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Combination of Atorvastatin–Endurance Training Has Positive Effect on Apoptosis and Protein Expression of SDF-1α/CXCR4 Axis after Myocardial Infarction in Rat's Heart Tissue

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Abstract

Background: *It is a well-known fact that both statins and exercise have beneficial effects in preventing cardiovascular disease; however, no information is available about their combinatorial effect on the cardiac cell apoptosis and protein expression of SDF-1α/CXCR4 after myocardial infarction (MI). This study aimed to evaluate the combined effect of endurance training and atorvastatin on the apoptosis and protein expression of SDF-1α/CXCR4 in the cardiac tissue of rats following MI.*

Methods: *In total, 30 rats (8–10 weeks old, weighing 220–240 g) were randomly divided into five groups. Myocardial infarction was induced by subcutaneous injection of isoprenaline (150 mg/kg) in 2 consecutive days. Drug and training intervention was initiated 2 days after infarction and was continued for 4 weeks. To assess apoptotis and protein expression of SDF-1α/CXCR4 axis, Tunnel and IHC staining were performed, respectively.*

Results: *The combination of endurance exercise and atorvastatin significantly reduced the number of apoptotic cells and increased the protein expression of SDF-1α compared with the other groups (P<0.001). Moreover, this combination significantly increased the protein expression of CXCR4 compared with the control and sham groups (P<0.001) and with the atorvastatin group (P<0.05). Endurance exercise training and atorvastatin, individually induced a significant decrease in the apoptotic cell count (P<0.001) and nonsignificant increase in the protein expression of SDF-1/CXCR4, compared with the control group.*

Conclusions: *Results of this study revealed that the combined effects of training and atorvastatin are more efficient in reducing the apoptosis and upregulation of SDF-1α/CXCR4 than exercise or atorvastatin alone.*

Keywords: Endurance training, Atorvastatin, Myocardial infarction, SDF-1α/CXCR4—apoptosis.

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Introduction

Cardiovascular disease is the leading cause of mortality worldwide.[1] Approximately, each year 17.1 million people die due to cardiovascular disease, of which about 7.2 million deaths result from myocardial infarction (MI).[2] MI occurs as a result of insufficient blood flow, hypoxia, and reduction of glucose availability to the cardiac tissue. The acute and chronic disorders of MI can lead to cell damage[3] and induce apoptosis.[4] Apoptosis occurs about 4.5 h after infarction; however, several months after the myocardial infarction, the cardiac cell death in the surroundings of the infarcted area is mainly attributed to apoptosis.[5]

Following ischemia, high speed angiogenesis begins.[6] CXC chemokine family, including the stromal cell-derived factor-1 alpha (SDF-1α) is a key regulator of angiogenesis that binds to the cognate CXCR4 receptor on the stem and progenitor cells and plays a crucial role in gathering and recruitment of stem cells into the infarcted area.[7] Studies reveal that SDF-1α induced mobilization of the bone marrow-derived stem cells to the ischemic area, leads to angiogenesis and improves cardiac function.[8] It is reported that the treatment by SDF-1α after myocardial infarction, exerts beneficial effects on the ventricular function by recruiting bone marrow or circulating hematopoietic stem cells and presumably by inhibiting the apoptosis of cardiac cells and enhancing angiogenesis.[8] Expression of SDF-1α and CXCR4 after myocardial infarction is a transitory process that peaks 2 days after infarction and returns to sham levels 28 days after infarction[9] Therefore, the brief and temporary upregulation of SDF-1α after acute myocardial infarction seems insufficient for complete recovery of cardiac function.[10] Till date, numerous methods are investigated to protect the heart against ischemic injury. Pharmacological intervention, especially using statin drugs for strengthening the expression of angiogenic factors such as the eNOS/NO and SDF-1α/CXCR4 axis, as well as for reducing the isoprenoide synthesis and apoptosis, are recognized as most effective methods in improving the body's innate ability to develop lateral vessels as well as in reducing the ischemic complications such as cardiac remodeling and extending the infarcted area.[10] It is reported that statins can be used to strengthen the SDF-1α expression instantly after ischemic damage. Moreover, studies report that these drugs stabilize eNOS mRNA and increased production of nitric oxide (NO), and thereby exert antiapoptotic effects in patients with cardiovascular disease.[10] Qiu et al. (2012) also reported that the expression of SDF-1α and CXCR4 was significantly increased after 7 days of treatment by atorvastatin.[10]

