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# The Comparison of Methods for Measuring Oxidative Stress in Zebrafish Brains

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## Abstract

The zebrafish is a versatile model organism with the potential to contribute to our understanding of the molecular pathological mechanisms underlying Alzheimer's disease (AD). An early characteristic of AD brain pathology is lipid peroxidation resulting from oxidative stress. However, changes in lipid peroxidation have not yet been assessed in zebrafish brains, and an earlier attempt to observe changes in $F_2$-isoprostane levels in the brains of zebrafish exposed to hypoxia was unsuccessful. In this article, we examine the utility of various assays of lipid peroxidation and more general assays of intracellular oxidative stress to detect the changes in oxidative stress in the brains of adult zebrafish exposed to hypoxia or explanted into a sodium azide solution for chemical mimicry of hypoxia. Levels of $F_2$-isoprostanes and $F_4$-neuroprostanes were low and variable in zebrafish brains such that statistically significant changes due to hypoxia or chemical mimicry of hypoxia could not be observed. However, measurement of lipid hydroperoxides did reveal significant changes in lipid peroxidation under these conditions, while analyses of *catalase* gene expression and an assay based on 2′,7′-dicholorofluorescein oxidation also revealed changes in oxidative stress levels.

## Introduction

The human brain consumes ~20% of the body's energy budget.[1] Thus, supply of oxygen to the brain tissue is critical for maintaining energy production and neural function. Disruption of energy metabolism and especially mitochondrial function may be fundamental to many neurodegenerative diseases, including Alzheimer's disease (AD).[2] Neural hypoxia can be a direct consequence of hypoperfusion, a common vascular component among the AD risk factors.[3] Hypoxia causes the mitochondrial electron transport chain to emit increased amounts of reactive oxygen species (ROS) at the Qo site of complex III, which is consistent with increased oxidative stress.[4,5] Interestingly, a large body of evidence suggests that oxidative stress plays an important role in the development and progression of AD.[6–8] Oxidative damage induced by ROS has been extensively studied for neuronal lipids, proteins, nucleic acids, and sugars in AD.[9] The high lipid concentration of the neuronal membrane, especially with respect to polyunsaturated fatty acids, and the high energy and oxygen consumption rates of neurons make the brain susceptible to lipid peroxidation.

$F_2$-isoprostanes ($F_2$-IsoPs) are a family of prostaglandin (PG)-like bioactive compounds that are formed *in vivo* independently of cyclooxygenase. They are formed primarily through free radical-mediated peroxidation of arachidonic acid (AA[10,11]) and result in 32 isomers of $F_2$-isoprostanes, which are divided into four subfamilies (series 5, 8, 12, and 15).[10,12] Once formed, these compounds can be released from phospholipids by phospholipases A2 (PLA2) or by platelet activating factor acetylhydrolase (PAF-AH or lipoprotein [Lp]-PLA2).[13] $F_2$-isoprostanes are named as such because they contain F-type prostane rings and are isomeric to $PGF_{2\alpha}$.[12]

Docosahexaenoic acid (DHA) has been the subject of considerable interest due to the fact that it is highly enriched in the human brain, especially in the gray matter, where it makes up ~25%–35% of the total fatty acids in aminophospholipids.[14,15] Although DHA is present at high concentrations in neurons, these cells are unable to elongate or desaturate essential fatty acids to form DHA. Alternatively, DHA is synthesized mainly by astrocytes, then secreted, and taken up by neurons.[16] Although the precise function of DHA in the brain is not well understood, deficiency of DHA is associated with abnormalities in brain function.[17] Neuroprostanes (or $F_4$-neuroprostanes [$F_4$-NeuroPs]) are isoprostane-like compounds that are formed by free radical-induced peroxidation of DHA. $F_2$-isoprostanes and neuroprostanes have been implicated in the pathogenesis of many diseases and their potential roles in AD have also been

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investigated.[18–20] $F_2$-isoprostanes and neuroprostanes are the most reliable biomarkers of *in vivo* lipid oxidation and oxidative stress.[21]

