



Collagenase nanocapsules for the treatment of Peyronie's disease. Evaluation in fibrotic plaques obtained from human patients

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Abstract

Introduction: Intraplaque injection of collagenase *Clostridium histolyticum* (CCH) is a therapeutic approach for treating Peyronie's disease (PD) that may represent an alternative to surgery in some patients. However, the efficacy of collagenase injection is rather variable among patients. Nanocapsules (NC) provide therapeutic advantages by allowing sustained protein release and preserving enzymatic activity. The aim was to evaluate the *in vitro* efficacy of preparation of polymeric NC containing collagenase in reducing human PD plaque.

Methods: Polymeric nanocapsules containing collagenase type I (CoINC) or blank NC were synthesized. Enzymatic activity of CoINC and free-collagenase (Col) was determined. The impact of NC, CoINC, and Col on cell viability of commercially available fibroblasts and myofibroblasts was determined. The effects of treatments on differentiation of fibroblasts obtained from plaques of PD patients into myofibroblast and differential cell viabilities were evaluated. Plaque tissues from PD patients ($n = 13$) were injected with phosphate-buffered saline solution, Col (0.1%), CoINC (0.1%), or equivalent amount of NC. Seven days after, the reduction in plaque tissue weight, type 1 collagen content, and the release of protein to the medium were determined.

Results: Nanocapsules exerted protective effect for collagenase by preserving its activity for 10 days versus 48 h for Col. Although fibroblasts from PD patients were unaffected, CoINC decreased the viability of myofibroblasts by 80% compared to 20% reduction obtained by Col. Single injection of CoINC into PD plaque reduced weight by 30%, compared to 10% by Col. This was related to significant reduction in collagen content in the plaque and increased protein content in the medium.

Discussion: Extending CCH activity and efficacy by nanoencapsulation may reduce injection repeats and improve therapeutic response to CCH injection, potentially representing a therapeutic strategy. Superiority of CoINC was demonstrated with

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biochemical assays, cell culture procedures, and *in vitro* functional evidence in plaque tissue from PD patients. Main limitation was the relatively low number of human samples due to limited human tissue availability. Encapsulation of collagenase into polymeric NC extends enzymatic activity and efficiency to degrade fibrous PD plaque. These results suggest that treatment with ColNC may represent a potential therapeutic strategy to reduce injection repeats in the management of PD but requires validation in *in vivo* and clinical studies.

Keywords collagenase, nanocapsules, Peyronie's disease, fibrosis, fibroblast, myofibroblast, human tissue, α -smooth muscle actin, transforming growth factor- β 1.

Introduction

Peyronie's disease (PD) is a pathological condition of the penis in which the tunica albuginea (TA) undergoes abnormal scarring, resulting in the formation of a collagenous fibrous plaque.¹ Peyronie's disease begins with an acute phase characterized by pain mainly during erection and the deposition of collagenous tissue in the penile shaft. During the subsequent chronic phase, the plaque formed becomes fibrous or even calcified, acquiring rigidity and becoming persistent over time.² The consequence is clinical presentation characterized by pain and deformity during erection and sexual dysfunction.³

Despite the large number of research papers on this disease, the etiology of PD is not clearly established.⁴ It is assumed that plaque is produced by abnormal wound healing after trauma in TA during coitus.^{5,6} Such trauma leads to fibrin accumulation, reactive oxygen species production, and expression of profibrotic factors, mainly transforming growth factor beta 1 (TGF- β 1), causing increased deposition and disorganization of collagen fibers in the TA.^{7,8}

One of the key processes at the cellular level leading to abnormal healing and tissue fibrosis is the differentiation into myofibroblasts from fibroblasts present in TA, induced by oxidative stress and TGF- β 1.⁹ Myofibroblasts originate during wound healing to aid in wound repair and, under normal conditions, disappear by apoptosis when the injury has been repaired.¹⁰ In the case of PD, these cells persist and continue to actively produce collagen, resulting in local scarring. In parallel to scarring, myofibroblasts play a critical role in the development of tissue fibrosis.^{11,12} Myofibroblasts are virtually absent in normal penile TA, but are very abundant in the PD plaque.¹³

The most effective way to treat the penile deformity caused by PD is surgical intervention. However, treatment with intralesional injections of collagenase *Clostridium histolyticum* (CCH) directly into the plaque is a much less aggressive alternative and widely accepted by patients.¹⁴ Collagenase *Clostridium histolyticum* is a metalloprotein consisting of a mixture of two microbial collagenases AUX-I and AUX-II, which have the capacity to lyse type 1 and type 3 collagen, which are the most abundant in fibrotic plaques.¹⁵ Different treatment modalities have been evaluated to improve the efficacy of CCH administration¹⁶ but the CCH treatment regimen involves subjecting the patient to several cycles, commonly up to four cycles, of two injections spaced 6 weeks apart. The need for cyclic repeat injections is due to the rapid loss of CCH activity, which virtually disappears within 24 h. Although intralesional treatment with CCH has been shown to be safe, successive administration of CCH may result in adverse effects, the most serious of which is penile fracture, estimated to occur in the range of 0.5%-4.9% of treated patients¹⁷ and usually occurring after the second injection of the third or fourth cycle.¹⁸ On the other hand, the only

approved and commercialized CCH for PD treatment (Xiapex) is no longer available in Europe, which further justifies the search for alternative CCH-based therapies to cover a clinical unmet need.

In the last decade, interest in incorporating proteins into polymeric nanocapsules (NC) has been increasing.¹⁹ The main objectives are to protect the enzymatic activity of these proteins and/or to prolong circulation times. In 2018, Villegas et al.²⁰ proposed the encapsulation of CCH in NC obtained from aminated acrylic monomers. Using two types of crosslinkers, one of them being biodegradable, these authors achieved a sustained release of CCH that showed efficacy in a mouse model of local dermal fibrosis. On the other hand, clinical trials have shown that the dosing regimen is a determining factor in the efficacy of PD plaque treatment, as well as in the occurrence of adverse effects associated with intralesional injection of CCH. For this reason, the administration of CCH in the form of nanomedicines that preserve its activity and reduce the risks of penile fracture would represent a very important advance in the treatment of these patients. To this end, it is necessary to understand the effects of free and encapsulated CCH at the cellular level on the different cell lines involved in this process.

The aim of this work was to evaluate collagenase nanoencapsulation as a strategy to extend and preserve enzymatic activity. This would lead to improve its impact on cellular hallmarks of PD pathophysiology and to enhance its efficacy to reduce the size of fibrotic plaques obtained from human patients undergoing penile surgery for PD *in vitro*.

