

Preparation of microencapsulated VEGF gene-modified human umbilical cord mesenchymal stem cells and *in vitro* culture

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Abstract. – BACKGROUND AND OBJECTIVES: The application of microencapsulated stem cells has been shown to have many advantages in various fields of medical research. However, optimal modes for preparation of microencapsulate stem cells need to be improved, and expression and release of products of microencapsulated gene modified stem cells need to be studied *in vitro*.

AIM OF THE STUDY: To explore the optimal parameters when preparing microencapsulated stem cells, and to investigate the effect of microencapsulation on growth, secretion, and metabolism of genetically modified human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs).

MATERIALS AND METHODS: In this study, the parameters of preparation were regulated by observing the microcapsule shape and size. Live/dead cell viability kits and fluorescein isothiocyanate-labeled dextrans (FD) were used to detect the microencapsulated cell viability, and the permeability of microcapsules, respectively. Vascular endothelial growth factor (VEGF) production in the supernatant of microencapsulated and non-microencapsulated VEGF gene-modified hUCMSCs cultures was measured by ELISA.

RESULTS: The optimal parameters of preparing microcapsules were regulated as followed: bolus velocity was 6 ml/h, and airflow velocity was 3 L/min. The morphology of microcapsules was a spherical structure with a diameter of $450 \pm 30 \mu\text{m}$. More than 90% of the cells were viable after 21 days of culture. Low and middle molecular weight FD was able to pass through the microcapsules; however, high molecular weight FD was not. Also, the VEGF concentration in microencapsulated and non-microencapsulated cell culture supernatants exhibited no significant difference at each time point.

CONCLUSIONS: Microencapsulated stem cells can be ideally prepared via specifically regulated

preparation. Lastly, microencapsulation does not alter growth, secretion, and metabolism of the genetically modified hUCMSCs.

Key Words:

Vascular endothelial growth factor (VEGF), Human umbilical cord mesenchymal stem cells (hUCMSCs), Microencapsulation, Cell culture.

Introduction

Lim and Sun (1980) used the sodium alginate-polylysine-polyethyleneimine microencapsulation technique to produce biocompatible capsules that contained rat islet cells. These microencapsulated islet cells were efficacious in treating experimental diabetes¹, which shows the positive outcome of the microencapsulated cell transplantation technique. This technique increases the viability of donor cells in a recipient based on the immunoprotection contributed by the microcapsules. Due to the recently, fully developed technique of gene transfer, research studies and applications for the implantation of microencapsulated genetically modified cells have been successfully investigated². Recent studies have indicated that xenotransplantation of microencapsulated cells could induce a detectable immune inflammatory response³. However, the application of microencapsulated stem cells has been found to inhibit the immune response in the recipient; therefore, this technique is beneficial both for cell viability and for enhancing its biological value⁴. Human mesenchymal stem cells (hMSCs) are a better option

for microencapsulated cell therapy⁵. Moreover, umbilical cord tissue, which is rich in mesenchymal stem cells, is an ideal source for hMSCs.

Vascular endothelial growth factor (VEGF) is a major growth factor that stimulates angiogenesis in organisms and has, therefore, been used in many clinical studies⁶. However, we currently do not know much about the preparation of microencapsulated VEGF gene-modified human umbilical cord mesenchymal stem cells (hUCMSCs) or how to *in vitro* culture microencapsulated cells. Therefore, in this study, sodium alginate and barium chloride were chosen as materials to manufacture microencapsulated VEGF gene-modified hUCMSCs with a “one-step” method. Cell viability and target gene expression of VEGF gene-modified hUCMSCs were observed after microencapsulation. Our study establishes an experimental tool for further research on the application of microencapsulated VEGF gene-modified hUCMSCs.

