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Chronic Treatment with Ethanolic Extract of the Leaves of *Azadirachta indica* Ameliorates Lesions of Pancreatic Islets in Streptozotocin Diabetes

Tratamiento Crónico con Extracto Etanólico de Hojas de *Azadirachta indica* Disminuye las Lesiones de los Islotes Pancreáticos en Diabetes por Estreptozotocina

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SUMMARY: Botanical drugs are complementary therapies in the management of diabetes mellitus. In this work, we studied the effects of chronic treatment of diabetic rats with *A. indica* (neem) on blood glucose, pancreatic islet histopathology, and oxidative status of the pancreas. Fifty-four Wistar rats (5-8 weeks old) were randomly assigned to 5 treatment groups. Hyperglycemia was induced in 34 fasted rats with a single i.p. injection of STZ (70 mg/kg bw/d). Ethanolic extract of *A. indica* leaves (500 mg/kg bw/d) was given orally to diabetic rats (n=12) for 50d. Glibenclamide was given (p.o) at 600 µg/ kg bw/d. In each group, blood glucose, islet histopathology, and pancreatic oxidative status, were assessed. All hyperglycemic rats in the neem-treated group had become normoglycemic at the end of week 2. By 50d, the number of viable b cells was highest in the neem-treated diabetic rats (compared with the diabetic and glibenclamide groups). Similarly, islet histology showed marked improvement in this group, in addition to improved oxidative stress. Our findings confirmed the hypoglycemic effect of neem. Besides, the improved islet morphology and oxidative status in neem-treated diabetic rats suggest the potential of this herb at improving lesions of the pancreatic islet in diabetes mellitus.

KEY WORDS: *Azadirachta indica*; Pancreatic islet lesion; Diabetes mellitus.

INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterised by glucose intolerance and chronic hyperglycemia (Kántarová *et al.*, 2006). Type 1A diabetes describes the immune-mediated form of type 1 DM; while type 1B is the non-autoimmune idiopathic form (American Diabetes Association, 1997). Type 1 DM accounts for about 10% of all cases of DM and it affects approximately 20 million people worldwide (Libman *et al.,* 1993). In most patients, pancreatic islets are infiltrated by immunocytes (insulitis). At diagnosis, hyperglycemia is evident, and destruction of b cells of pancreatic islets is more than 90% complete (Ryu *et al.*, 2001). Thus, in long-standing type 1 DM, b cell mass is usually decreased to less than 1% of normal mass (Meier *et al*., 2005). On the other hand, insulin resistance, b-cell dysfunction and failure, are typical features of type 2 DM (Hayden, 2007). In such patients, the initial relative insulin deficiency may progress to absolute deficiency, and the individual may depend on exogenous insulin for survival (Raskin *et al.,* 2005). As the disease advances, the pancreatic islets are characterised by fibrosis (Ko *et al.*, 2004), amyloid formation by b cells (Marzban & Verchere, 2004), and b cell deficit from apoptosis (Butler *et al.,* 2003). Thus, structural deterioration of the pancreatic islets is a key factor in both type 1 and type 2 forms of DM. The link between chronic hyperglycemia and oxidative stress in b cells has been documented (Robertson *et al.*, 2004). Chronic hyperglycemia results in oxidative stress via autoxidation of glucose in the presence of transition metals (Wolff *et al*., 1989); decreased activities of antioxidant enzymes such as SOD and glutathione peroxidase (Blakytny & Harding, 1992); increased oxidative phosphorylation (Nishikawa *et al*., 2000), glycosylation of proteins (Wolff *et al*.); and activation of the hexosamine pathway (Kaneto *et al*., 2001). Such increased, hyperglycemia-induced, intra-

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islet oxidative stress has been hypothesized and demonstrated to result in b cell dysfunction and death (Donath *et al*., 1999; Robertson *et al*., 2003), as well as in fibrosis of pancreatic islets (Hong *et al*., 2007; Hayden *et al*., 2007). Thus, drugs that improve glycemia and/or oxidative stress have been reported to ameliorate or prevent islet lesions and fibrosis. In this regard, Ko *et al*. reported the beneficial effects of ramipril, an angiotensin converting enzyme (ACE) inhibitor, on the improvement of glycemia and prevention of islet fibrosis in Otsuka Long-Evans Tokushima fatty rats. And using a similar animal model, Yamabe & Yokozawa (2007) reported the protective effect of the polyherbal drug, Hachimi-jio-gan, on oxidative damage and fibrosis of the islets of Langerhans. Moreover, using islet cells isolated from Wistar rats, Beppu *et al*. (2003) reported that the free-radical scavenging activity of Aloe arborescens is associated with the preservation of b cells of pancreatic islets.

Previous studies had reported the beneficial effects of *Azadirachta indica* A. Juss in the management of DM and amelioration of the oxidative stress associated with this disease (Chattopadhyay, 1999a; Gupta *et al.*, 2004). *A. indica* is currently included in polyherbal antidiabetic drugs being subjected to controlled clinical trials in human (Hsia *et al*., 2004; Shekhar *et al*., 2002). However, histopathological changes of the pancreatic islets, which may be associated with chronic exposure to *A. indica* as an antidiabetic therapy, are yet to be reported. Thus, in this study, we report morphologic, morphometric and oxidative changes in the pancreatic islets of diabetic rats chronically exposed to ethanolic extract of *A. indica* leaves. The possible implications of these findings in humans are discussed.

MATERIAL AND METHOD

Animals. Fifty-four Wistar rats of both sexes (Harlan, Milan, Italy) were used. Animals weighed 125-199 g and were 5-8 weeks old on arrival. They consisted of 40 females and 14 males. Animals were kept in cages and housed in the animal room of the Faculty of Biological and Environmental Sciences and Technology, University of Salento, Italy. They were exposed to 12-hour light, 12-hour dark cycle at 21-23 0C. All animals were maintained on Harlan Global Diet 2018 (Harlan, Milan, Italy). Food and water were given freely. The study was approved by the Animal Use Committee of the Faculty of Biological and Environmental Sciences and Technology, University of Salento.

