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Original Article

DMT1 inhibitor ebselen inhibits iron-induced amyloidogenic APP processing

Ling Xie[1,2], Deshui Yu[3], Jiangping Hu[4], Yan Fang[2], Zhongfu Zuo[2], Yanting Gu[5], Dehua Li[2]

[1]Key Laboratory of Neurodegenerative Diseases, [2]Department of Anatomy, Jinzhou Medical University, Jinzhou, PR China; [3]Department of Spine Surgery, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, PR China; [4]Department of Histology and Embryology, Mudanjiang Medical University, Mudanjiang, PR China; [5]Department of Physiology, Life Science and Biopharmaceutical Institution, Shenyang Pharmaceutical University, Shenyang, PR China

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Abstract: Dyshomeostasis of iron is involved in β-amyloid (Aβ) deposition in Alzheimer's disease (AD). Divalent metal transport 1 (DMT1) is the transporter of iron participating in iron homeostasis. In a previous study, ebselen was confirmed as an effective inhibitor of DMT1 that can reduce cellular iron influx and affect tau hyperphosphorylation. To further demonstrate the effects of ebselen on APP processing, this present study hypothesized that ebselen could modulate amyloid precursor protein (APP) processing and inhibit iron-induced increase of amyloidogenic metabolism. Thus, human neuroblastoma SH-SY5Y cells, stably transfected with human APPsw, were treated with ferrous sulfate after ebselen pretreatment. The results demonstrated that ebselen, an inhibitor of DMT1, decreased iron influx and levels of reactive oxygen species (ROS), consequently repressing β-amyloid (Aβ) generation by inhibition of β-site APP cleavage enzyme (BACE1) and presenilin 1 (PS1) in iron-induced APPsw cells. Collectively, the present data suggests that ebselen treatment may be useful in AD and inhibiting DMT1 is a potential target for prevention and treatment of this disease.

Keywords: Ebselen, divalent metal transporter 1 (DMT1), amyloid precursor protein (APP), β-amyloid peptide (Aβ), Alzheimer's disease (AD)

Introduction

Accumulation of extracellular plaque and intracellular neurofibrillary tangles are predominant neuropathological hallmarks of Alzheimer's disease (AD). After amyloid precursor protein (APP) is hydrolyzed by α-, β-, and γ-secretase and produces β-amyloid (Aβ) peptide, the aggregation of Aβ forms extracellular plaque [1]. Accumulating evidence has demonstrated that iron homeostasis is associated with both Aβ and its precursor APP in brains with AD [2, 3]. Iron can accumulate in plaque and neurofibrillary tangles in AD brains and bind to Aβ, APP, and secretases [2-7]. Thus, iron accumulation not only contributes to metal-catalyzed protein oxidation but also to deposition of Aβ peptides and activation of the amyloid cascade, promoting hyperphosphorylated tau aggregation [8-12].

Iron in the brain is regulated by several factors. Divalent metal transporter 1 (DMT1) is the best characterized Fe^{2+} transporter and is involved in cellular iron uptake in mammals [13, 14]. A mutation in DMT1 could impair iron transport and protect mice against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neuronal death [15-17]. Previous studies have shown that high expression of DMT1 occurs in senile plaque in AD transgenic mice brains and postmortem brains with AD [18, 19]. Moreover, upon silencing the DMT1 gene, iron ion influx is reduced and amyloidogenic process and Aβ secretion is inhibited, *in vitro*, suggesting that DMT1 is a potential target for AD treatment [19].

Ebselen, 2-phenyl-1,2-benzisoselenazol-3[2H]-one, is a lipid-soluble low molecular weight seleno-organic compound. Some pieces of evidence have proven that ebselen can exhibit

