

## Original Article

# Reconstruction of the pelvic autonomic nerve using artificial nerve graft after rectal cancer surgery

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**Abstract:** The aim of the present study was to investigate the feasibility of reconstructing the severed pelvic autonomic nerve using an artificial nerve graft after rectal cancer surgery. In this study, Laparotomy was performed in Beagle dogs and nerves of 1 cm long were cut from the hypogastric nerve. Then Fifteen Beagle dogs with 30 hypogastric nerves were randomly assigned to five groups, Group A: nerves were reconstructed with copolymer of lactic and glycolic acids (PLGA) tube and Bone marrow stromal cells (BMSCs); Group B: nerves were reconstructed with PLGA tube; Group C: nerves were reconstructed with autologous peritoneum tube and BMSCs; Group D: nerves were reconstructed with autologous peritoneum tube; Group E: nerves were reconstructed with autologous nerve graft. Three months later, the grafted nerve segment was removed and a series of examinations was performed, including immunohistological staining of nerves, immunostaining of neurofilament, transmission electron microscope observation and axon counts. Results showed that the regenerated nerve fibers crossing the anastomosis of the hypogastric nerves in all groups. The number and density of nerve fibers in group A and group C was similar to group E, better than group B and group D. The difference was statistically significant ( $P < 0.05$ ). Therefore, we made a conclusion that reconstruction of the pelvic autonomic nerve using artificial nerve was technically feasible and BMSCs could promote nerve regeneration.

**Keywords:** Pelvic autonomic nerve, nerve reconstruction, rectal cancer

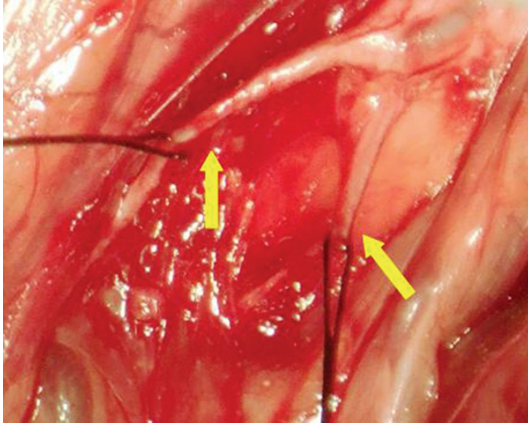
## Introduction

The primary goal of radical resection for rectal cancer is to achieve an oncologic cure while preserving function. Since the introduction of total mesorectal excision (TME), the oncologic outcome of rectal cancer has improved greatly in terms of local recurrence and cancer-specific survival [1]. However, urinary dysfunction remains around 0%-27% and sexual dysfunction around 11%-55% after radical resection for rectal cancer [2-5]. Intraoperative nerve damage is the main reason for sexual and urinary dysfunction and occurs due to lack of poor visualization of the pelvic autonomic nerve [6].

With the development of medical devices, laparoscopic TME is becoming more and more popular. However, some studies showed that laparoscopic TME was associated with increased

rates of sexual dysfunction than traditional TME, while the rates of urinary dysfunction did not differ [7-9]. The reason could be ineffective laparoscopic traction and countertraction, which cause difficulties in dissection around the neurovascular bundles. It seems more and more difficult to further reduce the injury of the pelvic autonomic nerve by the improvement of surgical techniques. How to deal with this problem? Reconstructing the severed pelvic autonomic nerve using an artificial nerve graft might be an optional method.

At present, satisfactory results had been achieved by using artificial nerve to reconstruct medullated nerves (such as ischiadic nerve, cubital nerve et al) [10, 11]. However, there was little study investigating pelvic autonomic nerve (non-medullated nerve) reconstruction using artificial nerve. In the present study, we aimed



**Figure 1.** Bilateral hypogastric nerves were exposed (yellow arrow).

to investigate the feasibility of reconstructing the severed pelvic autonomic nerve using an artificial nerve graft after rectal cancer surgery.

