



## Effect of lycium barbarum polysaccharide on human hepatoma QGY7703 cells: Inhibition of proliferation and induction of apoptosis

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Received 27 May 2004; accepted 9 November 2004

### Abstract

Lycium barbarum polysaccharide (LBP), extracted from *Lycium barbarum* that is a kind of traditional Chinese herb, is found to have anticancer activity. In this study, the effect of LBP on the proliferation rate, cell cycle distribution and apoptosis in the human hepatoma QGY7703 cell line were investigated. The effects of this compound were also tested on the concentration of calcium in cells. LBP treatment caused inhibition of QGY7703 cell growth with cycle arrest in S phase and apoptosis induction. The amount of RNA in cells and the concentration of intracellular Ca<sup>2+</sup> were increased. Moreover, the distribution of calcium in cells was changed. Taken together, the study suggests that the induction of cell cycle arrest and the increase of intracellular calcium in apoptotic system may participate in the antiproliferative activity of LBP in QGY7703 cells.

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**Keywords:** Lycium barbarum polysaccharide; Apoptosis; Cell-cycle; Calcium

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## Introduction

Lycium barbarum polysaccharide (LBP), extracted from traditional Chinese herb *Lycium barbarum*, is found to have bioactivities such as anticancer, antioxidant, hypoglycemic and immunological activities (Zang et al., 1989; Li et al., 1991; Luo et al., 1997). Both in vitro and in vivo studies, the results showed that LBP was an effective anticancer compound. In vivo, LBP could dose-dependently decrease tumor weight and increase the amount of splenocytes, proliferation of activated T cells, NK activity and TNF $\alpha$  levels in S180-bearing mice (Liu et al., 1996). In vitro, 20–1000 mg/L LBP could inhibit the growth of human leukemia HL-60 cells in dose-dependent manner and decrease the membrane fluidity (Gan et al., 2001).

Apoptosis has been characterized as a fundamental cellular activity to maintain the physiological balance of the organism. It is also involved in immune defense machinery and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells proliferated owing to various chemical agents' induction. Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer (Hengartner, 2000; Hsu et al., 2004). The anticancer activity mechanism of LBP is complicated. Cytokine had been studied as a way of mechanism. According to Geng's study, LBP could increase the IL-2 level in spleen cells of sixteen-month old mice to that of two-month old mice (Geng et al., 1989). And the levels of IL-2 and TNF- $\alpha$  mRNA in human peripheral blood mononuclear cells could be up regulated by LBP at the dose of 5–40 mg/L (Gan and Zhang, 2002). But more and more experiments established that apoptosis is one of the key pathways. Biochemical changes in apoptosis have been studied in cells other than hepatic cells, in particular in T lymphocytes (Wyllie, 1980). In vitro, LBP could affect the synthesis of DNA of cervix cancer cell. The cells of S-phase decreased from 56% to 49%. And the cells of G1-phase increased from 16% to 33% (Hu et al., 1994). However, results confirm that many biochemical changes recorded in these cells are also present in hepatic cells. In contrast to necrosis and despite the morphological nuclear changes, apoptosis is an active cell phenomenon characterized by an increase in RNA and protein synthesis (Wyllie et al., 1984). This fundamental fact is well established, even if the sequence of this process is still being debated (Martin, 1993). The increased synthesis is reflected by an increased activity of several nuclear and cytoplasmic enzymes some of which are Ca<sup>2+</sup>, Mg<sup>2+</sup>-dependent. Although the identification of these enzymes is not certain yet, several candidates such as NUC 18, or DNase I or DNase II have been suggested (Peitsch et al., 1994). So the role of Ca<sup>2+</sup> concentration in cell is very important in apoptosis.

Liver cancer can be considered as the commonest cancer in the world with an annual incidence of one million cases (Siu et al., 2002). Moreover, there are some limitations related to the current treatment of liver cancers. In this study, we determined the antiproliferative activity of LBP, and examined its effect on cell cycle distribution and apoptosis in the human liver cancer cell line, QGY7703. Furthermore, to establish the anticancer mechanism of LBP, we assayed the levels of the concentration of Ca<sup>2+</sup> in cells, which are strongly associated with the signal transduction pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents.

## Methods

### Materials

Fetal calf serum (FCS), penicillin G, streptomycin, amphotericin B, and RPMI-1640 were obtained from GIBCO BRL. Dimethyl Sulphoxide (DMSO), ribonuclease (RNase), propidium iodide (PI),

acridine orange, ethidium bromide and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) were purchased from Sigma Chemical.

