Hypoglycaemic activity and cardiac autonomic neuropathy amelioration of neem in alloxan-induced diabetic Wistar rats

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**ABSTRACT**

**Introduction:** Diabetes-related complications encompass injury to blood vessels, renal system, integuments, feet and nerves resulting from hyperglycaemia. As herbal remedy, the effects of aqueous extract of *Azadirachta indica* (Neem) leaves on hyperglycaemia and Cardiac Autonomic Neuropathy (CAN) in rats were assessed.

**Methods:** Thirty-five Wistar rats (both sexes) used in this study were assigned into two phases of three groups in each phase and a common control group; - Control (C), Diabetic group (DG), Glibenclamide group (AG), and Neem group (AN) with five rats in each group. All the groups in phase 1 except control were rendered diabetic by injection of 2% solution of alloxan monohydrate, intraperitoneally at 150 mg/kg. AG and AN rats were immediately given Glibenclamide (0.5mg/kg) and Neem extract (500mg/kg) respectively. The control group received 0.5ml of normal saline daily for 2 weeks. In phase 2, after injection of 2% alloxan monohydrate, rats were left till we confirmed that they were diabetic using glucometer before Neem and Glibenclamide were introduced to the corresponding groups. This was done at 48 hours when all rats were confirmed diabetic. At two weeks, Electrocardiography (ECG) was recorded.

**Results:** There were signs of Cardiac Autonomic Neuropathy (CAN) in the DG groups during both phases such as increased heart rate, myocardial infarction, increased R and T amplitudes, Atrial fibrillation and flutter.

**Significance:** It could be concluded that Neem reversed alloxan-induced hyperglycaemia but could not totally ameliorate the cardiovascular effects of CAN in diabetic rats once it is fully established.

**ARTICLE INFO**

Article history:

Received: March 09, 2020
Received in revised form: June 05, 2020
Accepted: June 17, 2020

**Keywords:**
Cardiac autonomic Neuropathy, Neem, Glibenclamide, Electrocardiography.

**Introduction**

In type 1 diabetes (T1D), previously known as juvenile diabetes, little or no insulin is produced by the pancreas; the hormone required for the body to utilise blood sugar as describe by Shehadeh et al., (2001). Symptoms which develop over short period of time include high blood sugar level (hyperglycaemia), frequent micturition, more fatigue, hunger and weight loss (Chiang et al., 2014) as well as blurry vision, tiredness, and poor wound healing (Shehadeh et al., 2001). This is related to long-term skin, liver, kidney, nerve, blood vessel damage and central nervous system degenerative diseases. This condition is characterised by lack of functional beta cells of the pancreas resulting in insulin insufficiency. Patients suffering from this condition therefore depend on external source of insulin. In type 2 diabetes (T2D), symptoms include high blood sugar, thirst, frequent urination, more fatigue, hunger and weight loss, relative lack of insulin (NIH, 2014). Other symptoms include increased hunger, tiredness, and sores that do not heal (NIH, 2014). Long-term complications which mostly follow increased blood sugar are heart disease, strokes, diabetic retinopathy that can lead to blindness, renal failure and poor blood flow in the limbs often leading to amputations. However, ketoacidosis is uncommon (Pasquel and Umpierrez, 2014; Fasanmade et al., 2008).

Diabetic Autonomic Neuropathy (DAN) is a severe and widespread diabetes complication, frequently neglected and misdiagnosed. It's a systemic-wide disease that in the early stages may show no symptoms. Cardiovascular Autonomic Neuropathy (CAN) is the most studied and clinically important form of DAN described as impairing the autonomic control of the cardiovascular system in patients (human or animals) with diabetes (Tesfaye et al., 2010). In the early years of diabetes, CAN is detectable with warning signs such as reduced heart rate variability (HRV) during deep breath, prolonged QT interval, temporarily followed by resting tachycardia, impaired exercise tolerance and reduced baroreflex sensitivity with consequent abnormal blood pressure regulation, and orthostatic hypotension (Pop-Busui, 2010). Increased sympathetic tone is associated with cardiac alterations in early stage (Krause, 2009).

