Inhibitory Effect of Hirudin on Hepatocellular Carcinoma Cells

Xintian HUANG[1#], Keyi ZHOU[1#], Dengfeng ZOU[1], Jinfeng ZHOU[1], Haijiao ZHANG[2], Xiaofan WANG[1], Jingyi JIANG[1], Zhiqiang CHEN[3*], Changfu HU[2*], Xin JIANG[3*]

1. Guilin Medical University, Guilin 541199, China; 2. Department of Vascular Surgery, Nanxishan Hospital of Guangxi Zhuang Autonomous Region, Guilin 541004, China; 3. The Affiliated Hospital of Guilin Medical University, Guilin 541001, China

Abstract [**Objectives**] This study was conducted to explore the proliferation inhibition of hirudin on hepatocellular carcinoma HepG 2 cells and Huh-7 cells. [**Methods**] Hirudin solutions of different concentrations (2.0, 2.5, 3.0, 3.5, 4.0 mg/ml) were used to treat HepG 2 cells and Huh-7 cells. The effects of different hirudin concentrations on the proliferative activity of HepG2 and Huh-7 cells were detected by CCK-8 assay, and the IC_{50} values were calculated. A living/dead cell double staining experiment was conducted to observe the fluorescence of cells under a fluorescent microscope, so as to assess the inhibitory effect of different concentrations of hirudin on the proliferation of HepG2 and Huh-7 cells. A cell scratch assay was carried out, and an inverted microscope was employed to observe the healing of the scratched areas, so as to assess the impact of hirudin on the migratory and invasive capabilities of hepatocellular carcinoma HepG2 and Huh-7 cells. [**Results**] (i) The results of CCK-8 assay indicated that compared with the blank control group, the proliferation inhibition rates of both hepatocellular carcinoma HepG2 and Huh-7 cells increased with the concentration of hirudin increasing, demonstrating that hirudin had an inhibitory effect on the proliferative activity of these cells. Specifically, the IC_{50} values for HepG2 and Huh-7 were found to be 3.5 and 4.0 mg/ml. (ii) The living/dead cell double staining experiment revealed that the number of living cells in the hirudin-treated group decreased significantly compared with the control group, while the number of dead cells increased markedly, indicating an inhibitory effect of hirudin on the proliferation of HepG2 and Huh-7 cells. (iii) The results of cell scratch assay showed that the healing degree of the scratched areas in the hirudin-treated groups was significantly lower than that of the control group, indicating a reduction in cell growth and migration capabilities. [**Conclusions**] Hirudin exhibited a significant inhibitory effect on the proliferation of hepatocellular carcinoma HepG2 and Huh-7 cells.

Key words Hirudin; HepG 2 cells and Huh-7 cells; Proliferation; Migration; Inhibition

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Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. According to the statistics of global cancer data updated by the International Agency for Research on Cancer (IARC) in January, 2021, there were 199.3 million new cancer cases and nearly 10 million deaths, in which the proportion of liver cancer reached 8.3%, and liver cancer is the third largest factor of cancer death in the world[1]. In China, liver cancer is the second most common tumor after other malignant tumors, and its morbidity and mortality also rank second[2]. Although there are many methods and means to treat liver cancer, the overall prognosis of patients with liver cancer is still not optimistic[3]. In the field of liver cancer treatment, finding a new safe and effective anti-liver cancer drug has become a hot and difficult problem in the field of liver cancer treatment research.

Leeches have a long history of medicinal use in China. They were first recorded in *Shennong's Herbal Classic* and have a long history in traditional Chinese medicine. In modern times, leeches are mainly dried and processed and then used as a medicine, which has the functions of breaking blood, removing blood stasis and dredging menstruation, and these functions have also been confirmed in modern medicine[4]. In the 1960s, due to the development and progress of science and technology, scientists innovatively purified hirudin, the active substance in the salivary gland of leeches, for the first time, and separation and purification of hirudin from leech saliva was thus more effectively[5], which greatly promoted the research on hirudin. Leeches contain a variety of bioactive components, including protein peptides such as hirudin, heparinoid, histamine and hementin[6], and hirudin is the main active component. It is a polypeptide compound extracted and separated from the salivary gland of medicinal leeches, consisting of 64 to 66 amino acid residues, and its relative molecular weight is about 7 000 Da[7]. Hirudin is a natural peptide active substance with remarkable anticoagulant and antithrombotic properties, so it has been widely used in the prevention and clinical treatment of cardiovascular and cerebrovascular diseases[8]. In recent years, scientific research has found that hirudin has anticancer properties and can inhibit the proliferation and metastasis of various cancer cells[9-11]. Therefore, in-depth study on the application prospect of hirudin in the treatment of liver cancer is of positive research significance for enriching the types of drugs for liver cancer treatment and alleviating the pain of patients.

