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Title: Study of injectable hydrogel microspheres loaded with bone marrow mesenchymal stem cells for the treatment of degenerative intervertebral disc

Study of injectable hydrogel microspheres loaded with bone marrow mesenchymal stem cells for the treatment of degenerative intervertebral disc

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Abstract: Intervertebral disc degeneration is the main cause of low back pain. Stem cell transplantation is an advanced medical technology, which brings hope for the treatment of intervertebral disc degeneration. Stem cell therapy is to transplant healthy stem cells or stem cell-related products into patients to repair or replace damaged cells or tissues, so as to achieve the goal of cure. However, stem cell therapy alone has its drawbacks. In order to improve the activity of stem cells, prevent leakage *in vivo*, we prepared photocross-linked hydrogel microspheres by microfluidic technology and loaded bone marrow mesenchymal stem cells into hydrogel microspheres. By injecting these microspheres into intervertebral disc, the progression of intervertebral disc degeneration was reversed. This project provides a strong theoretical basis for the final transformation and clinical application.

Keywords: Microfluidic technology, hydrogel microspheres, intervertebral disc degeneration, stem cell therapy, tissue engineering, injectable hydrogels, biomaterials

1 Introduction

Intervertebral disc degeneration (IVDD) is a primary cause of spinal degenerative disease, which can be manifested as herniation of intervertebral disc, instability of spinal segment, spinal stenosis, nerve root irritation and myelopathy. The motor dysfunction caused by IVDD is widespread in the middle-aged and elderly people, and there is a growing trend of younger and younger, which is one of the main causes of musculoskeletal disability. The United States spends more than $190 billion annually on disc degeneration-related diseases, and the incidence of low back and leg pain in industrialized countries is as high as 60-90%. The existing treatment methods for intervertebral disc degeneration include surgical treatment and non-surgical treatment, however, there is a common limitation of these methods that they can only relieve the symptoms of the end-stage instead of preserving or reconstructing the structure and mechanical function of the intervertebral disc tissue. Therefore, there is an urgent need for a method that can effectively prevent the process of intervertebral disc degeneration and restore its function.

Stem cell transplantation is an advanced medical technology, which brings hope for the treatment of some difficult and complicated diseases. Stem cell transplantation therapy is to transplant healthy stem cells into patients to repair or replace damaged cells or tissues, so as to achieve the goal of cure. Stem cell transplantation has a wide range of treatments. It can generally treat nervous system diseases, immune system diseases, and other medical and surgical diseases. Stem cells are called "multipurpose cells" in the medical field. They can differentiate into many functional cells or organs. Stem cells cultured in APSC pluripotent cell lab have the potential of unlimited proliferation, multidirectional differentiation, hematopoietic support, immune regulation and self-replication.

Stem cells have been shown to repair degenerative intervertebral disc and restore the function of intervertebral disc. However, there are defects of stem cell therapy alone. Firstly, the activity of stem cells is greatly reduced due to the deterioration of the

microenvironment in the degenerative intervertebral disc. Secondly, because of the limitation of administration, stem cells may leak out and osteophytes form in paravertebral tissues after injection.

Microfluidics refers to the science and technology involved in the use of microchannels (with sizes ranging from tens to hundreds of microns) to process or manipulate micro fluids (with volumes ranging from nanoliters to ascendants). It is a new interdisciplinary subject involving chemistry, fluid physics, microelectronics, new materials, biology and biomedical engineering. Because of its miniaturization and integration, microfluidic devices are often referred to as microfluidic chips, also known as Lab on a Chip and micro-Total Analysis System.

