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Assessment of Diabetic Cardiac Autonomic Neuropathy in Type I Diabetic Mice

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Abstract—**Diabetic cardiac autonomic neuropathy (DCAN) is one of the most common complications of diabetes. One reason why the pathogenesis of DCAN is unclear is that non-invasive assessment of DCAN in humans and animals has been problematic. To overcome this limitation, we utilized a sensitive and non-invasive method to assess cardiac autonomic dysregulation from ECG records. The method, which could be easily applied to humans, is based on principal dynamic mode (PDM) analysis of heart rate variability (HRV). The method is unique, in that is able to separately identify the activities of the parasympathetic and sympathetic systems without pharmacological intervention. In our study, ECG was measured via telemetry in ten sex- and age-matched (4 month old male) C57 (n=5) and Akita (n=5) mice, a model of insulin-dependent type 1 diabetes. The results indicate significant reduced cardiac autonomic function in the diabetic mice in comparison to the controls. Further, both immunohistochemical and Western blot analyses show a reduction in nerve density in Akita mice as compared to the control mice, thus, corroborating our PDM data analysis of HRV records.**

I. Introduction

Diabetic cardiac autonomic neuropathy (DCAN) is one of the most overlooked of all serious diabetes complications, and can cause abnormalities in heart rate control as well as central and peripheral vascular dynamics[1]. Consequences of DCAN include exercise intolerance, intraoperative cardiovascular lability, orthostatic hypotension, myocardial ischemia, increased risk of mortality, morbidity, and reduced quality of life for persons with diabetes. All of these symptoms are manifestations of autonomic neuropathy [1]. One useful noninvasive method to assess autonomic function in various physiological and pathophysiological conditions, including evaluation of the autonomic dysfunction in diabetic subjects, is the use of heart rate variability (HRV) [2]. HRV is a marker of sympathetic and parasympathetic (vagal) influences on the modulation of heart rate [3]. Interactions between the sympathetic and parasympathetic nervous activities are essential in the regulation of cardiovascular function. Autonomic imbalance, such as sympathetic hyperactivity, promotes life-threatening ventricular tachyarrhythmia, whereas augmented vagal tone exerts a protective and anti-fibrillatory effect [4]. Experimental evidence suggests that myocardial ischemia, acute myocardial infarction, sudden cardiac death, and chronic heart failure are associated with some degree of autonomic imbalance [5]. Further, patients who have had a myocardial infarction (MI) have a marked decrease in HRV due to an increase in sympathetic and a decrease in vagal neural activities [5, 6]. Reduced heart rate variability is known to be one of the earliest indicators of DCAN [7] as it has been shown to involve an imbalance of the autonomic nervous system (ANS) [1, 2]. A recent study examining the effect of sustained hyperinsulinemic hypoglycemia on cardiovascular autonomic regulation in type I diabetic and their non-diabetic counterparts has found reduced cardiac vagal outflow in all patients [2]. Another study, examining HRV changes in diabetes, has found that decreases in autonomic function are present early in the development of diabetes and that diabetes leads to a progressive decline in autonomic function [8]. Animal subjects, rats or mice, all show similar manifestation of DCAN for both type I and type II diabetes.

Young adult rats treated with streptozotocin (STZ) developed diabetes with significant reductions in both heart rate and HRV, suggesting disturbance in the ANS balance [9]. Further, insulin treatment of these STZ-treated diabetic rats showed no significant recovery of the autonomic nervous activity even though heart rate recovered to the pre-STZ treated state [10]. This suggests that heart rate itself is not a reliable diagnostic marker of DCAN [11]. The literature on diabetic complications subsequently leading to DCAN symptoms in mice is sparse. However, there has been much sophisticated work done on peripheral neuropathy complications using various mouse models [12-14]. One intriguing mouse model of Type I diabetes is the Akita mouse. Akita mice, which spontaneously develop insulin-dependent diabetes at about 4 weeks of age, express a mutant non-functional insulin isoform. A study has found the presence of peripheral sensory nerves impairment in 4 month old Akita mice. Another work has found significant increases in fasting blood glucose levels, gait disturbances, and sensory nerve conduction slowing as early as at 4 months of age in Akita mice [12]. However, it is not known if DCAN is present in Akita mice. In general, the pathogenesis of experimental DCAN is clearly understudied, likely because of the lack of adequate methodology.

The goal of this work was to determine if pathogenesis of DCAN in Akita mice can be monitored with PDM analysis of HRV. Our study involved male Akita and age-matched C57 controls from which ECG records were collected via telemetry. The results of the ECG analysis were correlated with the measurements of immunohistochemical staining study and Western blot assay assessments of autonomic

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nerves. The long-term goal of this project is developing this method to the point at which it can be used to detect DCAN in diabetic patients.

