Expansion of mouse sertoli cells on microcarriers

B. Shi*, S. Zhang*, Y. Wang*, Y. Zhuang*, J. Chu*, S. Zhang*, X. Shi†, J. Bi‡ and M. Guo*

*State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China, †Reproductive Medical Center of Capital Medical University, Beijing, China, and ‡School of Chemical Engineering, The University of Adelaide, Adelaide, SA, Australia

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Abstract

Background: Sertoli cells (SCs) have been described as the ‘nurse cells’ of the testis whose primary function is to provide essential growth factors and create an appropriate environment for development of other cells [for example, germinal and nerve stem cells (NSCs), used here]. However, the greatest challenge at present is that it is difficult to obtain sufficient SCs of normal physiological function for cell transplantation and biological medicine, largely due to traditional static culture parameter difficult to be monitored and scaled up.

Objective: Operational stirred culture conditions for *in vitro* expansion and differentiation of SCs need to be optimized for large-scale culture.

Materials and methods: In this study, the culturing process for primary SC expansion and maintaining lack of differentiation was optimized for the first time, by using microcarrier bead technology in spinner flask culture. Effects of various feeding/refreshing regimes, stirring speeds, seed inoculum levels of SCs, and concentrations of microcarrier used for expansion of mouse SCs were also explored. In addition, pH, osmotic pressure and metabolic variables including consumption rates of glucose, glutamine, amino acids, and formation rates of lactic acid and ammonia, were investigated in culture.

Results: After 6 days, maximal cell densities achieved were 4.6×10^6 cells/ml for Cytodex-1 in DMEM/FBS compared to 4.8×10^5 cells/ml in static culture. Improved expansion was achieved using an inoculum of 1×10^5 cells/ml and microcarrier concentration of 3 mg/ml at stirring speed of

30 rpm. Results indicated that medium replacement (50% changed everyday) resulted in supply of nutrients and removal of waste products inhibiting cell growth, that lead to maintenance of cultures in steady state for several days. These conditions favoured preservation of SCs in the undifferentiated state and significantly increased their physiological activity and trophic function, which were assessed by co-culturing with NSCs and immunostaining.

Conclusion: Data obtained in this study demonstrate the vast potential of this stirred culture system for efficient, reproducible and cost-effective expansion of SCs *in vitro*. The system has advantages over static culture, which has major obstacles such as lower cell density, is time-consuming and susceptible to contamination.

Introduction

Sertoli cells (SCs) are recognized as the ‘nurse cells’ of the testis whose function is to secrete essential growth factors, trophic factors, regulatory proteins and nutritive factors to create an appropriate environment for development of other cells (for example, germinal and nerve stem cells, as used here) (1). These factors include transforming growth factors (TGF- α and β), insulin-like growth factor I (IGF-I), basic fibroblast growth factor (bFGF or FGF2) (2), platelet-derived growth factor (PDGF) (3,4) and neurturin (NTN) (5). SCs express cytokines such as interleukin-1 α (IL-1 α) and interleukin-6 (IL-6) that have also been shown to have trophic effects on dopaminergic neurons (6,7). Trophic factors such as sulphated glycoproteins 1 (prosaposin) (8) and 2 (clusterin) (9), desert hedgehog (Dhh) (10) [a member of the Sonic hedgehog (Shh) family] is also produced by SCs, which seems to stimulate differentiation of dopaminergic neurons (11). Co-culturing of SCs with foetal ventral mesencephalon (VM) has resulted in a remarkable increase in number of surviving TH-positive neurons, and soma size and neuritic outgrowth were

Correspondence: M. Guo, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China. Tel.: +86 21 64251131; Fax: +86 21 64253702; E-mail: guo_mj@ecust.edu.cn

enhanced (12). Cameron and colleagues reported that 16-to 19-day-old SCs enhanced survival of foetal neurons or cells of a human teratocarcinoma line hNT neurons, upon thawing, when exposed to conditioned media from SCs (13). Moreover, number of tyrosine hydroxylase (TH+) neurons of both rat and human ventral mesencephalic tissues, together with human neuroteratocarcinoma cells (hNT), were significantly higher when co-cultured with SCs from 2-month-old pigs (14,15). Furthermore, soma size and neurite outgrowth significantly increased in the co-culture group compared to control cultures. When mouse SCs were transplanted alone (allograft) into the 6-hydroxydopamine-lesioned striatum (12), SCs survived and enhanced the rats' behavioural recovery. Moreover, SCs promoted graft survival of co-transplanted bovine adrenal chromaffin cells (xenografts) (16–18) in normal rat striatum.

