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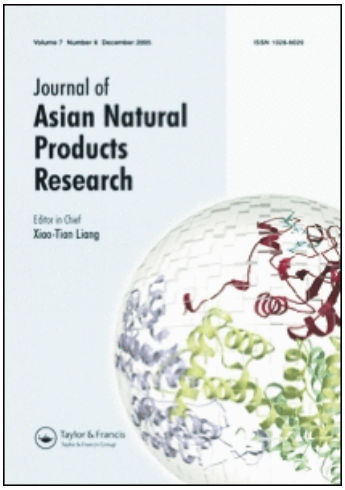

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Patensin-Induced Apoptosis is Accompanied by Decreased *bcl-2* Expression and Telomerase Activity in HL-60 Cells

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PATENSIN-INDUCED APOPTOSIS IS ACCOMPANIED BY DECREASED *bcl-2* EXPRESSION AND TELOMERASE ACTIVITY IN HL-60 CELLS

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The present study aimed to investigate the effect of telomerase activity in patensin-induced apoptosis and the regulation of B cell leukemia/lymphoma 2 (*bcl-2*) gene expression in human leukemia HL-60 cells. Apoptosis of HL-60 cells was induced by patensin ($100\,\mu mol\,L^{-1}$) for 3, 6, 12 and 24 h. Apoptosis and *bcl-2* were determined by flow cytometry analysis. A polymerase-chain-reaction-based telomeric repeat amplification protocol assay was used to detect the telomerase activity. Patensin induced growth arrest and apoptotic cell death in HL-60 cells. The telomerase activity was inhibited in a time-dependent manner during the patensin-induced apoptosis of HL-60 cells, and the expression of *bcl-2* was progressively down-regulated by patensin. Inhibition of the telomerase activity of HL-60 cells was closely related to the patensin-induced apoptosis. The present results indicate that inhibition in telomerase and reduced *bcl-2* gene expression may play a role in the patensin-induced apoptosis of HL-60 cells.

Keywords: Patensin; Telomerase; Apoptosis; Bcl-2 gene; HL-60 cell

INTRODUCTION

Telomerase is a ribonucleoprotein that adds repeated units of TTAGGG to the end of telomeres and thereby prevents their shortening. Telomerase activation has been reported in 98% of established immortal cell lines and in about 90% of tumor tissues tested, but not in most somatic cells [1,2], suggesting that activation of telomerase may play an important role in carcinogenesis and immortalization. Apoptosis, the physiological mode of cell death, is related to the regulation of development and homeostasis. Abnormal apoptosis is associated with many different disease states, including cancers, in which apoptosis is suppressed. Both increased telomerase activity and enhanced resistance to apoptosis have been widely reported in human tumor cells [3,4], but little is known about specific links between the regulation of telomerase activity and drug-induced apoptosis. Some studies have suggested that telomerase activity is mechanistically involved in the regulation of apoptosis in both physiological settings and pathological states [3–5].

Pulsatilla patens var. *multifida* has been used as a traditional Chinese medicine against amoebae, cancer, vaginal trichromoniasis and bacteria in China. To make out what

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compounds might be responsible for the pharmacological activities, we isolated one of the compounds, patensin, which was initially purified from the ethanolic extract of roots of the plant [6]. In the present study, we tried to study the cytotoxicity and apoptosis induced by patensin in human leukemia HL-60 cells. The telomerase activity during the apoptosis was examined by an advanced telomeric repeat amplification protocol (TRAP) assay, and B cell leukemia/lymphoma 2 gene (*bcl-2*) was analyzed to determine whether their expressions correlated with telomerase activity.

RESULTS AND DISCUSSION

Effect of Patensin on HL-60 Cells Growth

A concentration-dependent inhibition of cell growth was seen in cultures treated with patensin for 12 h at dosages ranging from 0.001 to 10 mmol L^{-1} (Table IA). At 100 μmol L^{-1}, patensin induced detectable effects within rather short exposure periods (3 to 6 h) (Table IB). Patensin at a concentration of 100 μmol L^{-1} revealed a cytotoxic effect against HL-60 cells even after short exposure periods. After the addition of patensin to the incubation medium for 3, 6, 12 and 24 h, cell viability progressively decreased by 1.96%, 10.28%, 22.84% and 38.68%, respectively (Table I).

