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Original Article

Carbamylated erythropoietin-Fc ameliorates Aβ25-35 induced neurotoxicity by modulating autophagy, apoptosis and necroptosis in Alzheimer's disease model rats

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ABSTRACT

Introduction: Alzheimer's disease (AD) is a progressive and chronic neurodegenerative disorder in which amyloid-β (Aβ) and hyperphosphorylated-tau (P-tau) are well-established pathological hallmarks. Carbamylated erythropoietin (CEPO-Fc) is one of the erythropoietin derivatives with neuroprotective properties against neurodegenerative disorders. However, the underlying molecular mechanism of CEPO-Fc has not been fully elucidated. Therefore, we investigated the therapeutic effects of CEPO-Fc on Aβ-induced neurotoxicity in the *in-vivo* rat model.

Methods: Adult male Wistar rats were cannulated in the dorsal hippocampus and $A\beta_{25-35}$ was microinjected for four consecutive days. CEPO-Fc was administered intranasally during the next six consecutive days. Learning and memory performance were examined (days 10-13) using the Morris water maze test. Furthermore, the hippocampal levels of critical proteins involved in apoptosis (Bax, Bcl-2 and caspase-3), necroptosis (phosphorylated-receptor-interacting serine/threonine-protein kinase 3) and autophagy (p-Beclinbeclin-1 and phosphorylated- 1A/1B-light chain 3) were assessed using immunoblotting.

Results: Behavioral analysis showed that CEPO-Fc treatment significantly improved Aβ-induced learning and memory impairment. Furthermore, the hippocampus's molecular analysis showed that CEPO-Fc induced up-regulation of the autophagic proteins, p-Beclin-1 and p-LC3-II, while decreased caspase-3, Bax/Bcl2 ratio as well as the necroptosis factor p-RIP3.

Conclusion: Our results indicate that the neuroprotective properties of CEPO-Fc in animal model of AD could be mediated by autophagy activation and inhibition of apoptosis and necroptosis processes. This study introduces CEPO-Fc as a potential protective compound against AD and other neurodegenerative disorders.

Keywords:
Alzheimer's disease
CEPO-Fc
Apoptosis
Autophagy
Necroptosis

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and one of the most common causes of dementia, affecting almost 10% of people over age 65 (Breijyeh and Karaman, 2020). Various factors are involved in the onset of AD, including diabetes, obesity, smoking, cognitive and physical inactivity, high blood pressure (in middle-age) and depression (Armstrong, 2019). AD is characterized by intracellular aggregation of neurofibrillary tangles (NFTs) containing hyperphosphorylated-tau (P-tau) and extracellular accumulation of β-amyloid (Aβ) plaques particularly in the memory- and cognitive-related regions such as the hippocampus (Tiwari et al., 2019). These pathological features are usually associated with memory deficits and impairment in reasoning, planning, speaking and perception, which eventually affects the patient's practical skills (Atri, 2019; Joe and Ringman, 2019).

A substantial body of evidence considers Aβ a leading cause of neuronal loss in AD (Liu et al., 2019; Zhang and Zheng, 2019). Analyzing the transgenic mice with an aberrant level of Aβ elucidated AD's pathological and behavioral symptoms (Bayer and Wirths, 2008; Ivins et al., 1999). Aβ peptide is highly toxic to neurons. It consists of 39-43 amino acid residues which is generated from the amyloid precursor protein (APP). APP cleaved sequentially by β- and γ-secretases (Pourhamzeh et al., 2020). Aβ toxicity is principally due to an 11 amino acid fragment between residues 25 and 35 ($A\beta_{25-35}$) (Chen et al., 2017). It is believed that Aβ neuronal toxicity could be mediated through local inflammation and apoptosis induction (Chen et al., 2006; Wu et al., 2014). Intracellular Aβ could be found in subcellular compartments including mitochondria, Golgi, endoplasmic reticulum, lysosomes and cytosol (Zheng et al., 2011). Ubiquitin-proteasome and autophagy-lysosome pathways are implicated in Aβ degradation, which is impaired in AD (Nilsson et al., 2013; Nilsson and Saido, 2014). Accordingly, when the autophagy regulator Beclin-1 was removed in the mouse model, both intracellular and extracellular aggregation of Aβ increased (Ma et al., 2017; Pickford et al., 2008).

Erythropoietin (EPO) is a glycoprotein with hematopoietic and neuroprotective activity (Hemani et al., 2021). EPO expression and its receptor (EPOR) are significantly enhanced in the CNS following ischemia, hypoxia and other stress conditions (Suresh et al., 2020). It has also been shown that EPO exerts neuroprotective effects on PC12 cells, which underwent $A\beta_{25-35}$ administration by stabilizing the mitochondrial membrane potential, decreasing the excessive generation of reactive oxygen species (ROS) and suppressing Bax/Bcl-2 ratio and caspase-3 level (Li et al., 2008). Evidence indicates that non-erythropoietic EPO derivatives have similar neuroprotective effects without common EPO side effects such as hypertension. Carbamylated erythropoietin-Fc (CEPO-Fc) is one of the EPO derivatives which stimulates EPOR/common β receptor (βcR) but not erythropoiesis (Brines et al., 2004; Coleman et al., 2006; Fantacci et al., 2006; Montero et al., 2007). Several studies on animal models of neurotoxicity have reported neural protection of CEPO-Fc (King et al., 2007; Lapchak et al., 2008; Wang et al., 2007); however, the underlying mechanisms are still under investigation. Hence, we herein studied the possible neuroprotective effects of CEPO in an AD rat model by evaluating neuronal apoptosis, necroptosis and autophagy.

Material and methods

Animals

Thirty-five Adult male Wistar rats, weighing 250-300g, were purchased from the Shahid Beheshti University of Medical Sciences, Tehran, Iran. Animals were housed (three per standard plastic cage) in a room with a 12h light/dark cycle (lights on at 07.00 AM) and controlled temperature (24±1°C). Unlimited access to water and standard food (27% protein, 32% fat and 41% carbohydrate) was provided.

All of the procedures that involved animals and their care were carried out under the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996) and confirmed by the Institutional Animal Care and Use Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran) (IR.SBMU.PHNS.REC.1397.109).

