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A new design of coculture microfluidic chip for HepG2 and LO2 cells

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ABSTRACT

Objective: In order to better screen targeted drugs, a microfluidic chip that can culture both diseased and normal cells is designed.
Methods: With Human hepatocellular carcinomas (HepG2) and Human normal liver cells (LO2) as the cell models, Polydimethylsiloxane-glass (PDMS-glass) chip as the carrier, and potential anticancer drug sanguinarine as the research object, two cells were co-cultured on the chip and the drug acted on both cells simultaneously in a diffusion manner.
Results: In co-cultured cell chips, the apoptosis rate of HepG2 cells was significantly higher than that of LO2 cells under the action of sanguinarine.
Conclusion: The chip diffusion perfusion form does not damage the cells, and can achieve cell staining and in situ observation more flexibly and conveniently, and more realistically reflects the selective effect of drugs on different cells, which has the advantages of simple operation and low cost.

1. Introduction

Whether a drug is safe and effective is a determinant of the success of drug development, and toxicity (safety) is one of the important reasons for terminating drug development throughout the drug development process.[1,2] In fact, a human cell culture model that is similar to its *in vivo* behavior offers significant advantages in eliminating species differences, saving time and cost in drug research. So far, several conventional *in vitro* cell-culture techniques have been developed. Among these techniques, cells are grown in a static macroscopic environment, making it difficult to detect toxicity to normal cells before clinical application when used for drug-targeted screening.[3,4] However, the co-culture system can provide physiological clues, better mimic the *in vivo* environment, facilitate better observation of cell-to-cell and cell-to-culture environment interactions, and explore the mechanism of action and possible targeting of drugs, filling the gap between traditional cell culture and overall animal experimental research.[5,6,7]

Cell co-culture methods mainly include direct contact co-culture, indirect contact co-culture, and three-dimensional cell co-culture. Co-culture means that two or more types of cells are seeded in the same well at the same time or separately, and different types of cells are in direct contact, such as culturing cells in a dish or 96-well plate, but direct co-culture will cause difficult cell separation, inconvenient observation and subsequent detection.[8] Indirect co-culture system, that is, two or more types of cells are seeded on different vectors, and then the two vectors are placed in the same culture environment, so that different types of cells share the same culture system without direct contact. For example, lv et al. successfully established a Transwell co-culture cell model of silicosis including RFL-6 (rat lung fibroblasts), RLE-6TN (rat alveolar epithelial cells type II) and NR8383 (rat alveolar macrophages) cells, but this model could not satisfy the co-culture of 3 or more cells.[9] T et al. cultured endothelial cells using slides in a dish and astrocytes in the medium to achieve co-culture of cells.[10] Three-dimensional co-culture is the use of microstructures or biodegradable materials to create three-dimensional co-culture microenvironment cells. Anne et al. used three-dimensional collagen gel culture to co-culture primary human osteocytes and mature human osteoclasts.[11] Among them, microfluidic technology is a powerful tool for studying cell behavior due to its simplicity, biocompatibility, and beneficial advantages of high-throughput detection.[12,13] This tool has been used by researchers to establish co-culture models of cells for physiological, toxicological, pathological, and pharmacokinetic studies.[14–19]

Over the past decade, microfluidics and cell co-culture techniques have provided valuable tools for the screening of miniaturized *in vitro* model drugs.[15,16] To date, reported microfluidic chip drug targeting screens often use different tumor cells.[20,21] Xu et al.[20] developed a drug

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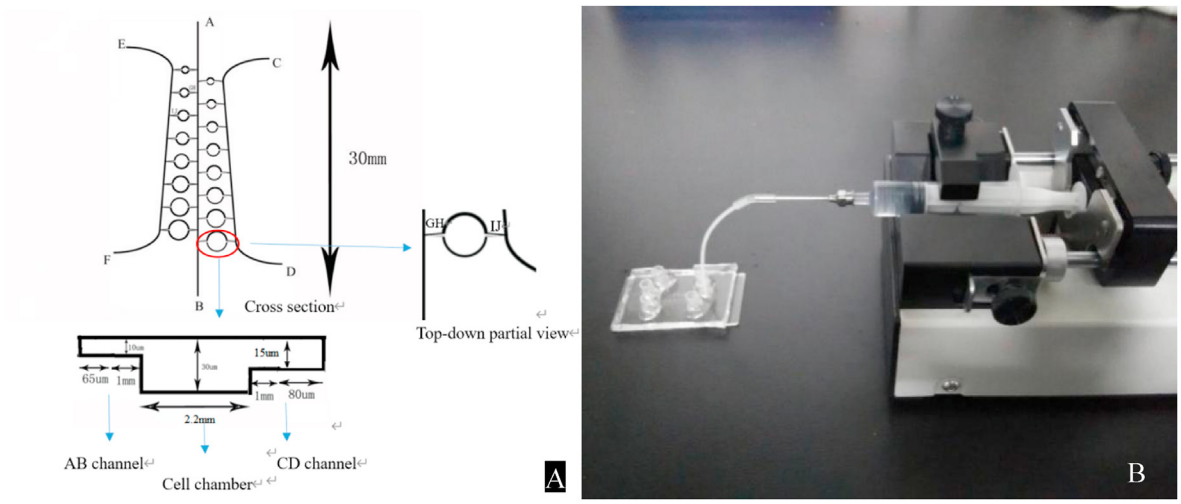

