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Original Article

Impacts of endurance training and crocin supplementation on the apoptotic pathways in rat skeletal muscle following doxorubicin administration

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Abstract

Background and aims: Doxorubicin (DOX), in addition to its anti-cancer properties, causes toxicity and increases the apoptosis of healthy tissues. The objective of the current research was to explore the concurrent influence of an eight-week aerobic training regimen combined with crocin supplementation on the apoptosis induced by DOX within the soleus muscle tissue of male rodents.

Methods: In this study, a cohort of 40 male Wistar rats, each weighing between 200 and 220 g and approximately 8 weeks old, were systematically distributed into five distinct experimental clusters, including a healthy control (normal saline) and a patient control (intraperitoneal injection of 2 mg/kg of DOX). The other clusters were DOX-exercise (intraperitoneal injection of 2 mg/kg of DOX with eight weeks of treadmill/5 days a week), DOX-crocin (intraperitoneal injection of 2 mg/kg) 2 kg of DOX along with 10 mg/kg of crocin extract/8 weeks), and DOX-exercise-crocin.

Results: The administration of DOX was associated with a notable elevation in Bax and a reduction in Bcl-2 expression ($P=0.001$ and $P=0.001$). In contrast, engaging in eight weeks of aerobic exercise, ingesting crocin, or a synergistic approach combining both interventions resulted in a marked upregulation of Bcl-2 expression ($P=0.001$). This combined treatment also led to a significant diminution in the Bax mRNA ($P=0.006$) and a decrease in the Bax/Bcl-2 ratio ($P=0.001$) within the soleus muscle tissue of male rodents subjected to DOX exposure.

Conclusion: Aerobic exercise with crocin supplementation could inhibit apoptosis caused by DOX in the soleus muscle of male rats.

Keywords: Crocin, Apoptosis, Aerobic exercise, Doxorubicin

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Introduction

Cell dysfunction caused by doxorubicin (DOX or adriamycin) increases apoptosis and oxidative damage, and some mitochondrial changes occur in mitochondria (1). Some researchers found that DOX treatment contributes to the inefficiency of the cell phenotype through the imbalance between mitochondrial fusion and fission and makes heart and muscle cells susceptible to apoptosis (1,2). In light of the diverse pathways through which DOX can precipitate mitochondrial impairment, with potential subsequent cardiovascular compromise or mortality, a spectrum of therapeutic strategies, encompassing both pharmacological and lifestyle interventions, such as exercise and phytotherapy, have been proposed to attenuate mitochondrial-targeted adverse effects associated with DOX (3). Adaptations within the mitochondria are likely to play a critical role in safeguarding the cardiovascular system during physical activity, which is attributed to the heightened requirement for oxygen as muscles contract more vigorously, necessitating an accelerated synthesis of adenosine triphosphate by the mitochondria (4,5). Moreover, significant metabolic adjustments within the mitochondrial oxidative phosphorylation system enhance the capacity for substrate oxidation (6), which may bolster the resilience of these cellular powerhouses against various pathophysiological states linked to mitochondrial impairment, such as those induced by DOX administration (7). This evidence shows that exercise and sports activity probably protect mitochondria from apoptosis caused by DOX treatment because an increase in apoptosis markers mediated by mitochondria has also been observed after DOX treatment (8). Studies have shown that the chronic exercise activity of apoptotic factors caused by DOX treatment, including the Bax/Bcl2 ratio (9), caspase 3, caspase 8, caspase 9, and TUNEL-positive nuclei, reduces the mitochondrial permeability transition pore (9).

The integration of phytotherapeutics with regular physical exercise is a pivotal approach to diminishing the apoptotic processes induced by pharmacological

