

L-Selenocystine induce HepG2 cells apoptosis through ROS-mediated signaling pathways

HAIYANG CHEN^{1,2,*}; JINGYAO SU^{1,#}; DANYANG CHEN^{1,#}; YUYE DU¹; RUILIN ZHENG¹; QINGLIN DENG²; QIANQIAN DU³; BING ZHU^{1,*}; YINGHUA LI^{1,*}

¹ Center Laboratory, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China

² Nanfang Hospital, Southern Medical University, Guangzhou, China

³ Department of Laboratory Medicine, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

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Abstract: At present, Hepatocarcinoma is one of the main causes of tumor related death all over the world. However, there are still many clinical restrictions on the treatment of liver cancer. Recently, L-Selenocystine has been shown to be a novel treatment for tumors, especially human glioma cells. But, the mechanism of L-Selenocystine against hepatocellular carcinoma remains unclear. Therefore, the main objective of this study was to investigate the effects of L-Selenocystine on HepG2 cell proliferation and activation of reactive oxygen species (ROS) mediated signaling pathway. L-Selenocystine can significantly inhibit HepG2 cell proliferation by activating caspase-3 and cleaving PARP to induce apoptosis. Moreover, the excessive production of ROS and the influence of Bax signaling pathway which can promote cell apoptosis are key factors for L-Selenocystine to induce HepG2 cell apoptosis. Therefore, the date of this study suggest that ROS mediated signal transduction mechanism may provide certain reference significance for L-Selenocystine induced HepG2 cell apoptosis.

Introduction

Hepatocellular carcinoma is one of the three majority common cancers in the general population and the fourth leading cause of cancer-related death (Fitzmaurice *et al.*, 2018; Mattiuzzi and Lippi, 2019). Although the prognosis of patients with early or intermediate liver cancer has improved significantly in recent years due to advances in diagnosis and treatment, most liver cancers are diagnosed as advanced when treatment is not feasible (Hilmi *et al.*, 2019). At the same time, the overall prognosis of HCC is also influenced by important factors such as its strong metastatic potential, strong resistance to traditional drugs and poor sensitivity to chemotherapeutic drugs. Thus, the development of effective chemotherapy has become a major challenge in clinical treatment and is imperative (Cao *et al.*, 2021; Marin *et al.*, 2020). In addition, most of the current anti-cancer drugs have some common shortcomings, such as short effective time and poor solubility in water, which affect the effectiveness of chemotherapy

(Yingchoncharoen *et al.*, 2016; Glasgow and Chougule, 2015). Lately, L-Selenocystine has been diffusely used because of its unique biological characteristics, including that it is the main form of selenium in proteins, the only amino acid containing metalloids elements, and is mostly located in the active centers of selenium-containing enzymes or selenoproteins (Seale, 2019; Evans *et al.*, 2019). With the further study of L-Selenocystine, people have hope for L-Selenocystine as an alternative anticancer drug (Radomska *et al.*, 2021; Álvarez-Pérez *et al.*, 2018).

L-Selenocystine is less toxic than other forms of selenium and is one of the forms of selenium that enter cells to perform biological functions. Compared with cysteine, L-Selenocystine has attracted extensive attention in consumer and medical products because of its selenium element. Selenium is also an essential trace element in human body, which plays an important role in immune regulation and antioxidant (Álvarez-Pérez *et al.*, 2018; Misra *et al.*, 2015; Spallholz, 1994). Furthermore, L-Selenocystine is very low toxic in humans and has different applications in *in vitro* and *in vivo* (Hoefig *et al.*, 2011; Wallenberg *et al.*, 2014; Kumar *et al.*, 2009; Ostadalová and Babický, 1980). Therefore, the emergence of L-Selenocystine as a promising anticancer selenium species has attracted increasingly attention.

