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The Interactive Effect of Swimming Training and Curcumin on Bcl- 2 and Bax Gene Expression in the Rat Cardiac Tissue during the Withdrawal Period of Excessive Ethanol Consumption

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Abstract

Introduction: Excessive consumption of ethanol can lead to development of apoptosis in cardiac tissue. Then, this study aimed to investigate anti-apoptotic effects of swimming training and curcumin during the withdrawal period of excessive ethanol consumption in rats.

Methods: In an experimental study, 40 rats were selected and exposed to ethanol (25% w/v) every eight hours for four days by gavage. After 7 days of quitting ethanol consumption, they were placed in 5 groups of 8 each, including 1- control, 2-curcumin, 3-swimming training, 4-curcumin and swimming training, and 5- DMSO (dimethyl sulfoxide). Groups 3 and 4 performed five swimming training sessions per week for two weeks and groups 2 and 4 were received curcumin (50 mg/kg body weight) five times a week for two weeks by intraperitoneal injection. The two-way ANOVA was used for statistical analysis of data ($P \leq 0.05$).

Results: Swimming training causes significant increase in Bcl-2 and significant decrease in Bax in the cardiac tissue of the rats received ethanol ($P \leq 0.05$). Curcumin also significantly increased Bcl- 2 and decreased Bax ($P \leq 0.05$). Curcumin and swimming training have interactive effects on the reduction of Bax in the cardiac tissue of rats ($P \leq 0.05$). However, the concurrence of these two factors did not have a significant interactive effect on Bcl-2 elevation and Bax/Bcl- 2 gene expression regulation ($P \geq 0.05$).

Conclusion: Based on the results, swimming training and curcumin consumption alone had a significant effect on reducing the ethanol-induced apoptosis in cardiac tissues of the rats during the withdrawal period of excessive ethanol use. Furthermore, results showed that swimming training with curcumin consumption had a significant interactive effect on reducing Bax gene expression. However the interactive effect of the combination of training and curcumin on Bcl- 2 and Bax/Bcl- 2 ratio was not significant, but it is more effective than the effect of each intervention alone. Consequently, it seems that the combination of swimming training and curcumin may be used during the withdrawal period of excessive ethanol consumption to modulate apoptotic process.

Keywords: Training, Curcumin, Ethanol, Apoptosis

Introduction

Extreme consumption of ethanol is one of the progressive problems facing current communities which is known as the third cause of death with an annual rate of 79,000 people in the United States (1). Although there are no accurate data on ethanol abuse in Iran, but excessive consumption of this addictive drink is a serious social issue (2). This issue, while changing the social, political, cultural and economic systems of societies, is one of the destructive factors on the function of multiple organs. Researchers believe that ethanol consumption through the progression of atherosclerosis and consequently cardiovascular diseases such as high blood

