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Original Article

Heart rate variability indices, biomarkers, and cardiac nerve density: Independent surrogate markers for diagnosis of diabetic cardiac autonomic neuropathy in type 2 diabetes mellitus animal model

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ABSTRACT

Objectives: Heart rate variability (HRV) has been shown to represents a promising quantitative marker of autonomic activity. Studies have shown that diabetic patients and animal models have derangements in certain biochemical parameters with reduced cardiac nerve density following development of diabetic cardiac autonomic neuropathy (DCAN). This study, therefore, aims to correlate HRV indices, cardiac histology, and cardiac nerve density with selected biochemical markers in the DCAN rat model using high fat diet (HFD) and streptozotocin (STZ) induction.

Methods: DCAN was induced in Wistar rats using HFD for 8 weeks with 25 mg/kg STZ daily for 5 days. DCAN features were then assessed using Holter electrocardiography (ECG), invasive biomarkers, and cardiac histology.

Results: DCAN group had significantly higher advanced glycated end product levels ($P < 0.0001$), noradrenaline ($P = 0.010$), and insulin resistance ($P = 0.016$) compared with controls. The level of antioxidants, sorbitol dehydrogenase activity ($P = 0.009$), nerve growth factors ($P < 0.0001$), and choline acetyl-transferase ($P = 0.031$) was, however, significantly reduced. Furthermore, HRV indices which were also reduced with DCAN induction correlated significantly with levels of biomarkers and cardiac nerve density.

Conclusion: HRV is a cheap and easy tool for assessing DCAN that significantly correlates with markers of autonomic activity. Holter ECG and HRV evaluation should be considered early in patients with diabetes.

Keywords: Cardiac nerve density, diabetes mellitus, diabetic cardiac autonomic neuropathy, heart rate variability

Introduction

Cardiovascular diseases are the primary cause of morbidity and mortality among patients with diabetes mellitus (DM).[1] Diabetic cardiac autonomic neuropathy (DCAN) is the impairment of autonomic control of the cardiovascular system in patients with diabetes after exclusion of other causes.[2,3] It is a major complication of diabetes yet the pathogenesis is not fully understood. Once DCAN patients become symptomatic, no form of management options has been shown to effectively reverse the progression; therefore, prevention and early diagnosis are key factors in the management.[4] There has been increasing recognition of the significant relationship between the autonomic nervous system with cardiovascular morbidity and mortality especially in DM patients. Experimental evidence for an association between the propensity for arrhythmogenesis and signs of either increased sympathetic or reduced vagal activity has spurred efforts for the development of quantitative markers of autonomic activity.[5-8] Heart rate variability (HRV) has been shown to represent one of the most promising of such markers.[9-11] Other methods that have been proposed for the diagnosis of DCAN include: The assessment of symptoms and signs, cardiovascular autonomic reflex test, and ambulatory blood pressure monitoring. HRV is largely used in clinical and for research purpose. Studies have shown that diabetic patients and animal models have reduced cardiac nerve density with the development of DCAN.[7,8,12-14] However, the relationship between the HRV, laboratory parameters of DCAN, cardiac histology, and cardiac nerve density has not been fully elucidated. This study therefore aims to correlate HRV indices, cardiac histology, and nerve density with selected biochemical markers in DCAN rat model using streptozotocin (STZ) induction.

Materials and Methods

Animals

The experimental protocol was approved and the research was conducted in accordance with the guiding principles of the University of Ilorin Ethical Review Committee (UERC) with approval number: UERC/ASN/2019/1912. Forty-two male Wistar rats were used for the experiment. The animals were acclimatized to their environment for 2 weeks before commencement of the experiments while been fed *ad libitum* and housed in pairs in wooden cages.

