·Basic Research·

Effect of Tetrandrine on proliferation of human pterygium fibroblasts

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Abstract

• AIM: To investigate the effect of Tetrandrine (Tet) on proliferation of human pterygium fibroblasts (HPF) *in vitro* and to search for a new method to prevent the recurrence after pterygium surgery.

• METHODS: With different concentrations (0 to 160μ mol/L) of Tet acting on HPF cultured *in vitro*, the impact was observed at 24, 48, 72 hours respectively after Tet intervention. The MTT method was used to assay the biologic activities of Tet and inhibitive rate of cell growth. The expression of proliferating cell nuclear antigen (PCNA) in each group was detected by immunohistochemistry before and after Tet intervention.

• RESULTS: With different concentrations of 20, 40, 80 and 160μ mol/L and acting for 24 to 72 hours, Tet could inhibit the proliferation of HPF in a dose- and time-dependent manner (P <0.05). After the intervention of Tet, the expression of PCNA protein declined. When the concentration of Tet was in the range of 20 to 160μ mol/L, it was able to inhibit the expression of PCNA in a concentration-dependent manner (P<0.05).

• CONCLUSION: Tet can significantly inhibit the proliferation of pterygium fibroblasts, and the inhibitive action is in a dose- and time-dependent manner within a certain range of concentration. But in high concentration (>160μ mol/L), Tet will have cytotoxity.

• KEYWORDS: Tetrandrine; pterygium; fibroblasts; proliferation

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INTRODUCTION

Pterygium is local fibrovascular tissue proliferation of bulbar conjunctiva, which violates the cornea. It is one of the most common clinical eye diseases, which could not only cause eye irritation signs and appearance defects but also influence the visual acuity to different extents, and at present, its pathogenesis is not clear. There are various surgical methods for pterygium, but it always recurs and the therapeutic effects are not satisfying. So the aim of current studies is to find the effective therapy with fewer side effects[1]. Through our experimental study about the inhibition of Tetrandrine (Tet) on fibroblasts of pterygium *in vitro*, we hope to find a new method as complementary therapy for pterygium.

MATERIALS AND METHODS

Materials Human pterygium fibroblasts (HPF) cultured *in vitro*, Tet was made by the pharmaceutical factory in Jinhua, Zhejiang Province, China (powder, purity> 98%); 1:250 trypsin, propidium iodide (PI), RNA enzymes, EDTA Hanks solution were provided by the Tongji Medical University; MTT was purchased from Sigma Company and DMEM medium and neonatal bovine serum was purchased from GIBCO Corporation; mouse anti-human keratin monoclonal antibody and mouse anti-human PCNA monoclonal antibody were supplied by Wuhan Boshide Company, Hubei Province.

Methods

Preparation of Tet storage solution A small amount of DMSO was joined to dissolve, and DMEM was added to make 10mmol/L solution of storage, and it was packed and preserved at -20℃ avoiding light. The period of validity lasted two weeks. When used, DMEM medium was diluted to the required final concentration, and the concentration of DMSO was less than 1‰.

Cultivation,identification and passage of HPF Pterygium tissues were obtained from ophthalmic surgical resection in our hospital. Mass culture was applied to observe block adherence, medium color, cell growth and morphological characteristics after inoculating for one day. It was observed that after 48 hours, some cells climbed out from organization

