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Functional recovery of injured cavernous nerves achieved through endogenous nerve growth factor-containing bioactive fibrous membrane

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ABSTRACT

Radical prostatectomy is a highly successful treatment for prostate cancer, among the most prevalent manifestations of the illness. Damage of the cavernous nerve (CN) during prostatectomy is the main cause of postoperative erectile dysfunction (ED). In this study, the capability of a personalized bioactive fibrous membrane to regenerate injured CN was investigated. The fibrous membrane bioactivity is conferred by the selectively bound nerve growth factor (NGF) present in the rat urine. In a rat model of bilateral CN crush, the implanted bioactive fibrous membrane induces CN regeneration and restoration of erectile function, showing a significantly increased number of smooth muscle cells and content of endothelial and neuronal nitric oxide synthases (eNOS; nNOS). In addition, the bioactive fibrous membrane promotes nerve regeneration by increasing the number of myelinated axons and nNOS-positive cells, therefore reversing the CN fibrosis found in untreated rats or rats treated with a bare fibrous membrane. Therefore, this personalized regenerative strategy could overcome the recognized drawbacks of currently available treatments for CN injuries. It may constitute an effective treatment for prostate cancer patients suffering from ED after being subject to radical prostatectomy.

Statement of significance

The present work introduces a unique strategy to address post-surgical ED resulting from CN injury during pelvic surgery (e.g., radical prostatectomy, radical cystoprostatectomy, abdominoperineal resection). It comprises a bioactive and cell-free fibrous implant, customized to enhance CN recovery. Pre-clinical results in a rat model of bilateral CN crush demonstrated that the bioactive fibrous implant can effectively heal injured CN, and restore penile structure and function. This implant selectively binds NGF from patient fluids (i.e. urine) due to its functionalized surface and high surface area. Moreover, its local implantation reduces adverse side effects. This tailored regenerative approach has the potential to revolutionize the treatment of ED in prostate cancer patients following radical prostatectomy, overcoming current treatment limitations.

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1. Introduction

Cavernous nerve (CN) injuries following pelvic surgeries, namely radical prostatectomy, radical cystoprostatectomy, and abdominoperineal resection are associated with a higher risk of erectile dysfunction (ED) and urinary incontinence [1]. ED is defined by the inability to acquire and/or sustain a satisfactory penile erection during sexual activity. Currently, ED is a significant problem following surgical treatments for prostate cancer. Prostate cancer is one of the most common cancers worldwide, being the second most commonly occurring cancer in males, affecting more than 16% of men [2]. Despite the continuous development of surgeon skills, namely technique, and experience with nerve-sparing prostatectomy, the prevalence of postprostatectomy ED is about 60-70% [1]. Clinically available treatment options for refractory ED rely on pharmacotherapy (e.g. phosphodiesterase-5 inhibitors), external devices, and surgical therapy, all having poor outcomes in postprostatectomy ED patients [3,4]. Current innovative strategies to treat postoperative ED, namely platelet-rich plasma, amnion/chorion membrane, stem cells, and neurotrophic factors, are focused on restoring the structure and function of injured CN [5–9].

The peripheral nerves are known to have the ability to regenerate and recover function after injury. However, this self-repair is relatively restricted and often does not provide a complete functional recovery [10,11]. Therefore, CN injury was thought to be treatable using adult stem cells and neurotrophic factors (i.e., nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF]) [8,12–15]. Among the neurotrophic factors, NGF was widely used to improve nerve regeneration [12,16–20]. For example, the NGF administration improved motor function recovery and increased survival of neurons in rat spinal cord lesions [19]. Human adipose-derived stem cells and NGF-loaded hydrogel were able to rescue erectile dysfunction in a rat model of CN crush damage [12]. In the field of peripheral nerve regeneration, the incorporation of trophic factors into a neural guiding conduit is an emerging strategy. Although enthusiastic, the cost and biological instability, as well as the uncontrolled delivery leading to supraphysiological doses, have limited the clinical use of trophic factors. Consequently, it is hypothesized that the local administration of an endogenous NGF immobilized at the surface of an implantable device may prevent instability and off-site deleterious effects.

Accordingly, in this study, we proposed the use of a bioactive electrospun fibrous membrane comprising an autologous neurotrophic factor (i.e. NGF), to facilitate a sustained and localized CN regeneration. The fibrous membrane bioactivity was achieved by its chemical and biological functionalization with NGF present in a rat biological fluid - urine. The personalized regenerative strategy herein presented was validated in a rat model of bilateral CN crush injury, since this model mimics neural damages associated with radical prostatectomy. In addition, numerous pathophysiological processes connected to radical prostatectomy-associated ED in humans were examined in rats with bilateral CN crush injuries.

2. Materials and methods

Preparation of bioactive fibrous membranes: The fibrous membranes (eFM) were produced by the electrospinning technique [21]. As previously reported, 15% polycaprolactone (PCL - Mn 70,000-90,000 by GPC, Sigma-Aldrich) in a chloroform/dimethylformamide (7/3, Sigma-Aldrich) solution was electrospun at a voltage of 12kV, a needle tip-to-ground collector distance of 20 cm, and a flow rate of 1mL/h [22,23].

Endogenous NGF was bound to the surface of activated and functionalized eFM through an immobilized anti-NGF antibody (provisional patent application nr. 116970) [24]. Briefly, the eFM surface was activated by exposing both sides to UV-ozone irradiation for two minutes (ProCleaner 220 system, Bioforce Nanoscience) and functionalized with amine groups (-NH_2) by incubation in a 1M 1,6-hexane-diamine solution (Sigma-Aldrich) for one hour at 37 °C [25,26]. The antibody against NGF (10 g/mL; EP1320Y; Abcam) was immobilized through the action of a cross-linking agent (1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide/hydroxysuccinimide combination; Sigma-Aldrich) for 2 h at room temperature (RT), after an inhibitory procedure using bovine serum albumin (BSA; Sigma). The anti-NGF antibody immobilized eFM was then soaked with 200 μL of a pool of rat urine for 1 h at RT, washed three times (5 min each) with phosphate-buffered saline (PBS; pH 7.4), and remained hydrated until further usage. To analyze the NGF binding capability of the activated and functionalized eFM, unbound protein solutions, including washing solutions, were collected and kept at -20 °C for subsequent NGF measurement.

Animal experimentation: All animal studies were approved by the institutional Animal Welfare Body (SECVS) and enforced by the University of Minho Ethics Commission, and the Portuguese National Authority for Veterinary Policies (DGAV). All experiments were conducted in ISO 9001:2015 certified animal facilities, following adequate technical protocols currently in use at the I3Bs, ensuring respect for animal life and sanitary laws.

Twenty-nine Male Sprague–Dawley (SD) rats (initial weight: 100–200 g; approximately 4-6 weeks old) were obtained from Charles River (Crl: CD (SD): 001). The rats had free access to food and water at all times while they were housed in their cages. After four weeks of acclimatization, the rats reached an age of approximately 8-10 weeks and an average weight of 425 ± 38 g. A group of at least 5 animals was used for biological fluid collection (Group BFC) and then euthanized with an intracardiac injection of pentobarbital sodium (200mg/Kg). The remaining 24 animals were divided into four groups (n = 6) according to their weight (400 – 450 g) and submitted to the surgical intervention (**Fig. S1**). For surgical procedures, Ketamine (75 mg/Kg) and Medetomidine (0.5 mg/Kg) were injected intraperitoneally into the animals, followed by the administration of Atipamezole (1mg/Kg) subcutaneously.

After surgery, the animals were kept separately and assessed once a day for changes in their overall health by qualified personnel. At 5 weeks post-surgery, erectile function was measured, and animals were euthanized by an overdose of pentobarbital sodium (200mg/Kg) through an intracardiac injection (**Fig. S2**). The penile and surrounding tissues of the prostate, including the implant, were collected for histological evaluation.

Five non-interventional SD rats were anesthetized (Group BFC), and the urinary bladder was exposed to collect urine. Urine samples were centrifugated for 10 min at 3000 rpm and 4 ºC, and then frozen at -80 °C for later use. A pool of urine was used, and the levels of NGF were determined by the Rat beta-NGF DuoSet® Enzyme-Linked Immunosorbent Assay (ELISA) (R&D Systems, Inc.), according to the manufacturer's protocol.