Furthermore, the animal studies suggest that regular sessions of aerobic exercise, protects the heart against ischemia or reperfusion injury.[11,12] Benefits of exercise on the metabolic, cardiovascular, and anti-inflammatory factors, has led many investigators to suggest exercise training as a nonpharmaceutical tool in the prevention and treatment of cardiovascular disease. The efficiency of exercise training as a powerful tool in the treatment of abnormalities associated with myocardial infarction is widely reported in clinical and

experimental conditions.[13] Some protective effects of endurance exercise training include lateral vessel formation, increase heat shock proteins (HSPs), expression of myocardial cyclooxygenase (COX), increased antioxidant and anti-inflammatory status, ER stress proteins, expression and activity of eNOS/NO system, and an increase in the angiogenic factors such as SDF-1α and VEGF;[11,13-15] however, the effects of exercise training after MI on cardiac cell apoptosis still remain unclear.[16]

As aforementioned, it is clear that statins (including atorvastatin), and also endurance exercise training may be effective in secondary prevention after myocardial infarction. Hence, it is likely that the simultaneous use of statin drugs and endurance training may be more efficient in the treatment and prevention of cardiovascular disorders such as myocardial infarction.

Materials and Methods

Thirty adult male Wistar rats (8–10 weeks old, 230±10 g body weight) were obtained from Tehran Pasteur Institute and housed in standard cages and controlled environment (22–24 °C, 45–50% humidity, and 12-h light/dark cycle), with free access to food and water. All experiments were performed in accordance with the Helsinki Declaration. Rats were randomly divided into five groups. Sham, control, endurance training, atorvastatin, and exercise+atorvastatin groups (6 rats per group).

MI Induction: Isoprenaline solution in normal saline was injected SC at a dose of (150 mg/kg) on two consecutive days with an interval of 24 h for MI induction.[17,18]

Study design and data collection: According to figure 1, training and pharmaceutical intervention began 2 days after MI. Training groups initiated the core training program at a speed of 10 m/min, for 10 min, once a day, for 5 sessions a week. The speed and duration of training were gradually increased to 16 m/min and 50 min/day (including a 5 min warm-up at 10 m/min) by the end of the second week; the intensity and duration were maintained constant throughout the experiment. Endurance exercise protocol continued for 4 weeks. The intensity of exercise is average about 55% of VO_2 max (the maximum volume of oxygen consumption and is the best index for determining the intensity of exercise training.[19,20] Other groups were placed on the device for 5 min once a day at a speed of 5 m/min daily. Moreover, the drug intervention groups, 48 h after induction of myocardial infarction, received daily atorvastatin dissolved in saline (1 mg/ml/) and were then orally gavaged by a dose of 10 mg/kg of body weight.[10] Other groups received similar amount of normal saline. On 48 h after the last training session (28 days after infarction), the rats were anesthetized using chloroform and their hearts were removed under deep anesthesia; they were washed by saline and were placed in formalin as a fixative. After 24 h, the hearts were cut longitudinally and were immersed in molten paraffin. After preparing the paraffin blocks using microtome, 6 μm thick sections were prepared and mounted on the glass slides. To evaluate the cell apoptosis, tunnel staining, and the protein expression of SDF-1α/CXCR4, IHC staining was performed. After staining, the samples were studied using an optical microscope (Olympus AX-70) at 400× magnifica-tion.
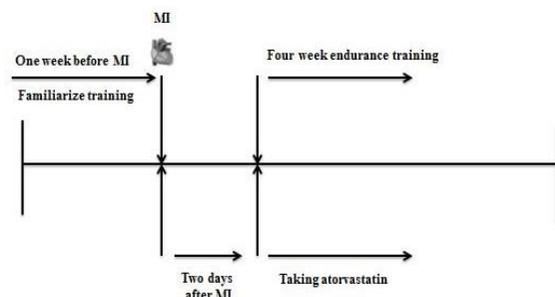

Figure 1. The outline of research

To detect the apoptotic cells, TUNEL assay (terminal deoxy nucleotidyl transferase mediated dUTP nick end labeling) was performed according to manufacturer's instructions (Roche, Germany). In brief, the sections were incubated at 60 °C, washed, and then rehydrated by successive series of alcohol, rinsed in PBS, and treated by 10 mM proteinase K for 30 min at room temperature. Subsequently, to block the endogenous peroxidase activity, the sections were washed and incubated with 3% H2O2 in methanol for 10 min in a dark place. Moreover, TUNEL reaction mixture was added to the sections and incubated for 60 min at 37 °C in a chamber with moderate humidity, followed by rinsing with PBS and visualization was executed using converter-POD for 30 min at 37 °C in a dark place with appropriate humidity. In the next stage, the sections were washed with PBS and 50 μl of DAB substrate (0.05% 3, 3-diaminobenzidine) as a chromogen was added to the sections and incubated for 10 min at room temperature. Eventually, the slides were washed with PBS, placed in hematoxylin for counter stain and were mounted by a cover slip. The visible TUNEL positive cells in dark brown were quantified by light microscope Olympus at 40× magnification. In each slide, five random fields were examined and the apoptotic cells were visually counted in each field. The average of five studied regions was considered as the number of apoptotic cells per slide. All counting procedures were blindly performed.

