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Effect of scopoletin on PC_3 cell proliferation and apoptosis[1]

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KEY WORDS scopoletin; *Lycium barbarum*; prostate hyperplasia; cultured cells; cell division; apoptosis; flow cytometry; fluorescence microscopy

ABSTRACT

AIM: To investigate the effect of scopoletin on cell proliferation and apoptosis of PC_3 cells. **METHODS**: Cell growth curve, MTT assay, and acid phosphatase activity (ACP) were used to determine cell proliferation. Coomassie brillient blue assay was used to measure the content of protein in cells. Light microscope, transmission electronmicroscope, and fluorescence microscope were used to observe scopoletin-induced morphological changes. Apoptosis rate and cell cycle distribution were determined by flow cytometry. **RESULTS**: The IC_{50} of scopoletin for inhibiting PC_3, PAA, and Hela cell proliferation was (157 ± 25), (154 ± 51), and (294 ± 100) mg/L, respectively. Scopoletin induced a marked time- and concentration-dependent inhibition of PC_3 cell proliferation. Scopoletin reduced the protein content and decreased the ACP level in PC_3 cells in a concentration-dependent manner. Cells treated by scopoletin showed typical morphologic changes of apoptosis by light microscope, fluorescence microscope, and transmission electronmicroscope. Apoptosis rate was 0.3 %, 2.1 %, 9.3 % and 35 % for scopoletin 0, 100, 200, and 400 mg/L, respectively, and cells in G_2 phase decreased markedly after being treated with scopoletin. **CONCLUSION**: Scopoletin inhibited PC_3 proliferation by inducing apoptosis of PC_3 cells.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is a disease of old men, and over 50 % of men older than 50 years have been found, at autopsy, to have histologic evidence of prostatic enlargement. With advancing age, there is a progressive increase in the incidence of the disease. Until recently, transurethral resection of the prostate represented the only recognized treatment for BPH. The medical therapies including female hormone, α-adrenoceptor blockade, and 5α-reductase inhibitor, *etc*, have different side effects[1]. Therefore to search a more effective medicine without any side effect is of most importance. Recently with the advanced study of apoptosis, it is reported that the apoptotic index of the prostate epithelium cells is lower in BPH tissue than that in the normal prostate, whereas there is a marked increase in the proliferative index in the hyperplastic prostate. Therefore to increase apoptosis and decrease proliferation is the aim of treatment of BPH[2,3].

BPH and primary prostate cancer are common diseases in old men and both diseases are sometimes presented in same gland. It seems that abnormal androgen is especially related with both of them[4]. Since human prostate cancer cell line grows more quickly and it consistently reacts to treatment, it is often used to study the effect of anti-BPH drug[5]. We have found scopoletin is the active component from the fruit of *Lycium barbarum* for inhibiting prostatic cell proliferation[6]. In this paper, We further investigated the effects of scopoletin on PC_3 cells (human androgen-independent prostate adenocarcinoma cell) both in proliferation and apoptosis.

MATERIALS AND METHODS

Materials Scopoletin, Trypan blue, MTT, RNase, and propidium iodide (PI) were purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from Gibco. Acridine orange (AO) was obtained from Edward Gurr. Coomassie brillient blue G-250 was purchased from Serva. New-born calf serum was purchased from Hangzhou Sijiqing Co. All other

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reagents were of analytical reagent quality.

Drug Scopoletin was dissolved in dimethyl sulfoxide (Me_2SO) to make a stock solution, which was then diluted as desired with the culture media (without new-born calf serum). The Me_2SO concentration was kept under 0.001 % in all the experiments and did not show any detectable effect on cell growth or apoptosis.

Cell culture and IC_{50} PC_3 cells were obtained from ATCC (American Type Culture Collection). PAA (human lung cancer cell) and Hela cells (human cervical squamous cell carcinoma) were kind gifts of Oncology Research Institute of Zhejiang University. All cells of above cell lines were cultured at 37 ℃ in a humidified CO_2(5 %) incubator in DMEM supplemented with 10 % heat-inactivated new-born calf serum and passaged at intervals of 3 – 4 d. For all experiments, cells were treated with various concentrations of scopoletin for 24 h after being seeded. The 50 % inhibitory concentrations (IC_{50}) of scopoletin on PC_3, PAA, and Hela cells were determined by MTT assay[7]. The experiment was performed at least three times.

