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Original Article

Selegiline protects against isoproterenol-induced myocardial ischemia injury: a potential mechanistic role of the PI3K/AKT/mTOR signaling pathway

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Abstract

Background and purpose: Selegiline, an irreversible monoamine oxidase B inhibitor, has been shown to have potential in reducing cell damage. The present study design focused on the cardioprotective effect of selegiline and its possible mechanism of action through phosphoinositide-3-kinase/serine-threonine kinase AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway.
Experimental approach: Myocardial ischemia was induced in male Wistar rats by isoproterenol injection. Selegiline was administered (2 and 5 mg/kg) for 14 days. Electrocardiogram (ECG) parameters and serum markers were measured. PI3K, AKT, and mTOR protein expression and histopathological examination of cardiac tissue were performed. All data were analyzed using GraphPad Prism.
Findings/Results: Pre-treatment with selegiline (5 mg/kg) effectively restored ECG parameters changes and cardiac serum markers elevation seen in isoproterenol receiving groups, with a reduction of lactate dehydrogenase by 55.2% and creatine kinase-myoglobin bind level by 80.1%. Histopathological examination of cardiac tissue revealed successful prevention of fibrosis and inflammation following isoproterenol administration in selegiline-treated groups. Furthermore, western blot analysis demonstrated that pre-treatment with selegiline (5 mg/kg) increased the proportion of phosphorylated to non-phosphorylated proteins involved in the PI3K/AKT/mTOR signaling pathway.
Conclusions and implications: Selegiline administration could protect against myocardial ischemia, induced following isoproterenol injection, which is mediated through PI3K/AKT/mTOR signaling pathways. However, future study needs to focus more on the exact protective route of selegiline action.

Keywords: Cardiovascular Diseases; Isoproterenol; Monoamine oxidase inhibitor; Myocardial ischemia; Selegiline.

INTRODUCTION

Cardiovascular diseases, particularly ischemic heart diseases, have become the leading cause of mortality on a global scale (1,2). The global prevalence of cardiovascular disease has increased twofold, from 271 million in 1990 to 523 million in 2019. Moreover, disability-adjusted life years and years of life lost have considerably increased as well (3).

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Ischemic heart disease is known as the most prevalent manifestation of cardiovascular disease, which clinically manifests as ischemic cardiomyopathy and myocardial infarction (MI) (4). Around one billion cardiomyocytes are permanently lost following MI, and 20 to 30% of MI survivors consequently develop congestive heart failure (5). Unfortunately, despite the large variety of treatments available, new drug innovation has remained limited in the context of the relatively high morbidity and disability associated with cardiovascular diseases. Therefore, developing novel cardiovascular drugs should be taken into account (6).

Selegiline (deprenyl) is a monoamine oxidase B (MAO-B) inhibitor that is frequently prescribed for the treatment of Parkinson's disease. However, a multitude of studies have investigated additional potential advantages of selegiline and its effect on cellular survival in various tissues (7-10). Selegiline is believed to significantly reduce reactive oxygen species (ROS) and enhance the antioxidant activity of neural cells in an ischemic stroke model. These observations suggest that selegiline may potentially protect against ischemia (7,11). Furthermore, selegiline can inhibit the amine oxidation reaction due to the catalytic activity of MAO, leading to a reduction in hydrogen peroxide production (12). As a result of the cell survival and antioxidant properties of selegiline, various studies have explored its potential cardiovascular benefits, revealing a promising protective effect against diabetic cardiomyopathy and congestive heart failure (13-15). On the other hand, MAO-B knockout mice have been shown to significantly reduce infarct size in ischemia-reperfusion injury compared to wild type, which can highlight its crucial role in ischemia injury (16). Even though selegiline has been proposed as a therapeutic agent for various pathological conditions owing to its ability to inhibit MAO-B, the precise cellular mechanisms underlying its enhancement of cell surveillance in cardiovascular diseases have yet to be elucidated (11). Moreover, several studies have suggested that specific protective attributes of selegiline may not be correlated with its role as an irreversible MAO-B inhibitor, as it has been shown to exhibit protective effects even at suboptimal doses for MAO inhibition (14).