Study design

Rats were randomly divided into five groups (n=7 in each group) as follows: healthy control group without any intervention; AD model group, which only received an intra-hippocampal injection of $A\beta_{25-35}$ (5μg/2.5 μl per side/day) in a four-day duration (days 0 – 3); CEPO-Fc treated groups in which AD rats received nasal administration of CEPO-Fc at two doses of 75 and 112 IU/kg

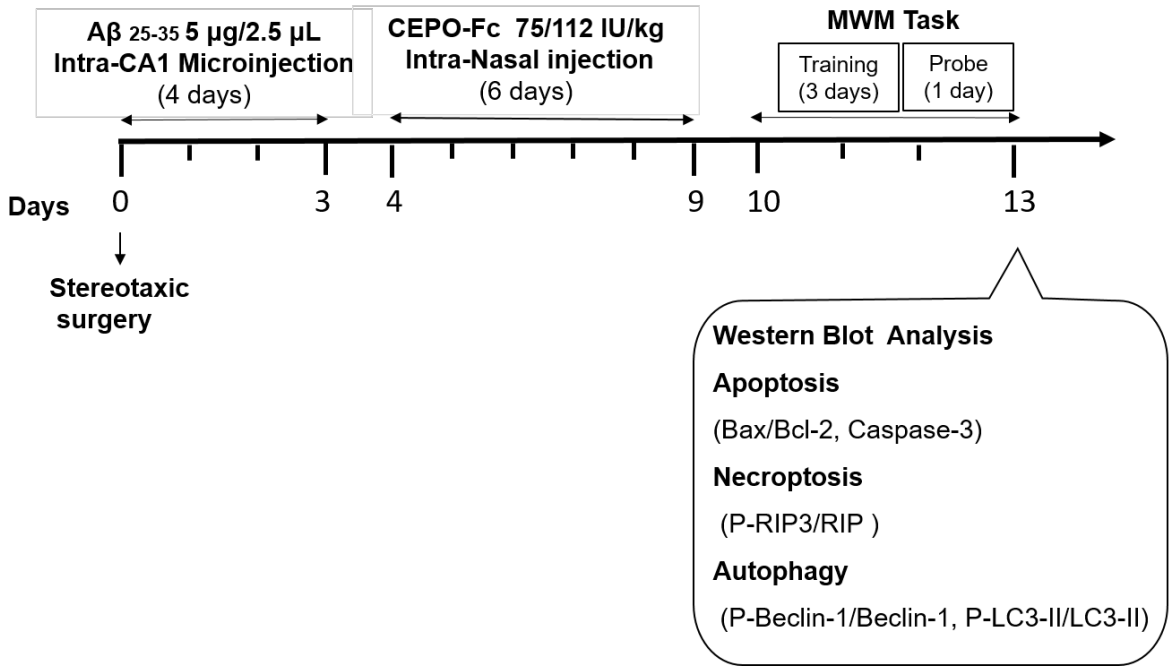

FIGURE 1. Graphical scheme to show experimental design of the study.

daily for six consecutive days (days 4 – 9) in two separate groups (AD-CEPO 75 and AD-CEPO 112); and a sham CEPO-Fc group, which only received the highest dose of CEPO-Fc (112 IU/kg per day) during days 4 – 9 (CEPO 112 group) to rule out the possible effects of CEPO-Fc. Then, animals were subjected to behavioral and molecular analysis (Figure 1).

Preparation and administration of drugs

$A\beta_{25–35}$ (Sigma-Aldrich, A4559) was prepared in a concentration of 2µg/µl in distilled water and kept at –20°C. According to our earlier studies (Hooshmandi et al., 2020; Hooshmandi et al., 2018), $A\beta_{25–35}$ aggregations was made by incubating at 37°C for four days. To induce AD model, $A\beta_{25–35}$ (5µg/2.5 µl, per side) was microinjected in the dorsal hippocampus during four days.

CEPO-Fc complex is made of two rhEPO molecules bound to the Fc domain of a human IgG1 antibody which underwent carbamylation to remove the erythropoietic potency (Schriebl et al., 2006). CEPO-Fc was prepared in our lab (Vienna, Austria). The main stock of CEPO-Fc was prepared in a concentration of 1.91mg/ml (2.3×10^5 IU) in phosphate-buffered saline and then the required dilutions (75 and 112IU) were prepared using the main stock. AD animals in the treatment groups received nasal CEPO-Fc in doses 75 or 112IU/kg for six consecutive days started one day following the last dose of Aβ.

Stereotaxic surgery

Rats were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). The animals were immobilized in a stereotaxic frame. Based on the Paxinos brain atlas guidelines (Paxinos and Watson, 2007), two guides cannulas bilaterally placed into the dorsal hippocampus (AP: -3.8, ML: ±2.2, DV: -2.7) (Hooshmandi et al., 2018) and fixed to the skull using a jeweler's screw and secured by dental cement.

$A\beta_{25–35}$ was directly microinjected to the dorsal hippocampus by a 5-µl Hamilton syringe with a 30-gauge microinjection needle inserted 0.5mm beyond the guide cannula's tip. Each 0.5µl of drug solution was infused during a 2-min period and the needle was left in the injection site for an additional minute to prevent regurgitation. During the infusion procedure, rats were allowed to move freely. The $A\beta_{25–35}$ microinjections were carried on continuously for four days (days 0-3).

Morris water maze test (MWM)

MWM was used to assess spatial memory and learning ten days following stereotaxic surgery (Hooshmandi et al., 2018). The apparatus is a dark circular pool (140cm in diameter and 55cm in high) filled with water (25±1°C) to a depth of 25cm. Four portions in the pool

area were considered; north-east (NE), south-east (SE), south-west (SW) or northwest (NW). An 11-cm-diameter escape platform was placed 1cm below the water's surface in the center of one of the quadrants (the target quadrant). The rats were supposed to use the distal spatial extra-maze cues in the training room, such as posters, to find the escape platform.

The MWM test consisted of a habituation phase, a training phase and a probe trial (days 10–13). All of the trials were performed during 9:00-11:00 AM. During habituation, rats were allowed to swim in the pool (without the platform) for 60s a day before starting the training phase. In the training phase, rats were given four trials daily for three days. The starting point was chosen randomly (NE, SE, SW and NW) and varied in each trial. Each rat had 60s to find the hidden platform in each trial and remained on the platform for the following 20s when it reached it. In case of failure, rats were guided to the platform gently. Before starting the subsequent trial, the rats were dried and kept in their home cages for 30s. The escape latency (spent time) and the traveled distance to reach the invisible platform were measured for each trial during days 1-3 of training.

On the fourth day, the rats underwent probe trials without the escape platform. The subjects were released from the opposite location of the target site and allowed to swim in the pool for the next 60s freely. Then, time spent in the target quadrant and swimming speed were measured. To identify rats with performance problems associated with motor abnormalities, swimming speed was evaluated at the probe trial. The rats' behaviors in the maze were tracked by a 3CCD video camera linked to the Noldus EthoVision (7.1 version, Noldus Information Technology, Netherlands) placed above the maze. At the end of the behavioral assessment, rats were sacrificed and the hippocampus was dissected and after snap-frozen in liquid nitrogen kept at -80°C for molecular assessment.

