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Deferiprone Reduces Amyloid- β and Tau Phosphorylation Levels but not Reactive Oxygen Species Generation in Hippocampus of Rabbits Fed a Cholesterol-Enriched Diet

Jaya R.P. Prasanthi^a, Matthew Schrag^{b,c}, Bhanu Dasari^a, Gurdeep Marwarha^a, Wolff M. Kirsch^b, and Othman Ghribi^{a,*}

^aDepartment of Pharmacology, Physiology and Therapeutics, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND, USA

^bDepartment of Biochemistry and Neurosurgery Center for Research, Training and Education, Loma Linda University, Loma Linda, CA, USA

^cDepartment of Neurology, Yale University, New Haven, CT, USA

Abstract

Accumulation of amyloid- β (A β) peptide and the hyperphosphorylation of tau protein are major hallmarks of Alzheimer's disease (AD). The causes of AD are not well known but a number of environmental and dietary factors are suggested to increase the risk of developing AD. Additionally, altered metabolism of iron may have a role in the pathogenesis of AD. We have previously demonstrated that cholesterol-enriched diet causes AD-like pathology with iron deposition in rabbit brain. However, the extent to which chelation of iron protects against this pathology has not been determined. In this study, we administered the iron chelator deferiprone in drinking water to rabbits fed with a 2% cholesterol diet for 12 weeks. We found that deferiprone (both at 10 and 50 mg/kg/day) significantly decreased levels of A β ₄₀ and A β ₄₂ as well as BACE1, the enzyme that initiates cleavage of amyloid- β protein precursor to yield A β . Deferiprone also reduced the cholesterol diet-induced increase in phosphorylation of tau but failed to reduce reactive oxygen species generation. While deferiprone treatment was not associated with any change in brain iron levels, it was associated with a significant reduction in plasma iron and cholesterol levels. These results demonstrate that deferiprone confers important protection against hypercholesterolemia-induced AD pathology but the mechanism(s) may involve reduction in plasma iron and cholesterol levels rather than chelation of brain iron. We propose that adding an antioxidant therapy to deferiprone may be necessary to fully protect against cholesterol-enriched diet-induced AD-like pathology.

Keywords

Alzheimer's disease; cholesterol; deferiprone; iron; oxidative stress; tau

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*Correspondence to: Othman Ghribi, Department of Pharmacology, Physiology and Therapeutics, University of North Dakota School of Medicine and Health Sciences, 501 North Columbia Road, Grand Forks, ND 58202, USA. Tel.: +1 701 777 2522; Fax: +1 701 777 4490; othman.ghribi@med.und.edu.

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INTRODUCTION

Iron is a biologically important element that plays a major role in many cellular functions [1, 2], but accumulation of iron at high levels is harmful to cells and may contribute to various neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease, Pick's disease, tauopathies, and other syndromes [3–7]. Iron dyshomeostasis and associated redox activity and oxidative stress are considered by some investigators as secondary pathological hallmarks of AD [8]. In AD brains, iron deposition was colocalized with deposits of amyloid- β (A β) [9, 10], neuritic plaques, and neurofibrillary tangles [11]. Recently, Smith and colleagues showed increased levels of redox active iron at the preclinical and mild cognitive impairment (MCI) precursor stages of AD [12].

Iron dyshomeostasis may be a major source of redox-generated free radicals in AD [13]. Redox-active iron may produce reactive oxygen species (ROS) by Fenton chemistry [14–16] and increased production of ROS may produce oxidative damage, leading to lipid peroxidation and neuronal degeneration in the brain [17]. Oxidative stress can lead to increased A β deposition [18, 19], however the exact mechanisms underlying A β deposition are yet to be determined. As redox-active iron overload can trigger or exacerbate neurodegenerative disorders, iron chelation may be considered as a therapeutic measure to protect against neurodegeneration.

Deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one) is an iron chelator that has the ability to prevent the production of toxic oxygen-activated species [20, 21]. Several studies have shown the beneficial effects of deferiprone treatment. Deferiprone protected cortical neurons and SHSY-5Y cells from ferric iron, hydrogen peroxide, MPP⁺, and A β -induced neuronal cell death [22]. Deferiprone also reduced atherogenesis in rabbits [23], oxidative stress in erythrocytes, platelets and polymorphonuclear leukocytes from myelodysplastic syndrome patients [24], suppressed experimental autoimmune encephalomyelitis and inhibited T-cell function in mice [25], and reversed glutathione depletion in erythrocytes isolated from patients with β -thalassemias who were exposed to tert-butyl hydroperoxide [26]. Deferiprone reduced brain iron accumulation in patients with pantothenate kinase-associated neurodegeneration, however this treatment did not induce clinical improvement in these patients [27]. Although one study demonstrated a protective effect of deferiprone against A β ₄₀ *in vitro* [22], very little has been done on the potential protective effects of deferiprone in an *in vivo* setting, either in animal models or in AD patients.

