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The effect of swimming exercise on age-dependent electrophysiological changes in female rat myocardium

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Abstract: The effects of swimming exercise on age-associated electrical changes in female rat hearts were investigated. Four- and 24-month-old Wistar female rats were divided into three groups as follows: sedentary young, sedentary old and exercise old. Swimming exercise was performed for 8 weeks (60 min/day, 5 days/week). All recordings were taken from freshly isolated left ventricular myocytes of rat heart. Aging caused a significant increase in the size of myocytes; swimming exercise did not affect this change. The repolarization period of the action potential was prolonged in aged myocytes, but exercise training had no effect on this prolongation. Exercise suppressed the transient outward potassium currents, while the inactivation and reactivation kinetics did not change between the groups. Moreover, aging caused suppression in the inward rectifier potassium currents, and exercise increased this suppression. Consequently, changes in the action potential and potassium currents may contribute to the impaired cardiac function in the elderly female myocardium, and swimming exercise is not an effective strategy in reversing these electrical changes.

Keywords: action potential; aging; exercise; potassium currents

INTRODUCTION

Aging is widely accepted as a primary risk factor for cardiovascular diseases, including coronary heart disease, stroke, peripheral vascular disease and heart failure [1]. It is believed to increase the likelihood of cardiovascular diseases by promoting negative changes in cardiac structure owing to the decrease in cardioprotective molecular mechanisms [2,3]. However, aging does not only directly change the structure of the heart, it also disrupts excitation-contraction coupling (ECC), Ca^{2+} homeostasis and contractile structure regulation [4,5]. The pathologic myocardial alterations seen during the aging process, such as left ventricular wall thickening, increase in the amount of fibrotic tissue, and cellular hypertrophy can functionally affect the contraction dynamics of the heart and thereby reduce the fractional shortening and lower cardiac output [6].

Abnormal ventricular functions and decreased reserve capacity in aging heart are associated with electrophysiological changes, including prolonged atrioventricular conduction, altered morphology of the action potential (AP) and decreased heart rate. The regulation of cardiac excitation and the contraction process largely depends on the shape of the AP in the myocytes. The depolarization and repolarization of AP occur through inward and outward currents that pass through specific ion channels. The activity of the K^+ channels (transient outward K^+ currents [I_{to}] and inward rectifier K^+ currents [I_{K1}]) is the main determinant of the AP duration (APD) and thus, the time course of the Ca^{2+}-mediated contraction, as it limits the repolarization and refractory periods [7]. In addition, the repolarization process, which ultimately returns the cell membrane to its resting potential, is mainly driven by the outward current through voltage-gated K^+ channels, and I_{K1} currents play an important role in stabilizing this resting level of the membrane potential [8]. The AP duration prolongation that has been demonstrated by numerous studies does not only alter myocardial cell excitability, but also increases the likelihood of arrhythmias [9-11]. Studies have suggested that this change in the AP is mainly caused by the reduction of I_{to} and by the prolonged recovery time from L-type Ca^{+2} channel inactivation [11,12]. On the

other hand, in some studies, the I_{to} and sustained (I_{sus}) current amplitudes in atrial myocytes were observed to increase during aging, and it was suggested that this may trigger atrial fibrillation [13,14].

Studies have shown that the changes in ECC in cardiac ventricular myocytes are sex-dependent, but these studies were mostly conducted on young subjects. It was found that in myocytes isolated from young female rats, contractions and Ca^{2+} transients were smaller, and their kinetics were slower than in young male myocytes [15,16]. However, the sarcoplasmic reticulum (SR) Ca^{2+} load was similar in female and male rats, and the Na^{+}/Ca^{2+}-exchange current did not vary depending on gender [17,18]. These results obtained in young adult cardiac myocytes indicate that ECC changes depend on gender. However, gender-dependent alterations in the electrical properties of elderly hearts have not been demonstrated unequivocally. In a study of elderly mice, in contrast to the results obtained in male myocytes, it was suggested that Ca^{2+} currents did not change in female myocytes, but the SR Ca^{2+} content increased, thereby maintaining SR Ca^{2+} release and fractional shortening. Moreover, it was suggested that neither the amplitude nor the voltage-dependent inactivation and time-dependent reactivation of I_{to} currents change, but that the channel density decreased in the older male myocytes [11]. In previous studies, mostly parameters related to the role of Ca^{2+} on age-dependent electrophysiological changes were examined, but the changes occurring in K^{+} currents, the major component of the AP responsible for the initiation and maintenance of ECC, remained unexplained [19-21].

