MOLECULAR AND ULTRASTRUCTURAL CHANGES IN THE PROXIMAL TUBULES OF WISTAR RATS TREATED WITH STREPTOZOTOCIN AND *ARTOCARPUS COMMUNIS* FORST. (MORACEAE) ROOT-BARK AQUEOUS EXTRACT

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ABSTRACT

The present study was prompted by the claim of some traditional health practitioners in Western Nigeria that decoctions and infusions of *Artocarpus communis* (Forst.) (family: Moraceae) root-bark are effective remedies for the management and/or control of diabetes mellitus. In our pilot study, however, we discovered that *A. communis* root-bark aqueous extract (ACE) raised blood glucose concentrations in rats. Therefore, we decided to evaluate the effects of ACE on structural changes in the glomeruli, glomerular tuft and renal parenchyma in order to determine the degree of renal injury caused by diabetes mellitus induced by ACE and streptozotocin (STZ). The effects of ACE have also been compared with those of STZ on renal ultrastructural changes, as well as on the activities of conventional marker enzymes of renal disease in the experimental animal paradigm used. Four (A, B, C and D) groups of Wistar rats, each group containing 10 rats, were used in this study. Group A rats received distilled water in quantities equivalent to the volume of ACE and STZ administered intraperitoneally. Diabetes mellitus was induced in the diabetic animal groups B and C by intraperitoneal injections of STZ (75 mg/kg body weight). The rats in group C were additionally treated with ACE (100 mg/kg body weight i.p.) daily from day 3 to day 10 after STZ treatment. Group D rats received ACE (100 mg/kg body weight i.p.) only. Urinary albumin, creatinine and microalbuminuria, renal tissue γ-glutamyltransferase (GGTase), alkaline phosphatase (AlkPase) and glycogen, serum creatinine, cholesterol, phospholipids, inorganic phosphate, insulin and blood glucose concentrations were measured in the four groups (A, B, C and D) of animals. Renal tissues were also processed for transmission electron microscopy. Groups B, C and D rats exhibited pronounced polyuria, significant albuminuria, hypoinsulinemia and hypercreatinemia. Group D rats developed significant hyperglycemia ($p<0.05$) immediately after ACE administration, while groups B and C rats became hyperglycemic 24-h post STZ and STZ + ACE treatment when compared with the control group A rats.

The activities of AlkPase and GGTase in the diabetic rats were significantly decreased ($p<0.05$) at the brush-border membrane vesicles. Glycogen markedly accumulated in the kidneys of groups C and D diabetic rats, but mild in group B rats. Electron microscopic examination of the kidneys revealed a spectrum of structural damages. The most striking deleterious alterations in the kidney structures of groups B, C and D rats included basement membrane thickening, loss of podocytic foot processes, disruption of tubular basal infoldings and their related mitochondria, when compared with the control group A rats. Localized large glycogen clusters were also noticed in the renal tubules of groups B, C and D diabetic rats. The findings of the present study indicate that *A. communis* root-bark aqueous extract induces hyperglycemia in the experimental animal model used just like STZ, and that the plant's extract disrupts ultrastructural characteristics and architecture of the kidney tubules as well as the activities of brush-border membrane marker enzymes.

Key Words:
Artocarpus communis; root-bark aqueous extract; streptozotocin; proximal tubule; microalbuminuria; brush-border membrane marker enzymes; ultrastructural changes; electron microscopy.

1. INTRODUCTION

From time immemorial, plants have been used in folk medicines worldwide to treat various human ailments, including diabetes mellitus. More than 400 traditional plant treatments for diabetes mellitus have been recorded, although only a few of these plant products have received scientific evaluation to assess their safety and efficacy (Satyavati *et al*., 1987; Bailey and Day, 1989). There is no doubt that some of these plants will be toxic to tissues and/or organs in the body. Sometimes, to the lay public, 'natural' is equivalent to 'safety', but this is a false assumption.

Artocarpus communis (Forst.) is a perennial, evergreen, terrestrial, single-stemmed, erect flowering plant, native to Pacific Islands, and popularly known in English as 'breadfruit' tree because of the 'bread-like texture' of its edible fruits. It is a member of the Moraceae family which consists of about 50 genera and over 1000 species (Tindal, 1965). The 'breadfruit' tree is a fast growing plant of up to 20–30 metres in height, with a trunk of up to 1–2 metres in diameter. All the morphological parts of the tree, including the unripe fruit, are rich in milky, gummy latex. The shoots, bark and latex of the plant have been reported to have many ethnomedical uses. For example, in the West Indies, a decoction of the plant's leaves is used to reduce elevated blood pressure and to relieve asthma. In Taiwan, the leaves are used for fever and liver disorders; sap is used for thrush, stomach pain, and dysentery. Root extracts of the plant are antibacterial against gram-positive bacteria and anticancer (Kasahara and Hemmi, 1986). In Nigeria, *A. communis* root-bark is traditionally used for a variety of human ailments, including management or treatment of diarrhea, dysentery and diabetes mellitus. Natural compounds isolated from *A. communis* leaves, stem- and root-barks include prenylflavonoids. Generally, flavonoids are a group of naturally occurring compounds widely distributed as secondary metabolites in the plant kingdom. Flavonoids have been

recognized for possessing many interesting pharmacological properties, including anti-inflammatory, antiallergic, antiviral, antibacterial, and antitumoral activities (Middleton, 1998). One member of the flavonoids, quercetin (3,5,7,3´,4´-pentahydroxyflavone), prevents oxidative injury and cell death by several mechanisms, such as scavenging oxygen radicals (Inal *et al*., 2002), protecting against lipid peroxidation, and chelating metal ions (Afanas've *et al*., 1989).

Nowadays, diabetes mellitus is considered a worldwide public health problem, either in terms of the number of people affected by the disease, or the costs involved in controlling it and treating its complications (DCCT, 1993). Diabetes mellitus is a heterogenous disease characterized by microvascular pathology leading to chronic complications, clinically and principally manifested in the kidney and retina (DeFronzo and Lilly, 1988). Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD), and is clinically characterized by proteinuria and progressive renal insufficiency (White and Bilous, 2000). Proteinuria has been established more recently as a key risk marker of renal function decline in individuals with primary renal disease and nephropathy from type 1 or type 2 diabetes (Remuzzi and Bertani, 1998). Risk factors for DN include hyperglycemia, hypertension, genetic predisposition, glomerular hyperfilteration, proteinuria, advanced glycation end-products, and possibly reduced nephron number and lipid disorders (Amri *et al*., 1999). Diabetic renal disease begins not with loss of nephrons, but rather, with glomerular hyperfilteration and increased glomerular filteration rate (GFR). This hyperfilteration is present in early phases of both type 1 and type 2 diabetes. It damages the glomerular capillary in subtle ways, produces mesangial cell and glomerular basement membrane injury, and stimulates release of cytokins (Lurbe *et al*., 2002)). Several studies have shown that hyperfilteration is associated with hyperglycemia, and in vulnerable patients destined for diabetic kidney disease, GFR begins to decrease as microalbuminuria appears (Bangstad *et al*., 2002). The genesis of proteinuria in diabetes could be due to alterations in glomerular filteration barrier, which is composed of the glomerular endothelium, the glomerular basement membrane (GBM), and the podocyte (glomerular visceral epithelial cell). Widespread endothelial dysfunction is believed to result in proteinuria (Deckert *et al*., 1989), which is exacerbated by intraglomerular hemodynamic stress (Zatz *et al*., 1985).

