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New basic insights on the potential of chitosan-based medical device for improving functional recovery after radical prostatectomy

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NEW BASIC INSIGHTS ON THE POTENTIAL OF CHITOSAN-BASED MEDICAL DEVICE FOR IMPROVING FUNCTIONAL RECOVERY AFTER RADICAL PROSTATECTOMY.

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Keywords:	chitosan, autonomic ganglia, prostate cancer, nerve regeneration

Abstract:	Objectives: To study the promising effects on potency recovery in patient that undergo nerve sparing radical prostatectomy of the use of chitosan membranes (CS-Me) aimed to protect neurovascular bundles. The aim of this study was two-fold: (1) to evaluate the neuro-regenerative potential of CS-Me on autonomic neurons; (2) to rule out the possibility that a pro-regenerative biomaterial could stimulate the proliferation of prostate cancer cell lines. Material and methods: As regards the neuro-regenerative potential, primary organotypic cultures derived from sympathetic ganglia were cultured on CS-Me over 3 days and neurite extension and axonal sprouting were evaluated. As regards the effects of chitosan (CS) on cancer cells, different human prostate cancer cell lines (PC3, DU-145, LN-Cap) were seeded on CS-coated plates or cultured in the presence of CS-Me dissolution products. Results: Results showed that the CS coating as well as the dissolution products of CS-Me lead to a significantly lower proliferation rate of prostate cancer cell lines. Concerning the pro-regenerative effects on autonomic ganglia, results showed that CS-Me significantly stimulate axon elongation in comparison to control conditions. Conclusion: Altogether, results of this study provide experimental data in support of the safety of CS-Me's clinical employment and of their potential efficacy for improving functional recovery after radical prostatectomy. Yet, our results provide the first experimental evidence that CS-Me stimulate posttraumatic

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	axon regrowth of autonomic neurons.

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NEW BASIC INSIGHTS ON THE POTENTIAL OF CHITOSAN-BASED MEDICAL DEVICE FOR IMPROVING FUNCTIONAL RECOVERY AFTER RADICAL PROSTATECTOMY.

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ABSTRACT

Objectives:

To study the promising effects on potency recovery in patient that undergo nerve sparing radical prostatectomy of the use of chitosan membranes (CS-Me) aimed to protect neurovascular bundles. The aim of this study was two-fold: (1) to evaluate the neuro-regenerative potential of CS-Me on autonomic neurons; (2) to rule out the possibility that a pro-regenerative biomaterial could stimulate the proliferation of prostate cancer cell lines.

Material and methods:

As regards the neuro-regenerative potential, primary organotypic cultures derived from sympathetic ganglia were cultured on CS-Me over 3 days and neurite extension and axonal sprouting were evaluated.

As regards the effects of chitosan (CS) on cancer cells, different human prostate cancer cell lines (PC3, DU-145, LN-Cap) were seeded on CS-coated plates or cultured in the presence of CS-Me dissolution products.

Results:

Results showed that the CS coating as well as the dissolution products of CS-Me lead to a significantly lower proliferation rate of prostate cancer cell lines. Concerning the pro-regenerative effects on autonomic ganglia, results showed that CS-Me significantly stimulate axon elongation in comparison to control conditions.

Conclusion:

Altogether, results of this study provide experimental data in support of the safety of CS-Me's clinical employment and of their potential efficacy for improving functional recovery after radical prostatectomy. Yet, our results provide the first experimental evidence that CS-Me stimulate posttraumatic axon regrowth of autonomic neurons.

Keywords: *chitosan, autonomic ganglia, prostate cancer, nerve regeneration*

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1. INTRODUCTION

Prostate cancer is the most common cancer among men. An incidence of 214/1000 cases is registered in Europe, surpassing the number of the lung and colorectal cancers (1) and representing the second leading cause of cancer mortality (2).

The current most popular treatment of localized prostate cancer in patients with a life expectancy longer than 10 years is radical prostatectomy (European Association of Urology Guidelines on Prostate Cancer, 2017 update). Unfortunately, in patients who undergo radical prostatectomy, frequently iatrogenic damage to the peri-prostatic neurovascular bundles (NVB) occurs, leading to erectile dysfunction (3).

There have been many efforts with the intent to promote a more rapid and efficient recovery of functions (namely potency and continence) after radical prostatectomy. Patel and colleagues conducted a pivotal study aimed to determine if the use of dehydrated human amnion/chorion membrane (dHACM) allograft wrapped around the NVB during robot-assisted radical prostatectomy is able to accelerate the return to the normal functionality. Interestingly, the authors could demonstrate that the application of dHACM in patients accelerates the recovery of potency and continence, compared to patients who did not receive the membranes (4).

Recently, the application on the neurovascular bundles after nerve-sparing robot-assisted radical prostatectomy of membranes made of another biomaterial of natural origin, namely the chitosan, has been reported to be safe. Authors also appreciated encouraging results regarding potency recovery (5).

Starting from the promising clinical evidences, the present study aims were divided into two different perspectives: 1) to investigate the neuro-regenerative effect of CS-Me on *ex vivo* cultures of autonomic ganglia; 2) to rule out that chitosan employment in oncological surgery might have negative side effects by promoting prostatic cancer cell growth.

