Effects of Vitamin D Receptor Knockout and Vitamin D Deficiency on Corneal Epithelial Wound Healing and Nerve Density in Diabetic Mice

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Diabetic keratopathy occurs in ~70% of all people with diabetes. This study was designed to examine the effects of vitamin D receptor knockout ($VDR^{-/-}$) and vitamin D deficiency (VDD) on corneal epithelial wound healing and nerve density in diabetic mice. Diabetes was induced using the low-dose streptozotocin method. Corneal epithelial wounds were created using an Algerbrush, and wound healing was monitored over time. Corneal nerve density was measured in unwounded mice. $VDR^{-/-}$ and VDD diabetic mice (diabetic for 8 and 20 weeks, respectively) had slower healing ratios than wild-type diabetic mice. $VDR^{-/-}$ and VDD diabetic mice also showed significantly decreased nerve density. Reduced wound healing ratios and nerve densities were not fully rescued by a supplemental diet rich in calcium, lactose, and phosphate. We conclude that $VDR^{-/-}$ and VDD significantly reduce both corneal epithelial wound healing and nerve density in diabetic mice. Because the supplemental diet did not rescue wound healing or nerve density, these effects are likely not specifically related to hypocalcemia. This work supports the hypothesis that low vitamin D levels can exacerbate preexisting ophthalmic conditions, such as diabetes.

Diabetes can result in a number ophthalmic complications, including retinopathy, cataract, and uveitis (1,2). Diabetic keratopathy, with symptoms including reduced corneal sensitivity and impaired epithelial wound healing, occurs in ~70% of all people with diabetes (3,4). Nerve changes in diabetic corneas have been well described and include reduced nerve density, thin nerve fibers, and impaired nerve migration (5–8). Neurotrophic keratopathy can lead to blindness (9–12). Clinical studies have demonstrated that corneal nerve regeneration can occur after interventions to improve diabetic control (13–15).

Vitamin D is a fat-soluble prohormone that is synthesized in the skin in response to sunlight (16) and is the precursor to the potent steroid hormone calcitriol [1,25(OH)$_2$D3] (17). Vitamin D is primarily involved in mineral ion homeostasis (18) and has been demonstrated to regulate a wide range of physiological and pathological processes, including cell growth, migration, immune response modulation, and differentiation (19–22). 1,25(OH)$_2$D3 is the naturally occurring ligand of the vitamin D receptor (VDR), a nuclear receptor and a clinically validated drug target. VDR has been found to be involved in controlling epidermal stem cells and progeny during cutaneous wound repair (20). Moreover, 1,25(OH)$_2$D3 and synthetic analogs (e.g., calcipotriol) are approved treatments for numerous diseases, including psoriasis, a common autoimmune disorder (16).

Low serum vitamin D concentration levels have been detected in those with type 1 and type 2 diabetes (23–27). Reduced serum vitamin D binding protein levels are also associated with diabetes (28). An inverse association between 25-hydroxy vitamin D [25(OH)D] concentration and incident diabetes has been proven to be highly consistent (29). A number of trials have examined the possibility that vitamin D supplementation has the capacity to modify the development of diabetes. Early studies focused mainly on the effects of vitamin D deficiency (VDD) in animal models and humans on insulin secretion and glucose tolerance (30,31). Recently, vitamin D was found to improve glucose homeostasis and reduce weight (32). Vitamin D reduces diabetes incidence in nonobese diabetic mice (33) and may have a combined role with VDR

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in children at increased genetic risk for type 1 diabetes (25). Moreover, vitamin D–catabolizing CYP24A1 was found to be elevated in diabetic mouse kidneys, leading to lower vitamin D levels in these mice (34).

Extrarenal tissue–specific local production of vitamin D has been demonstrated in a number of tissues (35). Our laboratory has previously demonstrated that corneal epithelium, which is directly exposed to the sun in the same manner as the skin, has the enzymatic components and ability to generate and activate vitamin D. The VDR, CYP24A1, CYP27B1, and five vitamin D metabolites (36,37) have all been detected in the cornea and anterior segment. $1,25(OH)_2D3$ has been found to inhibit neovascularization and modulate angiogenesis, inflammatory responses (21), age-related macular degeneration (38), myopia (39), diabetic retinopathy (40,41), and antitumor activity in retinoblastoma (42). VDR knockout ($VDR^{-/-}$) mice exhibit delayed skin wound healing (43–45). Our laboratory has demonstrated that $VDR^{-/-}$ mice have significantly slower corneal epithelial wound healing rates than wild-type (WT) mice (46). Interestingly, topically applied vitamin D has been found to decrease the corneal epithelial wound healing rate in a mouse model where acute inflammation is necessary for efficient wound closure (21).

Vitamin D is essential for calcium homeostasis. Serum calcium levels are reduced in $VDR^{-/-}$ mice (47). Calcium restriction has been found to enhance the deficit in skin wound healing caused by VDR deletion (43), and we found that a supplemental diet rich in calcium, lactose, and phosphate rescued the corneal wound healing deficit in $VDR^{-/-}$ mice (46). This same diet was found to rescue impaired mucin packaging in conjunctival goblet cells (48). The current study is the first in our knowledge to examine how VDD and $VDR^{-/-}$, along with combined calcium supplementation, affect diabetic corneas.

RESEARCH DESIGN AND METHODS

Animals

All animal studies were approved by the Augusta University institutional animal care and use committee, and animals were treated according to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. $VDR^{-/-}$ mice were bred from breeding pairs ordered from The Jackson Laboratory (stock no. 006133; Ellsworth, ME). All animals were housed in standard conditions with a 12-h dark-light cycle. If not otherwise stated, at the end of the experiments, the mice were killed with CO_2 inhalation and neck dislocation, and tissues were collected and frozen in liquid nitrogen.

Measurement of the Serum 25(OH)D

The serum level of 25(OH)D was measured using a 25(OH) D enzyme immunoassay kit (Immunodiagnostic Systems, Tyne and Wear, U.K.) according to the manufacturer's protocol.

Measurement of the Serum Calcium Concentration

Serum calcium concentration was measured using a calcium colorimetric assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol.

