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Original Article

Intraperitoneal Carbamylated erythropoietin improves memory and hippocampal apoptosis in beta-amyloid rat model of Alzheimer's disease through stimulating autophagy and inhibiting necroptosis

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ABSTRACT

Introduction: Alzheimer's disease (AD) is marked by the deposition of amyloid-β (Aβ) plaques and tau tangles. Although Erythropoietin (EPO) provides neuroprotective and memory-improving properties, its application has been limited due to the hematopoietic effects. Carbamylated Erythropoietin-Fc (CEPO-Fc) was developed as a non-erythropoietic EPO derivative that possesses neuroprotective potential. However, the molecular mechanisms behind the protective effects of CEPO-Fc's in AD are still under consideration. Therefore, herein investigated the therapeutic properties of intraperitoneal (i.p.) dose of CEPO-Fc on Aβ-induced neurotoxicity in adult male Wistar rats.

Methods: The rats received microinjections of Aβ25-35 (5 µg/2.5 µl, per side) in the dorsal hippocampus for four consecutive days. CEPO-Fc was injected intraperitoneally in two doses of 500 and 5000 IU during the next six days. Learning and memory performance were studied (days 10-13) using the Morris Water Maze task. Immunoblotting was also undertaken to assess the molecular levels of leading indicators of apoptosis (Bax, Bcl-2, and caspase-3), necroptosis (Phosphorylated-Receptor-interacting serine/threonine-protein kinase 3 (p-RIP3)), as well as autophagy (phosphorylated-Beclin-1 (p-Beclin-1) and phosphorylated-1A/1B-light chain 3 (p-LC3-II)) in the hippocampus.

Results: Behavioral analysis indicated that CEPO-Fc 500 and 5000 IU reversed memory impairment. Moreover, the hippocampus's molecular study showed upregulation of P-LC3-II/LC3-II and suppression of Bax/Bcl-2, Caspase-3, and P-RIP3/RIP3 processes.

Conclusion: Our findings imply that the neuroprotective characteristics of CEPO-Fc in the AD rats are mediated through autophagy activation and regulation of apoptosis and necroptosis processes. These results suggest that an i.p. dose of CEPO-Fc could be used to protect against AD-induced neurotoxicity.

Keywords:
Apoptosis
Autophagy
Alzheimer's disease
Necroptosis
Carbamylated Erythropoietin-Fc

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Introduction

Alzheimer's disease (AD), as a neurodegenerative disorder, is clinically featured by irreversible memory and behavioral deterioration. AD patients are suffering from the disease progressing to dementia due to progressive neuronal loss. It has a high mortality rate worldwide, and current trends estimate that the rate of AD will rise eighty-five-fold by 2050 (Hu et al., 2018). Microscopically, the AD brain is described by the accumulation of abnormal structures of intracellular tau neurofibrillary tangles (NFT) and extracellular amyloid-β (Aβ) plaques. The development of Aβ plaques and NFT in the brain is linked to behavioral symptoms of AD, which are caused by the destruction and loss of synapses (Bloom, 2014).

Evidence shows that neurotoxicity of Aβ is a primary pathogenic driver of AD, contributing to synaptic failure, dysfunction of neurons, and eventually neuronal loss when abnormally accumulates in the hippocampal formation and cortex of AD patients (Mohamed et al., 2016). Aβ is a peptide containing 39 to 43 amino acids formed by β- and γ-secretases that are sequentially cleaving the amyloid precursor protein (APP) (Mawuenyega et al., 2010; Pourhamzeh et al., 2020). $A\beta_{25-35}$ is an 11-amino-acid synthetic peptide corresponding to a fragment of $A\beta_{1-40}$ and $A\beta_{1-42}$ and is commonly used to develop AD cell models. Intracellular Aβ has been found in the cytosol and cellular compartments, including mitochondria, Golgi, endoplasmic reticulum (ER), and lysosomes, suggesting that these are Aβ development sites (Zheng et al., 2011). The ubiquitin-proteasome and autophagy-lysosome pathways, both are defective in AD patients, are involved in Aβ clearance by degrading Aβ (Subramanian et al., 2021). As the predominant clearance machinery, the autophagy-lysosome pathway is implicated in the degradation of Aβ (Nilsson & Saido, 2014). Autophagy suppression caused by the loss of the autophagy marker Beclin-1 in mice resulted in increased intracellular and extracellular Aβ accumulation, as well as neurodegeneration (Luo et al., 2021; Swaminathan et al., 2016). On the other hand, autophagy may be activated in AD (Kuang et al., 2020). Accordingly, lysosomal Aβ accumulation was discovered in neuroblastoma cells, resulting in oxidant-induced apoptosis (Zheng et al., 2011). Aβ-induced apoptosis is supposed to be the primary cause of neuronal death in AD (Calvo-Rodriguez et al., 2020). Necroptosis is also triggered in human AD brains and an AD mouse model with neuronal loss. Accordingly, RIPK1, one of the critical proteins participating in necroptosis execution, was found to be elevated in human AD brains across different cohorts (Caccamo et al., 2017). Thus, in addition to apoptosis (Lu et al., 2021), reduced necroptosis (Caccamo et al., 2017) appears to be a feasible therapy for slowing the onset and development of AD.

Neuroprotective and neuroregenerative properties of erythropoietin (EPO) have been demonstrated in various preclinical studies of traumatic brain injury (TBI) (Liu et al., 2020), amyotrophic lateral sclerosis (Kim et al., 2014; Lauria et al., 2015), stroke (Larpthaveesarp et al., 2021), chronic autoimmune encephalomyelitis (Moransard et al., 2017), and spinal cord injury (Zhong et al., 2020). The main concern in recommending EPO for neuroprotection in clinical practice is the possibility of a substantial increase in hematocrit with long-term treatment (Hwang, 2020; Sun et al., 2019). Carbamylated erythropoietin (CEPO-Fc) shows similar protective effects to EPO but without the erythrogenic properties because it does not bind to EPO receptors. As such, CEPO-Fc improves spatial learning in TBI rats just as well as EPO (Skrifvars et al., 2017). According to experimental studies, CEPO-Fc's protective effects are mediated by binding to a heteroreceptor EPOR-βcR (also known as CD131 or β-common receptor) (María Eugenia Chamorro et al., 2013; Maltaneri et al., 2017). CEPO-Fc has also been shown to stimulate neurite outgrowth and the development of neuronal spines (Miyeon Choi et al., 2014). Previously, it has been reported that CEPO-Fc restores $A\beta_{25-35}$-induced cell toxicity in isolated hippocampal neurons (Hooshmandi et al., 2020). In our recent *in-vivo* study, CEPO-Fc was found to prevent $A\beta_{25-35}$-induced learning and memory deficits in rats by modulating hippocampal Akt/GSK-3, MMP-2, and MAPKs activity (Hooshmandi et al., 2018).

The majority of investigations have focused on EPO and rhEPO, leaving the CEPO- Fc's protective activities at the molecular level unexplained. Thus, the goal of this investigation is to assess whether CEPO-Fc could protect rats against $A\beta_{25-35}$-induced neurotoxicity. We also assessed the impact of CEPO-Fc on $A\beta_{25-35}$-mediated apoptosis (Bax/Bcl-2 and cleaved caspase-3), as well as changes in necroptosis markers receptor-interacting protein 3 (RIP-3), and autophagy markers at the molecular level (Beclin-1 and LC3-II).

