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A novel effect of lobeline on vascular smooth muscle cell: inhibition of proliferation induced by endothelin-1

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Lobeline has a long history of therapeutic use ranging from an emetic and respiratory stimulant to a tobacco smoking cessation agent. *Lobelia chinensis* Lour, a traditional Chinese herb whose active ingredient is the alkaloid lobeline, demonstrated to antagonize the bioactive effect of endothelin-1 (ET-1) and prevent the proliferation of vascular smooth muscle cells (VSMCs) in hyperlipidemic rats. The objective of the present study was to determine the effects of lobeline on proliferation of cultured human umbilical VSMCs induced by ET-1. The results showed that the increased cell numbers and enhanced [^{3}H]thymidine incorporation induced by ET-1 were inhibited and the transition of cells from static phase (G_0/G_1) to DNA synthesis (S) and mitotic phase (G_2/M) was held back by lobeline in a concentration-dependent manner. Confocal microscopy demonstrated that lobeline markedly decreased the fluorescent intensity of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with a significant difference from ET group. Cytotoxicity was determined by Trypan blue exclusion. These results demonstrated a novel biological role of lobeline. Lobeline inhibited the proliferation of human umbilical VSMCs induced by ET-1 in a dose-dependent manner and the anti-proliferative effect was involved in the reduce of increased $[Ca^{2+}]_i$, rather than nonspecific cytotoxicity.

1. Introduction

Lobeline is one of several alkaloids found in Indian tobacco and other *Lobelia* species. Although lobeline was originally considered as a respiratory stimulant and nicotinic agonist, recent observations suggest that its pharmacological action is more complex than what was thought previously.

It is well-known that endothelin (ET) plays an important role in the pathogenesis of cardiovascular diseases such as hypertension, atherosclerosis, and restenosis after balloon angioplasty (Haak et al. 1994; Lerman et al. 1991). The sarafotoxin (SRTX) isolated from the venom of the Israeli burrowing asp *Atractaspis engaddensis* showed remarkable sequence homology of gene and polypeptide with ET. Both ET and SRTX act on the vascular system via identical receptors and have similar biologic actions (Klogg et al. 1988; Bdolsh et al. 1989). These interesting relationships between ET and SRTX led us to investigate the effect of Chinese anti-snake venom herbal medicines on ET bioactivity. Previous investigations demonstrated that some anti-snake venom herbal medicines had significant effects to improve cardiovascular function, prevent hypertension and modulate triglyceride levels (Yang et al. 1998).

The Chinese herb *Lobelia chinensis* Lour grows wildly throughout South China. This plant has commonly been used to cure sores, abscesses, poisonous snakebites etc. (Yeung 1985) for centuries, and is considered to be one of the 50 fundamental herbs (Duke et al. 1985). In our earlier study *Lobelia chinensis* Lour, whose active ingredient is lobeline, demonstrated to antagonize the bioactive effect of ET-1 and prevent the proliferation of vascular smooth muscle cells (VSMCs) in hyperlipidemic rats (Li and Hu 2003).

The aim of the present study was to demonstrate that ET-1 induces the proliferation of human umbilical VSMCs, and determine the effects of lobeline on the proliferation of cultured human umbilical arterial VSMCs induced by ET-1.

2. Investigations and results

2.1. Effects of lobeline on ET-1-induced cell proliferation

Fig. 1 shows the dose responses of lobeline on the proliferation of human umbilical arterial VSMCs induced by ET-1, measured by the WST assay 3 days after drug administration. Compared with the control cells, ET-1 significantly stimulated VSMCs proliferation ($P < 0.01$), lobeline significantly and dose-dependently suppressed the proliferation of VSMCs with $IC_{50} > 300\ \mu M$. Trypan blue staining showed that the cell viability was 85∼90% throughout the first 3 days in the presence of lobeline, suggesting that the reduced proliferation was not due to cell death (data not shown).