*Address correspondence to: Bing Zhu, zhubing@gzhmu.edu.cn; Yinghua Li, liyinghua@gzhmu.edu.cn

#These authors contributed equally to this work

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ROS is a single-electron reduction product of oxygen produced in cells, including hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen free radicals and singlet oxygen (Zorov *et al.*, 2014; Li *et al.*, 2016a). As an obvious imbalance, oxidative stress was often described between ROS consumption and cellular defense mechanisms. (Moloney and Cotter, 2018; Klaunig, 2018; Wu *et al.*, 2019). Previous studies have also shown that ROS have important effects on many physiological processes in the body. Redox imbalances have been linked to many serious diseases, such as cancer, skin disease, chronic kidney disease, neurodegenerative diseases, etc. (Bjørklund *et al.*, 2020; Limongi and Baldelli, 2016; Xian *et al.*, 2019; Poulianiiti *et al.*, 2016). Previous studies have shown that selenocysteine can interact with other compounds to induce apoptosis of HepG2 cells (Su *et al.*, 2013; Fan *et al.*, 2014). However, it has not been reported that different concentrations of selenocysteine can induce apoptosis of HepG2 cells under the premise of ensuring high survival rate of normal liver cells. It is greatly interesting to clarify the mechanism of L-Selenocystine. This study aims to investigate how changes in L-Selenocystine-related redox balance induced HepG2 cell apoptosis.

Materials and Methods

Materials

Human hepatocellular carcinoma cell line, HepG2 and hepatic cell line, L02 were maintained in our laboratory. Dulbecco modified Eagle Culture Medium (DMEM) was purchased from Gibco. Fetal Bovine serum (FBS) was purchased from ExCell. Bcl-2, Bax, Bid, c-PARP and caspase-3 and caspase-9 antibody were obtained from Cell Signaling Technology. L-Selenocystine were obtained from Sigma. 2',7'-dichlorofluorescein diacetate (DCF-DA), thiazolyl blue tetrazolium bromide (MTT) and bicinchoninic acid (BCA) were obtained from Sigma. RIPA (protein lysis buffer) and enhanced mitochondrial membrane potential assay kit with JC-1 was obtained from Beyotime. Milli-Q Water used in all experiments was provided by Water Purification System of Millipore Company.

IC50 of L-Selenocystine in HepG2 cell line and L02 cell line

This routine work for apoptosis research is only carried out in HepG2 cells, not in normal liver cells L02. The MTT experiment was used to detect cell survival rate, and GraphPad prism was used to calculate IC50 values and draw graphs.

Cell viability assay

HepG2 and L02 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (penicillin and streptomycin), and placed in a humidified incubator at 37°C and 5% CO₂. The cytotoxicity of L-Selenocystine was detected by MTT test as described by us earlier (Zhu *et al.*, 2016). In short, the classic MTT method was used to detect cell viability. HepG2 cells (6 × 10⁴ cells/well) and L02 cells (6 × 10⁴ cells/well) were inoculated in 96-well cell culture plate and incubated at 37°C with 5% CO₂ for 24 h. Then, the cells were treated with 8 μM, 16 μM, and 32 μM L-selenocystine for 24 h, respectively. After treatment, 180 μl/well DMEM containing

1% FBS and 20 μl/well MTT solution was added and incubated for another 4 h. The medium was removed and 150 μl/well DMSO was added to dissolve the formazan crystals. The 96-well plates were then placed on a 37°C shaker and shaken for 10 min. Finally, the color intensity of solution was measured at 570 nm using thermo Flyer's microplate reader (Varioskan). The test was repeated three times.

Determination of ROS production in HepG2 cells

As previously reported, ROS production in HepG2 cells treated with L-Selenocystine was detected by DCF fluorescence staining (Zhu *et al.*, 2016; Li *et al.*, 2016b; Li *et al.*, 2018). Briefly, DCFH-DA was added to fully cover the cells after the medium was removed, and incubated at 37°C for 20 min. The cells were repeatedly washed twice with DMEM to completely remove the extracellular DCFH-DA, and the fluorescence intensity was observed with a fluorescence microscope. The experiment was repeated three times.

Mitochondrial membrane potential measurement ($\Delta\Psi_m$)

Changes in mitochondrial membrane potential in HepG2 cells exposed to L-Selenocystine were assessed using JC-1. The experiment steps follow the instructions. HepG2 cells were inoculated on 6-well plates and cultured for 24 h. After 24 h, the medium was removed and the cells were washed once with PBS or other suitable solution as required by the specific experiment. Then 1 mL DMEM and JC-1 staining solution were added to the six-well plate and fully mixed, and the cells were kept at 37°C for 20 min. During incubation, 4 mL distilled water was added to each 1 mL JC-1 staining buffer (5X) to prepare an appropriate amount of JC-1 staining buffer (1X) and put it in an ice bath. After incubation at 37°C, the supernatant was removed and washed twice with JC-1 staining buffer (1X). Add 2 mL cell culture solution and observe under inverted fluorescence microscope.