### Materials and methods

#### *Animals*

Fifteen adult Beagle dogs, each weighing 15-17 kg were purchased from The Research Center of Experimental Animal Technology in Zhaoqin, Guangdong Province, China. The protocols in this study were approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal operations were performed in the Center of Experiment Animal of Sun Yat-sen University and under Animal Care and Use Committee guidelines. All efforts were made to minimize the animal's suffering.

#### *Cultivation of BMSCs from Beagle dog*

After anesthesia, 5 ml bone marrow was extracted from the posterior superior iliac spine of the Beagle dog. Cells were disaggregated by gentle pipetting several times, passed through 30- $\mu$ m nylon mesh, and centrifuged for 10 min at 1500 r.p.m. The pellet was resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 15% fetal bovine serum (FBS; Gibco, USA), 2 mM L-glutamine (Sigma, USA) and 100 mg/mL kanamycin sulfate (Sigma, USA). After 72 h, the

nonadherent cells were removed by replacing the medium. BMSCs were subcultured four times, and finally brought to the following experiments.

#### *Preparation of artificial nerve scaffolds*

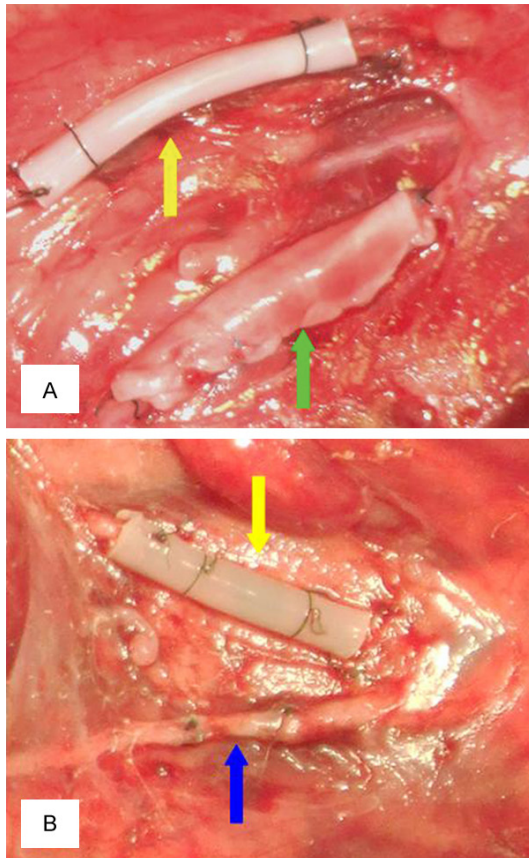
The 85:15 copolymer of lactic and glycolic acids (PLGA) tube was obtained from the High Polymer Chemistry Institute of the Sun Yat-Sen University. The tube was produced with 1.5-mm I.D., 2.5-mm O.D., 12-mm length, and the pore distribution ratio was 75%. The tubes were sterilized using 75% ethanol for 30 min and then washed three times with PBS (pH 7.4).

The collagen protein sponge (Kelaode, China) was put into the PLGA tubes carefully to facilitate the attachment and growth of BMSCs. In group A, 0.1 ml of BMSCs was injected (concentration of  $1.0 \times 10^7$  cells/mL) into the collagen protein sponge, while in group B, 0.1 ml of DMEM was injected as a control. The slice of autologous peritoneum was taken and sutured to be tubeworm using 9-0 PROLENE sutural line (Ethicon, USA). The serous membrane surface of peritoneum was made as the inner surface of peritoneum tube. Also, the collagen protein sponge was put into the autologous peritoneum tubes carefully to facilitate the attachment and growth of BMSCs. In group C, 0.1 ml of BMSCs (concentration of  $1.0 \times 10^7$  cells/mL) was injected into the collagen protein sponge and in group D, 0.1 ml of DMEM was injected as a control.

