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Full Length Research Paper

Measurement of oxygen consumption rate of osteoblasts from Sprague-Dawley rat calvaria in different *in vitro* cultures

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The oxygen consumption rates of Sprague-Dawley (SD) rat's osteoblasts cultured in different *in vitro* media were on-line measured by using precision dissolved oxygen and respirometry instrumentation. The osteoblasts, isolated from the calvaria of neonatal SD rat by an enzymatic digestive process of 0.25% trypsin solution and 0.1% type II collagenase, were diluted to 1 × 10^6 cells/ml in fresh media and passaged in T-25 flasks in an incubator. After two (2) passages, the cells were cultured directly in T-flasks and encapsulated medium by calcium alginate microcapsules, respectively. The cells were evaluated through live/dead assay, hematoxylin-eosin (HE) and alkaline phosphatase (ALP) staining. Moreover, Von-Kossa staining and Alizarin Red S staining were carried out for mineralized nodule formation. Following this, the oxygen consumption rates of osteoblasts in the earlier mentioned different cultures were measured on-line. The results showed that the osteoblasts performed well in its major functions after being examined by cellular morphology and viability, growth curve in growth kinetics, HE, ALP, Von-Kossa and Alizarin Red stains, which were capable of being used for further investigation as seed cells in bone tissue engineering. The osteoblasts cultured in static T-flask and encapsulated medium *in vitro* both proliferated well, and the oxygen consumption rates were 5.56 × 10^{-6} and 1.25 × 10^{-7} μmol/(min·cell), respectively. By measuring the oxygen consumption rates of osteoblasts in different cultures *in vitro*, it would provide significant instruction and model function for further fundamental investigation and clinical application in bone tissue engineering.

Key words: Bone tissue engineering, osteoblast, respiration rate, *in vitro* culture, encapsulation.

INTRODUCTION

Tissue engineering has been defined as the application of principles and methods of engineering and life sciences for the development of biological substitutes, to restore, maintain or improve tissue function (Langer et al., 1993; Song et al., 2006, 2007). It is a rapidly growing and multi-disciplinary field, showing great promise in generating generating biological and living alternatives for harvested tissues and organs for implantation and reconstructive surgery (Kedong et al., 2008a; Shastri 2009; Song et al., 2008). Because of the limitations of traditional treatment methods such as auto-genous bone graft and variant bone transplantation, in the treatment of external injury, inborn bone defect, the osteoma and other orthopedics illness, the method of bone tissue engineering hopefully become a novel way to repair sick or impaired bone tissues (Brown and Shaw, 2010; Oshima et al., 2010).

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Abbreviations: HE, Hematoxylin-eosin; **ALP,** alkaline phosphatase; **LT-HSCs,** long term hematopoietic stem cells; **FBS,** fetal bovine serum; **SD,** Sprague-Dawley.

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The essential factors of bone tissue engineering mainly include seed cells, supported material/biomaterial scaffolds, three-dimensional *in vitro* culture environment, and so on (Ushida et al., 2002; Kedong et al., 2008b; Song et al., 2008). Amongst them, the *in vitro* culture and efficient large-scale expansion of seed cells are the most fundamental problem. The osteoblasts being the cell types for *in vivo* bone formation are the typical origin of seed cell in bone tissue engineering. Its main function is to secrete bone matrix and facilitate the matrix mineralization to form the bone tissue. The content of alkaline phosphatase (ALP) within the osteoblasts is high, and they also secreted type I collagen, osteonectin, osteocalcin, fibronectin and a series of growth factors (Choi et al., 1996). When fabricated with biological/biomedical materials that can continue to proliferate and differentiate, they thus form new bone tissues, which has obtained a great deal of success in animal experiments and clinical trials (Perel et al., 2006). Accordingly, the *in vitro* culture, expansion and gene modification of osteoblasts have been the research hot spot in bone tissue engineering. In addition, the osteoblasts are the important members in the hematopoietic microenvironment of adult human bone marrow (Song et al., 2009; de Barros et al., 2010; Kedong et al., 2010). Osteoblasts are also crucial in maintaining and expanding long term hematopoietic stem cells (LT-HSCs) in the bone marrow hematopoietic niche (Calvi et al., 2003; Zhang et al., 2003). In respect to their described functions *in vitro* and *in vivo*, we believe that the *in vitro* oxygen consumption rate of osteoblasts in different cultures could be an important parameter for further investigation described earlier.

