

Beta Cell Regenerating Potential of *Azadirachta indica* (Neem) Extract in Diabetic Rats

G McCalla, O Parshad, PD Brown, MT Gardner

ABSTRACT

Objective: This study evaluated the ability of 0.8% neem leaf extract (NLE) to treat diabetes mellitus by assessing its effects on blood glucose, insulin levels and islet morphology in streptozotocin (STZ)-induced diabetic Sprague-Dawley rats.

Methods: Diabetes was induced in two to three-day old rat pups by STZ intraperitoneally (60 mg/kg), followed by a further 40 mg/kg dose 12–23 weeks later. The diabetic treated (DT) rats received 0.8% w/v NLE in tap water while diabetic control (DC) and normal control (NC) rats received water ad libitum. Body weight, water and chow consumption, and blood glucose were evaluated weekly. Blood and pancreas were collected at the end of the study to evaluate serum insulin and islet histology, respectively.

Results: Neem leaf extract (0.8%) improved weight gain and beta cell regeneration but did not reduce blood glucose. Serum insulin increased slightly in the treated group and three-fold in the DC group ($p < 0.05$).

Conclusion: The results suggest that NLE has beta cell regenerating potential.

Keywords: *Azadirachta indica*, diabetes, neem, serum insulin

Potencial del Extracto de la *Azadirachta indica* (Neem, ó Nim) para la Regeneración de las Células Beta en Ratas Diabéticas

G McCalla, O Parshad, PD Brown, MT Gardner

RESUMEN

Objetivo: Este estudio evaluó la capacidad del extracto de hojas de neem (EHN) al 0.8% para tratar la diabetes mellitus, a partir de sus efectos sobre la glucosa en sangre, los niveles de insulina, y la morfología de los islotes en ratas Sprague-Dawley con diabetes inducida por estreptozotocina (SZT).

Métodos: La diabetes fue inducida en crías de ratas, de dos a tres días de edad mediante STZ intraperitoneal (60 mg/kg), seguida de una dosis de 40 mg/kg 12–23 semanas más tarde. Las ratas diabéticas tratadas (DT) recibieron EHN al 0.8% p/v en agua corriente, mientras que las ratas diabéticas de control (DC) y las normales de control (NC) recibieron agua ad libitum. El peso corporal, el consumo de agua y comida, y la glucosa en sangre se evaluaron semanalmente. La sangre y el páncreas se recolectaron al final del estudio para evaluar la insulina sérica y la histología de los islotes, respectivamente.

Resultados: El extracto de hoja de neem (0.8%) mejoró la regeneración de células beta y el aumento de peso, pero no redujo la glucosa en sangre. La insulina sérica aumentó ligeramente en el grupo tratado y se triplicó en el grupo DC ($p < 0.05$).

Conclusión: Los resultados sugieren que el EHN tiene potencial para la regeneración de las células beta.

Palabras claves: *Azadirachta indica*, neem, diabetes, insulina sérica

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INTRODUCTION

Diabetes mellitus (DM) is a rapidly growing global health problem with an overall prevalence of 13.4% in Jamaicans who are 15 years and older (1), and 4.0% worldwide in adults who are 20 years and older (2). There are two major forms of DM: Type 1 and Type 2, with Type 2 DM being the most common, affecting over 90% of the DM population. Neem (*Azadirachta indica* A Juss [*Melia azadirachta* Linn]) leaves have been used as a folklore remedy to treat diabetes in Jamaica. There are, however, conflicting reports regarding the antidiabetic properties of Neem. This current study was, therefore, designed to determine the effectiveness of the leaves and associated stems of the neem plant as an antidiabetic agent.

Azadirachta indica A Juss (*Melia azadirachta* Linn) is a botanical cousin of the mahogany (family Meliaceae). This tropical tree is native to India and Myanmar but is now grown in many countries in Asia and in the tropical regions of the Western Hemisphere including the Caribbean islands, Central and South America, and Africa (3). The entire neem tree has been used for centuries worldwide, particularly in the Indian subcontinent, for its insecticidal, therapeutic and pharmacological efficacy. The twigs are used to clean teeth, the juice to treat skin disorders, the leaves to ward off bugs, and the tea as tonics (4). It has also been shown to possess anti-inflammatory, anti-arthritis, and antimalarial properties (5–8).

