [51]. Furthermore, Bradley et al. [28] showed levels of extractable 4-hydroxyhexenal were significantly increased in the CER of LAD subjects compared to age-matched NC subjects ina subset of subjects described in the current study. Additionally, Braak et al. [57] and Yamaguchi et al. [58] described the presence of diffuse amyloid plaques in the molecular layer of the cerebellar cortex of AD subjects and Weigel et al. [59] reported a volumetric loss in both the molecular and granular layers. Shrinkage of the posterior cerebellar region of AD has been associated with poorer cognitive performance [60]. Furthermore, elevated levels of 8-hydroxyguanine and FapyG were observed in the CER of DC subjects, consistent with FTD associated loss of the Purkinje cells [61] and detection of α-synuclein positive Purkinje cells in DLB subjects [62]. Significant oxidation of nucleic acids in the CER of DC subjects suggests the phenomenon is not AD specific.

Correlation analyses between median levels of RNA base adducts and neuropathological features including NP and NFT densities, and PBS-, SDS-, and FA-soluble Aβ1-40 and Aβ1-42 were determined for the SMTG, IPL, and CER. No significant correlations were detected between NP or NFT counts. Although multiple significant correlations between RNA adducts and specific pools of Aβ1-40 and Aβ1-42 in AD levels of multiple RNA lesions were also found to be significantly elevated in DC subjects whose levels of Aβ1-40 and Aβ1-42 were comparable between both early stages of AD progression and LAD and DC subjects suggesting that nucleic acid oxidation is a common feature of neurodegeneration rather than an AD exclusive feature.

Conclusion

Consistent with our previous study of nuclear and mitochondrial DNA, the current data suggest RNA oxidative damage is an early event in the pathogenesis of AD. Furthermore, levels of RNA oxidation are comparable between both early stages of AD progression and LAD and DC subjects suggesting that nucleic acid oxidation is a common feature of neurodegeneration rather than an AD exclusive feature.

Conflict of Interest

The authors have no conflicts of interest.

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