INTESTINAL LESIONS OF STREPTOZOTOCIN-INDUCED DIABETES AND THE EFFECTS OF *AZADIRACHTA INDICA* TREATMENT

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Summary

Streptozotocin (STZ) uptake into cells is mediated by GLUT-2 transporter. Besides pancreatic beta cells, GLUT-2 transporter is also expressed by epitheliocytes of the intestinal mucosa, hepatocytes, and cells of the renal tubules. In this work, we studied the effects of chronic streptozotocin-induced diabetes on the microanatomy of ileal epithelium. We also report the effects of chronic *Azadirachta indica* (neem) treatment on STZ-induced ileal lesions. Twenty-five male Wistar rats (8 weeks old, 150 g) were used. Diabetes was induced in 15 fasted rats with an intraperitoneal dose of 70 mg/kg body weight/day (70 mg/kg b.w/d) of STZ in citrate buffer (0.1 M; pH 4.5). Animals were randomly assigned to five groups of five rats each: control, diabetic, diabetic+neem, diabetic+glibenclamide, and neem only. *Azadirachta indica* was administered at 500 mg/kg b.w/d for 50 days (50d). By 50d, diabetic rats showed superficial erosion of ileal mucosa with necrosis of goblet cells. Such lesions were absent in hyperglycemic and normoglycemic rats treated with neem leaf extract. These findings suggest that (i) chronic streptozotocin diabetes is associated with intestinal lesions; (ii) neem prevents STZ-induced intestinal lesions; and (iii) chronic exposure of intestinal mucosa to the leaf extract of *Azadirachta indica* in healthy (non-diabetic) rats is not associated with deleterious effects.

Key words: *Azadirachta indica*, streptozotocin diabetes, intestinal lesions

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Introduction

Streptozotocin (STZ) is an antimicrobial and anticancer drug produced by *Streptomyces achromogenes* (1). Since 1963, STZ has been the agent of choice for the induction of diabetes in experimental animals (2). Streptozotocin acts by methylating DNA in islet beta cells, and by acting as a nitric oxide donor (3). However, the cytotoxicity of this drug is not limited to the beta cells of the pancreatic islets. Cells that express GLUT-2 transporter are likewise subject to STZ toxicity. In this regard, hepatocytes, cells of the renal tubules, and epitheliocytes of the intestine are candidates (4).

In this work, we studied the effects of chronic streptozotocin diabetes on the microanatomy of intestinal (ileal) epithelium. We also report the effects of the ethanolic extract of the leaves of *Azadirachta indica* on intestinal epithelial morphology in healthy and streptozotocin-induced diabetic rats.

Materials and Methods

Animals. Twenty-five male Wistar rats (Harlan, Milan, Italy) were used. Animals weighed 150 g on average and were 8 weeks old at the commencement of the work. Animals were kept in cages (5 animals per cage) and housed in the animal holdings of the Faculty of Biological and Environmental Sciences and Technology, University of Salento, Italy. Animals were exposed to 12 hr light, 12 hr dark cycle at 21-23 ^{0}C. They were maintained on Harlan Global Diet 2018 (Harlan, Milan, Italy). Water was given freely; and feed intake was quantified. The study was approved by the Animal Use Committee of the Faculty of Biological and Environmental Sciences and Technology, University of Salento, Lecce, Italy.

Collection of *Azadirachta indica* leaves. Fresh mature leaves of *Azadirachta indica* (A. Juss) were collected from neem trees in the premises of the University of Ilorin Mini Campus, Nigeria, between June and August 2007. A sample of the collection was compared to the Voucher specimen at the herbarium of the Botany Department of the same University (Voucher No. 542).

Extraction of *Azadirachta indica* Leaves. Fresh leaves of *A. indica* were air-dried and extracted by percolation as previously described (5). A total of 2.4 kg of the dry leaf powder was extracted at room temperature using 70% ethanol. The initial ethanolic solution of the extract was concentrated under vacuum in a Buchi Rotavapor R-114 (Buchi, Switzerland) at 50 ^{0}C (bath temperature). The residue was dissolved in bidistilled water and filtered with Whatman No.1 paper. The filtrate was concentrated in the rotavapor at 50 ^{0}C (bath temperature). The final residue (about 120 g) was a dark-brown sticky mass. This was stored at 4 ^{0}C.

