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论文题目：利用微流控技术构建细胞共培养平台探究紫草宁对伤口愈合过程的影响

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Using Microfluidic Technology to Construct a Cell Co-culture Platform to Investigate the Effect of Shikonin on the Wound Healing Process

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Abstract

Human skin is composed of multiple types of cell and plays important roles in protecting the body and sensing external stimuli. The quality of wound healing determines the risk of skin infection and scar formation and affects health. At present, the main models used for skin-related drug testing suffer from challenges such as high preparation barriers or relatively low physiological fidelity. In this study, with the help of microfluidic-hydrogel microsphere preparation technology, skin cells were spatially arranged in hydrogel microspheres, and a skin-on-a-microsphere (SOM) model was designed and constructed. The SOM model consumes few cells, is convenient for large-scale preparation, and can be used for high-throughput experiments. It has a clear and controllable structure, good permeability, high cell viability, and close and adjustable cell interactions. These advantages make the SOM model a high-throughput research tool for skin metabolism research and drug response evaluation. Shikonin is an important active component in a traditional Chinese medicine. This study applies the SOM model to investigate the mechanism by which Shikonin affects skin wound healing.

Author: Zhang Mengyin

Keywords: hydrogel microsphere, microfluidics, skin model, Shikonin

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1 Introduction

Human skin is composed of multiple types of cells and plays important roles in protecting the body and sensing external stimuli. The process of wound healing greatly affects health. Human skin contains endothelial cells, fibroblasts, and other cells[1]. When skin wounds occur, cell-cell interactions and cell migration play important roles in resisting oxidative damage, angiogenesis, and wound healing [2,3].

Existing in vitro skin models are diverse, and different models have their own advantages, but difficulties remain in their use. Animal models can reproduce human skin to some extent and have intact organisms, but species differences limit the assessment of drug toxicity and efficacy. Ethical considerations are also driving the development of alternative technologies to reduce animal experimentation. Two-dimensional (2D) cell culture models usually use a monolayer of a single cell type as the experimental subject for drug testing and are convenient to prepare, but such models lack interactions among different cell types. Three-dimensional (3D) cell culture models, such as microphysiological systems (MPSs), have well-developed structures, but their preparation requires high expertise, and their inter-laboratory reproducibility is limited[1,4,5,6]. Therefore, constructing a skin model that is stable in preparation, convenient to handle, and ensures fidelity is of practical significance.

This study proposes a skin-on-a-microsphere (SOM) model that mimics the structure and function of skin on hydrogel particles. This study uses microfluidic-hydrogel microsphere preparation technology to prepare multicompartmental sodium alginate microspheres, in which Human Umbilical Vein Endothelial Cells (HUVEC) and Human Dermal Fibroblasts (HDF) are embedded for cell co-culture. The SOM model can be prepared on a large scale, has a clear and controllable structure, good permeability, and close cell interactions. These advantages make the SOM model suitable for high-throughput drug experiments.

Lithospermum is a traditional Chinese medicine used in Shengji Yuhong Ointment for the treatment of burns, scalds, chronic ulcers, and other wounds. Its main active component is Shikonin, which is the world's first medicinal plant cell engineering product. It is a purplish-red natural pigment with antibacterial, anti-inflammatory, and antitumor activities. This study uses the SOM model to investigate the specific effects of Shikonin on skin wounds.

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2 Methods

2.1 Preperation of Multicompartmental Microspheres

The device for preparing multicompartmental microspheres in this study includes a microfluidic chip, a needle, and a gas shearer. A six-channel chip was used to prepare multicompartmental microspheres. Six streams of sodium alginate solution were simultaneously injected into the chip. Due to the laminar flow effect, a stable six-stream fluid was formed and pushed into the needle. After the liquid was pushed out of the needle, the gas shearer generated airflow around the needle, so that the resultant force of gas shear force and gravity exceeded the surface tension of the sodium alginate solution, and droplets detached. The droplets fell into $CaCl_2$ solution in a culture dish below, and calcium ions crosslinked with sodium alginate to form a gel. In this study, 1.5% sodium alginate in saline was used to embed cells, 0.1 mol/L $CaCl_2$ solution was used to crosslink sodium alginate droplets, the gas flow rate of the gas shearer was 5.50 μL/min, and the sodium alginate solution flow rate was 100 μL/min.

2.2 Cell Culture and Treatment

This study used Procell CP-H082 primary immortalized HUVEC cells and Procell CP-H103 primary immortalized HDF cells, passaged at 1:2 every 3 days, and used after passage 2 or more. Experiments were performed 3 d after the latest passage, and 1 mL sodium alginate solution was used to resuspend all cells from a single culture flask for microsphere preparation.

In this study, by introducing sodium alginate solutions with or without different cells into the six channels of the microfluidic chip, four microsphere models with different structures were prepared. In one channel, sodium alginate solution containing HUVEC cells was injected, and the other five channels were injected with cell-free sodium alginate solution to prepare HUVEC-only hydrogel microspheres (H). In two adjacent, alternate, and opposite channels, sodium alginate solutions containing HUVEC cells and HDF cells were injected respectively, and the remaining four channels were injected with sodium alginate solution without cells to prepare adjacent hydrogel microspheres (adjacent, A), intermediate hydrogel microspheres (intermediate, I), and opposite hydrogel microspheres (opposite, O) (Figure 1a).

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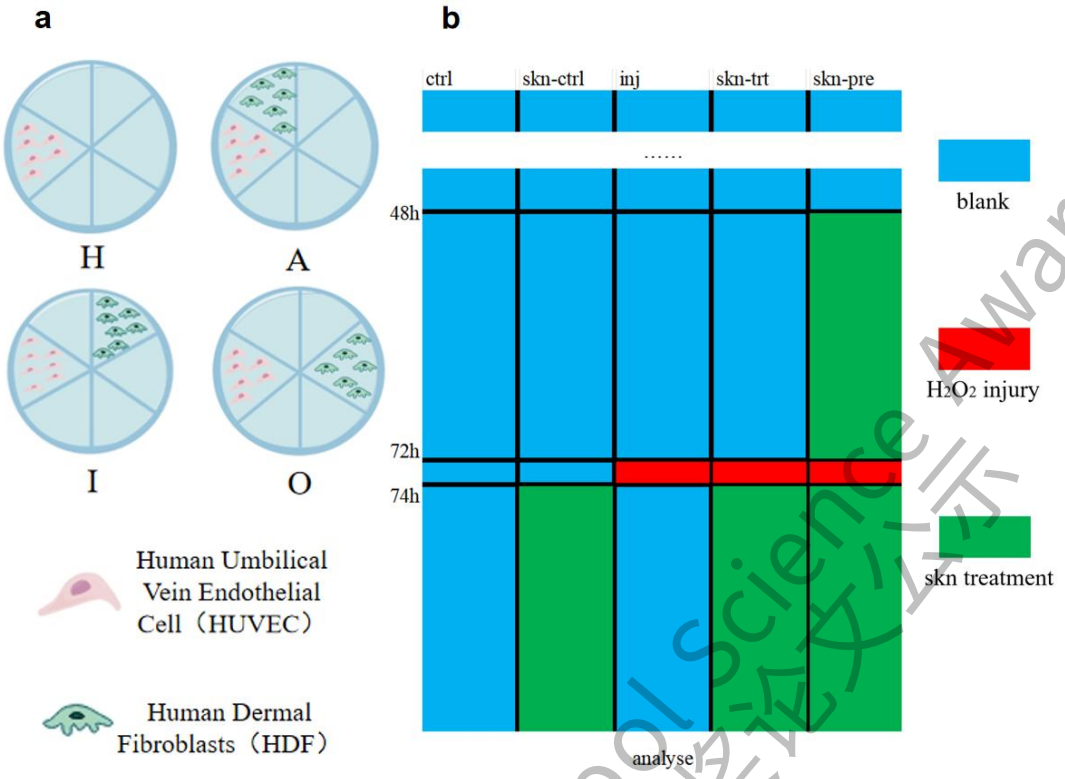


Figure 1 Different treatment methods for cells. (a) Structural diagrams of the four microsphere models. (b) Processing flowchart of the control and four experimental groups.

In the experiment, 24-well plates were used to culture hydrogel microspheres. After preparing hydrogel microspheres, a cell strainer was used to separate the hydrogel microspheres from $CaCl_2$ solution, and they were washed with saline. Then, the microspheres were placed into culture medium. Next, using a pipette with a cut pipette tip, 1 mL culture medium and approximately 15 microspheres were aspirated into each well.

According to different treatments, this study divided the samples into 5 groups: control group (ctrl), Shikonin control group (skn-ctrl), injury group (injury, inj), injury + Shikonin treatment group (Shikonin treatment, skn-trt), and Shikonin pre-treatment + injury + Shikonin treatment group (Shikonin pre-treatment, skn-pre) (Figure 1b). Through preliminary experiments, DMEM medium containing 50 μmol/L H_2O_2 was used to simulate oxidative damage of skin wounds; after 2 h culture, cell viability could be reduced to about 75%, so this concentration was selected for further experiments. To avoid prominent cytotoxicity caused by excessive Shikonin concentration, 0.01 μmol/L Shikonin solution was used to culture the microsphere models as treatment or pre-treatment.

2.3 Viability Assay

This study used the Live/Dead Cell Double Staining Kit to detect cell viability. The microspheres were incubated with the Calcein-AM/PI working staining solution for 20 min, and fluorescence was observed via inverted microscope.Cell viability was quantified using ImageJ. The calculation method of cell viability is: viability (%) = number of live cells/total number of cells.

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2.4 Metabolite Mass Spectrometry Detection and Evaluation Method

This study used a Shimadzu Liquid Chromatography-Ion Trap-Time Of Flight mass spectrometer (LCMS-IT-TOF) for metabolite mass spectrometry detection. In liquid chromatography-mass spectrometry, samples are first separated by liquid chromatography, then enter the mass spectrometry ion source for ionization, and finally are separated and detected by the mass analyzer according to mass-to-charge ratio. It combines strong substance separation and detection capabilities and can be widely used in the measurement and analysis of small organic molecules, drug metabolite identification, etc.