2. MATERIALS AND METHODS

2.1. Membranes preparation

CS-Me were produced from highly purified chitosan with a DA of 5% (Altakitin S.A.) as previously described (6). Briefly, CS was dissolved in 0.75% acetic acid to obtain a 1.5% solution, filtered, and poured into Petri dishes, followed by drying at room temperature. The resulting films were treated with a solution of ammonia in methanol/water, followed by intense washing with

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distilled water, and drying. Finally, the films were cut into the required size and sterilized by ethylene oxide.

2.2. Analysis of the regenerative potential of CS-Me on cultures of autonomic ganglia

2.2.1Autonomic ganglia dissection

For this study adult male Wistar rats (Envigo, Udine, Italy) weighing approximately 190-220g were used. All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/ EEC). Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress.

Animals were sacrificed by lethal injection of the anesthetic solution with Tiletamine + Zolazepam (Zoletil) i.m. (3 mg/kg).

Rats were placed under surgical microscope with the ventral side facing up and the caudal end oriented toward you. Using forceps neck skin and muscles were removed allowing to reach the carotid artery that runs alongside trachea. Carotid bifurcation at C2-C3 level, represents an important landmark to identify the superior cervical ganglia that appears as an almond-shaped structure surrounded by connective tissue capsule closely attached to the artery. Once the ganglion is identified, using fine forceps it can be dissected. Following the sympathetic trunk along the toracic level it is possible to identify the stellate ganglion, the cervicothoracic, a large ganglion probably formed by the fusion of the lower two cervicals and the first thoracic ganglia.

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2.2.2 Autonomic explants ganglia cultures

The experiment were performed using the organotypic cultures of superior cervical and stellate ganglia (cervicothoracic ganglion) harvested as a model for the sympathetic nervous segment.

The autonomic explants were cultured on two different substrate: chitosan membrane and glass coverslips (control).

After harvesting, the connective tissue capsule around the ganglia was removed and each ganglion was half cut to aid the attachment on the CS-Me , a 50 µl drop of Matrigel in F12 medium (50% v/v) was applied on the substrate before seeding the explants. After 2 hours of incubation at 37°C, SFM (7) culture medium with nerve grow factor (NGF, 50ng/ml) was slowly added to the plate.

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2.2.3. Immunofluorescence

After 3 days of culture, autonomic explants were fixed in 4% PFA for 15 min, washed in 0.1 M phosphate buffer (pH 7.2) and processed for immunofluorescence analysis.

Samples were permeabilized, blocked [0.1% triton X-100, 10% normal goat serum (NGS)/0.1% NaN_3, 1h] and incubated O/N in anti-βIII-Tubulin (mouse, monoclonal, 1:1000, Sigma-Aldrich) primary antibody; after incubation with secondary antibody goat anti-mouse IgG Alexa-Fluor-488-conjugated (1:200, Molecular Probes, Eugene, Oregon) for 1 hour, autonomic explants were mounted with a Dako fluorescent mounting medium.

2.2.4. Quantification of neurite outgrowth

Autonomic ganglia were scanned using a Zeiss LSM800 confocal laser microscopy system (Zeiss, Jena, Germany). For each sample, confocal Z-stacks were used and different images (10X) were captured in order to reconstruct the entire ganglia.

In order to evaluate the regenerative capability of the autonomic ganglia on the chitosan membranes, Neurite J (ImageJ plugin) was used and two different parameters were evaluated: neurite extension and axonal sprouting (8).

2.3. In vitro cell tests on CS based membranes

In vitro cell tests were performed using LN-Cap, DU-145 and PC3 cell lines. The cytotoxicity test was carried out with the dissolution products of CS-Me, while LN-Cap, DU-145 and PC3 adhesion, proliferation and protein expression were evaluated on a CS coating, as detailed below.

2.3.1. Dissolution products of CS-Me

The effect of the CS-Me-based material extracts was studied on LN-Cap, DU-145 and PC3 cell lines. Material extracts were prepared by incubating CS-Me in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12, Sigma Aldrich) supplemented with 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), 1 mM sodiumpyruvate (Sigma), 4 mM L-glutamine (Sigma) and stored at 37°C in a humidified atmosphere of 5% CO_2 for 13 days. As control media, samples of culture medium without CS were maintained in the same conditions as CS-Me samples and then collected after 15 days. Then, the proliferation assay was carried out using collected media. In detail, LN-Cap, DU-145 and PC3 cells were seeded and cultivated in the previously prepared extract media, at a density of $5x10^3$ cells/cm^2 on Petri dishes. After 1, 3, and 6 days in vitro (DIV), cells were trypsinized and counted in a Burker's hemocytometer chamber. Experiments were

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performed as technical triplicates. The counts obtained from assays were analyzed, averaged and expressed as logarithmic scale of viable cells/mm^2 ± SD.

