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Rapid Evaluation of Anti-inflammatory Effect of Geniopicroside

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Abstract: Skin acts as a barrier and is an important part of body immune system. External physical, biochemical and other stimuli might cause skin inflammation. Drugs and cosmetics have been developed for skin inflammation treatment. At present, the tests of skin inflammation are mainly conducted on animal and 2D cell models. The former might bring problems such as ethics and authenticity, while the latter cannot fully represent the complex micro-environment of the human body. Microfluidic based Organ-on- a- chips technology provides a new method for drug and cosmetic ingredient screening. Skin-on-a-chip (SOC) has been designed for constructing *in vitro* skin models. In this paper, a SOC was developed to culture skin-like models *in vitro*. We tested the differentiation of SOC cultured skin model, and the results showed that its stratum corneum was well differentiated. It indicates that the skin tissue cultured by the SOC bears some similarities to human skin, which can be used for subsequent drug testing. We tested the anti-inflammatory effect of gentiopicroside and compared with dexamethasone. The results showed that 5μg/ml~50μg/ml of gentiopicroside had similar anti-inflammatory effect to 1μM of dexamethasone.

Keywords: Skin, Microfluidic Technology, Anti-inflammatory

1. Introduction

The skin area of an adult is approximately 1.2-2.0 square meters, making it one of the largest organs in the human body [1]. The skin is the first barrier of immune system, protecting other organs. Skin is inevitably in contact with physicochemical substances and biological agents, which may cause various skin reactions, such as skin irritation, allergy, inflammation and even cancer. Therefore, research on drugs and cosmetics for skin inflammation continues to increase. However, a drug needs to do a lot of drug analysis and pharmacological experiments before clinical application. Among them, 2D cell experiments and animal experiments are the most commonly used methods for predicting drug efficacy and toxicity [2-4]. Animal experiments are of limited use in predicting drug efficacy and toxicity because there is still a large phylogenetic distance between animals and humans. Not only that, but the ethical controversies caused by experiments are becoming more and more serious [2-4]. However, 2D cell culture cannot cover the complex physiological microenvironment of the human body [5]. Therefore, it is an urgent task to develop more effective drug analysis platforms and pharmacological detection models. need. The perfusion cultured skin chip combined with microfluidic technology provides a new solution, which can simulate living tissue very closely and provide a platform for drug release testing and biological characterization.

Human skin is mainly composed of three tissue layers - epidermis, dermis and subcutaneous layer. The stratum corneum is the top layer of the epidermis, made up of dead skin cells, and acts as the main barrier. The epidermis is a dense and poorly vascularized area mainly composed of keratinocytes (KCs) with very few pigment-producing dendritic cells (DCs). The dermis consists of a highly vascularized fibrous layer that is low in cell density but rich in collagen and elastin fibers. The epidermis and dermis play major roles in the absorption, distribution, and metabolism of

foreign organisms and generate immune responses against them. Therefore, these two layers are the focus of skin chips to reconstruct better human-related skin tissue models [6]. At present, the research and modeling of skin chip can be roughly divided into three categories: skin tissue directly taken from biopsy (STFB), skin model formed on the chip with the help of supporting structures such as membranes (SMFM), and skin model formed on the chip by planting cells on collagen and combining microfluidic technology (SMFC).

The source of STFB is simple as it does not require perfusion of the system to be considered to create a skin equivalent on a chip. Wagner et al. [7] established a liver-skin co-culture system. They loaded slices of liver and skin into the respective culture chambers of each chip. Under perfused conditions, both tissues maintained metabolically active expression in culture for up to 28 days. They also tested the sensitivity of on-chip co-cultures of liver and skin equivalents to different assay levels of hepatotoxic substances. MaschmeyerI et al. [8] fabricated a multi-organ co-culture chip that can accommodate four human organ equivalents: reconstructed human 3D small intestine, skin biopsy tissue for detection of dermal substance absorption, and 3D liver for major substance metabolism. Equivalents and proximal tubular compartments of the kidney that support metabolite excretion. Their co-culture system was able to remain stable for more than 28 days. This multi-organ system has important implications for the evaluation of pharmacokinetic and pharmacodynamic parameters such as effective concentration, maximum tolerated dose, time course, and intensity of treatment and adverse effects.

