Biocompatibility of polylactic-co-glycolic acid with bone marrow stromal cells of rhesus monkeys *in vitro****☆◆

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Abstract

BACKGROUND: Biocompatibility of polylactic-co-glycolic acid (PLGA) with different cells has been reported except its biocompatibility with rhesus monkey bone marrow stromal cells (BMSCs).

OBJECTIVE: To observe the biological characteristics of rhesus monkey BMSCs, and to investigate the biocompatibility of PLAG with rhesus monkey BMSCs.

METHODS: The second passage BMSCs of rhesus monkeys were co-cultured with PLGA for biocompatibility investigation. Light and electron microscopy revealed that BMSCs adhered to and elongated along the PLGA fiber scaffold. BMSCs cultured alone served as controls.

RESULTS AND CONCLUSION: After 4 days of co-culture with PLGA, CD29-positive BMSCs adhered to the surface of PLAG fiber scaffold, forming long cell chains. Some of the attached BMSCs elongated along the scaffold. After 72 hours culture in PLAG extract fluid, neurotrophic factors of BMSCs had no difference from those cultured in plain Iscove's Modified Dulbecco's Medium ($P > 0.05$). After 7 days, there were no differences in morphology, viability and proliferation of BMSCs cultured in PLGA extract fluid and plain Iscove's Modified Dulbecco's Medium. The results confirmed a high biocompatibility between PLGA materials and BMSCs.

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INTRODUCTION

Severe traumatic injury or invasive surgical procedures on a peripheral nerve can result in a gap between two nerve stumps. The standard technique for peripheral nerve repair is the implantation of autologous nerve grafts, which, however, are associated with inevitable drawbacks, such as the limited availability of donor nerves, donor site morbidity and secondary deformities[1-2]. Tissue-engineered nerve grafts have become promising alternatives to autologous nerve grafts. This alternative method is typically composed of a neural scaffold with the incorporation of support cells and/or growth factors. The choice of suitable biomaterials is one of the critical requirements for preparing neural scaffolds, with the common synthetic biomaterials under consideration, including poly(glycolide) (PGA), poly(l-lactide) (PLLA), polylactic-co-glycolic acid (PLGA), poly(lactide-ε-caprolactone), polyurethanes, poly(organo)phosphazenes and their copolymers[3]. In our previous studies, a chitosan/PGA (or PLGA)-based scaffold was developed and used for bridging large peripheral nerve gaps, achieving varying degrees of success[4-6].

Successful axonal growth depends on not only physical guidance by a longitudinally oriented scaffold, but also a microenvironment created by support cells that can provide neurotrophic factors and an extracellular matrix for neuronal survival and neurite extension. Schwann cells are considered as the most important support cells for tissue-engineered nerve grafts, but the availability of Schwann cells limits clinical usage. Bone marrow stromal cells (BMSCs) are easily accessible as non-hematopoietic stem cells that have been proven to be good candidates for support cells in nerve-tissue engineering[7-8]. As shown in many studies, BMSCs express neural markers such as neuron-specific enolase, neuronal nuclei, thyrosine hydroxylase, nestin and glial acidic fibrilary protein on basal levels[9], and BMSCs-based therapy can be used to treat neurodegenerative diseases[10-12], brain and spinal cord injuries[13-14] as well as peripheral nerve damage[15-16]. It has been found that BMSCs can be induced to differentiate into Schwann-like and neuron-like cells *in vitro* and *in vivo*, producing many different growth factors, cytokines and chemokines that may positively impact neural cell survival and neuritogenesis[17-20], thereby promoting nerve regeneration after peripheral nerve injury[21-24]. The goal of this study was to investigate the *in vitro* biocompatibility of PLGA with BMSCs, and to validate the possibility of BMSCs as support cells, together with PLGA as a scaffold material, for generating tissue-engineered nerve grafts. After the BMSCs, harvested from rhesus monkeys, were cultured on PLGA fibers and in

PLGA extract fluid, the changes in morphology, cell viability and proliferation, and the neurotrophic factor expression of BMSCs were examined.

MATERIALS AND METHODS

Design

An *in vitro* observation experiment on nerve tissue engineering.

Time and setting

This study was performed at Jiangsu Key Laboratory of Neuroregeneration and Experimental Animal Center of Suzhou between July 2007 and December 2009.

