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Entacapone is an Antioxidant More Potent than Vitamin C and Vitamin E for Scavenging of Hypochlorous Acid and Peroxynitrite, and the Inhibition of Oxidative Stress-Induced Cell Death

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Entacapone (ENT), a clinical drug for the treatment of Parkinson's disease, has been shown to have antioxidant effects, but little is known about its antioxidant mechanisms. The objective of the current study was to determine the antioxidant activity of ENT against different species of oxidants and compared it with that of vitamin C and vitamin E. We also determined the effect of ENT on oxidative stress-induced cell death in human umbilical vein endothelial cells (HUVECs).

Material/Methods: The total antioxidant activities of ENT, vitamin C and vitamin E were determined with a standard DPPH-scavenging assay. Specific assays to determine ENT's scavenging activity on hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2), and the chelating effect on Fe(II) were used. H_2O_2-induced cell death in HUVECs was determined with the MTT assay.

Results: ENT (10 and 20 µM) scavenged 60% and 83% of DPPH activity, respectively. These percentages were greater than those resulting from using the same concentrations of vitamin C and vitamin E. ENT's HOCl-scavenging activity was concentration-dependent and 8 to 20 times stronger than those of vitamin C and vitamin E. ENT's $ONOO^-$-scavenging activity was 8% to 30% stronger than that of vitamin C. However, ENT, vitamin C, and vitamin E were not able to directly scavenge H_2O_2, and did not show any chelating effect on Fe(II). Importantly ENT, but not vitamin C or vitamin E, inhibited H_2O_2-induced cell death in HUVECs.

Conclusions: ENT is an antioxidant that can scavenge toxic HOCl and $ONOO^-$ species and inhibit oxidative stress-induced cell death more effectively than vitamin C and vitamin E. ENT may have new clinical applications as an antioxidant in the treatment of ROS-induced diseases including cardiovascular disease, cancer, and neurodegenerative diseases.

MeSH Keywords: **Antioxidants • Endothelial Cells • Entacapone • Peroxynitrous Acid**

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Background

Oxidative stress refers to a state in which the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the human body are higher than their physiological concentrations. These ROS and RNS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), nitric oxide (NO), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl). ROS comprise a heterogeneous population of molecules that can be interconverted from one form to another [1–3]. Homeostasis of ROS is highly regulated by multiple factors including oxidation processes and antioxidation mechanisms. Oxidative stress can result from overproduction of ROS and/or insufficient antioxidant molecules and enzymes. ROS can be generated from the electron transport chain (ETC) as by-products of glucose or free fatty acid metabolic processes in the mitochondria, and can be produced in the cytoplasm, peroxisomes and endoplasmic reticulum, via oxidation reactions with several enzymes [1,2]. Additionally, ROS can be produced from food additives, drugs, ionizing radiation, photo-oxidation, tobacco smoke, and many other environment pollutants. The human body has a variety of antioxidant mechanisms including those involving endogenous molecules such as thiols and glutathione (GSH), and enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Other main sources of antioxidants are diet or supplements such as vitamin E (Vit E), vitamin C (Vit C), carotenoids and trace metals (selenium, manganese, and zinc), and flavonoids. These antioxidant mechanisms can inhibit ROS formation or promote free radical scavenging. Physiological levels of ROS are involved in physiological responses as part of signaling processes, gene regulation, and defense mechanisms against pathogens [4]. However, excessive production of ROS and/or insufficient activity of antioxidant defense mechanisms may result in oxidative stress, which could cause the damage to DNA, lipids, proteins, and carbohydrates, and abnormal gene expression [5], thereby contributing to many inflammatory and chronic diseases including cancer, atherosclerosis, hypertension, diabetes mellitus, Alzheimer's disease, Parkinson's disease, and rheumatoid arthritis [6] (Figure 1). For instance, NO can reversibly bind to heme proteins; O_2^- can react with proteins, changing their redox state [7]; and H_2O_2, O_2^-, and NO species can be converted to OH, $ONOO^-$, and HOCl species, which are more toxic. In addition, the Fenton reaction between ferrous ions and H_2O_2 yields OH; the reaction of O_2^- with NO yields $ONOO^-$; and the enzymatic reaction from H_2O_2 and Cl^- forms HOCl [8–10].

Antioxidants reduce oxidative stress; therefore, they may prevent or reduce the risk of oxidative stress-related pathological conditions. Indeed, some reports have shown the beneficial effects of oral antioxidants on the survival of women with breast cancer [11] and on the improvement of endothelial function in patients with atherosclerosis, diabetes, and heart failure [12]. However, conflicting data are often reported [13–15]. The reasons for this are complex and multifactorial. For instance, most clinical trials examined a single antioxidant and specific mechanisms of ROS-mediated diseases are not completely understood. Measuring ROS accurately in patients is still challenging. In addition, different antioxidants may have different antioxidant mechanisms, pharmacokinetics, and tissue distribution. Considering the complex multifactorial nature of ROS-mediated chronic diseases, it could be more appropriate to treat these diseases with combination therapy of multiple antioxidants. The rational for discovering new antioxidant properties in established drugs is that it would increase the pool of drugs for combination therapy for ROS-mediated diseases. Examples of established drugs with pleiotropic antioxidant effects are statins, ACE-inhibitors, and AT1-receptor blockers [16]. In this study, we determined the antioxidant activity of ENT, an established drug for the treatment of Parkinson's disease.