Fig. 1. Schematic diagram of chip mask structure. A) Schematic diagram of chip structure; B) Cross-sectional schematic view of the chip channel; C) Image of the fabricated chip.

Table 1
Etch time for each channel (min).

Etch time	channels				
	AB	CD	EF	GH	IJ
1	15	15	15	15	15
2	15	15	15	10	10
3	10	15	15	10	15

Table 2
Number of cells loaded into each cell chamber.

Etch time group	cell chambers			
	1 mm	1.4 mm	1.8 mm	2.2 mm
1	23	64	130	340
2	33	85	190	870
3	56	119	230	1600

sensitivity platform to determine the sensitivity of different anticancer drugs by co-culturing SPCA-1 cells (Human lung adenocarcinoma 1) and HFL1 cells (Human fetal lung fibroblast 1) using a combination of four microfluidic chips. Mi et al.[21] successfully established a microfluidic co-culture system that mimics different regions of metastatic breast tumors to study cancer cell migration and anticancer drug screening. Chen et al. designed a hepatorenal model and co-cultured HepG2 cells and HRGEC (Human Renal Glomerular Endothelial Cells) to study the toxicity of emodin,[22] but the study found that emodin is more harmful to the kidneys than diseased liver cancer cells. Most scholars only use different cancer cells for targeted screening when screening antitumor drugs on microfluidic chips, thus ignoring the toxicity of normal cells. In addition, some scholars have studied the co-culture of a variety of tumor cells to study cell migration and drug metabolism.[23–26] However, the method of co-culturing diseased cells and human normal cells on microfluidic chips has been reported little in models for screening highly efficient and low-toxicity antitumor drugs.

Here, we design a novel PDMS-glass chip for co-culture and drug activity screening of HepG2 and LO2 cells. Seeding both types of cells by differential pressure and delivering nutrients using an external syringe pump and gravity effect enabled it to successfully culture both types of cells simultaneously on the same chip.

2. Materials and metheds

2.1. Instruments & reagents

10% Fetal bovine serum (FBS, HyClone), 1 mL of penicillin-streptomycin solution (HyClone, 10,000 units/mL penicillin and 10,000 μg/mL streptomycin), DAPI dye (C1006, Biyuntian Biotechnology Co., Ltd.), DMSO (Biotechnology, 500 mL), tetramethylzole blue (MTT, Amresco), inverted fluorescence microscope (BX41-32H02, Onlympus Co., Japan), Rodamine B (Aladdin Industries, Shanghai, China), Lithography Machine (JKG-2A, Shanghai Huayan Instrument Equipment Co., Ltd.), Bio Tek Microplate Reader (KQ3200DV, Bio Tek), CO_2 Incubator (TDL-40B, Thermo), Micro Syringe Pump (TJ-3A/W0109-1B, Baoding Lange Constant Flow Pump Co., Ltd.), Centrifuge (TDL-40B, Shanghai Anting Scientific Instrument Factory).

2.2. Cell culture

Human hepatoma cell line HepG2 (subcultured cells seeded from the School of Pharmacy, Guangdong Pharmaceutical University) and human

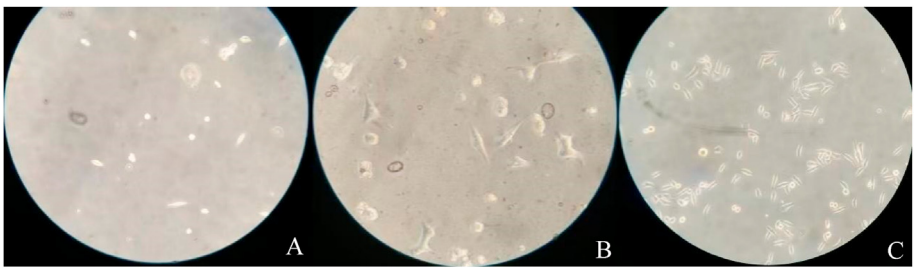

Fig. 2. Cell adherence status at different flow rates. Cell number and adherent state: A) at 10 μL/min flow rate; B) at 8 μL/min flow rate; C) at 3 μL/min flow rate.

