

Swimming exercise reverses aging-related contractile abnormalities of female heart by improving structural alterations

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Abstract

Background: *The objective of this study was to examine the effect of swimming exercise on aging-related Ca^{2+} handling alterations and structural abnormalities of female rat heart.*

Methods: *For this purpose, 4-month and 24-month old female rats were used and divided into three following groups: sedentary young (SY), sedentary old (SO), and exercised old (Ex-O). Swimming exercise was performed for 8 weeks (60 min/day, 5 days/week). Myocyte shortening, L-type Ca^{2+} currents and associated Ca^{2+} transients were measured from ventricular myocytes at $36 \pm 1^\circ C$. NOX-4 levels, aconitase activity, glutathione measurements and ultrastructural examination by electron microscopy were conducted in heart tissue.*

Results: *Swimming exercise reversed the reduced shortening and slowed kinetics of aged cardiomyocytes. Although the current density was similar for all groups, Ca^{2+} transients were higher in SO and Ex-O myocytes with respect to the SY group. Caffeine-induced Ca^{2+} transients and the integrated NCX current were lower in cardiomyocytes of SY rats compared with other groups, suggesting an increased sarcoplasmic reticulum Ca^{2+} content in an aged heart. Aging led to upregulated cardiac NOX-4 along with declined aconitase activity. Although it did not reverse these oxidative parameters, swimming exercise achieved a significant increase in glutathione levels and improved structural alterations of old rats' hearts.*

Conclusions: *We conclude that swimming exercise upregulates antioxidant defense capacity and improves structural abnormalities of senescent female rat heart, although it does not change Ca^{2+} handling alterations further. Thereby, it improves contractile function of aged myocardium by mitigating detrimental effects of oxidative stress. (Cardiol J 2017; 24, 1: 85–93)*

Key words: aging, swimming exercise, myocardial structure, oxidative stress, female heart

Introduction

Numerous studies in human and animal subjects have shown that aging may cause structural and functional alterations in the myocardium. These age-dependent changes lead to a prominent decline in cardiac function, which may cause elderly people to be more prone to various cardiovascular diseases. The cardiac contraction-

relaxation cycle has been shown to alter with age, most likely due to dysregulation of cytosolic calcium homeostasis [1, 2]. In cardiomyocytes, a typical contraction-relaxation cycle is initiated by opening of L-type Ca^{2+} channel (LTCC) which triggers the opening of the ryanodine receptor (RYR) and gives rise to a global intracellular Ca^{2+} increase. This is then terminated by removal of excess of cytosolic calcium via either the sarco-

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plasmic reticulum (SR) Ca^{2+} pump (SERCA2a) to SR or by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) to the extracellular space. In aging hearts, however, decreased Ca^{2+} removal rate and altered Ca^{2+} handling which precede slowed contraction may eventually result in ventricular dysfunction [1]. Despite this hypothesis, somewhat unchanged [3], depressed [4, 5] or increased [6] results have been reported for cell shortening and Ca^{2+} transient amplitude in animal models of senescent heart. The most likely mechanisms that account for these alterations in cardiac excitation-contraction (E-C) coupling are L-type Ca^{2+} current (I_{CaL}) and altered activity or expression of Ca^{2+} regulatory proteins which may lead to impaired SR function. However, previous studies performed in different species have reported [7, 8] unchanged [9] or increased [10] I_{CaL} densities with age in cardiac myocytes.

Although the exact mechanism by which aging impairs intracellular Ca^{2+} homeostasis and cardiac contractile function has not been fully elucidated, the accumulation of reactive oxygen species (ROS) has been raised as the contributing process [11]. In fact, apart from the leakage from mitochondrial electron transport chain (ETC), ROS can be generated through cellular enzymes including NOX-4, upregulation of which is suggested to inactivate aconitase and thereby play an important role in mitochondrial aging [12, 13].

On the other hand, the effect of chronic exercise on electrical and mechanical activities of heart is still an area of interest. It has been demonstrated that chronic exercise is capable of reversing the aging-related prolongation of relaxation time and Ca^{2+} transient in papillary muscle due to improvement of Ca^{2+} reuptake by cardiac SR [14, 15]. However, the influence of swimming exercise on aging-related changes in Ca^{2+} transients and underlying regulatory mechanisms is still uncertain. Secondly, current findings that address either age-related or exercise-induced changes in myocardial function have mostly been obtained from male subjects, despite the fact that recent studies have shown striking behavioral differences between male and female myocardium [7, 16]. Therefore, the purpose of this study was to determine whether swimming exercise elicits any alteration in ultrastructure and Ca^{2+} handling of senescent female rat myocardium and to delineate the underlying cellular mechanism. In order to examine the potential role of oxidative stress in aging-related changes of female myocardium NOX-4, GSH/GSSG levels and aconitase activity were also studied.