Detection of Apoptosis by Flow Cytometry

A typical "Apoptosis peak" was detected after exponentially growing HL-60 cells were treated with patensin (100 μmol L^{-1}) for 3, 6, 12 and 24 h. The percentage of apoptotic cells with fractional DNA content was 0.77%, 3.78%, 9.32% and 29.09%, respectively (Fig. 1).

Detection of *bcl-2* Immunofluorescence

The protein expression of the apoptosis-modulating gene *bcl-2* was analyzed to determine the mechanism of patensin (100 μmol L^{-1})-induced apoptosis in HL-60 cells. The fluorescence induced by *bcl-2* in control cells was high, but it was significantly down-regulated parallel with the increased proportion of apoptotic cells (Fig. 2).

TABLE I Effect of patensin on HL-60 cells growth

Patensin (mmol L^{-1})	*O.D. $_{570\,nm}$, Mean ± S.D.^{n}*		*Inhibition rate (%)*
(A) Dosage dependence study; duration of treatment was 12 h			
0	1.36 ± 0.12		–
0.001	1.31 ± 0.09[a]		3.67
0.01	1.26 ± 0.04[b]		7.35
0.1	1.19 ± 0.03[c]		12.50
1	0.99 ± 0.05[c]		27.21
10	0.78 ± 0.03[c]		42.64
Time (h)	− Patensin	+ Patensin	
(B) Time-course study			
0	0.958 ± 0.02	0.960 ± 0.03	–
3	0.97 ± 0.03	0.951 ± 0.04[a]	1.96
6	1.05 ± 0.05	0.932 ± 0.02[c]	10.28
12	1.16 ± 0.04	0.895 ± 0.011[c]	22.84
24	1.29 ± 0.05	0.791 ± 0.03[c]	38.68

$n = 4$ Wells in each experiment. 100 μmol of patensin was applied to each experiment. [a]$P > 0.05$, [b]$P < 0.05$, [c]$P < 0.01$ *vs.* control.

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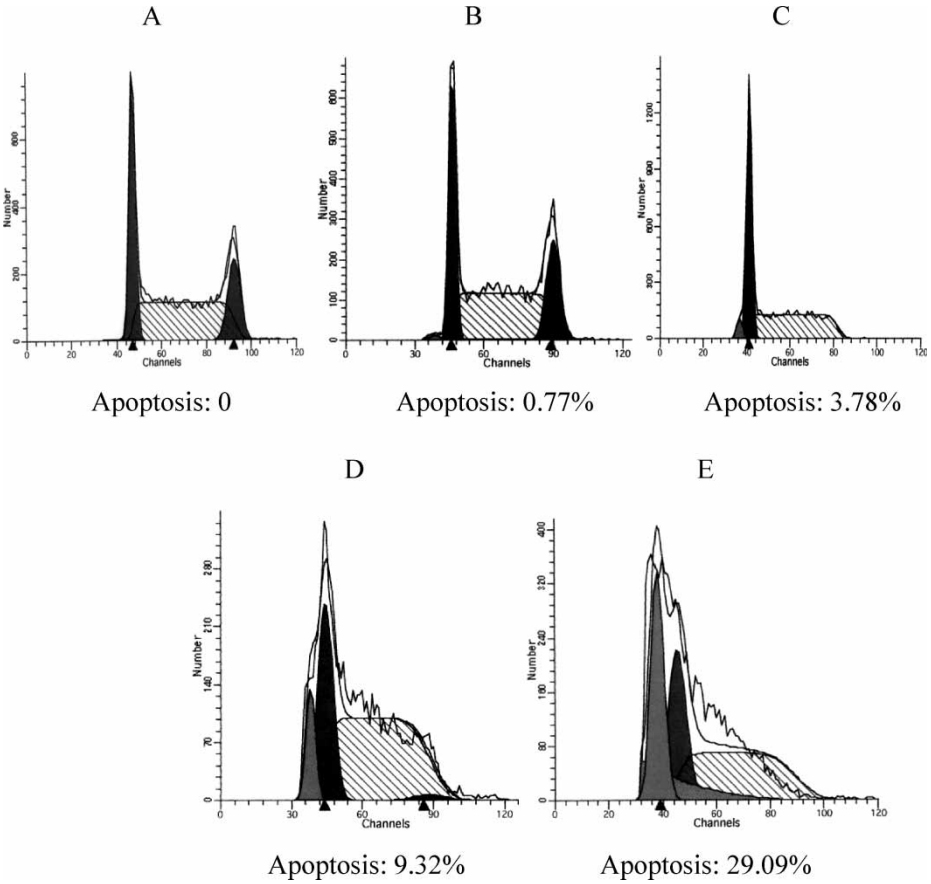