Animal Groups

The low-dose streptozotocin (STZ) injection method was used to induce diabetes in WT and $VDR^{-/-}$ mice. Males and females were used in our studies. Five sequential daily intraperitoneal injections of a freshly prepared solution of STZ in 0.1 mol/L citrate buffer (pH 4.5) at 60 mg/kg body weight were administered to 4-week-old mice. Blood glucose was measured 1 week after the final STZ injection. We limited the mice receiving corneal epithelial wounds to those with blood glucose levels >249 and <651 mg/dL (49).

The first group was composed of 8-week diabetic duration WT and $VDR^{-/-}$ mice. The second group was composed of 8-week diabetic $VDR^{-/-}$ mice fed a supplemental diet high in calcium, lactose, and phosphate (20% lactose, 2% calcium, 1.25% phosphate) (TD.96348 Diet; Envigo, Tampa, FL) previously shown to alleviate many of the $VDR^{-/-}$ phenotypical features (50,51). The supplemental diet was initiated at the end of the STZ protocol. The third group was composed of 10- and 20-week diabetic WT mice, which were fed a VDD diet (TD.89123 Diet; Envigo) initiated at the end of the STZ protocol. The control group for the VDD mice was fed the TD.89124 vitamin D control diet (Envigo).

Wound Healing

After anesthesia with isoflurane (Tec 4 vaporizer; Anesthesia Service and Equipment, Atlanta, GA), a 2-mm central epithelial wound was made with an Algerbrush. To monitor wound size, a drop of fluorescein sodium 0.25% with proparacaine hydrochloride 0.5% was applied to the wounded eye. An antibiotic ointment (bacitracin, neomycin, polymyxin) and 1 mg/kg buprenorphine (intramuscular) were used after every procedure. Wounds were photographed with a Topcon slit lamp (SL-D4; Topcon Medical Systems, Inc., Oakland, NJ) at 0, 18, 24, 40, 48, 72, and 96 h or until the wounds were healed. Wounds were manually traced using cellSens Dimension (Olympus, New Orleans, LA) software, and wound areas were measured at each time point. Wound areas were scaled to the initial wound area, and the wound healing ratio is defined as the percent change in wound area at a given time, where a ratio of 1 represents a fully healed wound.

Nerve Immunofluorescence Staining and Imaging

To stain corneas for sensory nerves (52), eyes were enucleated from nondiabetic 12-week-old WT and $VDR^{-/-}$ mice, 8-week diabetic WT and $VDR^{-/-}$ mice, 10-week diabetic VDD mice and their control counterparts WT mice. Eyes were fixed with Zamboni fixative (American MasterTech Scientific, Lodi, CA) for 75 min followed by washing with PBS (three times). Corneas were carefully excised along the sclera corneal rim and subjected to

a rehydration series with increasing concentrations of Triton X-100 in PBS. To block nonspecific binding, corneas were incubated with 10% normal goat serum plus 0.1% Triton X-100 solution in PBS for 60 min at room temperature. Tissue was then incubated with primary rabbit polyclonal anti-β III tubulin (ab18207; Abcam, Cambridge, MA) (1:500) antibody in PBS containing 5% goat serum plus 0.1% Triton X-100 for 24 h at room temperature and constantly shaken. After washing with PBS (three times for 10 min each), the corneas were incubated with the secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Thermo Fisher Scientific, Norcross, GA) for 24 h at 4°C and washed thoroughly with PBS. To image the corneas, three radial cuts were made on each cornea, and the tissue was mounted flat on a slide with the epithelium side up. Images were taken with a Zeiss LSM 780 Confocal Microscope (Zeiss, Oberkochen, Germany). Stromal and basal epithelium z-stack images were collected, and nerves were analyzed from the zone just under the basal epithelium.

Nerve densities were measured using the method described by He et al. (7). Images were taken just below the central cornea epithelium using a confocal microscope (four pictures relative to the center: left, right, top, bottom). The most central half of each photo was traced in Adobe Photoshop. The average percentage of pixels traced, among the four central images, was defined as the nerve density value of the image.

Statistical Analysis

Wound healing was assessed using the parallelism test after curve fitting with the Weibull growth model (JMP Pro 14 software; SAS Institute, Cary, NC), and comparisons were made between genotypes and diet type using each data set. One-way ANOVA and independent *t* tests were used to analyze all other data (GraphPad Software, La Jolla, CA).

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

Serum 25(OH)D Concentrations

Figure 1*A* shows 25(OH)D concentrations in individual VDD mice and their WT controls. 25(OH)D concentrations were undetectable in 11 of 15 mice fed the vitamin D–free diet for 10 weeks and in 12 of 16 mice fed the same diet for 20 weeks (assay lower detection limit 16.3 nmol/L). Figure 1*B* shows the effect of diabetes on serum 25(OH)D concentrations. The mean ± SE serum 25(OH)D concentration in WT mice was 121.7 ± 14.57 nmol/L ($n = 12$). Mean 25(OH)D concentrations for 8-, 10-, and 20-week diabetic duration WT mice were 104.2 ± 12.88, 103.9 ± 15.31, and 74.73 ± 4.512 nmol/L ($n = 12$, 6, and 20), respectively. Only the mean 25(OH)D concentration of the 20-week diabetic WT group was significantly different from that of the normal WT group (*t* test, $P < 0.05$). The 20-week diabetic WT value of 74.73 nmol/L would be considered vitamin D insufficient for mice (53).

