Scientific Research Publishing

Open Journal of Ophthalmology, 2026, 16(1), 44-51
https://www.scirp.org/journal/ojoph
ISSN Online: 2165-7416
ISSN Print: 2165-7408

The Impact of Vitamin D Supplementation on the Expression of Klotho Protein in Diabetic Mice Retina

Haneen Jabaly-Habib[1*], Ronit Heinrich[2], Inbal Dahan[1], Reem Taha[1], Farid Nakhoul[3], Jehard Hashoul[1]

[1]Ophthalmology Department, Tzafon Medical Center, Poriya, Faculty of Medicine, Bar Ilan University, Zfat, Israel
[2]Department of Neuroscience, Ruth and Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
[3]Nephrology Department, Tzafon Medical Center, Poriya, Faculty of Medicine, Bar Ilan University, Zfat, Israel
Email: *hjabaly@gmail.com

How to cite this paper: Jabaly-Habib, H., Heinrich, R., Dahan, I., Taha, R., Nakhoul, F. and Hashoul, J. (2026) The Impact of Vitamin D Supplementation on the Expression of Klotho Protein in Diabetic Mice Retina. *Open Journal of Ophthalmology*, **16**, 44-51.
https://doi.org/10.4236/ojoph.2026.161005

Received: November 19, 2025
Accepted: January 2, 2026
Published: January 5, 2026

Abstract

Klotho protein is a trans-membrane protein, with an antioxidant and anti-aging effect, in addition to its involvement in insulin resistance. Klotho was found to play a role in the normal retinal function in mice. Klotho -/- mice, have changes similar to those seen in age related macular degeneration. exogenous supplementation of vitamin D, increased the expression of Klotho protein in mice kidneys. In a meta-analysis, the results of 15 observational studies provided strong evidence that decreased serum 25(OH)D levels were associated with an increased risk of DR in type 2 diabetes patients. Four groups of DBA/2J mice, seven in each group, were included in our study. Group 1 was given citrate buffer only (the Streptozotozin solvent), group 2 had an induction of diabetes by infusion of Streptozotocin (STZ) as well as Propylene (Vitamin D solvent), group 3 had an induction of diabetes by STZ as well as vitamin D supplementation at the same time, and group 4 had diabetes induction by STZ and 3 weeks later given vitamin D. All the injections (of propylene glycol and of paricalcitrol) were repeated 3 times a week for 12 weeks or until mice were sacrificed. Seven mice were included in each group and all of them survived. 4 males and 3 females in each group except for group 3 that included 4 female and 3 male mice. All mice were sacrificed by ketamine, and 10 - 30 minutes later, their eyes were extracted including the optic nerve through disinsertion of lateral cantal ligament. The globe was later immersed in optimal cutting temperature (OCT) compound, and stored in −70˚C. From the second eye the retina was separated and fixated. slices were subjected to anti KLOTHO ab (ls-b7010, LSBio, France). Control slides were subjected to the

fluorescent antibody only, without the anti Klotho abs. The slices were filmed by fluorescent microscope with a number of filters; Bright field, DAPI, MCHERRY and MRGE. The analysis of these films was done by imaging analysis application FIJI Image. The florescence scores of all groups were compared between each other using Kruskal-Wallis test followed by post-hoc test (Dunn's test) The Mean fluorescence scores of groups 1, 2, 3 and 4 were 252995, 175420, 311890 and 311266, showing significant difference only between groups 2 and 4 ($p = 0.038$). In conclusion, late vitamin D supplementation significantly restored expression of Klotho protein in diabetic mice retina to its basal expression as in wild type mice.

Keywords

Diabetic Retinopathy, Klotho, Vitamin D, Protective Effect, Mice

1. Introduction

Diabetes mellitus (DM) is a common disease in the western world, associated with increase morbidity and mortality [1]. Persistently elevated blood glucose levels causes micro and macro-vascular abnormalities that lead to micro-angiopathy, atherosclerosis, nephropathy, neuropathy and retinopathy [2].

Approximately 90% of patients with type I DM and 60% with type II DM will develop some degree of diabetic retinopathy (DR), after 20 years of disease [3]. Strict glucose control in patients with type 1 DM, reduced all the microvascular complications including diabetic retinopathy [4]. The pathophysiology of DR is not fully understood. It is known that continuously elevated blood glucose levels, is associated with retinal capillary endothelial damage, that includes loss of pericytes and thickening of basement membrane, leading to capillary obstruction and ischemia with endothelial damage and leakage of serum resulting in retinal exudates [5].