pressure, atherosclerosis plaque formation, cardiac muscle dysfunction, cardiac arrhythmias, coronary artery disease and cell death, increases the risk of cardiac attacks and sudden death (3). Research has attributed ethanol-induced apoptosis to various factors including oxidative stress, free radical production, increased permeability, mitochondrial membrane, the release of cytochrome c, the activity of procaspases, the calcium fluctuations, production of TNF-α (tumor necrosis factor alpha), suppression of anti-apoptotic routes, cellular immune mechanisms, reducing antioxidant capacity and reducing the stimulation of adrenergic receptors, including PKA (protein kinase A) (1). Many cellular processes include cell metabolism, message transmission paths, regulatory pathways of gene expression, cell proliferation as well as programmed cell death are affected by oxidative stress. Researchers believe that oxidative stress from the signal pathway of the p53 suppressor gene and free radicals causes cell death by damaging the DNA (4). As a result, oxidative stress can lead to cell damage and induction of gene mutation. In most cases, an increase in antioxidants leads to repair of cell damage by blocking the pathway of apoptosis (4). Cells suffering from apoptosis indicate numerous morphological changes including cell shrinkage, chromatin density, nucleus breakdown, plasma membrane blebbing, and finally cell fragmentation and formation of apoptosis bodies (5). According to the existing research, two proteins Bax and Bcl-2 play effective roles in modulating cell death process. Bcl-2 family members control the pathways involved in stimulating the apoptotic process. Furthermore, the activation of Bax protein results in mitochondrial membrane permeability (5). A common approach to control the apoptosis process is to increase the antioxidant capacity of the tissue using supplementary consumption and physical activity. Kakalra *et al.* showed that exercise training reduces the oxidative stress caused by ethanol consumption in 3-month-old and 18-month-old rats (6). In this regard, studies have shown that long-term endurance training reduces significantly t-Bid, Bad, Bak, Bax, cytochrome c/cytosolic, activated caspase-9 and activated caspase-3 levels in rats (7). For example, after 12 weeks endurance training (10-60 m/min, 33-43 min/day, 15%), Bcl-2 gene expression has significantly elevated and the gene expression of apoptotic factor (Bax) reduced in trained rats (8). Also, the interactive effect of endurance training and ethanol consumption on rat cardiac tissue has been investigated by Aggarwal *et al.* (9). They reported increase in antioxidant capacity such as SOD (superoxide dismutase), catalase, xanthine oxidase and lipid peroxidation. In general, ethanol consumption, in addition to increasing oxidation, reduces the antioxidant enzymes capacity. In this study, the exercise training and ethanol consumption group showed an increase in antioxidants and decreased oxidants compared to the group receiving ethanol without exercise training (9). On the other hand, the use of antioxidants especially antioxidants of plant origin have widely been considered by the researchers due to biological properties (10). Curcumin is a natural product in turmeric. Due to its specific chemical structure, it has a surprisingly wide range of biological and medicinal properties including anti-inflammatory, antimicrobial, and antioxidant, and therefore it may be effective in diabetes, rheumatoid arthritis, psoriasis, Alzheimer's and cancer (11). Furthermore, various studies have shown the effective role of curcumin to protect pancreas, liver, and brain against the toxic effects of excessive ethanol consumption (11, 12). Curcumin has also been proven to affect 104 genes of 214 genes related to apoptosis (13). In several studies, in addition to confirming the protective role of curcumin to improve the apoptosis because of excessive use of ethanol, the protective role of this antioxidant has been reported to reduce apoptosis caused by various factors, including chloric acid in the

hippocampus (14) or increase the apoptosis due to acetaminophen use (15). Regarding the oxidative properties of ethanol and the beneficial effects of curcumin antioxidants and physical activity in reducing apoptosis, it is valuable to investigate the anti-apoptotic effects of physical exercises combined with curcumin on ethanol-induced apoptosis. To the best of the authors' knowledge, the existing research has not investigated the anti-apoptotic effects of combination of physical exercises and curcumin supplementation during the ethanol withdrawal period on cardiac tissue. Therefore, the present study aimed to examine the interactive effects of swimming training and supplementation of curcumin on suppression of apoptosis in cardiac tissue, induced by excessive consumption of ethanol during withdrawal period in rats.

Methods

This experimental study was performed on 40 male Wistar rats weighing about 200-250 g. These rats were purchased from the Animal Breeding Center, Department of Pharmacology, Tehran University of Medical Sciences and kept in the animal house of the university under standard conditions of temperature, humidity, nutrition and light, in 12/12 h light and dark cycles, for 10 days to adapt to environmental conditions. After 10-day environmental compatibility period, all rats consumed ethanol for four days. In this study, ethanol was administered by intragastric gavage according to the study conducted by Meynard and Leasure (16), as follows. The rats were gavaged with ethanol (25% ethanol w/v in vanilla EnsureTM; Abbot Laboratories, Columbus, OH) every 8 hours for 4 days, starting from the first day of the experiment (i.e., 12 doses total). This initial dose was 5 g/kg for each animal and subsequent doses were obtained using a 6-point behavioral intoxication scale (0- normal; 1-hypoactive; 2-ataxia; 3- ataxia with dragging abdomen and/or delayed righting reflex; 4-loss of righting reflex; 5-loss of eye blink reflex) (13). Each of these scale points is associated with a specified dose of ethanol, such that the greater the observed behavioral intoxication scale, the smaller the subsequent dose. Then, the rats were placed in their cages for 7 days to quit ethanol without any intervention. On the seventh day of abstinence, they were divided into 5 groups of 8 each, including 1- control (no intervention), 2- curcumin consumption, 3-swimming training, 4- curcumin consumption with swimming training, and 5- DMSO (dimethyl sulfoxide - a solvent for curcumin). The rats in groups 3 and 4 performed swimming training five sessions per week for two weeks (i.e., 10 sessions total). The swimming training was begun at a certain hour (11 o'clock). Each session took 20 minutes at first and reached an hour on session 3; then it took an hour for the next seven sessions. After each swimming training session, the animals were dried with a towel and returned to their cages. Rats in groups 2 and 4 received curcumin (50 mg/kg body weight) five times a week for two weeks by intraperitoneal injection (i.e., 10 times total). Curcumin (Merck, Germany 820354) was dissolved in DMSO with a concentration of 10% and injected inrtaperitoneally into the rats. Forty-eight hours after the last swimming training session, the rats were anesthetized with ketamine and xylazine, and then their cardiac tissue was removed to measure the variables of the research. Gene expression of Bcl-2 and Bax proteins in the cardiac tissue was measured by RT-PCR (real-time PCR) method as follows. First, total RNA was extracted in each group using Guanidine/phenol solution (QIAzol, Qiagen, USA) according to the manufacturer's protocol. Assessment of quantity and quality of RNA was performed by NanoDrop 2000 (Thermo scientific) instrument. Then, first strand cDNA (complementary DNA) synthesis was conducted by reverse transcription of 1 µg of total RNA using RT-PCR (Thermo Scientific, USA) according to the manufacturer's protocol. Finally, the analysis of the mRNA

