ORIGINAL ARTICLE

Lead-induced morphometric changes in the kidneys of albino rats ameliorated by ginkgo biloba extract (EGb 761)

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Abstract

Objective: To observe the effects of ginkgo biloba extract on lead-induced morphometric changes in the kidneys of albino rats.

Methods: This randomised controlled study was conducted at the Institute of Basic Medical Sciences, Dow University of Health Sciences, Karachi, from April 2009 to March 2010, and comprised male Wistar albino rats weighing between 150-180 gm who were randomly divided into three equal groups, A, B and C. These were further split into subgroups 1, 2, 3 and 4 according to the duration of the experiment (one, two, four and six weeks). Group A rats were given 1 ml normal saline intraperitoneally daily, group B rats were given lead acetate 8mg/kg intraperitoneally daily, while group C animals received 100mg/kg ginkgo biloba extract orally along with 8mg/kg lead acetate injection. The animals were sacrifised at the end of the prescribed period, and kidneys were retrieved, fixed, stained and examined under light microscope. SPSS 16 was used for data analysis.

Results: Of the 120 rats, there were 40(33.3%) in each group. Time-dependent deterioration was observed in the histological architecture of kidneys in group B animals compared to the group A animals, whereas less marked changes were observed in the protected group C animals. In group B animals, the diameter of proximal convoluted tubules increased, the number of proximal convoluted tubules and their nuclei decreased, whereas diameter of the nuclei decreased after an initial increase during the first and second weeks. These parameters remained largely undisturbed in group A animals, whereas changes in group C animals were comparable with those in the controlled group A animals.

Conclusion: Ginkgo biloba extract had a protective effect on lead-induced morphometric changes in the kidneys of albino rats.

Keywords: Lead acetate, Ginkgo biloba extract, EGb761, Kidney, Antioxidant. (JPMA 67: 58; 2017)

Introduction

Lead is a ubiquitous metal existing everywhere on the earth. Injurious effects of lead to the health of people are well known, and tremendous efforts are being made all over the world to prevent exposure to this toxic metal. However, in underdeveloped countries like ours, the problem of exposure still persists. The main sources of lead are leaded gasoline, paint flakes from old houses, ceramics, old sanitary pipes, calk, lead smelters, bullets and batteries. In spite of the phasing out of lead from gasoline, it has remained the most important source of chronic lead exposure, and developing Asian countries including Pakistan are the major sufferers in this respect.[1]

Kidneys are an easy target for toxicity by a number of drugs, chemicals and industrial wastes. Lead, cadmium, mercury, etc. are common industrial and environmental pollutants which can damage kidneys, and can ultimately lead to chronic renal failure.[2] Ample blood flow and diversity of actions performed by kidneys relative to their small size are probably the most important factors which make the kidneys vulnerable to these notorious agents.

Lead is excreted via kidneys, therefore, acute exposure can lead to damage to the proximal convoluted tubules (PCTs).[3] In fact, lead exposure can affect kidneys in two ways. Acute exposure to lead, particularly in children, can lead to reversible tubular damage, whereas chronic industrial exposure in adults can lead to insidious onset of irreversible interstitial nephropathy.[4] Therefore, end-stage renal disease (ESRD) is not an uncommon sequel to lead-induced nephrotoxicity.

It has been observed that damage to kidneys due to lead and other pollutants is associated with exhaustion of body's resources to overcome the oxidative stress produced by these agents.[5,6] Production of reactive oxygen species by these harmful elements leads to destruction of lipids, proteins and deoxyribonucleic acid (DNA) in the body.[7] Several studies have proved that these changes can be avoided, or even ameliorated by the use

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of antioxidants, either alone or in combination with the chelating agents. List of antioxidants is extensive and includes zinc, cysteine, N-acetylcysteine, methionine, alpha-lipoic acid, melatonin, vitamins B6, C and E, taurine and ginkgo biloba extract (EGb761).[6,8,9]

Ginkgo biloba, also known as maidenhair tree, is the most ancient tree found in the world. It is exceptional due to the fact that it does not have any other existing relative, and is identified as a living fossil.[10] Researchers are exploring its beneficial effects in different ailments. Its purified extract, known as EGb761, is widely being studied with regard to its antioxidant activity, both in vivo and in vitro.[9,11,12] The active ingredients in EGb761 are 24% flavonoids and 6% terpene lactones with 5ppm of an allergenic component, the ginkgolic acid.[13] The flavonoids component of the extract has been attributed to the scavenging activity against free radicals, especially those derived from oxygen.[12]