Hyperglycaemia leads to oxidative stress in diabetic neurons and triggers several biochemical pathways that are a major cause of damage, and are potential therapeutic targets in diabetic neuropathy (Edward, 2008). CAN is associated with complications which endanger life: such as arrhythmias, silent myocardial ischemia and sudden death (Tesfaye et al., 2010). Valensi et al.,

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https://doi.org/10.36759/svj.2020.082
(2003) reported that CAN is found early in the course of diabetes and should be considered as a prognostic marker of the associated complications. The autonomic innervation from parasympathetic nervous system is the primary extrinsic control mechanism that regulates cardiac performance (Gordan et al., 2015). Complications of diabetes significantly impair the sympathetic tone resulting in CAN (Pop-Busui et al., 2004; Taskiran et al., 2004). Lifestyle changes with increased physical activities are primary non-pharmacological interventions to reduce risk of cardiovascular dysfunction such as QTc interval prolongation, left ventricular (LV) hypertrophy that portrays the ECG recordings as distorted R wave amplitudes.

Electrocardiography is the process of producing an electrocardiogram, a recording of the electrical activities of the heart obtained through placement of electrodes on specific body surface of an animal or humans. It is a diagnostic device that is regularly used to test heart muscles and its electrical functions. The measurements are often displayed as PQRST as demographically set down by Einthoven; a very old Scientist that first worked on ECG. The P wave denotes atrial contraction while the QRS complex corresponds to ventricular contraction (depolarization) and T wave stands for relaxation of the ventricle. Other measurements include PR interval which denotes delay at AV bundle; ST segment and QT interval signs of what happens during ventricular relaxation. Sometimes QTc will be used (Corrected QT with RR interval). It is a non-invasive, inexpensive technique that yields useful information in diagnosis of arrhythmias, conduction abnormalities as well as prognostic and therapeutic considerations of the heart (Azeez et al., 2016; Azeez et al., 2017).

Alloxan monohydrate is a toxic glucose equivalent, which when given to rats, mice and many other animal organisms, selectively damages beta cells in the pancreas (Sreenivasa and Gangapatnam, 2017). The diabetes expert committee of the World Health Organization (WHO) has recommended further study of conventional herbal medicinal products for the management of diabetes and its complications (Nagashayana et al., 2014). Khosla et al., (2000) reported that when Neem leaves extract (500 mg / kg orally) was administered for 4 weeks after diabetes induced by alloxan in rabbits, blood glucose levels were significantly reduced. In addition to its hypoglycaemic effects, this current study was conducted to assess the ameliorative effect of aqueous extract of Neem leaves on CAN in alloxan-induced diabetic rats.

Materials and methods

Experimental animals

Thirty-five adult Wistar rats (150-250g) of both sexes were used for the experiment in two phases. They were obtained from the Animal House unit of the Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine, University of Ilorin. They were housed under standard laboratory environmental conditions; they were given feed (Vital grower pelletised feeds (Grand Cereals Limited, Jos, Nigeria) and water ad libitum. The animals were allowed one week to acclimatize before commencement of the study and were housed throughout the experiment in well ventilated cages. All the animals were handled according to the International Guiding Principles for biochemical research involving animals (CIOMS, 1995).

Dosing protocol

Induction of diabetes: Freshly prepared 2% solution of alloxan monohydrate in 0.9% normal saline was injected intra-peritoneally at 150 mg/kg body weight single dose (Bopanna et al., 1997). Glucose level was thereafter measured using Glucometer (Bionime GM 300 blood glucose monitoring system made by Bionime Corporation, GMBH; Switzerland). The level of serum glucose considered to be normal in Rattus norvegicus ranges from 50 to 135 mg/100 mL (Wang et al., 2010). In this study, rats with glucose levels above 175 mg/dL were considered as being diabetic.

Grouping of rats

Wistar rats were assigned to four groups as follows: Control (C), Diabetic group (DG), Alloxan/ Glibenclamide (AG) and Alloxan/Neem (AN).