II. METHODS

A. ECG transmitter implantation and data collection

The experiment was performed on 10 age-matched male C57 (wildtype) and Akita mice at 4 month old. All animal-related experimental protocols were approved by the Institutional Animal Care and Use Committee at Stony Brook University and Worcester Polytechnic Institute. The surgery was conducted in accordance with Division of Laboratory Animal Resources. Mice were first anesthetized in a chamber with isoflurane and maintained anesthesia with 0.8% to 1.5% isoflurane in 50% oxygen/50% nitrogen to keep animal unconscious and to prevent vital damage to the animal. The wireless ECG transmitter (sampled at 2000 Hz) was then embedded subcutaneously on the dorsal side of the animal, and wired electrodes were threaded under skin to reach the position of Einthoven bipolar lead II configuration and suture to the body wall. ECG and activity data were collected after one week's recovery and continuously recorded for 1 month (Columbus Instruments Inc., Columbus, OH).

B. Heart Rate Variability Analysis

We selected daily 15 minutes ECG data segment from all mice when they were in a resting state. Both heart rates and activity data were examined to make certain that a data segment selected was in a resting state. We also selected two additional 15 minute segments from each data set and compared amongst three segments in order to examine if our selection was unbiased and provided consistent results. For all measurements, 1 minute data were analyzed and averaged over 15 minutes. For time domain measures, we examined mean heart rate (HR), standard deviation of normal-to-normal R-R intervals (SDNN), and root mean square of successive differences in normal-to-normal R-R intervals (RMSSD). SDNN reflects overall autonomic nervous activities and RMSSD reflects the parasympathetic dynamics [4]. Prior to calculation of frequency domain measures, each HRV segment was zero meaned, detrended and normalized to unit variance. In addition, we computed spectral powers in both the LF (0.4-1 Hz) band and HF (1-4Hz) bands. It is well established that the LF band represents both sympathetic and parasympathetic nervous activities while the HF band represents the parasympathetic nervous activity [15]. We also computed LF/HF ratio to assess the autonomic balance of the wildtype and Akita mice.

C. Principle dynamic mode (PDM) analysis of HRV

The Principle Dynamic Mode (PDM) was compared to the traditional HRV measures in section B. The advantages of PDM have been well documented and its most salient feature is that it enables accurate separation of the sympathetic and parasympathetic dynamics [15].

D. Western Blot Analysis and Immunohistochemistry analysis

Ten sex- and age-matched (4 month old male) wild-type C57 (n=5) and Akita (n=5) mice were studied to quantify the development of nerve degeneration using Western blot. The atrial section of the heart was used for Western blotting of heart tissue since autonomic nerves are more prevalent in the sinoatrial (SA) and atrial ventricular (AV) nodes [16]. In this study, we focused on three autonomic nerve protein markers: tyrosine hydroxylase (TH, a marker for sympathetic nerves), choline acetyltransferase (ChAT, a marker for parasympathetic nerves) and synaptophysin (SYN, a marker for general nerves); actin was used as a loading volume control. To quantitatively compare protein density in C57and Akita mice, integrated density of each protein expression bar was recorded and compared after normalized to actin density in each column.

For immunohistochemistry, the atrial section of the heart was fixed in paraformaldehyde after dissection. The slides were prepared by sectioning the tissue into 6 micrometers thick). All samples were on the same blot and the image was subjected to overall contrast enhancement. We investigated the overall nerve density expression using synaptophysin; the HCN4, a membrane channel found only in SA and AV nodes in the heart was used to ensure that the excised atrial tissue section was correctly localized since the autonomic nerves are most abundant in these nodes.

E. Statistical Analysis

All numerical results were presented as mean ± STD. For the comparison of two groups, significance was determined using students' t-test (normality was tested in advance) at 95% confidence interval ($P<0.05$). Statistical analysis was performed using SigmaStat 3.0 (SPSS Inc.)

III. RESULTS

A. Time and Frequency Parameters in HRV Analysis

Comparisons of the traditional HRV time and frequency domain measures to PDM analysis are provided in Table I. We only show results on a representative 15 minutes data among the three independent segments as described in Part B of Methods section. In Table I, all comparisons are made between wildtype and Akita mice. As shown in Table I, mean HR is significantly depressed in Akita mice for all four weeks. Similarly, SDNN was also significantly lower in Akita mice but only in weeks 3 and 4. However, RMSSD increased in Akita mice in weeks 3 and 4.