Western blot assay

As mentioned earlier, the classical Western Blot method was used to detect the effect of L-Selenocystine on the expression levels of various intracellular proteins in HepG2 cells, including the classic P53 pathway, Bcl-2 family and caspase family, etc. (Guo *et al.*, 2017; Li *et al.*, 2016c). RIPA was used to digest HepG2 cells treated with L-Selenocystine to obtain total protein. The BCA assay was used to detect protein concentration. 10 μg protein was taken from each group. The transferred PVDF membrane was sealed in 5% skimmed milk powder for 2 h, then incubated overnight with 1:1000 diluted primary antibody at 4°C, and finally incubated with 1:1000 diluted secondary antibody at 4°C for 2 h. Enhanced chemiluminescence detection reagent (ECL) was used to visualize the protein bands.

Statistical analysis

Each experiment was repeated at least three times. All data were expressed as mean ± standard deviation. Two-tailed student *T* test was used to analyze the differences between the two groups. *P* < 0.05(*) or *P* < 0.01(**) were judged to be statistically significant. Multiple groups were compared using one-way analysis of variance. These analyses were performed using SPSS26.0 for Windows software.

Results

The IC₅₀ of HepG2 and L02 cell line

As shown in Fig. 1A, as the IC₅₀ of L-Selenocystine in L02 is >70 μM, it is not considered that L-Selenocystine is cytotoxic to normal liver cells. However, the IC₅₀ in HepG2 cells is 5.444 μM (Fig. 2B). This means that L-Selenocystine has a higher proliferation inhibitory activity on HepG2 cells while ensuring the survival of L02 cells. Therefore, 8, 16 and 32 μM L-Selenocystine were selected for follow-up studies.

Anticancer activity of L-Selenocystine in vitro

MTT assay was used to detect the toxicity of L-Selenocystine on HepG2 cells. As shown in Fig. 2A, L-Selenocystine significantly inhibited HepG2 cell growth with increasing

concentration. HepG2 cells were treated with 8 μM L-selenocystine for 24 h, the cell viability was decreased to 43.37%. At the concentration of 16 μM and 32 μM, L-Selenocystine obviously reduced the cell viability (29.50% and 18.04%). As shown in Fig. 2B, the anticancer activity of L-Selenocystine was further confirmed. L-selenocystine made HepG2 cells significantly smaller and round, intercellular contact disappeared, and intercellular space increased. In conclusion, these outcomes suggest that L-selenocystine inhibits tumor cell growth in a dose-dependent manner.

ROS production induced by L-Selenocystine

ROS is a well-known key stimulator of apoptosis, especially in apoptosis induced by many anti-tumor drugs (Srinivas *et al.*, 2019;

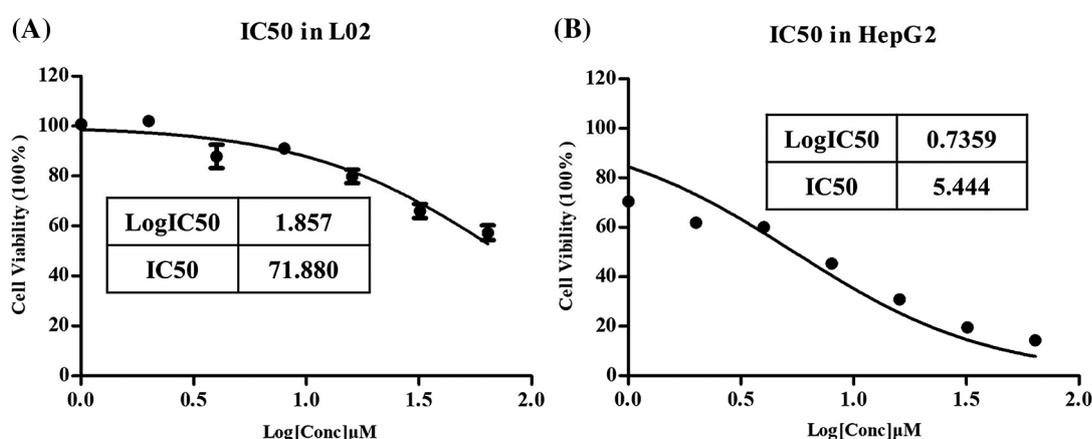


FIGURE 1. Percentages of cell viability of HepG2 cells treated with L-Selenocystine using serial dilutions from 64 to 1 μM.