Control (C): This group was composed of non-diabetic rats, used as study controls. During two weeks they received 0.5 ml of normal saline daily.

Alloxan (DG): In this group, diabetic was induced by injection of 2 % alloxan monohydrate solution, intraperitoneally at a dose of 150 mg/kg body weight. For two weeks this group also received 0.5ml of normal saline per day.

Alloxan/Glibenclamide (AG): This group served as positive control. Rats were rendered diabetic by injecting 2 % solution of alloxan monohydrate at a dose of 150 mg / kg body weight intraperitoneally. Glibenclamide was also given orally to the rats in this group at a dose of 0.5mg/kg body weight suspended in normal saline. This was administered orally and daily for two weeks (Patil et al., 2013).

Alloxan/Neem (AN): Rats in this group were made diabetic by injection of 2 % alloxan monohydrate solution at a dose of 150 mg / kg body weight, intraperitoneally. This group also received orally, daily for two weeks, aqueous extract of Neem at a dose of 500 mg / kg body weight. The dosage was calculated by a pilot study in which the diabetic rats were given a gradually increasing dose from 200 mg to 1000 mg of the extract, and the effect was observed (Nagashayana et al., 2014).

In the 1st phase of the study, rats were fasted for about 6 hours, before alloxan was administered in the morning. Glibenclamide and Neem (Azadirachta indica) were administered immediately after injection of alloxan (Nagashayana et al., 2014).

In the 2nd phase of the study, rats were fasted as above. Glibenclamide and Neem were given 48 hrs after injection of alloxan when rats in all treated groups were confirmed to have become diabetic and all other procedures follow.

Determination of blood glucose concentration

Blood glucose concentration was determined before the commencement of the experiment (Basal) and thereafter at 48 hours, seven days and 14 days post treatment.

Measurement of electrocardiographic parameters

At the end of the two weeks, each rat was anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) before the ECG recording with Veterinary Electrocardiographic Equipment.
(EDAN® 10, made in China). The detail of the procedure was reported by Azeez et al. (2016; 2017).

Statistical analysis

The data collected were expressed as Mean ± Standard Deviation (Mean ± SD) and subjected to one-way analysis of variance. This was followed by a multiple post-hoc comparison by Tukey to compare differences between the means obtained from the control and the rats tested, using GraphPad Prism version 5.3 for windows (San Diego, CA, USA). Differences were considered significant at p < 0.05.

Results

Following injection of alloxan monohydrate, rats were observed for 24–48 hours, the rats showed the following signs: polydipsia (anomalous thirst), polyuria (excessive urine volume), weight loss (due to lean mass loss), asthenia (weakness due to failure to use glucose as a source of energy) and some degree of dehydration.

Table 1 shows the variation in the blood glucose level at 48 hours, 7 days and 14 days after induction of diabetes. The mean blood glucose concentration in the control group varied from basal level (50.3±6.5 mg/dL) to two weeks (61.8± 2.2 mg/dL). There was a time dependent increase in blood glucose level in the AG group from a basal level of 55.3± 10.2 mg/dL to 291.6±26.2 mg/dL at 48 hours 323.8±30.8 mg/dL at 7 days and to 438.2± 15.6 mg/dL at 14 days.

In the AG group, there was a significant increase in blood glucose level from basal level (69.8±2.7 mg/dL) when compared with 48 hours (233.6±11.50 mg/dL) and 7 days post treatment (176.7±16.90 mg/dL). This was later followed by a steady decline through the study period to 14 days (89.3±6.70 mg/dL).

