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ORIGINAL

Erythropoietin (EPO) and Hippocampal Neurons: Effects on Sports Performance, Fitness, and Football Players with Vascular Cognitive Impairment

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ABSTRACT

Objective: This study delves into the impact of erythropoietin (EPO) on hippocampal neurons and its potential implications for sports performance, fitness, and the cognitive well-being of football players facing vascular cognitive impairment. **Methodology:** The study employs a comprehensive approach, utilizing a rat model of vascular dementia (VaD) induced by bilateral carotid artery ligation. Exogenous EPO is administered to the VaD rat model. Observations of EPO's influence on hippocampal neurons are made, and in vitro experiments are conducted to validate the specific mechanisms at play, particularly under oxygen/glucose-deprived conditions. **Results:** The results reveal several noteworthy findings. VaD rats treated with EPO demonstrate significantly shorter escape latency, increased neuronal populations, and enhanced preservation of Nissl bodies in hippocampal subfields, specifically cortical area 1 (CA1) and CA2. Moreover, these rats exhibit a lower count of TUNEL-positive cells compared to the model group, with higher doses of EPO

demonstrating more notable improvements in escape latency. Molecular analysis shows that EPO up-regulates key protein expressions, including phosphorylated EPO receptor (p-EPOR), p-phosphatidylinositol 3-kinase (p-PI3K), p-protein kinase B (Akt), and p-cyclic AMP response element binding protein (p-CREB). Simultaneously, it down-regulates expressions of apoptosis- and autophagy-related proteins, such as B-cell lymphoma-2-associated X protein (Bax), Cleaved-Caspase 3, Cleaved-Caspase 9, light chain 3β (LC3β), Beclin, autophagy-related gene 5 (ATG5), and ATG7. Notably, in vitro experiments confirm the role of the PI3K/AKT pathway in EPO's mechanisms, with implications for cognitive health. **Conclusion:** These findings suggest that EPO may hold promise as a potential intervention to suppress apoptosis and autophagy in hippocampal neurons affected by vascular cognitive impairment. The broader implications extend to sports performance and fitness, particularly for football players who rely on cognitive and physical prowess. Addressing cognitive health alongside physical fitness may yield innovative strategies for enhancing athletic performance and the well-being of athletes facing cognitive challenges. Further research in this direction could pave the way for novel approaches to optimize both physical and cognitive aspects of sports performance in football and beyond.

KEYWORDS: Football players, Athletes, Fitness, the PI3K/AKT signaling pathway, vascular dementia, hippocampus, apoptosis and autophagy, oxidative stress, miR-29

INTRODUCTION

In the world of sports, particularly in the physically demanding arena of football, athletes are revered not only for their physical prowess but also for their cognitive abilities, including quick decision-making, strategic thinking, and spatial awareness. Football, often dubbed "the beautiful game," places immense demands on players, requiring them to excel both physically and mentally. However, the cognitive well-being of athletes, particularly football players, can be challenged by conditions such as vascular cognitive impairment, which can impact their performance, fitness, and overall athletic journey.(Devan, Goad, & Petri, 1996).

Vascular cognitive impairment, associated with conditions like vascular dementia (VaD), poses a significant threat to cognitive health. As football players engage in rigorous training regimens and compete at elite levels, any compromise to their cognitive abilities can have profound implications for their careers and well-being. It is within this context that the potential role of erythropoietin (EPO), a naturally occurring hormone with known neuroprotective properties, comes into focus.(ARUNACHALAM, SIVAKUMAR, & MURUGAN, 2017).

EPO has garnered attention not only for its role in erythropoiesis but also for its impact on neural tissues, including the hippocampus—a region crucial for memory, spatial navigation, and cognitive functions. Recent studies have explored the protective mechanisms of EPO on hippocampal neurons, particularly in the context of vascular cognitive impairment(Kim et al., 2015). Understanding how EPO influences hippocampal neurons holds promise not only for individuals facing cognitive challenges but also for athletes, including football players, whose cognitive and physical abilities are intertwined(Dong & Venkatachalam, 2003). This study embarks on a journey to explore the intricate relationship between EPO and hippocampal neurons, with a unique focus on its potential implications for sports performance, fitness, and the cognitive well-being of football players. Through a comprehensive approach, including in vivo observations and in vitro experiments, this research aims to shed light on how EPO might serve as a protective agent for hippocampal neurons in the face of vascular cognitive impairment(Knudson & Korsmeyer, 1997; Namura et al., 1998).

The findings of this study may hold transformative potential, not only in the realm of cognitive health but also in the world of sports(Chu, 2008; B. Liu et al., 2014). Football players, celebrated for their mental agility as much as their physical prowess, could benefit from interventions that safeguard their cognitive health(Koukourakis et al., 2010; Maejima et al., 2013). Enhanced cognitive function can lead to better decision-making, strategic planning, and teamwork on the field, ultimately influencing their sports performance. Moreover, addressing cognitive health alongside physical fitness is paramount for athletes who strive for excellence in both arenas(Xingyong et al., 2013). As the study unravels the intricate mechanisms behind EPO's effects on hippocampal neurons, it invites us to envision a future where innovative strategies optimize both cognitive and physical aspects of sports performance. This research underscores the holistic well-being of athletes and the multifaceted nature of their athletic journey(Heid et al., 2017; Kiffin, Bandyopadhyay, & Cuervo, 2006; Shiau et al., 2022). Ultimately, it emphasizes that the pursuit of peak performance on the football field may indeed intersect with the quest for cognitive excellence—an intersection where EPO and hippocampal neurons play a pivotal role.

