Aerobic training and vitamin E administration ameliorates cardiac apoptosis markers in rats exposed to methamphetamine

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Abstract

Methamphetamine (MA) abuse is related to risks to the cardiovascular system. The present study aimed to compare the effects of moderate-intensity aerobic training (MIAT) and vitamin E (Vit.E) supplementation on markers of cardiac apoptosis following MA exposure. Fifty-four rats were randomly divided into six groups. CON group did not receive MA, while the others received MA alone or in combination with MIAT, Vit. E, MIAT+Vit E, or paraffin (PAR). These groups received MA incrementally for 23 consecutive days. Vit.E and MIAT+Vit.E groups received vitamin E three times a week for six weeks. MIAT and MIAT+Vit.E groups exercised for 25–40 min. Immunohistochemical and gene expression analyses were performed on the heart tissues. Bax and TGF-β expression was significantly higher, while Bcl-2 and VEGF expression was significantly lower in the MA and PAR groups than in the other groups ($p < 0.05$). Bcl-2 and VEGF expression was higher, and Bax and TGF-β expression was significantly lower in the MIAT and MIAT+Vit.E groups than in the other groups ($p < 0.05$). In Vit.E treated groups, Bax and TGF-β expression were lower, and VEGF was higher than that in the MA and PAR groups, but higher than those in the CON, MIAT and MIAT+Vit.E groups. MA increased the expression of Bax and TGF-β, and decreased the expression of Bcl-2 and VEGF, suggesting increased cardiac apoptosis. In contrast, MIAT and Vit.E decreased the expression of Bax and TGF-β, suggesting a reduction in cardiac apoptosis induced by MA.

Key Words: aerobic training; aerobic exercise; apoptosis; immunohistochemistry; drug abuse.

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Methamphetamine (MA) is a synthetic drug with potent and highly addictive stimulant effects, and its abuse is associated with severe health risks, especially to the cardiovascular system.[1,2] Previous studies demonstrated that MA leads to cellular oxidative stress, impairs mitochondrial function, affects cardiac contractility, and result in cardiomyocyte apoptosis.[3-5] One of the most important cytokines in the apoptotic pathway is the transforming growth factor beta (TGF-β), which upregulates the upstream controllers of Bcl-associated X (Bax) and BAK, two proapoptotic proteins, and downregulates B-cell lymphoma (Bcl-x), an anti-apoptotic protein.[6] In contrast, vascular endothelial growth factor (VEGF) is a cytokine that suppresses the apoptotic process through upregulation of anti-apoptotic components, like Bcl-2, helping to regulate DNA synthesis and phosphorylate intercellular endothelial adhesive components.[7-9] Considering the cardiac damage associated with MA abuse, it is important to propose therapies to counteract its cardiotoxic effects.[10-11] Among the possible therapies considered, physical exercise has been studied because of its positive effects on neurochemical imbalance, neurogenesis, oxidative stress and markers of cardiac apoptosis.[11-14] However, previous studies involving markers of cardiac apoptosis involved high-intensity interval training, which depends on the protocol and might not be convenient or possible for

Table 1. *Moderate intensity aerobic training protocol*

Week	Duration (min)	Intensity (m/min)
Firth	25 minutes	50% of the maximum speed (10 m per minute)
Second	30 minutes	50% of the maximum speed (10 m per minute)
Third	30 minutes	55% of the maximum speed (11 m per minute)
Fourth	35 minutes	55% of the maximum speed (11 m per minute)
Fifth	35 minutes	60% of the maximum speed (12 m per minute)
Sixth	40 minutes	60% of the maximum speed (12 m per minute)

some populations .[15-17] Therefore, it is important to test moderate-intensity aerobic training (MIAT), which is commonly performed and prescribed in different situations,[18-20] and is one of the preferred modes of exercise among people with a history of drug abuse.[21]
Nutritional interventions might also help counteract the negative effects of MA abuse,[21-26] especially antioxidant agents,[21-26] Among them, vitamin E might be useful since it mitigates inflammatory processes by suppressing the generation of reactive oxygen species (ROS) in cardiac cells following methylenedioxymethamphetamine exposure in mice.[27] Nevertheless, to the best of our knowledge, there are no other studies on the effect of vitamin E supplementation on markers of MA-induced cardiac cell apoptosis.
Therefore, considering the negative effects associated with MA and the need for therapeutic strategies to counteract these effects, the purpose of the current study was to compare the effects of MIAT and vitamin E administration on cardiac apoptosis markers, following MA exposure in rats. Our hypothesis is that both MIAT and Vitamin E will have positive effects and that their combination will result in additional benefits.

