Research Article

In Vitro Vitamin K\textsubscript{3} Effect on Conjunctival Fibroblast Migration and Proliferation

I. Pinilla, L. B. Izaguirre, F. J. Gonzalvo, E. Piazuelo, M. A. Garcia-Gonzalez, A. I. Sanchez-Cano, and F. Sopeña

1. Introduction

Antimetabolites and other fibroblast inhibitor drugs have been shown to enhance the success rate of filtering surgery although, depending on the dose, they can lead to severe complications and may result in the failure of the surgery.

Corticosteroids [1–6] antiproliferative agents (5-fluorouracil and other fluoropyrimidines, taxol, doxorubicin, mycophenolate mofetil... alone or in combination or with different delivery systems) [3–12], systemic, periocular, intracocular steroid, and nonsteroidal anti-inflammatory agents [5,13–16], colchicine [8], daunomycin [8], tissue plasminogen activator [17], heparin [12,18–20], interferon-gamma [21,22], calcium channel blockers [23], prolyl and lysyl hydroxylase inhibitors [19,24–26], retinoic acid [27,28], alpha-tocopherol [29–31], disintegrins [32], siRNA-PKC\textsubscript{\alpha} [33]... are some of the useful drugs that have been used in the treatment of conditions such as proliferative vitreoretinopathy, bleb scarring after trabeculectomy, and other disorders with cell proliferation (progressive conjunctival or extraocular cicatriztion).

Vitamin K\textsubscript{3} (menadione, 2-methyl-1,4-naphthoquinone) has been used as antihemorrhagic agent. Its ability to inhibit proliferation of tumor cells has already been reported; its activity has been demonstrated in human tumor stem cell and tissue in clinical trial for advanced malignancies acting in different pathways and has also been related to other oxidative stress processes at the eye level as cataract formation... [34–40]. Liu et al. reported that this drug could inhibit proliferation of rabbit conjunctival cells [41].
The Scientific World Journal

Figure 1: Phase-contrast microphotographs showing the process of wound healing in an ulcer treated with vitamin K₃ 1 mg/L. Lesion time, 0 hour: in vitro ulcer after being produced in a confluent monolayer; 24 hours fibroblasts migrations was evident; 48 hours: some fibroblast were filling the ulcer area.

The aim of this study was to evaluate and to compare the antiproliferative properties of vitamin K₃ in cultured human fibroblasts.

2. Methods

2.1. Material. All supplies for cell culture were purchased from Nunc (Roskilde, DK). Dulbecco's Modified Eagles Medium (DMEM), phosphate buffer saline (PBS), fetal calf serum (FCS), and antibiotics-antifungals were purchased from Gibco (Madison, WI). [methyl-³H]thymidine was purchased from Amersham Iberica (Madrid, Spain). 2-Methyl 1,4-Naphthoquinone (Menadione) (98%) was obtained from Sigma (St. Louis, MO). The drug was initially dissolved in 90% ethanol. This alcohol solution was then diluted into BSS to yield a final ethanol concentration of 0.1%.

2.2. Cell Cultures. Conjunctival fibroblasts were obtained from explants of a healthy adult subject who underwent ophthalmic surgery for retinal detachment. All subjects gave informed consent to participate in the study, which was conducted in accordance with the tenets of the Declaration of Helsinki, and the experimental protocol was approved by the local Ethics Committee of the Aragon Health Science Institute. Cells were cultured in uncoated plastic flasks in DMEM supplemented with antibiotics and antifungals (100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B), and 20% fetal calf serum (FCS) in a humidified atmosphere at 37 degrees Celsius and 5% CO₂. The culture medium was changed every 3 days and the experiments were performed with cells obtained between the 5th and 8th passages.

2.3. Wounding Assays. Wounding assays were performed using the method described by Sato and Watanabe [42]. An artificial wound was made by mechanical cell denudation with a rotating tip, as described in previous papers [26]. The wound repair process was monitored by two independent observers measuring the cell-free area (mm²) in a blind fashion, at different times: 0, 18, 24, and 48 hours (Figure 1). The cell-free area was quantified in the elliptic or circular shape wounds with homogeneous size. Then, the major and the minor axes were measured in a phase-contrast microscopy equipped with a calibrated visor. The area was calculated applying the mathematical formula: area = A × B × π/4, where A and B were the major and the minor axes, respectively.