Experimental groups/model development

The male Wistar rats were divided into two major groups, normal group (n = 10) and diabetic group calculated using prevalence of DCAN from previous studies.[15] Type 2 DM was induced with initial 8 weeks high-fat diet (HFD) intake, thereafter, low dose STZ at 25 mg/kg dose intra-peritoneally administered under ketamine anesthesia over 5 days. Ten days after STZ-injection, rats with plasma glucose >16 mmol/L were selected into the DCAN subgroup. Whole blood was taken from the proximal ventral tail vein for glucose measurement using a glucometer (Accu-Chek II Boehringer Mannheim Canada, Dorval, Quebec). Fasting morning plasma glucose levels, water consumption, and body weights were determined at weekly intervals while other parameters were determined at the beginning and end of the experiment. The effects of DCAN on all the subgroups of the model were studied. These entail both non-invasive and invasive assessment of autonomic neuropathy. Time-varying, nonlinear, and non-invasive methods were used to assess cardiac autonomic dysregulation from electrocardiography (ECG) records using Holter ECG. Blood samples were collected to measure serum biomarkers, before and after DM induction; moreover, T2DM was also confirmed with plasma insulin, c-peptide, and insulin resistance (IR) using homeostasis model assessment for IR (HOMA-IR = Fasting glucose (mmol/l) * fasting insulin (μIU/l)/22.5)[16] and histology of the pancreas.

ECG and measurement of time and frequency domain parameters

The ECG signals were continuously recorded for at least 15 min in the limb, augmented, and chest leads following subcutaneous administration of ketamine (75 mg/kg).[16] These recordings were stored and thereafter analyzed. Lead II was achieved in the Wistar rat by the placement of the negative electrode near the right shoulder and the positive electrode to the left of the xiphoid space, in the same way as the Einthoven triangle (right arm position in the negative electrode and left leg position in the positive electrode).[17,18] Frequency domain (low frequency [LF], high frequency [HF], and LF:HF) and time-domain parameters including mean HR, standard deviation of normal-to-normal R-R intervals (SDNN), root mean square of successive differences in normal-to-normal R-R intervals (RMSSD), and percentage of successive normal sinus RR intervals >50 ms (PNN50) were calculated from the HRV data.

Invasive assay

At the end of the experiment, the rats were humanely euthanized using cervical dislocation after been anaesthetized; blood samples were collected via cardiac puncture after the opening of the upper abdominal region. The blood samples were centrifuged at 1500× g 15 min and the plasma samples micro-pipetted into plain bottles and immediately stored at a temperature of 0–4°C.

For the invasive assay, protein expression markers, which have been correlated with DCAN and diabetes complications in previous studies and also recommended by the DCAN subcommittee of the Toronto autonomic neuropathy working group, were assayed.[19-23] These include nor-adrenalin, nerve growth factor (NGF), and choactase.

Histological studies

Paraffin sections from epicardial region of the heart (3 μm thickness) were cut; thereafter, cardiac nerve density was evaluated using standard staining protocol for H&E and histochemistry.[24] Morphometric analysis was, thereafter, done after histochemistry to quantify and evaluate the nerve density.

Statistical analysis

Data were analyzed using Statistical Products and service solutions (SPSS) software (Version 23.0; SPSS Inc., IL., USA) for windows. Results were expressed as the mean ± SEM. Comparisons among groups were done using one-way ANOVA. Group means for two independent samples were compared using Student's t-test. Correlation and regression analysis were also done step-wisely to identify variables that could predict the nerve density estimates as an outcome. $P < 0.05$ was taken as statistically significant.