The skin model formed by SMFM on the chip is usually simpler in structure and easier to model [9-11]. The model established by Ramadan et al [9] is typical. They use keratinocytes to establish the epidermis and introduce dendritic cells (DC) to establish an immune model. These two kinds of cells are isolated and co-cultured through porous membrane to represent the epidermal and subcutaneous immune system of the skin, respectively. Their study also showed that perfusion-based cell culture showed healthier skin barrier function and prolonged the cell culture cycle. In addition, they evaluated the barrier effect of the stratum corneum formed by co-culture of HaCaT cells, U937 cells and HaCaT/U937 cells with LPS, and measured the resistance across the epithelium. The results showed that keratinocytes formed a strong barrier against the invasion of endotoxin.

SMFC can more simulate the establishment of in vitro model [12-14]. Sriram [15] has developed a new microfluidic device for the internal culture and testing of full-layer human SE. The SE with good differentiation or full thickness was obtained by using this device. At the same time, the barrier function of the skin is enhanced and the permeability is decreased. LeeS [16] designed and fabricated a skin chip with a microfluidic channel structure that can be used for vascular endothelial cell culture and for 3D culture of skin cells (fibroblasts FBS and keratinocytes KCs). They use gravity to shake the platform for pump-free perfusion. Their chip supports the cultivation of the model for up to two weeks.

Depending on the type of skin model on the chip, the function of the skin chip is also different. STFB [7, 8, 17] is generally used to construct multi-organ co-culture chips to establish a circulatory system to study drug metabolism. SMFC is more committed to a more accurate simulation of full-thickness skin. SMFM is often used to simulate inflammation or immune response.

In this paper, a Skin-on-chip device (SOC) is proposed, which can simulate inflammation in order to greatly improve the screening efficiency of drugs and cosmetics. The platform is based on microfluidic chip technology and is made of PDMS, which is non-toxic to cell generation, low production cost and suitable for large-scale production. Human fibroblasts were selected to form dermis in vitro, and human keratinocytes were co-cultured on polyethylene terephthalate (PET) film as epidermis to establish an in vitro skin model. The skin model was treated with LPS to induce inflammation. We used the constructed skin model to test the anti-inflammatory effect of gentiopicroside. Gentiopicroside (GPS) is one of the effective ingredients of gentian, which is used to treat pain and inflammation. A number of studies have shown that GPS has anti-inflammatory, analgesic, antibacterial, and free-radical-scavenging activities [18-22]. The platform could be used in toxicity tests at a lower cost for efficient cosmetic and drug screening.

2. Method and Materials

2.1. Design and Fabrication of SOC Devices

Considering cytotoxicity and convenient fluorescence inverted microscope observation, the microfluidic device is composed of an elastomeric and transparent material, PDMS The device adopts a three-layer design structure. The upper and lower layers are prepared by casting PDMS prepolymer on a mold made of resin material (B9 Core 550 from B9 create company). Design of mold includes runner and culture chamber. PET porous membranes on which cells seeded and grow were placed between two layers to separate the chambers, which is obtained from Whatman company. The membrane acts as a support structure for the cell culture while allowing efficient transport of nutrients, metabolites and test compounds. Each chamber has a diameter of 6.2mm and a height of 1mm as shown in Figure 1a. The flow channel of each layer is designed to be divided into three parts. The liquid enters three independent culture chambers after entering from the inlet, and each chamber has an independent liquid outlet.

2.2. Cell Culture

The experimental device used HaCaT cells and HDF cells as substitutes for epidermal and dermal cells existing in skin tissue. Both HaCaT cell (DSMZ; ACC-771) and HDF cell (lnoherb) were cultured in high-glucose DMEM (I Gibco,

Grand Island, NY) supplement with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 1% penicillin/streptomycin solution (Gibco, Grand Island, NY). The passages of cells involved in the experiment didn't exceed 15.