The zebrafish is a versatile vertebrate model for studies of developmental biology, genetics, pharmacology, and other areas of neuroscience.[22–24] Zebrafish have many inherent advantages as a model organism, including low cost, easy handling, and maintenance compared with other vertebrate models, and around 70% protein sequence identity with humans.[25] Oxidative stress and, in particular, lipid peroxidation appears to be a critical part of the development of AD pathology. For our work, to develop the zebrafish as a model system in which to examine the molecular pathology of AD, we must find sensitive and reliable methods to measure oxidative stress, including lipid peroxidation. In the previous work, using zebrafish embryos and the brains from adult fish, we attempted to examine relative oxidative stress after exposure to hypoxia by examining $F_4$-isoprostanes and levels of *catalase* mRNA.[26] No significant difference in $F_4$-isoprostane levels could be observed, whereas very significant increases in *catalase* mRNA were seen under hypoxia. Chemical mimicry of hypoxia using exposure of embryos or explanted adult brains to a sodium azide ($NaN_3$) solution produces the same effects.[27] In this article, we have examined oxidative stress, including lipid peroxidation, using specific methods such as measurement of $F_2$-isoprostanes and $F_4$-neuroprostanes, and lipid hydroperoxides (LOOHs). We also examined more general assays for ROS such as quantitative PCR (qPCR) for *catalase* gene expression and dichlorofluorescein (DCF) measurement of intracellular ROS. We show that concentrations of $F_2$-isoprostanes and $F_4$-neuroprostanes are very low and variable, but lipid peroxidation can be revealed in zebrafish brains by measurement of LOOH. ROS is detectable by qPCR for catalase transcripts and by DCF measurement of intracellular ROS.

## Materials and Methods

### *Ethics*

This work was conducted under the auspices of the Animal Ethics Committee of the University of Adelaide and in accordance with the EC Directive 86/609/EEC for animal experiments and the Uniform Requirements for Manuscripts Submitted to Biomedical Journals.

### *Zebrafish husbandry and experimental procedures*

*Danio rerio* were bred and maintained at 28°C on a 14-h light/10-h dark cycle.[28] Wild-type zebrafish (AB strain) at ~1 year of age were used for all experiments. Both male and female fish were used (i.e., except for qPCR analysis of *catalyse* mRNA levels there was no differentiation on the basis of gender). For chemical mimicry of hypoxia, adult explant brain tissues were exposed to 100 $\mu$M $NaN_3$ (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in the DMEM. In studies of hypoxia, it is common to use chemical agents that can mimic (partially) hypoxic conditions (also known as chemical hypoxia). Agents commonly used are cobalt chloride ($CoCl_2$), nickel chloride ($NiCl_2$), and $NaN_3$. Azides, including $NaN_3$, have an action on the respiratory chain very similar to that of cyanide.[27] In the experiments conducted under low oxygen conditions, oxygen was depleted by bubbling nitrogen gas through the fish tank water. Oxygen concentrations were measured using a dissolved oxygen meter (DO 6+; Eutech Instruments, Singapore). The dissolved oxygen level in the hypoxia group was measured to be $1.15 \pm 0.6$ mg/L, whereas the normal ambient oxygen level was $6.6 \pm 0.45$ mg/L. All experiments were performed with three or six replicates (i.e., $n = 3$ or $n = 6$) as indicated. Briefly, after each hypoxia trial, the animals were killed by plunging into ice water and then brains removed.[29] For $F_2$-isoprostane and $F_4$-neuroprostane measurements, brains were removed and frozen immediately in solid $CO_2$ before eventual analysis using three adult zebrafish brains per sample. Other assays were conducted using freshly explanted brains with six brains per sample.