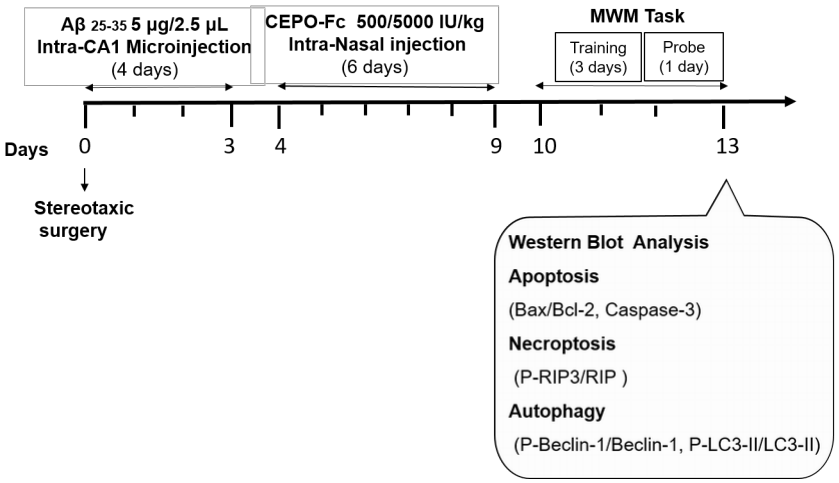

FIGURE 1. Graphical scheme to show the experimental design of the study.

Material and methods

Animals

The study was performed on 54 adult male Wistar rats (eight weeks old, 250-300 g), supplied by the Shahid Beheshti University of Medical Sciences, Tehran, Iran. This investigation was conducted on male rats because the fluctuations of sexual hormones in female rats impact memory performance (Frick et al., 2015). The animals were housed three per standard plastic cage under the standard laboratory conditions (12:12 h light/dark cycle, 24 ± 1˚C), and unlimited accessibility to traditional food and water ad libitum was provided. All research and animal care procedures followed the Care and Use of Laboratory Animals guidelines (National Institutes of Health Publication No. 80-23, revised 1996). In addition, all procedures were ratified by the Research and Ethics Committee of the School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.PHNS.REC.1397.109).

Drugs

In our research, $A\beta_{25–35}$ (2 µg/µl, Sigma-Aldrich, A4559) was dissolved in distilled water and kept at –20°C. In line with our prior studies, $A\beta_{25–35}$ aggregations were obtained by incubation at 37°C for four days (Hooshmandi et al., 2020; Hooshmandi et al., 2018).

The CEPO-Fc compound was developed in Professor Hermann Katinger's laboratory (Vienna, Austria). It comprises two rhEPO molecules coupled to the Fc domain of a human IgG1 antibody carbamylated to reduce its erythropoietic potency (Schriebl et al., 2006). The primary stock of CEPO-Fc was synthesized in phosphate-buffered saline (PBS) at a dose of 1.91 mg/ml (2.3 x 105 IU), and the required dilutions (500 and 5000 IU) were then made using the stock.

Experimental Design

The rats were allocated into six groups at random (n = 9): control group, which received no intervention; AD group, which underwent bilateral microinjections of $A\beta_{25–35}$ (5 µg/2.5 µl per side/day) in the dorsal hippocampus for four days (days 0–3); CEPO-Fc treatment groups, which received CEPO-Fc at two doses of 500 or 5000 IU intraperitoneal (i.p.)/daily for six days (days 4–9). To rule out any possible CEPO-Fc effects, two sham CEPO-Fc groups received a daily dose of 500 or 5000 IU/kg CEPO-Fc for four days (days 4–9, CEPO 500 and CEPO 5000 groups). The animals were then subjected to a series of behavioral and molecular tests (Figure 1).

Stereotaxic Surgery

An i.p. injection of ketamine and xylazine (100 and 10 mg/kg, respectively) was used to anesthetize rats. In a stereotaxic frame, the animals were immobilized, and two guiding cannulas were bilaterally implanted into the dorsal hippocampus (AP: -3.8, ML: ±2.2, DV: -2.7) (Pourhamzeh et al., 2020) and anchored to the skull using a jeweler's screw and secured by dental cement, according to the Paxinos brain atlas (Paxinos & Watson, 2007). $A\beta_{25–35}$ were directly infused into the dorsal hippocampus using a 5µl Hamilton syringe and a 30-gauge

microinjection needle placed 0.5 mm beyond the guide cannula's tip. Each 0.5 µl of $A\beta_{25-35}$ aggregations was microinjected over 2 minutes. The needle was maintained in the injection site for one more minute to reduce regurgitation. Rats were allowed to walk around freely during the infusion procedure. For four days (days 0-3), the $A\beta_{25-35}$ microinjections were performed continuously.

Morris Water Maze Test (MWM)

The MWM task was carried out to assess spatial learning and memory deficits by an investigator blind to the treatment status. The MWM test was carried out in a 1.4-m-diameter dark circular tank in a room with added maze clues. The swimming pool was separated into four sections: Northeast (NE), southeast (SE), southwest (SW), and northwest (NW). An 11-cm-wide escape platform was located 1.5 cm below the water's surface in the center of one of the quadrants (target quadrant), so mice could not see it during the test.

The MWM test consisted of a training phase and a probe trial (days 10 – 13). During the training phase, animals have undergone four trials per day for three consecutive days (10-12 days). Each trial began with a different starting point (NE, SE, SW, or NW). Each rat had 60 seconds to reach the hidden platform in each trial and then stayed on it for the next 20 seconds. If a rat could not discover the platform for 60 seconds, the researcher carefully placed it on the platform and remained there for 20 seconds. The rats were kept in a cage for 30 seconds at the end of each trial before moving on to the subsequent trial. During days 1-3 of training, the escape latency (spent time) and the distance traveled to reach the invisible platform were measured for each trial.

The probe trial was held 24 hours after the last training session (day 13) without the escape platform. After being released from the opposite side of the target quadrant, the rats were given 60 seconds to swim in the pool. The time spent in the target zone and the swimming velocity were then calculated. A 3CCD video camera connected to the Noldus EthoVision (7.1 version, Noldus Information Technology, Netherlands) situated above the maze recorded the behavior of each rat in the maze. Rats were sacrificed at the end of the behavioral evaluation. The hippocampus tissue was dissected and snap-frozen in liquid nitrogen and maintained at -80°C for molecular analysis.

Western blot

Lysis buffer [50 mM Tris-HCl, pH8.0; 150 mM NaCl; 1% Triton X-100; 0.5% Na-Deoxycholate; 0.1% SDS (sodium dodecyl sulfate)] containing protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, A32963) was utilized to homogenize frozen hippocampus tissues. The lysates were centrifuged at 14,000 rpm for 30 minutes at 4 °C. The protein content of the samples was determined by the Bradford test, which is based on bovine serum albumin (BSA). The protein samples were then loaded with loading buffer and heated for 5 minutes at 100°C. The samples with an equal concentration of protein (50 µg) were electrophoresed on a 12 % SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (iBlot, Thermo Fisher Scientific). The membranes were blocked for 60 min in 2 % skim milk in Tris-buffered saline with Tween (TBST; 0.1 M Tris, 0.15 M NaCl, and 0.1% Tween 20). After blocking, membranes were incubated overnight at 4 °C with the appropriate primary antibodies: Bax (1:1000, Cell Signaling Technology, #2772), Bcl-2 (1:1000, Cell Signaling Technology, #2876), caspase-3 (1:1000, Cell Signaling Technology, #9665), LC3-II (1:1000, Cell Signaling Technology, #3868), P-LC3 (Ser12) (1:500, Sigma-Aldrich, ABC466) and β-actin (1:1000, Cell Signaling Technology, #4970), Beclin-1 (1:1000, Abcam, #ab62557), P-Beclin-1 (1:1000, Cell Signaling Technology, #84966), RIP3 (1:1000, Abcam, #ab62344), and P-RIP3 (1:1000, Cell Signaling Technology, #93654) in blocking solution. Chemiluminescent detection was performed using horseradish peroxidase-conjugated anti-rabbit secondary antibodies diluted in blocking solution for 1 hour at room temperature. Immunoreactive protein bands were visualized by ECL select kit and autoradiography. Signal intensities were quantified by ImageJ software v1.43 (NIH, Bethesda, MD, USA).