The objective of this study was to evaluate the effect of 0.8% (w/v) neem leaf extract (NLE) on blood glucose, insulin level and islet morphology in streptozotocin (STZ)-induced diabetic Sprague-Dawley rats, thereby assessing its antidiabetic capacity. We hypothesize that aqueous NLE has antidiabetic effects on STZ-induced Type 2 diabetic rats through beta cell regeneration.

SUBJECTS AND METHODS

Ethical approval was granted by the University Hospital of the West Indies/University of the West Indies/Faculty of Medical Sciences (UHWI/UWI/FMS) Ethics Committee.

Aqueous neem leaf extract

A sample of the neem tree was collected and authenticated by Mr Patrick Lewis, a resident botanist and herbarium curator at The UWI, Mona herbarium. Voucher number 35688 was assigned to the specimen, which was deposited in the herbarium. Permission to harvest and use the plant was granted by the Ministry of Agriculture of Jamaica. Fresh, healthy neem leaves with their stems were collected from The UWI Mona campus, and placed in a solar drier (3–5 days, maximum temperature 40 °C). The dried leaves and their associated stems were milled and the powder obtained weighed then boiled in distilled water (100 g/L portions) under reflux for four hours, cooled and filtered using a strainer and muslin cloth. The filtrate was measured, made up to 1 L (stock neem concentration ranged from 0.011–0.048 g/mL) then diluted with distilled water to make a 0.8% stock solution (9) for oral feeding. Unused portions were freeze-dried and refrigerated at 4 °C for future use. The dia-

betic treated (n = 13) rats received 0.8 % freshly made NLE *ad libitum* for 5–13 weeks and thereafter fed orally daily (0.62 g/kg bw/mL) in the morning for three weeks using a syringe with a 20-gauge ballpoint needle to evaluate the effect of the NLE on their blood glucose levels.

Induction of diabetes

Sixty-six two and three-day old rat pups were injected intraperitoneally with 60 mg/kg STZ (Sigma, France) in 0.1 M citrate buffer, pH 4.5. Neonatal rat pups were re-united with their mothers after injection and weaned at four weeks of age at which time they were allowed free access to chow and water and kept at a constant light cycle of 12 hours on/12 hours off. A normal control group of nine rat pups was injected with an equivalent volume of citrate buffer. Twenty-four adult rats (6–10 weeks old) from this group of neonates were re-injected with 40 mg/kg STZ to ensure that diabetes developed and persisted as they could possibly revert to a normoglycaemic state (modified from Bonner-Weir *et al* (10)). Glucose levels were determined three days post-first and second STZ injection, following an eight-hour fast. Blood was collected from the tail vein and an Accu Chek Advantage glucometer (Roche Diagnostics, Germany) was used to analyse the blood glucose. Glucose assessment was repeated weekly from weeks 6–15 until diabetes was confirmed. Rats that developed hyperglycaemia were administered 15 mg/kg glibenclamide (11) dissolved in 1 mL dimethylsulphoxide [DMSO] (12) orally after an eight-hour fast using a syringe with a 20-gauge ballpoint gavage needle to assess the type of diabetes. The first glucose reading was obtained immediately after glibenclamide then at half-hour intervals thereafter for an hour and a half. The Type 2 diabetic rats (fasting blood glucose > 6.9 mmol/L) were separated into diabetic control (DC; n = 11) and diabetic test (DT; n = 13) groups one week after the re-injection, and fed NLE for three weeks *via* gavage.

The normal control (NC) and DC rats received standard rat chow and normal tap water, whereas DT rats received aqueous NLE *ad libitum* in place of water after developing diabetes. Glucose assessment continued weekly throughout the duration of the study. Body weight and consumption of both chow and water or NLE were evaluated on a weekly basis. Chow, water and NLE were measured prior to feeding rats.