### *Chemicals, reagents, and chromatography*

15-$F_{2t}$-IsoP (8-iso-$PGF_{2a}$) and 15-$F_{2t}$-IsoP-$d_4$ (8-iso-$PGF_{2a}$-$d_4$) were purchased from Cayman Chemicals (Ann Arbor, MI) and used without further purification. $F_4$-neuroprostane standard was kindly provided by Dr. Thierry Durand (Institut des Biomolécules Max Mousseron, Université Montpellier I, Montpellier France). Pentafluorobenzylbromide (PFBBr) and *N*,*N*-diisopropylethylamine (DIPEA) were purchased from Sigma Chemicals (St Louis, MO). The silylating agent *N*,*O*-*bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (BSTFA-TMCS, 99:1) was purchased from Pierce Chemicals (Rockford, IL). Certify II cartridges were from Varian (Lake Forrest, CA). All solvents were of HPLC grade. The 4(*RS*)-$F_{4t}$-NeuroP ($F_4$-IsoP) was synthesized as previously described.[30,31]

### *Measurement of $F_4$-NeuroPs and $F_2$-IsoPs*

$F_4$-NeuroPs and $F_2$-IsoPs were measured using a modification of our previously reported method.[32,33] Briefly, samples were spiked with 5 ng internal standard 15-$F_{2t}$-IsoP-$d_4$ (8-iso-$PGF_{2a}$-$d_4$), then hydrolyzed with 1 M potassium hydroxide in methanol, acidified, and applied to prewashed Certify II cartridges (Varian). After washing with methanol/water (1:1) and hexane/ethyl acetate (75:25), the metabolites were eluted with ethyl acetate/methanol (90:10) and dried under vacuum. Samples were derivatized using PFBBr and DIPEA (Sigma Chemicals), dried under nitrogen, and then treated with the silylating agent BSTFA with 1% TMCS (Pierce Chemicals). IsoFs, $F_2$-IsoPs, and $F_4$-NeuroPs were quantified by gas chromatography–mass spectrometry using electron capture negative ionization and selected ion monitoring. Ions monitored were *m/z* 569, 573, and 593, for $F_2$-IsoP, 15 $F_{2t}$-IsoP-$d_4$, and $F_4$-NeuroPs, respectively.

### *Measurement of LOOHs*

Lipid peroxidation in zebrafish adult brain was estimated using an LOOH assay kit (Cayman Chemical Company, Ann Arbor, MI). Zebrafish brain tissues from normoxic and actual hypoxic and chemical mimicry of hypoxia were homogenized in a buffer containing no transition metal ions [ice-cold Tris-HCl buffer (containing 0.25 M sucrose, pH 7.4), differential centrifugation (700 and 4500 *g*, 10 min, 4°C)]. Five hundred microliters of homogenized brain was placed in separate test tubes. An equal amount of Extract R saturated methanol was added and mixed by vortexing. One milliliter

of cold deoxygenated chloroform was added to all the control and sample tubes and centrifuged at 1500 *g* for 5 min at 0°C (Beckman XL-100 K, Ramsey, MN). After centrifugation, the chloroform layer (500 $\mu$L) was mixed with 450 $\mu$L of chloroform:methanol (2:1) and 50 $\mu$L of chromogen (thiocyanate ion). The reaction mixtures of all the control and sample tubes were incubated at room temperature for 5 min. The absorbance of each control and sample was recorded at 500 nm using a research spectrophotometer.

### Quantitative real-time PCR

The relative standard curve method for quantification was used to determine the expression of experimental samples compared with a basis sample. For experimental samples, target quantity was determined from the standard curve and then compared with the basis sample to determine fold changes in expression. Gene-specific primers were designed for amplification of target cDNA and the cDNA from the ubiquitously expressed control gene e*ef-1a1a*. The reaction mixture consisted of 50 ng/$\mu$L of cDNA. Eighteen micromolars of forward and reverse primers and *Power* SYBR green master mix PCR solution (Applied Biosystems, Foster City, CA).