Azadirachta indica Leaves. Mature leaves of *A. indica* A. Juss were collected from neem trees at the University of Ilorin Mini Campus, Nigeria, between June and July 2007. A sample of the collection was compared with the voucher specimen at the herbarium of the Botany Department of the same University (Voucher No 542).

Extraction of A. indica Leaves. Fresh leaves of A. Indica were air-dried and extracted by percolation as previously described (Chattopadhyay, 1999b). A total of 2000 g of the dry leaf powder was extracted. The final yield (about 120 g) was a dark-brown sticky mass. This was stored at 4 0C.

Induction of Diabetes Mellitus. Hyperglycemia was induced in 34 overnight-fasted, randomly selected animals by a single injection of streptozotocin (Sigma, MO, USA), at 70 mg/kg bw, in citrate buffer (0.1M, pH 4.5). Animals were allowed free access to food and water after injection. Sustained hyperglycemia developed about 72 hours STZ post-injection. Animals with fasting blood glucose of ≥250 mg/dl were considered hyperglycemic (Gupta *et al*.). Non-diabetic control animals (n=9) received a single i.p. injection of citrate buffer (1 ml/kg b w).

Azadirachta indica **and Glibenclamide Treatment**. The dose of A. indica used was based on the report of Mostofa *et al.* (2007). The extract (500 mg/kg b w/d) was dissolved in physiological saline and was administered by gavage to hyperglycemic rats (n=12) at 9.00-10.00 hour each day for 50d. For comparative studies, a sulphonylurea, glibenclamide (Sigma, MO, USA), was given at 600 µg/kg bw/d (Sathishsekar & Subramaniam, 2005) to another group of hyperglycemic rats (n=12) for 50d. Twelve (12) hyperglycemic rats, which received neither *A. indica* nor glibenclamide, constituted the diabetic group. Besides, a separate group of normoglycemic rats (n=10) were administered 500 mg/kg bw/d of *A. indica* only. Blood glucose, body weight, hepatic histopathology and oxidative stress biomarkers were studied in each group.

Blood Glucose. Blood glucose was estimated in fasted rats at 9.00-10.00 hr using One Touch Ultra 2 Glucometer (Lifescan, CA, USA). Blood was obtained from the dorsal vein of the tail. At day 0 of *A. indica* and glibenclamide treatment, blood glucose was monitored every alternate hour (1, 3, 5, 7, 9 & 11 hr); then each day in the first week; and thereafter, twice in a week, for 7 weeks.

Body Weight. Body weights of rats were taken prior to the induction of hyperglycemia, at day 0 of *A. indica* treatment, and on a daily basis thereafter, for 7 weeks.

Termination of Treatment. In each treatment group, animals (n=4) were killed at 7d, 21d, and 50d under

pentobarbital (60 mg/ kg bw) (Pharmacia & Upjohn, Milan, Italy). Laparatomy was performed and the pancreas was excised, trimmed free of fat, rinsed in PBS and fixed in 4% paraformaldehyde solution. Portions of the pancreas were also stored at -80 0C for analysis of oxidative stress markers. Tissue Processing for Microscopy – After fixation, paraffin embedding was done, and 3.5 µm sections were cut on a Reichert-Jung 2050 rotary microtome (Cambridge Instruments, Buffalo, Germany), followed by haematoxylin & eosin, and Masson's trichrome staining. Photomicrographs were taken with a Nikon digital camera DXM1200F (Nikon, Japan) mounted on a Nikon Eclipse 80i light microscope (Nikon, Japan).

Oxidative Stress Markers. The levels of lipid hydroperoxides, aqueous hydroperoxide, and SOD were estimated in homogenates of the pancreas. For each sample, a 10% homogenate was prepared with 0.15M KCl in a potter homogeniser (GPE, Bedfordshire, England). The homogenate was centrifuged at 1000 g for 10 min at 4 0C (Sumanth & Rana, 2006). The supernatant was used for the estimation of oxidative stress markers and protein assay. This was stored at –80 0C prior to analysis.

Lipid Peroxide. Lipid hydroperoxide levels of each sample of the pancreas were estimated with a PeroxiDetectTM kit (Sigma, MO, USA). tert-Butyl hydroperoxide served as standard. Absorbance was read at 560 nm in an Ultraspec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Aqueous Hydroperoxide. Aqueous hydroperoxide levels of each sample of the pancreas were estimated using PeroxiDetectTM kit (Sigma, MO, USA). Hydrogen peroxide served as standard, and absorbance was read at 560 nm in a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Superoxide Dismutase (SOD) – Pancreatic SOD activities were estimated using 19160 SOD Determination Kit (Fluka, Buchs, Switzerland). The assay uses a highly soluble tetrazolium salt, WST-1. Absorbance of the formazan dye was read at OD 450 nm in an ETI-System Fast Reader (SORIN Biomedica, Vicenzia, Italy).

Protein Assay. Protein levels of the samples were estimated by the BioRad Protein Assay Method (BioRad, CA, USA). Bovine serum albumin (BSA) (100 µg/ml) served as standard, and absorbance was read at 595 nm in a spectrophotometer.

Statistical Analysis. Data were analysed using SPSS 15.0 (SPSS Inc, Chicago, USA) and Excel 2007 (Microsoft Corporation, USA). Data were expressed as Mean±SEM. Means were compared using the student's t-test.