The phosphoinositide-3-kinase/serine-threonine kinase AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signalling pathway is well known to be pivotal for cell survival and death (7). In summary, this signalling pathway is initiated by PI3K activation in response to a stimulus, which converts phosphatidylinositol 4, 5-bisphosphate (PIP2) to phosphatidylinositol 3, 4, 5-trisphosphate (PIP3). Subsequently, AKT, a crucial intermediate factor, is activated and triggers mTOR activation as one of the final proteins in the pathway. Ultimately, mTOR can regulate autophagy (as a reverse moderator), apoptosis-related proteins such as Bax/Bcl2 and caspase-3, and suppress inflammatory response following ischemia-reperfusion injury (2,17,18). It appears to play a crucial role in myocardial infarction and is known to exert a significant impact on apoptosis, cell proliferation, and migration after myocardial ischemia (2). Also, the current understanding suggests that this signalling pathway experiences significant suppression during myocyte injury, further underscoring its critical role in myocardial ischemia (17).

Selegiline has been proposed as a potential protective agent against various pathological conditions, particularly in cardiovascular diseases. The myocardial MAO-B enzyme and the PI3K/AKT/mTOR signalling pathway both have significant roles in myocardial ischemic injury. Therefore, this study aimed to investigate the potential role of selegiline in isoproterenol-induced myocardial ischemia through modulation of the PI3K/AKT/mTOR signalling pathway.

MATERIALS AND METHODS

Animals

The present study was conducted following the guidelines for Care and Use of Laboratory Animals in Iran and was approved by the Ethics Committee of Shahrekord University of Medical Sciences (Ethic code: IR.SKUMS.REC.1396.261) (19). A total of 48 healthy Wistar (*Rattus norvegicus*) male rats weighing 200 to 250 g were enrolled in the study. The animals were housed in standard cages with unrestricted access to food and water at the conventional animal laboratory center of Shahrekord University of Medical Sciences,

where they were maintained under controlled environmental conditions (12/12-h dark/light cycle and temperature maintained at 22 ± 1 °C).

Study design and experimental groups

As depicted in Fig. 1, 48 male rats were divided into 4 groups, including ISO + saline (receiving isoproterenol and normal saline), ISO + Sel 2 mg/kg, and ISO + Sel 5 mg/kg: receiving isoproterenol and selegiline at 2 and 5 mg/kg, respectively, and the control group without any intervention. Rats were pretreated with 2 and 5 mg/kg of selegiline for 2 weeks through intraperitoneal injection based on previous evidence and a pilot study (20). Myocardial ischemia was induced by administering 2 doses of 100 mg/kg isoproterenol through subcutaneous injections over 2 days with a 24-h interval between injections (on days 13 and 14) (21). About 24 h after the injection of the second dose of isoproterenol, all experimental animals were anesthetized by administration of ketamine and xylazine (60 and 10 mg/kg, respectively), and blood samples and heart tissue were taken; then they were euthanized.

Myocardial ischemia protocol

Isoproterenol (Catalogue number: 16504, Sigma-Aldrich) was administered to induce myocardial ischemia in rats, as previously described (21). In summary, isoproterenol (100 mg/kg) was dissolved in normal saline and administered subcutaneously for 2 consecutive days with a 24-h interval. The development of myocardial ischemia was evaluated by single lead electrocardiogram (ECG) ST segment elevation and elevated amount of creatine kinase-myoglobin bind (CK-MB) and lactate dehydrogenase (LDH) 24-h following the second injection of isoproterenol in anesthetized animals (6 to 8 animals from each group) as they are frequently present in myocardial cells and are important serum markers in determining the degree of myocardial infarction (22-24). The levels of CK-MB and LDH were measured using specific rat enzyme-linked immunosorbent assay (ELISA) kits (Cat No. CSB-E14403r, CUSABIO Co., Cat. No. MBS269777, MyBio Source Co., respectively).