2.4. Assay of Cell Mitogenic Activity. Freshly trypsinized fibroblasts were seeded in 24-well plates at a density of 15 × 10⁴ cells/well and were incubated for 24 hours in fresh medium and the drugs to be tested. Cells were labeled for the last 3 hours period with 1μCi/mL of [methyl-³H]thymidine.
After removing the media, the cells were washed 3 times with ice-cold PBS and then 2 times with 5% ice-cold trichloracetic acid to precipitate the DNA. The precipitate was dissolved in 500 μL 0.1N NaOH and 0.1% sodium dodecyl sulfate. The extract was neutralized with 0.1N HCl and radioactivity was counted in a liquid scintillation counter (1900 TR, Packard Instrument Company, Meriden, CT).

2.5. Groups of Treatment. There were 6 different treatment groups: group 1/control group: no drugs added; group 2/0.5 μL of ethanol 10% (final ethanol concentration 0.1%); group 3/vitamin K₃ 1mg/L; group 4/vitamin K₃ 2mg/L; group 5/vitamin K₃ 4mg/L; group 6/vitamin K₃ 6mg/L.

2.6. Statistical Analysis. Each experiment was carried out in triplicate and at least 4 times. The results were expressed as mean ± standard deviation (x ± SD). Statistical significances between mean values were assessed with Mann-Whitney U-test. The probability level at which the Null Hypothesis was rejected was set at P < 0.05.

3. Results and Discussion

Wound healing in some ocular diseases and surgeries, as mucous-cutaneous diseases or after glaucoma filtering surgery, or complicated retinal detachment, is one of the problems that needs to be solved. We were studying the effect of vitamin K on wound healing and its possible toxicity.

The wound area is presented in Table 1. There were no differences among the groups at the initial time (Table 1). The mean size was 0.586 mm² ± 0.082. There were no differences between control group and ethanol group. Ethanol 0.1mg/mL did not show effect on fibroblast migration and proliferation. No toxic effect had been related to its use. Vitamin K is a liposoluble drug. This fact can be remarkable in order to use it to assist the effect of the silicone oil on complicated retinal detachments.

Wound healing process can be divided into three phases: inflammation, proliferation, and modulation of the scar. This process begins immediately after the injury. The fibroblast proliferation appears after 24 hours [43]. In this experimental model of wound healing, we evaluate the fibroblast migration 18 hours after the ulcer has been done. In the 24 hours’ time migration and proliferation are evident.

Vitamin K₃ at the doses 2, 4, and 6 mg/L significantly decreased the speed of wound repair during the experiment. There were no differences between control group and vitamin K₃ 1mg/L (Table 2). Vitamin K₃ at the doses 2, 4, and 6 mg/L inhibited the cell migration and proliferation and showed slower closure of the wounds than the other groups (from 18 hours on). Liu et al. found that most of the cells died at concentrations of 7.5mg/L; the concentration of 4.0mg/L inhibited fibroblast growth. The cellular border became clearer and some cells started to die at 5 mg/L [41].

Fibroblast mitogenic activity was significantly inhibited by all vitamin K₃ doses. There were differences between vitamin K₃ 1 mg/mL and all the others vitamin K₃ groups. In this study, vitamin K₃ at 1 mg/L did not show differences with the control group in the speed of wound repair. Fibroblast mitogenic activity was inhibited by all doses of vitamin K₃; differences were found among vitamin K₃ 1 mg/mL and the greater doses. Vitamin K₃ 1 mg/mL is able to inhibit fibroblast
mitogenic activity with no influence in wound repair; this effect has probably been counteracted by its no migration inhibitory effect.

In our study, doses of 4 mg/L induced great cellular alterations. Vitamin K₃, 4 mg/L and 6 mg/L induced cellular toxicity. Cells presented changes in their morphology, which characterized apoptosis, including nuclear and cytoplasmic condensation with intact plasma membrane cell. They lost their adherence to the plate, showing a growing ulcer throughout the time being the ulcer sizes larger than in the other groups. MMC and 5-FU are also able to induce apoptosis in cultured tenon's fibroblast [44].

The application of experimental data derived from cell cultures to clinical use has limitations. Variables such as bioavailability, diffusional barriers, metabolic inactivation, excretion, drug resistance, and enzyme induction prohibit simple extrapolation of cell culture data to human diseases. Nevertheless, this basic approach to drug selection is invaluable.

The mechanism of cytotoxicity of vitamin K₃ is not well known and it has been the focus of multiple papers. The drug is able to affect the cell by two mechanisms. One is its ability to disturb the intracellular calcium flux and calcium-dependent potassium flux [45]. The other is that its chemical transformation within the cell may generate reactive oxygen species and potentially deplete intracellular glutathione [46]. Effects on different cells have been described such as inhibition of PTP-1B in keratinocytes [47], induction of tumor cell death through hydrogen peroxide generation, and regulation of the expression of G1 phase-related cell cycle molecules [48, 49].