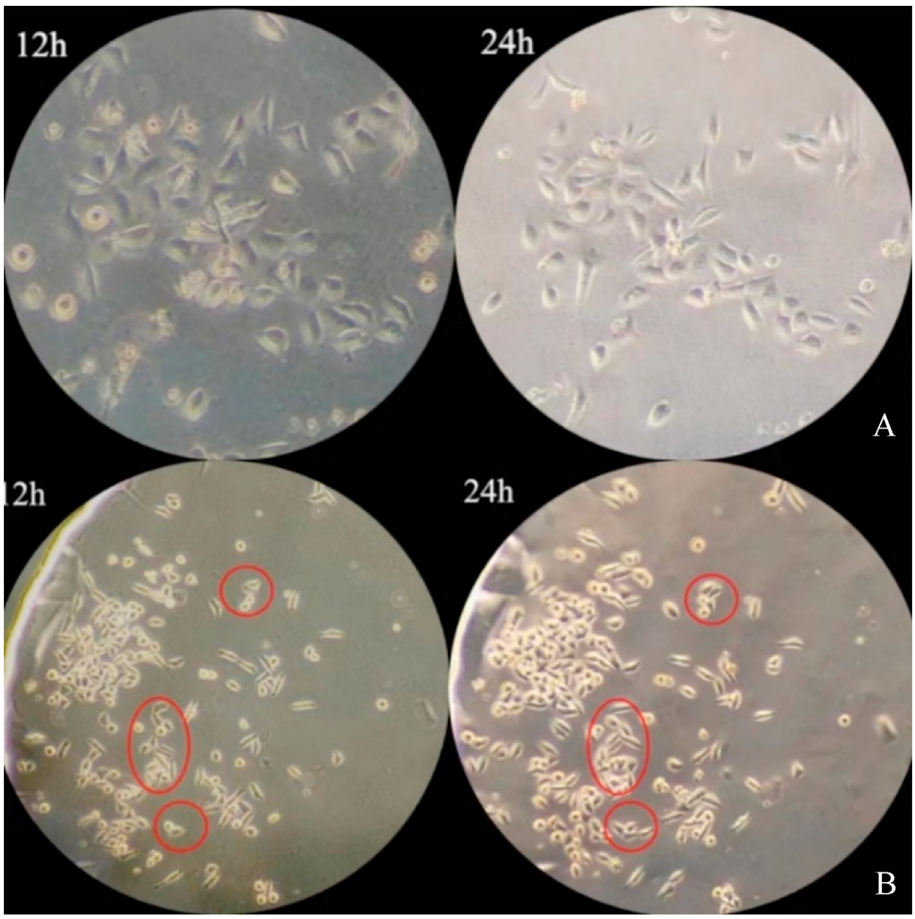

Fig. 3. Cell growth status on PDMS of different thicknesses. A) When the PDMS thickness is 2.0 mm, the cell growth state is cultured on the chip for 12 h and 24 h; B) Growth status of cells at 12 h and 24 h on PDMS molds with a thickness of 1.5 mm.

hepatocyte line LO2 cells (subcultured cells seeded from the School of Pharmacy, Guangdong Pharmaceutical University). HepG2 and LO2 cells were cultured in DMEM high-sugar medium supplemented with 10% fetal bovine serum and 1% streptomycin and penicillin in a humidified incubator at 37 °C under 5% CO_2 atmosphere. When the cells grew to 80%-90% confluency, cells were sub-cultured and resuspended in DMEM. The cells were washed with PBS, resuspended in a fresh culture medium to the desired densities, and cells are loaded into the microchip by a pump for cell experiments.

2.3. Microchannel fabrication and design

The microfluidic chip consists of two layers, one of which is a PDMS cover sheet and the other is a glass negative. First, the reference uses photolithography and chemical etching methods on glass negatives by step-by-step etching,[27] as shown in Fig. 1. The main channel AB of the chip is 30 mm (length) × 80 μm (width) × 10 μm (depth). The width and depth of EF and CD are 65 μm and 15 μm, respectively. The GH connecting the main channel AB to the culture cell and the IJ connecting the culture cell to the side channels EF and CD are isosceles triangular configurations with a height of 1 mm and a depth of 10 μm. Cell culture chips use eight cell culture chambers with diameters of 0.8 mm, 1 mm, 1.2 mm, 1.4 mm, 1.6 mm, 1.8 mm, 2 mm, and 2.2 mm and a depth of 30 μm. The six outlets and inlets of the chip are made of PDMS hole punches. The chip has 3 inlet reservoirs (0.7 mm diameter, 0.7 mm height) and 3 outlet reservoirs (0.7 mm diameter, 0.6 mm height): reservoir A is the perfusion port for the medium, reservoirs E and C are perfusion ports for both cells, and reservoirs F, B, and D are waste collection outlets (Fig. 1).