(messenger RNA) relative expression was done by providing reaction mixture with Power SYBR Green PCR Master Mix (2X) (Applied Biosystems, catalogue number 4309155) and gene specific primers with diluted cDNA. The final volume was made up to 10 µL with nuclease-free water. The primer sequences for genes are listed in Table 1. Quantitative RT- PCR reactions were carried out as a one-step qualitative PCR (Applied Biosystems Step One RT-PCR systems). The target gene expression was normalized to the house-keeping gene GAPDH and relative expression was obtained using DDCt (delta delta Ct) method. Data were described using mean and standard deviation and processed with SPSS 20.0. The two-way ANOVA (analysis of variance) was used for independent groups. P-values less than 0.05 indicated that difference was statistically significant.

Results

The results (means and standard deviations) related to the gene expression levels of Bax, Bcl-2 and Bax/Bcl- 2 ratio are presented in Table 2. Also, P-value, F-test, and effect size related to the studied groups are shown in Table 3. Swimming training and curcumin consumption significantly decreased the expression of Bax gene in the cardiac tissue of the rats during the withdrawal period of excessive consumption of ethanol (Figure 1a). Also, swimming training with curcumin had a significant interactive effect on reducing Bax gene expression in the cardiac tissue of rats during the withdrawal period (Figure 1a). Swimming training and curcumin consumption significantly increased the expression of Bcl- 2 in the cardiac tissue of the rats during the withdrawal period (Figure 1b). However, the consumption of curcumin with swimming training had no significant interactive effect on the increase of Bcl-2 gene expression in rat cardiac tissue during the withdrawal period (Figure 1b). Swimming training, curcumin consumption and combination of swimming training and curcumin consumption had no significant effect on gene expression Bax/Bcl- 2 ratio in rat cardiac tissue of the rats during withdrawal period (Figure 1c).

Discussion

This study demonstrated that swimming training resulted in a significant increase in Bcl-2 gene expression level and a significant decrease in Bax gene expression level in cardiac tissue of the rats during the withdrawal period of excessive alcohol use. However, there is no significant effect was observed in Bax/Bcl-2 ratio in rat cardiac tissue during withdrawal period. Previous research mostly has investigated many disorders associated with the adverse effects of ethanol consumption in various tissues, including cardiac tissue (3). During long-term high-dose ethanol consumption, cardiac tissue faces with abnormal conditions, including changes in size, intracellular edema, the presence of lipid droplets in muscle cells, and changes in the contraction process, which the reason of these conditions was attributed to cell death (17). Also, studies have shown that due to ethanol consumption, the gene expression of effective proteins in the apoptosis process including caspases and Bax increases and as a result fragmentation of DNA occurs (1). Ethanol-induced apoptosis was attributed to several factors, including enhanced acetaldehyde exposure, increased production of ROS (reactive oxygen species), increased production of FAEEs (fatty acid ethyl esters), the increase in tumor necrosis factor alpha production, stimulation of Fas, and reduction of antioxidant capacity (18). Regarding the effects of physical activity on the apoptotic process, the studies has shown that regular and prolonged physical activity is an appropriate strategy to reduce apoptosis (7). It was shown that 12-week endurance training significantly increased Bcl- 2 gene expression in trained rats and decreased Bax gene expression (8).

