Bull. Egypt. Soc. Physiol. Sci. Vol. (42), Issue (1), 101-117

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

The possible ameliorative effect of vitamin D3 and/or omega-3 fatty acids in a rat model of type I early diabetic nephropathy: A physiological and histological study

Nahed H. El-Sokkary[1], Alaaeldin Yusuf[1], Rehab A. Abdel-Moneim[2], Hanan Salah Abdulrazeg[1], Passainte S. Hassaan [1*]

[1] Department of Medical Physiology, Faculty of Medicine, University of Alexandria, Alexandria, Egypt
[2] Department of Medical Histology and Cell Biology, Faculty of Medicine, University of Alexandria, Alexandria, Egypt

Submit Date: 7 August 2021
Revise Date: 20 Spet 2021
Accept Date : 3 Oct 2021

Keywords

- Diabetic nephropathy
- vitamin D
- omega-3 fatty acids
- nephrin

Abstract

Diabetic nephropathy (DN) is one of the most common causes of chronic kidney diseases (CKDs) that have been associated with high morbidity and mortality. Recently, several studies have highlighted the protective role of vitamin D3 (VD3) and omega-3 fatty acids (O3-FAs) in CKDs. However, their effect on DN is still unclear. **Aims:** This study aimed to evaluate the effects of both VD3 and / or O3-FAs in slowing the progression of DN through their impact on indices of renal function, glycemic control, oxidative stress and markers of podocyte injury. **Materials and methods:** type I diabetes was induced in *albino* rats by single intraperitoneal injection of streptozotocin (65mg/kg). Rats received daily oral administration of VD3 and O3-FAs separately and in combination for 6 weeks. **Results:** VD3 and O3-FAs therapy improved significantly hyperglycemia, renal function tests, with concomitant decrease in total urinary protein content, urinary nephrin, a marker of podocyte injury and renal oxidative stress. However, the combined therapy was superior in its effect over VD3 and O3-FAs separately. Such results were confirmed by renal cortical tissue assessment using light microscopic examination of H&E and PAS stains in addition to transmission-electron microscopy. **Conclusions:** VD3 and O3-FAs have a potential beneficial effect on amelioration of structural changes in pedicels and glomerular basement membrane that was reflected as evident renal functional restoration.

Corresponding author: Passainte S. Hassaan , MD , Department of Medical Physiology, Faculty of Medicine, University of Alexandria E.mail: Passainte.saber@alexmed.edu.eg . Passaintehassaan@gmail.com. Tel. 01222271414

INTRODUCTION

Diabetic kidney disease **(**DKD), previously known as diabetic nephropathy (DN), is one of the most serious microvascular complications of diabetes mellitus (DM) with significant morbidity and mortality (1) . It takes place in 20% to 40% of all diabetic patients (2). DKD is defined as DM with albuminuria, impaired estimated glomerular filtration rate (GFR < 60 mL / min/ 1.73 m^2), or both (3). Some researches on DN have mainly focused on role of podocytes' pedicels in maintaining glomerular basement membrane (GBM) selectivity (4). Interestingly in DN, podocytes lose the integrity of their slit diaphragm, hence leading to disruption of the filtration barrier. Nephrin is a trans-membrane protein of the slit diaphragm that regulates the passage of plasma proteins into Bowman's space (5). Decreased nephrin usually leads to disruption of the glomerular filtration barrier integrity and its excretion could be an early indicator of podocyte injury, even before the onset of albuminuria (6). Hyperglycemia is the major risk factor for DN through the formation of advanced glycation end products that promote the overproduction of reactive oxygen species (ROS) (7).

Despite the available present therapies that can delay the onset of DN, numerous patients continue to suffer from progressive renal damage. So, So, it is important to discover new therapeutic regimens that could limit the progression of DN either by attenuating the podocyte injury or oxidative stress (8). The presence of Vitamin D3 receptors (VD3R) in pancreatic tissue raises the importance of VD3 in regulation of insulin synthesis and secretion (9). It causes inhibition of profibrotic growth factors and inflammatory cytokines and suppression of the renin angiotensin system (RAS) system (10). In addition, VD3 has been reported to reduce proteinuria in diabetic patients and attenuate oxidative stress by up-regulating the expression of genes encoding antioxidant enzymes (11).

Additionally, Omega-3 fatty acids (O3-FAs) are important to enable normal growth, and to reduce blood triglyceride levels (12). Their administration improves also insulin sensitivity (13) and has favorable effects on inflammation and oxidative stress (14). So far, no previous study has reported the synergistic effect of VD3 and O3-FAs in treatment of DN. Therefore, the present work aims to evaluate the effects of VD3 and O3-FAs separately and in combination, to limit hyperglycemia, impaired renal functions, oxidative stress and urinary nephrin excretion as a marker of early podocyte injury, which imparts novelty to the current study.

Materials and methods

Ethics statement

The protocol of this study was approved by the Ethics Committee of the Faculty of Medicine, Alexandria University constituted and operating according to ICH GCP guidelines. All applicable international, national and/or institutional guidelines for the care, use and sacrifice of animals were followed. Also, all performed procedures involving animals were according to the ethical standards of Alexandria University, Egypt. Serial no. 0105966 – 18/04/2019. (IRB NO: 00012098 – FWA NO: 00018699).

Experimental Animals

All experimental procedures including animal sacrifice in this study were approved by the Ethics Committee of the faculty of Medicine, Alexandria University. The study was conducted on 50 adult male albino rats weighing 200-250g supplied by the animal house of Medical Physiology Department. Rats were maintained in clean polypropylene cages, at room temperature 22-25°C, with free access to standard laboratory chow and tap water throughout the experiment with 12 hours light-dark cycle.

The rats were randomly distributed into five groups according to different treatment (n=10 rats per group); i. Control non-diabetic group (control group) that received 0.01 mmol/L Sodium Citrate buffer at pH=4.5 intraperitoneally (IP) ii: Diabetic non-treated group (DM group), iii. DM group treated with VD3 (VD3 group) in a dose of 0.2µg/kg cholecaliciferol (each 1ml contains 2800IU equivalent to 70µg of Vitamin D3), Cholecaliciferol (2.5 µg) was dissolved in 10 ml of corn oil (vehicle), (11) iv. DM group treated with O3-FAs (O3-FAs group) in a dose of 400mg/Kg omega-3 fatty acids 50% supplied as fish oil supplement (each 1000mg contains 300mg EPA and 200mg DHA). (15), v. DM group co-treated with VD3 and O3-FAs (VD3+O3-FAs group) in the same previous doses. VD3 and O3-FAs were administered via intragastric gavage once daily immediately after meal for 6 weeks.

DM was induced by a single IP injection of freshly prepared streptozotocin (STZ) (65mg/kg, Sigma, Chemicals, St. Louis, MO, USA), dissolved in 0.01 mmol/L Sodium Citrate buffer at pH=4.5. 72 hours after STZ injection, fasting blood glucose was assessed by tail vein prick using glucose-oxidase reagent strips (One Touch Ultra, Johnson & Johnson®, New Brunswick, NJ, USA). Only rats showing blood glucose level more than 250 mg/dl were considered diabetics and included in the study. Rats with less than that level were replaced by others.