We can conclude that all the studied doses of vitamin K₃ were able to inhibit fibroblast mitogenic activity. Vitamin K₃, at 2 mg/L or higher doses, interfere the mechanisms of cell repair, delaying the wound healing process in this in vitro model. Vitamin K₃ at 4 and 6 mg/L in cell culture showed fibroblast toxicity. The drug could be considered an alternative to the drug treatment and prevention of exaggerated scarring in some ocular diseases.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**References**

[1] S. J. Ryan, “Traction retinal detachment XLIX Edward Jackson Memorial Lecture,” The American Journal of Ophthalmology, vol. 115, no. 1, pp. 1–20, 1993.

[2] F. Koerner, A. Merz, B. Gloor, and E. Wagner, “Postoperative retinal fibrosis—a controlled clinical study of systemic steroid therapy,” Graefes Archive for Clinical and Experimental Ophthalmology, vol. 219, no. 6, pp. 268–271, 1982.

[3] A. García-Layana, M. T. Hernando, L. Manzanas, and J. C. Pastor, “Tratamiento profiláctico de la vitreoretinopatía proliferante,” Archives de la Sociedad Española de Ofthalmología, vol. 60, pp. 315–322, 1991.

[4] R. Fiscella, G. A. Peyman, J. Elvart, and B. Yue, “In vitro evaluation of cellular inhibitory potential of various antineoplastic drugs and dexamethasone,” Ophthalmic Surgery, vol. 16, no. 4, pp. 247–249, 1985.

[5] M. S. Blumenkranz, A. Clalin, and A. S. Hajek, “Selection of therapeutic agents for intraocular proliferative disease. Cell culture evaluation,” Archives of Ophthalmology, vol. 102, no. 4, pp. 598–604, 1984.

[6] A. S. Berger, C. K. Cheng, P. A. Pearson et al., “Intravitreal sustained release corticosteroid-5-fluorouracil conjugate in the treatment of experimental proliferative vitreoretinopathy,” Investigative Ophthalmology and Visual Science, vol. 37, no. 11, pp. 2318–2325, 1996.

[7] M. S. Blumenkranz, M. K. Hartzler, and A. S. Hajek, “Selection of therapeutic agents for intraocular proliferative disease. II. Differing antiproliferative activity of the fluoropyrimidines,” Archives of Ophthalmology, vol. 105, no. 3, pp. 396–399, 1987.

[8] C. Verdoorn, V. W. Renardel de Lavalette, J. Dalma-Weizhaus, G. M. Orr, N. Sorgente, and S. J. Ryan, “Cellular migration, proliferation, and contraction. An in vitro approach to a clinical problem—proliferative vitreoretinopathy,” Archives of Ophthalmology, vol. 104, no. 8, pp. 1216–1219, 1986.

[9] C. Heinz, T. Hudde, K. Heise, and K. P. Steuhl, “Antiproliferative effect of mycophenolate mofetil on cultured human Tenon fibroblasts,” Graefes Archive for Clinical and Experimental Ophthalmology, vol. 240, no. 5, pp. 408–414, 2002.

[10] C. Heinz, K. Heise, T. Hudde, and K. P. Steuhl, “Mycophenolate mofetil inhibits human Tenon fibroblast proliferation by guanosine depletion,” The British Journal of Ophthalmology, vol. 87, no. 11, pp. 1397–1398, 2003.

[11] M. Akimoto, T. Miyahara, J. Arai et al., “A new delivery system for 5-fluorouracil using prodrug and converting enzyme,” The British Journal of Ophthalmology, vol. 86, no. 5, pp. 581–586, 2002.

[12] V. Sundaram, A. Barsam, and G. Virgili, “Intravitreal low molecular weight heparin and 5-fluorouracil for the prevention of proliferative vitreoretinopathy following retinal reattachment surgery,” Cochrane Database of Systematic Reviews, no. 1, Article ID CD006421, 2013.

[13] M. Reibaldi, A. Russo, A. Longo et al., “Rhegmatogenous retinal detachment with a high risk of proliferative vitreoretinopathy treated with episceral surgery and an intravitreal dexamethasone 0.7-mg implant,” Case Reports in Ophthalmology, vol. 4, no. 1, pp. 79–83, 2013.

[14] P. Yang, B. S. McKay, J. B. Allen, and G. J. Jaffe, “Effect of NF-κ B inhibition on TNF-α-induced apoptosis in human RPE cells,” Investigative Ophthalmology and Visual Science, vol. 45, no. 7, pp. 2438–2446, 2004.