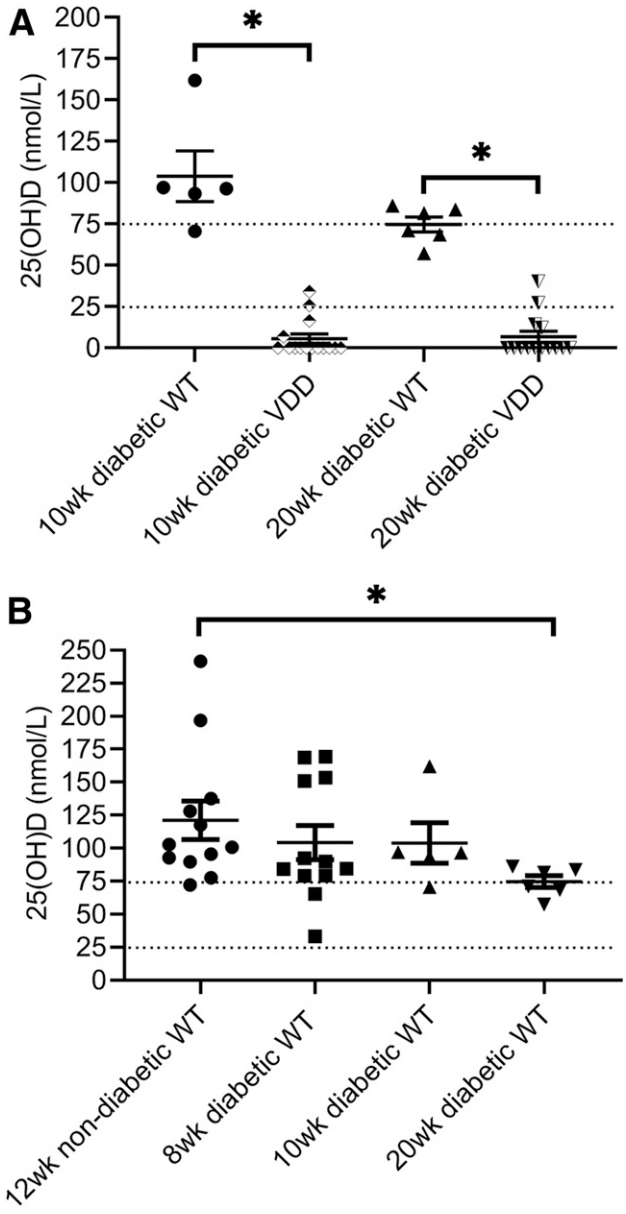

Figure 1—Serum vitamin D concentrations in diabetic mice. *A*: The vitamin D concentration was significantly decreased in mice fed the VDD diet for 10 ($n = 15$) or 20 ($n = 16$) weeks (wk) and in the majority of mice was below the assay's detection limit. *B*: Only the mean 25(OH)D concentration of the 20-week diabetic WT 25(OH)D concentration was significantly different from that of the normal WT value ($n = 12$, 6, and 20 for 8-, 10-, and 20-week diabetic duration WT mice, respectively). The dotted lines represent the vitamin D insufficiency range [serum 25(OH)D >25 nmol/L and <75 nmol/L]. *$P < 0.05$.

Serum Calcium Concentrations

Figure 2*A* shows calcium concentrations in individual diabetic VDD mice, and Fig. 2*B* shows calcium concentrations in individual $VDR^{-/-}$ mice fed the supplemental

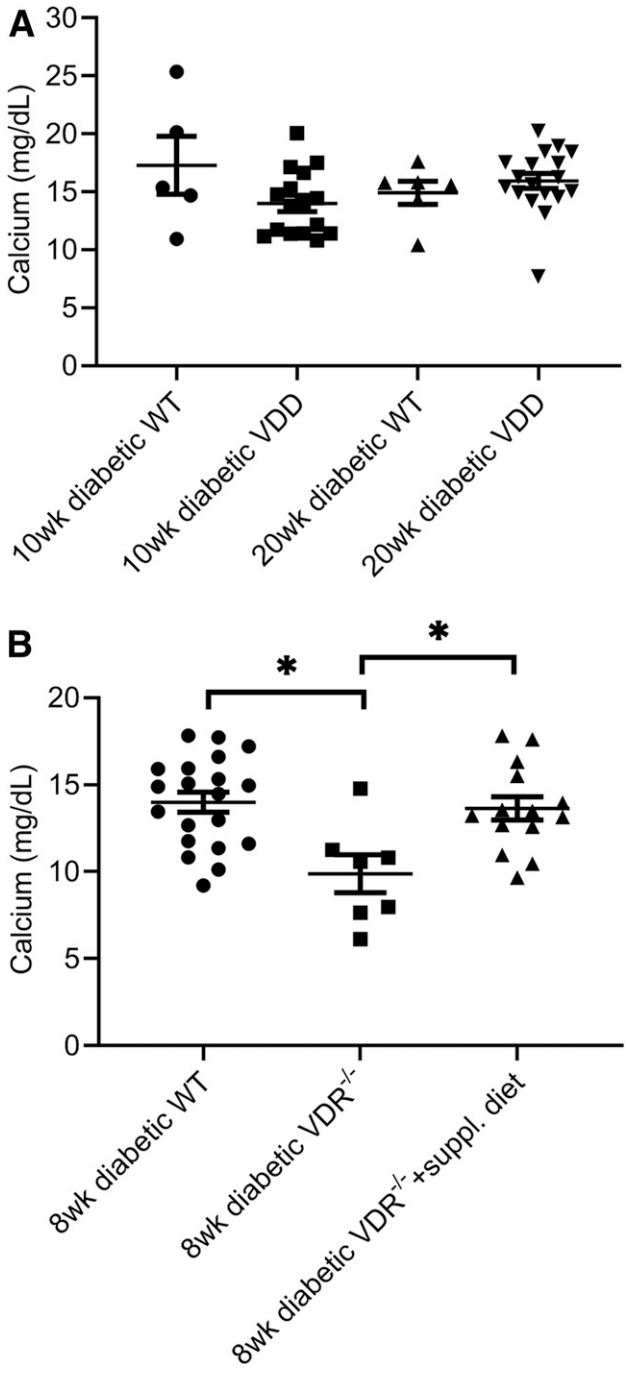

Figure 2—Effect of VDD and VDR$^{-/-}$ on diabetic mouse serum calcium and vitamin D concentrations. (All mice in figure were diabetic.) *A*: Serum calcium concentrations were not significantly different between diabetic WT mice ($n = 6$) and diabetic VDD mice fed the VDD diet for 10 ($n = 15$) or 20 ($n = 16$) weeks (wk). *B*: The serum calcium concentration was significantly decreased in 8-week diabetic VDR$^{-/-}$ mice ($n = 8$) and was increased to the control diabetic level when VDR$^{-/-}$ mice were fed the supplemental diet ($n = 15$). *$P < 0.05$.

mineral-rich diet. Mean ± SE values for VDD mice on the diet for 10 and 20 weeks were 13.99 ± 0.69 and 15.93 ± 0.65 mg/dL ($n = 15$ and 16), respectively (Fig. 2*A*). There were no significant differences in mean serum calcium concentration between 10- and 20-week diabetic VDD and WT mice or between 10- and 20-week diabetic WT mouse groups ($n = 6$ for both groups).