Morphological studies have shown that renal proximal tubule in general, and its brush-border membrane (BBM) in particular, are the major targets for diabetic injury (Dousa and Kempson, 1982). It has been demonstrated by Maedda *et al*., (1993) that DN decreases specific activities of certain marker enzymes of renal cortical BBM, and this is accompanied by an increase in enzyme activities in the urine (Herminghuysen *et al*., 1985). Toxic insults are also known to produce profound alterations in the structure and excretory function of the kidney, depending on the severity of the damage caused. Diabetic kidney disease is a serious diabetic complication, and its progression can be monitored by the presence of albumin in the urine or microalbuminuria.

In view of the above considerations, the core aims of the present study were to determine (i) the effects of *A. communis* root-bark aqueous extract on the subpopulations of glomeruli and proximal tubules, and (ii) the extract's effects on various biochemical components, including activities of AlkPase, GGTase and (Na^+-K^+)-dependent ATPase in homogenates, and in the brush-border membranes from kidney cortices in control, STZ-, STZ- +ACE-, and ACE-treated diabetic rats.

2. MATERIALS AND METHODS

The experimental protocol and procedures used in this study were approved by the Ethics Committee of the University of Durban-Westville, Durban 4000, South Africa; and conform with the *"Guide to the Care and Use of Animals in Research and Teaching"* [Published by the Ethics Committee of the University of Durban-Westville, Durban 4000, South Africa].

Animals

This study was carried out in healthy, male and female Wistar rats, weighing 240-260 g. The animals were housed under standard laboratory conditions of light, temperature and humidity. The animals were given standard rat pellets and tap water *ad libitum*. The rats were randomly divided into four (A, B, C and D) experimental groups, comprising Group A (distilled water-treated control), Group B (STZ-treated diabetic), Group C (STZ- + *A. communis* root-bark extract-treated diabetic), and Group D (*A. communis* root-bark extract-treated) rats. Each group consisted of 10 rats. All the animals were fasted for 16 hours, but still allowed free access to water, before the commencement of our experiments.

Plant material

The root-bark of *Artocarpus communis* (Forst.) [family: Moraceae] (locally known by its common English name of "Breadfruit" or "Gbere" in Yoruba language of Western Nigeria) were collected in Ile-Ife, Western Nigeria, between April and May, 2006. Pieces of the plant's root-bark were identified by the Taxonomist/Curator of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria, as those of *A. communis* Forst. (family, Moraceae). A voucher specimen of the plant has been deposited in the Herbarium of the University's Botany Department.

Preparation of *Artocarpus communis* root bark aqueous extract

Pieces of *Artocarpus communis* fresh root-bark were air-dried at room temperature. One kilogram (1 kg) of the air-dried root bark of the plant was milled into fine powder in a Waring commercial blender. The powdered root-bark was macerated in distilled water and extracted twice, on each occasion with 2.5 1itres of distilled water at room temperature for 48 h. The combined aqueous extract solubles were concentrated to dryness under reduced pressure at 60±1^{o}C in a rotary evaporator. The resulting aqueous extract was freeze-dried, finally giving 56.23 g (i.e., 5.62% yield) of a clay-colour, powdery, crude root-bark aqueous extract of *A. communis* (ACE). Aliquot portions of this crude extract residue were weighed and dissolved in distilled water for use on each day of our experiment.

Acute toxicity testing

The median lethal dose (LD_{50}) of *A. communis* root-bark aqueous extract (ACE) was determined in mice using a modified method of Lorke (1983). Mice fasted for 16 h were randomly divided into groups of 10 mice each. Graded doses of ACE (12.5, 25, 50, 100, 200, 400, 800 and 1600 mg/kg) were separately administered intraperitoneally to the mice in each of the test groups. Each of the mice in the control group was treated with distilled water (3 ml/kg, i.p.) only. The mice in both the 'test' and 'control' groups were then allowed free access to food and water, and observed over a period of 48 h for signs of acute toxicity. The number of deaths (produced by the extract) within this period of time was noted and recorded. Log dose-response plots were constructed for the plant's extract, from which the LD_{50} of the root-bark aqueous extract was determined.

Induction of experimental diabetes and ACE administration

Diabetes mellitus was induced (in groups B and C 'test' rats) by intraperitoneal injections of STZ (75 mg/kg i. p.), freshly dissolved in 0.1mol/l citrate buffer (Rossini *et al*; 1978). Group A 'control' rats were injected with volumes of distilled water equivalent to the volume of administered ACE intraperitoneally. The 'test' animals in groups B and C became diabetic within 48 hours after STZ administration. Diabetes was allowed to develop and stabilize in these STZ-treated rats over a period of 3–5 days. Group C rats additionally received intraperitoneal injections of ACE (100 mg/kg/day i.p.) daily from the 3rd to the 10th day after STZ treatment. Group D rats were treated with ACE (100 mg/kg i.p.) only. All the animals in groups A, B, C and D were kept and maintained under laboratory conditions of light, humidity and temperature. Before the commencement of our experiments, both the control, normal (normoglycemic) and STZ-treated, diabetic (hyperglycemic) 'test' rats were fasted for 16-h, but still allowed free access to water throughout. At the end of the 16-h fasting period – taken as 0 time (i.e., 0 h) – blood glucose levels (initial glycemia, G_0) of the fasted normal, STZ-, STZ- + ACE-, and ACE-treated, diabetic rats were determined and recorded.

Analysis of blood glucose and serum parameters

Blood samples were obtained from each rat by repeated needle puncture of the same tail tip vein. Blood samples were taken 1 day before STZ- and ACE-treatments, and also on various days after induction of diabetes mellitus. Blood glucose concentrations were determined by means of Bayer Elite® Glucometer and compatible blood glucose test strips (Atkin *et al*., 1991). Fasted STZ–treated rats with blood glucose concentrations ≥18 mmol/L were considered to be diabetic, and used in this study. Serum samples were deproteinized with 3% trichloroacetic acid in the ratio of 1:3. The samples were centrifuged at 2,000 x g (4,000 rpm) (Remi Centrifuge) for 10 min. The protein-free supernatant was used for the estimation of inorganic phosphate (Kates *et al*., 1997), whereas the pellet was used for phospholipids estimation (Jouanal *et al*., 1980). Total serum cholesterol was estimated directly in serum samples by the method of Allain *et al*., (1974). Serum insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA), using a commercial kit (Crystal Chem, Illinois, Chicago,

USA). Serum creatinine concentrations were also determined using Beckman Creatinine Analyzer II (Brea, CA) with the modified Jaffe rate method (Ruggenenti *et al*., 1998).

Measurement of urine albumin, creatinine and microalbumin

Urine was collected from individually-caged rats for a 24-h period in metabolic cages. A commercially available urine dipstick was used in this study (Gilbert *et al*., 1992). The Clinitek Microalbumin reagent strip (Bayer) is composed of 2 reaction pads that allow a visual estimation of albumin and creatinine concentrations, using color comparison charts. The dipstick can also be evaluated with a desktop reflectance photometer such as the Clinitek 50 or Clinitek 100 (Bayer), which reports urine albumin and creatinine concentrations, and calculates the albumin:creatinine ratio. The albumin:creatinine ratio is reported as <30 µg/mg, 30-300 µg/mg, or >300 µg/mg, which is interpreted by the manufacturer as negligible urine albumin concentration, possible microalbuminuria, or overt albuminuria (nephropathy), respectively. All the urine samples used in this study were processed immediately following collection according to manufacturer's recommendations. Dipstick results were evaluated with the Clinitek 50 urine chemistry analyzer.