Methods

Reagents and equipment

Collagenase Type I and EnChek Gelatinase/Collagenase Assay Kit were sourced from Life Technologies (Carlsbad, CA, USA). Acrylamide (AA) was obtained from Fluka (Seelze, Germany). 2-Aminoethyl methacrylate hydrochloride (Am), ethylene glycol dimethacrylate (EG), *N,N'*-methylenebis(acrylamide) (MBA), ammonium persulfate (AP), and *N,N,N',N'*-tetramethylethylenediamine (TMEDA) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Amicon Ultra-2 mL Centrifugal Filters Ultracel 10 K were acquired from Millipore (Burlington, MA, United States).

Reagents for *in vitro* assays: paraformaldehyde, Triton X-100, phalloidin-Atto 565, fluoroshield with DAPI, and Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM-F12) were acquired from Sigma-Aldrich. Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640), L-glutamine, and phosphate-buffered saline (PBS) solutions were purchased from Gibco (Waltham, MA, USA). Penicillin/streptomycin (13-0050) was obtained from ZellShield (Berlin, Germany). Cell counting kit-8 (CCK-8) was sourced from

Merck (Darmstadt, Germany). CoraLite Plus 488-conjugated smooth muscle actin (α -SMA) antibody and recombinant human TGF- β 1 protein were obtained from Proteintech (Rosemont, IL, USA).

The analytical methods employed to characterize the NC were as follows:

Transmission electron microscopy (TEM) analysis was conducted with JEOL TEM 2100 or 3000 instruments operating at 300 kV (JEOL Ltd., Tokyo, Japan). Samples were prepared by resuspending the NC in distilled water and depositing them on carbon-coated copper grids. To enhance visualization of the protein capsules, a 1% phosphotungstic acid (PTA) solution at pH 7.0 was used as the staining agent. The hydrodynamic size of the NC was determined by dynamic light scattering (DLS) and electrophoretic mobility measurements of the capsules resuspended in water were employed to determinate the zeta potential values (ζ) of the NC. For both techniques we used a Zetasizer Nano ZS, which is equipped with a 633-nm “red” laser (Malvern Instruments Ltd., United Kingdom).

On the other hand, fluorescence spectroscopy measurements were taken with a Synergy 4 Microplate Fluorescence Reader (Biotek, Winooski, VT, USA), using $\lambda_{\text{ex}} = 495$ nm and $\lambda_{\text{em}} = 515$ nm for collagen substrate digestion products.

Collagenase NC synthesis

The NaHCO_3 reaction buffer (0.01 M, pH 8.5) was first deoxygenated by several freeze-vacuum- N_2 cycles. Collagenase (3.1×10^{-5} mmol) was then dissolved in 1 mL of deoxygenated buffer. Within a vial, AA, Am, EG, and MBA were blended into 2 mL of deoxygenated buffer at ratios of 7:6:1:1, respectively. This solution was divided into two vials, one for the formation of blank NC and one was mixed together with the protein solution to form collagenase nanocapsules (ColNC). After this time, 0.013 mmol of AP and 0.02 mmol of TMDA dissolved in 1 mL of deoxygenated buffer were added to each of these mixtures. These solutions were stirred at 300 rpm for 90 min under inert atmosphere at room temperature. Finally, the NC were purified by centrifugal separation using 10 kDa cut-off filters (AMICON Ultra-2 mL 10 kDa) and washed five times with NaHCO_3 buffer. These capsules were preserved at 4°C.

Enzymatic activity assay

Collagenase activity present in the ColNC or Col was measured for 10 days using the EnzChek Gelatinase/Collagenase Assay Kit according to the manufacturer's protocols. Briefly, 50 μL of the samples and 50 μL of the collagenase substrate prepared above at a concentration of 100 $\mu\text{g}/\text{mL}$ were mixed. This substrate is conjugated with a fluorophore that is released upon degradation by the enzyme. These mixtures were incubated for 60 min and then fluorescence was measured at $\lambda_{\text{ex}} = 495$ nm and $\lambda_{\text{em}} = 515$ nm. Finally, the fluorescence values obtained were used to calculate the collagenase activity in the ColNC or Col.

Human tissues

Adult patients undergoing penile surgery involving plaque excision for the treatment of PD and giving their written informed consent were included. Patients with infectious diseases were

Table 1 Characteristics of human subjects.

<i>n</i>	13
Age (years $\pm$ SD)	54.6 $\pm$ 6.7
Diabetes (%)	3 (23.1)
Dyslipidemia (%)	4 (30.8)
Hypertension (%)	4 (30.8)
Penile curvature ($^\circ \pm$ SD)	73.4 $\pm$ 16.7
Dorsal plaque (%)	8 (61.5)
Dorsolateral plaque (%)	5 (38.5)

n indicates the number of subjects. SD: standard deviation.

excluded. Peyronie's disease plaques excised during the operation were used for *in vitro* procedures. The study followed the principles outlined in the Declaration of Helsinki. Protocol and consent form were approved by the Ethics Committee at Puerta de Hierro-Majadahonda University Hospital (PI 50/20, 9 May 2020).

Tissues from 13 patients were obtained. Table 1 shows main characteristics of the patients included in the study.

Isolation and cell culture of fibroblasts from PD plaque tissue

Plaque tissues extracted from different patients ($n = 3$) were sterilely divided into 1 mm² fragments. These fragments were incubated in DMEM-F12 complete culture medium in 6-well plates at 37°C and 5% CO_2 in humidified atmosphere for 7–10 days. This method, known as “explant technique,” enables the cultivation of pure fibroblast cultures.²¹ Tissues were removed from the 6-well plates once cells were observed growing out of the tissue. The cells were then washed several times with PBS, and fresh medium was added to each well. Cells were incubated at 37°C until 80% confluence was reached, followed by PBS washing and subsequent culturing in T75 flasks, where the cells were maintained until passage 10.

Transdifferentiation of fibroblasts obtained from plaque tissue

Transdifferentiation of fibroblasts into myofibroblasts was induced by exposing fibroblasts to TGF- β 1 (10 ng/mL) for 72 h and was confirmed through immunofluorescence assays for phalloidin (F-actin) and α -SMA for flow cytometry, as previously demonstrated.²²

Cell culture

Human dermal fibroblasts (HDFa), mouse embryonic fibroblasts (MEF), myofibroblasts, and fibroblasts extracted from PD plaque tissue were cultured in DMEM-F12 supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin–streptomycin at 37°C and 5% CO_2 .