Cell transplantation is a promising approach for restoration of function in neurodegenerative conditions such as Parkinson's disease (PD). Neural transplantation in PD is to provide dopamine-producing cells for the striatum to substitute those mesencephalic dopamine neurons that have been lost. The major obstacle to this approach is extensive cell death during isolation and grafting (19–21), co-transplantation of Sertoli cells with therapeutic neurons is a promising way to overcome this (22,23).

As SCs are only available in small numbers in the human body, it is necessary to optimize their culture process *in vitro* suspension to maximize primary population for cell transplantation or for other uses. Typically, multiplication of SCs is performed in two-dimensional (2D) cultures or plastic tissue culture, which has serious drawbacks such as time consumption and susceptibility to contamination due to increased number of cell passages necessary, although there is limited availability of surface area and nutrients. Furthermore, culture conditions tend to be suboptimal due insufficient monitoring and control of culture (24). To expand SCs in a controlled reproducible and cost-effective way, development of appropriate bioreactors could be an alternative option.

For development of a bioreactor system, it is important to understand the demands of the specific cells for optimal survival and expansion. Cell growth is inhibited by shortage of nutrients or by excess metabolites. To obtain optimal culture conditions, the feeding/refreshing regime is crucial (25–27), nutrients such as glucose, glutamine and other amino acids are consumed while, metabolites such as lactate and ammonia accumulate and are toxic to the cells. These could inhibit cell expansion at certain concentrations (28–31). To prevent growth inhibition by metabolite production and/or nutrient deficiency, the feeding regime always needs to be optimal. Cell number expansion is correlated to nutrient consumption and metabolite production,

so cell proliferation can be monitored in the bioreactor indirectly by monitoring metabolism (27,32,33). To date, investigation of metabolism related growth of SCs in a microcarrier bead-based culture system has, to the best of our knowledge, not been published. The main aim of this investigation has been to optimize the culture process of primary SCs by microcarrier technology in spinner flask culture and to evaluate effects of various feeding/refreshing regimes, stirring speeds and inoculation concentrations on expansion of mouse SCs. In addition, SCs have been co-cultured with neural stem cells (NSCs) to test nutritional functions of SCs after microcarrier culture.

Materials and methods

Sertoli cell isolation

ICR mice were purchased from the BIKAI experimental animal center (Shanghai, China) and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sertoli cells were isolated from the mice at 12 days according to the method of Steinberger, with some modifications (34). Briefly, testes were decapsulated, minced and rinsed twice in phosphate-buffered saline (PBS), then digested with 0.25% trypsin and 0.1% collagenase-1(1:1) (Sigma, St. Louis, MO, USA) at 31 °C for 30 min. Isolated testicular fragments were centrifuged at 99 g for 10 minutes and washed twice with DMEM supplemented with 10% foetal bovine serum (FBS; Gibco, Grand Island, NY, USA) to terminate digestion and fragments were filtered through a 200-mesh stainless steel filter. Cells were collected by centrifugation, supernatant was discarded and the collected cell pellet was resuspended in DMEM supplemented with 10% FBS, 1.5 g/l NaHCO₃ (Lingfeng, CA, China), 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) and 100 µg/ml streptomycin (Invitrogen). Finally, the dispersed cells were seeded into culture flasks and maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C for 10 min; cell culture fluid was transferred to other flasks to remove spermatogenic cells. Sertoli cells attached to the bottom of T-flasks 2 days later, and the supernatant was discarded. Then the purified Sertoli cells quickly spread to form a monolayer in the new medium. When cells on surfaces of T-flasks were nearing confluence, they were washed in PBS and enzymatically harvested using 0.25% trypsin in 1 mM EDTA solution (Invitrogen), followed by replating at 500 cells/cm² (passage 1).

Monolayer pre-culture

The SCs were plated at 2500 cells/cm² in DMEM proliferation medium which was replaced once every 3 days.

At near-confluence, cells were subcultured to passage 3 as described above. Harvested passage 3 cells were used for 3D microcarrier bead culture in spinner flasks.

Microcarrier culture in spinner flask

For stirred microcarrier bead cultures, 100 ml spinner flasks (Bellco Glass Inc., Vineland, NJ, USA; model #1965) were used with final medium volume of 80 ml and stirring speed of 20, 30 and 40 rpm respectively. Spinner flasks were siliconized before use with Sigmacote (Sigma) and in which SCs were also cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

Required quantities of Cytodex-1 were weighed (final concentrations 1.0, 3.0 and 5.0 mg/ml, respectively), hydrated and sterilized by autoclaving, as recommended by the manufacturers. Microcarriers were equilibrated in serum-free culture medium for at least 30 min prior to cell addition, to maximize cell attachment. Cells were inoculated on to microcarrier beads at 50% the final volume. Intermittent stirring (3 min every 1 h) was carried out for 6 h. After this, volume was increased to 75% and intermittent stirring was performed for another 6 h. Finally, volume of medium in the spinner was increased to 100% and constant stirring speed was initiated. During the culture period, 50% fresh medium was replaced every day starting from day 2, that is microcarriers were allowed to settle for 10 min, 50% supernatant was discarded and new medium was added.