The mean ± SE 8-week-old VDR$^{-/-}$ mouse serum calcium concentration was 9.876 ± 1.09 mg/dL ($n = 8$), which was significantly lower than the age-matched diabetic WT group (14.00 ± 0.58 mg/dL, $n = 20$) (t test, $P < 0.05$). Diabetic VDR$^{-/-}$ mice on the supplemental diet had a mean serum calcium concentration of 13.64 ± 0.66 mg/dL ($n = 15$), which was not significantly different from that of the diabetic WT group (t test, $P = 0.69$) and was significantly higher than the diabetic VDR$^{-/-}$ group (t test, $P < 0.05$).

Effects of VDR$^{-/-}$ and Supplemental Diet on Diabetic Mouse Corneal Wound Healing

Figure 3*A* shows representative wound healing photos of the different diabetic WT and VDR$^{-/-}$ mouse groups. Diabetic WT mice typically healed on average in <48 h, while diabetic VDR$^{-/-}$ mice and the VDR$^{-/-}$ mice on the supplemental diet were often not completely healed at 96 h after surgery. Total healing times were significantly different between diabetic WT and diabetic VDR$^{-/-}$ mice and between diabetic WT mice and diabetic VDR$^{-/-}$ mice fed the supplemental diet (8-week diabetes duration) (Fig. 3*B*) (one-way ANOVA, $P < 0.01$, $n = 10$). There was no difference in total healing time between the diabetic VDR$^{-/-}$ mice and the VDR$^{-/-}$ mice fed the supplemental diet.

To analyze the wound healing process, the Weibull growth model was used to test parallelism of the different growth curves among different groups. The parallelism test did show significant differences between the healing curves of 8-week diabetic duration WT mice and the diabetic VDR$^{-/-}$ mice ($P < 0.05$, $n = 10$) (Fig. 3*C*). While the supplemental diet shifted the healing curve to the left, it was still significantly different from that of the diabetic WT mice. The diabetic VDR$^{-/-}$ and VDR$^{-/-}$ supplemental diet groups were significantly different from each other ($P < 0.05$, $n = 10$), indicating that normalizing calcium levels did result in some reversal of the wound healing deficit.

Effects of VDD on Diabetic Mouse Corneal Wound Healing

Figure 4 illustrates the effects of the duration of hyperglycemia and VDD on epithelial wound healing. There was a significant difference in the wound healing curves between the diabetic WT and WT groups, and between the VDD and the WT groups, with no significant difference between the diabetic WT and diabetic VDD groups (Fig. 4*A*). The 10-week diabetic group data were then analyzed with the equivalence test, which analyzes the asymptotes, inflection points, and growth rate parameters of the fitted curves when the parallelism test itself is not significantly different between groups. If a ratio value falls below the 0.8 (lower decision limit) or above the 1.2 (upper decision limit) ratio threshold, that specific parameter is considered significantly different between the curves. Figure 4*B* shows each parameter level of the fitted curves for the VDD diet versus the diabetic WT mice groups. The test results shown in the graph demonstrate that the growth rate of the