Methods

Animals

In this study, 4- and 24-month-old female Wistar rats were used. They were housed at $23 \pm 2^\circ\text{C}$ and had free access to standard rat chow and drinking water. Rats were assigned to three groups as: sedentary young (SY, $n = 19$), sedentary old (SO, $n = 21$), and exercised old (Ex-O, $n = 20$). The animals in training group were subjected to swimming exercise (60 min/day, 5 days/week for 8 weeks) in a glass chamber (100 × 50 cm diameter and 50 cm depth) filled with tap water and maintained at $33 \pm 2^\circ\text{C}$. For adaptation, duration of the first swimming experience was limited to 10 min and increased by 10 min daily until 60 min was reached. The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University and in accordance with the Declaration of Helsinki and International Association for the Study of Pain (IASP) guidelines.

Cell isolation

The rats were anaesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneal) and their hearts were excised rapidly. The cell isolation was carried out as previously described [17, 18]. Briefly, the aorta was cannulated and perfused retrogradely through the coronary artery with a Ca^{2+} -free solution containing (in mM): 137 NaCl, 5.4 KCl, 1.2 MgSO_4 , 1.2 KH_2PO_4 , 6 HEPES, 20 glucose (pH 7.2) and bubbled with 100% O_2 at 37°C . This was followed by a perfusion of hearts with the same solution containing 0.7–0.8 mg/mL collagenase and 0.07 mg/mL protease. Left ventricles were then removed, minced into small pieces and gently massaged through a nylon mesh. Subsequently, the cell suspension was washed several times and Ca^{2+} was increased in a graded manner for adaptation. All experiments were performed at $36 \pm 1^\circ\text{C}$.

Myocyte shortening

Myocyte contraction was measured by detecting the length of two edges with contractility recording system (IonOptix LLC, Milton USA) during electrical field stimulation (5 V and 1 Hz). The Soft-edge software (IonOptix) was used to capture and analyze the changes in edge length (L/L_0) and results given as fractional shortening.

Voltage clamp recordings

Standard Tyrode solution which contained (in mmol/L) 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.5 CaCl₂, 11.8 HEPES, and 10 glucose (pH adjusted to 7.40 with NaOH) was used as external solution. For measurement of I_{CaL}, pipettes were filled with a solution containing (in mmol/L) 120 Cs-aspartate, 20 KCl, 10 NaCl, 10 HEPES, 5 MgATP, and 0.05 Fura-2 pentapotassium salt (pH = 7.2 with CsOH). During the measurement of I_{CaL}, K⁺ was replaced with Cs⁺ in the external Tyrode solution.

Currents were recorded by patch-clamp amplifier (Axon 200B, Molecular Devices, USA) using the whole-cell configuration mode. Following a pre-pulse from -70 mV to -45 mV for inactivation of Na⁺ currents, 300 ms steps ranging between -50 mV and +60 mV were applied at 0.2 Hz. I_{CaL} was calculated as the difference between the peak current and the end of pulse current. Current densities were normalized to the relevant cell capacitance and presented as pA/pF.

The ratio of the fluorescence emission at 510 nm in response to 340 nm and 380 nm excitation wavelengths was used as an indicator of intracellular Ca²⁺ (IonOptix, Milton, USA). The Ionwizard software was used to capture and analyze the Ca²⁺ transients.

The Ca²⁺ content of the SR were measured during application of 10 mmol/L caffeine via fast perfusion system (VC-6 Fast Perfusion System, Harvard Apparatus, USA).

Histopathological examination by transmission electron microscopy

For electron microscopic examination, left ventricular tissues were fixed in 4% glutaraldehyde, 0.1 M Sorensen phosphate buffer (pH = 7.4) at 4°C for 2 h and then in 1% phosphate-buffered osmium tetroxide for 2 h. The specimens were dehydrated in a graded series of ethanol and embedded in Araldite CY212 epoxy resin (TAAB, England). Semi-thin (1 mm) and ultra-thin sections cut by an ultra-microtome (Leica Ultracut UCT, Austria) were stained with uranyl acetate and lead citrate prior to examination by transmission electron microscope (Zeiss LEO 906E TEM, Oberkochen, Germany).