Induction of Diabetes Mellitus. Hyperglycemia was induced in 15 overnight-fasted, randomly selected animals by a single intraperitoneal injection of streptozotocin (STZ) (Sigma, MO, USA), at 70 mg/kg bw (6). STZ was dissolved in 0.1 M citrate buffer, pH 4.5, just prior to injection. Animals were allowed free access to feed and water after the injection. Hyperglycemia was allowed to develop over a minimum period of 72 hours (7). Animals with fasting blood glucose ≥250 mg/dl were considered hyperglycemic (8); and thus included in the study.

***Azadirachta indica* Treatment.** The dose of *Azadirachta indica* used was based on the report of Chattopadhyay (5). Ethanolic extract of *A. indica* was dissolved in physiological saline and was administered by gavage to a group of hyperglycemic rats (n=5) at 500 mg/kg bw/d (at 9.00-10.00 hour each day) for 50d. Fresh stock solution was prepared each week and stored at 4 ^{0}C. A standard antidiabetic drug, glibenclamide (Sigma, MO, USA) was given at 600 µg/kg bw/d (9) to another group of hyperglycemic rats (n=5) for 50d. Blood glucose and histopathological findings of the ileum in this treatment group were compared with those of neem-treated hyperglycemic animals.

Furthermore, a separate group of normoglycemic rats (n=5) were administered 500 mg/kg bw/d of *A. indica* only. A group of normoglycemic rats (n=5) that received neither STZ nor neem, constituted control. Control rats were given a single i.p dose of 0.1 M citrated buffer, pH 4.5. Five (5) hyperglycemic rats, which received neither *A. indica* nor glibenclamide, constituted the diabetic group.

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

Body Weight. Body weights of the rats were taken prior to the induction of hyperglycemia, and on a daily basis during treatment.

Feed and Water Intake. The quantities of feed and water consumed on a daily basis were documented each morning (9:00 hr) prior to neem treatment.

Termination of Treatment. Animals were anaesthetized with a single i.p. injection of pentobarbital (60 mg/kg bw) (Pharmacia & Upjohn, Milan, Italy). Part of the ileum was excised, trimmed free of adipose tissue, rinsed in phosphate buffered solution (PBS) and fixed in pre-cooled Bouin's fluid for paraffin embedding.

Microtomy. For each treatment group, 3.5 µm-thick sections of the ileum were cut on a Reichert-Jung 2050 rotary microtome (Cambridge Instruments, Germany). Sections were floated on water bath at 50 ^{0}C, and mounted on pre-washed, sterilised, 25.4 x 76.2 mm glass slides (Pearls, China).

Alcian Blue Staining. To study the effect of chronic streptozotocin diabetes and *A. indica* treatment on the epithelial lining of the ileum, intestinal sections were stained in Alcian blue. Briefly, paraffin sections of the ileum were taken to water and stained in Alcian blue for 20 minutes, followed by rinsing in water. Nuclear staining was done in Carazzi's haematoxylin (5 minutes), followed by staining of the cytoplasm in eosin Y. Sections were dehydrated and mounted in Eukit (Fluka, Buchs, Germany).

Photomicrography. Photomicrographs of ileal sections were taken with a Nikon digital camera DXM1200F (Nikon, Japan) mounted on a Nikon Eclipse 80i light microscope (Nikon, Japan).

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; while means were compared using paired sample student's t-test. All graphs were drawn with Excel 2007 (Microsoft Corporation, USA).

Results

Blood Glucose. Figure 1 shows details of blood glucose levels of the animals on a weekly basis. At day 0 of neem treatment, all animals in the diabetic, neem-treated diabetic and glibenclamide-treated diabetic groups were hyperglycemic. By the end of week 1, 87.5 % of hyperglycemic rats treated with 500 mg/kg bw/d of neem had become normoglycemic (P>0.05 vs. ctrl). By the end of week 2, normoglycemia had been established in all neem-treated hyperglycemic rats (P>0.05 vs. ctrl). This normoglycemic state was maintained till euthanasia at 50d.