To our knowledge, there is still no report about the *in vitro* oxygen consumption rate of osteoblasts in different cultures. In order to define this fundamental parameter of *in vitro* cell culture for further application toward bone tissue engineering, therefore, in this paper, the oxygen consumption and oxygen consumption rates of osteoblasts in the *in vitro* static and encapsulation media were respectively detected and compared. This can provide theoretical basis for the *in vitro* investigation of bone tissue engineering and stem cells.

MATERIALS AND METHODS

A total of 6 neonatal Sprague-Dawley (SD) rat, of either sex were provided by Experimental Animal Center of Dalian Medical University, with certification number of SCXK (Liao) 2002-0002. The treatment processes of animals were confirmed to be the standard of 'Instructive notions with respect to caring for laboratory animals' promulgated in 2006.

Fetal bovine serum (FBS), IMDM medium, D-Glucose, trypsin and type II collagenase were purchased from Gibco; Dulbecco-PBS was prepared by our laboratory. Several instruments were used in the experiments, including inverted phase contrast microscope (IX70-Olympus, Japan), scanning electron microscope (SEM, JEOL-1200EX, Japan), CO_2 incubator (HERA cell, Kendro Lab Products, Germany), UV/VIS spectrophotometer (V-560 SCOJL), digital color camera (Sony-3), dissolved oxygen meter (Model-782, Strathkelvin, British) and image analyzer (Image-pro-plus, Cold Spring, USA).

Cell culture

Static culture of osteoblasts in T-flask

The osteoblasts, isolated from the calvaria of neonatal SD rat by an enzymatic digestive process, were pooled, pelleted, and resuspended in a known amount of medium. The cells were counted by hemacytometer and diluted to concentrations of 1×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin). Cells were grown in an incubator, which was maintained at 37°C, 5% CO_2. Primary culture medium was changed at day 3. After two passages in 7 days, the cells were ready to be used in the experiments (Song et al., 2007).

Encapsulation culture of osteoblasts

Gelatin (0.2 g/l) and alginate (0.2 g/l) were separately prepared and filtered. Osteoblast suspension was added to the gelatin solution to give 8×10^5 cells/ml. Afterwards, 3 ml alginate solution was added to 1 ml cell suspension to obtain a cell-gelatin-alginate mixture with 2×10^5 cells/ml. Gelatin-alginate beads were produced using an electrostatic generator at 6 kV and liquid flow rate of 40 mL/h, resulting in beads with 800 μm diameter. Next, 1 ml beads was placed into 30 ml $CaCl_2$ (100 ml/L), and allowed to polymerize for 10 min at room temperature. The beads were then washed 3 times with phosphate-buffered saline (PBS) and cultured in an incubator for later use.

Biological detections

The osteoblasts were assayed with the following, staining after being cultured in static culture: Hematoxylin-eosin (HE) staining described by Song et al. (2008) was applied in this study; ALP staining: Von-Kossa staining and Alizarin red S staining described by Song et al. (2008) were all applied in this study.

Histomorphology detection

The adhesion, viability, spreading and distribution of osteoblasts both in static culture and encapsulation culture were observed under an inverted microscope. **Oxygen consumption rate of osteoblasts in different culture conditions**

Oxygen consumption rate in static culture

A certain density of single-cell suspension was prepared, and 3.4 ml of the suspension was taken and added to the chamber of the dissolved oxygen meter with a mixture at 37°C. Following this, the probe was put into the chamber to make it touch the surface of cell suspension. After that, the change of the oxygen levels at the same interval was recorded after the instrument was stabilized, and then the oxygen consumption of individual cell was therefore calculated.

Oxygen consumption rate in encapsulation culture

Encapsulation osteoblasts (3.4 ml) were added into the chamber of the dissolved oxygen meter with a mixture at 37°C. After that, the same method was used to detect and calculate the oxygen

