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Beat to Beat Variability of Embryonic Chick Heart Cells under Septic Conditions: Application and Evaluation of Entropy as well as Fractal Measures*

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Abstract— **Extracardiac factors of heart rate variability have commonly been investigated using linear and nonlinear methods for a long time. Recently, intracardiac mechanisms on an electrophysiological basis have been found to be also important. This work is focused on the evaluation of complex measures of temporal signals gained with microelectrode measurements of embryonic chick heart aggregates. Septic conditions were mimicked *in vitro* by lipopolysaccharide (LPS) administration in order to investigate the influence on beat to beat variability. Surrogate data analysis revealed high statistical significances for normalized complexity measures.**

I. Introduction

Heart rate variability (HRV) has extensively been studied for cardiovascular diseases and is meanwhile an important physiological parameter with high prognostic impact. The source of HRV can be autonomous or a baroreflex feedback loop, but recent reports have uncovered that intracardiac processes seem to be very important, too [1, 2]. In line with this it has been recently shown, that lipopolysaccharide (LPS), a major component of the outer wall of Gram-negative bacteria, impairs the pacemaker current I_f, which in turn potentially contributes to HRV reduction under septic conditions [3]. I_f, a determinant of diastolic depolarisation and pacing rate, is mainly encoded by the hyperpolarisation activated cyclic nucleotide gated HCN4 isoform in mammalian sinoatrial node [4]. Since beat to beat variability (BBV) of the cardiac tissue itself partly reflects HRV, it seems to be adequate to investigate BBV using methods commonly applied for HRV analysis. In order to investigate the effect of LPS on BBV we measured spontaneous action potentials in cultured embryonic chick heart cell aggregates, expressing HCN4 encoded pacemaker channels [5] and providing well defined biological conditions without any extrinsic influences.

Theoretical investigations of HRV mostly assume fractional Brownian motion (*fBm*) as the underlying process, but care has to be taken in order to separate the temporal signals from fractional Gaussian noise (*fGn*) [6]. Power spectral densities can be calculated and the slope of a double logarithmic plot enables to determine the Hurst coefficient. Other methods investigate long range correlations directly in the time domain. For instance, nonlinear methods such as entropy measures, particularly approximate entropy, sample entropy as well as the Higuchi dimension are well established. Although these time domain methods cannot distinguish between distinct types of signals (*fBm* or *fGn*), they can be used to investigate complexity or long range correlations, especially for short data series. Normalization of entropy and Higuchi dimension measures turned out to increase statistical significances, which were tested by applying a thorough surrogate data evaluation. The hypotheses of this report are that LPS reduces the beat to beat variability in embryonic chick cell aggregates and that entropy and fractal measures are able to discriminate these biologically motivated conditions. The pacemaker current I_f may be involved in the LPS effect of reducing beat to beat variability.

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II. Methods

A. Electrophysiological Measurements

Chick ventricular myocytes were isolated from 7-day old embryos by techniques previously described [7]. Spontaneous action potentials were recorded by conventional microelectrode technique [8]. Pacemaker current I_f was measured with the patch-clamp technique in the whole-cell voltage -clamp mode [3].

B. Power Spectral Analysis

The power spectrum was evaluated according to Eke et al. [6]. The discrete signals $X(i)$ with length N were preprocessed by parabolic windowing using following weighting factors

$$w(i) = 1 - \left(\frac{2i}{N+1} - 1\right)^2 \qquad i = 1,\ldots..,\ N\ , \tag{1}$$

and bridge detrending by subtracting the line connecting the first and the last point of the data series. Then, discrete Fourier transformation (DFT) was performed yielding the power spectral density $P(f)$. A double logarithm plot of the spectrum was created and a linear regression line was calculated. Assuming a power law

$$P(f) \propto f^{-\beta}\ , \tag{2}$$

the slope β (spectral index) was calculated. Two distinct regions of β can be distinguished:

$$fGn\ :\ -1 > \beta < 1 \tag{3}$$

$$fBm : 1 < \beta < 3 \tag{4}$$

Fractional Gaussian noise *fGn* can be negatively as well as positively correlated, whereas fractional Brownian motion *fBm* can only be positively correlated.