At the end of the 6th week, 24-hours urine samples were collected into separate metabolic cages. Then rats were weighed, anaesthetized with ketamine 100 mg/kg and xylazine 5mg/kg IP, and blood samples were collected from retro-orbital vein into heparinized collecting tubes. Rats were sacrificed and kidney weights were measured.

Samples' preparation and Biochemical analysis

Blood samples:

Blood samples were centrifuged at 3000 rpm for 10 min and plasma samples were stored at -80˚C until assayed for measuring i. Glucose concentrations using glucose oxidase (GOD)-peroxidase (POD) method, ii. Serum urea by Berthelot enzymatic colorimetric method (D-P international), iii. Serum creatinine by Jaffe Colorimetric kinetic method (D-P International).

24-hours urine samples:

24-hours urine samples were centrifuged at 1400 rpm for 5 min and the supernatant was stored at -80˚C until assayed for measuring 24h total protein by colorimetric assay using endpoint pyrogallol-red molybdate method (16), and nephrin level using Rat Nephrin ELISA Kit (Cat.no; E-01092Ra) (Cloud Clone-Corp, USA) according to the manufacturer's instructions (6).

Kidney tissues:

Kidney tissues: one kidney from all rats was dissected and washed with isotonic saline. About 0.1 gram from each kidney was homogenized in 1 ml 0.1M phosphate buffered

saline (PBS) at pH 7.4 and centrifuged at 12000g for 10 min at 4°C. The supernatant was stored at -80˚C until assayed for measuring the level of oxidative stress parameters including malondialdehyde (MDA), and glutathione peroxidase (GPX) using calorimetric kits (Bio-Diagnostic, Egypt).

Histological and histochemical assessment:

The other kidney samples from all rats were dissected and then subdivided into three parts. One part was fixed in 10% neutral-buffered formalin and a second part was fixed in absolute alcohol, then each part was processed to get 6 µm thick paraffin sections for Hematoxylin and Eosin (H&E) staining and Periodic Acid Schiff (PAS) reaction, respectively (17). These sections were examined by the light microscope (Olympus BX41) equipped with spot digital camera (Olympus DP20, Tokyo, Japan) in the Center of Excellence for Research in Regenerative Medicine and its Application (CERRMA), Alexandria Faculty of Medicine. The H&E stained sections were examined to assess histological changes in the renal corpuscles and cortical proximal and distal tubules. PAS stained sections were examined to assess glomerular basement membrane (GBM) thickening.

The third part was further cut into small pieces (1/2- 1 mm^3) and immediately fixed in 3% phosphate-buffered glutaraldehyde solution and processed to get ultrathin sections for transmission electron microscopic examination (TEM) (JEM-100 CX Electron Microscope, JEOL, Japan) (18). Electron microscopic examination was used to assess GBM and pedicels.

Histomorphometric analysis:

Histomorphometric analysis was done using NIH Fiji© program (NIH, USA), where the diameters of glomeruli and the PAS stained area percentage were measured in twenty randomly selected renal corpuscles from six different rats per group, in images captured from H&E and PAS stained sections, respectively, at magnification x200 to assess mesangial expansion and GBM thickening, respectively.

Statistical Analysis:

The results were represented as mean ± SD. Differences between groups were compared using one-way ANOVA followed by Dunnett's post hoc test (version 20.0 software, SPSS Inc.). Differences with a $P<0.001$ were considered to be statistically significant.

Results

Biochemical results

Effect on body and kidney weights (g)

. Diabetes was associated with reduced body weight when compared with the control rats. Changes in initial and final body weight of normal control and diabetic groups are shown in Table 1. There was also significant weight change between the final and initial weight in each group. In DM group, the % loss in body weight was 39.74%. Treatment with VD3 and O3-FAs reduced the % of weight loss to be 22.06% and 28.47% respectively. In the combined group, treatment with both supplements appeared to protect diabetic rats from marked weight loss which was only 8.37%.

The ratio of kidney to body weight shows the same findings detected with body weight changes. This ratio was 200% increase in diabetic rats compared to normal control ones. It decreased

to be 95% increase in VD3 group, 120% increase in O3-FAs group and 48% increase in the combined group compared to the normal control group (P< 0.001) (Table 1).

Effect on plasma glucose levels (mg/dl)

At the end of 6 weeks, plasma glucose level was significantly higher in diabetic rats as compared to normal rats, where it was ~~2.7 folds~~ 270% more.

Treatment of diabetic rats with VD3, O3-FAs separately and in combination attenuated this hyperglycemia. The elevation in plasma glucose level was only 99%, 127% and 73% in these groups respectively, as compared to normal control one (P< 0.001) (Table 1).

Effect on kidney function tests (mg/dl)

DM group showed an increase in serum levels of urea and creatinine by 130% and 86% respectively in relation to normal control group (P< 0.001). Improvement of kidney function tests was observed in all diabetic treated rats with different degrees. With VD3, the increase in serum urea and creatinine was only 65% and 37%. Meanwhile, O3-FAs increased serum urea and creatinine to 70% and 47% respectively. The combination therapy caused an increase of only 34% in serum urea and 28% in serum creatinine. (P< 0.001) (Table 1)

Table 1: Changes in body weight, % change in body weight, kidney weight, kidney/body weight ratio, blood glucose and kidney function tests in the studied groups

	Control	DM	VD3	O3-FAs	VD3+O3-FAs
Initial Body weight (g)	231.9 ± 15.72	237.0±10.94	222.90±15.22	233.40±9.23	230.8±10.67
Final body weight (g)	238.0±14.58	143.0 ±11.52 $^{\#*}$	174.0± 19.62$^{\#*}$	167.2 ± 17.03$^{\#*}$	211.6 ± 13.30$^{\#*}$
Body weight change	6.10 ± 2.47	-94.0 ± 3.59*	-48.90 ± 10.40*	-66.20 ± 11.17*	-19.20 ± 4.21*
% change in body weight	2.68 ±1.12	-39.74 ±2.35*	-22.06 ±4.83*	-28.47 ±5.24*	-8.37 ±1.99*
Kidney weight (g)	0.53 ± 0.05	0.95 ± 0.07*	0.75 ± 0.03*	0.83 ± 0.04*	0.69 ± 0.05*
Kidney weight/ body weight (%)	0.22 ± 0.03	0.66 ± 0.06*	0.43 ± 0.02*	0.49 ± 0.02*	0.32 ± 0.03*
Fasting blood glucose (mg/dl)	90.70 ± 5.98	340.0 ± 11.69*	180.9± 6.69*	206.5±25.81*	157.0±10.55*
Blood urea (mg/dl)	34.10 ± 2.18	79.80 ± 3.16*	56.30 ± 2.11*	57.90 ± 1.60*	45.60 ± 1.90*
Serum creatinine (mg/dl)	0.57 ± 0.06	1.06 ± 0.09*	0.78 ± 0.05*	0.84 ± 0.02*	0.73± 0.06*

Data is represented as mean ± SD (n=10).