agents and mitigating their adverse effects. Crocin stands out among many antioxidants for managing diverse pathologies (10-12). This hydrophilic carotenoid, predominantly extracted from Gardenia and Crocus species, is the principal component in saffron (13). Hoshyar et al (14) have documented that chromium (Cr) administration elevates markers of apoptosis within human gastric *adenocarcinoma* cancer cells, thereby inhibiting their proliferation. Conversely, research by Elsherbiny et al (15) has revealed that the nasogastric administration of Cr diminishes caspase-3 expression, a marker indicative of apoptosis in cardiac tissues, in rat models subjected to DOX-induced toxicity. Few studies have explored the combined impact of crocin intake and aerobic physical activity on diminishing the adverse reactions associated with pharmacological treatments. In their latest findings, Davoodi et al (7) found that combining aerobic exercise with Cr consumption improves DOX damage to spermatogenesis and the expression of luteinizing hormone receptors in male Wistar rats. In addition, Davari et al (16) demonstrated that the synergistic effect of interval training and continuous aerobic training with Cr consumption has a more favorable impact on mitochondrial biogenesis factors in the liver tissue of rats with type 2 diabetes. Recent research findings also revealed that the combination of exercise training and the consumption of antioxidant supplements (pistachio green skin extract and Cr) increased the anti-apoptotic activity of mice (17). In light of recent research, it has become evident that Cr is instrumental in safeguarding typical cellular structures and functions from oncogenic transformation by modulating the mechanisms of cell death and survival, mainly through apoptosis, which can be influenced by physical activity. Studies examining the impacts of sustained exercise and crocin supplementation, either independently or synergistically, on cellular markers that regulate apoptosis have yielded mixed results. Given the growing interest in non-pharmacological interventions such as physical training and botanical compounds for managing and mitigating the adverse effects associated with DOX toxicity, this investigation was designed to assess the influence of an 8-week regimen of continuous aerobic exercise combined with crocin administration on the gene expression levels of Bax and Bcl-2, as well as the Bax/Bcl-2 ratio, in the soleus muscle tissue of male rats subjected to DOX treatment.

Materials and Methods

In the context of this research, a cohort of 40 male Wistar rats, each exhibiting an approximate mass of 220 g with a standard deviation of 20 g, were acquired and conveyed to a controlled laboratory environment. Upon arrival, these specimens underwent a seven-day acclimatization period to align with the new environmental parameters. After this adjustment phase, the subjects were systematically allocated to five distinct clusters for the investigation. They included control, aerobic continuous exercise, crocin, aerobic continuous exercise + crocin, and healthy control. In the experimental design, excluding the group serving as the healthy baseline, subjects received an intraperitoneal injection of DOX at a dosage of 2 mg/kg, sourced from Ebewe, a Belgian pharmaceutical firm, over seven weeks. Concurrently, groups designated for aerobic exercise intervention and those allocated to crocin treatment were administered a daily dose of 10 mg/kg of crocin via oral gavage, procured from Sigma-Aldrich Company in the United States (18). Furthermore, the cohorts engaging in aerobic activity and the cohort combining physical exercise with crocin intake adhered to a regimen of sustained aerobic exercise at an intensity ranging from 60% to 75% of their maximal capacity. This exercise protocol was maintained for eight weeks. Forty-eight hours after the final exercise session, the rodents were sedated using an intraperitoneal administration of an anesthetic cocktail comprising ketamine at a dosage of 80 mg/kg of body weight and xylazine at a dosage of 10 mg/kg. Subsequently, the soleus muscle was meticulously removed and promptly secured within a cryogenic vial, which was then submerged in liquid nitrogen. To preserve their integrity for subsequent analysis, these specimens were stored at a temperature of -70 ˚C, with the intent to assess the mRNA levels of caspase-3, Bax, and Bcl-2. The quantitative polymerase chain reaction method was employed to quantify the transcriptional activity of these specific genes. Primer sequences were synthesized in alignment with the specifications listed in Table 1. Total RNA extraction from the muscle biopsies ensued, followed by its reverse transcription into complementary DNA. The resultant complementary DNA underwent amplification through a polymerase chain reaction, facilitating the investigation of gene expression profiles.

How to prepare and inject doxorubicin and saline

Acquired from Ebewe, a Belgian pharmaceutical entity, DOX was procured and subsequently formulated into seven separate administrations of 2 mg/kg, summing to an aggregate of 14 mg/kg. This preparation was achieved by diluting the compound with an isotonic saline solution to attain the requisite concentration. The administration

Table 1. Sequence of primers related to Bax Bcl-2 gene expression

Gene	Forward Primer 5' →3'	Reverse Primer 5'→3'
Gap	AAG TTC AAC GGC ACA GTC AAG G	CAT ACT CAG CAC CAG CAT CAC C
Bax	GCA AAC TGG TGC TCA AGG	CAG CCA CAA AGA TGG TCA
Bcl2	GAGTGGGATACTGGAGATGAAG	TGGTAGCGACGAGAGAAGTC

of DOX occurred via intraperitoneal injection, with each of the seven doses being dispensed weekly. This regimen spanned from the initial week's conclusion until the seventh week's termination, adhering to a schedule that placed the injections 48 hours after the final training and 24 hours before the ensuing session. Furthermore, to establish uniform conditions across all experimental cohorts and mitigate any physiological responses induced by the injection process, control groups were administered an equivalent volume of saline solution (0.9% sodium chloride). To minimize variations attributable to diurnal fluctuations, all injections were consistently conducted at approximately 10 AM (18).