2.3.2. Proliferation assay on CS coating

Culture wells were coated with a solution of 2 mg/ml chitosan in 0.1% acetic acid (EMD Biosciences) or with 0.1% acetic acid alone. Briefly, the solutions were distributed in an excess volume into each well to ensure the entire surface area was covered. Plates were placed at 4°C overnight. The next day, prior to plating prostate cancer cells, remaining chitosan or acetic acid solution was aspirated. Then the proliferation assay was carried out using LN-Cap, DU-145 and PC3 cells. Cell lines were seeded and cultivated in RPMI medium supplemented with 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), 1 mM sodiumpyruvate (Sigma), 4 mM L-glutamine (Sigma) and 2% fetal bovine serum (FBS), at a density of 5x10^3 cells/cm^2 on coated Petri dishes. After 2, 5, and 7 days in vitro (DIV), cells were trypsinized and counted in a Burker's hemocytometer chamber. Experiments were performed as technical triplicates.

2.3.3. Total protein extraction, and western blot

Total proteins were extracted by solubilizing cells in boiling Laemmli buffer (2.5% SDS and 0.125 M Tris-HCl pH 6.8), followed by 3 min at 100°C. The protein concentration was determined by the BCA method, and equal amounts of proteins (denaturated at 100°C in 240 mM 2-mercapto ethanol and 18% glycerol) were loaded onto each lane, separated by SDS-PAGE, transferred to a Hybond-C Extra membrane and blocked for 1 h at 37°C in 1× TBST (150 mM NaCl, 10 mM Tris–HCl (pH 7.4), and 0.1% Tween) plus 5% non-fat milk. Membranes were incubated overnight at 4°C in primary antibodies diluted in TBST plus 1% non-fat milk. The day after, they were rinsed four times with TBST for 5 min each at room temperature and incubated for 1 h at room temperature with peroxidase-linked secondary antibody (diluted in TBST plus 1% non-fat milk). Membranes were washed 4 times, 5 min each, with TBST at room temperature, and specific binding was detected by the enhanced chemiluminescence ECL system (Amersham Biosciences) using Hyperfilm™ (Amersham Biosciences).

Primary and secondary antibodies used are: rabbit polyclonal anti-Bcl2 (1:500, sc-492, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-actin (1:4.000, #A5316, Sigma); horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:10.000, Amersham Biosciences), sheep anti-mouse secondary antibody (1:100.000, Amersham Biosciences).

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2.4 Statistical analysis

For *in vitro* experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) and *post hoc* Bonferroni .

For explants ganglia neurites quantification, statistical analysis was performed using Two-Sample t-Test. Statistical analysis were performed using SPSS Software.

The level of significance was set at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Values were expressed as mean ± standard deviation (SD).

3. RESULTS

3.1. CS-Me represent a permissive substrate for neurite regeneration and axonal elongation of autonomic explants ganglia

To evaluate the pro-regenerative capability of CS-Me, we cultured autonomic explant ganglia over 3 days and determined the numbers of extending neurites at certain distance from the explant body according to the Sholl method (8).

Despite the neurites sprouting in both culture conditions is very enhanced (Figure6), it is possible to observe from the graphs (Figure1) that autonomic explant ganglia cultured on CS-Me exhibit an increased neurites length compared to control condition.

Morphological analysis on explants ganglia stained with βIII-Tubulin showed a high neurites outgrowth and a strong neurites arborization supporting the pro-regenerative effect of chitosan membrane (Figure 2).

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3.2. Dissolution products of CS-Me negatively affect proliferation of LN-Cap, DU-145 and PC3 cell lines

The proliferation of three different human prostate cancer cell lines: DU-145, LN-Cap and PC3 cultured in medium containing dissolution products of chitosan membranes was evaluated. After 15 days of CS film dissolution at a temperature of 37°C, media collected were used to cultivate three cell lines and proliferation assays were carried out. Cells were plated and cultured in presence of conditioned media and their proliferation was evaluated after 1, 3 and 6 days. As control media, samples of culture medium without dissolution products of chitosan membranes maintained in the same conditions were collected after 15 days. The proliferation assay was performed after 1, 3 and 6 days on DU-145, LN-Cap and PC3 cells cultured with this control condition medium.

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Results showed that cancer cells grown in conditioned medium with the dissolution products from CS-Me exibit a significant ($p < 0.05$) lower proliferation compared to control medium. (Figure 3).

3.3. CS coating direct contact with human prostate cancer cell lines: LN-CaP, DU-145 and PC3 substantially alter their morphology and proliferation rate

The anti-proliferative effect of CS was tested on three different human prostate cancer cell lines: DU-145, LN-Cap and PC3. Cells were plated in three different experimental condition: control condition, chitosan coating, acetic acid coating (the latter represents a control of the toxicity, since it is the substance in which CS is dissolved) and the proliferation assay was performed after 2, 5 and 7 days.

All cell lines tested have shown a significant reduction in proliferation when grown on the substrate represented by CS. No interference with proliferation was found by cultivating the cells in the presence of a coating of acetic acid at 0.1%. (Figure 4). Cell morphology reflects the results of the proliferation experiments. Cultured cells on the CS substrate lose their normal morphology and adhesion capacity, they appear rounded and form clusters (Figure 5).

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3.4. CS coating affects the expression of BAX and BCL2 proteins suggesting the activation of a pro-apoptotic program

BAX and BCL2 protein expression changes were evaluated on the prostate cancer cell lines: DU-145, LN-Cap and PC3 cells, to study pro-apoptotic and cell survival signaling after 2, 5 and 7 days of culture at three different experimental conditions, control substrate, acetic acid coating, CS coating. In cells cultivated on CS-coated substrates it is possible to observe an increase in the Bax pro-apoptotic protein expression and a simultaneous decrease in anti-apoptotic Bcl2 at the 7 days experimental time point (Figure 6). This pattern of expression suggests an activation of the CS inducted pro-apoptotic program.