Sampling

Spinner flasks were removed from the incubator and placed in a laminar flow hood. Agitation was interrupted and at 24-h intervals and samples were collected immediately to minimize variation occurring as a result of sedimentation of microcarriers. Then, collected samples were stored at -20 °C for prospective analysis of glucose, lactic acid, amino acids and ammonium in the medium; pH and osmotic pressure were also measured.

Cell number and viability

Cell density was determined using a haemocytometer and viability was assayed using standard trypan blue dye exclusion. For sample preparation, microcarriers attached to cells were washed twice in PBS followed by trypsin (0.25%) (v/v) addition, then incubated for 3 min in a 37 °C water bath. Occasional flicking was performed to facilitate detachment of cells from the beads. DMEM with 10% foetal bovine serum was added to stop trypsinization and supernatant with cells was collected into new tubes. Beads were washed twice to detach remaining cells and

supernatants were pooled. Cells were centrifuged and suspended in fresh medium. Samples were then taken for counting and cell staining. Each experiment was performed in triplicate.

Measurement of metabolite in media

Supernatant samples were collected throughout the experiments, centrifuged for 10 min at 197 g and stored at -20 °C for subsequent analysis. Samples were treated according to instructions provided on the assay kit before analysis and glucose, L-glutamine, lactate and ammonia concentrations were measured using the kit (Jiancheng Technology, Nanjing, China). Amino acid concentrations were measured using methods for quantitation of amino acids with *O*-phenyl isothiocyanate (OPA, Fluka, Switzerland). Resulting phenylthiocarbamyl derivatives were assayed by reverse-phase high-performance liquid chromatography (HPLC) (HP1100; Hewlett Packard, Waldbronn, Germany).

Estimation of specific growth rate and rate of metabolite formation

Specific growth rate (μ) was calculated by plotting logarithm of viable cell density *versus* culture time during exponential growth phase based on eqn (1):

$$\mu = \frac{1}{t} \ln \frac{C_t}{C_0} \quad (1)$$

where t is culture time (days), C_t is viable cell density (cells/ml) at time t and C_0 is inoculation concentration.

Specific consumption or production rates of glucose (q_{Glc}), L-glutamine (q_{Gln}), lactate (q_{Lac}) and ammonia (q_{Amm}) were calculated according to eqns (2) and (3) based on data collected during growth phase and evaluated from plotting substrate and product concentrations against integrated viable cell density.

$$q_{\text{Glc/Gln}} = -\frac{1}{C} \cdot \frac{dS}{dt} \quad (2)$$

$$q_{\text{Lac/Amm}} = \frac{1}{C} \cdot \frac{dP}{dt} \quad (3)$$

where C is viable cell density (cells/ml), S is substrate concentration, P is product concentration and t is time (days).

Lactate yield on glucose consumed ($Y_{\text{lactate/glucose}}$, mmol/mmol) was calculated by dividing specific lactate production rate by specific glucose consumption rate. Yield ($Y_{\text{ammonia/glutamine}}$, mmol/mmol) was calculated by dividing specific production rate (ammonia) by specific consumption rate (L-glutamine).

Neuronal stem cell isolation and culture

Primary neuronal cultures derived from day 14 mouse embryos (E14) were performed as previously described, with some modifications (Daadi *et al.*, 1999). In brief, dorsal-most aspect of medial and lateral ganglionic eminences, cortex and mesencephalon were dissected and mechanically dissociated (separately) using a fire-polished Pasteur pipette in DMEM/F12 (1:1) medium supplemented with 10% FBS. Cells were plated at density 10^6 cells/ml on poly-L-ornithine-coated (15 mg/ml; Sigma) glass coverslips in 24-well Nunclon culture dishes at 0.5 ml/well. Under these culture conditions, 98% of cells exhibited neuronal morphology [for detailed description of the bioassay, see Daadi *et al.* (1999)]. In cultures maintained for 1 week, half cell culture medium was replaced after 3 and 5 days *in vitro* (DIV), and incubated at 37 °C in 95% air/5% CO₂ humidified atmosphere.

