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Original Article

Effects of calcitriol on structural changes of kidney in C57BL/6J mouse model

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Abstract: Thisaim of the studyisto investigate the effects of calcitriol (vitamin D) on mouse kidneys under obese conditions. Male C57BL/6J mice were maintained on either low fat diet (LFD) or high fat diet (HFD) with/without calcitriol treatment (150 IU/kg/day) for 16 consecutive weeks. Results of HFD fed mice demonstrated more weight gain and showed numerous structural abnormalities in the corticomedullary region compared to those under control and LFD conditions. Near nephropathy condition in HFD mice were characterized by damage in renal tubules, including dilatation of interstitial cells and blood vessels. Furthermore, exfoliation and shedding of proximal tubular cells takes place. The conditions further worsen by thickening the basement membrane and interstitial inflammation, as evidenced by abundant interstitial debris. Additionally, a large number of degenerated mitochondria, fat droplets, lysosomal bodies' mesangial expansion, and cellular debris were found throughout the kidney. Sustained cell hypertrophy was also evident by transmission electron microscope confirming a marked increase in degeneration of cells within renal areas. No significant variances were detected in the glomerulus' area and diameter in both low and high fat diets with/without calcitriol treatment as well as inner and outer diameters of both distal and proximal tubule in all groups. Evidently, calcitriolcould act as a protective agent to normalize kidney structure in obese condition. This study suggests that calcitriol could normalize the function of kidney and protect its structural integrity in obesity.

Keywords: Calcitriol, C57BL/6J mouse, high fat diet, obesity, kidney, pathological changes

Introduction

Obesity is a common human health problem in many nations which can lead to a decreased life expectancy [1]. Co-morbidities of obesity include type 2 diabetes mellitus, cardiovascular disease, hepatic steatosis, and certain types of cancers [2, 3]. Indeed, the American Institute for Cancer Research states that 20% of all kidney cancer cases in males and 28% in females could be avoided by maintaining appropriate diet [4]. Furthermore diabetic nephropathy (DN) is a multifactorial condition involving hypertension, hyperglycemia, hyperinsulinemia, and hyperlipidemia [5, 6]. Recent studies have highlighted the role of obesity in renal damage patients as well as overweight subjects with diabetes [4, 7].

The role of vitamin D is no longer restricted to its classical functions in maintaining calcium and phosphate homeostasis [8, 9]. Vitamin D also appears to play a major role as a cell differentiating and anti-proliferative factor with actions at a variety of tissues including renal, cardiovascular, and immune systems [10, 11]. For example, in chronic kidney disease (CKD), vitamin D can help in the regulation of renin-angiotensin system (RAS) and the nuclear factor (NF) κB pathway, the two important pathways involved in a broad range of pathological processes [12]. Indeed, it appears that adequate replacement of vitamin D in deficient populations could potentially reduce premature morbidity and mortality. The implications of these new findings will serve to shift the approach to vitamin D replacement in CKD patients into a new era where use of vitamin D is no longer solely for the treatment of secondary hyperparathyroidism [8, 13]. Therefore the present study is designed to elucidate the

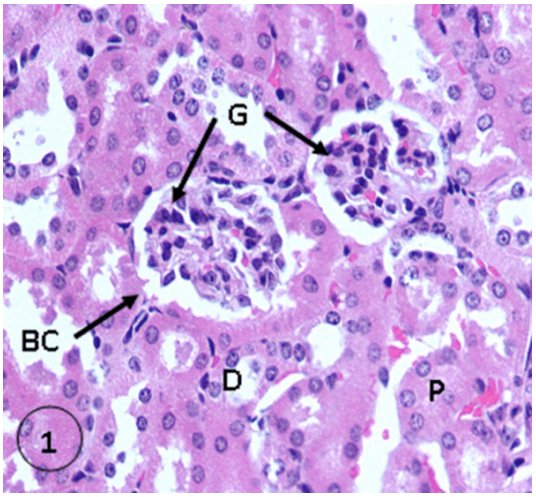

Figure 1. Mild dilation in Bowman's capsule (BC), with minimal disturbance in proximal (P) and distal (D) tubules (H&E 40×).

effects of calcitriol [1,25-(OH)2 vitamin D3] on structural changes of kidneys of mice served with two different diets. Thus, we hypothesized that calcitriol could reduce obesity-related risk and associated pathological changes in kidney. This study may perhaps shed some important changes that could be used in advanced studies.