There have been many studies in which histological and morphometric changes in the kidneys of lead-treated rats have been observed,[14,15] and different antioxidants have been tried to observe their ameliorating effects, but the efficacy of concomitant use of EGb761 on lead-induced nephrotoxicity has not been ascertained yet. The present study was planned to determine the effects of EGb761 on lead-induced histomorphometric changes in the kidneys of Wistar albino rats.

Materials and Methods

This randomised controlled trial (RCT) was performed at the animal house of the Institute of Basic Medical Sciences (IBMS), Dow University of Health Sciences (DUHS), Karachi, from April 2009 to March 2010, and comprised albino rats. Approval was obtained from the institutional ethics review committee. Young male Wistar albino rats weighing between 150-180 grams were included. Using web-based Research Randomiser software,[16] random assignment by blocks technique was applied to divide the animals into 3 equal groups, A, B and C. The three groups were further split into 4 subgroups according to the duration of treatment, i.e. one week (A1, B1, C1), two weeks (A2, B2, C2), four weeks (A3, B3, C3) and six weeks (A4, B4, C4). The animals were tagged by punching their ears, and were kept under observation for one week prior to starting the experiment, so that they could be acclimatised to the experimental environment. Throughout the experiment the animals were kept in standard laboratory environment at controlled temperature and 12-hour day and night cycle. They were offered ad libitum access to rat chow and water. The animals were weighed both initially as well as at the end of the experimental period.

Group A animals served as control and were given 1ml of normal saline/day intraperitoneally.

Group B animals received an intraperitoneal dose of 8 mg/kg/day of lead acetate[17] (Merck, Germany). Lead acetate solution was prepared by dissolving 160 mg of the salt in 100 ml of distilled water. The dose in ml was calculated using the formula:

100/160×8/1000×weight of the animal in grams

Group C animals received 100 mg EGb761/kg/day orally[18] in addition to the intraperitoneal 8 mg/kg/day of lead acetate. EGb761 solution was prepared by dissolving 25 tablets of Tanakan (Atco), each containing 40 mg of EGb761, in 50 ml of distilled water. The dose in ml for each animal was calculated using the formula:

50/(25×40)×100/1000×weight of the animal in grams

The animals were killed at the end of the assigned period, i.e. one, two, four and six weeks, by using an overdose of ether in a glass jar. A midline incision in the abdomen was given extending from xiphisternum to symphysis pubis. The kidneys were identified and removed. They were divided into two equal halves longitudinally, and were fixed in 10% neutral buffered formalin for a period of 24 hours. After that the kidneys were dehydrated with ascending strengths of alcohol, cleared with xylene, infiltrated with liquid paraffin and then embedded in molten paraffin. Then 5µm thick sections were cut from the paraffin blocks with the help of rotary microtome and mounted on gelatinised glass slides. The tissues were stained with haematoxylin and eosin to observe the general architecture of the renal parenchyma, particularly the PCTs. Periodic acid-Schiff (PAS) stain was used to examine the brush border while Gömöri's Methenamine Silver method (modified hexamine silver technique) was used to study changes in the basement membrane of the tubules.

An ocular micrometer and a counting reticule were calibrated with the help of a stage micrometer which had 1 mm wide scale divided into 10 equal parts, each representing a length of 10 µm. The calibration was done at 10×, 40× and 100× magnifications.

Data was analysed with SPSS 16. Mean ± standard error of mean (SEM) was calculated. Assumption of normality was tested by Shapiro-Wilk normality test and p-value was found to be more than 0.05 for all groups. Therefore we used one-way analysis of variance (ANOVA) to determine the mean difference, while post-hoc Tukey test was

applied for pair-wise comparison. P≤0.05 was considered significant.