Regular exercise has been recognized as an effective method to improve heart function and reduce cardiovascular disease and mortality. Different animal models and human epidemiological studies suggest that exercise can have significant cardioprotective effects [22-24]. These protective mechanisms can be listed as maximum oxygen consumption, improvements in cardiorespiratory capacity, lipid profile and endothelial functions, as well as increases in mitochondrial antioxidant capacity and the number of capillaries [25]. Moreover, a series of adaptations, such as enlargement of the ventricular chamber and an increase in heart muscle that resulted in an improved stroke volume, have been demonstrated in exercised hearts [26,27]. Depending on age, chronic exercise has been shown to reverse the prolongation in the relaxation of Ca^{2+} transients in papillary muscle by correcting Ca^{2+} uptake into the SR [28,29].

It is noteworthy that although the electrical properties of the heart vary depending on gender, studies related to aging that are mostly performed on male subjects, including the changes in K^{+} currents that are the prominent ionic components of the AP, have not been determined in myocytes of elderly females. Therefore, we investigated the AP-related ionic changes occurring in myocytes of elderly females and the effects of exercise on these changes.

MATERIALS AND METHODS

Preparation of animals

In this study, 4- and 24-month-old female Wistar rats were used. They were housed at 23±2°C and had free access to standard rat chow and drinking water. The rats were randomly assigned and age-matched to three different groups: sedentary young (SY, n=8), sedentary old (SO, n=8), and exercised old (Ex-O, n=7). In this study, swimming was preferred as the exercise model because it offers some advantages over treadmill exercises. Swimming is a natural ability of rats and eliminates the need to choose animals that comply with the protocol, as happened on the treadmill [30]. In addition, it eliminates the possibility of physical trauma in animals. Another advantage is that swimming provides uniform physical activity due to the use of ankle and flexor muscles [31]. Swimming for 8 weeks (60 min/day, 5 days/week), as we chose in this study, has been defined as a moderate-intensity exercise [32] (See Supplementary Fig. S1). The animals in the training groups were subjected to swimming exercise in a 100×50 cm glass tank with a 50-cm depth filled with tap water and maintained at 33±2°C. For the adaptation of the rats, the duration of the first swimming exercise was limited to 10 min and increased by 10 min daily until 60 min was reached [33]. The experimental protocol was approved by the Animal Care and Usage Committee of the Akdeniz University and was in accordance with the Declaration of Helsinki and International Association for the Study of Pain guidelines.

Cell isolation

At the termination of the training period, the rats were anesthetized with pentobarbital sodium (50 mg/kg) and the hearts were rapidly excised. Cell isolation procedure was performed as previously described [34,35]. Briefly, the aorta was cannulated on the Langendorff apparatus and retrogradely perfused through the coronary artery with a Ca^{2+}-free solution containing 137 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 6 mM HEPES, 20 mM glucose (pH=7.2) and bubbled with 100% O_2 at 37°C. This was followed by perfusion of the hearts with the same solution containing 0.7-0.8 mg/mL of collagenase (Collagenase A, Roche, Switzerland) and 0.07 mg/mL of protease (Sigma type XIV). The 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±1°C.