Results

Diabetes induction and laboratory parameters

Eighty-nine percent of the DCAN group had significantly higher blood sugar levels following diabetic induction. The plasma levels of insulin and c-peptide were significantly lower compared with the control. Total cholesterol was also seen to be significantly higher in the DCAN group compared with the control. In addition, HOMA, which is a method for assessing β-cell function and IR from basal (fasting) glucose and insulin or C-peptide concentrations, was also significantly higher in the DCAN group compared with control [Table 1]. Advanced glycated end product (AGEs) was significantly higher in the DCAN group compared with the control. In addition, biomarkers of anti-oxidants which included total

Table 1: Comparison of the laboratory parameters of DCAN and control group

Parameters	Baseline/ control	DCAN induction	*P* value
UREA (mg/dl)	10.90±4.73	15.71±3.59	0.698
TC (mg/dl)	66.61±2.52	69.28±2.71	0.001*
TG (mg/dl)	66.07±1.46	94.02±1.59	0.121
AGEs (ng/ml)	32.04±2.32	91.61±7.26	<0.0001*
C-PEPTIDE (ng/ml)	1.20±0.21	0.73±0.19	0.001*
INSULIN (µIU/mL)	2.89±0.26	1.14±0.19	<0.0001*
HOMA-IR	0.13±0.08	0.25±0.09	0.016*
NGF (pg/ml)	284.16 (91.18–381.34)	110.56 (89.27–169.58)	<0.0001*
Noradrenaline (pg/ml)	111.56±45.09	889.76±171.27	0.010*
GSH (mM)	0.35±0.09	0.25±0.04	0.001*
SD (U/L)	42.50±3.71	31.07±2.53	0.009*
TAC (mM)	1.44±0.25	0.44±0.08	<0.0001*
Choactase (ng/ml)	1.19±0.04	0.69±0.03	0.031*

TC: Total cholesterol, TG: Triglyceride, AGEs: Advanced glycation end product, HOMA-IR: Homeostatic model assessment for insulin resistance, NGF: Nerve growth factor, GSH: Glutathione, SD: Sorbitol dehydrogenase, TAC: Total antioxidant capacity, Choactase: Acetylcholine transferase. **P*<0.05 compared with normal control

antioxidant capacity and glutathione were significantly lower in the DCAN group compared to the control group. The study also showed that markers of NGF and parasympathetic expression (Choactase) were significantly lower in the DCAN group, while on the other hand nor-adrenaline was significantly higher in the DCAN group relative to control [Table 1].

Effect of DCAN on HRV indices

The evaluation of autonomic function using indices of HR variability (frequency and time domain spectral) showed a similar pattern with the laboratory markers. DCAN induction was observed to reduce time-domain parameters, which may reflect the progressive destruction of the parasympathetic nerve endings. PNN50, HRV index, triangular index, and RMSSD were significantly reduced compared to the control group. SDNN which has been shown to correlate with sympathetic outflow was, however, also seen to be significantly lower than the control group [Table 2]. The HF was significantly lower in the DCAN group while the LF and LF:HF were both higher after DCAN induction, as shown in Table 2.

Cardiac histology

Histological sections of the atrial/SA region of the heart using H&E stain showed a normal arrangement of cardiac muscle fibers with deeply staining basophilic nuclei in the control group. The DCAN group, however, showed varying pattern ranging from swelling of cardiac tissue and increased cellularity, graded to complete loss of normal cardiac morphology to outright disorganization of cardiac muscle architecture with degenerating myocyte nuclei and increased

Table 2: Mean heart rate variability parameters among the groups

HRV indices	Control group	DCAN group	*P* value
Minimum RR interval	305.0±25	420.0±36	0.001
Maximum RR interval	875.0±102	1720.0±201	<0.0001*
Average RR interval[#]	514.5±56	954.3±78	<0.0001*
Low frequency (LF)[#]	789.1±73	1052.4±96	<0.0001*
High frequency (HF)	12033.2±203	5743.3±189	<0.0001*
LF/HF[#]	0.067	0.187	0.0001*
HRV index (ms*ms)	100.8±7.1	47.5±5.3	<0.0001*
PNN50(ms*ms)	81.6±8.3	62.8±6.4	<0.0001*
SDNN (ms*ms)	334.2±13.5	118.3±10.3	<0.0001*
RMSSD (ms*ms)	442.2±35.2	172.6±24.9	<0.0001*
Triangular index (ms*ms)	224.5±15.6	47.5±5.2	<0.0001*