Results

Of the 120 rats, there were 40(33.3%) in each group. Histological examination of group A animals revealed normal renal cortical architecture in all the sub-groups (Figures-1-3). The PCTs were compactly arranged around the renal corpuscles and were cut in round, oblique or longitudinal sections because of their convoluted arrangement. Lining epithelium of the PCTs comprised cuboidal to low columnar cells having fine granular cytoplasm with basally or centrally located round nuclei and fine, uniformly dispersed chromatin. The epithelial

Table-1: Mean ±SD of diameter of PCTS in different groups of rats (in µm).

Groups	Treatment Received	Proximal Tubular Diameter(µM)			
		1st Week	2nd Week	4th Week	6th Week
A (n=40)	Control	41.95 1.234	41.81 0.475	40.78 0.722	41.62 1.108
		A1 (n=10)	A2 (n=10)	A3 (n=10)	A4 (n=10)
B (n=40)	Lead Acetate	43.14 0.962	44.82 0.677	45.81 0.874	48.23 0.774
		B1 (n=10)	B2 (n=10)	B3 (n=10)	B4 (n=10)
C (n=40)	Lead Acetate + EGb 761	41.12 0.729	42.1 0.667	43.00 0.764	43.13 0.313
		C1 (n=10)	C2 (n=10)	C3 (n=10)	C4 (n=10)

SEM: Standard error of mean
n: Number of animals
PCTs: Proximal convoluted tubules
SD: Standard deviation
EGb 761: Ginkgo biloba extract.

Table-2: Mean ±SEM of number of PCTS in cortical region of kidneys in different groups of rats.

Groups	Treatment Received	Number of PCTs per Unit Area under High Power			
		1st Week	2nd Week	4th Week	6th Week
A (n=40)	Control	17..576 0.356	18.176 0.310	18.28 0.300	18.433 0.510
		A1 (n=10)	A2 (n=10)	A3 (n=10)	A4 (n=10)
B (n=40)	Lead Acetate	15.53 0.334	14.244 0.458	13.075 0.504	11.422 0.422
		B1 (n=10)	B2 (n=10)	B3 (n=10)	B4 (n=10)
C (n=40)	Lead Acetate + EGb 761	17.022 0.456	16.699 0.355	16.09 0.260	15.98 0.290
		C1 (n=10)	C2 (n=10)	C3 (n=10)	C4 (n=10)

SEM: Standard error of mean
n: Number of animals
PCTs: Proximal convoluted tubules
EGb 761: Ginkgo biloba extract.

Table-3: Mean ± SEM of nuclear count in PCTs of kidneys in different groups of rats.

Groups	Treatment Received	Number of PCT Nuclei per Unit Area under High Power			
		1st Week	2nd Week	4th Week	6th Week
A (n=40)	Control	18.04 0.292	18.16 0.262	18.53 0.310	18.50 0.279
		A1 (n=10)	A2 (n=10)	A3 (n=10)	A4 (n=10)
B (n=40)	Lead Acetate	17.82 0.229	16.04 0.187	14.08 0.295	8.82 0.123
		B1 (n=10)	B2 (n=10)	B3 (n=10)	B4 (n=10)
C (n=40)	Lead Acetate + EGb 761	17.94 0.363	17.6 0.141	17.01 0.131	16.55 0.217
		C1 (n=10)	C2 (n=10)	C3 (n=10)	C4 (n=10)

SEM: Standard error of mean,
n: Number of animals
PCTs: Proximal convoluted tubules
EGb 761: Ginkgo biloba extract.

Table-4: Mean ±SEM of nuclear diameter in PCTs of kidneys in different groups of rats (in µm).

Groups	Treatment Received	Nuclear Diameter of PCTs			
		1st Week	2nd Week	4th Week	6th Week
A (n=40)	Control	6..576 0.052	6.583 0.047	6.859 0.061	6.919 0.069
		A1 (n=10)	A2 (n=10)	A3 (n=10)	A4 (n=10)
B (n=40)	Lead Acetate	7.199 0.044	7.200 0.047	6.027 0.017	5.447 0.094
		B1 (n=10)	B2 (n=10)	B3 (n=10)	B4 (n=10)
C (n=40)	Lead Acetate + EGb 761	6.847 0.098	6.921 0.079	6.548 0.039	6.476 0.070
		C1 (n=10)	C2 (n=10)	C3 (n=10)	C4 (n=10)

SEM: Standard error of mean,
n: Number of animals
PCTs: Proximal convoluted tubules
EGb 761: Ginkgo biloba extract.