There are some key proteins that are considered to play an important role in the development of diabetes complications. These proteins include nitric oxide synthase isoform, nuclear encoded mitochondrial transcription factor A, and Klotho protein [6].

Klotho protein is a trans-membrane protein with an antioxidant and anti-aging effect, in addition to its involvement in insulin resistance [7]. It is mainly expressed in the kidney and in the brain, and is also secreted into the blood. A decrease in Klotho levels is associated with diabetic nephropathy in patients with diabetes, as well as in atherosclerosis, including peripheral vascular disease [8]. In humans, the Klotho protein was detected and found to have a protective anti-oxidant effect on retinal pigmented epithelium cell culture as well as the prevention of vascular endothelial growth factor secretion from the basement membrane [7]. Klotho was found to play a role in the normal retinal function in mice. Klotho -/- mice had changes similar to those seen in age-related macular degeneration [9]. In another study, it was found that a genetic variant of Klotho, KL-VS gene, had a protective

effect against the development of DR in type I diabetes [10].

Classically, vitamin D is involved in calcium and phosphorus homeostasis. However, it was shown that exogenous supplementation of vitamin D, increased the expression of Klotho protein in mice kidneys [11]. Supplementation of vitamin D receptor agonists to mice with renal failure resulted in an increase in serum and urine Klotho protein levels [12]. In this meta-analysis, the results of 15 observational studies provided strong evidence that decreased serum 25(OH)D levels were associated with an increased risk of DR in type 2 diabetes patients [13].

In this study, we wanted to evaluate the effect of vitamin D supplementation on Klotho expression in the retina of diabetic mice and the development of DR.

2. Methods

Four groups of DBA/2J mice, seven in each group, were included in our study. Group 1 was given citrate buffer only (the streptozotocin solvent (STZ)), group 2 had an induction of diabetes by infusion of STZ as well as Propylene (Vitamin D solvent), group 3 had an induction of diabetes by STZ as well as vitamin D (Paricalcitrol) supplementation 0.3 μg/kg at the same time, and group 4 had diabetes induction by STZ and 3 weeks later given Paricalcitrol 0.3 μg/kg [14]. The mean age of the mice was 9 weeks, and all of them were weighed prior to diabetes induction. A concentration of 6.25 mg/ml STZ was prepared in a dark room in a citrate buffer. Group 1 mice were injected with 200 microliter of citrate buffer, and the rest of the groups had STZ injection daily for 5 consecutive days. The injection of STZ was performed within 15 minutes after its preparation, using 1 ml syringe with 25G needle, 35 mg/Kg dose. In the first day the right hip was injected, in the second day injection was given at the left hip, etc. 5 - 7 days following STZ injection, all the mice developed diabetes with glucose levels above 240 mg%, and these mice were followed for weight, dehydration, respiratory rate, mobility, alertness and kyphotic posture as an indication of distress for early sacrifice.

Group 2 mice, following development of diabetes were given propylene glycol only (Vitamin D solvent) of a volume similar to the injected paricalcitrol; group 3 mice were given 0.3 μg/kg of paricalcitrol injection (nearly one week following the last STZ injection) and group 4 mice were given 0.3 μg/kg of paricalcitrol 3 weeks after diabetes diagnosis.

All the injections (of propylene glycol and of paricalcitrol) were repeated 3 times a week for 12 weeks or until mice were sacrificed. The injections were given in alternating fashion, first on the right hip, then on the left hip .

All mice were sacrificed by ketamine, and 10 - 30 minutes later, their eyes were extracted including the optic nerve through disinsertion of lateral cantal ligament. One eye of each mouse was kept in −70˚C freezer. After enucleation, the periocular fat of the eyeball was removed, and the globe was immersed in a bottle of paraformaldehyde 4% for one hour. Then the globe was transferred into 15% sucrose solution for one hour, then into 20% concentration for 1 hour, then it was kept in 30% sucrose solution for 16 hours in a 4˚C refrigerator overnight. The

globe was later immersed in optimal cutting temperature (OCT) compound, and stored in −70˚C. From the second eye, the retina was separated and fixated in the following steps: Cornea, lens, and vitreous were extracted, the retina was gently removed and four longitudinal cuts were made through the retina, and then the retinal strips were stored in Eppendorf tube at −70˚C.

