Original Article

Aerobic Exercises Induce Antioxidant Pathways Activation in Rats

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Abstract

Background: Aerobic exercises induce adaptations that improve physiological function. However, aerobic exercises, oxidative reproduction may lead to injury and other health issues such as adverse cardiac effects. The aim of this study is to evaluate the effect of aerobic exercises on protein expression change in the heart left ventricle to determine the advantages and disadvantages related to this mode of exercise. **Methods:** Male Wistar rats were randomized into two groups; trained (T) and control (C). Animals from T group were trained for 8 weeks, and then 2D LC-MS/MS iTRAQ method was used for extracting and analyzing the left ventricular proteins. Certain proteins that were highlighted in the special process were selected for further analysis via protein-protein interaction network (PPI) method. The identified proteins were enriched via gene ontology (GO) to find biological terms. **Results:** We identify five overexpressed antioxidant proteins in T group compared with C group including extracellular superoxide dismutase [Cu-Zn], Frataxin, protein kinase C delta type, STE20/SPS1-related proline-alanine-rich protein kinase, and amyloid-beta A4 protein. **Conclusions:** Findings indicate that catalase and insulin are two exercise-related proteins. However, they were not included in the significant differentially expressed proteins. Finally it was found that enhancement of antioxidative activity is a direct effect of aerobic exercises.

Keywords: *Antioxidants, exercise therapy, heart ventricles, oxidative stress, proteomics*

Introduction

Exercise training has many benefits for health improvement, prevention, and treatment of diseases.[1,2] However, it also produces toxic substances in the body that can cause further damage to the body's organs. One of them is oxidative stress.[3] Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damages. Any kind of disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress from oxidative metabolism causes base damage, as well as a fracture in the DNA strand and disruptions in normal mechanisms of cellular signaling.[4,5] Increased production of ROS or decreased levels of antioxidants leads to variety of pathological conditions including cardiovascular diseases, neurological disorders, lung pathologies, and accelerated aging.[6,7] The exercise is an effective tool that can reduce long-term oxidative stress[8] and causes major changes in the body tissues, and these morphological changes are often associated with physiological and metabolic changes. In fact, these changes are based on molecular and cellular changes. Exercise can change the amount of protein inside the cells by changing their concentration and content, in a way that improves the functional capacity of the muscles and the heart as well as the entire body.[9] Various efforts have been made to provide possible efficacy of exercise activities by identifying the molecular and cellular processes[10] and in some cases, it has been an effort to control and conduct these biological processes by using regulators.[11] Nowadays, methods based on the comprehensive study of biological molecules, including genes, proteins, and metabolites, have attracted the attention of many researchers in different scopes.[12]

Regarding the role and importance of proteins in controlling all biochemical and biophysical activities of living organisms.[13] It seems that proteins exactly know what they do inside and outside the cell, therefore, the use of proteomics is

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important for the study of the biological activity of living organisms.[14] In the expression proteomics, which is a kind of proteomics, the change in the expression of proteins in a given state is examined compared to standard or control mode, and based on the profile, a large set of data is made available to understand molecular mechanisms of exercise activities.[15,16] Moreover, screening methods for biological molecules, including the use of protein-protein interaction (PPI) networks, have led to introduce a limited number of transformed proteins as key proteins in the formation of the studied state.[17,18]

The advantage of using the 2D LC-MS/MS iTRAQ method is that it can analyze complex biological samples and identify a large number of proteins with high precision and it allows us to identify and quantify several proteins even at low concentrations.[19] The relation between exercise and oxidative stress is extremely complicated and depends on the type, intensity, and duration of the exercise. It seems that regular exercises have beneficial effects on oxidative stress and health.[20] Also, the type of exercise activity is one of the main variables that determine the response and body tissue adaptation to exercise.[21,22] In this study, the 2D LC-MS/MS iTRAQ method is used for extracting and analyzing the left ventricular protein of the rats in the training group compared with the control group, attempted to introduce the key proteins that were effective in tissue and metabolism changes and understanding the adjustment of the molecular consistency of the heart muscle.