Table 1. Duration dependent change in blood glucose level in different rat groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Basal glucose</th>
<th>48 hours PT</th>
<th>7 days PT</th>
<th>14 days PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (mg/dL)</td>
<td>50.30±6.50</td>
<td>58.59±3.70</td>
<td>48.90±5.50</td>
<td>61.80±2.20</td>
</tr>
<tr>
<td>DG (mg/dL)</td>
<td>55.30±10.20</td>
<td>291.61±26.20*</td>
<td>323.80±30.80*</td>
<td>438.20±15.60*</td>
</tr>
<tr>
<td>AG (mg/dL)</td>
<td>69.80±2.70</td>
<td>233.60±11.50*</td>
<td>176.70±16.90*</td>
<td>89.30±6.70†</td>
</tr>
<tr>
<td>AN (mg/dL)</td>
<td>56.60±8.90</td>
<td>212.31±5.40*</td>
<td>98.56±2.50†</td>
<td>68.42±6.80†</td>
</tr>
</tbody>
</table>

C – Control, DG – Diabetic group, AG – Glibenclamide group, AN – Neem group, PT – Post treatment, * - Significantly increased, † - Significantly reduced . Level of significance – p < 0.05

Table 2. ECG parameters of different rat groups in phase 1 of the experiment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (C)</th>
<th>Diabetic Group (DG)</th>
<th>Alloxan/Giben. (AG)</th>
<th>Alloxan/Neem (AN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>250.40±4.90</td>
<td>266.40±2.60</td>
<td>251.60±4.60</td>
<td>242.20±1.90</td>
</tr>
<tr>
<td>P (ms)</td>
<td>34.80±3.40</td>
<td>115.00±1.60*</td>
<td>109.80±10.60</td>
<td>104.60±2.90</td>
</tr>
<tr>
<td>PR (ms)</td>
<td>47.00±1.50</td>
<td>130.40±1.70*</td>
<td>124.20±3.60</td>
<td>121.60±1.60</td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>21.20±1.30</td>
<td>35.80±1.90</td>
<td>28.60±2.90</td>
<td>20.00±4.80</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>142.80±6.30</td>
<td>167.40±3.40*</td>
<td>142.80±8.90</td>
<td>128.20±7.90</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>256.20±19.10</td>
<td>398.00±21.40*</td>
<td>293.60±31.50</td>
<td>258.00±15.70</td>
</tr>
</tbody>
</table>

HR – Heart rate, P – Duration of P wave, PR – Duration of PR interval, QRS – Duration of QRS, QT – Duration of QT interval, QTc – Duration of corrected QT interval, ms – milliseconds, Gilben – Glibenclamide
Phase 1 Electrocardiography

Figure 1. Control group: ECG record of waves in lead II of Control rat group showing normal P, QRS complex and T

Figure 2. Diabetic Group (DG): ECG record of waves in lead II of alloxan induced diabetic rats showing wider P, tall and wider ‘R’, taller and wider ‘T’, making T/R ration reduced, prolonged QT interval. ‘S’ is below the iso-electric baseline; RR interval is irregular

Figure 3. Glibenclamide group (AG): ECG record of waves in lead II of rats given Glibenclamide (0.5ml/kg) ‘R’ remains tall, ‘T’ height is reduced but still wide, QT still prolonged. RR interval is irregular

Figure 4. Alloxan/neem (AN): ECG record of waves in lead II rats given Neem (500ml/kg) there are reduced ‘R’ and ‘T’ amplitude, but with wider T/ prolonged QT. RR interval is regular.

Table 3. Variation (mean ± SD) glucose values of diabetic rats compared to the Neem and Glibenclamide treatment groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Basal glucose</th>
<th>24 hours PT</th>
<th>48 hours PT</th>
<th>7 days PT</th>
<th>14 days PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (mg/dL)</td>
<td>59.3±6.50</td>
<td>58.59±3.70</td>
<td>68.9±5.50</td>
<td>61.8±2.20</td>
<td>69.45±7.30</td>
</tr>
<tr>
<td>DG (mg/dL)</td>
<td>70.32±5.60</td>
<td>192.8±63.60*</td>
<td>289.3±88.70*</td>
<td>319.6±110.70*</td>
<td>345.6±125.90*</td>
</tr>
<tr>
<td>AG (mg/dL)</td>
<td>69.41±6.10</td>
<td>138.5±40.20*</td>
<td>188.5±48.60*</td>
<td>141.8±89.50*</td>
<td>101.9±41.90</td>
</tr>
<tr>
<td>AN (mg/dL)</td>
<td>62.34±5.40</td>
<td>177.4±58.00*</td>
<td>196.8±76.80*</td>
<td>112±61.90*</td>
<td>89 79±26.32†</td>
</tr>
</tbody>
</table>

