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ORIGINAL PAPER

Regeneration of sciatic nerve crush injury by a hydroxyapatite nanoparticle-containing collagen type I hydrogel

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Abstract The current study aimed to enhance the efficacy of peripheral nerve regeneration using a hydroxyapatite nanoparticle-containing collagen type I hydrogel. A solution of type I collagen, extracted from the rat tails, was incorporated with hydroxyapatite nanoparticles (with the average diameter of ~212 nm) and crosslinked with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to prepare the hydrogel. The Schwann cell cultivation on the prepared hydrogel demonstrated a significantly higher cell proliferation than the tissue culture plate, as positive control, after 48 h ($n = 3$, $P < 0.005$) and 72 h ($n = 3$, $P < 0.01$). For in vivo evaluation, the prepared hydrogel was administrated on the sciatic nerve crush injury in Wistar rats. Four groups were studied: negative control (with injury but without interventions), positive control (without injury), collagen hydrogel and hydroxyapatite nanoparticle-containing collagen hydrogel. After 12 weeks, the administration of hydroxyapatite nanoparticle-containing collagen significantly ($n = 4$, $P < 0.005$) enhanced the functional behavior of the rats compared with the collagen hydrogel and negative control groups as evidenced by the sciatic functional index, hot plate latency and compound muscle action potential amplitude measurements. The overall results demonstrated the applicability of the produced hydrogel for the regeneration of peripheral nerve injuries.

Keywords Collagen type I · Hydrogel · Hydroxyapatite · Nanoparticles · Peripheral nerve regeneration

Introduction

Treating peripheral nerve injuries often requires sophisticated surgical procedures followed by a rarely satisfactory functional recovery [1]. Peripheral nerve defects have been treated by a variety of methods, including nerve autografts, artificial neural guidance conduits and end-to-end cooptation of the impaired nerve stumps, but the obtained results have been rarely satisfactory [2, 3]. Recent studies indicated that the local treatment of peripheral nervous system

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damaged sites with signaling factors could enhance the outgrowth of axons and increase the survival of injured neurons [4, 5]. One agent that has been recognized as an important signaling factor for the appropriate regeneration of nerves is calcium [6]. Studies have shown that the calcium ions play a pivotal role in the formation of functional connections of the nervous system and development of individual nerve cells [6]. Calcium-mediated signaling pathways take part in the regulation of axonal outgrowth, the specification of neurotransmitter subtype and axonal migration and extension [7, 8]. Hydroxyapatite (HAP) is a known calcium ion supply with a slow-release pattern, which has excellent bioactivity, biocompatibility and desirable biologic properties [9, 10].

Collagen type I is the most expressed collagen in the human body and is widely dispersed in the peripheral nervous system [11]. It plays an important role in the formation of sheaths surrounding the axons and nerve fascicles [12]. The sciatic nerve crush injury followed by Wallerian degeneration results in the Schwann cell and fibroblast migration into the injured site and partial degradation of collagen [13]. Collagen type I administration has been shown to enhance the axonal regeneration following the peripheral nerve injuries [14–17].

The aim of current study was to treat the sciatic nerve crush injury with a collagen type I hydrogel containing hydroxyapatite nanoparticles (nHAP). The collagen matrix was enriched with hydroxyapatite nanoparticles and investigated in terms of in vitro biocompatibility and effectiveness for the nerve regeneration in vivo.

Materials and methods

In vivo studies

Animal experiments were approved by the ethics committee of Tehran University of Medical Sciences and were carried out in accordance with the university's guidelines. Sixteen healthy adult male Wistar rats (3 months old, weighing 250–270 g) were purchased from the Pasteur Institute (Tehran, Iran). The rats were randomly divided into four groups (4 rats per group): positive control (without injury), negative control (with injury but without interventions), collagen hydrogel and collagen + nHAP hydrogel. For the surgical procedure, the animals were anesthetized by intraperitoneal injection of ketamine 5%/xylazine 2% [(Alfasan Co., Woerden, Netherlands), 25% (v/v), 0.10 mL/100 g body weight]. The sciatic nerve crush was performed following the protocol reported by Beer et al. [18]. Briefly, a skin incision was made at the left lower limb of the rats, and a 3-mm-long crush injury, 10 mm above the bifurcation into the tibial and common peroneal nerves, was created by exerting a constant force using a non-serrated clamp for a period of 30 s. Each rat in both hydrogel groups was treated by the local injection of 0.50 mL of a single batch of each hydrogel to the site of crush injury using a 16-gauge needle. The muscle and skin were closed using no. 3-0 polyglycolic acid and silk sutures (SUPA Medical Devices, Tehran, Iran), respectively.