Materials

Reagents and instruments

Reagent and instrument	Source
Iscove's Modified Dulbecco's Medium (IMDM), fetal bovine serum (FBS), ethylene diamine tetraacetic acid (EDTA)	Gibco, Carlsbad, CA
Fluoresceine isothiocyanate (FITC) or PE-labeled monoclonal antibodies against CD90, CD29, CD73, CD11b, CD45 and CD34, mouse monoclonal anti-CD29	BD Bioscience, San Jose, CA
FITC -labeled CD105	AbD Serotec, UK
FITC-labeled goat anti-mouse IgG	Santa Cruz Biotechnology, Santa Cruz, CA
Rabbit-specific polyclonal antibodies directed against brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF)	Abcam, Cambridge, UK
Rabbit-specific polyclonal antibodies directed against nerve growth factor beta (NGF-β)	Chemicon, Temecula, CA
Murine-specific monoclonal antibody to β-actin	Sigma, St. Louis, Mo
Donkey anti-rabbit-IRDye, goat anti- mouse-IRDye	Rockland, Gilbertsville, PA
Hydroxyapatite	Sigma-Aldrich, Shanghai, China
Organotin	Hongding Chemicals Company, Nantong, China
PLGA fibers	Holycon Medical devices Co., Ltd., Nantong, China
Omniscript RT kit	Qiagen, Valencia, CA
Fast EvaGreen® Master Mix	Biotium, Hayward, CA
BCA protein assay kit	Biocolors, Shanghai, China
JFC-1100 unit	Jeol Inc., Japan
Flow cytometry	BD FACScalibur, San Jose, CA
Confocal laser scanning microscope	LEICA-SP2, LEICA, Germany
Scanning electron microscope	JEM-T300, Jeol Inc., Japan
EIX- 800 Microelisa reader	Bio-Tek Inc., USA
Real-time PCR machine	Applied Biosystems, Foster City, USA
Odyssey Infrared Imager	Licor, Lincoln, Nebraska, USA

Animals

Rhesus monkeys, both male and female, aged 6–8 years, weighing 6–8 kg, were provided by the Experimental Animal Center of Suzhou, Suzhou, China (license No. SYXK(su)2010-0002). The study was conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and under the approval of the Administration Committee of Experimental Animals, Jiangsu Province, China.

Methods

Culture and characterization of monkey BMSCs

Bone marrow extracts were taken from the iliac crests of rhesus monkeys and resuspended in IMDM containing 10% FBS and 1% penicillin/streptomycin after centrifugation. The culture was kept in a humidified 5% CO_2 incubator at 37 ℃ for 72 hours, when non-adherent cells were removed by changing the medium. After 10–12 days, the 80% confluent cells were treated with 0.25% trypsin/0.01% EDTA and then diluted 1:2 per passage for cell expansion. BMSCs at passage 2 (P2) were used for phenotypic analysis. The cells were incubated for 30 minutes with FITC or PE-labeled monoclonal antibodies against CD90, CD29, CD73, CD11b, CD45, CD34 and CD105, respectively, prior to analysis by flow cytometry.

Preparation of PLGA extracts fluid

PLGA extract fluid was obtained by placing 1 g PLGA fibers in an extraction container with 10 mL IMDM at (37±0.5) °C for (72±0.5) hours. For control, the extraction fluids of hydroxyapatite or organotin were prepared in the same manner.

Morphology observation

BMSCs at P2 were co-cultured with the scaffold of PLGA fibers. After 4 days, the specimens were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes followed by treatment with 0.1% Triton X-100 containing 10% normal goat serum and 3% bovine serum albumin for another 30 minutes. The samples were incubated with mouse monoclonal anti-CD29 antibody (1:10) overnight at 4 ℃, and with FITC-labeled goat anti-mouse IgG (1:200) for 2 hours at room temperature, and then observed under a confocal laser scanning microscope.

For electric microscope observation, BMSCs cultured on PLGA fibers for 4 days were fixed in 4% glutaraldehyde and then post-fixed with 1% OsO_4, dehydrated stepwise in increasing concentrations of ethanol. Afterwards, the samples were coated with gold in a JFC-1100 unit and observed under a scanning electron microscope.

Cell viability assay

After a 24-, 48-, 72-hour or 7-day incubation in the extraction fluids of PLGA, hydroxyapatite, organotin or plain IMDM, BMSCs were subjected to MTT assay. In brief, a 10 μL MTT solution (5 mg/mL) was added to

each well at 4 hours prior to the end of incubation. Formazan crystals that formed in the cells were dissolved by adding 100 μL of dimethyl sulfoxide. Absorbance values were measured by spectrophotometry at 490 nm with an EIX- 800 Microelisa reader.

