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Abstract: This study was designed to investigate the effect of curcumin (diferuloylmethane) on the proliferation and apoptosis of hepatic stellate cells (HSC). The cell line HSC-T6 (1.25×10^5 cells/mL) was incubated with curcumin and HSC proliferation was detected by a methyl thiazolyl tetrazolium colorimetric assay. HSC apoptosis was detected by flow cytometry, transmission electron microscope and agarose gel electrophoresis. HSC proliferation was significantly inhibited in a concentration-dependent manner (10.6 to 63.5%) after incubation with 20-100 μ M curcumin, compared with a control group. At 20, 40, and 60 μ M, after 24 h of incubation, curcumin was associated with a significant increase in the number of HSC in the G2/M phase, and a significant decrease in cell numbers in the S phase ($P < 0.05$). At these concentrations, curcumin was also associated with an increase in the apoptosis index of 15.3 ± 1.9 , 26.7 ± 2.8 , and $37.6 \pm 4.4\%$, respectively, compared to control ($1.9 \pm 0.6\%$, $P < 0.01$). At 40 μ M, the curcumin-induced apoptosis index at 12, 24, 36, and 48 h of incubation was 12.0 ± 2.4 , 26.7 ± 3.5 , 33.8 ± 1.8 , and $49.3 \pm 1.6\%$, respectively ($P < 0.01$). In conclusion, curcumin inhibits the *in vitro* proliferation of HSCs in the G2/M phase of the cell cycle and also induces apoptosis in a concentration- and time-dependent manner. The *in vivo* effect of curcumin on HSCs requires further investigation.

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Effect of Curcumin on the Proliferation and Apoptosis of Hepatic Stellate Cells

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Abstract

This study was designed to investigate the effect of curcumin on the proliferation and apoptosis of Hepatic Stellate Cells (HSC). A cell line, HSC-T6, was incubated with curcumin. Proliferation of HSCs was detected by methyl thiazolyl tetrazolium (MTT) colorimetric assay. Apoptosis of HSCs was detected by Flow Cytometry (FCM), transmission electron microscope (TEM) and agarose gel electrophoresis. Proliferation of HSC was inhibited significantly in a concentration-dependant manner after incubation with 20-60 $\mu\text{mol/L}$ curcumin, compared with control group ($P < 0.05$). At 20, 40 and 60 $\mu\text{mol/L}$, curcumin was associated with a significant increase in the number of HSCs in G₂/M phase ($P < 0.05$), and a significant decrease in cell numbers in the S phase ($P < 0.05$). At these concentrations, curcumin was also associated with an increase in the apoptosis index of $15.3 \pm 1.9\%$, $26.7 \pm 2.8\%$ and $37.6 \pm 4.4\%$, respectively, compared with that of the control group ($1.9 \pm 0.6\%$, $P < 0.01$). At 40 $\mu\text{mol/L}$, curcumin induced apoptosis index at 12 h, 24 h, 36 h and 48 h of incubation was $12.0 \pm 2.4\%$, $26.7 \pm 3.5\%$, $33.8 \pm 1.8\%$, and $49.3 \pm 1.6\%$ respectively ($P < 0.01$). In conclusion, curcumin inhibits the *in vitro* proliferation of hepatic stellate cells in the G₂/M phase of cell cycle. It also induces apoptosis in a concentration- and time-dependent manner. The *in vivo* effect of curcumin on the hepatic stellate cells requires further investigation.

Key Words: Curcumin; hepatic stellate cell; proliferation; apoptosis; hepatic fibrosis

Introduction

Hepatic fibrosis is a reversible complication of advanced liver disease and represents a major worldwide health care burden. Hepatic stellate cells (HSC) undergo activation into proliferative and fibrogenic myofibroblast-like cells during liver injury.¹ The imbalance between proliferation and apoptosis of HSC is the main pathogenesis of liver fibrosis.^{1,2} Therefore, inhibiting HSC activation and inducing their apoptosis may be important strategies to prevent or treat hepatic fibrosis.³

Curcumin, also known as Turmeric yellow or diferuloylmethane, is a phenol derived from a herb by the name of *Curcuma aromatica salisb.*⁴ Curcumin has a chemical structure of 1,6-Heptadiene-3,5-dione and a molecular weight of 368. For several decades curcumin has been widely used as a food coloring ingredient for its yellowish color. In the last 10 years, several *in vitro* and *in vivo* studies have found that curcumin possesses significant pharmacological actions, such as anti-tumor effects, anti-inflammatory effects and induction of apoptosis of cancer cells.⁵⁻¹⁰

The effect of curcumin on HSC and hepatic fibrosis are unknown. The primary aim of this study was to investigate effects of curcumin on the proliferation and apoptosis of HSC cell line *in vitro*.