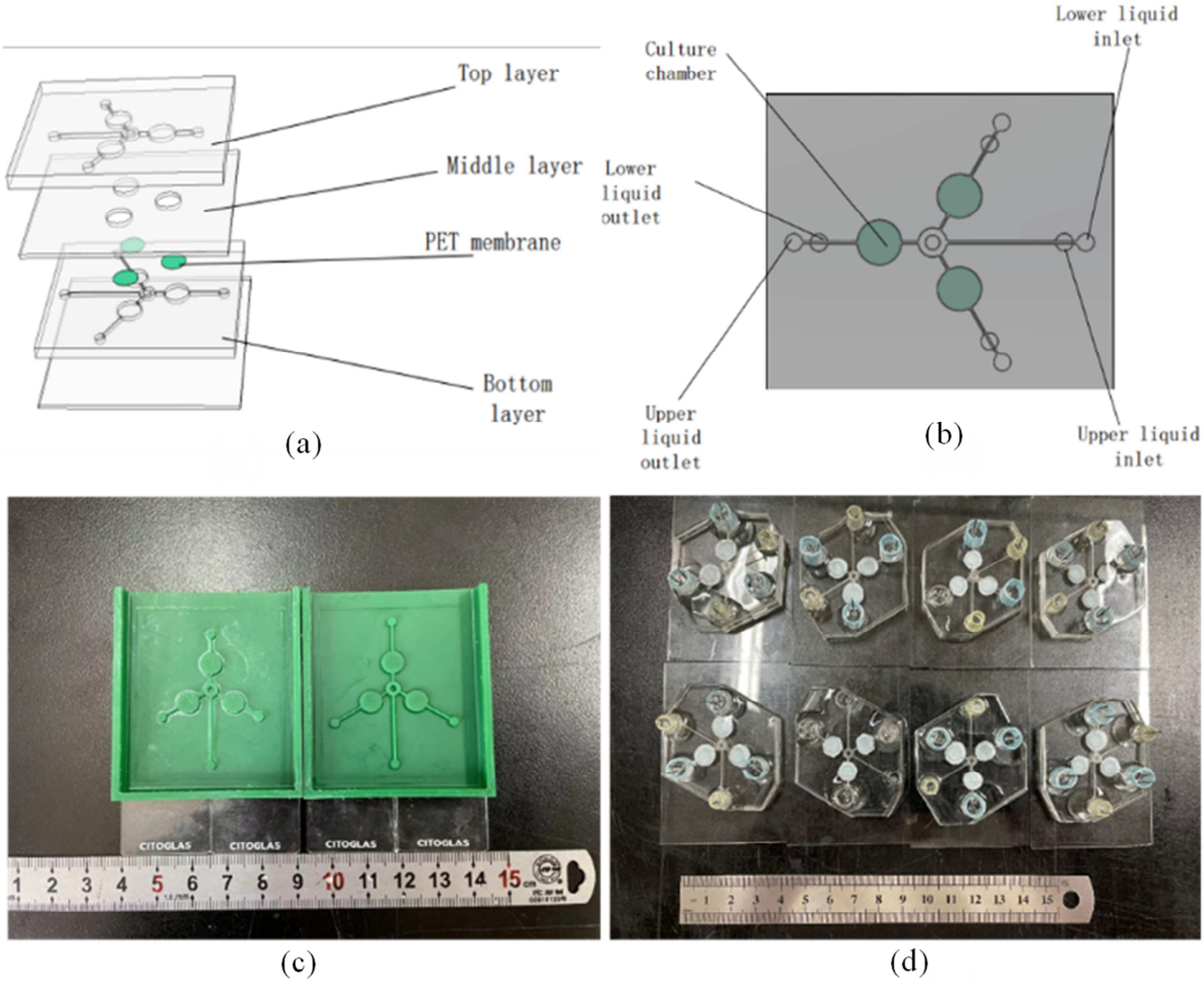

Figure 1. *(a) the structure of the skin-on-chip (b) Inlet layout (c) molds for making skin-on-chip (d) skin-on-chip physical.*

2.3. Build of the Skin-on-a-Chip

The PDMS chip is located on a glass plate. The bonding between the two layers of PDMS is the same as the bonding between the PDMS chip and the glass plate, which is cleaned by a plasma cleaner for plasma bonding.

The combined chip is irradiated with an ultraviolet sterilization lamp (30w) for more than 90 minutes, and then the joints and pipes are installed. The fittings, tubes and fittings used in the experiment were sterilized in the autoclave before before assembling them onto the chip on the set-up day.

Before introducing the cell suspension into the chip, some treatment needs to be done to the chip. First pass 70% alcohol to achieve the purpose of secondary disinfection. Pass PBS buffer for 3-5 times to ensure that there is no alcohol residue in the flow channel. Finally pass the culture media to provide an environment for cell fine growth.