The abdomen of the anesthetized rats was shaved and disinfected using povidone-iodine (Betadine®). A lower midline abdominal incision was performed to expose the bladder, the prostate gland, the major pelvic ganglia (MPG), and the CNs, being identified bilaterally as presented in **Fig. S3**. In the positive control group, no further pelvic surgical procedures were undertaken (Sham). In the other groups, the bilateral CNs were carefully isolated, and the crush injury was induced by compressing it with a clamp for 30 s. *Crush* served as a negative control and received no treatment. Electrospun fibrous membrane [*eFM*] and electrospun fibrous membrane biofunctionalized with NGF from rat urine [*eFM-uNGF*] (0.5 cm^2) were put over the injured CN crush in the remaining groups. Those meshes hold in place, as shown in Fig. S3. After-

Table 1
Primer sequences used for the qPCR procedure.

gene	forward (5'- 3')	reverse (5'-3')
rGAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA
rGAP-43	TTTCCTCTCCTGTCCTGCTC	TGGACTTGGGATCTTTCCTG
rMAP2	GGCACTCCTCCAAGCTACTCT	CTTGACGTTCTTCAGGTCTGG
rNF-160	AGCATTGAGCTCGAGTCGGTG	CTGCTGGATGGTGTCCTGGTAG
rNF-200	AAAGTGAACACGGATGCTATGC	GTGCTTTTCAGTGCCTCCAAC

rGAPDH: rat *glyceraldehyde 3-phosphate dehydrogenase; rGAP-43:* rat *growth-associated protein 43; rMAP-2:* rat *microtubule-associated protein 2; rNF-160:* rat *neurofilament 160; rNF-200:* rat *neurofilament 200.*

ward, the anesthesia was discontinued and the abdominal wall was sealed in two layers.

Erectile function measurement: Five weeks after CN crush injury surgery, erectile function was evaluated using a subcutaneous injection of apomorphine (0.05mg/kg) as described in the literature [27,28]. Briefly, anesthetized rats were injected with an apomorphine solution (Apo-go®; Britannia Pharmaceuticals, Ltd) in the loose skin of the back of the neck to induce an erection. The rats were observed for 30 min, especially for penile erection assessment. The glans exposure and penis erection were scored as mild (1), moderate (2), high (3), and total (4). A full erection was considered only when the emergence of an engorged glans and the distal shaft is notorious, and penile spines were observed along the glans and/or shaft.

For the intracavernous pressure (ICP) measurements, the animals were placed in the supine position. The penis was cleansed of skin and fascia before cannulating the left penile crus with a 25 G butterfly needle, filling it with citrate-dextrose solution (Sigma-Aldrich) and connecting it to a pressure transducer (Infinity® Gamma XL monitor, Dräger). The ICP measurements were recorded continuously during the experiment, ranging from 10 to 30 min after the apomorphine injection. To standardize the ICP reading, mean arterial pressure (MAP) was assessed by cannulating the carotid artery with a 25-gauge needle linked to the pressure transducer. The percentage of erectile function recovery was determined, firstly, by normalizing all ICP values by the median ICP value of non-stimulated (i.e. without apomorphine injection) and non-interventional SD rats. The median ICP value of the Sham group was then used as a reference (100%) for evaluating erectile function recovery.

Neurogenic gene expression analysis: After the surgical procedure, the midshaft of the penis was immediately frozen in liquid nitrogen and stored at -80ºC for real-time quantitative polymerase chain reaction (RT-qPCR). After PBS washing, the penile tissue was weight and immersed in 5% (w/v) Tri Reagent® (Life Science, VWR, USA). Total RNA was extracted following the protocol specified by the manufacturer. Employing a cDNA synthesis kit (qScript™;Quanta Biosciences, VWR), the cDNA was amplified from 100 ng of total RNA. The qPCR reactions were carried out in a Mastercycler® ep Gradient S realplex® thermocycler (Eppendorf; Hamburg, Europe) for neurogenic genes [rat *growth-associated protein 43* (*rGAP-43*), rat *microtubule-associated protein 2* (*rMAP-2*), rat *neurofilament 160* (*rNF-160*) and rat *neurofilament 200* (*rNF-200*), described at Table 1], according to the manufacturer's instructions of the PerfeCta™ SYBR® Green (Quanta Biosciences, VWR, USA).

The housekeeping gene rat *glyceraldehyde-3-phosphate-dehygrogenase* (r*GAPDH*) was utilized to normalize the transcript expression data, and quantification was carried out using the Livak technique ($2^{-\Delta\Delta CT}$ method), with the control (Sham) serving as a calibrator.

Histological and Immunohistochemical analysis: Tissue samples were fixed in 10% neutral buffered formalin, transferred to histological cassettes, dehydrated in an increasing graded ethanol series, and embedded in paraffin wax. The samples were then serially sectioned with 4 µm thickness using a microtome, collected on glass slides, submitted to deparaffinization, by placing the slides in xylene twice for 5 min, rehydrated through a decreasing graded ethanol series, and stained for routine histopathological diagnosis with Haematoxylin and Eosin (H&E) in automatic staining equipment. For the observation of smooth muscle and collagen content, the tissue sections were stained with Masson's trichrome.

Light microscopy was used to analyze all slides using a Zeiss Mod. Axioplan 2 microscope together with the image processing software - LAS Advanced Analysis Software Bundle (No: 12730448).

The histopathological analysis was performed blindly fashion by two expert observers (i.e. HV and CN), in a semi-quantitative rating, The data was expressed in ordinal scale units to each lesion, ranging from absent/normal to severe (extensive damage), adapted from Schackelford et al. [29] and Ferreira et al. [30]. Severity was graded as absent/normal, mild, moderate, and severe, and scoring was defined according to the extension occupied by the lesion (% area): absent/normal - 0; mild: $<$ 25%; Moderate: 25 – 50%; Severe: $>$ 50%.

For immunofluorescence analysis, tissues were fluorescently labeled for β-tubulin and α-SMA to identify the peripheral nerve fibers and confirm the presence of smooth muscle, respectively. The release of nitric oxide (NO) was evaluated by immunofluorescent staining of nNOS and eNOS, while the content of neuron-specific phospho-protein was by immunostaining of synapsin. To identify the remaining urine-derived NGF, the surrounding tissue was immunostained for NGF.

Thermal antigen retrieval was carried out in deparaffinized sections with citrate buffer (pH 6.0), after cell permeabilization with 0.2% (v/v) Triton X-100 for 15 min at RT and blocking with a 3% (w/v) BSA solution for 30 min at RT.Samples were then incubated overnight at 4°C with primary antibodies ([anti-beta III tubulin polyclonal antibody, 1:100 dilution; Abcam], [anti-nNOS (neuronal) antibody (EP1855Y), 1:200 dilution; Abcam], [anti-eNOS antibody (M221), 1:200 dilution; Abcam], [anti-α-smooth muscle (α-SMA), 1:500 dilution; Abcam], [anti-NGF antibody (EP1320Y), 1:300 dilution; Abcam] [anti-synapsin I polyclonal antibody, 1:200 dilution; Abcam]) diluted in 1% BSA. Alexa Fluor (594/488)-conjugated secondary antibodies (1:500; Molecular Probes) were incubated for 2 h at RT after washing with PBS. The samples were counterstained with DAPI (0.02 mg/ml, Biotium; 15 min, RT) for nuclei visualization. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end staining (TUNEL) was performed, according to the manufacturer's instructions using the TdT *In Situ* Apoptosis Detection Kit – Fluorescein (4812-30-K; R&D Systems, Inc.). All samples were mounted in an aqueous medium (Sigma) and analyzed by fluorescence microscopy (Axio Observer, Zeiss) and laser scanning confocal microscopy (TCS SP8, Leica).

Statistical analysis: The analysis relied on the usage of SPSS, a statistical package (release 24.0.0.0 for Mac). The data normality was ascertained with the Shapiro-Wilk test, and Levene's test to assess the homogeneity of variances. For group comparisons, non-parametric tests (Tukey's HSD test follows the Kruskal-Wallis test) were employed, and p-values less than 0.05 were deemed statistically significant.

