




## Exploring the effects of aerobic exercise combined with chitosan nanoparticle-encapsulated ginger on miRNA-214 and SERCA2a in isoproterenol-induced myocardial infarction in rats

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### Abstract

**Background:** Aerobic exercise and ginger after myocardial infarction (MI) modify calcium handling. Ginger has cardioprotective effects on cardiovascular disease. This study assessed the effects of aerobic exercise combined with ginger extract (GE) loaded into chitosan nanoparticles (CNPs) on miRNA-214, Serca2a, and Anp genes and cardiac fibrosis in myocardial infarction (MI) rat models.

**Methods:** Twenty-five male rats divided into 5 groups were subjected to ginger treatment and exercise. Aerobic exercises (AE) were performed on a rodent treadmill 5 days per week for 6 weeks. The GE-CNPs (500 mg/kg) were orally administered to the rats for 6 weeks. The expressions of miRNA-214, Serca2a, and Anp genes were assessed by real-time polymerase chain reaction (PCR). The histopathological assessments were performed using hematoxylin and eosin (H&E) and Masson's trichrome staining. The serum activities of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) were also measured by ELISA.

**Results:** The MI model and CNP groups had the highest rate of collagen deposition ( $P < 0.05$ ). The serum activities of both CK-MB and LDH were significantly elevated in the Isop group compared to the control ( $P < 0.05$ ), while following aerobic exercise and ginger treatment, their activity was significantly dropped in the Isop + AE + GE-CNPs group. The expression of miRNA-214 showed a significant increase in GE-CNPs ( $P < 0.01$ ) and GE-CNPs + AE ( $P < 0.001$ ) groups. Serca2a and Anp genes showed significant changes in the GE-CNPs + AE group ( $P < 0.05$ ).

**Conclusion:** Our findings revealed that aerobic exercise, along with ginger treatment, improved cardiac fibrosis, modulating the expression levels of miRNA-214, Serca2a, and Anp genes and serum levels of MI biomarkers.

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### Highlights

#### What is current knowledge?

Aerobic exercise combined with chitosan nanoparticle-encapsulated ginger regulated the miRNA-214 and SERCA2a in isoproterenol-induced myocardial infarction in rats.

#### What is new here?

Chitosan nanoparticle-encapsulated ginger enhanced the condition of myocardial infarction.

### Introduction

Cardiovascular disease (CVD) is a common disease worldwide that causes a high mortality rate due to its high prevalence (1). Studies have indicated that the growing population with coronary artery disease in 2008 was about 17 million people, which is expected to reach 23 million in 2030. There are several factors involved in the development of CVD, including high blood pressure, bad lifestyle, poor nutrition, and high stress (2, 3).

Myocardial infarction (MI) is a condition that occurs due to interruption of coronary artery blood flow that inhibits myocardial nutritional needs and oxygen (4). In myocardial infarction, oxidative stress also increases (5). Oxidative stress leads to changes in the structure of phospholipids and proteins, and this action causes lipid peroxidation and thiol group oxidation (5). Oxidative stress may be caused by a defect in the sarcoplasmic reticulum pump  $\text{Ca}^{2+}$ -ATPase (SERCA), thereby inhibiting the movement of calcium from the sarcoplasm to the cytoplasm of cardiomyocytes (6, 7). An increase in  $\text{Ca}^{2+}$  during ischemia converts xanthine dehydrogenase into xanthine oxidase and, consequently, increases  $\text{O}_2$  production (8, 9). Apart from these changes, MI also affects heart hormones such as natriuretic peptides (ANP), which reportedly suppresses the renin-angiotensin-aldosterone system, endothelin synthesis, and sympathetic nerve activity (10, 11). Treatment with atrial natriuretic peptide (ANP) has been shown to result in a relative reduction of approximately 15% in infarct size and a 2.2% absolute increase in left ventricular ejection fraction, so an increase in ANP synthesis in the early stage of MI is still unclear. Various cellular factors are also required to protect the heart cell, including the miRNA family.

MicroRNAs (miRNAs) are a class of unencoded single-stranded ribonucleic acids (RNAs) that are involved in the regulation of almost all major cellular functions, including development, differentiation, cell proliferation, and apoptosis (12). Recent studies have shown that miRNA-21 is involved in ischemic disease and has a protective effect on heart function (13). Overexpression of miRNA-494 has also been reported to protect against ischemia/reperfusion-induced heart damage, whereas miRNA-320 elevation was harmful (14). miRNA-214 was initially investigated due to its role in apoptosis (15). It has also been shown that miRNA-214 protects against  $\text{H}_2\text{O}_2$ -induced myocyte damage (16). However, changes in miRNA-214 with antioxidant boosters such as exercise and the use of nano-antioxidant supplements such as nano ginger are unknown.