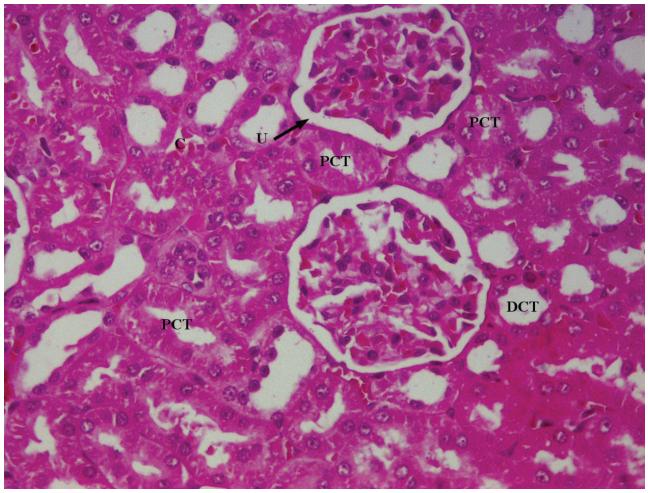

Figure-1: Photomicrograph of 5µm thick paraffin section of kidney from group A1 albino rat showing normal cortical architecture. Proximal convoluted tubule (PCT), distal convoluted tubule (DCT), urinary space (U) and peritubular capillaries (C) are seen.

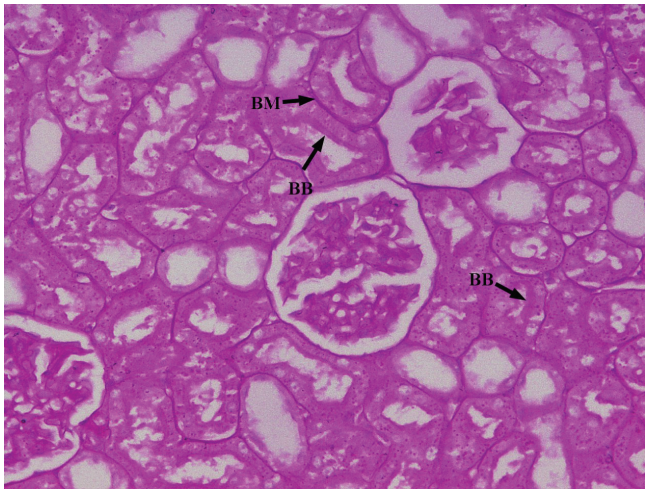

Figure-2: Photomicrograph of 5µm thick paraffin section of kidney from group A3 rat showing normal cortical architecture with intact basement membrane (BM) and narrow, clear lumina of proximal convoluted tubules (PCT) having intact brush border (BB). PAS).

basement membrane was intact and there was well-defined regular brush border towards the luminal aspect of the cells. Cellular or nuclear debris was not seen in the lumina of the PCTs.

The histological examination of group B animals revealed gradual deterioration in the cortical architecture of kidneys. There was a slight karyomegaly in some of the PCT cells in B1 and B2 animals (Figure-4), whereas in B3 and B4 animals, the nuclei in many of the cells were small and pyknotic. The cytoplasm of the cells showed vacuolation which was most marked in B4 animals (Figure-5). Deterioration in the continuity of the brush border and the basement membrane was also noted which was less marked in B1 animals and most marked in B4 animals (Figure-6). No inflammatory reaction was observed in any of the sub-groups.

Microscopic changes observed in group C were comparable to group A (Figure-7). Karyomegaly in the PCT cells was not much marked in sub-groups C1 and C2. Similarly the cytoplasmic vacuolation was also less prominent in group C. The deterioration of the brush border and discontinuity in the basement membrane was also less marked as compared to the corresponding group B animals (Figure-8).