Recording of the action potentials

All recordings were obtained at 1 Hz frequency using 2-2.5-MΩ resistance electrodes. Intra-pipette solution content was precisely prepared as follows: 120 mM K-aspartate, 20 mM KCl, 6.8 mM $MgCl_2$, 5 mM Na_2ATP, 0.4 mM Na_2GTP, 10 mM EGTA, 4.7 mM $CaCl_2$ and 20 mM HEPES (pH=7.4). Potential changes of the membrane were recorded in the current clamp configuration of amplifier by stimulating the cell by depolarizing pulses. AP traces were analyzed, and 25, 50, 75, and 90% ($APD_{25,\ 50,\ 75,\ 90}$, respectively) of the repolarization were determined.

Recording of potassium currents

Potassium currents were recorded using the voltage clamping mode of the patch-clamp amplifier (Axon 200B, Molecular Devices, USA) at the Digidata 1200 sampling rate at 5 kHz. The myocytes were evoked from a holding potential of -70 mV by 3 s test pulses between -60 mV and +60 mV. Depolarizing potentials were preceded by a step to -40 mV to inactivate the Na^+ currents [36]. Current densities were calculated, and the results were presented as pA/pF.

The extracellular medium was provided by applying the solutions directly over the cell by a fast perfusion system (VC-6 Warner Instruments LLC, Hamden, USA). The extracellular solution contained 137 mM NaCl; 5.4 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose and 11.8 mM HEPES (pH=7.35), whereas the pipette solution included 120 mM K-aspartate, 20 mM KCl, 10 mM NaCl, 5 mM MgATP and 10 mM K-HEPES (pH=7.2). To block the Ca^{2+} currents, 250 μM $CdCl_2$ were added to the perfusion solution. I_{to} was calculated by subtracting the current values in the last part of a 3-s pulse (I_{ss}) from the peak values of the total currents. The peak values measured for the currents were divided into cell capacitance and defined as current density to avoid deviations caused by cell size.

Next, the decay phase of the current at +60 mV was fitted to exponential function to identify the underlying components (I_{fast}, I_{slow}, and $_{Iss}$) of the K^+ current [36,37]. At the end of this process, the A_{fast}, A_{slow} and A_{ss} amplitudes of the I_{fast}, I_{slow},and I_{ss} current components were calculated, and the time constants τ_1 and τ_2 of the decay phase were determined.

The inactivation kinetics were determined by applying 400 ms conditioning step pulses ranging from -60 to +60 mV in 10 mV steps, followed by a 3-s test pulse to +60 mV where the holding potential was -70 mV. Subsequently, the current amplitude obtained with the test pulse was plotted as a function of the voltage command of the conditioning pulses and fitted to the Boltzmann equation ($I/Imax=[1+exp(V_{1/2}-V_m/k]^{-1}$).

The reactivation kinetics was attained by applying a protocol consisting of a 500-ms pre-pulse from -70 to -50 with a 400-ms conditioning pulse to +60 mV, followed by a repetitive test pulse with a 3-ms delay to +60 mV. The reactivation curves were analyzed by the estimation peak current of the test pulse with respect to the conditional peak current and fitted to the following equation: ($I/I_{max}=[1-exp(-t/\tau)]$).

The current-voltage (I-V) relationship of I_{K1} was determined by using a protocol of 1-s voltage steps in 10-mV increments ranging between -120 mV and +10 mV from a holding potential of -70 mV. The last 200-ms part of each current trace was measured as I_{K1}.

Statistical analysis

All results were subjected to a normality test by the Shapiro-Wilk test. For comparison of non-normally

distributed variables, a Kruskal-Wallis test was used, and one-way ANOVA was used for normally distributed variables. For comparison, $P<0.05$ were considered significant. Data are represented as mean±standard error of mean (SEM).

RESULTS

Physiological properties

The body weight of the young group was significantly lower than those of the elderly groups before the start of the swimming protocol (SY: 216.35±4.89 g, n=8 vs. SO: 297.87±7.23 g, n=8). At the end of the experimental period, there were no significant differences between the body weights of the matched groups (SY: 230.25±7.31 g, n=8 vs SO: 293.60±7.82 g, n=8 vs Ex-O: 265.25±9.48 g, n=7). As shown in Fig. 1A, a significant increase in myocyte capacitance, which indicated hypertrophy, was observed in elderly myocytes (SY: 142.62±10.81 pF vs SO: 200.03±11.09 pF, $P<0.01$, and n=20, 25 respectively). Swimming exercise did not elicit any significant change in cell capacitance in rats when compared with the age-matched controls. The heart weight/body weight (HW/BW) ratio did not change in the SO group, but significantly increased in the Ex-O group when compared with the SO group (Fig. 1B). These results show that exercise further increases cardiac hypertrophy in elderly rats.