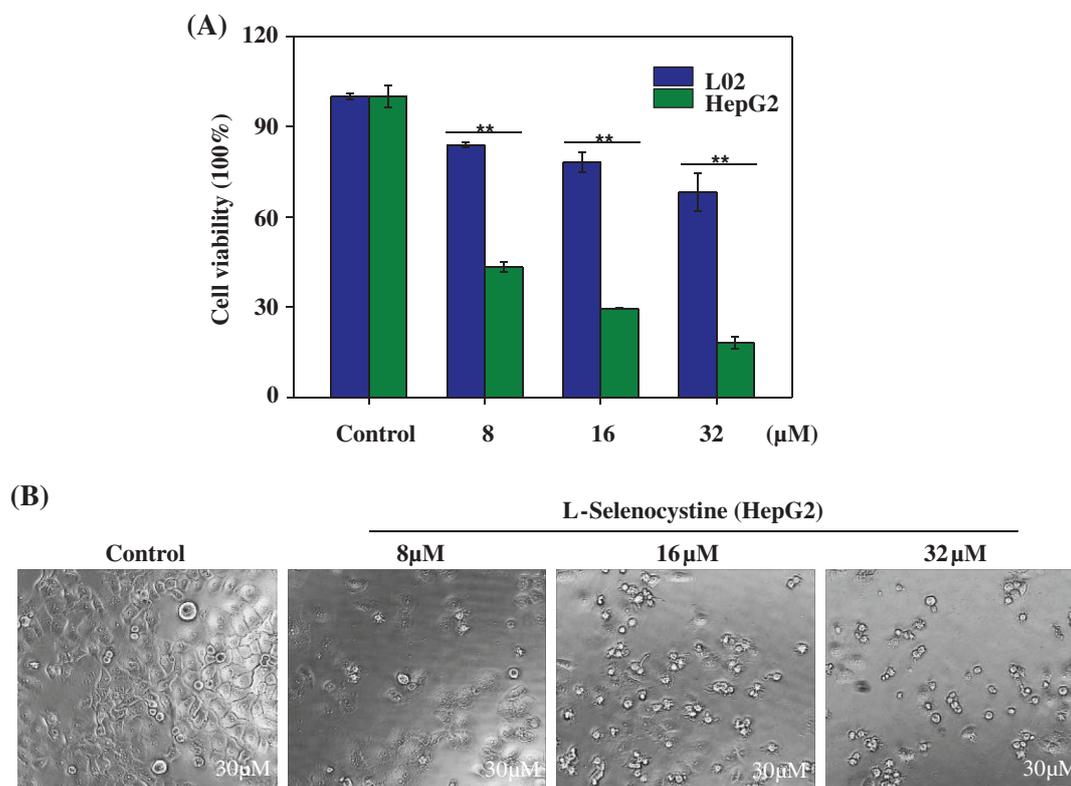


FIGURE 2. Cytotoxic effects of L-Selenocystine on HepG2 cells. (A) Cell viability of HepG2 and L02 cells with the treatment of L-Selenocystine were determined by MTT assay. (B) Morphological changes in HepG2 cells. Bars with different characters are statistically different at $P < 0.05$ (*) or $P < 0.01$ (**).

Perillo *et al.*, 2020). Consequently, the production of ROS was determined by DCF fluorescence method in this study to disclose its role in the antitumor activity of L-selenocystine. As illustrated in Fig. 3, the production of ROS in HepG2 cells was increased little by little under the action of L-Selenocystine, and the fluorescence intensity was found to be stronger in HepG2 cells with a concentration of 32 μM .

Depletion of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential (MMP, $\Delta\Psi_m$) was used to detect the occurrence of apoptosis by fluorescence inverted microscope (Ma *et al.*, 2018). As shown in Fig. 4, red fluorescence represents complete MMP, while green fluorescence represents lost MMP. Compared with the control group, the fluorescence of HepG2 cells treated with different concentrations of L-Selenocystine changed from red to green, indicating that mitochondrial depolarization of HepG2 cells was enhanced and MMP was significantly decreased.