Serum insulin and histological studies

Rats were anaesthetized with diethyl ether and sacrificed (13) at the end of the treatment period. Blood was obtained from the heart and the serum assayed for insulin *via* radioimmunoassay. The whole pancreas was removed from each rat and the tail region fixed in 40% formalin and processed using standard histological procedures. Tissues were stained with aldehyde-fuchsin according to Halmi and Scott's modified Gomori's aldehyde-fuchsin methods (14–16). Islets were analysed under oil immersion using a light microscope, and their cross-sectional areas were measured using the formula: SA = a x b x $\pi/4$, where SA is the sectional area, and 'a' and 'b' are the two

longest perpendicular diameters (17). The β cells were counted by identifying their nuclei and cell membranes. Islet images were taken with a Leica DMRME optical microscope fitted with a Leica DC 500 digital camera (Leica, Wetzlar, Germany). The β cells that showed positive stain reaction (fully or partially stained purple/purple-violet) were classified as viable. Of the viable cells, those with low granulation were regarded as having low activity. Beta cells that did not absorb the stain or appeared degranulated were classified as inactive.

Statistical analysis

The results were partitioned into “phases” at the end of the study based on observation and the nature of treatment received by each group of animals (Tables 1, 2). Results are expressed as means \pm SEM using SigmaPlot 2000 software, and SPSS for Windows software version 12 (Chicago, USA) was used to determine the significance of the mean difference between groups *via* one-way analysis of variance (ANOVA). The Bonferroni post hoc test was used for multiple comparisons. A *p*-value < 0.05 was considered statistically significant.

Table 1: Description of “phases” and nature of treatment of animals

“Phase”	Description	Treatment		
		NC	DC	DT
1	Baseline glucose for NC rats	CB	–	–
2	1 st glucose post STZ injection	–	STZ	STZ
3	Decreasing blood glucose	–	–	–
4	Rising blood glucose	–	–	NLE to all
5	Injection of 2 nd STZ dose	–	2 nd STZ	Post NLE + 2 nd STZ
6	Post-STZ injection	–	Post 2 nd STZ	Post 2 nd STZ
7	Sacrifice	S	S	S

NC: normal control; DC: diabetic control; DT: diabetic treated; CB: citrate buffer; STZ: streptozotocin; NLE: neem leaf extract; S: sacrifice

RESULTS

Diabetes induction

There was no mortality in the citrate buffer-injected control neonates but 50% of the STZ injected pups died within two weeks of injection. Twenty-seven (81.8%) of the 33 STZ treated pups that survived developed hyperglycaemia (> 6.9 mmol/L) between 6 and 14 weeks of age, predominantly 8 and 10 weeks of age.

Fasting blood glucose concentration

The mean fasting blood glucose (FBG) concentration of the normal control rats was 4.5 \pm 0.1 mmol/L. This was significantly higher (*p* < 0.01) in the diabetic groups [14.5 \pm 1.6 to 20.1 \pm 2.0 mmol/L] (Fig. 1). No significant difference was seen between the glucose concentrations in the diabetic groups after NLE treatment, and glucose concentration was higher in the treated (DT) group.

Table 2: Description of “phases” and duration of treatment of animals

“Phase”	Description	Duration (weeks post 1 st STZ injection)		
		NC	DC	DT
1	Baseline glucose for NC rats	2	–	–
2	1 st glucose post STZ injection	–	1	1
3	Decreasing blood glucose	–	2–7	2–7
4	Rising blood glucose	–	8–14	8–11
5	Injection of 2 nd STZ dose	–	15–23	12–23
6	Post-STZ injection	–	24–26	24–26
7	Sacrifice	17	27	27

NC: normal control; DC: diabetic control; DT: diabetic treated; STZ: streptozotocin

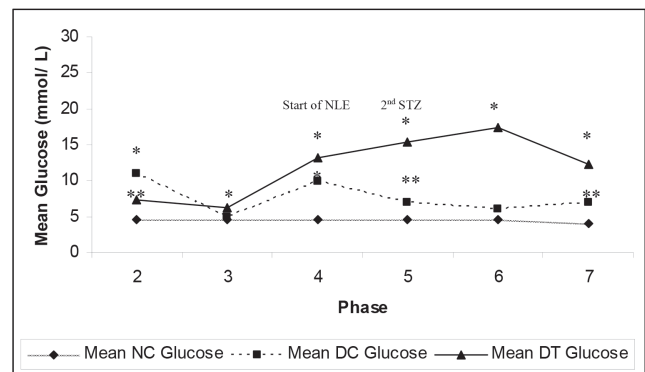


Fig. 1: Mean (\pm SEM) fasting blood glucose concentration (mmol/l) for normal control (NC), diabetic control (DC) and diabetic rats (DT). **p* < 0.0001 vs NC; ***p* < 0.01 vs NC

Insulin determination

There was no significant difference between mean insulin concentration for the DT and NC rats, however, DC rats showed a significantly higher insulin concentration compared to the NC rats (Table 3).