Renal glycogen assay

Glycogen levels in the kidney extracts were determined as described by Chung and Dao (1998). Kidneys were removed immediately after euthanization, rinsed with cold saline, and weighed. Thereafter, a small piece of the kidney tissue was isolated and frozen in liquid nitrogen. Renal tissue extract (0.1 g) was dissolved in 30% KOH and heated at 100^{o}C for 10 min, followed by 3-min room temperature incubation. The sample was diluted (1:10) with KOH and vortexed. Anhydrous ethanol (1.1-1.2 volumes) was added to precipitate glycogen from alkaline digestate, and samples were centrifuged at 5,700 rpm for 15 min. The supernatant was carefully removed, and the pellet was resuspended in 0.5 ml distilled water. One milliliter of a 0.2% anthrone reagent (0.2 g in 100 ml of 98% H_2SO_4) was quickly added and mixed, and the mixture was incubated at room temperature for 30 min. The samples were then measured at 620 nm with a spectrophotometer.

Brush-border membrane vesicle preparation (BBMV)

Kidneys were quickly removed from euthanized rats, decapsulated, and placed in ice-cold saline (0.9% NaCl). Brush-border membrane vesicles (BBMV) were prepared at 4^{o}C, using $MgCl_2$ precipitation method as described by Yusufi *et al*., (1983). Freshly minced cortical slices were homogenized in 50 mM mannitol and 5 mM tris(hydroxymethyl)aminomethane-N-2-hydroxyethylpiperazine-N`-2-ethanesulfonic acid (Tris-HEPES), pH 7.0 (20 ml/g), in a glass Telfon homogenizer with four complete strokes. The homogenate was then subjected to high-speed [20,500 revolutions/min (rpm)] homogenization in an Ultra-Turex Kunkel homogenizer for three strokes of 15 s each with an interval of 15 s between each stroke. $MgCl_2$ was added to the homogenate to

a final concentration of 10 mM, and slowly stirred for 20 min. The homogenate was spun at 2,000 g in J2-21 Beckman centrifuge, using JA-17 rotor for 10 min. The supernatant was recentrifuged at 35,000 g for 30 min. The pellet was resuspended in 300 mM mannitol and 5 mM Tris-HEPES, pH 7.4, with four passes by a loose-fitting Dounce homogenizer (Wheaton, Illinois, Chicago, USA), and centrifuged at 35,000 g for 20 min in 15-ml Corex tubes, using Beckman JA-20 rotor. The outer, white fluffy pellet was resuspended carefully in a small volume of buffered 300 mM mannitol. Aliquots of homogenates were also saved for enzyme analysis together with BBM preparations. Isolated basolateral membrane vesicles were prepared by free-flow electrophoresis as described by Reynolds *et al*., (1980). The supernatant after the first centrifugation in the basolateral-membrane preparation was the starting material for the isolation of brush-border vesicles. Protein determination was done by the method of Lowry *et al*., (1951), using bovine serum albumin as standard.

The purity of the membranes was assessed by analyzing the activities of BBM marker enzymes, Na^+-K^+-adenosine triphosphatase (Na^+-K^+-ATPase) (basolateral membrane enzyme) and acid phosphatase (lysosome enzyme). It was observed that the membrane preparations were several fold purified as the activities of BBM enzymes increased (9- to 11-fold). The activities of Na^+-K^+-ATPase and acid phosphatase were depleted to 0.9±0.1 and 0.8±0.1-fold, respectively, compared with their activities in the homogenates. Enrichment of the enzymes indicated that the preparation was predominantly of luminal brush-border membranes.

Enzyme assays

Enzyme activities were determined on samples of the initial cortical homogenate, BBMV and pooled basolateral membrane fractions after electrophoresis. These determinations were performed by standard procedures. The activity of alkaline phosphatase (AlkPase) was measured by the method of Yusufi *et al*., (1983). GGTase activity was determined by the method of Glossmann and Neville (1972) as reported by Yusufi *et al*., (1994). Acid phosphatase was determined according to Verjee (1969), whereas Na^+-K^+-ATPase activity was determined by the method described by Szczepanska-Konkel *et al*., (1986). Protein was estimated by the method of Lowry *et al*., (1951), using bovine serum albumin as standard.

Ultrastructural examination

For electron microscopy, six biopsy tissues from each group of sacrificed animals were fixed overnight in formaldehyde-glutaraldehyde fixative at 4^{o}C by the method of Karnovsky (1965). The tissues were subsequently post-fixed in 1% osmium tetroxide. After dehydration in graded concentrations of ethyl alcohol and propylene oxide, the tissues were embedded in spur medium. Samples were sectioned with an ultramicrotome Reichert Ultracut S, using a glass knife. Semi-thin sections were stained with methylene blue, and ultra-thin sections with 8% uranyl acetate dissolved in 50% methanol, and thereafter in lead citrate according to the method of Venable and Coggeshall (1966). All studies were performed under Transmission Electron Microscope (Jeol JEM 1011) operating at 100 kV, and Image Analysis System (Kontron 300 and AnalySIS). The

primary magnification of the Electron Microscopic (EM) examination was X6, 000 for proximal tubules and X30,000 for glomeruli.

Statistical analysis

Results are expressed as means (±SEM). Statistical evaluation of the data obtained was done by Students' t-test. P values less than 0.05 were considered to indicate statistically significant differences.

3. RESULTS

Acute toxicity testing

Intraperitoneal administrations of relatively low doses of *Artocarpus communis* (Forst.) root-bark aqueous extract (1–50 mg/kg) were found to be safe in mice (and rats). However, relatively moderate to high doses of the plant's extract (>100 mg/kg i. p.) were found to be toxic and/or lethal to the animals. The LD_{50} value of the plant's extract was calculated to be 130±12 mg/kg i. p. in mice. This relatively low LD_{50} value of 130±12 mg/kg suggests that *Artocarpus communis* (Forst.) root-bark aqueous extract is toxic to mice.

Diabetic state, blood glucose levels and body weights

Blood glucose levels were measured at various time points throughout the study period. Table 1 shows blood glucose levels and changes in body and kidney weights of the four animal groups studied. The three treated rat groups, B (STZ-treated), C (STZ-+ACE-treated) and D (ACE-treated), showed significant increases ($p<0.05$) in blood glucose levels when compared with the control, group A rats. After 3 weeks, the blood glucose concentrations of the ACE-treated group D rats started to drop, but it never attained group A rats normoglycemic level throughout the study period (Table 1). There was a progressive decrease in the body weights of the rats in groups B, C and D, whereas the body weights of the control group A rats showed moderate but insignificant ($p>0.05$) increases (Table 1). Increases in whole kidney and cortex weights of the diabetic animals were also observed when compared with the control rats (Table 1).

Table 1. Changes in blood glucose concentrations, body and kidney weights of control, STZ-, STZ- + ACE-, and ACE-treated rats.