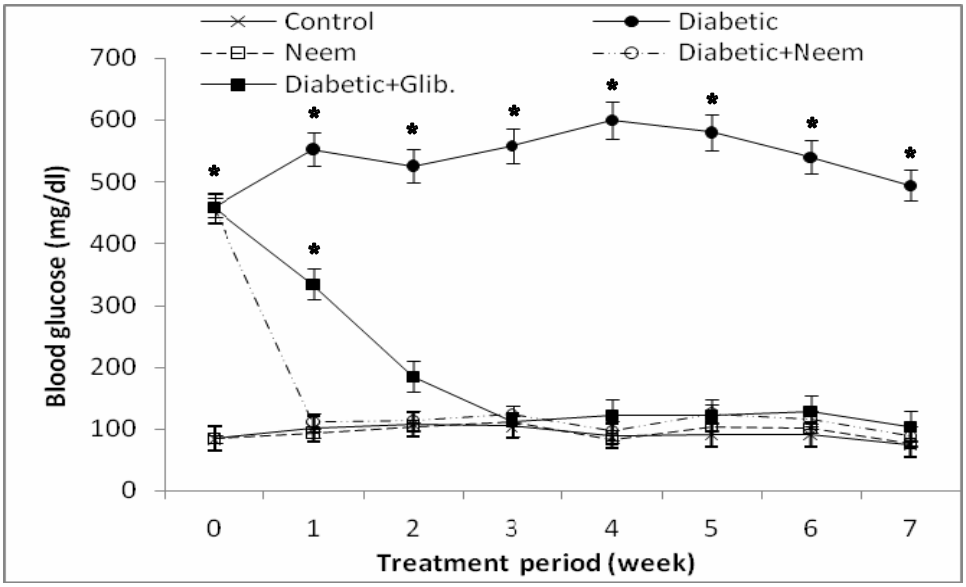

Figure 1: Weekly blood glucose of control and treated animals. Bar represents mean±SEM. * P<0.05 vs. non-diabetic control. (Glib. = Glibenclamide).

In glibenclamide-treated hyperglycemic rats, 100% of the animals remained hyperglycemic at the end of week 1 (P<0.05 vs. ctrl). By the end of week 2, normoglycemia was attained in 66.7% of these animals, with 53% fall in blood glucose (P>0.05 vs. ctrl). By week 3, all animals in this group had become normoglycemic, with 71.6% fall in blood glucose (P>0.05 vs. ctrl). Continued glibenclamide treatment maintained this normoglycemia till euthanasia at 50d. In contrast, hyperglycemia persisted in the diabetic rats from week 0 to week 7. Neem-treated non-diabetic rats remained normoglycemic throughout the treatment period (Fig. 1).

Body Weight. Figure 2 shows changes in body weights of animals at 7d, 21d and 50d. At the end of week 1 of treatment, 1.5% loss in body weight had occurred in neem-treated diabetic animals. Similarly, body weight gain (0.3%) in neem-treated non-diabetic rats was negligible. However, relatively higher increases in body weight occurred in control, diabetic and glibenclamide-treated diabetic rats (Fig. 2). By 21d, neem-treated non-diabetic rats had the least gain in body weight (13%); while the highest gain (29.9%) was obtained in glibenclamide-treated rats. This trend was maintained till 50d. By this time, weight gain in neem-treated non-diabetic rats remained the least (19.9%), while the highest weight increase of 49.9% occurred in glibenclamide-treated rats.