Materials and Methods

Main Reagents and Instruments

The following reagents and instruments were used in this study: VEGF₁₆₅ recombinant adenovirus (donated by the Research Institute of Cardio-Thoracic Surgery, Changhai Hospital of Shanghai, China); rabbit α -human VEGF antibodies (hVEGF purchased from Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA); Dulbecco's modified eagles medium (DMEM) (Gibco); fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA); trypsin (Gibco); ethylenediaminetetraacetic acid (EDTA) (Gibco); a live/dead kit, FD-4, FD-70 and FD-150 (Sigma, St Louis, MO, USA); an ELISA kit (Jingmei BioTech, China); chemically purified sodium alginate (Cambridge, MA, USA); 25 mmol/L barium chloride (prepared in this study); an inverted phase contrast microscope (Leica, Solms, Germany); a Biotek Multi-Detection Reader (SynergyTMz, Winooski, VT, USA); and an FACSort Flow Cytometer (BD Co. Ltd., Mill Creek, WA, USA).

Cell Culture and Gene Modification

The human umbilical cord-derived mesenchymal stem cells (hUCMSCs) that were used in this study were obtained from a previously grown cell culture in the institute. The cells were purified and then cultured in 12-well plates⁷. Once the cells had reached 90% confluence, the super-

natant was discarded. VEGF₁₆₅ recombinant adenoviruses were used to infect the hUCMSCs with the optimal multiplicity of infection (MOI). VEGF gene-modified hUCMSCs were cultured in an incubator at 5% CO₂ and 37°C.

Preparation of VEGF-Gene Modified Microencapsulated Cells

The VEGF gene-modified hUCMSCs were suspended in 2% sodium alginate solution at a cell concentration of 1×10^6 cells/mL, and then pumped through a dual-nozzle sprayer. The hUCMSCs suspension subsequently went into a 25 mmol/L barium chloride solution and were allowed to react for 15 min to produce microencapsulated VEGF gene-modified hUCMSCs. The optimal protocol for preparing microcapsules was set using shape, size, bolus velocity, and air-flow velocity. Microencapsulated cells were collected after centrifugation, washed with sterilized 0.9% sodium chloride three times and transferred into cell culture bottles with Dulbecco Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and penicillin-streptomycin. The cells were cultured in an incubator at 37°C under humidified conditions and an air atmosphere of 5% CO₂. Consequently, empty microcapsules were prepared. Inverted phase contrast microscopes were used to observe the microencapsulated cell morphology and the sizes of the microcapsules.

Detection of Microcapsule Permeability

For 24 hours, the empty microcapsules were suspended in 0.9% saline at room-temperature, which contained 100 μ g/mL of fluorescein isothiocyanate-labeled dextran at various molecular weights (FD-4, FD-70 and FD-150). The reaction was shielded from light and shaken; fluorescein-labeled markers were able to fully diffuse into each microcapsule. Fluorescence was detected using a laser scanning confocal microscope (LSCM). The proper amount of microcapsules was placed on a clean slide and was scanned and focused using visible light. The emitted light was then used to project fluorescent images of the microcapsules onto the monitor for further detection.

After 24 hours, different molecular weighted FD empty microcapsules were collected from the saline and counted. After adding sodium citrate and oscillating, the microcapsules ruptured and the optical density (OD) per 100 microcapsules could be measured under a 450 nm detection wavelength.

Live/dead Staining of Microencapsulated Cells

Based on the manufacturer's instructions, 5 parts solution A and 20 parts solution B were mixed with VEGF gene-modified microencapsulated cells (after 7 days of culturing) and then diluted with phosphate buffered saline (PBS) to become a final volume of 10 ml. The cells were stained in the dark at room temperature for 20 min and observed by a fluorescence microscope. Cells that expressed green fluorescence were considered living cells; cells that expressed red fluorescence were considered dead cells. The experiment was repeated three times.

***In vitro* Microencapsulated Cell Culture and Collection of Supernatant**

Under the conditions of 5% CO₂ and 37°C, The microencapsulated and non-microencapsulated VEGF gene-modified hUCMSCs were cultured in 6-well plates at an initial concentration of 1×10^4 cells/well. Each well contained 2 ml of cell culture medium. The culture medium was changed and collected every 2 days and stored at -20°C. Each group was replicated in six wells.

