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HENRIQUE RINALDI MATHEUS

Desenvolvimento, caracterização e avaliação in vivo de um filme de quitosana-sinvastatina sobre a superfície do titânio: avaliação do potencial de osseointegração sob a influência de nicotina

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Tese apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista (Unesp), como requisito para a obtenção do Título de Doutor em Odontologia – Área de Periodontia.

Orientador: Prof. Dr. Juliano Milanezi de Almeida

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Dedicatória

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"A gratidão é uma forma singular de reconhecimento,
e o reconhecimento é uma forma sincera de gratidão"
Alan Vaszatte

A Deus

Pela sua singularidade e presença irrestrita em todos os momentos da minha vida.

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"Deus é o nosso refúgio e a nossa fortaleza, auxílio sempre presente na adversidade"
Salmo 46:1

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O agradecimento é a memória do coração
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A ciência não é só compatível com a espiritualidade; é uma profunda fonte de espiritualidade. Quando reconhecemos nosso lugar na imensidão de anos-luz e no transcorrer das eras, quando compreendemos a complexidade, a beleza e a sutileza da vida, então o sentimento sublime, misto de júbilo e humildade, é certamente espiritual. (...) A noção de que a espiritualidade e a ciência são de alguma maneira mutuamente exclusivas presta um desserviço a ambas.

Carl Sagan

Resumo

Matheus HR. Desenvolvimento, caracterização e avaliação in vivo de um filme de quitosana-sinvastatina sobre a superfície do titânio: avaliação do potencial de osseointegração sob a influência de nicotina [tese]. Araçatuba: Universidade Estadual Paulista (Unesp), Faculdade de Odontologia; 2023.

RESUMO

A osseointegração sob influência sistêmica de nicotina (NIC) apresenta-se como um desafio à implantodontia atual, visto que a capacidade de reparo ósseo mediado por células residentes é negativamente influenciada por esse fatore. Neste projeto de pesquisa, objetiva-se desenvolver um filme de quitosana (CH)-sinvastatina (SV) sobre a superfície do titânio de forma a modular e otimizar o processo de reparo peri-implantar em modelos de comprometimento por NIC. Diferentes técnicas de funcionalização com quitosana (direta ou por meio de aminosilano [APTMS]) e incorporação de sinvastatina nas concentrações de 1, 2,5, e 5 μM foram aplicadas para obtenção de maior estabilidade e potencial modulatório do filme. Para caracterização da superfície, espectroscopia de fotoelétrons excitados por raios X (XPS), microscopia eletrônica de varredura (MEV) e ângulo de contato foram utilizados. O Sucesso na adsorção de CH e SV foi confirmado pelas alterações elementares observadas através de XPS. Todos os filmes apresentaram estrutura nanofibrilar e super-hidrofilicidade. Células tronco da medula ósseo suína (pBMSC) foram utilizadas para mapeamento inicial. Os filmes obtidos através da adsorção de CH por meio de APTMS e incorporação de SV nas concentrações de 1 e 2,5 μM (principalmente) apresentaram a melhor capacidade de induzir a deposição de matriz mineralizada por pBMSCs. APTMS-CH_SV 2,5 μM foi testada *in vitro* em cultura de SAOS-2 em meio suplementado com NIC. O filme testado foi capaz de bio-estimular as células ainda que na presença de nicotina (quando comparado com controles não funcionalizados). Sendo assim, o filme foi testado em modelo *in vivo* de administração sistêmica de NIC. Os efeitos deletérios da NIC no processo de reparo ósseo peri-implantar ao redor de implantes tratados por jateamento e ataque ácido (convencionais; NF) foi reafirmado.

Assim como o filme de CH_SV se provou eficaz em reverter o impacto negativo mediado por NIC no processo de osseointegração. O maior contato osso/implante (BIC) e área de tecido ósseo peri-implantar (BAFO) em CH_SV quando comparado com controle (NF) na presença de nicotina foi suportado pelos mecanismos de regulação positiva da proteína morfogenética óssea (BMP)-2 e BMP-7, angiogênese (regulação positiva do fator de crescimento endotelial vascular [VEGF]), renovação acelerada (osteocalcina), modulação da inflamação (expressão reduzida do fator de necrose tumoral [TNFα] e interleucina [IL] 1β) e redução da reabsorção óssea mediada por osteoclastos (expressão da fosfatase ácida resistente ao tartarato [TRAP]). Também, a maior formação óssea em período inicial (15 dias), que não diferiu com o período pós-osseointegração (45 dias) indica que o filme acelerou a formação óssea, tanto na presença quanto na ausência de NIC. O filme CH_SV é uma escolha eficaz para aumentar a taxa de sucesso de reabilitações mediadas por osseointegração sob influência sistêmica de NIC e amplia o escopo de aplicação de revestimentos bioativos na superfície de Ti.

Palavras-chave: Nicotina. Osseointegração. Estatina.

Abstract

Matheus HR. Development, characterization and in vivo assessment of a chitosan-simvastatin film for titanium coating: evaluation of the osseointegration potential under influence of nicotine [tese]. Araçatuba: Universidade Estadual Paulista (Unesp), Faculdade de Odontologia; 2023.

ABSTRACT

The osseointegration under systemic modification by nicotine (NIC) remains a great challenge to implantology since the bone reparative potential of resident cells is negatively influenced in the presence of this factor. In this research project, the objective is to develop a chitosan (CH)-simvastatin (SV) film on the surface of titanium in order to modulate and optimize the peri-implant repair process in models of systemic compromise by NIC. Distinct technique for immobilization of CH (directly os by means of an aminosilane [APTMS]) and incorporation of SV at the concentrations of 1, 2.5, and 5 μM were used to obtain greater stability and modulatory capacity. For surface characterization, X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), and contact angle measurement were applied. The success in CH and SV adsorption were confirmed by the elemental changes observed through XPS. All films exhibited nanofibrillar super hydrophilic characteristics. Porcine bone marrow stem cells (pBMSCs) were used for initial screening. The films obtained by adsorption of CH through APTM and incorporation of SV at 1 and e 2,5 μM (specially) showed the best capacity to induce mineralized matrix deposition by pBMSCs. APTMS-CH_SV 2.5 μM was tested in vitro in SAOS-2 cultures in medium supplemented with NIC. The tested film exerted a bio-stimulatory potential in the presence of NIC (as compared in no-functionalized controls). Therefore, the model was tested in an *in vivo* model of systemic delivery of NIC. The hazardous effects of NIC on peri-implant bone healing around sandblasted acid etched implants (conventional, NF) were reaffirmed. The CH_SV film proved to be effective in reverting the impact of NIC on peri-implant bone healing. The greater bone-to-implant contact (BIC) and bone area fraction occupancy (BAFO) in CH_SV,

when compared with control (NF) in the presence of NIC was underlined by the mechanisms of upregulation of bone morphogenetic protein (BMP)-2 and BMP-7, coupled angiogenesis (upregulation of vascular endothelial growth factor [VEGF]), accelerated turnover (osteocalcin), modulation of inflammation (reduced expression of tumor necrosis factor [TNFα] and interleukin [IL] 1β), and reduced osteoclast-mediated bone resorption (tartrate-resistant acid phosphatase [TRAP] expression). Additionally, the increased bone formation in initial stages (15 days) did not differ from post-osseointegration phases (45 days), thus indicating that the osseointegration process was accelerated by CH_SV in the presence or absence of NIC. CH_SV film is an effective choice for widening success rate of osseointegration-mediated rehabilitations under systemic influence of NIC and enlarges the application scope of bioactive coatings on the surface of Ti.

Keywords: Nicotine. Osseointegration. Statins.

Lista de Figuras

LISTA DE FIGURAS

CAPÍTULO 1

CATÍTULO 2

Lista de Abreviaturas e Símbolos

Lista de Abreviaturas e Símbolos

%.....percentage
®.....trademark
±.....plus-minus
≤.....less than or equal to
≥.....greater than or equal to
3Rs..... replacement, reduction, and refinement
AKT..... serine/threonine protein kinase
ANOVA.....analysis of variance
APTMS.....3-aminopropyltrimethoxysilane
ARRIVE..... Animal Research: Reporting of In Vivo Experiments
BA.....bone area
BAFO.....bone area fraction occupancy
BIC.....bone-to-implant contact
BMP.....Bone Morphogenetic Protein
C.....Carbon
CH.....Chitosan
CO_2.....carbon dioxide
d.....days
Da.....unified atomic mass unit
DAB.....3,3′-diaminobenzidine tetrahydrochloride
DAPI..... 4′,6-diamidino-2-phenylindole
DMEM.....Dulbecco's Modified Eagle Medium
DNA..... deoxyribonucleic acid
EA.....Emission angle
EC.....endothelial cells
ECM.....extracellular matrix
EDTA..... ethylenediaminetetraacetic acid
Erk..... extracellular signal-regulated kinase
Et al.....and others
eV.....electric vehicle
FAPESP.....Fundação de Amparo à Pesquisa do Estado de São Paulo
FBS.....Fetal bovine serum
g.....grams
h.....hour
HE.....hematoxylin and eosin
HIF-1α..... hypoxia-inducible factor 1α
HRP.....horseradish peroxidase
i.m. .....intramuscular
IACUC.....Institutional Animal Care and Use Committee
IgG.....immunoglobulin
IL-1β.....interleukin 1β
L.....liter
LACET.....Laboratório de Cultura de Células e Engenharia de Tecidos
M.....Molar
MC3T3-E1.....mouse calvarium osteoblastic cell line
Mg/kg.....milligram per kilogram
min.....minute

mm.....millimeters
mM.....micromolar
mm^2.....square millimeters
MPK..... mitogen-activated protein kinase
MSC.....mesenchymal stem cell
N.....Nitrogen
NaOH.....Sodium hydroxide
NEPPI.....Núcleo de Estudo e Pesquisa em Periodontia e Implantodontia
NF.....No functionalization
NIC.....nicotine
nM.....nanomolar
Nm.....nanometers
º.....degrees
O.....Oxygen
ºC.....Degrees Celsius
OCN.....osteocalcin
pBMSC.....porcine Bone Marrow Stem Cell
PBS.....phosphate buffered saline
pH..... potential of hydrogen
ROI.....region of interest
SAOS-2.....human osteosarcoma cell line
SD.....standard deviation
SEM.....Scanning Electron Microscopy
Si..... Silicon
SMAD..... suppressor of Mothers against Decapentaplegic
SV.....Simvastatin
TA.....total area
Ti-6Vl-4Al.....Titanium, Vanadium, Alluminium alloy
Ti.....Titânio
TiZr.....Titanium Zirconium alloy
TNFα.....tumor necrosis factor α
TRAP.....tartrate-resistant acid phosphatase
UNESP.....Universidade Estadual Paulista "Júlio de Mesquita Filho
v/v.....Volume per volume
VEGF.....vascular endothelial growth factor
VEH.....Physiological saline solution
X-ray.....x ray
x.....times
XPS.....X-ray Photoelectron Spectroscopy
µL.....microliter
µM.....micromolar

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CAPÍTULO 1

Artigo Normatizado de acordo com as normas do Periódico "Journal of Biomedical Materials Research Part A" (Anexo A)

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Title

Chitosan-simvastatin nanofibrillar super hydrophilic film on titanium alloy for biomedical applications

Short running title

Novel biofunctional film improves osseointegration

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Abstract

Functionalization of load-bearing metallic implants is important for assuring rapid osseointegration and improved cell-surface interactions. This study aimed to chemically modify the surface of titanium (Ti) through biofunctionalization with chitosan (CH) loaded with multiple concentrations of simvastatin (SV). For that, 2% CH was incorporated directly on the surface of hydroxylated Ti discs or by means of 3-aminopropyltrimethoxysilane (APTMS). Then, the discs were submerged in SV solution at concentrations of 1, 2.5, or 5 μM. Surface properties were evaluated with X-ray Photoelectron Spectroscopy (XPS), Scanning electron microscopy (SEM), and contact angle measurement. Porcine Bone Marrow Stem Cells (pBMSCs) supplemented with osteogenic medium were used to evaluate the films' bioactivity at a distance and the cell-surface interaction. The elemental analysis identified Ti 2p was completely undetectable in coated groups, this indicating successful functionalization. Regardless of the method of incorporation, a nanofibrillar super hydrophilic CH network was observed in functionalized discs. SV did not significantly alter the wettability or the 3-dimensional structure of the network. SV 5μM exerted cytotoxic effects at a distance. APTMS-CH, APTMS-CH_SV 1μM, and APTMS-CH_SV 2.5μM significantly increased the mineralized matrix deposition when compared with their respective controls (without silanization). The bio-stimulatory effect of SV was confirmed by the significant increase in mineralized matrix deposition in APTMS-CH_SV 1μM and APTMS-CH_SV 2.5μM, as compared with APTMS-CH. Biofunctionalization of Ti with CH and SV (1 and 2.5μM) improved surface characteristics and exerted a bio-stimulatory effect in pBMSCs.

Key-words: biofunctionalization, titanium, osseointegration.

Introdução

1 Introduction

The extensive use of titanium (Ti) alloys in orthopedics and dentistry may be reasoned by their bulk properties (e.g. mechanical strength, corrosion resistance, and biocompatibility), however, surface characteristics govern their ability to interact with surrounding host environments, thus dictating successful osseointegration and long-term survival. [1,2] Significant efforts in implantology research rely on surface modification techniques, either for accelerating osseointegration (herein improving loading protocols),[3] preventing/controlling microbial colonization,[4] or increasing their success rate in patients with impaired bone quality.[5]

Biofunctionalization of Ti reportedly improves the osteogenic capacity of implants.[6] Chitosan (CH) is a natural cationic biopolymer that, besides its biocompatibility, acceptable hemocompatibility and antimicrobial properties,[7] aids osteoconductivity *in vitro* and *in vivo*.[8] CH-biofunctionalized Ti enhances differentiation of osteoprogenitor cells and secretion of extracellular matrix *in vitro*.[9,10] Also, Fakry et al.[11] demonstrated that CH supports the preferential attachment and spreading of osteoblasts over fibroblasts. *In vivo*, CH-based coatings have shown mechanical stability, good degradability, and optimal osseointegration in multiple animal models.[12,13]

Owing to its biodegradability, polycation characteristic, bioadhesion, bacteriostasis, and simplicity of modification, CH is actively studied as a bioactive carrier for drugs.[14,15] Statins are the mainstay in the treatment of high cholesterol and are vastly prescribed for cardiovascular disease.[16] Rather than merely regulating angiogenesis, several studies have reported the pleiotropic effect of statins.[17,18] Simvastatin (SV) was first reported to upregulate the expression of BMP family members.[19] Later, research exploited its osteogenic effect in numerous cell-based and in vivo models.[20] SV has been loaded onto implant surfaces for

enhancing osseointegration. [21-23] Direct binding of SV to a titanium zirconium alloy (TiZr) promoted alkaline phosphatase, collagen type I, and osteocalcin gene expression in MC3T3-E1,[21] profiling features of osteogenic differentiation. Additionally, the benefits of SV in multilayered films[23] permit multiple combinations of SV with biopolymers to widen its beneficial effects around implants.

Poorly soluble drugs require a carrier for increasing their solubility and, hence, bioavailability.[24] CH has proven to be a suitable carrier for SV, as confirmed by promising osteogenic effects of CH-SV scaffolds *in vitro*[25] and *in vivo*.[26] Importantly, SV-induced inflammatory response[27] demands dosage screening for selecting optimal concentrations of SV that support osteogenesis. The objective of the present research was to develop, characterize, and evaluate the biological potential of a CH coating loaded with multiple concentrations of SV on the surface of titanium.