Immunoblotting analysis

Frozen hippocampal tissues were homogenized with lysis buffer (50mM Tris-HCl, pH8.0; 150mM NaCl; 1% Triton X-100; 0.5% Na-deoxycholate; 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Thermo Fisher Scientific, A32963). Lysates were centrifuged at 14,000 rpm for 30min at 4°C. The protein content of samples was quantified using the bovine serum albumin-based Bradford method. Then, loading sample buffer was added to the protein samples and heated at 100°C for 5min. Electrophoresis of samples with an equal concentration of protein (50μg) was performed on 12% SDS-PAGE gel. The obtained protein band was transferred to polyvinylidene fluoride membrane and then incubated with blocking buffer (2% skim milk) for an hour at room temperature. The membranes were incubated overnight at 4°C with specific monoclonal antibodies of Bax (1:1000, # 2772), Bcl-2 (1:1000, #2876), caspase-3 (1:1000, #9665), LC3-II (1:1000, #3868), p-RIP3 (1:1000, # 93654), p-Beclin-1 (1:1000, # 84966) and β-actin (1:1000, #4970) from Cell Signaling Technology, USA as well as Beclin-1 (1:1000, ab62557) and RIP3 (1:1000, ab62344) from Abcam, USA and anti-phospho-LC3 (Ser12) (1:500, abc466) from Merck, USA. Immunoreactive protein bands were visualized by ECL select kit and autoradiography. Densitometric scanning of the films and Image analysis by Image. J software (Version 1.48u, NIH, USA) were used to quantify the results.

Statistical analysis

Data analysis was performed using SPSS Statistics 21 and graphs were prepared by GraphPad Prism 7.01. All data are presented as the mean±SD. The Kolmogorov–Smirnov test was used for testing the normal distribution of continuous variables. Behavioral results of the training days were analyzed by two-way analysis of variance (ANOVA) repeated measures followed by post hoc Tukey's test. One-way ANOVA and post hoc Tukey's test were used to probe trial data and molecular test results. Statistical significance was considered as $P < 0.05$.

Results

Nasal administration of CEPO-Fc improved spatial learning and memory in AD rats

The MWM test was performed to evaluate CEPO-Fc nasal administration's effect on spatial learning and memory in AD rats. $A\beta_{25-35}$ significantly affected the time it takes to find the invisible platform during the acquisition phase (Figure 2A). Two-way repeated measures ANOVA analysis indicated significant main effects of day [$F(2, 53)= 76.71$; $P<0.001$] and treatment [$F(4, 28)= 11.01$; $P<0.001$] on the escape latency. However, no significant effect was observed by the interaction of day and

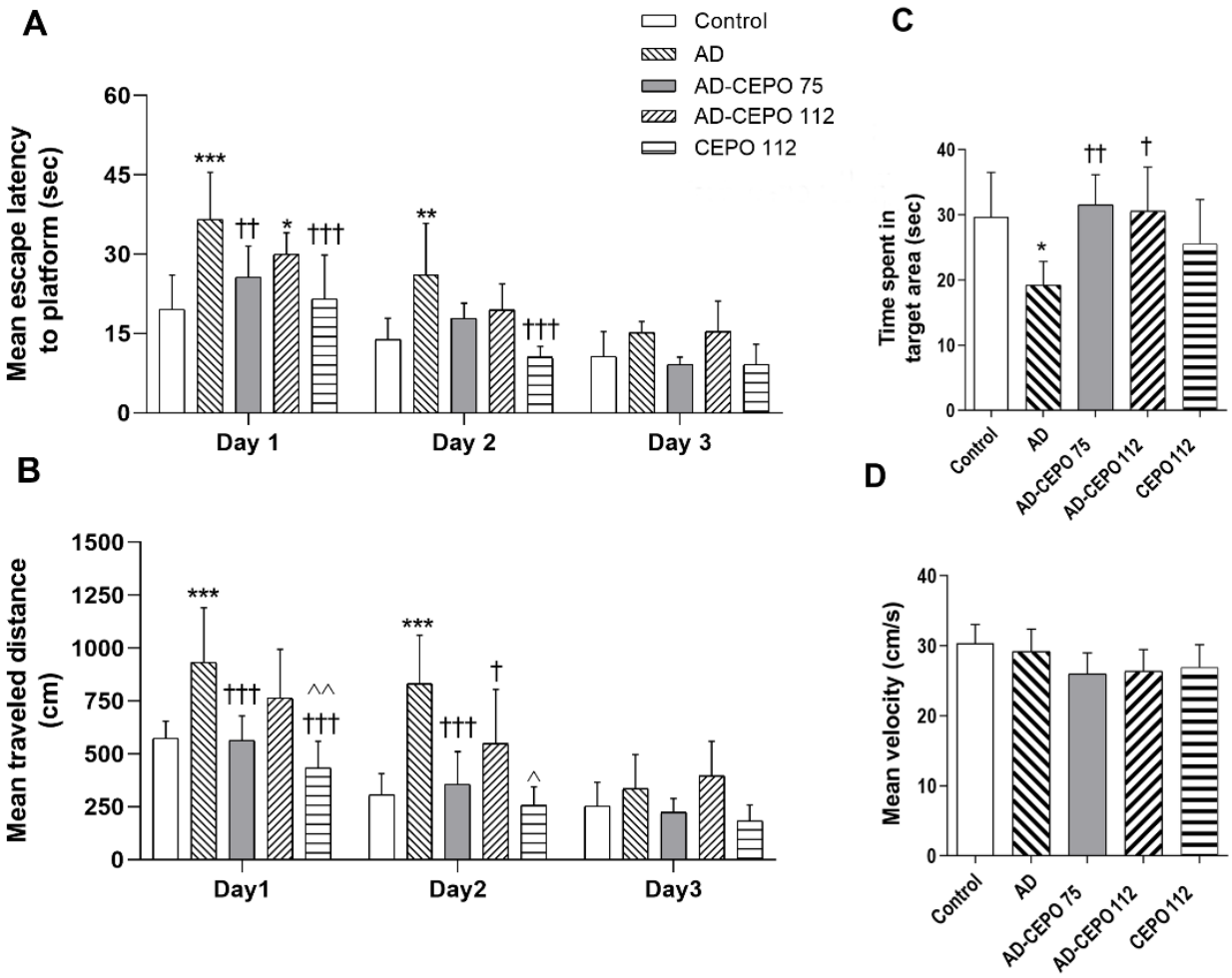

FIGURE 2. CEPO-Fc treated AD rats exhibited improved learning and spatial memory on Morris water maze performance. A-B illustrates the effect of nasal administration of CEPO-Fc on the (A) escape latency and (B) traveled distance to reach the invisible platform during training days in the AD rats. (C) represents the spent time (s) during a 60s period in the target quadrant (Q3) of the swimming pool in the no-platform probe test. (D) Mean swimming speed does not show a significant difference between the groups. The mean±SD is used to represent the data (n=7 in each time point). $^{\dagger\dagger\dagger}P<0.001$, $^{\dagger\dagger}P<0.01$ and $^{\dagger}P<0.05$ represents the difference between AD and AD-CEPO groups. $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ represents the difference between AD and control groups.

