

Insulin-like growth factor-1 improves postoperative cognitive dysfunction following splenectomy in aged rats

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Abstract. Postoperative cognitive dysfunction (POCD) is a serious complication following anesthesia and operations in aged patients undergoing surgical intervention. It is characterized by temporary or permanent cognitive decline, memory impairment and deterioration in language comprehension and social adaptation ability. Therefore, the development of POCD prevention and treatment tools has become an area of interest. The current study assessed the therapeutic effects of insulin-like growth factor-1 (IGF-1) on POCD in aged rats and explored the underlying mechanisms. Model rats underwent splenectomy under 1.5-2% isoflurane and mechanical ventilation. IGF-1 (50 $\mu\text{g}/\text{kg}$) was diluted in normal saline and administered by abdominal hypodermic injection daily from the operation to day 7 post-operation. Following splenectomy, the animals showed marked cognitive impairment as determined by the Morris water maze test. Hippocampal protein levels of amyloid precursor protein (APP), β -site APP-cleaving enzyme-1 (BACE-1), amyloid- β (A β), caspase3, Bax and Bcl-2 were assessed by immunoblotting. Neuronal apoptosis in the hippocampus was analyzed using a TUNEL assay. The results demonstrated that the levels of APP, BACE-1, A β , caspase3 and Bax were increased following splenectomy, while the levels of Bcl2 were reduced at days 1, 3 and 7 post-operation in aged rats. However, IGF-1 downregulated APP, BACE-1, A β , caspase3 and Bax, and upregulated Bcl2 at these time points following splenectomy. TUNEL staining revealed that administration of IGF-1 significantly reduced neuronal apoptosis in the hippocampal CA1 region following splenectomy. These

results indicated that IGF-1 decreased A β -protein production and inhibited neuronal apoptosis in the hippocampus following splenectomy, subsequently alleviating POCD.

Introduction

Postoperative cognitive dysfunction (POCD) represents a serious complication following anesthesia and surgical procedures for patients undergoing surgical intervention (1). POCD is characterized by temporary or permanent cognitive decline, memory impairment, deterioration in language comprehension and social adaptation ability, and particularly affects elderly people (>65 years) (2). POCD can lead to increased mortality, prolonged hospitalization, other complications such as Alzheimer's disease and higher treatment costs (3). Although the pathogenic mechanisms for POCD remain unknown, its risk factors comprise trauma surgery, postoperative pain and neuronal apoptosis (4). Therefore, the development of POCD prevention and treatment tools has become a focus of interest for research.

Amyloid precursor protein (APP) is hydrolyzed in two ways: i) Degradation by α -secretase during normal physiological conditions; or ii) generation of soluble β -APP8 and C99 by β -site APP-cleaving enzyme-1 (BACE-1), followed by C99 hydrolyzation by γ -secretase to generate insoluble amyloid- β (A β) (5). APP is distributed in neuronal synapses (6). A β , a 36-43-amino acid peptide, is the main constituent of amyloid plaques in Alzheimer's disease (AD) (7). It is widely accepted that A β oligomers are causally associated with the neurodegenerative processes accompanying AD (8). The most common isoforms of A β are A β 42 and A β 40 (9), which serve important roles in POCD (10). A previous study revealed that POCD was associated with apoptosis of hippocampal neurons in rats (11). Therefore, effective inhibition of A β and apoptosis of hippocampal neurons demonstrated potential for the prevention and treatment of POCD.

Insulin-like growth factor I (IGF-I) serves critical roles in regulating body growth and metabolism and affects multiple cerebral functions (12). IGF-I promotes brain development, neuronal excitability, myelin sheath production, angiogenesis, synaptogenesis and neuronal survival, growth and differentiation (13). Additionally, IGF-I stimulates cell proliferation and survival in multiple cell types (14-16) and is considered a

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universal cytoprotective molecule, protecting cells from free radicals and apoptosis (17). Notably, a reduction in the amount of IGF-1 markedly contributed to age-associated cognitive impairment (18). A previous study revealed that IGF-1 expression was negatively associated with the progression of cognitive impairment (19).

Therefore, the current study aimed to assess whether IGF-I improved POCD by mediating apoptosis and A β production. The present study studied cognitive function in aged rats following surgery with or without IGF-I administration to investigate the protective effects of IGF-I on splenectomy-induced POCD.

