

THE EFFECT OF PLGA-COLLAGEN I PATCH ON INGUINAL HERNIA

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Different approaches are used for the treatment of inguinal hernia, and patches play a central role. This study aimed to investigate the efficacy of a new patch with poly(lactic-co-glycolic acid) (PLGA) - Collagen I, on a murine model of inguinal hernia. 30 rats with induced abdominal wall defect were divided into 2 groups, an experimental group and a control group, 15 animals *per* group. The experimental group was treated with PLGA-Collagen I patch implanted with bone marrow mesenchymal stem cells (BMSCs) and the control group was treated with cell-free PLGA-Collagen I patch. After implantation each group was divided in 3 subgroups, 5 animals *per* subgroup intended to evaluate the changes at 1, 2 and 6 months. The pro-inflammatory factors IL-6 (interleukin -6) and IL-10 (interleukin -10) were investigated at 7, 14 and 28 days after implantation. The peritoneal inflammatory reactions were evaluated by identifying the macrophage surface antigen modifications in tissue. The regeneration of the tissue in the patch and the degradation state of the material was evaluated by haematoxylin-eosin (HE) staining. SEM was used to observe the integrity of peritoneum and the angiogenesis was detected by immunohistochemistry. After a week, the macrophages of both groups were significantly increased; after one month, the inflammatory reaction gradually subsided and the number of macrophages decreased. One month after surgery, there was no swelling in the abdominal wall of the experimental group and the control group. The PLGA-Collagen I patch of BMSCs can promote cell infiltration, angiogenesis and tissue regeneration. The degradation rate of PLGA increased. In sixth months, the BMSCs PLGA-Collagen I was almost completely degraded compared with cell free PLGA-Collagen I patches, in which the degradation was slower. PLGA-Collagen I tissue engineering materials planted with BMSCs can promote the repair of abdominal injury and can be a good candidate for the treatment of abdominal wall defects.

Rezumat

În abordarea terapeutică a herniei inghinale platurii joacă un rol central. Acest studiu a urmărit investigarea eficacității unui platură nou, conținând o asociere a colagenului de tip I cu poli(acid lactic-co-glicolic) (PLGA), pe un model murin de hernie inghinală. 30 de șobolani cu defect de perete abdominal indus au fost împărțiți în două grupuri, un grup experimental și un grup de control, 15 animale/grup. Grupul experimental a fost tratat cu platură PLGA-colagen I implantat cu celule stem mezenchimale (BMSC) din măduva osoasă, iar grupul martor a fost tratat cu platură PLGA-colagen I fără celule. După implantare, fiecare grup a fost împărțit în 3 subgrupuri, 5 animale/subgrup, destinate evaluării modificărilor după 1, 2 și 6 luni. Au fost determinați factorii proinflamatori IL-6 (interleukina -6) și IL-10 (interleukina -10) la 7, 14 și 28 de zile după implantare. Reacțiile inflamatorii peritoneale au fost evaluate prin identificarea modificărilor antigenului de suprafață a macrofagelor. Regenerarea țesutului și starea de degradare a materialului a fost evaluată prin colorație hematoxilina-eozină (HE). Microscopia electronică a fost utilizată pentru a observa integritatea peritoneului, iar angiogeneza a fost detectată prin imunohistochimie. Testele pe termen lung au arătat că platurile ar putea promova sinteza fibrelor de colagen, regenerarea vaselor de sânge și repararea leziunii peretelui abdominal.

Keywords: PLGA-Collagen I patch; inguinal hernia; macrophage; inflammatory response, IL-6, IL-10**Introduction**

