Int. J. Morphol.,
37(1):54-58, 2019.

Morphological Observation of Bone Marrow Mesenchymal Stem Cells under Matrigel Three Dimensional Culture Conditions

Observación Morfológica de Células Madre Mesenquimales de Médula Ósea en Condiciones de Cultivo Tridimensional con Matrigel

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LIU, L. L.; ZHANG, B. B.; GAO, Q. W.; BING, L.; SHA, H. & BIN, Y. Morphological observation of bone marrow mesenchymal stem cells under matrigel three dimensional culture conditions. *Int. J. Morphol., 37(1)*:54-58, 2019.

SUMMARY: Matrigel is a basement membrane matrix extracted from the EHS mouse tumor containing extracellular matrix protein,its main components are laminin, type IV collagen, nestin, heparin sulfate, growth factor and matrix metalloproteinase.At room temperature, Matrigel polymerized to form a three dimensional matrix with biological activity. It can simulate the structure, composition, physical properties and functions of the cell basement membrane in vivo, which is beneficial to the culture and differentiation of the cells in vitro, and can be used for the study of cell morphology, biochemical function, migration, infection and gene expression. In this study,Matrigel three-dimensional culture model of bone marrow mesenchymal stem cells(BMSCs) was established, and its morphology, proliferation and survival were observed. BMSCs were isolated and cultured with whole bone marrow adherence method. The Second generation BMSCs with good growth condition were selected and mixed with Matrigel to form cell gel complexes. The morphology and proliferation of mesenchymal stem cells were observed by phase contrast microscope and HE staining,Live/Dead staining was used to evaluate the cell activity.Phase contrast microscopy showed that BMSCs were reticulated in Matrigel and proliferated well, After 7 days, the matrix gel gradually became soft and collapsed, a few cell reticular crosslinking growth was seen at 14 days; HE staining showed that the cytoplasm of the cells was larger on the fourth day and the cells were elongated and cross-linked on the seventh day; Live/dead staining showed that most cells showed green fluorescence with the prolongation of culture time, on the first, 4 and 7 days, the activity of bone marrow mesenchymal stem cells in Matrigel gradually increased, and the percentages were 92.57 %, 95.54 % and 97.37 %, respectively. Matrigel three-dimensional culture system can maintain the morphology, function and proliferation ability of bone marrow mesenchymal stem cells.

KEY WORDS: Morphology; Tissue engineering; Bone marrow mesenchymal stem cells(BMSCs); Matrigel.

INTRODUCTION

Bone marrow mesenchymal stem cells (BMSCs), a type of adult stem cells with self-replicating and multidirectional differentiation potential from bone marrow, are characterized by strong proliferation, low immunogenicity, differentiation to osteoblasts, and migration to the lesion after transplantation. In recent years, it has been widely used as the seed cell of tissue engineered bone (Elsayed & Sausville, 2011; Beyth *et al*., 2011). Scaffold materials provide a good three-dimensional space for material exchange, which is an important part of bone tissue engineering. There are many kinds of research on scaffold materials at home and abroad, the commonly used scaffold materials are chitosan, polyglycolic acid, calcium alginate gel, collagen, polylactic acid, fibrin. The injectable gel scaffold material has the advantages of easy to plastic, can be mixed with cells evenly, and can be in good chimerism, safety and efficiency with the repair tissue. It has become a new scaffold material in recent years.

Matrigel is a kind of extracellular matrix complex, mainly composed of laminin, type IV collagen, heparin sulfate, heparin, growth factor and so on, it is similar to the basal membrane of mammalian cells. Matrigel, as an extracellular matrix complex, can promote cell proliferation, differentiation and collagen secretion in cell culture system (Kleinman & Martin, 2005; Mondrinos *et al*., 2006). The aim of this study was to observe the proliferation, morphology and survival of BMSCs on Matrigel scaffold, and lay the foundation for the next step of injection of Matrigel in BMSCs *in vivo.*

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Funding: Beijing Natural Science Foundation of China, No. 7144249.

MATERIAL AND METHOD

The study was conducted in the experimental animal center of the First Affiliated Hospital of PLA General Hospital and the Basic Medical Research Institute of the PLA General Hospital from October 2016 to August 2017.