Oxidative stress plays an important role in Parkinson's disease [17–19]. The main pathology of this disease is the presence of a decreased number of dopamine-secreting cells in the brain. Currently, there is no cure for Parkinson's disease, but medications and other treatments can provide relief from the symptoms. For example, L-DOPA is the most commonly used drug which, when converted into dopamine, diminishes the motor symptoms [20]. However, L-DOPA can be degraded by the enzyme catechol-O-methyl transferase (COMT) in the body, reducing L-DOPA effectiveness. To increase the effect of L-DOPA, a COMT inhibitor, entacapone (ENT), is often used. Previous studies have found that ENT has antioxidant effects [21–23]; however, its antioxidant mechanisms are not yet known. Our study has addressed this important issue by determining ENT's antioxidant activities against diverse forms of ROS and comparing them with those of the most commonly used antioxidants, Vit C and Vit E. We determined the antioxidant effects and mechanisms of ENT in a cell free system, and examined the effect of ENT on oxidative stress-induced cell death in human umbilical vein endothelial cells (HUVECs). This study may provide a significant rationale for using ENT as a power antioxidant in combination therapy for ROS-induced diseases, including Parkinson's disease, cancer, and cardiovascular diseases.

Material and Methods

Reagents

The following reagents were obtained from Sigma-Aldrich (St Louis, MO): ENT, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), pyruvate, Vit C, Vit E, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), iron (III) chloride ($FeCl_3$), EDTA, ferrous ammonium sulfate, hydrogen peroxide (H_2O_2),

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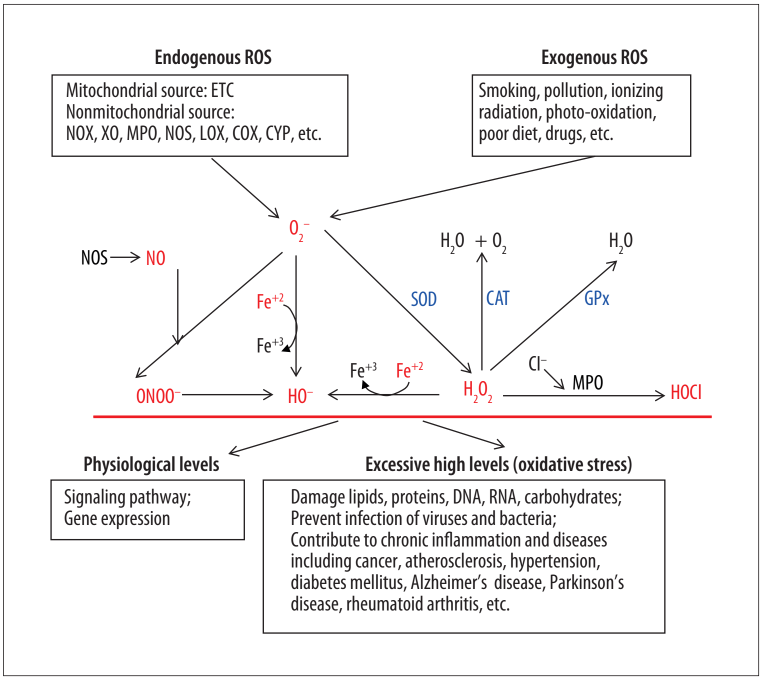

Figure 1. Overview of ROS. The free radical O_2^- can be produced from endogenous and exogenous sources: it can be converted to $ONOO^-$ through reaction with NO, to HO^- through transition Fe(II), and to H_2O_2 via SOD enzymatic reaction. H_2O_2 can be converted to HOCl through an MPO enzymatic reaction and to HO^- through the Fenton reaction. $ONOO^-$ can be converted to HO^-. SOD, CAT, and GPx are major antioxidant enzymes in the body. The physiological levels of ROS play important roles in signal transduction pathways; however, excessive high levels of ROS damage biomolecules (DNA, RNA, lipids, and carbohydrates), prevent infection by viruses and bacteria, and contribute to chronic inflammation and diseases. ROS – reactive oxygen species; RNS – reactive nitrogen species; ETC – electron transport chain; XO – xanthine oxidase; MPO – myeloperoxidase; NOS – nitric oxide synthase; LOX – lipoxygenase; COX – cyclooxygenase; CYP – cytochrome; NO – nitric oxide; O_2^- – superoxide radical anion; $ONOO^-$ – peroxynitrite; HO^- – hydroxyl radical; H_2O_2 – hydrogen peroxide; HOCl – hypochlorous acid; H_2O – water; O_2 – oxygen; SOD – superoxide dismutase; CAT – catalase; GPx – glutathione peroxidase; NOX – NADPH oxidase.

sodium hypochlorite and ferrozine. We purchased the following reagents from VWR: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and nitroblue tetrazolium (NBT). Dihydrorhodamine (DHR123) was obtained from Cayman Chemical (Ann Arbor, MI). Hydroxyphenyl fluorescein (HPF, Hydroxyl/Peroxynitrite Detection Kit™) was obtained from Cell Technology, Inc (Mountain View, CA).