In this study, the inhibitory effect of hirudin on hepatocellular carcinoma HepG2 cells and hepatocellular carcinoma HH-7 cells was explored, aiming to provide some research basis for

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Xintian HUANG (1998 -), female, P. R. China, master, devoted to research about photodynamic therapy of tumor.

Keyi ZHOU (2006 -), female, P. R. China, devoted to research about photodynamic therapy of tumor.

#These authors contributed equally to this work.

* Corresponding author.

further exploring its molecular mechanism. Through CCK-8 assay, living/dead cell staining experiment and scratch assay, the effects of hirudin on the proliferation, survival and migration of hepatocellular carcinoma cells were evaluated, hoping to provide an experimental basis for the application of hirudin in the treatment of liver cancer, as well as new ideas and methods for the treatment of liver cancer.

Materials and Methods

Experimental materials

Cell line Hepatocellular carcinoma HepG2 cells and Huh-7 cells were purchased from Wuhan Pricella Biotechnology Co., Ltd.

Main experimental reagents The main experimental reagents and manufacturers were as follows: hirudin, Lemaitian Medicine; phosphate buffer solution, Wuhan Pricella Biotechnology Co., Ltd.; DMEM medium, Wuhan Pricella Biotechnology Co., Ltd.; penicillin-streptomycin (double antibody), Wuhan Pricella Biotechnology Co., Ltd.; FBI fetal bovine serum, Wuhan Pricella Biotechnology Co., Ltd.; pancreatic enzyme, Wuhan Pricella Biotechnology Co., Ltd.; cell freezing medium, Wuhan Pricella Biotechnology Co., Ltd.; CCK-8 cell proliferation assay kit, Shanghai Beyotime Biotechnology Co., Ltd.; Calcein AM/PI live/dead cell double staining kit, Shanghai Beyotime Biotechnology Co., Ltd., China; 10 × Aassay Buffer solution, Shanghai Beyotime Biotechnology Co., Ltd.

Main experimental instruments The main experimental instruments and manufacturers were as follows: Super clean bench/biosafety cabinet, Qingdao Haier Biomedical Co., Ltd.; carbon dioxide incubator, Shanghai Boxun Medical Biological Instrument Corp.; constant-temperature water bath pot, Shanghai Boxun Medical Biological Instrument Corp.; analytical balance, Shanghai Weichuan Precise Instrument Co., Ltd.; centrifuge, Guangzhou Jidi Instrument Co., Ltd.; pipette gun, Guangzhou Kaichuang Biotechnology Co., Ltd.; enzyme labeler, Guangxi Nanning Bomei Technology Co., Ltd.; optical inverted microscope, Shanghai Pooher Optoelectronic Technology Co., Ltd.; fluorescent inverted microscope, Beijing Precise Instrument Co., Ltd.

Experimental methods

Cell culture

Cell lines HepG2 and Huh-7 cell lines were selected for subsequent cytological experiments. The two cell lines were cultured in DMEM complete medium containing 10% fetal bovine serum, and then placed in an incubator with constant temperature of 37 ℃, 5% CO_2 and saturated humidity. Change of culture medium and cell passage were performed in time to ensure the continuity and accuracy of the experiment.

Cell recovery

(i) After opening a super clean bench and spraying 75% ethanol for disinfection, centrifuge tubes, pipettes, pipette tips and culture bottles needed for experiments were placed in the super clean bench. Ultraviolet radiation was used for disinfection for 30 min.