Table 3: Mean (\pm SEM) insulin in normal and diabetic rats at “phase” 7

Group	n	Insulin concentration (mIU/ml) Mean \pm SEM
NC	9	1.27 \pm 0.74
DC	10	4.26 \pm 1.07*
DT	13	1.94 \pm 0.54

**p* < 0.05 DC vs NC

NC: normal control; DC: diabetic control; DT: diabetic treated

Islet morphology and beta cell count

Purple or purple-violet beta cells formed the core and vast majority of the islets. Few green delta and yellow alpha cells formed a peripheral zone around the core β cell mass (Fig. 2). Islets were reduced in size in the diabetic groups but not to a significant extent (Table 4), with the DT group having the smallest sectional area (4524.26 \pm 676.02 μ m² vs 8404.25 \pm ...)

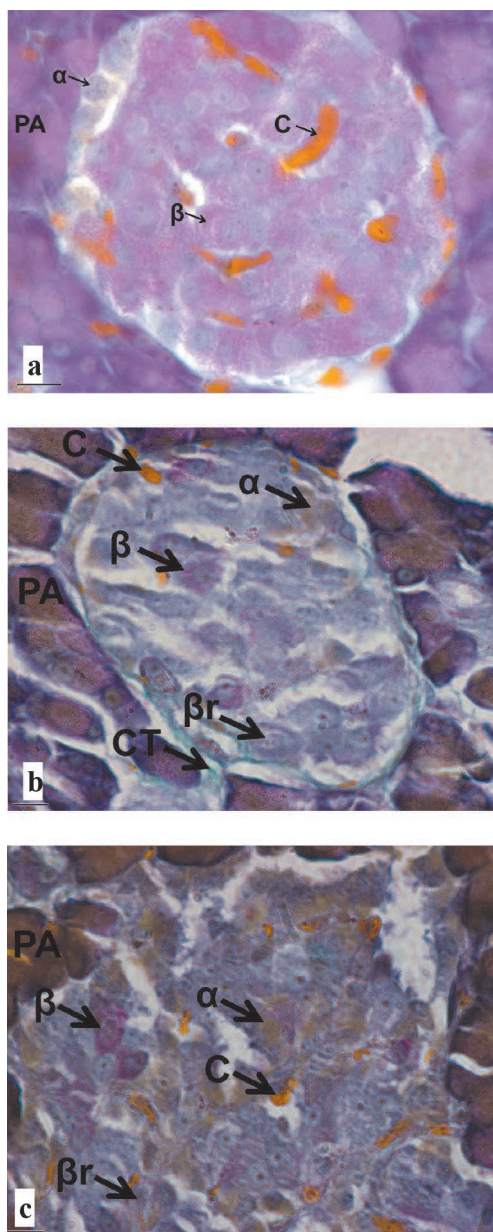


Fig. 2: Pancreatic islets of normal control (a), diabetic control (b) and diabetic treated (c) rats (10 μm scale bar).

C: Capillary; PA: pancreatic acinus; CT: connective tissue; α : alpha cell; β : beta cell; βr : beta cell with reduced staining intensity

2365.59 μm^2 in NC and $6209.88 \pm 610.92 \mu\text{m}^2$ in DC rats). All islets in the diabetic groups had less intense stain reaction in most of their β cells and the number of β cells per unit area was lowest in the DC group ($6.98 \pm 0.65 \times 10^{-3}/\mu\text{m}^2$) and highest in the DT group [$12.28 \pm 1.41 \times 10^{-3}/\mu\text{m}^2$] (Fig. 2, Table 4). However, there was no significant difference between the diabetic groups (DC or DT) and NC rats ($11.55 \pm 4.34 \times 10^{-3}/\mu\text{m}^2$). More than 80 % of the β cells in all groups were viable, based on their ability to absorb the AF stain and show purple or purple-violet granulation. No significant difference in viability was observed. The remaining cells appeared degranulated and did not stain purple but there was no significant difference between the groups. There was also a higher percentage of β cells with low/reduced granulation (activity) per islet sectional area in the diabetic groups *versus* the NC group ($4.91 \pm 4.91\%$) with the DC group showing significantly ($p < 0.02$) reduced activity ($43.5 \pm 7.42\%$). The difference was not significant when comparing the DT group ($35.5 \pm 8.38\%$) with the NC group.