FIGURE 1 Flow cytometry analysis of apoptotic cells treated of patensin. 10^6 cells were treated with patensin, stained with propidium iodide, and analyzed on a flow cytometer to identify sub-G1 peak representing apoptotic cells. (A) normal HL-60 cells; (B-E) show cells treated with patensin ($100\,\mu mol\,L^{-1}$) for 3, 6, 12 and 24 h, respectively. The number below the *y*-axis represents the percentage of apoptosis cells.

Effect of Patensin on Telomerase Activity of HL-60 Cells

After incubation with patensin ($100\,\mu mol\,L^{-1}$) for 3, 6, 12, 24 h the telomerase activity of HL-60 cells was pronouncedly down-regulated (Fig. 3). The percentage of relative activity of telomerase in the treated cells at each incubation time was significantly decreased to 62.98%, 44.23%, 32.32% and 23.30%, respectively, from the 100% of the control group.

The view that apoptosis is a tightly regulated cell self-destruction mechanism is widely accepted. Many studies suggested that telomerase may be an important factor in suppressing apoptotic signaling cascades. Peptide nucleic acid and 2′-*O*-meRNA oligomers inhibited telomerase, leading to progressive telomere shortening and inducing immortal human breast epithelial cells to undergo apoptosis with increasing frequency until no cells remain [7]. Other reports revealed that treatment of pheochromocytoma (PC12) cells with telomerase inhibitor (oligodeoxynucleotide TTAGGG or 3,3′-diethyloxadicarbocyanine) pronouncedly enhanced mitochondrial dysfunction and apoptosis induced by staurosporine, Fe^{2+}, and amyloid β-peptide [5]. Moreover, inhibition of telomerase with an antisense telomerase expression vector not only decreased telomerase activity but also increased susceptibility to cisplatin-induced apoptotic cell death in human malignant glioblastoma cell line [3]. In addition, the stable overexpression of *bcl-2* has been demonstrated to result in increased telomerase activity and