Activation of ROS-mediated signaling pathways by L-Selenocystine

Intracellular ROS overproduction could trigger DNA damage and cell apoptosis by activating of Bax signaling pathways.

The western blot assay was used to detect its effects on ROS-mediated downstream pathways. As illustrated in Fig. 5A, the expression level of Bax protein in HepG2 cells was significantly increased after treated with L-Selenocystine. Moreover, the effects of L-Selenocystine treatment on Bcl-2 and Bid were different, which significantly increased Bid and inhibited Bcl-2. Meanwhile, the expression levels of caspase family and cleaved PARP with the group of L-Selenocystine treatment were significantly higher than those in the control group. The signaling pathway of ROS-mediated regulation of DNA damage and Bax as shown in Fig. 5B.

Discussion

HCC is a primary cancer with a very high mortality rate. It is the most common malignancy worldwide, especially in Asia, Africa and parts of Europe. There are many factors leading to HCC, such as chronic HBV/HCV infection, epigenetic changes, aflatoxin exposure, etc. (Wu *et al.*, 2021). For the treatment of HCC, there are no effective drugs to curb the progression of the disease, and the prognosis is mostly very poor, prone to recurrence and metastasis, and eventually

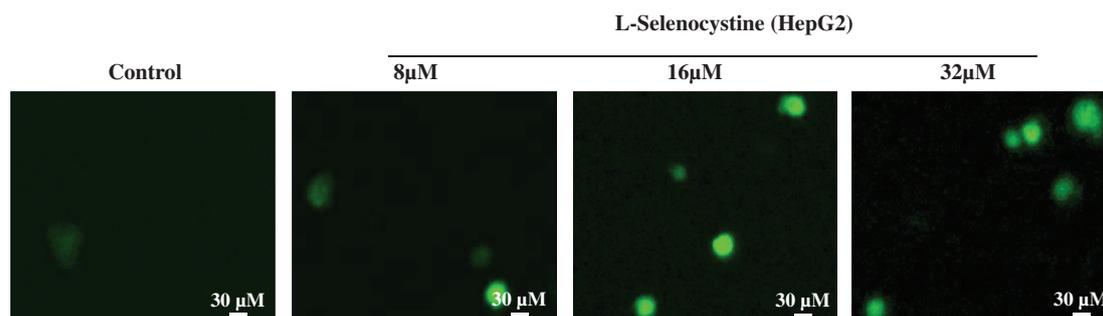


FIGURE 3. ROS overproduction induced by L-Selenocystine in HepG2 cells. HepG2 cells were incubated with 10 μM DCF-DA in PBS for 30 min and then the fluorescence intensity was observed under microscope.