Materials and methods

Animals and groups. A total of 150 male Wistar rats (age, 16-18 months; weight, 350-550 g) were purchased from the SPF Beijing Biotechnology Co., Ltd. (license no. SCXK-2016-0002) and examined. The animals were housed under a 12-hour light/dark cycle with free access to water and rodent chow. All animal experiments followed the Guidance Suggestions for the Care and Use of Laboratory Animals by the Ministry of Science and Technology of the People's Republic of China (20). Approval was obtained from the Animal Ethics Committee of Qingdao Municipal Hospital (Qingdao, China). Rats were housed under standard conditions with food and water available *ad libitum* and were allowed to acclimatize at 24-26°C for 1 week prior to experiments. The living environment of the rats was clean and tidy and suitable for survival. The rats were randomized into five groups (n=30/group), as follows: i) Control (C); ii) isoflurane (I); iii) splenectomy (S); iv) S + normal saline (S + NS) and v) S + IGF-1 (S + IGF-1).

Surgery and injection. The control group underwent no treatment. Rats in the I group were given continuous inhalation of 1.5-2% isoflurane for intubation and given 1.5% isoflurane and mechanical ventilation with 100% oxygen for anesthesia maintenance. This anesthetic procedure was selected due to its clinical relevance; additionally, anesthetics are considered to play a role in cognitive impairment (21). Rats in Group S underwent splenectomy following the same anesthesia as those in Group I. Briefly, the animals were placed in a supine position followed by skin shaving. After disinfection, an incision was made 1.5-2.0 cm below the costal margin for spleen removal. The splenic artery and vein were ligatured with silk threads. The abdominal cavity was closed after hemostasis and bupivacaine (0.25%) was administered subcutaneously prior to wound closure to ensure that the rats did not undergo pain during and following the operation (22). IGF-1 (50 μ g/kg; cat. no. 50437-MNAY; Sino Biological) was diluted in NS and administered via abdominal hypodermic injection every day from splenectomy to 7 days post-surgery in the S + IGF-1 group. In the S + NS group, equal volumes of NS were administered via abdominal hypodermic injection every day from splenectomy to 7 days post-surgery.

Current POCD models are achieved by splenectomy (23), partial hepatectomy (24) and limb orthopedic surgery (25). Splenectomy results in postsurgical reversible learning and memory dysfunction, subsequently inducing POCD (26). Furthermore, splenectomy shares significant similarity with

clinical abdominal surgery (short surgery time, controllability, high success rate and reduced mortality) (27). Therefore, splenectomy is often performed to induce a model of POCD (28).

After the rats were anesthetized with 4-5% isoflurane for 5-7 min, reflexes disappeared. When the monitor indicated cardiac arrest, respiratory arrest and pupil dilation, the rats were euthanized by cervical dislocation.

Morris Water Maze (MWM). To assess learning and memory abilities, MWM tests were performed as described previously (29). This assay can identify animals presenting with sensorimotor dysfunction and was used to assess cognitive function.

The MWM system (Shanghai XinRuan Information Technology Co., Ltd.) comprised an imaging device for swimming tracking and a circular test pool (diameter, 120 cm; height, 40 cm) with a cylindrical platform (diameter, 10 cm; height, 30 cm) 2 cm below the water surface. For the swimming test, animals were allowed to adapt to the platform for 30 sec before entering the water from four different quadrants on the pool wall. The time spent before finding the platform (escape latency) was recorded to assess learning and memory abilities prior to surgery. Animals that could not find the platform were guided within 60 sec to it and allowed a 10-sec adaptation. The swimming test was performed 4 trials/day for 5 consecutive days starting from 1 week prior to surgery. In the spatial test, the platform was removed prior to animal placement in the water for 60 sec and the ratio of swimming time in the target quadrant was evaluated. The spatial test was performed on the day preceding the operation and at postoperative days 1, 3 and 7. Escape latency and swimming distance in the target quadrant were analyzed.

