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RESEARCH PAPER

# Adropin contributes to the nephro-protective effect of vitamin D in renal aging in a rat model via MAPK/HIFα/VEGF/eNOS mechanism

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**Abstract**

Great efforts have been established to promote healthy aging and prevent illnesses connected to aging. The prevalence of elderly population increases and this will be associated with an increase in disability and illness. This study analyzed renal aging process following administration of D-galactose (120 mg/kg/d ip for 8 weeks), and the potential protective effects of vitamin D administration (1,000 and 10,000 IU/kg/d by oral gavage for 8 weeks) in animals with D-galactose induced aging. The renal function tests (serum urea and creatinine, creatinine clearance, and 24 hours urinary albumin excretion levels), oxidative stress (MDA, TCA), renal tissue expression of TNFα, HIFα, Adropin, MAPK, eNOS, and VEGF as well as histopathological and EM alterations were assessed. D-galactose administration resulted in noticeable changes in renal histopathology, deteriorated renal function tests, elevated oxidative stress and inflammation, and significantly diminished Adropin, MAPK, eNOS, HIFα and VEGF expression. Vitamin D effectively reversed these alterations and enhanced histopathological and EM ultrastructure changes triggered by D-galactose administration.


*Keywords:* Anti-aging; D galactose; Oxidative stress; Adropin; Geriatric Rehabilitation.

## 1. Introduction

Aging is a progressive loss of functional reserve that manifests as a substantial decrease in the adaptive homeostatic ability to external or internal stress, leading to an elevated risk of death and disease [1]. With the significant increase in the elderly population, it is imperative to acknowledge the substantial physiologic changes that occur in all organ systems as they age [2]. An appropriate assessment of renal function in the context of aging is particularly important due to the fact that the kidney is a frequently targeted organ for a range of disorders [3]. The kidneys undergo a number of abnormalities as they age, such as glomerulosclerosis development, the loss of tubules, increasing interstitial fibrosis [4], and vascular alterations, such as endothelium-dependent vasodilation dysfunction and a lack of angiogenic capacity [5]. Currently, renal fibrosis lacks an effective therapy due to the incomplete elucidation of the pathophysiologic mechanisms [6]. Consequently, it is imperative to investigate strategies that can impede the progression of kidney injury.

Oxidative stress and inflammation are among the numerous cellular signaling pathways that have been consistently linked to renal aging [7]. The oxidative/inflammatory cascade plays a central pathogenic role in the destruction of renal tissue, irreversible loss of kidney function, and the progression of age-related kidney fibrosis [8]. Therefore, boosting antioxidant activity could potentially alter age-related kidney fibrosis by reducing oxidative stress and promoting tissue regeneration.

Vitamin D is recognized as a factor in preservation of proper calcium metabolism, but it could also be essential for a wide variety of "non-classical" functions [9]. Its deficiency can disrupt

*Abbreviations:* eNOS, endothelial nitric oxide synthase; HIFα, hypoxia-inducible factor alpha; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; TCA, total capacity of antioxidant; TNFα, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor.

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mitochondrial function, boost oxidative stress, and intensify systemic inflammation. Therefore, the correlation between vitamin D levels and the risk of age-related disorders may be attributed to its impact on oxidative stress and antioxidant status [10].

It is crucial to observe that elderly populations may have low levels of vitamin D as a result of various factors, including lower consumption, absorption, or decreased exposure to sunlight [11]. Furthermore, the kidney as it ages is likewise less capable of synthesizing 1,25-dihydroxyvitamin D from 25-hydroxyvitamin D. (25[OH] D) [12].

The vitamin D receptor (VDR) is a nuclear transcription factor with diverse biological effects and is abundantly expressed in renal tubular epithelial cells, indicating a role for vitamin D in maintaining kidney homeostasis [13]. In addition to its classical role, the binding of vitamin D to VDR affects a variety of signaling cascades, such as apoptosis, inflammation, and oxidative stress [14].Nevertheless, the potential role of vitamin D in alleviating renal aging has not been fully clarified.

The energy homeostasis-associated (Enho) gene encodes a secreted peptide known as Adropin which is produced by various body tissues including kidney, heart, brain, and the liver [15]. Adropin has received a lot of attention due to its tight relationship to insulin resistance and glucose and lipid homeostasis [16]. Itwas also found to stimulate antioxidant reactions to facilitate reactive oxygen species (ROS) clearance [17].In addition, it has been indicated that Adropin enhances the bioavailability of nitric oxide (NO) via activating mitogen-activated protein kinase (MAPK) signaling in endothelial cells through the VEGF receptor 2 (VEGFR2), which lead to the upregulation of endothelial NO synthase (eNOS) [18]. New evidence suggests that aging is closely linked to reduced plasma levels of Adropin [19,20]. It has been shown that age-related decrease in plasma Adropin levels and protein expression in the brains of Sprague Dawley rats correlates with the expression of protein markers linked to oxidative stress [21]. Additionally, in non-human primate tissues, the expression of the Adropin transcript is linked to genes associated with age-related neurodegeneration [22]. However, the impact of Adropin in renal aging is largely unknown.

Hypoxia inducible factor $\alpha$ (HIF$\alpha$) is a key transcription factor that mediates transcriptional activation of VEGF, a central mediator of angiogenesis and vital player of vascular aging. According to Wong et al. [23], the deletion of HIF $\alpha$ in lysosome M-positive cells in a mouse model of type 1 diabetes mellitus prevented the vitamin D –induced vascular healing through VEGF. It also controls redox balance, inflammation, and glucose metabolism to eventually maintain cellular homeostasis. Since the discovery of HIF-1$\alpha$, several studies have identified the changes in HIF associated with age and the development of age-related disorders [24].

Studies investigating vitamin D effect on crucial signaling pathways impacted in biological aging are limited, therefore, our study aims to identify a novel mechanism through which vitamin D may protect against renal aging. This research tended to determine whether vitamin D administration could effectively ameliorate renal aging in male rats via modulating Adropin expression and MAPK/HIF$\alpha$/VEGF/eNOS signaling.

## 2. Materials and methods

### 2.1. Drugs and chemicals

D- galactose (D- gal) (CAS No. 59-23-4) in the form of white powder was obtained from LOBA chemie Pvt. Ltd., Mumbai, India. Vitamin D was available in the form of cholecalciferol obtained from Memphis Pharmaceuticals & Chemical Industries, Cairo, Egypt.

### 2.2. Animals and ethical considerations

Thirty adult male Wistar rats, aged 8–10 wk and weighing between 150 and 200 g, were procured from the Experimental Animal Breeding Farm in Helwan, Cairo.

Animals were maintained in controlled laboratory environments with a temperature range of 20–25°C and a light-dark cycle of 12/12 h. Animals were fed commercially available standard rodent diet composed of 20% crude protein, 4% crude fat, 3.5% crude fiber, 6% ash, 0.5% salt, 1% calcium, 0.6% phosphorus, 20 IU/g vitamin A, 2.2 IU/g vitamin D, and 70 IU/g vitamin E [25–27]. This diet was supplied by Al-Magd Feed Company, Quesna Industrial Zone, Qalyubia Governorate, Egypt.

Prior to the trial, all rats were acclimatized to the laboratory environment for 1 week. Rats were caged (3/cage) in a fully ventilated room in the Physiology Department, Benha Faculty of Medicine. The research followed guidelines intended for maintenance and utilization of laboratory animals [28], in addition to being accepted by Research Ethics Committee of the Faculty of Medicine at Benha University, Egypt (Approval No. RC: 13-3-2024).

### 2.3. Experimental design

Sample size calculated using epi info soft calculator version 3 based on Yang et al. [21]. The anticipated mean ±SD adropin levels among aging group and young group were 1.0±0.1 and 1.5±0.2 respectively. The study had a power of 80%, a 95% confidence level, and an alpha error of 5%, therefore the minimal calculated sample size is 3 rats in each group and increased to 6 rats in each group for fear of dropout.