Immune-florescence staining: 12Micron slices were performed in cryostat through the frozen globes and each slice was loaded on a slide and stored at −20˚C temperature for 16 hours. The next day, the slices were taken out from −20˚C freezer to room temperature, and after 30 minutes, washed 3 times with PBSX for 5 minutes each. Blocking was done with par film after irrigation with FCS5%. These slices were subjected to anti KLOTHO ab (ls-b7010, LSBio, France), and kept in 4˚C overnight, then flushed with PBSX1 for 5 minutes several times. Fluorescent antibody drops (Donkey Anti-Rabbit IgG, Alexa Flour 595 AffiniPur, France) were added to the slides and covered with Parafilm for 75 minutes. In addition, in each group, control slides were subjected to the fluorescent antibody only, without the anti Klotho abs. The slices were filmed by fluorescent microscope with a number of filters: Bright field, DAPI, MCHERRY and MRGE. The analysis of these films was done using the imaging analysis application FIJI Image.

Statistical analysis: The fluorescence scores of all groups were compared with each other using the Kruskal-Wallis test followed by a post-hoc test (Dunn's test).

3. Results

Seven mice were included in each group and all of them survived. 4 males and 3 females in each group, except for group 3, which included 4 female and 3 male mice. Klotho protein existed in mice retinal tissue, particularly in the nuclear layers of the retina.

Table 1 shows the mean fluorescence scores of groups 1, 2, 3 and 4 that were 252995, 175420, 311890 and 311266, respectively.

Table 2 showed the results of the Kruskal-Wallis test analysis with post-hoc test (Dunn's test), showing significant difference only between groups 2 and 4 ($p = 0.038$).

Table 1. Fluorescein cores in the different groups of mice.

	Group 1	Group 2	Group 3	Group 4
	164141.14	231966.34	217211.84	420078.51
	225152.76	173127.27	215117.82	346219.12
	253313.81	205011.58	319582.42	374476.36
	619974.80	143689.53	247508.74	123867.77
	154380.90	167931.34	789649.76	334746.86
	112513.14	102987.88	151137.68	282723.79
	241488.91	203230.33	243024.01	296753.98
Mean	252995	175420	311890	311266

Table 2. Kruskal-Wallis with post-hoc test (Dunn's test).

Comparison	Mean Rank1	Mean Rank2	Rank Diff	Z-statistic	P value	P value bonferroni
Group1 vs Group2	13.00	8.29	4.71	1.0722	0.283645	1.000000
Group1 vs Group3	13.00	16.43	−3.43	−0.7798	0.435533	1.000000
Group1 vs Group4	13.00	20.29	−7.29	−1.6570	0.097522	0.585134
Group2 vs Group3	8.29	16.43	−8.14	−1.8519	0.064036	0.384219
Group2 vs Group4	8.29	20.29	−12.00	−2.7292	0.006350	0.038099
Group3 vs Group4	16.43	20.29	−3.86	−0.8772	0.380363	1.000000

Note: P-values are adjusted using Bonferroni correction for multiple comparisons (6 comparisons total). Significance level: $\alpha = 0.05$.

4. Discussion

This study investigated the impact of vitamin D supplementation on Klotho protein expression in the retina of diabetic mice. Our results demonstrate that delayed vitamin D supplementation (administered 3 weeks after diabetes induction) (group 4) significantly increased Klotho expression in the diabetic retina compared to control group (group 2) ($p = 0.038$). Interestingly, early vitamin D supplementation (concurrent with diabetes induction) (group 3) showed a trend toward increased Klotho expression that did not reach statistical significance ($p = 0.38$).

The timing-dependent effect observed in our study warrants careful consideration. The significant increase in Klotho expression with delayed vitamin D supplementation (Group 4) compared to the non-significant trend with early supplementation (Group 3) suggests that the diabetic microenvironment may need to be established before vitamin D can effectively upregulate Klotho expression. This could reflect several mechanisms: (1) diabetes-induced cellular stress may prime retinal cells to respond more robustly to vitamin D; (2) early metabolic chaos during acute hyperglycemia may interfere with vitamin D signaling pathways; or (3) the vitamin D receptor system may require time to adapt to the diabetic state before effectively responding to supplementation.