1. MATERIALS AND METHODS

1.1 Establishment of animal model and grouping

Adult male Wistar athletes, weighing (170±10) g, were purchased from the Laboratory Animal Center of Hebei Medical University. The VaD model was established by permanent ligation of bilateral common carotid arteries in athletes with reference to the Olsson method (Olsson, Brun, & Englund, 1996). After anesthesia, a medial incision was made at the neck to bluntly separate

subcutaneous connective tissue, anterior cervical muscles and separate and expose the left and right common carotid arteries. Finally, the bilateral common carotid arteries were ligated using 0 silk sutures. All the animals were randomized into four groups control group, model group, low-dose EPO group and high-dose EPO group. At 1 h after operation, EPO (Shanghai Kexing Biopharm Co., Ltd.) was intraperitoneally injected for 7 d at 3,000 U/kg/once. No drug injection was conducted in control group and model group.

1.2 Cell treatment and culture

Hippocampal neurons were routinely cultured (Ludueña, 1973). The bilateral hippocampi were aseptically separated from 1-day-old Wistar athletes, and digested and dispersed in 0.125% trypsin (37℃, 30 min). Then, they were prepared into cell suspension at the density of 5×10^8 cells/L, seeded into 35 mm plastic petri dishes coated with calfskin collagen at 2 mL/dish, which was then cultured in an incubator with 10% CO_2 and air at 36℃. After being cultured for 12 d, the hippocampal neurons were taken, and all the cells were divided into control group and model group (oxygen/glucose-deprived group) according to the experiment requirements.

Serum-free glucose-free artificial cerebrospinal fluid (ACSF) composed of 124 mmol/L NaCl, 3.3 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 44 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, and 2.4 mmol/L $MgSO_4$ was used in oxygen/glucose-deprived group. The cells were treated with EPO and Dactolisib (BEZ235) in control group and model group, respectively, while those in high-dose EPO group were treated with EPO + Dactolisib (BEZ235). Then, the cells in model group were transferred to a thermostatic airtight container, and constantly filled with oxygen-free gas (90% N_2, 10% CO_2) for culture, and those in control group were normally cultured.

1.3 Morris water maze test

After the rat model was constructed, the spatial learning and memory capacities of athletes were determined using Morris water maze test. According to the method of Shan *et al.*, the assessment was conducted for six times each in the morning and afternoon after the athletes received escape latency and platform-crossing training for five times. At the same time, the escape latency and the times of athletes crossing the original platform after removing the platform were recorded, and the recorded values were averaged (Shan et al., 2007).

1.4 Nissl staining

The well-fixed cerebral tissues were placed in an embedding box, washed using running water and then stored in 75% alcohol overnight. On the next day, the tissues were dehydrated in gradient alcohol, transparentized using

xylene, and embedded in paraffin. The embedded tissues were serially sectioned with a microtome (4 μm), conventionally deparaffinized, and subjected to Nissl staining by means of methyl violet stain. After that, the differentiated sections were conventionally dehydrated and mounted. Finally, the morphology of Nissl bodies in the hippocampal brain regions was observed and photographed under a light microscope and counted.

1.5 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

The TUNEL staining was conducted in accordance with the instructions of the TUNEL staining kit. The TUNEL-positive cells were microscopically brown and normal cells blue. The TUNEL-positive cells and total cells were counted in 5 randomly-selected fields under a fluorescence microscope. The number of the apoptotic cells was calculated using the ImageJ software based on the formula apoptosis index (AI)= the number of positive cells in each field/total cells in the fields ×100%.

1.6 Western blotting

The hippocampal tissues and cells were collected, from which the total proteins were extracted using the bicinchoninic acid (BCA) kit. Then the protein content was determined. An equal amount of protein sample was extracted and denatured at 100°C for 5 min. The protein sample was isolated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane.

Subsequently, the resulting protein was incubated with the corresponding primary antibodies against phosphorylated EPO receptor (p-EPOR), p-phosphatidylinositol 3-kinase (p-PI3K), p-protein kinase B (Akt), p-cyclic AMP response element binding protein (P-CREB), Bcl-2-associated X protein (Bax), Cleaved-Caspase 3, Cleaved-Caspase 9, light chain 3β (LC3β), Beclin, autophagy-related gene 5 (ATG5), ATG7, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 4℃ overnight. Then the horseradish peroxidase-labeled secondary antibodies were added for 2 h of incubation at 4°C. Finally, the ECL solution was added for exposure. The gray value was calculated using the ImageJ software.