3. Results

3.1. Bioactive fibrous membrane

The electrospun fibrous membranes (eFM) are composed of nanofibers with diameters in the submicron range, from 0.4 to 1.4 μm having standard-sized pores of 72.67 $\pm$ 31.48 µm (**Fig. S4**). Its high specific surface area and flexibility in surface functionality allow the successful immobilization of an anti-NGF antibody at the surface of activated and functionalized eFM, at the maximum con-

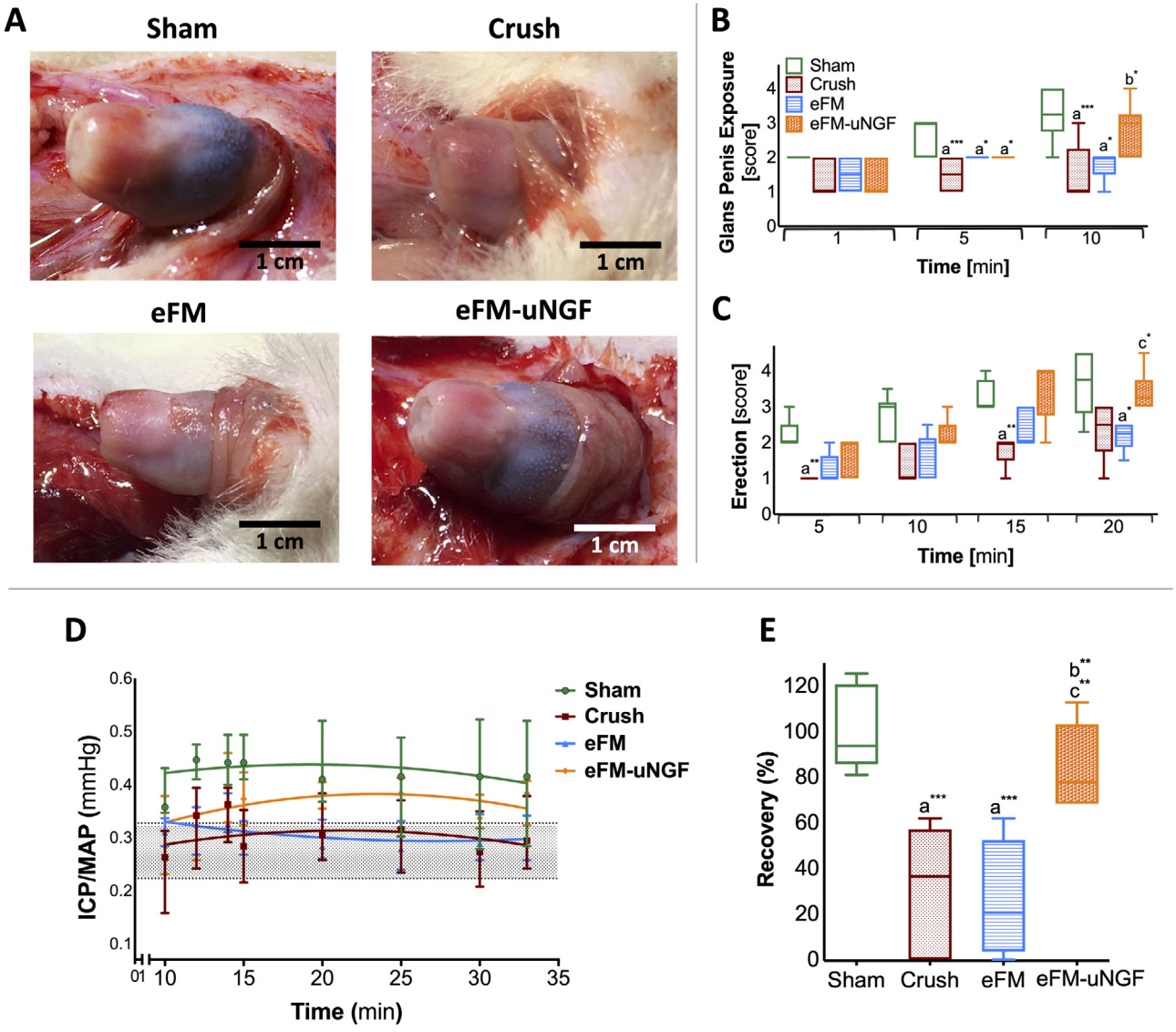

Fig. 1. Penile erection after apomorphine injection (A). A semi-quantitative rating of glans exposure (B) and penile erection (C). Intracavernous pressure-to-mean arterial pressure (ICP/MAP) ratio with a baseline between 0.22 and 0.33 mmHg (D) and percentage of erectile function recovery (E). Tukey's HSD test follows the Kruskal-Wallis test ($p < 0.01$) to assess the data: *a, b,* and *c* indicate significant differences compared to *Sham, Crush,* and *eFM*, respectively; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

centration of 10 μg/mL (**Fig. S5**). A pool of rat urine from 5 individuals contained 171 ± 21 ρg/mL of NGF. Anti-NGF immobilized at the surface of activated *eFM* may selectively retrieve 146 ± 30 ρg/mL, which corresponds to about 85% binding efficiency. A NGF release study conducted for 28 days demonstrated that the bioactive fibrous membrane retained NGF throughout the entire time period (**Fig. S6**).

The effectiveness of the bioactive fibrous membrane (*eFM-uNGF*) was assessed *in vitro* using PC12 cells derived from a pheochromocytoma of the rat adrenal medulla. At day 7, the PC12 cells cultivated on the bioactive fibrous membrane (*eFM-uNGF*) demonstrated considerably greater metabolic activity than the negative control conditions (*eFM*) as seen in **Fig. S7**a. Furthermore, the bioactive fibrous membrane (*eFM-uNGF*) was favorable for cell proliferation and protein synthesis, since the levels of DNA content (as shown in Fig. S7b) and protein synthesis (as shown in Fig. S7c) are comparable to those observed on the control culture condition (*eFM*) along the time. Since NGF stimulation causes PC12 cells to differentiate into neuron-like cells, the bioactivity of urine-derived NGF immobilized at the surface of the fibrous membrane was assessed by measuring the expression of genes involved in neuronal function using RT-qPCR. With the exception of *GAP-43*, all other genes were not expressed on the 1st day of PC12 cell culture on the *eFM-uNGF* condition (**Fig. S8**). On the 3rd and 7th days, PC12 cells cultured on the *eFM-uNGF* condition presented significantly higher *GAP-43, MAP2, NF160, NF200* and *Syn1 (synapsin 1)* expression when compared to the control condition (*eFM*), confirming the bioactivity of the fibrous membrane along time.

3.2. Erectile function

As shown in Fig. 1A, rats in the positive control group (*Sham*) showed observable penile erections, characterized by an engorged glans penis exposure and a full erection with a distal shaft and development of penile spines. Conversely, the CN crush injury group (*Crush*) did not present glans penis exposure and a lower penile erection scoring (Fig. 1B and C), which represented a state of erectile dysfunction. The rats treated with the bioactive fibrous membrane (*eFM-uNGF*) showed a recovery of penile erection, an erectile response comparable to the one observed in *Sham* animals. The erection score of the *eFM* group at 20 min was comparable to the CN crush injury group, being significantly lower when compared

to the score of the *eFM-uNGF* group and uninjured animals (*Sham*) (Fig. 1C).

The ICP/MAP ratio after apomorphine injection was the lowest in rats subject to CN injury (*Crush*) or treated with the bare fibrous membrane (*eFM*), being the highest values observed for uninjured *Sham* animals (Fig. 1D). Treatment of injured CN with the bioactive fibrous membrane (*eFM-uNGF*) enhanced the ICP/MAP ratio. Consistent with the ICP/MAP values, the percentage of erectile function recovery of *Crush* and *eFM* groups was significantly lower when compared with the positive control group (*Sham*) (Fig. 1E). However, the recovery in the *eFM-uNGF* treated group was comparable to the *Sham* group and significantly higher than either *Crush* or *eFM* groups.