To generate the standard curve, cDNA was serially diluted (100, 50, 25, 12.5 ng). Each sample and standard curve reactions were performed in triplicate for the control gene and experimental genes. Amplification conditions were 2 min at 50°C followed by 10 min at 95°C, and then 40–45 cycles of 15 s at 95°C and 1 min at 60°C. Amplification was performed on an ABI 7000 Sequence Detection System (Applied Biosystems) using 96-well plates. Cycle thresholds obtained from each triplicate were averaged and normalized against the expression of e*ef-1a1a*, which has previously been demonstrated to be suitable for normalization for zebrafish qPCR.[34] Each experimental sample was then compared with the basis sample to determine fold changes of expression. The primers used for quantitative real-time PCR analysis of *catalase* mRNA levels were *cat* F: (5′-TAAAGGAGCAGGA GCGTTTGGCTA-3′) and *cat* R: (5′-TTCACTGCGAAACC ACGAGGATCT-3′).

### Evaluation of intracellular ROS production

ROS production was measured following the method based on 2′,7′-dichlorofluorescein oxidation.[35] Zebrafish brain tissues from normoxic and hypoxic conditions were homogenized in an ice-cold Tris-HCl buffer (containing 0.25 M sucrose, pH 7.4). Differential centrifugation (700 and 4500 *g*, 10 min, 4°C) was employed to isolate cytosol and mitochondria from the brain tissue. Homogenates from total zebrafish brain (60 $\mu$L) were incubated for 30 min at 37°C in the dark with 240 $\mu$L of 100 $\mu$M 2′,7′-dichlorofluorescein diacetate ($H_2$DCF-DA) solution in a 96-well plate. $H_2$DCF-DA is cleaved by cellular esterases and the $H_2$DCF formed is eventually oxidized by ROS present in samples. The last reaction produces the fluorescent compound DCF, which was measured at 488 nm excitation and 525 nm emission, and the results were presented as DCF fluorescence intensity. A calibration curve was produced using purified DCF as a standard. To exclude the possibility of false signals arising from reactions with $NaN_3$, a volume of 100 $\mu$M $NaN_3$ equivalent to the volume of zebrafish brain in the assay (100 $\mu$L) was incubated with DCF and measured at 488 nm excitation and 525 nm emission, with the results presented as DCF fluorescence intensity.

### Statistical analysis of data

Means and standard deviations were calculated for all variables. A Student's *t*-test was used to evaluate significant differences between controls and samples from different treatments. *p*-Values, degrees of freedom (*df*), and *t*-values are shown within the figures and figure legends. A criterion alpha level of $p < 0.05$ was used for all statistical comparisons. Values given in figures represent means ± SEMs of brains from three animals per biological replicate (three replicates per assay) for qPCR, DCF, and LOOH assays. For IsoPs and NeuroPs, values represent means ± SEMs of brains from one animal per biological replicate (six replicates per assay). All data were analyzed using GraphPad Prism version 6.0 (GraphPad Prism, La Jolla, CA).

## Results

### Catalase expression validates oxidative stress conditions

To support that oxidative stress had been produced under our experimental conditions, we examined the relative expression of the gene encoding Catalase *cat* that has previously been used as a marker of oxidative stress in zebrafish.[36,37] We previously reported that *cat* mRNA levels are increased in the brains of zebrafish exposed to actual hypoxia.[26] For the work described in this article, we analyzed relative *cat* mRNA levels by qPCR in explanted zebrafish brains under chemical mimicry of hypoxia and actual hypoxia from both female and male zebrafish. We found that, under chemical mimicry of hypoxia and actual hypoxia, *cat* mRNA levels are increased more than twofold in the brains of both female and male zebrafish. This supports that incubation of explanted adult brains in 100 $\mu$M $NaN_3$ for 6 h is a valid method for mimicry of hypoxia in this tissue (Fig. 1). No significant differences were seen due to brain gender. Subsequent analyses did not differentiate between the gender of the individuals.