Cell co-culture procedures

NSCs, NSCs + SCs_{2D} (obtained from two-dimensional culture) and NSCs + SCs_{3D} (obtained from microcarrier culture) were seeded at density of 200 000 cells/cm² on poly-L-lysine coated six-well chamber slides (10 µl/ml; Sigma) in DMEM with 10% FBS and 50 µg/ml gentamicin (Sigma). In the co-culture, cells were plated 1:1 and plating density was maintained at 200 000 cells/cm². After 24-h incubation, media changes (50%) were performed each day. Cells were then incubated at 37 °C in a 95% air/5% CO₂ humidified atmosphere.

Microscope observation

Cell expansion on control plates and on Cytodex-1 was observed on a daily basis using an inverted microscope. Samples of microcarrier beads were taken on days 2, 4, 6 and 8 and incubated at 37 °C for 45 min with MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) to assess metabolic activity. MTT, a soluble yellow salt, is converted by mitochondria into insoluble purple formazan salt. Meanwhile, samples of microcarri-

ers were checked daily using a scanning electron microscope (SEM).

Immunohistochemistry

To identify SCs and NSCs, immunohistochemistry was performed using a Fas-L staining kit (Boshide Technology, Wuhan, China); NSCs were identified using a nestin immunofluorescence kit (Boshide Technology). Cells were processed and stained according manufacturers instructions.

Results

Effects of stirring speed and inoculum on cell expansion

Viscosity of fresh culture media with 3 mg/ml Cytodex-1 at 37 °C was determined using a Couette viscometer (Brookfield, Stoughton, MA, USA). Media were found to be Newtonian fluids with viscosity of approximately 1cPa. Specific density of fresh media was determined using a balance and graduated cylinder, and found to be approximately 1.01. Rheology of media in the spinner was simulated by computational fluid dynamic (CFD) (ANSYS ICFD10.0, UK) at stirring speed of 20, 30 and 40 rpm, respectively. Corresponding shear stresses (τ) were 0.8025, 1.3402 and 1.8025 Pa. Samples of microcarriers were checked using an inverted microscope on day 2, and it was observed that the majority of SCs had successfully attached to microcarriers at stirring speed of 30 rpm, resulting in almost all beads being covered by cells in the spinner; however, only 40% of cells attached to microcarrier beads at stirring speed of 20 rpm, and almost 10% of microcarrier beads were crushed by the impeller at rotation rate of 40 rpm (shown in Fig. 1).

Effect of inoculum on expansion of SCs was investigated by performing control experiments in both six-well plates and in spinner flasks. Cells were seeded at three different concentrations, 0.5×10^5 , 1×10^5 and 2×10^5 cells/ml in spinners with microcarrier Cytodex-1 concentration of 3 mg/ml and six-well plates respectively.

Figure 1. Microscopic observation of Sertoli cells (SCs) grown at different stirring speeds in spinner flask culture. SCs plated at 1×10^5 cells/ml in 80 ml of DMEM in the presence of 3 mg Cytodex-1 per ml at the stirring speed of 20, 30 and 40 rpm.

Viable cell densities, in terms of cells per ml, and viabilities were obtained by three independent runs; this indicated that maximum density of SCs grown in spinner flasks with microcarrier reached 4.6×10^6 cells/ml, which was approximately 10 times that of controls. Cells grown in spinner flasks consistently showed high viabilities (above 90%), while with those on control plates an increase in cell death was observed (viabilities decreased as far as 80% at some points) (Fig. 2).

Typically after seeding, cells exhibited classical expansion trends consisting of death or lag phase in the first 24 h followed by an exponential phase leading to maximum cell density of $11.5\text{--}45.8 \times 10^5$ cells/ml in stirred microcarrier cultures and $4.5\text{--}4.8 \times 10^5$ cells/ml on control plates. For inocula of 0.5×10^5 , 1×10^5 and 2×10^5 cells/ml, 23-, 44.6- and 22.7-fold increases respectively were obtained from stirred microcarrier cultures, while increases were significantly lower (9-, 4.6- and 2.4-fold) on control plates. Interestingly, maximum cell number per cm^2 in stirred microcarrier culture was also higher than that of control plates (3.38 versus 1.84×10^5 cells/ml), as SCs grow layer by layer on microcarriers compared to those on control plates (Table 1).

The death or lag phase occurred within the first 24 h, due to decrease in cell number caused by adaptation of cells to the culture system. This drop in cell density was more pronounced in stirred cultures possibly because of poor microcarrier colonization and agitation of unattached cells, which in turn were a result of shear stress to cells. The exponential phase continued for an extended time period for stirred cultures (5–6 days for stirred culture versus 2–3 days for six-well plate cultures), yielding higher final cell densities ($11.5\text{--}45.8 \times 10^5$ cells/ml from stirred culture versus $4.5\text{--}4.8 \times 10^5$ cells/ml from six-well plate cultures); this was probably due to greater available surface area available compared to control plates (Table 2).

