

Efficacy and Safety of Biodegradable Microparticles in the Regeneration of Injured Rabbit Corpus Cavernosum: Primary Report

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= Abstract =

Purpose: This study analyzed the effectiveness of poly (lactic-co-glycolic acid) (PLGA) as a tissue recovery agent and determines the in vivo safety and efficacy of microparticle-based PLGA.

Materials and Methods: Fifteen 3-month-old male white rabbits were used. Allogenic adipose tissue derived stromal vascular fraction (SVF) was cultured and labeled with the fluorescent dye PKH26. The rabbits were divided into 4 groups: the SVF group, the PLGA group, the normal control group, and the disease control group. The right corpus cavernosal tissue of the rabbits was surgically removed in the selected portion, except in the normal control group. The defect space of each rabbit was replaced with 10^6 SVF cells in the SVF group and 0.1 g of biodegradable polymer solution in the PLGA group. Microscopic confirmation and analysis of tissue regeneration were performed after 8 weeks. Using confocal microscopy, the nuclei of the smooth muscle cells and SVF migration were examined. The composition of smooth muscle and fibrosis of the injured corpus cavernosum were compared and analyzed by Masson's trichrome stain.

Results: There were no signs of migration or rejection of the injected materials in any of the experimental groups. The mean amount of smooth muscle in the normal control group was $15.25 \pm 1.34 \mu\text{m}^2$ (right) and $13.90 \pm 0.703 \mu\text{m}^2$ (left); in the disease control group it was $11.10 \pm 0.87 \mu\text{m}^2$ (right) and $12.80 \pm 1.01 \mu\text{m}^2$ (left); in the SVF group it was $13.82 \pm 4.10 \mu\text{m}^2$ (right) and $13.96 \pm 3.94 \mu\text{m}^2$ (left); and in the PLGA group it was $12.89 \pm 1.39 \mu\text{m}^2$ (right) and $13.24 \pm 1.43 \mu\text{m}^2$ (left). Only the disease control group showed significant decreased smooth muscle in the left cavernosum ($p < 0.05$). No significant difference was found between the left and right side of each rabbit's cavernosal smooth muscle in the SVF or PLGA group ($p > 0.05$). Furthermore, no difference was found between any two groups (normal control versus SVF ($p = 0.705$), normal control versus PLGA ($p = 0.88$), SVF versus PLGA ($p = 0.23$)).

Conclusions: PLGA microparticles had the same tissue restoring effect when compared with SVF and no adverse effect or migration of particles was found through the injection of PLGA or SVF. PLGA is safe and has the proper tissue recovery effect, saving additional tissue harvesting.

Key Words: Penis, Poly (lactic-co-glycolic acid), Regeneration

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Introduction

Good tissue restoration without anatomical or functional damage is a surgical challenge in the penile corpus cavernosum. Adipose tissue autografts or implantable prostheses are used for treating penile tissue defects or anatomical corrections of the penis; however, none of them has provided satisfactory results. Poly (lactic-co-glycolic acid) (PLGA) has been successful as a biodegradable polymer because it undergoes hydrolysis in the body to produce original monomers, lactic acid, and glycolic acid. These two monomers are by-products of various metabolic pathways in the body. As a result, there is minimal systemic toxicity or local foreign body reaction associated with the use of PLGA for mediating tissue regeneration.^{1,2}

During the past decades, various approaches to tissue engineering for the penile corpus cavernosum have been explored. Kershen et al.³ successfully reconstructed human corporal smooth muscle tissue from cultured human corporal smooth cells seeded onto biodegradable PLGA scaffolds. Perovic et al.⁴ used a PLGA scaffold in penile girth enlargement surgery. Although stem cell studies have limitations, the data are promising.

Urologically injectable agents consisted of PLGA microparticles are usually bulking agents commonly used as a therapy for urinary incontinence.^{5,6} Previous studies of biodegradable materials for penile tissue engineering mainly dealt with scaffolds that need to be inserted. If injectable microparticles are as effective as scaffolds without tissue migration, the process of manipulation of the injured corpus cavernosum would become much easier.

There is a lack of data about the safety and tissue regenerating effect of biodegradable PLGA microparticles in animal or human studies.

Therefore, in this study, we aimed to preliminarily investigate the efficacy and safety of microparticles of PLGA in the tissue recovery of the injured penile corpus cavernosum of rabbits.