Diameter of PCTs was measured and compared. The mean values in control groups A1, A2, A3 and A4 were 41.95±1.234, 41.81±0.475, 40.78±0.722 and 41.62±1.108. Statistically, there was no change in the diameter of PCTs in group A rats. However, the diameter gradually

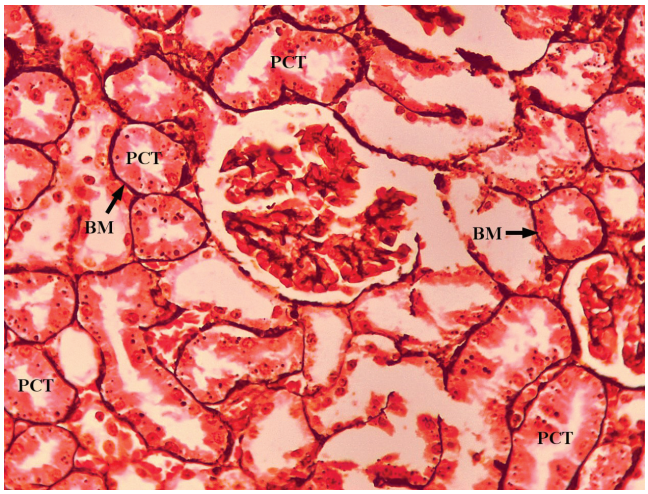

Figure-3: Photomicrograph of 5µm thick paraffin section of kidney from group A1 rat showing normal cortical architecture with intact basement membrane (BM) and narrow, clear lumina of proximal convoluted tubules (PCT). GMS stain. ×400.

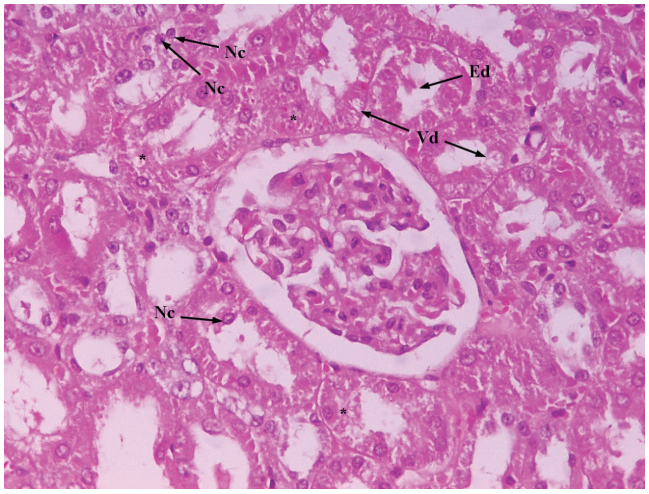

Figure-5: Photomicrograph of 5µm thick paraffin section of kidney from group B4 rat showing tubular dilatation, vacuolar degeneration (VD) of the cells and epithelial debris (Ed) in the lumen of PCTs. Small contracted nuclei can be seen in some of the PCT.

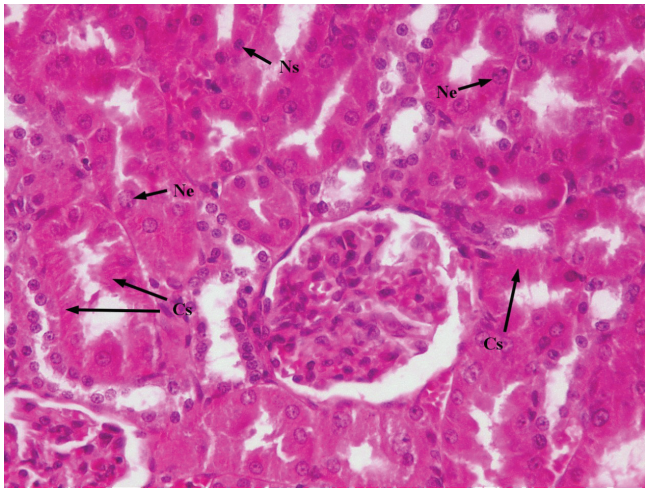

Figure-4: Photomicrograph of 5µm thick paraffin section of kidney from group B1 rat showing cellular swelling (Cs) in some of the proximal tubules. Some of the nuclei are enlarged (Ne), while others are shrunken (Ns). H&E stain. ×400.

