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Effects of glutamine on tumor growth and apoptosis of hepatoma cells[1]

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KEY WORDS glutamine; free radicals; apoptosis; nitric oxide; glutathione; hepatocellular carcinoma

ABSTRACT

AIM: To explore the effects of glutamine on growth and apoptosis of hepatoma cells. **METHODS**: Mice inoculated with hepatoma cell (H22) suspension subcutaneously at right axilla were orally administered with glutamine (GLN) solution. Human hepatoma cell culture (SMMC-7721) was treated with different concentrations of GLN solution. The content of malondialdehyde (MDA) and nitric oxide (NO) was detected in mice plasma and cell culture, and that of glutathione (GSH) was decected in cells. The inoculated tumor's growth in the mice and hepatoma cells' proliferation and apoptosis were observed. **RESULTS**: When mice were administered orally with GLN solution (300 mg/kg), the growth of inoculated hepatoma was suppressed in the mice. When different concentrations of GLN solution were added in human hepatoma cell culture, the hepatoma cells' proliferation was inhibited and cells were induced to apoptosis, which was dependent on GLN concentration; meanwhile the contents of NO rose both in mice plasma and in cell culture, however MDA contents were slightly lowered in both, and the activity of GSH increased in the cells which had been ultrasonically shattered. **CONCLUSION**: Hepatoma cell apoptosis and tumor growth inhibition by GLN may be associated with its antioxidative activity and its intervention in hepatoma cell proliferation, and simultaneous release of NO.

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INTRODUCTION

Glutamine (GLN) can offer energy by being decomposed in the course of body's metabolism. It can also protect human body against oxidative damages as it is involved in synthesis of Glutathione (GSH)[1-3]. It is known that free radicals are involved in the hypoxic or ischaemic stress and carcinogenesis, and accompany the change in the activity of the antioxidases *in vivo*[4,5]. GLN is reported to have an antioxidative potential, however it is still uncertain whether GLN influences tumor growth and leads to proliferation of hepatoma cells, so we explored this contention in this paper using both animal and cell model.

MATERIALS AND METHODS

Materials Hepatoma cells (H22) were provided by Shanghai Institute of Materia Medica, Chinese Academy of Sciences; SMMC-7721 human hepatoma cell line (provided by Zhongshan Hospital Institute of Hepatoma, Shanghai, China); Cyclophosphamide (CTX, provided by Shanghai Twelfth Pharmaceutical Factory); *L*-Glutamine (provided by Second Military Medical University, Shanghai); "1640" culture containing 10 % new bovine serum (SABC, Sino-American Biotechnology Co Shanghai Branch); Normal saline with 0.2 % trypan blue; RNase A (SABC), Proteinase K (Merck, Germany). All reagents were of analytical quality.

Apparatuses SCHIMADZU UV-240 ultraviolet spectrophotometer (Japan); LEICA DMLB fluorescence microscope (Germany).

Animal experiments Mice were obtained from the Experimental Animal Center of Shanghai Medical University, Shanghai, China. They were housed in an air-conditioned (23 ± 3) ℃ environment with a light-dark cycle of 12 h, and were allowed access to water and a standard diet *ad libitum*.

Thirty male Kunming mice weighing 22 - 25 g were divided into three groups: normal saline control (10

mice), positive control group (10 mice), and experimental group (10 mice). Under aseptic conditions, we tapped ascites from the abdomen of mouse which was used to preserve hepatoma cells (H22) and then diluted it into the hepatoma cells suspension with equal volume of normal saline. Each mouse was inoculated subcutaneously at its right axilla with 0.2 mL hepatoma cell suspension (2×10^6 cells/mL). Ten days before the inoculation mice of the control group were begun to be administered orally, normal saline and mice of the experimental group were to be administered orally GLN solution (300 mg/kg) everyday. Mice of the positive control group were administered orally CTX ($15\ mg \cdot kg^{-1} \cdot d^{-1}$) after the inoculation. Two weeks after the inoculation, the mice were killed and their subcutaneous tumors were weighed. The content of NO in the mouse plasma was determined using the improved nitrite method[6]. The content of MDA was determined by assaying thiobarbiturate (TBA) spectrophotometrically[7].

