

*Full Length Research Paper*

## Effect of the neurosphere size on the viability and metabolism of neural stem/progenitor cells

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Accepted 8 February, 2012

The objective of this study was to investigate the metabolic activity and viability of the mouse neural stem/progenitor cells (NSPCs) affected by the size of neurospheres. NSPCs dissociated from the forebrain of embryonic 14 days (E14) mice were cultured in flask for 120 h. During cultivation, the diameter distribution of neurospheres, cell viability and metabolic activities were monitored, together with the concentrations of glucose, lactate, glutamine and ammonia in the media. The results show that cell activity decreased with the increment of neurospheres size. When the diameter reached about 100  $\mu\text{m}$  and the concentration of glucose and glutamine were 36.38 and 1.33 mmol/L, the growth of central cells in neurospheres began to surface. Furthermore, when the diameter reached about 100 to 150  $\mu\text{m}$  and the concentrations of glucose and glutamine were 31.11 and 1.15 mmol/L, simultaneously, the death rate of NSPCs was larger than that of the newly born cells within the neurospheres. The metabolic activity of the cells declined to a very low level. This observation can be explained by diffusion limitation of nutrients and metabolic waste inside neurosphere. In conclusion, the mass transfer will be limited when the neurospheres size reaches a critical value of 100 to 150  $\mu\text{m}$  and beyond this critical value, serious impact of nutrient supply and metabolites on the cell viability and metabolism occurs.

**Key words:** Neural stem/progenitor cells (NSPCs), neurospheres, critical size, metabolism, mass transfer.

### INTRODUCTION

In recent years, neural stem/progenitor cells (NSPCs) have attracted great interest due to their multipotency and potential repair capacity towards the nervous system. Culture of NSPCs *in vitro* mainly relies on static suspension culture in the form of neurospheres (Cai et al., 2002; Gritti et al., 1996; Johansson et al., 1999; Reynolds and Weiss, 1996; Svendsen et al., 1998). Limitations of static cell culture are well recognized; among them, the main limitation is the difficulty in exchanging the oxygen and nutrients, which compromises cells and leads to cell death (Ma et al. 2008). Bioreactors such as spinner flasks and rotating, wall vessels have also been attempted to culture NSPCs but

with limited success (Kehoe, 2010; King and Miller, 2007). One of the reasons is the lack of knowledge and data of metabolism and growth characteristics of *in vitro* cultured neural stem cells, which are the prerequisite for design and operation of appropriate bioreactors.

Measurements of cell metabolism in suspension and monolayer cultures can be routinely done and such data have been employed to assess and improve bioreactor design and operation (Zeng and Deckwer, 1995). However, NSPCs are relatively new cells, and more importantly they have the natural tendency to form strong heterogeneous spheres which makes the measurement of metabolism parameters quite difficult. As a result, data with respect to NSPCs metabolism is quite limited. The glycometabolism of NSPCs was qualitatively investigated recently (Horie et al., 2004), and the study of metabolism of cells differentiated from neural stem cells was also studied (Abe et al., 2006). More systematic and quantitative metabolic analysis of suspended growing

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neurospheres is needed, in particular, considering the size effect of the neurospheres.

It is well known that cells growth and metabolism mainly depend on glucose, the oxidation of which can provide the highest efficient energy for cell metabolism, and it can also supply sufficient carbon source for the cell growth (Vander et al., 2010). Similarly, glutamine, another important nutrient substance, provides the cells with nitrogen source and participates in the synthesis of purine, pyrimidine, polypeptide and protein (Maranga and Goochee, 2006). Glucose and glutamine are regarded as the main nutrients; hence most of the serum-free mediums used to culture NSPCs belong to the category of high glucose and glutamine. The main metabolic products are lactate and ammonia. Therefore, glucose, glutamine, lactate and ammonia are all significant in studying the metabolism of NSPCs.

The size of the neurospheres is important to the viability and metabolism of NSPCs as transfer of nutrients gradually reduces along with increasing size of neurosphere, potentially leading to the formation of a necrotic core inside neurospheres and the neurospherers become hollow inside (Moeller and Dimitrijevic, 2004). In such cases, NSPC neurospheres consist of apoptotic, necrotic and phagocytic cells (Milosevic and Schwarz, 2004). The transport of nutrients and metabolites, mainly by diffusion, the size of neurospheres thus, become one of the most significant factors that affect the cell growth inside neurosphere.

In this paper, NSPCs from the embryonic forebrain tissues of embryonic 14 days (E14) mice were isolated and culture in suspension to form the neurospheres in serum-free medium. Neurosphere size, cell viability and the concentrations of glucose, lactate, glutamine and ammonia were on-line monitored during the culture process. After that the metabolic characteristics of neurospheres were further analyzed. Moreover, this research could provide a foundation for optimizing NSPCs culture conditions *in vitro*, designing the bioreactor for NSPCs large-scale expansion and providing the parameters for substance metabolism mathematic model with which the substance distribution can be simulated from the surface of neurosphere to the center.

## MATERIALS AND METHODS

### NSPCs isolation and culture

E14 Kunming strain mice (from the Animal Centre, Dalian Medical University, Dalian, China) were isolated following the procedure approved by the relevant ethical committee, sacrificed by cervical dislocation, and the embryonic forebrain tissues were dissociated for primary culture. The methods were described in previous literatures (Cai et al., 2002; Gritti et al., 1996; Reynolds and Weiss, 1996; Svendsen et al., 1998). Dissected tissue samples were transferred into a 15 ml test tube with 3 ml Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, without L-glutamate, glucose, phenol red, sodium pyruvate and sodium bicarbonate) and

triturated gently using a Pasteur pipette until the suspension looked cloudy; however, 20 to 30 strokes were sufficient for this purpose. The solution was allowed to stay for 10 min in ice bath, and then the top cell suspension was transferred to another test tube. For the undissociated tissue pieces, 2 ml DMEM culture medium was added and the step was repeated until the cell suspension looked homogeneous. The cell suspensions in the two test tubes were mixed and pelleted by centrifugation at 75 g for 5 min; supernatant with much cell debris were discarded. Cells were resuspended in 1 ml serum-free primary culture medium consisting of DMEM/F12/RPMI1640 mixture (1:1:1) (F12 and RPMI1640, Gibco) supplemented with Gibco (B27) and 2 mM glutamax (Gibco) containing 10 ng/ml of basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NJ) and 20 ng/ml of epidermal growth factor (EGF) (PeproTech, Rocky Hill, NJ), and counted using a hemocytometer.