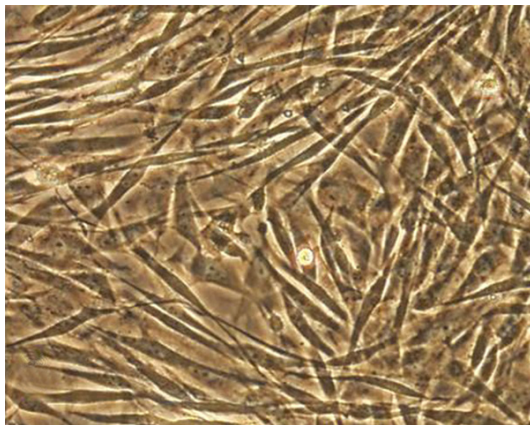
#### *Surgical procedure*

General anesthesia was induced with intravenous injected propofol (10 mg/kg). An endotracheal tube with a balloon cuff was placed and connected to a circular anesthesia circuit. The anesthesia was sustained with sevoflurane. Multimodal analgesia was employed in the perioperative (ketorolac 1 mg/kg; tramadol 1.7 mg/kg; buprenorphine 0.01 mg/kg). Laparotomy was performed and hypogastric nerves were exposed (**Figure 1**). For the tube repairs, the nerve was sharply transected, allowed to retract, and transected again to create a 10-mm defect. The transected nerve was then repaired. End-to-end anastomosis was performed between the broken ends of the nerves and artificial nerve scaffolds, using three sutures of 9-0 PROLENE line (Ethicon, USA) for each anastomosis.

## Reconstruction of the pelvic nerve



**Figure 2.** A. PLGA tube (yellow arrow) and autologous peritoneal canal (green arrow) bridge nerve defect. B. PLGA tube (yellow arrow) and autologous nerve graft (blue arrow) bridge nerve defect.



**Figure 3.** ( $\times 200$ ). BMSCs present fusiform shape, mix together, uniformly distribution like vortex.

Fifteen Beagle dogs with 30 hypogastric nerves were randomly divided into five groups. There were six nerves in each group. In group A, PLGA tubes containing BMSCs and collagen protein sponge were used to bridge the nerve defects;

In group B, PLGA tubes containing DMEM and collagen protein sponge were used to bridge the nerve defects; In group C, autologous peritoneum tubes containing BMSCs and collagen protein sponge were used to bridge the nerve defects; In group D, autologous peritoneum tubes containing DMEM and collagen protein sponge were used to bridge the nerve defects; In group E, autologous nerve graft was used as positive control. A 10-mm section of the nerve was reversed 180° to suture (**Figure 2A** and **2B**).

All dogs received intravenous antibiotics. After two days of surgery, the dogs were given a liquid diet, after which a solid diet was provided. Twelve weeks after the operation, laparotomy was performed in dogs and the nerve grafts were taken out for observation. After surgery, the dogs were euthanized with a lethal dose of sodium pentothal.