1.7 Immunofluorescence staining of p-PI3K, p-AKT and oxidative stress-probe

Semiquantitative analysis of hippocampal neurons cell climbing sheets which gathered after 72h by immunocytochemistry for p-PI3K, p-AKT, and briefly the PBS climbing sheets were flushed 3 times by PBS, and fixated by 4% paraformaldehyde, goat serum blocked for 30 minutes and then incubate the first antibody of p-PI3K and p-AKT (1:50 dilution), 4 °C overnight and washed

by PBS for 3 times, and then incubated with corresponding secondary antibodies. The oxidative stress-probe were in cubed for 30 minutes and then washed with PBS for 3 times. Image acquisition under fluorescence microscope for all sheets.

1.8 Statistical analysis

SPSS 17.0 software was employed for statistical analysis. Data were expressed as ($\bar{\chi}\pm s$). Pairwise comparisons were made using independent *t*-test, while the intergroup comparisons were conducted using one-way analysis of variance. $p<0.05$ was considered to be statistically significant.

2. Experimental results

2.1 Comparison of escape latency among all the groups of athletes

Compared with that in control group, the escape latency was obviously prolonged at each time point in model group and low-dose EPO group. Moreover, EPO groups had greatly shorter escape latency at each time point than model group.

2.2 Nissl staining results

There were more neurons and Nissl bodies in the hippocampal subfields cortical area 1 (CA1) and CA2 in control group, and the neuronal cells had round morphology and dark color and were neatly arranged. Model group had fewer neurons and Nissl bodies in the CA1 and CA2 than control group, and the neuronal cells were lightly stained and vesicular. In comparison with those in model group, neurons and Nissl bodies were markedly increased and the neuronal cells had darker color and rounder morphology in the CA1 and CA2 in EPO group.

2.3 EPO decreased the apoptosis of hippocampal neurons in athletes

After TUNEL staining, typical positive cells had brown-stained nucleus, while some cells lost normal morphology due to karyopyknosis, and nucleus distortion and rupture. After operation, TUNEL-positive cells were observed in the hippocampi in all the groups. Low-dose EPO group and high-dose EPO group had significantly fewer TUNEL-positive cells than model group, and the number of these cells was the smallest in high-dose EPO group. The above results suggest that EPO can repress the apoptosis of hippocampal neurons in VaD rats.

2.4 EPO decreased the apoptosis of autophagy of hippocampal neurons in athletes

According to the Western blotting results, the protein expressions of p-

EPOR, p-PI3K, p-AKT, and p-CREB obviously rose, and the expressions of the apoptosis- and autophagy-related proteins Bax, Cleaved-Caspase 3, Cleaved-Caspase 9, LC3β, Beclin, ATG5, and ATG7 were also considerably increased after operation. Compared with those in model group, the protein expressions of p-PI3K, p-AKT, and p-CREB were further raised, while the expressions of the apoptosis- and autophagy-related proteins declined markedly. Besides, high-dose EPO had a more significant effect on the expressions of these proteins.

2.5 EPO activated the PI3K/AKT signaling pathway to decrease the apoptosis of oxygen/glucose-deprived hippocampal neurons

The hippocampal neurons cultured *in vitro* under oxygen/glucose-deprived conditions had evidently higher protein expressions of p-EPOR, p-PI3K, p-AKT and p-CREB, as well as expressions of Bax, Cleaved-Caspase 3 and Cleaved-Caspase 9 than those in normal group. After treatment with EPO, the protein expressions of p-EPOR, p-PI3K, p-AKT and p-CREB were further increased, while the expressions of Bax, Cleaved-Caspase 3 and Cleaved-Caspase 9 declined in the hippocampal neurons cultured *in vitro* without oxygen and glucose.

PI3K phosphorylation was repressed in the hippocampal neurons treated with Dactolisib (BEZ235), followed by decreased protein expressions of p-AKT and p-CREB but raised expressions of Bax, Cleaved-Caspase 3 and Cleaved-Caspase 9, suggesting that EPO may decrease the apoptosis of oxygen/glucose-deprived hippocampal neurons through activating the PI3K/AKT signaling pathway.

2.6 EPO activated PI3K/AKT signaling and suppressed the oxidative stress in hippocampal neurons cell via inhibiting miRNA-29

Under hypoxic environment, EPO groups significantly increased the immuno- fluorescence intensity vs control group, and the dactolisib treated groups showed the least immuno- fluorescence intensity; the oxidative stress was negatively associated with activated PI3K/AKT signal, therefore the EPO remarkably suppressed the ROS intensity vs control groups and the dactolisib significantly increased the ROS. And the miRNA-29 is the key regulator for PI3K/AKT signals thus the relative miRNA-29 were also testeb by Q-PCR, the EPO inhibited the expression of miRNA-29, data shwon in figure 6C.

3. DISCUSSION

VaD refers to cerebrovascular disease-related brain dysfunction, which is an important etiology of senile dementia[1]. One of cause of VaD is chronic cerebral ischemia, hypoxia and hypoperfusion (Rojas-Fernandez & Moorhouse, 2009). The messenger ribonucleic acid (mRNA) and protein of EPO and its receptors can be expressed in cerebral nerve cells at various sites, such as in

multiple mammalian neurons, glial cells, and vascular endothelial cells, and EPO and its receptors have been found to have a strong activity of secreting EPO in an oxygen-deficient environment (Moore, Bellomo, & Nichol, 2011). Previously, EPO was traditionally regarded as a macromolecular substance that theoretically cannot pass through blood brain barrier (BBB). However, in recent years, it has been found that exogenous EPO can enter the brain through BBB probably by the mechanism that EPO binds to specific receptor and is then selectively transported into the brain through BBB by endocytosis.