Material e Métodos

2 Materials and Methods

Sandblasted and acid etched titanium discs (Ti-6Vl-4Al; 8 mm ⌀ and 3 mm height) were provided by DSP Biomedical® (Campo Largo, Parana, Brazil), and were used as controls (and substrates) for all tested coatings. Following surface hydroxylation, the process for coating the titanium alloy with CH and SV was performed directly on the surface of the metal or by means of an aminosilane, as described below.

2.1 Coating protocols

2.1.1 Surface Hydroxylation

All groups (except control [NF]) were subjected to surface hydroxylation with sodium hydroxide (NaOH) 5M. For that, the discs were submerged in NaOH 5M for 6 hours at 60ºC under stirring. Discs were washed in deionized water and vacuum dried overnight.

2.1.2 Silanization process

Hydroxylated discs were submerged in an aminosilane solution (3-aminopropyltrimethoxysilane [APTMS], high-performance liquid chromatography; 281778, Sigma Aldrich) diluted in anhydrous ethanol (v/v, 3/97) for 24 h.[28] Discs were washed in anhydrous ethanol and vacuum dried for 12h.

2.1.3 Functionalization of Ti with Chitosan

Low molecular weight (50,000-190,000 Da) CH (448869, Sigma Aldrich, St. Louis, MO, USA) was dissolved in 2% acetic acid to obtain a final solution of 2% CH. Discs, both only hydroxylated or hydroxylated and silanized with APTMS were submerged in 2% CH[29] for 12 hours. All discs were washed 3 times in deionized water and vacuum dried for 72h.

2.1.4 Incorporation of Simvastatin

The discs were submerged in SV (≥97% high-performance liquid chromatography, solid; Supelco, Bellefonte, PA, USA) at 1, 2.5, or 5 µM for 24 h, washed in anhydrous ethanol and vacuum dried overnight.

2.1.5 Tested groups

Finally, the following groups were tested: NF; CH; CH_SV 1µM; CH_SV 2.5µM; CH_SV 5µM; APTMS-CH; APTMS-CH_SV 1µM; APTMS-CH_SV 2.5µM; and APTMS-CH_SV 5µM.

2.2 Surface characterization

2.2.1. X-ray Photoelectron Spectroscopy

The chemical compositions of the material surfaces were determined using X-ray Photoelectron Spectroscopy (XPS; Scienta Omicron ESCA) based on the standards described by Dias et al.[30] For that, XPS was equipped with an EA125 Xm1000 monochromator and an Al Kα (1486.7 eV) X-ray source. The survey spectra were collected using a pass energy of 50 eV and an energy step of 0.500 eV. The spectra were fitted using CasaXPS software, and the binding energy was corrected using the aliphatic hydrocarbon C 1 s reference signal at 285.0 eV.

2.2.2. Scanning Electron Microscopy

For Scanning Electron Microscopy (SEM; Helios NanoLab 600i; FEI, Hillsboro, OR, United States), analysis of the discs' surface morphology was carried at 10 000x and 50 000x magnification for emphasizing the differences between control and tests.

2.2.3 Contact angle measurement

Wettability was measured by means of the sessile drop method through contact angle goniometry (Ramé-Hart 100-00; Succasunna, NJ, USA) with ultrapure water as the wetting fluid. A drop (10 µL) of ultrapure water was deposited on the sample surface and the angle measured with the instrument software.

2.3 In vitro biological analyzes of the CH_SV coatings

2.3.1 Obtention of porcine Bone Marrow stem cells

Femur and tibiae from 6- to 8-month-old porcine donors (IACUC #2022N000088) were sawn and the bone marrow extracted under sterile conditions. The protocol for isolation and expansion of porcine bone marrow stem cells (pBMSCs) was conducted as described by Ringe et al.[31]

2.3.2 Cell response at a distant – extract assay

For the initial screening of all groups and evaluation of the compounds released by the samples, the extract assay was performed as described de Melo et al.[32] Discs from groups NF, CH_SV 1µM, CH_SV 2.5µM, CH_SV 5µM, APTMS-CH_SV 1µM, APTMS-CH_SV 2.5µM, and APTMS-CH_SV 5µM were incubated in osteogenic medium (DMEM supplemented with 10% Fetal Bovine Serum [FBS], 100 nM/L dexamethasone, ascorbic acid 50 mg/mL, and 10 mM/L β-glicerophosphate) at 37ºC and 5% CO_2 for up to 14 days to obtain a conditioned medium (extracts; n=8). On the day of incubation of the discs, pBMSCs ($3x10^3$ cells) were seeded in 96-well plates and cultured in complete DMEM at 37ºC and 5% CO_2 for 24 h. Then, the cell medium was replaced with the 24-h extracts and refreshed every 48 h for the acquisition of new extract applications. Cell metabolism assay and quantification of mineralized matrix deposition were performed.

2.3.3 Cell metabolism

The Alamar Blue assay was performed at 1, 3, 7, and 14 days of culture. In each analysis, the cells were incubated for 4 h at 37ºC and 5% CO_2 with the Alamar Blue reagent® (Life Technologies) in DMEM without FBS (1:10). The reagent was transferred to 96-well plates and analyzed in a fluorescent reader (530nm excitation—590nm emission; Varioskan™ LUX, Thermo Fisher Scientific, Walthan, MA, USA). The data were normalized based on the control (NF) and its metabolism was considered 100% in the first period of analysis.

2.3.4 Mineralized matrix deposition

After 14 days of culture, the cells were washed with PBS and fixed in 70% ethanol at 4ºC for 1 h. Then, they were washed in PBS and incubated for 15 min in Alizarin Red solution (40 mM, pH 4.2; Sigma-Aldrich). Following incubation, the cells were washed in PBS three times and the nodules of mineralization dissolved in 10% cetylpyridinium chloride solution under stirring. Absorbance was ridden at 560 nm (Varioskan™ LUX, Thermo Fischer Scientific). The NF group was considered as 100% of mineralized matrix deposition.

2.3.5 Direct contact analysis

The 5 µM SV was excluded from direct contact analysis based on its toxicity in the extract assay. For the direct contact analysis, pBMSCs were seeded ($2.5x10^4$) onto NF, CH, CH_SV 1µM, CH_SV 2.5µM, APTMS-CH, APTMS-CH_SV 1µM, and APTMS-CH_SV 2.5µM discs (n=6) and cultivated in osteogenic medium for 14 days. Measurement of mineralized matrix deposition (as described above) and assessment of the adhesion and spreading were performed at 14 days. Cell metabolism was quantified by means of the Alamar Blue assay, as previously described.

2.3.6 Adhesion and Spreading

In order to evaluate the cytoarchitecture of the cells, samples (n=2) obtained at 14 days of culture were fixed in 4% buffered formaldehyde solution for 10 min, washed in PBS, permeabilized in 0.1% triton X (Thermo Fisher Scientific) and incubated for 30 min with Alexa Fluor 555 Phalloidin (Thermo Fisher Scientific, Carlsbad, CA, USA) in bovine serum albumin (BSA) (1:40). The nuclei were stained with DAPI (Thermo Fisher Scientific). The discs were mounted with fluorescence mounting medium (Dako Laboratories, CA, EUA) and images were recorded under confocal microscopy (FLoid®, Life-Technologies).

2.4 Statistical analysis

The numerical data of the experiments were compiled and analyzed by 1- or 2-way analysis of variance (ANOVA) followed by Tukey's test for observation of significant differences between the study groups ($p \leq 0.05$ was statistically significant).

Resultados

3 Results

3.1. X-ray photoelectron spectroscopy

The elemental composition indicated significant changes to O 1s and C 1s in all tested groups. The presence of Si 2p was observed in APTMS and APTM CH. In all test groups (excluding control, NF), it was possible to identify N 1s (Figure 1).

3.2. Surface topography

SEM analysis revealed that, regardless the method of functionalization (direct or the surface by means of APTMS), the bio-functionalized discs exhibited a layer of CH-fibrillar network in its surface (Figure 2). Interestingly, the images in higher magnification (50 000x) indicate that innumerous fibrils fit within the 1 μm scale, thus indicating that the methods used for incorporation of CH onto the surface of Ti resulted in a nanofibrillar network. SV appeared not to interfere on the diameter or morphology of the nanofibrils.

3.3 Wettability

Ti substrats (NF) displayed a relatively hydrophobic surface with a water contact angle of 93.06±0.05º. After being hydroxylated and coated with CH and SV (either directly on the surface or through the aminosilane), the hydrophilicity of the discs increased dramatically, as appreciated in Figure 3. The 10 μL water drop was completely adsorbed onto the surface of the tested films, precluding the measurement of the contact angle. The inability to measure the contact angle by means of the goniometer indicates that the contact angle with the surface is lower than 10º, thus characterizing the tested films as super hydrophilic.[33]

3.4 Cell response at a distance

The intragroup analysis indicated that cell metabolism increased over time in all experimental groups ($p \leq 0.05$). At 7 days, APTMS-CH_SV 1μM and APTMS-CH_SV 2.5μM exhibited lower cell metabolism as compared with NF, CH, CH_SV 1μM, CH_SV 2.5μM, and CH_SV 5μM ($p \leq 0.05$). APTMS-CH_SV 1μM and APTMS-CH_SV 2.5μM showed the lowest mean values for cell metabolism (Figure 4a).

Higher mineralized matrix deposition (%) was observed in APTMS-CH_SV 2.5μM as compared with NF, CH, CH_SV 1μM, CH_SV 5μM, and APTMS-CH_SV 5μM ($p \leq 0.05$). (Figure 4b).

3.5 Direct contact analysis

All groups exhibited greater cell metabolism at 14 days when compared with 1, 3, and 7 days ($p \leq 0.05$). No statistically significant differences in cell metabolism were observed when comparing 1, 3, and 7 days within the same group. Also, no intergroup differences were identified when comparing groups in the same periods of analysis (Figure 5a).

Groups NF, CH, CH_SV 1μM, and CH_SV 2.5μM showed the lowest values for mineralized matrix deposition ($p \leq 0.05$). Increased mineralized matrix deposition was observed in APTMS-CH_SV 1μM and APTMS-CH_SV 2.5μM as compared with APTMS-CH ($p \leq 0.05$) (Figure 5b).

The analysis of the cytoarchitecture of cells indicated a similar adhesion and spreading among groups. Groups CH_SV 1μM (Figure 5e) and APTMS-CH (Figure 5g) exhibited a lower density of cells as compared with other groups.

Discussão

4 Discussion

Early success and long-term survival of orthopedic and dental load-bearing rehabilitations rely on anchoring an implant to the bone, a phenomenon termed osseointegration,[34,35] which is defined as ‘‘a direct – on light microscopic level – contact between living bone and implant”.[36] The intimate contact between bone and implant is the conclusion of an overlapping cascade of events of communicating cells.[37] The temporal sequence of the osseointegration process is highly impacted by the implant surface.[38] It has been demonstrated by the present experiment that the biofunctionalization of Ti with CH and SV resulted in a nano fibrillar super hydrophilic film capable of stimulating BMSCs at a distance and improving cell-surface interaction.

The extracellular matrix (ECM) is the nanofibril protein network surrounding and supporting the many functions of cells in all tissues.[39] Within the meso-micro-nano continuum, mineralized collagen fibrils form the structural basis of the bone and provide its hierarchical structure in the bone-implant interface.[40] Cell-ECM interactions affect signaling pathways that dictate cell responses, such as adhesion, proliferation, differentiation, and tissue neogenesis,[42] thus, tailored materials that mimic the natural cell environment of the ECM might be beneficial for wound healing. Interestingly, nanofibrous scaffolds, mainly when associated with bioactive molecules, widened the efficacy of tissue-engineered approaches for regeneration of multiple tissues.[41] The 3-dimentional CH fibril network combined with the bio stimulatory effect of SV might have favored the beneficial effects of the tested films *in vitro*.

Among the many surface properties (topography, charge, pore structure) capable of manipulating cells and downstream signaling pathways, wettability is critical for the initial attachment of cells onto the surface of biomedical materials.[38] Jo et al.[46] demonstrated that the adhesion of pre-osteoblasts MC3T3-E1 increased proportionally with the increase on the

hydrophilicity of the tested poly e-caprolactone scaffolds. Protein absorption inevitably occurs on both hydrophilic and hydrophobic surfaces; however, serum proteins acquire distinct conformation depending on wettability. Fibronectin is transformed into an active conformation when absorbed into hydrophilic surfaces and might be the underlining mechanism facilitating cell adhesion.[38,43] Indeed, our cell culture findings indicate that super hydrophilicity alone is not sufficient to excel the stimulatory capacity of Ti (group NF), but the benefits of this property ought not to be neglected.

The modern era of drug delivery has put considerable effort into developing optimal nanovehicles. Nanofibers are among the many effective nanovehicles used for drug delivery of therapeutic agents at a cellular level.[44] Inefficient cellular uptake, endosomal escape, and premature release are the main shortcomings attributed to nano-scale carriers so far.[45] To adjust the binding strength of polymers and drug molecules might ensure their optimal retention and controlled release. Tran & Lee[46] found that APTMS was key for achieving optimized release of ibuprofen in mesoporous silica nanoparticles and hypothesized that the chemical strategy used in their study is a prominent “anchoring” approach that can kinetically control the release of various drug molecules with different sizes and surface charges. Although not at a distance, the higher amount of mineralized matrix deposition in APTMS-CH, APTMS-CH_SV 1μM, and APTMS-CH_SV 2.5μM as compared with their respective controls (without silanization) indicates that cell-surface interactions were significantly impacted by APTMS. Despite the confirmed elemental alterations among groups (XPS analysis), further high-resolution analysis of binding strength, degradation rate, and SV release kinetics are necessary to fully appreciate the role of APTMS in this scenario.

Regardless of the carrier (e.g. hydroxyapatite, CH, gelatin) or method for coating deposition onto the surface of Ti, improvements in cell response and in vivo osseointegration have been attributed to statins (SV and pitavastatin).[47,48] Guided endogenous MSCs

recruitment and osteogenic differentiation are essential for proper bone regeneration and enhanced osseointegration.[49] As confirmed by the greater deposition of mineralized matrix in the presence of SV, our study corroborates with other studies supporting the capacity of statins in inducing an osteogenic profile to osteoprogenitors[47] and completely undifferentiated MSCs.[48]

Albeit considerably high success rates are attributed to endosseous titanium implants, peri-implant infections[50] and early failure in systemically compromised patients[5] demand research to improve cell-implant interactions. Given the promising results presented in this study and the antimicrobial property of CH,[51] we encourage further research to evaluate the effects of APTMS-CH_SV films in compromised systems.

Conclusão

5 Conclusions

Biofunctionalization with CH resulted in a super hydrophilic nanofibrillar film on the surface of Ti. The highest concentration of SV (5µM) significantly reduced the mineralization capacity of cells at a distance. Surface silanization with APTMS improved the cell-surface interaction. When in silanized discs, the incorporation of SV at 1 and 2.5µM increased mineralized matrix deposition as compared with CH alone.