Maximum specific growth rates were estimated from slope of growth curves during the exponential phase

Table 1 Initial and final cell densities, and fold increase of Sertoli cells grown in stirred microcarrier cultures and six-well control plates

Culture type	Initial cell density		Cells per bead	Final cell density		Fold increase
	10^5 cells/ml	10^5 cells/ cm^2		10^5 cells/ml	10^5 cells/ cm^2	
Stirred	0.5	0.039	4	11.5 ± 1.0	0.87 ± 0.075	23
microcarrier	1.0*	0.078*	8*	$44.6 \pm 3.7^*$	$3.38 \pm 0.28^*$	44.6*
cultures	2	0.156	16	45.4 ± 4.1	3.44 ± 0.31	22.7
Control plates	0.5	0.13		4.5 ± 0.48	1.80 ± 0.128	9
	1	0.26		4.6 ± 0.53	1.84 ± 0.192	4.6
	2	0.52		4.8 ± 0.53	1.86 ± 0.212	2.4

For stirred cultures, 3 mg of Cytodex-1 per ml was used for the different inocula. Values denoted with an asterisk (*) obtained when using inoculum of 1.0×10^5 cells/ml. All experiments were averages of three independent runs ($\bar{x} \pm S$, $n = 3$).

Table 2 Available surface areas estimated in for stirred microcarrier bead cultures and control plates

Culture type	Cytodex-1 concentration		Media (ml)	Total available area (cm^2)
	mg/ml	cm^2/ml		
Stirred	1	4.4	80	352
microcarrier	3	13.2	80	1056
culture	5	22	80	1760
Control plates	–	3.84	2.5	9.6

Table 3. Apparent (μ_{app}) and maximum (μ_{max}) specific growth rates calculated using first order kinetic model for Sertoli cells (SCs) grown in stirred microcarrier cultures and control plates.

Culture type	Initial cell density (10^5 cells/ml)	Apparent growth rate (μ_{app}) (h^{-1})	Maximum growth rate (μ_{max}) (h^{-1})
Stirred	0.5	0.022 ± 0.001	0.028 ± 0.001
microcarrier	1	0.026 ± 0.0012	0.029 ± 0.002
culture	2	0.022 ± 0.0009	0.026 ± 0.0011
Control plates	0.5	0.017 ± 0.0008	0.023 ± 0.0013
	1	0.027 ± 0.0013	0.03 ± 0.002
	2	0.028 ± 0.0014	0.031 ± 0.0021

In stirred cultures, 3 mg of Cytodex-1 per ml was used for the different inocula. Maximum specific growth rates (μ_{max} , h^{-1}) and apparent specific growth rate (μ_{app} , h^{-1}) estimated using first order kinetic model for cell expansion and death applied to slope of curves during exponential phase. All experiments were averages of three independent runs, represented as ($\bar{x} \pm S$, $n = 3$).

(Table 3). Interestingly, calculated maximum growth rates (approximately 0.029 h^{-1}) were very similar in stirred cultures and the plate cultures (Table 3), indicating that shear stress was not one of the crucial factors affecting cell expansion in the spinner flasks (36–38).

During cell culture, activity of the enzyme lactate dehydrogenase (LDH), normally used to characterize viability status of the culture (39), was also detected. In this case, LDH levels were all below 21 U/l (data not shown),

Figure 2. Effect of inoculum on viable cell density of Sertoli cells (SCs). Effect of inoculum on viable cell density for SCs grown in: (a) stirred microcarrier cultures and (b) control plates. Cells were plated at (■) 0.5×10^5 , (●) 1×10^5 and (▲) 2×10^5 cells/ml in 80 or 2.5 ml of DMEM, for the stirred and control cultures, respectively. For stirred cultures, 3 mg of Cytodex-1 per mL was used. □, ○, △ stand for viabilities (%) of SCs, plated at (■) 0.5×10^5 , (●) 1×10^5 and (▲) 2×10^5 cells/ml in the figure. Media (50%) was changed every day from day 2.

which is in accordance with percentage low cell death observed in stirred cultures. In particular, LDH level for inoculum 1.0×10^5 cells/ml was lowest (15 U/l). Inoculum 1.0×10^5 cells/ml was optimal, taking into account inoculum maximum cell density and fold increase in cell population.