Continuous aerobic exercise protocol

In the initial phase of the experiment, male rodents underwent a preparatory phase on the treadmill, engaging in a moderate activity that reached 50%–60% of their peak velocity for five minutes, followed by a session of sustained aerobic exertion. The exercise routine was concluded with a deceleration period, where the intensity was reduced to 30%–40% of their maximum capability. The sustained aerobic activity entailed treadmill running at an intensity ranging between 65% and 75% of the rodent's highest attainable speed (Table 2). The procedures for the care and humane euthanasia of the rodents were conducted in strict accordance with the protocols approved by the Animal Ethics Committee at Jundishapur University of Medical Sciences in Ahvaz.

How to prepare and consume crocin

Crocin, a potent antioxidant, was procured as a pre-prepared powder in one-gram vials at a purity level of 98%, sourced from the Sigma Company based in the United States. During an eight-week regimen on designated training days, the subjects assigned to the supplement-plus training cohort and the supplement-only group were administered an oral dose of 50 mg per kg of body mass via gavage. Similarly, the saline-treated cohorts were given an equivalent dosage of the crocin supplement through the same gavage method (18).

Data analysis

The Shapiro-Wilk test was employed to assess the distribution of data for normality. A one-way analysis of variance and Tukey's post hoc test were conducted to explore the primary and interaction effects among groups. The collected data were statistically evaluated using the SPSS analytical tool, version 22. Statistical significance was determined at a 95% confidence threshold.

Results

Analysis of Tukey's post hoc test data demonstrated a statistically significant elevation in Bax expression ($P=0.001$) within the group of patients compared to their healthy counterparts. Conversely, expression levels of Bcl-2 were markedly reduced in the patient cohort relative to the healthy control group ($P=0.001$). The statistical measures for Bcl-2 showed an associated $P=0.001$. Similarly, the Bax expression yielded a P value of 0.001, and the ratio of Bax to Bcl-2 reached statistical significance ($P=0.001$). These significance levels across all measured variables point to a discernible disparity between the average values of the patient group and those of the healthy control group. This disparity suggests that the administration of DOX has a quantifiable impact on these biomarkers (Figures 1-3, Table 3).

The result revealed the statistically significant effect of eight weeks of continuous aerobic training ($P=0.001$), consumption of crocin ($P=0.001$), and the interaction or combined effect of exercise and crocin ($P=0.001$) on Bcl-2 sole muscle tissue of male rats subjected to apoptosis induction. In addition, eight weeks of continuous aerobic exercise ($P=0.004$), consumption of crocin ($P=0.001$), and the interaction or combined effect of exercise and crocin ($P=0.006$) had an impact on Bax. The soleus muscle tissue of male rats subjected to apoptosis induction was statistically significant. Therefore, with a 95% confidence interval, continuous aerobic training and crocin, each alone, as well as a combination of these two interventions,

Table 2. Steps of continuous aerobic exercise

Exercise Steps	Warm up	Training				Cooling Down
Intensity	40–50% maximum speed	1 Week	2 Weeks	3 Weeks	After 4 weeks	40%–50% of maximum speed
		60% of maximum speed	65% of maximum speed	70% of maximum speed	75% of maximum speed	
Time	5 minutes	16 minutes	24 minutes	32 minutes	40 minutes	5 minutes

Table 3. Mean ± standard deviation expression of all genes

Groups	Markers		
	Bax	Bcl-2	Caspase-3
Healthy	0.00001 ± 0.000005	0.1 ± 0.2	0.0000025 ± 0.0000012
Control	0.000055 ± 000002	0.03 ± 0.01	0.0002 ± 0.0001
Crocin	0.000015 ± 0.000005	0.02 ± 0.01	0.00005 ± 0.0000025
Training	0.00003 ± 0.00002	0.13 ± 0.03	0.00016 ± 0.00005
Training + Crocin	0.000005 ± 0.0000025	0.26 ± 0.15	0.0001 ± 0.00005