Materials and Methods

Rat hepatic stellate cell line (HSC-T6), with SV40 transfection showing activated phenotype,¹¹ was a generous gift from Professor Lie-Ming Xu (Division of Liver Diseases, Shanghai University of TCM, Shanghai, China). Curcumin and methyl thiazolyl tetrazolium

(MTT) were purchased from Sigma-Aldrich Company (USA); New-born calf serum (NBCS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone, Gibco (USA). 200bp DNA marker was obtained from Sino-American Biotechnology Company (China).

Preparation for curcumin stock solution

Dimethyl sulfoxide (final concentration <1%) was used to dissolve curcumin. Dulbecco's modified Eagle's medium was added into the stock solution at the concentrations of 10 mmol/L. A 0.22 μ m micropore filter was employed to filtrate bacteria. Subpackages of the stock solution were stored and kept away from light at -20°C.

HSC-T6 culture

Cells were cultured in Dulbecco's modified Eagle's medium culture solution, supplemented with 100U/ml penicillin, 100U/ml streptomycin and 10% new-born calf serum. The cell culture was performed at 37°C under 5% CO₂, 95% air and saturated humidity in cell culture flask. Culture medium was changed every two days. Cells were digested by 0.25% trypsinase and 0.02% EDTA as cell density grew to 80% to 90%.

Effect of curcumin on cell proliferation

Influence of curcumin on HSC proliferation was detected by MTT methods. The cell concentration of logarithmic growth phase was adjusted to 1×10^5 /ml. Then cells were transferred into 96-well plate with 100 μ l in each well. Serum-free of Dulbecco's modified Eagle's solution was added to every well after 24 h and incubated for 24 h to make cells synchronize to resting

stage. Supernatant was removed. Different concentrations (0, 20, 40, 80, 100 $\mu\text{mol/L}$) of curcumin containing Dulbecco's modified Eagle's medium, and 2% serum were added and co-cultured for 24 h, with 5 repeat holes. Supernatant was removed again. 10 μl MTT and 100 μl serum were added to every well and cultured for 4 h. Culture solution was removed. Remaining cells were dissolved with 100 μl dimethyl sulfoxide. Absorbance was detected by Enzyme sign machine (ELSIA Reader, Japan) with 630nm reference wavelength and 570nm measure wavelength.

$$\text{Inhibition ratio} = 1 - (\text{absorbance of drug group} / \text{absorbance of control group}) \times 100\%.$$

Flow cytometry test for apoptosis

Cells in logarithmic growth phase were seeded into T-25 culture flasks at $7 \times 10^5/\text{mL}$ density and incubated for 24 h. Medium was removed. The serum-free of Dulbecco's modified Eagle's medium was added into T-25 culture flasks and incubated for 24 h. Medium was removed. Five different concentrations (0, 10, 20, 40, 60 $\mu\text{mol/L}$) of curcumin were added and incubated for 24 h. In an additional set of experiment, curcumin of 40 $\mu\text{mol/L}$ was added and cultured for 12 h, 24 h, 36 h and 48 h, respectively. Suspended and adherent cells were collected and washed with cold phosphate-buffered saline (PBS) once. Then cells were fixed with 70% alcohol. Single cell suspension was prepared and preserved at 4°C. The fixation fluid was washed with PBS before test. 20 μl RNase A was added and incubated for 30 min at 37°C. 800 μl propidium iodide staining solution was added for staining at night away from light at 4°C.

Apoptosis of HSC-T6 was detected by flow cytometry (BD FACS Calibur, USA). Images were quantitated using ModFIT software to analyze cell generation cycle.

Morphology observation of cell apoptosis

Cells were co-cultured with 40 $\mu\text{mol/L}$ curcumin for 24 h. Suspended and adherent cells were collected and washed with PBS once, and then they were fixed with 2.5% glutaraldehyde at 4°C for 1 h, and postfixed with 1% osmic acid for 30 min. The cells were stained with lead-uranium and the changes in ultrastructural organization were observed under a Hitachi-600 (Tokyo, Japan) transmission electron microscope.