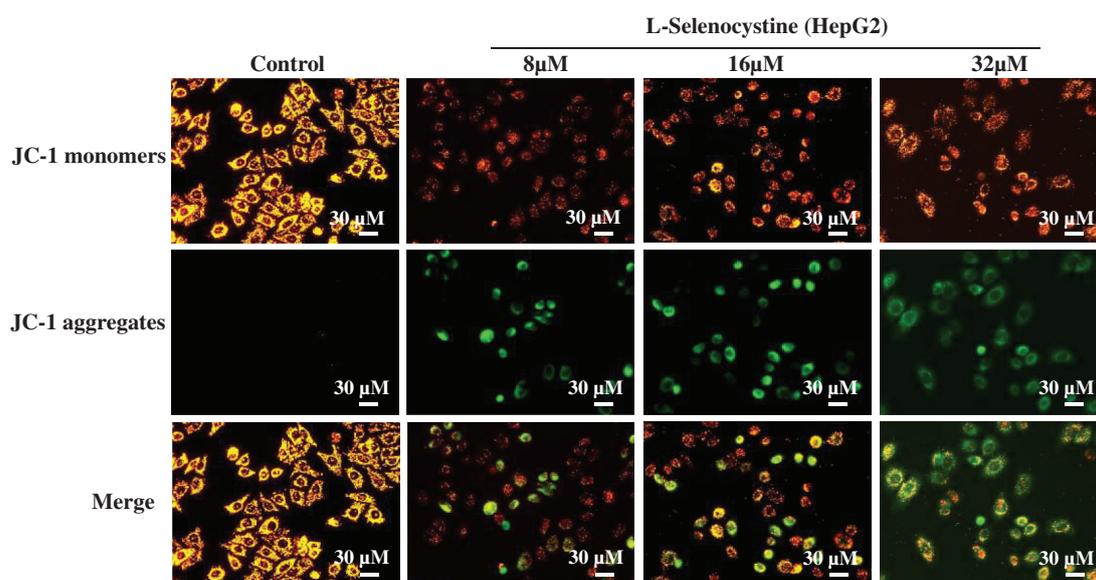


FIGURE 4. Depletion of mitochondrial membrane potential induced by L-Selenocystine. JC-1 exists in the mitochondria of normal HepG2 cells in the form of polymer, showing bright red fluorescence and weak green fluorescence. After treatment with L-Selenocystine, JC-1 could not exist in the mitochondrial membrane potential in the form of polymer, so the red fluorescence intensity in mitochondria was significantly decreased, while the green fluorescence was significantly enhanced.