Epidemiological studies have suggested that hypercholesterolemia is a risk factor for AD [28]. High cholesterol diet induces a set of major pathological changes including A β accumulation, tau phosphorylation, and behavioral impairment in rabbits [29, 30]. We have also demonstrated that rabbits fed with a 1 or 2% cholesterol diet exhibit high levels of ROS, and iron accumulation within cortical A β plaques [31–33]. Oxidative stress can increase A β PP processing leading to increased production of A β , which in turn can exacerbate oxidative stress [34]. In the present study, we determined the extent to which the iron chelator deferiprone protects against a 2% cholesterol-enriched diet-induced AD pathological hallmarks in rabbit hippocampus, a brain region involved in learning and memory and severely affected in AD.

MATERIALS AND METHODS

Animals and treatments

New Zealand white male rabbits, 1.5 to 2 years of age weighing about 3–5 kg, were purchased from Charles River Laboratories and used for our studies. Rabbits were assigned randomly to 6 groups ($n = 9$ each), fed normal chow or 2% cholesterol-enriched diet (Harlan

Teklad Global Diets, Madison, WI), and simultaneously treated with deferiprone for 12 weeks (Table 1). Deferiprone (Fisher Scientific) was prepared daily and given in drinking water at 10 or 50 mg/kg/day. Deferiprone has been administered at a 100 mg/kg to rabbits [35] and 75–100 mg/kg/day to β -thalassaemia patients for a period ranging from 1–4.8 years [36, 37]. At necropsy, animals were perfused with Dulbecco's phosphate-buffered saline at 37°C and the brains were promptly removed. Hippocampi were quickly dissected and homogenized on ice and used for Western blot, ELISA, and ROS assays. All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of North Dakota.

Tissue sample preparation for quantification of A β levels

Tissue samples were processed in Tris buffer saline (TBS) (for soluble fraction) and lysis buffer (for membrane fraction) containing protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) and as described by Lim et al. [38] and Ma et al. [39]. Briefly, samples were homogenized and sonicated in 10 volumes of TBS containing a cocktail of protease and phosphatase inhibitors and centrifuged at 100,000 g for 20 min at 4°C to generate a TBS-soluble fraction. The TBS insoluble pellet was sonicated in 10 volumes of lysis buffer (150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, 0.5% deoxycholate, and protease and phosphatase inhibitors cocktail). The resulting homogenate was centrifuged at 100,000 g for 20 min at 4°C to produce a lysis buffer-soluble fraction (membrane fraction).

To analyze the detergent-insoluble A β , the lysis-insoluble amyloid-laden pellets were sonicated in 8 volumes of 5 M guanidine and 50 mM Tris-HCl and solubilized by agitation at room temperature for 3–4 h. The samples were diluted with cold reaction buffer (Dulbecco's phosphate-buffered saline with 5% BSA and 0.03% Tween-20 supplemented 1X protease inhibitor cocktail) and centrifuged at 16,000 g for 20 min at 4°C. The supernatant was decanted, stored in ice until use, diluted at 1 : 2 with standard diluent buffer, and quantified by calorimetric sandwich ELISA kits. Soluble A β ₄₀ and A β ₄₂ levels were measured in the TBS-soluble fraction and detergent-insoluble A β ₄₀ and A β ₄₂ was measured in the guanidine fraction from hippocampi of all rabbits using kits from Invitrogen (Carlsbad, CA, USA) as per the manufacturer's protocol. The values of A β levels obtained by ELISA were normalized to the amount of protein in the samples. The values were expressed as means $\pm$ standard error. The changes in the levels of A β were considered significant at $p < 0.05$. Levels of A β ₄₀ and A β ₄₂ were expressed as pg/mg of protein.

Western blot analyses

Hippocampi samples were homogenized using tissue protein extraction reagent containing protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA). Protein concentrations were determined by BCA protein assay (Thermo Scientific). Proteins (10 μ g) were separated by SDS-PAGE (10 and 12.5% gels), followed by transfer to a polyvinylidene difluoride membrane (Biorad, Hercules, CA, USA), and were incubated overnight at 4°C with antibodies to amyloid- β protein precursor (A β PP; 1 : 100; Millipore, Temecula, CA, USA); β -secretase (BACE1) enzyme (1 : 100; Millipore); sA β PP α (1 : 100; IBL-America, Minneapolis, MN, USA); total tau (1 : 200; Calbiochem, La Jolla, CA, USA); phosphorylated tau (CP13 and PHF-1, 1 : 500; gift from Dr. Peter Davis, Albert Einstein College of Medicine); GSK-3 β and pTyr²¹⁶GSK-3 β (1 : 250; BD Biosciences, San Jose, CA, USA), a kinase that can phosphorylate tau; tumor necrosis factor (TNF- α , 1 : 100; Abcam, Cambridge, MA, USA); heme oxygenase-1 (HO-1, 1 : 200; Abcam), iron regulatory proteins (IRP-1, 1 : 500; Millipore; IRP-2, 1 : 100; Chemicon International); transferrin receptor (TfR, 1 : 100; Abcam); and ferritin light (FLC) and heavy (FHC) chain (1 : 100;

Santa Cruz Biotechnology). β -Actin (1 : 5000; Santa Cruz Biotechnology) was used as a gel-loading control. The blots were developed with enhanced chemiluminescence (Immun-Star horseradish peroxidase (HRP) chemiluminescence kit; Bio-Rad). The results were quantified by densitometry and represented as total integrated densitometric values. The densitometry analyses for proteins were normalized against their respective β -actins.