RESULTS

Body weight. Figure 1 shows changes in body weights of animals at 7d, 21d and 50d. At the end of week 1, 1.5% loss in body weight had occurred in the neem-treated diabetic rats. Similarly, body weight gain in the neem-treated non-diabetic rats was negligible. In contrast, higher increases in body weight were obtained in the control, diabetic and glibenclamide-treated diabetic rats at this time. By 50d, weight gain in the neem-treated normoglycemic rats was the least (19.9%); while the highest weight increase of 49.9% (P<0.05) occurred in the glibenclamide-treated rats.

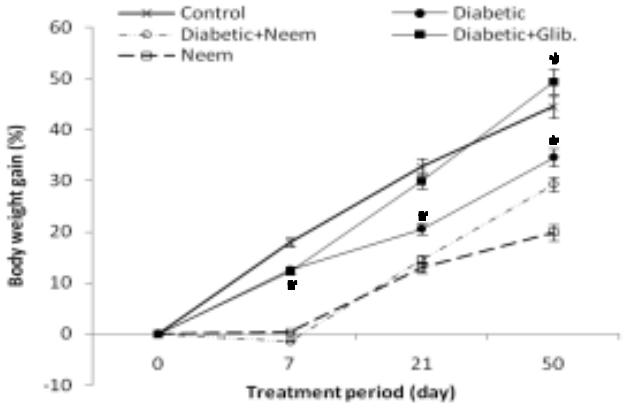


Fig. 1.Change (%) in body weights of animals at 7d, 21d, and 50d of treatment. Bar represents mean±SEM. *P<0.05 vs. control. (Glib. = Glibenclamide).

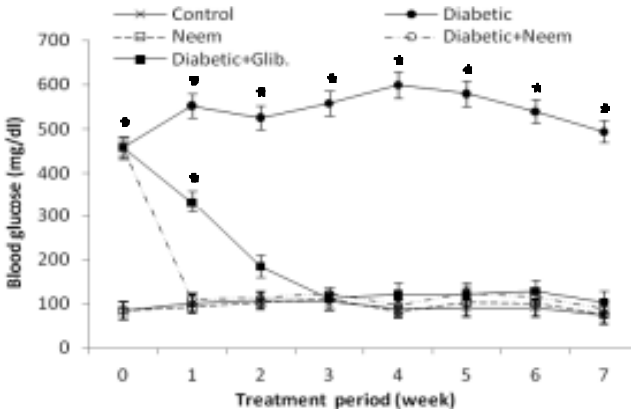


Fig. 2. Weekly blood glucose of control and treated animals. Bar represents mean±SEM. * P<0.05 vs. non-diabetic control. (Glib. = Glibenclamide).

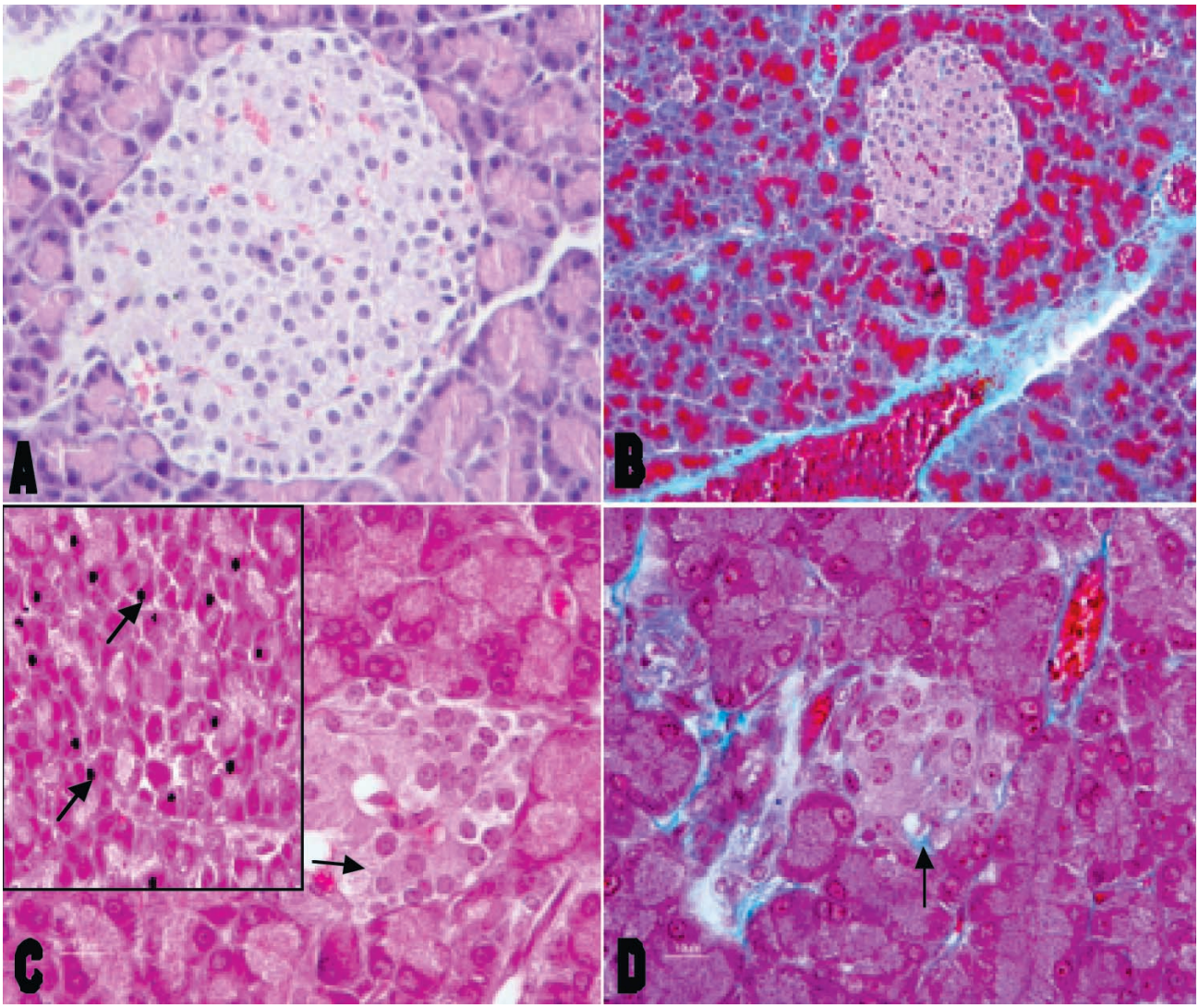
Blood Glucose. Figure 2 shows details of weekly blood glucose. At week 0, all animals in the diabetic, neem-treated diabetic and glibenclamide-treated groups were hyperglycemic. By the end of week 2, normoglycemia had been attained in all hyperglycemic rats treated with A. indica; and this status was maintained till euthanasia at 50d. In hyperglycemic rats treated

