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ORIGINAL ARTICLE

N^6-(2-hydroxyethyl)-adenosine from *Cordyceps cicadae* attenuates hydrogen peroxide induced oxidative toxicity in PC12 cells

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Abstract

N^6-(2-hydroxyethyl)-adenosine (HEA), is one of the active molecule found in *Cordyceps cicadae*. The protective effect of HEA against H_2O_2 induced oxidative damage in PC12 cells and the mechanism of action was investigated. The cells were exposed to varying concentrations of HEA (5–40 μM) for a period of 24 h and further incubated with 100 μM of H_2O_2 for an another 12 h. Cell viability, LDH release, MMP collapse, Ca^{2+} overload, antioxidant parameters (reactive oxygen species generation (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), inflammatory mediators (interleukins 6 and 1β (IL-6 and IL-1β), tumor necrosis factor alpha (TNF-α) and NF-kB were evaluated. The results obtained showed that cells exposed to H_2O_2 toxicity showed reduced cell viability, increased LDH, ROS and Ca^{2+} overload. However, prior treatment of PC12 cells with HEA increased cell viability, reduced LDH release, MMP collapse, Ca^{2+} overload and ROS generation induced by H_2O_2 toxicity. Furthermore, HEA also increased the activities of antioxidant enzymes and inhibited lipid peroxidation as well as reduced IL-6, IL-1β, TNF-α and NF-kB. Thus, our results provided insight into the attenuative effect of HEA against H_2O_2 induced cell death through its antioxidant action by reducing ROS generation, oxidative stress and protecting mitochondrial function.

Keywords *Cordyceps cicadae*; oxidative stress · N^6-(2-hydroxyethyl)-adenosine · Neurodegeneration · PC12 cells

Introduction

Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease and cerebral ischemia are characterized by a gradual and continuous loss of the functional properties of the neurons in the brain (Yu et al. 2017). The etiology of most neurodegenerative disorders is still evasive and complex, however recent research evidences have implicated oxidative stress as a major contributor to the onset and progression of these incurable and debilitating disorders (Bhat et al. 2015; Halliwell 2006; Xiong et al. 2016). Oxidative stress arises when there is a disruption in the balance between oxidant and anti-oxidant systems due to excessive production of reactive oxygen species (ROS) which exceeds the scavenging capacity of the antioxidant system of the cells. Excessive levels of ROS have the ability to damage DNA, lipids and proteins in the cells leading to cell apoptosis or death (Circu and Aw 2010; Halliwell and Aruoma 1991; Xiong et al. 2016). The brain is easily susceptible to the harmful effect of ROS and oxidative stress due to the high metabolic rate involving the use of oxygen as well as its modest antioxidant defense (Cobley et al. 2018).

Hydrogen peroxide (H_2O_2) is produced during normal physiological functioning of the body as byproduct of enzymatic oxidases. Its concentration is relatively low in normal conditions, however, if in excess H_2O_2 could cause oxidative stress leading to apoptosis and necrosis of neuronal cells (Feng et al. 2016; Hu et al. 2015). Hydrogen peroxide has been widely used as an experimental toxic agent for inducing oxidative stress related cellular damage as well as evaluating

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the potential antioxidant effects of new neuroprotective therapeutic substances (Guo et al. 2016; Song et al. 2015).

Natural products have continued to be a promising alternative treatment for several diseases including neurodegenerative diseases due to their multiple mechanism of action as well as their synergistic properties. In addition, natural products are been considered as potential neuroprotective agents against oxidative damages due to their ability to scavenging excessive ROS and augment the antioxidant system capacity. *Cordyceps cicadae* is a fungus that parasitizes on the larvae of *Cicada flammata*. *C. cicadae* is an edible mushroom which is considered as a valuable traditional medicine, widely used as medicinal and functional food in many Asian countries (Ng and Wang 2005). *C. cicadae* have attracted much attention in recent times due to the multiple pharmacological actions displayed including hypoglycemic, antitumor, immunomodulatory, antibacterial, sedative and renoprotective activities. Nucleosides are considered as one of the major class of bioactive constituents in *C. cicadae*. N^6-(2-hydroxyethyl)-adenosine (HEA) is one of the major active nucleosides in *C. cicadae*. Previous studies have indicated that HEA displayed anti-inflammatory, renoprotective, Ca^{2+} antagonist, antitumor, sedative, insecticidal, radiation resistance and antidiabetic properties (Fang et al. 2016; Furuya et al. 1983; Hermann and Feigl 1992; Li et al. 2019; Lu et al. 2015; Wang et al. 2013; Zhu et al. 2013a, b). However, there are no report on the protective properties of HEA against oxidative neuronal cell death caused by toxicity. Thus, the aim of the study is to investigate the antioxidant effect of HEA against H_2O_2 induced toxicity in PC12 cells and the mechanisms of action.

Materials and methods

Chemical and reagent

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and annexin V-FITC/PI apoptosis detection kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to Bcl-2, Bax as well as caspase-3 were supplied by Santa Cruz Biotechnology (Santa Cruz, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) for cell culture were obtained from Gibco Co. (Grand Island, NY, USA). Malondialdehyde (MDA), lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). DCFH-DA ROS, Rhodamine-123, Fluo-3-AM assay kits were procured from Beyotime Biotechnology Institute (Nanjing, China). All other reagents used were of analytical grade.