3.3. Penile tissue evaluation

Examination of penile tissue, namely corpus cavernosum and dorsal nerves, revealed significant histopathological changes after CN crush in the experimental groups *Crush, eFM* and *eFM-uNGF*, when compared to the uninjured *Sham* animals (Fig. 2). The penile cavernous smooth muscle and fibroelastic tissue density appeared normal in the uninjured *Sham* group, followed by the *eFM-uNGF* group, whereas the *eFM* group revealed the most notorious fibroelastic tissue density and smooth muscle atrophy, approaching that observed at the *Crush* group, that reached the highest score in this parameter, as represented in Fig. 2A and B. Vascular lesions were observed only in rats subjected exclusively to CN injury (*Crush*), being significantly different from the other testing groups (Fig. 2A).

Dorsal nerve lesions, including degeneration with axonal loss, demyelination, and fibrous tissue deposition in supporting framework were reported in rats subject to CN injury, being observed a recovery in rats treated with the bare and the bioactive fibrous membranes (*eFM and eFM-uNGF*) (Fig. 2A and B).

Immunofluorescence staining of α-SMA confirms the presence of smooth muscle in the corpus cavernosum of all animals (Fig. 2C), being possible to observe intense staining in the uninjured sham animals (*Sham*), as well as in rats treated with the bioactive fibrous membrane (*eFM-uNGF*). Interestingly, the expression of *Collagens type I and type II* was downregulated in the penile tissue of rats treated with the bioactive fibrous membrane (*eFM-uNGF*) (Fig. 2D), which indicates that the developed implantable system prevents penile fibrosis. The lower expression of *Collagen type I* was considerably similar in rats subject to CN injury and further treated with the fibrous membranes *eFM* or *eFM-uNGF*. Intracavernous apoptosis was enhanced after CN crush injury, namely in *Crush* and *eFM* groups, as shown in Fig. 3. While CN crush injury treated with the bioactive fibrous membrane (*eFM-uNGF*) presented a decreased number of TUNEL-positive cells.

Genotypic data of the penile tissue also confirm that only rats treated with the bioactive fibrous membrane (*eFM-uNGF*) overexpressed neurogenic-related genes (Fig. 4A). *GAP-43, NF200, NF160*, and *MAP2* expression are all markedly elevated in the *eFM-uNGF* group compared to the *Crush* condition. Likewise, this condition (*eFM-uNGF*) showed a significantly higher expression of *NF200* and *MAP2* than rats subject only to CN injury or further treated with *eFM*. The CN regeneration score was observed only in rats treated with the bioactive fibrous membrane (*eFM-uNGF*), being significantly different from the other groups.

The penile sections were stained with β-tubulin and nNOS to identify the peripheral nerve fibers, namely dorsal nerves, and evaluate the release of NO, respectively. The nNOS staining revealed a loss of nNOS-positive fibers after CN crush injury when compared to uninjured animals (*Sham*) (Fig. 4B). However, the bioactive fibrous membrane (*eFM-uNGF*) treatment seems to preserve nNOS-positive fibers. In the corpus cavernosum was observed a decreased expression of eNOS-positive vessels in the CN crush injury group (*Crush*) (Fig. 4C). The expression of eNOS was increased in rats treated with *eFM-uNGF*.

3.4. Cavernous nerve tissue evaluation

The histological sections of CN tissue from the four groups corroborate the previous results (Fig. 5A), as the CN of uninjured animals (*Sham*) and of rats treated with bioactive fibrous membranes (*eFM-uNGF*) were preserved. Atrophy, distortion, and hypercellularity of CN, as well as fibrous tissue deposition in the supporting framework, were markedly increased in rats subject only to CN injury (*Crush*) or treated with the bare fibrous membrane (*eFM*), being significantly different from all other testing groups. Interestingly, the bioactive fibrous membranes (*eFM-uNGF*) treatment restored the CN crush injury, namely improving degrees of above-indicated lesions (atrophy, distortion, hypercellularity, and fibrous tissue deposition), similar to the positive control group (*Sham*) and with significantly lower severity degrees than the *Crush* or even *eFM* groups. Furthermore, in the bioactive fibrous membrane (*eFM-uNGF*) group is observed Schwann cells proliferation as longitudinal columns termed Büngner's bands or concentric whirls called onion bulbs (Fig. 5A).

CN tissues were fluorescently labeled for β-tubulin (in red) and nNOS (in green) to identify the CN fibers and their content, respectively. Fig. 5B shows the β-tubulin-positive CN fibers in all experimental groups. The nNOS content in rats subject only to CN injury (*Crush*) was significantly lower than in all other animal groups. Both fibrous membranes (*eFM* and *eFM-uNGF*) treatments increased the number of nNOS-positive nerve fibers relative to the negative control group (*Crush*). The apoptosis was remarkable after CN crush injury, presenting a high number of TUNEL-positive cells in the *Crush* and *eFM* groups (Fig. 5C). Animals treated with the bioactive fibrous membrane (*eFM-uNGF*) demonstrated an anti-apoptotic effect, with a low number of TUNEL-positive cells.

3.5. Host response at the injury site

The histological evaluation of the host reaction to both fibrous membranes (*eFM* and *eFM-uNGF*) revealed inflammatory tissue reactions in surrounding tissues of the prostate at low degrees of intensity. Acute diffuse inflammation with mast cells was reported in all CN injured groups (*Crush, eFM,* and *eFM-uNGF*), while chronic focal granulomatous inflammation was observed only in rats subject to CN injury (*Crush*). Moreover, the bioactive fibrous membranes (*eFM-uNGF*) present significantly higher cellular permeation and lower density when compared to the bare fibrous membranes (*eFM*). Capsule thickness of both fibrous membrane conditions (*eFM* and *eFM-uNGF*) although similar, showed a slight tendency to be thinner in *eFM-uNGF* (Fig. 6A).

The implanted fibrous membranes (*eFM* and *eFM-uNGF*) and the surrounding tissue were stained for NGF (in red) and synapsin (in green) to identify the remaining urine-derived NGF and the content of neuron-specific phospho-protein, respectively. As shown in Fig. 6B, the NGF was observed mainly in the bioactive fibrous membrane condition (*eFM-uNGF*), indicating that the immobilized NGF contributed to the CN regeneration through a paracrine effect. Interestingly, the co-expression of NGF and synapsin was observed in the CNs of uninjured animals (*Sham*) and rats treated with bioactive fibrous membranes (*eFM-uNGF*).

4. Discussion

ED remains a significant morbidity following surgical and radiation therapies for pelvic malignancies, namely prostate cancer [1,2]. Mechanical damage to the CN during the surgical procedure

