ARRHYTHMIA ANALYSIS IN LANGENDORFF PERFUSED HEARTS: SIGNIFICANT CONTRIBUTION TO MODEL CHARACTERISATION AND VALIDATION OF ARRHYTHMIA DEFINITIONS

Summary of PhD thesis

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THE THESIS IS BASED ON THE FOLLOWING PAPERS

I. Takács, H; Kui, P; Farkas, A S; Sarusi, A; Forster, T; Papp, J Gy; Varró, A; Curtis, M J; Shattock, M J; Farkas, A; Ventricular cycle length irregularity affects the correlation between ventricular rate and coronary flow in isolated, Langendorff perfused guinea pig hearts. *Journal of Pharmacological and Toxicological Methods. 77 pp. 45-52., 8 p. (2016).*
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II. Regev, A; **Takacs, H**; Farkas, A S; Rarosi, F; Polyak, A; Papp, H; Ivany, E; Papp, J Gy; Varro, A; Farkas, A; Application of ventricular tachyarrhythmia definitions of the updated Lambeth Conventions provides incompatibility with earlier results, masks antifibrillatory activity and reduces inter-observer agreement. *Journal of Physiology and Pharmacology 70: 1 (2019).*
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FURTHER RELEVANT PAPERS

III. Kui, P; Orosz, S; **Takács, H**; Sarusi, A; Csík, N; Rárosi, F; Csekő, C; Varró, A; Papp, JG; Forster, T; Farkas AS; Farkas A; New in vitro model for proarrhythmia safety screening: IKs inhibition potentiates the QTc prolonging effect of IKr inhibitors in isolated guinea pig hearts. *Journal of Pharmacological and Toxicological Methods 80 pp. 26-34., 9 p. (2016).*
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IV. Papp, H; Sarusi, A; Farkas, AS; **Takacs, H**; Kui, P; Vincze, D; Ivany, E; Varro, A; Papp, JG; Forster, T; Farkas A; Hyperventilation assists proarrhythmia development during delayed repolarization in clofilium-treated, anaesthetized, mechanically ventilated rabbits. *Journal of Physiology and Pharmacology 67: 5 pp. 731-737. , 7 p. (2016).*
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QUOTABLE ABSTRACTS

I. Takács, H; Farkas, AS; Forster, T; Varró, A; Farkas, A; Az új Lambeth convenciós módosított kamrafibrilláció definíciója megváltoztathatja az aritmiavizsgálatok konklúzióját. (The new definition of ventricular fibrillation of the upgraded Lambeth conventions may substantially alter the conclusions of arrhythmia investigations); *Cardiologia Hungarica 44: Suppl. E pp. E38-E39. (2014)*

II. Takács, H; Kui, P; Farkas, A; Forster, T; Varró, A; Farkas, A; A kamrai irregularitás okozta koronáriaáramlás-növekedés mechanizmusa izolált, langendorff perfundált tengerimalac szívben (The mechanism of increased coronary flow caused by ventricular irregularity in isolated, langendorff perfused guinea pig hearts); *Cardiologia Hungarica 45: Suppl. D pp. D25-D26. (2015)*

III. Kui, P; **Takács, H**; Morvay, N; Leprán, I; Farkas, A; Varró, A; Forster, T; Farkas, A; Sportszív kamrahipertrófia modell beállítása nyulakban (Setting up an athlete's heart model in rabbits); *Cardiologia Hungarica 45: Suppl. D p. D34 (2015)*

IV. Prorok, J; Kui, P; **Takács, H**; Oravecz, K; Hézső, T; Polyák, A; Farkas, AS; Papp, JGy; Varró, A; Tóth, A; Acsai K; A szelektív NCX-gátlás csökkenti a hypokalaemia és a kamrafibrilláció által okozott miokardiális diszfunkciót. (Selective NCX inhibition reduces myocardial dysfunction associated with hypokalaemia and ventricular fibrillation); *Cardiologia Hungarica 46: Suppl. F p. F60 (2016)*