HEA isolation and identification

The sample specimens (*C. cicadae*) was obtained from Jurong, Jiangsu province, China and was authenticated by Prof. Zhen Ouyang and a reference specimen (CC-0015) was kept at the herbarium of the School of Pharmacy, Jiangsu University. *C. cicadae* was grinded to powder and extracted with methanol. The extract obtained was filtered and evaporated under reduced pressure. The crude methanolic extract was re-suspended in water and partitioned with hexane, petroleum ether, ethyl acetate and butanol. HEA (Fig. 1a) was obtained from the butanol fraction using repeated silica column chromatography and RP-C18 HPLC. The identity of HEA was authenticated by spectroscopic analysis and by comparing with previous data.

Cell culture and treatment

The cells were cultured in DMEM medium supplemented with FBS (10%), 100 U/ml of penicillin and 100 U/ml streptomycin. The cell cultures were incubated at 37 °C and 5% CO_2 in a humidified environment. After the cells have reached 80% confluence, they were treated with HEA ranging from 5, 10, 20 to 40 μM for 24 h. Thereafter, the cells were further incubated with 100 μM of H_2O_2 for an additional period of 12 h. The cells designated as control cells were treated with DMEM.

Measurement of cell viability

In other to determine the protective effect of HEA on PC12 cells, the MTT assay was assessed to determine the cell viability. PC12 cells were grown in 96-well plates and treated with the various concentration of HEA and H_2O_2. After the treatment, 10 mg/ml of MTT was added to each well containing the treated cells and incubated for 4 h at 37 °C in a humidified environment. DMSO was added to each well to solubilize the formazan and the absorbance of the solution was measured at 540 nm with a microplate reader.

Measurement of LDH release

The measurement of LDH release was determined by LDH assay kit according to the manufacturer's instruction. After cell treatment as previously mentioned above, the cell culture was collected and the supernatant obtained after centrifuge was used for the LDH activity by determining the absorbance at 440 nm using a microplate reader.

Measurement of ROS, MDA, GSH-Px and SOD activity

The amount of ROS generated was assessed using the DCFH-DA fluorescent probe assay. After the treatment of PC12 cells