Materials and instruments. Beagle dogs (Beijing Maas Biotechnology Co, Ltd, license: SCXK 2016-0001).Low sugar DMEM medium, 0.25 % trypsin (American Gibco company); Matrigel (American BD company); Living / dead cell staining kit (Life Technologies, USA); BX51 fluorescence microscope (Olympus Corporation, Japan); water proof CO_2 incubator (American Thermo); enzyme labelling (Olympus Corporation); frozen section Machine (German Leica company).

Co-culture of BMSCs and Matrigel. The second generation BMSCs with good growth state were selected, and when the cells reached 80-90 % fusion, 0.25 % trypsin was digested, centrifuged, and the cell counts were collected. The cell density of 2×106/mL was rapidly inoculated into 24 well plates after suspension of Matrigel,they were incubated at 37 °C incubator and incubated with 30 min to form gel. A suitable amount of low sugar DMEM medium was added to each hole, the volume was 5 % CO_2 at 37 °C and incubated in saturated humidity incubator.

Cell morphology and proliferation ability. Bone marrow mesenchymal stem cells were cultured with Matrigel for first, 4, 7,14 days, the morphology and proliferation of bone marrow mesenchymal stem cells (MSCs) were observed by fluorescence microscope. Cell gel complexes were extracted on fourth and 7 days and washed with PBS for 2 times, 40 g/L paraformaldehyde was fixed at room temperature for 15 min, washed with PBS for 3 times, frozen sectioning, hematoxylin eosin staining, and finally observed under phase contrast microscope and collected images.

Detection of cell activity by live/dead staining. BMSCs were cultured with Matrigel for first, 4, 7 days,the samples were stained with live/dead cell staining kit. At high magnification, 4 visual fields were randomly selected to count living cells and dead cells, the percentage of cell activity was calculated, and the average value was repeated 3 times.

Observation index. The morphology and proliferation of BMSCs in Matrigel; Cell viability of BMSCs in Matrigel.

RESULTS

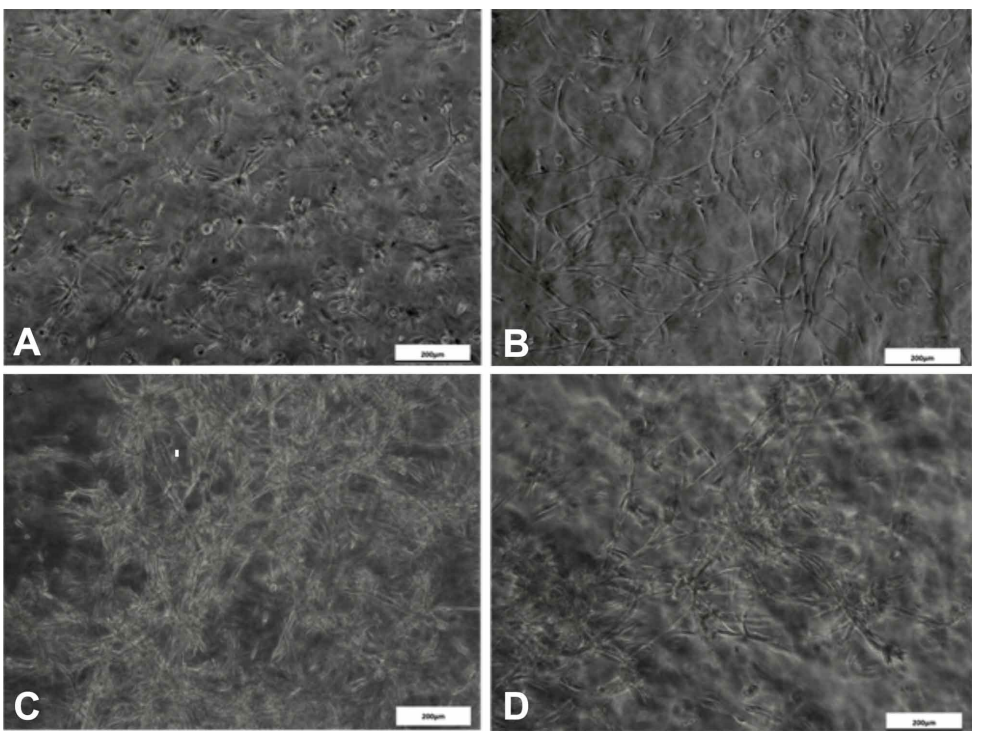

Fig. 1. Growth of BMSCs in Matrigel under phase contrast microscope (×100). A. On the first day, the cells were evenly distributed in the matrix material, and some cells could be elongated. B. On the fourth day, most of the cells were obviously elongated. C. On the seventh day, cell crosslinking to form reticulation. D. On the fourteenth day, most of the matrix glue softened and some of the cells were cross-linked.