Table 1. The sequence of primers

Gene	Sequence	Melting temperature
Bax R	CAG CCA CAA AGA TGG TCA	87.08
Bax F	GCA AAC TGG TGC TCA AGG	87.08
r-Bcl-2-f	GAGTGGGATACTGGAGATGAAG	91.68
r-Bcl-2-r	TGGTAGCGACGAGAGAAGTC	91.68
rGap R	CAT ACT CAG CAC CAG CAT CAC C	81.27
rGap F	AAG TTC AAC GGC ACA GTC AAG G	81.27

Table 2. The mean and standard deviation of Bax and Bcl-2genes and Bax/Bcl-2 ratio in the studied groups

Gene	Group	Mean	Standard deviation
Bax	Curcumin + Training	0.0000022	0.0000013
	Training	0.0000037	0.0000250
	Curcumin	0.0000321	0.0000120
	Control	0.0001374	0.0000562
	DMSO	0.0001091	0.0001321
Bcl-2	Curcumin + Training	0.0012339	0.0006886
	Training	0.0004500	0.0002122
	Curcumin	0.0002573	0.0001991
	Control	0.0000010	0.0000011
	DMSO	0.0000019	0.0000017
Bax/Bcl-2	Curcumin + Training	0.0025243	0.0023361
	Training	0.0088109	0.0046675
	Curcumin	0.2368777	0.2593492
	Control	280.1409545	274.5593207
	DMSO	162.6301342	273.3380211

Table 3. The P-value, two way ANOVA test, and effect size related to the studied groups

Group	Gene	F	P-value	Effect size
Training	Bcl-2	14.64	0.002	0.55
	Bax	32.32	0.0001	0.72
	Bax/Bcl	4.14	0.064	0.25
Curcumin	Bcl-2	7.81	0.016	0.39
	Bax	13.77	0.003	0.53
	Bax/Bcl-2	4.15	0.064	0.25
Curcumin + training	Bcl-2	2.02	0.180	0.14
	Bax	12.98	0.004	0.52
	Bax/Bcl-2	4.15	0.064	0.257