* Significantly different from normal control rats (P< 0.001).

Effect on total urinary protein content (mg/24h)

Total urinary protein increased significantly in all diabetic rats versus the normal control ones. The increase in diabetic untreated rats was 370%. Meanwhile, treatment with VD3, O3-FAs and both drugs together limited this increase to 65%, 120% and 22% respectively (P< 0.001) (Fig 1A).

Effect on urinary nephrin (ng/ml)

At the end of the experimental period, the level of urinary nephrin increased significantly in DM group by 49% as compared to normal control one. In the diabetic treated groups, the level of nephrin in urine was significantly improved (P< 0.001). It decreased to be only 17% and 24% increase in VD3 and O3-FAs groups respectively, as compared to control level. However, no significant difference was observed between the

combined group and the normal control one (Fig 1B).

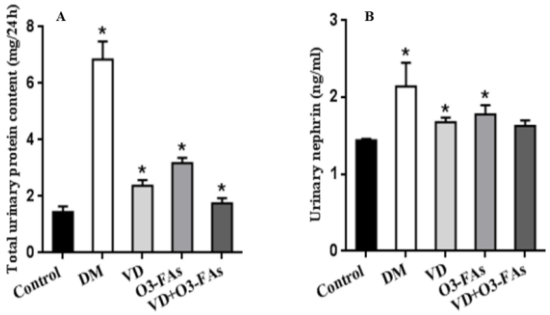

Fig 1. Effect of VD3 and O3-FAs on: A- total urinary protein content (mg/24h) and B- urinary nephrin (ng/ml). * Significant versus control group (P< 0.001).

Correlation between different parameters of the study

Pearson correlation analysis revealed significant positive correlations between the mean levels of urinary nephrin with plasma glucose level (r=0.776), with serum urea and creatinine where r=0.788 and r=0.756 respectively and finally with total urinary protein content (r=0.784), at P< 0.001).

Effect on renal oxidative stress parameters

The diabetic state caused a profound imbalance between oxidants presented by MDA as a marker of lipid peroxidation and antioxidants presented by GPX as one of the most important scavengers against oxidative damage. In comparison to the normal control group, MDA was increased by 220% in DM group, 68% in VD3 group, 89% in O3-FAs group and 41% in the combined group. By contrast, GPX was significantly reduced by 190% in diabetic untreated group as compared to normal control one.

Treatment with VD3, O3-FAs separately and in combination improved the reduction in GPX to be less only by 47% in VD3 group, 95% in O3-FAs group and 14% in the combined group versus the normal control one (P< 0.001) (Fig 2).

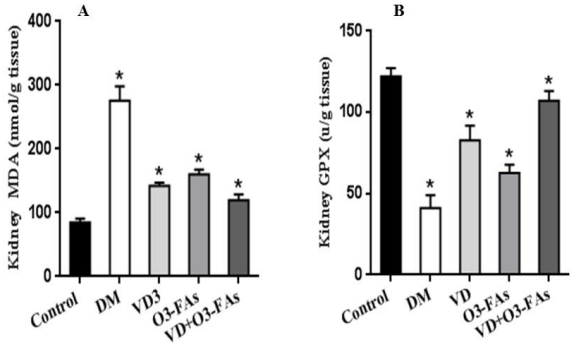

Fig 2. Effect of treatment with VD3 and O3-FAs on oxidative stress markers (MDA and GPX). *Significant versus control group (P< 0.001). Abbreviations: MDA: malondialdehyde, GPX: glutathione peroxidase.

Histological results

Light microscopic results

H&E results

Examination of kidney cortex from H&E stained sections of control group (CG), revealed normal structure of different cortical components. Renal corpuscles appeared formed of looped

glomerular blood capillaries and mesangium. Well defined Bowman's capsule was seen with narrow Bowman's space. Proximal convoluted tubules appeared lined by few pyramidal cells that exhibited acidophilic cytoplasm, euchromatic nuclei and prominent apical brush border. Distal tubules appeared lined by cuboidal cells with less acidophilic cytoplasm and vesicular nuclei (Fig 3A).

Examination of renal cortex of rats of DM group revealed marked changes. Some glomeruli exhibited apparent mesangial expansion, in comparison to glomeruli in control rats. Other glomeruli revealed splitting between their capillaries. Proximal and distal tubular cells exhibited variable degenerative changes in the form of hydropic swelling or extensive cytoplasmic vacuolization. Some other tubules depicted cell lysis and attenuation with widening of tubular lumina. Some nuclei appeared dark, shrunken and extruded into tubular lumen. (Fig 3B-D).

Limited histological improvement was noticed in renal cortex of rats in VD3 group, where some glomeruli still exhibited mesangial expansion. Some proximal and distal tubules appeared almost normal, while other tubular cells revealed degenerative changes. (Fig 3E and 3F).

On the other hand, extensive degenerative changes were still observed in O3-FAs' sections. The glomeruli exhibited mesangial expansion and glomerular capillaries splitting (Fig 4A and 4B). Marked histological improvement of kidney cortical tissue was evident in rats of combined treatment group. Renal corpuscles exhibited limited splitting of glomerular capillaries. Most of the proximal and distal tubules appeared with nearly classical histological features. (Fig 4C and 4D).

