

Challenges in Culturing *Macaca fascicularis* Bone Marrow Stem Cells

(Cabaran di dalam Pengkulturan Sel Stem Sum-Sum Tulang *Macaca fascicularis*)

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ABSTRACT

Culturing Macaca fascicularis bone marrow stem cells in fetal bovine serum (FBS) resulted in low proliferation and long period of incubation. Therefore, its potential uses are exhausted. Here we report the establishment of culturing the Macaca fascicularis bone marrow stem cells using the FBS in combination with autologous serum. Five percent autologous serum was added to the Minimum Essential Medium (MEM) alpha medium and 10% FBS while 0.2 mM acid ascorbic 2-phosphate, 10 mM β -glycerolphosphate, 10⁻⁸ molar dexamethasone were used for osteogenic induction. Following this combination, our results showed higher growth kinetic i.e. 1.41% growth rate higher compared to only 0.46% growth rates of the cells using FBS alone and shorter population doubling time (4 to 7 days) compared to the culture without the combination of FBS and autologous serum (30 days). Thus, the combination of the FBS and autologous serum permits fast cell growth and tissue construction.

Keywords: Bone marrow stem cells; Macaca fascicularis; tissue engineering

ABSTRAK

Pengkulturan sel stem sum-sum tulang Macaca fascicularis di dalam (FBS) fetal bovine serum (FBS) menghasilkan proliferasi sel yang rendah serta tempoh inkubasi yang panjang. Kami melaporkan penambahbaikan teknik pengkulturan sel stem sum-sum tulang Macaca fascicularis ini dengan menggunakan FBS bersama-sama dengan serum Macaca fascicularis. Serum (5%) ditambah kepada Minimum Essential Medium (MEM) alpha medium dan 10% FBS manakala 0.2 mM asid askorbik 2-fosfat, 10 mM β -glyserolfosfat dan 10⁻⁸ molar deksamethason digunakan untuk pengaruhi tulang. Kajian ini menunjukkan pertumbuhan kinetik sel yang tinggi iaitu 1.41% lebih tinggi jika dibandingkan dengan 0.46% pengkulturan sel tanpa kehadiran serum Macaca fascicularis manakala masa untuk populasi sel berganda pula mengambil masa di antara 4 hingga 7 hari di dalam kultur yang menggunakan serum berbanding dengan 30 hari kultur tanpa serum. Ini menunjukkan kombinasi serum ini dapat mempercepatkan pertumbuhan sel dan pembentukan konstruk tisu tulang.

Kata kunci: Kejuruteraan tisu; Macaca fascicularis; sel stem sum-sum tulang

INTRODUCTION

Tissue engineering is a multidisciplinary field that combines engineering, physical sciences, biology, and medicine to restore or replace tissues and organs functions (Pancrazio et al. 2007). These approaches have proven to be very effective in bone regeneration and successful repair of bone defects have been demonstrated in large animals like canines (Csaki et al. 2009), goats (Wang et al. 2010) and sheep (Viateau et al. 2007). Tissue engineering has evolved to the use of bone marrow stem cells (BMSC) as a source of progenitor cells. In recent years, there has been an increase in research of the BMSC using animal models such as the primates (Viateau et al. 2007). BMSC has been proven to be the most readily available source of osteoblast progenitor cells, which is able to differentiate into osteogenic lineage *in vitro* (Angela et al. 2005) and provide potential source of osteogenic material (Schecroun Delloye 2004). Several studies using a variety of animal models have shown that BMSC may be useful in the repair

or regeneration of cartilage, damaged bone, tendon and meniscus (Kokemueller et al. 2010) as well as have the capability to differentiate into cardiomyocytes, skeletal muscle and neural precursors (Shahdadfar et al. 2005).