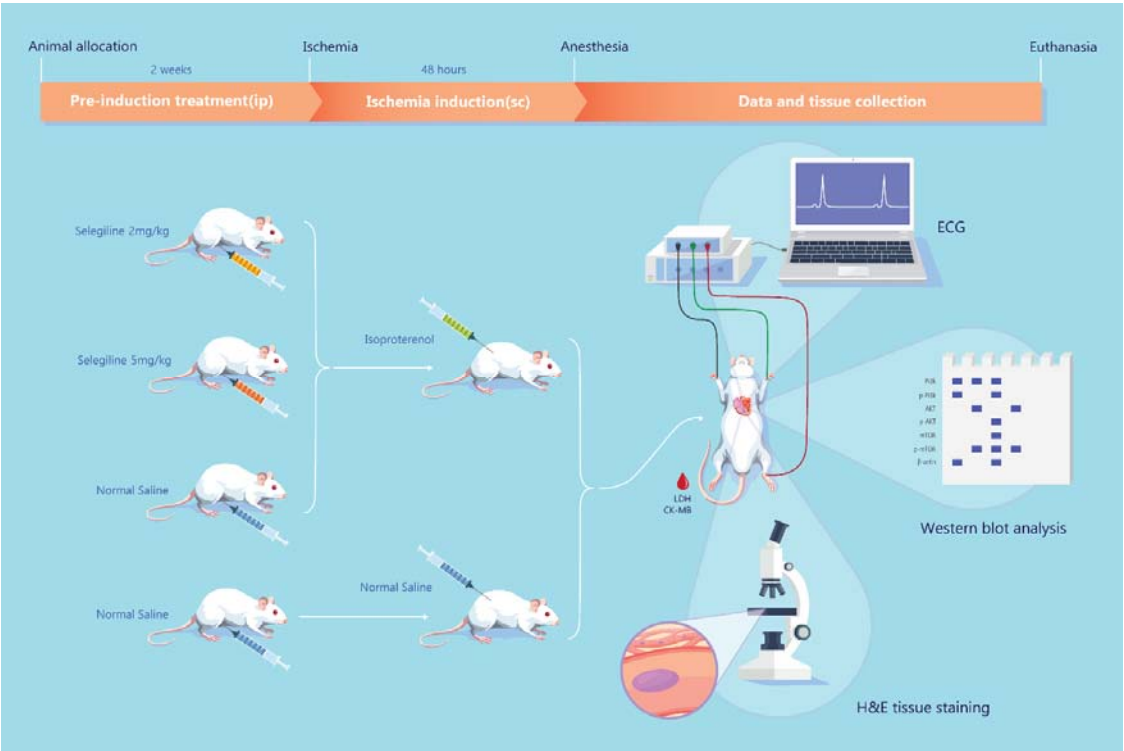

Fig. 1. This figure summarizes the study design and method. ip, Intraperitoneal; sc, subcutaneous; H&E, hematoxylin and eosin; ECG, electrocardiogram; LDH, lactate dehydrogenase; CK-MB, creatine kinase-myocardial band.

Tissue collection and histopathologic evaluation

Fresh cardiac apex tissue was dissected from the heart tissue following euthanasia. All samples (3 samples from each group) were initially fixed in 10% formalin for 48 h. Then, samples were processed automatically in ascending alcohol, cleared in xylene, and embedded in paraffin. Sections with 4 µm thickness were prepared from paraffin blocks using a microtome. Samples were subjected to hematoxylin and eosin (H&E) staining. The sliced photographs were obtained by a microscope camera (Leica K3C) (25). Finally, a histopathologist examined different sections for signs of inflammation and tissue damage in a blinded manner (26).