The protective effects of increased Klotho expression in diabetic retinopathy likely involve multiple pathways. Klotho's antioxidant properties may counteract oxidative stress, a key pathogenic mechanism in diabetic retinopathy [6] [7]. The protein's ability to suppress VEGF secretion from retinal pigment epithelium (RPE) cells by inhibiting insulin-like growth factor-3 (IGF-3) signaling and phosphorylation of VEGF receptor 2 (also referred to as VEGF receptor 4 in some sources). This ultimately prevents neovascularization and helps maintain normal morphol-

ogy of the choroidal layer [7] [15].

The retinal microvascular complications in diabetes, including pericyte loss, basement membrane thickening, capillary obstruction, and vascular leakage [5], may be mitigated by Klotho's vascular protective effects. By maintaining endothelial integrity and reducing inflammation, increased Klotho expression could interrupt the pathophysiological cascade leading to diabetic retinopathy.

Several limitations should be acknowledged. First, our sample size was relatively small (seven mice per group), which may have limited statistical power, particularly for detecting the effect in Group 3. Second, we assessed Klotho expression at a single time point; longitudinal assessment would provide more comprehensive information about the temporal dynamics of vitamin D's effect on Klotho expression. Third, we did not assess functional outcomes such as retinal vasculature integrity, electroretinography findings, or histological markers of diabetic retinopathy, which would strengthen the clinical relevance of our findings.

Additionally, the study utilized STZ-induced diabetes, which primarily models type 1 diabetes. The applicability of these findings to type 2 diabetes, which has different pathophysiology, remains to be established. Nevertheless, these findings likely extend to type 2 diabetic retinopathy, as both forms share common downstream mechanisms including oxidative stress, chronic inflammation, and pathological neovascularization driven by VEGF upregulation, regardless of the underlying diabetic etiology [8].

The dose and route of vitamin D administration (paricalcitol at 0.3 μg/kg intraperitoneally) may also differ from typical human supplementation strategies.

Future research should investigate several key questions. First, dose-response studies are needed to determine optimal vitamin D supplementation regimens for maximizing retinal Klotho expression. Second, functional studies assessing retinal vascular permeability, neuronal function, and histological changes would establish whether increased Klotho expression translates to improved retinal outcomes. Third, studies in type 2 diabetes models would broaden the applicability of these findings.

Long-term studies examining whether sustained vitamin D supplementation prevents or delays diabetic retinopathy development would be particularly valuable. Mechanistic studies elucidating the molecular pathways through which vitamin D regulates Klotho expression in retinal cells could identify additional therapeutic targets. Finally, clinical trials in diabetic patients examining the relationship between vitamin D supplementation, serum Klotho levels, and retinopathy progression are warranted.

5. Conclusion

In conclusion, this study demonstrates that delayed vitamin D supplementation significantly increases Klotho protein expression in the diabetic mouse retina. These findings provide experimental support for the epidemiological association between vitamin D deficiency and diabetic retinopathy risk, suggesting that Klotho

upregulation may represent a mechanistic link. The timing-dependent effect observed highlights the complexity of vitamin D's effects in the diabetic state and suggests that supplementation strategies may need to be tailored to disease duration. Further research is needed to translate these findings into clinical applications for preventing or treating diabetic retinopathy.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Wong, J., Constantino, M. and Yue, D.K. (2014) Morbidity and Mortality in Young-Onset Type 2 Diabetes in Comparison to Type 1 Diabetes: Where Are We Now? *Current Diabetes Reports*, **15**, Article No. 566. https://doi.org/10.1007/s11892-014-0566-1

[2] Henning, R.J. (2018) Type-2 Diabetes Mellitus and Cardiovascular Disease. *Future Cardiology*, **14**, 491-509. https://doi.org/10.2217/fca-2018-0045

[3] Song, S.H. (2016) Significant Retinopathy in Young-Onset Type 2 vs. Type 1 Diabetes: A Clinical Observation. *International Journal of Clinical Practice*, **70**, 853-860. https://doi.org/10.1111/ijcp.12789

[4] Sun, S., Hisland, L., Grenet, G., Gueyffier, F., Cornu, C., Jaafari, N., *et al.* (2022) Reappraisal of the Efficacy of Intensive Glycaemic Control on Microvascular Complications in Patients with Type 2 Diabetes: A Meta-Analysis of Randomised Control-Trials. *Therapies*, **77**, 413-423. https://doi.org/10.1016/j.therap.2021.10.002