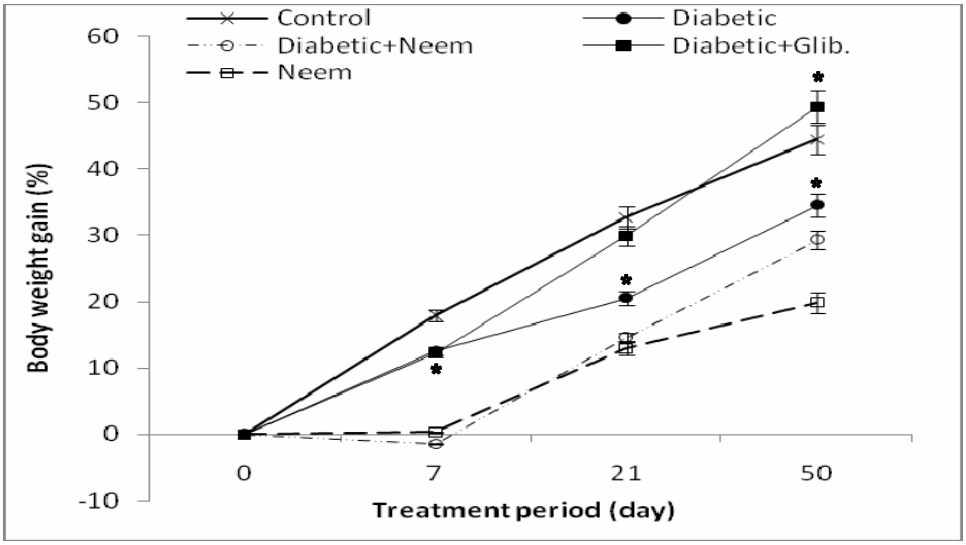

Figure 2: Change (%) in body weights of animals at 7d, 21d, and 50d of treatment. Bar represents mean±SEM. *P<0.05 vs. control. (Glib. = Glibenclamide).

Feed Intake. Figure 3 shows feed intake/g bw of the animals per day. At day 0, feed intake was comparable in all the groups. However, in the diabetic group, 1.8-fold increase in feed consumption had occurred as early as 7d, and by 50d, feed intake had increased by 2.4 fold (P<0.05 vs. ctrl). Neem-treated diabetic rats also had a slight increase (1.3 fold) in feed intake at 7d; thereafter, feed consumption declined gradually and by 50d, 1.3-fold decrease (P>0.05 vs. ctrl) in feed intake had occurred. A similar trend was observed in the neem-treated normoglycemic rats. In these animals, more than 1.5-fold decrease (P>0.05 vs. ctrl) in feed intake had occurred by 50d. In contrast, glibenclamide-treated diabetic rats were hyperphagic by the end of the first week (1.8-fold increase in feed intake); thereafter, the hyperphagia declined; and by 50d, feed intake was 1.5-fold higher than non-diabetic control (P<0.05).

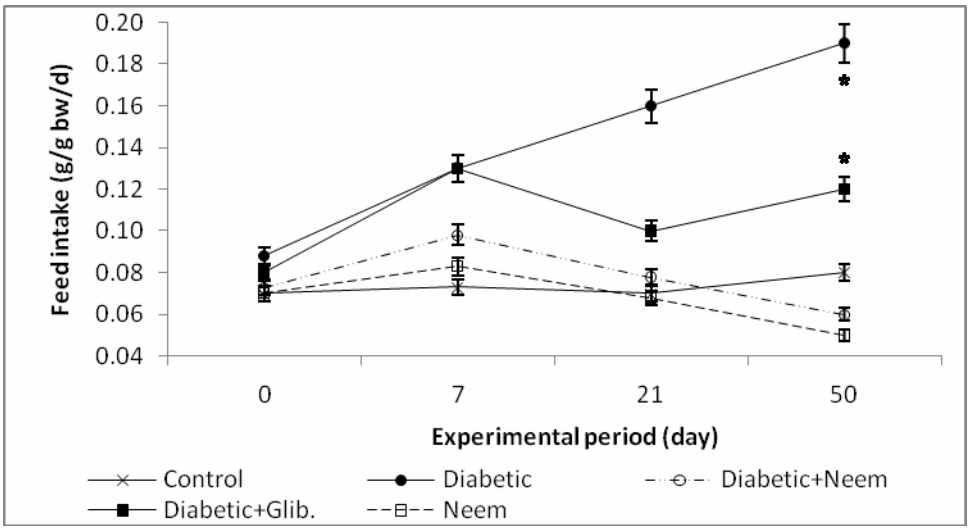

Figure 3: Feed intake per gram body weight of animals. P<0.05 vs. control. (Glib. = Glibenclamide).

