Effect of chronic exercise on myocardial electrophysiological heterogeneity and stability. Role of intrinsic cholinergic neurons: A study in the isolated rabbit heart

Luis Such-Miquel1,2, Laia Brines2,3, Antonio M. Alberola2,3, Manuel Zarzoso1,2, Francisco J. Chorro2,4, Juan Guerrero5, Germán Parra2,3, Nathalia Gallego2, Carlos Soler2,3, Irene Del Canto6, Antonio Guill6, Luis Such2,3*

1 Department of Physiotherapy, Universitat de València, Valencia, Spain, 2 Health Research Institute (INCLIVA), Valencia, Spain, 3 Department of Physiology, Universitat de València, Valencia, Spain, 4 Department of Medicine, Universitat de València, Valencia, Spain, 5 Department of Electronic Engineering, Universitat de València, Valencia, Spain, 6 ITACA, Universitat Politècnica de València, Valencia, Spain

* luis.such@uv.es

Abstract

A study has been made of the effect of chronic exercise on myocardial electrophysiological heterogeneity and stability, as well as of the role of cholinergic neurons in these changes. Determinations in hearts from untrained and trained rabbits on a treadmill were performed. The hearts were isolated and perfused. A pacing electrode and a recording multielectrode were located in the left ventricle. The parameters determined during induced VF, before and after atropine (1μM), were: fibrillatory cycle length (VV), ventricular functional refractory period (FRPVF), normalized energy (NE) of the fibrillatory signal and its coefficient of variation (CV), and electrical ventricular activation complexity, as an approach to myocardial heterogeneity and stability. The VV interval was longer in the trained group than in the control group both prior to atropine (78±10 vs. 68±10 ms) and after atropine (76±8 vs. 67±10 ms). Likewise, FRPVF was longer in the trained group than in the control group both prior to and after atropine (53±8 vs. 42±7 ms and 50±6 vs. 40±6 ms, respectively), and atropine did not modify FRPVF. The CV of FRPVF was lower in the trained group than in the control group prior to atropine (12.5±1.5% vs. 15.1±3.8%) and, decreased after atropine (15.1±3.8% vs. 12.2±2.4%) in the control group. The trained group showed higher NE values before (0.40±0.04 vs. 0.36±0.05) and after atropine (0.37±0.04 vs. 0.34±0.06; p = 0.08). Training decreased the CV of NE both before (23.3±2% vs. 25.2±4%; p = 0.08) and after parasympathetic blockade (22.6±1% vs. 26.1±5%). Cholinergic blockade did not modify these parameters within the control and trained groups. Activation complexity was lower in the trained than in the control animals before atropine (34±8 vs. 41±5), and increased after atropine in the control group (41±5 vs. 48±9, respectively). Thus, training decreases the intrinsic heterogeneity of the myocardium, increases electrophysiological stability, and prevents some modifications due to muscarinic block.
Introduction
It has been reported that aerobic physical exercise can protect against cardiac sudden death, which in most cases is produced by ventricular fibrillation (VF) [1,2], and experimental studies have evidenced that exercise leads to reduced VF [3]. However the exact underlying cardiovascular protective mechanisms are not fully known [4,5]. In this regard, research has been conducted to elucidate whether electrophysiological modifications produced by training could represent a possible protective mechanism. Reports have shown that training increases ventricular electrical stability [6], decreases electrophysiological heterogeneity in in vivo canine models [7], and increases the VF threshold during coronary occlusion in isolated rat hearts [8]. We have also reported that physical training increases ventricular refractoriness in trained rabbits [9,10]. Additional electrophysiological modifications produced by training have been investigated [11]. However, the effect of chronic physical exercise upon the intrinsic electrophysiological heterogeneity of the myocardium has been less studied in in vitro models. Myocardial heterogeneity is a well known proarrhythmic factor [12].

On the other hand, the cardiac nervous system, which has been suggested to act as the final coordinator of regional cardiac indices [13], can generate spontaneous activity [14] independently of central neuronal inputs [15], and in the absence of vagal impulse activity, acetylcholine is released from cholinergic neurons in mammals in a non-quantal manner [16]. Although many studies have focused on the participation of the extrinsic nervous system in the electrophysiological modifications caused by training, there is less knowledge about the direct influence of cholinergic myocardial neurons upon intrinsic electrophysiological heterogeneity and stability modifications due to physical training. Thus, in the present study conducted in the isolated, perfused and fibrillating rabbit heart, we have investigated the effects of chronic physical exercise upon ventricular electrophysiological heterogeneity and stability through the analysis of fundamental electrophysiological parameters and their ventricular dispersion, together with the complexity of myocardial activation during VF (which is also an associated parameter). Likewise, we have investigated the participation of myocardial cholinergic neurons in the modifications of these parameters. We hypothesize that myocardial physical training decreases electrophysiological heterogeneity and increases myocardial stability, and furthermore that intrinsic myocardial cholinergic neurons are implicated in these changes.