Cell growth curve of scopoletin PC_3 cells (5×10^7/L) 1 mL in exponential growth were seeded into four 24-well plates (NUNC). The plates were incubated at 37 ℃ in a humidified 5 % CO_2 atmosphere. After 24 h, scopoletin 33, 66, 133, 266, and 533 mg/L were added to wells (3 wells for each concentration for each plate). For control cells (3 wells for each plate), only DMEM was added. The plates were incubated continually. The viable cells were counted by hemocytometer every day in the frist 4 d by Trypan blue dye exclusion method.

Cell protein determination The cell protein of PC_3 cells treated with or without scopoletin was measured as described previously[7].

Assay for acid phosphatase (ACP) The activity of acid phosphatase was determined by modified phenyl phosphate method of Kind and King and modified method of Lowry[8,9]. Briefly, PC_3 cells were treated with scopoletin 0, 100, and 200 mg/L at 24 h after plating. After 72 h, 1×10^6 cells for each concentration were harvested and washed by PBS, then the cell pellets reacted with 0.05 % Triton X-100 0.5 mL on ice bath for 30 min, which resulted in rude enzyme solution. The above solution 60 μL reacted with citrate buffer 38 mmol/L (pH 4.8, containing 0.1 % Triton X-100 and *p*-nitrophenylphosphata 4.2 mmol/L) at 37 ℃ exactly for 15 min. Then NaOH 0.1 mol/L 1.2 mL was added to stop the reaction. After being kept at 23 ℃ – 25 ℃ for 30 min – 60 min, absorbance at 405 nm was measured with an UV-754 spectrophotometer. Protein content was measured with the Folin phenol reagent by Lowry's method. According to the amount of released *p*-nitrophenol and the protein content, the ACP activity was calculated ($mmol \cdot min^{-1} \cdot g^{-1}$ protein).

Morphological determination of apoptosis

Light microscopic and fluorescence microscope observation After a 4-d exposure to scopoletin 0, 100, 200, 400 mg/L, the cover slides in each culture dish were taken out, washed with PBS, stained with HE or 0.4 % AO, and put upside down onto a slide prior to microscopic evaluation. The cells were observed under the light microscope and the fluorescence microscope.

Electron microscopic observation After a 4-d exposure to scopoletin 0, 100, 200 mg/L, cells (1×10^6) were fixed with 2.5 % glutaraldehyde, and postfixed with 2 % osmium tetroxide. After dehydration, the samples were embedded in Epon 812, and then ultramicrotomed. The sections were routinely stained and examined by electron microscope.

Flow cytometric analysis of cell cycle After a 3-d treatment with scopoletin, cellular DNA content was detected by flow cytometry via determination of PI. Briefly, after trypsinization, cells (1.0×10^6 per sample) were washed with PBS and cell pellets were fixed in 70 % ethanol at 4 ℃ overnight. After being washed twice with PBS, RNase (the final concentration was 50 mg/L) was added. After reacting at 37 ℃ for 60 min, the cells were stained with PI (the final concentration was 50 mg/L) at 4 ℃ in dark for 60 min before cytofluorometry.

Statistics Data were expressed as $\bar{x} \pm s$, and compared with *t*-test. IC_{50} was calculated by NDST program. Flow cytometry (FCM) results were expressed as percentages and statistical comparisons were made with *t* test. $P < 0.05$ is considered significant.

RESULTS

Effect of scopoletin on cell proliferation According to MTT assay, the IC_{50} of scopoletin for inhibiting PC_3, PAA, and Hela cell proliferation were (157 ± 25), (154 ± 51), and (294 ± 100) mg/L, respectively. Scopoletin induced a marked concentration-dependent inhibition of PC_3 cell proliferation (Fig 1). Scopoletin reduced the protein content in PC_3 cells