In this study, after all treatments were completed, the culture medium where the hydrogel microspheres were located was extracted for detection. To avoid protein molecule adsorption and blockage of the injection system, methanol solution was added to the culture medium before detection, followed by centrifugation and filtration to remove protein molecules.

After LC-MS detection, extracted ion chromatograms were obtained. Extracted ion chromatograms with obvious single peaks were analyzed, and the peak area of the single peak was measured to reflect the detected amount of the corresponding metabolite (Figure S1). Using the control group of hydrogel microspheres with different structures as the baseline, the log2FC value of each metabolite peak area in the four treatment groups was calculated and made into a heatmap. To judge the degree of metabolic level response of each microsphere model to Shikonin treatment and oxidative damage, for each microsphere model, the log2FC values of all metabolites in each group were squared, summed, and square-rooted to obtain the degree index of the microsphere response to oxidative damage and Shikonin treatment.

2.5 Cell Migration Detection Method

This study used the minimum covering circle diameter of cells to measure cell migration. The photos taken in 2.3 were used for analysis. For each hydrogel microsphere in the image, the smallest circle that could cover all cells in the microsphere was drawn, and the diameter of this circle was measured. Compared with the control group, changes in the minimum covering circle diameter of cells can reflect changes in cell aggregation degree, thereby reflecting cell migration toward the same point.

3 Results

3.1 Construction and Evaluation of the SOM model

The SOM model has a controllable and stable structure. Microspheres were prepared as described above. Sodium alginate solutions containing nanoscale Cobalt Blue and SiC particles were injected into the six channels of the chip alternately (Figure 2a). Under a microscope, the microsphere compartments were clearly divided and highly consistent. To make the effect clearer, sodium alginate solutions containing red and green fluorescent dyes were alternately injected into the chip

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(Figure 2b). Under a fluorescence microscope, the compartments were clearly separated overall, slightly mixing the edges. The microsphere diameter was stable, with a coefficient of variation CV < 3%. The microsphere diameter can be adjusted by adjusting the gas flow rate. Increasing the gas flow rate can effectively reduce the microsphere diameter.

The SOM model consumes few cells. In this study, there were 48 culture systems in preliminary and formal experiments, with approximately 720 microspheres. In this study, only one culture flask each of HUVEC and HDF was used to prepare all microspheres required for the experiment, with the remaining amount exceeding the required quantity.

The SOM model has high cell viability. Hydrogel microspheres with four structures were cultured. After 4 days of culture, the cell viability was over 90%.

The SOM model has good permeability. Twelve small-molecule metabolites, including asparagine, proline, and succinic acid, were effectively detected in the culture medium. After oxidative damage and Shikonin treatment, the metabolic levels of cells changed significantly. This indicates that sodium alginate hydrogel microspheres have good permeability and can be directly administered through the culture medium and used to measure cell metabolic levels.

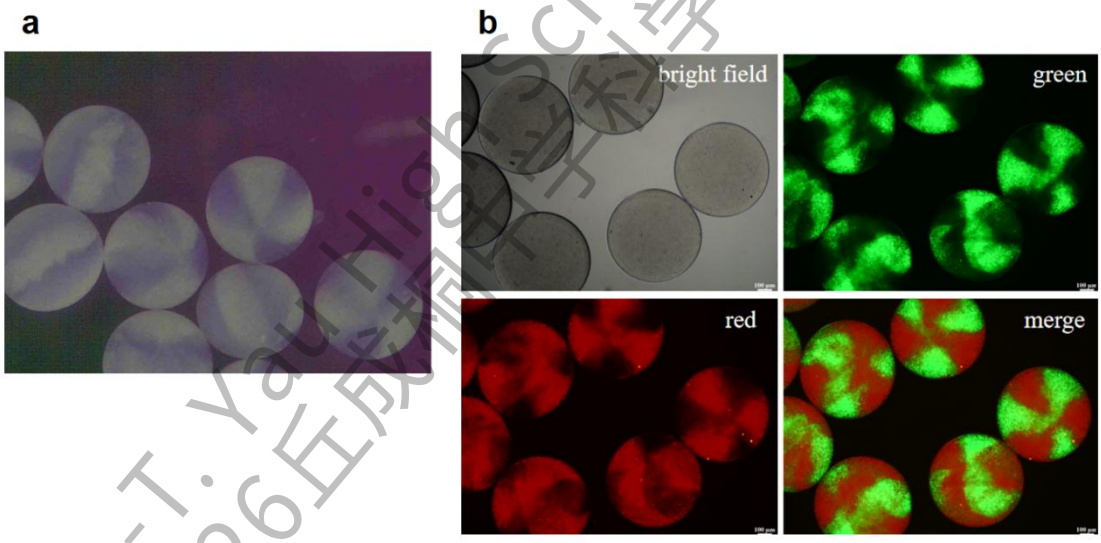


Figure 2 Images of sodium alginate hydrogel microspheres. (a) Multicompartmental hydrogel microspheres stained with nanoscale Cobalt Blue (blue) and SiC (white) particles. (b) Multicompartmental hydrogel microspheres stained with Red polystyrene-based fluorescent particles and green polystyrene-based fluorescent particles.

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3.2 Shikonin Increases the Viability of Oxidatively Damaged Cells

The death of oxidatively damaged cells affects their ability to secrete vascular endothelial growth factor (VEGF), collagen and other factors, thereby further affecting subsequent wound healing processes [2,9,10]. Shikonin treatment affects the viability of oxidatively damaged cells (Figure 3a,b). In A-type and O-type hydrogel microspheres, Shikonin treatment alone reduced cell viability; in I-type hydrogel microspheres, Shikonin treatment had little effect on cell viability. In A-type, I-type, and O-type hydrogel microspheres, oxidative damage reduced cell viability to a certain extent, while after Shikonin treatment, cell viability recovered and approached the control group viability. In O-type hydrogel microspheres, adding Shikonin pre-treatment on the basis of oxidative damage and Shikonin treatment had little effect on cell viability; in A-type and I-type hydrogel microspheres, adding Shikonin pre-treatment reduced cell viability. This indicates that shikonin treatment after oxidative injury can protect damaged cells, and shikonin's improvement of cell viability is not direct.

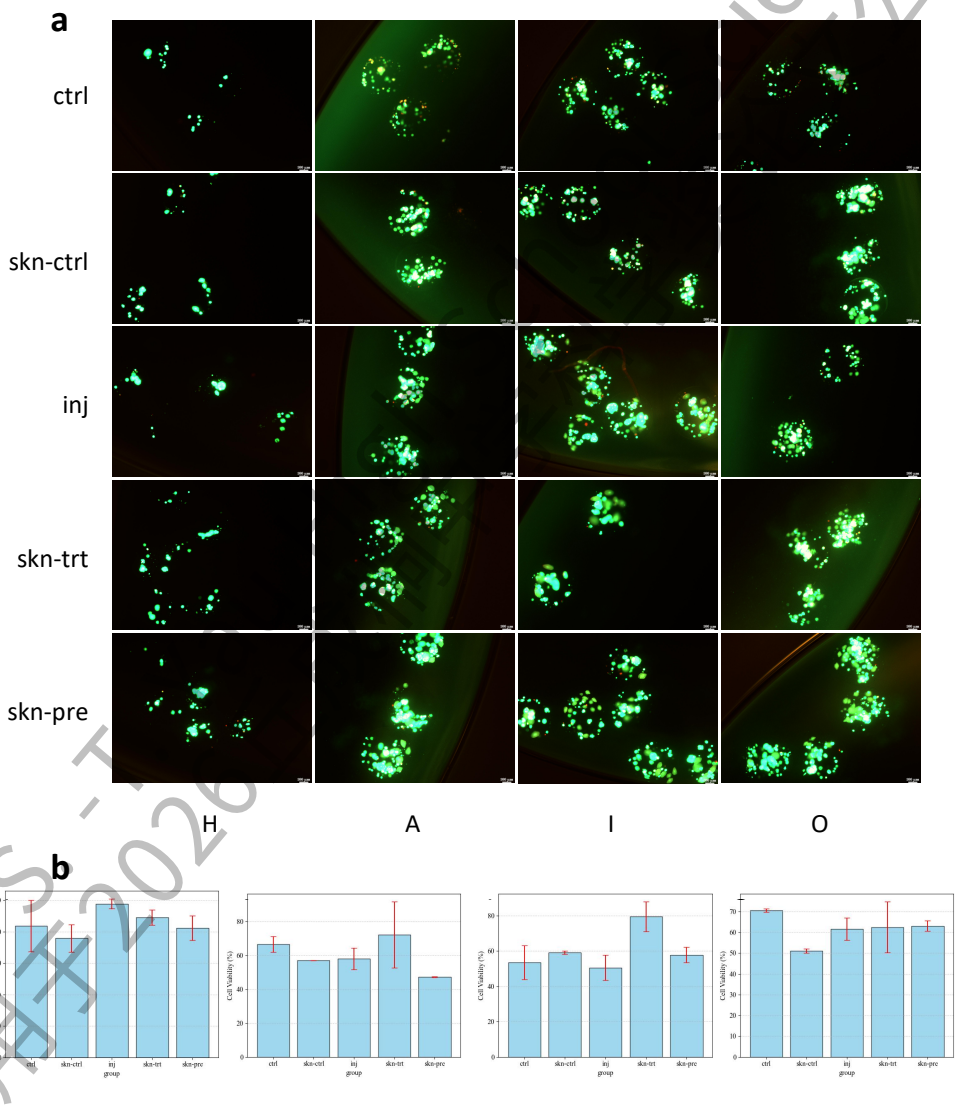


Figure 3 Analysis of cell viability. (a) Calcein-AM/PI staining images of the microsphere models. Red indicates dead cells, green indicates live cells. (b) Relationship between cell viability and treatment method in microsphere models with different structures.