Most BMSC-based studies were performed in lower animals (mice, rats and rabbits) instead of higher animals (non-human primates) due to diminished potential for bone regeneration in primates (including humans) compared with phylogenetically lower animals but results obtained from lower animal models is not comparable to human. The rhesus monkey on the other hand, is closely related to human in genetic homology (98% of human DNA is found in non-human primates), making it particularly suitable for experimental purposes (Wang et al. 2005). In lower animal studies, the fetal bovine serum (FBS) has been used as a supplement in culture media without any setbacks. FBS is an established supplement for tissue culture media; supplying growth factors, attachment proteins and essential proliferation factors for culturing and proliferation of

cells *in vitro* (Gstraunthaler et al. 2008). Culturing human bone marrow stem cells, on the other hand, posed several problems especially when the FBS supplemented culture media is used. It has resulted in low proliferation rate and slow expansion of BMSC, depriving its potential use. To date, literature describing culture techniques of BMSC in this non-human primate group is very limited. The autologous serum has been used as a supplement as it contains platelets derived growth factors that stimulate osteoprogenitor cells proliferation and osteoblastic differentiation (Shahdadfar et al. 2005). The autologous serum may be combined with FBS in order to provide better cell growth. Some of the cultures may also be treated with specific osteogenic induction factors in the medium to promote osteogenesis (Csaki et al. 2009). Without proper culture techniques, the osteogenic potential of the mesenchymal stem cells from these non-human primates will not be able to be stabilized, optimized before reaching pertinent definitive conclusions and used for future regenerative medicine research.

This study aimed to show that addition of 5% of autologous serum into the conventional BMSC culture provides better results in relation to proliferation rate and population doubling time of the bone marrow stem cells isolated from *Macaca fascicularis*.

MATERIAL AND METHODS

Animal Preparation

All animal procedures were conducted under a protocol approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC;FD/OMS/2008/ROSZALINA/12-AUGUST/228-SEPT-2008-JUNE-2009). Six Cynomolgus monkeys (*Macaca fascicularis*) weighing between 2.0 and 5.0 kg were used for this study. The animals were provided by the Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN) and housed in the Animal House, Universiti Kebangsaan Malaysia. The animals were quarantined for two weeks before any procedures or experiments to ensure that they were not carrying any infestation parasites. All surgical procedures were performed under general anaesthesia using intramuscular injection of 10 mg/kg ketamine hydrochloride and 0.1 mg/kg medetomidine hydrochloride.

ISOLATION OF BONE MARROW STEM CELLS

The hair around the femur was shaved to enhance the vision of the veins and femur. The skin was swabbed with alcohol swab prior to needle insertion. General anaesthesia was administered using the above drugs and maintained throughout the procedure using pentobarbital sodium (1-2% intravenously). Bone marrow stem cells (BMSC) was aspirated from bilateral greater trochanter of the femurs of each *Macaca fascicularis* using 18- and 19-gauge needles and transferred into the sodium heparin tube. Approximately 5-10 mL sample of the aspirated BMSC

was isolated and diluted with fetal bovine serum (FBS) and Phosphate Buffer Saline (PBS). An additional of 30mL of whole blood was collected for the autologous serum.

PREPARATION OF AUTOLOGOUS SERUM

From each *Macaca fascicularis*, about 20-30 mL of blood was drained into the plain tube and the blood was allowed to clot for 4 h at 4°C to 8°C. Subsequently, the blood was centrifuged at 3000 rpm at 4°C for 15 min. The serum was collected, filtered into a sterile tube and stored at -20°C.

PREPARATION OF BMSC FOR TISSUE CULTURE

The bone marrow stem cells (BMSC) were separated from the red blood cells by adding the sample onto Ficoll-paque layer in a tube and centrifuged at 5000 rpm for 30 min. The isolated cells were then cultured in the culture medium consisted of α -minimum essential medium (GIBCO, Invitrogen Co., NY, USA.), supplement with 1X antibiotic-antimycotic (GIBCO), glutaMAX-1 (GIBCO), 10% fetal bovine serum, (FBS; GIBCO), 50 μ g/mL acid L-ascorbic (Sigma-Aldrich CO., St. Louis, MO, USA), 0.02M HEPES buffer (GIBCO), 0.2 mM acid ascorbic 2-phosphate (Sigma-Aldrich), 10 mM β -glycerolphosphate (Sigma-Aldrich CO), 10^{-8} molar dexamethasone (Sigma-Aldrich CO) and cultured in the six-well plate (9.6 cm² each well).