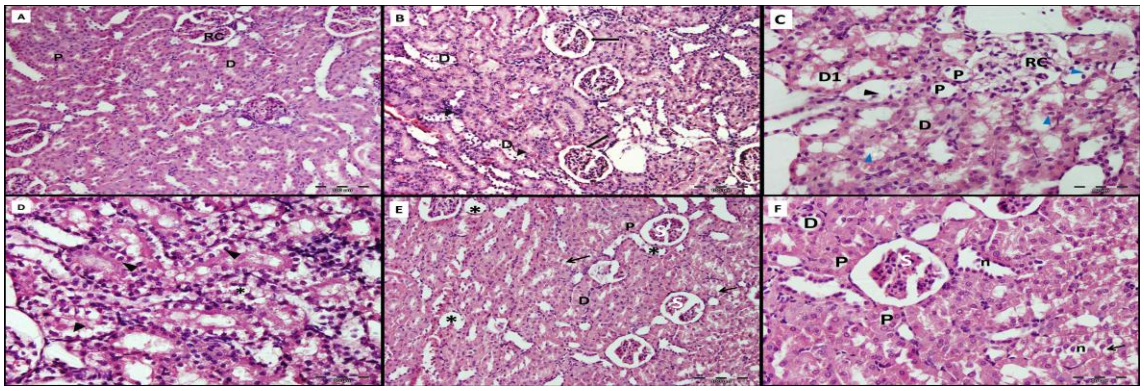

Fig 3. Representative H&E light microscopic images of the control & diabetic groups. A (CG); Normal histological structure of kidney cortex is observed. Proximal tubules (P) appear rounded with narrow lumen, distal tubules (D) appear with a wider lumen. Both appear lined by eosinophilic cuboidal epithelium. RC; renal corpuscle. B-D (diabetic groups). B; Splitting of capillaries in some renal corpuscles (arrows). B & C; Some distal tubules (D) show cytoplasmic vacuolization (blue arrowheads) with extrusion of dark nuclei (black arrowheads). C; Cellular attenuation in proximal (P) & distal (D1) tubules, mesangial expansion of (RC). D; Some tubular cells reveal hydropic swelling with vacuolated cytoplasm (arrowheads). Extruded dark nuclei (asterisk) are seen. E & F (VD3 group). E & F; Renal corpuscles exhibit glomerular capillaries splitting (S). Some proximal (P) & distal (D) tubules appear almost normal. Some other distal tubular cells show cytoplasmic vacuolization (arrows) with extrusion of dark shrunken nuclei (n) E; Some renal tubules appear with cellular attenuation (asterisk).
A, B & E; magnification X 200, C, D & F; magnification X 400.

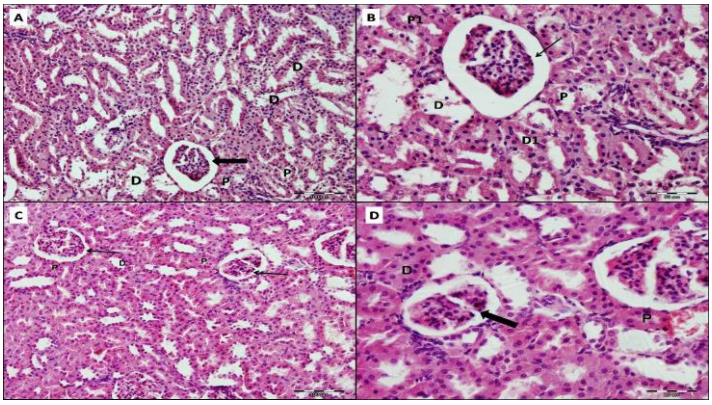

Fig 4. Representative H&E light microscopic images of O3-FAs and combined groups. A & B (O3-FAs group); Some distal (D) & proximal tubules (P) show extensive cytoplasmic vacuolization with extrusion of dark shrunken nuclei. Few distal (D1) & proximal (P1) tubules are apparently normal. Some renal corpuscles (arrows) show mesangial expansion & glomerular capillaries splitting. C & D (combined group); Most of proximal (P) & distal (D) tubules are observed with nearly normal structure. Few renal corpuscles (arrows) show limited glomerular capillaries splitting.
A & C; magnification X 200, B & D; magnification X 400.

PAS results

Normal pattern of reaction with PAS was observed in the kidney cortex of control group, where PAS positive reaction was seen as dark pink color delineating the basement membranes of renal tubules and the brush border of proximal tubules, as well as defining the GBM.

Apparent increased reaction with PAS was noticed in DM group, however, the intensity of PAS reaction was apparently decreased in VD3, O3-FAs and the combined groups (Fig 5A-E).

Histomorphometric analysis

PAS area percent:

PAS stained area percentage increased significantly in DM group, 120% when compared to normal control one. Treatment with vitamin D3 as well as O3-FAs decreased PAS area percentage to be only 32% and 53% (P <0.001) respectively, as compared to the control group.. The combination therapy decreased PAS stained area percentage to become finally insignificant from normal control group (Fig 5F) and (Table 2).

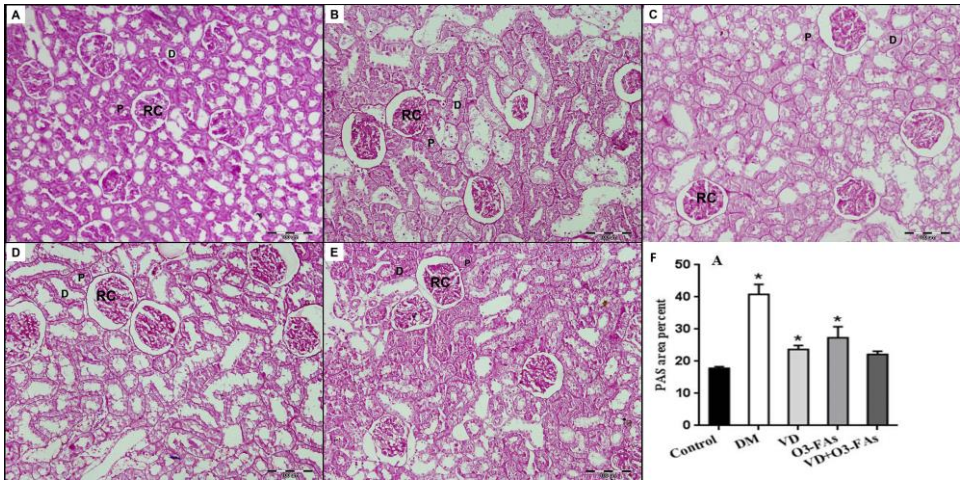

Fig 5. Representative PAS stained light microscope images. A (CG); normal pattern of reaction is observed. B (DM); Apparent intense reaction is noticed in renal corpuscles (RC) of diabetic untreated rats. C (VD3), D (O3-FAs) & E (combined group);

Apparently decreased reaction is observed within renal corpuscles (RC) in all diabetic-treated groups being significantly decreased in E. F; P; proximal tubules, D; distal tubules. Graphical presentation of PAS stained area percentage in different groups compared to normal control one. Data are presented as means ± SD using one-way ANOVA test (n=4). * Significant versus control group (P< 0.001). A-E; magnification X 200.

Glomerular diameter

Glomerular diameter was used to assess the degree of mesangial expansion. Glomerular diameter increased significantly by 80% in DM group as compared to normal control one. However, in O3-FAs group, this increase became only 38% (P <0.001). No statistical difference was present between both VD3 and the combined groups with the normal control one denoting the restoration of glomerular diameter (Table 2).

Table 2: Histomorphometric analysis concerning PAS area percentage and measurement of glomerular diameter by H&E stain in the studied groups

Histological morphometric analysis	Control	DM	VD3	O3-FAs	VD3+O3-FAs
PAS area percentage	17.84 ± 0.47	40.80 ± 3.19*	23.68 ± 1.28*	27.31 ± 3.45*	22.15 ± 0.99
Measurement of glomerular diameter (H & E stain)	56.14 ± 6.56	101.45 ± 0.61*	70.13 ± 2.62*	77.64 ± 3.28*	62.61 ± 2.03

Data is represented as mean ± SD (n=10).
* Significantly different from normal control rats (P< 0.001).