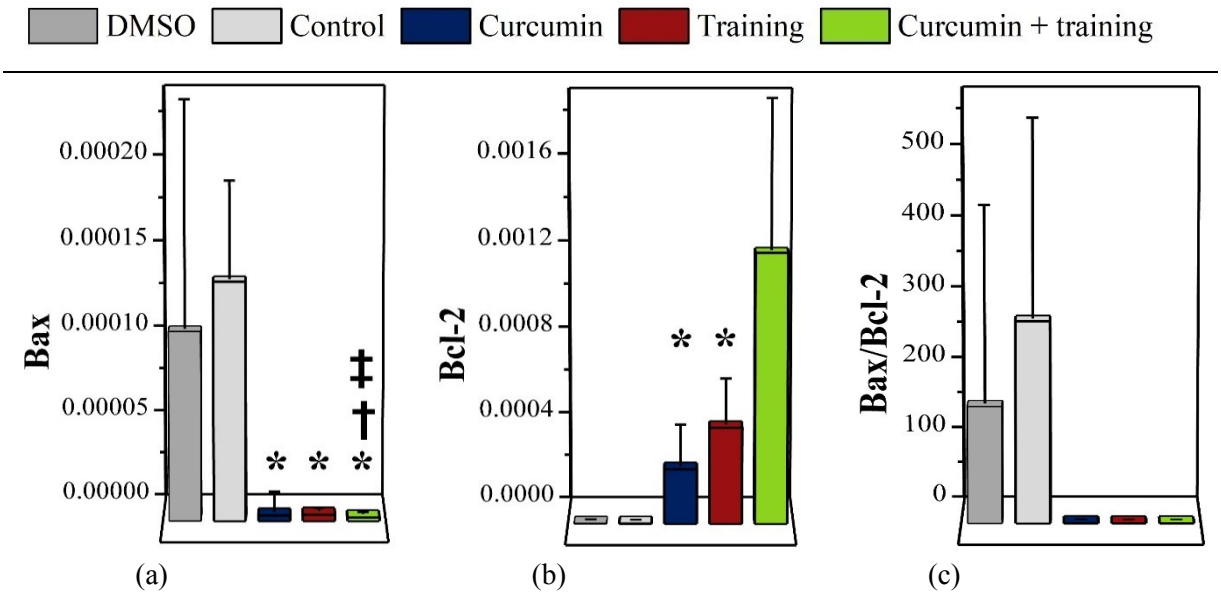

Figure 1. The relative level of mRNA expression of Bax and Bcl-2 genes and Bax/Bcl-2 ratio in the studied groups (1- control, 2-curcumin, 3-swimming training, 4-curcumin and swimming training, and 5-DMSO). Bar graphs show the mean gene expression levels and error bars represent the standard deviations. Significance was considered at $p < 0.005$; * indicates significant difference compared with control group; † indicates significant difference compared with swimming training group; ‡ indicates significant difference compared with curcumin group. (a) the expression level of Bax gene in the studied groups. (b) the expression level of Bcl-2 gene in the studied groups. (c) the expression level of Bax/Bcl-2 ratio in the studied groups.

Also, 13 weeks of aerobic training significantly elevated the expression of anti-apoptotic gene (Bcl- 2) and significantly reduced the gene expression of apoptotic protein (Bax) (19). Additionally, 10- week endurance training reduced apoptosis, increased Bcl- 2 in older rats and improved memory and learning (20). Long-term endurance training significantly decreased levels of t-Bid, Bad, Bak, Bax, Cytochrome- C cytosolic, activated caspase-9 and activated caspase-3 in rats (7). The findings of the above mentioned studies are consistent with the present study. The possible reasons could be the mechanism of training effect on apoptosis process and the same statistical population. Different mechanisms involved in apoptosis process. As an example, mitochondria are organelles which play a key role in the regulation of apoptosis; and its membrane depolarization leads to the production of pro-apoptotic molecules (5). It has been shown that physiological NO (nitric oxide) concentration reversibly inhibits cytochrome c oxidase, which leads to the hyperpolarization of mitochondrial membrane and consequently prevents apoptosis (21). Also, NO directly and indirectly upregulates different anti- apoptotic molecules including Mcl-1. The researchers in (22) concluded that exercise training inhibits the neutrophil apoptosis through the elevating of iNOS (inducible NO synthase) expression and NO-regulated anti-apoptotic molecules including Mcl-1 (a key anti-apoptotic member of the Bcl-2 family). Physical activity upregulates PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-α) and SIRT1 (sirtuin 1) pathway and consequently suppresses the pro-apoptotic effect of ROS in the cardiac and skeletal cells (23). It is worth mentioning that PGC-1α and SIRT1 are involved in mitochondrial biogenesis that is vital for cell survival (23). Many researchers believe that exercise training could improve