with glibenclamide, all rats had become normoglycemic by the end of week 3 (Fig. 2). In contrast, hyperglycemia persisted in all the rats in the diabetic group from week 0 to 7. Control and neem-treated normoglycemic rats remained normoglycemic throughout the study period.

Histopathological Observations. At 50d of treatment, islets of diabetic rats were markedly diminuted, with necrosis and paucity of cells (Fig. 3 C). In addition to these changes, marked eosinophilia was seen in both the exocrine and endocrine pancreas. Besides, characteristic darkly-pigmented intracytoplasmic bodies were observable in acinar cells of exocrine pancreas (Fig. 3 C, inset). Furthermore, extensive necrosis that characterised the islets of neem-treated diabetic rats at 7d and 21d (figures not shown) was largely absent at 50d. By this time (50d), necrosis was restricted to islet periphery, while the central part of the islet was vascularised and possessed viable cells (Fig. 3 E).

In contrast, diffuse necrosis that characterised the islets of glibenclamide-treated diabetic rats at 21d (figure not shown) was also noted at 50d (Fig. 3 G). Fibrosis and necrosis were absent in the islets of control and neem-treated normoglycemic rats (Fig. 3 A-B & I-J). In diabetic animals treated with neem, islet fibrosis was restricted to the periphery at 50d (Fig. 3 F). Diabetic rats showed fibrotic spots at islet periphery (Fig. 3 D); while islet fibrosis was absent in glibenclamide-treated diabetic rats (except for extensive necrosis).

Morphometric Analysis of Pancreatic Islets. Figure 4 shows the morphometric parameter of the pancreatic islets. At 7d of treatment, islet b-cell density (number of b-cells/mm2 of the islet) had decreased significantly in the diabetic, neem-treated diabetic, and glibenclamide-treated diabetic rats ($P<0.05$ vs. ctrl). Only 24.4 %, 29.8 % and 24.0 %, respectively, of b cells remained viable in these groups.