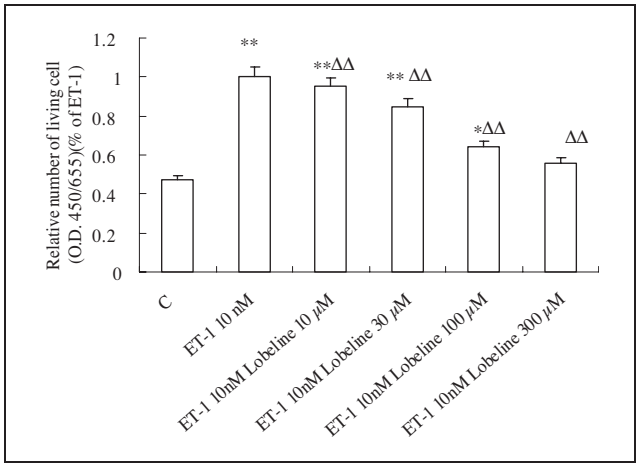

Fig. 1: Dose effect of lobeline on the proliferation of human umbilical arterial VSMCs induced by ET-1. Cell proliferation was assessed by a spectrometric method using the WST-8 assay system. The ratio of O.D. 450/655 nm represents mitochondrial activity reflecting the number of living cells. The values are means ±S.E.M. (n = 6)
** Significantly different from corresponding values in control group ($P < 0.01$)
△ Significantly different from corresponding values in ET-1 group ($P < 0.05$)
△△ Significantly different from corresponding values in ET-1 group ($P < 0.01$)

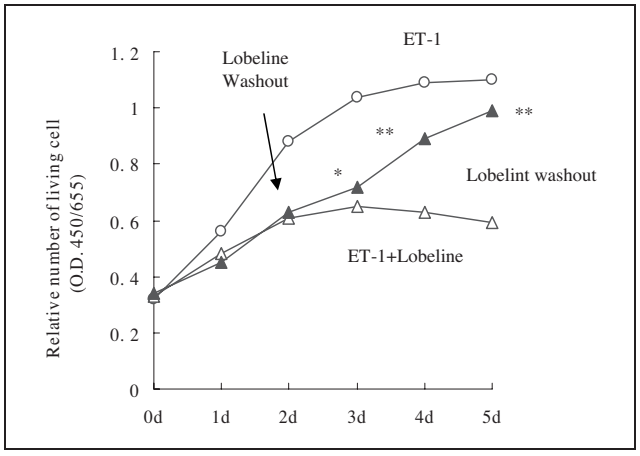

Fig. 2: Washout effects on the proliferation of lobeline-treated human umbilical arterial VSMCs. 10 nM ET-1 treated-cells (○), 10 nM ET-1 and 300 μM lobeline-treated (△) cells were cultured for 5 days. In washout experiments in lobeline-treated cells, the medium was changed to lobeline-free one on 2d (▲). The values are means ±S.D. (n = 4)
* Significantly different from corresponding values in ET-1 + Lobeline group ($P < 0.05$)
** Significantly different from corresponding values in ET-1 + Lobeline group ($P < 0.01$)

Fig. 2 shows the time course of VSMCs proliferation. The growth rate began to slow down from 24 h by 300 μM lobeline and 10 nM ET-1 treatment, but washout of the lobeline from the culture medium on day 2 significantly restored the rate of proliferation thereafter, indicating that lobeline suppressed the growth rate, but did not kill the cells.

2.2. Measurement of [^{3}H] thymidine incorporation into DNA of VSMCs

As shown in Fig. 3, ET-1 (10 nM) stimulated [^{3}H] thymidine uptake in serum-starved cells. The value of [^{3}H] thymidine incorporation in the ET-1 group was increased 1.88 times higher than that in the control group (n = 6). 10 μM lobeline did not change VSMCs incorporation of