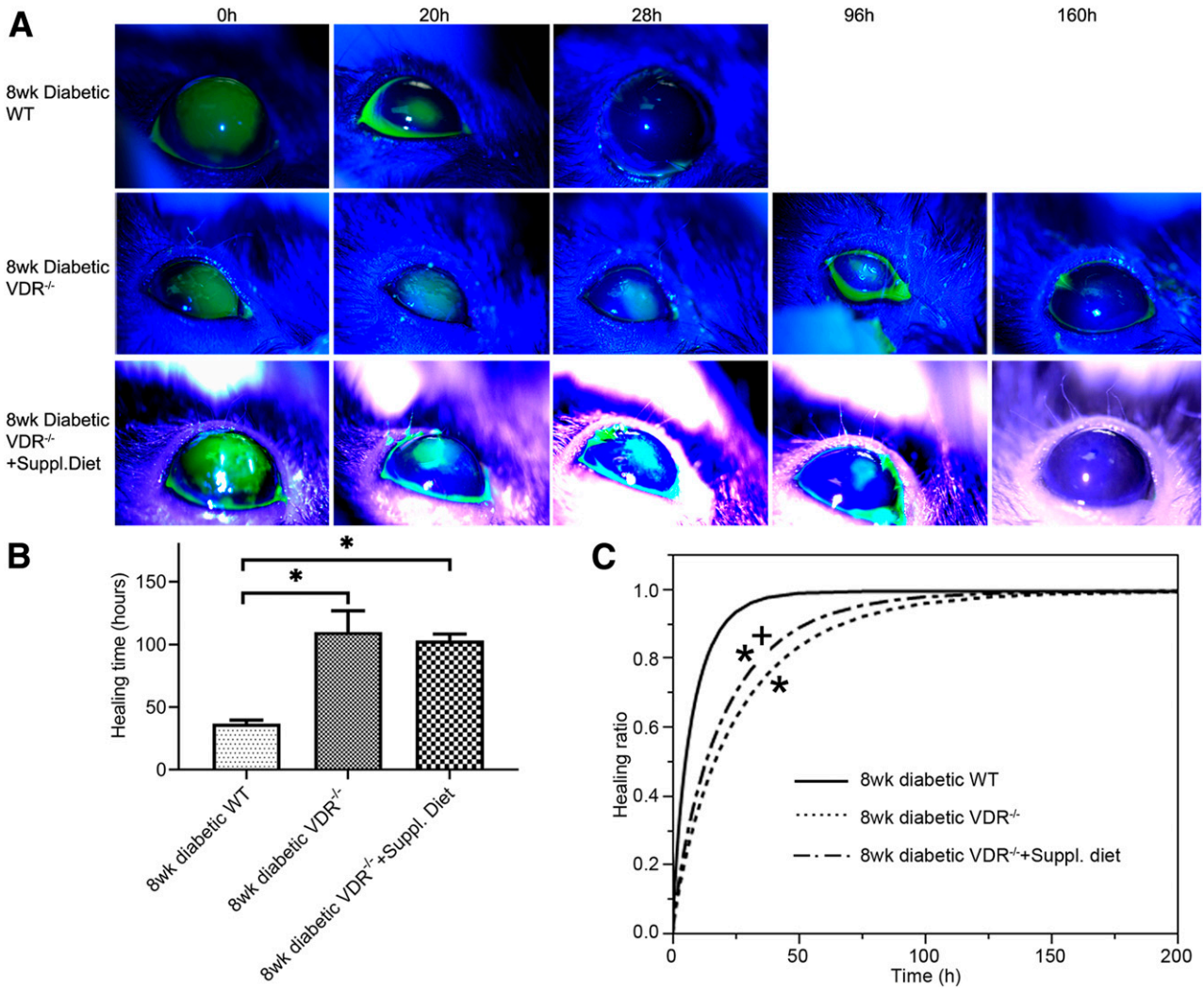

Figure 3—Epithelial wound healing in diabetic VDR$^{-/-}$ and supplemental diet mice. *A*: Representative corneal wound healing slit lamp photographs of diabetic WT, VDR$^{-/-}$, and VDR$^{-/-}$ mice on the supplemental diet (n = 10). *B*: Graph showing the mean time to complete wound healing in both diabetic VDR$^{-/-}$ and VDR$^{-/-}$ mice on the supplemental diet was slower than in diabetic WT controls. Data are mean ± SE. *C*: Healing ratio data were fitted with the Weibull growth model. Healing curves for both diabetic VDR$^{-/-}$ and VDR$^{-/-}$ mice on the supplemental diet were significantly different from the diabetic WT controls (*$P < 0.05$), while the VDR$^{-/-}$ and VDR$^{-/-}$ mice on the supplemental diet were significantly different from each other (+$P < 0.05$), indicating that the supplemental diet only partially reversed the effects of VDR$^{-/-}$.

diabetic 10-week VDD mice (n = 15) was significantly different from that of the diabetic WT mice (n = 10). Figure 4*C* demonstrates that parallelism testing of the wound healing curves of the 20-week diabetic VDD mice (n = 16) versus the diabetic WT mice (n = 10) showed significant differences between the curves ($P < 0.05$). Parallelism testing demonstrated significant differences between normoglycemic WT mice and all diabetic groups at both time points (Fig. 4*A* and *C*).

Effects of VDR$^{-/-}$ and Supplemental Diet on Diabetic Mouse Corneal Nerve Density

These was no significant difference in the nerve density of nondiabetic 12-week-old WT (n = 9) versus VDR$^{-/-}$ mice (n = 10) (Fig. 5*B* and *C*), indicating that VDR$^{-/-}$ alone may not affect corneal nerve density. However, for diabetic mice, the nerve density was significantly decreased in the VDR$^{-/-}$ (n = 6) versus WT (n = 7) groups. The nerve density was also significantly decreased in diabetic VDR$^{-/-}$ (n = 6) versus nondiabetic VDR$^{-/-}$ (n = 10) groups. Feeding the diabetic VDR$^{-/-}$ mice the supplemental diet (n = 10) did not rescue the reduced nerve density in these mice (Fig. 5*A* and *C*).

Effects of VDD Diet on Diabetic Mouse Corneal Nerve Density

Nerve density was significantly decreased in 10-week diabetic VDD mice (n = 8) compared with WT or diabetic WT controls (n = 6) (t test, $P < 0.01$) (Fig. 6*A* and *B*).

DISCUSSION

There have been a number of clinical studies demonstrating a correlation between serum vitamin D levels and diabetes, and vitamin D replacement has been shown to