Reactive oxygen species assay

ROS generation was determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA) and fluorimetric detection of H_2O_2 as we have described previously [33]. Briefly, for the DCFH-DA assay, protein samples (25 μ g) from hippocampi of all animals were diluted in PBS and incubated with 5.0 μ M DCFH-DA (Sigma, St. Louis, MO, USA) in the dark for 15 min at 37°C. Fluorescence was measured every 15 min for 1 h with excitation and emission wavelengths of 488 and 525 nm, respectively, using a SpectraMax Gemini-EM microplate reader (Molecular Devices, Sunnyvale, CA, USA). Values were expressed as percentage increase in fluorescence compared to controls.

H_2O_2 was measured using the HRP-linked fluorimetric assay (Amplex Ultra red; Invitrogen, Carlsbad, CA, USA). Briefly, protein samples from hippocampus (20 μ g) of control and treated-rabbits was added to a 96-well plate containing 100 μ l of reaction buffer containing 0.1 U/ml HRP, 50 μ M Amplex Ultra red. Resorufin fluorescence was followed using a Spectra-Max Gemini-EM (Molecular Devices) with excitation at 530–560 nm and emission at 590 nm.

Plasma cholesterol levels

Total plasma cholesterol in rabbits was measured after overnight fasting in blood collected from an ear vein immediately before euthanasia. The measurements were carried out using a Flex reagent cartridge and the Dimension clinical chemistry system (Dade Behring, Inc.). Brain cholesterol was shown previously to be unchanged in cholesterol-fed rabbits and therefore was not measured here [31, 32, 40].

Plasma and brain iron levels

Iron levels in plasma were analyzed by direct colorimetric method using Ferene (Multigent Iron Liquid Assay) on the Architect cSystems and the Aeroset System. Iron forms a stable colored complex with ferene and read at 604 and 700 nm as primary and secondary wavelengths. The intensity of the color formed is directly proportional to the amount of iron in the sample. For free iron concentrations in the brain, no fixative or metallic instruments were used to handle the tissue. Specimens from hippocampi were wet-ashed immediately after weighing as previously described [41]. Briefly, the tissue was covered in 300 μ L 30% nitric acid and allowed to incubate overnight. The resulting mixture was heated to 80°C for 20 min then allowed to cool to room temperature for 10 min. Hydrogen peroxide was then added to dissolve lipid components (300 μ L of 10% solution). After 30 min at room temperature, the solution was heated to 70°C for 15 min and allowed to cool. The resulting clear to slightly yellow transparent solutions were stored at –80°C until ready for analysis. Atomic absorption spectra were acquired on a Varian SpectrAA 220Z graphite furnace atomic absorption spectrometer. The standard curve was produced from 25, 50, 75, and 100 parts per billion solutions of standardized iron in nitric acid (Arcos Organics). The instrument was zeroed to a maximum of 0.005 mean absorbance. Samples were diluted 1 : 30 prior to analysis. All values of total iron reported were acquired as the mean of six measurements.

Statistical analysis

The results were analyzed for statistical significance with two-way analysis of variance, using Bonferroni's post hoc test with GraphPad Prism software 4. All values in each group were expressed as the mean values $\pm$ SEM. All group comparisons were considered significant at $p < 0.05$.

RESULTS

Deferiprone reversed cholesterol-induced increase in TBS-soluble and detergent-insoluble A β

The two-way ANOVA revealed a significant interaction between cholesterol and deferiprone treatments for TBS-soluble and detergent-insoluble A β_{40} and A β_{42} levels. The 2% cholesterol-enriched diet induced a significant increase in the levels of both TBS-soluble and detergent-insoluble A β_{40} and A β_{42} in rabbit hippocampus. An increase of about 28% in the levels of TBS-soluble A β_{40} and of 145% in the levels of TBS-soluble A β_{42} were observed in hippocampus of cholesterol-fed rabbits compared to normal chow-fed rabbits (Fig. 1a, c). A 14% and 212% increase were also found in the levels of detergent-insoluble A β_{40} and A β_{42} levels, respectively (Fig. 1e, g). Deferiprone treatment, both at 10 and 50 mg/kg/day, significantly reduced the cholesterol-induced increase in both TBS-soluble and detergent-insoluble A β_{40} and A β_{42} levels (Fig. 1b, d, f, h). At 10 mg/kg/day, deferiprone reduced the cholesterol-enriched diet-induced increase in TBS-soluble A β_{40} and A β_{42} levels by 24% and 66%, respectively; whereas a decrease of 22% and 59% in TBS-soluble A β_{40} and A β_{42} levels were observed in the cholesterol-fed rabbits administered 50 mg/kg/day of deferiprone compared to cholesterol-fed rabbits (Fig. 1b, d). Deferiprone at 10 mg/kg/day reduced the cholesterol-enriched diet-induced increase in detergent-insoluble A β_{40} and A β_{42} levels by 5% and 54%, respectively. At 50 mg/kg/day, deferiprone reduced detergent-insoluble A β_{40} and A β_{42} levels by 9% and 67%, respectively, in comparison to cholesterol-fed rabbit levels (Fig. 1f, h).