LO2 cells are perfused first, followed by HepG2 cells. Remove the cleaned PDMS glass chip and place it in a clean bench. Draw a volume of medium into a syringe, wash the chip for half an hour at a flow rate of 10 μL/min and seal reservoirs A, E and F. 6×10^5 cells/mL density of LO2 cell suspension is perfused from port C at a flow rate of 3 μL min^{-1} for 3 min. After the end of cell perfusion, it can be seen that the cells in the 8 cell culture cells on the right side of the chip are evenly distributed. Then aspirate the excess cells in the B and D tanks, place the chip in the incubator for 12 h, and wait for the cells to fully adhere to the wall. Add 100 μL of medium to the three tanks of C, B and D, and perfuse HepG2 cells symmetrically as before.

The culture medium was exchanged for each 24 h. Fresh media was perfused into the chip by syringe pump in reservoir A at a flow rate of 6 μL/min for 10min. After the end of perfusion, a certain volume of medium was added into reservoirs A, B, C, D, E and F with a pipet-gun to make the liquid level of reservoirs A, C and E higher than that of reservoirs B, D and F, and then the chip was transferred in an incubator for further cultivation. The culture medium in the chip is continuously updated by gravity drive.

2.4. MTT test of sanguinarine on HepG2 and LO2 cells

In this experiment, HepG2 and LO2 cells in logarithmic phase were taken and counted to a density of 1×10^4 cells/mL with a blood cell counting board. The different cells were seeded in different 96-well plates with a pipette gun. After incubating the cells for 12 h, sanguinarine (Aladdin Industrial Corporation, Shanghai, China) was added to 96-well plates at a concentration of 0, 2, 3, 4, 5, and 6 μmol/min. To determine cell activities, 10 μL of MTT solution (5 mg/mL) was added to each well after LO2 and HepG2 cells were cultured in an incubator for 24

