Collagen/nano-sized β-tricalcium phosphate conduits combined with collagen filaments and nerve growth factor promote facial nerve regeneration in miniature swine: an in vivo study

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Objective. The aim of this study was to investigate the efficiency of a novel biomedical system that repairs facial nerve gaps in a miniature swine model.

Study Design. A collagen (COL)/nano-sized β-tricalcium phosphate (nβ-TCP) conduit combined with COL filaments and nerve growth factor (NGF) was prepared and used to bridge a 35-mm-long facial nerve gap in miniature swine. The functional recovery and axonal regeneration were evaluated by electrophysiologic and histologic assessments in the different groups at 6 months postoperatively.

Results. Morphologic analysis revealed that the COL+NGF, COL/nβ-TCP+NGF, and autograft groups exhibited a superior recovery compared with the COL and COL/nβ-TCP groups. The compound muscle action potential ratios were significantly greater in the COL/nβ-TCP+NGF group compared to the COL+NGF and COL/nβ-TCP groups ($P < .01$). Moreover, transmission electron microscopy demonstrated significantly larger axon diameters and myelin sheath thicknesses in the COL/nβ-TCP+NGF group compared with the COL, COL+NGF, and COL/nβ-TCP groups ($P < .05$). The expression of S-100 was significantly greater in the COL/nβ-TCP+NGF group than in the COL+NGF and COL/nβ-TCP groups ($P < .05$).

Conclusions. The functional nerve biomedical system containing the COL/nβ-TCP conduit combined with COL filaments and NGF could promote facial nerve regeneration, thus offering promising potential for clinical applications. (Oral Surg Oral Med Oral Pathol Oral Radiol 2019;128:472−478)

Peripheral nerve injury caused by infection, trauma, or surgical intervention is a common clinical problem.[1,2] Although nerve injury can occur in any part of the human body, nerve injury in the face is a particularly great challenge because it influences a person's appearance.[3] Unlike the central nervous system, the peripheral nerve shows an intrinsic capability for axonal regeneration. However, self-regeneration is always imperfect, with unsatisfactory results, especially in longer gaps. Therefore, bridging nerve defects is a significant challenge in regenerative medicine. Autologous nerve grafting is the gold standard in bridging peripheral nerve gaps. However, morbidities of the related donor sites, complications, and limited amount of available grafts, as well as mismatches in nerve diameter, architecture, and function remain inexplicable problems in autologous nerve grafting.[1,2] Therefore, great efforts have been made to find alternatives for autologous nerve grafting, such as allografts and vein, artery, skeletal muscle, and artificial nerve grafts. The development of artificial nerve grafts is currently considered a promising alternative to autologous nerve grafts.[1,4-6]

The design of optimal biomaterials serving as an appropriate scaffold plays an important role in nerve tissue engineering and regenerative medicine. Over the past few decades, conduits comprising type I collagen (COL) have been confirmed as promising nerve conduits and applied in different clinical and experimental settings.[7-10] The addition of neurotrophic factors to the conduits is the next step in the field of nerve tissue regeneration. Nerve growth factor (NGF) is a neurotrophic factor that positively regulates Schwann cell myelination and supports the development of axons during the nerve regeneration process. Accumulating evidence has demonstrated that the addition of NGF to nerve conduits can significantly promote the recovery of

Statement of Clinical Relevance

Facial nerve injury is particularly challenging because it influences a person's appearance. We aimed to evaluate the efficiency of a nerve guidance conduit combined with collagen filaments and nerve growth factor for bridging a long facial nerve gap.

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injured peripheral nerves.[10-13] Recently, we developed a new type of artificial nerve conduit comprising COL and nano-sized β-tricalcium phosphate (nβ-TCP), the latter of which enhances cell attachment and retards the degradation of COL conduits.[13,14]

Nevertheless, most of the work addressing peripheral nerve regeneration has utilized rodent models, which have a short nerve gap. However, there are significant anatomic differences in the facial nerves between rodents and humans. Interestingly, the facial nerve of miniature swine is similar to that of humans in terms of its anatomy, and it is long enough to be used for extensive investigation.[15,16] In our previous work, we successfully established a facial nerve gap model in miniature swine and demonstrated that COL conduits combined with linear-ordered COL filaments and neurotrophic factors could promote nerve regeneration.[7,9]

In the present study, we aimed to evaluate the efficiency of a new nerve guidance conduit combined with COL filaments and NGF for bridging a long facial nerve gap in miniature swine. First, a COL/nβ-TCP conduit was fabricated and characterized by scanning electron microscopy (SEM). Then, the conduits were combined with COL filaments and NGF and used to bridge a 35-mm-long facial nerve gap in miniature swine. The functional recovery and axonal regeneration were further analyzed by performing electrophysiologic and histologic assessments.