Inguinal hernia is a protrusion of the abdominal canal through the abdominal contents of the abdominal wall defect, known as direct hernia and oblique hernia [1, 2]. Direct hernia occurs mainly in adults. Compared with the oblique hernia, it is more likely to recur [3]. Most hernia do not have high risk, and can be operated to repair the defect. If hernia seriously affects the supply of blood flow in the intestines, surgical intervention is needed [4-6]. Currently, more and more studies show that the formation of abdominal hernia does not come from abdominal wall wear [7-9], but it is a group of histological lesions caused by disturbed collagen

metabolism and systemic connective tissue damage [10]. The disease has a certain degree of correlation with the heredity and acquired factors. Therefore, the incidence of hernia is not formed independently. It is a systemic disease caused by a variety of factors. Hernia repair is a common general surgery. The aim is to reduce the patient's pain and complications, to repair the defect and the weak point. Inguinal hernia has many treatments and approaches, such as open surgical suture, open patch repair, laparoscopic patch repair, etc. [12, 13]. There are various patch materials available on the pharmaceutical market, some of them being expensive [17]. This study aimed to investigate

the repair effect of a new patch material, poly(lactic-co-glycolic acid) (PLGA) - Collagen I patch on inguinal hernia.

Materials and Methods

Reagents

Foetal bovine serum (Shenzhen Kangchuyuan Co., Ltd., Shenzhen, China); immunohistochemistry kits (Thermo Fisher Scientific (China) Co., Ltd., Shanghai, China); EDTA (ethylenediaminetetraacetic acid) (China bioengineering Biotechnology (Shanghai) Co., Ltd., Shanghai, China); DAB (diaminobenzidine) colour box (Beijing Baiaolaibo Technology Co., Ltd., Beijing, China); PLGA75/25 (Sigma, USA); HFIP (hexafluoroisopropanol) (Sigma, USA); collagen I (Sigma, USA); chloroform, acetone, phosphate buffer solution (PBS) and heparin sodium (Wuhan Biocar Biological Medicine Co., Ltd., Wuhan, China); tween-20 (Hubei Xinxin Jiali Biotechnology Co., Ltd., Wuhan, China); normal saline (Foshan City Comfort Medical and Health Supplies Co., Ltd., Foshan, Guangdong); polyoxymethylene (POM) (Shandong West Asia Chemical Industry Co., Ltd., Linyi, Shandong); vWF antibody and F4/80 antibody (Abcam, USA); CCR7 (CC receptor 7) antibody, CD206 antibody, BSA (bovine serum albumin), 3,3'-diaminobenzidine (DAB) chromogenic solution and Masson's stain (Wuhan BOSTER Biological Technology Co. Ltd, Wuhan, China); PKH26 Kit (Red Fluorescent Cell Linker Kit; Sigma, USA); IL-6 and IL-10 ELISA Kits (Shanghai Meilian Biotechnology Co., Ltd., Shanghai, China); DMSO (dimethylsulfoxide) (Changzhou City Chemical Co., Ltd., Changzhou, China).

Equipment

Ultra-quiet workbench (Shanghai Qiao Yue Electronics Co., Ltd., Shanghai, China); carbon dioxide cell culture box (Shandong Boke Biological Industry Co., Ltd., Jinan, China); digital electron microscope (DEM) inverted fluorescence microscope (Shunyu Optical Technology Co., Ltd., Ningbo, China); pH-meter and scanning electron microscope (Nantong Water Environmental Protection Technology Co., Ltd., China); high-voltage electrostatic spinning machine (Tianjin Yunfan Tech. Co., Ltd., Tianjin, China); electronic balance (Shanghai Jingpie Instrument Co., Ltd., Shanghai, China); Telstar freeze dryer (Telstar, Spain); paraffin embedding machine and paraffin slicer (Leica, Germany); automatic microplate reader (Thermo, USA); scanning electron microscopy (Olympus, Japan); laser scanning confocal microscope (Leica, Germany).

Preparation of PLGA collagen scaffolds

The PLGA was dissolved in HFIP (hexafluoroisopropanol) at a concentration of 20%. Collagen I was also dissolved in HFIP at a concentration of 6.66%. The ratio of PLGA: Collagen I was 1:1 (v/v). The mixture was loaded into the spinning tube (diameter 21). The PLGA-Collagen I scaffold was prepared by

electrostatic spinning using a roller rotating at a uniform speed using a voltage of 18 kV, a distance of 153 cm and a flow velocity of the spinning solution of 1 mL/h. Then the PLGA-Collagen I scaffold was freeze-dried for 24 h.