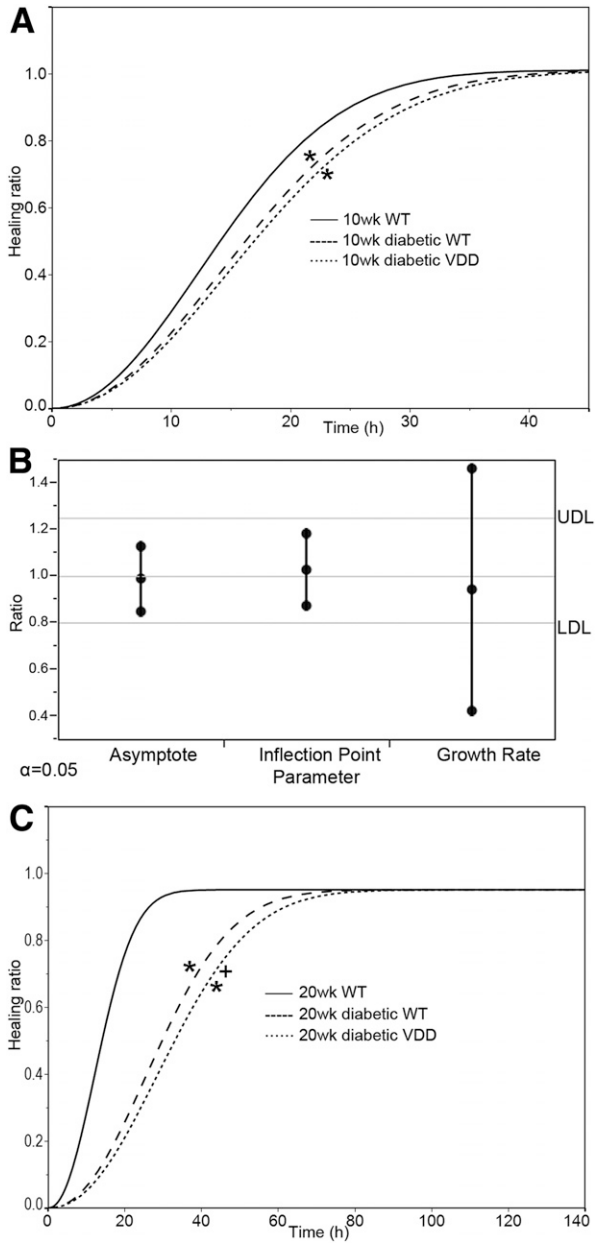

Figure 4—The effects of hyperglycemia and VDD duration on epithelium wound healing. *A*: Wound healing ratio in WT, diabetic WT, and diabetic mice fed VDD diet for 10 weeks (wk) (n = 5, 10, and 15, respectively). Healing ratio data were fitted with the Weibull growth model. The parallelism test showed a significant difference in the overall wound healing curves (i.e., the curves were not considered parallel) for the diabetic WT or VDD group compared with the WT group, and no significant difference between the diabetic WT and diabetic VDD groups. *B*: Follow-up equivalence test on the parallelism data demonstrates a significant difference in the growth rate (equivalent to slope) between diabetic WT mice and 10-week diabetic VDD mice as indicated by both the lower decision limit (LDL) and the upper decision limit (UDL) being exceeded. *C*: Wound healing in diabetic WT mice fed the VDD diet for 20 weeks (n = 5, 10, and 16 for WT, diabetic WT, and VDD, respectively). Parallelism test results showed a significant difference in the wound healing curves of the diabetic WT and VDD groups compared with WT group (*$P < 0.05$), and a significant difference in the wound healing curves of diabetic mice and mice with the same duration of diabetes fed the VDD diet for 20 weeks (+$P < 0.05$).

have beneficial effects in people with type 2 diabetes (54). The Prospective Metabolism and Islet Cell Evaluation (PROMISE) study found that higher baseline 25(OH)D independently predicted better β-cell function and lower area under the curve (glucose) at follow-up, supporting a role for vitamin D in the type 2 etiology of diabetes (55). Cooper et al. (56) demonstrated that three key vitamin D metabolism genes show consistent evidence of association with type 1 diabetes risk, and Kodama et al. (57) provided evidence that vitamin D binding protein is an autoantigen in type 1 diabetes. The current study provides additional links between vitamin D and diabetes, demonstrating that diabetic $VDR^{-/-}$ and VDD mice have impaired corneal epithelial wound healing and reduced corneal nerve densities.

$VDR^{-/-}$ Impairs Diabetic Mouse Corneal Epithelial Wound Healing

A previous study from our laboratory found impaired corneal epithelial wound healing in $VDR^{-/-}$ mice that was rescued by the calcium-enriched supplemental diet examined in the current study (46). The same supplemental diet has also been shown to rescue mucin packaging problems found in $VDR^{-/-}$ mouse conjunctiva cells (48). $VDR^{-/-}$ has been found to delay cutaneous wound healing (20,58) and mucosal wound healing (59). In the current study, corneal epithelial wound healing in diabetic $VDR^{-/-}$ mice was significantly slower than in diabetic WT mice, and the supplemental diet only partially rescued the diabetic $VDR^{-/-}$ mouse healing ratio. This result in diabetic mice supports the hypothesis that loss of vitamin D will exacerbate primary corneal pathologies, in this case diabetic keratopathy. In addition, the partial rescue by the supplemental diet demonstrates that while some of the effects of $VDR^{-/-}$ result from the hypocalcemia associated with $VDR^{-/-}$ (47), there are also calcium-independent issues contributing to the wound healing deficit.

VDD Impairs Diabetic Mouse Corneal Wound Healing

VDD was examined in this study as a less dramatic and more realistic model of vitamin D interactions with diabetic keratopathy than the $VDR^{-/-}$ model. VDD is associated with clinical pathological conditions, including osteomalacia in adults and rickets in children, and much of the work exploring VDD has centered around bone repair and delayed bone mineralization and remodeling (60). The most recent estimates of the prevalence of the U.S. population at risk for VDD [serum 25(OH)D <30 nmol/L] is ~5%, while the prevalence at risk for vitamin D inadequacy [serum 25(OH)D 30–49 nmol/L] is 17.7% (61). VDD was found to aggravate the decreased bone mineral density of diabetic mice (62). In our study, VDD significantly impaired the corneal epithelial healing ratio of 10-week diabetic mice, with a more significant impairment at 20 weeks diabetic duration. This demonstrates that the duration of VDD is an important contributor to