The power spectral density (PSD) LF power of Akita mice was observed to be consistently lower than C57 mice throughout the month (2.97±0.26 compare to 3.48±0.18 in week 1). However, the HF power was significantly higher in Akita mice in weeks 2-4. The LF/HF ratio was significantly lower in Akita mice in weeks 3-4. The same observations

TABLE I
COMPARISON OF DIFFERENT METHODS FOR HRV ANALYSIS

		Week 1	Week 2	Week 3	Week 4
HR	Wildtype	505±35	531±21	549±31	530±19
	Akita	421±56 *	417±66*	396±36*	375±26*
SDNN	Wildtype	8.25±1.13	7.96±1.12	8.78±1.61	8.34±1.26
	Akita	6.31±1.94	6.37±0.96	6.26±1.01*	5.88±1.18*
RMSSD	Wildtype	1.66±0.73	1.73±0.39	1.81±0.29	1.94±0.38
	Akita	1.69±0.64	2.01±0.77	2.24±0.21*	2.28±0.30*
PSD LF	Wildtype	3.48±0.18	3.61±0.11	3.62±0.21	3.55±0.22
	Akita	2.97±0.26*	3.07±0.40*	2.93±0.34*	3.05±0.13*
PSD HF	Wildtype	0.73±0.14	0.69±0.14	0.73±0.12	0.76±0.08
	Akita	0.77±0.08	0.92±0.18*	0.99±0.09*	0.97±0.11*
LF/HF	Wildtype	5.99±0.95	6.49±0.55	6.74±1.35	6.12±0.54
	Akita	5.76±0.77	5.15±2.5	4.43±0.80*	4.73±0.39*
PDM Symp.	Wildtype	0.86±0.04	0.86±0.04	0.90±0.06	0.88±0.03
	Akita	0.76±0.06*	0.77±0.07*	0.72±0.05*	0.72±0.06*
PDM Para.	Wildtype	5.04 ±0.49	5.27±0.51	5.07±0.42	4.89±0.35
	Akita	4.26±0.34*	4.19±0.62*	4.38±0.41*	4.27±0.30*
Symp. / Para.	Wildtype	0.21±0.01	0.22±0.03	0.23±0.04	0.24±0.02
	Akita	0.18±0.02*	0.19±0.02*	0.19±0.01*	0.18±0.03*

Mean ± STD, * denotes for statistical significance ($p<0.05$);

were found for 2 other data segments (not shown) suggesting that our results are consistent and are not based on the choice of where the data segments were selected among each day's measurement.

B. PDM Analysis of HRV

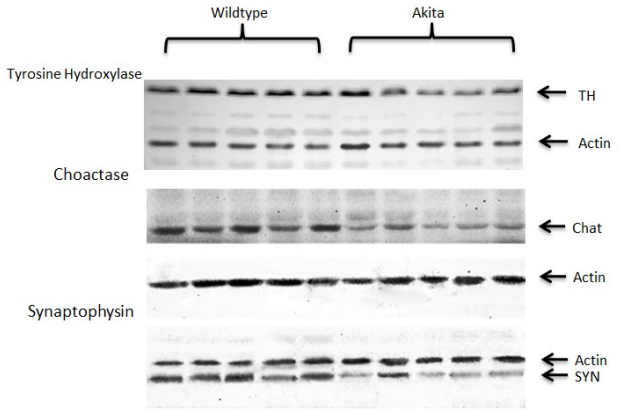
As shown in Table I, both sympathetic and parasympathetic dynamics for all four weeks were found to be significantly reduced in Akita mice. Note that the decrease in parasympathetic dynamics in Akita mice using PDM method is in contrast to the PSD and RMSSD results. Moreover, the Symp./Parasym. ratio decreased significantly (0.18±0.017 compare to 0.21±0.014 in week 1) which demonstrates autonomic imbalance in diabetic mice. We did not see a progressive degradation of the autonomic nervous activities with the increased time duration (e.g., from week 1 to 4). Finally, there was no difference among the three different data segment trials.

C. Western Blot Assessment of autonomic nerves density

Our HRV analysis using PDM approach indicates significantly reduced autonomic activity in Akita mice when compared to C57 mice. To corroborate our noninvasive PDM approach finding of significantly depressed autonomic nervous dynamics, we performed Western blot analysis to quantify nerve rarefaction in the SA and AV nodes in both strains of mice (N=5 for each strain). Certainly, we expect no autonomic nerve rarefaction in wildtype but certainly expect it in Akita mice. As shown in the top panel of Fig. 1, the autonomic nerve protein markers, SYN, TH, and ChAT all show significantly lower presence in Akita than wildtype. For all results, equal protein loading was confirmed with actin antibody. For example, there is no difference in intracellular actin abundance in normal and Akita mice.