The special case $\beta = 0$ indicates Gaussian noise without any correlation and $\beta = 2$ indicates Brownian motion (Random walk). Actually, the slope β was not calculated over the whole frequency spectrum. Only the power spectral values of the lower half of the spectrum (depending on the actual signal lengths) were included in order to avoid influences of high frequency noise components.

C. Approximate Entropy ApEn and Sample Entropy SampEn

ApEn was introduced by Pincus [9] in order to estimate the information content of a signal. First, with a fixed integer $m = 2$, $(N\text{-}m\text{+}1)$ sequences are created according to

$$x(1), x(2),..., x(N - m + 1), \tag{5}$$

and

$$x(i) = [X(i), X(i+1),..., X(i - m - 1)]. \tag{6}$$

Next, distances in between the data series are calculated with

$$d[x(i), x(j)] = \max_{k = 1,2,..., m} \left(\left| X(i + k - 1) \right| - \left| X(j + k - 1) \right| \right). \tag{7}$$

For each $i, j \quad 1 \le i, j \le N - m + 1$ the normalized sum of distances smaller than a predefined maximal distance r = 0.15*SD*, where *SD* is the standard deviation of the signal, is calculated according to

$$C_i^m(r) = \frac{[\#\ of\ \ j\ \ with\ \ d[x(i), x(j)] \le r]}{N - m + 1}. \tag{8}$$

For each *m* the following sum can be calculated

$$\Phi^m(r) = \frac{1}{N - m + 1} \sum_{i = 1}^{N - m + 1} \ln C_i^m(r). \tag{9}$$

Then, *ApEn* is defined as

$$ApEn\ (m, r, N) = \Phi^m(r) - \Phi^{m+1}(r). \tag{10}$$

ApEn is sensitive to the data point number and to the distance *r*, which prevents comparisons of *ApEn* values of different signal lengths. Eliminating these drawbacks, Richman and Moorman [10] introduced the sample entropy statistics. Contrary to *ApEn*, *SampEn* does not count self matches $i = j$ and takes the logarithm in the very last step. Accordingly, (5)-(7) are valid for *SampEn* without any changes. Even (8) is still valid, only $i = j$ is omitted. Instead of (9) the sums are calculated without the logarithm for *m* and *m* + 1

$$B^m(r) = \frac{1}{N - m} \sum_{i = 1}^{N - m} C_i^m(r), \tag{11}$$

$$A^m(r) = \frac{1}{N - m} \sum_{i = 1}^{N - m} C_i^{m+1}(r). \tag{12}$$

Finally, (10) is replaced by

$$SampEn\ (m, r, N) = -\ln\left(\frac{A^m(r)}{B^m(r)} \right). \tag{13}$$

D. Higuchi Dimension D_H

Without phase space constructions, Higuchi [11] proposed a method to calculate the fractal dimension of the temporal signal itself. With an initial data point $m = 1,2,\ldots,d$ and a delay interval $d = 1,2,\ldots,30$ following data point series are constructed:

$$S_m(d) : x(m),\ x(m + d),\ x(m + 2d),..... ..x\left(m + \left\lfloor \frac{N - m}{d} \right\rfloor d \right) \tag{14}$$

Next, the individual lengths of these series are calculated with

$$L_m(d) = \frac{1}{d} \left\{ \left(\sum_{i=1}^{\left\lfloor \frac{N-m}{d} \right\rfloor} \left| x(m + id) - x(m + (i-1)d) \right| \right) \frac{N - 1}{\left\lfloor \frac{N - m}{d} \right\rfloor d} \right\}, \tag{15}$$

where $\lfloor\ \rfloor$ denotes the floor function. For each *d,* the mean length is determined by

$$L(d) = \frac{1}{d} \sum_{m = 1}^{d} L_m(d). \tag{16}$$

D_H was calculated with the slope of a regression line of the linear part of a double logarithmic plot of $L(d)$ and *d*.