Extraction and identification of seed cells

Six healthy, four weeks old Sprague Dawley male rats with an average weight of 110 g were selected. The rats were acclimatized to the new laboratory conditions for one week: constant temperature of $24 \pm 1^\circ\text{C}$, relative humidity of $50 \pm 5\%$ and 12 h light - dark with free access to feed and water. The rat bone marrow mesenchymal stem cells were extracted from the bone marrow of the rats and identified by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and immunohistochemistry. The animal study was approved by the Ethics Committee of the Hangzhou First People's Hospital, Hangzhou and the animal handling methods involved in the experiment were also in line with the International Animal Health Regulations.

Isolation and culture of rat bone marrow mesenchymal stem cells. Rats were sacrificed using pentobarbital (Shanghai Xinya Pharmaceutical Co., Ltd, 40 mg/kg bw) injected intraperitoneally. Afterwards, the body was immersed in 90% benzalkonium bromide solution for 2 minutes and then disinfected with 75% alcohol for 3 - 5 minutes. The skin of hind limbs was cut and tibia and femur were removed for extracting the bone marrow from the cavity. After extraction of the bone marrow it was cleaned with 2 mL PBS until the bone became white. After washing all the bones, the cells were dispersed in the liquid by gentle blow repeatedly with a 1 mL transporter. Then the cell suspension was transferred into a 15 mL centrifugal tube with 200 mesh nylon grid filters placed at the orifice of the tube. The filtrate of the centrifugal tube was collected and centrifuged at 1000 rpm for 6 minutes. When the supernatant was discarded, the cells were read. The cells were mixed with 2 - 3 mL erythrocyte lysate and kept for 2 minutes at room temperature. After lysis, the same volume of FBS was added to stop the lysis and centrifuged at 1000 rpm for 6 minutes. The cells were cultured in CO_2 with 9 mL for 1 mL of cell suspension. After 24 hours of culture, half of liquid was changed and again changed after the third day. Cells were sub-cultured when the bottom of the dish was about 80%. Medium was absorbed and 800 μL 0.25% digestive juice containing EDTA was added. The trypsin was slightly shaken to cover the whole dish. Microscopically, the cells contracted and became round. When most of the cells contracted and there was a moderate amount of floating liquid, the digestion was terminated by adding a complete medium.

MTT assay [11]

The enlarged third-generation bone marrow mesenchymal stem cells were mixed with 10% foetal bovine serum full medium and inoculated into 96-well plate. The

cells were cultured in a constant temperature incubator with 5% CO₂ at 37°C for nine days. On the fourth day, the complete culture medium was replaced according to the conventional method to avoid cell death due to insufficient nutrition and increased metabolic waste. Starting from the first day after inoculation, one plate was taken daily for testing. 20 µL MTT solution was added to each plate hole and incubated for 4 hours in a constant temperature incubator at 37°C and 5% CO₂. Subsequently, the liquid in the hole was carefully sucked out with a pipette, and then 150 µL DMSO was added and let to react for 10 minutes on the oscillator to dissolve the crystals. The absorbance values (OD values) of each hole were measured with an automatic enzyme labelling instrument, $\lambda = 492$ nm.

Immunohistochemical identification of the cells

The immunohistochemical identification of the cells standard haematoxylin stained was used and the expression of CD34, CD44, CD45 and CD105 was established.

The animal model

In this study, an animal model of abdominal wall defect was used to investigate the effect of PLGA-Collagen I Patch on the repair of abdominal wall defects. 30 healthy, four weeks old CD-SD male rats were selected. Ketamine (Qigihar Second Pharmaceutical Co., Ltd., China) in doses of 100 mg/kg bw intraperitoneal was used to anesthetize the rats. The lower abdomen of the rat was sterilized with 70% alcohol. Then, the abdominal wall fascia and abdominal muscle, internal oblique was reserved and next to the spermatic cord a defect of abdominal wall was made. After the model was obtained, the rats were divided into two groups: the experimental group (15 rats) and the control group (15 rats). The experimental group: BMSCs (bone marrow mesenchymal stem cells) labelled with PKH26 (Red Fluorescent Cell Linker Kit) was planted on the PLGA-Collagen I collagen scaffold. The planted of the cells on the scaffold was made as follows: 500 µL 2×10^6 cell suspension was cultured for 2 h at 37°C and 5% CO₂ with the scaffold. After complete adsorption, the complete culture medium was added to up to 5 mL to continue the culture for 22 h. the control group: the same size PLGA-Collagen I collagen scaffold was placed directly in the complete medium for soaking 24 h.