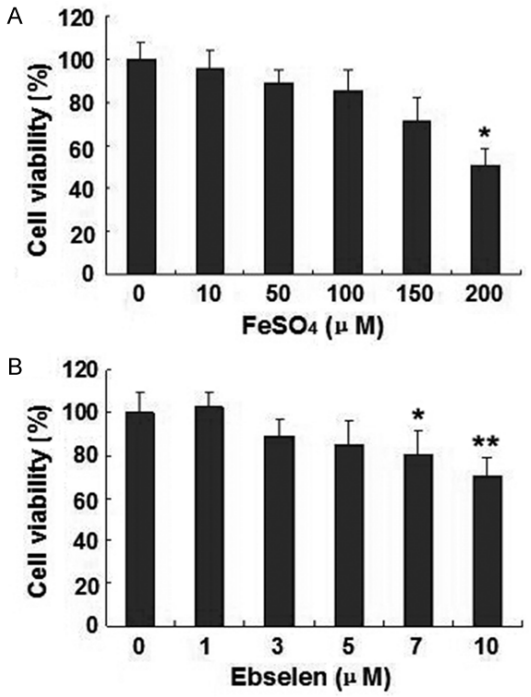

Figure 1. Cell viability of APPsw cells after treatment with ferrous sulfate or ebselen. (A and B) To select appropriate concentrations of $FeSO_4$ and ebselen for follow up experiments, methyl-thiazolyl-tetrazolium (MTT) analyses were performed on SH-SY5Y cells, stably transfected with human APPsw, and 50 μM $FeSO_4$ (A) and 5 μM ebselen (B) were chosen. Mean ± SEM. **p*<0.05, ***p*<0.01, *vs.* the control.

antioxidant and neuroprotective effects [20-24]. Interestingly, ebselen has been demonstrated to be an inhibitor of DMT1 transport [25-27]. A previous study confirmed that ebselen, an inhibitor of DMT1, can inhibit ferrous iron-induced tau hyperphosphorylation in human neuroblastoma SH-SY5Y cells [28].

This present study investigated a cell model of AD wherein cells were transfected with APP harboring the Swedish mutation (APPsw cells). APPsw cells were pretreated with ferrous sulfate ($FeSO_4$) and/or ebselen. The effects of ebselen on Aβ peptide and proteolytic pathways of APP were evaluated. It was found that $FeSO_4$ could accelerate the influx of iron ions, increase production of reactive oxygen species (ROS) and secretion of Aβ, and elevate expression of APP protein and APP cleavage enzymes. Intriguingly, ebselen is able to inhibit these changes. Thus, this study's results indicate that ebselen, an inhibitor of DMT1, inhibits iron-induced amyloidogenic APP processing in APPsw cells.

Materials and methods

Human neuroblastoma SH-SY5Y cells, stably transfected with human APPsw (APPsw cells), were kindly provided by Professor Baolu Zhao at the Institute of Biophysics, Chinese Academy of Sciences in Beijing. Cells were cultured in Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture (DMEM/F12; Gibco) supplemented with 10% heat-deactivated fetal calf serum (FBS; Gibco), 100 IU/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 200 μg/mL G418 (Sigma, USA) at 37°C in the presence of 5% CO_2 [5, 19, 28].

To detect cell viability after treatment with $FeSO_4$ or ebselen and to select appropriate concentrations of $FeSO_4$ or ebselen, methyl-thiazolyl-tetrazolium (MTT) analyses were performed on APPsw cells, with 50 μM $FeSO_4$ and 5 μM ebselen selected. Before treatment, serum-free medium was added for 2 hours. Upon reaching 70-80% confluency, cells were treated with 5 μM ebselen (ebselen dissolved in DMSO) (Sigma) for 12 hours, followed by 50 μM $FeSO_4$ ($FeSO_4$ dissolved in ascorbic acid solution, 1:44 molar ratio, pH 6.0) for 24 hours. Consecutively, the cells were harvested for analysis.

Ferrous iron transport assay by quenching the calcein fluorescence

Calcein is an iron chelator. It binds imported iron and becomes quenched. The quenching of calcein fluorescence can determine the influx of ferrous ions into APPsw cells.

Cells were treated with 5 μM ebselen for 12 hours, followed by incubation with 0.5 μM calcein-AM (Dojindo Laboratories, Japan) for 30 minutes at 37°C. After removing excess calcein-AM, cells were washed three times with phosphate-buffered saline (PBS, pH 7.4), mixed with 500 μL calcein, and evaluated.

First, the initial baseline of fluorescence intensity was recorded. Cell fluorescence intensity was then measured every 100 seconds. At 500 seconds, 50 μM $FeSO_4$ was added to the cells. To investigate the transport of ferrous ions, 50 μM BIP (2,2'-bipyridyl) (Sigma), a high-affinity cell-permeable ferrous chelator, was added to cells at 900 seconds. An F-4500 fluorescence spectrophotometer, equipped with a stirring cuvette holder (Hitachi, Japan) [excitation wave-