Cell culture studies

Primary rat Schwann cells were isolated from the sciatic nerves of adult male Wistar rats according to Terraf et al. [19]. The cells were cultured in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, Grand Island, USA), 100 unit/mL of penicillin and 100 mg/mL of streptomycin in a humidified incubator at 37 °C with 5% CO_2. The proliferation of Schwann cells on the hydrogels was assessed by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The hydrogels in a 96-well tissue culture plate were seeded with 5×10^3 Schwann cells. The cells in the wells of the plate without hydrogels were treated as positive control. All experiments were performed in triplicate, and the absorbance values were read at 570 nm by an Awareness Technology microplate reader (Palm City, FL, USA).

Chemicals

The materials and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively, unless otherwise noted.

Preparation of hydroxyapatite nanoparticles

The nHAP were synthesized following the protocol described by Ragu et al. [20]. At 33 °C, 7.48 g of $Ca(OH)_2$ was dissolved in 100 mL of an ethanol-water mixture (1:1, v/v) and stirred for 3 h. A solution of 6.70 g $NH_4H_2PO_4$ in 100 mL water was added to the $Ca(OH)_2$ solution over a period of 24 h. The pH of the resulting slurry was adjusted to 11 by adding NaOH 1 M. The slurry finally was frozen at −80 °C for 24 h and freeze-dried (Telstar, Terrassa, Spain) for 48 h. The average diameter of the nHAP was determined after three runs using a dynamic light-scattering device (K-One, Seoul, South Korea).

Preparation of hydrogels

Type I collagen was extracted from the rat tails (purchased from Pasteur Institute, Tehran, Iran) using the modified

method described by Techatanawat et al. [21]. The sterilized extracted collagen was dissolved in an autoclaved 0.60% acetic acid in phosphate-buffered saline at a final concentration of 2 mg/mL. The pH of resulting solution was adjusted to 7 by adding autoclaved 1 M NaOH. The collagen + nHAP hydrogel was prepared by adding the autoclaved nHAP to the collagen solution at the weight ratio of 1:3 (nHAP: collagen) according to Shen et al. [22]. The collagen and nHAP-containing collagen solutions were crosslinked with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide powder (EDC; 0.10 mM) 5 min before use.

Functional analysis

Walking-footprint analysis

Four, 8 and 12 weeks post-surgery, the rats' footprints were recorded for the analysis of the sciatic functional index. The rats' hind paws were soaked in the ink, and they were placed inside an acrylic corridor (43 cm length, 8.70 cm width and 5.50 cm height) lined with a millimeter paper and ended with a darkened goal box. The sciatic functional index was calculated using the following equation where PL is the distance from the heel to the top of the third toe, TS is the distance between the first and the fifth toe, and IT is the distance from the second to the fourth toe. NPL, NTS and NIT were the measures from the non-operated foot, and OPL, OTS and OIT were from the operated one [23]. In the positive control, the measures for the right foot were compared with those from the left foot. An index of 0 represented the normal function while -100 represented the complete loss of function. The sciatic functional index values for four rats in each group were averaged.

$$\begin{aligned}\mathrm{SFI} = &-38.30 \times [(\mathrm{OPL} - \mathrm{NPL}) \,/\, \mathrm{NPL})] + 109.50 \\ &\times [(\mathrm{OTS} - \mathrm{NTS}) \,/\, \mathrm{NTS}] + 13.30 \\ &\times [(\mathrm{OIT} - \mathrm{NIT}) \,/\, \mathrm{NIT}] - 8.80.\end{aligned}$$

Hot plate latency test

Twelve weeks post-surgery, the rats were evaluated for the hot plate latency by placing their injured limb on a hot plate (56 °C) and recording the time until they jumped or licked their paws [24]. Following the response, the rats were removed from the plate. The cutoff time for their reaction was set at 12 s. Latencies from four rats in each group were averaged.