Material and methods

Animals and study design
Housing conditions and experimental procedures were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No. 123, Strasbourg 1985) and European Union Directive 2010/63 on the protection of animals used for scientific purposes, and were approved by the Institutional Animal Care and Use Committee of the University of Valencia as promulgated by Spanish legislation (RD 53/2013). Two groups of male New Zealand rabbits, nine weeks old and weighing 2.02 ± 0.11 kg, were studied. One group of eleven rabbits was trained on a motor-driven treadmill following a previously described protocol [9], and the other group of ten rabbits (control group) was housed in the animal quarters for the same period. Briefly, in the trained group, rabbits underwent a running program after four days of becoming familiarized with the treadmills, and they ran 5 days a week for 6 weeks at a speed of 0.33 m/s. Each of the training sessions was divided into 6 periods consisting of four minutes of running and one minute of rest between periods.
Preparation and perfusion

Following heparinization and anesthesia (ketamine 10 mg/kg i.v.), the animals were euthanized, and after a midsternal thoracotomy, the heart was quickly removed and immersed in cold Tyrode solution for further preparation. The aorta was cannulated and connected to a Langendorff system to provide the heart with the warmed and oxygenated Tyrode solution containing (millimolar): NaCl 130, KCl 4.7, CaCl$_2$ 2.2, MgCl$_2$ 0.6, NaH$_2$PO$_4$ 1.2, NaHCO$_3$ 24.2, and glucose 12. The pH was maintained at 7.4 by equilibration with a mixture of 95% O$_2$ and 5% CO$_2$. Tyrode temperature was constant throughout the experiment (37˚C), and the perfusion pressure was kept at 60 mmHg.

One bipolar surface electrode (Teflon-coated silver wire, with an inter-electrode distance of 1 mm) was positioned on the right atrium for recording, and another on the left ventricle for pacing. Ventricular recordings were made by means of a plaque with 240 unipolar stainless steel electrodes (0.125 mm electrode diameter and 1 mm inter-electrode distance) positioned on the epicardial surface of the posterior wall of the left ventricle. The indifferent electrode was a silver plaque located over the cannulated aorta. Recordings were obtained with a cardiac electrical activity mapping system (MAPTECH, Waalre, The Netherlands). The electrograms were amplified with a gain of 100–300, broad-band (1–400 Hz) filtered, and multiplexed. The sampling rate in each channel was 1 kHz.

Ventricular fibrillation was induced by pacing at increasing frequencies from 4 to 20 Hz, and coronary perfusion was maintained during the arrhythmia. Ventricular fibrillation was triggered when the stimulation frequency reached 816±52 beats per minute in the control group and 773±86 beats per minute in the trained group, the difference not being statistically significant. Muscarinic receptor blockade with atropine did not produce modifications in the stimulation frequency triggering VF in either group. Electrical stimuli for inducing VF (2 ms duration and three times the diastolic threshold) were delivered by a Grass S-88 stimulator (Grass Instruments, Inc., Quincy, MA, USA) fitted to two stimuli isolation units. Data analysis was carried out involving a data block of 4096 points. Data processing was performed with Matlab software on a Hewlett-Packard platform (Hewlett-Packard Co., Palo Alto, CA, USA).