Rats were split into five groups at random:

- **Group I (Control group; n=6):** This group obtained normal saline via an intra-peritoneal injection. They also received distilled water using an oral gavage daily at 1 mL/rat for 8 weeks.
- **Group II (Vitamin D group; n=6):** This group received vitamin D (10,000 IU/kg/d by oral gavage for 8 weeks) [29].
- **Group III (Aging group; n=6):** This group obtained D-galactose (120 mg/kg/d) dissolved in saline by intraperitoneal injection for 8 weeks [30].
- **Group IV (Vitamin D 1,000+ Aging; n=6):** This group obtained D-galactose (120 mg/kg/d) by intraperitoneal injection for 8 weeks and vitamin D 1,000 IU/kg/d by oral gavage for 8 weeks [29].
- **Group V (Vitamin D 10,000+ Aging; n=6):** This group obtained D-galactose (120 mg/kg/d) by intraperitoneal injection for 8 weeks and vitamin D 10,000 IU/kg/d by oral gavage for 8 weeks [29].

By the conclusion of the 8th week, rats were individually placed in a homemade metabolic cage for 24 h to calculate urine output (starting from 10:00 AM to 10 AM next day). Rats were let to habituate and acclimatize to changes in location and caging prior to the 24-h urine collection. Urine collecting funnels of the appropriate size were placed at the base of the metabolic cages, and fecal matter was retained in the funnels by placing special perforated plastic discs inside [31]. The urine volume was measured and centrifuged for 5 minutes at 1,500 rpm. At a temperature of –20°C, the clear supernatant was stored for further biochemical analysis.

After an overnight fast, rats were placed on an operating table, given urethane anesthesia (1.5 g/kg; i.p.), and had their hearts punctured to draw blood. The rats were then euthanized via decapitation. Samples of blood were permitted to clot at room temperature before being centrifuged for 15 min to separate the

serum, which was then kept at -20°C for use in the biochemical examination of creatinine and urea.

To completely remove extra blood, the two kidneys were washed in ice-cold PBS (0.02 mol/L, pH 7.0–7.2).

The right kidney of each animal was divided in 2 part. One part was homogenized in a certain amount of PBS using a Mixer Mill MM400 (Retsch GmbH). Tissue homogenates were centrifuged for 15 min at 4,000 rpm, 4°C and Supernatant was used for quantitative detection of Malondialdehyde (MDA), total capacity of antioxidant (TCA), Adropin, mitogen-activated protein kinase (MAPK) and eNOS. The 2nd part of right kidney was placed in RNAlater stabilization solution (Catalog No: AM7021; Thermo Fisher Scientific, MA) at 10 $\mu$L per 1 mg of tissue, and kept at -80°C for real time PCR analysis of TNF$\alpha$ and HIF$\alpha$ mRNA expression.

The left kidney was kept in formalin for histopatholgical and immunohistochemical examination of VEGF. Ultrathin sections from kidney about (1 $mm^3$) were put in 2.5% solution of glutaraldehyde on 0.1 M phosphate buffer (pH = 7.4) mixed with 4.0% paraformaldehyde as primary fixative for electron microscopy tissue processing.

### 2.4. Biochemical analysis

#### 2.4.1. Evaluation of serum urea, creatinine, creatinine clearance, and 24 h urinary albumin excretion levels

Quantitative determination of rat serum urea and creatinine was done employing Urea Colorimetric Assay Kit II (Catalog # K376-100; BioVision, Milpitas, CA, USA) and Creatinine Colorimetric/Fluorometric Assay Kit (Catalog # K625-100; BioVision, Milpitas, CA, USA), respectively. Creatinine clearance was determined using the following formula: Creatinine concentration in urine (mg/dL) × Volume of urine (mL/min) /Concentration of creatinine in serum (mg/dL) [32]. The 24 h urinary albumin excretion was measured in the centrifuged urine as described by [33].

#### 2.4.2. Assessment of the oxidative stress markers

Renal levels of MDA and TAC were estimated using Lipid Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit (Catalog # K739-100; BioVision, Milpitas, CA, USA) and Rat Total antioxidant capacity (TAC) Elisa kit (Catalog # AMS.E02T0028, amsbio, MA, USA), respectively in compliance with manufacturer's guidelines.

#### 2.4.3. Assessment of the tissue adropin, MAPK and eNOS

The levels of renal Adropin, MAPK and eNOS were estimated using Rat AD (Adropin) ELISA Kit (Catalog # orb1088203; Biorbyt, NC, USA), Rat MAPK (P38) ELISA Kit (Catalog # ER1138; FineTest, Wuhan, China), Rat eNOS ELISA Kit (Catalog # NBP2-80136; Novus Biologicals, CO, USA) respectively in compliance with the manufacturer's guidelines.

### 2.5. Real–time PCR analysis for mRNA expression of TNFα&HIFα

#### 2.5.1. RNA purification and cDNA synthesis

Direct-zol RNA Total RNA was extracted and purified from cell homogenate utilizing Miniprep Plus kit following guidelines provided by the manufacturer (Catalog # R2072; ZYMO RESEARCH CORP., CA, USA). Following evaluation of the extracted mRNA's amount and quality employing a Beckman dual spectrophotometer (USA), SuperScript IV One-Step RT-PCR kit was utilized. (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA, USA) was employed for reverse transcription of extracted RNA following manufacturer instructions.

Table 1
Primer sequences employed for RT-PCR analysis.

| Gene name | Primer sequences (5' → 3') (F: Forward; R: Reverse) | Accession number |
|---|---|---|
| ***HIFα*** | F: TGCTTGGTGCTGATTTGTGA<br>R: GGTCAGATGATCAGAGTCCA | NM_024359.2 |
| ***TNFα*** | F: TGCCTCAGCCTCTTCTCATT<br>R: GAGCCCATTTGGGAACTTCT | NM_012675.3 |
| ***GAPDH*** | F: GGTCGGTGTGAACGGATTTGG<br>R: ATGTAGGCCATGAGGTCCACC | NM_017008.4 |

#### 2.5.2. mRNA quantification and PCR reaction

StepOne Real-Time Cycler (Applied Biosystem, CA, USA) employed to perform amplification utilizing SensiFAST SYBR Hi-ROX One-Step Kit (Catalog # BIO-73001, meridian BIOSCIENCE, USA).Primer sequence for studied target genes (HIF$\alpha$ and TNF$\alpha$)and reference housekeeping gene (GAPDH) were shown in Table 1.The mRNA expression of each sample was calculated after correcting for GAPDH expression. $2^{-\Delta\Delta Ct}$ technique was utilized to calculate relative expression [34]. The results are given in terms of n-fold difference from the controls.

### 2.6. Histopathologic study

Samples of kidney tissue were preserved for 48 hours in 10% formalin. Sections of paraffin (5 $\mu$m thick) were produced, processed, and stained with hematoxylin and eosin (H&E) [35]. Additional sections were put on positively charged slides so that VEGF could be detected by immunohistochemistry [36].

### 2.7. Immunohistochemical study

Immunohistochemical staining for identifying VEGF. The rabbit anti-VEGF monoclonal antibody served as the main one (Santa Cruz Company, California, USA) (1:500 with PBS). Cytoplasmic brown was the hue of the reaction's cellular location [37]. After doing an immunohistochemical analysis employing avidin–biotin peroxidase method, a chromogen called diaminobenzidine (DAB) (Dakopatts, Glostrup, Denmark) was utilized on the slides. After that, distilled water was utilized to wash slides. Hematoxylin was then employed as a counterstain for the sections.