(ii) The required freezing tube cells were taken out from a low-temperature refrigerator at −80 ℃, quickly transferred to a constant-temperature water bath that was preheated to 37 ℃ in advance, and gently shaken repeatedly. During the shaking process, the action was fast and gentle, and the liquid was thawed within 1 min as soon as possible.

(iii) The thawed frozen tube was sterilized with 75% ethanol, and put on a super clean bench. The cell suspension in the freezing tube was transferred with a 1 ml pipette to a 15 ml centrifuge tube, which was then added with 5 ml of culture medium, and put in a centrifuge. The liquid was centrifuged at 1 000 r/min for 5 min. After discarding the supernatant, 1 − 3 ml of culture medium was added, and the liquid was gently blown to re-suspend the cells, so as to prepare a single cell suspension. The cells were transferred to T25 or T75 culture bottles with a pipette and cultured in an incubator with 5% CO_2 and saturated humidity at 37 ℃.

(iv) After the resuscitated cells were cultured overnight, the adherence of cells was observed. The culture medium was replaced with fresh complete culture medium in time, and the growth status of cells was regularly checked and recorded.

Change of culture medium

(i) Cell culture bottles were taken out to observe whether the cell culture medium became turbid. Meanwhile, the growth state of cells was observed by an optical microscope to judge whether the cells were pollution-free.

(ii) The culture bottle in which the culture medium should be changed was disinfected with 75% ethanol. Meanwhile, operator's hands were also sprayed. A pipette was used to draw the cell supernatant in the culture bottle in a super clean bench, and it was moved gently to avoid scratching the cells at the bottom of the bottle. Next, PBS buffer was added to wash the cells gently for two or three times.

(iii) Finally, new complete medium was supplemented, and the cell culture bottle was put back into the incubator to continue the culture.

Cell passage

(i) Liver cancer HepG2 cell culture bottles and Huh-7 cell culture bottles were taken out from the incubator to observe the growth state of cells in the culture bottles through an optical microscope. After excluding the situation that the cells were not polluted, cell passage was carried out for cells growing well with a cell density reaching 80% − 90%.

(ii) The culture bottle chosen for cell passage was disinfected with 75% ethanol. The supernatant was drawn and discarded in a super clean bench, with gentle movements to avoid scratching the cells at the bottom of the bottle. Then, PBS buffer was added to clean the cells for two or three times and then discarded.

(iii) Next, 1 ml of trypsin was added for digestion. The culture bottle was gently shaken to make the trypsin evenly cover the surface of cells and left in a still state to allow digestion for a few seconds.

(iv) The cells were observed under a microscope, and when they became round and were floated in a wrinkled state, complete culture medium with twice the volume was added to terminate the digestion. The bottle wall was blown slowly and gently with a pipette to blow off the cells attached to the bottle wall and re-suspend them into a single cell suspension.

(v) Cell counting was performed for subsequent cell plating experiments. Then, the cells were inoculated into new culture bottles and cultured in an incubator.

Cell cryopreservation Referring to the above-mentioned operation steps of "cell passage", the supernatant was discarded after digestion and centrifugation. Next, 1 ml of cell freezing medium was transferred to the 15 ml centrifugal tube, and gently blown with a pipette to form a uniformly-dispersed single cell suspension. The cell suspension was transferred into freezing tubes, and the cover of each freezing tube was screwed tightly. All freezing tubes were labeled, and the cell name, freezing date and other information were recorded with a marker. Finally, the cells were stored in a refrigerator at −80 ℃.