Electron microscopic results

In the control group, GBM appeared with well-defined filtration slits between the interdigitating pedicels of podocytes. Fenestrated endothelium was seen on the inner side of GBM (Fig 6A and 6B). However, in DM group, marked thickening of GBM was observed together with evident effacement of the pedicels, where they exhibited flattened appearance and were broadly applied to GBM. In addition, interdigitation of pedicels was less frequently encountered (Fig 6C and 6D).

VD3 group revealed partial improvement, where apparent decreased thickening of GBM was noticed in comparison to DM group while some pedicels still depicted effacement (Fig 6E and 6F). O3-FAs group exhibited limited improvement, where apparent thickening of GBM and effacement of pedicels were frequently observed (Fig 7A and 7B). Evident improvement was mostly noticed in the combined treatment group, where GBM thickness was apparently decreased in comparison to DM group, as well as VD3 and O3-FAs groups. In addition, regularly arranged, interdigitating pedicels on GBM were frequently seen (Fig 7C and 7D).

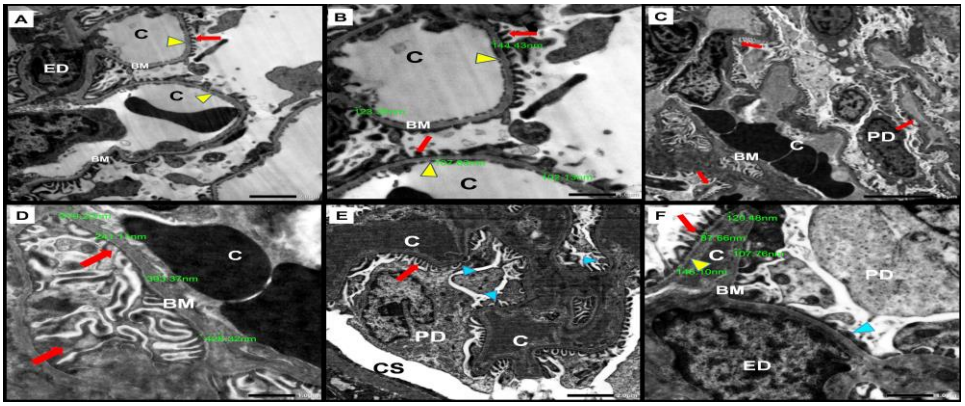

Fig 6. Representative transmission electron microscope (TEM) photomicrographs. A & B (control group); Glomerular blood capillaries (C) lined by endothelium (ED). Pedicels (red arrow) appear regularly applied to the glomerular basement membrane (BM). Fenestrated endothelium at yellow arrow heads. A; magnification x2500, B; x5000. C & D (DM group); Glomerular basement membrane (BM) appears thickened, pedicels (red arrows) display limited interdigitations & apparent effacement. C; magnification x1500, D; x5000. E & F (VD3 group); GBM (BM) is apparently less thickened as compared to diabetic group, some pedicels (red arrows) appear separated by narrow filtration slits, others (blue arrow heads) still show effacement, fenestrated endothelium (yellow arrow heads) is apparent. E; magnification x2500, F; x5000 C; blood capillary, PD; podocyte, CS; Bowman's capsule space, ED; endothelium.

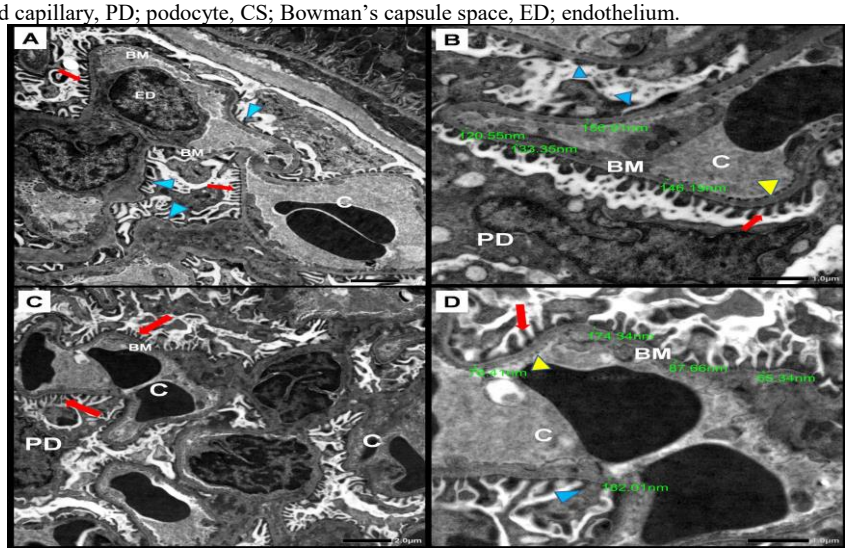

Fig 7. Representative transmission electron microscope (TEM) photomicrographs. A & B (O3-FAs group); Pedicels (blue arrow heads) mostly display effacement, other few pedicels (red arrows) appear regular & separated by narrow filtration slits, GBM (BM) appears moderately thickened. C & D (combined treatment group); GBM (BM) appears less thickened, most of the pedicels appear interdigitating (red arrows) & separated by narrow filtration slits, few pedicels show effacement (blue arrowhead). PD; podocyte, ED; endothelium, C; blood capillary, yellow arrowhead; fenestrated endothelium. A; magnification X 2000, B; X 5000. C; magnification X 2000, D; X 5000.

Discussion

DN is characterized by the presence of abnormally increased quantities of urine albumin excretion, diabetic glomerular lesions, and decreased GFR. Globally, DN is a significant cause of CKD and end-stage renal disease (ESRD).

In the present study, induction of DN was confirmed on biochemical and histological basis. Treatment of rats with VD3 and O3-FAs either separately or in combination caused a significant decrease in fasting blood glucose (FBG) level as compared to DM group without reaching normal level. The most significant effect was noticed by the combined treatment followed by VD3. Similarly, Derakhshanian et al. (19) and El-Sayed et al. (20) found that VD3 supplementation in STZ-induced diabetic rats significantly reduced FBG and facilitated glycemic control. The beneficial effect of VD3 could be referred to the increased sensitivity of insulin receptors. Moreover, VD3 enhances glucose transport and

inhibits fatty acid induced insulin resistance via reduction of c-Jun N-terminal kinases (JNK) activation (21). In addition, the potential effect of O3-FAs supplementation may result from decreased inflammation and subsequently improvement of insulin sensitivity in pre-diabetic rats as reported by Molinar-Toribio et al. (22). Concerning both supplements, their beneficial effect could be attributed to their influence on beta cell functions as shown by Baidal et al (23). They proved that both supplements together resulted in a significant decrease in pancreatic beta-cell inflammatory response leading to preservation of beta-cell mass and inhibition of adaptive and cell-mediated immune response. Combined therapy had also a synergistic effect on glycemic control in islet transplantation (24). In addition, O3-FAs supplementation may result in increased levels of VD3. This may be due to either extrarenal 1α-hydroxylase stimulation and subsequent activation of VD3 or suppression of the enzyme 24-hydroxylase that catabolizes 1,25(OH)2D as reported by An et al (25).