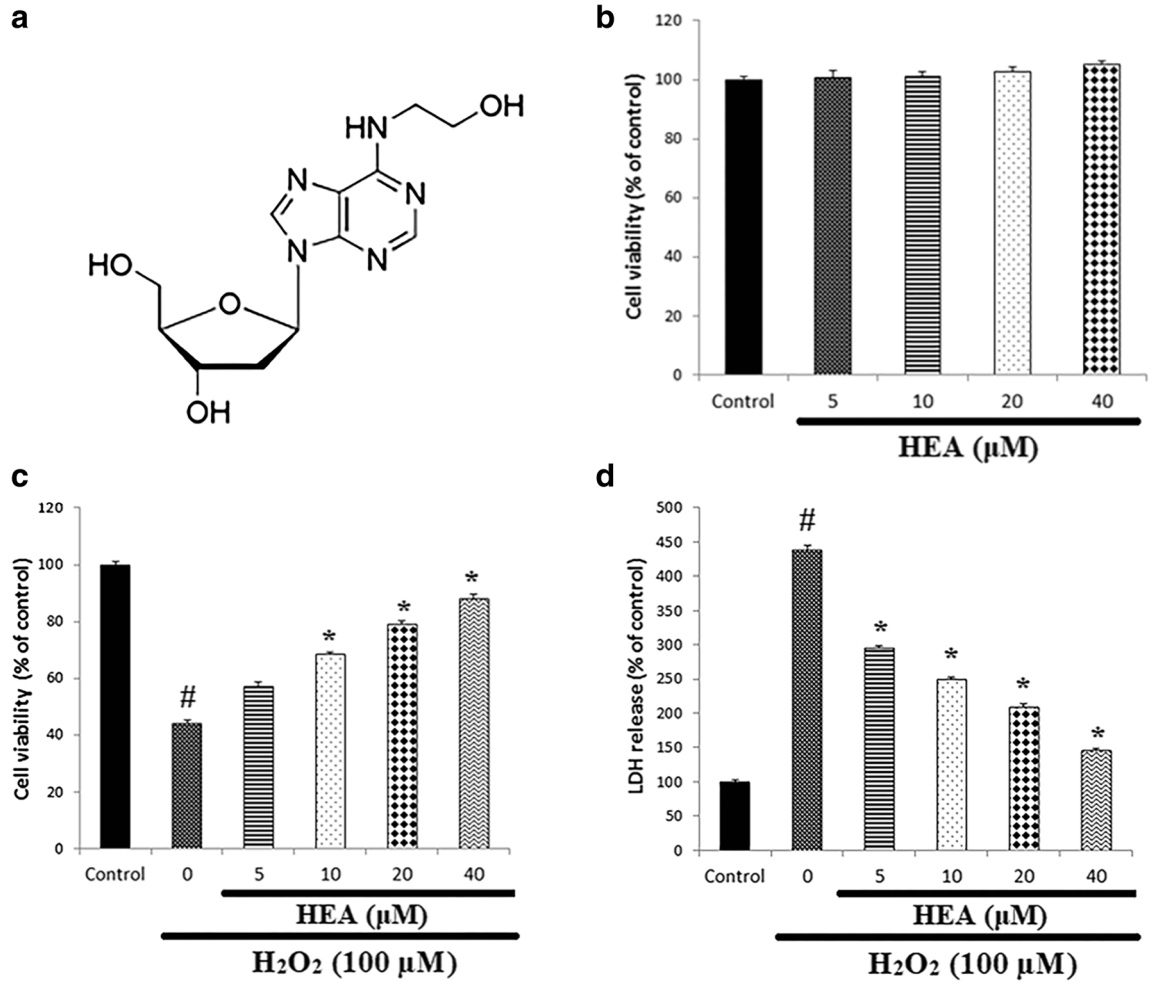

Fig. 1 **a** Structure of HEA; **b** Effect of HEA on PC12 cell viability. Cells were incubated with different concentrations of HEA (0–40 μM) for 24 h. **c** Effect of HEA on PC12 cell viability **d** Effect of HEA on LDH release from PC12 cells exposed to H_2O_2 toxicity. Cells were pretreated with HEA (0, 5, 10, 20 and 40 μM) for 24 h and then exposed to 100 μM of H_2O_2 for 12 h. After treatment, cell viability was determined by MTT assay. Data are expressed as mean ± SD ($n = 5$). #$p < 0.05$ as compared to control; *$p < 0.05$ as compared to H_2O_2 treated group

with HEA or H_2O_2, the medium was removed and the cells were washed with PBS. The cells were further incubated in the dark with DCFH-DA dissolved in DMEM at 37 °C for 30 mins. The fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

MDA content, GSH-Px and SOD activities were measured according to the manufacturer's protocol in the assay kit. After cell treatment and washing thrice with PBS, lysed with lysis buffer (0.5% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.0), centrifuged and the supernatant obtained was used for the determination of the activities of the antioxidant enzymes and MDA levels based of specified protocols in the assay kit.

Measurement of inflammatory mediators

PC12 cells (1×10^6) were seeded in in 6-well plate at 37 °C and incubated for 24 h. After treatment with HEA and H_2O_2, the cells were washed twice with PBS, lysed with lysis buffer (0.5% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.0) and centrifuged. The supernatant obtained was used for the determination of inflammatory cytokines namely tumor necrosis factor-α (TNF-α), interleukins (IL)-1β, IL-6 and NF-kB using commercial ELISA kits (Beyotime Institute of Biotechnology and Elabscience Biotechnology, Wuhan, China).

Measurement of cell apoptosis

Cell apoptosis was assessed with the aid of Annexin V-FITC/PI apoptosis assay. In brevity, after the treatment of cells as previously described, the cells were harvested and washed with PBS twice. After washing the cells were re-suspended in buffer and incubated with 5 μl of Annexin V/FITC and propidium iodide for 30 min in the dark at room temperature. The cells were analyzed using flow cytometry (FAC Scan, BD Biosciences).

Measurement of intracellular calcium (Ca^{2+})

The intracellular calcium was determined with the aid of Fluo-3 AM dye florescence based on the instructions in the manufacturer's manual. Briefly, Fluo-3-AM solution was added to post treated PC12 cells and incubated in the dark for 1 h at 37 °C. After incubation, the cells were washed thrice with PBS to remove extracellular dye. The fluorescent intensity was measured at an excited wavelength of 488 nm and an emission wavelength of 525 nm using a spectrofluorometer.

Measurement of mitochondrial membrane potential (MNP)

Mitochondrial membrane potential was detected through rhodamine-123 fluorescence method. In brief, after cell treatment rhodamine-123 solution was loaded on the treated PC12 cells for 15 min. The fluorescence was determined at an excitation wavelength of 490 nm and an emission wavelength of 530 nm. MMP was expressed as the percentage of fluorescence intensity when compared to control group.

Western blot analysis

The treated PC12 cells were analyzed for the expression of apoptotic related proteins using western blot. Concisely, post treated cells were lysed using ice-cold RIPA lysis buffer for 30 min. After lysis, the cells were subjected to centrifugation (12,000 g for 20 min at 4 °C) and the supernatant obtained was used for the quantification of total protein content with the aid of a BCA protein assay kit. The proteins samples were subjected to electrophoresis on a 10–15% SDS polyacrylamide gel and further transferred to a PVDF membranes. The membranes were subjected to blocking with 5% non-fat dry milk and incubated through the whole night at 4 °C with primary antibodies of Bcl-2 (1:1000), Bax (1:1000) and cleaved caspase-3 (1:1000). The membranes were washed three times and further probed with secondary antibodies (1:5000) for 1 h and detection was carried out using ECL substrate.

Statistical analysis

Data are shown as mean ± SD of at least three independent experiments. SPSS (version 16.0, SPSS Inc., Chicago, IL, USA) was used for result analyses. One way Analysis of Variance (ANOVA) followed by Tukey Post hoc analysis was used for the analysis of statistical differences. Differences were considered statistically significant when $p < 0.05$.