Morphology and proliferation of bone marrow mesenchymal stem cells in Matrigel. Phase contrast microscopy showed that BMSCs were reticulated in Matrigel and proliferated well, After 7 days, the matrix gel gradually became soft and collapsed, while 14 days showed a few cells reticulated and crosslinked (Fig. 1); hematoxylin and eosin staining showed that the cytoplasm of the cells was larger on the fourth day and the cells were elongated and cross-linked on the seventh day (Fig. 2). It is suggested that the establishment of the Matrigel composite

model makes BMSCs form a multi cell group with three-dimensional gradient in the matrix microenvironment, and the cell proliferation ability is strong.

Cell viability of bone marrow mesenchymal stem cells in Matrigel. Live/dead staining showed that most cells showed green fluorescence with the prolongation of culture time (Fig. 3), on the first, 4 and 7 days, the activity of BMSCs in Matrigel gradually increased, and the percentages were 92.57 %, 95.54 % and 97.37 %, respectively (Fig. 4). It is suggested that after the combination of BMSCs with Matrigel in beagle dogs, the cells with different gradients have good activity and proliferation ability, and there is a good biocompatibility between the cells and the materials.

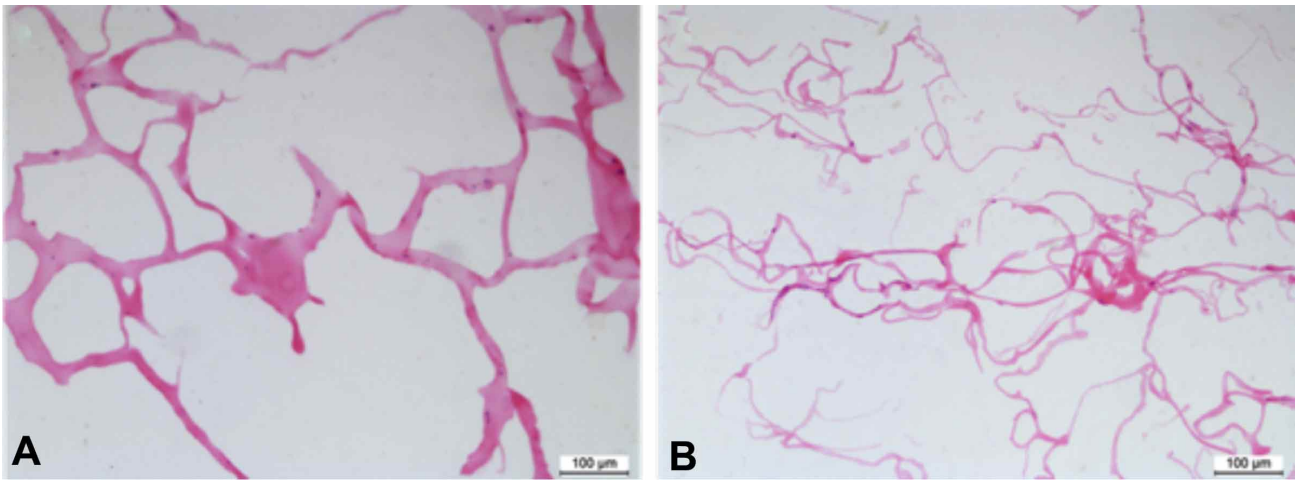

Fig. 2. Morphological characteristics of BMSCs in Matrigel composite culture (HE, x100). A. On the fourth day, the cells elongated, the cytoplasm was large and began to crosslink growth. B. On the seventh day, the cell elongation was obvious, the cytoplasm was slender, and network crosslinking is more compact.