Materials and methods

Animals and study protocol

Male C57BL/6J mice aged 4-5 weeks and weighing 20-25 g were obtained from the Animal Care Facility, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were housed in a temperature controlled room on a 12-hour light/dark cycle and were maintained in accordance with the recommendations from the Care and Use of Laboratory Animals approved by the faculty. The mice were acclimatized for a week before being introduced into the study. After conditioning, animals were randomly divided into four groups (11 animals/group). Group I was a control group fed on a 10 kcal (4.3 g fat) low fat diet (LFD) (Research Diets Inc., New Brunswick, NJ, USA) and coconut oil (1 ml/day). Group II was fed on LFD and received calcitriol (1,25-(OH)2D3, (Rocaltrol®, Hoffman-LaRoche Ltd, Basel, Switzerland) diluted with coconut oil given at a dose of 150 IU/kg/day by oral gavage (1 ml). [14] Group III served as controls and received 1 ml coconut oil/day and supplied with 45 kcal (24 g fat) high fat diet (HFD) (Research Diets Inc., New Brunswick, NJ, USA). Group IV was an experimental group fed on HFD and treated with 150 IU/kg/day calcitriol orally (delivered as 1 ml in coconut oil by oral gavages). Treatment was carried out for 16 weeks. A separate control group, without any treatment, was also kept to compare histopathological changes in all groups. The dose and duration was based on our previous studies [16].

Histology and morphometric study

At the end of the treatment, mice were anesthetized with mild ether and immediately euthanized by cervical dislocation. Kidney tissues were harvested and fixed in 10% formal-saline buffer and then processed to get paraffin sections (2-3 μm) for the histological study using hematoxylin and eosin stain for examination under light microscopy. A morphometric analysis including glomerulus area, glomerulus diameter, and outer and inner proximal and distal tubule diameter was performed using Leica camera and IM500 image manager software (Leica, Wetzlar, Germany).

Transmission electron microscope (TEM) Study

Immediately after removal of the kidney from the dissected mice, tissues were diced into proper size (1 mm^3) and fixed by immersion in 3% buffered glutaraldehyde (Sodium cacodylate buffer, pH 7.2) for 10-12 hrs at 4°C. Tissue specimens were then fixed in 1% Osmium tetraoxide (OsO4) in cacodylate buffer (pH 7.2) for 90mins at 30°C. Dehydration of the fixed tissue was performed using ascending grades of ethanol and the tissues were subsequently transferred to epoxy resin via propylene oxide. After impregnation with the pure resin tissue specimens were embedded in the same resin mixture. Semi-thin sections (300 nm thickness) were prepared for the purpose of tissue orientation and stained with toluidine blue. Accordingly, ultra-thin sections (60-70 nm) were cut on an ultra-microtome (Leica Ultracut UCT, Tokyo, Japan) with a diamond knife and stained with uranyl acetate and lead citrate. Stained sections were observed under the TEM (JEOL 1100, Japan) operating at 80 kV.

Data analysis

Data were represented as mean ± standard deviation (S.D.) Variables were appropriately

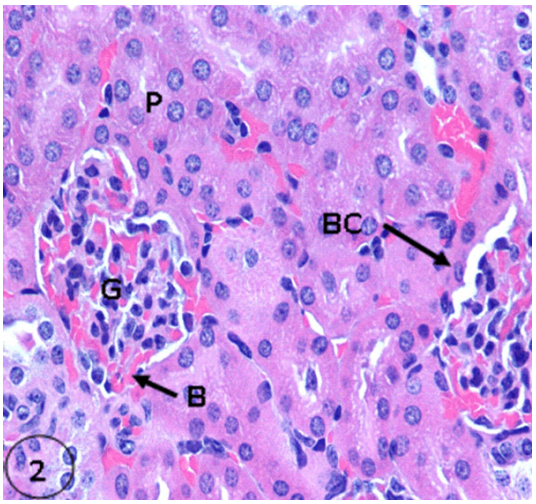

Figure 2. There was recovery in the structure treated with calcitriol. The structure looks like normal in its appearance (H&E 40×).

