# CHARACTERIZATION OF POSSIBLE ARRHYTHMIA MECHANISMS:

# THE IMPORTANCE OF ATTENUATED REPOLARIZATION RESERVE

**Ph.D. thesis**

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# THE IMPORTANCE OF ATTENUATED REPOLARIZATION RESERVE

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II. Zsófia Kohajda*, László Virág*, Tibor Hornyik, Zoltán Husti, Anita Sztojkov-Ivanov, Norbert Nagy, András Horváth, Richárd Varga, János Prorok, Jozefina Szlovák, Noémi Tóth, Péter Gazdag, ***Leila Topal***, Muhammad Naveed, Tamás Árpádffy-Lovas, Bence J. Pászti, Tibor Magyar, István Koncz, Szilvia Déri, Vivien Demeter-Haludka, Zoltán Aigner, Balázs Ördög, Márta Patfalusi, László Tálosi, László Tiszlavicz, Imre Földesi, Norbert Jost, István Baczkó, András Varró (2022). In vivo and cellular antiarrhythmic and cardiac electrophysiological effects of desethylamiodarone in dog cardiac preparations
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V. Khadija Belasri, ***Leila Topal***, Matthias Heydenreich, Andres Koch, Erich Kleinpeter, Ferenc Fülöp, István Szatmári (2020). Synthesis and Conformational Analysis of Naphthoxazine-Fused Phenanthrene Derivatives
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# 1. INTRODUCTION

## 1.1 Athletic training-induced structural and electrocardiographic cardiac remodeling

Regular physical activity and competitive sports have undeniable benefits; however, recent decades have witnessed that athletes who perform high-level training beyond an optimal level may be more prone to unpleasant cardiac events such as atrial and ventricular arrhythmias, and even sudden cardiac death (SCD) in certain circumstances. The long-term vigorous training or overexertion can lead to harmful structural (e.g., enhanced level of myocardial fibrosis) and electrophysiological changes (i.e., extreme bradycardia, widened QRS interval, and prolonged QT interval) of the heart. These alterations may act as a trigger for arrhythmogenesis especially in pre-existing cardiac conditions or in the presence of silent attenuated repolarization reserve. Presumably, the coexistence of altered cardiac structure and attenuated repolarization reserve may enhance the susceptibility to arrhythmogenicity among athletes. It is important to emphasize that the prolonged repolarization-induced repolarization inhomogeneity, alone is not sufficient to elicit cardiac arrhythmias but it may establish a potential substrate. In this case, a "arrhythmia trigger" like an extrasystole in a vulnerable period with unfortunate timing can traverse the ventricle where the repolarization inhomogeneity was augmented, so it can form a reentry pathway.

## 1.2 The importance of repolarization reserve and the arrhythmogenic consequences of its attenuation and increased dispersion of repolarization

The concept of repolarization reserve was originally proposed by Roden and later this concept was proven experimentally. Based on these, the repolarization is adequately over-secured to protect the heart against extremely lengthened action potential duration and consequently prolonged QT interval, which can provide a potential arrhythmia substrate. Based on this, the loss of one repolarization component does not lead to a marked lengthening of action potential duration (APD) and excessively prolonged QT interval. The underlying mechanism behind this observation is that various potassium channels are able to substitute and/or supplement the function of the missing link, therefore, the repolarization remains seemingly intact. Consequently, the properly functioning heart is defended by the orchestrated cooperation between the different repolarizing ionic currents against malignant cardiac arrhythmias. The repolarization capacity has an important role in reducing transmural APD dispersion and thereby preventing life-threatening arrhythmias. The repolarization reserve can be attenuated due to several causes like drug exposures (including cardiac and non-cardiac drugs), different conditions evoked cardiac remodeling (i.e., heart failure, long-term vigorous training, diabetes

mellitus), extreme bradycardia, or genetic disorders (long QT syndromes). In these conditions, even a moderate potassium current inhibition may lead to potentially proarrhythmic prolongation of ventricular APD. Consequently, inhibition of multiple potassium channels or the failure of these channels is associated with increased proarrhythmic risk since it can result in excessive repolarization lengthening by eliminating the repolarization reserve, additionally, the spatial repolarization heterogeneity is increased. In this case, a "trigger" like an extrasystole in a vulnerable period with unfortunate timing can traverse the ventricle where the repolarization inhomogeneity was augmented, so it can form a re-entry pathway.