Assessment of NOX-4 content and aconitase activity

For assessment of the NOX-4 level in heart tissue, an ELISA kit (USCN Life Science Inc., China) was used. Briefly, the tissue homogenate was centrifuged at 5000 × g and the supernatants were collected and assayed immediately. 100 μL

samples were added to the wells and incubated for 2 h at room temperature. Following addition of the reagents reaction was stopped and the absorbance measured by spectrophotometer at wavelength of 450 nm.

Aconitase activity was measured by using the Aconitase Assay Kit (Cayman Chemical Co., USA). Citrate is isomerized by aconitase to isocitrate which is then converted to alpha-ketoglutarate in a reaction catalyzed by isocitric dehydrogenase. These reactions are monitored by measuring the increase in absorbance at 340 nm.

Tissue glutathione and oxidized glutathione levels

Tissue glutathione (GSH) and oxidized glutathione (GSSG) levels were measured by a commercial assay kit (Cayman Chemical Ann Arbor, MI). Briefly, supernatants were deproteinated in 10% metaphosphoric acid (Sigma Aldrich, Steinheim, Switzerland) and the GSSG was reduced to GSH in the provided assay cocktail of the kit. The resultant yellow color of this reaction was measured at an absorbance of 405 nm. Tissue levels of GSSG were determined by first derivation of GSH with 2-vinylpyridine (Sigma Aldrich, Steinheim, Switzerland). The values of GSH and GSSG were calculated from their respective slopes for each sample by using a GSSG or GSH standard curve.

Statistical analysis

Statistical analysis of data was performed by using a one-way ANOVA followed by a post-hoc Bonferroni test for comparison of the relevant groups. Where non-parametric analysis of the groups was more appropriate Kruskal-Wallis test was used. Values smaller than 0.05 (p < 0.05) were accepted as significant. Data are represented as mean ± standard error of mean (SEM).

Results

Morphological parameters

As it can be seen in Figure 1, body weights of aged groups were significantly higher before the training program and these differences were maintained at the termination of swimming protocol. Swimming exercise did not elicit any significant change in body weights of rats when compared with age-matched controls. The apparent cardiac hypertrophy in the old rats was demonstrated by greater heart weight and heart weight/tibia ratio and this difference did not change with swimming exercise.

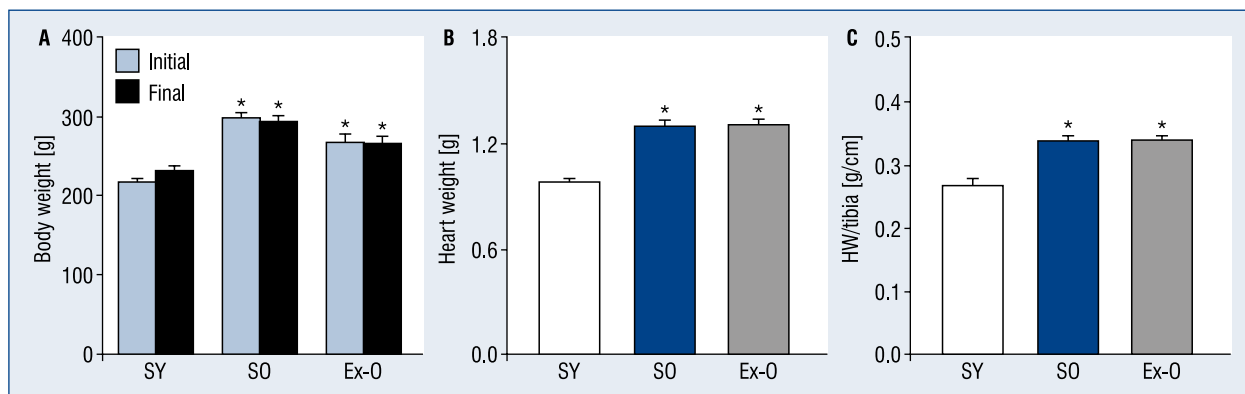


Figure 1. General features of animals studied. Body weight, heart weight (HW), and heart weight/tibia length ratio (HW/tibia) of sedentary young (SY), sedentary old (SO), and exercised old (Ex-O) female rats. Heart weight and HW/tibia increase significantly with age and exercise training. Data are presented as mean ± SEM; *p < 0.05 vs. SY group (n = 19, 21, 20 rats for SY, SO and Ex-O groups, respectively).