DNA ladder of cell apoptosis test

Cells were treated with four different concentrations (0, 20, 40,60 $\mu\text{mol/L}$) of curcumin for 24 h. DNA was extracted as described previously.¹² 1.5% agarose gel electrophoresis was performed for 3 h at constant voltage of 50 volt, and observed with gel imaging system (Multi Genius Bio-imaging system, SYNGENE, USA).

Statistical analysis

Results were expressed as Means \pm SD. Statistical difference were examined by One-way analysis of variance or student *t* test. Differences were considered to be statistically significant at $P<0.05$.

Results

Curcumin and HSC proliferation.

After curcumin treatment, hepatic stellate cell proliferation reduced significantly compared to the control group (Table 1, $P<0.05$). Inhibition ratio increased along with the increment of the

curcumin concentration ($P<0.01$). The 50% inhibiting concentration (IC_{50}) was $89\mu\text{mol/L}$ after computing with Probit method.

Effects of curcumin on cell cycle

As shown in Table 2, curcumin treatment at a concentration between 10 and $60\mu\text{mol/L}$ curcumin was associated with no significant changes cell numbers in the G_0/G_1 ($P>0.05$ compared with that of the control group). There was a gradual decrease in the number of cells in the S phase, and a gradual increase in the cells in the G2/M phase ($P<0.05$ and 0.01 , respectively), as the curcumin concentrations increased from 10 to $60\mu\text{mol/L}$.

Effects of curcumin on cell apoptosis

A significant peak of hypodiploid of apoptosis could be found by flow cytometry when HSCs were incubated with curcumin at 20, 40 and $60\mu\text{mol/L}$ (Fig 1), whereas no such peak could be found in the control group. Apoptosis indexes (%) of the curcumin groups were $15.3\pm 1.88\%$, $26.7\pm 2.79\%$ and $37.6\pm 4.38\%$, respectively, which were higher than that of control group ($1.9\pm 0.64\%$, $P<0.01$).

Apoptosis index of HSCs elevated gradually along with the treatment time when the HSCs were treated with $40\mu\text{mol/L}$ curcumin (Fig 2).

Morphology of HSC

After incubated with $40\mu\text{mol/L}$ of curcumin for 24h, HSCs became smaller under the contrast phase microscope (Fig 3). Nuclear chromatin enriched to globular, and chromatin condensation ranked along inside of nuclear membrane. Condensed organelle and apoptotic body could be observed by transmission electron microscope (Fig 4).

DNA fragment detection

A high molecular weight strap was found on the top of the agarose gel of the control group (Fig 5). Meanwhile, DNA ladder could be found after treatment at 20, 40 and 60 μ mol/L of curcumin (Fig 5).

Discussion

The major findings of our study are: 1) Curcumin concentration-dependently inhibits HSC proliferation *in vitro*; 2) Curcumin concentration-dependently reduces the number of cells in the S phase, and increases the cells in the G2/M phase; 3) Curcumin induces apoptosis of hepatic stellate cells in a concentration- and time-dependent manner.

Because of the critical role of HSC in the pathogenesis of hepatic fibrosis, induction of HSC apoptosis has become potentially important prevention or treatment of hepatic fibrosis. Enhanced HSC apoptosis may reduce the substrate of collagen genesis and increase extracellular matrix degradation, assisting the recovery of hepatic fibrosis. Our *in vitro* study clearly demonstrated that curcumin can induce HSC apoptosis, and suppress the proliferation of HSCs in the S phase. Curcumin not only inhibits HSC proliferation and induces apoptosis, but also inhibits the transformation of HSC from quiescent phenotype to activated phenotype, and decreases the expression of α -SMA, a marker of HSC activation.^{13,14} Curcumin also reduces the secretion of collagetype I in HSC *in vitro*, and the collagen deposition in the liver of hepatic fibrotic rats induced by carbon tetrachloride.^{13,14} An earlier human study showed that in a group of volunteer treated with 8 g curcumin each day for three months, no significant side effects were observed.¹⁵ These results suggest that curcumin may be safely used to treat hepatic fibrosis

through several different mechanisms.