Cell cycle analysis

After BMSCs were cultured in plain IMDM or PLGA extract fluid for 72 hours, the cells were collected, washed twice with PBS, and fixed with 70% ethanol at −20 ℃ for 3 days. Cell pellets were washed twice with PBS and stained with PI/RNase staining buffer for 30 minutes in the dark at 4 ℃. The stained cells were analyzed by flow cytometry.

Quantitative real-time RT-PCR

To determine the mRNA levels of the NGF, BDNF, CNTF, and bFGF produced by the BMSCs that were cultured in different mediums, total RNA was extracted from the BMSCs and reverse-transcripted to cDNA using the Omniscript RT kit, following the manufacturer's instructions. The reaction in a 20 μL volume containing a 1 μL cDNA template was prepared using Fast EvaGreen® Master Mix and run with a Stepone Real-Time PCR machine. The procedures consisted of incubation at 95 ℃ for 2 minutes, and amplification with 45 cycles of denaturation at 95 ℃ for 15 seconds, followed by annealing and extension at 60 ℃ for 1 minute. The Ct values for each target gene (repeated in triplicate) were analyzed by the $2^{-\Delta\Delta Ct}$ method, using GAPDH as the housekeeping gene, and normalized to controls[25]. The sequences of the primers are shown in Table 1.

Table 1 Oligonucleotide primers used in real-time quantitative PCR

Target	Sequence (5' to 3')	Length (bp)
GAPDH	F: TCC GAC TTC AAC AGC GAC A R: CCA CCA CCC TGT TGC TGT AG	127
BDNF	F: ATC GAA AGG CCA ACT GAA G R: CCC GCA CAT ACG ACT GG	134
bFGF	F: GAA GAG CGA CCC TCA CAT CA R: TCT TCC ATC TTC CTT CAT AGC A	109
CNTF	F: TCC ACT TCA GGG TTC TAA TCT TT R: TCA GAC CGA GTC ATC CAG AAC	113
NGF	F: AGA CCC GCA ACA TTA CTG TGG AC R: ATG TCT GTG GCG GTG GTC TTA TC	258

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, BDNF: brain-derive neurotrophic factor; CNTF: ciliary neurotrophic factor; bFGF: basic fibroblas growth factor; NGF: nerve growth factor

Western blot analysis

After 48 hours, the BMSCs cultured in plain IMDM and PLGA extract fluid were respectively collected and lysed at 4 ℃ in a protein extraction reagent. Protein concentrations were determined with the BCA protein assay kit. Cell lysates were separated by SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes, which were then blocked with 5% nonfat dry milk in a TBST buffer. The membranes were then incubated with rabbit-specific polyclonal antibodies directed against various markers overnight at 4 ℃: BDNF (1:250), NGF-β (1:500), CNTF (1:200) and bFGF (1:500). The murine-specific monoclonal antibody to β-actin (1:1 000) was used as a lane loading control. After washing three times with a TBST buffer, the membranes were incubated with secondary antibodies donkey anti-rabbit-IRDye (1:10 000) or goat anti-mouse-IRDye (1:10 000,) at room temperature for 2 hours. Images were captured on an Odyssey Infrared Imager and analyzed using the Odyssey densitometry program.

Main outcome measures

Changes in morphology, viability and cell cycle of BMSCs cocultured with PLGA as well as expressions of NGF, BDNF, CNTF, bFGF mRNA and proteins.

Statistical analysis

Data were expressed as mean±SD. A one-way analysis of variance was used to conduct statistical analysis by the help of the Stata 7.0 software package (Stata Corp., College Station, TX, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of monkey BMSCs

The BMSCs isolated from the bone marrow of monkeys displayed a spindle shape. Flow cytometric analysis demonstrated that the BMSCs were positive for CD73 (99.89%), CD105 (92.16%), CD29 (99.35%), and CD90 (86.69%), and negative for CD34 (4.69%), CD45 (5.89%), and CD11b (5.87%) (Figure 1), which conformed to previous results[26-27].