Then, the responded cells were transferred to 75 cm<sup>2</sup> T-flask with a seeding density of 5×10<sup>4</sup> viable cells/ml in serum-free medium for primary culture. bFGF and EGF were replenished every three days when 1/4 medium was changed, allowing neurospheres to grow at 37°C in 95% air (21% O<sub>2</sub>) and 5% CO<sub>2</sub> in a humidified chamber until they were about 150 μm. Then neurospheres were harvested by centrifugation at 75 g for 3 min and then dissociated with Accutase™ (Sigma, 1×) for 15 min in 37°C water bath, with gently shaking of the centrifuge tube.

Fresh passage medium, N<sub>2</sub> substitute for B27 of the primary culture medium was added to the cells and seeded the cells at a density of 1×10<sup>5</sup> viable cells/ml to the 75 cm<sup>2</sup> T-flask for passage culture. The passage 3 (P3) NSPCs were used for the following analysis.

### Double-immunostaining for Hoechst and nestin

For immunostaining, NSPCs were incubated in PORN/laminin coated cover-slips until 50 to 70% confluent. The cells were fixed for 10 min in 4% paraformaldehyde (Sigma, St.Louis, MO, USA) and then washed twice in 0.01M phosphate buffered saline (PBS). After blocked with 10% goat serum containing 0.25% TritonX-100, cells were stained with mouse anti-nestin (Chemicon, Temecula, CA, USA) which were diluted (1:1,000) in antibody diluent (DAKO, USA) overnight at 4°C.

The cells were washed three times with PBS and incubated for 2 h in the dark with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated secondary antibody against mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After that cells were washed twice in 0.01 M PBS and incubated in PBS containing Hoechst33342 (10 ng/ml) (Sigma, St.Louis, MO, USA) for 15 min, then the cover slips were sealed with glycerol and visualized under the fluorescent microscope (Olympus, Tokyo, Japan).

### 5-Bromo-2-deoxyuridine (BrdU) incorporation

To identify actively proliferating cells, cells were incubated on PORN/laminin coated cover slips which were placed into 24-well plates. 5-Bromo-2-deoxyuridine (BrdU) (30 μg/ml) (Sigma, St.Louis, MO, USA) was added into the cultures and incubated for 24 h before fixation with 4% paraformaldehyde. Formamide was used for DNA denaturation at 100°C for 5 min. Then cells were washed several times with PBS and incubated for 1.5 h with a 1:50 dilution of the monoclonal mouse IgG anti-BrdU (Sigma, St.Louis, MO, USA) at 37°C. Cells incorporating BrdU were identified by rhodamine-conjugated secondary antibody against mouse IgG (1:500 dilution) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

### Analysis of viability

Cell viability was measured by the Cell counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) as described previously (Guan et al., 2009; Kanemura et al., 2002). Briefly, cell suspension ( $1 \times 10^6$  cells/ml) were diluted into a serial different grads concentrations (from  $1 \times 10^6$  to  $1.5 \times 10^4$  cells/ml), after adding 10  $\mu$ l CCK-8 solution into the cell suspension incubated at 37°C in 5% CO<sub>2</sub> for 4 h. Absorbance was read at 450 nm using a microplate reader (Microplate reader, Bio-Tek, USA) to make a standard curve about the absorbance and the viable cell number. To assay the NSPCs growth status, cells were detected using the CCK-8 every 12 h, and the cell viability was determined according to the standard curved made previously.

### Determination of neurospheres size

To determine size of the neurospheres at different culture time, 10 bright field images were randomly captured, respectively at each of the predefined time points of 12, 36, 60 and 72 h under the inverted microscope (Olympus, Tokyo, Japan). The size of the neurospheres was determined using image analysis and number of the neuroshperes were counted and grouped according to their diameters, 0 to 50  $\mu$ m, 50 to 100  $\mu$ m and 100 to 150  $\mu$ m.

### Metabolite analysis

To detect changes in glucose, lactate and glutamine concentrations during cell culture, culture samples were collected at each predefined time points and determined by using a semi-automatic analyzer (Shanghai ANTAI Diagnostics Co. Ltd., China) with glucose, lactate and glutamine concentration detecting kits (Nanjing Institute of Biological Engineering, China) for every 12 h in the time of culture. Ammonia concentration was determined by using indophenol blue colorimetry assay.

### Metabolic parameter calculation

Cell-specific growth rate is one of the most important parameters in biotechnological processes. The relationships between cell-specific growth rate, substrate consumption and product formation are crucial for monitoring and controlling cell culture processes. The cell-specific growth rate was calculated for any given time point using the following equation:

$$\mu = \frac{dQ(t)}{Q(t)dt} \quad (1)$$

Cell-specific consumption rates of the substrates (glucose and glutamine) were calculated for any given time point using the following equations:

$$q_{glu} = V \frac{dC_{glu}}{Q(t)dt} \quad q_{gln} = V \frac{dC_{gln}}{Q(t)dt} \quad (2)$$

The equation describing the cell-specific product rate is given as:

$$P_{lac} = V \frac{dC_{lac}}{Q(t)dt} \quad P_{NH_3} = V \frac{dC_{NH_3}}{Q(t)dt} \quad (3)$$

The cell yield coefficient equations describing the relationship between the number of cells and consumption of the substrates (glucose and glutamine) are given as:

$$Y_{\mu/q_{glu}} = \frac{\mu}{q_{glu}} \quad Y_{\mu/q_{gln}} = \frac{\mu}{q_{gln}} \quad (4)$$

The product yield coefficient equations describing the relationship between the yield of products (lactate and ammonia) and consumption of the substrates (glucose and glutamine) were given as:

$$Y_{lac/q_{glu}} = \frac{P_{lac}}{q_{glu}} \quad Y_{NH_3/q_{gln}} = \frac{P_{NH_3}}{q_{gln}} \quad (5)$$

Where, Q is the number of the living cells; V is the volume of the culture system (L) and C is the concentration (mmol/L).