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3.3 Shikonin Affects the Metabolic Profile of Oxidatively Damaged Cells

Cell metabolic levels can reflect the physiological state of cells more finely and accurately [11].

The metabolite measurement results of the H-type microsphere model (Figure 4a) show that Shikonin treatment had little effect on the basal metabolic level of the microsphere model. After oxidative damage, the overall metabolic profile of the microsphere model was downregulated. Shikonin treatment after injury increased the metabolic levels of various substances; the metabolic amounts of substances such as succinic acid recovered, substances such as tryptophan and phenylalanine returned to healthy levels, and substances such as leucine and histidine rose slightly above control levels. In the group with Shikonin pre-treatment, the overall metabolic level of the microsphere model was higher than that of the control group. These results reflect that Shikonin treatment after injury has a protective effect on oxidatively damaged HUVEC cells.

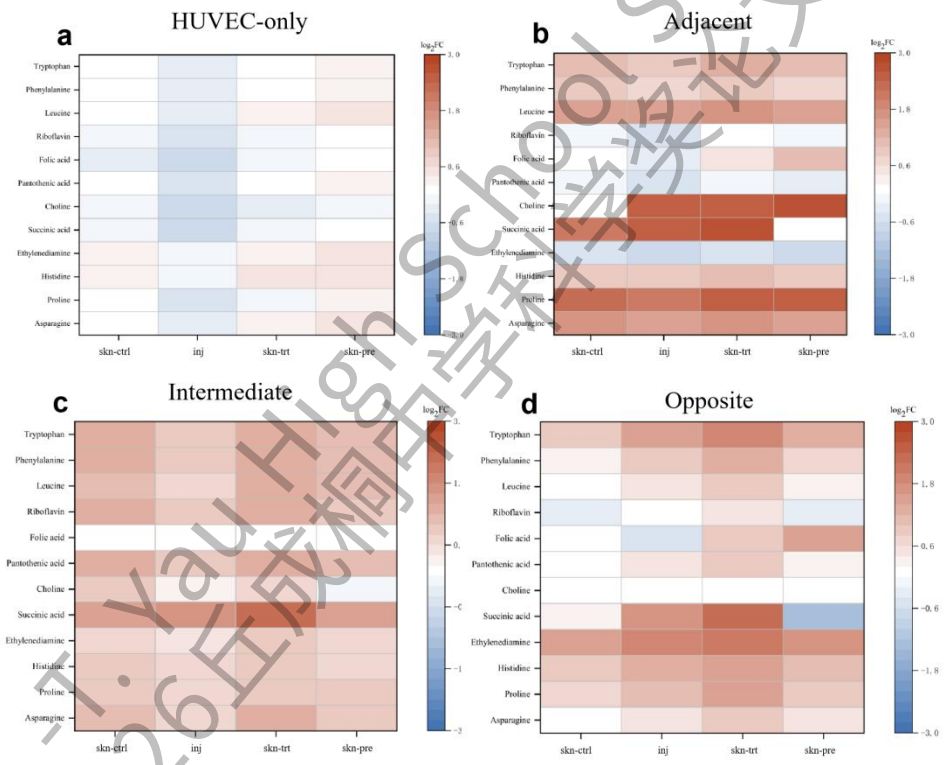


Figure 4. Heatmaps of cell metabolites. Red indicates upregulation, blue indicates downregulation. (a) Metabolite heatmap of HUVEC-only hydrogel microspheres. (b) Metabolite heatmap of A-type microspheres. (c) Metabolite heatmap of I-type microspheres. (d) Metabolite heatmap of O-type microspheres.

In A-type, I-type, and O-type hydrogel microspheres, both Shikonin treatment and oxidative damage led to significantly increased metabolic levels of multiple amino acids. In these three hydrogel microspheres, among the 12 metabolites, except for folic acid and choline, 10 substances reached their maximum values in the skn-trt group. Compared with Shikonin treatment alone, additional Shikonin pre-treatment caused the overall metabolic level to fall back. Folic acid metabolism was inhibited by oxidative damage; Shikonin treatment could increase folic acid content, and Shikonin

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pre-treatment could further increase folic acid content. These results reflect that Shikonin boosts cellular metabolism in the SOM model with oxidative damage. These results also show that Shikonin treatment has more complicated effects than cellular protection in this cell co-culture.

Following the method described in 2.4, the degree index of microsphere response to oxidative damage and Shikonin treatment was calculated. A-type microspheres had the highest index, O-type microspheres the second, and I-type microspheres the lowest. In addition, from the heatmaps, the metabolite changes in A-type microspheres were larger in magnitude, while the changes in various metabolites in O-type and I-type microspheres were more balanced. This suggests that the closer the arrangement of the two cell types in the microsphere, the stronger the metabolite changes when the two cell types are stimulated by drugs. This may be brought about by closer cell-cell interactions.

In the metabolite data, changes in proline were relatively stable and prominent (Figure 5). In H-type microspheres, proline metabolism remained at a low level. In A-type, I-type, and O-type microspheres, Shikonin could increase basal proline metabolism, oxidative damage promoted proline secretion, and Shikonin treatment could further increase proline metabolism in oxidatively damaged cells. Adding Shikonin pre-treatment reduced proline metabolism. Combined with the data in 3.2, this suggests that Shikonin may protect oxidatively damaged HDF cells and promote the secretion of collagen and extracellular matrix (ECM), promoting wound healing [12,13,14].

Among the biological replicates set up in the experiment, the levels of some metabolites differed greatly. It was observed that in all 20 groups of replicates, 15 groups showed relatively stable ratios for several metabolites between their replicates. It is speculated that this may be caused by uneven distribution of the number of microspheres in each well.

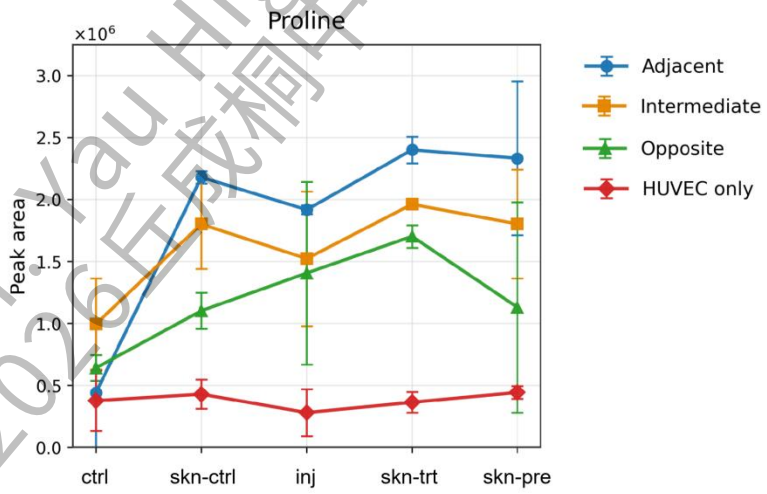


Figure 5 Line graph of proline metabolite levels in different treatment groups. The y-axis represents the peak area of a single peak in the extracted ion chromatogram, indicating the relative abundance of the substance. ctrl is the control group, skn-ctrl is the shikonin control group, inj is the injury group, skn-trt is the injury + shikonin treatment group, and skn-pre is the shikonin pre-treatment + injury + shikonin treatment group.

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3.4 Determination of Cell Migration in Hydrogel Microspheres

At wounds, the migration rate of cells such as vascular endothelial cells directly affects the speed of angiogenesis, thereby affecting the speed and effect of wound healing [2,3]. To investigate the effect of Shikonin on cell migration, this study analyzed the images of live/dead cell staining of hydrogel microspheres. ImageJ was used to draw the smallest-diameter circle in the image that could cover all cells in a single microsphere, and the diameter was recorded (Figure 6a). The diameter of the smallest covering circle can reflect the degree of cell aggregation in the microsphere, and changes in cell aggregation degree can reflect cell migration.

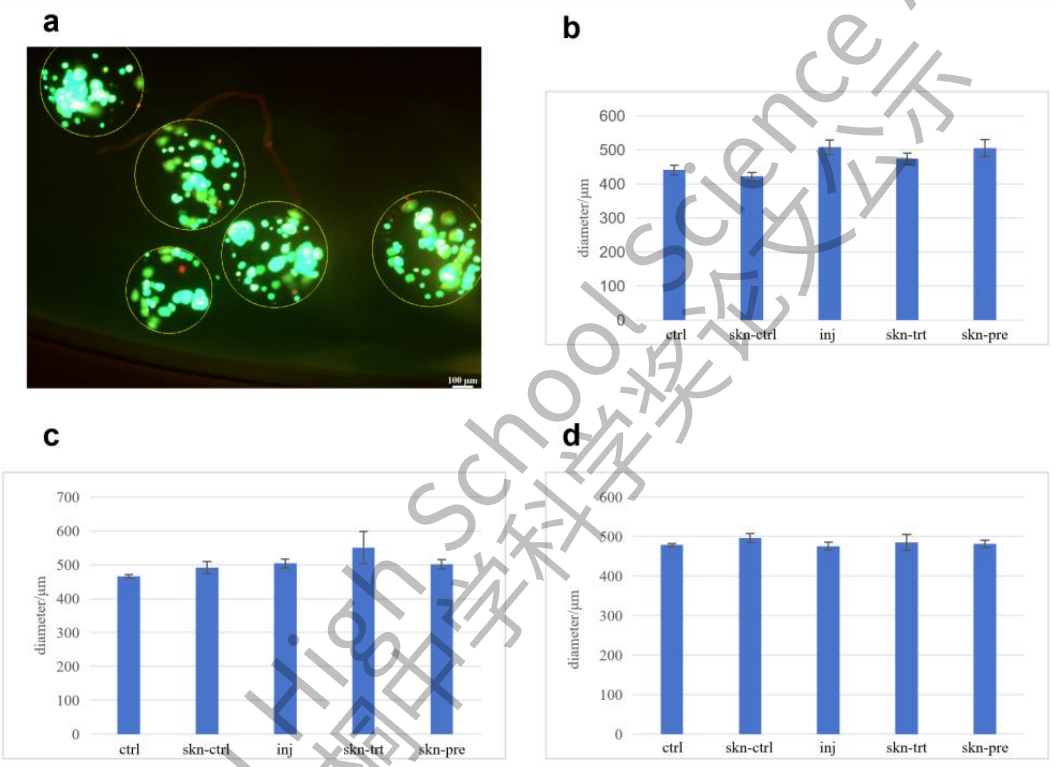


Figure 6 Analysis of cell migration. (a) Method for drawing the minimum covering circle of cells. (b) Minimum covering circle diameter of cells in A-type microspheres. (c) Minimum covering circle diameter of cells in I-type microspheres. (d) Minimum covering circle diameter of cells in O-type microspheres.