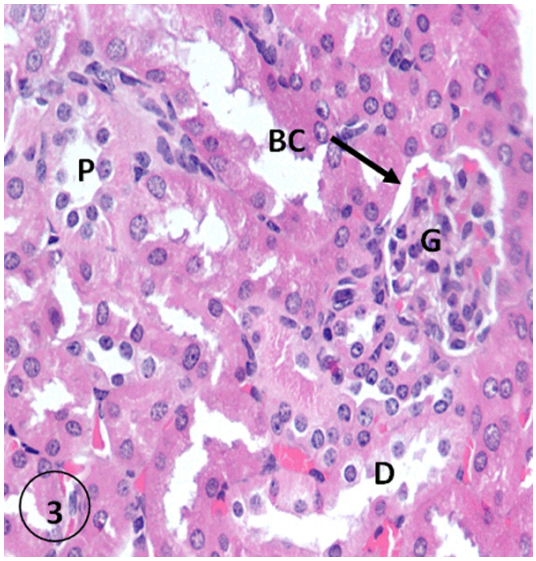

Figure 3. Dilation in Bowman's capsule was clear, with large disturbance in proximal (P) and distal (D) tubules. There was mesangial glomerular hypercellularity and lymphocytes infiltration in the glomerulus region. We also noticed dilation of blood capillaries (B) and mild degeneration of cells (H&E 40×).

either log transformed or square root transformed. Analysis of variance (ANOVA) was performed to assess differences across groups followed by Tukey's HSD or Bonferroni post hoc test. Non-Gaussian variables were compared using nonparametric Kruskal-Wallis H and Mann-Whitney U-test. Significance was set at P0.05, and all statistical analyses were carried

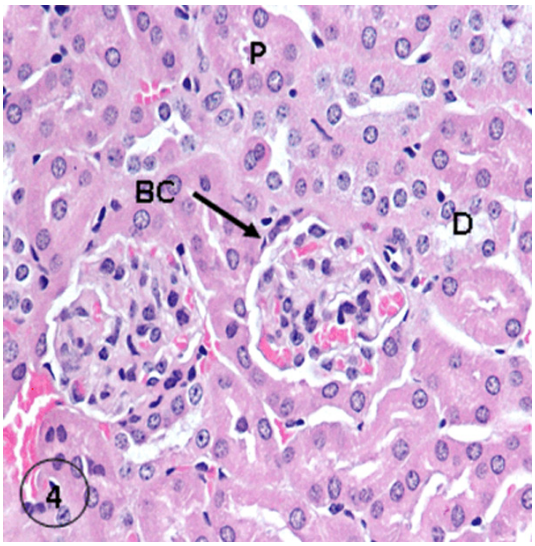

Figure 4. There was a near complete recovery in the structure induced with calcitriol. The structure appears normal in morphology (H&E 40×).

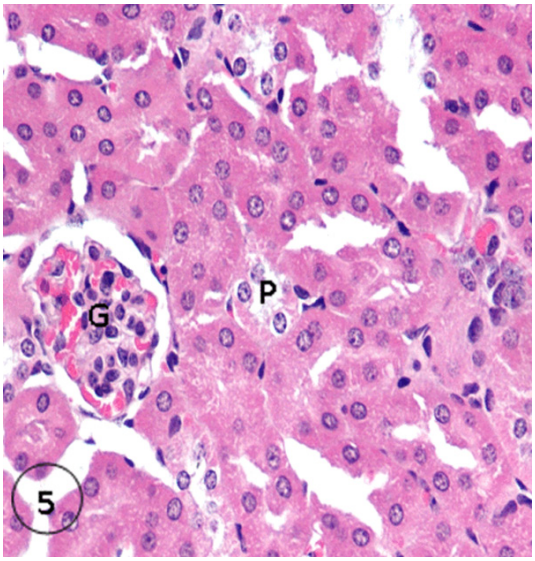

Figure 5. Photomicrographs of healthy mice showing normal glomerulus with other structure at renal cortex site (H&E 40×).

out using SPSS for Windows (version 16.0, Chicago, IL, USA).