D. Immunohistochemistry of cardiac autonomic nerves

We also performed immunohistochemical staining analysis to examine if indeed SYN is less expressed in Akita as compared to wildtype mice. The bottom panel of Fig. 1 shows deconvolved optical sections of immunostaining of SA nodes for HCN4 (red) channels and SYN (green). The negative control was obtained by omitting both primary antibodies and the image was not deconvolved. The tissue sections were stained concurrently and imaged with the same settings. As shown, the SA node in this Akita mouse is clearly less densely innervated than the C57 control mouse.



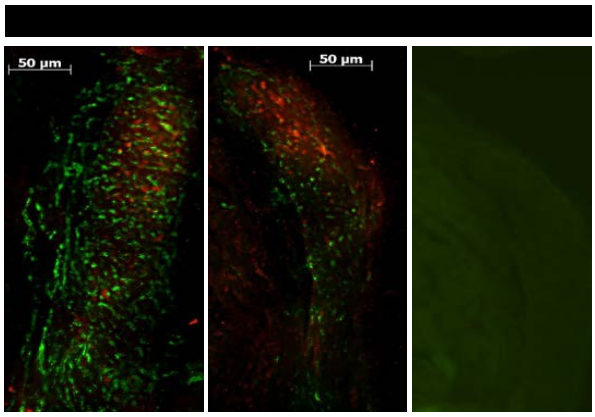


Figure 1, Top panel: Western blot of 4 month old mice for TH, CH and SYN. Actin was used as volume control for each protein; bottom panel shows immunohistochemistry staining of SYN (green) and HCN4 (red)

IV. Discussion

Diabetic autonomic neuropathy (DAN) is a disease involving the entire autonomic nervous system and it is a common complication of diabetes. Cardiovascular autonomic neuropathy is one of the most studied and a clinical subset of DAN. Timely diagnosis of DCAN is needed as individuals with abnormal autonomic function are

found to have a greater risk for severe hypoglycemia [2]. Diagnosis of DCAN currently involves many different autonomic function tests and involves testing of RR variability, Valsalva maneuver and postural blood pressure testing. It has been suggested that regular HRV testing provides early detection of DCAN which subsequently can lead to timely therapeutic interventions [17]. For example, it has been suggested that peripheral neuropathy can be reversed if timely detection and appropriate subsequent therapeutic interventions are administered [18].

To this end, our present work is to demonstrate an alternative to the traditional HRV measures involving both time- and frequency-domain parameters which have been well documented to have many shortcomings. This was the case with our results as we found significantly reduced parasympathetic dynamics via PDM method but not via HF power as well as RMSSD values in Akita as compared to wildtype mice. Because parasympathetic activity consists both LF and HF power thus HF doesn't reflect the complete parasympathetic dynamics. The gold-standard approaches such as immuohistochemical staining and Western blot analysis of mice heart clearly showed a significant decrease in the parasympathetic and sympathetic activities in Akita as compared to wildtype mice, corroborating our PDM results. Thus, our current work demonstrate a more effective and sensitive approach to HRV analysis in the diagnosis of DCAN than the traditional HRV measures. The advantage of using PDM method over the traditional HRV measures has been already demonstrated in our previous study involving healthy subjects with the aid of pharmacological blockades [15].

We have primarily studied 4 month old Akita mice because peripheral neuropathy is known to occur after mice have reached this age. Our work suggests DCAN also occurs at 4 months old. However, we do not know if DCAN occurs earlier than 4 months. Thus, we are interested in investigating the onset of DCAN in Akita mice at younger ages. The main roadblock to recording telemetry ECG earlier than 4 months is that a telemetry sensor is too big and heavy (~5 gms). Note that a 1 month old Akita mouse weighs ~16 gms. To circumvent this limitation, we are currently investigating the use of a tail-cuff pulse oximeter as our preliminary results suggest that accurate RR intervals can be obtained in 1 month old mice. This will allow us to monitor the progression and onset of DCAN in Akita mice.

V. CONCLUSIONS

In this study, ECG was measured via telemetry in conscious 4 month old C57 controls and in Akita mice, a model of insulin-dependent type I diabetes. Our results indicate a significant cardiac autonomic impairment in the diabetic mice. Further, both immunohistochemical and Western blot analyses show a reduction in nerve density in Akita mice as compared to the control mice, thus, corroborating our PDM data analysis of HRV records. The long-term goal of this project is to develop this method to the point at which it can be used to detect DCAN in diabetic patients. The present study provides a sound basis for future clinical and basic science studies of our new non-invasive high-throughput tool for the detection of DCAN.

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