### 2.8. Transmission electron microscopic specimen study

At Mansoura University's Faculty of Medicine, Mansoura, Egypt, ultrathin sections were made [38]. In Electron Microscope unit of Mansoura Faculty of Medicine at Mansoura University, grids were inspected and electron micrographs were acquired utilizing a transmission electron microscope called JOEL (JEM-100 SX, Akishima, Tokyo, Japan).

### 2.9. Computer-assisted digital image analysis (Digital morphometric study)

Employing morphometrics employing a Leica Qwin 500 computer system for image analysis (Leica Microsystems Ltd, Cambridge, UK) at the Pathology department, Faculty of Medicine, Mansoura University. Each group's five slides were analysed, and ten non-overlapping fields were determined from each slide. Interstitial space, average tube roundness, average glomular diameter, and the quantity of inflammatory cells in H&E-stained sections were also evaluated. At a × 200 magnification, positive immunoreactivity for VEGF was also evaluated. They were computed in

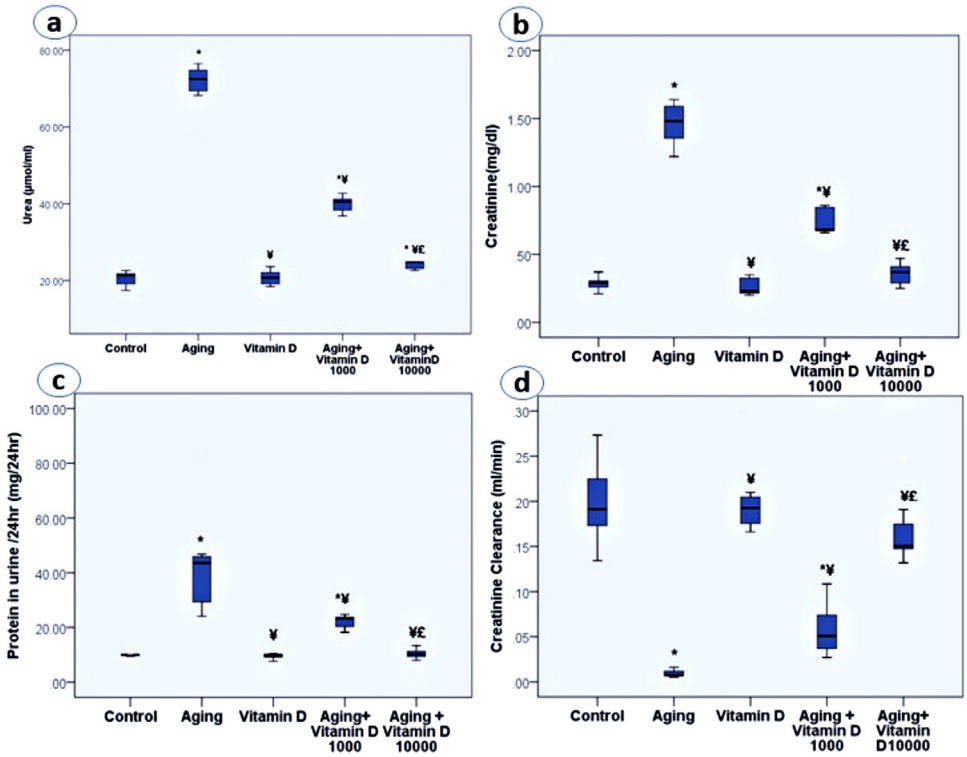


**Fig. 1.** Vitamin D impact in different doses on D-Galactose -induced altered renal function tests. The data are displayed as means ± SD (n=6) using one way ANOVA then Scheffe's test as post-hoc test. *: significant in contrast to the control and Vitamin D group. #: significant in contrast to the aging group. ϵ: significant opposed to Vitamin D 1,000+Aging group. Significance: $P<.05$.

respect to a common measurement frame and utilizing the color detect menu.

### 2.10. Data management and statistical analysis

The normality of distribution for the analyzed variables were tested using Shapiro test assuming normality at $P>.05$. The collected data were summarized in terms of median and Inter Quartile Range (IQR) as appropriate for nonparametric data. The statistical significance of the difference between the treated groups and the control group was evaluated using Kruskal–Wallis test as followed by Mann–Whitney test for comparison between two groups appropriate for nonparametric data. Statistical Package for the Social Sciences (SPSS) 25.0 for windows SPSS Inc., Chicago, IL, USA). All tests were two sided. A $P$ value less than .05 was considered significant.

## 3. Results

### 3.1. The impact of Vitamin D in different doses on aging-induced altered kidney function parameters

As presented in Figure 1, D-Galactose administration (120 mg/kg/d, ip) for 8 weeks was enough to alter kidney function. In the Aging group, D-Galactose administration induced significant elevation in the median (IQR) of serum urea (72.5 [68.2–75.8]) (Fig. 1a), serum creatinine (1.48 [1.23–1.63]) (Fig. 1b) and 24-h urine albumin excretion (43.54 [24.64–46.78]) (Fig. 1c) levels by with subsequent decrease in creatinine clearance level (0.0075 [0.0065–0.01]) (Fig. 1d) contrasting them with control group's ([21.3 {18.7–21.8}], [0.29 {0.25–0.31}, [9.88 {9.55–10.22}] and [0.19 {0.16–0.24}], respectively) ($P<.05$). Interestingly, Vitamin D treatment significantly improved the kidney functions. A substantial decrease in serum urea, serum creatinine and 24-h urine albumin excretion levels and a significant rise in creatinine clearance level were demonstrated with Vitamin D treatment in both the 1,000 IU Vitamin D + D-Galactose ([40.50 {37.80–41.70}], [0.68 {0.67–0.85}], [23.23 {18.64–23.98}] and [0.05 {0.03–0.08}], respectively) and 10,000 IU Vitamin D + D-Galactose treated groups ([24.76 {22.60–24.76}], [0.37 {0.27–0.41}], [10.16 {8.75–12.32}] and [0.15 {0.14–0.19}], respectively) compared with the aging group. Moreover, Vitamin D at a dosage of 10,000 IU/kg/d significantly exerted more protective effects against D-Galactose -induced alteration in kidney function parameters when compared to Vitamin D at a dosage of 1,000 IU/kg/d therapy ($P<.05$).

### 3.2. The impact of Vitamin D in different doses on D-Galactose -induced oxidative stress and inflammation

As presented in Figure 2, administration of D-Galactose enhanced the oxidative stress as demonstrated by the significantly increased in the median (IQR) of MDA levels (1.45 [0.99–1.48]) (Fig. 2b) coupled with the substantial decrease in the TAC levels (0.47 [0.38–0.57]) (Fig. 2a) in aging group as opposed to control group ([0.41 {0.38-0.42}] and [2.27 {2.21-2.37}], respectively) ($P<.05$). Vitamin D treatment significantly diminished MDA and enhanced TAC levels in both the 1000 IU Vitamin D + D-Galactose ([1.04 {0.80–1.13}] and [1.54 (1.39-1.64)], respectively) and 10,000 IU Vitamin D + D-Galactose treated groups ([0.52 {0.51–0.63}] and [2.22 {1.89-2.27}], respectively) contrary to aging group.