Nerve conduction test

Twelve weeks post-surgery, the amplitudes of the compound muscle action potential of the sciatic nerves were measured. The animals were anesthetized by intraperitoneal injection of ketamine 5%/xylazine 2% [25% (v/v), 0.10 mL/100 g body weight]. The sciatic nerve proximal to the site of the injury was stimulated with an electric stimulus (3-5 mA) using needle electrodes. The compound muscle action potential amplitudes were recorded from the needle and cap electrodes placed on the gastrocnemius muscle (filtering frequency of 10 Hz to 10 kHz, the sensitivity of 2 mV/division and sweep speed of 1 ms/division) using an electromyographic recorder (Negarandishegan, Tehran, Iran). The measurements for four rats in each group were averaged.

Gastrocnemius muscle wet weight-loss

At the end of 12th week post-surgery, the animals were sacrificed under anesthesia, and the posterior gastrocnemius muscles on the injured and uninjured hind limbs were harvested and immediately weighed to determine the wet weight-loss of muscles using the following equation [25]. The percentages for four rats in each group were averaged.

$$\begin{aligned}&\text{Gastrocnemius muscle wet weight} - \text{loss}\ (\%) \\ &= \left(1 - \frac{\text{Wet weight of muscle on the injured side}}{\text{Wet weight of muscle on the uninjured side}}\right) \\ &\times 100.\end{aligned}$$

Histopathologic examination

The rats' sciatic nerves and gastrocnemius muscles at the end of 12th-week post-surgery were fixed in 10% buffered formalin and after processing and embedding in paraffin were cross-sectioned and stained with hematoxylin-eosin (H&E). The prepared samples were examined under a light microscope (Carl Zeiss, Thornwood, NY, USA) with a digital camera (Olympus, Tokyo, Japan).

Statistical Analysis

The results were statistically analyzed by Minitab 17 software (Minitab Inc., State College, PA, USA) using one-way ANOVA, with Tukey's post hoc test, and the data were expressed as the mean ± standard deviation (SD). In all evaluations, $P < 0.05$ was considered as statistically significant.

Results

The synthesized nHAP had an average diameter of 212.33 ± 33.02 nm with the polydispersity index of 0.02. The injectable hydrogels were obtained after crosslinking their solutions with EDC at room temperature (Fig. 1). The

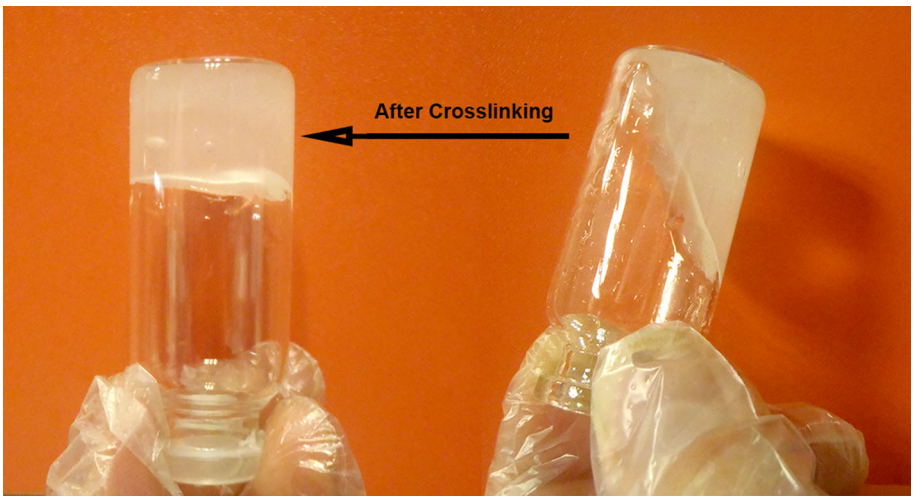

Fig. 1 Optical image of the hydroxyapatite nanoparticle-containing collagen type I hydrogel before and after crosslinking with EDC