[15] R. G. Williams, S. Chang, M. R. Comaratta, and G. Simoni, “Does the presence of heparin and dexamethasone in the vitrectomy infusate reduce reproliferation in proliferative vitreoretinopathy?” Graefes Archive for Clinical and Experimental Ophthalmology, vol. 234, no. 8, pp. 496–503, 1996.

[16] W. Chen, H. Chen, P. Hou, A. Fok, Y. Hu, and D. S. C. Lam, “Midterm results of low-dose intravitreal triamcinolone as adjunctive treatment for proliferative vitreoretinopathy,” Retina, vol. 31, no. 6, pp. 1137–1142, 2011.

[17] G. A. Williams, F. H. Lambrou, G. A. Jaffe et al., “Treatment of postvitrectomy fibrin formation with intraocular tissue activator,” Archives of Ophthalmology, vol. 106, no. 8, pp. 1055–1058, 1988.

[18] R. N. Johnson and G. Blankenship, “A prospective, randomized, clinical trial of heparin therapy for postoperative intraocular fibrin,” Ophthalmology, vol. 95, no. 3, pp. 312–317, 1988.
D. G. Charteris, G. W. Aylward, D. Wong, C. Groenewald, R. L. Izaguirre, I. Pinilla, I. Gonzalvo, S. Pérez, and F. M. Honrubia, J. W. Doyle, R. K. Dowgiert, and S. M. Buzney, "Factors J. J. Araiz, M. F. Refojo, M. H. Arroyo, F. L. Leong, D. M. Albert, and A. Danielsson, "Inhibition of hepatic fibrogenesis: a review of pharmacologic candidates," Scandinavian Journal of Gastroenterology, vol. 29, no. 5, pp. 385–391, 1994.

L. Iazguirre, I. Pinilla, F. Gonzalvo, S. Pérez, and F. M. Honrubia, "Effect of doxorubicin on fibroblast migration and proliferation," Annals of Ophthalmology, vol. 35, no. 1, pp. 48–52, 2003.

J. J. Araiz, M. F. Refojo, M. H. Arroyo, F. L. Leong, D. M. Albert, and D. M. Albert, "Antiapoptotic effects of retinoic acid in in vitro studies on keloid tissue fibroblasts," Investigative Ophthalmology and Visual Science, vol. 34, no. 3, pp. 567–575, 1993.

J. Wu and A. Danielsson, "Inhibition of hepatic fibrogenesis: a review of pharmacologic candidates," Scandinavian Journal of Gastroenterology, vol. 29, no. 5, pp. 385–391, 1994.

L. Iazguirre, I. Pinilla, F. Gonzalvo, S. Pérez, and F. M. Honrubia, "Effect of doxorubicin on fibroblast migration and proliferation," Annals of Ophthalmology, vol. 35, no. 1, pp. 48–52, 2003.

J. J. Araiz, M. F. Refojo, M. H. Arroyo, F. L. Leong, D. M. Albert, and D. M. Albert, "Antiapoptotic effects of retinoic acid in in vitro studies on keloid tissue fibroblasts," Investigative Ophthalmology and Visual Science, vol. 34, no. 3, pp. 567–575, 1993.

J. W. Doyle, R. K. Dowgiert, and S. M. Buzney, "Factors modulating the effect of retinoids on cultured retinal pigment epithelial cell proliferation," Current Eye Research, vol. 11, no. 8, pp. 753–765, 1992.

J. M. Larrosa, A. A. S. Veloso Jr., F. L. Leong, and M. F. Refojo, "Antiproliferative effect of intravitreal α-tocopherol and α-tocopheryl acid-succinate in a rabbit model of PVR," Current Eye Research, vol. 16, no. 10, pp. 1030–1035, 1997.

J. M. Larrosa, V. Polo, T. Ramírez, I. Pinilla, L. E. Pablo, and F. M. Honrubia, "Alpha-tocopherol derivatives and wound healing in an experimental model of filtering surgery," Ophthalmic Surgery and Lasers, vol. 31, no. 2, pp. 131–135, 2000.

I. Pinilla, J. M. Larrosa, V. Polo, E. Piazuelo, P. Jiménez, and F. M. Honrubia, "Comparison of fibroblast inhibitory effect of α-tocopherol succinate and 13-cis retinol," Annals of Ophthalmology, vol. 34, no. 2, pp. 108–112, 2002.

C. H. Yang, T. F. Huang, K. R. Liu, M. S. Chen, and P. T. Hung, "Inhibition of retinal pigment epithelial cell-induced retinal detachment by disintegrins, a group of Arg-Gly-Asp-containing peptides from viper venom," Investigative Ophthalmology and Visual Science, vol. 37, no. 5, pp. 843–854, 1996.

