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Resveratrol Supplement Could Reverse the Effect of Resistance Training on Muscular eMHC and PAX7 in BALB/C Mice Bearing CT-26 Tumor

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Abstract

Introduction: Although resistance training and resveratrol is recommended as a stimulant for muscle regeneration in healthy subjects their effects on cancer-related cachexia indices are unclear. This study aimed to investigate the effect of a course of resistance training with resveratrol supplement on PAX-7 and eMHC in Gastrocnemius muscle tissue of mice bearing colon cancer induced cachexia.

Methods: This was an experimental study conducted on 20 six-week-old BALB/c mice implanted with CT-26 tumor. The mice were randomly divided into four groups: resistance training, resveratrol, resveratrol with resistance training, and control. The resistance training group performed 6 weeks of progressive resistance training, the resveratrol group received 100 mg/kg resveratrol per day. 48 hours after the last experimental session, the mice were sacrificed and PAX-7 and eMHC protein levels were measured using ELISA test through specimen taken from gastrocnemius muscles. Statistical test of one way ANOVA was used for data analysis.

Results: PAX-7 level was significantly lower in resveratrol with resistance training group compared to control (P=0.009), resveratrol (P=0.005), and resistance training groups (P=0.002). eMHC was significantly lower in the resistance training group compared to the control (P=0.08), resistance training, and resveratrol groups (P=0.002).

Conclusion: Resistance training was not an appropriate intervention for treating reduced muscular regeneration due to cachexia. However, regarding reducing effect of resveratrol supplementation with resistance training on PAX-7 level, it may be recommended to improve muscular regeneration.

Keywords: Resistance Training, Resveratrol, PAX-7, eMHC, Cancer, Cachexia

Introduction

Cachexia (wasting syndrome) is an abnormal condition in cancer and nearly half of cancer patients suffer from cachexia. Patients with gastrointestinal and pancreatic cancers show the highest incidence of cachexia (1), which is the mortality factor of 22- 40 % of cancer patients accounting for over1500000 deaths per year (2). Unfortunately, none of the therapeutic methods has been so far able to completely inhibit muscle atrophy caused by cachexia (3). Several factors such as altered muscle microenvironment and inflammation (4), increased degradation and decreased synthesis of proteins (5), and disruption of muscular regeneration (6) have been suggested as factors of cachexia. The results of several studies have shown that dysfunction of satellite cells is involved in cancer-induced cachexia (7). In the process of muscle regeneration, paired box 7 (Pax-7) and MyoD are increased as indicators of stem cell proliferation; however, in differentiation stage of stem cells, the level of Pax-7 decreases but myoenzyne, MyoD, and Mrf4 increase. In this regard, different interventions in various ways

can increase muscle cell regeneration (8), including exercise activities, food and drug supplements. Resveratrol, a polyphenolic compound, has anti-inflammatory and antioxidant properties. Saini *et al.* (2012) in their study showed that resveratrol supplement could reduce TNF-**α**-induced apoptosis by increasing SITR1, which results in differentiation of myoblasts (9). Montazenau *et al.* (2013) in their study on the effect of resveratrol on hypertrophy and myogenesis in cultured rodent cell myoblasts showed that resveratrol increased myoblast differentiation to myotube and inhibited cell proliferation via expression of MRFs such as Myf-5, MyoD, and myogenin as well as byreducing various types of cyclins (10). Regeneration of muscle is different in cachexia with natural conditions; nevertheless, no study was found to investigate the effect of resveratrol on muscle regeneration in patients with cachexia. A number of studies have also indicated that sports activities stimulate different mechanical and metabolic variables as well as hypoxia. Exercise also leads to secretion of different growth factors, cytokines, and hormones, activating satellite cells in adult skeletal muscle and leading to their differentiation and muscle hypertrophy(11). Nederveen *et al.* (2016) showed that during long-term resistance training, muscle regeneration rate, paired box 7 (Pax-7) and MyoD are increased, which are indicators of stem cell proliferation (12). In general, the differentiation of muscle cells is dependent on myogenic regulating genes, but in the final stage of differentiation, myogenin, MyoD, and Mrf4 are required coupled with a decrease in Pax-7 (8).Considering the importance of treatment of cachexia and its mentioned complications, it is necessary to investigate therapeutic interventions to improve this condition. Because limited study was found related to the effect of resistance training and resveratrol supplement on skeletal muscle regeneration, the present study was conducted to investigate the combined effect of resistance training and resveratrol supplementation on PAX-7 and eMHC as regeneration indices.