[5] Leley, S.P., Ciulla, T.A. and Bhatwadekar, A. (2021) Diabetic Retinopathy in the Aging Population: A Perspective of Pathogenesis and Treatment. *Clinical Interventions in Aging*, **16**, 1367-1378. https://doi.org/10.2147/cia.s297494

[6] Wang, K., Mao, Y., Lu, M., Liu, X., Sun, Y., Li, Z., *et al.* (2022) Association between Serum Klotho Levels and the Prevalence of Diabetes among Adults in the United States. *Frontiers in Endocrinology*, **13**, Article 1005553. https://doi.org/10.3389/fendo.2022.1005553

[7] Saito, Y., Yamagishi, T., Nakamura, T., Ohyama, Y., Aizawa, H., Suga, T., *et al.* (1998) Klotho Protein Protects against Endothelial Dysfunction. *Biochemical and Biophysical Research Communications*, **248**, 324-329. https://doi.org/10.1006/bbrc.1998.8943

[8] Tang, A., Zhang, Y., Wu, L., Lin, Y., Lv, L., Zhao, L., *et al.* (2023) Klotho's Impact on Diabetic Nephropathy and Its Emerging Connection to Diabetic Retinopathy. *Frontiers in Endocrinology*, **14**, Article 1180169. https://doi.org/10.3389/fendo.2023.1180169

[9] Kokkinaki, M., Abu-Asab, M., Gunawardena, N., Ahern, G., Javidnia, M., Young, J., *et al.* (2013) Klotho Regulates Retinal Pigment Epithelial Functions and Protects against Oxidative Stress. *The Journal of Neuroscience*, **33**, 16346-16359. https://doi.org/10.1523/jneurosci.0402-13.2013

[10] Słomiński, B., Ryba-Stanisławowska, M., Skrzypkowska, M., Myśliwska, J. and Myśliwiec, M. (2018) The KL-VS Polymorphism of KLOTHO Gene Is Protective against Retinopathy Incidence in Patients with Type 1 Diabetes. *Biochimica et Biophysica Acta* (*BBA*)—*Molecular Basis of Disease*, **1864**, 758-763. https://doi.org/10.1016/j.bbadis.2017.12.015

[11] Chen, H., Zhang, Y., Miao, Y., Song, H., Tang, L., Liu, W., *et al.* (2024) Vitamin D Inhibits Ferroptosis and Mitigates the Kidney Injury of Prediabetic Mice by Activating the Klotho/p53 Signaling Pathway. *Apoptosis*, **29**, 1780-1792. https://doi.org/10.1007/s10495-024-01955-4

[12] Lau, W.L., Leaf, E.M., Hu, M.C., Takeno, M.M., Kuro-o, M., Moe, O.W., *et al.* (2012) Vitamin D Receptor Agonists Increase Klotho and Osteopontin While Decreasing Aortic Calcification in Mice with Chronic Kidney Disease Fed a High Phosphate Diet. *Kidney International*, **82**, 1261-1270. https://doi.org/10.1038/ki.2012.322

[13] Lu, L., Zou, G., Chen, L., Lu, Q., Wu, M. and Li, C. (2021) Elevated NLRP3 Inflammasome Levels Correlate with Vitamin D in the Vitreous of Proliferative Diabetic Retinopathy. *Frontiers in Medicine*, **8**, Article 736316. https://doi.org/10.3389/fmed.2021.736316

[14] Nakhoul, N., Thawko, T., Farber, E., Dahan, I., Tadmor, H., Nakhoul, R., *et al.* (2020) The Therapeutic Effect of Active Vitamin D Supplementation in Preventing the Progression of Diabetic Nephropathy in a Diabetic Mouse Model. *Journal of Diabetes Research*, **2020**, Article ID: 7907605. https://doi.org/10.1155/2020/7907605

[15] Ahmed, O.M., Ali, T.M., Abdel Gaid, M.A. and Elberry, A.A. (2019) Effects of Enalapril and Paricalcitol Treatment on Diabetic Nephropathy and Renal Expressions of TNF-α, P53, Caspase-3 and Bcl-2 in STZ-Induced Diabetic Rats. *PLOS ONE*, **14**, e0214349. https://doi.org/10.1371/journal.pone.0214349