treatment [F(8, 56)= 2.03; P=0.058]. Post hoc Tukey's test exhibited that the escape latency was markedly increased in the Aβ group in comparison to the control group on day 1 (36.58±8.89 *vs.* 19.59±6.44, $P<0.001$) and day 2 (26.02±9.81 *vs.* 13.86±4.01, $P<0.01$). We observed that the nasal administration of CEPO-Fc at the concentration of 75IU reversed the learning and spatial memory impairment in AD rat models compared to the untreated AD rat on day 1 (25.64±5.90 *vs.* 36.58±8.89, $P<0.01$). No significant difference was observed on day 2 and day 3 following CEPO-Fc 75 IU treatment ($P>0.05$). Likewise, no statistical improvement was detected in the spatial learning and memory of AD rats which underwent nasal administration of CEPO-Fc at the dose of 112IU at days 1-3. Also, administration of 112IU CEPO-Fc in rats did not affect their behavior during days 1-3.

Figure 2B demonstrates the traveled distance results of the studied groups. The two-way repeated measures ANOVA analysis indicated the significant impact of day [F(2, 49)= 62.82, $P<0.001$], treatment [F(4, 28)= 13.69, $P<0.001$] and treatment x day interaction [F(8, 56)= 3.32, $P<0.01$]. Tukey's post hoc analysis showed that the traveled distance in AD rats was significantly higher than healthy control rats on day 1 (933.42±257.14 *vs.* 573.08±80.71, $P<0.001$) and day 2 (831.41±228.61 *vs.* 305.89±101.09, $P<0.001$). On the contrary, no significant difference was detected between normal rats receiving 112IU CEPO-Fc and the normal control group on days 1, 2 or 3 (Figure 2B). On the other hand, we ob-

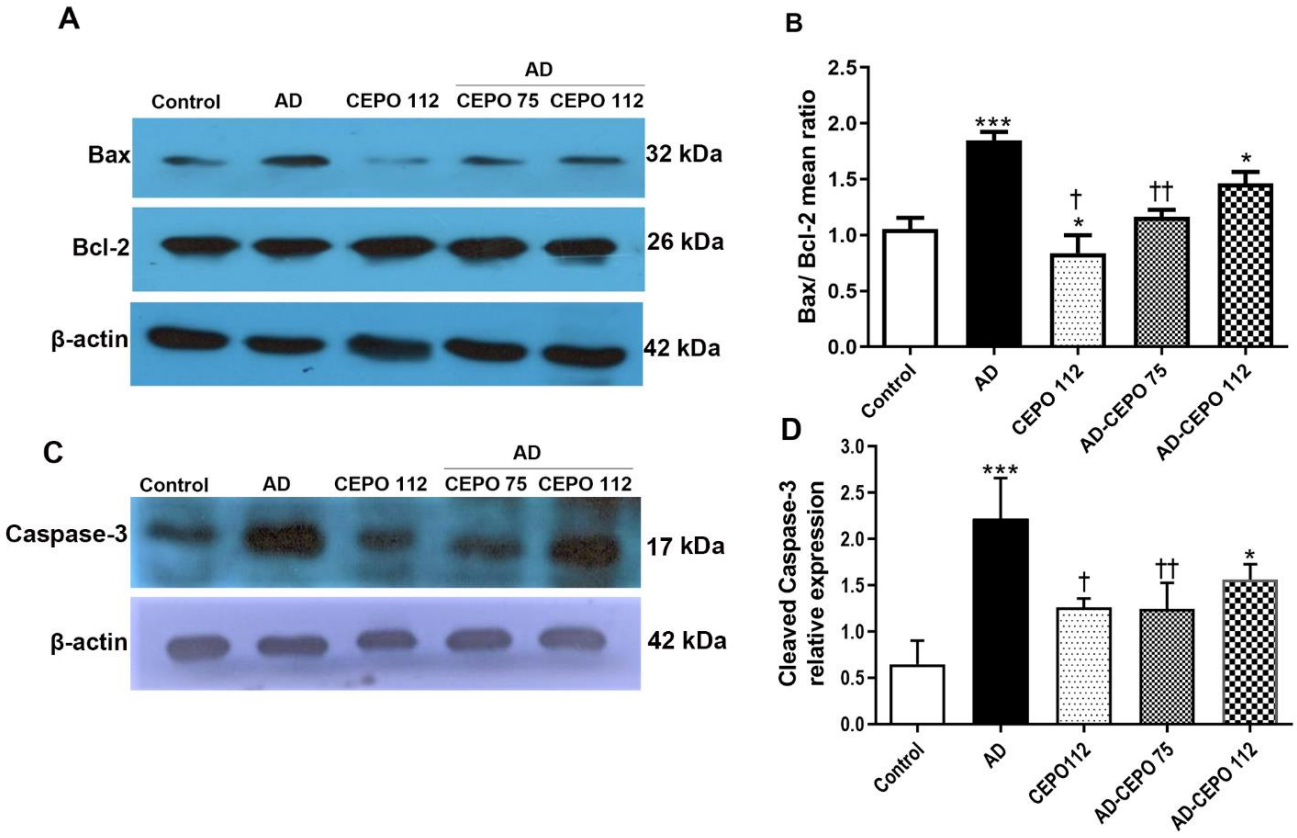

FIGURE 3. CEPO-Fc treatment exerted anti-apoptotic effects in the hippocampus of AD rats. Western blot analysis revealed that Aβ25-35 microinjection induced severe apoptosis in the hippocampus. Nasal administration of CEPO-Fc at two doses of 75IU (AD-CEPO 75) and 112IU (AD-CEPO 112) decreased (A-B) Bax/Bcl-2 ratio and (C-D) caspase-3 level in the AD rat model. The densitometry values were normalized as a ratio to β-actin ($^{***}P$<0.001 and $^{*}P$<0.05 vs. control group; $^{\dagger\dagger}P$<0.01 and $^{\dagger}P$<0.05 vs. AD group). The mean±SD is used to represent the data (n=3).

served that the nasal administration of CEPO-Fc at the dose of 75IU noticeably reduced $A\beta_{25-35}$ induced deficits compared to the AD rats at day 1 (564.45±115.21 *vs*. 933.42±257.14, P<0.001) and day 2 (355.66±155.35 *vs*. 831.41±228.61, P<0.001). CEPO-Fc administration at the higher dose of 112IU statistically decreased the traveled distance only at day 2 (548.27±255.82 *vs*. 831.41±228.61, P<0.05). No significant difference was detected between AD-CEPO 75 and AD-CEPO 112 groups. These results suggested that nasal administration of CEPO-Fc might rescue learning and memory deficits following $A\beta_{25-35}$ microinjection.