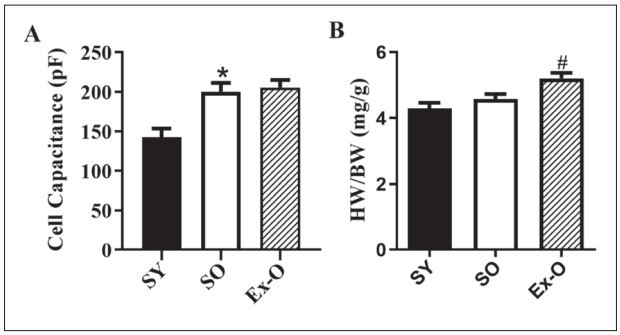

Fig. 1. Physiological parameters. **A –** Cell capacitance (pF); **B –** Heart weight/body weight (HW/BW). Data are presented as the mean±SEM; *$P<0.05$ vs SY and #$P<0.05$ vs SO (n=8, 8, 7 rats for the SY, SO and Ex-O groups, respectively); SY – sedentary young, SO – sedentary old; Ex-O – exercised old.

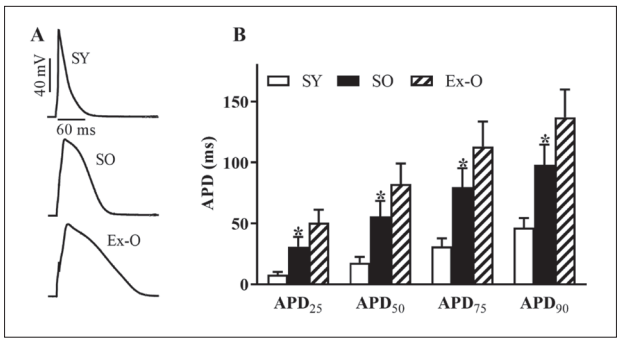

Fig. 2. A – Current clamp recordings from cardiac myocyte cells demonstrated action potential duration in all groups. **B –** Action potential duration (APD) at 25%, 50%, 75%, 90% of the repolarization phase. Data are presented as the mean±SEM; *$P<0.05$ vs the SY group (the number of cells, n=18 (5 hearts), 21 (8 hearts), 16 (5 hearts) for the SY, SO and Ex-O groups, respectively); SY – sedentary young, SO – sedentary old; Ex-O – exercised old.

Action potentials

The four phases of repolarization were evaluated for the duration of AP analysis. These phases were selected to reach 25, 50, 75 and 90% of the repolarization phase from the peak of the AP, and they are represented as APD_{25}, APD_{50}, APD_{75} and APD_{90}, respectively. Sample recordings of the APs are provided in Fig. 2A. Compared with young myocytes, the APD was significantly prolonged in elderly myocytes (APD_{25}; SY: 7.99±2.26 ms vs SO: 30.96±8.05 ms, $P<0.01$, APD_{50}; SY:17.84±4.73 ms vs SO: 55.71±12.91 ms, $P<0.01$, APD_{75}; SY:31.10±6.62 ms vs SO: 79.74±15.57 ms, $P<0.01$, APD_{90}; SY:46.74±7.67 ms vs SO: 98.11±16.59, $P<0.01$ and n=18, 21 respectively). On the other hand, the APD in the Ex-O group was longer than in the SO group, but this increase did not reach a significant level (Ex-O-APD_{25}: 50.65±10.61 ms, $P<0.237$, Ex-O-APD_{50}: 82.42±16.80 ms, $P<0.829$, Ex-O-APD_{75}: 113.15±20.44 ms, $P<0.76$, Ex-O-APD_{90}: 137.25±22.64 ms, $P<0.299$, n=16; Fig. 2B)