Statistical Analysis

Graph Pad Prism (Version 7.01, USA) was used to create graphs, and SPSS software was used to conduct statistical analyses (Statistical Package for the Social Sciences, version 21, USA). The Kolmogorov–Smirnov test was used for testing the normal distribution of continuous variables. Data from training days were analyzed using a two-way repeated measure analysis of variance (ANOVA) followed by a post hoc Bonferroni's test. Data from retention day and molecular tests were

evaluated using a one-way ANOVA followed by a post hoc Tukey's test. All results are presented as means with SD and $P < 0.05$ is considered a significant difference in all statistical comparisons.

Results

Intraperitoneal injection of CEPO-Fc improved spatial learning and memory in AD rats

The MWM test was used to investigate rats' spatial learning and memory following $A\beta_{25\text{-}35}$ and CEPO-Fc treatment. The learning pattern revealed a negative linear correlation between escape latency and training days in all experimental groups. The performance of $A\beta_{25\text{-}35}$-treated animals, however, was lower than that of the other groups. We observed a significant main effect of days [F (2, 144) = 70.83, $P < 0.001$] as well as treatment [F (5, 144) = 10.68, $P < 0.001$] using a two-way ANOVA repeated measure analysis. However, there was no evidence of a significant effect of days × treatment interaction [F (10, 144) = 1.793, $P = 0.0667$]. The Post hoc analysis by Bonferroni's test represented that escape latency in the $A\beta_{25\text{-}35}$ treated group is statistically higher than the control group on day 1 ($P < 0.001$) and day 2 ($P < 0.01$), demonstrating Aβ-induced learning and memory impairment. In both doses of 5000 IU and 500 IU, the i.p. administration of CEPO-Fc could restore the

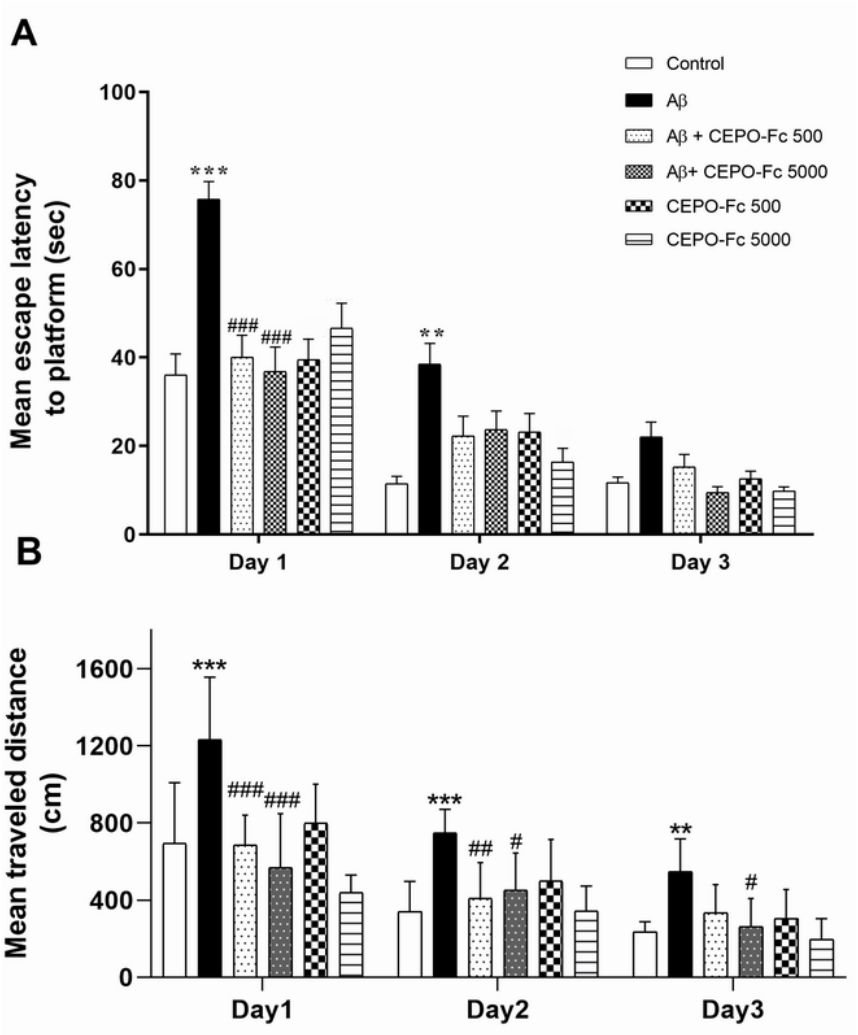

FIGURE 2. Intraperitoneal administration of CEPO-Fc in AD rats led to enhanced learning and spatial memory on the Morris Water Maze test. This figure depicts (A) the escape latency and (B) the traveled distance to reach the invisible platform during training days in the AD model rats. A rat model of AD was developed using intra-CA1 microinjection of 5 µg/2.5 µL Aβ25-35 for four days. The results demonstrate that the Aβ25–35 treated group has a prolonged escape latency and a higher displacement rate than the control group. Intraperitoneal injection of CEPO-Fc in both doses of 5000 IU and 500 IU for six days reduced learning and memory impairment in the AD rats compared to the untreated AD rats. Data are represented as mean ± SD (n = 9 in each group; $^{***}P < 0.001$ and $^{**}P < 0.01$ represents the difference with the control group; $^{###}P < 0.001$, $^{##}P < 0.01$, and $^{#}P < 0.05$ represents the difference with the AD group; two-way ANOVA test).