Western blot analysis

Following tissue harvest, the extraction of samples (3 samples from each group) was achieved through homogenization with a lysis buffer containing Tris-hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate, sodium dodecyl sulfate (SDS), protease inhibitor cocktail, and nonidet P-40 (NP-40, 1%). Subsequently, the total protein concentration of the extractions was measured using the Bradford assay. Then, the samples containing equivalent protein concentrations were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 2 wells were assigned to each sample) and subsequently transferred onto an activated polyvinylidene difluoride (PVDF) membrane. After blocking using non-fat milk in Tris-buffered saline with Tween 20 (TBST) buffer, the membranes were incubated at 4 °C with specific primary antibodies targeting PI3K (Cat. No. PA5-86628, 1:200), Akt (Cat. No. E-AB-30471, 1:40000, Elabscience), mTOR (Cat. No. sc-517464, 1:300), p-PI3K (Cat. No: PA5-118549, 1:1000), p-Akt (Cat. No: sc-271966, 1:300), p-mTOR (Cat. No: sc-293089, 1:1000), and β-actin (Cat. No. sc-47778, 1:300) proteins for 24-h. Afterwards, the membranes were incubated with a secondary antibody (mouse anti-rabbit IgG-HRP; Cat. No. sc-2357, 1:1000) for 75 min at room temperature. Finally, the target proteins were detected using a chemiluminescence kit (ECL advanced reagents, Amersham, Piscataway, NJ) and exposed to X-ray film. To measure the expression of beta-actin protein as an internal standard, the blots were incubated 3 times in the stripping solution to wash the primary and secondary antibodies and the ECL kit, then the steps of incubation with the primary antibody (β-actin Cat. No. sc-47778, 1:300) and the secondary antibody were repeated. The intensity of the protein bands was quantified using National Institutes of Health (NIH) ImageJ software, and the relative expression levels of the target protein were compared between different samples (27).

Statistical analysis

All data were presented as mean ± SEM. The statistical analysis was performed using GraphPad Prism software, 9th version. The differences among experimental groups were evaluated using ordinary one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant in all analyses.

RESULTS

Selegiline alleviated isoproterenol-induced ECG changes

About 24 h after the second injection of isoproterenol, significant ECG changes were observed (Table 1). ISO + saline had a markedly higher heart rate, ST segment elevation, narrower QRS complex, and wider QT interval compared to the control group.

Selegiline treatment significantly reduced ST elevation relative to the ISO + saline group. Selegiline showed a dose-response efficacy in decreasing ST-segment elevation; specifically, the 5 mg/kg dose demonstrated greater efficacy than the 2 mg/kg dose. At 5 mg/kg, selegiline's effects were nearly indistinguishable from the control group, suggesting substantial cardio-protection.

Isoproterenol administration significantly increased heart rate compared to the control group, while selegiline notably mitigated this effect. Both doses of selegiline significantly decreased QT interval prolongation compared to the ISO + saline group, where selegiline

at 5 mg/kg was considerably more effective, and it was almost equal to the control group. In addition, selegiline pretreatment significantly restored the QRS complex narrowing effect of isoproterenol compared to the ISO + saline group; it seems that selegiline at 5 mg/kg was more effective.

Selegiline mitigated myocyte injury

As depicted in Fig. 2, our findings suggest that isoproterenol increased CK-MB and LDH significantly compared to the control. Selegiline decreased serum CK-MB and LDH at 2 mg/kg by about 25.4% and 63.6%, respectively, and at 5 mg/kg by about 80.1% and 55.2%, respectively, relative to the group that merely received isoproterenol.

Selegiline mitigated inflammation in the cardiac apex tissue

Heart apex tissue H&E staining showed that the ISO + saline group had inflammation and lower integrity compared to the control, and cells showed fibrotic appearance. In contrast, selegiline preconditioning decreased inflammation and fibrosis and increased cell integrity. However, selegiline at 5 mg/kg was more impactful compared to 2 mg/kg (Fig. 3).

Table 1. The effect of selegiline on isoproterenol-induced electrocardiogram changes. The data are expressed as mean ± SEM. $^{*}P < 0.05$ indicates significant differences compared to the control group; $^{\#}P < 0.05$ versus ISO + saline group; and $^{\dagger}P < 0.05$ against ISO + Sel (2 mg/kg) group.