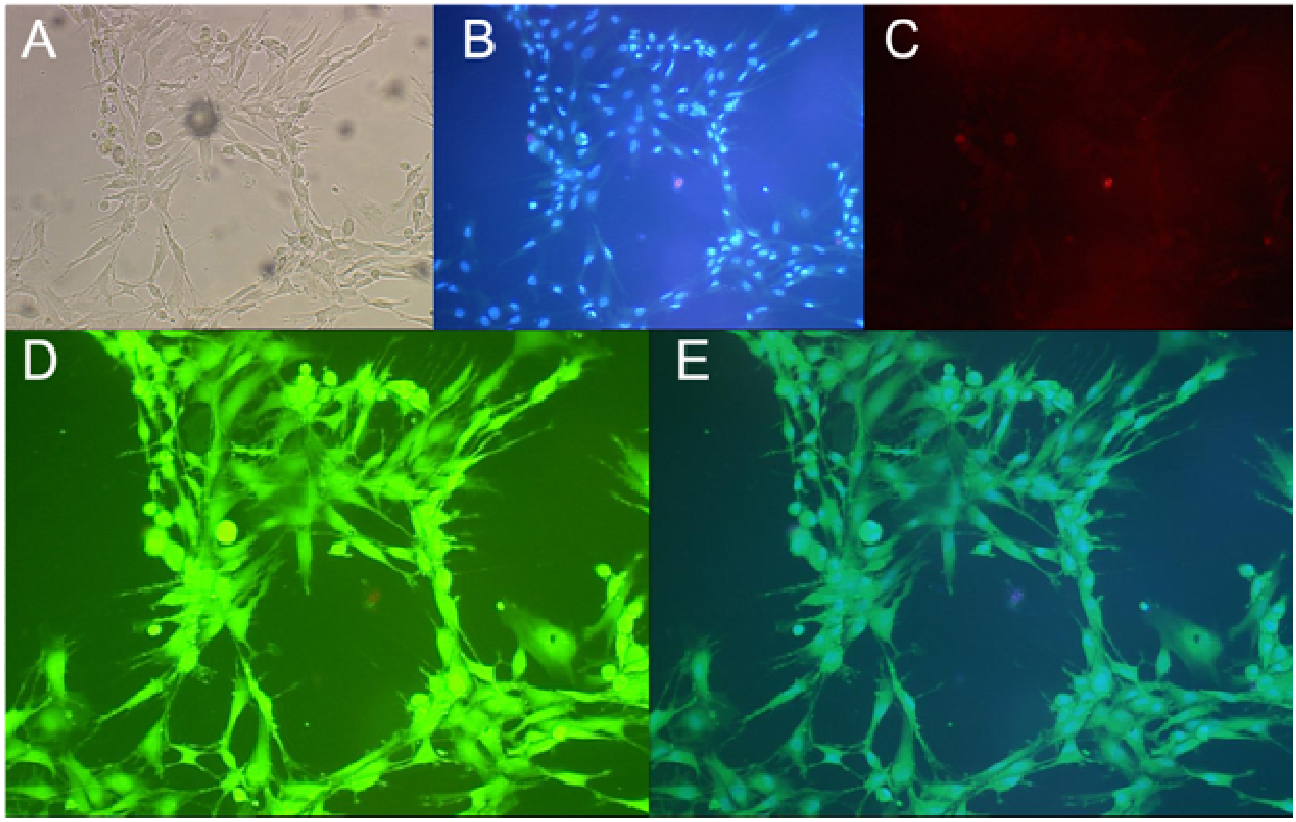

Figure 1. Live/dead assay of passaged osteoblasts at day 7. A, Bright field; B, blue nuclei of live cells stained with Hoechst 33342; C, dead cells stained with PI; D, live cells stained with Calcein AM; E, overlayed by B-D; A to E: magnification 100x. The pictures showed that the osteoblasts still had very high cellular viability.

consumption of individual cell within microbeads.

Statistical analysis

Results are expressed as the mean ± SD. The significant differences were assessed by either the Student t test or the ANOVA test.

RESULTS

In vitro culture of osteoblasts

Figure 1 A to E shows the cellular morphology and viability of passaged osteoblasts at day 7. The osteoblasts were fusiform or polygon, and cells extend out with quantity of protuberances which are different from each other in quantity, length and configuration with a tendency of forming cell colonies. The cells in the center of the colonies grew and overlapped with clear extracellular matrix. Figure 1 also shows that, the passaged cells have very high cellular viability. Figure 2 shows the cellular morphology and viability of encap-sulated osteoblasts within alginate microbeads. There was a large number of channels with a diameter of about 15 μm within a bead along its radius, through which the transfer of nutrients, cytokines and signaling mole-cules for cell growth and communication was facilitated. In addition, this figure also indicates that the cells are well distributed within microbeads with very high viability.