### *Preparation of LBP*

LBP was a kind of polysaccharide-protein complex extracted from *Lycium barbarum*, which was supplied by our laboratory. LBP was prepared as a stock of 5000 mg/L in basal medium RPMI-1640 and kept at  $-20\text{ }^{\circ}\text{C}$ . For all experiments, final concentrations of the tested compound were prepared by diluting the stock with RPMI-1640. Control cultures received the same volume of RPMI-1640.

### *Cell line and culture*

Human hepatoma cell line, QGY7703, was supplied by Shanghai Institute of Biochemistry. It was maintained in monolayer culture at  $37\text{ }^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in RPMI-1640 supplemented with 10% FCS, 10 U/mL penicillin, 10 mg/mL streptomycin, 200 mg/L L-Glutamine (Sigma, USA) and 5 ml/L insulin (Sigma, USA).

### *Cell proliferation assay*

Inhibition of cell proliferation by LBP was measured by MTT assay. Briefly, QGY-7703 cells were plated in 96-well culture plates ( $2.0 \times 10^3$  cells per well). After 24-hr incubation, the cells were treated with LBP (0 mg/L, 50 mg/L, 100 mg/L, 200 mg/L and 400 mg/L) for 2, 4, and 6 days. The medium was replaced every other day. Dye solution (40  $\mu\text{l}$ ) specific for the MTT assay was added to each well for an additional 4-hour incubation at  $37\text{ }^{\circ}\text{C}$ . After adding stop solution DMSO (100  $\mu\text{l}$ /well), the absorbance at 490 nm was measured on an ELISA reader (Multiskan EX, Labsystems). The percent viability of the treated cells was calculated as follows:  $(A_{490\text{ nm}})_{\text{sample}} / (A_{490\text{ nm}})_{\text{control}} \times 100\%$ .

### *Morphological observation*

QGY7703 cells were cultured with basal medium and medium contains 100 mg/L LBP for 4 days. Then they were examined with fluorescence microscope (OLYMPUS IX70) and laser scanning confocal microscope (LSCM) (LSM510, ZEISS, GER). The cells were dyed by acridine orange (AO) and ethidium bromide (EB) before examined by fluorescence microscope and were dyed by acridine orange before examined by LSCM.

### *Analysis of cell-cycle phase distribution by flow cytometry with PI staining*

After have been cultured for 4 days with the medium contains 100 mg/L, 200 mg/L and 400 mg/L LBP, QGY7703 cells were collected, washed by PBS and were fixed with ice-cold 70% ethanol for at least 24 hours. The cells were centrifuged at  $1500 \times g$  and the cell pellet was resuspended in 400  $\mu\text{l}$  of PBS, 50  $\mu\text{l}$  of Rnase A (10 mg/ml; Sigma) and 10  $\mu\text{l}$  of PI (2 mg/ml; Sigma). The mixture was incubated in the dark at  $37\text{ }^{\circ}\text{C}$  for 30 minutes and was then analyzed by flow cytometer (Becton

Dickinson). The percentage of cell population at a particular phase was estimated with ModFit LT for Mac V.3.0.

#### *Determination of the concentration of calcium in cells*

QGY-7703 cells were cultured for 4 and 6 days with the medium containing 100 mg/L LBP. Then the fluo-3/AM (Sigma) was added to cells for an additional 40-min incubation. Finally, the cells were washed by PBS for 3 times and examined by LSCM.

#### *Statistical analysis*

The results were expressed as mean  $\pm$  standard deviation. The difference between control and LBP-treated cells was evaluated using Student's t test. P value less than 0.05 was considered statistically significant.

## Results

#### *Effects of LBP on QGY7703 cells proliferation*

QGY7703 cells were cultured in 10% FCS-containing medium with or without LBP (50–400 mg/L) during 6 days and cell proliferation was evaluated by the MTT test. Under our experimental conditions, a dramatic decrease in proliferation was observed until 4 days after LBP treatment (50, 100, 200 and 400 mg/L) (Fig. 1), especially at 4 days for 100 mg/L LBP where the percentage of inhibition was 30 % ( $P < 0.05$ ). As the percentage of inhibition did not increase for 50, 200 or 400 mg/L LBP, we choose 100 mg/L for the following experiments.