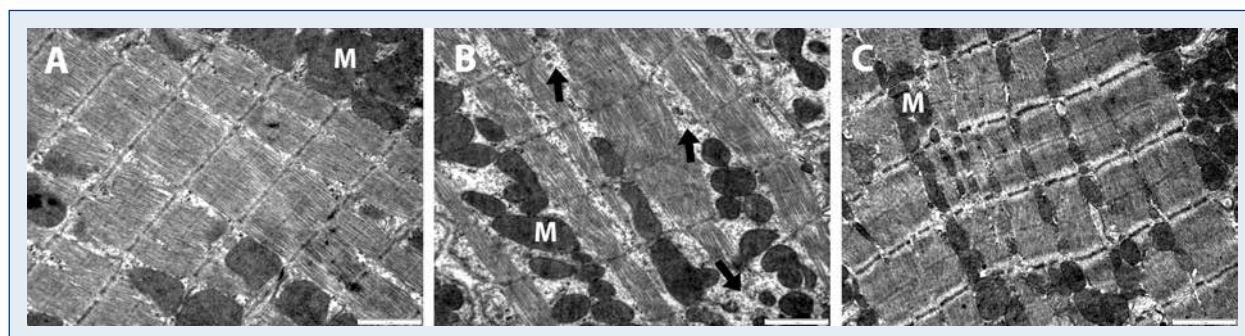


Figure 2. Ultrastructural examinations of hearts by electron microscopy. The micrographs show ultrastructural analysis of myocardium in panels A–C; uranyl acetate/lead citrate stained (scale bar 1 μm) sections from left ventricles of sedentary young (SY), sedentary old (SO), and exercised old (Ex-O) groups. Poorly organized and disrupted myofibrils and thinning of the Z lines were observed in the SO group. Swimming exercise significantly improved these morphological and ultrastructural changes; M — mitochondria; black arrow — myofibrillar spacing.

Effects of aging and exercise on cardiac muscle ultrastructure

Ultrastructural examination by electron microscopy demonstrated that SO group rats show randomly distributed mitochondria between poorly organized and disrupted myofibrils with increased myofibril spacing in an electron-lucent sarcoplasm (Fig. 2B). These abnormalities were significantly reduced in Ex-O rats (Fig. 2C). Sedentary young hearts, however, showed coherent myocardial structure, with myofibrils composed of regular and uninterrupted sarcomeres delimited by Z lines and adjacent myofibrils (Fig. 2A). Swimming exercise induced electron-dense sarcoplasm with prominent rows of mitochondria intervenes between myofibrils in old rats (Fig. 2C).

Contractile functions

Fractional shortening dramatically diminished in aging group myocytes (SO: 4.56 ± 0.45 , n = 35 cells from 9 rats; p < 0.01) when compared with the young group (SY: 8.18 ± 0.53 , n = 35 cells from 9 rats) (Fig. 3A, B). However, exercise training reversed the aging-related contractile deficit (Ex-O: 7.60 ± 0.51 , n = 35 and p < 0.01). Moreover, rates of contraction (+dL/dt) and relaxation (−dL/dt) of the SO group cardiomyocytes were significantly reduced, while swimming exercise restored these impaired kinetics of aged myocytes (Fig. 3C, D). We therefore conclude that exercise training improves contractile parameters of myocardium in aged females.