Blood glucose concentrations (mmol/L)

	Control	←	Treated	→	
Days / Treatments	0	15	30	45	60
STZ-treated	4.1±0.5	18.6±0.2[a]	19.2±0.3[a]	19.3±0.4[a]	18.5±0.7[a]
STZ + ACE-treated	4.4±0.2	22.6±0.5[b]	21.6±0.2[b]	20.5±0.3[b]	19.9±0.4[b]
ACE-treated	4.2±0.3	22.8±0.4[c]	18.6±0.2[c]	14.2±0.1[c]	10.7±0.5[c]

Parameters	Control	STZ-treated	STZ + ACE-treated	ACE-treated
Body weights (g)	258±10	217±14[a]	216±06[a]	212±11[a]
Kidney weights (g)	0.98±0.8	1.27±0.5[b]	1.43±0.4[b]	1.33±0.2[b]
Cortex weights (g)	0.45±0.2	0.56±0.4[c]	0.63±0.7[c]	0.58±0.3[c]

Values are expressed as means (±SEM) of 10 rats for all groups. [a,b,c] Significant difference ($p<0.05$) in the same row between various treatments and control group A rats.

Serum parameters

The results summarized in Table 2 indicate that serum insulin levels of the rats in the three treated groups B, C and D significantly decreased ($p<0.05$) when compared with group A control rats. Other serum parameters measured significantly increased ($p<0.05$) when compared with the control rats. A linear decrease and increase in serum insulin and creatinine respectively were observed among the diabetic rats for the duration of our study. The values for control rats were not significantly different; and hence, data were expressed from pooled mean values observed at various time points. The serum levels of inorganic phosphate, phospholipids and cholesterol also increased to some extent (Table 2).

Renal glycogen contents

Renal glycogen levels significantly increased ($p<0.05$) in the diabetic animals treated with STZ, STZ + ACE, and ACE when compared with the control group of rats. Glycogen levels became markedly elevated in these rats, especially at longstanding diabetic states. Glycogen values for the control, STZ-, STZ- + ACE- and ACE-treated rats were found to be 0.57±1.32, 3.15±1.56, 4.32±0.35 and 3.59±2.26 mg/g tissue, respectively (Fig 1B).

Table 2. Effects of STZ-, STZ- + ACE-, and ACE-induced diabetes mellitus on serum parameters

Serum insulin concentrations (μU/ml)					
	Control ←		Treated	→	
Days / Treatments	0	15	30	45	60
STZ-treated	14.6±1.2	8.9±1.6[a]	7.5±1.3[a]	6.2±1.7[a]	5.8±1.4[a]
STZ + ACE-treated	13.9±1.5	9.9±1.8[b]	8.0±1.7[b]	6.9±1.0[b]	6.2±1.3[b]
ACE-treated	13.5±1.3	10.1±1.5[c]	9.7±1.2[c]	9.0±1.7[c]	8.9±1.4[c]
Serum creatinine (μg/ml)					
STZ-treated	24.74±0.6	32.62±0.2[a]	36.82±0.7[a]	39.43±0.5[a]	39.98±0.7[a]
STZ + ACE-treated	25.26±0.3	39.42±0.7[b]	48.19±0.2[b]	54.09±0.1[b]	55.72±0.6[b]
ACE-treated	23.90±0.5	34.83±0.2[c]	46.20±0.1[c]	52.17±0.8[c]	54.83±0.3[c]
Serum cholesterol (mg/dl)					
STZ-treated	78.42±1.26	178.32±3.52[a]	193.46±2.37[a]	199.60±4.25[a]	213.65±1.47[a]
STZ + ACE-treated	76.39±2.35	198.54±1.27[b]	214.68±5.72[b]	223.94±3.22[b]	228.12±7.16[b]
ACE-treated	77.32±1.54	187.38±5.32[c]	197.62±4.19[c]	218.80±2.38[c]	219.94±6.53[c]
Serum phospholipids (mg/dl)					
STZ-treated	105.18±1.32	135.32±2.12[a]	146.14±5.07[a]	152.28±3.43[a]	159.40±7.21[a]
STZ + ACE-treated	108.16±2.51	157.61±7.25[b]	166.78±1.24[b]	172.74±3.16[b]	178.38±1.19[b]
ACE-treated	107.17±4.04	146.41±3.15[c]	153.52±1.37[c]	159.59±2.84[c]	162.64±3.20[c]
Serum inorganic phosphate (μmol/ml)					
STZ-treated	0.99±0.11	2.58±0.32[a]	2.73±0.64[a]	2.83±0.25[a]	2.98±0.71[a]
STZ + ACE-treated	1.10±0.21	2.81±0.51[b]	2.89±0.24[b]	3.24±0.19[b]	3.92±0.29[b]
ACE-treated	1.02±0.14	2.69±0.05[c]	2.82±0.32[c]	2.92±0.24[c]	3.19±0.92[c]

Values are expressed as means (±SEM) of 10 rats for all groups. [a,b,c] Significant difference ($p<0.05$) in the same row between various treatments and control group A rats.

Albuminuria, microalbuminuria and albumin-creatinine ratio

Table 3 contains the values obtained for urinary albumin, microalbumin and albumin-to-creatinine ratios (ACR). Microalbuminuria was diagnosed if albumin-to-creatinine ratios were ≥ 3 mg/mmol in at least two sequential, sterile, early-morning urine samples, and if the urinary albumin excretion rate was between 30 and 300 mg/day in a 24-h urine collection. Urine albumin concentration ranged from 0.1 to >500 μg/ml as determined by albumin Clinitek. Of the 40 urine samples tested (10 from control, non-diabetic rats and 30 from diabetic rats), a total of 17 from diabetic rats with an albumin excretion >30 mg/24-h had an albumin-to-creatinine ratio >2.5 mg/mmol. Eight of the diabetic rats with

an albumin excretion >300 mg/24-h also had albumin-to-creatinine ratio >25 mg/mmol. Another 5 diabetic rats with an albumin excretion <30 mg/24-h had albumin-to-creatinine ratio <2.5 mg/mmol. Urine samples from control, group A rats did not indicate proteinuria, but diabetic rats showed significant elevation of ACR, especially the animals that received ACE.

Table 3. Urinary albumin (U_A), urinary creatinine (U_{Cr}), microalbumin (U_{MA}) and albumin-creatinine ratio (ACR) in the control and diabetic rat groups

Parameters	Control	STZ	STZ + ACE	ACE
U_A (µg/ml)	5.6	14.8±0.45[a]	19.6±0.14[a]	16.7±0.32[a]
U_{Cr} (mg/dl) mmol	12.6	135.3±1.5[b]	168.4±2.7[b]	157.6±4.0[b]
U_{MA} (≥30-300) %	<30	3(10%)	21(70%)*	6(20%)
ACR (mg/mmol)	0.66	19.82±0.25[c]	26.09±0.32[c]	22.41±0.75[c]

Values are expressed as means (± SEM) of 10 rats for all groups. [a,b,c]Significant increase (p<0.05) in the same row when compared with the control group A animals. *Significant increase (p<0.05) in the same row between STZ- and ACE-treated groups of rats.