In MI, there is a disturbance in the balance of oxidative stress and the level of antioxidants in the body. Therefore, the use of antioxidants can minimize the cellular destruction of the heart. Ginger [*Zingiberaceinale roscoe* (Zingiberaceae)] is one of the most famous spices in the world and has been used throughout history for its health benefits (17). A study on the cardiovascular effects of ginger has reported that ginger extract can regulate the amount of superoxide and hydroxyl anion radicals since it can have antioxidant properties (18). Ginger extract is a potent inhibitor of LDL lipoprotein peroxidation and platelet aggregation induced by arachidonic acid and adenosine diphosphate (19). It has been shown that the use of this supplement can also have beneficial effects on the heart tissue. However, for better effect, this supplement can be used in the nanoform.

Applying the knowledge of encapsulation of drugs and anti-cancer substances has been considered in the treatment of various cancers (20). One of the benefits of encapsulation is that it prevents the drug from inducing damage before reaching the target tissue. Chitosan is widely used in pharmaceutical systems due to its non-toxic properties, biocompatibility, biodegradability, and mucosal adhesion (21). Chitosan forms hydrogel particles from a large scale to nanometers under relatively mild gel formation conditions and can, therefore, be used to confine biologically active compounds.

Hydrogels are widely recognized as crosslinked hydrophilic polymer-based systems that can absorb and retain substantial quantities of water without dissolution (22). Consequently, they are extensively utilized as the predominant form of scaffolds (23). Chitosan nanoparticles have been recognized as viable platforms for delivering cardioprotective medicines in the treatment of myocardial ischemia-reperfusion injury (22). As reported by previous research, chitosan nanoparticles administered intravenously exhibit no discernible accumulation in the intact myocardium, irrespective of the  $\zeta$ -potential. However,

Hwang et al. have provided empirical support for the retention of chitosan hydrogel nanoparticles in the damaged heart in vivo (24).

Research has demonstrated that in a sample of human subjects, engaging in exercise training following myocardial infarction (MI) yields beneficial outcomes regarding left ventricular remodeling (25). Furthermore, it leads to improvements in left ventricular function, injection fraction, and diastolic capacity (26, 27). Furthermore, it has been observed that exercise training in animal specimens decreases the collagen content, enhances intracellular calcium ion (Ca<sup>2+</sup>) handling and calcium sensitivity, and eventually improves the contraction of cardiomyocytes (28, 29).

Regular exercise is an effective factor in controlling heart damage after MI (30). Since regular exercise could increase the antioxidant capacity, it seems that along with the nano ginger supplement, it might have good effects and prove a proper treatment modality for MI. Therefore, in this study, we considered the effects of aerobic exercise with ginger loaded into chitosan nanoparticles on miRNA-214, SERCA2a, atrial natriuretic peptide, and cardiac fibrosis change after isoproterenol-induced myocardial infarction in rats.

## Methods

### Animals and designing the study groups

All animal studies were performed in accordance with the standard ethical instructions for working with laboratory animals approved by the Azad University of Isfahan (Khorasgan, Iran) and after receiving the code of ethics IR.IAU.KHUISF.REC 1399.041. The rats were offered unrestricted access to food and water throughout the study. Thirty male Wistar rats (8 weeks old) were purchased from Pasteur Institute, Tehran, Iran. The rats were acclimated at the animal laboratory of the department, equipped with all required environmental conditions, including suitable temperature ( $22 \pm 2^\circ\text{C}$ ), 30-70% humidity, and regular 12-hour light/dark cycles. During the study phase, the rats were treated according to standard ethical instructions described for working with laboratory animals (Islamic Azad University, Isfahan, Khorasgan, Iran). The rats were randomly divided into 6 groups (n=5): isoproterenol (Isop), isoproterenol + chitosan nanoparticles (Isop + CNPs), isoproterenol + ginger extract loaded into chitosan nanoparticles (Isop + GE- CNPs), isoproterenol + aerobic exercise (Isop + AE), isoproterenol + ginger extract loaded into chitosan nanoparticles + aerobic exercise (Isop + GE- CNPs + AE), and control (normal group). Following the final training session, the rats were euthanized utilizing xylazine and ketamine anesthesia. Subsequently, the samples were meticulously segregated and preserved for subsequent analyses.