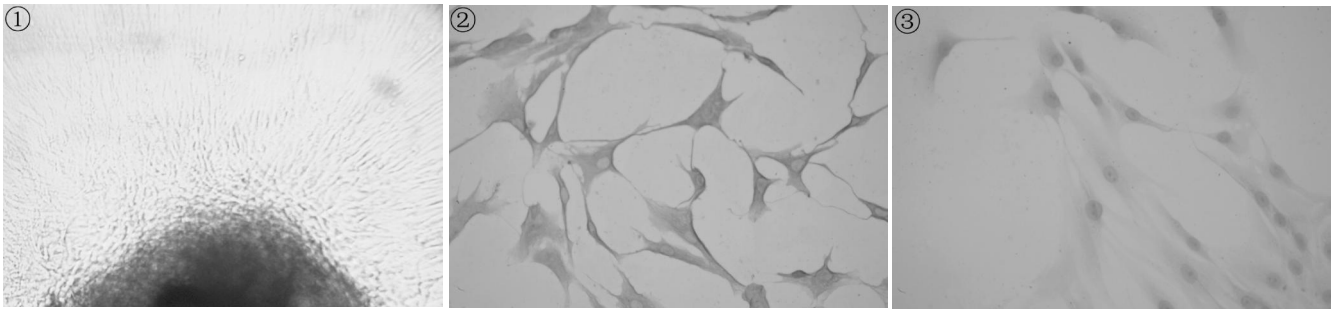

Figure 1 Primary cells of HPF: the brim of tissue section is brush-like and the cells are fire flame-shaped or radial extension; the contour is clear and cell body is translucent

Figure 2 Cell identification: SP staining found the positive expression of vimentin, and the positive expression lied in the cytoplasm, which showed brown fascicular or network structure that was consistent with the direction of fibroblast long axis

Figure 3 HE staining of passage cells: cell contour is clear and appears flat multilateral epithelium-like shape; cytoplasm is of light red color; nuclei have clear borders, and some can be seen "dual-core"

block (Figure 1) and every three to four days the culture solution was replaced once. After the cells grew to 80% from the bottom of bottle, 2.5g/L trypsin was used to digest cells, and passage culture was carried out in the proportion of 1:3. Subculturing cells were inoculated in six preset orifices of glass coverslips. When cells overgrew on glass coverslips, cells were fixed by cold acetone and ethanol mixed 1:1 for 15 minutes. Then after they were dried, we dyed them with HE staining (Figure 2), and with anti-vimentin and keratin antibody immunohistochemical appraisal was performed (Figure 3).

Effect of Tet on proliferation of HPF MTT colorimetric assay was adopted. HPF cells were cultured with 100mL/L bovine serum, 100U/mL penicillin, and 100U/mL streptomycin in DMEM medium at 37℃, 50mL/L CO_2 incubator. Then, the cells were digested and subcultured with 2.5g/L trypsin. HPF cells of logarithmic growth phase were taken and inoculated in the 96-hole boards with 1×10^5/mL, and each was plus 200μL solution. Blank group without inoculation, Tet group and control group were established. The fluid was changed after 24 hours, and Tet was not added to control group. The final concentrations of Tet group were 20μmol/L, 40μmol/L, 80μmol/L and 160μmol/L, respectively. A six-point hole was set up for each concentration at each time point. 20μL of 5g/L MTT was added at 24, 48, and 72 hours respectively after Tet intervention. It was continued to culture for 4 hours and then the culture solution was aspirated. 150μL DMSO was added to each hole and the solution was vibrated for 10 minutes until the crystallization was completed dissolved. In ELX800 Reader 490nm wavelength was elected and blank group was adjusted to zero, and the absorbance (A) of each hole was determined. The inhibition rate of cell growth was calculated as follows: inhibition rate = (A value of the control group-A value the experimental group/A value of the control group)×100%.

PCNA expression of HPF Immunocytochemistry method was employed. HPF cells of logarithmic growth phase were taken and inoculated in the 6-hole boards with 1×10^5/mL. The solution was changed after 24 hours. Tet was not added to control group. In Tet group the final concentrations of Tet were 20μmol/L, 40μmol/L, 80μmol/L, and 160μmol/L respectively. After it was cultured for 48 hours, it was washed three times with 0.01mmol/L PBS and fixed with cold acetone plus ethanol in the proportion of 1:1. SP method was adopted and operation proceeded according to the manual. The dilution of mouse anti-PCNA monoclonal antibody was 1:50. PCNA-positive nucleus was of brown color. The expression intensity of PCNA was determined with high-resolution color image analysis system (Tongji Technology Group, Qianping Image Corporation). The optical density of five visual fields was measured and the average was adopted. One hundred cells were randomly selected (magnification 20×10) and all of the positive and negative cells were counted and listed.