Measurements and calculations

The study parameters were: a) Mean fibrillatory cycle length (VV). Activation times in each electrode were determined by identifying the moment of maximum negative slope of the electrograms. Accordingly, marks were placed on each slope corresponding to the activation waves of the electrograms, thus allowing identification of the VV intervals. The minimal threshold for dV/dt to be considered as a local deflection was a percentage (20%) of the maximal negative slope in each channel. In this way, the fibrillation interval (VV) histograms and the mean of the consecutive VV intervals were determined (Fig 1); b) Ventricular functional refractory period (FRPVF) as the 5th percentile of the VV intervals histogram, constructed from 4500 to 5000 consecutive VF cycles (detailed explanation in Fig 1); c) The coefficient of variation (CV) of FRPVF in each of the 240 unipolar electrodes; d) Normalized energy (NE) of the fibrillatory signal in each of the electrodes, defined as the spectral energy in a window centered on the dominant frequency (DF ± 1 Hz) and normalized by the spectral energy in the band of interest (5–35 Hz). This latter parameter was obtained by processing the recordings in consecutive segments of 4 s each (4000 samples). For each segment and channel we obtained the spectrum from the modified Welch periodogram using Hanning’s window and two non-overlapping stretches with a resolution of 0.5 Hz (see Appendix); e) The CV of NE in each of the electrodes; and e) The epicardial complexity of electrical activation of the ventricular myocardium during the arrhythmia. To determine this latter parameter, epicardial activation maps
during VF were generated. Activation maps during VF every 100 ms for one second in windows of 50 ms width were constructed. The number of windows analyzed was 11 in one second. Isochrones were drawn, and in each map the wavefronts, reentrant activations, breakthrough patterns, etc., were analyzed (Fig 2). A numerical value, depending on the number of activation processes observed in the analyzed time windows was assigned to each activation map. When only one activation process appeared (e.g., a reentry, a single front, or breakthrough), the assigned value was one. The sum of the numbers indicating the activation complexity detected in each of the analyzed windows was then calculated.

**Experimental series and protocol**

Two experimental groups (control and trained) were studied. The experimental protocol in each group was as follows: once the hearts were isolated and perfused (Langendorff system), VF was induced by pacing at increasing frequencies from 4 Hz up to the onset of VF. Coronary perfusion was maintained during the arrhythmia. Once VF was triggered, recordings were obtained for 5 minutes, after which we proceeded to defibrillation of the hearts. At this time an atropine solution was infused through the root of the aorta, reaching 1 μM in the heart, until the end of the experiment. Ventricular fibrillation was again induced and recordings were obtained 5 minutes after onset of the arrhythmia. Perfusion pressure was controlled during the experiment, and the hearts were weighed at the end of the experiments. Measurements of the parameters were determined 5 minutes after VF onset and after atropine administration.

**Data analysis**

Two-way analysis of variance (ANOVA) with repeated measures on one factor and an unpaired t test were used when appropriate. Values of $p<0.05$ were considered statistically significant.
Results

VV intervals

The mean of the VV intervals was longer in the trained group than in the control group prior to atropine administration (78 ± 10 vs. 68 ± 10 ms) and after atropine administration (76 ± 8 vs. 67 ± 10 ms). No significant differences were found before and after atropine administration in any group.

Functional refractory period during ventricular fibrillation

The FRPVF was longer in the trained group than in the control group prior to atropine administration (53 ± 8 vs. 42 ± 7 ms, respectively). This parameter did not change after atropine versus the pre-atropine values in either the control group (40 ± 6 vs. 42 ± 7 ms, respectively) or in the trained group (53 ± 8 vs. 52 ± 6 ms, respectively).
Coefficient of variation of FRPVF

The CV of FRPVF was higher in the control group than in the trained group prior to atropine administration (15.1 ± 3.8% vs. 12.5 ± 1.5%), but not after atropine (12.2 ± 2.4% vs. 10.8 ± 2.1%). This parameter decreased after atropine in the control group (15.1±3.8% vs. 12.2 ±2.4%) but not in the trained group (12.5 ± 1.5% vs. 10.8 ± 2.1%).

Normalized energy of the fibrillatory signal

Hearts from trained animals showed higher NE values before (0.40±0.04 vs. 0.36±0.05; p<0.05) and after atropine administration (0.37±0.04 vs. 0.34±0.06; p = 0.08) (Fig 1C). Physical training decreased the heterogeneity of this parameter as shown by the CV of NE, both before (23.3±2 vs. 25.2± 4%; p = 0.08) and after parasympathetic blockade (22.6±1 vs. 26.1 ±5%; p <0.05) (Fig 1D). On the other hand, cholinergic blockade did not modify these parameters within the control and trained groups.

Coefficient of variation of NE of the fibrillatory signal

Physical training decreased the heterogeneity of NE of the fibrillatory signal as shown by the CV of NE, both before (23.3±2 vs. 25.2±4%; p = 0.08) and after parasympathetic blockade (22.6±1 vs. 26.1±5%; p<0.05) (Fig 1D). On the other hand, cholinergic blockade did not modify these parameters within the control and trained groups.