Cell experiments In thirty-six culture flasks were added 4×10^5 human hepatoma cells and 10 mL "1640" culture containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 % bovine serum inactivated for 1 h at 56 ℃. The control group comprised of nine flasks without the addition of GLN solution, and in the other three experimental groups each of which comprised nine flasks was added 5 mmol/L, 10 mmol/L, and 20 mmmol/L GLN solution respectively.

Cells cultured for 1, 3, and 5 d were digested with 0.25 % trypisn and made into cell suspension (3 flasks from each group). Cell viability of the total cell population was examined by 0.2% trypan blue[8]. The survival rate of the cells was calculated by the following formula:

$$\text{Survival rate of cells} = \frac{\text{Number of total cells} - \text{Number of dyed cells}}{\text{Number of total cells}} \times 100\ \%$$

300 μL cell culture was taken to determine NO content with the improved nitrite method[6].

200 μL cell culture was taken to determine MDA content with the TBA assay[7].

500 μL cell suspension which had been shattered by ultrasonic wave was taken to determine GSH content fluorimetrically[9].

Determination of cell apoptosis One hundred μL cell suspension (8×10^5/mL) was treated with 10 μL acridine orange (1 g/10 L), and homogenized. A drop of sample was spread on a slide and was observed under fluorescence microscope, and photographed.

Flow cytometry analysis was applied to determine cell cycle and apoptosis. After cultured for 5 d, 41×10^6 cells washed with PBS, added with 75 % cold alcohol, fixed at −20 ℃ overnight, added with splitting solution (0.2 mol/L Na_2HPO_4, 0.1 mol/L citric acid, 0.1 % Triton X-100, pH 7.8), placed stationarily for 45 min at 20 ℃, digested by RNaseA (50 μg/mL) for 10 min, stained by the DNA binding dye propidium iodide (PI) 50 μg/mL for 30 min, and determined flow cytometry. A cell was considered as apoptotic when its DNA content was less than that of a cell in G1 phase[10].

Statistical analysis Results are expressed as $x \pm s$ and were analysed with Students t-test. Statistical significance was designated as $P < 0.05$.

RESULTS

Determination of animal samples The average weight of tumors in the GLN groups was markedly less than that in the normal saline control group and close to that in the positive control group (CTX). So GLN treatment could inhibit the growth of tumor markedly.

The plasma MDA content in GLN groups tended to be lower than that in the normal saline group which was close to that in the positive control group.

The plasma NO content in the experimental groups tended to be higher than the normal saline group which was slightly higher than the positive control group. So GLN treatment improved NO content in the plasma (Tab 1).

Tab 1. Effects of GLN on tumor growth and MDA and NO content. $n = 10$ **mice.** $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* **control.**

	Control NS	CTX	GLN
Tumor weight (g)	4.0 ± 1.2	2.1 ± 0.9	2.8 ± 1.0[b]
MDA (nmol/L plasma)	2.5 ± 1.1	2.5 ± 1.0	1.8 ± 1.0[a]
NO (μmol/L plasma)	3.1 ± 1.8	3.0 ± 1.0	4.4 ± 2.2[a]

Cell samples Cell proliferation in the GLN groups was inhibited. NO and GSH contents in the experimental group treated with higher concentration of GLN (20 mmol/L) were markedly higher than that of the other groups. MDA content in experimental group decreased dose dependently (Tab 2).