### Statistical analysis

All of the data were expressed as the mean  $\pm$  SD from the three parallel experiments and significant differences were assessed by either the Student t-test or the analysis of variance (ANOVA) test. Differences were considered significant when the p-value was less than 0.05.

## RESULTS

### Growth morphology of isolated mouse NSPCs

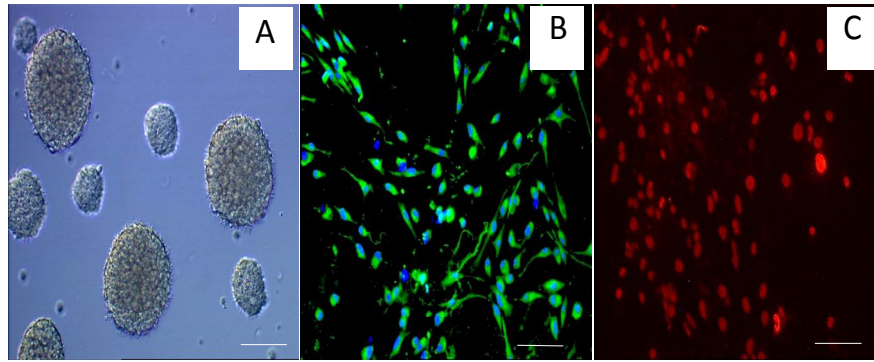
NSPCs derived from the embryonic forebrain tissues of E14 mouse began to form the suspended neurospheres 2 days after passage culture. After 5 to 6 days, average diameter of the floating spheres grew up to around 150  $\mu$ m and all of the neurospheres could be identified by their phase bright appearance with obvious diffraction rings (Figure 1A).

### Identification of NSPCs proliferation

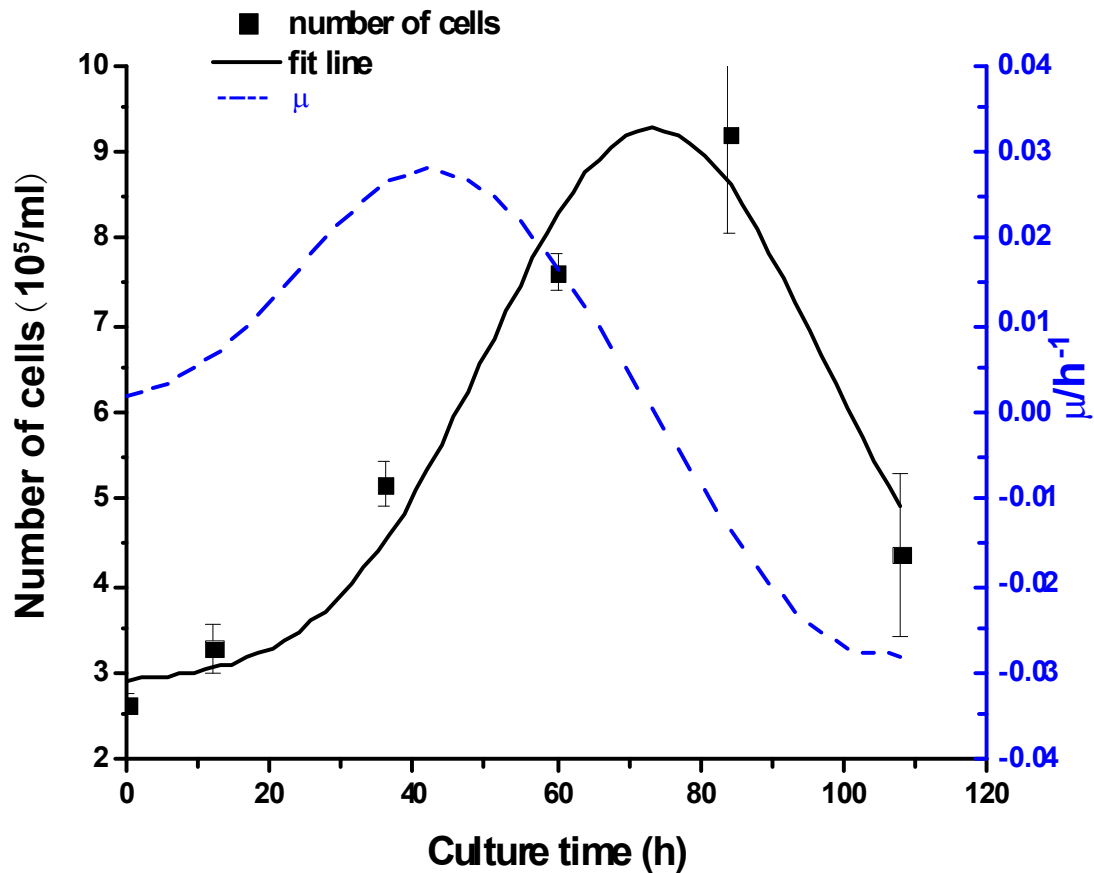
Neurospheres of P3 was stained for identification of the NSPCs marker protein nestin and proliferative capacity. The results in Figure 1B showed that a large number of cells in neruospheres were nestin-positive. In addition, BrdU positive result is shown in Figure 1C. Therefore, the cultured cells were NSPCs and can be used in our next study.

### Cellular activity in different sized of neurospheres

To investigate the correlation between viable cells in neurospheres and neurosphere size during proliferation, the growth curve of neurosphere cells in 5 days and the diameter of neurospheres were measured at the same



**Figure 1.** The morphology, differentiation and proliferation of neurospheres. A, Neurospheres formed by the passage of 3 NSPCs after 5 days culture; B, NSPCs stained by Nestin/Hoechst fluorescence; C, BrdU fluorescence stained cells; A-C, x100.