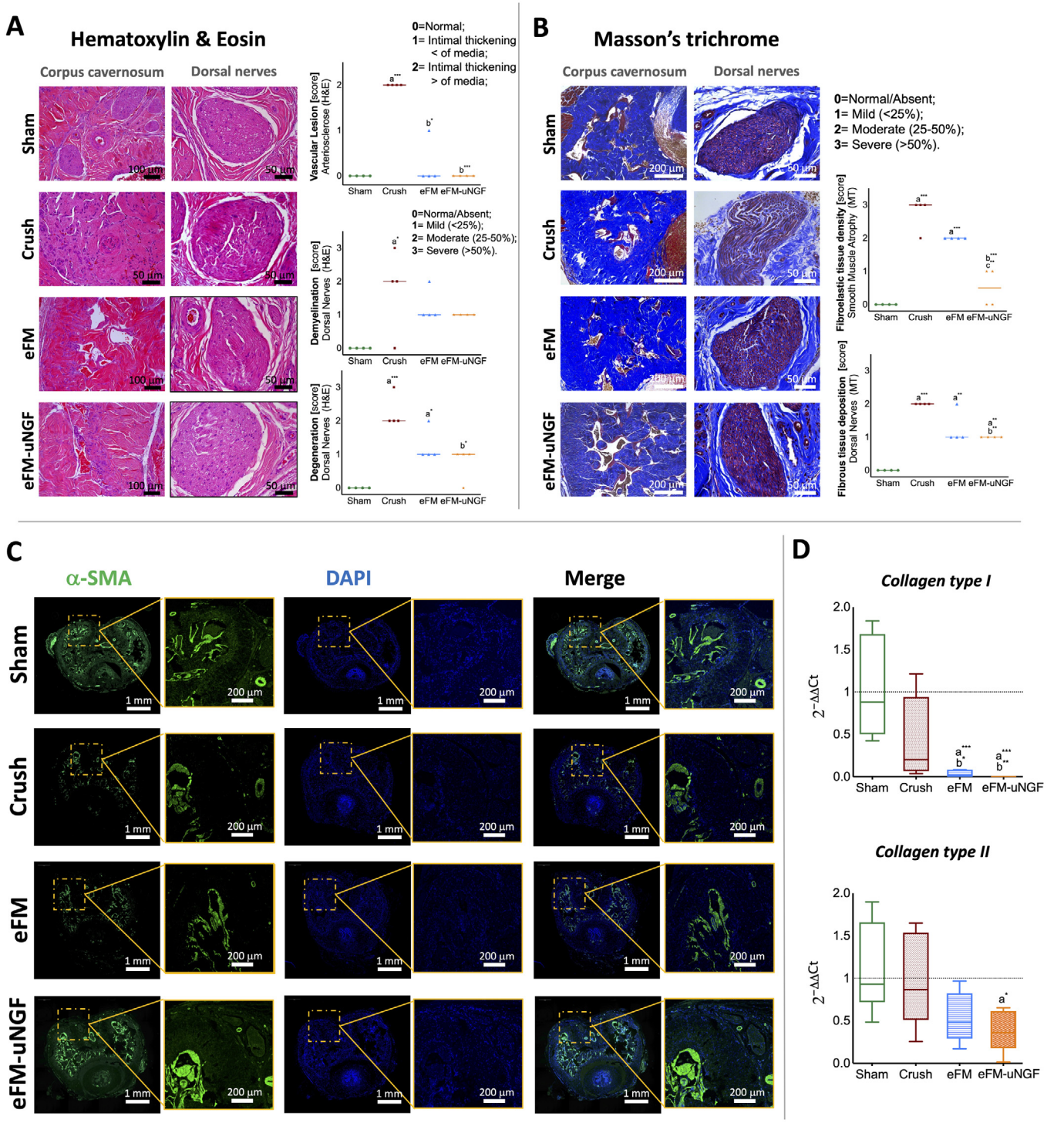

Fig. 2. Histological analysis of the corpus cavernosum and dorsal nerves stained with H&E (A) and Masson's trichrome (B). A semi-quantitative rating of smooth muscle and fibroelastic tissue density (A and B). A semi-quantitative rating of dorsal nerve degeneration with axonal loss, demyelination, and supporting framework fibrous tissue deposition (A and B). Immunofluorescence for the α-smooth muscle actin (α-SMA) (green) and the nuclei stained with DAPI (blue) (C). Relative expression of *Collagen type I* and *type II* in penile tissue. The expression was normalized against the GAPDH gene and quantified using the *Livak* technique, with the control condition (*Sham*) serving as a calibrator. (D). Tukey's HSD test follows the Kruskal-Wallis test ($p < 0.01$) to assess the data: *a, b*, and *c* indicate significant differences compared to *Sham, Crush*, and *eFM*, respectively; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

is predictable and could result in permanent erectile function loss, which is difficult to recover with currently available treatments [31]. Therefore, there is a need for new effective treatments to preserve and restore erectile functions, highlighted by the increasing number of younger patients undergoing radical prostatectomy. Herein, it is proposed a bioactive fibrous membrane to be applied at the injured CN. The fibrous membrane bioactivity was conferred by its functionalization with an endogenous bioactive factor (i.e. NGF) with neurotrophic and neuroregenerative properties. Besides, NGF is a small protein secreted by urothelial and smooth muscle cells in the urinary tract [32], being the levels in human urine among 0.85 - 100 ρg/mL [33–35], while in rats vary between 5

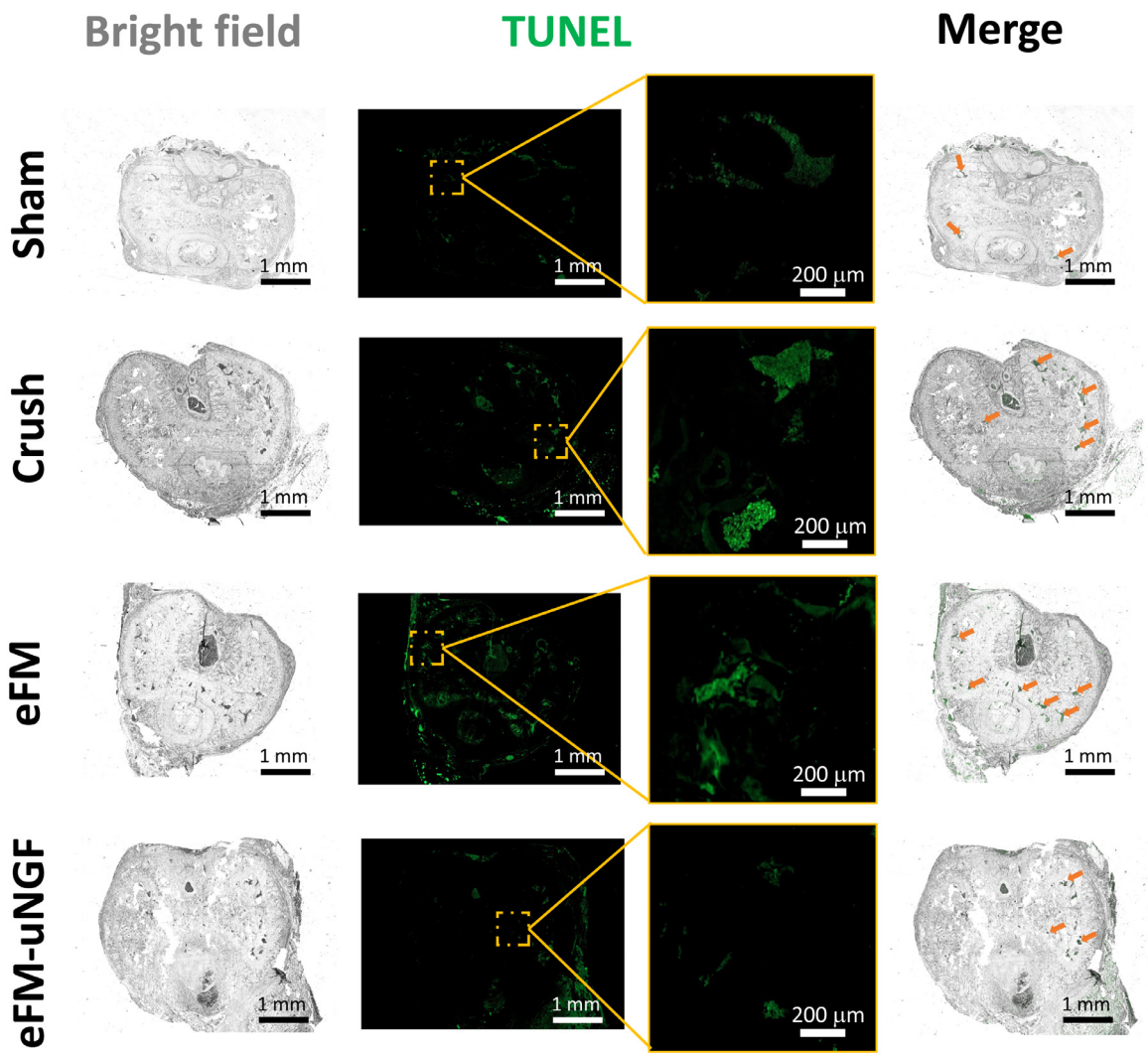

Fig. 3. Bright-field and TUNEL staining (green) of the corpus cavernosum in a penile midshaft specimen of *Sham, Crush, eFM,* and *eFM-uNGF* groups. Apoptotic cells have TUNEL signals (arrows). Quantification of TUNEL-positive signals yielded Fig. S9 in the Supplementary Information.

and 200 ρg/mL [36,37]. Herein the levels of NGF present in a pool of rat urine presented a concentration (171 $\pm$ 21 ρg/mL) within the reported range. Furthermore, the functionalized fibrous membrane with a non-neutralizing NGF antibody allows the selective retrieval of the corresponding growth factor from a pool of rat urine, displaying a high binding efficiency (approximately 85%).