Results

In this study, minor histopathological changes were observed in LFD mice (**Figure 1**). In EM (electron microscope) studies the nucleus was slightly indented with few lysosomal bodies, degenerating vacuoles and disintegrating

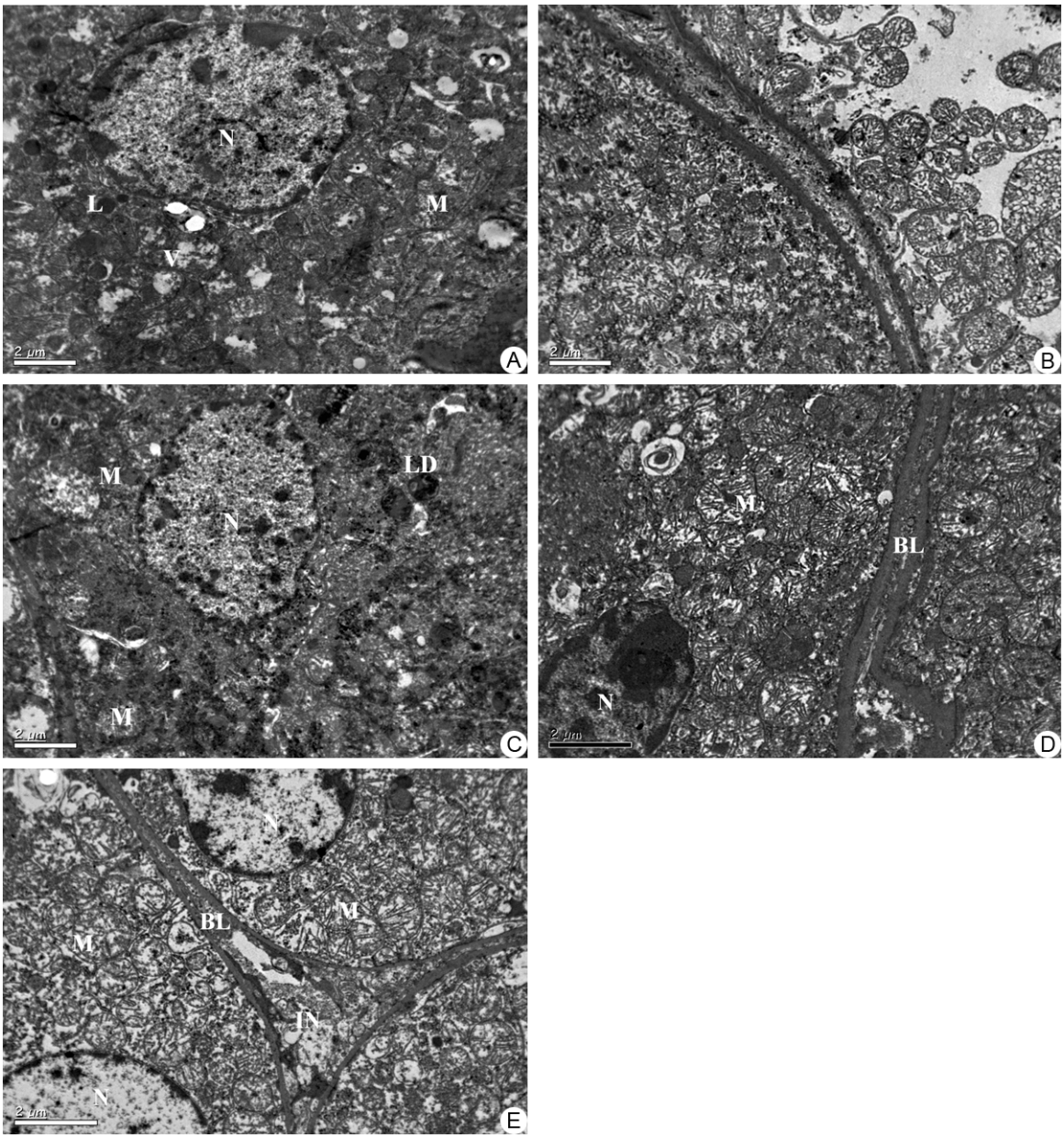

Figure 6. A. An intended nucleus with its irregular membrane, vacuoles (V), lysosomes (L), swollen mitochondria (M) with damaged cristae was seen throughout the region. Large debris was accumulated in the cell region (6000×). B. Mitochondria (M) were normal in the cells and the cells were regaining their structure to calcitriol treatment. Basal lamina appears normal (6000×). C. Damaged mitochondria (M), more lysosomal activity (L) were seen in the region. The structure became blur and mushy with more fat droplets (FD). Many cell organelles were on degenerative stages (6000×). D. Mitochondria (M) were big in size and appear normal in the region. The cells were normal with no sign of degenerative structure, they were responding to calcitriol treatment. Basal lamina appears normal with other structures (6000×). E. Photo showing control kidney tubules with normal mitochondria (M), nucleus (N) with normal basal lamina and interstitial cells (6000×). BL-Basal Lamina; N-Nucleus; L-Lysosomes; V-Vacuoles; M-Mitiochondria; IN-Interstitial Cells; LD-Lipid Drop lets.

mitochondrion (**Figure 6A**). Significant changes were developed in the corticomedullary region of mice fed with HFD. In this group, the glomerulus has dilated, including glomerular blood vessels, exfoliation and shedding of proximal tubular cells into the tubular lumen. Furthermore, in EM studies the mitochondria have significantly degenerated, an interstitial inflammation was evident by abundant interstitial debris. The cellular damage was accompanied