MATERIALS AND METHODS

Preparation of nerve conduits, COL filaments, and NGF

COL filaments were prepared from bovine COL, as described previously.[7,9] The COL conduit was fabricated by wrapping a COL membrane around a cylindrical mold. The COL/nβ-TCP composite was fabricated by the solvent volatilization method. Briefly, bovine COL was dissolved in ethyl acetate at a concentration of 5% (by weight); then, nβ-TCP particles (0.08 g) were added and mixed thoroughly into the solution. The COL/nβ-TCP composite was fabricated into conduits. All conduits were cut to size and sterilized by radiation before the surgical procedures. For the COL/nβ-TCP+NGF conduits, 80 μg of NGF was added to the COL filaments and incubated for 30 minutes. Finally, the functional biomedical systems were obtained by inserting the COL filaments combined with NGF inside the conduits.

SEM evaluation

The inner surface topography of the COL and COL/nβ-TCP conduits was examined by using SEM, according to standard protocols. Briefly, samples were fixed with 2.5% glutaraldehyde, dehydrated in a graded series of ethanol solutions, and dried with carbon dioxide at the critical point. Finally, samples were sputter coated with a thin layer of gold and visualized by SEM (Inspect F, FEI, The Netherlands).

Experimental design and surgical procedure

All experimental procedures were revised and approved by the Animal Experiment Ethics Committee of Shanghai Ninth People's Hospital (affiliated to Shanghai Jiaotong University, Shanghai, China). The study design followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

A total of fifteen 10-month-old male miniature swine (weighing 35-40 kg) were purchased from China Agricultural University (Beijing, China) and were kept in separate cages under standard conditions (room temperature, 22 ± 2°C; humidity, 55 ± 5%; light-dark cycle, 12 h/12 h), with food and water available ad libitum.

Before the surgical procedures, the animals were anesthetized with an intramuscular injection of ketamine (10 mg/kg) and xylazine (2 mg/kg). Subsequently, a 7-cm subcutaneous incision was made to expose the buccal branch of the facial nerve. After exposure, the nerve was transected at a constant point (1 cm from the anterior edge of the masseter muscle), and a 35-mm section from the distal end was removed by microsurgical scissors. Once the defects were created, the proximal and distal nerve stumps were bridged by the prepared conduits with COL filaments. The conduits were sutured with 10/0 monofilament nylon into the respective nerve end (Figure 1). The 15 animals were randomly divided into 5 groups: (1) COL group, collagen conduit with collagen filaments; (2) COL/nβ-TCP group, collagen/nβ-TCP conduit with collagen filaments; (3) COL+NGF group, collagen conduit with collagen filaments and NGF; (4) COL/nβ-TCP+NGF group, collagen/ nβ-TCP conduit with collagen filaments and NGF.; and (5) autograft group (positive control). For the COL+NGF and COL/nβ-

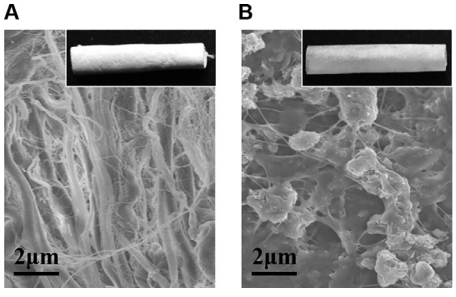

Fig. 1. Experimental design and outline of the surgical procedure. **(A)** Anatomy of the buccal branch of the facial nerve in miniature swine. **(B)** Representative diagram representing the use of the functional nerve conduit biomedical system to bridge a 35-mm-long facial nerve gap in miniature swine.

TCP+NGF groups, the COL filaments were incubated with NGF, as described above, and for the COL and COL/nβ-TCP groups, the filaments were incubated with an equal volume of phosphate-buffered saline. In the autograft group, the excised nerve segments were reversed and sutured back together.