The processed materials were transplanted into the inguinal hernia model, and the animals were subdivided into 3 respectively groups (5 animals *per* group) and observed for 1, 2 and respectively 6 months. The abdominal wall of the experimental group and the control group were slightly swollen after operation. However, this does not affect the activity.

We evaluate the plasma pro-inflammatory factors IL-6 and IL-10 using ELISA kits according to manufacturer instructions. Immunohistochemistry was used to detect macrophage surface antigen F4/80, CCR7 and CD206. The number of cells in macrophages, M1 macrophages and M2 macrophages were assessed to analyse the inflammatory reaction. HE staining was used to observe the regeneration of the tissue and the degradation state of the patch.

At each observation point (1, 2 and 6 months) 5 animals from each group were sacrificed using pentobarbital injected intraperitoneally in doses of 40 mg/kg bw. The peritoneal tissues were collected and stained with HE (haematoxylin-eosin). SEM (scanning electron microscope) was used to observe the integrity of peritoneum, the angiogenesis state was detected by immunohistochemistry and the formation of collagen fiber was detected by Masson's trichrome staining.

Statistical analysis

The data obtained in this study were statistically analysed with SPSS19.0 software. The analysis of variance and q test were used in each group. The paired Student's t test was used among the groups. The difference was statistically significant at $p < 0.05$.

Results and Discussion

The expression of IL-6 and IL-10

In the experimental group and the control group, IL-6 increased continuously in the first 28 days. In the experimental group, IL-6 increased less than in the control group and the difference between the two groups was statistical significant at the 28th day ($p < 0.05$) (Figure 1A). IL-10 levels increased till the 14th day and after started to decrease. In the experimental group IL-10 level was higher than that of the control group, and the difference was statistical significant in the 28th day ($p < 0.001$) (Figure 1B).

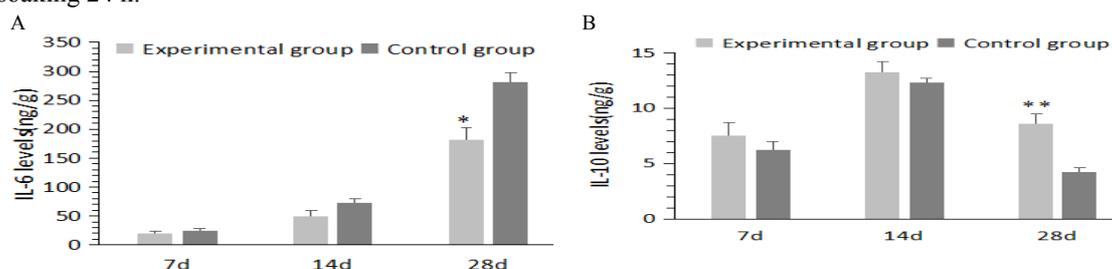


Figure 1.

IL-6 and IL-10 levels in the two groups at 7, 14 and 28 days after treatment (* $p < 0.05$, ** $p < 0.001$, experimental vs control group)

Immunohistochemical detection of macrophages

As shown in Figure 2, the distribution of macrophage infiltration stents in the experimental group was higher than that in the control group. The total macrophages in the two groups reached the maximum on the seventh day. However, on the fourteenth and the 28 day, they all decreased gradually (Figure 2A). The control group began to decrease when the M1 type

macrophage reached the maximum at 14th day, while the experimental group presented a descendent trend from the 7th day (Figure 2B). In the 28th day, the M2 cells in both the control group and the experimental group were gradually reduced, and the M2 cells in the experimental group increased compared to the control group (Figure 2C).