Methods

Before preparing samples for the experiment, first 4-week-old male BALB/c mice were prepared and injected in the left flank with 3×10^6 CT-26 cells purchased from Pasteur Institute of Tehran. The tumor was allowed to grow within 4 weeks. After this step, a sample of 20 male 6-8 week old BALB/c mice with an average weight of 17-18 g was provided by Pasteur Institute and transferred to animal house. The cages holding the mice were of plexiglas. The mice were kept in 12:12 darkness- light cycle under standard laboratory conditions, and the room temperature was maintained at 22-24°C in 45% humidity. The mice had free access to chow ad libitum and water and were kept for one week (4 mice per cage) to be adapted to the laboratory environment. After experimental period, ketamine and xylazine (10:1 ratio) (13) were combined and 1 ml of their mixture was intraperitoneally injected per kilogram body weight of mice. The left flank hair of the mice was shaved, which was also performed for previously malignant mice whose tumors were transplanted to other mice by punch biopsy. Two weeks after tumor implantation and development, the mice were randomly assigned into four groups of equal size (n=5) including: 1) control, 2) resveratrol, 3) resistance training, 4) resistance training with resveratrol. The exercise protocol included resistance training involving climbing a ladder. The initial ladder included a length of 1 m with a 2-cm clearance between steps which was modified manually by researcher to be appropriate for mice. The training protocol consisted of three sessions per week for 6 weeks, which started one week after adaptation with training. At the beginning of each session, the mice went up and down the ladder without any weight to warm up. On the first day of each week, the maximum weight carrying capacity of mice was measured. The first repetition was done without any weight attached to mice tails, repeats of 2-5 with 50%

maximum capacity and repeats of 6-10 with 70% maximum capacity of mice, and each training session included 10 repetitions with two minutes of rest between each repetition. During this period, the control group was not subjected to any training program(14). Resveratrol (Nutrivitashop Co.) groups received 100 mg/kg of resveratrol in 1% methylcellulose by gavage(15)but the mice in other groups received only methylcellulose solution. The mice were weighted at the beginning of the week, and the level of received supplement was calculated based on the new weight**.** The mice were sacrificed in accordance with ethical principles after completing the exercise protocol and 48 hours after the last training session. Initially, the mice were anesthetized with a combination of xylazine (1 mg) and ketamine (10 mg) at a rate of 1 mg/kg of body weight. The tumor tissue of the mice was removed using forceps and scissors and placed in sterile plates containing PBS. The blood vessels and adipose tissue around the tumor were removed and the tumor was divided into smaller parts by scissors. Gastrocnemius muscle of animal's left leg was placed in a microtube after rinsing with sodium chloride, immediately frozen in liquid nitrogen and stored at -70°C**.** ELISA test was used for measuring PAX-7 and eMHC using commercial kits of Mybiosource(Made in USA). Data analysis was performed using SPSS software. After approving normal distribution of findings with the Shapiro- Wilk test, statistical test of one way ANOVA was used to compare the study groups and in the case of significant finding, Tukey's pot-hoc test was used to compare the paired groups.

Result

The results of one way ANOVA showed that PAX-7 level was significantly different between study groups (F =5.87, p=0.007). The results of Tukey's post-hoc test indicated that there was lower PAX-7 in resveratrol with resistance training group (5.1±3ng/ml) compared to the control (19.17±5.8ng/ml) (P=0.009), resveratrol (21.5±8.3) (P=0.005), and resistance training (19.8±9.1) (P = 0.002) groups. One way ANOVA results indicated that eMHC was significantly different between the study groups (F=3.157, p=049). Also, the results of Tukey's post-hoc test showed that there was lower eMHC in the resistance training group (3.94±0.6ng/ml) compared to the control (4.22±0.2ng/ml) (P=0.082), resistance training with resveratrol (4.66٠0.20) (P=0.002) groups**.**

Table 1. One-way ANOVA results for evaluating the effect of resistance training and Resveratrol on PAX-7 and eMHC in cachexic mice

Variable	Source of changes	Degree of freedom	Mean square	F	P
PAX-7 (ng/ml)	Intergroup	3	869.40	5.87	0.007
	Intragroup	16	789.59		
	Total	19	1658.99		
eMHC (ng/ml)	Intergroup	3	3.78	3.157	0.049
	Intragroup	16	6.39		
	Total	19	10.17		