DISCUSSION

This study was geared toward evaluating the effect of 0.8% (w/v) neem leaf and stem extract (NLE) on blood glucose, insulin level and islet morphology in STZ-induced diabetic Sprague-Dawley rats. We found that STZ induced diabetes in the majority (81.8 %) of the rat pups ($p < 0.0001$ vs NC) but NLE (0.8%) did not alleviate it. Neem leaf extract, however, improved weight gain and β cell regeneration. We also found that serum insulin concentration in the neem treated group was not significantly different from the normal control but increased three-fold in the untreated DC group ($p < 0.05$ vs NC).

The arrangement of cells in the islets with a core of purple or purple-violet β cells seen in this study has been previously reported (18, 19). Streptozotocin altered the morphological features of the islets, which was also seen by Aybar *et al* (20). These changes were evidenced by reduction in the size of islets, perhaps due to apoptosis (21), size and number of cells, possibly due to necrosis (22, 23), as well as alteration in their shape.

The effect of the NLE was seen in improvement in the size, number and granular appearance of the β cells, a sign of β cell regeneration. The hypertrophic β cells seen in the dia-

Table 4: Mean (\pm SEM) beta cell parameters in normal and diabetic rats at "phase" 7

Parameter	Group (Mean \pm SEM)		
	NC	DC	DT
Islet sectional area, SA (μm^2)	8404.25 \pm 2365.59	6209.88 \pm 610.92	4524.26 \pm 676.02
Total # of β cells ($\times 10^{-3}/\mu\text{m}^2$)	11.55 \pm 4.34	6.98 \pm 0.65	12.28 \pm 1.41
Viable β cells (%)	85.21 \pm 4.60	84.24 \pm 5.86	83.88 \pm 6.49
β cells with low activity (%)	4.91 \pm 4.91	43.5 \pm 7.42*	35.5 \pm 8.38
Inactive β cells (%)	14.79 \pm 4.60	15.04 \pm 5.88	16.29 \pm 6.55
Low activity + inactive β cells (%)	19.7 \pm 5.84	58.54 \pm 7.21*	51.80 \pm 8.91

* $p < 0.02$ vs NC

NC: normal control; DC: diabetic control; DT: diabetic treated

betic rats were perhaps due to a compensatory mechanism by the cells to produce more insulin to meet the body's demand for it. Yin *et al* (24) associated the restoration of β cells with cell hypertrophy and modest levels of proliferation. Luscombe and Taha (25) reported that the antidiabetic activity of NLE was hinged on the presence of functional pancreatic β cells. This study shows that there were functional β cells in the diabetic rat pancreases, evidenced by production of slightly more insulin in the treated (DT) group than in the normal control (NC) group (1.94 ± 0.54 vs 1.27 ± 0.74 mIU/mL) and significantly ($p < 0.05$) more in the untreated DC group (4.26 ± 1.07 mIU/mL) at the end of the study (Table 3). The DC group also had a significantly ($p < 0.02$) higher percentage of β cells with low activity compared to the NC group (Table 4). Since insulin was still produced, we therefore suggest that NLE stimulated β cell recovery or proliferation and hypertrophy, but diabetes was due to insulin resistance.

From this study, we noted that diabetes was induced by STZ in rats predominantly between 8 and 10 weeks and impaired β cell functionality. Diabetes was due to reduced β cell activity and possibly cellular insulin resistance, with untreated diabetic animals having a significantly higher percentage of low β cell activity. The 0.8% NLE did not alleviate diabetes nor restore glucose homeostasis but improved β cell regeneration. We therefore conclude that 0.8% NLE is not potent enough to treat diabetes but its β cell regenerating capacity may be useful. Neem leaf extract (0.8%) also results in insulin concentrations that are comparable to the normoglycaemic condition.

Further work should involve assessment of insulin at multiple intervals coinciding with glucose assessment. The active principle(s) in NLE as well as the mechanism of action by which neem reduces blood glucose and regenerates β cells are also areas in need of investigation.

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