Statistical Analysis The experimental results and data were expressed with mean±SD, and analysis of variance was used to analyze the relationship between MTT inhibition rate and concentration and time. SPSS11.0 software was used in statistical analysis, and t-test was employed to analyze the effects of Tet of different concentrations on cells compared with control group. The expression of PCNA- positive cells was analyzed through Chi-square test. P <0.05 was regarded as statistically significant.

RESULTS

The inhibition rate of HPF growth changed after the intervention of Tet, which was seen in Table 1. Cells in

Table 1 Effect of Tet on HPF proliferation

Concentration (μmol/L)	24h		48h		72h	
	A value	inhibition rate(%)	*A* value	inhibition rate(%)	*A* value	inhibition rate(%)
0	0.416±0.012	—	0.485±0.079	—	0.531±0.031	—
20	0.284±0.020	31.7	0.254±0.032[e]	47.6	0.156±0.031[a, c, e]	71.1
40	0.229±0.023[a, c]	44.8	0.132±0.013[a, c, e]	72.8	0.097±0.021[a,e]	81.5
80	0.214±0.018	48.6	0.073±0.011[a, e]	85.0	0.055±0.016[a]	89.9
160	0.167±0.019[a, c]	59.9	0.040±0.004[e]	91.8	0.052±0.008[a]	90.6

[a]*P*<0.05 *vs* control group; [c]*P*<0.05 *vs* adjacent low concentration group; [e]*P*<0.05 *vs* adjacent short time group

Table 2 Impact of Tet on PCNA expression of HPF at 48 hour

concentration (μmol/L)	Cell number of PCNA expression		Labeling index (%)	*P* value (compared with the adjacent group)	
	(−)	(+)			
0	0	0	0	-	+
20	26	74	74	[a]	[c]
40	40	60	60	[a]	[c]
80	56	44	44	[a]	[c]
160	77	23	23	[a]	[c]

[a]*P*<0.05 *vs* control group; [c]*P*<0.05 *vs* adjacent short time group

control group were shaped like paving-stones, neatly arranged and growing adherent to the wall, while cells in Tet group became round and distributed loosely. Moreover, the number of cells in high dose group decreased markedly. When the concentration of Tet exceeded 20μmol/L, Tet could significantly inhibit the proliferation of HPF(P<0.05). In the range of 20μmol/L to 160μmol/L, the inhibitory intensity increased with the rising of concentration(P<0.05). During 24 to 72 hours of drug acting, the inhibitory intensity increased with the prolonging of time (P <0.05). Forty-eight hours later, the inhibition rate did not increase, which showed the feature of saturated pharmaco-dynamics. At 48 hours with the concentration of 160μmol/L, the inhibition of Tet on HPF proliferation was significant (P<0.05) and the inhibitive rate reached 91.0%. At 72 hour, the inhibitive rates of HPF proliferation were respectively 89.9% and 90.6% with the concentrations of 80μmol/L and 160μmol/L. With the increase of Tet concentration and the prolongation of time, the inhibition rate of fibroblast growth increased (P <0.05). Within the range of 20-60μmol/L acting for 24 to 72 hours, Tet could be in a dose- and time-dependent manner. Based on the inhibition rates of HPF proliferation under various concentrations, the time-dose effect curve was produced (Figure 4).

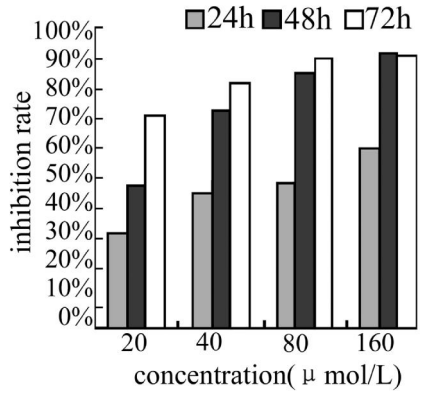

Figure 4 Inhibition of Tet of different concentrations to HPF proliferation

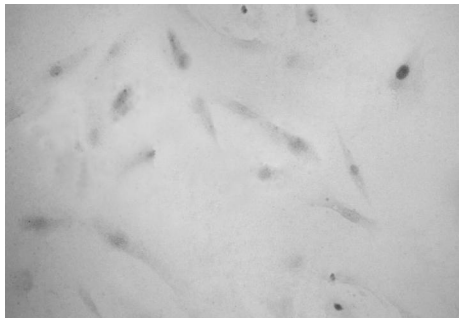

Figure 5 PCNA expression of cells in Tet group (×200)