4. DISCUSSION

Chitosan (CS) is a derivative of chitin, obtained from the exoskeleton of crustaceans, which is achieving significant interest both in basic research and in clinical settings. Its chemical structure (polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine linked with β (1-4) bond) gives it a remarkable hypoallergenicity, making it an excellent candidate for the development of innovative applications in the field of medicine and surgery, also thanks to its biocompatibility,

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bioavailability and lack of toxicity. For these reasons, CS potential clinical applications range from orthopedic and drug-delivery systems, to scaffolds for regeneration of nerve, skin, bone and cartilage (9-14).

The aim of this of this study was two-fold: (1) addressing the neuro-regenerative potential of chitosan membranes on autonomic ganglia; (2) excluding the possibility that a pro-regenerative biomaterial stimulates cell proliferation in a tumor site.

As regards the first study aim, we have used organotipic cultures that allow to obtain a multicellular *ex vivo* model that preserves both the cytoarchitecture of the tissue and the interactions among cells, providing a closer approximation to *in vivo* conditions (15, 16). Since the prostatic plexus is innervated by autonomic nerve fibers, the regenerative potential of chitosan membrane was assessed through organotipic sympathetic ganglion cultures that represents an innovative and original experimental model for testing autonomic nervous system regenerative properties.

We demonstrated, for the first time in the literature, that CS-Me not only exert a pro-regenerative effect on somatic nervous ganglia (6, 14) but also on autonomic nervous ganglia. This observation is important since it provides a possible mechanistic explanation about the positive effects of CS-Me application on functional recovery after radical prostatectomy (5), namely that the faster recovery of potency is due to the post-surgical stimulation of axonal regrowth in the peri-prostatic neurovascular bundles.

The second aim of this study, regarded the safety of the employment of CS-Me in surgical oncology of prostate cancer. Given the intrinsic cell supportive capacity of CS we wanted to rule out that CS might have any negative side due to a proliferative stimulation of prostate cancer cells. Alongside the pro-regenerative effects, of great interest is the anti-proliferative activity of CS reported on different tumor cell lines. Indeed, the anticancer activity of CS was proved in human breast cancer cell lines (17), in a human gastric carcinoma cell line MGC803 and in a human monocytic leukemia cell line (18, 19). Gibot and colleagues reported a study in which demonstrated the mechanisms underlying the anti-proliferative effect of CS on human melanoma cell lines, suggesting a cell line dependent effect on the apoptosis and proposing it as a future instrument for assessing cancer therapies in the field of melanoma (20). The tumor cells used in this work are cell lines derived from prostate adenocarcinomas: lymph node, brain and bone metastasis, respectively LN-Cap, DU-145 and PC3 cells [9-11].

We wanted to test the effect of CS-Me dissolution products on the proliferation of tumor cells. It is known from the literature that CS products are able to stimulate regeneration and therefore the positive effect on the neuronal population is not only due to direct contact with the material but also

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from its degradation (21). Interestingly, we observed that cancer cells grown in conditioned medium with the dissolution products from CS-Me exhibit a significantly lower proliferation compared to control medium.

The direct contact of the CS with cancer cell lines was tested with the concern of the proliferation, morphology and protein expression of apoptotic markers. This experiments revealed a significant reduction in proliferation of the prostate cancer cell lines grown on the CS substrate. The analysis of the cell morphology also reflected this aspect: cultured cells on the CS substrate lose their normal morphology and adhesion capacity, appearing rounded and forming clusters. From the molecular point of view CS-coated substrates led to an increase in the Bax pro-apoptotic protein expression and a simultaneous decrease in anti-apoptotic Bcl2 at the 7 days experimental time point in all cancer cells tested. This pattern of expression suggests an activation of the CS inducted pro-apoptotic program.

Prostate cancer, whose surgical therapy, radical prostatectomy, remains gold standard therapy today, is the tumor that has an increasing incidence in the male every year, exceeding the number of lung cancer and colorectal cancer [1]. Following the removal of the prostate, the neurovascular bundles may be damaged, leading to functional deficits such as impotence and incontinence. There have been many clinical research efforts designed to minimize side effects and limit residual functional deficiencies [3]. Recently, Porpiglia and colleagues reported the preliminary results of a clinical trial in which they tested chitosan, already known for its effectiveness in promoting nerve regeneration (6), in form of membranes aimed to protect the NVB following radical prostatectomy (5). They showed the feasibility of the application and the safety of the material, meanwhile observing a promising effect on the recovery of the potency in the patients.

Taken together, results of this study provide a strong evidence, from basic science point of view, in favour of the previous promising clinical results and further support the view that application of CS-Me can be a simple, safe and effective strategy to protect the periprostatic plexus following radical prostatectomy. Yet, these results provide the first experimental evidence about the mechanism of action which underlies the effects of CS-Me on autonomic neurons, namely the promotion of posttraumatic axon regrowth, a key factor in neuro-regenerative processes.