Detection by cell counting Kit-8 (CCK-8) assay HepG2 cells and HH-7 cells growing well in the logarithmic growth phase were selected. After discarding the old culture medium, the cells were washed with PBS buffer for three times, and digested with trypsin. Next, centrifugation was performed at 1 000 r/min for 5 min to collect cell precipitate, which was added with DMEM complete culture medium containing 10% fetal bovine serum and gently blown to form a single-cell suspension. Referring to the operation steps of "cell plating", after calculating the cell concentration by using cell counting plates, the cell suspension was inoculated into 96-well plates, and each group of experiments was set with four replicate wells, each containing about 4 000 cells. Then, the cells were cultured for 24 h. After 24 h, different concentrations of hirudin (2.0, 2.5, 3.0, 3.5, 4.0 mg/ml) were used to treat hepatocellular carcinoma HepG2 cells and HH-7 cells. In addition, several wells were reserved in each 96-well plate and added with only culture medium and no other substances to serve as a blank control group. The culture plates were placed in an incubator for 24 h. After 24 h, the plates were taken out and placed in a super clean bench, and 10 μl of CCK-8 detection reagent was added to each well for cell activity analysis. Then, they were placed back in the incubator for 40 − 42 min. Subsequently, the plates were taken out, and the absorbance (OD) value of each well was measured at the wavelength of 450 nm by a microplate reader (the measurement should be carried out in dark place as much as possible). Each experiment was set with four replicate wells and repeated three times. The inhibitory concentration of cell activity was detected to calculate its inhibitory concentration (IC_{50}), so as to determine the best dosage, and the subsequent experiment was carried out under this dosage.

Detection of living/dead cell staining experiment HepG2 cells and Huh-7 cells with suitable density were inoculated into 24-well plates with about 40 000 cells for each well, and then cultured in an incubator with 5% CO_2 and saturated humidity at 37 ℃ for 24 h. The HepG2 cell group was treated with hirudin solutions at concentrations of 0 and 3.5 mg/ml, while the Huh-7 cell group was treated with hirudin solutions at concentrations of 0 and 4 mg/ml. After incubation for 24 h, the culture medium was discarded, and 1 ml of 1 × Aassay Buffer and 1 μl of Calcein AM staining solution mixture were added to each well. After incubation in a dark incubator for 20 min, 0.5 μl of propidium iodide (PI) was added, and incubation was continued in the incubator for 5 min. Each plate was observed and photographed under an inverted fluorescence microscope. Calcein-AM dye can penetrate the membrane of living cells, and after being cleaved by esterase in the cell, it shows green fluorescence, thus identifying living cells. PI dye can't penetrate the membrane of living cells, but can only enter dead cells, and after binding with nuclear DNA, it shows red fluorescence, thus identifying dead cells.

Detection of cell scratch assay HepG2 and HH-7 cells with suitable density were cultured in 6-well plates respectively. When it was observed that the coverage rate of cells reached or exceeded 90%, a vertical line was gently drawn with a 10 μl sterile pipette tip. Then, the cells were washed with PBS for three times, and DMEM medium was added to the wells. An inverted microscope was used for observation and recording. After the cell plates were put back into the constant temperature incubator for 24 h, the position of the corresponding field of view under the microscope was observed and photographed again by the same observation and recording method after the culture time ended. In order to carry out quantitative analysis, five different visual field regions were randomly selected from each sample, and the data of these regions were processed and subjected to the corresponding statistical analysis.

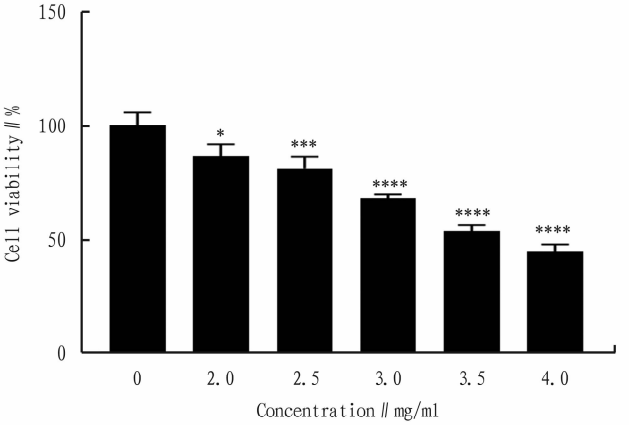
Statistical processing In the data analysis stage of this study, version 16.0 of SPSS software was adopted as the analysis tool. The analysis results of quantitative data were presented in the form of mean ± standard deviation ($\bar{x} \pm s$). For the comparison on the mean values of two groups of independent samples, we adopted the statistical method of independent sample t-test. For the comparison on the mean values of three or more groups of samples, One-way ANOVA was adopted, with $a = 0.05$ as the test threshold for evaluating whether the differences between groups were statistically different. $P < 0.05$ indicated there was a significant statistical difference. According to the obtained result data, GraphPad Prism 10 and ImageJ software were employed for drawing and calculation.

