Glaucoma

The Effect of Dietary Vitamin K1 Supplementation on Trabecular Meshwork and Retina in a Chronic Ocular Hypertensive Rat Model

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Received: October 6, 2019
Accepted: June 22, 2020
Published: July 28, 2020


PURPOSE. The pathophysiologic relationship between vitamin K and glaucoma remains largely unknown. The aim of the study was to explore the effect of dietary vitamin K supplementation in a rat glaucoma model.

METHODS. Rats were randomly divided into two groups: standard diet and high vitamin K1 (VitK1) diet (300 mg VitK1/kg diet). Induction of chronic ocular hypertension by episcleral vein cauterization was performed on the right eye. The left eye with sham operation served as controls. Rats received standard or high VitK1 diets for 5 weeks after surgery until the end of experiment. Immunohistochemistry analyses of the retina and trabecular meshwork were performed. The change in coagulation function and IOP were evaluated.

RESULTS. We observed a significant declined IOP at 2 weeks after surgery in the high VitK1 group compared with the control group. High VitK1 showed no significant effect on the body weight, rat phenotypes, or coagulation function. High VitK1 significantly inhibited the loss of retinal ganglion cells in the retina and increased the expression of matrix gla protein. High VitK1 also ameliorated the collapsed trabecular meshwork structure and increased collagen staining in the trabecular meshwork.

CONCLUSIONS. High VitK1 intake inhibited the loss of retinal ganglion cells during glaucomatous injury, probably by increasing the expression of matrix gla protein. A transient decrease in the IOP was observed in the high VitK1 group, implying a potential effect of VitK1 on aqueous outflow. Retinal ganglion cells protection by high VitK1 supplementation may be due to the IOP-lowering effects as well as neuroprotective effect. Further research is required to delineate these processes.

Keywords: vitamin K1, glaucoma, neuroprotection, matrix gla protein

Glaucoma is a leading cause of irreversible blindness and characterized by progressive damage of retinal ganglion cells (RGCs). Although the pathologic mechanisms of glaucomatous neuropathy have not been fully elucidated yet, it is known that apoptosis of RGCs is the hallmark of glaucoma. The mechanism of RGCs death in glaucoma is complicated, including glial cells activation, mitochondrial dysfunction, oxidative stress, inflammation, autophagy, and so on. Abundant experimental and clinical studies have been carried out to pursue and develop effective neuroprotection therapeutics in glaucoma, which is highly significant and valuable in the field of glaucoma treatment. Hence, innovative approaches of neuroprotection are necessary to develop more potential options for glaucoma treatment.

Oxidative stress is presumed to play a crucial role in the neurodegenerative process of glaucoma. Apart from its neurodegenerative effect on the optic nerve, oxidative stress has also been shown to damage another major target tissue of glaucoma: trabecular meshwork (TM). Oxidative damage to the TM is much greater in patients with glaucoma, which eventually leads to outflow resistance of aqueous humor and an increase in the IOP. Thus, oxidative stress is relevant to the pathophysiology of glaucoma and may contribute to damage in the retina and the TM. For these reasons, the nutrients with antioxidant activity are of great interest in the treatment of glaucoma, and the intake of nutrients is modifiable. Recently, it has been shown that vitamin B3 can modulate mitochondrial vulnerability and prevent RGCs death in acute and chronic glaucoma model, which highlights the importance of some vitamins.

Fat-soluble vitamin K is an essential micronutrient, which is important for the γ-carboxylation of specific glutamic acid residues in a number of vitamin K-dependent proteins within the body, such as the coagulation factors (II, VII, IX, and X, and protein C and protein S), osteocalcin (a bone-forming protein), and matrix Gla protein (MGP).
Dietary supplementation of vitamin K has been shown to significantly delay the progression of coronary artery calcification in the elderly people, indicating the beneficial effect of vitamin K intake for the health of cardiovascular system. A recent study demonstrated that circulating dp-ucMGP (an inactive form of MGP protein that indirectly reflects the low vitamin K status) is a long-term predictor of smaller retinal arteriolar diameter in the general population, which highlights the potential beneficial effect of vitamin K supplementation in retinal health. However, the pathophysiologic relationship between vitamin K and the optic neuropathy in glaucoma remains largely unknown.