Measurement of VEGF Production in Supernatant

Every 48 hours, 1 ml of culture medium was collected and centrifuged [3000 r/min ($r = 10$ cm)] for 3 min. The cell debris was discarded. VEGF content of the supernatant was measured based on the manufacturer's instructions for the ELISA kits. The OD of the sample was measured using a 450 nm detection wavelength and was calibrated using a 570 nm wavelength. The VEGF levels in the supernatant were calculated by comparing them to a standard curve.

Statistical Analysis

The results in this study were reported as the means $\pm$ the standard deviation (SD). The data was analyzed using SPSS10.0 statistical software (SPSS Inc., Chicago, IL, USA). A *t*-test was used, and $p < 0.05$ was considered significant.

Results

Observation of Microencapsulated VEGF Gene-modified cell Morphology

Using a light microscope, the microcapsules appeared to be symmetrical bodies with similar sizes. The outlines of microcapsules were clear, the surfaces of microcapsules were smooth, and no adhesion between two microcapsules was observed. Each microcapsule (diameter 400-500 μ m) was comprised of 50 to 80 cells. The cells were able to proliferate within the microcapsules after 7 days of *in vitro* culture. As the cells proliferated within the microcapsules the outlines became gradually unclear. In a portion of the cells, cell fusion and cell division were found to cause the formation of asymmetrical cell masses. However, no cells were found to grow outside the microcapsules (Figure 1).

The relationship between bolus velocity, airflow velocity and microcapsule diameter was summarized as well (Figure 2). Through observation of microcapsule size and shape, an optimal parameter of preparing microcapsules was regulated as follows: bolus velocity was 6 ml/h, and airflow velocity was 3 L/min.

Permeability of Sodium Alginate-Barium Chloride-Made Microcapsules

Low and middle molecular weight dextrans (4 kDa and 70 kDa) were able to pass through the

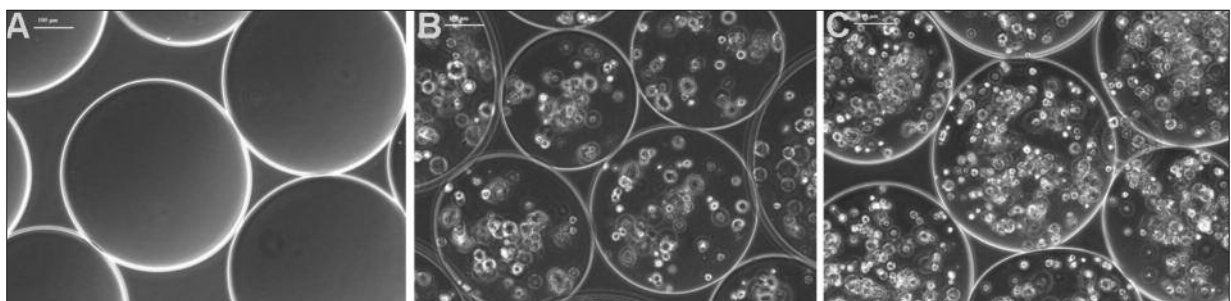


Figure 1. Observation of cell morphology: no free cells were found after microencapsulation of hUCMSCs. In addition, microencapsulation showed no effects on cell proliferation. **A**, An empty microcapsule in a spherical shape with a smooth surface. **B**, The morphology of microencapsulated cells after 1 day of culture. **C**, The morphology of microencapsulated cells after 7 days of culture. A-C: $\times 100$. Abbreviation: hUCMSCs, human umbilical cord mesenchymal stem cells.