Experimental groups	Heart rate (beats per min)	ST-segment elevation (mV)	QT interval (s)	QRS complex (s)
Control	225 ± 3.6	0.0053 ± 0.00001	0.0504 ± 0.0008	0.025 ± 0.008
ISO + saline	326 ± 3.8*	0.1537 ± 0.0025*	0.0854 ± 0.0035*	0.015 ± 0.0002*
ISO + Sel (2 mg/kg)	253 ± 2.47$^{\#}$	0.0649 ± 0.0165$^{\#}$	0.0638 ± 0.0025$^{\#}$	0.016 ± 0.0004*$^{\#}$
ISO + Sel (5 mg/kg)	238 ± 6.28$^{\#}$	0.0059 ± 0.0003$^{\#\dagger}$	0.05025 ± 0.0007$^{\#\dagger}$	0.019 ± 0.0005*$^{\#\dagger}$

ISO, Isoproterenol; Sel, selegiline.

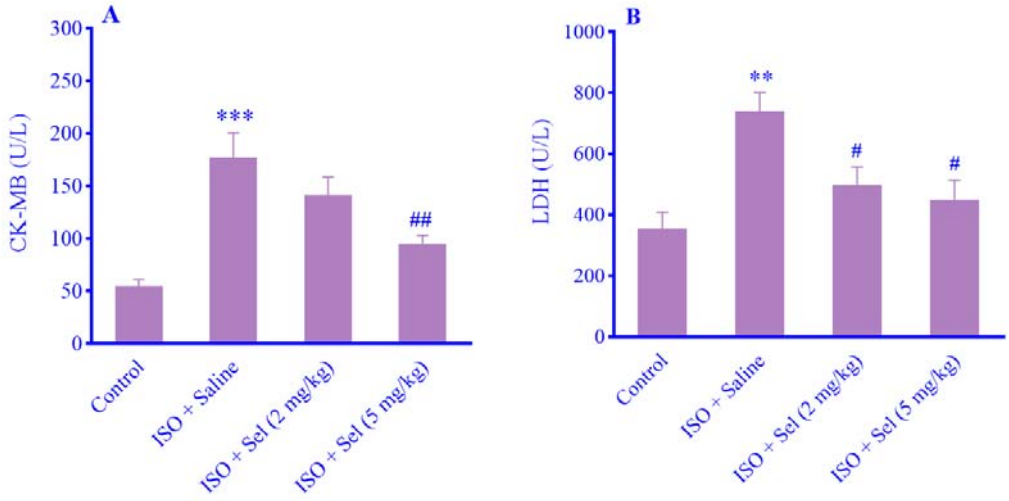

Fig. 2. The impact of selegiline on (A) CK-MB and (B) LDH serum levels. Results are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus ISO + saline group. ISO, Isoproterenol; Sel, selegiline; CK-MB, creatine kinase-myocardial band; LDH, lactate dehydrogenase.

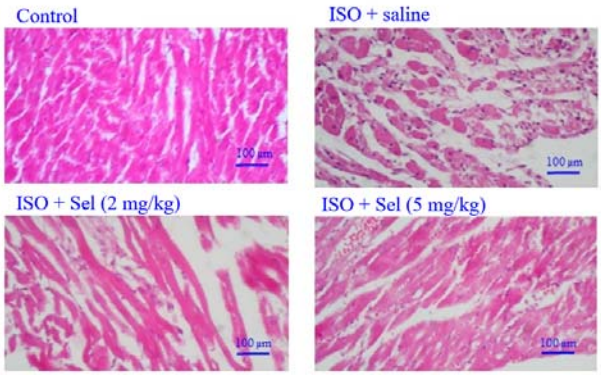

Fig. 3. Selegiline preconditioning on histopathological changes in heart apex tissue decreased inflammation and fibrosis formation, and increased cell integrity. Light microscopy (H&E staining, 40 ×). ISO, Isoproterenol; Sel, selegiline