Furthermore, aging group indicated a statistically significant rise in the median (IQR) of TNF$\alpha$ mRNA expression levels as opposed to controls ([2.94 {1.96- 3.87}] and [1.13 {1.11–1.23}], respectively) ($P<.05$) (Fig. 2c) indicating enhanced inflammatory status that was further confirmed by the marked interstitial inflammation

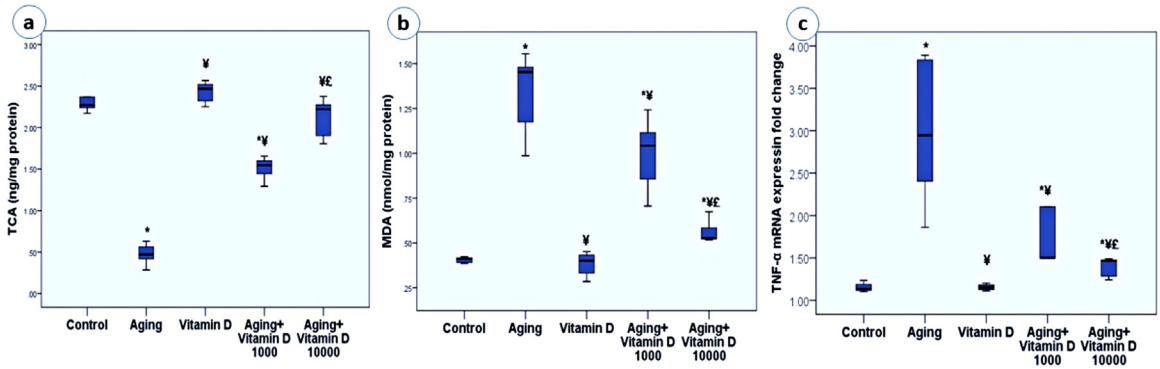


**Fig. 2.** The impact of Vitamin D in different doses on D-Galactose -induced oxidative stress and inflammation. The data are exhibited as means ± SD (n=7) using one way ANOVA then Scheffe's test as post-hoc test. Significance (*): in contrast to control and Vitamin D group. *: significant contrary to control and Vitamin D group. #: significant in contrast to Aging group. €: significant as opposed to the Vitamin D 1000+aging group. Significance: $P<.05$. MDA, malondialdehyde; TCA, total capacity of antioxidant; TNF$\alpha$, tumor necrosis factor alpha.

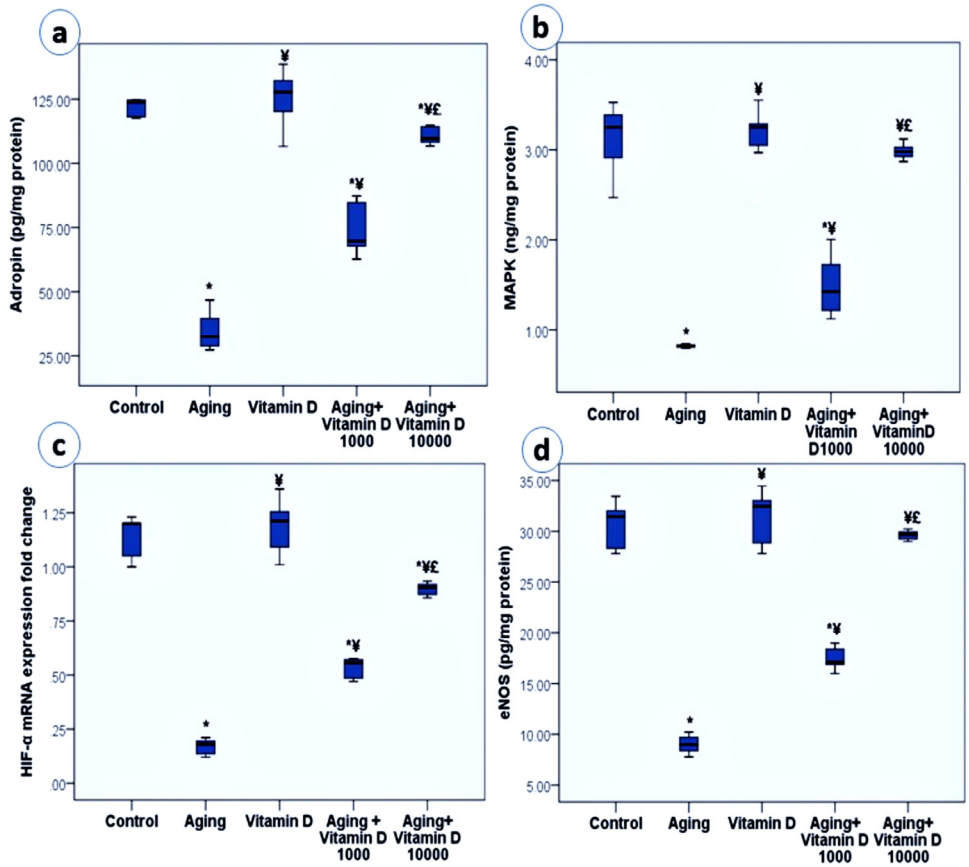


**Fig. 3.** Vitamin D impact in different doses on D-Galactose -induced altered Adropin/ MAPK/HIF $\alpha$/ eNOS expression. The data are exhibited as means ± SD (n=7) using one way ANOVA then Scheffe's test as post-hoc test. *: significant in contrast to the control and Vitamin D group. #: significant in contrast to aging group. €: significant contrary to Vitamin D 1,000+aging group. Significance: $P<.05$. eNOS, endothelial nitric oxide synthase; HIF $\alpha$, hypoxia-inducible factor alpha; MAPK, mitogen-activated protein kinase.

detected histologically (Fig. 5). Vitamin D treatment significantly reduced TNF$\alpha$ mRNA expression levels in both the 1000 IU Vitamin D + D-Galactose and 10000 IU Vitamin D + D-Galactose treated groups ([1.50 {1.50–2.10}] and [1.46 {1.28–1.47}], respectively) contrary to the aging group. Notably, Vitamin D at a dose of 10,000 IU/kg/d significantly exerted more preventive benefits against D-Galactose -induced oxidative stress and inflammation when compared to Vitamin D at a dosage of 1,000 IU/kg/d therapy ($P<.05$).

### *3.3. The impact of Vitamin D in different doses on D-Galactose -induced altered Adropin/ MAPK/ HIFα/ VEGF/ eNOS protective pathway*

#### *3.3.1.* The impact of Vitamin D in different doses on D-Galactose -induced altered Adropin/ MAPK *expression*

As presented in Figure 3, the administration of D-Galactose significantly decreased in the median (IQR) of Adropin (32.50 [28.30–