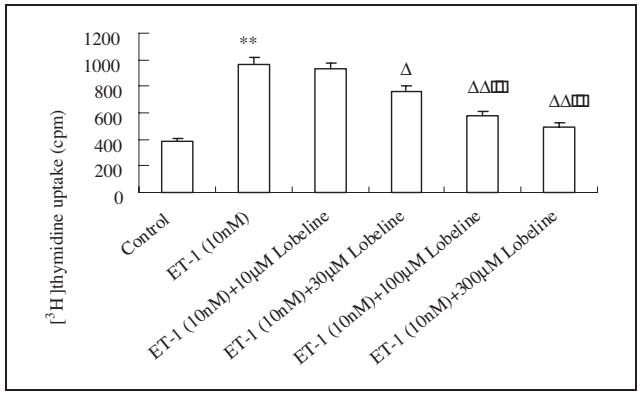

Fig. 3: Effects of lobeline on [^{3}H] thymidine uptake of human umbilical arterial VSMCs. The value of [^{3}H] thymidine incorporation in ET-1 group was increased 1.86 times compared with that in control group. Treatment of human umbilical arterial VSMCs with lobeline resulted in a reduction of [^{3}H] thymidine uptake induced by ET-1 in a concentration-dependent manner. Experiments were performed 6 times independently in duplicate
** Significantly different from corresponding values in control group ($P < 0.01$)
△ Significantly different from corresponding values in ET-1 group ($P < 0.05$)
△△ Significantly different from corresponding values in ET-1 group ($P < 0.01$)

[^{3}H] thymidine in the absence of ET-1, lobeline at the concentrations of 30 μM, 100 μM and 300 μM lowered [^{3}H] thymidine incorporation by 12.58 ± 4.30% (n = 6), 23.81 ± 8.14% (n = 6), and 32.62 ± 9.81% (n = 6), respectively, compared with that of cells treated only with ET-1. These results suggest that lobeline antagonized proliferation of human umbilical arterial VSMCs induced by ET-1 in a concentration-dependent manner.

2.3. Effects of lobeline on cell cycle of human umbilical arterial SMCs

As shown in the Table, DNA content of nuclei in human umbilical arterial VSMCs were measured by flow cytometry analysis after 48 h of exposure to chemical compounds. ET-1 propelled human umbilical arterial VSMCs from static phase (G_0/G_1) to DNA synthesis (S) and mitotic phase (G_2/M). The S and G_2/M phase ratios in ET-1 group increased to 15.9 ± 0.6% and 11.8 ± 0.5% (n = 3), respectively, while G_0/G_1 phase ratio decreased to 72.3 ± 1.5% (n = 3) compared with control group. When the cells were treated simultaneously with ET-1 (10 nM) and lobeline (10 μM), the ratios of G_0/G_1, S, and G_2/M phase were similar to the values of the cells treated with ET-1 only. In the groups treated with both ET-1 and lobeline at the concentrations of 30 μM, 100 μM and 300 μM, the S and G_2/M phase ratios were both decreased ($P < 0.05$, n = 3), whereas G_0/G_1 phase ratio was increased to 74.2 ± 1.3% (n = 3), 80.2 ± 1.1% (n = 3), and 84.9 ± 1.5% (n = 3), respectively, compared with the value of the cells treated with ET-1 only. Similar to WST assay and [^{3}H] thymidine incorporation test, these results also indicated that lobeline antagonized the proliferation of human umbilical arterial VSMCs induced by ET-1 in a concentration-dependent manner.

2.4. Effects of lobeline on [Ca^{2+}]$_i$ in human umbilical arterial VSMCs

As shown in Figs. 4 and 5, ET-1 at the concentration of 10 nM induced a biphasic increase of fluo-3 fluorescence

Table: Effect of lobeline on cell cycle progression of human umbilical arterial VSMCs ($x \pm S.D., n = 3$)

Groups	Cell cycle ratios (%)		
	G0/G1	S	G2/M
Controls	91.2 ± 2.1	5.7 ± 0.4	3.2 ± 0.2
ET-1 (10 nM)	$72.3 \pm 1.5^{*}$	$15.9 \pm 0.6^{*}$	$11.8 \pm 0.5^{*}$
ET-1 + Lobeline (10 pM)	71.8 ± 1.1	15.8 ± 0.4	11.4 ± 0.4
ET-1 + Lobeline (30 pM)	$74.2 \pm 1.3^{\triangle}$	$14.9 \pm 0.4^{\triangle}$	$10.9 \pm 0.5^{\triangle}$
ET-1 + Lobeline (100 pM)	$80.2 \pm 1.1^{\triangle\triangle}$	$12.1 \pm 0.2^{\triangle\triangle}$	$7.7 \pm 0.2^{\triangle\triangle}$
ET-1 + Lobeline (300 pM)	$84.9 \pm 1.5^{\triangle\triangle}$	$8.8 \pm 0.3^{\triangle\triangle}$	$6.3 \pm 0.2^{\triangle\triangle}$