Materials and Methods

1. Isolation of allogenic rabbit stromal vascular fraction (rSVF) cells from adipose tissue

Allogenic rabbit SVF cells were obtained from rabbit intra-abdominal adipose tissue. Adipose tissues for rSVF cell isolation were harvested from same kind of New Zealand white rabbits (3 months old with an average body weight of 3.0 kg). They were discarded after collecting intra-abdominal adipose tissues. The rabbit adipose tissue was washed extensively with phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. The washed adipose tissue was finely minced and treated with 0.1% collagenase I (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Invitrogen, Life Technologies Co., Grand Island, NY, USA) for 60 min at 37°C with gentle agitation. The collagenase I was neutralized by adding an equal volume of DMEM that contained 10% fetal bovine serum (FBS; GIBCO, Invitrogen, Life Technologies, Co., Grand Island, NY, USA), 100 µg/ml penicillin and 100 µg/ml streptomycin (control medium, Sigma-Aldrich Co., LLC, St. Louis, MO, USA), and filtered through a 100 µm cell strainer (BD Biosciences Co., Ltd., San Jose, CA, USA) to remove the debris. The filtrate was centrifuged at 1,000 rpm for 10 min and the pellet was suspended with DMEM/10% FBS. The rSVF cells were cryopreserved, labeled with PKH26 dye, and plated onto conventional culture plates. After culture for 24 h, the cells adhered to the culture plates were further cultured for 6 days.

2. PLGA microparticle preparation

Microparticles were prepared by a new solvent spray technique. One point seven grams of polylactideglycolic acid (PLGA; Lakeshore Biomaterial, Co., Ltd., Dallas, TX, USA) was dissolved into 10 ml of methyl sulfoxide (DMSO; Merck, Co., Ltd., Darmstadt Germany). The solution was sprayed into a cold hexane (−5°C) to create frozen microparticles. After transferring the frozen microparticles to a two-fold

cold solution (-20°C) containing 25% NaCl (Duksan, Co., Ltd., Ansan, Korea), the solution was stored in a freezer (-20°C) for 48 h to remove the DMSO. The microparticles were washed three times with distilled water and dried. Two grams of $50\sim 100\ \mu\text{m}$ microparticles were suspended in 3 ml of 3% carboxymethyl cellulose (CMC; Bolak Co., Ltd., Hwasung, Korea).

3. Cell labeling with PKH26 fluorescent dye

The rSVF cells isolated from rabbit adipose tissue were labeled with the fluorescent dye PKH26 (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) according to the manufacturer's protocol. Briefly, 2×10^6 rSVF cells were suspended in 1 ml of dilution buffer from the manufacturer's labeling kit. The cell suspension was mixed with an equal volume of a labeling solution containing 4×10^{-6} M PKH26 dye in the dilution buffer and incubated for 4 min at room temperature. The reaction was terminated by adding 2 ml of FBS. After washing with a control medium, 5×10^5 rSVF cells labeled with PKH26 dye were incubated for 1 day in control medium at 37°C in 5% CO_2 . The attachments of rSVF cells were observed by confocal fluorescence microscopy (Olympus FV1000, Olympus Co., Ltd., Tokyo, Japan).

4. Animals and surgical procedures

The subject animals were 15 male New Zealand

white rabbits (3 months old with an average body weight of 3.0 kg). All rabbits were kept in an aseptic environment with a room temperature of $22\sim 24^{\circ}\text{C}$ at $40\sim 60\%$ humidity. Sufficient food and water were provided for all the rabbits.

The 15 New Zealand white rabbits were divided into 4 groups: the normal control group ($n=3$), disease control group ($n=2$), SVF group ($n=5$), and PLGA group ($n=5$). General anesthesia was accomplished by intramuscular injection of xylazine ($0.15\ \text{mg/kg}$ bodyweight, Bayer Korea Co., Ltd., Seoul, Korea) and tiletamine ($0.05\ \text{mg/kg}$ bodyweight, Virbac Korea Co., Ltd., Seoul, Korea) solution. After anesthesia, shaving and povidone draping was performed from the umbilicus to the knee including the genital area. All surgical procedures were conducted under sterile conditions. In the supine position, a 1 cm incision was made at the penile dorsum of the rabbit. Except in the normal control group, a further incision was made at the right tunica albuginea and a $3\times 3\times 5\ \text{mm}$ rectangular area was removed from the corpus cavernosum by scalpel and Metzenbaum scissors. In the normal control, we only incised the penile skin without making a cavernosal defect. In the disease control, the corpus cavernosal tissue was removed as in the other experimental groups, without adding any biodegradable material. 10^6 SVF was injected into the right corpus cavernosum for the SVF group, and 0.1 g PLGA was