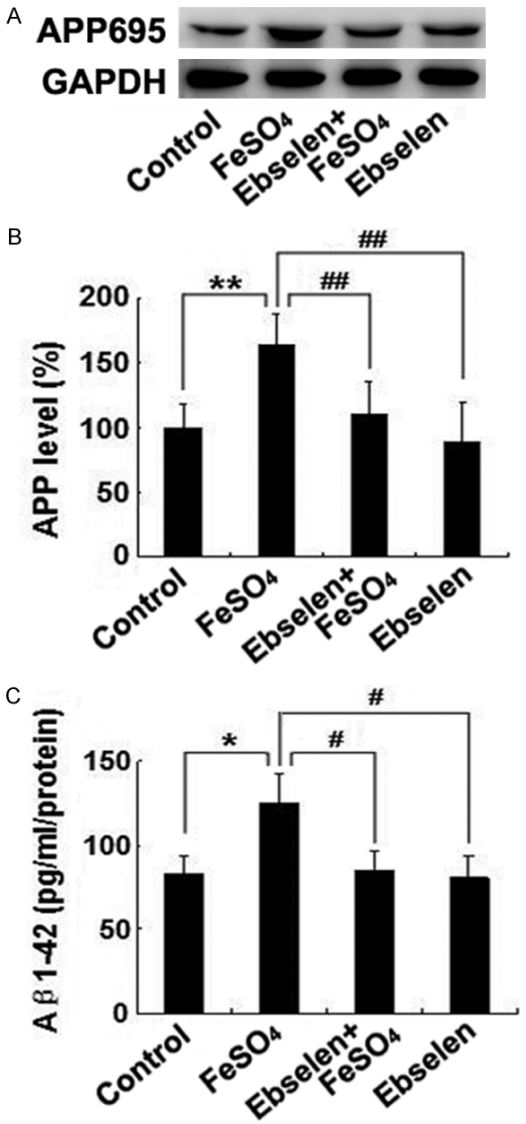

Figure 2. Expression levels of amyloid precursor protein (APP) and Aβ in APPsw cells. A and B. Western blot analysis showed levels of APP695 protein. C. Levels of secreted Aβ1-42 from APPsw cells were analyzed using an ELISA Kit. *$p<0.05$, **$p<0.01$; #$p<0.05$, ##$p<0.01$.

length (λex) 490 nm, emission wavelength (λem) 515 nm, 37°C], was utilized. Data were normalized to steady-state (baseline) values of fluorescence.

Flow cytometry-based estimation of ROS

APPsw cells were treated by control, $FeSO_4$, ebselen + $FeSO_4$, and ebselen. Production of intracellular ROS was assessed using dihydroethidium (DHE, Vigorous, China) by flow cytometry analysis, according to methods described previously. After the above treatments, 10 μM DHE was incubated with the cells for 30 minutes at 37°C in DMEM/F12. Cells were then washed with PBS and harvested. Fluorescence was estimated at λex of 488 nm and λem of 585 nm; further evaluation was conducted based on the mean fluorescence intensity of 20,000 cells.

Enzyme-linked immunosorbent assay (ELISA)

Supernatant from the APPsw cells was collected. Using a human Aβ1-42 Colorimetric Immunoassay ELISA Kit (Biosource International, USA), Aβ1-42 contents were assessed, according to manufacturer instructions.

Western blot analysis

Cells were lysed, directly, using chilled lysis buffer [150 mM NaCl, 0.1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/mL leupeptin, 1 mM Na_3VO_4, and 1 mM NaF]. Homogenates were centrifuged at 12,000 rpm for 30 minutes at 4°C and supernatants were collected. Total protein content was measured on a UV 1700 PharmaSpec ultraviolet spectrophotometer (Shimadzu, Japan).

The equivalent of 80 μg protein, from each sample, was resolved on 8-12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) by electroblotting (50 V, 2.5 h). Membranes were blocked with 5% BSA for 1 hour. Blots were then probed with the following primary antibodies: rabbit anti-APP695 (1:1000; Millipore), rabbit-anti-DMT1-IRE (1:1000; Alpha Diagnosis), rabbit-anti-DMT1+IRE (1:1000; Alpha Diagnosis), rabbit anti-ADAM10 (1:1000; Abcam, UK), rabbit anti-BACE1 (1:1000; Abcam), rabbit anti-PS1 (1:800; Abcam), mouse anti-sAPPα (1:500; IBL, Gunma, Japan), mouse anti-sAPPβ (1:500; IBL), rabbit anti-APP C-terminal fragments (1:1000; Sigma), and GAPDH (1:3000; Abcam, UK), at 4°C overnight. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, USA) for 2 hours at room temperature. Using an enhanced chemiluminescence (ECL) Kit (Pierce, USA), immunoreactive bands were visualized by ChemiDoc™ XRS using Quantity One software (Bio-Rad, USA).

