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Experimental Research Article

Ferulic acid mitigates H_2O_2-induced oxidative stress by hindering the activity of reactive oxygen species and programmed cell death in rat PC12 cells

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ABSTRACT

Introduction: Oxidative stress (OS) is related to the onset and development of different disorders including neurodegenerative diseases. Attenuation of OS may be an appropriate way to combat such a situation. Ferulic acid (FA) as a natural antioxidant compound has been shown to have potent free radical scavenging activity. Hence, the present study aimed to investigate the effects of FA to inhibit the intrinsic apoptosis pathway evoked by H_2O_2 in rat pheochromocytoma (PC12) cells.

Methods: PC12 cells were treated with various concentrations of FA at different times. Then, H_2O_2 (300 µM for 2h) was added. Afterward, cell viability was assessed by MTT assay followed by determining the levels of total antioxidant power (TAP), and malondialdehyde (MDA) level. The protein expressions of Caspase-3, Bax, and Bcl-2 were also measured by western blotting.

Results: Current results indicate that after 72 hours, FA significantly protected PC12 cells against H_2O_2-induced damage by reducing the generation of MDA levels as well as increasing TAP. H_2O_2-induced caspase-3 overexpression and the increase of the Bax/BCL-2 protein ratio were also diminished by FA treatment.

Conclusion: Taken together, FA may be considered a protective agent to prevent or postpone the progression of oxidative neurodegenerative diseases through its anti-oxidant and anti-apoptotic effects.

Keywords:
Apoptosis
Ferulic acid
H_2O_2
Oxidative Stress
PC12 cells

Introduction

Under physiological conditions, reactive oxygen species are generated 1-2% of consumed O_2 causing to induction of OS and normally cells can combat such insults by their antioxidant systems (Chen et al., 2012). Neurons have higher oxygen consumption, metal ions,

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polyunsaturated fatty acids, and lower antioxidants that make them more susceptible to OS compared to other cells (Chen et al., 2012; Gandhi and Abramov 2012). Reactive oxygen species mediate neural loss due to protein aggregation, mitochondrial dysfunction, and apoptosis (Li and Li 2015). There are two basic apoptotic pathways including intrinsic and extrinsic pathways, among which intrinsic pathway shows important roles in reactive oxygen species-mediated neuronal apoptosis (Franklin 2011). Regards the close relation between reactive oxygen species production and the onset of neurodegenerative diseases, therefore, the inhibition of reactive oxygen species formation may be considered as a promising approach to postpone the progression of these diseases. Natural plant products have shown promising antioxidant and neuroprotective activities (Bastin et al., 2021; Uddin et al., 2013). Ferulic acid (FA) (4-hydroxy-3-methoxy cinnamic acid) is a natural phenol compound that is found in many plants (Ou and Kwok 2004). Previous reports have shown that FA possesses free radical scavenging activity (Srinivasan et al., 2007). Several lines of evidence have described the potential neuroprotective effect of FA in different models of neurotoxicity both in in vitro and in vivo experiments (Cheng et al., 2008; Sultana et al., 2005). It has been also demonstrated that the treatment of primary neuronal cell cultures with FA could protect cells against hydroxyl and peroxyl radicals mediated OS (Kanski et al., 2002). Moreover, through its powerful anti-inflammatory and anti-oxidant actions, FA exerts an excellent protective effect in the progression of some disorders (Srinivasan et al., 2007). FA could exert a protective effect against a transient focal cerebral ischemia model by reducing the activation of several apoptotic pathways. They are related to the activation of glutamate receptors and subsequent Bax translocation and cytochrome c release (Cheng et al., 2010). In a recent study, FA could inactivate SMAC/Diablo and Bad, inhibit phosphorylation of the extracellular signal-regulated kinase (ERK), and restore expression levels of brain-derived neurotrophic factor (BDNF) by regulating microRNA-10b expression in PC12 cells (Nakayama et al., 2020). The current study evaluated the pre-treatment effect of FA by affecting the mitochondrial apoptotic pathway in undifferentiated PC12 cells against H_2O_2-induced damage. Subsequently, we assessed some of the underlying mechanisms involved in the protective effects of FA.