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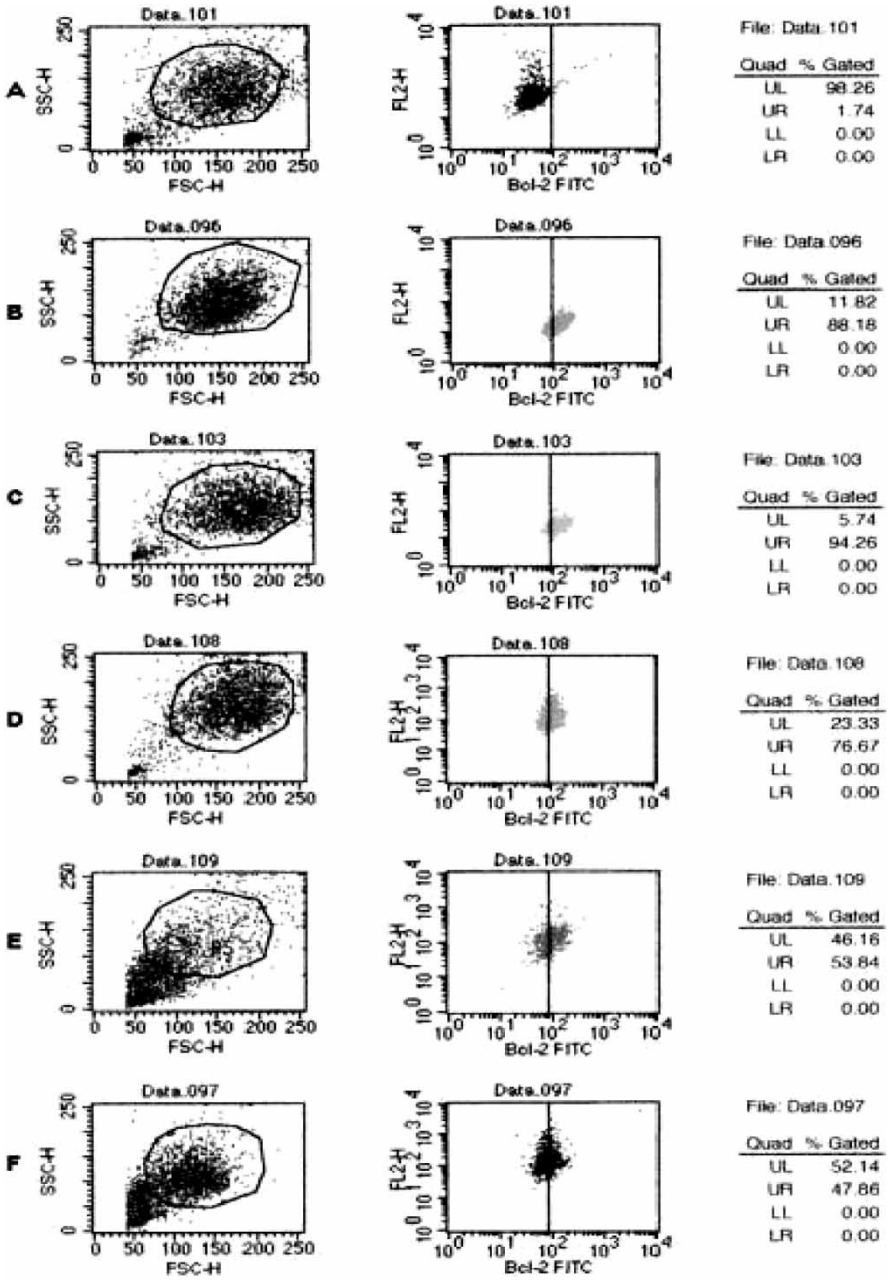

FIGURE 2 Detection of *bcl-2* immunofluorescence by flow cytometry. (A) Control (2nd antibody alone), (B) normal HL-60 cells, (C-F) show the treatment of patensin (100 μmol L^{-1}) for 3, 6, 12 and 24 h, respectively.

decreased in apoptosis [4], and an enhancing in apoptosis has been associated with a decrease in telomerase activity in quiescent and terminal differential cells [8,9]. Our results also indicated that an increased apoptotic population caused by patensin was accompanied by a significant inhibition of telomerase activity, and were consistent with these reports. All these data implied that telomerase might be playing an important role in modulating apoptotic cell death.

The regulation of apoptosis is a complex process and involves a number of gene products, including the survival factor *bcl-2*, which has been found to be frequently deregulated in

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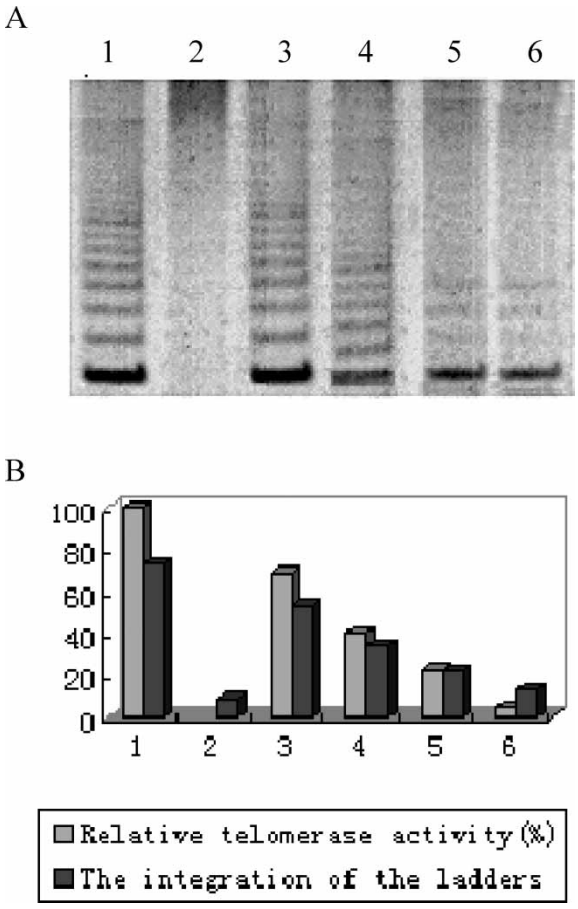

