

## **L-asparaginase exerts anti-leukemia activity.**

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### **Abstract**

**This study aimed to investigate the effects of L-Asparaginase (L-ASP) on leukemia cell proliferation, cell cycle distribution and geminin expression. Jurkat cells were treated with different concentration of L-ASP for 24 h, 48 h or 72 h. Cell proliferation was analysed by the CCK-8 assay, cell cycle distribution was examined by flow cytometry, and mRNA and protein expression levels of geminin were assessed by PCR and Western blot analysis. The results showed that L-ASP inhibited the proliferation of Jurkat cells in a dose- and time-dependent manner. In addition, L-ASP at 0.5-10 U/ml induced G1 phase arrest of Jurkat cells. Geminin mRNA and protein expression remarkably decreased in Jurkat cells treated with L-ASP at 1.0-10 U/ml. In conclusion, our study suggests that the anti-leukemia mechanism of L-ASP is related to the arrest of leukemia cells in G1 phase and the downregulation of geminin expression.**

**Keywords:** L-ASP, Jurkat cells, Cell cycle, Geminin.

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### **Introduction**

L-Asparaginase (L-ASP) has been widely accepted as one of basic regimens used in the treatment of Acute Lymphoblastic Leukemia (ALL) [1,2]. The efficacy of L-ASP is generally thought to result from a rapid and complete depletion of asparagines in the plasma by hydrolysing this amino acid to aspartic acid. Leukemic cells have reduced expression of asparagine synthetase and thus need asparagines from the blood. However, asparagines deamination alone may be insufficient to induce cell death [1]. Additional mechanisms may account for anti-leukemia activity of L-ASP. For example, a recent study showed that L-ASP induced apoptosis and cytoprotective autophagy in leukemia cells [3].

In this study we mainly focused on the effects of L-ASP on leukemia cell proliferation and cell cycle progression. Our results suggest that the anti-leukemia mechanism of L-ASP is related to the arrest of leukemia cells in G1 phase and the downregulation of geminin expression.

### **Material and Methods**

#### ***Cells and reagents***

The Jurkat cell line (Human T-lymphoblastic leukemia cell line) was kindly provided by the Cell Culture Laboratory of College of Animal Science of Zhejiang University. Jurkat cells were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1X PenStrep. L-ASP was purchased from Qianhong Biopharma Co. Ltd (Changzhou, China).

#### ***CCK-8 assay***

The cells in the logarithmic growth phase were plated in 96-well plates with 100 µl of cell suspension (at a concentration of  $1 \times 10^5$ /ml) in each well. The wells for test groups were added with 0.1, 0.5, 1.0, 5.0 and 10 U/ml L-ASP for treatment with 24 h, 48 h and 72 h, respectively. The wells for blank control group were added with the equal volume of medium. The CCK-8 kit (Sigma) was used to assess cell proliferation. The experiments were performed in triplicate.

#### ***Cell cycle analysis***

The cells in test groups were treated with different concentration of L-ASP for 24 h, 48 h and 72 h. The G1 phase rate of the cell cycle was analysed by Flow Cytometry (FCM) based on the measurement of the DNA content of nuclei labelled with propidium iodide (Sigma). Samples were analysed using a FACSCanto II flow cytometer (Becton-Dickinson). The ratio of cells in G1 phase was expressed as mean  $\pm$  SD. The experiments were performed in triplicate.

#### ***Western blot analysis***

Total protein was extracted from cells and quantified using a protein assay kit. Aliquots of protein (50 µg) were separated by SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked and then incubated overnight at 4°C with geminin or GAPDH antibody (Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology). GAPDH was used as loading control. Immunoreactive bands were detected by ECL Western blotting

detection system (Amersham Biosciences) and the density was analysed using imageJ software.