Endogenous NGF captured from rat urine provides a more clinically relevant approach comparing to a commercial protein that would be administered freely. Free NGF would not serve as a fair study control, as it would have a different source, that may not be directly equivalent to the endogenous NGF utilized in the *eFM-uNGF* group. Additionally, the bioactivity of NGF rapidly declines when administered freely, requiring repeated administration at specific time intervals to maintain therapeutic efficacy. Even injected locally, NGF in the free form could have unintended effects over other visceral nerves, not necessarily only the CN, potentially impacting the study outcomes. Conversely, the incorporation of endogenous NGF into the fibrous membrane allows for controlled and sustained release specifically at the injury site, enhancing CN regeneration and erectile function recovery. Comparing the *eFM* group (a crush defect treated with a bare fibrous membrane) with the *eFM-uNGF* group adequately demonstrates the benefits of endogenous NGF in the bioactive fibrous membrane.

The Sprague-Dawley rat model of ED was extensively used to evaluate the neuro-integrity of CN after injury, being the ICP measurement the most common technique for assessing erectile function [12,15,27,38]. Herein the erectile function was stimulated by using the apomorphine challenge, which has been also used in previous studies [27,39]. Apomorphine activity is mediated by dopamine receptors in the hypothalamic paraventricular nucleus, which causes penile erection in rats [40]. In the present study, all uninjured rats (*Sham*) recorded penile erections with high ICP/MAP levels. Conversely, the rats subject to CN injury without treatment had no erection, suggesting that bilateral CN injury can lead to ED. Interestingly, only the rats treated with the bioactive fibrous membrane (*eFM-uNGF*) presented erectile responses at levels near the positive control group (*Sham*), corresponding to the functional penile recovery of 65% at least.

Molecular stimulators, including the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), NGF, and BDNF, have been reported in several studies on ED [9,12,13,41,42]. The intracavernous injections of VEGF have shown a penile corporal endothelium protective effect in a rabbit model of ED [41]. In a rat model of postprostatectomy ED, the application of BDNF-membranes [12,13], NGF-hydrogel [12], NGF-graft [42], and bFGF-hydrogel [13] into the corpus cavernosum resulted in a virtually normal erectile function. Thus, the use of growth fac-

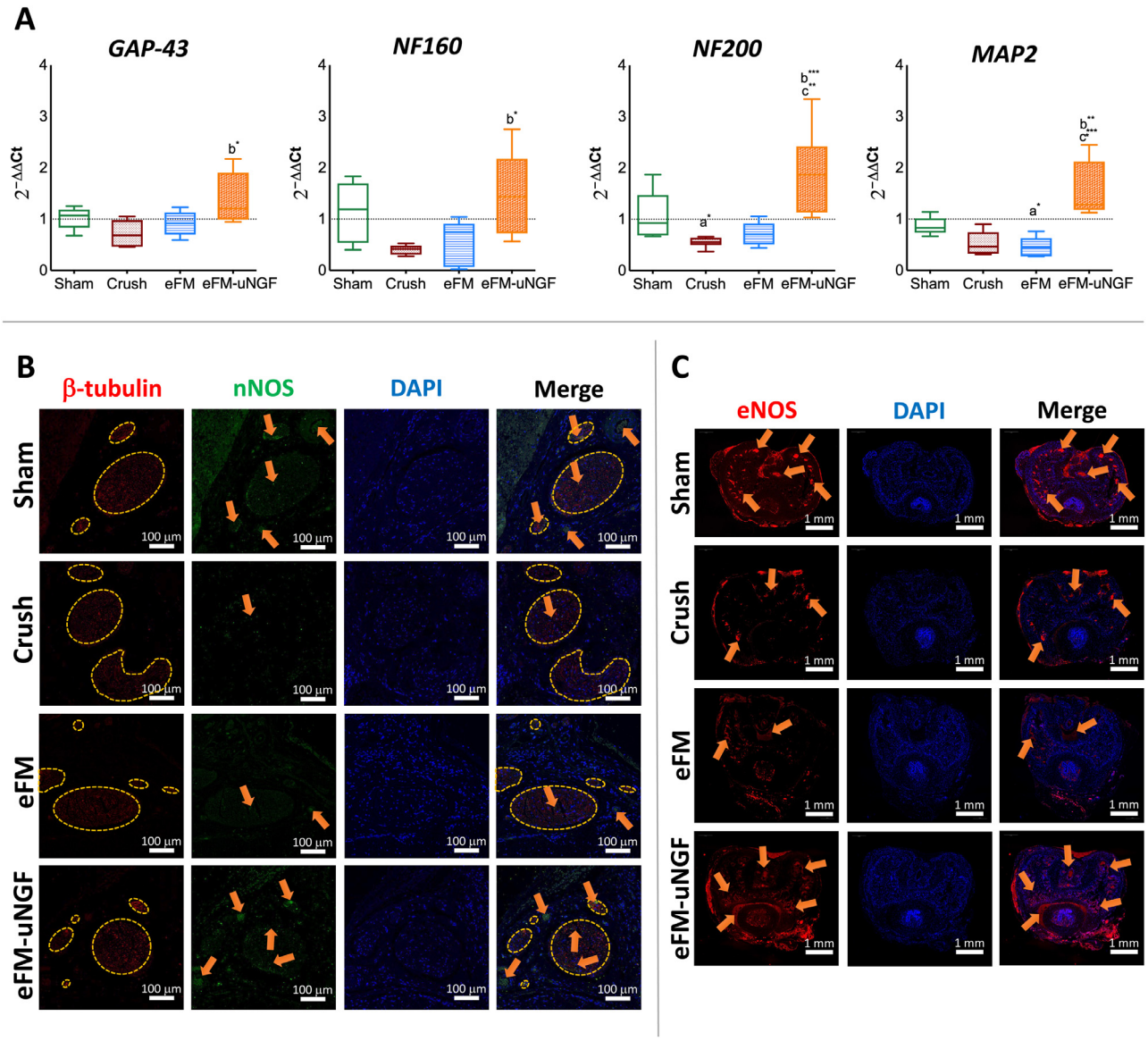

Fig. 4. Several neurogenic markers in penile tissue were analyzed to determine their expression levels, including the growth-associated protein 43 regulation gene (GAP-43), the neurofilament protein regulation genes NF160 and NF200, and the microtubule-associated protein 2 regulation gene (MAP2)(A). The expression was normalized against the *GAPDH* gene and quantified using the Livak technique, with the control condition (*Sham*) serving as a calibrator. Tukey's HSD test follows the Kruskal-Wallis test ($p < 0.01$) to assess the data: *a*, *b*, and *c* indicate significant differences compared to *Sham*, *Crush*, and *eFM*, respectively; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. Immunofluorescence for the β-tubulin (red), neuronal nitric oxide synthase (nNOS) (green), and the nuclei stained with DAPI (blue) in the dorsal penile nerve (B). The dorsal nerves are delimited with dashed lines. Immunofluorescence of the endothelial nitric oxide synthase (eNOS) (red) and the nuclei stained with DAPI (blue) in the penile tissues (C). Arrows indicate positive cells.

tors leads to enhanced neural regeneration, contributing to CN repair or reconstruction [12,42]. Our results show that the bioactive fibrous membranes containing endogenous NGF (retrieved from urine) were more effective to restore erectile function (>65%). Indeed, the proposed bioactive fibrous membrane (*eFM-uNGF*) has the advantage of a local exposure of NGF with more prolonged activity, avoiding their degradation by physiological means.