### Measurement of intracellular ROS production

In their analysis of the effects of respiratory burst response in zebrafish, Hermann *et al.* measured intracellular ROS in embryos by evaluating changes in the fluorescence intensity of the dye DCF.[38] Embryos at various developmental stages were found to oxidize the probe to different extents. Significant amounts of ROS were produced only in embryos of 3 dpf and older.[38] Seibt *et al.* also described changes in intracellular ROS by evaluating changes in the fluorescence intensity of the dye DCF after treatment with antipsychotic drugs on zebrafish brains.[39] We tested whether DCF might be useful for assessing ROS induced by actual hypoxia and/or chemical mimicry of hypoxia in zebrafish brains. When zebrafish were exposed to hypoxia for 3 h (Fig. 2A) or explanted brains were incubated with 100 $\mu$M of $NaN_3$ at 28°C for 6 h (Fig. 2B), both revealed significantly increased intracellular ROS by DCF fluorescence. A control in which a volume of 100 $\mu$M of $NaN_3$ equivalent to the zebrafish brain volumes used in the assay

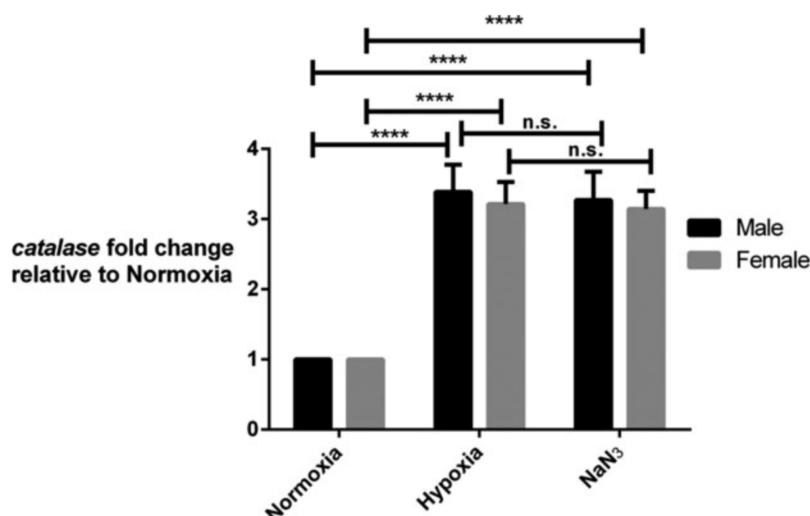


**FIG. 1.** Quantitative PCR analysis of zebrafish *cat* mRNA levels in explanted zebrafish brains [male and female ($n=3$)] under normoxia relative to under chemical mimicry of hypoxia. Fold changes in gene expression are shown relative to normoxia ($^{****}p<0.0001$).

incubated with DCF showed no evidence of any false signal due to the reaction of $NaN_3$ and DCF (Fig. 2B).

### *$F_2$-isoprostane and $F_4$-neuroprostane levels in zebrafish brain tissue under hypoxic stress*

To determine whether $F_2$-isoprostane and/or $F_4$-neuroprostane levels might reveal changes in lipid peroxidation under oxidative stress conditions, we first examined their levels in brains from adult zebrafish exposed to hypoxia for 3 h or in explanted zebrafish brains subjected to chemical mimicry of hypoxia by incubation in 100 $\mu$M $NaN_3$ for 6 h. No significant differences from normoxia were seen for $F_2$-IsoPs (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/zeb) or $F_4$-NeuroPs (Supplementary Fig. S2) levels under either actual hypoxia or chemical mimicry of hypoxia. The lack of observable changes may be due to the variability seen for the levels of these molecules between individuals. We found that $F_2$-isoprostane and $F_4$-neuroprostane levels were generally low in zebrafish brain ($\sim$4 and $\sim$15 pg/mg, respectively) relative to the brains of mammals. The levels of AA and DHA in fish brain tissue are very low (1% and 5% of total fatty acids, respectively) compared with the levels in the human brain (11% and 25% of total fatty acids, respectively), which may contribute to the low levels of $F_2$-isoprostanes and $F_4$-neuroprostanes observed.[40,41]