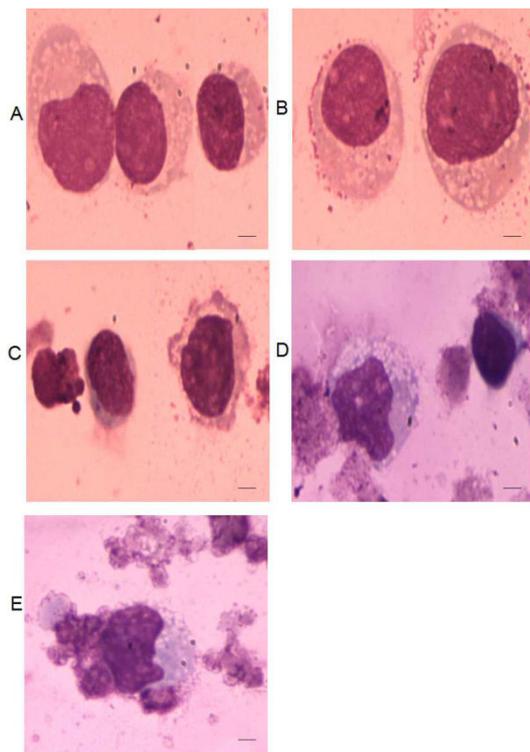
### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation. The difference in means among multiple groups was compared using one-way ANOVA. The difference in means between two groups of multiple groups was compared using LSD-t test. A value of  $P < 0.05$  was considered statistically significant.

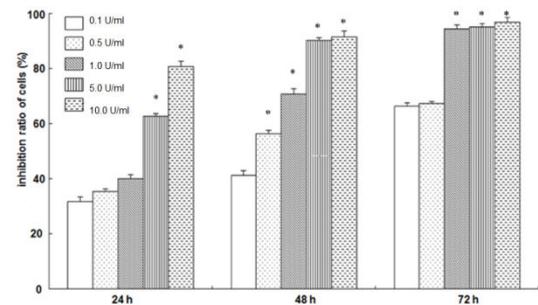
## Results

### L-ASP inhibited Jurkat cell proliferation

As shown in Figure 1, the morphology of Jurkat cells began to change after treatment with 0.5 U/ml L-ASP. The cytotoxicity effect of 10 U/ml L-ASP on Jurkat cells was most obvious, showing nuclear membrane shrinkage and deeply stained nuclei. The inhibition of cell proliferation was higher with increased exposure time and increased concentration ( $P < 0.01$ , Figure 2). These data indicate that L-ASP inhibited Jurkat cell proliferation in a dose and time dependent manner.



**Figure 1.** Effect of L-ASP on Jurkat cell morphology. Cells were treated with different concentration of L-ASP for 48 h and stained with haematoxylin and eosin. A-E: L-ASP concentration was 0.1 U/ml, 0.5 U/ml, 1 U/ml, 5 U/ml and 10 U/ml, respectively. Scale: 10  $\mu$ m.



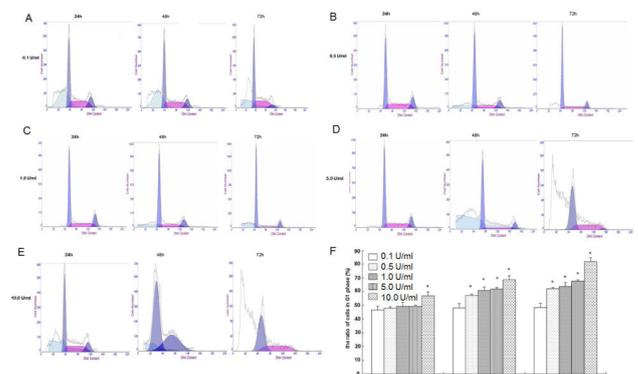
**Figure 2.** L-ASP inhibited the proliferation of Jurkat cells. Cells were treated with the indicated concentration of L-ASP for 24 h, 48 h and 72 h, respectively. The inhibition was the strongest at the concentration of 10 U/ml L-ASP for 72 h. \* $P < 0.05$  compared to 0.1 U/ml at respective time point.

### L-ASP arrested Jurkat cells in G1 phase

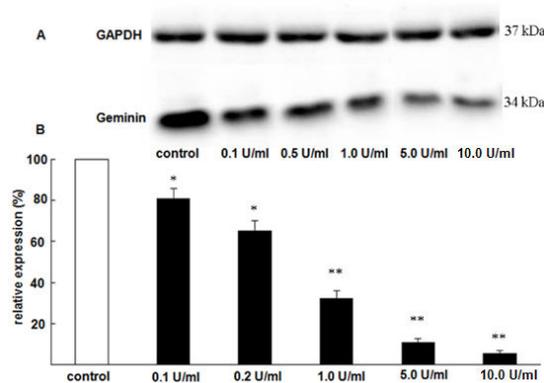
We found no difference in cell cycles of Jurkat cells treated with 0.1 U/ml L-ASP at different time points. 48 h after exposure to L-ASP at the concentration of 0.5 U/ml, the proportion of Jurkat cells in G1 phase increased markedly, and reached the peak in cells treated with 10 U/ml L-ASP for 72 h, showing a dose and time dependent manner (Figure 3).