Acknowledgments

We thank Monarch Bioimplants for supplying CS-Me for the study of autonomic neurons regeneration and for the dissolution products study on prostate cancer cell lines.

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Conflicts of Interest

Stefano Geuna and Francesco Porpiglia are scientific consultant for Monarch Bioimplants.

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Figure legends

Figure 1. Quantification of sprouting and neurites elongation.

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Bar Graph and line graph depicting quantification of organotipic autonomic ganglia cultured on CS-Me (red) and control condition (blue).

Figure2. Morphology.

Axonal outgrowth of organotipic autonomic ganglia cultured, stained with anti-β tubulin, on CS-Me (B-D) and control substrate represented by glass slides (A-C).

Figure 3. Effects of dissolution products of CS-Me. Line graph depicting the proliferation of different human prostate cancer cell lines: LN CaP, DU-145 and PC3 cultured in medium collected after 15 days of CS film dissolution.*: $p < 0.05$.

Figure 4. Effects of cell direct contact with CS coating. Proliferation curve experiment: LN-Cap, Du-145 and PC3 cell lines were cultivated on the control, on 0.1% acetic acid coating and on CS coating. Results showed a significant lower proliferation for cell lines cultured on Chitosan. $^{\$/\#}$: $p < 0.05$; $^{\$\$/\#\#}$: $p \leq 0.01$; $^{\$\$\$/\#\#\#}$: $p \leq 0.001$. $ indicates the comparison between CTR and CS coating treatment; # indicates the comparison between acetic acid coating and CS coating treatment.

Figure 5. Effects of cell direct contact with CS coating. Morphology: Representative panel depicting PC3 cell lines cultivated on the control, on chitosan coating and on 0.1% acetic acid coating. Cultured cells on the CS substrate lose their normal morphology and adhesion capacity, they appear rounded and form clusters.

Figure 6. Protein expression analysis. A representative Western blot analysis performed on PC3 cell line cultured in the three experimental conditions showed, at days 5 and 7 of CS coating, an over-expression of the pro-apoptotic protein BAX and a simultaneous down-regulation of BCL2, an anti-apoptotic marker.

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1. Boyle P, Ferlay J. Mortality and survival in breast and colorectal cancer. Nat Clin Pract Oncol. 2005 Sep;2(9):424-5.
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008 Mar-Apr;58(2):71-96.
3. Porpiglia F, Fiori C, Bertolo R, Manfredi M, Mele F, Checcucci E, et al. Five-year Outcomes for a Prospective Randomised Controlled Trial Comparing Laparoscopic and Robot-assisted Radical Prostatectomy. Eur Urol Focus. 2016 Nov 23.
4. Patel VR, Samavedi S, Bates AS, Kumar A, Coelho R, Rocco B, et al. Dehydrated Human Amnion/Chorion Membrane Allograft Nerve Wrap Around the Prostatic Neurovascular Bundle Accelerates Early Return to Continence and Potency Following Robot-assisted Radical Prostatectomy: Propensity Score-matched Analysis. Eur Urol. Jun;67(6):977-80.
5. Porpiglia F, Bertolo R, Fiori C, Manfredi M, De Cillis S, Geuna S. Chitosan membranes applied on the prostatic neurovascular bundles after nerve-sparing robot-assisted radical prostatectomy: a phase II study. BJU Int. 2017 Jul 15.
6. Wrobel S, Serra SC, Ribeiro-Samy S, Sousa N, Heimann C, Barwig C, et al. In vitro evaluation of cell-seeded chitosan films for peripheral nerve tissue engineering. Tissue Eng Part A. 2014 Sep;20(17-18):2339-49.
7. Fueshko S, Wray S. LHRH cells migrate on peripherin fibers in embryonic olfactory explant cultures: an in vitro model for neurophilic neuronal migration. Dev Biol. 1994 Nov;166(1):331-48.
8. Torres-Espin A, Santos D, Gonzalez-Perez F, del Valle J, Navarro X. Neurite-J: an image-J plug-in for axonal growth analysis in organotypic cultures. J Neurosci Methods. 2014 Oct 30;236:26-39.
9. Martins EA, Michelacci YM, Baccarin RY, Cogliati B, Silva LC. Evaluation of chitosan-GP hydrogel biocompatibility in osteochondral defects: an experimental approach. BMC Vet Res. 2014 Aug 27;10:197.
10. Xu B, Jiang G, Yu W, Liu D, Liu Y, Kong X, et al. Preparation of poly(lactic-co-glycolic acid) and chitosan composite nanocarriers via electrostatic self assembly for oral delivery of insulin. Mater Sci Eng C Mater Biol Appl. 2017 Sep 01;78:420-8.
11. Haastert-Talini K, Geuna S, Dahlin LB, Meyer C, Stenberg L, Freier T, et al. Chitosan tubes of varying degrees of acetylation for bridging peripheral nerve defects. Biomaterials. 2013 Dec;34(38):9886-904.
12. Brown MA, Daya MR, Worley JA. Experience with chitosan dressings in a civilian EMS system. J Emerg Med. 2009 Jul;37(1):1-7.
13. Fregnan F, Ciglieri E, Tos P, Crosio A, Ciardelli G, Ruini F, et al. Chitosan crosslinked flat scaffolds for peripheral nerve regeneration. Biomed Mater. 2016 Aug 10;11(4):045010.
14. Meyer C, Stenberg L, Gonzalez-Perez F, Wrobel S, Ronchi G, Udina E, et al. Chitosan-film enhanced chitosan nerve guides for long-distance regeneration of peripheral nerves. Biomaterials. 2016 Jan;76:33-51.
15. Geuna S, Raimondo S, Fregnan F, Haastert-Talini K, Grothe C. In vitro models for peripheral nerve regeneration. Eur J Neurosci. 2016 Feb;43(3):287-96.
16. Morano M, Wrobel S, Fregnan F, Ziv-Polat O, Shahar A, Ratzka A, et al. Nanotechnology versus stem cell engineering: in vitro comparison of neurite inductive potentials. Int J Nanomedicine. 2014;9:5289-306.
17. Jiang M, Ouyang H, Ruan P, Zhao H, Pi Z, Huang S, et al. Chitosan derivatives inhibit cell proliferation and induce apoptosis in breast cancer cells. Anticancer Res. 2011 Apr;31(4):1321-8.
18. Qi LF, Xu ZR, Li Y, Jiang X, Han XY. In vitro effects of chitosan nanoparticles on proliferation of human gastric carcinoma cell line MGC803 cells. World J Gastroenterol. 2005 Sep 07;11(33):5136-41.
19. Salah R, Michaud P, Mati F, Harrat Z, Lounici H, Abdi N, et al. Anticancer activity of chemically prepared shrimp low molecular weight chitin evaluation with the human monocyte leukaemia cell line, THP-1. Int J Biol Macromol. 2013 Jan;52:333-9.
20. Gibot L, Chabaud S, Bouhout S, Bolduc S, Auger FA, Moulin VJ. Anticancer properties of chitosan on human melanoma are cell line dependent. Int J Biol Macromol. 2015 Jan;72:370-9.
21. Zhao Y, Wang Y, Gong J, Yang L, Niu C, Ni X, et al. Chitosan degradation products facilitate peripheral nerve regeneration by improving macrophage-constructed microenvironments. Biomaterials. 2017 Jul;134:64-77.