* Significantly different from corresponding values in control group ($P < 0.01$).
$^{\triangle}$ Significantly different from corresponding values in ET-1 group ($P < 0.05$).
$^{\triangle\triangle}$ Significantly different from corresponding values in ET-1 group ($P < 0.01$)

intensity; in the presence of external Ca^{2+}, ET-1 elicited an transient peak of $[Ca^{2+}]_i$, in which fluorescence intensity increased by $202 \pm 18.16\%$ (vs. baseline, $n = 6$) in 60 s, and then fell to a subsequent sustained higher plateau of $210 \pm 12.16\%$ vs. baseline. Pretreatment of human umbilical arterial VSMCs with lobeline (300 μM), in the highest concentration used in the above experiments for 10 min and which significantly prevented the transient increase and sustained increase of $[Ca^{2+}]_i$ elicited by ET-1. The fluorescence intensity in these cells only increased by $115.78 \pm 12.25\%$ vs. baseline ($P < 0.01$, $n = 6$), and the sustained plateau only increased to $151.05 \pm 13.56\%$ vs. baseline ($P < 0.01$, $n = 6$). These results suggest that the inhibition of ET-1-induced proliferation by lobeline is due to suppression of Ca^{2+} influx.

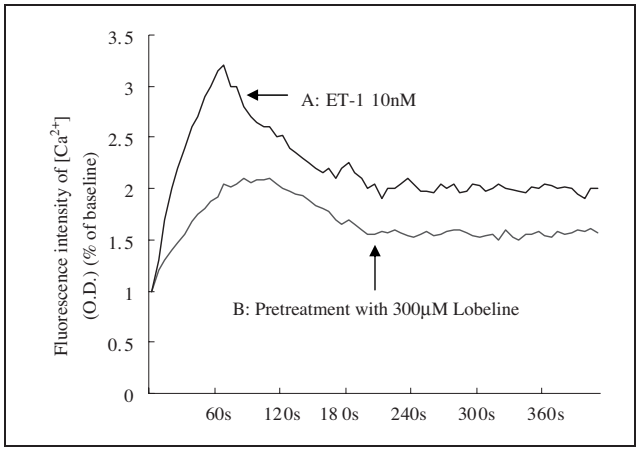

Fig. 4: Effects of 300 μm lobeline on the ET-1-induced transient and sustained increase in $[Ca^{2+}]_i$ in human umbilical arterial VSMCs. The experiments were performed in Ca^{2+}-containing buffer. (A) 10 nM ET-1 induced the increase of $[Ca^{2+}]_i$. (B) Pre-incubate of cells with 300 μm Lobeline for 10 min followed by the addition of 10 nM ET-1. The increases in $[Ca^{2+}]i$ are presented as a percentage of baseline values. Each point represents the mean ±S.D. ($n = 6$)

3. Discussion

VSMCs proliferation induced by various growth factors can develop a variety of pathological processes including atherosclerosis, hypertension and restenosis after balloon angioplasty (Ross 1986), of which ET-1 is one of the most important cytokines. Consequently, inhibition of VSMCs proliferation induced by ET-1 represents a potentially important therapeutic strategy for the treatment of above diseases. In the present study, we have first demonstrated the effects of lobeline on the proliferation of human umbilical arterial VSMCs in a dose-dependent manner. Lobeline inhibited ET-1-induced the increased cell numbers as well as the enhanced [^{3}H] thymidine incorporation, as well as the transition from static phase (G_0/G_1) to DNA synthesis (S) and mitotic phase (G_2/M), and this effects were independent of cell cytotoxin.

$[Ca^{2+}]_i$ is essential in ET-1-induced cell proliferation and contraction. Indeed, removal or chelation of extracellular $[Ca^{2+}]$ significantly inhibits the growth of human pulmonary arterial VSMCs in media containing serum and growth factors (Platoshyn et al. 2000). A rise in $[Ca^{2+}]_i$ increases intranuclear $[Ca^{2+}]$ rapidly (Allbritton et al. 1994), propelling the quiescent cells into cell cycle and through mitosis, and thereby promoting cellular proliferation (Means 1994; Hardingham et al. 1997). In this study, we found that ET-1 (10 nM) induced a biphasic increase in the $[Ca^{2+}]_i$ consisting of a transient peak and a subsequent sustained increase. It is generally accepted that the sustained increase in $[Ca^{2+}]_i$ requires the persistent entry of extracellular Ca^{2+}, whereas the transient increase results