Materials and Methods

Study protocol

All research methods and procedures followed the necessary regulations (DC 86/609/EEC, 2003/65/EC, 2010/63/EU) and were approved by the Regional Animal Care and Use Committee of the pertinent institution (Ref no: IR.MUQ.AEC.1400.007). Sixty male Wistar rats (8–10 weeks old) weighing 200–210 g each were used in this study. Animals were allowed to acclimate to the testing environment for seven days, receiving water and food *ad libitum.*, The animals were then divided into six groups: 1) MA-dependent rats (n = 10, MA); 2) MA-dependent rats performing MIAT (n = 10, MIAT); 3) MA-dependent rats performing MIAT and receiving vitamin E (n = 10, MIAT+Vit.E); 4) MA-dependent rats receiving vitamin E (n = 10, Vit.E); 5) MA-dependent rats consuming oral paraffin (n = 10, PAR); and 6) rats receiving the MA vehicle saline solution (n = 10, CON). Unfortunately, two animals in the MA group, one in PAR and one in the MIAT+Vit.E group were omitted because of death. Moreover, one animal in the MIAT group and one in the MIAT+Vit.E group were excluded because they became unable to run on the motorized treadmill.

MA administration

MA hydrochloride (purity > 96%) was dissolved in normal saline (0.9%). Animals in the MA, MIAT, Vit.E, MIAT+Vit.E and PAR groups received MA incrementally under an escalating regimen to mimic human MA abuse (2.5-10 mg/kg, IP injection, daily, for 23 consecutive days) as reported earlier.[28] The CON group received injections of saline of the same volume daily for 23 consecutive days.

Vitamin E supplementation

Vit.E and MIAT+Vit.E groups received vitamin E (150 mg/kg, three times a week for six weeks), dissolved in oral paraffin (1.5 mg/kg), and supplemented to the groups by gastric gavage.[27] Oral paraffin was administered to the PAR group by gastric gavage (150 mg/kg three times a week for 6 weeks).

Moderate-intensity aerobic training protocol

Maximum running speed was evaluated on a motorized treadmill (Navid, Pishroo Andishe Sana't Co., Iran) according to a standardized procedure previously described.[29] Concisely, after a 5 minute warm up period at 0.2 m/s, the treadmill speed was progressively increased by 0.3 m/s every two minutes until the rat was unable to run. MIAT and MIAT+Vit.E initiated exercise with a 10-minutes warm up at 50% of maximum speed, followed by the MIAT training session that started from 25 min in the first week and progressed to 40 min in the sixth week (approximately 50-60% of the maximum speed, 20 m/min; Table 1). MIAT was performed six times per week for 6 weeks.

Tissue preparation

Rats were anesthetized with ketamine and xylazine injections and then sacrificed by decapitation. After anesthesia and removal, the heart was placed in neutral buffered formalin. After washing, the hearts were fixed in paraffinand then cut using a microtome to a thickness of 5 µm. The cut tissues were placed on slides. The slides and then stained a described below.

Immunohistochemistry-Paraffin (IHC-P).

The fixed, paraffin-embedded heart sections were deparaffinized and rehydrated. The sections then

Table 2. *The designed primers sequences for TGF-β and VEGF genes*

Gene	Reverse Primer	Forward Primer
TGF-β	5′-GTAACGCCAGGAATTGTTGCTA-3′	5′-CTTCAATACGTCAGACATTCGGG -3′
VEGF	5′-CGCCTCGGCTTGTCACAT -3’	5′-AGAGATGAGCTTCCTACAGCAC -3′
β-Actin	5′-CACCATTGGCAATGAGCGGTTC-3′	5′-AGGTCTTTGCGGATGTCCACGT -3′

underwent antigen retrieval and immunohistochemical staining. Where desired the sections were counter stainied, dehydrated, and stabilized with a cover slip and mounting medium before being viewed under a microscope.