Histopathology of Ileal Mucosa. At 50d, ileal mucosa of diabetic rats was characterised by necrosis, low mucin content and poor secretory activity of goblet cells; as well as erosion of the surface epithelium. The epithelium was devoid of mucous lining; and desquamated cells and tissue debris were seen in the intestinal lumen (Fig. 4 B). On the other hand, ileal mucosae of neem-treated diabetic and neem-treated normoglycemic rats were comparable to control (Fig. 4 C). The epithelium was intact and most goblet cells were actively secretory. The glibenclamide group also had normal mucosal morphology except for the poor secretory function of goblet cells (Fig. 4 D). The surface epithelium, though intact, was largely devoid of mucous lining.

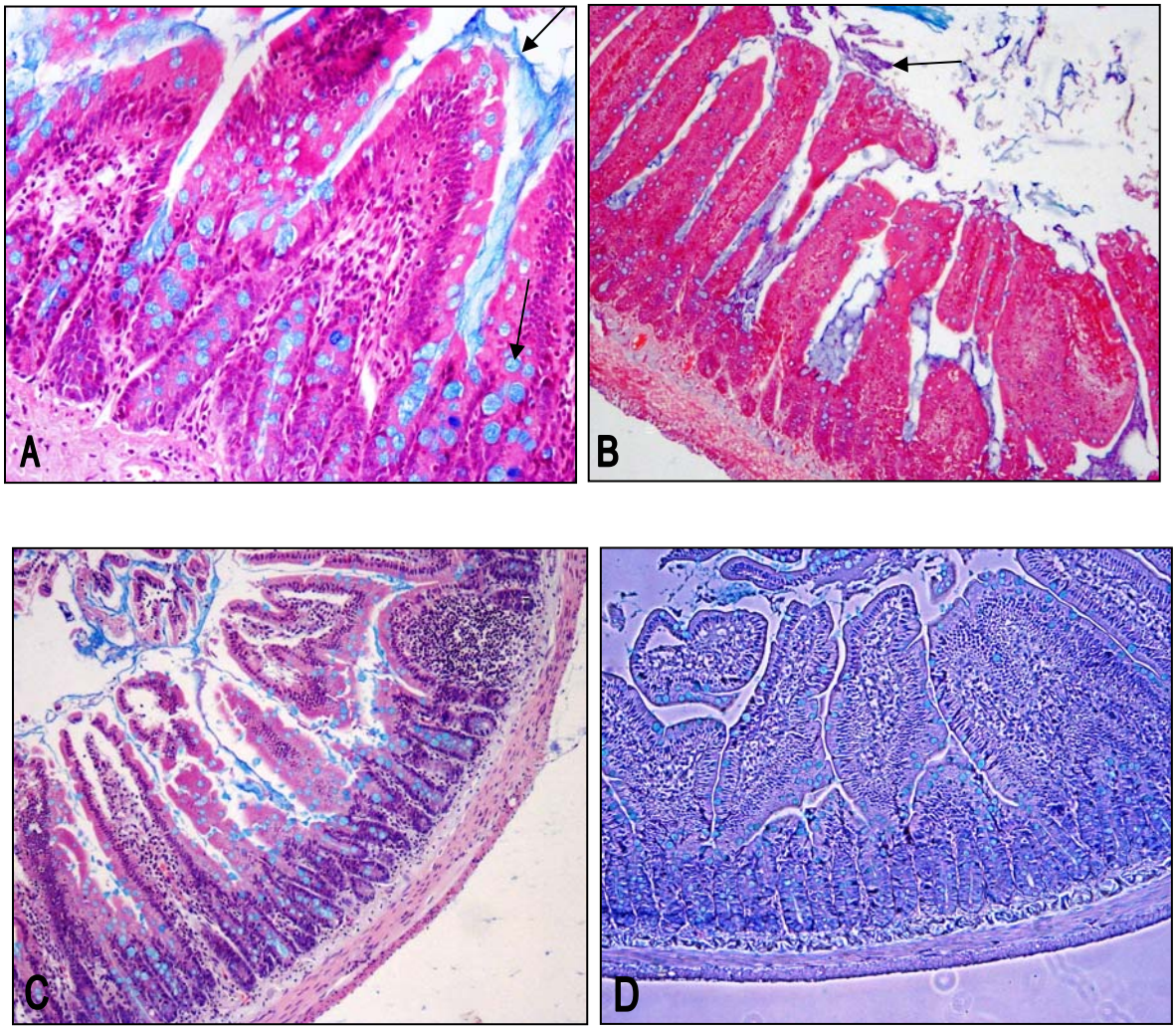

Figure 4 (A-D): Intestinal (ileal) sections at 50d of treatment. (***A***) Control group; note the intact epithelium and its lining of mucus (arrow). (***B***) Diabetic group; note erosion of the surface epithelium, and the necrosis and poor secretory activity of most goblet cells. Tissue debris is seen in the intestinal lumen (arrow). (***C***) Neem-treated diabetic group; intestinal mucosa is largely normal, with unimpaired secretory function of goblet cells. (***D***) Glibenclamide-treated diabetic group; note the low mucin content and the poor secretory activity of goblet cells. Alcian blue stain, x10. Bar represents 10µ.

Discussion

In this study, exposure of STZ-induced hyperglycemic rats to a chronic regimen of the ethanolic extract of *Azadirachta indica* leaves, at a dose of 500 mg/kg bw/d, produced normoglycemia in 87.5% of the animals by the end of week 1 of treatment; and all animals had become normoglycemic by week 2 (Fig. 1). Hypoglycemic activity of *A. indica* leaves had been reported in dogs (10); rats (5, 11), and rabbits (12). At day 0 of exposure, *A. indica* produced its maximum effect on blood glucose 5 hrs after the first dose of the drug (Figure not shown). A similar study by Murty *et al* (10) showed that the hypoglycemic effect of *A. indica* leaves was most pronounced 1-2 hrs post-dose. In this instance however, the drug was given intravenously to adrenaline-induced hyperglycemic dogs.