### Myocardial infarction-induced models

Isoproterenol was purchased from Sigma-Aldrich (St. Louis, USA). Isop solution at a dose of 85 mg/kg was subcutaneously injected into rats for 2 consecutive days, at a 24-hour interval, to induce the myocardial infarction (MI) rat models (31). After the Isop injection, 5 rats died and were replaced the next day.

### Ginger extract preparation

The rhizomes of the ginger plant were rinsed with water and cut into small pieces. The rhizome pieces were then left in the oven for 24 hours to dry. The dried rhizomes were ground into powder form and dissolved in a hydroalcoholic solution (50% ethanol and 50% water). Thereafter, the resulting solution was subjected to the Soxhlet extraction method for 6 hours and then concentrated using a rotary evaporator at  $45^\circ\text{C}$  for 45-50 min. Finally, the resulting chocolate ginger extract was covered with aluminum foil and stored at  $-20^\circ\text{C}$  until used. In this study, the ginger extract encapsulated using chitosan nanoparticles at a dose of 500 mg/kg body weight was used for the treatment of MI rat models (32).

### Preparation of chitosan nanoparticles

Chitosan nanoparticles (CS-NPs) were fabricated based on the modified ionotropic gelation technique as described by Ibrahim et al., 2017 (33). In brief, chitosan was dissolved in 1% (v/v) acetic acid, and the resulting solution was allowed to stir for 24 hours. The pH was adjusted to 5.5 with 0.01 N NaOH solution. The sodium tripolyphosphate (TPP) aqueous solution was added to the chitosan solution while stirring at room temperature to provide the stability of chitosan nanoparticles. The formed CS-NPs were then precipitated by centrifuging at  $8000 \times g$  and  $4^\circ\text{C}$  for 30 min. To remove biopolymers and excessive chemicals, the separated CS NPs were washed with deionized water several times, lyophilized at  $-60^\circ\text{C}$ , and stored at  $4^\circ\text{C}$ .

### Characterization of ginger-chitosan nanoparticles (GE-CNPs)

The morphological structure of the prepared ginger-chitosan nanoparticles (GE-CNPs) was examined by scanning electron microscopy (SEM, LEO 1430VP, Germany and UK). Moreover, the size distribution and zeta potential of GE-CNPs were determined by dynamic light scattering (DLS) (34) using a Zetasizer Nano ZS (Malvern, UK). To this end, 30  $\mu\text{L}$  of the sample was passed through a 0.2- $\mu\text{m}$  filter. The sample was then transferred into a cuvette and assessed by the Zetaseizer Nano-series instrument.

### Exercise training protocol

Before MI induction, exercise rats were familiarized with exercise training protocols by running on a treadmill at a speed of 5-8 m/s with no grade for 10

min each day for 5 days. Two days after MI induction, the exercise groups were subjected to the main training protocol for 6 weeks (Table 1). As previously described by Azamian Jazi et al. (2017), in the first week, the training groups began treadmill running at a speed of 10 m/min for 10 min each day. The running and time were gradually increased by 1m/min and 10 min every week and finally enhanced to 15m/min and 60 min a day (including 5 min warm-up and 5 min cool-down) at the end of the sixth week (35).

**Table 1.** Exercise training protocol

Week	Frequency (sessions/day)	Exercise speed (m/min)	Exercise intensity (%)	Recovery intensity (%)	Recovery speed (m/min)	Total exercise time (min)
1	1	10	45	25	5	10
2	1	11	50	30	5	15
3	2	12	55	30	6	25
4	2	13	60	35	6	30
5	3	14	65	40	6	35
6	3	15	70	45	7	40

### ELISA Assay

Blood samples were taken from all the groups, and the blood serums were obtained by centrifuging at  $300 \times g$  for 10 min to assess the serum activities of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) in different study groups. The serum creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) levels were detected by ELISA assay using LDH Assay Kit (Sigma Aldrich, Germany) and Creatine Kinase MB ELISA Kit (Sigma Aldrich, Germany). The procedures were performed according to each kit's instructions.