Purity of marker enzymes

The purity of the brush-border membrane vesicle preparation was assessed by analyzing the activities of BBMV marker enzymes, AlkPase and GGTase. As shown in Table 4, there was a 10-fold enrichment in the specific activity of AlkPase, and a 9-fold enrichment of GGTase activity in the brush-border preparation from control rats compared with the homogenate. An 11-fold enrichment of AlkPase and a 10-fold enrichment in GGTase activity in the brush-border preparation from diabetic rats compared with the homogenate were observed (Table 4 and Fig. 2). There was a small but insignificant increase (p>0.05) in the activity of (Na^+-K^+)-dependent ATPase in the brush-border vesicle preparations from both control and diabetic rat groups, compare with homogenate. There was a 16-fold enrichment of (Na^+-K^+)-dependent ATPase in the basolateral-membrane preparation compared with the homogenate from control animals, and an 11-fold enrichment in the basolateral-membrane preparation from diabetic rats, demonstrating the purity of this preparation (Table 4 and Fig. 3). The specific activities of the brush-border membrane markers, AlkPase and GGTase, in the basolateral membrane preparation from both control and diabetic rats were not significantly increased (p>0.05) compared with the homogenates (Fig. 3).

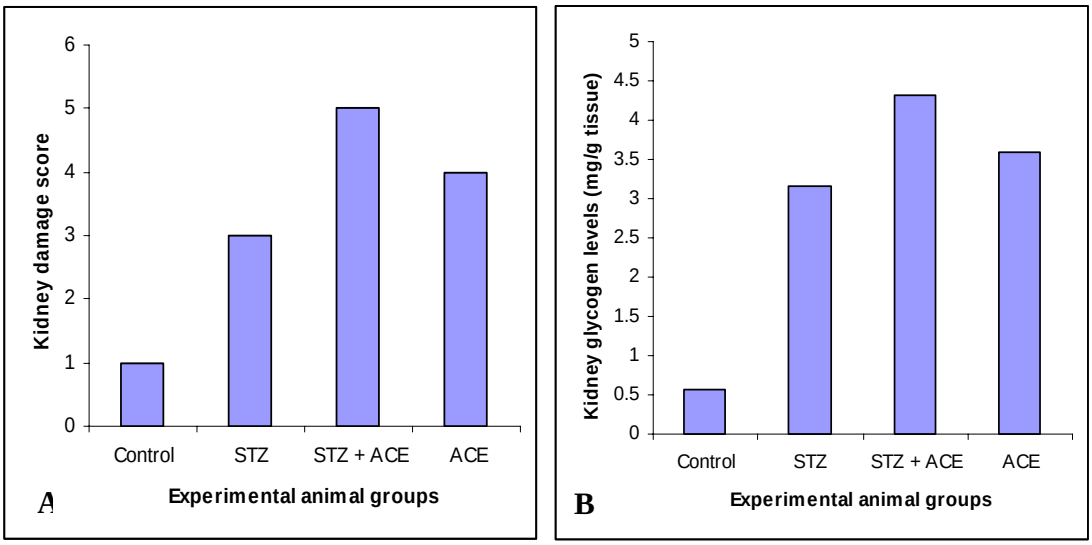

Fig. 1. Effects of STZ-, STZ- + ACE-, and ACE-induced diabetes on kidney tissue: (A) quantification of kidney tubule damage (scale 1-5), carried out under double-blind conditions. (B) chemical quantification of glycogen levels in the kidney. Values are means (±SEM) of 6 rats.

Table 4. Marker enzyme activities (alkaline phosphatase, γ-glutamyltransferase and (Na^+-K^+)-dependent ATPase) of kidney's homogenate, brush-border membranes (BBMV) and basolateral membranes (BLM).

Alkaline phosphatase (μmol . mg protein^{-1} . h^{-1}) of AlkPase								
Kidneys	Control		STZ		STZ + ACE		ACE	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Homogenate	121±11	126±09	97±15	92±21[a]	86±22	81±25[a]	86±19	83±21[a]
BBMV	1248±7	1254±9	1121±1	997±5[a]	981±4	958±3[a]	983±5	967±6[a]
BLM	99±12	98±25	69±22	62±29[a]	67±19	57 ±27[a]	66 ±21	59±29[a]
γ-Glutamyltransferase (μmol . mg protein^{-1} . h^{-1}) of GGTase								
Homogenate	268±4	269±7	225±9	212±5[a]	207±9	189±4[a]	203±2	194±25[a]
BBMV	2511±5	2513±3	2221±1	2199±5[b]	2212±2	2128±3[b]	2231±7	1998±6[b]
BLM	152±22	151±25	84±27	75±24[a]	73±29	67 ±17[a]	76 ±25	71±26[a]
(Na^+ -K^+)-dependent ATPase (mmol . mg protein^{-1} . h^{-1}) of Pi								
Homogenate	7.6±0.4	7.8±0.7	8.7±0.3	9.5±0.5[b]	8.7±0.9	9.6±0.4[b]	8.9±0.2	9.6±0.6[b]
BBMV	8.7±1.3	8.8±1.6	9.6±1.4	10.7±1.5[b]	9.2±1.2	9.9±1.5[b]	9.7±1.7	9.9±1.6[b]
BLM	122.5±2	121.3±7	118.6±3	114.1±4[a]	114.0±9	97.1±7[a]	116. ±5	112.9±6[a]

Values for the enzyme activities are expressed as means (±SEM) of 10 rats for all groups. [a]Significant decrease (p<0.05) when compared with the control group A animals. [b]Significant increase (p<0.05) in the same row between treated and control group A rats. The values of enzyme specific activities in the membrane preparation to those in the cortical homogenate before isolation of the membranes were: AlkPase, (control =10.3, diabetics = 11.4), GGTase (control = 9.3, diabetics = 10.6), Na^+ -K^+-dependent ATPase (control = 1.3, diabetics = 1.0).

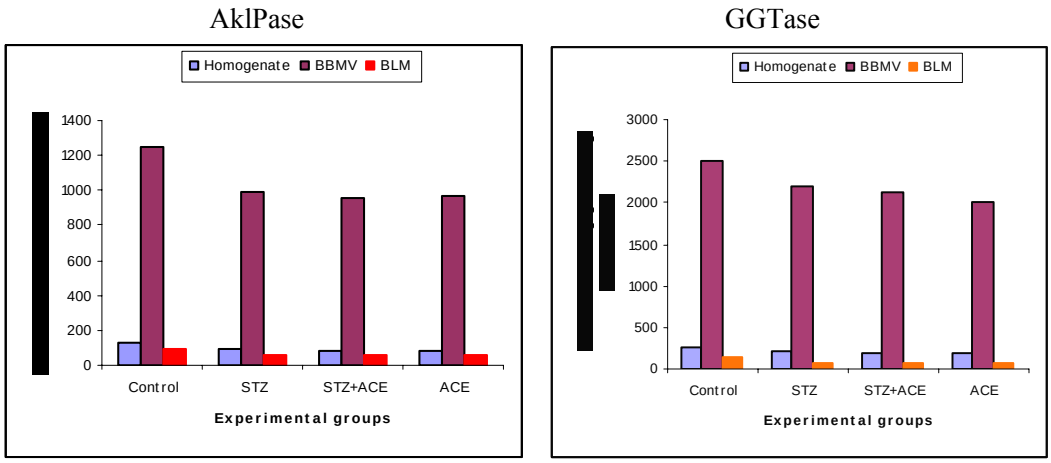

Fig. 2. Specific activities of AlkPase and GGTase in homogenate, BBMV and BLM at the end of the 8-week study period. Values are means (± SEM) of 6 different experiments.