E. Surrogate Data

In order to statistically test the gained values for D_H, *ApEn, SampEn* and β, surrogate data was generated. Three methods were used. (i) *SurrSH*: shuffled surrogates, created by randomly shuffling the data points,

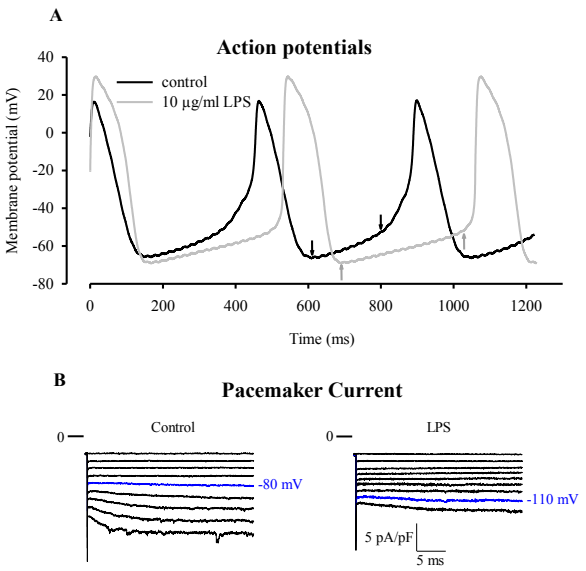

Figure 1. Effect of 10 µg/ml LPS on embryonic chick ventricular myocyte excitability. A: Spontaneous action potentials, the regions between the arrows indicate diastolic depolarisation phase. B: Pacemaker current I_f in control (left panel) and after LPS administration (right panel), current traces in blue mark the activation voltage.

(ii) *SurrG*: Gaussian surrogates with same mean and variance as the original signal, and (iii) *SurrRP*: randomized phase surrogates with same power spectrum as the signal but randomly shuffled phases. For every signal and every surrogate generation method, 100 surrogates were generated. Values for surrogate data are denoted by $ApEn^{SurrSH}$, $ApEn^{SurrG}$,..., D_H^{SurrRP},....

III. Results

Fig.1A illustrates spontaneous action potentials recorded in a small cluster of embryonic chick ventricular myocytes under control conditions (black line) and after administration of 10 µg/ml LPS (gray line). Under LPS conditions diastolic depolarisation phase (displayed by the region between arrows) is prolonged resulting in a reduction of beating frequency. Investigation of the pacemaker current I_f shows that LPS prominently shifts current activation to more negative membrane potentials (from -80 mV to -110 mV, Fig.1B). This in turn explains well the slowing of diastolic depolarisation phase and hence reduction of beating frequency.

D_H, *ApEn*, *SampEn* and β were calculated for control signals as well as for signals measured under LPS conditions. First, the influence of signal lengths was determined, because experimentally it was not possible to gain data with constant signal lengths. Theoretically, Gaussian noise signals with infinite data length should yield following values: $\beta = 0$, *ApEn = 2.5, SampEn = 2.5* and $D_H = 2$. Table I shows actual values for Gaussian surrogates of some recorded signals.

TABLE I. Signal Length Dependency

Signal length	Mean of Gaussian surrogates (n = 100)			
	β^{SurrG}	$ApEn^{SurrG}$	$SampEn^{SurrG}$	D_H^{SurrG}
513	0.0135	1.12	2.47	2.02
748	0.0224	1.38	2.48	2.01
1123	0.020	1.56	2.48	2.01
1981	0.0033	1.84	2.47	2.00
2575	-0.0303	1.96	2.47	2.00

The length dependency of *ApEn* is quite obvious and in accordance to the literature [10]. β, *SampEn* and D_H showed practically only a negligible length dependency. Data for shuffled surrogates is not shown, because the results were quite identical to the Gaussian surrogates ($x^{SurrSH} \approx x^{SurrG}$).