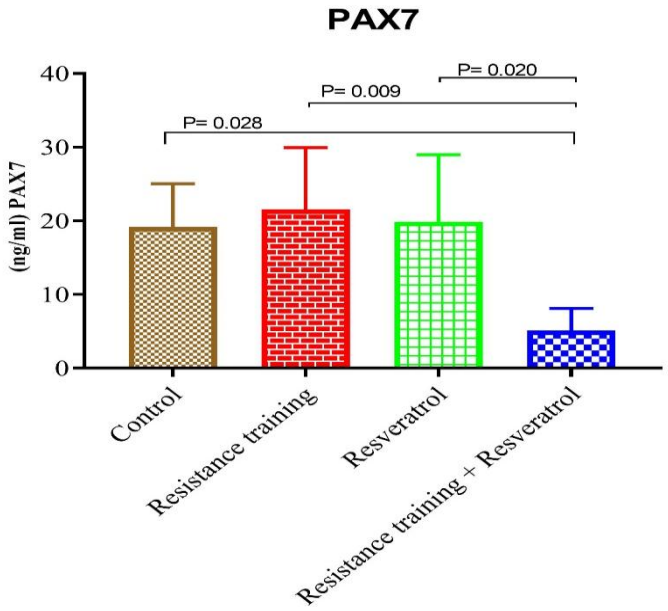

Figure 1. Comparing PAX-7 between four study groups in cachexic mice.

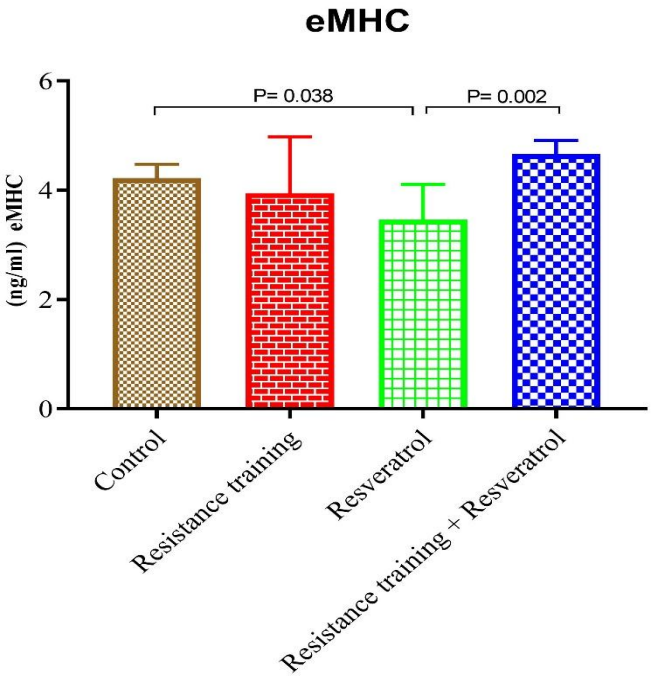

Figure 2. Comparing eMHC between four study groups in cachexic mice

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Discussion

The findings of the present study indicated that resistance training with resveratrol decreased PAX-7 compared to all other groups, while it caused no significant effect on eMHC compared to other groups. Resistance training did not affect PAX-7 significantly, while it reduced eMHC significantly compared to the resistance training with resveratrol and control groups. Resveratrol caused no significant