concentration of 0.10 mM EDC was chosen according to the previous study by Priyadarshani et al. [26], in which they demonstrated that this concentration is optimal for maximal cytocompatibility. Since the positive effect of obtained hydrogels on the physiology of Schwann cells, as the major cells to support the repair and regeneration of injured peripheral nerves, is desirable [27, 28], the effect of hydrogels on the proliferation of Schwann cells was investigated in vitro. Incorporation of nHAP not only did not induce any toxic effect toward Schwann cells, it enhanced their proliferation (Fig. 2). The collagen + nHAP hydrogel displayed significantly higher absorbance values than the positive control (tissue culture plate) and collagen hydrogel after 48 h ($P < 0.005$) and 72 h ($P < 0.05$).

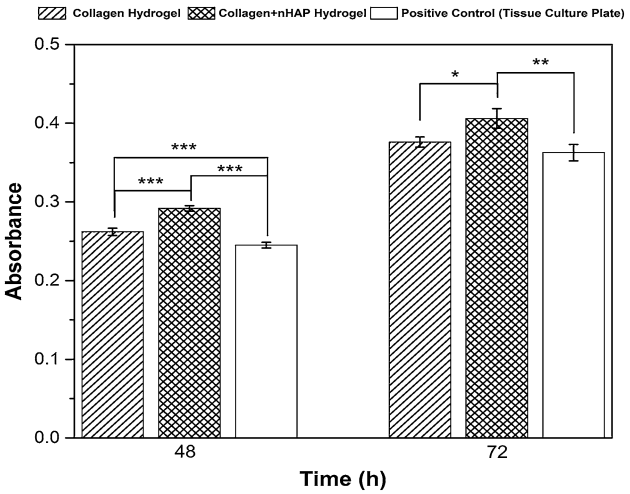

Fig. 2 Effect of the prepared hydrogels on the proliferation of Schwann cells 48 and 72 h after cell seeding. Values represent the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ (obtained by one-way ANOVA)

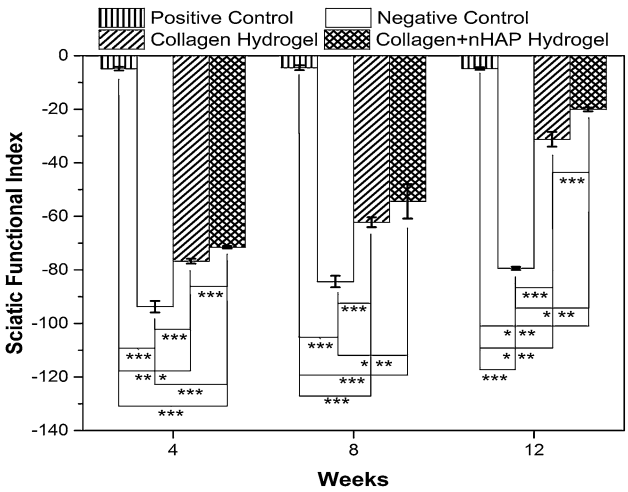

Fig. 3 Sciatic functional index results. Values represent the mean ± SD, $n = 4$, $^{***}P < 0.005$ (obtained by one-way ANOVA)

Figure 3 displays the average sciatic functional index (SFI) values of all study groups. The positive control had an average SFI value of −4.80 ± 0.49 after 12 weeks. The average SFI values for the negative control had very little improvement and increased from −93.73 ± 2.15 after 4 weeks to −84.37 ± 2.12 and −79.44 ± 0.56 at the end of 8th and 12th weeks, respectively. The collagen + nHAP hydrogel treatment considerably enhanced the SFI value from −71.55 ± 0.48 at the end of 4th week to −54.41 ± 6.44 and −20.03 ± 0.72 after 8 and 12 weeks, respectively. These values for the collagen hydrogel group were −76.77 ± 0.92, −62.20 ± 1.81 and −31.24 ± 2.76 after 4, 8 and 12 weeks, respectively. The SFI values for the collagen + nHAP hydrogel were significantly ($P < 0.005$) higher than those of the collagen hydrogel at the end of 4th and 12th weeks and negative control in all time points.