Electrophysiologic assessment

Six months postoperatively, the facial nerves from both sides were re-exposed for electrophysiologic assessments (n = 6 nerves/group) under systemic anesthesia before sacrifice. An electrical stimulus (10 mA) was sequentially applied to the facial nerve trunk at 10 mm from the proximal and distal nerve ends. The recording electrode was placed in the buccinator muscle. Subsequently, the compound muscle action potentials (CMAPs) were recorded, and the ratio of CMAPs was calculated to assess the functional recovery of the nerve.

General observation and animal sacrifice

Nerve regeneration and degradation of the graft as well as the surrounding tissues were observed by gross morphology. Specimens of the regenerated segment were harvested, and all animals were sacrificed humanely in accordance with the animal protection rules.

Transmission electron microscopy and morphologic analysis

A portion of the regenerated segment was fixed in 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide solution, dehydrated in an ascending ethanol series, embedded in epoxy resin, and serially cut into 700-nm semi-thin sections and 70-nm ultrathin sections. The semi-thin sections were stained with Luxol fast blue to examine the remyelinated axons. Briefly, the samples were deparaffinized, incubated in solvent blue solution (Solvent blue 38; S3382; Sigma, Carlsbad, CA) at 37° C overnight, destained with 0.05% lithium carbonate, and counterstained with cresyl violet. The density of the myelinated axons was determined on the Luxol fast blue-stained samples under a light microscope. To calculate the density of myelinated axons, the total number of myelinated axons in 5 random images per sample were counted and divided by the total area, and a total of 4 samples were examined in each group (n = 6). The ultrathin sections were stained with lead citrate and uranyl acetate, and then they were visualized under a transmission electron microscopy (TEM) (JSM-6460LV, JEOL, Japan). Image-Pro Plus software (Media Cybernetics) was used to quantify the diameter and thickness of the regenerated myelin sheath. A total of 10 random axons from 5 random TEM images were analyzed, and the data were averaged for each sample.

Immunohistochemical analysis

Another portion of the regenerated segment was analyzed by performing immunohistochemistry. Samples were fixed in 4% buffered paraformaldehyde solution at 4° overnight, washed with distilled water, dehydrated in an ascending ethanol series, embedded in paraffin, and cut into 5-μm-thick sections. Subsequently, the sections were incubated with anti-S100 (1:2000, Abcam, Cambridge, MA) at 4° overnight and visualized. For the negative control samples, we omitted the primary antibody to avoid signals generated from nonspecific binding. Three fields per sample were randomly selected, and the percentages of S100-positive areas (positively stained area/total area) were quantified by Image-Pro Plus software (Media Cybernetics, Rockville, MD).

Statistical analysis

All numerical data are expressed as mean ± standard deviation. Data were analyzed by 1-way analysis of variance followed by the Student's t test using GraphPad Prism Software version 7. 0 (GraphPad Software Inc., La Jolla, CA). Differences were considered to be statistically significant at $P < .05$.

RESULTS

Characterization of the nerve conduits

In this study, the nerve conduits were 40 mm long, with a lumen diameter of 4.5 mm and a wall thickness of 80 μm. In the COL conduit, SEM revealed pronounced nanofibers showing a hierarchically preferential orientation on the inner surface (Figure 2A). In the COL/nβ-TCP conduit, the nβ-TCP particles were randomly deposited around the fibrils on the inner surface (Figure 2B).

Postoperative gross morphology

All surgical sites were re-exposed and examined after 6 months. In the functional conduit groups, we did not observe debris of the nerve conduits, indicating the degradation and absorption of the conduits. In the COL and COL/nβ-TCP groups, we observed relatively severe scars surrounding the regenerated nerve as well as adhesion of both stumps to the epimysium. In the COL+NGF and COL/nβ-TCP+NGF groups, we observed that the size, color, and appearance of the regenerated nerve were similar to those of the autograft group. Visual inspection revealed that the COL+NGF, COL/nβ-TCP+NGF, and autograft groups exhibited better recovery compared with the COL and COL/nβ-TCP groups.