V. Kui, P; **Takács, H**; Morvay, N; Leprán, I; Tiszlavicz, L; Nagy, N; Ördög, B; Farkas, A; Forster, T; Varró, A; Farkas AS; Repolarizációs érzékenység vizsgálata nyúl sportszív-modellben. (Examination of the repolarization sensitivity in a rabbit athlete's heart model); *Cardiologia Hungarica 46: Suppl. F p. F40 (2016)*

VI. Kui, P; **Takács, H**; Morvay, N; Leprán, I; Tiszlavicz, L; Nagy, N; Ördög, B; Farkas, A; Forster, T; Varró, A; Farkas AS; Proaritmia érzékenység vizsgálata nyúl sportszív modellben (Assessment of proarrhythmia sensitivity in a rabbit athlete's heart model); *Sportorvosi Szemle 57: 1 p. 7 (2016)*

VII. Kui, P; **Takács, H**; Polyák, A; Morvay, N; Leprán, I; Nagy, N; Ördög, B; Farkas, A; Forster, T; Varró, A; Farkas AS; Investigation of electrophysiolological abnormalities in a rabbit athlete's heart model; *Cardiovascular Research 111: Suppl. 1 p. S101 (2016)*

Introduction

Cardiac arrhythmias and their impact to the heart and its function are important questions in cardiovascular research. Sudden cardiac death caused by ventricular arrhythmias is still a major public health problem in modern industrialized countries. On the other hand many patients live with irregular ventricular rate caused by either frequent ventricular or atrial arrhythmias. Irregular ventricular rate may be harmful in the long term, e.g. it is well documented that frequent ventricular premature beats (VPB) can lead to development of cardiomyopathy.

Isolated, Langendorff perfused mammalian hearts, which was described by Oscar Langendorff more than 100 years ago, are valuable tools in experimental heart research even today. In our investigations, we used the Langendorff perfused heart model for arrhythmia investigations, and the data of the arrhythmia analysis served for clarifying and characterising the physiology of the model and also, to validate arrhythmia definitions.

Our first study was about understanding the relationship between ventricular rhythm and coronary flow autoregulation in experimental preparations such as the Langendorff. In *in vivo* conditions coronary flow is regulated by a combination of many factors. Irregular ventricular rhythm may have an independent effect on coronary flow via modifying the work-induced autoregulation of the coronary arteries. However, this has never been examined, and data about the well-known positive correlation between ventricular rate and coronary flow have been obtained from hearts free of arrhythmias.

Currently, there are clinical and experimental guidelines dealing with arrhythmia research and management, but these guidelines do not use unified arrhythmia definitions, and that is a notable problem when we try to objectively evaluate and compare results of arrhythmia investigations. The Lambeth Conventions (LC I), a guidance for research on arrhythmias published in 1988, had a substantial impact on the experimental arrhythmia research; the paper has been cited more than 1000 times since its publication 30 years ago according to the database of Web of Science. However, the advances in technology, development of monitoring and pharmacologic solutions and of course the extensive research about arrhythmias had finally led to the realization that LC I was in need to be updated. The revised Lambeth Conventions (LC II) were intended to be applied in preclinical and clinical research. Authors of LC II invited investigators to state whether or not they had used the conventions in their studies, and to test their validity by experiment. Importantly, there are

substantial changes in the definitions of the ventricular tachyarrhythmias between the original and the updated Lambeth Conventions.

Aims of the studies

The aim of the first study was to determine the effect of beat-to-beat variability of ventricular cycle length on coronary flow in isolated, Langendorff-perfused guinea pig hearts. Under these conditions coronary flow is regulated independently of perfusion pressure and autonomic nervous system, and only intramural pressure during the cardiac cycle and work-dependent autoregulation determine coronary resistance. As ventricular irregularity was found to significantly affect coronary flow in the present investigation, the mechanism was examined in a further set of experiments performed in Langendorff perfused guinea pig hearts.

