·Basic Research·

Inhibition of human pterygium fibroblasts *in vitro* by vitamin E succinate

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Abstract

• AIM: To study the antiproliferation of vitamin E succinate (VES) on pterygium fibroblasts *in vitro* and to find a potential agent for prevention and treatment of primary and recurrence pterygium.

• METHODS: Primary culture and subculture of pterygium fibroblasts were established *in vitro*, and different concentrations of VES (0, 10 and 20mg/L) were added to subcultured fibroblasts, respectively. Influence of VES on the growth curve of fibroblast was observed at day 2, 4 and 7 after treatment of VES. 3- [4,5-Dimethylthiazolzyl]-2,5-Diphenyl Tetrazolium Bromide (MTT) assay at 490nm was used to evaluate the effect of the cells proliferation.

• RESULTS: The addition of VES to culture caused the marked descent of growth curve in comparison with the control group, and the inhibiting rate of 10 and 20mg/L of VES was 33.2% and 46.7%, 67.9% and 76.8%, 81.7% and 89.3% at day 2, 4 and 7, respectively. VES could obviously inhibit the fibroblast proliferation in dose-dependent manner by MTT assay.

• CONCLUSION: VES can significantly inhibit the proliferation of pterygium fibroblast *in vitro*.

• KEYWORDS: vitamin E succinate; ptergium; fibroblasts; cell culture

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INTRODUCTION

Pterygium consists of epithelium of bulbar conjunctiva and hypertrophied subconjunctival connective tissue. Although described and recognized before 1000 B.C, its pathogenesis still remains an ophthalmic enigma. Generally it is considered to be a cause of corneal blindness. The treatment of pterygium is still quite controversial, although the methods of pterygium surgery changes from the simplest bare sclera excision to stratified keratoplastics and transplantation technique [1,2]. Unfortunately, there are very few well-conducted controlled clinical trials of treatments. Moreover, years of anecdotal and noncontrolled studies have confirmed that some methods such as bare scleral closure are no longer acceptable in the treatment of pterygium and that other methods are likely to be more useful [3]. In the future, it will be important to develop a grading system, and surgeons will need to be conservative in the treatment of pterygium until such time as a single treatment provides lower recurrence rate and complication rate. Vitamin E, known for its antioxidative properties, has been shown the inhibition of cell proliferation in a variety of tumor cell lines *in vitro* studies[4]. However, the value of vitamin E succinate (VES) against pterygium has not been previously examined. The purpose of this study was to examine the effect of VES on the pterygium fibroblasts *in vitro*, providing a basis for new potential adjuvant and palliative drugs for pterygium.

MATERIALS AND METHODS

Materials and Reagents VES (+)-α-tocopherol acid succinatel was purchased from Sigma (St. Louis, MO, USA). Cell culture dishes, plates, centrifuge tubes and other plastic ware were purchased from Becton Dickinson (Lincoln Park, NJ, USA). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, fetal bovine serum(FBS) and EDTA were purchased from Life Technologies (Burlington, ON, Canada). Cell identity markers and antibodies were from Sigma (rabbit-anti-human vimentin and goat anti-rabbit IgG- Cy3), and 3- [4,5-Dimethylthiazolzyl]-2,5-Diphenyl Tetrazolium Bromide (MTT) was also from Sigma.

Cell Culture and Identification Human pterygium tissues samples from the patients undergoing pterygia excision surgery resection ptergium tissues were from the department

of ophthalmology of our hospital (Zhongshan Ophthalmic Center, Guangzhou, China). These tissues were separated from the epithelium and were preserved in DMEM at 4℃ until they were processed for culture. Pterygium fibroblasts were grown from explants using a modification of previously described method [5]. In brief, pterygium tissues were rinsed with Hank's balanced solution containing 1×10^5U/L penicillin and 100mg/L streptomycin. After carefully removing the fascia, the remaining pterygium was cut into several equal pieces (1mm×1mm) and directly placed into a well of 6-well-culture plate or into a 35-mm dish. They were covered with a drop of FBS for 6 hours. The explants were then cultured in DMEM supplemented with 100mL/L FBS, penicillin(1×10^5U/L) and streptomycin(100mg/L) , 2mmol/L L-glutamine at 37℃ under 50mL/L CO_2 and 950mL/L humidity. The medium was renewed every 2-3 days. When the cells became confluent, cells were detached by incubation with 2.5g/L trypsin-EDTA solution and reseeded for subcultures. In this study, cells between passages 3 or 4 were used. Fibroblasts were identified by their characteristic fusiform appearance in confluent cell culture and by positive immunofluorescence staining with vimentin antibody by fluorescence microscope.

