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The polysaccharides from *Ganoderma lucidum*: Are they always inhibitors on human hepatocarcinoma cells?

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ABSTRACT

The antitumor activity of intracellular polysaccharides from submerged fermentation of *Ganoderma lucidum* was investigated focusing on the inhibition on human liver cancer cells. The polysaccharides inhibited human hepatocarcinoma cell HepG2 during earlier phase with lower dosage but obviously became less functional in later phase regardless of the dosage applied. However, apoptosis of the drugged HepG2 cells appeared in later incubation phase with high dosage, and the apoptosis could be enhanced by supplemental dose of the intracellular polysaccharides. Nevertheless, the intracellular polysaccharides inhibited other human hepatocarcinoma cells such as BEL-7402 and Huh-7 but luckily stimulated human normal liver cell L02 only in a positive dose- and time-dependent manner; so did the sulfated extracellular polysaccharides when it inhibited HepG2 and L02 cells. However, the toxicity of sulfated extracellular polysaccharides to L02 cells can be eliminated by the intracellular polysaccharides.

1. Introduction

Ganoderma lucidum plant or its extract has been prescribed to treat various human diseases in Asia for thousand years. *G. lucidum* polysaccharide (GLP) and its sulfated derivatives (sulfated GLP) have been reported as inhibitors on tumor growth (Abidin, Lai, Abdullah, & Sabaratnam, 2010; Chen, 2011; Chiu, Luk, Qin, & Gong, 2009; Fukuzawa et al., 2008; Hsieh & Wu, 2011; Hsu, Huang, Chen, Wong, & Juan, 2011; Joseph, Sabulal, George, Antony, & Janardhanan, 2011; Sun et al., 2011; Trajkovic et al., 2009; Xie et al., 2012), especially on liver cancer (Chen, Xu, Chen, Zhong, & Wang, 2010; Chen, Wang, et al., 2010; Li, Fang, & Zhang, 2007; Xu, Chen, Zhong, Chen, & Wang, 2011; Yu et al., 2010; Zhang, Liu, Park, Xia, & Kim, 2012). GLP may suppress tumorigenesis or inhibit tumor growth through direct cytotoxic effect or anti-angiogenic actions (Abidin et al., 2010; Chen, 2011; Juan et al., 2011; Lin et al., 2011; Wong, Chen, Yang, & Shih, 2010; Yu et al., 2010; Zhou et al., 2011), or, affecting immune cells or immune-related cells (Chen, 2011). In most of cases, positive dose-dependences of inhibition profiles have been reported (Chen, Xu, et al., 2010; Chen, Wang, et al., 2010; Synytsya et al., 2010; Tao, Zhang, & Cheung, 2006), but the time dependences have been seldom reported.

The submerged fermentation of *G. lucidum* is an efficient technology to harvest GLP gross, supplying polysaccharides with constant or possible desired components (Berovic, Habijanic, Svagelj, Boh, & Wraber, 2009). The fermentation product contains mycelia polysaccharides (intracellular polysaccharides) and extracellular polysaccharides, in which the mycelia polysaccharides possess similar compositions and structure with the polysaccharides extracted from fruiting bodies or spores of *G. lucidum*. Moreover, the mycelia polysaccharides have been commonly believed to be more active than the extracellular polysaccharides (Li et al., 2007; Petre, Teodorescu, Tuluca, Bejan, & Andronescu, 2010; Tang, Zhang, Liu, Zhu, & Zhong, 2011). However, questions still remained before GLP can be absolutely trusted or used as antitumor agent, although many commercial GLP products are often used as nonprescription medicine to help treating patients carrying liver cancer in China or Southern Asia (Gordan et al., 2011; Hanaoka et al., 2011; Kozarski et al., 2011; Soccol et al., 2010; Thomas & Bernd, 2011; Yu et al., 2010). Until now, it is not very clear that if GLP are always active on cancer cells. Sometimes the inactive results were ignored or accounted on the complexity of the sample or cells. Most experiments about the antitumor activity of polysaccharides or their derivatives usually record the data at a specific time point, say, 48 h or a week, rather than recording the time course. Nevertheless, there are also comments questioned about the role of sulfated polysaccharides (Chen, Wu, & Wen, 2008) on their immune response. For instance, sulfation is always taken without doubt to improve antitumor activity of polysaccharides; but Chen et al. (2008) declared that sulfated GLP play two edged-roles, promoter or inhibitor, depending on their structures or where they came from. Therefore, a question has been raised: could GLP or sulfated GLP do any harm in the medication? Or, is it the more the better to take them? In fact, GLP or