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Figuras

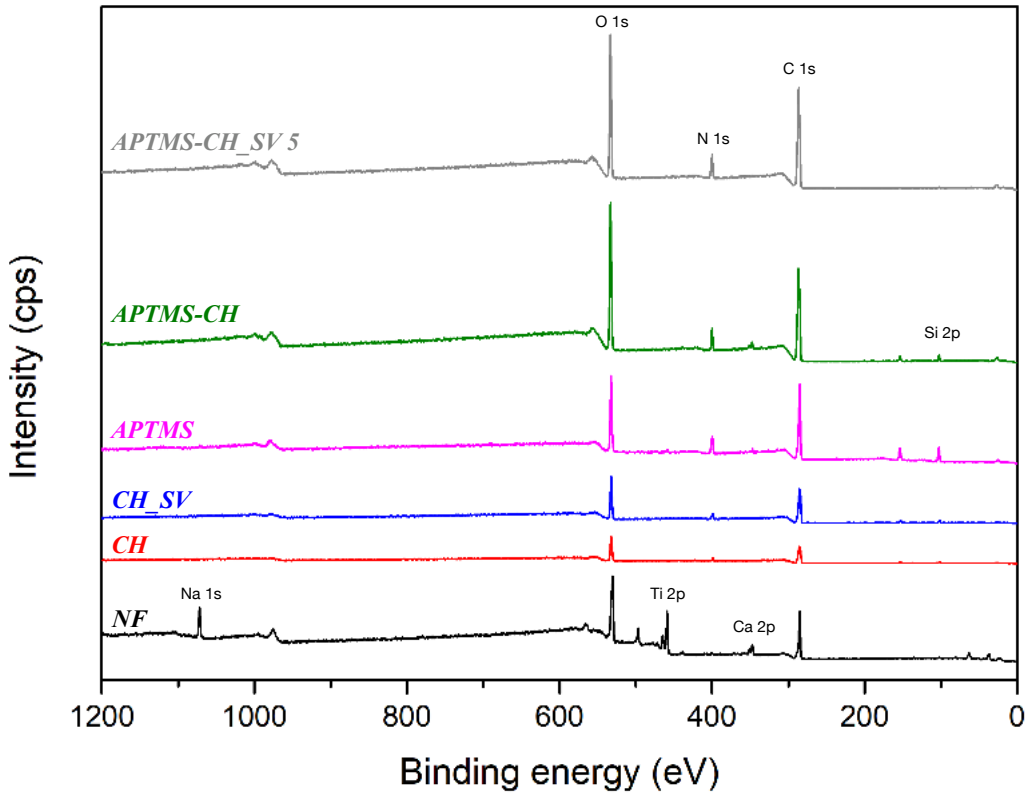

Figure 1: XPS widescan spectra. The concentration of the SV solution used for the preparation of the CH_SV and APTM-CH_SV substrates was 5 µM. Fonte: Autor, 2023.

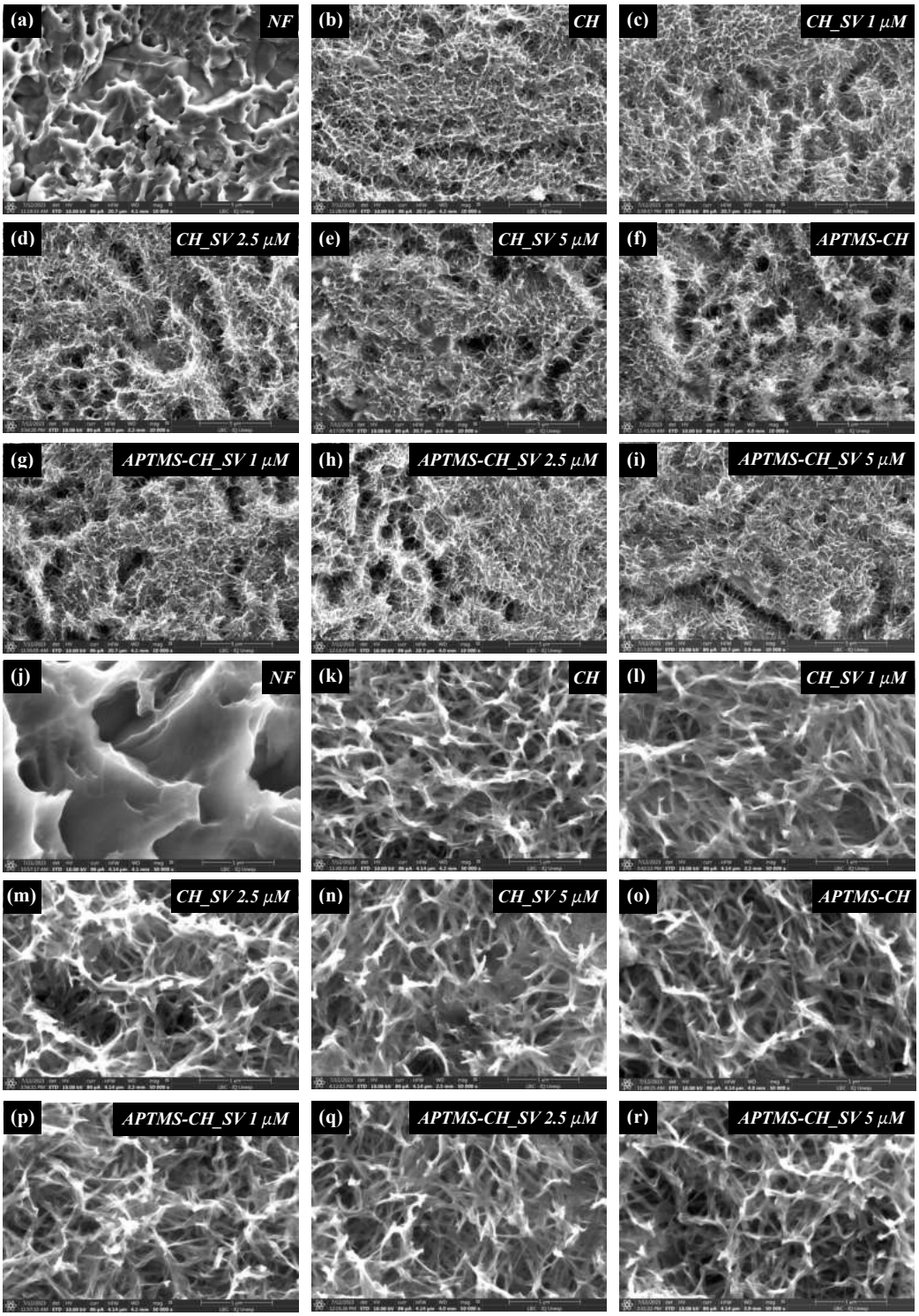

Figure 2: Scanning electron microscopy images of the specimen's surface morphology. Images (a) to (i) provide an overview of the films at 10 000x. Higher magnification images ("j" to "r") allow the appreciation of the fibril network. Fonte: Autor, 2023.

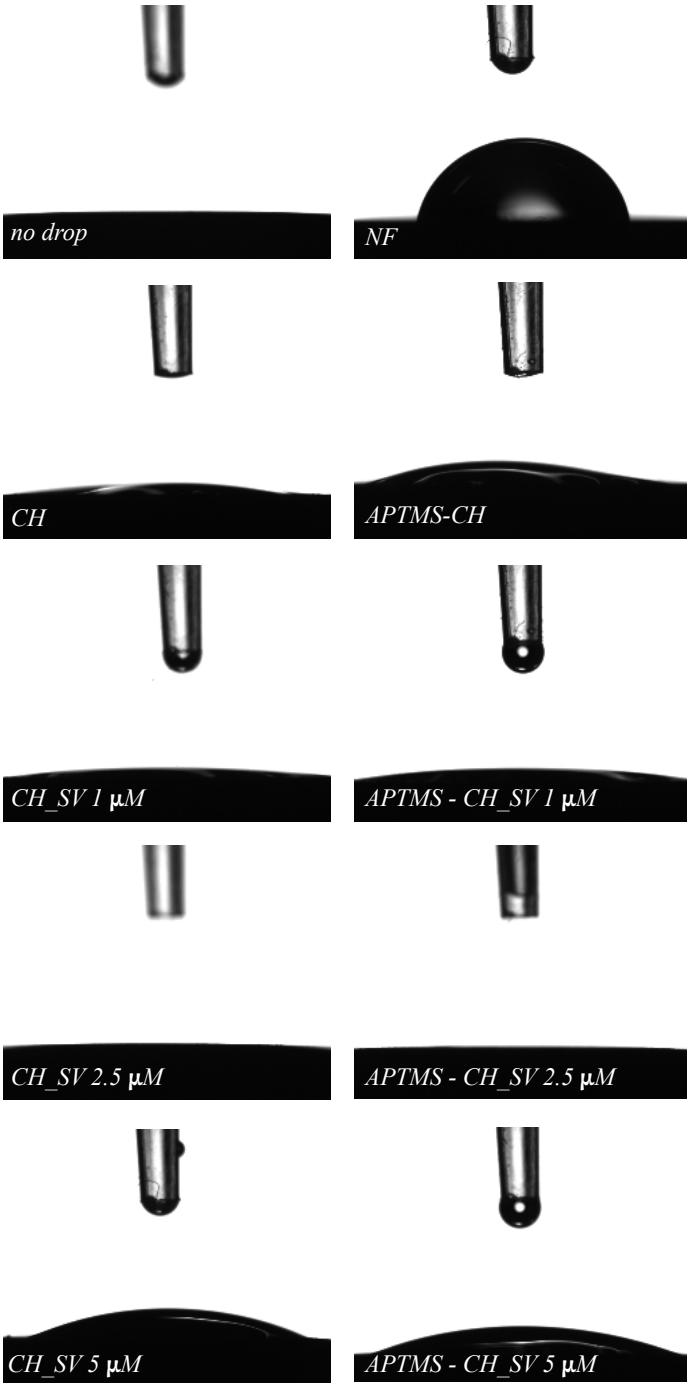

Figure 3: Wettability test results obtained for investigated films. Fonte: Autor, 2023.

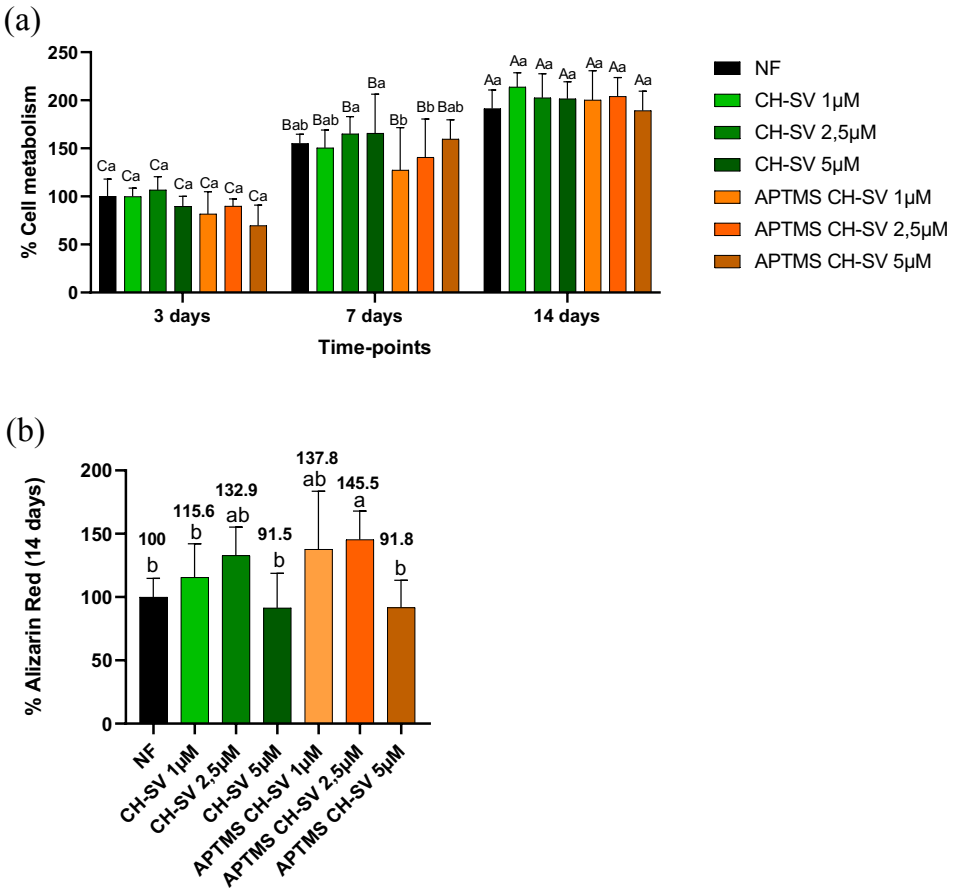

Figure 4: Cell response at a distance. (a) Percentage of cell metabolism for each group and period. (b) quantification of mineralized matrix deposition. Statistical tests: ANOVA and Tukey. Distinct uppercase letters indicate statistically significant intragroup differences among periods. Distinct lower-case lattes indicate statistically significant intergroup differences in the same period of analysis. Fonte: Autor, 2023.

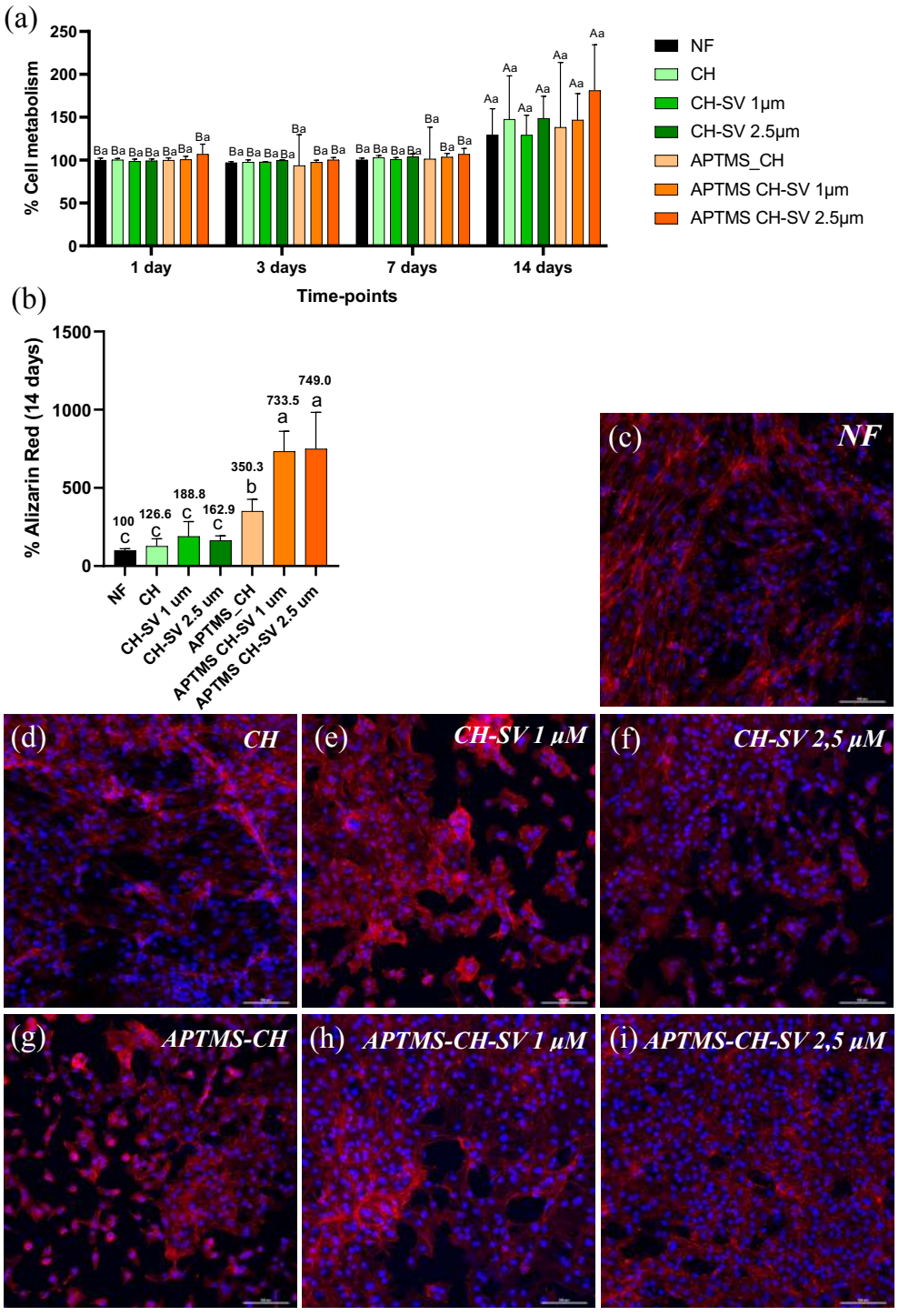

Figure 5: Direct contact analysis. (a) Percentage of cell metabolism for each group and period. (b) quantification of mineralized matrix deposition. Statistical tests: ANOVA and Tukey. Distinct uppercase letters indicate statistically significant intragroup differences among periods. Distinct lower-case lattes indicate statistically significant intergroup differences in the same period of analysis. (c - g) photomicrographs of cell adhesion and speeding on the surface of the discs. Fonte: Autor, 2023.