Effect of microcarrier bead concentration on cell expansion

Maximum cell density achieved in microcarrier culture not only depended on adequate culture conditions but also on available surface area for cell population growth (40). Based on recommended concentrations of Cytodex-1 ranging from 0.5 to 5 mg/ml final volume, 1.0×10^5 cells/ml were inoculated with different microcarrier bead concentrations of 1, 3 and 5 mg/ml, respectively. Microscopic observation of cultures showed highest cell density of 45.8×10^5 cells/ml, which was achieved with microcarrier concentration of 3 mg/ml with inoculum of 1.0×10^5 cells/ml. However, there were very many empty beads at concentration of 5 mg/ml; even by end of the experiments, when cells

reached the plateau stage, and final achieved cell density was only 41.2×10^5 cells/ml. At microcarrier concentration of 1 mg/ml, no empty beads were observed in the medium, but final cell density was very low (21.5×10^5 cells/ml) (Fig. 3). This indicated that there was insufficient surface area for attachment of SCs at 1 mg/ml Cytodex-1 concentration, whereas excess surface area for increase in number of SCs was available at 5 mg/ml Cytodex-1 concentration.

Metabolic characterization of SCs in stirred cultures

Concentration of glucose, glutamine, lactate and ammonia as well as their consumption or production rates, throughout the culture, is depicted in Fig. 4. In general, medium replacement was efficient in supplying nutrients (glucose and glutamine). Indeed, in our experiments, glucose and glutamine concentrations did not decrease to 15 and 1 mM, respectively. Moreover, maximal lactate concentration was 13.4 mM during the culture process, which was far lower than 20 mM. It has been reported that population growth of haematopoietic cells and further mammalian cells were inhibited when lactate

Figure 3. Microscopic observation of Sertoli cells grown at different microcarrier concentrations. Sertoli cells (SCs) plated at 1×10^5 cells/ml in 80 ml of DMEM with different microcarrier bead concentrations, observed on the 2nd day after inoculation.

Figure 4. Substrate concentration and metabolite profiles during culture of Sertoli cells (SCs) on Cytodex-1 microcarrier beads. Concentration (■, ●, ▲, ▼) and specific production/consumption (□, ○, △, ▽) rates of glucose (a), lactate (b), glutamine (c) and ammonia (d) in culture represented. Values displayed represent average of three independent experiments. Error bars indicate the standard deviation of duplicate cultures. SCs (1×10^5 cells/ml) cultured with microcarrier bead concentration of 3 mg/ml. Media (50%) was changed every day, starting on day 2.

concentration was above 20 mM (41,42). Ammonia is also detrimental to well-being of cells, being an order of magnitude more toxic than lactate. In this study, ammonia concentration was higher than 4 mM after 7 days culture, which may have been the main factor leading SCs entering a stable phase. Similarly, it has also been reported that ammonia accumulation inhibited cell expansion when its concentration was above 4 mM (41). Overall apparent yields of lactate from glucose ($Y_{\text{lact./gluc.}}$) and ammonia from glutamine ($Y_{\text{ammo./gln}}$) were calculated. $Y_{\text{lact./gluc.}}$ provides an estimate of the fraction of glucose converted to lactate by glycolysis (43); mean $Y_{\text{lact./gluc.}}$ was $1.65 \text{ mol}_{\text{lac}}/\text{mol}_{\text{gluc}}$ and calculated $Y_{\text{ammo./gln}}$ was $1.39 \text{ mol}_{\text{ammo}}/\text{mol}_{\text{gln}}$.

Concentrations of amino acids in the medium were measured, and there were no obvious changes in their concentrations apart from glutamate and alanine (data not shown). Final concentrations of glutamate and alanine were 0.56 and 1.12 mM respectively. pH and osmotic pressure of media were also determined. Variation in pH was in the range of 6.8–7.2, and the range of osmotic pressure values was 305–320 mOsm/kg.

Visualization of SCs using optical microscopy and scanning electron microscopy

SCs grown on microcarrier beads in spinner flasks were visualized with MTT staining, using an optical microscope and also were visualized using a scanning electron microscope (SEM) at 2-day intervals. Gradual increase in microcarrier occupancy by SCs was observed and cells remained viable and metabolically active in culture (Fig. 5). In general, successful cell adhesion of SCs to microcarriers was achieved after 2 days of culture (Fig. 5a,e); at day 4, number of viable cells on beads significantly increased and some microcarrier aggregation was observed (Fig. 5b,f). At days 6 and 8, a high percentage of beads was totally covered by SCs and intense microcarrier aggregation became more evident (Fig. 5c,d,g,h, respectively).