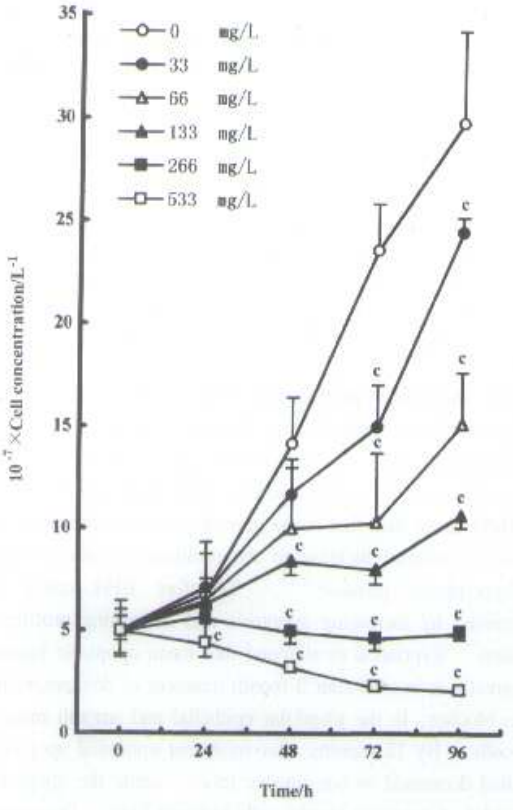

Fig 1. Effect of scopoletin in PC_3 cell growth. $n = 3$ experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control (0 mg/L).

in a concentration-dependent manner (Tab 1) and markedly reduced ACP activity in PC_3 cells (Tab 2).

Tab 1. Effects of scopoletin on PC_3 cells protein content. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Concentration/ mg·L^{-1}	Absorbance	Reduced protein/%
0	0.310 ± 0.003	
33	0.29 ± 0.03^a	7.8
66	0.269 ± 0.023^b	13.2
133	0.22 ± 0.03^c	29.1
266	0.194 ± 0.017^c	37.2
533	0.030 ± 0.010^c	90.4

Effect of scopoletin on apoptosis of PC_3 cells

After exposure to scopoletin, under both light microscopic and fluorescence microscope, PC_3 cells showed typical apoptosis features: volume reduction, chromatin condensation, nuclear fragmentation, and apprearence of apoptotic bodies (Fig 2). Under electron microscopy, the chromatin of PC_3 cells was located along the nuclear envelope, or formed irregularly shaped crescents at the nuclear edges (Fig 3).

Tab 2. Effect of scopoletin on ACP in PC_3 cells. $n = 2$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Concentration/ mg·L^{-1}	10^{-3} × mmol·min^{-1}·g^{-1}	Inhibition rate/%
0	20.08 ± 0.21	0
100	12.38 ± 0.17^c	39.8
200	7 ± 3^b	65.4

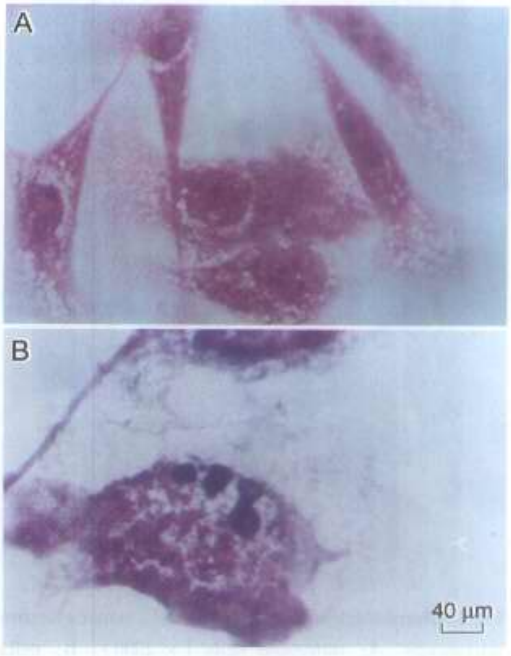

Fig 2. Morphological change of PC_3 cells stained by propidium iodide under fluorescence microscope. A: untreated cells; B: cells treated with scopoletin 100 mg/L. × 270.