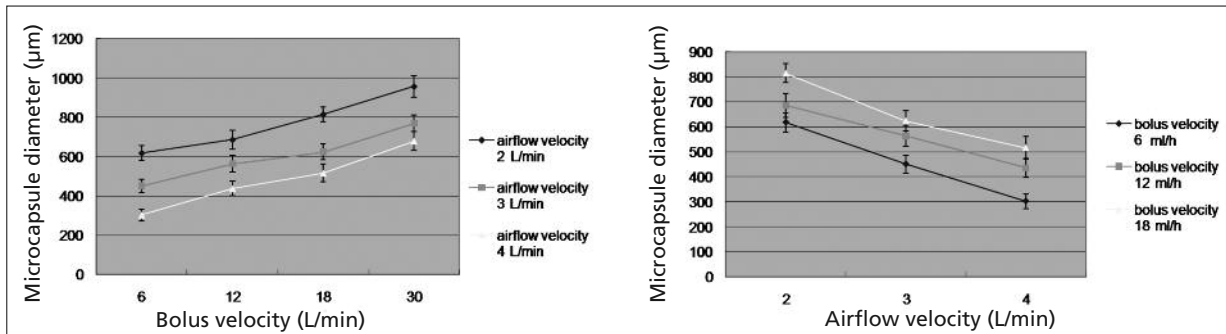


Figure 2. The relationship between microcapsule diameter and bolus velocity, airflow velocity.

microcapsules, and green fluorescence expressed in microcapsules was visible by fluorescence microscopy (Figure 3). Optical density value per 100 microcapsules was 3.06 ± 0.51 and 2.89 ± 0.46 , respectively. However, high molecular weight dextran (150 kDa) was not able to pass through the microcapsules, preventing green fluorescence to be visible in the microcapsules (Figure 3). Optical density value (0.02 ± 0.01) per 100 microcapsules was significantly lower than the above two ($p < 0.01$). This result demonstrated that the membrane of sodium alginate-barium chloride-made microcapsules is impermeable to material of molecular weight greater than 70 kDa.

Live/Dead Staining for Microencapsulated VEGF Gene-Modified Cells

LIVE/DEAD staining was performed after the microencapsulated cells were cultured for seven, fourteen and twenty-one days. About 95% of the cells that expressed green fluorescence were observed by fluorescence microscopy. This result indicated that sufficient nutrition was able to enter the microcapsules in order to support cell metabolism (Figure 4).

Measurement of VEGF in the Supernatant

VEGF gene expression obtained from the microencapsulated VEGF gene-modified hUCMSCs was maintained at a steady-state level during an *in vitro* 14 day culture period. VEGF gene expression increased after 48 hours of culturing. The maximum expression level was observed on day eight. Although the expression of the VEGF gene showed a gradual decrease, it was still detectable on day 14. No statistically significant difference was found between the two groups at each time point ($p > 0.05$) (Figure 5).

Discussion

Lim et al¹ first proposed the microencapsulation technique (which provides immunoprotection) to study the implantation of microencapsulated islet cells. Based on modifications to this study, O'Shea et al⁸ utilized sodium alginate-barium chloride as the material to manufacture sodium alginate-poly-L-lysine-sodium alginate (APA) membranes rather than polyethyleneimine as the outer membrane layer. An APA membrane is processed into a gelatinous form, which has