### 1.3 The impact of seemingly harmless drugs among athletes

Athletes often seek different avenues to reach greater sporting achievements. A lot of products in the market, for instance, various dietary supplements (e.g., cannabidiol; CBD) and even some medicines (e.g., non-steroidal anti-inflammatory drugs; NSAIDs) offer several benefits for athletes to accomplish their goals. Surprisingly, the cardiovascular safety profile of some compounds found in these products, like the impact on the duration of the QT interval and/or different transmembrane ionic currents, has not been properly investigated yet. The interplay between the effect of these compounds and chronic high-intensity exercise evoked cardiac structural-electrophysiological remodeling may attenuate the repolarization reserve, thus creating a potential arrhythmogenic substrate in athletes. Therefore, accurate characterization of the effects of seemingly harmless compounds on ventricular repolarization would be necessary, regardless of their seemingly marginal effects.

## 2. AIMS

Since the different changes occurring during repolarization present a very complex issue affected by several factors, such as structural and functional cardiac remodeling associated with long-term endurance training, as well as drug-induced alternations of repolarization reserve, the aims of the present study were the following:

1. To develop a small animal model that was relevant to the human athlete's heart.
2. To assess the underlying mechanism of structural–electrical cardiac changes due to long-term endurance training.
3. To investigate the cardiac electrophysiological effects of cannabidiol and ibuprofen at the cellular level in native left ventricular myocytes obtained from larger animals that closely mimic human basic cellular electrophysiology (rabbit and canine). To elucidate their proarrhythmic side effects, their attenuating impacts were studied on various transmembrane ionic currents, which play a key role in the repolarization reserve.

# 3. RESULTS

## 3.1 Examination of long-term endurance training-induced cardiac remodeling in a small animal model

### 3.1.1 Echocardiographic changes after long-term endurance training

The 15-week-long training program led to significant changes in diastolic parameters in the exercised (EXE) group compared to sedentary (SED) guinea pigs: greater internal dimension of the left ventricle and increased interventricular septum thickness. Additionally, the thickness of the left ventricular posterior wall in systole was significantly increased in the „EXE" group. It is important to highlight that „EXE" animals showed an increasing tendency in the left-ventricular end-diastolic dimension. The ejection fraction and fractional shortening did not differ significantly between the groups.

### 3.1.2 Long-term endurance training-induced cardiac fibrosis in guinea pig athlete's heart model

The long-term endurance training indicated greater fibrosis scores and the greater presence of scarring tissue in „EXE" animals compared to their "SED" counterparts. The histopathological study identified a significantly greater degree of fibrosis in the subendocardial region of the right atrium, the right ventricle, and the septal wall of the hearts in the „EXE" group. In addition, enhanced fibrosis was also presented in the left ventricle of the „EXE" animals.

### 3.1.3 The effect of long-term endurance training on heart rate in conscious animals and in *ex vivo* isolated Langendorff perfused guinea pig hearts

In conscious "EXE" animals the endurance training program resulted in significantly lengthened RR intervals indicating training-induced bradycardia. In contrast with the *in vivo* data, during *ex vivo* Langendorff experiments, the RR intervals did not differ significantly between the groups. Various beat-to-beat variability parameters, including the root mean square of the successive differences (rmsSD) and the standard deviation of successive differences (sdSD) of the RR intervals, significantly increased by the end of the training protocol in conscious "EXE" guinea pigs compared to the "SED" animals.

### 3.1.4 The effect of long-term endurance training on ECG parameters and their variability parameters

The ECG recordings at the 15$^{th}$ week in conscious animals revealed no significant difference in the PQ interval between the examined groups. However, the QRS interval significantly widened in the "EXE" group. Contrarily, the lengths of PQ and QRS intervals did not differ significantly

between the groups in *ex vivo* Langendorff experiments. At the end of the training protocol, the QT interval was significantly prolonged in the "EXE" group. Besides that, the heart rate corrected QT interval (QTc) of conscious "EXE" animals was significantly longer. The short-term variability of the QT intervals seemed elevated in the "EXE" group; however, it did not reach statistical significance. Additionally, an increased tendency was observed in other beat-to-beat variability parameters of repolarization in "EXE" animals (including long-term variability, long-term instability, instability), but none reached a statistically significant difference. Similar to the unchanged RR intervals, the duration of QT intervals did not differ between the groups in *ex vivo* Langendorff experiments.

Cardiac arrhythmias were not detected during in *in vivo* ECG studies in either group. Although a few cardiac arrhythmias developed in both groups during *ex vivo* Langendorff experiments, there were no differences in the number and complexity of these arrhythmias between the groups.

