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In vitro culture and oxygen consumption of NSCs in size-controlled neurospheres of Ca-alginate/gelatin microbead

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ABSTRACT

Neural stem cells (NSCs) forming neurospheres in a conventional culture tend to develop necrotic/apoptotic centers due to mass transport limitations. In this study, the internal pore structure of calcium-alginate/gelatin (CAG) microbeads was tuned and controlled to provide a suitable three-dimensional environment supporting NSC proliferation. Direct impact of three-dimensional space availability was quantified by oxygen consumption rates of NSCs and cells were cultured in three different methods: neurospheres, single cell suspension of NSCs, and encapsulated NSCs in microbeads. Our results showed that encapsulated NSCs in CAG microbeads maintained higher cell viability than in conventional culture. In addition, NSCs encapsulated in CAG microbeads preserved their original stemness and continued to express nestin, CNPase, GFAP and β-tubulin-III post-encapsulation. Oxygen consumption rates of encapsulated NSCs in CAG microbeads were the lowest as compared to the other two culture methods. The optimal cell density supporting high cell proliferation in CAG microbeads was found to be 1.5×10^5 cells/mL. The glucose consumption curve suggests that encapsulated NSCs in microbeads had a slower growth profile. This study presents an alternative method in hybrid microbead preparation to generate a highly favorable three-dimensional cell carrier for NSCs and was successfully applied for its effective *in vitro* expansion.

1. Introduction

Neural stem cells (NSCs) are multipotent stem cells, which can differentiate into astrocytes, neurons and oligodendrocytes under induced conditions [1–5]. Recently, NSC transplantation as a cell therapy has attracted more and more attention worldwide [6–9]. However, sources of NSCs are so limited that insufficient cell number greatly restricts rapid advancement for further clinical applications. Conventionally, expansion of NSCs can be achieved by generating neurospheres *in vitro*. However, the neurospheres gathered by NSCs in an *in vitro* culture procedure undergo uncontrolled size increase [10–12]. While single NSCs dissociated from neurospheres with a diameter of less than 150 μm could obtain considerable cell numbers with excellent viability [13–15], neurospheres that exceed this diameter would develop a necrotic center [16,17]. Improved culture methods for generating NSCs are therefore much needed.

Several studies have looked into the utilization of encapsulated NSCs in calcium-alginate microbeads [18–22]. These studies confirm that calcium-alginate microbeads possess many advantages including biocompatibility, tunable mechanical properties, good toughness, and a three-dimensional porous structure, which ensures favorable and efficient mass transfer [23–27]. Gelatin has been widely used as scaffold material in tissue engineering because it has good biocompatibility and biodegradability as the hydrolyzate of collagen; one of the main components of the extracellular matrix and tissue. Moreover, many researchers have shown that collagen is conducive to cell adhesion and promotes the proliferation of NSCs [28–30]. In addition, the most important virtue is that the microbead size and internal pore structure can be controlled by changing the microbead preparation parameters such as the concentration of calcium chloride, sodium alginate and gelatin as well as reaction conditions.

Oxygen consumption rates of cells in different culture conditions can reflect cellular viability and growth status. As compared to traditional detection methods for cellular viability such as growth curve and measurements of glucose and/or metabolite concentrations in the media, the measurement of oxygen consumption rate is not only simple and economical, but also more accurate or consistent compared to glucose/lactate measurements. This study provides a practical method using oxygen consumption rates to evaluate NSC activity in different *in vitro* culture processes. The average oxygen consumption rates of individual cells can be obtained by dividing the total cell number; this

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value can provide an insightful correlation to the central physiological process of ATP production.

In this study, the internal pore structure of calcium-alginate/gelatin (CAG) microbeads was adjusted and controlled to provide a suitable three-dimensional biophysical environment for NSC growth and proliferation. These physical properties were tuned to ensure sufficient expansion of NSCs and maintenance of excellent viability. To quantify the impact of three-dimensional space availability on NSC growth, oxygen consumption rates of NSCs cultured in three different methods: neurospheres, single cell suspension and encapsulated NSCs in microbeads were studied and evaluated. The consumption rate of glucose and the expression of specific protein markers of NSCs pre and post-encapsulation were simultaneously evaluated for neural stemness.

2. Materials and methods

2.1. Isolation and culture of NSCs

This study was approved by the Ethics Committee of Dalian University of Technology and by an Administrative Method of Experimental Animal License (2001-545). Neural stem cells were dissociated from the forebrain and hippocampus of embryonic 14-day (E14) Kunming mouse as Zheng et al. described [21]. Briefly, following trituration, the cell suspension was plated onto T-25 flasks at a concentration of 8×10^4 cells/mL in suspension and inoculated in 37 °C, saturated humidity, and 5% CO_2 incubator, and 50% of the media were replaced every 3 days for later use. The essential components of media were DMEM (Sigma, solid powder, USA), F12 (Gibco, solid powder, USA), RPMI-1640 (Sigma, solid powder, USA), $NaHCO_3$ (Sigma, solid powder, USA), HEPES (Roche, solid powder, Germany), glucose (Gibco, solid powder, USA), GlutaMax (Gibco, solid powder, USA), heparin (Jiangsu Biochemistry Institute, solid powder, China), N-2 (Gibco, liquid, USA), EGF (Invitrogen, solid powder, USA) and bFGF (Invitrogen, solid powder, USA).