LF: Low frequency, HF: High frequency, HRV: Heart rate variability, pNN50: Percentage of successive RR intervals that differ by more than 50 ms. SDNN: Mean of the standard deviations of all the NN intervals for each 5 min segment of a 24 h HRV recording. RMSSD: Root mean of squared successive differences. **P*<0.05 compared with normal control, [#](means higher value is worse)

cellularity [Figure 1a and b]. Bielschowsky histochemical stain of the control group shows normal cardiac tissue, with axons innervating the intercalated disks staining black. Cardiac connective tissue in staining various shades of gray; nuclei seen as round to oval-shaped cells staining gray to black. The DCAN group shows a varying degree of axonal dystrophy characterized by; excessive vascular tissue reaction, disorganization of the cardiac tissue, inflammatory cells, clumping together of nerve fibers, and thickening of the nerves and axons supplying the intercalated disks [Figure 1c and d].

Nerve density estimates and correlation with HRV and laboratory parameters

Morphometric nerve estimates showed significantly lower nerve density in the DCAN group compared to the control group, as shown in Figure 2. It was also observed that there was a significant correlation between the nerve density and both laboratory and HRV parameters [Table 3]. Positive correlation was found with sorbitol dehydrogenase ($P = 0.048$), NGF ($P = 0.018$), glutathione ($P = 0.025$), and choactase ($P = 0.048$). However, there was significant negative correlation with AGE ($P = 0.026$) and noradrenaline ($P = 0.049$). The time spectral-domain showed significant positive correlate with nerve density, unlike the frequency domain that did not correlate significantly with nerve density.

Discussion

The study shows the induction of T2DM in a rat model using STZ in combination with HFD. High-fat feeding has been proposed to cause obesity, hyperinsulinemia, and altered glucose homeostasis due to insufficient compensation by the beta cells of the pancreatic islets.[25] The development of significantly lower insulin with higher total cholesterol and HOMA confirms the establishment of T2DM model.

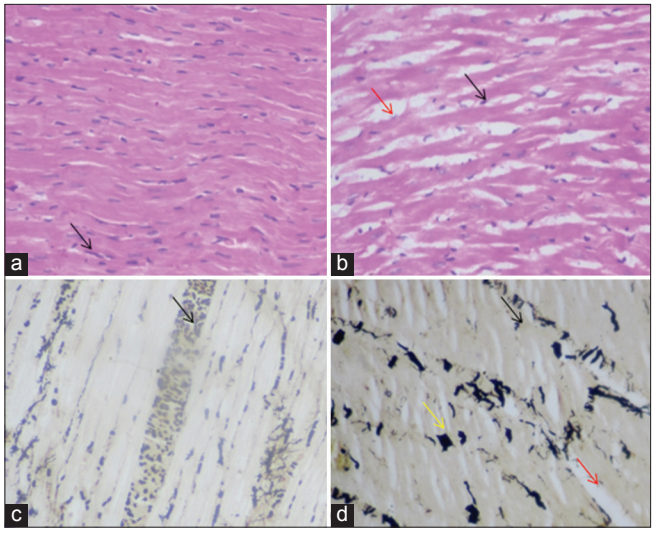

Figure 1: (a and b) Photomicrographs representative of hematoxylin and eosin-stained slides of the atrial/SA region of the heart in the DCAN 2 rat model. (a) Control groups showing normal cardiac muscle fibers and basophilic nuclei, (b) DCAN group shows a waxy pattern, increased cellularity, swelling with disorganized muscle architecture. Red arrows=Edema, Black arrow=Nuclei. (c and d) Photomicrographs representative of Bielschowsky histochemical silver-stained slides of the atrial/SA region of the heart of the DCAN 2 rat model. (c) Control groups showing normal cardiac muscle fibers and basophilic nuclei, (d) shows swelling with disorganized muscle architecture and degenerating myocyte nuclei (axonal dystrophy). Red arrows=Edema, Yellow arrow=Axonal dystrophy, Black arrow=Nerve sheath