### 3.1.5 Electrophysiological findings at the cellular level following long-term endurance training

The applied training protocol and experimental measuring conditions revealed unchanged magnitudes of slow delayed rectifier potassium current ($I_{Ks}$), the late sodium current ($I_{NaL}$), and the sodium-calcium exchanger ($I_{NCX}$) in native guinea pig left ventricular myocytes between the groups. In agreement with the patch clamp data, there was no significant difference in the length of APD measured at 90% repolarization in myocytes isolated from the left ventricle of the "EXE" group compared to the sedentary group. The short-term variability of APD did not differ significantly between the groups.

## 3.2 Investigating the electrophysiological effects of cannabidiol on repolarizing transmembrane potassium currents

### 3.2.1 Effects of cannabidiol on rapid delayed rectifier potassium current ($I_{Kr}$) in native rabbit left ventricular myocytes

The possible effect of CBD on $I_{Kr}$ was examined in enzymatically isolated left ventricular myocytes from a rabbit heart at 37 °C. The whole-cell patch clamp studies already showed significant inhibition of $I_{Kr}$ after 3–5 minutes of acute drug superfusion of 1 μM CBD. Higher concentrations of CBD, namely 2.5, 5, and 10 μM CBD led to further significant inhibition of $I_{Kr}$. These results support the concentration-dependent inhibition on $I_{Kr}$ by CBD. The concentration-dependent patch clamp studies revealed an estimated half-maximal effective concentration ($EC_{50}$) value of 4.9 μM.

### 3.2.2 Investigating the inhibitory effect of cannabidiol on slow delayed rectifier potassium current ($I_{Ks}$) in native rabbit left ventricular myocytes

The inhibitory effect of CBD on $I_{Ks}$ was examined in enzymatically isolated left ventricular myocytes from a rabbit heart at 37 °C. The patch clamp studies revealed a significant reduction after the acute superfusion of 1 μM CBD. The current amplitude decreased further after acute superfusion of 2.5 and 5 μM CBD, additionally, the $I_{Ks}$ tail current seemingly vanished after the application of 10 μM CBD. On this basis, CBD depressed the $I_{Ks}$ tail current amplitude in a concentration-dependent manner. The concentration-dependent patch clamp studies revealed an estimated $EC_{50}$ value of 3.1 μM.

### 3.2.3 Examination of the cannabidiol-induced changes on transient outward potassium current ($I_{to}$) and inward rectifier potassium current ($I_{K1}$) in native canine and rabbit left ventricular myocytes

The impact of CBD on $I_{to}$ was studied in canine and rabbit left ventricular myocytes. The whole-cell patch clamp experiments revealed that the $I_{to}$ amplitude in enzymatically isolated canine left ventricular myocytes was significantly reduced by different concentrations of CBD (1, 2,5, 5, 10 μM). However, no change in the current amplitude of native rabbit left ventricular myocytes was detected even in the presence of 10 μM CBD. Various CBD concentrations were applied in isolated canine left ventricular myocytes to estimate the $EC_{50}$ value, including 1 μM, 2.5 μM, 5 μM, and 10 μM. The $I_{to}$ current amplitude was significantly depressed by 5 μM CBD, aligning closely with the estimated $EC_{50}$ value. To exclude the influence of DMSO, a solvent control experiment was performed which revealed that 0.01 % DMSO had no effect on $I_{to}$. Our cellular electrophysiological studies reveled that CBD had no impact on $I_{K1}$.

## 3.3 Investigating the electrophysiological effects of cannabidiol on different inward transmembrane ionic currents: L-type calcium ($I_{CaL}$) and late sodium current ($I_{NaL}$)current in native rabbit left ventricular myocytes

The potential effects of CBD were also studied on $I_{CaL}$ in enzymatically isolated left ventricular myocytes. 10 μM CBD markedly reduced the amplitude of $I_{CaL}$ in a frequency-dependent manner. The potential effect of CBD was studied on $I_{NaL}$ in native rabbit left ventricular myocytes as well. The application of 10 μM CBD led to a significant reduction of the $I_{NaL}$.

### 3.4 Investigating the electrophysiological effects of ibuprofen on transmembrane ionic currents

#### 3.4.1 Effects of ibuprofen on transient outward potassium current ($I_{to}$) and inward rectifier potassium current ($I_{K1}$) in native canine left ventricular myocytes

The amplitude of $I_{to}$ was slightly but significantly reduced after the acute superfusion of 250 μM ibuprofen. To exclude the influence of DMSO, a solvent control experiment was performed. Based on our findings, 0.01 % DMSO did not affect $I_{to}$. As the steady-state current-voltage curves indicate, the $I_{K1}$ remained unchanged after the application of both the solvent and 250 μM ibuprofen.