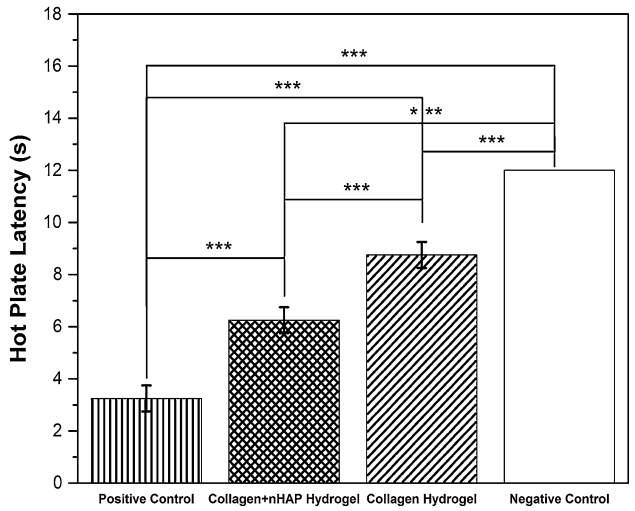

Fig. 4 Histogram comparing the hot plate latency results 12 weeks post-surgery. Values represent the mean ± SD, $n = 4$, ***$P < 0.005$ (obtained by one-way ANOVA)

The hot plate latency test was carried out to evaluate the nociceptive function of the injured limb, and the results are presented in Fig. 4. The rats in the negative control group did not withdraw their paws from the hot plate within 12 s. The positive control had the smallest hot plate latency (3.25 ± 0.50 s), and its time was significantly ($P < 0.005$) smaller than those of other groups. The latency for the collagen + nHAP hydrogel was recorded to be 6.25 ± 0.50 s, which was significantly ($P < 0.005$) smaller than the latency of the collagen hydrogel (8.75 ± 0.50 s) and negative control group.

The compound muscle action potential amplitudes as the indirect assessment of regenerated motor neurons [29] were reported in Fig. 5. The collagen + nHAP hydrogel group possessed significantly ($P < 0.005$) higher amplitude (19.09 ± 1.49 mV) than the collagen hydrogel (10.04 ± 2.55 mV) and negative control (5.98 ± 0.06 mV). The amplitude of the positive control (31.50 ± 0.41 mV) was significantly ($P < 0.005$) higher than those of the other groups.

Finally, the gastrocnemius muscle wet weight-loss as an indirect assessment of sciatic nerve regeneration efficacy [30] was assessed. As Fig. 6 shows, the treatment with both hydrogels significantly ($P < 0.005$) prevented the muscle weight-loss compared with the negative control group. The difference between the weight-loss percentages of collagen + nHAP hydrogel (8.45 ± 2.45%) and collagen hydrogel (15.69 ± 2.80%) were not statistically significant. The negative control had the highest weight-loss percentage (48.44 ± 6.59%) after 12 weeks.

Figure 7 illustrates the histopathologic examination of the sciatic nerves and the gastrocnemius muscles of all study groups after 12 weeks. Examination of the sciatic nerve sections of positive control showed well-arranged myelinated fibers without any histopathologic changes. In