The aim of the present study was to explore the influence of dietary vitamin K1 (VitK1) that have anti-inflammatory and antioxidative effects on the progressive damage of RGCs in a rat model of glaucoma. We also determined the effects and possible mechanisms of VitK1 on TM, whose pathology is responsible for the increased IOP and neuropathy in glaucoma.

**Methods**

**Animals and VitK1 Administration**

Adult 6- to 8-week-old male Sprague-Dawley rats, weighing 200 to 250 g, were purchased from the model animal research center of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All animals were raised in a 12-hour light/dark cycle environment with free access to water at the model animal research center of Tongji Hospital. Rats were randomly divided into two groups of 20 rats: standard diet group and VitK1-rich group. The VitK1 was purchased from MedChemExpress Company (HY-N0684; Monmouth Junction, NJ) and diets were synthesized by WQIXBIO Technology Company (Wuhan Wqianjiaxing Biological Technology Company, Wuhan 430030, China). Standard diet ingredients were as follows: 20.7% crude protein, 4.15% crude fat, 2.31% crude fibers, 0.2% compound vitamin, 1.24% calcium, 0.83% inorganic phosphorus, and others. In addition, 3 g VitK1 was added to 10k g standard diet, producing the VitK1-rich diet (300 mg VitK1 per kg diet). Rats had free access to the standard diet and VitK1-rich diet 2 weeks before chronic ocular hypertension (COH) surgery until killing at 5 weeks after the surgery. Rats had free access to the standard diet and VitK1-rich diet 2 weeks before chronic ocular hypertension surgery until killing at 5 weeks after the surgery. Animals were cared for and handled according to the ARVO Statement for the Use of Animals in Vision and Ophthalmic Research and the Animal Care and Use Committee of Huazhong University of Science and Technology.

**COH Glaucomatous Model**

The COH model was established by cauterizing three episcleral vessels of right eyes. The left eyes were sham operated and used as controls. The rats were anesthetized by using intraperitoneal injection of 10% chloral hydrate. The corneas and conjunctivas were topically anesthetized with 2% lidocaine. After making a 2-mm-long incision through the conjunctiva and Tenon’s capsule, three episcleral veins near the superior and temporal rectus muscles were cauterized. The ophthalmic cautery was applied to a point for 1 second on the trunk and branch of the veins. The eyes were treated with antibiotic ointment at the end of the operation.

**IOP Measurements**

The IOP of both eyes was measured using a TONO-PEN XL Tonometer (Reichert, NY) in rats. IOP measurements were always performed between 9 AM and 11 AM on days 0, 7, 14, 21, and 35 after the COH surgery. Eyes were topically anesthetized with 2% lidocaine once before IOP measurements and at least three measurements were taken from each eye. All repeated measurements reached the level with a coefficient of variation of less than 5%.

**Coagulation Assays**

Cardiac punctures were made for blood collection to determine the prothrombin time and activation of partial thrombin time before the rats being killed. Assays were performed using an automated coagulation analyzer (sysmex CA 7000, Siemens Healthineers, Milan, Italy) following standard procedures after thawing the samples at 37°C.

**Serum VitK1 and Total MGP Measurements**

Rat serum or standard solutions were diluted in isopropanol for injection in high-pressure liquid chromatography (HPLC; Nexera UHPLC LC-30A, Japan). VitK1 was separated in reversed-phase HPLC (with a maximum RP C12 column; Phenomenex, Aschaffenburg, Germany). VitK1 was detected at 246 nm and quantified by external standard. All procedures were performed according to established protocol described previously.

Rat MGP ELISA kit (Signalway Antibody LLC, College Park, MD 20740, USA) was used to measure rat plasma total MGP concentrations. All procedures were performed according to the ELISA instructions and the manufacturers’ instructions.