Activation complexity

In terms of the characteristics of the activation maps during VF, the activation complexity was greater in the control group than in the trained animals prior to atropine (41 ± 5 vs. 34 ± 8) and after atropine (48±9 vs. 36±7). This parameter increased in the control group after atropine versus the prior to atropine values (41 ± 5 vs. 48 ± 9 ms, respectively). No differences were observed in the trained group (36 ± 7 vs. 34 ± 8 ms, respectively). See Fig 3.

Fig 3. Myocardial activation complexity during ventricular fibrillation. (A) Bidimensional representation of two activation maps obtained in both situations (pre- and post-atropine), in an experiment of the control group and another experiment of the trained group. In each map, the number of activation processes observed determines activation complexity level: in map 2 (M2), arrows represent each one a wavefront and circle with arrows represent breakthrough activation, resulting in a total of 4 activation processes and the assigned activation complexity is 4; map 8 (M8) shows 3 simultaneous wavefronts, and the assigned activation complexity is 3. The more simple activation maps are seen to predominate in trained group before and after atropine infusion. (B) Myocardial activation complexity in control and trained group, before and after atropine administration. *p<0.05 vs. control; #p<0.05 vs. pre-atropine. A.T., activation time; M, map.

https://doi.org/10.1371/journal.pone.0209085.g003
Heart and body weight of rabbits and heart–to–body weight ratio

No differences in cardiac mass were found between the control and trained groups (14.4 ± 1.9 g; n = 11 vs. 14.1 ± 2.2 g; n = 12). Likewise, there were no differences in the weight of the rabbits (3.63 ± 0.25 kg; n = 11 vs. 3.54 ± 0.41 kg; n = 12) or in the heart-to-body weight ratio (0.0037 ± 0.0004; n = 11 vs. 0.0040 ± 0.0003; n = 12) on comparing the control group with the trained group.

Discussion

An isolated and fibrillating rabbit heart model has been used to study the effects of chronic physical exercise upon the ventricular myocardium functional refractory period, normalized energy of the fibrillatory signal, and ventricular dispersion of these parameters as an index of myocardial heterogeneity. We have also determined the frequency of activation during ventricular fibrillation, analyzed in the time domain, and ventricular activation complexity during ventricular fibrillation these parameters being indexes of refractoriness, electrical stability and heterogeneity, respectively. The study was made before and after atropine administration in order to investigate the contribution of myocardial cholinergic neurons to the mentioned parameters and their modification through chronic exercise, which remain unknown to date. This experimental study has been performed using the rabbit, which is an ideal model for examining the effects of aerobic physical training. Indeed, it has been reported that by using the proper intensity, duration, and frequency of exercise, the rabbit achieves a documented cardiovascular training effect rather easily [17]. We have previously reported that using the training protocol of the present study, the in vivo heart rate was lower in the trained animals than in the non-trained animals. Moreover, sinus cycle length analyzed in the isolated heart, likewise as a cardiovascular parameter of training, was longer in the trained rabbits than in the controls. Not only the cardiovascular parameters evidenced the effect of the applied protocol on a treadmill, but also the biochemical parameters HS60 and iNOS were modified [9]. Since we have determined these parameters during VF (without interrupting perfusion), the rabbit also provide a good model in contrast to other species such as the mouse, because VF in rabbits can be easily induced and sustained [1]. The present model simulates sedentary humans that perform moderate exercise as cardiovascular protection against life-threatening arrhythmias [1].

One of the results in our study is that chronic physical exercise increased the ventricular functional refractory period, and cholinergic blockade did not modify this parameter in trained and non-trained animals. The results also showed a longer mean VV interval in the trained group versus the control group, and no modifications of this interval were observed after cholinergic blockade. The mean VV interval is known to be closely related to refractoriness [18]. The increase in refractoriness due to chronic physical exercise which we have observed has been previously reported using different methodology for assessing this property [9,19,20]. Our results also showed ventricular functional refractory period dispersion to be greater in the control group than in the trained group before atropine administration. Cholinergic blockade did not modify the CV of FRPVF after atropine in the trained group, though a slight but significant decrease was observed in the control group. The determination of the ventricular functional refractory period constructed from multiple consecutive VF cycles from unipolar left ventricle electrograms offers several advantages [20], allowing estimation of the refractory period at over 200 different points of the myocardium, and not only at the site of pacing, as occurs when the extrastimulus test is used. Moreover, by using the mentioned methodology we can also estimate myocardial heterogeneity, analyzing refractory period dispersion through the ventricular myocardium.
In a way similar to the dispersion of refractoriness, the greater NE values and lower CV of the NE found in the trained animals also reinforces the idea that training decreases myocardial electrophysiological heterogeneity. As is known, NE informs about the spectral concentration of the fibrillatory signal. Indeed, a perfectly periodic signal will have all its power spectrum concentrated in the dominant frequency and its harmonics, while an irregular signal will have a lower percentage of the spectrum area under the dominant frequency. Thus, the calculation of this percentage provides information about the organization and regularity of the VF signals, and therefore about electrophysiological heterogeneity, which in turn is associated with the facility of establishing and/or ending VF. The absence of changes in NE and its CV, after atropine administration, also allows us to conclude that cholinergic neurons do not play a direct role in relation to these properties.