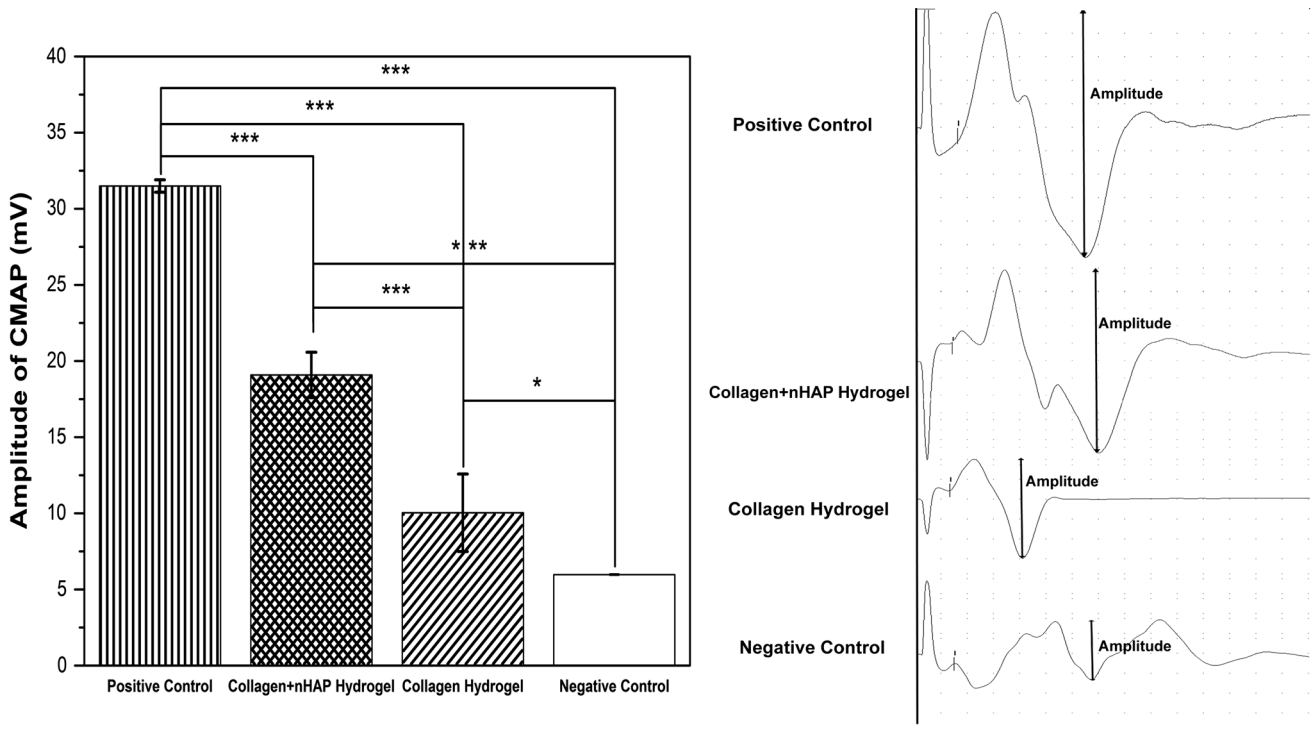

Fig. 5 Histogram comparing the amplitudes of the compound muscle action potential (CMAP) and the electromyographic waves of all study groups 12 weeks post-surgery. Values represent the mean ± SD, $n = 4$, *$P < 0.05$, ***$P < 0.005$ (obtained by one-way ANOVA)

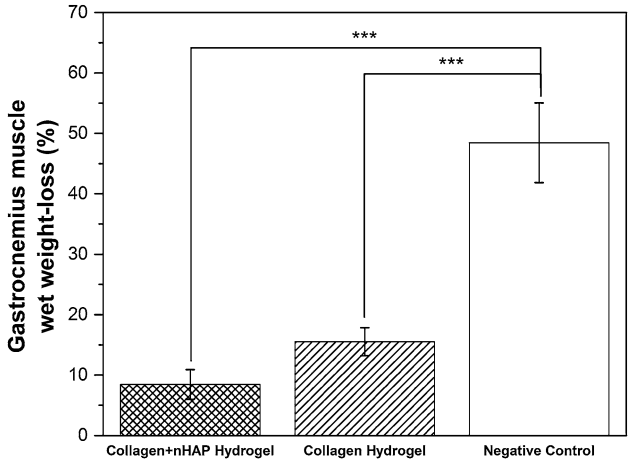

Fig. 6 Histogram comparing the gastrocnemius muscle wet weight-loss percentages 12 weeks post-surgery. Values represent the mean ± SD, $n = 4$, ***$P < 0.005$ (obtained by one-way ANOVA)

the negative control, the arrangement of fibers was disturbed, and they had swollen or missing axons with various degrees of edema and vacuolation. Severe histopathologic damages were seen in this group, including disintegration of the myelin sheath, degenerated nerve fibers, axonopathy and notable edema of the nerve fibers. The histopathologic examination of the collagen hydrogel group showed severe vacuolation, perineural fibrosis and mild edema. In the collagen + nHAP hydrogel group, the arrangement of fibers was the most similar to that of the positive control; however, there was a mild vacuolation in this group. The myelin sheath and fibers were well arranged, and there was no sign of fibrosis or inflammatory cell infiltration in this group.