The VaD athletes model experiment and in vitro cell assay results have demonstrated that EPO has a neuroprotective effect against cerebral ischemia (R. Liu, Suzuki, Guo, Mizuno, & Urabe, 2006). The above studies have suggested that endogenous and exogenous EPOs have the protective effect against cerebral ischemic injury and provide feasible molecular bases for the clinical treatment of VaD with EPO These are consistent with the findings of the present study that EPO had a protective effect on hippocampal neurons in VaD as proven by *in vivo* and *in vitro* experiments. Compared with that in model group, the escape latency was obviously shortened at each time point after intraoperative injection of EPO and high-dose EPO group showed a more significant decrease in the escape latency. At the same time, neurons and Nissl bodies were markedly increased and the neuronal cells had darker color and rounder morphology in the CA1 and CA2 after EPO treatment.

The PI3K/AKT signaling pathway is a classic pro-survival and anti-apoptosis pathway, and once activated, it plays an important role in cerebral ischemic-hypoxic neuronal injury. A study has demonstrated that the PI3K/AKT signaling pathway can inhibit the apoptosis of hippocampal neurons to improve the cognitive disorder in VaD athletes (Si et al., 2019). Mammalian target of rapamycin (mTOR), the downstream signaling factor of the PI3K/AKT signaling pathway, after being activated, can modulate cell autophagy. According to a study (Yeh et al., 2016), the PI3K/AKT signaling pathway plays a direct or indirect regulation effect on the cerebral ischemic neuronal injury, thereby mitigating the learning, memory and other cognitive disorders of the animal model. In the present study, consistent experimental results showed that EPO obviously up-regulated the postoperative protein expressions of p-PI3K, p-PI3K and p-CREB in the hippocampal tissues, thus exerting a protective effect against VaD-caused neuronal injuries.

Cell apoptosis, one mode of cell death in chronic ischemic-hypoxic injuries, has not fully clear pathogenesis. However, multiple studies have implied that cell apoptosis is regulated by specific genes. Of them, Bcl-2 and Bax are considered to be the two most important anti-apoptotic and pro-apoptotic genes, respectively, and the gene family is composed of the anti-apoptotic genes represented by Bcl-2 and the pro-apoptotic genes represented by Bax, which play an important role in apoptosis and serve as indicators of

assessing the degree of apoptosis (Wang, 2001; Zarch et al., 2009). The results of this study showed that TUNEL-positive cells were seen in all groups of hippocampi after operation and EPO reduced the number of TUNEL-positive hippocampal neurons in athletes. Additionally, the Western blotting results displayed that EPO down-regulated the expressions of the apoptosis-related proteins Bax, Cleaved-Caspase 3 and Cleaved-Caspase 9 in postoperative rat hippocampal tissues.

There are no obvious neuronal apoptosis and necrosis in the cerebral ischemic-hypoxic model mice with the deficiency of ATG7, an indispensable gene for autophagy induction, indicating that in the process of cerebral ischemia pathological damage, autophagy is a necessary mechanism of action for neuronal necrosis (Koike et al., 2008). Based on the results of the present study, EPO significantly downregulated the expression of autophagy-related proteins LC3β, Beclin, ATG5, and ATG7 in the hippocampal neurons of athletes. This illustrates that EPO can protect VaD rat hippocampal neurons by inhibiting autophagy.

The hippocampal neurons were cultured *in vitro* under oxygen/glucose-deprived conditions to further validate the mechanism by which EPO suppresses the apoptosis and autophagy in postoperative rat hippocampal tissues. After treatment with Dactolisib (BEZ235), PI3K phosphorylation was repressed in the hippocampal neurons, followed by decreased protein expressions of p-AKT and p-CREB but raised expressions of Bax, Cleaved-Caspase 3 and Cleaved-Caspase 9, suggesting that EPO may decrease the apoptosis of hippocampal neurons cultured without oxygen and glucose through activating the PI3K/AKT signaling pathway. In summary, EPO inhibits the apoptosis and autophagy of hippocampal neurons in athletes through activating the PI3K/AKT signaling pathway.

4. Conclusion:

In the pursuit of athletic excellence, especially in sports as demanding as football, the convergence of physical prowess and cognitive acuity is paramount. This study, exploring the intricate relationship between erythropoietin (EPO) and hippocampal neurons in the context of vascular cognitive impairment, uncovers a tapestry of potential implications for sports performance, fitness, and the well-being of football players.

The findings of this study underscore the importance of addressing cognitive health alongside physical fitness, highlighting the symbiotic nature of these two dimensions in the athletic journey. Cognitive health is not merely an adjunct concern; it is a foundational pillar upon which athletic prowess is built. Football players, celebrated for their strategic thinking, quick decision-making, and spatial awareness, stand to gain immensely from interventions that

safeguard their cognitive well-being. EPO, a hormone primarily known for its role in erythropoiesis, reveals a new facet of its potential—one that could transform how athletes, particularly football players, approach their training and competition. The study's observations of EPO's protective mechanisms on hippocampal neurons hold promise. The neuroprotective properties of EPO suggest that it may serve as a shield against cognitive decline, especially in the face of conditions like vascular cognitive impairment.