The results showed that cells were most densely distributed in the initial state in A-type microspheres and least densely distributed in O-type microspheres. After treatment, the change in the degree of cell aggregation was more pronounced in A-type microspheres and less pronounced in O-type microspheres. This reflects the effect of different spacing between HUVECs and HDFs on their migration (Figure 6 b–d).

In hydrogel microspheres with O, I, and A structures, it was observed that after 98 h of culture, the cells in the microspheres had a smooth, round appearance and a low degree of spreading (Figure 6a), which is presumed to be due to the lack of cell attachment sites in the gel.

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4 Conclusion

The SOM model fabricated via microfluidic technology requires fewer cells, facilitates large-scale preparation, and can be used for high-throughput experiments; the microsphere structure is clear and controllable and has high stability; cell viability is high and can be maintained for more than 96 h; and the hydrogel microspheres have good permeability, allowing drug administration and metabolite detection without destroying the microsphere model. These factors make the microspheres effective for evaluating viability and metabolites in cell co-culture and for drug response assessment.

Compared with models containing only one cell type, co-culture models can reflect more aspects of drug mechanisms. Compared with HUVEC-only microsphere models, the two-cell co-culture models showed more obvious changes in cell metabolism after treatments. The microsphere structure and the spacing between the two cell types are related to the strength of cell interactions. In A-type microspheres, in which the two cell types are closer together, post-treatment metabolite changes and cell migration were more obvious; the opposite was observed in O-type microspheres, in which the cells were farther apart. In healthy human skin, the distance between dermal cells and capillary cells is approximately 200 μm[15]; therefore, the cell spacing in I-type microspheres is more consistent with physiological conditions.

Based on the experimental results, it is speculated that shikonin protects oxidatively damaged cells at the wound site and stimulates their metabolic level, thereby promoting ECM synthesis and VEGF secretion, accelerating endothelial cell migration, promoting angiogenesis, and improving wound healing.

5 Discussion

This study demonstrates that the SOM model has multiple advantages. However, the SOM model also has limitations. Firstly, cells lack attachment sites in hydrogel microspheres, and their migration ability is weak. Secondly, microspheres are difficult to distribute evenly, affecting metabolite-related measurement results. These problems remain to be solved. In follow-up research, I plan to find more effective and more precise methods for microsphere model distribution, so as to obtain more stable and effective experimental data. After the data are stable, I plan to measure VEGF secretion of cells, test the feasibility of using the SOM model to measure macromolecular protein secretion, and provide further support for investigating the mechanism of Shikonin. In addition, studies have shown that adding collagen to hydrogels and other methods can allow cell migration. I plan to use these methods to improve the SOM model, making it usable for research in more fields.

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Appendix

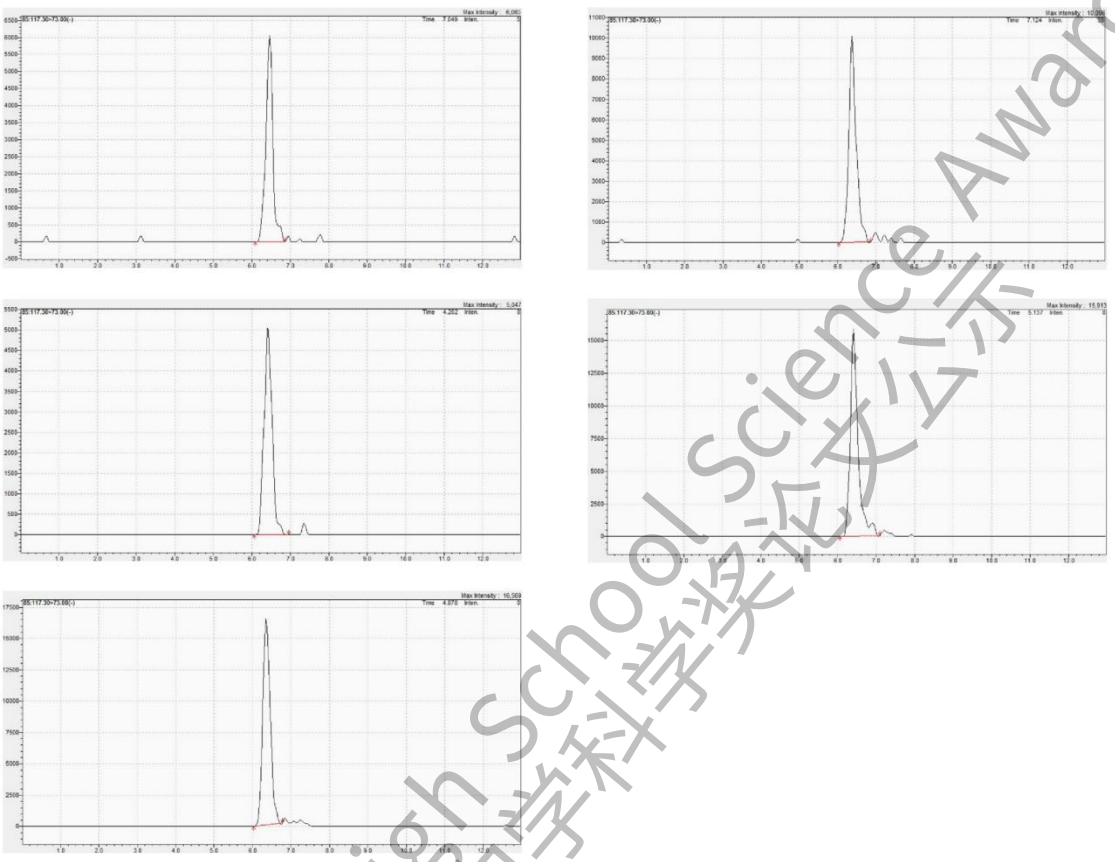


Figure S1 Example of LC-MS extracted ion chromatogram analysis method. The x-axis is retention time, and the y-axis is ion abundance. Substances with identifiable, baseline-separated chromatographic peaks in the extracted ion chromatogram were analyzed. During analysis, the target peak was integrated to obtain the peak area. The peak area can qualitatively reflect the content of the corresponding substance.

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Acknowledgements

This study was conducted in lab D101 in the Department of Chemistry, Tsinghua University, using the laboratory's advanced microfluidic technology and mass spectrometry detection technology.

This study was conducted under the guidance of Professor Jin-Ming Lin and Doctor Shiqi Wang. Professor Jin-Ming Lin is a professor at Tsinghua University, and Doctor Shiqi Wang is the biology teacher at my high school. The two supervisors provided detailed unpaid guidance in topic selection, theoretical knowledge, data analysis, experimental methods, and paper writing. Two doctoral students in Professor Jin-Ming Lin's laboratory, Xiaojian Feng and Junting Lin, also provided guidance in experimental design and data analysis methods. The topic selection, experimental design and implementation, data acquisition and analysis, and paper writing in this study were all carried out by myself under the guidance of the four teachers. In topic selection, Professor Jin-Ming Lin provided guidance on the focus of the topic, and Doctor Shiqi Wang provided guidance on methods for consulting materials, inquiry-based learning, and confirming the specific direction. In experimental design and implementation, Doctor Shiqi Wang taught me the arrangement of preliminary and formal experiments and common experimental operations; Professor Jin-Ming Lin pointed out defects and parts that could be optimized in the experimental design and provided experimental equipment; the two doctoral students offered instructions on experimental arrangement, operation methods, and precautions of the experiment. In data acquisition and analysis, Professor Jin-Ming Lin suggested the content of data collection, and Doctor Shiqi Wang and the two doctoral students provided guidance on data collection methods, processing methods, and presentation methods. In paper writing, Doctor Shiqi Wang taught me the methods of paper writing and put forward revision opinions on the logic and clarity of the paper. I am very grateful to the four teachers for their careful teaching. Under the guidance of the four teachers, I not only learned a lot of new knowledge and new methods, but also enhanced my ability for independent inquiry and hands-on practice. I also sincerely thank Professor Jin-Ming Lin for providing the equipment and reagents needed for the experiment, enabling me to complete the study.

During the inquiry process, I encountered many difficulties. During topic selection, I had difficulty determining the specific direction of the topic. Under the guidance of the teachers, on the basis of in-depth learning of relevant knowledge, I searched for the research status of each direction and accordingly selected skin wound healing as the research direction. In experimental design and implementation, the drug concentration in my initial experiment was inappropriate, causing extremely low cell viability. After guidance from the teachers, I rearranged the experiment according to preliminary and formal experiments, made sufficient preparations before the formal experiment, and improved the formal experiment results. In data acquisition and analysis, to investigate cell migration, the data acquisition method I initially determined required confocal microscopy for photography, and all microsphere models had to be oriented in the same direction. I initially added heavier substances to one side of the hydrogel microspheres to adjust the orientation, but the effect was not obvious. After discussion with the teachers, I placed Fe_3O_4 particles on one side of the microspheres and used a magnet to attract them, achieving orientation adjustment and directional movement of the microspheres. However, this method reduced the clarity and stability of the microsphere structure, weakening the advantages of the microsphere model in these aspects.