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sulfated GLP is still not that popular in western society as it is in Asia.

In our earlier experiments, a phenomenon was noticed but also ignored primarily until it appeared repeatedly. That is, the mycelia GLP initially inhibited the HepG2 cells but seemed to stimulate the HepG2 cells in later phase comparing to the undrugged cells. Since most patients think GLP could help in any case, so people usually do not concern how many GLP should be taken and how often should take. Therefore, to clarify that if one can always trust GLP and the sulfated GLP for their antitumor activity, the inhibition activities of GLP and the sulfated GLP were investigated on human liver cancer cells with the time dependence, dose dependence, cell strains and so on.

2. Materials and methods

2.1. Materials and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma, USA. RPMI 1640 medium, phosphate-buffered saline (PBS) and fetal bovine serum were purchased from Gibco, USA. Aminosulfonic acid, pyridine, and N,N-dimethylformamide (DMF) were from Sinopharm Chemical Reagent Co., Ltd. All other chemical reagents were of analytical grade unless further stated.

2.2. Cell lines

The human hepatocytes L02 and human hepatocarcinoma cell lines (HepG2, BEL-7402, Huh-7) were provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The cells were cultured in DMEM medium containing 10% fetal bovine serum, 1% glutamine (200 mmol/L), penicillin (100 IU/mL), and streptomycin (100 mg/L) in a humidified 5% CO_2 atmosphere at 37 °C before use.

2.3. Preparation of mycelia Ganoderma lucidum polysaccharides and sulfated extracellular Ganoderma lucidum polysaccharides

The wet mycelia *of G. lucidum* were obtained by incubating *G. lucidum* SB1997 (obtained from the Laboratory of Biomass Resources, Jiangnan University, China) in a 20 L fermenter in the medium at 30 °C for 3 days. The mycelia were crashed with a high-pressure homogenizer (Constant Systems TS 20, UK), and then extracted in hot water to obtain the raw intracellular polysaccharides. The optimal extraction condition was decided with response surface analysis as follows: extraction in twice volume of water at 95 °C for 3.5 h for three times. The extract solutions were centrifuged. The supernatants were combined and concentrated, and then precipitated with 3 volume of 95% ethanol at 4 °C and remained for 24 h to harvest the crude polysaccharides. The yield of intracellular polysaccharides was up to 11.56 mg/g dry mycelia. The precipitate was then dissolved in distilled water and deproteinated using the Savage assay. Subsequently, the solution was dialyzed against distilled water for 3 days (M_w cut off = 3500 Da). Finally, the resulting polysaccharides solution was lyophilized to obtain the polysaccharides for subsequent experiments.

The sulfated extracellular *G. lucidum* polysaccharides were prepared as described previously (Zhang et al., 2012). Samples with DS (degree of substitution) of 1.18 and DS of 1.28 were employed in this experiment, respectively.