Electrophysiologic assessment

We assessed the recovery of nerve conduction (CMAP index) by performing electrophysiologic analysis

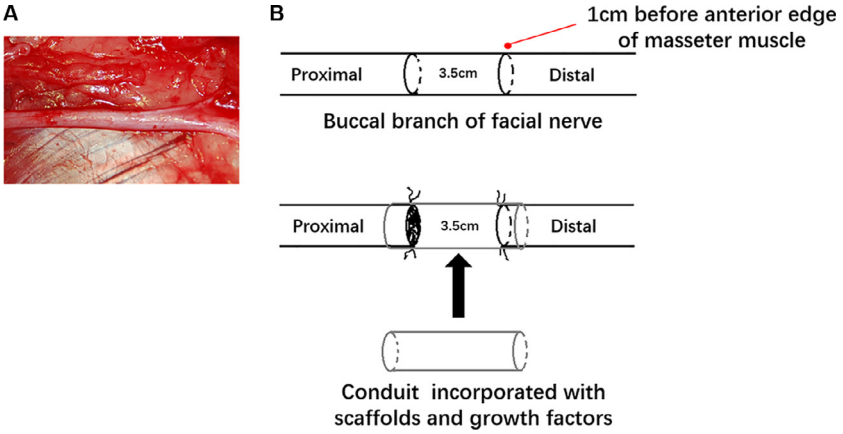

Fig. 2. Scanning electron microscopy images. **(A)** The COL conduit and its inner surface topography. **(B)** The COL/nβ-TCP conduit and its inner surface topography. *COL,* collagen; *nβ-TCP,* nano-sized β-tricalcium phosphate.

(Figures 3A and 3B). Compared with the COL group, the CMAP was significantly greater in both the proximal stump and distal stump of all the examined groups (n = 6; $P < .01$; see Figure 3B). In the proximal stump, the CMAP ratio was significantly greater in the COL/nβ-TCP+NGF group (7.76 ± 1.178) compared with the COL+NGF (2.25 ± 0.271) and COL/nβ-TCP (2.00 ± 0.151) groups, but it was significantly less than that in the autograft group ($P < .01$; see Figure 3B). Similarly, the CMAP ratio in the distal stump was significantly greater in the COL/nβ-TCP+NGF group (3.96 ± 0.163) compared with the COL+NGF (1.295 ± 0.093) and COL/nβ-TCP (1.83 ± 0.158) groups ($P < .01$; see Figure 3B).

Morphologic evaluation of the regenerated facial nerve

Cross-sections of the regenerated segment were examined by light microscopy and TEM (Figures 4A and 4B). Luxol Fast blue staining revealed that the myelinated nerve fibers in the COL/nβ-TCP+NGF and autograft groups were more structurally organized compared with the other groups examined (see Figure 4A). The myelinated nerve fibers were significantly more densely packed in the COL/nβ-TCP+NGF group than in the COL+NGF and COL/nβ-TCP groups (Figure 4C).

Next, we quantitatively calculated the axonal diameter and the myelin sheath thickness based on the TEM images (Figures 4D and 4E). Among all of the examined groups, the greatest nerve fiber density and myelin sheath thickness as well as the largest axon diameter were observed in the autograft group. Nevertheless, larger axon diameters and myelin sheath thicknesses were observed in the COL/nβ-TCP+NGF group compared with the COL, COL+NGF, and COL/nβ-TCP groups ($P < .05$), indicating enhanced remyelination of the regenerated nerves in the COL/nβ-TCP+NGF group. Furthermore, there were no statistically significant differences between the COL+NGF and COL/nβ-TCP groups and the COL group, respectively.

Immunohistochemistical analysis

Anti-S100 antibodies were used to analyze axon regeneration (Figure 5). S100-positive cells were detected in all groups. Immunohistochemical analysis indicated