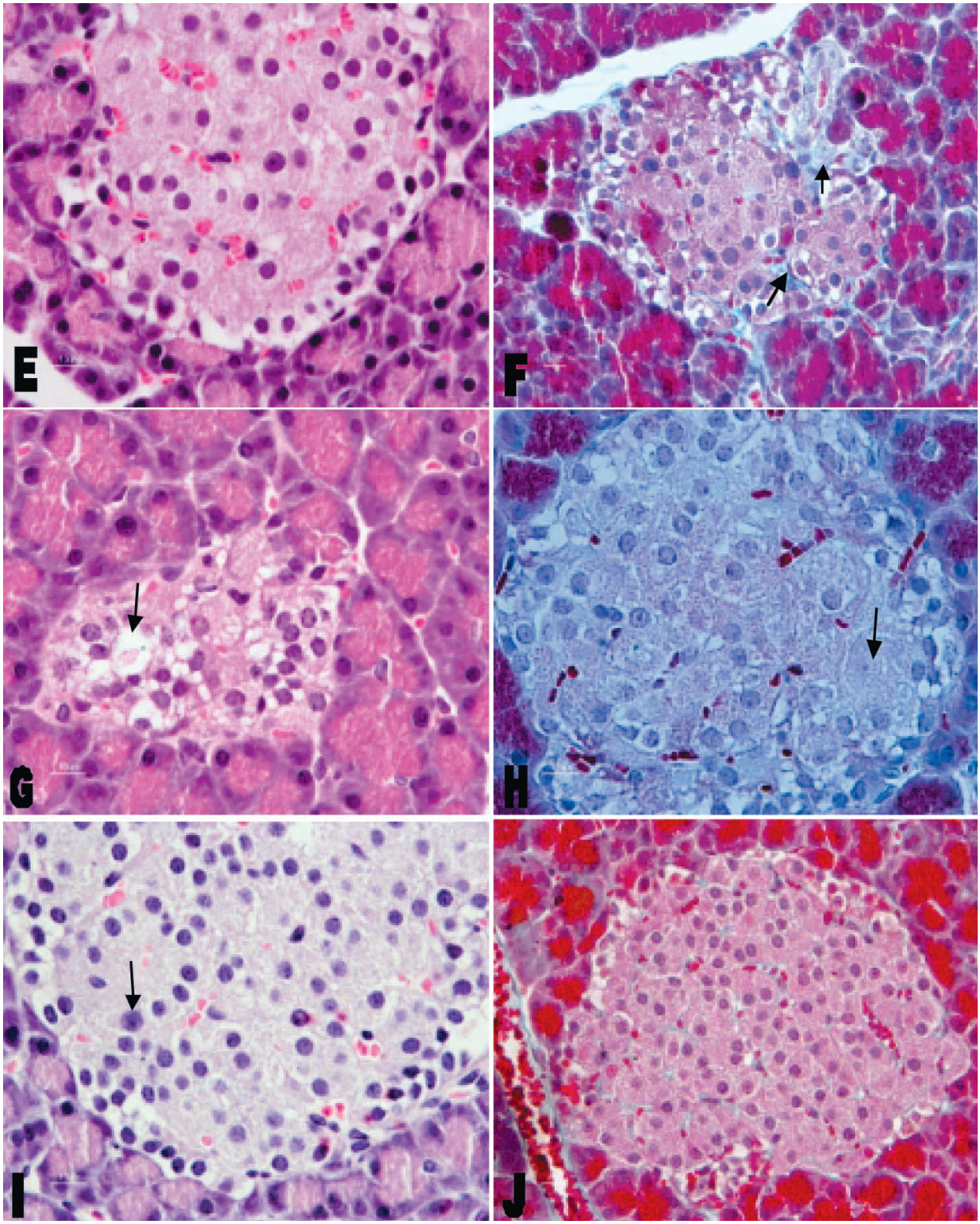


Fig. 3 (A-J): Pancreatic islets at 50d of treatment. (A&B) Control. A, islet appears normal; H&E, x40. B, fibrosis and necrosis are absent; trichrome, x20. (C&D) Diabetic group. C, islet is diminuted with paucity of cells and necrosis (arrow); (Inset) exocrine pancreas of diabetic rats. Darkly-pigmented bodies are seen in many acinar cells. Eosinophilia of exocrine pancreas has increased; H&E, x60. D, there is paucity of islet cells and presence of fibrous spots at islet periphery (arrow); trichrome, x60. (E&F) Neem-treated diabetic group. E, islet is well-vascularised, with viable cells; H&E, x60. F, viable cells are observable in the central part of the islet; necrosis and fibrosis (arrows) are confined to the periphery; trichrome, x60. (G&H) Glibenclamide-treated diabetic group. G, islet necrosis is persistent (arrow); H&E, x60. H, fibrosis is negligible, while necrosis is observable; trichrome, x60. (I&J) Neem-treated normoglycemic group. I, islet necrosis is absent; a few cells are hypertrophic (arrow); H&E, x60. J, fibrosis and necrosis are absent, while islet cells are intact. Masson's trichrome, x40. Bar represents 10 μ.

The larger percentage had been lost to necrosis arising from STZ toxicity. Islet b-cell density in neem-treated normoglycemic rats did not differ significantly from control (P>0.05).

By 50d, neem-treated diabetic rats had had the highest percentage (50%) of viable b cells (when compared with diabetic and glibenclamide-treated diabetic groups), although this was still significantly less than control (P<0.05) (Fig. 4).

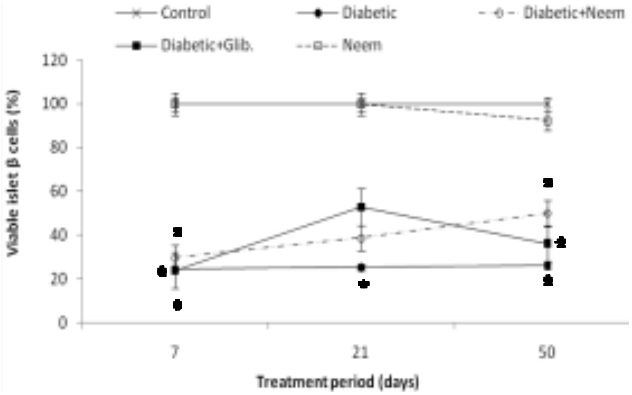


Fig. 4. Percentage of viable islet b-cells relative to control. Bars represent Mean±SEM of 5 animals;*P<0.05 vs. control.

Pancreatic Lipid Hydroperoxide Levels. Figure 5 shows changes (%) in pancreatic lipid peroxide (LPO) levels in the various treatment groups versus control. Pancreatic lipid peroxide levels were significantly low in all the treatment groups at 7d (P<0.05). By 21d of treatment, a 5-fold increase (P<0.05) in LPO levels had occurred in the pancreas of glibenclamide-treated rats. At this time, peroxide levels had also risen significantly in the diabetic rats; while a 35% fall had occurred in the neem-treated diabetic rats (P<0.05). By the 50th day of exposure, significant increases in lipid peroxide levels (P<0.05) was obtained in the diabetic rats; while lipid peroxide levels in the neem-treated diabetic rats had fallen markedly (P<0.05).

Pancreatic Aqueous Hydroperoxide Levels. Figure 6 shows changes (%) in aqueous hydroperoxide levels in the various treatment groups versus control. Time-based changes in aqueous hydroperoxide levels were comparative to the trend obtained for lipid peroxides (Fig. 5). At 50d, a significant rise (29%) in aqueous hydroperoxide levels had occurred in the diabetic rats. However, by this time, pancreatic aqueous hydroperoxide levels in neem-treated diabetic and neem-treated normoglycemic rats had fallen below the control by 61% and 32% respectively (P<0.05) (Fig. 6).

Pancreatic Superoxide Dismutase (SOD) Activity. Figure 7 shows pancreatic SOD activities in the various treatment groups versus control. By 50d, no statistically significant differences in SOD activity were obtained in all the groups compared with control. However, while SOD activity in the diabetic and neem-treated diabetic rats showed slight increases at this time, decreases in activity occurred in the glibenclamide-treated and neem-treated normoglycemic groups (Fig. 7).