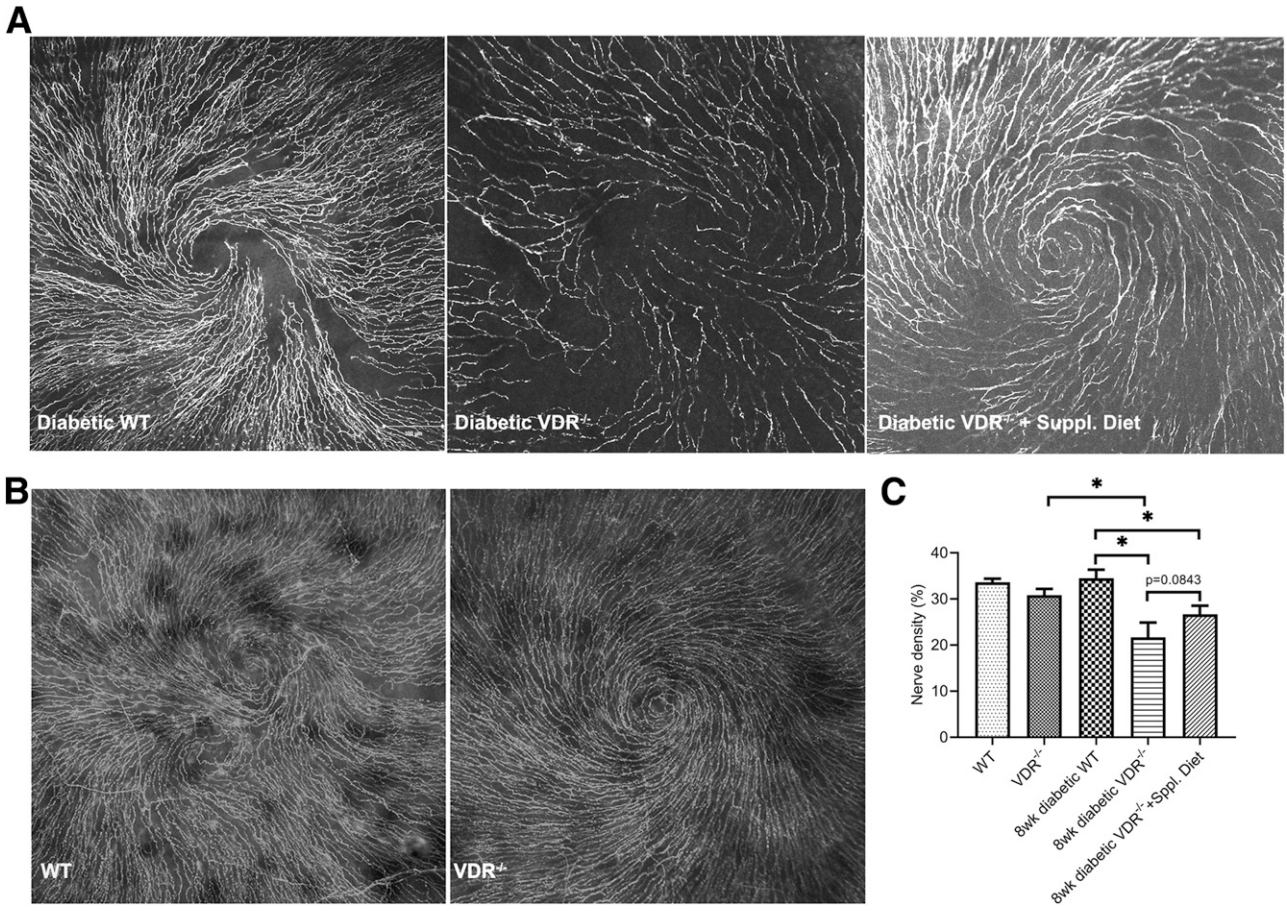

Figure 5—Effect of diabetes, $VDR^{-/-}$, and supplemental diet on mouse corneal nerve densities. *A*: Representative images showing corneal subbasal nerves of 8-week diabetic duration WT, diabetic $VDR^{-/-}$, and diabetic $VDR^{-/-}$ mice on the supplemental diet. *B*: Representative images showing corneal subbasal nerves of 12-week-old nondiabetic WT and $VDR^{-/-}$ mice. *C*: There was no significant difference in nerve densities between WT ($n = 9$) and $VDR^{-/-}$ ($n = 10$) mice or WT and diabetic WT mice ($n = 7$). Diabetic $VDR^{-/-}$ mice ($n = 6$) had a significantly reduced nerve density compared with $VDR^{-/-}$ or diabetic WT mice, and the supplemental diet ($n = 10$) did not correct this reduced nerve density. Data are mean ± SE. $^*P < 0.05$

the impact of VDD on corneal epithelial wound healing. Interestingly, for all these studies, the relatively short duration of diabetes in these mice was long enough to affect the vitamin D interaction with diabetes. Serum calcium was unchanged in these mice compared with their control counterparts, supporting the $VDR^{-/-}$ findings of a calcium-independent effect of low vitamin D and diabetes on corneal epithelial wound healing.

$VDR^{-/-}$ and VDD Reduces Nerve Density in Diabetic Mice

Corneal nerves have important trophic effects on the cornea and play a significant role in the maintenance of a healthy ocular surface through the stimulation of corneal wound healing after corneal injuries (4,63). Corneal nerves can stimulate epithelial cell growth, proliferation, differentiation, and type IV collagen formation through the release of neurotrophins and neuropeptides (64). Decreased corneal nerve function is closely related to poor epithelial healing, reduced lacrimal gland secretion, and epitheliopathy (65,66). Reduced nerve density in the diabetic cornea has been well documented and has been related to diabetic keratopathy (5–7). Human corneas from donors with type 1 diabetes show reduced nerve density that was significantly affected by the duration of diabetes (11). Ten-week diabetic mice have been shown to have a significant decrease in epithelial nerve density with stromal nerve neuropathies (7). Interestingly, the current study found no difference in the nerve density between WT and 8-week diabetic mice (Fig. 5) or in 10-week diabetic mice (Fig. 6). One difference in the previous study was the age of STZ treatment (8 weeks) versus the current study (4 weeks). It is possible that the earlier age of diabetes initiation could have allowed for more plasticity and capacity to produce nerves. It is also possible that this difference is the result of the congenic strain of these mice because all were bred from VDR heterozygotes. The current study did reveal that nerve density was significantly reduced in diabetic $VDR^{-/-}$ and VDD mice compared with their diabetic control counterparts. It is likely that this