Enhanced cognitive function, as facilitated by EPO, extends beyond the football field. It contributes to better decision-making, strategic planning, and teamwork, all critical elements in sports performance. Football players who are mentally sharp can excel in their positions, elevate team dynamics, and navigate the complexities of the game with greater finesse. Moreover, the implications of this research reach beyond sports, encompassing the broader landscape of cognitive health and well-being. As we unlock the mechanisms through which EPO interacts with hippocampal neurons, we open doors to innovative strategies that optimize both physical and cognitive dimensions of sports performance. These strategies may benefit athletes in various disciplines and individuals facing cognitive challenges.

In conclusion, the pursuit of peak performance in sports, exemplified by football, intersects profoundly with the quest for cognitive excellence. EPO and its protective influence on hippocampal neurons stand at this intersection, offering a glimpse into a future where athletes, including football players, can thrive not only physically but also cognitively. The holistic well-being of athletes is a multifaceted endeavor, where the fusion of mind and body is celebrated. As we continue to unravel the mysteries of EPO and cognitive health, we pave the way for a new era of athletic excellence—one where the beautiful game of football and the brilliance of cognitive prowess unite in harmony.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

Compliance with Ethical Standards.

Data Availability

The DATA TYPE data used to support the findings of this study are available from the corresponding author upon request.

Authors' Contributions

The first draft of the manuscript was written by Zhipeng Tang and all authors commented on previous versions of the manuscript. Qian Zhang, Yangqing Tie, Nan Yin and Lei Gaoto the study conception and design. Xiaoli Niu: Material preparation. Sheng Chang: Data curation. Xiaozheng Gu: Software. Peiyuan Lv: Project administration. All authors read and approved the final manuscript.

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subjected to ischemia-reperfusion-induced injury. *International journal of neuroscience, 119*(9), 1346-1361.

Figures Legends

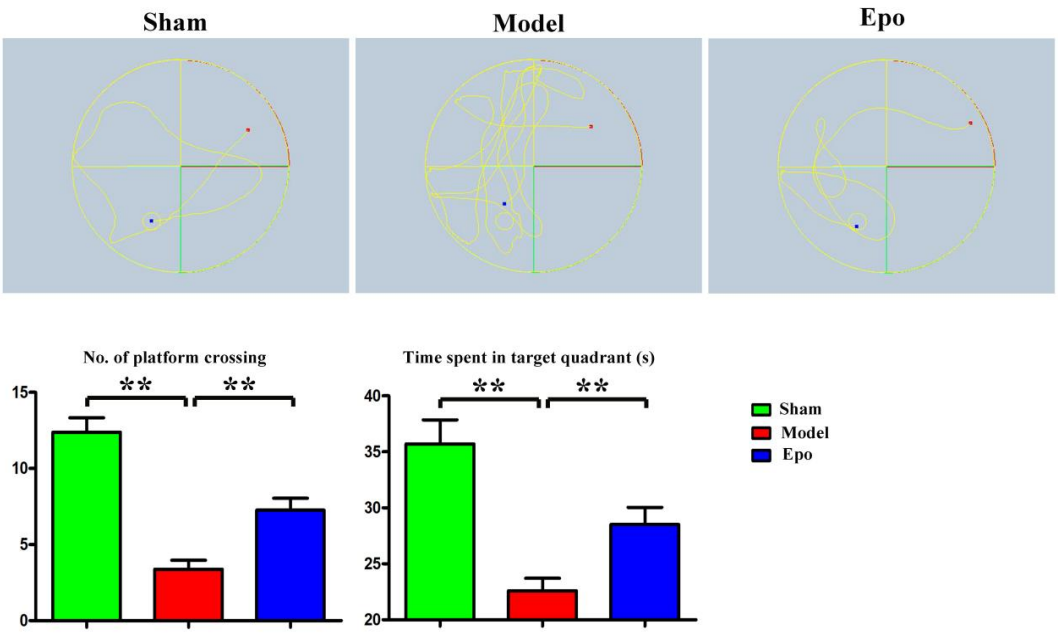

Figure 1: EPO treatment improved neurological behavior. A: EPO increased the numbers of platform crossing and time in target quadrant. B: statistic data.