2.2. Growth curve of NSCs

CCK-8 kit (Nanjing Jiancheng, China) was used to detect *in vitro* cellular proliferation and to determine the growth curve of NSCs. A substance called 2-(2-methoxy-4-nitrobenzene)-3-(4-nitrobenzene)-5-(2,4-dithiabenzo)-2H-water-soluble tetrazolium-8 contained in the kit is reduced to formazan dye by cellular dehydrogenase within the mitochondria *via* an electrical sub-carrier, 1 Methoxy PMS. The amount of formazendye produced is directly proportional to cell activity. Therefore, it can be used to detect cell activity and a standard curve for cell densities of $0.2–1.6 \times 10^5$ cells/mL was created for this study. Briefly, neurospheres were digested into single cell suspension and seeded at respective cell densities onto a 96-well plate. For cell assessment, 10 μL of CCK-8 was added into each well and the cells were cultured for 3.5 h in a 37 °C incubator. The absorbance of each well was measured at 450 nm using a microplate reader, keeping the reference wavelength of 600 nm.

For this comparative study, NSCs at a density of 1×10^5 cells/mL were inoculated in a 96-well culture plate and incubated with 10 μL of CCK-8 for 3.5 h in the incubator. Cellular absorbance was then measured every 24 h to obtain the growth profile of NSCs.

2.3. Encapsulation culture of NSCs

Gelatin (0.2 g/L) (Sigma, solid powder, USA) and alginate (0.2 g/L) (Sigma, solid powder, USA) were separately prepared and filtered. The single-cell suspension was added to gelatin solution at a density of 2×10^6 cells/mL. Afterwards, 3 mL of alginate solution was added to 1 mL of cell suspension to obtain a cell–gelatin–alginate mixture with 0.5, 1.5, 2.5 and 3.5×10^5 cells/mL, respectively. Next, the mixture was dropped into a $CaCl_2$ solution, which was continuously stirred at 100 rpm by a magnetic stirrer, using a #5 needle and allowed to polymerize for 10 min at room temperature. Residual $CaCl_2$ solution was discarded 10 min after polymerization. The beads were then washed 3 times with PBS for later use.

2.4. Assays of NSCs pre- and post-encapsulation

Cellular morphologies of NSCs were observed under an inverted bright-field microscope. As described by Zheng et al. [21], NSCs cultured in suspension and monolayer were dissociated to a single-cell suspension by Accutase. The single cells were placed on glass coverslips that had been coated with poly-L-ornithine (PORN, 0.01%, Sigma, USA)/laminin (0.01%, Sigma). Two days after culturing in induction medium, lysosome activity of adhered NSCs was detected by staining with a novel fluorescent probe, LysoZn-1. The main components of differentiation medium were DMEM, F12, RPMI-1640, $NaHCO_3$, HEPES, glucose, GlutaMax, heparin, N-2 and albumin bovine (Sigma, liquid, USA). Lysosomes are key degradative compartments of NSCs. Lysosomal cathepsins, which are enclosed within lysosomes, help to maintain homeostasis of NSC metabolism by participating in the degradation of heterophagic and autophagic material [31,32]. Therefore, assaying the lysosomes of NSCs is an important step in evaluating their fundamental and clinical basis for further investigations. In this study, laser confocal scanning microscopy was performed to assay the distribution of lysosomes that were marked with a novel fluorescent probe, LysoZn-1 within NSCs.

Nestin is widely used for the positive identification of NSCs and regarded as a NSC-specific protein marker, while CNPase, β-tubulin-III and GFAP were used to identify differentiated NSCs towards oligodendrocytes, neurons and astrocytes, respectively. Cells cultured in the different methods were stained by immunochemistry for the respective proteins. Live/Dead Viability–Cytotoxicity Kit staining and Hoechst 33342 (Sigma, liquid, USA) staining were used to detect cellular viability pre- and post-encapsulation.

2.5. Measurement of glucose consumption

Glucose concentration in the culture medium was directly measured by a semi-automatic biochemical analyzer (758, Antai Instrument Co., Ltd., Shanghai) and a corresponding glucose quantification kit (glucose kit, Nanjing Jiancheng Bioengineering Institute) throughout the whole culture procedure. Operating steps for the latter are as follows: the working solution was prepared by mixing the buffer with an enzyme reagent at a ratio of 1:1. Afterwards, the sample solution was added into the working solution. This liquid system was then incubated in a water bath at 37 °C for 20 min and was subsequently detected using an automatic biochemistry analyzer.

2.6. Determination of oxygen consumption rate

In this study, Mitocell S200 mitochondrial respiratory instrument (S200, Strathkelvin, Britain) was used to detect the oxygen consumption rates in three different culture methods. The mitochondrial respiratory instrument was calibrated before the detection of samples. To create the standard curve, sodium sulfite was slowly added to 50 mL ultrapure water under stirring until it did not dissolve any longer; roughly 2 h with a magnetic stirrer until it reaches saturation. Because sodium sulfite could spontaneously react with O_2 in an aqueous state, this solution could be used for the zero point calibration while saturated dissolved oxygen at room temperature was used to calibrate the highest point.

Neurospheres, single NSC suspension and encapsulated NSCs within microbeads with the same cell number, were added to a mitochondrial respiration chamber in a waterbath at 37 °C. Changes in oxygen content in the culture media were monitored for 30 min with continuous stirring.

The oxygen consumption rate was calculated by taking three segments from the oxygen curve that was randomly selected. The timescale