Growth Studies Harvested cells were plated onto 24-well plates at a density of 2×10^7 cells/L and allowed to preincubate in normal medial at 37℃ for 24 hours. After preincubation, cells were exposed to medium containing various doses (0-20mg/L) of VES diluted in normal media (with 5mL/L ethanol as carrier) for 2, 4 and 7 days, respectively. The medium was renewed every day. On the day of the assay, the cell numbers were counted with a hemocytometer every 4 wells. The same experiments were repeated for 3 times. Growth curves were done by the mean of cell numbers, and cell proliferation inhibitory rate was calculated by the following formula: (1-cell numbers in VES/cell numbers in control)×100%.

MTT Assay The viability of cells in the presence or absence of VES was analyzed in microplates using an assay based on the reduction of MTT. It has been widely assumed that mitochondrial dehydrogenases in living cells convert soluble MTT into an insoluble blue formazan product that can be dissolved in isopropanol and color intensity measured spectrophotometrically [6]. Briefly, MTT was dissolved in PBS at 5g/L and filtered to remove a small amount of insoluble residue. Harvested cells were plated onto 96-well plates at a density of 3×10^7 cells/L and allowed to preincubate in normal medial at 37℃ for 24 hours. After preincubation, cells were exposed to various doses (0-20mg/L) of VES into a formazan product by metabolically active cells for 2, 4 and 7 days, respectively. On the day of the assay, 20mL of MTT solution was added to each well, and the plates were incubated at 37℃ for 4 hours. The wells were drained and 150μL of DMSO was added to each well to dissolve the crystalline formazan product. The plate was gently mixed for 3 minutes or until all crystals were dissolved. Absorbance was then measured at 490nm. This assay was used to test the effects of different culture conditions on cell viability and growth and to establish a dose-response relationship for the effect of VES. In all plates cell-free controls were run in the presence and absence of VES.

Statistical Analysis Data were presented as mean±SEM. Significance was tested using a two-sample t-test with SPSS 11.0 statistical soft ware (SPSS Inc, Chicago, IL, USA). $P<0.05$ was deemed significant.

RESULTS

Cultivation of Human Pterygium Fibroblasts After the explants inoculated for 48 hours, some cells were derived from areas of pterygium tissue. On the 4-5 days, cells grew around the tissues showing aristate formation (Figure 1). The cells were mainly fusiform with a small quantity of round or polygonal cells. At 2 weeks, the cells showed reticular forming (Figure 2). After the 3 passages, the cells were fusiform approximately on the whole and grew faster. Immunohistochemical studies were carried out to investigate the characteristic of cells. Immunofluorescence of vimentin exhibited positive and the staining located in cell cytoplasm with red fluorescence, which was parallel with the long axis of the cell (Figure 3).

Growth Curve of Pterygium Fibroblasts Experiments on the growth curve were done at the 3-4 passages of cells which were cultured in VES (0, 10 and 20mg/L) (Figure 4). The growth curve contained logarithmic growth phase (24-96 hours after inoculation) and plateau phase (after 96 hours) in control. The cell proliferated more rapidly than in VES, but the growth curve down-regulated and rose slowly in VES. There was no significant logarithmic growth phase or plateau phase. Moreover, various time point exposure to VES inhibited pterygium fibroblasts proliferation in a concentration-dependent manner. In general, greater inhibition of cellular proliferation occurred with increased exposure time. The inhibition rate were 33.2%, 67.9% and 81.7% on 2, 4 and 7 days in 10mg/L respectively and 46.7%, 76.8% and 89.3% on 2, 4 and 7 days in 20mg/L, respectively.