After perfusion, cell suspension containing HDF was injected with a planting density of 105 / ml. The chip was incubated in a 37°C incubator to allow the cells to attach to the pet membrane. After 4 hours of cell attachment, the cell culture medium was introduced. Four days after the HDF cells cultured in PET membrane, HaCaT cell suspension with a planting density of 2x105 / ml was introduced to co- culture with HDF. The upper and lower layers of perfusion were opened at a flow rate of 1.0μL/min at the same time.

After 3 days, the culture at the air-liquid interface was set-up by interrupting the perfusion of the upper culture media and pumping air at 1.0μL/min. The lower compartment was perfused with culture media at a flow rate of 1.0μL/min for two weeks to promote differentiation, stratification, and cornification.

The perfusion culture medium and air are filtered by a

bacterial sieve before operation.

2.4. The Differentiation of HaCaT

Human skin tissue contains well-differentiated epidermal tissue, which consists of basal, spinous, granular and cornified layers from the bottom. In order to verify the correct formation of the air-liquid interface on the skin tissue on the skin-on-a-chip, we compared the expression of Keratin-5 [23-26] in the chip without the air-liquid interface and the chip with the air-liquid interface added. Keratin-5 is mainly expressed in the basal layer and is one of the markers of keratinocyte differentiation.

We conducted a comparative experiment on the chip with the air-liquid interface and the chip without the air-liquid interface. Both of them were injected with X-100 lysis solutionto lyse the cells in the chip for 1 hour. Afterwards, the supernatant of the cell fluid was tested for BCA total protein concentration and Keratin-5 concentration. to characterize the differentiation of HaCAT cells.

The BCA protein concentration kit used in the experiment was obtained from Kingmorn. Under alkaline conditions, the protein reduces Cu^{2+} to Cu^{+}, and Cu^{+} forms a violet-blue complex with BCA reagent, and its absorption value at 562 nm is measured and compared with the standard curve to calculate the protein concentration. The quantitative determination method of Keratin-5 is Elisa, and the selected kit comes from Enzyme-linked Biotechnology, shanghai.

2.5. Establishment of Inflammation Model Screening of Anti-inflammatory Drug Concentration

We selected Lipopolysaccharide (LPS) LPS to induce inflammatory response to establish an inflammatory model. Lipopolysaccharide is a common endotoxin, which can activate mononuclear macrophages, endothelial cells, epithelial cells, etc. through the cell signal transduction system in vivo, synthesize and release a variety of cytokines and inflammatory mediators, and then cause a series of reactions in the body. In order to choose an appropriate LPS concentration, a preliminary experiment of concentration screening was performed in a 48-well plate. 10,000 cells were seeded in each well, and media containing 1 mg/ml LPS, 2 mg/ml LPS, 4 mg/ml LPS and 8 mg/ml LPS were added respectively. After 3 days, the cell viability was determined by staining the cells with CAM and PI stains. The final LPS concentration chosen was 1 mg/ml, which induced an inflammatory response without affecting cell survival.

2.6. Screening of Anti-inflammatory Drug Concentration

After 7-10 days of air-liquid interface culture, medium containing 1 mg/ml LPS was passed into the chip. After incubating in a 37°C incubator for 24h, the culture medium containing different concentrations of Gentoopicroside (GPS) was introduced. In the process, we took the culture medium after adding LPS and adding GPS for testing. The experimental results characterized the anti-inflammatory effects of different concentrations of GPS by comparing the changes in the concentration of inflammatory factor IL-1α.

3. Results

3.1. Permeability of Membranes

We tested the permeability of porous membranes before seeding cells on them. Because the two types of skin cells of the skin-on-a-chip model are seeded on the upper side of the porous membrane, when the subsequent air-liquid interface culture is carried out, only the lower culture chamber passes through the culture medium, and the permeability of the membrane has a great influence on whether the upper side cells can reach A continuously perfused medium is essential. In order to test the permeability of the membrane in the skin chip, we took the finished skin chip for membrane penetration test. Bright red ink is fed into the lower inlet to differentiate it from the blue porous membrane. As the ink slowly advances, the color of the upper side of the porous membrane gradually changes as shown in Figure 2. This shows that the membrane structure of the skin-on-a-chip device can support the growth of cells while ensuring the exchange function of substances, which can be used for the subsequent construction of skin-on-a-chip models.