FCM was accomplished using a FACScan and repeated for 3 times. Nearly 1×10^6 cells were analyzed by excitation and emission of PI at 488 nm and 575 nm, respectively. Examination of untreated PC_3 cells by FCM revealed essentially a population of cells that had a normal rate of apoptosis. After treatment of PC_3 cells with scopoletin, a subdiploid peak (apoptotic peak) of DNA characteristic of apoptosis was observed. After

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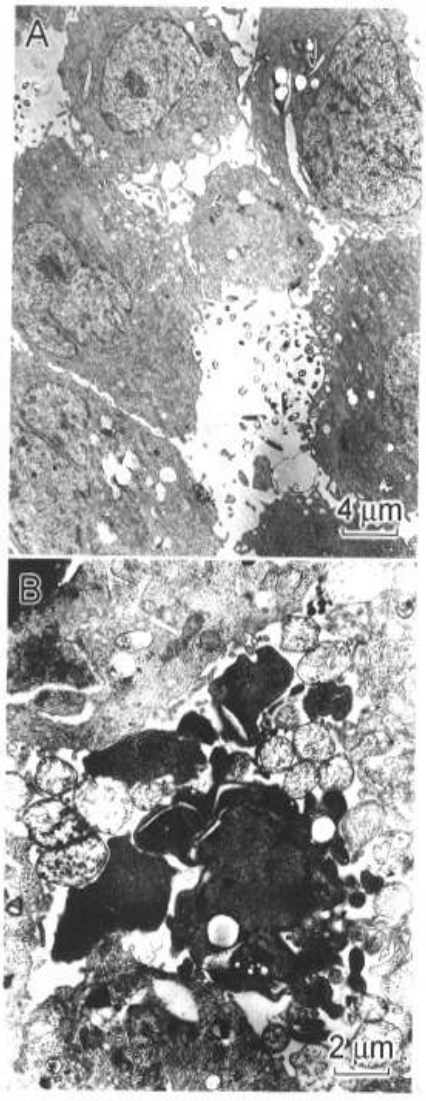

Fig 3. Morphological change of PC_3 cells under electron microscopy. A: untreated cells (× 2500); B: cells treated with scopoletin 100 mg/L (× 6000).

treatment of PC_3 cells with scopoletin 100, 200, and 400 mg/L for 96 h, the cells in G_1 phase and S phase were slightly increased, whereas cells in G_2 phase were markedly decreased. The percentage of apoptotic cells increased with scopoletin concentration (Tab 3).

Tab 3. Effect of scopoletin on cell cycle distribution and apoptotic index of PC_3 cell after 96 h treatment. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Concentration/ mg·L^{-1}	Cell cycle/% G_0/G_1	S	G_2/M	Apoptosis rate/%
0	51 ± 4	24 ± 7	28 ± 4	0.3 ± 0.6
100	49 ± 7^a	31 ± 8^a	20 ± 3^b	2.1 ± 0.4^c
200	50 ± 7^a	29 ± 7^a	18 ± 3^b	9.3 ± 2.8^c
400	54 ± 18^a	38 ± 12^a	8 ± 7^b	35 ± 13^c

DISCUSSION

Cell growth in the normal prostate is regulated by a delicate balance between cell death and cell proliferation (ie, apoptotic *vs* proliferative activity). Disruption of the molecular mechanisms that regulate these two processes may underline the abnormal growth of the gland leading to BPH. The apoptotic index of the secretory and basal cells of the prostate epithelium was lower in BPH tissue than that in the normal prostate, whereas there was a marked increase in the proliferative index in the hyperplastic prostate[2,3]. Therefore BPH could be treated by increasing apoptosis and decreasing proliferation. Kyprianou et al found that mean apoptotic indices greatly increased after 3-month treament of doxazosin, an α blocker, in the glandular epithelial and smooth muscle cells. By 12 months after treatment epithelial apoptosis had decreased to constitutive levels, while the apoptotic index of prostatic stroma cells remined high. Doxazosin induced apoptosis of smooth muscle cells which correlated with prostatic stromal degeneration, and decreased α-smooth muscle actin expression and improved BPH symptoms[10]. Saez *et al* reported that finasteride, a 5α-reductase inhibitor, might modulate the TGF-β (transforming growth factor β) signaling system to promote changes leading to apoptosis of epithelial cells and prostatic glandular atrophy[11]. In our exeriment, based on FCM, the apoptosis index of PC_3 cells was increased with scopoletin concentration. Previous reports indicated that when ACP activity decreased, the protein synthesis was inhibited in prostate[8,9]. Our results indicated ACP activity and protein content in PC_3 cells were decreased with scopoletin concentrion. According to PC_3 cell growth curve, the protein content and ACP activity in PC_3 cells, it was found that scopoletin inhibited PC_3 cell proliferation. The PC_3 cells were mainly arrested in G_0/G_1 and S phases. Therefore scopoletin might be possessed of anti-benign prostatic hyperplasia actions both by decreasing proliferation and increasing apoptosis of prostatic cells.