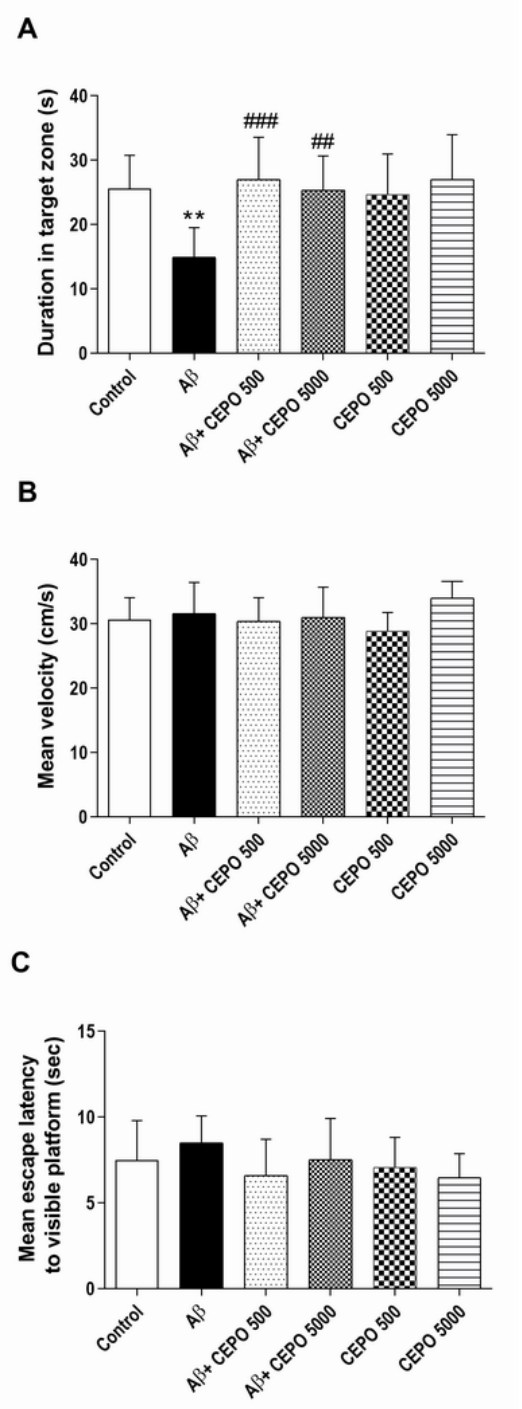

FIGURE 3. Intraperitoneal dose of CEPO-Fc in AD rats reversed memory impairment on the retention day of the Morris Water Maze test. (A) Represents the spent time (s) in the target zone of the swimming pool during a 90 s in the no-platform probe trial. A rat model of AD was developed using intra-CA1 microinjection of 5 µg/2.5 µL Aβ25-35 for four days. The AD group showed a lower time spent in the target zone than the control rats. These findings show that AD rats were given CEPO-Fc intraperitoneally in both 5000 IU and 500 IU doses for six days and spent more time in the target zone than the AD group. (B) This depicts that there is no significant difference in mean swimming speed between the groups. (C) This shows that the animals' performances in the visible platform test do not differ statistically between the groups. Data represent mean ± SD (n = 9 in each group; $^{**}P < 0.01$ represents the difference with the control group; $^{###}P < 0.001$, $^{##}P < 0.01$, and $^{#}P < 0.05$ represents the difference with the AD group; One-way ANOVA test).

between Aβ+CEPO-Fc and $A\beta_{25-35}$-treated groups on days 2 and 3, no significant difference was also detected between control and Aβ+CEPO-Fc treated groups on these days at both 5000 IU and 500 IU doses, demonstrating a neuroprotective impact of CEPO-Fc against $A\beta_{25-35}$-induced toxicity.

The results of the traveled distance analysis are displayed in Figure 2B. A significant main effect of days [$F(2, 24) = 112.60$, $P < 0.001$], treatment [$F(5, 120) = 22.83$, $P < 0.001$], and interaction of days × treatment [$F(10, 120) = 1.95$, $P = 0.044$] were found in a two-way ANOVA repeated measure analysis. Post hoc Bonferroni's test demonstrated that the traveled distance in $A\beta_{25-35}$ treated group is considerably elevated compared with the control group on day 1 ($P < 0.001$), day 2 ($P < 0.001$), and day 3 ($P < 0.01$). CEPO-Fc treatment reversed $A\beta_{25-35}$-induced deterioration at two doses of 500 IU (day 1, $P < 0.001$ and day 2, $P < 0.01$) and 5000 IU (day 1, $P < 0.001$; day 2, $P < 0.05$; and day 3, $P < 0.05$). Together, these findings indicated that CEPO-Fc reverses $A\beta_{25-35}$-induced learning and memory deficits, while there was not any significant difference between the 500 and 5000 IU doses of CEPO-Fc.

Intraperitoneal injection of CEPO-Fc enhanced memory retention in AD rats

The protective impacts of the i.p. injection of CEPO-Fc were investigated on memory retention in AD rats. Figure 3A depicts the time spent in the target zone on probe day (day 4). One-way ANOVA revealed that the i.p. injection of CEPO-Fc in $A\beta_{25-35}$-treated rats significantly extended the time spent in the target zone [$F(5, 48) = 5.556$, $P = 0.0004$]. The following Post hoc analysis by Tukey's test revealed that the time spent in the target zone dwindled considerably in the $A\beta_{25-35}$ received group than the control group (14.87 ± 4.66 *vs.* 25.58 ± 5.18, $P < 0.01$) while both CEPO-Fc 500 IU (27.00 ± 6.55, $P < 0.001$) and CEPO-Fc 5000 IU (25.35 ± 5.31, $P < 0.01$) significantly prevented Aβ-induced memory impairment.

The swimming speed of animals was measured in the probe trial to determine the potential impact of drugs on motor performance (Figure 3B). One-way ANOVA followed by Tukey's test displayed that administration of $A\beta_{25-35}$ and/or CEPO-Fc did not affect swimming speed [$F(5, 48) = 1.79$, $P = 0.131$]. A visible platform test was also conducted on day four after the probe trial to assess