Nasal administration of CEPO-Fc improved memory retention in AD rats

The protective impacts of nasal administration of CEPO-Fc were examined on memory retention in AD rats. We calculated the time spent in the target quadrant (Q3) during a 60-s period by each animal on the probe day. It was observed that $A\beta_{25-35}$ microinjection resulted in apparent learning and memory dysfunction. The one-way ANOVA followed by Tukey's post hoc test showed that the AD group spent less time in the target area than the control group (19.23±3.62 *vs*. 29.69±6.84, P<0.05, Figure 2C). On the other hand, the spent time in the Q3 zone was increased in the AD-CEPO 75 (31.53±4.60, P<0.01) and AD-CEPO 112 (30.55±6.76, P<0.05) groups in comparison to the untreated AD group. To investigate the impact of drugs on motor performance, rats' swimming speed was analyzed, and no significant difference was observed among groups (P>0.05, Figure 2D).

Anti-apoptotic impact of CEPO-Fc in AD rats was mediated through decreasing caspase-3 level and Bax/Bcl-2 ratio

Apoptosis is a molecular process that is well-protected during evolution. Although apoptotic cells' apparent characteristics may differ, their morphological features such as cell wrinkling, degeneration of cytoplasm, DNA

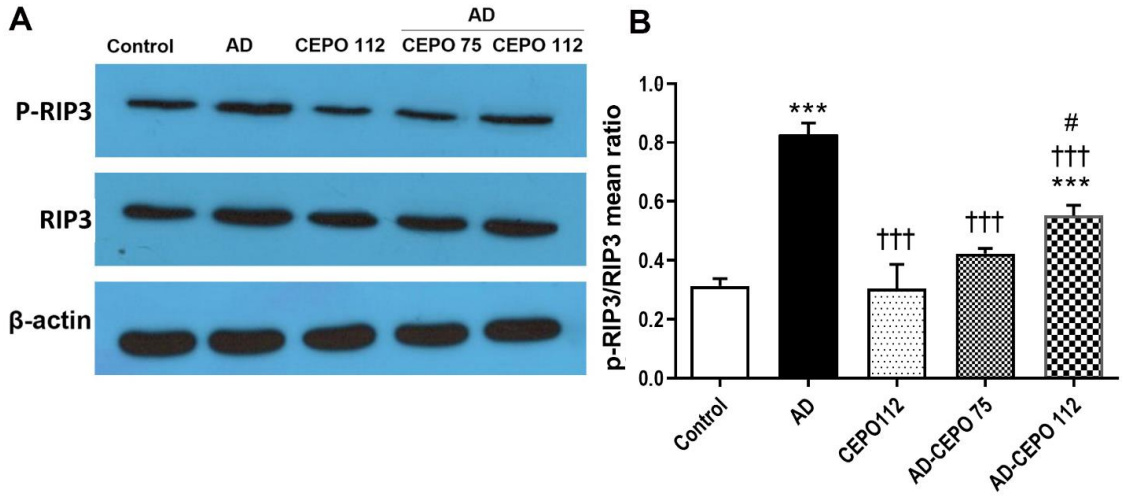

FIGURE 4. Nasal administration of CEPO-Fc induced anti-necroptotic effect in the hippocampus of AD rats. Western blot analysis represented a noticeable increase in the p-RIP3 protein (Ser227) level in the AD rat model. The densitometry values were normalized as a ratio to β-actin (***P<0.001 vs. control group; †††P<0.001 vs. AD group; #P<0.05 vs. CEPO-Fc 75). The mean±SD is used to represent the data (n=3).

fragmentation and membrane permeability are similar (Elmore, 2007). From the molecular aspects, analysis of apoptotic mediators could identify the apoptosis status of cells. The Bax/Bcl-2 ratio is the main indicator of the intrinsic and extrinsic apoptosis pathways, which affects the effector caspase-3 (Elmore, 2007). We herein examined the impact of CEPO-Fc on hippocampal apoptosis in AD rats using immunoblotting (Figure 3). One-way ANOVA followed by Tukey's test revealed that CEPO-Fc treatment reduced Bax/Bcl-2 ratio [$F(4, 10)= 13.80$, P=0.0004] and caspase-3 level [$F(4, 10)= 13.00$, P=0.0006] which was induced by $A\beta_{25-35}$. Quantitative analysis of the caspase-3 level and Bax/Bcl-2 ratio by Western blotting showed that $A\beta_{25-35}$ microinjection induced severe apoptosis in hippocampal neurons of AD rats compared to the control group (P<0.001, Figures 3B and D). However, apoptosis was prevented by decreasing the caspase-3 level and Bax/Bcl2 ratio in AD-CEPO 75 group (P<0.01) in comparison to untreated AD rats. The anti-apoptotic effect of CEPO-Fc at the dose of 75IU (P<0.01) was higher than 112IU on AD rats; but there was no noticeable difference between these two groups. Furthermore, in comparison to control rats, the Bax/bcl-2 ratio and caspase 3 level were significantly higher in AD rats who received CEPO-Fc 112 treatment. While CEPO-Fc 112 treatment decreased the Bax/bcl-2 ratio and caspase3 level in AD rats, the reduction was not statistically significant compared to untreated AD rats (P>0.05, Figures 3B and D).