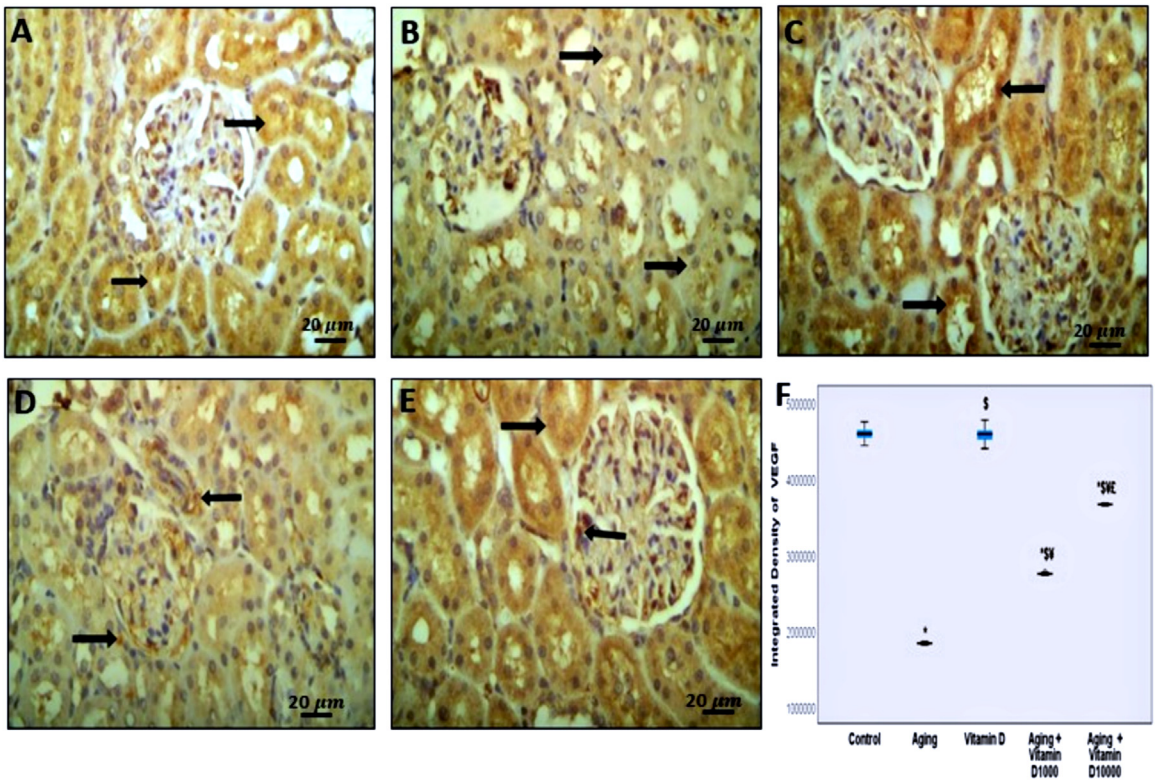


**Fig. 4.** Photomicrographs of sections in the kidney tissues of rats stained with anti-VEGF antibody. (a–f) (A) Control group: intense expression and brown stained cytoplasm of renal glomeruli and tubules cells indicating positive reaction (black arrows). (b) Aging group: downregulated VEGF expression and most cells are showing weak reactions. (c) Vitamin D group: similar to control group (d) Vitamin D 1,000+aging group: mild increase in VEGF expression (black arrows). (e) Vitamin D 10,000+aging group: high increase in VEGF expression (magnification power,400x). The data are exhibited as means ± SD (n=7) using one way ANOVA followed by Scheffe's test as post-hoc test. *: significant in contrast to the control and Vitamin D group. #: significant in contrast to aging group. €: significant contrary to Vitamin D 1,000+Aging group. Significance: $P<.05$.

45.70]) (Fig. 3a) and MAPK (0.82 [0.79–0.84]) (Fig. 3b) levels in aging group as opposed to control group ([123.70 {117.60– 124.70}] and [3.25 {2.67-3.42}], respectively) ($P<.05$). While, Vitamin D treatment significantly raised Adropin and MAPK levels in both the 1,000 IU Vitamin D + D-Galactose ([69.70 {66.70–85.26}] and [1.42 {1.16–1.82}], respectively) and 10,000 IU Vitamin D + D-Galactose treated groups ([109.70 {107.70–114.80}] and [2.98 {2.88–3.04}], respectively) contrary to aging group. Notably, Vitamin D at a dosage of 10,000 IU/kg/d significantly exerted more protective impacts against D-Galactose-induced decrease in Adropin and MAPK levels when compared to Vitamin D at a dosage of 1,000 IU/kg/d therapy ($P<.05$).

### 3.3.2. Vitamin D impact in different doses on D-Galactose -induced altered HIFα/ eNOS

As presented in Figure 3, administration of D-Galactose significantly suppressed decreased in the median (IQR) mRNA expression of HIFα (0.18 [0.13–0.20]) (Fig. 3c) and decreased eNOS (8.98 [7.98–9.75]) (Fig. 3d) level in aging group contrary to control group ([1.20 {1.00–1.20}] and [31.45 {27.80–32.11}], respectively) ($P<.05$). While, Vitamin D treatment significantly raised HIFα mRNA expression and eNOS level in both the 1,000 IU Vitamin D + D-Galactose ([0.55 (0.48–0.57)] and [17.09 {16.87– 18.43}], respectively) and 10,000 IU Vitamin D + D-Galactose treated groups ([0.90 {0.86-0.92}] and [29.67 {29.12–30.02}],respectively) contrary to aging group. In addition, Vitamin D at a dosage of 10,000 IU/kg/d significantly enhanced HIFα mRNA expression and eNOS levels compared to Vitamin D treatment at a dosage of 1,000 IU/kg/d ($P<.05$).

### 3.3.3. The impact of Vitamin D in different doses on D-Galactose -induced altered VEGF protein expression in kidney tissues

Compared to control's median (IQR) of immunostaining for VEGF in the kidney tissues (4588500.50 [4537778.5–4641083.0]), there is a significant decreased in mean area percentage of VEGF expression with D-Galactose administration in aging group (1832898.0 [182377602–1838064.5]) ($P<.05$) (Fig. 4). While, Vitamin D treatment significantly raised mean area percentage, median (IQR) of VEGF expression in both the 1000 IU Vitamin D + D-Galactose (2745952.5 [2741158.2–2751060.5]) and 10,000 IU Vitamin D + D-Galactose (3660362.5 [3654591.5–3667142.7]) treated groups in contrast to aging group. In addition, a significant rise in mean area percentage of VEGF expression was observed with Vitamin D treatment at a dosage of 10,000 IU/kg/d compared to Vitamin D treatment at a dose of 1,000 IU/kg/d ($P<.05$).

### 3.4. The impact of Vitamin D in different doses on D-Galactose -induced altered morphological changes of the kidney tissues

The kidney sections of rats in control group and Vitamin D group displayed normal median (IQR) of renal glomeruli and tubules roundness ([209.00 {197.7500–222.00}] and [0.87 {0.86–0.89}]), respectively without interstitial inflammation (4.00 [2.00–5.00]). On the contrary, in the aging group, most of the glomeruli and tubules were necrotic and distorted with a significant decline ($P<.05$) in median (IQR) of glomerular diameter and tubular roundness ([112 {104.00–117.75}] and [0.42 {0.39–0.44}]), as well as a significant degeneration and inflammation were also obvious and a significant rise ($P<.05$) in inflammatory cells and interstitial spaces ([258.00 {247.25–270.750}] and [929.50 {864.75–