C – Control, DG – Diabetic group, AG – Glibenclamide group, AN – Neem group, PT – Post Treatment, * - Significantly increased, † - Significantly reduced, Significant level – p <0.05
Table 4. Variation in the ECG indices of the different rat groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DG</th>
<th>AG</th>
<th>AN</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>250.4±4.90</td>
<td>277±13.60</td>
<td>254.3±9.40</td>
<td>224.3±4.08†</td>
</tr>
<tr>
<td>P (ms)</td>
<td>25±1.70</td>
<td>45±1.70</td>
<td>34.7±3.10</td>
<td>35.7±2.08</td>
</tr>
<tr>
<td>PR (ms)</td>
<td>47.0±1.50</td>
<td>56±3.90</td>
<td>61.7±4.70</td>
<td>48.7±2.08</td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>21.2±1.30</td>
<td>35±2.10*</td>
<td>25.3±2.90</td>
<td>26.7±3.80</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>142.8±6.30</td>
<td>187.3±13.80*</td>
<td>108±11.50*</td>
<td>144.7±9.80</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>256.8±19.10</td>
<td>461.3±44.20*</td>
<td>327±24.80</td>
<td>287.3±31.78</td>
</tr>
</tbody>
</table>

C – Control, DG – Diabetic group, AG – Glibenclamide group, AN – Neem group, HR – Heart rate, P – Duration of P wave, PR – Duration of PR interval, QRS – Duration of QRS, QT – Duration of QT interval, QTc – Duration of corrected QT interval, ms – milliseconds, * - Significantly increased, † - Significantly reduced, Significant level – p < 0.05

Phase 2 Electrocardiography

Figure 5. C (control group): ECG record of rats in Control group showing normal P, QRS complex, QT and QTc

Figure 6. Diabetic Group (DG): ECG record of waves in lead II of alloxan induced diabetic rats showing the P, ‘R’ wider ‘T’ wide and tall. ‘S’ is on the iso-electric baseline. There is infarction, fibrillation with flutter and hypokalaemia

Figure 7. Glibenclamide group (AG): ECG record of waves in lead II of rats that were given Glibenclamide (0.5ml/kg) ‘R’ remains significantly tall, Height of ‘T’ is reduced, QT and QTc prolongation reduced insignificantly

Figure 8. Neem Group (AN): ECG record of waves in lead II of rats given Neem (500ml/kg). ‘R’ remains tall, Height of ‘T’ height has reduced but still wide, QT and QTc, reduced insignificantly. RR interval irregular
Discussion

The ECG offers a quick, early and non-invasive opportunity for the detection of cardiovascular changes. These changes in cardiovascular functions in diabetic patient include changes in duration of P wave, PR interval, QRS wave and so on. These variations result in syncpe, flutter, arrhythmia, and fibrillation which are characteristics of Cardiac Autonomic Neuropathy (CAN) (Voulgari et al., 2011). The observed increased in amplitude of R wave in the 1st phase is a sign of Left Ventricular Hypertrophy (LVH), increase in the QRS width indicates blockage of bundle branch. The amplitude of R wave was reduced in the AN group (Figure 4). This showed that Neem reduced the height to minimal level close to that of the control (Figure 1). In 2nd phases, R amplitude remained high in AG and AN rat groups suggesting left ventricular hypertrophy. RR interval remained irregular (arrhythmia persist). This suggested that once the diabetes becomes established and chronic, Neem may not be adequate to ameliorate the neuropathy associated with alloxan-induced diabetes (Pop-Busui, 2010).