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Therefore, I improved the data processing method, used the minimum covering circle diameter of cells to reflect cell migration, and completed the data measurement without adjusting the orientation of the hydrogel microspheres. In addition, when I first collected the raw data, it was difficult to find patterns in the data by directly observing the raw data. After learning various processing and analysis methods from the teachers, with their help I made the data into heatmaps, bar charts, etc., and found patterns in the data. This made me understand the importance of visual analysis and quantitative analysis.

During the inquiry process, I used two AI tools, Doubao (version number not found) and DeepSeek-V4, for language polishing, grammar checking, and translation assistance within a reasonable extent. After writing the full paper independently, I used the two AI tools to check grammatical errors and used the two tools for assistance in translation.

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报告名称：利用微流控技术构建细胞共培养平台探究紫草宁对伤口愈合过程的影响

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摘要

人的皮肤由多种细胞组成，在保护人体、感受外界刺激等方面起到重要作用。伤口愈合质量决定皮肤感染风险与瘢痕形成，影响健康。目前进行皮肤相关药物实验的主要模型具有制备门槛高或还原性略低等难点。本探究借助多腔室海藻酸钠凝胶微球制备技术，将皮肤细胞在水凝胶微球中进行空间排布，设计并构建了人皮肤微球模型。人皮肤微球模型实验所耗细胞数量少，便于大规模制备，可用于高通量实验，结构清晰可控，通透性良好，细胞存活率高，细胞相互作用密切且可调节。这些优点使人皮肤微球模型可为皮肤代谢研究与药物响应评估提供高通量研究手段。紫草宁是一种传统中药中的重要有效成分，本探究应用人皮肤微球模型探究紫草宁对皮肤伤口愈合的影响机理。

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关键词：水凝胶微球、微流控、皮肤模型、紫草宁

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1 引言

人的皮肤由多种细胞组成，在保护人体、感受外界刺激等方面起到重要作用。伤口愈合效果对健康有较大影响。人皮肤处包含内皮细胞、成纤维细胞等多种细胞[1]。在皮肤出现伤口时，细胞间的相互作用以及细胞的迁移对于抵抗氧化损伤、血管新生、伤口愈合起到重要作用[2],[3]。

现有的体外皮肤模型种类多样，不同的模型各有优势，但在使用中也存在难点。动物模型可以在一定程度上还原人体皮肤，并且动物具有完整的机体，但物种差异导致药物毒性及有效性的判断有限。伦理层面也在推动替代技术的开发，以减少动物实验。二维细胞模型通常采用一层单种细胞作为实验对象进行药物实验，模型制备便捷。但这种模型缺乏不同细胞相互作用。三维皮肤微生理系统（MPSs）细胞模型结构完善，但制备门槛高、实验室间重复性有限[1],[4],[5],[6]。因此，构建一种制备稳定、处理便捷且保证保真度的皮肤模型具有实用意义。

本探究提出人皮肤微球模型，在水凝胶颗粒上模仿皮肤的结构与功能。本探究采用多腔室海藻酸钠凝胶微球制备技术，合成多腔室海藻酸钠微球，内部包埋人脐静脉内皮细胞（HUVEC）和人真皮成纤维细胞（HDF），进行细胞共培养。人皮肤微球模型具有可大规模制备、结构清晰可控、通透性良好、细胞相互作用密切等优点。这些优点使人皮肤微球模型适用于高通量药物实验。

紫草是一种传统中药，应用在生肌玉红膏中，用于烧伤、烫伤、慢性溃疡等创面的治疗。其主要有效成分为紫草素[7]。紫草宁是紫草素的一种手性异构体，是世界上首例药用植物细胞工程产品[8]，是一种紫红色天然色素，具有抗菌、消炎和抗肿瘤活性[7]。本探究使用人皮肤微球模型，对紫草宁在皮肤伤口的具体作用进行探究。

2 研究方法

2.1 多腔室凝胶微球的制备

本探究中制备多腔室微球的装置包含微流控芯片、针头、气体剪切器。实验中采用六通道芯片制备多腔室微球。将六股海藻酸钠溶液同时注入芯片，依靠层流效应，可以形成稳定的六股流体，并导入针头。液体由针头推出后，气体剪切器从针头四周均匀产生气流，使气体剪切力与重力合力超过海藻酸钠溶液表面张力，液滴发生脱离。液滴落入下方培养皿内的$CaCl_2$溶液中，钙离子与海藻酸钠交联，形成凝胶。本探究中使用 1.5%海藻酸钠的生理盐水溶液包埋细胞，使用 0.1 mol/L $CaCl_2$ 溶液使海藻酸钠液滴发生交联，气体剪切器气体流速 5.50 μL/min，海藻酸钠溶液流速 100 μL/min。

2.2 细胞培养及处理

本探究采用普诺赛 CP-H082 原代永生化 HUVEC 细胞，以及普诺赛 CP-H103 原代永生化 HDF 细胞，每 3 日进行一次 1:2 传代操作，传至 2 代以上后使用。实验在距离上一次传代 3d 时进行，使用 1 mL 海藻酸钠溶液重悬单个培养瓶中的全部细胞，进行微球制备。

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本探究中通过在微流控芯片的六个通道中通入含有不同细胞的海藻酸钠溶液或无细胞海藻酸钠溶液，制备了 4 种不同结构的微球模型。在一个通道注入含 HUVEC 细胞的海藻酸钠溶液，另外五个通道注入无细胞海藻酸钠溶液，制成仅 HUVEC 凝胶微球（H）。在相邻、相间、相对的两个通道中分别注入含有 HUVEC 细胞和 HDF 细胞的海藻酸钠溶液，剩余四个通道注入无细胞海藻酸钠溶液，制成邻位凝胶微球（adjacent,A），间位凝胶微球（intermediate,I），对位凝胶微球（opposite,O）（图 1a）。

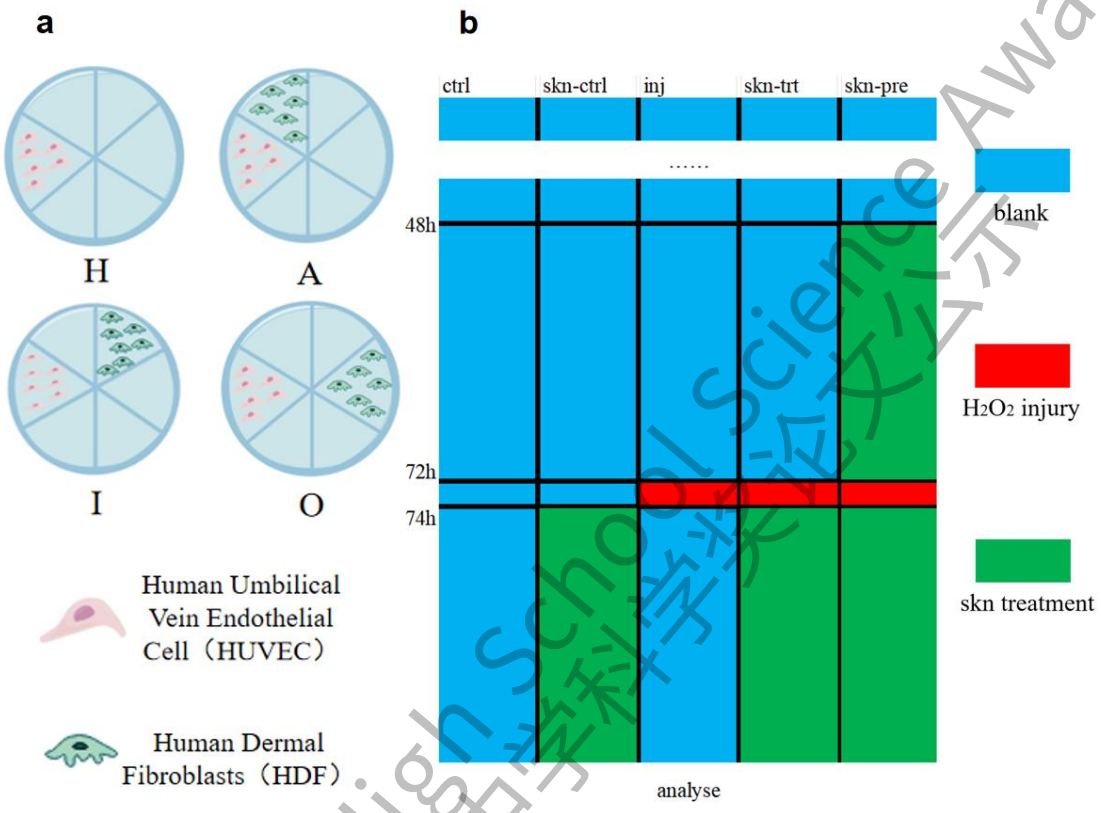


图 1 细胞的不同处理方式。（a）四种微球模型的结构图。（b）对照组和四个实验组的处理流程图。

实验中使用 24 孔板培养凝胶微球。制备凝胶微球后，使用细胞滤网将凝胶微球与 $CaCl_2$ 溶液分离，使用生理盐水洗涤。随后将微球放入培养基中，将移液枪枪头尖端削去，吸取 1 mL 培养基和大约 15 个微球置于每个孔中。

本探究中按照不同处理方式分为 5 组，分别为对照组（ctrl），紫草宁对照组（Shikonin control,skn-ctrl），损伤组（injury,inj），损伤+紫草宁处理组（Shikonin treatment,skn-trt），紫草宁预处理+损伤+紫草宁处理组（Shikonin pre-treatment,skn-pre）（图 1b）。经过预实验，含有 50 μmol/L H_2O_2 的 DMEM 培养基模拟皮肤伤口氧化损伤，在 2h 培养后可使细胞存活率降至 75%左右，选用该浓度进行进一步实验；为避免紫草宁浓度过高导致细胞毒性突出，采用 0.01 μmol/L 紫草宁溶液对微球模型进行培养以作为处理或预处理。