Western blotting. Western blotting was performed as previously reported (30) to detect proteins. The hippocampus was lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology) and protease inhibitors (PMSF; Beyotime Institute of Biotechnology), homogenized and placed in ice for full lysis for 40 min, followed by 20 min of centrifugation (1,600 x g; 4°C). Total protein was quantified using a bicinchoninic acid assay kit. Proteins (30 μ g/lane) were resolved by 10% SDS-PAGE and electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore), which were blocked with 10% skimmed milk containing TBS-Tween-20 (TBS-T; 0.1%) in ambient conditions for 1 h at room temperature. This was followed by overnight incubation at 4°C with the following primary antibodies: Anti- β -actin (1:2,000; cat. no. ab8226; Abcam), anti-APP (1:2,000; cat. no. ab32136; Abcam), anti-BACE1 (1:1,000; cat. no. ab183612; Abcam), anti-caspase-3 (1:200; cat. no. ab13847; Abcam), anti-Bax (1:200; cat. no. ab32503; Abcam), anti-Bcl2 (1:200; cat. no. ab32124; Abcam) and anti-A β (1:2,000; cat. no. ab126649; Abcam). Following overnight incubation, the membranes were washed with TBS-T three times (10 min each) and were further incubated with goat anti-mouse secondary antibody (1:5,000; cat. no. SAA544Mu19; Cloud-Clone Corp.) and goat anti-rabbit secondary antibody (1:5,000; cat. no. bs-0295G-HRP; Bioss) for 1 h at room temperature. membranes were then washed with TBS-T three times (10 min each). The generated immune complexes were detected with an enhanced chemiluminescence detection system (Amersham; Cytiva), and visualization

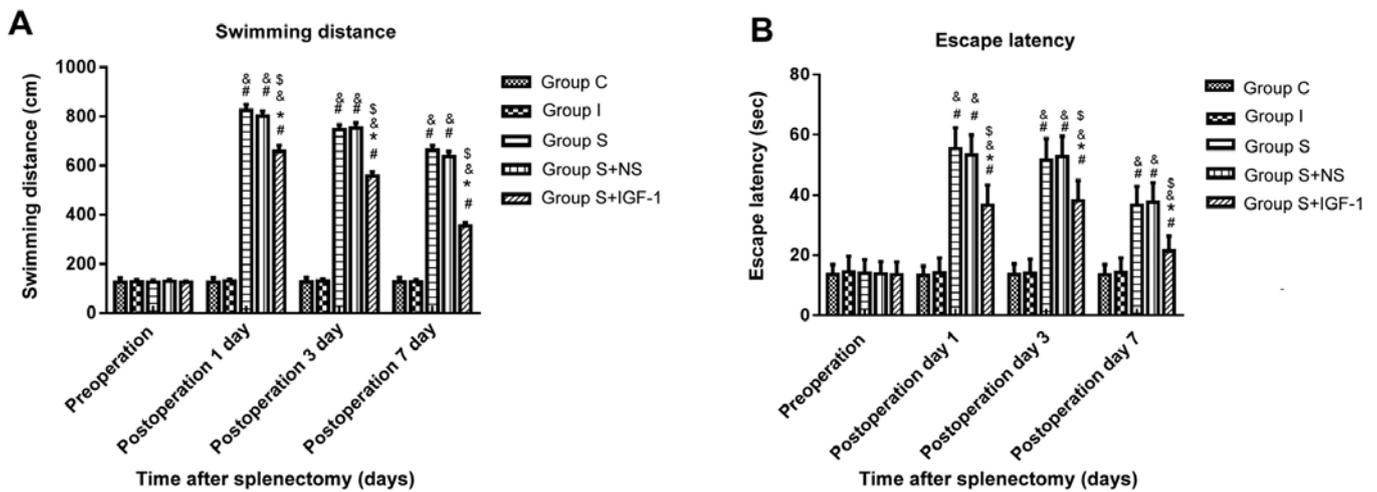


Figure 1. Neurocognitive tests were performed using the Morris water maze. The results revealed that (A) Swimming distance and (B) Escape latency increased in the S, S + NS and S + IGF-1 groups compared with the C and I groups at days 1, 3 and 7 post-surgery. [#]P<0.05 vs. C and [&]P<0.05 vs. I. ^{*}P<0.05 vs. S and [§]P<0.05 vs. S + NS. C, control; I, isoflurane; S, splenectomy; NS, normal saline; IGF-1, insulin-like growth factor-1.