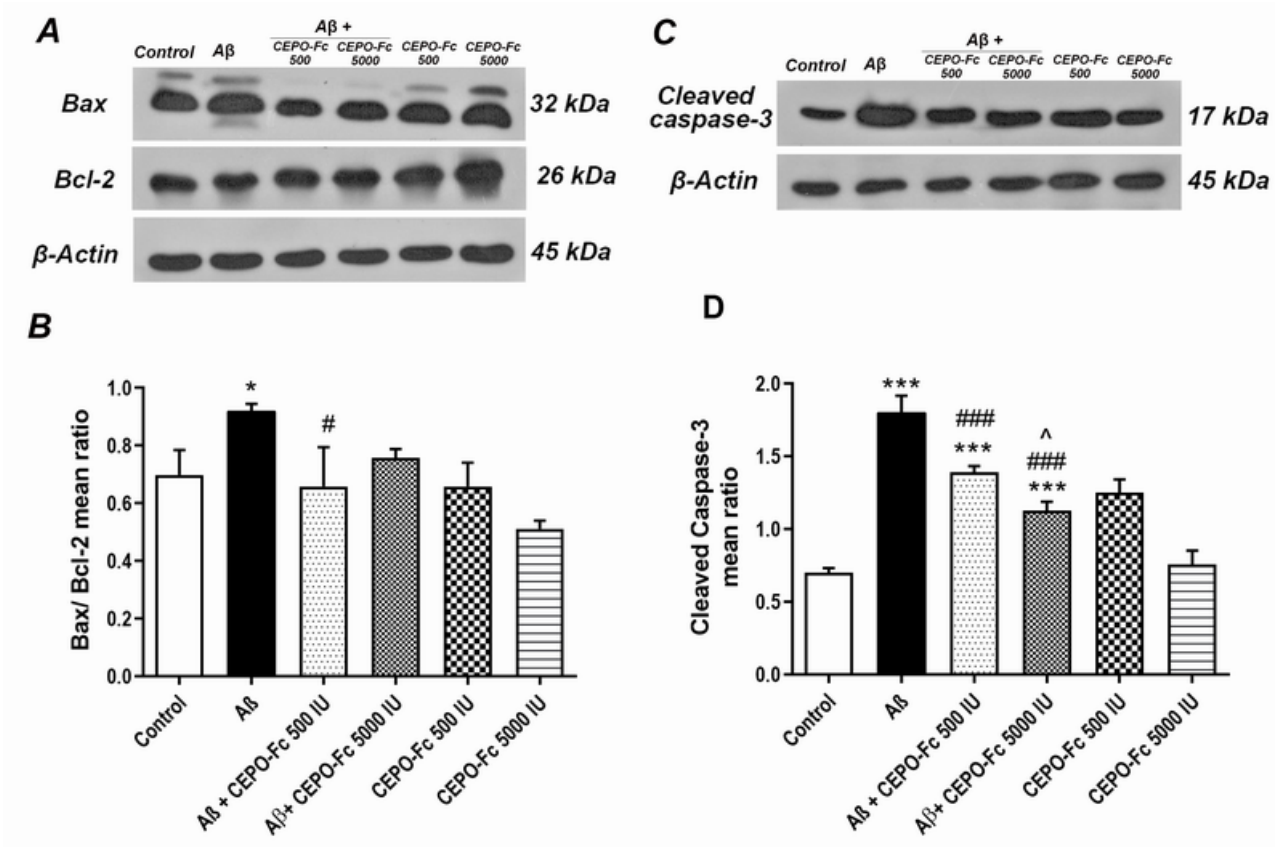

FIGURE 4. Intraperitoneal dose of CEPO-Fc inhibited apoptosis in the hippocampus of AD rats. Immunoblotting analysis shows the effects of Aβ25–35 and/or CEPO-Fc administration on the Bax/Bcl-2 ratio (A-B) and caspase-3 level (C-D) in the hippocampus of AD rats. An AD model was developed by intra-CA1 microinjection of 5 μg/2.5 μL $A\beta_{25-35}$ for four consecutive days. $A\beta_{25-35}$ microinjection increased the Bax/Bcl-2 ratio and caspase-3 level in the hippocampus of rats, which was inhibited by i.p. injection of CEPO-Fc at two doses of 500 IU (AD-CEPO 500) and 5000 IU (AD-CEPO 5000) for six days. The densitometry values were normalized as a ratio to β-actin. Data represent mean ± SD (n = 3 in each group; $^{***}P < 0.001$ and $^{*}P < 0.05$ represents the difference with control group; $^{\#\#\#}P < 0.001$, and $^{\#}P < 0.05$ represents the difference with the AD group; $^{\wedge}P < 0.05$ represents the difference between Aβ + CEPO-Fc 5000 IU and Aβ + CEPO-Fc 500 IU; One-way ANOVA test).

learning and memory deficits on day 1 ($P < 0.001$, Figure 2A). Although there was no significant difference the animals' sensory-motor coordination, vision, and motivation. One-way ANOVA followed by Tukey's test did not demonstrate a significant main effect of $A\beta_{25-35}$ and CEPO-Fc on escape latency to the visible platform [F (5, 48) = 1.36, $P = 0.255$, Figure 3C]. These findings suggest that the effects of $A\beta_{25-35}$ and CEPO-Fc administration on learning and memory are not due to visual/motor impairment or swimming velocity.

Intraperitoneal dose of CEPO-Fc exerted anti-apoptotic effect in AD rats through decreasing caspase-3 level and the Bax/Bcl-2 ratio in the hippocampal neurons

Investigation of apoptotic mediators could be used to determine the apoptosis status of cells on a molecular level. The Bax/Bcl-2 ratio impacts the effector caspase-3, a key indicator of intrinsic and extrinsic apoptosis pathways (Zhang et al., 2016). Using immunoblotting, we examined the effect of CEPO-Fc on hippocampal cell apoptosis in AD rats (Figure 4). One-way ANOVA analysis showed that CEPO-Fc treatment could reduce Bax/Bcl-2 ratio [F (5, 12) = 9.31, $P = 0.0008$] and caspase-3 level [F (5, 12) = 83.03, $P < 0.001$] which were induced by $A\beta_{25-35}$. Post hoc analysis by Tukey's test exhibited that $A\beta_{25-35}$ injection up-regulated hippocampal Bax/Bcl-2 ratio ($P < 0.05$) and caspase-3 level ($P < 0.001$). Treatment with CEPO-Fc 500 IU prevented Aβ-induced increases in the Bax/Bcl-2 ratio ($P < 0.05$, Figure 4A&B) and caspase-3 level ($P < 0.001$, Figure 4C&D). CEPO-Fc 5000 IU reduced both Bax/Bcl-2 ratio and caspase-3 level, but only the reduction of caspase-3 was statistically significant ($P < 0.001$). Interestingly, CEPO-Fc at a dose of 5000 IU reduces caspase-3 levels in AD rats more effectively than 500 IU ($P < 0.05$).

Intraperitoneal injection of CEPO-Fc protects AD rats from Aβ-induced necroptosis by reducing P-RIP3/

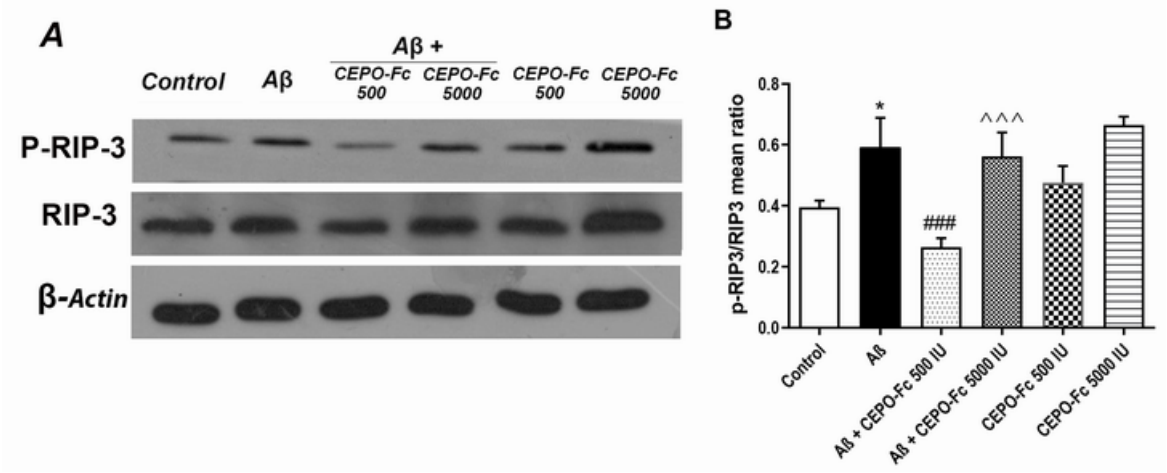

FIGURE 5. Intraperitoneal administration of CEPO-Fc induced anti-necroptotic effect in the hippocampus of AD rats. (A) Western blot analysis revealed a noticeable increase in the P-RIP-3 protein (Ser227) level in the AD rat model. AD model was induced by the intra-CA1 microinjection of 5 µg/2.5 µL $A\beta_{25\text{-}35}$ for four consecutive days in rats. Intraperitoneal administration of CEPO-Fc 500 IU significantly reduced P-RIP-3/RIP-3 ratio. (B) The densitometry values were normalized as a ratio to β-actin. Data represent mean ± SD (n = 3 in each group; $^{*}P < 0.05$ represents the difference with control group; $^{\#\#\#}P < 0.001$ represents the difference with the AD group; $^{\wedge\wedge\wedge}P < 0.001$ represents the difference between Aβ + CEPO-Fc 5000 IU and Aβ + CEPO-Fc 500 IU; One-way ANOVA test).