#### 3.4.2 Effects of ibuprofen on the L-type calcium current ($I_{CaL}$) and the late sodium current ($I_{NaL}$) in native canine left ventricular myocytes

250 μM ibuprofen reduced the $I_{CaL}$ moderately but significantly. The amplitude of $I_{CaL}$ returned to baseline levels after an approximately ten-minute-long drug washout period. To exclude the influence of DMSO, a solvent control experiment was performed. Our findings show that 0.01 % DMSO did not change the current amplitude. In addition, $I_{NaL}$ was also affected by 250 μM ibuprofen. The current amplitude was moderately but significantly reduced. No effect of the 0.01 % DMSO application was observed on $I_{NaL.}$

## 4. DISCUSSION

The main goals of my thesis were the attempt to investigate possible changes of the repolarization reserve in certain situations like chronic endurance training-induced cardiac remodeling and to identify the cannabidiol (CBD) and ibuprofen treatment-evoked cellular cardiac electrophysiological alterations, since these certain situations may be associated with a higher risk of the development of cardiac arrhythmias.

### 4.1 Considerations for the appropriate choice of experimental animal models for the human heart

Since cardiac arrhythmias continue to be a major health burden worldwide, research into the underlying mechanisms is important for the development of novel preventive and therapeutic strategies. The proper choice of an animal model depends mainly on the investigated research question and the nature of arrhythmia. Small rodents are widely used laboratory animals for their advantages; however, they present substantial differences from the cardiac electrophysiological features of the human heart. The heart rate of larger laboratory animals and humans is considerably lower than that of small rodents, such as mice and rats. Some

electrophysiological characteristics of other laboratory animals, like dogs, rabbits, and guinea pigs, share similarities with the human heart. Based on the literature, the left ventricular region of the canine heart has similar cellular electrophysiological properties to human hearts. It is important to note that the guinea pig and rabbit animal models offer the same economic benefits and large sample sizes similar to rats and mice but provide additional advantages. Besides the lower heart rate, the characteristics of QT interval and action potential are similar to larger laboratory animals such as dogs. Therefore, comparable to the canine model, rabbits and guinea pigs are often employed as 'drug-induced long-QT' models in safety pharmacology and toxicology research. However, it should be noted that the $I_{to}$ in native left ventricular myocytes of guinea pig and rabbit hearts is distinct from those of dogs and humans.

## 4.2 The impact of endurance training-induced cardiac remodeling in a small animal athlete's heart model

### 4.2.1 Long-term endurance training-induced structural changes

In our small animal training model, the main structural change involved the dilation and enlargement of the left ventricular myocardium following long-term endurance training. This finding is consistent with the results in larger animal models. Similar to elite athletes, ejection fraction and fractional shortening remained unchanged in the exercised group. Our structural and hemodynamic echocardiographic results correspond to the high dynamic demand training-induced cardiac adaptations among endurance athletes. It has been reported that regular isotonic exercises related to endurance training (i.e., swimming, long-distance running,) commonly lead to cardiac enlargement (increased left ventricular cavity dimension), albeit obvious cardiac hypertrophy (increased left ventricular wall thickness) is not present in every case. Even though some of our measurements turned out to be not statistically significant, the observed overall trends could be valuable to understanding the structural adaptations in the athlete's heart model and can facilitate further investigations.

The prevalence of myocardial fibrosis among athletes who engaged in intense endurance training is an important observation. Our histopathological findings align with the human studies: higher levels of cardiac fibrosis developed after chronic endurance training in the hearts of exercised guinea pigs. An increased level of fibrosis was documented in the left and right atrial, septal, and right ventricular regions of the heart, additionally, a similar nearly significant trend was observed in the left ventricle. Although this type of structural abnormality is undetectable in most cases under the generally used non-invasive methods, it is important to keep in mind that it has an impact the development of re-entry cardiac arrhythmias. Therefore, myocardial fibrosis may predispose to potentially life-threatening cardiac arrhythmias, as an

arrhythmia substrate. Since our working hypothesis focused on electrophysiological alteration due to long-term vigorous training, we did not determine the biological markers or underlying causes of cardiac fibrosis. Additionally, it is noteworthy that highly trained endurance athletes are prone to atrial fibrillation. Fibrotic changes within the atria may contribute to the occurrence of supraventricular arrhythmias.