### Quantitative gene expression analyses

For evaluation of the expression levels of miRNA-214, *Serca2a*, and *ANP* genes, the total RNA was isolated from the heart tissues according to the QIAzol lysis reagent protocol (QIAGEN Inc., Valencia, CA), and the cDNAs were synthesized using the cDNA synthesis kit (Thermoscientific, UK). For real-time PCR, miRNA-214, *Serca2a*, and *ANP* primers were first designed by the Gene Runner software v. 6.5.52 (Oberlin, San Diego, CA), and the expression levels of the genes of interest were quantitatively measured by the ABI system. The *Gapdh* gene was used as the housekeeping gene. The PCR thermal program was described as follows: an initial denaturation at  $95^\circ\text{C}$  for 15 min, 40 cycles of 3 steps, including a denaturation step (15 sec at  $95^\circ\text{C}$ ), an annealing step (30 sec at  $60^\circ\text{C}$ ), and an extension step (30 sec at  $72^\circ\text{C}$ ). The relative expression of the genes of interest was calculated by the REST software (versions REST 2009) according to the  $2^{-\Delta\Delta\text{Ct}}$  method. The sequences of primers are shown in Table 2.

**Table 2.** The sequences of primers used for real-time polymerase chain reaction

Genes	Sequences (5'-3')
<i>Serca2-f</i>	AGTGGCTGATGGTGCTGAAA
<i>Serca2-r</i>	GCACCCGAACACCCCTTACAT
<i>Gapdh-f</i>	AAGTTCACGGCACAGTCAAGG
<i>Gapdh-r</i>	CATACTCAGCACCAGCATCACC
U6-f	TGCTTCGGCAGCACATATAC
U6-r	AGGGGCCATGCTAATCTTCT
miR-214-f	ACAGCAGGCACAGACA
miR-214-r	GGCAAAGTGCTTACAGTGC

### Statistical analyses

All experiments were performed in triplicate, and the data were depicted as the Mean  $\pm$  SD. Statistical analyses were performed in SPSS v. 23 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) and Tukey's tests were used to compare the differences among the studied groups. A P-value  $\leq 0.05$  was statistically significant.

## Results

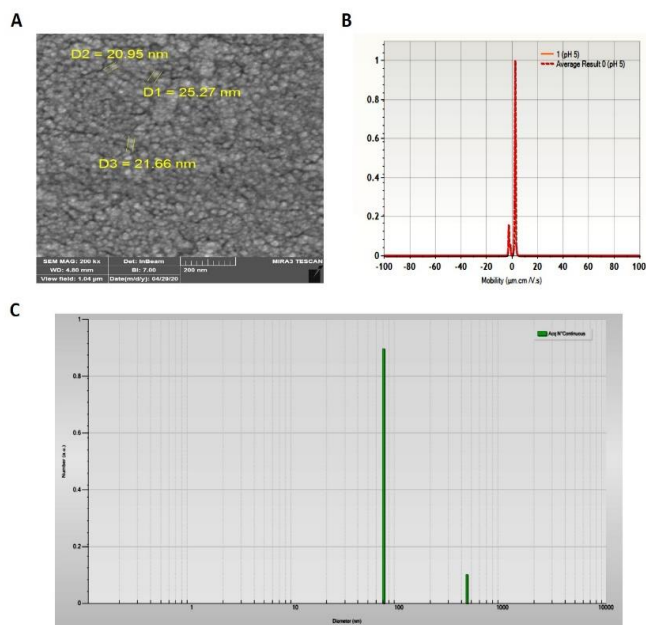
### Size and morphology analyses of the prepared ginger-chitosan nanoparticles

The morphological properties of the prepared ginger-chitosan nanoparticles (GE-CNPs) were examined by SEM. As shown in Figure 1A, the GE-CNPs displayed spheroidal shapes and coherent structures. They were homogeneous in size and shape. Furthermore, the DLS analyses indicated that the mean sizes of the prepared Nano-liposomes were in the range of 20-70 nm.

### Zeta potential assessment

As illustrated in Figure 1B and C, GE-CNPs showed a zeta potential of  $0.2 \pm 0.8$  mV. The closer the zeta potential value to negative, the more likely the GE-CNP's

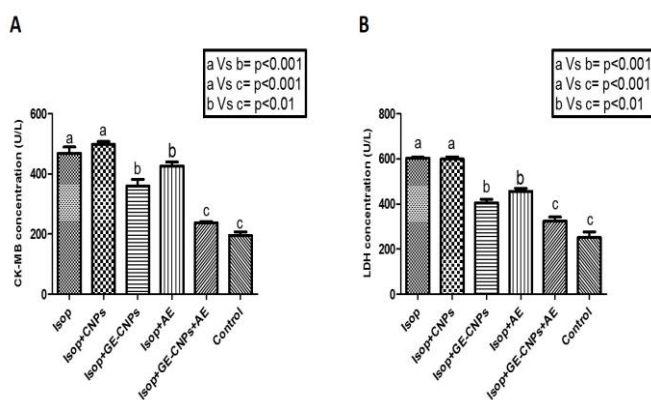
entry to cells. Our result showed that the GE-CNPs could pass the blood vessels in heart attack groups based on particle charge. The pH of the solution was 5 during measurement under precise temperature control (25 °C) in a 3-mm light path cuvette.