The signaling pathways by which curcumin affects cell proliferation and apoptosis are not entirely clear. In rat HSC, the inhibition of cell proliferation and induction of apoptosis appears to be related to the activation of peroxisome proliferator,¹⁶ a key regulator of the cell cycle and apoptosis. In Hodgkin's lymphoma cells, the effect of curcumin on the cells is associated with the inhibition of constitutively active NF-kappaB and STAT3 pathways, leading to a decreased expression of proteins involved in cell proliferation and apoptosis, e.g. Bcl-2, Bcl-xL.¹⁷ Also in Hodgkin's lymphoma cells, curcumin triggers apoptosis by activating caspase-3 and caspase-9, and by changing the nuclear morphology and phosphatidylserine translocation.¹⁷ In human acute myelogenous leukemia HL-60 cells, curcumin is also found to induce apoptosis through mitochondrial pathway involving caspase-8, BID cleavage, cytochrome C release, and caspase-3 activation.⁷ It is uncertain how these signaling pathways or their interplay would have contributed to the significant effect of curcumin on cell growth and apoptosis in our study. The precise signaling pathways leading to proliferation inhibition and apoptosis induction needs to be further investigated.

In summary, we found that curcumin significantly inhibits proliferation and induces apoptosis of hepatic stellate cells *in vitro*. Further clinical studies are warranted to evaluate the safety and efficacy of this agent in the management of hepatic fibrosis.

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Table1. Effect of curcumin on hepatic stellate cell proliferation.

| <i>Concentration</i> ($\mu\text{mol/L}$) | <i>Absorption</i> | <i>Inhibition ratio (%)</i> |
|---|--------------------|-----------------------------|
| 0 | 1.33 \pm 0.04 | 0 |
| 20 | 1.19 \pm 0.02 * | 10.6 |
| 40 | 1.01 \pm 0.05 ** | 24.2 |
| 60 | 0.93 \pm 0.03 ** | 30.1 |
| 80 | 0.75 \pm 0.02 ** | 43.9 |
| 100 | 0.49 \pm 0.03 ** | 63.5 |

* $P < 0.05$; ** $P < 0.01$ vs control group.

Table 2. Effects of curcumin on cell cycle.

| <i>Concentration (μmol/L)</i> | <i>G0/G1 (%)</i> | <i>S (%)</i> | <i>G2/M (%)</i> |
|-------------------------------|------------------|--------------|-----------------|
| 0 | 43.7±1.61 | 50.3±1.51 | 6.0±0.53 |
| 10 | 46.5±1.58 | 46.9±1.18 | 6.6±1.08 |
| 20 | 48.6±1.08 | 43.6±0.92 * | 7.8±0.78 * |
| 40 | 48.1±1.73 | 33.0±0.64 ** | 18.9±1.16 ** |
| 60 | 48.4±1.71 | 30.4±1.28 ** | 21.2±0.92 ** |

* $P < 0.05$, ** $P < 0.01$ vs control group

Figure legends

Fig 1. Flow cytometry examination of apoptosis before (control group, Fig 1a), and after treatment of curcumin at 20 (Fig 1b), 40 (Fig 1c) and 60 $\mu\text{mol/L}$ (Fig 1d).

Fig 2. Comparison of apoptosis index of hepatic stellate cells treated with 40 $\mu\text{mol/L}$ of curcumin for different duration.

Fig 3. Effect of curcumin on the apoptosis of hepatic stellate cells (examined by inverted microscope) ($\times 100$)

Fig 4. Effect of curcumin on the apoptosis of hepatic stellate cells (examined by electron microscope) ($\times 7000$)

Fig 5. Detection of DNA fragmentation by agarose gel electrophoresis. A: 200bp DNA marker; B: Control group; C: curcumin 60 $\mu\text{mol/L}$; D: curcumin 40 $\mu\text{mol/L}$; E: curcumin 20 $\mu\text{mol/L}$