The absence or decreased capacity for penile erection after CN injury could result in neurogenic ED [43]. In the current work, we revealed a correlation between reduced penile erection capability and lower neurogenic-related mRNA levels. Indeed, the RT-qPCR results complement the histological analysis, as the presence of neurofilament and GAP-43 is crucial to confirm axonal growth and nerve maturation [44]. The application of the bioactive fibrous membranes (*eFM-uNGF*) at the injured CN resulted in neurogenic-related mRNA upregulation in the penile shaft, contributing to CN recovery and erectile function. Neural integrity is essential to normal erectile function [45]. The role of innervation in penile erection is mainly related to the release of NO that acts on sinusoidal relaxation, arterial dilatation, and venous compression, being crucial to initiate the hemodynamic changes in erection and maintaining tumescence [46]. The corpus cavernosum's smooth muscle layer can relax because NO is transported across the cell membrane. [45]. Vascular relaxation is initiated by neuronal nitric oxide synthase (nNOS), and it is maintained by endothelial nitric oxide synthase (eNOS), which promotes blood flow into cavernous tissue. Nerve fibers containing nNOS which innervate the penis derive mainly from the major pelvic ganglion followed by the CN [47]. Our results are consistent with the literature since the nNOS was absent or decreased in both the CN and the dorsal penile nerve of rats subject only to CN injury (*Crush*), whereas the bioactive fibrous membrane (*eFM-uNGF*) treatment reversed the axon content after

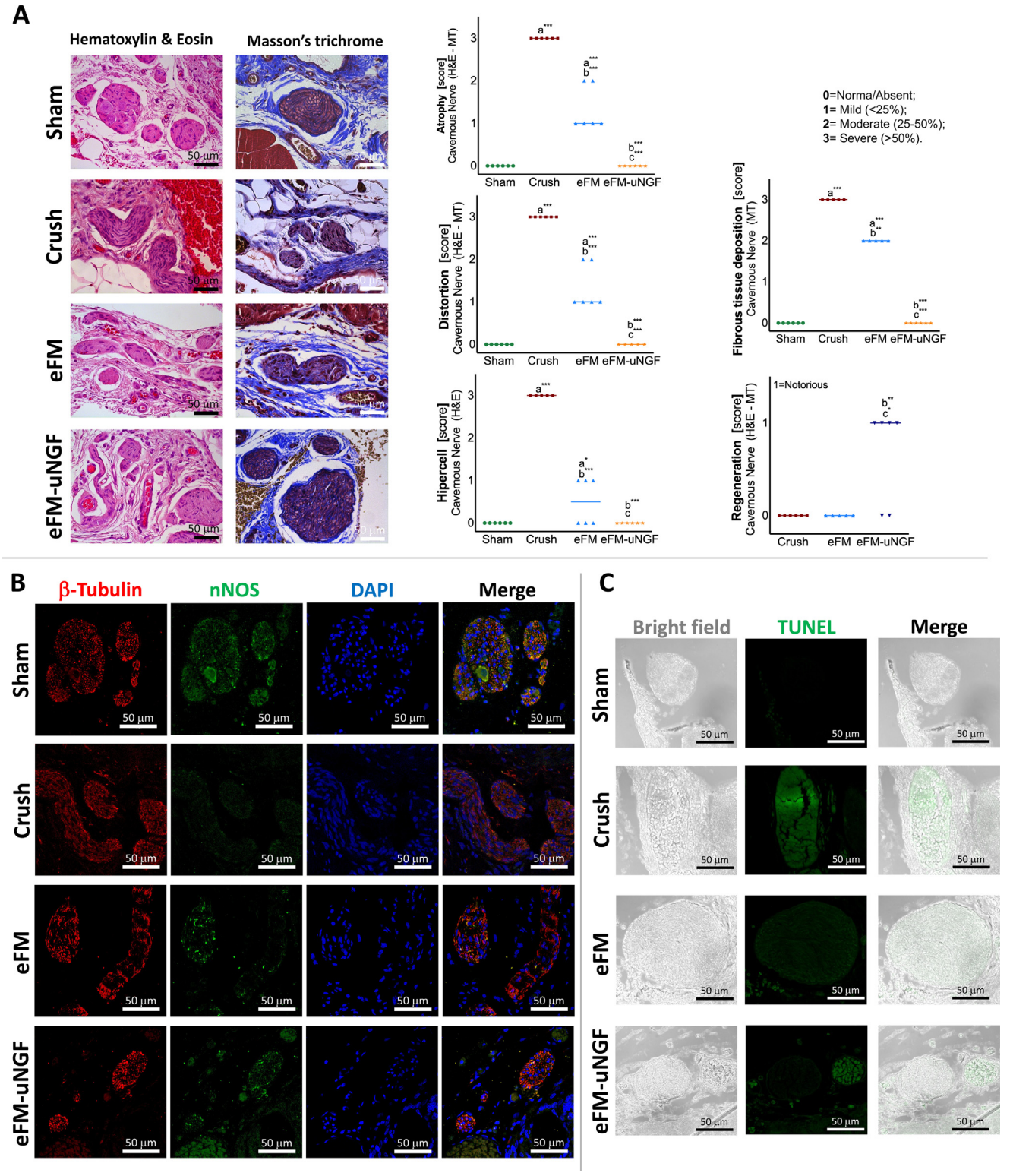

Fig. 5. Histological analysis of the cavernous nerve stained with H&E and Masson's trichrome (A). A semi-quantitative rating of cavernous nerve atrophy, distortion, hypercellularity, supporting framework fibrous tissue deposition, and regeneration. Tukey's HSD test follows the Kruskal-Wallis test ($p < 0.01$) to assess the data: *a, b*, and *c* indicate significant differences compared to *Sham, Crush*, and *eFM*, respectively; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. Immunofluorescence for the β-tubulin (red), the neuronal nitric oxide synthase (nNOS) (green), and the nuclei stained with DAPI (blue) in the cavernous nerve (B). Bright-field and TUNEL staining (green) of cavernous nerve (C).

CN injury. Moreover, the eNOS expression in the *eFM-uNGF* group was higher than in the other testing groups (*Crush* and *eFM*), although comparable with the uninjured rats (*Sham*). According to these findings, CN injury may affect erectile function by reducing the number of nNOS-positive nerve fibers in the dorsal penile nerve and inducing veno-occlusive dysfunction in the corpus cavernosum.

Erectile tissue is structured through numerous blood-filled venous spaces delimited by connective tissue and bundles of smooth muscle [45]. Veno-occlusive dysfunction resulting from CN dam-