Results

HEA prevented H_2O_2 induced PC12 cell viability loss

In other to determine whether HEA was cytotoxic to PC12, cell viability was determined using MTT assay. As indicated in Fig. 1b, HEA had no cytotoxic effect on PC12 cell viability even up to the highest concentration of 40 μM (>100%). We further evaluated the protective effect of HEA on H_2O_2 induced cytotoxicity in PC12 cell. As shown in Fig. 1c, HEA significantly increased cell viability in a dose dependent fashion as compared to the H_2O_2 group. The cell viability of the untreated PC12 cells after exposure to H_2O_2 was 44.86%.

Effect of HEA on H_2O_2 induced cell cytotoxicity

The effect of HEA on the amount of LDH released after H_2O_2 induced toxicity is as shown in Fig. 1d. After the exposure of cells to H_2O_2 toxicity, the LDH release was markedly increased (4.5 fold) in the H_2O_2 treated group when compared to the normal control cells. In contrast, treatment with various concentration of HEA significantly inhibited the release of LDH. These results suggests that HEA could protects PC12 cells from H_2O_2 induced LDH release.

Effect of HEA on H_2O_2 induced cell apoptosis

The cells apoptotic rate was evaluated by flow cytometry analysis using Annexin V/PI double staining method. As shown in Fig. 2a, the results unveiled a significant reduction in the apoptotic rate in cells pretreated with 40 μM HEA before exposure to H_2O_2 toxicity. The rate of apoptosis significantly increased to 20.06% after H_2O_2 treatment, but decreased to 16.08% and 14.26% when pretreated with HEA (20 and 40 μM, respectively).

Effect of HEA on H_2O_2 induced ROS generation

ROS is an important parameter that has been implicated in oxidative and ER dysfunction of neuronal cells. We determined the effect of HEA on intracellular ROS generation after cells were exposed to H_2O_2. As shown in Fig. 2b, a significant elevation of ROS generation was observed in PC12 cells upon exposure to H_2O_2. The level of ROS produced following the exposure to H_2O_2 (100 μM) in the untreated cells was of 3.5-fold magnitude when compared to the control cells. HEA dose dependently inhibited the fluorescent intensity in the pretreated cells when compared with the H_2O_2 treated group. These findings unveil that HEA can decrease H_2O_2 induced ROS production.