2.4. Cell growth inhibition assay (MTT test)

The in vitro inhibition effects of polysaccharide sulfate on the cells were evaluated using MTT assay. The cells in logarithmic growth phase were digested with 0.25% trypsin and adjusted to 5×10^4/mL using RPMI1640 complete medium respectively. Before the drug exposure, 150 μL of the cell sample was pipetted into each well of 96-well plates and cultured for 24 h at 37 °C in 5% CO_2. Then, cells were cultured with GLP or sulfated GLP for 72 h at 37 °C in 5% CO_2. After drug exposure, the culture medium was removed and 100 μL of MTT reagent (diluted in culture medium, 0.5 mg/mL) was added. After 4 h of incubation, the MTT/medium was removed and DMSO (150 μL) was added to dissolve the formazan crystals. Absorbance of the solution was recorded at 570 nm to calculate the inhibition rate of cell growth. Cell viability was determined for each assay including blank wells that did not contain cells. All measurements were performed in triplicate. The inhibition rate was calculated according to the formula as following:

$$\text{Cell growth inhibition rate (\%)} = \frac{A_{570}\ \text{of control} - A_{570}\ \text{of sample}}{A_{570}\ \text{of control}} \times 100$$

2.5. Cell cycle (flow cytometry analysis)

Cells stimulated with 2000 μg/mL polysaccharides for 24, 48, and 72 h were assayed for cell cycle and cell apoptosis analysis using the PI staining assay. Briefly, cells were trypsinized, washed with cold PBS buffer twice and centrifuged at 1000 rpm for 5 min; and then fixed in 80% ethanol at 4 °C for 1 h, stained with PI staining buffer (50 μg/mL PI and 10 μg/mL RNase in PBS). Flow cytometry analysis was performed using a FACS Caliber (Becton-Dickinson Immunocytometry Systems, USA). Approximately 2×10^4 counts were made for each sample. The percentage of distribution of cell cycle phase and apoptosis were analyzed using Modfit LT (V3.2.1, Verity Software House). Microscope images were captured using microscope (IX51, Olympus) and a charged-coupled device camera. The images were opened, sized, and placed into figures using Photoshop (7.0, Adobe).

3. Results and discussion

3.1. Inhibition time course of the intracellular polysaccharides on HepG2 cell line

In general, most chemicals perform a positive dose-dependence until reaching their best performance and then stay the best afterwards. However, as shown in Fig. 1a, after drugging HepG2 with the

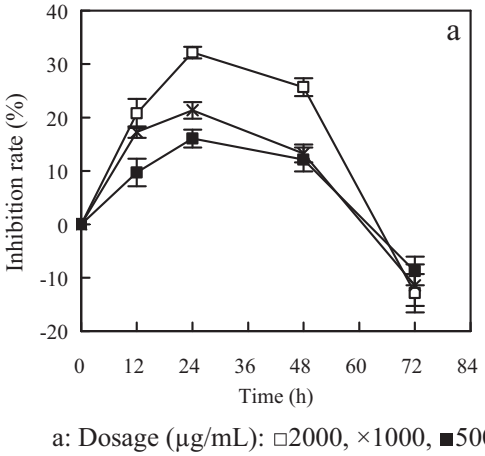


Fig. 1. The time course of HepG2 growth in the presence of intracellular polysaccharides. (a) Dosage (μg/mL): □, 2000; ×, 1000; ■, 500; (b): ×, control; ▲, 2000 μg/mL).

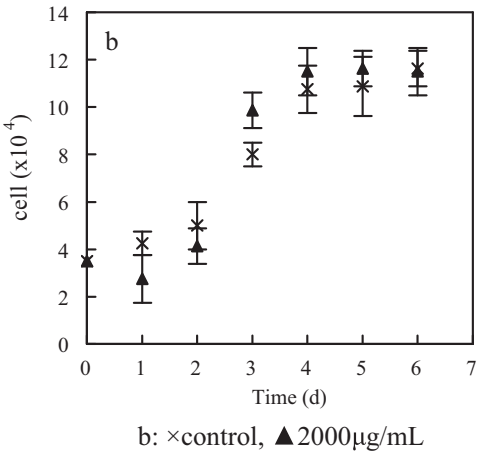


Fig. 1. (*Continued*).

polysaccharides for 48 h, the polysaccharides became less active no matter how many doses were applied. The drugged cells grown even more than the undrugged cells did since the third day (Fig. 1b), but eventually they both stayed at similar levels. The cell images (Fig. 2) also indicate this turning.