Materials and methods

Chemicals

All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Ferulic acid, protease, and phosphatase inhibitor cocktails, Dulbecco's Modified Eagle's Medium F12 (DMEM/F12), DCF, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Sigma Aldrich, St Louis, MO, USA). Fetal bovine serum (FBS), and penicillin/streptomycin were from Gibco (Invitrogen, Carlsbad, CA, USA).

Cell culture and treatment

PC12 cells (pheochromocytoma of the rat adrenal medulla) were purchased from Pasture Institute Cell Bank (Tehran, Iran) and cultured in DMEM F12 medium supplemented with 10% FBS, and 1% penicillin/streptomycin. They were incubated at 37 °C in 5% CO_2 and humidity of 80%. The culture medium was changed every 3 days. PC12 cells were used after 3 consecutive passages. Ethanol 96% was used as the solvent for FA, ensuring that its final concentration was lower than 0.1%. The medium was used as the solvent for H_2O_2. Cells were treated with FA at 0, 10, 50, 100, and 400 µM concentrations for 24, 48, and 72 h for evaluation of cytotoxicity of FA. Then, to evaluate the protective effect of FA against H_2O_2-induced toxicity, the PC12 cells were treated with FA, and then H_2O_2 (300 µM for 2h) was added. The control contained a similar concentration of solvents.

Measurement of cell viability

MTT assay is an established colorimetric method for determining the viability of cells in cytotoxicity and proliferation studies. At first, PC12 cells were seeded in 96-well plates at the density of 5000 cells per well. After 24 h, they were treated as noted in the previous section and MTT was added to each well and the cells were incubated for 4 h in a humid incubator at 37°C. Afterward, the medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added (Mehrabani et al., 2020b; Sargazi et al., 2021).

Measurement of malondialdehyde (MDA) level

Briefly, cells at the density of 1×106 were resuspended at 400 µL PBS and lysed by sonication. 400µL of the solution and 1000 µL of TCA 20% were incubated

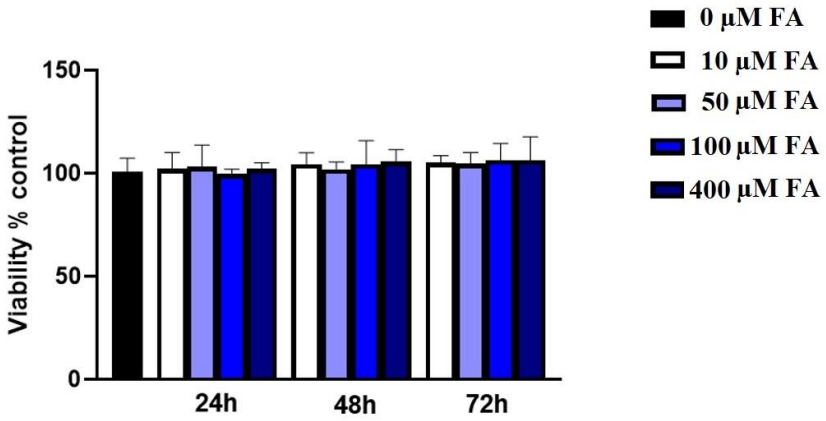

FIGURE 1. Measuring viability of PC12 cells after exposure to different concentrations of Ferulic acid (FA) by MTT assay. Viability of cells is reported in comparison with control. Data are represented as mean ± SD (n = 3).

for 10 min. Then it was centrifuged at 1500 ×g for 10 min. The precipitate was mixed with sulfuric acid (0.05 M) and 0.8 ml TBA (0.2% in 2 M sodium sulfate) was incubated for 30 min in boiling water. After adding 0.8 ml n-butanol the absorbance was determined at 532 nm by a spectrophotometer (UV-160A, Shimadzu, Japan) (Juybari et al., 2018).