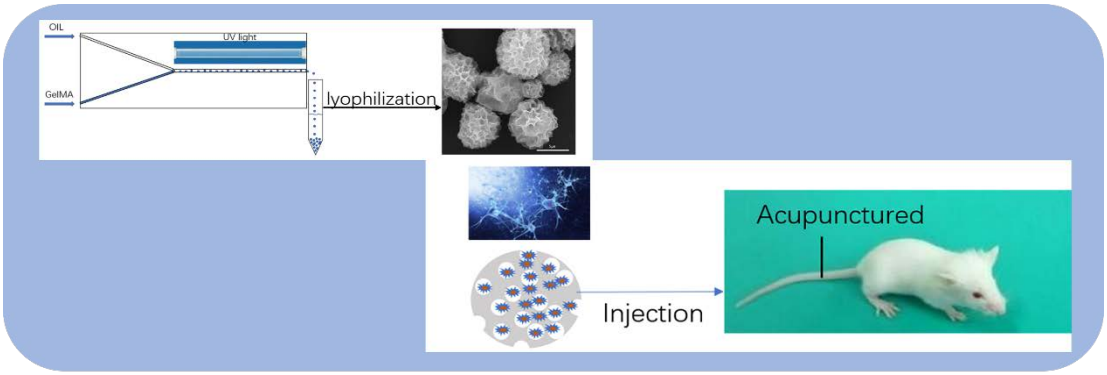

Fig. 1 Scheme of project

2 Materials and Methods

2.1 Preparation of photo-crosslinked hydrogel particles by microfluidic technology

A microchannel device with a square glass tube with a side length of 1 mm is designed and fabricated, and a circular glass tube with an outer diameter of 1.0 mm and an inner diameter of 0.7 mm is drawn into a tip of 100 micron by a capillary pull-out apparatus, and inserted into the main channel to guide the intermediate fluid. The external fluid is introduced through the gap between the square glass tube and the circular glass tube. The flow of the two fluids is regulated by two injection pumps, so that the inner and the intermediate fluids first form stable laminar flow, and then form two or three fluids

laminar flow together with the external fluids in the main channel. The hydrogel microspheres are constructed by means of light curing and chemical curing.

2.2 Effects of hydrogel microspheres on physiological behavior of BMSCs and its mechanism

2.2.1 Micromorphology of hydrogel microspheres

The morphological characteristics of hydrogel microspheres are observed by scanning electron microscopy (SEM) from different angles (material surface, transverse section, longitudinal section), and the microspheres diameter is measured.

2.2.2 Cell viability

Microspheres were stored in cell culture medium, and cell viability is measured once a day within 1-7 days. Live / dead kit is used for detection. Then fluorescence microscope is used for observation. The results are analyzed by image-J software to calculate the ratio of surviving cells to total cells; CCK-8 kit is used to characterize the number of cells, and the effect of different drug loading GelMA hydrogel scaffolds on the growth of BMSCs is investigated to evaluate the effect of the system on BMSCs

2.3 Puncture model of intervertebral disc degeneration in rats

Three-month-old male SD rats weighing 250-300g are selected. After satisfactory anesthesia, the puncture of nucleus pulposus in intervertebral disc is performed with 18G needle after the center of the target intervertebral disc was determined. The depth of puncture needle is 5mm. Immediately after the puncture operation, X-ray is used to confirm the location of the puncture needle in the nucleus pulposus. At the same time, the puncture needle is pulled out to observe whether the vacuum tissue is nucleus pulposus tissue to further confirm the correct puncture position.

2.4 Radiological evaluation of the effect of BMSC loaded hydrogel microspheres on repairing degenerative intervertebral disc in rats

2.4.1 MRI evaluation

MRI coccygeal vertebra scanning is performed on all experimental animals at 4, 8 and 12 weeks after puncture. The sagittal T2WI images of coccygeal spine are evaluated. The disc changes are classified into 5 grades by Pfirrmama classification. The setting of MRI parameters: According to the pre-experiment, the switching rate of gradient

field is 150MT/m/ms and the gradient field intensity is 30mT/m. The spin echo sequence T2WI: TR, TE = 3500 MS / 120 ms, scan matrix 256 * 256, reconstruction matrix 512 * 512, FOV (mm) = 100.00, RFOV (%) = 100.00, layer thickness 3 mm, layer spacing 0.3 mm.

2.4.2 X-ray evaluation

X-ray lateral lumbar lateral scan is performed on all experimental animals at 4, 8 and 12 weeks after puncture. The height of vertebral intervertebral space (DH) is measured by lateral radiograph.

2.5 Histological evaluation of the effect of BMSC loaded hydrogel microspheres on repairing degenerative intervertebral disc in rats

Tissue specimens were collected 8 weeks after operation. All specimens were collected under sterile condition. The specimens were fixed at 4% paraformaldehyde for 48 hours and decalcified by EDTA for 4 weeks. After decalcification, tap water was washed overnight, gradient alcohol was dehydrated, xylene was transparent and embedded in paraffin. After embedding, the wax blocks were frozen overnight in the refrigerator at - 20 C. Continuous slices with a thickness of 5 microns were made by Leica tissue microtome. Slices were baked overnight in a constant temperature oven at 60 C. Alcian-Blue staining was used to compare the structural changes of intervertebral discs.