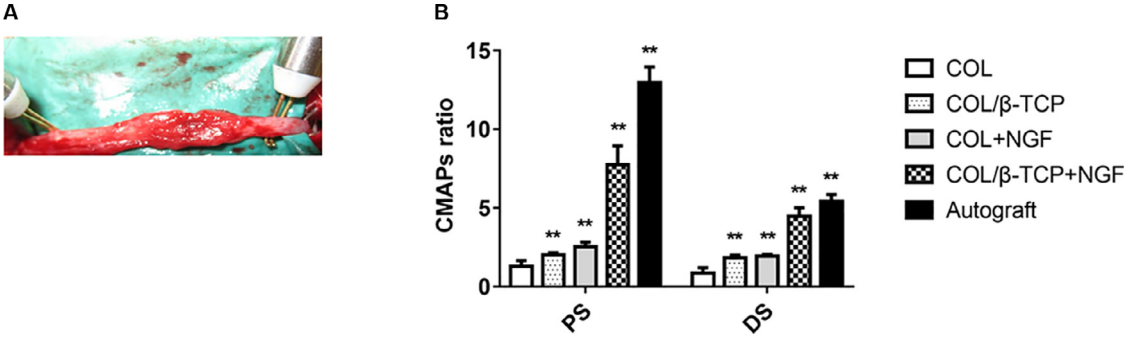

Fig. 3. Electrophysiologic assessments. **(A)** Evaluation of electrophysiology in the autograft group. **(B)** Bar chart representing the CMAP ratio analyzed at 6 months postoperatively, ** $P < .01$ vs the COL group (n = 6). *CMAP,* compound muscle action potential; *COL,* collagen; *DS,* distal segment; *nβ-TCP,* nano-sized β-tricalcium phosphate; *NGF,* nerve growth factor; *PS,* proximal segment.

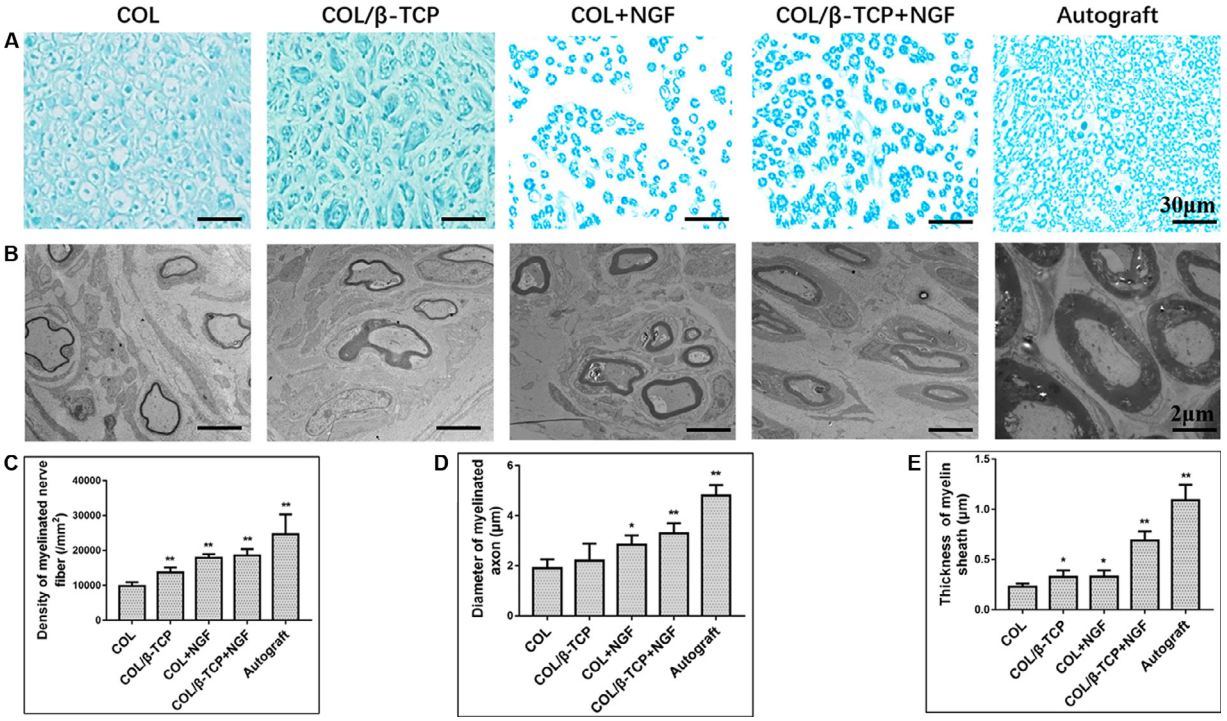

Fig. 4. Evaluation of myelinated nerve fibers in regenerated segments at 6 months postoperatively. **(A)** Luxol fast blue-stained sections. **(B)** TEM images. **(C)** Histomorphometric evaluation of regenerated axons through calculating the density of myelinated axons based on Luxol fast blue staining; analysis of **(D)** myelinated axon diameters and **(E)** myelin sheath thicknesses. *$P < .05$, ** $P < .01$ vs the COL group (n = 6). *COL,* collagen; *n*β-*TCP,* nano-sized β-tricalcium phosphate; *NGF,* nerve growth factor; *TEM,* transmission electron microscopy.