Fig 1

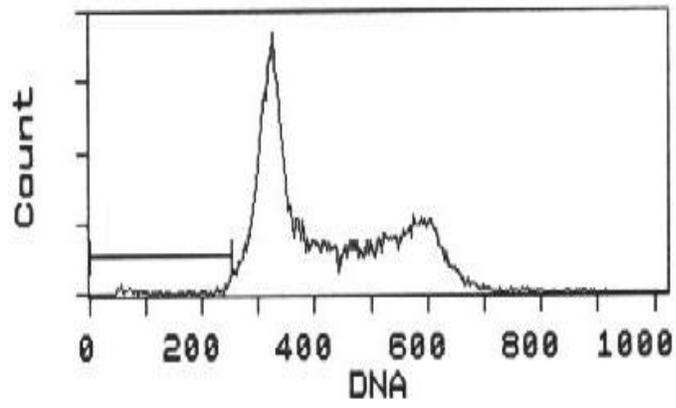


Fig 1a

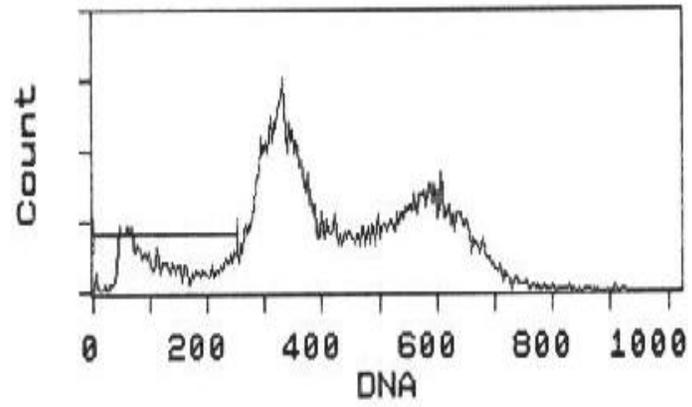


Fig 1b

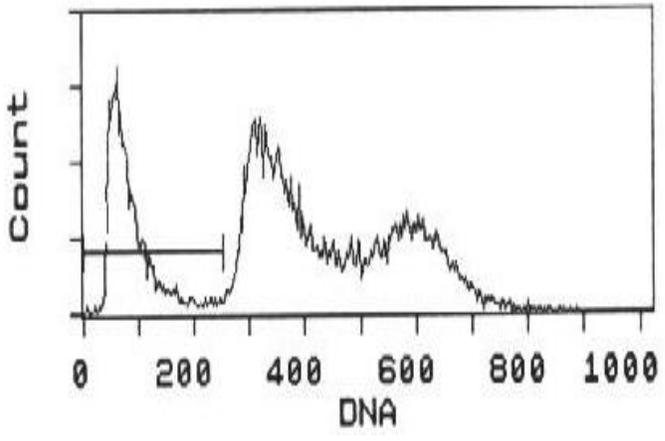


Fig 1c

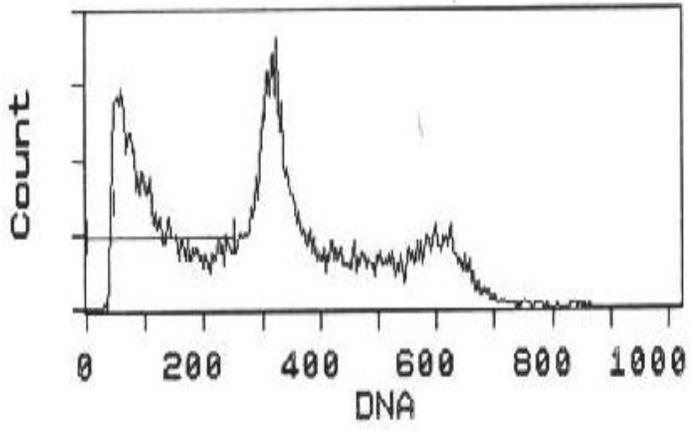


Fig 1d

Fig 2

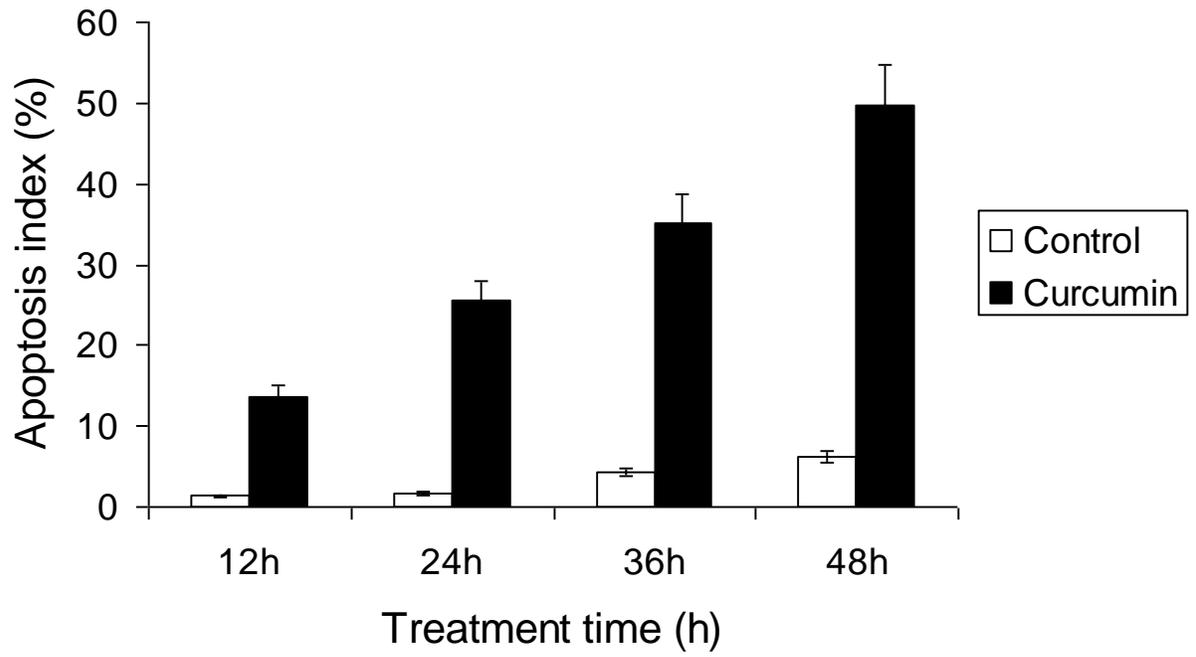
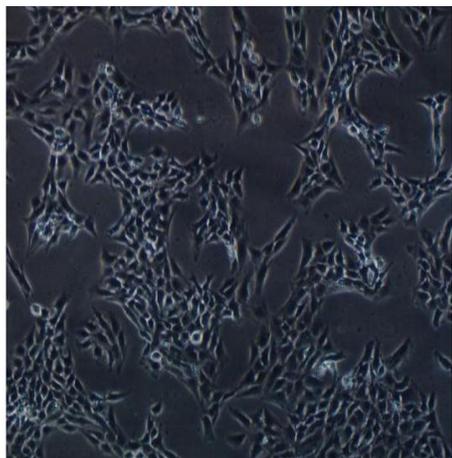
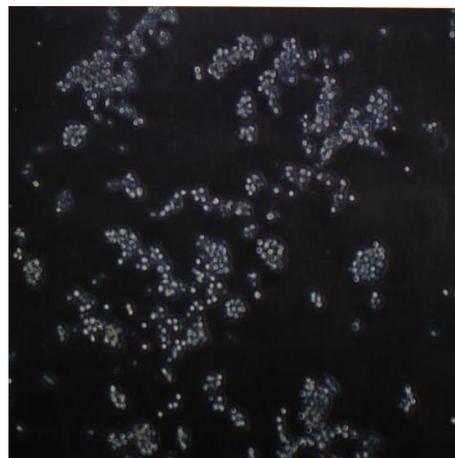


Fig 3