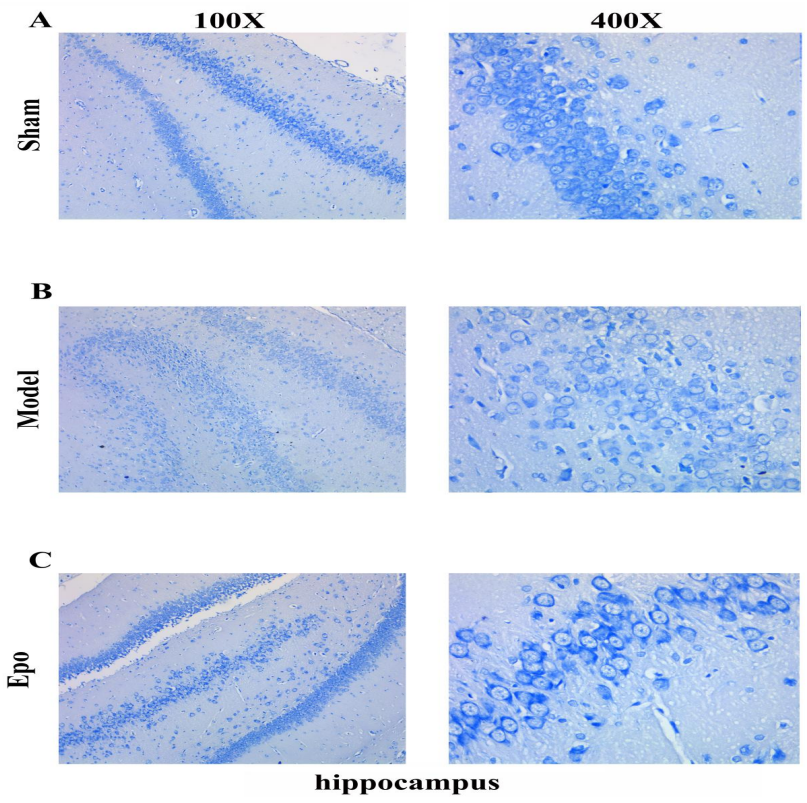

Figure 2: EPO improved hippocampal structure in vascular diementia rat brains. A: hippocampal in control rat; B: hippocampal structure in vascular diementa; C: hippocampal structure of EPO treated vascular diementa.

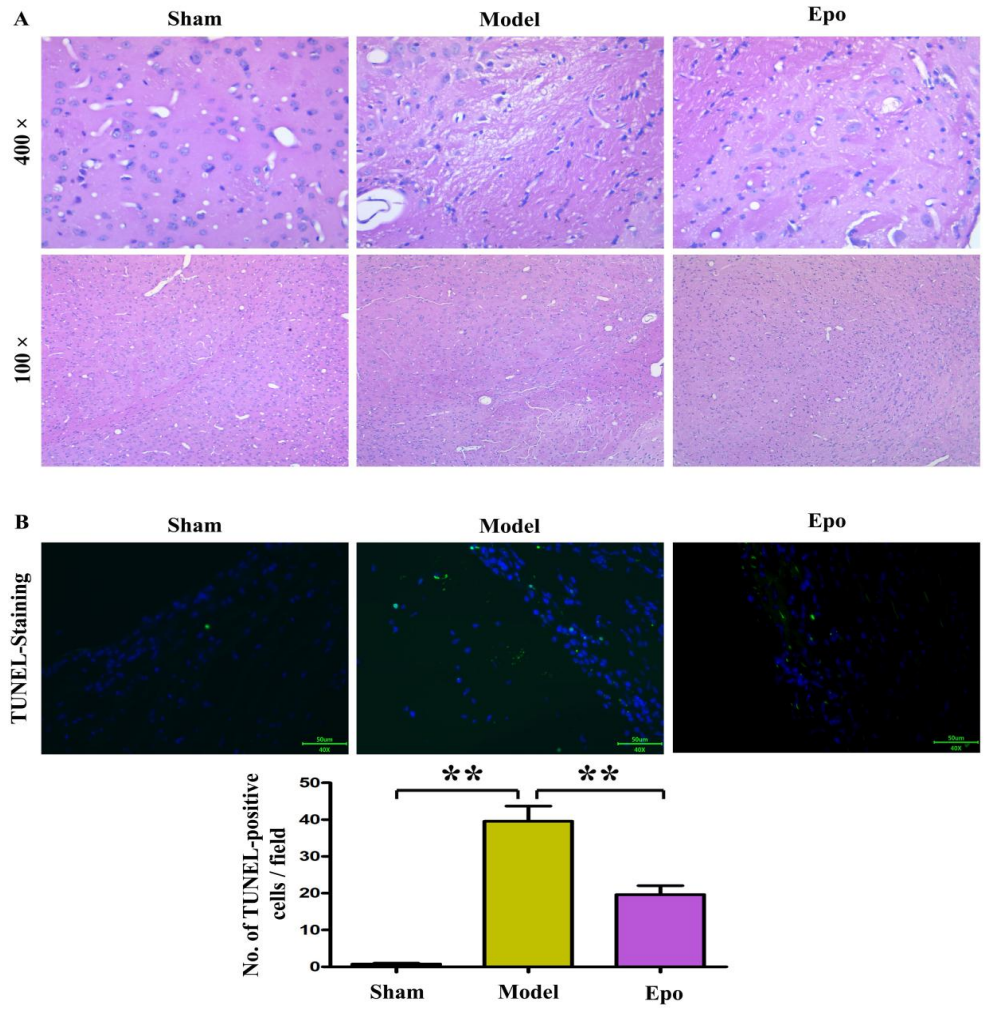

Figure 3: EPO suppressed the apoptosis in mouse brain. A: HE staining showed the less nucleus distortion and rupture after EPO treatment. B: EPO suppressed the TUNEL-positive numbers in brain.