that the autograft group exhibited the highest S100-positive staining areas, whereas the lowest S-100-positive staining areas were observed in the COL group. Furthermore, the expression levels of S100 were significantly higher in the COL/nβ-TCP+NGF group compared with the COL+NGF and COL/nβ-TCP groups (see Figure 5).

DISCUSSION

Peripheral nerve injury is a common problem encountered in the clinic. When the head and neck areas are exposed to infection, trauma, or tumors, the nerve that is most susceptible to peripheral nerve injury is the facial nerve. The facial nerve plays a key role in numerous functional capacities, such as mastication, baseline symmetry, eye lubrication, and both verbal and nonverbal communication[3,17]; thus, facial nerve injuries can cause devastating psychological and socioeconomic consequences. Clinically, autologous nerve grafting is still the gold standard for the treatment of peripheral nerve injury in long gaps; however, its disadvantages and limitations have led to efforts toward improvements in tissue engineering for peripheral nerve regeneration.[1,2] Various nerve conduits, combined with axon-guidance filaments and neurotrophic factors or cells, have been studied as alternatives for the repair of nerve defects.[1,18] In this study, we evaluated the efficiency of a COL/nβ-TCP conduit combined with COL filaments and NGF in bridging a long facial nerve gap in miniature swine.

Synthetic nerve conduits have been considered promising solutions for the repair of nerve defects. Functional COL nerve conduits are biocompatible and biodegradable, and they have been demonstrated to facilitate nerve regeneration.[7,9,10] Therefore, they can potentially serve as a promising alternative to autografts. β-TCP is expected to enhance cell attachment and to retard the degradation of COL conduits, so we combined it with COL to obtain a better outcome in terms of nerve regeneration. Indeed, the regenerative effects achieved in the COL/nβ-TCP groups were superior to those in the COL groups. This result is consistent with previous reports, in which the addition of β-TCP to poly{(lactic acid)-co-[(glycolic acid)-alt-(l-lysine)]}/poly(d,l-lactic acid) conduits has been demonstrated to promote peripheral nerve regeneration.[13,14]

The initial nerve conduit models were shaped in a simple hollow tube. However, this hollow conduit displayed a limited functional recovery and deficient axon regeneration, which may be attributed to the poor intraluminal microenvironments.[18,19] To enhance the effect of the nerve conduit, fillers of the intraluminal guidance cues