CEPO-Fc therapy had a negative impact on necroptosis in AD rat

Prior research has found that programmed necroptosis plays a significant role in various morphological or pathological mechanisms. The necroptosis signaling cascades can be identified via measuring the enzymatic activity of particular mediators such as RIP3 and its phosphorylated form (Ser227) (Dhuriya & Sharma, 2018). Investigating the necroptosis status induced by $A\beta_{25-35}$ microinjection in the hippocampal neurons and examining the protective effect of CEPO-Fc, the levels of total and phosphorylated forms of RIP3 (p-RIP3) were measured. One-way ANOVA revealed the significant main effect of treatment [$F(4, 10)= 66.70$; P<0.001] in the level of p-RIP3/RIP3 in the experimental groups (Figure 4). We observed a noticeable increase in the ratio of p-RIP3/RIP3 in the AD rats compared to the healthy control group (P<0.001). Nevertheless, intranasal administration of CEPO-Fc considerably decreased the ratio of p-RIP3/RIP3 at the doses of 75 and 112IU (P<0.001) in comparison to the AD group (Figure 4). Significant difference was detected between the two CEPO-Fc 75 and 112IU treated groups (Figure 4, P<0.05). It seems that administration of 75IU CEPO-Fc more effectively reduced the p-RIP3/RIP3 ratio in compare to the 112IU CEPO-Fc. These observations indicated the inhibitory impact of CEPO-Fc against neuronal necrosis in AD rats.

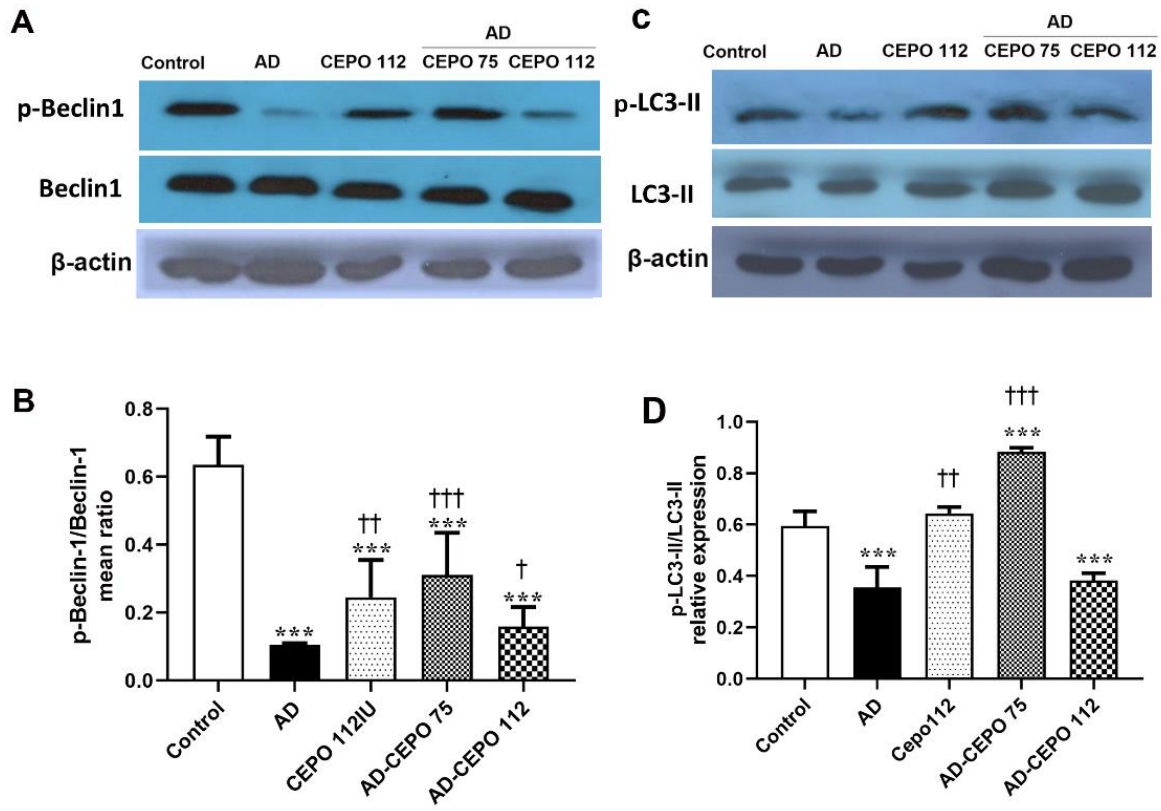

FIGURE 5. Nasal administration of CEPO-Fc increased autophagy in the hippocampus of AD rats. Western blot analysis revealed a significant decrement in the p-Beclin-1/Beclin-1 and p-LC3-II/LC3-II ratio in the hippocampus of AD rats as compared to the control group. The densitometry values were normalized as a ratio to β-actin ($^{***}P$<0.001 vs. control group; $^{†††}P$<0.001, $^{††}P$<0.01, and $^{†}P$<0.05 vs. AD group). The mean±SD is used to represent the data (n=3).

CEPO-Fc provoked the autophagy in AD rat hippocampal neurons by activation of Beclin-1 and LC3-II proteins

Autophagy is defined as the catabolic degradation of cytoplasmic organelles in response to the accumulation of defective structures, metabolic stress and nutrient starvation (Parzych and Klionsky, 2014). To investigate the impact of CEPO-Fc on the autophagy process in the hippocampal neurons of AD rats, we evaluated the level of Beclin-1, phosphorylated-Beclin-1 (p-Beclin-1), LC3-II and phosphorylated-LC3-II (p-LC3-II) proteins. Since post-translational phosphorylation of Beclin-1 at Ser15 leads to autophagy activation in response to various intracellular and extracellular signals, thereby we herein reported the P-Beclin-1/Beclin-1 ratio (McKnight and Yue, 2013). p-LC3-II is also necessary for autophagy's cellular recycling process and loss of phosphorylation blocks autophagy by impairing the autophagosome's fusion with lysosome (Wilkinson et al., 2015). So, we herein analyzed p-LC3-II/LC3-II ratio in evaluating the autophagy process. Using one-way ANOVA analysis indicated the remarkable main effect of treatment on the P-Beclin-1/Beclin-1 [$F(4, 10)= 66.06$; P<0.001] and p-LC3-II/LC3-II ratio [$F(4, 10)= 60.51$; P<0.001] in the studied groups. Tukey's post hoc test revealed that there is a significant decrease in the P-Beclin-1/Beclin-1 (P<0.001, Figures 5A and B) and p-LC3-II/LC3-II ratio (P<0.001, Figures 5C and D) in AD rats compared to the control group. Interestingly, treating with CEPO-Fc at the dose of 75IU elevated both p-Beclin-1/Beclin-1 (P<0.001) and p-LC3-II/LC3-II (P<0.001) ratio. The ratio of p-Beclin-1/Beclin-1 was also enhanced in AD rats treated with a dose of 112IU CEPO-Fc (1.51-fold, P=0.0372, Figures 5A and B). However, the results obtained for p-LC3-II/LC3-II ratio in the AD-CEPO 112 were not statistically different from the AD group (P=0.11, Figure 5B). These results indicated the stimulatory effect of CEPO-Fc on autophagy signaling mediators in the hippocampal neurons of AD rats.