Different concentrations (50, 100, and 200 $\mu\text{g}/\text{mL}$) of collagenase present in ColNC or Col were used in the viability assay of commercial HDFa and myofibroblasts. As the NC did not contain collagenase, the same concentrations were applied as with the other samples. However, only the 50 $\mu\text{g}/\text{mL}$ concentration of

these samples was used for cell viability of fibroblasts and myofibroblasts obtained from patients, as well as for flow cytometry and confocal microscopy.

Viability assay

For this test the CCK-8 (Merck) commercial kit was used. 6×10^5 fibroblasts, myofibroblasts, and HDFa cells were seeded on 12-well plates. Following 24 h, cells were treated with the above concentrations of NC, ColNC, or Col at 37°C and 5% CO₂. Viability was measured on days 1, 2, 4, and 7 after exposure to treatments in commercial fibroblasts and myofibroblasts. However, viability was assessed on days 1, 4, and 7 for fibroblasts and myofibroblasts extracted from patients. The CCK-8 solution contains (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (WST-8), a compound that is bio-reduced by cellular dehydrogenases into an orange formazan product, which is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of viable cells. Cell counting kit-8 solution was analyzed by measuring absorbance ($\lambda_{\text{max}} = 450 \text{ nm}$) using the same spectrofluorimeter mentioned above.

Flow cytometry and immunofluorescence staining

In both assays, fibroblasts and myofibroblasts (1×10^6 cells/well) were seeded and cultured in 6-well plates in the presence of 50 $\mu\text{g/mL}$ of NC, ColNC, or Col for 1, 4, or 7 days. For immunofluorescence staining, after exposure to the different treatments, cells were then washed with PBS and fixed with 4% paraformaldehyde in $1 \times$ PBS (w/v) for 30 min at 37°C. The cells were then washed with $1 \times$ PBS and permeabilized with 0.5% Triton X-100 (v/v) at 4°C for 10 min. Finally, cells were stained with phalloidin (10 μL in 1 mL of PBS) and DAPI (3 μM in PBS) for 20 and 5 min, respectively. Images were captured with a FV1200 laser scanning microscope (Olympus Corporation, Tokyo, Japan).

For flow cytometry assays, fibroblasts and myofibroblasts were collected and washed with $1 \times$ PBS by centrifugation at 1000 rpm for 5 min on days 1, 4, and 7 following the above treatment. The cells were then fixed and permeabilized as described for immunofluorescence staining. Subsequently, the pellets were washed again and blocked with staining buffer (SB) (5% FBS in PBS) for 15 min at room temperature in the dark. Afterward, the cells were centrifuged at 1000 rpm for 5 min, and the pellets were incubated with anti-SMA antibody (1:200 μL dilution) for 1 h at 4°C in the dark. Finally, the cells were washed and resuspended in 200 μL of SB until analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Evaluation of the efficacy of collagenases in PD plaque size reduction

Tissues obtained in surgical procedures were immediately immersed in ice-cold tissue transportation medium (M-400) and transported to laboratory within 24 h under refrigerated conditions (0–4°C). Immediately after arrival at the laboratory, plaque specimens were divided into portions of 150–250 mm³.

Each piece of tissue was weighed and then immersed in cell culture medium (RPMI 1640) and maintained in sterile conditions. Using a laminar flow hood, plaque portions were injected with Col or ColNC and maintained in incubators at 37°C for 7 days. Col and ColNC were diluted in PBS to obtain a 1 mg/mL concentration. Volume injected was 1 $\mu\text{L}/\text{mg}$ tissue, ie, 0.1 mg collagenase/100 mg tissue or 0.1%. This dose would not be presumably higher than that recommended for each CCH injection with Xiaflex (0.58 mg/plaque). Controls consisted of equivalent volume injections of PBS or NC without collagenase (NC). After the incubation period, plaque fragments were again weighed and both conditioned media and plaque tissues were frozen and stored at -80°C until determination of protein and collagen 1 contents, respectively.

Determination of protein content in conditioned medium

Total protein concentration in media conditioned by PD plaque portions was determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA). The protein content in the medium was normalized by the initial weight of the respective plaque tissue portion.

Measurement of remaining type 1 collagen content in plaque tissue

Total proteins were extracted from plaque tissues by homogenization in T-PER lysis buffer (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. A Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA) was included. Protein extracts were assayed for type 1 collagen determination by using a commercial kit (Cloud-Clone Corp., Katy, TX, USA, cat# SEA571Hu) and following manufacturer's instructions. Type 1 collagen content was normalized by the initial weight of the respective plaque tissue portion.

Immunofluorescence assays

Plaque fragments were cleaned of blood and immersed in saccharose at increasing percentages (10%–30% w/v). After 1 h at the higher percentage of saccharose, tissues were embedded in optimal cutting temperature compound (OCT) and stored at -80°C until used for immunofluorescence assays. Then, OCT blocks were sectioned into 6 μm thick transverse slices using a cryostat and mounted onto polylysine-coated glass slides. For the immunodetection of type 1 collagen, OCT was removed from the sections, which were then fixed in a mixture of acetone and methanol to eliminate autofluorescence. Sections were then incubated with polyclonal rabbit antibody against Type 1A collagen (Invitrogen by ThermoFisher Scientific, Waltham, MA, USA, cat.# PA5-29569, 1:200 dilution) overnight at 4°C. Sections were then washed in 0.3% Triton X-100 in PBS and incubated for 1 h at room temperature with a secondary Alexa Fluor 488-conjugated goat anti-mouse antibody (dilution 1:250; SouthernBiotech, Birmingham, AL, USA, cat.# 1031-30). The sections were prepared for visualization and examined under a fluorescence microscope (Olympus BX51).