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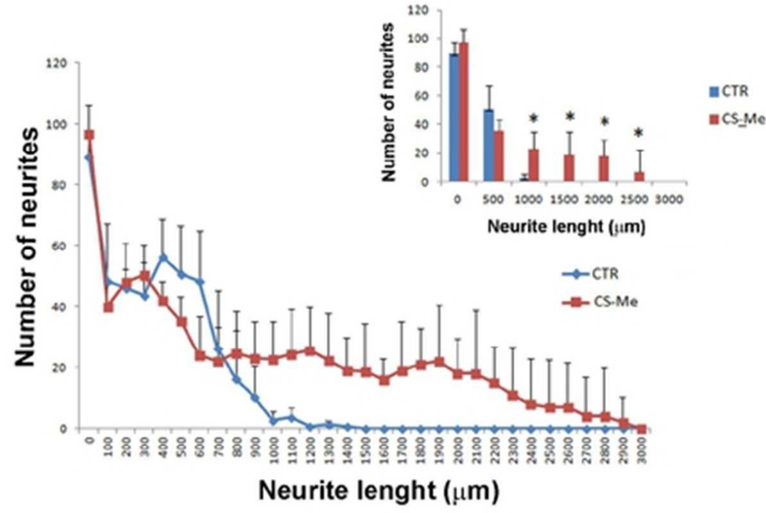

26 Figure 1. Quantification of sprouting and neurites elongation.
27 Bar Graph and line graph depicting quantification of organotipic autonomic ganglia cultured on CS-Me (red)
28 and control condition (blue).
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30 47x27mm (300 x 300 DPI)

Review

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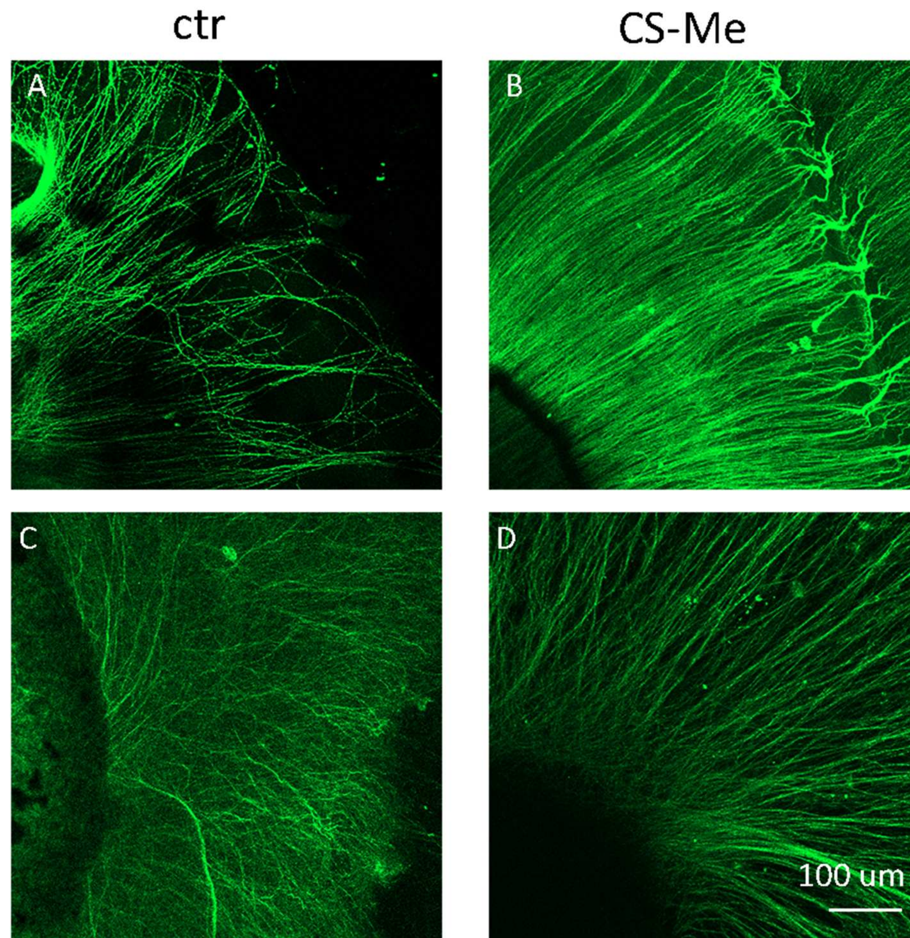