MTT Assay As showed in Table 1, compared with the control, VES inhibited pterygium fibroblasts proliferation in a concentration-dependent manner. Greater inhibition of cellular proliferation was seen with longer VES exposure.

DISCUSSION

The impact of pterygium on vision is evident. Although surgical resection following stratified keratoplastics and transplantation technique is potentially curative, approximately

Table 1 Effect of VES on human pterygium fibroblast (mean±SEM, n=12)

VES(mg/L)	*A* value			Inhibition rate (%)		
	day 2	day 4	day 7	day 2	day 4	day 7
0	0.284±0.03	0.402±0.01	0.476±0.02	-	-	-
10	0.195 ±0.02[b]	0.157±0.02[b]	0.103 ±0.02[b]	31.5	61.2	78.4
20	0.195 ±0.02[b]	0.116± 0.02[b]	0.086 ±0.01[b]	45.1	71.3	81.9

[b]P<0.01 *vs* 0 concentration group

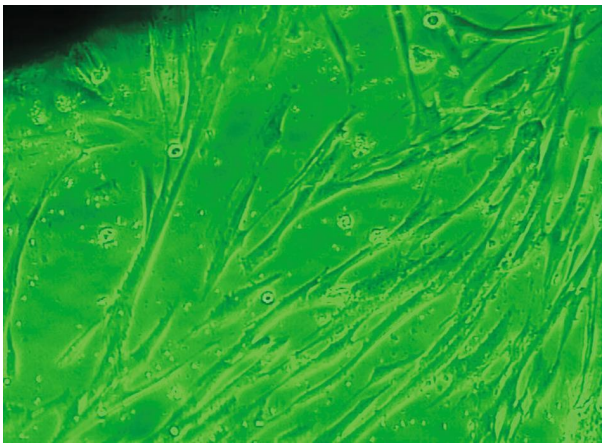

Figure 1 Primary culture of human pterygium fibroblast: fusiform–shape cells emigrate from the pterygium tissue×100

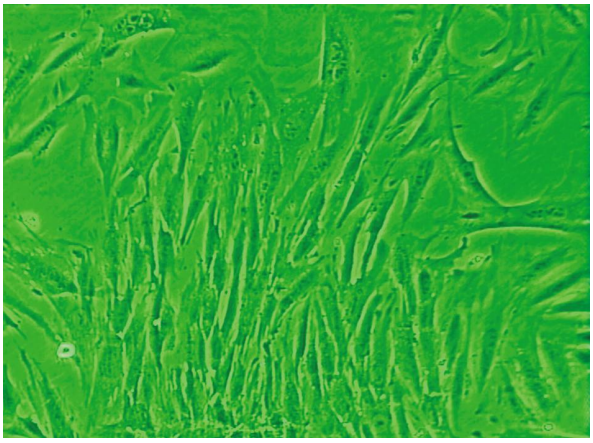

Figure 2 Passage culture of human pterygium fibroblasts: cells show the connection of mass or reticula×100