#### *Results of morphological observation*

Stained by EB and AO, the color of normal cell is green while the color of apoptosis cell is yellow or kelly under fluorescence microscope. After 4 days treatment of LBP at the dose of 100 mg/L, the cells

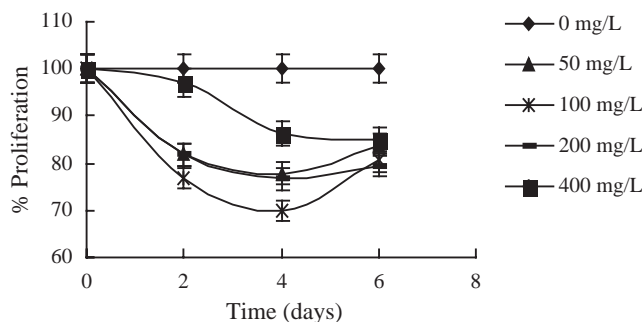


Fig. 1. Effect of LBP on QGY7703 cell growth. After 24 h adherence, a cell line was cultured in 10% FCS medium and treated with LBP (50–400 mg/L) for 2–6 days. Results are presented as percentage of control (untreated cells). Values were expressed as means  $\pm$  S.D. of six experiments ( $n = 6$ ) ( $P$ -value relative to control group:  $P < 0.05$ ).

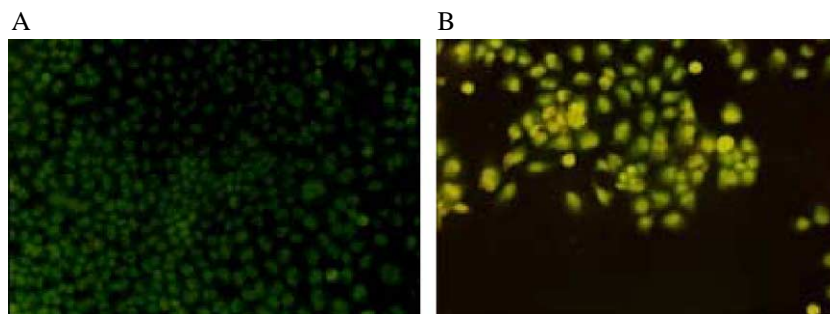


Fig. 2. Morphological study with fluorescence microscope. Cultured QGY7703 cells were stained with ethidium bromide (EB), acridine orange (AO) before (control) (A) and after treatment with LBP (100 mg/L) for 4 days (B). The representative cells were counted as apoptosis in fluorescence microscopy with  $\times 200$  magnification.

were much brighter than the control cells. Almost all the control cells were green and seldom were yellow (Fig. 2A). But many treatment cells were yellow or kelly (Fig. 2B).

Observed by LSCM, the karyon appears bright yellow and the profile of cells appears dark yellow (Fig. 3). The treatment of LBP induced apoptosis at the dose of 100 mg/L. In the control group cells, the karyon was bigger and smoother than that of cells treated by LBP (Fig. 3A). While among the LBP treated cells, many “apoptotic bodies” were observed. The compact masses of chromatin aggregated along the nuclear membrane. Round, compact granular masses appeared nearer the center of the nucleus and there was a reduction in nuclear volume. At the same time the cytoplasm displayed condensation. And some of the nucleus degenerated into discrete spherical or ovoid fragments of highly condensed chromatin (Fig. 3B).

Excited by the light at the wavelength of 460 nm, RNA appeared red while DNA was yellow observed under the excitation wavelength of 502 nm (Fig. 4). After the cells were cultured for 4 days, the amount of RNA in LBP (100 mg/L) treated cells was more than that in control cells (Fig. 4A, B).

Three-dimensional structure of cells was made by LSCM (Fig. 5). The karyon of control cell was flat while that of LBP treated cell was coarse. And the center part of the latter was sunken.

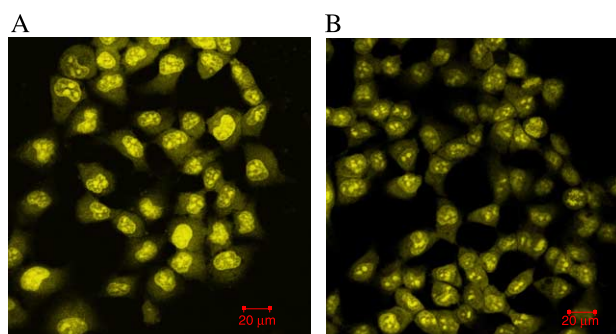


Fig. 3. Morphological study with LSCM. Cultured QGY7703 cells were stained with ethidium bromide (EB), acridine orange (AO) before (control) (A) and after treatment with LBP (100 mg/L) for 4 days (B). The representative cells were counted as apoptosis in LSCM with  $\times 200$  magnification.