DPPH scavenging assay

We measured the ability of ENT to scavenge stable radical cationic DPPH spectrophotometrically by monitoring the reduction in absorbance at 515 nm, as we previously described [3,24]. Briefly, various concentrations of antioxidants (ENT, Vit C, and Vit E) were added separately to DPPH solutions, and absorption readings were recorded with a UV-Vis spectrophotometer (Agilent Technologies Inc, Santa Clara, CA). DPPH has a deep violet color in solution, and it becomes colorless or pale yellow as it is neutralized by antioxidants. The absorbance of each reading at 515 nm was corrected with a blank (water) control. The change in the optical absorption at 515 nm in the solution of DPPH with each antioxidant was calculated against the DPPH alone control. Final data were presented as percentage of DPPH scavenging activity.

HOCl scavenging assay

The HOCl-scavenging assay is based on the inhibition of the oxidation of thio-2-nitrobenzoic acid (TNB) to 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), induced by HOCl. TNB has a chromophore that has maximal absorbance at 412 nm, but DTNB is colourless [3]. Briefly, HOCl and TNB were freshly prepared as previously described [24]. We determined the HOCl-scavenging activity of ENT by measuring the inhibition of HOCl-mediated oxidation of TNB to DTNB. We calculated the percentage of HOCl-scavenging activity for each antioxidant according to the difference of absorbance readings between the reaction of HOCl and TNB and the reaction of HOCl, TNB, and antioxidant. We then compared the scavenging activity of ENT with that of Vit C and Vit E.

Peroxynitrite scavenging assay

Peroxynitrite ($ONOO^-$) readily oxidizes dihydrorhodamine (DHR 123), a fluorescence molecule, to rhodamine 123. This reaction can be measured with a spectrophotometer at 502 nm. We determined the ability of ENT to scavenge $ONOO^-$ by measuring the inhibition of $ONOO^-$-induced oxidation of DHR 123 to rhodamine 123. For this assay, we prepared fresh batches

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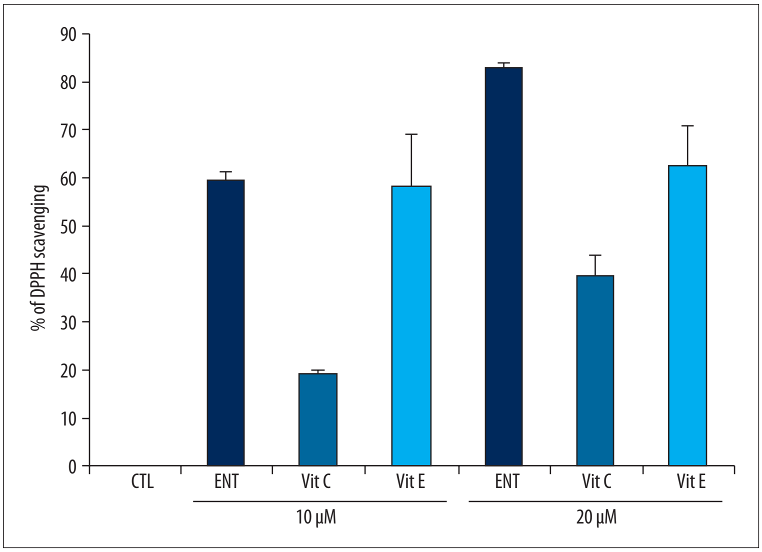

Figure 2. DPPH-scavenging activities of ENT, Vit C, and Vit E. The DPPH-scavenging assay was performed to determine the antioxidant activity of ENT. Vit C and Vit E were used as controls. Two concentrations (10 μM and 20 μM) of ENT, Vit C, and Vit E were tested in these assays. The reduction of DPPH was monitored spectrophotometrically at 515 nm. n=3.

of H_2O_2 and $NaNO_2$, as previously described [3]. We determined the concentration of $ONOO^-$ before each experiment with spectrophotometric readings at 302 nm. We measured the $ONOO^-$-scavenging activity of each antioxidant by calculating absorbance differences between the reaction of $ONOO^-$ and DHR 123 and the reaction of $ONOO^-$, DHR 123, and antioxidant. Then, we compared ENT's $ONOO^-$ scavenging activity with that of Vit C. Due to a solubility issue, Vit E could not be used in this assay.