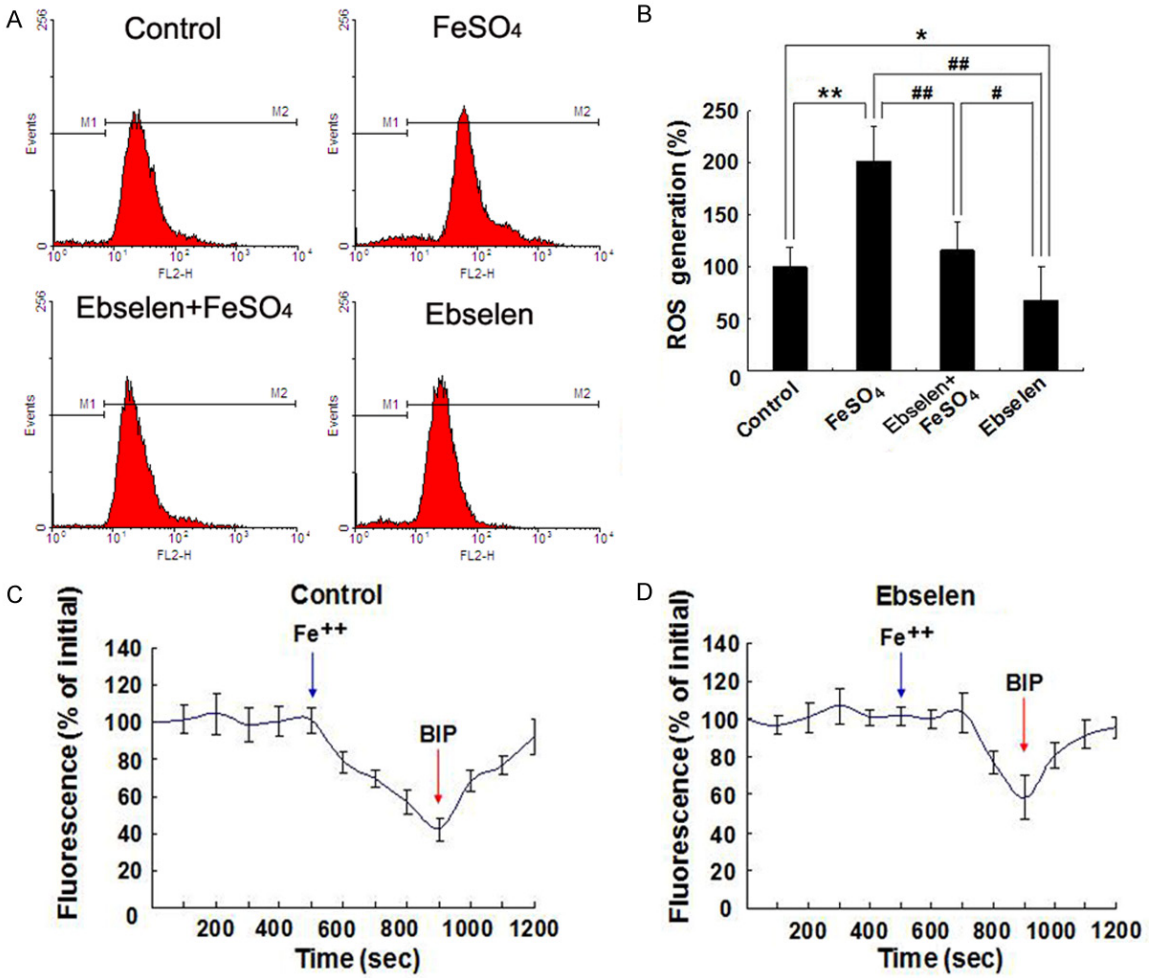

Figure 3. Ebselen pre-treatment reduced ROS production and cellular iron influx in ferrous iron-treated APPsw cells. A and B. Production of ROS in ferrous sulfate group was increased compared with control. However, cells pre-treated with ebselen showed deceased levels of ROS compared with ferrous iron-treated cells. The fluorescence of the control was set 100%. C and D. Intracellular calcein-fluorescence-quenching that occurs in APPsw cells in response to 50 μM ferrous sulfate (added at 500 s) with or without ebselen pre-treatment. Calcein-fluorescence, depressed by ferrous-binding, was finally dequenched by the addition of BIP (a strong ferrous chelator), at a final concentration of 50 μM (added at 900 s). The fluorescence intensity of calcein (λex of 490 nm; λem of 515 nm) was stronger in ebselen-treated cells than that in control cells. *p<0.05, **p<0.01; $^{\#}p$<0.05, $^{\#\#}p$<0.01.