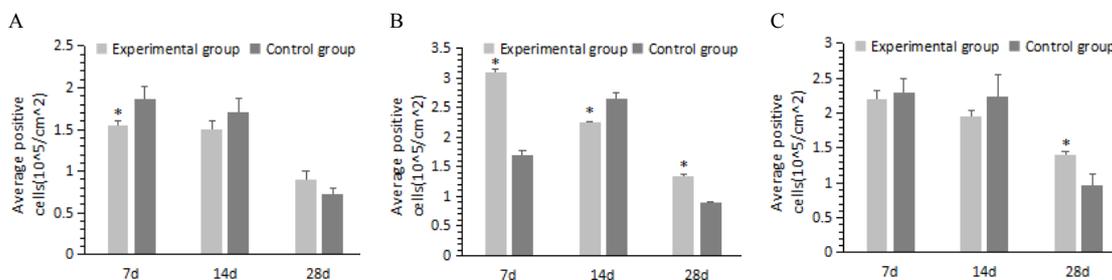


Figure 2.

Macrophage distribution in the 2 groups at 7, 14 and 28 days after treatment (A: anti-F4/80, B: anti-CCR7, C: anti-CD206; *p < 0.05, experimental vs control group)

The regeneration of the tissue in the patch and the degradation state of the patch by HE staining

The results of HE staining showed that the boundary of the patch material in the experimental group grew tightly together with the surrounding tissue of the animal, while the control group was not close to the experimental group. As shown in Figure 3, in both groups it could be seen a degradation of the material in the first and second months. In the sixth month,

for the experimental group, the patch materials were almost completely degraded, while in the control group, there was still PLGA in the sixth month, and the degradation rate was low.

The integrity of the peritoneum

As shown in Figure 4, the peritoneal integrity of the experimental group was superior to that of the control group.

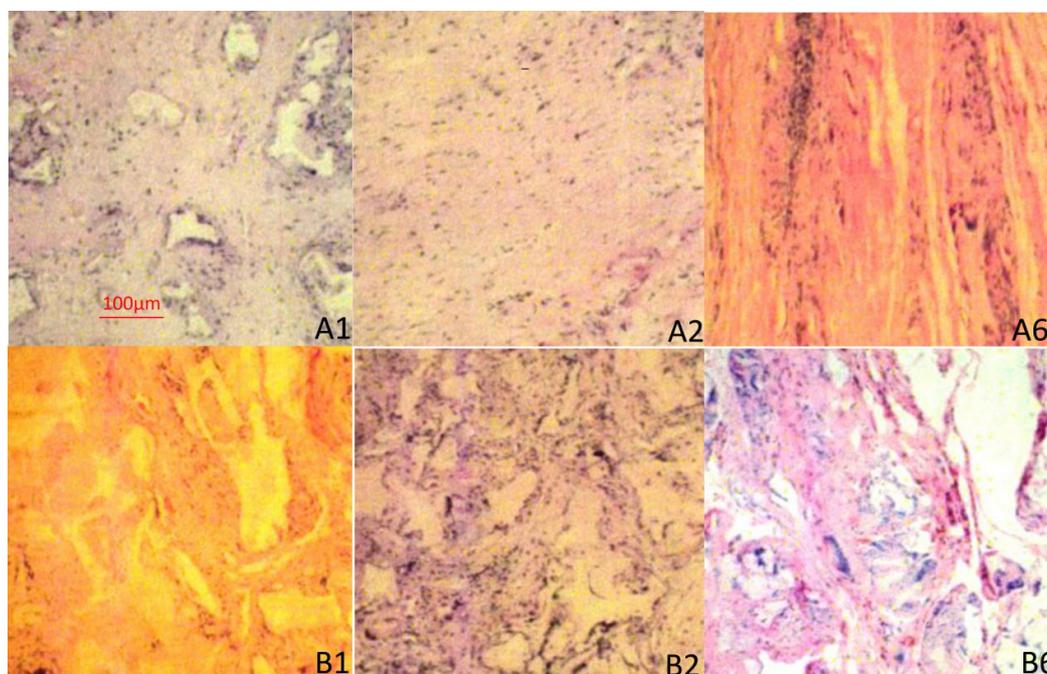


Figure 3.