The aim of the second study was to examine whether the arrhythmia definitions of LC I and LC II are compatible, and yield the same qualitative arrhythmia results. Also, it was tested whether arrhythmia definitions of LC I or LC II allow better inter-observer agreement. The arrhythmia data obtained by applying arrhythmia definitions of LC I and LC II were compared to test the compatibility between the arrhythmia definitions of LC I and LC II. Also, inter-observer agreement was determined to investigate whether arrhythmia definitions of LC I or LC II allow better agreement on the arrhythmia results between the two independent observers.

Methods

ECG analysis, measurement of the RR intervals and calculation of variability of the ventricular cycle length

In the first study female guinea pigs were used. Their hearts were perfused according to Langendorff with modified Krebs-solution. Two sets of experiment were performed. In the first set, ECG was recorded and coronary flow was measured by timed collection of coronary effluent and the flow value was corrected to the ventricle weight. The RR intervals were measured irrespective of rhythm even during arrhythmias in a 30-second-long sampling period. The beat-to-beat variability of the RR intervals was quantified by the root mean square of the successive differences of the RR intervals (RMSSD). These hearts were divided into two groups based on RMSSD value as the ‘Low’ RR variability group (RMSSD < 3 ms; n=50 hearts), and the ‘High’ RR variability group (RMSSD > 3 ms; n=37 hearts). The beat-to-beat variability of

the cycle length (RMSSD), the per cent frequency of the arrhythmic beats, the mean ventricular rate and the coronary flow were compared between the 'Low' and 'High' RR variability groups.

Measurement of the duration of perfused and non-perfused intervals in every cardiac cycle

In order to examine whether elevated RR interval variability increased the ratio of durations of perfused intervals to non-perfused intervals in the Langendorff preparation, a second set of experiments was performed. Left ventricular pressure was recorded via a thin medical needle stuck through the apex of the heart, and attached to a plastic cannula filled with saline. Perfusion pressure (aortic pressure) was recorded via a side arm at the bottom of the perfusion column where perfusion cannula was connected to the aorta. "Real-time" aortic flow was measured by an ultrasonic flow meter implanted to the bottom of the perfusion column, closely above the aortic stump. Volume-conducted ECG, real-time aortic flow, aortic and left ventricular pressures were recorded continuously. RR interval variability (RMSSD of the RR interval), mean ventricular rate and mean aortic flow were determined in every 30-second-long interval during the 60-minute-long perfusion period in each heart. 18 hearts thus replicated the RR interval variability and mean ventricular rate of the 'Low' and 'High' RR variability groups in the first set of experiments, with 9 hearts allocated into a 'Low' RR interval variability group (RMSSD < 3 ms), and the other 9 to a 'High' RR interval variability group (RMSSD > 3 ms). An analysis of the durations of perfused and non-perfused intervals in the 18 hearts was performed in a blinded manner. The number of beats in which left ventricular pressure did not exceed perfusion pressure during systole, and the duration of the non-perfused intervals during each systole were determined. The ratio of the perfused to non-perfused intervals was calculated by dividing the cumulative duration of the perfused intervals by the cumulative duration of the non-perfused intervals in the 30-second-long sampling interval at the time point of the measurement.

Reanalysing ischaemic arrhythmias according to definitions of LC I and LC II, measurement of the number and incidence of tachyarrhythmias

In the second study we reanalysed the ECG recordings of a previous investigation utilizing isolated, Langendorff perfused rat hearts subjected to a 30-min-long local ischaemia in the left ventricle. The ECG recordings of the original investigation done by Farkas and Curtis were reanalysed by two independent investigators using LabChart7. Ventricular arrhythmias were defined according to the LC I for the primary evaluation. The number of VT and VF episodes, the incidence and the time to onset of VT and VF were determined from the ECG

recorded during the 30-min-long ischaemia. Then the whole analysis was repeated according to the arrhythmia definitions of LC II. Arrhythmia incidences were expressed as percent values. The intra-observer agreement on arrhythmia incidence data obtained according to LC I and LC II was assessed with Cohen's kappa statistic. Similarly, the inter-observer agreement on VT and VF incidence data among independent observers was calculated with Cohen's kappa statistic.