CAPÍTULO 2

Artigo Normatizado de acordo com as normas do Periódico "Biomaterials" (Anexo B)

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Title

Chitosan-simvastatin nanofibrillar film improves cell response and enhances osseointegration in nicotine-compromised environments

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Abstract

The negative impact of nicotine (NIC) to the osseointegration process around dental and orthopedic implants is irrefutable. Here, the capacity of a novel chitosan (CH) loaded simvastatin (SV, 2.5 μM) nanofibrillar super hydrophilic film to suppress the NIC-induced peri-implant bone damages was tested *in vitro* and *in vivo*. CH_SV film was not cytotoxic, permitted better adhesion and spreading, and dramatically increased the mineralization capacity of SAOS-2 in the presence or absence of NIC. The CH_SV 2.5 μM film was effective in increasing bone-to-implant contact (BIC) and bone are fraction occupancy (BAFO) in rats receiving NIC (comparable with control implants installed in rats not receiving NIC), while accelerating the osseointegration process in non-compromised animals. The underlining mechanisms of upregulation of bone morphogenetic protein (BMP)-2 and BMP-7, coupled angiogenesis (upregulation of vascular endothelial growth factor [VEGF]), accelerated turnover (osteocalcin), modulation of inflammation (reduced expression of tumor necrosis factor α [TNFα] and interleukin [IL] 1β), and reduced osteoclast-mediated bone resorption (tartrate-resistant acid phosphatase [TRAP] expression) were found to support rapid and improved peri-implant bone formation in NIC and non-compromised animals. CH_SV film is an effective choice for widening success rate of osseointegration-mediated rehabilitations under systemic influence of NIC and enlarges the application scope of bioactive coatings on the surface of Ti.

Key-words: Simvastatin, biofunctionalization, titanium, nicotine, osseointegration

Introdução

1. Introduction

Successful rehabilitation of lost functions, including hearing, mastication, and movement (joint, hip, limbs) rely on the phenomenon of osseointegration. Orthopedic [1], cochlear [2], and dental implants [3] demand intimate contact with the surrounding bone to be functional. The anchorage of an implant to the host's bone (osseointegration) is the conclusion of an overlapping cascade of events of communicating cells through mediators secreted in the extracellular matrix. Molecular adsorption onto the metallic surface, controlled inflammation, mesenchymal to endothelial transition, and osteogenesis dictate the early success (or failure) of osseointegration [4].

Despite the high success rates of endosseous implants in dentistry [5] and orthopedy [6], patient-related conditions that alter bone metabolism are reportedly related to failures in osseointegration [7]. During smoking, many chemicals are released into the bloodstream and trigger undesired effects in multiple tissues, including bone [8]. Early failure of dental [9] and orthopedic [10] osseointegration-mediated rehabilitations is considerably higher in smokers than non-smokers. The mechanisms through which smoking compromises bone healing, and thereby osseointegration, are not completely elucidated. Nicotine (NIC) alone has been shown to exert major negative effects on bone metabolism and skeletal remodeling *in vivo* [11, 12].

Multiple cell types (e.g. mesenchymal stem cells [MSCs], endothelial cells [ECs], inflammatory cells, osteoprogenitors, osteoblasts, and osteoclasts) are involved in the spatio-temporal model of osteogenesis or ossification [13,14]. Suppressed human MSC-derived osteogenesis [15], persistent and exacerbated inflammatory response [16], and uncoupled angiogenesis [17] are some of the jeopardizing effects attributed to NIC on bone healing. The confirmed negative impact of NIC on transcription factors (e.g. core-binding factor α-1, runt-related transcription factor-2) and osteogenic genes at peri-implant sites [18,19] reason the dramatically lower bone-to-implant contact (BIC) *in vivo* [18-20]. Given the biomedical

relevance of osseointegration-mediated rehabilitations and the high prevalence of smoking worldwide (around 1.18 billion smokers in 2020) [21], to excel the success rate of endosseous implants achieved in smokers so far is of great interest for improving life quality for these patients.

Titanium (Ti) functionalization has proven to be an effective approach capable of altering the local microenvironment and improving osseointegration in compromised systems [22]. Recently, a functional nano fibrillar super hydrophilic film of chitosan (CH) and simvastatin (SV) enhanced cell-Ti interactions and significantly induced an osteogenic profile to porcine bone marrow MSCs [23]. CH has been reported to increase hemocompatibility [24], while providing an ideal substrate for adhesion, spreading, and proliferation of osteoblastic lineage cells when adsorbed onto Ti surface [25,26].

Rather than merely controlling high cholesterol, the pleiotropic effects of statins on the modulation of chemotaxis, proliferation, angiogenesis, inflammation, and cell differentiation [27] have been vastly exploited for biomedical reasons. SV was first reported to upregulate the expression of bone morphogenetic protein (BMP) family members [28]. Further research unraveled that SV promotes osteoblast differentiation via the membrane-bound Ras/Smad/Erk/Bone morphogenetic protein (BMP)-2 pathway [29] or by expression of osteocalcin and osterix genes [30]. Importantly, osteoclast activity (via MAPK, AKT, and Src signaling) [31], inflammation [32], and angiogenesis (via increased expression of vascular endothelial growth factor [VEGF]) [33] are vital processes related to bone healing also positively modulated by SV. Irrespective of the method of incorporation onto the surface of Ti, SV enhanced osseointegration *in vivo* [34 - 36].

Substantiated by the negative impact of NIC in bone healing and the promising effects of CH and SV on the many players orchestrating the intricate osseointegration process, the current study aimed to explore the feasibility of a nanofibrillar super-hydrophilic film of

CH_SV to improve cell response *in vitro* and enhance osseointegration *in vivo*, under the influence of NIC.

Material e Métodos

2. Materials and Methods

2.1 Surface Biofunctionalization of Ti Discs

Sandblasted and acid-etched titanium discs (8 mm ⌀ and 3 mm height) were provided by DSP Biomedical® (Campo Largo, Parana, Brazil) and were used as controls (and substrates) for all tested coatings. The process for biofunctionalization with CH and SV was carried in accordance with Matheus & Almeida [23]. In summary, following hydroxylation with sodium hydroxide (NaOH) 5M, the discs were submerged in an amino silane solution (3-aminopropyltrimethoxysilane [APTMS], high-performance liquid chromatography; 281778, Sigma Aldrich) diluted in anhydrous ethanol (v/v, 3/97) for 24 h. Silanized discs were submerged in 2% low-molecular-weight CH (448869, Sigma Aldrich, St. Louis, MO, USA) for 12 h. Finally, SV (≥97% high-performance liquid chromatography, solid; Supelco, Bellefonte, PA, USA) was incorporated into CH by submerging the discs in a solution of SV at 2.5 µM for 24h. The discs were dried under vacuum between all steps.

2.2. In vitro experiment

2.2.1 Primary cell culture

Human osteosarcoma cell lines (SAOS-2) were cultivated in cell culture flasks (75 cm^2, Costar Corp., Cambridge, MA, EUA) in Dulbecco´s Modified Eagle´s Medium (DMEM, SIGMA Chemical Co., St. Louis, MO, EUA) supplemented with 15% Fetal Bovine Serum (FBS), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine (GIBCO) at a 37ºC and 5% CO_2 atmosphere, until being confluent.

2.2.2 Cell seeding

The cells reached 90% confluence, were digested with 0.25% trypsin, washed, resuspended, and seeded ($2.5x10^4$) onto tested discs (control [NF], CH, and CH_SV) in a volume of 50µl.

The discs were incubated for 30 min to permit the initial adhesion of the cell to the substrates and then, 1mL of either the conventional medium (described above) or the same medium supplemented with NIC 1mM [37] was added to the 48-well plate. The media was changed every 48 h.

2.2.3 Cell viability and metabolism

For assessing the vital cells after seeding, the discs (n=2) were cultivated for 1 d, washed in PBS, and incubated with a culture medium supplemented with Calcein AM and Ethyl Homodimer-1 (1:1000; Live/Dead® Viability/Cytotoxicity Kit; Invitrogen) for 10 minutes. The presence of live and dead cells was evaluated under fluorescence.

The Alamar Blue assay was performed at 1, 7, and 14 days of culture. In each analysis, the discs (n=6) were incubated for 4 h at 37ºC and 5% CO_2 with the Alamar Blue reagent® (Life Technologies) in DMEM without FBS (1:10). The reagent was transferred to 96-well plates and analyzed in a fluorescent reader (530nm excitation—590nm emission). The data from NF on day 1 was considered 100%.

2.2.4 Mineralized matrix deposition

At 7 and 14 days of culture, the discs (n=6) were washed with PBS and fixed in 70% ethanol at 4ºC for 1 h. Then, they were washed in PBS and incubated for 15 min in Alizarin Red solution (40 mM, pH 4.2; Sigma-Aldrich). Following incubation, the cells were washed in PBS three times and the nodules of mineralization dissolved in 10% cetylpyridinium chloride solution under stirring. Absorbance was ridden at 560 nm. The group NF on day 7 was considered as 100% of mineralized matrix deposition.

2.2.5 Cell adhesion and spreading

In order to evaluate the cytoarchitecture of the cells, samples (n=3) obtained at 4 days of culture were fixed in 4% buffered formaldehyde solution for 10 min, washed in PBS, permeabilized in 0.1% triton X (Thermo Fisher Scientific) and incubated for 30 min with Alexa Fluor 555 Phalloidin (Thermo Fisher Scientific, Carlsbad, CA, USA) in bovine serum albumin (BSA) (1:40). The nuclei were stained with DAPI (Thermo Fisher Scientific). The discs were mounted with a fluorescence mounting medium (Dako Laboratories, CA, EUA) and images were recorded under confocal microscopy.

2.2.6 Cell migration

The scratch wound assay was used to investigate the migration of SAOS-2 onto the substrates. For that, following 3 days of culture, the cell layer was scratched by a P1000 pipette tip to create a straight wound. The detached cells were washed, and the remained cells were further cultured in a conventional or NIC-supplemented medium for 24 h. Then, the same protocol for appreciating the cytoarchitecture of the cells was conducted. Images were recorded under fluorescence microscopy and the total area of the wound was measured.

2.3 In vivo experiment

Male rats (Rattus norvegicus, albinus, Wistar), aged 3 months, weighing 300 to 350 g were kept under 12/12- hours light/dark cycles, 22◦ C ± 2◦ C ambient temperature, 20 air changes per hour, and air humidity about 55% ± 5% and housed in plastic cages in groups of three and monitored daily, receiving feed and water ad libitum. The experimental protocol was approved by the Ethics Committee in Animal Use (#403-2023; Anexo C) at the Araçatuba Dental School, São Paulo State University. This study was conducted according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines [38].

2.3.1 Sample size calculation and randomization protocol

The sample size was estimated according to the previous literature [39], experience, and execution of a pilot study. A 0.8 power and 0.05 alpha error based on a 12% potential SD, and the assumption that a 10% difference between groups/periods would be relevant, indicated that 8 animals were sufficient to assure statistical significance while standing for the 3Rs of *in vivo* research.

A total of 32 animals were used in this study, following a randomized, single-blind, controlled design. Numbers from 1 to 32 were labeled in the upper tail of the rats with a nankin drawing pen. The number sequence was uploaded to the software Minitab® 17 (Minitab Inc., State College, PA, USA). Simple randomization of the animals (1:1 allocation ratio) to groups VEH 15d, VEH 45d, NIC 15d, and NIC 45d was performed through a computer-generated random number table by a blinded staff external to the study.

2.3.2 Experimental protocol

All animals began to receive either physiological saline solution (VEH) or NIC hemissulfate twice a day in the 30 days preceding implant surgery, which was continued until euthanasia. At day zero, test (CH_SV) or control (NF) implants were installed in both tibiae of the rats. Euthanasia was performed at 15 and 45 days after surgery (Figure 1a).

2.3.3. Nicotine administration

NIC (Sigma, St. Louis, MO, EUA) was dissolved in VEH to obtain a solution at the concentration of 5 mg/mL. Then, the corresponding volume to a dose of 3 mg/kg was subcutaneously administered twice a day (12-h interval) [19,40] during the whole experiment to the animals belonging to group NIC. Aiming to subject control animals to the stress of

subcutaneous injection, 0.1 mL of VEH was administered twice a day for all animals from group VEH.

2.3.4 Implants placement

Surgical interventions were stated by sedation and general anesthesia, obtained by a combination of 70 mg/kg ketamine hydrochloride (Vetaset; Zoetis, Florham Park, NJ, USA) and 6 mg/kg xylazine hydrochloride (Coopazine; Coopers, São Paulo, Brazil), intramuscularly, on the biceps femoris of their right legs. The surgery for implants placement was conducted in accordance with Matheus et al [39] and is shown in Fig. 1(b-j). In this experiment, each animal (VEH or NIC) received both control and CH_SV implants. The titanium implants (2.2 mm × 4 mm) (DSP Biomedical®, Campo Largo, Parana, Brazil) used as controls (NF) were the substrate (same macro- and microgeometry) for biofunctionalization with CH and SV 2.5 μM (test). The decision of whether to install a control or test implant in the right of the left tibia was randomly conducted for each animal. One test implant (CH_SV 2.5 μM) was inserted and immediately retrieved from the alveolus to confirm whether the 3-dimentional topography was conserved following installation (Fig. 1k). After the surgical intervention, the animals were allowed to move freely and feed themselves with pellets and water ad libitum. During three postoperative days, with 24-hr time interval, 2.5 mg/kg morphine i.m. was administered for analgesia.