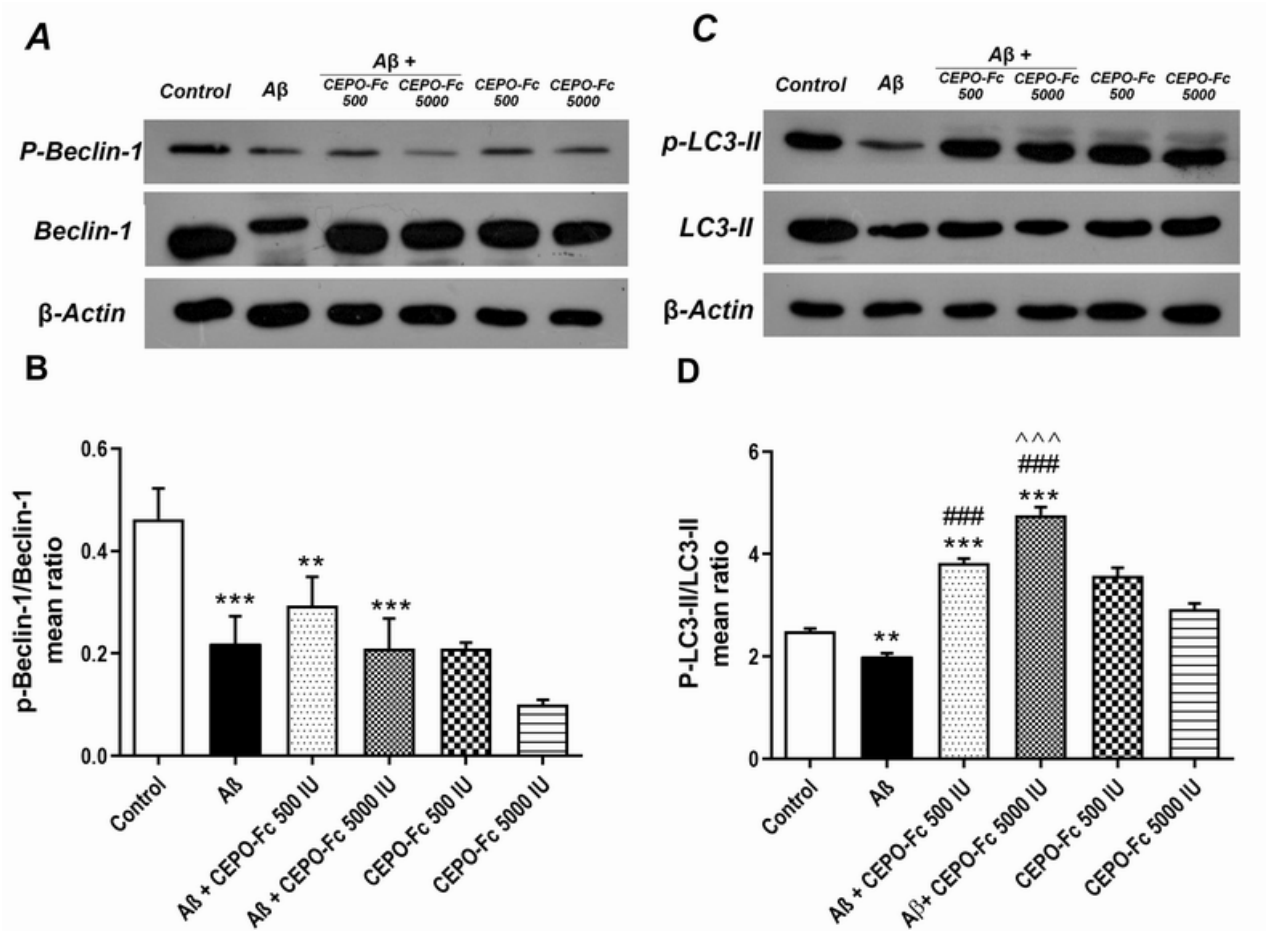

FIGURE 6. Intraperitoneal injection of CEPO-Fc increased autophagy in the hippocampus of AD rats. (A-C) Western blot analysis showed a significant decrement in the P-Beclin-1/Beclin-1 and P-LC3-II/LC3-II ratio in the hippocampus of AD rats compared with the control group. AD model was induced by the intra-CA1 microinjection of 5 µg/2.5 µL $A\beta_{25\text{-}35}$ for four consecutive days in rats. Treating with CEPO-Fc 500 and 5000 IU did not affect the P-LC3-II/LC3-II and P-Beclin-1/Beclin-1 ratios. Treating with CEPO-Fc 500 and 5000 IU elevated P-LC3-II/LC3-II ratio. (B-D) The densitometry values were normalized as a ratio to β-actin. Data represent mean ± SD (n = 3 in each group; $^{***}P < 0.001$ and $^{**}P < 0.01$ represents the difference with control group; $^{\#\#\#}P < 0.001$ represents the difference with the AD group; $^{\wedge\wedge\wedge}P < 0.001$ represents the difference between Aβ + CEPO-Fc 5000 IU and Aβ + CEPO-Fc 500 IU; One-way ANOVA test).

RIP3 ratio in the hippocampal neurons

To assess the protective properties of CEPO-Fc against $A\beta_{25\text{-}35}$ toxicity, a western blot analysis of the alterations in the enzymatic activity of RIP-3 and its phosphorylated form (Ser227) was used. The P-RIP-3/RIP-3 ratio was normalized to control and displayed in Figure 5. One-way ANOVA analysis exhibited a significant difference between the groups [F (5, 12) = 9.31, $P = 0.0008$]. Tukey's post-hoc test revealed that $A\beta_{25–35}$ significantly increased P-RIP3/RIP3 ($P < 0.05$), which was dramatically reduced by treatment with CEPO-Fc at dose 500 IU ($P < 0.001$, Figure 5). The results also revealed that CEPO-Fc 5000 IU treatment failed to reduce the activation of P-RIP-3/RIP-3 induced by $A\beta_{25–35}$. According to the statistical analysis, a significant difference was observed between Aβ + CEPO-Fc 500 IU and Aβ + CEPO-Fc 5000 IU groups ($P < 0.001$). Collectively, these findings indicate that the i.p. administration of CEPO-Fc 500 IU could efficiently reverse the necroptosis effects of $A\beta_{25–35}$.

Intraperitoneal dose of CEPO-Fc triggered autophagy by activating the Beclin-1 and LC3-II proteins in the hippocampal neurons of AD rats

The microtubule-associated protein light chain 3 (LC3-I) and its phospholipid conjugate (LC3-II) control autophagosome formation. LC3-II is used as a marker for the double-membrane vesicles, and autophagosome. Therefore, the LC3-II level can indicate the activation of autophagic flux (Rahman et al., 2021). We tested the levels of Beclin-1 and its phosphorylated form (p-Beclin-1) as well as LC3-II, and its phosphorylated form (p-LC3-II) proteins in the hippocampal neurons of AD rats to see how CEPO-Fc affected the autophagy process. Figure 6 presents the results of a western blotting analysis of the P-Beclin-1/Beclin-1 and p-LC3-II/LC3-II ratios in hippocampus cells. Analysis by one-way ANOVA revealed a significant difference between groups in the levels of P-Beclin-1/Beclin-1 [F (5, 12) = 9.31, $P = 0.0008$] and p-LC3-II/LC3-II [F (5, 12) = 18.47, $P < 0.001$]. Tukey's post hoc test disclosed that $A\beta_{25–35}$ significantly decreased P-Beclin-1/Beclin-1 ($P < 0.001$) and p-LC3-II/LC3-II ($P < 0.01$) ratios in hippocampal cells. CEPO-Fc at doses of 500 IU and 5000 IU could significantly reverse the Aβ-induced decrement of p-LC3-II/LC3-II ($P < 0.001$). Furthermore, CEPO-Fc 5000 IU was shown to be more effective than CEPO-Fc 500 IU at reversing Aβ-mediated p-LC3-II/LC3-II decrement ($P < 0.001$, Figure 6 C&D). However, CEPO-Fc did not substantially affect the P-Beclin-1/Beclin-1 ratio (Figure 6 A&B). Therefore, the findings show that CEPO-Fc at two doses of 500 IU and 5000 IU can successfully initiate autophagy by increasing p-LC3-II/LC3-II but not P-Beclin-1/Beclin-1 levels.