Nevertheless, how do the intracellular polysaccharides act on the other human hepatocarcinoma cells? As Fig. 3 indicates, the intracellular polysaccharides presented a clear positive dose- and time-dependent inhibition on human hepatocarcinoma cell lines BEL-7402 and Huh-7.

Therefore, the anti-liver cancer activity of GLP is up to the cell species but active on all assayed human hepatocarcinoma cell lines. To verify if the aforementioned effect on HepG2 was due to the drug consuming, a supplemental dose was applied to each case when the drugged cells were incubated 24 h or 48 h, respectively (Fig. 4, each supplemental dose was equal to the initial dose).

However, as shown in Fig. 4, a supplemental dose also did not continue or enhance the inhibition after 48 h. That implies that the living cells cannot take the GLP any more after the first 24 h. Therefore, a cell cycle analysis was applied (Table 1, Fig. 5), which indicates the cell apoptosis appeared at 72 h and was enhanced by supplemental dose of the intracellular polysaccharides.

Cell apoptosis induced by the extract from *G. lucidum* has been found in inhibition of tumor growth (Shang et al., 2011; Wu et al., 2012; Zhao et al., 2011). For example, the extract from *G. lucidum*

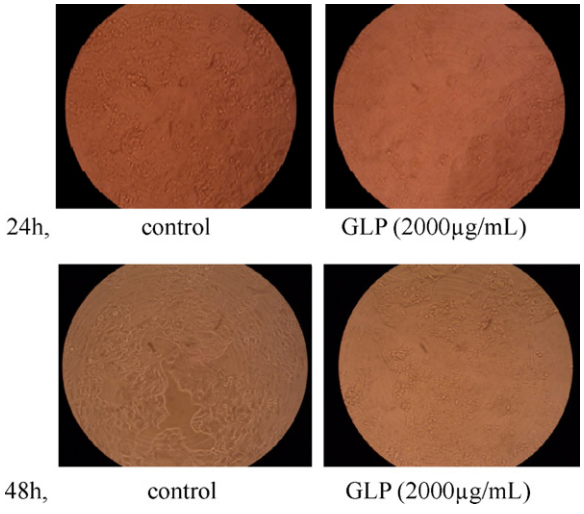
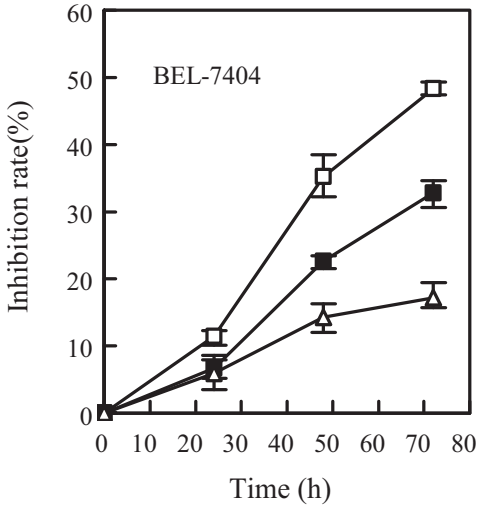


Fig. 2. HepG2 cells exposed to the intracellular polysaccharides.



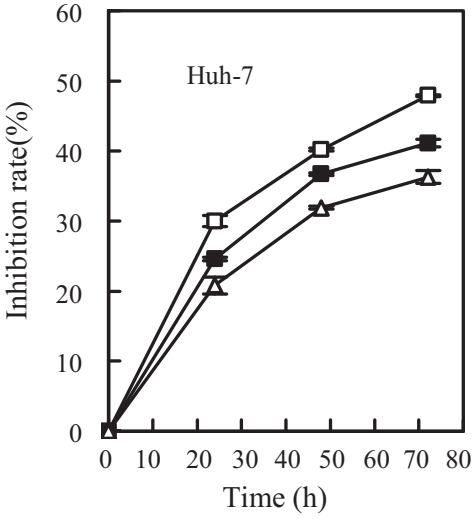


Fig. 3. Effect of the intracellular polysaccharides on human hepatocarcinoma cell lines BEL-7402 and Huh-7 (Dosage applied in μg/mL: □, 2000; ■, 1000; △, 500).