The fixed, paraffin-embedded heart sections were deparaffinized, placed in a rack, and washed as follows: 1. Xylene: 2 × 3 min; 2. Xylene 1:1 with 100% ethanol for 3 min; 3. 100% ethanol: 2 × 3 min; 4. 95% ethanol for 3 min; 5. 70% ethanol for 3 min; 6. 50% ethanol for 3 min, and 7. running cold tap water. Immunohistochemical staining was performed for two days. Day 1. The slides were placed in citrate buffer containing 0.05% Tween-20 for 11 minutes at 100-1200 in a microwave. The slides were then washed in phosphate buffered saline containing 0.025% Triton-Xl00 for 3 min. To prevent non-specific staining between the primary antibody and the tissue, 6 µM of 0.3% Triton was dissolved in 200 µM of 10% goat serum in 2000 µmol of phosphate buffered saline. Incubation was performed with primary antibodies against Bax (#ab32503; Abbexa Ltd., UK) and Bcl-2 (#ab59348; Abbexa Ltd., UK) in a dark, cool, and moist environment overnight. Day 2. The tissue sections were washed with PBS for 5 min and incubated with rabbit IgG secondary antibody conjugated with HRP for 90 min at room temperature (RT). The cells were then washed with PBS buffer for 5 min. The sections were incubated with DAB substrate solution for 15 min at 37°C in the dark. The cells were then washed with PBS for 5 min. The slides were then placed in hematoxylin for 30–60 s and finally washed with water. Dehydration was performed with 70, 90, 96%, and 100 percent ethanol for 60 s each. Clarification with xylene was performed twice for 60 s each. The slides were mounted and examined under a light microscope (Hund-WETZLAR, Germany).

QRT-PCR protocol

Heart tissue (50 mg) total RNA was isolated using TRIzol solution (#YT9065, Yektatajhiz Azma Co., IR, USA) and a tissue homogenizer (IKA, Germany). RNase-free DNase was used to remove DNA contaminants and the RNA of all samples was measured using a NanoDrop device (NanoDrop One, Thermo Scientific, USA) at wavelengths of 260/230 and 260/280 nm. An RNase inhibitor was added to stabilize RNA and cDNA synthesis was completed using a PCR device

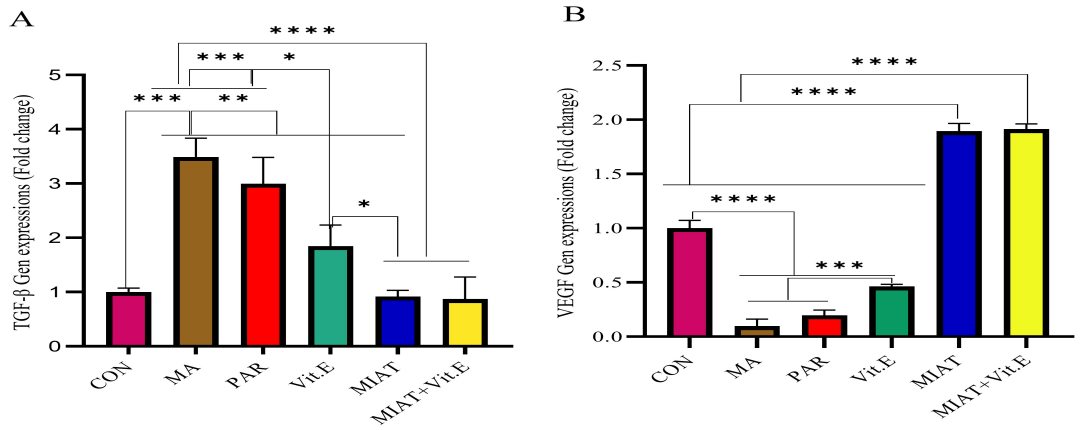

Fig 1. *Changes in TGF-β (A) and VEGF (B) gene expression levels in the cardiac tissues of methamphetamine-dependent rats (MA). * represents p < 0.05, **** represents p < 0.0001. Data are shown as mean ± SD. For TGF-β, Welch's ANOVA was followed by Dunnett's T3 post-hoc test, and for VEGF, one-way ANOVA was followed by Bonferroni post-hoc test. MA-dependent rats (MA); MA plus moderate-intensity training (MIAT); MA plus Vit.E supplementation (Vit.E); MA with MIAT and Vit.E (MIAT+Vit.E); MA plus paraffin (PAR); rats that did not receive MA (CON).*

manufactured by Analitik Jena (Germany) and a cDNA Synthesis Kit (#YT4500, Yektatajhiz Azma Co., IR). The expression levels of the relevant genes were determined by real-time PCR (qRT-PCR, Real-time PCR of Rotor-Gene, StepOnePlus™, Applied Biosystems, USA) using Real Q Plus 2 × Master Mix Green enzyme (#YT2551, Yektatajhiz Azma Co., IR). The temperature protocol involved an initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. The primer sequences were designed using Primer-BLAST (NCBI) online software, and β-actin was used as an internal control gene (Table 2). Data analysis was performed using threshold cycle comparison (ΔCT). The amplification curve of each primer pair was accurately normalized to the amplification curve of the corresponding β-actin reference gene. Finally, when the control samples were calculated, the CT difference was obtained from the TGF-β/VEGF samples, and the target gene to the reference gene ratio was calculated using the ΔCT formula.