H_2O_2 scavenging assay

To determine the ability of ENT to scavenge H_2O_2, we mixed a solution of 75 μM H_2O_2 with solutions of different concentrations of ENT (1:1 v/v) and incubated the mixtures for 1 hour at room temperature. Then, we mixed H_2O_2 with the FOX reagent and measured the absorbance at 560 nm [24]. Scavenging of H_2O_2 by the antioxidant leads to a reduced absorbance. We then compared the ability of ENT to scavenge H_2O_2 with that of Vit C and Vit E. Pyruvate was used as a positive control.

Fe(II) chelating assay

The ability of ENT to chelate Fe^{2+} ions was measured with a method previously described [24]. Briefly, 0.2 mL of solutions containing increasing concentrations of ENT or other antioxidants was added individually to 0.74 mL of H_2O. This mixture was then combined with 0.02 mL of 2 mM $FeCl_2$ and 0.04 mL of 5 mM ferrozine. After 20 minutes, we read the absorbance at 562 nm. A reduction in absorbance indicated higher chelating activity. EDTA was used as a positive control.

Oxidative stress-induced cell death

HUVECs (Lonza Inc, Alendale, NJ) were grown in EGM-2 culture medium (Lonza) at 37°C in a humidified atmosphere with 5% CO_2. Fifth passages of HUVECs were used for this study. We determined oxidative stress-induced cell death with the MTT assay, as previously described [25]. Briefly, HUVECs (5×10^3 cells/well) were seeded into 96-well plates in regular growth medium and maintained in this medium for one day. We then pre-treated the cells with DMSO (1%), ENT (20 μM), Vit C (20 μM) or Vit E (20 μM) for 2 hours, and incubated them in 0.7 mM H_2O_2 for 4 hours. Subsequently, we treated the cells with MTT (250 μg/mL) at 37°C for 4 hours and lysed them in 100 μL lysis buffer. After solubilizing MTT to a blue formazin dye with DMSO, we read the absorbance at 570 nm. The cells incubated with control medium were considered to be 100% viable.

Results

ENT is a DPPH scavenger more potent than Vit C or Vit E.

DPPH is one of the most commonly used reagents to determine the total antioxidant activity for potential antioxidants. Chemically, the odd electron of the nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants [26]. Using the DPPH assay we determined that ENT at 10 μM and 20 μM significantly reduced DPPH activity by 60% and 83%, respectively. At 20 μM, Vit C and Vit E reduced DPPH activity by 40% and 63%, respectively (Figure 2). Thus, ENT has a potent DPPH-scavenging activity, which is stronger than that of Vit C and Vit E.

ENT scavenges hypochlorous acid more effectively than Vit C and Vit E

HOCl is a highly toxic species of ROS and it reacts with a wide variety of biomolecules including proteins, lipids, and DNA. We performed an HOCl-scavenging assay based on HOCl being able to oxidize TNB to DTNB. ENT strongly inhibited HOCl-induced

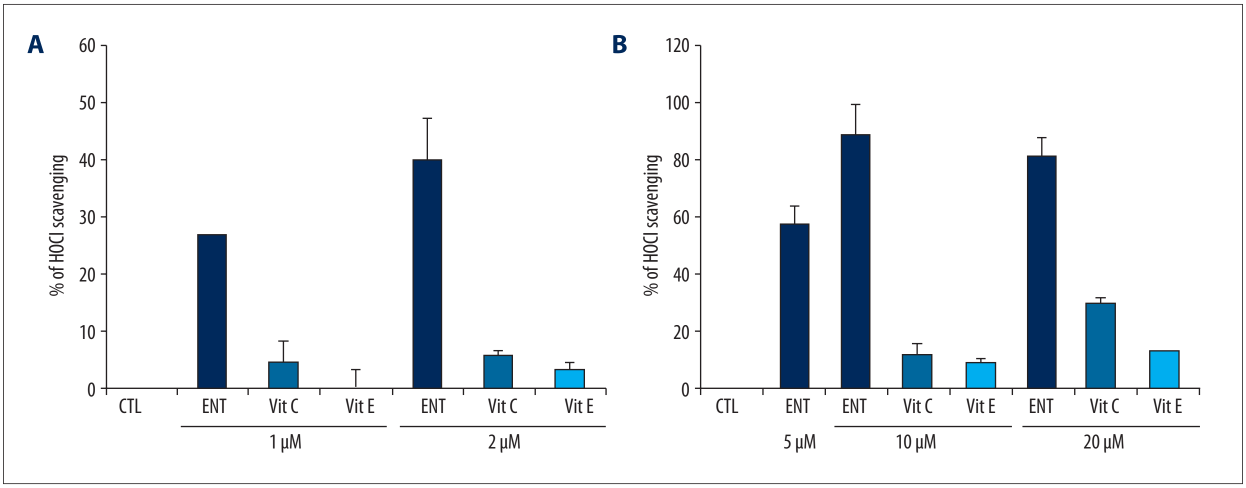

Figure 3. Hypochlorous acid (HOCl)-scavenging activities of ENT, Vit C, and Vit E. To determine HOCl-scavenging activity, different concentrations of ENT, Vit C, Vit E, and a control reagent were added into the HOCl-scavenging assay. The HOCl-scavenging activity was monitored spectrophotometrically at 414 nm. n=3. (**A**) Lower concentrations of antioxidants (1 μM and 2 μM). (**B**) Higher concentrations of antioxidants (5 μM, 10 μM, and 20 μM).