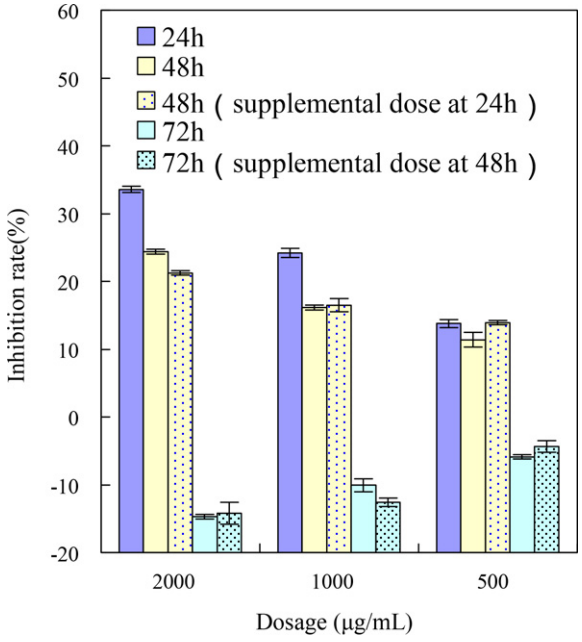


Fig. 4. HepG2 growth in the presence of intracellular polysaccharides.

Table 1
Cell cycles of HepG2 drugged by the intracellular polysaccharides.

Cycles	Control	24 h	48 h	48 h with supplemental dose	72 h	72 h supplemental dose
G1 (%)	58.13	47.28	82.85	83.41	80.73	80.18
S (%)	30.6	29.2	12.46	12.68	16.51	17.69
G2 (%)	11.27	23.52	4.69	3.90	2.76	2.13