Fig. 1. Surgical features of rabbit corpus cavernosum. (A) An incision was made and tissue was partially removed in the right dorsum of the rabbit corpus cavernosum. (B) Nylon 5-0 suture was performed as a marker and the tunica albuginea was closed so that it was watertight. Poly (lactic-co-glycolic acid) (PLGA) microparticle (0.1 g) solution was injected into the defect of the corpus cavernosum at the final step of the closure. This figure shows the placement of the PLGA in the corpus cavernosum defect before closure.

inserted for the PLGA group (Fig. 1A). Nylon 5-0 suture was performed as a marking to ensure the incised and injected region in the cavernosum (Fig. 1B). The tunica albuginea was closed so that it was watertight. PLGA or SVF was injected into the corpus cavernosum defect at the final step of the closure to prevent solution leakage. To prevent operation site infection, 1 g Triaxone (Hanmi Pharmaceutical Co., Ltd., Seoul, Korea) was injected intramuscularly for 7 days to all rabbits. Two rabbits (1 from the SVF group and 1 from the PLGA group) were sacrificed on the 8th postoperative day for detecting any acute rejection. The bodyweight was measured twice, before the experiment and 8 weeks later. Blood sampling tests including complete blood cell count (CBC), aminotransferase (ALT), alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) were processed on the 8th week before sacrifice. To confirm the presence of PLGA particles, an additional rabbit from the PLGA group was sacrificed on the 4th week. All the other rabbits were sacrificed in a CO₂ chamber on the 8th week. Corpus cavernosal tissue was harvested from the proximal penile shaft in all the sacrificed rabbits.

All procedures were approved by the Ewha Institutional Research Board and Committee of Animal Models.

5. Histological analysis

The penile specimens were fixed with formalin in a neutral buffer. Sections (6 μ m) were processed for Masson's trichrome staining according to a standard procedure. Digital images were obtained and analyzed using Image Pro 6.2 (Media Cybernetics, Inc., Bethesda, MD, USA). In each section, 10 fields of 600 \times 400 μ m were randomly assisted. The smooth muscle density of each field was checked under a 100 \times microscopic magnification.

6. Statistical analysis

The data was analyzed through a Mann-Whitney test; a p-value of less than 0.05 was considered to be statistically significant.

Results

The bodyweights of the rabbits slightly decreased in all of the four groups. Although the average bodyweight of all the groups decreased, the weight loss was statistically insignificant when compared with the normal control group. In 8th week's blood tests, there were no significant serologic changes or signs of infection or inflammation associated with material injections in any of the groups. To detect signs of acute rejection of SVF or PLGA injection, we closely observed the rabbits' behavioral changes and performed daily physical examinations. No sign of acute rejection were found in the 2 rabbits which were sacrificed on the 8th day.

At the surgical wound sites, no signs of necrosis or infection were found in any of the 15 rabbits over the 8 weeks. No migration of PLGA particles to the main organs (lung, kidney, liver, heart, or brain) was found after the autopsies. There was no evidence of migration to adjacent tissue from the injection site in the penile corpus cavernosum.

In the SVF group, fluorescence was confirmed in

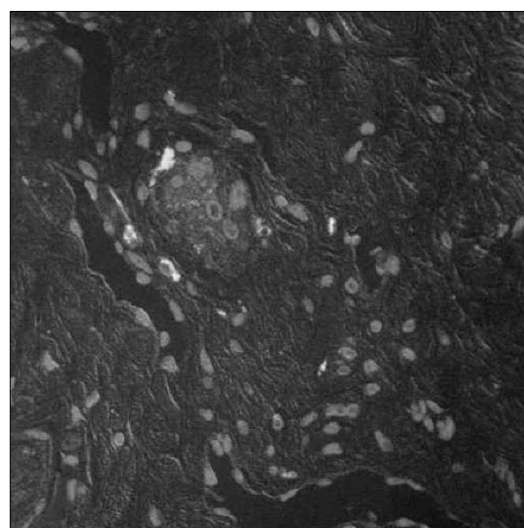


Fig. 2. Fluorescence was confirmed in the nuclear area of the corpus cavernosal smooth muscle cells in the injected site of the stromal vascular fraction (SVF). Smooth muscle cells with fluorescence-positive nuclei were differentiated from injected SVF, and there was no evidence of abnormal differentiation from or migration of SVF.

