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Original Research

Pyridoxine exerts antioxidant effects in cell model of Alzheimer's disease via the Nrf-2/HO-1 pathway

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Abstract: Pyridoxine is a water- soluble pyridine derivative. The effect of pyridoxine in cell models of Alzheimer's disease (AD), and the potential mechanisms involved, are not fully understood. In this study, the anti-AD effects of pyridoxine were studied in an AD cell model using a combination of techniques viz MTT assay, western blotting and assays for reactive oxygen species (ROS). Assays were also carried out to determine the mechanism underlying the antioxidant effects of pyridoxine. The results obtained revealed that pyridoxine exerted a protective potential against AD, attenuated ROS levels, decreased the expressions of cytoplasmic Nrf2, and upregulated whole-cell HO-1 expression. These results suggest that the anti-AD effect of pyridoxine may be attributed to its anti-oxidant property elicited via stimulation of the Nrf2/HO-1 pathway.

Key words: Pyridoxine; Alzheimer's disease; Reactive oxygen species; Anti-oxidation; Nrf2.

Introduction

Alzheimer's disease (AD) is a neurodegenerative syndrome that results in progressively increasing poor memory, spatial disorientation, and dramatic slowdown in intellectual potency. These symptoms are the primary causes of dementia and disabilities in old age, which impose enormous socio-economic burdens on many countries with rapidly growing elderly populations (1). Although the pathogenesis of this disease is fairly well understood, several competing hypotheses have been proposed. These include amyloid-beta (Aβ) protein accumulation, cholinergic neurotransmitter deficiency, neurofibrillary tangles (NFTs) of tau proteins, increased inflammatory response, elevated reactive oxygen free radicals, and disordered ion dynamic equilibrium.

Increasing evidence indicate that excess oxidative stress is an important event in the etiology of neurodegenerative diseases, including AD (2, 3). As products of aerobic metabolism, the levels of reactive oxygen species (ROS) are controlled by the endogenous antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) (4, 5, 6). Under normal physiological conditions, low ROS function in a transitory manner as signaling molecules. However, excess ROS causes peroxidation, leading to severe oxidative damage to DNA, phospholipids, and proteins. These damages can reduce the oxidase potency of mitochondrial cytochrome C and result in metabolic disturbance and cell apoptosis (7). Consequently, considerable research interest has been focused on the suppression of oxidative stress as a novel strategy for prevention and treatment of AD (8, 9).

The multifunctional regulator nuclear factor erythroid 2-related factor (Nrf2) is considered as a cytoprotective factor regulating the expression of genes coding for anti-oxidant, anti-inflammatory and detoxifying proteins, and heme oxygenase-1 (HO-1), which besides removing toxic heme, produces biliverdin, iron ions and carbon monoxide, exerts beneficial effects through the protection against oxidative injury (10-12). As a result, Nrf2/HO-1 signaling pathway was a key field for anti-oxidation. However, the current anti-oxidative drugs used for AD patients have not achieved satisfactory efficacies in clinical trials. Thus, attention is shifting towards natural products as hopeful sources of drug candidates for treating AD (13, 14).

Pyridoxine (PYR, Figure 1A) is derived from pyridoxal phosphate, the biologically active form of vitamin B6 which is a coenzyme for several amino acid-metabolizing enzymes necessary for vital physiological processes such as immunity and growth. Pyridoxine is crucial for gluconeogenesis, biosynthesis of niacin, and one-carbon metabolism (15). Moreover, studies have demonstrated that PYR has antioxidant properties (16). The present study was designed to investigate the inhibitory potency of PYR administration on the development of AD-related pathologies in HEK293/20E2 cell model of AD.

Materials and Methods

Cell culture and chemical reagents

Normal neuroblastoma SH-SY5Y cells were obtained from Shanghai Cell Bank of the Chinese Academy of Sciences. Stable amyloid precursor protein (APP) gene bearing the Swedish mutation-expressing HEK293/20E2 cells were donated by Professor Xiulian Sun of Shandong University. The cells were grown in Dulbecco's modified eagle medium with 5% heat-inac-

tivated fetal bovine serum (FBS) at 37°Cin an atmosphere of 5% CO_2. In addition, G418 (Geneticin, Sigma) was added to the HEK293/20E2 culture medium at a concentration of 1μL G418/mL to screen the cells, but G418 was not used in the experiment to avoid affecting the final results.