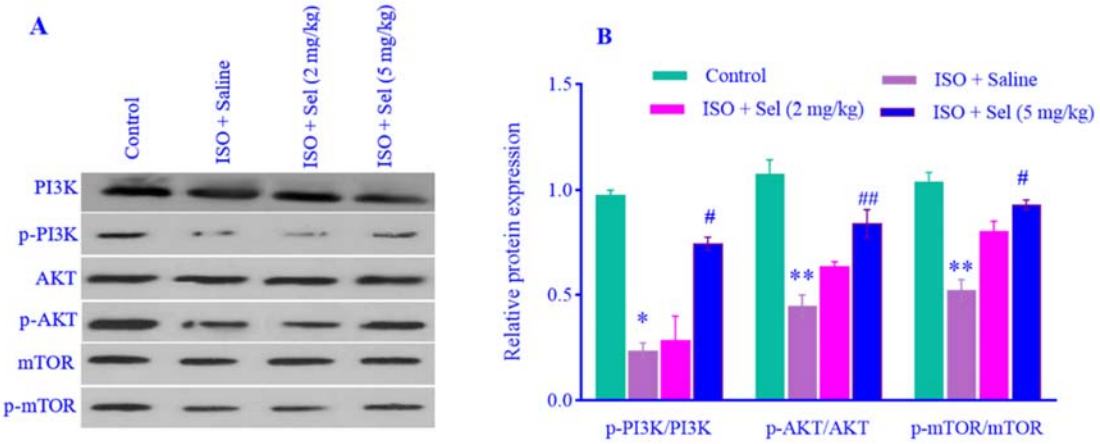

Fig. 4. The impact of selegiline on PI3k/AKT/mTOR signaling. (A) Western blotting and (B) the analysis of relative protein expression across experimental groups. Data are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicates significant differences compared to the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the ISO + saline group. ISO, Isoproterenol; Sel, selegiline.

Selegiline facilitated PI3K/AKT/mTOR signalling

As illustrated in Fig. 4, ISO + saline decreased activation to inactive protein form density (phosphorylated to non-phosphorylated) of PI3K/AKT/mTOR compared to control. In addition, the administration of selegiline at 2 mg/kg resulted in a non-significant alteration in the expression of phosphorylated to non-phosphorylated form of PI3K and mTOR proteins; nevertheless, it led to a significant increase in phosphorylated to non-phosphorylated AKT protein expression when compared to the ISO + saline group. Moreover, the administration of selegiline at 5 mg/kg increased considerably the phosphorylated to non-phosphorylated forms of PI3K, AKT, and mTOR proteins compared to the ISO + saline group.

DISCUSSION

The present study shows that 2 consecutive days of isoproterenol administration increased heart enzymes (CK-MB and LDH), produced ST-segment elevation, prolonged QT interval, and induced pathological myocardial alterations (notably apical fibrosis as seen on H&E staining). These results confirm that isoproterenol did induce myocardial ischemia. As a nonselective β-adrenergic receptor agonist, isoproterenol increases heart rate,

contractility, and oxygen consumption, contributing to myocardial ischemia and generation of ROS, consequently damaging cardiac cells (21,23,28). Moreover, isoproterenol exposure led to a significant shortening of the QRS complex- a finding consistent with previous reports, likely reflecting enhanced ventricular conduction and rapid repolarization due to activation of ATP-sensitive K^+ channels (29,30).