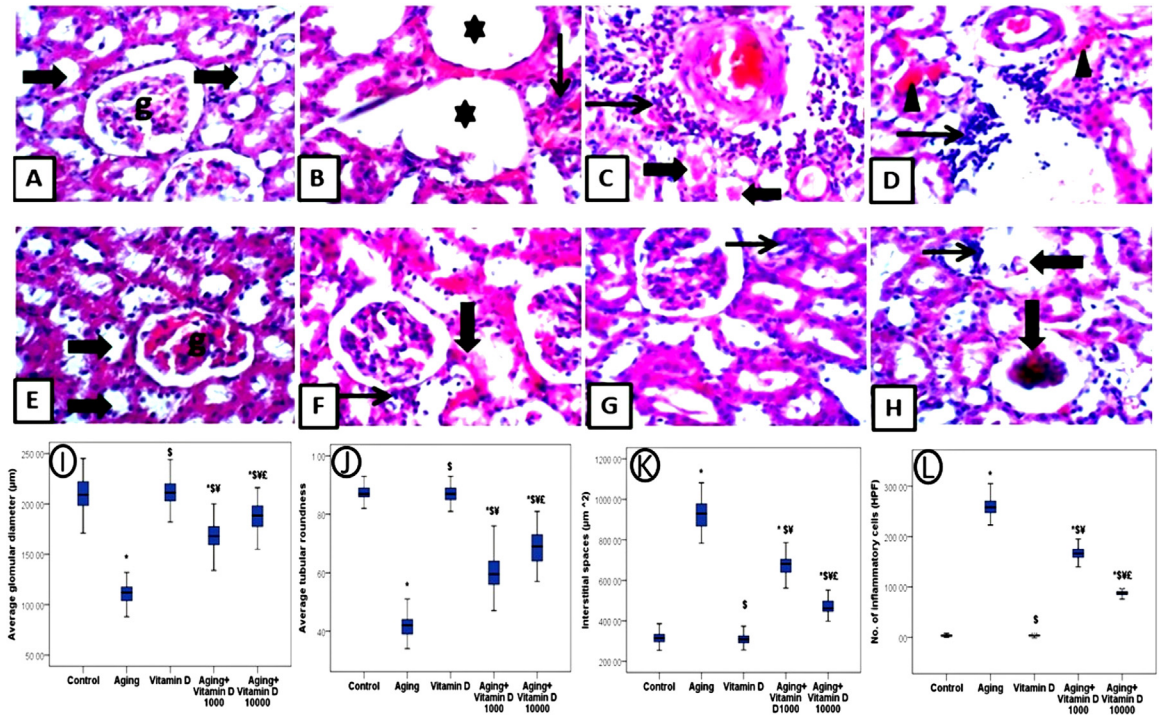


**Fig. 5.** (Resolution=300dpi) Effect of co-administration of Vitamin-D in different doses with D-galactose on histological alterations in kidney tissues of albino rats. Photomicrographs of the kidney tissues stained with hematoxylin-eosin. (a) Control group: normal histological appearance of glomeruli (g) and renal tubules (black arrow). (b–d) Aging group: showing distorted and empty glomeruli (stars), ectatic, and necrotic renal tubules are lined with attenuated epithelium and have intraluminal necrotic debris (thick arrows) with marked interstitial inflammation (thin arrows) characterized by high perivascular cellular infiltrates admixed with hemorrhage (arrowheads). (e) Vitamin D group: showing normal histological appearance of renal parenchyma. (f) Vitamin D 1,000+aging group: showing focal tubular necrosis (thick arrow) with focal periglomerular aggregation of inflammatory cells (thin arrow). (g, h) Vitamin D 10,000+aging group: showing restoration of most normal architecture, but few, minimal aggregation of inflammatory cells (thin arrows) are still present and some glomerular tufts are still shrunken (thick arrows). The magnification power is X=400. (I) the data are exhibited as means ± SD (n=7) employing one way ANOVA then a Kruskal–Wallis's test then Mann Whitney.

979.00}]),respectively compared to control group ([4.00 {2.00–5.00}], [314.50 {295.50–334.00}]).These pathological findings were enhanced after co-administration of Vitamin D in different doses (1,000, 10,000IU) respectively with D-galactose in the form of reducing distortion, necrosis with a significant rise ($P<.05$) in the median (IQR) of glomerular diameter and tubular roundness ([168.00 {159.25–177.75}, [0.59 {0.56–0.64}]), ([188.50 {177.25–198.00}], [0.69 {0.64–0.73}])], respectively and returning glomeruli and tubules relatively to normal histology. Besides, in vitamin D treated groups, there is a significant reduction in interstitial inflammation illustrated by a significant decreased no. of inflammatory cells ([166.50 {159.00–175.00}], [88.50 {85.00–91.000}], respectively contrary to aging group ($P<.05$). Notably, Vitamin D at a dosage of 10,000 IU/kg/d significantly exerted more protective effects against D-Galactose-induced altered morphological alterations of the kidney tissues (Fig. 5).

### *3.5. Vitamin D improved renal tissue ultrastructure pathological changes induced by D-galactose exposure*

The TEM examination of proximal and distal convoluted tubules cells and renal glomeruli from each group of rats supported the histopathological findings. Examination of control group revealed normal proximal convoluted cells with characteristic large euchromatic rounded nuclei, numerous elongated mitochondria, luminal closely-packed microvilli and thin basement membrane as displayed in (Fig 6a). This group also showed normal cells of distal convoluted tubules (Fig 6b) and normal podocyte with irregular euchromatic nucleus and its primary processes. Thin fenestrated endothelium lined the glomerular capillary, and glomerular basement membrane had a thin, uniform, trilaminar appearance (Fig 6c). The vitamin D group showed the same normal ultrastructural morphology (Fig 6g–i). The aging group showed an obvious deterioration in the ultrastructural morphology. Proximal and distal convoluted tubules cells showed irregular contour, shrinked nuclei, scattered small rounded, degenerated mitochondria, and cytoplasmic vacuolations. Apical part of cells displayed distorted irregular microvilli (Fig 6 d and e). Moreover, the same group displayed podocyte with dark nucleus and dilated foot processes. Some areas showed lost foot processes. Glomerular blood capillaries showed areas of lost endothelial fenestrations (Fig 6f). Vitamin D 1,000 and 10,000 IU coadministartion with D-galactose decreased this deterioration. The improvement in the ultrastructure changes was obvious with Vitamin D treatment at a dosage of 10,000 IU/kg/d compared to Vitamin D treatment at a dosage of 1,000 IU/kg/d (Fig 6 j–o).

## 4. Discussion

Aging populations pose an increasing challenge to therapeutic procedures as long as people live longer. A number of organ systems, comprising kidney, experience molecular, structural, and functional alterations as people age. The kidney undergoes microscopic histological changes and a steady functional deterioration as we age. These changes are made worse by systemic comorbidities like DM and hypertension, and by underlying or previous renal disorders. Human health depends on vitamin D endocrine system, and the system's ability to function properly depends on a kidney that is anatomically sound [9]. Herein, we aimed to look into a possible reno-protective effect of vitamin D during aging