As shown in Fig 1, under fluorescence microscope, it was observed that there were some apoptotic cells in

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Tab 2. Effects of GLN on hepatoma cell growth and MDA, NO, GSH content in cell culture suspension. * cell survival rate. $n=3$ flasks. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

	Control NS	5 mmol/L	10 mmol/L	20 mmol/L
1 d cells ($\times 10^5$)	7.88±0.21 (*98.2)	7.72±0.22 (*98.1)	7.49±0.18 (*96.2)	5.55±0.13 (*94.3)
3 d cells ($\times 10^5$)	15.1±0.4 (*97.5)	14.5±0.3 (*97.2)	12.9±0.3 (*95.1)	9.62±0.25 (*94.2)
5 d cells ($\times 10^5$)	40.4±1.1 (*95.3)	37.3±1.2 (*94.8)	28.2±1.0 (*89.2)	24.1±0.6 (*83.1)
5 d MDA (nmol/10^6 cells)	1.44±0.15	1.38±0.14[a]	1.36±0.13[a]	1.20±0.12[a]
5 d NO (μmol/L)	2.8±0.3	2.41±0.15[b]	2.85±0.23[a]	3.97±0.21[c]
5 d GSH (μg/10^6 cells)	25.1±0.6	27.0±0.7[b]	29.4±0.5[c]	34.4±0.8[c]

GLN groups in which nucleus or cytoplasm showed densely dyed, greenish-yellow lumps and grains, and many greenish-yellow fragments, and that some cell's membrane stuck out forming apoptotic bodies in partial visual field; whereas in the control group the cells' nucleus showed an even fluorescence, that is no apoptotic cells (Fig 1).

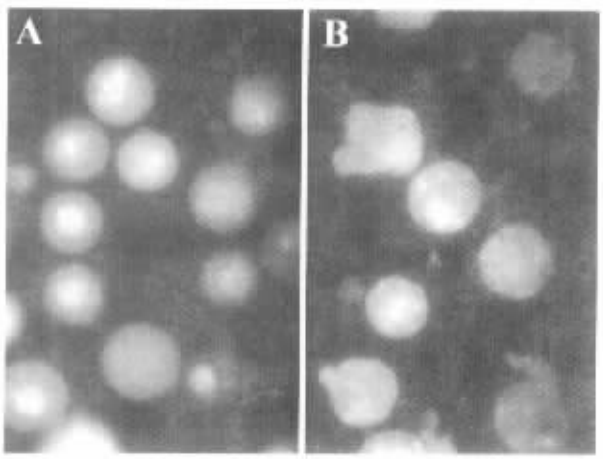

Fig 1. The photonicrography of SMMC-7721 human hepatoma cell line cultured for 5 d by fluorescent dyeing. × 400. A) control, B) 20 mmol/L GLN treatment for 5 d.

Flow cytometric Observations on merging three flasks of cells suspension from the same group, cells in each group which had been cultured for 5 d were assayed for the percentage of the apoptotic cells. The apoptosis percent of each group was 4.24 %, 7.91 %, 8.07 %, 24.97 %, respectively (Fig 2).

DISCUSSION

After being administered orally with GLN solution, the average tumor weight was observed to be less than that of control group. It is known that GLN, a precursor of glutathione, can protect body against oxidative damage[11]. It was found that if GLN concentration reached a certain level, it could inhibit the tumor growth and the hepatoma cell proliferation, thus promoting tumor cells apoptosis. In animal and cell models, the content of MDA in GLN experimental group tended to be lower than that in other groups. This implies that apoptosis in tumor cells may be not caused by the stress caused by oxidative injury. Otherwise, in GLN group, the cell's raised GSH content, could get rid of free radicals and hold back the chain reaction of lipid hyperoxidation. So it can be inferred that the inhibitory effect of GLN on the growth of tumor cells may be possibly related with its ability to eliminate the free radicals, as free radicals intervene in the proliferation of tumor cells. The mechanism may be related to previous studies which state that antioxidants by acting against oxidative damage can inhibit the transmission of cell growth signal in tumor cells[12,13].

Besides, it was also found that NO content both in mice blood and in cells culture was raised in high concentration GLN group. However, NO content in the positive control group was slightly lower. The possible explanation is that excess GLN can be converted into glutamic acid, a kind of excitatory amino acid, which can act upon the NMDA receptor, inducing Ca^{2+} influx into cells; and when Ca^{2+} in cells reaches a certain concentration, NOS can be activated, which improves NO generation[14].