### *Measurement of lipid peroxidation in zebrafish brain tissue under hypoxic stress using a LOOH assay*

LOOHs are derived from unsaturated lipids and cholesterol. LOOHs are prominent intermediates of peroxidative reactions induced by ROS, including hydroxyl radicals and singlet oxygen.[42] LOOH has previously been used to measure changes in lipid peroxidation in metabolic studies of caudal fin muscle of zebrafish by Rosa *et al.*[43] We measured LOOH levels in explanted zebrafish brains subjected to chemical mimicry of hypoxia by incubation in 100 $\mu$M $NaN_3$ for 6 h (Fig. 3A) in the brain tissue of zebrafish exposed to actual hypoxia for 3 h (Fig. 3B). LOOH levels were significantly increased approximately twofold in both cases indicating that this assay can be used to detect changes in lipid peroxidation in zebrafish brain for studies of the mechanisms underlying AD pathology.

## Discussion

A growing number of studies have exploited zebrafish to examine the role of hypoxia in brain dysfunction. For example, Braga *et al.* studied the effect of hypoxia on fish behavior.[29] They confirmed that decreased oxygen levels reduced the mitochondrial metabolic activity using a Triphenyl tetrazolium chloride-based assay. Hypoxia also causes increased production of ROS by mitochondria and we have previously monitored this indirectly by measuring relative *catalase* mRNA levels using qPCR.[26] However, for modeling of the oxidative stress associated with AD, more direct measures of ROS are desirable. In this study, we have sought to continue our development of the zebrafish as a model for analysis of the molecular pathology of AD by examining other, more direct, assays of ROS in the brains of zebrafish exposed to hypoxia and chemical mimicry of hypoxia.

The role of hypoxia-induced oxidative damage in the pathogenesis of neurodegenerative disorders has been firmly established.[44,45] Increases in oxidative stress occur when there is an imbalance between ROS generation and antioxidant defenses. The increase in ROS production is due to mitochondrial dysfunction caused by hypoxia.[4] There are multiple pathological consequences of increased ROS production.[46] In particular, markers of lipid peroxidation have been found to be elevated in brain tissues and body fluids in several neurodegenerative diseases, including AD, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and Down syndrome.[47–49]

The brain is highly vulnerable to oxidative stress because of its high rate of oxygen consumption, its low level of antioxidant defenses, and also its high lipid concentration providing substrates for lipid peroxidation.[46] In AD brain, increased lipid peroxidation has been identified by measuring levels of free and protein-bound 4-hydroxy-2-nonenal (HNE), $F_2$-isoprostanes, and $F_4$-neuroprostanes.[50,51] Pratico *et al.* showed increased levels of the isoprostane 8, 12-iso-i$PF_2\alpha$-VI, in cerebrospinal fluid in AD and mild cognitive