2.3 细胞存活率检测方法

本探究采用 Calcein-AM/PI 染色检测细胞存活率。使用生理盐水将 Calcein-AM 染液和 PI 染液按照 1:1000 进行稀释，抽取凝胶微球所在的培养基后，向孔板中每个孔加入 100 μL 稀释染液，避光孵育 20 min，采用徕卡 DMI4000 B 自动倒置显微镜进行拍照，使用 ImageJ 进行细胞计数，并按照如下方式计算细胞存活率：细胞存活率=活细胞数量/细胞总数。

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2.4 代谢物质谱检测和评估方法

本探究采用岛津液相色谱-离子阱-飞行时间质谱联用仪（LCMS-IT-TOF）进行代谢物质谱检测。液相色谱-质谱联用中，样品先经液相色谱分离后，再进入质谱离子源进行离子化，最后由质量分析器按质荷比分离检测，兼具较强的物质分离能力和检测能力，可以广泛应用于有机小分子的测量分析，药物代谢物鉴定等。

本探究中，在全部处理结束后吸取凝胶微球所在培养基进行检测。为避免蛋白质分子吸附、堵塞进样系统，在检测前向培养基中加入 2 倍体积的甲醇溶液，离心，过滤，以将蛋白质分子聚沉并去除。

进行 LC-MS 检测后，获得提取离子流色谱图。对有明显单峰的提取离子流色谱图进行分析，测量单峰的峰面积，以反映对应代谢物的检出量（图 S1）。以不同结构凝胶微球的对照组为基准，计算四个处理组各代谢物峰面积的 $\log_2$FC 值，制成热图。为了判断每种微球模型代谢水平响应紫草宁处理和氧化损伤的程度，对于每种微球模型，将各组所有代谢物的 $\log_2$FC 值求平方和并求二次根，得到微球响应氧化损伤和紫草宁处理的程度指标。

2.5 细胞迁移检测方法

本探究使用细胞最小覆盖圆直径对细胞迁移进行测定。采用 2.3 拍摄的照片进行分析，对于画面中的每一个凝胶微球，画出能够覆盖这个微球中所有细胞的最小的圆，并测定这个圆的直径。和对照组进行对比，细胞最小覆盖圆直径的变化可以反映细胞聚集程度的变化，从而反映细胞向同一点迁移的情况。

3 结果分析

3.1 人皮肤微球模型的构建与评价

人皮肤微球模型结构可控且稳定。按照前述方式制备微球。将含有纳米级钴蓝、碳化硅颗粒的海藻酸钠溶液相间注入芯片（图 2a），在显微镜下观察到微球腔室划分清晰，一致性高。为使效果更清晰，将含有红色、绿色荧光染料的海藻酸钠溶液相间注入芯片（图 2b），在荧光显微镜下观察到腔室整体分割清晰，有轻微串扰。微球直径稳定，变异系数 CV<3%。微球直径可以通过调整气体流速进行调节。增大气体流速可以有效减小微球直径。

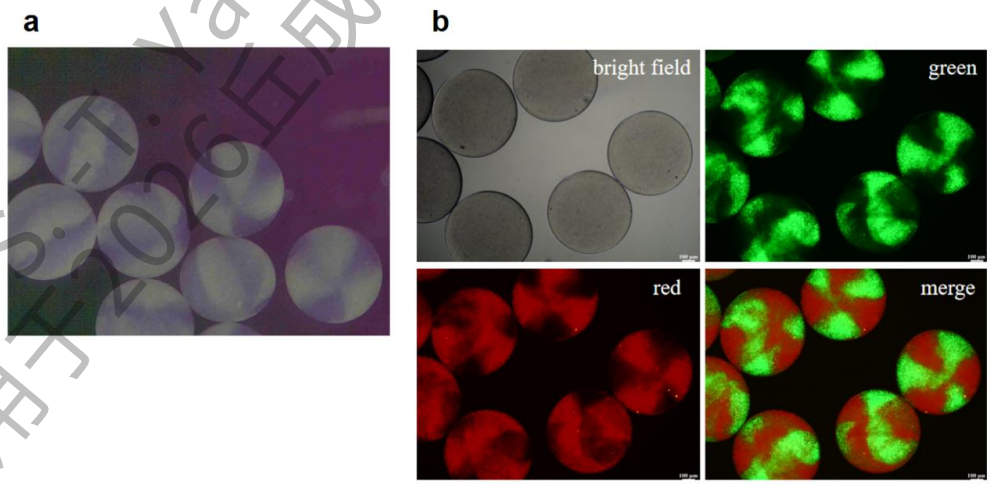


图 2 海藻酸钠凝胶微球效果图。（a）使用纳米级钴蓝（蓝）和碳化硅（白）颗粒染色的多腔室凝胶微球。（b）使用红色聚苯乙烯基荧光颗粒和绿色聚苯乙烯基荧光颗粒染色的多腔室凝胶微球。

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人皮肤微球模型耗费细胞少。本探究中，预实验、正式实验共有 48 个培养体系，大约 720 个微球。本探究中仅使用 HUVEC 和 HDF 各一培养瓶就制备出实验所需的全部微球，且留有超过一倍的冗余。

人皮肤微球模型细胞存活率较高。对 H、A、I、O 四种结构的凝胶微球进行培养。4 天培养后，观察到超过 90%的细胞存活。

人皮肤微球模型通透性良好。在培养基中有效检出了天冬酰胺、脯氨酸、琥珀酸等 12 种小分子代谢物，在进行氧化损伤和紫草宁处理后，细胞的代谢水平均发生明显变化。这说明海藻酸钠凝胶微球通透性良好，可直接通过培养基给药、测量细胞代谢水平。

3.2 紫草宁提高氧化损伤细胞存活率

氧化损伤细胞的死亡情况会影响其分泌血管内皮生长因子（VEGF）等的能力，从而进一步影响后续伤口愈合进程[2],[9],[10]。紫草宁处理对于氧化损伤细胞的存活率具有影响（图 3a,b）。在 A 型、O 型凝胶微球中，单独紫草宁处理会降低细胞存活率；在 I 型凝胶微球中，紫草宁处理对细胞存活率影响较小。在 A 型、I 型、O 型结构凝胶微球中，氧化损伤均在一定程度上降低了细胞存活率，而在进行紫草宁处理后，细胞存活率回升，接近对照组存活率。在 O 型凝胶微球中，在氧化损伤和紫草宁处理的基础上增加紫草宁预处理对于细胞存活率影响小；在 A 型和 I 型凝胶微球中，增加紫草宁预处理降低了细胞存活率。这说明在氧化损伤后进行紫草宁处理可以保护损伤细胞，且紫草宁对细胞存活率的提升不是直接的。

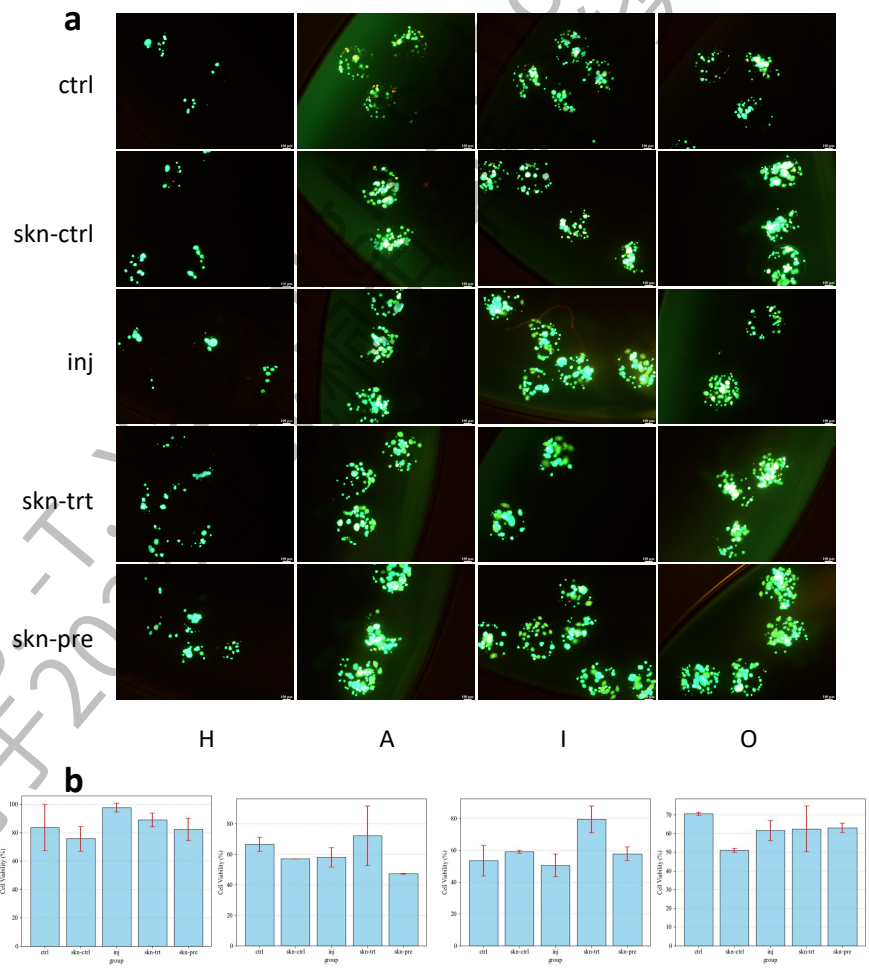


图 3 对于细胞存活率的分析。（a）微球模型 Calcein-AM/PI 染色效果图。红色表示死细胞，绿色表示活细胞。（b）不同结构的微球模型中细胞存活率与处理方法的关系图。