Pyridoxine (30 mg; Sigma-Aldrich, St. Louis) was dissolved in 10 mL of normal saline used for dilutions. The activities of CAT, SOD and GSH-Px were determined with appropriate assay kits provided by Jiancheng Institute of Bioengineering (Nanjing, China).

Drug treatment Groups

Four treatments were conducted on cells in this study. The normal control (NC) group consisted of SH-SY5Y cells without any treatment, while un-treated HEK-293/20E2 cells served as model group. The treatment groups comprised two HEK-293/20E2 cell groups given PYR at two separate doses (0.5 and 3 μg/ml) for 48 h.

Cytotoxicity Assay by MTT

The anti-proliferative effect of PYR on SH-SY5Y and HEK-293/20E2 cells was determined with MTT assay. At logarithmic phase, the cells were incubated in 96-well (1×10^4 /well) for 48 h with different levels of PYR. After 44 h, 2.5 mg/mL solution of MTT (10 μL) was put into each well, and incubation was continued for the next 4 h, after which the resultant formazan crystals were recovered by centrifugation at 1500 rpm for 15 min. The crystals were solubilized in DMSO (150 μL) and their absorbance values were read at 490 nm in a Thermo microplate reader. Thereafter, the IC_{50} values of PYR for SH-SY5Y and HEK-293/20E2 cells were computed using San Diego GraphPad Prism 6.0 software. Experiments were conducted thrice for each concentration of PYR.

Reactive oxygen species assay

Cellular levels of ROS were assayed using ROS Assay kits (Beyotime Inst. Biotech., Nanjing). Cells in the various groups were exposed to 10 μM of dichloro-dihydro-fluorescein diacetate (DCFH-DA) at 37 °C. After 20 min, the cells were rinsed thrice in PBS, and examined using fluorescence microscopy at excitation and emission wavelengths of 488 nm and 525 nm, respectively. The cells were also subjected to flow cytometric analysis after rinsing them twice in PBS.

Assay of GSH-Px, SOD and CAT activities in AD cells

After 48 h-treatment, the cells were lysed and centrifuged for 10 min at 2500 rpm. Following centrifugation at 2500 rpm for 10 min, the supernatant was assayed for the activities of GSH-Px, SOD and CAT using their respective ELISA kits in line with the manufacturer's protocol.

Separation of nuclear protein and cytoplasmic protein

Based on the instructions of the protein separation kits (Beyotime Biotechnology), PMSF was added to appropriate cytoplasmic protein extraction reagent A and the nuclear protein extract reagent before use, so that the final concentration of PMSF was 1nM. After 48 -h treatment, the medium was discarded, and the cells were washed once with pre-warmed PBS. The cells were scraped down, transferred to the EP tube, and centrifuged for 5 min at 800 rpm at room temperature. The supernatant was discarded, and the cells in the EP tube were fully dispersed with high speed vortexing for 10 sec. The EP tube was placed in ice bath for 15 min, and l0 uL extractive reagent B was added, followed by high speed vortexing for 10 sec. The tube was placed in ice bath for 60 sec, and then centrifuged at 12,000 – 16,000g for 5 min at 4°C. The supernatant from the EP tube which was the target cytoplasmic protein was immediately quantitatively transferred into another pre-cooled EP tube, and 50 μL nuclear protein extraction reagent was added, along with a small amount of PMS. The tube contents were vortexed at high velocity for 20 seconds for full dispersion. Then, the EP tube was put back into the ice bath, and vortexed for 20 sec every 2 min for 30 min. Finally, the tube was spun at 12,000 - 16000g for 10 min at 4°C, and the supernatant from the EP tube which was the target nuclear protein was immediately transferred into another pre-cooled EP tube.