3 Results

3.1 Preparation of photo-crosslinked hydrogel particles by microfluidic technology

The microfluidic device was established. Injectable hydrogel microspheres were prepared by microfluidic technology. Under microscope, microspheres showed monodisperse size distribution (Fig 2a,2b). Scanning electron microscopy (SEM) showed that the microspheres were spherical structure with porous microstructures. And the diameter of the microspheres were 450 μm approximately (Fig 2c,2d).

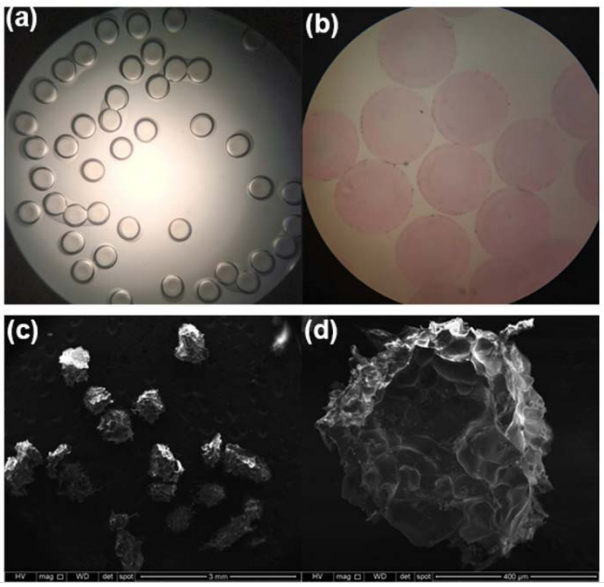

Fig 2. the morphology of microspheres. (a,b) Microscopic images of the microspheres. (c,d) Scanning electron microscopy (SEM) images of the microspheres.

3.2 Effects of hydrogel microspheres on physiological behavior of BMSCs

CCK-8 kit assay and cell viability staining indicated that cells and microspheres could be co-cultured and the microspheres had high biocompatibility (Fig 3).

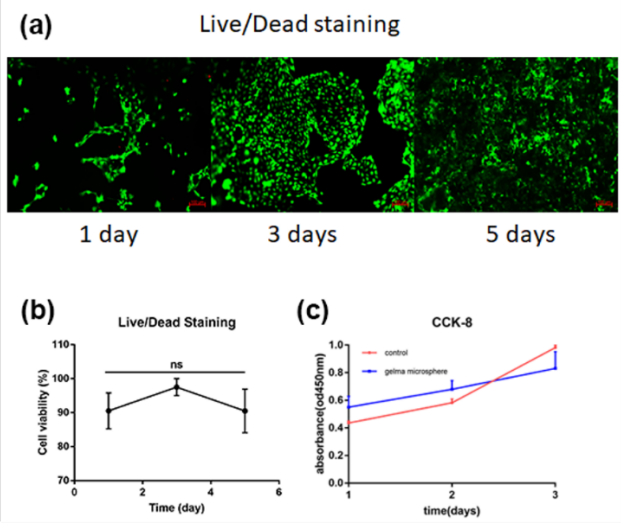

Fig 3. The biocompatibility of microspheres.(a) the images of Live/Dead Staining. (b) The quantitative data of Live/Dead Staining. (c) CCK-8 assay of microspheres

3.3 Radiological evaluation of the effect of BMSC loaded hydrogel microspheres on repairing degenerative intervertebral disc in rats

Puncture model of intervertebral disc degeneration in rats was successfully established as previous reported. The degeneration of intervertebral disc with the decrease of intervertebral space height appeared 8 weeks after the tail vertebra was needled. After 8W, MRI showed that there was water loss in the intervertebral disc. X-ray showed the height of intervertebral space after injection of hydrogel microspheres is higher than

that of puncture alone (Fig 4a). MRI showed water content of intervertebral disc after injection of hydrogel microspheres was higher than that of puncture alone (Fig 4b).

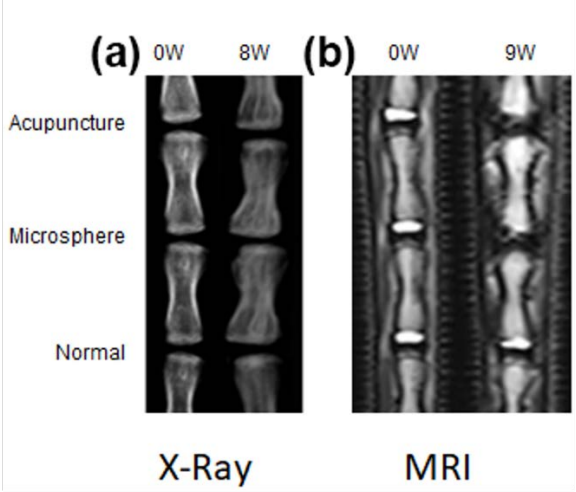

Fig 4. Imaging of coccygeal vertebral of rats. (a) The X-Ray images of pre-operation and 8W after operation. (b) The MRI images of pre-operation and 8W after operation.