Results

Results of the first study – Understanding the relationship between ventricular cycle length irregularity and coronary flow in Langendorff perfused guinea pig hearts

The beat-to-beat variability of the ventricular cycle length was tested as an independent variable influencing coronary flow. RMSSD values were significantly greater in the 'High' RR variability group, and the per cent frequency of arrhythmic beats was significantly greater in the 'High' RR variability. Importantly, the mean ventricular rate did not differ significantly between the two groups, whereas the coronary flow was significantly greater in the 'High' RR variability group. The relationship between mean ventricular rate and coronary flow was tested, too. A significant positive linear correlation was found between these variables in the 'Low' RR variability group, but with a low correlation coefficient. A similar relationship was seen in the 'High' RR variability group. However, the slope of the regression line was significantly greater in the 'High' RR variability group, with consequentially greater coronary flow values in the 'High' RR variability group in the physiological heart rate range for guinea pig (210-280 1/min).

The RMSSD, mean ventricular rate and coronary flow values measured in the first set of experiments were reproduced in the second set of experiments. The analysis of the real time aortic flow signal, the perfusion pressure and the left ventricular pressure in each cardiac cycle revealed that left ventricular pressure exceeded perfusion pressure in most beats in hearts with 'Low' RR variability, associated with a transient reversal of the direction of aortic flow. The average (per beat) duration of the non-perfused interval (in which left ventricular pressure exceeded perfusion pressure) did not differ significantly between the 'High' and 'Low' RR variability groups. However, cumulative number of beats that lacked non-perfused interval was greater in the 'High' RR variability group. Consequently, the cumulative duration of the perfused intervals was significantly greater in the 'High' RR variability group. Thus, ventricular irregularity significantly increased the ratio of the perfused to non-perfused intervals. Usually,

the beats that lacked non-perfused interval were atrial or ventricular premature beats and beats that follow 'post-extrasystolic potentiation' beats.

Result of the second study – Comparing arrhythmia results obtained according to the old and the updated Lambeth Conventions, validation of arrhythmia definitions

When the arrhythmia definitions of LC I were applied, both of the two independent investigators found that only flecainide was able to reduce VF incidence significantly at the applied low concentration. Also all three drugs reduced VF incidence significantly at the applied high concentrations. Both investigators found that the elevation of the K^+ concentration from 3.0 mM to 5.0 mM significantly reduced the incidence of ischaemic VF. These results are in a good accordance with the arrhythmia results of the original investigation. When arrhythmia definitions of LC II were applied, both of the two independent investigators found that none of the drugs affected significantly VF incidence at the applied low concentrations. Observer B found that the high concentrations of all three drugs reduced the incidence of ischaemic VF. However, Observer A found that the applied high concentration of flecainide did not significantly reduce the incidence of ischaemic VF, which qualitatively differs from the results obtained according to LC I, and also the results of Observer B. Furthermore, the marked antiarrhythmic effect of the increased K^+ concentration was also masked when arrhythmia definitions of LC II were applied.

Intra-observer agreement was tested; Observer A achieved good intra-observer agreement on VT incidence. However, Observer B achieved only moderate intra-observer agreement in VT incidence. Both observers found that VF was diagnosed in a significantly greater proportion of hearts, when the arrhythmia definitions of LC II were applied. Intra-observer agreement on VF incidence data obtained according to LC I and LC II was only moderate in case of both observers. The inter-observer agreement on VT and VF incidences among independent observers was calculated. Applying VT definition of LC II did not remarkably improve the inter-observer agreement on VT incidence between the observers. Importantly, when VF incidence obtained according to LC I was tested, very strong inter-observer agreements were found among the two independent investigators and the investigator of the original investigation. When arrhythmia definitions of LC II were applied, a good agreement was found in VF incidence between the two independent investigators; however, the kappa value was lower than that obtained according to LC I.