Table 1. Glomerulus area and glomerulus diameter in C57BL/6J mice of treatment and control group (Mean ± SD)

Group	Glomerulus diameter (μm)	Glomerulus area (μm^2)
LFD	61.38±13.01	77.41±21.18
LFD+Calcitriol	60.21±10.12	77.33±17.12
HFD	62.19±17.43	78.45±23.16
HFD+Calcitriol	63.25±19.14	78.23±22.41
Control	63.22±27.15	78.28±34.17
P-value	0.99	0.98

by tubules (tubule interstitial fibrosis). Additionally, we also observed more fat droplets; vacuole formations, mesangial expansion, glomerular epithelial injury, and cell debris throughout the region (**Figures 3**, **6C**). In LFD and HFD mice kidneys treated with calcitriol, substantial and sustained regenerative renal cell proliferationwas observed, resulting in simple tubular hyperplasia as indicated by a broad increase of cell number and multilayered tubules (**Figures 2**, **6B**), (**Figures 4**, **6D**). Even the mitochondria and nucleus were seen normal with distinct basement membrane and were similar to controls. Sustained cell proliferation was further visualized by EM studies confirming a marked increase of proliferating cells within areas presenting with the highest severity of pathological changes in HFD group than-calcitriol-treated mice (**Figure 6D**). In the non-treated controls all structural integrity is intact (**Figures 5**, **6E**). No significant variances were detected in morphometric analysis across all groups as shown in **Tables 1** and **2** (P>0.05).

Discussion

This study was undertaken to evaluate the effects of calcitriol treatment on kidney ultra-structural and histopathological changes caused by HFD in C57BL/6J mouse model. This mouse strain has especially been used as a human obesity model because it develops obesity, insulin resistance and hyperlipidemia when raised on a high-fat and high-sucrose diet; however, it remains lean if the fat content of the diet is limited [15, 16].