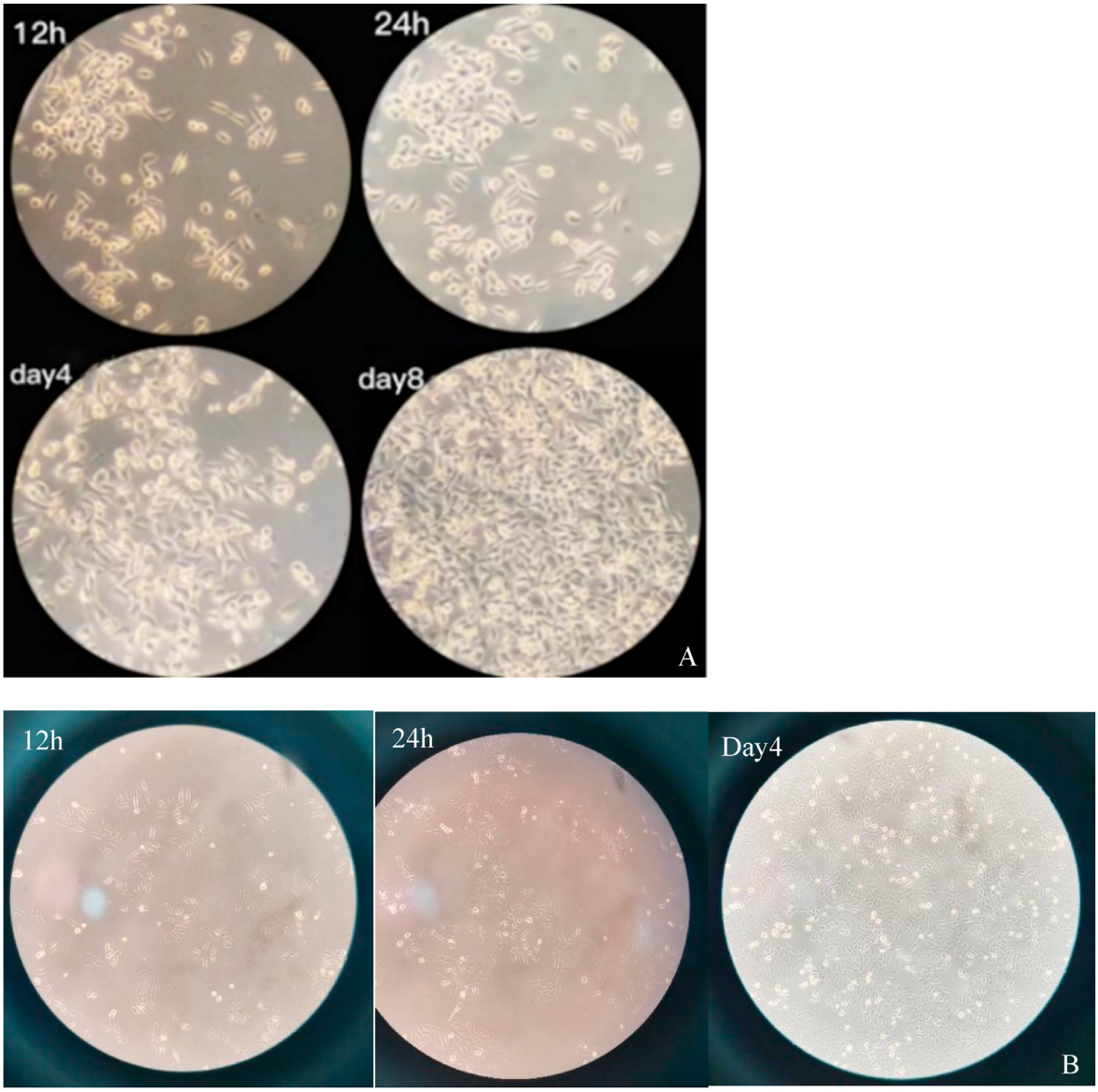

Fig. 4. A) HepG2 cells cultured on cell culture chips can be cultured continuously for 8 days in a cell culture chamber.
B) HepG2 cells cultured in flasks begin to enter the aging phase on the fourth day.

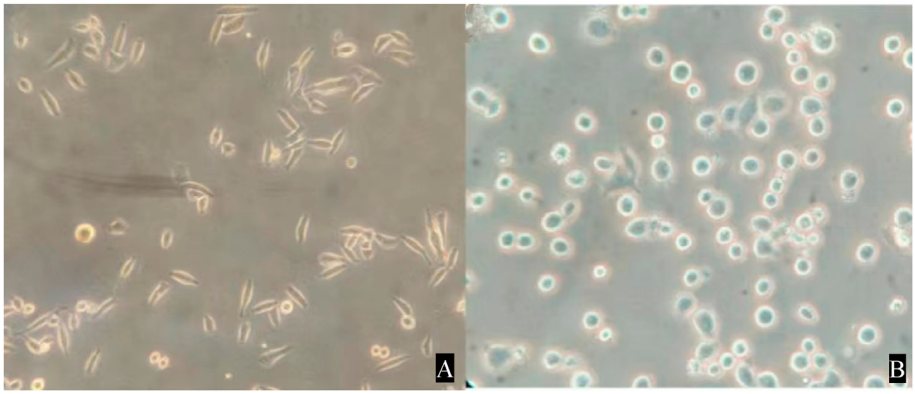

Fig. 5. Cell growth states perfused by different sequences. A) LO2 is perfused before HepG2; B) HepG2 is perfused before LO2.

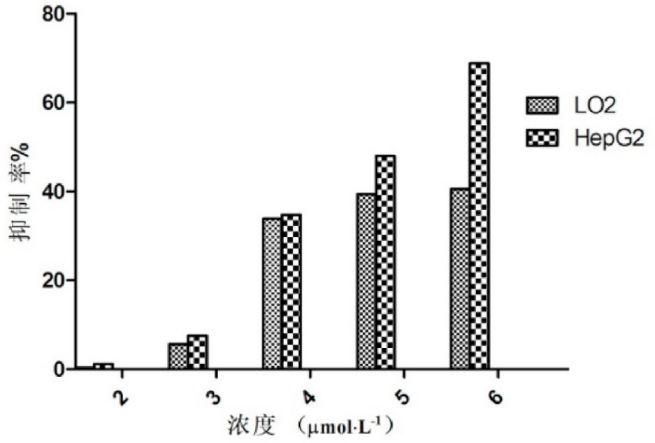

Fig. 6. Comparison of growth inhibition rates of HepG2 and LO2 cells with different concentrations of sanguinarine.

h at 37 °C. Four hours later, it was detected with a microplate reader at a wavelength of 490 nm. All assays were performed in triplicate.