Western blot assay

Cells in the various groups were harvested after the respective treatments, and lysed. The total protein contents of the cell lysates were determined with bicinchoninic acid (BCA) protein assay kit. The primary antibodies for APP, Nrf2, HO-1 and β-actin were bought from Cell Signalling Technology (Beverly, MA, USA), with β-actin as internal reference. The total protein extract was subjected to gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were adsorbed on polyvinylidene difluoride membrane, and blocked with 5 % non-fat milk at room temperature for 1 h in TBST buffer, pH 8. Following overnight incubation with primary antibody at 4 °C, the membrane was rinsed thrice with TBST buffer prior to incubation with the secondary antibody for 2 h. Thereafter, the membrane was rinsed three times in TBST buffer, and the resultant protein bands were detected using enzyme-linked chemiluminescence kits according to the kit instructions. Band intensity was quantified with ImageJ and protein expression was estimated as target protein band intensity relative to that of β-actin.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences between means were analyzed with one-way ANOVA and Tukey's post hoc test using GraphPad Prism 6.0 software. Values of $p < 0.05$ were assumed to indicate statistically significant differences.

Results

Expressions of APP and $A\beta_{1-40}$ proteins in SH-SY5Y and HEK293/20E2 cells

The expressions of $A\beta_{1-40}$ and APP proteins in SH-SY5Y and HEK293/20E2 cells were first compared, to establish the pathological features of AD in the cell model of AD used (17, 18). As shown in Figure 1B, the protein expression of $A\beta_{1-40}$ in the HEK293/20E2 group

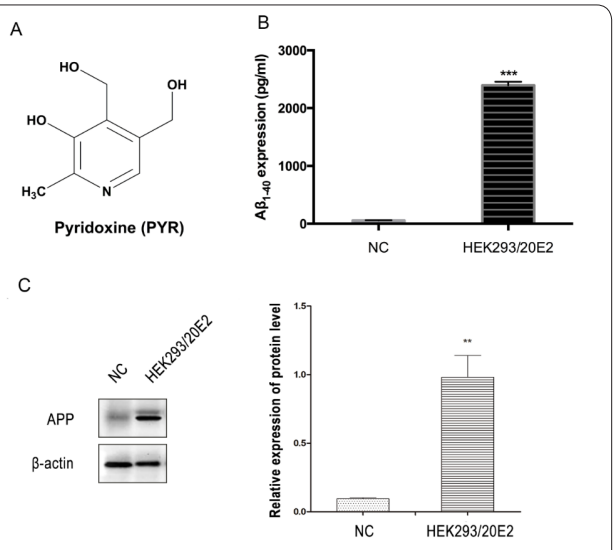

Figure 1. (A) Structure of pyridoxine; **(B)** expression of $A\beta_{1-40}$ in the two cell lines; **(C)** relative expression of APP. Values are mean ± SD (n = 3, one-way analysis of variance followed by Tukey's multiple range test), $^{**}p < 0.01$, $^{***}p < 0.001$, compared with NC.

(2392.71 ± 63.49 pg/mL) was significantly higher ($p < 0.01$) than that in the NC group (55.28 ± 4.81pg/mL), indicating that the expression of the APP downstream product $A\beta_{1-40}$ was increased in HEK293/20E2 cells. The APP expression in HEK293/20E2 cells (0.98 ± 0.19) was significantly higher than that in SH-SY5Y (0.13 ± 0.01; $p < 0.01$), implying that the APP protein expression was stable in HEK293/20E2 cells. Thus, HEK293/20E2 was employed as a model of AD (Figure 1C).

In vitro cytotoxicity of PYR against HEK293/20E2 cells

The MTT method was utilized in assessing the cytotoxicity of PYR on the two cell lines. Figure 2 shows that 48-h incubation with PYR had no obvious inhibitory effects on the two cell lines even at the high dose of 64 μg/ml, indicating that PYR concentration of 0.5 or 3 μg/ml was suitable for subsequent experiments.