A β reduction by deferiprone is associated with a decrease in BACE1 levels

Increased levels of A β_{40} and A β_{42} with high cholesterol diet are accompanied by an increase in the levels of A β PP as well as BACE1 and a decrease in the levels of the non-amyloidogenic sA β PP α (Fig. 2). The two-way ANOVA analysis showed a significant interaction between cholesterol and deferiprone treatments for A β PP as well as BACE1 but not for sA β PP α levels. A 39% increase in A β PP and 42% increase in BACE1 levels, together with a 29% decrease in sA β PP α levels were observed in hippocampi from rabbits fed with the high cholesterol diet compared to control rabbits (Fig. 2a, b, d, f). These results suggest that cholesterol-enriched diet increased A β levels by, at least in part, increasing processing from A β PP through the amyloidogenic pathway. At 10 mg/kg/day, deferiprone did not affect A β PP, increased sA β PP α and reduced BACE1 levels in cholesterol-fed rabbits (Fig. 2a, c, e, g). At 50 mg/kg/day, deferiprone reduced both A β PP and BACE1 levels but did not affect sA β PP α levels (Fig. 2a, c, e, g). On the other hand, deferiprone administration to rabbits fed normal chow did not alter levels of A β , A β PP, sA β PP α , or BACE1 levels (Figs. 1 and 2).

Deferiprone reduces the phosphorylation of tau

Two-way ANOVA analysis showed a significant interaction between cholesterol and deferiprone treatments for phosphorylated but not for total tau levels. The cholesterol-enriched diet induced a significant increase in phosphorylated tau levels in the hippocampus as determined with PHF-1 and CP13, antibodies that detect tau phosphorylation at Ser^{396/404} and Ser²⁰² respectively (Fig. 3a, b, d). Deferiprone at 10 mg/kg/day did not affect tau

phosphorylation at Ser^{396/404} but significantly decreased tau phosphorylation at Ser²⁰². At 50 mg/kg/day, deferiprone significantly reduced the cholesterol-enriched diet-induced increase in phosphorylation of tau at both Ser^{396/404} and Ser²⁰² sites (Fig. 3a, c, e). No significant alterations in total tau levels have been observed in the hippocampus of rabbits fed with cholesterol-enriched diet and deferiprone treated to cholesterol-fed rabbits (Fig. 3a, f, g). In normal chow-fed rabbits, deferiprone administration at 10 or 50 mg/kg/day did not alter total or phosphorylated tau levels (Fig. 3a–g).

GSK-3 β is an enzyme that plays an active role in the phosphorylation of tau and is suggested to mediate tau hyperphosphorylation in humans with AD. Two-way ANOVA analysis showed a significant interaction between cholesterol and deferiprone treatments for active pTyr²¹⁶ GSK-3 β but not for GSK-3 β levels. Our results show that the cholesterol-enriched diet increased levels of the active pTyr²¹⁶ GSK-3 β (Fig. 4a, b) and deferiprone treatment significantly reduced active pTyr²¹⁶ GSK-3 β levels at 50 mg/kg/day but not at 10 mg/kg/day (Fig. 4a, c). No significant changes were observed in levels of total GSK-3 β following deferiprone treatments in rabbits fed normal chow or cholesterol-enriched diet (Fig. 4a, d, e).

Deferiprone does not reduce levels of ROS

ROS generation causes oxidative stress, a process that is involved in various diseases including AD. No significant interaction was found between cholesterol and deferiprone treatments for ROS and H₂O₂ production in the present study. Cholesterol-enriched diet increased levels of ROS by 37% and H₂O₂ by 62% (Fig. 5a, c). Deferiprone, at both concentrations, did not prevent or reduce the cholesterol-enriched diet-induced increase in ROS and H₂O₂ levels (Fig. 5b, d). Additionally, deferiprone administration to rabbits fed normal chow did not alter basal levels of ROS and H₂O₂.