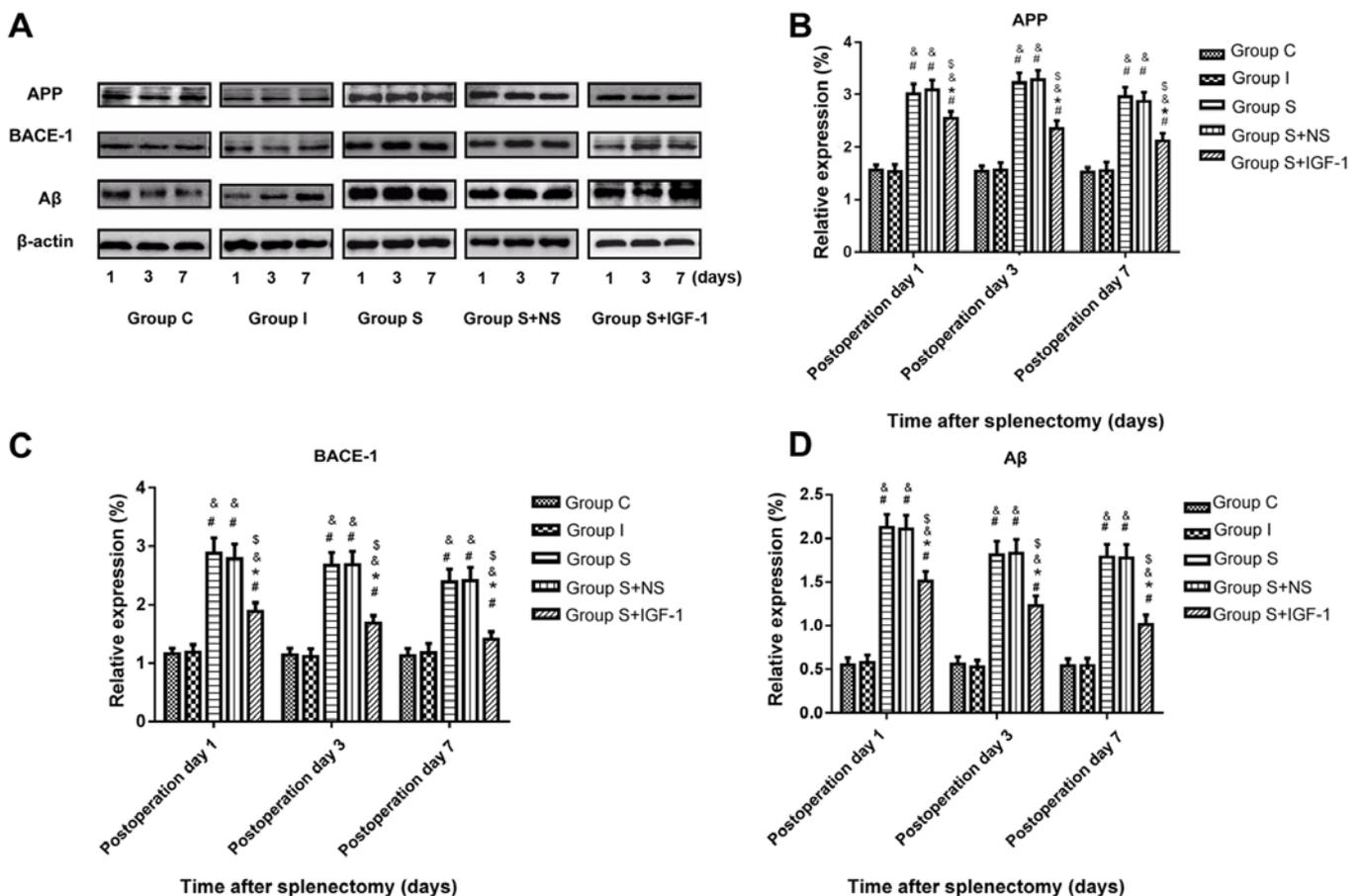


Figure 2. Hippocampal levels of APP, BACE-1 and Aβ after splenectomy at different timepoints. (A) IGF reduces the levels of (B) APP, (C) BACE-1 and (D) Aβ in the hippocampus of aged rats at days 1, 3 and 7 post-surgery. [#]P<0.05 vs. C and [&]P<0.05 vs. I. ^{*}P<0.05 vs. S and [§]P<0.05 vs. S + NS. IGF-1, insulin-like growth factor-1; APP, amyloid precursor protein; BACE-1, β-site APP-cleaving enzyme-1; Aβ, amyloid-β; C, control; I, isoflurane; S, splenectomy; NS, normal saline.

was performed with the Sygene Bio Image system (Vilber). Gray values for APP, BACE-1, Aβ, caspase3, Bax and Bcl-2 were detected by ImageJ 1.8.0 (National Institutes of Health). The ratio of the target protein to the internal control, β-actin, was used in the final analysis.