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3.3 紫草宁影响氧化损伤细胞代谢谱

细胞代谢水平可以更细致、准确地反映细胞的生理状态[11]。

H 型微球模型代谢物测量结果（图 4a）显示，紫草宁处理对于微球模型基础代谢水平影响较小。进行氧化损伤后，微球模型代谢谱整体下调。损伤后紫草宁处理提高了各种物质的代谢水平，琥珀酸等物质的代谢量回升，色氨酸、苯丙氨酸等物质恢复至健康水平，亮氨酸、组氨酸等物质升至高于对照水平。增加紫草宁预处理的组别中，微球模型代谢水平整体高于对照组。这些结果反映了损伤后紫草宁处理对于氧化损伤 HUVEC 细胞具有保护作用。

在 A、I、O 型凝胶微球中，紫草宁处理与氧化损伤均导致多种氨基酸的代谢水平显著提高。在这三种凝胶微球里，12 种代谢物中除叶酸和胆碱外，10 种物质在 skn-trt 组中达到最大值。与只进行紫草宁处理相比，额外进行紫草宁预处理会使整体代谢水平回落。叶酸的代谢量受氧化损伤抑制，紫草宁处理可以提高叶酸的含量，而紫草宁预处理可以进一步增加叶酸含量。这些结果说明紫草宁提高氧化损伤的 SOM 模型中细胞的代谢。这些结果也说明紫草宁在该共培养体系中除了保护细胞以外还有更复杂的作用。

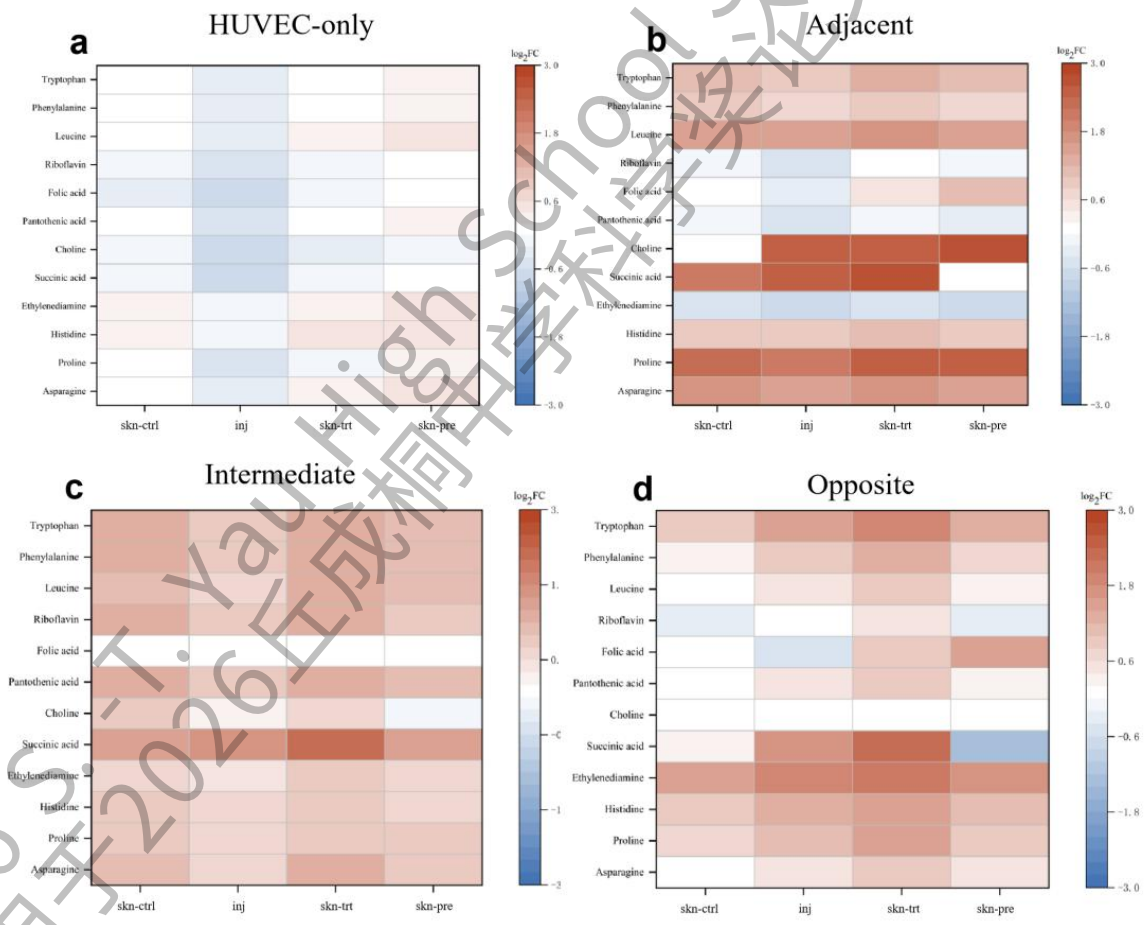


图 4 细胞代谢物热图。红色表示上调，蓝色表示下调。（a）仅 HUVEC 凝胶微球代谢物热图。（b）A 型微球代谢物热图。（c）I 型微球代谢物热图。（d）O 型微球代谢物热图。

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按照 2.4 所述方法求微球响应氧化损伤和紫草宁处理的程度指标，A 型微球指标最高，O 型微球指标其次，I 型微球指标最低。另外，由热图可以看出，A 型微球代谢物变化幅度更大，而 O、I 型微球各种代谢物幅度变化较为均衡。这提示微球中两种细胞排布越近，两种细胞受到药物刺激时的代谢物变化越强烈。这可能是由更紧密的细胞相互作用带来的。

在代谢物数据中，脯氨酸的变化较为稳定、突出（图 5）。在 H 型微球中，脯氨酸代谢量一直维持在较低位置。在 A、I、O 型微球中，紫草宁可提高脯氨酸基础代谢量，氧化损伤促进脯氨酸的分泌，而紫草宁处理可以进一步提高氧化损伤细胞的脯氨酸代谢量。增加紫草宁预处理会降低脯氨酸代谢量。结合 3.2 数据，这提示紫草宁可能通过保护氧化损伤 HDF 细胞，促进其分泌胶原蛋白和细胞外基质（ECM），从而促进伤口愈合[12],[13],[14]。

在实验中设置的生物复孔之间，部分代谢物的水平差异较大。观察到在全部 20 组复孔中，有 15 组孔间若干种代谢物呈现较为稳定的倍数关系，推测这可能是每一孔中微球数量分配不均导致的。

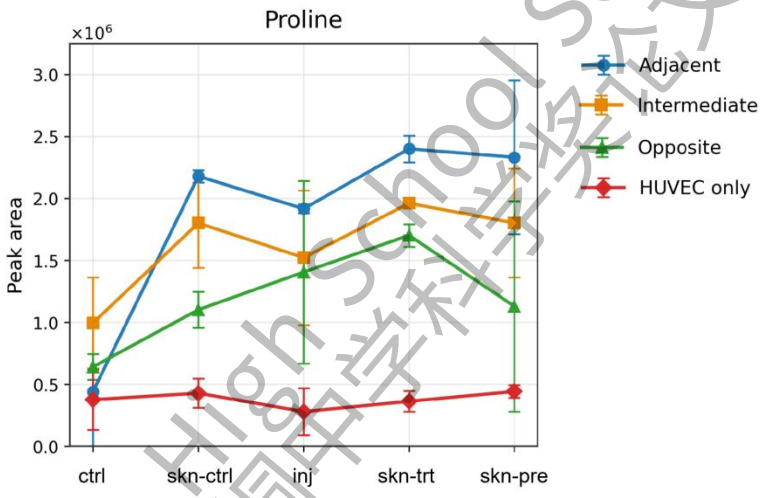


图 5 不同处理组中脯氨酸代谢量折线图。该图纵坐标为提取离子流色谱图中单峰峰面积，表示物质的相对丰度。ctrl 为对照组，skn-ctrl 为紫草宁对照组，inj 为损伤组，skn-trt 为损伤+紫草宁处理组，skn-pre 为紫草宁预处理+损伤+紫草宁处理组

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3.4 凝胶微球中细胞迁移情况的测定

在伤口处，血管内皮细胞等细胞的迁移速率直接影响血管新生速度，从而影响伤口的愈合速度和效果[2],[3]。为了探究紫草宁对细胞迁移的影响，本探究中对凝胶微球细胞活死染色结果图进行分析。使用 ImageJ 在图片中画出直径最小的圆，使这个圆能够覆盖单个微球中全部细胞，并记录直径（图 6a）。直径最小圆的直径可以反映微球中细胞的聚集程度，细胞聚集程度的变化可以反映细胞的迁移情况。

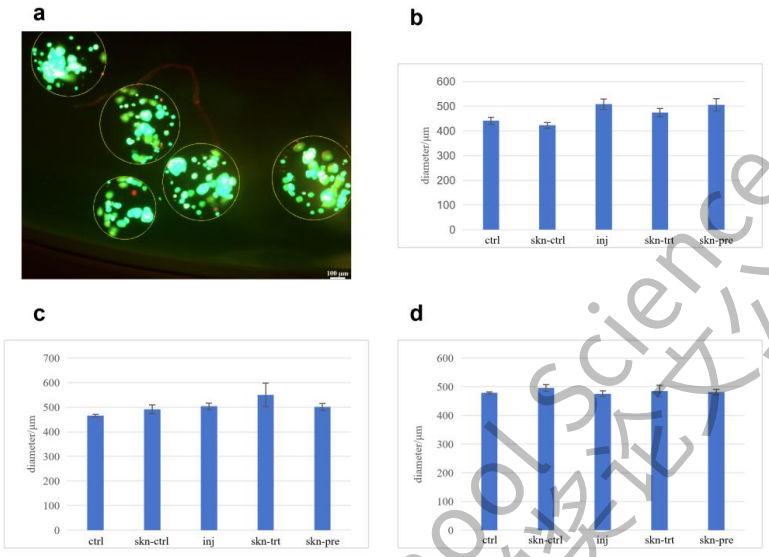


图 6 对于细胞迁移的分析。（a）细胞最小覆盖圆绘制方法。（b）A 型微球细胞最小覆盖圆直径图。（c）I 型微球细胞最小覆盖圆直径图。（d）O 型微球细胞最小覆盖圆直径图。