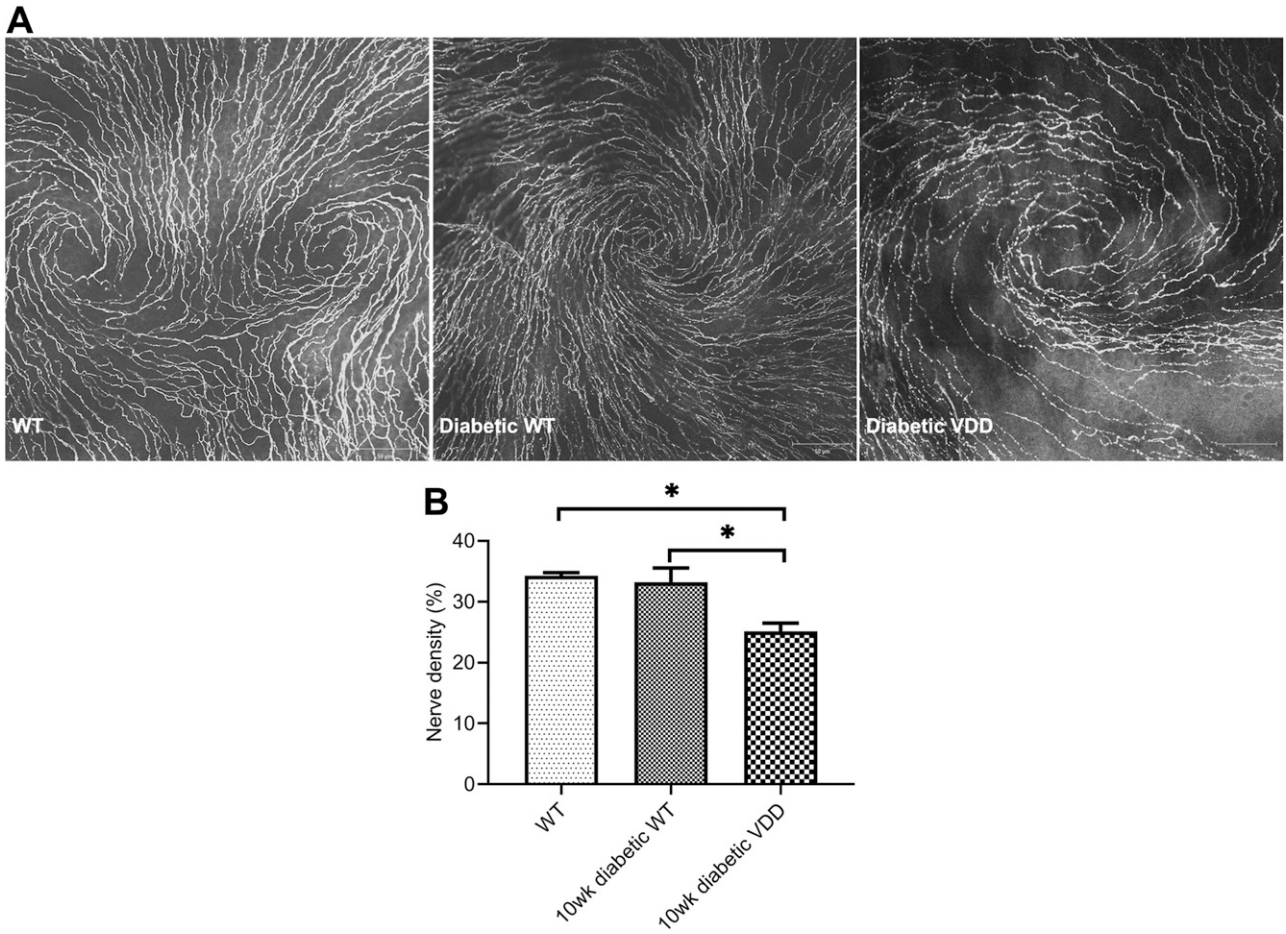

Figure 6—Effects of VDD on mouse corneal nerve densities. *A*: Representative images showing corneal subbasal nerves of 10-week diabetic WT and diabetic VDD mice. *B*: Diabetic VDD mice ($n = 8$) had a significantly reduced average nerve density value compared with WT ($n = 9$) or diabetic WT ($n = 6$) mice. There was no difference observed between the WT and diabetic WT mice. Data are mean ± SE. *$P < 0.05$.

reduced nerve density contributed to the delayed wound healing in these mice. Additional studies will be required to provide data to further support this possibility.

VDR is present in neurons and glial cells (47,67), and $1,25(OH)_2D3$ has been shown to be a potent inducer of nerve growth factor, glial cell line–derived neurotropic factor, and neurotropin-3 (68,69). In addition, the expression of nerve growth factor and glial cell line–derived neurotrophic factor were both found to be reduced in VDD newborn rats (70), and gene expression of nerve growth factor, neurofilament, and γ-aminobutyric acid-A was reduced in the brains of rats that were transiently VDD (47,71). In the current study, there was no significant change in the nerve density of nondiabetic $VDR^{-/-}$ mice compared with their WT controls.

Vitamin D and the VDR play a significant role in calcium homeostasis, and a phenotypical characteristic of $VDR^{-/-}$ mice is hypocalcemia (47). A number of studies have found that placing $VDR^{-/-}$ mice on the supplemental diet high in calcium, lactose, and phosphate used in the current study can reverse this hypocalcemia along with a number of other phenotypical characteristics of these mice (72,73).

In the current study, WT diabetic mice were found to have elevated serum calcium levels compared with their nondiabetic counterparts. Interestingly, the 10-week diabetic mice fed the VDD diet had lower calcium levels than the controls, while the 20-week diabetic mice had normal calcium levels. Diabetic $VDR^{-/-}$ mice on the supplemental diet had significantly higher serum calcium levels than did the diabetic $VDR^{-/-}$ mice on the normal diet. Both the diabetic $VDR^{-/-}$ and VDD diet mice had lower nerve densities than their respective control groups, and the supplemental diet did not reverse the low nerve density in the diabetic $VDR^{-/-}$ mice. We conclude that as in the wound healing response, serum calcium is not a major contributor to the reduced nerve density found in diabetic $VDR^{-/-}$ or VDD mice.

In summary, corneal epithelial wound healing is negatively affected by VDD and $VDR^{-/-}$ in diabetic mice. These results may be related to the reduced nerve density in the diabetic versus nondiabetic mice, which is a widely used clinical marker for early detection of diabetic neuropathy. The impaired wound healing and reduced nerve densities appear to be independent of serum calcium levels. These

results support the hypothesis that VDD can exacerbate pathologies associated with primary ophthalmic pathologies, such as diabetic keratopathy, suggesting that vitamin D levels should be assayed and normalized in patients with corneal pathologies.

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Duality of Interest. No potential conflicts of interest relevant to this article were reported.
Author Contributions. X.L. carried out experiments and drafted the manuscript. S.V. carried out the nerve analysis. Z.C. carried out detection of calcium and vitamin D. J.C. performed the biostatistics analyses and edited the manuscript. M.A.W. designed the study and edited and revised the manuscript. All authors approved the final manuscript. M.A.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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