Effects of PYR on reactive oxygen species

There was a marked increase in green fluorescence in HEK293/20E2 cells prior to treatment with PYR (Figure 3A). However, with PYR treatment, the fluorescence intensity decreased dose-dependently and significantly. Flow cytometric analysis showed that the relative content of ROS after treatment with 0.5 or 3μg/ml of PYR decreased significantly, when compared with the AD model cells (Figure 2B). There was consistency

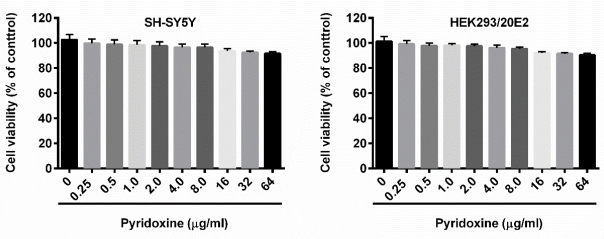

Figure 2. Anti-proliferative effects of PYR against SH-SY5Y and HEK293/20E2 cells at doses (0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 and 64 μg/ml). Values are mean ± SD (n = 3).

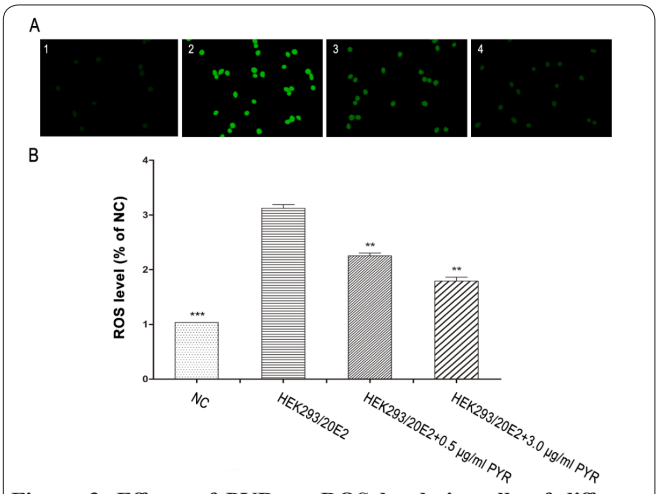

Figure 3. Effects of PYR on ROS levels in cells of different groups. **(A)** ROS images visualised from fluorescence microscopy (x200), 1: NC; 2: HEK293/20E2; 3: HEK293/20E2 + 0.5μg/ml PYR; 4: HEK293/20E2 + 3.0 μg/ml PYR; **(B)** Quantitative analysis of the level of ROS by flow cytometry. Values are mean ± SD (n = 3, one-way analysis of variance followed by Tukey's multiple range test). $^{**}p < 0.01$, compared with HEK293/20E2, $^{***}p < 0.001$, compared with HEK293/20E2.

between the results from flow cytometric analysis and those from the fluorescent images, indicating the ability of PYR to inhibit ROS production in HEK293/20E2 cells.

PYR inhibited SOD, CAT, and GSH-Px activities in HEK293/20E2

There was significant inhibition of CAT, SOD, and GSH-Px activities in HEK293/20E2 cells, when compared with SH-SY5Y cells. However, these inhibitions were significantly and dose-dependently reversed by PYR administration, when compared with corresponding activities in untreated HEK293/20E2 cells ($p < 0.01$, $p < 0.001$; Figures 4A - 4C).