2.3.5 Euthanasia

Animals were submitted to euthanasia with a lethal dose of sodium thiopental (150 mg/kg) within 15 and 45 days after implants placement, consisting of 8 animals per group/period, thus totaling 64 specimens (NF and CH_SV). Immediately after euthanasia, the tibiae were removed and fixed with buffered 4% formaldehyde solution for 48 h.

2.3.6 Sample processing

After fixation, the 64 samples were demineralized in buffered 10% ethylenediaminetetraacetic acid (EDTA; E5134, Sigma-Aldrich) for 4 weeks, followed by subsequent routine histological processing and embedding in paraffin. The implants were retrieved, and the samples re-infused with paraffin. Semi-serial sections with 4 μm thickness were performed longitudinally along the implant alveolus. Six equidistant sections comprising the central portion of the alveolus from each specimen were stained with hematoxylin and eosin (HE) for histological and histometric analysis of BIC and bone area fraction occupancy (BAFO). Seven additional sections comprising the same region were subjected to immunohistochemistry for the detection of BMP-2, BMP-7, VEGF, tumor necrosis factor α (TNFα), interleukin (IL) 1β, osteocalcin (OCN), and tartrate-resistant acid phosphatase (TRAP).

2.3.7 Immunohistochemistry technique

The indirect immunoperoxidase technique was performed in accordance with the protocol described by Matheus et al [41]. In summary, the sections were divided into seven batches, each of the incubated with the respective primary antibodies (1:100). For signal amplification, universal biotinylated secondary antibodies (anti-goat IgG + anti- rabbit IgG + anti-mouse IgG) (2 hr) and streptavidin conjugated with horseradish peroxidase (HRP) (1 hr) (Universal Dako Labeled HRP Streptavidin-Biotin Kit®; Dako Laboratories, CA, EUA) were used. The reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB chromogen Kit®; Dako Laboratories). Harris hematoxylin was used as counter-staining to all biomarkers.

2.3.8 Analysis of the results

Based on the necessity of evaluating all pre-existing and newly formed tissues or to strictly evaluate the bone tissue formed as a result of the placement of the implant within the medullary cavity, a certified histologist E.E. determined two threads placed in cancellous bone and one in compact bone as the region of interest (ROI) for all outcome parameters.

2.3.8.1 Bone-to-implant contact

Images comprising the ROI were taken from the sections stained with H&E. The images were recorded through a digital camera (AxioCam®; Carl Zeiss, Gottingen, Germany) attached to a light microscope (AxioLab®) connected to a computer. Linear measurements (mm) of the perimeter of bone occupying the void space underlining the region occupied by the retrieved implant [42] was performed using the software ImageJ. BIC is expressed as a percentage of the total length of the three threads.

2.3.8.2 Bone area fraction occupancy

Images comprising the region of interest were taken from the sections stained with H&E. In this case, the total area (TA) between threads was measured, in mm^2, and considered 100% of the area to be analyzed. The entire bone area (BA) was delineated within TA. The ratio of BA to TA was calculated and expressed as BAFO.

2.3.8.3 Histological analysis of the peri-implant tissues

Histological analysis was carried under light microscopy evaluating the following parameters: (1) intensity of the local inflammatory response; (2) extension of the inflammatory process; (3) structural pattern of the newly formed compact bone; and (4) structural pattern of the newly formed cancellous bone.

2.3.8.4 Immunohistochemical analysis

For the immunohistochemical analysis of TRAP, the perimeter of the bone within the TA was measured (in mm). The immunoreactive cells in contact with bone were counted, and the results are expressed as TRAP-positive cells/mm.

Images of histological sections immunolabeled for BMP-2, BMP-7, VEGF, TNFα, IL-1β, and OCN were recorded as previously described. The percentage area corresponding to immunolabeling was obtained through the density of immunolabeling using a color threshold tool of Image J [43].

2.3.9 Statistical analysis

Data were analyzed using the software BioStat (BioStat version 5.0, Belém, PA, Brazil). Normality of all outcome measures was analyzed using Shapiro–Wilk test followed by ANOVA and post-test of multiple comparisons of Tukey ($p \leq 0.05$).

Resultados

3. Results

3.1 In vitro analyses

3.1.1 Cell viability and metabolism

The Live/Dead assay at day 1 indicated that cells remained vital following seeding (Fig. 2b). Cell metabolism increased over time in all groups ($p \leq 0.05$). At 1 and 7 days, no intergroup differences were observed, regardless the supplementation with NIC ($p \leq 0.05$). At 14 days, the harmful effect of NIC was evidenced by the lower cell metabolism in NIC-NF, NIC-CH, and NIC-CH_SV as compared with their respective controls (without NIC) ($p \leq 0.05$) (Fig. 2a).

3.1.2 Mineralized matrix deposition

At 7 days, CH_SV exhibited the highest values for mineralized matrix deposition as compared with NF and CH ($p \leq 0.05$). The same was observed for NIC-CH_SV as compared with NIC-NF and NI-CH ($p \leq 0.05$). No differences were observed when comparing groups supplemented with NIC (NIC-NF, NIC-CH, and NIC-CH_SV) and their respective controls.

At 14 days, the harmful effect of NIC was evidenced by the lower mineralized matrix deposition in NIC-NF, NIC-CH, and NIC-CH_SV as compared with their respective controls (without NIC) ($p \leq 0.05$). Yet, biofunctionalization with CH and SV resulted in the highest values of mineralized matrix deposition as compared with CH and NF, in the presence or absence of NIC ($p \leq 0.05$) (Fig. 2c). An overview of the alizarin staining on the surface of the discs for each group and period can be appreciated in Fig. 2d.

3.1.3 Wound healing and adhesion and spreading

Although the lowest wound area is to be observed in NF ($p \leq 0.05$) (Fig. 3a,b), it can be appreciated in higher magnification that cells are clustered (Fig. 3c), instead of spread onto the surface of the discs, as observed for CH, CH_SV, NIC-CH, and NIC-CH_SV (Fig. 3c).

3.2 In vivo experiment

All animals were healthy and showed no complications (signs of pain or discomfort) throughout the execution of the experiment, irrespective of the experimental group.

3.2.1 Bone-to-implant contact

BIC increased over time in all experimental groups, except for NIC-NF (15d, 30.38±4.20%; 45d, 35.20±6.91%) ($p \leq 0.05$). The lowest values of BIC were observed in NIC-NF as compared with the other groups in each period of analysis ($p \leq 0.05$). BIC did not differ among groups neither at 15 nor 45 days.

3.2.2 Bone area fraction occupancy

Early bone formation was observed in VEH-CH_SV (64.99±15.54%) and NIC-CH_SV (45.47±9.33%), once at 15 days, higher BAFO was observed in these groups as compared with VEH-NF (34.57±8.12%) and NIC-NF (24.37±5.16%), respectively ($p \leq 0.05$). The lowest values of BAFO were observed in NIC-NF as compared with the other groups in each period of analysis ($p \leq 0.05$). NIC-CH_SV (15d, 45.47±9.33%; 45d, 57.45±13.37) exhibited higher BAFO as compared with NIC-NF (15d, 24.37±5.16%; 45d 30.40±3.71) in both periods of analysis ($p \leq 0.05$). Importantly, BAFO did not differ between VEH-NF and NIC-CH_SV in any of the periods of analysis (Fig. 4b).

3.2.2 Histopathologic analysis of the peri-implants tissues

Groups VEH_NF 15d and VEH-CH_SV 15d exhibited thin and moderately thick bone trabeculae, respectively, with the space between threads mainly occupied by primary bone tissue. In VEH-NF 45d, the space between threads was occupied by a greater amount of trabeculae of moderate thickness, with equivalency on the proportion of primary and secondary bone tissue. In VEH-CH_SV 45d, the space between threads was occupied by thick trabeculae of secondary bone tissue (predominantly). In NIC-NF 15d, was observed thin trabeculae of primary bone and underlining immature connective tissue proper and hematopoietic tissue. In NIC-CH_SV 15d, was observed greater amount of bone and the trabeculae were slightly thicker than in NIC-NF 15d. In NIC-NF 45d and NIC-CH_SV 45d days, mainly quantitative differences were observed, once in NIC-CH_SV 45d was observed an evident increase in the amount of area occupied by bone, mainly due to the increased thickness of the trabeculae (predominantly primary bone tissue) (Fig. 4c-j).

3.2.3 Immunohistochemical analyses

The technique for detection of BMP-2, BMP-7, VEGF, TNFα, IL-1β, OCN and TRAP yielded high specificity for all antigens. As confirmed by the immunolabeling confined to the cytosolic compartment inf TRAP and to the cytosolic compartment and poorly to the extracellular matrix in BMP-2, BMP-7, VEGF, TNFα, IL-1β and OCN.

3.2.3.1 Bone morphogenetic proteins 2 and 7

The pattern of immunolabeling for BMP-2 (Fig. 5a-i) and BMP-7 (Fig. 5j-r) was very similar among groups. The lowest density of immunolabeling for BMP-2 (15d, 3.59±1.74%; 45d, 2.54±1.34%) and BMP-7 (15d, 5.10±1.6%; 5d, 3.64±2.18%) were observed in NIC-NF as compared with the other groups in each period of analysis ($p \leq 0.05$). VEH-CH_SV exhibited

higher density of immunolabeling for BMP-2 (15d, 13.11±1.98%; 45d, 12.47±3.19%) and BMP-7 (15d, 14.17±4.75%; 45d, 12.36±4.04%) than VEH-NF ([BMP-2; 15d, 8.37±4.54%; 45d, 6.33±2.12%][BMP-7; 15d, 8.63±1.84%; 45d, 7.27±2.58%]) in both periods of analysis ($p \leq 0.05$). NIC-CH_SV exhibited higher density of immunolabeling for BMP-2 (15d, 11.42±2.4%; 45d, 11.97±2.72%) and BMP-7 (15d, 15.51±3.09%; 45d, 10.57±2.68%) than NIC-NF in both periods of analysis ($p \leq 0.05$). Interestingly, NIC-CH_SV exhibited higher density of immunolabeling for BMP-2 than VEH-NF at 45 days ($p \leq 0.05$).

3.2.3.2 Osteocalcin

For OCN (Fig. 6), the intragroup analysis showed a lower density of immunolabeling for OCN at 45 days (3.47±1.92%) as compared with 15 days (7.43±2.17%) in VEH-NF ($p \leq 0.05$). At 15 days, higher density of immunolabeling for OCN was observed in VEH-NF, VEH-CH_SV (10.84±3.29%), and NIC-CH_SV (7.63±1.34%) as compared with NIC-NF (1.87±1.45%) ($p \leq 0.05$). Importantly, higher density of immunolabeling for OCN was observed in NIC-CH_SV (45d, 7.33±2.34%) as compared with NIC-NF (45d, 3.08±1.23%) in both periods and VEH-NF at 45 days ($p \leq 0.05$).

3.2.3.3 Vascular endothelial growth factor

For VEGF (Fig. 7), the lowest density of immunolabeling for VEGF was observed in NIC-NF (15d, 2.95±0.72%; 45d, 2.62±1.117%) as compared with the other groups in each period of analysis ($p \leq 0.05$). Importantly, the biofunctionalization with CH and SV significantly increased the density of immunolabeling of VEGF irrespective of exposure to NIC at 15 days ($p \leq 0.05$). At 45 days, NIC-CH_SV (7.38±1.25%) exhibited higher density of immunolabeling of VEGF than NIC-NF and VEH-NF (4.69±1.16%) ($p \leq 0.05$).

3.2.3.4 Inflammatory profile

For TNFα (Fig. 8a-i), the density of immunolabeling was lower at 45 days in NIC-CH_SV (2.08±1%) as compared with 15 days (5.97±2.1%) in the same group ($p \leq 0.05$). The highest density of immunolabeling for TNFα was observed in NIC-NF (15d, 8.76±1.59%; 45d, 7.88±1.12) as compared with the other groups in each period of analysis ($p \leq 0.05$). Importantly, NIC-CH_SV exhibited lower density of immunolabeling for TNFα than VEH-NF at 45 days (3.66±1.56%) ($p \leq 0.05$).

For IL-1β (Fig. 8j-r), the density of immunolabeling was lower at 45 days in NIC-NF (5.71±1.59%) and NIC-CH_SV (1.05±0.35%) as compared with 15 days in the same group (NIC-NF 15d, 7.83±2.05%; NIC-CH_SV 15d, 5.24±1.3%) ($p \leq 0.05$). VEH-NF (4.39±2.37%) exhibited higher density of immunolabeling for IL-1β as compared with VEH-CH_SV (2.3±1.77%) at 15 days ($p \leq 0.05$). NIC-NF exhibited higher density of immunolabeling for IL-1β as compared with NIC-CH_SV in both experimental periods ($p \leq 0.05$). Importantly, NIC-CH_SV exhibited lower density of immunolabeling for than IL-1β VEH-NF (3.07±1.99%) at 45 days ($p \leq 0.05$).

3.2.3.5 Resorptive activity

For TRAP (Fig. 9), the intragroup analysis showed that the number of TRAP-positive cells/mm was lower at 45 days than at 15 days in all experimental groups ($p \leq 0.05$). The highest number of TRAP-positive cells/mm was observed in NIC-NF (15d, 30.85±3.8; 45d, 14.28±4.19) as compared with all other groups in the same periods of analysis ($p \leq 0.05$). Importantly, the biofunctionalization with CH and SV significantly decreased the number of TRAP-positive cells irrespective of exposure to NIC at 15 days ($p \leq 0.05$). NIC-NF exhibited higher number of TRAP-positive cells/mm as compared with NIC-CH_SV (15d, 14.57±2.07; 45d, 6.28±1.38) in both periods of analysis ($p \leq 0.05$). The lowest number of TRAP-positive

cells/mm was observed in VEH-CH_SV at 45 days (2.5±1.19) when compared with the other groups (VEH-NF 45d, 5.5±2.32) in the same period of analysis ($p \leq 0.05$).

Discussão

4. Discussion

The hazardous effects of NIC on bone healing and its hierarchical structure around endosseous implants, both in dentistry and orthopedy, are irrefutable [5,6]. Given the high prevalence of smokers in the world population [21] and the relevance of osseointegrable metallic devices in restoring lost functions to individuals, to suppress the local harm of NIC to the osseointegration process might benefit the lives of many. The *in vitro* stimulatory capacity of the CH_SV 2.5 μM nanofibrillar super hydrophilic film on SAOS-2 was confirmed in an *in vivo* model of osseointegration under the influence of systemic NIC.

Surface characteristics govern the interaction of Ti with the surrounding environment [44]. The *in vitro* component of the present study evidenced that the tested CH_SV film was not cytotoxic (Fig.2b) and increased mineralized matrix deposition (Fig. 2c) while providing an optimal substrate for cell adhesion and spreading. In contrast, wounds were smaller in NIC- NF (Fig. 3a). Interestingly, the super hydrophilicity and the nanofibrillar extracellular mimic permitted SAOS-2 to occupy the whole surface of the discs, instead of favoring cells to cluster, as shown in Fig.3c and confirmed by the focal mineralization onto NF discs (Fig. 2d).