Identification of SCs by immunocytochemistry

SCs cultured both on control plates and in stirred culture conditions were identified by immunocytochemical

Figure 5. Optical and scanning electron micrographs of Sertoli cells (SCs) cultured on microcarrier beads under stirred culture conditions. SCs visualized using an optical microscope, day 2 (a), day 4 (b), day 6 (c) and day 8 (d) after MTT staining (200 $\times$ amplification) for Cytodex-1, respectively. SCs visualized using a scanning electron microscope, day 2 (e), day 4 (f), day 6 (g) and day 8 (h).

reaction labelled with anti-Fas-L antibody. Strongly positive expression Fas-L in cytoplasm was observed when SCs were cultured with both culture conditions, but expression of Fas-L was absent in negative control cells (Fig. 6), indicating that SCs obtained from microcarrier culture were undifferentiated and had strong physiological activity.

Co-culture of SCs with neuronal stem cells

To explore bioactivity/biofunction of SCs cultured in spinner flasks, co-culture of SCs with NSCs was performed. When NSCs were cultured alone, they tended to remain in small groups with a few scattered individual neurons; no neurite outgrowth was observed (Fig. 7a). In co-cultures of SCs with NSCs from the same isolation group however, the NSCs dispersed over the culture plate in close proximity to the SCs; extensive neurite outgrowths were observed in co-cultures where long neurites connected neighbouring individual cells and groups of cells (Fig. 7b,c). In particular, size of NSCs increased

significantly; number of neurite outgrowth was highest in stirred culture conditions in all tested modes; This indicated that NSCs in 3D co-culture systems have strongest physiological activity and potential biofunction. Meanwhile, neurospheres were identified by nestin antibody immunofluorescence staining. Results revealed that no neurospheres were observed on control plate; however, a mass of neurospheres appeared 5 days after inoculation in co-culture systems (Fig. 8b,c). It was clear that the number of neurospheres in NSCs + SCs_{3D} group was greater than from NSCs + SCs_{2D} group (Table 4), and number of cells gradually declined in the latter case.

Discussion

Co-transplanting SCs with grafts (cells or tissue) could be an ideal way to enhance graft survival, by delivering an appropriate biological cocktail of trophic and immunosuppressant factors. Currently, the requirement for large numbers of SCs for research and therapeutic applications is evident. Static cultures have several drawbacks (for

Figure 6. Identification of Sertoli cells (SCs) using immunocytochemistry with anti-FAS-L antibody. (a) SCs cultured on control plates. (b) Sertoli cells (SCs) cultured in stirred spinner flasks.

Figure 7. Photomicrograph of neuronal stem cells (NSCs) cultured alone and NSCs co-cultured with Sertoli cells (SCs). (a) NSCs cultured in six-well chamber slides. (b) NSCs co-cultured with SCs obtained from two-dimensional culture. (c) NSCs co-cultured with SCs obtained from microcarrier bead suspension culture. All photographed on day 3 after inoculation (the scale bar is 5 μ m). Neurite outgrowth was marked with.

Figure 8. Nerve stem cells (NSCs) immunocytofluorescently labelled with anti-nestin antibody to identify neurospheres (day 6). (a) NSCs cultured on six-well chamber slides. (b) NSCs cultured with Sertoli cells (SCs) obtained from two-dimensional culture. (c) NSCs cultured with SCs obtained from microcarrier bead culture.

example, concentration gradients, difficult monitoring and control) that can be alleviated by using suspension culture systems such as in stirred bioreactors (44). Thus, population growth of these cells can constitute an increasingly important bioprocess with scaling-up feasibility. Use of stirred bioreactors facilitates expansion of cells under controlled conditions and the 3D microcarrier bead system offers advantages in terms of cell yield, compared to

conventional cultures on plates. For expansion of SCs, no previous report has been published regarding use of a stirred system. As SCs are usually expanded as anchorage-dependent cells, they require support and microcarrier beads appear to be a promising solution. Cytodex-1 (GE Healthcare, Milwaukee, USA), a microporous microcarrier made up of a dextran matrix with a collagen layer at the surface, has been successfully used for expansion of primary cells as well as established cell lines [Amersham Biosciences, Baie d'Urfé, Quebec, Canada; (43,45)]. One gram dry weight of this support has an approximate surface area of 4400 cm^2 (Microcarrier Cell Culture Technology, GE Healthcare, Milwaukee, USA). Thus, Cytodex-1 microcarriers were used in this study and the effect of several culture parameters was analysed. Its ability to support population growth of SCs in spinner flasks was investigated here. Improved cell number expansion was obtained using an inoculum of 1×10^5 cells/ml, 3 mg of Cytodex-1 per ml, and agitation rate of 30 rpm, leading to maximum cell density of $2.1\text{--}4.6 \times 10^6$ cells/ml. Therefore, stirred culture appeared to be a promising system for expansion of SCs. Such similar culture systems have also been used for the expansion of bone marrow mononuclear cells (46), human natural killer cells (47), haematopoietic progenitor cells (48,49), neural stem cells (50), mammary