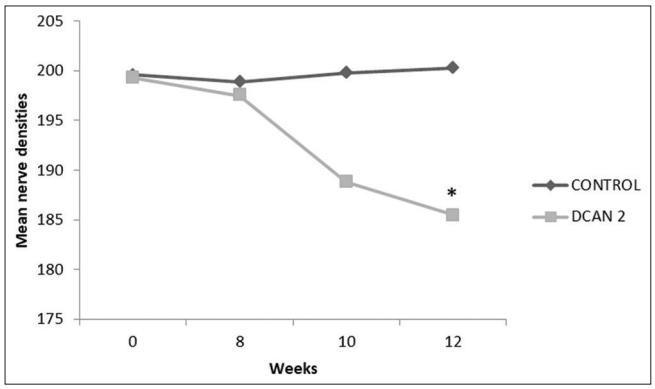

Figure 2: Morphometric estimates of the nerve densities of the control and DCAN group. Nerve estimate was done at the start of the experiment (week 0), after HFD (week 8), during induction of diabetes (week 10) and 4 weeks after diabetes induction (week 12). *P<0.05 significantly lower compared with control. Control: Normoglycemic control (+10 ml/kg normal saline), DCAN 2: type 2 DM group (+10 ml/kg normal saline)

The current study showed significantly increased AGEs after DM induction in the rats. Previous studies have shown that accumulations of AGEs are associated with neuronal fiber loss in the human diabetic peripheral nerve. AGEs interfere with axonal transport, thus contributing to the development of atrophy and degeneration of nerve fibers.[14,26,27] AGEs contribute to diabetic complications through (i) formation of cross-links between key molecules in the basement membrane of the extra-cellular matrix (ECM), permanently altering cellular structure and architecture and (ii) interaction of AGEs with its receptors on cell surfaces, altering the signaling cascades and cellular function.[14,27]

Table 3: Correlation of morphometric nerve densities with laboratory parameters and heart rate variability indices

Parameters	r*	*P* value
TG	−406	0.133
AGEs	−0.571	0.026*
SD	0.469	0.048*
NGF	0.363	0.018*
NA	−191	0.049*
CHOACTASE	0.203	0.048*
GSH	0.575	0.025*
SDNN	0.351	0.019*
RMSSD	0.231	0.048*
PNN50	0.235	0.039*
HRV INDEX	0.311	0.259
LF	−0.236	0.398
HF	−0.343	0.211

TG: Triglyceride, AGEs: Advanced glycation end product, SD: Sorbitol dehydrogenase, NGF: Nerve growth factor, NA: Nor-adrenaline, Choactase: Acetylcholine transferase, GSH: Glutathione, SDNN index (SDNNI): Mean of the standard deviations of all the NN intervals for each 5 min segment of a 24 h HRV recording. RMSSD: Root mean of squared successive differences, pNN50: Percentage of successive RR intervals that differ by more than 50 ms. LF: Low frequency, HF: High frequency. *P<0.05 compared with normal control

Noradrenaline was significantly higher in the DCAN group compared with the normoglycemic control. This may indicate over stimulation of the sympathetic nerves since noradrenaline has been proposed as a surrogate of muscle sympathetic activity and one of the recommended test by the Toronto Consensus committee on the diagnosis of DCAN.[28-33] Choline acetyltransferase (choactase), a protein marker of parasympathetic nerves, had been shown to correlate with autonomic nerve densities. Yang *et al*. found a decreased concentration of choactase in Akita diabetic rat model similar to what was observed in this study. He further showed that the reduced choactase correlated with lower western blot choactase antibodies and autonomic nerve rarefaction.[34] The above findings may support the fact that while there is the destruction of both autonomic nerves, there seems to be an early reduction in parasympathetic activity, unlike the sympathetic system that is overexpressed. This may explain the resting tachycardia seen in diabetic patients with cardiac autonomic neuropathy.