BIC (mm) is the primary outcome measure to assess successful osseointegration *in vivo* [45], however, it can be properly inferred that the amount (mm^2) of bone formed is essential to reduce focused overload and, hence, to increase the longevity of load-bearing rehabilitations. Osteogenesis after injury is a multifaceted complex process involving multiple regulators. BMP signal transduction and crosstalk with other major signaling pathways results in tightly regulated outcomes, including bone formation [46]. BMP-2 conditional knockout mice [47] and inactivated BMP-7 gene (by insertional mutagenesis) [48] are associated with severe defects in bone development, which highlights their vital role in osteogenesis. The capacity of BMPs in inducing an osteoblastic profile to MSCs [49] and their proliferation and activity might have accelerated the bone formation (Fig. 4) in VEH-CH_SV and NIC-

CH_SV, evidenced by the higher amount of BAFO at 15 days (as compared with VEH-NF and NIC-NF) and no significant difference with 45 days. This finding is supported by the increased expression of OCN (Fig. 6), one of the most fundamental regulators of mineralization and bone turnover [50], at 15 days in VEH-CH_SV and NIC-CH_SV. Interestingly, the overexpression of BMP-2 in the presence of SV was predictable [51], however, upregulation of BMP-7 was rather unexpected. The stimulation of BMP-7 production (but not BMP-2) under mechanical loading [52] indicates that, although not functional in the present study, the proposed film might benefit the mechanical adaptation of bone around load-bearing rehabilitations, even in smokers.

Excepting for the transportation function of blood vessels, there has been increasing evidence indicating that they originate angiocrine signals to regulate the process of bone defect healing [53]. The complex and well-organized cellular-molecular events orchestrating the process of peri-implant bone healing largely recapitulate the temporal and spatial formation patterns of bone growth, formation, and repair [54]. Coupling angiogenesis and osteogenesis is one of the most important aspects for assuring functional bone formation [55]. In general, VEGF may couple angiogenesis to osteogenesis both indirectly through its effects on endothelial cells, and directly by modulating cells expressing VEGF receptors [56,57]. Ma et al. [17] demonstrated that NIC exposure harmed angiogenesis and osteogenesis by altering gene expression of VEGF, hypoxia-inducible factor 1α (HIF-1α), and BMP-2. The present experiment corroborates with Ma's [17], as observed by the downregulation of BMPs (Fig. 5), VEGF (Fig. 6), and impaired bone formation (Fig. 4) in NIC-NF. Interestingly, the increased density of VEGF in NIC-CH_SV infers that tissue ischemia in nicotine-compromised animals was reversed by the local delivery of SV. VEGF-BMPs interaction enhances neo-angiogenesis and promotes the release of osteogenic factors [58], which might sustain the improved vascularization and higher (and rapid) BAFO in VEH-CH_SV and NIC-CH_SV.

Although inflammation after injury is modular in nature, tissue homeostasis is only restored when inflammatory cells either exit the site or are eliminated through apoptosis [59]. If targeted destruction and assisted repair are not properly phased, persistent inflammation can lead to persistent tissue damage [60]. A previous work of our group [19] has demonstrated that, in the same model of subcutaneous delivery of NIC, exacerbated inflammation after implant surgery perpetuates until later stages, exerting irreversible damage to osseointegration. Efficient modulation of inflammation in VEH and potent control of NIC-induced super-inflammatory state following injury in VEH-CH_SV and NIC-CH_SV, respectively (Fig. 8), might be underlined by the pro-resolving profile of statins [61]. Additionally, TNFα and IL-1β are potent regulators of osteoclasts differentiation and function [62,63]. TNFα-mediated engagement of phosphatidylinositol 3-kinase Akt and MEK/ERK signaling pathways promotes the survival of osteoclasts [64]. Intriguingly, SV inhibits osteoclast differentiation through regulating MAPK, AKT and Src signaling [31]. Hence, the dramatically lower number of active osteoclasts (Fig. 9) in the peri-implant bone of animals from groups VEH-CH_SV and NIC-CH_SV is either directly (through regulation of AKT signaling) or indirectly (modulation of inflammation) regulated by SV.

Osteoporosis findings suggest that surface-related local strategies are likely to overcome systemic debilitating conditions that might affect peri-implant healing [66]. Although consolidated for osteoporosis [65], the loading of bioactive agents onto the surface of Ti to promote osseointegration in smokers has been neglected and merits investigation. SV undergoes significant first-pass metabolism, resulting in very low systemic availability (<5%) and, eventually, toxicity [33]. Once the therapeutic effect of SV (or any drug) depends on the local concentration, the current oral regimen for SV precludes its desired bone-related effects. Promisingly, the adsorption of SV to a CH nanofibrillar super hydrophilic film provided a tissue concentration not only capable of excelling at the osseointegration rate of conventional

Ti implants under normal conditions but also suppressing the damages caused by NIC on the peri-implant bone formation *in vivo*.

Indeed, smoking cessation would be ideal for the patient. However, it was proven that smoking leads to extensive genome-wide changes in DNA methylation [66]. As investigations whether these changes are strict facilitators of cancer promotion or also regulating genes that orchestrate functions in distinct tissues are yet to come, the present study provides a solid foundation for increasing the success rate of endosseous dental and orthopedic Ti implants in smokers.

Conclusão

5. Conclusions

In summary, the nano fibrillar super hydrophilic film of CH and SV (2.5 μM) accelerates peri-implant bone formation under normal conditions and can be successfully used to overturn the negative impact of NIC on the osseointegration process around Ti implants. The CH_SV film improved cell-surface interaction and substantially increased BIC and BAFO *in vivo* by modulating inflammation, inducing differentiation and activity of osteoblasts, enhancing angiogenesis, and controlling TRAP-mediated bone resorption.

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6. References

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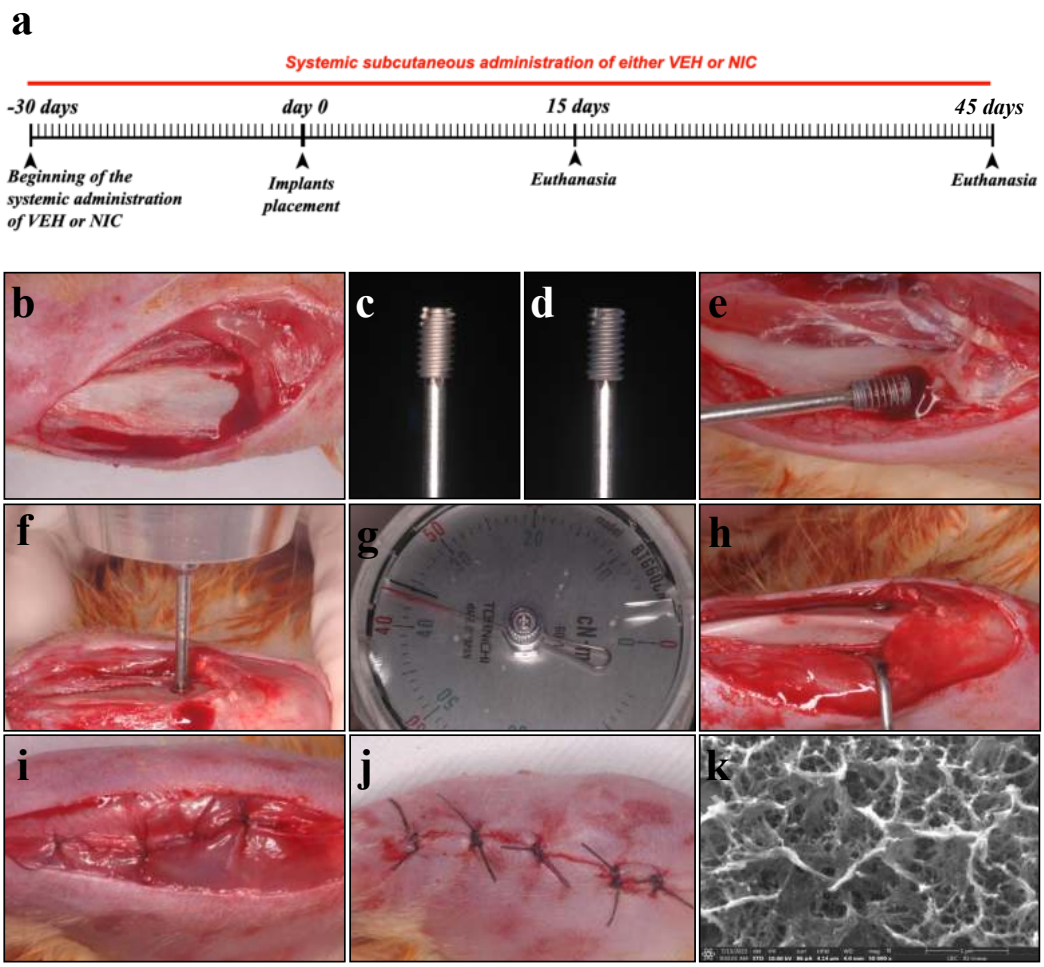

Figure 1: Experimental design and animal surgery. Images showing the exposure of the tibial metaphysis (a), macroscopic aspect of the control (NF) (c) and CH_SV (d) implants, implant insertion (e), adaptation of the torque wrench (f), insertion torque (g), bicortical fixation of the implant with sufficient amount of bone at the site (h), internal suture with reservable thread (i), and external suture with nylon. It can be appreciated that following implant insertion and immediate removal, the 3-dimension nanofibrilar network is maintained (k). Original magnification: 50 000x (k).

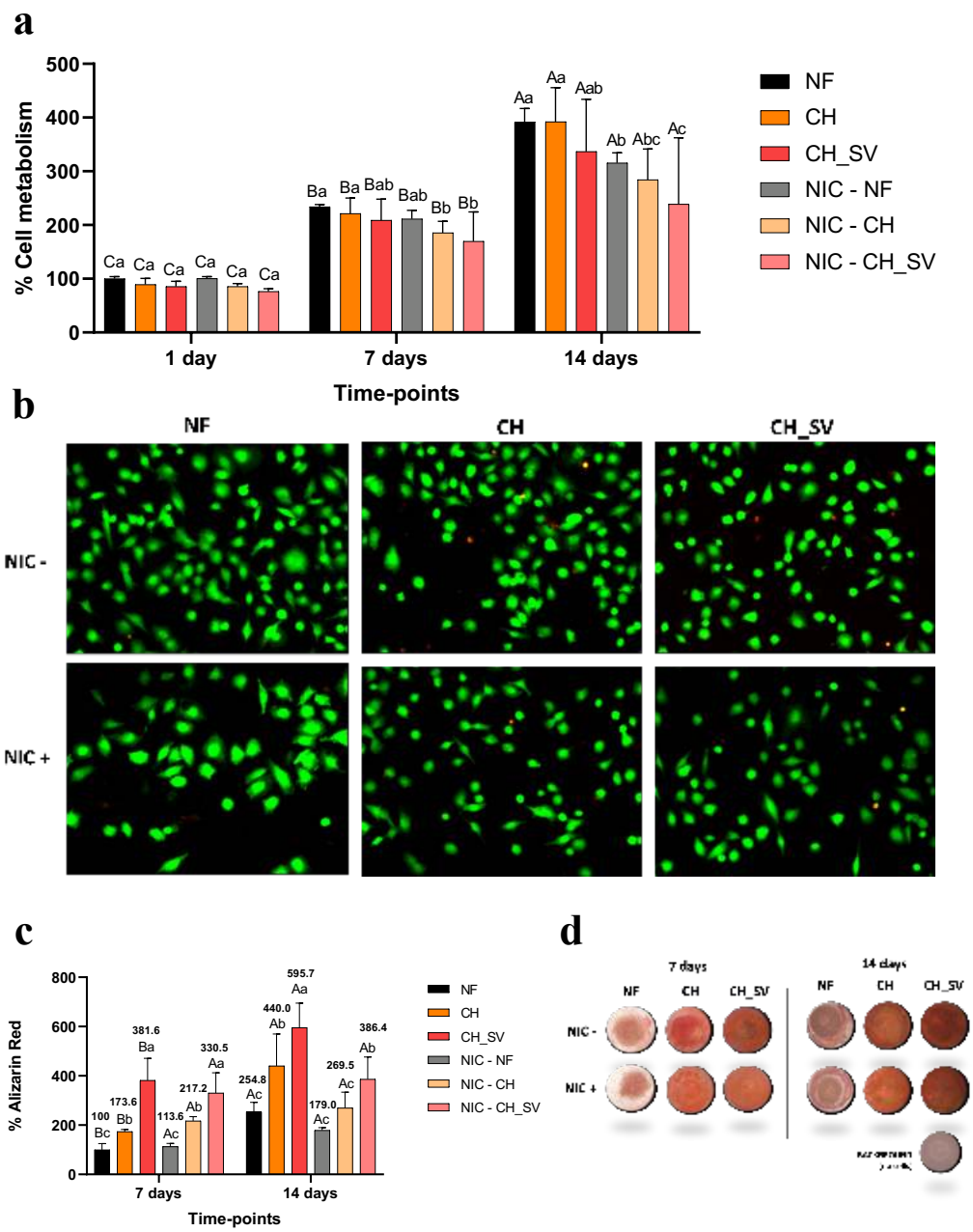

Figure 2: *In vitro* assays to determine metabolism, viability and mineralized matrix deposition by SAOS2. (a) Percentage of cell metabolism for each group and period. (b) Live/Dead assay at day 1. (c) quantification of mineralized matrix deposition. (d) representative images of the Alizarin Red stating of the discs from each group and period. Statistical tests: ANOVA and Tukey. Distinct uppercase letters indicate statistically significant intragroup differences among periods ($p \leq 0.05$). Distinct lower-case lattes indicate statistically significant intergroup differences in the same period of analysis ($p \leq 0.05$) . Fonte: Autor, 2023.

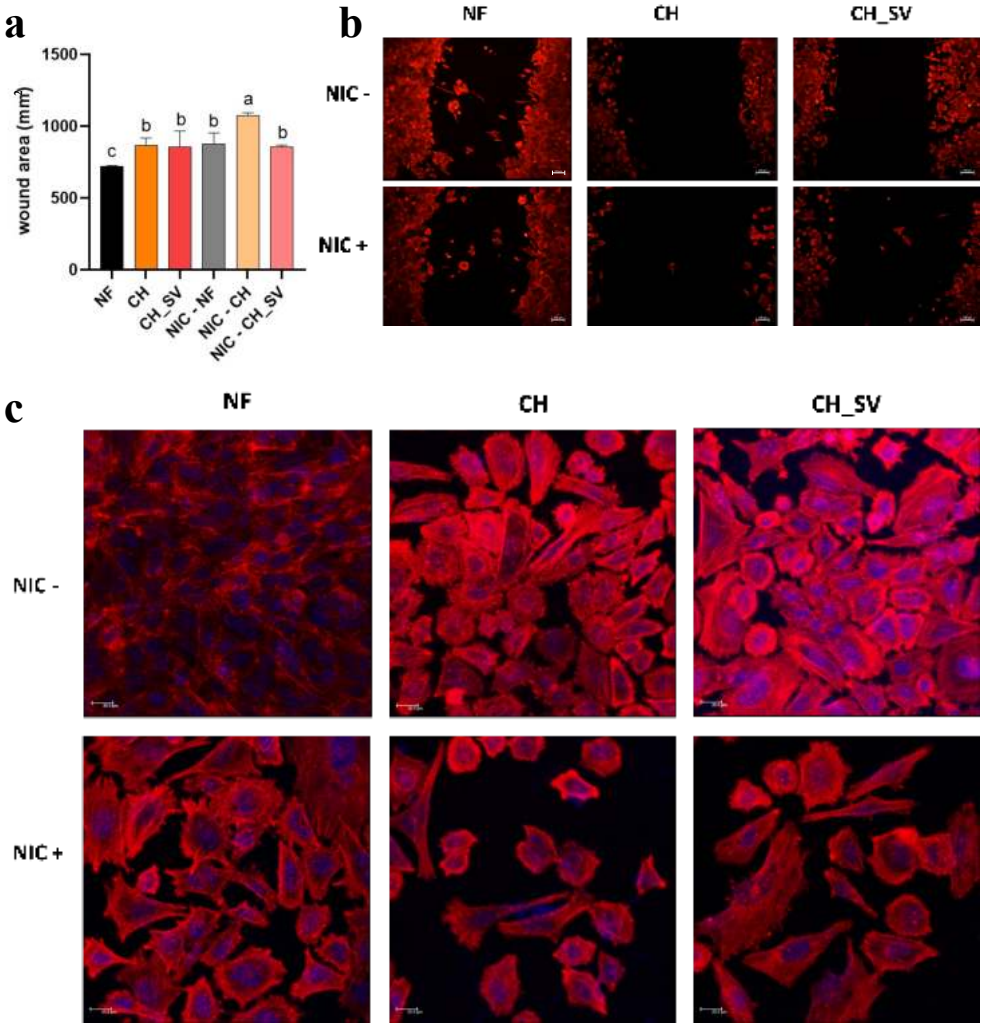

Figure 3: Wound healing and cell adhesion and spreading. (a) Wound area (mm^2). (b) Representative photomicrographs of the wound and (c) adhesion and spreading of SAOS2 onto the surface of the discs. Statistical tests: ANOVA and Tukey. Distinct lower-case lattes indicate statistically significant intergroup differences($p \leq 0.05$) . Fonte: Autor, 2023.