2.5. DAPI staining of HepG2 and LO2 cells

Cells were fixed by immersing in 75% ethanol for 10 min at 6 μL/min for 10 min which eliminated the medium in the cell culture chamber and played the role of fixing cells. After the termination of perfusion, the chip was left for 5 min and then incubated in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at the same flow rate. Subsequently, 50 μL of DAPI dye (C1006, Biyuntian Biotechnology Co, LTD) was accurately measured with a pipet-gun in the dark environment and injected into the chip with a syringe pump at a flow rate of 6 μL/min for 5 min. Due to the continuous fluid environment, the coculture chip should be placed for 3 min to allow the dye to fully interact with the cells, and then imaged using an inverted fluorescence microscope (BX41-32H02, Onlympus Co., Japan).

3. Results and discussion

3.1. Optimization of the chip

3.1.1. Effect of channel depth on cell perfusion efficiency

Since the depth of the cell pool affects the retention capacity of the cells, we designed different channel etch times. The cells culture in the chamber at different depth were designed at Table 1, and the results of cells culture are shown in Table 2. In the case of 10 min etching of AB channel, 15 min etching of cell perfusion channel CD and EF, 15 min of channel IJ connecting cell perfusion channel and cell culture chamber, and 15 min of channel GH connecting main channel and cell culture cell indicates that the cell culture chamber captures the appropriate numbers.

3.1.2. Effect of cell perfusion flow rate on cell perfusion efficiency

To screen out suitable cell perfusion flow rates, we established 7 different flow rate models. Inoculate from the microarray inlet E and inoculate and perfuse HepG2 cells in the logarithmic stage under 7 flow rate conditions (10, 8, 6, 4, 3, 2, 1 μL/min) for 5 min. Fig. 2 shows an image of the cells and observe the adherent state of the cells after 12 h in the incubator at a flow rate of 3 μL/min (Fig. 2C). At this time, the adhesion effect of cells is better than that of 8 μL/min and 10 μL/min, and

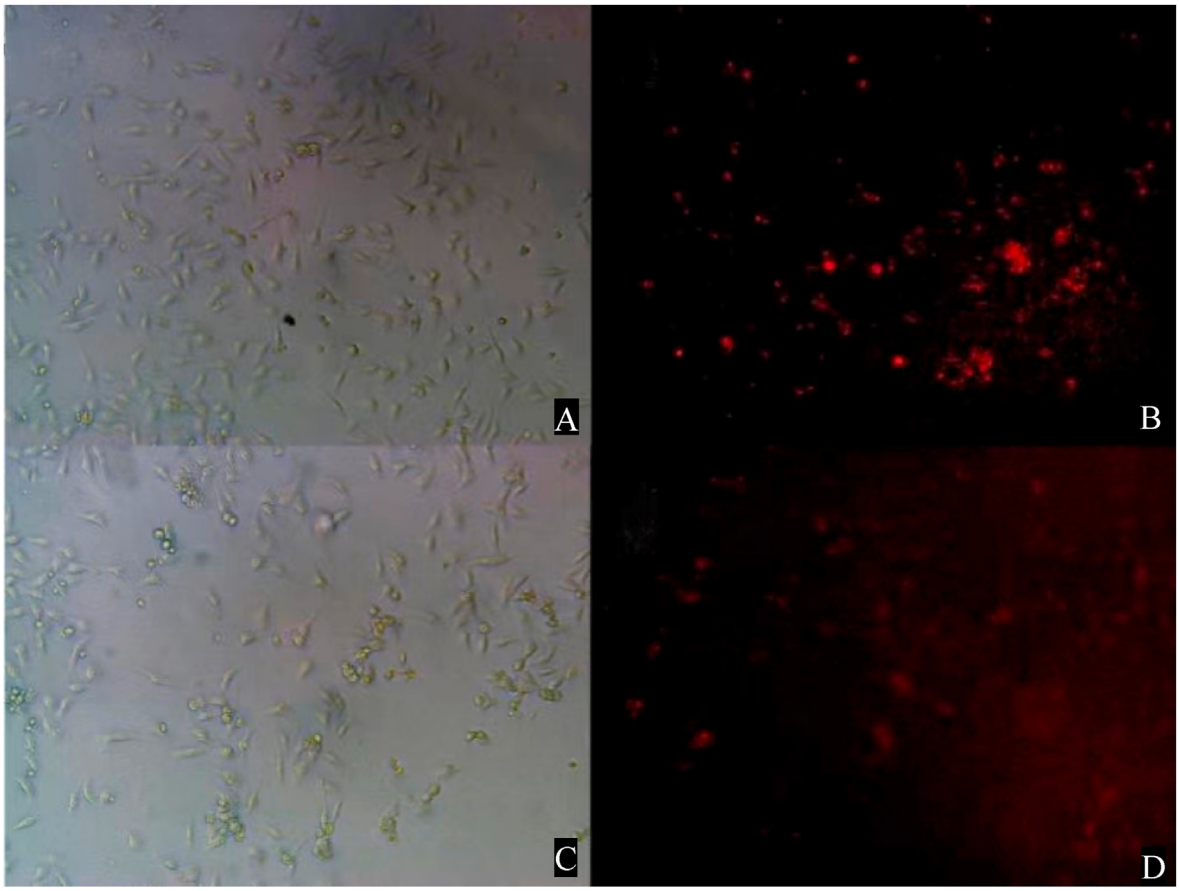

Fig. 7. SAN acts on the fluorescence map of the cell.
A—B. SAN acts on the LO2 cells for 24 h; C—D. SAN acts on the HepG2 cells for 24 h. SAN: sanguinarine.