Potassium currents (I_{to}, I_{sus} and I_{K1})

Potassium currents are leading ionic currents that play a pivotal role in AP repolarization phase formation. Therefore, in our study, all major potassium currents (I_{to}, I_{sus} and I_{K1}) were measured. Sample current recordings and the average current densities of the groups according to the membrane potential are presented in Fig. 3A, B. The transient outward potassium currents were significantly reduced in the SO group

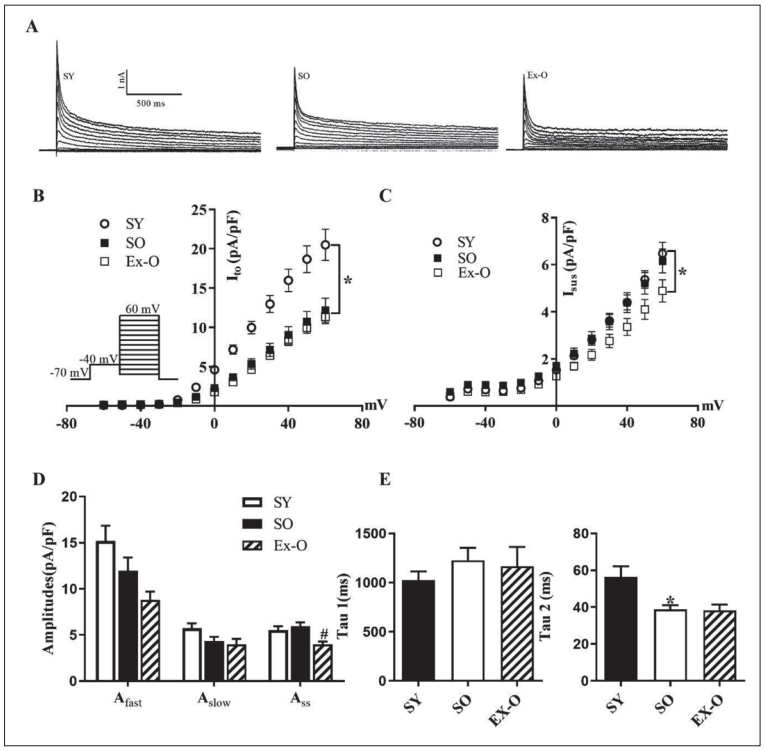

Fig. 3. Repolarizing K^+ currents of experimental groups recorded by 3s duration test pulses between -60 mV and +60 mV following a 200 ms pre-pulse to inactivate Na^+ currents. Current densities were plotted as a function of voltage. **A –** Original traces of SY, SO, Ex-Y and Ex-O; **B –** Amplitude of Ito was calculated as the difference between peak and the last part of the current which was defined as **C** – I_{sus}; **D** – The decay phase of the current trace obtained at +60 mV was fitted with two exponentials and a non-inactivating component. The average data of amplitudes of the fast, the slow, and the steady-state current; A_{fast}, A_{slow}, A_{ss}, **E** – The time constants. Data are given as the mean±SEM; **P*<0.05 vs the SY group (the number of cells, n=20 (5 hearts), 25 (5 hearts), 24 (5 hearts) for the SY, SO and Ex-O groups, respectively); SY – sedentary young, SO – sedentary old; Ex-O – exercised old.