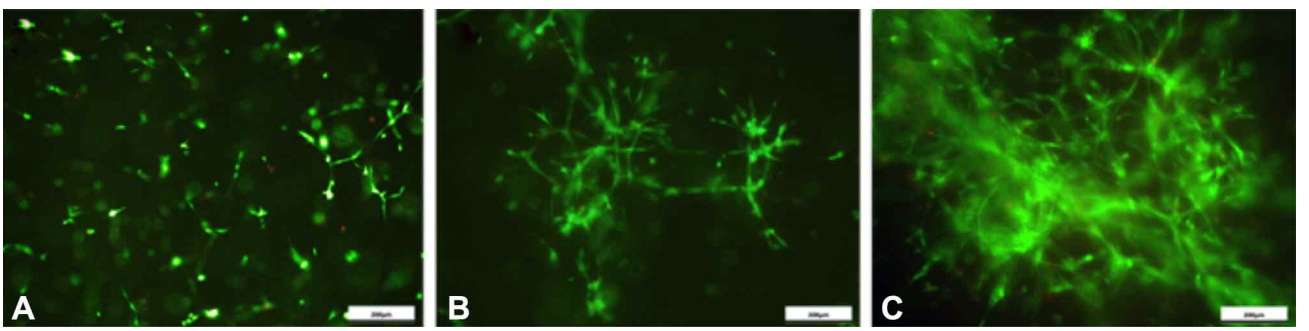

Fig. 3. Cell viability of BMSCs in Matrigel (live/dead staining,x100). A-C. With the prolongation of culture time, the cells cross linked to form three dimensional network cross-linked growth.

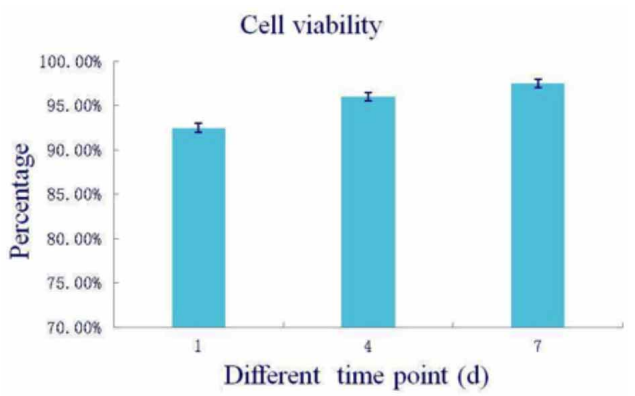

Fig. 4. Cell viability of BMSCs in Matrigel.

DISCUSSION

In adult stem cells, the research on mesenchymal stem cells is a hot topic at present.Mesenchymal stem cells have been isolated and cultured in some tissues, such as umbilical cord blood, embryo blood, liver and amniotic fluid, but its acquisition and research are still mainly concentrated in bone marrow tissue (Campagnoli *et al*., 2001; Jiang *et al*., 2002; Lee *et al*., 2004). BMSCs are the components of bone marrow stroma in fresh bone marrow, only 0.01-0.0001 % of bone marrow cells is found (Noël *et al*., 2004; Silva *et al*., 2005; Yue *et al*., 2008). The methods of obtaining BMSCs mainly include density gradient centrifugation, flow cytometry,

immunomagnetic beads sorting and all bone marrow adherent culture, among them, whole bone marrow adherent culture is the simplest way to obtain the cells based on the adhesion characteristics of BMSCs (Neuhuber *et al*., 2008; Lin *et al*., 2018; Liu *et al*., 2018). In this experiment, BMSCs were isolated, purified and amplified by the method of full bone marrow adherence, and the heterocells were removed through passages and fluid transfer methods. BMSCs with long spindle shape and polygon with high purity were obtained. The growth curve of bone marrow mesenchymal stem cells in beagle dogs is "S" shape, which is consistent with the general cell growth characteristics.