Proposed mechanisms of hypoglycemic effect of *A. indica* include inhibition of adrenalin-induced glycogenolysis (13); and an insulinotropic effect (5). In the present study, we observed a modulatory effect of *A. indica* on feed intake and body weight of diabetic (and non-diabetic) rats exposed to the leaf extract. By 50d, increases in body weight were least in neem-treated rats (Fig. 2). Histological examination of intestinal mucosae of these animals showed intact epithelium (Fig. 4 C); and this suggests that the reduced feed intake and relatively low weight gain in these animals were 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, might be involved. A similar mechanism was reported for exendin-4, an antidiabetic substance from the salivary gland of the Gila monster lizard (14). Future research on *A. indica* may thus test this hypothesis by quantifying the levels of leptin in the plasma or serum of neem-treated rats.

A plethora of phytochemicals is obtainable in neem leaf (15). Among these, quercetin, myricetin, kaempferol, rutin and their glycosides, have been implicated as responsible for the antidiabetic effect of neem leaves (5). Interestingly, the work of Adewole *et al* (16) showed the hypoglycemic activity of quercetin (25 mg/kg bw/d) in Wistar rats induced to hyperglycemia with 75 mg/kg bw of STZ. Similarly, Srinivasan *et al* (17) reported the beneficial effect of rutin (200 mg/ kg bw) in male Swiss mice induced to diabetes with multiple low dose STZ (40 mg/kg bw) given intraperitoneally for five consecutive days. These findings suggest that the hypoglycemic effect of neem leaves may be mediated by its bioflavonoids. In contrast, Sanders *et al* (18) and Dias *et al* (19) reported negative effect of quercetin on blood glucose in STZ-induced hyperglycemic rats treated with 10 mg/kg bw/d and 150 µmol /kg bw/d of quercetin respectively. This negative finding may however be due to the use of relatively low doses of quercetin, when compared to the work of Adewole *et al* (16), where the dosage of quercetin was much higher (25 mg/kg bw/d).