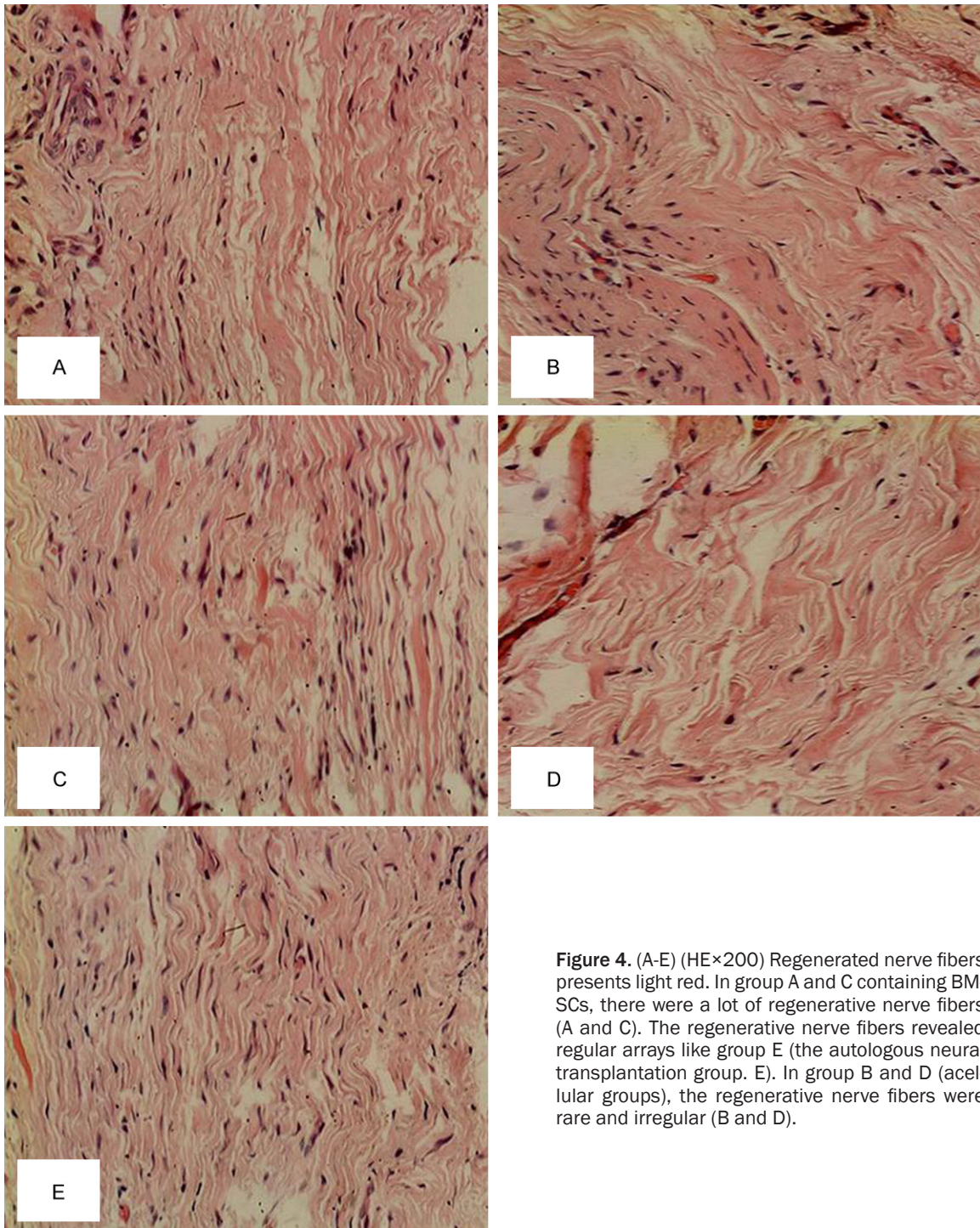
### *Histomorphometric evaluation*

Twelve weeks after the reconstructive surgery, artificial nerve graft were collected for examination. Longitudinal sections and transverse sections were cut in the proximal and distal anastomosis sites, which were then stained with hematoxylin and eosin (H&E) and Neurofilament (NF) immunohistochemistry.

Transverse sections were cut in the distal anastomosis sites for electron microscope observation. The specimens were fixed by immersion in a 2.5% Na-cacodylate-buffered glutaraldehyde solution for 24 h at pH 7.4 and postfixed in 2% Na-cacodylate-buffered osmium tetroxide for 2 h, serially ethanol dehydrated, infiltrated, and embedded in Epon. Ultrathin sections (70 nm) were cut, stained with 5% uranyl acetate in 70% methanol for 5 min, dried, stained with lead citrate for 5 min, dried, and examined on a Hitachi H-600 transmission electron microscope (Japan).

Transverse sections were cut in the middle field of grafted nerve, which were then stained with NF immunohistochemistry and observed with light microscopy. The slides were analyzed by High Definition auto-Image Analysis System (Qianping, China). Axon measurements were obtained in blinded fashion for nerve fiber quantity, density, and percentage of neural tissue in the total transverse section.





**Figure 4.** (A-E) (HE×200) Regenerated nerve fibers presents light red. In group A and C containing BMSCs, there were a lot of regenerative nerve fibers (A and C). The regenerative nerve fibers revealed regular arrays like group E (the autologous neural transplantation group. E). In group B and D (acellular groups), the regenerative nerve fibers were rare and irregular (B and D).