Deferiprone reduced cholesterol-enriched diet-induced disturbances in iron-regulatory proteins

Disturbances in iron metabolism has been suggested to mediate the neurodegenerative processes that characterize AD [42–46]. We determined levels of transferrin receptor (TfR) which regulate iron uptake, ferritin light (FLC) and heavy (FHC) chains that regulate iron storage, and the iron-regulatory proteins IRP-1 and IRP-2 which are RNA-binding proteins that control iron metabolism by binding to conserved RNA motifs known as “iron-responsive elements” (IREs). A significant interaction was observed between cholesterol and deferiprone treatments for TfR, IRP-2, FLC, and FHC but not for IRP-1. Cholesterol-enriched diet reduced TfR as well as IRP-2 levels (Fig. 6a, b, f) and increased levels of FLC and FHC (Fig. 7a, b, d). Treatment with 10 mg/kg/day of deferiprone increased levels of TfR as well as IRP-2 (Fig. 6a, c, g) and reduced both FLC and FHC levels (Fig. 7a, c, e) in cholesterol-fed rabbits. At 50 mg/kg/day, deferiprone did not alter TfR or IRP-1 but reduced IRP-2 as well as FLC and FHC levels in cholesterol-fed rabbits (Fig. 6a, c, e, g; Fig. 7a, c, e). Treatment with 10 or 50 mg/kg/day of deferiprone did not alter TfR, IRP-1, IRP-2, FLC, or FHC in rabbits fed normal chow (Figs. 6 and 7).

Deferiprone reduced HO-1 and TNF- α levels

HO-1 is an oxidative stress sensor that is increased in response to stress conditions. Induction of HO-1 by oxidant stress usually accompanies increase in the levels of ferritin [47]. A significant interaction was found between cholesterol and deferiprone treatments for HO-1 and TNF- α . Our results show that cholesterol-enriched diet increased HO-1 levels and this increase was reversed by treatment with deferiprone at 10 and 50 mg/kg/day (Fig. 8a–c). Activation of TNF- α is a marker of inflammation and has been shown to induce the expression of ferritin in a variety of cell lines [48] and thus could dysregulate iron

homeostasis. We determined the level of TNF- α which was significantly increased in the cholesterol-enriched diet group compared to controls (Fig. 8a, d). Deferiprone at 10 or 50 mg/kg/day significantly reduced the cholesterol-enriched diet-induced increase in TNF- α levels (Fig. 8a, e). Deferiprone administration to rabbits fed normal chow did not alter expression levels of HO-1 or TNF- α (Fig. 8).

Deferiprone reduced plasma iron and cholesterol levels

High cholesterol diet significantly increased plasma cholesterol levels but not plasma or brain iron levels (Table 2). At both concentrations, deferiprone reduced plasma cholesterol levels induced by the cholesterol-enriched diet. Cholesterol plasma levels in rabbits fed normal chow were not affected by deferiprone treatments. On the other hand, deferiprone at 10 or 50 mg/kg/day reduced plasma iron levels in cholesterol-fed rabbit but did not affect iron levels in brains of cholesterol or normal chow-fed rabbits (Table 2).

DISCUSSION

In the present study, we showed that cholesterol-enriched diet increases A β levels, tau phosphorylation, and oxidative stress in rabbit hippocampus. The increase in A β was associated with increased A β PP and BACE1 levels, suggesting that the cholesterol-enriched diet enhanced the amyloidogenic pathway by promoting the turnover of A β PP by BACE1, thereby increasing A β production. Increased tau phosphorylation was associated with increased levels of p-Tyr²¹⁶ GSK3 β , the active form of GSK-3 β , an enzyme which mediates tau phosphorylation in AD. In addition to increased A β production and tau phosphorylation, the cholesterol-enriched diet increased ROS generation and disturbed iron-regulatory protein levels.

We demonstrated in this study that the iron chelator deferiprone reduced levels of both TBS-soluble and detergent-insoluble A β ₄₀ and A β ₄₂ in cholesterol-fed rabbits. The cellular mechanisms by which deferiprone regulate A β levels are unclear. We found that deferiprone reduced BACE1 at both 10 and 50 mg/kg/day in cholesterol-fed rabbits. This suggests that reduction in BACE1 likely plays a role in the mechanism of deferiprone effects on A β levels. Additionally, deferiprone increases sA β PP α at 10 mg/kg/day and reduces A β PP at 50 mg/kg/day in hippocampus of cholesterol-fed rabbits. The increase in sA β PP α and reduction of A β PP levels may also contribute, in addition to BACE1 reduction, to the decrease in A β levels. A β PP is tightly linked to iron metabolism. A β PP mRNA has an IRE in the 5'-untranslated region with sequence homology to the IRE for TfR and ferritin. IRPs bind to A β PP IRE and regulate A β PP translation as they do for ferritin. This translation effect has been shown to be selectively down-regulated in response to intracellular iron chelation [49, 50]. It may be possible that the reduced A β PP levels in the 50 mg/kg/day deferiprone-treated group could be due, at least in part, to the effects of IRP-2 as our results show reduced levels of this IRP in cholesterol-fed rabbits treated with 50 mg/kg/day deferiprone.