**Figure 2.** Growth curve and the cell-specific growth rates ( $\mu$ ) of NSPCs.

time. The results in Figures 2 and 3 showed that within the first 80 h, the cells showed typical exponential growth phase and the cell number increased about 3-fold. At the same time, the diameter of neurospheres increased from 0 to 50  $\mu m$  to 50 to 100  $\mu m$ . All these results indicated that the diameter of neurospheres was less than 100

$\mu m$ 108 h, the diameter of most neurospheres was in 100 to 150  $\mu m$ , and the activities of NSPCs represented a rapid declination, which was not observed in other cells that within 0 to 80 h, and the growth curve of neurosphere cells was relatively stable. However, in the period of 80 to 108 h, the neurospheres grew in non-spherical pattern. This special

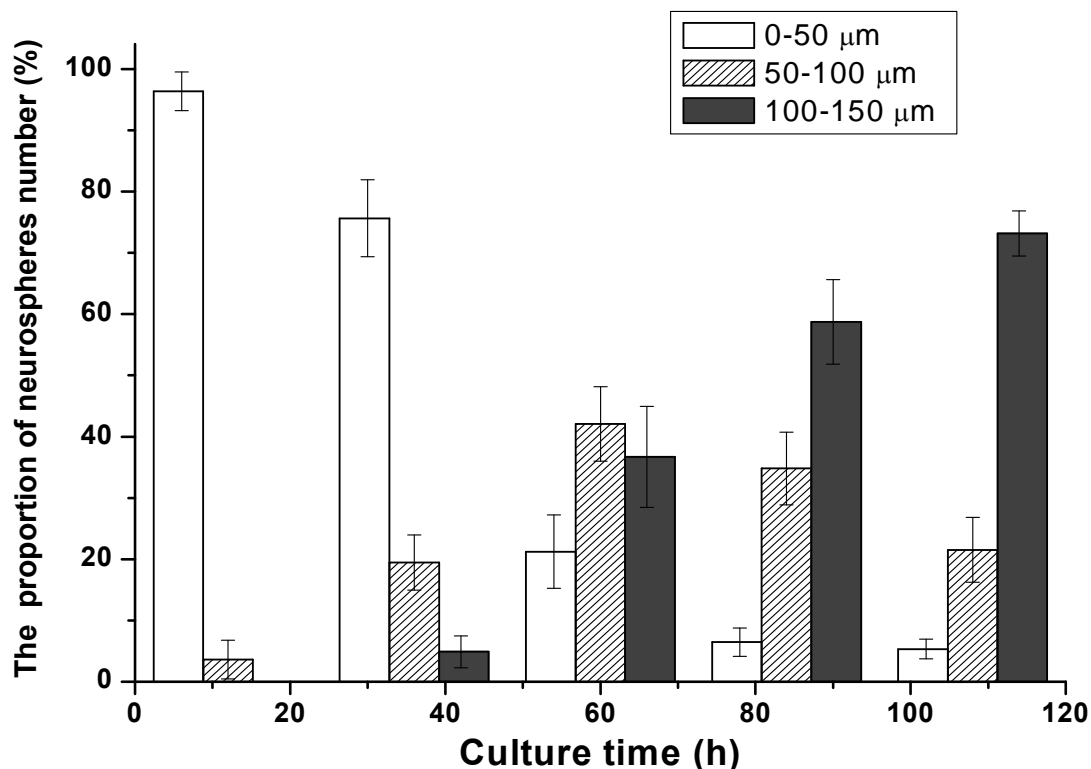


Figure 3. Distribution of neurospheres diameter at different culture time.

growth curve of NSPCs resulted from the neurosphere pattern. With the increase in neurosphere size, nutrient supply and metabolic waste removal became limited, resulting in large and rapid death of neurospheres in the cells.

It can also be figured out that cell-specific growth rate ( ) and proliferative ability of cells in the neurospheres continued to increase from 0 to 48 h of the culture time, and the diameters of most neurospheres were less than 100 μm in this period. Within 48 to 72 h, the neurospheres diameter reached about 100 μm; the proliferative ability of cells began to decrease although cell-specific growth rate remained positive. After 80 h, the diameter of most neurospheres exceeded 100 μm and the cell-specific growth rate turned negative value indicating that the number of cell began to reduce.

### Metabolism of glucose and lactate

Glucose consumption and lactate production during the time of NSPCs culture are shown in Figures 4 and 5. The concentration of glucose decreased steadily over time from the initial 40.8 to 27.3 mmol/L. Consistently, the special rate of glucose consumption (qglu) firstly increased to the maximum value  $3.27 \times 10^{-10}$  mmol/cell/h up to 36 h, then, qglu began to decrease to minimum value  $1.15 \times 10^{-10}$  mmol/cell/h. On the other hand, the

concentration of lactate increased gradually all over the culture time, but the ascending trend became slower after 48 h in the process of culture. At the end of 120 h culture, the lactate concentration was 7.4 mmol/L which was still lower than the inhibitory lactate concentration of 20 mM for mammalian cells (Ozturk et al., 1997; Patel et al., 2000). Meanwhile, the cell-specific lactate production rate (Plac) decreased rapidly from 4.00 to 0.31 mmol/cell/h.

### Metabolism of glutamine and ammonia

The consumption of glutamine and production of ammonia during the neurosphere culture are shown in Figures 6 and 7. It can be observed that the concentration of glutamine decreased steadily from 2.09 to 0.8 mmol/L and the cell-specific glutamine consumption rate (qgln) also decreased all the time during culture from  $5.02 \times 10^{-11}$  to  $1.67 \times 10^{-11}$  mmol/cell/h. The qgln showed that the decrease in qgln was slow during the first 24 h, while it became rapid between 24 to 48 h, after 48 h it was slow again.