Figure2. Morphology. Axonal outgrowth of organotipic autonomic ganglia cultured, stained with anti-β tubulin, on CS-Me (B-D) and control substrate represented by glass slides (A-C).

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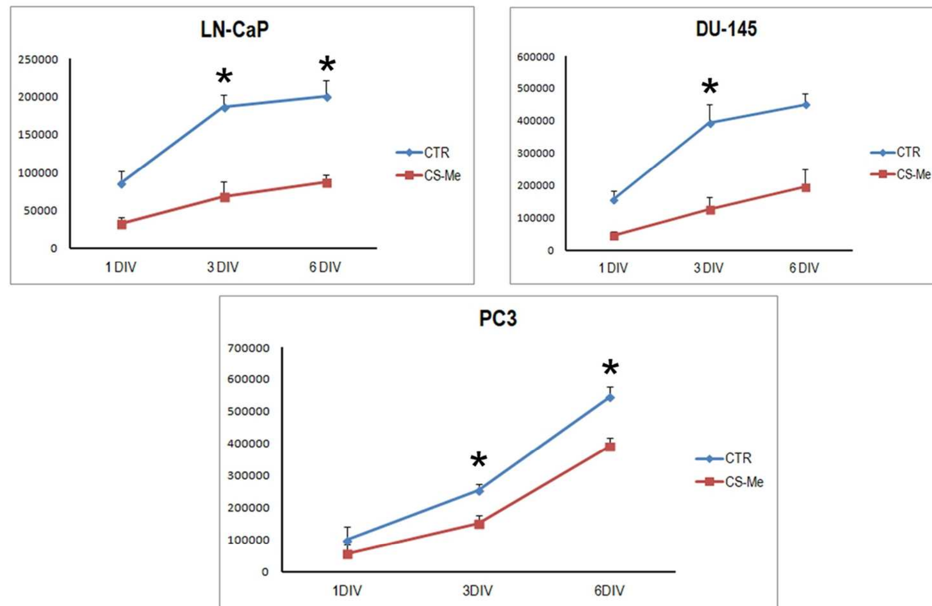

Figure 3. Effects of dissolution products of CS-Me. Line graph depicting the proliferation of different human prostate cancer cell lines: LN CaP, DU-145 and PC3 cultured in medium collected after 15 days of CS film dissolution.*: $p < 0.05$.

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review

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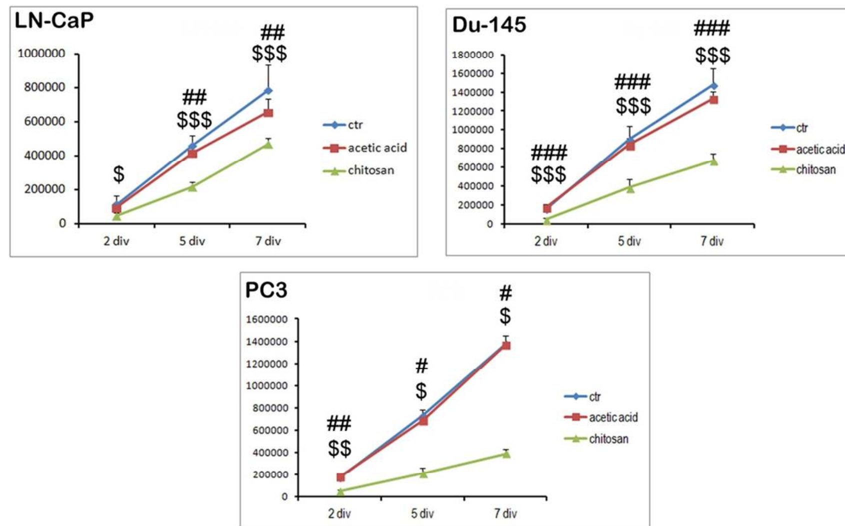

Figure 4. Effects of cell direct contact with CS coating. Proliferation curve experiment: LN-Cap, Du-145 and PC3 cell lines were cultivated on the control, on 0.1% acetic acid coating and on CS coating. Results showed a significant lower proliferation for cell lines cultured on Chitosan. $/#: p < 0.05; $$/##: p ≤ 0.01; $$$/###: p ≤ 0.001. $ indicates the comparison between CTR and CS coating treatment; # indicates the comparison between acetic acid coating and CS coating treatment.