TUNEL assay. TUNEL staining was performed according to a previous study (31). Neuronal apoptosis in hippocampal samples was analyzed by TUNEL assays. The samples were fixed with 4% paraformaldehyde at 4°C for 4 h. Following this, samples were treated with 3% hydrogen peroxidase

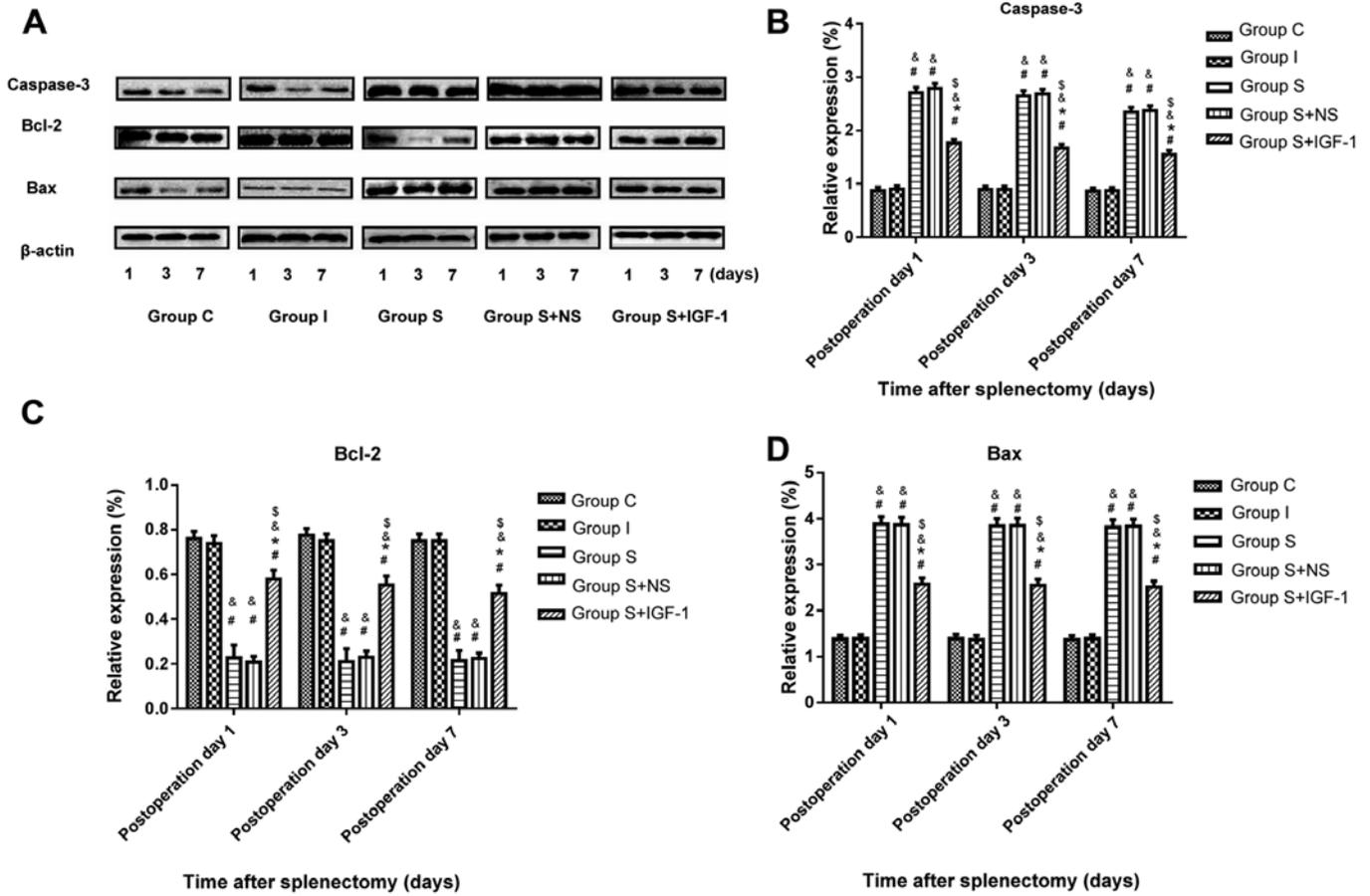


Figure 3. Hippocampal levels of caspase-3, Bax and Bcl-2 after splenectomy at different timepoints. (A) IGF reduces the expression amounts of (B) caspase-3, (C) Bax and (D) Bcl-2 in the hippocampal samples of aged rats at days 1, 3 and 7 post-surgery. #*P*<0.05 vs. C and I, **P*<0.05 vs. S and &*P*<0.05 vs. S + NS. IGF-1, insulin-like growth factor-1; C, control; I, isoflurane; S, splenectomy; NS, normal saline.

and incubated in a labeling reaction mixture comprised of terminal deoxynucleotidyl transferase and deoxynucleotides overnight at 4°C. Sections were then subjected to further incubation with horseradish peroxidase (1:500; Shanghai Macklin Biochemical Co., Ltd.) for 30 min and treatment with 3,3'-diaminobenzidine for 15 min at 37°C in the dark. Reactions were stopped with running water and counterstaining was performed with hematoxylin at 37°C for 10 min. Following dehydration with a graded ethyl alcohol series and xylene treatment, tissue samples were mounted on coverslips with neutral gum. Apoptotic nuclei appeared as dark brown dots. Apoptotic cells in the CA1 region were assessed by light microscopy (magnification, x400) in a blinded manner in five random high-power fields.

Statistical analysis. Data are presented as the mean ± SD. All parameters were assessed by one-way ANOVA followed by Tukey's post hoc test. SPSS (version no. 20.0; IBM Corp.) was used for data analysis. *P*<0.05 was considered to indicate a statistically significant difference.