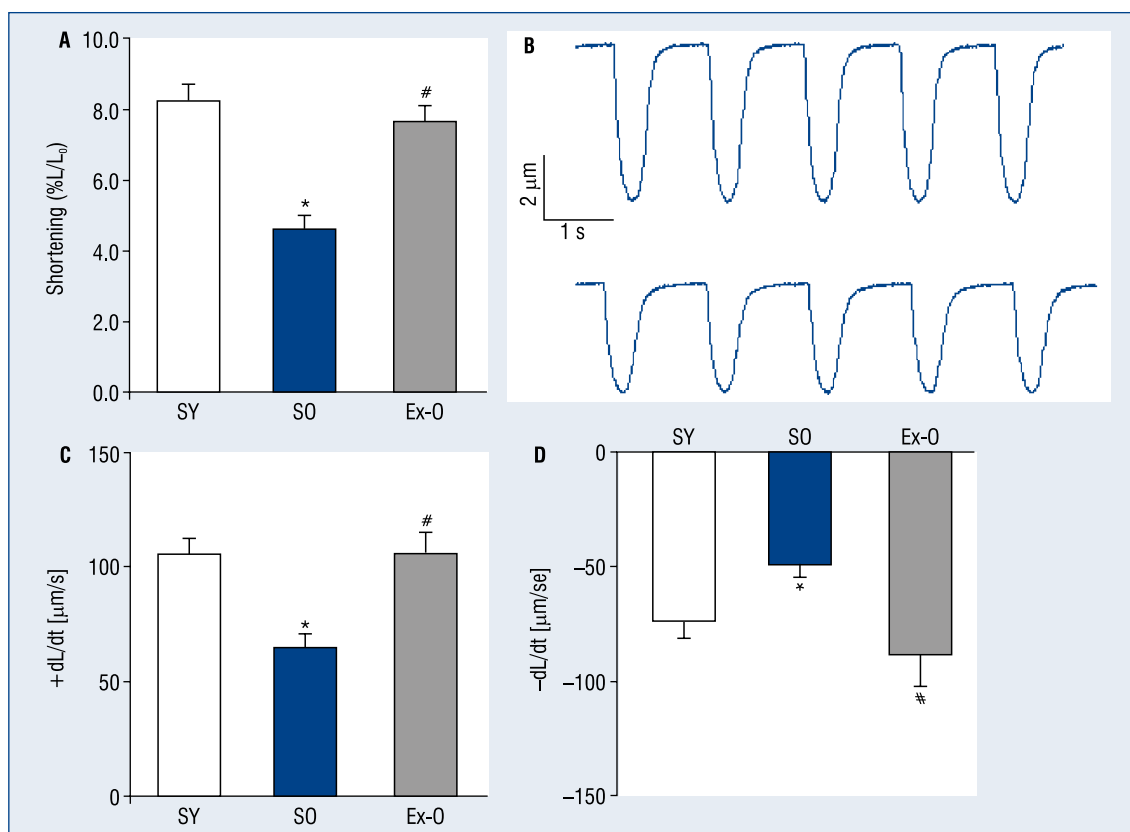


Figure 3. Effect of swimming exercise on aging-related changes in contractile function of myocytes presented as fractional shortening (A), and representative traces (B). Reduced rates of systolic contraction (C), and diastolic relaxation (D) were also improved in Ex-O group ventricular myocytes. Data are presented as mean \pm SEM; * $p < 0.01$ vs. SY group; # $p < 0.01$ vs. SO group (number of cells, $n = 35$ [9 hearts], 35 [7 hearts], 30 [7 hearts] for SY, SO and Ex-O groups, respectively); SY — sedentary young; SO — sedentary old; Ex-O — exercised old.

Effect of aging and exercise on Ca-handling in ventricular myocytes

Ca²⁺ transients elicited in response to Ca²⁺ currents at different potentials were recorded in order to assess the efficiency of LTCC-RyR coupling in left ventricular myocytes. I_{cal} parameters were not significantly different between SY, SO and Ex-O ventricular myocytes (Fig. 4B). However, a significant increase in Ca²⁺-transients was characterized with advanced age both in exercised and non-exercised old groups' myocytes which implicate a higher gain of calcium-induced calcium release in both SO and Ex-O groups with respect to sedentary young myocytes, (Fig. 4A).

SR Ca²⁺ content was estimated by measurement of caffeine-induced Ca²⁺ transient amplitude and calculation of the integral of the caffeine-induced NCX current (I_{NCX}) obtained during 10 mM caffeine application for 10 s (Fig. 5). Aging led to considerable increase in caffeine-induced Ca²⁺-transient amplitude and integrated current

which infers a greater Ca²⁺ content in SR stores of SO group cardiac myocytes. Swimming exercise did not change further the I_{NCX} current and SR content of ventricular myocytes in old rats.

NOX-4 content, aconitase activity, and GSH/GSSG levels

To examine whether oxidative stress parameters change with aging or swimming exercise NOX-4 concentration and aconitase activity were quantified by enzyme immunoassay kit (Fig. 6A–C). Compared to hearts from young female rats, NOX-4 was significantly increased in aged hearts. Exercise training did not change the elevated level of NOX-4 in aged hearts. However, aconitase activity was found to be significantly declined in elder groups and swimming exercise did not induce any remarkable alteration in cardiac aconitase activity of the aged female rats.