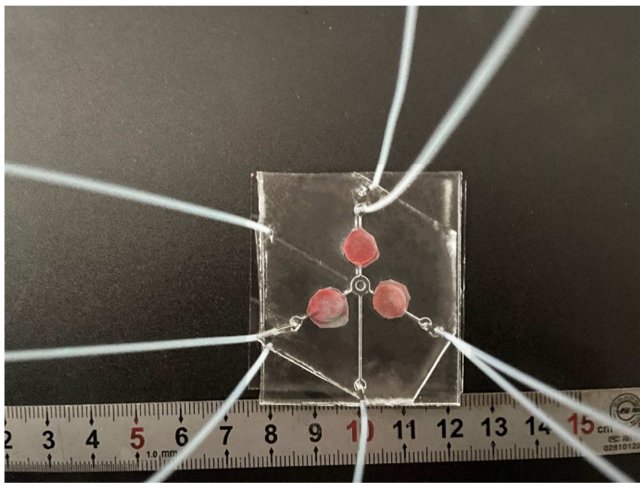

Figure 2. *Permeability of membranes.*

3.2. Cell Morphology

As shown in the Figure 3, the fluorescent images of HDF cells and HaCaT cells were taken under a fluorescent inverted microscope using a mercury light source after being planted on the chip. HDF cells are stained with CAM stain, which emits green fluorescence. Cell morphology was normal spindle-shaped. As can be seen from the images, the cells are in good condition and grow fast during days 1-4. HaCaT cells were stained with DiI stain, and the cell membrane fluoresced orange. It can be seen from the image that the cells grow well, and gradually grow from a relatively scattered distribution to a relatively dense sheet-like distribution, which is in line with the normal growth law of HaCaT cells.

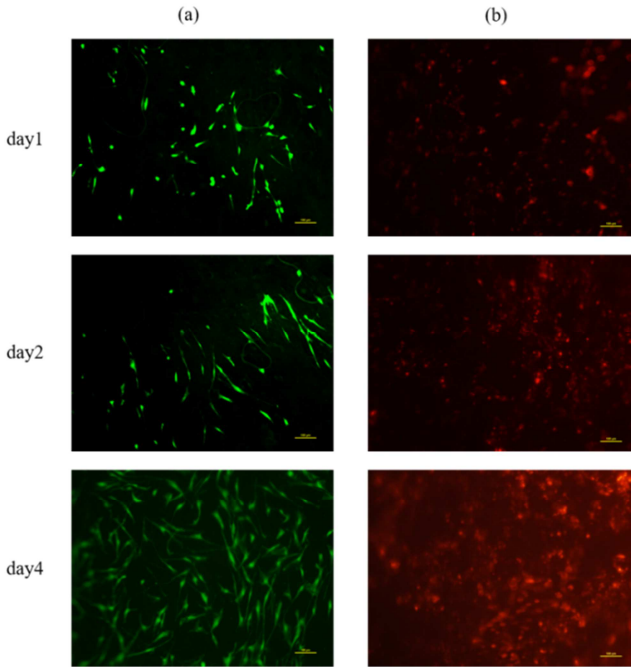

Figure 3. *HDF and HaCaT staining observation (a) HDF (b) HaCaT.*

3.3. Cell Morphology

We performed a CCK-8 cell viability test on the tissue in the chip. The configured CCK-8 working solution is injected into the culture chamber of the chip by a syringe, and after the HaCaT cells are introduced into the chip. After the chip was incubated in the incubator for 30 minutes, the working solution was discharged. Afterit, medium is passed through to continue culturing of the tissue within the chip. We took the discharged working solution for absorbance test, as shown in the figure is the growth rate of cells in the chip obtained by CCK-8 test. The value of the absorbance of the working solution in the chip minus the absorbance of the blank control on the first day is 100%. On the second day and the fourth day, the growth rate of the skin tissue cells in the chip is the ratio of the absorbance of the working solution in the chip minus the absorbance of the blank control on the second day and the fourth day to the first day. It can be seen from the figure that the cells are in good condition and proliferate rapidly. This means that microfluidic-based on-chip skin tissue growth is stable and can be used for long-term culture and subsequent testing.