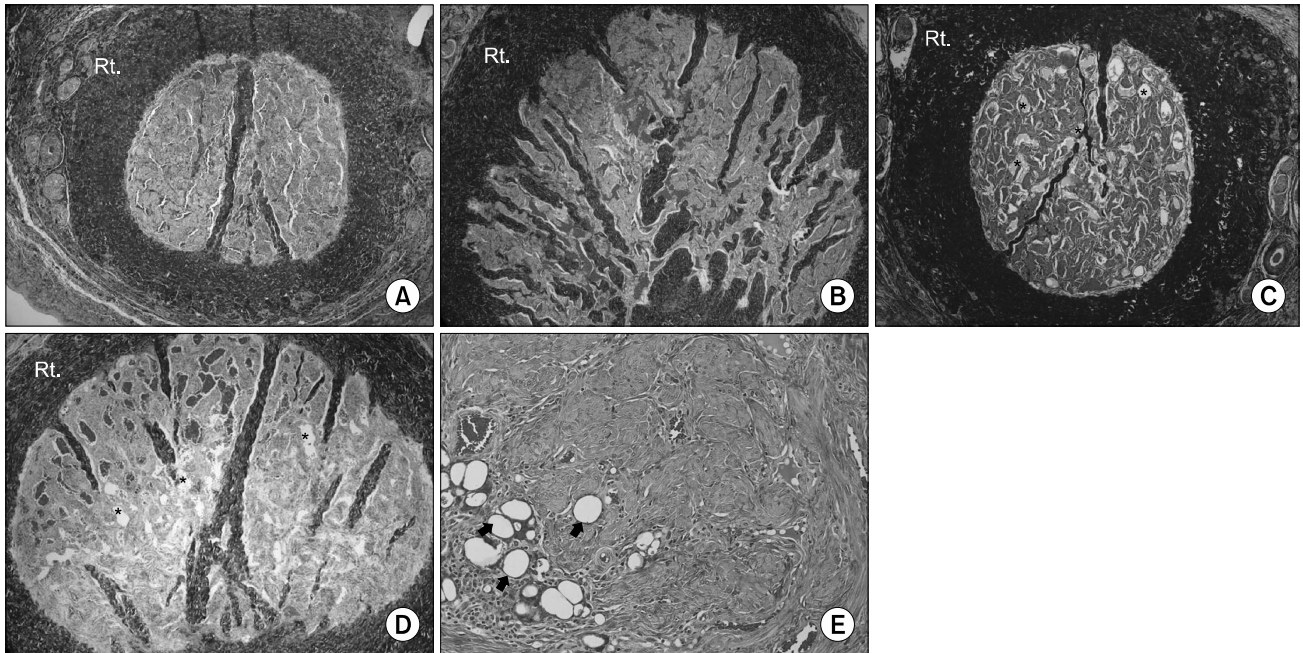


Fig. 3. Section of corpus cavernosum with Masson-trichrome stain. (A) Normal control group. (B) Disease control group. (C) Stromal vascular fraction (SVF) group, 8 weeks after the SVF injection. (D) Poly (lactic-co-glycolic acid) (PLGA) group, 8 weeks after the injection. PLGA particles were almost completely resolved 8 weeks after implantation (40 \times each). Whitish empty spaces (*) in the corpus cavernosal area of the SVF (C) and PLGA (D) groups are artifacts that developed during processing. (E) The PLGA group, 4 weeks after the injection. Distinguishable PLGA particles (arrows) were present (400 \times).

the nuclear area of the smooth muscle cells in the injected site of the corpus cavernosum, showing that these smooth muscle cells were differentiated from injected SVF (Fig. 2). Masson's trichrome staining of the four groups revealed that cavernosal smooth muscle of the four groups was successfully restored (Fig. 3A ~ D). PLGA particles were almost completely resolved after 8 weeks from implantation (Fig. 3D). To observe the nature of PLGA degradation, we additionally observed the status of PLGA particles after 4 weeks of injection. PLGA particles were observed at the injection site of the corpus cavernosum at week 4 (Fig. 3E).