Methods

Animals

Eight-week-old male Wistar rats were randomized into control (C) and trained (T) groups (n = 10/group). The ventricular proteome of the trained and control groups at the end of eight weeks of experience was compared. They were housed in a temperature-controlled room (22 ± 2°C) with light on from 6 a.m. to 6 p.m. and received commercial rodent chow and filtered water. All procedures were approved by Research Ethics Committee of University of Isfahan (Ethics Identity: IR.UI.REC.1396.042).

Training protocol

Swimming endurance training included 8 weeks and 5 sessions per week in a specific rodent pool (60 cm × 60 cm × 90 cm) and in the water at 31 ± 1°C. Details of protocol are tabulated in Table 1. Loaded weight was applied as 60% of 6% of each rat's weight[23,24] and tied at the beginning part of its tail [Figure 1]. Before starting training protocol, a functional test was performed on the training group. The test was repeated at the end of training procedure at the eight weeks of experience.[25]

Table 1: Swimming endurance training protocol for T group rat. The training was done without loading any mass in the first week

Week of training	1	2	3	4	5	6	7	8
Exercise duration (Minute)	30	30	35	40	45	50	55	60

Sample preparation

Two days after training activity, the rats were anesthetized by 60 mg sodium pentobarbital/kg body weight and the heart tissue rapidly removed by surgery and the left ventricle was cut and quickly transferred to a freezer at -80°C, after weighing and washing with a buffered saline phosphate solution (PBS: phosphate-buffered saline). The samples which were in combination with protease inhibitor were sent to the ADDTEC Laboratory, University of Macquarie, Australia, for Proteome Analysis.

Heart samples sent on dry ice were immediately placed at -80°C. The heart samples (containing 5–6 rat heart pieces) were washed sufficiently with PBS at 4°C. Samples were homogenized with Precellys ceramic beads with additional glass beads. All 10 samples were combined, acidified, and concentrated with SepPak prior to high pH fractionation. Later, 17 fractions were prepared from the fractionation plate. Samples dried, reconstituted in 30 µL, 0.1% formic acid then 10 µL was injected on QExactive.

Data acquisition and processing

Each sample was analyzed by reversed-phase nano-LC-MS/MS. The raw data files were submitted to Proteome Discoverer (version 2.1, Thermo Scientific). The data were processed using Sequest HT against the *Rattus norvegicus* Uniprot database and Mascot (Matrix Science, London, UK) against *Rattus* Swissprot database.

Statistical analysis

Preliminary statistical analysis performed using automated analysis pipeline TMTPrePro showing differentially expressed proteins between the two conditions, and based on ANOVA of protein log ratios, based on fold change >1.5 and P value < 0.05. Statistical analysis for cardiac

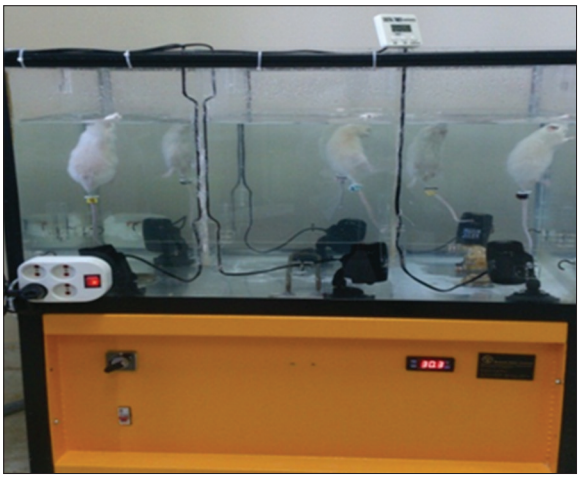

Figure 1: The rats swimming with loaded weight on the tail, inside the rodent pool

hypertrophy was performed using SPSS for Windows software, version 19. Data normality was determined by the Kolmogorov-Smirnov test. An independent *t*-test was used for groups' comparison. $P \leq 0.05$ was considered.

Bioinformatics analysis

The antioxidant proteins were included in PPI network analysis via the STRING database[26] and Cytoscape software.[27] The main connected component was identified as network and was analyzed by network analyzer plugin of Cytoscape.