Measurement of total antioxidant power (TAP) level

290 µL of the fresh working solution (25 mL acetate buffer [300 mM], 2.5 mL TPTZ [2, 4, 6 tripyridyl-s-triazine] solution [10 mM in 40 mM HCL], and 2.5 mL $FeCl3.6H_2O$ [20 mM]) was added to 10µL of the PC12 cells supernatants and was incubated at 37°C for 10 min. The absorbance of the blue ferrous complex was determined by a microplate reader at 593 nm (Bio-Tek ELX800, USA) (Mehrabani et al., 2020a).

Western blot analysis

The cells were separated and then were lysed by RIPA lysis buffer. Afterward, the mixture was centrifuged for 30 min at 12000 xg at 4°C. Total protein concentration was determined by the Bradford method. An equal concentration of samples (70 µg) was separated on SDS-PAGE gel. The proteins were transferred to a PVDF membrane followed by incubation with primary antibodies (1:1000 dilution) overnight at 4°C. Antibodies included polyclonal antibody anti- Caspase-3, anti-Bax, anti-Bcl2, and anti-β-actin. After this step, the membranes were incubated with the appropriate secondary antibody (1:2000 dilution) for 1 h at 4°C. An enhanced chemiluminescence kit was used to visualize the protein bands. Then, band density was quantified by Image J (Amirkhosravi et al., 2023).

Statistical analysis

The data were examined from at least three independent experiments and presented as the mean ± standard deviation (SD). One-way ANOVA with Dunnett's post hoc test was performed for multiple comparisons. Statistically, the differences were assumed significant at $P<0.05$.

Results

FA showed a non-cytotoxic effect on the viability of PC12 Cells

To investigate the effect of FA on the viability of PC12 cells, they were incubated with different concentrations of FA (0-400 µM). As demonstrated in Figure 1, treatment of PC12 cells with FA (0, 10, 50, 100, and 400 µM) showed a non-cytotoxic effect after 24, 48, and 72 h (Fig. 1).

FA protected cells from H_2O_2-induced damage

Treatment with FA (100 µM and 400µM only for 72h) significantly protected PC12 cells against H_2O_2-induced toxicity (300 µM for 2h) (100µM; $P<0.05$, 400µM; $P<0.01$). Hence, FA at these concentrations was applied for 72 h in the next steps of the current study (Fig. 2).

FA conditioning modulated MDA and TAP level

As shown in Figure 3, a significant increase in MDA level ($P<0.01$) and a decrease in TAP ($P<0.01$) were seen in H_2O_2-exposed groups. Conditioning of PC12

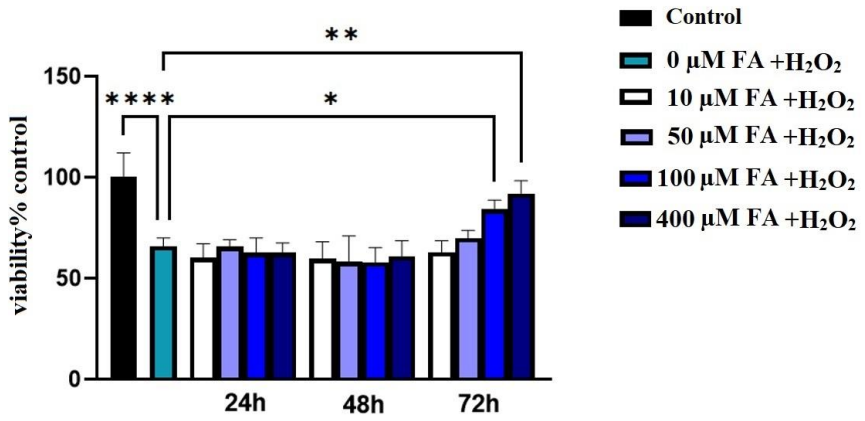

FIGURE 2. Protective effects of Ferulic acid (FA) against H_2O_2-induced damage in PC12 cells measured by MTT assay. After 72h, FA at concentrations of 100 and 400 µM could prevent H_2O_2-induced damage in PC12 cells. Data are represented as mean ± SD (n = 3; *P < 0.05, ** P < 0.01, **** P < 0.0001 compared with H_2O_2).