the cell survival proteins including MnSOD (manganese SOD), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), ERK (extracellular receptor kinase), IGF1/Akt (insulin-like growth factor 1/ Protein Kinase B) pathway, HSPs (heat shock proteins) in the cardiac, which are important upstream regulators of apoptosis (7). As a result, physical activity reduces the apoptosis induced by various factors through changes in different signaling pathways including upregulation of iNOS-NO-cGMP-Mcl-1, SIRT1, SOD, and HSP pathways, increase in expression of PGC1-α, PI3K (phosphatidylinositide 3-kinase), and Bcl-2 proteins, reduction in expression of ROS, Fas, Bax, and cytochrome C proteins, and as well as changes in the pathway of caspase activity. Therefore, according to the reviewed studies, it can be expected that endurance activity reduces the apoptosis process. However, there are a few opposite studies that suggest exercise training can induce apoptosis. As an example, voluntary exercise (wheel running) did not have a significant effect on the level of Bcl- 2, HSP70 and P53 proteins in rats under restrain stress condition (24). Sandri *et al.* demonstrated that physical activity increases the fragmentation of DNA and the apoptotic process after eccentric exercise in rats (25). Also, Qiguan showed that after exhaustive swimming training for 8 weeks, the level of Bcl- 2 levels reduced significantly and the level of Fas protein increased in skeletal cells (26). Perhaps one possibility to reconcile these opposite studies is the difference in intensity of exercise, duration of exercise and type of physical activity. We used curcumin as an antioxidant supplement in this study. The results showed that curcumin consumption led to a significant elevation in Bcl- 2 gene expression and a significant reduction in Bax gene expression in the cardiac tissue of rats during withdrawal period of excessive ethanol consumption. However, curcumin consumption had no significant effect on the reduction of Bax/Bcl- 2 ratio in the cardiac tissue of rats during the withdrawal period. Curcumin is a yellow compound extracted from the turmeric rhizome. It is known as a strong antioxidant due to the presence of diketone (27). Studies have shown that it could reduce a variety of ROS such as superoxide anion radicals, hydroxyl radicals and nitrogen dioxide radicals (27). Also, curcumin has attracted considerable attention in the research as a cardiac muscle protector because of its wide spectrum of therapeutic activities (28). Furthermore, studies have demonstrated that curcumin effectively protects pancreas, liver and brain against adverse effects of excessive ethanol consumption (11, 12). Some researchers believed that the anti-apoptotic effects of curcumin depend on its dosage (29, 30). Somasundaram *et al.* indicated that curcumin exhibits antioxidant properties and inhibits activation of JNK (c-Jun N-terminal kinase) pathway and mitochondrial release of cytochrome c in a concentration-dependent way. Note that JNK is also involved in stimulating apoptotic signaling (29). It has been shown that protective effects of curcumin (1μM) on ethanol-induced apoptosis in the liver tissue is attributed to the decrease in release of cytochrome C (30). Tiwari and Chopra demonstrated that curcumin (30-60 mg/kg), in addition to behavioral, biochemical, and molecular changes in different brain regions of ethanol-exposed rats, causes significant inhibition of TNF-α and thereby reduction in apoptosis in the brain tissue of the rats (12). Wang showed the consumption of curcumin (50 mg/kg) for 6 weeks protect the mitochondria structure, and reduce the oxidative stress and the gene expression of apoptotic genes including Fas/FasL in liver tissues of NASH (non-alcoholic fatty hepatitis) rats (31). The Fas/FasL also constitutes an important pathway of apoptosis. The protective effect of curcumin (10 μM/L) on reducing apoptosis induced by high glucose in cardiac muscle cells has been investigated by Yu *et al.* (32). They illustrated curcumin considerably decreases Bax/Bcl- 2 ratio and

significantly increases Akt in cardiac cells. This anti-apoptotic effect of curcumin was mainly attributed to the PI3K-Akt signaling pathway, where it is an intracellular important signaling pathway in regulating cell survival (32). It has been shown that curcumin (25 μM) significantly reduces the apoptosis in the ethanol-exposed embryonic hearts by downregulation of activated forms of caspase 3 and caspase 8, and upregulation of Bcl- 2 gene expression (33). Based on the study done by Tao *et al.* curcumin (100 mg/kg, dissolved in saline to 10 mL/kg) suppresses hepatocyte apoptosis caused by mitochondrial permeability transition through inhibition of caspase- 3 activation, mitigation of pro-apoptotic Bax gene expression, and promotion of anti-apoptotic Bcl- 2 gene expression (34). According to the reviewed studies, curcumin prevents the apoptosis caused by various factors through several signaling pathways. The results showed that combination of curcumin consumption and swimming training did not have significant interactive effects on increasing the expression of Bcl- 2 gene and Bax/Bcl- 2 ratio in rat cardiac tissues during withdrawal period of excessive ethanol use. However, it had significant interactive effects on reducing the expression of Bax gene in the cardiac tissue of the rats during withdrawal period. Similar to this study, Hosseini *et al.* investigated the interactive effect of exercise training (forced treadmill running, 5 days per week for six weeks and voluntary wheel running for six weeks) with Nano-curcumin supplement (100 mg/kg for 14 days) on doxorubicin-induced hepatotoxicity in mice (35). They measured the levels of SOD and AIF (apoptosis inducing factor). However, there was no significant decrease compared with doing exercise training alone. Considering these results, it seems that exercise training and curcumin might control apoptosis through different signaling pathways.

Conclusion

According to the results of this study, it seems that physical activity and curcumin consumption can modulate apoptosis process through different signaling pathways during ethanol poisoning. Therefore, it is recommended that these two interventions were used as a protective mechanism against myocardial damage. However, there are some limitations in this study, in particular short-term training and lack of measurement of the studied variables at serum level. Therefore, in future research, changes in these variables at serum level could be investigated, while long-term training periods are considered.

Ethical issues

No applicable.

Authors' contributions

All authors equally contributed to the writing and revision of this paper.

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