Action maps including expression, activation, inhibition, and binding actions were provided for elements of the main connected component via CluePedia.[28] The biochemical pathways related to all nodes of the main connected component were identified by clueGO. More information related to the central nodes were obtained via functional analysis in terms of biological process (BP) by ClueGO+CluePedia. This plug-in is provided by Cytoscape and can offer annotations including pathway analysis and gene ontology. Gene ontology (GO) includes molecular function (MF), cellular component (CC), and biological process (BP). GO information for many organisms including *Rattus norvegicus* are available and can be downloaded in the ClueGO app.[29]

Results

Cardiac hypertrophy

Bodyweight was similar between groups at the beginning of the protocol (C = 168.30 ± 7.88 g; T = 173 ± 11.46 g). At the end of the exercise training protocol, T group presented lower body weight as compared with C group (T = 256.80 ± 20.16 vs. C = 302.50 ± 38.86; $P < 0.05$). Because the left ventricle weight of T and C groups were (T = 0.311 ± 0.038 g vs. C = 0.313 ± 0.048 g), respectively; cardiac hypertrophy index was significantly higher in T group (T = 1.218 ± 0.137 mg/g vs. 1.029 ± 0.137 mg/g; $P < 0.05$).

Proteins

The statistical matching of protein samples in the form of a normal curve is presented in Figure 2.

Nearly 2113 proteins have been identified with False Discovery Rate (FDR) ≤0.05. Among the identified protein groups, 2008 were quantified. Five up-regulated proteins that are involved in the antioxidant activity in the T group were selected for more analysis [Figure 3].

The network including the five query proteins and ten neighbors are shown in Figure 4. Except for one of the query proteins, Sod3, App, Fxn, and Stk39 were included in the main connected component. The constructed subnetwork indicates that there are closed relationships between the query proteins, except one of them the others are connected. Action maps of 14 nodes of the main

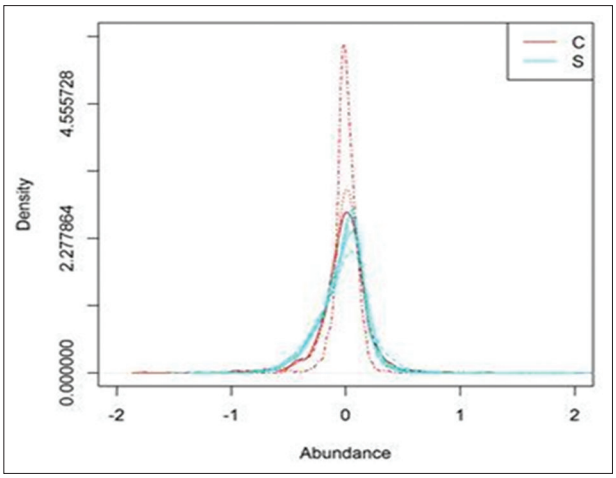

Figure 2: Normal curve of sample densities, the red curve represents the control group (C) and the blue curve, the training group (S)

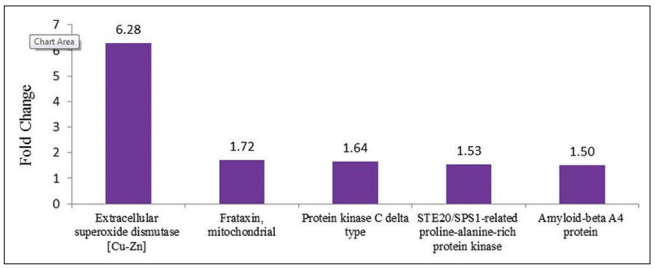

Figure 3: Fold change >1.5 and *P* value <0.05. Five up-regulated proteins in the training group

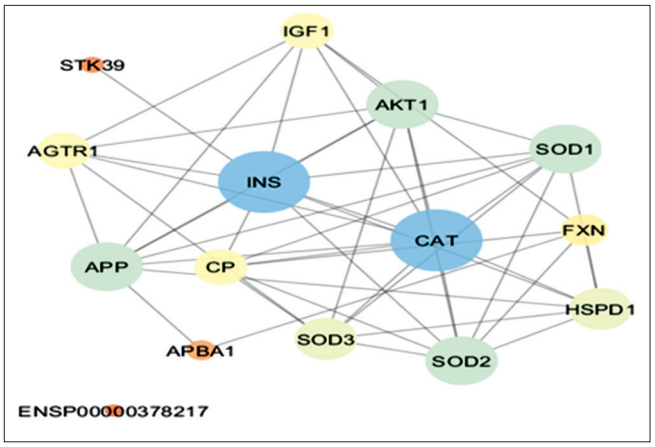

Figure 4: PPI network of 5 query proteins that are involved in the antioxidant activity and 10 added relevant neighbors. The neighbors are obtained from STRING database

connected components including expression, activation, inhibition, and binding actions were created, which is illustrated in Figure 5. In this map the nodes are connected via different types of edges. Insulin as central node has formed more connections with others while APBA1, CP, and HSPD1 are involved in single connection.