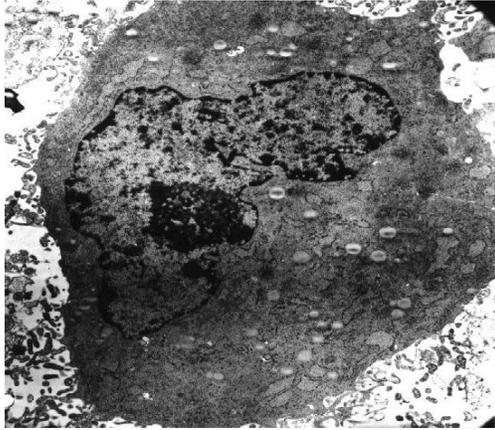
Control group ($\times 100$)



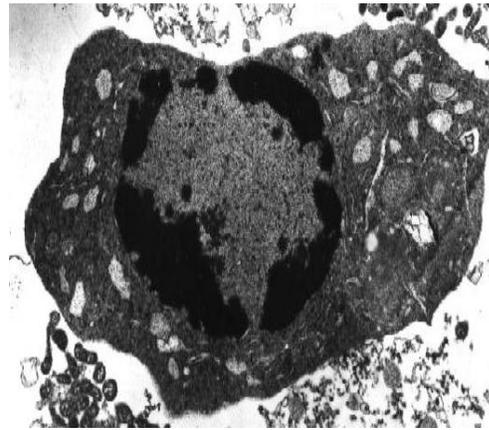
Curcumin $40\mu\text{mol/L}$ ($\times 100$)

在每个图中插入放大倍数的表尺(μm)，不能只标明 $\times 100$. 并用箭头标出调亡的细胞

Fig 4



Control group ($\times 7000$)



Curcumin 40 $\mu\text{mol/L}$ ($\times 7000$)

在每个图中插入放大倍数的表尺(μm)，不能只标明 $\times 7000$.

并用箭头标出相关的结构，附加文字说明

Fig 5