A detailed analysis of the values for the spectral index β was performed. The type of signal (*fGn* or *fBm*) may be determined with (3) and (4) but the experimental data showed very inconsistent results for β. Control and LPS signals were negatively as well as positively correlated, in some cases they were *fGn* and in other cases *fGm*. The reason for this inconsistency seems to be the limited signal length in accordance to Eke et al. [6]. Because of these conceptual problems of β as well as of *ApEn*, further investigations concentrated on *SampEn* and D_H.

Except one phase randomized surrogate data set, all surrogates for *SampEn* and *Dh* were normally distributed (Kolmogorov-Smirnov-test, $\alpha = 0.05$, n=100). Furthermore, except for one case and particularly for the phase randomized surrogates, each measured time signal was significantly not equal to the corresponding surrogates (One sample Student's t-test, $\alpha = 0.01$, n=100). Calculated values for *SampEn* can be seen in Fig.2A. The lower values for the LPS signals are clearly visible, indicating a loss of complexity. In order to see differences to the corresponding phase randomized surrogate data, Fig.2B shows normalized values $SampEn/SampEn^{SurrRP}$. A value of about 1.5 for the control signals indicates that the entropy or complexity is about 50% higher than the corresponding phase randomized surrogates. For the LPS signals the values were slightly below 1. This indicates that complexity is reduced to values slightly lower than the corresponding phase randomized signals.

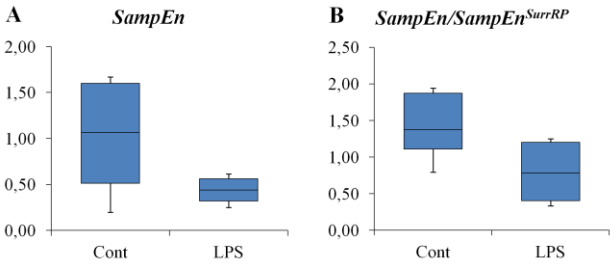

Figure 2. Sample entropy for control and LPS signals. A: Sample entropy, B: Sample entropy normalized to sample entropy of corresponding random phase surrogates.

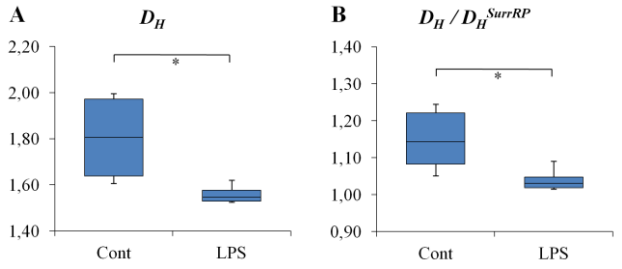

Figure 3. Higuchi dimension for control and LPS signals. * $p \leq 0.05$ of Kruskal-Wallis-test. A: Higuchi dimension. B: Higuchi dimension normalized to Higuchi dimension of corresponding random phase surrogates.

Similarly to *SampEn,* the Higuchi dimension D_H (Fig.3A) and a normalized Higuchi dimension D_H/D_H^{SurrRP} (Fig.3B) were calculated. Now, LPS signals show statically significant lower values (Kruskal-Wallis-test, $\alpha = 0.05$, $n = 4$). The normalized values confirm this result. The control signals show an about 15% higher value than the corresponding phase randomized surrogate signals. For the LPS signals the percentages are about 5% and therefore they are still different from the corresponding phase randomized surrogate signals.

The differences between the control group and the LPS group are consistent and clearly evident for *SampEn* as well as for D_H. Furthermore, the normalized values show higher significances.