The terms are clustered in the eight groups including small groups (as a single term) and a large group contains eight terms. Numbers of 23 biochemical pathways

related to the elements of the main connected component which are clustered in eight groups are presented in Figure 6 and Table 2.

Discussion

There are documents about the balance between produced ROS level and endogenous antioxidants during normal cellular metabolism. This equilibrium is a part of a process that protects tissue damage versus oxidative stress.[6,30] Therefore, imbalance between oxidative reagents and antioxidants is unfavorable condition to maintain right cellular function. This phenomenon can lead to blood pressure increment and heart remodeling.[31] Exercises usually are accompanied by oxidative product increment in the body and tissue. It is reported that oxidative stress is a normal result of exercise activity; by increasing enhancement of hormones levels such as catecholamine, prostanoids metabolites, xanthine oxidase, and NADH oxidase which lead to increased lipid peroxidation process. It has been reported that macrophage activity also increases.[32] Reactive oxygen species such as superoxide ($O_2^{\bullet-}$), peroxides (ROOR), singlet oxygen, peroxynitrite (ONOO-), and hydroxyl radical (•OH) that generate by cellular processes,[33] and levels of redox enzymes which are needed to reduce them are elevated and cause damages due to their high reactivity.[34,35] Several pathways are proposed to decrease toxicity of oxidative products. Aerobic cells convert reactive oxygen species to less reactive products to protect themselves.[36]

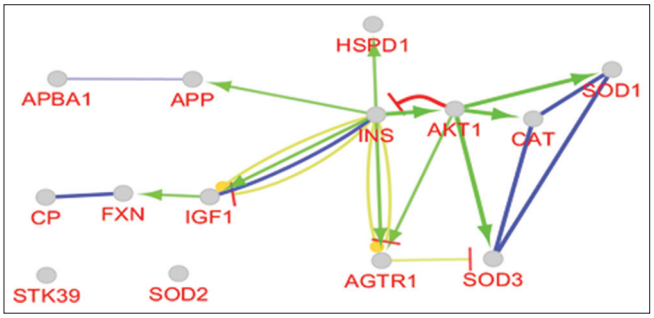

Figure 5: The action map of nodes is illustrated. Yellow, Green, red and blue colored arrow refer to expression, activation, inhibition and binding actions, respectively

In this study, a protective mechanism against oxidative stress, products are investigated in the trained rats.

Since proteomics is a useful method to detect biomarkers and differential express proteins (DEPs), possible DEPs in the trained rats are evaluated as it is shown in Figure 2. Most of the expressed proteins are remind on differentially and expression of DEPs is distributed normally. It can be concluded that maximum value of expression is reduced in the trained group relative control group. At the same time, distribution of expression values of the trained group is expended compared with control samples. Hence, it means that number of differentially expressed proteins increased in the training group. Extracellular superoxide dismutase[Cu-Zn] (SOD3), Frataxin (FXN), protein kinase C delta type (PRKCD), STE20/SPS1-related