The standard culture mediums were added with or without 5% autologous serum. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air and the medium were changed every two days. Viability of the cells was counted at passage 1. Cell number was counted using a haemocytometer.

RESULTS

We encountered two main problems associated with the culturing of BMSC; low proliferation and long cell expansion time.

As a result, the osteogenic potential of these cells was not able to be tested and the cells had no practical and clinical benefits for bone tissue engineering research. To overcome the problems, we added autologous serum to improve cell growth.

The cell growth in the culture medium with 5% autologous serum were higher compared to the cells in media with 10% FBS only. The cells in media with added 5% autologous serum have 1.41% compared to 0.46% (\pm 3 time) higher in growth rate compared to the cells in the media with 10% FBS only.

The population doubling time for the cells in the medium added with 5% autologous serum was decreased. The proliferation of these cell were rapid compared to the cells in the media that used 10% FBS only. BMSC in media with added 5% autologous serum scored population doubling of 4-7 days compared to the cells in media with 10% FBS only which took about 30 days to double its cell number.

DISCUSSION

BMSC are attractive adult stem cell source, possessing the capacity for self-renewal and differentiation into a variety of mesodermal cells under appropriate conditions, providing a prospective therapeutic approach for tissue regeneration (Ren et al. 2010). Non-human primate BMSC have been invaluable in developing and assessing new therapeutic transplant approaches for the treatment of human diseases (Ren et al. 2010) due to its closeness to human genetic homology.

Seto et al. (2006) in their study of mandibular reconstruction via combination of rhesus bone graft and BMSC expanded *in vitro* stressed the importance of serum choice to be used in a culture medium. In our study, the primary culture of BMSC from *Macaca fascicularis* showed low proliferation rate and did not confluent well. In addition, these primary cells also required longer time for expansion. As a result, these cells lost their ability to differentiate.

This condition was improved by adding 5% autologous serum into the culture media. Higher growth rate within shorter period of time in the BMSC with 5% autologous serum and 10% FBS compared to BMSC cultured in 10% FBS only. This study showed that appropriate choice of serum and culture condition is able to induce and enhance the potential of *Macaca fascicularis* BMSC for tissue engineering purpose. Our justification for using 5% autologous serum is because of the limited amount of blood that can be withdrawn from each animal before it became dangerous.

In the routine culture technique, culture medium is commonly supplemented with FBS. However, direct contact of cells with FBS should be avoided as it can increase the risk of viral or prion-related disease transmission and foreign protein contamination (Schecroun & Delloye 2004). It has been shown that culture medium supplemented with FBS lowered the proliferation rate of the BMSC (Thomas et al. 2007). This is because the growth factors in the FBS may not be suitable for the cells proliferation compared to the autologous serum (Shahdadfar et al. 2005). Seti et al. (2006) in their study using Japanese monkeys (*Macaca fuscata*) were able to induce cell proliferation and create three dimensional cell cultures. In their study, the source of BMSC was surgically obtained from the cancellous bone and marrow of the tibia. In our study, the source of the multipotent cells was obtained from aspiration of the marrow at the greater trochanter of the femurs.

Autologous serum has been shown to contain growth factors, such as the platelet derived growth factor (PDGF), epithelial growth factor (EGF) and transforming growth factor β (TGF β) that can stimulate osteoprogenitor cells proliferation and osteoblastic differentiation (Schecroun & Delloye 2004). These growth factors also stimulate extracellular matrix (collagen I) synthesis (Schecroun & Delloye 2004). The age of the BMSC donor also contributes to the cell proliferation rate. Older BMSC donors will give shorter life-span, diminished proliferative rate and reduced differentiation potential of the cells (Ren et al. 2010).

BMSC expanded in autologous serum proliferated faster but differentiated slower than BMSC expanded in the FBS. This was supported by a study that showed BMSC in autologous serum were less differentiated but remain transcriptionally more stable over time in a culture (Shahdadfar et al. 2005).

CONCLUSION

The 5% autologous serum plus the FBS supplementation was able to increase BMSC growth rate in a shorter period of time. The advantage of using 5% autologous serum is to provide more cells for tissue engineering purpose such as bone construction.

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