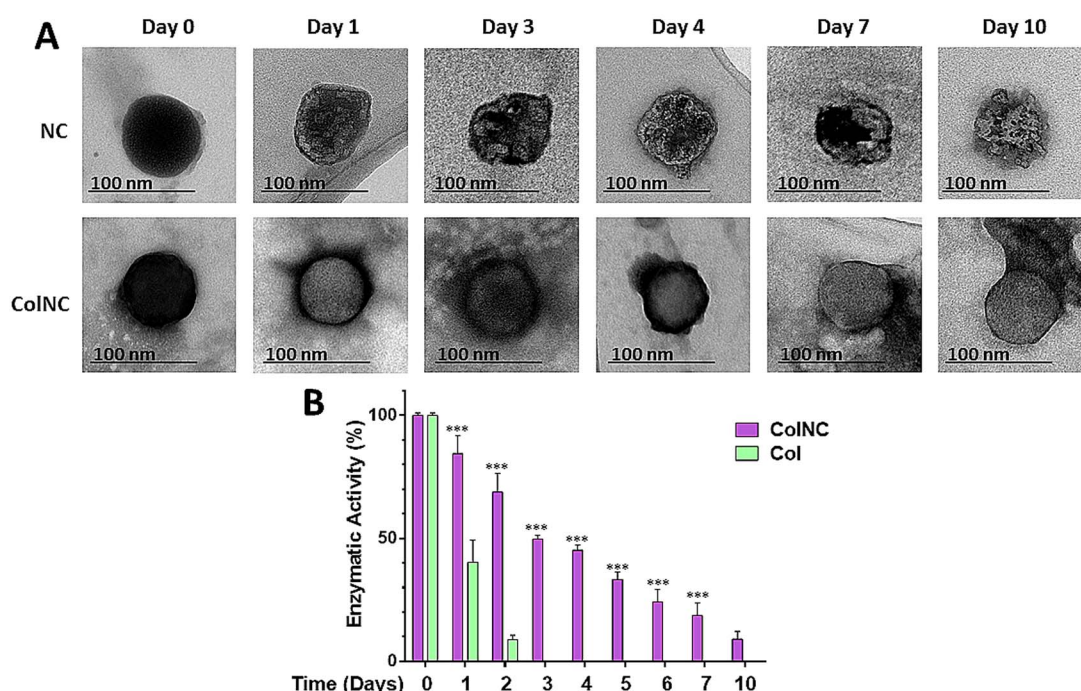


Figure 1 Transmission electron microscopy (TEM) images of collagenase-loaded nanocapsules (CoINC) and empty nanocapsules (NC) incubated under physiological conditions at different times (A). All samples were stained with a 1% PTA solution in water. Stability study comparing CoINC with free collagenase (Col) (B). Means $\pm$ SD were calculated from three independent tests. Statistical significance: *** $P < .001$, compared to Col.

Statistical analysis

Cell culture results and collagenase activity were displayed as the mean $\pm$ SD from three independent experiments. In experiments with human tissues, individual data from each patient and mean $\pm$ SD were provided. Shapiro–Wilk test was used for checking normal distribution of data. Statistical significance was determined by using one-way analysis of variance (ANOVA) followed by Holm–Sidak’s post hoc test for multiple comparisons. For the experiments with human plaque tissues, a mixed-effects model that accounts for repeated measures with missing values was applied, followed by a Tukey’s test for multiple comparisons. $P < .05$ was considered statistically significant across all analyses. Experimental data were analyzed using GraphPad Prism software (8.2 version, GraphPad Software, San Diego, CA, USA).

Results

Characterization and enzymatic activity of collagenase-loaded NC

In Figure 1A, TEM images revealed that the nanosystems exhibited a morphology of spherical NC (NC and CoINC), with an approximate size of 80 nm which and zeta potential values of -7.9 and -14 mV, for NC and CoINC, respectively, as determined by DLS and zeta potential measurements (data not shown). On day 0, both types of NC displayed a well-defined and intact structure. After day 3, a progressive degradation process was observed, characterized by deformations and fragmentation, which was more evident for NC. In contrast, CoINC presented a greater resistance to degradation compared to the empty NC.

Figure 1B showed the enzymatic activity (%) of collagenase over time (days) for CoINC NC and free collagenase (Col). The

enzymatic activity of collagenase was sustained for a longer duration when encapsulated in CoINC compared to its unencapsulated form. Specifically, free collagenase activity decreased to 40% after 24 h and further to 10% at 48 h, becoming undetectable by 72 h. In contrast, collagenase activity within CoINC retained values of 85% and 70% at 24 and 48 h, respectively, and still exhibited 10% activity after 10 days.

Evaluation of the biocompatibility of CoINC and NC

The biocompatibility of collagenase-loaded nanocapsules (CoINC) and empty NC was evaluated through cell viability studies. The effect of these NC was assessed in three cell lines (HDFa, myofibroblasts differentiated from these fibroblasts, and MEF). Cells were seeded in 12-well plates and treated with different concentrations of collagenase (50, 100, and 200 $\mu\text{g/mL}$) present in CoINC and free Col at specific time points (1, 2, 4, and 7 days). For NC lacking collagenase, equivalent volumes to those of CoINC were used.

Studies with HDFa cells showed that cell viability was minimally affected under any of the three conditions studied. For CoINC, no significant decrease was observed at any of the exposure times considered (1, 2, 4, and 7 days) (Figure 2A). This finding was further confirmed in mouse fibroblasts (Figure S1). In contrast, myofibroblast viability was significantly decreased, particularly in the presence of CoINC, reaching 50% after 7 days. Meanwhile, NC produced only a slight reduction in cell viability and free collagenase decreased myofibroblast viability by only 20% (Figure 2B).

Considering that collagenase treatment did not affect fibroblasts but significantly decreased myofibroblast viability, the dose of 50 $\mu\text{g/mL}$ was considered to provide the best risk/benefit ratio. Therefore, this concentration was selected for studies with cell