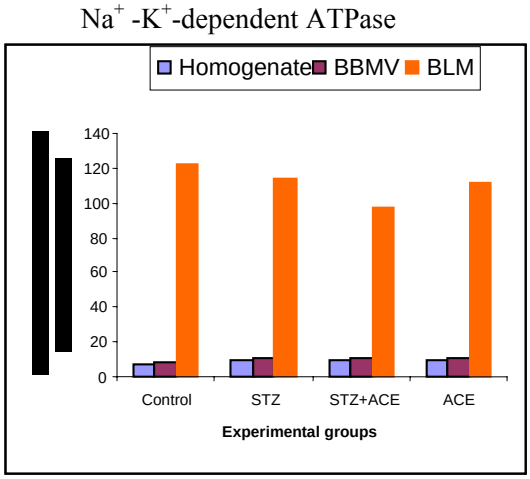

Fig. 3. Specific activity of Na^+ -K^+-dependent ATPase in homogenate, BBMV and BLM at the end of the 8-week study period. Values are means (± SEM) of 6 different experiments.

Effects of *A. communis* on the activities of BBM marker enzymes

The effects of *A. communis* were first determined on the activities of BBM marker enzymes in the homogenates, BBMV and in BLM fractions isolated from whole cortex. Specific activities of both AlkPase and GGTase significantly decreased with increase in the duration of diabetic state in BBMV (Table 3). However, the enzyme activities insignificantly decreased ($p>0.05$) in the homogenates of all the three treated diabetic rat groups compared with the control group A rats. The specific activities (enzyme unit/mg protein) of both enzymes in the cortical homogenates from control and diabetic kidneys were not significantly different, and enzyme-specific activities were found to be altered only in BBM fractions and not in the homogenates (Table 4). Further analysis showed that total enzyme activities greatly declined in the membrane-bound fractions, while

simultaneously increasing in the supernatant fractions (i.e., free enzymes released due to diabetes). The changes observed were found to be in proportion to the duration of diabetes, i.e., the decrease in the membrane-bound enzyme, as well as the corresponding increase in supernatant fraction, was much greater at the 8th week of study period (Fig. 4). The activity of (Na^+-K^+)-dependent ATPase (basolateral membrane) was determined in the BBMV and cortical homogenate samples. Results obtained indicate that the activity of the enzyme was lower at BBM, suggesting an overall effect of hyperglycemia on the proximal tubular cells. The activity of (Na^+-K^+)-dependent ATPase markedly increased at BLM, suggestive of increase in Na^+ retention (Fig. 3).

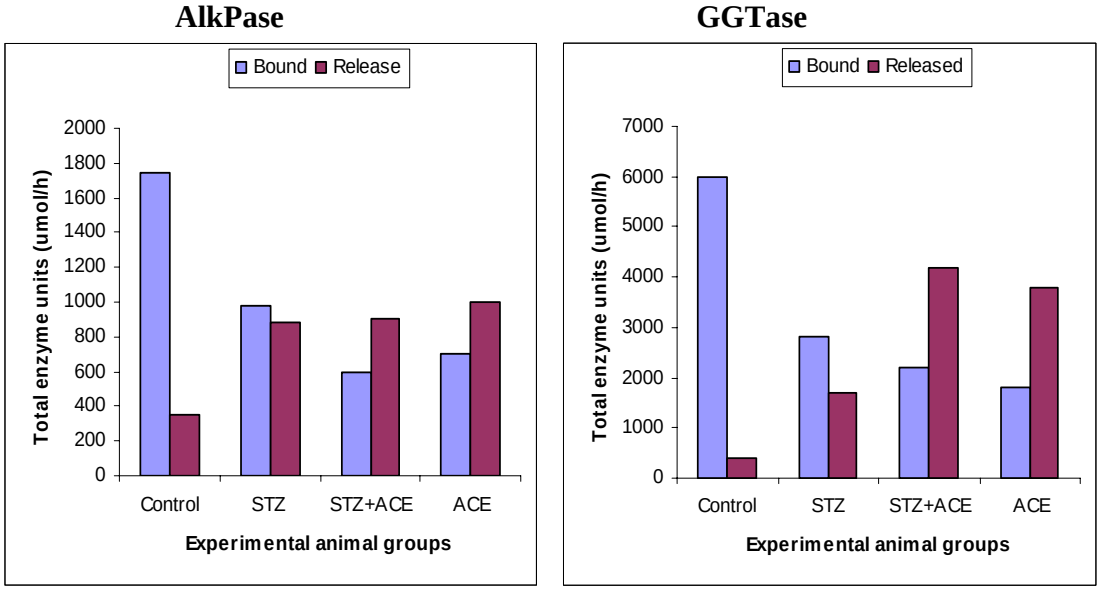

Fig. 4. Total enzyme activities of AlkPase and GGTase, membrane bound (blue) and released (purple), in cortical homogenates. Values are means (± SEM) of 6 different experiments.

Electron microscopy

Ultrastructurally, the kidney tissues of diabetic rats revealed increases in mesangial matrix, thickened and irregular basement membrane that was pronounced in ACE-treated groups of animals (Fig. 5C and 5D). Focal and extensive fusions of foot processes (Fig. 5B and 5D) were observed. Also noticed were decreased numbers of podocyte and broadening of foot processes (Fig. 5C and 5D). At the PCT of diabetic rats were glycogen deposits, abundant lysosomes and cytoplasmic accumulation of lipid droplets (Fig. 6B, 6C and 6D). Also observed were disruption of tubular basal infoldings and their related mitochondria in groups B, C and D rats when compared with group A control rats (6D). Quantification of kidney tubule damage was carried out under double-blind conditions (Fig. 1B).