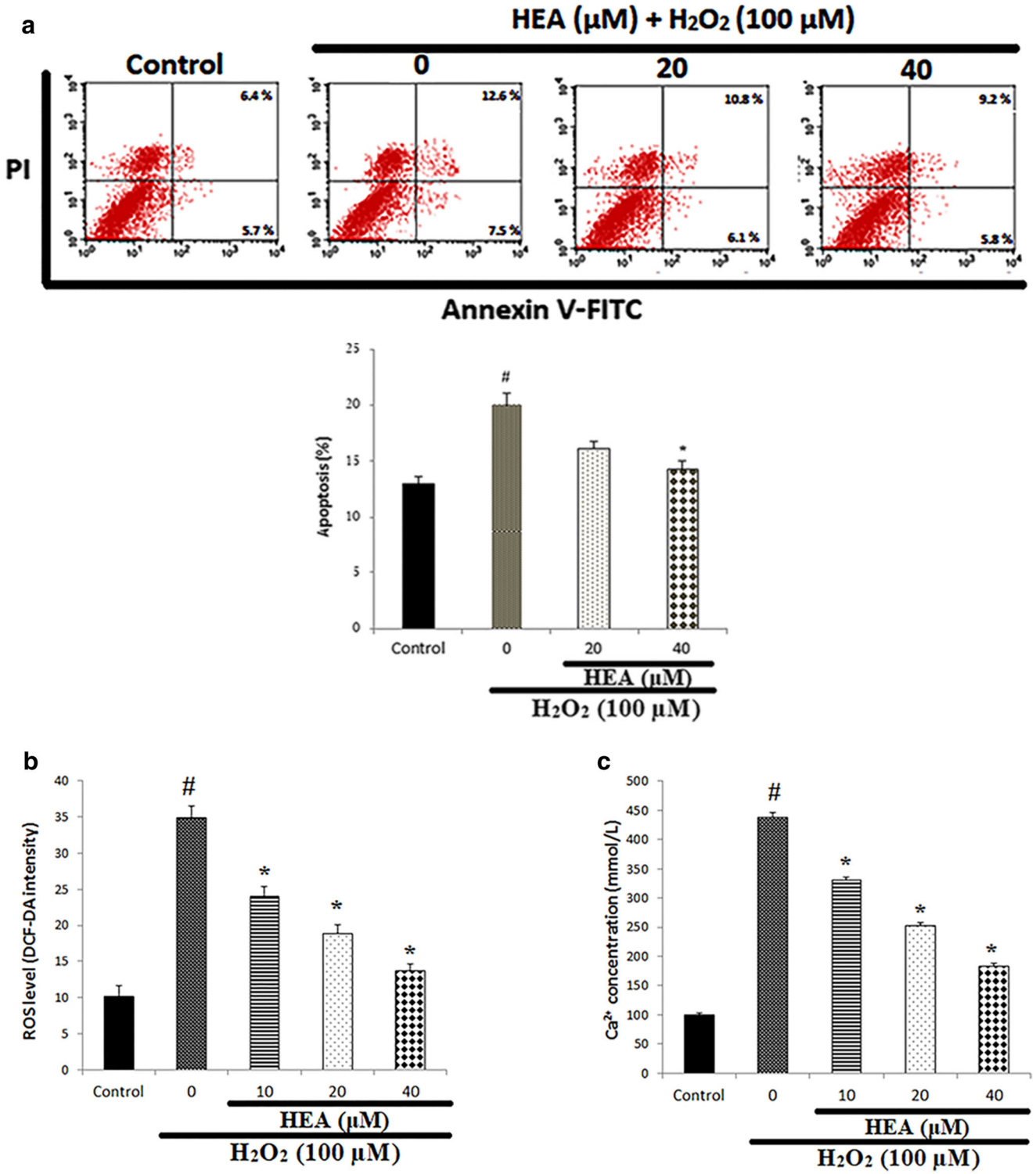

Fig. 2 **a** Effect of HEA on H_2O_2-induced apoptosis in PC12 cell. Cells were pretreated with HEA (0, 20 and 40 μM) for 24 h and then exposed to 100 μM H_2O_2 for 12 h. After treatment, cells were treated with Annexin V-FITC/PI and incubated at room temperature for 30 min in the dark; **b** Effects of HEA against H_2O_2-induced ROS production in PC12 cells. Cells were pretreated with HEA (0, 10, 20 and 40 μM) for 24 h and then exposed to 100 μM H_2O_2 for 12 h. After treatment, cells were incubated with DCFH-DA at 37 °C for 30 min and the fluorescence of ROS-oxidized DCFH-DA probe was measured **c** Effect of HEA against H_2O_2-induced intracellular Ca^{2+} influx in PC12 cells. Cells were pretreated with HEA (0, 10, 20 and 40 μM) for 24 h and then exposed to 100 μM H_2O_2 for 12 h. After treatment, cells were incubated with Fluo-3-AM incubated in the dark for 1 h at 37 °C and the fluorescent intensity was measured. The data are represented as means ± SD ($n = 5$). #$p < 0.05$ as compared to control; *$p < 0.05$ as compared to the H_2O_2 treated group

Effect of HEA on Ca^{2+}influx in H_2O_2 induced PC12 cells

The level of intracellular Ca^{2+} was observed to be significantly increased in untreated cells after exposure to H_2O_2 toxicity in comparison to the control group, while the pretreatment of different concentration of HEA markedly reduced the intracellular level of Ca^{2+} in a concentration dependent manner (Fig. 2c).

Effect of HEA on MDA content, GSH-Px and SOD activities

As illustrated in Fig. 3a, PC12 cells exposed to H_2O_2 treatment without prior treatment with HEA have significantly higher level of MDA, which was approximately 4 fold when compared to the normal control cells. Furthermore, GSH-Px and SOD activities were decreased in the untreated cells after been exposed to H_2O_2. However, increase in the antioxidant enzymes activities were observed in the cells pretreated with HEA (Fig. 3b-c). These results demonstrates that HEA can attenuate oxidative stress via reducing MDA and increasing GSH-Px and SOD activities.

Effect of HEA on inflammatory mediators in H_2O_2 induced PC12 cells

The effect of HEA on the release of inflammatory mediators in H_2O_2 induced PC12 cells was examined. As indicated in Fig. 4, the exposure of PC12 cells to H_2O_2 toxicity significantly increased the concentration of TNF-α, IL-1β and IL-6, while the pretreatment of cells with HEA significantly reduced TNF-α, IL-1β and IL-6 concentrations in the H_2O_2 treated cells (Fig. 4a-c). Furthermore, the expression of NF-κB protein in the PC12 cells pretreated with HEA was significantly decreased when compared to the cells exposed to H_2O_2 toxicity alone (Fig. 4d).

Effect of HEA on MMP loss in H_2O_2 induced PC12 cells

The results clearly show that a significant loss in the MMP of cells subjected to H_2O_2 toxicity, while on the contrary pretreatment of PC12 cells with HEA ameliorated the loss in MMP. The recovery in the MMP of cell pretreated with 20 and 40 μM was 77.30% and 83.10%, respectively (Fig. 5a).

Effect of HEA the expression of caspase-3, Bcl-2 and Bax levels in H_2O_2 induced PC12 cells

As the result indicated in Fig. 5b-c, Bax and cleaved caspase-3 levels were significantly increased after PC12 cells exposure to H_2O_2, while Bcl-2 levels was down regulated. Nonetheless, the treatment of cells with various concentrations of HEA increased the level of Bcl-2, while Bax and cleaved caspase-3 levels were reduced.

Discussion

Oxidative stress is key to the pathological processes leading to neurodegenerative disorders such as Parkinson's disease, ischemic stroke and Alzheimer's disease. Therapeutic intervention geared towards enhancing antioxidant potential could be of beneficial clinical importance (Kim et al. 2012). Cells have an inbuilt mechanism for fighting against ROS, which is directly linked to the actions of antioxidants enzymes such as GSH-Px and SOD. Thus, inducing the activities of these enzymes can afford protection against oxidative stress induced neuronal cell death (Hseu et al. 2012).