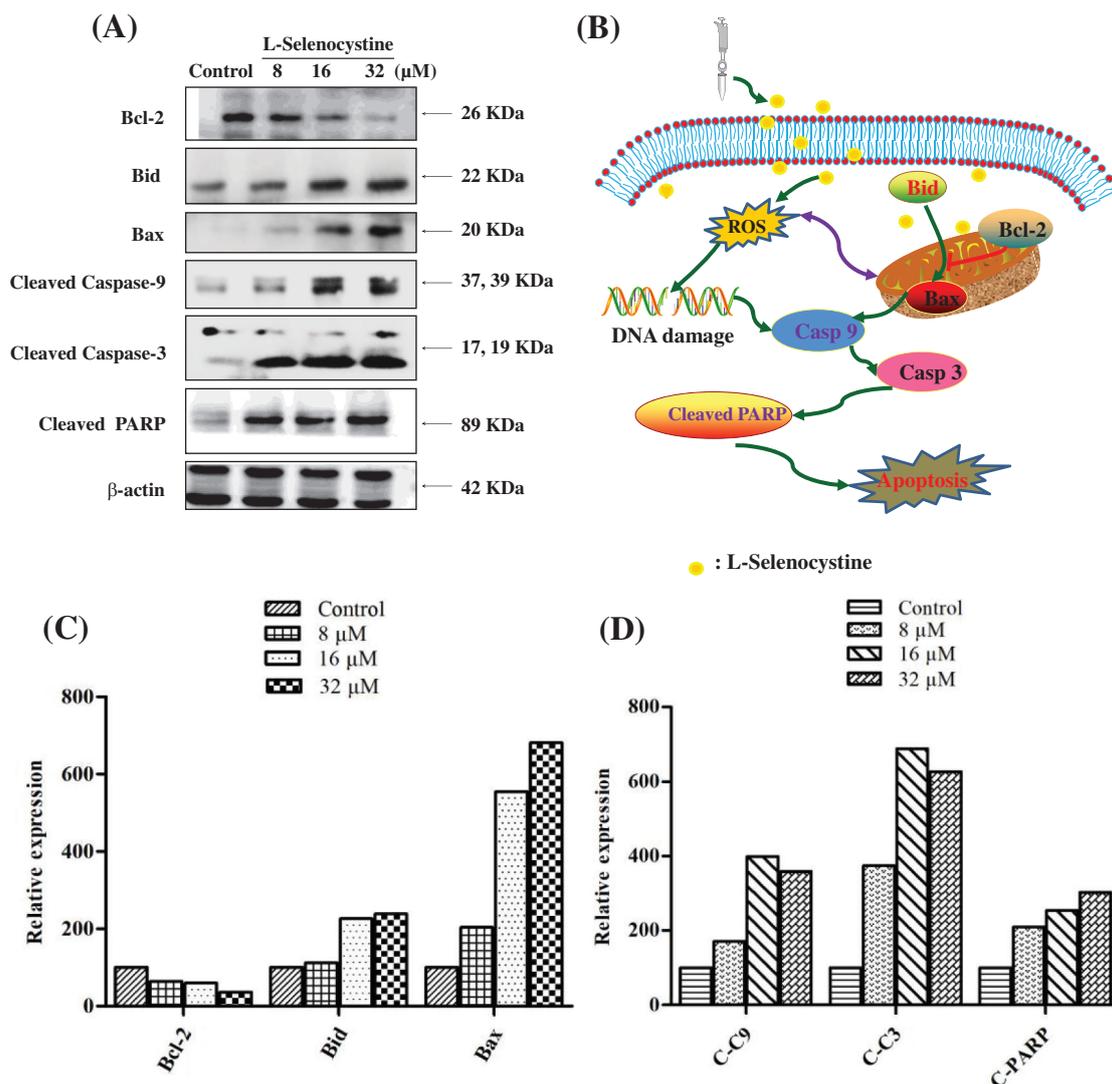


FIGURE 5. Activation of apoptotic signaling pathways by L-Selenocystine in HepG2 cells. The expression level of Bax, Bcl-2, Bid and caspase in HepG2 cells (A). The signaling pathway of ROS-mediated regulation of DNA damage and Bax (B). The relative expression levels of apoptosis-related proteins in L-selenocystine treatment group and blank control group (C) and (D). The expression of β -actin was measured as control. Abbreviations: PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species. Casp 9, Caspase 9; C-C9, Cleaved Caspase 9; Casp 3, Caspase 3; C-C3, Cleaved Caspase 3.

ends in death. Therefore, it is necessary to explore new and effective drugs to resist the growth of HCC cells.

Our results shown that the cytoplasm of HepG2 cells treated with L-Selenocystine was significantly atrophied, the contact between cells was lost, and the number of cells was evidently reduced. These data indicate that L-Selenocystine could significantly inhibit the replication and proliferation of HepG2 cells in a dose-dependent manner *in vitro*. In addition, MMP decreased and ROS increased in HepG2 cells with L-Selenocystine treatment group, which are the key factors to induce apoptosis. ROS is a kind of substance with free radical chemical activity. In cells, ROS is mainly produced by mitochondria through the respiratory chain (Wang *et al.*, 2020). Its balance plays an important role in maintaining the normal physiological function of cells (Mirzaei *et al.*, 2021). Previous study show that the increase of intracellular ROS production will break the redox balance, induce changes in cell function and participate in

the occurrence and development of diseases (Butterfield and Halliwell, 2019). Mitochondrial membrane potential is a necessary index to evaluate mitochondrial function. As one of the key organelles in cells, mitochondria have a wide range of physiological functions, including energy metabolism, signal transduction, maintaining intracellular redox balance and so on. Mitochondrial damage plays a very important role in triggering cell apoptosis (Romero-Cordero *et al.*, 2021). These data intensively make clear that L-Selenocystine can induce HepG2 cell apoptosis through mitochondrial damage and ROS production.

Bcl-2 protein family is an important protein regulating cell survival, including pro-apoptotic proteins bid, Bad, Bax and pro-survival proteins Bcl-2, BFL-1, Bcl-XL (Fairlie and Lee, 2021). Under normal circumstances, these two kinds of proteins will maintain a certain balance in cells. If this balance was broken, it will lead to cell apoptosis (Ye *et al.*, 2021). Compare with the control group, the expression of Bid

and Bax protein in HepG2 cells treated with L-Selenocystine increased significantly, while the expression of Bcl-2 protein decreased significantly in this study. The Caspase family plays a critical role in the modulation of apoptosis. In particular, Caspase-3 plays an important central regulatory role in the signal network (Liu *et al.*, 2022; Wang *et al.*, 2010). Therefore, Caspase-3 and Cleaved PARP were also detected to evaluate their role in apoptosis in Figs. 5A and 5D. L-Selenocystine significantly activated the expression of caspase-3 and the downstream effect of PARP cleavage. Take it all together, these dates confirm that L-Selenocystine induces HepG2 cells apoptosis by regulation of ROS-mediated Bax signaling pathways (Fig. 5B).

Conclusion

In summary, this study describes the ability of L-Selenocystine to inhibit HepG2 cell proliferation through cellular oxidative stress. L-Selenocystine significantly increased apoptosis in a dose-dependent manner. Molecular mechanism studies showed that L-Selenocystine promotes caspase-3-mediated apoptosis through its involvement in ROS production. Further studies showed that Bax signaling pathway was the main apoptotic signaling pathway triggered by L-Selenocystine in HepG2 cells. In a word, our results suggest that L-Selenocystine is an amino acid with potential anticancer properties.

Availability of Data and Material: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author's Contribution: Study design: BZ and YHL; experiments carry out: JYS, DYC and YYD; data collection: RLZ and QLD; analysis of results: QQD; draft manuscript preparation: HYC. All authors reviewed the final version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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