Tau is mostly a neuronal protein, almost 20% of which can be phosphorylated because of its serine, threonine, and tyrosine rich sequences [51, 52]. Numerous protein kinases have been suggested to phosphorylate tau, however, the signaling processes that activate these protein kinases and cause tau phosphorylation are not well known. A fibrillogenic form of tau is formed when tau is phosphorylated at Ser³⁹⁶ and Ser⁴⁰⁴ [53], and phosphorylation at Ser⁴²² promotes tau filaments [52]. Phosphorylation of tau at Ser²⁶² (and Ser²¹⁴) decreases the affinity of tau for microtubules and inhibits polymerization of tau into filaments [54]. Regarding its effects on tau phosphorylation, deferiprone 10 mg/kg/day did not affect tau phosphorylation at Ser^{396/404} but significantly reduced tau phosphorylation at Ser²⁰². At 50 mg/kg/day, deferiprone significantly decreased tau phosphorylation at both Ser^{396/404} and

Ser²⁰² sites. We also showed that reduction in tau phosphorylation by deferiprone at 50 mg/kg/day, but not 10 mg/kg/day, is associated with reduced levels of active pTyr²¹⁶-GSK-3 β . These latter results suggest that GSK-3 β is not the only enzyme that phosphorylates tau in the cholesterol-fed rabbits. Phosphorylation of Tyr²¹⁶ increases the catalytic activity of GSK-3 β , which is required for biological function [55]. GSK-3 β and casein kinase 1 δ can phosphorylate Ser²⁵⁸, Ser²⁶², Ser²⁸⁹, and Ser³⁵⁶ sites of phosphorylation present at the microtubule binding repeat region in PHF-tau [56]. Co-localization of phospho-Tyr²¹⁶ GSK-3 β and phospho-tau epitopes has been observed in a double transgenic mice obtained by crossing P25-overexpression mouse with FTDP-17 P301L-mutant tau [52]. A study by Sperber et al. [57] reported that transient transfection of human GSK-3 β in Chinese hamster ovary cell caused an increase in tau phosphorylation at Ser²⁰², Ser³⁹⁶, Ser⁴⁰⁴, Thr¹⁸¹, and Thr²³¹ sites.

Iron overload in the brain has been suggested to increase the risk for AD. Interestingly, our data show that the cholesterol-enriched diet did not increase plasma or brain levels of free iron in rabbit hippocampus. These results suggest that the cholesterol-enriched diet disturbed iron metabolism rather than affecting total iron concentration. However, it may be possible that although the total iron concentration is unchanged, the cholesterol-enriched diet altered cellular and/or compartmental distribution of iron. A population-based cohort study demonstrated that the risk of developing AD is much greater in patients with elevated cholesterol and iron than in patients having either high cholesterol or elevated iron levels [58]. However, a recent meta-analysis study found little evidence of increased iron in AD [59].

Iron homeostasis in cells is maintained by interactions of IRPs, ferritin, transferrin, and transferrin receptor proteins. Disturbances in IRPs can induce cellular damage. Circulating iron bound to transferrin is transported into cells by transferrin receptor and is stored by ferritin in cells [60–63]. The levels of iron in the cells are sensed by IRPs [64–69]. We show here that high cholesterol diet reduces transferrin receptors and IRP-2 but not IRP-1 levels and increases levels of both ferritin L and H chain. Reduced transferrin receptor and increased ferritin levels were observed in AD brains compared to matched-control brains [70]. Alterations in the IRP-2 but not IRP-1 localization were also reported in AD [71]. Ferritin L and H chains levels can be modulated by a variety of factors that generate oxidative stress and preferentially affect the L or H chain in hippocampus [72]. Treatment with deferiprone increases levels of transferrin receptors as well as IRP-2 at 10 mg/kg/day and reduces these levels at 50 mg/kg/day. The mechanisms behind the opposite effects of deferiprone at the low and high doses are unclear. Treatment with deferiprone on the other hand reduced the cholesterol-induced increase in ferritin L and H chains at both doses.

HO-1 is an enzyme that catalyzes the degradation of heme. HO-1 is an inducible isoform that functions as a sensor of oxidative stress and has been shown to also have anti-inflammatory properties [73]. Cholesterol is known to induce inflammation [74] so we speculate that HO-1 induction in the present study may be an anti-inflammatory response to increased cholesterol levels. The effect of deferiprone on HO-1 may be mediated by reduced plasma cholesterol. Induction of HO-1 by oxidative stress is usually accompanied by an increase in ferritin [47]. We showed here that increased levels of ferritin are associated with increased levels of HO-1 in the hippocampus in the cholesterol-enriched group. Deferiprone at both doses reduced both ferritin and HO-1 levels. Activation of the inflammation marker TNF- α may potentially contribute to dysregulation of iron homeostasis as TNF- α has been shown to induce the expression of ferritin in a variety of cell lines [48, 75]. We show here that cholesterol-enriched diet also increased levels of TNF- α and deferiprone at both doses reversed the cholesterol-induced increase in TNF- α levels.