Our results concerning ventricular refractoriness as well as the VV interval should be interpreted as a protective mechanism against reentrant arrhythmias. It is well known that myocardial refractoriness is a property, along with conduction velocity, that determines the wavelength of the myocardial activation process, which in turn is a factor closely related to the appearance and stability of reentrant arrhythmias over time [21]. As noted above, cholinergic blockade does not modify refractoriness in trained or non-trained animals, suggesting that myocardial cholinergic neurons do not have a direct effect upon this property under sedentary and trained conditions. The lower values of the parameters of electrophysiological heterogeneity produced by physical training, observed in the present study, and as has been discussed for refractoriness, indicate that endurance physical training could exert an antiarrhythmic effect at least in part by decreasing myocardial electrophysiological heterogeneity. Indeed, the importance of heterogeneity and the refractory period in the induction of cardiac fibrillation has been well established [12]. Thus, if the changes in refractoriness and heterogeneity could participate as an additional basic mechanism in which aerobic exercise exerts a protective effect against reentrant arrhythmias such as VF, which can produce cardiac sudden death [1], then cholinergic neurons do not seem to play a direct role in the electrophysiological adaptations found in the present study.

Regarding the slight decrease in refractoriness dispersion on ventricular myocardium after cholinergic blockade, observed in the control group, although we have not investigated the mechanisms capable of explaining this effect, some considerations should be taken into account. The data accumulated in the last few years give an account of the complexity of intracardiac neurons in modulating heart function. It has been reported that intrinsic cardiac neurons generate spontaneous activity [14] and can function independently of central neuronal inputs [15]. Moreover, recent reports show that in the absence of vagal impulse activity, acetylcholine is released from cholinergic neurons in mammals [16]. In a way similar to the effects exerted by acetylcholine on the atria, shortening the atrial refractory period and increasing its dispersion [22], we speculate that acetylcholine release in the isolated heart ventricle and thus not under central nervous system control, would also increase the dispersion of ventricular refractory periods. This would explain the decrease in ventricular heterogeneity in terms of functional refractory period dispersion during VF after cholinergic blockade.

With respect to electrical activation during VF, we observed lesser electrical activation complexity during VF in the trained group than in the control group. Cholinergic blockade did not modify this parameter in the trained group, though it increased after atropine in the control group. This result should be interpreted as a beneficial effect. Indeed, a decrease in activation complexity implies an increase in myocardial electrical stability, and thus represents a protective effect against the generation of reentrant arrhythmias. The mechanisms by which cholinergic blockade increases the complexity of myocardial activation during VF in the control group have not been investigated in the present study. Although it seems paradoxical that
cholinergic blockade decreased electrophysiological heterogeneity in the control group despite an increase in the complexity of activation, no such paradox actually exists. Indeed, it has been reported that drugs like flecainide, sotalol and verapamil, with different electrophysiological actions (i.e., upon refractoriness and the spectral characteristics of ventricular fibrillation), produce the same effect upon the complexity of ventricular activation [18]. It has also been reported that ventricular stretching and verapamil administration increase the dominant frequency of ventricular fibrillation, and consequently the coefficient of variation of the VF dominant frequency [23]. However, ventricular stretch increased the complexity of the myocardial activation during arrhythmia, while verapamil decreased it [18].

On the basis of the results obtained, some considerations should be taken in account. It has been reported that intracardiac neurons can operate with some degree of independence from extrinsic neuronal inputs [24], and that spontaneous activity can occur if the ganglia have been acutely decentralized [25]. This could explain why myocardial cholinergic neurons seem to exert some cholinergic control of electrophysiological heterogeneity as well as of electrical activation complexity during VF, even though these neurons do not seem to influence myocardial refractoriness. Our results suggest the need for further research to explain the mechanisms by which cholinergic neurons directly modify some electrophysiological parameters and also explain the apparent paradox of the changes in these parameters.