Table 4. Distribution of the number of neurospheres in different co-culture systems

Days	The number of neurospheres	
	NSCs + SCs _{2D} *	NSCs + SCs _{3D} **
6	22 $\pm$ 0.54	34 $\pm$ 0.39
10	20 $\pm$ 0.34	33 $\pm$ 0.27
14	19 $\pm$ 0.59	36 $\pm$ 0.49
18	13 $\pm$ 0.44	35 $\pm$ 0.51

All experiments were averages of three independent runs, represented as ($\bar{x} \pm S$, $n = 3$).

*Neuronal stem cells (NSCs) co-cultured with Sertoli cells (SCs), obtained from two-dimensional cultivation.

**Neuronal stem cells (NSCs) co-cultured with Sertoli cells (SCs), obtained after microcarrier culture.

epithelial stem cells (38), primary brain astrocytes (43) and human embryoid bodies (51,52).

Comparison of maximum proliferation rates in static and microcarrier suspension cultures can be used to evaluate effects of shear stress caused by impeller rotation. Results indicate that maximum growth rates were similar, indicating that shear stress was not affecting cell population growth in the spinner flasks, which was further confirmed by estimating maximum shear stress ($\tau_{\max}$), assuming that $\tau_{\max}$ was 1.342 Pa. Maximum shear stress values of 1.5–3.0 Pa have previously been reported to cause damage to cells attached to such surfaces (53).

In addition, SCs exhibited typical growth curves in spinner flask cultures with microcarrier beads, which was characterized by a short death or lag phase, followed by exponential and stationary phases. In other mammalian cell cultures, as well as in microbial growth, a subsequent death phase is usually observed when medium change is not able to provide nutrients and remove toxic metabolic byproducts. As a preliminary study to assess whether chosen media renewal was necessary and would be sufficient to sustain cell needs, batch experiments were also performed (data not shown). For this purpose, cells were grown over 8 days with no medium change, both in spinner flasks and on the control plates. Contrary to results from re-feed assays (50% medium changed everyday, starting on day 2), of batch experiments, the exponential phase was followed by a marked death phase. This phenomenon could be ascribed to average lower final maximum cell densities (2.68×10^6 cells/ml), indicating that medium renewal is required to maximize cell population growth, and in this case, the chosen re-feeding system was sufficient to provide adequate culture conditions. However, it has been shown that concentration of glucose at 18.34 mmol/l and concentration of glutamine at 1.12 mmol/l, far lower than limited concentration (54–56)). This indicates that there is need for further feeding protocols or design of the new media. For example, dilution-feeding protocols have been reported to increase extent of total cell expansion for cultures of umbilical cord blood cells and mobilized peripheral blood mononuclear cells in stirred and static conditions (49,57).

Testing physiological activity and functional maintenance of Sertoli cells cultured by this newly developed stirred culture system is essential. Morphological analysis and biochemical monitoring of the cells grown on the microcarrier beads indicate that optimized conditions are able to sustain physiological activity of the SCs. It is surprising that nutritional function of SCs enhanced through culturing them under the microcarrier culture system, was identified for co-culturing with NSCs.

In conclusion, in this study, we have successfully optimized the culture process for primary SCs by microcarrier technology in spinner flasks and obtained highly purified cell populations of undifferentiated cells. Viable cell concentration of SCs improved from 4.8×10^5 cells/ml in static culture to 4.6×10^6 cells/ml with normal physiological function. These SCs were also successfully co-cultured with neuronal stem cells *in vitro* and significantly promoted nerve cell growth. This study demonstrates the vast potential of stirred culture systems for efficient, reproducible, and cost-effective expansion of SCs *in vitro* suspension culture. This culture system has advantages over static culture (which has major obstacles such as lower cell density achieved, is time-consuming and susceptibility to contamination). In this study, we overcame the major obstacle of obtaining sufficient SCs with normal physiological function for cell transplantation. For SCs to proceed into clinical trials for treatment of neurodegenerative diseases (for example Parkinson's disease and Huntington's disease), further investigation is needed to characterize trophic factor secretion by SCs during postnatal development and within transplants. On the other hand, maximal passage number of primary SCs is usually not more than 15, and quantities of bioactive factors secreted by primary SCs is lower than 0.1 g/l. To overcome these drawbacks, it is important to establish and develop immortalized and high-level protein-producing cell lines.

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