Cell culture scaffolds are one of the key factors affecting three-dimensional culture *in vitro*. Matrigel matrix gum is an extract from EHS tumor,its main components are laminin, type IV collagen, basilar proteoglycan, growth factor and so on. It is a hydrogel matrix with a diameter of 20-50 nm, Therefore, cells attached to multiple microporous scaffolds can be fully contacted with materials to form a three-dimensional culture microenvironment (Hill & Sarkar, 2017; Han *et al*., 2018). In addition to promoting cell proliferation, differentiation and collagen secretion, Matrigel matrix adhesive, as a kind of extracellular matrix complex, also has good mechanical properties and good gelation Matrigel matrix colloid exists in a liquid form at 4°C. The gel is formed by 30 min at 37 °C and forms a crosslinking network in the form of solid state, so that the cells are dispersed evenly and better simulate the three-dimensional microenvironment of cell growth in the body. The results of the experiment show that BMSCs were reticulated in Matrigel and proliferated well, After 7 olays, the matrix gel gradually became soft and collapsed, Live/dead staining showed that most cells showed green fluorescence with the prolongation of culture time, the activity of BMSCs in Matrigel gradually increased. On the first day day, cell viability was relatively low, probably due to the excessive cell death due to mechanical action when the cells were mixed. On the seventh days, cell viability was higher, the possible reason is that cell proliferation is faster and cell proliferation rate exceeds cell death rate. Second: with the prolongation of time, some dead cells or apoptotic cells gradually separated the three-dimensional structure with the degradation of matrix glue, which formed the increase of living cell count in the single field of vision under high magnification, which led to the increase of cell activity, indicating that BMSCs have good biocompatibility with matrix glue, and have strong proliferative capacity and good activity.

In recent years, more and more scholars have used Matrigel matrix glue in cell culture of beagle dogs. The extracellular matrix, which is grown on the culture medium containing Matrigel gel, forms many cell aggregation regions and tube like structures, indicating that Matrigel gel can promote the differentiation and formation of extracellular matrix (Kang *et al*., 2012; Park *et al*., 2012; Du *et al*., 2016). This study found that BMSCs can proliferate and survive in matrix glue, and gradually grow into three-dimensional reticular crosslinking growth, forming many cell aggregation areas, the possible reason is that the cells grow along the porous structure of matrix glue to form a three-dimensional network crosslinking structure, and may also be matrix glue to promote cell differentiation and tissue formation. Experts found that the control group injected with rabbit autologous BMSCs and the blank group injected with saline did not change significantly in the quality of osteogenesis and osteogenesis.

This study laid the foundation for further study of matrix gelatin combined with BMSCs transplantation *in vivo* to promote distraction osteogenesis.

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RESUMEN: Matrigel es una matriz de membrana basal extraída del tumor de ratón EHS que contiene proteína de matriz extracelular. Los componentes principales son laminina, el colágeno tipo IV, nestina, sulfato de heparina, factor de crecimiento y metaloproteinasa de matriz. A temperatura ambiente, Matrigel se polimerizó para formar una matriz tridimensional. Es posible simular la estructura, la composición, las propiedades físicas y las funciones de la membrana basal celular *in vivo*, lo que es beneficioso para el cultivo y la diferenciación de las células *in vitro*, y se puede utilizar para el estudio de la morfología celular, la función bioquímica, la migración, infección y expresión génica. En este estudio, se estableció el modelo de cultivo tridimensional Matrigel de células madre mesenquimales de médula ósea (BMSC), y se observó su morfología, proliferación y supervivencia. Las BMSC fueron aisladas y cultivadas con el método de adherencia de la médula ósea completa. Se seleccionaron las BMSC de segunda generación con buenas condiciones de crecimiento y se mezclaron con Matrigel para formar complejos de gel de células. La morfología y la proliferación de las células madre mesenquimales se observaron con microscopio de contraste de fase y se tiñó con Hematoxilina-Eosina (HE); para evaluar la actividad celular se usó la tinción Live/Dead. La microscopía de contraste mostró que las BMSC se reticularon en Matrigel y proliferaron bien. Después de 7 días, se observó que el gel de matriz gradualmente se volvió blando y colapsó, y se visualizó un cruce transversal de algunas células reticulares a los 14 días. La tinción mostró que la mayoría de las células mostraron una fluorescencia verde con la prolongación del tiempo de cultivo; en los primeros 4 y 7 días, la actividad de las células madre mesenquimales de la médula ósea en Matrigel aumentó gradualmente y los porcentajes fueron de 92,57 %, 95,54 % y 97,37 %, respectivamente. El sistema de cultivo tridimensional de Matrigel puede mantener la morfología, la función y la capacidad de proliferación de las células madre mesenquimales de la médula ósea.

PALABRAS CLAVE: Morfología; Ingeniería de tejidos; Células madre.

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Received: 09-07-2018
Accepted: 13-09-2018