Discussion

The goal of this work was to discover the mechanism behind CEPO-Fc's neuroprotective effects on $A\beta_{25\text{-}35}$-induced toxicity. Following the i.p. injection of CEPO-Fc, we measured the levels of central regulators of apoptosis (Bax/Bcl-2 and caspase-3), necroptosis (P-RIP-3/RIP-3), and autophagy (P-Beclin-1/Beclin-1 and P-LC3-II/LC3-II) in the hippocampal cells of the AD rats. CEPO-Fc 500 and 5000 IU doses restored spatial learning impairment, although CEPO-Fc 5000 IU had a more prolonged impact, extending until the third day. The immunoblotting analysis demonstrated that CEPO-Fc 500 and 5000 IU predominantly affected caspase-3 levels, whereas the Bax/Bcl-2 ratio was down-regulated after CEPO-Fc 500 IU. We also showed that in response to CEPO-Fc 500 IU, the level of P-RIP-3/RIP-3 protein reduced. In addition, the ratios of P-Beclin-1/Beclin-1 and P-LC3-II/LC3-II following CEPO-Fc 500 and 5000 IU treatment revealed a considerable increase in the autophagy process. These data imply that i.p. injections of CEPO-Fc at 500 and 5000 IU can have neuroprotective effects in the AD rat model, which may be mediated in part by autophagy activation and suppression of the apoptosis and necroptosis pathways.

The FDA has approved only a few medications for AD treatment, which work only moderately and for a short time (Bloom, 2014). Therefore, developing new approaches for treating AD is desperately required (Liu et al., 2021). The well-known neuroprotective properties of EPO are frequently accompanied by undesired erythrocyte stimulating effects, which can lead to thromboembolic problems (Adembri et al., 2008). CEPO-Fc cannot simulate erythropoiesis because EPO with all lysines carbamylated to homocitrulline does not attach to the homodimeric EPOR (Macias-Velez et al., 2019). The typical β chain of the IL-3/IL-5/GMCSF (CD131) receptor, which can functionally interact with EPOR, appears to be required for CEPO-Fc–induced tissue protection (Mennini et al., 2006; Xu et al., 2009). However,

the exact mechanism of CEPO-Fc neuroprotection is still an open issue.

Because only 0.5-1% of systemically administered EPO can cross the blood-brain barrier (Castañeda-Arellano et al., 2014), higher doses of EPO are required to reach effective brain concentrations (Leyland-Jones, 2003; Wun et al., 2003). In animal studies, up to 5000 IU/kg systemic doses have been utilized (Genc et al., 2011; Zhou et al., 2020). Accordingly, Yu *et al*., reported protective effects of i.p. administration of rhEPO 5000 IU/kg on acute injury after focal cerebral ischemia (Yu et al., 2005). Due to these reports and based on earlier investigations in our group (Hooshmandi et al., 2018; Moosavi et al., 2020), we selected 500 and 5000 IU/kg of CEPO-Fc for i.p. administration in rats.

CEPO-Fc has been shown to regulate several genes that have been linked to the modulation of long-term potentiation (LTP) (Tiwari et al., 2019). It has been revealed that following TBI induction, CEPO-Fc therapy increased spatial learning and memory in rats (Mahmood et al., 2007). Furthermore, CEPO-Fc treatment after TBI reduces hippocampal neuronal loss and lesion volume, stimulates neurogenesis and angiogenesis, and enhances functional sensorimotor recovery (Xiong et al., 2011). Likewise, CEPO-Fc has been found to have significant cognitive properties in a social defeat rat model and influence hippocampal neurotrophic gene expression (Sathyanesan et al., 2018). In line with these findings, we discovered that $A\beta_{25-35}$ received rats had increased escape latencies during training sessions and traveled long distances to reach the hidden platform, confirming a negative impact of Aβ on spatial learning and memory. However, a six-day i.p. injection of CEPO-FC reversed the learning deficits induced by $A\beta_{25-35}$. Furthermore, in the probe trial, intra-CA1 microinjections of $A\beta_{25-35}$ resulted in memory retention abnormalities, whereas CEPO-Fc treatment at 500 IU and 5000 IU concentrations prevented the impairment, with no significant difference between the two doses. No significant differences in animal performance were found in the visible platform task, showing that the animals' visual-motor skills and motivation were unaffected. The underlying mechanisms of CEPO-Fc 's neuronal protection have yet to be fully understood. Several receptors and signaling pathways have been described as being involved in CEPO-Fc 's neuronal protection. The βcR, a heterodimer comprised of one EPOR monomer and CD131, is thought to be involved in EPO's extra-hematopoietic action (M. E. Chamorro et al., 2013).

Oxidative stress is a well-known hallmark of AD, which leads to structural and functional abnormalities in the brain and eventually neuronal cell death (Xilouri & Stefanis, 2010). The apoptosis process is regulated by caspase family members and protease cascades that induce apoptosis by stimulating several death-signal transduction proteins (Fan et al., 2005). CEPO-Fc treatment has been linked to anti-apoptotic properties in various conditions, such as spinal cord injury (King et al., 2007), TBI (Liao et al., 2008), and focal cerebral ischemia (Wang et al., 2007). Previously, it has been reported that daily CEPO-Fc injections (10 μg/kg, 10 days) reduce the apoptotic index and suppress hypoxia-inducible factor 1α (HIF-1α) upregulation under chronic hypoxia conditions (Fantacci et al., 2006).

Antiapoptotic signaling pathways induced by CEPO-Fc appear to be comparable to those induced by EPO (Chen et al., 2015). Similar Jak2- and PI3K-mediated pathways for the antiapoptotic effects of EPO and CEPO-Fc were found in SH-SY5Y neuroblastoma cells (Chamorro et al., 2013; Tóthová et al., 2021). By upregulating CREB-binding protein (CBP)/E1A-associated protein (p300), CEPO-Fc promotes neurite outgrowth and neuronal spine formation by upregulating the expression of two well-characterized postsynaptic molecules, Shank2 and Shank3, which controls neuronal activities. The phosphorylation of the signal transducer and activator of transcription (STAT)-3, the extracellular signal-regulated kinase (Erk), and Akt is involved in the signaling pathways from CEPO-Fc to CBP/p300 (Choi et al., 2014), while in UT-7 and TF-1 cells, CEPO-Fc induced Jak2 phosphorylation but did not result in significant activation of cell-proliferating signals such as Erk1/2, nuclear factor-κB, and STAT-5. Antiapoptotic signaling pathways induced by CEPO-Fc appear to be comparable to those induced by EPO. Similar Jak2- and PI3K-mediated pathways for the antiapoptotic effects of EPO and CEPO-Fc were discovered in SH-SY5Y neuroblastoma cells (Chamorro et al., 2013). According to Ma *et al*., CEPO-Fc possesses anti-apoptotic activities in myocardial cells that are not dependent on JAK2/STAT5 signaling, which was previously thought to involve EPO's impact (Ma et al., 2015). Chamorro *et al*., found that CEPO-Fc prevented FOXO3a phosphorylation but did not result in p27kip1 downregulation in

UT-7 and TF-1 cells. Another possible explanation for the varied effects of CEPO-Fc and EPO was the degree and time course of phosphorylation of certain signal factors, such as Jak2, Akt, Erk1/2, and FOXO3a (Chamorro et al., 2015).