Results and Analysis

Effects of hirudin on proliferation activity of hepatocellular carcinoma HepG2 cells and Huh-7 cells

In order to explore the effects of hirudin on the cell activity of hepatocellular carcinoma HepG2 cells and Huh-7 cells, and to determine the best dosage in subsequence experiment, a series of hirudin solutions with different concentrations were set up, namely

0, 2.0, 2.5, 3.0, 3.5, and 4.0 mg/ml, respectively. These hirudin solutions with different concentrations were added to the culture systems containing HepG2 and Huh-7 cells, respectively. CCK-8 cell activity detection reagent was added at 24 h after drug administration, and the cell activity was detected after 40 – 42 min of continuous culture. The results of the assay showed that after the intervention of hirudin solution treatment, the cell survival rates of the two groups showed a downward trend, and the proliferation activity of hepatocellular carcinoma HepG2 cells and HH-7 cells was inhibited, and the inhibitory effect was enhanced with the concentration of hirudin solution increasing, as shown in Fig. 1 and Fig. 2. With the concentration of hirudin increasing, cell survival rate decreased gradually, and the cell survival rates of hirudin groups with different concentrations were significantly different from that of the blank control group ($P<0.05$, $P<0.01$). According to the survival rates of the two groups of cells, the inhibitory concentrations (IC_{50}) of the two groups of cells were calculated respectively. After calculation, the IC_{50} value of HepG2 was at the concentration of 3.5 mg/ml with an obvious inhibitory effect, and the IC_{50} value of Heh-7 was at the concentration of 4.0 mg/ml with an obvious inhibitory effect. Therefore, in subsequent experiment, 3.5 and 4.0 mg/ml were chosen as the main research targets, and the effects of hirudin on the proliferation of HepG2 cells and Huh-7 cells after 24 h of unified treatment with the control group were observed. The experiment was repeated three times, and similar results were obtained each time (Table 1).

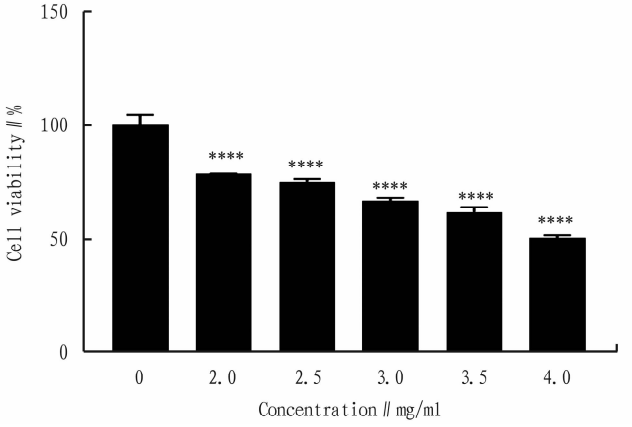


Comparing different concentrations of hirudin groups with the control group, $^{*}P<0.05$, $^{***}P<0.001$, $^{****}P<0.0001$.

Fig. 1 Effects of different concentrations of hirudin on survival rate of HepG2 cells

Detection of killing effects of hirudin on hepatocellular carcinoma HepG2 cells and Huh-7 cells by living/dead cell staining experiment

In order to further verify the inhibitory effects of hirudin on HepG2 cells and Huh-7 cells, we adopted Calcein AM/PI kit to stain the cells. Calcein AM/PI kit is used for distinguishing living cells from dead cells by the fluorescence double staining technology. In specific, Calcein AM can penetrate living cells and produce green fluorescence, and propidium iodide (PI) specifically binds to the nucleic acid of dead cells and produces red fluorescence. On this basis, the living state of cells could be distinguished, and the killing effects of hirudin solution on hepatocellular carcinoma HepG2 cells and HH-7 cells were further evaluated. As shown in Fig. 3, compared with the control group, the number of living HepG2 cells and Huh-7 cells in the hirudin-treated group decreased, and the number of dead cells increased significantly, with a significant difference ($P<0.01$), as shown in Fig. 4. It indicated that hirudin had an inhibitory effect on the proliferation and growth of hepatocellular carcinoma HepG2 cells and Huh-7 cells.