In summary, the paraffin-embedded sections were moved to the slides after preparation. In the next step, the sections were placed in three containers with xylol for deparaffinization of each sample for 5 min. Furthermore, by descending the concentration of alcohol in series, the samples were rehydrated, washed in Tris buffer (PH 7.4), and antigens were retrieved by heating the sections (autoclave 120 °C) in citrate buffer solution (PH 6). In order to reduce the activity of the endogenous enzymes, the sections were placed in a solution of 10% hydrogen peroxide (H2O2/methanol) for 10 min. Moreover, the sections were incubated with primary antibody (UK, Biorbyt) overnight at 4 °C. Optimal dilution was found to be 1/100 for SDF-1α (1/100) and 1/200 for CXCR4 (1:200). Then the sections were washed in PBS buffer and incubated for 30 min at room temperature with secondary antibody HRP (1/1000), following incubation with 1% DAB solution (3, 3'diaminobenzidin, Sigma, USA) in the dark for 10 min and counter staining with hematoxylin solution. Finally, the sections were dehydrated in ascending alcohol series and were cleared with xylene. Regions of tissue that reveal protein expression of the relevant markers are visible in brown.

To analyze the protein expression of SDF-1α and CXCR4, the images of four randomly selected fields in each section were prepared by light microscope Olympus (400×). In each field, the cells revealing positive protein expression of relevant markers were counted. Moreover, using image analyzer software ImageJ, the ratio of positive cells to the total cells in each image was extracted. Average of four assessed areas was recorded as the percentage of protein expression of relevant marker in each slide.

For data analysis, SPSS version 16 and descriptive and inferential statistics were used. All results are reported as a mean±SD. The Kolmogorov-Smirnov test was used to verify the normality of the distribution. One-way analysis of variance (ANOVA) test was used to compare the differences between the groups. When a significant difference was revealed, the Scheffe's post hoc test was used to specify where the difference occurred. A value of P≤0.05 was considered as statistically significant. All data were analyzed by the SPSS software (SPSS for windows; version 16.00). A value of P≤0.05 was considered as statistically significant. All data were analyzed by the SPSS software (SPSS for windows; version 16.00).

Results

The results indicated that experimental myocardial infarction induces cell apoptotis in the cardiac muscle. The results also revealed that the number of apoptotic cells in the exercise training (16.17±1.47), atorvastatin (16.17±0.75), and exercise+atorvastatin (12±1.41) groups significantly decreased compared to the control group (22±0.63, P<0.001). The difference between the training and atorvastatin groups was not significant in terms the apoptotic cell count; however, the difference between the exercise+atorvastatin group and the atorvastatin and training groups alone is significant (P<0.001; figure 2).

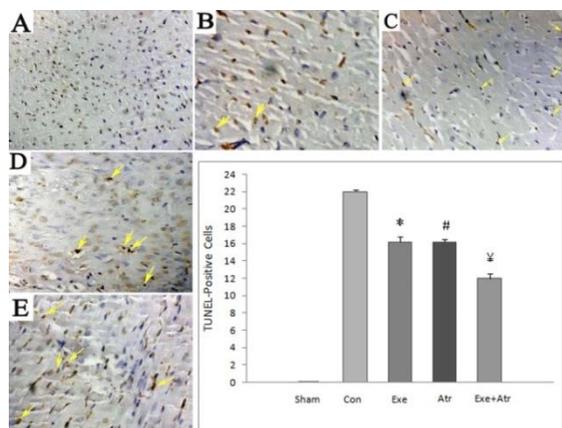Figure 2. The number of TUNEL-positive cells in the study groups.(A) sham, (B) control, (C) exercise, (D) atorvastatin, (E) exercise+atorvastatin. The number of apoptotic cells in exercise training, atorvastatin, and exercise+atorvastatin groups decreased significantly compared to the control group. (Arrows indicate the active TUNEL-positive cells, Magnifications 400×).
* Significantly different compared with control and sham groups (P<0.001).
# Significantly different compared with control and sham groups (P<0.001).
¥ Significantly different compared with sham, control, exercise, and atorvastatin groups (P<0.001).