Twelve weeks after sciatic nerve injury, the muscle fibers in the negative control group were shrunk, broken and wiggly. In addition, the striation of myocytes disappeared, the shrinkage of muscle fibers was evident, and the fibrosis considerably increased between the muscular cells. Hyalinization of the muscle bundles was also observed in this group. In the collagen hydrogel group, the muscular cells were disorganized, and collagen fibers proliferated between the muscular fibers. Muscular atrophy also existed in this group, but its severity was less than in the negative control. Muscular fibers in the collagen + nHAP hydrogel group were considerably regenerated with reduced fibrosis and muscular shrinkage. In the positive control group, muscle fibers were plump and red in color, which indicated an intact gastrocnemius muscle. The cross-sectional area of the gastrocnemius muscle was analyzed by Image-Pro Plus software (version 6.0, Media Cybernetics, Rockville, MD, USA), and the results are depicted in Fig. 8. Treating the sciatic nerve injury with collagen + nHAP hydrogel significantly ($P < 0.005$) increased the cross-sectional area compared with the collagen hydrogel and negative control groups. The positive control had the highest cross-sectional area, and its value was significantly ($P < 0.005$) higher than those of the other groups.

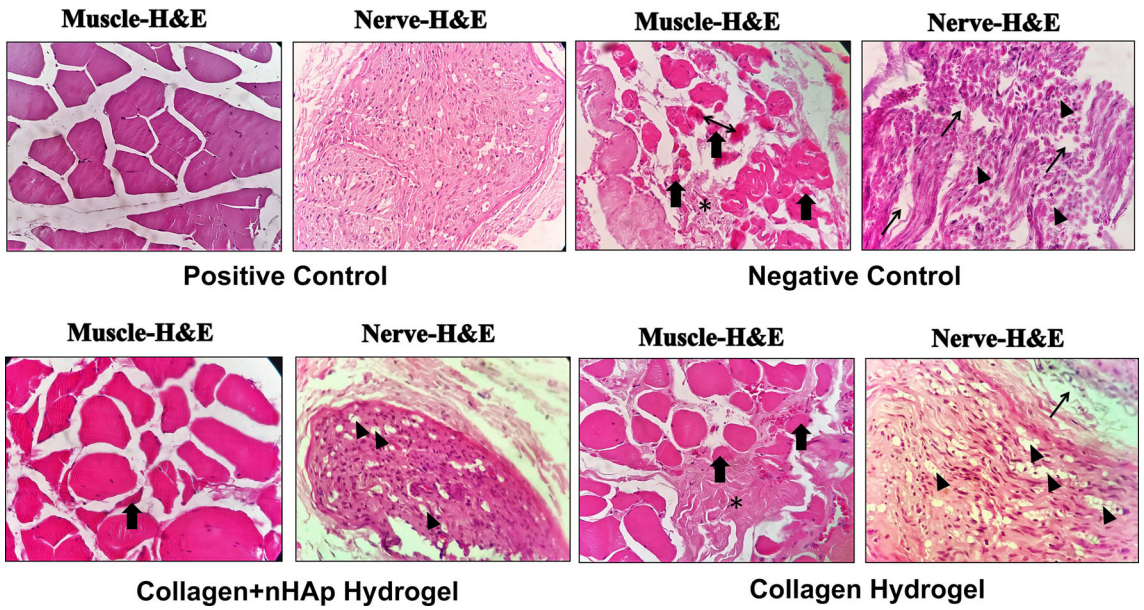

Fig. 7 Histopathologic examination of the sciatic nerve and gastrocnemius muscle cross-sections stained by hematoxylin-eosin (H&E) at the end of the 12th week post-surgery. *Arrowheads* vacuolation, *asterisks* collagen hyperplasia, *thick arrows* atrophied muscle fiber, *thin arrows* edema, *double-head arrow* hyalinization. The magnification for muscle cross-sections: ×400, the magnification for nerve cross-sections: ×200