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REFERENCES

1 Sun ZY, Wu HY, Wang MY, Tu ZH. The mechanism of epristeride against benign prostatic hyperplasia. Eur J Pharmacol 1999; 371: 227-33.
2 Kyprianou N, Tu H, Jacobs SC. Apoptotic versus proliferative activities in human benign prostatic hyperplasia. Hum Pathol 1996; 27: 668-75.
3 Deng F, Gu F, Xia T. Proliferaion and apoptosis status in benign prostatic hyperplasia. Chin J Surg 1996; 34: 620-2.
4 Zhou ZY. The relationship between BPH and prostate cancer. In: Guo YL, editor. Prostate hyperplasia and prostate cancer. Beijing: The People's Medical Publishing House; 1998. p 116-28.
5 Habib FK, Ross M, Buck AC, Ebeling L, Lewenstein A. *In vitro* evaluation of the pollen extract, cernitin T-60, in the regulation of prostate cell growth. Br J Urol 1990; 66: 393-7.
6 Liu XL, Sun JY, Li HY, Zhang L, Qian BC. Extraction and isolation of active component for inhibiting PC_3 cell proliferation *in vitro* from the fruit of *Lycium barbarum* L. Chin J Chin Mater Med 2000; 25:481-3.
7 Liu XL, Chen K, Ye YP, Peng XY, Qian BC. Glutathione antagonized cyclophosphamide and acrolein-induced cytotoxicity of PC_3 cells and immunosuppressive actions in mice. Acta Pharmacol Sin 1999; 20; 643-6.
8 Zhang CQ, Zhuang LZ, Yang CR. Effect of prolactin on production of acid phosphatase in rat prostatic cells and its mechanism. Acta Biol Exp Sin 1993; 26: 215-9.
9 Yamada K. Effects of melatonin on reproductive and accessory reproductive organs in male rats. Chem Pharm Bull 1992; 40: 1066-8.
10 Kyprianou N, Litvak JP, Borkowski A, Alexander R, Jacobs SC. Induction of prostate apoptosis by doxazosin in benign prostatic hyperplasia. J Urol 1998; 159: 1810-5.
11 Saez C, Gonzalez-Baena AC, Japon MA, Giraldez J, Segura DI, Miranda G, *et al*. Regressive changes in finasteride-treated human hyperplastic prostate correlate with an upregulation of TGF-beta receptor expression. Prostate 1998; 37: 84-90.

莨菪亭对 PC_3 细胞增殖和凋亡的影响[1]

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关键词　莨菪亭；枸杞子；前列腺增生；培养的细胞；细胞分裂；细胞凋亡；流式细胞术；荧光显微镜检查

目的：研究莨菪亭对人前列腺癌细胞 PC_3 增殖的作用和莨菪亭是否能引起 PC_3 细胞的凋亡．**方法**：用细胞生长曲线，MTT 试验和酸性磷酸酶(ACP)活性来测定细胞增殖，考马斯亮蓝法测细胞内蛋白质的含量，光镜、透射电镜和荧光显微镜观察莨菪亭引起的形态学变化．用荧光显微镜和流式细胞仪确定凋亡率和细胞的周期分布．**结果**：莨菪亭对 PC_3，PAA 和 Hela 细胞的 IC_{50} 分别为(157 ± 25)，(154 ± 51)和(294 ± 100) mg/L，莨菪亭时间和浓度依赖性地抑制 PC_3 细胞的增殖，并引起细胞内蛋白质含量减少和 ACP 活性降低．经莨菪亭处理后，在光镜、透射电镜和荧光显微镜下可观察到莨菪亭引起的典型的凋亡形态学变化，流式细胞仪测定显示经莨菪亭 0，100，200 和 400 mg/L 处理后 PC_3 细胞的凋亡率分别为 0.3 %，2.1 %，9.3 %和 35 %，G_2 期细胞显著减少．**结论**：莨菪亭抑制 PC_3 细胞增殖且可引起 PC_3 细胞的凋亡．

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