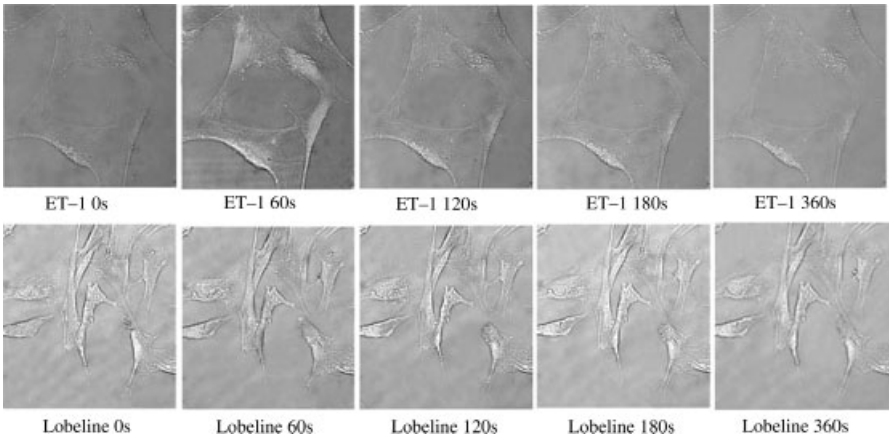

Fig. 5:
Time courses of changes in confocal images of fluo-3-loaded VSMCs (×200)

from mobilization of Ca^{2+} from the intracellular Ca^{2+} store (Gardner 1989). Pretreatment of human umbilical arterial VSMCs with lobeline (300 μM) for 10 min significantly prevented the transient increase of $[Ca^{2+}]_i$ and sustained increase elicited by ET-1. These observations provide clues that lobeline (300 μM) inhibited both intracellular Ca^{2+} release and extracellular Ca^{2+} influx induced by ET-1. Santha et al. (2000) found that lobeline inhibited Ca^{2+} influx induced by KCl in cultured sympathetic neurons measured by the Fura-2 technique, which supported the former observations that lobeline (10∼300 μM) antagonized the high voltage-operated Ca^{2+} current (VOCC) in a dose-dependent manner using whole-cell patch clamp in rat sympathetic neurons (Toth and Vizi 1998). However, ET-1 induced the increase of $[Ca^{2+}]_i$ through three types of voltage-independent Ca^{2+} channels (VICC), as well as through VOCC. The latter include two types of Ca^{2+}-permeable nonselective cation channels (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC) (Iwamuro et al. 1998, 1999). However, it is not known which types of Ca^{2+} channels are involved in the lobeline effect on inhibition of increased $[Ca^{2+}]_i$ induced by ET-1. This remains to be determined.

In summary, lobelin added to the cells for the initial incubations prior to addition of ET-1 substantially inhibited cell numbers, DNA synthesis, increased G_0/G_1 phase ratio, and decreased S and G_2/M phase ratio of human umbilical arterial VSMCs in a concentration-dependent manner. Lobeline antagonized proliferation of human umbilical arterial VSMCs induced by ET-1 through inhibition the increase of $[Ca^{2+}]_i$.

These results provide insights into the pharmacological action of lobeline for controlling VSMCs proliferation. Furthermore, lobeline can abolish the VSMC contraction evoked by electrical field stimulation consistently with its suggested presynaptic VOCC inhibitory effect (Santha et al. 2000). All this suggests that lobeline might be used as a potential medicine for the treatment of atherosclerosis, hypertension, restenosis after balloon angioplasty and so on.

4. Experimental

This study was approved by the Human Ethics Committee of our University. All umbilical cords used in the experiments were remnant tissues which would have otherwise been discarded.

4.1. Cell culture and preparation

Samples of human umbilical cord were obtained from normal pregnancies and normal deliveries, immediately after delivery. VSMCs were prepared by the explant method from human umbilical artery. Briefly, the artery was freed from connective tissue and adherent fat, the endothelial cell layer of the intima was removed, and the artery was cut into about 3-mm cubes. They were placed in DMEM supplemented with 20% neonatal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. VSMCs exhibited a typical "hill and valley" growth pattern and the identity was confirmed by morphological examination and by staining for α-actin. Medium was replaced twice a week. The cell became confluent, followed by subculture using trypsinization. Confluent cell at passage of 3∼6 was used for the experiments.