## Statistical analysis

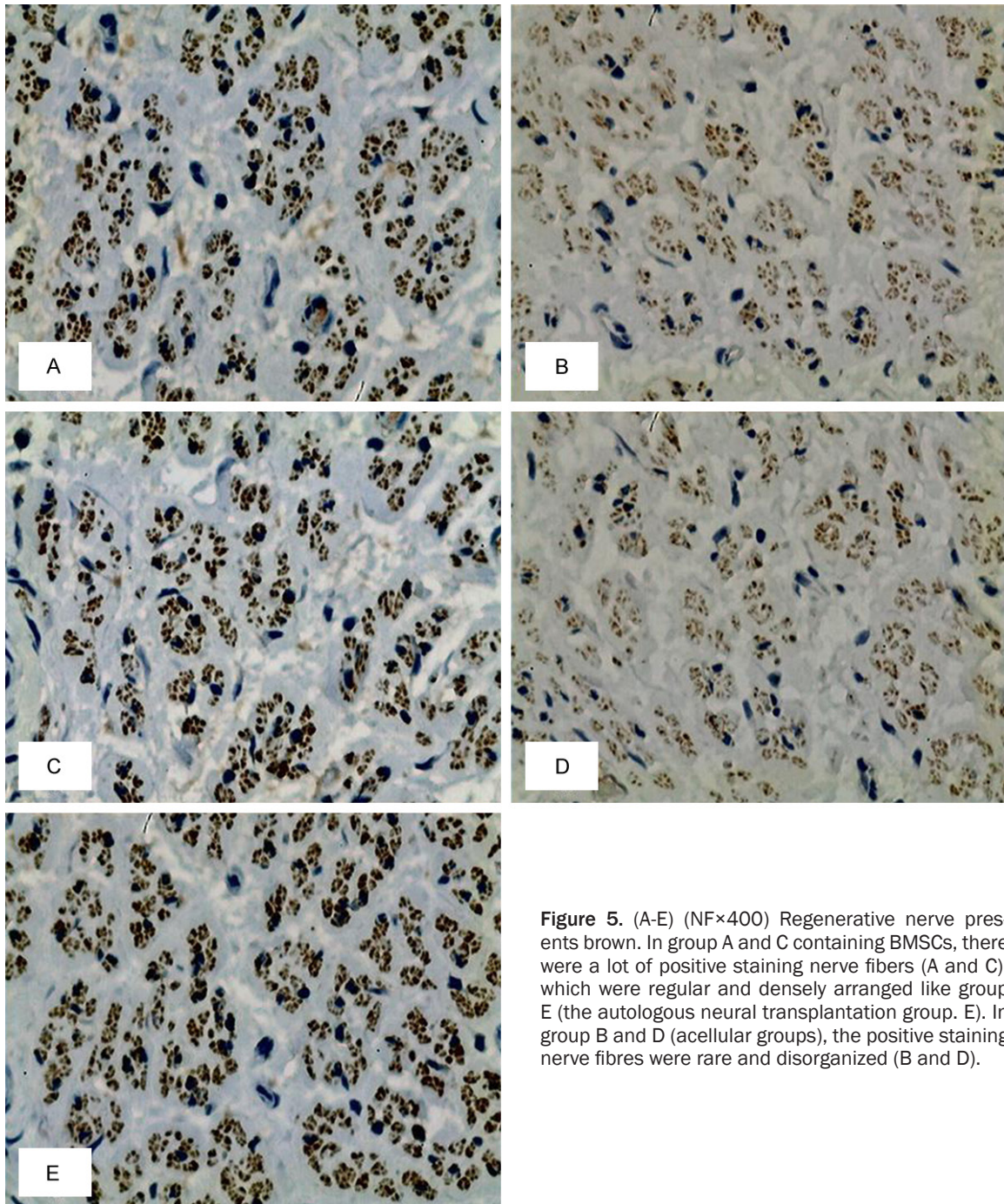
Statistical Product and Service Solutions (SPSS) 10.0 was used for statistical analysis. Measurement data were reported as means  $\pm$  standard deviation ( $\bar{X} \pm s$ ). The significance of difference between groups was calculated by one-way analysis of variance (one-way ANOVA) and a  $P$  value  $< 0.05$  was considered statistically significant.