Statistical analysis

All results are expressed as mean ± SD with SPSS16.0 software. Differences among groups were analyzed by two-way analysis of variance (ANOVA). p<0.05 was considered statistically significant.

Results

Ebselen inhibits protein levels of APP and secretion of Aβ1-42 in iron-treated APPsw cells

Based on cell viability MTT assay, optimal concentrations of $FeSO_4$ and ebselen were selected to avoid the effect of cell death in follow up experiments (**Figure 1A**, **1B**).

To assess the effects of ebselen on levels of APP protein, protein levels of APP695 were measured after ebselen pretreatment. Western blot analysis showed that iron treatment alone significantly increased APP695 protein levels by 164.5 ± 23.3% (p<0.01, **Figure 2**) compared to controls. Ebselen pretreatment significantly decreased APP695 levels by 110.7 ± 25.0%, compared to $FeSO_4$ treatment alone (p<0.01, **Figure 2A** and **2B**). Simultaneously, ELISA evaluated secretion of Aβ1-42. In control cells, Aβ1-42 was 83.0 ± 10.6 pg/mL/protein, whereas in

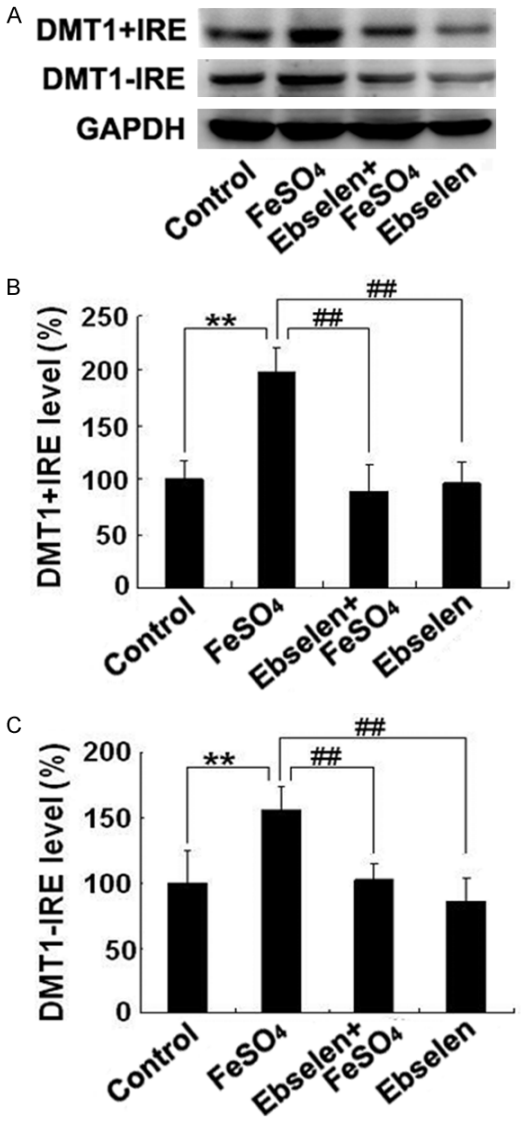

Figure 4. Expression levels of DMT1 isoforms in APPsw cells. A. Western blot was performed to detect levels of DMT1-IRE and DMT1+IRE proteins. B and C. In ferrous sulfate group, levels of DMT1+IRE and DMT1-IRE were increased, compared with control (p<0.01). In the ebselen pre-treated and ebselen alone treated groups, levels of DMT1+IRE and DMT1-IRE were decreased, compared with ferrous sulfate group. $^{**}p$<0.01; $^{\#\#}p$<0.01.

the iron treatment alone groups, it was 125.6 ± 16.3 pg/mL/protein, indicating a significant increase (p<0.05) compared to controls. In ebselen pretreatment groups, Aβ1-42 was 85.6 ± 11.0 pg/mL/protein and decreased significantly (p<0.05) compared to the iron-treated groups. Ebselen-treated groups were 80.8 ± 12.7 pg/mL/protein (**Figure 2C**).

Ebselen reduces production of ROS and decreases cellular iron influx

To validate the activity of ebselen against oxidation and its influence on ferrous iron transport in cells, intracellular ROS content and iron ion transport in iron-induced APPsw cells were tested after ebselen pretreatment.