On the other hand, GSH/GSSG ratio was up-regulated in hearts from Ex-O rats with respect to SO group. However, there was only a modest and

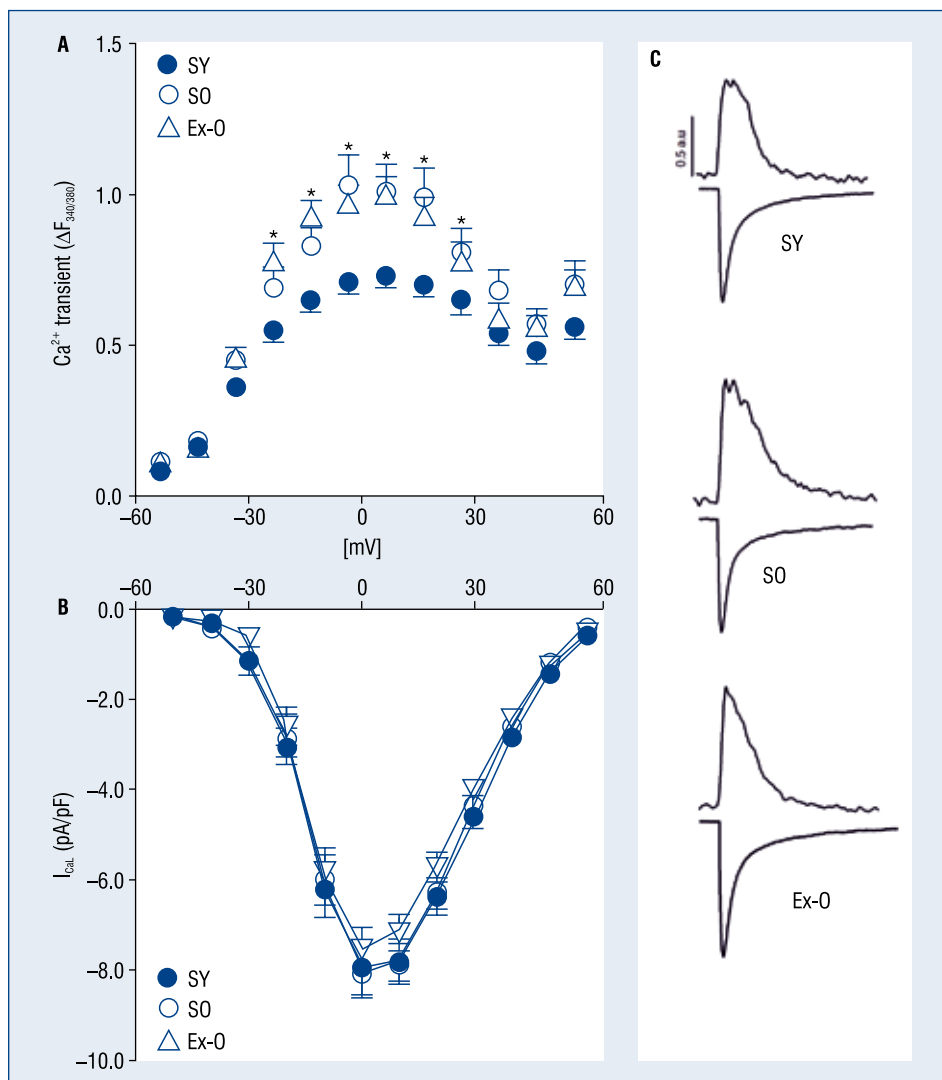


Figure 4. L-type Ca²⁺ current (I_{CaL}) and associated Ca²⁺ transients obtained from ventricular myocytes in response to step pulses between -50 mV and +60 mV; **A.** Average amplitudes of intracellular Ca²⁺ transients; **B.** Peak I_{CaL} densities as a function of membrane potential; **C.** Representative traces of current and associated Ca²⁺ transients induced by a depolarizing step from to 0 mV in sedentary young (SY), sedentary old (SO), and exercised old (Ex-O) group ventricular myocytes. Data are presented as mean ± SEM; *p < 0.05 vs. SY group (number of cells, n = 33 [9 hearts], 27 [11 hearts], 29 [6 hearts] for SY, SO and Ex-O groups, respectively).

non-significant reduction in GSH/GSSG values of SO group with respect to SY group.

Discussion

There are several major findings of this study. First, the contractile function of aged myocytes decreased significantly despite elevated Ca²⁺ transients and SR-Ca²⁺ load. Second, although swimming exercise did not change further the increased values of Ca²⁺ parameters in myocytes of aged rats, it improved cardiac contractile function remarkably. Third, moderate swimming exercise

improved the impaired myofibril fine structure of senescent rats' myocardium. Fourth, exercise training did not change the elevated NOX-4 content and the depressed aconitase activity of aged hearts, while it elicited significant increase in GSH/GSSG ratio. Therefore, the present study demonstrated that swimming exercise does not exhibit significant effects on age-related alterations of intracellular Ca²⁺ handling in left ventricular myocytes of female rats, although it improves structural and contractile abnormalities of aged myocardium.