### 4.2.2 Investigation of chronic endurance training-induced resting bradycardia and beat-to-beat heart rate variability parameters

Sinus resting bradycardia is considered to be a well-recognizable electrophysiological hallmark of long-term high-intensity aerobic training among humans, as well as in animal models of athlete's heart. Our results well correlate with the literature, since the exercised guinea pigs developed significant resting bradycardia. Although resting bradycardia is a general finding, there is considerable debate about the underlying mechanism of training-induced resting bradycardia in recent years. Earlier studies suggested that resting bradycardia is a consequence of increased vagal activity. While other studies took a critical look at the exclusive role of enhanced parasympathetic tone in the development of training-induced resting bradycardia. They proposed that intense exercise induces morphological and electrical intrinsic remodeling of the sino-atrial node (SAN) which plays a crucial role. According to this hypothesis, the role of vagal tone is most probably secondary, or non-existent. In our previous study, we demonstrated that SAN remodeling occurred in the canine model of the athlete's heart. However, our results in guinea pigs seem inconsistent with the latter perspective since no statistically significant difference was detected in spontaneous heart rate between the groups during *ex vivo* Langendorff experiments. These outcomes further support the hypothesis of a notable increase in parasympathetic tone due to long-term endurance training in this model. Although we do not know the appropriate reason of the observed differences between our results and other studies, these can be accounted for methodological and species differences.

Athletes usually have greater heart rate variability than untrained counterparts: an increased heart rate variability, accompanied by decreased resting heart rate, typically indicates an individual's ability to cope well with the training. The present study revealed that exercised guinea pigs, with significant resting bradycardia, developed significantly enhanced heart rate variability parameters, suggesting the effectiveness of the applied training protocol. Heart rate variability parameters are often considered surrogate markers of parasympathetic tone. Consequently, our findings support the idea that resting bradycardia accompanied by the increased heart rate variability parameters in conscious exercised guinea pigs may result from higher parasympathetic activity induced by endurance training.

### 4.2.3 The effect of long-term endurance training on ventricular depolarization and repolarization under in vivo and in vitro circumstances

Our in vivo ECG results correlate with the human data since profound QT interval prolongation was presented in the exercised group by the end of the training protocol. The QTc of conscious exercised animals was slightly but significantly enhanced and a seemingly greater STV-QT variability parameter was observed in conscious exercised guinea pigs at the end of the training protocol. Interestingly, the imprint of QT interval prolongation detected in conscious exercised guinea pigs was not observed in in vitro experiments. The duration of the QT interval did not differ between the groups in ex vivo Langendorff studies. Therefore, the detected *in vivo* QT interval prolongation most likely can be attributed to exercise-induced resting bradycardia resulting from increased parasympathetic tone. Further support of this statement, neither the APD nor the STV-APD was altered in enzymatically isolated left ventricular myocytes of exercised guinea pigs compared to sedentary animals. Our recently published study of canine model of the athlete's heart showed that besides significantly lengthened QT and QTc intervals, prolonged APD and STV-APD developed in exercised dogs. In this study, it was pointed out that decreased amplitude of $I_{to}$ may contribute to the observed prolongation in *in vivo* and *in vitro* experiments as well. Since the $I_{to}$ lacks in the native left ventricular myocytes of the guinea pig's heart, it can limit the electrophysiological translational value to the human hearts. In guinea pig left ventricular myocytes the major repolarizing rate-dependent current is the $I_{Ks}$, which may have a more prominent role than $I_{Kr}$ in repolarization of the guinea pig heart. Based on our cellular electrophysiological data, the amplitude of $I_{Ks}$ did not differ between the examined groups. However, similar to the results of our previous study in the large animal model of athlete's heart, the amplitude of IKs, INaL, and INCX did not differ significantly between the groups. Although it cannot be excluded the control of other uninvestigated currents may have an impact on the altered QT interval and its variability parameters in conscious exercised guinea pigs. Presumably, the electrophysiological differences among the species strongly limit the correlation between the human heart and our small animal model from the perspective of repolarization.

The PQ interval in conscious exercised guinea pigs although showed some tendency to increase but did not reach the level of statistical significance. This may be attributed to the enhanced vagal tone after endurance training; however, further investigations are needed. Similar to our previous study conducted on dogs, long-term training evoked a significantly widened QRS complex in conscious exercised guinea pigs. A widened QRS complex on the ECG suggests an electrical conduction abnormality, and it is associated with a higher risk of arrhythmias. Interestingly, this finding diminished in *ex vivo* Langendorff experiments. Since the observed

broad QRS complex of the conscious exercised guinea pigs and the absence of this phenomenon in *ex vivo* Langendorff experiments are not entirely understood, it would be essential to examine this issue in more depth.