Statistical Analysis

The Shapiro–Wilk test confirmed the normality of the data, and the results are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was used to perform between-group comparisons. Dunnett's test was performed to compare the means of the experimental groups with the control group. Statistical analysis was performed using SPSS software version 16 (IBM Corp., USA). GraphPad Prism software (version 6.0) was also used to produce

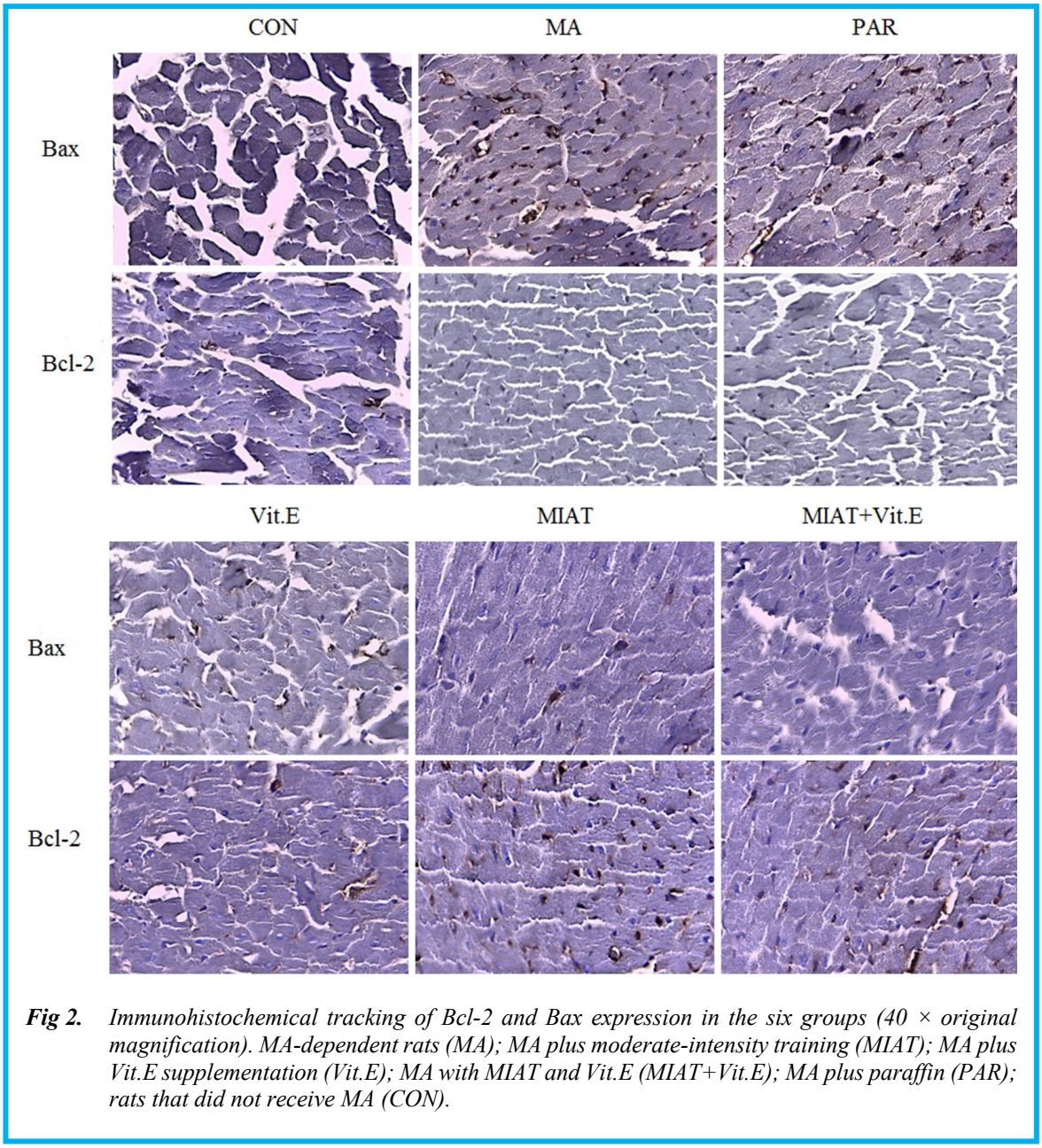

Fig 2. *Immunohistochemical tracking of Bcl-2 and Bax expression in the six groups (40 × original magnification). MA-dependent rats (MA); MA plus moderate-intensity training (MIAT); MA plus Vit.E supplementation (Vit.E); MA with MIAT and Vit.E (MIAT+Vit.E); MA plus paraffin (PAR); rats that did not receive MA (CON).*

graphs. Statistical significance was set at $p<0.05$ (two-tailed).

Results

The expression levels of Bcl-2 and Bax are shown in Figure 2. Brown pigments showed an increased expression of Bcl-2 and Bax. Bcl-2 expression was not detected in MA and PAR; however, Bax expression increased in both groups. In the Vit.E group, Bax expression was higher than in the CON, MIAT, and MIAT+Vit.E groups, but it was lower than that in the PAR and MA groups. In this group, Bcl-2 expression was higher than that in the PAR and MA groups, but lower than that in the CON, MIAT, and MIAT+Vit.E groups. In the MIAT+Vit.E group, the expression of Bax was lower and the expression of Bcl-2 was higher than those in the other groups (except CON). In the MIAT group, BCL-2 expression conditions were similar to those in the MIAT+Vit.E group, but in this group, a small amount of Bax expression was observed in some areas, as indicated by the red arrow (Figure 2).