## Results

### Characterization of the BMSCs from Beagle dogs

Phase-contrast microscopy was used to observe the features of the BMSCs. There were numerous round cells suspending on karyocyte suspension when inoculated. Most cells adherent after 12 hours. Adherent cell mitosis and





**Figure 5.** (A-E) (NF×400) Regenerative nerve presents brown. In group A and C containing BMSCs, there were a lot of positive staining nerve fibers (A and C), which were regular and densely arranged like group E (the autologous neural transplantation group. E). In group B and D (acellular groups), the positive staining nerve fibres were rare and disorganized (B and D).

proliferation was observed after 3 days and dispersed cell colony formed 5 days later. Ninety percent of cell colony mixed together to be monolayer in 10~15 days. Cells displayed fusiform or polygon shape as collagenoblast. There were no significant morphological changes in cells after subcultured several times. BMSCs grew in a homogeneous vortex fashion and the directionality was good (Figure 3).

## Histomorphometric evaluation

Twelve weeks after surgery, the nerve grafts shape kept well. The surface of the artificial nerve graft was surrounded by well-vascularized connective tissues. Adhesion between the tube and its periphery tissue was insignificant. PLGA tube was almost absorbed. The regenerated nerves went through defect area to distal



**Figure 6.** ( $\times 10000$ ) Regenerative nerve fibers were close to normal. Schwann cells (yellow arrow) wrapped around the regenerated nerve fiber axon (green arrow).

end in every group and the two-end of regenerative nerve was not degenerative atrophy. No neuroma was found in each group.

Histologically, the HE staining of the regenerative nerve was shown in **Figure 4A-E**. The collagen protein sponge was almost absorbed. In group A and C containing BMSCs, there were a lot of regenerative nerve fibers presenting wave shape within many elliptic Schwann nucleus deeply stained. The regenerative nerve fibers revealed regular arrays. Morphologically, it was similar to autologous neural transplantation (group E). Regenerative nerve fibers were also found in acellular groups (group B and D), but the number of regenerative nerve fibers was rare. The longitudinal section revealed a disorganized regeneration.

The brown part of the figures indicated NF-positive nerve fibers (**Figure 5A-E**). Regenerative nerve fibers revealed sarciniform in cross section. In group A and C containing BMSCs, A lot of regular and densely arranged regenerative nerve fibers could be observed in cross section. While in acellular groups (group B and D), positive staining nerve fibers were rare and disorganized.

The ultrastructure of regenerative nerve fibers was similar to normal while observed by transmission electron microscopy. There were nerve fibers crossing the anastomosis site in the distal grafted nerve. Regenerative nerve fiber axon was covered by Schwann cell (**Figure 6**).

## Statistical analysis

One-way ANOVA was chosen to analysis the difference between each group. The result showed that the quantity, density and area percentage of regenerative nerve fiber in group A and group C was similar to group E, better than that of group B and group D. The differences were statistically significant ( $P < 0.05$ ). In addition, There was no significant difference between group A and group C or between group B and group D ( $P > 0.05$ ) (**Table 1**).

## Discussion

The primary aim of rectal cancer surgery is survival of the patient and to reduce the risk of local recurrence. The second aim is to maintain function by minimizing injury to the pelvic nerves. In some cases, bladder and sexual dysfunction could be avoided by identifying and preserving the nerves subserving them. However, difficulties could arise during anterior and lateral dissection where the anatomical planes might not be clear. In spite of the continuous improvement of surgical methods, it seems more and more difficult to reduce the incidence of pelvic autonomic nerve injury in rectal cancer surgery [12, 13].

At present, autologous neural transplantation is still a “gold standard” for the repair of peripheral nerve injury. However, clinical application of autologous neural transplantation was strictly limited because of the numbness and pain in the nerve donor region. Application of artificial nerve transplantation to repair peripheral nerve defect was a new therapeutic method [14, 15]. Current researches on peripheral nerve repair were mainly concentrated on medullated nerve fibers (like sciatic nerve or cubital nerve) currently [16, 17]. There was no relevant study reported about autonomic nerve reconstruction. In this study, we used artificial nerve to bridge pelvic autonomic nerve defect to explore a new therapeutic method for this injury, in order to provide an experimental evidence for clinical application in the future.