### 4.3 Effects of cannabidiol on cardiac transmembrane ionic channels

The clinical use of CBD has risen in the past few years and, in addition, CBD is becoming a more fashionable dietary supplement worldwide. Although its pharmacokinetic properties have been investigated in several studies, its electrophysiological features, namely its electrophysiological effects on cardiac transmembrane ion currents, which may be associated with potential cardiovascular side effects, are only partially known. Thus, our goal was to investigate the effects of CBD on various transmembrane ionic currents. Application of lower (1, 2.5, 5 μM) and higher (10 μM) concentrations resulted in significant depression of $I_{Kr}$ in native rabbit left ventricular myocytes. Besides the $I_{Kr}$ inhibition, our study showed that $I_{Ks}$ was also inhibited significantly by CBD at lower and highest concentrations as well. Moreover, our experimental design revealed that CBD significantly inhibited $I_{to}$ in isolated canine left ventricular cells at every applied concentration (1, 2.5, 5, 10 μM), but even the highest concentration of CBD (10 μM) did not show any effect in isolated rabbit myocytes. This difference can be attributed to the marked species-dependent expression of the pore-forming protein subunits. The canine model is believed to show a greater level of translational value to humans. Our studies pointed out that CBD can attenuate the repolarization reserve: the use of this cannabinoid may result in APD prolongation and consequent QT lengthening because of the multiple outward potassium channel inhibition. Other major findings of our study were that $I_{CaL}$ and $I_{NaL}$ were inhibited by 10 μM CBD. These observations are in line with the study of others. Therefore, the suppression of these inward currents with 10 μM CBD can counterbalance the inhibited multiple potassium channel-induced repolarization lengthening.

### 4.4 Potential adverse cardiovascular events associated with cannabidiol consumption

Nowadays, CBD is widely used dietary supplement in various formats among amateur and elite athletes because of its alleged or real positive effects since CBD is not prohibited by the World Anti-Doping Agency. Despite its growing popularity, some studies warn athletes to avoid the use of CBD until further research into the efficacy and safety of supplementation is available. Taking into account our experiments, the $EC_{50}$ values for $I_{Kr}$, $I_{Ks,}$ and $I_{to}$ inhibition were 4.9, 3.1, and 5 μM, respectively. These $EC_{50}$ values are higher than the $C_{max}$ values observed in enrolled individuals in the clinical studies. Our findings suggest a small or negligible pro-arrhythmic risk in physiological conditions in healthy individuals. Nevertheless, higher $C_{max}$

values may occur in certain individuals with markedly slower drug elimination due to concomitant diseases or with concomitant use of other drugs that inhibit the metabolism of CBD, therefore caution should be advised. When the intake of CBD is combined with pharmacological agents that affect cardiac repolarization, as well as in certain pathophysiological situations in which cardiac repolarization reserve is attenuated or drug metabolism is impaired, CBD may have an additive effect, further increasing the proarrhythmic risk and the possible incidence of SCD.

Evidence from several clinical studies suggests that athletes who engage in chronic high-intensity exercise appear to experience irregular heart rhythm, which in rare and sudden cases can be fatal. Considering the experiments in guinea pigs presented in my thesis, as well as our results in trained dogs in our previous work, it is important to note that the effects of electrophysiological cardiac remodeling induced by long-term heavy endurance exercise and the use of CBD can be additive, and may result in life-threatening cardiac arrhythmias. Therefore, CBD-containing products should be used with appropriate caution.

The cardiovascular effects of CBD may only be partially due to its impacts on transmembrane ion channels, therefore, further studies are needed to evaluate the adverse cardiovascular effects of CBD and other cannabinoids both *in vivo* and *in vitro* studies, with a particular focus on the benefit-risk assessment of products with different cannabinoid content.

### 4.5 Investigating the alterations in transmembrane ionic current magnitude induced by therapeutic concentration of ibuprofen

Interestingly ibuprofen has long been one of the most commonly used over-the-counter (OTC) and prescribed non-steroidal anti-inflammatory drug (NSAID) on the market, its electrophysiological effects are poorly investigated yet. Therefore, we aimed to investigate the electrophysiological characteristics of ibuprofen under *in vitro* circumstances.