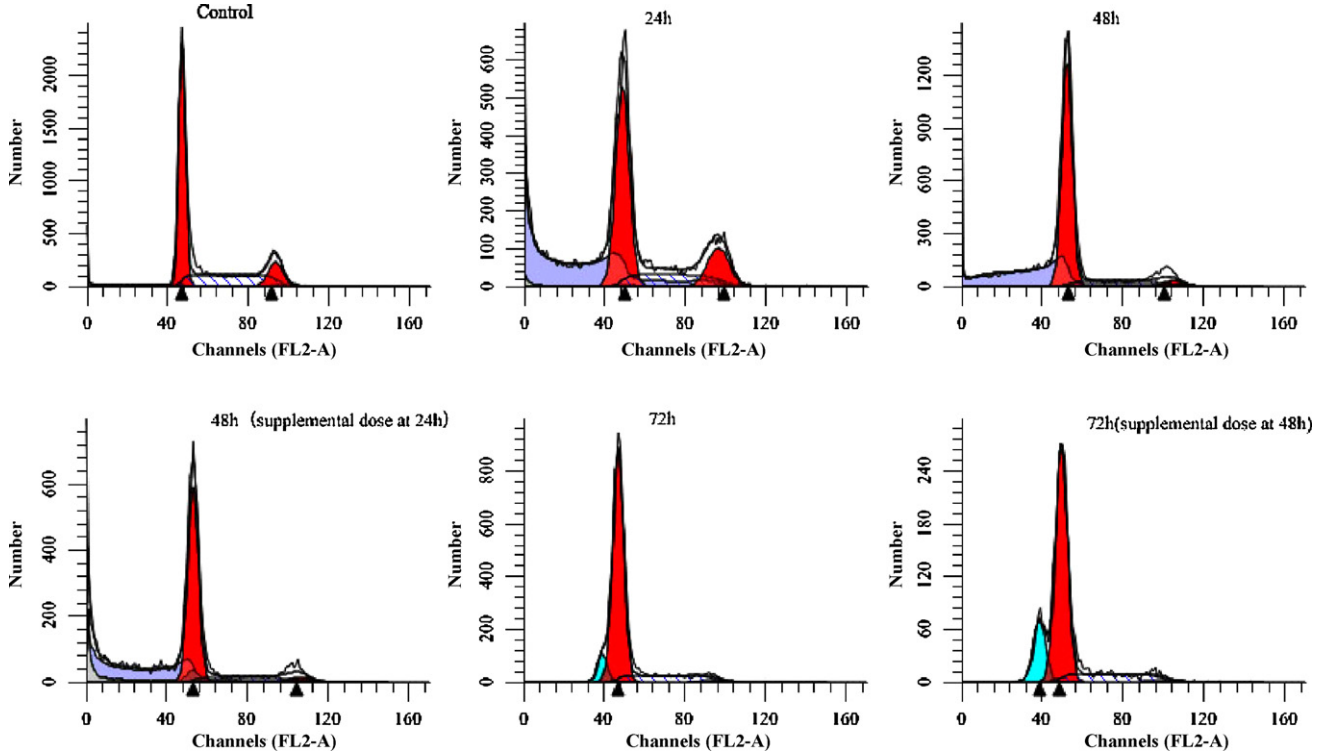


Fig. 5. Flow cytometry analysis of the drugged HepG2 cells (GLP: 2000 μg/mL).

inhibited epithelial ovarian cancer and induced the cell cycle arrest at G2/M phase (Zhao et al., 2011). Ganoderic acids from mycelia of *G. lucidum* affected HeLa cells in different ways: the ganoderic acid S caused cell cycle arrest at S phase, while another ganoderic acid Mf caused cell cycle arrest at G1 phase (Liu et al., 2011). Nevertheless, to our knowledge, there is no report about apoptosis of the liver cancer cells caused by GLP from fermented mycelia of *G. lucidum*. In this experiment, GLP treatment induced G1 phase arrest of HepG2.

3.2. Inhibition of the sulfated extracellular polysaccharides on HepG2 cell line

As indicated above, the intracellular GLP is active on HepG2 cells, but it is well-known that the extracellular GLP has weaker or invisible inhibition activity on most of cancer cells. Luckily, the sulfation is a popular and effective approach to improve the anti-cancer activity of polysaccharides (Chen, Xu, et al., 2010; Chen, Wang, et al., 2010; Tao et al., 2006; Volpi, 2010; Ye et al., 2009). As

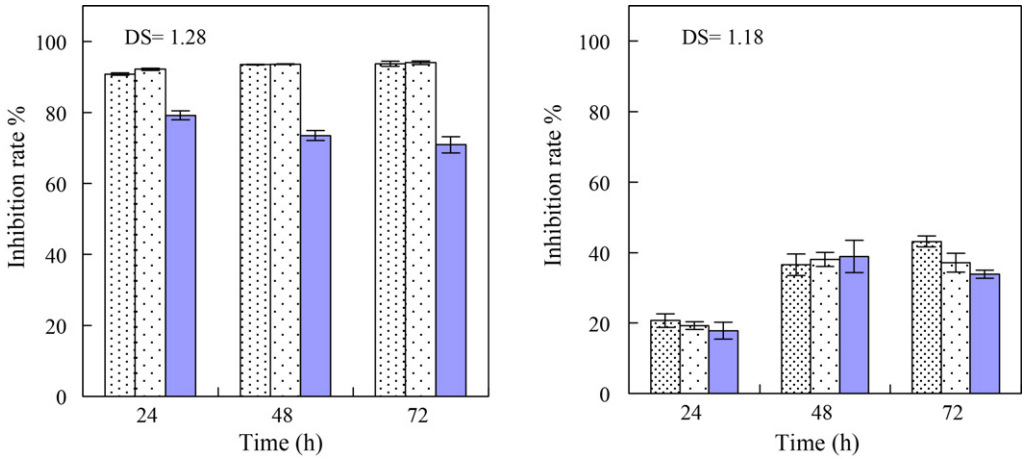


Fig. 6. HepG2 growth in the presence of sulfated extracellular polysaccharide (Dosage applied in μg/mL: ⊡, 2000; ⊡, 1000; ■, 500).