Effect of PYR on the expressions of Nrf-2 and HO-1

The cytoplasmic expression of Nrf2 in HEK293/20E2

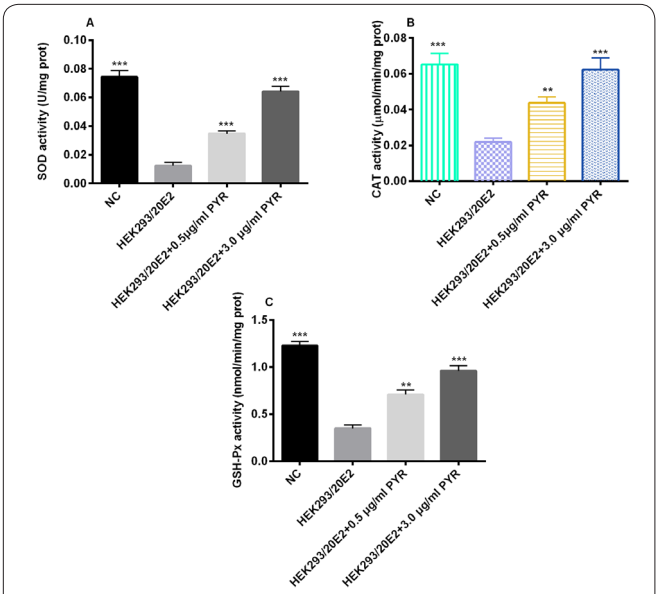

Figure 4. Effect of PYR on activities of antioxidant enzymes in the various groups, as assessed by ELISA. Data are mean ± SD (n = 5, one-way analysis of variance followed by Tukey's multiple range test). $^{*}p < 0.01$, $^{***}p < 0.001$, compared with AD model.

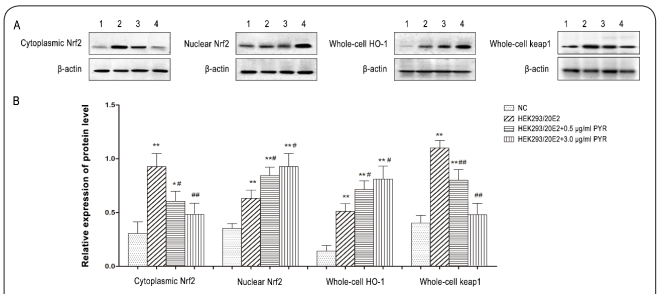

Figure 5. (A) Expression of cytoplasmic and nuclear Nrf2, whole-cell HO-1 whole-cell Keap1 in the various groups 1: NC; 2: HEK293/20E2; 3: HEK293/20E2 + 0.5μg/ml PYR; 4: HEK293/20E2 + 3.0 μg/ml PYR; (B) quantitative analysis of Nrf2 and HO-1, with β-actin as internal control. Data are mean ± SD (n = 3, one-way analysis of variance followed by Tukey's multiple range test). $^{**}p < 0.01$, compared with NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with model group.

cells was significantly decreased, relative to the AD model group, while nuclear Nrf2 expression showed a reversal of this trend. As the negative regulator of Nrf2, the whole-cell Keap1 expression was decreased significantly after PYR treatment relative to AD model control cells. When compared with model cells, whole-cell HO-1 expression in low or high dose-PYR-treated AD model cells increased significantly.

Discussion

Under normal conditions, α- secretase catalyzes the conversion of p-amyloid precursor protein (APP) to soluble APP peptide, which is then further decomposed by γ-secretase (19). However, under pathological conditions, mutation in the APP gene makes the APP protein more easily degraded by β-secretase and -secretase, resulting in the production of insoluble β-amyloid (amyloid peptide, Aβ) (20). In this study, the AD cell model used was HEK293 cells carrying the Swedish mutant APP gene, which produces a large number of APP protein, relative to normal nerve cells (21). These APPs are highly susceptible to hydrolysis into insoluble Aβ peptide. The HEK293/20E2 cells used are characterized by significantly increased levels of APP protein, $A\beta_{1\text{-}40}$ protein, and oxidative stress, when compared with normal neuroblastoma cells SH-SY5Y. These cells are ideal models for studying the mechanistic processes involved in AD.

Increasing attention has been focused on the beneficial effect of fighting oxidative stress on AD development, especially the effect of diverse natural nutrient products (22, 23). As a result, the present study investigated the effect of pyridoxine on AD cells. An MTT assay was carried out first, to assess the cytotoxicity of PYR on both cell lines. Even at the highest concentration, 48-h PYR exposure had almost no effect on the viabilities of these cells.