Hydrogen peroxide toxicity is known to be critically involved in the pathophysiological process leading to several neurodegenerative diseases via oxidative stress and excessive free radical production which can cause injury and death to the cells by

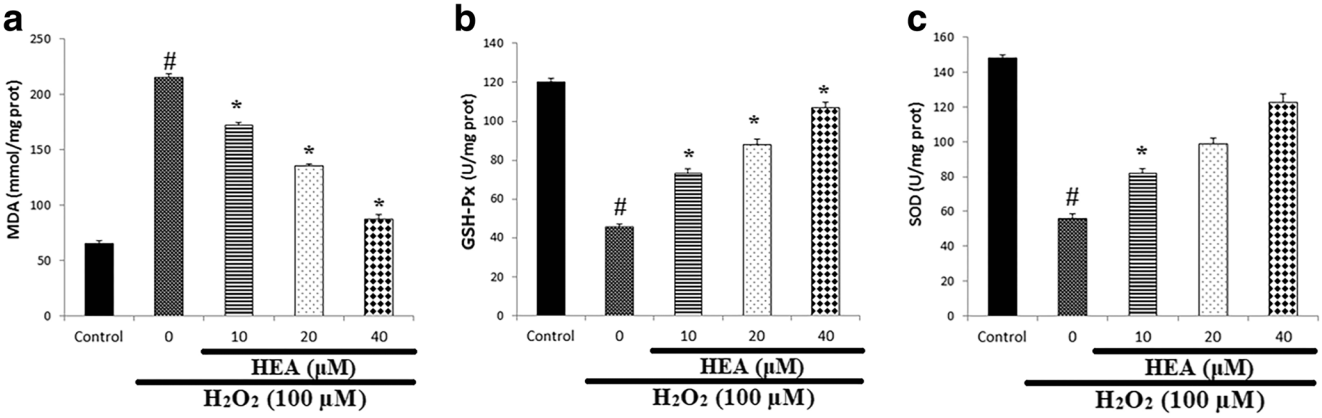

Fig. 3 Effect of HEA on **a** MDA levels activity in H_2O_2 treated PC12 cells; **b** GSH-Px activity in H_2O_2 treated PC12 cells; **c** SOD activity in H_2O_2 treated PC12 cells. Cells were pretreated with HEA (0, 10, 20 and 40 μM) for 24 h and then exposed to 100 μM H_2O_2 for 12 h. After treatment, MDA, GSH-Px and SOD levels were determined by commercial ELISA kits. The data are represented as means ± SD (n = 5). #$p < 0.05$ as compared to control; *$p < 0.05$ as compared to the H_2O_2 treated group