Thus the mechanism of GLN induced tumor-cell apoptosis, may be, on one hand due to GLN acting as an antioxidant, blocking signaling transmision and reducing free-radical intervention in the growth of tumor cells[15]; on the other hand, it may be associated with excessive release of NO due to NOS activation, which inhibits the growth of tumor cells, and even directly damages the tumor cells, resulting in cell apoptosis[16].

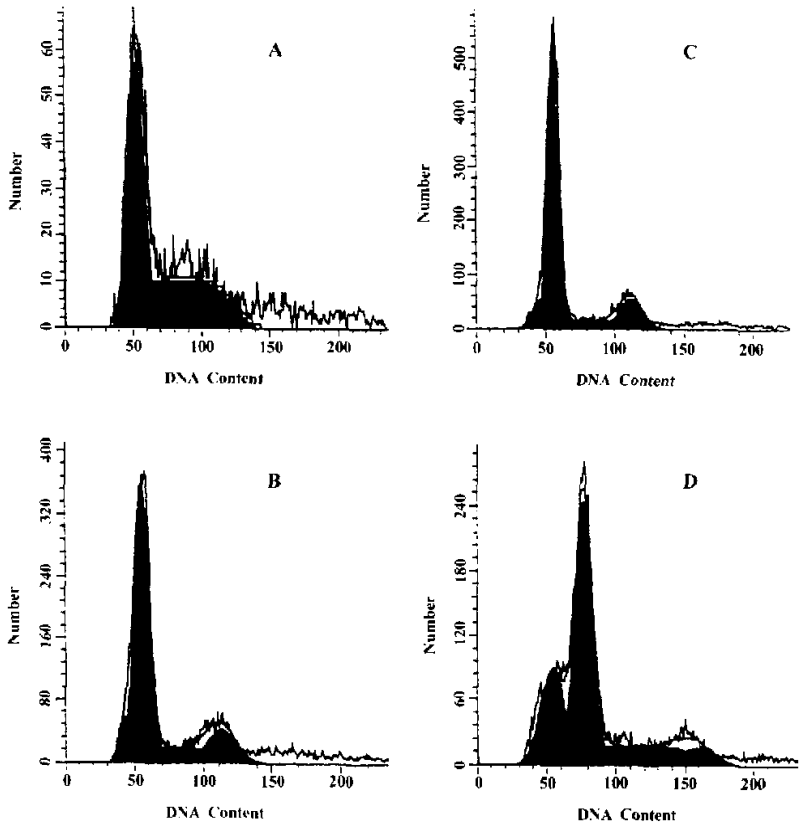

Fig 2. DNA fragmentation of SMMC-7721 human hepatoma cell line cultured for 5 d. a) control, b) with 5 mmol/L GLN treatment, c) with 10 mmol/L GLN treatment, d) with 20 mmol/L GLN treatment.

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谷氨酰胺对肿瘤生长和肝癌细胞凋亡的影响[1]

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关键词 谷氨酰胺；自由基；细胞凋亡；一氧化氮；谷胱甘肽；肝细胞癌

目的：探索谷氨酰胺对肿瘤生长及肝癌细胞凋亡的影响. **方法**：在小鼠右腋皮下接种 H22 肿瘤细胞悬液，灌服含谷氨酰胺（GLN）液；在人肝癌细胞培养液中加入不同浓度的 GLN 液. 分别检测小鼠血浆及细胞培养液中 MDA、NO 及细胞中 GSH 含量，观察小鼠右腋皮下肿瘤生长及肝癌细胞增殖和凋亡情况. **结果**：灌服 GLN 液，有抑制皮下肿瘤块生长的作用；在人肝癌细胞培养液中加入一定浓度的 GLN 液，有抑制肝癌细胞增殖的作用并促使肝癌细胞凋亡. 同时小鼠血浆和细胞培养液中 NO 含量升高，MDA 稍有下降；细胞粉碎液中，GSH 升高. **结论**：GLN 对肿瘤生长的抑制作用及对肝癌细胞凋亡的影响可能与使抗氧化活性的提高、阻抑自由基对癌细胞增殖的介导及促使 NO 释放有关.

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