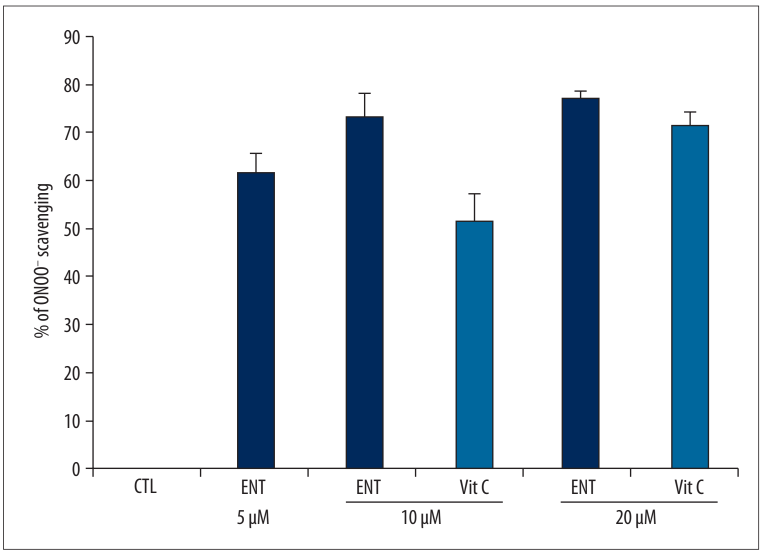

Figure 4. Peroxynitrite ($ONOO^-$)-scavenging activities of ENT and Vit C. To determine $ONOO^-$-scavenging activity, different concentrations of ENT, Vit C, and a control reagent were added to the $ONOO^-$ scavenging assay. The $ONOO^-$-scavenging activities of ENT and Vit C were monitored spectrophotometrically at 500 nm. n=3.

TNB oxidation in a concentration-dependent manner (1, 2, 5, 10 and 20 μM). For example, at a lower concentration (2 μM), ENT was able to scavenge 40% of HOCl, while Vit C and Vit E scavenged 3% and 2% of HOCl, respectively, under the same conditions (Figure 3A). At a higher concentration (10 μM), ENT scavenged 88% of HOCl, while Vit C and Vit E scavenged 11% and 10%, respectively (Figure 3B). Thus, ENT can effectively scavenge HOCl in a cell-free system with an activity that is 8 to 20 times stronger than that of Vit C and Vit E.

ENT scavenges peroxynitrite more effectively than Vit C

The reaction of NO with superoxide forms peroxynitrite ($ONOO^-$), which is an oxidant much more powerful than superoxide [27,28]. $ONOO^-$ reacts directly with amino acids and lipids to form nitrotyrosine, nitrotryptophan, and nitrated lipids [29]. The current *in vitro* $ONOO^-$-scavenging assay is based on the ability of $ONOO^-$ to oxidize the fluorescence molecule dihydrorhodamine (DHR 123). ENT at the concentrations of 5 μM, 10μM, and 20 μM effectively scavenged 61%, 73%, and 76% of $ONOO^-$, respectively. The effect of ENT was stronger than that of Vit C by 8% to 30%, at the same concentrations (Figure 4). Vit E was not studied in this assay because of its solubility issue.

ENT does not directly scavenge hydrogen peroxide nor chelate Fe(II)

Hydrogen peroxide (H_2O_2) is a precursor of several ROS such as OH^- and HOCl. The transition metal ion Fe(II) possesses the ability to perpetuate the formation of free radicals by gain or

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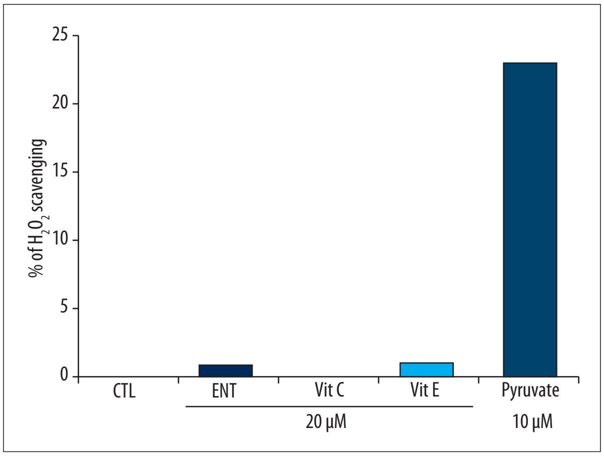

Figure 5. Hydrogen peroxide (H_2O_2)-scavenging activities of ENT, Vit C, and Vit E. To determine H_2O_2-scavenging activity, different concentrations of ENT, Vit C, Vit E, pyruvate (positive control), and a negative control reagent were added to the H_2O_2-scavenging assay. The H_2O_2-scavenging activities of ENT, Vit C, Vit E, and controls were monitored spectrophotometrically at 560 nm. n=3.