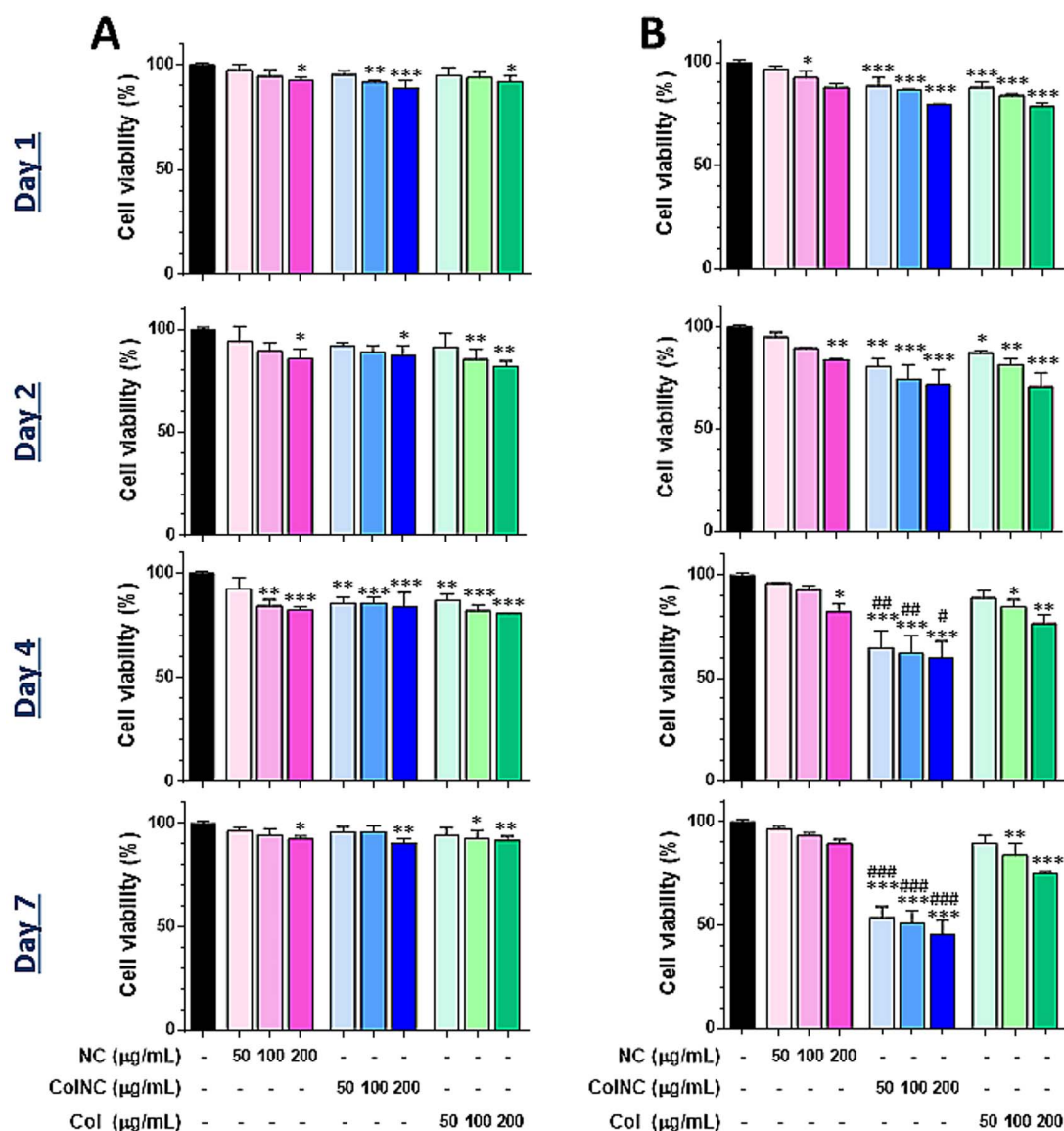


Figure 2 Cell viability in human fibroblasts (HDFa) (A) and myofibroblasts (B) treated with collagenase-containing or empty nanocapsules. Both cell lines were incubated with NC, ColNC, or free Col at different concentrations (50, 100, and 200 µg/mL) for 1, 2, 4, or 7 days. After each time point, cell viability was assessed in all samples. Cells cultured without nanocapsules were used as controls (-). Data are means $\pm$ SD of three independent experiments. Statistical significance: * P < .05, ** P < .01, *** P < .001, compared to control. Samples containing collagenase were also compared with statistical significance denoted by # P < .05, ## P < .01, ### P < .001, compared to Col.

cultures derived from Peyronie's plaque samples obtained from patients, as well as for immune response studies.

Fibroblast-to-myofibroblast differentiation in Peyronie's plaque samples

Fibroblasts extracted from patient plaques were stimulated with 10 ng/mL of TGF- β 1 for 72 h to promote their differentiation into myofibroblasts before conducting the different assays with the NC. Flow cytometry and confocal microscopy were subsequently used to analyze this differentiation (Figure 3). Confocal images demonstrated that fibroblasts changed from a fibroblast-like morphology to a stellate morphology over time after stimulation, with this transformation stabilizing at 72 h (Figure 3A).

To support this observation, flow cytometry studies were performed to assess α -SMA levels in these cells (Figures 3B and S2), which is a characteristic marker of activated myofibroblasts. Figure 3B shows that α -SMA levels increased in fibroblasts from the patient sample following treatment with TGF- β 1, reaching their highest levels at 72 h. Furthermore, an increase in cell size (Figures 3C and S2) and complexity (Figures 3D and S2) was observed over this period, with differentiation being most pronounced at 72 h.

Effects on fibroblasts and myofibroblasts derived from Peyronie's plaque samples

Once differentiation was optimized, cell viability, confocal microscopy, and flow cytometry assays were performed on fibroblasts and myofibroblasts obtained from patient plaques