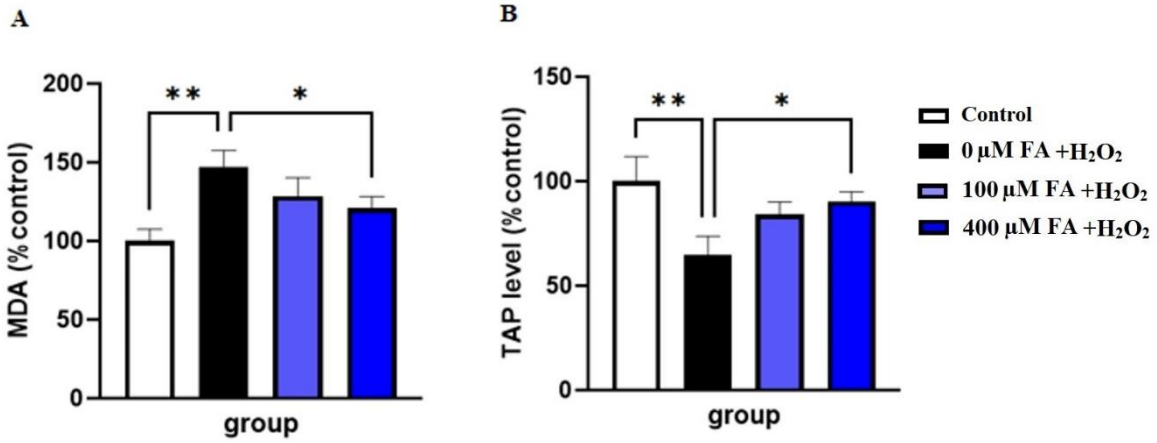

FIGURE 3. Protective effects of Ferulic acid (FA) on MDA formation (A) and TAP level (B) in PC12 cells that are exposed to H_2O_2. Cells were treated with FA for 3 days and then exposed to H_2O_2. Data are represented as mean ± SD (n = 3; * P < 0.05, ** P < 0.01).

cells with FA could significantly reduce MDA level (400 µM; P<0.05) when compared to the H_2O_2-exposed group. Also, a significant increase in TAP level (400 µM; P<0.05) was observed in groups treated with FA when compared to H_2O_2-treated groups (Fig. 3).

FA treatment modulated Caspase-3 and Bcl-2/Bax ratio protein levels

Western blotting analysis was used to determine changes in protein expression of caspase-3 and Bcl-2/Bax in PC12 cells. As the current result demonstrated H_2O_2-exposure remarkably increased cleaved Caspase-3 in PC12 cells compared to control (P<0.01). However, FA pre-conditioning significantly decreased the effect of H_2O_2 on PC12 cells (P<0.05). H_2O_2-exposure markedly decreased the Bcl-2/Bax ratio in PC12 cells (P<0.0001). However, FA significantly increases the Bcl-2/Bax ratio (P<0.01). (Fig. 4).

Discussion

In the current study, the protective effect of FA against some disturbing mechanisms evoked by H_2O_2 was evaluated. The study demonstrated that exposure to H_2O_2 decreased the viability of PC12 cells, indicating the induction of OS. Previous research has shown that H_2O_2 at a concentration of 200 µM for 2h significantly reduces the viability of PC12 cells (Lv et al., 2017). This exposure leads to excessive reactive oxygen species attacking cellular components, resulting in the overproduction of apoptosis mediators (Nita and Grzybowski 2016). lipid peroxidation (LPO), resulting from reactive oxygen species attacking lipids, particularly polyunsaturated