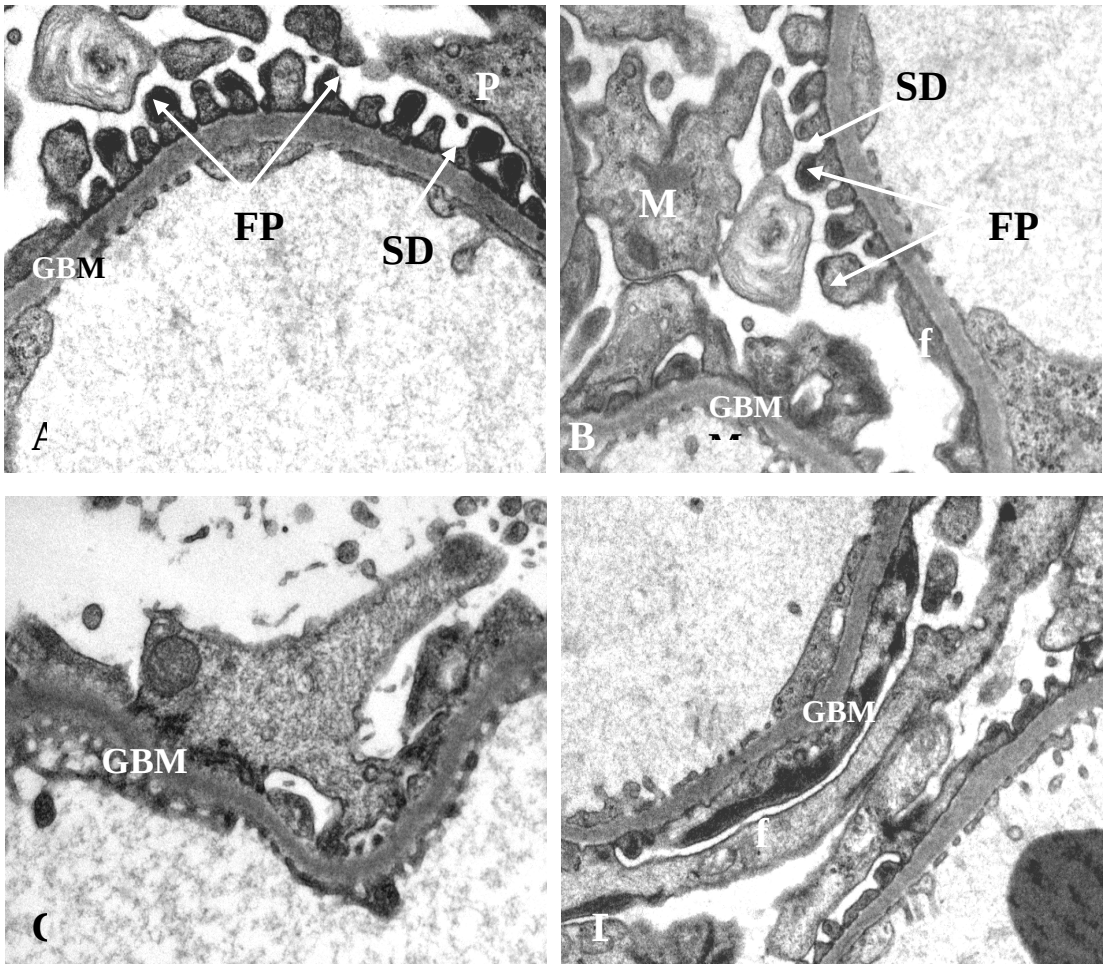

Fig. 5. Electron micrographs of kidney sections from control (A), and diabetic rats (B, C and D), illustrating glomerular basement membrane (GBM), podocyte (P), slit diaphragm (SD), mesangial (M) and foot processes (FP). (A) Control, non-diabetic rats, showing normal glomerular structure. (B) STZ-treated diabetic rats showing minimal focal fusion of foot processes (f). (C) STZ- + ACE-treated diabetic rats with extensive fusion of foot processes, thickening of GBM with deposits and complete absence of slit diaphragm. (D) ACE-treated diabetic rats with distorted glomerular structures. ACE caused significant structural damages to the glomeruli of groups C and D rats, consisting of GBM, podocyte and slit diaphragm. Magnification X30, 000.

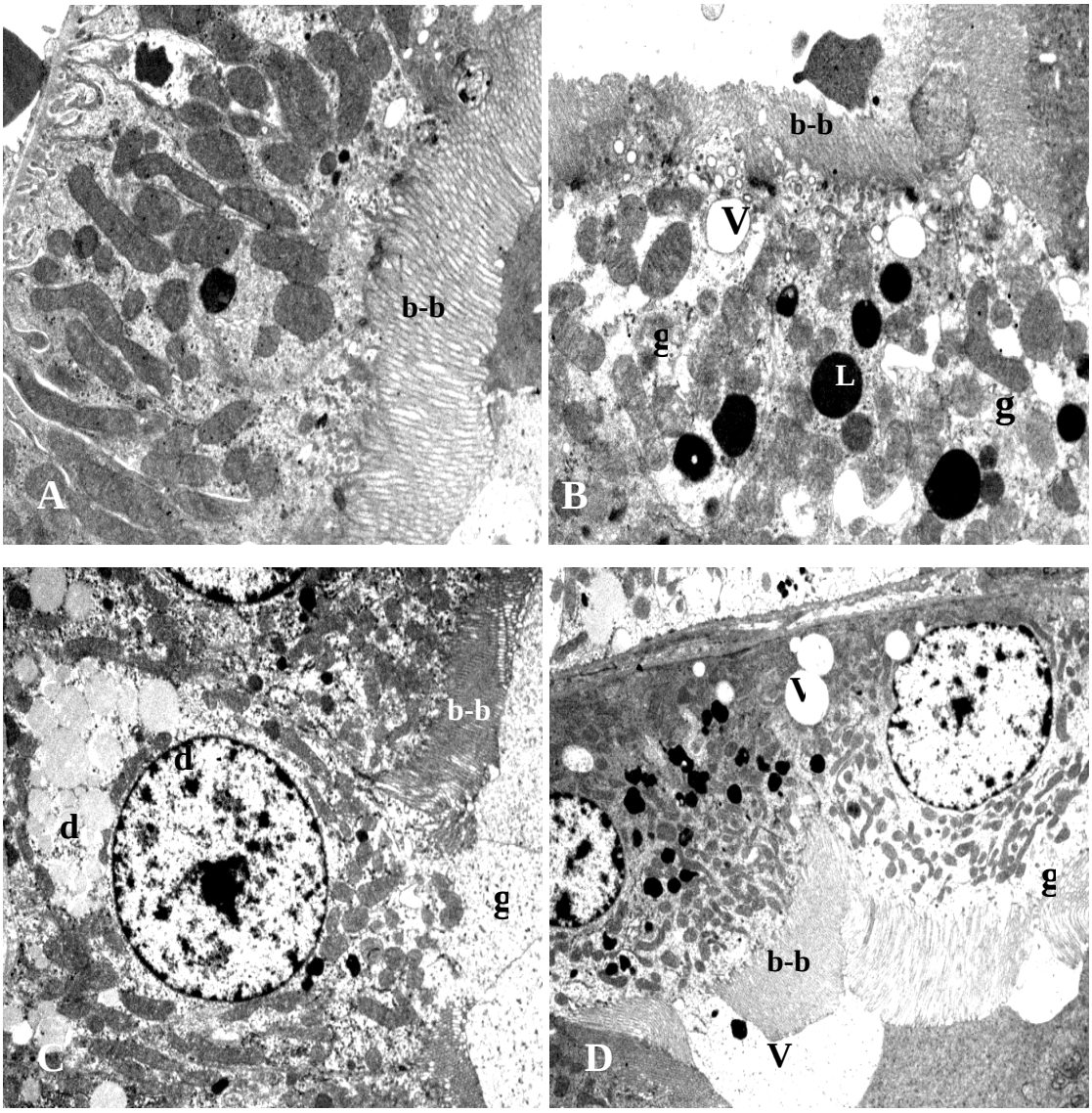

Fig. 6. Electron micrographs of kidney sections from control (A) and diabetic rats (B, C and D), illustrating proximal convoluted tubule (PCT), with brush-border (b-b), lysosmes (L), glycogen accumulation (g), vesicles and vacuoles (V), and lipids droplets. (A) Control, non-diabetic rats, showing normal epithelial cells of PCT (b-b) with prominent elongated mitochondria. (B) STZ-treated diabetic rats showing glycogen deposits (g), abundant lysosomes (L) and lipid droplets (d). (C) STZ- + ACE-treated diabetic rats with abundant lipid droplets (d) and glycogen deposits. (D) ACE-treated rats with vesicles and large vacuoles. ACE caused significant structural changes in the proximal tubules of groups C and D rats, consisting of aggregates of lysosomes, a common feature of cells in the early stages of necrosis. Magnification X 6, 000.

4. DISCUSSION

Previous studies in our laboratories have shown that *Artocarpus communis* (Forst.) root-bark aqueous extract is toxic to, and induces hyperglycemia in, mice and Wistar rats. This finding is quite intriguing because decoctions and infusions of *A. communis* root-bark are traditionally used by the Yoruba-speaking people of Western Nigeria as effective folk remedy for the management, control and/or treatment of adult-onset, type 2 diabetes mellitus. In view of the observed hyperglycaemic and toxic effects of *A. communis* root-bark aqueous extract (ACE) in rats, the present study was undertaken to compare the glycaemic, metabolic and ultrastructural effects of ACE with those of streptozotocin (STZ) in the renal cortex of Wistar rats.