Our findings showed that intra-CA1 microinjected-Aβ_{25-35} causes a substantial rise in the Bax/Bcl-2 ratio and cleavage of caspase-3, which were consequently reduced after the i.p. injection CEPO-FC 500 IU, showing that it alleviated hippocampal apoptosis and learning and memory impairment. CEPO-Fc appears to have direct metabolic effects, such as increasing the threshold for reactive oxygen species (ROS) at the mitochondrial permeability transition pore (Moon et al., 2006). Since mitochondria are O2 sensors, the rise in the threshold for ROS produced within mitochondria may destabilize HIF-1α (Guzy, 2005). CEPO-Fc also limits mitochondrial permeability transition pore opening, which reduces mitochondrial swelling and the release of ROS and cytochrome c (Moon et al., 2006). We found that CEPO-Fc 5000 IU only affects the caspase-3 level, which is widely considered the critical marker of hippocampus apoptosis. Similarly, Moosavi *et al.* found that CEPO-Fc therapy at a concentration of 5000 IU/kg/i.p. for ten days prevented learning and memory deficits and inhibited caspase-3 cleavage in the hippocampus following intracerebroventricular streptozotocin injection (Moosavi et al., 2020). As CEPO-Fc at a lower dose of 500 IU decreased both the Bax/Bcl-2 ratio and the caspase-3 levels, which are implicated in both the extrinsic and intrinsic routes of apoptosis, more exploration is required to assess the efficacy of lower doses of CEPO-Fc.

The involvement of necroptosis in neurodegenerative disorders has just been considered (Zhang et al., 2017). Necroptosis is a type of cell death controlled by signaling pathways marked by cell swelling and rupture. Necroptosis is conducted by the mixed lineage kinase domain-like (MLKL) protein, which is induced by receptor-interactive protein kinases (RIPK) 1 and 3(Caccamo et al., 2017). Despite apoptosis, necroptosis is not essential in normal development or adult homeostasis. Accordingly, RIPK3-null animals and mice with various RIPK1 kinase-dead knock-in mutations are expected in development and adulthood (Yuan et al., 2019). Necroptosis has been detected in AD, providing an effective mechanism for neuronal cell death. In the current investigation, Aβ_{25-35} microinjection increased RIP3 levels in hippocampal neurons. Similarly, necroptosis markers in human AD brains correlate favorably with the clinical manifestation and adversely with brain mass and cognition. Indeed, activated RIPK1 and RIPK3 produce Aβ-like fibrils as part of the necroptosis induction signaling pathway (Li et al., 2012). Moreover, there is much overlap between the gene sets controlled by AD and RIPK1 (Yuan et al., 2019). Ofengeim *et al.*, also demonstrated that TNF suppression by RIPK3 depletion could cause necroptosis of mature primary rodent oligodendrocytes (Ofengeim et al., 2015). The appearance of insoluble activated MLKL, RIPK1, and RIPK3, in human neurodegenerative disorders, presents the intriguing notion that necroptosis activation increases necrotic cell death and inflammation, seeding the mechanism of pathogenic protein aggregation, and eventually mediates neurodegeneration (Yuan et al., 2019). This emerging evidence highlights the importance of developing a viable therapy to modify the necroptosis process in AD patients. In this regard, *in vitro* studies demonstrated that pre-treatment of neurons with the RIPK1 inhibitor Necrostatin1 (NEC-1) prevented necroptosis and neuronal loss (Li et al., 2008; Xu et al., 2007). In line with previous investigations, we found that i.p. injection of CEPO-Fc 500 IU dramatically lowered the RIP3 marker in the hippocampus neurons of AD rats.

The presence of autophagy vacuoles in the brains of AD animal models and AD patients suggests that a dysfunctional autophagy-lysosome proteolysis pathway may account for the accumulation of Aβ and tau proteins in AD (Cataldo et al., 2004; Yang et al., 2011). Mice lacking the essential autophagy-related genes display gradual neuronal loss, abnormal intracellular protein accumulation, and the development of a massive amount of aggregates and inclusions (Hara et al., 2006; Komatsu et al., 2006). However, it is still unclear if autophagy plays a causal, protective, or merely a result of the disease pathology in AD (Liu & Li, 2019; Metaxakis et al., 2018). Autophagy appears to have a protective impact in the early stages of AD development, but it appears to trigger neurodegeneration in the advanced stages, according to a significant body of research (Liu & Li, 2019). Accordingly, studies suggest that autophagy-related proteins such as Beclin-1, Atg5, and Atg7 diminish as people grow older (Boland et al., 2008; Lipinski et al., 2010), possibly contributing to AD's late-onset (Harris & Rubinsztein, 2012). Pickford *et al*.,

discovered that in the brains of AD patients, Beclin-1, a crucial protein for autophagy induction, is lower than in healthy people (Pickford et al., 2008). Similarly, our data indicated that the $A\beta_{25-35}$ suppressed the autophagy process by decreasing the levels of p-Beclin-1/Beclin-1 and P-LC3-II/LC3-II ratios. Increased caspase 3 activity, which happens in the brains of Alzheimer's patients, is the main cause of Beclin-1 loss (Rohn et al., 2011). Treatment with Aβ peptide causes defective autophagy in astrocytes with reduced LC3-I/LC3-II transformation (Derk et al., 2018). Accordingly, in an APP transgenic mice model with Beclin-1 deletion, autophagy is disturbed, and intracellular Aβ accumulation increases, while injection of lentiviral vectors expressing Beclin-1 causes autophagy to be induced, and both extracellular and intracellular Aβ accumulation is reduced (Pickford et al., 2008). Considering autophagy modulation as an AD therapy, our findings supported the neuroprotective properties of i.p. injection of CEPO-Fc 500 and 5000 IU, which was mediated by activating autophagy by increasing the ratio of p-LC3-II/LC3-II. Similarly, beclin1F121A-mediated hyperactive autophagy in AD mice models significantly reduces Aβ accumulation, prevents cognition impairment, and restores the survival rate (Rocchi et al., 2017). In contrast, $A\beta_{42}$-induced cell death can be prevented by inhibiting rather than stimulating autophagy (Wang et al., 2010). Although baseline autophagy is needed for neuronal survival, it seems that the efficacy of increased autophagy activation is context-dependent (Bernard & Klionsky, 2014). The current study's findings align with the results of our unpublished data in which intranasal CEPO administration provided protective effects in the beta-amyloid rat model via stimulating autophagy and inhibiting necroptosis.

Our findings revealed that CEPO-Fc 500 IU was more effective than 5000 IU. Similarly, Maurice *et al.,* investigated the effects of Neuro-EPO in a range of doses (62, 125, and 250 g/kg) on $A\beta_{25-35}$-induced neurotoxicity and found that the neuroprotective effects of Neuro-EPO were bell-shaped, and the 250 g/kg dose had no impact (Maurice et al., 2013). Similarly, Yu *et al*. discovered that rhEPO might have a therapeutic benefit on acute injury following localized cerebral ischemia at lower levels than systemic treatment (Yu et al., 2005). While high levels of CEPO-Fc are beneficial for neuroprotection, they can also stimulate ROS generation, resulting in a loss of potential benefits and even toxicity (Tayra et al., 2013).

Conclusion

We compared the efficacy of intraperitoneally administered CEPO-Fc 500 IU and 5000 IU in terms of apoptosis, necroptosis, autophagy, and spatial learning. Although both doses were efficient, functional recovery via apoptosis reduction and enhanced autophagy was significantly improved in AD rats treated with CEPO-Fc 5000 IU. Our research provided novel information about AD pathology and opened up new research and treatment options for AD. Our findings strongly imply that i.p. injection of CEPO-Fc could be a viable therapeutic option for AD by reducing apoptosis and necroptosis while also activating autophagy. Our findings are consistent with earlier research and reveal a new aspect of CEPO-Fc's neuroprotective mechanism. However, more studies are required to employ various doses and delivery methods to clarify the molecular mechanism that underlies the CEPO-Fc's neuroprotective effects in AD. Also, further investigation is necessary to properly comprehend the possible biological effects of CEPO-Fc in neurodegenerative disorders besides considering when to intervene and how long/strong the modulation should be exerted.

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Conflict of interest

The authors have declared that no competing interests exist.

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