结果显示，A 型微球中细胞初始状态下最密集，O 型微球中细胞初始状态下最稀疏。经过处理后，A 型微球中细胞聚集程度变化较明显，O 型微球中细胞聚集程度变化较小。这体现了 HUVEC 与 HDF 间距不同对其迁移的影响（图 6 b-d）。

在 O、I、A 三种结构的凝胶微球中，观察到经过 98h 培养，微球中的细胞外观平滑，呈圆球状，铺展程度较低（图 6a），推测为凝胶中缺乏细胞附着位点所致。

4 结论

通过微流控技术制备的皮肤微球模型实验所耗细胞数量少，便于大规模制备，可用于高通量实验；微球结构清晰可控，稳定性高；细胞存活率高，细胞高存活率可维持超过 96h；凝胶微球通透性良好，可在不破坏微球模型的情况下进行给药和代谢物检测。这些因素使微球可有效用于细胞共培养中存活率和代谢物的评估及药物响应评价。

和仅一种细胞的模型相比，共培养模型可以反映药物机理的更多方面。与仅加入 HUVEC 的微球模型相比，两种细胞共培养的模型中各项处理后细胞代谢变化更明显。微球结构、两种细胞的间距与细胞相互作用强度有关。与在两种细胞距离较近的 A 型微球中，进行处理后的代谢物变化与细胞迁移都更加明显；两种细胞距离较远的 O 型微球则相反。在健康人的皮肤处，真皮层细胞与毛细血管细胞间距约为 200μm[15]，因此 I 型微球中细胞的间距更符合实际。

根据实验结果，推测紫草宁保护创口处氧化损伤的细胞并刺激其代谢水平提高，以此促进 ECM 合成以及 VEGF 分泌，加快内皮细胞迁移，促进血管生成，改进创口愈合效果。

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5 讨论

本探究表明了海藻酸钠凝胶微球用于构建人皮肤模型具有多种优势。但皮肤微球模型也具有局限。细胞在海藻酸钠凝胶中缺乏附着位点，迁移能力弱。微球难以分配均匀，影响代谢物相关测定结果。这些问题有待解决。在后续探究中，本人计划寻找更有效、更精准的微球模型分配方法，从而获得更稳定、更有效的实验数据。在数据稳定后，对细胞的 VEGF 分泌量进行测定，检验用人皮肤微球模型测定大分子蛋白质分泌量的可行性，并为紫草宁药物机理的探究提供进一步支持。另外，有研究显示通过在水凝胶中添加胶原蛋白等方式可以允许细胞迁移，本人计划利用这些方法改进人皮肤微球模型，使人皮肤微球模型具有更高的还原度，并可以用于更多方面的研究。

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附录

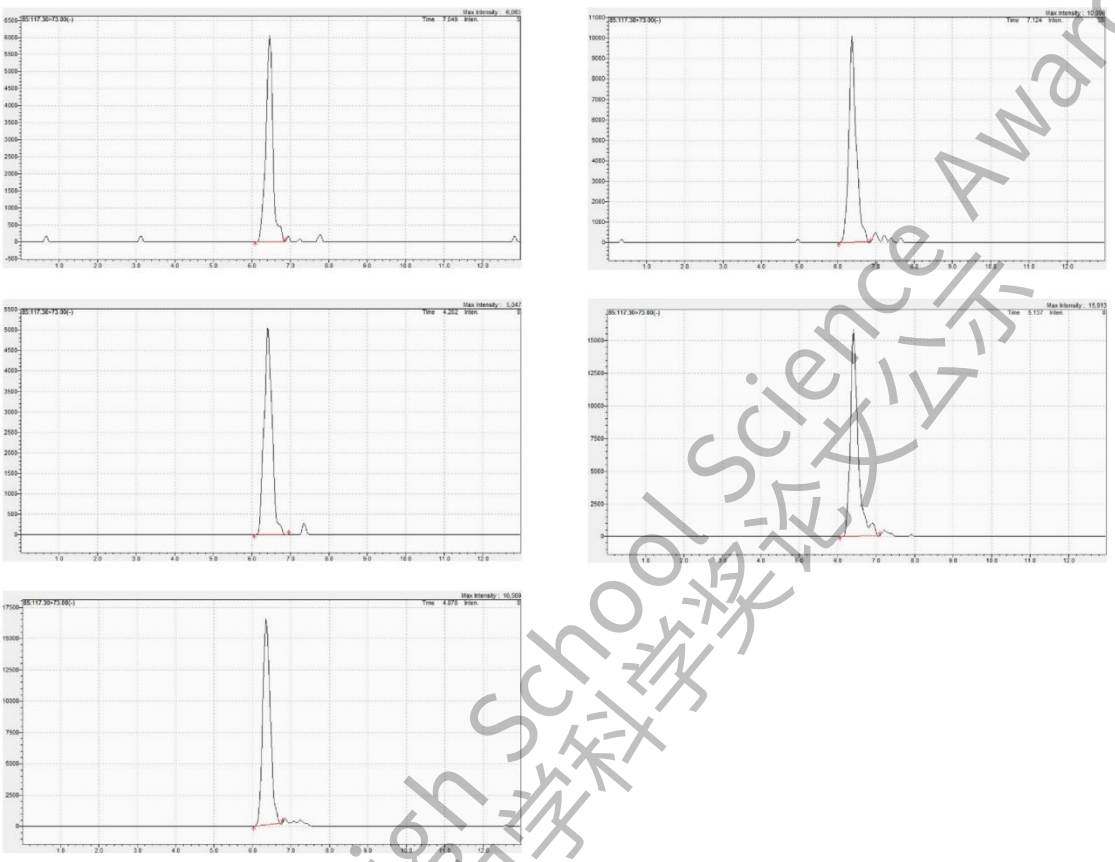


图 S1　LC-MS 提取离子流色谱图分析方法示例。本图横坐标为保留时间，纵坐标为离子丰度。对提取离子流色谱图中具有可识别、基线分离色谱峰的物质进行分析。分析时，目标峰进行积分，得到峰面积。峰面积大小可定性反映相应物质的含量多少。

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致谢

本探究在微量分析测试方法与仪器研制北京市重点实验室，借助实验室先进的微流控技术、质谱检测技术进行。

本探究在林金明教授、王诗琦老师的指导下进行。林金明教授为清华大学教授，王诗琦博士为本人所在高中的生物老师，两位指导教师在选题、理论知识、数据分析、实验方法、论文写作方面给予细致的无偿指导。林金明教授实验室中的两位博士研究生，冯晓健老师和林峻廷老师也在实验设计、数据分析方法等方面给予指导。本探究中的选题、实验设计及实施、数据获取与分析、论文写作都由本人在四位老师的指导下进行。在选题中，林金明教授对于选题的侧重点给予了指导，王诗琦博士对于查阅资料、研究性学习、确认具体方向的方法给予了指导。在实验设计及实施中，王诗琦博士对预实验和正式实验的安排、常用实验操作等方法进行了教学；林金明教授指出了实验设计中出现的缺陷和可优化的部分，并提供了实验设备；两位博士研究生对于实验的具体安排、操作方法、注意事项等给予了教学。在数据获取与分析中，林金明教授对收集数据的内容提出了建议，王诗琦博士和两位博士研究生对数据的采集方法、处理方法、呈现方式给予了指导。在论文写作中，王诗琦博士对论文写作的方法进行教学，并对论文的逻辑性、清晰性等方面提出修改意见。我非常感谢四位老师对我的悉心教导。在四位老师的指导下，我不仅学到了很多新的知识、新的方法，还增强了自主探究、亲手实践的能力。我也非常感谢林金明教授为我提供了实验所需的场所、设备、试剂，使我能够完成探究。

在探究过程中，我遇到了很多困难。在选题过程中，我遇到选题具体方向难以确定的问题。在老师的指导下，我在深入学习相关知识的基础上，了解各个方向的研究现状，据此挑选了皮肤伤口愈合作为探究方向。在实验设计与实施中，我在起初的实验中药物浓度不合适，导致细胞大量死亡，经老师指导，我按照预实验和正式实验重新安排实验，在正式实验前做好充足准备，改善了正式实验效果。在数据获取与分析中，为了探究细胞迁移情况，我起初确定的数据采集方式需要使用共聚焦显微镜进行拍照，且所有微球模型的朝向必须一致。我最初在凝胶微球的一侧加入较重物质来调整方向，但效果不明显。与老师讨论后，将 Fe_3O_4 微粒放在微球一侧，并用磁铁吸引，实现了微球的方向调整与定向移动，但这种方法会降低微球结构的清晰程度和稳定性，削减了微球模型在这些方面的优势。于是，我改进了数据处理方法，使用细胞最小覆盖圆直径反映细胞迁移，在不调整凝胶微球方向的情况下完成了数据的测量。另外，在刚收集到原始数据时，我直接观察原始数据，很难找出数据间的规律。我向老师学习了多种处理分析方法后，在老师帮助下将数据制成热图、柱状图等，找出了数据间的规律，这令我感受到了可视化分析和定量分析的重要性。

在探究过程中，我使用豆包（未查找到版本号）、DeepSeek-V4 两款 AI 工具进行了合理范围内的语言润色、语法检查、翻译辅助工作。我在自主写完论文全文后，使用两款 AI 工具检查语法的错误，并在翻译中使用两款工具进行辅助。

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