Any glomerular injury that increases the permeability of glomerular basement membrane will allow plasma protein to escape into the urine, resulting in proteinuria. Some of these proteins are ingested by proximal tubular cells, initiating an inflammatory response that contributes to interstitial scaring. Recent studies, however, suggest that proteinuria itself may contribute to progression of renal disease (Deanne *et al*., 2004). The STZ-diabetic experimental model has been used in a variety of research studies for the characterization of treatments associated with diabetes mellitus in humans. STZ causes hyperglycemia by specifically inducing DNA strand breaks in pancreatic islet β-cells, and stimulating nuclear poly(ADP-ribose) synthetase, thus depleting intracellular NAD^+ and $NADP^+$ levels. Reduction of intracellular NAD^+ and $NADP^+$ inhibits proinsulin synthesis, leading to a diabetic state (Wilson *et al*., 1988). It has been suggested that production of activated oxygen species (superoxide) by hydroxyl radical and singlet oxygen, plays a major role in the development of STZ-induced diabetes (Sato *et al*., 1979).

The renal proximal tubular segment is considered to be the chief nephron site for the damage that occurs as a result of persistent hyperglycemia or toxic insult (Glaumann and Trump, 1975). The present study was aimed at comparing the hyperglycemic effects of STZ and ACE on renal proximal tubules at different durations of diabetic state. The activities of BBM marker enzymes, vis, AlkPase and GGTase, and Na^+-K^+-dependent ATPase were determined to evaluate the structural, molecular and functional integrity of proximal tubules under short- and long- term periods of diabetic conditions.

In this study, we observed a decreased body weight of the treated diabetic rats, and increased whole kidney and cortex weights in the treated diabetic animals when compared with the control animals. These findings are in agreement with those of Seyer-Hansen (1976) who reported a 15% rise in whole kidney weight within 72-h of induction of STZ experimental diabetes in rats. The later observation could be due to glomerular cell proliferation accompanying glomerular enlargement in the early phase of STZ-induced diabetes mellitus. Furthermore, the serum insulin levels of the treated animals sharply and significantly decreased. The serum concentrations of creatinine, inorganic phosphate, phospholipids and cholesterol increased progressively with increased duration of diabetic state. An increase or a decrease in serum creatinine levels reflects the degree of damage caused to the kidney by diabetes. Increased level of serum cholesterol is probably due to decreased level of high-density lipoprotein-cholesterol.

This, in turn, will result in decreased removal of cholesterol from extra-hepatic tissues by high-density lipoprotein-cholesterol (Prince *et al*., 1999). Phospholipids are vital components of biomembranes, and cholesterol is responsible for increased synthesis of diabetic phospholipids. In this context, higher levels of cholesterol and phospholipids have been observed in diabetic liver and kidney (Pari and Venkateswaran, 2003). Therefore, diabetic complications associated with renal tissue may be partly due to abnormalities in lipid metabolism.

Nephropathy is a major complication of diabetes mellitus. The gradual and progressive kidney damage that occurs in diabetic nephropathy is reflected in an increasing urinary albumin, which is detected initially as persistent microalbuminuria, and subsequently as persistent macroalbuminuria. Our findings in this regard are in agreement with those of White *et al*., (2002). Severe diabetes (or toxin) equally induces elevation of urinary albumin and albumin-creatinine ratios which are considered as significant markers of renal dysfunction. Tojo *et al*., (2001) observed absolute tubular reabsorption of albumin to be decreased in STZ-treated rats, while at the same time, ultrastructural studies have shown a decrease in albumin uptake, and a reduction in the levels of megalin in the kidneys of STZ-treated rats. Albumin leakage indicates a general renal vascular dysfunction, particularly to the blood vessel walls (Meyer *et al*., 1999). In our study, morphological evaluation and chemical assays showed that STZ and ACE are capable of inducing renal glycogen accumulation in the treated animals, possibly suggesting decreased glucose utilization. Extensive renal glycogen levels observed in our study can be attributed to renal dysfunction and development of diabetic nephropathy, observations that are in agreement with the findings of Monica *et al*., (2001).

The activities of AlkPase and GGTase in the BBMV isolated from the kidney cortex declined markedly in diabetic rats that received ACE by the end of the 8th week of our study. These enzymes are closely associated with cell membranes and transport processes in the kidney brush-border (Guder and Ross, 1984). Because specific enzyme activities (activity/mg protein) were not significantly changed in the homogenates, as also reported by Paddock *et al*., (1981), we hypothesized that the activities of AlkPase and GGTase actually decreased in the pellet of cortical homogenate (membrane-bound enzyme), whereas the dissociated enzymes were traced in the supernatant where the activities increased. This observation implies that BBM might have been severely damaged during persistent hyperglycemia with the enzymes and other proteinous components, after dissociation from the BBM, released in the supernatant and later excreted in the urine (as has been observed by Herminghuysen *et al*., 1985). The distribution of the enzyme pattern in BBMV showed that the activities of these marker enzymes decreased due to hyperglycemia. As observed in the whole cortical homogenates, the activities of AlkPase and GGTase declined only in the membrane-bound fraction with the corresponding increase in the supernatant as free or dissociated enzymes. The data on enzyme activities clearly uphold the results of our urinalysis and morphological findings, suggesting greater damage to the nephrons, especially *pars recta* of the proximal tubule, as indicated by greater reductions of both AlkPase and GGTase in the BBMV.

In the diabetic group of rats, the glomerular basement membrane (GBM) inner layer was diffusely enlarged, and the meshwork structures in the GBM middle layer, as well as the mesangial matrix (MM), were markedly irregular due to rupturing of the fine fibrils.

These irregularities and enlargements of the mesh pores in the diabetic rats were significantly different from those in the control animals. Also noticed in the GBM was an enlarged subendothelial space, suggestive of diabetic nephropathy. It has been reported by Nishikawa *et al*., (2000), and Wolf and Thaiss (1995), that diabetes-induced glomerular and tubular cell damage occurs as a consequence of prolonged exposure to high glucose concentrations. It has also been suggested that hyperglycemia might be responsible for the series of biochemical events leading to diabetic nephropathy. Heilig *et al*., (1997) demonstrated immense pathological features of mesangial cell grown in high glucose concentrations, which included increased collagen and fibronectin synthesis.

It is abundantly clear that uncontrolled diabetes mellitus may cause kidney hypertrophy with deleterious consequences. The findings of the present study indicate that *A. communis* root-bark aqueous extract has toxic, non-specific deleterious renal effects, and affects the activities of conventional marker enzymes of brush-border membranes. Furthermore, the lipid environment that is essential for normal functioning of the membrane proteins, urinary albumin and creatinine, were also adversely affected by ACE. Our data also show that ACE-induced diabetic state caused significant structural changes in the glomerular barrier, consisting of GBM, podocytes and slit diaphragm, resulting in proteinuria. In conclusion, the results of the present study demonstrate that *A. communis* root-bark aqueous extract inhibits physiologically-important features of various renal biochemical components, including activities of AlkPase, GGTase and $(Na^{+}\text{-}K^{+})$-dependent ATPase in homogenates and the brush-border membranes from kidney cortices of ACE-treated rats.

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