**RGCs Labeling and Quantification**

Retrograde labeling of RGCs was performed according the protocol described by Chiu et al. Briefly, rats were anesthetized and their scalps were shaved. They were placed in a stereotactic apparatus (RWD Life Science, Guangdong, China). Stereotaxic coordinates of superior colliculi were 0.5 mm lateral and 0.5 mm anterior to lambda at a depth of 3.0 to 4.2 mm. Fluro-Gold (Fluorochrome, LLC, Denver, CO) diluted in distilled water (1 µl per injection of 4% wt/vol) was injected into both superior colliculi. Rats were allowed 4 to 5 days for retrograde transport of Fluro-Gold before retina collection to ensure RGCs labeling. At 1 month after COH surgery, retinal flat mount were prepared and Fluro-Gold positive RGCs were identified with a fluorescent microscope (Olympus BX51, Olympus Co. LTD, Tokyo, Japan). Nine pictures were captured in the central retina (1–2 mm from the optic disc), in the middle retina (2–3 mm from the optic disc), and in the peripheral retina (3–4 mm from the optic disc), and the counts were averaged. Surviving RGCs (gold dots) were counted using Image Pro Plus (Version 6.0, Media Cybernetics, Rockville, MD).
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**FIGURE 1.** Schematic representation of the study design. Rats were randomly divided into two groups and received a standard diet or standard diet+VitK1 (300 mg/kg diet) upon initiation. After 2 weeks of accommodation, rat glaucoma model was performed: episcleral vein cauterization was performed on the right eye and the left eye with sham operation served as controls. The rats continued the standard or high VitK1 diets for another 5 weeks. At the end point of the experiment, the eye specimens were collected for histologic analysis and the peripheral blood samples for coagulation assays, ELISA, and HPLC.

**FIGURE 2.** Dietary VitK1 supplementation has no significant effect on the body weight, IOP, and coagulation function. (A) Time course of IOP status after the induction of glaucoma. At 2 weeks after surgery, the IOP showed a significant decrease in the COH+VitK1 group, compared with the COH group. The IOP between the control group and control+VitK1 group also demonstrated a declined trend. No significant difference was observed in IOP either between the control group and control+VitK1 group, or between the COH group and the COH+VitK1 group at other time point after the operation. \( n = 9 \) for each group. (B) The body weight between control and high VitK1 group showed no significant difference. \( n = 9 \) for each group. (C) The effect of VitK1 on the coagulant levels in the normal or high VitK1 group. Prothrombin time (PT), thrombin time (TT), fibrinogen (FIB), activation of partial thrombin time (APTT), and international standardized ratio (INR) exhibited no significant difference. \( n = 5 \) for the Normal VitK1 group. \( n = 6 \) for the High VitK1 group. Two-tailed unpaired \( t \) tests with Welch's correction were used for the statistical analysis. Data are presented as mean ± standard deviation, \(* P < 0.05, ** P < 0.01, *** P < 0.001.\) NS, no significance.
**Figure 3.** Dietary VitK1 supplementation protects RGC loss in COH retina. (A) The representative image of Fluo-Gold labeled RGC cell in control, COH, and high VitK1 retinas. Original magnification ×20. Scale bar = 100 μm. (B) The quantitative analysis of RGC survival. The surviving number of RGCs were counted manually by an independent investigator and automatically by Image J. n = 6 retinas for each group. One-way ANOVA with Dunnett’s multiple comparisons was used. Data are presented as mean ± standard deviation, *P < 0.05, **P < 0.01, ***P < 0.001. (C) The representative immunofluorescence staining of RGC marker Brn3a. A significant reduction of Brn3a+ cells in the COH retina, whereas in the dietary VitK1-supplemented group, the density of Brn3a+ cells returned to levels similar to the contralateral control eyes. Original magnification, ×40. Scale bar = 100 μm. (D) The quantitative analysis of Brn3a+ cells in a 1mm linear region of retina. The Brn3a-labled RGCs were counted manually by independent investigator and automatically by Image J. n = 5 retinas for each group. One-way ANOVA with multiple comparisons was used. Data are presented as mean ± standard deviation, *P < 0.05, **P < 0.01, ***P < 0.001.

**Histologic Examination and Immunofluorescence**

At 4 weeks after COH surgery, rats were sacrificed and the eyes were fixed in 4% formalin and embedded in paraffin. Paraffin sections (5 μm) through the optic disk of each eye were prepared in a standard manner. Sections were stained with hematoxylin and eosin, and Masson according to a modified protocol. For immunohistochemistry analysis, sections were treated with 5% donkey serum albumin for 1 hour at room temperature and incubated at 4°C overnight with primary antibodies (Brn3a, sc-8429, Santa Cruz Biotechnology, Santa Cruz, CA; 1 :50; Iba1, CST17918, Cell Signaling Technology, Danvers, MA; 1:800; GFAP, Ab53554, Abcam, Cambridge, UK; 1:1000; MGP, 10734-1-AP, Proteintech Group, Rosemont, IL; 1 :50; BMP2, A2031, ABclonal Technology, Woburn, MA, 1 :50), then subsequently incubated with DAPI and donkey anti-mouse Alexa Fluor 594 (Thermo, Rockford, IL, 1:200) in PBS at room temperature for 2 hours. The sections were examined with a fluorescent microscope (Olympus Bx51).