In addition to A β accumulation, tau phosphorylation, and iron dyshomeostasis, oxidative stress is an important hallmark of AD and may represent an early event that triggers AD pathology [76–79]. Oxidative stress can be estimated by several markers including ROS generation and increased intracellular H₂O₂ levels. The high cholesterol diet increased the production of ROS and H₂O₂; treatment with deferiprone at 10 or 50 mg/kg/day failed to reduce the diet-induced increase in levels of ROS and H₂O₂. It is intriguing that deferiprone did not prevent the cholesterol-enriched-diet induced ROS and H₂O₂ overproduction as this agent has been shown to reverse oxidative damage in iron-loading and non-iron-loading conditions. It may be possible that doses higher than 50 mg/kg/day are necessary to fully reduce oxidative stress in the cholesterol diet-fed rabbits as plasma cholesterol levels remained more than ten times higher in the 50 mg/kg deferiprone treated, high-cholesterol group than in controls. Deferiprone has a low molecular weight, is known to penetrate the cells [80], crosses the blood brain barrier in less than 7 minutes after systemic administration [81], yields a peak plasma concentration one hour after oral administration [36], and its cellular uptake is superior to that of deferoxamine [82], another widely known metal chelator.

The mechanisms by which cholesterol-enriched diet triggers AD pathology are currently unclear as plasma but not brain concentrations of cholesterol are increased in rabbits fed the cholesterol diet [31, 32]. Furthermore, the extent to which oxidative stress, iron dyshomeostasis, A β , and tau phosphorylation are functionally linked and the order at which they are generated by the cholesterol-enriched diet are still to be determined. Our results show that iron chelation with deferiprone was sufficient to reduce soluble A β overproduction, decrease tau phosphorylation, and corrected levels of IRPs but did not affect ROS generation. These results suggest that ROS formation may emanate, at least in large part, from pathways independent of iron dyshomeostasis or A β and phosphorylated tau accumulation. Although there was an increase in the levels of iron in the plasma of rabbits fed with high cholesterol diet, this increase was not statistically significant; both cholesterol-treated groups exposed to the chelator had significantly reduced plasma iron levels compared to the cholesterol-treated group. Brain levels of total iron were unaltered by the cholesterol-enriched diet. Our data demonstrate that the protective effects of deferiprone are likely independent from reducing iron levels in the brain. It may be possible that the deferiprone effects are related to reductions in plasma cholesterol and plasma iron and maintenance of IRP homeostasis. In fact, a previous study showed that deferiprone reduced atherogenesis by reducing total plasma cholesterol, LDL, and VLDL in rabbits fed a cholesterol-enriched diet [80]. However, the mechanism by which deferiprone reduces cholesterol levels is unclear.

In summary, our data shows that treatment with the iron chelator deferiprone opposes several pathological events induced by a cholesterol-enriched diet in rabbit hippocampus. Deferiprone reduced the generation of A β and lowered levels of tau phosphorylation. In addition, deferiprone prevented dysregulation of IRPs and reduced the increase in levels of TNF- α , an inflammation marker and contributor to iron dyshomeostasis. Nevertheless, at concentrations that prevent the formation of the pathological hallmarks of AD, deferiprone failed to reduce levels of ROS and intracellular H₂O₂ levels subsequent to cholesterol-enriched diet feeding to rabbits. Oxidative stress in cholesterol-fed rabbit may result from pathways independent from increased A β and tau phosphorylation. It is possible that a higher dose of deferiprone, or combination therapy of deferiprone together with an antioxidant to prevent ROS generation would more-fully protect against the deleterious effects of cholesterol-enriched diet that are relevant to AD pathology.

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Fig. 1.

ELISA shows that levels of TBS-soluble A β_{40} (a, b) and A β_{42} (c, d) as well as detergent-insoluble A β_{40} (e, f) and A β_{42} (g, h) are increased in hippocampi of rabbits fed a 2% cholesterol-enriched diet for 12 weeks. Treatment with 10 mg/kg/day or 50 mg/kg/day deferiprone in drinking water for 12 weeks reduced levels of both TBS-soluble and detergent-insoluble A β_{40} and A β_{42} in cholesterol-fed rabbits (a–h). * $p < 0.05$; *** $p < 0.001$.

Fig. 2. Western blots (a) and optical density (OD) of AβPP (b, c), sAβPPα (d, e), and BACE1 (f, g) in rabbit hippocampus. Feeding rabbits a 2% cholesterol-enriched diet increased AβPP and BACE1 and reduced sAβPPα levels. Treatment with deferiprone reduced AβPP at 50 mg/kg/day increased sAβPPα at 10 mg/kg/day and reduced BACE1 at both 10 and 50 mg/kg/day in hippocampus of cholesterol-fed rabbits. Deferiprone treatments did not alter AβPP, sAβPPα, or BACE1 levels in control rabbit brains. * $p < 0.05$; ** $p < 0.01$.

Fig. 3.