3.4. Differentiated Keratinocytes

Figure 4 shows the relative content test results of keratin-5. The relative concentrations of keratin-5 in the chip with air-liquid interface and the chip without air-liquid interface are 3517pg/mg and 2327pg/mg respectively. The relative concentration of keratin-5 in the chip cultured by the air-liquid interface was significantly higher than that of the chip not cultured by the air-liquid interface, which indicated that the keratinocytes in the skin tissue cultured by the air-liquid interface had obvious differentiation and keratinization. The cultured skin tissue is relatively close to human skin tissue. At this time, the skin-like tissue cultured in the chip is initially formed, which can be used for subsequent inflammation research and drug effect testing.

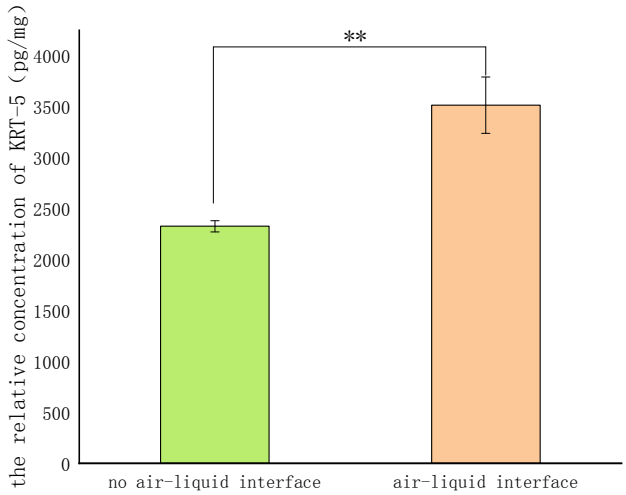

Figure 4. *Relative concentration of keratin 5 cultured with or without gas-liquid interface.*

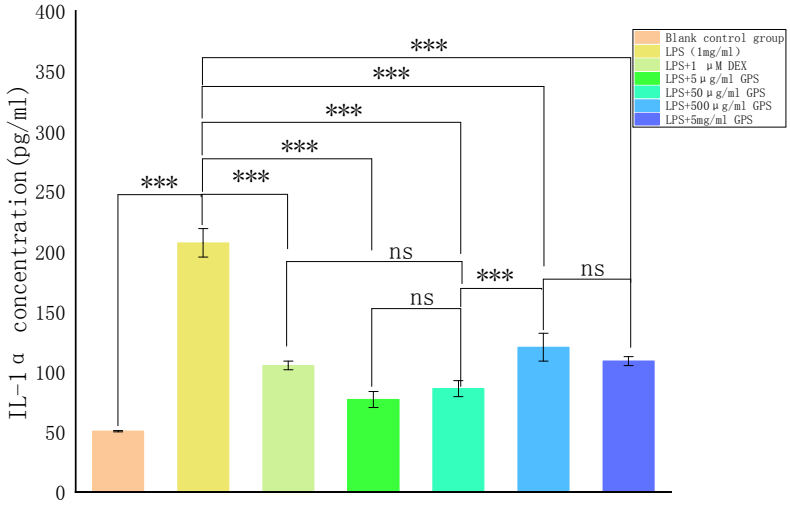

Figure 5. *Anti-inflammation effect of Gentiopicroside.*

3.5. Anti-inflammatory Effect of Gentianaside

We tested the anti-inflammatory effects of 5 μg/ml gentiopicroside, 50 μg/ml gentiopicroside, 500 μg/ml gentiopicroside, and 5 mg/ml gentiopicroside. The results are shown in Figure 5. The results showed that the concentration of IL-1α was the lowest at 50μg/ml gentiopicroside. There is no statistically significant difference between 50μg/ml gentiopicroside, 5μg/ml gentiopicroside and the experimental control group administered with dexamethasone. This indicates that gentiopicroside at 5-50 μg/ml has similar anti-inflammatory properties to 1 μM dexamethasone and has a better anti-inflammatory effect.

4. Conclusion

In this paper, we successfully constructed an in vitro SOC system to obtain inflammation skin models and investigate the anti-inflammatory effect of GPS. We developed a microfluidic chip with a three-layer structure to simulate the structure of the skin. HDF cells and HaCaT cells were used to represent the endothelial and dermal layers of the skin, respectively, and LPS was used to create the inflammatory model. The results showed that 5μg/ml~50μg/ml of gentiopicroside had a similar anti-inflammatory effect to 1μM of dexamethasone. This platform could be used in screening of drugs and cosmetic ingredients.

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