A growing body of evidence suggests that modification of the cardiac contraction with advanced age is

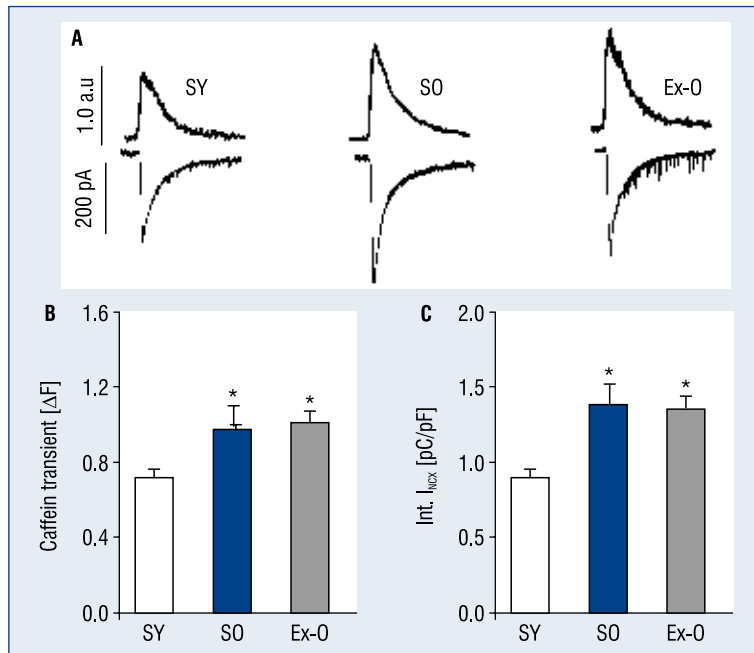


Figure 5. Caffeine-induced Ca^{2+} release and estimation of SR Ca^{2+} content; **A.** Representative raw tracings of Ca^{2+} fluorescent signals and Na^+ - Ca^{2+} exchanger currents (I_{NCX}) in myocytes after application of 10 mM caffeine; **B.** Caffeine-induced Ca^{2+} transient amplitudes; **C.** Integral of the associated NCX current (I_{NCX}) after caffeine application. Data are presented as mean \pm SEM; * $p < 0.05$ vs. SY group (number of cells, $n = 23$ [8 hearts], 18 [9 hearts], 18 [6 hearts] for SY, SO and Ex-O groups, respectively).

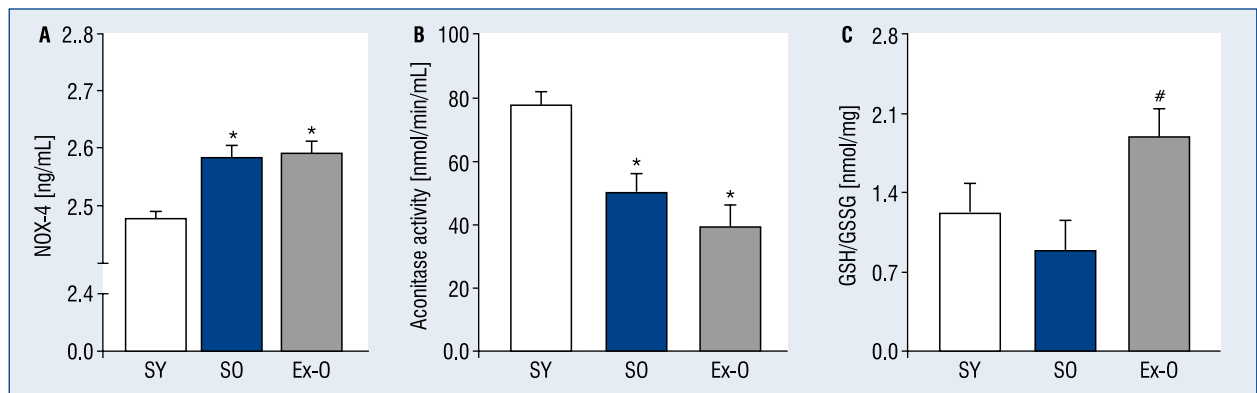


Figure 6. Myocardium NOX-4 content, aconitase activity, and tissue glutathione (GSH) and oxidized glutathione (GSSG) ratio presented as mean \pm SEM; * $p < 0.05$ vs. sedentary young (SY) group; # $p < 0.05$ vs. sedentary old (SO) group ($n = 7$ SY, 6 SO, 7 exercised old hearts).

associated with structural, functional and biochemical changes in the myocardium [1, 19]. Exercise training is suggested in order to protect against age-induced remodeling of the left ventricle that includes myocyte loss, reactive hypertrophy of the remaining cells and increased myocardial fibrosis [20]. Consistent with these findings, we observed a reduction in aging-dependent myofibril loss and impaired fine structure of myocardium in exercised rats.