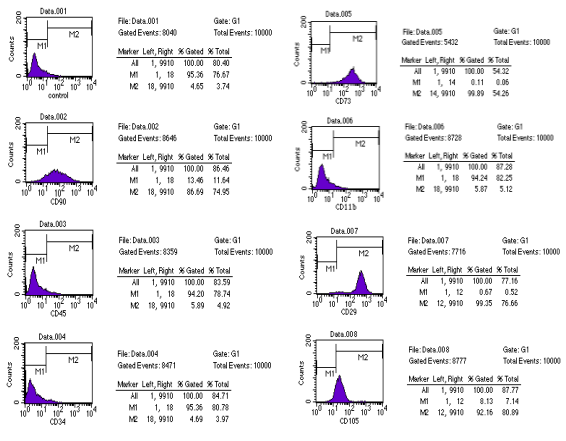
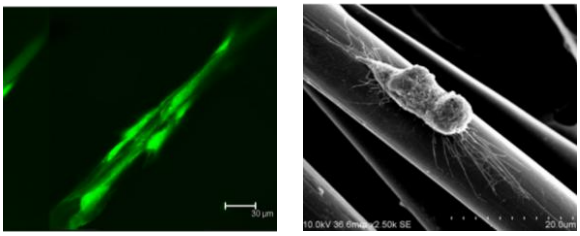


Figure 1 Phenotype analysis of cultured rhesus monkey bone marrow stromal cells by flow cytometry. The cells are strongly positive for CD29, CD105, CD73, CD90, and negative for CD34, CD45 and CD11b

Morphology of BMSCs cultured on PLGA scaffolds

After 4 days on the PLGA scaffolds, BMSCs enwrapped the PLGA fiber scaffold in cell groups, some of them encircled and elongated along the scaffold. The attached BMSCs exhibited a spindle- or spherical-shape, forming long cell chains along the scaffold (Figure 2).



a: Laser scanning confocal micrographs showing CD29-positive BMSCs extended along PLGA filament (Scale bar=30 μm)

b: Scanning electron micrographs showing attachment of BMSCs on PLGA fiber scaffolds (Scale bar= 20 μm)

Figure 2 Morphology of monkey bone marrow stromal cells (BMSCs) cultured on polylactic-co-glycolic acid (PLGA) fiber scaffolds

Survival and proliferation of BMSCs in PLGA extract fluid

Cell morphology: BMSCs cultured in the plain IMDM, PLGA extract fluid and hydroxyapatite extract fluid displayed spindle-shaped, fibroblast-like morphology. There were no significant differences in cell morphology among the cells cultured in these different mediums during the 7-day cultivation. In contrast, BMSCs cultured in organotin extract retracted their processes, were round shaped, and detached from the culture plate after 6 hours (Figure 3).

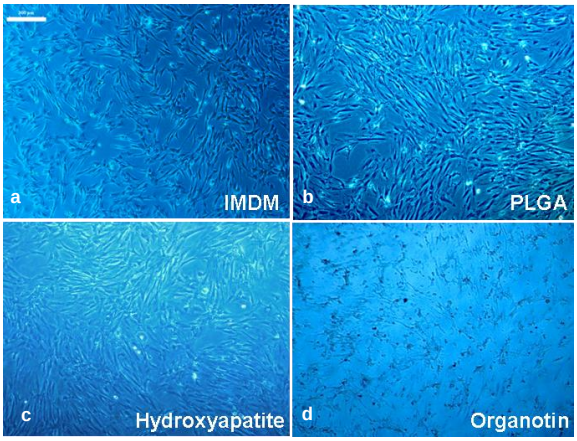
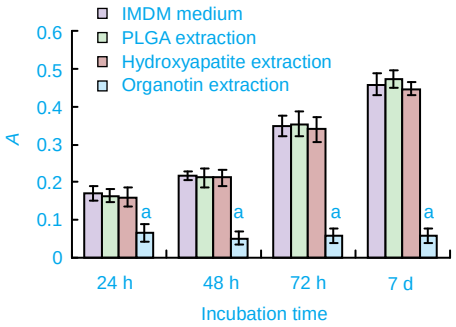


Figure 3 Phase contrast micrographs showing monkey bone marrow stromal cells cultured in the plain Iscove's Modified Dulbecco's Medium (IMDM) (a), polylactic-co-glycolic acid (PLGA) extract fluid (b), hydroxyapatite extract fluids for 72 h, and in the organotin extract fluids (c) for 4 h (d) (Scale bar=300 μm)

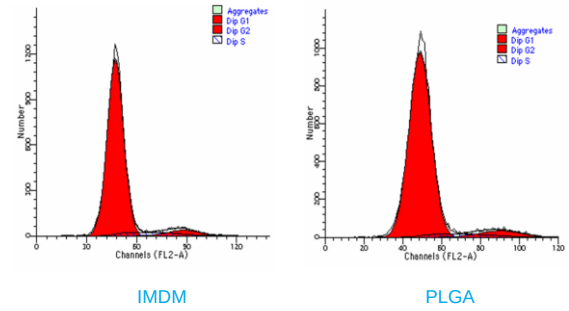
Cell viability: MTT assay showed that the cell viability of monkey BMSCs cultured in the PLGA extract fluid was not significantly different from that in the plain IMDM after 24, 48, 72 hours or 7 days culture. The viability of BMSCs cultured in the organotin extract fluid was, however, significantly lower than that in other mediums at all designated time points (Figure 4).