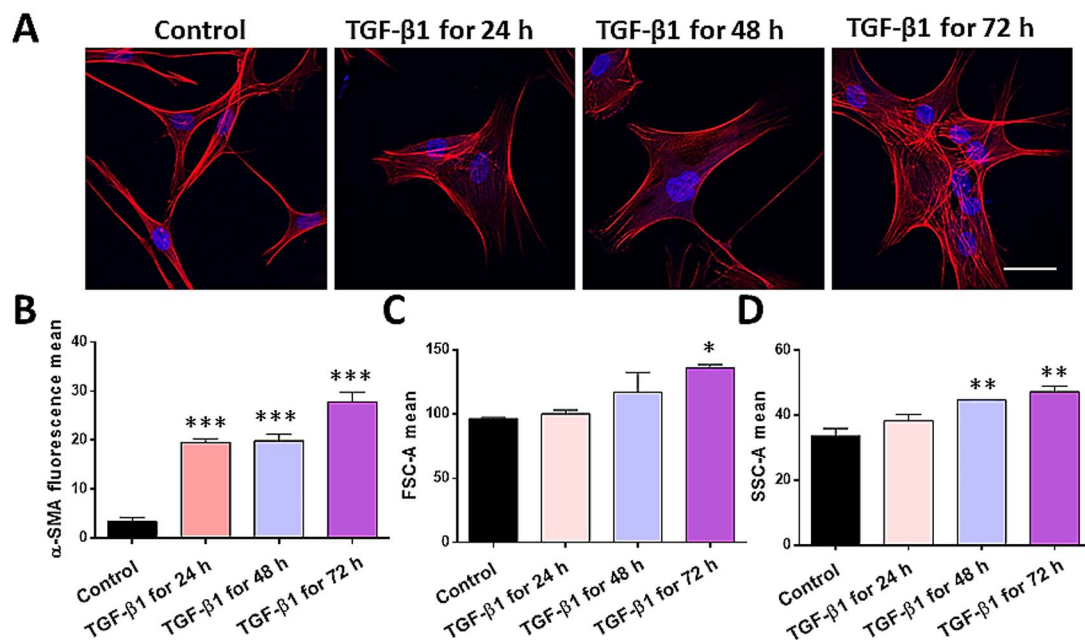


Figure 3 Differentiation of fibroblasts into myofibroblasts obtained from the PD patients' plaques ($n=3$) evaluated by confocal microscopy and flow cytometry after treatment with TGF- β 1 (10 ng/mL) for 24, 48, and 72 h. Morphological changes were analyzed by immunofluorescence staining for F-actin (red) (A). Scale bar = 20 μ m. α -SMA content (B), cell size (C) (FSC-A: forward scatter area), and cell complexity (D) (SSC-A: side scatter area) were evaluated over time. Untreated cells were used as controls (-). Data are means $\pm$ SD of three independent experiments. Statistical significance: * $P < .05$, ** $P < .01$, *** $P < .001$, compared to control.

after exposure to 50 μ g/mL of ColNC, Col, and NC for 1, 4, and 7 days, respectively.

Biocompatibility studies and confocal images showed a minimal effect of all treatments on fibroblasts after 1 or 4 days (Figures S3 and S4A and B), with cell viability recovering by day 7 (Figures 4A, E, and G). On the other hand, flow cytometry studies indicated that α -SMA fluorescence mean in fibroblasts remained constant and unchanged throughout the 7-day period (Figure 4C and S5). In contrast, ColNC treatment in myofibroblasts led to a decrease in viability from the first day (Figures S3 and S4C and D) reaching a reduction of over 75% by day 7, compared to only 20% observed with free collagenase treatment (Figure 4B, F, and H). In addition, α -SMA levels decreased on day 7, which was associated with reduced myofibroblast viability following ColNC treatment (Figure 4D and S5B).

Nanoencapsulation increases efficacy of collagenase to reduce plaque size *in vitro*

Exposure to 0.1% collagenase for 7 days caused a significant reduction in plaque weight *in vitro*. However, the treatment with 0.1% ColNC resulted in greater efficacy in reducing plaque size, yielding a significantly further reduction in plaque weight at 7 days after intra-plaque injection with respect to the effect driven by Col (Figure 5). Treatment with the vehicle (PBS) or the NC did not significantly modify plaque weight (Figure 5).

The significant reduction of plaque exerted by the treatment with ColNC is related to the removal of collagen from the plaque tissue as suggested by the marked loss in type 1 collagen immunodetection in plaque tissue sections treated with ColNC (Figure 6A–D). Immunofluorescence assays show detection of disorganized type 1 collagen fibers in the plaque tissue at 7 days

after injection of PBS (Figure 6A) or NC (Figure 6B). Some collagen fibers are still observable in plaque tissue treated with Col (Figure 6D) while collagen fibers are practically undetectable in plaque tissue treated with ColNC (Figure 6C). This observation supported by immunofluorescence assays is confirmed by the quantification of type 1 collagen content by ELISA in homogenates of plaque tissue. In this sense, only the treatment with ColNC caused a significant decrease in the remaining content of type 1 collagen in plaque tissues at 7 days after injection (Figure 6E). These results suggest enhanced efficacy of ColNC in removing collagen accumulation in PD plaque, thereby promoting plaque size reduction. This idea was further reinforced by the significant increase in protein content in the media conditioned by the plaque tissues treated with ColNC (Figure 6F).

Discussion

The present results evidenced the ability of collagenase nanoencapsulation to preserve collagenase activity over time. We have demonstrated for the first time the evident advantage of using ColNC versus Col, showing a greater efficacy on human tissue samples, as well as the induction of events at the cellular level favorable for a normal healing of the lesions caused by PD.

We were able to generate well-defined spherical NC which were more resistant to degradation when loaded with collagenase. This higher stability may be attributed to the fact that empty NC are more easily hydrolyzed in the surrounding medium and are also susceptible to internal degradation as described for other nanoparticle systems.²³ Results on enzymatic activity confirmed that encapsulation of collagenase in NC significantly improved its enzymatic stability and provided a controlled release of collagenase over time. For the treatment of PD, this