Comparing different concentrations of hirudin groups with the control group, $^{****}P<0.0001$.

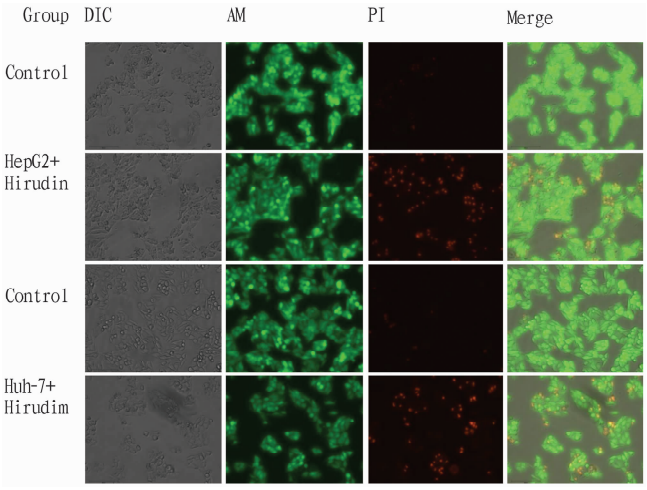
Fig. 2 Effects of different concentrations of hirudin on survival rate of Huh-7 cells

Table 1 Effects of hirudin concentration on proliferation of HepG2 and Huh-7 cells

Group	Dose mg/ml	HepG2 proliferation rate after 24 h // %	Huh-7 proliferation rate after 24 h // %
Control	0.0	100.00 ± 0.00	100.00 ± 0.00
Hirudin administration group	2.0	87.03 ± 5.04	78.39 ± 0.25
	2.5	81.36 ± 5.48	75.11 ± 1.17
	3.0	68.18 ± 1.91	66.89 ± 1.25
	3.5	53.89 ± 2.49	62.83 ± 2.26
	4.0	44.54 ± 3.62	50.26 ± 1.33

Detection results of hirudin to migration capacities of hepatocellular carcinoma HepG2 cells and Huh-7 cells

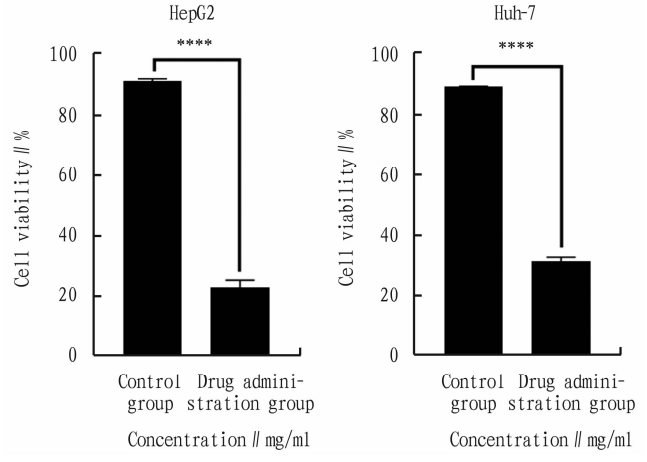
The results of cell scratch test showed that after 24 h treatment with hirudin, the growth and migration capacities of cells were weakened. The scratch filling areas of hepatocellular carcinoma HepG2 cells and Huh-7 cells were significantly lower than that of the control group, showing weakened growth and migration capacities of cells. The differences were significant ($P<0.01$), as shown in Fig. 5 and Fig. 6. It indicated that hirudin affected the migration capacities of hepatocellular carcinoma HepG2 cells and Huh-7 cells.



DIC is the cell image in the bright field state; AM is the cell image under the green fluorescence channel; PI is the cell image under the red fluorescence channel; and Merge is the cell image in the bright field superimposed with green fluorescence and red fluorescence.