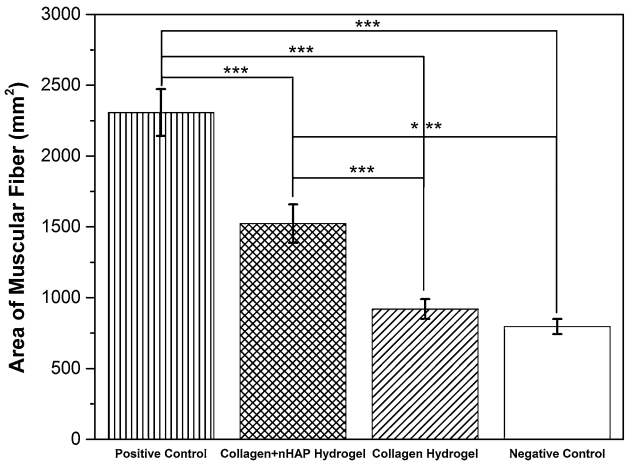

Fig. 8 Histogram comparing the cross-sectional area of the gastrocnemius muscle. Values represent the mean ± SD, $n = 4$, ***$P < 0.005$ (obtained by one-way ANOVA)

Discussion

The enhanced Schwann cell proliferation on both collagen hydrogels compared with the tissue culture plate was in contrast with the result obtained by Alberti et al. [31]. They claimed that the collagen hydrogel hydrates over time and loses its cell anchoring sites. However, we hypothesize that the higher proliferation of cells on the collagen hydrogel can be attributed to the most physiologic and biomimetic environment that it provided for the Schwann cells [32]. Incorporation of nHAP enhanced the proliferation of Schwann cells cultured on the collagen hydrogel even further. We hypothesize that the following reasons are behind this observation: first, the expected increase of the collagen + nHAP hydrogel roughness as a result of nHAP incorporation [33]. A rougher surface normally has more area for the cell attachment and consequently better proliferation [34]. Second, the internalized nHAP could be partially dissolved during the lysosomal digestion and release the calcium ions into the cytoplasm and consequently enhance the proliferation of cultured Schwann cells [35, 36]. It is worth mentioning that treating the cancer cells with nHAP led to their apoptosis [37–39]. This observation was mainly attributed to their high endocytosis and consequently accumulation of nHAP in them, inhibiting the synthesis of key proteins for the cell cycle progression [40].

The walking-footprint analysis, hot plate latency, nerve conduction and gastrocnemius muscle wet weight-loss are the most popular postoperative tests to assess sciatic nerve regeneration [41, 42]. Both collagen hydrogel groups significantly enhanced the nerve regeneration in all postoperative tests. The regenerative activity of collagen in neural injury treatment has been demonstrated previously [43–46]. The collagen can act as a tropic factor and guides the elongation of growth cones [47]. Calcium-mediated signaling pathways have been shown to be responsible for the axonal and dendritic outgrowth in neural cells, as well as the angiogenesis in the endothelial cells [6, 48]. Increased angiogenesis is crucial for the repair and regeneration of neural injuries [49]. The calcium ions released from the collagen + nHAP hydrogel could be easily accepted by the membranes of cells [50]. The accepted calcium ions may enhance the neural injury by enhancing the axonal outgrowth and angiogenesis in the defective area [6, 48]. Liu et al. [51] also demonstrated that the nHAP can induce axonal outgrowth by upregulating the netrin-1 expression. Netrin-1 is a protein that regulates the neuronal migration, axon guidance and synaptogenesis [52]. Madison et al. [53] showed that following the peripheral nerve injury, the netrin-1 gene is overexpressed in the Schwann cells and induces their migration [54], and proliferation [55].

Conclusion

In this study, we successfully prepared and examined a hydroxyapatite nanoparticle-containing collagen type I hydrogel for the treatment of sciatic nerve crush injury in rats. Our results revealed that the prepared hydrogel significantly enhanced the regeneration of the created defect. However, it was unsuccessful in fully restoring the complete function, indicating the need for further optimization of the produced hydrogel.

Compliance with ethical standards

Animal experiments were approved by the ethics committee of Tehran University of Medical Sciences and were carried out in accordance with the university's guidelines.

Conflicts of interest The authors declare that they have no conflicts of interest.

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