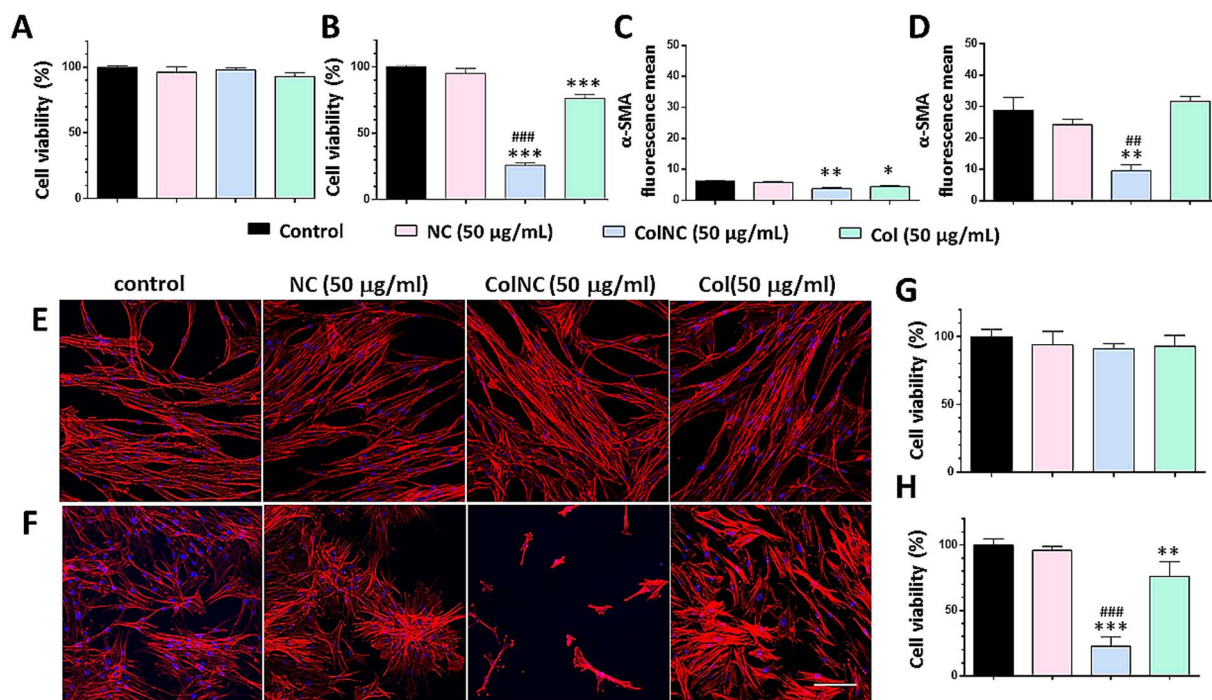


Figure 4 Biocompatibility and α -SMA level studies in fibroblasts and myofibroblasts obtained from the PD patients' plaques ($n = 3$) treated with NC, ColNC, or Col (50 μ g/mL) for 7 days. Cell viability was assessed in fibroblasts (A) and myofibroblasts (B). α -SMA content was also tested in fibroblasts (C) and myofibroblasts (D) by flow cytometry. Representative confocal microscopy images of fibroblasts (E) and myofibroblasts (F) were stained with phalloidin (red) and nuclei with DAPI (blue). Scale bar: 20 μ m. Quantification of fibroblasts (G) and myofibroblasts (H) were performed by using four independent images. Cells cultured without treatment were used as controls (-). Data are means $\pm$ SD of three independent experiments. Statistical significance: * $P < .05$, ** $P < .01$, *** $P < .001$, compared to control. Samples containing collagenase were also compared with statistical significance denoted by ### $P < .01$, ### $P < .001$, compared to Col.

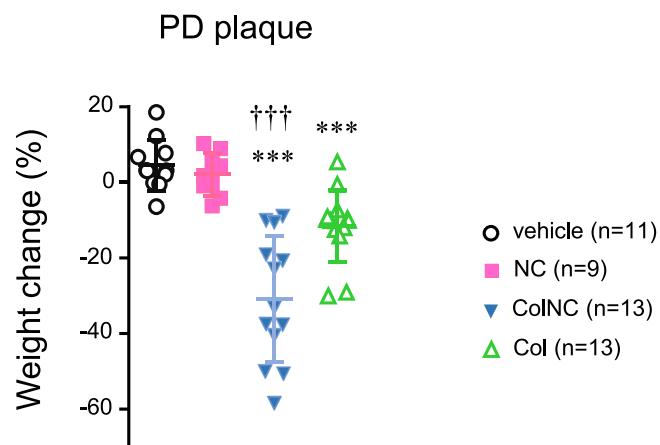


Figure 5 Weight change of plaque tissue obtained from patients with Peyronie's disease (PD) at 7 days after intraplaque injection of vehicle (PBS), empty nanocapsules (NC), nanoencapsulated collagenase (ColNC 0.1%), or free collagenase (Col 0.1%). Individual data and mean $\pm$ SD of the percentage of weight change from baseline are shown. n indicates the number of patients from whom the tissues were collected. *** $P < .001$ vs. vehicle; ††† $P < .001$ vs. Col.

sustained release has the potential to allow prolonged exposure of fibrotic plaques to collagenase, which could reduce the need for repeated injections and improve treatment tolerability. This finding is consistent with previous studies that have shown that degradable polymeric NC are able to protect therapeutic enzymes

and release their contents in a sustained manner at the site of action.²⁰

Prior research has demonstrated that deregulated activation of the TGF- β 1 signaling pathway is closely linked to the progression of PD.²⁴ TGF- β 1 plays a key role in driving the transformation of fibroblasts into myofibroblasts, contractile cells responsible for the overproduction of extracellular matrix (ECM) components, including collagen and fibronectin. Myofibroblasts are major contributors to the development of fibrotic tissue and scar tissue formation.²⁵ The evaluation of the impact of ColNC versus Col on myofibroblast cultures suggests that the selective activity of ColNC towards myofibroblast viability with respect to fibroblast could have important implications regarding the efficacy and safety of intralesional treatment of PD. Fibroblasts are a cell type widely present in penile TA. Maintaining the viability of these cells is critical to the safety of CCH treatment, avoiding potential treatment-related adverse events such as corporal rupture or hematoma formation.²⁶ On the other hand, the persistence of myofibroblasts and the continued production of collagen in PD cases are the main cause of fibrous plaque formation. As mentioned above, myofibroblasts are virtually absent in normal TA, but are very abundant in PD plaque. The marked decrease in cell viability in ColNC-treated myofibroblasts may allow that ColNC would not only be effective in degrading fibrous plaque in the chronic phase of PD, but that its use in the early acute stage could prevent plaque development by inhibiting the proliferation of the cells causing the fibrotic process. The impact of ColNC on myofibroblast viability is supported by the higher efficacy of