FIGURE 3 Effect of patensin on telomerase activity in HL-60 cells. (A) Lane 1, normal HL-60 cells. Lane 2, negative lysis buffer control, Lanes 3–6 show 3, 6, 12 and 24 h treatment of patensin (100 μmol L^{-1}), respectively. (B) The signal intensity in each lane was measured by area integration of the first six ladders from the bottom of the gel using the Furi Electrophoresis Image Analysis System. Relative telomerase activities were quantified by comparing the signal intensity in each lane, and using normal HL-60 cells as 100%.

human cancers. The report that stable overexpression of *bcl-2* results in up-regulation of both the telomerase activity and resistance to apoptosis established a link between *bcl-2* expression and telomerase activity [4]. This seems to be confirmed by the experimental result that diminished telomerase activity was accompanied by down-regulation of *bcl-2*.

In conclusion, patensin induced growth arrest and apoptotic cell death in HL-60 cells. Its mechanism is associated with the inhibition of telomerase activity and the down-regulation of *bcl-2* during the apoptosis. Hence, this experimental observation indicates the necessity of further study of the anti-cancer activity of patensin.

EXPERIMENTAL

Materials

The ethanolic extract of the roots of *Pulsatilla patens* var. *multifida* were concentrated and defatted. The defatted extract was partitioned with *n*-butanol–water. The *n*-butanol layer was evaporated and then subjected to silica gel column to afford patensin (1) (Fig. 4).

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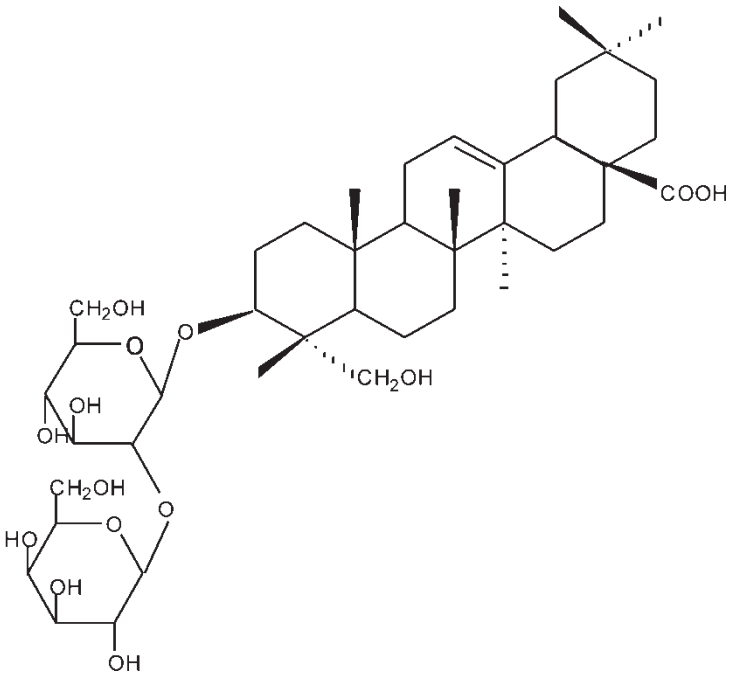

FIGURE 4 Chemical structure of patensin (**1**).

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Patensin (purity $>$ 98%) was dissolved in dimethyl sulfoxide (Me_2SO). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Fluka (USA), and RPMI-1640 medium was the product of Gibco (USA). Telomerase activity was measured by using a PCR-based telomeric repeat amplification protocol (TRAP) kit from the Department of Heredity, Shangdong Medical University, China. All other chemicals were of analytical grade.

Cell Culture and Viability Assay

Human leukemia HL-60 cell line was obtained from the Shanghai Institute Cell Biology, China. The cells were cultured at 37°C in an humidified CO_2 (5%) incubator in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin (100,000 Unit L^{-1}) and streptomycin (100 mg L^{-1}).