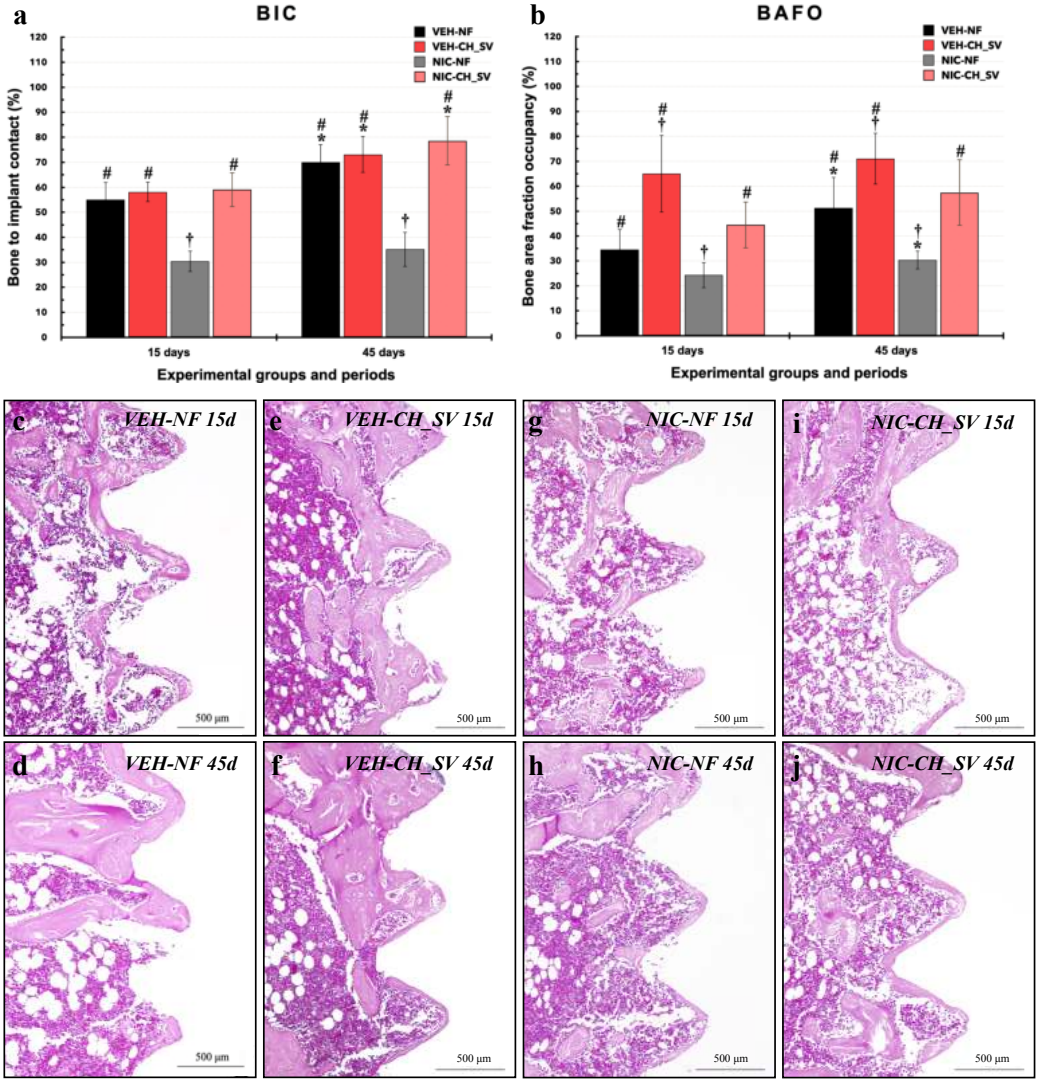

Figure 4: Bone formation and histological features of the peri-implant tissues. Graphs showing BIC (a) and BAFO (b). Statistical tests: ANOVA and Tukey. Symbols: *, statistically significant difference with 15 days in the same group ($p \leq 0.05$); †, statistically significant difference with VEH-NF in the same period ($p \leq 0.05$); #, statistically significant difference with NIC-NF in the same period ($p \leq 0.05$). (c-j) Photomicrographs showing the histological features of the peri-implant tissues for each group and period. Satining: Hematoxylin and Eosin. Scale bars: 500 µm. Fonte: Autor, 2023.

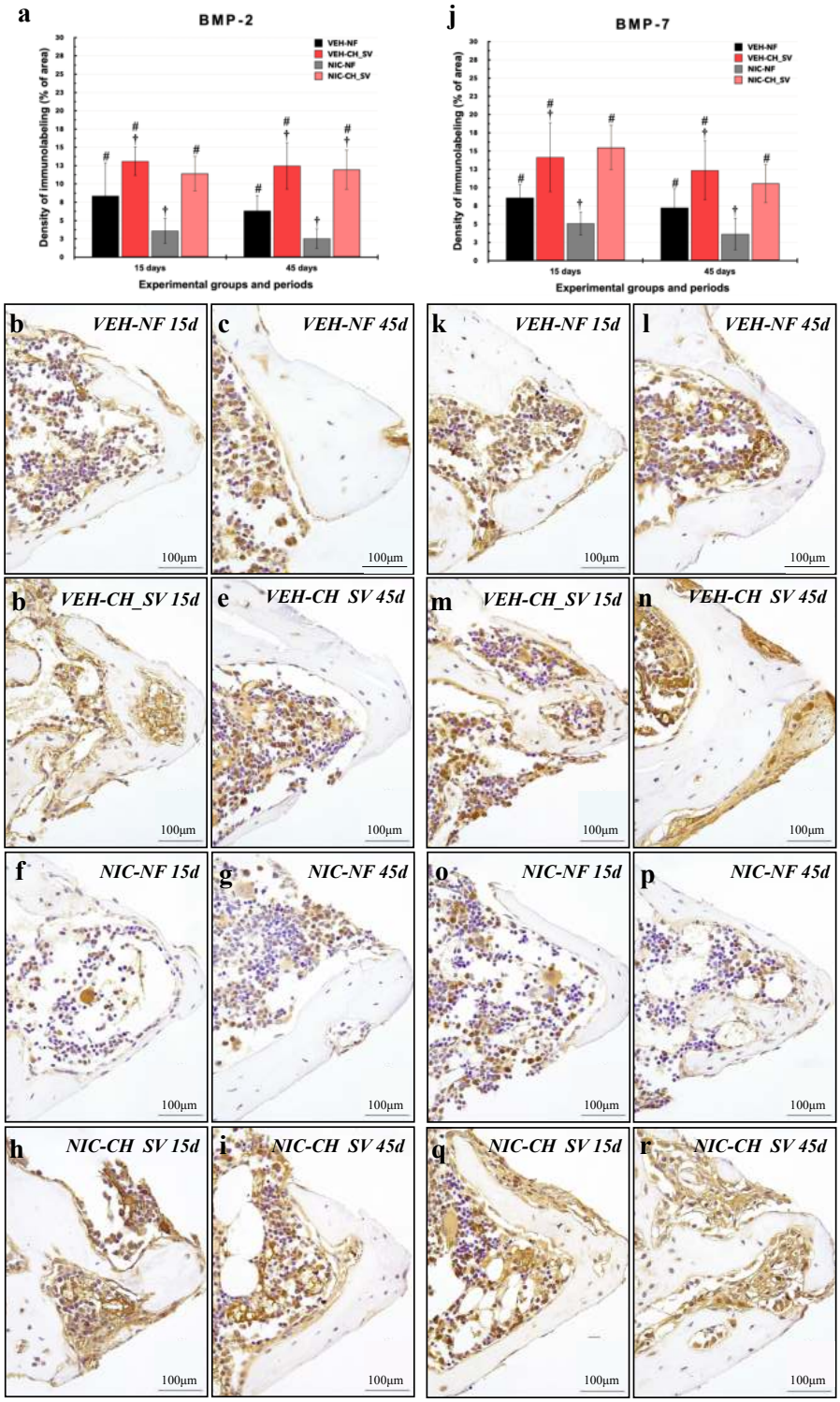

Figure 5: Immunolabelling pattern for BMP-2 (a through i) and BMP-7 (j through r). Graph showing the density of immunolabelling of BMP-2 (a) and BMP-7 (j). Statistical tests: ANOVA and Tukey. Symbols: †, statistically significant difference with VEH-NF in the same period ($p \leq 0.05$); #, statistically significant difference with NIC-NF in the same period ($p \leq 0.05$). Photomicrographs representing the immunolabelling pattern of BMP-2 (b-i) and BMP-7 (k-r). Counterstaining: Harry's hematoxylin. Scale bars: 100 µm. Fonte: Autor, 2023.

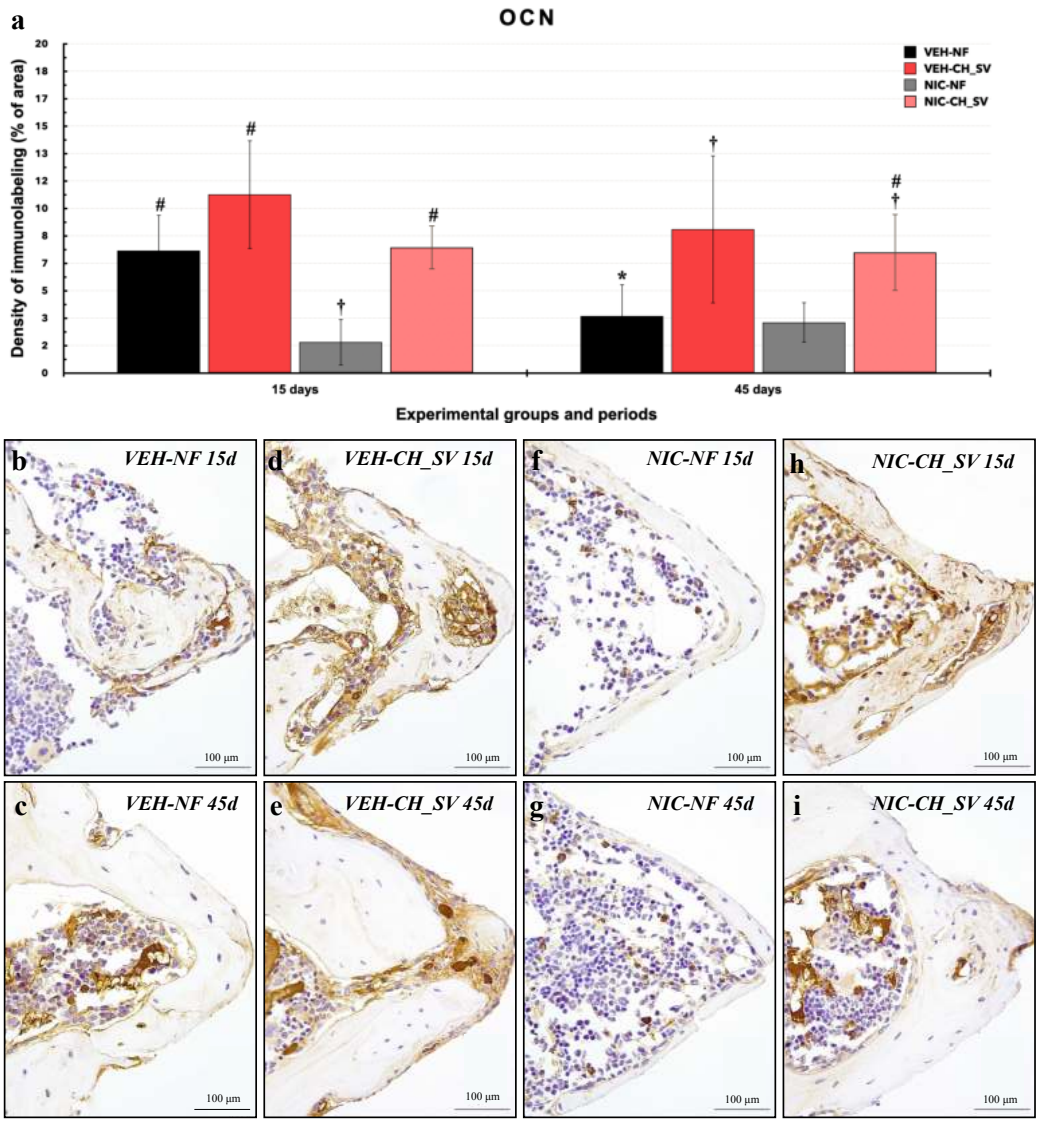

Figure 6: Immunolabelling pattern for OCN. Graph showing the density of immunolabelling of OCN (a). Statistical tests: ANOVA and Tukey. Symbols: *, statistically significant difference with 15 days in the same group ($p \leq 0.05$); †, statistically significant difference with VEH-NF in the same period ($p \leq 0.05$); #, statistically significant difference with NIC-NF in the same period ($p \leq 0.05$). Photomicrographs representing the immunolabelling pattern of OCN (b-i). Counterstaining: Harry's hematoxylin. Scale bars: 100 µm. Fonte: Autor, 2023.