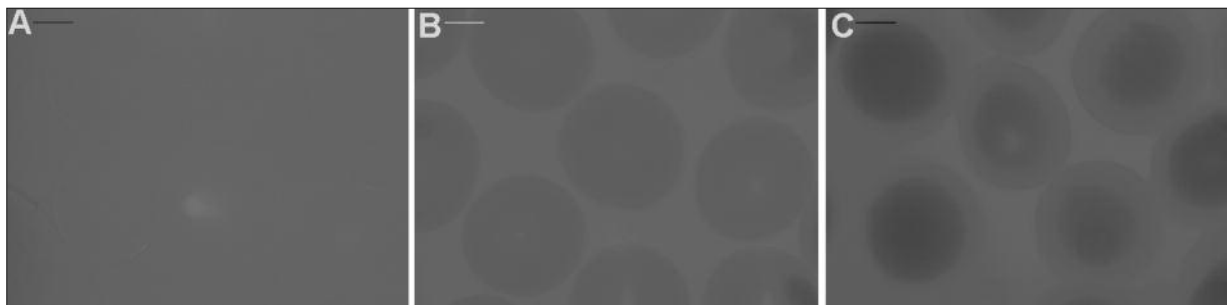


Figure 3. Detection of microcapsule permeability of the microcapsules made with various molecular weights FD. **A**, FD-4. **B**, FD-70. **C**, FD-150. FD, fluorescein isothiocyanate-labeled dextran. Scale bar: 100 μm.

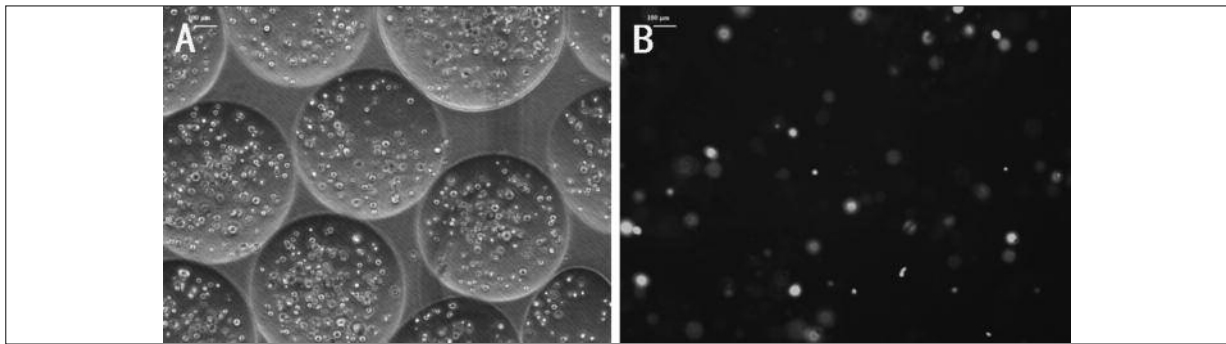


Figure 4. LIVE/DEAD staining for microencapsulated VEGF gene-modified hUCMSCs. **A**, Observation using an inverted phase contrast microscope. **B**, LIVE/DEAD staining: Green fluorescence represents live cells and red fluorescence represents dead cells. A-B: $\times 100$. Abbreviation: hUCMSCs, human umbilical cord mesenchymal stem cells.

better biocompatibility. Recently, this technique has started to be applied to xenotransplantation. Due to the well-developed sodium alginate-barium chloride-made microencapsulation technique, better forms of transplantation has been acquired^{9,10}. Therefore, this technique was used to preparing the microcapsules in this study. Microcapsules prepared using this technique showed higher stability. Moreover, the microcapsules, which consisted of semi-permeable membranes, were relatively easy to preparing using this technique. Low molecular weight compounds (*e.g.* O_2 , CO_2 and nutrients) were able to transverse the membranes in contrast to high molecular weight compounds (*e.g.* immune cells and immunoglobulins)¹¹. The experimental findings showed that microcapsules provide a greater permeability for the VEGF protein, and that microencapsulated VEGF gene-modified hUCMSCs could release VEGF freely.

The purity of sodium alginate is another factor that may affect the biocompatibility of microcap-

sules¹². In this study, we used purified sodium alginate and barium chloride to preparing the microcapsules. The protocol and materials for the preparation of microcapsules were different from those previously used when manufacturing alginate/PLL/alginate (APA) microcapsules¹. We used the “one-step” method to prepare the sodium alginate solution combined with $BaCl_2$ to manufacture the microcapsules¹⁰. This method simplifies the process for manufacturing microcapsules. Recently, studies regarding materials for manufacturing microcapsule reported that APA microcapsules (a diameter of a microcapsule is approximately 500 μm) exhibit great physical tolerance, a smooth surface, less friction and good biocompatibility in organisms¹¹. In this study, the microcapsules which enclosed genetically modified cells were prepared using the sodium alginate-barium chloride-made microencapsulation technique. The VEGF expression levels that were measured from the microencapsulated hMSCs did not decrease compared to the non-microencapsulated hMSCs.