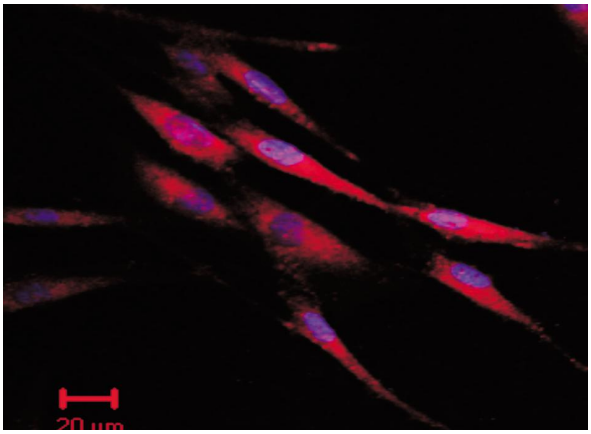

Figure 3 Fibroblasts from human pterygium are vimentin staining positive×100

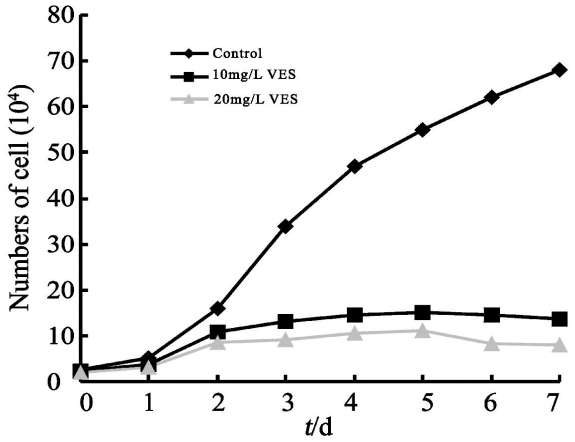

Figure 4 Effect of VES on growth curve of human pterygium fibroblasts

7.7%-15.4% of patients present with significant recurrence rates [3]. Therefore investigation of potential adjuvant and palliative therapies is necessary to prevent this disease from people.

In our previous study, it has been confirmed that pterygial fibroblasts have acquired many of the properties of the transformed phenotype[5]. Hence, it appears that it may be of considerable value in the prevention of pterygium growth and recurrence if some drugs have a marked inhibitory effect on pterygium fibroblasts proliferation, such as mitomycin C, 5-fluorouracil, etc. However, the clinic applications of these adjunctive therapies are often restricted because of their potential risk of toxic effects on the eye or body[7].

Vitamin E, known for its antioxidative properties, is composed of a group of 8 naturally occurring tocopherols and tocotrienols of subtypes α, β, γ and δ. Of these, the α-tocopherol subunit is the most abundant and biologically active. The VES derivative of α-tocopherol has received increasing attention for its potential use against cancer. *In vitro* studies of VES have shown inhibition of cell proliferation in a variety of tumor cells. Previous *in vivo* studies also suggest that VES may promote dormancy and decrease metastatic spread in numerous cancers, including breast [8], melanoma [9] and colon [10]. Moreover, VES has no inhibitory effect on normal cells. Because of the transformed phenotype of pterygium fibroblasts and reliability of VES on

normal cells, we examined the value of VES against pterygium in this study. The results suggest that VES inhibited pterygium fibroblasts proliferation in a dose and time-dependent manner. These *in vitro* findings are comparable with previously published VES studies on tumor cells. The mechanism by which VES decreases the cells proliferation is not entirely clear. However, several possible pathways have been studied: ① VES has been shown to induce cells apoptosis by inhibition of protein kinase C [11] and suppression of nuclear factor-kappaB activation [12]. Subcellular organelle destabilization, namely mitochondria, is a key component of VES induced apoptosis. Mitochondrial destabilization is followed by the formation of reactive oxygen subtypes, which leads to the release of cytochrome C. This cascade results in activation of executioner caspases, which ultimately leads to cells death [13]. ② VES cooperates *in vitro* with the TNF-related apoptosis-inducing ligand in killing transformed cells but not normal cells. ③ VES has been shown to suppress cells proliferation at sub-apoptotic levels by deregulating expression of genes involved in cell cycle transition [14], including inhibition of the E2F trans-activation activity[15]. The E2F family of transcriptional factors controls cell cycle transition, with implication for cell proliferation. One important family of genes, whose expression is controlled specifically by E2F1, is the receptor tyrosine kinases of the fibroblast growth factor receptor (FGFR) family. Of these, E2F1, which has been shown to be modulated by VES, controls transcription of FGFR and FGFR2 which are activated upon interaction with their congnate ligands, FGF1 and FGF2. We have known that fibroblast growth factors and their receptors are expressed in pterygium [16]. Thus we reasoned that VES could selectively modulate proliferation of cells in pterygium fibroblasts by down-regulation of FGFRs via the E2F1 pathway. It needs further studies to make clear whether the two former mechanism taking part in the effect of VES against pterygium.

In conclusion, the results of our study appear to suggest that pterygium fibroblasts response to VES, although significant, will probably not be sufficient to allow VES to serve as primary treatment in patients with clinically detectable disease. In this regard, one might consider VES as a preventative agent against either primary disease or recurrence after curative resection. Alternatively, VES may have some efficacy as palliative agent to slow the progression of pterygium burden on patients with unresectable diseases. With further study, this route of delivery may prove this to be a simple and effective method of drug administration for pterygium lesion. To our knowledge, the optimal dose and route of VES administration in human subjects has yet to be established.