Ammonia, a by-product of glutamine metabolism has a more harmful effect to cells than lactate. It can be observed from Figure 7 that the concentration of ammonia increased rapidly from 0 to 1.11 mmol/L and then became stable. At the same time, the cell-special ammonia production rate (PNH3) decreased sharply from

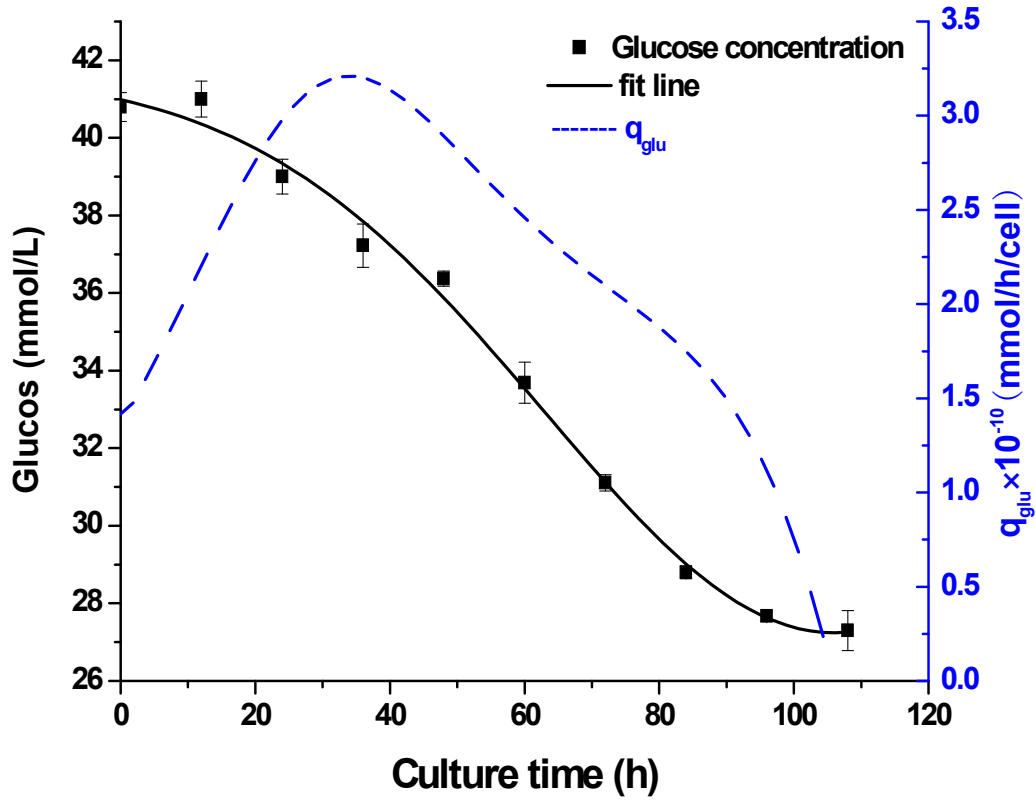


Figure 4. Glucose concentration curve and the cell-special glucose consumption rate ( $q_{glu}$ ) curve.

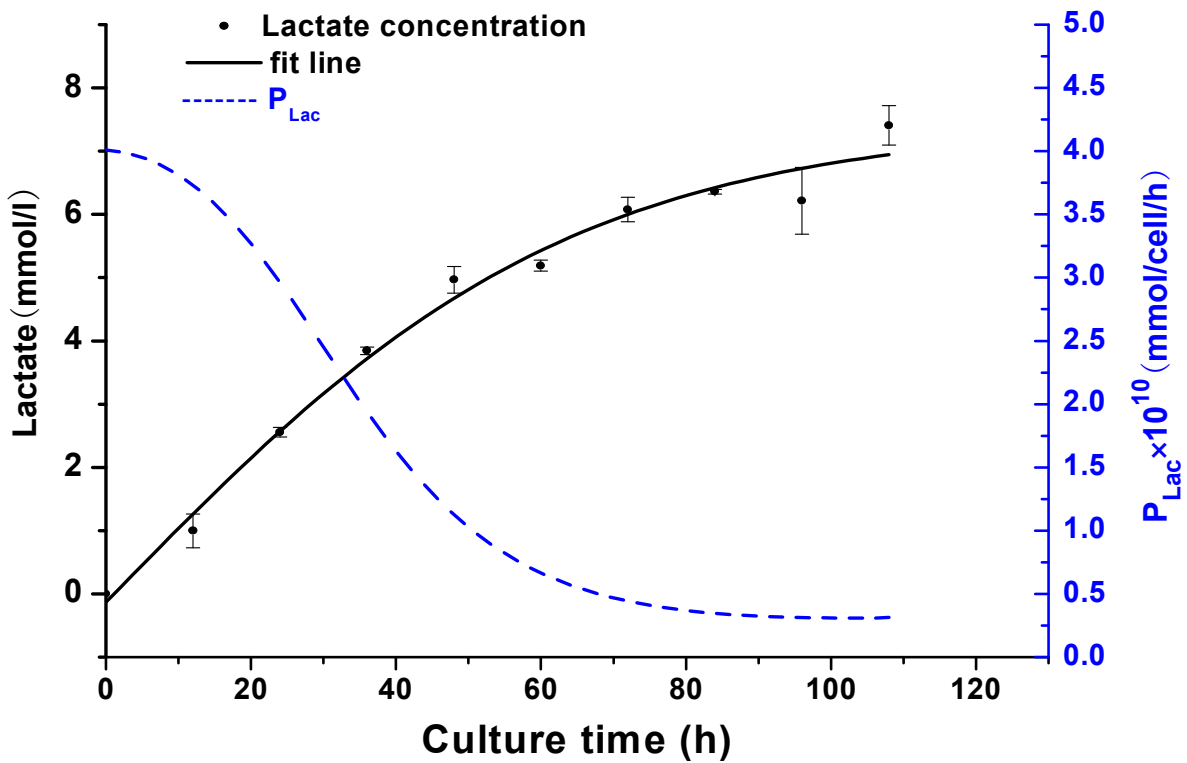


Figure 5. Lactate concentration curve and the cell-specific lactate production rate ( $P_{Lac}$ ) curve.