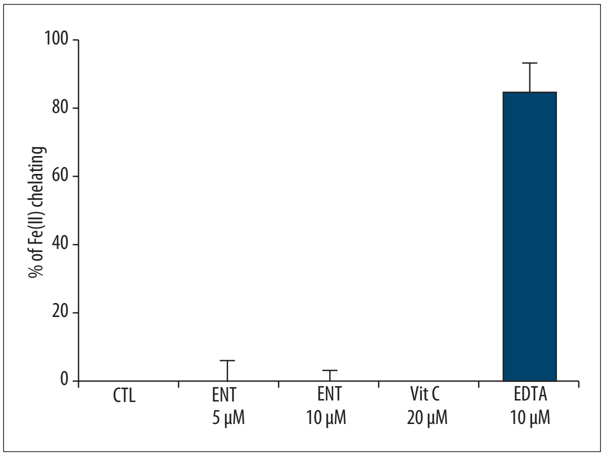

Figure 6. Fe(II)-chelating activities of ENT and Vit C. To determine Fe(II) chelating activity, different concentrations of ENT, Vit C, EDTA (positive control), and negative control reagents were added to the Fe(II)-chelating assay. The Fe(II)-chelating activities of ENT, Vit C, and controls were monitored spectrophotometrically at 562 nm. n=3.

loss of electrons. Therefore, agents that chelate metal ions could play an antioxidant role in reducing the formation of ROS. In the current study, we determined whether ENT had H_2O_2-scavenging and Fe(II)-chelating activities. Neither ENT (20 µM), Vit C, nor Vit E showed significant H_2O_2-scavenging activity with the sensitive Fox reagent method (Figure 5). Pyruvate was used as a positive control because it is able to directly scavenge H_2O_2 [30]. In our experiment, pyruvate (10 µM) scavenged 23% of H_2O_2. In addition, ENT, Vit C, and Vit E showed no Fe(II)-chelating activity, while the positive control with EDTA (10 µM) chelated 82% of Fe(II), under the same experiment condition (Figure 6). Thus, ENT has no H_2O_2-scavenging or Fe(II)-chelating activities *in vitro*.

ENT significantly inhibits oxidative stress-induced cell death in HUVECs

Excessive ROS leads to oxidative damage and cell death. One of the major contributors to oxidative damage is H_2O_2. H_2O_2 is produced when O_2^-, a byproduct of cellular metabolism, is simultaneously reduced and oxidized (dismutated). H_2O_2 readily diffuses out of the mitochondria and reacts with ferrous iron (Fe^{2+}) or other transition metal ions to produce OH^-, which are highly toxic. H_2O_2 is also quickly converted to toxic HOCl species. It is well known that H_2O_2-mediated oxidative stress can cause cell death [31,32]. We tested whether ENT could inhibit H_2O_2-induced toxicity in HUVECs. Indeed, treatment with H_2O_2 induced 48% of cell death in HUVECs; however, if the cells were pre-treated with ENT (20 µM), cell death was 16% (Figure 7). Under the same experimental conditions, Vit C and Vit E did not protect HUVECS from of H_2O_2-induced cell death. Thus, ENT significantly prevents HUVECs from oxidative stress-induced cell death.

Discussion

In the current study, we have explored the antioxidant capabilities of ENT, a clinical drug for Parkinson's disease. ENT is a potent scavenger of DPPH, a free radical indicator, as well as specific ROS, HOCl and $ONOO^-$. Accordingly, ENT is able to effectively inhibit H_2O_2-induced cell death in human endothelial cells. These antioxidant effects of ENT are even much stronger than those of Vit C or Vit E, under the same experimental conditions. Thus, ENT may have clinical applications as a novel antioxidant in the treatment of oxidative stress-induced diseases.

We first showed the antioxidant effect of ENT with the DPPH-scavenging assay because it is one of the most commonly used assays to determine the antioxidant capability of compounds. DPPH is a stable free radical, and its odd electron in the nitrogen atom can be reduced by receiving a hydrogen atom from antioxidants. The reduced form of DPPH has a different color, which is used for its detection. This assay has been successfully utilized for investigating antioxidant properties of foods and medicines and to examine both hydrophilic and lipophilic antioxidants [33,34]. ENT and Vit C are hydrophilic; while Vit E is lipophilic. Current studies use the DPPH assay as the first option for screening the antioxidant capacity of multiple compounds according to their chemical structure analyses. It is surprising that ENT has a stronger DPPH scavenging