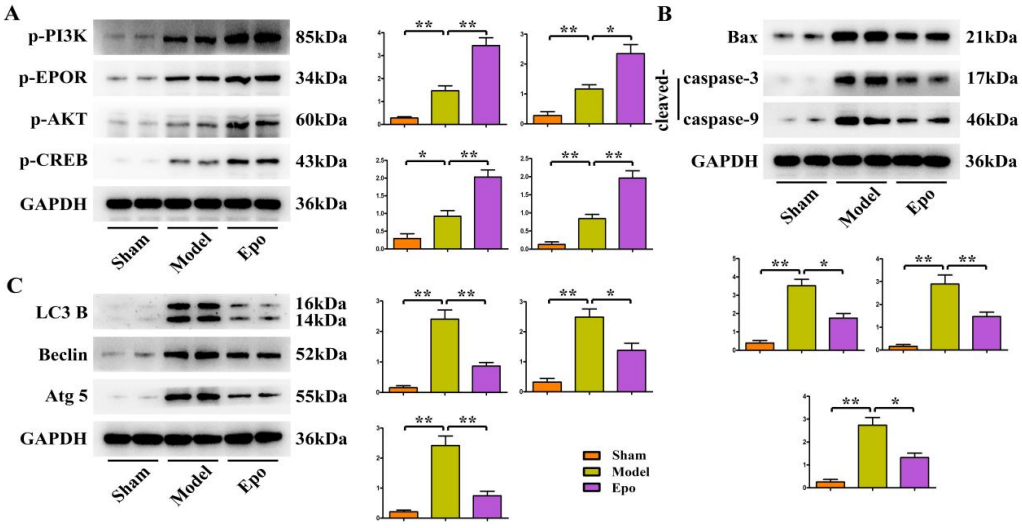

Figure 4: EPO inhibited autophagy and apoptosis by increasing PI3K/AKT signal. EPO activated the PI3K, EPO, AKT and CREB (A) and inhibited LC3B, beclin, Atg5 (C) as well as Bax, cleaved -caspase-3/-9 (B) vs model group, tested by western blot.