**Statistical Analysis**

Statistical analyses were performed using two-tailed pair/unpaired t tests and ordinary one-way ANOVA with Dunnett’s multiple comparisons test by GraphPad Prism software (version 8.0, GraphPad, San Diego, CA). Data were presented as mean ± standard deviation. A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**High VitK1 Supplementation Induced a Transient Decrease of IOP at 2 Weeks After Surgery, While Showing No Significant Effect on Body Weight or Coagulation Function**

The schematic representation of study design is illustrated in Figure 1. Briefly, rats were randomly divided into two groups and received either a standard diet or a standard diet supplemented with VitK1 (300 mg/kg diet) upon initiation. After 2 weeks of accommodation, the right eye was operated to induce COH. After surgery, 5 weeks of the control diet or VitK1-supplemented diet was administrated to respective groups. At the end point of the experiment, the TM and retina were collected for histologic analysis and peripheral blood samples for coagulation assays, ELISA, and detection of VitK1 by HPLC.

During the 5 weeks of the experimental period, both the COH group and the COH+Vit K1 group showed a significant increase in IOP compared with the control group through weekly measurements as illustrated in Figure 2A. At 2 weeks after surgery, the IOP showed a significant decrease in the COH+VitK1 group, compared with COH group (16.25 ± 0.99 mm Hg vs 18.69 ± 1.74 mm Hg; P < 0.0001) (Fig. 2A). Interestingly, the IOP between the control group and control+VitK1 group also demonstrated a decreasing trend (14.49 ± 1.05 vs 13.49 ± 0.86; P = 0.072) (Fig. 2A). However, no significant difference was observed in IOP either between the control group and control+VitK1 group.
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**FIGURE 4.** Dietary VitK1 supplementation protects retinal damage caused by the elevation of IOP. (A) The representative immunofluorescence staining of vitamin K-dependent protein MGP. In the COH retina, the expression of MGP was decreased significantly. High VitK1 intake increased the expression of MGP in the RGC layer of retina. (B) The representative immunofluorescence staining of microglial markers Iba1 and astrocyte activation maker GFAP. A significant increased expression of Iba1 was determined in the retina of COH group. There was no significant effect of high VitK1 supplementation in the expression of astrocyte activation maker GFAP and microglial markers Iba1 in the retina. Original magnification ×40. Scale bar = 100 μm. (C) The quantitative analysis of MGP expression in the retina. High VitK1 significantly increased the expression of MGP in the retina of COH group. The intensity of positive staining area was analyzed by Image J. n = 5 retinas for each group. Unpaired t test with Welch’s correction was used. Data are presented as mean ± standard deviation, *P < 0.05. (D) The quantitative analysis of Iba1 and GFAP expression in the retina. No significant effect of high VitK1 was noticed on the expression of Iba1 and GFAP in COH retina. The intensity of positive staining area was analyzed by Image J. n = 5 retinas for each group. Unpaired t test with Welch’s correction was used. Data are presented as mean ± standard deviation, *P < 0.05, ns, no significance.

Group, or between the COH group and COH+VitK1 group at other time point after the operation. The result indicated that our COH modeling method effectively maintained a significant IOP elevation, and that dietary VitK1 supplementation seemed to have no major effect on IOP. In the high VitK1 group, no increased appetite or food intake, and no other abnormal phenotypic changes, were observed. We monitored weight changes of the rats and found no significant differences between the groups (Fig. 2B).

Vitamin K is known to be important for blood coagulation, as it is essential for the γ-carboxylation of a number of vitamin K-dependent proteins, such as clotting factors II, VII, X, and XII. In clinic, the well-known vitamin K antagonist warfarin has been used for anticoagulation therapy. In this study, VitK1 has shown reliable safety and does not cause hypercoagulability. We have analyzed the blood coagulation function in our model, which confirmed the safety of dietary VitK1. As presented in Figure 2C, the prothrombin time, thrombin time, fibrinogen, activation of partial thrombin time, and international standardized ratio showed no significant difference between the control and high VitK1 groups.