This study confirms the premises that STZ-diabetic rats showed changes in HRV indices early in the course of diabetes, although autonomic functions evaluated by time-domain indices of HR variability were more pronounced about 28 days after STZ injection. Interestingly, all of these changes showed a negative correlation with plasma glucose and AGEs while positively correlated with sorbitol dehydrogenase and antioxidant markers.

Earlier studies have suggested that DCAN has both parasympathetic and sympathetic dysfunction with early defective parasympathetic control, represented by persistent resting tachycardia and loss of beat-to-beat variation during deep respiration.[29,35-37] Similar to what Faran *et al*. documented, this study observed early changes in the PNN50 in the DCAN model while SDNN showed significant changes 28 days after DCAN induction.[35] SDNN had been shown to provide an estimate of overall HR variability and sympathetic balance while PNN 50 correlates with overall parasympathetic events. The findings that parasympathetic dysfunction occurs early in DCAN may be due to the fact that it takes a longer time for the sympathetic dysfunction to manifest in abnormality of HR variability. Since blood parameters showed a significant increase in nor-adrenaline levels early after DCAN induction, it may then indicate that HR variability indices lag behind plasma markers of sympathetic dysfunction. SDNN has important correlates with sympathetic function and in fact has been proposed as a prognostic marker for myocardial infarction and sudden death.[37-43] Studies have also shown significant derangement in PNN50 in heart failure subjects with autonomic dysfunction.[44-46]

Structural changes occurring in peripheral nerves may typify that of human diabetic neuropathy and usually preceded by hyperglycemia-induced biochemical abnormalities. Non-enzymatic glycosylation of myelin components, reduction of endoneurial blood flow, increased oxygen free radical activity, or production and deprivation of the NGF have been implicated in the process of axonal dystrophy.[47] The present study confirmed the afore-mentioned showing impaired antioxidants activity and reduced NGF, which correlates with the HR variability spectral events shown.

The finding of disorganized cardiac muscular architecture and swelling in the study may be a pointer to the early development of diabetic cardiomyopathy. This may lead to diastolic dysfunction and a high incidence of heart failure in patients with diabetes. Previous studies have shown that AGEs can affect the physiological properties of proteins in the ECM by inducing the formation of cross-links, thereby causing intracellular changes in vascular and myocardial tissue.[48]

The results of the current study corroborate the fact that cardiac autonomic neuropathy and axonal dystrophy seems to be closely associated with biochemical derangements in STZ-diabetic rat model, such as increased AGEs, glucose, and norepinephrine, with reduced SD activity, choactase, anti-oxidants, and NGF, which all correlate with HR autonomic indices derangements. In agreement with some early data reported by researchers who showed that autonomic nerve structural lesions do not appear as early as these functional changes. The functional changes seen could be attributed to the early development of autonomic neuropathy in this model, which if treated early, may prevent permanent structural neurological dysfunction. The correlation observed between cardiovascular HR variability dysfunction and plasma biomarkers are in accordance with these observations.

Conclusion

At the moment, management options have been geared toward prevention of DCAN; however, once developed no treatment has been proven to reverse the DCAN features, thus the need for early routine checks for diabetic patients. The correlation of cardiac nerve densities with HRV indices and biomarkers in this study further strengthens the assertion that routine ECG Holter will be very useful and indeed recommended for patients with DM for early detection and follow-up of neuropathy before interventions may be impossible.

Authors' Declaration Statements

Ethics approval and consent to participate

All experimental protocols were approved and performed in accordance with the guiding principles of the UERC.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

No conflicts of interest.

Funding

The cost of this study was borne by the investigators.

Authors' Contributions

AOM designed, performed the project work, analyzed, and interpreted the data. He was the main contributor in writing the manuscript. BVO supervised the work. He contributed to designing, analyzing, and interpretation of the data, also a major contributor in writing the manuscript. SAO supervised the work. He contributed to designing, analyzing, and interpretation of the data, also a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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