**Figure 1.** Characterization of ginger-chitosan nanoparticles (GE-CNPs) by SEM and DLS assay. SEM (A) and DLS (C) results showed that the size distribution in the ginger-chitosan nanoparticles was approximately 25 nm. Zeta potential result (B) showed that the particles could pass the blood vessel in heart attack groups based on particles charge.

#### Activity assessment of serum creatine kinase-MB and lactate dehydrogenase

The serum activities of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) were evaluated using ELIZA assay. The data obtained from all study groups indicated that the serum levels of creatine kinase-MB and lactate dehydrogenase were significantly increased in the Isop group compared to the control (normal) group ( $P < 0.05$ ). After aerobic exercise and treatment with ginger-chitosan nanoparticles or chitosan nanoparticles alone, the serum LDH level was significantly reduced in Isop + AE, Isop + GE-CNPs, and Isop + AE + GE-CNPs groups compared to the Isop group. The serum level of creatine kinase-MB was also significantly decreased in Isop + GE-CNPs and Isop + AE + GE-CNPs groups compared to the Isop group. The lowest activity of both LDH and creatine kinase-MB was observed in the Isop + AE + GE-CNPs group (Figure 2).



**Figure 2.** The effects of AE and GE-CNPs on serum activities of creatine kinase MB fraction (CK-MB) (A) and lactate dehydrogenase (LDH) (B) in different groups of study. All data were shown as Mean  $\pm$  SD. Isop; Isoproterenol, CNPs; Chitosan nanoparticles, AE; Aerobic exercise, GE: ginger.

#### Discussion

Lifestyle modification and regular exercise lead to positive physiological cellular adaptations that are suitable for reducing cardiovascular risk factors (36, 37). Aerobic exercise can reduce fibrosis and improve cardiovascular function by strengthening the heart muscle (38). The use of some antioxidant herbal supplements seems to increase the positive effects of exercise training on cardiomyocytes. Therefore, this study aimed to investigate the effect of aerobic exercise along with an herbal ginger supplement (in the nanoform) on improving cardiac tissue, miRNA-214, SERCA2a, and ANP in male Wistar rats with myocardial infarction.

The results of the present study showed that the Isop and Isop + CNPs (Sham) groups had the highest increase in collagen deposition ( $P < 0.05$ ). However, Isop + GE-CNPs, Isop + AE, and Isop + GE-CNPs + AE groups showed a significant reduction in cardiac tissue damage (collagen deposition). Examination of cellular and hormonal variables also showed that Isop + GE-CNPs and Isop + GE-CNPs + AE groups showed a significant increase in miRNA-214 and ANP gene expression, while SERCA2a mRNA significantly increased only in the combination therapy group (Isop + GE-CNPs + AE). Regarding the effect of ginger on heart tissue, it can be said that the positive effects of ginger on the cardiovascular system have been proven (39, 40). The results of the present study also showed that consumption of ginger, especially in the nano form, improves the structure of heart tissue and reduces collagen deposition. Consistent with the results of the present study, there is evidence that ginger extract may have a positive effect on heart tissue damage (41). Fibrosis and inflammation of the heart cells can be a feature of cardiovascular diseases, such as MI, which decreases the contractile force of the heart muscle. Collagen and fibroblasts change the structure of the heart after fibrosis and cause impaired cardiac function (42). It seems that aerobic exercise with strengthening heart muscles reduces tissue damage caused by MI. In the present study, the greatest reduction in collagen deposition was related to the combination therapy (nano-ginger supplement with aerobic exercise). Consumption of nano-ginger supplement with aerobic exercise seems to be effective in increasing the effects of exercise on heart tissue, including a reduction in cardiac apoptosis and cardiac fibrosis, increased PI3K activity, improvement in calcium in cardiac muscle, enhancement of endothelial function as a result of increased nitric oxide production, increased parasympathetic tone, and dramatic improvements in antioxidant defense (43, 44).