Q. Gao, W. Wang, Y. Lan et al., "The inhibitory effect of small interfering RNA protein kinase C-α on the experimental proliferative vitreoretinopathy induced by dispase in mice; International Journal of Nanomedicine, vol. 8, pp. 1563–1572, 2013.

R. T. Chlebowski, S. A. Akman, and J. B. Block, "Vitamin K in the treatment of cancer," Cancer Treatment Reviews, vol. 12, no. 1, pp. 49–63, 1985.

D. Lim, R. J. Morgan Jr., S. Akman et al., "Phase I trial of menadiol diphosphate (vitamin K3) in advanced malignancy," Investigational New Drugs, vol. 23, no. 3, pp. 235–239, 2005.

P. Oztopçu, S. Kabadere, A. Mercangöz, and R. Uyar, "Comparison of vitamins K1, K2, and K3 effects on growth of rat glioma and human glioblastoma multiforme cells in vitro," Acta Neurologica Belgica, vol. 104, no. 3, pp. 106–110, 2004.

M. Ishibashi, M. Araí, S. Tanaka, K. Onoda, and T. Hirano, "Antiproliferative and apoptosis-inducing effects of lipophilic vitamins on human melanoma A375 cells in vitro," Biological and Pharmaceutical Bulletin, vol. 35, no. 1, pp. 10–17, 2012.

B. R. Acharya, D. Choudhury, A. Das, and G. Chakrabarti, "Vitamin K3 disrupts the microtubule networks by binding to tubulin: a novel mechanism of its antiproliferative activity," Biochemistry, vol. 48, no. 29, pp. 6963–6974, 2009.

A. M. Marchionatti, G. Picotto, C. J. Narvaez, J. Welsh, and N. G. T. de Talamoni, "Antiproliferative action of menadione and 1, 25(OH)2D3 on breast cancer cells," Journal of Steroid Biochemistry and Molecular Biology, vol. 113, no. 3-5, pp. 277–232, 2009.

K. R. Hegde and S. D. Varma, "Combination of glyceric and oxidative stress in lens: implications in augmentation of cataract formation in diabetes," Free Radical Research, vol. 39, no. 5, pp. 513–517, 2005.

X. H. Liu, X. W. Song, Y. Xu, and C. Zhang, "The inhibition of vitamin K3 on rabbit fibroblast proliferation in vitro," Ophthalmologica, vol. 210, no. 3, pp. 180–182, 1996.

N. Sato and S. Watanabe, "Influence of extracellular matrix in gastric mucosal repair in vitro," Biochemical and Biophysical Research Communications, vol. 202, no. 1, pp. 285–292, 1994.

R. J. Kirnsner and H. Eaglestein, "The wound healing process," Dermatologic Clinics, vol. 11, no. 4, pp. 629–640, 1993.

J. G. Crowston, A. N. Akbar, P. H. Constable, N. L. Occleston, J. T. Daniels, and P. T. Khaw, "Antimetabolite-induced apoptosis in Tenon's capsule fibroblasts," Investigative Ophthalmology and Visual Science, vol. 39, no. 2, pp. 449–454, 1998.

E. Kawamura, N. Hirashima, T. Furuno, and M. Nakanishi, "Effects of 2-methyl-1,4-naphthoquinone (Menadione) on cellular signaling in RBL-2H3 cells," Biological and Pharmaceutical Bulletin, vol. 29, no. 4, pp. 605–607, 2006.

H. Morrison, B. Jerndtrom, and M. Nordenskjold, "Induction of DNA damage by menadione (2-methyl-1,4-naphthoquinone) in primary cultures of rat hepatocytes," Biochemical Pharmacology, vol. 33, no. 11, pp. 1763–1769, 1984.

J. I. Beier, C. von Montfort, H. Sieg, and L. O. Klotz, "Activation of ErbB2 by 2-methyl-1,4-naphthoquinone (menadione) in human keratinocytes: role of EGFR and protein tyrosine phosphatases," FEBS Letters, vol. 580, no. 7, pp. 1859–1864, 2006.

C. Lin, J. Kang, and R. Zheng, "Vitamin K3 triggers human leukemia cell death through hydrogen peroxide generation and histone hyperacetylation," Pharmazie, vol. 60, no. 10, pp. 765–771, 2005.

S. Kuriyama, M. Hitomi, H. Yoshii et al., "Vitamins K2, K3 and K5 exert in vivo antitumor effects on hepatocellular carcinoma by regulating the expression of G1 phase-related cell cycle molecules," International Journal of Oncology, vol. 27, no. 2, pp. 505–511, 2005.