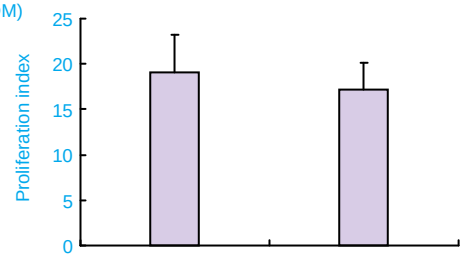
[a]*P* < 0.05, *vs*. BMSCs in the plain Iscove's Modified Dulbecco's Medium (IMDM)

Figure 4 Cell viability of bone marrow stromal cells (BMSCs) cultured in different culture mediums for 24, 48, 72 h and 7 d as measured by MTT assay

Cell circle: BMSCs cultured in the PLGA extract fluid for 72 hours showed a cell-cycle progression similar to that of BMSCs cultured in the plain IMDM for the same time period. No significant difference in cell proliferation, as indicated by the proliferation index ($(G_2M+S)/G_0G_1+G_2M+S$), was found between BMSCs cultured in two different mediums (Figure 5).



a: Flow cytometric analysis on the cell cycle of BMSCs cultured in the PLGA extract fluid and in the Iscove's Modified Dulbecco's Medium (IMDM)



b: Comparison of proliferation index [$(G_2M+S)/(G_0G_1+G_2M+S)$, %] of BMSCs cultured in different media

Figure 5 The effects of polylactic-co-glycolic acid (PLGA) on cell survival and proliferation of bone marrow stromal cells (BMSCs)

Growth factor expression by BMSCs cultured in PLGA extraction fluid

According to quantitative real-time RT-PCR and Western blot analyses, NGF, BDNF, CNTF and bFGF produced by BMSCs that were cultured in the plain IMDM and PLGA extract fluid exhibited no significant differences at the mRNA and protein levels (Figure 6).

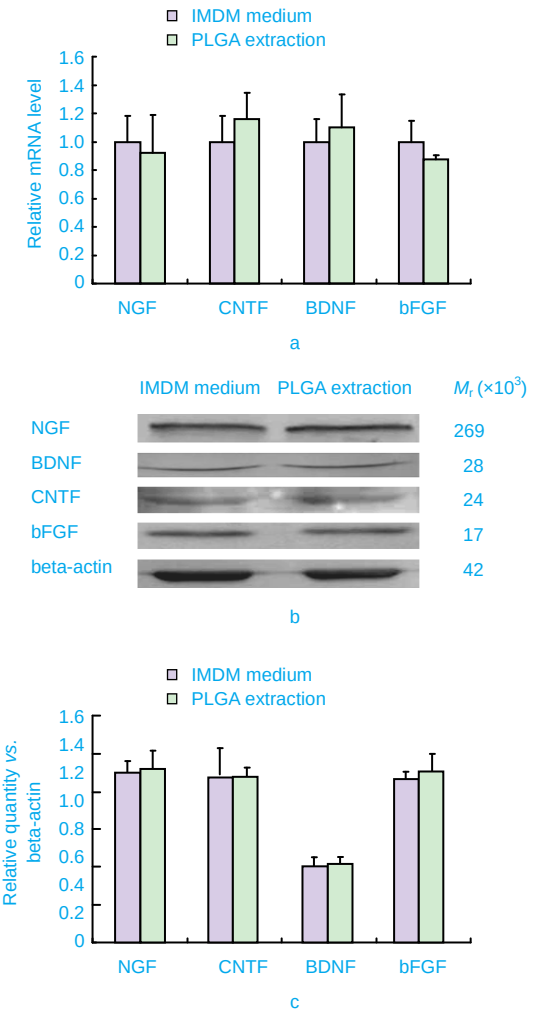


Figure 6 The effects of polylactic-co-glycolic acid (PLGA) on neurotrophic factors expressions of bone marrow stromal cells (BMSCs). The mRNA levels (a) and protein levels (b, c) of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF) expressed by BMSCs treated with the plain Iscove's Modified Dulbecco's Medium (IMDM) and PLGA extract fluid, respectively