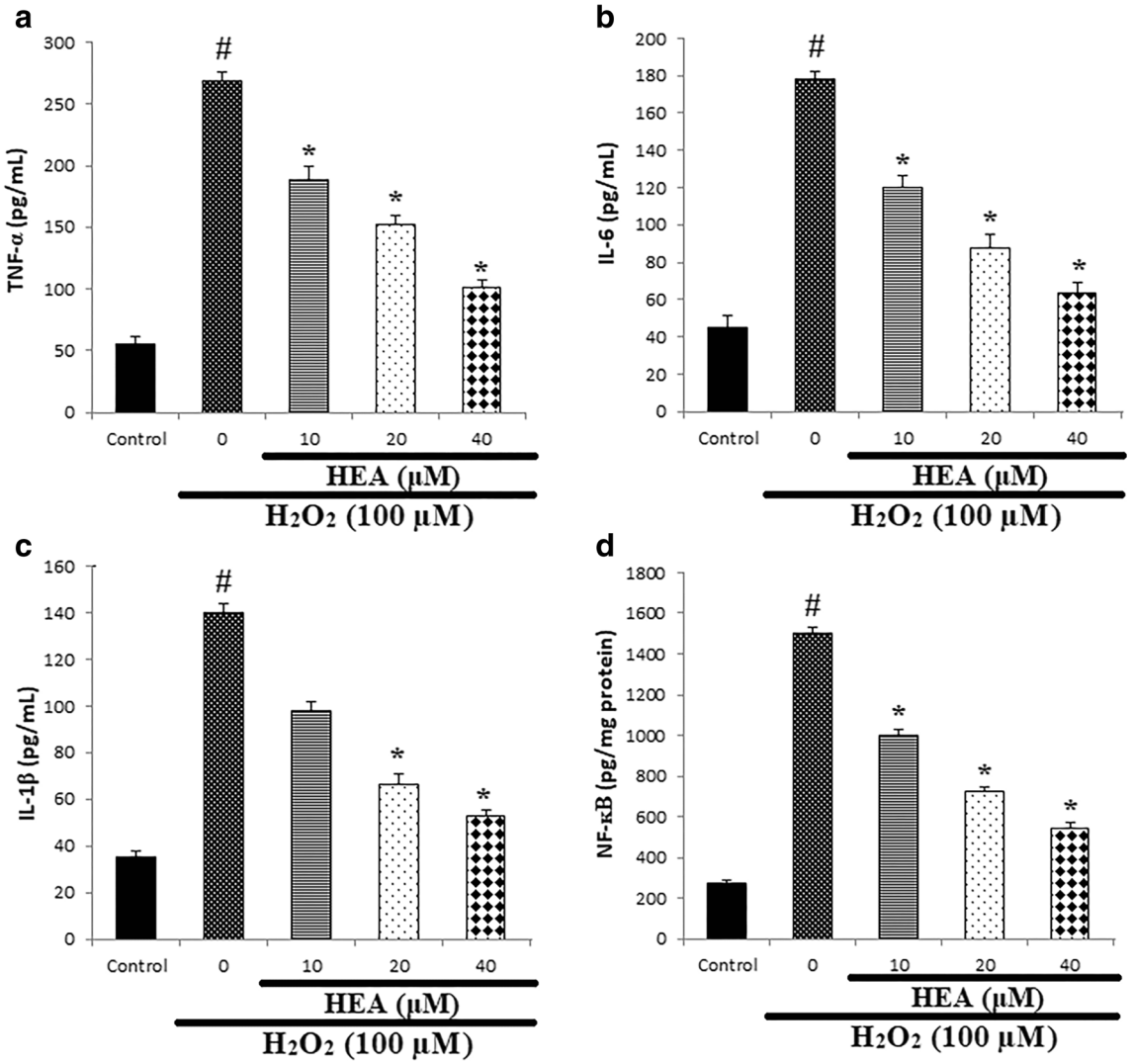

Fig. 4 Effect of HEA on **a** TNF-α levels in H_2O_2 treated PC12 cells; **b** Effects of HEA on IL-6 levels in H_2O_2 treated PC12 cells; **c** Effect of HEA on IL-1β levels in H_2O_2 treated PC12 cells **d** Effect of HEA on NF-κB levels in H_2O_2 treated PC12 cells. Cells were pretreated with HEA (0, 20 and 40 μM) for 24 h and then exposed to 100 μM H_2O_2 for 12 h. After treatment, TNF-α, IL-6, IL-1β and NF-κB levels were determined by commercial ELISA kits. Data are expressed as mean ± SD ($n = 5$). #$p < 0.05$ as compared to control; *$p < 0.05$ as compared to H_2O_2 treated group

regulating biochemical changes that causes neurodegenerative disorders (Divate et al. 2017; Hu et al. 2015; Uttara et al. 2009). Indisputable evidences have indicated that exposure of in vivo and in vitro models to H_2O_2 toxicity led to a marked increase in ROS generation and oxidative stress, causing damages and impairment to cell (Chansriniyom et al. 2018; Stanić et al. 2016; Zhu et al. 2013a, b). Antioxidants from natural products have been shown to have the ability in preventing the development and progression of several neuronal disorders (Escribano et al. 2018; Li et al. 2018; Singhrang et al. 2018; Xiong et al. 2016). This study revealed the neuroprotective effects of HEA against H_2O_2 induced oxidative cell apoptosis.

Upon the exposure to H_2O_2, the viability of PC12 cells was decreased indicating an increase in cell death, however after treating cells with HEA, cell viability was restored indicating the protective effect on PC12 cells against H_2O_2 cytotoxicity. LDH is an enzyme that is released when the integrity of cell membrane is compromised leading to cell death (Li et al. 2018). Increased LDH release in cell culture medium signifies cell oxidative damage. The present results illustrated that H_2O_2 toxicity elevated LDH content in the cell culture medium, whereas HEA effectively inhibited the leakage of LDH induced by H_2O_2 which correlates with the increase in cell survival after pretreatment.

During redox reaction in the cells, H_2O_2 is produced as one of the major ROS and it is involved in a number of intracellular signaling cascades (Finkel 1998; Rhee 1999; Wang et al. 2018). It is undoubtedly recognized that ROS such as hydroxyl radicals, peroxynitrite and superoxide anion damages biological molecules resulting in cell apoptosis or necrosis, which ultimately cause neurological disorders. Furthermore, H_2O_2 increases lipid peroxidation, MMP dysfunction due to impaired energy