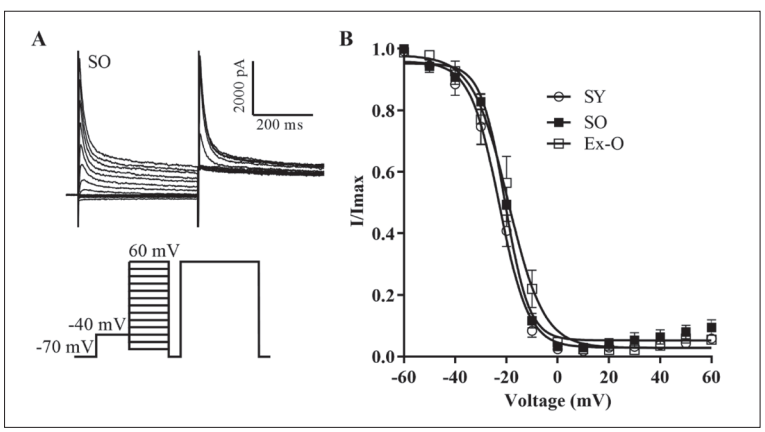

Fig. 4. Effects of aging on the steady-state inactivation of Ito. **A –** Original trace of SO; **B –** Steady-state inactivation of Ito in SY, SO and Ex-O ventricular myocytes were fitted using the Boltzmann equation. Boltzmann distributions, ($I/Imax=[1+exp(V_{1/2}-V_m)/k]^{-1}$), where I/I_{max} is the ratio of the current to the maximum current, $V_{1/2}$ is the half-maximal potentials of inactivation, k is the slope factor. Data are given as the mean±SEM; **P*<0.05 vs the SY group; the number of cells, n=20 (5 hearts), 18 (5 hearts), 18 (5 hearts) for the SY, SO and Ex-O groups, respectively; SY – sedentary young, SO – sedentary old; Ex-O – exercised old.

(+50 mV I_{to}; SY: 18.68±1.72 pA/pF vs SO: 10.74±1.30 pA/pF, p<0.01 and n=20 and 25, respectively) and in Ex-O group myocytes (Ex-O: 9.95±0.73 pA/pF, n=24). There was no significant reduction in the I_{sus} of the SO group, whereas swimming exercise caused a significant decrease in these currents (Fig. 3C). A similar change was observed in the I-V curves when the fast, slow and steady-state components of the potassium currents were examined. The amplitude of the rapid component of the current decreased with aging and this reduction increased with exercise. While no difference was observed between the groups in the slow component, the suppressive effect of exercise was observed in the steady-state component (Fig. 3D). Furthermore, the time constant of the rapid component of the current was accelerated by aging and exercise was not effective in this change (Fig. 3E). This decrease in the A_{fast} and A_{slow} amplitudes of the I_{fast} and I_{slow} current components confirmed that one of the main mechanisms underlying the prolongation of APD is the potassium current and that swimming exercise does not have a corrective effect on these changes.

The steady-state inactivation kinetics of I_{to} were compared between SY and elderly myocytes. The data were fitted using the Boltzmann distribution function with a least squares method, where I/I_{max} is the relative amplitude of I_{to}, V_m is the conditioning voltage, $V_{0.5}$ is the half-inactivation voltage and k is the slope factor. Neither the half-activation potential $V_{0.5}$ nor the slope factor k of the inactivation were significantly altered between the groups (Fig. 4).

The time course of recovery from the I_{to} inactivation was also compared between the control myocytes and the elderly myocytes by use of a double-pulse method. Double pulses to +60 mV from the holding potential of -70 mV (each pulse duration was 400 ms) were applied