3.4 Histological evaluation of the effect of BMSC loaded hydrogel microspheres on repairing degenerative intervertebral disc in rats

The degree of intervertebral disc degeneration can be compared by Alcian-Blue staining. After 8W, the degeneration of intervertebral disc without microsphere treatment was obvious, the structure of nucleus pulposus disappeared completely, the cells inside nucleus pulposus were almost invisible, and the demarcation between nucleus pulposus and annulus fibrosus was not obvious (Fig 5a). The discs treated with microspheres had good properties, which was similar to the normal discs (Fig 5b, 5c).

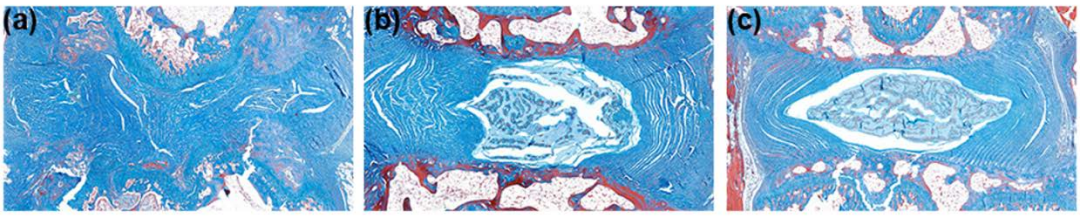

Fig 5. Histological sections of rat coccygeal intervertebral disc after 8W. (a) The acupuncture group, (b) The microspheres group, (c) The normal group.

4 Discussion

IVDD is the initial cause of degenerative diseases of the spine. It can be manifested as

herniation of the intervertebral disc, instability of the spinal segment, spinal stenosis, nerve root irritation and myelopathy. The motor dysfunction caused by it exists widely in the middle-aged and elderly people, which is one of the main reasons leading to musculoskeletal disability. There is an urgent need to find a way to protect intervertebral disc from degeneration. IVDD is mainly caused by the lose of nucleus pulposus cells. It is reported that stem cells can not only differentiate into nucleus pulposus cells but also improve nucleus pulposus cells proliferation. However, there are several shortcomings using stem cells only *in vivo*. Stem cells are less resistant to the environment. It's possible that stem cells would all die soon when inject into the intervertebral disc because of the low pH microenvironment. Furthermore, it's reported that stem cells are liable to leak while injecting. So it's important to developed a cell delivery system can overcome the shortcomings of stem cells.

In this project, photo-crosslinking hydrogel microspheres prepared by microfluidic technique are used to deliver stem cells to intervertebral disc. Hydrogel microspheres have multi-void structure, which is capable of accommodating stem cells to grow in. The diameter of microspheres is about 450 μm, which is easy to go through the syringe. And because the microspheres crosslinked by ultraviolet lamp are solid, they are not easy to leak. A cell delivery system which is made of photo-crosslinking hydrogel microspheres prepared by microfluidic technique and loaded with BMSCs is able to maximize the efficacy of stem cells and reduce the weakness of stem cells.

To evaluate whether a biomaterial can be used in vivo, we should pay attention to whether it has good biocompatibility. CCK-8 kit assay and L/D staining showed good biocompatibility of the microspheres.

Imaging data prompts that photo-crosslinking hydrogel microspheres loaded with BMSCs is good for intervertebral disc. They can reduce the decrease of intervertebral disc height and lose of water. The microspheres are able to protect intervertebral disc for degeneration *in vivo*.

Histological sections suggest that the discs undergoing puncture degenerate significantly after 8W. At the same time, the microenvironment of the discs improved

after microsphere treatment, which provides a strong support for the regeneration of degenerated discs.

5 Conclusion

We have developed a cell delivery system which is made of photo-crosslinking hydrogel microspheres prepared by microfluidic technique and loaded with BMSCs. This project provide scientific and technological support for the effective prevention IVDD, and provide a strong theoretical basis for the final conversion into clinical application.

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