REFERENCES

1 Liu DJ, Chen XM, Bao J, Pan RG, Li M, Liu YL. Effects of extensive Tenon's membrane excision combined with mitomycin C treatment in the pterygium surgery. *Int J Ophthalmol (Guoji Yanke Zazhi)* 2006;6(3):700-701

2 Onwasigwe EN, Ezegwui IR. An overview of management of pterygium in Nigeria. *Int J Ophthalmol (Guoji Yanke Zazhi)* 2006;6(3):552-554

3 Busin M, Halliday BL, Arffa RC, McDonald MB, Kaufman HE. Precarved lyophilized tissue for lamellar keratoplasty in recurrent pterygium. *Am J Ophthalmol* 1986;102(2):222-227

4 Rutkowski M, Grzegorczyk K, Chojnacki J, Kedziora J. Antioxidative properties of vitamin E as a new approach to its applications in therapy. *Pol Merkuriusz Lek* 2006;20(119):609-614

5 Chen JK, Tsai RJ, Lin SS. Fibroblasts isolated from human pterygia exhibit transformed cell characteristics. *In Vitro Cell Dev Biol Anim* 1994;30(4):243-248

6 Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65(1-2):55-63

7 Morrow GL, Stein RM, Heathcote JG, Ikeda-Douglas JV, Feldman F. Ocular toxicity of mitomycin C and 5-fluorouracil in the rabbit. *Can J Ophthalmol* 1994;29 (6):268-273

8 Malafa MP, Neitzel LT. Vitamin E succinate promotes breast cancer tumor dormancy. *J Surg Res* 2000;93(1):163-170

9 Malafa MP, Fokum FD, Smith L, Louis A. Inhibition of angiogenesis and promotion of melanoma dormancy by vitamin E succinate. *Ann Surg Oncol* 2002;9(10): 1023-1032

10 Barnett KT, Fokum FD, Malafa MP. Vitamin E succinate inhibits colon cancer liver metastases. *J Surg Res* 2002;106(2):292-298

11 Neuzil J, Weber T, Schroder A, Lu M, Ostermann G, Gellert N, Mayne GC, Olejnicka B, Negre-Salvayre A, Sticha M, Coffey RJ, Weber C. Induction of cancer cell apoptosis by alpha-tocopherol succinate: molecular pathways and structural requirements. *FASEB J* 2001;15(2):403-415

12 Dalen H, Neuzil J. Alpha-tocopheryl succinate sensitises a T lymphoma cell line to TRAIL-induced apoptosis by suppressing NF-kappaB activation. *Br J Cancer* 2003;88(1):153-158

13 Weber T, Dalen H, Andera L, Negre-Salvayre A, Auge N, Sticha M, Lloret A, Terman A, Witting PK, Higuchi M, Plasilova M, Zivny J, Gellert N, Weber C, Neuzil J. Mitochondria play a central role in apoptosis induced by alpha-tocopheryl succinate, an agent with antineoplastic activity: Comparison with receptor-mediated pro-apoptotic signaling. *Biochemistry* 2003;42(14):4277-4291

14 Ni J, Chen M, Zhang Y, Li R, Huang J, Yeh S. Vitamin E succinate inhibits human prostate cancer cell growth via modulating cell cycle regulatory machinery. *Biochem Biophys Res Commun* 2003;300(2):357-363

15 Turley JM, Ruscetti FW, Kim SJ, Fu T, Gou FV, Birchenall-Roberts MC. Vitamin E succinate inhibits proliferation of BT-20 human breast cancer cells: increased binding of cyclin A negatively regulates E2F transactivation activity. *Cancer Res* 1997;57(13):2668-2675

16 Kria L, Ohira A, Amemiya T. Growth factors in cultured pterygium fibroblasts: immunohistochemical and ELISA analysis. *Graefes Arch Clin Exp Ophthalmol* 1998;236(9):702-708