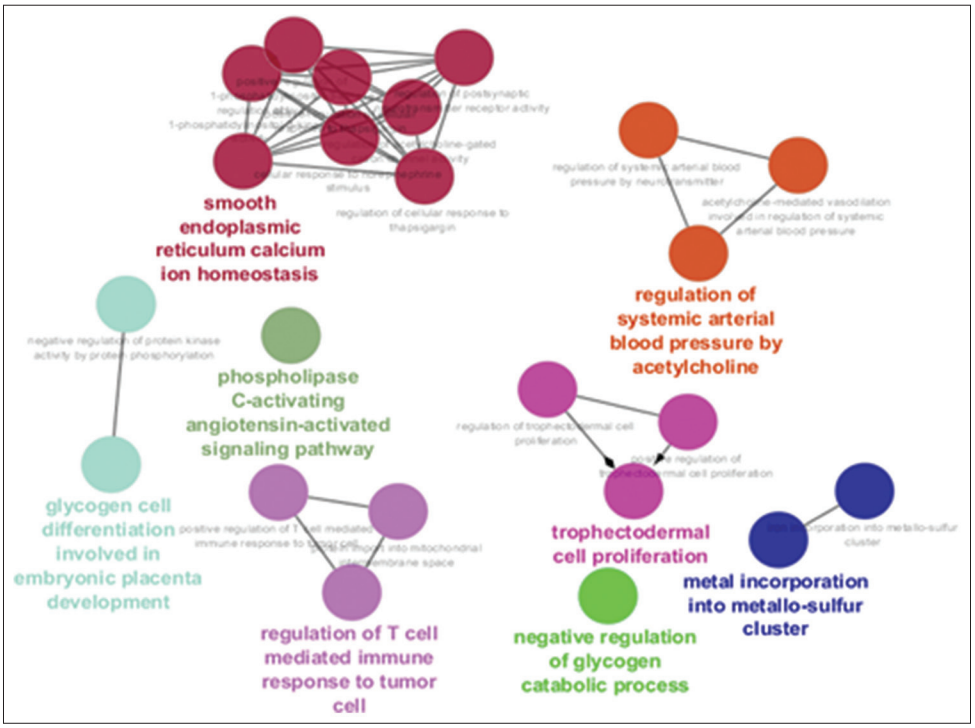

Figure 6: Biochemical pathways relative to nodes of proteins involved in the antioxidant activity are shown. The terms are grouped based on the Kappa score of more than 0.6 except the term that grouped is characteristic with its name the other terms are backgrounded. The pathways are retrieved from GO_Biological Process-EBI-QuickGO GOA_20.11.2017_00h00

Table 2: Biochemical pathways and their associated genes among nodes of proteins involved in the antioxidant activity. The proteins that are involved in the pathway are tabulated are in the last column. The pathways are retrieved from GO_BiologicalProcess-EBI-QuickGO GOA_20.11.2017_00h00 Corrected with Bonferroni step down genes per term

R	GOTerm	Group	Associated Genes Found
1	phospholipase C-activating angiotensin-activated signaling pathway	1	[AGTR1]2
2	negative regulation of glycogen catabolic process	2	[INS]
3	glycogen cell differentiation involved in embryonic placenta development	3	[AKT1]
4	negative regulation of protein kinase activity by protein phosphorylation		[AKT1]
5	metal incorporation into metallo-sulfur cluster	4	[FXN]2
6	iron incorporation into metallo-sulfur cluster		[FXN]2
7	trophectodermal cell proliferation	5	[IGF1]2
8	regulation of trophectodermal cell proliferation		[IGF1]
9	positive regulation of trophectodermal cell proliferation		[IGF1]
10	regulation of systemic arterial blood pressure by the neurotransmitter	6	[SOD2]
11	regulation of systemic arterial blood pressure by acetylcholine		[SOD2]
12	acetylcholine-mediated vasodilation involved in the regulation of systemic arterial blood pressure		[SOD2]
13	regulation of T cell-mediated immune response to tumor cell	7	[HSPD1]2
14	positive regulation of T cell-mediated immune response to tumor cell		[HSPD1]
15	protein import into mitochondrial intermembrane space		[HSPD1]2
16	smooth endoplasmic reticulum calcium ion homeostasis	8	[APP]2
17	regulation of cellular response to thapsigargin		[APP]2
18	positive regulation of cellular response to thapsigargin		[APP]
19	regulation of postsynaptic neurotransmitter receptor activity		[APP]
20	cellular response to norepinephrine stimulus		[APP]2
21	regulation of acetylcholine-gated cation channel activity		[APP]
22	regulation of 1-phosphatidylinositol-3-kinase activity		[APP]
23	positive regulation of 1-phosphatidylinositol-3-kinase activity		[APP]