Moreover, in case of SDF-1α protein expression, the results revealed that the difference between the exercise training

(5.80±0.31), control (5.58±0.28), sham (5.21±0.23), and atorvastatin (5.61±0.09) groups was not statistically significant; however, the difference between the exercise+atorvastatin group (6.52±0.38) compared with other groups was significant (P<0.001; figure 3).
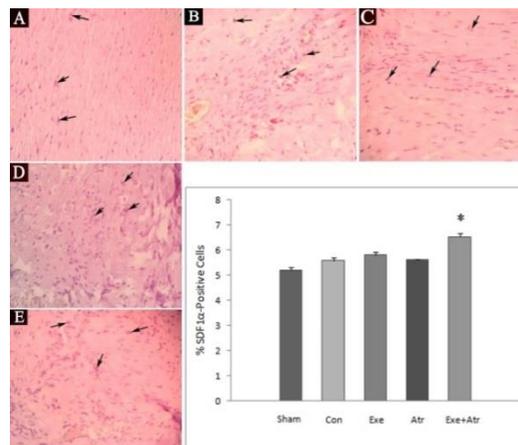

Figure 3. SDF1α-positive cells in the study groups. (A) sham, (B) control, (C) exercise, (D) atorvastatin, (E) exercise +atorvastatin. The combined intervention of endurance training and atorvastatin significantly increased the amount of SDF-1α-protein expression. (Arrows indicate the SDF1α-positive cells, Magnifications 400×).
* Significantly different compared with other groups (P<0.001).

Considering the protein expression of CXCR4, results indicated that the difference between the exercise training (5.47±0.39), control (5.09±0.20), sham (4.92±0.38), and atorvastatin (5.40±0.48) groups was not statistically significant; however, the difference between the exercise+atorvastatin group (6.19±0.41) compared with control and sham groups (P<0.001) as well as with the atorvastatin group (P<0.05) was statistically significant. Difference between exercise+atorvastatin group with training group alone was not statistically significant (figure 4).
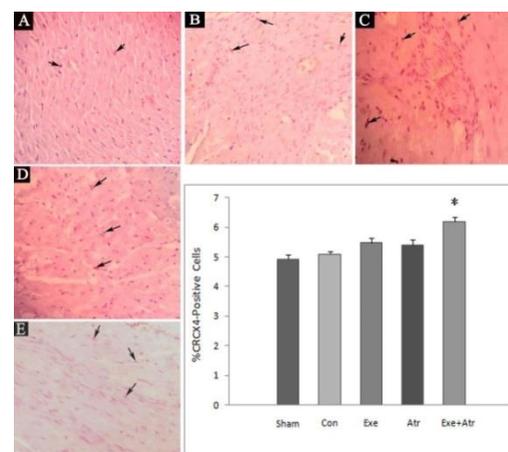

Figure 4. CRCX4-positive cells in the study groups. (A) sham, (B) control, (C) exercise, (D) atorvastatin, (E) exercise+atorvastatin. The combined intervention of endurance training and atorvastatin significantly increased the amount of CRCX4-protein expression. (Arrows indicate the CRCX4-positive cells, Magnifications 400×).
* Significantly different compared with control and sham groups (P<0.001) and atorvastatin group (P<0.05).

Discussion

In this study, the SC injection of isoprenaline (150 mg/kg) significantly increased the number of apoptotic cells compared with the healthy control group (normal saline). Four weeks of endurance training and intake of atorvastatin both alone and in combination, significantly reduced the number of apoptotic cells compared to the control group; however, the combination of exercise and atorvastatin caused a greater reduction in the apoptotic cell count. Moreover, the results of IHC revealed that induced myocardial infarction in rats by isoprenaline, increased the protein expression of SDF-1α/CXCR4 axis in the cardiac tissue of rats 2 days after infarction. Compared with the control group, only the combined intervention of endurance training and atorvastatin significantly increased the amount of SDF-1α/CXCR4 protein expression. This upregulation of SDF-1α/CXCR4 axis is considered as a protective process in response to the tissue damage caused by infarction, and as a compensatory response to restore the cardiac function and prevent the progression of infarction.