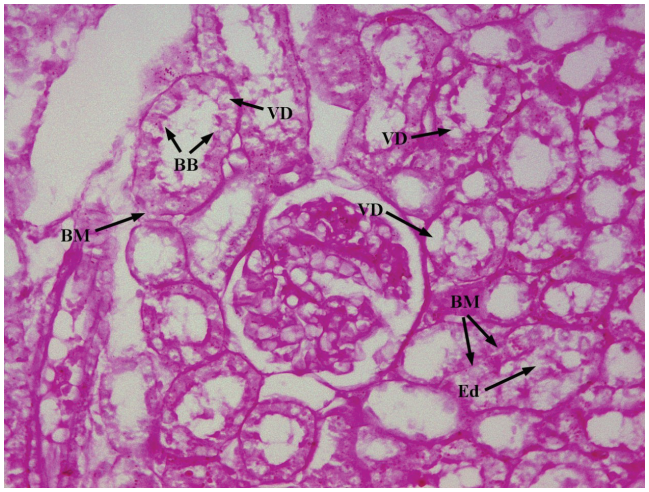

Figure-6: Photomicrograph of 5µm thick paraffin section of kidney from group B4 rat showing tubular dilatation, vacuolar degeneration (VD) of the PCT cells, epithelial debris (Ed) in the lumen, discontinuous, broken basement membrane (BM) and scanty brush.

increased in the sub-groups B1, B2, B3 and B4 (43.14±0.962, 44.82±0.677, 45.81±0.875 and 48.23±0.774). When the diameter of PCTs of B1, B2 and B3 was compared with the corresponding subgroups of control animals, the change was insignificant ($p>0.05$). However, the increase in diameter was highly significant ($p<0.001$) when B4 animals were compared with A4 animals. Similarly, the diameter of the PCTs in all the subgroups of group C animals increased, but to a lesser extent as compared to the group B animals (41.12±0.729, 42.1±0.667, 43.00±0.764 and 43.13±0.313). Statistically, the increase in the diameter was insignificant when B1, B2 and B3 animals were compared with C1, C2 and C3 animals ($p>0.05$). However, the increase in diameter was highly significant when B4 animals were compared with C4 animals ($p<0.01$). The change between all the corresponding subgroups of group A and C animals was statistically non-significant ($p>0.05$) (Table-1).

The mean number of PCTs per field of reticule in the control group A animals was 17.576±0.356, 18.176±0.310, 18.28±0.300 and 18.433±0.510, with no statistical difference ($p>0.05$). However, there was gradual reduction

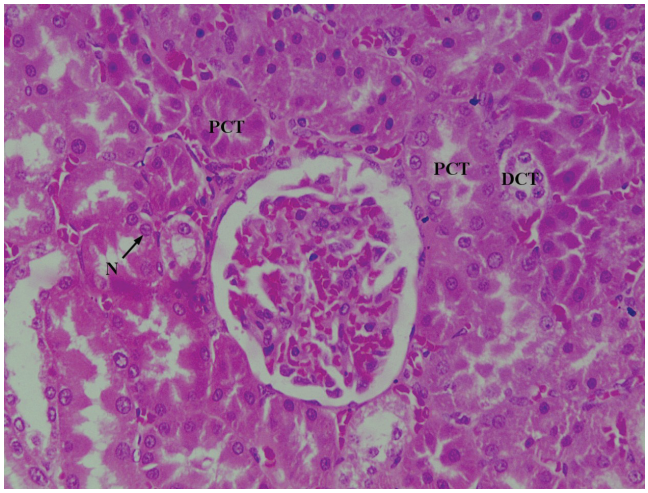

Figure-7: Photomicrograph of 5µm thick paraffin section of kidney from group C4 rat showing almost normal PCT, DCT with intact cells and nuclei (N), comparable to control. H&E stain. ×400.