Considering that homogeneity of variance was not confirmed, Welch's ANOVA (with Dunnett's T3 post-hoc test) was used to analyze TGF-β gene expression. TGF-β gene expression was higher in the MA and PAR groups than that in the CON group ($p < 0.05$; Figure 2-A). TGF-β expression in MIAT, MIAT+ Vit.E, and Vit.E was significantly lower than that in MA and PAR ($p < 0.05$; Figure 1-A). However, there was no significant difference between the CON and MIAT ($p > 0.85$), CON and MIAT+ Vit.E groups ($p > 0.95$), and MA and PAR groups ($p > 0.53$; Figure 1-A).

Our results also showed that VEGF expression was significantly lower in the MA and PAR groups than in the other groups ($p < 0.05$; Figure1-B). VEGF expression was significantly higher in the MIAT and MIAT+Vit.E groups than in the other groups ($p < 0.05$). There was no significant difference between the MA and PAR levels ($p > 0.05$; Figure 1-B). VEGF expression for Vit.E higher than that in MA and PAR ($p < 0.05$; Figure 2-B), but it was lower than that in CON, MIAT, and MIAT+Vit.E ($p < 0.05$; Figure 1-B). There was no significant difference between the MIAT and MIAT+Vit.E groups ($p > 0.05$; Figure 1-B).

Discussion

The present study aimed to compare the effects of MIAT and Vit.E, alone or in combination, on the expression of cardiac apoptosis-related genes following MA exposure in rats. Our results demonstrate that MA exposure increased Bax and decreased Bcl-2 expression in cardiac cells, suggesting an increase in cardiac damage. Nevertheless, MIAT and Vit.E, alone or in combination, decreased Bax and increased Bcl-2 expression, suggesting a protective role in cardiac muscle cells. The fact that the combination of MIAT and Vit.E resulted in greater changes than all other groups suggests that they act in different and complementary ways. This might be of clinical importance, because decreases in Bcl-2 have been associated with various pathological processes, such as cancer, non-alcoholic fatty liver disease, brain injury, neurodegenerative diseases, myocardial infarction, dilated cardiomyopathy, and ischemic heart diseases.[31-38]