The cytotoxicity of patensin was measured by the MTT assay [10]. Briefly, 90 μl of the cell suspension containing 5×10^4 exponentially growing HL-60 cells were planted in flat-bottomed 96-well plates. Patensin (10 μl each well) was added immediately to achieve the desired concentration of 0.001 – 10 mmol L^{-1}. The plates were cultured at 37°C for 3, 6, 12 and 24 h. MTT (5 mg ml^{-1}; 20 μl) was added and the plates were incubated for 4 h. A "lysis solution (10% sodium dodecyl sulfate-5% isobutanol–HCl, 12 mmol L^{-1})" was added and the cells were incubated for 12 h. The optical density at 570 nm was then measured using an ELISA plate reader (Hua Dong Electronic Co., Nanjing, China). All of the cell viability assays were performed in triplicate.

Flow Cytometry Analysis

10^6 Cells were treated with patensin (100 μmol L^{-1}) for 3, 6, 12 and 24 h, and then were collected and fixed in 70% ethanol at 4°C overnight. Subsequently, cells were treated

with Tris–HCl buffer (pH 7.4) containing 1% Rnase A and were stained with propidium iodide (PI, $5\,\mu g\,ml^{-1}$). The distribution of cells with different DNA contents was determined by flow cytometry (Facscalibur, Becton Dickinson, USA) and the data were analyzed by multicycle DNA content and cell cycle analysis software (Modfit LT 2.0) [11].

Indirect Immunofluorescence Assay of *bcl*-2 Expression

The protein *bcl-2* level in cells was measured by flow cytometry [12]. Each sample contained 1.0×10^6 cells. Briefly, cells were washed twice in cold phosphate-buffer saline (PBS) before the addition of 20 μl of FITC (fluorescein isothiocyanate)-conjugated mouse anti-human *bcl-2* antibody. After incubation (30 min) at room temperature in the dark, cells were rinsed twice and resuspended in PBS (500 μl) for flow cytometry analysis. A FACScan flow cytometry (Becton Dickinson, USA) equipped with an argon laser (488 nm) was used to measure fluorescence. Data were analyzed using CellQuest software (Becton Dickinson, USA).

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Telomeric Repeat Amplification Protocol (TRAP) Assay

Telomerase activity was assayed by the modified TRAP methods as described previously [1,13], with minor modifications. Generally, HL-60 cells treated with patensin ($100\,\mu mol\,L^{-1}$) for 3, 6, 12 and 24 h were washed twice in PBS (pH 7.4), and suspended in cold 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) lysis buffer. After incubation (30 min) on ice, the lysates were centrifuged at 12,000*g* for 30 min at 4°C and the supernatant was rapidly frozen and stored at − 80°C. The concentration of protein was measured with the use of a Bradford protein assay; an aliquot of extract containing 1 μg of protein was used for each telomerase assay.

Each extract (2 μl) was assayed in 50 μl of reaction mixture containing Tris–HCl ($20\,mmol\,L^{-1}$, pH 8.3), $MgCl_2$ ($1.5\,mmol\,L^{-1}$), KCl ($63\,mmol\,L^{-1}$), 0.005% Tween-20, egtazic acid ($1\,mmol\,L^{-1}$), deoxynucleoside triphosphates ($50\,\mu mol\,L^{-1}$), TS primer (5′-AATCCGTCGAGCAGAGTT-3′) (0.1 μg) and 2 units of Taq DNA polymerase. After a 30 min incubation at 30°C for telomerase-mediated extension of the TS primer, CX oligonucleotides (5′-CCCTTACCCTTACCCTTACCCTAA-3′) (0.1 g) was added. The reaction mixture was then subjected to polymerase chain reaction (PCR) amplification in a thermal cycler with 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The PCR products were examined by electrophoresis on 12% polyacrylamide gel in 1 × Tris-borate–EDTA (TBE) buffer at 200 V for 2 h. The gels were then stained with 0.2% silver nitrate for 10 min, scanned and analyzed by a Smart View Bioimge Analysis System (Furi Company, Shanghai, China).

Statistical Analysis

Data were expressed as means ± standard deviation of *n* experiments. A statistical difference ($P < 0.05$) was analyzed using the Student's *t*-test.

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