Results

Cognitive function declines in aged rats following splenectomy. The MWM was performed to assess spatial learning and memory abilities. In Group S, the swimming distance (Fig. 1A)

and escape latency (Fig. 1B) were significantly longer at days 1, 3 and 7 post-surgery compared with the C and I groups. The C and I groups presented similar values for swimming distance and escape latency throughout the experiment. These results indicated that surgery aggravated cognitive impairment. Swimming distance and escape latency in the S + IGF-1 group were significantly shorter at days 1, 3 and 7 post-surgery compared with the S and S + NS groups, indicating that IGF-1 improved cognitive function following splenectomy.

IGF-1 decreases Aβ protein production in the hippocampus of aged rats following splenectomy. Protein levels of APP, BACE-1 and Aβ were assessed in hippocampal specimens following surgery by immunoblotting. Splenectomy significantly upregulated APP, BACE-1 and Aβ expression at the protein level in hippocampal samples from aged rats at days 1, 3 and 7 compared with the C and I groups (Fig. 2). IGF-1 administration following splenectomy significantly decreased the protein amounts of APP, BACE-1 and Aβ in the hippocampus of aged rats at days 1, 3 and 7 compared with the S + NS group.

IGF-1 inhibits the apoptosis of neurons in the hippocampal CA1 region in aged rats following splenectomy. Immunoblotting was performed to assess the protein levels of caspase-3, Bax and Bcl2 in rat hippocampi following surgery.