The degradation state of materials by HE staining (A: experimental group, B: control group, the numbers denote the month)

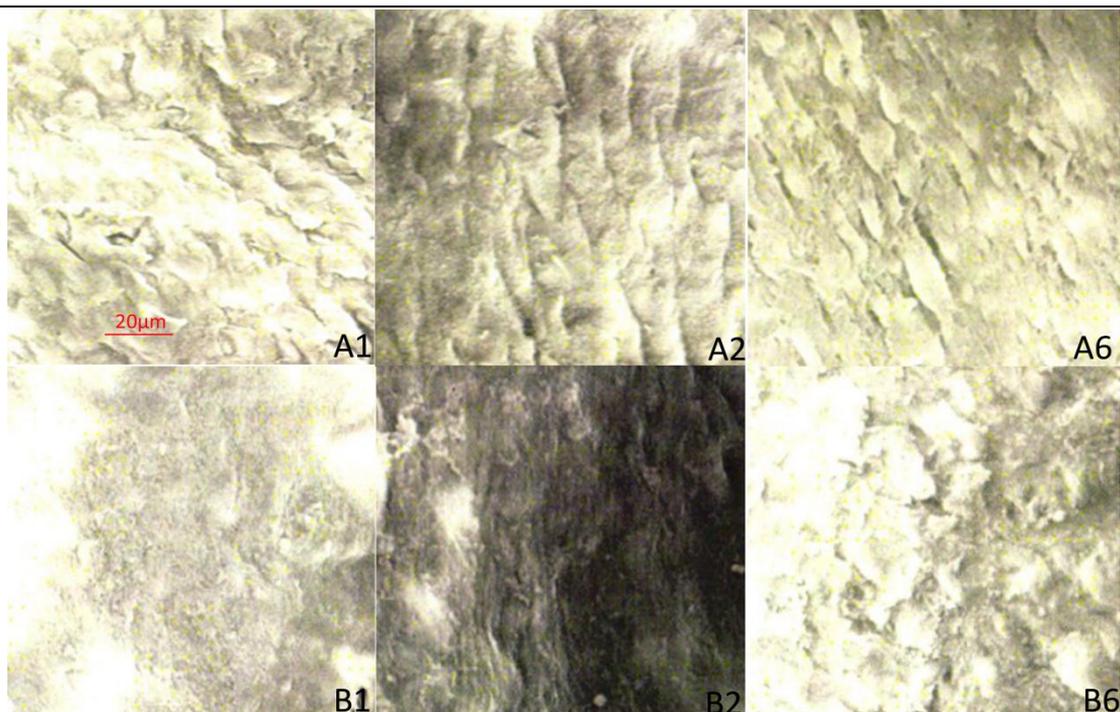


Figure 4.

The peritoneal integrity observed by scanning electron microscopy (20µm)(A: experimental group, B: control group, the numbers denote the month)

Formation of blood vessels and collagen fibbers

As can be seen from Figure 5A and Figure 5B, the capillary density in the experimental group was significantly higher than the control group, and the number of mature blood vessels in the experimental group was significantly higher than that of the control group.

The number of collagen fibbers in the experimental group was increased compared with the control group (Figure 5C). Moreover, in the study, it was found that the collagen fibber dimension of the experimental group increased gradually over time, and was more

regular and coherent. On the contrary, the collagenous fibbers in the control group were arranged in an irregular state. In the experimental group, the PLGA-Collagen I scaffold was degraded gradually and was replaced by new tissue and collagen fibbers. After six months, the PLGA-Collagen I scaffold was almost completely degraded and replaced by its own tissue. In the control group, the PLGA-Collagen I scaffold was degraded. It does not produce more new-born tissues and collagen fibbers. After six months, there was still no complete degradation of PLGA-Collagen I stents and no substitute for new tissue.