Discussion

The goal of this study was to determine the mecha-

nism behind CEPO- Fc's neuroprotective activities against neurotoxicity induced by $A\beta_{25-35}$. To this aim, we evaluated the level of core regulators of apoptosis (Bax and Bcl2), necroptosis (RIP3) and autophagy (Beclin-1 and LC3-II) in the hippocampus of the AD rat model following the nasal administration of CEPO-Fc. Nasal administration of CEPO-Fc was performed at two doses of 75 and 112IU. We observed that CEPO-Fc improved cognitive function mainly at the dose of 75IU. The immunoblotting assessment revealed that the Bax/Bcl2 ratio was down-regulated in the hippocampus as an apoptosis indicator. Accordingly, we also provided evidence of the decreased level of RIP3 protein as a marker of necroptosis in response to CEPO-Fc. We found a significant modulation in the autophagy process by evaluating the ratio of p-Beclin-1/Beclin-1 and p-LC3 II/ LC3 II after CEPO-Fc treatment. These results showed that CEPO-Fc neuroprotective effect is partially mediated by triggering the autophagy and inhibition of apoptosis and necroptosis mechanisms.

Neuronal loss is a principal feature of AD that inevitably affects multiple brain regions. Despite extensive considerations, the precise mechanism of neurodegeneration has not explicitly been elucidated (Caccamo et al., 2017). Amyloidopathy is one of the prevailing theories for AD pathocascade. Several lines of evidence indicate that the misprocessing of APP and accumulation of Aβ is an early driver of AD preceding the onset of dementia. The progression and severity of AD correlate with Aβ accumulation in the intracellular and extracellular spaces (Duyckaerts et al., 2009). Following the microinjection of misfolded Aβ in animals' brains, Aβ can deposit through a prion-like mechanism (Jucker and Walker, 2013).

Intranasal administration of medications using nose drops or nasal spray has considerable therapeutic potential in compare to systemic administration, including simplicity and non-invasive drug delivery, rapid drug absorption and CNS delivery, ability to repeat dosing easily, no requisite for drug modification and reduced systemic exposure. After nasal instillation, medications may reach the CNS via olfactory, trigeminal and systemic pathways involving the olfactory and trigeminal neurons, respiratory and olfactory mucosa, the nasal vasculature, cerebrospinal fluid and the lymphatic system. Intranasal administered medicines can enter the systemic circulation by passing across the nasal epithelium into the submucosal tissue's blood capillaries. The fast dispersion of therapeutic chemicals throughout the brain can be attributed to the perivascular pump (Genc et al., 2011). Due to the literatures, intranasal rhEPO doses range from 16 to 80U/kg, which is equivalent to the recommended clinical doses (75 to 150U/kg) for subcutaneous or intravenous injection, but significantly lower than those used in animal studies with intraperitoneal injections (usually 1000 to 5000U/kg) (Yu et al., 2005). Moreover, Fletcher et al. (2009) used EPO 5000U/kg for systemic injection and 100U for intranasal delivery in both normal and ischemic mouse brains and reported the largest and earliest peak concentrations following the intranasal administration. Similarly, neuro-EPO is a new EPO formulation which has been investigated at three doses of 62, 125 and 250μg/kg (Maurice et al., 2013). According to these results, we selected 75IU as the lower dose and 112IU as the higher dose.

In the present study, we investigated the neuroprotective effects of CEPO-Fc on the neurotoxicity caused by microinjection of $A\beta_{25-35}$ aggregates into the CA1 region, which is a commonly used approach for studying AD (Hooshmandi et al., 2018; Pourhamzeh et al., 2020). In the structure of CEPO-Fc, all lysine residues in CEPO-Fc undergo carbamylation and are converted to homocitrulline (Leist et al., 2004). Instead of interacting to the classic EPO receptor, CEPO-Fc links to the common receptor (Chamorro et al., 2013), preserving the tissue-protective qualities. It has been suggested that CEPO improves proliferation in both *in vitro* (Wang et al., 2004) and *in vivo* (Xiong et al., 2011) models and enhances neurite growth and formation of the neuronal spine (Choi et al., 2014). Studies have shown that AD patients' cognitive dysfunction derives from impaired synaptic plasticity by misfolded Aβ peptide (Jha et al., 2017). Frequently, it has been shown that Aβ microinjection leads to memory deficits (Choi et al., 2011; Christensen et al., 2008). Similarly, our study's findings showed increased escape latency in the MWM probe test of AD rats. However, the lower escape latency in CEPO-Fc treated groups revealed that CEPO-Fc could ameliorate Aβ-induced spatial learning and memory deficits in AD rats.

It is widely accepted that amyloidopathy activates the neuroinflammatory process critical in AD progression (Ising et al., 2019). Increased active cleaved caspase-1 has been found in the Aβ-plaque containing mice and

AD patients (Heneka et al., 2013). Oxidative stress is also the known feature of AD, which results in structural and functional impairments, eventually death of neuronal cells (Walker and LeVine, 2000; Xilouri and Stefanis, 2010). Aβ plaques have been linked to mitochondrial membrane potential in the manner that mitochondrial depolarization raises the generation of oxidative stress, which causes neuronal apoptosis (Guglielmotto et al., 2010; Lee et al., 2019; Pourhamzeh et al., 2020) and consequently worsens the AD progression (Huang et al., 2016). Likewise, Aβ deposition in the mitochondria induces toxicity and subsequently activates the caspase-3-dependent apoptosis pathway (Lustbader et al., 2004), which up-regulates the generation of Aβ and NFTs (Rohn, 2010). The Bcl-2 family includes pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins, which are involved in the induction of the apoptotic process in mitochondria. Bax/Bcl-2 ratio is suggested as a determining indicator of apoptosis. One of the promising therapeutic strategies for neuronal disorders is preventing programmed cell death by upregulation of anti-apoptotic proteins such as Bcl-2 and to avoid ROS generation, inhibition of cytochrome c release and inhibition of caspase-3 activation (Kim and Suh, 2009). Our results showed that $A\beta_{25-35}$ increased caspase-3 level and Bax/Bcl-2 ratio at the protein level in the hippocampal neuronal cells of $A\beta_{25–35}$ treated animals, while nasal administration of CEPO-Fc relieved the level of apoptosis dose-dependently. It seems that Aβ-induced mitochondrial malfunctioning triggered the translocation of pro-apoptotic protein Bax and the stimulation of the caspase cascade, which CEPO-Fc eventually blocked. These results showed the anti-apoptotic effects of CEPO-Fc by investigating caspase-3, Bax, and Bcl-2, paving the way for further assessment of the apoptotic signaling pathway.