In our study, 250 μM (51.5 μg/mL) moderately but significantly decreased the amplitude of $I_{to}$. Our previous study found that 250 μM ibuprofen significantly inhibited the $I_{Kr}$ in canine left ventricular myocytes. Neither the kinetic nor the measured amplitude of $I_{K1}$ was affected by the applied concentration of ibuprofen. Since cardiac repolarization is determined not only by outward potassium currents, the effect of ibuprofen was also studied on $I_{NaL}$ and $I_{CaL}$ in isolated canine left ventricular myocytes. At a concentration of 250 μM, ibuprofen moderately, but statistically significantly decreased the amplitude of both inward transmembrane ionic currents. Based on our study, in normal situations and at therapeutically relevant concentrations, ibuprofen exerted no or only a moderate prolongation of repolarization, but in situations where

the repolarization reserve was attenuated, the degree of repolarization lengthening was further increased.

A previous study pointed out that ibuprofen dose-dependently shortened the APD and the effective refractory period on fast and slow response action potentials in guinea pig ventricular papillary muscle preparations. It might be speculated that $Na^+$ and $Ca^{2+}$ transmembrane ionic channels are dose-dependently suppressed by ibuprofen. In our present study, we could confirm the hypothesized inhibition of $I_{CaL}$ and $I_{NaL}$. Contrarily, another study found that diclofenac, but not ibuprofen inhibited $I_{NaL}$ and $I_{CaL}$ in a dose-dependent manner in rat ventricular myocytes.

The dissimilarities between the studies can be attributed to the different baseline electrophysiological features of the used species in respective studies (i.e., rabbit, neonatal rat, or dog), and differences in the experimental conditions (room temperature vs 37 °C). Unlike dogs, left ventricular guinea pig myocytes are unique due not only to their total lack of native $I_{to}$ but the very strong expression of $I_{Ks}$ as well. The ibuprofen-induced inhibition of $I_{CaL}$ and $I_{NaL}$ in the guinea pig ventricle would alter the balance of outward and inward currents, and favor a relative enhancement of outward currents, resulting in shortened repolarization. The opposite effect is expected in the mid-myocardial region of the left ventricle of rabbit and canine hearts, where the density of $I_{Ks}$ is weaker than in guinea pigs, therefore, $I_{Kr}$ should make a stronger contribution to the repolarization. Additionally, it is well-established that $I_{to}$ is a key regulatory current in the generation of action potentials and repolarization in cardiac midmyocardial cells with appropriate plateau phase morphology. Consequently, the co-inhibition of $I_{Kr}$ and $I_{to}$ can lead to attenuated repolarization reserve.

### 4.6 Increased risk of arrhythmic events associated with ibuprofen intake

A recent nationwide case-time-control study reported that the use of two NSAIDs, namely diclofenac and ibuprofen, is associated with an increased risk of out-of-hospital cardiac arrest and consequent sudden cardiac death. Athletes are also frequent consumers since they often seek new ways to enhance their athletic performance, therefore different NSAIDs provide opportunities to amateur and professional athletes not just for pain relief but also for increased pain tolerance and faster recovery. Some studies reported that NSAIDs, especially ibuprofen, are common among endurance athletes (i.e., marathon runners, and soccer players) who usually take them daily to achieve the desired effect. According to the literature, athletes frequently use higher doses of ibuprofen than the recommended therapeutic dose. For instance, based on a recently published survey, nearly 90 % of the runners who participated in Parkrun UK used NSAIDs, usually in the form of OTC ibuprofen. Moreover, most of them used NSAIDs directly before, during, and after the race to relieve pain and to compete more effectively. Despite

valuable studies joining the growing evidence that ibuprofen and other NSAID consumption before a workout does not offer any benefit and causes disagreeable damage, particularly to the intestines, adverse cardiovascular events are poorly highlighted. Despite that, some studies have drawn attention to the greater risk of adverse cardiovascular events, such as myocardial infarction, and sudden cardiac arrest, associated with larger doses and/or chronic use of these drugs. Although the mechanism of these tragic events can be diverse, the possible direct modulation of these drugs on the transmembrane ionic channels should also be considered.

In our study, the applied concentration of ibuprofen in our experiments was relatively well-fitted to the therapeutic range in patients.

Based on our cellular electrophysiological findings, ibuprofen seems a relatively safe drug under normal circumstances. However, under certain conditions characterized by attenuated repolarization reserve, like the athlete's heart presented also in this thesis, ibuprofen may enhance proarrhythmic risk and may even contribute to the incidence of adverse cardiovascular events, such as arrhythmias and even sudden cardiac arrest. This possibility should be considered and addressed in clinical practice, given that ibuprofen is a commonly used OTC drug taken daily by millions of people without any medical supervision.