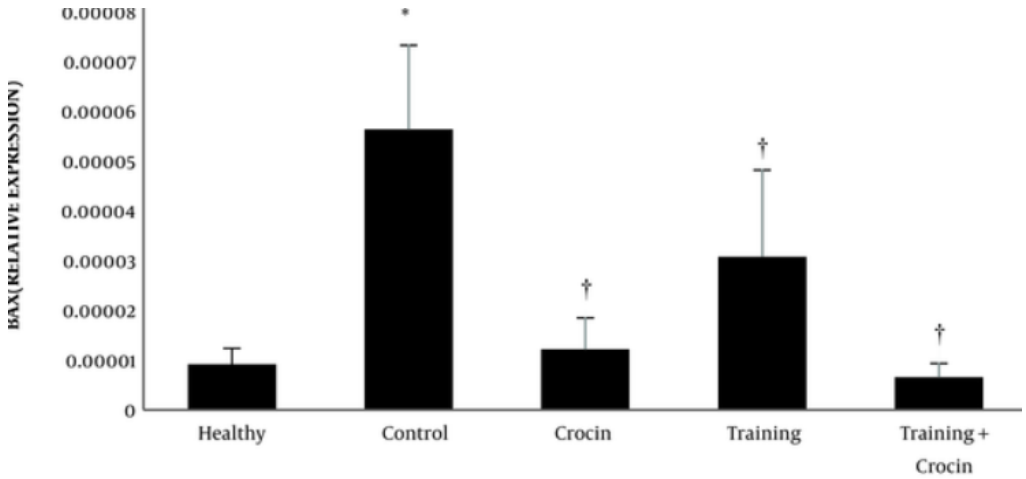

Figure 1. Bax mRNA. *Note*. The symbol * shows significant differences in healthy control, and the symbol † indicates a significant difference from DOX-control.

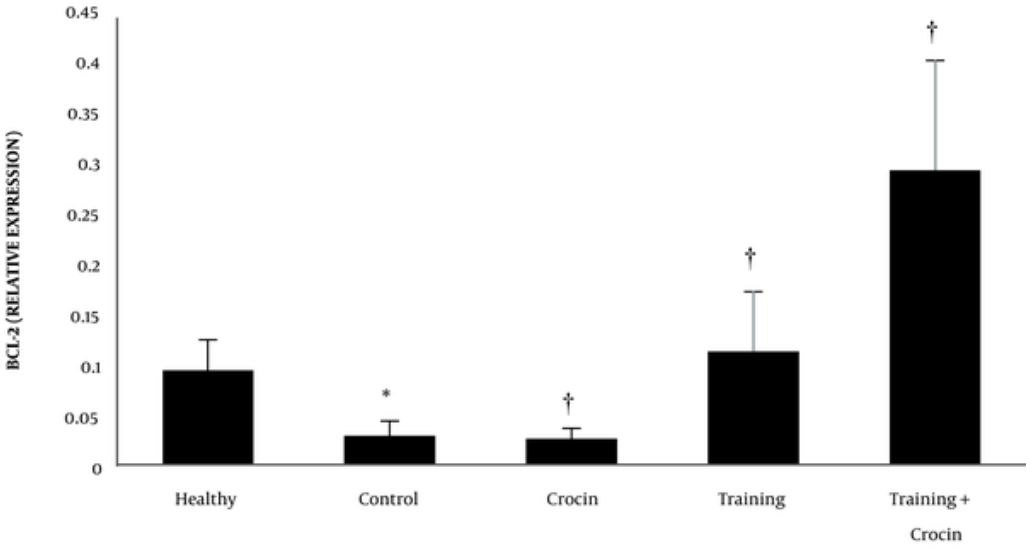

Figure 2. Bcl-2 mRNA. *Note*. Symbol * displays significant differences in healthy control, and symbol † illustrates a significant difference from DOX-control.