The role of necroptosis has been recently investigated in neurodegenerative diseases (Zhang et al., 2017). Necroptosis is a regulated type of cell death under signaling pathways, featured by cell swelling and rupture. One of the critical mechanisms of necroptosis is related to the activation of RIP1, RIP3 and mixed lineage kinase domain-like protein (MLKL) (Cho et al., 2009). Activated RIP1 forms the necrosome complex with RIP3, which is followed by MLKL phosphorylation and oligomerization, highlighting the initiation of caspase-independent cell death (Sun et al., 2012). Increased RIP1 level has been reported in human AD brains (Caccamo et al., 2017) and a mouse model of AD (APP/PS1) (Ofengeim et al., 2017). Our data indicate that $A\beta_{25-35}$ microinjection increased RIP3 level in the hippocampal neurons. Caccamo et al. (2017) indicated that RIP3 was associated with RIP1 and MLKL in AD brains; however, this difference was not statistically different between AD and healthy controls. Emerging evidence focuses on the importance of developing a practical therapeutic approach to modify the necroptosis process in AD. Accordingly, *in vitro* evidence showed that pre-treatment of neuronal cells with a RIPK1 inhibitor necrostatin-1 blocked necroptosis and protected neurons against degeneration (Y. Li et al., 2008; Xu et al., 2007). Also, mTOR inhibitors, such as rapamycin, CCI-779, RAD001 and AP23573, promoted autophagy and prevented apoptosis and necrosis in neurons (Ravikumar et al., 2006; Wu et al., 2008). Our results revealed that nasal administration of CEPO-Fc significantly reduced RIP3 marker in the hippocampal neurons of AD rats in line with these studies.

Autophagy is an essential degradation mechanism for protein homeostasis and neuronal health. Ample evidence has indicated that autophagy dysfunction occurred in the early AD stage and affected Aβ and P-tau's metabolism. The autophagy process consists of phagophore sequestration, autophagosome formation and autolysosome maturation (Mizushima et al., 2010), which could be considered a therapeutic target to prevent AD progression (Colacurcio et al., 2018; Li et al., 2017). LC3-II and Beclin-1 are two biomarkers for evaluating autophagy alterations which their turnover must be analyzed together (Glick et al., 2010; Orhon and Reggiori, 2017). In differentiated SK-N-BE neuroblastoma cells, Guglielmotto et al. (2014) found that an oligomers blocked autophagy and promoted necrotic and apoptotic cell death. Indeed, Aβ monomers suppress apoptosis and promote autophagy, resulting in the formation of autophagosomes within the cell and an increase in LC3-II levels. Similarly, our data indicated that the CEPO-Fc neuroprotective effect was mediated by activating autophagy by enhancing the ratio of p-LC3-II/LC3-II and p-Beclin-1/Beclin-1. Tan et al. (2018) reported that helix B surface peptide as an EPO-derived peptide, protected C57BL/6 mice cells against hepatic ischemia/reperfusion injury via activating Akt, improving the mTOR-autophagy pathway. Accordingly, Hooshmandi

et al. (2018) showed that CEPO-Fc administration increased survival of hippocampus in $A\beta_{25-35}$ induced AD rats via modulating Akt/GSk-3β, ERK, MMP-2 and p38 activities.

Furthermore, our findings have revealed that CEPO-Fc provided neuroprotection via multiple pathways, such as induction of autophagy and inhibition of apoptosis and necroptosis. It could be occurred due to the role of Beclin-1 in the regulation of both autophagy and apoptosis mechanisms through interacting with anti-apoptotic molecules (Cao and Klionsky, 2007; Edinger and Thompson, 2004; Erlich et al., 2007; Funderburk et al., 2010). As a member of the BH3-only protein family, Beclin-1 binds with the anti-apoptotic proteins Bcl-2 or Bcl-xL and deactivated. This medium leads to suppression of autophagosome formation and triggers endogenous apoptosis (He and Levine, 2010; Itakura et al., 2008; Maiuri et al., 2007; Morselli et al., 2009; Robert et al., 2012). The other known Beclin-1 association with apoptosis is the mitochondrial insertion of the C-terminal fragment of Beclin-1 cleaved by various caspase enzymes such as caspase-3 or caspase-8, and subsequent mitochondrial membrane permeability transition and apoptosis (Wirawan et al., 2010). Besides, caspase-3-dependent apoptosis activation prevents autophagy by enzymatic cleavage of autophagic proteins such as Beclin-1 (Rohn et al., 2011).

Our results showed that the efficacy of 75IU CEPO-Fc was better than 112IU. Similarly, Maurice et al. (2013) tested the neuro-EPO effects at dosages of 62, 125 and 250g/kg following intranasal administration on $A\beta_{25-35}$ -induced neurotoxicity. They reported that the neuroprotective effects of neuro-EPO was bell-shaped and the 250μg/kg dose seemed to have no effect. Likewise, Yu et al. (2005) found that intranasal rhEPO may provide a therapeutic benefit on acute injury after focal cerebral ischemia at smaller doses than those in systemic administration. Through their study, the range of effective doses of intranasal rhEPO is approximately 32–160 U/kg totally and the doses of 4.8 and 12U are most effective but the larger dose of 24U seems to be weaker. While high levels are ideal for neuroprotection and neurorescue, they can also cause the production of ROS, which can lead to a loss of positive effects and even toxicity (Erbayraktar et al., 2006; Thomas Tayra et al., 2013; Wu et al., 2010).

Conclusion

Our study showed that CEPO-Fc provokes autophagy in hippocampal neurons as a protective action against $A\beta_{25-35}$ induced neurotoxicity and apoptosis. Our study revealed new insights into AD's pathogenesis and opened new avenues of research and AD treatment interventions. From a therapeutic perspective, our data strongly suggest that CEPO-Fc -by reducing apoptosis and necroptosis besides inducing autophagy- may be a valid therapeutic intervention for AD. Our findings are in line with previous reports and elucidated part of the underlying neuroprotective mechanism of CEPO-Fc. However, more exploration is required to fully understand the potential biological effects of CEPO-Fc in neurodegenerative disorders. More research employing RT-PCR to examine the gene expression of βcR in the hippocampus is needed to better clarify the underlying mechanism of CEPO-Fc's neuroprotective effects.

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Conflict of Interest

The authors have declared that no competing interests exist.

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