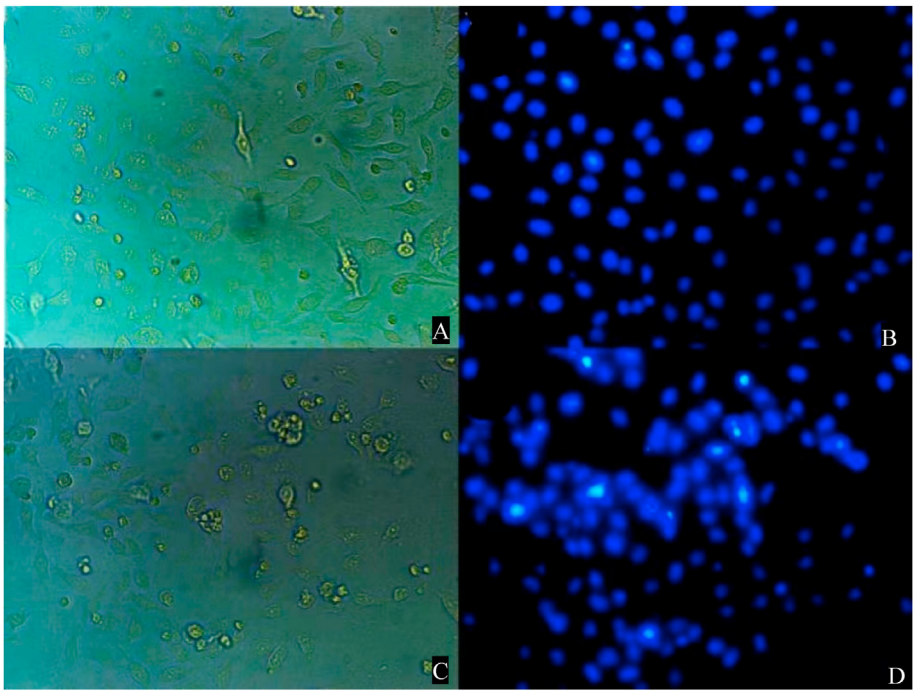

Fig. 8. DAPI staining results. A—B. The growth status of LO2 cells and the fluorescence map of DAPI staining were indicated after the root line was applied to LO2 cells for 24 h; C—D. The growth state of HepG2 cells and DAPI staining fluorescence patterns were shown after the hepG2 cells were applied to HepG2 cells for 24 h, respectively.

the number of cells in the same cell culture chamber is significantly higher than that of high flow rate perfusion conditions.

3.1.3. *Effect of PDMS thickness on cell growth*

PDMS thickness affects cell growth. As shown in Fig. 3, cells can grow successfully on both PDMS thicknesses of 1.5 mm and 2 mm, and the growth rate is better at 2 mm than at 1.5 mm, the number of cells increases, and the number of cell divisions is obvious.

3.2. *Coculture of HepG2 and LO2 cells*

In the traditional culture method, both HepG2 and LO2 cells are cultured in cell culture flasks, and the two cells are adhered to the wall for about 6 h and 8 h, respectively. The cells cultured on this chip are introduced into the cell culture chamber of the chip using a syringe pump, and then continuously cultured in the cell culture chamber by a steady and slow liquid flow under the action of gravity difference. It can be observed that the adhesion time of HepG2 is shortened by about 2 h, and the adhesion time of LO2 is shortened by about 1h.

In the chip configuration design of this study, the drug is introduced from port A, and in the process of flowing to port B, it spreads to both sides at the same time and acts on both cells at the same time. Taking HepG2 cell culture as an example, HepG2 cells cultured on a cell culture chip can be cultured continuously for 8 days in a cell culture chamber, as shown in Fig. 4. Due to the limitation of cell culture chamber space, the cell density reaches the maximum limit after 8 days of cell culture in the chip, and continued culture is not conducive to cell growth.