Biological assays for osteoblasts

Biological assays of cultured osteoblasts are shown in Figure 3. As shown in Figure 3A, ALP, a transient early marker of osteoblasts (Sun et al., 2004), is usually used to assess the osteoblastic characteristics of the isolated cells (Chaudhary et al., 2004). The cells cultured *in vitro* had higher ALP active expression. Normal phenotype, biggish bulk, numerous pseudopodia, rich cytoplasm and bulky nuclei are all situated on one side. Many blue-black granules of ALP positive reactions existed in cellular matrix and the majority of the positive cells presented squamate. Figure 3B shows that the *in vitro* cultured osteoblasts grow well and presented clear morphology.
Von-Kossa staining of mineralized nodules can present the end differentiation of osteoblasts (Sun et al., 2004). Mineralized and non-mineralized nodules could be distin-guished (Figure 3C). Mineralization and nodule formation

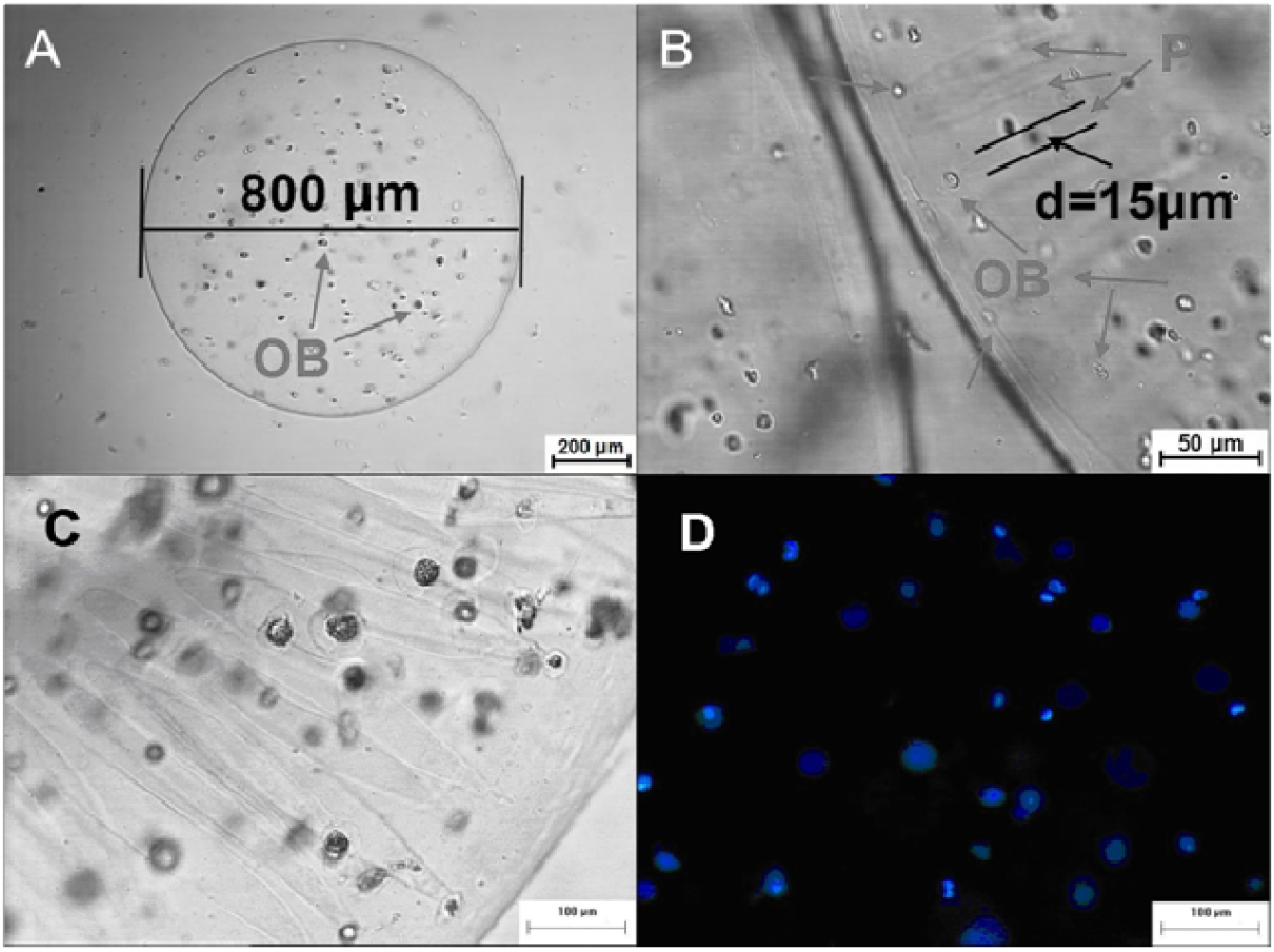

Figure 2. Cellular morphology and viability of osteoblasts within GAC microbeads. A, Osteoblasts within a whole microbead with 800μm of diameter, B, there were lots of porous channel towards the edge of the beads from the center, C, the cellular distribution of osteoblasts in part of microbeads, D, blue nuclei of live cells stained with Hoechst 33342 in part of microbeads.

could be determined also with Alizarin Red S staining (Figure 3D). Alizarin Red S staining showed obvious aggregates after 3 weeks of *in vitro* culture of osteoblasts. It therefore indicates that the osteoblasts cultured *in vitro* is suitable for use as seed cells for bone tissue engineering application and/or as trophoblast cells to expand hematopoietic stem/progenitor cells *ex vivo*.

In vitro growth curve of osteoblasts

Figure 4 shows the *in vitro* growth curve of passaged osteoblasts in static culture. After adaptation period of primary culture *in vitro*, the cells growth rate was up-regulated, and the exponential phase was achieved at the second day, resulting in three days of completed growth cycle. Therefore, the osteoblasts cultured in this study are capable of being used as seed and feeder cells for tissue engineering and stem cell investigations.

Oxygen consumption rates in different culture methods

The density of cell suspension was tested by a hemacytometer, and the oxygen consumption in different cultures was obtained via the dissolved oxygen meter. Three sections of consecutive time with the same interval were selected from the curve of oxygen consumption rate. Therefore, the oxygen consumption rate could be calculated according to Formula 1.

$$V = \frac{\frac{B-A}{t} \times V_c}{M} \quad \text{.....1}$$

Where, V is the oxygen consumption rate, B is the terminal value of oxygen consumption, A is the initial value of oxygen consumption, Vc is the cell volume and