It seems especially remarkable that unlike in the control group, the trained rabbits exhibited no modifications in the heterogeneity and complexity of the activation produced by cholinergic blockade. These results lead us to postulate that training seems to reduce sensitivity to cholinergic muscarinic blockade, making the ventricle less susceptible to the direct influence of intrinsic cholinergic neurons upon the electrophysiological parameters investigated. The absence of effects of cholinergic blockade upon the parameters analyzed in fibrillatory rhythm in the trained group and on some parameters in the control group seems to be closely related to the results referred to heart rate and other parameters obtained in sinus rhythm, previously reported by our group [10].

Despite the results obtained in the present study, we must emphasize the interest which the use of M2R-knock-out-rabbits would have in investigating not only the electrophysiological parameters analyzed in this study but also other parameters previously analyzed using our experimental model. This, together with studies based on cholinergic stimulation and/or beta-adrenergic blockade, would make it possible to clarify and probably reinforce our findings referred to the participation of cholinergic neurons in the electrophysiological modifications produced by low intensity training in a sedentary animal such as the laboratory rabbit. We recommend the conduction of such studies in rabbits and not in other species, due to the above-mentioned reasons. On the other hand, the effects of physical exercise upon the activity of the sinus node and the underlying mechanisms have been investigated and attributed to the down-regulation of hyperpolarization-activated cyclic nucleotide-gated (HCN), Na+, Ca2+ and K+ channel mRNA, and a significant correlation between heart rate and HCN4 mRNA expression has been described as well [26]. Nevertheless, although some of the mentioned components, such as the HCN channels, are present in the ventricular myocardium [27, 28], as far as we know, no information is available about their influence upon the effects induced by exercise training on ventricular electrophysiology. Accordingly, some of these mechanisms should be investigated in the ventricles in order to clarify and obtain basic knowledge about the effects of chronic physical exercise upon the myocardial electrophysiological properties related to establishment of the life-threatening arrhythmias, which we have investigated in the present study.

In conclusion, training by means of a physical exercise protocol in rabbits has produced an increase in ventricular refractoriness, a decrease in ventricular electrophysiological heterogeneity, and a reduction of the complexity of electrical activation during ventricular fibrillation.
in the isolated and perfused heart. Training seems to reduce sensitivity to cholinergic muscarinic blockade, which does not alter the intrinsic refractoriness, heterogeneity and stability modifications produced by training. The results obtained in the present study raise the need to pursue this research in order to elucidate the mechanisms underlying the observed modifications through both training and the cholinergic blockade S1 Appendix.

Appendix

For each segment and channel, we obtained the power spectrum by means of the Welch [29] periodogram, which uses the averaging of modified periodograms. In sum, the process is as follows:

1. Divide the data segment, x, into sections that may overlap. In this case, it has been divided into two non-overlapping sections, t1 and t2:

   \[ x(n), \quad n = 1, \ldots, L \]

   \[ x_{t1}(i), \quad i = 1, \ldots, L/2 \]

   \[ x_{t2}(j), \quad j = L/2 + 1, \ldots, L \]

   \( L = \text{number of samples.} \)

2. A window is applied to each signal section, resulting in a modified periodogram. Hanning’s window is used, defined by:

   \[ w(n) = \frac{1}{2} \left( 1 - \cos \left( \frac{2\pi n}{L/2} \right) \right), \quad n = 1, \ldots, L/2 \]

3. The periodogram of each section is calculated:

   \[ P_{x_t}^e(f) = \frac{1}{L/2} \sum_{n=0}^{L/2-1} x_t(n) w(n) e^{j2\pi fn}, \quad t = 1, 2 \]

4. The power density spectrum of each segment of the record is given by:

   \[ P_{xx}(f) = \frac{1}{2} \sum_{i=1}^{2} P_{x_t}^e(f) \]

5. The spectral energy is calculated in the FD \( \pm 1 \) Hz band, and in the band of interest (5–35 Hz) by integrating the power spectrum in these bands.

6. Finally, we obtain the normalized energy (NE) as the ratio between the spectral energy in the FD \( \pm 1 \) Hz band and the energy of the band of interest.

Supporting information

S1 Appendix. S1_Minimal_Data_Set.xlsx. Data obtained to calculate each parameter. (XLSX)
Acknowledgments

The authors thank Carmen Rams, Ana Díaz, Pilar Navarro and César Avellaneda for their excellent technical assistance.