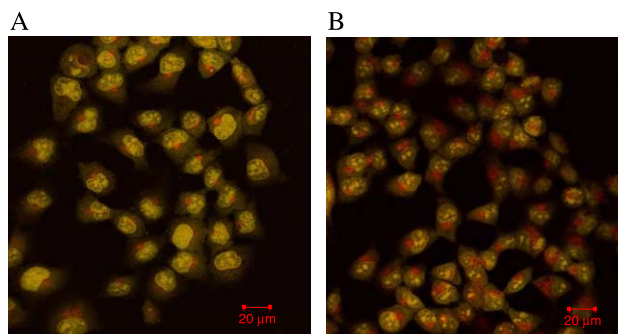


Fig. 4. RNA distribution analysis in QGY7703 cells. Cultured QGY7703 cells were stained with ethidium bromide (EB), acridine orange (AO) before (control) (A) and after treatment with LBP (100 mg/L) for 4 days (B). The representative cells were counted as apoptosis in LSCM with  $\times 400$  magnification.

#### *Effect of LBP on cell cycle progression of QGY7703*

Cell-cycle phase distribution was analyzed by flow cytometry with PI staining (Fig. 6). The percentage of cells in G0/G1, S, and G2/M phases were calculated using Multicycle software and were indicated on the Table 1. QGY7703 cells were treated with 100 mg/L, 200 mg/L and 400 mg/L of LBP for 4 days. A significant accumulation of cells in the S phase (32.47% to 46.57%) was observed in various LBP concentrations ( $P < 0.01$ ) (Table 1). A sub-G1 population, normally associated with apoptotic cells, appeared compared to controls (Fig. 2C).

#### *Effect of LBP on the concentration of calcium in cells*

To investigate whether  $\text{Ca}^{2+}$  signaling is involved in LBP-induced apoptosis in QGY7703 cells, the concentration of  $\text{Ca}^{2+}$  in cells was determined by LSCM. The results showed that 100 mg/L of LBP could enhance the intracellular  $\text{Ca}^{2+}$  concentration in QGY7703 cells significantly. When cells were cultured for 4 days, the concentration of  $\text{Ca}^{2+}$  in control cells was  $20.7 \pm 11.8$ , while the

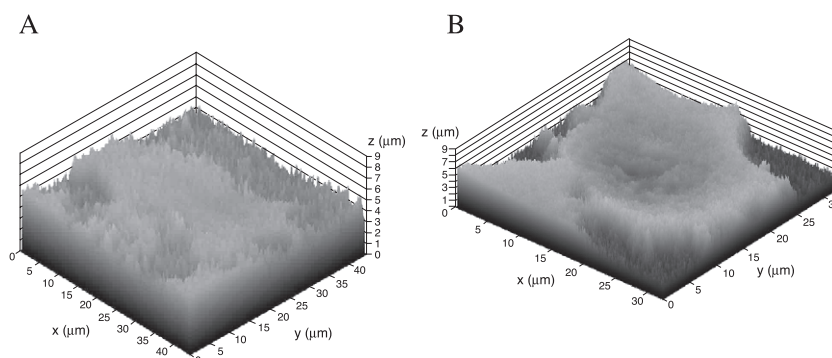


Fig. 5. Three-dimensional structure of QGY7703 cells. Cultured QGY7703 cells before (A) and after treatment with LBP (100 mg/L) for 4 days (B).