proline-alanine-rich protein kinase (STK39), and Amyloid-beta A4 protein (APP) were determined as DEPs in the training group. Extracellular superoxide dismutase [Cu-Zn] shows about six-fold change expression alteration. This enzyme which predominantly expresses in heart, represents the first line of defense against ROS, catabolic pathway of activated oxygen species, and free radical detoxification and protects the extracellular space from toxic effect of reactive oxygen intermediates by converting superoxide radicals to hydrogen peroxide and water.[7,37] High values of fold change for the five proteins confirm that all of them are involved in antioxidation activities significantly.[38-54] Except SOD3 which is differentiated from the other DEPs by fold change value, network interaction can discriminate proteins based on centrality parameters in an interactome unit. As shown in Figure 4, protein kinase C delta type is excluded from the constructed network and the others are included in the network counting the four query protein and ten neighbors. As highlighted in Figure 4, insulin and catalase are the two important central nodes in the constructed network. As it is known, catalase has the highest turnover numbers relative to the other enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen per second.[55] Therefore, it acts as a protective agent against oxidative stress products.[56] Insulin is a known key element in sugar metabolism and also the other metabolic processes.[57] The critical regulatory role of insulin is shown in Figure 5. In this figure, insulin plays as an activator that reacts directly to APP and indirectly to FXN and SOD3. Catalase is activated indirectly by insulin.

It can be concluded that insulin is a critical protein that is involved in the maintenance of the internal medium of the trained rats. Biochemical pathway analysis revealed that smooth endoplasmic reticulum calcium ion homeostasis is the main class of pathways that is related to the DEPs. Regulation of systemic arterial blood pressure by acetylcholine is the other pathway group that is concerned with the training group. Glycogen metabolic regulation and immunologic response to tumors cells are the pathways which are highlighted in the train rats. As shown in Table 2, APP is an associated protein in the smooth endoplasmic reticulum calcium ion homeostasis. In this regard, this method is applied to evaluate connections between the five DEPs. Network analysis can provide precise information about investigated samples.

In the athlete, the amount of balance between antioxidants and oxidative stress (as an index) is greater than nonathletes, and regular exercise increases antioxidant activity and improves their performance, making the body more adaptable and resistant to increased production of radicals from exercise.[58] About the role of SOD, numerous studies have concluded that the role of this enzyme is

critical for achieving cardioprotective adaptations from exercise against arrhythmias and myocardial infarction.[59] Adachi *et al.* developed an immunoassay system to measure EC-SOD levels in the serum of subjects. They reported a higher level of serum SOD3 in the people who exercised relative to the controls.[60] This finding is consistent with results of our study. Previous studies indicate that Frataxin plays a role in iron storage and detoxification, stimulation of oxidative phosphorylation, activation of antioxidant defenses, regulation of iron metabolism, tumor suppressor, and protection against proapoptotic stimuli in the body.[53] It is suggested that Frataxin may be directly involved in mitochondrial iron binding and detoxification. It has also been reported that oxidative damages lead to reduction of Frataxin level.[39] Amyloid-beta A4 protein (also known as APP or A4) is involved in copper homeostasis/oxidative stress through copper ion reduction.[44,45] One of the most important mechanisms for copper toxicity is increasing the production of free radicals and oxidative stress.[49] In the presence of transition of metals such as iron or copper; H_2O_2 can give rise to the indiscriminately reactive and toxic hydroxyl radical.[51,52] H_2O_2 has the ability to produce hydroxyl radicals in the presence of iron and copper ions.[50] APP in human and mouse cortical tissue interacted with ferroprotein to facilitate iron transport. Studies show that APP as a functional ferroxidase plays a role in preventing iron-mediated oxidative stress.[46] As discussed antioxidative activity, metabolism regulation, and iron transport are the main processes that are affected in the trained group.

Conclusions

Results show that aerobic exercises change the left ventricle proteome; the main part of changes is characterized by antioxidant activities. In this investigation, protective and compensation effects of exercises are highlighted in the heart tissue. It seems health promotion especially heart health is a consequence of exercise and regular exercise increases antioxidant activity and improves heart function. The role of these antioxidant proteins seems to be critical in achieving exercise-induced cardiac protection adaptations against diseases such as arrhythmias and myocardial infarction.

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Conflicts of interest

There are no conflicts of interest.

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