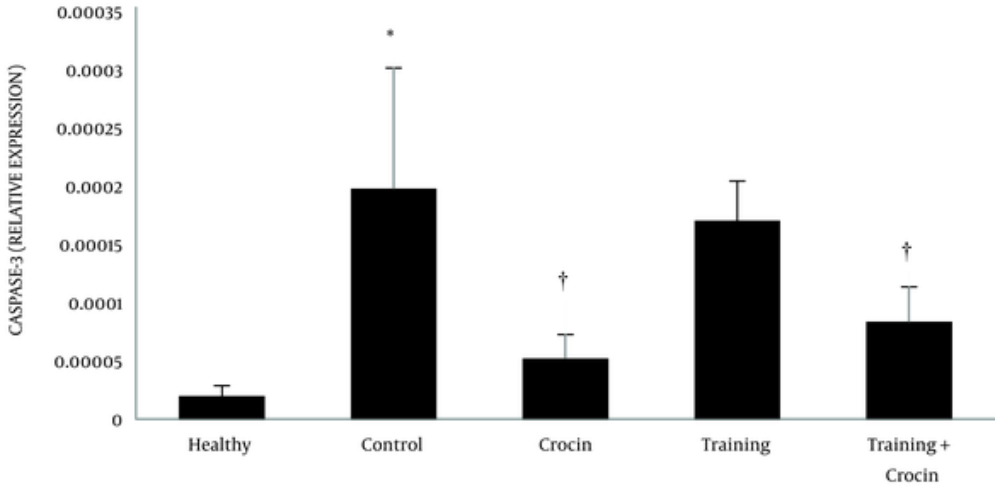

Figure 3. Caspase-3 mRNA. *Note*. The symbol * implies significant differences in healthy control, and the symbol † represents a significant difference from DOX-control.

compared to the DOX group, led to significant changes in the levels of Bcl-2 and Bax in the soleus muscle, due to the induction of apoptosis in male rats ($P=0.045$). Based on the findings of the present research, a period of continuous aerobic training, consumption of antioxidant crocin, and a combination of these two interventions could significantly change the levels of Bcl-2 in the soleus muscle of male rats subjected to apoptosis induction (Figures 1-3, Table 3).

Discussion

DOX is recognized for its efficacy in treating cancer. Nonetheless, it is not without adverse effects on vital organs, including the heart, liver, kidneys, and muscles. Current interventions to alleviate the toxicity induced by DOX in oncology patients are limited, often lack substantive clinical success, and may introduce additional side effects. Consequently, there is a growing interest in exploring non-pharmacological strategies, such as botanical therapeutics and consistent exercise, to mitigate these toxic effects. In this context, incorporating crocin, a potent antioxidant constituent of saffron, into the diet as a supplement has gained attention. Moreover, aerobic exercise has become increasingly popular to support the body's natural detoxification processes in response to DOX treatment (19). In this investigation, the administration of DOX was observed to initiate apoptotic processes in the soleus muscle of Wistar rats, as evidenced by the upregulation of *Bax* gene expression and the elevation of the Bax/Bcl-2 ratio, alongside a reduction in Bcl-2 expression when compared to a healthy control cohort. However, the study also revealed that three distinct interventions—namely, sustained aerobic exercise, intake of crocin, and a synergistic approach combining both—effectively counteract the pro-apoptotic influence of DOX on murine skeletal muscle. The synergistic intervention of consistent aerobic activity paired with crocin supplementation notably led to an upsurge in *Bcl-2* gene expression while concurrently downregulating *Bax* gene expression and diminishing the Bax/Bcl-2 ratio in the soleus muscle of male rats that were subjected to induced apoptosis. Furthermore, it was demonstrated that both aerobic exercise and crocin consumption independently serve as protective agents against DOX-induced apoptosis within the skeletal muscle tissue.

DOX interacts with DNA to inhibit topoisomerase II transcription and replication (20). Although no precise mechanism has been reported for DOX-induced cytotoxicity, there is a view that this process occurs through the formation of free radicals, oxidative damage, and apoptosis (21). Apoptosis triggered by DOX operates through dual mechanisms, namely, the extrinsic death receptor cascade and the intrinsic mitochondrial route. The latter is a critical and primary conduit for DOX-induced cell apoptosis. Beyond their role in orchestrating apoptosis via caspase activation, mitochondria also play a pivotal role in modulating the Bcl-2 family of proteins, which are integral to apoptosis and proceed independently of caspases (21). Modifications in mitochondrial dynamics have been identified as critical regulators of apoptosis, mainly through mechanisms that do not involve BCL proteins or caspases. The proteins Bcl-2 and BAX are pivotal in modulating the permeability of the mitochondrial membrane, which is a crucial step in the apoptotic process. Furthermore, the role of mitochondrial integrity and the subsequent release of cytochrome c are instrumental in apoptosis induction. This research examined the impact of DOX on apoptotic pathways, aligning with the results from multiple investigations within this domain (22). Recent findings indicate that a regimen of consistent aerobic exercise paired with crocin intake selectively mitigates DOX-induced cell death in muscle tissues. There is a limited body of research exploring the detrimental effects of DOX on muscular structures. This investigation pioneers exploring the synergistic protective role of sustained physical activity and crocin, an antioxidant, against the adverse outcomes and toxic responses prompted by DOX in a healthy murine model. The researchers have also discerned that intense, sporadic training coupled with crocin supplementation curtails the apoptotic response triggered by DOX in male rodents. This protective mechanism involves a reduction in the levels of the pro-apoptotic protein Bax and an elevation in the anti-apoptotic protein Bcl-2, thereby attenuating the toxic impact on muscle fibers (19). Similarly, Shekarriz et al demonstrated that the combination of aerobic physical activity and the intake of crocin offers a safeguarding influence against cardiotoxicity precipitated by DOX (23).