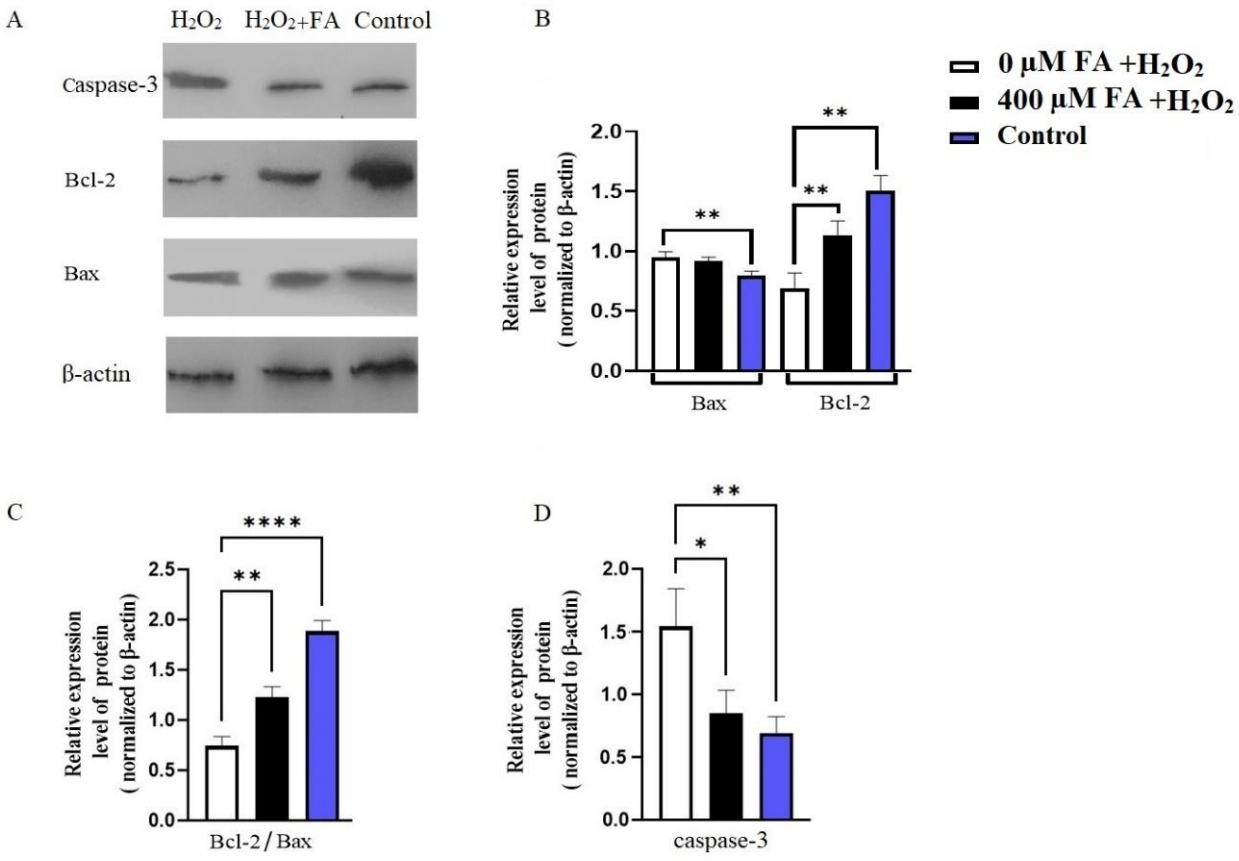

FIGURE 4. Western blotting analysis to determine the effects of Ferulic acid (FA) on Bax, Bcl-2, and Caspase-3 protein expression in PC12 cells. (A) Representative blots and; (B) Semi-quantitative data of Bax and Bcl-2; (C) Semi-quantitative data of Bcl-2 /Bax ratio; (D) Semi-quantitative data of cleaved Caspase-3 protein expression in PC12 cells. Data are represented as mean ± SD (n = 3; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

fatty acids, generates MDA, an indicator of increased OS (Draper and Hadley 1990). LPO products have been implicated in promoting apoptosis (Choudhary et al., 2002). Antioxidants can prevent LPO by scavenging reactive oxygen species, thereby protecting cells from damage (Chen et al., 2023). The study revealed that H_2O_2 increased MDA and decreased TAP levels, consistent with prior research. Yu et al. found that H_2O_2 raised reactive oxygen species and LPO levels while reducing antioxidant enzymes (Yu et al., 2010).