DISCUSSION

An ideal biomaterial for preparing a neural scaffold for tissue-engineering nerve grafts requires good biocompatibility as well as biodegradation capacity. Biocompatibility covers not only non-cytotoxicity, non-carcinogenesis and non-mutagenesis, but also cell-affinity, such as the promotion of cell survival, adhesion, proliferation and differentiation, and even activation of the expression of certain intracellular genes or the transduction of intracellular signals. Because different types of support cells may exhibit different reactions on the scaffold materials, it is important to investigate the related biocompatibility.

In this study, we chose rhesus monkey BMSCs as support cells, whose biologic characteristics are similar to those of human BMSCs. We found that monkey BMSCs adhered to and migrated along the PLGA fiber scaffold, forming long cell chains and exhibiting multiple processes. In order to examine the effects of the PLGA fiber scaffold on survival, growth and proliferation of BMSCs, we performed a cytotoxicity test in accordance with International Standards ISO 10993-12 and ISO 10993-5. The morphology and viability of monkey BMSCs cultured in the PLGA extract fluid were similar to those cultured in the plain IMDM after 7 days. Also, no significant difference in cell proliferation was found between the cells cultured in different media according to the results of cell-cycle analysis. The results indicated that the PLGA scaffold had no adverse effect on cell growth and proliferation.

To determine whether the scaffold materials influenced the paracrine ability of the BMSCs, we investigated the expression of NGF, BDNF, CNTF and bFGF produced by the BMSCs cultured in PLGA extraction fluid. NGF and BDNF are known to be important for the development, maintenance and response to injury of the peripheral nervous system, CNTF and FGF may improve remyelination and promote axonal regeneration after peripheral nerve injury[28-31]. The results demonstrated that the PLGA extraction fluid did not influence expressions of these neurotrophic factors at either the mRNA or protein levels, providing evidence that the PLGA fiber scaffold was suitable for promoting the biologic activities of the BMSCs.

It is reasonable for us to conclude that the morphology, viability, proliferation, and growth factor production of BMSCs were not altered by the PLGA fiber scaffold, indicating a high biocompatibility of PLGA material with the BMSCs of rhesus monkeys. This study might provide a necessary prerequisite for an *in vivo* study of the joint use of PLGA scaffolds and BMSCs of non-human primate origin in peripheral nerve regeneration.

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聚乳酸乙醇酸与猕猴骨髓基质细胞的生物相容性***☆◆

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文章亮点：

以灵长类动物猕猴作为实验对象，证明支架材料聚乳酸乙醇酸在体外与猕猴骨髓基质细胞有良好的生物相容性，且不会影响猕猴骨髓基质细胞神经营养因子的分泌。

摘要

背景：聚乳酸乙醇酸与不同细胞的生物相容性的报道较多，但探讨其与猕猴骨髓基质细胞生物相容性的研究极少。

目的：观察猕猴骨髓基质细胞的生物学特性以及其与支架材料聚乳酸乙醇酸在体外的生物相容性。

方法：将经流式细胞检测证实的第 2 代猕猴骨髓基质细胞与聚乳酸乙醇酸支架材料联合培养，以单独培养的骨髓基质细胞作为对照。

结果与结论：猕猴骨髓基质细胞与聚乳酸乙醇酸共培养 4 d 后，激光共聚焦显微镜下可见 CD29 阳性的骨髓基质细胞黏附包绕在聚乳酸乙醇酸纤维表面，形成长的细胞链。扫描电镜下可见骨髓基质细胞于聚乳酸乙醇酸上贴附良好，且有较多细长的突起从骨髓基质细胞伸出，沿材料伸展。骨髓基质细胞在聚乳酸乙醇酸浸出液中培养 72 h，骨髓基质细胞神经营养因子表达量与对照组比较差异无显著性意义($P > 0.05$)；培养 7 d，其形态、细胞活力、细胞增殖指数与对照组亦无明显差异。提示猕猴骨髓基质细胞与聚乳酸乙醇酸支架材料有良好的生物相容性。

关键词：骨髓基质细胞；聚乳酸乙醇酸；生物学特性；生物相容性；猕猴；生物材料

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伦理要求：实验过程中对动物的处置符合科技部颁布的《关于善待实验动物的指导性意见》的相关要求。

(Edited by Li YK, Su LL/Wang L)

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