Such results were further supported biochemically, where different renal function parameters came in agreement with blood glucose levels in different groups. Treatment with VD3 and/or O3-FAs caused an improvement in the level of serum urea and creatinine with the most significant ameliorative effect observed in the group treated with both supplements. Concerning O3-FAs, Wong et al. (26) proved that a three months supplementation with fish oil in diabetic patients showed lowered serum creatinine level when compared with controls suggesting a protective role for O3-FAs against renal dysfunction.

The body and kidney weights were affected also by the diabetic state; where there was a significant decrease in body weight and an increase in kidney weight in DM group as compared to normal control one. This resulted in an increased kidney/body weight ratio. In diabetes, loss of body weight could occur as a result of increased muscle wasting secondary to loss of tissue proteins. In addition, the body uses free fatty acid from stored fats instead of using glucose in aerobic glycolysis, [(27)]. Increased kidney weight may be attributed to the effect of some inflammatory cytokines such as TGF-β1, which is a potent fibrogenic factor contributing to glomerular hypertrophy and progressive accumulation of extra cellular matrix (ECM) through promoting fibronectin synthesis and inhibiting ECM-degrading enzymes, ultimately resulting in glomerulosclerosis and increase in kidney weight (28). However, the weight loss and the increase in kidney weight were significantly less observed in diabetic rats treated with VD3 and O3-FAs resulting in a decrease in kidney/body weight ratio. Rats treated by the combined therapy showed the most significant effect. Also, in diabetic patients with DN and hypertriglyceridemia, O3-FAs supplementation improved significantly the micro albuminuria without altering body weight, blood glucose levels, or blood pressure (14). This variation observed concerning the effect of both supplements may be related to different dose given or duration of study.

Light microscopic results of H&E stained sections of different groups, came in accordance with the biochemical results. Herein, diabetic group rats exhibited significant mesangial expansion and GBM thickening in comparison to

control group, as was demonstrated by histomorphometric analysis. These results came in agreement with Ha and Lee (29) who reported an upregulation of ECM proteins by glomerular mesangial cells in response to increased ROS in diabetic kidney, leading to mesangial expansion. Such enhanced effect can also be referred to some factors such as angiotensin II (ANG II), vasoactive endothelial factors and TGF-β that are released in response to the state of induced chronic hyperglycemia. Subsequently, the hemodynamics of glomerular filtration is altered thus exposing mesangial cells to a sort of stress. Hence, resulting in their proliferation and overproduction of mesangial cell matrix, as well as, GBM thickening and podocyte injury that facilitate albumin leakage from the glomerular capillaries (30).

In this context, albuminuria correlates with renal injury in STZ-induced DM (31). In the present study, diabetic rats showed a significantly higher urinary protein excretion in comparison to normal control ones. Although, proteinuria appeared to be significantly lower in VD3 and/or O3-FAs groups as compared to DM group, yet control levels were not restored.

Partial histological improvement was noticed in VD3 and O3-FAs treated groups, where glomerular diameters, as well as, PAS stained area percentage were still significantly increased as compared to normal control group but to a lesser extent than that observed in the diabetic one. However, there was no significant difference between the combined and normal control groups denoting a more potent ameliorative effect for the combined therapy.

Presence of proteinuria suggests damage to the components of the glomerular filtration barrier (32). However, detection of podocyte-specific proteins such as nephrin in urine indicates damage of podocytes (33). Hence, nephrin is considered as an early and more specific marker for diagnosis of glomerular injury in comparison to microalbuminuria (6).

In the present study, urinary nephrin level increased significantly in the diabetic group as compared to the control one. The increase in VD3 and O3-FAs groups was significantly less with more ameliorative effect with VD3. No statistical difference was observed between the combined and normal control groups. In addition, there were positive correlations between levels of urinary nephrin and FBG, urinary protein, serum urea, and creatinine. Zhang et al. (34) found that, protein expression of both nephrin and podocin was significantly reduced in DN rats compared with that in non-DN ones and they were significantly increased by calcitriol treatment.

Electron microscopic results came in agreement with biochemical results of urinary nephrin level, thus further confirming the suggested mechanisms. In DM group, marked thickening of GBM was observed together with evident effacement of the pedicels. As nephrin is an integral protein of pedicels that share in the filtration barrier, any structural derangement to it, disturbs filtration process with subsequent deposition of some molecules in GBM & its thickening. VD3, O3-FAs treated groups revealed partial improvement. However, the combined group showed more evident improvement, where GBM depicted more pronounced decreased thickening with decreased effacement in comparison to the other two groups.

The inverse association between nephrin gene expression and urinary protein excretion signifies nephrin as an entrusting biomarker that has good diagnostic and therapeutic potential (35). Urinary nephrin is excreted in 100% of diabetics with microalbuminuria, as well as in 54% of diabetic patients with normoalbuminuria (36).

Supporting our results, Zhao et al.(37) showed that VD3 supplementation ameliorates proteinuria and protects patients with DN from kidney injury, which was independent of the blood glucose reduction. The renoprotective property of VD3 may be attributed to its regulatory effect on RAS, where intrarenal RAS is increased in diabetes and plays a key role in the development of DN. Moreover, regulation of renin activity or ANG II activity as with VD3 supplementation has been reported to ameliorate DN in both animals and humans (34). Concerning O3-FAs, Han et al.(14), stated that high dose of O3-FAs significantly decreased albuminuria in diabetic patients with DN and hypertriglyceridemia. O3-FAs could inhibit inflammation, by blocking some signaling cascades, such as those downstream of Toll-like receptor4 (TLR4). This signaling pathway ends in ROS generation and expression of proinflammatory proteins including inducible nitric oxide synthase and TNF. In addition, O3-FAs could shift the production of mediators towards the less inflammatory ones like PGE3 or towards those which resolve inflammation like resolvins resulting in attenuation in the expression of markers of tissue inflammation such as interleukin IL-6, MCP-1 and TGF-β implicated in end-organ complications associated with DM [(38)].

As regards the ROS, STZ-induced diabetes caused significant decrease in renal GPX with significant increase in MDA levels in diabetic untreated group. Basically, chronic hyperglycemia induces excessive formation of these reactive species that might diminish antioxidant activity, leading to domination of the oxidative stress. This causes further production of more ROS, resulting subsequently in the development of different pathological conditions in DM and its secondary complications [(39)]. The histological results of the diabetic group came in accordance with such biochemical results, where evident degenerative changes were noticed in proximal and distal cortical tubular cells.. VD3 and O3-FAs administration significantly increased kidney GPX and decrease MDA levels. In VD3 group, amelioration of oxidative stress status was significantly better than in O3-FAs group. Meanwhile, the combined group demonstrated more pronounced ameliorative effect on the oxidative stress state.