**VitK1 Supplementation Alleviated RGCs Apoptosis in COH Rats**

RGC counting is essential to evaluate retinal degeneration especially in glaucoma and retrograde labeling of RGCs by application of Fluoro-Gold is a reliable RGC labeling method for evaluating the effects of any treatment. We labeled the RGCs with Fluoro-Gold and confirmed the protective effect of VitK1 (Fig. 3A). The control rat retina had an average of 2174 ± 199 RGCs/mm² (n = 6 retinas; Fig. 3B). Compared with the contralateral control eyes, COH eyes (mean 1587 ± 156 RGCs/mm²) had a 27% RGC loss in the retina 5 weeks after IOP elevation (n = 6 retinas; P = 0.0002; Fig. 3B). VitK1 supplementation significantly increased RGC survival by 22% in the retina (mean, 1939 ± 99 RGCs/mm²) compared with those COH retinas (n = 6 retinas; P = 0.0002; Fig. 3B). Brn3a has been shown to be specifically expressed by RGCs. When analyzing the retina with histologic sections, we found a significant reduction of Brn3a⁺ cells in the COH retina compared with that in control retina. Dietary VitK1 supplementation could significantly increase the the density of Brn3a⁺ cells in the COH retina (Fig. 3C, D), indicating that VitK1 supplementation might have potential antiapoptotic effect on RGCs in experimental glaucoma.

In addition, we found a significant decreased expression of vitamin K-dependent protein MGP in the retina of COH group. VitK1 supplementation can significantly increase the expression of MGP in the RGC layers of retinas in COH+VitK1 group (Figs. 4A, C). However, we did not detect significant effect of VitK1 in the expression of astrocyte activation maker GFAP and microglial markers Iba1 in the retina (Figs. 4B, D).
**Figure 5.** Dietary VitK1 supplementation ameliorates the lesions of TM in COH rat. (A) The representative HE staining and Masson staining of anterior angle tissue showed the changes of TM structure in control and high VitK1 supplemented group. We observed the partial collapsed TM and reduced collagen fiber staining in the TM. SC, Schlemm’s canal. Original magnification ×20. Scale bar = 50 μm. (B) The quantitative analysis of collagen staining in the TM. High VitK1 significantly increased the collagen expression in the TM of COH group. The intensity of positive staining area was analyzed by Image J. n = 5 retinas for each group. Unpaired t test with Welch’s correction was used. Data are presented as mean ± standard deviation, *P < 0.05, **P < 0.01, ***P < 0.001. (C) The representative immunofluorescence staining of MGP in TM showed the significant decreased expression of MGP in the TM of COH group, and partially restored expression of MGP in COH+VitK1 group. CB, ciliary body; Co, cornea; Ir, iris; SC, Schlemm’s canal; Sc, sclera. Original magnification ×20. Scale bar = 100 μm. (D) The quantitative analysis of MGP expression in the TM. No significant effect of high VitK1 was noticed on the expression of MGP in the TM of COH group. The intensity of positive staining area was analyzed by Image J. n = 5 retinas for each group. Unpaired t test with Welch’s correction was used. Data are presented as mean ± standard deviation, *P < 0.05. ns, no significance.

**VitK1 Supplementation Ameliorates the Morphology of TM in COH rats**

The TM is a soft tissue formed by different types of endothelial-like cells, which controls the extracellular matrix composition and deposition levels, that have a direct correlation with increased flow resistance and glaucoma. We observed the partial collapsed TM and a significant reduced collagen fiber staining in the TM of COH group (Figs. 5A, B).

Early studies had identified the MGP among the 10 most highly expressed genes in the human TM, and expression of the gene could be altered by the mechanical forces of pressure or by other glaucoma risk factors such as TGF-β and dexamethasone. In the COH eyes, we also detected the significant decreased expression of MGP in the TM (Figs. 5C, D). High VitK1 supplementation improved the collagen fiber staining (Figs. 5A, B), but did not increase the expression of MGP in the TM of COH group (Figs. 5C, D). In addition, the expression of BMP2 and F-actin in the TM showed no significant change between control and high VitK1 group (data not shown). Thus, with dietary VitK1 supplementation, the morphology of the TM seemed to be ameliorated, although the potential mechanism requires need further investigation.

We measured the level of VitK1 in plasma by the method of HPLC, and found that the concentration of VitK1 was nonsignificant increased in the high VitK1 group, compared with that in the control group (0.4 ± 0.52 ng/mL vs 7.62 ± 5.98 ng/mL; P = 0.054) (Figs. 6A, B, C). However, when we detected the level of MGP in the serum by ELISA, no significant difference was found between control and high VitK1 group (Fig. 6D). All these results together indicate the potential neuroprotective role of VitK1 in the RGCs may be a direct effect via circulation system.