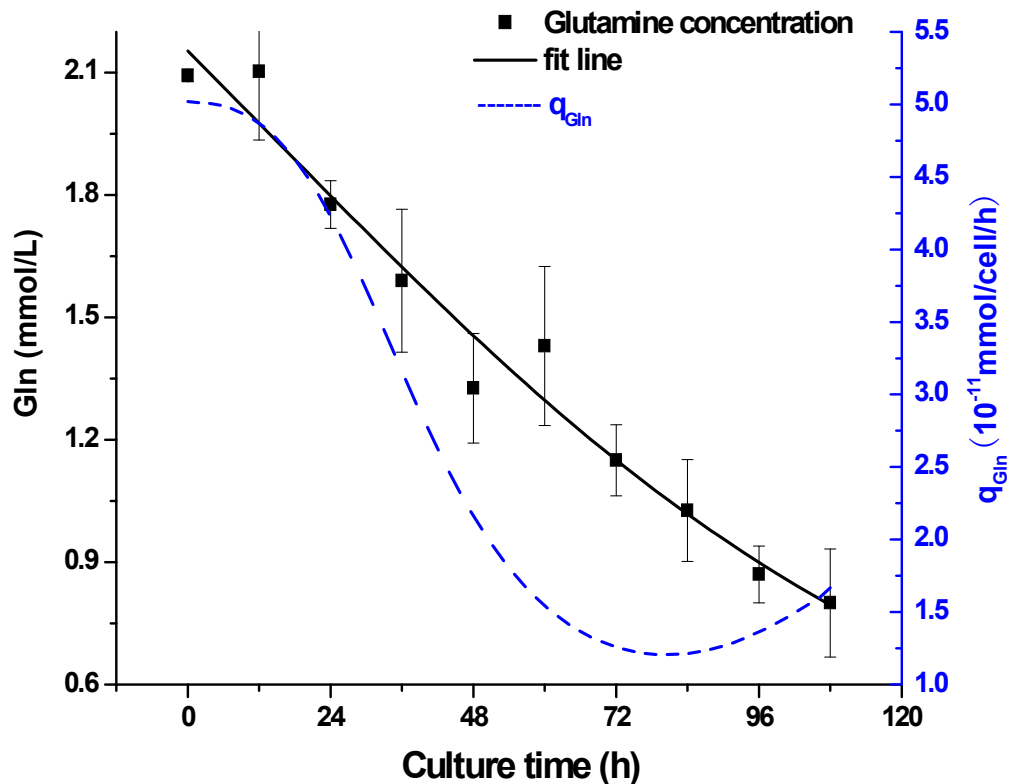


Figure 6. Glutamine concentration curve and the cell-specific glutamine consumption rate.

$2.95 \times 10^{-11}$  to  $0.25 \times 10^{-11}$  mmol/cell/h.

### Cell yield coefficient and product yield coefficient

As shown in Figures 8 and 9, the cell yield coefficient  $Y_{/qglu}$  and  $Y_{/qgln}$  had a similar trend in the neurosphere continuous culture process. They were both climbing up the peaks of the curves at 48 h ( $0.88 \times 10^8$  cells/mmol,  $12.19 \times 10^8$  cells/mmol), then decreased rapidly to a negative value at 72 h. As for the product yield coefficient,  $Y_{lac/glu}$  decreased rapidly from 3.09 to 0.22;  $Y_{NH3/gln}$  also decreased, but the downtrend was gradual relative to  $Y_{lac/glu}$  after reaching a peak value of 0.59 then decreased to the lowest point 0.15.

### DISCUSSION

The application of NSPCs for the treatment of neurological disorders (for example, Parkinson's disease) is a significant program in regenerative medicine field (Conti et al., 2006; Kim, 2007; Lim et al., 2007). It is well known that NSPCs *in vitro* usually form spheres from a single cell with suspension culture technique (Deleyrolle and Reynolds, 2009; Ostenfeld et al., 2002; Svendsen et al., 1998). Lots of investigations have been reported about

mouse NSPCs proliferation, differentiation and spontaneous apoptosis *in vitro* in neurospheres (Abe et al., 2006; Milosevic et al., 2006; Nelson et al., 2008) as well as morphological and ultra-structural characterization of neurospheres. However, little is known about the metabolism characteristics of *in vitro* cultured NSPCs, which compels us to optimize the NSPCs culture conditions *in vitro* and design appropriate bioreactor for NSPCs large-scale expansion. The main objective of this study was to investigate the NSPCs metabolic activity with respect to the sizes of neurospheres. Toward this end, we monitored the changes in neurospheres sizes, cell viability and the change in concentrations of glucose, lactate, glutamine and ammonia in the media during the whole cells cultivation. At the same time, we calculated the  $\mu$ ,  $q_{glu}$ ,  $Plac$ ,  $q_{gln}$ ,  $PNH3$ ,  $Y_{/qglu}$  and  $Y_{/qgln}$ . The results show the mass transfer of the nutrients and metabolic waste was limited when the neurospheres size reaches a critical value of 100 to 150  $\mu m$ , and beyond this critical value, serious impact of nutrient supply and metabolites on the cell viability and metabolism occurs. This conclusion was verified by the calculations of  $\mu$ ,  $q_{glu}$ ,  $Plac$ ,  $q_{gln}$ ,  $PNH3$ ,  $Y_{/qglu}$  and  $Y_{/qgln}$ .