Smooth muscle components of each side of the corpus cavernosum were measured with an image analyzer program with a repeat measurement of a 6 \times 4 μm field under a light microscope. The mean amount of smooth muscle in the normal control group was 15.25 \pm 1.34 μm^2 (right) and 13.90 \pm 0.703 μm^2 (left); in the disease control group, it was 11.10 \pm 0.87 μm^2 (right) and 12.80 \pm 1.01 μm^2 (left); in the SVF group,

it was 13.82 \pm 4.10 μm^2 (right) and 13.96 \pm 3.94 μm^2 (left); in the PLGA group, it was 12.89 \pm 1.39 μm^2 (right) and 13.24 \pm 1.43 μm^2 (left). Only the disease control group showed significantly decreased smooth muscle in the left cavernosum ($p < 0.05$). When comparing the amount of smooth muscle in the right cavernosum, which was experimental side, among the groups, it was significantly lower only in the disease control group (Fig. 4, $p < 0.05$).

Discussion

There have been various reports on replacing tissue with biodegradable polymers and initial experiments were carried out by Kershen et al.⁷ in the late 1990s. The results showed that cultured human corporal smooth muscle cells may be used in conjunction with biodegradable polymers to create corpus cavernosum tissue de novo. When grown on collagen, corporal cavernosal endothelial cells formed capillary structures that created complex three-dimensional capillary

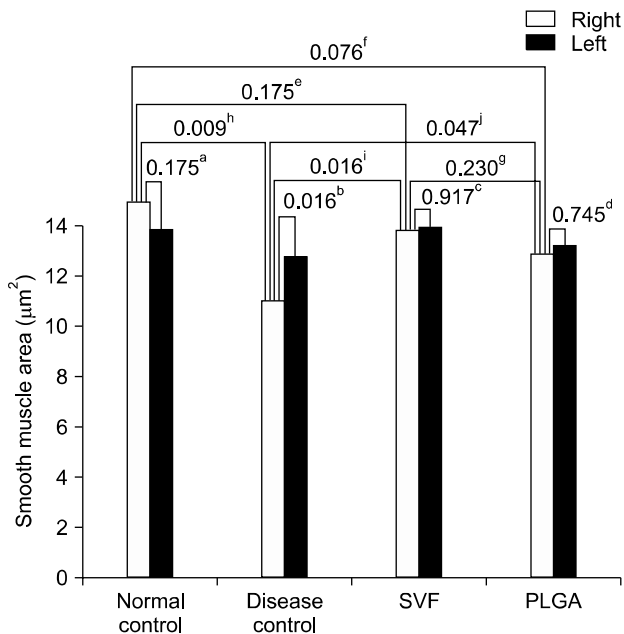


Fig. 4. Only the disease control group showed a significantly decreased amount of smooth muscle in the left cavernosum ($p < 0.05$ in Mann-Whitney test). When comparing the amount of smooth muscle content among the groups, in the right cavernosum, which was experimental side, it was significantly decreased only in the disease control group ($p < 0.05$ in Mann-Whitney test). a: normal control group left vs. right $p=0.175$, b: disease control group left vs. right $p=0.016$, c: SVF group left vs. right $p=0.917$, d: PLGA group left vs. right $p=0.745$, e: normal control group vs. SVF group $p=0.175$, f: positive group vs. PLGA group $p=0.076$, g: SVF group vs. PLGA group $p=0.230$, h: normal control group vs. disease control group $p=0.009$, i: disease control group vs. SVF group $p=0.016$, j: disease control group vs. PLGA group $p=0.047$.

networks. In addition, PLGA is studied in many other fields. In a recent study performed by Kuzyk et al.⁸ 18 canine tibiae were reamed to 7.0 mm and fixed with a 6.5 mm statically locked intramedullary nail, after the creation of an 8.0 mm diaphyseal defect. The results showed a greater percent vasculature volume in the scaffold group than the autograft group.