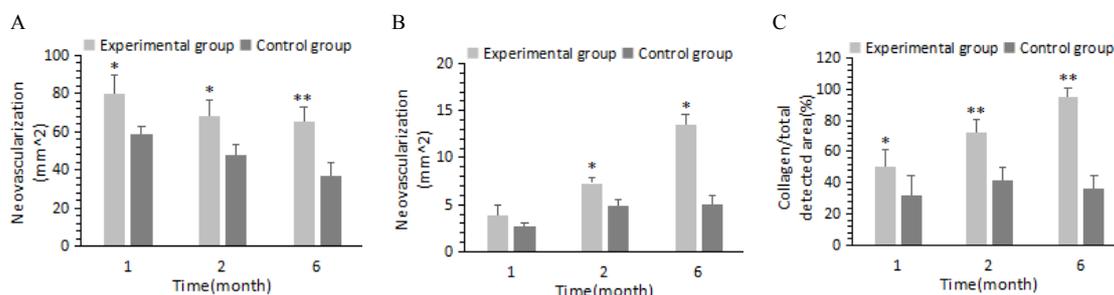


Figure 5.

Formation of blood vessels and collagen fibbers in the two groups after 1, 2 and 6 months of treatment (A: capillary, B: mature vessel, C: collagen fibbers formation; * p < 0.05, ** p < 0.001, experimental vs control group)

Abdominal wall defects and treatment of abdominal hernia focus on to restoring the integrity of the abdominal wall. However, the pathophysiological and pathological factors make that the simple tissue suture repair, in order not to achieve long-term treatment

[14-16]. Therefore, combined with the anatomy of the inguinal region, the patch material may be used to repair the abdominal wall. It can provide the growth support for the patient's fibrous tissue [18]. Currently, most of the patch materials are not bio-

degradable and have a high price, which make them inappropriate for wide use. PLGA is a nontoxic, non-antigenic, safe and adjustable degradation rate polymer [19, 20]. However, the adsorption capacity of cells is weak. It may cause the decrease of the local pH in the process of degradation, which leads to aseptic inflammation [21-23]. In this study, rat BMSC cells were used as model. The PLGA-Collagen I tissue engineering patch was synthesized by high voltage electrostatic spinning machine. It was found that the BMSCs and PLGA-Collagen I materials have good compatibility *in vitro*. Afterwards, an animal model of abdominal wall defect was established. After a week of implantation on the abdominal wall tissue of rats, the two groups of animals developed stimulate the inflammatory response in the local tissue. Macrophages were significantly increased, which is consistent with the body's response to foreign body. If the implants are not compatible with the body, there will be produced gradual and intense rejection reactions [24, 25]. The number of inflammatory cells increased gradually. The part of the cell is necrotic and suppurative. Until the material was removed, the local and systemic inflammatory response was subsided [26]. In this study, after one month, the inflammatory response gradually subsided and the number of macrophages decreased. Moreover, the BMSCs patch can promote the synthesis of collagen fibers, regeneration of blood vessels and repair of abdominal wall injury. After long-term experimental observation, the abdominal wall of the rats in the experimental group and the control group had no swelling, and the rats were healthy. At 1 month, 2 months and 6 months after operation, the morphological and SEM micrographs were observed. The PLGA-Collagen I patch with BMSCs can promote the fusion of the tissue and the material. It can promote cell infiltration, angiogenesis and tissue regeneration. The degradation rate of PLGA materials was faster. After sixth months, the cell-based patch was almost completely degraded. However, PLGA-Collagen I patches on which there were no planted cells were degraded slower. The degradation in this case was done in pieces and remained in the body as a foreign material. The PLGA-Collagen I tissue engineering material with BMSCs is a biodegradable polymer material. After the operation, with the continuous growth of the abdominal wall tissue, it can accelerate the repair of the abdominal wall injury, so as to achieve the purpose of treating the abdominal wall defect. In this process, the material is eventually completely degraded and replaced by the new tissue.

Conclusions

This study shows the efficacy of PLGA-Collagen I patch with BMSCs in the treatment of abdominal hernia, by restoring the integrity of the abdominal

wall. This can be a non-expensive and safe alternative for the treatment of inguinal hernia.

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