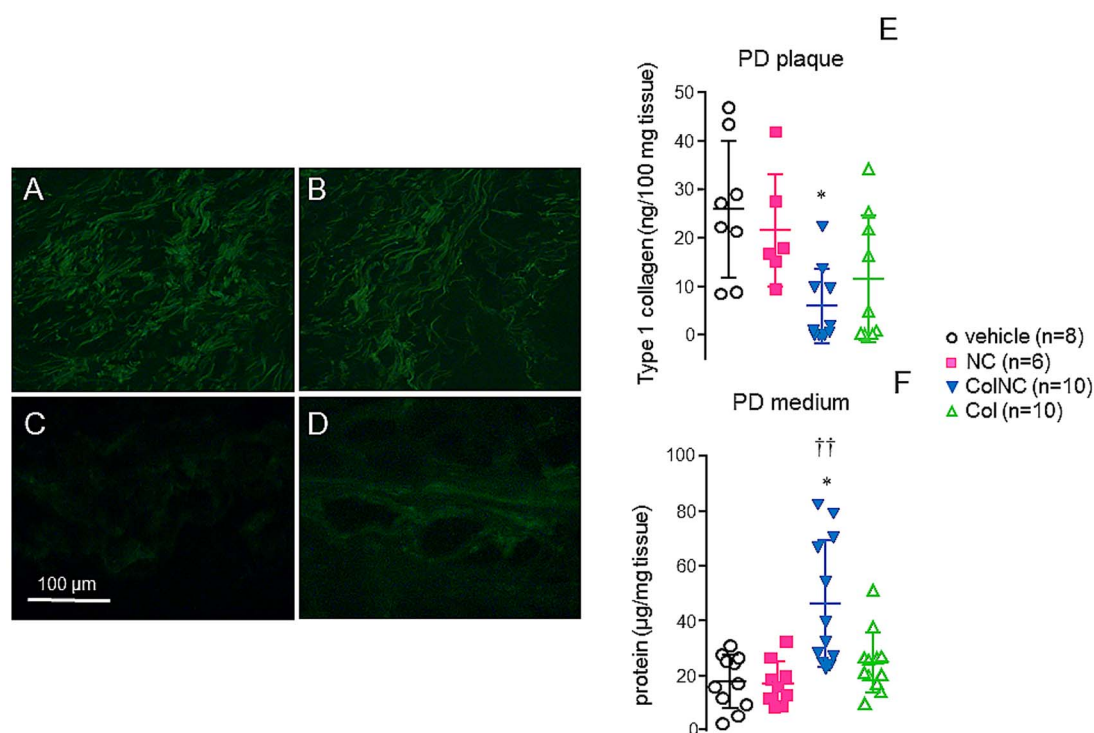


Figure 6 Representative images of immunodetection of type 1 collagen in sections of Peyronie's disease (PD) plaques from PD patients at 7 days after intraplaque injections of vehicle (PBS) (A), NC (B), ColNC (0.1%) (C), or Col (0.1%) (D). Quantification of type 1 collagen content in plaque tissues (E) and protein content in conditioned media (F) after the different treatments. Individual data and mean $\pm$ SD normalized by baseline tissue weight are shown. * $P < .05$ vs. vehicle; †† $P < .01$ vs. Col.

ColNC to reduce α -SMA levels. Activated myofibroblast display abundant presence of enhanced stress fibers, which are part of the actomyosin cytoskeleton and predominantly contain α -SMA, a characteristic marker of myofibroblasts.²⁷ The selective effect of ColNC on myofibroblasts is probably based on the dependence of these cells on the density of collagen in the ECM, as demonstrated by Helary et al. in 2006.²⁸ Thus, the sustained proteolytic activity of collagenase released from ColNC has a marked deleterious effect on the proliferation of myofibroblasts, as well as on the expression of their characteristic spindle shaped morphology after 7 days as can be seen in Figure 4.

The better profile of ColNC to influence cellular alterations related to PD pathophysiology was improved by demonstrating its superiority with respect to Col for reducing the weight of the plaque tissues obtained from patients with PD, 7 days after a single intraplaque injection. This ability to effectively reduce PD plaque was consistent with the higher efficacy of ColNC to remove collagen from plaque tissue that resulted in increased content of peptide derivatives in the medium outside the tissue. In agreement with our observations, previous evidence has shown that *in vitro* exposure of PD plaque tissue to CCH caused a reduction in collagen content and an increase in soluble collagen.²⁹ Although PD plaque may involve accumulation of other ECM proteins in addition to collagen, the observed superiority of ColNC to reduce the weight of the plaque together with the loss of collagen suggests a potential larger dissolution of the PD plaque when the administered collagenase is nanoencapsulated.

We propose that nanoencapsulation of collagenase may allow for a more controlled and sustained release of the enzyme at the injection site, thereby avoiding high local peak concentrations

that could lead to abrupt and excessive weakening of the TA. In addition, nanoparticle-based delivery systems may enhance intralesional retention and reduce nonspecific diffusion into adjacent healthy tissues, potentially limiting collateral degradation of structurally relevant collagen. Furthermore, modulation of the release profile may reduce the effective maximum concentration (C_{max}) of active collagenase, which could be a contributing factor in adverse events such as corporal rupture. Depending on their design, certain nanocarriers may also exhibit preferential interaction with fibrotic ECM components, further supporting a more localized therapeutic effect. Importantly, we acknowledge that there is currently no direct clinical evidence demonstrating a reduction in penile fracture rates with nanoparticle-based CCH formulations. Therefore, this concept should be interpreted as a biologically plausible hypothesis that warrants further investigation in preclinical and clinical studies.

In summary, nanoencapsulation of collagenase offers enhanced capacity of decreasing myofibroblast content and inflammatory response, two key events in PD pathophysiology.³⁰ Furthermore, nanoencapsulation increases the efficacy of collagenase injection to reduce human plaque size and collagen content *in vitro*. Certainly, *in vitro* evidence confers limitations to the clinical translation of the present study. For instance, the *in vitro* explant lacks systemic blood flow, vascularization, macrophage clearance, immune response, and biomechanical factors present in the *in vivo* environment. In a living patient, the immune system might clear the NC faster than the 10 days observed in the laboratory. On the other hand, despite former approval of CCH for PD, new formulations with nanoparticle-based delivery systems need additional efficacy and safety evaluations to be considered by

drug regulatory institutions. In addition, the relatively small sample size may limit the generalizability of the present findings. However, the use of multiple approaches and the utilization of human target tissue from patients suffering from PD support potential therapeutic relevance of the results reported here. In this sense, although CCH injections generates treatment satisfaction in an important percentage of PD patients,³¹ even with superiority with respect to surgery,³² a significant number of patients discontinue CCH treatment before completing the prescribed cycles of administrations.³³ This is important because the therapeutic response increases when the number of cycles administered progresses.³⁴ Furthermore, patients treated with CCH therapy may require up to eight injections, followed by penile remodeling to break down collagen fibers, which is time consuming, very painful and increases the risk of penile fracture. Furthermore, the higher stability and controlled release of collagenase achieved through nanoencapsulation, could help reduce the number of repeated injections required in the treatment of PD with CCH, thereby potentially decreasing treatment discontinuation rates, improving therapeutic response and reducing the risk of adverse events, including penile fracture.

Conclusions

In the present study, polymeric nanosystems in the form of NC containing collagenase have been prepared for the intralesional treatment of PD. For the first time, the efficacy of these NC has been studied with fibrotic plaques extracted from human patients undergoing surgical intervention. Our study suggests that encapsulation of collagenase protects the enzyme and increases its period of activity, which translates into a substantial improvement of its *in vitro* efficacy in the degradation of collagen that constitutes the fibrotic plaque. On the other hand, the treatment of PD-derived cells with nanoencapsulated collagenase shows a decrease in the cell viability of myofibroblasts, as well as a decrease in the production of enhanced stress fibers, without affecting the viability of fibroblasts. These results suggest a potential therapeutic benefit of nanoencapsulation of collagenase in the treatment of PD.

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Supplementary material

Supplementary material is available at *The Journal of Sexual Medicine* online.

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Conflicts of interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this paper.

Ethics approval

The study followed the principles outlined in the Declaration of Helsinki. Protocol and consent form were approved by the Ethics Committee at Puerta de Hierro-Majadahonda University Hospital (PI 50/20, 9 May, 2020).

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