Similarly, VD3 supplementation enhanced renal and hepatic activity of GPX, tissue catalase and superoxide dismutase (SOD), and reduced lipid peroxidation when given to diabetic rats as a preventive measure or therapeutic strategy (40). Also, the levels of oxidative stress markers including MDA was reduced in serum. In addition the level of antioxidant defenses including GPX, catalase and SOD activity were increased after administration of paricalcitol given as VD3R activator treatment in hemodialysis patient (41) and STZ-induced diabetic rats (42). Aligned with our results, in an adenine-induced rat chronic renal failure model, O3-FA supplementation reduced MDA and ROS levels in kidney tissue while GPX and SOD levels were increased, indicating that O3-FAs intervention can reduce oxidative stress

products and increase anti-oxidative substances in the lesioned tissues (43). Jamilian et al. (44), also reported that co-supplementation of VD3 and O3-FAs had beneficial effects on GPX and MDA levels on women with gestational DM. These results suggest that treatment with VD3 and/or O3-FAs exerts a renoprotective effect via increasing GPX and reducing MDA.

Light microscopic results of H&E stained sections came in agreement with oxidative stress markers. In VD3 group, some proximal and distal tubular cells appeared almost normal, while few others revealed limited degenerative changes. In O3-FAs group, limited histological improvement was observed. The most evident histological improvement was noticed in the combined group, where most of the proximal and distal tubular cells restored nearly normal features.

Conclusions

The current study revealed that combined treatment using VD3 and O3-FAs have the most potent therapeutic effect in DN as proved by different parameters. Moreover, nephrin ~~assessment proved to~~ is suggested to be an accurate indicator to monitor progression of DN. However, more investigation about nephrin expression and localization in glomerular membrane in relation to the duration and severity of diabetes and the later onset and progression of DN are needed to elucidate its precise role in this disease. This represents a limitation in the search for factors underlying DN and the development of new therapeutic strategies to prevent progressive renal disease in diabetes.

Acknowledgment

The authors wish to thank the University of Alexandria for the unlimited support.

References

1. **Alicic RZ, Rooney MT, Tuttle KR.** Diabetic Kidney Disease: Challenges, Progress, and Possibilities. Clin J Am Soc Nephrol. 2017;12(12):2032-45. doi: 10.2215/CJN.11491116.
2. **Gheith O, Farouk N, Nampoory N, Halim MA, Al-Otaibi T.** Diabetic kidney disease: world wide difference of prevalence and risk factors. J Nephropharmacol. 2016;5(1):49-56.
3. **Macisaac RJ, Ekinci EI, Jerums G.** Markers of and risk factors for the development and progression of diabetic kidney disease. Am J Kidney Dis. 2014;63(2 Suppl 2):S39-62. doi: 10.1053/j.ajkd.2013.10.048.
4. **Anil Kumar P, Welsh GI, Saleem MA, Menon RK.** Molecular and cellular events mediating glomerular podocyte dysfunction and depletion in diabetes mellitus. Front Endocrinol (Lausanne). 2014;5:151. doi: 10.3389/fendo.2014.00151.
5. **Martin CE, Jones N.** Nephrin Signaling in the Podocyte: An Updated View of Signal Regulation at the Slit Diaphragm and Beyond. Front Endocrinol (Lausanne). 2018;9:302. doi: 10.3389/fendo.2018.00302.
6. **Kandasamy Y, Smith R, Lumbers ER, Rudd D.** Nephrin - a biomarker of early glomerular injury. Biomark Res. 2014;2:21. doi: 10.1186/2050-7771-2-21.
7. **Nowotny K, Jung T, Hohn A, Weber D, Grune T.** Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. Biomolecules. 2015;5(1):194-222. doi: 10.3390/biom5010194.

8. **Breyer MD, Susztak K.** The next generation of therapeutics for chronic kidney disease. Nat Rev Drug Discov. 2016;15(8):568-88. doi: 10.1038/nrd.2016.67.
9. Seshadria KG, Tamilselvana B, A R. Role of Vitamin D3 in Diabetes. . Journal of Endocrinology and Metabolism. 2011;1(2):47-56.
10. **Li YC.** Renoprotective effects of vitamin D analogs. Kidney Int. 2010;78(2):134-9. doi: 10.1038/ki.2009.175.
11. **Deng X, Cheng J, Shen M.** Vitamin D improves diabetic nephropathy in rats by inhibiting renin and relieving oxidative stress. J Endocrinol Invest. 2016;39(6):657-66. doi: 10.1007/s40618-015-0414-4.
12. **Chauhan S, Kodali H, Noor J, Ramteke K, Gawai V.** Role of Omega-3 Fatty Acids on Lipid Profile in Diabetic Dyslipidaemia: Single Blind, Randomised Clinical Trial. J Clin Diagn Res. 2017;11(3):OC13-OC6. doi: 10.7860/JCDR/2017/20628.9449.
13. **Lalia AZ, Lanza IR.** Insulin-Sensitizing Effects of Omega-3 Fatty Acids: Lost in Translation? Nutrients. 2016;8(6). doi: 10.3390/nu8060329.
14. **Han E, Yun Y, Kim G, Lee YH, Wang HJ, Lee BW, et al.** Effects of Omega-3 Fatty Acid Supplementation on Diabetic Nephropathy Progression in Patients with Diabetes and Hypertriglyceridemia. PLoS One. 2016;11(5):e0154683. doi: 10.1371/journal.pone.0154683.
15. **Zararsiz I, Sonmez MF, Yilmaz HR, Tas U, Kus I, Kavakli A, et al.** Effects of omega-3 essential fatty acids against formaldehyde-induced nephropathy in rats. Toxicol Ind Health. 2006;22(5):223-9. doi: 10.1191/0748233706th260oa.
16. **Orsonneau JL, Douet P, Massoubre C, Lustenberger P, Bernard S.** An improved pyrogallol red-molybdate method for determining total urinary protein. Clin Chem. 1989;35(11):2233-6.
17. **Drury R, E. W.** Light microscope and slide preparation. Carleton's histological technique. London: Oxford University Press; 1980.
18. **J. B.** Specimen staining and contrast methods for transmission electron microscopy. Electron Microsocopy Principles and Techniques for Biologists. 1992:16-51.
19. **Derakhshanian H, Javanbakht MH, Zarei M, Djalali E, Djalali M.** Vitamin D increases IGF-I and insulin levels in experimental diabetic rats. Growth Horm IGF Res. 2017;36:57-9. doi: 10.1016/j.ghir.2017.09.002.
20. **El-Sayed LA, Tork OM, Seddiek H, Taha RM, NI. G**. Vitamin D3 protects diabetic rats from neuropathic changes by improving insulin sensitivity and upregulating Vitamin D3 receptors. Kasr Al Ainy Med J. 2015; 21(3):115-12.
21. **Eltablawy N, Ashour H, Rashed LA, Hamza WM.** Vitamin D protection from rat diabetic nephropathy is partly mediated through Klotho expression and renin-angiotensin inhibition. Arch Physiol Biochem. 2018;124(5):461-7. doi: 10.1080/13813455.2018.1423624.
22. **Molinar-Toribio E, Perez-Jimenez J, Ramos-Romero S, Romeu M, Giralt M, Taltavull N, et al.** Effect of n-3 PUFA supplementation at different EPA:DHA ratios on the spontaneously hypertensive obese rat model of the metabolic syndrome. Br J Nutr.