Regarding the increase in miRNA214, it seems that this increase also has positive and cardio-protective effects in groups consuming nano-ginger. Consistent with the results of the present study, Yang et al. (2015) showed that overexpression of miR-214 significantly improved left ventricular (LV) hemodynamic function and LV regeneration in the AMI model rats. The researchers suggested that a possible mediating mechanism of miR-214 was related to the suppression of myocyte apoptosis by suppression of PTEN (45). In the present study, although the changes in apoptosis were not evaluated, it seems that the use of increasing oxidative capacity due to nano-ginger supplementation along with aerobic exercise is effective in inhibiting cardiac cell apoptosis, which needs further investigation because the miRNA 214 might reduce apoptosis which lead to a new treatment strategy.

Calcium changes and calcium ion exchange can also affect cardiac function after MI. Based on the results of the present study, combination therapy (nano-ginger and aerobic exercise) significantly increased SERCA2a levels. However, aerobic exercise alone could not improve post-MI SERCA2a levels. Contrary to the results of the present study, Bo et al. (2018) showed that intense intermittent exercise with increased NRG1 activates the NRG1/SERCA2a signaling pathway, which improves cardiac function after myocardial infarction (46). It seems that the differences in the type (interval vs. continuous) and duration of exercise (6 weeks in the present study vs. 8 weeks in Bo et al.'s research) are among the reasons for the difference in the expression of SERCA2a in the present study and the study of Bo et al. However, taking nano-supplementation along with aerobic exercise caused significant changes in SERCA2a after MI. It seems that taking this nano-supplement can be better adapted to aerobic exercise in less time.

The expression of miR-214 is dysregulated in ischemia injury and heart failure (47). However, its precise involvement in these pathological conditions remains unclear. Given that the sodium/calcium exchanger 1 (NCX) gene has been confirmed as a target for miRNA-214, it has been proposed that dysregulated levels of miRNA-214 might serve as a mechanism for the downregulation of NCX in a pathological state. However, an increase in NCX was identified in the S-INF group, but it was not found to be associated with miRNA-214. One possible explanation for the elevated activity of the sodium-calcium exchanger (NCX) in the sinoatrial node (S-INF) is that, under certain pathological conditions, the NCX might operate in reverse mode, thereby facilitating the influx of calcium ions ( $\text{Ca}^{2+}$ ) into the cell. This influx of  $\text{Ca}^{2+}$  can trigger a phenomenon known as  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum, resulting in an excessive accumulation of  $\text{Ca}^{2+}$  and subsequent impairment of cardiac function (45-47). In contrast, the T-INF group exhibits a suppression of miRNA-214, a molecule that has been linked to the production of NCX in the S-SHAM group. This effect is consistent with the established mechanism by which ET mitigates  $\text{Ca}^{2+}$  excess (28).

Moreover, in the present study, the amount of ANP mRNA in the groups consuming nano-ginger and the combined group of nano-ginger and aerobic exercise showed a significant increase compared to the MI group. Atrial natriuretic peptide (ANP) is a member of the natriuretic peptide family, which exerts its protective functions on the heart not only as a circulating hormone but also as a paracrine hormone. This factor also reduces the size of infarction so incremental changes in this factor in nano-ginger groups and aerobic exercise can indicate the protective effect of this therapeutic modality.

As a limitation, we did not assess the gain and loss of function of miRNA-214 in the condition of myocardial infarction. Moreover, we did not use the antagonism and antagomir of the miRNA-214. In addition, different doses of chitosan nanoparticle-encapsulated ginger were not compared in this study.



Furthermore, the different intensity, duration, and repetition of aerobic exercise training were not evaluated.

## Conclusion

It seems that the use of new technology in medicinal plants, such as nano-ginger, along with exercise training, can have a better protective effect on the cardiovascular system. According to the results of the present study, the simultaneous application of nano-ginger with aerobic exercise in MI model rats resulted in positive regulation of miR-214, SERCA2a, and cardiac ANP, which also improved the structure of the cardiac tissue. However, more studies are required on human samples.

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## Ethical statement

All animal studies were performed in accordance with the standard ethical instructions for working with laboratory animals approved by the Azad University of Isfahan (Khorasgan, Iran) and after receiving the code of ethics (IR.IAU.KHUISEF.REC 1399.041).

## Conflicts of interest

The authors declare that they have no conflict of interest.

## Author contributions

VF carried out the experiments and drafted the manuscript. FT participated in this study as a supervisor and contributed to study design, conceptualization, data analysis and validation, and revising the manuscript. KJD participated in data validation and revising the manuscript. All the authors read and approved the final manuscript.

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