In recent studies, animals receiving HFD and calcitriol had the least positive weight changes than untreated animals [19]. Additionally, it is also observed that calcitriol treatment reduces the levels of various inflammatory markers suchas TNF-α, CRP and IL-6, which could lead to insulin resistance reduction in animals as indicated by lower calculated HOMA-IR values [16].

In the present study, mice fed on HFD exhibited more pathological alterations than the LFD group. However, in LFD and HFD treated with calcitriol groups, the kidneys demonstrated a massive and sustained regenerative renal cell proliferation. Under normal conditions, tubular regeneration serves to restore the loss of damaged cells by a transient increase in cell proliferation. Similar changes at the molecular level were also reported for the liver and the skeletal muscle tissues of mice fed on HFD and were diminished by calcitriol treatments [17]. Further, we also observed that calcitriol reinstated mitochondrial biogenesis and oxidative metabolism in the skeletal muscles caused by HFD [17]. Therefore, the influence of calcitriol on the kidney and liver structural abnormalities under HFD-fed conditions may underline its role as an organ-protective agent as published in earlier studies [17].

The mechanism of these effects is perhaps related to calcitriol's ability to reduce cytokine release. The intracellular Toll-like Receptors (TLRs) are differentially regulated by vitamin D, with TLR9 being down-regulated. This decrease in TLR9 expression in monocytes has a downstream functional effect as these cells subsequently secrete less IL6 in response to TLR9 challenge [18]. Worth noting is that calcitriol decreases serum PTH levels through direct and indirect suppressive effects and prevents abnormal growth of the parathyroid glands which may contribute to its anti-inflammatory action and tissue protective effect [9]. Further, the exogenous administration of calcitriol can modulate both the classical endocrine renal 1-α-hydroxylase vitamin D pathway and the autocrine intracellular 1-α-hydroxylase pathway through which vitamin D has been shown to function [19].

Obesity has been associated with increased risk of kidney diseases including cancer. However, the mechanism(s) remain largely unknown [20, 21]. In an earlier study [22], diet-induced obesity (DIO) was found to promote the earliest stages of renal carcinogenesis. Using chow-fed lean rats and two groups of HFD fed

Table 2. Outer and inner proximal tubule diameter, outer and inner and distal tubule diameter in C57BL/6J mice of treatment and control group (micrometer) (Mean ± SD)

Group	Inner distal tubule diameter (μm)	Outer distal tubule diameter (μm)	Inner proximal tubule diameter (μm)	Outer proximal tubule diameter (μm)
LFD	13.43±31.21	23.33±14.17	13.75±22.14	41.18±28.32
LFD+Calcitriol	12.51±21.28	23.17±21.14	13.86±26.12	41.32±27.14
HFD	13.45±29.14	24.41±19.24	13.82±27.26	40.28±22.16
HFD+Calcitriol	13.26±20.19	23.40±21.11	13.80±28.65	40.55±24.31
Control	13.23±18.13	24.30±21.16	13.79±25.23	41.43±29.27
P-value	0.99	0.98	0.99	0.99

rats with either pronounced sensitivity to develop full or partial resistance to DIO, a correlation between body adiposity and the severity of kidney pathology was demonstrated. DIO rats had increased renal inflammation and a higher incidence of nephropathies and pre-neoplastic lesions. Those earlier findings also support the current study as similar morphological changes were observed in C57BL/6J mice fed on HFD. Additional reports have also clearly associated renal lipid accumulation with the pathogenesis of CKD in animal models of DIO and diabetes [22-25]. These changes were on par with our findings where lipid depositions in the kidney were a serious pathological change at the histological and as well as the ultrastructural levels of the HFD group. In contrast, the lipid droplets were absent or in a degenerative stage in the calcitriol treatment groups. To our best knowledge the current work is the first to determine a causal relationship between calcitriol intake and structural changes in renal tubules under HFD conditions.

Conclusion

In summary, HFD induces histopathological changes in C57BL/6J mice kidney, whereas these changes are ameliorated by calcitriol treatment. Further studies are underway to explore the exact molecular mechanisms at target cells.

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Disclosure of conflict of interest

None.

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