### L-ASP downregulated geminin expression

To understand the mechanism how L-ASP arrested Jurkat Cells in G1 phase, we examined the expression of geminin, a well-known cell cycle regulatory protein. Geminin expression was significantly downregulated by L-ASP in a dose dependent manner (Figure 4).



**Figure 3.** L-ASP induced cell cycle arrest of Jurkat cells. Jurkat cells were treated with different concentration of L-ASP for 24 h, 48 h and 72 h, respectively. Cell cycle distribution was determined by flow cytometry. A-E. Representative FACS graphs of Jurkat cells treated with L-ASP at 0.1 U/ml, 0.5 U/ml, 1.0 U/ml, 5.0 U/ml and 10.0 U/ml, respectively. The x-axis was DNA content and the y-axis was cell number. F. Quantitative analysis of the ratio of cells in G1 phase. \* $P < 0.05$  compared to 0.1 U/ml at respective time point.



**Figure 4.** *L*-ASP inhibited geminin expression in Jurkat cells. Jurkat cells were treated with different concentration of *L*-ASP for 72 h. Geminin protein level was determined by Western blot analysis. A. Representative blots showing geminin protein expression in different groups. GAPDH was loading control. B. Densitometry analysis of geminin expression. \* $P < 0.05$ , \*\* $P < 0.01$  compared to control.

## Discussion

ALL is a disease more frequent in children and greatly threatens the health of children [4,5]. The incidence of ALL in China has been on the rise recently [6,7]. *L*-ASP is widely used in the treatment of multiple malignant blood system diseases, especially in ALL in children. In this study we aimed to understand the mechanism by which *L*-ASP exerts inhibition on leukemia cell proliferation.

CCK-8 assay is a very good method to determine cell proliferation and toxicity [8,9]. Using CCK-8 assay we found that *L*-ASP substantially inhibited Jurkat cell proliferation in a dose and time dependent manner. Our results are consistent with previous report that *L*-ASP inhibited the proliferation of eight haematological malignant tumor cell lines [10].

The progression of cell cycle is closely associated with cell differentiation, growth, apoptosis and carcinogenesis. There are two cell cycle checkpoints during cell cycle progression, including checkpoint in G1/S phase and checkpoint in G2/M phase [11,12]. The distribution in cell cycle determined by FCM showed that the proportion of cells in S phase decreased after treatment with *L*-ASP, demonstrating that DNA synthesis of leukemia cells was inhibited. The arrest of cells in G1 phase resulted in prolonged cell cycle and decreased cell proliferation. Similar results showed that *L*-ASP induced cell cycle arrest of tumor cells [13,14]. Furthermore, human leukemia cell lines such as Molt-4 and HL-60 had higher proportion of cells in G1 phase after treatment with *L*-ASP [15]. Taken together, these data suggest that *L*-ASP regulates cell cycle progression of human leukemia cells to inhibit their proliferation.

Geminin acts as initiating signal for the transition from G1 phase to S phase, and plays important role in cell growth and proliferation [16]. Abundant studies showed that geminin was overexpressed in multiple types of tumor cells [17,18]. The length of transition from G0 phase to G1 phase was decreased

in cells overexpressing geminin [19]. In this study, we found that the expression of geminin protein was substantially decreased in Jurkat cells after treatment with *L*-ASP, suggesting that the arrest of cell cycle in G1 phase by *L*-ASP is probably mediated by the downregulation of geminin expression.

Interestingly, a recent study reported that *L*-ASP induced the apoptosis of glioblastoma cells by the dissipation of mitochondrial membrane potential and the activation of effector caspases [20]. Another study showed that *L*-ASP induced the apoptosis of human leukemia MOLT-4 cells via the activation of intrinsic apoptotic pathway [21]. Further studies are needed to investigate the mechanism by which *L*-ASP induces the apoptosis of leukemia cells *in vivo*.

In summary, this study investigated the effect of *L*-ASP on leukemia cell proliferation and explored the underlying mechanism. Our data revealed a potential new mechanism that *L*-ASP inhibits leukemia cell proliferation by downregulating geminin expression and inducing G1 cell cycle arrest. These findings suggest that *L*-ASP could be utilized as novel drugs for the treatment of leukemia.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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