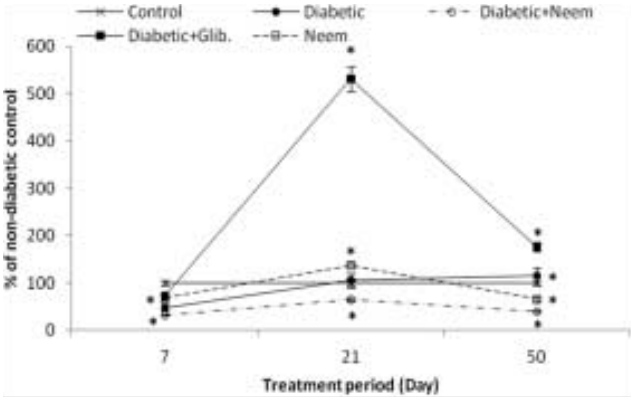


Fig. 5. Lipid hydroperoxide levels of the pancreas. Bars represent Mean±SEM of 5 animals;*P<0.05 vs. control.

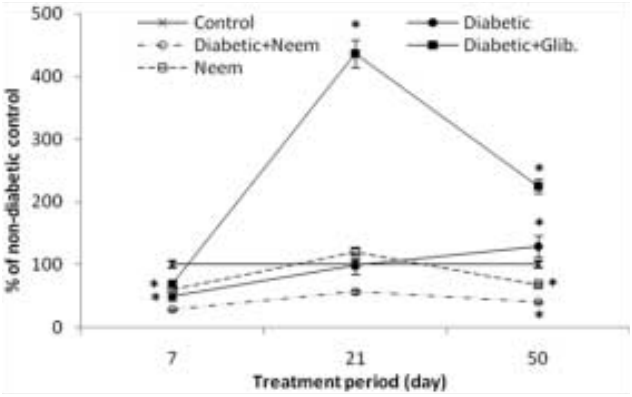


Fig. 6. Aqueous hydroperoxide levels of the pancreas. Bars represent Mean±SEM of 5 animals; *P<0.05 vs. control.

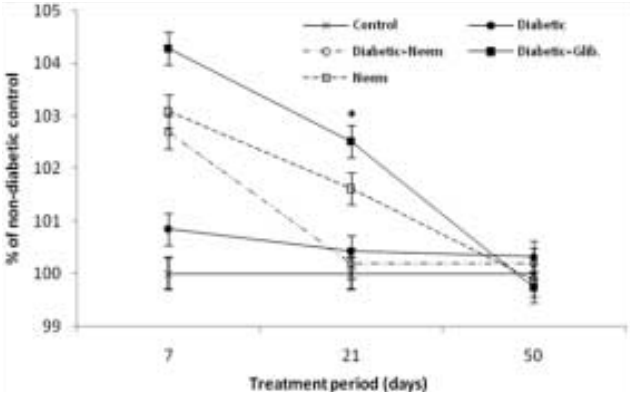


Fig. 7. SOD activity of the pancreas. Bars represent Mean±SEM of 5 animals;*P<0.05 vs. control.

DISCUSSION

Our findings in the present study showed that A. indica treatment (500 mg/kg bw/d) improves hyperglycemia in diabetes. Exposure of STZ-induced diabetic rats to a chronic regimen of the ethanolic extract of *A. indica* produced normoglycemia in 87.5% of the animals by the end of week (wk) 1, and all animals had become normoglycemic by wk 2. Comparison of the data from these animals to those from glibenclamide-treated diabetic rats showed a relative advantage of A. indica over glibenclamide (at 600 µg/kg bw/d) in the maintenance of glucose homeostasis (Fig. 2). Findings comparable to our data had been reported in different animal models of diabetes (Chattopadyhyay, 1999a; Khosla *et al.*, 2000; Ebong *et al.,* 2008). At day 0 of exposure, A. indica produced its maximum effect on blood glucose 5 hrs post-dose (Fig. not shown). A similar study by Murty *et al.* (1978) showed that the hypoglycemic effect of *A. indica* was most pronounced 1-2 hrs post-administration. However, in this instance, the drug was given intravenously to adrenaline-induced hyperglycemic dogs.

Propositions on the mechanisms of the hypoglycemic action of *A. indica* are rather inconclusive (Chattopadyhyay, 1996; 1999b). In the present study, we observed a modulating effect of *A. indica* on body weight of diabetic (and non-diabetic) rats exposed to the leaf extract. By 50d, increases in body weight were least in the neem-treated rats (Fig. 1). Histological examination of the intestinal mucosae of these animals showed intact epithelium (Figures not shown); and this suggests that the relatively low weight gain in these animals was not a function of compromised nutrient absorption. Rather, a modulatory effect of the drug on leptin production, secretion and/or action, or effects on body adiposity, may be involved. A similar mechanism was reported for exendin-4, an antidiabetic substance from the salivary gland of the Gila monster lizard (Szayna *et al.*, 2000). Future research on *A. indica* may thus consider this hypothesis.

Intraperitoneal injection of 70 mg/kg bw/d of STZ to fasted rats resulted in sustained hyperglycemia starting about 72 hrs post-injection. Histopathological examination of the pancreatic islets at 21d showed extensive necrosis that was most noticeable in the central parts of the islets in the diabetic, diabetic+neem, and diabetic+glibenclamide rats (figure not shown). Less than 25% of islet b cells (in each of these groups) remained viable at this time (Fig. 4). Uptake of STZ into b cells is mediated by GLUT-2 transporter (Tjalve *et al.,* 1976). In these cells, STZ mediates its cytotoxicity via the DNA-alkylating activity of its N-methyl-N-nitrosourea moiety, especially at position O6 of guanine (Murata *et al.,* 1999). The transfer of the methyl group from STZ to DNA results in DNA fragmentation and b-cell death (Yamamoto *et al.*, 1981). Additional mechanisms involved in b-cell toxicity of STZ include over-activation of the poly (ADP-ribose) polymerase (in an attempt to repair damaged DNA), with the resultant depletion of cellular NAD+ and ATP (Uchigata *et al*., 1982); as well as glycosylation of proteins (Konrad & Kudlow, 2002).