IV. Conclusion

Intrinsic heart tissues properties may be one important factor for HRV *in vivo*. This presentation gives evidence that this hypothesis cannot be rejected. BBV of embryonic chick heart aggregates was quite lowered under septic conditions and therefore confirms prior reports using rat cells [1]. Moreover, the results show an LPS induced impairment of pacemaker current in embryonic chick heart cells which may underlie this described reduction of BBV. Since I_f of embryonic chick ventricular myocytes is encoded by the HCN4 isoform, which is also the major isoform in mammalian sinus node, our results may contribute to a better understanding of LPS induced reduction of heart rate variability observed in humans.

Whereas power spectral analysis as well as approximate entropy did not yield reliable results, sample entropy as well as the Higuchi dimension turned out to be robust and statistically significant, despite the low number of data points and samples. Surrogate data analysis emphasized these findings. Of all parameters tested, Higuchi dimension normalized to the randomized phase surrogate value (Fig.3B) turned out to be the best parameter in order to describe BBV quantitatively.

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References

[1] H.Schmidt, J.Saworski, K. Werdan, U. Müller-Werdan, "Decreased beating rate variability of spontaneously contracting cardiomyocytes after co-incubation with endotoxin," *J. Endotoxin Res*., vol. 13, no.6, pp.339-342, 2007.

[2] V.E. Papaioannou, A.O. Verkerk, A.S. Amin, and J.M.T. de Bakker, "Intracardiac Origin of Heart Rate Variability, Pacemaker Funny current and their Possible Association with Cardiac Illness," *Current Cardiology Reviews*, vol.9, no.1, pp.82-96, 2013.

[3] K. Zorn-Pauly, B. Pelzmann, P. Lang, H. Mächler, H. Schmidt, H. Ebelt, K. Werdan, B. Koidl, and U. Müller-Werdan, "Endotoxin impairs the human pacemaker current I_F," *Shock*, vol. 28, no. 6, pp. 655-661, 2007.

[4] C. Wahl-Schott, and M. Biel, "HCN channels: Structure, cellular regulation and physiological function, "Cell. Mol. Life Sci, vol. 66, pp. 470–494, 2009.

[5] A. Sarre, S. Pedretti, S. Gardier, E. Raddatz, "Specific inhibition of HCN channels slows rhythm differently in atria, ventricle and outflow tract and stabilizes conduction in the anoxic-reoxygentaed embryonic heart model,"Pharmacol. Res., vol. 61, pp85-91, 2010.

[6] A. Eke, P. Hermán, J.B. Bassingthwaighte, G.M. Raymond, D.B. Percival, M. Cannon, I. Balla, and C. Ikrényi, "Physiological time series: distinguishing fractal noises from motions," *Pflügers Arch. – Eur. J. Physiol.*, vol. 439, pp. 403-415, 2000.

[7] T. Krogh-Madsen, P. Schaffer, A.D. Skriver, L.K. Taylor, B. Pelzmann, B. Koidl, M.R. Guevara, " An ionic model for rhythmic activity in small clusters of embryonic chick ventricular cells," *Am. J. Physiol.,* vol. 289, no. 1, pp. H398-H413, 2005.

[8] B. Pelzmann, " Die Wirkung von Bariumionen auf Spontanaktivität und Ionenströme isolierter embryonaler Hühnerherzventrikelzellen," Doctoral dissertation, Inst. Med. Physics and Biophysics, Karl-Franzens University, Graz, Austria, 1996.

[9] S.M. Pincus, "Approximate entropy as a measure of system complexity," *Proc. Natl. Acad. Sci. USA*, vol.88, pp.2297-2301, 1991.

[10] J.S. Richman, and J.R. Moorman, "Physiological time-series analysis using approximate entropy and sample entropy," *Am. J. Physiol. Heart Circ. Physiol.*, vol.278, pp.H2039-H2049, 2000.

[11] T. Higuchi, "Approach to an irregular time-series on the basis of the fractal theory." *PhysicaD*, vol. 31, pp. 277-283, 1988.