The expression of PCNA of HPF cells was seen in Figure 5 and Table 2. Cell positive expression showed brown, yellow or pale yellow uniform fine particles, distributing in the whole nucleus. No nuclear staining and very pale yellow color of cytoplasm were characteristics of negative control group. The nuclei of HPF cells appeared brown, and the expression of PCNA was strongly positive; after the intervention of Tet, the color of nuclear staining became light, and in a dose-dependent manner (P <0.05).

DISCUSSION

Pterygium was a common disease of ophthalmology. Pathological study indicated that its main ingredient was abnormal proliferating fibroblasts and neovascularization [2], with the nature of pre-tumorigenesis. The scope and extent of fiber proliferation and neovascularization of pterygium were reliable morphological indicators for prediction of recurrence after pterygium resection surgery[3]. In the present study, cell culture was directly from human pterygium tissue, so its biological characteristics were in accordance with human eye diseases. Furthermore, the immunohistochemical method was used to identify the cells. Its expression of vimentin was positive, and its expression of keratin was negative. In view of organization sources and cell growth characteristics, they could be identified as fibroblasts. There are many surgical methods to treat pterygium currently, but we are unable to effectively control the relapse rate. In clinic the immunosuppressive agents, anti-proliferative and anti-metabolic drugs were used as adjuvant therapies, such as dexamethasone, mitomycin C and 5-fluorouracil alone or in combination to reduce the recurrence rate after pterygium surgery. Clinical observation showed that they had certain effects on delaying the development of pterygium, but these drugs had great local and systemic side-effects. Therefore, it is a research topic facing many scholars to find safe and effective anti-proliferation drugs.

Tet is a kind of alkaloid separated from traditional Chinese medicine such as Menispermaceae, Tetrandrine or Stephania, derivative of dual-benzyl-isoquinolin. It had anti-inflammatory, analgesic, antipyretic, anti-allergic, anti-free radicals, anti-cancer and other pharmacological effects. It could also inhibit fibroblast proliferation and collagen synthesis [4]. Researches found that Tet in the concentration of above 5μg/mL could significantly inhibit the proliferation and collagen synthesis of human scar fibroblasts cultured *in vitro* [5]. The anti-scar function of Tet may be accomplished by directly inhibiting the DNA synthesis of fibroblast, regulating and controlling collagen metabolism, indirect acting on cytokines and other aspects[6].

In this experiment, we used Tet of different concentrations to intervene human pterygium fibroblasts and found that Tet could inhibit the growth of human pterygium fibroblast. With the increase of concentration and prolongation of time, the inhibition rate increased. In addition, the expression of PCNA protein was inhibited and the degree of its decrease was negatively correlated with Tet concentration. PCNA is a nuclear protein, coenzyme of D polymerase in the DNA replication. It is directly involved in the nuclear DNA synthesis and mainly expressed in the S phase of cell proliferation cycle, which was closely related to cell proliferation activities. Therefore it could be used as an accurate and simple indicator of cell proliferation status [7]. Our study found that when the concentration of Tet ⩾ 20μmol/L, it could dose-dependently inhibit the expression of cell PCNA. It was consistent with the results of Tet inhibiting cell proliferation activity detected by MTT in this experiment. The present experimental study showed that Tet could inhibit the proliferation of HPF and induce the apoptosis in time- and dose-dependent manner, suggesting that the treatment of pterygium by Tet has potential application prospect. In short, this experiment has provided a new way of thinking for adjuvant therapies of pterygium, but the current study is limited in vitroexperiments and mechanisms of Tet are complex, so further studies are still expected.

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