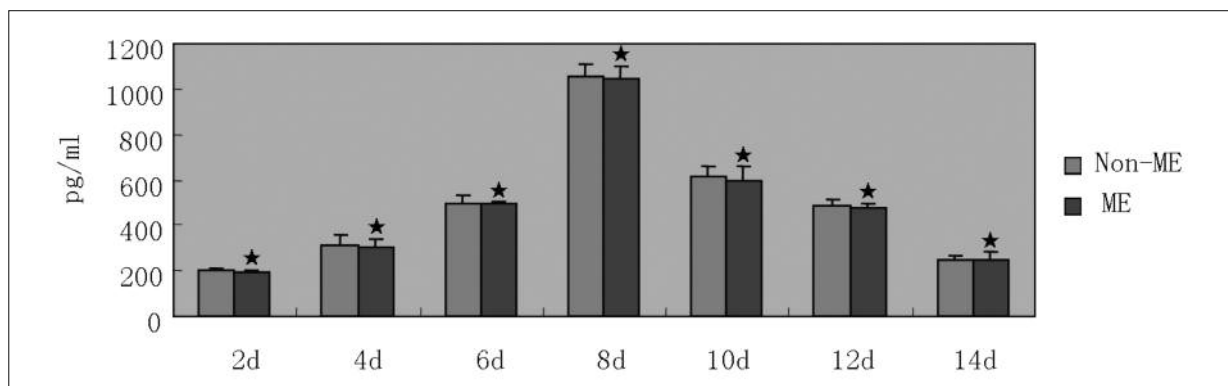


Figure 5. VEGF gene expression levels obtained from microencapsulated and non- microencapsulated VEGF gene-modified hUCMSCs. *Denoting $p > 0.05$.

This phenomenon was consistent with previous studies¹³. Furthermore, the cells were able to decelerate their metabolism rates to proliferate longer after microencapsulation¹⁴.

Recently, investigations regarding the implantation of microencapsulated genetically modified cells have made a great progress worldwide, especially in the fields of retina research, dwarfism, family genetic disorders, and cancer research^{5,15,16}. Studies have indicated that immunoprotection provided by microcapsules allowed hepatocytes to survive longer in a recipient compared to non-microencapsulated cells¹⁷. Thus, implantations of microencapsulated cells are superior to traditional implantations. However, according to other studies, microencapsulated xenogenic somatic cells have also been known to trigger the immune inflammation response. Conversely, microencapsulated stem cells could inhibit the immune response, improve cell viability, and promote their biological functions⁴. Taken together, these findings demonstrate that the implantation of microencapsulated stem cells prove more advantages compared to somatic cells. Goren A et al.⁵ also reported that stem cells are an ideal cell type for gene transfection. Moreover, human mesenchymal stem cells (hMSCs) are a good choice for microencapsulated cell therapy. The umbilical cord is the best source from which to isolate hMSCs because these cells are easy to acquire, surgery is not required and there are no bioethical concerns. Therefore, umbilical cords could be widely used for research. Cell proliferation and the multipotency of hMSCs that are obtained from umbilical cords and bone marrow have also been investigated. The results have shown that hMSCs obtained from the umbilical cord are more practical for further applications compared to those obtained from bone marrow¹⁸.

Conclusions

Overall in this study, we demonstrated that microencapsulated stem cells can be ideally prepared well via certain preparation regulations. Microencapsulation did not affect secretion of the target gene product, cell growth, or cell proliferation. Moreover, we identified more advantages for using hMCSs as target cells. To conclude, this study established a stable experimental basis for further *in vivo* research on implantation of microencapsulated genetically modified stem cells.

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