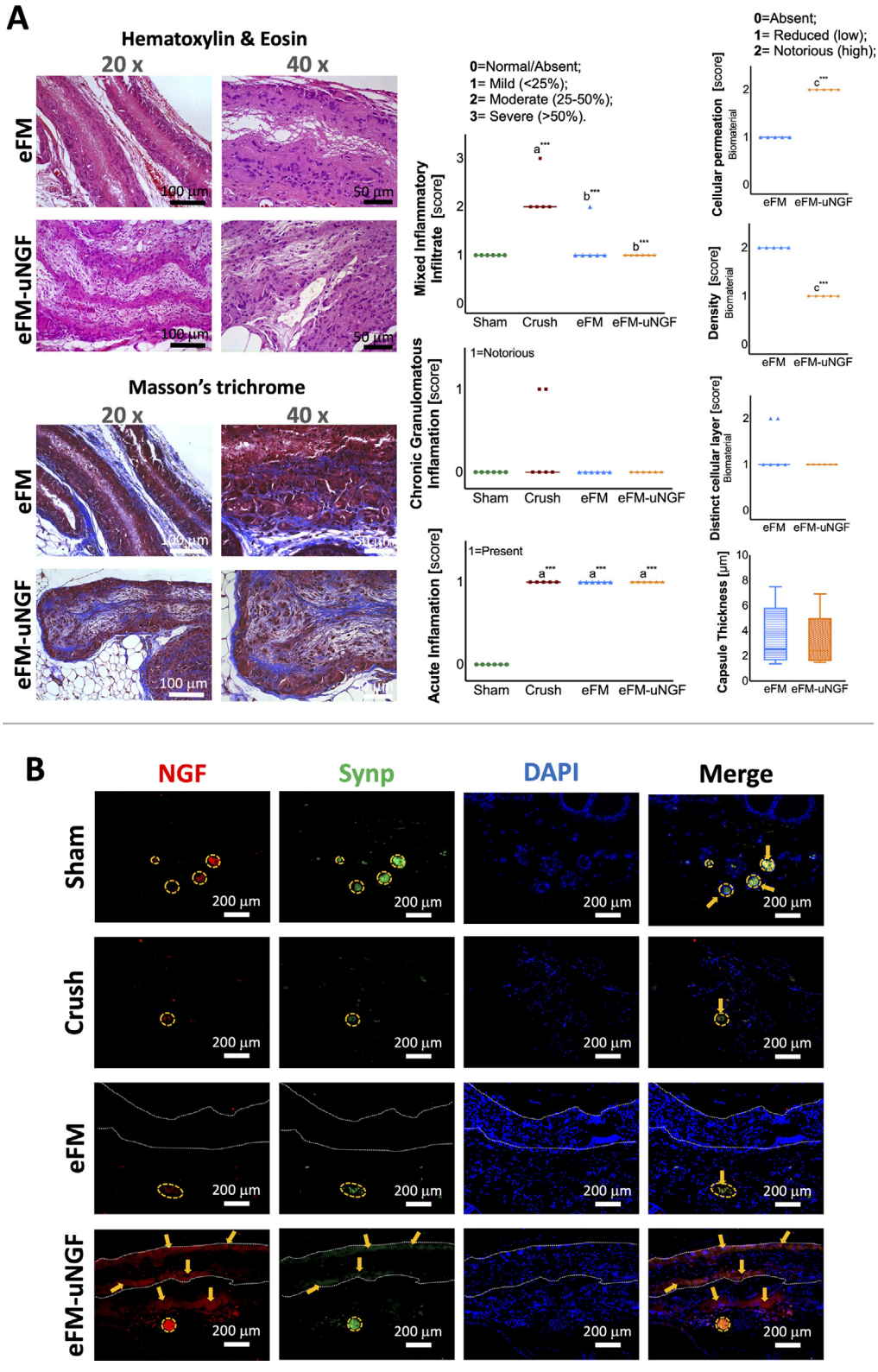

Fig. 6. Histological analysis of implanted fibrous membranes (*eFM* and *eFM-uNGF*) with H&E and Masson's trichrome (A). A semi-quantitative rating of fibrous membranes' cellular permeation, density, distinct cellular surrounding layer, and capsule thickness. Tukey's HSD test follows the Kruskal-Wallis test ($p < 0.01$) to assess the data: *a*, *b*, and *c* indicate significant differences compared to *Sham*, *Crush*, and *eFM*, respectively; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. Immunofluorescence staining for the nerve growth factor (NGF) (red), the synapsin (synp) (green), and the nuclei stained with (DAPI, blue) (B). The implanted fibrous membranes and cavernous nerves (CN) are outlined with dashed lines. Positive signals for NGF and synp on the implanted fibrous membranes are highlighted with arrows.

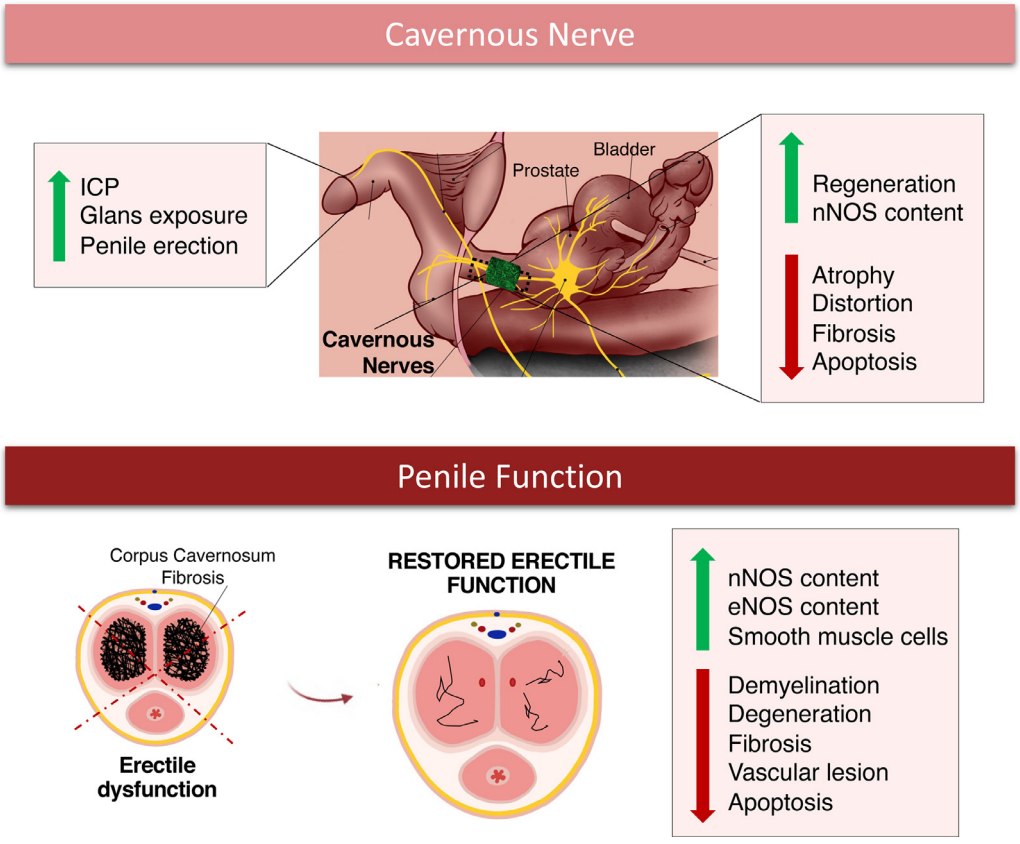

Fig. 7. Outline on the role of the bioactive fibrous membrane (eFM-uNGF) related to cavernous nerve regeneration, penile structure, and erectile function. This diagram represents the pivotal role of endogenous NGF, harnessed by the eFM-uNGF, in supporting cavernous nerve regeneration and the subsequent recovery of erectile function. ICP: intracavernous pressure; nNOS: neuronal nitric oxide synthase; eNOS: endothelial nitric oxide synthase. Illustration adapted from Dorin Novacescu [49].

age causes the death of smooth muscle cells in the corpus cavernosum, which is the primary cause of ED following CN injury. [48]. Accordingly, the present study shows a decrease in smooth muscle cells and increased apoptosis in the corpus cavernosum of rats subject only to CN injury (*Crush*). In contrast, the *eFM-uNGF* group showed high smooth muscle density, supported by an increased number of α-SMA-positive cells, similar to the control group (*Sham*). These data suggested that the proposed bioactive fibrous membrane (*eFM-uNGF*) plays a key role in preventing smooth muscle atrophy in the corpus cavernosum, preventing penile fibrosis as confirmed by the low expression of Collagens mRNA, as well as by Masson's trichrome staining.

Wholly, the present study shows that the implantation of the bioactive fibrous membrane (*eFM-uNGF*) into the injured CN improved erectile function. Those physiological results were supported by the absence of atrophy, distortion, and fibrous tissue deposition in the supporting framework of CN, supported by decreased apoptotic cells and nNOS-positive cells, reflecting nerve regeneration. These results suggested that the bioactive fibrous membrane (*eFM-uNGF*) provides a neuroprotective effect by controlling the wound healing process (namely injured CN). Urine-derived NGF bound to the surface of a fibrous membrane may act as an activator of CN regeneration via the paracrine effect. The fibrous structure of the membranes may act as a physical barrier to prevent connective fibrotic tissue invasion, supporting nerve regeneration. The bioactive fibrous membrane (*eFM-uNGF*) was able to maintain the sustained delivery of urine-derived NGF, as well as its original structure, during the implantation time. These qualities indicate that bioactive fibrous membranes may be useful for the permanent treatment of prostate cancer patients with ED after radical prostatectomy.

5. Conclusions

A bioactive fibrous membrane containing endogenous NGF, implanted into the injured CN, improved its regeneration and restores erectile penile function in a rat model of postprostatectomy ED. The underlying mechanism's function restoration (Fig. 7) may involve: (i) CN recovery: low histological score of atrophy, distortion, and fibrosis, absence of apoptosis, regeneration evidence, and high nNOS content; (ii) Penile function: low histological score of dorsal nerves degeneration, demyelination, fibrosis, and high nNOS-positive dorsal nerves, a low histological score of fibroelastic tissue density and vascular lesions, high smooth muscle density with high cavernous smooth muscle cells relaxation, low/absent apoptosis and, high eNOS content in corpus cavernosum; (iii) Increased blood flow to the penis: an increase in the ICP and a high macroscopic score of glans exposure and penile erection. These findings suggest that the bioactive fibrous membrane may be a promising treatment for postprostatectomy ED, overcoming the recognized limitations in the efficacy of currently available treatments, and using pioneering and personalized strategies.

Data availability

The data supporting the conclusions of this investigation are accessible upon simple request from the corresponding author.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

This work was developed under the scope of the provisional patent application nr. 116970 “Polymeric substrates for nerve regeneration, methods and uses thereof”, priority date 3 December 2020.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2023.07.015.

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