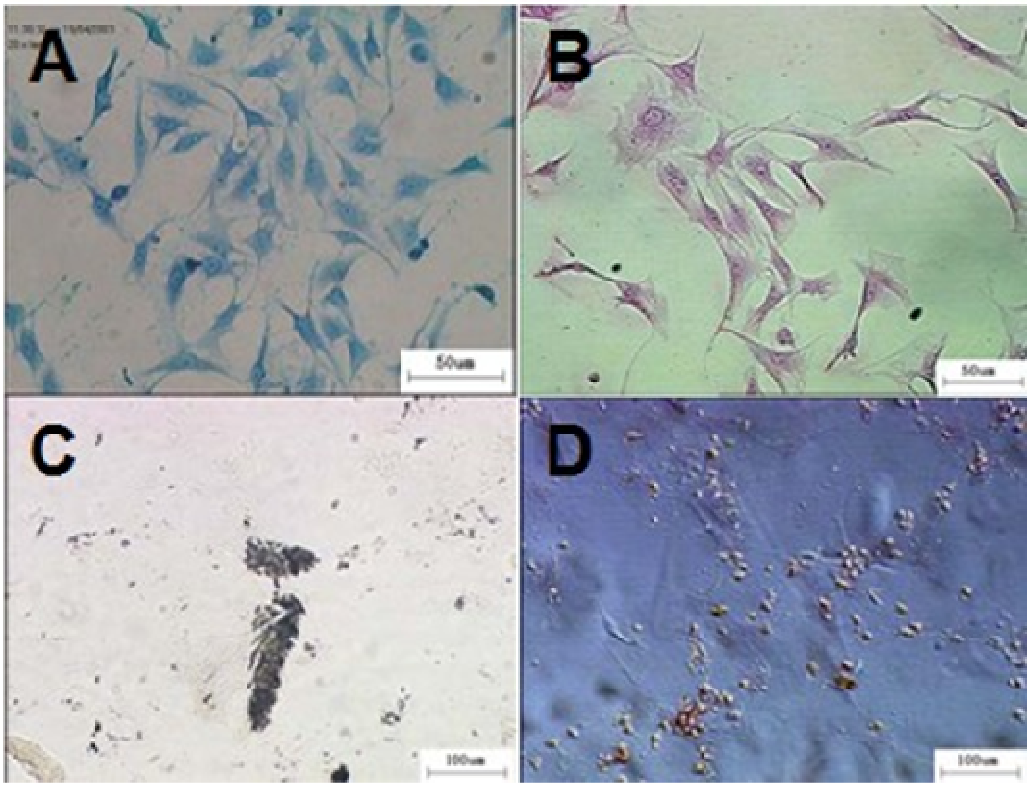

Figure 3. Biological assays of cultured osteoblasts. A, ALP staining; B, HE staining; C, Von-Kossa staining; D, Alizarin Red S staining. A to D: magnification 100×.

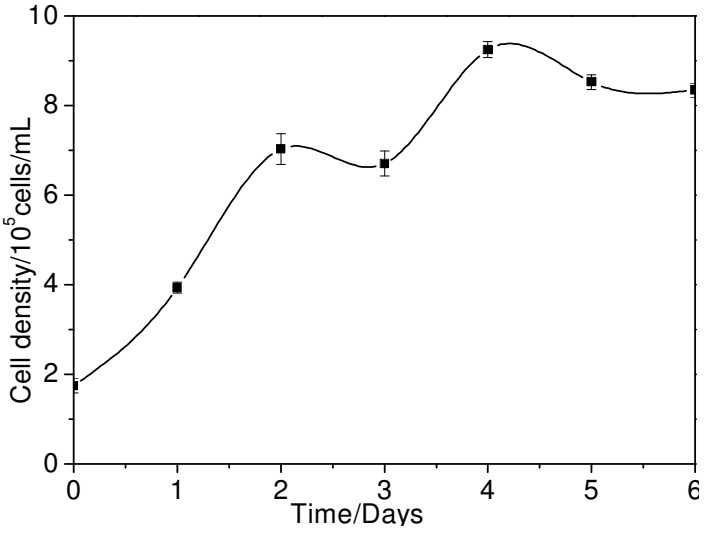

Figure 4. Growth curve of osteoblasts in static culture *in vitro*.

M is the cell number.

DISCUSSION

In this study, the oxygen consumption and oxygen consumption rates of osteoblasts cultured in different ways were detected by dissolved oxygen meter. The oxygen consumption rate of cells cultured in suspension and encapsulation medium were about 5.56×10^{-6} and 1.25×10^{-7} μmol/(min·cell), respectively (Table 1). These experimental data may provide a very significant

Table 1. Oxygen consumption and velocity of osteoblasts in different *in vitro* cultures.

Parameter	Culture	
	Flask culture	**Encapsulation culture**
Original value of oxygen consumption (μmol/L)	306.1	290.7
	294.8	253.1
	282.4	217.1
Terminal value of oxygen consumption (μmol/L)	296.3	253.1
	284.9	217.1
	272.0	180.5
Cell number (cells)	9.9×10^5	2×10^5
Cell volume (mL)	3.4	3.4
Interval time (min)	6	5
Oxygen consumption rate (μmol/min·cell)	5.56×10^{-6}	1.25×10^{-7}

parameter for *in vitro* culture, expansion and gene modification under different conditions (Heywood et al., 2010). Besides, the experimental results also indicate that the oxygen consumption rate of static culture is much higher than that of encapsulation culture. Mass transfer resistance caused by encapsulation can explain this phenomenon, but it confirms that the cells within micro-beads can still get enough oxygen supply since they show very high viability.

Up to now, the application of seed cells for tissue engineering and stem cell research combined with encapsulation techniques has been more and more widespread (Song et al., 2008, 2010; Zhu et al., 2008; Kd et al., 2009; Kedong et al., 2010), so parameters collection concerned with *in vitro* metabolism are therefore very crucial. In respect to the encapsulated microbeads, the oxygen dissolved in culture media can enter into them through those micro channels in bead walls, since the walls have minor thickness, which results in much lower oxygen consumption rate in encapsulation culture as compared to static culture. Meanwhile, the length of measuring time, to some extent, also affects the measuring result. If the testing time is too long, the temperature of liquid chamber of dissolved oxygen meter can not be maintained at 37°C, and the temperature of cell suspension will thus be changed with the environment, which might cause the changes of physiological activity with it, leading to the fluctuation of oxygen consumption rate, so the measuring time should not be too long.

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