Cell-specific growth rate ( $\mu$ ) is a global measurement of proliferation performance of a cell culture population providing a quantitative parameter for cell growth during the exponential phase (Dos et al., 2010). So we first

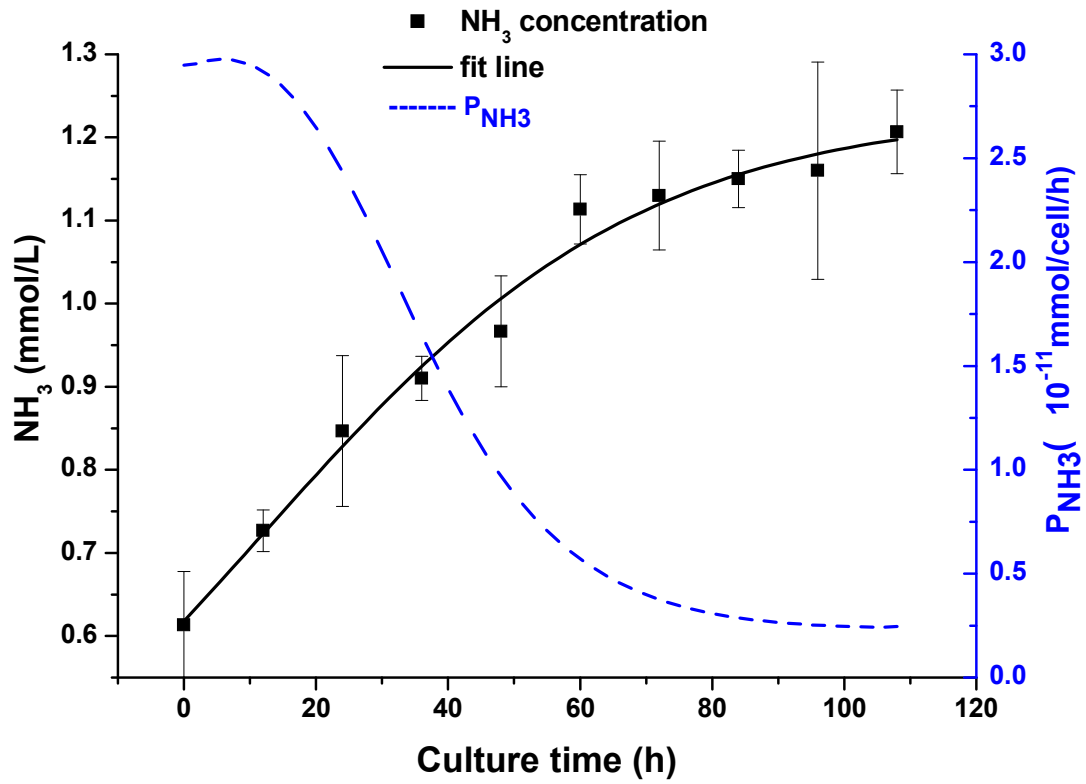


Figure 7. NH<sub>3</sub> concentration curve and the cell-special ammonia production rate (P<sub>NH3</sub>) curve.

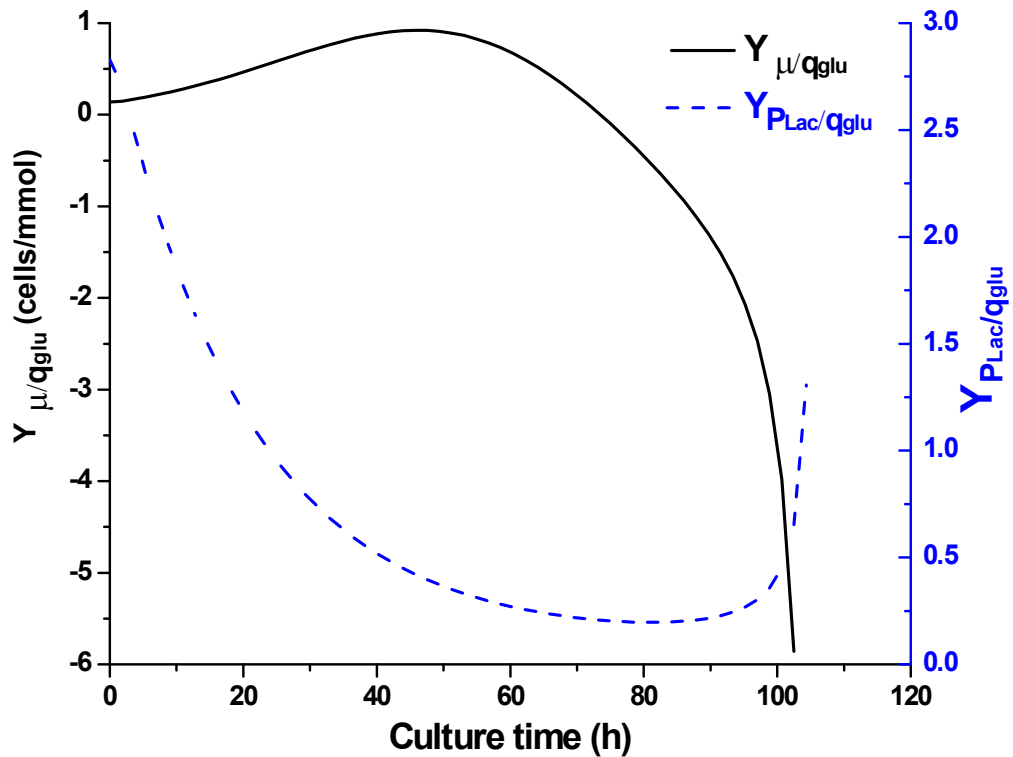


Figure 8. Curves of Y<sub>μ/qglu</sub> and Y<sub>Lac/qglu</sub>.