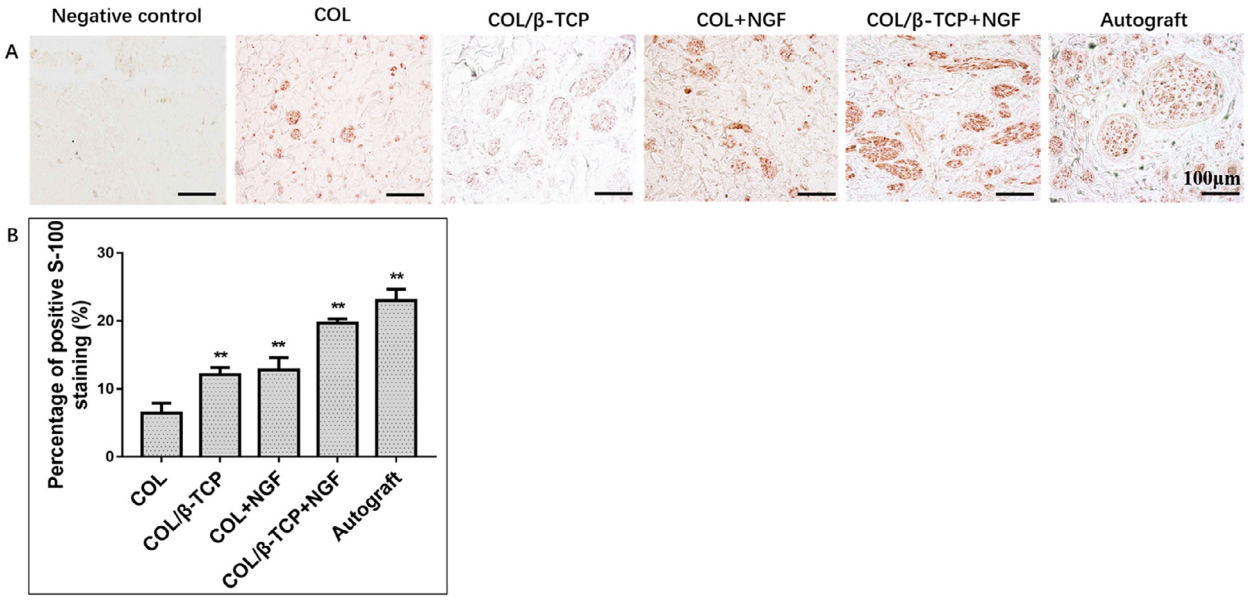

Fig. 5. Immunohistochemical staining analysis. **(A)** S100 in the regenerated segments at 6 months postoperatively. **(B)** Bar charts demonstrating the percentage of positive staining among the different groups. *$P < .05$, ** $P < .01$ vs the COL group (n = 6). *COL,* collagen; *nβ-TCP,* nano-sized β-tricalcium phosphate; *NGF,* nerve growth factor.

have been proposed to provide additional topographic signals for axon regeneration and Schwann cell migration.[18-20] At present, many types of fillers, in the form of fibers,[7,9,21], gels,[6,22,23] or sponges,[24] have been widely used in the lumen of nerve conduits to offer a scaffold for promoting nerve regeneration. COL filaments are superior in terms of semipermeability because they allow the diffusion of neurotrophic factors into the lumen. In our previous study, we demonstrated that linear-ordered COL filaments combined with factors filled in the COL conduit could facilitate nerve regeneration.[7,9]

Neurotrophic factors are important for nerve regeneration. Many neurotrophic factors, such as brain-derived neurotrophic factor, basic fibroblast growth factor, and ciliary neurotrophic factor, have been demonstrated to promote nerve tissue regeneration.[7,9] NGF is a well-characterized neurotrophic factor.[11-13] NGF has a dual function; it not only provides the neurons with nutrition, but it also regulates the development, differentiation, and regeneration of neurons.[25,26] Numerous studies have demonstrated that this soluble exogenous neurotrophic factor can be directly incorporated into nerve conduits to promote the morphologic and functional recovery of injured nerves.[10,11,13] In this study, we combined nerve conduits with COL filaments and NGF to evaluate their effects in bridging a long nerve gap facial nerve in a miniature swine model. Our results indicated that nerve conduits combined with NGF could serve as a promising therapeutic intervention for peripheral nerve injury.

Nerve tissue engineering studies usually use a rodent animal model to evaluate the characteristics of biomaterials and to explore mechanisms of nerve regeneration or development.[6,11,16,27] However, in clinical settings, most strategies cannot reproduce the outcomes achieved in rodent models because of the shorter gaps and different structures in rodent nerves compared with those in humans. The miniature swine model is considered suitable for studying facial nerve injury because the structures of the facial nerve in miniature swine resemble those of humans, with a relatively long and robust segment for multiple procedures.[15,16] In this study, we investigated the effects of the functional nerve conduit biomedical system in a 35-mm-long facial nerve defect representing the actual situation in most human nerve injuries with longer gaps. We demonstrated that the COL/nβ-TCP nerve conduits were a better substitute for facial nerve injury repair compared with pure COL, and the COL/nβ-TCP conduits combined with COL filaments and NGF could significantly promote facial nerve regeneration. However, the functional recovery and axonal regeneration results were still not totally satisfactory compared with those of autologous nerve grafts. Therefore, further efforts are necessary to improve this nerve biomedical system for potential clinical applications in the future.

CONCLUSIONS

The present study verified that the COL/nβ-TCP conduit can achieve better outcomes when used to bridge a 35-mm-long nerve gap in miniature swine compared with the COL conduit. Additionally, we observed that the COL/nβ-TCP conduit combined with NGF could

further promote facial nerve regeneration, thus offering a promising potential use for applications in the clinic.

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