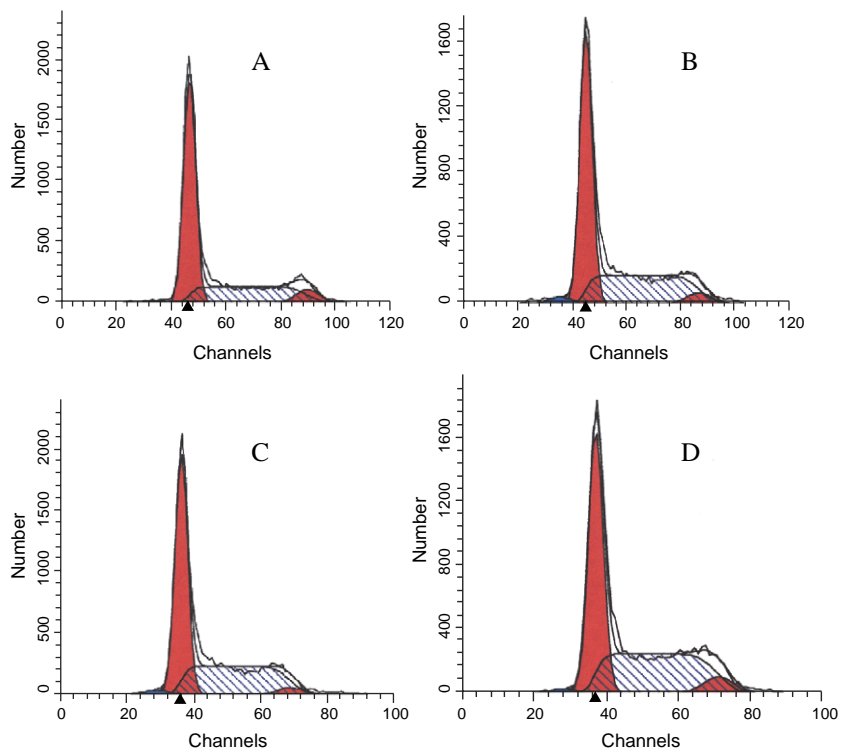


Fig. 6. Inhibition of cell cycle progress in QGY-7703 cells by treatment with LBP. (A) Cell cycle analysis of QGY-7703 cells following treatment without LBP for 4 days. (B) Cell cycle analysis of QGY-7703 cells following treatment with 100 mg/L LBP for 4 days. (C) Cell cycle analysis of QGY-7703 cells following treatment with 200 mg/L LBP for 4 days. (D) Cell cycle analysis of QGY-7703 cells following treatment with 400 mg/L LBP for 4 days. Cells were fixed with ethanol and stained with PI, and then cell cycle distribution was analyzed by flow cytometry.

concentration of  $\text{Ca}^{2+}$  in treated cells increased to 4.5 times of that of the control cells. When cells were cultured for 6 days, intracellular  $\text{Ca}^{2+}$  concentration in control cells became  $38.2 \pm 12.6$ , while concentration of  $\text{Ca}^{2+}$  in LBP treated cells became  $47.3 \pm 13.6$ , which was higher than that of control

Table 1  
Effect of LBP on cell cycle distribution of QGY-7703 cells (n=3)

Group	Sub-G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
A	$0.72 \pm 0.03$	$62.98 \pm 2.75$	$32.47 \pm 1.80$	$4.56 \pm 1.06$
B	$2.01 \pm 0.20^{**}$	$54.31 \pm 1.51^{**}$	$41.46 \pm 1.96^{**}$	$4.23 \pm 1.23$
C	$1.63 \pm 0.21^{**}$	$53.00 \pm 1.90^{**}$	$43.97 \pm 1.80^{**}$	$3.03 \pm 1.38$
D	$1.06 \pm 0.15^*$	$47.58 \pm 1.90^{**}$	$46.57 \pm 1.30^{**}$	$5.84 \pm 3.55$

(A) Cell cycle analysis of QGY-7703 cells following treatment without LBP for 4 days. (B) Cell cycle analysis of QGY-7703 cells following treatment with 100 mg/L LBP for 4 days. (C) Cell cycle analysis of QGY-7703 cells following treatment with 200 mg/L LBP for 4 days. (D) Cell cycle analysis of QGY-7703 cells following treatment with 400 mg/L LBP for 4 days. Compared with A.

\*  $P < 0.05$ .

\*\*  $P < 0.01$ .

Table 2  
Effect of LBP on the concentration of  $\text{Ca}^{2+}$  in QGY7703 cells

Days	n	Control	Treated	Treated/Control
4	20	20.7 $\pm$ 11.8	93.1 $\pm$ 26.9**	4.5
6	20	38.2 $\pm$ 12.6	47.3 $\pm$ 13.6*	1.2

Cells treated with 100 mg/L LBP for 4 days and 6 days and were visualized by confocal microscopy. Cells were labeled with Fluo-3 AM, mounted in a small chamber and incubated in standard buffer for imaging, to indicate the intracellular calcium (excitation at 488 nm). The concentration of calcium was expressed in the relative fluorescence intensity of cells. Compared with control.