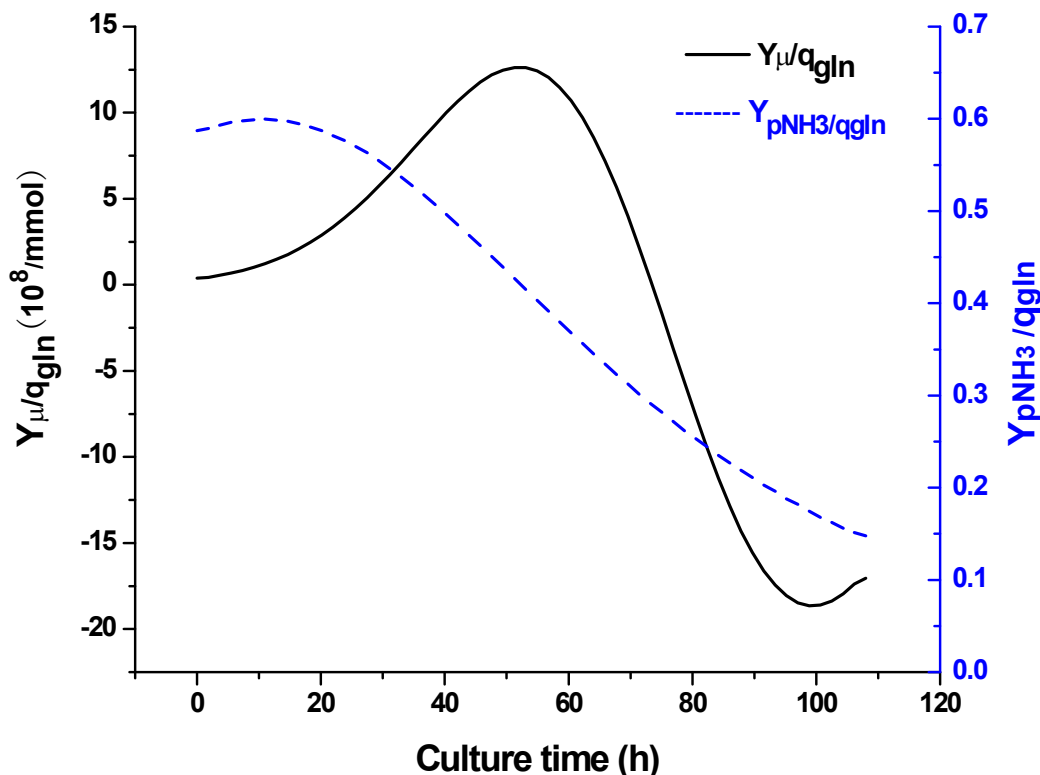


Figure 9. Curves of  $Y_{\mu}/q_{gln}$  and  $Y_{pNH3}/q_{gln}$ .

analyzed the change of  $\mu$  with different size of neurospheres and found it varied and was considerably influenced by the size of neurospheres, unlike non-aggregating cells. Quite obvious and distinct distributions of nutrients and metabolites as well as oxygen inside neurospheres can affect the cell metabolism, which in turn can result in the variation of cellular growth and viability.

Glucose and glutamine are the major energy sources for animal cells in standard culture medium. The fate of glucose in cells is to generate the lactate by anaerobic glycolysis or acetyl-CoA into the tricarboxylic acid (TCA) cycle by a number of intermediate metabolites (Xie and Wang, 1994). The switch between the glycolysis and the TCA cycle is closely linked to the availability of oxygen. Oxygen is required for the electron transport chain to function which recycles nicotinamide adenine dinucleotide (NADH) back to  $NAD^+$  and flavin adenine dinucleotide (FADH<sub>2</sub>) back to FADH, providing  $NAD^+$  and FADH are required by enzymes in the TCA cycle. If the oxygen supply to cells is low,  $NAD^+$  and FADH levels fall, the TCA cycle cannot proceed forward, and the cell must resort to anaerobic glycolysis to continue making adenosine-5'-triphosphate (ATP). On the other hand, the accumulation of metabolic byproducts, lactic acid and ammonia, will limit cell yields. In our experiment, we found that high glucose concentration was needed as carbon source and energy source for cell proliferating

during the first 0 to 36 h. At this time, the diameter of neurospheres were mainly under 50  $\mu$ m and small enough for nutrient and  $O_2$  transfer as well as the metabolic by-product removal. As a result, the  $q_{glu}$  rapidly increased. But on the other hand, the high concentration of glucose in the culture medium would cause the cells to use the glucose via glycolysis pathway. Therefore, the utilization of glucose was incomplete, only a fraction of glucose entered the TCA cycle for supplying the energy and a mass of lactate was produced. With the decrease of glucose concentration, the more glucose switch to the higher-efficient TCA cycle, and the  $Plac$  decreased markedly. After 36 h, with the increase of the size of neurospheres and the decrease of glucose concentration inside and outside neurospheres, both  $q_{glu}$  and  $Plac$  also declined.

Additionally, the initial concentration of glutamine of about 2 mmol/L was lower than the initial concentration of glucose in the culture medium; glycolysis has provided a majority of energy and the complete oxidation of glutamine for energy was limited in cells. For this reason, most of the glutamine was used for biosynthesis through incomplete oxidation metabolism pathway and more ammonia was produced as the metabolic by-products during this period. As the main energy supplier, the concentration of glucose could not meet the demands of NSPCs within the neurospheres due to the increase of their size. Following to this, the metabolic pathway

concerned with glutamine switched from incomplete oxidation to a complete one. As a result, less ammonia was produced in late stage of the culture (Xie and Wang, 1994, 1996).

Furthermore, the concentration of glucose and glutamine in the medium were both further descending with the increase of most neurosphere size after 72 h. The concentrations of glucose and glutamine within the neurospheres would also decrease rapidly due to the resistance of mass transfer, which could result in the decrease of the proportion of glycolysis in glucose metabolism and the proportion of glutamine incomplete oxidation in glutamine metabolism within the neurosphere. Therefore, the lactate and ammonia yield coefficient remained at a lower level.

NSPC-based therapy has received much attention due to its potential as a therapeutic strategy for neurodegenerative disorders. Sufficient number of cells is the fundamental guarantee for clinical treatment. Many of the molecular mechanisms involved in neurosphere formation, growth and cells metabolism are gradually clear, therefore, further research is needed to operate NSPCs large-scale expansion *in vitro* based on the understanding of the NSPCs characteristics. Our findings provide a method to evaluate the NSPCs metabolic activity with respect to the sizes of neurospheres involved in glucose (or glutamine) consumption and the effects of lactate (or ammonia) production, and then we could create a mathematical model based on the data from our experiments for predicting nutrient/metabolic waste distribution inside the neurospheres.

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