Both observers found that VF onset time was significantly reduced in the groups, when arrhythmia definitions of LC II were used as compared with the respective values obtained by

applying arrhythmia definitions of LC I. The two independent observers also found that applying arrhythmia definitions of LC II significantly reduced the onset time of the first episode of VF as compared with the value obtained by using arrhythmia definitions of LC I.

We also examined whether applying arrhythmia definitions of LC II causes an 'arrhythmia shift' from the VT class to the VF class. Those eight hearts of the flecainide treated group of the first set of experiments were analysed, in which neither Observer A nor Observer B found VF according to LC I, but both of them identified VF according to LC II. Our results suggest that when arrhythmia analysis was performed according to LC II, VT episodes were not only shifted to the VF class, but were also fragmented (i.e. broken up into shorter episodes due to containing diastolic pauses).

Discussion

Understanding the correlation between increased beat-to-beat variability of ventricular cycle length and coronary flow in Langendorff perfused hearts

The linear correlation between mean ventricular rate and coronary flow reflects the established relationship between coronary blood flow in vivo and myocardial oxygen and nutrient demand. One interesting implication from the present study is confirmation that autoregulation is largely autonomic-independent (Langendorff hearts are denervated). The relationship between cardiac work and the oxygen supply is likely to be of critical importance to this. Exercise-induced tachycardia results in an increased coronary blood flow due to decreased coronary vascular resistance, as shown in several species. Increased production of adenosine and other endogenous vasodilator substances, such as bradykinin, atrial natriuretic peptide or nitric oxide have been reported to contribute to this. Beat-to-beat variability of the ventricular cycle length is referred to '*heart rate variability*' which is a biomarker of vagal nerve activity *in vivo*. However, the present experiments were performed in isolated, Langendorff perfused hearts (denervated, in the absence of the rest of the animal). Thus, the effect of cycle length variability on coronary flow was not mediated by parasympathetic activity or any effect of the autonomic nervous system.

The results of the second set of experiments showed that the positive effect of ventricular irregularity on coronary flow in the physiological heart rate range was mediated via changes in left ventricular pressure, presumably via ventricular compression of coronary arterioles. In regular rhythm, left ventricular (and intramural) pressures overcame perfusion pressure in systole, reversing the direction of aortic flow for a short period during systole in

each beat. In contrast, a single premature beat produced at least two beats, in which left ventricular pressure remained below the perfusion pressure in systole. This suggests that the positive effect of ventricular irregularity on coronary flow is specific to the isolated heart preparation perfused at constant pressure, and it does not necessary involve any change in work-induced autoregulation. Real-time aortic flow, perfusion pressure and left ventricular pressure signals imply that left ventricle ejected during systole in regular rhythm. A crucial question emerges: how does the perfusion fluid enter into the left ventricle in Langendorff perfused hearts? According to the original description by Langendorff, when perfusion solution flows retrogradely to the aorta, the aortic valve is closed, therefore the left ventricle should not be filled with perfusion solution. One explanation is that the aortic valve was incompetent in the present experiments. Technical failure (e.g., valve damage during insertion of the aortic cannula) is unlikely as special care was taken during preparation to avoid this. Also, the dicrotic notch in the aortic pressure signal implies that aortic valves were functioning normally. A more plausible explanation is that that the intact (not disrupted) aortic valve does not close properly in isolated hearts perfused with constant pressure, and there is a considerable leakage of perfusion fluid into the left ventricle in normal circumstances. Our results show that the Langendroff perfused guinea pig heart is loaded. Furthermore, results of the present investigation imply that ventricular irregularity disturbs the balance between load and ejection, which consequently affects left ventricular and intramural pressures, and thus, influences coronary flow in isolated hearts.