Results of ROS measurements by flow cytometry showed a significant increase in level of ROS (179.6 ± 43.1%) in ferrous iron-treatment (p<0.01, **Figure 3A** and **3B**). However, APPsw cells with ebselen pretreatment exhibited a decrease (130.3 ± 26.9%) in ROS production compared to ferrous iron treatment (p<0.05, **Figure 3A** and **3B**). No remarkable variations were found in cells treated with ebselen alone, compared to controls (**Figure 3A** and **3B**).

In addition, ferrous iron influx was measured after ebselen pretreatment to verify whether reduction in ROS was associated with the effect of ebselen inhibiting DMT1, using fluorescent dye calcein-AM method [19, 28]. The consequence in APPsw cells showed that calcein fluorescence was quenched by $FeSO_4$ in a time-dependent manner (**Figure 3C** and **3D**). Addition of 50 μM BIP (the membrane-permeable ferrous-specific chelator) could reverse the intracellular quenching of calcein-fluorescence (**Figure 3C**, **3D**).

Importantly, in the ebselen-pretreatment group, fluorescence intensity was stronger than controls, suggesting that ebselen could reduce ferrous iron uptake in APPsw cells.

Ebselen inhibits expression of DMT1 in iron-induced APPsw cells

To assess ebselen's influence on DMT1, Western blot was utilized to detect protein levels of DMT1 (**Figure 4**). Results showed that levels of DMT1+IRE and DMT1-IRE were increased to 198.4 ± 22.4% and 156.3 ± 18.3%, respectively, in the ferrous sulfate groups compared to controls (p<0.01) (**Figure 4A-C**). However, ebselen pretreatment could markedly decrease levels of DMT1+IRE (88.2 ± 25.4%; p<0.01) and DMT1-IRE (102.4 ± 12.4%; p<0.01), compared to the iron groups (**Figure 4A-C**). Thus, it was speculated that ebselen pretreatment could significantly inhibit expression of DMT1.