In the present study, selegiline treatment significantly mitigated the myocardial pathologic effect of isoproterenol at 5 mg/kg, which seems more effective against myocardial injury, as previously shown; higher doses may be more potent antioxidants (20,31). Selegiline treatment in our study revealed a dose-dependent protective effect in ECG parameters in rats that underwent ischemia. Notably, 5 mg/kg was more effective in decreasing ST-segment deviation. ST-segment elevation representing ischemia decreased in a dose-dependent manner, as shown in other histopathological and cardiac biomarkers studies. Additionally, isoproterenol administration resulted in significant QT interval prolongation. It is well established that QT prolongation can predispose cardiac rhythm to lethal arrhythmias, such as torsades de pointes (32). Conversely, selegiline mitigated this pathological effect in a dose-dependent manner in isoproterenol-induced ischemia conditions. However, the impact of selegiline on the QT interval was beyond the scope of this study and warrants further investigation in future research. In addition, selegiline administered at 5 mg/kg notably prolonged the QRS complex; however, its effect did not restore the QRS duration to normal levels observed in the control group. Furthermore, isoproterenol significantly increased heart rate because of the β-adrenergic effect on the SA node, increasing heart rate as expected (33). The administration of selegiline has the potential to reduce heart rate in groups receiving isoproterenol. Although this study does not delve into the underlying mechanism, it is noteworthy that patients with Parkinson's disease who are being treated with selegiline may develop orthostatic hypotension. This effect could stem from the decreased sympathetic response of the cardiovascular system, which was also observed in the current investigation (34). The PI3K/AKT/mTOR signaling pathway was notably suppressed in the isoproterenol-induced injury group, whereas selegiline treatment resulted in its profound activation. This observation suggests that the aforementioned signaling pathway may play a significant role in the protective effects of selegiline.

Selegiline is prescribed for the treatment of Parkinson's disease as an MAO-B inhibitor. Nevertheless, recent pre-clinical research has shifted attention to other potential benefits of selegiline on the liver and cardiovascular tissue due to its antioxidant properties (13,35). Low dose (0.3 mg/kg) treatment with selegiline for 8 weeks in rabbits with congestive heart failure has significantly ameliorated myocyte apoptosis and increased the Bax/Bcl-2 ratio (14). Also, selegiline could significantly reduce hydrogen peroxide production, possibly through MAO inhibitory properties, and reduce oxidative stress in aortic ring and neural cells (12,13). This evidence suggests that selegiline could decrease the myocardial injury at least partially through decreasing the large amount of reactive oxygen production following myocardial injury (36).

On the other hand, PI3K/AKT/mTOR signaling is well known in myocardial infarction pathophysiology, and it contributes to various physiological and pathophysiological processes such as proliferation, survival, migration, apoptosis, and autophagy (2,23,37). Specifically, it is believed to regulate apoptosis following myocardial infarction, and is supposed to decrease large amounts of ROS production following myocardial ischemia (38). In our previous study (7), administering selegiline at 5 mg/kg to normal rats indicated no significant impact on the PI3K/AKT/mTOR signaling pathway compared to untreated controls. However, in an ischemia model focusing on hippocampal tissue, selegiline modulated ischemia-related changes in this pathway. This finding suggests that, in the absence of pathological conditions, selegiline does not markedly influence the PI3K/AKT/mTOR pathway. Conversely, under stress conditions

such as myocardial injury, selegiline appears to modulate this pathway, indicating its potential role in disease-specific mechanisms. Our recent findings demonstrated that this signaling pathway was significantly inactivated following isoproterenol injection, and selegiline significantly increased PI3K/AKT/mTOR active forms compared to inactive indicating an important role in myocardial injury. A study conducted by Ke *et al.* revealed that LY294002 usage for inhibiting this signaling pathway can significantly interfere with the protective effect of their intervention following isoproterenol-induced heart injury (17). Furthermore, inhibition or knockdown of PI3Kγ, which belongs to the PI3K family, could significantly decrease infarction recovery or increase infarct size following myocardial infarction (39,40). On the other hand, the PI3K/AKT pathway is an upstream signaling that can modulate nuclear factor-kappa B (NF-κB) transcription and NF-κB-transcribed chemokines and cytokines (41). As a result, PI3K/AKT/ NF-κB activation is believed to protect myocardial ischemia-reperfusion injury through activation of NF-κB as a pro-inflammation marker and immune response (17). However, overactivation of PI3K/AKT/NF-κB seems to induce higher amounts of inflammation, which consequently results in higher apoptosis and injury (41,42). In summary, the protective effect of selegiline against myocardial injury following PI3K/AKT/mTOR activation may be attributed to its impact on apoptosis, inflammation, and ROS production.