Author Contributions

**Conceptualization:** Antonio M. Alberola, Francisco J. Chorro, Luis Such.

**Data curation:** Luis Such-Miquel, Laia Brines, Manuel Zarzoso, Francisco J. Chorro, Juan Guerrero.

**Formal analysis:** Luis Such-Miquel, Manuel Zarzoso, Carlos Soler.

**Funding acquisition:** Antonio M. Alberola, Luis Such.

**Investigation:** Luis Such-Miquel, Laia Brines, Antonio M. Alberola, Manuel Zarzoso, Francisco J. Chorro, Germán Parra, Nathalia Gallego, Carlos Soler, Irene Del Canto, Antonio Guill, Luis Such.

**Methodology:** Luis Such-Miquel.

**Project administration:** Antonio M. Alberola, Francisco J. Chorro.

**Supervision:** Luis Such-Miquel, Antonio M. Alberola, Manuel Zarzoso, Francisco J. Chorro, Luis Such.

**Writing – original draft:** Luis Such-Miquel, Luis Such.

**Writing – review & editing:** Luis Such-Miquel, Luis Such.

References

1. Billman GE. Aerobic exercise conditioning: a nonpharmacological antiarrhythmic intervention. J Appl Physiol. 2002 Feb; 92(2):446–454. https://doi.org/10.1152/japplphysiol.00874.2001 PMID: 11796650

2. Billman GE. A comprehensive review and analysis of 25 years of data from an in vivo canine model of sudden cardiac death: Implications for future anti-arrhythmic drug development. Pharmacol Ther. 2006 Sep; 111(3):808–835. https://doi.org/10.1016/j.pharmthera.2006.01.002 PMID: 16483666

3. Dor-Haim H, Berenfeld O, Horowitz M, Lotan C, Swissa M. Reduced Ventricular Arrhythmogeneity and Increased Electrical Complexity in Normal Exercised Rats. PLoS One. 2013 Jun;18:8(6):e66658. https://doi.org/10.1371/journal.pone.0066658 PMID: 23825553

4. Hamer M, Stamatakis E. Physical Activity and Cardiovascular Disease: Directions for Future Research. The Open Sports Sciences Journal. 2008; 1, 1–2.

5. Powers SK, Smuder AJ, Kavazis AN, Quindry JC. Mechanisms of exercise-induced cardioprotection Physiology. 2014 Jan; 29(1): 27–38. https://doi.org/10.1152/physiol.00030.2013 PMID: 24382869

6. Hull SS Jr, Vanoli E, Adamson PB, Verrier RL, Foreman RD, Schwartz PJ. Exercise training confers anticipatory protection from sudden death during acute myocardial ischemia. Circulation. 1994 Feb; 89 (2): 548–552. PMID: 8315642

7. Hajnal A, Nagy O, Litvai A, Papp J, Parratt JR, Végh A. Nitric oxide involvement in the delayed antiarrhythmic effect of treadmill exercise in dogs. Life Sci. 2005 Sep; 77(16):1960–1971. https://doi.org/10.1016/j.lfs.2005.02.015 PMID: 15907332

8. Noakes TN, Higginson L, Opie LH. Physical training increases VF thresholds of isolated rat hearts during normoxia, hypoxia, and regional ischemia. Circulation. 1983 Jan; 67:24–30. PMID: 6291806

9. Such L, Alberola AM, Such-Miquel L, López L, Trapero I, Pelechano F, et al. Effects of chronic exercise on myocardial refractoriness: a study on isolated rabbit heart. Acta Physiol. 2008 Aug; 193:331–339.

10. Zarzoso M, Such-Miquel L, Parra G, Brines-Ferrando L, Such L, Chorro FJ, et al. The training-induced changes on automatism, conduction and myocardial refractoriness are not mediated by parasympathetic postganglionic neurons activity. Eur J Appl Physiol. 2012 Jun; 112(6):2185–2193. https://doi.org/10.1007/s00421-011-1219-x PMID: 21968799
11. Billman GE. Cardiac autonomic neural remodeling and susceptibility to sudden cardiac death: effect of endurance exercise training. Am J Physiol Heart Circ Physiol. 2009 Oct; 297(4):H1171–1193. https://doi.org/10.1152/ajpheart.00534.2009 PMID: 19684184

12. Han J, Moe GK. Nonuniform Recovery of Excitability in Ventricular Muscle. Circ Res. 1964 Jan; 14:44–60. PMID: 14104163

13. Beaumont E, Salavatian S, Southerland EM, Vinet A, Jacquemet V, Armour JA, et al. Network interactions within the canine intrinsic cardiac nervous system: implications for reflex control of regional cardiac function. J Physiol. 2013 Sep; 591(18):4515–4533. https://doi.org/10.1113/jphysiol.2013.259382 PMID: 23818689

14. Arora RC, Hirsch GM, Johnson Hirsch K, Hancock Friesen C, Armour JA. Function of human intrinsic cardiac neurons in situ. Am J Physiol. 2001 Jun; 280(6):1736–1740.