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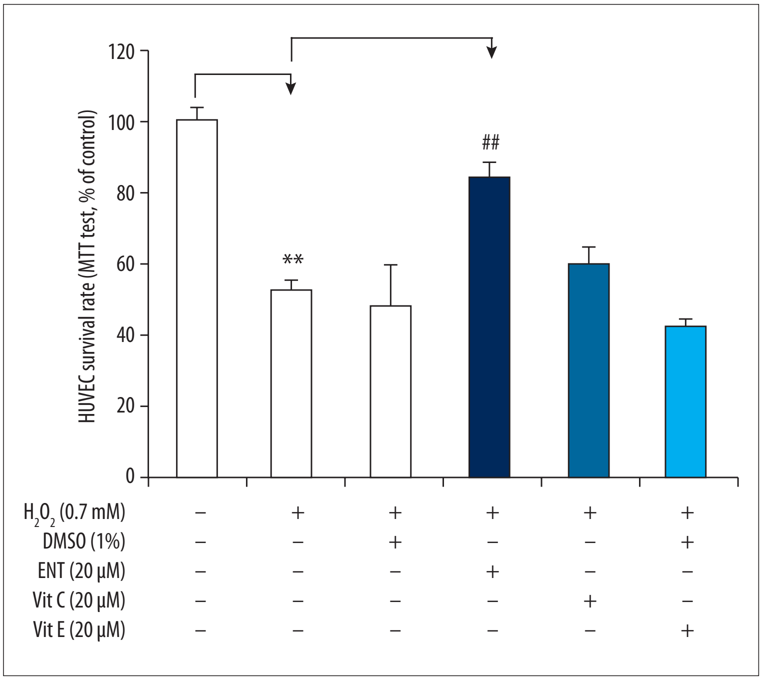

Figure 7. The effects of ENT, Vit C, and Vit E on the inhibition of oxidative stress-induced cell death in HUVECs. To determine the ability of ENT to protect HUVECs from cell death induced by oxidative stress, HUVECs were pretreated with 20 µM ENT, Vit C, Vit E or control reagents and then challenged with a high concentration of H_2O_2 (0.7 mM) for 4 hours. Cell viability was determined with the MTT assay. n=3. ** *P*<0.01 comparing negative control with H_2O_2 treated cells. ## *P*<0.01 comparing H_2O_2 and H_2O_2+ENT-treated cells.

activity than Vit C and Vit E. For example, ENT at 20 µM reduces 83% of DPPH activity; while Vit C and Vit E only reduced this activity by 40% and 63% of DPPH, respectively, at the same concentration.

We then determined the antioxidant activity of ENT on HOCl. HOCl is a strong oxidant that results from MPO-mediated peroxidation of chloride ions from H_2O_2 in activated neutrophils and macrophages, and contributes to the destruction of bacteria and viruses. HOCl reacts strongly with many biologically important molecules, such as DNA, proteins, lipids, and carbohydrates. The reactivity of HOCl with most substrates exceeds that of other ROS, such as H_2O_2 and $ONOO^-$, by several orders of magnitude [35]. HOCl has been implicated in inflammation as a mediator of oxidative tissue damage and cellular dysfunction leading to the initiation and acceleration of many diseases such as cystic fibrosis [36], chronic obstructive pulmonary disease [37], gout [38], atherosclerosis [39], rheumatoid arthritis [40], and cancers [41,42]. Several biomarkers have been used for detecting the production of HOCl *in vivo,* for instance the detection of 3-chlorotyrosine for proteins, 2-chloradipic acid for lipids, and 8-chloroadenine for DNA [43–45]. In our study, we show that ENT has an antioxidant effect against HOCl that is dose-dependent and between 13 and 20 times stronger than that of Vit C and Vit E. These results suggest that ENT could be indicated for the treatment of HOCl-induced diseases.

In biological systems, peroxynitrite ($ONOO^-$) is formed by the reaction of the free radical O_2^- with NO. $ONOO^-$ is a powerful oxidant that can damage a wide array of molecules in cells. For instance, the reaction of $ONOO^-$ with amino acids results in the formation of nitrated amino acids, such as 3-nitrotyrosine. $ONOO^-$-mediated protein modification is irreversible and may have a pathological effect on cellular function. $ONOO^-$ can also oxidize unsaturated fatty acids in biological membranes to form nitrated fatty acids such as oleic, linolenic, and arachidonic acids [46,47]. Furthermore, $ONOO^-$ can react with DNA and produce damaged DNA molecules such as 8-hydroxy-2-deoxyguanosine (8- OHdG) and 8-nitroguanine [48]. In addition, $ONOO^-$ is able to oxidize the NOS cofactor BH4, thereby leading to eNOS uncoupling and O_2^- production [49]. $ONOO^-$ can be decomposed to yield HO^-, independently on the presence of transition metals [50]. This evidence indicates that $ONOO^-$ is highly toxic to cells and strongly supports the need for antioxidants that can potently scavenge $ONOO^-$. In this study, we provide a contribution to satisfy that need. We show that ENT effectively scavenges $ONOO^-$ in an *in vitro* system under the same conditions in which Vit C is less effective. Vit E could not be compared with ENT because assay conditions limited the solubility of Vit E. In other ROS scavenging assays, ENT, Vit C, and Vit E did not scavenge H_2O_2, and did not have any Fe(II)-chelating activity.