\*  $P < 0.05$ .

\*\*  $P < 0.01$ .

cells, but lower than that of LBP treated cells cultured for 4 days (Table 2). These results suggest that in apoptotic QGY7703 cells intracellular calcium accumulated. In control cells, calcium calculated in peri-nuclear area. However, cells treated with LBP for 4 days present a very different pattern of Fluo-3 staining, i.e. disappearance of the peri-nuclear higher  $[\text{Ca}^{2+}]_i$  region and accumulation of calcium in the nucleus (Fig. 7).

## Discussion

LBP was a kind of polysaccharide-protein complex, which has anticancer and immunologic enhancement activities (Gan et al., 2003, 2004). This study showed that LBP inhibited the proliferation of liver cancer cell line QGY7703.

Fluorescence microscopy was the most common method for morphological observation of cells. Apoptosis cells could be differentiated from normal cells by the difference colour of cells. But this method could not tell the difference of the profile of chromatin. Scanning electronic microscopy (SEM) and laser scanning confocal microscopy (LSCM) could observe the morphological difference of chromatin. But the complicated and time wasted sample preparation of SEM limited its application in the field of apoptosis research. LSCM has been wildly applied to cell biology including morphological

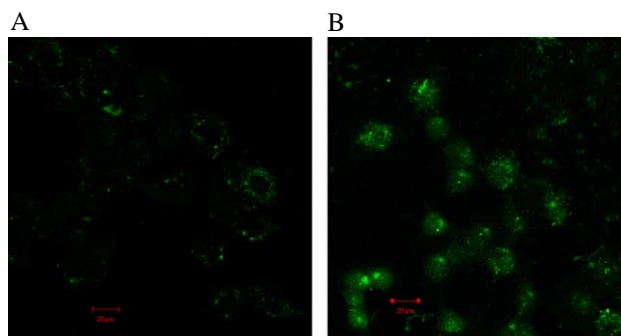


Fig. 7. Effects of LBP on intracellular  $\text{Ca}^{2+}$  distribution in apoptotic QGY7703 cells at the dose of 100 mg/L for 4 days. QGY7703 cells were labeled with Fluo-3 AM, attached to a coverslip. (A) Calcium distribution in control cells cultured in a small chamber with RPMI 1640 medium for 4 days at 37 °C. (B) Calcium distribution in LBP treated cells cultured in a small chamber with RPMI 1640 medium supplemented with 100 mg/L LBP for 4 days at 37 °C.

identification of apoptosis, organization of chromatin, apoptotic DNA fragmentation, endonuclease activity and the concentration of  $\text{Ca}^{2+}$  (Tattona and Rideout, 1999). A double-labeling method with EB and AO was used in this study to observe the morphological changing of apoptosis cells. Excited by light of wavelength of 502 nm, the compact mass of chromatin in karyon of LBP treated cells was visible directly. When the excited light wavelength turn to 460 nm, RNA could be seen in the colour of red. The amount of RNA in LBP treated cells increased, which suggested that cells were arrested in S-phase.

In the studies of cell cycle distribution, the results of FACS confirmed that cell cycle progression of LBP treated cells was blocked in S-phase, which was in according with the results of LSCM. LBP treated cells' apoptosis rate was increased, but not strongly. This might own to the LBP treated cells were blocked in S-phase, which was between diploid and tetraploid and could not presented in sub-G1 phase.

Early studies from many laboratories have demonstrated that the most important signal element,  $\text{Ca}^{2+}$ , might be involved in triggering and regulating apoptosis. Working with immature thymocytes, McConkey shows that glucocorticoid-stimulated apoptosis is associated with enhanced  $\text{Ca}^{2+}$  influx. Apoptosis in other systems also appears to involve alterations in intracellular  $\text{Ca}^{2+}$  (Fang et al., 1998; Kaneko and Tsukamoto, 1994). The present study demonstrated that apoptosis induced by LBP was accompanied by an increase in  $\text{Ca}^{2+}$  ions. Furthermore, the effect of LBP on the concentration of  $\text{Ca}^{2+}$  ions was in accordance with the effects on the proliferation of QYG7703 cells. The distribution of intracellular calcium was also changed by the treatment of LBP.

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