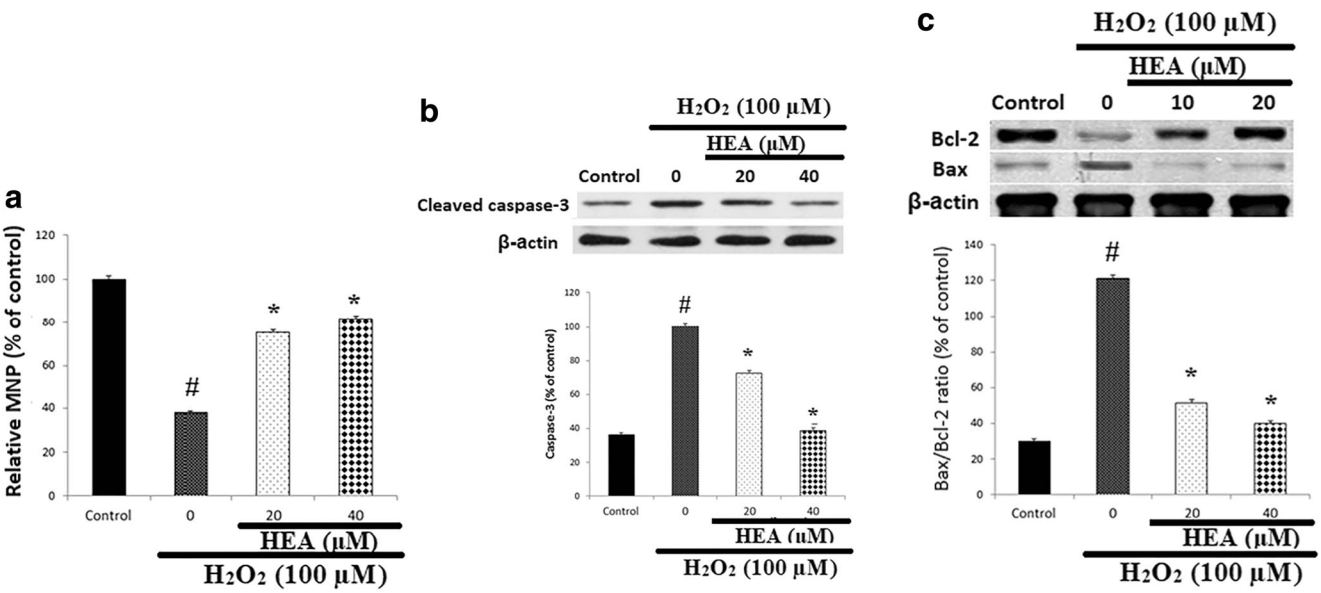

Fig. 5 Effect of HEA on **a** MMP in H_2O_2 treated PC12 cells; **b** Cleaved caspase-3 expression in H_2O_2 treated PC12 cells; **c** Bcl-2/Bax protein expression in H_2O_2 treated PC12 cells. Cells were pretreated with HEA (0, 20 and 40 μM) for 24 h and exposed to 100 μM H_2O_2 for 12 h. MMP quantitative analysis was determined by measuring the fluorescence intensity. The levels of cleaved caspase-3, Bax and Bcl-2 were determined by western blot analysis. Data are expressed as mean ± SD ($n = 5$). #$p < 0.05$ as compared to control; *$p < 0.05$ as compared to H_2O_2 treated group

production and DNA damage resulting in cell death (He et al. 2018; Kwon et al. 2015; Satoh et al. 1997). The fluorescent intensity of DCF in PC12 cells was significantly increased after exposure to H_2O_2 treatment. Interestingly, pretreatment with HEA markedly decreased H_2O_2 induced ROS production, which suggested that the protective effects of HEA might be related to the inhibition of intracellular ROS production.

Oxidative cellular damage caused by ROS is normally accompanied by an increase in lipid peroxide which reduces membrane fluidity and enzyme activity. Cells are equipped with endogenous protective mechanisms against insults from ROS and oxidative stress. Endogenous antioxidants including SOD and GSH-Px are actively involved the neutralization and detoxification of ROS (Mates 2000; Xiong et al. 2016). The content of the end product of lipid peroxidation (MDA) is an indication of the level of oxidative stress and the impediment of the antioxidant system in an organism (Kwon et al. 2015). The data from this study demonstrated a reduction in the activities of SOD and GSH-Px after the exposure of PC12 cells to H_2O_2. However, HEA augmented the activities of these antioxidant enzymes in pretreated cells. These results suggests that the protective property of HEA could be possibly related to the activation of antioxidant defense enzymes.

Oxidative stress has been shown to enhance the release of several inflammatory mediators which in turn contributes to the progression of degenerative diseases (Guo et al. 2012; Hsieh and Yang 2013; Liu et al. 2006). Several proinflammatory cytokines and proteins have been reported to positively correlate to oxidative stress in neurodegenerative disorders (Chiurchiù and Maccarrone 2011; Li and Li 2015). NF-κB, a vital inflammatory transcription factor, plays a vital role in neuronal cell survival and this transcriptional factor is involved in the pathogenesis of several neurodegenerative diseases (Kaltschmidt et al. 2005; Liu et al. 2011; Mattson 2005). Our study portrayed that H_2O_2 exposure to PC12 cells induced an increase in the levels of TNF-α, IL-1β, IL-6 and NF-κB, however, HEA pretreatment significant reduced the levels of these inflammatory mediators.

A number of mitochondrial apoptosis related proteins are triggered during oxidative stress induced mitochondrial dysfunction. Notable among these are the Bcl-2 proteins family, which play a paramount role in the regulation of cell apoptosis (Antonsson 2004; Beal 2005; Li et al. 1999). Bcl-2 (anti-apoptotic protein) and Bax (pro-apoptotic protein) regulates cell death by controlling mitochondrial function, which is linked to cytochrome c release from the mitochondrial. Blc-2 can inhibit the activation of caspases and the release of cytochrome c, while Bax can induce the release of cytochrome c, thus enacting the downstream apoptosis cascade (Chen et al. 2015). Our results portrayed that H_2O_2 toxicity led to an increase in the levels of caspase-3 and pro-apoptotic protein Bax, while the level of Bcl-2 was down regulated, while HEA pretreatment decreased caspase-3 and Bax level and increased the amount of Bcl-2.

In conclusion, the results from this present study demonstrated that HEA could protect against H_2O_2 induced oxidative damage in PC12 cells through its ROS scavenging ability. Furthermore, HEA significantly enhanced the antioxidant enzymes GSH-Px and SOD, prevented mitochondrial membrane dysfunction by increasing Bcl-2, decreasing Bax and caspase-3 levels. These results clearly support the protective effect of HEA is mediated through its antioxidant properties.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest associated with this manuscript.

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