Isolated Langendorff-perfused hearts are frequently used for proarrhythmia investigations, and ancillary readout such as coronary flow helps form an integrated risk assessment. The present results indicate that drug-induced arrhythmias may affect coronary flow independently of heart rate in isolated hearts, meaning any observed flow changes will include a component that is entirely independent of direct coronary vascular actions and rate-dependent vascular tone changes.

Comparing arrhythmia results obtained according to the old and the updated Lambeth Conventions by using arrhythmia data from Langendorff perfused hearts

VF definition of LC II diagnosed VF in all hearts, in which VF was identified according to LC I. Moreover, there were many other hearts, in which VF was detected only according to LC II. Also, applying VF definition of LC II did not increase VF onset time in any of the hearts, however reduced this variable in the majority of the hearts. These results show that the VF class of LC II incorporated and enlarged the VF class defined by LC I. The reason behind this is that

VF definition of LC II not only identified VF episodes detected by LC I, but also identified 'de novo' VF episodes not detected according to LC I.

Comparing tachyarrhythmia definitions of LC I and LC II explains the mechanism of detection of 'de novo' VF episodes according to LC II. While arrhythmias containing 4 or more consecutive ventricular premature beats with non-progressive variation in peak-peak interval, height and intrinsic shape should be classified as VTs according to LC I, the same arrhythmias are now shifted to the VF category of LC II. These polymorphic VTs of LC I form the 'de novo' VF episodes detected by LC II. This 'arrhythmia shift' of LC II is also indicated by the data showing that applying arrhythmia definitions of LC II reduced the number of VT episodes and simultaneously increased the number of VF episodes.

The analysis of the VF onset times in the first set of experiments showed that application of the VF definition of LC II increases VF detection in all groups including control and drug treated groups, too. These results imply that the change in VF definition may retrospectively change the results of previous pharmacological studies analysed according to LC I.

The physiological and pathophysiological effects of potassium in the development of arrhythmias have been intensively studied in earlier investigations. Arrhythmias following coronary occlusion may depend on factors such as serum potassium. Hyperkalaemia is antiarrhythmic during myocardial ischemia and infarction in man. An association between low serum potassium concentrations and ventricular arrhythmias has also been observed by a number of investigators and an increased frequency of ventricular fibrillation in patients with low serum potassium concentrations was also demonstrated. When we applied the VF definition of LC II, the VF incidence was very high in the control groups irrespective of the K^+ concentration of the Krebs solution. This suggests that increased sensitivity of VF detection according to LC II coincided with markedly reduced specificity. Consequently, application of VF definition of LC II substantially changed the conclusion about the physiological and pathophysiological effects of K^+ concentration on VF development.

Our results show that application of the VF definition of LC II may invalidate a widely known and accepted concept of pathophysiology of arrhythmia development, and also invalidate earlier investigations based on this concept. This is a warning sign, which suggests that the tachyarrhythmia (VT and VF) definitions of LC II should be abandoned or at least amended in order to provide greater compatibility with the results of earlier investigations.

Present investigation provides a strong evidence that VF results are well reproducible and the inter-observer agreement is high, when the VF definition of LC I is applied. This suggests that the VF definition of LC I is clear and it is not necessary to be changed in order to

provide greater objectivity during arrhythmia evaluation. This aim was clearly not fulfilled by the new VF definition of LC II, as applying VF definition of LC II resulted in less inter-observer agreement on VF incidence. Furthermore, VT definition of LC II did not remarkably improve inter-observer agreement on VT incidence.

Our results provide the first clear cut evidence that by changing the definition of VF the analysis of the same ECG recordings may yield a completely different conclusion. We also provide the first clear-cut evidence that applying VF definition of LC II may change the conclusion not only in pharmacological but also in physiological and pathophysiological arrhythmia investigations. These suggest that applying VT and VF definitions of LC II does not improve compatibility of the results of various research groups and investigators, which further emphasizes the need for an amendment of the VT and VF definitions of LC II.

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