The findings of the current investigation revealed that sustained aerobic activity may attenuate DOX-induced apoptosis in Wistar rat skeletal muscle through the upregulation of BCL-2 and downregulation of Bax. These findings are consistent with those of prior studies, suggesting the protective role of aerobic exercise in cellular apoptosis in this context (19). The study's conclusions indicate that engaging in voluntary physical activity and treadmill running before and concurrent with DOX therapy can mitigate disturbances in mitochondrial structure, diminish oxidative stress, bolster antioxidant defense mechanisms, and reduce signals that promote apoptosis (18). Based on the results of the current research, regular aerobic exercise over eight weeks reduced the levels of pro-apoptotic markers (Bax and the Bax/BCL-2 ratio) following the administration of DOX. This observation aligns with the findings of prior investigations that have explored a variety of exercise modalities and athletic disciplines (18). Marques-Aleixo et al (18) found that exercise before and during DOX treatment reduces the ratio of Bax/BCL-2 and sensitivity to the mitochondrial permeability transition pore and increases the level of Bcl-2 as an anti-apoptotic factor. The findings of the present study contradict those of the above study. Among the possible reasons for the inconsistency with the mentioned study are the type of tissue examined and the intensity of the exercise.

Physical activity exerts specific constraints on skeletal muscle tissue, attributable to mechanical and metabolic demands accompanying heightened functional and energetic needs. Such demands activate inflammatory cascades via several processes. These processes encompass the modulation of apoptotic signaling pathways, characterized by an elevation in Bcl-2 protein levels and a concomitant reduction in Bax protein expression, thereby mitigating the cytotoxic effects of DOX (18). Conversely,

the concurrent engagement in aerobic exercise and intake of crocin yields a combined effect that attenuates the harmful impact of DOX on skeletal muscles. This amelioration is facilitated through the modulation of mitochondrial signaling networks and the regulation of cellular apoptosis via the Bcl-2/Bax pathway. However, this study has some constraints that warrant further exploration in subsequent research endeavors. The intricate relationship between autophagy and apoptosis is particularly noteworthy, given that DOX exposure has been linked with an upsurge in autophagic processes. Indeed, it has been observed that autophagy plays a role in the cardiotoxicity induced by DOX. This is underscored by the interaction between Bcl-2 and Beclin-1 (a key autophagic indicator), where Bcl-2's complex formation with Beclin-1 serves as an autophagic inhibitor (18); it is suggested that autophagy factors be used in future research to further investigate the possible mechanisms of physical activity. Herbal medicines should be studied for the reduction of toxicity caused by DOX treatment. Furthermore, it is advisable to consider additional laboratory techniques (e.g., Western blot analysis and histochemical assays) to conduct a more comprehensive assessment of interventions' effects on DOX-triggered apoptotic processes.

Conclusion

In general, the findings of our investigation substantiated the hypothesis that apoptotic pathways are implicated in the cytotoxicity provoked by DOX. Moreover, it was observed that both sustained aerobic physical activity and the intake of crocin, independently or in combination, could impede the apoptotic processes instigated by DOX in skeletal muscle tissues. Notably, the concurrent application of exercise regimens and crocin supplementation exhibited a superior advantage in diminishing apoptotic occurrences. Consequently, the strategic integration of regular exercise and phytotherapeutic agents emerges as a potent approach to lessening the harmful effects of DOX chemotherapy.

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Authors' Contribution

Conceptualization: Kavous Eydivandi.
Data curation: Kavous Eydivandi.
Investigation: Kavous Eydivandi, Parvin Farzanegi.
Methodology: Kavous Eydivandi, Mohammad Ali Azarbayjani.
Supervision: Mohammad Ali Azarbayjani.
Writing–original draft: Kavous Eydivandi.
Writing–review & editing: Mohammad Ali Azarbayjani, Parvin Farzanegi.

Competing Interests

None to be declared.

Ethical Approval

This study was approved by the Institute of Physical Education and Sports Sciences with code IR.SSRC.REC.1399.057.

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