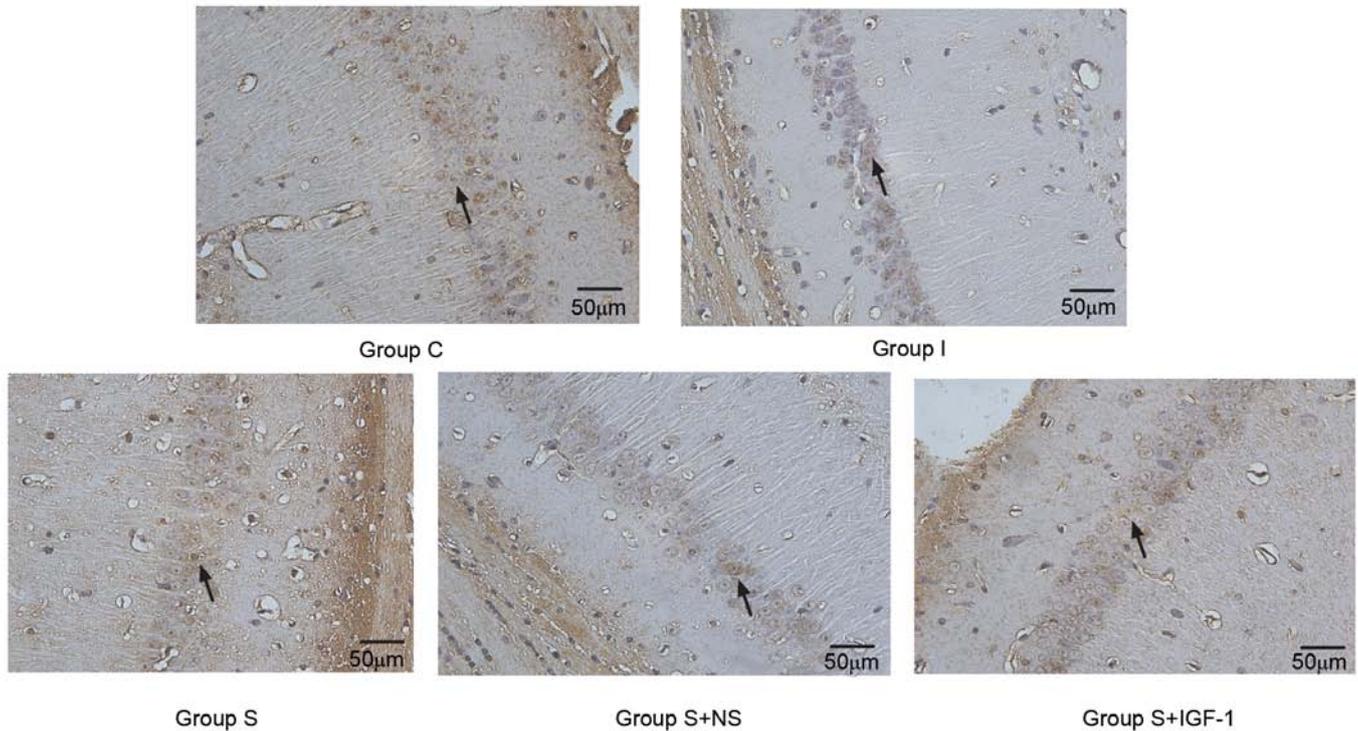


Figure 4. TUNEL staining results of the hippocampal CA1 region of rats. The numbers of neurons were reduced in the C and I groups. Markedly increased numbers of apoptotic neurons and neuron shrinking were observed in S and S + NS groups. The number of apoptotic neurons markedly decreased in the S + IGF-1 group compared with the S and S + NS groups. Black arrows indicate apoptotic neurons. Scale bar, 50 μ m. C, control; I, isoflurane; S, splenectomy; NS, normal saline; IGF-1, insulin-like growth factor-1.

The results demonstrated that splenectomy significantly upregulated caspase-3 and Bax expression, and significantly downregulated Bcl2 expression in the hippocampal CA1 region of aged rats at days 1, 3 and 7 post-surgery compared with the C and I groups (Fig. 3). Furthermore, IGF-1 administration following splenectomy significantly reduced caspase-3 and Bax protein levels, and significantly increased Bcl2 levels in hippocampal samples in aged rats at days 1, 3 and 7 post-surgery compared with the S and S + NS groups.

The TUNEL assay revealed that IGF-1 administration markedly reduced neuronal apoptosis associated with surgery in the hippocampal CA1 region of animals in the S + IGF-1 group compared with the S and S + NS groups (Fig. 4).