3.3. *LO2 and HepG2 are co-cultured*

Perfusion sequencing of HepG2 and LO2 cells was studied. Load LO2 cells first followed by HepG2 cells, or HepG2 cells first and then LO2 cells. When HepG2 cells were loaded first and LO2 cells were loaded onto the chip, poor adherent growth of LO2 cells after 12 h in the chip was observed, with low perfusion rate and adhesion rate, as shown in Fig. 5B. When LO2 cells were loaded first and HepG2 cells were loaded onto the chip, both cells showed normal growth, as shown in Fig. 5A. LO2 cells are more difficult to culture than HepG2 cells compared to traditional culture methods. Therefore, LO2 cells are first injected into the chip and re-infused with HepG2 cells after the LO2 cell growth is stable, which is conducive to the co-culture of the two types of cells in the chip.

3.4. *The MTT assay detects the cytotoxicity of sanguinarine*

Sanguinarine (SAN), a naturally occurring benzophenanthridine alkaloid isolated from Macleaya cordata and Chelidonium majus, elicits an ample array of pharmacological activities such as anti-bacterial, antifungal, and antiinflammatory properties, and is a potential anti-cancer reagent.[28] The growth inhibition effect of SAN on HepG2 and LO2 cells (cell concentration 2×10^5/mL) is shown in Fig. 6. MTT assay of SAN on HepG2 cells in a 96-well plate showed that the IC_{50} values of SAN on HepG2 cells was 5.0239 μmol/L. MTT results showed that the inhibition rate of SAN on human hepatocellular carcinoma cell line HepG2 was higher than that of human normal liver cell line LO2 at the same concentration. In a certain range, the inhibition rate of cell growth also increased gradually with the increase of SAN concentration. Moreover, the growth inhibition of LO2 cells by SAN was less intense than that of HepG2 cells within a certain concentration range. Therefore, SAN was selected as the drug to study the inhibition of cell growth.

3.5. *Assess the apoptosis of HepG2 and LO2 cells by SAN on a chip*

As shown in Fig. 7 and A and B respectively represented the cell image of LO2 cells after SAN treatment and the fluorescence image of SAN in the cell culture chamber. Fig. 7C and D respectively showed the cell image of HepG2 cells after SAN treatment and the fluorescence image of SAN in the cell culture chamber. It was observed that under this perfusion condition, the culture medium containing SAN could completely enter the cell culture chamber and interact with cells. Moreover, the growth inhibition effect of SAN on HepG2 cells was stronger than that of LO2 cells.

Fig. 8A—B and Fig. 8C—D were the original and fluorescence images of LO2 cells and HepG2 cells in the cell culture chamber under DPAI

staining, respectively. Fig. 8B and D respectively showed the apoptosis of LO2 cells and HepG2 cells after SAN treatment, the nuclei of apoptotic cells were shrunkenized and presented a highlighted blue fluorescence. It was observed that the number of apoptosis of HepG2 cells after drug treatment was higher than that of LO2 cells after drug treatment.

4. Conclusion

In summary, this study proposed a method for coculture of HepG2 and LO2 on a microfluidic platform. The chip allows flexible and convenient for cell analysis operations, such as cell staining, in situ observation, and drug screening. In this study, the main channel (length, 30 mm; depth, 30 μm), the cell culture chamber (AB depth, 10 μm), the channel (CD、EF depth, 15 μm) on both sides, and the connection channel (GH depth, 10 μm and IH depth, 15 μm) between the two sides of the channel and the cell culture chamber were selected as the chip size for cell culture and drug preliminary screening. PDMS with a thickness of 1.5 mm was selected as the cover slip of chip, and 3 μL/min and 6 μL/min were selected as cell perfusion flow rate and culture medium perfusion flow rate, respectively. By perfusing the two cells in different sequences, HepG2 and Lo2 were cultured simultaneously on both sides of the main channel in the same chip. After the two cells co-cultured on the chip were simultaneously treated by sanguinarine and stained with DAPI stain, the results observed by fluorescence microscopy were consistent with the results of MTT experiments on 96-well plates, and the inhibitory effect of sanguinarine on HepG2 cells was greater than that of Lo2 cells.

In addition, our microfluidics-based approach requires only a small number of cells for the assay. This method has the characteristics of simple system setup, low cost and flexible operation, and can be applied to cell co-culture, preliminary drug screening, and preclinical drug toxicity test. In the future, in the face of clinical personalized treatment, it can be used to analyze rare and precious cell samples to provide better medication guidance. In short, our method combines modern Chinese medicine with advanced scientific and technological means, and uses cell co-culture to simulate the process of Chinese medicine *in vivo* and the correlation between pharmacodynamic activity, and can be used to use the microarray method for pharmacological evaluation of Chinese medicine in the future.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yuan LI reports financial support was provided by National Natural Science Foundation of China.

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