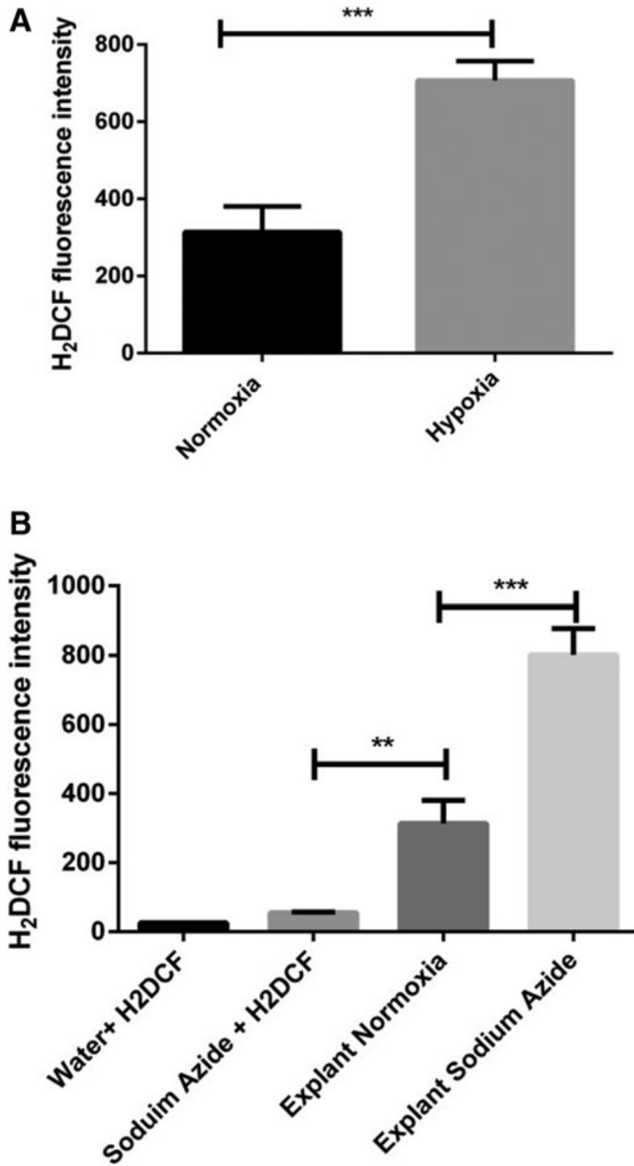


**FIG. 2.** **(A)** Measurement of intracellular reactive oxygen species (ROS) production in the brains of zebrafish after exposure to hypoxia. ROS are significantly increased. Error bars show standard deviation (***$p<0.0012$, $t=8.170$, $df=4$). **(B)** Measurement of intracellular ROS in explanted zebrafish brains under chemical mimicry of hypoxia. Treatment with 100 $\mu$M sodium azide ($NaN_3$) significantly increased ROS after 6 h of exposure. No fluorescence signal was observed for a negative control experiment in which the assay was performed using a volume of 100 $\mu$M $NaN_3$ equivalent to the volume of zebrafish brain normally used in the assay ($NaN_3+H_2DCF$) (***$p<0.0011$, $t=8.383$, $df=4$, **$p<0.0025$, $t=6.747$, $df=4$).