Representative western blots (a) and optical density of phosphorylated tau, as detected with anti-PHF-1 (b, c) and anti-CP13 (d, e) antibodies, as well as total tau (f, g). The cholesterol-enriched diet increased phosphorylated tau levels, and while deferiprone at 10 mg/kg/day did not reduce tau phosphorylation at Ser^{396/404} (as detected with PHF-1 antibody), there was a significant decrease in tau phosphorylation at Ser²⁰² (as detected with CP13 antibody). Deferiprone at 50 mg/kg/day significantly decreased tau phosphorylated at both sites in cholesterol-fed rabbit hippocampus. Total tau levels were unchanged in all animal groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 4. Western blot (a) and optical density of pTyr²¹⁶ GSK-3β (b, c) and GSK-3β (d, e). Cholesterol-enriched diet increased the ratio pTyr²¹⁶ GSK-3β: GSK-3β. Deferiprone treatment at 50 mg/kg/day, but not at 10 mg/kg/day, significantly reduced the cholesterol-induced increase in pTyr²¹⁶ GSK-3β: GSK-3β ratio. Levels of GSK-3β were not affected by cholesterol feeding or deferiprone treatments (d, e). **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

Fig. 5. Levels of reactive oxygen species (ROS) (a, b) and H_2O_2 (c, d) in rabbit hippocampus. High cholesterol diet increased levels of ROS, including H_2O_2 levels. Deferiprone treatment at either concentration did not have any significant effect on the levels of ROS or H_2O_2 in control or cholesterol-fed rabbits. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 6. Western blot (a) and optical density (b–g) of the iron-regulatory proteins transferrin receptor (TfR), iron-regulatory protein-1 (IRP-1), and iron-regulatory protein-2 (IRP-2) in rabbit hippocampus. Cholesterol-enriched diet significantly reduced TfR (a–c) and IRP-2 levels (a, f, g). Treatment with deferiprone reversed the effect of cholesterol diet on TfR (a–c) and IRP-2 (a, f, g) at 10 mg/kg/day. Deferiprone at 50 mg/kg/day did not prevent the decrease in TfR but reduced IRP-2 levels. No changes were observed in IRP-1 levels in cholesterol or deferiprone treated rabbits (a, d, e). ** $p < 0.01$; *** $p < 0.001$.

Fig. 7. Western blots (a) and optical density (b–e) of ferritin light chain (FLC) and ferritin heavy chain (FHC) in rabbit hippocampus. Cholesterol-enriched diet increased levels of FLC (a–c) as well as FHC (a, d, e). Treatment with deferiprone reversed the effect of cholesterol diet on FLC and FHC at 10 and 50 mg/kg/day. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 8. Western blots (a) and optical density of HO-1 (b, c) and TNF-α (d, e) in rabbit hippocampus. Cholesterol-enriched diet increased levels of both HO-1 and TNF-α and treatment with deferiprone at 10 or 50 mg/kg/day reversed the effects of the cholesterol-enriched diet on HO-1 and TNF-α levels. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 1Rabbits groups ($n = 9$ per group) and corresponding treatments

Groups	Treatment
Group 1	Control (normal chow)
Group 2	2% (w:w) cholesterol-enriched diet
Group 3	2% Cholesterol diet + 10 mg/kg/day deferiprone in drinking water
Group 4	2% Cholesterol diet + 50 mg/kg/day deferiprone in drinking water
Group 5	Normal chow + 10 mg/kg/day deferiprone in drinking water
Group 6	Normal chow + 50 mg/kg/day deferiprone in drinking water

Table 2

Plasma cholesterol and iron levels and hippocampus iron levels of rabbits fed normal chow or a 2% cholesterol-enriched diet and receiving or not deferiprone in drinking water for 12 weeks

	Plasma cholesterol (mg/dl)	Plasma iron (μ g/dl)	Hippocampus iron (μ g/g tissue)
Control	57.75 $\pm$ 20.0	235.5 $\pm$ 6.5	23.3 $\pm$ 5.3
Cholesterol	1050 $\pm$ 115 ***	261.3 $\pm$ 15.2	24.7 $\pm$ 1.7
Cholesterol + deferiprone 10 mg/kg/day	650 $\pm$ 136.7 ***##	194.3 $\pm$ 17.9 [#]	22.6 $\pm$ 2.4
Cholesterol + deferiprone 50 mg/kg/day	640 $\pm$ 56 ***##	191.8 $\pm$ 13.7 [#]	23.4 $\pm$ 3.3
Deferiprone 10 mg/kg/day	54 $\pm$ 6	220.7 $\pm$ 8.0	28.7 $\pm$ 4.4
Deferiprone 50 mg/kg/day	48 $\pm$ 11	194 $\pm$ 12.0	27.2 $\pm$ 4.4

Measurements in hippocampus are normalized to tissue wet weight.

 $p < 0.001$ versus control;

[#]
 $p < 0.05$ and

 $p < 0.01$ versus cholesterol.