As was also obtained in the present work (Fig. 5, 6), increased pancreatic oxidative stress is associated with the progression of diabetes mellitus (Kakkar *et al*., 1998); and high ROS levels had been hypothesized as an additional mechanism of b-cell death in the pathogenesis of this disease (Robertson, 2004). This mechanism may thus contribute to the death of b cells from STZ treatment in this study; and also to the failure of islet morphology to improve in the diabetic rats in the latter part of the experiment.

At 50d of exposure, morphometric analysis of the islets of *A. indica*-treated diabetic rats showed increased number of viable b-cell nuclei (Fig. 4). Histopathological assessment of the same tissue showed improved islet morphology (Fig. 3). This was in addition to the improved glycemia observed in these animals starting at week 1 (Fig. 2). In contrast, islet b-cell density in the diabetic and glibenclamide-treated diabetic rats remained relatively low at this time (50d) (Fig. 4), and islet morphology did not improve (Fig. 3). These suggest that the test extract ameliorates islet lesions, possibly by enhancing b-cell proliferation. The latter may be owing to the upregulation of glucose-6-phosphate dehydrogenase (G6PD) in b cells exposed to neem-leaf extract. G6PD is the first key enzyme in the pentose phosphate pathway (Stryer, 1988). Its activity ultimately results in the synthesis of ribose-5-phosphate (R-5-P) (Kuo & Tang). The latter is converted to 5-phosphoribosyl-1-pyrophosphate that acts as the donor of the ribose phosphate unit in nucleotide biosynthesis (Kuo & Tang). Thus, G6PD plays an important role in DNA synthesis and cell proliferation (Kuo & Tang). Certain phytochemicals in neem may thus mediate upregulation of this enzyme (G6PD), with increased production of R-5-P and the resultant increased DNA synthesis and b-cell proliferation. The report of Kuo & Tang showed that proliferation of NIH 3T3 cells was associated with overexpression of G6PD. Furthermore, upregulation of this enzyme had also been shown to be associated with enhanced redox status of cells. In addition to the synthesis of the precursor of DNA (R-5-P), G6PD also generates NADPH. The latter is critical for maintaining

glutathione (GSH) in its reduced form. Glutathione is essential for the detoxification of reactive free radicals and lipid hydroperoxides (Halliwell & Gutteridge, 1989). NADPH also maintains the catalytic activity of catalase, and thus, the reduction of H_2O_2 (Halliwell & Gutteridge). In this work, it is possible that the low levels of lipid hydroperoxide and H_2O_2 obtained in neem-treated diabetic rats (at 50d) arose from enhanced GSH and/or catalase activity, though the levels of SOD (assayed in this work) were not significantly different from control (Fig. 7). Thus, upregulation of G6PD may be the key factor mediating increased islet b-cell density and improved oxidative status in the pancreas of neem-treated diabetic rats (at 50d). This may be one mechanism by which A. indica mediates long-term normalization of glycemia in rodent models of diabetes mellitus. Histochemical and biochemical analysis of this enzyme (G6PD) in the pancreas of diabetic rats on chronic neem regimen may therefore be a subject of future studies.

However, current opinions on the regenerative capacity of the b cells are rather conflicting. The long-held dogma that these cells are post-mitotic had been challenged by recent findings. In mice, the work of Dor *et al*. (2004) showed that terminally-differentiated b cells retain a significant proliferative capacity in vivo. A similar finding had been reported by Nir *et al*. (2007) in a murine model of diabetes. Furthermore, in vivo evidence that human b cells possess regenerative potential, even in the diabetic state, had been reported by Meier *et al.* (2006a). However, recent in vitro studies of Parnaud *et al*. (2008) on human adult b cells yielded contrary findings. This notwithstanding, their report on mice b cells provided additional evidence that these cells could, at least, divide in rodents.

Moreover, duplication of pre-existing adult b cells is not the only possible mechanism by which b-cell mass may be improved in diabetes. Extra-islet stem cells from the liver, spleen, bone marrow, and exocrine pancreas have been identified as potential insulin-producing cells (Meier *et al.*, 2006a).

Though the mechanisms of improved islet morphology and enhanced cell mass (seen in diabetic rats exposed to a chronic regimen of *A. indica* in this work) are yet to be elucidated, it is noteworthy that such morphological improvement of the islet was absent in the diabetic and glibenclamide-treated diabetic rats within a similar time-frame. Indeed, chronic exposure of diabetic patients to sulphonylureas (such as glibenclamide) had been associated with eventual deterioration of b-cell mass (Aston-Mourney *et al.*, 2008). In contrast, based on the present morphometric and morphological findings, we suggest that the test botanical drug (*A. indica*) is capable of enhancing b-cell proliferation in vivo. Our findings provide morphologic evidence supporting the tradomedical use of this drug as an antidiabetic therapy (Abo *et al*., 2008), as well as its current use in certain antidiabetic formulations (Shekhar *et al*., 2002; Hsia *et al*.). Similar morphological improvement of the pancreatic islets had been reported in studies that employed the tetrapeptide pancreagene (Kvetnoi *et al*., 2007), the angiotensin-converting enzyme inhibitor ramipril (Ko *et al*.), and the herbal drugs dangnyosoko (Kim *et al*., 2007), hachimi-jiogan (Yamabe & Yokozawa), and Aloe vera (Noor *et al.*, 2008). With respect to *A. indica* leaf and islet b-cell proliferation, future work may consider BrdU or Ki67 staining of the islets of Langerhans of diabetic rats exposed to a chronic regimen of this drug.

Furthermore, a number of the viable nuclei observed at 50d in each of the treatment groups (except control) had become hypertrophic (Fig. 3), with the highest frequency of such nuclei being observed in the glibenclamide-treated rats. It is noteworthy that hypertrophic nuclei were observed in the islets of these rats as early as 7d. Hypertrophy of b cells is a common finding in conditions of increased insulin demand, e.g. chronic hyperglycemia, as was demonstrated in vitro (Chan *et al*., 2002) and in vivo (Jonas *et al*., 1999).