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view

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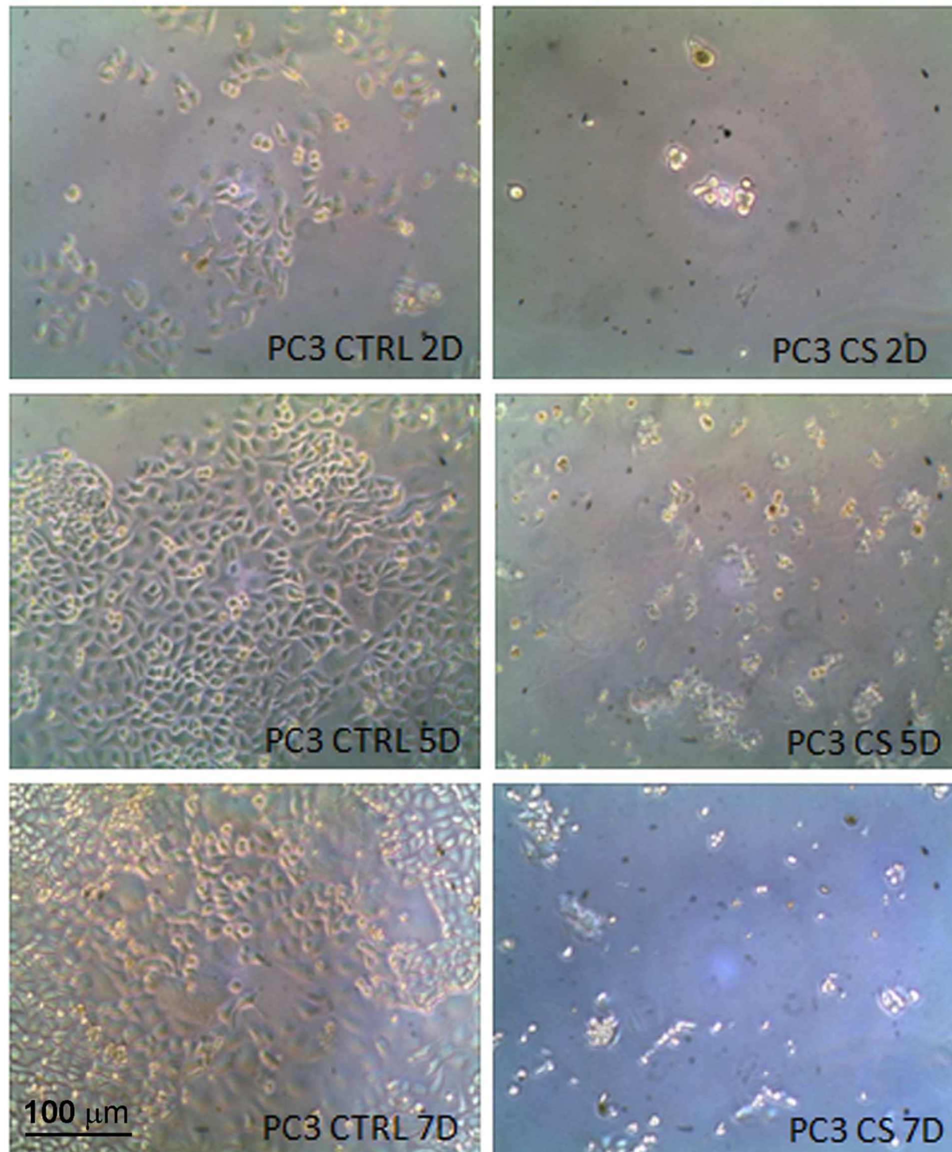

Figure 5. Effects of cell direct contact with CS coating. Morphology: Representative panel depicting PC3 cell lines cultivated on the control, on chitosan coating and on 0.1% acetic acid coating. Cultured cells on the CS substrate lose their normal morphology and adhesion capacity, they appear rounded and form clusters.

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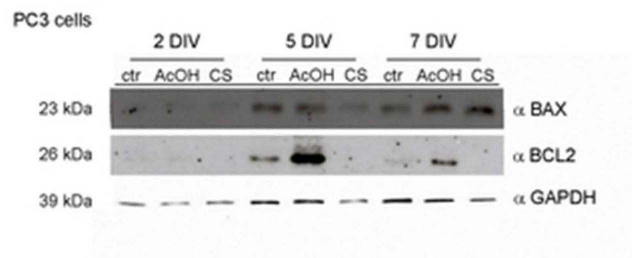

Figure 6. Protein expression analysis. A representative Western blot analysis performed on PC3 cell line cultured in the three experimental conditions showed, at days 5 and 7 of CS coating, an over-expression of the pro-apoptotic protein BAX and a simultaneous down-regulation of BCL2, an anti-apoptotic marker.

30x11mm (300 x 300 DPI)

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