Vit C and Vit E as antioxidants have been used extensively in the clinic, with mix outcomes. For example, intra-arterial administration of high doses of Vit C resulted in improved endothelium-dependent vasodilation in the forearm microcirculation of hypertensive patients [51]. However, prolonged oral administration of Vit C did not result in improved endothelial function in hypertensive patients. Vit E supplementation had no effect on endothelial function in aged individuals [51]. Large clinical antioxidant trials of Vit C or Vit E have also failed

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to show significant cardiovascular benefits [52], and it is controversial whether Vit C and Vit E have therapeutic effects in cancers [53–55]. Different antioxidants may act in different ways. Vit C is water-soluble and reacts rapidly with a variety of ROS; it can play an antioxidant role in the cytosol and the extracellular matrix. However, Vit C may have a prooxidant activity and reduce trivalent iron to its divalent form, which enhances ROS formation [56,57]. On the other hand, Vit E is lipophilic and located in the biological membrane where it can react with lipid peroxyl radicals produced during lipid peroxidation process. Our results and those of others strongly support research to develop new antioxidants with different antioxidant mechanisms and to consider combination therapy of multiple antioxidants for ROS-induced diseases, such as cardiovascular disease and cancer.

ROS can modify biomolecules, such as proteins, DNA, and lipids, leading to cell death and disease formation. For example, high levels of ROS-mediated oxidative stress can damage the structure and functions of endothelial cells, and contribute to pathogenesis of hypertension, diabetes, inflammation and atherosclerosis [58,59]. The assays of H_2O_2-induced death of endothelial cells or neuronal cells are commonly used *in vitro* models for determining antioxidant effects on oxidative stress [31,32,60,61]. Using these assays, other have shown that H_2O_2 is highly permeable to cell membrane [62] and can increase production of O_2^- by activating NADPH oxidase and eNOS uncoupling [63,64]. H_2O_2 can also be converted to HOCl by MPO, and O_2^- is readily converted to $OHOO^-$ through reaction with ON. Both HOCl and $OHOO^-$ are highly toxic to cells. To test ENT's antioxidant properties against HOCl and $OHOO^-$, we first used a cell-free system and found that ENT has a scavenging activity that is more potent than that of Vit C and Vit E. Therefore, we expected that ENT would be able to protect cells from an H_2O_2 challenge. Indeed, ENT (20 µM) effectively reduced H_2O_2-induced cell death in HUVECs by 67%, while, under the same experimental conditions, Vit C and Vit E did not show a protective effect. These data strongly encourage considering ENT as a clinical antioxidant medicine.

ENT is a drug currently used in the treatment of Parkinson's disease (PD). ENT inhibits catechol-O-methyltransferase (COMT) which results in increased levels of levodopa/carbidopa in the brain, enhancing its effectiveness. Previous *in vitro* investigations showed that ENT can effectively inhibit alpha-synuclein and beta-amyloid oligomerization and fibrillogenesis, which play a central role in the pathogenesis of PD and related synucleinopathies. The catechol moiety of ENT is essential for its anti-amyloidogenic activity [65]. In addition, a previous study has shown that ENT has an antioxidant effect on alkaline phosphatase (ALP) oxidation *in vitro*. In this assay, ALP is oxidized by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and loses its enzymatic function of hydrolyzing 4-methylumbelliferyl phosphate (4-MUP) to fluorescent 4-methylumbelliferone (4-MU). ENT can protect ALP from AAPH-induced oxidation and functional loss [21]. ENT is also able to scavenge NO in an *in vitro* assay [23]. In this study, we show the total antioxidant capacity of ENT in the DPPH assay. We also show, for the first time, that ENT can directly scavenge HOCl and $ONOO^-$. A previous study has shown that ENT has Fe(III)-chelating activity, as shown by the electromotive force titration method [22]; however, the functional role of this Fe(III)-chelating activity is not yet clear. It is well known that transition metal ions such as Fe(II) play a critical role in ROS production via Fenton or Haber-Weiss chemistry. Any compound which has a Fe(II)-chelating property may be an effective antioxidant [66,67]. In the current study, we observed no Fe(II) chelating activity for ENT, Vit C, or Vit E.

Conclusions

We show, for the first time, that the antioxidant mechanisms of the clinical drug ENT are involved in its ability to scavenge HOCl and $ONOO^-$, two highly toxic ROS. ENT's antioxidant potential is significantly higher than that of Vit C and Vit E, as shown by the DPPH-, the HOCl-, and the $ONOO^-$-scavenging assays. Functionally, ENT effectively protects HUVECs from oxidative stress-induced cell death. These new data suggest that ENT may have new indications for the treatment and/or prevention of oxidative stress-induced diseases, including cardiovascular disease, cancer, neurodegeneration disease, and inflammatory disease. New clinical trials to confirm these new indications of ENT are warranted.

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Conflict of interest statement

No conflict of interest.

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