4.2. Cell proliferation assay

Human umbilical arterial VSMCs were counted and seeded into 96-well culture plates at a density of 2×10^4 cells/well. After 24 h, the medium was changed for DMEM containing 0.2% neonatal calf serum to make them quiescent for 48 h. The cells were then exposed to ET-1 (10 nM) in the absence or presence of the indicated concentration of lobeline (0, 10, 30, 100, 300 μM) in DMEM containing 0.2% neonatal calf serum. The cells stained with 0.4% Trypan blue were counted and the cell viability (total cell number-stained cell number)/total cell number × 100% was calculated. The number of living cells was also assessed by 2-(2-methoxy-4nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) reduction for mitochondrial activity using Cell Counting Kit-8 (WST-8, Dojindo, Tokyo, Japan) (Jiang et al. 2004). The O.D. (optical density) value represents the relative number of living cells.

4.3. Measurement of DNA synthesis

Human umbilical VSMCs were seeded (10^5 cells/well) in 24-well plates for 24 h and starved in DMEM containing 0.2% neonatal calf serum for 48 h. Then agents to be tested, ET-1 alone or in combination with 10, 30, 100, 300 μM lobeline, were added into DMEM containing 0.2% neonatal calf serum. VSMCs were exposed to [^{3}H] thymidine at a concentration of 1 μCi/ml for the last 6 h in the 24 h incubation period. At the end of the incubation period, the incorporated radioactivity (counts per minute, cpm) was measured after fixation and solubilisation of cells with a liquid scintillation counter (LS6500, Beckman). The results were expressed as percentages relative to the mean cpm/10^5 cell of the control for each experiment. Experiments were performed 5 times independently in duplicate.

4.4. Analysis of cell cycle progression

Changes in the VSMC cell cycle were assayed by flow cytometry (Beckman Coulter, USA). Quiescent vascular smooth muscle cells were treated with ET-1 (10 nM) in the absence or presence of lobeline (10, 30, 100, 300 μM) for 48 h. Cells were harvested into tubes and fixed with 70% ethanol, then washed with phosphate-buffered saline (PBS), re-suspended in 1 ml of PBS containing 1 mg/ml RNase and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by flow cytometer.

4.5. Measurements of $[Ca^{2+}]_i$ in human umbilical arterial SMCs

$[Ca^{2+}]_i$ was measured using the fluorescent dye fluo-3/AM by standard methods (He et al. 2004). VSMCs (10^6 cells/ml) were loaded with 5 μM fluo-3/AM and incubated for 30 min at 37 °C in a dark environment. After loading, cells were washed three times and incubated with HEPES buffer solution (concentration in mM: NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, HEPES 5, and glucose 10. The pH was brought to 7.4 with NaOH). Fluorescence image of $[Ca^{2+}]_i$ was measured using a laser-scanning confocal microscope (Zeiss LSM510, Germany) and qualitative changes of $[Ca^{2+}]_i$ was inferred from the fluorescence intensity by SimplePCI Imaging Systems (SimplePCI, Compix Inc., USA).

4.6. Drugs and reagents

Lobeline hydrochloride, ET-1, Pluronic F-127, trypsin, dimethyl sulfoxide (DMSO), trypan blue were purchased from Sigma-Aldrich. Fluo-3 AM (Molecular Probes, USA) was dissolved in DMSO and stored at −20 °C. Dulbecco's modified Eagle's medium (DMEM) and neonatal calf serum (NCS) were obtained from Gibco. α-Actin antibody and PCNA antibody were obtained from Maixin-Biotechnology Inc (Fuzhou, China). Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). [^{3}H] Thymidine (specific activity, 1 mCi/mL) was obtained from the Beijing Institute of Nuclear Research (Beijing, China). All other chemicals used were of the highest grade available.

4.7. Statistical analysis

Statistical comparison was carried out with three or more groups using one-way analysis of variance (ANOVA) and Dunnett's test. The data represent means ±S.D. The values of $P < 0.05$ were considered statistically significant, the values of $P < 0.01$ were considered highly significant.

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