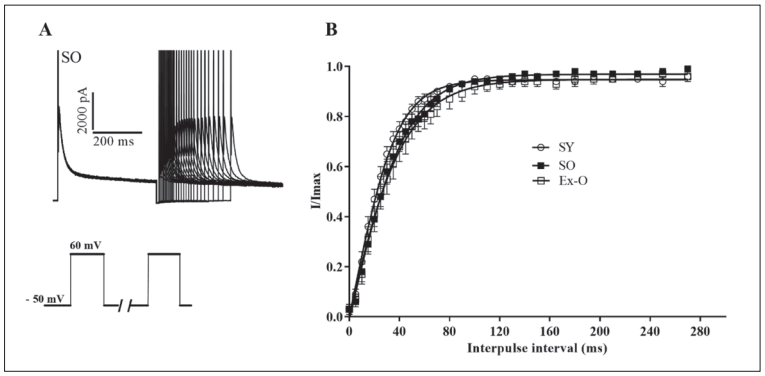

Fig. 5. Effects of aging on the steady-state reactivation of Ito. **A –** Original trace of SO; **B –** Steady-state reactivation of Ito in SY, SO and Ex-O ventricular myocytes was fitted by a single exponential function to give the time constant for recovery. The single exponential function is $I/I_{max}=[1-exp(-t/\tau)]$, where I/I_{max} is the ratio of current to maximum current and τ is the time constant for recovery. Data are given as the mean±SEM; **P*<0.05 vs the SY group; the number of cells, n=20 (5 hearts), 18 (5 hearts), 18 (5 hearts) for the SY, SO and Ex-O groups, respectively; SY – sedentary young, SO – sedentary old; Ex-O – exercised old.

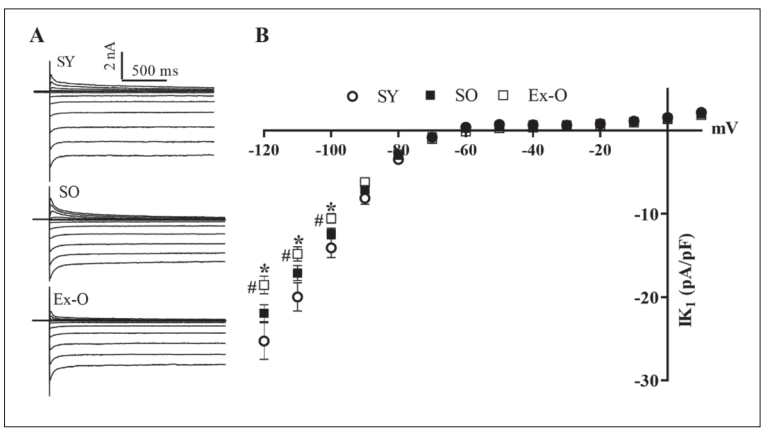

Fig. 6. Inward rectifier K^+ current of the experimental groups recorded by 1 s duration test pulses ranging between -120 mV and +10 mV. **A –** Original traces of SY, SO, Ex-Y and Ex-O; **B.** Current-voltage relationship of currents. Data are given as the mean±SEM; **P*<0.05 vs SY, #*P*<0.05 vs SO; the number of cells n=22 (5 hearts), 25 (6 hearts), 22 (5 hearts) for the SY, SO and Ex-O groups, respectively; SY – sedentary young, SO – sedentary old; Ex-O – exercised old.

every 5 s, while the inter-pulse interval was increased from 5 ms to 92 ms. Recovery from the inactivation was not different between the groups, as shown in Fig. 5. On the other hand, the I_{K1}, which was dominant in the late phase of the AP, showed little change with aging, while exercise caused a significant decrease of these currents in elderly rats (Fig. 6).

DISCUSSION

There are four major findings in the current study. First, there was no apparent cardiac hypertrophy at the tissue level, although a significant increase in cell capacitance was observed in aged female cardiomyocytes. However, chronic swimming exercise caused a significant increase in the total heart weight. Second, the APD was prolonged with aging and exercise further increased this prolongation, although not significantly. Third, aged female myocytes exhibited a significant decrease in I_{to} currents and exercise did not correct this suppression. Finally, aging had no effect on the inactivation and reactivation kinetics of the K^+ currents. Therefore, this study showed that swimming exercise did not remarkably reverse the age-related ionic changes in the ventricular myocytes of older female rats.

Previous reports have suggested that ventricular dysfunction is associated with structural, functional and biochemical alterations in older ages [19,38]. Myocardial cell loss and fibrosis are observed in the elderly myocardium as well as hypertrophy [39]. Exercise has been shown to protect against age-induced left ventricular remodeling, such as increased myocardial fibrosis, and to promote physiological hypertrophy in the remaining cells [40]. It increases the cardiomyocyte proliferation rate to contribute to this compensated physiological hypertrophy [41,42]. According to our results, exercise did not engender further change in myocyte size caused by aging, but it generated an increase in the total heart weight. Considering that exercise improves contractile functions, as shown in our previous study [21], it can be said that exercise increases myocyte proliferation in aged rats.