## 5. CONCLUSION

The most important new results in this Ph.D. thesis are the followings:

1. In exercised guinea pigs, long-term endurance training decreases the repolarization reserve, but unlike other experimental animal models of the athlete's heart, it is mainly attributed to the chronic endurance training-induced enhanced vagal tone, not to the electrophysiological remodeling.
2. Similar to other animal models of athlete's heart and human data, long-term heavy endurance training evoked a significant level of cardiac fibrosis in the exercised guinea pigs, which may enhance proarrhythmic risk.
3. Cannabidiol (CBD) depresses the amplitude of several transmembrane ionic currents, such as $I_{to}$, $I_{Kr}$, $I_{Ks}$, $I_{CaL}$, and $I_{NaL}$, which can attenuate the repolarization reserve in the ventricle.
4. Ibuprofen decreases different transmembrane ionic currents, such as $I_{to}$, $I_{CaL}$, and $I_{NaL}$ which can lead to decreased repolarization reserve in the ventricle.

The present study introduced a novel guinea pig exercise-induced athlete's heart model. In our model, similar to the endurance-trained human athlete's heart, long-term endurance training led to increased left ventricular end-diastolic diameter and moderate enlargement of cardiac muscle due to increased volume load. In addition, mild fibrosis was also present that may also occur in human athlete's hearts according to recently published studies. The training program also led

to electrophysiological changes in conscious exercised guinea pigs. The *in vivo* resting bradycardia and increased heart rate variability parameters together with unchanged *ex vivo* results indicated a higher resting vagal tone in exercised animals. The widened QRS interval in conscious exercised animals may be associated with structural and functional remodeling of the heart. The significantly prolonged QT interval due to decreased resting heart rate can be related to the increased vagal tone in exercised animals. Besides the observed QT interval prolongation, conscious exercised guinea pigs developed slightly increased QT variability parameters, which may indicate impaired repolarization and higher repolarization instability.

These observations do not necessarily indicate that at a competitive level, endurance exercise is harmful since the evidence regarding the beneficial effect of exercise is overwhelming. However, in certain individuals or in situations where the repolarization reserve is impaired due to hidden diseases, such as hypertrophic cardiomyopathy, long QT-syndromes, electrolyte imbalances, doping substances, or any seemingly harmless drugs, the observed significant resting bradycardia, alterations of *in vivo* depolarization and repolarization, and mild fibrosis induced by endurance training in our study may present additional potential risk factors to be considered in the prevention of possible adverse events in competitive sport.

Athletes often seek different avenues to reach greater sporting achievements. Therefore, they often use different agents, for instance, CBD or NSAIDs, like ibuprofen to accomplish their goals. CBD has a prominent impact on the cellular level of the heart. It decreases the amplitude of various potassium currents each of which plays a prominent role in left ventricular repolarization. CBD significantly depressed the amplitude of $I_{to}$, $I_{Kr}$, and $I_{Ks}$ of enzymatically isolated left ventricular myocytes at lower (1, 2.5, 5 μM) and higher (10 μM) concentrations. The inhibition of multiple potassium currents can evoke prolonged action potential duration and consequently prolonged QT interval. Our study suggests that potential prolongation of the repolarization can be counterbalanced by the 10 μM CBD evoked inhibition of $I_{CaL}$ and $I_{NaL}$.

The alterations of transmembrane ionic currents at lower concentrations of CBD could decrease the repolarization reserve of the cardiac action potentials contributing to the proarrhythmic risks of CBD resulting in cardiac arrhythmias or even sudden cardiac death.

Based on our study, ibuprofen has a slight impact on different transmembrane ionic currents, including $I_{to}$, $I_{NaL}$, and $I_{CaL}$ in the therapeutic concentration. Consequently ibuprofen is a relatively safe drug in normal situations. Despite that, because of the easy availability of different OTC products, ibuprofen can be easily administered above the therapeutic range. In certain conditions characterized by attenuated repolarization reserve, ibuprofen may enhance proarrhythmic risk, and may even contribute to the incidence of SCD observed in clinical

studies. This possibility should be taken into account in clinical practice since ibuprofen is taken every day by several million people without medical control.

In conclusion, these agents should be consumed with caution by athletes, as they can attenuate the repolarization reserve and, together with the cardiac structural-electrophysiological changes that can result from long-term vigorous training, may lead to life-threatening arrhythmias.

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