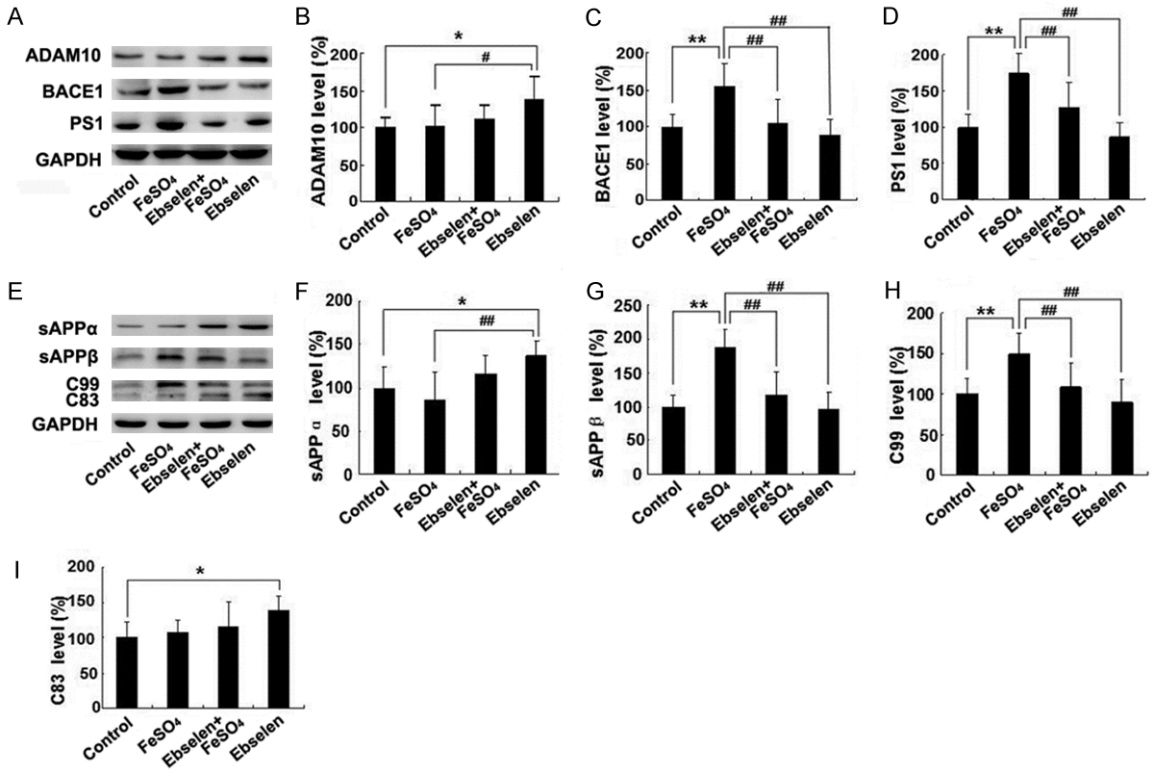

Figure 5. Expression levels of amyloid precursor protein (APP) cleavage enzymes and products in iron- and/or ebselen-treatment APPsw cells. A-D. Western blot analysis showed expression levels of metalloproteinase domain-containing protein 10 (ADAM10), β-secretase 1 (BACE1), and presenilin 1 (PS1) in APPsw cells. In the ebselen-treatment group, levels of ADAM10 were significantly increased, compared with control and Fe-treatment group. Levels of BACE1 and PS1 in the Fe-treatment group were markedly increased. However, in the ebselen pre-treatment, levels of BACE1 and PS1 were significantly reduced, compared with Fe-treatment group. E-I. Levels of sAPPα and C83 were significantly increased in the ebselen-treatment group, compared with control. Levels of sAPPβ and C99 in the Fe-treatment group were markedly increased, compared with control. Moreover, levels of BACE1 and PS1 in the ebselen pre-treatment group were significantly reduced, compared with the Fe-treatment group. $^{*}p<0.05$, $^{**}p<0.01$; $^{\#}p<0.05$, $^{\#\#}p<0.01$.

Ebselen regulates APP processing and expression levels of APP cleavage enzymes in iron-induced APPsw-transfected cells

To further study the effects of ebselen on APP hydrolysis, key enzymes of APP processing and expression levels of cleavage fragments of APP (including sAPPα, sAPPβ, C83, and C99 (the cleavage products of APP)) were tested (**Figure 5**).

Western blot results showed the effects of ebselen on α-secretase of APP a-disintegrin and metallopetidase 10 (ADAM10). Protein levels of ADAM10 (137.4 ± 30.4%; $p<0.05$), sAPPα (136.5 ± 19.7%; $p<0.05$), and C83 (138.1 ± 20.8%; $p<0.05$) in ebselen alone treated groups increased, compared to control and ferrous groups (**Figure 5A**, **5B**, **5E**, **5F**). However, these protein levels did not significantly alter between ferrous groups and ebselen pretreated groups (**Figure 5E**, **5I**). Moreover, in the ferrous groups, levels of BACE1 and PS1 significantly increased to 156.3 ± 30.1% and 174.4 ± 26.8% ($p<0.01$), respectively, compared to controls. After ebselen pretreatment, levels of BACE1 and PS1 remarkably decreased to 104.2 ± 32.6% ($p<0.01$) and 126.7 ± 34.8% ($p<0.05$), compared to the ferrous groups (**Figure 5A**, **5C**, **5D**). In ebselen alone treatment groups, levels of BACE1 and PS1 significantly decreased to 89.4 ± 19.6% and 86.2 ± 20.3% ($p<0.01$), respectively, compared to controls (**Figure 5A**, **5C**, **5D**). Additionally, levels of β-secretase-generated fragments (sAPPβ) and C99 in ferrous groups significantly increased to 186.2 ± 28.4% and 148.7 ± 26.2% ($p<0.01$), respectively, compared to controls (**Figure 5E**, **5G**,

5H). After ebselen pretreatment, sAPPβ and C99 decreased to 117.7 ± 34.7% and 108.9 ± 30.1% ($p<0.01$), respectively, compared with the ferrous groups.

Discussion

AD is a severe progressive brain disorder affecting a significant subset of the elderly human population. Extracellular Aβ aggregates and intracellular neurofibrillary tangles comprise the neuropathological hallmarks of AD [31]. Production of Aβ is a key in the pathological processing of AD. Growing evidence has shown the deposit of metal ions in senile plaque and tangles of neurons in brains with AD [3, 13, 16, 19]. Metal ions combined with Aβ peptide, APP, and secretases promotes APP hydrolysis and Aβ production [3, 19, 32]. Iron ions produced ROS by Fenton reaction, however, excess ROS can damage neurons and aggravate AD [3, 33, 34].