Ca^{2+} handling alterations due to changes in the expression and/or function of Ca^{2+} regulatory proteins may also contribute to functional abnormalities observed in myocytes from aged hearts [1, 6, 16]. Accordingly, training dependent enhancement in systolic function of the aging heart has been ascribed to the improvement in Ca^{2+} handling of ventricular myocytes [15, 19]. Despite discrepant findings reported for I_{CaL} as

a function of sex or animal species [2, 4, 8, 9, 10] in the present study, neither age nor exercise was shown to alter the peak I_{CaL} of left ventricular myocytes. However, I_{CaL} -triggered Ca^{2+} transients were higher in aged myocytes and swimming exercise did not change them further. Consistent with this, we observed significantly higher SR Ca^{2+} content in cardiomyocytes as a function of age and exercise. Fares and Howlett [1] and Howlett [16] have also demonstrated an increase in SR Ca^{2+} content with aging in female cardiomyocytes, conversely to male rat myocytes. They [1] further demonstrated that contractile function does not deteriorate with age in myocytes both from female rats and mice, and thus suggested a likely mechanism by which cardiac contractile function is preserved in senescent females. Although we have observed similar increase in Ca^{2+} parameters of aged myocytes, the contractile function was reduced significantly. These results implicate that this increase in SR Ca^{2+} content of aged myocytes is a compensatory response against the risk of contractile deficit, although it cannot counteract the effect of aging on systolic function. These data led us to suggest that intracellular Ca^{2+} changes cannot account for either diminishing effect of aging or improving effect of exercise on contractile function. Alternatively, increased myofilament Ca^{2+} -sensitivity due to exercise may be the underlying mechanism of the improved contractility [21].

Reactive oxygen species, accumulation of which has been substantially accentuated with advanced age, can also lead to functional abnormalities [6]. Due to its high metabolic demand, heart is rich in mitochondria, and therefore susceptible to oxidative damage [22]. Apart from the ETC enzymes, NOXs may also actively contribute to ROS production in mitochondria. Of the NOXs currently known, NOX-4 has been suggested to drive oxidative stress in the mitochondria either alone or by stimulating ROS-induced ROS release, thereby exacerbating mitochondrial dysfunction which may result in cardiac dysfunction [13]. Recently, one study showing augmented NOX-4 expression in aged myocytes has gone further to propose a causal link between NOX-derived oxidative stress and decelerated contraction kinetics of cardiac myocytes from aged mice [6]. Consistently, in our study, NOX-4 was significantly increased in aged hearts but exercise training did not change the elevated level of NOX-4.

Furthermore, overexpression of cardiac NOX-4 in mice has been shown to inactivate aconitase [12] which is a mitochondrial enzyme specifically subjected to oxidative attack during aging [23]. Oxidative inactivation of aconitase can initiate

a cascade resulting in dramatic increase in the oxidative damage to macromolecules including proteins [24]. With our study, we further support the idea that age-related accumulation of ROS may causally contribute to inactivation of aconitase and swimming exercise is unable to reverse this effect. However, the upregulation of GSH in exercised rats may protect the macromolecules against oxidative damage and reverses the structural abnormalities of myocardium in aged heart. As a consequence, swimming exercise stimulates the upregulation of antioxidant enzyme activity which counteracts the detrimental effects of ROS in myocardium and thereby improves the contractile function [6].

Conclusions

In summary, swimming exercise reverses the diminished myocyte contractile function despite the fact that it does not change further the altered Ca^{2+} homeostasis in aged cardiomyocytes. The cause of this is that contractile dysfunction of aged myocardium might result from structural abnormalities which are associated with elevated ROS and outweigh compensatory effect of upregulated Ca^{2+} parameters. Thus, it is likely that improved antioxidant capacity due to exercise training restores structural changes of myocardium and preserves contractile function of aged myocytes. On the other hand, increased myofilament Ca^{2+} -sensitivity of exercised rats may also play role in restored contractile activity of aged myocardium. Further studies are required to fully characterize the exact mechanisms of exercise-induced improvements in aged myocardium.

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Conflict of interest: None declared

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