**Discussion**

In the present study, we found that dietary VitK1 supplementation is beneficial for glaucoma by targeting the two glaucoma relevant tissues, namely, the RGCs and TM.

First, dietary VitK1 presented protective effect on retina, as the apoptosis of RGCs is partially alleviated confirmed by Fluoro-Gold quantitative analysis. Basic and clinical studies...
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FIGURE 6. Dietary VitK1 supplementation increased the VitK1 level in the circulation, but had no significant effect on serum MGP concentration. Representative chromatograms of VitK1 concentration in the serum of control rats (A) and high VitK1 rats (B), n = 5 for each group. (C) Determination of serum total MGP protein concentration in control and high VitK1 rats by ELISA assay. n = 10 for control, n = 12 for high VitK1 group. Unpaired t tests with Welch’s correction were used for the statistical analysis. Data are presented as mean ± standard deviation. *P < 0.05. ns, no significance.

have demonstrated the importance of appropriate vitamin K status for the health of the vascular system23 and musculoskeletal tissues.24 A recent study showed that circulating inactive dp-ucMGP is a long-term predictor of smaller retinal arteriolar diameter in the general population,15 which highlights the potential role of vitamin K in retinal health. In the present study, dietary VitK1 supplementation can partially protect the apoptosis of RGCs from COH, which further confirmed the neuroprotective role of vitamin K. The beneficial effect of VitK1 may act through its antioxidative and anti-inflammatory effect, which warrants further investigation.

Second, VitK1 supplementation was shown to ameliorate the morphology of TM in COH rats. The physiopathologic changes in the TM are responsible for the main outflow resistance in open-angle glaucoma. Previous studies have confirmed the calcification process in glaucomatous TM22 and aging or other proglaucoma insults can provoke the pathologic calcification by decreasing the expression of vitamin K-dependent protein MGP.25 We found significant
declined IOP at 2 weeks after surgery in the high VitK1 group compared with the control group, and the morphology of TM is obviously ameliorated at 5 weeks in the COH+VitK1 group compared with the COH group (partial alleviation of collapsed TM and decreased collagen fiber staining). We also found a nonsignificant increased expression of MGP in the TM of VitK1 supplemented group. These results reveal the potential long-term effect of VitK1 in ameliorating the lesions of TM, which is important for the regulation of aqueous outflow.

Noticeably, the change of IOP in the COH eyes is not affected significantly by dietary VitK1 supplementation (except at 2 weeks after surgery). The reason may be the limited numbers of rats used in our study. We may also need to increase the concentration of dietary VitK1 to visualize its effect on IOP. Hence, we can also add vitamin K2 to compare and make clear the specific effect of vitamin K subgroup in the future. Importantly, the safety of VitK1 is guaranteed in our study; because of that, (1) the coagulation function is not affected, (2) the biological phenotypes of high VitK1 fed rats showed no abnormal change, and (3) no rats died in the high VitK1 group.

In the future, local administration of VitK1 needs to be tested, because the ocular system is rather a closed system and the topical use of medication is more effective and convenient. The advantage of local use can also avoid the potential systemic side effects of vitamin K, such as hemorrhage. The difficulty is to seek the appropriate delivery system, like nanoparticles. Importantly, the vitamin K regulated proteins, such as MGP, should be highlighted, which opened up an option for the targeted and gene therapy of glaucoma, which warrants further investigation.

In summary, our results demonstrated the beneficial effect of dietary VitK1 supplementation on TM and retina in the rat glaucoma model. Dietary intake of VitK1 may be recommended for glaucoma patients, if the coagulation function is monitored and the safety is guaranteed.

Acknowledgments
The authors thank Hervé Kempf, Chargé de Recherche, CNRS INSERM UMR 7505 CNRS-Université de Lorraine, for his constructive comments and suggestions in revising the manuscript, and for checking and revising the grammar and punctuation error.

Supported by the grants from National Natural Science Foundation of China (No. 81974135).

Disclosure: C. Deng, None; K. Yao, None; F. Peng, None; B. Zhao, None; Z. Chen, None; W. Chen, None; Y. Zhao, None; H. Zhang, None; J. Wang, None

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