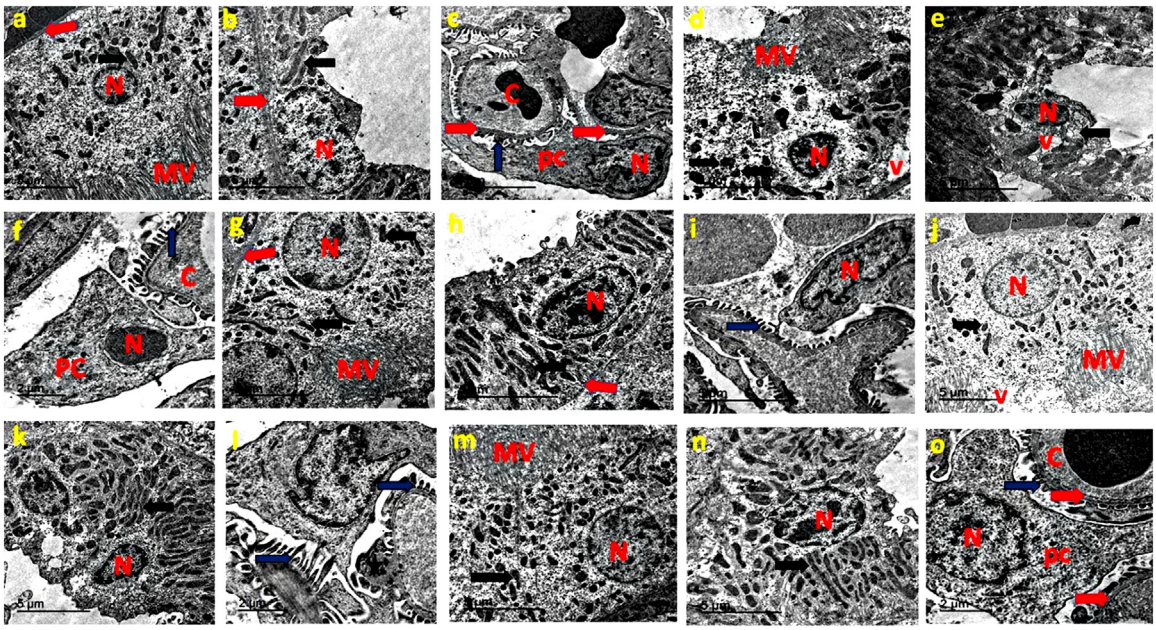


**Fig. 6.** (a–c): Electron micrographs of the renal cortex in the control group reveal: (a) densely packed microvilli (MV) on the luminal surface and proximal tubular epithelial cells containing rounded, euchromatic nuclei (N). The basement membrane appears thin (red arrow), with multiple elongated mitochondria (black arrow) present. (b) Epithelial cells in the distal tubules displaying euchromatic nuclei (N) and a thin basement membrane (red arrow), along with numerous elongated mitochondria (black arrow). (c) A podocyte (PC) with an irregular, euchromatic nucleus (N) and its processes highlighted (blue arrow). The glomerular capillary (C) is lined by a thin, fenestrated endothelium, and the glomerular basement membrane is thin, uniform, and shows a trilaminar structure (red arrows). **(d–f):** Electron micrographs of the renal cortex in the aging group show: (d) basal cytoplasmic vacuoles (v) and small, rounded mitochondria (black arrows) scattered within the proximal convoluted tubular epithelial cells, alongside a small, shrunken nucleus (N) with an obscured internal structure. Microvilli (MV) in the apical cell region appear irregular and deformed. (e) Distal convoluted tubular epithelial cells containing a pyknotic,shrunken nucleus (N), along with noticeable cytoplasmic vacuoles (v) and degeneration and loss of mitochondria (black arrows) without basal infoldings. (f) Podocytes (PC) with dilated, absent foot processes and a darkened nucleus (N) (blue arrow) are also observed. In some regions, endothelial fenestrations are lost in glomerular capillaries (C)**. (g–i): Vitamin-D** group showing as control group. **(j–l): Vitamin D 1,000+aging** group showing (j) In a proximal convoluted tubule cell, the euchromatic nucleus (N) appears rounded, and the cytoplasm contains numerous predominantly rounded mitochondria (black arrow). The apical region of the cell is filled with densely packed microvilli (MV), with a few cytoplasmic vacuoles (v) also present. (k) Distal convoluted tubular cells show shrunken nuclei (N) in some cells with nearly normal elongated mitochondria. (black arrow) (l) Irregularities and focal thickening are observed in certain podocyte foot processes (black star), while other foot processes (blue arrows) appear to have a normal structure. **(m–o):Vitamin D 10,000+aging** In the proximal convoluted tubules, the nuclei (N) are rounded and euchromatic, with numerous mitochondria dispersed throughout the cytoplasm, especially enlarged mitochondria in the basal region (black arrow). The apical surface is lined with densely packed, elongated microvilli (MV). (n) In the distal convoluted tubule, prominent rounded euchromatic nuclei (N) are seen along with abundant mitochondria (black arrow). The basement membrane is thin. (o) Regularly structured foot processes (blue arrow) of podocytes (PC) present. The glomerular capillaries (C) exhibit normal fenestrations, while the glomerular basement membrane maintains a consistent thickness and characteristic trilaminar structure (red arrows).

and the underlying pathophysiology driving vitamin D supplementation effects on aged kidneys. Experimentally, chronic administration of D-galactose produces alterations that mimic natural aging in rats [39]. In this research, D-galactose administration caused alterations at the gene, protein and tissue levels. Nevertheless, these alterations were reversed by vitamin D treatment, indicating the renoprotective consequences of vitamin D during aging. The deterioration in kidney function was obvious with D-galactose administration in the aging group as demonstrated by the significantly increased serum urea, creatinine and 24-h urine albumin excretion levels associated with decreased creatinine clearance level. In addition, the histopathological and TEM findings of this study were consistent with the deteriorated biochemical results, showing damaged podocytes with missing foot processes and endothelial fenestrations, which result in glomerular basement membrane damage, ultimately leading to albuminuria and kidney dysfunction. These results further support what is currently recognized about D-galactose as a deteriorating agent that can induce degenerative and biochemical changes similar to nature kidney aging [40–43].

Oxidative stress has been established as paramount in promoting the aging process of the kidney [44,45]. It has been proposed also that oxidative stress and its associated processes, including excess of free radicals, lipid peroxidation, and the depletion of antioxidant enzymes, are the primary processes in charge of consequences of D-galactose-induced aging. The galactose oxidase enzyme has the ability to convert large quantities of D-galactose into aldose and hydroperoxide that alters redox homeostasis and causes oxidative stress. In this study, the oxidative stress was obvious as demonstrated by the significantly increased MDA levels associated with significant lower TAC levels in the aging group compared to controls. These results further support what was previously reported by several studies that established a connection between enhanced oxidative stress and aging and between altered redox homeostasis and D-galactose administration [46–50]. Oxidative stress state observed in this study explains the demonstrated disrupted histological findings. Further explaining the decline in kidney function state seen in this study is the fact that oxidative stress has been linked to disruptions in nephron excretory function [51], which leads to an imbalance in homeostasis and the accumulation of metabolic waste products.

Increased levels of TNF$\alpha$ have been reported with D-galactose administration in various organs [52–54]. Consistent with that, results of this study revealed a significantly increased level of TNF$\alpha$ mRNA expression in renal tissues of aging group as opposed to controls. The interplay between oxidative stress and inflammation reported in several researches [55,56] further support the idea that D-galactose induced oxidative stress observed in this study contributed to the demonstrated elevation in TNF$\alpha$ mRNA expression.