effect on PAX-7 and eMHC. PAX-7 is one of the most important molecules in the process of musculoskeletal regeneration in mice afflicted with cancer, and several studies have examined this matter in various types of cancer(16, 17). For example, Penna *et al.* (2010) in their research reported that PAX-7 expression increased in mice bearing C-26 tumor, which was a major cause of decreasing muscle regeneration (17). In contrast to our research, the study of Coletti *et al.* (2016) involving optional medium-intensity wheel running exercise increased muscle regeneration. Optional training decreased PAX-7 levels by reducing the activity of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-KP), leading to increased regeneration because PAX-7 expression should cease to enable the differentiation of satellite cells into myocytes(16). These results have been obtained while a majority of studies have reported resistance training as a stronger stimulus compared to low-intensity aerobic exercises for muscle regeneration in normal conditions (12, 18, 19); nevertheless, a variety of reasons could have caused this difference. For instance, TNF-**α** is an important inflammatory factor in muscular microenvironment, which its increase both in vitro and in vivo increases PAX-7 expression and decreases muscle regeneration (20). Resistance training, however, has a weaker anti-inflammatory role than aerobic exercises (21) and the effect of resistance training on TNF-**α** levels has not been well-established (22), with some studies reporting an increase(23) and others a decrease in its levels (24). In another investigation, reduced muscle regeneration has been attributed to ERK protein(17),and most studies have shown increasing activity of this protein after a period or even a single session of resistance training (25), which may be a mechanism for decreasing muscle regeneration due to resistance training. Resistance training is of course a strong stimulus for secretion of various growth factors such as insulin-like growth factor (IGF-1) (26), which can contribute to tumor malignancy and more inflammation in tumor microenvironment (27). Nonetheless, in the present study, the growth factor levels were not investigated and resistance training did not result in increasing tumor growth. On the other hand, resveratrol, which is known as an anti-inflammatory factor, could not change PAX-7 level by itself. In two other studies consistent with the present study, resveratrol did not significantly change PAX-7 levels (28, 29). These researches have been conducted on elderly people, and although old age is different from cancer, only papers on this subject were found. In this research, the interaction of resistance training with resveratrol decreased PAX-7 levels in gstrocnemius muscle, although this decrease could not improve muscle regeneration in CT-26 tumor-bearing mice. In line with the present study, Ballak *et al.* (2014) showed the decrease of PAX-7 in resistance training and resveratrol groups compared to control (28), although the mentioned study examined the combined effect of resistance training and resveratrol on elderly mice. However, Alvaia *et al.* (2017) used resveratrol and combined (resistance and aerobic) exercise in elderly people, and their results were inconsistent with ours and showed increasing PAX-7 levels (30). This difference can be ascribed to a few reasons. Firstly, this study was conducted on elderly people and the exercises were combined training with different signaling pathways and effects of these two types of exercise. Finally, the resveratrol dose in the mentioned study was five times higher than the present study, which may have contributed to differences in results concerning PAX-7. Another result of this study was the reduction in eMHC levels, which is an indicator of muscle regeneration in resistance training group. However, many investigations have indicated that this factor is decreased in muscles of tumor-bearing mice compared to healthy mice in which resistance training increases MHC levels (31). Although the results of the present study were unexpected

and contrary to research presumption, such outcome may not be unpredictable if we consider PAX-7 levels following resistance training because PAX-7 (which is a main factor of impaired regeneration) has been insignificantly increased whilst resistance training, especially at the beginning of training, causes some damage in muscle fibers (32). In justifying this issue, the theory of delayed bruising or minor injuries after resistance training could be helpful, as inflammation has been suggested to cause these damages and many studies have recommended anti-inflammatory drug to avoid injury after exercise. A number of studies have investigated the extent of muscle regeneration in tumor-bearing mice after injury and reported impairment in muscle regeneration. In our study, minor injuries of resistance training stimulating and increasing muscle regeneration are likely to have decreased the muscle regeneration index (eMHC) in tumor-bearing mice. These exercises may have damaged newly differentiated fibers expressing eMHC and impaired the differentiation of satellite cells due to cancer, leading to the decrease in eMHC levels. Nonetheless, eMHC was increased in combined resistance training and resveratrol group. On the other hand, the anti-inflammatory effect of resveratrol has been confirmed by a large number of papers. In the present study, an increase in eMHC was observed when resistance training was combined with resveratrol. As an anti-inflammatory agent, resveratrol has inhibited injuries to eMHC due to resistance training, but as PAX-7 was shown to decrease in this group, this result was not unexpected because (as noted above) PAX-7 expression inhibits muscle regeneration in tumor-bearing mice (33). Considering the results and discussion, it can be concluded that while the combination of resistance training and resveratrol decreased PAX-7 (as a muscle regeneration inhibitor) in Gastrocnemius muscle of tumor-bearing mice, the regeneration rate and its index (muscle eMHC) was not significantly changed. Therefore, resistance training alone does not have favorable outcomes to improve muscle regeneration in cachexic mice and may even reduce the regeneration rate, but the combination of resistance training and resveratrol inhibited the negative effects of resistance training on muscle regeneration factors. Nonetheless, further studies investigating various regeneration indices seem to be necessary for definitive results.

Conclusion

Resistance training was not an appropriate intervention for treatment of reduced muscular regeneration due to cachexia. However, regarding reducing effect of resveratrol supplementation with resistance training on PAX-7 level, it may be recommended to improve muscular regeneration.

Ethical issues

The study proposal and procedures were approved by ethic committee of animal studies in Shiraz University in Iran and followed the ethical guidelines for the care and use of animal labs, published by the National Institute of Health

Authors' contributions

All authors participated in study design and approved the final manuscript. Besides, EA participated in study performance and writing the manuscript, MS cooperated in study performance and MKJ participated in editing the manuscript, too.

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