PI3K/AKT/mTOR signaling restoration following selegiline administration could be justified by the MAO-B inhibitory activity of selegiline. Recently, Canin *et al.* concluded that MAO-B inhibition by pargyline can significantly restore insulin-like growth factor 1 receptor (IGF1R) and its downstream signaling pathway, as PI3K and AKT, possibly through different miRNA activation in diabetic cardiomyopathy (43). Furthermore, cardiac-specific MAO-B knocked-out mice revealed significantly lower infarct size and ROS production following myocardial ischemia-reperfusion injury. In this study, the addition of the MAO-B substrate (β-phenylethylamine) increased ROS production in wild-type mice but did not have any effect on MAO-B-deficient mice, which further suggests an important effect of MAO-B activation on the myocardial injury regardless of its substrate's direct effect (14,16). The study conducted by Bianchi *et al.* also showed that MAO-B and MAO-A inhibition in rats receiving pargyline and clorgiline, respectively, resulted in a significant reduction in infarct size following ischemia/reperfusion (44). However, some studies believe that some beneficial effects of selegiline are independent of MAO-B inhibition since a subeffective dose (0.3 mg/kg) of selegiline for MAO-B inhibition is also effective in diminishing apoptosis in myocardium (14). On the other hand, selegiline is primarily a selective MAO-B inhibitor; however, at higher doses, it can also inhibit MAO-A (45). This dual inhibition may explain the dose-dependent effects observed in our study.

In addition to its importance as an MAO-B inhibitor, the activation of the PI3K/AKT/mTOR signaling pathway by selegiline may be partly attributed to the dopamine 4 receptor, as a previous study demonstrated that dopamine 4 receptor activation can significantly trigger this pathway and ameliorate myocardial apoptosis and injury resulting from ischemia-reperfusion. Furthermore, the protective effect of this pathway was significantly eliminated by wortmannin, a PI3K/AKT/mTOR signaling suppressor (46). Besides the ability of selegiline to activate PI3K/AKT/mTOR signaling, its metabolites have also been found to possess this property. Selegiline undergoes metabolism to methamphetamine in the liver. While high doses of methamphetamine abuse have been shown to adversely affect cardiac histology and induce ROS production in the brain and heart, previous studies suggest that it may activate the PI3K/AKT/mTOR pathway through its interaction with D1, D2, or norepinephrine receptors, thereby promoting neuronal survival (47-49).

Finally, to elucidate the precise mechanism underlying selegiline’s impact on myocardial ischemia, we suggest the utilization of a transdermal form of selegiline to bypass liver metabolism and prevent amphetamine production (50). Furthermore, to assess the

impact of selegiline on the activation of the PI3K/AKT/mTOR pathway, dopaminergic receptor activity in cardiac tissue should be directly evaluated, and a proper control group should be established. In addition, future investigations should explore selegiline's effects on molecular mechanisms beyond mTOR, particularly examining the roles of autophagy and apoptosis.

CONCLUSION

Chronic selegiline administration provides significant protection against myocardial ischemia following isoproterenol injection. The present study suggests that protection is mediated through activation of the PI3K/AKT/mTOR signaling pathway. While the MAO-B inhibitory effect of selegiline appears to be important for its cardiovascular protective effect, further investigation is required to determine whether this effect is attributed to the MAO-B inhibitory effect of selegiline, its metabolites' effect, or other factors independent of MAO-B inhibition.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contribution

E. Saghaei contributed to the design and development of the study, supervision, project administration, and editing of the manuscript; H. Ataei-Goujani contributed to performing the experiments and drafting the manuscript; H. Amini-khoei analyzed final data, drafted and edited the manuscript; M. Anjomshoa was responsible for histopathological analysis; S. Najafi-chaleshtori carried out the experiments and drafted the manuscript. All authors read and approved the finalized article.

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