15. Armour JA. Potential clinical relevance of the "little brain" on the mammalian heart. Exp Physiol. 2008 Feb; 93(2):165–176. https://doi.org/10.1113/expphysiol.2007.041178 PMID: 17981929

16. Abramochkin DV, Nurullin LF, Borodinova AA, Tarasova NV, Sukhova GS, Nikolsky EE, et al. Non-quantal release of acetylcholine from parasympathetic nerve terminals in the right atrium of rats. Exp Physiol. 2010 Feb; 95(2):265–273. https://doi.org/10.1113/expphysiol.2009.050302 PMID: 19767403

17. DiCarlo SE, Bishop VS. Exercise training enhances cardiac afferent inhibition of baroreflex function. Am J Physiol. 1990 Jan; 258(1 Pt 2):H212–220. https://doi.org/10.1152/ajpheart.1990.258.1.H212 PMID: 1967912

18. Chorro FJ, Cánoves J, Guerrero J, Mainar L, Sanchis J, Soria E, et al. Opposite Effects of Myocardial Stretch and Verapamil on the Complexity of the Ventricular Fibrillatory Pattern: An Experimental Study. Pacing Clin Electrophysiol. 2000 Nov; 23(11 Pt 1):1594–1603. PMID: 11138295

19. Such L, Rodríguez A, Alberola A, Lopez L, Ruiz R, Artal L, et al. Intrinsic changes on automatism, conduction, and refactoriness by exercise in isolated rabbit heart. J Appl Physiol. 2002 Jan; 92(1):225–229. https://doi.org/10.1152/jappl.2002.92.1.225 PMID: 11744664

20. Duyschaever M, Mast F, Killian M, Blauw Y, Wijffels M, Allessie M. Methods for determining the refractory period and excitable gap during persistent atrial fibrillation in the goat. Circulation. 2001 Aug; 104(8):957–962. PMID: 11514386

21. Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. Circulation. 1995 Oct; 92(7):1954–1968. PMID: 7671380

22. Liu L, Nattel S. Differing sympathetic and vagal effects on atrial fibrillation in dogs: role of refractoriness heterogeneity. Am J Physiol. 1997 Aug; 273(2 Pt 2):H805–H816. https://doi.org/10.1152/ajpheart.1997.273.2.H805 PMID: 9277498

23. Zaitsev AV, Berenfeld O, Mironov SF, Jalife J, Pertsov AM. Distribution of Excitation Frequencies on the Epicardial and Endocardial Surfaces of Fibrillating Ventricular Wall of the Sheep Heart. Circ Res. 2000 Mar; 86(4):408–417. PMID: 10700445

24. Armour JA, Collier K, Kember G, Ardell JL. Differential selectivity of cardiac neurons in separate intrathoracic autonomic ganglia. Am J Physiol Regulatory Integrative Comp Physiol. 1998 Apr; 274(4 Pt 2):R939–R949.

25. Armour JA, Hopkins DA. Activity of in vivo canine ventricular neurons. Am J Physiol Heart Circ Physiol. 1990 Feb; 258(2 Pt 2):H326–H336.

26. D’Souza A, Bucchi A, Johnsen AB, Logantha SJRJ, Monfredi O, Yanni J, et al. Exercise training reduces resting heart rate via downregulation of the funny channel HCN4. Nat Commun. 2014 May; 5:3777. https://doi.org/10.1038/ncomms4775 PMID: 24825544

27. Sartiani L, Romanelli MN, Mugelli A, Cerbai E. Updates on HCN Channels in the Heart: Function, Dysfunction and Pharmacology. Curr Drug Targets. 2015; 16:868–876. PMID: 26028050

28. Herrmann S, Layh B, Ludwig A. Novel insights into the distribution of cardiac HCN channels: an expression study in the mouse heart. J Mol Cell Cardiol. 2011; 51:997–1006. https://doi.org/10.1016/j.yjmcc.2011.09.005 PMID: 21945247

29. Welch PD. The use of Fast Fourier Transform for the estimation of power spectra: A method based on time averaging over short, modified periodograms. IEEE Transactions on Audio and Electroacoustics. 1967; AU-15(2):70–73.