Moreover, anti-apoptotic Bcl-2 proteins have therapeutic potential for heart disease because they have been shown to protect myocardial cells from various stresses by blocking p53-mediated apoptosis in cardiac myocytes.[39] In contrast, Bax is highly expressed under ischemic conditions and oxidative stress, which might be associated with increased cardiac damage, cancer, and neurodegenerative diseases,[35,40,41] As MA reduces antioxidant enzymes,[42] the beneficial effects reported in the present study may be related to the antioxidant potential of both exercise and Vit.E.[43-47] Especifically regarding MA, Shaifei et al. reported that exercise increased antioxidant activity in rats exposed to the drug.[42] In addition, Ghafori et al. showed that the Vit.E administration reduces the number of reactive oxygen species, phosphocreatine kinase, and lactate dehydrogenase in cardiac cells of mice following MA exposure.[27] Sedaghat reported that Bax expression was reduced more in rats that performed exercise with taurine supplementation than in rats that consumed taurine or exercised alone,[48] suggesting that a combination of exercise and nutritional strategies might act in different and complementary ways. Although Vit.E reduced TGF-β expression in rats exposed to MA, these values were still higher than those in the CON group. Moreover, the decrease in TGF-β for MIAT was higher than that for Vit.E and similar to MIAT+Vit.E, suggesting that exercise has a central role in controlling TGF-β and no additional effect is obtained with Vit.E. Our results showed that MA increased TGF-β expression, which is in agreement with the results of Spender et al.[6] The decrease in TGF-β for MIAT agrees with a systematic review by Ayary et. al. (2023), which reported that physical exercise decreases TGF-β in both human and animal models.[49,50]

Changes in TGF-β might also be clinically relevant, since disruption in TGF-β-mediated apoptosis signaling may be associated with some pathological conditions such as inflamatory bowel disease, cardiovascular diseases, non-alcaholic fat liver disease, fibrotic disorders, arteriovenous malformation, multiple sclerosis, muscle diseases, aneurysm, atherosclerosis, myocardial fibrosis, cancer, and heart valve disease.[6,51-56] VEGF levels increased in Vit.E relative to MA and PAR, but the values were still lower than those in the non-MA treated CON group. On the other hand, VEGF expression increased in MIAT, reaching higher values than those in CON. The absence of a difference between MIAT and MIAT+Vit.E shows that exercise has a large effect on VEGF, and Vit.E brings no additional benefits. In agreement with this, Misiou et al. (2023) reported that exercise stimulates the mobilization of VEGF in both patients with

cardiovascular diseases and in healthy individuals.[57] The potential clinical importance of VEGF is related to its ability to stimulate angiogenesis,[58] restore cardiovascular circulation in vascular injury,[59] and stimulate neuroplasticity and brain repair.[60,61] Moreover, previous studies have suggested that VEGF protects cells from apoptosis by increasing the expression of Bcl-2,[7,9,62] which is consistent with the results of our study, since the abuse of MA increased the expression of Bax and decreased the expression of Bcl-2 and VEGF.

This study has several novelties and advantages over previous studies, such as the performance of MIAT, which is a popular and feasible form of exercise, the simultaneous comparison of different apoptosis biomarkers, and the use of immunohistochemistry and gene expression measurements to detect both gene expression and protein levels. However, this study had some limitations.

Apart from the inherent apoptosis biomarker changes that are expected in a small sample, gene expression processes and protein abundance in rats may differ from those in humans. Despite exercise and vitamin-related amelioration following the study's interventions, the degree of MA-induced cardiotoxicity and cardiac dysfunction may not have been sufficient to elucidate the full impact of exercise training and vitamin E administration on cardiac apoptosis-related gene expression and protein levels in chronic exposure to MA.

In conclusion, our findings show that MA increases the expression of Bax and TGF-β, while decreasing the expression of Bcl-2 and VEGF, which suggests an increase in cardiac cell apoptosis. However, MIAT reversed these changes, resulting in a more positive state than that observed in animals exposed to MA. While Vit.E resulted in the same benefits, its effects were not as pronounced as those of MIAT. However, considering that the combination of MIAT and Vit.E resulted in many additional benefits, we suggest that they should be used in combination to prevent or restore cardiac damage from MA abuse.

List of acronyms

ANOVA - One-way analysis of variance
Bax - Bcl-associated X
Bcl-2 - B-cell lymphoma
CON - control
MA - Methamphetamine
MIAT - moderate-intensity aerobic training
PAR - paraffin
TGF-β - transforming growth factor beta
VEGF - vascular endothelial growth factor
Vit.E - vitamin E

Contributions of Authors

HS: Conceptualization, methodology, writing–original draft preparation, and software; AHH: Conceptualization, data curation, investigation, supervision, writing-original draft preparation; SA and HM: Visualization, investigation, supervision; SA: Software, validation, writing-original draft preparation, writing-review and editing; ALP and PG: Writing - final preparation, writing- review and editing.

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Conflict of Interest

The authors declare no conflicts of interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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