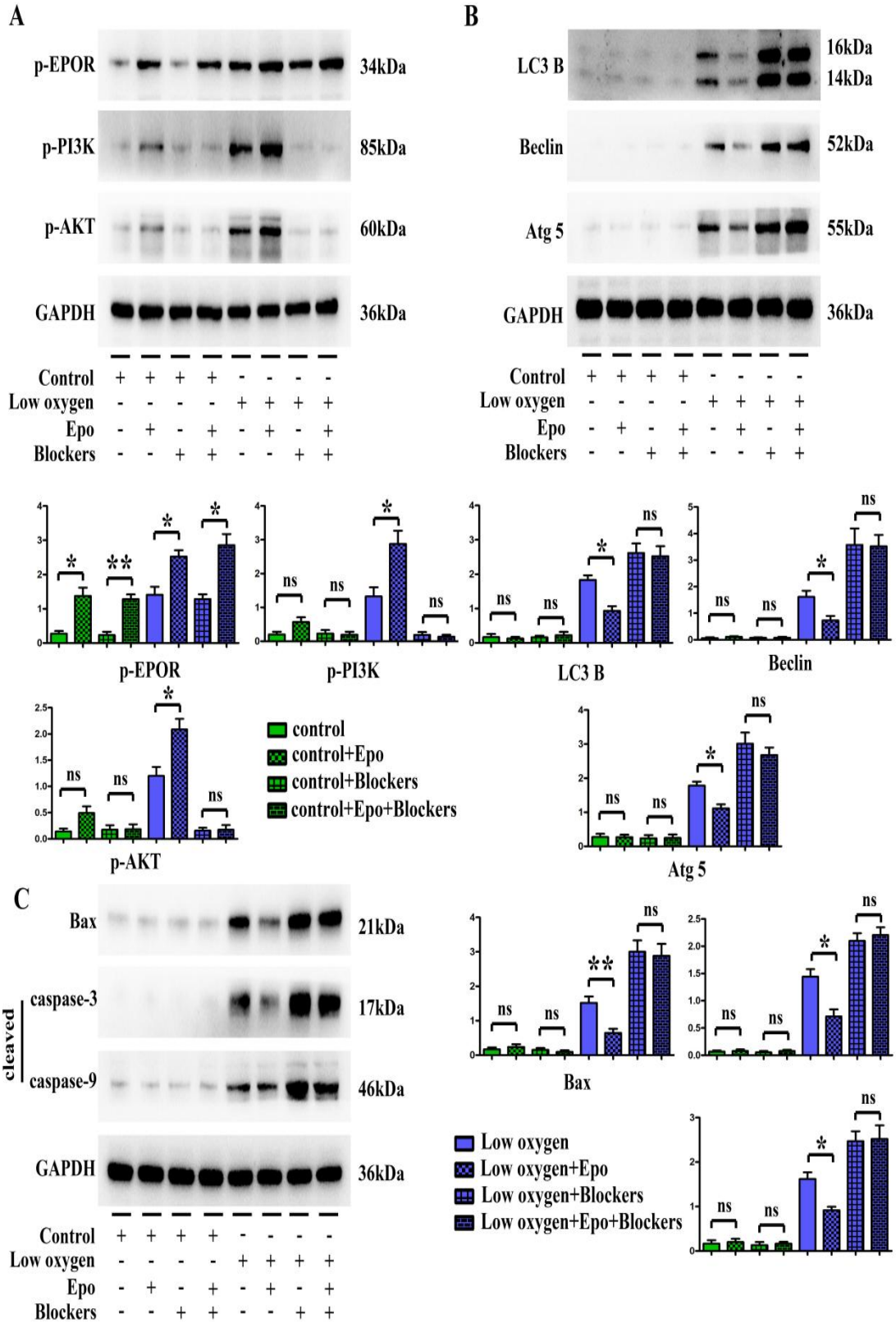

Figure 5: EPO suppressed autophagy and apoptosis through PI3K signal in hippocampal neurons. EPO activated PI3K/AKT (A), and suppressed LC3B, becli, Atg5 (B), as well as Bax, cleaved-caspase-3/-9 (C), and these effects were corrected by dactolisib, tested by western blot.

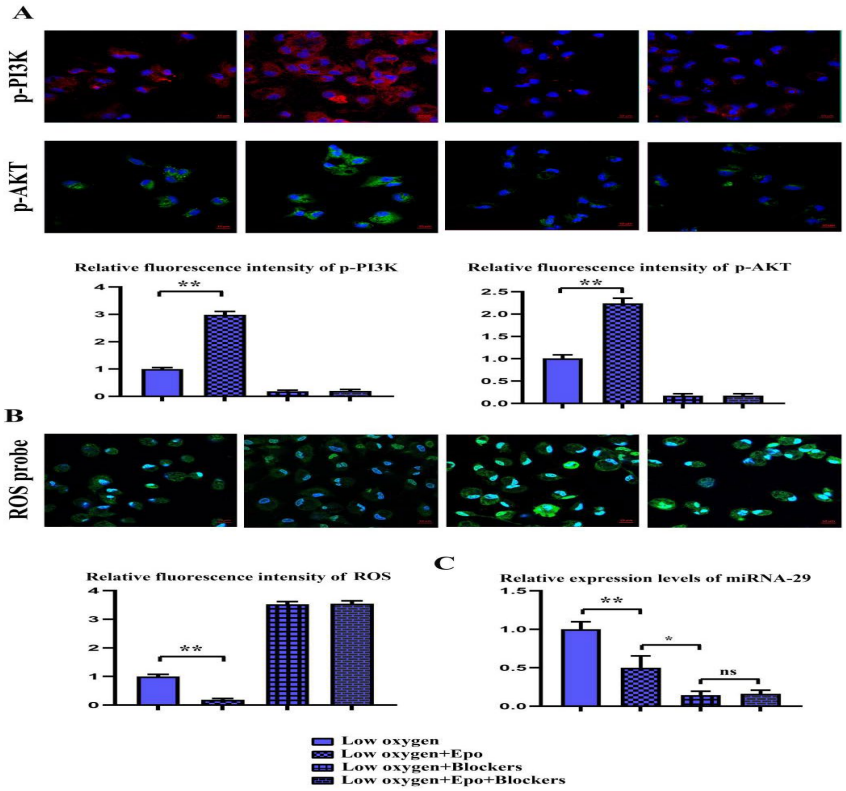

Figure 6: EPO activated PI3K/AKT signal and inhibited oxidative stress by suppressing the expression of miR-29. Hippocampal neurons cell climbing sheets, EPO increased the immunofluorescence intensity of p-PI3K and p-AKT and inhibited the ROS the dactolisib corrected these effects of EPO, data were shown in A and B; EPO suppressed the expression of miR-29 (C).

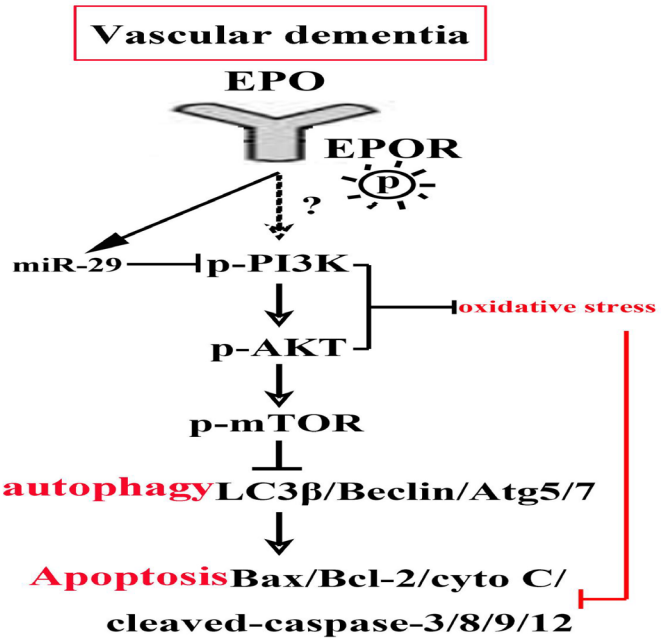

Figure 7: Diagrammatic sketch of EPO' effects in vascular dementia.