Oxidative stress is a confounding factor in diabetes mellitus, and it contributes to the pathogenesis (Robertson) and complications (Baynes, 1991) of this disease. Beta cells are especially vulnerable to oxidative insult in that they possess a relatively poor complement of antioxidants (Grankvist *et al.*, 1981). In chronic hyperglycemia, oxidative stress is induced via several mechanisms (Wiernsperger, 2003; Wolff *et al*.; Kennedy & Lyons, 1997; Kawamura *et al.*, 1992). In streptozotocin diabetes, increases in pancreatic oxidative stress are associated with the progression of the disease (Kakkar *et al*.), as was also obtained in this work (Figs. 5, 6).

STZ is a nitric oxide (NO) donor; and NO had been reported to mediate the destruction of pancreatic islet cells, probably via DNA damage (Kroncke *et al.*, 1995). In addition to NO, STZ also generates ROS (from the action of this drug on the mitochondria and from increased xanthine oxidase activity) (Szkudelski, 2001). Thus, scavengers of NO (Kroncke *et al.,* 1995) and ROS (Szkudelski) have been reported to possess beneficial effects against DNA damage and b-cell toxicity induced by these substances. In this work, lipid peroxide and aqueous hydroperoxide levels of the pancreas of diabetic rats had increased significantly above control at 50d (Figs. 5, 6). This indicates increased production of ROS in this organ. Besides, while lipid peroxidation products were also significantly higher than control in the glibenclamide-treated diabetic rats at this time, significantly

low levels were obtained in the A. indica-treated diabetic (and non-diabetic) rats (Figs. 5, 6). Given that the activities of SOD in all the treatment groups were not significantly different from control in the latter phase of exposure (Fig. 7), it is suggested that attenuation of oxidative stress in the pancreas of A. indica-treated diabetic rats was not a result of enhanced endogenous SOD activities. Rather, this effect may partly be attributable to phytochemicals in the administered extract; and might be a factor contributing to the improved histological findings in the islets of *A. indica*-treated diabetic rats at 50d. Active components of *A. indica* leaves had been reported to include quercetin, rutin, and their glycosides, etc (Chattopadyhyay, 1999b). Adewole *et al*. (2006) reported that quercetin, at 25 mg/kg bw, given to adult Wistar rats for 30 days, resulted in improved morphology of the pancreatic islets. Similarly, Prince & Kamalakkannan (2006) reported the beneficial effects of rutin on islet morphology, oxidative status and glycemia in diabetic rats.

Moreover, in addition to the possible role of *A. indica* phytochemicals (antioxidants) in the amelioration of oxidative stress in the pancreas of experimental animals, the prompt reversal of hyperglycemia in *A. indica*-treated diabetic rats may also be a contributory factor. As early as week 1 of treatment, normoglycemia had been restored in the larger percentage of these animals. Thus, hyperglycemia-induced, pancreatic ROS generation in this group could be minimized or abolished earlier. However, the normoglycemic status of the glibenclamide-treated diabetic rats (which at 50d showed high pancreatic lipid peroxidation products) suggests a possible role for *A. indica* phytochemicals in the attenuation of pancreatic oxidative stress in diabetic rats treated with this herb.

In conclusion, our findings in this work confirm the hypoglycemic activity of *A. indica* leaves. Besides, we report the potential of this herb at attenuating islet lesions associated with established diabetes. The mechanisms of these findings are however a subject of further studies. In addition, the low levels of lipid peroxidation products in the pancreas of diabetic rats exposed to A. indica also suggest the beneficial potential of this herb in the amelioration of ROS-associated pancreatic islet lesions in diabetes mellitus.

AKINOLA, O. B.; CAXTON-MARTINS, E. A. & DINI, L. Tratamiento crónico con extracto etanólico de hojas de Azadirachta indica disminuye lesiones de islotes de Langerhans en diabetes por estreptozotocina. ***Int. J. Morphol., 28(1)***:291-302, 2010.

RESUMEN: Los medicamentos a base de plantas son terapias complementarias en el manejo de la diabetes mellitus. En este trabajo se estudiaron los efectos del tratamiento crónico de ratas diabéticas con *A. indica* (Neem) sobre la glucosa de la sangre, la histopatología de los islotes pancreáticos, y el estado oxidativo del páncreas. Cincuenta y cuatro ratas Wistar (5-8 semanas de edad) fueron asignadas aleatoriamente a 5 grupos de tratamiento. La hiperglucemia fue inducida en 34 ratas en ayunas con una única inyección IP de STZ (70 mg/kg peso corporal/d). El extracto etanólico de hojas de A. indica (500 mg/kg de peso corporal/día) fue administrado por vía oral a ratas diabéticas (n=12) por 50d. Glibenclamida fue dada (PO) a 600 mg/kg peso corporal/d. En cada grupo, la glucosa en la sangre, la histopatología de los islotes, y el estado oxidativo de páncreas, se evaluaron. Todas las ratas de hiperglucemia en el grupo tratado con el Neem se habían convertido en normoglucémicas al final de la semana 2. Por 50d, el número de células b viables fue mayor en el Neem ratas tratadas con diabetes (en comparación con los grupos de diabéticos y glibenclamida). Del mismo modo, la histología de los islotes mostró una notable mejoría en este grupo, además de mejorar el estrés oxidativo. Nuestros resultados confirman el efecto hipoglucemiante de Neem. Además, la mejora de la morfología de los islotes y el estado de oxidación en el neem tratados con ratas diabéticas sugieren el potencial de esta hierba en la mejora de las lesiones de los islotes pancreáticos en la diabetes mellitus.

PALABRAS CLAVE: Azadirachta indica; Lesión de islotes pancreeáticos; Diabetes mellitus.

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