Excessive reactive oxygen species production and mitochondrial overload can lead to cell injury and apoptosis. Mitochondria play a crucial role in determining cell fate. OS triggers the activation of death signals, translocating Bax to mitochondria, where it forms pores, releasing cytochrome c and initiating apoptosis. Cytochrome c nucleates the formation of the apoptosome that activates Caspase 9, 3, and the execution phase of apoptosis (Czabotar and Garcia-Saez 2023). Conversely, overexpression of Bcl-2 inhibits pore formation (Adams and Cory 2018; Poustforoosh et al., 2022). In this study, H_2O_2 decreased the Bcl2/Bax ratio and increased Caspase-3 levels, activating the intrinsic apoptotic pathway. A previous report also has suggested that H_2O_2 conditions markedly increase OS and cleaved caspase-3 ratio in PC12 cells (Ao et al., 2014). The phenolic nucleus and unsaturated side chain in FA contribute to its potent antioxidant properties by preventing LPO and scavenging free radicals (Srinivasan et al., 2007). FA can also upregulate nuclear factor-E2-related factor (Nrf2), activating the antioxidant-responsive element (ARE), and boosting antioxidant enzyme expression (Yeh and Yen 2006). Current results demonstrated that Pre-treating PC12 cells with 400μM FA for 72 hours significantly protected them from H_2O_2-induced damage. In agreement with us, FA demonstrated a suppressive effect against peroxide-induced toxicity in PC12 cells, reducing lipid peroxidation and enhancing TAP levels. Addi-

tionally, FA increased the Bcl-2/Bax ratio and cleaved Caspase 3 levels (Pavlica and Gebhardt 2005). Balasubashini et al. found that FA administration decreased thiobarbituric acid reactive substances (TBARS) and hydroperoxides while increasing reduced glutathione, Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) levels in diabetic rat livers (Balasubashini et al., 2004). It was also suggested by Gupta et al. that FA exerted a protective effect against MPTP/MPP+-related toxicity by decreasing ROS production in PC12 cells and subsequently resulted in reduced Bax/Bcl-2 ratio (Gupta and Benzeroual 2013). FA also exerted a marked protective effect against glutamate toxicity in the cortical neuron through induction of PI3K/Akt and direct up-regulation of Bcl-2 (Jin et al., 2007). Furthermore, FA could bind to Cytochrome c and inhibit apoptosis mediated by Cytochrome c in the SMMC-7721 human hepatoma cell line (Yang et al., 2007). On the contrary, although FA (100 and 400 µM for 18 h) was able to decrease LPO and DNA damage induced by hydrogen peroxide (H_2O_2) (5 mM for 2 h) in peripheral blood mononuclear cells, it did not change the expression of Bcl-2. This discrepancy may arise from the differences between cell types or time of treatment (Khanduja et al., 2006). In a previous study, FA at a lower concentration (40µM) and time (24h) than the concentration used in the current study attenuates H_2O_2-induced injury (1mM for 1 h) by the inhibition of phosphorylation of the extracellular signal-regulated kinase (ERK) and restoring of brain-derived neurotrophic factor (BDNF) (Nakayama et al., 2020). In contrast, in the current study, FA at higher concentration (400µM) and time (72 h) could decrease H_2O_2-induced damage in the PC12 cell line.

In summary, the current results revealed that H_2O_2 significantly decreased the viability of PC12 cells partly via mitochondrial pathway and FA conditioning attenuated OS, Bax/Bcl-2 ratio, and cleaved Caspase-3 protein and, therefore, protected PC12 cells against apoptotic insult. These results suggested that FA might be applied as a promising therapeutic tool to attenuate neuronal damage in Oxidative neuropathy.

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