The significant increase in QRS intervals in DG group is a sign of abnormal intraventricular conduction. In the 2nd phase, it increased significantly in DG group (35±2.10) compared to control (21.2±1.30) but insignificantly with AG (25.3±2.90) and AN (26.7±3.80). The ventricular hypertrophy, right and left branch bundle block (RBBB/LBBB) was not ameliorated by Neem. This is due to the diabetic complications that resulted in the dysfunction of the autonomic nervous system in cardiac regulation. Zhang et al., (1999) also observed hypertrophy of left ventricle and QT interval alterations coupled with decreased cardiac function are commonly observed in diabetes related CAN.

There was significant reduction in RR intervals and increased heart rates observed in DG rat group of 1st phase, in comparison to control (Figure 2) and other groups (Figures 3 and 4). The RR interval was irregular (presence of arrhythmia) in DG (Figures 2) and AG (Figure 3) but became regular in AN group (Figure 4) an improvement in the rhythmicity of the heart. In the 2nd phase, RR remained irregular in AG (Figure 7) and AN (figure 8); an indication that arrhythmia persisted in the groups after administration of Glibencamamide and Neem. This implied that CAN was not reversed by the administration of neem.

PR intervals in the 1st phase revealed significant prolongation of PR intervals in DG rat groups (130.4±1.70) compared to control (47.0±1.50). The difference was insignificant in the AG (124.2±3.60) and AN (121.6±1.60) rat groups. Excessive prolongation of PR interval is an indication that movement of impulse was further delayed at the atrio-ventricular node by the effect of the diabetes (Aro et al., 2014).

T wave increased in amplitude and width significantly in DG of the two phases (Figures 2 and 6). This made the T/R ratio comparatively lower and it’s a sign of myocardial infarction and hypokalaemia. There was significant reduction in T height and width of AN group (Figure 4) in the 1st phase study. The height was not lowered/reduced in AN group of the 2nd phase study. The myocardial infarction, valvular problems and hypokalaemia persisted in 2nd phase AG (Figure 7) and AN groups (Figure 8).

From this result, it appears that Neem could not remove all cardiovascular neuropathy syndromes once diabetes is already established or chronic (Fabiyi-Edebtor, 2020). Significant exaggeration of QT and QTc intervals (a measure of ventricular repolarization) (Tables 1 and 2) was observed in DG group compared to control, AG and AN rat groups. The abnormalities of ventricular repolarization such as hypokalaemia and valvular impediment present in the DG group appeared to have been ameliorated by Neem in the AN rat group (Figure 4). This implies that some of the signs of CAN were ameliorated during the first phase of the experiment.

In this study, autonomic nerves function was not measure directly. However, ECG indicators of autonomic dysfunction such as alterations of increased heart rate, R and T wave height and width as well as QT interval prolongation were observed in all the treatment groups. Van Hoose (2010) in his study observed that imbalance of sympathetic and parasympathetic responses stimulates hyperglycaemia and when prolonged resulted in elevated levels of catecholamine and a decrease in adrenergic receptors; this was corroborated by Scott and Kench, (2004). The corresponding imbalance in sympathetic and parasympathetic stimulation resulted in denervation of the vagus nerve. According to (Vinik et al, 2003), these developments are believed to occur within one year of type 1 diabetes in humans; while only years into the diagnosis may clinical presentations emerge (Ewing et al., 1985). According to Bergstrom et al., (2009) symptoms of CAN in type 1 diabetic patients can be seen in as low as two months. This is an indication that CAN symptoms can be seen in acute diabetic situation as seen in our results.

Conclusion

Neem has negative chronotropic effect (decreasing heart rate) on diabetic rats, able to ameliorate myocardial infarction, bundle branch block and ventricular hypotrophopy seen DG rats of the 1st phase. However, Neem was unable to remove all these dysfunctions when diabetes is already established or chronic. We know that human beings only go to hospital for treatment when they have health challenges which may be diagnosed as diabetes. Neem is good in bringing down the blood glucose level but unable to totally remove all the cardiovascular neuropathy that comes with it. We therefore recommend that Neem be used along with other drugs if CAN symptoms already ensued.

Conflict of interest

No authors declare conflicting interest.

References