Increased production of free radicals and decreased endogenous antioxidants may accelerate membrane phospholipid breakdown, leading to lipid peroxidation and cellular dysfunction. Therefore, the contents of ROS indirectly reflect the levels of free radicals (24-26). In this study, ROS level was markedly reduced by PYR treatment in AD model cells as indicated in results from fluorescence images and flow cytometric analysis. These results demonstrate that PYR ameliorated oxidative stress in HEK293/20E2 cells. Physiological antioxidants are present in several tissues, including brain tissues. These include CAT, SOD, and GSH-Px, which scavenge ROS, thereby protecting brain neurons from oxidative damage (27). The results obtained in this study showed that the activities of these antioxidant enzymes were significantly increased by PYR exposure in model cells. This finding suggests that PYR exerts antioxidative stress effects through elevation of antioxidant enzyme activity.

Many polyphenols regulate the expression of HO-1 via antioxidant activity through the Nrf2/HO-1 pathway. We investigated the likelihood of involvement of this pathway in antioxidant effects of PYR, because Nrf2/HO-1 is important in endogenous antioxidant defense, and is indeed the core antioxidant pathway (28-31). Nuclear factor erythroid2-related factor2 (Nrf2) is an important transcription factor which regulates oxidative stress, and its specific receptor is Keap1 (32, 33). In the healthy state, Nrf2 and Keap1 form a cytoplasmic complex which inhibits the oxidative activity of Nrf2. However, under oxidative conditions, Keapl is modified, leading to dissociation of Nrf2 from the complex. The dissociated Nrf2 enters the nucleus from the cytoplasm, and combines with the antioxidant response element (ARE) and its transcription factors. The resultant complex activates the expression of the related antioxidant enzyme genes, thereby strengthening the ability to combat oxidative stress (34, 35).

Heme oxygenase -l (HO-l), one of the most important antioxidant enzymes, belongs to the family of heat shock proteins; it protects the neurons from oxidative damage (36). Under stress stimulation, the expression of HO-1 is stimulated and highly expressed, and under pathological conditions such as AD and Parkinson's disease, HO-1 expression in brain is significantly increased (37, 38). The elevated expression of HO-1 seen in the present study indicates that oxidative damage was evident, and cells were trying to maintain redox homeostasis. A study has suggested that the significant elevation in HO-1 protein in the brain of patients with AD plays a protective role by mopping up iron, recovering damaged mitochondrial function, and inhibiting the formation of Aβ (39, 40). Exogenous HO-1 can induce the formation of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), and accelerate the clearance of Aβ (41). Therefore, we believe that the increase in HO-1 is an adaptation by the body aimed at alleviating the oxidative damage induced by Aβ deposition.

In the present study, the nuclear Nrf2, cytoplasmic Nrf2, whole cell Keap1 and whole cell protein HO-1 were determined. The results showed that compared to normal cells, the content of Nrf2 protein in the nucleus of AD cells and the expression of HO-1 protein were increased, while Keap1 expression was reduced, indicating increased oxidative stress in the AD cell model, and the activation of the Nrf2/HO-1 pathway. When compared with the model group, PYR reduced the expression of cytoplasmic Nrf2 protein in the AD cell model, and increased the nuclear expression of Nrf2. It is likely that PYR enhanced the rate of nuclear transposition of Nrf2, thereby increasing the expression of HO-1 and strengthening the antioxidant capacity of the cells.

Incubation of AD model cells with PYR confers protection against ROS damage. This protective effect may be attributed to its potential anti-oxidant property and activation of the Nrf2/HO-1 pathway. These findings indicate the potential for clinical application of PYR in the treatment of Alzheimer's disease.

Acknowledgements
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Conflict of interest
The authors have no conflicts of interest to declare.

Author's contribution
This work was done by the authors named in this article and the authors accept all liabilities resulting from claims relating this article and its contents. The study was conceived and designed by Chen Li; Ruili Wang, Chunting Hu, Hui Wang, Qiaoya Ma, Songsheng Chen, Ya He collected and analysed the data; Chen Li wrote the manuscript and all authors read and approved the manuscript for publication.

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