The results of the present study suggest beneficial effects of endurance training in reducing the number of apoptotic cells. This has been confirmed in the previous studies.[21-25] The underlying mechanism of cardioprotective effects of physical exercise still remains unclear, and it presumably, the antiapoptotic effects of endurance training, probably due to the positive effect of endurance training on the antioxidant capacity, increased the expression and activity of antiapoptotic factors such as Bcl-2, and inhibited the activity of preapoptotic factors such as Bax and Caspases3 and 9. The exercise increases the production of ROS by increasing the oxygen consumption, whereas in the long term it strengthens the antioxidant defense and reduces oxidative stress.[21-25] Formation of mitochondrial ROS is one of the crucial factors in necrotic and apoptotic cardiac cell death after ischemia reperfusion injury. Several evidences reveal that endurance exercise training increases various components of the tampon system in the heart. For example, it is known that endurance training increased the levels of superoxide dismutase enzymes 1 and 2 (SOD 1, 2) in the intermembrane space and mitochondrial matrix. These enzymes are known as the first line of defense against superoxide radicals in cells, which through mutations in the superoxide radicals, convert them to the nonradical species, hydrogen peroxide (H2O2).[26] These findings suggest that reduced apoptosis in muscles after exercise, was potentially associated with the increasing antioxidant capacity and modulating of oxidative stress levels.[27]

Another possible cardioprotection mechanism of endurance training against apoptotis is the strengthening levels of heat shock proteins (HSPs). Siu et al. (2004) reported that the exercise-induced decrease in apoptosis is linked with increased levels of heat shock protein 70 (HSP-70).[27] Recent findings have reported that regular endurance training protects the heart against ischemia-reperfusion induced apoptosis.[21] It is also reported that exercise training increases the HSP-72 level 3–5 times in the cardiac muscle.[11] Previous studies suggest that HSP-72 mainly prevents apoptosis and increases the antioxidant capacity by protecting the heart against ischemia-reperfusion injury.[11,28]

Another finding of this study is that atorvastatin significantly reduced the apoptotic cell count.

The gradual loss of cardiac cells as a result of apoptosis is one of the most important pathogenic factors in heart failure. Therefore, the reduced loss of heart cells through inhibition of apoptosis has important implications in the treatment of heart failure.

Stress may lead to apoptosis in the endoplasmic reticulum. In the heart tissue, factors such as hypoxia, ischemic/reperfusion injury, hypertrophy, and pressure overload may activate the ER stress.[29] It is reported that atorvastatin exerts possible mechanism in protecting the heart against heart failure via downregulation of ER stress response.[30] Moreover, atorvastatin exert antiapoptotis by inhibiting the synthesis of mevalonate, which is the source of several isoprenoides involved in various intracellular events such as apoptosis and inflammation.[31] Another possible cardioprotection mechanism of atorvastatin against apoptotis is the increased expression of CXC chemokines which are the two N-terminal cysteines of CXC chemokines (or α-chemokines) are separated by one amino acid, represented in this name with an "X", such as SDF-1α and receptor CXCR4. Qiu et al. (2012) reported that the increased expression of SDF-1α/CXCR4 axis, as a result of taking atorvastatin in infarcted rats, confers antiapoptotic effects.[10] This finding is consistent with results of this study. In this study, the protein expression of SDF-1α/CXCR4 axis significantly increased compared to the control group as a result of atorvastatin intake. Thus, it can be claimed that some of the antiapoptotic effects of atorvastatin are presumably caused by the increased protein expression of SDF-1α/CXCR4 axis.

Nevertheless, the combination of exercise and atorvastatin significantly increased the protein expression of SDF-1α/CXCR4 axis. One of the associated mechanisms with boosting effects of combined therapy by exercise and atorvastatin in the expression of SDF-1α/CXCR4 is presumably due to their effects in increased eNOS activity and strengthening of NO production. It is reported that the overexpression of the eNOS/NO system via genetic or pharmaceutical interventions, leads to increased protein and mRNA expression of SDF-1α/CXCR4 axis.[10] Thus, it is likely that, the effects of endurance training and atorvastatin in the upregulation of the eNOS/NO expression aggregate together and lead to additional positive effects on the protein expression of SDF-1α/CXCR4.

In conclusion, the results of this study reported that the combined intervention of endurance training and atorvastatin in case of both apoptosis and protein expression has better efficacy than either drug or training individually. Thus, it is suggested that patients with cardiovascular disease along with medical therapy use the aerobic exercises as a powerful tool in strengthening their heart capacity and function; however, further investigation is warranted.

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Conflict of Interest

The authors declared that they have no conflict of interest.

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