Furthermore, the negative reports of Sanders *et al* (18) and Dias *et al* (19) on the hypoglycemic effects of quercetin suggest that phytochemicals other than flavonoids may mediate or contribute to the hypoglycemic activity of neem leaves. This hypoglycemic activity may as well be owing to the synergistic effect of the flavonoid phytochemicals (quercetin, rutin, etc) in the leaves of *A. indica,* in contrast to the use of isolated flavonoids.

Diabetic rat that were treated with neither neem nor glibenclamide showed intestinal lesions characterised by superficial erosion of mucosal epithelium, blunting of villi, necrosis of goblet cells and absence of mucous coat at 50d of treatment (Fig. 4 B). Such lesions were absent in glibenclamide- and neem-treated hyperglycemic and neem-treated normoglycemic rats.

In human, diabetic mellitus may or may not be associated with histological changes in the small bowel (20). In diabetic patients presenting with diarrhoea, with or without steatorrhoea, biopsy from a number of such patients showed microangiopathy of the small bowel, with atrophic, thickened and reduced villi, and stromal infiltration of the mucosa (21). Blood vessels were seen to display prominent mural thickening and luminal narrowing secondary to accumulation of hyaline material. In the present work, though staining of intestinal vessels for markers of microangiopathy was not done, this pathology might account partly for necrosis of intestinal epithelial and goblet cells seen in this study. Hyperglycemia had been reported to trigger arteriosclerosis in vessels via AGE- and ROS-mediated mechanisms (22). Thus, chronic hyperglycemia in the diabetic animals may result in arteriosclerosis of vessels of the small bowel, with thickening of vessel wall, narrowing of their lumen, and the resultant mucosal injury. In addition, intestinal lesions seen in diabetic rats at 50d may as well arise from STZ toxicity. Epitheliocytes of the intestine possess GLUT-2 transporter (23). Cells expressing this transporter, including islet β cells, hepatocytes, renal tubular cells and epitheliocytes of the intestine, are able to take up STZ (7). Intestinal uptake of STZ might result in cell necrosis via DNA methylation and increased oxidative stress (7). In gastroduodenal lesions (ulceration), oxidative damage of the mucosa by ROS (24), and apoptotic cell death (25), had been reported as major causative factors. Oxidative damage of mucosa epitheliocytes by ROS (especially hydroxyl radical) is characterised by increased lipid peroxidation and protein oxidation, with depletion of thiols (e.g. glutathione) (26).

The work of Chattopadhyay *et al* (27) on SD rats induced to gastric ulceration with indomethacin, stress, and ethanol, showed the beneficial effect of neem leaf extract against mucosal ulceration. Similar findings were reported by Ofusori *et al* (28) against ethanol-induced gastric lesions. One mechanism proposed for this effect was the prevention of oxidative damage of gastric mucosa by attenuation of lipid peroxidation by neem; the extract was also reported to prevent apoptotic cell death from DNA damage (27). Thus, the beneficial effect of neem leaf extract reported in the present work could arise from attenuation of STZ- and hyperglycemia-induced oxidative stress in the epitheliocytes and goblet cells of ileal mucosa.

Furthermore, Garg *et al* (29) reported that the extract of neem leaves could inhibit depletion of mucous lining of intestinal mucosa. In our model, neem leaf extract enhanced mucus secretion in hyperglycemic and normoglycemic rats (Fig. 4 C). Thus, our findings suggest that neem can enhance the secretory activity of goblet cells, thereby conferring protection on intestinal mucosa. Besides, the beneficial activity of neem leaves against STZ-induced (diabetic) intestinal lesions may arise from the antioxidant activity of its phytochemicals, but this is subjected to further studies.

The absence of intestinal lesions in neem-treated normoglycemic rats suggest that exposure of intact animals to neem is not associated with any deleterious intestinal effects. However, phytochemicals essential for the beneficial importance of neem in the GIT are yet to be reported. Additional work is therefore required to isolate the active principles of the leaf extract as a potential therapy in diabetes-associated intestinal lesions. In conclusion, the current body of evidence, as reported in this work, suggests that the leaves of *Azadirachta indica* confer protection on intestinal mucosa, and prevents intestinal injury induced by diabetes mellitus.

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