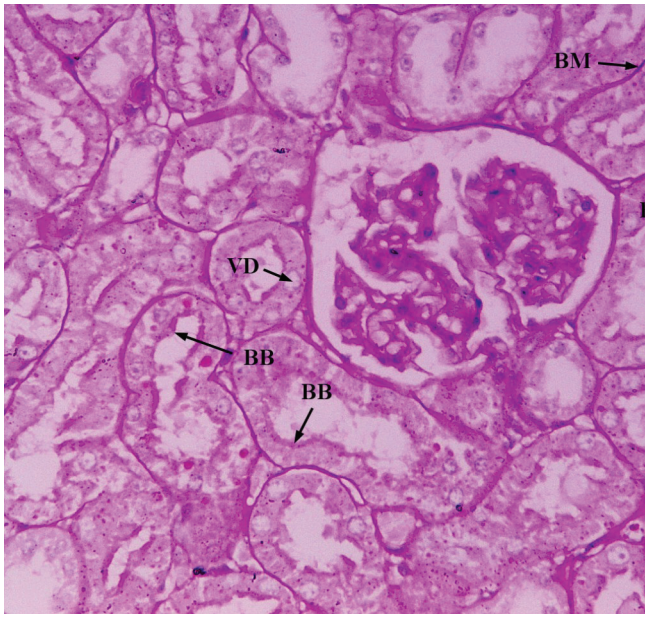

Figure-8: Photomicrograph of 5µm thick paraffin section of kidney from group C4 rat showing PCTs with an architecture that is close to control. Basement membrane (BM) and brush border (BB) are mostly intact. Vacuolar degeneration (VD) can be seen sparsely.

in the number of PCTs in group B animals (15.53±0.334, 14.244±0.458, 13.075±0.504 and 11.422±0.422), and the difference between B1 and B4 animals was highly significant ($p<0.001$). When group B animals were compared with the corresponding group A animals, there was statistically significant difference between subgroups B1 and A1 ($p<0.05$), whereas the difference was highly significant when B2, B3 and B4 animals were compared with A2, A3 and A4 animals, respectively ($p<0.001$). The number of PCTs in group C animals also decreased, but to a lesser extent as compared to group B animals (17.022±0.456, 16.699±0.355, 16.09±0.260 and 15.98±0.290). The change was insignificant between B1 and C1 animals ($p>0.05$), significant between B2 and C2 animals ($p < 0.01$) and highly significant when B3 and B4 animals were compared with C3 and C4 animals, respectively ($p<0.001$). The difference between all the corresponding subgroups of group A and C animals was insignificant ($p>0.05$) (Table-2).

The mean nuclear count in the PCTs of group A animals did not show any difference throughout the length of the experiment (18.04±0.292, 18.16±0.262, 18.53±0.310 and 18.50±0.279). However, in group B animals it decreased gradually (17.82±0.229, 16.04±0.187, 14.08±0.295 and 8.82±0.123). The change was insignificant when we compared group B1 animals with group A1 animals ($p>0.05$), whereas it was highly significant when we compared group B2, B3 and B4 animals with the corresponding group A2, A3 and A4 animals ($p<0.001$). The number of nuclei also decreased in group C animals, but to a lesser extent (17.94±0.363, 17.6±0.141, 17.01±0.131 and 16.55±0.217). The change in the number of nuclei in B1 animals was insignificant as compared to C1 animals ($p>0.05$), whereas it was highly significant in B2, B3 and B4 animals as compared to C2, C3 and C4 animals, respectively ($p<0.001$) (Table-3).

The nuclear diameter of the PCT cells was also measured. The diameter remained consistent throughout the experiment in group A animals (6.576±0.052, 6.583±0.047, 6.859±0.069 and 6.919±0.069). However, it increased in B1 and B2 animals (7.199±0.044, 7.2±0.047) and decreased in B3 and B4 animals (6.027±0.017, 5.447±0.094). The change was highly significant ($p<0.001$) between all the corresponding sub-groups of A and B animals. Changes in the nuclear diameter of group C rats were similar to, but less marked as compared to the group B rats (6.847±0.098, 6.921±0.079, 6.548±0.039 and 6.476±0.070). When group B1 and B2 animals were compared with C1 and C2 animals, the change was significant ($p<0.01$), whereas the change was highly significant ($p<0.001$) when B3 and B4 animals were compared with the corresponding C3 and C4 animals (Table-4).