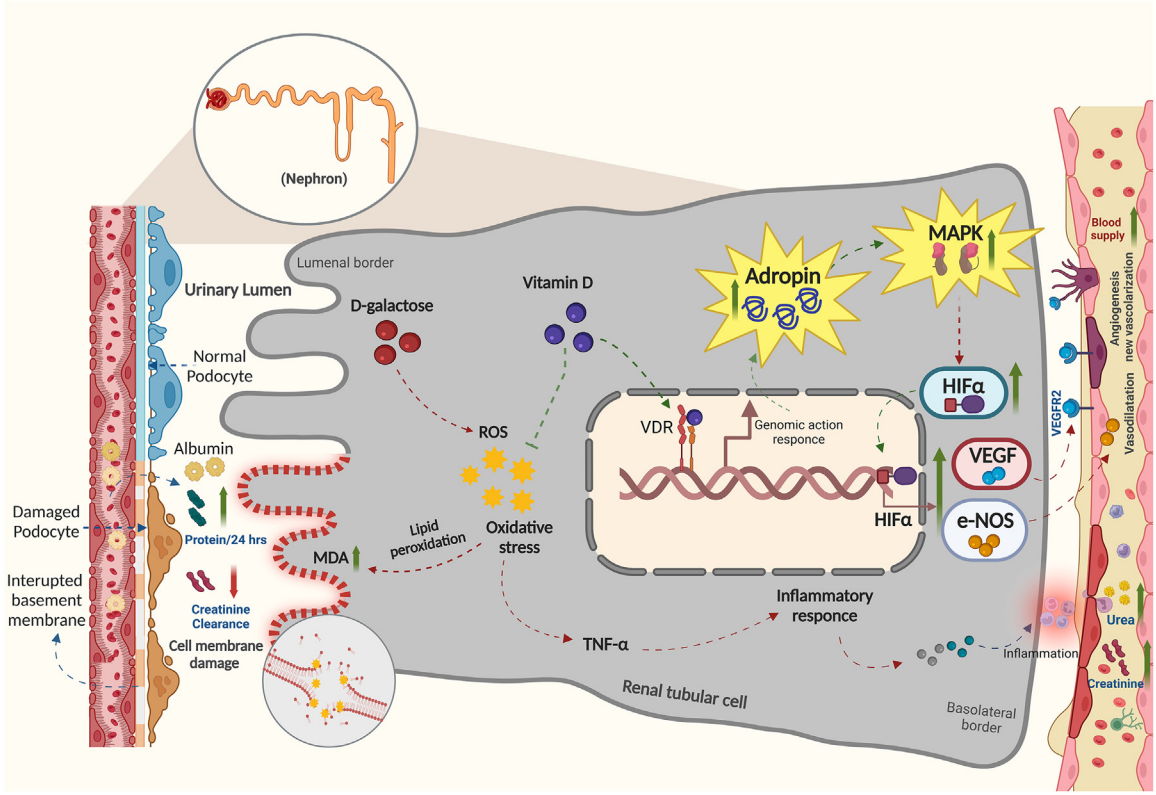


**Fig. 7.** A diagrammatic scheme summarizing the modulatory impact of vitamin D in different doses on the altered renal function in aging caused by D-galactose in rats. Beside the ROS-quenching and anti-inflammatory action of vitamin D, it binds to VDR. The vitamin D/VDR complex causes a genomic action response that upregulates the expression of adropin. Adropin next causes up-regulation in MAPK that results in phosphorylation and activation of HIF$\alpha$. HIF$\alpha$ causes upregulation in the expression levels of VEGF that binds to VEGFR2, enhancing the angiogenesis, besides increasing e-NOS that causes vasodilatation. eNOS; endothelial nitric oxide synthase, HIF$\alpha$; hypoxia-inducible factor alpha, MAPK; mitogen-activated protein kinase, MDA, malondialdehyde; TCA, total capacity of antioxidant; TNF$\alpha$, tumor necrosis factor alpha; VDR, Vitamin D receptor; VEGF, vascular endothelial growth factor; VEGFR2; VEGF receptor 2.

There is growing evidence linking endothelial damage and aging to low plasma levels of Adropin [20,21]. Additionally, it has been indicated that MAPK signaling is one of the several pathways that mediate Adropin actions and that Adropin modulates the activity of MAPK pathways [18,57]. MAPK signaling has also been shown to influence eNOS regulation [58,59], with one proposed mechanism involving the transcription factors FoxO1 and FoxO3a. Reports indicate that MAPK signaling phosphorylates these transcription factors, thereby diminishing their repressive influence on eNOS gene expression [60,61].Supporting the above mentioned interconnected signaling pathways, data from this research demonstrated decreased renal expression of Adropin, MAPK and eNOS in the aging group compared to controls.

Both Adropin and HIF$\alpha$ are crucial for maintaining endothelial function via enhancing VEGF expression through a mechanism involving MAPK signaling [18,62,63]. Consistent with decreased MAPK expression observed in this study and what previously reported about decreased VEGF production with aging in other article [64,65], our findings revealed significantly decreased VEGF protein expression in the aging group compared to controls.

Since HIF$\alpha$ is a key transcription factor that stimulates the production of VEGF and eNOS [63], and considering that VEGF and eNOS production is decreased with aging, we hypothesized that HIF$\alpha$ expression may be altered with aging. Therefore, we evaluated the mRNA expression of HIF$\alpha$ and found a significantly diminished expression level in the aging group contrary to controls. In contrast, Guo et al. [66] documented no significant variation in HIF$\alpha$ buildup in brains of young and old rats.

Deciphering the causes of aging and developing novel treatment approaches to cure age-related illnesses and extend lifespan are the main goals of extensive research being conducted nowadays. Many physiological processes in the human body are crucially regulated by the vitamin D endocrine system. In addition to preserving calcium homeostasis and bone health, it has been discovered to have a variety of non-skeletal benefits [67]. Numerous studies have recommended that vitamin D may also prevent the aging process and regulate inflammation, angiogenesis, and immunological function. The preventive benefits of vitamin D supplementation are highlighted by the link between vitamin D insufficiency and atypical vitamin D-related illnesses. Furthermore, the fact that VDR is found in the heart and vascular cells indicates that vitamin D's advantageous effects extend beyond bone health and may even have therapeutic advantages [68].

In this study vitamin D administration exerted an obvious dose-dependent enhancement in the antioxidant capacity as demonstrated by the substantial decline in the MDA levels and the substantial rise in the TAC. In addition, vitamin D administration effectively reduced TNF$\alpha$ mRNA expression levels. Although the potential function of vitamin D as an antioxidant could not be confirmed [69], our findings are consistent with what previously documented in several studies on vitamin D antioxidant as well as anti-inflammatory effects [70–73].

Vitamin D administration exerted also a dose-dependent amelioration of the renal dysfunctions as evidenced by the significantly elevated creatinine clearance levels and the decreased serum creatinine, serum urea, and 24-h urine albumin excretion levels.

Whether vitamin D supplementation benefit patients with chronic kidney diseases continues to be contentious [74,75]. Most of these research have reported on vitamin D effects in diabetic nephropathy, however studies investigating its effects on kidney functions in the context of renal aging are scarce.

Although the mechanisms by which vitamin D targets fundamental pathways in biological aging are still lacking, our study attempts to elucidate an innovative mechanism in parallel to the antioxidant and anti-inflammatory pathways by which vitamin D protects against D galactose-induced renal aging.

Herein, we provide an evidence on vitamin D significant ability to enhance D-galactose induced suppression of Adropin, MAPK, HIF$\alpha$, VEGF and eNOS. The significant positive association of Adropin with vitamin D was also documented by Kaur et al. and Zorlu et al. [76,77]. In addition, it has been reported that vitamin D plays a significant function in the production and secretion of VEGF [68,78]and is observed to be beneficial in enhancing the expression of eNOS gene [79] which support our findings. Contradictory to our findings regarding the increased MAPK expression exerted by Viamin D administration, other studies demonstrated decreased p38 MAPK expression [80] and suppressed MAPK pathway signaling with vitamin D treatment [81,82]. Hence, we believe that Adropin may be the contributing factor to the increased MAPK levels in this experimental setting. Moreover, we add to the existing knowledge that vitamin D could promote VEGF production through upregulating HIF$\alpha$ mRNA expression since VEGF represents the main target of HIF$\alpha$ [83]. As opposed, Gharib et al. [84] reported that vitamin D levels demonstrated a significant inverse relationship with HIF$\alpha$ levels. These seemingly conflicting reports raise the possibility that vitamin D's functions may vary depending on the pathological conditions and call for more research on the vitamin's impact on the kidneys in relation to renal aging to further support or contradict our results.

This study had some limitations as it would be more precise to measure the phosphorylated of p38 rather than assessing total expression of p38. The study also did not address the molecular basis underlying the reported positive association of Adropin with Vitamin D, which would be recommended in the future.

## 5. Conclusion

To conclude, in this research we utilized a D-galactose-induced aging rat model to demonstrate the potential renoprotective effect of vitamin D on aging kidneys. The present research exhibited that vitamin D administration, in this setting, exerted a renoprotective impacts demonstrated by significantly improved kidney functions. Furthermore, we have delineated the involved molecular mechanism underlying the renoprotective effect of vitamin D and shown that this effect is brought on by enhancing Adropin, MAPK, HIF$\alpha$, VEGF and eNOS levels Figure 7.

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This research received no external funding.

## Data availability

All relevant raw data will be freely available by the authors.

## Declaration of competing interest

The authors declare that there are no conflicts of interest.

## CRediT authorship contribution statement

**Heba S. Youssef:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Walaa H. Mohammed:** Writing – original draft, Methodology, Investigation. **Walaa Bayoumie El Gazzar:** Writing – original draft, Writing – review & editing, Validation, Investigation. **Amina A. Farag:** Validation, Resources, Investigation. **Esraa H. Khairat:** Writing – original draft, Methodology, Investigation. **Neama E. Abdelmaksoud:** Resources, Methodology, Investigation. **Manar A. Elkholy:** Resources, Investigation. **Amira Elalfy:** Writing – original draft, Methodology, Investigation. **Tayseer A. Ibrahim:** Writing – original draft, Supervision, Methodology, Investigation.

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