2015;113(6):878-87. doi: 10.1017/S0007114514004437.

23. **Baidal DA, Ricordi C, Garcia-Contreras M, Sonnino A, Fabbri A.** Combination high-dose omega-3 fatty acids and high-dose cholecalciferol in new onset type 1 diabetes: a potential role in preservation of beta-cell mass. Eur Rev Med Pharmacol Sci. 2016;20(15):3313-8.
24. **Gurol AO, Okten-Kursun A, Kasapoglu P, Suzergoz F, Kucuksezer UC, Cevik A, et al.** The synergistic effect of omega3 and Vit D3 on glycemia and TNF-alpha in islet transplantation. Cell Mol Biol (Noisy-le-grand). 2016;62(1):90-8.
25. **An WS, Lee SM, Son YK, Kim SE, Kim KH, Han JY, et al.** Omega-3 fatty acid supplementation increases 1,25-dihydroxyVitamin D3 and fetuin-a levels in dialysis patients. Nutr Res. 2012;32:495–502.
26. **Wong CY, Yiu KH, Li SW, Lee S, Tam S, Lau CP, et al.** Fish-oil supplement has neutral effects on vascular and metabolic function but improves renal function in patients with Type 2 diabetes mellitus. Diabet Med. 2010;27(1):54-60. doi: 10.1111/j.1464-5491.2009.02869.x.
27. **El-Hafiz HI, El Batsh MM, Mohamed WMY, Omar AH, Yassin AA, Mansour MA, et al.** Effect of Vitamin D3 on diabetic nephropathy in rats. Menouf Med J. 2018;31(1):324-32.
28. **Tian Y, Lv G, Yang Y, Zhang Y, Yu R, Zhu J, et al.** Effects of vitamin D on renal fibrosis in diabetic nephropathy model rats. Int J Clin Exp Pathol. 2014;7(6):3028-37.
29. **Ha H, Lee HB.** Reactive oxygen species and matrix remodeling in diabetic kidney. J Am Soc Nephrol. 2003;14(8 Suppl 3):S246-9. doi: 10.1097/01.asn.0000077411.98742.54.
30. **Vallon V, Komers R.** Pathophysiology of the diabetic kidney. Compr Physiol. 2011;1(3):1175-232. doi: 10.1002/cphy.c100049.
31. **Garman JH, Mulroney S, Manigrasso M, Flynn E, Maric C.** Omega-3 fatty acid rich diet prevents diabetic renal disease. Am J Physiol Renal Physiol. 2009;296(2):F306-16. doi: 10.1152/ajprenal.90326.2008.
32. **Shahid MA, Baig S, SNN S.** Nephrin: An emerging biomarker for detecting damage of glomerular filtration barrier. Pak J Med Dent 2014;3(4):83-8.
33. **Al-Rubeaan K, Siddiqui K, Al-Ghonaim MA, Youssef AM, Al-Sharqawi AH, D. A.** Assessment of the diagnostic value of different biomarkers in relation to various stages of diabetic nephropathy in type 2 diabetic patients. Sci Rep. 2017;7(1):2684.
34. **Zhang Z, Sun L, Wang Y, Ning G, Minto AW, Kong J, et al.** Renoprotective role of the vitamin D receptor in diabetic nephropathy. Kidney Int. 2008;73(2):163-71. doi: 10.1038/sj.ki.5002572.
35. **Kostovska I, Tosheska-Trajkovska K, Topuzovska S, Cekovska S, Spasovski G, Kostovski O, et al.** Urinary nephrin is earlier, more sensitive and specific marker of diabetic nephropathy than microalbuminuria. J Med Biochem 2020 39(1):83-90.
36. **Patari A, Forsblom C, Havana M, Taipale H, Groop PH, Holthofer H.** Nephrinuria in diabetic nephropathy of type 1 diabetes.

Diabetes. 2003;52(12):2969-74. doi: 10.2337/diabetes.52.12.2969.

37. **Zhao J, Dong J, Wang H, Shang H, Zhang D, Liao L.** Efficacy and safety of vitamin D3 in patients with diabetic nephropathy: a meta-analysis of randomized controlled trials. Chin Med J (Engl). 2014;127(15):2837-43.
38. **Nielsen SE, Schjoedt KJ, Astrup AS, Tarnow L, Lajer M, Hansen PR, et al.** Neutrophil Gelatinase-Associated Lipocalin (NGAL) and Kidney Injury Molecule 1 (KIM1) in patients with diabetic nephropathy: a cross-sectional study and the effects of lisinopril. Diabet Med. 2010;27(10):1144-50. doi: 10.1111/j.1464-5491.2010.03083.x.
39. **Trohatou O, Tsilibary EF, Charonis A, Iatrou C, Drossopoulou G.** Vitamin D3 ameliorates podocyte injury through the nephrin signalling pathway. J Cell Mol Med. 2017;21(10):2599-609. doi: 10.1111/jcmm.13180.
40. **Sezgin G, Ozturk G, Guney S, Sinanoglu O, Tuncdemir M.** Protective effect of melatonin and 1,25-dihydroxyvitamin D3 on renal ischemia-reperfusion injury in rats. Ren Fail. 2013;35(3):374-9. doi: 10.3109/0886022X.2012.760409.
41. **Lee WC, Mokhtar SS, Munisamy S, Yahaya S, Rasool AHG.** Vitamin D status and oxidative stress in diabetes mellitus. Cell Mol Biol (Noisy-le-grand). 2018;64(7):60-9. doi: 10.1038/ki.2009.175.
42. **Ahmed OM, Ali TM, Abdel Gaid MA, Elberry AA.** Effects of enalapril and paricalcitol treatment on diabetic nephropathy and renal expressions of TNF-alpha, p53, caspase-3 and Bcl-2 in STZ-induced diabetic rats. PLoS One. 2019;14(9):e0214349. doi: 10.1371/journal.pone.0214349.
43. **Xu J, Feng ZP, Peng HY, Fu P.** Omega-3 polyunsaturated fatty acids alleviate adenine-induced chronic renal failure via regulating ROS production and TGF-beta/SMAD pathway. Eur Rev Med Pharmacol Sci. 2018;22(15):5024-32. doi: 10.26355/eurrev_201808_15645.
44. **Jamilian M, Samimi M, Ebrahimi FA, Hashemi T, Taghizadeh M, Razavi M, et al.** The effects of vitamin D and omega-3 fatty acid co-supplementation on glycemic control and lipid concentrations in patients with gestational diabetes. J Clin Lipidol. 2017;11(2):459-68. doi: 10.1016/j.jacl.2017.01.011.