Discussion

The current study demonstrated that swimming distance and escape latency increased post-operatively, indicating splenectomy induced POCD in aged rats. This was consistent with previous studies which indicated markedly aggravated cognitive dysfunction in rats that underwent splenectomy (27,32). Furthermore, the results of the current study revealed that splenectomy induced the overexpression of APP, BACE-1 and A β in the hippocampus. This indicated that changes in A β -protein may be associated with early POCD, which is consistent with findings published by Canet *et al* (33). Furthermore, IGF-1, a multifunctional polypeptide essential for normal growth and development (12), inhibited the production of upstream proteins APP and BACE-1, attenuated A β production and improved surgery-induced POCD. These

results indicated IGF-1 had a protective role in POCD by attenuating A β production in aged rats. Cognitive dysfunction persists transiently due to the acute production of APP and A β (34). Neuroinflammation associated with A β aggregation is an essential factor in cognitive dysfunction (35). Cleavage of APP by BACE-1 produced soluble β -APP8 and C99, and C99 is hydrolyzed by γ -secretase to produce insoluble A β (36). Research has demonstrated that A β is located in the brain as metastable monomeric A β is constantly produced by APP under conditions of catalysis by secretases (37). AD and POCD have similar neuropathogenesis (38). Given that cognitive function is unavoidably impaired by major surgeries in aged patients, developing efficient therapeutic tools is of high significance (39). The present work recommended IGF-1 as a novel potent drug as it improved POCD by reducing the generation of A β . A previous study reported that IGF-1 inhibited JNK activity (40) and enhanced APP phosphorylation at Thr668 in rat hippocampal tissue (41). Furthermore, IGF-1 increased α -secretase expression in the hippocampus and lowered the levels of BACE1 and γ -secretase, thereby reducing the levels and deposition of A β in hippocampal tissue (42).

Additionally, the results of the current study revealed that IGF-1 downregulated caspase-3 and Bax, and upregulated Bcl2 following splenectomy in hippocampal samples at days 1, 3 and 7 post-surgery. IGF-1 administration markedly reduced neuronal apoptosis associated with surgery in the hippocampal CA1 region of rats. Apoptosis is another important mechanism for POCD development (43). The present study demonstrated that in the hippocampus of aged rats following splenectomy, caspase-3 and Bax were significantly increased,

while Bcl-2 was significantly decreased. The Bcl-2 family protein is located upstream of the mitochondria and is an important regulator of mitochondrial membrane permeability, which controls the release of cytochrome c and activates downstream caspase-3 proteases, mediating cell survival or death (44,45). Under the condition of apoptosis inducer signals, caspases are activated by the combination of specific cofactors (46). Once caspases are activated, degradation of cellular proteins occurs, eventually causing irreversible cell death (47). Bcl-2 localization in the outer membrane of mitochondria is mediated by the indirect action of the caspases (48). Apoptotic protease activating factor 1 is targeted to the mitochondrial membrane by Bcl-2, which blocks the activation of the apoptotic protease by regulating its structure and regulates the action of cry-e (49). IGF-1 prevents apoptosis by inducing the signaling pathway mediated by PI3K and its downstream target Akt (50). The interaction between IGF-1 and the IGF-1 receptor phosphorylates tyrosine kinase or activates PI3K by activating the insulin receptor substrate (51). When activated, PI3K subunits phosphorylate phosphoinositide-dependent protein kinases and activate gene expression and protein translation of downstream target Akt. When the upstream signal activates the main target enzyme Akt, anti-apoptotic genes are upregulated, and Akt regulates Bcl-2 protein expression and enhances Bcl-2 activity (52). Peruzzi *et al.* (53) demonstrated that PI3K inhibitors prevented IGF-1 from upregulating Bax and downregulating Bcl-2. Therefore, IGF-1 inhibited the apoptotic pathway (54).

Certain limitations of the current study must be discussed. Firstly, the number of animal experiments was limited. Sample sizes will be increased in future experiments. Secondly, the behavioral memory of rats can be measured comprehensively using behavior tests, including contextual fear conditioning test and the elevated plus maze test. Thirdly, since animal models cannot completely reproduce complex clinical situations, it remains essential to confirm whether similar changes occur in patients following surgery.

Overall, the current study reported a neuroprotective role for IGF-1 for POCD in aged rats. The mechanism involved decreased A β -protein production and inhibited neuronal apoptosis in the hippocampus. The present results indicated the use of IGF-1 for preventing POCD in aged patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YB and MW conceived and designed the current study and drafted the manuscript. CX, CL and JZ performed the experiments at the physical laboratory of Qingdao University, China. RD, XL and XS analyzed data. GZ and BW performed the experiments and wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics and approval and consent to participate

The present study followed the recommendations of the National Institute of Health guidelines for the care and use of laboratory animals and obtained approval from the Clinical Trial Ethics Committee of Qingdao Municipal Hospital, Qingdao, China.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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