In cardiac muscle cells, the AP, aside from initiating contraction, provides a control mechanism for its maintenance. Electrophysiological studies have demonstrated that cardiac AP was prolonged in the elderly;

however, in these studies, gender-dependent changes were generally ignored [43,44]. In the present study, we observed that the APD was significantly prolonged in aging myocytes of female rats, but exercise did not reverse this prolongation. It has been previously reported that in young female rats, exercise significantly increased the APD in left ventricular myocytes [45,46]. Furthermore, in aged male rats, it was demonstrated that moderate exercise causes a significant prolongation in APD measured as the time to 80% repolarization, while it reduces the contraction and relaxation time of the myocardium [28]. Consistent with these findings, in our study, the swimming exercise further increased the APD value in aged female myocytes, although not significantly.

The voltage-activated outward K^+ currents are involved in both early and late phases of AP repolarization in rat ventricular myocytes. We suggest that it consists mainly of two components. The first is a component of early repolarization, which is called the I_{to}, and it is a fast-activating and inactivating current. The other component is the slowly inactivated or the non-damped component, which is called the I_{sus} (or I_{late}) and contributes to the late phase of the AP [47]. In a study using 3- and 9-month-old male and female rats, neither the APD nor the amplitude and kinetics of the I_{to} and I_{K1} currents were changed in the groups [16]. Conversely, Liu et al. [43], who used 6-month-old and 27-28-month-old male F344 rats and 29-36-month-old male Long-Evans rats, observed an increase (rapid inactivation) in the I_{to} in addition to cellular hypertrophy in elderly ventricular myocytes. The authors also demonstrated that the I_{K1} did not change in aged F344 rat myocytes but increased in the Long-Evans rat myocytes. Thus, they suggested that alterations in currents as well as channel kinetics and properties might be responsible for the age-related changes in AP configuration, contractile function and heart rate. In our study, the I_{to} was suppressed by aging, and exercise was ineffective against this suppression. Additionally, aging did not affect the voltage-dependent inactivation or recovery from the inactivation of channels, which implies that the reduction of I_{to} density was not associated with alterations in its inactivation and reactivation kinetics. In addition, although aging had no effect on the I_{sus} currents, which is another component of outward K^+ currents, exercise caused a significant decrease in these currents, and this suppression may be one of the main reasons for the prolonged APD in Ex-O myocytes. We did not observe a significant effect of aging on the I_{K1} currents, while exercise caused a significant reduction in elderly female rat myocytes.

In conclusion, we observed a decrease in the I_{to} and inward I_{K1} in aging myocytes, which started early after depolarization and increased the propensity for arrhythmias. Previously published reports and our data led us to propose that the age-induced changes in the electrophysiological properties of the myocardium may vary depending on gender, age and species of animals. On the other hand, although clinical and experimental studies illustrate that exercise has beneficial effects on cardiac functions, there are specific conditions where it may lead to unexpected death, especially in patients with cardiovascular diseases [48-50]. There is also evidence of remarkable electrocardiogram abnormalities in some people who exercise normally, and therefore sudden death associated with cardiac hypertrophy and altered repolarization has been suggested in trained athletes even in the absence of pathologic cardiac conditions [46]. Hence, the intensity, type, frequency and duration of exercise are critical factors for a successful outcome in exercise training procedures. The exercise schedule we selected in our study is referred to as moderate exercise and although it showed an improving effect in contractile functions, it did not restore the age-associated ionic current changes. Accordingly, in the clinical aspect, a lighter exercise schedule should be more beneficial in older age.

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Author contributions: Both authors were involved in the planning and performing of the experiments, data analysis and writing of the manuscript.

Conflicts of interest disclosure: The authors do not have any conflicts of interest to disclose.

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Supplementary Material

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