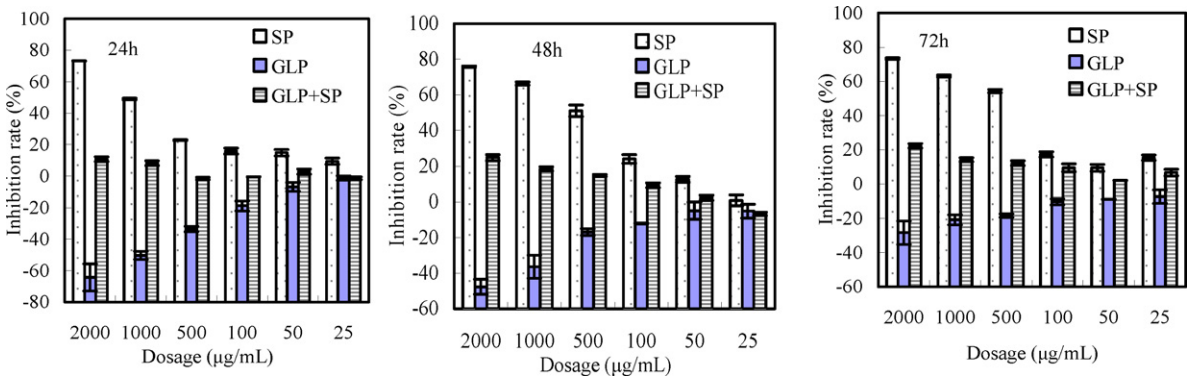


Fig. 7. Effect of the intracellular polysaccharides (GLP) and sulfated extracellular polysaccharides (SP, DS = 1.28) on human liver cell line L02 (total dosage applied: 2000 μg/mL).

the derivative of GLP, will the sulfated GLP perform same activity as the intracellular GLP presented? Fig. 6 provides a negative answer to that question by a positive dose-dependent and time-dependent inhibition on HepG2 cells; moreover, the inhibition rates are even much higher than that of GLP. This result is one more proof to support that sulfation is an effective strategy to improve the anticancer activity of polysaccharides.

3.3. Effect of the intracellular polysaccharides and sulfated extracellular polysaccharides on human liver cell line L02

The excellent activity of sulfated extracellular polysaccharides induces the concern of its toxicity to human normal cells, especially when they are applied in high dosages although it is safe in lower dosages (Zhang et al., 2012). Fig. 7 does indicate the remarkable toxicity of sulfated extracellular polysaccharides on human normal liver cell L02. Fortunately, the intracellular polysaccharides presented excellent stimulation on L02. Moreover, the harm was decreased dramatically with the help of intracellular polysaccharides (Fig. 7).

4. Conclusions

The intracellular polysaccharides from submerged fermentation of *G. lucidum* inhibited human hepatocarcinoma cell HepG2 in the first 48 h but stimulated the cell growth after 72 h regardless the dosages applied.

Apoptosis of GLP drugged HepG2 cells appeared in 72 h and was enhanced by supplemental dose of the intracellular polysaccharides. For the other human hepatocarcinoma cell lines, such as BEL-7402 and Huh-7, the intracellular polysaccharides presented an obvious positive dose- and time-dependent inhibition activity. In the other hand, the intracellular polysaccharides accelerated the growth of the normal human liver cell L02.

The sulfated extracellular polysaccharides performed excellent inhibition as high as 93.5% on HepG2 cell line but it also presented toxicity on human normal liver cell L02 with an inhibition rate up to 75.8% at the dosage of 2000 μg/mL. Blending the intracellular polysaccharides with the sulfated extracellular polysaccharides dramatically reduced the harm to L02 cells.

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