The forms of PLGA are the most widely used biodegradable polyesters having biocompatibility and a predictable degradation rate for medical applications.^{9,10} The form of PLGA used currently is mostly macroporous scaffold.^{4,11,12} However, the researchers in this study used a microparticulate form of PLGA with a solution to be injected that is more convenient to handle. Microparticle-dispersed PLGA has been studied in var-

ious fields, such as drug delivery or injected as a bulking agent.^{6,13-15} We investigated tissue regeneration and the migration of injected PLGA microparticles to compare with SVF cells.

Proper smooth muscle regeneration was confirmed in the injured site of the corpus cavernosum after the injection of a PLGA microparticle solution. There was no tissue overgrowth or fibrosis in the regenerated defect of the corpus cavernosum, with a similar consistency compared to normal corpus cavernosum of the contralateral side and the normal control.

Studies have revealed that SVF is multipotent and shares numerous features with mesenchymal stem cells derived from bone marrow. SVF has also shown a strong pro-angiogenic potential.¹⁶ With these advantages, previous studies have shown that it can be used for repair of tendons and bones as well as skeletal muscle.¹⁷⁻¹⁹

In this study, SVF showed a good tissue regenerative effect in the injured corpus cavernosum, and the PLGA microparticle solution also had a satisfactory effect in regenerating the corpus cavernosum.

Tissue regeneration after the injection of PLGA showed the same results as the SVF injection: focused locally on the site of the injection and without any migration. This suggests that the injection of the PLGA solution into the corpus cavernosum should be safe from microparticle migration or the formation of emboli.

The injection of PLGA and SVF through 24 gauge needles proved to be simple during the experiment and no sign of wound infection or polymer leakage was observed. No foreign body reaction was observed after injecting the polymers. We created the corpus cavernosal injuries by removing the cavernosal tissue after opening the tunica albuginea. Although PLGA or SVF were injected at the final step after water-tight suturing of the cleaved tunica albuginea, this process might provide some possibilities for leakage of injected materials leading to observational error.

The main advantage of PLGA in urological surgery is the absence of a tissue harvesting procedure that is necessary to provide enough bulking or graft material.^{7,10} Some reports have shown unsatisfactory results of tis-

sue regeneration with PLGA or biodegradable material only, and better results with cell seeding, but others have not.^{6,20} *In vitro* and *in vivo* reconstitution of human corpus cavernosum from cultured human corporal smooth muscle celled with scaffolds became clinically available.³ Adipose-derived SVF or SVF seeded scaffolds are currently being used in penile plastic surgeries with good cosmetic results.^{4,6} However, in our experiment, we performed similar tissue regeneration with biodegradable microparticles without the cellular component. In this study, PLGA particles were still present until the 4th week after injection, but later on, at the 8th week, they had been almost completely absorbed. We did not observe the whole process of PLGA absorption over time, but we could confirm that the PLGA particles degrade enough within a matter of months.

Although the microparticle form of PLGA might be advantageous compared to other scaffold forms of biodegradable material, we need further study to confirm whether the same results are possible in a larger tissue areas such as the human corpus cavernosum.

The PLGA microparticle solution used in this study has the advantage of application by direct injection to the area of concern without the skin incision that was an unavoidable procedure for the insertion of a tissue or graft/block form of PLGA. In addition, PLGA without additional SVF showed similar tissue regeneration compared to the control and the SVF group, with a complete degradation of the PLGA material over time. This shows that it has the advantage of avoiding the unnecessary extra processes of adipose tissue harvesting.

To further the application of the microparticulate form of PLGA to humans in tissue recovery or organ augmentation, it will be necessary to develop a proper microparticle size and a safe buffer to ensure their safety and from the lack of migration or embolization even in organs with larger vessels.

This study has clear limitations in that we could not perform a functional evaluation. Based on the results of morphological restoration by PLGA microparticle injection, further research about proper functional restoration could be performed.

Conclusions

This study was performed to investigate the *in vivo* safety and efficacy of microparticle-type PLGA injection in injured corpus cavernosum. Microparticle-type PLGA did not show any complications related to injection and had the advantage of simple injection with proper tissue regeneration.

This study is a preliminary report supporting that the injectable biodegradable polymer (PLGA) has a tissue recovery effect without distant migration in the rabbit penile corpus cavernosum, and further study including proof of functional restoration and reproducibility in larger tissues will be required.

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