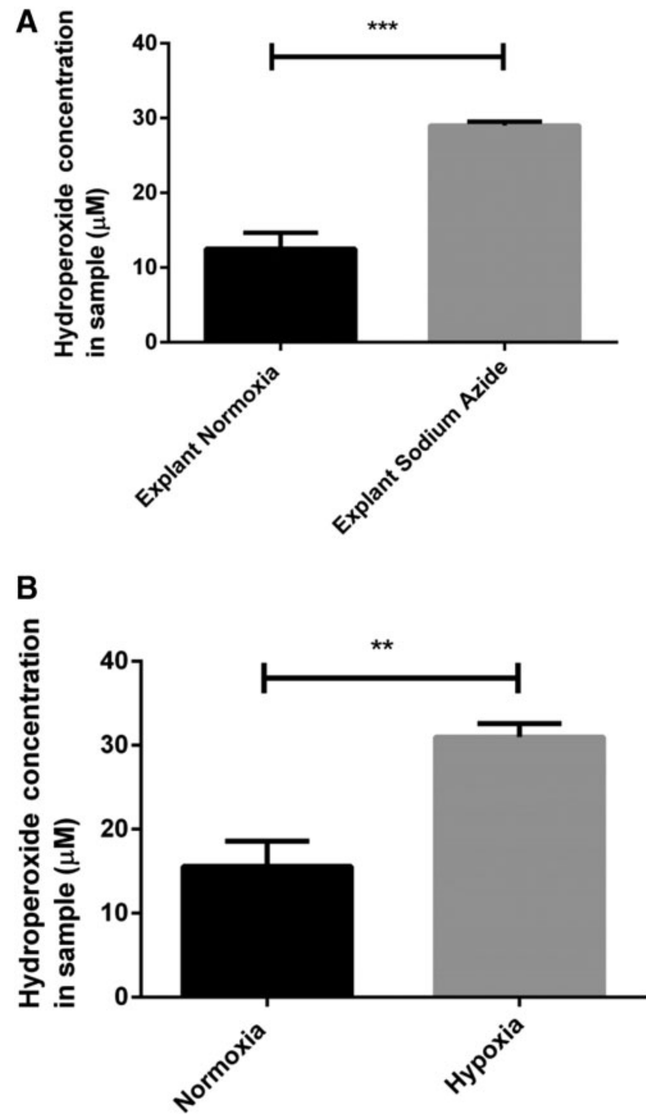


**FIG. 3.** **(A)** Levels of lipid hydroperoxide (LOOH) in explanted brains of zebrafish exposed to $NaN_3$ for chemical mimicry of hypoxia. Error bars show standard deviation (***$p<0.0002$, $t=13.17$, $df=4$). **(B)** Levels of LOOH in explanted brains of zebrafish *Danio rerio* after exposure to actual hypoxia (**$p<0.0014$, $t=7.849$, $df=4$).

impairment (MCI) and suggested that this lipid peroxidation product conceivably could be used as a marker to identify MCI patients who are at increased risk of progression to symptomatic AD.[52,53]

To verify that hypoxia causes oxidative stress and lipid peroxidation in zebrafish brain tissue, $F_2$-isoprostane and/or $F_4$-neuroprostane levels were measured. We also performed qPCR analysis of catalase expression, LOOH assay, and DCF to assess ROS. Our results revealed that exposure to acute levels of actual hypoxia or chemical mimicry of hypoxia has no consistently measurable effect on highly specific markers of lipid peroxidation, $F_2$-isoprostane, and/or $F_4$-neuroprostane levels in zebrafish brain tissue. The lack of observable significant changes may be due to the variability seen for the levels of these molecules between individuals. We found that $F_2$-isoprostane and $F_4$-neuroprostane levels were generally low in zebrafish brain (4 and 15 pg/mg, respectively) relative to the brains of mammals. As previously suggested in human studies[54] and fish studies,[55] the timing of sampling after a treatment is important. Some fish may have a faster response to ROS to release oxidized lipid products (isoprostanes, neuroprostanes), some may take time, and some are even suppressed (7$\beta$-hydroxycholesterol).[54] However, exposure to actual hypoxia or chemical mimicry of hypoxia significantly increased lipid

peroxidation as measured by the LOOH assay. LOOHs are primary products of lipid peroxidation mainly generated from phospholipids, cholesterol esters, and unsaturated fatty acids, which are degraded to reactive aldehydes, such as HNE malondialdehyde and alkenals as secondary peroxidation products.[42] Once formed, LOOHs undergo reductive degradation, enhancing cytotoxic potential. In addition, LOOHs trigger signal transduction pathways that lead to apoptosis.[42] As far as the behavior of hydroperoxide is concerned, our data are in agreement with those of Hayashi *et al.* showing that formation of hydroperoxides from phospholipids can be increased by the presence of an amyloid$\beta_{1-42}$-$Cu^{2+}$ complex.[56]

We also observed that intracellular ROS levels were significantly increased in zebrafish brains exposed to actual hypoxia or $NaN_3$. These data are consistent with the idea that insufficient oxygen supply may cause oxidative stress and alterations in antioxidant enzymes observed early in the development of AD.[7] Indeed, Markesbery *et al.* and Pratico *et al.*[51,53] showed that levels of lipid peroxidation increased significantly in brains of patients with AD when compared with controls.

In summary, we have presented evidence from a number of different assays that acute exposure to hypoxia or chemical mimicry of hypoxia increases oxidative stress in zebrafish brain tissue. Although $F_2$-isoprostane and $F_4$-neuroprostane levels did not show significantly measurable alteration, lipid peroxidation appeared to increase as demonstrated by the lipid hydroperoxidase assay. Our findings show that exposure of zebrafish to actual hypoxia or chemical mimicry of hypoxia can produce biochemical changes similar to those observed in AD. This supports the use of zebrafish as a useful animal model for future investigation of the causes of, and potential treatments for, AD.

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## Disclosure Statement

No competing financial interests exist.

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