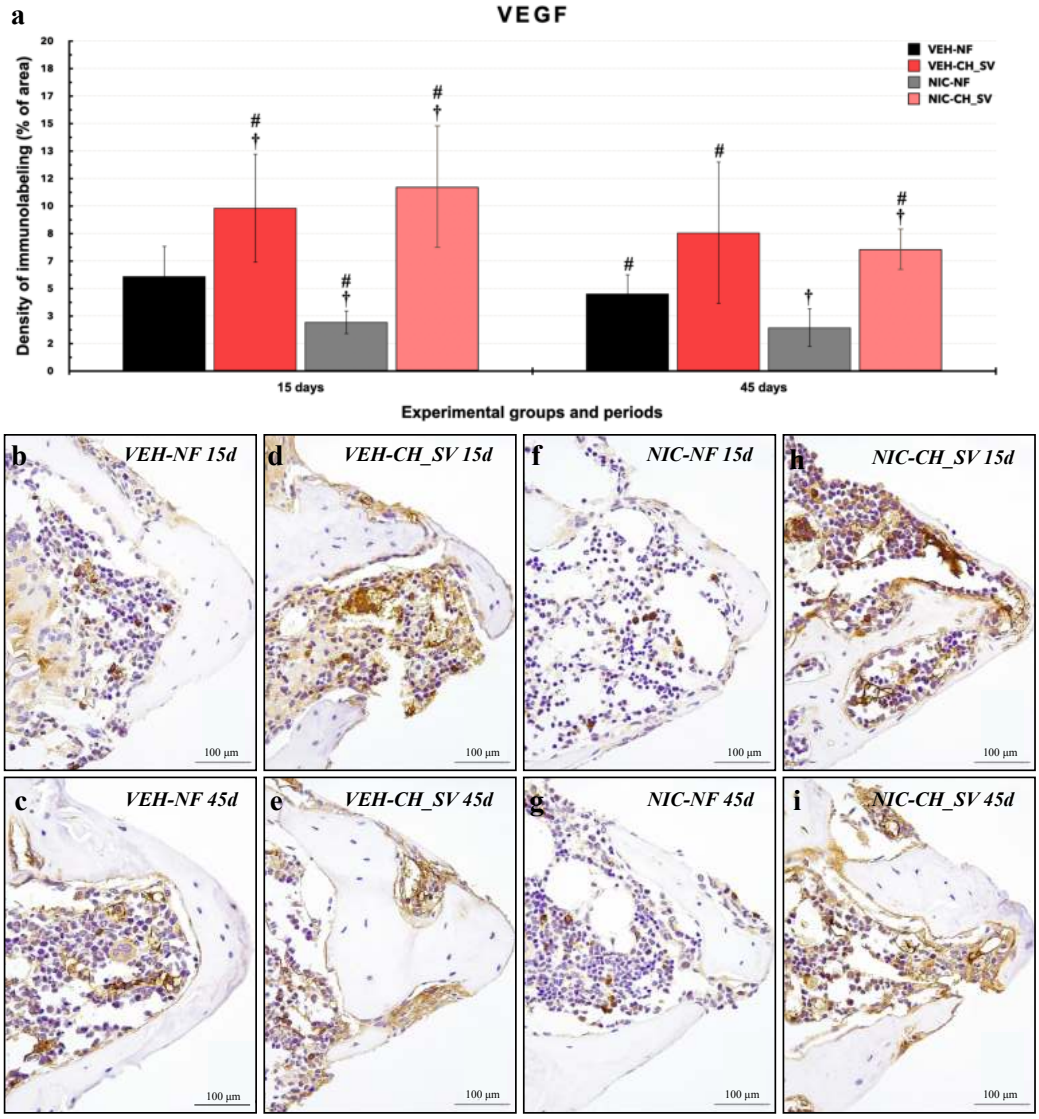

Figure 7: Immunolabelling pattern for VEGF. Graph showing the density of immunolabelling of VEGF (a). Statistical tests: ANOVA and Tukey. Symbols:†, statistically significant difference with VEH-NF in the same period ($p \leq 0.05$); #, statistically significant difference with NIC-NF in the same period ($p \leq 0.05$). Photomicrographs representing the immunolabelling pattern of OCN (b-i). Counterstaining: Harry's hematoxylin. Scale bars: 100 µm. Fonte: Autor, 2023.

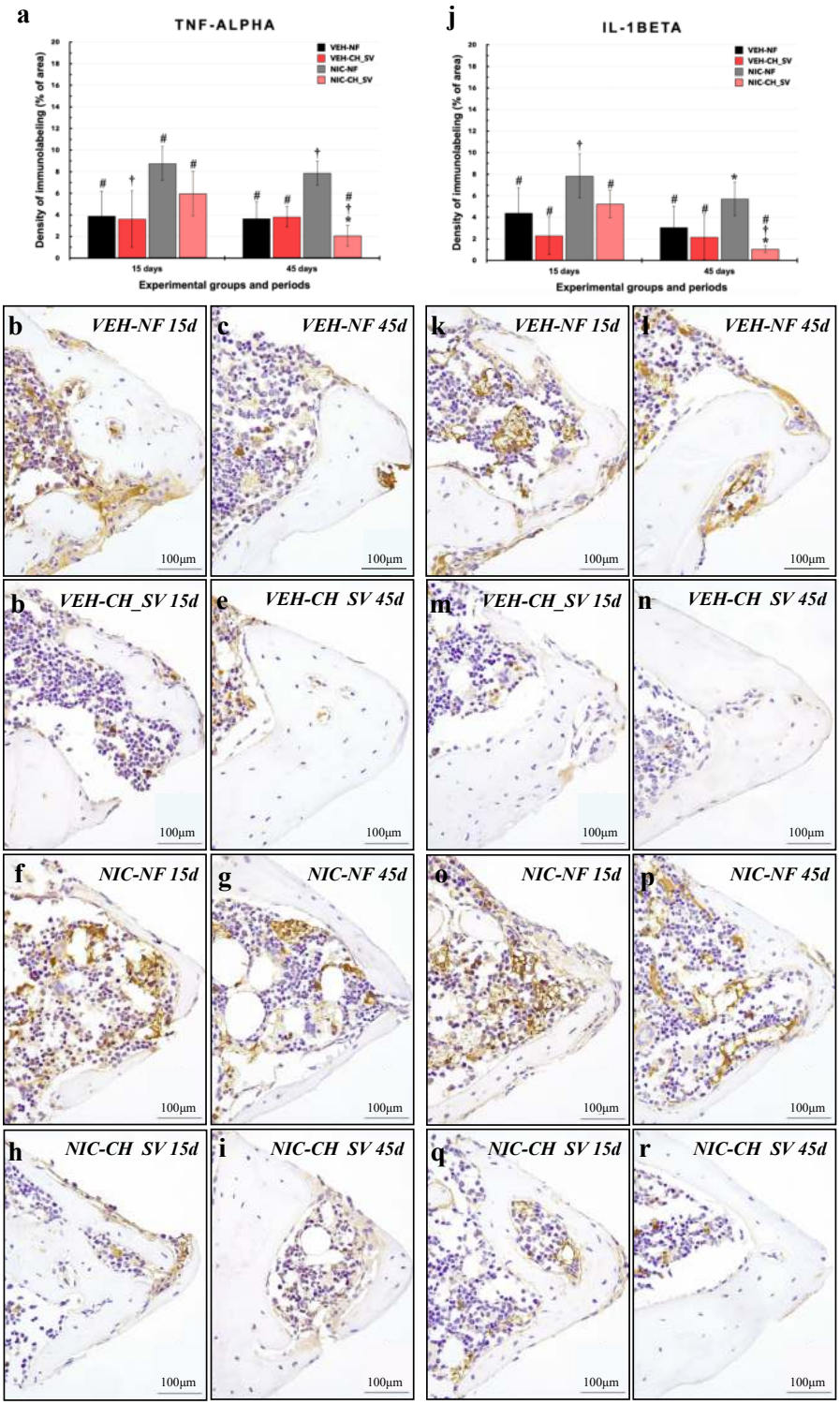

Figure 8: Immunolabelling pattern for TNFα (a through i) and IL-1β (j through r). Graph showing the density of immunolabelling of TNFα (a) and IL-1β (j). Statistical tests: ANOVA and Tukey. Symbols: *, statistically significant difference with 15 days in the same group ($p \leq 0.05$); †, statistically significant difference with VEH-NF in the same period ($p \leq 0.05$); #, statistically significant difference with NIC-NF in the same period ($p \leq 0.05$). Photomicrographs representing the immunolabelling pattern of TNFα (b-i) and IL-1β (k-r). Counterstaining: Harry's hematoxylin. Scale bars: 100 µm. Fonte: Autor, 2023.

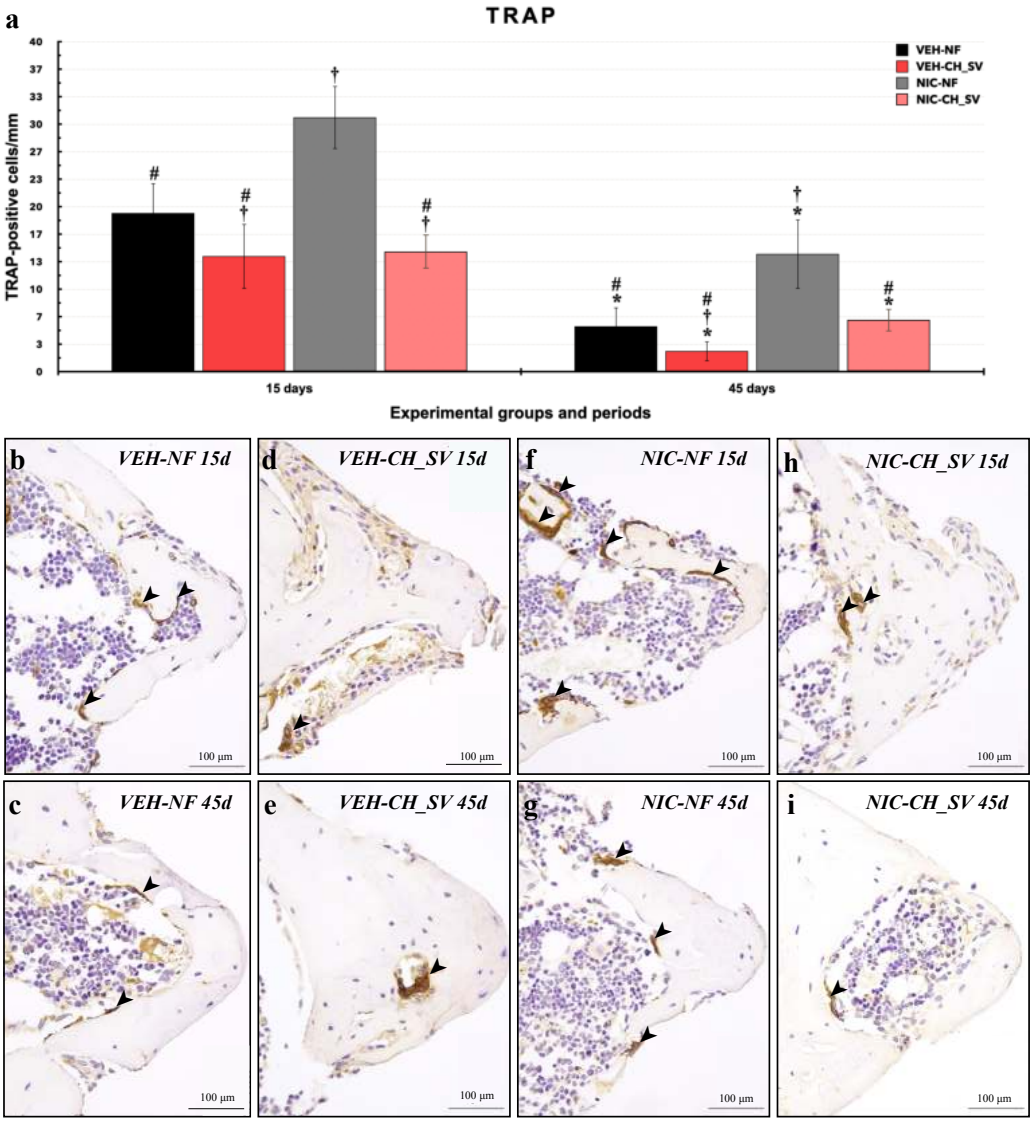

Figure 9: Immunolabelling pattern for TRAP. Graph showing the number of TRAP-positive cells/mm (a). Statistical tests: ANOVA and Tukey. Symbols: *, statistically significant difference with 15 days in the same group ($p \leq 0.05$); †, statistically significant difference with VEH-NF in the same period ($p \leq 0.05$); #, statistically significant difference with NIC-NF in the same period ($p \leq 0.05$). Photomicrographs representing the immunolabelling pattern of TRAP (b-i). Black arrowheads, immunoreactive cells. Counterstaining: Harry's hematoxylin. Scale bars: 100 µm. Fonte: Autor, 2023.

Anexos

ANEXO A - normas de Publicação do Periódico “Journal of Biomedical Materials Research Part A”

Journal of Biomedical Materials Research PART A

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Sections

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- Statistical methods used to assess significant differences (name of the statistical test including one- or two-sided testing, testing level (i.e., alpha value, *P* value), if applicable post-hoc test or any alpha adjustment, validity of any assumptions made for the chosen test),
- Software used for statistical analysis.

All relevant figure and table legends should contain information on sample size/number of replicates (*n*), probability (*P*) value, the specific statistical test for each experiment, data presentation, and the meaning of the significance symbol.

Note to NIH Grantees

ANEXO B - Normas de publicação do Periódico “Biomaterials”

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To find out more, please visit the Preparation section below.

Introduction

Types of article

Original Research: Provide a full length account of original research.
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2. Wellcome Trust. Economic Analysis of Scientific Research Publishing. Histon, UK: Wellcome Trust. Online. 2003. Available from URL: http://www.wellcome.ac.uk/doc_wtd003181.html (http://www.wellcome.ac.uk/doc_wtd003181.html)
3. Keeney M, Lai JH, Yang F. Recent progress in cartilage tissue engineering. Curr Opin Biotechnol. 2011. Available from URL: http://www.ncbi.nlm.nih.gov/pubmed/21531126 (http://www.ncbi.nlm.nih.gov/pubmed/21531126) (DOI: 10.1016/j.copbio.2011.04.003).

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
[2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
Reference to a dataset:
[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/xwj98nb39r.1.
Reference to software:
[7] E. Coon, M. Berndt, A. Jan, D. Svyatsky, A. Atchley, E. Kikinzon, D. Harp, G. Manzini, E. Shelef, K. Lipnikov, R. Garimella, C. Xu, D. Moulton, S. Karra, S. Painter, E. Jafarov, S. Molins, Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88), Zenodo, March 25, 2020. https://doi.org/10.5281/zenodo.3727209.

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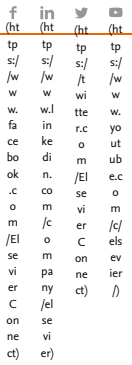

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UNIVERSIDADE ESTADUAL PAULISTA
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CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Desenvolvimento, caracterização e avaliação in vivo de um filme de quitosana-sinvastatina sobre a superfície do titânio: avaliação do potencial de osseointegração sob a influência de nicotina"**, Processo FOA nº 403-2023, sob responsabilidade de Juliano Milanezi de Almeida apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 25 de Abril de 2023.

VALIDADE DESTE CERTIFICADO: 05 de Maio de 2024.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 06 de Junho de 2024.

CERTIFICATE

We certify that the study entitled **"Development, characterization and in vivo assessment of a chitosan-simvastatin film for titanium coating: evaluation of the osseointegration potential under influence of nicotine"**, Protocol FOA nº 403-2023, under the supervision of Juliano Milanezi de Almeida presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on April 25, 2023.

VALIDITY OF THIS CERTIFICATE: May 05, 2024.
DATE OF SUBMISSION OF THE FINAL REPORT: June 06, 2024.

Prof. Dr. Fellippo Ramos Verri
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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