Discussion

In our study it has been observed that administration of lead acetate in albino rats produces histomorphometric changes in the kidneys, particularly in the PCTs, which can

be prevented by the concomitant use of EGb761. The damaging effects of lead on the kidneys are well established in other animal and human studies. In a recent study by Jarad AS, significant changes in the histopathological and biochemical parameters of kidneys were observed in lead-exposed rats.[19] Similarly, Alasia D. et al. in their cross-sectional study conducted in Nigeria found elevated levels of serum creatinine and blood uric acid, and decreased creatinine clearance in occupationally exposed adult lead workers. According to them, there was an increased risk of developing hyperuricaemia and impaired renal function in people with occupational exposure to lead.[20] However, in another cross-sectional study performed on storage battery repair unit workers, no significant change in the renal function parameters was observed in spite of their blood lead levels being significantly higher than the control population.[21] The possible explanation might be that although the duration of exposure was long, but the magnitude of exposure was not enough to cause severe functional damage to the kidneys. However, this does not preclude the possibility of structural damage to the kidneys because of its large functional reserve.[3]

In the current study, the diameter of PCTs in the lead-treated group B animals was significantly increased. The most significant increase ($p<0.001$) was observed in group B4 animals. This is in agreement with the study conducted by Mohamed et al., who reported increase in the diameter of PCTs of male albino rats exposed to lead acetate both pre- as well as post-natally.[14] One of the reasons for increase in the diameter of PCTs may be the loss of brush border due to oxidative stress produced by lead. This argument is favoured by a study in which significantly increased loss of brush border antigen was observed in urine of lead workers, particularly those in whom blood lead levels were more than 40mg/dl[2]. Increase in the diameter of PCTs was also observed in streptozotocin-induced diabetic mice where it was proposed to be due to the hyperglycaemia-induced oxidative stress which produces tubular atrophy and apoptotic changes in the cells of PCTs.[8]

Decrease in the number of PCT nuclei in group B rats reflects oxidative stress-induced necrosis and apoptosis by lead acetate, as suggested by Wang et al.[22] when they treated primary cultures of rat's PCTs with different doses of lead to observe its cytotoxic mechanism. Similar findings were also observed by Massanyi et al. who gave different doses of lead nitrate to young albino rats and noted reduced number of nuclei in their testes and kidneys, proposed to be due to the oxidative stress-induced necrosis and apoptosis.[23] The dilated PCTs and the necrosis and apoptosis of the cells may be responsible for the decreased number of PCTs in group B rats, as suggested by Khan et al.[24]

Increase in the size of nuclei which we observed in B1 and B2 rats is in conformity with the investigation of Loumbourdis, who observed the effects of lead nitrate on the kidneys of the frog Rana ridibunda at a dose of 14 ppm for 4, 10 and 30 days.[25] He observed karyomegaly in the PCTs of the frog which was most marked in the 10-day treatment group. This initial increase in the size of nuclei probably reflects pseudo-inclusions and invaginations of cytoplasmic contents into the nuclei and the accumulation of lead as intranuclear inclusion bodies. The decrease in the size of nuclei in group B3 and B4 rats in our study is in agreement with the study of Khan et al. who observed decrease in the size of PCT nuclei of rats after giving 8mg lead acetate intraperitoneally daily for four to six weeks.[26] These small and pyknotic nuclei appear most probably due to oxidative stress-induced necrosis and apoptosis due to persistent and prolonged exposure to lead.[22]

In the present study, the histomorphometric parameters of PCTs of group C rats, which were concomitantly treated with EGb761, were comparable to changes in group A (control) rats. However, when compared with the parameters of group B rats, there was statistically significant difference. This preservation of the histomorphometric features in group C rats was most probably due to the antioxidant effects of EGb761, as suggested in many experimental studies.[27,28] In a recent study it has been proved that EGb761 protects kidneys from the cytotoxic effects of cisplatin by its anti-oxidative as well as anti-inflammatory properties.[29] In another study it was observed that EGb761 delays the development of glomerulosclerosis by preventing hypertrophy and accumulation of extracellular matrix in the mesangial cells of diabetic rats. These effects were associated with elevated levels of catalase, superoxide dismutase and glutathione peroxidase in the cells thus confirming the antioxidative activity of EGb761.[30]

Conclusion

Lead acetate had damaging effects on the kidneys leading to morphometric changes, especially in the PCTs. EGb761, on the other hand, had a protective effect, keeping the morphometric parameters near to normal when given concomitantly with lead acetate. These protective effects of EGb761 were most probably due to the anti-oxidative activity of the extract. Thus the use of EGb761 in people in whom lead exposure cannot be prevented, can at least decrease or delay the damaging

effects of lead on the kidneys.

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Conflict of Interest: None.

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