Seed cell is the key issue in tissue-engineering fields. Perineural regenerative capacity mainly relies on Schwann cells. It could not only provide physical support for axonal elongation and growth, but also secrete many neurotrophic factors to axonal restoration and extracellular



# Reconstruction of the pelvic nerve

**Table 1.** Image analysis of regenerative nerve fiber in each group (mean  $\pm$  SD)

| Groups                     | Sample size | Total count of fibers (n) | Fiber density (n/10 <sup>4</sup> $\mu$ m <sup>2</sup> ) | Neural tissue (%) |
|----------------------------|-------------|---------------------------|---|-------------------|
| A (PLGA + BMSCs)           | 6           | 1718.8 $\pm$ 264.6        | 170.7 $\pm$ 13.1  | 0.34 $\pm$ 0.05   |
| B (PLGA)                   | 6           | 1216.7 $\pm$ 172.6*       | 116.7 $\pm$ 13.9*                                       | 0.21 $\pm$ 0.03*  |
| C (peritoneum + BMSCs)     | 6           | 1742.7 $\pm$ 185.3        | 167.8 $\pm$ 14.4  | 0.32 $\pm$ 0.07   |
| D (peritoneum)             | 6           | 1131.0 $\pm$ 262.2*       | 124.3 $\pm$ 16.8*                                       | 0.21 $\pm$ 0.08*  |
| E (autologous nerve graft) | 6           | 1920.3 $\pm$ 131.4        | 183.5 $\pm$ 14.1  | 0.39 $\pm$ 0.06   |

The number, density and area percentage of regenerative nerve fiber in group A and group C was similar to group E ( $P>0.05$ ), better than that of group B and group D ( $P<0.05$ )\*. There was no significant difference between group A and group C or between group B and group D ( $P>0.05$ ).

matrix to shape the tubiform of axonal medullary sheath. However, it was difficult to obtain Schwann cells and the bioactivity of the cells might decrease after repeated passage [18]. This restricts the application of Schwann cells as a seed cell in tissue-engineering fields. Nowadays, researchers tended to use BMSCs as seed cells [19, 20]. While the peripheral nerve injured, Schwann cells at the nerval broken ends secret, many promoting nerve growth factors [21], including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) etc. to induce BMSCs differentiate into Schwann cells [22, 23]. In this study, BMSCs were used as seed cells and transferred to experimental animals directly. Twelve weeks after operation, nerve grafts were taken out and stained with HE and NF immunohistochemistry. Results showed a lot of Schwann cells in groups containing BMSCs and the effect of nerve recovery was similar to autologous neural transplantation. While in acellular groups, Schwann cells and regenerative nerve fibers were rare. This result confirmed BMSCs could differentiate to Schwann cells and promote axonal regeneration.

Materials of tissue-engineering nerve included native material and artificial synthetic material. Each had advantages respectively. Native materials mainly included biological membranes (anadesma, peritoneum, spinal dura mater, et al), blood vessel, hydrogel, chitosan, acellular xenogenic nerve, et al [24, 25]. It contained cell identification signal which could facilitate the cell attachment. However, immunogenicity and bad mechanical function of native material would cause the difficulty of graft growth and remodeling. Artificial synthetic materials included polylactic colactic coglycolic acid (PLGA), polydioxanone (PDX), Poly-3-

hydroxybutyrate (PHB) et al [26, 27]. It was easy to proceed and biodegrade. But the surface of this material was lack of cell identification sites and the acid metabolite released in degradation process would impede nerve regeneration. In this study, the two kind of tissue-engineering materials (autologous peritoneum for native material and PLGA for synthetic material) were used to construct the artificial nerves. Our result showed there was no statistically significant between these two materials. Both of native material and artificial synthetic material would be applied in clinical in future.

Our study has established the feasibility of repair the pelvic autonomic nerve injury using artificial nerve grafting during radical resection of rectal cancer. Further studies are merited to investigate the functional evaluation of regenerative nerves [28]. Our ultimate goal is to apply artificial nerve graft in the treatment of pelvic nerve injury in rectal cancer surgery.

## Disclosure of conflict of interest

None.

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