Metal ions have been implicated in the pathogenesis of AD [35-37]. Altered iron homeostasis may be a major factor leading to the pathogenesis of AD, as they have been demonstrated to affect APP expression, Aβ generation, and production of oxidative compounds [3, 38]. Therefore, it is reasonable to speculate that metal transporters may play vital roles in the pathogenesis of AD by altering metal homeostasis [39, 40]. In the brain, metabolism of iron ions is regulated by a variety of proteins. DMT1 is an across-the-membrane transporter that transports divalent metal ions, especially iron, to cells [13]. Previous studies have shown increased DMT1 expression levels in the AD brains of transgenic mice and in autopsies of brains with AD [16, 19]. Dysfunction of DMT1 in mk mice can protect the dopaminergic neurons [15]. Thus, it was speculated that raised DMT1 may promote and participate in occurrence and development of AD. Herein, this study attempted to identify DMT1 inhibitors in order to inhibit DMT1 expression, thereby reducing the pathogenesis of AD.

Ebselen, a compound containing selenium, has an antioxidant effect. Some studies have shown that ebselen can protect neurons [41]. Interestingly, other studies have found that ebselen can inhibit the activity of DMT1 and, thus, it is an inhibitor of DMT1 [25-27]. Importantly, this present study adopted iron-induced SH-SY5Y cells and found that ebselen pretreatment effectively reduced excessive phosphorylation of tau protein by modulating CDK5 and GSK3β pathways [28].

In this study, APPsw cells served as an *in vitro* model to detect production of Aβ amyloid and APP protein expression levels, after ebselen pretreatment and $FeSO_4$ treatments. Data showed that secretion levels of Aβ1-42 amyloid protein and expression levels of APP protein increased significantly in the $FeSO_4$-treated group. Bivalent iron ions can increase production of Aβ through various metal ion binding sites [2]. Simultaneously, APP also has metal ionic binding sites that could combine with iron ions and increase expression levels. After ebselen pretreatment, expression of both was significantly reduced, suggesting that ebselen could suppress secretion of Aβ in iron-induced APPsw cells. To further verify if ebselen affects the intracellular transport of iron ions as a DMT1 inhibitor, cellular iron influx was tested by flow cytometry using calcein-fluorescence-quenching method and ROS generation in cells. The results showed that ebselen reduced intracellular iron ion flux and ROS production in cells. Importantly, in the present study, ferrous ion treatment increased DMT1 levels and ebselen pretreatment decreased DMT1 levels, suggesting that ebselen pretreatment could reverse levels of iron-induced DMT1.

Ferrous ion promotes the Fenton reaction and generates ROS [16]. Ebselen serves as a DMT1 inhibitor, thereby reducing the cellular influx of ferrous ion. Thus, the rate of iron ion coupled with Aβ and APP was decreased. Finally, secretion of Aβ and expression levels of APP protein decreased. These phenomena illustrate that ebselen might inhibit expression of DMT1, reduce intracellular iron ion flow, and decrease ROS generation.

Two APP degradation pathways are: (1) Wherein α hydrolyzes APP, secretes the enzyme, and forms soluble short chains of Aβ, sAPPα, and C83 under normal conditions; (2) APP is hydrolyzed by β-secretase enzymes to produce insoluble long chain Aβ (mainly Aβ1-42) forming extracellular fibrous polymers, sAPPβ, and C99, during AD. The insoluble long chain Aβ is the core of senile plaque and its neurotoxicity can cause neuronal degeneration and death. For further investigation of ebselen as a DMT1

inhibitor affecting the APP hydrolysis process, this study examined APP cleavage enzymes. Results showed that ebselen pretreatment did not significantly alter ADAM10 (as α-secretase enzyme) and its products (sAPPα and C83). However, after pretreatment, ebselen substantially reduced levels of BACE1 and PS1 and secretion of sAPPβ and C99 in iron-induced APPsw cells. This decrease suggests that, by inhibiting DMT1, ebselen reduces intracellular iron ions and interaction with BACE1 and PS1 and further decreases levels of BACE1 and PS1. Thus, levels of Aβ1-42, sAPPβ, and C99 declined.

In summary, this current study demonstrated that ebselen, an effective inhibitor of DMT1, suppresses the APP hydrolysis process in iron-induced APPsw cells, thereby reducing APP and generation of insoluble Aβ. Therefore, ebselen may be an effective drug-based treatment for AD and administration of a DMT1 inhibitor might be an effective therapeutic strategy for AD.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ling Xie, Key Laboratory of Neurodegenerative Diseases, Jinzhou Medical University, Jinzhou, PR China; Department of Anatomy, Jinzhou Medical University, No. 40, Section 3, Songpo Road, Linghe District, Jinzhou City 121000, Liaoning Province, PR China. Tel: 86-15004226160; E-mail: xi.eling2009@hotmail.com

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