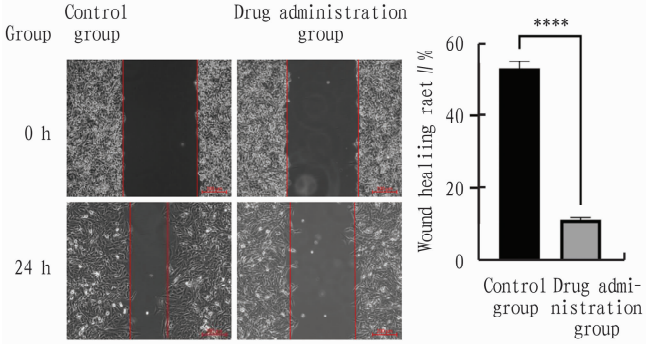
Living cells are marked in green and dead cells are marked in red.

Fig. 3 Living/dead cell fluorescent images of HepG2 cells and Huh-7 cells



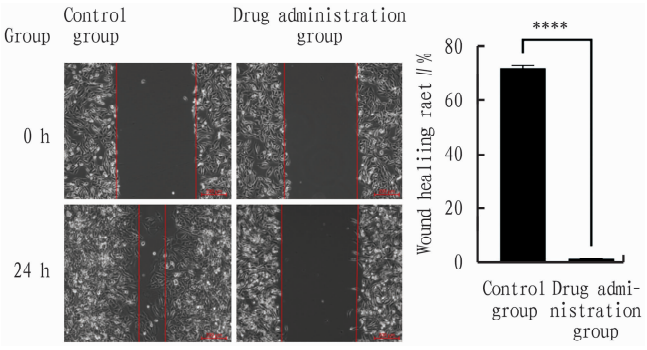
Comparing the drug administration group with the control group, $^{****}P < 0.000\ 1$.

Fig. 4 Living/dead cell quantitative analysis on HepG2 cells and Huh-7 cells



Comparing the drug administration group with the control group, $^{****}P < 0.000\ 1$.

Fig. 5 Effects of hirudin on migration of hepatocellular carcinoma HepG2 cells



Comparing the drug administration group with the control group, $^{****}P < 0.000\ 1$.

Fig. 6 Effects of hirudin on migration of hepatocellular carcinoma Huh-7 cells

Conclusions and Discussion

In this study, the inhibitory effect of hirudin on liver cancer was preliminarily discussed, and two cell lines of human hepatocellular carcinoma HepG2 cells and human hepatocellular carcinoma HH-7 cells were selected to study the preliminary inhibitory effect of hirudin on liver cancer. The effects of different concentrations of hirudin on the proliferation of two kinds of liver cancer cells were observed, aiming to provide a new idea for the possibility of treating liver cancer with hirudin. The results of the CCK-8 assay showed that with the concentration of hirudin solution increasing, the survival rates of HepG2 cells and HH-7 cells exhibited a downward trend, as shown in Fig. 1 and Fig. 2, indicating that hirudin had a preliminary inhibitory effect on the proliferation of the two kinds of cells. The living/dead cell staining experiment showed that compared with the control group, the number of living cells in the two groups decreased and the number of dead cells increased obviously after the intervention of hirudin, and the differences were statistically significant ($P < 0.01$), as shown in Fig. 3 and Fig. 4, which indicated that hirudin had a killing effect on these two kinds of cells. The results of cell scratch assay showed that the scratch filling areas of HepG2 cells and Huh-7 cells were lower than that of the control group, and the differences were statistically significant ($P < 0.01$), as shown in Fig. 5 and Fig. 6. Therefore, hirudin can inhibit the proliferation and migration of HepG2 cells and Huh-7 cells. Therefore, hirudin can inhibit the proliferation and migration of HepG2 cells and Huh-7 cells. In this study, the inhibitory effect of hirudin on hepatocellular carcinoma HepG2 cells and HH-7 cells was preliminarily discussed through *in-vitro* experiments. However, due to the lack of *in-vivo* experimental study of hirudin on liver cancer, the inhibitory effect of hirudin on liver cancer needs further experimental verification, and the mechanism by which hirudin induces apoptosis of hepatocellular carcinoma HepG2 cells and HH-7 cells needs further in-depth study.

In a word, hirudin, as a potential drug for treating liver cancer, has broad development prospects. In this study, the inhibitory effect of hirudin on hepatocellular carcinoma HepG2 cells and Huh-7 cells were preliminarily discussed, which provides a new research perspective for the treatment of hepatocellular carcinoma with hirudin.

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